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 α 1 ADRENOCEPTOR AND D1 DOPAMINERGIC
RECEPTOR PATHWAYS IN THE DIET-INDUCED
OBESE RAT**

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TESOFENSINE, A NOVEL TRIPLE MONOAMINE REUPTAKE INHIBITOR, INDUCES APPETITE SUPPRESSION BY INDIRECT STIMULATION OF α_1 ADRENOCEPTOR AND D₁ DOPAMINERGIC RECEPTOR PATHWAYS IN THE DIET-INDUCED OBESE RAT

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Running title: Tesofensine-induced hypophagia

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ABSTRACT

Tesofensine is a novel monoamine reuptake inhibitor (MRI) which inhibits both norepinephrine, 5-HT and dopamine reuptake function. Tesofensine is currently in clinical development for the treatment of obesity, however, the pharmacological basis for its strong effect in obesity management is not clarified. Using a rat model of diet-induced obesity (DIO), we characterized the pharmacological mechanisms underlying the appetite suppressive effect of tesofensine. DIO rats treated with tesofensine (2.0 mg/kg, s.c.) for 16 days showed significantly lower body weights than vehicle-treated DIO rats, being reflected by a marked hypophagic response. Using an automatized food intake monitoring system during a 12h nocturnal test period, tesofensine-induced hypophagia was investigated further by studying the acute interaction of a variety of monoamine receptor antagonists with tesofensine-induced hypophagia in the DIO rat. Tesofensine (0.5-3.0 mg/kg, s.c.) induced a dose-dependent and marked decline in food intake with an ED₅₀ of 1.3 mg/kg. The hypophagic response of tesofensine (1.5 mg/kg, s.c.) was almost completely reversed by co-administration of prazosin (1.0 mg/kg, α_1 adrenoceptor antagonist) and partially antagonized by co-administration of SCH23390 (0.03 mg/kg, dopamine D₁ receptor antagonist). In contrast, tesofensine-induced hypophagia was not affected by RX821002 (0.3 mg/kg, α_2 adrenoceptor antagonist), haloperidol (0.03 mg/kg, D₂ receptor antagonist), NGB2904 (0.1 mg/kg, D₃ receptor antagonist), ritanserin (0.03 mg/kg, 5-HT_{2A/C} receptor antagonist). Hence, the mechanism underlying the suppression of feeding by tesofensine in the obese rat is dependent on the drug's ability to indirectly stimulate α_1 adrenoceptor and dopamine D₁ receptor function.

Key words: Diet-induced obesity, monoamine reuptake inhibitor, food intake, anti-obesity effect, α_1 adrenoceptor; dopamine D₁ receptor.

INTRODUCTION

Tesofensine is a novel centrally acting triple monoamine reuptake inhibitor (MRI) with intrinsic inhibitory activity on norepinephrine (NE), serotonin (5-HT) and dopamine (DA) transporter function (Lehr et al., 2008). Results from recent clinical trials show that tesofensine effectively produces a substantial weight loss in obese patients (Astrup *et al.*, 2008a; Astrup *et al.*, 2008b). When corrected for placebo and diet effects, long-term tesofensine treatment produces a maximal weight loss of 10.6% in obese patients, which is twice that achieved by other anti-obesity agents. The robust weight loss produced by tesofensine is explained by a dose-dependent hypophagia due to stimulation of satiety (Astrup *et al.*, 2008b), suggesting that tesofensine predominantly acts as an appetite suppressant to produce a negative energy balance. However, the pharmacological basis for the hypophagic effect is not resolved.

Monoaminergic neurotransmission is centrally involved in the homeostatic control of appetite function, and it has become increasingly clear that there is a close association between dietary obesity and altered monoaminergic neurotransmission. For example, pharmacological manipulation studies have shown that stimulated central monoaminergic activity induces profound effects on feeding behavior and thus energy intake, and agents which enhance synaptic levels of NE, 5-HT or DA by stimulating release or reducing reuptake can decrease feeding and weight gain (Nelson and Gehlert, 2006).

The hypothalamus is a key site of action for the anorexic effect of monoamine receptor agonists, as increased monoaminergic activity within the hypothalamus can markedly influence feeding behavior by triggering satiety signals (Meguid *et al.*, 2000b; Wellman, 2000). Monoaminergic modulation of appetite function at the hypothalamic level is complex as several hypothalamic NE, DA and 5-HT receptor subtypes are involved in the control of feeding activity. Notably, α_1 adrenoceptor and 5-HT_{2C} agonists inhibits food intake, and these monoaminergic signaling pathways are strongly implicated in the anorexic action NE and

5-HT (Clifton and Kennett, 2006). In addition, D₁ and D₂ receptors agonists can suppress feeding, and it is thought that these DA receptor subtypes can produce synergistic anorexic effects (Meguid *et al.*, 2000b; Wellman, 2005).

There is a growing notion that mesolimbic dopaminergic neurotransmission contributes to the effect of DA on feeding behavior (Volkow and Wise, 2005). Food intake and food deprivation has opposing effects on extracellular DA levels in the nucleus accumbens, as feeding stimulates DA release and turnover whereas food deprivation causes the opposite effects (Nelson and Gehlert, 2006). Several DA receptors may be effectors of increased DA availability during feeding episodes, as both D₁, D₂ and D₃ receptor agonists reduce food intake in animal models of obesity (Scislawski *et al.*, 1999; McQuade *et al.*, 2003; Davis *et al.*, 2008).

Correspondingly, there are several indices of defective central monoaminergic neurotransmission in preclinical models of obesity, including reduced hypothalamic α_1 adrenoceptor binding (Wilmot *et al.*, 1988), enhanced electrophysiological responsiveness to intrahypothalamic NE application (Kraszewski and Cincotta, 2000), decreased basal hypothalamic 5-HT and DA levels (Meguid *et al.*, 2000a), as well as reduced mesolimbic DA activity (Geiger *et al.*, 2008).

Therefore, we reasoned that the anti-obesity effect of tesofensine treatment in obese individuals could be a consequence of combined modulation of several central monoaminergic pathways. In the present study, the pharmacological mechanisms underlying the anti-obesity effect of tesofensine was investigated in a rat model of diet-induced obesity (DIO). Tesofensine induced a robust weight reduction in DIO rats during chronic tesofensine treatment which was accompanied by a strong hypophagic response. In order to identify the principal monoamine receptor(s) being critically involved in hypophagic effect of tesofensine, we investigated whether tesofensine-induced hypophagia could be reversed by co-administration of various monoaminergic receptor antagonists.

MATERIALS AND METHODS

Animal care and housing

Five-weeks old male Sprague-Dawley rats (150-180 g, Taconics, Ry, Denmark) were initially group housed (4 rats per cage) in solid bottomed Plexiglas cages with dust free wood chippings and a cardboard tube. Holding rooms were maintained under a 12-h light/dark cycle (lights off: 1500 h). Ambient temperature was 18.0 to 22.0°C and relative air humidity of 40 to 60%. A dim red light was the sole source of illumination during the dark period. The rats had ad libitum food and water, and they were made obese by switching to high-fat diet for a minimum of 10 weeks (#D12492; 60% kcal from fat, energy density 5.24 kcal per g, Research Diets, New Brunswick, NJ) in pellet form. After 3-4 weeks on high-fat diet, the rats were randomly matched (2 rats per cage) with free access to animal diet throughout all experiments. Initially, an optimization study (see Fig. 1) indicated that the rats obtained a significantly higher body weight gain, as compared to rats receiving standard chow (Altromin 1324, 10% kcal from fat, energy density 2.85 kcal per g, Altromin GmbH, Lage, Germany). Thus, after 10 weeks (day 70) on high-fat diet, the rats had a 22% higher net body weight gain (365 ± 15 g, $n=9$) as compared to the net body weight gain (300 ± 12 g, $n=9$) of rats on standard chow (repeated measure two-way ANOVA, $p < 0.001$). The outbred rats in the present experiments were therefore after at least 10 weeks on high-fat diet considered to be diet-induced obese (DIO) as a group, according to the term introduced by Levin and co-workers (Levin and Keeseey, 1998). However, no discrimination was made between rats on high-fat diet with lowest or highest weight gain. Thus, rats were assigned randomly to experimental groups irrespectively of any individual differences in net weight gain.

All experiments were approved (permission no. 2007/561-1343) and conducted in accordance with the guidelines of the Animal Experimentation Inspectorate, Ministry of Justice, Denmark.

Drugs

Tesofensine (8-Azabicyclo[3.2.1]octane,3-[3,4-dichlorophenyl)-2-(ethoxymethyl)-8-methyl-[1R-(2-endo,3-exo)]-2-hydroxy-1,2,3-propanetricarboxylate) is a derivative of an azabicyclooctane citrate, synthesized at the Department of Medicinal Chemistry, NeuroSearch A/S. Prazosin, RX821002, SCH23390 and ritanserin were purchased from Sigma-Aldrich (St. Louis, MI). Haloperidol and NGB2904 were from Janssen-Cilag (Beerse, Belgium) and Tocris (Ellisville, MO), respectively. All stock solutions were prepared daily and diluted to working concentration with the relevant vehicle. Tesofensine was dissolved in 0.9% saline solution, all other compounds were dissolved in 15% HP- β -cyclodextrine. All drug solutions were brought to pH 7.4 with 0.1 N NaOH.

