

Metabolic and endocrine alterations in histamine-deficient HDC-KO mice

PhD thesis booklet

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Budapest
2010

Introduction

It has been almost a hundred years since the discovery of histamine, first described by Dale and Laidlaw. However, this small molecule with many roles is still subject of intensive research. It has several central and peripheral functions: it mediates inflammatory processes and allergic reactions, regulates acid secretion of the stomach, affects smooth muscle contraction, increases blood vessel permeability, and acts as a neurotransmitter, neuromodulator and a regulator of metabolic processes. Four histamine receptors are known to date: H1, H2, H3 and H4 receptors, which may be expressed on distinct cells or coexpressed on the same cells. They use distinct signaling pathways and thus mediate a wide range of histamine effects.

Histamine is formed by decarboxylation of L-histidine while releasing CO₂, a reaction catalyzed by a single enzyme, histidine decarboxylase (HDC). The fact that histidine synthesis is a single step catalyzed by a single enzyme, and thus can be blocked by inhibiting the enzyme, has always had an important role in histamine research. The most widely used histamine inhibitors are α -methyl-histamine and α -fluoromethyl-histidine (α -FMH), which are classified as enzyme activated irreversible inhibitors.

Even though histamine seems to be so crucial for life, HDC deficiency is not incompatible with life since a number of mechanisms exist to compensate for the lack of histamine. The lack of HDC enzyme leads to complete histamine deficiency in histidine decarboxylase knock-out (HDC^{-/-}) mice. HDC knock-out mice were created by Canadian and Japanese scientists in a joint effort with the Department of Genetics, Cell and Immunobiology at Semmelweis University. When histamine deficient mice are kept on a low-histamine diet, histamine will be barely detectable in tissues, and therefore will have no physiological effects.

The pleiotropic effects of histamine are underscored by the fact that its deficiency will lead to a significantly altered phenotype in mice. As expected, the immunophenotype of histamine deficient mice is significantly altered as well. Previous literature data, as well as experiments with HDC^{-/-} mice show that histamine shifts the immune response to Th2. HDC^{-/-} mice show alterations in allergic skin reaction and other immune responses compared to wild type, as well as in the morphology, number and granulation of their mast cells. H1 receptor expression is unchanged in HDC^{-/-} mice, whereas H2 histamine receptor expression in brain, stomach, ileum, heart, liver and skin tissue samples is reduced, but the decrease could be compensated by the administration of exogenous histamine.

The role of histamine in gastric acid secretion is demonstrated by the fact that baseline gastric acid secretion is reduced compared to control, but it can be increased to two-fold by administering exogenous histamine. Histamine deficiency causes significant alterations in the bone formation of

mice. Higher bone density was observed in HDC^{-/-} mice, bone thickness and bone formation increased, and the number of osteoclasts decreased significantly. HDC knock-out mice were more protected against ovariectomy-induced osteoporosis due to osteoclast inhibition and an increase in calcitriol synthesis.

In terms of central histamine effects, a number of differences were noted between the two genotypes. Prolonged histamine deficiency caused alterations in the behavior and the sleep-wake cycle of mice, their time spent awake significantly decreased. Locomotory activity of HDC^{-/-} mice significantly reduced, their circadian rhythm changed, the expression of “clock genes” in the cortex and the striatum decreased. Results were different in various kinds of memory tests: e.g., in the water maze test, which is based on negative feedback, HDC^{-/-} mice performed better, whereas in the nonreinforced relational object memory, HDC^{-/-} mice performed worse than wild type controls. In HDC^{-/-} mice treated with cocaine, it was shown that their exploration behavior decreased, but they were hyperactive. These results do not support a role for histamine in the inhibition of reward behavior.

Our animal model, the HDC^{-/-} mouse enables the study of the metabolic phenotype resulting from life-long chronic histamine deficiency and of the central and peripheral physiological mechanisms involved in its regulation, and of the role of histamine in the regulation of sexual behavior and reproduction.

Several factors are involved in the regulation of energy homeostasis, some of which regulate food intake, while others energy expenditure. Elements of regulatory circle responsible for the development of the metabolic phenotype act in part on the periphery, while on the other hand are involved in central regulation. Deficiencies in the feedback mechanisms involved in the process may result in weight gain (leptin and insulin resistance) or in anorexia. Below I will give a description of the elements of these regulatory circles which I studied in my PhD thesis in more detail.

