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Involvement of cocaine–amphetamine regulated transcript in the differential feeding responses to nociceptin/orphanin FQ in dark agouti and Wistar Ottawa Karlsburg W rats

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ABSTRACT

Wistar Ottawa Karlsburg W (WOKW) rats and their controls, dark agouti (DA), present different features: in particular, DA rats are lean, while the WOKW are obese and present symptoms of hypertension, dyslipidemia, hyperinsulinemia, and impaired glucose tolerance. The present study tested the hypothesis that these two strains would demonstrate different sensitivity to nociceptin/orphanin FQ (N/OFQ). N/OFQ was injected into the lateral brain ventricle (LBV) of sated DA and WOKW rats, and corticosterone levels in both strains were measured after LBV injection of N/OFQ. LBV N/OFQ injections dose-dependently produced a significant increase in food intake (4 h) in DA rats, but not in WOKW. However, corticosterone levels were increased by N/OFQ to a greater degree in WOKW than in DA rats. Gene sequencing and gene expression of ORL1 receptor and cocaine–amphetamine regulated transcript (Cart) peptide were evaluated to study the difference in N/OFQ-induced feeding behavior in the two strains. WOKW rats had a different amino acid sequence of Cart peptide and a significantly higher expression of Cart in the hypothalamus. The present data show that DA and WOKW rats demonstrate different sensitivity to N/OFQ, and suggest that Cart peptide might be the underlying mechanism of this difference.

1. Introduction

There is strong evidence that the feeding responses to central injection of orexigenic or anorectic neuropeptides are different in lean and obese rats [3,5,24] as well as in lean and obese mice [25].

Within the neuropeptide family, nociceptin/orphanin FQ (N/OFQ), the endogenous ligand for the NOP receptor, previously referred to as opioid receptor-like 1 receptor (ORL1) or OP4 receptor [18,21], has also been shown to increase food intake in normal rats [19,20], while antisense oligodeoxynucleotides directed against either exons 1, 2 or 3 of the ORL1/KOR-3 clone reduces N/OFQ-induced hyperphagia [13]. No data are available so far on how the N/OFQ system affects feeding behavior in obese rats. In normal rats, N/OFQ-containing neurons and NOP receptors are widely distributed in the brain, especially in hypothalamic areas that regulate food intake [14]. These observations provide a solid neuroanatomical foundation in support of the notion that N/OFQ might regulate feeding behavior through central mechanisms. Our previous papers have suggested that N/OFQ might elicit feeding behavior through inhibition of an inhibitory mechanism [22]. These findings are partially confirmed by Bewick, who showed that N/OFQ produces a decrease in cocaine–amphetamine regulated transcript (Cart) release; this peptide decreases food intake in rodents and has been shown to

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mediate leptin anorexic effects [9]. Other data further support that N/OFQ is present in the arcuate nucleus, as well as Cart. So we can hypothesize that N/OFQ could work through the inhibition of the release of Cart.

Phenotypic characterization after more than 50 generations of the Wistar Ottawa Karlsburg W (WOKW) rats has shown that these animals develop the main features of the metabolic syndrome, such as moderate hypertension, dyslipidemia, hyperinsulinemia, obesity, and impaired glucose tolerance [8,26,27,29]. On the other hand, the dark agouti (DA) strain does not show any of these characteristics and has been considered as the control strain for the WOKW rats [28]. Indeed, a cross-sectional study with DA rats confirmed that WOKW rats provide a good animal model expressing the metabolic syndrome [26]. WOKW, compared to DA rats, show hyperphagia, are heavier and fatter [29]. The WOKW animals are especially useful because their metabolic syndrome is under polygenic control, as in humans, and not due to a single gene mutation [12].

The present study evaluated the hypothesis that the two strains would respond differently to the hyperphagic effect of N/OFQ and investigated the underlying mechanism of the different responses.

2. Materials and methods

DA/K (>F80) and WOKW rats (F69) from the Department of Laboratory Animal Science of the University of Greifswald (Karlsburg, Germany), were individually housed in the animal facility of the Department of Experimental Medicine and Public Health of the University of Camerino, in hanging stainless-steel cages with grid floors in a room with an artificial 12:12 h light/dark cycle (dark onset at 9:00 p.m.) and a constant temperature of 20–22 °C. They were offered free access to chow pellets (Mucedola Diets, Settimo Milanese, Italy) in hoppers hung on the cage wall and to tap water at libitum.

Rats were food deprived overnight and anesthetized by intramuscular injection of Tiletamine chlorahydrate (200 mg/kg) and Zolazepam chlorahydrate (200 mg/kg) (Laboratoires Virbac, Carros, France). A prophylactic dose of Rubrocillin 200 μl/rat (Farmaceutici Gellini Spa, Aprilia, Italy) was also given by intramuscular injection. Then, a 22-gauge guide cannula for intracerebroventricular (ICV) injections was stereotaxically implanted into the right lateral ventricle (LV): 1 mm posterior to the bregma, 1.8 mm lateral to the sagittal suture and 2 mm ventral to the surface of the skull. A stainless-steel obturator of the same length was placed into the guide cannula at the end of surgery. A 30-gauge injector that was 2.5 mm longer than the guide cannula was used for ICV injections. The volume injected into the LV was of 1 μl.

