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REVIEW

The role of relaxin-3 and its receptor RXFP3in defense of elevated body weight in diet-induced obesity

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Incidence of overweight and obesity has dramatically increased during the past three decades. Treatment of this serious clinical problem is hindered by the fact that once obesity has developed, the elevated body weight is defended against weight-decreasing treatment strategies by mechanisms that are not yet fully understood. This review focuses on the neuronal mechanisms that contribute to the maintenance of obesity after it development in the DIO rat model. Among the neuronal factors regulating energy intake, orexigenic neuropeptide relaxin-3 and its cognate receptor RXFP3 may play an important role in the defense of elevated body weight in DIO. The levels of expression of relaxin-3 mRNA in the brainstem nucleus incertus (NI) were significantly increased in thead libitum feeding state in DIO rats compared to DR rats. However, the effects of relaxin-3 in the DIO ad libitum-fed rats may be compensated by a significant decrease in the levels of expression of RXFP3 mRNA in the food intake-regulating brain regions of DIO rats including the paraventricular hypothalamic nucleus (PVN), central amygdala (CeA), NI, and nucleus of the solitary tract (NTS). Remarkably, the DIO rats showed an immediate rebound in food intake at refeeding and regained all body weight lost during starvation. This significant increase in food intake during refeeding was accompanied by an increase in the levels of expression of RXFP3 in the parvocellular PVN, CeA, NI, and NTS in the DIO rats to the levels of the DR rats. Moreover, the expression of RXFP3 in the paraventricular thalamic nucleus was significantly higher in the refed DIO rats compared to the DR counterparts. A constitutive increase in the expression of relaxin-3 accompanied by a relative increase in the expression of RXFP3 in food intake-regulating brain regions during refeeding after food deprivation may contribute to the mechanisms of defense of elevated body weight in the DIO phenotype.

Keywords: Relaxin-3; RXFP3; diet-induced obesity; rat model; food intake

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Introduction

Incidence of overweight and obesity has dramatically increased during the past three decades. In fact, the population of overweight and obese individuals in the world has more than doubled from1980 to reach 2.1 billion in 2013 [1]. Despite multiple prevention and treatment initiatives against this serious problem threatening public health, no country has succeeded in significantly decreasing obesity [1-3]. Dietary therapy remains the first-line treatment for the

majority of obese patients to provide the health benefits of a decrease in body weight and adiposity [4, 5]. However, numerous studies have shown that dietary restriction produces an effective weight loss within a relatively short initial period of several months but not in longer periods [6-8]. In addition, for one to two thirds of dieters calorie-restricted diets are counterproductive because in the long-term the patients regain more weight than they lose on their diets [9,10]. This difficulty maintaining body weight loss suggests functioning of mechanisms that strongly defend elevated body weight in obese patients [11-14]. These mechanisms originate from a monogenic or polygenic basis. Although the monogenic forms of obesity induce the most extreme and early-developed obese phenotype, human obesity in the vast majority of cases is polygenic in origin [15-17].

An animal model closely representing human obesity developed on a polygenic basis is diet-induced obesity (DIO) in rodents. DIO rats fed a high-energy (HE) diet develop the obese phenotype in contrast to diet-resistant (DR) rats that maintain normal body weight on an HE diet [18-23].This review focuses on the neuronal mechanisms that contribute to the maintenance of obesity after it is established in the DIO rat model. Among the neuronal factors regulating energy intake, a recently discovered orexigenic neuropeptide relaxin-3 [24, 25] and its cognate receptor RXFP3 may play an important role in the defense of elevated body weight.

Relaxin family peptides and elaxin family peptides and its receptors

Relaxin-3, a 6 kDa peptide discovered in 2001 [24], belongs to the relaxin peptide family [26]. This family includes3relaxins and 4 insulin-like peptides (INSL 3-6), which are heterodimeric peptides with two disulfide bonds linking the A- and B-chains and an additional intra-A chain connection [26]. Unlike the other relaxins, which show considerable interspecies heterogeneity, relaxin-3 homology is well-conserved across species suggesting highly preserved and critical biological functions [27]. Relaxin family peptides bind to G protein-coupled receptors (GPCR). So far, four receptors of relaxins, the relaxin family peptide receptors (RXFP) 1-4, have been identified [28]. RXFP3 and RXFP4 are classic peptide ligand GPCRs and bindrelaxin-3 and INSL5 with high affinity [26 ,29]. The receptors RXFP1 and RXFP2 have a large extracellular N-terminal leucine-rich repeat domain and are the cognate receptors for relaxin (relaxin-2 in humans and primates and relaxin-1 in other mammals) and INSL3 [26]. Although relaxin-3 binds with high affinity to its cognate receptor RXFP3, it also binds to RXFP1 [30, 31] and RXFP4 [32]. In contrast to humans, the RXFP4 receptor gene is a non-functional pseudogene in rats [32, 33]. Therefore, in the rat brain the relaxin-3 signals may be mediated by RXFP1 and RXFP3 receptors. However, the affinity of relaxin-3 to its cognate receptor RXFP3is about 10 times higher than to RXFP1 [32, 34]. Importantly, RXFP3, the cognate receptor of

