

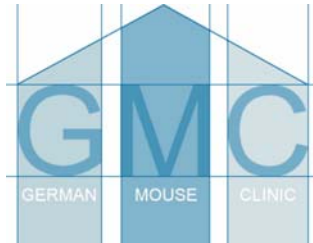
The

GERMAN MOUSE CLINIC

Report for MCHR1 KO

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The German Mouse Clinic



The German Mouse Clinic (GMC) was founded January 2002 at the GSF research center in Munich/Neuherberg to provide an open access platform for standardized mouse phenotyping. The GMC is supported by the National Genome Research Network (NGFN, <http://www.ngfn.de/>) and is a partner of the EUMORPHIA research program (<http://www.eumorphia.org/>).

In the GMC, experts from various fields of mouse genetics, physiology and pathology in close collaboration with clinicians work side by side at one location. We offer a primary phenotypic analysis of mouse mutants (more than 240 parameters/mouse) in the areas of allergy, behavior, bone and cartilage, cardiovascular diseases, clinical chemistry, energy metabolism, eye development and vision, immunology, lung function, molecular phenotyping, neurology, nociception, and pathology. Additional screens for host-pathogen interaction can be performed at the GBF Braunschweig. Secondary and tertiary screening for in depth analysis is offered by the different screens and is available on demand.

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1 Summary

1.1 Primary Screening

In a primary screen 56 animals of the MCHR1 KO mouse line (29 controls, 27 mutants) were analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Metabolism, Expression Profiling, and Pathology.

1.1.1 Overall Assessment of the Results

The results suggest a sex-specific phenotype affecting female mice more than males. For more detailed information please read the summaries from the individual screens below.

1.1.2 Results from the Individual Modules

Behavior Screen: The mutant females demonstrated hyperactivity during the test, which was undertaken during the light phase. These changes might be due to a hyper-reactivity revealed by the novel environment test situation. In mutant males no phenotype could be detected.

Dymorphology Screen: DXA analysis showed decreased bone mineral density (BMD) and bone mineral content as well as differences in the body composition of the mutants. We expect that at least part of the differences in the bone related parameters BMD, pBMD and BMC are due to body weight differences.

Clinical Chemical Screen: The most striking findings of the clinical chemical screening were decreased triglyceride and cholesterol concentrations in mutant animals. These results go with the finding of a lower body weight of mutant animals compared to the controls.

Immunology Screen: We were able to detect some minor, but statistically significant differences in the frequencies of Natural Killer (NK) cells, which tended to be higher in male mutant mice. In addition, the level of IgA was significantly lower in female mutant mice. These slight alterations are probably caused by physiological variation.

Lung Function Screen: The results suggest a sex-specific phenotype affecting female mice, i.e., the mutant animals showed significantly lower breathing rates accompanied by lower absolute and specific tidal volumes and minute ventilations, as well as lower flow rates compared to control mice. These differences were mainly pronounced during sleeping phase. However, due to the high tidal volumes measured in female controls, interpretation of the data re-

quires some precaution. Therefore, we recommend the study of a second batch of mice to verify the present findings.

Metabolic Screen: Results of the primary metabolic screening could not confirm any metabolic phenotype in this mutant mouse line. In case of the body weight, only mutant females showed a significantly reduced body weight compared to control females. Both mutant sexes showed a tendency of elevated food consumption, but this could not be determined as statistically significant. It could be demonstrated that mutant females showed a 49.2% higher rasping activity than the controls, mutant males even 56.3%, which might be deduced from the hyperactive behavior.

Pathology Screen: Both mutant and control groups developed dermatitis with abscess formation. This was more frequently found in the mutant male mice, and the difference was statistically significant. In conclusion, we found a specific pathological phenotype consisting of low weight and skin lesions in mutant males, most probably secondary to the more aggressive behavior or hyperactivity of this mutant mouse line.

In the screens **Neurology, Eye, Allergy, Nociception, and Expression Profiling**, no genotype-specific differences could be found.

1.2 Recommendations for Secondary Screening

Secondary screening is suggested from the screens **Behavior** and **Energy Metabolism**.

Behavior Screen: Due to the supposed influence of MCH on learning and memory, *Mchr1* mutants are currently bred in the behavioural screen and tested for learning and memory deficits. As discussed with the mouse provider, results will be presented and discussed when the analysis is finished.