One week after intracranial surgery, all the animals were ICV treated with saline to familiarize them with the ICV injection.

2.1. Experiment 1: effect of ICV injection of N/OFQ on food intake in WOKW and DA rats

A week after the ICV familiarization, N/OFQ (0, 2.1, 4.2 or 8.4 nmol/rat) was injected into both strains (at 9:00 a.m.) and the animals' food intake was frequently measured using scales with a precision of 0.1 g for the following 24 h (Table 1).

Table 1 – Mean body weight (g) of WOKW and DA rats at the beginning of the experiments

WOKW	370 ± 4.8
DA	259 ± 6.4
LEW.1W	255 ± 3.5
F344	250 ± 4.0
BB/OK	265 ± 3.7
SHR	250 ± 6.1
KWR	258 ± 3.7
HTG	253 ± 4.4

2.2. Experiment 2: effect of ICV injection of SHU 9119 on food intake in WOKW and DA rats

The same DA and WOKW group of rats used for N/OFQ injection was injected 2 weeks later with SHU 9119 (0 and 1 nmol/rat) at 9:00 a.m. and the animals' food intake was measured at 4, 12, and 24 h.

2.3. Experiment 3: effect of ICV injection of N/OFQ on plasma corticosterone levels in WOKW and DA rats

Since it has been shown that N/OFQ hyperphagia is due to the increase of blood corticosterone [11,16], plasma corticosterone levels (PCL) were measured. Intracerebroventricular injection of N/OFQ (8.4 μg/rat) was given to the animals that had been used previously for feeding studies and thus were not naive to N/OFQ injections; 30 min later, blood samples (500 μl) were collected by cutting the tip of the tail of both animal strains. Corticosterone levels were measured using the CAYMAN Corticosterone EIA Kit (INALCO S.P.A., Italy).

2.4. Experiment 4: gene sequencing of the nociceptin/orphanin FQ receptor (Oprl1 gene) and Cart in WOKW and DA rats

The gene sequencing of the NOP receptor (still called the *Oprl1* receptor in the NCBI GenBank data base) and *Cart* was performed to determine whether *Cart* is involved in the mechanism of action of the N/OFQ hyperphagic effect, as suggested in a recent study [1]. Genomic DNA of one male and one female WOKW and DA rat was extracted from liver tissue using a commercially available DNA isolation kit (Wizard®, Genomic DNA Purification Kit, Promega, Mannheim, Germany). To identify genetic variants, DNA was sequenced according to the standard protocol of the ABI PRISM® BigDye Terminators v3.0 (Applied Biosystems, Foster City, CA, USA) as recently described [7]. Primers were designed to perform PCR experiments based on published *Oprl1* sequence (GenBank acc. no.: NC_005102; forward 5′-atgctcatggaggcgttc-3′ and reverse 5′-gaatttatctgcagctccaagc-3′;) and *Cart* sequence (GenBank acc. no.: NC_005101; forward 5′-aagtccagcaccatggagag-3′ and forward 5′-gatgcaaggatctgggtga-3′, and reverse 5′-ttcagccacctgctagagtaaa-3′, and reverse 5′-ttgcacacataccaacacc-3′).

BioBreding Ottawa Karlsburg (BB/OK) rats, spontaneous hypertensive rats (SHR), Lewis 1W rats (LEW.1W) (animals which are not obese) [10], Fisher 344 (F344) rats, Karlsburg wild

Table 2 – Gene sequencing of the nociceptin/orphanin FQ receptor (*Oprl1* gene)

Rat strains	Position Intron 897	Change SNP	Position Intron 898	Change SNP	Position Intron 4145	Change SNP
BB/OK	TTCTT	T → C	TTCTT	C → T	CCCAT	C → T
SHR	TTCTT		TTCTT		CCCAT	
WOKW	TCTTT		TCTTT		CCCAT	
DA	TTCTT		TTCTT		CCCAT	
LEW.1W	TTCTT		TTCTT		CCCAT	
F344	TTCTT		TTCTT		CCCAT	
KWR	TTCTT		TTCTT		CCCAT	
HTG	TTCTT		TTCTT		CCTAT	

Gene: *Oprl1*; location: Chr.3; 3q43; and accession no.: NC_005102.

