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Effects of cannabinoids on neuropeptide Y and β-endorphin expression in the rat hypothalamic arcuate nucleus

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Abstract

The control of appetite and satiety is extremely complex and involves a balance between neurotransmitters and neuropeptides to stimulate and/or inhibit feeding behaviour. The effect of cannabinoids on food intake is well established, but little is known about the mechanism of action underlying their activity. In the present report, the effect of pharmacological manipulation of the cannabinoid receptor on the expression of hypothalamic neuropeptides is investigated. We used an immunohistochemical approach to examine the effect of intracerebroventricular administration of the cannabinoid receptor agonist WIN55,212-2 and the inverse agonist AM251 on neuropeptide Y (NPY) and the β-endorphin (β-end) neuronal hypothalamic systems. Double immunohistochemistry (c-fos/β-end) was used to assess the number of β-end neurons activated by the cannabinoid agonist. The present results showed that 1 μg WIN 55,212-2 increases β-end immunoreactivity within the arcuate nucleus while no significant changes were noted in the NPY-immunoreactive nerve fibres network in comparison to the control group. Injection of 1 μg AM251 decreases both NPY and β-end immunoreactivity within the arcuate nucleus. The number of β-end neurons exhibiting c-fos increased significantly in WIN 55,212-2 compared with the control group. These results suggest that cannabinoids affect the expression of hypothalamic neuropeptides, notably the NPY and β-end systems, which may have implications in the orexigenic action of cannabinoids.

Key words: Cannabinoids: Neuropeptide Y: β-Endorphin: Arcuate nucleus of hypothalamus

Endocannabinoids are a group of lipid ligands acting in the central nervous system primarily as neuromodulators rather than 'classical' neurotransmitters[1]. The cannabinoid receptors (CB1 and CB2) and their specific biosynthetic and degradation pathways have been described[1]. The CB1 receptors are expressed in the brain and peripheral organs involved in the control of food intake, including the hypothalamus, gastrointestinal tract and adipose tissue[1]. Furthermore, the endocannabinoid ligands have been shown to stimulate appetite[2], while the CB1 cannabinoid receptor antagonists, SR 141716 and AM251, reduce food intake in animals and humans[3]. Accumulating evidence supports a role for the cannabinoid system in food intake; however, little is known about the mechanism underlying their effect. On the other hand, there is a general agreement that modulation of food intake is controlled by the hypothalamus[4].

We have previously shown that intracerebroventricular administration of the cannabinoid receptor agonist WIN 55,212-2 is associated with a significant increase in food intake, whereas the administration of the antagonist AM251 caused a significant reduction in food intake. These effects were accompanied by considerable changes in serotonin and 5-hydroxyindoleacetic acid levels compared with vehicle-injected control rats[5].

Neuropeptide Y (NPY) is a potent orexigenic marker in the hypothalamus, where it shows high expression, suggesting its implication in food intake control. Indeed, intracerebroventricular injection of NPY potently stimulates food intake[6]. β-Endorphin (β-end) neurons are mainly present in the hypothalamic arcuate nucleus (ARC)[7] and their stimulation by hypothalamus microinjection stimulates feeding behaviour. Studies looking at the expression of the immediate early gene product c-fos suggested that

Abbreviations: β-end, β-endorphin; AgRP, agouti-related peptide; ARC, arcuate nucleus; GABA, γ-aminobutyric acid; NPY, neuropeptide Y.

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c-fos labelling represents an anatomical tool for identifying activated neurons[8,9]. Indeed, previous studies have shown that cannabinoid agonists induce c-fos expression in a variety of neural sites, including the hypothalamus[8].

Few studies have examined the interactions between cannabinoids, NPY and β-end, in the hypothalamus; however, their functional similarities as well as their localisation in structures implicated in feeding imply the existence of such an interaction. It is likely that cannabinoids affect the expression of NPY and β-end, as cannabinoids are orexigenic in nature and have been shown to modulate transmitter release[5,10]. Therefore, the purpose of the present study is to examine the possible interactions between cannabinoids, NPY and β-end.

Materials and methods

Male Wistar rats (250–300 g) were maintained under controlled conditions of temperature (24°C) and photoperiod (12 h light–12 h dark cycles). Food and water were supplied *ad libitum*. The experiments were performed according to the recommendations of the University's Animal care and Ethics Committee whose approval is in agreement with the international guidelines.

