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Effects of Ovariectomy and 17β-Estradiol Replacement on Dopamine D2 Receptors in Female Rats: Consequences on Sucrose, Alcohol, Water Intakes and Body Weight

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Abstract

Background: Mechanisms underlying overeating-induced obesity in post-menopausal woman include functional lack of 17β-estradiol dysregulating dopamine D2 receptors, thereby inducing food addiction, glucose craving or alcohol dependence through reward circuitry. This study aimed at further understanding 17β-estradiol and dopamine D2 receptors interferences in the etiology of woman obesity. ***Method***: Seventy-two Wistar female rats weighing 200 - 205 g, individually-housed, were divided into non-ovariectomized control (C = 6 groups) and ovariectomized rats (OVX = 6 groups) which were concurrently subjected to the following treatments: Non-drug-treated (DMSO vehicle), 17β-estradiol (E2, 5 µg/kg, s.c.), sulpiride (SUL, 20 mg/kg, i.p.), bromocriptine (BR, 0.1 mg/kg, i.p.), E2 + SUL or E2 + BR, designating the 6 constitutive groups of either control or ovariectomy. Within each experimental group, consumption of different solutions (10% alcohol, 10% sucrose and water) as well as food intake and body weight were daily measured, for 10 consecutive days. ***Results***: This study indicated that D2S was a specific inducer of alcohol and food intakes, but reduced sugar consumption. In addition, 17β-estradiol regulated the body weight set point, modulating D2S functions towards increased food intake at lower weights and decreased food intake at higher weights. D2S met the slow genomic actions induced by 17β-estradiol. Conversely, D2L inhibited alcohol and food intakes, but induced specifically sugar consumption, thereby regulating blood glucose levels and promoting energy expenditure in reducing body weight. Indeed, 17β-estradiol exerted a tonic inhibition on D2L which was released by OVX, exacerbating sugar intake and increasing body weight. D2L mediated the rapid metabolic effects of

17β-estradiol. ***Conclusion***: Our results supported physiological data reporting that activation of the mostly expressed presynaptically D2S-class autoreceptors decreased dopamine release stimulating food intake, whereas activation of the predominantly postsynaptic isoform D2L receptors increased dopamine activity inhibiting food intake. Our studies indicated that 17β-estradiol acted on the two types of D2 receptors showing opposite functions to equilibrate energy intake vs. expenditure for weight set point regulation. Our data also supported biochemical findings reporting that 17β-estradiol induced D2 genes transcriptional regulation, thereby involving both types of D2 receptors in the etiology of obesity. The combined dysregulated effects of D2L and D2S receptors, as 17β-estradiol was lacking, would be causal factors underlying the etiology of obesity.

Keywords

17β-Estradiol, Dopamine D2 Receptors, Bromocriptine, Sulpiride, Water, Sucrose, Alcohol Intakes, Obesity

1. Introduction

Obesity as a result of overeating and excessive weight gain, was defined as abnormal or excessive fat accumulation in adipose tissue, thereby becoming a major public health concern. It resulted from a dysfunction of the appetite normal regulation loop where an unbalance occurred between overeating and low energy expenditure, leading to fat accumulation [1]. Many studies were carried out for the understanding of mechanisms underlying food intake and weight gain.

One of the main mechanisms involved the dopamine-modulated reward circuits in both normal and pathologic eating behaviors [2] [3] [4]. Indeed, functional studies closely involved dopamine D2 receptors in several addiction patterns including sugar, alcohol, food and drugs [5] [6] [7] [8]. The D2 receptor existed as two alternatively spliced isoforms differing in the insertion of a stretch of 29 amino acids in the third intracellular loop [9]. This loop seemed to play a central role in receptor coupling, implying functional diversity [10] [11]. For instance, the two isoforms (D2L and D2S) played differential roles in rewards related to alcohol and sugar consumptions. On the one hand, dopamine (DA) D2 receptors were involved in palatable food consumption predicting a food addiction which may lead to excessive weight increase [12] [3]. In addition, Smith and Schneider [13] reported that sucrose feeding in rats significantly increased the most significant dopamine metabolite DOPAC in the hypothalamus, compared with water intake; this result was interpreted as activation of mesolimbic dopaminergic terminals in the hypothalamus. Similar results showed release of nucleus accumbens DA in response to sucrose licking [14]. The findings of Hajnal *et al.* [15] involved D2 receptors in heightened consumption of sucrose observed in obese rats. On the other hand, literature involved recurrently dopamine media-

tion in alcohol-related rewarding effects [16] [17]. Evidence from previous studies suggested that human alcoholics tended to have fewer D2 than non-alcoholics [18] [19] [20] [21]. Genetic association studies generally supported the hypothesis that polymorphisms which reduced D2 expression increased alcoholism risk [22] [23] [24]. This role of D2 receptors in attenuating ethanol drinking behavior was consistent with both clinical [19] [25] [26] and preclinical [27] [28] [29] evidence revealing the importance of these receptors for therapeutic strategies in alcoholism treatment. Taken together, the human studies suggested that reduced expression of D2 receptors in brain reward circuits predisposed to excessive alcohol consumption.

Other mechanisms which influenced food consumption and body weight, related to steroidal hormones, in particular the 17β-estradiol (estradiol or E2) in females. There was tangible evidence that the 17β-estradiol decreased food consumption, body weight and fatty tissue [30]. However, recent studies suggested that estradiol increased palatable food consumption [31]. Consumption of highly palatable and energy-dense food was a critical variable leading to excessive gain in weight and increasing risk factors for obesity. Correlatively to the circulating levels of the 17β-estradiol in woman, the incidence of obesity greatly increased after menopause [32] [33] [34]. Physiological effects of estrogens were mediated by two different signaling pathways: slow genomic actions versus rapid non-genomic actions. Rapid non-genomic actions of 17β-estradiol were promoting glucose transport into the brain and neural cells, regulating metabolism and mitochondrial function to sustain the energetic demands during neuronal activation and physical activities [35] [36] [37]. These signaling cascades were triggered when 17β-estradiol bound to transmembrane G protein-coupled estrogen receptors, localized either in the plasma membrane or endoplasmic reticulum [37] [38]. Slow genomic actions of 17β-estradiol passed through its direct binding to nuclear estrogen receptors ERα and ERβ, followed by receptor translocation into nucleus and transcription of hormonally regulated genes [37] [39]. For instance, the 17β-estradiol regulated through its slow genomic actions, the transcription and synthesis of both D2L and D2S isoforms [40] [41].

From literature review, despite of sequential difference between the two isoforms (D2L and D2S), the specific function related to each isoform was poorly understood. Thus, mechanisms by which dopamine D2 receptors induced glucose craving or alcohol dependence were far from being clarified. In addition, mechanisms of 17β-estradiol-modulated D2 receptors to control fluid consumption (e.g. sugar, alcohol and water) and to regulate food intake and body weight were also unknown. For further understanding of these mechanisms, this study investigated the effects of 17β-estradiol without or with cotreatment by either dopamine receptor D2 agonist (bromocriptine), or antagonist (sulpiride) on: 1) daily consumption of different solutions (10% alcohol, 10% sucrose and tap water); 2) daily measurements of food intake and body weight gain, during a 10-day treatment period. Animals had free access to food and different solutions.

