Dopamine D2 receptors in the pathophysiology of insulin resistance

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Blocking dopamine D2 receptors by haloperidol curtails the beneficial impact of calorie restriction on the metabolic phenotype of high fat diet induced obese mice

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3

Abstract

Calorie restriction is the most effective way of expanding life-span and decreasing morbidity. It improves insulin sensitivity and delays the age-related loss of dopamine receptor D2 (DRD2) expression in the brain. Conversely, high fat feeding is associated with obesity, insulin resistance and a reduced number of DRD2 binding sites. We hypothesized that the metabolic benefit of calorie restriction involves preservation of appropriate DRD2 transmission.

The food intake of wild type C57Bl6 male mice was restricted to 60% of *ad libitum* intake while they were treated with the DRD2 antagonist haloperidol or placebo using subcutaneously implanted pellets. Mice with *ad libitum* access to food receiving placebo treatment served as controls. All mice received high fat food throughout the experiment. After 10 weeks an intraperitoneal glucose tolerance test was performed and after 12 weeks a hyperinsulinemic euglycemic clamp. Hypothalamic DRD2 binding was also determined after 12 weeks of treatment.

Calorie restricted (CR) placebo mice were glucose tolerant and insulin sensitive compared to *ad libitum* (AL) fed placebo mice. CR mice treated with haloperidol were slightly heavier than placebo treated CR mice. Haloperidol completely abolished the beneficial impact of calorie restriction on glucose tolerance and partly reduced the insulin sensitivity observed in CR placebo mice. The metabolic differences between AL and CR placebo mice were not accompanied by alterations in hypothalamic DRD2 binding.

In conclusion, blocking DRD2 curtails the metabolic effects of calorie restriction. Although this suggests that the dopaminergic system could be involved in the metabolic benefits of calorie restriction, restricted access to high fat food does not increase (hypothalamic) DRD2 binding capacity, which argues against this inference.

Introduction

Calorie restriction is the most effective way to extend lifespan and reduce morbidity. The maximal lifespan of rodents can be prolonged up to 60% by lifelong calorie restriction[1]. In mice, carcinogenesis is efficiently decreased by calorie restriction[2,3] and in humans it is associated with a reduced risk for atherosclerosis development[4]. Calorie restriction is also highly effective in reversing insulin resistance, both in humans and rodents[5-9]. The mechanisms responsible for the benefits of calorie restriction on morbidity and mortality are not yet fully elucidated. In the context of improving metabolic features though, the impact of calorie restriction on the dopaminergic system may be involved. In rats, calorie restriction delays the age-related loss of dopamine receptor D2 (DRD2) and tyrosine hydroxylase expression[10-12], while it enhances the affinity of DRD2 to ligands[13].

The dopaminergic system plays a complex dual role in feeding behaviour and profoundly affects glucose and lipid metabolism[14-16]. Dopamine action is mediated by 5 distinct G-protein coupled receptor subtypes, functionally classified into 2 receptor families according to their effect on target neurons. Activation of dopamine receptor D2, D3 or D4, comprising the D2 family, inhibits adenylyl cyclase. Activation of the receptors belonging to the D1 family (DRD1 and DRD5) stimulates adenylyl cyclase[17].

Dopaminergic transmission is altered in obese and insulin resistant animals. Basal and feeding evoked dopamine release is exaggerated in several nuclei of the hypothalamus of obese Zucker rats[18-20], whereas DRD2 expression is reduced in hypothalamic nuclei of obese animal models[21,22]. The number of DRD2 binding sites in the striatum of obese humans is reduced and inversely correlated with body mass index[23].

Modulation of DRD2 activity profoundly affects energy homeostasis. Drugs that block DRD2 enhance appetite and induce weight gain in animals and humans[24-27]. Conversely, DRD2 agonist drugs reduce body weight, increase energy expenditure and improve glycemic control in obese individuals[28-31].

High fat feeding induces obesity, insulin resistance and type 2 diabetes in rodents. A reduction in DRD2 transmission due to the high fat diet may be involved in this deleterious metabolic profile. We hypothesized that restricting access to high fat food would curtail the diet's effects on glucose metabolism by maintaining appropriate hypothalamic DRD2 binding capacity (and thereby DRD2 mediated neurotransmission).

To test this hypothesis, wild type C57Bl6 mice were fed a high fat diet, either with *ad libitum* or restricted access. Half of the calorie restricted (CR) mice were continuously treated with the DRD2 antagonist haloperidol to pharmacologically reduce dopaminergic neurotransmission via this receptor. The other CR mice and the *ad libitum* (AL) fed mice received continuous placebo

treatment. We speculated that the haloperidol treatment would counteract the benefits of calorie restriction on the metabolic phenotype of high fat fed mice. A glucose tolerance test was performed after 10 weeks of treatment and a hyperinsulinemic euglycemic clamp after 12 weeks. In addition, the hypothalamic DRD2 binding capacity was determined after 12 weeks of treatment.