Animals were anaesthetised with an intraperitoneal injection of sodium pentobarbital (400 mg/kg) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). A stainless steel cannula guide was implanted into the brain above the lateral ventricle according to the Paxinos & Watson method[11] (anteroposterior: −0·8 mm, lateral: ±2 mm, posterior: −3 mm). As we have previously described[5], a 10 d post-surgical recovery period was allowed to stabilise food intake before the experimental period.

Cannabinoids were purchased from Tocris (Ellisville, MO, USA). WIN55,212-2 and AM251 were prepared in a 5 μl mixture of dimethylsulphoxide, Tween 80 (polyoxyethylene sorbitan mono-oleate) and saline; this mixture was used as a vehicle and was administered to the control animals.

The effects of cannabinoids on hypothalamic neuropeptides were analysed in partially satiated rats; to this end, the rats were deprived of food but not of water for 24 h before the beginning of the experiments. At the end of the 24 h, the rats were given free access to food for 60 min[5]. We used a presatiation procedure in which rats were allowed to eat a meal before drug administration to permit a easier observation of the drug-inducing feeding effect[5]. Infusions of cannabinoid (1 μg) solutions were made at a rate of 1 μl/min and the volume injected into the lateral ventricle was 5 μl. The injector remained in place for 1 min to allow diffusion of the drugs into the brain and to reduce backflow through the cannula track. At 1 h after drug administration, the animals were anaesthetised and perfused through the aorta with 50 ml saline followed by 300 ml of a fixative solution containing 4% paraformaldehyde and 0·2% picric acid in 0·1 M-phosphate buffer, pH 7·4. The brains were dissected, cut into 5 mm-thick slabs and post-fixed for 24 h at 4°C with the same fixative. Coronal sections (60 mm) thick throughout the ARC of the hypothalamus were obtained using a vibratome and then processed for indirect immunohistochemistry.

The expression of NPY and β-end and the coexpression of c-fos/β-end within the ARC were determined using rabbit polyclonal antibodies raised against NPY, β-end or c-fos and β-end. The specificity of the antibodies was previously characterised and checked for in different animals[12,13].

The brain slices were incubated overnight at 4°C with the primary NPY or β-end antiserum (1:5000) in PBS containing 0·3% Triton-100. Then the slices were incubated for 2 h at room temperature with a biotinylated goat anti-rabbit antiserum (Vector Laboratories, Paris, France) (1:400); the sections were then rinsed in PBS and incubated for 2 h at room temperature with standard avidin–biotin peroxidase (Vector Laboratories) (1:400). Peroxidase activity was revealed according to the method of Shu *et al.*[14] using diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO, USA) as a chromogen intensified with nickel ammonium sulphate (Sigma-Aldrich). The reaction was stopped and the section was mounted on gelatine-coated slides using a phosphate buffer–glycerol (1:1) solution.

For Fos and β-end double labelling, ARC sections from each animal in the vehicle- and WIN 55,212-2-treated groups were processed as described earlier. Briefly, the sections were incubated with a rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:3000 in PBS. The diaminobenzidine tetrahydrochloride intensified with nickel ammonium sulphate reaction[14] produces a dark-blue nuclear staining. The Fos-labelled sections were also processed for β-end and were incubated for 48 h at 4°C with a rabbit anti-β-end antibody (1:5000). Cytoplasmic β-end labelling was detected using diaminobenzidine tetrahydrochloride and H_2O_2 in 0·05 M-Tris–HCl buffer as a chromogen, without intensification with nickel sulphate, which produces a brown reaction product.

The sections were mounted on gelatine-coated slides, air dried, dehydrated and cover-slipped before examination with a Nikon Microphot-FX microscope (Nikon, Inc., Tokyo, Japan). The photomicrographs were taken with a Leitz microscope (Leica, Heidelberg, Germany) coupled to a Canon 630 video camera.

Quantification of the number of β-end immunopositive cell bodies per section within the ARC was performed at the light microscope level as we have previously described[13]. Cell counts were performed by direct microscopic observation in a double-blinded fashion, where the counts were made by two different investigators unaware of the animal group assignments. The neuroanatomical identification of the hypothalamic structure (ARC) was based on the atlas of the rat brain by Paxinos &

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Watson[11]. The results were expressed as the mean number of neurons counted on four rostrocaudal sections per animal (n 6).

