Brain circuitries in control of feeding behaviors

Focus on Neuropeptide Y

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Chapter IX.
A 24-hour fast differentially affects dopamine- and opioid-related gene expression

Abstract

The need to understand food intake regulation has increased during the last decades as the prevalence of obesity due to overeating is still increasing. Food deprivation through dieting or fasting, leads to adaptive physiological changes that increase food seeking behavior and the motivation to obtain food that make it difficult to consume lower caloric intake. The dopamine and opioid system are two components of the reward circuitry that, when activated, increase motivation to obtain food or palatable intake. Two areas of the reward system, the ventral tegmental area (VTA) and nucleus accumbens (NAc), express G-protein-coupled receptors for blood borne factors that signal energetic status, such as insulin, leptin and ghrelin. Effects of chronic food restriction on the reward circuitry have been studied extensively, but little is known about the effects of acute deprivation.

We here determined the effects of acute deprivation on reward system gene expression by measuring dopaminergic and opioid gene expression in VTA and NAc brain punches of 24 hours food deprived and *ad libitum* fed male Wistar rats using RT-qPCR at two time points. In addition, the arcuate nucleus (Arc) and lateral hypothalamus (LHA) were also studied, as these are hypothalamic areas with reciprocal connections with reward centers.

Interestingly, a short acute deprivation affected dopaminergic gene expression in the VTA but not in the NAc, and the changes in gene expression were consistent with decreased basal dopamine signaling as is seen after chronic food deprivation. Gene expression related to the opioid system was affected only in the Arc, and only observed for the delta-opioid receptor. Our data demonstrate that dopaminergic gene expression is affected after a short period of deprivation in the input structure, and not in the output structure, of the mesolimbic dopamine system.

Introduction

Fasting leads to adaptive changes in humoral and neural systems to increase motivation to enable food-seeking and subsequent hyperphagia to restore energy balance. The mesolimbic dopamine system, originating in the ventral tegmental area (VTA) and its target the nucleus accumbens (NAc), mediates motivational and reward-related processes (Boekhoudt et al., 2018; Morton et al., 2006). The VTA and NAc express G-protein coupled receptors for peripheral signals related to energetic status (Abizaid et al., 2006; Batch et al., 1992; Figlewicz et al., 2003; Krugel, 2003; Werther et al., 1987; Zigman et al., 2006). In addition, the VTA and NAc receive information related to energetic status from the arcuate nucleus (Arc) of the hypothalamus via Neuropeptide Y (NPY) projections (M. C. R. Gumbs et al., 2019; van den Heuvel et al., 2015). Arc NPY neurons are activated by fasting, and can increase feeding as well as reward-related behaviors via G-protein coupled receptors (Jewett, Cleary, Levine, Schaal, & Thompson, 1995; Michel et al., 1998; Pandit, Luijendijk, Vanderschuren, la Fleur, & Adan, 2014). Fasting could thus lead to changes in reward-related behavioral processes via changes in gene expression.

The increased motivation to procure food after chronic food restriction is accompanied by a general increased sensitivity of the dopaminergic system and decreased basal dopamine levels (K. D. Carr, 2007). Though acute caloric deprivation also increases the motivation for food in a manner that scales with the duration (i.e. longer deprivation is accompanied by more motivation; [Hanlon et al., 2004; Jewett et al., 1995; Scheggi et al., 2013]), only a few studies have studied the dopamine system after acute food deprivation. For instance, a short acute food deprivation of 24 hours decreased *dopamine transporter* (*DAT*) mRNA in the VTA/substantia nigra area in rats, which was accompanied by decreased DAT function in the dorsal striatum, but not by changes in DAT binding (Patterson et al., 1998). No changes were observed in DAT binding in the NAc (Patterson et al., 1998). However, 48 hours of food deprivation did not change the expression of several dopamine-system-related genes in the VTA or NAc, including the *DAT* (Lindblom et al., 2006), indicating that the effects of acute deprivation on the dopamine system might be duration dependent. Yet, the effects of 24 hours of acute food deprivation on dopamine-related gene expression have not been described previously.

Fasting also leads to increased food intake to compensate for energy loss, such as temporary hyperphagia or bingeing of calorie-rich foods in which the opioid system also plays a role (D. R. Brown & Holtzman, 1979; Smith et al., 1997; Will et al., 2003). The opioid system consists of three opioid peptides (enkephalin, dynorphin and ß-endorphin) and three opioid receptors (mu-, delta- and kappa-receptors). Food deprivation induces changes in opioid gene expression. For example, in the hypothalamus, 24 hours of food deprivation decreased Arc pro-opio-melanocortin (*POMC*) mRNA, the precursor for enkephalin and ß-endorphin, but did

not affect pro-dynorphin (*ppDyn*) or prepro-enkephalin (*ppENK*) mRNA levels in the Arc, whereas longer food deprivation decreased both Arc *POMC* and *ppDyn* mRNA levels in rats (Kim, Welch, Grace, Billington, & Levine, 1996). In addition, opioid receptor mRNA levels were unaffected in the Arc and lateral hypothalamic area (LHA) after 24 hours of food deprivation, whereas longer food deprivation increased mRNA expression of Arc and LHA *mu*-opioid receptors, but not of *delta*- or *kappa*-receptors, in rats (Barnes, Primeaux, & Bray, 2008). Though studies on chronic food restriction indicate changes in opioid gene expression in the reward system, studies on acute food deprivation have not included reward structures or opioid-receptor gene expression as of yet (Berman, Devi, & Carr, 1994; Berman, Devi, Spangler, Kreek, & Carr, 1997; Ikeda et al., 2015).

This study was designed to broaden our knowledge on the effects of a short acute food deprivation on reward-related gene expression. Gene expression was measured in the Arc, LHA, VTA and NAc of 24-hour-food-deprived and *ad libitum* fed male Wistar rats using real-time quantitative polymerase chain reaction (RT-qPCR). Gene expression was measured at the start and end of the light period, as the opioid system displays a strong circadian rhythm (Przewlocki et al., 1983; Takahashi et al., 1986). We report on the detection of mRNA's of the enzymes involved in dopamine synthesis, metabolism, receptors and key regulators, as well as the mRNAs of the opioid peptides and receptors.

Experimental procedures

Animals and housing

Male Wistar rats (Charles River breeding Laboratories, Sulzfeld, Germany), weighing 240-280 g at arrival, were habituated to the temperature- (21-23 ˚C) and light-controlled room (12:12h light/dark cycle, 07:00-19:00 lights on) in the animal facility of The Netherlands Institute of Neuroscience. Rats had *ad libitum* access to a container with standard high-carbohydrate diet (Teklad global diet 2918, 18.6% protein, 44.2% carbohydrate, and 6.2% fat, 3.1 kcal/g, Envigo) and a bottle of tap water. The animal care committee of the Netherlands Institute for Neuroscience approved all experiments according to Dutch legal ethical guidelines.

Effects of 24 hours of food deprivation on mRNA expression

After seven days in the animal facility, naïve rats were food-deprived for 24 hours or kept under *ad libitum* conditions in two groups; 10:00 – 10:00 (ZT3; N = 8 per group) and 17:00 – 17:00 (ZT10; N = 8 per group), and decapitated after 33%CO_2/66%O_2 gas anesthesia. Brains were rapidly dissected, frozen on dry ice and stored at -80 ˚C.

Materials and methods, and data obtained from these tissue samples have been reported 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), the VTA (Bregma -4.68 till -6.24 mm) and the NAc (Bregma 3.00 till -0.84 mm) according to the rat brain atlas (Paxinos & Watson, 2007). Sections were placed in RNAlater (Ambion, Waltham, MA, USA) and punched with a 1mm-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. 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, London, 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. In Arc samples, the absence of *steroidogenic factor* (*Sf1*) gene expression was used to verify the absence of ventromedial hypothalamic material in Arc punches. PCR products were analyzed on a DNA agarose gel for qPCR product size.

Primers

Primers were designed using the Primer-Blast program (www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers had a maximum amplicon size of 250 bp, showed melting temperatures between 55 – 66 °C, with no greater difference than 1.5 °C between forward and reverse primers, a G/C content of 45 – 65 %, and a maximal self-complementarity of six nucleotides. Primer specificity was analyzed using the reference sequence database of *rattus norvegicus* (taxid: 10116). If unintended targets were present, primers had at least four mismatches with unintended targets. Were possible, primers were intron-spanning. All primers were purchased from Roche Molecular Biochemicals (04897030001; Mannheim, Germany). See Table 1 for all primer sequences.

Table 1. Primer information.