Materials and Methods

Animals

Fifty-four male C57BL/6J mice (Charles River, Maastricht, The Netherlands), 10 or 11 weeks old, were individually housed in a temperature- and humidity-controlled room on a 12-h light–dark cycle (lights on at 7.00 am) with free access to water.

All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare, and the experimental protocol was approved by the Animal Ethics Committee of the Leiden University Medical Center.

Experimental design

Mice were randomly assigned to one of the following groups (n=18 mice per group): 1) *ad libitum* access to food combined with placebo treatment, 2) restricted access to food combined with placebo treatment and 3) restricted access to food combined with haloperidol treatment (1 mg/kg/day). This dose was chosen as it is frequently used in rodent experiments and it has proven to be effective in several behavioral paradigms[32]. Chronic administration of this dose in mice yields serum concentrations of 8.2 mM, which is comparable to the haloperidol concentration in humans occupying 75% of DRD2[33].

After a run-in period (3 or 7 weeks for the autoradiography and the clamp experiment respectively) in which all mice were allowed to get accustomed to the high fat (HF) diet (45 energy% of fat derived from palm oil, 35 energy% of carbohydrate and 20 energy% of protein; Research Diet Services, Wijk bij Duurstede, The Netherlands) and the basal food intake was determined, food restriction and drug treatment were started simultaneously. All mice remained on the HF diet throughout the study, either with *ad libitum* or restricted access. The CR mice received 60% of the amount of food the AL mice consumed. Food intake of the AL mice was measured twice a week. The CR mice received half of their daily food in the beginning of the dark phase and the other half in the middle of the dark phase.

All mice received treatment by means of subcutaneous implantable pellets (Innovative Research of America, Florida, USA), ensuring continuous release of the medication. Haloperidol and placebo pellets were implanted under

isoflurane anesthesia. Mice were treated for 12 weeks, meanwhile having *ad libitum* or restricted access to the HF food. Non-fasted body weight of AL and CR mice was measured weekly.

After 10 weeks of treatment, a group of mice was subjected to an intraperitoneal glucose tolerance test and after 12 weeks of treatment the body composition of these mice was determined. Subsequently the mice were subjected to a hyperinsulinemic euglycemic clamp. Another group of mice was sacrificed after 12 weeks of treatment for analysis of hypothalamic DRD2 binding.

Intraperitoneal Glucose Tolerance Test

Eight mice per group were fasted for 16 hours after food withdrawal at 5.00 pm. That day, the CR mice received their food at 3.00 pm and if anything was left at 5.00 pm, this was discarded. The glucose tolerance test (GTT) started at 9.00 am the following day. An initial blood sample (t=0) was taken, immediately followed by an intraperitoneal injection of 2 g/kg D-glucose, provided as a 20% solution. Additional blood samples were taken via tail bleeding at 5, 15, 30, 45, 60, and 120 minutes after glucose injection for measurement of plasma glucose and insulin levels.

DEXAscan

The body composition of 10 mice per group was measured by dual-energy X-ray absorptiometry (DEXA) using the Norland pDEXA Sabre X-Ray Bone Densitometer (Norland, Hampshire, UK). Before measuring, mice were anesthetized with a combination of 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands) and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Mice were scanned *in toto*, yet the heads were excluded from the analysis due do the inability of the DEXAscan to accurately determine the composition of the tissue below the skull.

Hyperinsulinemic euglycemic clamp

Ten mice per group were fasted for 16 hours after food withdrawal at 5.00 pm. That day, CR mice received their food at 3.00 pm and if anything was left at 5.00 pm, this was discarded. Hyperinsulinemic euglycemic clamp studies started at 9.00 am the following day and were performed as described earlier[34]. During the experiment, mice were anesthetized with a combination of 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands) and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). First, the basal rate of glucose turnover was determined by giving a primed (0.5 µCi) continuous (0.9 µCi/h) intravenous (i.v.) infusion of D-[3-^{3}H]-glucose (37 MBq) (GE Healthcare, Little Chalfont, UK)

for 60 minutes. Subsequently, insulin (Novo Nordisk, Bagsværd, Denmark) was administered in a primed (3.7 mU) continuous (6.1 mU/h) i.v. infusion for 90 minutes to attain steady-state circulating insulin levels of ~6 ng/ml. Every 10 min the plasma glucose concentration was determined via tail vein bleeding (< 3 µl) (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany) and the i.v. infusion rate of a 12.5% D-glucose solution was adjusted to maintain euglycemia. Blood samples (60 µl) were taken during the basal period (after 50 and 60 min) and during the hyperinsulinemic period (after 70, 80, and 90 min) to determine plasma concentrations of glucose, insulin and ^{3}H-glucose specific activities. At the end of the clamp mice were sacrificed.