Optical density of NPY nerve fibres was measured using National Institutes of Health ImageJ software (National Institutes of Health, Bethesda, MD, USA). Quantitative analysis was performed for each brain at both sides of the third ventricle under 10 × objective. The quantification of data was based on eight measurements in four sections of the medial ARC. Each measurement was made in the sub-area of interest (0·5661 mm^2/field). The immunostaining optical density was expressed in arbitrary units corresponding to grey levels. To calculate the optical density, the background intensity of staining was subtracted from the intensity of staining in the middle ARC. The background intensity was measured in an area devoid of NPY fibres in the same coronal section as in the ARC analysis. The data from each section of each hypothalamus were pooled to represent different groups and an average value was calculated for each animal (n 6).

Double-labelled neurons with c-Fos and β-end neurons were identified as cells with brown cytoplasmic deposits for β-end staining and dark-blue nuclear staining for c-fos. The number of single- and double-labelled β-end neurons observed was counted in four to five sections from each animal (n 4). The data were expressed as the percentage of Fos-positive β-end neurons compared to the total number of neurons counted.

The results were expressed as mean values with their standard errors and statistical significance was determined using Student's unpaired t test. The differences with a P value $<0·05$ were considered significant.

Results

Immunostaining for NPY neurons was first attempted in the vehicle-injected group. In those animals, fibre staining was seen through the ARC but few cell bodies were visibly distinguishable (Fig. 1(a)). However, WIN 55,212-2 injections increase NPY immunoreactivity within the ARC fibres, which displayed numerous immuno-labelled varicosities as shown in Fig. 1(b).

Microscopic analysis of ARC sections from AM251- pretreated animals showed a faint immunostaining in the fibres and a lower density of punctuate profiles compared with vehicle-injected animals (Fig. 1(c) and (d))).

Densitometry analysis (Fig. 2) showed that the density of NPY immunoreactive nerve fibres was increased, though non-significantly ($P>0·05$), in the WIN 55,212-2 group compared with the vehicle group. However, a significant decrease ($P<0·05$) in nerve fibre network density was noted in the AM251 group and the difference was significantly higher between AM251 and WIN55,212-2 rats (38·02 (SEM 7·89) v. 93·74 (SEM 3·62); $P<0·01$).

Concerning the β-end system, the distribution pattern of both cell bodies and axons follows previous descriptions

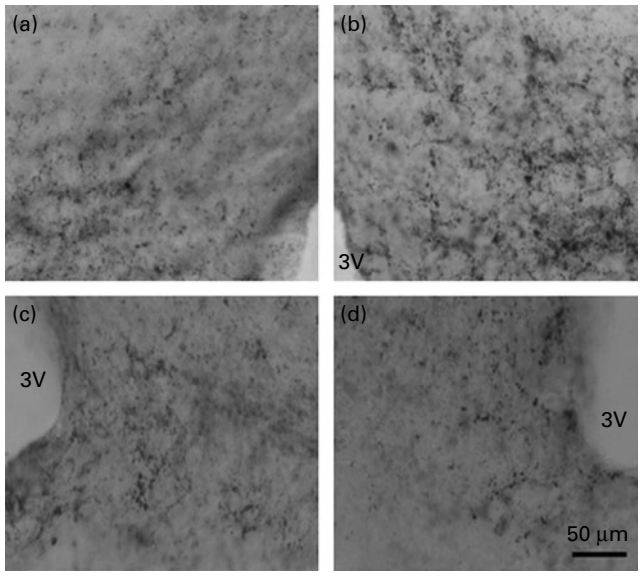

Fig. 1. Photomicrographs showing 60 μm sections of the mediobasal hypothalamus immunostained for neuropeptide Y. Rats treated with vehicle (a and c), WIN 55,212-2 (b) and AM251 (d). 3V, third ventricle. Scale bar = 50 μm.

of the hypothalamic β-end system[13]. In the WIN 55,212-2 group, an increase in β-end cell bodies was observed in the ARC, the cells were round or elongated and their dendrites were rarely seen compared with those of the NPY cells (Fig. 3(b)). Conversely, in the AM251 group, most of the β-end immunoreactive cells displayed faint staining (Fig. 3(c)).

Quantitative analyses (Fig. 2) show that the number of the β-end immunoreactive neurons in the ARC was increased in the WIN 55,212-2 group compared with the vehicle-treated controls (44·33 (SEM 4·43) v. 19·5 (SEM 2·42)); $P<0·001$). AM251 administration significantly decreased β-end immunoreactivity compared with WIN 55,212-2 (24·67 (SEM 3·6) v. 44·33 (SEM 3·58); $P<0·01$) and had no effect compared with vehicle-treated rats.