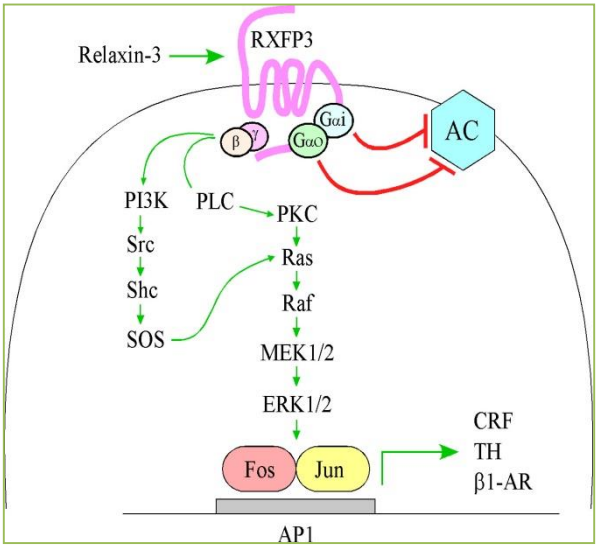

Figure 1. Intracellular signaling from relaxin-3 via its receptor RXFP3. RXFP3, the cognate receptor of relaxin-3, is a G protein-coupled receptor. Activation of RXFP3 by relaxin-3 leads to inhibition of adenylyl cyclase (AC) by $G\alpha_{i/o}$ subunits. Release of subunits from the G protein triggers activation of PI3K and PLC/PKC-dependent pathways leading to phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. Activation of ERK1/2 leads to the expression of immediate early gene c-*fos* and an increase in the AP1 complex formation (Fos-Jun complex, originally termed activator protein 1, AP1). The AP1 complex activates transcription of neuropeptides containing the AP1 binding motive in the gene promoter such as corticotropin-releasing factor (CRF), tyrosine hydroxylase (TH), β1-adrenergic receptor (β1-AR). ERK 1/2 - extracellular signal-regulated kinase; MEK 1/2 - mitogen-activated protein kinase; PI3K - phosphoinositide 3-kinase; PKC - protein kinase C; PLC - phospholipase C; Raf - serine/threonine-specific protein kinase; Ras - rat sarcome proto-oncogene; Shc - SH-containing proto-oncogene; SOS - son of sevenless guanine nucleotide exchange factor; Src - proto-oncogene tyrosine-protein-kinase.

relaxin-3, does not bind human relaxin or any other members of the insulin/relaxin peptide family [32]. RXFP3 is coupled to $G\alpha_{i/o}$ protein, and functional activation of RXFP3 by relaxin-3 results in inhibition of adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP) generation (Fig. 1) [26, 32, 34, 35]. RXFP3 activation and release of β and γ subunits from $G\alpha_{i/o}$ protein triggers activation of the protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)-dependent pathways leading to phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (Fig. 1) [26, 35]. Activation of ERK1/2 leads to induction of the expression of immediately early gene c-fos and an increase in the AP1 complex formation (Fos-Jun complex, originally termed activator protein 1, AP1) [36]. The AP1 complex activates transcription of a number of peptides that function as neurohormones, receptors of neurotransmitters, or neurotransmitter-producing enzymes such as corticotropin-releasing factor (CRF), tyrosine hydroxylase (catalyzing a rate-limiting step in the synthesis of catecholamine neurotransmitters), and β1-

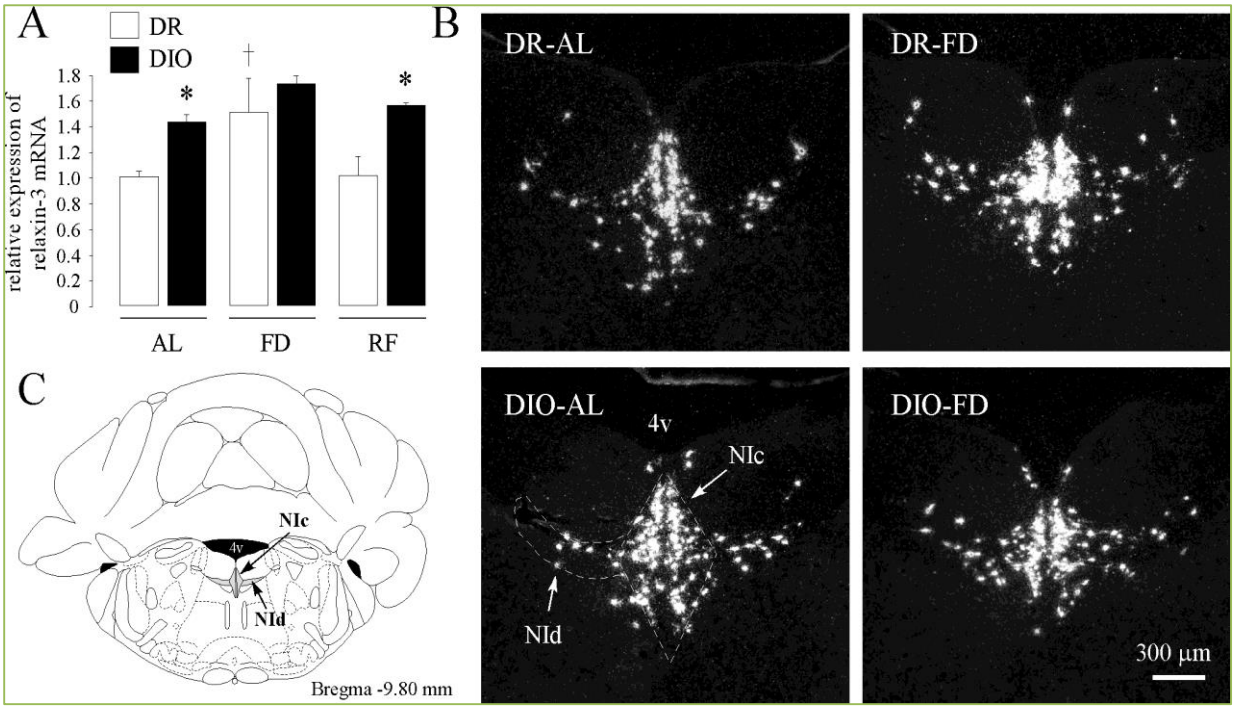

Figure 2. Expression of relaxin-3 in the diet-induced obese (DIO) model. The DIO rats show higher expression of relaxin-3 in the compact part of the nucleus incertus (NIc, pars compacta) at feeding (fed *ad libitum*, AL; or refed for 1 h after 12 h of food deprivation, FD) states compared to the diet-resistant (DR) rats. In contrast, 12 h of food deprivation (FD) significantly increased relaxin-3 expression in the NIc in the DR rats but not the DIO rats. A, The relative levels of expression of relaxin-3 mRNA in the NIc.B, Dark-field micrographs showing the positive hybridization signal of relaxin-3 mRNA in the NIc and pars dissipata of NI (NId) of the DR and DIO rats fed *ad libitum* (DR-AL, DIO-AL; left micrographs) or after 12 h of food deprivation (DR-FD, DIO-FD; right micrographs). C, Diagram of the coronal rat brain section [154] 9.80 mm caudal to the bregma showing the location of the NIc and NId. *Significantly ($p<0.05$) different compared to the DR rats in the same feeding condition. †Significantly different compared to the AL rats in the same phenotype. 4v–fourth ventricle. Modified from [67]. Reprinted with permission (Elsevier; 3416091293348).

adrenergic receptor [37-39]. Acute intracerebroventricular (icv) injection of relaxin-3 induced an increase in Fos protein expression, a molecular marker of neuronal activation, in the hypothalamic regions such as the paraventricular hypothalamic nucleus (PVN), arcuate hypothalamic nucleus (ARC), supraoptic nucleus(SON), and lateral hypothalamus [40, 41]. Relaxin has been classically known for its role in reproduction and parturition as well as in vasodilation and cardiac stimulation [42]. Accordingly, expression of relaxin and RXFP1 is widely distributed in the central and peripheral tissues including the brain, heart, skin, liver, ovaries, and testes [26]. Conversely, the expression of relaxin-3 and its cognate receptor RXFP3 is almost exclusively confined to the brain [24, 25, 34, 43-45].

