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The effect of leptin receptor deficiency and fasting on cannabinoid receptor 1 mRNA expression in the rat hypothalamus, brainstem and nodose ganglion

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ABSTRACT

Despite ample evidence for the involvement of the endocannabinoid system in the control of appetite, food intake and energy balance, relatively little is known about the regulation of cannabinoid receptor 1 (CB_1R) expression in respect to leptin signalling and fasting. In the present study, we examined CB_1R mRNA levels in lean (Fa/?) and obese (fa/fa) male Zucker rats under basal and food-restricted conditions. Using stereological sampling principles coupled with semi-quantitative radioactive in situ hybridization we provide semi-quantitative estimates of CB_1R mRNA expression in key appetite regulatory hypothalamic and brainstem areas, as well as in the nodose ganglia. Whereas no effect of fasting were determined on CB_1R mRNA levels in the paraventricular (PVN) and ventromedial hypothalamic (VMH) nucleus, in the brainstem dorsal vagal complex or nodose ganglion of lean Zucker rats, CB_1R mRNA levels were consistently elevated in obese Zucker rats pointing to a direct influence of disrupted leptin signalling on CB_1R mRNA regulation.

During the past two decades the importance of the endogenous cannabinoid system in the regulation of food intake has been extensively documented [4,6,17,28]. It is now well known that endocannabinoids and CB_1 receptor agonists increase appetite [26,27] whereas CB_1R antagonists decrease food intake and body-weight [2,4–6,19]. In addition, reports of altered endocannabinoid levels in response to fasting, feeding and satiety [12] and altered endocannabinoid levels in rats with defective leptin signalling [5,23] have further supported a physiological role of the brain endocannabinoid system in the regulation of appetite and body-weight.

Although the location of CB_1R expressing neurons in the CNS is well characterized (for review, see Ref. [14]) relatively little is known as to which neurons harbouring CB_1 receptors are specifically mediating the anorectic effects of CB_1 antagonists and inverse agonists. CB_1 receptors are widely expressed in the forebrain [16] including several hypothalamic nuclei involved in appetite regulation (e.g. the paraventricular nucleus (PVN), the ventromedial hypothalamus (VMH) and the lateral hypothalamic area (LHA)). CB_1R are also widely expressed in the brainstem [10] and in the sensory nodose ganglion on the vagus nerve [3]. Interestingly, it was recently demonstrated by the use of RT-PCR and in situ hybridization that fasting increases CB_1R mRNA levels in the nodose ganglion pointing to a role for vagal CB_1 receptors in appetite regulation [3]. However, there seems to be no available information about the possible regulation of CB_1R mRNA in key hypothalamic and brainstem appetite regulatory nuclei. The present study was undertaken to shed further light on fasting-induced CB_1R mRNA regulation in normal lean Zucker rats and in the obese strain with impaired leptin signalling, in an attempt to link the expression of CB_1R to central brain areas important for the modulation of appetite control.

Two separate experiments were performed: Experiment A using 20 male lean (Fa/?) and 20 obese (fa/fa) Zucker rats with clear difference in phenotype, and Experiment B using 20 male Sprague-Dawley (SPD) rats. All animals were transferred from Charles River (Germany) to the Rheoscience stables and kept from 9 weeks of age until the age of 12 weeks. Rats were housed individually (1 rat/cage) under a 12:12 light–dark cycle (lights on at 0600 a.m.) at controlled temperature conditions with food and water available ad libitum. At the initiation of the experiment each group of rats were assigned based on body-weight to two equally large groups, one of which was fasted for 48 h. Water was provided ad libitum to all groups. Cage bedding material was changed prior to fasting. After 48 h of food deprivation, animals were anaesthetised with CO_2 and killed by decapitation. In both experiments the brain and the left nodose ganglia was rapidly removed, frozen on crushed dry ice and stored at −80 °C.

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All animal experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and were approved by the Danish Committee for Animal Research.

The brain was divided into three parts by a dorsoventral cut at level with the optic chiasm and the pons (Fig. 1). The central forebrain (including the hypothalamus) was mounted with tissue-tek on a freezing microtome (Leitz, model 1720) and trimmed by a razor blade to cover only the telencephalon. The central forebrain and hindbrain was then cut exhaustively into 12 μm thick coronal sections and mounted directly on Superfrost Plus slides. In order to obtain representative series of sections from all animals, sampling was performed in a systematic uniform random way. All sections were sampled and arranged into 12 complete series covering the full rostral-dorsal extent of the hypothalamus or 18 series covering the dorsal vagal complex (i.e. the nucleus of the solitary tract (NTS) and area postrema (AP)). Each series was collected on 1–2 object slides with the main areas of interest being represented on at least 4 sections (PVN) to as much as 18 sections (arcuate/NTS) in each series (Fig. 1). Sections were allowed to dry at room temperature and kept at −80 °C until hybridizations were performed.

The nodose ganglion was embedded in tissue-tek and cut exhaustively into 12 μm sections. Sections were sampled in six series providing 12–18 sections per series for final estimation (Fig. 1).

In situ hybridizations were performed using 33P-labeled RNA probes directed against CB_1R cDNA (bp 2073–2840; GenBank accession number X55812). Sense RNA probes were used as negative controls – as a positive control for the fasting regimen, an additional in situ hybridization was performed against CART cDNA (bp 226–411; GenBank accession number U10071). Antisense and sense probes were generated by in vitro transcription from the linearized plasmid DNA containing the cDNA clones mentioned above. The RNA probes were synthesized using T3 and T7 polymerases as described in detail elsewhere [25].