2. Materials and Methods

2.1. Animals

Nulliparous females of Wistar rats, bred in our colony, which were 12 weeks old and weighing 200 - 205 grams, were used in our experiments. They were maintained under standard laboratory conditions at an ambient temperature of 30 ± 2˚C, with light/dark cycles of 12 hours each and relative humidity reaching 85 ± 3%. Rats were individually housed in polypropylene cages (27 × 37 × 18 cm) with the floor covered by wood shavings and fed with pellet chow diet and water ad libitum. One week prior to the onset of the tests, they were acclimated to the experimental conditions. All experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. Drugs and Chemicals

Drugs and Chemicals used in these experiments were: 17β-estradiol (estradiol or E2), bromocriptine mesylate, sulpiride, dimethyl sulfoxide (DMSO) manufactured by Sigma-Aldrich Chemie GmbH (Eschenstrasse 5, 82,024 Taufkirchen, Germany). DMSO was the solvent used for all drug dilution. Bromocriptine mesylate and sulpiride were used as selective agonist and antagonist respectively, at central dopamine-D2 receptors [42] [43].

2.3. Procedures

***Experiment* 1: *Control* (*non-ovariectomized rats*)**

Non-ovariectomized rats were treated for 10 days respectively with 17β-estradiol (5 µg/kg: [44]), bromocriptine mesylate (0.1 mg/kg: [45]), sulpiride (20 mg/kg: [46]) and a concomitant administration of "17β-estradiol + bromocriptine" or "17β-estradiol + sulpiride", and DMSO vehicle (0.7%: [47]). Precisely, thirty-six (36) nulliparous female rats, individually housed, were divided into 6 treatment groups (6 rats/treatment group) and were administered for 10 consecutive days as follows:

- Group C (control): Six females non-drug-treated, injected with DMSO vehicle.

- Group C + E2 (17β-estradiol): Six females subcutaneously injected (s.c.) with 17β-estradiol (5 µg/kg body wt).

- Group C + BR (bromocriptine): Six females intraperitoneally injected (i.p.) with bromocriptine mesylate (0.1 mg/kg body wt), a D2 agonist.

- Group C + SUL (sulpiride): Six females treated with intraperitoneal injection (i.p.) of sulpiride (20 mg/kg body wt), a D2 antagonist.

- Group C + E2 + BR (17β-estradiol + bromocriptine): Six females treated with concomitant administration of 17β-estradiol (5 µg/kg body wt, s.c.) and bromocriptine (0.1 mg/kg body wt, i.p.).

- Group C + E2 + SUL (17β-estradiol + sulpiride): Six females treated with concomitant administration of 17β-estradiol (5 µg/kg body wt, s.c.) and sulpi-

ride (20 mg/kg body wt, i.p.).

Within each experimental group, drinking solutions (water, 10% sucrose and 10% alcohol) were measured daily during an experimental period of 10 days. Food consumption and body weight were also daily measured in each cage. Drugs injections and the various measurements of weight or food and solutions intakes were performed every day at the same hour, 17:00 pm, corresponding to the beginning of activities, as the rats were nocturnal animals. Solution intake was measured by direct reading of the volume absorbed on graduated bottles, every 24 h. Fluids intake were measured in a four-bottle preference condition. After daily drug injection, the remaining content of the vial (one drug for a non-interchangeable 5 ml vial), as well as drinking solution contained in a 150 ml bottle were refreshed every day.

Experiment* 2: *Ovariectomy and* 1*7β-estradiol* (*E*2) *replacement

Thirty-six (36) nulliparous female rats, individually housed, were divided into 6 treatment groups (6 rats/treatment group). For surgical procedure, rats were immersed in a glass jar and anesthetized by ethyl ether inhalation. Then, the rats underwent bilateral ovariectomy (OVX group) via midline dorsal incision. After ligation, the ovaries were excised from a longitudinal incision made on the dorsolateral body region. The risk of postoperative infection was eliminated by applying an antibiotic cream and betadine alcoholic solution twice weekly for 4 weeks. After 4 weeks of recovery period, all the OVX rats were divided into 6 groups (n = 6/group) including, OVX (six ovariectomized females, non-drug-treated, injected with DMSO vehicle), OVX + 17β-estradiol (E2, s.c.), OVX + Bromocriptine (BR, i.p.), OVX + Sulpiride (SUL, i.p.), OVX + E2 + BR and OVX + E2 + SUL, for 10 consecutive days. Drug administration pattern in OVX rats was identical to that of the control. During the 10-day treatment period, all animals were recorded for daily consumption of different solutions (10% alcohol, 10% sucrose and tap water) and daily measurements of food intake and body weight. Animals had free access to food and different solutions.

2.4. Statistical Analysis

The two-way ANOVA was used to compare the effects of treatments on ovariectomy (6 factors) and control (6 factors) × 10-day treatment period (10 factors). *Post hoc* testing (p's = 0.05) was carried out using the Protected Least Significant Difference (PLSD) for means comparison [48]. The factors for each variable are the average value for the 10 day period, so that figures show only the average values for the 10 day experimental period within each treatment group.

3. Results

3.1. Effects of 17β-Estradiol, Sulpiride and Bromocriptine on Water Intake

A two-way ANOVA on water intake (**Figure 1**) yielded the main treatment effects [$F(11, 600) = 430.997$, $p < 0.01$], a significant change over days [$F(9, 600) =$