Histamine is an anorexigenic factor. In the central nervous system, histamine plays an important role in the regulation of homeostatic functions such as energy metabolism (thermoregulation), food intake and metabolic processes (lipolysis), sleep-wake cycle, and cardiovascular control. It has additional roles in the regulation of behavior, learning and memory functions and emotions.

Food intake and energy metabolism are complex: numerous neuropeptides and neurotransmitters are involved in its central regulation. Hypothalamus has a central role in its regulation, but brain stem structures also have an important role in mediating the signals from the periphery, and in the formation of appropriate visceromotor responses. Emotional, rewarding and aversive stimuli related to feeding are all important. In their regulation, the modulatory roles of cortical and limbic structures are important.

Histamine acts as a neurotransmitter in the central nervous system. Histaminergic neurons are found in the tuberomamillary region of the posterior basal hypothalamus in five well-defined clusters (E1–E5), sending axons to the entire central nervous system. Histaminergic neurons in the tuberomamillary region project to almost every area in the brain that is involved in the regulation of energy homeostasis. Thus, histamine positive axons can be found in nucleus arcuatus, in the paraventricular nucleus, around the ventromedial and lateral hypothalamus and in the spinal cord.

Insulin and leptin are the two most important metabolic signals whose main function is to regulate energy homeostasis in the central nervous system, in the hypothalamus. Insulin, produced by the pancreas, was the first hormone that was shown to regulate body weight when acting in the central nervous system, causing decreased energy intake. Insulin receptors can be found in nucleus arcuatus, a major component in feeding regulation. Leptin is an anorexigenic factor produced by adipocytes. Leptin receptor belongs to the type I cytokine receptor family. The *ob* gene encodes 6 leptin receptor isoforms (Ob-Ra-f), most of which are formed by alternative splicing. The JAK-STAT signaling pathway is activated in response to the full-length Ob-Rb isoform only. A considerable Ob-Rb mRNA expression was shown in nucleus arcuatus, dorsomedial hypothalamus, ventromedial hypothalamus and ventral premamillar nucleus. Ob-Rb mRNA expression was also shown in the periventricular hypothalamic nucleus and lateral hypothalamic area, and, to a lesser extent, in the paraventricular nucleus as well. Ob-Rb mRNA expression was found in a number of extrahypothalamic sites as well: in thalamus, Purkinje cells, cerebellar granular cells, piriform cortex, ventral tegmental area, and in the NTS and DMV areas of the brainstem. Ob-Rb protein was detected where neuropeptides and neurotransmitters regulating food intake, appetite and energy balance were produced. The shorter Ob-Ra isoform is also expressed in high quantities in brain, mostly in choroid plexus and in capillaries, where it may play a role in the passing of leptin through the blood-brain barrier.

Obesity is often caused by the development of leptin resistance, which can have a number of reasons: increased leptin expression, decrease in receptor numbers due to elevated leptin levels, receptor desensitization, inhibition of the JAK-STAT signaling pathway by SOCS3, decreased STAT3 activation, reduced passing of leptin through the blood-brain barrier, and a change in the number of soluble leptin receptors (Ob-Re) in the plasma.

The connection between leptin and histamine is mutual, but probably indirect, since the tuberomamillary nucleus, the localization of histaminergic neurons, is not sensitive to leptin. However, histaminergic neurons in the tuberomamillary nucleus project to virtually all areas of the brain that are involved in the regulation of energy homeostasis. Neuropeptides involved in feeding regulation can be largely divided into two groups: orexigenic peptides that increase food intake and decrease energy expenditure, and anorexigenic peptides which have opposite effects. In the nucleus

arcuatus of hypothalamus there are orexigenic NPY/AgRP-neurons and anorexigenic POMC neurons that express leptin receptors. Leptin has opposite effects on these cells: it stimulates POMC neurons whereas inhibits NPY neurons. Similar to NPY, orexin, which is produced in the lateral posterior hypothalamus, has orexigenic effects. The fact that tuberomammillary neurons are densely innervated with orexin containing axons, implies the existence of a feedback with functional relationship.