Assessment of feeding kinetics using real-time feeding monitoring

The DIO rats were re-housed after 9-10 weeks on high-fat diet (mean body weight 493 ± 49 g, weight gain with high-fat diet 343 ± 4.3 g) and transferred with its partnering rat to fully automated food intake monitoring cages (HM-2, MBRose Aps, Faaborg, Denmark; for further information, see <http://www.mbrose.dk/products.htm>). The HM-2 food intake monitoring cages were placed in a modified ventilated cabinet with lightproof doors and a light kit for cabinet-based control of light-dark cycle (Scanbur BK, Karslunde, Denmark) being similar to that in the holding rooms. Cabinet temperature was 24.0 to 26.0°C and relative air humidity of 40 to 60%. The animals were habituated to the HM-2 food intake monitoring system for at least 5 days prior to drug treatment. The feeding behavior of each animal was monitored on a constant basis during the habituation period in order to assure that the food intake of each animal returned to the pre-rehousing level. Food intake typically declined during the first day of habituation, but returned to the pre-rehousing level during the remainder of the habituation period (data not shown). On the day of re-housing to the fully automated food intake monitoring cages, the rats were subcutaneously

injected with a microchip (#402575, eVet, Haderslev, Denmark). The tagging did not affect feeding behavior or body weight gain (data not shown) and allowed the HM-2 food intake monitoring system to simultaneously identify and in real-time mode to track feeding behavior of each individual animal throughout the entire experiment. Food intake was monitored on a constant basis following entry of the tagged animal into the tag-reading food channel. Upon exit of the animal from the food channel, the net food intake was calculated by the HM-2 control unit software (HMLab, MBRose, Faaborg, Denmark). Animal-allocated food intake was discriminated from spillage by defining a minimum bout size (≥ 0.1 g) per entry. Food intake was measured on a constant basis and calculated as total food intake (g) and cumulated food intake (g per 2h interval), respectively. In addition, microstructural analysis of nocturnal food intake was carried out by analyzing various meal consumption parameters, *i.e.* total number of meals, mean meal size, average meal duration, latency to first meal and first meal size. Meals were defined using a minimum meal size criterion of 0.3 g, a minimum meal duration criterion of 30 seconds, and a minimum intermeal interval of 15 min., measured from the last measured intake of a meal (23). With the use of these criteria, all nocturnal feeding activity was resolved into meals.

All experimental compounds and vehicles were administered subcutaneously (1 ml/kg). The tesofensine dose-response and monoamine receptor antagonist interaction experiments were designed as between-subject studies with at least 6 (tesofensine dose-response study) or 8 DIO rats (monoamine receptor antagonist interaction study) per group. In all acute dosing experiments, food intake was measured on a constant basis throughout the 12h period.

When performing drug interaction experiments, bilateral subcutaneous injections were given within one minute between each injection. Drugs were administered 30 to 60 min. before dark onset. The home cage was removed from the HM-2 food intake monitoring system during the drug administration procedure and returned immediately after completion of the drug injections, whereafter automated monitoring of the feeding behavior of each individual animal was started.

In addition, a time–response study of longer duration was conducted in DIO rats with a single dose of tesofensine (1.5 mg/kg, s.c., n=6) or saline vehicle (n=6) in order to assess the temporal dynamics on food intake over a 72h measurement period. Data on food consumption were collected in 2h or 12h intervals.

Body weight and microstructural food intake analysis was performed using a data reporting software (HMView, MBRose, Faaborg, Denmark). The data were imported into a standard graphic and statistical analysis programme (GraphPad Prism v.4.03 or SigmaStat v.3.5). All data were expressed mean \pm S.E.M of n individual animals. The ED₅₀ of tesofensine-induced hypophagia was calculated using a four parameter logistic equation for non-linear curve-fitting of a sigmoidal dose-response curve. Statistical comparisons between groups were performed by a one-way ANOVA with Tukey's post-hoc test (body weight gain, total food intake and feeding kinetics) or a repeated measure two-way ANOVA with Bonferroni's multiple comparison test (cumulated food intake). A p-value less than 0.05 was considered statistically significant.

Chronic treatment with tesofensine

In a separate study, DIO rats (11 weeks on high-fat diet, mean body weight 556 ± 12.1 g, weight gain with high-fat diet 406 ± 4.8 g) were housed in the pair environment until one week before the study when they were singly housed in cages equipped with a food hopper allowing assessment of net food intake by manual weighing. The light-dark cycle was similar to that used in the acute drug treatment experiments. Tesofensine (2.0 mg/kg, n=5) or saline vehicle (n=5) was administered subcutaneously to the DIO rats once daily (approx. 1 hr before dark onset) for 16 days. Body weight and food intake was monitored once daily prior to the daily drug treatment procedure. Body weight data were expressed as daily body weight gain relative to the first day of drug administration. The data were fed into a standard graphic and statistical analysis programme (GraphPad Prism v.4.03). Body weight gain and food intake were expressed as means \pm S.E.M of n individual animals, and a repeated measure two-way ANOVA with Bonferroni's

multiple comparison test was applied to perform statistical comparisons between treatment groups. A p-value less than 0.05 was considered statistically significant.

RESULTS

Acute treatment with tesofensine

Tesofensine dose-dependently induces hypophagia in the DIO

A single dose of tesofensine (0.1–3 mg/kg, s.c.) robustly and dose-dependently inhibited food intake in DIO rats over the 12h nocturnal observation period ($p < 0.0001$, Fig. 2A). The threshold dose for inhibition of total food intake was 1.0 mg/kg ($p < 0.01$). The ED_{50} for tesofensine-induced inhibition of total food intake in DIO rats was estimated to be 1.3 mg/kg.

During the 12h-observation period, vehicle-treated rats consumed an amount of food corresponding to 54.1 ± 2.1 kcal, whereas acute treatment with the highest dose of tesofensine (3.0 mg/kg) resulted in a total food intake amounting to 12.6 ± 3.9 kcal, being equivalent to a 77% reduction of energy intake.

Analysis of the temporal effect of tesofensine indicated that doses of ≥ 0.5 mg/kg significantly affected cumulated food intake ($p < 0.001$, Fig. 2B). The onset and duration of tesofensine-induced hypophagia was dose-dependent. For doses ≥ 2.0 mg/kg, the hypophagic effect was introduced instantly after drug administration ($p < 0.05$) and maintained throughout the nocturnal observation period. Lower doses (0.5-1.5 mg/kg) showed a progressive increase in the lag-time for onset of food intake suppression. Thus, whereas treatment with intermediate doses resulted in significant inhibitory effect on cumulative food intake at 2-12h post-injection (1.0 mg/kg, $p < 0.01$; 1.5 mg/kg, $p < 0.001$), the inhibitory effect of lower doses on food intake was further delayed, being only significant at 10-12h post-injection (0.5 mg/kg, $p < 0.05$).

While tesofensine treatment (1.5 mg/kg, s.c.) affected food intake during the initial 12h post-injection, feeding activity was re-established to vehicle control level in the remainder 12-72 hrs of light-dark cycle period (Fig. 3), thus indicating that acute tesofensine treatment did not result in compensatory feeding activity.

The effects of tesofensine on nocturnal food consumption was subjected to microstructural analysis (Fig. 3). Tesofensine significantly affected several parameters of feeding kinetics, *i.e.* acute tesofensine treatment resulted in a decrease in the total number of meals (Fig. 4A), average meal size (Fig. 4B), average meal duration (Fig. 4C). In addition, tesofensine had a pronounced impact on first meal latency and size (Fig. 4D, 4E).

The lower food intake in tesofensine exposed rats had an impact on body weight gain during the 12hr observation period. Vehicle-treated DIO rats gained $+5.0 \pm 1.0$ g ($+1.0 \pm 0.2\%$ of initial body weight) during the experiment. In contrast, tesofensine significantly reduced weight gain in the dose range of 1.0-3.0 mg/kg. Thus, treatment with doses of 1.0 and 3.0 mg/kg induced a weight loss of -2.0 ± 1.0 g ($-0.4 \pm 0.3\%$ of initial body weight, $p < 0.05$) and -7.3 ± 1.8 g ($-1.5 \pm 0.4\%$ of initial body weight, $p < 0.001$), respectively.

In order to assess the effect of tesofensine on monoamine neurotransmission pathways, a dose of 1.5 mg/kg was selected for further analysis. This dose inhibited food intake corresponding to approximately 50% of basal food consumption in the DIO rat, and was thus suitable for interaction studies with various monoamine receptor antagonists. Preliminary dose-response experiments were performed with each monoamine receptor antagonist in the DIO rat (data not shown), and the highest dose of each antagonist producing no effect on total food intake *per se* was selected.

Involvement of adrenoceptor function in tesofensine-induced hypophagia

The reduction in food intake induced by tesofensine was almost completely abolished by co-administration of the α_1 -adrenoceptor antagonist, prazosin (1.0 mg/kg, s.c., Fig. 5A). Whereas tesofensine significantly reduced total food intake ($37\pm 4.9\%$ of vehicle control, $p < 0.001$), combined prazosin treatment reversed the feeding level to $80\pm 4.6\%$ of the vehicle control level ($p < 0.01$ and $p < 0.001$ as compared to vehicle and tesofensine alone, respectively). The antagonizing effect of prazosin had a fast onset (0-2h, $p < 0.05$) and was maintained throughout the 12h nocturnal period, although the counteracting effect slightly wore off during the 6-12h period (Fig. 5B).

By contrast, co-administration of the α_2 -adrenoceptor antagonist, RX821002 (0.3 mg/kg, s.c.) did not affect the hypophagic effect of tesofensine (Fig. 7, panel A). Neither prazosin nor RX821002 administration alone influenced baseline total or cumulated food intake in the DIO rat.

Involvement of dopamine function in tesofensine-induced hypophagia

The D_1 receptor antagonist SCH23390 (0.03 mg/kg, s.c.) partially antagonized the suppression of food intake with tesofensine ($p = 0.007$). The level of total food intake following tesofensine alone or in combination with SCH23390 was $35\pm 5.2\%$ and $65\pm 6.7\%$, respectively, of the vehicle control level (Fig. 6A). Based on real-time analysis of cumulated food intake, onset of the antagonizing effect of SCH23390 was delayed, thus partially antagonizing the inhibitory effect of tesofensine on cumulative food intake at 10-12h ($p < 0.01$) post-injection, also signifying that the efficacy of SCH23390 at intermediate time points was less robust (Fig. 7B).

The D_2 receptor antagonist haloperidol (0.03 mg/kg, s.c.) did not significantly ($p = 0.088$) affect tesofensine's ability to inhibit food intake in the DIO rat (Fig. 7, panel B). In addition, the D_3 receptor antagonist NGB2904 did not have any antagonizing effect on tesofensine-induced hypophagia ($p = 0.492$, Fig. 7, panel C).

SCH23390, haloperidol and NGB2904 alone had no effect on baseline total or cumulated food intake in the DIO rat.

No involvement 5HT_{2A/C} receptor function in tesofensine-induced hypophagia

Ritanserin (0.03 mg/kg, s.c.), a 5HT_{2A/C} receptor antagonist, did not affect tesofensine-induced inhibition of food intake in the DIO rat ($p=0.843$, Fig. 7, panel D). Ritanserin treatment *per se* did not display a significant effect on total food consumption or cumulative food intake.