Analytical procedures

A commercially available kit was used to determine the plasma concentration of glucose (Instruchemie, Delfzijl, The Netherlands). The plasma insulin concentration was measured by an ELISA (Crystal Chem Inc., IL, USA). The ^{3}H-glucose concentration was determined in plasma and in supernatant after trichloroacetic acid (20%) precipitation and water evaporation.

Calculations

The rate of glucose uptake (µmol/min/kg) was calculated during the basal period and under steady-state hyperinsulinemic conditions as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of ^{3}H-glucose (dpm/µmol). Endogenous glucose production (µmol/min/kg) was calculated as the difference between the tracer-derived rate of glucose uptake and the glucose infusion rate. Both glucose uptake and production measures were corrected for body weight.

Tissue preparation

Eight mice per group, used for the analysis of hypothalamic DRD2 binding, were sacrificed by cervical dislocation between 9.00 and 12.00 am, to minimize effects of the circadian rhythm. Brains were rapidly dissected, snap frozen in ice-cold isopentane (cooled in ethanol which was placed in dry ice) and stored at -80°C until further use.

Sections of 16 µm were cut on a Cryostat (Microm HM 500 M, Adamas Instruments, Leersum, The Netherlands) and mounted on Polysine Slides (Menzel-Gläser, Braunschweig, Germany). Sections were taken at the level of the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) (Bregma -0.70), ventromedial hypothalamus (VMH) (Bregma -1.34) and dorsomedial hypothalamus (DMH) and nucleus arcuatus (ARC) (Bregma -1.94) according to the brain atlas of Paxinos and Franklin[35]. Slides were stored at -20°C until further use.

DRD2 autoradiography

Sections were pre-incubated in a 50 mM Tris buffer (pH 7.0), containing 5.7 mM ascorbic acid, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, for 60 min at room temperature. Subsequently, sections were incubated with 0.7 ml of the Tris buffer containing 0.1 nM ^{125}I-Iodosulpride (2200 Ci/mmol, 100 µCi/ml; Perkin Elmer, Waltham, MA, USA) for 30 min at room temperature. Non-specific binding was determined in the presence of 2 µM haloperidol (Sigma-Aldrich, St. Louis, MO, USA). After incubation, sections were drained, dipped in distilled water (4°C), washed twice in Tris buffer (4°C) for 3 min, dipped in distilled water and air-dried. Sections were exposed to a Kodak BioMax MR film (Perkin Elmer, Waltham, MA, USA) for 4 weeks.

Analysis of DRD2 binding

The autoradiography films were digitized with an Epson Perfection V350 Photo scanner and the grey values on the scans were measured using ImageJ Software (NIH, Bethesda, USA). Counterstaining of the sections with Toluidine Blue O (Sigma-Aldrich, St. Louis, MO, USA) was performed to visualize individual hypothalamic nuclei. Hypothalamic nuclei that were damaged were excluded from the analysis. Accordingly, the arcuate nuclei of several mice were excluded. Grey values measured in the hypothalamic nuclei were corrected for background values.

Statistical analysis

Data is presented as mean ± standard deviation (SD). Statistical analysis was conducted using SPSS 16.0 software. A General Linear Model for repeated measures was used to analyze the differences in body weight over time, in glucose and insulin concentration and glucose infusion rate during the ipGTT and the clamp respectively. Only if the overall F-test indicated significant differences between the groups, a LSD post-hoc test was used to determine differences between specific groups. Analysis of the rest of the data was performed using a one-way ANOVA. Only if the overall F-test indicated significant differences between the groups, a LSD post-hoc test was used to determine differences between specific groups. Differences were considered statistically significant when $p<0.05$.

Results

Body weight and plasma metabolites

Body weight was similar in all groups of mice after the run-in HF diet (AL + Placebo: 29.6 ± 3.0 g; CR + Placebo: 30.4 ± 4.2 g; CR + Haloperidol: 30.3 ± 3.6 g; n=18 mice per group). During the 12 weeks of treatment, AL mice consumed on average 2.9 ± 0.2 g of HF food daily and accordingly, both groups of CR mice