Histamine has a role in the regulation of both sexual behavior and reproduction. Both central and peripheral effects have been reported. Histamine deficient mice can be maintained by homozygous crossing and the crossing of heterozygous animals will yield Mendelian ratios. Even though $HDC^{-/-}$ mice are fertile and births follow Mendelian ratios, their reproductive capacity is reduced. When homozygous $HDC^{-/-}$ mice are crossed, few pregnancies will occur within a couple of days after mating. Behavioral alterations in male mice underlie the reduced reproductive capacity. This problem will also be studied in my thesis.

Aims

To construct a histaminergic neuron in situ hybridisation probe.

To describe the metabolic phenotype of the histamine-deficient, histidine decarboxylase (HDC) knock-out mice.

- Quantification of food intake, body weight and fat pads
- Determination of metabolic signals in the blood

To study the functional role of histamine in the regulation of energy homeostasis by investigating the mechanisms that regulate the development of metabolic phenotype in HDC^{-/-} mice

- Description of the ability to metabolize energy stores
- Examination of the insulin- and leptin system
- Examination of the leptin level and leptin resistance
 - Determine leptin gene expression in the epididymal, subcutaneous, and brown adipose tissue of HDC knock-out and wild type (WT) mice
 - Determine leptin receptor (Ob-Rb) gene expression in hypothalamus and liver
 - Investigate the presence of functional leptin receptor and the Jak-STAT signal transduction pathway by detecting phosphorylated STAT
- Mapping of histaminergic neurons
- Mapping of neuronal activation following histidine challenge
- Description of orexigenic (NPY, orexin) and anorexigenic (POMC) gene expression in the hypothalamus

Investigation of the role of histamin in reproductive functions in male HDC^{-/-} animals

- Quantification of the level of androgen hormones
- Investigation of brain GnRH neurons
- Description of testis morphology

Materials and methods

Animals

Male wild-type and HDC^{-/-} mice (CD1/ 129Sv background) were kept under controlled temperature and lighting conditions. They were maintained on histamine-free diet (<0.6 nmol histamine/g nutrition, Altromin, Germany) for 14 days before experiments were performed. The HDC^{-/-} animals were generated by Ohtsu and coworkers in 2001. To confirm the genotype of the mice, genomic DNA was obtained from tail samples and analysed by PCR.

Metabolic experiments

To prevent coprophagy, the animals were housed individually in cages with wire bottom for the metabolic tests.

-Nutrition was followed by measuring *body weight* weekly from weaning till euthanasia. The weight of the adipose tissues (subcutaneous, epididymal and brown) were measured with milligram accuracy.

-To assess metabolic responses in a challenge situation, WT and HDC^{-/-} mice were fasted for 5 hours, and then transported to a cold room and kept at 4 °C for 90 min. Their rectal temperature was measured every 30 min, and their body weight was registered before and after the challenge.

Analysis of blood samples

-*Blood samples* were obtained by puncture of the retrobulbar venous plexus. Larger quantities were obtained by decapitation.

-*Blood glucose levels* were determined using the One Touch glucose monitoring system (Lifescan, Milpitas, CA).

-Before *glucose tolerance test* the animals were fasted for 18 hours and given an ip injection of D-glucose (1 mg/g body weight). Blood samples were obtained from the retrobulbar plexus immediately before and 30, 60, and 120 min after glucose injection and blood glucose levels were measured. Insulin secretory response to glucose challenge was determined from the same samples using the Mouse Insulin Ultrasensitive ELISA kit (DRG Instrument GmbH, Marburg, Germany).

-To assess the glucose response to insulin injection, mice received an ip injection of insulin (Actrapid HMge, Novo Nordisk, Bagsværd, Denmark) at a dose of 1 U/kg body weight immediately after obtaining blood sample to determine basal glucose level. Further samples were withdrawn 15, 30, and 60 min after insulin challenge.

-From plasma and serum we measured the blood level of *leptin, corticosterone, triglycerides, cholesterol, HDL, testosterone* and *estradiol* with test kits.

-To measure the steroid levels, testis and adrenal glands were homogenised, extracted with dichloromethane and separated with paper chromatography in order to eliminate crossreaction of different steroids during RIA measurements.

Morphologic examination/ Histological procedures

-The weight of the *brown and epididymal adipose tissues* were measured, and then tissues were fixed in Bouin's fixative, and embedded into paraffin. Ten-micrometer sections were cut and stained with haematoxylin-eosin.