Chronic treatment with tesofensine

Daily administration of a moderate dose of tesofensine (2.0 mg/kg, s.c.) over 16 days triggered a significant reduction in body weight after 4 days of administration ($p<0.05$) relative to vehicle-treated controls (two-way ANOVA, overall $p<0.0001$), see Fig. 8A. The weight lowering effect was sustained from day 4 throughout the remainder of the experiment, reaching a plateau from day 10 and onwards. The average relative decrease in the body weight of tesofensine-treated DIO rats over the entire treatment period was $8.6\pm 1.4\%$. When comparing to vehicle controls, the relative weight loss with tesofensine was $13.8\pm 1.4\%$.

Tesofensine also had a strong impact on food intake over the 16-day administration period. In accordance with the acute dosing experiments, tesofensine significantly reduced daily food intake on the first day of treatment, an effect that lasted to day 14 of treatment (2-way ANOVA, overall $p<0.0001$), see Fig. 8B. The percentage of suppression of daily food intake induced by tesofensine over days 1 to 14 averaged $49.4\pm 4.7\%$ relative to the vehicle control group. The hypophagic response to tesofensine was thus transient, eventually returning to levels not significantly different from vehicle controls on treatment day 15.

DISCUSSION

Tesofensine dose-dependently suppressed food intake in DIO rats within 12h of administration. The lowest dose which decreased the quantity of food consumed during nocturnal feeding episodes was 1.0 mg/kg with a strong hypophagic effect being observed after administration of more than 2.0 mg/kg. The hypophagic effect of a single dose of tesofensine (≥ 1.0 mg/kg) was sustained throughout the 12h observation period. As a consequence of the strong hypophagic response, acute tesofensine administration resulted in a corresponding dose-dependent weight loss in the DIO rat.

Analysis of the time-response effect and microstructural feeding activity revealed that tesofensine had a pronounced effect on several behavioral aspects of food intake. Most strikingly, the highest dose of tesofensine (3.0 mg/kg, s.c.) strongly increased the latency time (571% increase) to the first meal and reduced the total number of meals and average meal size by 60% and 69%, respectively.

Given the strong acute effects of tesofensine on body weight and food consumption, the maintenance of tesofensine's activity after a longer duration of administration was investigated in the DIO model. The reduction in body mass of DIO rats produced by tesofensine could be sustained by daily dosing throughout the 16-day treatment regimen. The decrease in body mass was accompanied by a significant suppression of daily food intake, although the hypophagic effect gradually returned to control levels after 14 days of treatment. In both normal (acute treatment) and DIO rats (acute or chronic treatment), tesofensine administration did not cause side-effects which could potentially influence feeding behavior, *e.g.* negative reinforcement or gastrointestinal upset (data not shown). We therefore conclude that tesofensine specifically reduces the drive to obtain food in DIO rats as a consequence of enhanced satiety signaling. This is also in close correspondence with observations from clinical settings, as the significant weight loss

(10.6%) observed after 6 months with tesofensine treatment in obese patients is associated with suppression of hunger and increased satiety measures (Astrup *et al.*, 2008a).

Considering that DIO rats gradually developed tolerance to the hypophagic effect of tesofensine during chronic dosing, this indicates that other factors than appetite regulation contributed to sustain the maximal weight loss. This notion is supported by the observation that stimulation of monoaminergic neurotransmission also impacts the metabolic rate by increasing energy expenditure, either directly by increasing thermogenesis via activation of peripheral β_3 adrenoceptors in brown adipose tissue, or indirectly by increasing central DA receptor-dependent motor activity. These combined effects are reported for a number of MRIs, including the dual NE/5-HT and NE/DA reuptake inhibitors, sibutramine and bupropion, respectively (Connoley *et al.*, 1999; Liu *et al.*, 2004; Golozoubova *et al.*, 2006; Billes and Cowley, 2008). Although it should be noted that no tolerance to the appetite suppressing effect of tesofensine is observed in clinical settings (Astrup *et al.*, 2008a), one possibility that warrants further investigation is whether sustained weight loss with tesofensine may involve increased energy expenditure.

Since appetite suppression was an important factor for triggering and initially establishing the anti-obesity effect of tesofensine in the DIO rat, it was of particular interest to identify the principal monoaminergic system(s) underlying tesofensine-induced suppression of food intake in the DIO rat.

To this end, the interaction of acute tesofensine administration with various monoamine receptor antagonists was investigated in the DIO rat. While prazosin and SCH23390 were able to produce a significant reversal of tesofensine-induced hypophagia in the DIO rat, all other antagonists tested in this study with distinct monoamine receptor profiles had no effect. It should be noted that the individual doses of RX821002, haloperidol, NGB2904 and ritanserin, respectively, used in the present study lie within ranges

previously reported to be active in the rat in other *in vivo* settings (Pfeffer and Samson, 1988; Rittenhouse *et al.*, 1994; Deupree *et al.*, 2008; Spiller *et al.*, 2008), thus excluding the possibility that the lack of an effect of these antagonists on tesofensine-induced hypophagia could be due to the use of physiologically inactive doses.

The hypophagic effect of tesofensine was strongly reversed by co-administration of prazosin. Correspondingly, prazosin also prevented the acute hypophagic effect of sibutramine (Jackson *et al.*, 1997). In addition, selective NE reuptake inhibitors, such as nisoxetine, desmethylimipramine and LY368975 produces anorexic effects in the rat (Durcan *et al.*, 1988; Gehlert *et al.*, 1998; Billes and Cowley, 2007). The triple MRI profile of tesofensine therefore suggests that its hypophagic effect to a major degree occurs after blockade of NE reuptake function, reflecting a subsequent activation of α_1 adrenoceptors as a consequence of increased synaptic availability of NE. The relevance of stimulated α_1 adrenoceptor function by tesofensine is supported by the consistent finding of anorexic effects following systemic administration of various α_1 adrenoceptor agonists (Wellman and Davies, 1992; Morien *et al.*, 1993; Racotta and Soto-Mora, 1993). In agreement, long-term α_1 adrenoceptor antagonist treatment causes overeating and weight gain in clinical settings (Bray, 2000), indicating that positive modulation of α_1 adrenoceptor activity is a clinically important target in obesity management.

The precise site of feeding modulatory action of adrenoceptor modulators in the CNS is not known. Both α_1 and α_2 adrenoceptors are localised within the hypothalamus (Young and Kuhar, 1980), and direct injections of prazosin into the hypothalamic paraventricular nucleus (PVN) can reverse the anorexic effect of a systemically administered α_1 adrenoceptor agonist (Wellman and Davies, 1992). Also, there is evidence that NE efflux increases in the hypothalamus, including the PVN, during food consumption (Stanley *et al.*, 1989; Morien *et al.*, 1995). Because extracellular NE levels in the rat PVN peaks just before onset of dark being closely correlated to subsequent feeding activity (Morien *et al.*, 1995), this region may

therefore be important in providing satiety/hunger signals downstream of NE secretion. Interestingly, DIO rats exhibit reduced hypothalamic α_1 adrenoceptor binding (Wilmot *et al.*, 1988), which may thus reflect a reduced α_1 adrenoceptor function. In addition, obese mice display enhanced electrophysiologic responsiveness to iontophoretically applied NE in the ventromedial hypothalamus as compared to lean mice, and high-fat feeding stimulates hypothalamic NE turnover in the obese rat (Shimizu *et al.*, 1994; Kraszewski and Cincotta, 2000). This could possibly indicate that the hypothalamic NE system is primed in DIO rats, potentially facilitating an increased responsiveness to tesofensine-induced NE effects subsequent to NET blockade.

The lack of an effect of the selective α_2 adrenoceptor antagonist RX821002 on tesofensine-induced hypophagia indicates that only α_1 adrenoceptor function was affected, presumably at the hypothalamic level. Notably, the PVN is among the brain regions expressing the highest NE transporter (NET) binding level (Kung *et al.*, 2004), which argues for tesofensine produced a blockade of NET function within the PVN, resulting in efficient accumulation of PVN synaptic NE favouring anorexic α_1 adrenoceptor-induced signaling. Specificity for α_1 adrenoceptor effects in the PVN following NET inhibition is further supported by the finding that sibutramine elevates synaptic NE levels in the PVN of lean rats, which is thought to be linked to its α_1 adrenoceptor antagonist-sensitive hypophagic effect (Jackson *et al.*, 1997; Wortley *et al.*, 1999).

The selective D₁ receptor antagonist SCH23390 partially blocked tesofensine-induced hypophagia. This suggests that tesofensine-treatment promoted stimulated D₁ receptor activity secondary to DA transporter (DAT) blockade. The finding that positive modulation of D₁ receptor function contributed to the anorexic effect of tesofensine is in agreement with the role of D₁ receptor receptors on feeding behavior. Accordingly, D₁ receptor stimulation reduces food intake and weight gain in a mouse model of obesity

(Scislowski *et al.*, 1999; Bina and Cincotta, 2000; Kuo, 2002). The inhibitory effect of D₁ receptor activation on feeding is most likely linked to stimulated hypothalamic DA function which can lead to suppression of hypothalamic orexigenic signaling (Kuo, 2002; Alberto *et al.*, 2006). Also, changes in hypothalamic D₁ receptor expression may contribute to the hyperphagic behavior of obese Zucker rats (Fetissov *et al.*, 2002).

Hypothalamic DA neurotransmission plays an important role in the control of the duration of feeding (Meguid *et al.*, 1997). Interestingly, DA levels accumulate in the ventromedial hypothalamus during the initial period of food intake, reaching a peak and declining towards baseline levels at the end of the feeding episode (Orosco and Nicolaidis, 1992; Orosco *et al.*, 1995). Also, intrahypothalamic DA infusions suppress feeding due to a reduction in the number of meal sizes (Yang *et al.*, 1997). Interestingly, obese rats exhibit reduced basal hypothalamic DA levels (Meguid *et al.*, 2000a). In this regard, it is noteworthy that tesofensine administration had a strong impact on the meal pattern of DIO rats, including a robust reduction in the average meal number and meal size. The close temporal association between hypothalamic DA kinetics and feeding suggests that high intrahypothalamic DA concentrations during feeding episodes are required to promote satiety. This may suggest that the effect of DA function in tesofensine-induced hypophagia is associated with an increase in hypothalamic DA tonus which could enhance satiety responses in the DIO rat.

