Published in final edited form as:

Neuropharmacology. 2012 June ; 62(8): 2447–2454. doi:10.1016/j.neuropharm.2012.02.019.

Feeding and Reward: Ontogenetic changes in an animal model of Obesity

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Abstract

Given that food is a natural reinforcement, deficits in the reward system can lead to disordered eating behavior, inducing or worsening an already existing pre-obese phenotype. In order to evaluate developmental, food-reward-related measures we used the OLETF rat, an animal model of early-onset overeating-induced obesity, and a natural CCK-1 receptor knockout. Dopamine-like-receptor type 1 (D1R) and D2R levels were examined in a reward-related brain area (Nac shell) and sucrose preference was assessed at selected time points from weaning to adulthood (postnatal day [PND]90). In addition, a group of OLETF was pair fed (PF) to the amount of food consumed by same age LETO controls (from weaning to PND90) to examine the contribution of overweight to the alteration in DR expression. In addition, we examined food “craving”-like behavior by analyzing microstructural patterns of licking a palatable liquid diet. OLETF rats expressed significantly lower D2R levels than LETO controls only on PND90. In PF OLETF, weight and D2R levels were normalized. In addition, OLETF presented exaggerated preference for the high sucrose concentration. After 30-day abstinence, OLETF rats presented significant increased initial rate of licking, suggesting food “craving”. Thus, adult OLETF rats demonstrated altered D2R signaling similar to drug-induced sensitization, suggesting a link with their avidity for sucrose and their abnormal craving response. However, the current findings of a late deficit appearance and the novel PF results suggest that deficits in the motivation/regulatory systems of the OLETF rat are a developing process (at least from weaning and on) depending on the overeating and obese phenotype of the rats and not only on the CCK mutation.

Keywords

D2R; Nucleus accumbens; OLETF; diet-restriction; Reward; obesity

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Conflicts of interest

The authors do not have any conflict of interest in the conduct and reporting of this research

The research reported in this paper was completed as part of the first author's graduate research, in the Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel.

Portions of this research were presented at the Annual Meetings of the Israel Society for Neuroscience (ISFN), Eilat, 2009, 2010.

1. Introduction

Given that food is a natural reinforcer, deficits in the reward system can lead to disordered eating behavior, inducing or worsening an already existing pre-obese phenotype. Previous studies on human subjects and rat models have shown that overweight individuals often **display** abnormal **feeding** behavior e.g., **a** high avidity for sucrose or **for** high fat food (De Jonghe et al., 2005; Figlewicz et al., 2007). Additional defects include a negative correlation between body mass index (BMI), Dopamine-like-receptor type 2 (D2R) (Wang et al., 2001) and hyposensitivity of the Dopamine, Opioid, and Acetylcholine systems (Avena et al., 2008; Davis et al., 2009), which all reflect defects in food-reward related measures. There are several possible mechanisms for the development of food-reward disruptions. These include genetic predisposition to reward deficiency and epigenetic modifications that are caused by environmental changes at different developmental periods. Pathological eating behavior which leads to alterations in gene expression of peptide hormones and neurotransmitters may alter brain mechanisms (such as dopamine signaling). Therefore overeating individuals are at risk for developing disruptions in the reward system, making them more vulnerable to develop obesity in an environment rich in palatable food.

In this research we studied the Otsuka Long Evans Tokushima Fatty (OLETF) rat, an animal model of early-onset overeating-induced obesity. Due to a spontaneous mutation, the OLETF strain lacks the expression of functional Cholecystokinin receptors type 1 (CCK-1). CCK is a brain-gut peptide that acts as a peripheral satiety signal and elicits the appearance of the behavioral satiety sequence (Smith and Gibbs, 1992a, b). OLETF rats exhibit hyperphagia and eventually become obese, an effect primarily due to the lack of CCK-1 receptors on their genotypic background. OLETF rats show pre-obese characteristics from infancy and sex-differences in patterns of obesity emergence (Schroeder et al., 2009a; Schroeder et al., 2009b). Research has showed that adult OLETF rats demonstrate high avidity for sucrose (De Jonghe et al., 2005; Hajnal et al., 2005), greater dopamine (DA) response to cocaine and amphetamine in the Nucleus accumbens (Nac) region (Feifel et al., 2003) and low D2R function and binding in the Nac shell area (Hajnal et al., 2008). Previous research in our lab revealed that OLETF males developed obesity in a gradual manner **until** PND 60-70, with specific “turning points” when their body weight diverged dramatically from controls. We now hypothesize that some of the factors responsible for the obesity turning points observed in the OLETF strain may be associated with disruptions in the mesolimbic dopamine-mediated reward system. **If this is so**, deficits in dopamine receptor expression **should** appear around the same time **that an outburst** of obesity **is** observed in developmental studies, i.e., before PND 90 (Schroeder et al., 2009a; Schroeder et al., 2009b).