-For *morphologic examination of the brain*, perfusion fixation was used. Serial sections (20-25 µm) in the frontal plane were cut on freezing microtome.

-For *light microscopic* examination *testes* were embedded in paraffin, and haematoxylin-eosin staining was performed. For *electron microscopy*, samples were fixed by immersion in Karnovsky fixative, postfixed with osmium tetroxide, and then embedded in Araldite.

Protein expression studies

-To identify *histamine, c-Fos* and *p-STAT3* expression in the brain, *immunohistochemistry* was performed. To intensify histamine staining, male mice were injected with L-histidine 2 hours before perfusion and fixations was performed according to Wang and Nakai with some modifications. The standard immunohistochemistry protocol for p-STAT3 staining was performed according to Munzberg et al.

-The expression of *UCP-1* protein in brown adipose tissue was measured by *immunoblotting* after tissue extraction with a chloroform: methanol mixture.

Gene expression studies

-As part of my work, a *HDC probe* was constructed for *in situ hybridisation*. The other probes used for *GnRH, POMC, NPY, and orexin* were obtained from various laboratories as plasmids. For the HDC probe, RNA was isolated from the hypothalamus of wild type male mice with TRI Reagent, and complementary DNA (cDNA) was synthesised. The cDNA was used as template to synthesize HDC PCR fragments with touch-down PCR technique. These fragments were ligated into the pGEM-T Easy vector. Competent E. coli DH5α bacteria line were used for the transformation. Correct insert orientation was checked with enzymatic digestion. For *in situ* hybridisation the vectors containing the intron and/or exon sequences were linearised, and transcribed in antisense direction with RNA polymerase (T3, T7, and SP6) *in vitro*. The probes were labelled with uridine 5'-[α-³⁵S] triphosphate (NEN, USA), and hybridisation was performed according to Simmons and coworkers. After post-hybridisation steps an Amersham β Max film or an Image Plate was placed

on the slides. The Image plates were read on an FLA-3000 Phosphoimager device, with BAS Reader (3.01) and evaluated with its own software (AIDA). Slides were exposed to X-ray films and dipped in NTB-2 nuclear emulsion (Kodak) and exposed for 3-5 days, developed in D-19 developer. The results were evaluated by semi-quantitative densitometry with NIH Image software. Nissl dye stained subdivisions were selected under a light microscope and the density was then measured under darkfield microscope.

-The level of *UCP-1 mRNA* in brown adipose tissues was measured by *Northern blot* analysis. Total RNA from brown adipose tissue was purified by the method of Chomczynski and Sacchi, separated on a denaturing gel, then transferred onto a Hybond N membrane (Amersham, UK) using the capillary transfer method. The probes were labeled by random priming (Multiprime Labeling Kit, Amersham, UK) using ^{32}P -dCTP (Izinta). All comparisons were made between RNA samples hybridised on the same filter and normalised to the content of β -actin mRNA detected in each individual sample. After hybridisation, blots were exposed to X-ray films (Kodak XAR, USA) for 1-2 days using intensifying screens. Data were analysed with the ScionImage analysis software (Scion Corporation, USA).

-*Leptin expression* in epididymal, subcutaneous and brown adipose tissue; and *leptin-receptor expression* (Ob-Rb) in the hypothalamus was measured by *real-time PCR*. RNA was isolated from the samples with TRI Reagent and then cDNA was synthesised (RevertAid First Strand cDNA kit, Fermentas). The expression levels of mRNAs were verified using a TaqMan assay run on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Branchburg, NJ, USA).

Statistical analysis

Statistical analysis was performed using repeated measures STATISTICA 6.0 software. Differences between the groups was demonstrated using ANOVA followed by Dunnett's *post hoc* tests. When comparisons were restricted to two experimental groups, a *t* test was used. Plasma glucose concentrations after ip injections of glucose or insulin and insulin secretory response to ip glucose were analyzed by repeated-measures of ANOVA.

Results

Cell clusters in the tuberomamillary region corresponding to E1-E5 cell groups were identified by HDC *in situ* hybridization, using our RNA probe. No hybridization signal in the tuberomamillary region of HDC knock-out animals could be detected.