Analysis of double-labelled sections (Fig. 4) showed that the WIN55,212-2 group significantly increased the percentage of β-end-containing cells expressing Fos

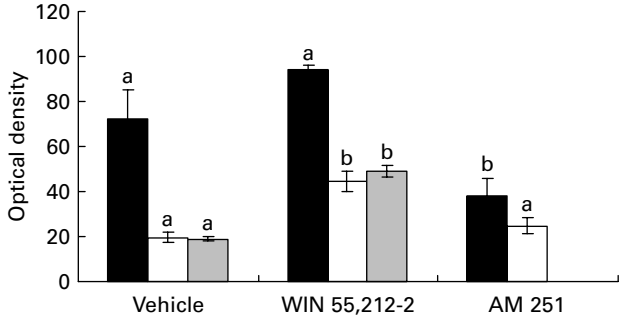

Fig. 2. Densitometry analysis of neuropeptide Y (■; n 6), β-endorphin (□; n 6), Fos/β-endorphin (▩; n 4) immunoreactive neurons in the arcuate nucleus of rats injected with vehicle, WIN55,212-2 and AM251. Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different.

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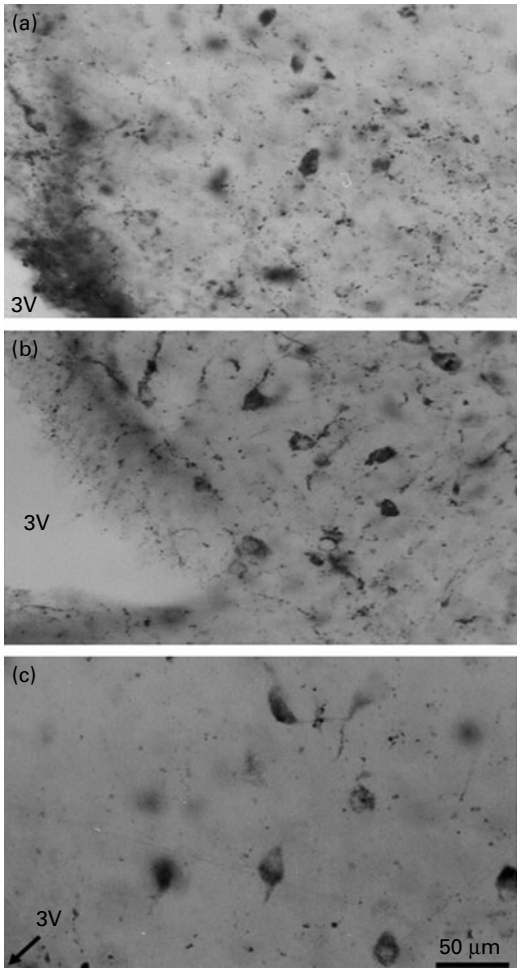

Fig. 3. Photomicrographs from arcuate nucleus sections showing the immunoreactivity of β-endorphin in vehicle (a), WIN55,212-2 (b) and AM251 (c) groups. 3V, third ventricle. Scale bar = 50 µm.

(49·15 (SEM 1·818); $P<0·05$) compared with the vehicle-injected controls (19·67 (SEM 1·178)) (Fig. 2).

Discussion

The effects of cannabinoids on appetite are well documented[15] and the evidence shows that the endocannabinoid system is strongly implicated in feeding behaviour[5,16]. However, the mechanisms by which cannabinoids stimulate food intake are unknown. The brain cannabinoid system controls food intake at two levels; first, it reinforces the motivation to consume food by interacting with the mesolimbic pathways involved in reward mechanisms; second, it is activated 'on demand' in the hypothalamus after food deprivation to regulate the levels and/or action of other mediators to induce appetite[15].

The localisation of CB1 receptors in the hypothalamus is suggestive for its involvement in feeding behaviour. Moreover, administration of anandamide into the hypothalamus has been shown to increase food intake, providing evidence that cannabinoid activity is at least partly hypothalamic[2].