In order to assess the *development of food-reward* and **its possible** contribution **to** the rats' obese phenotype, we studied OLETF and their lean Long-Evans Tokushima Otsuka (LETO) controls in 3 tests throughout development: 1. Sucrose preference tests (in order to obtain behavioral measures of the hedonic response). 2. Examination of DA-like-receptor type 1 (D1R) and D2R levels in a reward-related brain area. 3. Testing for food “craving”-like behavior – using palatable liquid food and analyzing microstructural patterns of licking after a cue reminder. Our aim was to examine possible differences in the OLETF rats' D1R and

D2R expression, compared to LETO controls and whether these differences appear at a specific developmental time window. In addition, by microstructural analysis of data collected with the lickometer device, we examined whether “compulsive” or “addictive-like” eating behavior follows these changes.

2. Material and methods

2.1 Subjects

OLETF and LETO rats were raised in the Specific Pathogen Free (SPF) colony of the Gonda Brain Research Center at Bar-Ilan University, Ramat-Gan, Israel. The progenitor rats were received as a generous gift from the Tokushima Research Institute, Japan. OLETF and LETO male offspring were housed together in sibling pairs from weaning and on (with the exception of the pair feeding group, where they were housed individually). In all of the experiments, polycarbonate cages (18.5 cm height × 26.5 cm width × 43 cm length) were used, with stainless steel wire lids and wood shavings as bedding material. In addition, large plastic tubes were present in all of the cages, available for use as a “house” or a hiding place for the rats. Standard chow (2018SC+F Teklad Global 18% Protein 6% Fat Rodent Diet HARLAN, Madison USA) and water were freely available (except when indicated for experimental manipulation). The animals were on a 12:12 hr light: dark cycle, with lights on at 06:00. Room temperature was maintained at 22±2°C. Food consumption was measured every day and the animal’s weight was measured every 5 days. The research protocol was approved by the Institutional Animal Care and Use Committee, and it adhered to the guidelines of the American Psychological Association and the Society for Neuroscience. All efforts were made to minimize animal suffering, to reduce the number of animals used.

2.2.1 Lickometers—To examine the microstructural pattern of sucrose solution intake in OLETF and LETO rats, animals were tested in lickometry cages (for details see Marco et al 2009). At the end of the test session, data were transferred for later analyses using a Matlab program.

2.2.2 Matlab program analysis—Licks were divided into meals and clusters. We used the criteria of 3 licks with interlick intervals of less than 0.25 s to initiate a meal/cluster (Davis and Smith, 1992). The intermeal interval (IMI) was defined as at least 300 sec without licking (Moran et al., 1998), and the criterion for ending a cluster was an interlick interval of 0.5 s or longer without licking (Davis and Smith, 1992). We used only the Clusters parameters in order to evaluate the positive and negative feedbacks according to the Davis and Smith parameters (Smith, 2001). The microstructural measures assessed were: total licks in the test, mean number of clusters per meal and mean number of licks in a cluster (cluster size). **Weibull function (WBL)** – this function was fit to quantify changes in the rate of licking during the test using the Levenberg-Marquardt nonlinear least squares method (Davis and Levine, 1977; Davis and Smith, 1988). The Weibull function has the form $y=A*\exp[-(B*t)^C]$ where t is the time in seconds, the A parameter is the initial rate of licking at $t=0$, the B parameter is the slope of the decline, and the C parameter provides a shape parameter that indicates how the function deviates from an exponential. The Weibull function was fit to the lick rate in licks per minute with a 1-min bin size, across 30 min of all

meals larger than 50 licks. Meals smaller than 50 licks were excluded because of the poor resolution (Rushing, 1997; Rushing et al., 1997). In the current study, all groups at all ages provided data from several meals, within this size limit, group mean number of meals ranged from 3-5.