Table 3 – Gene sequencing of *Cart*

Rat strains	Position Intron 499	Change SNP	Position Intron 510	Change SNP	Position Intron 526	Change SNP	Position Exon 1560	Change AS
BB/OK	CTACC	A → T	TTGTA	G → C	CCCTA	C → T	CTT	*Leu → Pro*
SHR	CTACC		TTGTA		CCTTA		CCT	
WOKW	CTTCC		TTCTA		CCCTA		CCT	
DA	CTACC		TTGTA		CCTTA		CTT	
LEW.1W	CTTCC		TTCTA		CCCTA		CCT	
F344	CTACC		TTGTA		CCCTA		CTT	
KWR	CTACC		TTGTA		CCCTA		–	
HTG	CTACC		TTGTA		CCCTA		–	

Gene: *Cart*; location: Chr.2; 2q12; and accession no.: NC_005101.

rats (KWR) [28], and hypertriglicemic (HTG) rats [26] of the same age were also used to compare the gene sequence of the nociceptin/orphanin FQ receptor and the *Cart* gene.

2.5. *Experiment 5: gene expression of the nociceptin/orphanin FQ receptor (Oprl1 gene), Pnoc and Cart in WOKW and DA rats*

Gene expression of the NOP receptor (*Oprl1*), prepronociceptin (*Pnoc*), and *Cart* peptide was performed on the hypothalamus and in adipose tissue.

Gene expression analysis was conducted on seven males of each strain (see Tables 2 and 3, respectively) at an age of 4 weeks. At the time of euthanasia, the hypothalamus and the subcutaneous and epididymal fat pads were removed. The total RNA of organs was isolated, transcribed in cDNA and used for real-time PCR as detailed before [6]. Each quantitative PCR was performed in duplicate. Target cDNA was amplified by primer sets of *Oprl1* (GenBank acc. no.: NC_005102; forward 5′-tgtatggcagccactttcaa-3′ reverse 5′-agtccaaggggcaggaag-3′), *Cart* (GenBank acc. no.: NC_005101; forward 5′-ccctactgctgctgctacct-3′ reverse 5′-ttctcatgggacgcatcat-3′), and of *Pnoc* (GenBank acc. no.: NM_013007; forward 5′-ctctctggactctttgcacca-3′ reverse 5′-cgaggctttcgactggtaaa-3′).

The rat 18S rRNA gene (eukaryotic 18S rRNA endogenous control; FAM™ Dye/MGB Probe, Applied Biosystems) served as the endogenous reference gene. The standard curve method was used for relative quantification. For each experimental sample, the amount of targets and the endogenous reference, 18S rRNA, were determined from the calibration curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value.

2.6. *Experiment 6: effect of ICV injection of N/OFQ on food intake in LEW.1W and F344 rats*

Based on the results of experiments 1 and 3 (specifically those of the *Cart* gene expression difference between DA and WOKW), F344 and LEW.1W rats, which have the same modification of the *Cart* gene sequence as the DA and WOKW, respectively, were ICV injected with N/OFQ (0, 2.1, 4.2 or 8.4 nmol/rat) as described in experiment 1.

3. Results

3.1. *Experiment 1: effect of ICV injection of N/OFQ on food intake in WOKW and DA rats*

Basal food intake differed significantly ($p < 0.05$) between both rat strains (Table 4). The ANOVA revealed a significant effect due to the strain factor (between factor) with an $F(1, 20) = 7.98$ ($p < 0.01$) and the dose factor (within factor) with an $F(3, 60) = 5.7$ ($p < 0.005$).

