Dopamine D2 receptors in the pathophysiology of insulin resistance

Leeuw van Weenen, J.E. de

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Dopamine D2 receptors in the pathophysiology of insulin resistance

Judith Carlier-de Leeuw van Weenen

Dopamine D2 receptors in the pathophysiology of insulin resistance

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Promotor: Prof. dr. H. Pijl

Overige leden: Prof. dr. A.J.W. Scheurink (Universiteit Groningen)
Prof. dr. J.A. Romijn (AMC, Amsterdam)
Prof. dr. E.R. de Kloet
Dr. B. Guigas

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Colophon

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General Introduction

1

Diabetes

Characteristics

The prevalence of Diabetes Mellitus Type 2, also known as non-insulin-dependent diabetes or adult-onset diabetes, is rising alarmingly. In 1985 approximately 30 million people worldwide suffered from diabetes. In 2007 this number had escalated to 246 million and by 2030 it is expected that ~ 438 million people (7.8% of the adult population) will be affected by diabetes[1]. At present, especially the developed world is coping with the diabetes epidemic, the prevalence in the US being 12.3% and in the Netherlands 7.7%, yet the developing countries are rapidly catching up[1]. It is estimated that, in the developing countries, the prevalence of diabetes will more than double in the years 2000-2030, compared to an increase of merely 50% in the western world[2].

Diabetes is a major cause of mortality. According to the WHO, diabetes has reached the top 10 of death causes in middle and high income countries[3]. It is predicted that in 2010 almost 4 million deaths will be attributed to diabetes, which represents 6.8% of global all-cause mortality[4]. The mortality risk for individuals with diabetes is 2.3 times higher than the risk for people with normal glucose homeostasis[5]. Cardiovascular disease, which is a frequently encountered complication of diabetes, is the main reason for the elevated mortality risk. Compared to the general population, diabetic people younger than 45 years are 10 times more likely to display cardiovascular disease, ranging from relatively mild (hypertension and atherosclerosis) to severe (stroke and myocardial infarction)[6]. Approximately 16% of diabetic patients suffer from severe cardiovascular incidents leading to hospital admission; this risk is ~ 2.3 fold higher than for non-diabetic subjects[7,8]. In addition, the risk of mortality due to cardiovascular disease is 2.6 times higher in diabetic patients[5].

Long term diabetes and poor glycemic control also lead to several other seriously disabling disorders. Diabetic nephropathy e.g. is one of the major causes of end-stage renal failure in the Western world[9]. Approximately 1.2% of diabetic patients develop renal failure, which represents a ~ 4 times higher risk than observed for people without diabetes[7,8]. Also, diabetes is the leading cause of new cases of blindness among adults aged 20-74 years[10]. The risk of developing any ophthalmologic complication, including cataract, glaucoma and diabetic retinopathy, is ~ 3 times elevated in diabetic versus nondiabetic individuals[8]. And, ~ 50% of diabetic patients develop neuropathy, which might manifest as sensory loss, muscle weakness, pain and/or erectile dysfunction[10,11].

Aetiology

Type 2 diabetes originates from a complex interplay between genetic and environmental factors. The contribution of a genetic component in the development of diabetes is undeniable, given the observation of an

extremely high diabetes prevalence among certain population groups like the Pima Indians[12,13]. Likewise, the high concordance rate of diabetes among both monozygotic and dizygotic twins suggests a genetic component to the disease[14,15]. And first-degree relatives from diabetic patients display several defects in energy and nutrient metabolism[16,17].

Some forms of type 2 diabetes, such as the different types of MODY (Maturity-Onset Diabetes of the Young), are of monogenic origin, meaning that one gene is responsible for the disease[18]. These forms of diabetes are characterized by a single gene mutation, an autosomal dominant inheritance pattern and an early onset of the disease. These cases however, represent only about 1-5% of all type 2 diabetes cases[18]. The majority of type 2 diabetes is of polygenic origin, meaning that several susceptibility genes additively increase the risk of disease onset. The contribution of single susceptibility genes to the diabetes risk is generally small; with odds ratios between 1.10 and 1.30. However, if several susceptibility loci are present, the risk of developing diabetes may increase substantially, as was shown for a Japanese population in which the risk of developing diabetes increased ~ 3.7 fold in the presence of a combination of 7 specific susceptibility loci[19]. Association studies in large population cohorts revealed several susceptibility genes, including PPARγ, TCF7L2, KCNJ11, CDKAL1, CDKN2A/CDKN2B, IGF2BP2, SLC30A8 and HHEX[19-21].

The contribution of the genetic predisposition is believed to remain stable throughout time; therefore it can not explain the recent rapid increase in diabetes incidence. Rather, this has been triggered by advances in health care and lifestyle changes. The prevalence of obesity, which is a major risk factor for diabetes development, has increased considerably the last decennia. In the US, the prevalence of adult obesity rose from 13.4% in 1960 to 30.9% in 2000[22] and the number of overweight children aged 6-11 and 12-19 increased from 4% and 6% in 1971 to 15.3% and 15.5% respectively in 2000[23]. The rise in diabetes incidence may greatly be accounted for by the recent rise in number of obese subjects. An objective measure to describe obesity is the body mass index (BMI), which is calculated as weight (in kilogram) divided by the square of the height (in meters); a BMI of < 18.5 represents underweight, 18.5-25 normal weight, 25-30 overweight, 30-35 obesity and > 35 morbid obesity. The lifetime risk for developing diabetes rises dramatically with increasing BMI. For an 18-year old person with normal weight, the risk of developing diabetes was calculated to be ~ 18.5%, if this person was morbidly obese though, the risk would increase to ~ 72%[24]. The predisposition of obesity to turn into diabetes is also reflected by the observation that in the US ~ 55% of type 2 diabetic patients is obese[25].

The change from an active to a sedentary lifestyle, promoted by the industrialization, the availability of easy transportation and the introduction of computers, television and video games, also independently adds to the elevated diabetes prevalence. A prospective cohort study in the US showed that with

every additional 2 hours of TV watching daily, the risk of diabetes increases with 14% and for every 2 hours/day increase in sitting at work, the risk for diabetes rises with 7%[26]. On the opposite, the impact of physical activity on reducing the risk of diabetes development has also firmly been established[27-31]. It is calculated that each 500 kcal increment in energy expenditure per week leads a 6% decrease in diabetes risk[27]. Even in people with impaired glucose tolerance, representing a pre-diabetes stage, physical activity is beneficial and reduces the risk of overt diabetes with 46%[32]. Clinical trials in patients with overt diabetes also indicate that physical activity, without weight loss, is able to improve the diabetic phenotype[33-35].

Also, the altered dietary pattern participates in the increased incidence of diabetes. With the introduction of highly palatable, energy-dense, food, total caloric intake increased and the dietary preferences shifted away from the traditionally "healthy" diet, including vegetables, fruits, low-fat dairy products and whole grain products, towards the "western type" diet, comprised of red and/or processed meat, high fat diary products, refined grain products, fried products and sweet beverages. Analyses of the health risk/benefit of both types of diets indicated that consumption of the "western type" diet is associated with a 28-60% higher risk of developing diabetes, while the "healthy" diet is associated with a modestly protective effect of 11-27%[36-39].

Finally, improved health care, which dramatically increased life expectancy the past decades, accounts for part of the elevated diabetes incidence. Aging is associated with an increased prevalence of diabetes; in the US, in the period of 2005-2006, the prevalence of previously diagnosed diabetes was 2.1% in the age group 20-39, 7.9% in the age group 40-59 and 17.6% in the age group 60-74[40]. Therefore increased longevity will greatly enlarge the number of diabetes patients. It is still a matter of debate whether the increased diabetes risk for elderly people is the result of aging per se or the result of age-related alterations in lifestyle and body composition. Unhealthy diets, decreased physical activity, increased adiposity and an altered fat distribution are all phenomena associated with aging and independent risk-factors for the development of diabetes. Accordingly, several studies showed an age-related deterioration of insulin action, yet in some, the reported differences between young and old individuals were diminished, or even completely lost, when corrected for age-related risk factors[41-45].

Glucose homeostasis

Physiology

Plasma glucose levels are maintained within a narrow range of 4-7 mmol/l. If glucose levels fall below the threshold of 3 mmol/l, energy supply to the brain becomes inadequate. The brain is unable to use substrates other than

glucose for energy and is only equipped with glycogen stores sufficient for a few minutes. Therefore, hypoglycemia rapidly leads to functional brain failure, seizures and coma. If the hypoglycemia is severe and prolonged it might even lead to brain death[46]. Conversely, elevated glucose levels can damage organs leading to macrovascular disease, nephropathy, retinopathy and neuropathy[47].

Insulin and glucagon are the key hormones regulating glucose homeostasis. Insulin is secreted by pancreatic β-cells in response to a physiological rise in glucose levels, e.g. after a meal. The net effect of insulin is to reduce the elevated glucose levels by promoting glucose uptake and simultaneously inhibiting glucose production. The liver is the main site responsible for the production of glucose. It can either convert stored glycogen into glucose or synthesize glucose de novo from non-carbohydrate substrates including lactate and amino acids. In insulin sensitive tissues like muscle, adipose tissue and also liver, glucose can be taken up and subsequently converted into glycerol for storage or oxidized to supply energy.

Glucagon is secreted by pancreatic α-cells in response to a reduction in blood glucose concentrations, e.g. during fasting. Opposing the action of insulin, glucagon increases glucose levels. It promotes the production of glucose by the liver, leading to an induction in both the conversion from glycogen to glucose and de novo glucose synthesis. Concomitantly glucagon inhibits the synthesis of glycogen and the oxidation of glucose in the liver[48]. During conditions of hyperglycemia glucagon production is suppressed by the combined action of the elevated glucose levels and the concomitantly raised insulin levels[49]. Other physiological regulators of glucose homeostasis include glucose, which can regulate its own disposal and release, catecholamines, cortisol and growth hormone.

Pathophysiology

Hyperglycemia is an important hallmark of diabetes and is the direct corollary of dysregulation of insulin and glucagon action. Impaired insulin action involves both insulin resistance, a reduced ability of tissues to respond to insulin, and defects in insulin secretion. In the early development of insulin resistance the diminished efficacy of the hormone is overcome by elevated insulin production by pancreatic β-cells; hyperinsulinemia therefore is a marker for diabetes development. Eventually, β-cells are no longer able to produce sufficient amounts of insulin to compensate for the resistance. Consequently, the biological function of insulin is undermined and hyperglycemia becomes manifest.

Insulin resistance can be demonstrated as a reduction in whole body glucose uptake and diminished suppression of glucose production during conditions of hyperinsulinemia. Several organ-specific mechanisms are thought to underlie the impaired insulin sensitivity.

Together, muscle, adipose tissue and liver are responsible for glucose disposal in response to insulin. As muscle tissue is the major contributor, insulin resistance of this tissue will greatly impair the ability of the body to remove glucose from the circulation. In response to insulin, GLUT4, the insulin-responsive transporter mediating the diffusion of glucose across the cell membrane, is translocated to the cell membrane. Once glucose has entered the cell, it is rapidly phosphorylated in order to maintain a concentration gradient for glucose across the cell membrane. In muscle cells from diabetic patients both the insulin induced transport of glucose across the cell membrane and the subsequent phosphorylation of intracellular glucose are diminished[50-52]. Several alterations in the intracellular signaling pathways downstream of the insulin receptor have already been described[53-55]. Together these might lead to a diminished recruitment of GLUT4 from intracellular storage vesicles to the cell membrane giving rise to the reduced glucose uptake[51].

Also in adipose tissue from diabetic individuals several defects have been noted. Diminished binding of insulin to its receptor in combination with a reduced receptor kinase activity greatly impairs the insulin action on adipocytes[56,57]. Concomitantly, both the basal expression of GLUT4 transporters on the cell membrane and the insulin stimulated translocation of GLUT4 to the surface is decreased in adipocytes from diabetic patients[57,58]. These latter observations might be ascribed to an enhanced turnover of glucose transporters and/or a diminished transporter gene expression[57,58].

Glucose uptake by the liver is mainly relevant after a meal, when both plasma glucose and insulin concentrations are elevated[59]. In diabetic patients, the capacity of the liver to extract glucose from the circulation under these postprandial conditions is compromised[60,61] as well as its ability to synthesize glycerol[62]. In contrast to muscle, where glucose transport across the plasma membrane is the rate-limiting step for glucose uptake, in liver, phosphorylation of glucose, by the enzyme glucokinase, is rate-limiting. Therefore, decreased activity of this enzyme, found in diabetic subjects[63,64], might be responsible for the reduced glucose uptake.

Concomitantly, the role of the liver as main producer of glucose is affected. Total, as well as directly measured hepatic, glucose production is higher in diabetic patients, both during basal, fasting, conditions[60,65-67] and hyperinsulinemic, fed, conditions[67,68]. The direct corollary is fasting and postprandial hyperglycemia. The contribution of increased gluconeogenesis to the elevated glucose production in diabetic subjects has firmly been established, but the contribution of glycogenolysis is still a matter of debate[65,66,68]. An increased ratio of the activity of the enzyme glucose-6-phosphatase to glucokinase, measured in diabetic patients, might contribute to the elevated glucose production[64].

Another contributory factor to the pathophysiology of hyperglycemia is an elevation in glucagon levels. In type 2 diabetic patients the postprandial suppression of glucagon production is impaired[69,70] leading to hyperglucagonemia and, regarding the nutritional status, an inappropriate stimulation of glucose production by the liver[71]. Possibly, resistance of pancreatic α-cells to the inhibitory action of insulin underlies this phenomenon.

Defective insulin secretion, which is, in addition to insulin resistance, an obligatory step in the development of type 2 diabetes, is the result of both a decrease in β-cell mass and β-cell malfunction. The reduced β-cell mass observed in type 2 diabetic patients is presumably the net effect an accelerated apoptosis rate in combination with normal β-cell replication and neogenesis[72,73]. Physiological signs of insulin secretion defects include an absence of the first phase insulin response[74,75], alterations in the pulsatility of insulin secretion[76,77] and an increased proinsulin to insulin ratio[78,79]. Intracellular defects underlying this β-cell malfunction include a reduction in the expression of glucose transporters GLUT1 and 2, impaired intracellular glucose processing[75] and a loss of insulin gene expression[80]. Damage and death of β-cells may be the consequence of hyperglycemia per se, as stated by the glucotoxicity theory. Accordingly, it was shown that prolonged hyperglycemia, either in combination with high circulating FFA levels or alone, promotes apoptosis and alterations in key components of cellular functioning through long-term increases in cellular Ca^{2+} concentrations[81] and oxidative stress[80]. Alternatively, or additionally, hyperglycemia may induce defects indirectly by promoting hypersecretion of insulin, leading to β-cell exhaustion[82].

Diabetic rodent models

Currently, several different rodent models have been established for diabetes research. Although most of these animal models fail to develop overt hyperglycemia and diabetes related complications, they do develop a diabetes-like phenotype characterized by obesity and insulin resistance. Some of these rodents models are genetic models; the result of single gene alterations. Three frequently used genetic models for diabetes research are the obese Zucker rat, the ob/ob mouse and the db/db mouse, all of which are characterized by mutations in genes involved in leptin signaling. The hormone leptin, predominantly synthesized by adipose tissue, serves as a regulator of long-term energy balance. Leptin is secreted in proportion to the amount of body fat and is therefore able to convey information about peripheral energy reserves. The long isoform of the leptin receptor is expressed in several regions of the brain, including the hypothalamus, and transmits the 'anti-obesity' action of leptin on food intake and energy expenditure[83]. In obese Zucker rats and db/db mice, respectively, a mutation in, and a deletion of, the leptin receptor were

found[84,85], whereas in ob/ob mice a mutation in the leptin gene was discovered[86]. Resistance to the physiological action of leptin, as well as the absence of leptin, leads to the development of a diabetogenic phenotype, including hyperphagia, reduced energy expenditure, obesity and insulin resistance[87-90].

Another genetic rodent model is the OLETF rat. These rats, presenting several of the characteristic features of diabetes, are naturally occurring CCK-1 receptor knockouts[91,92]. Cholecystokinin (CCK) is a peptide released from the gastrointestinal tract in response to food intake. CCK action is mediated by 2 distinct receptors which are both expressed in the periphery as well as in the brain. CCK regulates digestive function and promotes satiety. The CCK-1 receptor is responsible for the latter function[93].

Although the animal models described above, displaying spontaneous mutations in essential metabolic pathways, have provided us with important information concerning energy and nutrient balance, they only represent a small portion of the heterogeneous human diabetes population, since only a small percentage of diabetes cases are the result of single gene mutations. Diet induced obese (DIO) rodents better reflect the complex physiological alterations underlying the disease in the majority of obese type 2 diabetic patients. Several wild type (wt) rodents, such as the C57BL/6J mice, develop a diabetic phenotype after being fed a high fat diet for several weeks[94,95]. This DIO animal model is often used in diabetes research, yet, it is still a heterogeneous group; on average, DIO rodents develop a diabetic phenotype, but, there are large differences in the adaptation of individual animals to high fat feeding. It was shown that, after being maintained on a high fat diet for 9 months, 45% of a group of C57Bl6 mice became obese and diabetic, 12% remained lean and non diabetic, 12% was lean and diabetic and 30% showed an intermediate phenotype[96]. The insulin resistance phenotype of lean diabetic mice resembled more the phenotype of lean non-diabetic mice than of obese diabetic mice, so, simplified, the C57Bl6 mice could be divided into a diet induced obese (DIO), a lean diet resistant (DR) and an intermediate group. For experimental purposes wt rodents, while still maintained on a chow diet, can be divided into DIO and DR groups according to the amount of norepinephrine they excrete[97]. Alternatively, wt rodents can be classified according to their weight gain following several weeks of high fat feeding; the rodents with the highest weight gain are designated DIO and those with the least weight gain, DR[98].

Considering the heterogeneous response of humans towards the diabetogenic western type diets, we believe the DIO/DR rodent model accurately represents the human situation and is therefore best suited for analyzing the complex metabolic alterations associated with the development of obesity and diabetes.

Dopaminergic system

Physiology

Dopamine is the predominant catecholamine neurotransmitter in the mammalian central nervous system. It is synthesized in dopamine neurons and stored in synaptic vesicles until release. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the conversion of tyrosine into dopamine. Activation of dopamine neurons promotes fusion of the synaptic vesicles with the neuronal membrane, and dopamine is secreted. Upon release, dopamine binds to its receptor, located either on pre- or postsynaptic neurons, and initiates an intracellular signaling cascade. Dopamine transporters (DAT) take up dopamine from the extracellular fluid, thereby rapidly limiting the activity of secreted dopamine. Back in the neuron, dopamine is either transported into synaptic vesicles by a vesicular monoamine transporter (VMAT2) to be re-used or it is metabolized by monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT)[99,100].

Dopamine neurons are present in distinct areas of the brain, giving rise to three main dopaminergic pathways. The nigrostriatal pathway contains dopamine neurons originating in the substantia nigra and projecting to the dorsal striatum. Dopaminergic signaling in this pathway controls locomotor activity. The mesocorticolimbic pathway consists of dopamine neurons projecting from the ventral tegmental area to the ventral striatum, the limbic system and the cortex and is involved in emotion, cognition, motivation and reward. The dopamine neurons comprising the tuberoinfundibular pathway originate in the hypothalamus and project to the pituitary where they control hormone secretion and cell survival[101,102].

In addition to their role in the tuberoinfundibular pathway, dopamine neurons located in the hypothalamus control ingestive behavior. These neurons receive signals concerning energy homeostasis and nutrient availability from the periphery through afferent nerves, circulating hormones, nutrients and small peptide mediators. They integrate the information and relay it to the classical food intake-related neurons including NPY/ AGRP producing neurons (stimulators of food intake) and POMC producing neurons (inhibitors of food intake) to direct energy intake[103].

Dopamine receptors

Dopamine action is mediated by 5 distinct receptors which are categorized into 2 receptor families based on sequence homology and pharmacological characteristics. The D1-like family consists of the dopamine receptors D1 (DRD1) and D5. Activation of these receptors leads to stimulation of adenylyl cyclase and the subsequent production of cyclic AMP. Activation of the D2-like family on the contrary, inhibits adenylyl cyclase activity and the concomitant

production of cyclic AMP. The receptors DRD2, DRD3 and DRD4 represent the D2-like family[101]. Apart from different functional characteristics, the dopamine receptors also differ in spatial expression patterns. DRD1 and DRD2 are the most widely expressed receptors; they are found in all brain areas receiving dopaminergic innervation. The other dopamine receptors display more restricted expression patterns[101,102]. Although dopamine D1 and D2 receptors are present in the same brain areas, they are only occasionally expressed on the same neurons[101].

Dopamine receptors belonging to the DRD2 family exist both as pre- and postsynaptic receptors. Presynaptic receptors, or autoreceptors, which are believed to be mainly DRD2 and DRD3, are part of a dopaminergic feedback mechanism regulating neuronal activity and neurotransmitter release. Accordingly, stimulation of autoreceptors can alter firing rate of the neuron, dopamine synthesis and secretion. Postsynaptic receptors, which can be either DRD2, DRD3 or DRD4, modulate the action of second order neurons in response to dopamine[100,101].

Peripheral dopaminergic system

Dopamine receptors are highly expressed in the central nervous system, yet they are also present in several peripheral tissues, orchestrating a variety of biological functions. In the cardiovascular system, dopamine receptors are involved in the regulation of blood pressure. In the heart, up till now, D1, D2 and D4 dopamine receptors have been described. The role of the individual receptors has not yet been defined, but overall, a low concentration of dopamine is associated with an increased cardiac output due to improved contractility of the heart[104-106]. All dopamine receptors are expressed in the systemic blood vessels where they control vascular resistance by regulating vasodilatation[107-109].

In the kidney, dopamine, in general, increases renal blood flow and the excretion of water and ions such as sodium and calcium. The participation of the individual dopamine receptors in this effect is complex and varies depending on several factors such as systemic water and sodium balance[109,110]. Dopamine receptors D1, D2, D4 and D5 are also present in the adrenal glands[111,112]. The D2-like receptors are known to control aldosterone production, but the function of the D1-like receptors hasn't been clarified yet[112-114].

All dopaminergic receptors are expressed in the gastrointestinal tract. The dopamine D2 receptor is involved in the inhibition of gastric acid production[115] and gastrointestinal motility[116,117]. The role of the other dopaminergic receptors remains unclear.

Furthermore, all dopamine receptors, except DRD1, are expressed on peripheral blood leukocytes[118,119]. The action of dopamine on immune cells has best been studied in lymphocytes. In these cells dopamine exerts a dual role;

activating resting lymphocytes and inhibiting activated ones[120,121].

Recently dopamine receptors were also discovered on pancreatic β-cells. The dopamine D2 receptor is clearly involved in the modulation of insulin secretion, but the role of the other receptors remains to be elucidated[122].

DRD2 and diabetes

DRD2 polymorphisms

Several lines of evidence link the dopaminergic system to obesity, insulin resistance and type 2 diabetes in humans and animal models. An important indication for a functional relationship between dopamine and metabolic disturbances came from epidemiological studies. Several groups have examined the association of DRD2 polymorphisms and energy and nutrient metabolism. Although in general the impact is small, there is an interaction between DRD2 variants and energy homeostasis. The polymorphism Ser311Cys, which impairs the DRD2 signal transduction pathway[123], is associated with a higher BMI and lower resting energy expenditure in Pima Indians[124,125]. The TaqIA1 allele, resulting in lower DRD2 binding[126], is associated with obesity[127,128]. And, a haplotype consisting of 2 SNP's located in intron 5 and exon 6 of the DRD2 gene is associated with obesity as well[129]. Recently, proof for a role of DRD2 in the regulation of glucose and insulin metabolism was provided by Guigas et al. who showed that, in humans, the rate of glucose stimulated insulin secretion is associated with a 4-SNP haplotype (including TaqIA1 SNP) of the DRD2 gene[130].

DRD2 neurotransmission

More evidence came from the analysis of the dopaminergic system in obese and diabetic animals and humans. The expression of DRD2 is reduced in specific brain areas of obese Zucker and OLETF rats compared to lean control rats[131-133]. This decreased DRD2 expression is also observed in the striatum of obese humans. Moreover, in these individuals the number of DRD2 binding sites is inversely related to the body mass index[134]. Additionally, basal dopamine levels are increased in the hypothalamus of obese diabetic rats and the dopamine release in response to food intake is exaggerated and longerlasting[135-138]. A higher dopamine concentration was also measured in post mortem brains of diabetic patients compared to controls[139]. The reduction in dopaminergic neurotransmission elicited by a decreased DRD2 expression is thought to induce a “reward deficiency syndrome”, which might be compensated by elevated dopamine release and additionally, or alternatively, by “reward seeking behavior”, such as increased food intake[140].

DRD2 antagonists

Another indication that dopamine D2 receptors might be involved in energy

and nutrient metabolism came from the clinical observation that the use of antipsychotic medication is associated with obesity, insulin resistance and diabetes. Although numerous different antipsychotic drugs are used in clinic, the common denominator of these drugs is their affinity for dopamine D2 receptors. In general, the newer, second-generation 'atypical' antipsychotics have a broader range of action and a slightly lower affinity for the D2 receptor compared to first-generation 'typical' antipsychotics, but they are still (at least partial) DRD2 antagonists. In fact, it has been suggested that the clinical efficacy of these drugs to alleviate psychotic symptoms depends on the interaction of the drugs with dopamine D2 receptors[141].

Most antipsychotic drugs induce some degree of weight gain, yet the atypical, second generation, drugs clozapine and olanzapine are associated with the most severe increase in body weight[142-144]; in a meta-analysis it was calculated that both drugs can induce weight gain of up to 4.5 kg in 10 weeks in schizophrenic patients[142]. Other antipsychotic drugs, such as the typical drug haloperidol, induce much less weight gain[142,144].

The use of antipsychotic drugs is also linked to the development of diabetes[143,145]. Again, treatment with clozapine or olanzapine is associated with the greatest risk of developing diabetes[145]. One study even showed that, in a health care center, 36.6% of patients on clozapine treatment were newly diagnosed with diabetes within 5 years after the start of treatment[146]. Although the metabolic side effects of antipsychotic drugs have been observed in schizophrenic patients and schizophrenia itself contributes to the increased risk of developing diabetes[147,148], it is generally accepted that antipsychotics can directly affect energy and nutrient metabolism. This is confirmed by studies in animal models and healthy humans.

As in schizophrenic patients, weight gain is consistently observed in healthy volunteers treated with the antipsychotics olanzapine and risperidone[149-153]. The impact of antipsychotic drugs on glucose metabolism in healthy individuals though, is less clear; some studies report a reduction in insulin sensitivity following drug treatment[149,151,152], whereas others fail to observe an effect on insulin sensitivity[150,153]. In rodents the ability of antipsychotics to induce weight gain seems to be gender specific; female rats are sensitive to the weight inducing effect of the drugs, whereas in most studies using male rodents, body weight is not affected, or even decreased, by drug treatment[154-158]. The impact of antipsychotics on glucose metabolism, however, is consistent in animals. Both chronic and acute antipsychotic drug treatment is highly associated with the development of glucose intolerance and insulin resistance[156,159-164].

Alterations in several pathways might underlie these antipsychotic induced metabolic abnormalities. In most animal experiments, antipsychotics induce a defect in insulin stimulated glucose uptake during hyperinsulinemia[161-164]. Accordingly, it was observed that several antipsychotic drugs reduce glucose

uptake in neuronal cells[165]. The inability of tissues to appropriately respond to insulin stimulation might depend on an antipsychotic induced defect in insulin signaling, as is described in muscle cells after incubation with olanzapine[166].

In addition, an abnormally high endogenous glucose production during hyperinsulinemia is found in several animal models on antipsychotic drug treatment[159,161,164]. The underlying mechanism might be the inability of the liver to respond to the inhibitory action of insulin and/or the ability of antipsychotic drugs to acutely stimulate the endogenous glucose production, as is shown in rats[160].

A defect in insulin release might further add up to the metabolic alterations induced by antipsychotics. Several studies have reported an antipsychotic drug induced reduction in insulin response during hyperglycemia[162,163]. Accordingly, it was found that antipsychotic drugs can directly affect insulin release from isolated pancreatic islets[167-169].

DRD2 agonists

Considering the impact of the dopaminergic system on energy and nutrient metabolism, several groups have examined the efficacy of DRD2 agonists in ameliorating the adverse metabolic conditions associated with diabetes. The best studied DRD2 agonist in relation to obesity and diabetes is bromocriptine, clinically used in the treatment of Parkinson's disease and hyperprolactinemia. In humans, several trials have been performed with this DRD2 agonist. The most consistent impact of such treatment in obese individuals is normalization of elevated plasma glucose levels[170-173]. In addition, in several studies, bromocriptine treatment diminished basal plasma insulin levels in obese individuals[170,173]. The impact of bromocriptine on body weight though, is inconsistent among studies; in some the body weight and fat percentage of subjects decreased upon treatment[174], while in others body weight remained stable throughout the experiment[171-173]. The impact of bromocriptine on glucose metabolism is more consistent; it improves glucose tolerance and insulin sensitivity in obese people[172,174].