2.3 Western Blot

The samples were homogenized on ice with homogenization buffer (EDTA 100mM, EGTA 50mM, Tris 100mM pH=7.4 and protease inhibitor (Sigma), NaVO₄ 100mM, NaF 500mM). Protein concentrations of the lysates were determined by Thermo protein assay kit (BCA™ Protein Assay Kit, PIR-23225, ORNAT, Rehovot, Israel). Equal amounts of protein (30µg) were separated by SDS-PAGE gel (upper gel - 6%, lower gel -7.5%) and transferred to nitrocellulose membranes (Membrane, 0.45µm, BioRAD Pure Nitrocellulose). After blocking (5% BSA) the membranes were incubated with either; D2 Receptor antibody (Rabbit polyclonal to Dopamine D2 Receptor, IgG, ab21218, Abcam) or D1 Receptor antibody (Rabbit polyclonal to Dopamine D1 Receptor, IgG, ab20066, Abcam) (1:2500) and GAPDH (Rabbit polyclonal to GAPDH, IgG, ab9485, Abcam) as a loading control (1:5000) at 4° C overnight. We used secondary antibody (Goat anti-rabbit IgG, SC-2004, Santa Cruz) for one hour at room temperature. The blots were developed by enhanced chemiluminescence (Thermo Scientific - Pierce ECL kit, Ornat, Rehovot, Israel) and detected by gel documentation equipment. The signal intensity was determined by the public domain NIH Image J program. For data analysis, within each gel, the OLETF Optical Density (OD) readings at each particular age (e.g. PND22) were expressed as the percent of the OD obtained from same-age LETO samples. The antibody was tested for its specificity with SVG cells. This analysis showed a *single* 52Kd “band”. Only “bands” of equal size were taken for analysis

3. Experimental Procedures

3.1 General procedure

All experiments were conducted at postnatal days (PND) 22, 38, 60 and 90. This was based on our previous work demonstrating significant steps in the development of OLETF obesity at these time points, compared to same age LETO controls (Schroeder et al., 2009a).

3.2 Experiment 1 (sucrose preference)

After weaning (PND 21-22), rats were transferred to a housing room next-door to the lickometer testing room. These rooms were outside of the SPF facility. The rats were maintained with filtered cage covers, except during the periods of lickometer testing. Eight rats from each strain (total N=16) were placed in the lickometer cages in the “Lickometer room” and were adapted to consumption of sucrose, for 5 consecutive days (day one-housed in pairs, day two-housed alone). On days 3-5, after 2 hr of food and water deprivation, animals were individually transferred again to the lickometry cages and their licking behavior during the 2 hrs was monitored. Thus test days from which data were analyzed at the first age ranged from PND 24-27. The rats were next tested repeatedly, at 3 additional time-points beyond weaning: PND 38, 60 and 90. Pilot studies showed that in these ages there was no need for the adaptation period, therefore at each of these ages the test lasted

only 3 consecutive days. The actual testing days ranged from PND 38-41, PND 58-62 and PND 88-92. Between these time-points the rats were housed under the conditions mentioned above. In test sessions, a fresh supply of sucrose was presented each day for an individual subject and each rat was examined in 3 different tests: 0.03M vs. 0.1M – *test A*, 0.03M vs. 1M – *test B* and 0.1M vs. 1M – *test C*. To avoid conditioning a place preference we placed the higher concentration in each test in different licking spouts (left/right). In addition, each rat received the 3 tests (**A-C**) in a random order. At the end of the 2 hr test, rats were returned to their home cages, where chow and water were available *ad libitum*.

3.3.1 Experiment 2a (expression of dopamine receptors)—At each time point naïve LETO & OLETF rats (n=6 per strain) were sacrificed and DA-like-receptor type 1 (D1R) and D2R levels were examined in a reward-related brain area (Nac shell) by western blot analysis. Animals were sacrificed by decapitation (between 12-14 hrs) and brains were removed and immediately frozen on dry ice and stored at –80° C. Coronal brain sections were cut using a cryostat and the Nac shell punches were extracted from the brains according to Paxinos & Watson stereotaxic coordinates atlas (1.7 to 1.1mm from Bregma).

3.3.2 Experiment 2b (pair-feeding)—In order to examine the contribution of the rats' obese phenotype to the alteration in D2R expression a group of OLETF rats were pair fed (PF) the same amount of food consumed by same age LETO controls. Pair feeding was conducted from PND 22 up to PND 90. Rats were housed individually in the same conditions as in the other experiments. Food consumption was measured daily and the animal's weight was measured every 5 days. At PND 90 the animals were sacrificed and D2R levels were examined in the Nac shell by western blot analysis.