Metabolic phenotype of HDC^{-/-} animals

Body weight, fat pads, energy balance

Up to 10 weeks of age, the body weights of WT and HDC^{-/-} mice were indistinguishable. The difference in body weight increased with age and knock-out mice weighed 13% more than WT control at the age of 16 weeks. Knock-out mice older than 30 weeks were dramatically overweight compared with WT controls (WT: 39.10 ± 1.90 g, HDC^{-/-}: 46.40 ± 2.31 g; n=5).

HDC^{-/-} animals (age 13 wk) gained more weight (WT: 0.74 ± 0.3 g / week vs. HDC^{-/-}: 2.1 ± 0.58 g / week), even though their food intake was smaller than that of wild type animals (WT: 43.2 ± 1.52 g / week vs. HDC^{-/-}: 40.8 ± 1.36 g / week). Thus, the HDC^{-/-} animals had higher caloric efficiency than aged-matched controls.

At the dissection we observed that the overweight of the HDC^{-/-} mice was due to the increased amount of epididymal adipose tissue. Measurements showed that the amount of white epididymal and interscapular brown adipose tissues were twice greater in the knock-out animals compared with controls, without significant differences in the thickness of subcutaneous tissue. At the same time, epididymal adipose tissue underwent structural alterations, the size of the individual cells had increased (WT: 329.75 ± 25.26 μm², HDC^{-/-}: 551.15 ± 25.56 μm²). Significant histological alterations were not observed in the brown adipose and subcutaneous tissue.

Description of the ability to mobilize energy

Change in the core body temperature of WT and HDC^{-/-} mice

To measure the *metabolic rate*, we followed the protocol described by Forbes and coworkers by detecting the weight loss after 5 hours of fasting. Although mice lost 6–9% of their initial weight, the differences in body weights of the histamine deficient and WT control group were not statistically significant.

As an indirect measurement of the ability to mobilize energy, we followed the thermoregulatory responses to cold. The baseline core temperature of WT and knock-out animals were not different (WT: 37.50 ± 0.34 °C; HDC^{-/-}: 37.49 ± 0.29 °C). When mice fasted for 5 hours were cold challenged at 4 °C, the core temperature of histamine-deficient mice dropped by 3.82 ± 1.02 °C, whereas that of WT animals fell by only 1.34 ± 0.40 °C by the end of the 90-min test period. Hence,

the histamine-deficient mice have an impaired ability to metabolize energy stores compared to wild type animals.

UCP-1 mRNA and protein in the brown adipose tissue

The energy mobilising ability is characterized by the quantity of the uncoupling protein-1 (UCP-1, thermogenin). Measured by Northern blotting, there was no difference between the expression of UCP-1 in HDC^{-/-} and WT mice kept under standard laboratory circumstances. Immunoblotting also revealed no difference in the amount of UCP-1 protein between the two groups.

The quantity of UCP-1 mRNA increased in both groups when animals were kept at 4 °C for 18 hours, but the rate of the elevation was 3-fold larger in WT animals.

Blood glucose level and insulin secretion

To determine whether the increased adiposity in is accompanied by abnormalities in carbohydrate metabolism, ***glucose tolerance test*** was performed. Basal glucose levels after 18 hours of starvation were slightly but not significantly different between the knock-out and wild type groups (WT: 4.18 ± 0.18 mM, HDC^{-/-}: 3.83 ± 0.24 mM). 30 min after a single ip. injection of D-glucose (1 mg / g body weight), blood glucose level was significantly higher in knock-out mice than in wild-type animals. 30, 60 min after injection plasma glucose levels plateaued, in HDC^{-/-} mice 32, 30% higher, than in wild type animals.

To assess the effects of histamine deficiency on insulin action in vivo (***insulin test***), we gave a single ip. injection of insulin (1 U/kg body weight) to non-fasted animals and then measured plasma glucose concentration every 15 min for an hour. Although basal glucose levels were slightly higher in adult male non-fasted HDC^{-/-} animals than in WT mice, the difference was not significant. Insulin decreased blood glucose levels both in HDC^{-/-} and in WT animals and their glucose clearance curves were comparable.

Glucose-induced insulin response

After 18 hours of fasting, HDC^{-/-} mice had insulin levels almost twice higher than WT mice. 30 minutes after D-glucose was given ip. (1 mg / kg body weight), insulin levels of both HDC^{-/-} and WT animals were elevated and so remained for 120 min, until the end of experiment. In WT and HDC^{-/-} animals, maximum plasma insulin levels were 334% and 85.7% higher, respectively, than measured after fasting.