DIO rats have several indices of lowered striatal DA activity. These include reduced DA concentrations, impaired response to electrically evoked accumbal DA release, decreased basal tyrosine hydroxylase and DAT expression, as well as lower levels of D₂ receptor binding (Pothos *et al.*, 1998; Geiger *et al.*, 2008). Correspondingly, striatal D₂ receptor binding is inversely correlated to the degree of obesity in humans (Volkow and Wise, 2005), and D₂ receptor agonists produce hypophagia and weight loss in animal models of obesity (Scislowski *et al.*, 1999; Davis *et al.*, 2008). In addition, mesolimbic D₃ receptor function may also

play a role in obesity, as suggested by findings of D₃ receptor agonist-induced hypophagia in DIO mice and increased adiposity in D₃ receptor knock-out mice (McQuade et al., 2003; McQuade et al., 2004). Hence, the DAT inhibitory component of tesofensine would suggest that also D₂ and D₃ receptor could play a role in tesofensine-induced hypophagia. However, the D₂ and D₃ receptor antagonists, haloperidol and NGB2904, did not influence tesofensine-induced attenuation of food intake. In the current experimental settings, these data therefore indicate that only the D₁ receptor played a significant role in tesofensine-induced hypophagia.

The involvement of serotonin function in feeding behavior have been extensively studied, and there is strong evidence for anorexic effects of several 5-HT receptor subtypes (Halford *et al.*, 2007). In particular, the 5-HT_{2C} receptor has gained considerable interest as a potential anorexic target, and a number of 5-HT_{2C} receptor agonists have anti-obesity effects in preclinical and clinical settings (Clifton and Kennett, 2006; Halford *et al.*, 2007). However, the observation that ritanserin did not influence tesofensine's capacity to induce hypophagia indicates that 5-HT_{2A/C} receptor function is not enhanced by tesofensine-induced 5-HT transporter inhibition.

In contrast to tesofensine, other MRIs are known to produce anorexic responses via stimulation of serotonergic activity, including fluoxetine, sertraline and sibutramine (Clifton and Kennett, 2006; Halford *et al.*, 2007). For sibutramine, suppression of food intake could be partially reversed by simultaneous ritanserin or SB206553 administration, thus implicating 5-HT_{2A/C} or 5-HT_{2B/C} receptor activation (Jackson *et al.*, 1997; Grignaschi *et al.*, 1999; Balcioglu and Wurtman, 2000). It is suggested that the serotonergic effects of sibutramine on feeding and body weight may likely be ascribed to its primary and secondary amine metabolites, which are triple MRIs, rather than sibutramine *per se* (Heal *et al.*, 1998; Glick *et al.*, 2000; Nelson and Gehlert, 2006). Since tesofensine and the bioactive metabolites of sibutramine are almost equipotent 5-HTT inhibitors *in vitro* (Heal *et al.*, 1998; Glick *et al.*, 2000; Lehr *et al.*, 2008), this

suggest that methodological differences may account for this discrepancy between tesofensine and sibutramine on acute food intake. The observation that the hypophagic effect of tesofensine was not sensitive to ritanserin co-administration may be ascribed to the observation that higher doses (0.1-1.0 mg/kg) have been used in studies on sibutramine-induced hypophagia (Jackson *et al.*, 1997; Grignaschi *et al.*, 1999). However, the use of doses higher than 0.03 mg/kg were precluded due to an inhibitory effect of the ritanserin *per se* on food intake in the present DIO rat model (data not shown).

It is also noteworthy that all studies on the monoamine receptor-associated hypophagic effect of sibutramine have been conducted in lean rats, making it uncertain whether sibutramine displays similar effects on serotonergic receptor function in DIO rats. This is relevant, as obese rats have indices of altered hypothalamic 5-HT homeostasis, including reduced basal 5-HT levels, altered 5-HT turnover and increased 5-HT responsiveness during high-fat feeding (Gardier *et al.*, 1989; Mori *et al.*, 1999; Meguid *et al.*, 2000a).

In conclusion, tesofensine is an anorexic agent which induces a strong acute hypophagic effect in a rat model of diet-induced obesity. The mechanism underlying the robust and long-lasting suppression of acute feeding by tesofensine in the obese rats fed on a high-fat diet is dependent on the drug's ability to indirectly stimulate α_1 adrenoceptor and dopamine D₁ receptor function. Presumably, this reflects additive effects of increased NE and DA activity, which is consistent with tesofensine's ability to inhibit the reuptake of both NE and DA. Although it should be taken into account that the sustained weight loss obtained with long-term tesofensine treatment in DIO rats may in addition involve modulation of other monoaminergic receptor systems as well as stimulation of energy expenditure, the present data suggests that stimulated α_1 adrenoceptor and D₁ receptor function could contribute significantly to the robust weight loss observed with tesofensine treatment in obese patients (Astrup *et al.*, 2008a; Astrup *et al.*, 2008b).

DISCLOSURE/CONFLICT OF INTEREST

This study was funded by NeuroSearch. The authors (AMDA, JDM, HHH) declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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TITLES AND LEGENDS TO FIGURES

Figure 1. High-fat diet (Research Diets #D12492, 60% fat kcal., energy density 5.24 kcal/g) induces a significantly higher body weight gain over 10 weeks, as compared to rats receiving standard chow (Altromin 1324, 10% fat kcal, energy density 2.85 kcal/g). Data are shown as means \pm S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Tesofensine induces hypophagia in the DIO rat. (A) Dose-response effect of tesofensine (0.1-3.0 mg/kg, s.c., $n \geq 6$ per group) on total food intake in the DIO rat during a 12h nocturnal period. Total food intake over the entire observation period is shown in gram (left y-axis) as well as relative to vehicle-treated DIO rats (right y-axis). (B) Time-response effect of incrementing doses of tesofensine (0.1-3.0 mg/kg, s.c.) on cumulated nocturnal food intake (2h intervals). Data are shown as means \pm S.E.M. ** $p < 0.01$; *** $p < 0.001$.

Figure 3. Temporal pharmacodynamics of acute tesofensine treatment in the DIO rat. Time-response effect of tesofensine (1.5 mg/kg, s.c., $n=6$) or saline vehicle ($n=6$) on food intake during a 72h monitoring period. Upper panel, 12 hr interval data points. Lower panel, 2 hr interval data points with dark and white horizontal bars below the x-axis indicating dark and light phases, respectively. The arrow denotes when tesofensine or vehicle was administered. Data are shown as means \pm S.E.M. * *** $p < 0.001$ (compared to vehicle controls).

Figure 4. Feeding kinetics of tesofensine-induced hypophagia in the DIO rat. Microstructural food intake analysis of tesofensine-induced hypophagia (0.1-3.0 mg/kg, s.c., $n \geq 6$ per group, overall dose-response effects on food intake in the same animals are shown in Fig.1). (A) total number of meals, (B)

average meal size, (C) average meal duration, (D) latency to first meal, and (E) first meal size during a 12h nocturnal period. Data are shown as means \pm S.E.M. * p <0.05, ** p <0.01, *** p <0.001 (compared to vehicle controls).

Figure 5. Tesofensine-induced hypophagia is dependent on stimulated α_1 adrenoceptor function. Effect of prazosin (1.0 mg/kg, s.c.) on tesofensine-induced (1.5 mg/kg, s.c.) hypophagia in the DIO rat. (A) Total food intake (gram, left y-axis; relative to vehicle controls, right y-axis) and (B) cumulated food intake (gram) during 12h nocturnal period. Data are shown as means \pm S.E.M. * p <0.05, ** p <0.01, *** p <0.001 (compared to vehicle controls); ### p <0.001 (compared to vehicle+tesofensine).

Figure 6. Tesofensine-induced hypophagia is dependent on stimulated dopamine D₁ receptor function. Effect of SCH23390 (0.03 mg/kg, s.c.) on tesofensine-induced (1.5 mg/kg, s.c.) hypophagia in the DIO rat. (A) Total food intake (gram, left y-axis; relative to vehicle controls, right y-axis) and (B) cumulated food intake (gram) during 12h nocturnal period. Data are shown as means \pm S.E.M. * p <0.05, ** p <0.01, *** p <0.001 (compared to vehicle controls); # p <0.05, ## p <0.01 (compared to vehicle+tesofensine).

Figure 7. Tesofensine-induced hypophagia does not involve α_2 adrenoceptor, dopamine D₂, dopamine D₃, or 5-HT_{2A/C} receptor function. Effect of (A) RX821002 (0.3 mg/kg, s.c.), (B) haloperidol (0.03 mg/kg, s.c.), (C) NGB2904 (0.1 mg/kg, s.c.) or (D) ritanserin (0.03 mg/kg, s.c.) on tesofensine-induced (1.5 mg/kg, s.c.) hypophagia in the DIO rat. Data are shown total nocturnal food intake (g) as means \pm S.E.M. ** p <0.01, *** p <0.001 (compared to vehicle controls).

Figure 8. Chronic tesofensine treatment causes a sustained body weight reduction with a temporal suppressive effect on food intake. Effect of a daily dose of tesofensine (2.0 mg/kg, s.c.) on body weight gain (A) and food intake (B) over a 16-day treatment period. Data are shown as means \pm S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to vehicle controls).