Gene	NCBI ref.	Forward primer 5'- 3'	Reverse primer 5' – 3'
Dopaminergic and GABAergic primers			
TH	NM_012740.3	TGGGGAGCTGAAGGCTTATG	CAGAGAATGGGCGCTGGATA
DrD1	NM_012546.3	CCGAGATTGCTGGCTTTTGG	GGTTCAGAATGGACGCCGTA
DrD2	NM_012547.1	CACTCAAGGGCAACTGTACC	AGGGTTGCTATGTAGGCCGT
DrD3	NM_017140.2	GAAGAGCCTGATTTAGCCCAC	TAGGATGAGAGCACAGTAGGA
DrD4	NM_012944.2	ACTGCAAATCCCCAGGCTCAG	AAGTCCGGTGCCAGTACCTAA
DAT	NM_012694.2	TGCTGTCTCTCTTCTGCGTC	GCTGCCCTGTCATTTGCTTG
COMT	NM_012531.2	CGTCGGGATCGGACCT	CAGAAAATAGTCACCAAGCCC
MAOa	NM_033653.1	GGGTAGATGTTGGTGGAGCC	TGCACCACGGAATGGGTAAG
MAOb	NM_013198.1	AAGCAGTGTGGGGGTACAAC	AAAACAGGTGGGATGGCACT
DARPP32	NM_138521.1	CAGCTCGACCCCCGACAGGT	TCGACTTTGGGTGGTGCCCCT
Synuclein-a	NM_019169.2	AACTAAGGAGGGAGTCGTTC	TCATAAGCCTCACTGCTAGG
GAD65	NM_012563.1	AGCTGGAACCACTGTGTACG	ACCAGGAGAGCCGAACATTG
GAD67	NM_017007.1	CTCCCTGTGGCTGAATCGAG	GGCTACGCCACACCAAGTAT
vGat1	NM_031782.1	GGGTCACGACAAACCCAAGA	TAGGGTAGACCCAGCACGAA
ClC3	NM_053363.2	ACTGGGTGCGAGAAAAGTGT	AGCCATCCTGACCAAGCATC
vMAT2	NM_013031.1	GGACTCATCGCTCCCAACTT	CAAAAGGCCACGTCTGCAAT
Opioid primers			
Mu-r	NM_001038597.2	GTGGGCCTCTTCGGAAACTT	AAAGGGCAGTGTACTGGTCG
Kappa-r	NM_001318742.1	TCTCTCCAGCCATCCCTGTTAT	AGATGTTGGTTGCGGTCTTC
Delta-r	NM_012617.1	TTCACCAGCATCTTCACTGCT	ATGAGAATGGGCACCACGAA
ppENK	NM_017139.1	CTTGTCAGAGACAGAACGGGT	CCTTGCAGGTCTCCCAGATTT
ppDyn	NM_019374.3	CTTCTGAATCTTGGATCGGC	CATTCTGTATCACCCTTCCTG
POMC	NM_139326.2	CGTGTGGAGCTGGTGCCTGG	GCGGTCCCAGCGGAAGTGAC
PC1	NM_017091.2	CTGATCTTGCTTCTTTTCTCT	GTGATTTTCAAGTGATCCAAT
PC2	NM_012746.1	GAGTTGCATAAAGACGGAGA	TTGATGTCTCTGTACCCTCT
Reference gene primers			
Ubiquitin-C	NM_017314.1	TCGTACCTTTCTCACCACAGTATCTAG	GAAAACTAAGACACCTCCCCATCA
HPRT	NM_012583.2	CCATCACATTGTGGCCCTCT	TATGTCCCCCGTTGACTGGT
Cyclo-A	NM_017101.1	TGTTCTTCGACATCACGGCT	CGTAGATGGACTTGCCACC
*Sf1**	NM_001191099.1	CCAGTGTCCACCCTTATCCG	ACCTTGTCACCACACACTGG

ClC3 = chloride voltage-gated channel 3, *COMT* = catecholamine-o-methyl tranferase, *Cyclo-A* = Cyclophilin-A, *DARPP32* = dopamine and cAMP-regulated neuronal phosphoprotein, *DAT* = dopamine transporter (Slc6A3), *Delta-r* = delta opioid receptor, *DrD1/DrD2/DrD3/DrD4* = dopamine receptor subtype D1/D2/D3/D4, *GAD65* = glutamate decarboxylase isoform 65 kDa, *GAD67* = glutamate decarboxylase isoform 67 kDa, *HPRT* = Hypoxanthine guanine phosphoribosyl transferase, *Kappa-r* = kappa opioid receptor, *MAOa* = monoamine oxidase A, *MAOb* = monoamine oxidase B, *Mu-r* = mu opioid receptor, *PC1* = prohormone convertase 1, *PC2* = prohormone convertase 2, *ppDyn* = preprodynorphin, POMC = pro-opiomelanocortin, *ppENK* = prepro-enkephalin, *Sf1* = steroidogenic factor, *TH* = tyrosine hydroxylase, *vGat1* = vesicular GABA transporter 1 (SLc32A1), *vMAT2* = vesicular monoamine transporter 2 (Slc18A2), *absence of SF1 product was used to determine the exclusion of ventromedial hypothalamic tissue in Arc punches.

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 using the geometric mean of three reference gene values (*Ubiquitin-C*, *Hypoxanthine guanine phosphoribosyl transferase*, and *Cyclophilin-A*; see Table 1). As most reference genes showed regulation by time of the day, but not by food deprivation (assessed using Two-way ANOVA analyses), data are presented per time point. Differences between food deprived- and *ad libitum*-fed gene expression levels were determined using two-tailed Mann-Whitney tests for unpaired nonparametric data. Holms-Bonferroni method was used per region to correct for multiple testing. All data are presented as mean ± *SEM* and as adjusted p-values (significance level is $p_{adjusted} \leq 0.05$). Statistical details are provided in Supplemental Table 1.

Results

Body weight after fasting

Body weight was measured after 24 hours of fasting and was significantly decreased in the fasted (313.9 ± 2.5 grams) *vs.* the *ad libitum*-fed (324.9 ± 2.4 grams) group when measured at the beginning of the light period ($t_{14} = 3.17$, $p < 0.01$). Body weight was, however, similar in the fasted (343.5 ± 4.0 grams) *vs.* the *ad-libitum*-fed (352.6 ± 4.3 grams) group when measured at the end of the light period ($t_{14} = 1.55$, $p > 0.05$).

Effects of 24 hours of food deprivation on dopamine-related gene expression

A 24-hour fast affects VTA TH and DrD2 mRNA levels

At ZT10, 24 hours of food deprivation decreased VTA *TH* mRNA ($p_{adjusted} = 0.04$, N[*ad libitum*/food-deprived] = 7/8) and *DrD2* mRNA ($p_{adjusted} = 0.05$; N[*ad libitum*/food-deprived] = 6/8) significantly compared to gene expression in *ad libitum*-fed controls (Figures 1A, B). A trend for a reduction in *Synuclein alpha* mRNA ($p_{adjusted} = 0.1$; $p_{unadjusted} = 0.02$; N[*ad libitum*/food-deprived] = 7/8) and *GAD67* mRNA ($p_{adjusted} = 0.09$; $p_{unadjusted} = 0.02$; N[*ad libitum*/food-deprived] = 5/8) was also observed after 24 hours of food deprivation *vs. ad libitum*-fed controls (Figures 1C, E2). Gene expression of dopamine degradation enzymes (*COMT*, *MAOa*, *MAOb*), dopamine uptake mechanisms (*DAT/Slc6A3*), or the GABA system (*GAD65*, *vMat2*, *vGat1*, *ClC3*) were not significantly altered after 24 hours of food deprivation (all $p > 0.05$; Figure 1). VTA *DrD1*, *DrD3*, and *DrD4* mRNA expression levels were at detection level and therefore unreliable.

At ZT3, expression of the genes measured in the VTA were not significantly altered by 24 hours of food deprivation (supplemental Figure 1, and supplemental Table 1 for a summary of all p-values).