Expression of relaxin-3 and RXFP3 in the brain

Relaxin-3is expressed by neurons in the brainstem, but broadly innervate the forebrain including the entire limbic system and hypothalamus [26]. Neuroanatomical studies conducted in the rat and mouse have revealed that relaxin-3 is strongly expressed within the neurons of the pontine nucleus in certus (NI), while smaller populations are also present in the pontineraphé, periaqueductal gray, and in a region dorsal to the substantia nigra [25, 44-46]. The majority of the NI relaxin-3 neurons produces inhibitory neurotransmitter-aminobutyric acid (GABA) and co-expresses the CRF type 1 receptor [46]. CRFicv administration induces expression of the immediate early gene c-fos in NI relaxin-3 neurons [46]. Identification of dense-core peptide vesicles in the neuronal perikarya and presynaptic terminals of relaxin-3 neurons strongly suggests that relaxin-3 plays the role of neurotransmitter [46]. The NI relaxin-3 neurons widely project to the hypothalamus, septum, cortical, and limbic brain regions [45,46]. Histochemistry, in situ hybridization, and autoradiography have shown that distribution of RXFP3 mRNA largely overlaps the binding sites of relaxin-3 and distribution of relaxin-3-positive axonal terminals [44-47]. Thus, the density of relaxin-3 fibers as well as RXFP3 mRNA and binding sites is high in the brainstem and hypothalamic regions such as the nucleus of the solitary tract (NTS), NI,PVN, SON, the periventricular and lateral hypothalamic areas as well as in the septum, hippocampus, central and medial amygdala, and paraventricular thalamic nucleus (PVT) [43]. This large brain distribution of the relaxin-3/RXFP3 system suggests multiple functional implications for relaxin-3. There is evidence that relaxin-3

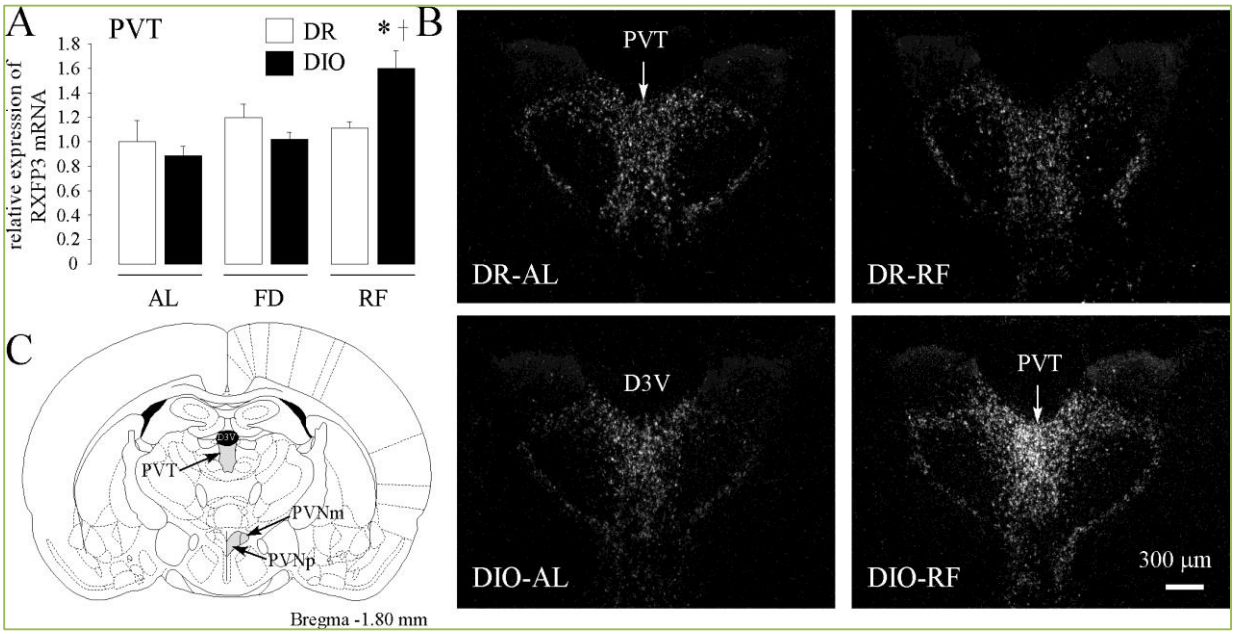

Figure 3. Expression of RXFP3 in the paraventricular thalamic nucleus (PVT) in the diet-induced obese (DIO) model.RXFP3 expression increased significantly in the PVT of the DIO rats but not the diet-resistant (DR) rats during refeeding. A, Relative levels of expression of relaxin-3 mRNA in the PVT of DIO and DR rats fad *ad libitum* (AL), food deprived for 12 h (FD), or refed for 1 h after 12 h of food deprivation (RF). B, Dark-field micrographs showing the positive hybridization signal of RXFP3 mRNA in the PVT of *ad libitum*-fed (-AL) or refed (-RF) DR rats (top micrographs) and DIO rats (bottom micrographs). C, Diagram of the coronal rat brain section [154] 1.80 mm caudal to the bregma showing the location of the PVT as well as the magnocellular (PVNm) and parvocellular (PVNp) parts of the paraventricular hypothalamic nucleus. *Significantly ($p<0.05$) different compared to the DR rats in the same feeding condition. †Significantly different compared to the AL rats in the same phenotype. D3V – dorsal third ventricle. Modified from [67].Reprinted with permission (Elsevier;3416091293348).

signaling is implicated in stress and anxiety [41,48-50], feeding and metabolism [48, 51-53], motivation, reward, and arousal [49, 54, 55].