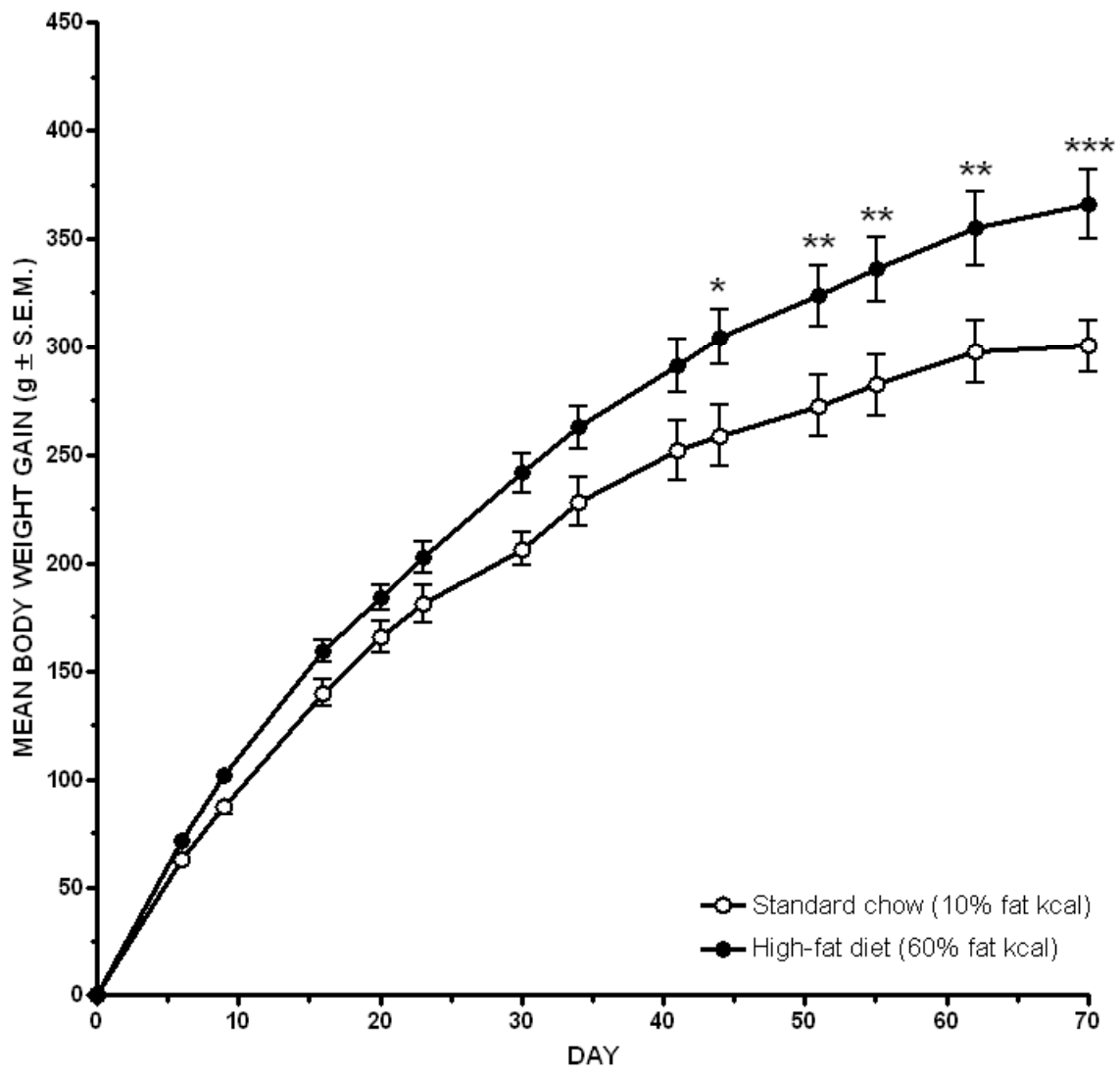
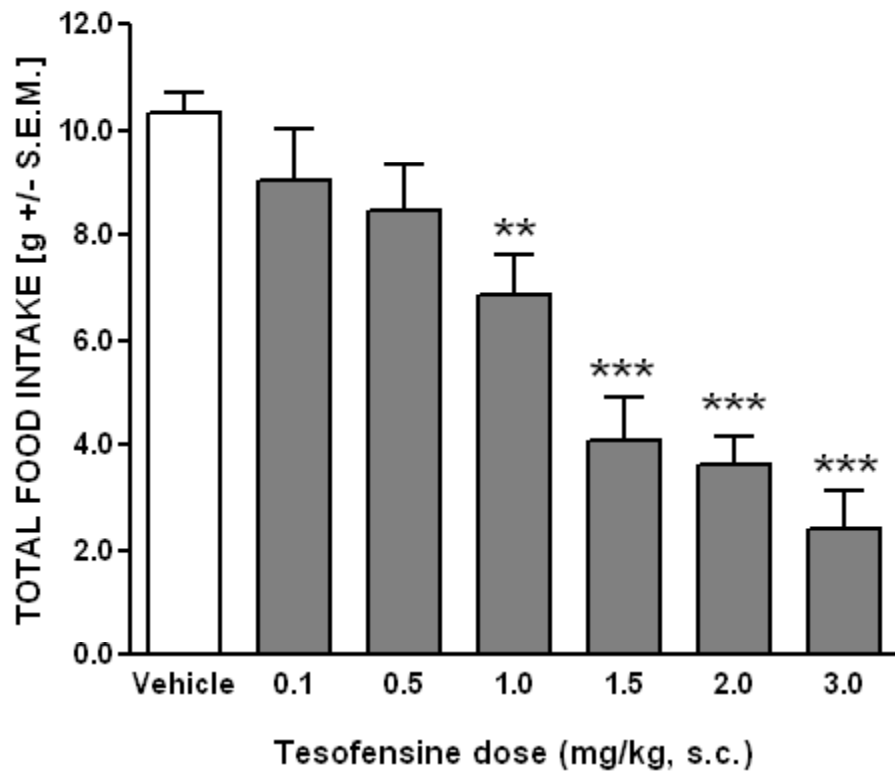
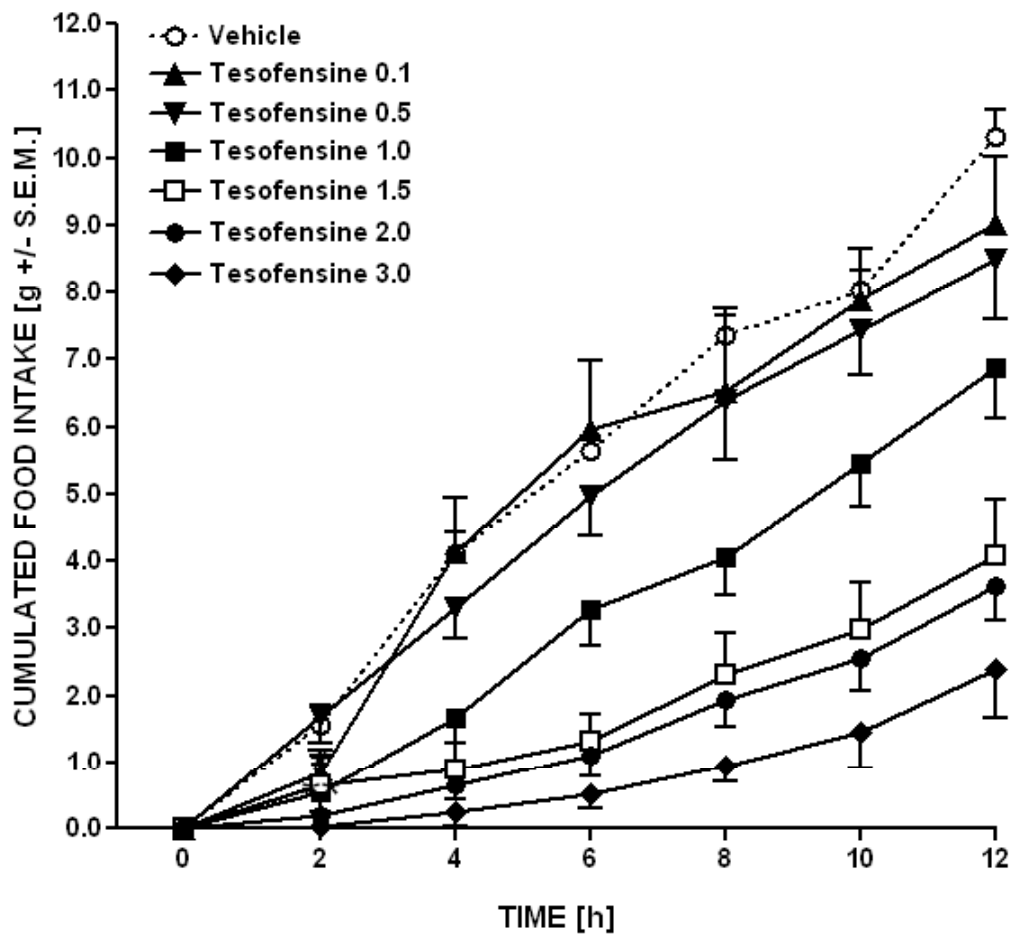