In most animal studies bromocriptine was given in combination with the DRD1 agonist SKF38393, as this latter drug enhances the efficacy of bromocriptine[175,176]. Unlike in humans, treatment of obese diabetic animal models with the combination of bromocriptine and SKF38393 consistently decreases food intake, fat mass and overall body weight[175,177-180]. Surprisingly, the decrease in food intake was only moderately involved in body weight reduction, as pair feeding was only able to partly reproduce this effect[179,180]. The impact of enhanced DRD2 stimulation on food intake and body weight has also been confirmed with quinpirole, another DRD2 agonistic drug[176]. Furthermore, like in humans, bromocriptine/SKF38393 treatment normalizes elevated plasma glucose and insulin levels in obese diabetic animals[175,177-180].

The underlying mechanism(s) for this improvement is not yet fully elucidated, but bromocriptine/SKF38393 treatment reduces the activity of 2 key enzymes involved in hepatic gluconeogenesis in obese insulin resistant mice[179] and glucose production is diminished in bromocriptine treated hamsters[181]. Also, bromocriptine improves glucose tolerance and insulin sensitivity[177,182]. This might be mediated by a restoration of the aberrant β-cell function by bromocriptine/SKF38393, resulting in a reduction of the elevated basal insulin release, an increase in insulin content and an improved glucose-stimulated insulin release[178,183,184].

Injection of bromocriptine directly into the brain of diabetic hamsters, in a concentration that does not have an effect when administered systemically, also diminishes body weight and improves glucose tolerance and insulin sensitivity[185]; suggesting that (part of) the observed effects of DRD2 stimulation on metabolism are mediated by dopamine receptors in the brain.

Outline of this thesis

The dopaminergic system in general and the dopamine receptor D2 specifically are functionally linked to diabetes-associated metabolic derangements. Genetic variations in the DRD2 gene are associated with altered energy and nutrient homeostasis. Inhibition of DRD2 promotes a diabetes-like phenotype, while activation of DRD2 restores a normal metabolic profile. Also several components of dopaminergic signaling are modified in obese and diabetic humans and animals. Despite the established interaction between DRD2 and disturbances in the energy and nutrient homeostasis, several questions regarding the exact role of DRD2 in the aetiology of diabetes and the mechanism underlying the metabolic corollary of DRD2 transmission modulation remain unanswered. The research described in this thesis is conducted in order to unravel the characteristics of the interplay between the DRD2 and glucose metabolism as well as to understand the underlying mechanism(s).

The aim of chapter 2 was to determine the role of the dopaminergic system in the aetiology of high fat diet induced obesity and insulin resistance. Therefore, glucose metabolism and several indicators of dopaminergic neurotransmission were evaluated after 4 weeks of high fat feeding in wt mice and compared to mice maintained on a low fat diet.

Calorie restriction is the most effective way to extend lifespan and reduce morbidity. As such, it also improves insulin sensitivity and delays the age-related loss of DRD2 expression in the brain. Considering this, together with the role of the dopaminergic system in glucose metabolism, it can be suggested that the dopaminergic system is involved in the beneficial impact of calorie restriction on insulin action. This hypothesis was addressed in chapter 3. Wt mice were maintained on a high fat diet, either with unlimited or restricted

access, for 12 weeks. During the entire experiment half of the calorie restricted mice also received continuous haloperidol treatment. After the treatment period glucose metabolism was evaluated and the hypothalamic DRD2 binding was determined.

In general, high fat feeding induces obesity, insulin resistance and a type 2 diabetic phenotype in rodents, but there is a large diversity in response within single strains of rodents. Based on weight gain, the phenotype of rodents on a high fat diet can be characterized as diet induced obese (DIO), intermediate or diet resistant (DR). DIO and DR rodents differ in several components of the dopaminergic system, even before the onset of obesity. This led to the suggestion that variation in dopaminergic neurotransmission participates in the development of the divergent DIO and DR phenotypes. Therefore, in chapter 4 we maintained wt mice on a high fat diet for 10 weeks to classify them as DIO and DR. Subsequently we treated DIO and DR mice with, respectively, the DRD2 agonist bromocriptine and the DRD2 antagonist haloperidol and performed indirect calorimetric measurements and characterized glucose metabolism. Placebo treated DIO and DR mice served as controls.

Antipsychotic drugs are associated with the development of insulin resistance and dyslipidemia. It is, however, still unclear if these drugs directly modify glucose and lipid metabolism or if they promote weight gain which may lead to the disturbed metabolic profile. Therefore, in chapter 5, the short-term impact of the typical antipsychotic drug haloperidol and the atypical drug olanzapine were studied in order to unravel the mechanism underlying the deregulation of nutrient metabolism. The carbohydrate and lipid metabolism of healthy men was evaluated before and after 8 days of antipsychotic drug treatment.

The DRD2 agonistic drug bromocriptine is highly effective in improving glucose metabolism and β-cell function, yet, the underlying mechanism remains unclear. In chapter 6 we studied the acute impact of bromocriptine on insulin secretion and action in wt mice and the impact on intracellular signaling in INS-1E cells.

In chapter 7 the results obtained with these studies and their implications are discussed.

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Four weeks high fat feeding induces insulin resistance without affecting dopamine release or gene expression patterns in the hypothalamus of C57Bl6 mice

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Judith E. de Leeuw van Weenen
Lihui Hu
Karola Jansen-van Zelm
Martin G. de Vries
Jouke T. Tamsma
Johannes A. Romijn
Hanno Pijl

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Abstract

Obesity is associated with diminished dopaminergic neurotransmission. It remains unclear whether this is a cause or a consequence of the obese state. We hypothesized that high fat feeding, a well-known trigger of obesity in diet sensitive mice, would blunt dopaminergic neurotransmission prior to the development of insulin resistance.

We monitored in vivo dopamine release in the dorsomedial region of the hypothalamus and determined hypothalamic gene expression patterns of dopamine receptors 1 and 2 (DRD1 and 2), tyrosine hydroxylase (TH) and the dopamine transporter (DAT) in C57Bl6 mice maintained on a high fat diet for 4 weeks. Also, a hyperinsulinemic euglycemic clamp was performed to evaluate the metabolic status of the mice. Mice maintained on a low fat diet served as controls.

The high fat diet did not alter dopamine release in the dorsomedial hypothalamus of fed or fasted mice or the dopaminergic response to refeeding. Furthermore, gene expression levels of DRD1, DRD2, TH and DAT were not affected by high fat feeding. However, the high fat diet did hamper insulin action as evidenced by diminished glucose disposal during hyperinsulinemia ($p<0.05$).

We show here that short term high fat feeding does not affect dopaminergic neurotransmission in the hypothalamus, whereas it does impair insulin action. This suggests that reduced dopaminergic neurotransmission in the hypothalamus of obese animal models is due to mechanism(s) that are not directly triggered by diet composition.

Introduction

Dopamine is intimately involved in the regulation of energy balance. Genetically engineered dopamine-deficient mice fail to initiate feeding and consequently die of starvation, unless L-DOPA, the precursor of dopamine, is provided daily[1]. Conversely, dopamine release in response to food intake induces satiety and reward[2]. Thus, dopamine plays an important dual role in the complex physiology driving meal initiation and termination. Moreover, dopaminergic neurotransmission profoundly affects glucose and lipid metabolism[3].

Dopamine action is mediated by at least 5 distinct G-protein coupled receptor subtypes, which are functionally classified into 2 receptor families. Dopamine receptor D1 (DRD1) and DRD5 activate adenylyl cyclase in target neurons and belong to the D1-like family. The others (DRD2, DRD3 and DRD4) are D2-like receptors, which inhibit adenylyl cyclase[4].

Drugs that block DRD2 enhance appetite and induce weight gain in animals and humans[5-8]. Conversely, DRD2 agonist drugs reduce body weight, increase energy expenditure and improve glycemic control in obese animals and individuals[9-12]. DRD1 agonistic drugs reduce food intake, body weight and plasma glucose levels in obese mice[10,13]. Thus, dopamine impacts on energy balance through activation of both DRD1 and DRD2 receptors.

The hypothalamus plays a critical role in the control of food intake and metabolism[14]. Compelling evidence indicates that dopaminergic neurotransmission is altered in the hypothalamus of obese animals. Basal and feeding evoked dopamine release is exaggerated and longer-lasting in several nuclei of the hypothalamus of obese Zucker rats[15-17], whereas DRD2 expression is reduced in hypothalamic nuclei of obese animal models[18,19]. Lack of DRD2 may induce a so called “reward deficiency syndrome”, eliciting exaggerated dopamine release in response to large meals to induce reward in the face of diminished signal transduction[20]. The number of DRD2 binding sites is reduced in the striatum of obese humans and inversely correlated with body mass index[21]. This supports the view that reward deficiency may be involved in the pathogenesis of human obesity.

Dopaminergic neurotransmission has particularly been studied in chronically obese animals and humans. Therefore, it remains unclear whether the observed changes are a cause or a consequence of the obese state. However, activation of DRD2 receptors redresses various pathologic features of obesity[12,18], which suggests that down regulation of DRD2 may be a primary characteristic. Therefore, we hypothesized that high fat (HF) feeding, a well known inducer of obesity and insulin resistance in C57Bl6 mice, would reduce DRD2 receptor expression and, via the mechanism of reward deficiency, enhance food intake and associated dopamine release in the hypothalamus of these mice. To test our hypothesis, we monitored in vivo dopamine release in animals maintained

on a HF diet for 4 weeks. In addition, we measured gene expression levels of the DRD1 and DRD2, tyrosine hydroxylase (TH), the enzyme catalyzing the rate-limiting step in dopamine synthesis and the dopamine transporter (DAT), which is responsible for presynaptic re-uptake of dopamine. Finally, a hyperinsulinemic euglycemic clamp was performed to evaluate the metabolic status of the animals. All these parameters were compared to those obtained in animals receiving a low fat (LF) control diet.

A relatively short intervention period of 4 weeks was chosen to run ahead of overt obesity and/or insulin resistance, as these metabolic features may impact on dopaminergic neurotransmission by themselves. Since the hypothalamus is a critical player in the control of energy balance and fuel metabolism[14], we decided to focus on this particular brain area in the current study.

Materials and Methods

Animals

Male 12-week-old C57BL/6J mice (Charles River, Maastricht, The Netherlands) were housed in a temperature- and humidity-controlled room on a 12-h light–dark cycle with free access to food and water, unless mentioned otherwise.

All mice were randomly assigned to a group receiving either a high fat (HF) diet (45 energy% of fat derived from palm oil; Research Diet Services, Wijk bij Duurstede, The Netherlands) or a low fat (LF) control diet (10 energy% fat derived from palm oil; Research Diet Services) for 4 weeks. The exact composition and caloric content of both diets is described in table 1.

All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare, and the protocol was approved by the Institutional Ethical Committee on Animal Care and Experimentation.

Plasma analysis

Blood samples were drawn from the tail vein before onset of the dietary pretreatment and again at the end. Before sampling, mice were fasted for 10 hours; from 11.00 pm until 9.00 am. Plasma glucose levels were measured using a commercially available kit (INstruchemie, Delftzijl, The Netherlands). A commercially available ELISA (Mercodia, Uppsala, Sweden) was used to measure plasma insulin levels.

Experiment 1. Effect of diet on in vivo dopamine release in the hypothalamus

Experimental design

Fourteen mice were randomly assigned to a group receiving either a HF diet or LF control diet. At the end of the 4-week dietary pretreatment, microdialysis probes were surgically implanted.

Table 1 - Composition and caloric content of the low and high fat diets used.

Low fat diet		High fat diet	
Ingredients	Mass (g/kg)	Ingredients	Mass (g/kg)
Casein	189.6	Casein	189.6
Cornstarch	298.6	Cornstarch	69.0
Maltodextrin DE10	33.2	Maltodextrin DE10	94.8
Sucrose	331.8	Sucrose	163.8
Cellulose (Arbocel B800)	47.4	Cellulose (Arbocel B800)	47.4
Palm oil	19.0	Palm oil	168.2
Soy oil	23.7	Soy oil	23.7
Mineral premix S10026	9.5	Mineral premix S10026	9.5
Dicalciumphosphate	12.3	Dicalciumphosphate	12.3
Calciumcarbonate	5.2	Calciumcarbonate	5.2
Potassiumcitrate monohydrate	15.6	Potassiumcitrate monohydrate	15.6
Vitamin premix V10001	9.5	Vitamin premix V10001	9.5
L-Cystein	2.8	L-Cystein	2.8
Choline Bitartrate	1.9	Choline Bitartrate	1.9
Energy Content (kcal/kg)	3845	Energy Content (kcal/kg)	4728

Microdialysis started 24 hours after surgery. On the first day, basal dopamine output in fed mice was measured. The microdialysis probe was connected to the pump and perfusion was started at 8.30 am. After a 2 h stabilization period, 4 baseline samples were collected at 30 minute intervals. At 11.00 pm, food was removed and the mice were fasted. The next day microdialysis was reinitiated at 08.30 am. After a 2 hour stabilization period, 4 baseline samples were collected at 30 minute intervals in fasted mice. Subsequently, food was provided, both groups of mice receiving their respective diets, and, while mice had ad libitum access to this food, 6 additional samples were collected at 30 minute intervals.

Surgery

Mice were anesthetized using isoflurane (2%, 1000 ml/min O_2). Lidocaine was used for local anesthesia and fynadine as analgesic. The animals were placed

in a stereotaxic frame (Kopf instruments, CA, USA), and I-shaped probes (PES membrane, 1 mm exposed surface; BrainLink, Groningen, The Netherlands) were inserted into the dorsomedial region of the hypothalamus. Coordinates for the tips of the probes were: posterior (AP) = - 1.5 mm to bregma, lateral (L) = 0.6 mm to midline and ventral (V) = - 5.1 mm to dura[22].

Microdialysis procedure

During the experiment, the probes were connected with flexible PEEK tubing to a microperfusion pump (Syringe pump UV 8301501, TSE, Bad Homburg, Germany) and perfused with artificial cerebrospinal fluid, containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$, at a flow rate of 1.5 µl/min.

Microdialysis samples were collected at 30 min intervals into mini-vials which already contained 15 µl 0.02 M acetic acid. The samples were collected by an automated fraction collector (CMA 142), and stored at -80° C awaiting analysis. After the experiment, the mice were sacrificed and the brains were removed. The brains were incubated for 3 days in a 4% (w/v) solution of paraformaldehyde. The position of each probe was histologically verified according to the stereotaxic atlas of Paxinos and Franklin[22].

Analysis of dopamine

Separation:

Samples (20 µl) were injected onto the HPLC column by a refrigerated microsampler system, consisting of a syringe pump (Gilson, model 402), a multi-column injector (Gilson, model 233 XL), and a temperature regulator (Gilson, model 832). Chromatographic separation was performed on a reverse-phase 150 x 2.1 mm (3 µm) C_{18} Thermo BDS Hypersil column (Keystone Scientific). The mobile phase (isocratic) consisted of a sodium acetate buffer (4.1 g/l) with methanol (2.5 % v/v), Titriplex (EDTA; 150 mg/l), 1-octanesulfonic acid (150 mg/l), and tetramethylammonium (150 mg/l) and adjusted with glacial acetic acid to pH = 4.1. The mobile phase was run through the system at a flow rate of 0.35 ml/min by an HPLC pump (Shimadzu, model LC-10AD vp).

Electrochemical detection:

Dopamine was detected electrochemically using a potentiostate (Antec Leyden, model Intro, Zoeterwoude, The Netherlands) fitted with a glassy carbon electrode set at +500 mV vs. Ag/AgCl (Antec Leyden).

Data were analyzed by Chromatography Data System software (Shimadzu, class-vp). The concentration of dopamine was quantified by external standard method.

Experiment 2. Effect of diet on hypothalamic expression of genes involved in dopaminergic neurotransmission

Experimental design

Another twelve mice were randomly assigned to a group receiving either a HF or a LF diet. After 4 weeks of dietary intervention, fed mice were sacrificed for the analysis of hypothalamic expression patterns of dopaminergic genes. All mice were sacrificed between 9.00 and 12.00 am, to minimize effects of circadian rhythm. The hypothalamus was rapidly dissected from the brain by making 2 coronal incisions, one caudal to the optic chiasm and the other rostral to the mammillary bodies. The hypothalamus was then isolated from this coronal section using the internal capsules as lateral boundaries and the thalamus as dorsal boundary. The tissue was immediately frozen in liquid nitrogen and stored at -80°C awaiting analysis.

RT-PCR

Total RNA was extracted from the hypothalamus using TRIzol reagent (Invitrogen, Breda, The Netherlands) and an additional phenol–chloroform (Invitrogen, Breda, The Netherlands) extraction after the phase separation step of the TRIzol protocol (C.M.A. Reijnders et al., *submitted*). Total RNA was further purified by treatment with RNase-free DNase (Promega, Leiden, The Netherlands) to circumvent DNA contamination. Reverse transcription was performed with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot ,Germany).

For RT-PCR commercially available primer sets were used: DRD2 and TH (Qiagen, Venlo, The Netherlands), DRD1a, Slc6a3, Rpl13a and Ppia (SuperArray, MD, USA). PCR amplification was performed in a total volume of 25 µl, containing 5 ng of cDNA, 1x primer mix, 1x SYBER Green mix (Qiagen or Bio-Rad, Veenendaal, The Netherlands) and RNase free water. The SYBER Green mix from Qiagen was used in combination with the DRD2 and TH primer sets, whereas the Bio-Rad SYBER Green mix was used in combination with the other primer sets. Conditions for the amplification of DRD2 and TH genes were 15 min at 95°C followed by 45 cycles of 10 sec 95°C, 30 sec 55°C and 30 sec 72°C. Conditions for the amplification of DRD1a and Slc6a3 were 3 min at 95°C followed by 45 cycles of 10 sec 95°C, 30 sec 55°C and 30 sec 72°C. Finally, the conditions for the amplification of Rpl13a and Ppia were 3 min at 95°C followed by 45 cycles of 10 sec 95°C, 30 sec 60°C and 30 sec 72°C.

Specificity of the amplification reaction was confirmed by analysis of the dissociation curve. Each sample was amplified in triplicate. Data was analyzed using the IQ5 software (Bio-Rad).

Gene expression levels in HF mice were expressed relative to gene expression levels in LF mice following normalization of the DRD2, DRD1, TH and DAT expression levels to those of the reference genes Rpl13a and Ppia.

Experiment 3. Effect of diet on in vivo insulin resistance

Experimental design

Another thirteen mice were randomly assigned to a group receiving either a HF or a LF control diet for 4 weeks. At the end of the dietary intervention, mice were subjected to a hyperinsulinemic euglycemic clamp procedure for evaluation of in vivo insulin resistance.

Hyperinsulinemic euglycemic clamp

Mice were fasted for 16 hours after food withdrawal at 5.00 pm on the day before the clamp. Hyperinsulinemic euglycemic clamp studies started at 9.00 am and were performed as described earlier[23]. During the experiment, mice were sedated 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 (0.2 μCi) continuous (0.3 μCi/h) intravenous (i.v.) infusion of D-[U-^{14}C]-glucose (37 MBq) (GE Healthcare, Little Chalfont, UK) for 60 minutes. Subsequently, insulin (Novo Nordisk, Bagsværd, Denmark) was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for 90 minutes to attain steady state circulating insulin levels of ~4 μg/l. A variable i.v. infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding (< 3 μl) (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). 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 ^{14}C-glucose specific activities. At the end of the clamp mice were sacrificed.

Analytical procedures

Plasma levels of glucose were determined using a commercially available kit (INstruchemie, Delfzijl, The Netherlands). Plasma insulin concentrations were measured by a mouse insulin ELISA (Mercodia AB, Uppsala, Sweden). Total plasma ^{14}C-glucose was determined in 7.5 μl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation.

Calculations

The rate of glucose disposal (Rd) (μmol/min/kg) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of ^{14}C-glucose (dpm/μmol).

The ratio was corrected for body weight. Hepatic glucose production (HGP) was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Statistical evaluation

Data is presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS. Metabolic data or data concerning the basal dopamine output and gene expression was analyzed using an independent sample t-test. The non-parametric Mann-Whitney test was used to analyze the hyperinsulinemic euglycemic clamp data. For the dopamine output in response to refeeding, four consecutive fasting microdialysis samples with less than 50% variation were taken as baseline and their mean was set at 100%. Treatment effects were expressed as percentages of basal level within the same animal. For statistical analysis raw dopamine output levels were compared to mean fasting baseline values using a two-way ANOVA for repeated measures. The LSD method was used as post-hoc test to determine differences at single time points. Differences were considered statistically significant when $p \leq 0.05$.

Results

Basal metabolic data

Body weight was significantly increased in mice maintained on a HF diet compared to mice maintained on a LF diet for 4 weeks (table 2). Fasting plasma glucose and insulin levels were not different in HF mice compared to LF mice after 4 weeks of dietary intervention.

Table 2 - Weight, fasting plasma glucose and insulin levels measured in mice at the start and end of the 4-week dietary intervention.

	Before diet		After diet	
	LF group	HF group	LF group	HF group
Weight (g)	24.7 ± 0.60	24.2 ± 0.61	27.3 ± 0.46	29.3 ± 0.51*
Glucose (mM)	6.99 ± 0.57	6.85 ± 0.63	7.53 ± 0.39	8.47 ± 0.42
Insulin (μg/l)	0.47 ± 0.05	0.63 ± 0.12	0.38 ± 0.04	0.44 ± 0.05

Data is measured in 19 HF and 20 LF mice and presented as mean ± SEM
* p< 0.01 vs. LF group after diet

Basal dopamine output

Mice had fully recovered from anesthesia when microdialysis was started, as indicated by complete body weight recovery (weight before vs. 24h after surgery; HF mice: 27.7 ± 1.4 vs. 28.0 ± 1.9 g; LF mice: 27.6 ± 1.4 vs. 27.5 ± 1.4 g).

Basal dopamine levels did not differ between animals maintained on a HF or LF diet, neither in fed (fig 1A) nor in fasted state (fig 1B).

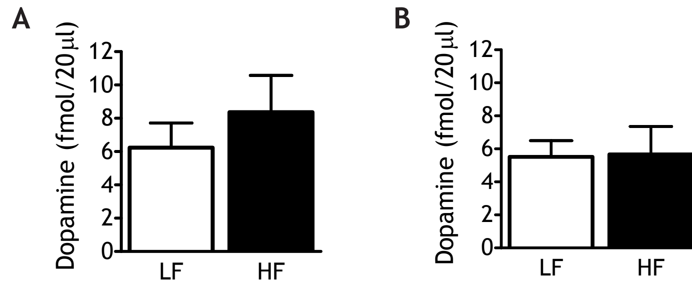

Figure 1 - Basal dopamine output in the dorsomedial region of the hypothalamus of fed (A) or fasted (B) mice maintained on a HF vs. LF diet during 4 weeks (n=7 mice per group). Value per mouse is the mean of 4 consecutive baseline measurements. Data is presented as mean ± SEM.

Dopamine output in response to refeeding

Dopamine levels rose to approximately 150% of baseline (p = 0.024) within 30 min after the return of food in both HF and LF mice and decreased to baseline again within the next 60 min (fig 2). These findings agree with previous observations by others[15,16,24], indicating that our experimental procedure adequately detects changes in dopamine levels. The dopamine response to refeeding was not different between HF and LF mice.

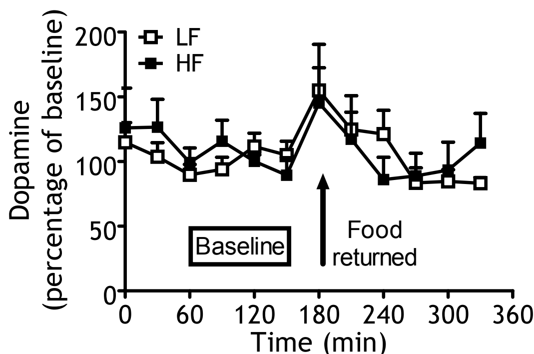

Figure 2 - Dopamine output in the dorsomedial region of the hypothalamus in response to refeeding after a 13.5-h fast in mice maintained on a HF vs. LF diet for 4 weeks (n=7 mice per group). Data is presented as mean ± SEM.

Expression levels of genes involved in dopaminergic neurotransmission

The hypothalamic expression patterns of DRD2, DRD1, TH and DAT were not different in mice maintained on a HF or LF diet (fig 3).

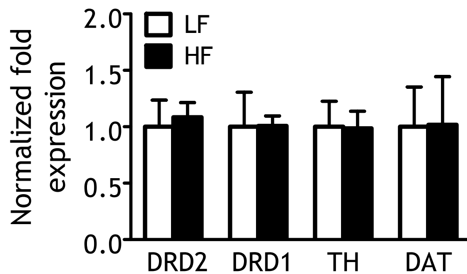

Figure 3 - Normalized, relative expression of DRD2, DRD1, TH, and DAT genes in the hypothalamus of mice maintained on a HF vs. LF diet during 4 weeks (n=6 mice per group). Data is presented as mean ± SEM.

Hyperinsulinemic euglycemic clamp

Glucose and insulin concentrations measured during basal and hyperinsulinemic clamp conditions are shown in table 3. Stimulation of the glucose disposal rate by insulin was significantly reduced in HF mice compared to LF mice (fig 4A). In contrast, the inhibitory effect of insulin on hepatic glucose production was not affected by diet composition (fig 4B).

Table 3 - Plasma glucose and insulin levels measured in mice during the basal and hyperinsulinemic conditions of the hyperinsulinemic euglycemic clamp.

	Basal		Hyperinsulinemia	
	LF group	HF group	LF group	HF group
Glucose (mM)	5.71 ± 0.31	6.19 ± 0.26	5.78 ± 0.28	6.21 ± 0.42
Insulin (µg/l)	0.29 ± 0.04	0.45 ± 0.11	2.81 ± 0.56	4.03 ± 0.94

Data is measured in 6 HF and 7 LF mice and presented as mean ± SEM.

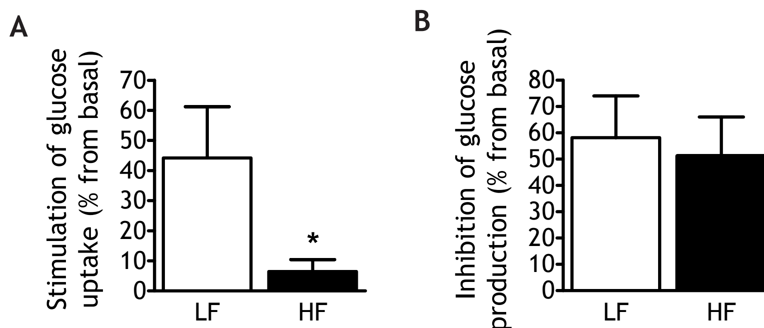

Figure 4 - Stimulation of glucose disposal (A) and inhibition of glucose production (B) during a hyperinsulinemic euglycemic clamp in mice maintained on a HF (n=6 mice) vs. LF diet (n=7 mice) for 4 weeks. Data is presented as mean ± SEM.
* $p < 0.05$ vs. LF diet

Discussion

In the present work we determined the impact of HF feeding on dopamine release and the expression of genes involved in the control of dopaminergic neurotransmission in the hypothalamus of diet-susceptible C57Bl6 mice. HF feeding, in these animals, recapitulates many of the metabolic and endocrine features of human obesity. We hypothesized that a HF diet would diminish the expression of DRD2 and thereby trigger a "reward deficiency syndrome" that might underlie weight gain and impaired insulin action. However, our results do not support this hypothesis. Four weeks of HF (45 energy% fat derived from palm oil) or LF (10 energy% fat derived from palm oil) feeding was associated with similar dopamine release in the dorsomedial hypothalamus and equivalent gene expression levels of DRD1, DRD2, TH and DAT in the whole hypothalamus of C57Bl6 mice. Nonetheless, HF feeding hampered insulin action in the current experimental context, as evidenced by diminished glucose uptake during the hyperinsulinemic euglycemic clamp. Thus, our results argue against the role of reduced dopaminergic neurotransmission in the hypothalamus as causal intermediate between HF feeding and the pathogenesis of obesity and/or insulin resistance in C57Bl6 mice.

A host of papers document a decrease of DRD2 expression and a compensatory rise in dopamine levels in the brain of obese animal models and humans. OLETF rats, which gradually develop obesity and diabetes as a consequence of cholecystokinin (CCK) receptor-1 deficiency, are characterized by increased striatal dopamine release[25]. A loss-of-function mutation in the leptin receptor gene, leading to a morbid obesity syndrome and diabetes, is associated with a reduction in DRD2 expression and exaggerated dopamine levels in the hypothalamus of Zucker rats[15,16,19,26]. Treatment with the DRD2 agonist bromocriptine ameliorates the metabolic phenotype of these animals[27,28]. Likewise, DRD2 availability is significantly reduced in the striatum of obese humans and inversely correlated with their body mass index[21,29], while bromocriptine treatment also ameliorates various metabolic anomalies of obese women[12].

Virtually all studies evaluating dopaminergic neurotransmission in obesity have used chronically obese animal models and human subjects. Therefore, current knowledge does not provide an answer to the question whether a deficiency in dopaminergic neurotransmission is a primary defect underlying obesity syndromes or rather a consequence of the metabolic state. At least one study documents a rise in dopaminergic tone in hypothalamic nuclei of diet sensitive rats prior to high energy diet exposure, suggesting that elevated dopamine levels lead to the development of obesity in these animals[30]. However, our data do not support the hypothesis that HF feeding blunts dopaminergic neurotransmission in a diet-sensitive mouse strain before the onset of obesity

and insulin resistance. In particular, they suggest that diets of quite distinct composition in terms of fat and carbohydrate do not, in the short term, impact on hypothalamic dopaminergic neurotransmission. Obviously, our data do not rule out the possibility that diet composition affects dopaminergic transmission in other brain areas. Intermittent (excessive) sugar intake, for example, has been shown to increase extracellular dopamine in the mesolimbic system[31].