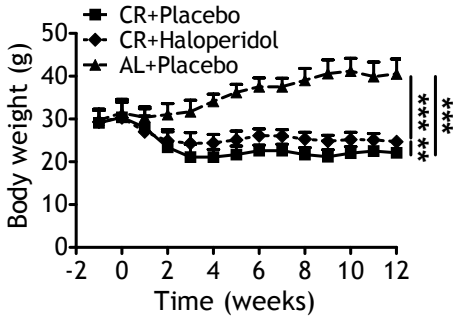

Figure 1 - Non-fasted body weight of AL and CR mice treated with placebo or haloperidol during the experiment.
Data is presented as mean ± SD for 18 mice per group.
** p<0.01; *** p<0.001

received 1.8 ± 0.1 g of HF food daily. During the first few weeks of treatment, CR mice lost weight until a new equilibrium was reached and the weight remained relatively stable for the rest of the experiment (fig 1). At the end of the treatment period, AL mice had a significantly higher body weight compared to both CR groups (p<0.001; AL + Placebo: 40.4 ± 2.9 g; CR + Placebo: 22.1 ± 1.2 g; CR + Haloperidol: 24.7 ± 0.8 g; n=18 mice per group). Haloperidol treatment resulted in a slightly, but significantly, higher body weight compared to placebo treatment. The difference in body weight between the CR and the AL mice was primarily accounted for by fat mass, but lean body mass was also different (table 1). The fasting plasma glucose concentration was not different between AL and CR placebo mice (table 2), yet the fasting plasma insulin concentration was significantly elevated in the AL mice compared to the CR placebo mice (table 2). Haloperidol treatment did not affect fasting plasma glucose or insulin concentrations in CR mice.

Glucose tolerance

After 10 weeks of treatment mice were subjected to an ipGTT. CR placebo mice had significantly lower glucose levels during the ipGTT (fig 2A) and a decreased area under the glucose curve (fig 2B) compared to AL mice, indicating improved

Table 1 - Fasted body weight, lean body mass and fat mass determined by DEXAscan analysis of AL and CR mice treated with placebo or haloperidol for 12 weeks.

	AL mice Placebo	CR mice Placebo	CR mice Haloperidol
Body weight (g)	38.4 ± 3.3	21.2 ± 1.5***	23.9 ± 0.7***$
Lean body mass (g)	22.09 ± 1.77	18.04 ± 1.48***	18.94 ± 1.08***
Fat mass (g)	10.68 ± 2.19	0.04 ± 0.13***	0.29 ± 0.40***

Data is presented as mean ± SD for 9 or 10 mice per group.
*** p<0.001 compared to AL placebo mice
$ p<0.05 compared to CR placebo mice

glucose tolerance. Accordingly, plasma insulin levels and the area under the insulin curve were significantly reduced in CR placebo compared to AL mice (fig 2C,D). Haloperidol treatment disrupted the glucose tolerance observed in CR placebo mice, as indicated by elevated plasma glucose levels and an increased area under the glucose curve. Surprisingly though, plasma insulin concentrations in CR haloperidol mice were not different from plasma insulin concentrations in CR placebo mice.

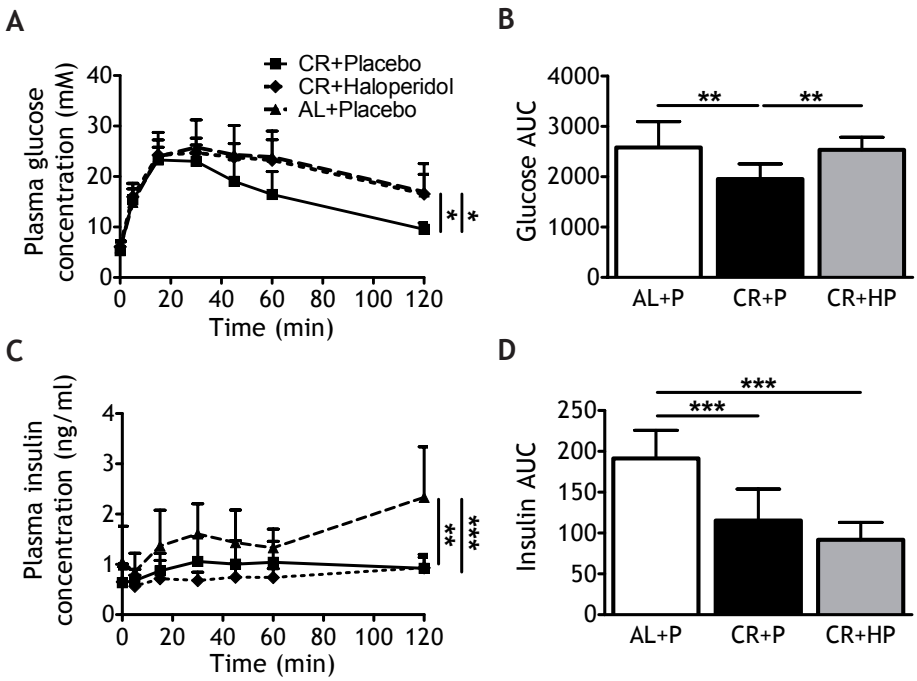

Figure 2 - Plasma glucose (A) and insulin (C) concentration during the intraperitoneal glucose tolerance test in AL and CR mice treated with placebo (P) or haloperidol (HP) for 10 weeks. Area under the glucose (B) and insulin (D) curve.
Data is presented as mean ± SD for 7 or 8 mice per group.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$