Figure 1

A**B****Figure 2**

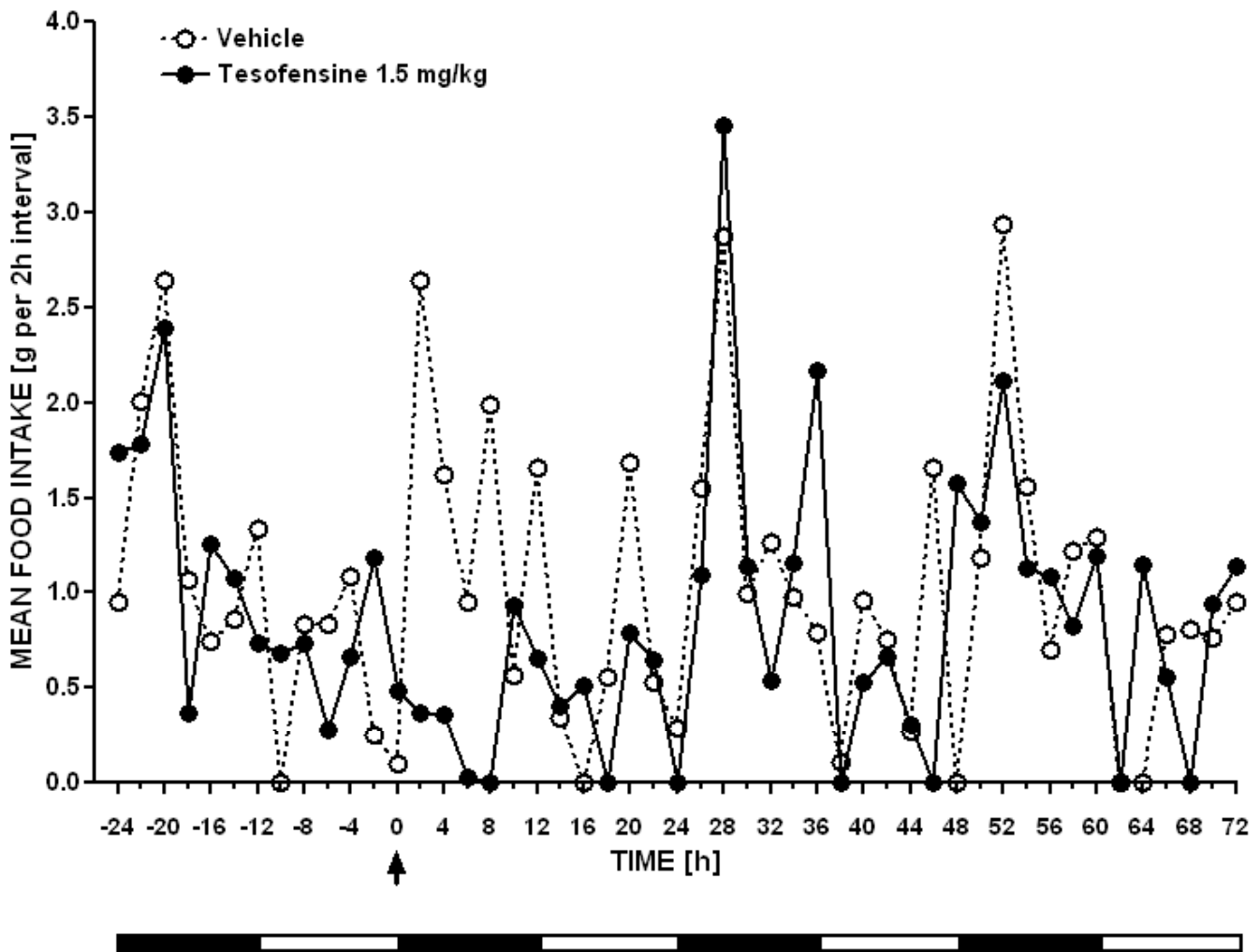
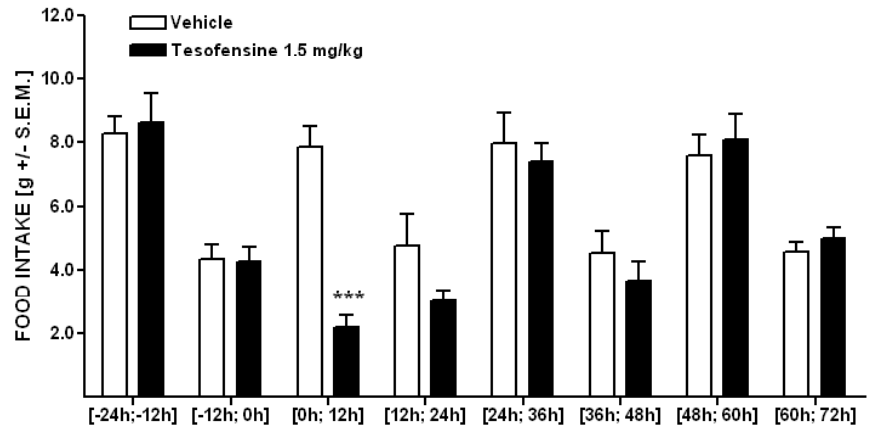


Figure 3

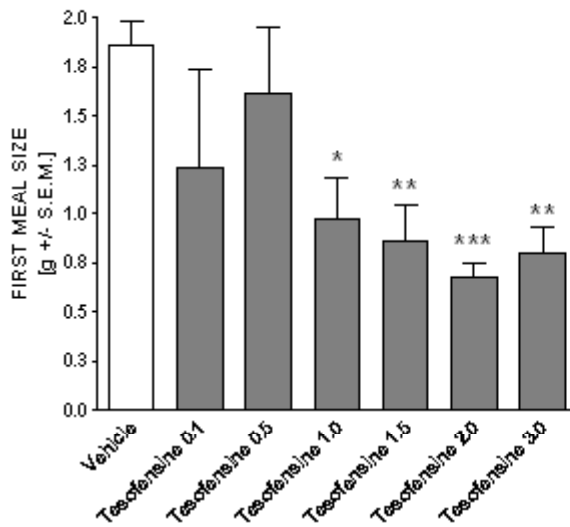
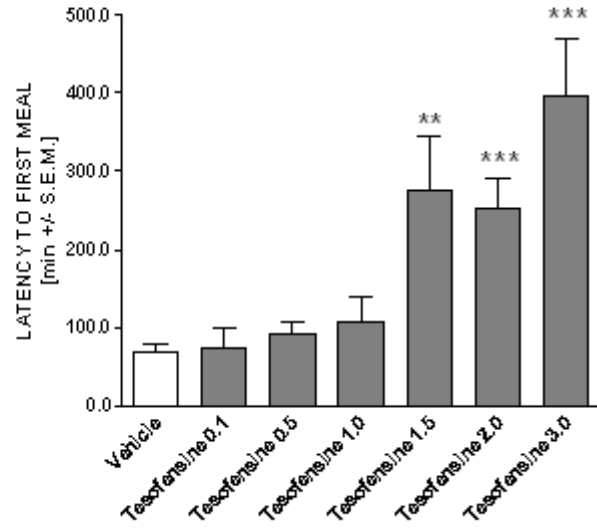
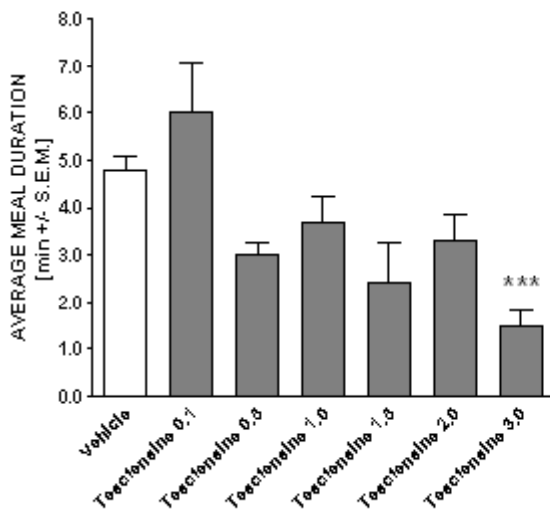
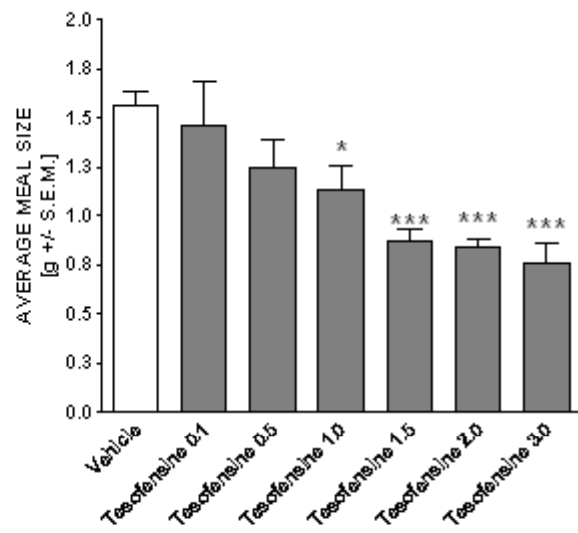
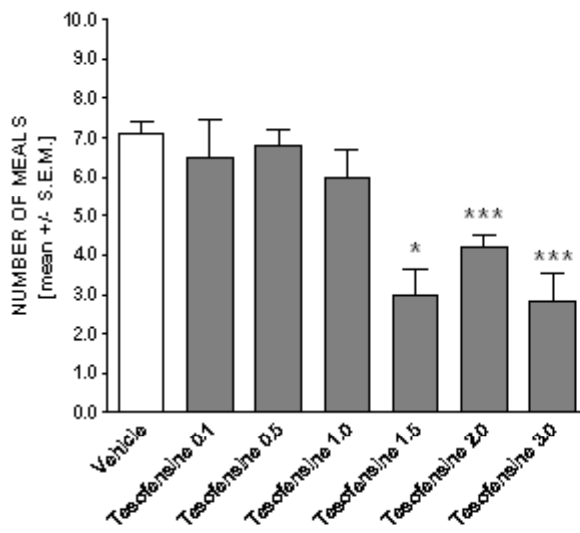