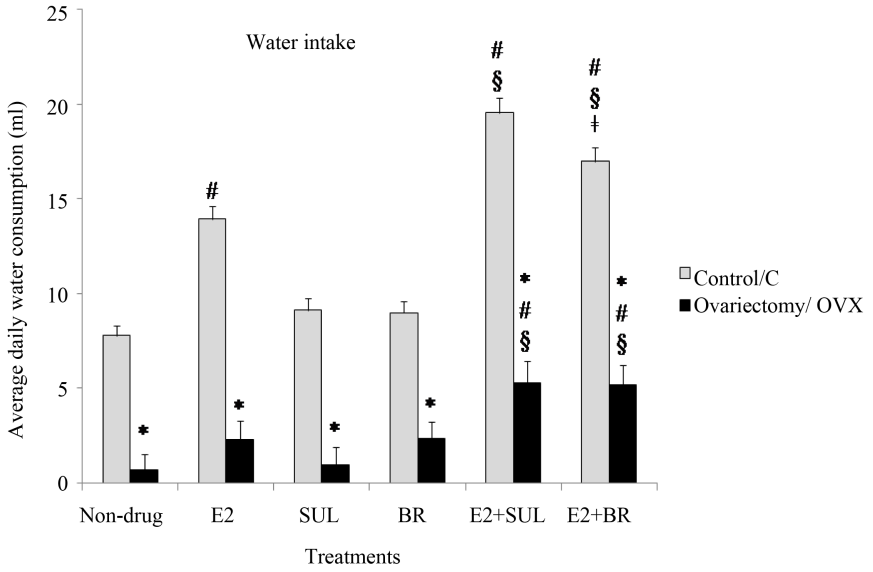

Figure 1. Effects of ovariectomy and 17β-estradiol (E2) replacement on bromocriptine (BR) and sulpiride (SUL) regulating water intake. The consecutive 10-day Average values (ml/rat/day ± SEM) of water intake within each experimental group (N = 6 female rats), were represented in non-ovariectomized control (C = 6 groups) and ovariectomized rats (OVX = 6 groups) concurrently subjected to the following treatments: Non-drug-treated (DMSO vehicle), 17β-estradiol (E2), sulpiride (SUL), bromocriptine (BR), E2 + SUL or E2 + BR, designating the 6 constitutive groups of control and ovariectomy respectively. "*" denoted a significant difference between control (C) and ovariectomized (OVX) rats subjected to the same treatments, $p < 0.01$; "#" significantly different from their corresponding control group C or control group OVX, non-drug-treated rats, $p < 0.01$; "§" denoted a significant difference between hormone-D2 receptors association effects (E2 + SUL and E2 + BR) *vs* individual D2 receptors stimulation effects (SUL and BR) in both control and ovariectomized rats $p < 0.01$; "ǂ" significantly different from E2 + SUL ($p < 0.01$).

4.128, $p < 0.01$], showing water consumption collapse in all ovariectomized groups relative to the control ones. However, there were no treatment x day interactions [$F(99, 600) = 0.315$, $p > 0.99$]. Indeed, *Post hoc* comparisons, using Fisher's PLSD test (p's = 0.05) showed a drastic inhibition of water consumption in ovariectomized rats (OVX: 0.68 ± 0.36 ml/rat/day), compared to the control rats (C: 7.78 ± 0.55 ml/rat/day), ($p < 0.01$), suggesting that 17β-estradiol (E2) exerted a tonic action activating water consumption. Supporting that hypothesis, administration of E2 to the females doubled the mean volume of water ingested daily (C + E2: 13.93 ± 0.68 ml/rat/day), compared to the control ($p < 0.01$). Likewise, the average daily water intake was amplified in female rats co-administered with either "17β-estradiol + sulpiride" (C + E2 + SUL: 19.55 ± 0.81 ml/rat/day) or "17β-estradiol + bromocriptine" (C + E2 + BR: 16.97 ± 0.77 ml/rat/day) compared to the controls ($p < 0.01$, respectively). However, water consumption was strongly inhibited in ovariectomized rats, and none of the treatments carried out with 17β-estradiol (OVX + E2: 2.29 ± 0.41 ml/rat/day), sulpiride (OVX + SUL: 0.96 ± 0.39 ml/rat/day) or bromocriptine (OVX + BR: 2.34 ± 0.45 ml/rat/day), could restore water consumption relative to their re-

spective controls ($p < 0.01$ in the three cases), showing the inability of these drug-mechanisms to support the observed tonic activation of water consumption in OVX rats. The most credible drug-mechanisms supporting the tonic activation of water intake were related to hormone-D2 receptors interactions effects, while comparing the C + E2 + BR group (16.97 ± 0.77 ml/rat/day) to the OVX + E2 + BR group (5.17 ± 0.50 ml/rat/day), ($p < 0.01$) showing that the E2 replacement partially restored water intake by means of bromocriptine-activated D2 receptors (D2/BR) residual activities, after ovariectomy. Likewise, the C + E2 + SUL group (19.55 ± 0.81 ml/rat/day) compared to the OVX + E2 + SUL group (5.31 ± 0.51 ml/rat/day), ($p < 0.01$), also indicated that E2 replacement in ovariectomized rats partially restored water intake by means of sulpiride-activated D2 receptors (D2/SUL) residual activities, after ovariectomy. The results indicated that E2 controlled both D2/BR and D2/SUL receptors for activating tonic water intake.

3.2. Effects of 17β-Estradiol, Bromocriptine and Sulpiride on a 10% Sucrose Solution Intake

A two-way ANOVA test on daily sucrose solution intake, comparing OVX versus control groups, yielded the main treatment effects [$F(11, 600) = 213.03$, $p < 0.01$], with significant changes over days [$F(9, 600) = 3.13$, $p = 0.001$], without any significant treatment × day interactions [$F(99, 600) = 0.22$, $p > 0.99$], showing an exacerbation of sucrose intake in the six ovariectomized groups relative to their corresponding control groups (**Figure 2**). Thus, Fisher's PLSD post hoc test (p's = 0.05) indicated that sucrose consumption strongly increased in the OVX group (non-drug treated) (46.96 ± 2.46 ml/rat/day) compared to the group C (26.58 ± 1.89 ml/rat/day), ($p < 0.01$), expressing a 17β-estradiol inhibitory tone on sugar intake. Supporting that hypothesis, 17β-estradiol administration to the females (C + E2: 19.58 ± 1.69 ml/rat/day) reduced significantly sucrose intake relative to the control ones (C: 26.58 ± 1.89 ml/rat/day), ($p < 0.01$). Indeed, neither 17β-estradiol replacement (OVX + E2: 46.77 ± 2.71 ml/rat/day), nor sulpiride treatment (OVX + SUL: 47.69 ± 2.45 ml/rat/day) have triggered sugar intake relative to OVX non-drug treated rats (46.96 ± 2.46 ml/Rat/day), ($p = 0.85$ and $p = 0.41$, respectively), suggesting that these drug mechanisms were not individually involved in the exacerbated sucrose intake. In contrast, bromocriptine alone (OVX + BR: 60.28 ± 2.73 ml/rat/day) significantly increased sugar consumption in OVX rats ($p < 0.01$), demonstrating that bromocriptine was a specific inducer of sugar intake. The OVX + BR group (60.28 ± 2.73 ml/rat/day) showed 3 times higher sugar intake than C + BR group (22.72 ± 1.83 ml/rat/day), ($p < 0.01$), indicating that E2 exerted a tonic inhibition on the D2/BR receptors which was released by OVX. Control and ovariectomized rats treated respectively with either E2 + SUL or E2 + BR, compared with control and ovariectomized rats treated with either SUL or BR ($p < 0.01$ in any statistical comparison) indicated that under hormonal induction of 17β-estradiol, both D2/BR and D2/SUL receptors reduced efficiently sucrose intake.