In the present study, we provide data supporting the notion that the endocannabinoid system may influence food intake by regulating the expression and/or action of several hypothalamic neuropeptides, such as NPY and β-end, implicated in feeding behaviour. Our findings provide morphological evidence that cannabinoid CB1 receptors alter NPY and β-end expressions in the ARC. Other studies have shown that pretreatment with colchicine, a drug that blocks axonal transport, intensifies the immunoreactivity of NPY-containing neuronal cell bodies[16]. However, in the present study, while NPY immunoreactive cell bodies were invisible in the absence of colchicine pretreatment, colchicine pretreatment was unsuitable for the present study and might have resulted in misleading results. However, the present comparative study assessing the immunoreactivity of NPY nerve fibres between vehicle, WIN 55,212-2 and AM251 groups showed changes in the medial ARC (Fig. 1).

The density of ARC fibres in the AM251 group was significantly reduced compared with that in the vehicle-injected controls, while the density of NPY immunoreactive fibres in the WIN55,212-2 group appeared increased, though non-significantly, compared with the vehicle-treated animals.

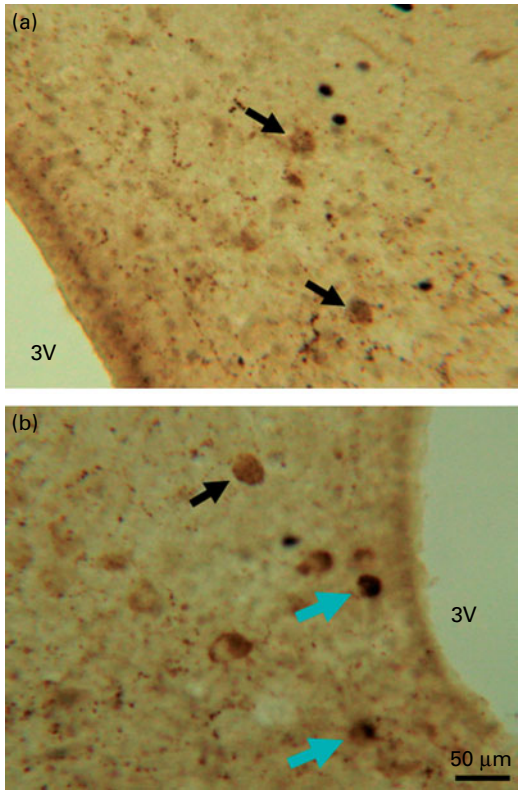

Fig. 4. Representative photomicrographs of coronal sections of arcuate nucleus showing double immunohistochemical staining for Fos (blue-dark nuclei) and β-endorphin (brown cytoplasm) in vehicle (a) and WIN55,212-2 (b) groups. →, Fos + /β-end positive neurons; ➔, Fos-negative β-end neurons. 3V, third ventricle. Scale bar = 50 µm.

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The fact that the intracerebroventricular application of WIN55,212-2 (1 μg), an agonist of CB1 receptors, did not significantly affect NPY immunoreactivity may be related to the fact that CB1 does not colocalise with NPY in ARC neurons[17], indicating the independence of the two orexigenic systems. NPY is primarily produced in the ARC, which sends NPY-containing projections to other feeding control centres, including the paraventricular, dorsomedial and lateral hypothalamus[6]. CB1 receptors have been shown to be present in all of these brain areas; however, no co-expression with CB1 and NPY was found[17]. The NPY system in the ARC does not seem to be targeted by endocannabinoid action; however, the significant decrease in the density of fibres immunoreactive to NPY in the AM251-treated group implies that the observed effect with WIN55,212-2 may be mediated by CB1 cannabinoid receptors. The decrease obtained from the AM251-treated group is consistent with the anorectic action of AM251 observed in presatiated rats[5] and in the well-documented orexigenic action of NPY[6]; these data are also in agreement with a previous study showing that AM251 significantly decreased NPY release in a rat hypothalamic explant model[18] and a study showing a decrease in NPY mRNA expression and protein level within the hypothalamus in response to acute administration of another CB1 receptor inverse agonist, rimonabant[19]. It would appear that the well-known anorectic effect of CB1 receptor blockade is due at least in part to inhibition of NPY production in the hypothalamus. However, the ability of rimonabant to affect food intake in wild-type as in NPY-knockout mice also indicates that the cannabinoid action is not mediated by NPY[20] (in agreement with the lack of co-expression of CB1 receptors and this neuropeptide). On the basis of these data, we cannot conclude that the observed effect on NPY is caused by a direct action of cannabinoids, which suggests the existence of a possible intermediate factor.