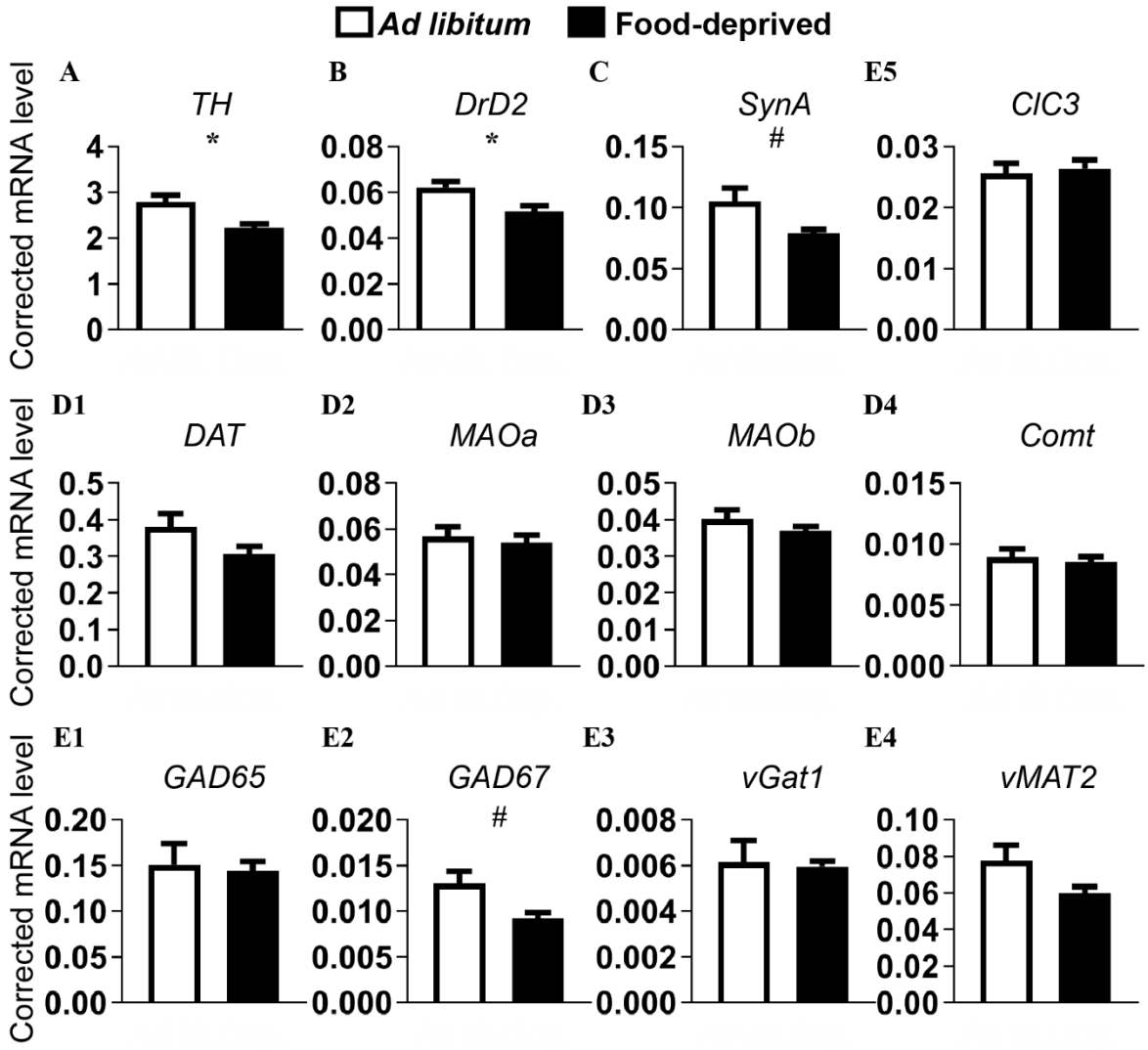


Figure 1. A 24-hour fast decreases VTA *TH* and *DrD2* mRNA expression at ZT10. Deprivation decreased **A)** VTA *TH*, and **B)** *DrD2* mRNA expression. **C)** A trend for a decrease was found in *Synuclein alpha*, and **E2)** *GAD67* mRNA expression. **D1 – D4)** No effect of deprivation was seen on genes related to dopamine uptake or metabolism, nor **E1, D3 – E5)** in other GABAergic genes. Gene expression for *Drd1*, *DrD3*, and *DrD4* is not shown as expression level was at detection level. * $p_{adjusted} < 0.05$, # $p_{adjusted} < 0.1$ and $p_{unadjusted} < 0.05$. See text for details and Table 1 for abbreviations.

A 24-hour fast does not affect NAc dopamine-related gene expression

At ZT10, NAc gene expression of dopamine receptors (*DrD1*, *DrD2*, *DrD3*), dopamine degradation enzymes (*COMT*, *MAOa*, *MAOb*), neuronal activity (*DARPP32*), or the GABA system (*GAD65*, *GAD67*, *vGat1*) were not significantly altered after 24 hours of deprivation (all $p > 0.05$; Figure 2). NAc *DrD4* mRNA expression was at detection level, and therefore excluded from analysis.

At ZT3, the expression levels of the genes measured in the NAc were also not altered by 24 hours of food deprivation (supplemental Figure 2, and supplemental Table 1 for a summary of all p-values).

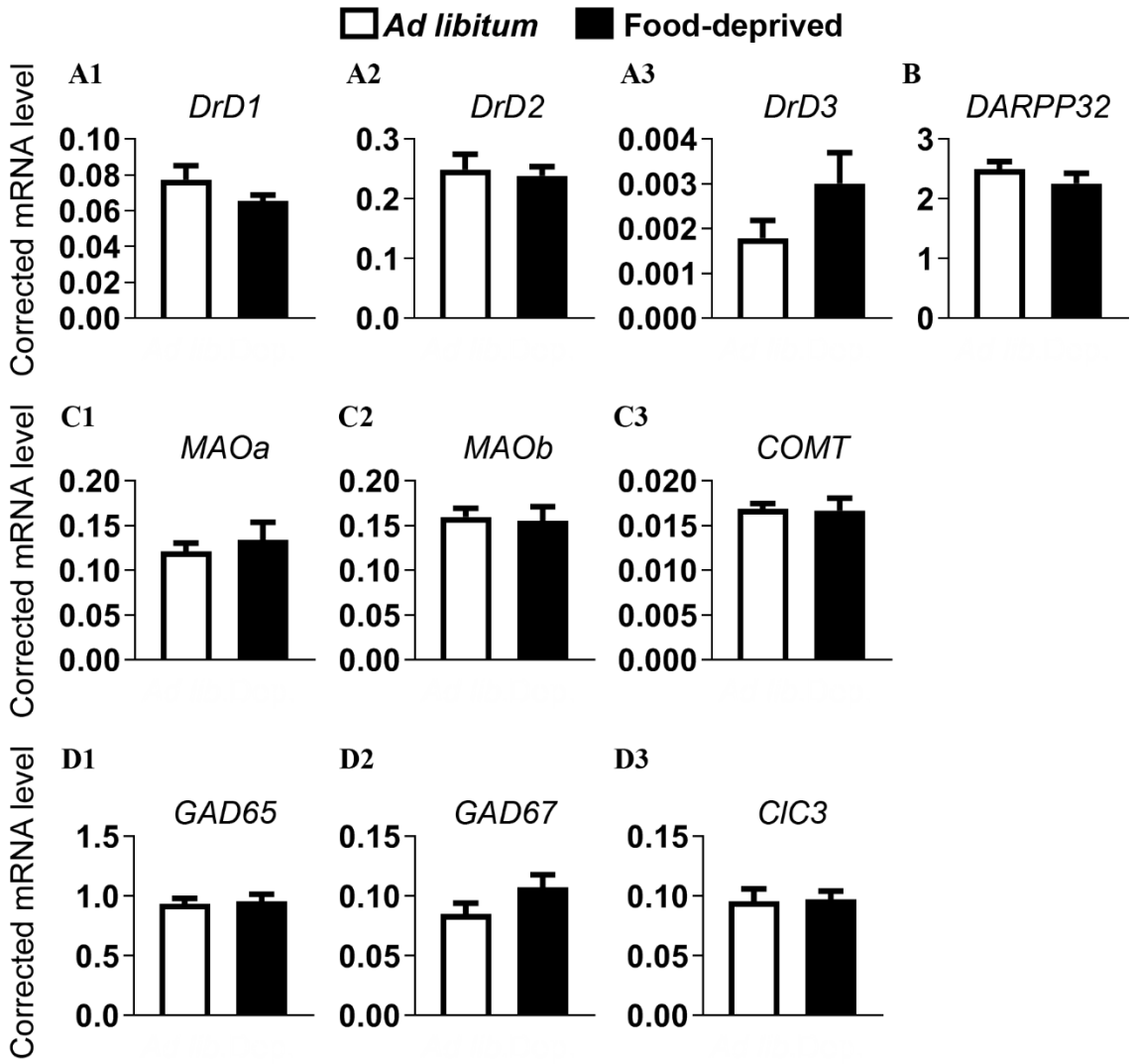


Figure 2. A 24-hour fast does not alter NAc dopamine-related gene expression at ZT10. A1-A3) Acute food deprivation did not affect expression of dopamine receptor genes, **B)** *DARPP32* **C1-C3)**, dopamine degradation enzymes, nor **D1-D2)** GABA-synthesizing enzymes, or **D3)** *ClC3*, an inhibitory channel associated with GABAergic transmission. Gene expression for *DrD4* is not shown as expression was at detection level. See text for details and Table 1 for abbreviations.