The question then arises whether the metabolic abnormalities associated with obesity and insulin resistance could change brain dopaminergic neurotransmission to explain the multitude of data documenting reductions of dopamine neurotransmission in the brain of obese animals and humans. The answer may be yes. For example, glucose dose-dependently enhances dopamine release by PC-12 neuroendocrine cells[32], hyperinsulinemia combined with hyperglycemia induces exaggerated dopamine release in vivo in the nucleus accumbens of rats[33], and dopamine release is diminished in hippocampal areas of spontaneously hypoinsulinemic diabetic rats[34]. Furthermore, intracerebroventricular administration of insulin increases DAT mRNA in the ventral tegmental area of rats[35], and insulin enhances dopamine uptake in striatal cells and human DAT transfected cells in vitro[36,37]. Conversely, food deprivation, which is accompanied by low circulating insulin levels, blunts DAT mRNA expression and activity in the ventral tegmental area of rats[38]. Thus, circulating metabolites and hormones clearly impact on dopaminergic neurotransmission, but it remains unclear whether insulin resistance, accompanied by hyperinsulinemia and hyperglycemia, affects dopaminergic signaling in brain areas involved in the control of food intake and metabolism.

Alternatively, reduction of dopaminergic neurotransmission in obese animal models and humans could be due to mechanism(s) that are not directly triggered by diet composition or metabolic cues. For example, elevated dopamine levels found in OLETF rat might be the consequence of CCK receptor-1 deficiency[25,39]. Reduced DRD2 expression and high dopamine levels in hypothalamic areas of Zucker rats[15,19] could be a direct corollary of their genetic resistance to the inhibitory effect of leptin on dopamine release[40-42]. In analogy, leptin resistance might also be the primary cause of reduced DRD2 binding in obese humans[43].

Whatever the biological underpinnings, reduction of central dopaminergic tone may modulate neuroendocrine activity so as to impair insulin action[5,7,8]. In a scenario where reduced brain dopamine signal transduction is not caused by hyperinsulinemia and/or hyperglycemia, but rather underlies these metabolic anomalies, it is easier to understand the undisputable benefits of dopamine DRD2 activation for glucose metabolism in various obese animal models and humans[12,44,45].

It is important to point out that our study did not allow evaluation of dopaminergic neurotransmission in individual hypothalamic nuclei. In particular, our method of dissecting the hypothalamus for RNA isolation only

permitted determination of gene expression levels in the whole hypothalamus. Furthermore, the size of the microdialysis probe did not allow determination of dopamine levels in one specific nucleus. We chose to position the probe in the dorsomedial region of the hypothalamus, as this region contains nuclei critical for the control of food intake and metabolism[46,47]. However, we realize that dopamine turnover may be differentially affected by obesity in distinct hypothalamic nuclei[19,48]. Our study does not exclude the possibility that HF feeding does impact on dopaminergic neurotransmission in a nucleus specific way.

In conclusion, we show here that HF feeding does not affect dopaminergic neurotransmission in the hypothalamus of a diet-sensitive mouse strain, even though it does impair insulin action. The data suggest that reduced dopaminergic neurotransmission in the hypothalamus of obese animal models and humans is due to mechanism(s) that are not directly triggered by diet composition.

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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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Judith E. de Leeuw van Weenen
Hanna E. Auvinen
Edwin T. Parlevliet
Claudia P. Coomans
Janny P. Schröder-van der Elst
Onno C. Meijer
Hanno Pijl

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

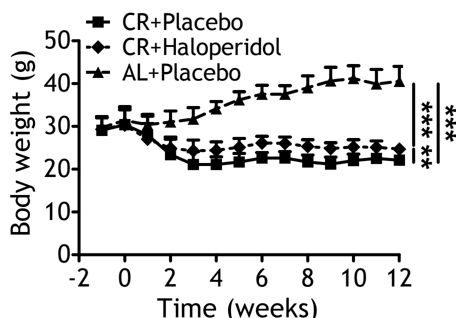


Figure 1 - Non-fasted body weight of AL and CR mice treated with placebo or haloperidol during the experiment. Data is presented as mean ± SD for 18 mice per group.
** p<0.01; *** p<0.001

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.

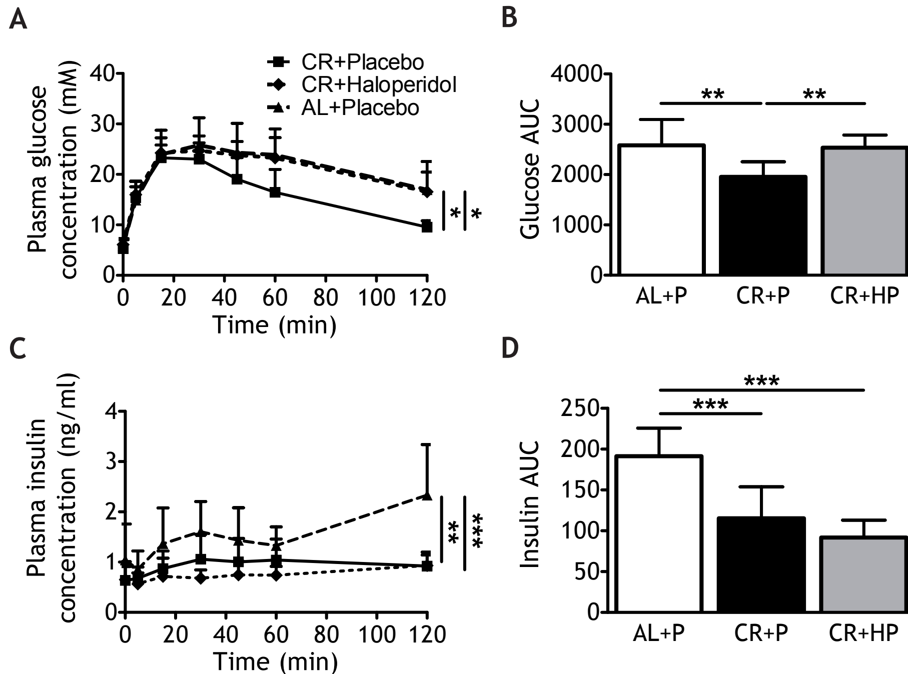


Figure 2 - Plasma glucose (A) and insulin (C) concentration during the intraperitoneal glucose tolerance test in AL and CR mice treated with placebo (P) or haloperidol (HP) for 10 weeks. Area under the glucose (B) and insulin (D) curve.
Data is presented as mean ± SD for 7 or 8 mice per group.
* $p<0.05$;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

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

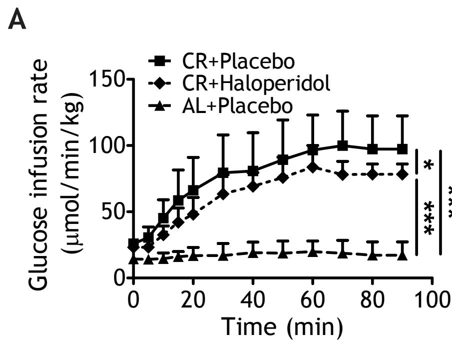
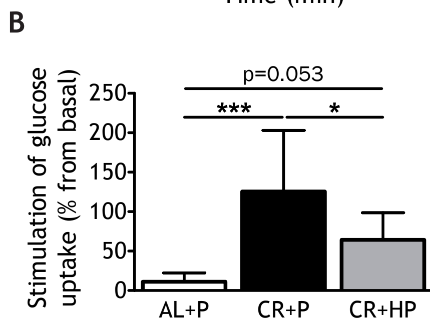
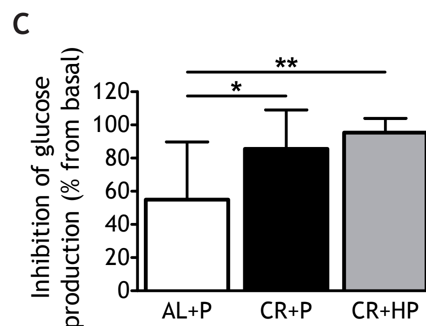


Figure 3 - Glucose infusion rate (A), stimulation of glucose uptake (B) and inhibition of glucose production (C) during a hyperinsulinemic euglycemic clamp in AL and CR mice treated with placebo (P) or haloperidol (HP) for 12 weeks.
Data is presented as mean ± SD for 7 or 8 mice per group.
* p<0.05;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

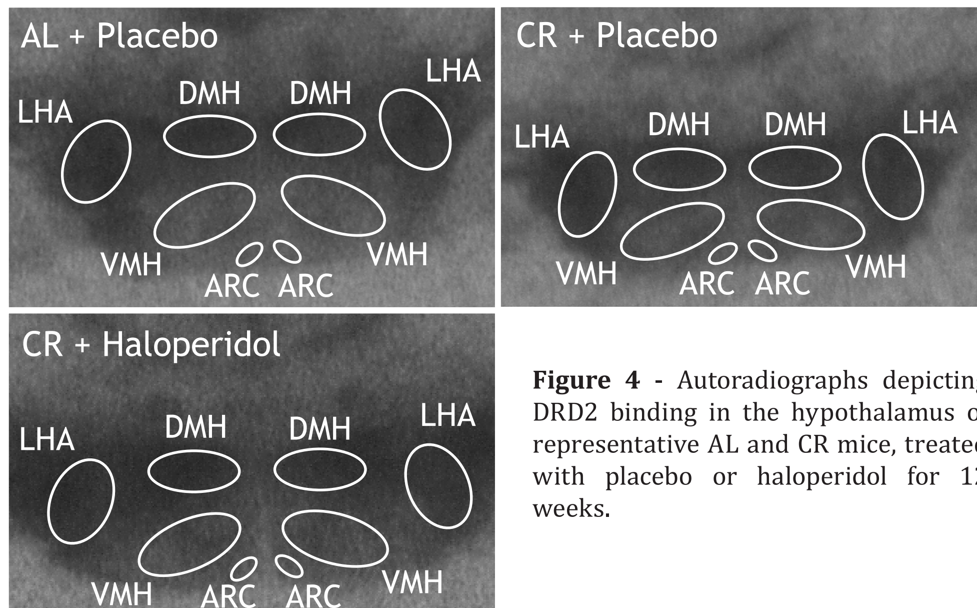


Figure 4 - Autoradiographs depicting DRD2 binding in the hypothalamus of representative AL and CR mice, treated with placebo or haloperidol for 12 weeks.

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

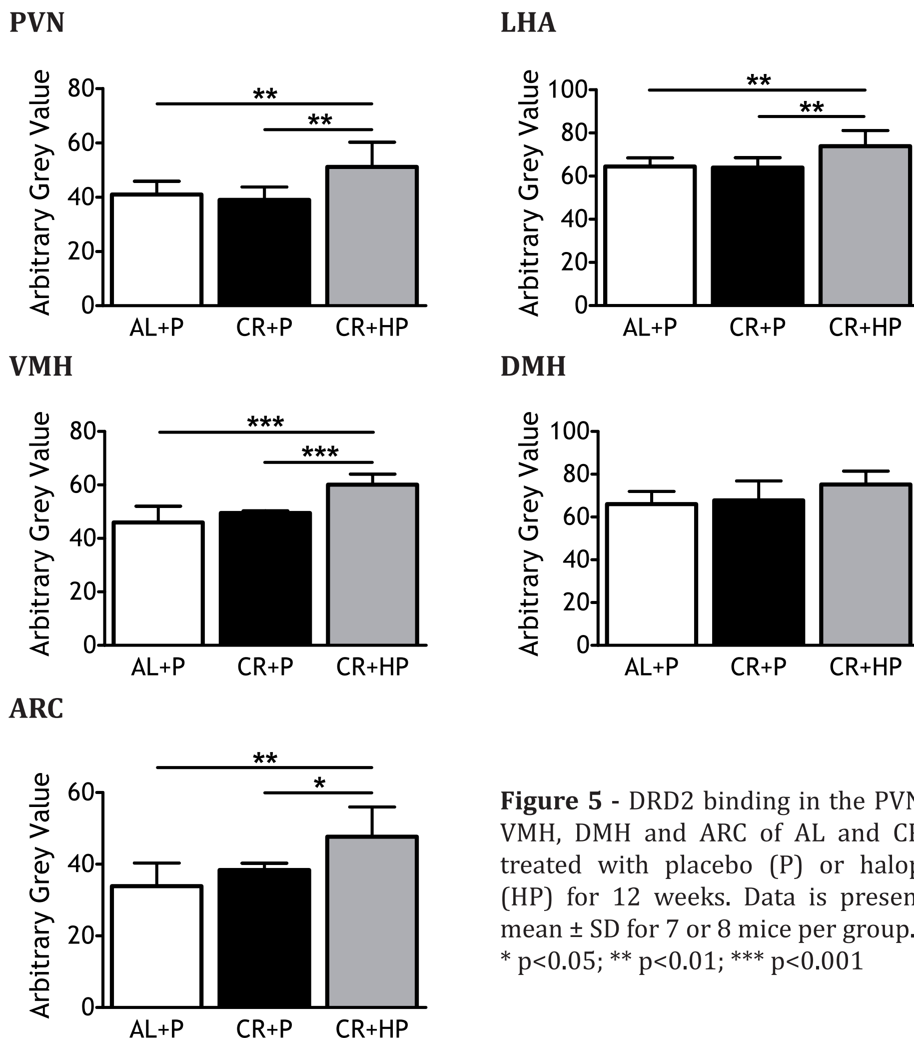


Figure 5 - DRD2 binding in the PVN, LHA, VMH, DMH and ARC of AL and CR mice treated with placebo (P) or haloperidol (HP) for 12 weeks. Data is presented as mean ± SD for 7 or 8 mice per group.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$

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.

Acknowledgements

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4

Pharmacological modulation of dopamine receptor D2 mediated transmission alters the metabolic phenotype of diet induced obese and diet resistant C57Bl6 mice

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Judith E. de Leeuw van Weenen
Edwin T. Parlevliet
Janny P. Schröder-van der Elst
Sjoerd A. van den Berg
Ko Willems van Dijk
Johannes A. Romijn
Hanno Pijl

Abstract

High fat feeding induces a variety of obese and lean phenotypes in inbred rodents. Compared to Diet Resistant (DR) rodents, Diet Induced Obese (DIO) rodents are insulin resistant and have a reduced dopamine receptor D2 (DRD2) mediated tone. We hypothesized that this differing dopaminergic tone contributes to the distinct metabolic profiles of these animals.

C57Bl6 mice were classified as DIO or DR based on their weight gain during 10 weeks of high fat feeding. Subsequently DIO mice were treated with the DRD2 agonist bromocriptine and DR mice with the DRD2 antagonist haloperidol for 2 weeks.

Compared to DR mice, the body weight of DIO mice was higher and their insulin sensitivity decreased. Haloperidol treatment reduced the voluntary activity and energy expenditure of DR mice and induced insulin resistance in these mice. Conversely, bromocriptine treatment tended to reduce body weight and voluntary activity, and reinforce insulin action in DIO mice.

These results show that DRD2 activation partly redirects high fat diet induced metabolic anomalies in obesity-prone mice. Conversely, blocking DRD2 induces an adverse metabolic profile in mice that are inherently resistant to the deleterious effects of high fat food. This suggests that dopaminergic neurotransmission is involved in the control of metabolic phenotype.

Introduction

Dopamine is intimately involved in the regulation of energy balance. Genetically engineered dopamine-deficient mice fail to initiate feeding and consequently die of starvation, unless L-DOPA, the precursor of dopamine, is provided daily[1]. Conversely, dopamine release in response to food intake induces satiety and reward[2]. Thus, dopamine plays an important dual role in the complex physiology driving meal initiation and termination. Moreover, dopaminergic neurotransmission profoundly affects glucose and lipid metabolism[3].

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 (DRD2), 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[4].

Dopaminergic transmission is altered in insulin resistant and obese animals. Basal and feeding evoked dopamine release is exaggerated in several nuclei of the hypothalamus of obese Zucker rats[5-7], whereas DRD2 expression is reduced in hypothalamic nuclei of obese animal models[8,9]. The number of DRD2 binding sites in the striatum of obese humans is reduced and inversely correlated with body mass index[10].

Modulation of DRD2 activity profoundly affects energy homeostasis in humans and animals. Drugs that block DRD2 enhance appetite and induce weight gain in animals and humans[11-14]. Conversely, DRD2 agonist drugs reduce body weight, increase energy expenditure and improve glycemic control in obese animals and individuals[15-18].

High fat feeding induces obesity, insulin resistance and diabetes in rodents. However, the amount of weight gained in response to a high fat diet varies considerably, even among animals with a genetically identical background[19-21]. Indeed, diet sensitive (diet induced obese, DIO) rodents display several alterations in pathways regulating energy homeostasis compared to diet resistant (DR) rodents[21,22], and DIO and DR rodents differ with respect to various components of their dopaminergic system, even before the onset of obesity[23,24]. In particular, DIO mice and rats are characterized by an increased expression of dopamine transporter and reduced DRD2 expression[23]. In view of the evidence summarized above, altered DRD2 mediated neurotransmission could contribute to the metabolic phenotype of these animals. We hypothesized that modulation of dopaminergic transmission in DIO and DR mice with DRD2 agonist or antagonist drugs would redirect the metabolic phenotypes of these mice. We particularly postulated that stimulation of DRD2 would ameliorate insulin resistance of DIO C57Bl6 mice, whereas DRD2 antagonism would induce insulin resistance in DR animals of the same strain. To address this hypothesis, DIO and DR mice were treated with bromocriptine, a DRD2 agonist, or

haloperidol, a DRD2 antagonist, respectively. After 1 week of treatment, energy metabolism was measured in a Comprehensive Laboratory Animal Monitoring System and after 2 weeks a hyperinsulinemic euglycemic clamp was performed to quantify insulin action, in particular with respect to its propensity to inhibit lipolysis.

Materials and Methods

Animals

Seventy-two male C57BL/6J mice (Charles River, Maastricht, The Netherlands), 11 or 12 weeks old, were housed in a temperature- and humidity-controlled room on a 12-h light–dark cycle with free access to food and water, unless mentioned otherwise.

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

All mice were maintained on a high fat 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). After 10 weeks of high fat feeding, the 24 mice with the highest weight gain were classified as DIO mice and the 24 mice with the lowest weight gain were classified as DR mice. The 24 mice with intermediate weight gain were not further used in this study.

DIO and DR mice were randomly divided into a placebo and treatment group. DR treated mice received haloperidol (1 mg/kg/day), DIO treated mice received bromocriptine (10 mg/kg/day) and DIO and DR placebo mice received placebo treatment. Subcutaneous implantable haloperidol, bromocriptine and placebo pellets (Innovative Research of America, Florida, USA), ensuring continuous release of the medication were used. Pellets were implanted under isoflurane anesthesia. Mice were treated for 2 weeks, meanwhile maintained on the high fat diet.

Measurement of energy metabolism

Mice were subjected to indirect calorimetric measurements for a period of 3 consecutive days using a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Ohio, USA). Due to a limited number of cages, eight mice per group were measured. Mice were allowed to acclimatize to the cages for a period of 14 hours prior to the start of the experiment. Measurements started at 7.00 am and continued for 72 hours. The CLAMS system enables real time continuous monitoring of food intake, drinking behavior, activity

and metabolic gas exchange. Oxygen consumption (VO_2) and carbon dioxide production rates (VCO_2) were measured at 7 minute intervals. The respiratory exchange rate (RER), as a measure for metabolic substrate choice, was calculated using the following formula:

$$RER = VCO_2/VO_2$$

Carbohydrate and fat oxidation rates were calculated from VO_2 and VCO_2 using the following formulas[25]:

$$\text{Carbohydrate oxidation (kcal/h)} = ((4.585*VCO_2)-(3.226*VO_2))*4/1000$$

$$\text{Fat oxidation (kcal/h)} = ((1.695*VO_2)-(1.701*VCO_2))*9/1000$$

VO_2 and VCO_2 are in ml/h.

Total energy expenditure was calculated as the sum of carbohydrate and fat oxidation. Activity was monitored by infrared beam breaks across the x- and y-axis. All energy metabolism data was calculated separately for day and night time.

DEXAscan

Body composition 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).

Hyperinsulinemic euglycemic clamp

Prior to the experiment, mice were fasted for 16 hours after food withdrawal at 5.00 pm. Hyperinsulinemic euglycemic clamp studies started at 9.00 am and were performed as described earlier[26]. 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 glycerol turnover was determined by giving a primed (0.6 µCi) continuous (0.9 µCi/h) intravenous (i.v.) infusion of [1-(3)-^{3}H]-Glycerol (GE Healthcare, Little Chalfont, UK) for 60 minutes. Subsequently, insulin (Novo Nordisk, Bagsværd, Denmark) was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for 90 minutes to attain a steady state circulating insulin concentration of ~6 µg/l.

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 accordingly 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 (at 50 and 60 min) and during the hyperinsulinemic period (at 70, 80, and 90 min) to determine plasma concentrations of glucose,

insulin, Non-Esterified Fatty Acids (NEFA), free glycerol and ^{3}H-Glycerol specific activities. At the end of the clamp mice were sacrificed.

Analytical procedures
Commercially available kits were used to determine the plasma concentration of glucose (Instruchemie, Delfzijl, The Netherlands), NEFA (Wako, Nuess, Germany) and free glycerol (Sigma, MO, USA). The plasma insulin concentration was measured by an ELISA (Mercodia AB, Uppsala, Sweden). Total plasma ^{3}H-Glycerol was determined in plasma and in supernatant after trichloroacetic acid (20%) precipitation and water evaporation.

Calculations
The turnover rate of glycerol (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-Glycerol (dpm/μmol). The turnover rates were corrected for body weight.

Statistical analysis
Data is presented as mean ± standard deviation. Statistical analysis was performed using SPSS. A one-way ANOVA was used for analysis of the data. If significant differences were found, the LSD method was applied as post-hoc test to determine differences between 2 groups. Statistical differences are only shown when apparent between DIO and DR placebo groups, between DIO placebo and bromocriptine groups or between DR placebo and haloperidol groups. Differences were considered statistically significant when $p<0.05$.

Results

Body weight and basal plasma metabolites
Mice were designated DIO or DR according to their weight gain following a 10-week high fat diet. By definition, DIO mice had a significantly higher body weight compared to DR mice after this dietary pre-treatment (35.4±1.5 vs. 30.6±1.9; $p<0.001$), which was completely accounted for by a difference in fat mass (fig 1B). Lean body mass did not differ (not shown). Two weeks of placebo treatment did not alter the difference in body weight between DIO and DR mice (fig 1A). Two weeks of bromocriptine treatment tended to induce weight loss in DIO mice (primarily fat mass, fig 1B), although the effect did not reach statistical significance. Haloperidol did not impact on the body weight of DR mice.

The fasting plasma glucose concentration was not different between placebo treated DIO and DR mice (fig 2A), whereas the fasting plasma insulin concentration was significantly elevated in DIO mice (fig 2B). Haloperidol significantly increased fasting plasma glucose and insulin concentrations in DR mice, while the insulin and glucose concentrations in DIO mice remained

unchanged upon bromocriptine treatment. The fasting plasma NEFA concentration didn't differ between the groups (fig 2C).

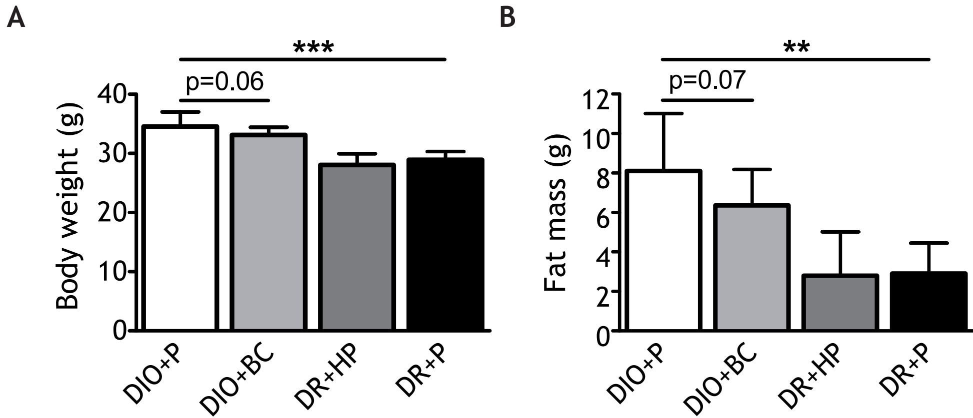

Figure 1 - Body weight (A) and fat mass (B) of DIO and DR mice after treatment with bromocriptine (BC), haloperidol (HP) or placebo (P) for 2 weeks. Data is presented as mean ± SD for 12 (A) or 10 (B) mice per group.
** $p<0.01$, *** $p<0.001$

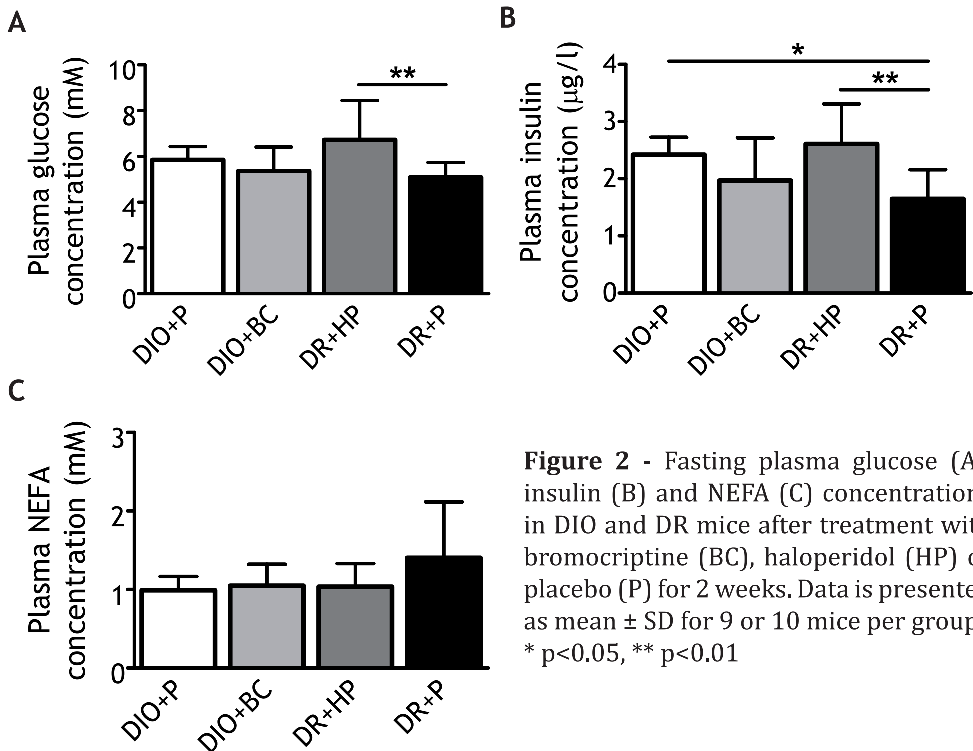

Figure 2 - Fasting plasma glucose (A), insulin (B) and NEFA (C) concentrations in DIO and DR mice after treatment with bromocriptine (BC), haloperidol (HP) or placebo (P) for 2 weeks. Data is presented as mean ± SD for 9 or 10 mice per group.
* $p<0.05$, ** $p<0.01$

Energy metabolism

After 1 week of treatment, whole body energy metabolism of mice was assessed with a Comprehensive Laboratory Animal Monitoring System using indirect calorimetry. Individual food intake, activity and respiratory gas exchange was monitored for 3 consecutive days. Cumulative food intake (fig 3A), voluntary activity (fig 3B,C), energy expenditure (fig 3D) as well as the carbohydrate oxidation rate (data not shown) did not differ between placebo treated DIO and DR mice. The diurnal fat oxidation rate tended to be higher in DIO mice, but this failed to reach statistical significance (fig 3E). Diurnal and nocturnal voluntary

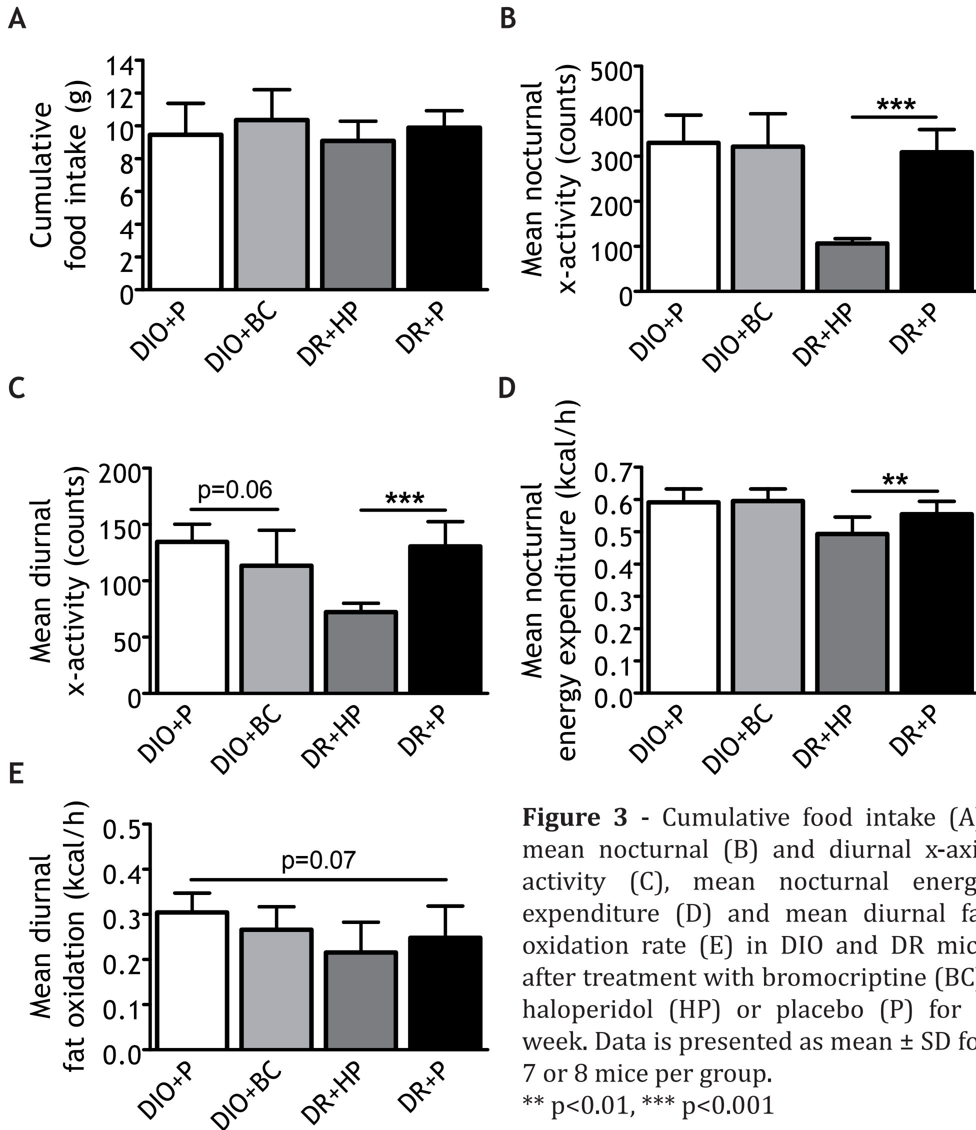

Figure 3 - Cumulative food intake (A), mean nocturnal (B) and diurnal x-axis activity (C), mean nocturnal energy expenditure (D) and mean diurnal fat oxidation rate (E) in DIO and DR mice after treatment with bromocriptine (BC), haloperidol (HP) or placebo (P) for 1 week. Data is presented as mean ± SD for 7 or 8 mice per group.
** $p<0.01$, *** $p<0.001$

activity in DR mice was dramatically reduced by haloperidol (fig 3B,C), and this was accompanied by a reduction in whole body nocturnal energy expenditure (fig 3D). The impact of haloperidol on fat (fig 3E) and carbohydrate oxidation (data not shown) did not reach statistical significance. Food intake was not affected by haloperidol treatment (fig 3A). The diurnal voluntary activity tended to be lower in DIO mice receiving bromocriptine, but this also failed to reach statistical significance (fig 3C). Furthermore, bromocriptine treatment had no significant effect on food intake (fig 3A), energy expenditure (fig 3D), fat oxidation (fig 3E) or carbohydrate oxidation (data not shown).