3.4 Experiment 3 (Food “craving”-like behavior)

A palatable diet was presented with intermittent access, followed by a 30-day “abstinence” period, **during which they were kept in their home cages** with no access to the lickometers or to Ensure. Eight rats from each strain were placed in the lickometer cages at the same ages (PND 22, 38, 60 and 90) as described in Experiment 1. At each age, the rats received two bottles for 3 consecutive nights: The right-side bottle contained tap water and the left-side bottle contained a liquid palatable diet (Ensure), as in Marco et al (Marco et al., 2009). After an abstinence period of 1 month, rats were placed again in the lickometer cages at age 120, only to be provided with two bottles of tap water and a cue reminder on the left-side bottle (a smear of Ensure was dabbed on the inner wall of the niche, near the spout). We analyzed the microstructural patterns of licking during a 2h test.

4. Results

4.1 Statistical approach

Western blots were analyzed as follows: One-sample t-tests compared the mean OLETF D1R and D2R values (normalized to same-age LETO controls) with 100%. In addition, Pearson correlations were performed between rats' age and D1R or D2R levels, in OLETF rats. Western blots for the pair feeding test were analyzed in the same manner - D2R levels of the LETO PND90 were set as 100%, and pair fed or *ad libitum* OLETFs were compared

to them. *Sucrose preference test*: In order to examine the correlation between the sugar preference and the age parameter, developmental changes in each strain were analyzed using linear regression, with age as the independent variable. This was followed-up by one-sample t-tests in each group at all of the ages, in order to test for the existence of a significant preference (>50%) for the higher concentration. The microstructural variables were assessed in relevant groups by MANOVA comparing the 2 strains, separate comparisons on each variable were taken from the MANOVA output. *Body-weight* (BW) in the pair-feeding test was analyzed by repeated measures ANOVA comparing free-fed OLETF with PFD rats on BW from weaning to adulthood. The significant interaction was followed-up by t-tests at each age.

4.2 Experiment 1

4.2.1 Sucrose preference—In *test A (0.03M vs 0.1M)* LETO rats (Fig 1a) **displayed** changes over time expressed mainly by **an increased** preference for the 0.1M concentration throughout development ($R=0.43$, $F(1,29)=6.75$, $p=0.015$). On the other hand the OLETF rats (Fig 1b) showed no developmental changes. Both strains presented clear preference (>50%) to the 0.1M concentration starting from PND 38 and on (Fig 1 - white bars). In *tests B (0.03M vs 1M)* there were no significant developmental changes in **either** strains. Nevertheless, while the LETO rats **displayed** clear preference to the high concentration (1M) only at PND 22, the OLETF rats **displayed** clear preference to the high concentration at all development stages (Fig 1b, gray bars). In *test C (0.1M vs 1M)* the LETO rats displayed a tendency ($p=0.06$) to decrease the preference for the high concentration as they matured. At PND 90 the LETO rats showed a significant aversion to the high concentration (1.0 M). The OLETF did not show a clear preference for any of the concentrations throughout development, although they seem to prefer the high concentration to some extent (Fig 1b, diagonal lines bars).

4.2.2 Microstructural analysis of sucrose feeding—When the sugar concentration is high, rats eat more intensively and maintain fewer intervals between the periods of eating (Davis and Smith, 1992). Therefore, in order to obtain more accurate results of the sucrose preference, we analyzed the microstructural patterns of intake of LETO and OLETF rats. We chose to focus on the analysis of the WBL function. As described previously, in this formula, the B parameter is the slope of decline, representing the rate of satiation. The A parameter is the initial rate of licking and has been shown to be a measure of the palatability of the test solution. Overall, the OLETF rats tended to lick at a higher initial rate at some sporadic time points (Table 1). Nevertheless, at PND 90 OLETFs showed a significantly higher initial rate compared to LETO in test C, both in the high and low concentrations (Table 1, $p<0.05$). Interestingly, in test C, LETO rats selectively reduced the rate of licking in the high (1M) but not the low (0.1M) concentration from PND 60 to PND 90, a developmental hedonic transition not found in the OLETF rats.

4.3.1 Experiment 2a (D1R and D2R expression in free-fed rats)—**D2R**: OLETF rats showed significantly less ($t(7)=3.05$, $p<0.05$) D2R on PND 90 compared to control LETO rats, but no strain difference was found at the other ages (Fig 2A). Moreover, in the OLETF group the D2R level decreased significantly with age ($r(25)=-.47$, $p<0.05$). **D1R**:

there were no significant differences between the strains in each of the time-points (Fig 2B). The correlation between age and D1R levels was not significant.