Table 4 – Baseline food intake (g) of WOKW and DA rats

WOKW	28.8 ± 1.0
DA	24.1 ± 0.5

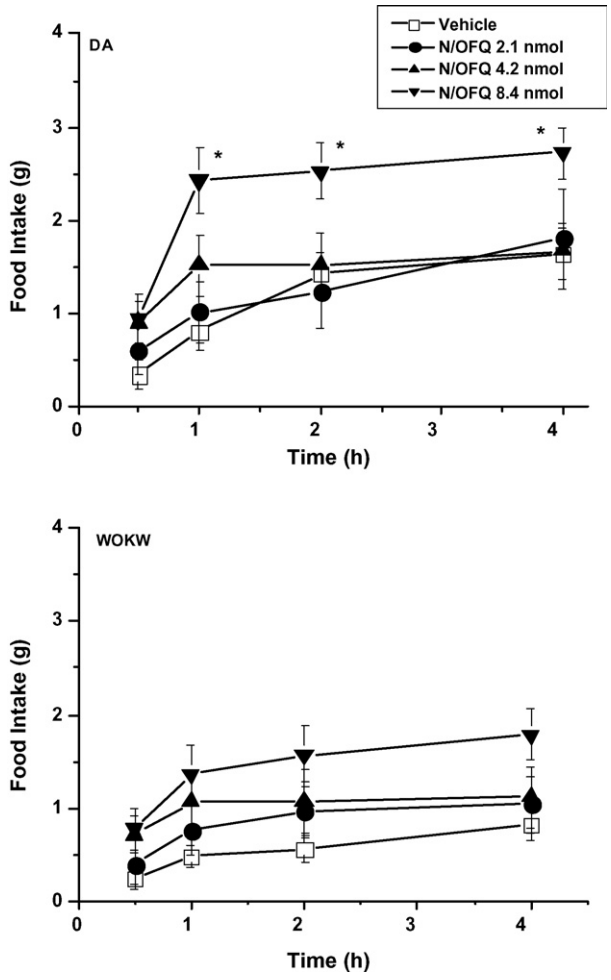

Fig. 1 – Four-hour food intake (g) following injection of N/OFQ (0, 2.1, 4.2 or 8.4 nmol) into the LV of DA and WOKW rats. Data represent the mean ± S.E.M. of 12 DA and 12 WOKW rats, respectively. Difference from controls: $^{*}p < 0.05$; where not indicated, difference was not statistically significant.

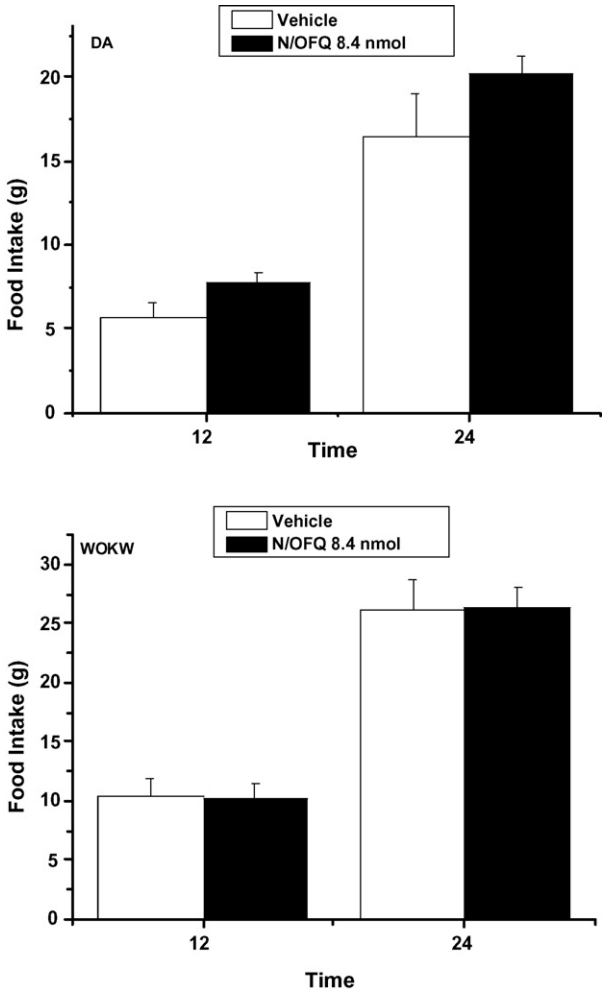

Fig. 2 – Twelve and twenty-four hour food intake (g) following injection of N/OFQ (0 or 8.4 nmol) into the LV of DA and WOKW rats. Data represent the mean ± S.E.M. of 12 DA and 12 WOKW rats, respectively. Difference from controls: $^{*}p < 0.05$; where not indicated, difference was not statistically significant.

When DA rats were injected with N/OFQ (8.4 nmol), they significantly increased their food intake during the first 4 h of observation ($F(3, 30) = 3.55$; $p < 0.05$). ANOVA also demonstrated significance for the time [$F(3, 30) = 34.9$; $p < 0.0001$] and the treatment–time interaction [$F(3, 30) = 2.34$; $p < 0.05$] (Fig. 1). On the other hand, WOKW rats did not significantly respond to any of the tested doses of N/OFQ during the same period of time (Fig. 1). Food intake 12 and 24 h after injection of N/OFQ did not differ significantly between DA rats and their vehicle controls, nor was there a difference between WOKW with their controls, though DA rats treated with N/OFQ tended to eat more than their controls (vehicle) (Fig. 2).

3.2. *Experiment 2: effect of ICV injection of SHU 9119 on food intake in WOKW and DA rats*

The ANOVA show that in both strains, SHU9119 increased significantly food intake for the entire period of observation (4, 12 and 24 h). In particular the effect for the dose in DA rats was $F(1, 9) = 32.3$; $p < 0.001$ while in WOKW it was $F(1, 11) = 18.81$; $p < 0.001$ (Fig. 7).

3.3. *Experiment 3: effect of ICV injection of N/OFQ on plasma corticosterone levels in WOKW and DA rats*

PCLs were elevated in both strains after ICV injection of N/OFQ ($F(3, 17) = 39.5$; $p < 0.001$). In particular, WOKW rats treated with N/OFQ had higher levels of corticosterone ($p < 0.001$) compared to the DA rats (Fig. 3).

3.4. *Experiment 4: gene sequencing of the nociceptin/orphanin FQ receptor (Oprl1) and Cart in WOKW and DA rats*

The gene sequencing analysis for the *Oprl1* gene has revealed a difference between WOKW and DA rats at the level of the 897 and 898 positions in the gene sequencing of chromosome 3 described in the GenBank.