Insulin action

After 2 weeks of treatment, mice were subjected to a hyperinsulinemic euglycemic clamp. Basal and hyperinsulinemic plasma glucose, insulin, free glycerol and NEFA concentrations are shown in table 1. The plasma NEFA concentration was reduced to the same extent in all groups during hyperinsulinemia.

The glucose infusion rate necessary to maintain euglycemia was significantly higher in DR compared to DIO mice (fig 4), which indicates that DIO mice were insulin resistant compared to DR animals. Haloperidol significantly diminished the glucose infusion rate in DR mice, reflecting a deterioration of insulin action, whereas bromocriptine tended to increase glucose infusion required to maintain euglycemia in DIO mice (indicating improved insulin action). The capacity of insulin to inhibit glycerol turnover was not different between DR and DIO mice and it was not affected by either drug (data not shown).

Table 1 - Plasma glucose, insulin, free glycerol and NEFA concentrations during the basal and hyperinsulinemic conditions of the hyperinsulinemic euglycemic clamp in DIO and DR mice after treatment with bromocriptine, haloperidol or placebo for 2 weeks.

		DIO mice		DR mice	
Clamp condition		Placebo	Bromocriptine	Placebo	Haloperidol
Glucose (mM)	B	5.9 ± 0.6	5.3 ± 1.1	5.0 ± 0.6	6.7 ± 1.7
	HI	5.4 ± 0.7	5.6 ± 0.6	6.0 ± 0.7	4.8 ± 1.1
Insulin (μg/l)	B	2.4 ± 0.3	2.0 ± 0.8	1.6 ± 0.5	2.6 ± 0.7
	HI	6.8 ± 1.9	7.2 ± 1.4	6.7 ± 1.3	7.1 ± 0.6
Free Glycerol (mM)	B	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	HI	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
NEFA (mM)	B	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.2	1.0 ± 0.3
	HI	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.1

B, Basal; HI, Hyperinsulinemia
Data is measured in 9 or 10 mice per group and presented as mean ± SD.

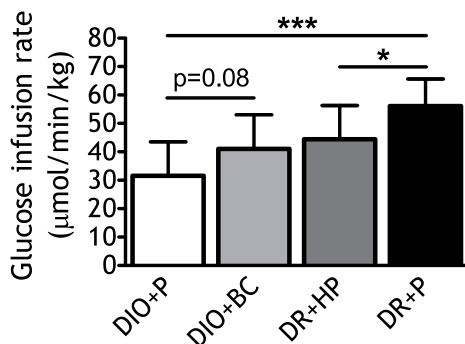

Figure 4 - Glucose infusion rate during a hyperinsulinemic euglycemic clamp in DIO and DR mice after treatment with bromocriptine (BC), haloperidol (HP) or placebo (P) for 2 weeks. Data is presented as mean ± SD for 9 or 10 mice per group. * $p<0.05$, *** $p<0.001$

Discussion

The results presented here demonstrate that pharmacological modulation of dopaminergic transmission by a DRD2 agonist or antagonist can partly redirect the divergent metabolic phenotypes of DIO and DR mice. In particular, blocking dopaminergic transmission by means of haloperidol induces insulin resistance of glucose metabolism in DR mice. Conversely, activation of dopaminergic neurotransmission by bromocriptine tends to ameliorate insulin resistance in DIO animals. These data suggest that DRD2 mediated neurotransmission is involved in the control of glucose and insulin metabolism.

Although they have a genetically identical background, individual C57Bl6 mice show distinct susceptibility to develop obesity and insulin resistance when maintained on a high fat diet. We classified mice as DIO or DR based on the amount of weight gained during 10 weeks of high fat feeding. DIO mice were insulin resistant compared to DR mice, as evidenced by higher fasting plasma insulin levels and a lower glucose infusion rate required to maintain euglycemia during insulin infusion. These findings are in accordance with other rodent studies[19-21,24,27]. Remarkably, there was no measurable difference in food intake, energy expenditure or voluntary physical activity in DIO compared to DR mice.

DIO mice have significantly lower DRD2 expression levels in certain brain areas compared to DR mice[23]. Also, dopamine turnover is reduced in hypothalamic nuclei of DIO rats even before the onset of obesity[24] and the hypothalamus is intimately involved in the control of glucose and lipid metabolism[28,29]. Since pharmacological activation of DRD2 ameliorates insulin resistance in various obese animal models[17,30], we hypothesized that modulation of DRD2 mediated neurotransmission could reverse the metabolic phenotypes of DIO and DR mice. In keeping with this hypothesis, blocking DRD2 by haloperidol induced insulin resistance in DR mice, whereas activation of DRD2 by bromocriptine tended to improve insulin sensitivity in DIO mice. In concert, these data suggest that DRD2 activation is involved in the control of glucose metabolism and that reduced dopaminergic transmission via DRD2 contributes to the metabolic

phenotype (insulin resistance) of obese animals.

However, we can not exclude the possibility that the observed effects of bromocriptine and haloperidol are (partly) mediated by receptors other than DRD2. Haloperidol is also known to have a high affinity for DRD3, DRD4 and adrenergic α1 receptors[31] and bromocriptine also possesses high affinity for DRD3, the serotonergic 5-HT1A and 1D receptors and the adrenergic α1 and α2 receptors[32]. Each of these receptors might participate in the impact of haloperidol and/or bromocriptine on energy and nutrient homeostasis. Adrenergic receptors (AR) are involved in the control of energy expenditure and glucose metabolism. Stimulation of α2-AR reduces spontaneous physical activity[33] and impairs insulin secretion[34-36]. Accordingly, overexpression of α2A-AR is associated with glucose intolerance[37]. Stimulation of α1-AR, on the other hand, has a positive impact on glucose homeostasis by promoting glucose uptake by adipose and muscle tissue[38-40] and absence of the α1B-AR leads to hyperinsulinemia and insulin resistance[41]. Acute stimulation of the 5-HT1A receptor increases food intake[42,43], reduces plasma insulin levels and induces a concomitant rise in plasma glucose levels[44,45]. As far as we know, the specific impact of DRD3, DRD4 and 5-HT1D receptors on the regulation of energy and nutrient homeostasis is still unknown. Thus, the effects of bromocriptine and haloperidol we observe here may be the ultimate result of modulation of various of these receptor activities.

The fact that haloperidol induced insulin resistance is consistent with literature reporting an increased incidence of diabetes among individuals treated with haloperidol[46]. Interestingly, treatment with haloperidol is not associated with (massive) weight gain in humans[47], which also fits with our data and suggests that the drug hampers insulin action via mechanistic routes other than obesity. First, haloperidol dramatically reduced physical activity of DR mice. This is in agreement with a wealth of data from other animal experiments[48,49]. Diminished locomotor activity hampers insulin action in muscle[50,51]. Second, a major (side) effect of haloperidol treatment is elevation of prolactin levels[52,53] which may contribute to the induction of glucose intolerance and insulin resistance[54,55]. Third, haloperidol may alter glucose metabolism by modifying plasma levels of peptide hormones. The data documenting effects of haloperidol on leptin levels are inconsistent; increased[56] as well as unchanged leptin levels in response to haloperidol treatment have been reported[57,58]. But, haloperidol seems to increase plasma ghrelin levels, while leaving levels of adiponectin, resistin and visfatin unaffected[56]. Both leptin and ghrelin may impact on insulin sensitivity directly[59,60]. Fourth, haloperidol may diminish glucose induced insulin secretion by blocking D2 receptors on pancreatic β-cells[61,62], which leads to (postprandial) hyperglycemia. In the long run, hyperglycemia diminishes insulin action through "toxic" effects on insulin sensitive tissues[63]. Fifth, blockade of central DRD2 may induce insulin resistance via modulation

of autonomic nervous output to peripheral tissues (including muscle, adipose tissue and liver)[64].

Bromocriptine treatment tended to improve insulin sensitivity of glucose metabolism in DIO animals, but its effect on glucose infusion rate did not reach statistical significance. It is important to note that the route of bromocriptine administration we used here may have diminished the efficacy of the drug. Indeed, it has been shown that subcutaneous, compared to intraperitoneal, administration of the drug limits its metabolic impact[65]. The tendency we observed though, is in line with data obtained in diet induced obese hamsters[66], and genetically engineered obese mice[67]. In accordance, short term administration of bromocriptine ameliorates various metabolic anomalies in obese humans without affecting body weight[18] and longer term treatment improves glycemic control and serum lipid profiles in patients with type 2 diabetes[68]. In addition, DRD2 agonists improve glucose and lipid metabolism in patients with hyperprolactinemia[69,70] and acromegaly[71-73]. Although DRD2 agonists generally benefit nutrient metabolism, the use of these drugs is sometimes associated with the development of impulse control disorders, including binge and compulsive eating, in patients with Parkinson's disease, which may lead to excessive weight gain and insulin resistance[74,75].

The effects of bromocriptine on metabolism may be mediated by central dopamine receptors, as is suggested by Luo et al.[17] who showed that intracerebroventricular administration of low dose bromocriptine during 14 days improves insulin sensitivity in obese, insulin resistant, hamsters. However, peripheral receptors might also be involved. We previously reported that bromocriptine acutely impairs insulin secretion by stimulating the α2-AR on β-cells[36]. To explain that (sub)chronic bromocriptine treatment improves glucose metabolism[15,66,76], we hypothesized that suppression of insulin secretion induces β-cell 'rest', which might allow β-cells to replenish insulin stores, thereby enhancing the secretory capacity in the long run[77,78]. It might also increase the number of organ specific insulin receptors leading to improved insulin sensitivity[79,80]. In addition, bromocriptine may alter glucose metabolism via modulation of circulating peptide levels. In obese women bromocriptine reduces leptin concentrations[81]; the biological relevance of this for the results reported by us is questionable however, as leptin *improves* insulin sensitivity[59]. The impact of bromocriptine on other regulatory peptide hormones remains to be determined.

In summary, activation of DRD2 tends to ameliorate the metabolic profile of DIO mice, whereas antagonism of these receptors induces insulin resistance in DR mice. In concert with previous findings by other groups indicating that dopaminergic (DRD2 mediated) neurotransmission is reduced in the brain of DIO mice, our data suggest that DRD2 mediated dopaminergic mechanisms may be involved in the development of the divergent metabolic phenotypes in response to high fat feeding in C57Bl6 mice.

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Effects of olanzapine and haloperidol on the metabolic status of healthy men

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Solrun Vidarsdottir
Judith E. de Leeuw van Weenen
Marijke Frölich
Ferdinand Roelfsema
Johannes A. Romijn
Hanno Pijl

5

Abstract

Background: A large body of evidence suggests that antipsychotic drugs cause body weight gain and type 2 diabetes mellitus, and atypical (new generation) drugs appear to be most harmful. The aim of this study was to determine the effect of short-term olanzapine (atypical antipsychotic drug) and haloperidol (conventional antipsychotic drug) treatment on glucose and lipid metabolism.

Research Design and Methods: Healthy normal weight men were treated with olanzapine (10 mg/day, n=7) or haloperidol (3 mg/day, n=7) for 8 days. Endogenous glucose production, whole body glucose disposal (by 6,6 $^2H_2$-glucose dilution), lipolysis (by $^2H_5$-glycerol dilution) and substrate oxidation rates (by indirect calorimetry) were measured before and after intervention in basal and hyperinsulinemic condition.

Results: Olanzapine hampered insulin-mediated glucose disposal (by 1.3 mg/kg/min), while haloperidol did not have a significant effect. Endogenous glucose production was not affected by either drug. Also, the glycerol rate of appearance (a measure of lipolysis rate) was not affected by either drug. Olanzapine, but not haloperidol, blunted the insulin-induced decline of plasma free fatty acid and triglyceride concentrations. Fasting free fatty acid concentrations declined during olanzapine treatment, while they did not during treatment with haloperidol.

Conclusions: Short-term treatment with olanzapine reduces fasting plasma free fatty acid concentrations and hampers insulin action on glucose disposal in healthy men, whereas haloperidol has less clear effects. Moreover, olanzapine, but not haloperidol, blunts the insulin-induced decline of plasma free fatty acid and triglyceride concentrations. Notably, these effects come about without a measurable change of body fat mass.

Introduction

Typical antipsychotic drugs (AP) have been the cornerstone of the medical management of patients with schizophrenia for a long time. The advent of atypical AP drugs has brought clear benefits for schizophrenic patients, as these compounds have less extrapyramidal side effects and ameliorate negative symptoms[1]. However, a large body of evidence suggests that the use of these drugs is associated with obesity[2,3] and diabetes mellitus[4]. Several studies have looked at the metabolic effects of AP drugs in non-diabetic schizophrenic patients. The results consistently show that these drugs induce (euglycemic) hyperinsulinemia and impaired glucose tolerance[5,6]. Treatment with atypical AP drugs appears to be more harmful for glucose/lipid metabolism than treatment with conventional AP drugs[5,7].

As obesity is a major risk factor for insulin resistance and type 2 diabetes[8], it is tempting to postulate that weight gain induced by atypical AP drugs is primarily responsible for their unfavourable impact on these pathologies. However, this does not appear to be the case in studies evaluating this possibility[2,9]. Moreover, in a review of case reports, diabetes often developed after a short treatment period, in some cases without significant weight gain[10]. The metabolic profile often improved upon drug discontinuation, while re-challenge with the same drug resulted in recurrence of hyperglycemia[10]. Thus, AP drugs may act directly to induce insulin resistance and diabetes. Atypical AP drugs antagonize a broad range of monoamine neurotransmitter receptors. In addition to their relatively week affinity for dopamine D2 receptors, they have a strong affinity for serotonin 5-HT2, histamine H1, α1 adrenergic, and muscarinic M3 receptors, while typical AP drugs particularly antagonize dopamine D2 receptors. Indeed, various neurotransmitters whose signals are blocked by atypical but not typical AP drugs, are involved in the control of glucose metabolism[11-15], which could mechanistically explain direct actions of olanzapine on insulin sensitivity.

We hypothesized that short-term treatment with AP drugs induces insulin resistance through a mechanistic route that is independent of weight gain and that atypical drugs exert stronger effects than typical compounds in this respect. To evaluate this hypothesis, we treated healthy non-obese men with olanzapine (atypical AP) or haloperidol (typical AP) for 8 days, and studied the impact of these interventions on glucose and lipid metabolism by hyperinsulinemic euglycemic clamp, isotope dilution technology and indirect calorimetry.

Subjects and Methods

Subjects

Fourteen healthy men between 20 and 40 years were recruited through advertisements in local newspapers. Subjects were required to have a normal

weight, normal fasting plasma glucose concentration (<6.0 mmol/l) and normal physical examination. Subjects who had ever used antipsychotic medication, and subjects who where currently smoking or using medication affecting the central nervous system were excluded. Subjects who dropped out (because of side effects) were replaced by other volunteers. All subjects provided written informed consent after the study procedures and possible adverse effects of the treatment had been explained. The protocol was approved by the medical ethics committee of the Leiden University Medical Center.

Clinical protocol

Subjects underwent a hyperinsulinemic euglycemic clamp at baseline and on the last day (day 8) of treatment with either olanzapine (10 mg once daily) or haloperidol (3 mg once daily). The drugs were taken at 8.00 am. The drug doses prescribed are in the low range of doses used for the treatment of patients with schizophrenia. On both study days, substrate oxidation was measured by indirect calorimetry (Oxycon ß; Jaeger Toennies, Breda, The Netherlands) in basal (after a 10 hr overnight fast) and hyperinsulinemic conditions. Body fat percentage was determined by bioelectrical impedance analysis (Bodystat® 1500, Bodystat Limited, Dougles, Isle of Man, UK). Body mass index (weight/length^2) and waist/hip circumference were measured according to WHO recommendations. The subjects were asked to refrain from vigorous physical exercise for one week before each clamp. When the study drug was not tolerated, treatment was discontinued. Food intake was not monitored.

Hyperinsulinemic euglycemic clamp

[6,6-$^2H_2$]-glucose was infused in the basal state and during a hyperinsulinemic euglycemic clamp to determine the effect of insulin on peripheral glucose disposal and endogenous glucose production. Lipolysis was monitored by a primed continuous infusion of [$^2H_5$]-glycerol. At 7.30 am, after an overnight (10 h) fast, subjects were admitted to the clinical research unit and asked to lie down in a semi-recumbent position. An i.v. catheter was placed in an antecubital vein for infusions. Another catheter was placed in the contra-lateral hand for blood sampling. This hand was placed in a heated box (60°C) to obtain arterialised venous blood samples.

The subjects were asked to take their last drug dose at 8.00 am. Thereafter, basal blood samples for glucose, insulin, FFA, lipid spectrum and background isotope enrichment of [6,6-$^2H_2$]-glucose and [$^2H_5$]-glycerol were taken. At t=0, a primed (26.4 μmol/kg) continuous (0.33 μmol/kg/min) infusion of [6,6-$^2H_2$]-glucose (enrichment 99.9%; Cambridge Isotopes, Cambridge, MA, USA) was started and continued throughout the clamp (4 h) to monitor glucose metabolism. At 9.00 am (t=60), a primed (1.6 μmol/kg) continuous (0.11 μmol/kg/min) infusion of [$^2H_5$]-glycerol (Cambridge Isotopes) began and continued

throughout the clamp (3 h) to monitor lipolysis.

At t=90-120 min, 4 blood samples were taken with 10 minute intervals for determination of plasma glucose, insulin, glycerol, and enrichment of [6,6-$^{2}H_{2}$]-glucose and [$^{2}H_{5}$]-glycerol. Subsequently (t=120), a primed continuous (40 mU/m^{2}/min) infusion of insulin (Actrapid, Novo Nordisk Pharma BV, Alphen aan de Rijn, The Netherlands) was started. Insulin was infused for 2 h. Blood glucose concentrations were measured every 5 minutes, and a variable infusion of 20% glucose (enriched with 3% [6,6-$^{2}H_{2}$]-glucose) was adjusted to maintain a stable blood glucose concentration (~5.0 mmol/l). By the end of the hyperinsulinemic clamp (t=210-240), blood was drawn every 10 minutes for determination of plasma glucose, insulin, glycerol, and enrichment of [6,6-$^{2}H_{2}$]-glucose and [$^{2}H_{5}$]-glycerol. Indirect calorimetry was performed for determination of resting energy expenditure, respiratory quotient (RQ), glucose and fat oxidation in basal condition (t=60-90) and during hyperinsulinemia (t=180-210).

Assays

Each tube, except the serum tubes, was immediately chilled on ice. Samples were centrifuged at 4000 rpm at 4° C for 20 min. Subsequently, plasma was divided into separate aliquots and frozen at –80° C until assays were performed.

Serum glucose, total cholesterol (TC) and HDL-cholesterol were measured in the laboratory for Clinical Chemistry at the Leiden University Medical Center, using a fully automated Hitachi Modular P800 system. LDL-cholesterol was measured with COBAS INTEGRA 800 (Roche Diagnostics, Mannheim, Germany).

Serum insulin was measured by immuno-radiometric assay (INS-IRMA; BioSource Europe S.A., Nivelles, Belgium) and serum glucagon was measured by radioimmunoassay (RIA; Medgenix, Fleurus, Belgium). Serum prolactin (PRL) concentrations were measured with a sensitive time-resolved fluoroimmunoassay with a detection limit of 0.04 μg/l (Delfia, Wallac Oy, Turku, Finland).

Plasma levels of free fatty acids (FFA) and triglycerides (TG) were determined using commercially available kits (Wako Pure Chemical Industries, Osaka, Japan and Roche Diagnostics).

Glucose and [6,6-$^{2}H_{2}$]-glucose enrichment as well as glycerol and [$^{2}H_{5}$]-glycerol enrichment were determined in a single analytical run, using gas chromatography coupled to mass spectrometry (Hewlett-Packard, Palo Alto, CA, USA) as previously described[16,17].

Calculations

In isotopic steady state condition, the rate of glucose disappearance (Rd) equals the rate of glucose appearance (Ra). Ra, which represents endogenous glucose production (EGP), was calculated by dividing the [6,6-$^{2}H_{2}$]-glucose infusion rate (mg/min) by the steady state plasma [6,6-$^{2}H_{2}$]-glucose tracer/tracee ratio.

During insulin infusion, Rd was calculated by adding the rate of exogenous glucose infusion to the Ra. The Ra of glycerol was calculated by dividing the [$^{2}H_{5}$]-glycerol infusion rate (µmol/min) by the steady-state plasma [$^{2}H_{5}$]-glycerol tracer/tracee ratio. Total lipid and carbohydrate oxidation rates were calculated as previously described[18]. Data are expressed per kilogram body weight.

Statistical analysis

The study was powered to detect a difference in glucose infusion rate before and after treatment with either drug. Eight subjects per group allowed detection of a 30% difference with 80% power at a 2-sided significance level of 0.05. Data is presented as mean ± standard error of the mean. Data were logarithmically transformed when appropriate. Comparisons were made within groups with two-tailed dependent Student's t-test. To compare the effect of olanzapine and haloperidol treatment (between groups) an independent Student's t-test was used; the difference of the values before and after each intervention was compared. When the distribution of data was not normal after logarithmic transformation they were analysed using non-parametric Wilcoxon signed-rank test. Significance level was set at 0.05. All analyses were performed using SPSS for Windows, version 12.0 (SPSS Inc, Chicago, IL, USA).

Results

Subjects, anthropometric measures and plasma metabolites

Fourteen subjects were included in the study. Four subjects discontinued haloperidol treatment: 1 subject because of a vasovagal reaction when basal blood samples where taken at the first study day; 3 subjects because of the occurrence of side effects. Of those subjects, 2 subjects had acute dystonia, which was treated with anticholinergic drugs (Akineton® i.m.) and 1 subject discontinued treatment because of restlessness. All of these subjects were replaced by other volunteers. None of the subjects using olanzapine had major side effects. Five were somewhat drowsy during the first day of treatment only. The father of one subject in the haloperidol group was of Mediterranean origin (ethnicity may have impact on insulin sensitivity); all other subjects were of Caucasian origin. In the haloperidol group one subject had a father with type 2 diabetes and in the olanzapine group one subject had a second degree family member with type 2 diabetes.

Table 1 summarizes anthropometric measurements and biochemical parameters in fasting condition on day 0 and day 8 in both groups. Baseline characteristics, including risk factors for insulin resistance (i.e. anthropometrics, ethnicity, family history of type 2 diabetes, fasting insulin and glucose levels), did not differ between the treatment groups. Body weight and waist-hip ratio did not change from day 0 to day 8 in either group. Fat percentage decreased

Table 1 - Subject characteristics; before and after treatment with olanzapine or haloperidol.

	Olanzapine (n=7)		Haloperidol (n=7)	
	Day 0	Day 8	Day 0	Day 8
Age (yr)	25.7 ± 1.3		23.7 ± 1.3	
Body weight (kg)	76.7 ± 3.4	77.4 ± 3.3	76.8 ± 2.2	76.6 ± 2.2
BMI (kg/m^2)	22.3 ± 0.7	22.5 ± 0.6	22.9 ± 0.8	22.8 ± 0.9
WHR	0.82± 0.02	0.83 ± 0.02	0.79 ± 0.02	0.80 ± 0.02
Fat (%)	9.1 ± 0.7	9.3 ± 1.2	10.6 ± 1.3	8.9 ± 1.5*
Glucose (mmol/l)	4.9 ± 0.2	5.1 ± 0.2	5.1 ± 0.1	4.9 ± 0.1
Insulin (mU/l)	10.2 ± 1.2	11.5 ± 1.9	8.3 ± 0.8	7.6 ± 0.6
Glucagon (pg/ml)	54.3 ± 4.7	68.5 ± 6.6*	50.9 ± 5.2	53.9 ± 4.3
Prolactin (μg/l)	9.0 ± 1.9	16.3 ± 3.1**	8.8 ± 1.5	15.2 ± 1.9*
TG (mmol/l)	1.22 ± 0.20	1.33 ± 0.18	1.16 ± 0.12	1.32 ± 0.30
FFA (mmol/l)	0.58 ± 0.10	0.43 ± 0.10*	0.51 ± 0.08	0.53 ± 0.08
Total cholesterol (mmol/l)	4.3 ± 0.3	4.2 ± 0.2	3.8 ± 0.3	3.9 ± 0.2

BMI, body mass index; FFA, free fatty acids; TG, triglycerides; WHR, waist hip ratio. Values are expressed as mean ± SEM.
* $p < 0.05$, ** $p < 0.01$ vs baseline

slightly during treatment with haloperidol. Fasting plasma insulin and glucose levels did not change during treatment in either group. FFA concentrations significantly declined during olanzapine treatment (p=0.03). This effect did not differ significantly from the effect of haloperidol treatment. Serum glucagon concentrations were significantly elevated by olanzapine treatment, but the difference with the effect of haloperidol did not reach statistical significance. Plasma prolactin concentrations were increased during treatment in both groups (olanzapine p=0.002 and haloperidol p=0.01), which indicates that the drugs were properly taken.

Endogenous glucose production and whole body glucose disposal

Basal condition

Serum glucose and insulin concentrations in basal condition did not change in response to either treatment (table 1). Accordingly, endogenous glucose production was not affected by olanzapine or haloperidol (table 2 and figure 1).

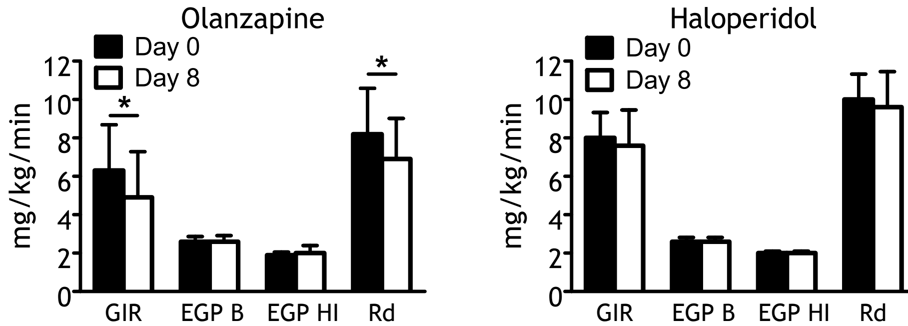

Figure 1 - GIR, EGP basal (B), EGP hyperinsulinemia (HI) and glucose disposal (Rd) before and after 8-d treatment with olanzapine and haloperidol. Values are expressed as mean ± SD, n=7 per group.
* $p < 0.05$

Hyperinsulinemic euglycemic clamp

Data on glucose metabolism during insulin infusion is shown in table 2. Serum glucose concentrations were clamped at similar levels on both study days. Also, plasma insulin concentrations during insulin infusion were in the postprandial range and similar on both days (table 2). Background enrichment of 6.6-$^2H_2$ glucose (% of total glucose) was similar on both study occasions (olanzapine day 0: 1.33 x 10^{-2} ± 0.07 x10^{-2}, day 8: 1.29 x 10^{-2} ± 0.04 x 10^{-2}; haloperidol day 0: 1.31 x 10^{-2} ± 0.02 x 10^{-2}, day 8: 1.32 x 10^{-2} ± 0.03 x 10^{-2}).