Changes in hormone levels in response to fasting

When mice were not fasted and kept on normal chow, steady-state plasma insulin levels were comparable between wild-type and knock-out groups (345 ± 53 pM and 361 ± 48 pM, respectively). After a 12-hour fasting, insulin levels decreased in both groups, however insulin levels of HDC^{-/-}

animals remained 20-fold higher than those of WT mice (212.5 ± 46.3 pM and 11.6 ± 2.3 pM, respectively).

Serum leptin levels were significantly higher in $HDC^{-/-}$ than in WT mice (3.60 ± 0.80 ng/ml and 0.64 ± 0.07 ng/ml, respectively) when mice were not fasted and kept on normal chow. In response to 12 hours of fasting, leptin levels decreased by about 50% in wild-type animals, while it remained elevated in the knock-outs.

Morning levels of plasma **corticosterone** were elevated in $HDC^{-/-}$ mice, although the difference was not statistically significant, presumably due to the high variance of the values (WT: 23.03 ± 7.34 ng/ml, $HDC^{-/-}$: 42.68 ± 6.88 ng/ml; $p=0.068$). After 12 hours fasting, corticosterone levels increased to 8-10 times in both genotypes, but difference was not significant.

Serum **triglyceride, cholesterol, and HDL levels** did not differ significantly between $HDC^{-/-}$ and wild-type controls.

Leptin gene expression in adipose tissues of wild type and $HDC^{-/-}$ mice

Plasma leptin levels were found to be higher in histamine deficient mice than in WT animals, most probably due to the lack of negative feedback of histamine. The cause of hyperleptinaemia can be the overexpression of leptin in the adipose tissues. To investigate leptin expression, epididymal, subcutaneous and brown adipose tissues were collected, and leptin expressions were measured by real-time PCR.

In both wild type and $HDC^{-/-}$ mice, the highest leptin expression was detected in epididymal adipose tissue. The expression of leptin in epididymal adipose tissue was about thirteen times higher than in BAT, and three times higher than in subcutaneous adipose tissue. When adipose tissue leptin mRNA expression was compared between wild type and $HDC^{-/-}$ mice, there was a tendency of decreased leptin mRNA expression in the adipose tissue of HDC knock-out animals, which could be a compensation. However, the difference was not significant, which, taken together with the increased amount of adipose tissue in $HDC^{-/-}$ mice, results in an overall increased amount of leptin mRNA, providing a likely explanation for elevated plasma leptin levels.

Leptin receptor (Ob-Rb) expression in the hypothalamus and liver of WT and $HDC^{-/-}$ mice

Possible mechanisms of leptin resistance include downregulation and/or desensitization of leptin receptors, and defects in signalling (pSTAT, SOCS3). The mRNA expression of the signalling form of leptin receptor, Ob-Rb, was measured by real-time PCR. There was no difference in Ob-Rb mRNA expression in hypothalamus or in liver between WT and HDC-knock-out animals.

Changes in STAT3 phosphorylation in WT and HDC^{-/-} mice treated with leptin

There were no significant changes in the expression of leptin or leptin receptors, which could have explained the leptin resistance of HDC deficient mice. This raised the possibility of a decrease in leptin receptor function or in STAT3 phosphorylation, or that of a rapid pSTAT3 dephosphorylation. Therefore, we studied the extent of STAT3 phosphorylation in response to leptin treatment in the hypothalamus of WT and HDC^{-/-} mice. No p-STAT3 immunoreactivity was observed in the hypothalamus of vehicle (saline) treated wild type (WT) and HDC^{-/-} animals. Systemic injection of leptin (1 mg/kg bw.) resulted in STAT3 phosphorylation in various hypothalamic nuclei (nucleus arcuatus, ventromedial, dorsomedial nuclei, a premamillary region, weakly in lateral hypothalamus) in both WT and HDC^{-/-} mice. Systemic injection of Leptin (5 mg/kg bw.) resulted in STAT3 phosphorylation in the dorsal vagal complex (DVC) of WT animals, but only few scattered cells were p-STAT3 positive in HDC^{-/-} animals. In the DVC of WT animals significantly more immunopositive cells occurred than in HDC^{-/-} mice (38.7 ± 4.6 and 14.3 ± 4.9 , respectively, n=5).