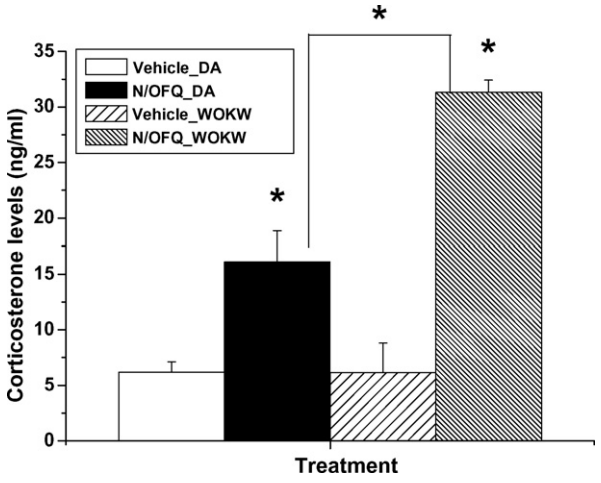

Fig. 3 – Plasma corticosterone levels (ng/ml) 30 minutes after the ICV injection of N/OFQ (8.4 nmol/rat) in DA and WOKW rats. Data represent the mean ± S.E.M. of 9 DA and 12 WOKW rats, respectively. Difference from controls: *$p < 0.05$; where not indicated, difference was not statistically significant.

All the other strains tested showed the same gene sequencing as the DA at intron 897 and intron 898. HTG rats showed a different gene sequence at intron 4145, while all the others tested showed the same nucleotide sequence.

The gene sequencing analysis for the *Cart* gene has revealed a difference between WOKW and DA rats at the level of position 499, 510, and 526 of the gene sequence described in the GenBank corresponding to a intron sequence, and at the level of position 1560 of the gene sequence described in the GenBank corresponding to an exon sequence.

In particular, the *Cart* gene sequencing data shows that at intron 499, 510 and exon 1560, WOKW and LEW.1W have the same sequence, as do the DA and F344.

3.5. *Experiment 5: gene expression of the Oprl1 and Cart in WOKW and DA rats*

The *Oprl1* receptor gene expression in the hypothalamus of WOKW and DA did not differ significantly $F(1, 12) = 0.024$; $p = 0.88$. The *Pnoc* gene expression also was not statistically different between the two strains ((F1, 12) = 2.22; $p = 0.16$). On the other hand, hypothalamic *Cart* gene expression was significantly higher in the WOKW rats ($F(1, 12) = 5.46$; $p < 0.05$).

The *Oprl1* receptor gene expression in subcutaneous fat pads of WOKW and DA did not significantly differ between the two strains ($F(1, 12) = 2.32$; $p = 0.15$). Also, *Cart* gene expression in subcutaneous fat pads of WOKW and DA did not differ between the two strains ($F(1, 12) = 0.92$; $p = 0.35$).

On the other hand, the *Oprl1* receptor gene expression in epididymal fat pads of WOKW and DA differs significantly ($F(1, 12) = 18$; $p < 0.001$). The gene expression of *Pnoc* in epidydimal fat pads of WOKW and DA did not differ significantly ($F(1, 12) = 4.38$; $p = 0.058$) (Figs. 4 and 5).

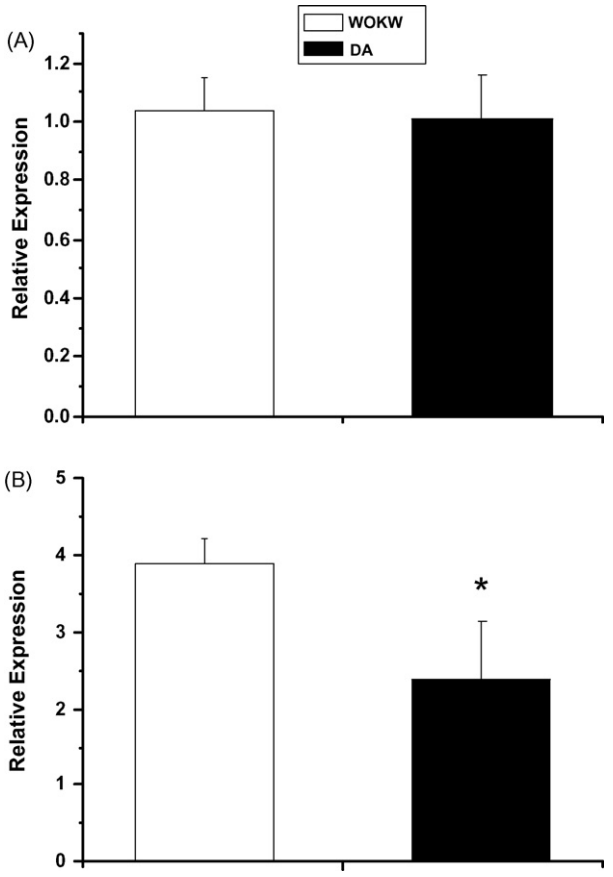

Fig. 4 – Relative gene expression of the Oprl1 receptor (panel A) and of the Cart peptide (panel B) of WOKW and DA rats at hypothalamic level. Data represent the mean ± S.E.M. of 7 WOKW and 7 DA rats. Difference: *$p < 0.05$; where not indicated, difference was not statistically significant.