4.3.2 Experiment 2b (D2R in OLETF pair-fed)—Whereas male OLETF rats expressed significantly lower D2R levels ($t(10)=4.33$, $p=0.001$) than LETO controls on PND90, PF OLETF rats showed similar levels to those of the LETO controls, which were significantly higher ($t(16)=2.78$, $p<0.05$) than free-fed OLETF rats (Fig 3A). As in previous studies (Schroeder et al., 2010), the body weight of PF OLETF rats was normalized to that of lean LETO controls, and it was significantly lower than free-fed OLETF from PND 30 and on $F(13,143)=148.14$, $p<0.001$; Fig 3B).

4.4 Experiment 3 (Food “craving”-like behavior)

After a two-hour test, the data collected were analyzed in two ways: 1. The first meal data - representing the “food-craving or bingeing” of the first encounter with the “Ensure cued” bottle. 2. Total means of the parameters during the test, from onset of 1st meal to termination of the last meal, which represents the “craving”-like behavior or when the rats had “given up” licking the bottle. During the *first meal* the OLETF rats licked the bottle containing water with the cue reminder in a more enhanced manner (Number of licks per meal, $p<0.05$), over a longer period (Meal duration (min), $p<0.05$), and they tended to lick at a higher rate ($p<0.1$) (MANOVA on these 3 variables, $F(3,9)=3.14$, $p<0.1$; See Table 2). When we analyzed the average of all the meals there were no significant differences between the OLETF and the LETO strains in the “number of licks per meal” and the “meal duration” parameters. Nevertheless, the OLETF’s total rate was higher ($p<0.05$) (MANOVA on these 3 variables, $F(3,10)=2.05$, NS; See Table 1). MANOVAs on the 3 WBL parameters (A,B,C) in the first meal and over all meals were not significant ($p<0.33$ and $p<0.06$, respectively). **There was also** a tendency in the A parameter of the WBL function from the 1st meal ($p=0.06$), and a significant difference in the A parameter over all meals (Fig 4A; $P<0.05$), reflecting a higher A parameter in OLETF compared to LETO rats (Table 1). In the global feeding parameters of 2h test the OLETF rats had more licks (Fig 4B; Total number of licks, $p<0.05$) and a longer total eating duration (Fig 4C; $p<0.05$) (MANOVA on these 2 variables, $F(2,10)=10.21$, $p<0.01$).

5. Discussion

In this study OLETF rats exhibited increased avidity for sucrose consumption which was associated with a significant reduction in NAcc D2R expression. This effect only emerged in adulthood. In addition, food restriction normalized both the OLETF’s weight and their D2R expression in the Nacc shell area. Adult OLETF rats have been shown to have a reward deficit and a significant reduction in D2R binding and function (De Jonghe et al., 2005; Hajnal et al., 2005; Hajnal et al., 2008). We attempted here to measure both types of dopamine receptors at different developmental time points in the same study. In addition, we present a novel approach to the study of “compulsive” or “addictive-like” eating behavior by microstructural analysis of the data from the lickometer device.

The analysis of the data from the sugar preference test was performed in two ways to neutralize the effects of satiety and weight as much as possible. First, the preference

parameter was calculated as percentage from total intake. Secondly, we analyzed the Weibull function (WBL), to quantify changes in the rate of licking during the test. Fig 3 presents the rats' preference between the different sucrose concentrations, throughout the developmental stages of the LETO and OLETF rats. Observation of the two graphs shows that during normal (LETO-like) development, the taste preference toward the sucrose solution changes. Overall, LETO presented increasing preference to the medium concentration and preferred the higher concentrations (~1M) only in the early stages of their lives (like children's preference for sweet taste). Towards maturity the 1M concentration became relatively aversive or "too sweet" for them. In contrast, the OLETF strain presented deficits in orosensory response expressed by "ignoring" the 0.03M concentration and high hedonic response expressed by an exaggerated preference for the relatively higher concentration (0.1M or 1M). These findings suggest that the OLETF avidity for sucrose appears only after sexual maturation, in early adulthood. The preference for the high concentrations is exacerbated with development, probably related to the increasing obesity-related factors that appear in this strain at the same time point. Variables such as: energy balance control, memory and maturation of the orosensory and the hedonic system are likely to be involved. Research suggested that DA is one of the neurotransmitters that mediate the orosensory positive feedback of sweet taste and other foods (Smith, 2004). For example, rats that consumed sucrose solution in real and sham feeding procedures had the same extracellular DA concentration in the Nac (Hajnal et al., 2004). It has been suggested that DA secretion is mainly affected by the sucrose concentration instead of the volume. Therefore disruptions in the dopamine system can lead to abnormal preference of sweet taste. Interestingly, in the early stages of their lives, the OLETF and LETO rats had similar sucrose preference. The abnormal sensitivity/response mainly occurred in the OLETF rats at later stages of development. We can only speculate that the decrease in the D2R levels found at PND 90 is involved in mediating the abnormal sweet taste response that the OLETF rats presented.