Insulin sensitivity

After 12 weeks of treatment mice were subjected to a hyperinsulinemic euglycemic clamp. The coefficient of variation of the specific activity was 10.5% during the basal period and 16.3% during the hyperinsulinemic period of the clamp. Basal and hyperinsulinemic plasma glucose and insulin concentrations as well as glucose uptake and production levels are shown in table 2. The basal glucose turnover was significantly lower in AL mice compared to both groups of CR mice (table 2). The glucose infusion rate, necessary to maintain

Table 2 - Plasma concentration of glucose and insulin, glucose uptake and production during the basal and hyperinsulinemic condition of the hyperinsulinemic euglycemic clamp in AL and CR mice treated with placebo or haloperidol for 12 weeks.

Clamp condition		AL mice Placebo	CR mice Placebo	CR mice Haloperidol
Glucose (mM)	B	5.6 ± 1.3	5.1 ± 1.3	6.5 ± 1.1
	HI	5.6 ± 1.6	4.5 ± 1.4	5.1 ± 0.8
Insulin (ng/ml)	B	1.5 ± 0.4	1.0 ± 0.2**	1.0 ± 0.3*
	HI	5.6 ± 0.6	6.6 ± 1.8	6.8 ± 1.3
Glucose Uptake (µmol/min/kg)	B	31.6 ± 7.8	44.4 ± 9.9*	46.5 ± 14.9*
	HI	34.5 ± 12.3	96.7 ± 37.7***	73.4 ± 15.5**
Glucose Production (µmol/min/kg)	B	31.6 ± 7.8	44.4 ± 9.9*	46.5 ± 14.9*
	HI	17.6 ± 18.8	6.2 ± 11.0	2.9 ± 5.8

B, Basal; HI, Hyperinsulinemia
Data is presented as mean ± SD for 7 or 8 mice per group.
* p<0.05; ** p<0.01; *** p<0.001 compared to AL placebo mice

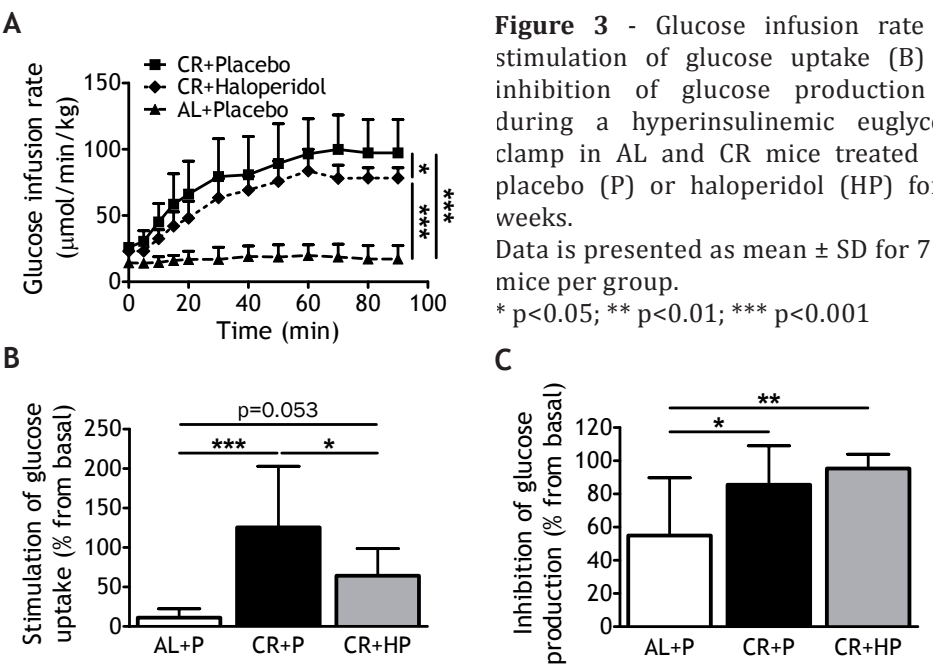

Figure 3 - Glucose infusion rate (A), stimulation of glucose uptake (B) and inhibition of glucose production (C) during a hyperinsulinemic euglycemic clamp in AL and CR mice treated with placebo (P) or haloperidol (HP) for 12 weeks.
Data is presented as mean ± SD for 7 or 8 mice per group.
* p<0.05; ** p<0.01; *** p<0.001

euglycemia, was significantly increased in CR placebo mice compared to AL mice (fig 3A), indicating improved whole-body insulin sensitivity. Haloperidol treatment significantly reduced the glucose infusion rate in CR mice, reflecting a decrease in insulin sensitivity. The improved insulin sensitivity of CR placebo mice compared to the AL mice was reflected by enhanced stimulation of glucose uptake (fig 3B) and stronger inhibition of glucose production (fig 3C). The decline of insulin sensitivity in CR mice treated with haloperidol was mainly due to a decreased ability of insulin to stimulate glucose uptake, whereas insulin's capacity to inhibit glucose production was unaffected (fig 3B,C).