From each animal one series of sections was selected using a random number within the section sampling interval. Prior to *in situ* hybridization, sections were fixed in 4% paraformaldehyde in phosphate buffered saline, acetylated in triethanolamine (0.1 M) and dehydrated through an ethanol gradient (from water to absolute ethanol). Hybridization was performed according to a protocol previously described [25]. Briefly, a viscous hybridization mixture containing the RNA probe was added to the dry sections (36 μl per slide/18 μl per section) after which the sections were coverslipped. Hybridization was carried out in a sealed polypropylene box at 47 °C and 100% humidity over-night. Post-hybridization washes were performed at 62 °C and 67 °C in 50% formamide (1 h at each temperature). Finally, single stranded RNA was removed by RNAse digestion (30 min at 45 °C). After hybridization, sections were exposed to autoradiographic films, exposed for 1 week (4 days for CART) and subsequently developed in Kodak D19 developer.

The in situ hybridization signals were evaluated using NIH image software (Image 1.60b). The hybridization signals were quantified using a frame covering the hypothalamic or brainstem region under examination. The signals were quantified as the sum product of frame area (in square millimetres) and mean pixel intensity within the actual frame. A local background subtraction method was applied. Levels of specific gene expression levels were estimated as the sum of measurements performed on all sections throughout the structure of interest. The mean expression in fed SPD rats or fed lean Zucker rats was used as a baseline and given the value 1. Statistical analyses were performed using a two-way ANOVA for the lean and obese Zucker rat study in order to examine the effects of genotype, treatment and interactions in the Zucker rats. Pair-wise comparison of groups was performed using one-way ANOVA followed by Fisher's post hoc test. For the SPD rats and unpaired Student's *t*-test was used. Values are expressed as means ± SEM. $P < 0.05$ was considered as significant.

Microscope images were acquired using a Nikon E1000M microscope fitted with a Nikon DCM1200 camera or scanned using a high resolution flat-bed scanner. To obtain final images as true to the microscopic view as possible, and to achieve optimal presentation, the digitally acquired images were reoriented and/or adjusted for brightness, contrast, or color balance in photoimaging software (Adobe Photoshop).

The systematic, uniform random sampling of sections allowed for a clear assessment of the mean hybridization signals in all regions of interest (Fig. 1). The hybridization signal intensity was assessed on all sections through the region of interest, i.e. 4–6 sections for the PVN to as much as 18 sections covering the full rostrocaudal extent of the arcuate nucleus and NTS. The coefficient of error (CE) of the sampling distribution was estimated by sub-sampling [22] and found to be below 10%, an ample precision compared to the overall observed coefficient of variation.

The results from the semi-quantitative ISH assessments of CB_1R mRNA levels in food-restricted male lean and obese Zucker rats are given in Fig. 2A and Table 1. Two-way ANOVA analyses of lean and obese Zucker rats revealed a significant effect of fasting and a significant genotype by fasting interaction in the VMH, in addition to a significant effect of genotype in the NTS and AP (Table 1). However, as seen in Fig. 2 fasting only affected CB_1R expression fasted in fa/fa rats. No changes were demonstrated in the nodose ganglion (Table 1 and Fig. 2A).

Two-way analysis of CART expression revealed a main effect of treatment and genotype, as well as a genotype by fasting interaction in the lean and obese Zucker rats (Table 1 and Fig. 2B). In the hypothalamic arcuate nucleus, the group-wise comparisons revealed a clear effect of fasting on CART expression of both lean and obese Zucker rats. In addition, CART mRNA was virtually absent in the arcuate of obese rats lacking a functional leptin receptor. In the brainstem, CART expression did not differ between genotypes, whereas a slight fasting-induced decrease in expression was observed across all genotypes.

The results from the semi-quantitative ISH assessments of CB_1R mRNA levels in food-restricted male SPD rats are given in Table 1 and Fig. 3. No significant CB_1R alterations could be established in the hypothalamic PVN or VMH, in the brainstem NTS or AP, nor in the vagal nodose ganglion hereby corroborating the findings from lean Zucker rats.

Despite an extensive body of literature describing the central location of CB_1 receptors and a wealth of studies demonstrating a potent appetite suppression and body-weight reduction following acute or chronic administration of CB_1R antagonists [2,5,14,24] very little information exists on the possible regulation of central CB_1R mRNA expression in respect to deficiency of food and the leptin receptor. In the present study we demonstrate that CB_1R transcription is unaffected during fasting conditions in the hypothalamus, brainstem and nodose ganglion whereas CB_1R mRNA are generally affected by leptin receptor deficiency reaching significance in the hypothalamic VMH and brainstem NTS.

Recently, several lines of evidence have converged, indicating that the effects of CB_1R blockade on food intake and body-weight may not be limited to a central mode of action. The expression of CB_1 receptors in the nodose ganglion has been reported to be up- and down-regulated during fasting and feeding, respectively indicating that CB_1 receptors on afferent vagal neurons may be involved in the transmission and processing of gut food-stimulated signals important in the control of food intake and meal size [3]. In the present study, however, we were unable to detect any CB_1R regulation in the nodose ganglion of food-restricted Zucker rats, as corroborated in fasted SPD rats. One may speculate that the fasting regimen used in the present study was ineffective, but as CART mRNA expres-