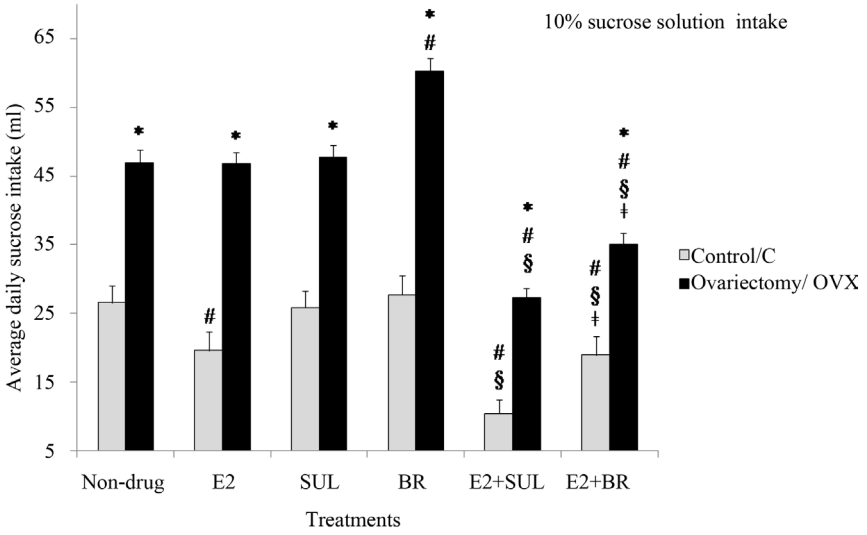

Figure 2. Effects of ovariectomy and 17β-estradiol (E2) replacement on bromocriptine (BR) and sulpiride (SUL) regulating sucrose intake. The consecutive 10-day average values (ml/rat/day ± SEM) of sucrose intake within each experimental group (N = 6 female rats), were represented in non-ovariectomized control (C = 6 groups) and ovariectomized rats (OVX = 6 groups) concurrently subjected to the following treatments: Non-drug-treated (DMSO vehicle), 17β-estradiol (E2), sulpiride (SUL), bromocriptine (BR), E2 + SUL or E2 + BR, designating the 6 constitutive groups of control and ovariectomy respectively. "*" denoted a significant difference between control (C) and ovariectomy (OVX), $p < 0.01$; "#" significantly different from their corresponding control group C or control group OVX, non-drug-treated rats, $p < 0.01$; "§" denote a significant difference between hormone-D2 receptors association effects (E2 + SUL and E2 + BR) vs individual D2 receptors stimulation effects (SUL and BR) in both control and ovariectomized rats $p < 0.01$; "ǂ" significantly different from E2 + SUL ($p < 0.01$).

3.3. Effects of 17β-Estradiol, Bromocriptine and Sulpiride on a 10% Alcohol Solution Intake

Overall analysis on alcohol intake (**Figure 3**) indicated a main difference among treatment groups [$F(11, 600) = 458.42$, $p < 0.01$], with no significant changes over days [$F(9, 600) = 0.44$, $p = 0.91$] and no reliable treatment × day interactions [$F(99, 600) = 0.60$, $p = 0.99$], showing a remarkable weakness of alcohol intake in ovariectomized rats relative to control females. Indeed, post-hoc comparisons using Fisher's PLSD test indicated that alcohol consumption was 10 times lower in the OVX group (0.28 ± 0.15 ml/rat/day), compared with the group C (2.87 ± 0.21 ml/rat/day), ($p < 0.010.01$), signifying that 17β-estradiol maintained an activating tonus on baseline consumption of alcohol. Bromocriptine, which already decreased alcohol consumption in the control (C + BR: 1.18 ± 0.15 ml/rat/day), further reduced alcohol consumption in OVX rats (0.095 ± 0.12 ml/rat/day), ($p < 0.010.01$) demonstrating a direct inhibition of D2/BR receptors on alcohol consumption. However, there was no significant difference when comparing treatment groups C + BR and C + E2 + BR (1 ± 0.19 ml/rat/day), ($p = 0.110$), suggesting that the inhibition of alcohol consumption induced by bromocriptine was independent from E2 and may be carried out by

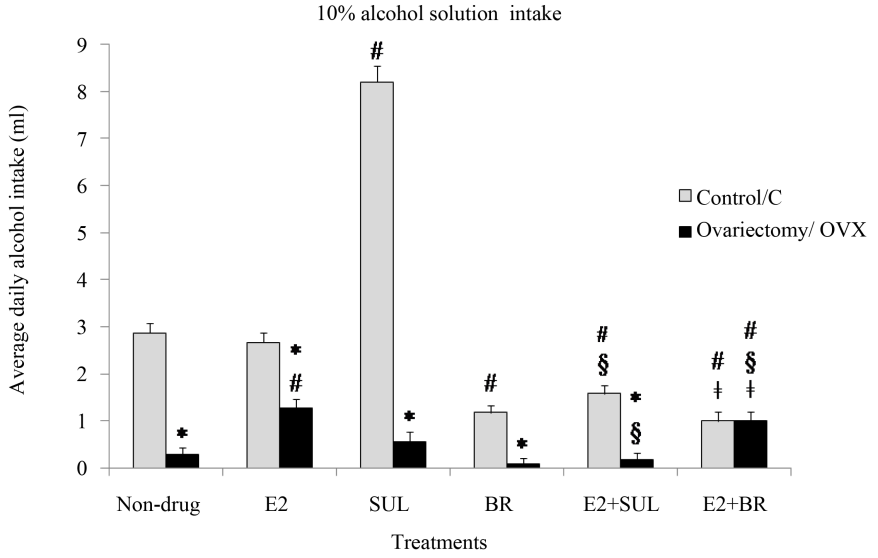

Figure 3. Effects of ovariectomy and 17β-estradiol (E2) replacement on bromocriptine (BR) and sulpiride (SUL) regulating alcohol intake. The consecutive 10-day average values (ml/rat/day ± SEM) of alcohol intake within each experimental group (N = 6 female rats), were represented in non-ovariectomized control (C = 6 groups) and ovariectomized rats (OVX = 6 groups) concurrently subjected to the following treatments: Non-drug-treated (DMSO vehicle), 17β-estradiol (E2), sulpiride (SUL), bromocriptine (BR), E2 + SUL or E2 + BR, designating the 6 constitutive groups of control and ovariectomy respectively. "*" denoted a significant difference between control (C) and ovariectomy (OVX), $p < 0.01$; "#" significantly different from their corresponding control group C or control group OVX, non-drug-treated rats, $p \leq 0.002$; "§" denoted a significant difference between hormone-D2 receptors association effects (E2 + SUL and E2 + BR) vs individual D2 receptors stimulation effects (SUL and BR) in both control and ovariectomized rats $p \leq 0.01$; "ǂ" significantly different from E2 + SUL ($p < 0.01$).