The glucose infusion rate (GIR) required to maintain euglycemia during hyperinsulinemia was reduced after olanzapine treatment (figure 1). Although haloperidol did not affect the GIR to a significant extent, the magnitude of its effect did not differ significantly from that of olanzapine. The capacity of insulin to suppress endogenous glucose production (EGP) was not affected by either treatment (figure 1). Glucose disposal during hyperinsulinemia was significantly blunted by olanzapine treatment (figure 1). Again, although haloperidol did not affect glucose disposal to a significant extent, the magnitude of its effect did not differ significantly from that of olanzapine.

Lipid metabolism

Basal condition

In fasting condition plasma FFA concentrations significantly decreased during olanzapine treatment, and this effect did not differ from that of haloperidol despite the fact that haloperidol's impact did not reach statistical significance. Fasting TG concentrations (table 1) and basal glycerol Ra (table 2) were not affected by either drug.

Table 2 - Metabolic variables during hyperinsulinemic euglycemic clamp.

	Olanzapine (n=7)		Haloperidol (n=7)	
	Day 0	Day 8	Day 0	Day 8
Glucose (mmol/l)	4.6 ± 0.2	4.5 ± 0.4	5.1 ± 0.1	4.9 ± 0.1
Insulin (mU/l)	71.6 ± 2.3	72.6 ± 2.8	61.6 ± 5.8	61.2 ± 5.7
Background enrichment of 6.6-$^2H_2$ glucose (% of total glucose)	$1.33 \times 10^{-2} \pm 0.07 \times 10^{-2}$	$1.29 \times 10^{-2} \pm 0.04 \times 10^{-2}$	$1.31 \times 10^{-2} \pm 0.02 \times 10^{-2}$	$1.32 \times 10^{-2} \pm 0.03 \times 10^{-2}$
GIR (mg/kg/min)	6.3 ± 0.9	4.9 ± 0.9*	8.0 ± 0.5	7.6 ± 0.7
Glucose disposal (mg/kg/min)	8.2 ± 0.9	6.9 ± 0.8*	10.0 ± 0.5	9.6 ± 0.7
EGP basal (mg/kg/min)	2.6 ± 0.10	2.6 ± 0.12	2.6 ± 0.08	2.6 ± 0.08
EGP hyperinsulinemia (mg/kg/min)	1.9 ± 0.06	2.0 ± 0.15	2.0 ± 0.04	2.0 ± 0.04
EGP % inhibition	25.9 ± 1.8	21.0 ± 4.7	21.6 ± 2.0	20.1 ± 2.3
Ra glycerol basal (µmol/kg/min)	2.5 ± 0.27	2.5 ± 0.30	3.0 ± 0.26	3.2 ± 0.24
Ra glycerol hyperinsulinemia (µmol/kg/min)	2.1 ± 0.10	2.1 ± 0.14	2.1 ± 0.17	2.0 ± 0.22
Ra glycerol % decline	16.4 ± 5.9	15.4 ± 7.1	28.2 ± 8.1	35.2 ± 6.8
FFA % decline	83.2 ± 2.2	65.3 ± 6.9*	81.4 ± 2.6	84.7 ± 2.4
TG % decline	17.0 ± 3.6	8.1 ± 3.6**	19.4 ± 6.2	28.2 ± 3.9

EGP, endogenous glucose production; EGP % inhibition, decline of EGP during hyperinsulinemia expressed as percentage of basal value; FFA % decline, decline of circulating FFA during hyperinsulinemia expressed as percentage of basal value; GIR, glucose infusion rate; Ra, rate of appearance; TG % decline, decline of circulating TG during hyperinsulinemia expressed as percentage of basal value. Values are expressed as mean ± SEM.
* $p < 0.05$, ** $p < 0.01$ vs day 0

Table 3 - Fuel oxidation before and after treatment with olanzapine or haloperidol.

	Olanzapine (n=7)		Haloperidol (n=7)	
	Day 0	Day 8	Day 0	Day 8
RQ				
B	0.83 ± 0.015	0.87 ± 0.026	0.82 ± 0.011	0.83 ± 0.012
HI	0.86 ± 0.020	0.92 ± 0.034	0.86 ± 0.018	0.86 ± 0.017
Glucose oxidation (mg/kg/min)				
B	1.92 ± 0.25	2.62 ± 0.52	1.85 ± 0.18	2.07 ± 0.18
HI	2.92 ± 0.37	3.60 ± 0.68	2.63 ± 0.28	2.87 ± 0.28
Lipid oxidation (mg/kg/min)				
B	1.04 ± 0.09	0.76 ± 0.14	1.06 ± 0.07	1.04 ± 0.09
HI	0.83 ± 0.11	0.48 ± 0.20	0.94 ± 0.12	0.93 ± 0.14
REE (kcal/day)				
B	1344.3 ± 36.4	1351.7 ± 79.9	1303.9 ± 35.3	1341.8 ± 26.6
HI	1382.7 ± 38.3	1457.1 ± 64.9	1430.6 ± 54.0	1493.1 ± 56.1

B, basal; HI, hyperinsulinemia; RQ, respiratory quotient; REE, resting energy expenditure. Values are expressed as mean ± SEM.

Hyperinsulinemic euglycemic clamp.
Data on lipid metabolism during insulin infusion is shown in table 2. Insulin significantly suppressed the glycerol Ra in the haloperidol treated group (p=0.028 on day 0 and p=0.018 on day 8) , but not in the olanzapine treated group (p=0.071 on day 0 and p=0.379 on day 8). During hyperinsulinemia, the decline of circulating FFA and TG levels, expressed as percentage of basal value, was significantly blunted by olanzapine, whereas the decline of circulating FFA and TG was not affected by treatment with haloperidol. Thus, the propensity of olanzapine to blunt the decline of circulating FFA and TG by hyperinsulinemia differed significantly from the effect of haloperidol.

Glucose and lipid oxidation rate
Table 3 provides an overview of the effects of both drugs on substrate oxidation. Resting energy expenditure, RQ, and lipid and glucose oxidation rate were not affected by either drug.

Discussion

To establish the early effects of antipsychotic drugs on glucose and lipid metabolism, we treated healthy young men with 10 mg olanzapine or 3 mg

haloperidol once daily for only 8 days. Olanzapine significantly reduced the glucose infusion rate required to maintain euglycemia during insulin infusion, indicating that the drug induces whole body insulin resistance. Specifically, olanzapine reduced insulin mediated glucose disposal, whereas it did not affect insulin's capacity to suppress EGP. These effects did not differ from those of haloperidol to a significant extent, although the glucose infusion rate and disposal during haloperidol treatment were not significantly different from baseline. Olanzapine also curtailed the decline of circulating FFA and TG during hyperinsulinemia, whereas it did not affect the glycerol rate of appearance or the ability of insulin to inhibit this measure of the rate of lipolysis. Notably, these metabolic effects occurred without a measurable effect on body weight or body fat mass, although the waist circumference increased slightly in response to olanzapine treatment. In clear contrast, haloperidol did not affect the insulin-induced decline of FFA and TG concentrations.

Effects on glucose metabolism

These data indicate that olanzapine hampers insulin action on glucose disposal, while the effect of haloperidol was less clear. This inference is consistent with data from large epidemiological studies[19-21], showing that patients treated with atypical antipsychotic drugs are more likely to develop diabetes mellitus than patients treated with typical AP drugs. Also in line with our data, Newcomer et al[7] reported that schizophrenic patients treated with olanzapine are more insulin resistant than patients treated with typical AP drugs, as estimated by i.v. glucose tolerance test. Relatively few studies have looked at the metabolic effects of AP drugs in healthy subjects. Sowell et al[22] assessed meal tolerance and insulin sensitivity, using a 2-step hyperinsulinemic euglycemic clamp and a mixed meal tolerance test (MMTT), in normal subjects after 3 weeks of olanzapine (10 mg/day; n=22), risperidone (4 mg/day; n=14) or placebo (n=19) treatment. The glucose infusion rate required to maintain euglycemia during hyperinsulinemia was not affected by either treatment, suggesting that the drugs did not impact on insulin action. However, treatment with olanzapine significantly increased fasting insulin and glucose levels, while treatment with risperidone or placebo did not. Also, there was a significant increase of the glucose area under the plasma concentration curve in response to the MMTT in the group treated with olanzapine. These data are quite difficult to reconcile. Moreover, glucose disposal and EGP were not determined in this study. In full agreement with our data, 10 days of olanzapine treatment was recently reported to decrease the glucose infusion rate required to maintain euglycemia in healthy men[23]. EGP and glucose disposal were not determined in this study.

The (sub)acute nature of the inhibitory impact of olanzapine treatment on glucose disposal is consistent with clinical data indicating that atypical AP drugs can induce hyperglycemia within a couple of weeks, before significant weight

gain has occurred[10]. Moreover, it corroborates papers reporting that proximate measures of insulin resistance do not correlate with BMI in schizophrenic patients treated with atypical AP drugs[2,9]. Also, Dwyer et al.[24] reported that atypical AP drugs acutely (<3 h) induce hyperglycemia in mice, while typical AP drugs do not. The ability of these medications to induce hyperglycemia *in vivo* was tightly correlated with their effect on glucose transport in pheochromocytoma (PC12) cells *in vitro*[24]. However, PC12 cells do not express the GLUT4 transporter, which is abundant in muscle and responsive to insulin[25], and the concentration of drugs required to block glucose uptake in these cell systems is generally very high[26]. Thus, although clozapine and fluphenazine were shown to also block glucose transport in a rat muscle cell line *in vitro*[27], the relevance of these findings for the mechanistic explanation of our data remains uncertain.

Alternatively, our observations may be explained by the distinct receptor affinity profiles of olanzapine and haloperidol. Haloperidol particularly antagonizes dopamine D2 receptors, whereas olanzapine also blocks serotonin 5-HT2, histamine H1, α1 adrenergic, and muscarinic M3 receptors[28]. Activation of all of these receptor (sub)types, including the dopamine D2 receptor[29], generally inhibits food intake, reduces body weight and/or enhances insulin secretion[30-33]. Notably, various receptors blocked by olanzapine appear to be directly (i.e. independent of their effects on body weight) involved in the regulation of glucose metabolism. Indeed, imipramine induces hyperglycemia in mice by blocking 5-HT2 receptors[14], and a single dose of ketanserin, a 5-HT2A receptor antagonist, impairs insulin action on glucose metabolism in healthy humans[12]. Blocking H1 receptors in cardiac muscle tissue impairs glucose uptake[15], whereas, in apparent contradiction, activation of H1 receptors in the brain acutely elevates plasma glucose levels[34]. Thus, the H1 receptor has multiple, apparently opposite roles in the control of glucose metabolism. Activation of dopamine D2 receptors ameliorates insulin resistance in obese women through a mechanism that is independent of body weight[13] and D2 receptor binding sites are reduced in the brain of obese animal models and humans[29]. Finally, α1-adrenergic receptor knock out mice are glucose intolerant[11] and α1-adrenergic receptors stimulate glucose uptake in muscle cells[35]. Thus, antagonism of either one of these receptors, alone or in combination, by olanzapine may hamper insulin action and explain our findings.

Effects on lipid metabolism

Neither drug affected insulin's capacity to suppress lipolysis. Olanzapine, but not haloperidol decreased FFA concentrations in fasting condition (although group differences did not reach statistical significance). Moreover, it curtailed the decline of circulating FFA and TG concentrations during hyperinsulinemia, which indeed clearly differed from the effect of haloperidol. In agreement with

our findings, olanzapine was shown to reduce FFA concentration in a recent comprehensive evaluation of lipid changes in schizophrenia[36]. The cause of these changes in lipid metabolism remains to be established. We speculate that olanzapine inhibits lipoprotein lipase (LPL) activity in muscles and impairs the stimulatory action of insulin on LPL in adipose tissue. LPL hydrolyses the triacylglycerol component of circulating lipoprotein particles, chylomicrons and very low density lipoprotein, to provide FFA for tissue utilisation. In fasting condition, LPL is active in muscle and inhibited in adipose tissue, whereas (postprandial) hyperinsulinemia stimulates LPL in adipose tissue and inhibits LPL activity in muscle[37,38]. Reduced LPL activity in muscle may therefore reduce plasma FFA concentrations and impair fatty acid oxidation in fasting condition. Reduced LPL activity in adipose tissue would explain the blunted decline of plasma FFA and TG during hyperinsulinemia. Inhibition of LPL activity could either result from direct effects of the drug or be secondary to its effect on circulating prolactin levels. Hyperprolactinemia has been reported to inhibit LPL activity in adipose tissue in humans[39] and rodents[40].

In aggregate, these data suggest that olanzapine impairs insulin action on glucose and lipid disposal in muscle and adipose tissue, whereas it does not affect insulin's capacity to inhibit glucose production or lipolysis. Notably, these are early metabolic effects of olanzapine, which occur without a measurable change of body fat mass. Our findings may explain the property of olanzapine to induce dyslipidemia and diabetes mellitus in the long term. Short term haloperidol treatment does not appear to affect lipid metabolism, which corroborates the notion that typical antipsychotic drugs are less harmful in a metabolic context.

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The dopamine receptor D2 agonist bromocriptine inhibits glucose-stimulated insulin secretion by direct activation of the α2-adrenergic receptors in beta cells

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Judith E. de Leeuw van Weenen
Edwin T. Parlevliet
Pierre Maechler
Louis M. Havekes
Johannes A. Romijn
D. Margriet Ouwens
Hanno Pijl
Bruno Guigas

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Abstract

Treatment with the dopamine receptor D2 (DRD2) agonist bromocriptine improves metabolic features in obese patients with type 2 diabetes by a still unknown mechanism. In the present study, we investigated the acute effect of bromocriptine and its underlying mechanism(s) on insulin secretion both in vivo and in vitro.

For this purpose, C57Bl6 mice were subjected to an intraperitoneal glucose tolerance test (ipGTT) and a hyperglycemic (HG) clamp 60 min after a single injection of bromocriptine or placebo. The effects of bromocriptine on glucose-stimulated insulin secretion (GSIS), cell membrane potential and intracellular cAMP levels were also determined in INS-1E beta cells.

We report here that bromocriptine increased glucose levels during the ipGTT in vivo, an effect associated with a dose-dependent decrease in GSIS. During the HG clamp, bromocriptine reduced both first-phase and second-phase insulin response. This inhibitory effect was also observed in INS-1E beta cells, in which therapeutic concentrations of bromocriptine (0.5-50 nM) decreased GSIS. Mechanistically, neither cellular energy state nor cell membrane depolarization was affected by bromocriptine, whereas intracellular cAMP levels were significantly reduced, suggesting involvement of G-protein-coupled receptors. Surprisingly, the DRD2 antagonist domperidone did not counteract the effect of bromocriptine on GSIS, whereas yohimbine, an antagonist of the α2-adrenergic receptor, completely abolished bromocriptine-induced inhibition of GSIS.

In conclusion, acute administration of bromocriptine inhibits GSIS by a DRD2-independent mechanism involving direct activation of the pancreatic α2-adrenergic receptors. We suggest that treatment with bromocriptine promotes beta cells rest, thereby preventing long-lasting hypersecretion of insulin and subsequent beta cell failure.

Introduction

Type 2 diabetes, which is often associated with obesity and dyslipidemia, is characterized by insulin resistance, glucose intolerance and a progressive deterioration of beta cell mass and function[1]. The central and peripheral dopaminergic system is involved in the regulation of whole-body fuel and energy homeostasis[2,3]. Besides its role in the control of complex processes driving feeding behavior, dopaminergic neurotransmission affects both glucose and lipid metabolism. Alterations of this central regulatory pathway have been reported in insulin-resistant rodent models[4-6] and patients with type 2 diabetes[7,8].

Dopamine action is mediated by 5 distinct G-protein coupled receptor subtypes belonging to 2 receptor families according to their effect on target neurons. Activation of dopamine receptors D2, D3 or D4, classified as the D2 family, inhibits adenylyl cyclase activity and cyclic AMP (cAMP) synthesis[9]. By contrast, activation of the receptors belonging to the D1 family (DRD1 and DRD5) leads to stimulation of adenylyl cyclase and cAMP generation[9].

Modulation of DRD2 activity profoundly affects energy homeostasis. Indeed, antipsychotic drugs antagonizing DRD2 enhance appetite and body weight gain in both animals[10] and humans[11]. Conversely, the DRD2 agonist bromocriptine, a semi-synthetic ergot alkaloid used in the treatment of Parkinson's disease and hyperprolactinemia, exerts opposite beneficial effects on energy homeostasis. For instance, prolonged administration of bromocriptine normalizes elevated plasma glucose and insulin concentrations and improves both glucose tolerance and insulin sensitivity in obese insulin-resistant rodents[12,13] and humans[14,15]. Furthermore, long-term treatment with bromocriptine improves pancreatic beta cell function and increases the islet insulin content in obese insulin-resistant mice[12,16,17]. However, the underlying mechanism(s) associated with these beneficial effects remains poorly understood. Interestingly, it has been recently demonstrated that all dopaminergic receptor subtypes are widely expressed on pancreatic beta cells, suggesting that at least some of the metabolic effects of bromocriptine could result from direct interaction with DRD2 on beta cells[18]. Accordingly, dopamine, as well as several synthetic analogues and the DRD2 agonist quinpirole, inhibit insulin secretion in beta cell lines and isolated islets from rodents[18-20].

Here, we hypothesized that one of the early steps involved in the beneficial effects of bromocriptine on glucose homeostasis could be linked to modulation of insulin secretion following direct activation of DRD2 in pancreatic beta cells. To address this issue, the acute effect of bromocriptine on insulin secretion was investigated by means of a glucose tolerance test and hyperglycemic clamp in C57Bl6 mice and the underlying molecular mechanism(s) was studied in INS-1E beta cells.

Materials and methods

Chemicals

Bromocriptine (2-Bromo-α-ergocryptine methanesulfonate salt), domperidone, yohimbine, hGLP-1 7-36 amide and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the in vivo experiments, bromocriptine was dissolved in water containing 5% (v/v) DMSO. Domperidone was dissolved in water containing 5% (v/v) DMSO and acidified with HCl until pH 4. Water containing 5% (v/v) DMSO was used as placebo for both drugs.

Animals

Twelve-week-old male C57BL/6J mice (Charles River, Maastricht, The Netherlands) were housed in a temperature- and humidity-controlled room on a 12-h light–dark cycle with free access to standard laboratory chow (RM3; Special Diets Services, Witham, UK) and water. All animal experiments were performed in accordance with the principles of laboratory animal care and the 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

For determination of the acute effects of bromocriptine on glucose and insulin homeostasis, mice were randomly assigned to groups receiving either bromocriptine (10 or 25 mg/kg BW[12,21]) or placebo. They were first subjected to an intraperitoneal glucose tolerance test (ipGTT) and subsequently, after a wash-out period of 2 weeks, to a hyperglycemic clamp (HG clamp). One hour before the start of the experiments (t=-60 min), a baseline blood sample was taken, immediately followed by an i.p. injection of either bromocriptine or placebo. At t=0 min the ipGTT or the HG clamp was started, as described below. The blood samples taken at t=-60 min and t=0 min were used to determine the basal effect of bromocriptine on plasma glucose and insulin levels.

Another group of mice was used to determine the impact of the DRD2 antagonist domperidone on bromocriptine-induced metabolic effects. The design of these experiments was similar to the one described above, except that domperidone (5 mg/kg BW[22,23]) or placebo was injected 30 min before bromocriptine administration (t=-90 min).

Intraperitoneal Glucose Tolerance Test

Nine mice per group were fasted for 16 hours after food withdrawal at 5.00 pm. At 9.00 am the next day, mice were injected with the drugs as described above and subjected to an ipGTT. This procedure began with the collection of a blood sample (t=0 min), immediately followed by i.p. injection of 2 g/kg D-glucose,

provided as a 20% solution in PBS. Additional blood samples (30 µl) were taken via tail bleeding at 5, 15, 30, 45, 60, and 120 min after glucose injection for measurement of plasma glucose and insulin concentrations.

Hyperglycemic clamp

Eleven mice per group were fasted for 16 hours after food withdrawal at 5.00 pm. At 9.00 am the next day, mice were injected with the drugs as described above and then subjected to a HG clamp. During the clamp, 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). An initial blood sample (t=0 min) was taken, immediately followed by a primed (30 or 20 mg for placebo and bromocriptine groups, respectively), continuous (10 mg/h) intravenous (i.v.) infusion of a 20% D-Glucose solution. To maintain a steady state blood glucose concentration of ~20 mM, the continuous glucose infusion rate was adjusted according to the glucose concentration measured via tail bleeding (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany) at 1, 5, 20, 35, 50, 70 and 90 min after the glucose bolus. Blood samples (30 µl) for the measurement of plasma glucose and insulin concentrations were taken at 1, 5, 20, 50, 70 and 90 min after the start of the clamp. The blood sampling at 90 min was immediately followed by an i.v. injection of 2.1 mmol/kg L-arginine (Sigma-Aldrich), in order to assess maximal insulin secretion. Two additional blood samples were taken at 5 and 20 min after the injection.

Analytical procedures

Plasma insulin and glucose concentrations were measured using commercially available kits (Insulin: Crystal Chem Inc., Downers Grove, IL, USA; Glucose: Instruchemie, Delfzijl, The Netherlands).

INS-1E cells

The rat insulinoma-derived INS-1E cell line was used as a well differentiated beta cell clone[24] and cultured in a humidified atmosphere containing 5% CO2 in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Breda, The Netherlands). The maintenance culture was split once a week by gentle trypsinization, and $3x10^6$ cells were seeded in 75-cm^2 Falcon bottles (BD Biosciences, Breda, The Netherlands) with 20 ml complete medium. For experiments, INS-1E cells were seeded at $2x10^5$ cells/1 ml in Falcon 24-well plates (insulin secretion, cell membrane potential and cAMP determination) or $9x10^5$ cells/2 ml in Falcon 6-well plates (Western blot) and used 4 days later, with one medium change on day 3.

Insulin secretion

Insulin secretion in response to glucose or other secretagogues was measured in INS-1E cells between passages 53-67. Before the experiments, cells were maintained for 2 h in glucose-free culture medium. The cells were then washed twice and pre-incubated for 30 min at 37°C in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1% BSA and 10 mM HEPES, pH 7.4) in the presence or absence of various drugs, as indicated. Next, cells were washed once with glucose-free KRBH and incubated for 30 min in KRBH with or without various drugs and secretagogues, as indicated. Then, plates were placed on ice and the supernatants were collected for determination of insulin secretion. Cellular insulin content was measured in acid-ethanol extracts[24]. Insulin concentrations were measured after appropriate dilution (1/20 and 1/400 for supernatant and acid-ethanol extract, respectively) using a rat/mouse ELISA kit (Millipore, Nuclilab, Ede, The Netherlands).

Cell membrane potential and cAMP levels

Cell membrane potential was monitored in INS-1E cells using 100 nM of the fluorescent probe bis-oxonol (bis-(1,3-diethylthiobarbituric acid)trimethine oxonol) (Molecular Probes, Leiden, The Netherlands) in a temperature-controlled (37°C) plate reader fluorimeter with excitation and emission wavelengths of 544 and 590 nm, respectively[18]. For cAMP measurements, INS-1E cells were incubated in the same conditions as for GSIS experiments, but in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM). At the end of incubation, cells were lysed following the manufacturer's instructions and cAMP levels were determined using an enzyme immunoassay kit (Amersham Biosciences, Roosendaal, The Netherlands).

Western blot analysis

INS-1E cells were incubated in the same conditions as for GSIS experiments. At the end of incubation, cells were washed once in PBS and then lysed in 400 ml of a buffer containing 10% (w/v) glycerol, 3% (w/v) SDS and 100 mM Tris-HCl (pH 6.8). The homogenates were immediately boiled for 5 min and then centrifugated (13.200 rpm; 2 min). Protein content of the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Proteins (10 mg) were separated by SDS-PAGE followed by transfer to a PVDF membrane. Membranes were blocked for 1 h at room temperature in TBST buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 20 containing 5% (w/v) fat free milk) and incubated overnight with primary antibodies (all from Cell Signalling, Danvers, MA, USA). Blots were then washed in TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After washing, blots were

developed using enhanced chemiluminescence and quantified by densitometry analysis using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Data is presented as mean ± SEM. Statistical analysis was conducted with SPSS 16.0 software. The basal effect of treatment on glucose and insulin concentrations was analyzed with a univariate General Linear Model, with pre-injection values as a covariate. Analysis of the remaining in vivo data was performed using a one-way ANOVA. Only if the overall F-test indicated significant differences between the groups, a Bonferroni post-hoc test was used to determine differences between specific groups. Statistical analysis of most of the in vitro data was performed using a two-tailed unpaired Student's t-test. The membrane potential data was analyzed with a General Linear Model for repeated measures. Differences were considered statistically significant when $p<0.05$.

Results

Acute effects of bromocriptine on glucose tolerance and insulin secretion in mice

To investigate the effects of bromocriptine on glucose homeostasis, overnight-fasted C57Bl6 mice received a single i.p. injection of the drug (10 or 25 mg/kg BW) or placebo and were subjected to an ipGTT one hour later. As shown in table 1, bromocriptine induced a dose-dependent rise in basal plasma glucose levels. During the ipGTT, plasma glucose and insulin levels were determined at different time points and their respective areas under the curve (AUC) were calculated (fig 1). Glucose levels were significantly elevated by bromocriptine (fig 1A,B; +48% and +50% for AUC glucose at 10 and 25 mg/kg BW, respectively;

Table 1 - Plasma glucose and insulin levels in fasted mice before and 60 min after a single i.p. injection of bromocriptine or placebo.

	Glucose (mM)		Insulin (ng/ml)	
	Before injection	60 min after injection	Before injection	60 min after injection
Placebo	3.3 ± 0.2	4.2 ± 0.2	0.16 ± 0.04	0.19 ± 0.05
Bromocriptine 10 mg/kg	3.9 ± 0.2	5.1 ± 0.3	0.13 ± 0.03	0.17 ± 0.05
Bromocriptine 25 mg/kg	3.7 ± 0.3	5.8 ± 0.2**	0.13 ± 0.04	0.27 ± 0.06

Data is expressed as mean ± SEM, n=8-9 mice per group.
** $p<0.01$ vs placebo

p<0.01), indicating impaired glucose tolerance. Concomitantly, bromocriptine delayed and reduced the rise in plasma insulin levels after the glucose challenge (fig 1C). Total insulin release, calculated as AUC, was thus decreased by bromocriptine (fig 1D; -19% (NS) and -58% (p<0.05) at 10 and 25 mg/kg BW, respectively).