The main reason that OLETF rats have been previously investigated in the context of DA deficits was because of the known CCK-DA interactions. CCK and DA are co-localized and co-released in several regions in the brain (Vaccarino, 1994) and play an important role in mediating the reward of feeding (Beinfeld et al., 2001; Feifel et al., 2003). Moreover, while CCK-1 receptors enhance mesolimbic DA neurotransmission, activation of the CCK-2 receptor subtype attenuates it (Beinfeld et al., 2001). Therefore, it has already been suggested by Hajnal and Covasa that the OLETF's mutation is the main cause for the DA deficit in this strain. Another, not necessarily competing explanation for the DA deficit that has been found in obese individuals is that it may result from high levels of hormones **such as** leptin and insulin that alter DA signaling. There is strong evidence suggesting that these two hormones interact with the DA system (Palmiter, 2007) and mediate reward-related eating behaviors. For example, rats that needed to press a lever a gradually increasing number of times to obtain a sucrose reward, decreased the number of presses after they received ICV leptin (Figlewicz et al., 2006). In addition, it was found that ICV insulin increased DA re-uptake transporter (DAT) mRNA levels, hence DA signaling decreased (Figlewicz et al., 2007). Previous research in the OLETF strain found both hyperleptinemia and hyperinsulinemia from young ages, which becomes drastic in adulthood (Kawano et al.,

1992; Schroeder et al., 2009b; Schroeder et al., 2009c). Moreover, studies have shown that male OLETF rats develop leptin resistance around week 8 (Niimi et al., 1999) and insulin resistance later in life (Zhao et al., 2008). It is not clear if the reduction in D2R that we found is in D2 presynaptic [the short form] or D2 postsynaptic receptors [the long form]. Geiger et al. (2008) reported that dopamine impairment in inbred obesity-prone rats was apparent at birth and associated with changes in expression of several factors regulating dopamine synthesis and release, including the (presynaptic) D2 short-form receptor (Geiger et al., 2008). This is in accordance with several reports of deficits in mesolimbic dopamine neurotransmission that are linked to dietary obesity.

In order to evaluate the contribution of the rats' obese phenotype to the reduction in D2R expression, we performed a follow-up experiment in which we examined D2R expression in pair-fed OLETF versus *ad libitum*-fed animals. The PF animals did not show reduction in the D2R levels as did the *ad libitum*-fed OLETF rats (Fig 2A). This extends the findings of a study by Bi et al. (2001) reporting that pair feeding OLETF rats normalized their elevated POMC and decreased NPY mRNA expression in the arcuate nucleus. In addition, other studies found that pair-fed male OLETFs presented normal leptin levels and body fat percentages (Moran and Bi, 2006; Schroeder et al., 2010). Thus we now add that D2R levels were also normalized. The only remaining measure so far found that indicates the "chronic hunger" status of pair-fed OLETF rats is the upregulation of NPY in the dorsomedial hypothalamus (DMH) in pair-fed OLETF rats (Bi et al., 2001). This in turn could affect other elements of the reward system not measured in the present study. However, the current findings of a late deficit appearance and the novel PF results suggest that deficits in the motivation/regulatory systems of the OLETF rat is a developing process (at least from weaning and on) depending on the overeating and obese phenotype of the rats and not only on the CCK mutation. It is possible that there are epigenetic changes that occur during pregnancy and lactation in these systems and then these differences are compensated for during the interaction with the dam and the associated consumption of breast milk. If so, its reappearance only at adulthood is probably triggered by obesity related factors (such as leptin) since this process does not occur in PF OLETF rats.

Our findings suggest that dopaminergic deficits are acquired after, or during the onset of obesity. Literature showing that brain dopamine and/or D2 receptor deficits are acquired after or irrespective of the onset of obesity in wild-type strain rats on a high-fat diet exists, alongside with findings in the other direction (Davis et al., 2008; Geiger et al., 2009; Johnson and Kenny; Pothos et al., 1998). For example, chronic food deprivation and diet-induced obesity were shown to induce similar changes in mesolimbic DA systems (Pothos et al., 1998). In a more recent study **from** this group, Geiger et al. (2009) reported that deficits in mesolimbic dopamine neurotransmission are linked to dietary obesity. Specifically they conclude that "bepressed dopamine release may lead obese animals to compensate by eating palatable "comfort" food, a stimulus that released dopamine when laboratory chow failed". Using a pair-feeding protocol, Davis et al. (2008) further reported that rats consuming a high-fat diet, independent of the development of obesity, exhibited decreased dopamine turnover in the mesolimbic system [In addition, diet-induced obesity with a high-fat diet also attenuated mesolimbic dopamine turnover]. Johnson & Kenny (2010) showed that striatal