D2/BR receptors only. The last results were confirmed when comparing OVX + BR (0.095 ± 0.12 ml/rat/day) with OVX + E2 + BR (1.07 ± 0.19 ml/rat/day), revealing no synergized actions between BR and E2 ($p = 0.533$). These observations showed that after ovariectomy, a residual basal activity of the D2/BR receptors persisted, which continued to have a direct and tonic inhibitory action on alcohol consumption, without any synergy with E2. Conversely, the C + SUL group significantly increased its alcohol consumption (8.2 ± 0.33 ml/rat/day) compared to the OVX + SUL group (0.579 ± 0.19 ml/rat/day), ($p < 0.01$), suggesting that sulpiride receptors functioning necessitated a baseline tone of E2. In addition, alcohol consumption was efficiently inhibited in the OVX + E2 + SUL group (0.18 ± 0.13 ml/rat/day) compared to the OVX + SUL group (0.579 ± 0.19 ml/rat/day), ($p < 0.01$), showing a residual activity of the D2/SUL receptors which acted synergistically with E2 to inhibit the persisting alcohol intake in OVX rats. Furthermore, control and ovariectomized rats treated respectively with either E2 + SUL or E2 + BR, compared with control and ovariectomized treated with either SUL or BR ($p < 0.01$ in most cases) indicated that E2 modulated more efficiently D2/SUL receptors in reducing alcohol intake than D2/BR

receptors.

3.4. Effects of 17β-Estradiol, Bromocriptine and Sulpiride on Food Consumption and Body Weight

A two-way ANOVA on food intake (FI) and body weight (BW), (Figure 4 and Figure 5) yielded the main treatment effects [$F(11, 600) = 50.29$, $p < 0.01$; $F(11, 600) = 116.49$, $p < 0.01$, respectively], significant changes in food intake over days [$F(9, 600) = 4.86$, $p < 0.01$], but no reliable variations in body weight over days [$F(9, 600) = 1.15$, $p = 0.32$]. However, Both FI and BW did not show any significant treatment x day interactions [$F(99, 600) = 0.17$, $p = 0.99$; $F(99, 600) = 0.41$, $p = 0.99$, respectively]. Post-hoc comparisons using Fisher's PLSD test indicated that OVX increased FI and BW relative to the control ($p < 0.01$ in both cases). Conversely, E2 administration to both OVX and control females decreased more efficiently FI and BW, comparatively to the controls non-drug-treated rats ($p < 0.01$ for any comparison). Similarly, bromocriptine administered to the females reduced more severely FI and BW in control rats than in OVX rats, relative to their respective control non-drug treated rats ($p < 0.01$ for any comparison), suggesting that OVX abolished the tonic inhibition of E2 on

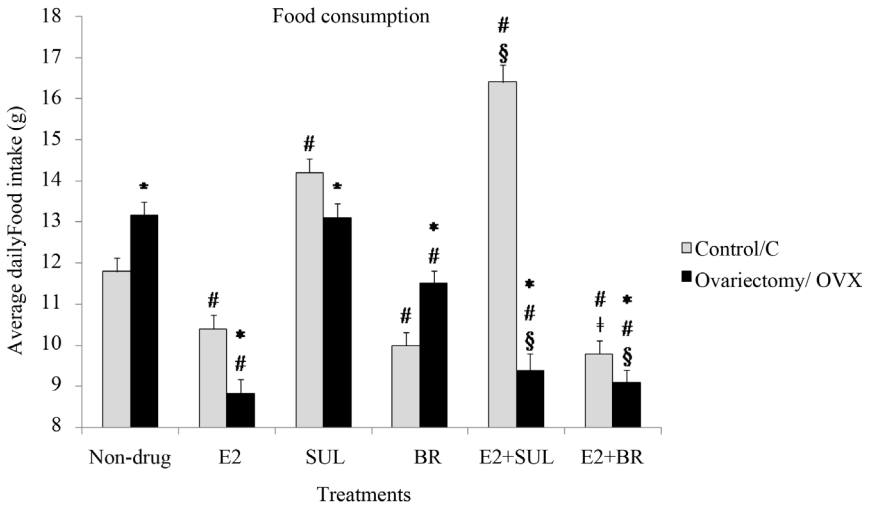

Figure 4. Effects of ovariectomy and 17β-estradiol (E2) replacement on bromocriptine (BR) and sulpiride (SUL) regulating Food consumption. The consecutive 10-day average values (ml/rat/day ± SEM) of food consumption within each experimental group (N = 6 female rats), were represented in non-ovariectomized control (C = 6 groups) and ovariectomized rats (OVX = 6 groups) concurrently subjected to the following treatments: Non-drug-treated (DMSO vehicle), 17β-estradiol (E2), sulpiride (SUL), bromocriptine (BR), E2 + SUL or E2 + BR, designating the 6 constitutive groups of control and ovariectomy respectively. "*" denoted a significant difference between control (C) and ovariectomy (OVX), $p < 0.01$; "#" significantly different from their corresponding control group C or control group OVX, non-drug-treated rats, $p < 0.01$; "§" denoted a significant difference between hormone-D2 receptors association effects (E2 + SUL and E2 + BR) vs individual D2 receptors stimulation effects (SUL and BR) in both control and ovariectomized rats $p < 0.01$; "ǂ" significantly different from E2 + SUL ($p < 0.01$).

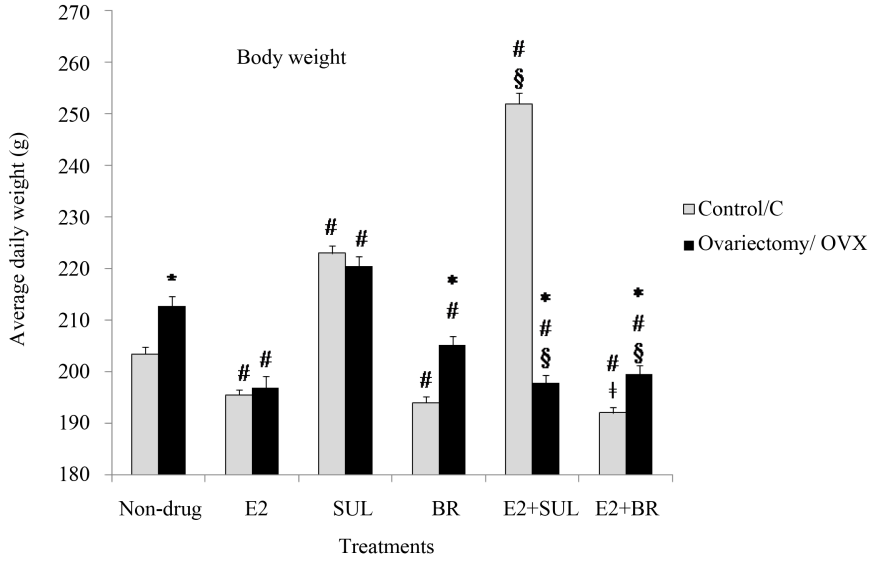

Figure 5. Effects of ovariectomy and 17β-estradiol (E2) replacement on bromocriptine (BR) and sulpiride (SUL) regulating body weight. The consecutive 10-day average values (ml/rat/day ± SEM) of body weight within each experimental group (N = 6 female rats), were represented in non-ovariectomized control (C = 6 groups) and ovariectomized rats (OVX = 6 groups) concurrently subjected to the following treatments: Non-drug-treated (DMSO vehicle), 17β-estradiol (E2), sulpiride (SUL), bromocriptine (BR), E2 + SUL or E2 + BR, designating the 6 constitutive groups of control and ovariectomy respectively. "*" denoted a significant difference between control (C) and ovariectomy (OVX), $p < 0.01$; "#" significantly different from their corresponding control group C or control group OVX, non-drug-treated rats, $p < 0.01$; "§" denoted a significant difference between hormone-D2 receptors association effects (E2 + SUL and E2 + BR) vs individual D2 receptors stimulation effects (SUL and BR) in both control and ovariectomized rats $p < 0.01$.