3.6. *Experiment 6: effect of ICV injection of N/OFQ on food intake in LEW.1W and F344 rats*

Lateral ventricle injection with N/OFQ (2.1–8.4 nmol) in Fisher 344 rats increased their food consumption during the first 4 h of observation ($F(3, 27) = 32.4$; $p < 0.001$). ANOVA also demonstrated significance for the time [$F(3, 27) = 13.6$; $p < 0.0001$] and the treatment–time interaction [$F(9, 81) = 2.3$; $p < 0.05$] (Fig. 6, bottom panel). On the other hand, LEW.1W rats did not significantly increase their food intake after any of the N/OFQ-injected doses during the same period of time of observation (Fig. 6 top panel).Discussion

Numerous neuropeptides that affect reward, stress, and intestinal status have been found to influence food consumption as well [17]. As shown by several researchers, a single neuropeptide does not act alone in the process of food intake regulation, but rather stimulates or inhibits a neural network that only in part has been cleared [17].

As is well known, brain neurotransmission seems to be altered in obese humans [15] and rats [23], leading to altered regulation of feeding behavior. Obesity research has dedicated considerable attention to brain leptin and its receptor, while

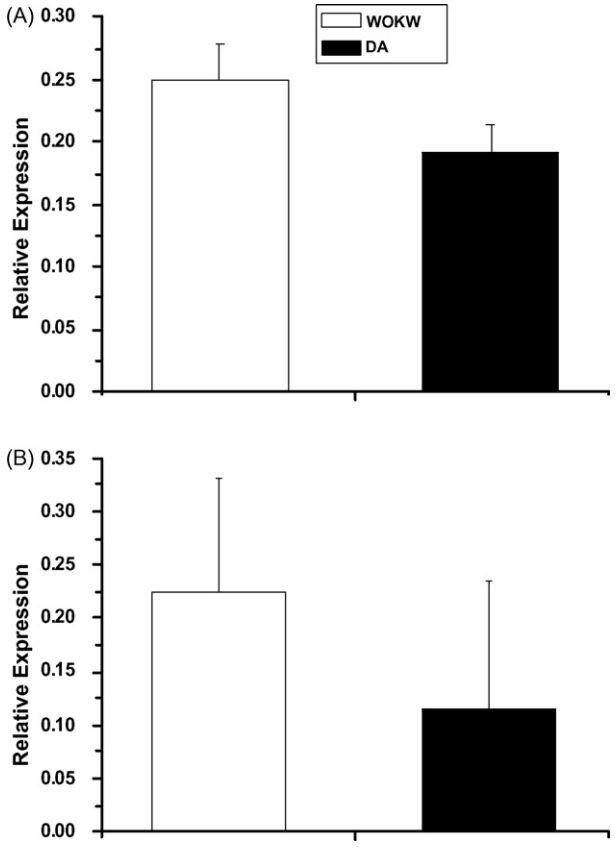

Fig. 5 – Relative gene expression of the *Oprl1* receptor (panel A) and of the *Cart* peptide (panel B) of WOKW and DA rats in the subcutaneous fat. Data represent the mean ± S.E.M. of seven WOKW and seven DA rats. Difference: $^{*}p < 0.05$; where not indicated, difference was not statistically significant.