D2Rs were downregulated in obese rats and lentivirus-mediated knockdown of striatal D2Rs rapidly accelerated the onset of “compulsive-like” food seeking in rats with extended access to palatable high-fat food. Finally, the evidence that D2R and dopamine deficits precede the obesity phenotype in inbred obesity-prone rats (Geiger et al., 2008), further suggests that the D2R deficiency in obese animals may not necessarily be ontogenetically regulated and it could very much depend on the animal model of obesity used in each study. Altogether, we believe that many (but not all) of these findings on dietary effects are in accordance with the OLETF findings reported in the current paper.

In the current study, OLETF rats demonstrated altered Nac D2R levels, similar to drug-induced sensitization (Bello et al., 2002). Therefore, we wanted to examine whether “compulsive” or addictive-like eating behavior follows these changes. After inducing a “food-craving”-like paradigm by a 30-day abstinence period following intermittent access to a palatable diet, OLETF rats showed a stronger response to the cued bottle. Since this bottle contained only tap water the animal did not receive any satiety or palatable signals that could have affected its licking behavior. We can therefore assume that the microstructural pattern was mainly influenced by signals such as: motivation to lick, reward, memory and craving, which appeared after placement in the lickometer cage and exposure to the cue reminder. Weibull function (WBL) was fit to quantify changes in the rate of licking during the test (Davis and Levine, 1977; Davis and Smith, 1988). The initial rate parameter (A) has been shown to be a measure of the palatability of the test solution, and the decay parameter (B) has been interpreted as a measure of the post-ingestive negative feedback that leads to meal termination. OLETF rats showed a stronger response to the cue reminder as they presented an initial rate of licking twice as high as the control strain (Fig 4A). In addition, it appears that it took OLETF rats more time to “give up” licking the water bottle which was reflected by significantly more licks (Fig 4B) and a longer licking time (Fig 4C). This kind of “craving-like behavior” after a period of abstinence indicates an increased hedonic response and maybe also suggests a high addiction potential and physiological disruption, similar to drug-induced sensitization, when a rat is presented with a cue reminder of the drug (Lu et al., 2004). It is not clear whether the exaggerated response is directly associated with the decrease in D2 receptors but nevertheless, this finding reinforces the link between mechanisms of addiction and obesity.

Limitations

The feeding data were collected during the animal’s light cycle when rats do not normally feed much. Replication in the dark phase of the cycle is needed.

We note that although it is customary to compare sucrose to water, in the present study we chose to give the rats two-bottle choices with two of the 3 different concentrations of sucrose solution 0.03M, 0.1M or 1M. This was because when the rat is presented with a choice between a bottle of water and a bottle of sucrose, the rat always prefers the sucrose to some extent. However, when presented with two concentrations of sucrose the rat must make a real preference between **solutions**. We are well aware that in the current approach, intake of the 1st concentration depends, to some extent, on the choice option presented in the

2nd concentration, thus complicating data analysis and interpretation. Therefore, we used lickometers to attain a more accurate profile of the preference pattern.

6. Conclusion

As hypothesized, the appearance of changes in D2 receptors was parallel to the changes in the adiposity profile. We suggest that the mutation in the CCK system in the OLETF strain is not the only factor responsible for the DA deficit. Abnormal levels of hormones like leptin and insulin, resulting from overeating, may represent key factors in the deterioration of the OLETF rats' reward system, eventually exacerbating the obesity problem. Although there is no evidence for the existence of a CCK-1 mutation in human obesity, overeating is a very frequent health risk-factor. Moreover, downregulation of D2R has also been found in human obesity (Wang et al., 2001). Therefore, the results of this paper reinforce the argument that uncontrolled eating can lead to neuronal changes in the reward and hedonic systems which in turn may exacerbate obesity.

Acknowledgments

The authors thank Dr. Kawano of the Tokushima Research Institute (Otsuka Pharmaceutical, Japan) for the generous gift of the OLETF and LETO rats.

This work was supported by NIH/NIDDK – RO1 DK57609.