Morphological and functional studies in the CNS

Neuronal activity following L-histidine challenge

To test the function of HDC gene in the central nervous system, mice were treated with L-histidine, substrate of the HDC enzyme. L-histidine can pass through the blood-brain barrier, and it has been shown to increase the activity of HDC and concentration of histamine in the CNS. We took advantage of this finding to reveal histamine-responsive profiles in the hypothalamus using c-fos as an immediate-early gene marker of neuronal activation. Both WT and HDC^{-/-} animals injected with PBS displayed weak to moderate c-Fos immunoreactivity throughout the hypothalamus, limited to scattered cell clusters in the anterior hypothalamic area, lateral hypothalamus, and suprachiasmatic, periventricular, lateroanterior hypothalamic, and ventromedial nuclei. Immunohistochemical staining patterns for c-Fos after ip. L-histidine administration was compared in the hypothalamus of WT and HDC^{-/-} animals. A significant increase of c-Fos signal was detected in the arcuate nucleus and lateral hypothalamic area of L-histidine-injected WT mice, but in contrast, HDC^{-/-} animals failed to display any c-Fos induction in these cell clusters.

Mapping of histaminergic neurons and their innervations

Histamine-immunoreactive profiles in the tuberomamillary region of WT mice were detected and dense histaminergic innervation of arcuate nucleus was revealed in WT mice. Histamine immunocytochemistry also confirmed the absence of histamine-containing neurons and fibers throughout the brain of HDC^{-/-} animals, even when pre-treated with systemic injection of L-histidine.

Comparison of the hypothalamic gene expression of different neuropeptides regulating nutrition in wild-type and histamine-deficient mice

Because strong histaminergic innervation is missing in the hypothalamus of the $HDC^{-/-}$ mice, changes in the regulation of orexigenic and anorexigenic neuronal groups in the hypothalamus can influence the development of the metabolic phenotype of these animals. Also, in histamine-deficient animals high levels of leptin influence POMC, NPY and orexin gene expression of orexigenic and anorexigenic neurons.

According to our *in situ* hybridisation data, the POMC, orexin and NPY expression sites (in the hypothalamus) and the density measurement of autoradiographic gains over individual neurons were similar in WT and $HDC^{-/-}$ mice.

NPY mRNA expression level was most intense in the hypothalamus and nucleus arcuate of the mice, and was detectable in the cortex and in the hippocampus. The knock-out mice showed higher expression than WT animals in the nucleus arcuatus, but the difference was not significant (WT: $100 \pm 8.7\%$, $HDC^{-/-}$: $143.5 \pm 28.7\%$; n=5).

POMC mRNA signals were detected in the nucleus arcuatus, as well as in the pituitary in both genotypes. There was no POMC gene expression in other regions of the brain. POMC expression was not significantly lower in knock-out animals (WT: $100 \pm 14.1\%$, $HDC^{-/-}$: $74 \pm 18.6\%$, n=8).

Gene expression of *orexin* was detected in the lateral hypothalamus. There was only a slight difference in the extent between the genotypes (WT: $100 \pm 5.7\%$, $HDC^{-/-}$: $110.6 \pm 6.3\%$; n=10).

Reproductive function of male $HDC^{-/-}$ mice

Changes in serum hormone levels

Serum *testosterone* levels showed 2-fold elevation in $HDC^{-/-}$ mice compared with wild-type littermates (WT: 7.15 ± 1.28 ng/ml, $HDC^{-/-}$: 13.8 ± 2.41 ng/ml).

Serum estradiol levels showed no significant difference between knock-out (29.75 pg/ml) and WT (23.14 pg/ml) animals.

The levels of tissue steroids, such as dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstendione (AD), testosterone and dhydro-testosterone (DHT), showed significant increases in the testes of $HDC^{-/-}$ mice, while the level of 17-OH-progesterone was similar in the two groups of mice. As compared with the control group's steroid levels, the percent increases of tissue steroid concentrations were 214% for DHEAS, 329% for DHEA, 193% for AD, 167% for testosterone and 155% for DHT. Tissue steroid levels in adrenal gland failed to show any significant changes between the two genotypes.