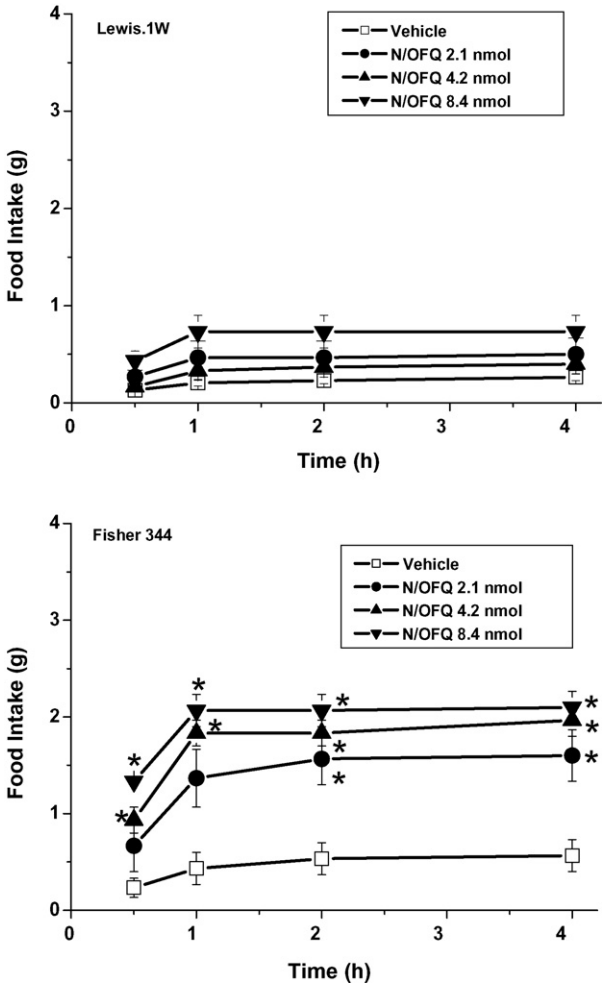

Fig. 6 – Four-hour food intake (g) following injection of N/OFQ (0, 2.1, 4.2 or 8.4 nmoles) into the LV of Lewis.1W (top panel) and Fisher 344 (bottom panel) rats. Data represent the mean ± S.E.M. of 10 LEW.1W and 10 F344 rats, respectively. Difference from controls: $^{*}p < 0.05$; where not indicated, difference was not statistically significant.

among the neurotransmitters, N/OFQ and its receptor have not yet been investigated thoroughly in obese rats.

Several laboratories have shown, in normal rats, that N/OFQ stimulates food intake through central mechanisms [19,20] by activation of hypothalamic and brain stem NOP receptors [17]. It has been postulated that N/OFQ, like the opioid peptides, could be involved in the prolongation of a meal, or may inhibit central signaling responsible for termination of food consumption [17,22].

The present feeding behavior study shows that the WOKW rats, which exhibit the metabolic syndrome, are less sensitive to central stimulation by N/OFQ than normal rats, such as the dark agouti, which are resistant to this polygenic disease [26,28]. To explore the underlying mechanism of this difference, this work evaluated two possibilities regarding the thus-far known effects of N/OFQ in the whole animal [16,22] or in *in vitro* studies [1].

The first possibility concerns the involvement of Cart in the regulation of N/OFQ-induced feeding behavior. Perhaps N/OFQ works by curtailing the activity of a peptide that inhibits feeding behavior [17]. Indeed, a recent study [1] on cultured cells has shown that N/OFQ reduces the release of the Cart neuropeptide while it increases the release of the agouti-related protein (AgRP) at the hypothalamic level [1]. Based on the result in experiment 2 with SHU9119, we did not perform any AgRP analyses, since both strains had a highly significant increase of food intake. Therefore, we partially set aside the possible involvement of alpha-MSH as described in Bomberg et al. [2]. These observations form the basis of our understanding of why DA rats and WOKW rats exhibit different feeding responses after an ICV injection of N/OFQ. Indeed, our data show that hypothalamic *Cart* gene expression in DA rats is significantly lower than that of WOKW rats. Therefore, it could be postulated that the increased food intake in DA rats might be due to the fact that N/OFQ needs to inhibit less *Cart* in these animals than that found in the WOKW rats. This hypothesis is further supported by the data obtained in our fifth feeding experiment with other two rat strains; indeed, the LEW.1W rat, which has an identical *Cart* gene sequence modification as the WOKW rat, in the same introns and exon, shows low food intake even at the highest dose of N/OFQ used, while the Fisher 344 rats, with a *Cart* gene sequence