Effects of 24 hours of food deprivation on opioid gene expression

A 24-hour fast does not affect opioid gene expression in the Arc or LHA

At ZT10, Arc expression of *ppENK*, *ppDyn*, *POMC*, *PC1*, *PC2*, or of the *mu-*, *kappa-* or *delta-*receptor was not significantly different between 24-hour food deprived rats and *ad libitum* fed controls (all $p > 0.05$; supplemental Figure 3). Unfortunately, expression of the opioid genes could not be performed at this time point for LHA samples, as these were not available.

At ZT3, Arc expression of the *delta*-receptor was decreased by 24 hours of food deprivation ($p_{adjusted} = 0.03$; N[*ad libitum*/food-deprived] = 6/5; supplemental Figure 4A3). In addition, Arc *ppDyn* mRNA expression showed a trend to decrease after 24 hours of food deprivation ($p_{adjusted} = 0.1$; $p_{unadjusted} = 0.02$; N[*ad libitum*/food-deprived] = 8/8; supplemental Figure 4B2). Arc expression of the other opioid genes was not significantly altered by 24 hours of food deprivation (all $p > 0.05$; supplemental Figure 4). In the LHA, opioid gene expression of all measured genes was unaltered by 24 hours of food deprivation *vs. ad libitum*-fed consumption (supplemental Figure 5). See supplemental Table 2 for a summary of all p-values related to opioid gene expression in the Arc and LHA.

A 24-hour fast does not affect opioid gene expression in the VTA and Nac

At ZT10, 24 hours of food deprivation did not significantly affect VTA or NAc expression of opioid-related genes (all $p_{adjusted} > 0.05$; supplemental Figures 6, 7).

At ZT3, 24 hours of food deprivation also did not affect VTA or NAc expression of opioid-related genes (all $p_{adjusted} > 0.05$; supplemental Figures 8,9). See supplemental Table 2 for a summary of all p-values related to opioid gene expression in the VTA and NAc.

Discussion

Our aim was to determine if acute food deprivation affected dopamine- and opioid-related gene expression in the VTA and NAc in male Wistar rats. Dopamine-related mRNA expression was affected by short-term food deprivation in the VTA, however only at the end of the light period. In the NAc, short-term food deprivation did not affect gene expression of the dopamine-related genes. Furthermore, opioid-related gene expression was unaffected by short-term food deprivation, except for *delta*-receptor mRNA expression in the Arc, which was decreased after short-term food deprivation. Taken together, our data indicate that gene expression modulation of the reward system by acute food deprivation occurs mainly in the dopamine system as opposed to the opioid system. In addition, our data indicate that the input structure of the mesolimbic dopamine system (i.e. the VTA) is sensitive to acute deprivation, whereas dopamine-related gene expression in the output structure (i.e. the NAc) is not.

Short food deprivation may decrease dopaminergic-output

We observed that VTA *TH* and *DrD2* mRNA were decreased after a short acute fast, and that this was only observed when measured at the end of the light period and not when measured at the beginning of the light period. A previous study did not observe changes in *TH* and *DrD2* gene expression in the VTA after a longer duration of food deprivation (i.e. 48 hours; [Lindblom et al., 2006]). However, this study did not mention when the measurements took place, therefore it could be that they missed effects due to timing. The *TH* and *DrD2* genes encode two principal components of the dopamine system. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the production of catecholamine synthesis, including dopamine synthesis, and the DrD2 is implicated in the regulation of various behaviors, including (food) reward and motivation (Soares-Cunha, Coimbra, Sousa, & Rodrigues, 2016). Though gene expression data should be interpreted with caution as, for instance, changes in *TH* mRNA do not always correlate with protein levels or enzyme activity (Daubner, Le, & Wang, 2011; Kumer & Vrana, 1996), changes in the expression levels for *TH* and the *DrD2* may thus indicate altered dopamine signaling. In reviewing the literature, no studies were found that directly measured dopamine output in the NAc after an acute fast in adult rats. One study indicated decreased basal extracellular dopamine in the striatum of acutely food-deprived neonatal rats (Ishida, Nakajima, & Takada, 1997), and Pages et al. (1993) indicated no changes in striatal dopamine content in 48- or 72 hour food deprivation, but both did not report on VTA or NAc dopamine content, where dopamine levels have been shown to increase after feeding (Yoshida et al., 1992).

The DrD2 primer used in this study anneals to the long form of this receptor, which is the post-synaptic signaling variant (Giros et al., 1989; Monsma, McVittie, Gerfen, Mahan, & Sibley, 1989). However, the short isoform, which encodes a pre-synaptic auto-receptor, dominates in the dopaminergic cells of the VTA (Ford, 2014; Giordano, Satpute, Striessnig, Kosofsky, & Rajadhyaksha, 2006), and is the main dopamine receptor involved in the regulation of dopamine release in the NAc (Anzalone et al., 2012). Indeed, increased local dopamine release in the VTA after acute deprivation has been reported in mice as well as changes in DrD2-evoked inhibitory postsynaptic currents (IPSCs) in VTA neurons, which may lead to decreased dopamine output in the projection areas (Elsworth & Roth, 1997; Roseberry, 2015). Behaviorally, midbrain DrD2 auto-receptor knockdown by short-hairpin mRNAs or knockout increases the motivation for sucrose pellets (Bello et al., 2011; de Jong et al., 2015). Though technically challenging, future studies could assess whether fasting affects expression of the DrD2 auto-receptor in the VTA, and the functional implications of decreased *DrD2* mRNA for the long isoform.

Apart from *TH* and *DrD2* mRNA, we also observed trends for decreased *Synuclein alpha* and *DAT* mRNA in the VTA of food-deprived rats at the end of the light period. Synuclein alpha is involved in the regulation of dopamine transmission and biosynthesis, and

the DAT is the major mechanism for uptake of dopamine from the synaptic cleft in the NAc and VTA (Butler, Sambo, & Khoshbouei, 2017). Decreased *TH*, *DrD2*, and *DAT* mRNA may together point to decreased dopaminergic output in food deprived-conditions, and decreased *Synuclein alpha* might indicate decreased dopamine biosynthesis, which would be similar to the effects of chronic food restriction on the dopamine system (K. D. Carr, 2007). Interestingly, all changes in dopamine-related gene expression were found at the end of the light period. Dopamine activity shows a circadian rhythmicity with a tendency towards increased activity during the dark period when rodents are active (Webb, Lehman, & Coolen, 2015). Fasting thus affects the dopaminergic machinery when it is becoming active, whereas the effects of fasting are not apparent when the dopamine system is relatively silent.

Interestingly, a previous study indicated decreased *DAT* mRNA in the VTA/substantia nigra area in food-deprived rats, which was associated with decreased DAT activity in the dorsal striatum (Patterson et al., 1998). The dorsal striatum is targeted preferentially by the substantia nigra as opposed to the VTA, and changes in *DAT* mRNA levels may thus be apparent only in the substantia-nigra->dorsal striatum projection, and, possibly, only in the VTA dopaminergic cells that project towards the dorsal striatum. Indeed, no changes in DAT functionality were found in the NAc (Patterson et al., 1998). In addition, fasting-induced changes in DAT functionality were most likely mediated through posttranslational effect on DAT protein (Patterson et al., 1998). Future studies could look into projection-specific changes in TH enzymatic activity, posttranslational changes in the DrD2 auto-receptor and the DAT, and how these changes correlate with changes in physiological factors and behavior.

Acute fasting: a role for the VTA GABAergic system?

Our data also suggest a change in the GABAergic system of the VTA, with a trend for decreased *GAD67* mRNA levels in the VTA after food deprivation at the end of the light period. GAD65 and GAD67 are encoded by different genes (Erlander, Tillakaratne, Feldblum, Patel, & Tobin, 1991), and though both encode the rate-limiting enzyme in the synthesis of GABA, they are thought to have different functional roles. GAD65 is concentrated in the nerve terminal and synthesizes GABA for neurotransmission (Martin & Rimvall, 1993), whereas GAD67 is spread evenly throughout the neuron and thought to have various functions including the synthesis of GABA for energy via the GABA shunt of the tricyclic-acid cycle, and as a regulator of redox potential during oxidative stress (Pinal & Tobin, 1998; Waagepetersen, Sonnewald, & Schousboe, 1999). Interestingly, the GAD67 promoter is flanked by sequences that are sensitive to insulin levels (Pedersen, Videbaek, Skak, Petersen, & Michelsen, 2001). In addition, though the glucose supply to the brain is protected as it is the most important fuel for the brain, a 24-hour fast may increase dependence on ketone bodies, which can affect GABA levels (A. A. Morris, 2005). Indeed, exposure to a ketogenic diet or chronic caloric restriction altered GAD65/67 levels in various brain regions, but the VTA or NAc were not

specifically measured (C. M. Cheng, Hicks, Wang, Eagles, & Bondy, 2004). Future studies could determine the implications of decreased VTA *GAD67* mRNA, whether this change is also reflected at the protein level, and for which metabolic or intracellular functions it is relevant.