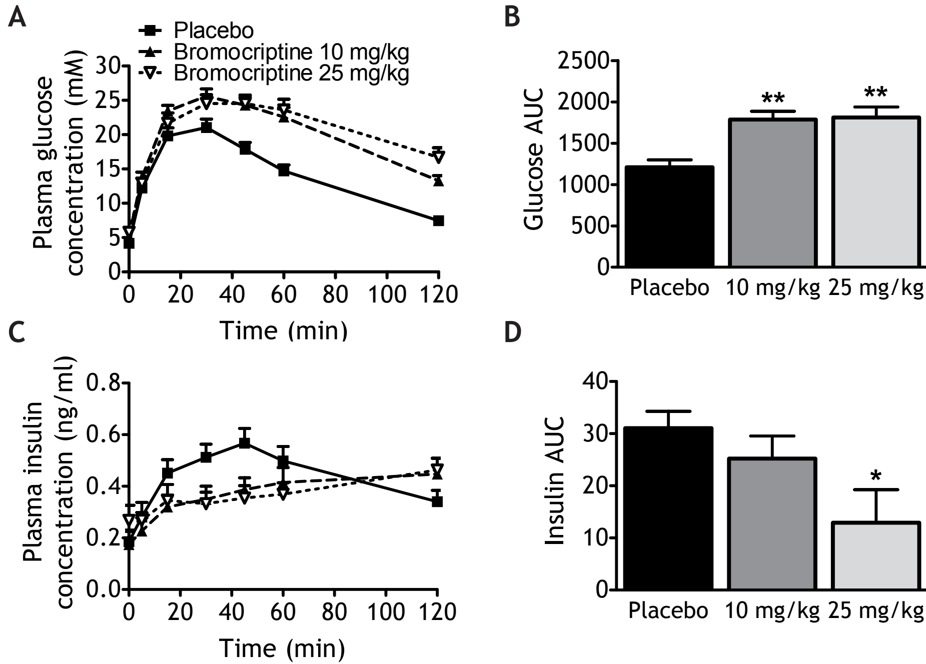


Figure 1 - Effects of acute injection of bromocriptine on plasma glucose and insulin kinetics in mice during an intraperitoneal glucose tolerance test.
An ipGTT was performed in overnight fasted C57Bl6 male mice one hour after a single i.p. injection of bromocriptine (10 or 25 mg/kg BW) or placebo. The glucose (A) and insulin (C) levels were measured and their respective areas under the curve (AUC) were calculated (B,D). Data is expressed as mean ± SEM, n=8-9 mice per group.
* p<0.05; ** p<0.01 vs placebo

To further assess the effect of bromocriptine on glucose-stimulated insulin secretion (GSIS), we performed a hyperglycemic (HG) clamp in similar conditions, i.e. one hour after i.p. administration of bromocriptine or placebo in overnight-fasted C57Bl6 mice. Plasma glucose levels were successfully clamped at 20 mM in both placebo and bromocriptine-injected mice (fig 2A). We confirmed that bromocriptine strongly affected GSIS (fig 2B). Indeed, insulin release was significantly and dose-dependently decreased during both first-phase (fig 2D; -58% (p=0.064) and -79% (p<0.01) for AUC insulin at 10 and 25 mg/kg BW, respectively) and second-phase GSIS (fig 2E; -51% and -70% for AUC insulin

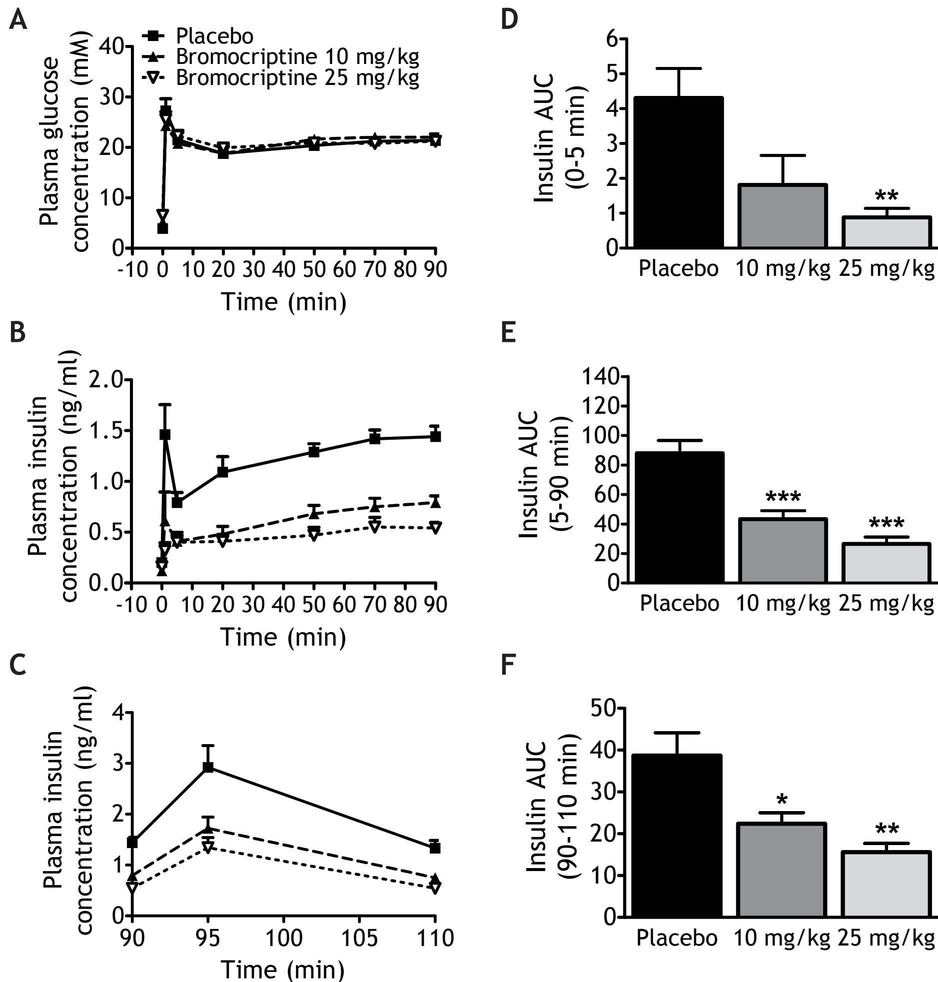


Figure 2 - Effects of acute injection of bromocriptine on glucose and arginine-induced insulin secretion in mice subjected to a hyperglycemic clamp.
A HG clamp (~20 mM) was performed in overnight fasted C57Bl6 male mice one hour after a single i.p. injection of bromocriptine (10 or 25 mg/kg BW) or placebo. The glucose (A) and insulin (B) levels were measured at different time points during 90 min and the areas under the curve (AUC) corresponding to the first (D, from 0 to 5 min) and second phase (E, from 5 to 90 min) of insulin secretion were calculated. At t=90 min, an i.v. bolus of arginine (2.1 mmol/kg BW) was administered to the mice and both insulin levels (C) and AUC (F) were determined during the next 20 min. Data is expressed as mean ± SEM, n=8-9 mice per group.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs placebo

at 10 and 25 mg/kg BW, respectively; p<0.001). Consistent with reduced GSIS, insulin secretion in response to arginine was still present but decreased in bromocriptine-injected mice (fig 2C,F).

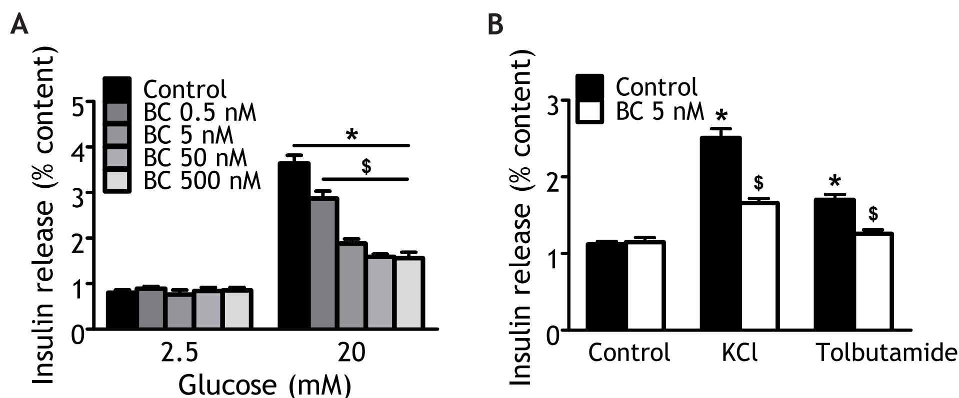
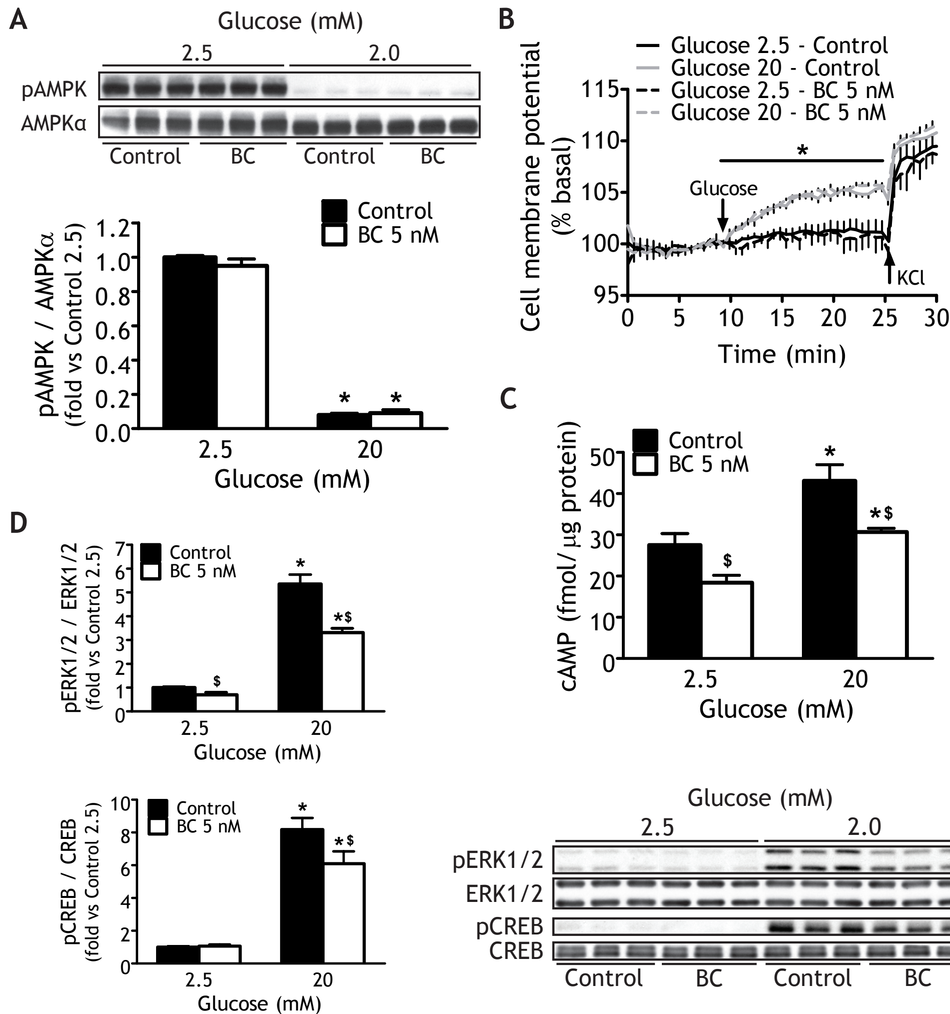


Figure 3 - Effects of bromocriptine on insulin secretion in INS-1E cells.
The insulin secretion in response to glucose and non-nutrient secretagogues was determined over a 30-min stimulation period in INS-1E cells pre-incubated for 30 min with various concentrations of bromocriptine (BC) or vehicle (0.05% (v/v) DMSO; black bars). (A) Dose-response effect of BC on insulin secretion in response to basal (2.5 mM) and stimulatory (20 mM) glucose concentrations. (B) Effect of BC (5 nM; open bars) on insulin secretion in presence of 2.5 mM glucose and 30 mM KCl or 250 µM tolbutamide. Data is expressed as mean ± SEM, n=3-4 independent experiments performed in triplicate.
* p<0.05 vs 2.5 mM glucose; $ p<0.05 vs control-vehicle

Figure 4 - Effects of bromocriptine on AMPK activity, cell membrane potential and intracellular cAMP levels in INS-1E cells.
Various metabolic parameters were determined at low (2.5 mM) and stimulatory (20 mM) glucose concentrations in INS-1E cells treated for 30 min with bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; black bars). (A) The phosphorylation state of AMPK was assessed by Western blot using anti-phospho-Thr172 antibody. Total AMPK was used as loading control. The quantitative results are expressed in arbitrary units as a ratio over the control-basal glucose group. (B) Cell membrane potential was monitored before and after successive addition of glucose (20 mM) and KCl (30 mM) in INS-1E cells treated from t=0 with BC (5 nM) or vehicle. (C) Intracellular cAMP levels were determined in the same conditions as in panel A except for the additional presence of 1 mM of the phosphodiesterase inhibitor IBMX. (D) The phosphorylation states of the PKA-downstream targets ERK1/2 and CREB were assessed by Western blot using anti-phospho-Thr202/Tyr204 and anti-phospho-Ser133 antibodies, respectively. Total ERK1/2 and CREB were used as loading controls. The quantitative results are expressed in arbitrary units as a ratio over the control-basal glucose group. Data is expressed as mean ± SEM, n=3-5 independent experiments.
* p<0.05 vs 2.5 mM glucose; $ p<0.05 vs control-vehicle



Effects of bromocriptine on insulin secretion in response to glucose and non-nutrient secretagogues in INS-1E cells

To elucidate the underlying molecular mechanism(s), we used the insulinoma-derived INS-1E cell line. The cells were pre-treated for 30 min with increasing concentrations of bromocriptine (from 0.5 to 500 nM) or vehicle (DMSO) before determination of insulin secretion over a 30-min stimulatory period with low (2.5 mM) or high (20 mM) glucose concentration. Figure 3A shows that addition of high glucose induced a 4.5-fold increase in insulin secretion in vehicle-treated INS-1E cells ($p<0.05$), in line with previous results obtained with this beta cell line[18,24]. Bromocriptine treatment did neither change cellular insulin content (data not shown) nor basal insulin secretion, but it dose-dependently inhibited GSIS (from -21% at 0.5 nM to -57% at 500 nM; $p<0.05$). In vehicle-treated INS-

1E cells, insulin secretion was also stimulated by non-nutrient secretagogues eliciting cell membrane depolarization, such as KCl or tolbutamide, leading to a 2.2-fold ($p<0.05$) and 1.5-fold ($p<0.05$) increase in insulin release, respectively (fig 3B). Bromocriptine also inhibited insulin secretion in these conditions (fig 3B; -34% and -26% in KCl- and tolbutamide-stimulated cells, respectively; $p<0.05$), suggesting that its inhibitory action is located at a step distal to ATP-sensitive potassium (K_{ATP}) channels and cell membrane depolarization.

Effects of bromocriptine on ATP level, cell membrane potential and cAMP generation in INS-1E cells

We assessed the effects of bromocriptine on basal and glucose-induced changes in cellular energy state, membrane depolarization and cyclic AMP (cAMP) levels in INS-1E cells. The AMP-activated protein kinase (AMPK) acts as a cellular fuel gauge and is activated in response to a drop in ATP levels or increase in AMP/ATP ratio[25]. As shown in figure 4A, challenging the cells with 20 mM glucose, which results in increased intracellular ATP levels, is associated with a significant decrease in AMPK activation, i.e. phosphorylation on its Thr172 residue. Bromocriptine did not change the AMPK phosphorylation state in both basal and glucose-stimulated conditions (fig 4A), suggesting that inhibition of GSIS by the drug is not mediated by impairment in glucose-induced mitochondrial ATP generation. In line with our results showing that the inhibitory effect of bromocriptine is still present when insulin secretion is triggered by KCl and tolbutamide (see above), we confirmed that the drug did not affect glucose- and KCl-induced cellular membrane depolarization measured using bis-oxonol fluorescence (fig 4B).

Because cAMP is a key player in the amplifying pathway involved in insulin secretion by beta cells[26], we also measured intracellular cAMP levels in INS-1E cells. As expected, upon a 30-min stimulatory period with 20 mM glucose in the presence of the phosphodiesterase inhibitor IBMX, intracellular cAMP levels were increased compared to 2.5 mM glucose (fig 4C; 28 ± 3 vs 43 ± 4 fmol/mg protein, respectively; $p<0.05$). Bromocriptine at 5 nM significantly decreased cAMP levels at both 2.5 and 20 mM glucose (fig 4C; -33% and -29% respectively; $p<0.05$). Accordingly, the phosphorylation state of two of the main cAMP-dependent protein kinase (PKA) downstream protein targets, CREB-Ser133 and ERK1/2-Thr202/Tyr204, were also significantly reduced by bromocriptine (fig 4D) in a dose-dependent manner (supplementary fig 1).

Effects of bromocriptine on GLP-1-stimulated insulin secretion in INS-1E cells

GLP-1 is among the most effective agents potentiating glucose-dependent insulin secretion in beta cells. It exerts its stimulatory effect via an increase in cAMP levels resulting from G-protein-coupled receptor-mediated activation

of adenylyl cyclase[27]. As expected, GLP-1 increased intracellular cAMP content and phosphorylation of CREB and ERK1/2, leading to amplified GSIS (fig 5A-C). In this condition, addition of bromocriptine reduced GLP-1-induced increase in cAMP level and GSIS, suggesting that the drug could interact with some G-protein-coupled receptors inhibiting adenylyl cyclase activity, such as DRD2[28].

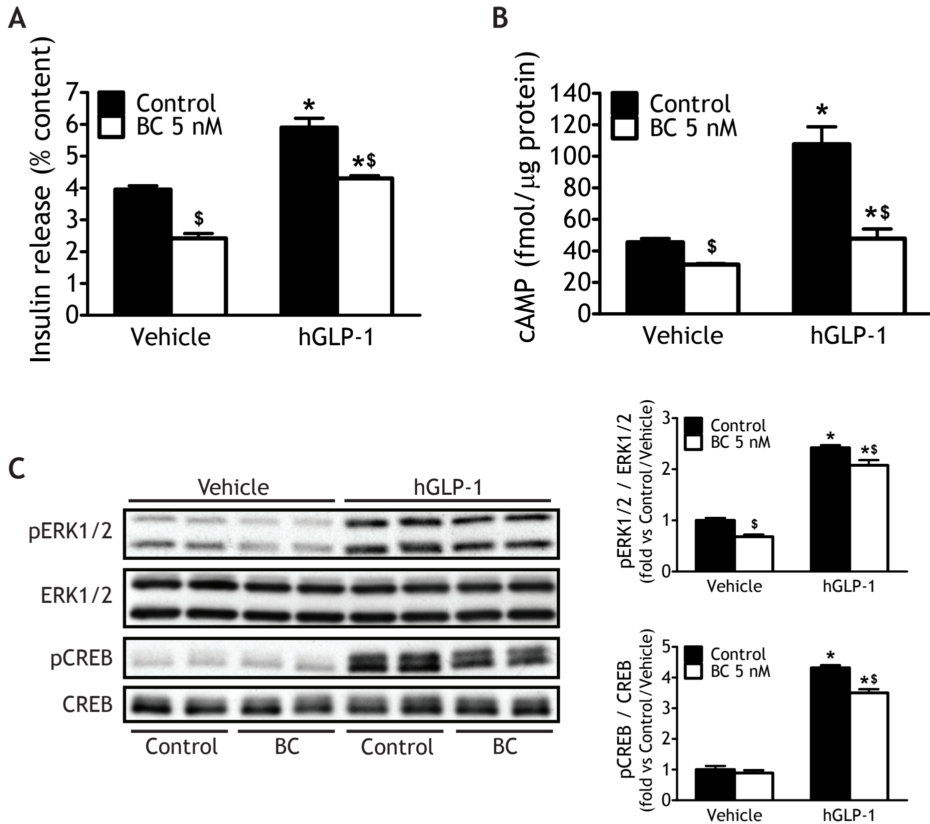


Figure 5 - Effects of bromocriptine on insulin secretion and intracellular cAMP levels in INS-1E cells pre-treated with GLP-1.
The insulin secretion was determined in INS-1E cells pre-incubated with hGLP-1 7-36 amide (10 nM) or vehicle (KRBH buffer) for 30 min and subsequently challenged with glucose (20 mM) for 30 min in the presence of bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; control, black bars). (A) Effects of BC (5 nM) on glucose-induced insulin secretion in basal (vehicle) and GLP-1-stimulated INS-1E cells. (B) Intracellular cAMP levels were determined in the same conditions except for the additional presence of 1 mM IBMX. (C) The phosphorylation states of the PKA-downstream targets ERK1/2 and CREB were assessed by Western blot as described in figure 4D. Data is expressed as mean ± SEM, n=3 independent experiments performed in duplicate. * $p<0.05$ vs vehicle; $ $p<0.05$ vs control

Effects of the DRD2 antagonist domperidone on bromocriptine-induced inhibition of GSIS in mice and INS-1E cells

We therefore tested whether the in vivo effects of bromocriptine on glucose tolerance and insulin secretion could be prevented by administration of the peripheral DRD2 antagonist domperidone in C57Bl6 mice. Pre-treatment with domperidone did not prevent the rise in basal plasma glucose levels induced by bromocriptine (table 2). During the ipGTT, domperidone did neither affect the bromocriptine-induced impairment of glucose disposal (fig 6A,C) nor its inhibitory effect on glucose-stimulated insulin response (fig 6B,D). In addition, domperidone did not prevent the bromocriptine-induced decrease in first- and second-phase GSIS during the HG clamp (fig 6E-H).

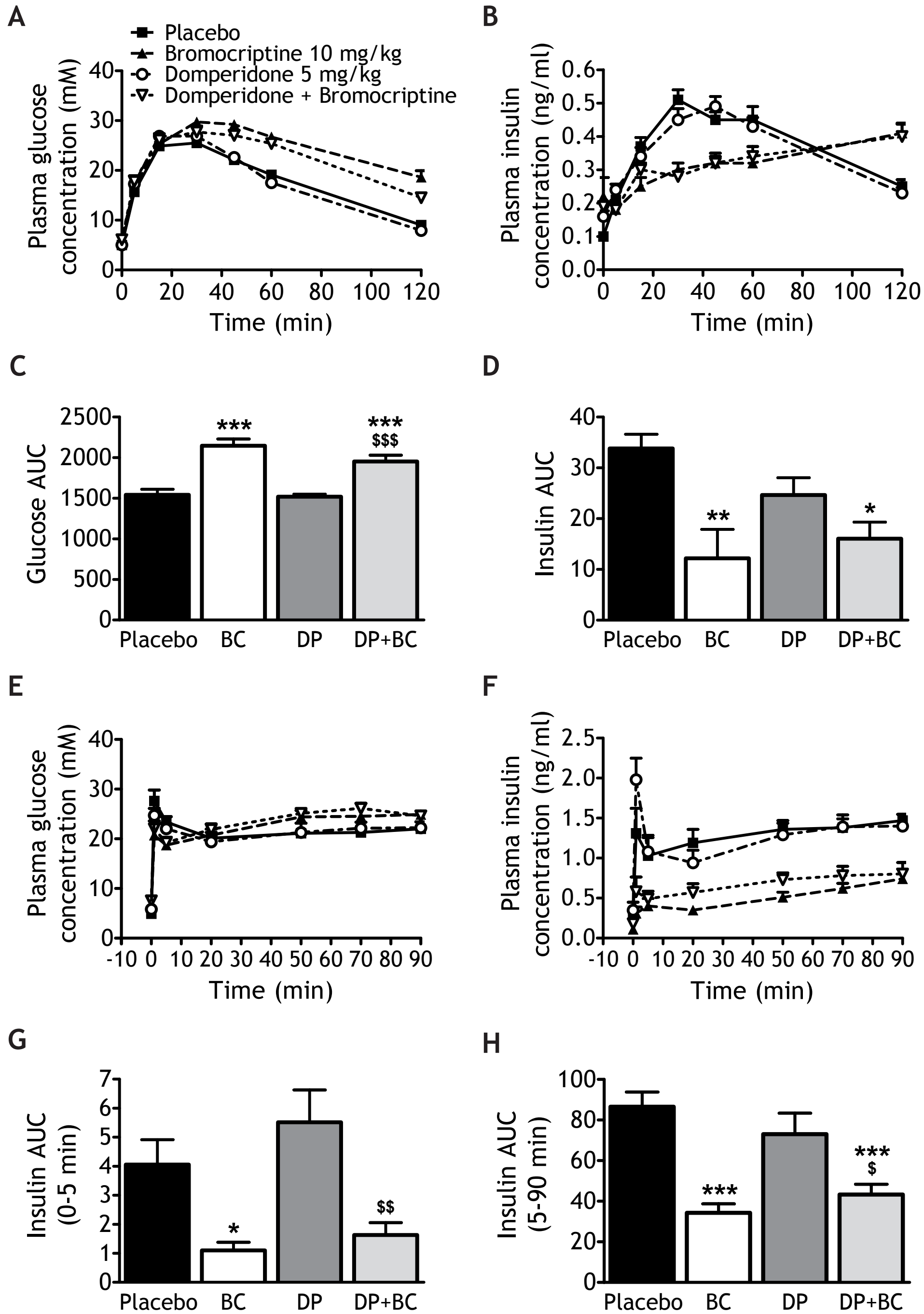
Table 2 - Plasma glucose and insulin levels before and 60 min after a single i.p. injection of bromocriptine or placebo in fasted mice pre-treated with or without domperidone.

	Glucose (mM)		Insulin (ng/ml)	
	Before injection	60 min after injection	Before injection	60 min after injection
Placebo	4.9 ± 0.3	5.0 ± 0.2	0.08 ± 0.01	0.10 ± 0.00
Bromocriptine 10 mg/kg	4.8 ± 0.2	6.3 ± 0.2***	0.13 ± 0.02	0.22 ± 0.06
Domperidone 5 mg/kg	5.0 ± 0.3	5.0 ± 0.3	0.09 ± 0.01	0.16 ± 0.02
Domperidone + Bromocriptine	4.8 ± 0.3	6.1 ± 0.3** $$	0.10 ± 0.01	0.19 ± 0.01

Data is expressed as mean ± SEM, n=9 mice per group
** p<0.01; *** p<0.001 vs placebo and $$ p<0.01 vs domperidone

Figure 6 - Effects of the DRD2 antagonist domperidone on bromocriptine-induced alterations of glucose tolerance and insulin secretion in mice.

An ipGTT was performed in overnight fasted C57Bl6 male mice pre-treated for 30 min with domperidone (i.p., DP, 5 mg/kg BW) or placebo one hour after a single i.p. injection of bromocriptine (BC, 10 mg/kg BW) or placebo. The glucose (A) and insulin (B) levels were measured and their respective areas under the curve (AUC) were calculated (C,D). A HG clamp (~20 mM) was performed in the same conditions. The glucose (E) and insulin (F) levels were measured at different time points during 90 min and the areas under the curve (AUC) corresponding to the first (G, from 0 to 5 min) and second phase (H, from 5 to 90 min) of insulin secretion were calculated. Data is expressed as mean ± SEM, n=6-9 mice per group.
* p<0.05, ** p<0.01; *** p<0.001 vs placebo and $ p<0.05; $$ p<0.01; $$$ p<0.001 vs domperidone →


A
Placebo
Bromocriptine 10 mg/kg
Domperidone 5 mg/kg
Domperidone + Bromocriptine
Plasma glucose concentration (mM)
Time (min)
B
Plasma insulin concentration (ng/ml)
Time (min)
C
Glucose AUC
***
***
$$$
Placebo BC DP DP+BC
D
Insulin AUC
**
*
Placebo BC DP DP+BC
E
Plasma glucose concentration (mM)
Time (min)
F
Plasma insulin concentration (ng/ml)
Time (min)
G
Insulin AUC (0-5 min)
*
$$
Placebo BC DP DP+BC
H
Insulin AUC (5-90 min)
***
***
$
Placebo BC DP DP+BC

Accordingly, we found that domperidone only modestly rescued the inhibitory effect of bromocriptine on GSIS in INS-1E cells whatever the antagonist concentrations used (-41% for bromocriptine alone vs -29% and -33% when pre-treated with 10 and 25 nM domperidone, respectively; $p<0.05$; supplementary fig 2), suggesting that a DRD2-independent signaling pathway(s) plays a more prominent role. Since it has been suggested that bromocriptine, in addition to its D2-dopaminergic activity, can stimulate the G-protein-coupled α2-adrenergic receptors (α2-AR)[29], we investigated the effect of yohimbine, a selective α2-AR antagonist, on cAMP levels and GSIS in INS-1E cells. Interestingly, yohimbine completely prevented the bromocriptine-induced decrease in intracellular cAMP levels and GSIS (fig 7). Taken together, our results indicate that the major effect of bromocriptine on insulin secretion results from modulation of the α2-AR rather than direct activation of the pancreatic DRD2.

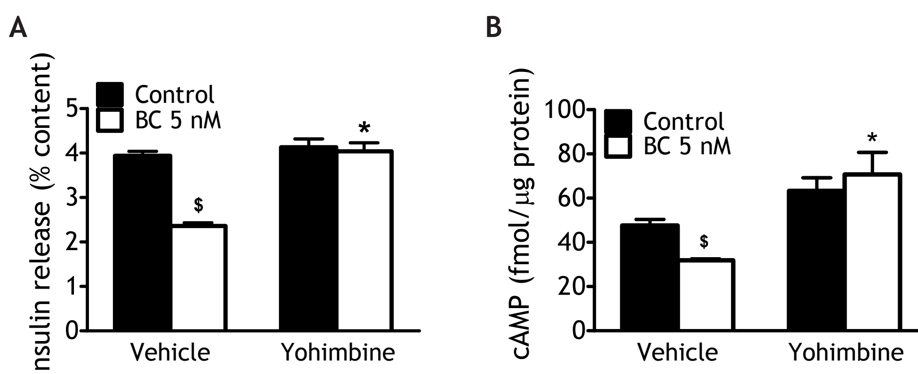


Figure 7 - Effects of the α2-adrenergic receptor antagonist yohimbine on bromocriptine-induced decrease in intracellular cAMP levels and insulin secretion in INS-1E cells.
The insulin secretion was determined in INS-1E cells pre-incubated with the α2-adrenergic receptor antagonist yohimbine (1 µM) or vehicle (KRHB buffer) for 30 min and subsequently challenged with glucose (20 mM) for 30 min in the presence of bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; black bars). (A) Effects of BC (5 nM) on glucose-induced insulin secretion in vehicle- and yohimbine-treated INS-1E cells. (B) Intracellular cAMP levels were determined in the same conditions except for the additional presence of 1 mM IBMX. Data is expressed as mean ± SEM, n=3 independent experiments.
* $p<0.05$ vs vehicle; $ $p<0.05$ vs control

Discussion

Here we report for the first time that a single administration of the DRD2 agonist bromocriptine inhibits GSIS in C57Bl6 mice at concentrations in the range of those used for chronic treatment in rodents[12,16,17,21]. This acute effect was also observed in INS-1E beta cells at therapeutic plasma concentrations, suggesting that bromocriptine affects insulin secretion through a direct effect on pancreatic beta cells. We further demonstrated that the underlying molecular mechanism of bromocriptine is not mediated by DRD2 activation but rather by modulation of the α2-AR.