Figure 4

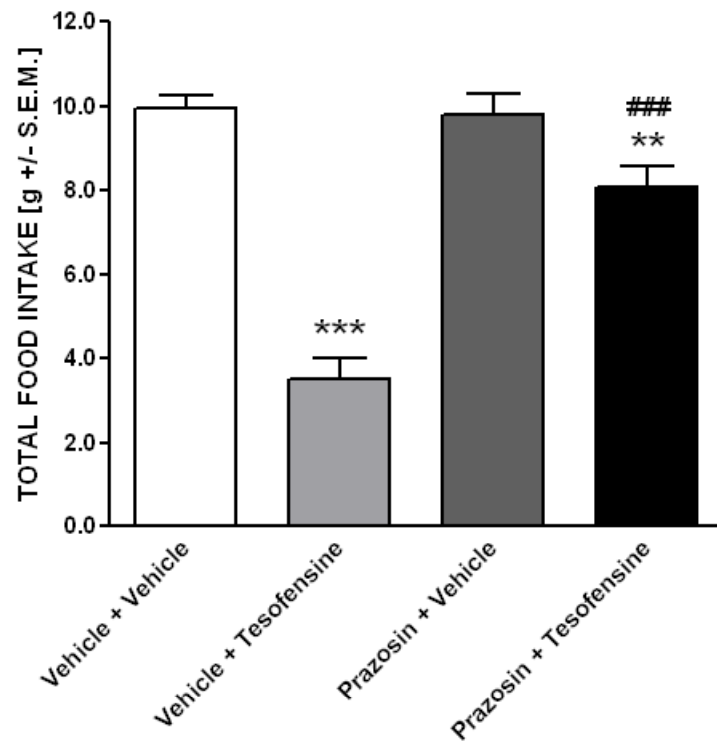
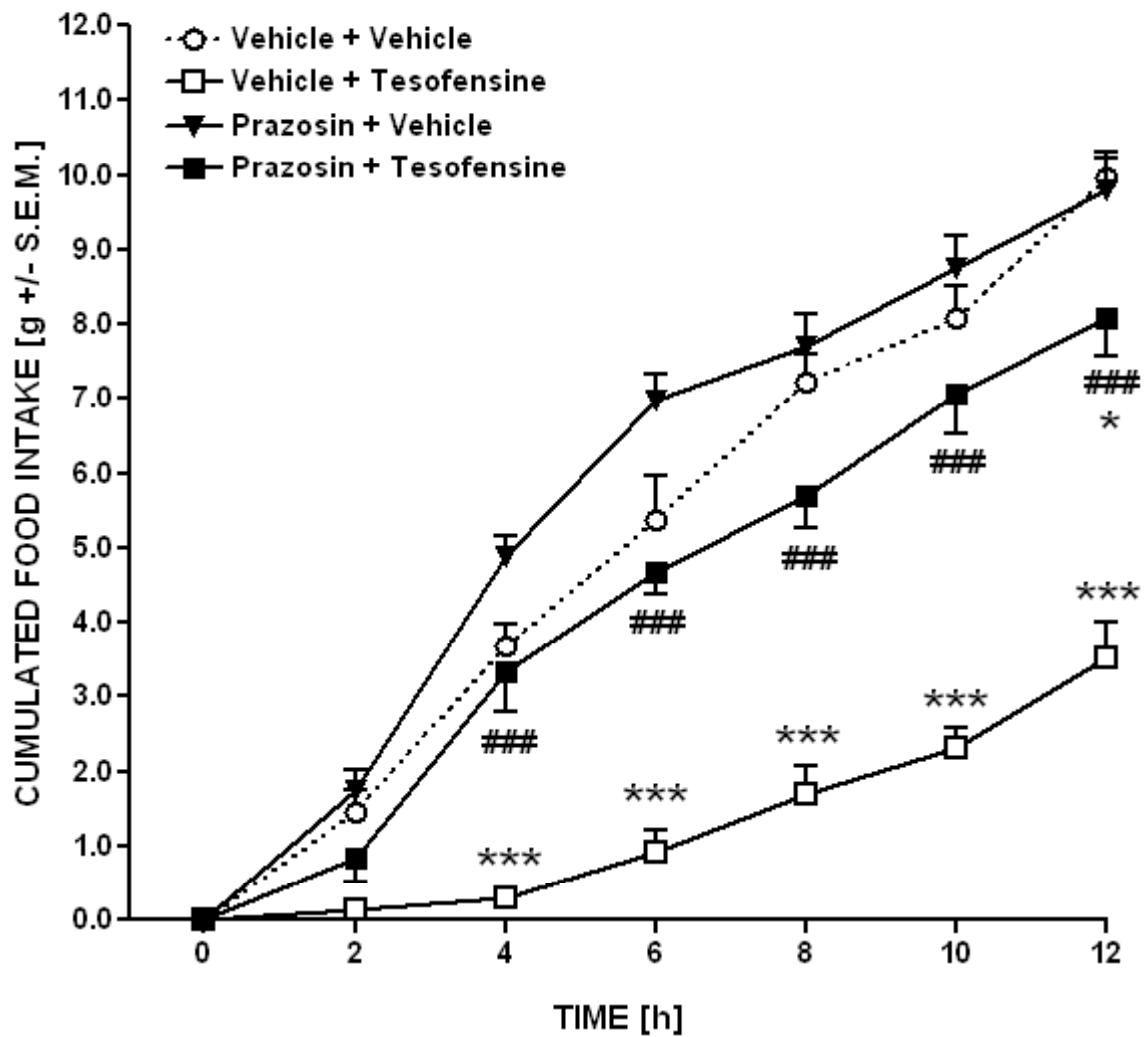
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Figure 5