A short period of food deprivation marginally affects opioid gene expression

Our data indicate that a 24-hour period of food deprivation only significantly decreased Arc mRNA for the *delta*-opioid receptor, and a trend for decreased *ppDyn* was observed. No significant changes in any of the other opioid system components in the Arc, LHA or the VTA and NAc of the reward system were found. Previous studies in rodents had indicated that Arc *POMC* mRNA might be decreased after 24 hours of food deprivation, however not all studies report this effect (Kim et al., 1996; Lauzurica, Garcia-Garcia, Pinto, Fuentes, & Delgado, 2010; Mizuno et al., 1998). Here, we also do not find altered Arc *POMC* mRNA after a short acute period of deprivation. After longer deprivation Arc *ppDyn* mRNA was also decreased (Kim et al., 1996). However, no change was seen in opioid receptor mRNA levels in the Arc or LHA after 24 hours of food deprivation (Barnes et al., 2008), but increased *mu*-opioid receptor mRNA levels were found in both regions after longer food deprivation (Barnes et al., 2008). Importantly, these rats could consume a high-fat diet in addition to standard chow, which may limit comparison (Barnes et al., 2008). In contrast, Ikeda et al. (2015) report decreased *POMC*, *ppENK*, and *ppDyn* mRNA levels in whole hypothalamic of male mice after only 16 hours of food deprivation. The discrepancy between these data and ours could be the result of differences in experimental setups such as timing, deprivation duration, dietary factors or experimental techniques, which have all been shown to play a role in determining the effect of fasting on opioid peptide levels (McLaughlin, Baile, & Della-Fera, 1985; Przewlocki et al., 1983; Takahashi et al., 1986).

The effect of fasting on the opioid system in the reward-related circuitry has received less attention than the hypothalamus although it has been reported that pharmacological manipulation of the opioid-receptors in the NAc and VTA can alter food intake (Ikeda et al., 2015; Noel & Wise, 1995; M. Zhang et al., 1998), and 16 hours of food deprivation was shown to decrease *POMC*, *ppENK*, and *ppDyn* mRNA levels in the midbrain of mice (Ikeda et al., 2015). However, our data indicate that 24 hours of food deprivation does not alter opioid-system-related gene expression in the VTA or the NAc either at the beginning or end of the light period in male Wistar rats. Interestingly, Scheggi et al. (2017) indicate that mu-opioid receptor signaling in the NAc is altered in 18-hour food-deprived mice. We therefore suggest that future studies focus on posttranslational modifications in opioid-receptor signaling in response to food deprivation.

Technical considerations

In this study, RTqPCR was used to measure gene expression in brain punches of the Arc, LHA, VTA, and NAc. There are of course several limitations to the use of gene expression measurements. As mentioned above, adaptive changes in neuronal activity or circuitries might be established at the protein level, which allows for more rapid modifications. In addition, though RT-qPCR is a very reliable and sensitive technique (Bustin, 2000), the use of brain punches decreases the spatial resolution and RT-qPCR limits the number of genes that can be tested efficiently. Here, the selection of genes was chosen based on regional expression levels. Assessment of posttranslational modifications are therefore warranted, and single-cell microarray analysis could provide more extensive information on gene expression alterations after acute food deprivation.

Of particular interest, the reference genes chosen in this study showed modulation by time of day effects, which precludes analysis of absolute gene expression changes between data from two time points. Therefore, time of day effects and possible interaction effects with fasting are therefore inaccessible in our data set. The dopamine and opioid system as well as other physiological responses to fasting show circadian fluctuations; therefore, future studies should identify reference genes that are not regulated by fasting or time of day to enable these analyses.

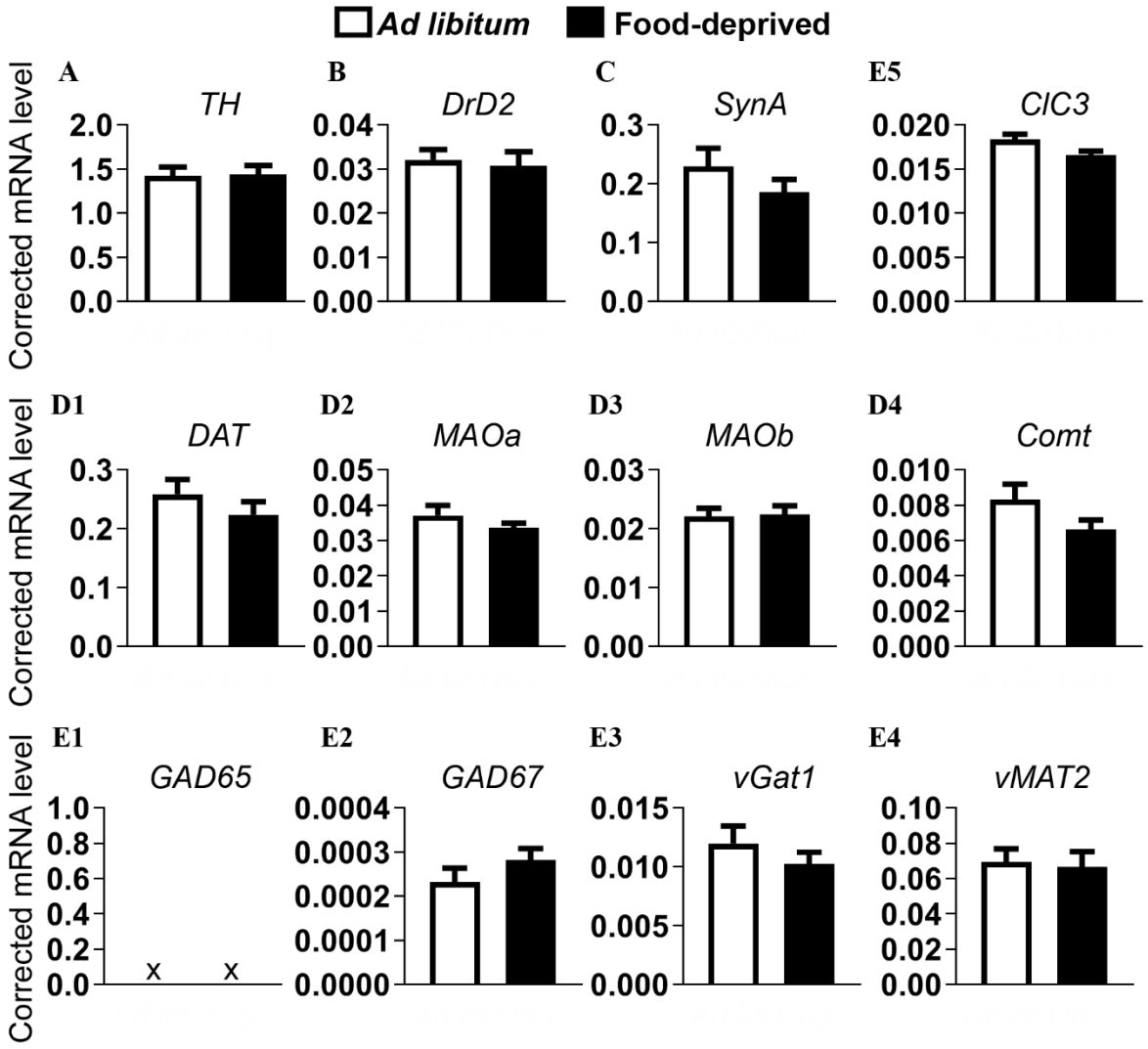
Summary

This study investigated the effects of short acute deprivation on principal dopamine- and opioid-related gene expression in the VTA and NAc of the reward system. Our data show that acute deprivation decreases *TH* and *DrD2* mRNA levels in the VTA, and point to decreased dopaminergic output, as is seen after chronic food restriction. Dopamine-related gene expression in the NAc was unaffected, as was opioid gene expression in the VTA and NAc. Fasting causes a variety of physiological changes, including a stress response, therefore further studies are required to determine via which pathways fasting changes dopamine-system related gene expression, and the functional implications of these changes for adaptive behaviors.