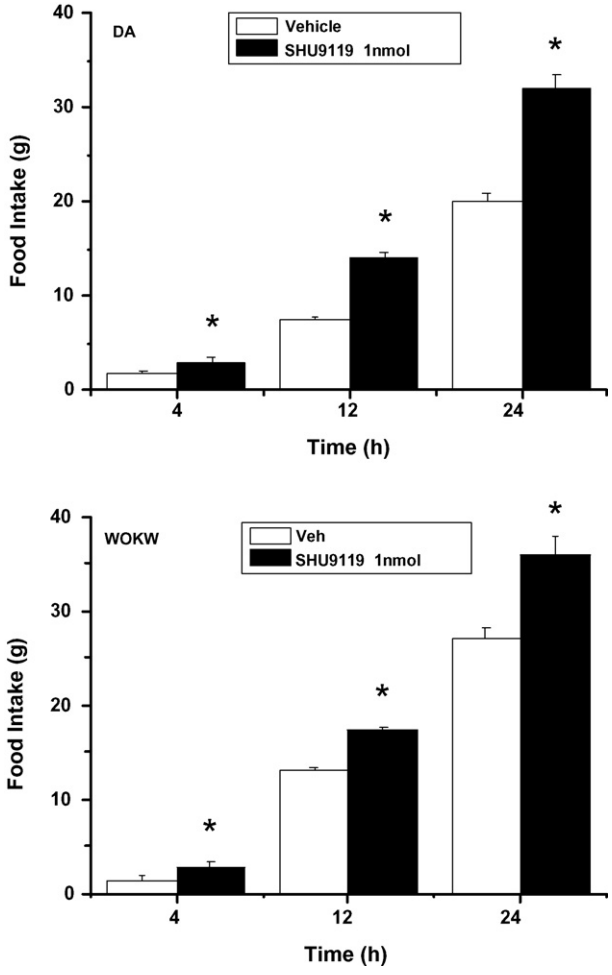

Fig. 7 – Four, twelve and twenty four-hour food intake (g) following injection of SHU9119 (0, 1 nmole) into the LV of DA (top panel) and WOKW (bottom panel) rats. Data represent the mean ± S.E.M. of 11 DA and 13 WOKW rats, respectively. Difference from controls: $^{*}p < 0.05$; where not indicated, difference was not statistically significant.

modification identical to the DA rats, respond to the N/OFQ injection with increased food intake, similar to the DA rats. Furthermore, the gene expression study of the *Oprl1* receptor shows that its expression in the hypothalamus does not differ at all between the WOKW and DA rats, suggesting that the difference does not involve the stimulation of different receptors, but occurs in the following step, where Cart may be involved. A consideration not to be overlooked in this regard is that the WOKW rats have a different amino acid sequence of the Cart peptide, a fact that might be responsible for the lower feeding response, owing to a lower affinity or capacity to activate its receptor. It would be interesting to validate this hypothesis by working with the different Cart peptides in both strains. Another possible hypothesis is that these strains have different Cart receptor sequences. Some might argue that the underlying mechanism for the differential responses to N/OFQ administration is simply the epiphenomenon related to the overall metabolic disturbances in the WOKW rats, and not, as we posit, due to Cart involvement in the WOKW rats. Instead, our N/OFQ data demonstrate that the WOKW show less sensitivity than the DA. We are lead to believe that this is not simply a metabolic epiphenomenon, because our experiment 2 data with SHU 9119 shows that they respond fairly similarly to this melanocortin antagonist. Our data also show that in the visceral fat, the *Oprl1* receptor gene expression in DA rats is higher than that in the WOKW rats, suggesting for the first time that the N/OFQ system might play a role in regulating energy status at the peripheral level as well.

The second possibility that might explain the different feeding behavior in response to central N/OFQ stimulation in DA and WOKW rats regards elevated release of corticosterone in DA animals. It is well known that N/OFQ stimulates the hypothalamic–pituitary–adrenocortical axis. Indeed, Nicholson et al. [16] have demonstrated that the presence of circulating corticosterone is necessary for the mediation of N/OFQ-induced hyperphagia in rats. On the other hand, Le Cudennec et al. [11] have shown, in mice, a decrease in plasma corticosterone levels within 30 min of ICV N/OFQ injection. Our data show that while both strains treated with saline showed the same PCL levels, the animals treated with N/OFQ did respond with a significantly different sensitivity. Though Nicholson et al. conclude that the hyperphagic effect of N/OFQ depends on the presence of corticosterone in the bloodstream, our data suggest that the corticosterone blood level cannot explain the difference in feeding behavior between WOKW and DA rats because the latter have lower corticosterone levels. It could be argued that WOKW do not eat because they are in an elevated state of stress/anxiety, as shown by their higher level of corticosterone. We do not think that this is the case, because the elevated plus maze technique of measuring anxiety has shown that N/OFQ is anxiolytic. Our previous work with the elevated plus maze technique (Vitale et al. [30]) has shown, unlike Fernandez et al. [4], who used only one injection, that when the animals are injected two or more times with N/OFQ, its effect is mainly anxiolytic. The corticosterone measurements taken in our experiments were done after the animals have been injected with N/OFQ three times. On the other hand, our data confirm previous discoveries about the interaction of N/OFQ with hypothalamic–pituitary–adrenocortical axis regulation.

These data and those in the literature can suggest that a central mechanism of control of food intake is regulated by Cart. In particular, the effect of leptin is mediated by Cart. Our data could suggest that N/OFQ and leptin could act on the same Cart-containing neuron. Therefore, further studies are called for to test whether N/OFQ is a functional antagonist of leptin.

In conclusion, our data show that the DA rats respond to the hyperphagic effect of N/OFQ, while the WOKW do not; this difference can be imputed to the *Cart* peptide's different expression or different affinity to its receptor, at hypothalamic level. In addition, PCLs are not correlated to N/OFQ-induced hyperphagia. Our data therefore strongly suggest that N/OFQ may exert its hyperphagic effect through an inhibition of a central signal responsible for termination of food consumption [17,22]. Therefore, it would be of great interest to test whether Cart antagonists could block N/OFQ's effect on food intake. An intriguing possibility for future study would be to

explore whether there is any difference in the sequence of the Cart receptor itself.

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