Role of relaxin-3 and RXFP3 in food intake regulation

Behavioral studies have demonstrated an important role of relaxin-3 in food intake regulation [56-59]. Central administration ofrelaxin-3 strongly stimulated feeding in satiated rats. Acute icv injections of relaxin-3 increased food consumption during 1 h after administration during early light and dark phases [59]. A similar increase in food intake was demonstrated after icv administration of specific agonists of RXFP3 (e.g., R3/I5, RXFP3 analogue 2) [60]. Blockade of the orexigenic effects of relaxin-3 by specific RXFP3 antagonists (e.g., R3 (BΔ23-27) R/I5 or RXFP3 analogue 3) provided evidence that an increase in food intake induced by relaxin-3 was specifically mediated by RXFP3 [60-62]. The neuron populations that mediate orexigenic effects of relaxin-3 include several RXFP3-expressing hypothalamic regions because microinfusions of relaxin-3 into the PVN, ARC, SONas well as in the anterior preoptic area strongly stimulated feeding [52, 59, 63]. In contrast to rats, icv or intra-PVN injections of RXFP3 agonists did not increase feeding in satiated or mildly food deprived mice, whereas administration of an RXFP3 antagonist reduced food intake [54]. These between-species variations indicate a possible difference at the basal tone of relaxin-3 or at the levels of RXFP3 expression in the food intake-regulating areas of the mouse and rat brains.

In addition to significant hyperphagia, chronic and subchronic icv or intra-PVN administration of human relaxin-3 or a specific RXFP3 agonist induced increased fat accumulation and body weight gain [51, 52, 64]. Bilateral intra-PVN injections of recombinant adeno-associated virus (rAAV) expressing a specific RXFP3 agonist R3/I5 increased daily food intake and body weight gain in adult rats [53]. Specific knockdown of relaxin-3 expression induced by infusion of rAAV silencing relaxin-3 expression did not alter the rats' food intake or body weight on regular chow [65]. However, a specific strain of relaxin-KO mice fed a high fat diet were leaner than congenic controls [64] suggesting that relaxin-3 may be specifically involved in diet-induced obesity. The expression ofralaxin-3may be perturbed by physiological challenges such as stressful conditions, food restriction, ordiet-induced obesity [46, 48, 66, 67]. The levels of expression of relaxin-3 mRNA in the compact part of the NI (NIc) were significantly increased in the DIO rats compared to the DR rats maintained on free-feeding (ad libitum) access to an HE diet since weaning (Fig. 2).In these rats, the expression of relaxin-3 mRNA in the NIc was estimated at

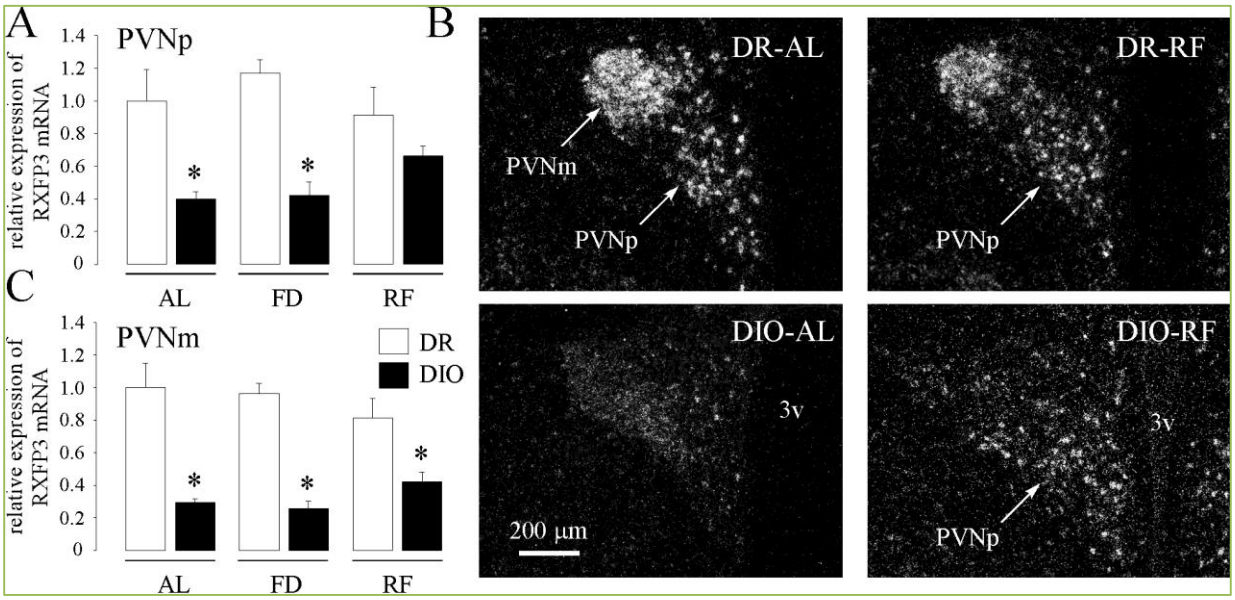

Figure 4. Expression of RXFP3 in the paraventricular hypothalamic nucleus (PVN) in the diet-induced obese (DIO) model. The levels of expression of RXFP3 mRNA were significantly decreased in the parvocellular (PVNp; panel A) and magnocellular (PVNm; panel C) parts of the PVN in DIO rats fed *ad libitum* (AL) and food deprived (FD) for 12 h compared to the DR rats in similar feeding conditions. One hour of refeeding (RF) increased the expression of RXFP3 in the PVNp but not the PVNm of the DIO rats to the levels of the DR rats. B, Dark-field micrographs showing the positive hybridization signal of RXFP3 mRNA in the PVNp and PVNm of the*ad libitum*-fed (-AL) or refed (-RF) DR rats (top micrographs) and the DIO rats (bottom micrographs). *Significantly ($p<0.05$) different compared to the DR rats in the same feeding condition. 3v – third ventricle. Modified from [67].Reprinted with permission (Elsevier; 3416091293348).

the 8th week of age after they had been maintained on an HE diet for 5 weeks, and the obese phenotype and hyperinsulinemia developed in the DIO but not the DR rats [67]. Overnight food deprivation significantly increased the levels of expression of relaxin-3 in the NIc in the DR rats but not the DIO rats suggesting that the expression of relaxin-3 in the DR rats but not the DIO rats was regulated by a negative metabolic state [67].