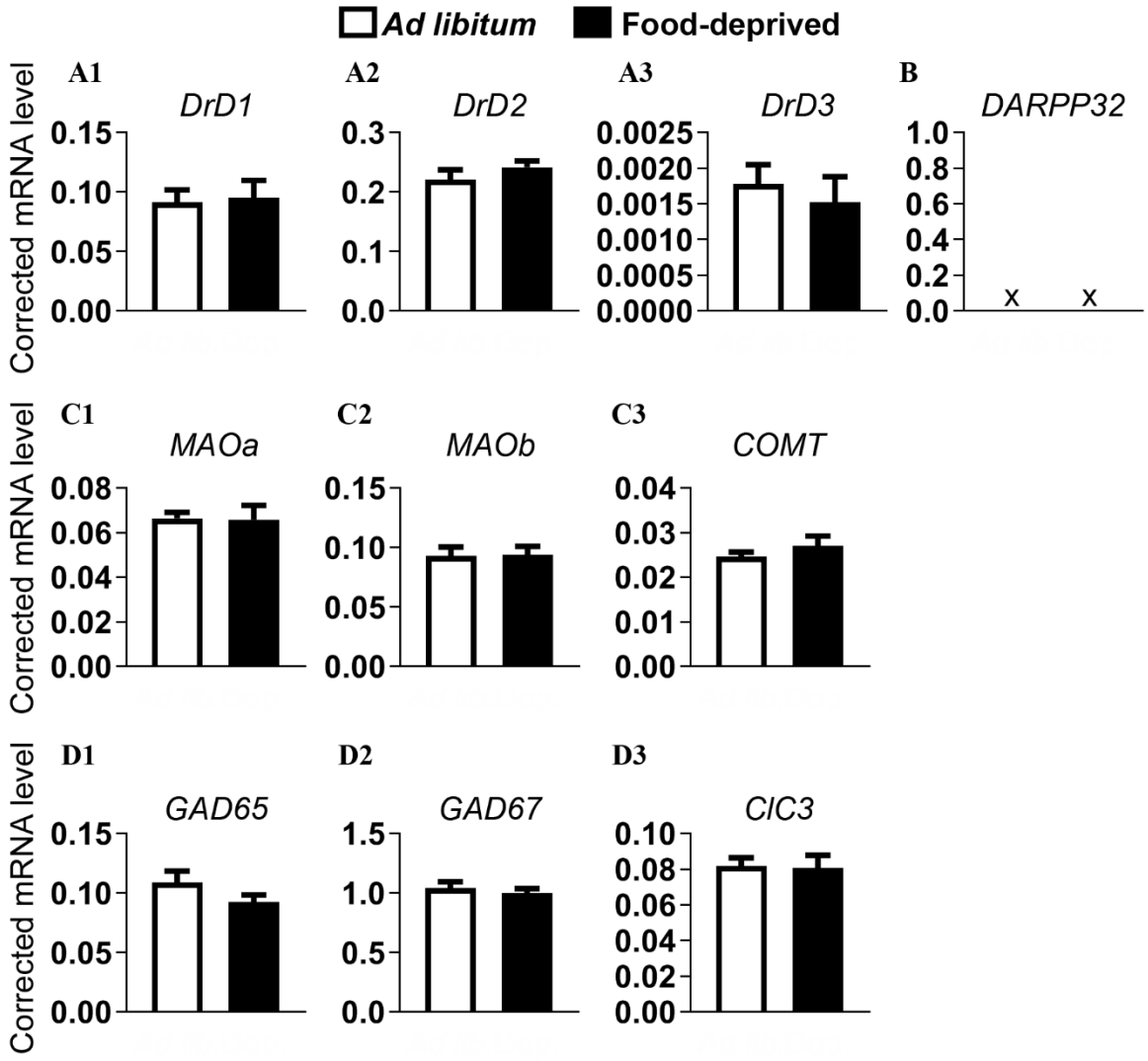
Acknowledgements

Myrtille Gumbs designed the experiment. Myrtille Gumbs, Ewout Foppen, Unga Unmehopa, and Khalid Lamuadni performed the experiments. Myrtille Gumbs prepared the manuscript. Joram Mul, Jan Booij, and Susanne la Fleur reviewed the manuscript.

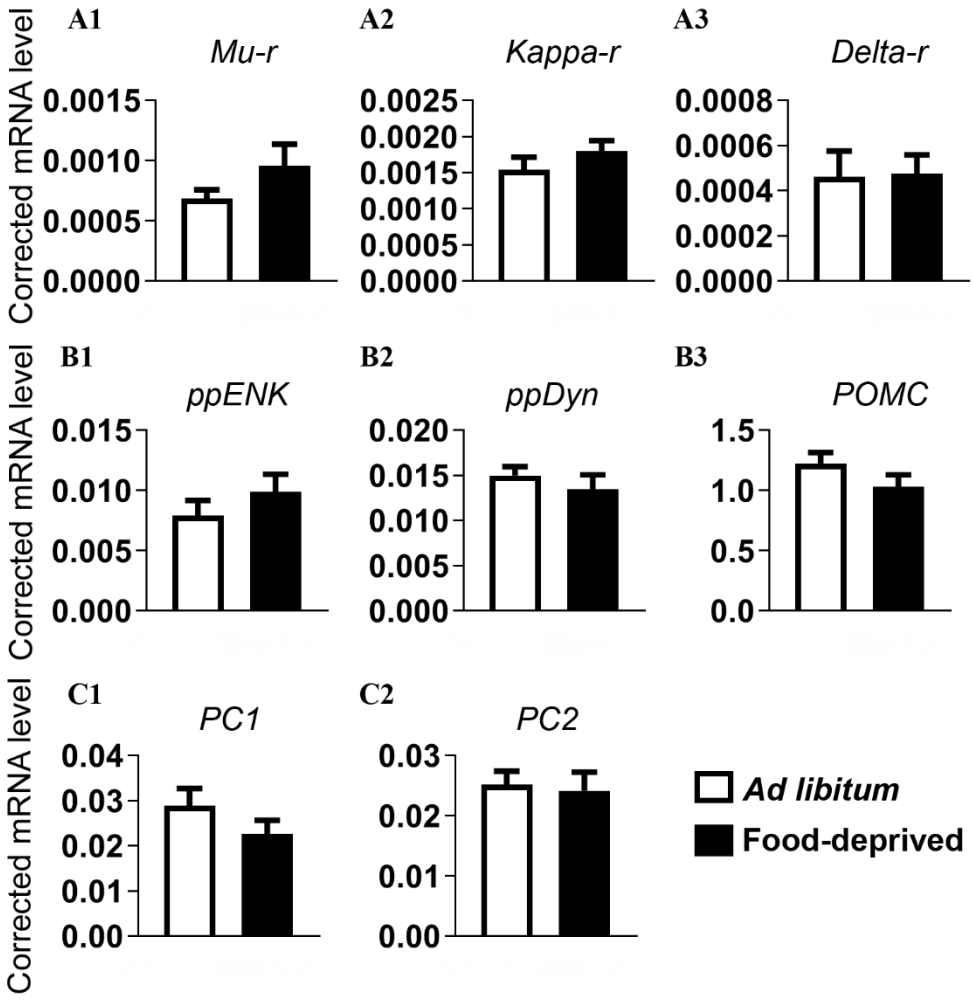
Supplemental figures and tables



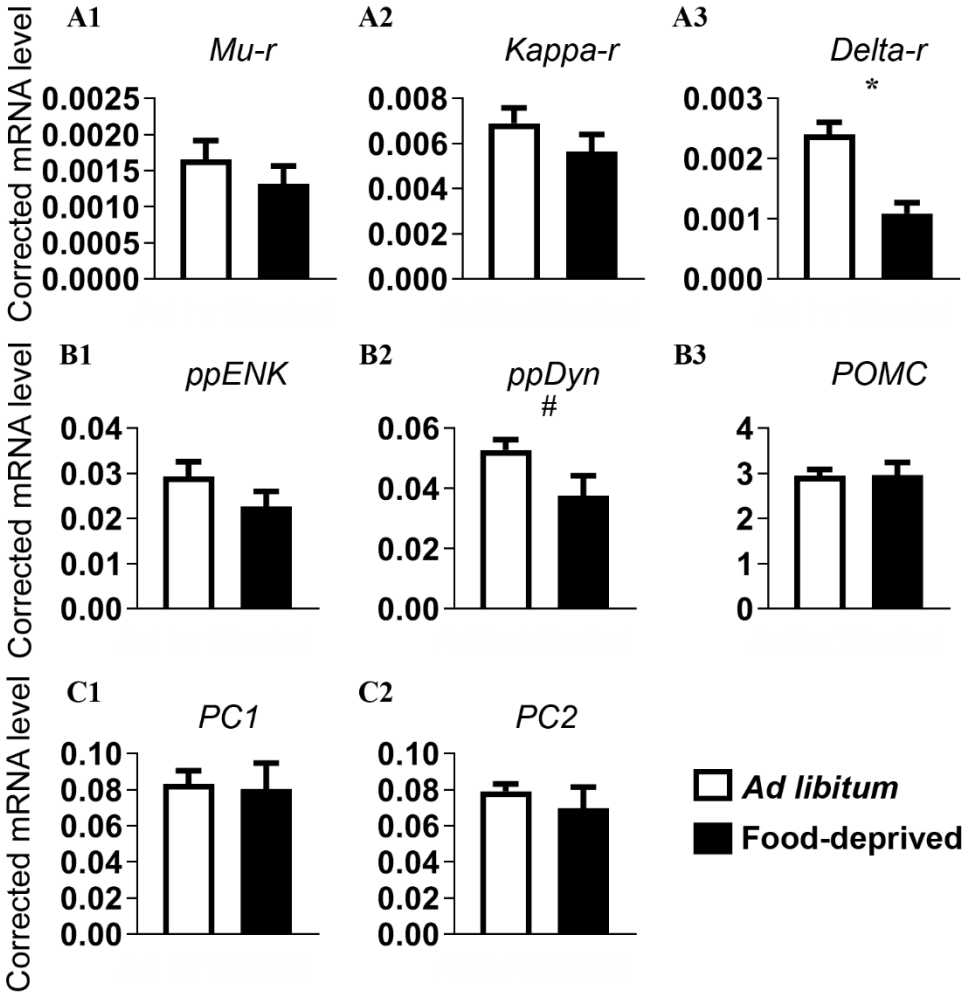
Supplemental Figure 1. A 24-hour fast does not affect VTA dopaminergic gene expression at ZT3. Food deprivation did not affect gene expression of the **A-D4)** dopamine-related, or **E1-E5)** GABAergic genes tested. **E1)** Gene expression for *GAD65* could not be measured at this time point. Gene expression levels for *Drd1*, *DrD3*, and *DrD4* are not shown as expression levels were at detection level. See text for details and Table 1 for abbreviations.