DRD2 binding

After 12 weeks of treatment, DRD2 binding in several hypothalamic nuclei was determined. Representative autoradiographs are shown in figure 4. There was no difference in DRD2 binding between AL and CR placebo mice in any region of the hypothalamus (fig 5). Haloperidol treatment though, significantly increased DRD2 binding in all hypothalamic nuclei, except in the DMH.

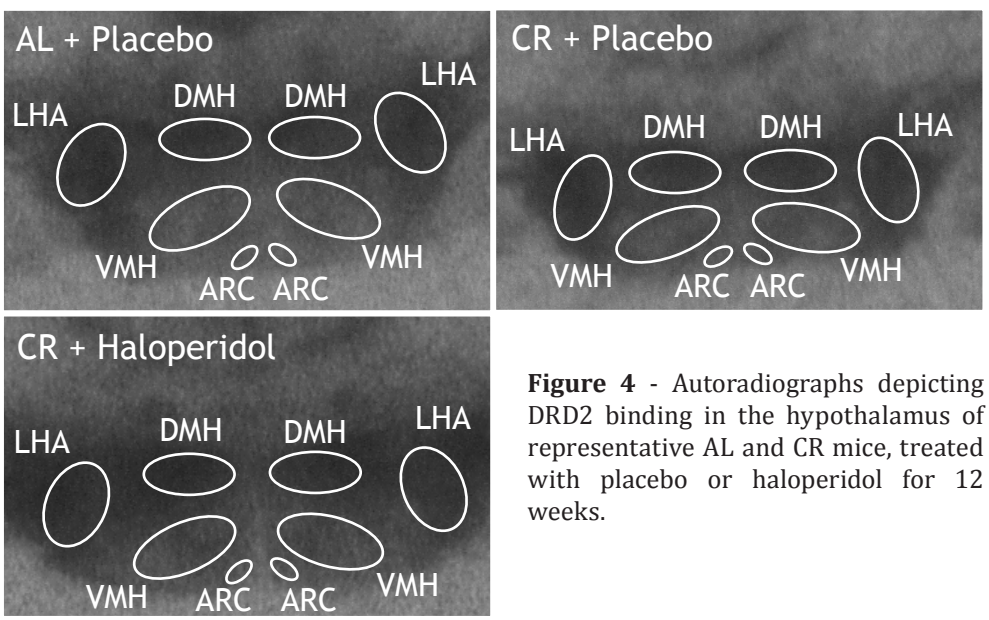

Figure 4 - Autoradiographs depicting DRD2 binding in the hypothalamus of representative AL and CR mice, treated with placebo or haloperidol for 12 weeks.

Discussion

The results presented here demonstrate that restricted access to a high fat diet strongly attenuates the diet's (detrimental) effect on glucose metabolism in mice. Simultaneous administration of haloperidol, a DRD2 antagonist, partially prevents this effect, suggesting that dopaminergic signalling is involved in