DIO model: development and behavioral and physiological characteristics

The monogenic forms of obesity such as those caused by proopiomelanocortin (POMC) or melanocortin-4 receptor gene mutations lead to early-onset severe obesity [68, 69]. However, monogenic forms of obesity in the human population are relatively rare [70]. The genetic predisposition to obesity is mainly based on a polygenic basis that in the obesogenic environment triggers an increase in energy intake and body weight gain [15-17]. An animal model of diet-induced obesity constitutes a reliable approach for studying the most common human obesity syndrome. In this model, the animals predisposed to develop DIO gain body weight at rates comparable to animals fed a low-energy diet and do not become obese unless they are fed a HE diet [71-73]. In outbred Sprague-Dawley (SD) rats, about one-half of the rats develop DIO on an HE diet. The rest of the rats are diet-resistant rats that gain weight and fat at the rate comparable to the chow-fed controls. The obese phenotype was exacerbated by selective breeding of DIO rats during 3-5 generations[74] and gestational obesity [75].

A highly palatable HE diet with high fat content increased food intake in DR and DIO rats compared to the chow-fed control group; however, DIO rats showed higher hyperphagia compared to DR rats [76]. In addition to increased food intake, feed efficiency (the ratio of weight gained to calories consumed) was significantly increased in DIO rats compared to DR rats [76,77]. Because of the increased food intake and feed efficiency, only the HE-diet-fed DIO rats but not the HE-diet-fed DR rats developed visceral obesity, hyperleptinemia, hyperinsulinemia, hypercortisolemia, and dyslipidemia [20]. The increased body weight gain in DIO rats compared to DR rats was primarily due to differences in adiposity, because these phenotypes have similar lean body mass [23, 74, 78]. Differential body composition between the DIO and DR phenotypes is based on the profound metabolic changes in the DIO rats. An assessment of the respiratory quotient in DIO rats showed that they preferentially use carbohydrates as their main energy substrate while DR rats might preferentially consume fat [79]. Therefore, the dyslipidemia and visceral obesity of DIO rats depend on higher efficiency for storing fat and a lower efficiency in consuming it.

Analyses of the feeding microstructure of DIO rats showed that the hyperphagia of DIO rats was produced by an

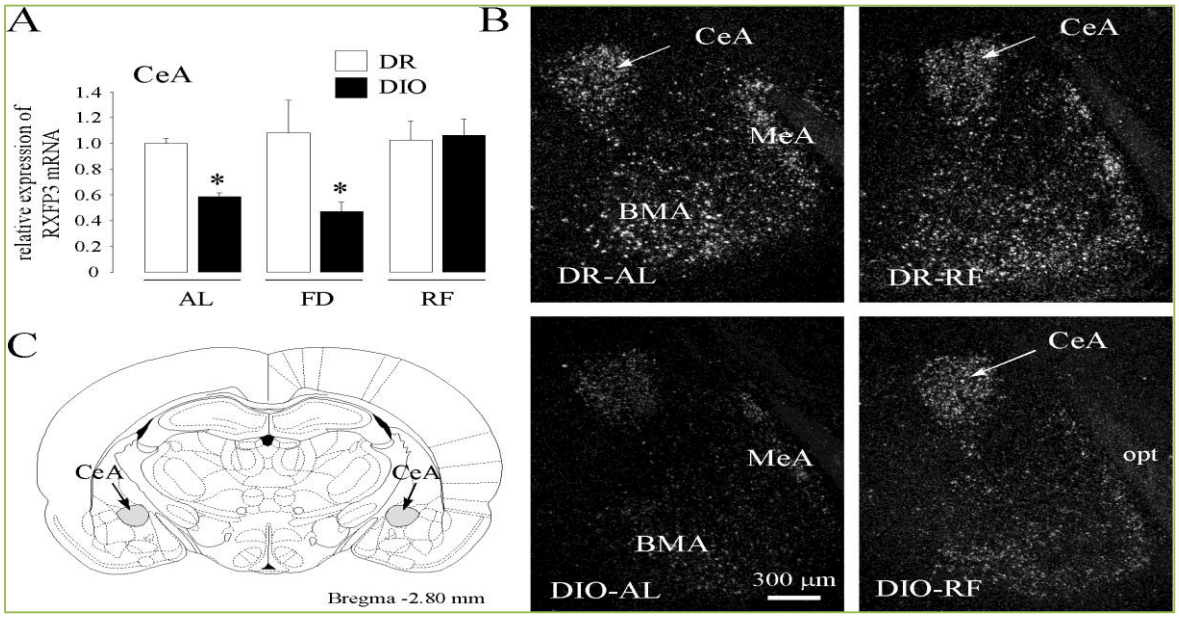

Figure 5. Expression of RXFP3 in the central amygdala (CeA) in the diet-induced obese (DIO) model. The levels of expression of RXFP3 mRNA were significantly lower in the CeA in the DIO rats fed *ad libitum* (AL) and food deprived (FD) for 12 h compared to the DR rats in similar feeding conditions. One hour of refeeding (RF) increased the expression of RXFP3 in the CeA in the DIO rats to the levels of the DR rats. B, Dark-field micrographs showing the positive hybridization signal of RXFP3 mRNA in the CeA of the*ad libitum*-fed (-AL) or refed (-RF) DR rats (top micrographs) and the DIO rats (bottom micrographs). C, Diagram of the coronal rat brain section [154] 2.80 mm caudal to the bregma showing the location of the CeA. *Significantly ($p<0.05$) different compared to the DR rats in the same feeding condition. BMA – basomedial amygdala; MeA – medial amygdala; opt – optic tract.Modified from [67]. Reprinted with permission (Elsevier; 3416091293348).

increase in meal size, but not number [80]. Meal size is generally enhanced by the increased hedonic value of food [81-83] suggesting that DIO rats have abnormalities in the functioning of the brain reward system. The nocturnal satiety ratio, defined as the intermeal interval divided by the energetic value of the previous meal, was significantly lower in the HE-diet-fed DIO rats compared to the HE-diet-fed DR rats and the chow-fed controls [80,84]. A deficit in the maintenance of postmeal satiety was revealed by shorter latencies to initiate feeding, faster eating reinitiating after meal completion, and consumption of larger meal bouts in DIO rats [80, 84].

Dysfunction in the integration of peripheral signals in DIO

There is no convincing evidence that increased effects of ghrelin, the only peripheral orexigenic peptide known to date [85, 86], is a proximate cause of diet-induced obesity. DIO rats showed significantly lower levels of plasma ghrelin at dark onset and similar levels 6 h later compared to the DR rats [87]. In addition, the arcuate and dorsomedial hypothalamic nuclei in the DIO rats expressed lower levels of the ghrelin receptor, growth hormone secret agogue receptor (GHS-R), compared to the DR rats [87].