GnRH neurons in WT and HDC^{-/-} mice

The number of cells showing a positive hybridisation signal for GnRH was slightly higher in HDC^{-/-} animals, although the difference was only significant in the GnRH neuron population found in the proximity of the vascular organ of the lamina terminalis (WT: $14,3 \pm 0,6$, HDC^{-/-}: $25,3 \pm 1,76$ cells/section). However, the density measurements of autoradiographic grains over individual GnRH neurons revealed less intense labeling in knock-out than in WT mice (WT: 28.57 ± 2.72 , HDC^{-/-}: 21.25 ± 1.73).

Morphology of the testis

At the age of 7 days and adult (3 months old) the testis weight was lower in HDC^{-/-} animals and this difference was significant at the age of 7 days.

The light microscopic morphology of the testis appeared normal in the knock-out mice. However, Leydig cell ultrastructure suggested signs of elevated steroid synthesis in the HDC^{-/-} animals. Larger amounts of lipid droplets and more tightly coiled membranous whorls were found in these cells of the HDC^{-/-} animals than in the WT.

Conclusions

We draw the following conclusions from our studies on histamine deficient $HDC^{-/-}$ mice:

- ✓ Histamine deficiency leads to perturbations in energy homeostasis:
 - Mice grow fat with age, the amount of epididymal adipose tissue increases, even though food intake is considerably lower than that of wild type animals. Caloric efficiency is thus increased.
 - The capability to mobilize energy is impaired in the absence of histamine: thermoregulatory response in response to cold and starvation is inappropriate, and ability to mobilize fat stores is also reduced compared to wild type animals.

We studied the interrelated factors that are at different levels of the regulatory circle of energy metabolism and are involved in the development of metabolic phenotype.

At the periphery:

- ✓ In $HDC^{-/-}$ mice, as a result of histamine deficiency, perturbations are observed in metabolic signals, presumably due to the lack of negative feedback between leptin and insulin and histamine:
 - We observed a perturbation of anorexigenic insulin response and a reduction in insulin sensitivity. However, both the response to glucose tolerance test and the insulin secretion response to starvation were similar to that observed in wild type animals.
 - Pathologically increased leptin levels were measured in the blood, which did not decrease even in response to starvation.
- ✓ High leptin levels are in part explained by the increased amount of epididymal adipose tissue in $HDC^{-/-}$ mice.
- ✓ The main cause of hyperleptinaemia is not leptin gene overexpression in adipose tissue.

In the central nervous system:

- ✓ Hyperleptinaemia is not due to a decrease in the expression of the long signalling form of leptin receptor in hypothalamus.
- ✓ We studied the functionality of leptin receptor in the central nervous system by measuring the amount of phosphorylated STAT, a member of the JAK-STAT signaling pathway.
 - There was no difference in pSTAT concentration in the nucleus arcuatus between wild type and $HDC^{-/-}$ animals.
 - In the DVC of the brainstem we found no immunopositive cells in $HDC^{-/-}$ animals. Therefore, DVC cells are in part responsible for the defects in the mediation of leptin effects.

- ✓ In the hypothalamus, there was no significant difference between wild type and $HDC^{-/-}$ animals in the expression of neuropeptides involved in feeding regulation: the anorexigenic POMC and orexigenic NPY and orexin.

Further studies will be necessary to investigate additional elements in the regulatory circle (neuropeptides, SOCS3, Ob-Re) in order to identify the mechanisms involved in the development of the metabolic phenotype of histamine deficient mice.

Fertility and reproduction of $HDC^{-/-}$ mice are different from those of wild type since, even though litters show Mendelian distribution, the increment and the number of successful reproductions are smaller.

We investigated the correlation between histamine and the regulation of major androgens.

- ✓ Blood testosterone level of male histamine deficient mice is twice higher than that of wild type animals.
- ✓ This elevation in testosterone level is concomitant with ultrastructural changes in Leydig cells: fat accumulation and the formation of more pronounced rough-surfaced endoplasmic reticulum.
- ✓ Hypothalamic expression of GnRH is not different between wild type and $HDC^{-/-}$ mice. Thus, elevated androgen levels in males are presumably not due to the histaminergic regulation of GnRH neurons in $HDC^{-/-}$ mice.

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