A single i.p. injection of bromocriptine acutely elevated fasting plasma glucose levels in mice. This is in agreement with several other studies reporting that short-term administration of L-DOPA, dopamine or dopamine agonists induces hyperglycemia in rodents[30-33]. The exact underlying mechanism remains unknown, although stimulation of glycogenolysis and/or gluconeogenesis through modulation of the sympatho-adrenal axis might be involved[19,32].

We also found that administration of bromocriptine acutely impairs glucose tolerance. This finding is in apparent contradiction with the improvements of fasting plasma glucose and insulin levels, glucose tolerance and insulin sensitivity observed in obese insulin-resistant animals and humans treated with bromocriptine[12,14,34]. However, this discrepancy might be explained by the difference between single/acute and multiple/chronic treatment and also possibly by the use of healthy/lean C57Bl6 mice instead of insulin-resistant/obese animals in the present study. It is interesting to note that the pancreatic K_{ATP} activator diazoxide, which also decreases insulin secretion, also exerts opposite effects on glucose tolerance in lean and obese Zucker rats[35]. Whether acute administration of bromocriptine differentially affects glucose tolerance and insulin secretion in rodent models of insulin resistance and obesity requires further investigation.

Our most striking finding is the clear demonstration that bromocriptine acutely inhibits GSIS in both mice and INS-1E beta cells. This confirms previous in vitro reports showing a negative impact of dopamine and several dopaminergic agonists on insulin secretion[18,20,36]. GSIS from beta cells is primarily controlled by metabolism-secretion coupling[26]. Following an increase in its circulating levels, glucose rapidly equilibrates across the plasma membrane due to the presence of GLUT2 and is phosphorylated by glucokinase to enter the glycolysis pathway. Subsequently, mitochondrial metabolism generates ATP, which promotes the closure of K_{ATP} channels and, as a consequence, depolarization of the plasma membrane. This leads to intracellular calcium (Ca^{2+}) influx through voltage-gated Ca^{2+} channels resulting in increased free cytosolic Ca^{2+} levels, which ultimately triggers insulin exocytosis[26]. Although this triggering pathway is essential for GSIS, activation of additional metabolic signals involved in the so-

called amplifying pathway, are also required to fully stimulate insulin secretion[26].

Interestingly, it has been shown that all dopamine receptor subtypes, including DRD2, are expressed in rat, mouse, and human islets as well as in INS-1E cells[18]. In addition to the early demonstration that dopamine inhibits GSIS in various cellular models[18,20], a role for dopaminergic receptors in the regulation of insulin secretion was supported by a recent large-scale compound screening showing that a substantial number of dopaminergic agonists and antagonists were able to modulate GSIS[37]. Furthermore, the siRNA-mediated knockdown of DRD2 in INS-832/13 beta cells enhances GSIS, which is consistent with the involvement of this receptor in the negative regulation of insulin secretion[37]. In the present study, we showed that the inhibition of GSIS by bromocriptine is apparently not the consequence of an impaired triggering signal since neither intracellular energy state nor glucose- and KCl-induced cell membrane depolarization are affected in INS-1E cells. In contrast, bromocriptine significantly decreases intracellular cAMP levels, affecting one of the main metabolic amplifying pathways involved in GSIS, suggesting that activation of G-protein-coupled DRD2 receptors could mediate its effect. However, pre-treatment with domperidone, a peripheral DRD2 antagonist which does not cross the blood brain barrier[38], did not prevent the inhibition of insulin secretion or the impairment of glucose tolerance induced by bromocriptine in vivo. Although we can not exclude that part of the bromocriptine-induced inhibition of GSIS is mediated by a DRD2-dependent central effect in vivo, the experiments performed in INS-1E cells confirmed that domperidone only marginally prevented inhibition of GSIS, pointing to a DRD2-independent mechanism.

Our in vitro results provide clear evidence that activation of G-protein-coupled adrenergic receptors primarily accounts for the inhibition of GSIS by bromocriptine since yohimbine, a α2-AR antagonist, completely prevented its action. Interestingly, activation of α2-AR has already been shown to inhibit insulin secretion by a still incompletely understood effect on the exocytosis machinery[39] following receptor-coupled inhibition of adenylyl cyclase and decrease in cAMP synthesis[36,40-43]. However, this effect on cAMP-mediated pathways seems to be ascribed to selective activation of the α2A-, but not α2C-AR, in isolated islets[44], suggesting that part of the inhibition of GSIS by bromocriptine could also be mediated by cAMP/PKA-independent effects through α2C-AR activation. Further studies in receptor-specific α2-AR knock-out mice would be crucial to clarify this point.

Strikingly, overexpression of α2-AR results in impaired insulin secretion and glucose intolerance[45,46], whereas α2-AR knockout mice display higher basal insulin levels and improved glucose tolerance[47]. Finally, an α2-AR-mediated inhibition of GSIS by bromocriptine is further supported by in vitro binding studies showing that the drug exhibited high affinity for α2-AR[48] and that yohimbine can counteract some of its effects on other biological systems[29,49].

It is also tempting to speculate that the reduction of blood pressure observed in patients treated with bromocriptine could be ascribed to its peripheral α2-adrenergic action[15].

Although counter intuitive, the ability of bromocriptine to acutely suppress insulin secretion might be at the basis of its beneficial effect on glucose homeostasis observed after long-term treatment in obese insulin resistant rodents and humans[13,15,34]. Indeed, it has been suggested that the progressive deterioration of beta cell mass and function in patients with type 2 diabetes might not only be the consequence of hyperglycemia per se, but could also be due to the compensatory insulin secretion itself. Thus, inhibition of GSIS could avoid long-lasting insulin hypersecretion and therefore prevent subsequent development of insulin resistance and beta cell failure[50,51]. Accordingly, it has been shown that chronic increase of insulin release using tolbutamide impairs GSIS in insulin sensitive rats[52], whereas long-term pharmacological inhibition of insulin secretion improves beta cell glucose responsiveness in hyperglycemic rats[53] and patients with type 2 diabetes[54,55]. While several mechanisms have been proposed to explain the beneficial impact of beta cell rest on glucose homeostasis[50,51], future studies will have to clarify this point.

In conclusion, we report here that acute administration of bromocriptine inhibits insulin secretion both in vivo and in vitro mainly by a DRD2-independent mechanism involving direct activation of the pancreatic α2-AR. Taking into account that long-term treatment with bromocriptine has been shown to improve glucose homeostasis in patients with type 2 diabetes, we suggest that inhibition of GSIS by the drug could be one of its early effects, preventing long-lasting hypersecretion of insulin and subsequent beta cell failure.

Acknowledgements

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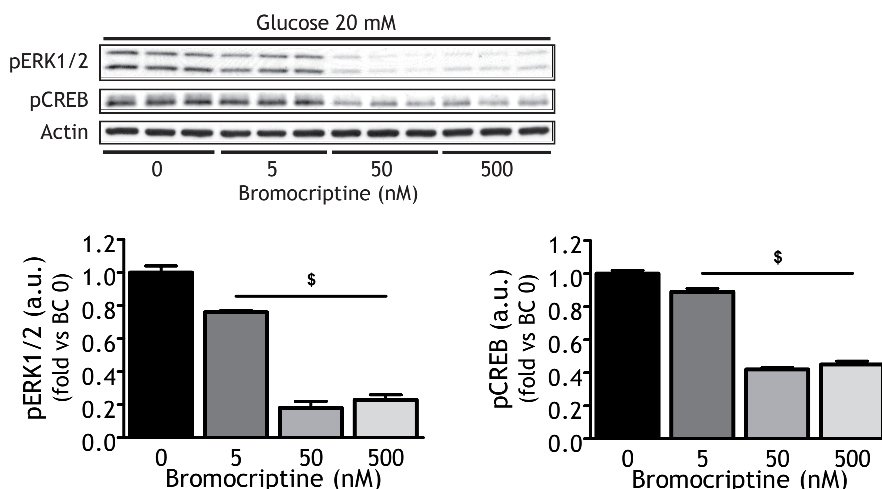
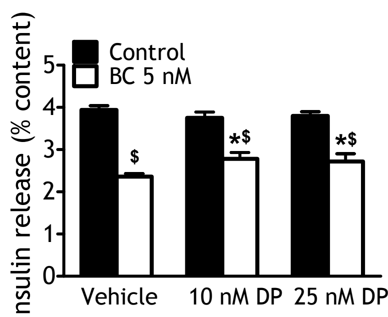


Supplementary Figure 1 - Dose-dependent effects of bromocriptine on phosphorylation state of PKA-downstream targets in INS-1E cells.

The phosphorylation states of the PKA-downstream protein targets ERK1/2 and CREB were assessed by Western blot in INS-1E cells treated for 30 min with increasing concentrations of bromocriptine (grey bars) or vehicle (0.05% (v/v) DMSO; black bars) in presence of 20 mM glucose. Actin was used as loading control. The quantitative results are expressed in arbitrary units as a ratio over the control-vehicle glucose group. Data is expressed as mean ± SEM, n=3 independent experiments.

$ p<0.05 vs control-vehicle



Supplementary Figure 2 - Effects of the dopamine D2 receptor antagonist domperidone on bromocriptine-induced inhibition of insulin secretion in INS-1E cells.

The insulin secretion was determined in INS-1E cells pre-incubated with the dopamine D2 receptor antagonist domperidone (DP, 10 or 25 nM) or vehicle (0.05% (v/v) DMSO) for 30 min and subsequently challenged with glucose (20 mM) for 30 min in the presence of bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; black bars). Data is expressed as mean ± SEM, n=3 independent experiments.

* p<0.05 vs vehicle; $ p<0.05 vs control

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7

General Discussion

The dopaminergic system controls a multitude of physiological functions, ranging from motor activity to hormone secretion and feelings of reward. Previously, it has also been implicated in glucose and insulin metabolism. Disruption of the glucose and insulin metabolism leads to insulin resistance and diabetes mellitus type 2. During the initial stages of diabetes development, insulin resistance will be compensated by an elevated pancreatic insulin production. When this compensatory mechanism fails, plasma glucose levels will rise and overt diabetes will develop.

A multitude of literature has firmly established the impact of modified dopaminergic transmission on glucose and insulin metabolism, yet several questions still remain unanswered. With the research described in this thesis, we sought to answer 2 main questions: is the altered dopamine signaling causally related to the development of diabetes? And, what is the mechanism underlying the ability of dopaminergic drugs to modify glucose metabolism? Knowledge of the developmental mechanisms of diabetes will hopefully assist in reducing morbidity and mortality by preventing the onset of diabetes as well as improving treatment.

In this chapter the major conclusions and implications of our findings are discussed in light of current knowledge.

Pharmacological modification of the dopaminergic system

To unravel the underlying mechanisms we examined the impact of DRD2 activation and inhibition on nutrient and energy metabolism. Inhibition of DRD2 by means of haloperidol and olanzapine induced glucose intolerance and insulin resistance (chapters 3-5) and activation of dopamine D2 receptors by means of bromocriptine led to improved insulin sensitivity (chapter 4). Although the results presented here show that activation and inhibition of dopamine D2 receptors lead to opposite metabolic profiles, the underlying mechanisms are distinct.

We showed in chapter 4 that subchronic treatment with bromocriptine leads to a reduction in body weight and fat mass, which is consistent with other experiments in rodents and humans[1-3]. The mechanism responsible for the decrease in body weight and fat mass is still unknown. The most straightforward explanation would be a reduction in energy intake and/or increase in energy expenditure, but, neither of these mechanisms occurred in our experiments. In accordance with our findings, Cincotta et al. showed that hamsters on bromocriptine treatment lost body weight and fat mass without alterations in food intake and energy expenditure[4]. Also, mice treated with bromocriptine displayed a significantly greater weight loss than pair fed mice[5,6]. Therefore, one must conclude that bromocriptine modifies adiposity and body weight via mechanisms other than food intake and energy expenditure.

As obesity and the associated increase in fat mass represent a significant risk factor for the development of insulin resistance[7], and loss of body fat and weight improves insulin sensitivity[8,9], the impact of bromocriptine on body weight and adiposity might participate in its positive effect on insulin sensitivity. However, several studies in humans show that bromocriptine beneficially alters the diabetic phenotype without implicating body weight and fat mass[10,11], indicating that alterations in adiposity are not necessarily involved in the positive action of the drug on glucose and insulin metabolism.

Bromocriptine also controls insulin secretion; in chapter 6 the drug acutely inhibits glucose-stimulated insulin secretion, which results in glucose intolerance. In agreement with this, mice injected with the DRD2 agonist cabergoline also acutely displayed glucose intolerance[12]. One can assume that suppression of insulin secretion leads to a diabetes-like phenotype. Initially, this assumption is true, as mice acutely develop glucose intolerance; however, in apparent contrast, we also showed that bromocriptine treatment for 2 weeks improved insulin sensitivity (chapter 4). This is in accordance with a wealth of literature showing that (sub)chronic bromocriptine treatment improves insulin secretion, glucose tolerance and insulin resistance in humans and animals[1,2,10,13]. To explain the discrepancy between acute and chronic treatment, we propose that bromocriptine promotes β-cell 'rest', leading to short-term deterioration and long-term improvement of glucose metabolism.

Beta-cell dysfunction is crucial in the development of diabetes; insulin resistance only progresses to overt diabetes when β-cells fail to secrete sufficient amounts of insulin to overcome whole body insulin resistance. This malfunction of β-cells is the corollary of an increased rate of apoptosis and changes in the intracellular pathway controlling insulin secretion. It has been hypothesized that the high glucose levels, fundamental in diabetes, might be, indirectly, responsible for β-cell degeneration by promoting insulin hypersecretion and consequently β-cell exhaustion and death[14,15]. Indeed, pharmacologically increasing insulin release for 48 h subsequently decreased insulin secretion in rats[16]. Accordingly, suppression of insulin secretion, preventing hypersecretion and death, might in the long-term, improve β-cell function. This concept has been verified in several experiments. Treatment of diabetic rats with the insulin secretion inhibitor diazoxide enhanced the diminished glucose-stimulated insulin response[17]. And, short-term treatment of diabetic patients with insulin secretion inhibitors attenuated the defective insulin release characteristic for type 2 diabetes[18,19]. Two mechanisms might explain the long-term beneficial impact of the initially deleterious impact of the suppression of insulin secretion: 1) inhibition of insulin secretion increases β-cell insulin stores, thereby enhancing the secretory capacity[17,20], and 2) inhibition of insulin secretion increases the number of organ specific insulin receptors leading to improved insulin sensitivity[21,22].

Finally, bromocriptine might also directly improve insulin sensitivity. In chapter 4 we showed that 2 weeks of bromocriptine treatment reduced insulin resistance. However, as up till now, no studies examining the acute impact of the drug on insulin action have been performed, it remains to be determined whether bromocriptine directly modulates insulin action or indirectly via its effect on insulin secretion[21,22].

The disruption of insulin action by inhibition of dopamine D2 receptors is achieved via other mechanistic routes than the improvement of insulin action by stimulation of D2 receptors. However, like bromocriptine, haloperidol treatment also participates in body weight regulation; although after subchronic treatment (2 weeks) body weight was not affected (chapter 4), after chronic treatment (12 weeks) body weight of treated mice was significantly increased compared to control mice (chapter 3). Keeping in mind that, due to the experimental setup of the latter study, food intake of haloperidol and control mice was identical, obviously the impact of haloperidol on weight is independent of alterations in food intake. In accordance with our findings, Pouzet et al. reported that an increased food efficiency, indicating an enhanced ability of food to increase body weight, was responsible for haloperidol induced weight gain[23]. In our experiments, mice treated with haloperidol for 1 week displayed a tremendous reduction in physical activity (chapter 4); in fact, this is a common phenomenon in rodents treated with antipsychotic drugs[24-26]. This reduced activity and concomitant reduction in energy expenditure might well account for the enhanced food efficiency and the body weight gain induced by the drug. If haloperidol indeed induces weight gain as we propose, an intriguing question is why we did not observe an increased body weight in mice treated with haloperidol for 2 weeks (chapter 4). Two explanations can be thought of: 1) the treatment period was too short to reveal differences in body weight. This would be in line with the chronic experiment in which alterations in body weight were not observed before the third week of treatment, or 2) haloperidol may have slightly, albeit not significantly, reduced food intake in the 2-week experiment, thereby preventing weight gain.

Besides its possible deleterious impact on insulin sensitivity via the development of obesity[7], the reduction in physical activity might also directly affect insulin sensitivity, independent of weight gain. 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[27-29]. 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[30,31]. Typically, this inactivity induced insulin resistance is restricted to tissues responsible for glucose-uptake, as glucose production remains adequately suppressed by insulin[27,28,32]. It has been suggested that a reduction in GLUT4 expression might underlie the inactivity

induced impairment of glucose uptake[31], but more research is warranted to confirm this.

In addition, haloperidol, and other DRD2 antagonists, might directly affect insulin sensitivity, independent of their impact on physical activity and body weight gain. In fact, it is known that several antipsychotic drugs, other than haloperidol, are able to acutely induce insulin resistance[26,33-35]. This insulin resistance seems to involve both glucose uptake and glucose production[33-35], although the acute impact of antipsychotic medication on glucose production is not always observed[26]. Interestingly, the antipsychotic drug induced inability of tissues to take up glucose during hyperinsulinemia seems largely confined to muscle tissue, as glucose clearance by adipose tissue is even enhanced[26]. Even though the direct impact on insulin sensitivity has not yet been confirmed for haloperidol, the drug does acutely impair glucose tolerance[36,37]. This implies that haloperidol is able to reduce insulin secretion and/or promote insulin resistance. As the glucose intolerance was accompanied by elevated insulin levels[36], defective insulin secretion can not (solely) explain the glucose intolerance. This provides evidence that haloperidol, like other antipsychotics is able to acutely decrease insulin sensitivity.

Finally, haloperidol might also impair insulin secretion; after 10 weeks of treatment, haloperidol and control mice had, despite significantly increased glucose levels in the former, similar insulin levels during a glucose tolerance test (chapter 3). This indicates an insulin secretion malfunction. Likewise, the low basal insulin levels in the face of elevated basal glucose levels observed in these mice after 12 weeks of drug treatment, confirm the hypothesis that β-cells are unable to produce sufficient amounts of insulin. These findings are in accordance with studies in DRD2 deficient mice, which also show inappropriately low insulin levels during an i.p. glucose tolerance test[12]. In vitro experiments with isolated islets from these mice showed that glucose was unable to stimulate insulin secretion from these islets compared to islets from wt mice. Further examination of the pancreata of DRD2 deficient mice revealed a reduced β-cell mass and insulin concentration[12]. According to these results it is conceivable that in our chronically treated haloperidol mice, the malfunctioning insulin secretion is due to a reduced β-cell mass and/or intracellular β-cell defects. The mechanism underlying the deregulation of β-cell function has not been resolved yet, but it has been suggested that DRD2 activation is essential for β-cell proliferation[12]. Consequently, chronically blocking DRD2 could reduce β-cell proliferation and eventually lead to a diminished β-cell mass. Alternatively, one might speculated that, in analogy with the hypothesized impact of bromocriptine on insulin secretion, haloperidol may initially promote glucose-stimulated insulin secretion, which may lead to insulin hypersecretion and consequently to β-cell damage and death. Several papers document a reduced responsiveness of β-cells towards insulin secretagogues following prolonged stimulation of

insulin secretion[16,38], confirming the last part of the hypothesis. The initial part though, the acute effect of haloperidol on insulin secretion, remains to be verified as the literature on this subject is controversial. In an in vitro study by Best et al. haloperidol induced a depolarization of β-cell membrane potential and, although this might be expected to enhance insulin secretion, such effect could not be detected[39]. Two other studies reported a diminished and an unaltered insulin secretory response of β-cells following incubation with haloperidol[40,41].

All together, we have provided evidence that modulation of glucose homeostasis by activation or inhibition of dopamine D2 receptors is achieved via different mechanistic routes. Presumably, bromocriptine mainly improves glucose metabolism by suppressing insulin secretion which, paradoxically, leads to enhanced insulin action. Weight reduction, as a result of bromocriptine treatment, might additionally improve insulin sensitivity, but it is not a prerequisite for the beneficial impact of the drug. Haloperidol, on the other hand, most likely disrupts physiological glucose metabolism by reducing physical activity, which, directly, or via weight gain, reduces insulin sensitivity. In addition, the drug probably also directly promotes insulin resistance and gradually impairs insulin secretion.

Dopaminergic system and the aetiology of diabetes

With the experiments described in this thesis, we also wanted to gain more insight into the role of dopaminergic neurotransmission in the course of diabetes development. Several cross-sectional studies suggest that alterations in dopaminergic neurotransmission are involved in the pathogenesis of type 2 diabetes. In obese humans and insulin resistant animals the expression of dopamine D2 receptors in certain brain areas is reduced[42-45]. In obese humans the decrease in dopamine D2 receptors is even inversely related with BMI[42]. And, in brains of diabetic patients and type 2 diabetic animal models, increased dopamine levels are measured[46-48]. As cross-sectional research does not provide details about the cause-effect relationship, two hypotheses, based on the observations above, can be postulated: 1) altered dopaminergic neurotransmission is the *cause* of metabolic derangements or 2) altered dopaminergic neurotransmission is the *consequence* of metabolic derangements.

Considering the indisputable positive impact of DRD2 activation on glucose and insulin metabolism and the detrimental effect of blocking DRD2, described in chapters 3-5 and discussed above, the first hypothesis is more likely. This hypothesis requires that components involved in dopaminergic signaling are altered prior to the initiation of metabolic derangements. Genetic variations of dopaminergic genes may be responsible for the initial alterations. This is supported by the observed association between DRD2 polymorphisms which diminish dopaminergic transmission[49,50] and disturbed energy homeostasis[51-54].

However, as diabetes is also associated with obesity[7], reduced physical activity[55], aging[56], an altered dietary pattern[57-60] and the use of antipsychotics[61,62], we proposed that the initial modifications in dopaminergic activity might also be triggered by nutritional, environmental, pharmaceutical or physiological factors.

In chapter 2 we examined the hypothesis that high fat feeding, which is a well-recognized trigger for the development of a diabetes-like phenotype in rodents, induces these metabolic anomalies via modifications in dopaminergic neurotransmission. In contrast to our hypothesis, though, wt C57Bl6 mice, maintained on a high fat diet for 4 weeks, were insulin resistant compared to control mice without detectable alterations of dopaminergic features. Consequently, we concluded that the reduced dopaminergic neurotransmission observed in obese humans and animals is not due to dietary factors. There are several ways to explain the discrepancy between the literature, showing an altered dopaminergic phenotype in obese animals and humans and the absence of dopaminergic alterations in our experiment.

We hypothesized that nutritional cues will diminish dopaminergic action, thereby inducing insulin resistance, but, we did not consider the existence of dopaminergic gene variations that might be present in our mice. However, these polymorphisms could alter dopaminergic action, which might set the stage for high fat diet induced insulin resistance. This is supported by the finding that body weight gain in schizophrenic patients on antipsychotic drug treatment is associated with certain DRD2 gene variations[63,64]. Also, compared to diet-resistant rats, rats prone to become obese on a high fat diet already display alterations in dopamine metabolism when still maintained on a regular low fat diet[65,66]. In addition, already prior to the onset of food intake and body weight alterations, the expression of DRD2 in the striatum of obese Zucker rats is reduced compared to lean Zucker rats[67]. These observations strongly suggest that genetic variations in dopaminergic parameters determine the susceptibility of individuals to develop an unfavorable metabolic phenotype in response to pharmacological or nutritional cues. One may even speculate that these dopaminergic variations are a prerequisite for the development of metabolic alterations. This genetic predisposition might explain why only some rodents develop massive weight gain on a high fat diet (DIO; Diet Induced Obese) and others remain relatively lean (DR; Diet Resistant)[68-70].

If this theory is true, it is understandable that we found metabolic, but not dopaminergic, differences between the mice maintained on a high vs. low fat diet. Rodents prone to become obese (DIO prone) or remain lean (DR prone) on a high fat already have a different dopaminergic profile when still on the control diet[65,66]. This suggests that, in a random population of rodents, various dopaminergic phenotypes are present. We showed in chapter 4 that some C57Bl6 mice become more obese and insulin resistant on a high fat diet than

others, so it is possible that the C57Bl6 mice in our experiment initially already have different dopaminergic phenotypes. We believe this might be true. As we divided our mice in chapter 2 randomly into a high and low fat group, both groups could have contained mice with a 'normal' dopaminergic phenotype as well as mice with a, genetically-determined, 'deterimental' phenotype. It goes without saying that if both phenotypes were equally represented in both the high and low fat group, there would be, on average, no measurable difference in dopaminergic parameters between these groups. The corollary of the presence of these different dopaminergic profiles in the high fat group should have been the development of different degrees of weight gain and insulin resistance. Unfortunately, due to the small sample size, we were unable to divide the high fat mice into DIO and DR mice according to their dopaminergic and metabolic phenotype. So, obviously, more research is warranted to confirm this hypothesis.

If, however, the first assumption that dopaminergic neurotransmission is the *cause* of metabolic derangements is incorrect, is it then possible that dopaminergic alterations are the *consequence* of changes in the hormonal environment in diabetic individuals? In other words, is it possible that in our experiment dopaminergic alterations would have developed *after* insulin resistance was established? This might be true. Hyperglycemia, a hallmark of diabetes, promotes elevated brain dopamine levels[71-73]. NPY, which's levels are elevated in obese and diabetic individuals[74], stimulates dopamine output[75]. PYY (3-36) suppresses dopamine release[76] and its levels are reduced in obese subjects[77]. Leptin also reduces dopamine output[78,79] while chronic obesity is characterized by a resistance to the actions of this hormone[80]. In apparent contrast, insulin acutely increases dopamine uptake by promoting the surface expression of the dopamine transporter[81] and chronic insulin stimulation upregulates dopamine transporter mRNA[82]. In conclusion, these results indicate that disturbances of several internal regulators of energy balance might account for the alterations in dopaminergic neurotransmission observed in obese diabetic animals and humans. Yet, the physiological role and relevance of these processes in the course of obesity and diabetes development remain to be determined.

All together, we presented evidence that alterations in dopaminergic signaling may be either cause or consequence of the diabetic phenotype. Polymorphisms in dopaminergic genes may determine the susceptibility of a subject to develop obesity and diabetes in response to nutritional of pharmacological cues. On the other hand, diabetes-associated disturbances of hormone levels, or action, may promote alterations in dopamine homeostasis.

Humans versus rodents

Interestingly, in our experiments we observed a discrepancy between the impact of haloperidol in humans and mice. In humans haloperidol did not

modify glucose and insulin metabolism (chapter 5) whereas in mice haloperidol clearly induced glucose intolerance and insulin resistance (chapter 3 and 4). Several explanations can be thought of: 1) different dose, 2) different mode of administration, 3) different treatment period and 4) species-specific sensitivity to the effect of haloperidol.

First, the difference in dose; in mice we used 1 mg/kg/day, whereas the human volunteers were treated with 3 mg haloperidol per day, which corresponds to 0.04 mg/kg/day given the average weight of the subjects (~75 kg). When comparing drug doses between humans and rodents, though, one must take into account that the metabolism of drugs in rodents is faster than in humans, resulting in a, in general, 4 - 6 times shorter half-life of drugs in rodents[83]. So for rodents, the dose comparable to the one used in humans would be 0.16 - 0.24 mg/kg/day. The dose given to the human volunteers is in the low range of doses prescribed to schizophrenic patients, whereas the dose given to the mice is in the high range of doses used to treat schizophrenia. Possibly, this difference in medication dose can explain the dissimilarities in efficacy of haloperidol in mice and man. Experiments performed in rodents using a low dose of haloperidol (0.25 mg/kg/day), equivalent to the dose used in our human study, showed no impact of the drug on glucose metabolism after 7 and 28 days of daily injections, although, interestingly, glucose intolerance was observed 1 hour after the first injection[36].

Another variable in these studies is the mode of drug administration which might further affect the plasma levels of the drug. In our experiments mice received haloperidol through subcutaneous implanted pellets, while human individuals received haloperidol tablets. Two issues regarding the mode of administration are relevant in this discussion: the frequency of drug administration (continuous vs. once daily) and the route of drug entry (subcutaneous vs. oral). As described above, the half-life of drugs in rodents is considerably shorter than in humans. Specifically, in humans, the half-life of haloperidol is 12-36 hours; in rodents, the half-life is only approximately 1.5 h[83]. According to these figures, in humans a ‘single administration a day’ regiment is sufficient to achieve a relatively stable plasma concentration of haloperidol and level of DRD2 receptor occupancy throughout the day. The short half-life of haloperidol in mice though, imposes that the drug should be administered approximately 8 times a day in order to achieve a similar stable drug concentration and receptor occupancy. The most practical way to ensure stable plasma haloperidol concentrations in mice is to use pellets or minipumps continuously releasing the drug. So, most likely, the frequency of drug delivery does not explain the discrepant impact of haloperidol in humans and mice, but the route of drug delivery may. Compared to subcutaneous drug administration, which we used in our mice experiments, the efficacy of orally administered drugs is limited by the so-called ‘first pass effect’. Orally ingested drugs are

absorbed by the gastrointestinal tract and are transported, via the portal vein, to the liver before entering the systemic circulation. The liver metabolizes part of the drug, in case of haloperidol, into inactive compounds, thereby limiting the bioavailability of the drug. So, the oral delivery route may have further amplified the impact of the different doses used in the human and mice studies.

Another variable was the treatment period; the volunteers were treated for 8 days (chapter 5), while the mice were treated for 14 days (chapter 4) or 12 weeks (chapter 3). One can propose that 8 days of treatment is too short for metabolic alterations to emerge, yet, this is most likely not true. Haloperidol already induces glucose intolerance as soon as 1 hour after injection in mice[36]. This strongly suggests that 8 days of haloperidol treatment should suffice to uncover the metabolic consequences of treatment if any were present.