An increase in food intake in DIO rats was maintained despite significantly higher plasma levels of leptin and insulin [76, 88]. Leptin and insulin have been suggested as the "adiposity signals" to the brain in the long-term regulation of body weight [89]. An increase in adiposity raises the plasma levels of leptin and insulin that inhibit the neurons producing orexigenic neuropeptide Y (NPY) and activate the neurons producing anorectic neuropeptide POMC leading to a decrease in food intake [89-92]. However, despite the2-3 times increase in plasma leptin levels, DIO rats maintained persistent hyperphagia and became more obese compared to chow-fed DIO rats and DR rats fed chow oran HE diet [88]. It seems that the low efficiency of leptin and insulin in decreasing food intake in DIO rats depends on the decreased capacity of the hypothalamic regions of the DIO rats to bind leptin and insulin [93]. Moreover, even before DIO-prone rats develop obesity on an HE diet, they had a pre-existing decrease in central leptin and insulin sensitivity [93].Pre-obese DIO-prone rats showed a lower decrease in chow intake in response to icv insulin injections compared to DR rats [94]. Adolescent pre-obese DIO-prone rats had less expression of leptin receptor mRNA in the ARC compared to the DR counterparts [95]. Peripheral injection of leptin produced significantly less anorexia and lower hypothalamic phosphorylation of the signal transducer and activator of transcription 3 (pSTAT3), a signal molecule downstream to the leptin receptor, in the DIO-prone rats compared to the

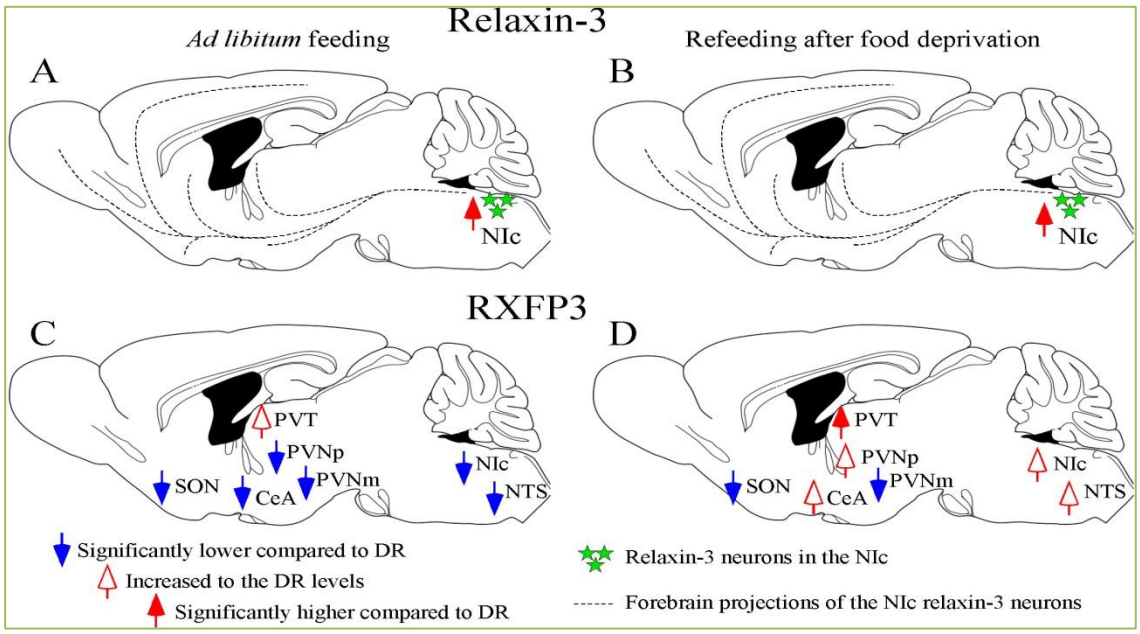

Figure 6. Dynamics of expression of relaxin-3 (A, B) and its receptor RXFP3 (C, D) in the brain of diet-induced obese (DIO) rats fed *ad libitum* (A, C) or refed after food deprivation (B, C) shown inthe sagittal rat brain sections[154]. DIO rats have increased levels of expression of relaxin-3 in the NIc at *ad libitum* feeding (panel A) and refeeding after food deprivation (panel B) compared to diet-resistant (DR) rats. However, in the*ad libitum*-fed state the effects of increased relaxin-3 may be compensated in DIO rats by a significant decrease in the levels of expression of RXFP3 in several brain regions (panel C). Conversely, during refeeding the levels of RXFP3 expression increased in DIO rats to the levels detected in the DR rats in the PVNp, CeA, NIc, and NTS. In addition, at refeeding the levels of expression of RXFP3 in the PVT were significantly higher in the DIO rats compared to the DR rats (panel D). An increase in RXFP3 expression during refeeding accompanied by constitutive overexpression ofrelaxin-3 may contribute to the mechanism of rapid regainingof body weight lost during food deprivation in the DIO model. CeA – central amygdala; NIc - compact part of the nucleus incertus; NTS – nucleus of the solitary tract; PVNm – magnocellular part of the paraventricular hypothalamic nucleus; PVNp – parvocellular part of the paraventricular hypothalamic nucleus; PVT – paraventricular thalamic nucleus; SON – supraoptic nucleus.

DR-prone rats [95]. This lower sensitivity of young DIO rats to leptin was not due to a defective blood-brain barrier (BBB) since leptin transport across the BBB was comparable between the DIO- and DR-prone rats [95]. In contrast, leptin BBB transport was decreased by the full development of the obese phenotype [95]. However, a decrease in BBB transport in DIO rats with fully developed obesity does not entirely account for the blunted response to leptin. Indeed, brain overexpression of leptin by icv administration of rAAV encoding leptin produced anorectic effects in the chow-fed control and DR rats maintained on an HE diet. Conversely, the DIO rats fed a HE diet were completely unresponsive to central rAAV-induced leptin expression [96]. These experiments suggest that responsiveness to the leptin receptor is significantly decreased in the brains of DIO rats. Indeed, direct measurements of leptin receptor mRNA expression in the DIO rats maintained on an HE diet showed a decrease in the levels of leptin receptor expression in the arcuate, dorsomedial, and ventromedial hypothalamic nuclei in the DIO rats compared to the DR rats [87]. Therefore, hyperphagia of DIO rats seems to depend onseveral pre-existing or developed on HE diets abnormalities in orexigenic and anorectic brain mechanisms.