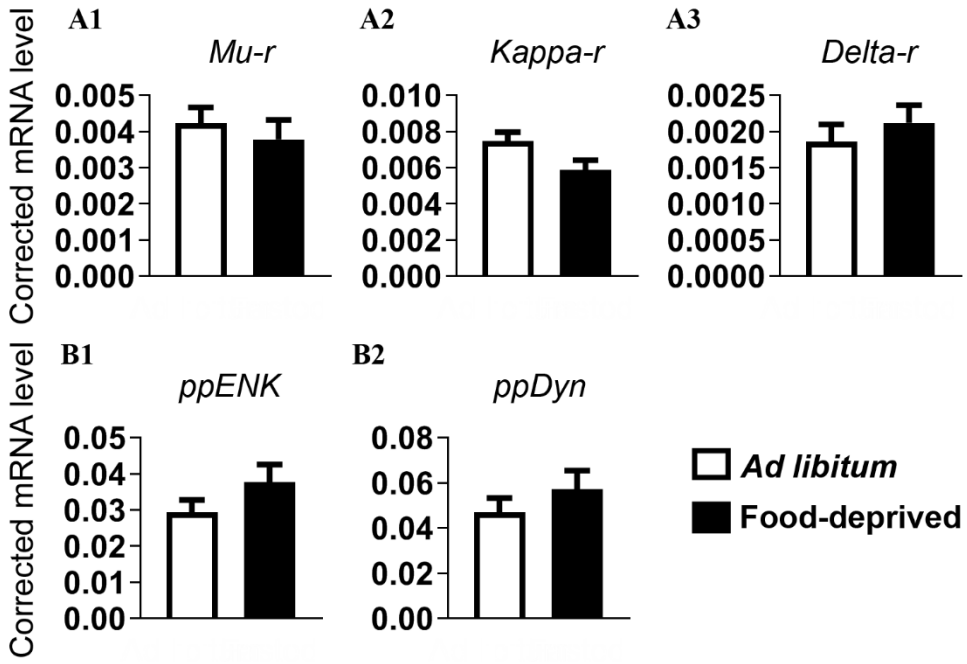
Supplemental Figure 2. A 24-hour fast does not affect NAc dopaminergic gene expression at ZT3. Food deprivation did not affect gene expression of the **A-C3)** dopamine-related, or **D1-D3)** GABAergic genes tested. **B)** Gene expression for *DARPP32* could not be measured at this time point. Gene expression for *DrD4* is not shown as expression level was at detection level. See text for details and Table 1 for abbreviations.



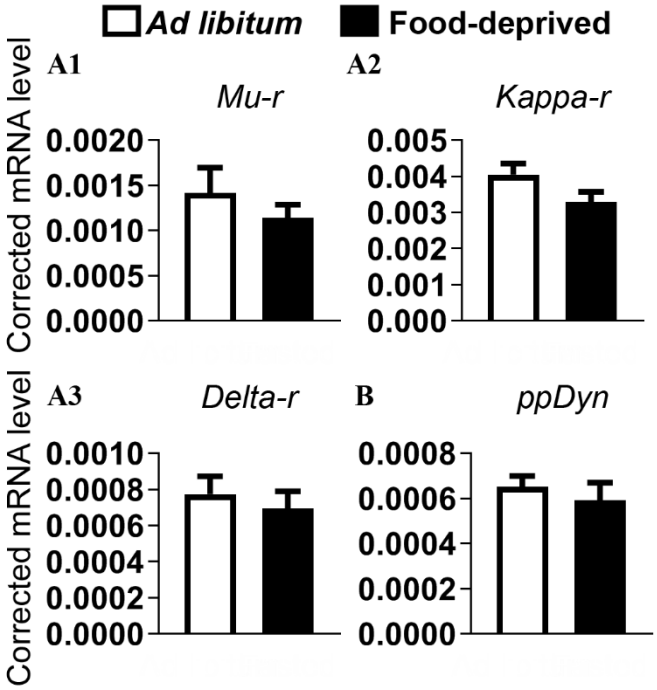
Supplemental Figure 3. A 24-hour fast does not affect Arc opioid gene expression at ZT10. Food deprivation did not affect gene expression of the **A1-A3)** opioid receptors, **B1-B3)** opioid peptide precursor mRNA, or **C1-C2)** mRNA for the cleavage-enzymes for POMC. See text for details and Table 1 for abbreviations.



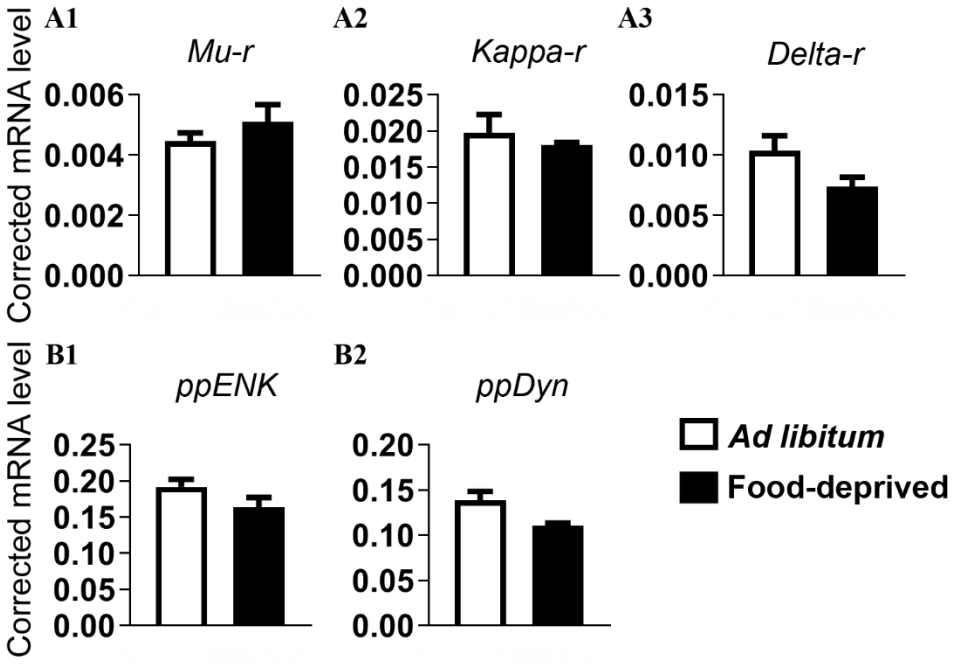
Supplemental Figure 4. A 24-hour fast does not affect Arc opioid gene expression at ZT3. Food deprivation did not affect gene expression of the **A1)** *Mu-*, or **A2)** *Kappa*-opioid receptor, but **A3)** decreased *Delta*-opioid receptor mRNA ($p = 0.03$). **B1, B3)** Opioid peptide precursor mRNA for *enkephalin* or *POMC* was not affected by food deprivation, but **B2)** opioid peptide precursor mRNA for *dynorphin* showed a trend to decrease after food deprivation ($p = 0.1$). **C1-C2)** mRNA for the cleavage-enzymes for POMC were also unaffected by food deprivation. See text for details and Table 1 for abbreviations. * $p_{adjusted} < 0.05$, # $p_{adjusted} < 0.1$ and $p_{unadjusted} < 0.05$.