D2/BR receptors which increased both FI and BW. Conversely, sulpiride maintained or increased efficiently FI and BW in both control and OVX rats compared to their respective control non-drug treated rats ($p < 0.01$ for any comparison). In control rats, treatment with E2 + SUL increased exponentially FI and BWT comparatively to SUL treatment ($p < 0.01$). Inversely, in OVX rats, treatment with E2 + SUL decreased exponentially FI and BWT comparatively to SUL alone ($p < 0.01$). These results indicated a tonic activation of E2 on the D2/SUL receptor which appeared to be a specific inducer of FI and BW. Indeed, D2/SUL receptors appeared to be more severely inhibited by E2 than D2/BR receptors, while comparing effects of E2 + SUL and E2 + BR with individual treatments of SUL and BR respectively on control and OVX rats ($p < 0.01$ for any comparison).

4. Discussion

Our results indicated that administration of 17β-estradiol doubled the mean volume of water ingested daily in control rats, while sulpiride or bromocriptine administrations did not show any significant effects. However, the average daily water intake was amplified by concomitant administration of either "17β-estradiol

+ sulpiride" or "17β-estradiol + bromocriptine" in control rats, indicating that 17β-estradiol activated D2 receptors to induce water intake. Conversely, ovariectomy collapsed water intake. Replacement of 17β-estradiol partially restored water intake, demonstrating that 17β-estradiol (E2) exerted a tonic action activating water consumption.

In control rats, 17β-estradiol administration reduced the average volume of a 10% sucrose solution consumed per day. However sulpiride or bromocriptine administrations had no effect on sucrose intake. Under hormonal induction of 17β-estradiol, sulpiride reduced more significantly sucrose intake than bromocroptine in the controls. Indeed, sulpiride actions on sucrose intake were E2-dependent, because the drug administered to OVX rats had no effect on sugar appetite. Conversely, ovariectomy exacerbated sucrose consumption, expressing a 17β-estradiol inhibitory tone on sugar intake. Likewise, bromocriptine alone significantly increased sugar consumption in OVX rats, demonstrating that bromocriptine is a specific inducer of sugar intake. However, 17β-estradiol replacement in OVX rats did not restore sugar consumption to a normal level. Indeed, E2 exerted a tonic inhibition on the D2/BR receptors which was released by OVX.

In our studies, administration of 17β-estradiol alone did not influence significantly alcohol intake in control female rats. However, ovariectomy collapsed alcohol intake indicating that 17β-estradiol exerted an activating tonus on baseline alcohol consumption. Replacement of 17β-estradiol generated a residual alcohol intake in OVX rats. In control female rats, sulpiride administration induced excessive alcohol intake, while bromocriptine treatment reduced efficiently alcohol consumption. However, E2 controlled both sulpiride and bromocriptine activities to reduce alcohol intake in physiological conditions. Indeed, our results indicated that E2 modulated more efficiently sulpiride activities in reducing alcohol intake than bromocriptine, in both control and OVX rats. For instance, E2 administered to OVX rats revealed no synergized actions with BR to reduce alcohol intake, while such a synergy was exhibited with SUL. Rather, bromocriptine inhibited specifically alcohol intake in both control and ovariectomized rats independently from E2.

Our results indicated that OVX increased food intake (FI) and body weight (BW) relative to the control. Conversely, E2 administration to both OVX and control females decreased efficiently FI and BW. Similarly, bromocriptine administered to the female rats reduced severely FI and BW in both control and OVX rats. OVX abolished the tonic inhibition of E2 on D2/BR receptors which increased both FI and BW. Conversely, sulpiride increased efficiently FI and BW in both control and OVX rats. In control rats, treatment with E2 + SUL increased exponentially FI and BWT. Inversely, in OVX rats, treatment with E2 + SUL decreased exponentially FI and BWT. These results assigned to E2 a main modulatory role on D2/SUL receptors regulating both FI and BW.

Following our experimental design, female rats had free access to three condi-

tions of solutions intakes (e.g. 10% sucrose, 10% alcohol and water). In these conditions, administration of 17β-estradiol to control females substantially increased water intake. It appeared that direct activation of dopamine D2 receptors by bromocriptine or blockade of these D2 receptors by sulpiride did not influence significantly water intake in control rats. However, 17β-estradiol powerfully amplified water intake (2 - 2.5 times) in co-administration with either sulpiride or bromocriptine which individually had no effect on water consumption in control rats. Previous studies reported that both systemic [49] or intrahypothalamic [50] injections of sulpiride, a specific D2 receptor blocker, induced dipsogenic effects in rats. In addition, increased water intake has been also reported in bromocriptine-treated rats [51]. These observations suggested the involvement of two different isoforms of dopamine D2 receptors modulated by 17β-estradiol to regulate water intake. In addition, our results indicated that ovariectomy collapsed water intake suggesting that 17β-estradiol exerted a tonic action activating water consumption. Replacement of 17β-estradiol partially restored water intake by means of both bromocriptine-activated D2 receptors (D2/BR) and sulpiride-activated D2 receptors (D2/SUL). It appeared that 17β-estradiol controlled both D2/BR and D2/SUL receptors for tonic activation of water intake.

Indeed, previous studies involved estrogen in genomic mechanisms underlying the cyclicity of water intake in female rodents [52]. In addition, central actions of estrogen influenced body fluid regulation [53]. Stachenfeld *et al.* [54] reported that estrogen therapy retained body water in menopausal women to avoid dehydration. Thus, estradiol lowered the operating point for osmoregulation of arginine vasopressin and thirst and increased plasma volume [55]. However, it has been reported that spontaneous water consumption fluctuated during the estrous cycle [56]. Indeed, ovariectomy abolished fluctuations in water consumption [56]. Thus, estrogen should be an important faster factor in modifying spontaneous water consumption [57]. Moreover, there was evidence that estrogen also should influence stimulated water intake. For instance, female rats have been shown to consume more water than do male rats after water deprivation [58]. Sex difference in deprivation-induced water consumption suggested hormonal mediation [57]. According to these authors, there were two different mechanisms that underlie stimulated water intake: intracellular dehydration associated with increased plasma sodium concentration, and extracellular dehydration related to a sudden decrease in blood pressure. It was possible that estrogen may influence water intake trough its action on one or both pathways [57].