Neuronal characteristics of the DIO model

At the neuronal level, diet-induced obesity was associated with synaptic remodeling and misbalance between excitatory and inhibitory neurotransmission [97]. There is evidence that some of these neuroadaptations take place to normalize a number of abnormalities in neuronal functions detected in pre-obese chow-fed DIO-prone rats [98]. For example, DIO-prone rats showed abnormalities in brain serotonin turnover that may predispose them to become obese when fat and caloric density of diet is increased. However, once diet-induced obesity developed, these abnormalities in serotonin turnover were normalized [98]. Pre-obese chow-fed SD rats may be separated as being prone to become DIO or DR by their high versus low 24-h urine norepinephrine (NE) output, respectively [22, 99]. In addition, the pre-obese DIO-prone rats had a greater intra-carotid glucose-induced plasma NE increase than the DR-prone rats [22, 100, 101], and their plasma NE levels increased more vigorously during refeeding after food deprivation [21]. However, the central response of the DIO-prone rats to glucose was generally lower compared to that in the DR-prone rats [101-104]. Indeed, 1-h intra-carotid glucose infusion selectively increased hypothalamic Fos expression in the inbred DR rats but not in the DIO rats [105].

In addition, the DIO rats showed an impaired counter regulatory response to insulin-induced hypoglycemia [106], and bilateral NE infusions in the PVN increased plasma NE in the DR-prone rats but not the DIO-prone rats [107]. Hypothalamic NE turnover and endogenous NE in the PVN were significantly decreased, whereas in the median eminence, a circumventricular organ with disrupted BBB, NE turnover was increased by 100% in DIO-prone rats [99]. Similar to normalization of serotonin turnover, 3 months on an HE diet resulted in comparable 24-h urine NE in DIO and DR rats [22].

Not all pre-existing neuronal abnormalities are normalized by the development of obesity. Thus, DIO rats showed lower basal and postprandial brain glucose utilization even after 3 months on an HE diet [108]. In addition, DIO rats regardless of their diet had lower hippocampal glucocorticoid receptor (GR) and central amygdala (CeA)CRF mRNA expression than DR rats [109]. A decrease in the expression of the anorectic and the rmogenic neuropeptide CRF in DIO rats may contribute to a higher body weight gain and higher feed efficiency in DIO rats submitted to a session of restraint stress compared to their unstressed DIO counterparts [110]. Indeed, stress was associated with reduced expression of the leptin receptor in the dorsomedial nucleus and CRF in the CeA specifically in the DIO rats fed a HE diet [110]. Therefore, the acutely stressed DIO rats showed a decrease in the anorectic effects of stress and an increase in the lipogenic and hyperphagic potential of circulating corticosterone [110]. The DIO rats submitted during 5 weeks to daily unpredictable stress, showed a lower increase in PVN CRF mRNA expression and a higher increase in food intake and body weight gain compared to the non-stressed DIO rats and the stressed and non-stressed DR counterparts [111]. In the brain, the anorectic effects of CRF are mainly mediated by the CRF type 2 receptor (CRF-2R) [112-114]. In addition to lower expression of CRF in the CeA in basal conditions [109] and in the PVN in stressful conditions [110,111], DIO rats have reduced sensitivity to the anorectic effects of a specific CRF-2R agonist urocortin 2 [84].

There is evidence that the intrinsic tone of another stress- and food intake-regulating neurohormone oxytocin expressed in the magnocellular neurons of the PVN and SONis substantially decreased in diet-induced obesity [115]. A decrease in oxytocin effects may contribute to the development of obesity because central and peripheral administration of oxytocin reduces food intake in fasted normal-weight rats [116] and decreases food intake and body weight gain in DIO rats [117]. Furthermore, oxytocin and oxytocin-receptor deficient mice were characterized by development of late-onset obesity [118, 119].

In the ARC, cocaine and amphetamine-regulated transcript (CART), POMC, and a product of POMC enzymatic cleavage, α-melanocyte-stimulating hormone (α-MSH), were recognized as anorectic neuropeptides suppressing feeding behavior [120-122]. DIO rats maintained on an HE diet expressed lower levels of POMC, α-MSH, and CART in the ARC compared to the DR counterparts [123,124]. However, this effect may be modified by diet composition and metabolic state:Feeding of a highly palatable cafeteria diet during 2 months increased POMC but not α-MSH expression in the ARC of the DIO rats compared to the DR rats [125]; conversely, when the DIO rats fed a highly palatable diet switched to low-energy chow, the levels of ARC expression of POMC mRNA decreased [126]. Such modulation of the expression of these anorectic neuropeptides suggests functioning of the neuronal mechanisms defending elevating body weight in DIO rats when caloric value of a diet changes.

Defense of elevated body weight in DIO rats: the NPY and relaxin-3 related mechanisms

There is good evidence that an individual organism maintains a certain level of homeostatic stability by all ostatic (i.e. "remaining stable by being variable") adjustments to the immediate conditions [127,128]. In the DIO model, diet composition and genetic background interact to reveal the underlying weight gain phenotype, which is manifested only if rats are maintained on an HE diet. After establishing, the higher set points in body weight regulating mechanisms are difficult to revoke [71, 73, 129]. It seems that with developed diet-induced obesity several neuronal mechanisms come into play to adjust food intake and energy expenditure against metabolic challenges to defend the elevated body weight in DIO rats.

Pre-obese chow-fed DIO-prone rats have physiological abnormalities that contribute to the development of obesity on an HE diet. Increased 24-h urine NE excretion [22] and hypothalamic NPY mRNA expression are examples of pre-obese abnormalities of DIO-prone rats [74]. NPY is a 36-amino acid neuropeptide that potently stimulates food intake in satiated states [130]. Food deprivation increases and refeeding restores the basal expression of prepro-NPY mRNA in the ARC in normal-weight rats [91]. Interestingly, during the pre-obese period, constitutively increased levels of NPY expression in DIO-prone rats did not show up-regulation by food deprivation that was normally detected in DR-prone rats [131]. Similarly, DR rats but not DIO rats with fully developed obese phenotype showed an increase in relaxin-3 expression in response to food deprivation (Fig. 2).