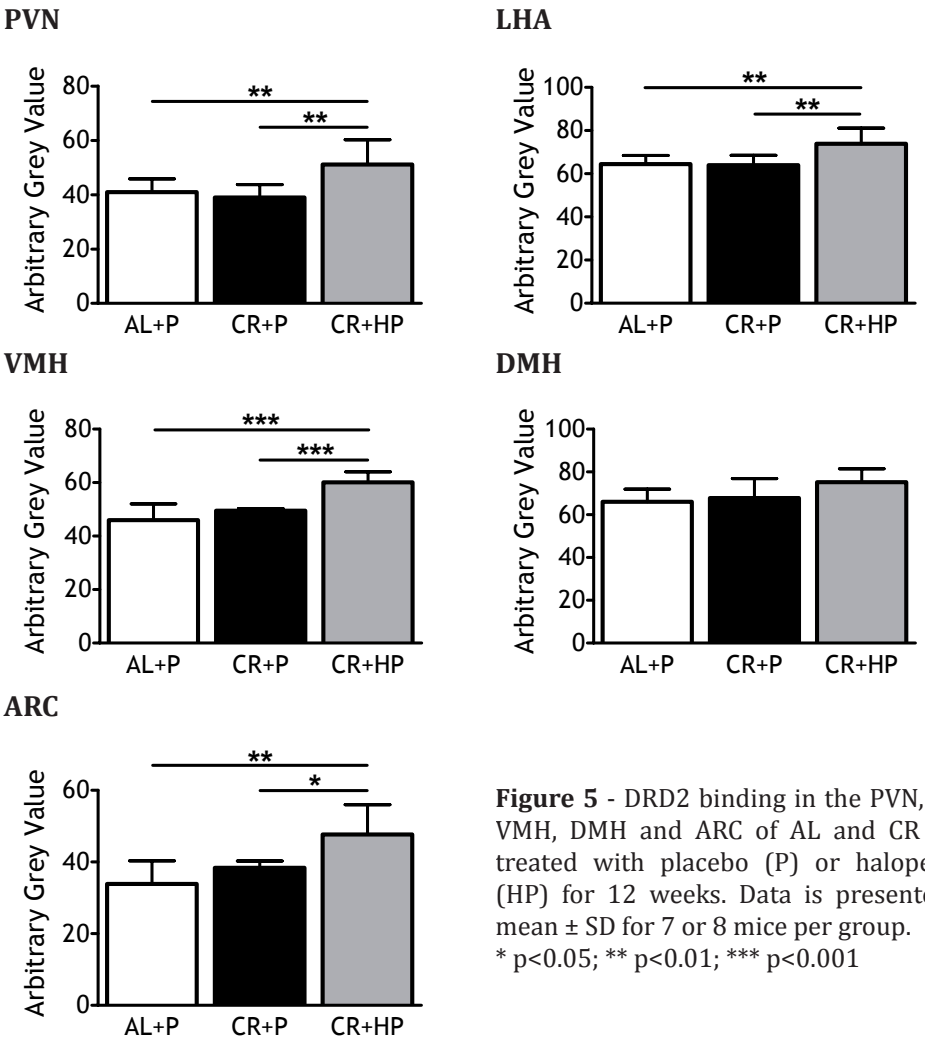

Figure 5 - DRD2 binding in the PVN, LHA, VMH, DMH and ARC of AL and CR mice treated with placebo (P) or haloperidol (HP) for 12 weeks. Data is presented as mean ± SD for 7 or 8 mice per group.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$

the beneficial impact of calorie restriction on glucose metabolism. However, restricted access to high fat food did not increase the availability of DRD2 binding sites in the hypothalamus, which does not support this inference.

Calorie restriction unequivocally reverses the metabolic derangements associated with obesity in humans[5-7]. Likewise, in rats several calorie restriction paradigms, ranging from 20% to 45% restriction, improve glucose metabolism during aging[9,36,37]. In addition, 30% or 40% calorie restriction prevents the development of diabetic characteristics in genetic rat models[8,38,39]. In mice, 40% calorie restriction reduces glucose and insulin concentrations[40]. Here, we extend these findings and show that, in C57Bl6 mice, restricted access to high fat food curtails the diet's impact on glucose metabolism. A high fat diet is well

known to induce obesity, insulin resistance and a type 2 diabetes-like phenotype in rodents. Restricted access to this diabetogenic diet clearly scales down these metabolic corollaries, as reflected by improved glucose tolerance and insulin sensitivity observed in CR placebo mice compared to AL mice. It seems important to note that we are unable to determine whether calorie restriction fully abolishes the high fat diet induced disturbance of glucose metabolism, as we did not include a low fat fed control group. However, we do show that calorie restriction efficiently curtails the metabolic corollaries of high fat feeding.

Although the beneficial impact of calorie restriction on glucose metabolism is unequivocal, the underlying mechanism(s) are still incompletely understood. Reduction of body weight and associated changes in ectopic lipid storage obviously play a critical role, but some of the metabolic benefits of calorie restriction are immediate and occur way before substantial weight loss has occurred[41]. Also, intermittent fasting recapitulates the beneficial impact of calorie restriction without altering body weight[40,42]. Therefore, other mechanisms than weight loss per se must contribute as well.

Calorie restriction delays the age-related loss of DRD2 expression and enhances the sensitivity of this receptor[10-13]. The dopaminergic system is critically involved in glucose and insulin metabolism. Activation of DRD2 normalizes elevated plasma glucose and insulin concentrations, improves islet function, ameliorates glucose intolerance and enhances insulin sensitivity in obese diabetic rodent models and humans[28-30,43,44]. Conversely, blocking DRD2 elevates plasma insulin levels and induces insulin resistance[45-47]. In concert, these data suggest that the dopaminergic system may mediate the beneficial effects of calorie restriction on metabolism. Specifically, we hypothesized that restricting the access to high fat food would prevent any reduction in DRD2 binding sites associated with high fat feeding. This would ensure appropriate hypothalamic DRD2 neurotransmission during restricted intake of the high fat diet and thereby curtail the development of high fat diet induced alterations in glycemic control.