The ARC is thought to play a pivotal role in the integration of several signals regulating food intake. This region contains two distinct populations: a subpopulation of the neurons in the medial ARC, which expresses the orexigenic neuropeptides NPY/agouti-related peptide (AgRP), and a second population that inhibits food intake via the expression of cocaine and amphetamine-regulated transcript (CART)/pro-opiomelanocortin. Interactions exist between these two populations as has been demonstrated[21]. Thus, the ARC has become a major focus for energy balance research.

The neurons expressing NPY in the ARC are also those expressing AgRP and γ-aminobutyric acid (GABA) (NPY/AgRP/GABA); a lack of GABA signalling from NPY neurons leads to the blockade of feeding behaviour[22]; furthermore, basal inhibition of GABA release by endocannabinoids may serve as a tonic regulatory mechanism in the ARC[23]. Menzies *et al.*[24] suggested an explant hypothalamic model, where rimonabant inhibits K^+-evoked GABA release in a tetrodotoxin-dependent mechanism; the K^+-evoked GABA release from the hypothalamus was sensitive to leptin, insulin and PYY(3-36), indicating that GABA was released by arcuate NPY/AgRP/GABA neurons. Nevertheless, the study proposes that the effect of rimonabant on NPY/AgRP/GABA is indirect and involved a disinhibition of a cannabinoid-sensitive inhibitory input onto NPY/GABAergic neurons, mostly by the opioid peptides released from neighbouring neurons[24].

The slow increase in the density of NPY nerve fibres of the WIN55,212-2 group *v.* control suggests an eventual increase in NPY synthesis, which may be counterbalanced by an increase in NPY release. This is not surprising since it has been shown that cannabinoid receptor agonists, anandamide and CP55, 940 increased the release of NPY in the rat hypothalamus[18]. The present results support those findings since we observed a high density of NPY-immunoreactive fibres in the inner layer of the median eminence in the WIN55,212-2 group compared with the controls (data not shown). Therefore, NPY may be considered – among others – as a 'downstream' peptidergic effector of CB1 receptor activation.

Interactions between endocannabinoid and opioid systems have been described to be implicated in the regulation of feeding at the level of motivational responses for food[25]. However, within the hypothalamus, the specific mechanisms involved in the endocannabinoid and opioid interactions are still to be defined.

The results of the present study provide evidence for a possible functional interaction between cannabinoids and opioids, particularly the β-end neuropeptide in the ARC.

Intracerebroventricular administration of WIN 55,212-2 induced a significant increase in β-end expression within the ARC, where both the number and the intensity of stained neurons increased. Conversely, AM251 was able to reverse the effect of WIN55,212-2 and to decrease the expression of β-end. These results corroborate other studies reporting that acute treatment with tetrahydrocannabinol elevated the concentration of β-end-like immunoreactivity in the plasma and in the hypothalamus[26] and a study showing that chronic administration of tetrahydrocannabinol can increase the synthesis of pro-opiomelanocortin, a precursor molecule of β-end in the ARC and stimulates the release of endogenous opioids[27]. To further support the data suggesting that β-end neurons in the ARC are responsive to cannabinoids, we used double immunohistochemistry for Fos/β-end as Fos is a marker of neuronal activity; we observed that WIN 55,212-2 increases Fos/β-end double labelling, confirming the neurochemical identity of cannabinoid-activated neurons in the ARC. This result is consistent with the orexigenic nature of β-end. In fact, β-end stimulates food intake following microinjection into the hypothalamic ventromedial nucleus[28], and hypothalamic β-end is decreased in chronically food-restricted rats[29]. We demonstrate that activation of β-end neurons in the

hypothalamus plays an important role in response to cannabinoids, suggesting that these neurons probably form – among others – the hypothalamic substrate for cannabinoids.

The present report examines directly the effects of cannabinoids on NPY and β-end expression, supporting a role of the hypothalamus in mediating the orexigenic effects of cannabinoids. Based on the present study, we cannot conclude if the observed effects on these neuropeptides are a result of a direct action of cannabinoids on hypothalamic neurons, or if cannabinoids are acting via other factors to influence the expression of hypothalamic neuropeptides. The present data should incite further investigations to elucidate the precise mechanisms and pathways involved in the recruitment of hypothalamic neurons in response to cannabinoids.

In summary, the present report suggests that cannabinoid orexigenic action involves a possible interaction between the hypothalamic NPY and β-end neuronal systems.

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