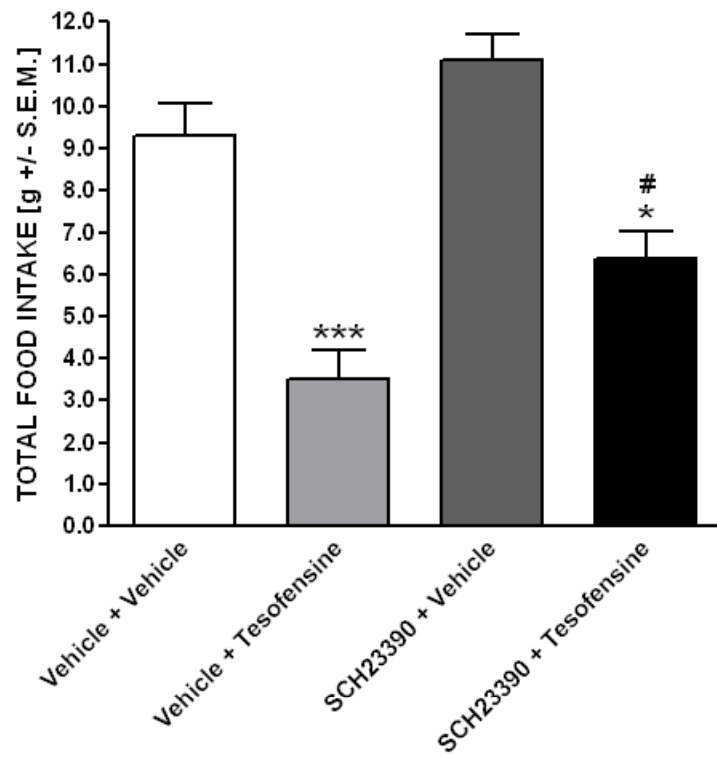
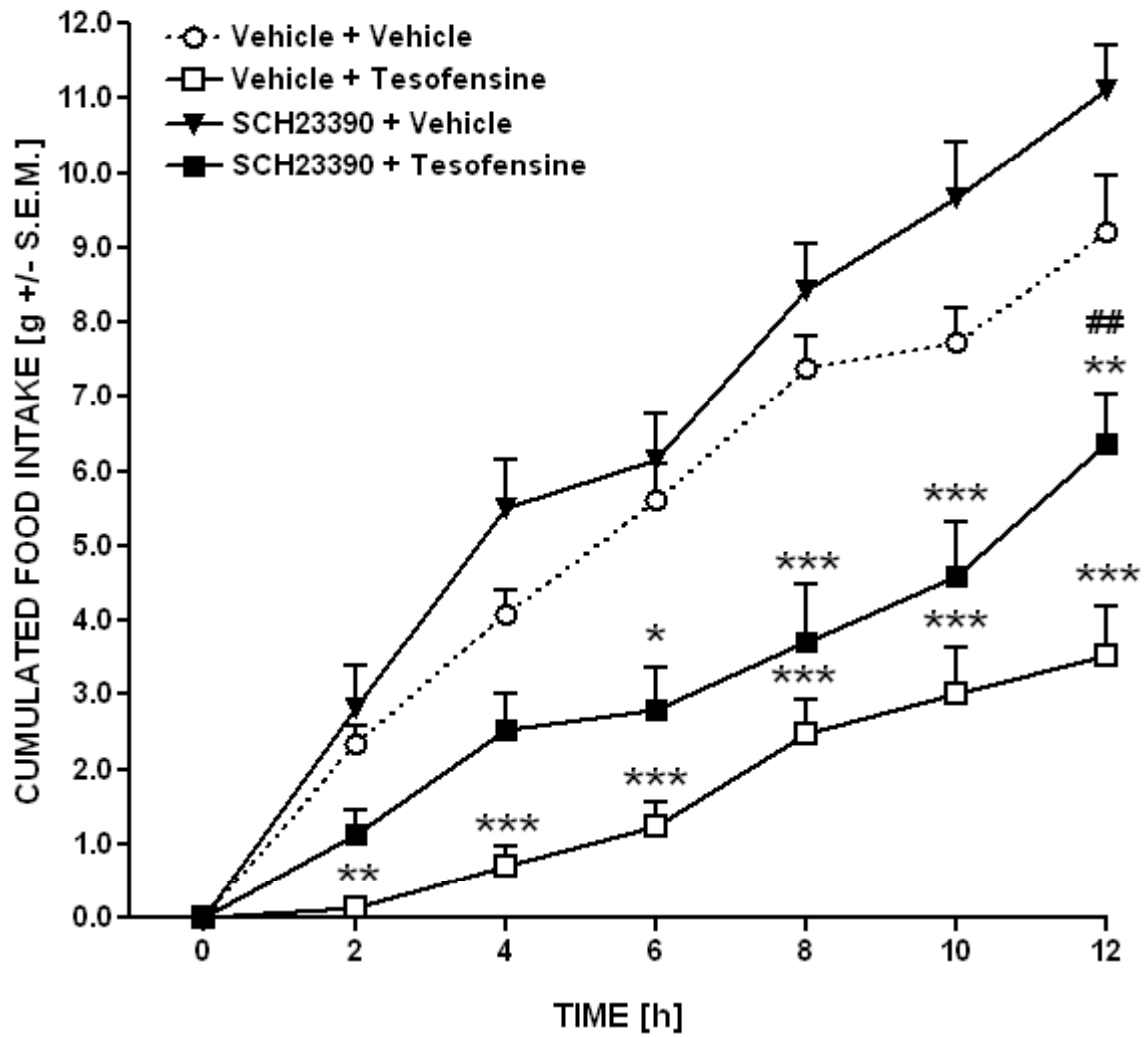
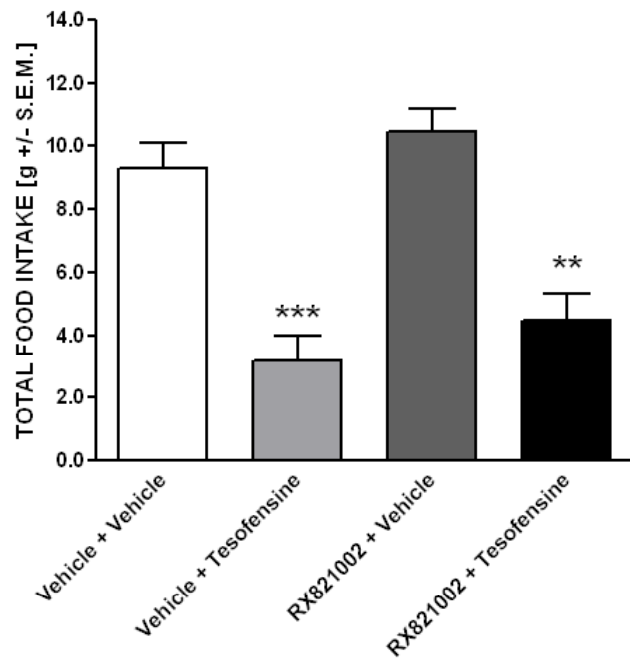
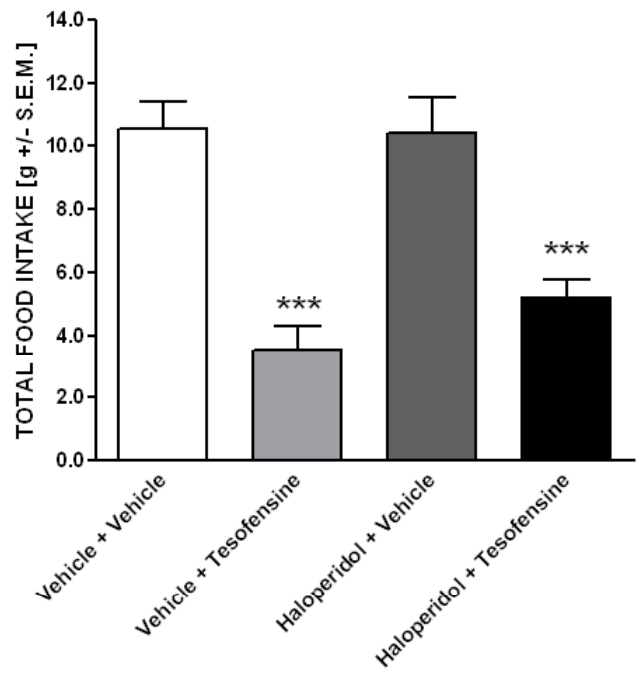
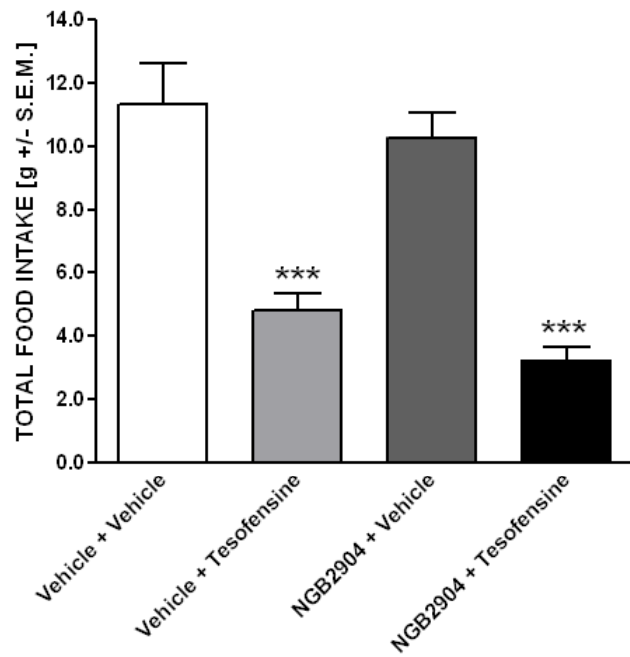
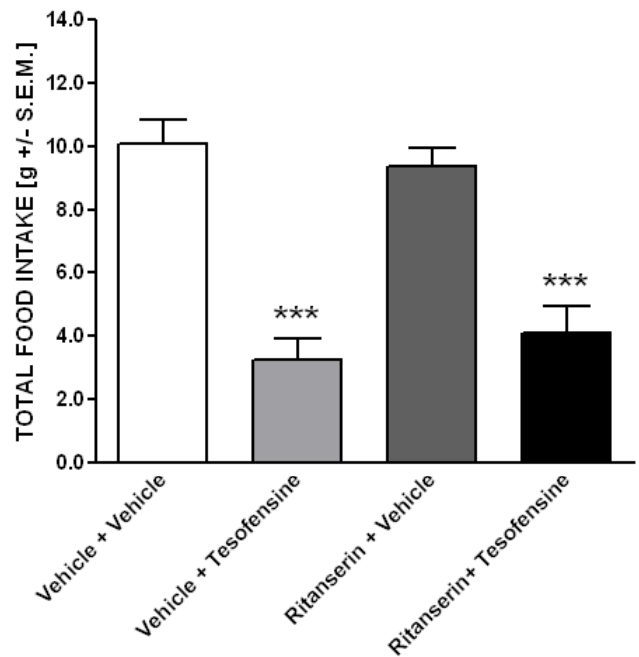
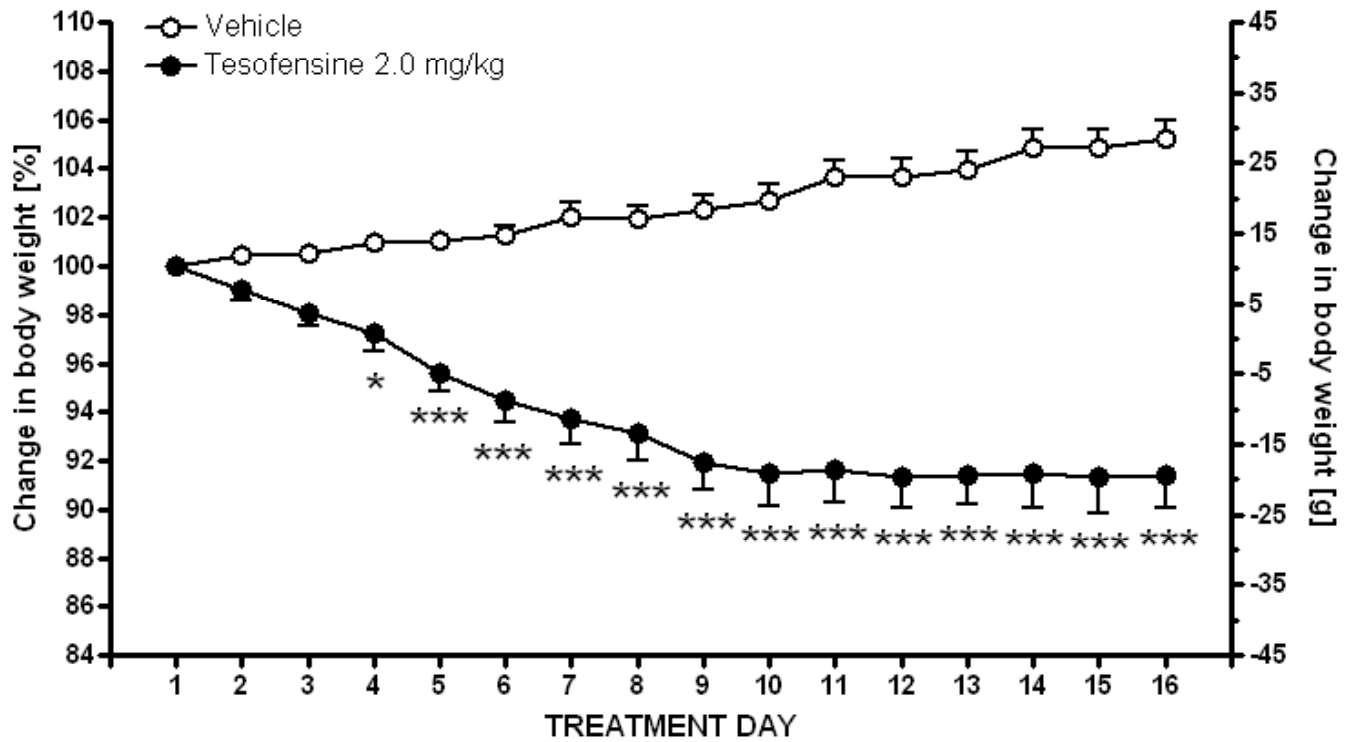
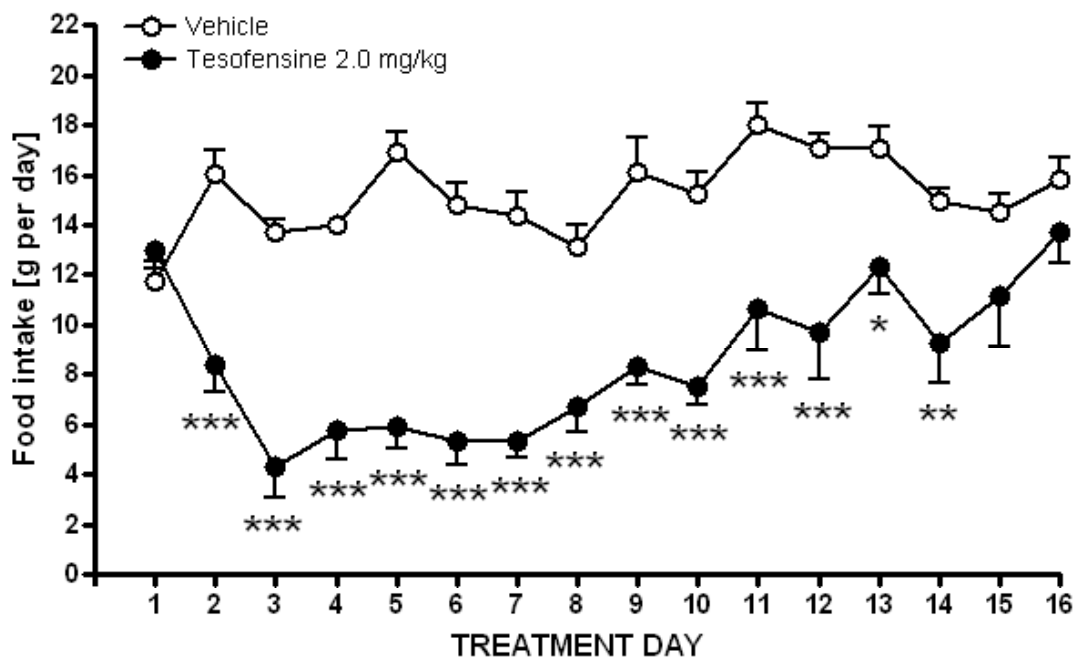
A**B**

Figure 6

A**B****C****D****Figure 7**

A**B****Figure 8**