While ovariectomy collapsed water intake, in the contrary, it exacerbated sucrose intake, showing a 17β-estradiol inhibitory tone on sugar consumption. The drugs mechanisms supporting the 17β-estradiol inhibitory tone on sugar consumption were related to hormone-D2 receptors interactions effects. Indeed, bromocriptine increased 3-fold sugar consumption in ovariectomized rats, indicating that 17β-estradiol exerted a tonic inhibition on the D2/BR receptors

which was released by ovariectomy. Thus, D2 receptors-binding bromocriptine (D2/BR) appeared to be specific inducers of sugar intake. Conversely, sulpiride failed to show any significant effect on sucrose intake, either in control or ovariectomized rats. In addition to the permanent inhibitory role played by 17β-estradiol on sucrose intake, it appeared also that under hormonal induction of 17β-estradiol, both D2/BR and D2/SUL receptors reduced efficiently sucrose intake. 17β-estradiol involvement in reduced sucrose intake was currently reported previously. Thus, Kenney and Redick [59] showed that removal of the ovaries enhanced intake of glucose solutions while replacement of estradiol reduced the glucose intake of ovariectomized rats. However, D2 receptors involvement in sucrose intake was not elucidated. On the one hand, previous studies reported that systemic [60] [61] [62] and intracerebral [63] bromocriptine administration in insulin-resistant animals, declined hepatic glucose production and gluconeogenesis, reduced adipose tissue lipolysis, and improved insulin sensitivity [43]. On the other hand, Hajnal *et al.* [15] reported that D2 receptors were involved in heightened sucrose intake observed in the OLETF obese rat. Indeed, OLETF obese rat showed exaggerated preference for the high sucrose concentration, expressing lower D2 receptors and exhibiting altered D2 receptors signaling [64]. From these observations, the D2 isoform involved in the exacerbated sucrose intake was unknown. An initial explanation was given by Caccavelli *et al.* [65] reporting that the D2 receptor blockade by sulpiride released another D2 isoform able to reduce sucrose intake actively. According to Martres *et al.* [66], treatment with a dopamine antagonist preferably increased the shorter isoform (D2S) in the brain or the pituitary gland. Hajnal *et al.* [15] reported D2 receptors to be efficient inhibitors reducing sucrose intake in rats, during exposure to high sucrose intake. These observations suggested that the D2 receptors-binding sulpiride (D2/SUL), likely D2S (short chain) inhibited sucrose intake in control rats during exposure to high sucrose intake, and that inhibition was induced under hormonal activation of 17β-estradiol on D2/SUL receptors. Indeed, as shown in our studies, 17β-estradiol activates more efficiently sulpiride than bromocriptine in reducing sucrose intake in control rats, showing functional differences between the two receptors [67]. Therefore, we can assume that 17β-estradiol exerted a tonic inhibition on the D2 receptors-binding bromocriptine, like D2L (long chain), which appeared to be specific inducers of sugar intake. In physiological conditions, 17β-estradiol would blockade D2/BR receptors (long chain) to maintain blood sugar level. When sucrose needs were physiologically expressed *i.e.* during hypoglycemia, the tonic inhibition of 17β-estradiol on D2/BR receptors (D2L) was temporally released, thereby triggering sugar intake. Conversely, during hyperglycemia, 17β-estradiol would induce D2/SUL receptors (D2S) to reduce sucrose intake. Consequently, both D2/SUL and D2/BR receptors participated in the homeostasis of sugar baseline level regulation under 17β-estradiol control.

Moreover, alcohol intake was powerfully increased in control rats, following

sulpiride administration, contrary to bromocriptine that reduced it. The 17β-estradiol which had no effect on alcohol consumption, specifically induced both D2/BR and D2/SUL isoforms enhancing inhibition of alcohol intake in non-ovariectomized rats. In particular, 17β-estradiol counteracted efficiently the effects of D2/SUL-induced excessive alcohol drinking, while it acted synergistically with D2/BR to inhibit alcohol intake in control females. In the same control females, D2/SUL isoform primarily decreasing sugar consumption, is going to amplify alcohol consumption; D2/BR receptors were acting exactly in the opposite way. These observations highlighted the divergent nature of the two isoforms. Our results were consistent with the findings of Bulwa *et al.* [68] who reported that both D2L and D2S isoforms played differential roles in alcohol and sugar intakes. On the one hand, Chen *et al.* [4] reported that the specific D2 receptor antagonist, sulpiride, stimulated consumption of ethanol. On the other hand, biased expression of D2S and D2L receptors has been associated with drug abuse. Thus, the loss of D2L receptors and concomitant overexpression of D2S receptors in D2L-deficient mice was associated with altered drug-taking [69]. These authors reported that overexpression of D2S helped increasing ethanol intake. From our studies, the D2/SUL isoform (revealed by sulpiride blockade) was the instigator of alcohol intake, like D2S, while the D2/BR isoform (activated by bromocriptine) would be the inhibitor of alcohol intake, like D2L. By analogy, D2S matched D2/SUL while D2/BR would be the equivalent of D2L in our studies.

Our results also showed that the consumption of alcohol collapsed in the ovariectomized group showing that 17β-estradiol exerted a tonic action facilitating the basic consumption of alcohol. Priddy *et al.* [70] reported that female rats drank more alcohol than males when given either continuous or intermittent access to alcohol; but they did not find any effect of estrous cycle on female alcohol drinking pattern. However, previous studies supported that estrus cycle influenced alcohol consumption: In alcoholic rats, the estrous cycle phase was correlated with blood alcohol levels, which were found to be lower in the estrus + proestrus than in the other phases of the cycle [71]. Our results also indicated functional alteration of either 17β-estradiol or D2 receptors, or both types of receptors in ovariectomized rats. In selectively bred lines of mice, the high alcohol preferring line displayed reduced expression of D2 in the nucleus accumbens as compared to the low alcohol preferring line [27]. Likewise, Ludlow *et al.* [16] reported a significant down-regulation in the expression of D2 receptors in ethanol-consuming rats *versus* pair-fed controls. Moreover, our studies indicated that subsequent 17β-estradiol replacement in ovariectomized rats restored partially ethanol consumption towards baseline levels. Similarly, Ford *et al.* [72] reported that administration of 17β-estradiol to ovariectomized rats restored ethanol consumption to baseline levels, showing 17β-estradiol efficacy to elicit ethanol intake. Indeed, previous studies reported that pharmacological doses of estradiol induced marked, enduring changes in appetite for alcoholic beverages

[73] [74]. These observations suggested that women should be more vulnerable to ethanol abuse which rewarding effects were enhanced by ovarian hormones [75]. Furthermore, bromocriptine efficiently inhibited alcohol consumption regardless of 17β-estradiol. After ovariectomy, functional residual activity of the D2/BR receptors persisted, which continued to have a direct and efficient inhibitory action on alcohol consumption, but showing non-synergy effects with 17β-estradiol. Similarly, in ovariectomized females, a residual activity of D2/SUL receptors also persisted, but undergoing synergized actions with 17β-estradiol to inhibit alcohol consumption.