Pre-obese abnormalities of DIO-prone rats such as the increased hypothalamic expression of NPY contribute to the DIO-prone rats to develop obesity on an HE diet. Once the DIO phenotype is established, the increased weight gain persists even when rats are switched back to a low-energy chow diet [71, 73]. For example, the increased body weight and fat deposition in DIO rats fed an HE diet for 3 months

persisted for up to 3-4 months after the rats were switched to chow [71,73]. To lose body weight and carcass fat to levels comparable to those in the HE diet-fed DR counterparts, the energy intake of DIO rats has to be forcibly restricted.

Full development of the obese phenotype in DIO rats on an HE diet was accompanied by normalization of or compensation for physiological parameters such as urine NE levels [98]. Maintaining DIO rats on an HE diet for 12 weeks that induced the development of obesity and hyperleptinemia, decreased the expression of ARC NPY to levels that were even lower than those detected in the HE- and chow-fed DR rats [132]. The decrease in hypothalamic NPY expression in DIO rats with an established obese phenotype was accompanied by a compensatory increase in the binding capacity of the NPY receptors in the hypothalamus, CeA, and hippocampus [133]. Interestingly, in DIO rats with established obesity, ARC expression of NPY became sensitive to metabolic challenges and up-regulated by food restriction [132] or switching to a low-fat diet after an HE diet[134]. Such adaptations may contribute to the immediate rebound in food intake and regaining all lost body weight during food restriction when DIO rats are given ad libitum access to chow after food restriction [129, 135].

The orexigenic effects of relaxin-3 constitutively over expressed in ad libitum-fed DIO rats with an established obese phenotype may be compensated by a significant decrease in the levels of expression of RXFP3 in specific brain regions such as the parvocellular and magnocellular parts of the PVN (PVNp and PVNm, respectively), SON, CeA, as well as in the brainstem in the NIc and NTS [67]. Conversely, in thead libitum-fed state the PVT expressed comparable levels of RXFP3 between the DIO and DR phenotypes. During refeeding, the expression of RXFP3 in the PVT was significantly higher in DIO rats compared to the DR counterparts (Fig. 3). The PVT integrates the visceral and hypothalamic signals and relays them to the limbic regions and ventral striatum [136, 137], thus regulating arousal and feeding. The role of relaxin-3 in arousal and circadian activity has been suggested based on the anatomical and behavioral studies [44, 138]. In the magnocellular brain regions, the PVNm and SON, relaxin-3 regulates water intake [40] and expression of oxytocin and vasopressin [53, 139]. However, our study demonstrated a significant decrease in the levels of expression of RXFP3 mRNA in the PVNm (Fig. 4, C) and SON [67] in all experimental feeding conditions including ad libitum feeding, 12 h of food deprivation, and 1 h of refeeding in the DIO rats compared to the DR counterparts. The stable decrease in the RXFP3 expression in the magnocellular brain regions in the DIO rats regardless of feeding conditions does not indicate a particular role of these areas in food intake regulation in the DIO model according to the feeding states at least in the short-term food deprivation and refeeding treatment used in this study [67]. In contrast to the PVNm and SON, the levels of expression of RXFP3 in the PVNp (Fig. 4) as well as in the CeA (Fig. 5), NIc, and NTS increased by 1-h refeeding in the DIO rats to the levels detected in the DR rats [67]. These brain regions that show a relative increase in the levels of RXFP3 expression in the refed DIO rats are directly involved in the food intake-regulating network. Postprandial activation of the PVNp, CeA, and NTS but not the PVNm and SON depends on the integrity of the visceral vagal afferents [140]. The NTS is the first brain relay for the vagal sensory signals, and the NTS neurons directly project to the NI, CeA, and PVNp [140-142]. The direct effects of relaxin-3 in the CeA and NTS have not yet been shown, but given the important role of these brain structures in feeding behavior [143-147], increased RXFP3 expression in the CeA and NTS in DIO rats during refeeding may contribute to an increase in sensitivity to the orexigenic effects of relaxin-3. In the NI, RXFP3 may be involved in autoregulating the expression of relaxin-3 or other neuropeptides expressed in this brain nucleus such as galanin, cholecystokinin, and somatostatin [56,148]. A relative increase in RXFP3 expression in the PVNp (Fig. 4A, B), the key regulator of the hypothalamic pituitary adrenal (HPA) axis activity [149], may play a role in the stronger activation of the PVNp and HPA axis to food deprivation and refeeding in the obese phenotype [150-152]. In addition to an important role in neuroendocrine hypophysiotropic activation of the HPA axis, the PVNp is directly involved in food intake regulation [153], and direct administration of relaxin-3 or a specific RXFP3 agonist in the PVN significantly increased feeding [53].

Therefore, the relative increase in the expression of the cognate relaxin-3 receptor RXFP3 in DIO rats in key brain regions involved in food intake regulation (Fig. 6) concomitantly with a constitutive increase in relaxin-3 expression in the NIc in the DIO rats may contribute to a significant increase in food intake in the DIO rats during refeeding after food deprivation compared to the DR rats [67] and rapid regaining of body weight lost during food deprivation [129, 135].

Conclusions

Investigating the neuronal mechanisms related to the development and maintenance of the DIO phenotype has revealed that DIO rats have pre-obese abnormalities such as increased NE plasma and urine levels, increased ARC NPY expression, and decreased sensitivity to leptin and insulin. Some of these abnormalities such as low sensitivity to leptin and insulin persist while obesity develops on an HE diet. However, some pre-obese abnormalities such as increased peripheral NE or ARC NPY normalize or compensate to levels similar to (such as the NE levels) or even lower than (such as the expression of NPY) those of the DR controls. This “normalization” seems to occur when the body weight and adipose tissue parameters reach a certain elevated, compared to the DR rats, level. Such “normalization” or

"compensation" of same food intake-regulating factors may help in maintaining relative dynamic (allostatic) stability in body weight regulation. Similarly, a constitutive increase in relaxin-3 expression in DIO rats with fully developed obesity may be "compensated" to some degree by a significant decrease in the expression of RXFP3 in the strategic food intake-regulating brain regions. At this point, any metabolic challenge would trigger allostatic adjustments to return to relative stability (i.e., the elevated body weight in the DIO model). These allostatic adjustments apparently include an increase in NPY expression during food deprivation and an increase in RXFP3 expression during refeeding after food deprivation that may contribute to immediate rebound in food intake and rapid regaining of body weight lost during an energy shortage in DIO rats. These allostatic mechanisms defending the elevated body weight set point in DIO rats against food restriction or against switching to a low-energy diet make the diet-induced obese phenotype hardly reversible after full establishment on a HE diet.

Conflicting interests

The authors declare that they have no conflicting interests.

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