Indeed, haloperidol partially abolished the beneficial impact of calorie restriction on glucose metabolism; compared to CR placebo mice, haloperidol treated mice developed both glucose intolerance and insulin resistance. But, in contrast to our hypothesis, we found no difference in DRD2 binding in the hypothalami of AL and CR placebo mice. We specifically measured DRD2 binding in the hypothalamus since this brain region is involved in the modulation of glucose and insulin homeostasis[48]. The lack of impact of CR on DRD2 binding is in apparent contrast with the study of Thanos et al., in which an increased DRD2 binding was detected in the striatum, cingulate and frontal cortex of CR obese Zucker rats compared to AL control rats[11]. Several differences between the study of Thanos et al. and ours might explain the discrepancy. First of all, the examination of different brain regions; it is possible that the impact

of calorie restriction is specific for certain regions, e.g. altering DRD2 binding in the striatum but not in the hypothalamus. Another difference is the animal model used; a diet-induced obese mouse model versus a genetic obese rat model. Furthermore, we used a slightly different protocol to measure DRD2 binding. Yet, we detected elevated DRD2 binding in the hypothalamus of CR haloperidol mice, which is consistent with a wealth of literature[49-51], indicating that our experimental protocol is well suited to accurately determine DRD2 binding patterns.

The detrimental impact of haloperidol is not constrained to calorie restricted mice, the drug affects glucose metabolism of ad libitum fed animals as well. Haloperidol acutely induces glucose intolerance in rats[52,53]. In a previous experiment we showed that haloperidol promotes insulin resistance in mice after 2 weeks of treatment (De leeuw van Weenen et al., submitted) and 4 weeks of haloperidol treatment increases basal insulin levels in rats[54]. The similar impact of haloperidol in calorie restricted and ad libitum fed animals makes it difficult to establish the importance of dopaminergic neurotransmission in the beneficial effect of calorie restriction. The fact that we were unable to detect differences in DRD2 binding between CR and AL mice argues against an important role for DRD2 receptors.

Haloperidol might affect glucose homeostasis through several distinct mechanisms. First, haloperidol, as well as other antipsychotics, dramatically reduces physical activity[55-57] and impaired physical activity might directly diminish insulin sensitivity. It has consistently been shown that 6-10 days of bed rest, representing severe physical inactivity, impairs insulin sensitivity in healthy man without affecting body weight[58-60]. Also in trained volunteers refraining from exercise for 10-14 days, representing a milder protocol for inactivity, insulin resistance is observed, again without alterations in body weight and fat mass[61,62]. Secondly, haloperidol consistently elevates serum concentrations of prolactin[63,64] and this is associated with glucose intolerance and insulin resistance[65,66]. Thirdly, haloperidol might, irrespective of its impact on physical activity and prolactin levels, acutely reduce insulin sensitivity. Several antipsychotic drugs are able to acutely induce insulin resistance[57,67-69]. Although this has not yet been confirmed for haloperidol, the drug does acutely impair glucose tolerance[52,53]. As the glucose intolerance was accompanied by elevated insulin levels, defective insulin secretion can not (solely) explain the observed glucose intolerance. So, this suggests that haloperidol, like other antipsychotic drugs, can acutely alter insulin sensitivity. Finally, haloperidol might impair insulin secretion. During the glucose tolerance test, the insulin levels were inappropriately low in the face of high glucose levels. This indicates defective insulin secretion. Likewise, DRD2 deficient mice are glucose intolerant in the face of low circulating insulin levels[70]. In vitro experiments with islets from these mice revealed that the islets were unable to secrete insulin in response to

glucose. The pancreata of these mice showed a reduced β-cell mass and insulin concentration. Considering these results, Garcia-Tornadu et al. suggested that DRD2 activation is essential for β-cell proliferation[70]. All together this supports that chronic inhibition of DRD2 neurotransmission might suppress insulin secretion.

Interestingly, in the current experiment, CR haloperidol mice were more insulin sensitive than AL mice but equally glucose intolerant. Glucose tolerance is the net effect of the production of insulin by pancreatic β-cells and the ability of peripheral tissues to respond to insulin by increasing glucose uptake. Insulin sensitivity was only modestly decreased in CR haloperidol mice compared to CR placebo mice, suggesting that a defect in glucose-stimulated insulin secretion was the main cause of the observed glucose intolerance in our experiment.

In summary, calorie restriction strongly limits the deleterious impact of high fat feeding on glucose and insulin metabolism in C57Bl6 mice. DRD2 inhibition, by means of haloperidol, curtails this effect of calorie restriction, suggesting that DRD2 mediated neurotransmission could be involved in the control of the metabolic benefits of calorie restriction. However, the fact that restricting access to high fat food does not increase hypothalamic DRD2 binding capacity does not support this inference.

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