Investigating body weight and food intake, daily administration of sulpiride and bromocriptine to female rats showed opposite effects. In control rats, sulpiride significantly increased food intake and body weight, while bromocriptine caused a significant decrease in food intake and body weight. These results demonstrated the direct involvement of dopamine D2 receptors in the regulation of food intake and body weight. Previous studies indicated that D2 receptor KO mice showed reduced food intake and body weight along with an increased basal energy expenditure level compared to their wild type littermates [76]. It has also been reported that consumption of a high-fat, high-sugar diet leaded to the down regulation of D2 receptors [77] and reduced dopamine turnover [78]. Availability of D2 receptors was decreased in obese individuals in proportion to their body mass index [79], suggesting that dopamine deficiency in obese individuals should perpetuate pathological eating [6]. However, these studies did not give any precise role to D2 isoforms in regulating food intake and body weight. Indeed, our results indicated that contrasted differential functioning of D2 receptors were hormonally induced by the 17β-estradiol. On the one hand, it appeared that bromocriptine binding receptors (D2/BR like D2L) mediated appetite and weight reductions. In addition, 17β-estradiol and bromocriptine exerted synergistic actions reducing both appetite and weight. Thus, 17β-estradiol enhanced bromocriptine-induced decreased food intake and body weight. Our results were in accordance with the findings of Eckel [80] indicating that 17β-estradiol reduced body weight and adipose tissue. Likewise, bromocriptine decreased food intake and body fat in both animal and human models, thereby showing anti-obesity properties [81] [82]. On the other hand, sulpiride significantly increased food intake and body weight gain in our studies. Previous studies reported that intrahypothalamic injections of sulpiride elicited feeding, even in satiated rats [50]. According to Baptista [83] sulpiride significantly increased body weight, fat gain and food efficiency and did not modify energy expenditure. Indeed, sulpiride induced substantial increase of dopamine D2 receptor mRNAs [84]. Consequently, D2S isoform appeared to be the main mediator of food intake and weight gain. Therefore, how 17β-estradiol modulated dopamine D2S receptors in regulating food intake and body weight?

Ovariectomy increased food consumption and body weight in the present study. Subsequent 17β-estradiol replacement in ovariectomized rats blockaded

food consumption and reduced their weight, confirming that 17β-estradiol regulated food intake and body weight. Literature has recurrently involved the 17β-estradiol in reduced food intake and body weight [85] [86]. Our studies indicated that in control females, 17β-estradiol activated D2/SUL receptors which appeared to be specific inducers of food consumption and weight increase. Conversely, in ovariectomized rats exhibiting increased body weight, administration of 17β-estradiol also inhibited D2/SUL receptors. These observations conferred to the 17β-estradiol a key modulatory role of D2S receptors. Thus, 17β-estradiol appeared to be a typical modulator of sulpiride binding receptors (D2/SUL like D2S), playing a pivotal role in the regulation of the body weight set point, increasing food intake at lower weights and decreasing food intake at higher weights. According to Clifton [87], stimulation of D2 receptors produced increases and decreases in food intake and meal size, in terms of dose-dependent stimulation of presynaptic (autoreceptor) and posysynaptic D_2 receptors. Furthermore, our studies indicated that ovariectomy prevented the tonic inhibition exerted by 17β-estradiol on D2/BR receptors (D2L), thereby exacerbating sucrose intake with moderate increase of food consumption. Conversely, these D2/BR receptors were blocked again in OVX rats by 17β-estradiol replacement, showing the efficiency of 17β-estradiol blockade on D2/BR receptors (long chain) in physiological conditions. Our data supported the findings of Wu *et al.* [41] reporting that 17β-estradiol acted to modulate the ratio of the two isoforms of the dopamine D2 receptor (D2L/D2S), by transcription of their respective genes, through nuclear estrogen receptors induction, thereby highlighting functional difference between the two isoforms. The two isoforms coexisted in most brain tissue analyzed and the ratio of D2L *versus* D2S mRNA expressions varied from region to region [88] [89]. Indeed, previous studies showed that dopamine D2 receptors existed in two isoforms, D2 long (D2L) and D2 short (D2S) chains, produced by alternative splicing of the same gene [90]. These variants of the D2 dopamine were G protein-coupled receptors which had distinct anatomical, physiological, signaling, and pharmacological properties [9] [10]. Structural studies indicated that D2L has a 29 amino acid insertion in the third cytoplasmic loop of the protein which was absent in D2S; this loop seemed playing a central role in receptor coupling, implying functional diversity [10] [11]. From anatomical investigations, D2S has been shown to be mostly expressed presynaptically and to be mostly involved in autoreceptor functions, whereas D2L seemed to be predominantly a postsynaptic isoform [91] [92] [93]. This should explain why sulpiride blockade released the D2S isoform exerting opposite effects to the D2L isoform directly activated by bromocriptine. Functional findings reported that presynaptically localized autoreceptors generally provided an important negative feedback mechanism that adjusted neuronal firing rate, synthesis, and release of the dopamine in response to changes in extracellular dopamine levels [94] [95] [10]. It appeared that activation of presynaptic D2-class autoreceptors generally caused a decrease in dopamine release whereas activation of postsynaptic recep-

tors increased dopamine activity [10].

Collectively, our studies highlighted a direct involvement of 17β-estradiol in the regulation of body weight set point, either controlling sucrose intake via D2/BR receptors (D2L) for glucose homeostasis and energy expenditure, or modulating food intake via D2/SUL receptors (D2S) for regulating body weight. From these observations, the effects of D2/SUL receptors (D2S) appeared to meet the slow genomic actions induced by 17β-estradiol, whereas D2/BR receptors (D2L) effects met the rapid non-genomic actions of 17β-estradiol, thereby involving both types of D2 receptors in the etiology of obesity. Indeed, removal of the ovaries produced an efficient and lasting elevation in food reward and food-seeking behavior, suggesting that ovarian sex steroids were critical for the maintenance of normal food reward behavior [96]. It appeared also that 17β-estradiol influenced spontaneous water intake; it acted most likely as a volumetric regulator of water intake, involving both D2L and D2S receptors: It should increase water consumption during the lower volumetric absorptions periods and vice versa, with additional tuning on body weight regulation. The combined effects of altered control of D2/BR and D2/SUL receptors, when 17β-estradiol was lacking, were contributory factors underlying the etiology of obesity.

Authors Contributions

A.B designed the study and wrote the manuscript; S.S., B.B., L.B and S.-V.G. performed experiments.

Disclosures

The authors have no conflict of interest to declare relating this work.

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