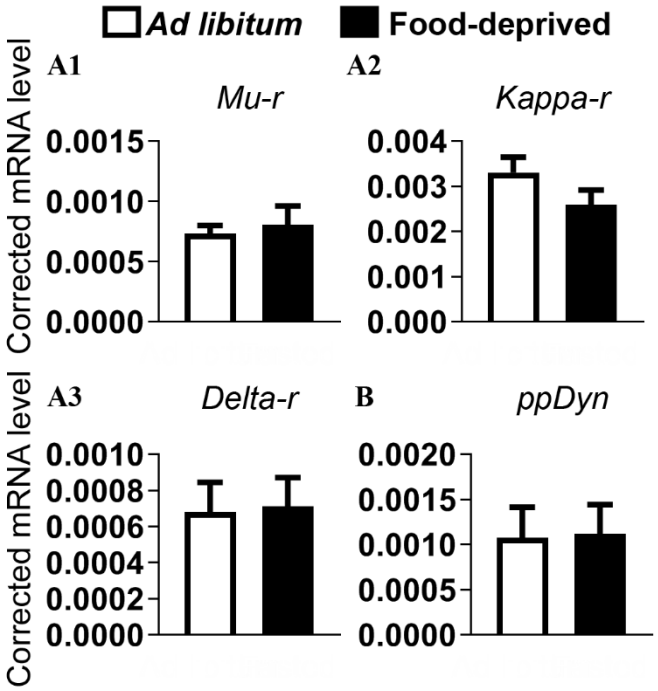
Supplemental Figure 5. A 24-hour fast does not affect LHA opioid gene expression at ZT3. Food deprivation did not affect gene expression of the **A1-A3)** opioid receptors, or of **B1-B2)** opioid peptide precursor mRNA. See text for details and Table 1 for abbreviations.



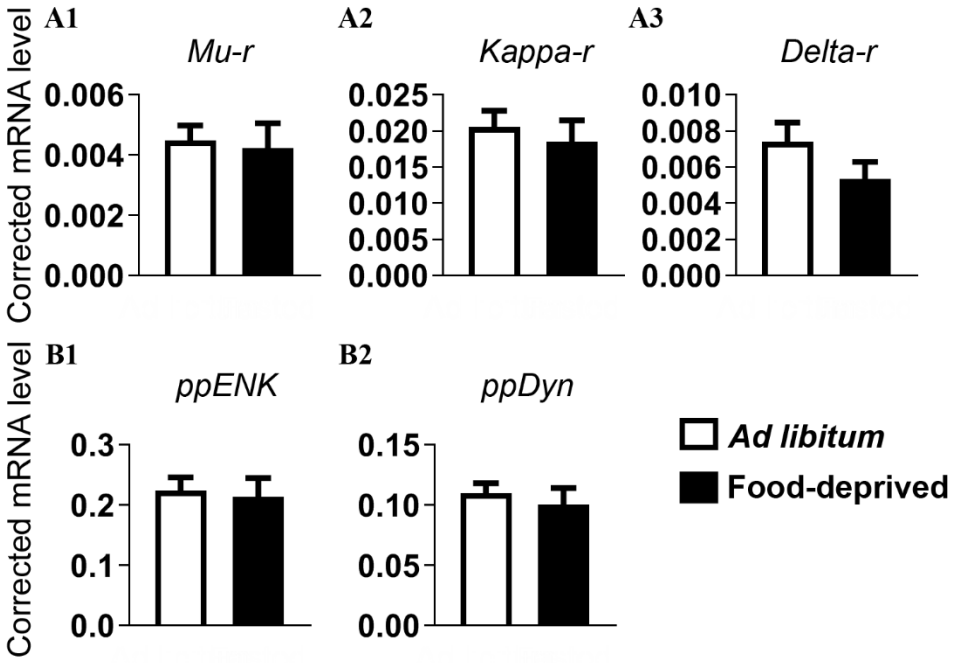
Supplemental Figure 6. A 24-hour fast does not affect VTA opioid gene expression at ZT10. Food deprivation did not affect gene expression of the **A1-A3)** opioid receptors, or of **B1-B2)** opioid peptide precursor mRNA for dynorphin. See text for details and Table 1 for abbreviations.



Supplemental Figure 7. A 24-hour fast does not affect NAc opioid gene expression at ZT10. Food deprivation did not affect gene expression of the **A1-A3)** opioid receptors, or of **B1-B2)** opioid peptide precursor mRNA for enkephalin and dynorphin. See text for details and Table 1 for abbreviations.



Supplemental Figure 8. A 24-hour fast does not affect VTA opioid gene expression at ZT3. Food deprivation did not affect gene expression of the **A1-A3)** opioid receptors, or **B1-B2)** opioid peptide precursor mRNA for *dynorphin*. See text for details and Table 1 for abbreviations.



Supplemental Figure 9. A 24-hour fast does not affect NAc opioid gene expression at ZT3. Food deprivation did not affect gene expression of the **A1-A3)** opioid receptors, or **B1-B2)** opioid peptide precursor mRNA for *enkephalin* and *dynorphin*. See text for details and Table 1 for abbreviations

Supplemental Table 1. Adjusted p-values for dopamine-related gene expression per region

	At the end of the light period		At the beginning of the light period	
Gene	**VTA**	**NAc**	**VTA**	**NAc**
TH	**p = 0.03 (7/8)*# ↓**	-	p = 1 (6/8)	-
DrD1	d.l.	p = 1 (7/7)	d.l.	p = 1 (5/4)
DrD2	**p = 0.048 (7/8)* ↓**	p = 1 (7/7)	p = 1 (7/8)	p = 1 (5/4)
DrD3	d.l.	p = 1 (6/7)	d.l.	p = 1 (5/4)
DrD4	d.l.	d.l.	d.l.	d.l.
DAT	p = 0.48 (7/8)	-	p = 1 (7/8)	-
COMT	p = 1 (7/8)	p = 1 (7/7)	p = 1 (7/8)	p = 1 (5/4)
MAOa	p = 0.61 (7/8)	p = 1 (7/7)	p = 1 (7/8)	p = 1 (5/4)
MAOb	p = 0.57 (7/8)	p = 1 (6/7)	p = 0.96 (7/8)	p = 0.90 (5/4)
DARPP32	-	p = 1 (7/8)	-	p = 1 (5/4)
Synuclein A	p = 0.1 (7/8)	-	p = 1 (7/8)	-
GAD65	p = 1 (6/8)	p = 1 (5/6)	p = 1 (7/8)	p = 1 (4/4)
GAD67	p = 0.09 (5/7)	p = 1 (6/7)	p = 1 (7/7)	p = 1 (5/4)
vGat1	p = 1 (6/8)	-	p = 1 (7/7)	-
ClC3	p = 1 (7/8)	p = 1 (7/6)	p = 0.27 (7/8)	p = 1 (5/4)
vMAT2	p = 0.66 (7/7)	-	p = 1 (6/8)	-

#(N[*ad libitum*]/N[food-deprived]), * = statistically significant, ↓ = decreased compared to *ad libitum*-fed rats, d.l. = at detection level, DAT/Slc6A3, vGat1/SLc32A1, vMAT2/Slc18A2.

Supplemental table 2. Adjusted p-values for opioid gene expression per region.

Gene	**Arc**	**LHA**	**VTA**	**NAc**
At the end of the light period				
Mu-r	p = 1 (8/8)#	-	p = 0.38 (8/8)	p = 0.56 (5/4)
Kappa-r	p = 1 (8/8)	-	p = 0.42 (8/8)	p = 0.97 (4/4)
Delta-r	p = 0.84 (6/7)	-	p = 0.66 (6/8)	p = 0.44 (5/4)
ppENK	p = 1 (8/8)	-	-	p = 0.57 (5/4)
ppDyn	p = 1 (8/8)	-	p = 0.98 (8/8)	p = 0.16 (5/4)
POMC	p = 1 (8/8)	-	-	-
PC1	p = 1 (8/8)	-	-	-
PC2	p = 0.84 (8/8)	-	-	-
At the beginning of the light period				
Mu-r	p = 1 8/8)	p = 1 (8/7)	p = 1 (7/8)	p = 1 (8/8)
Kappa-r	p = 0.72 (8/7)	p = 0.36 (8/7)	p = 0.75 (7/8)	p = 1 (8/7)
Delta-r	**p = 0.03 (6/5)* ↓**	p = 0.65 (8/8)	p = 0.94 6/6)	p = 1 (8/8)
ppENK	p = 0.65 (8/8)	p = 0.33 (8/8)	-	p = 0.88 (8/8)
ppDyn	p = 0.14 (8/8)	p = 1 (8/8)	p = 1 (7/8)	p = 1 (8/8)
POMC	p = 0.96 (7/8)	-	-	-
PC1	p = 1 (8/8)	-	-	-
PC2	p = 1 (8/8)	-	-	-

#(N[*ad libitum*]/N[food-deprived]), * = statistically significant, ↓ = decreased compared to *ad libitum*-fed rats.