Finally, a species-specific sensitivity should also be considered. The existence of such species-dependent sensitivity to antipsychotic drugs is perhaps best illustrated by the effect of those drugs on weight gain. In contrast to the human situation where both males and females develop obesity in response to antipsychotic drug treatment, in rats, only females seem to be susceptible for the weight inducing ability of these drugs[23,84]. Up till now, only one group, using a specific treatment protocol, has been able to induce obesity in male rats in response to olanzapine[85]. Also the impact of haloperidol on weight gain is different in rats and humans; low concentrations of haloperidol already trigger body weight gain in female rats[23], while this drug is associated with no, or very limited weight gain in the humans[86,87]. This discrepant body weight regulation in response to antipsychotic drugs suggests that glucose metabolism might also be differentially affected in humans and rodents, but to confirm this, thorough dose-response experiments should be performed in both species.

In conclusion, the absence of metabolic consequences in haloperidol treated humans in spite of the insulin resistance observed in mice treated with haloperidol is most likely due to a combination of factors: the bioavailability of the drug, determined by the drug dose and the route of administration, together with the species-specific sensitivity to the drug.

Conclusion

With the experiments described in this thesis we attempted to unravel the intricate relationship between diabetes and DRD2 mediated dopaminergic transmission. We provided evidence that although both DRD2 agonistic and DRD2 antagonistic drugs affect glucose metabolism, the mechanistic routes are distinct. Unlike bromocriptine, which beneficially affects insulin action by, paradoxically, suppressing insulin secretion, haloperidol disturbs insulin action by diminishing physical activity and directly disrupting insulin sensitivity.

We also discussed that dopaminergic dysfunction might be cause or consequence in the aetiology of diabetes. Genetic variations in dopaminergic

genes, leading to diminished dopaminergic transmission, may predispose individuals to develop a diabetes-like phenotype in response to physiological or pharmacological cues. Alternatively, dopaminergic transmission may be disturbed by diabetes-induced alterations in the hormone profile.

Although caution is warranted when extrapolating the results of drug experiments obtained in animals to humans, especially with regard to the dose-effect relationship, we believe the general mechanisms we observed in these animals are also applicable to humans.

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8

Summary

The dopaminergic system controls a multitude of physiological functions, ranging from motor activity to hormone secretion and feelings of reward. Previously, the dopaminergic system has also been implicated in glucose and insulin metabolism. Blood glucose levels are maintained within a narrow range to prevent glucose toxicity and, in the meantime, provide the necessary fuel for glucose-dependent tissues like the brain. Insulin is one of the key players mediating glucose control. Diabetes mellitus type 2 is characterized by insulin resistance and impaired insulin secretion. During the initial stages of diabetes development, insulin secretion is increased to maintain insulin action in spite of insulin resistance. When β-cells are no longer able to produce sufficient amounts of insulin to overcome the resistance, blood glucose levels rise and overt diabetes is established. This will, if left untreated, lead to significant morbidity and mortality.

Extensive literature links the dopaminergic system, and more specifically the dopamine receptor D2, to insulin resistance and diabetes. Polymorphisms of the DRD2 gene are associated with alterations in energy and nutrient metabolism. DRD2 antagonists induce weight gain and diabetes whereas DRD2 agonistic drugs improve glucose and insulin homeostasis. Also, dopaminergic neurotransmission is altered in obese and diabetic humans and animal models. Despite the evidence, many aspects of the functional relationship between diabetes and the dopaminergic system remain unclear. In this thesis we sought to unravel the characteristics of the interplay between dopamine D2 receptors and glucose metabolism as well as to understand the underlying mechanism(s).

In our studies we used wild type C57Bl6 mice. When maintained on a high fat diet for several weeks, these mice develop obesity, insulin resistance and a metabolic phenotype closely resembling type 2 diabetes in humans. Therefore, this mouse strain is a valuable animal model to study the development of diabetes. We also used the INS-1E cell line, which is derived from a rat insulinoma. Physiological β-cell functions are preserved in this cell line, making it a valuable model to study insulin secretion in vitro.

High fat feeding induces obesity and insulin resistance. Given the fact that obesity is associated with reduced DRD2 expression and increased dopamine release, we reasoned that high fat feeding could alter dopaminergic transmission and via this route stimulate weight gain and insulin resistance. Therefore, in **chapter 2** we examined the role of the dopaminergic system in the aetiology of high fat diet induced obesity and the deregulation of glucose metabolism. Wt C57Bl6 mice were maintained on a high fat diet for 4 weeks. The high fat feeding increased body weight of these mice and reduced insulin sensitivity. Despite the metabolic impact, the high fat diet did not alter hypothalamic dopamine

release in fed or fasted mice. Also, hypothalamic expression of dopamine receptors D1 and D2, dopamine transporter and tyrosine hydroxylase genes was not affected by high fat feeding. So, as high fat diet-induced metabolic corollaries are independent of changes in parameters of dopaminergic activity, we concluded that the alterations in dopaminergic parameters observed in obese animal models and humans are probably due to mechanisms other than dietary composition.

Calorie restriction is the most effective way of increasing life-span and decreasing morbidity. It improves insulin sensitivity and delays the age-related loss of DRD2 expression. Obesity and insulin resistance is associated with a reduction in DRD2 binding sites. Blocking DRD2 induces weight gain and promotes insulin resistance whereas activating DRD2 improves insulin sensitivity. Considering the impact of DRD2 on metabolism, we hypothesized that dopamine receptors might be involved in the beneficial effect of calorie restriction on glucose metabolism. We examined this hypothesis in **chapter 3**. Wt C57Bl6 mice were maintained on a high fat diet, either with ad libitum or restricted access. Half of the calorie restricted mice also received continuous haloperidol treatment to inhibit DRD2 activation. Mice with restricted access to the high fat diet were glucose tolerant and insulin sensitive compared to mice with ad libitum access to the diet. Haloperidol slightly increased the body weight of calorie restricted mice. Also, the drug completely abolished the beneficial impact of calorie restriction on glucose tolerance and partly reduced the insulin sensitivity observed in calorie restricted mice. The metabolic differences between ad libitum fed and calorie restricted mice were not accompanied by alterations in hypothalamic DRD2 binding. So, calorie restriction offers protection against the deleterious impact of high fat feeding, and blocking DRD2 curtails these metabolic benefits. Although this suggests that dopamine receptors are part of the mechanism underlying the beneficial effect of calorie restriction, the unchanged hypothalamic DRD2 binding in response to restricted access to high fat food argues against this suggestion.

Although in general high fat feeding promotes obesity and insulin resistance in rodents, there is a great diversity in the response of individual animals from a single strain to such challenge. Based on the body weight gain after several weeks of high fat feeding, rodents can be divided into Diet Induced Obese (DIO) and Diet Resistant (DR). Interestingly, dopaminergic neurotransmission differs in DIO and DR rodents, even before the onset of high fat diet induced weight gain. Specifically, DRD2 expression and dopamine turnover are decreased in DIO compared to DR rodents. This led us to believe that inherited alterations in dopaminergic transmission might mediate the differential corollaries of high fat feeding in these animals. We examined this in **chapter 4**. Based on the weight

gain of individual wt C57Bl6 mice on a high fat diet, these mice were classified as DIO or DR. Subsequently, half of the DIO mice were given bromocriptine to stimulate DRD2 activation and half of the DR mice were given haloperidol to inhibit DRD2 activation. Compared to DR mice, DIO mice were heavier, had elevated plasma insulin levels and were insulin resistant. Haloperidol treatment increased plasma glucose levels and impaired insulin sensitivity in DR mice. Furthermore, haloperidol decreased physical activity and energy expenditure in these mice. Conversely, bromocriptine tended to reduce body weight and physical activity and improve insulin sensitivity in DIO mice. In conclusion, blocking DRD2 induces a deleterious metabolic profile in mice that are resistant to the impact of a high fat diet, whereas activating DRD2 tends to restore a beneficial metabolic profile in mice are that highly susceptible to high fat diet induced corollaries. This suggests that dopaminergic transmission might indeed be involved in the control of metabolic phenotype.

Antipsychotic drugs are associated with the development of insulin resistance in humans and animals. Most reports though have been complicated by weight gain, making it difficult to determine any direct impact of the drugs on glucose and lipid metabolism. Therefore, in **chapter 5** we analyzed the short-term effects of 2 antipsychotic drugs to determine the mechanism(s) underlying deregulation of glucose and lipid metabolism. Healthy, normal weight, men received olanzapine or haloperidol treatment for 8 days. Olanzapine hampered insulin sensitivity, while haloperidol did not have a significant impact. Olanzapine specifically reduced insulin-stimulated glucose disposal, while endogenous glucose production was not affected. Also, lipolysis was not affected by either drug. Olanzapine, but not haloperidol, decreased fasting plasma free fatty acids and hampered the insulin-induced decline of plasma free fatty acids and triglyceride concentrations. Neither drug induced body weight gain or an increase in adiposity. In conclusion, short-term olanzapine promotes deregulation of glucose and lipid metabolism without changes in body weight and adiposity.

Long-term bromocriptine treatment improves glucose and insulin metabolism in obese and insulin resistant animal models and humans; the mechanism underlying the beneficial impact of bromocriptine treatment however is not known. Therefore, the aim of **chapter 6** was to elucidate this mechanism. Bromocriptine acutely induced glucose intolerance in wt C57Bl6 mice. This effect was associated with decreased insulin levels. Furthermore, bromocriptine reduced both the first- and second phase glucose-stimulated insulin response in mice. Also, in INS-1E cells, bromocriptine inhibited glucose-stimulated insulin secretion. Mechanistically, neither cellular energy state nor cell membrane depolarization were affected by bromocriptine, but intracellular cAMP levels

were significantly reduced. Surprisingly, the DRD2 antagonist domperidone was not able to counteract the effect of bromocriptine either in mice or INS-1E cells; yohimbine, an α2-adrenergic antagonist however, abolished the bromocriptine-induced inhibition of insulin secretion in INS-1E cells. In conclusion, bromocriptine acutely suppresses insulin secretion by a (mainly) DRD2-independent mechanism, involving direct activation of pancreatic α2-adrenergic receptors. We believe bromocriptine treatment promotes β-cell 'rest', thereby preventing prolonged insulin hypersecretion and subsequent cell death. In the long-term, this may improve insulin secretion.

All together, the studies described in this thesis contribute to our understanding of the complex interaction between dopaminergic signaling and disturbances in glucose metabolism. We showed that altered dopaminergic parameters associated with obesity are due to mechanisms other that diet composition. But changes in dopaminergic signaling may set the stage for metabolic corollaries of high fat feeding and may be involved in the beneficial impact of calorie restriction. We also demonstrated that inhibiting DRD2 activation may affect glucose homeostasis independent of its impact on body weight. The underlying mechanisms include a reduction in physical activity and a direct effect on insulin sensitivity. In addition we provided evidence that the mechanism by which long term stimulation of DRD2 activation improves glucose metabolism is, paradoxically, inhibition of insulin secretion. We believe these findings may offer new ideas for strategies to prevent or treat diabetes mellitus type 2

Samenvatting

Het dopaminerge systeem is betrokken bij de controle over een heel scala aan fysiologische functies, variërend van motorische activiteit tot de productie van hormonen en het teweeg brengen van gevoelens van beloning. Recentelijk is het dopaminerge systeem ook in verband gebracht met het glucose en insuline metabolisme. Glucose niveaus in het bloed worden strikt gereguleerd om enerzijds te voorkomen dat organen beschadigd worden door te hoge glucose niveaus en anderzijds om te voorkomen dat de niveaus te laag worden waardoor organen die van glucose afhankelijk zijn, zoals de hersenen, niet meer voldoende brandstof hebben. Insuline is een van de belangrijkste spelers in deze regulatie van bloed glucose niveaus; het remt de productie van glucose en stimuleert tegelijkertijd de opname van glucose uit het bloed. Het netto effect van insuline is daarom een verlaging van het bloed glucose niveau. Diabetes mellitus type 2 wordt gekenmerkt door insuline resistentie en een verminderde insuline productie. Gedurende de beginfase van de ontwikkeling van diabetes, als weefsels al ongevoelig zijn voor de werking van insuline, is de productie van dit hormoon, door β-cellen in de pancreas, verhoogd om te zorgen dat het effect van insuline gehandhaafd blijft ondanks de resistentie van de weefsels. Als β-cellen echter niet langer in staat zijn om voldoende insuline te produceren om het effect van de resistentie op te heffen, zullen de bloed glucose niveaus stijgen en is diabetes een feit. Dit zal, als het onbehandeld blijft, leiden tot velerlei complicaties en mogelijk zelfs de dood.

Een grote hoeveelheid literatuur laat zien dat er een verband is tussen het dopaminerge systeem, en dan met name de dopamine receptor D2 (DRD2), en insuline resistentie en diabetes. Zo zijn genetische variaties in het DRD2 gen geassocieerd met veranderingen in het energie en voedingsstoffen metabolisme. Verder leidt blokkering van DRD2 activatie tot gewichtstoename en diabetes, terwijl stimulering van de DRD2 activatie de glucose en insuline stofwisseling verbetert. Daarnaast is ook de dopaminerge transmissie in dikke en insuline resistente mensen en diermodellen veranderd. Ondanks alles wat al bekend is, blijven veel aspecten van de functionele relatie tussen diabetes en het dopaminerge systeem onduidelijk. In dit proefschrift hebben wij geprobeerd de wederzijdse beïnvloeding van dopamine D2 receptoren en het glucose metabolisme te ontrafelen en de onderliggende mechanismen te begrijpen.

Bij onze studies hebben wij gebruik gemaakt van wild type C57Bl6 muizen. Als deze muizen een aantal weken hoog vet voer te eten krijgen, ontwikkelen zij overgewicht, insuline resistentie en een fenotype dat erg veel lijkt op diabetestype 2 bij mensen. Daarom is deze muizen stam een waardevol diermodel om de ontwikkeling van diabetes in te bestuderen. We hebben ook gebruik gemaakt van de INS-1E cellijn die voort gekomen is uit een insuline producerende β-cel tumor van een rat. De fysiologische functies van β-cellen

zijn in deze cellijn bewaard gebleven, wat dit tot een waardevol model maakt om de insuline secretie te bestuderen in vitro.

Hoog vet voeding bevordert het ontstaan van overgewicht en insuline resistentie. Omdat overgewicht geassocieerd is met een verminderde DRD2 expressie en een verhoogde dopamine productie en blokkering van DRD2 activatie leidt tot overgewicht, speculeerden wij dat hoog vet voeding de dopaminerge transmissie zou kunnen veranderen en via deze route overgewicht en een verstoring van het glucose metabolisme zou kunnen induceren. Om dit te onderzoeken, hebben we in **hoofdstuk 2** de rol van het dopaminerge systeem bij het ontstaan van hoog vet dieet geïnduceerde gewichtstoename en insuline resistentie bestudeerd. Wild type C57Bl6 muizen kregen hiervoor gedurende 4 weken een hoog vet dieet. Dit dieet leidde tot een gewichtstoename in deze muizen en een afname van de insuline gevoeligheid. Ondanks het effect op het metabole profiel, veroorzaakte het hoog vet dieet geen veranderingen in de dopamine productie in de hypothalamus van, gevoede of gevaste, muizen. Ook de genexpressie van de dopamine receptor D1 en D2, de dopamine transporter en tyrosine hydroxylase in de hypothalamus was niet beïnvloed door de hoog vet voeding. Hoog vet dieet geïnduceerde metabole veranderingen zijn dus onafhankelijk van veranderingen in verschillende parameters van het dopaminerge systeem. Daarom hebben wij geconcludeerd dat de veranderingen in dopaminerge parameters die gevonden worden in dikke mensen en diermodellen, waarschijnlijk een andere oorzaak hebben dan de dieet samenstelling.

Beperking van de inname van calorieën is de meest effectieve manier om langer en gezonder te leven. Calorische beperking is ook in staat de insuline gevoeligheid te verbeteren en het leeftijdsgebonden verlies van DRD2 receptoren te vertragen. Overgewicht en insuline resistentie zijn geassocieerd met een afname van DRD2 expressie. Blokkade van de DRD2 induceert gewichtstoename en insuline resistentie, terwijl activatie van de DRD2 insuline gevoeligheid stimuleert. Gezien deze invloed van de dopamine receptor D2 op het metabolisme veronderstelden wij dat dopamine receptoren betrokken zouden kunnen zijn bij het gunstige effect van calorische beperking op het glucose metabolisme. We hebben deze hypothese onderzocht in **hoofdstuk 3**. Wild type C57Bl6 muizen kregen hiervoor een hoog vet dieet; een deel van de muizen kon ongelimiteerd eten, terwijl het andere deel van de muizen slechts een beperkte hoeveelheid van dit voer kreeg. De helft van de calorisch beperkte muizen werd ook nog continu behandeld met het antipsychoticum haloperidol om de DRD2 activatie te remmen. De calorisch beperkte muizen waren gevoelig voor de effecten van zowel glucose als insuline in vergelijking tot de muizen die onbeperkt hoog vet voer kregen. Haloperidol leidde tot een kleine

gewichtstoename bij de calorisch beperkte muizen. Daarnaast heeft haloperidol de positieve invloed van de calorische beperking op de glucose gevoeligheid compleet teniet gedaan en de insuline gevoeligheid van de calorisch beperkte muizen gedeeltelijk gereduceerd. De metabole verschillen tussen de calorisch beperkte of niet-beperkte muizen werden niet vergezeld door veranderingen in DRD2 binding in de hypothalamus. Calorische beperking biedt dus bescherming tegen de negatieve metabole effecten van hoog vet voeding; gelijktijdige blokkade van de DRD2 is echter in staat deze beschermende werking sterk te beperken. Hoewel dit suggereert dat dopamine receptoren betrokken zijn bij de positieve invloed van calorische beperking op de glucose stofwisseling, pleit de onveranderde DRD2 binding na beperkte inname van het hoog vet dieet toch tegen deze suggestie.

Hoewel hoog vet voeding over het algemeen overgewicht en insuline resistentie veroorzaakt in knaagdieren, kunnen individuele dieren van eenzelfde stam toch heel verschillend reageren. Aan de hand van de gewichtstoename na een aantal weken hoog vet voeding, kunnen knaagdieren onderverdeeld worden in de groepen 'dieet gevoelig' (Diet Induced Obese; DIO) en 'dieet resistent' (Diet Resistent; DR). Heel interessant is dat de dopaminerge neurotransmissie verschilt bij deze DIO en DR dieren, zelfs al voordat hoog vet geïnduceerde gewichtstoename optreed. In vergelijking tot DR dieren, hebben DIO dieren een verlaagde DRD2 expressie en dopamine turnover. Dit suggereert dat aangeboren veranderingen in dopaminerge transmissie een rol zouden kunnen spelen bij de gevoeligheid van individuele dieren voor de negatieve metabole effecten van hoog vet voeding. Wij hebben dit onderzocht in **hoofdstuk 4**. Uitgaande van de gewichtstoename van individuele wild type C57Bl6 muizen op een hoog vet dieet, hebben wij deze muizen geclassificeerd als DIO of DR. Vervolgens kreeg de helft van de DIO muizen bromocriptine toegediend om DRD2 activatie te stimuleren en kreeg de helft van de DR muizen haloperidol om DRD2 activatie te remmen. Vergeleken met de DR muizen, waren DIO muizen zwaarder, hadden ze een verhoogde insuline concentratie in hun bloed en waren ze insuline resistent. De haloperidol behandeling verhoogde de bloed glucose concentratie en verminderde de insuline gevoeligheid in DR muizen. Daarnaast verminderde haloperidol ook drastisch de fysieke activiteit en het energie verbruik van deze dieren. Bromocriptine leek, aan de andere kant, een gewichtsafname te veroorzaken en een verbetering van de insuline gevoeligheid in DIO muizen. Samenvattend leidt blokkade van DRD2 activatie tot een slecht metabool profiel in muizen die van nature ongevoelig zijn voor de effecten van een hoog vet dieet, terwijl stimulatie van DRD2 activatie een gunstig metabool profiel lijkt te herstellen in muizen die zeer gevoelig zijn voor de consequenties van een hoog vet dieet. Dit suggereert dat dopaminerge transmissie inderdaad betrokken is bij de controle van metabole profielen.

Het gebruik van antipsychotische medicijnen, die met name gericht zijn op het blokkeren van DRD2 activatie, is geassocieerd met het ontstaan van insuline resistentie in mensen en dieren. Omdat de meeste studies echter ook gewichtstoename rapporteren, blijft het onduidelijk of deze medicamenten een direct invloed hebben op de glucose en vet stofwisseling of dat ze enkel een gewichtstoename veroorzaken, wat dan vervolgens leidt tot een verstoring van de glucose en vet huishouding. Om hier meer duidelijkheid over te krijgen, hebben wij in **hoofdstuk 5** de korte termijn effecten van 2 antipsychotische medicijnen bestudeerd om zo het mechanisme te ontdekken dat ten grondslag ligt aan de verstoring van het voedingsstoffen metabolisme. Gezonde mannen met een normaal lichaamsgewicht kregen hiervoor gedurende 8 dagen een behandeling met olanzapine of haloperidol. Olanzapine verlaagde de insuline gevoeligheid, terwijl haloperidol geen significant effect had. Met name de insuline gestimuleerde glucose opname was verlaagd door olanzapine. Zowel olanzapine als haloperidol hadden geen invloed op de afbraak van vetten. Maar, olanzapine, in tegenstelling tot haloperidol, verlaagde de hoeveelheid vrije vetzuren in het bloed tijdens vasten en verstoorde de insuline geïnduceerde afname van vrije vetzuur- en triglyceride concentraties in het bloed. Geen van de medicamenten resulteerde in gewichtstoename of een toename van de vetmassa. Olanzapine is dus in staat om de glucose en vet stofwisseling te verstoren zonder dat daar veranderingen in gewicht of vetmassa voor nodig zijn.

Langdurige behandeling met bromocriptine (stimuleert DRD2 activatie) verbetert het metabole profiel van dikke insuline resistente mensen en diermodellen; het onderliggende mechanisme is echter nog onbekend. Het doel van **hoofdstuk 6** was daarom om meer inzicht in te krijgen in de gunstige invloed van dit medicament. Bromocriptine leidde acuut tot glucose intolerantie in wild type C57Bl6 muizen. Dit effect ging gepaard met een verlaagde insuline concentratie. Verder reduceerde bromocriptine zowel de eerste als tweede fase insuline respons na glucose stimulatie. Ook in INS-1E cellen remde bromocriptine de glucose-gestimuleerde insuline afgifte. Wat betreft het mechanisme was noch de energie status van de cel noch de celmembraan depolarisatie aangedaan door bromocriptine, maar de intracellulaire cAMP niveaus waren significant lager. Tot onze verrassing was de DRD2 antagonist domperidone niet in staat het effect van bromocriptine in zowel muizen als INS-1E cellen op te heffen; yohimbine, een α2-adrenerge antagonist, was daarentegen wel in staat de bromocriptine geïnduceerde remming van de insuline secretie in INS-1E cellen op te heffen. Bromocriptine onderdrukt dus acuut de insuline productie door middel van een (voornamelijk) DRD2-onafhankelijk mechanisme waar directe activatie van α2-adrenerge receptoren bij betrokken is. Onze hypothese is dat bromocriptine op deze wijze β-cellen

beschermt tegen langdurige insuline 'overproductie' en daarmee geassocieerd β-cel falen, waardoor op de langere termijn de insuline secretie juist verbeteren zal.

Samengenomen dragen de studies, beschreven in dit proefschrift, bij aan ons begrip van de complexe interactie tussen dopaminerge activiteit en verstoringen van het glucose metabolisme. We hebben laten zien dat veranderingen in dopaminerge parameters, geassocieerd met overgewicht, het resultaat zijn van andere zaken dan dieet samenstelling. Veranderingen in dopaminerge activiteit zouden echter wel ten grondslag kunnen liggen aan de metabole consequenties van hoog vet voeding en ze zouden ook betrokken kunnen zijn bij het gunstige effect van calorische beperking. We hebben ook laten zien dat remming van de DRD2 activatie het glucose metabolisme kan beïnvloeden onafhankelijk van veranderingen in het lichaamsgewicht. Onderdeel van het onderliggende mechanisme is een afname van de fysieke activiteit en een directe negatieve invloed op de insuline gevoeligheid. Daarnaast hebben we ook bewijs geleverd dat het mechanisme waardoor langdurige stimulatie van DRD2 activatie leidt tot een verbeterd glucose metabolisme, in tegenstelling tot de verwachting, een remming van de insuline secretie is. Wij geloven dat deze observaties aanknopingspunten bieden voor nieuwe maatregelen en strategieën voor het voorkomen dan wel genezen van diabetes mellitus type 2.

List of Abbreviations

AGRP	Agouti-Gene-Related Protein
AL	Ad Libitum
AMPK	AMP-activated Protein Kinase
AP	Antipsychotic
AR	Adrenergic Receptor
ARC	Nucleus Arcuatus
AUC	Area Under the Curve
BC	Bromocriptine
BMI	Body Mass Index
BW	Body Weight
cAMP	cyclic AMP
CCK	Cholecystokinin
CR	Calorie Restriction
CREB	cAMP Response Element Binding protein
DAT	Dopamine Transporter
DIO	Diet Induced Obese
DMH	Dorsomedial Hypothalamus
DP	Domperidone
DR	Diet Resistant
DRD1	Dopamine Receptor D1
DRD2	Dopamine Receptor D2
EGP	Endogenous Glucose Production
ERK1/2	Extracellular Signal-Regulated Kinases 1/2
FFA	Free Fatty Acids
GIR	Glucose Infusion Rate
GLUT	Glucose Transporter
GSIS	Glucose Stimulated Insulin Secretion
HF	High Fat
HG clamp	Hyperglycemic clamp
HP	Haloperidol
ipGTT	intraperitoneal Glucose Tolerance Test
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
KRBH buffer	Krebs-Ringer Bicarbonate HEPES buffer
LHA	Lateral Hypothalamic Area
LF	Low Fat
LPL	Lipoprotein Lipase
MMTT	Mixed Meal Tolerance Test
mRNA	messenger RNA

NEFA	Non-Esterified Fatty Acids
NPY	Neuropeptide Y
NS	Not Significant
P	Placebo
PKA	cAMP dependent Protein Kinase A
POMC	Pro-opiomelanocortin
PVN	Paraventricular Nucleus
PYY	Peptide YY
Ra	Rate of Appearance
Rd	Rate of Disappearance
RQ	Respiratory Quotient
RT-PCR	Real-Time Polymerase Chain Reaction
SD	Standard Deviation
SEM	Standard Error of the Mean
SNP	Single-Nucleotide Polymorphism
TG	Triglyceride
TH	Tyrosine Hydroxylase
VMH	Ventromedial Hypothalamus
WHO	World Health Organization
wt	wild type

Curriculum Vitae

Judith Elisabeth de Leeuw van Weenen werd geboren op 11 juli 1980 te Zürich (Zwitserland). Na het behalen van haar VWO diploma aan het Eckart College te Eindhoven in 1998, startte zij met haar studie Medische Biologie aan de Universiteit van Utrecht. In 1999 behaalde zij haar propedeuse. Tijdens haar studie heeft zij een drietal stages gedaan, waarvan twee bij het Universitair Medisch Centrum Utrecht. Zij heeft eerst onderzoek gedaan bij de Afdeling Experimentele Neurologie onder leiding van drs. G.J. Groeneveld en Prof. dr. P.R. Bär naar de effecten van zink en TCH 346 in een transgeen muismodel voor ALS. Haar tweede onderzoeksstage heeft zij gedaan bij het Eijkman Winkler Instituut onder leiding van Dr. C.J. de Haas waar zij gekeken heeft naar de aminozuren verantwoordelijk voor de binding van CHIPS aan de C5a en de formylated peptide receptor. Daarnaast heeft zij nog een extra stage gedaan bij het Department of Microbial Pathogenesis and Vaccine Research van het German Research Centre for Biotechnology te Braunschweig, Duitsland, onder leiding van Dr. E. Medina. Ze heeft hier onderzoek gedaan naar de effecten van veroudering op de gevoeligheid van muizen voor Groep A Streptokok infecties. In december 2003 behaalde zij haar doctoraal diploma. Aansluitend startte zij met haar promotieonderzoek bij de afdeling Endocrinologie & Stofwisselingsziekten van het Leids Universitair Medisch Centrum (LUMC) onder leiding van Prof. dr. H. Pijl. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sinds 2009 is zij tevens werkzaam als informatie wetenschapper voor Orphanet Nederland.

List of Publications

de Leeuw van Weenen JE, Parlevliet ET, Schröder-van der Elst JP, van den Berg SA, Willems van Dijk K, Romijn JA, Pijl H. Pharmacological modulation of dopamine receptor D2 mediated transmission alters the metabolic phenotype of diet induced obese and diet resistant C57Bl6 mice. *Exp Diabetes Res* 2011;2011:928523

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Dankwoord

Eindelijk is het zover: mijn proefschrift is af! En daarmee komt er ook een einde aan mijn promotietraject. Het was zeker niet altijd makkelijk, maar, mede dankzij de hulp van velen, heb ik alles goed af kunnen ronden en kan ik nu terugkijken op een leuke, uitdagende en leerzame tijd.

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Liefs,
Judith

Notes

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