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Highlights

- We studied feeding & reward in OLETF rats, an animal model of obesity
- OLETF presented exaggerated preference for high sucrose concentration
- After 30-day preferred diet abstinence, OLETF showed food “craving”-like behavior.
- OLETF rats expressed lower D2R levels than controls only in adulthood
- Diet-restriction normalized OLETF weight & D2R levels.

Fig 1.

(*Sucrose preference*): Percentage of preference for the high concentration of sucrose calculated by dividing the number of licks of the high concentration into the total number of licks. LETO (A) and OLETF (B) means are presented throughout all of the developmental stages (PND 22, 38, 60 and 90) in 3 different tests; *Test A* (White bars) 0.03M vs. 0.1M, *Test B* (Gray bars) 0.03M vs. 1.0M and *Test C* (Slanted bars) 0.1M vs. 1.0M. Data are presented in means and SEM. Significant correlations between age and sucrose preferences (calculated with the individual data) are shown as a line connecting the bar graphs. The correlation coefficient appears in a box above.

# Indicates that preference for the high concentration was significantly different from 50%, $p < 0.05$;

* Indicates that aversion for the high concentration was significantly different from 50%, $p < 0.05$.

Fig 2.

Dopamine receptor type 2 (D2R) (A) and type 1 (D1R) expression (B), measured by Western blot analysis at 4 different time points (PND 22, 38, 60 and 90) in both strains. Within each gel, we normalized the LETO OD levels to 100% and compared the OD levels of the OLETF age-matched sample to them. Data are presented in means and SEM. Significant correlations between age and D2R levels (calculated with the individual data) are shown as a line above the bars. The Pearson correlation coefficient appears in a box above. * Significant difference from the LETO (100%) sample at each particular age, $p < 0.05$.

Fig 3.

(Pair-feeding): D2R levels were measured by Western blot analysis. Mean levels of the OLETF (free-fed) and OLETF PF were compared to LETO PND 90 samples that were set as 100% (A). Mean body-weight in grams of the three groups (LETO, OLETF free-fed and OLETF pair-fed) from weaning to adulthood (B). Data are presented in means and SEM.

*Significant difference from the LETO (100%) sample, $p < 0.05$.

Fig 4.

(*Food “craving”-like behavior*): *WBL function* parameters (A), *Total number of licks* (B) and *Meal duration* (C) of licking (reaching the criteria, see Matlab analysis 2.3.2) was recorded during the test from onset of 1st meal to termination of the last meal.

*Significant difference from the LETO, $p < 0.05$.

Table 1

The Weibull function ($y=A*\exp[-(B*t)^C]$) was fit to the lick rate in licks per minute with a 1-min bin size, across 30 min of all meals larger than 50 licks. A parameter is the initial rate of licking at t=0.

		Initial rate of licking (A parameter from the WBL function) PND 38 - 90					
		Test A (0.03 Vs. 0.1M)		Test B (0.03 Vs. 1M)		Test C (0.1 Vs. 1M)	
		High con.	Low con.	High con.	Low con.	High con.	Low con.
PND 38	LETO	32.2	11.47	72.9	17.3	44.5	30.88
	OLETEF	195.06 **	86.76 \$	142.4	60.71 \$	148.03	33.5
PND 60	LETO	85.6	28.75	48.9	20.96	182.6	90.4
	OLETEF	115.06	31.27	139.4 *	40.24	124.5	154.9
PND 90	LETO	117.6	35.7	110.5	46.2	37.02	86.3
	OLETEF	196.4 \$	33.6	119.6	66.7	135.29 **	166.18 *

! Significant difference from the LETO

* p< 0.05

** p< 0.01

\$ Tendency, p< 0.1

Table 2

Total number of licks, rate and meal duration was recorded from the bottle with a cue reminder (smear of Ensure near the spout). LETO and OLETF 1st meal and total means are presented in table below. The Weibull function was fit to the lick rate in licks per minute with a 1-min bin size, across 30 min of all meals larger than 50 licks. A, b and C parameters were calculated and presented as means.

	Number of licks per meal		Rate (lick/sec)		Meal duration (min)	
	1st-meal	mean	1st-meal	mean	1st-meal	mean
LETO	80.75	73.31	4.95	4.79	7.05	7.02
OLETF	220.17 *	136.96	5.53 \$	5.18 *	16.92 *	6.93
WBL function parameters						
	1st-A	1st-B	1st-C	mean-A	mean-B	mean-C
LETO	32.78	0.01	4.58	33.11	0.01	5.27
OLETF	58.68 \$	0.01	2.28	78.38 *	0.01	3.21

/ Significant difference from the LETO

* p< 0.05

** p< 0.01

\$_{Tendency}, p< 0.1