Metabolic Screen: We would recommend analyzing the published hyperphagic phenotype in more detail. It might be possible that the “hyperphagia” described by several groups is due to the measurement of food consumption without subtraction of the rasped food. It is therefore strongly recommended to analyze basal metabolic rate (BMR), core body temperature and telemetrical activity pattern to confirm metabolic characteristics described in the literature.

Please contact Valérie Gailus-Durner to discuss further steps and details.

2 General Part

2.1 The Role of the Gene

MCHR1 (Melanin-Concentrating-Hormone-Receptor-1) is a G protein-coupled receptor, which binds highly selective to MCH (Melanin Concentrating Hormone). The gene is highly expressed in the brain, and at lower levels, in various peripheral tissues such as spleen, thymus, testis, and adipocytes.

MCH is an orexigenic hypothalamic neuropeptide, which plays an important role in regulating energy balance, body weight and body temperature.

→ Suggested role: Regulation of bone mass.

MCHR1 antagonists have antidepressant and anxiolytic as well as anorectic effects.

→ Suggested role for MCH in the regulation of mood and stress.

2.2 Known Phenotypes

- Hyperactive during dark phase
- Hyperphagic on regular chow, but body weight not altered (lower fat mass and increased lean mass)
- Resistant to diet-induced obesity: On a high fat diet, lower body weight due to lower fat mass, lower plasma levels of leptin and insulin
- Osteoporosis

(Marsh *et al.*, 2002; Chen *et al.*, 2002).

The provider confirmed the lean phenotype and the lack of difference in ambulatory activity during the light phase (unpublished results; Lakaye *et al.*, 2004)

All further findings which will be shown in this report we consider as new.

2.3 Expected Phenotypes according to the overview

The provider supposes possible effects of the knockout of *Mchr1* on several physiological processes and expects:

- an impaired memory,
- a reduced anxiety behavior,
- an altered sleep behavior, and
- an effect on the immune system.

2.4 Possible Disease Models

Up to now there are no data that demonstrate that *Mchr1* is mutated in any particular human disease. In the Vitiligo syndrome, autoantibodies against MCHR1 have been detected (Kemp *et al.*, 2002). Based on published data by Varas *et al.* (2003), MCH could be involved in feeding, anxiety, memory and/or sleep disorders (see overview).

2.5 Mice

2.5.1 Number and kind of mice

As described by the sender, the MCHR1 KO mutant mouse line was generated by deletion of a large coding region within exon 2, which was demonstrated to be essential for MCH binding. The mice analyzed were a 4th in-breeding generation on a mixed 129/Sv and C57BL/6 genetic background. The animals arrived healthy, some animals developed an abscess.

Table 1: MCHR1 KO mice provided for analysis.		
Numbers in brackets indicate animals which were kept in reserve.		
Genotype / Sex	Number of Animals	
Mutant female	12	
Mutant male	15 (+1)	2 died
Control female	14	1 died
Control male	15 (+3)	1 died

2.5.2 Housing conditions

In the GMC mice are housed in type II polycarbonate cages in individually ventilated caging (IVC) systems (VentiRack Bioscreen TM, Biozone, Margate, UK) on wood fibre (Altromin, Lage, Germany). The IVCs operate with positive pressure. Mice are transferred in weekly intervals to new cages with forceps in Laminar Flow Class II changing stations. Mice are fed with irradiated standard rodent high energy breeding diet (Altromin 1314) and given semidemineralized filtered (0.2 µm) water *ad libitum*. Light is adjusted to a 12h/12h light/dark cycle; temperature and relative humidity are regulated to 22 ± 1°C and 55 ± 5%, respectively. In specified modules husbandry conditions are adjusted according to the experiment requirements (See corresponding sections). All people attending the facility completely change their garment (jackets and trousers autoclaved) and shoes and wear caps and masks before entering the GMC (Brielmeier *et al.*, 2002).

Outbred 8-week-old male SPF Swiss mice are used as sentinels and kept on a mixture of new bedding and aliquots of soiled bedding (50:50) from all cages of the IVC rack. In addition, the sentinels were also exposed to soiled air from all “upstream” cages of the IVC rack. Health monitoring is car-

ried out by on-site examination of the sentinel mice by certified laboratories according to FELASA recommendations (www.felasa.org).

Mice are kept according to the German laws. Tests were carried out by authority of the Regierung von Oberbayern.

2.6 Workflow

2.6.1 Standardized workflow for the primary screen in the German Mouse Clinic

Mouse mutants entering the GMC are examined in a primary screen according to the following standard workflow (Fig. 1; Gailus-Durner, Fuchs *et al.*, 2005). Analyzed parameters are listed in Table 2.

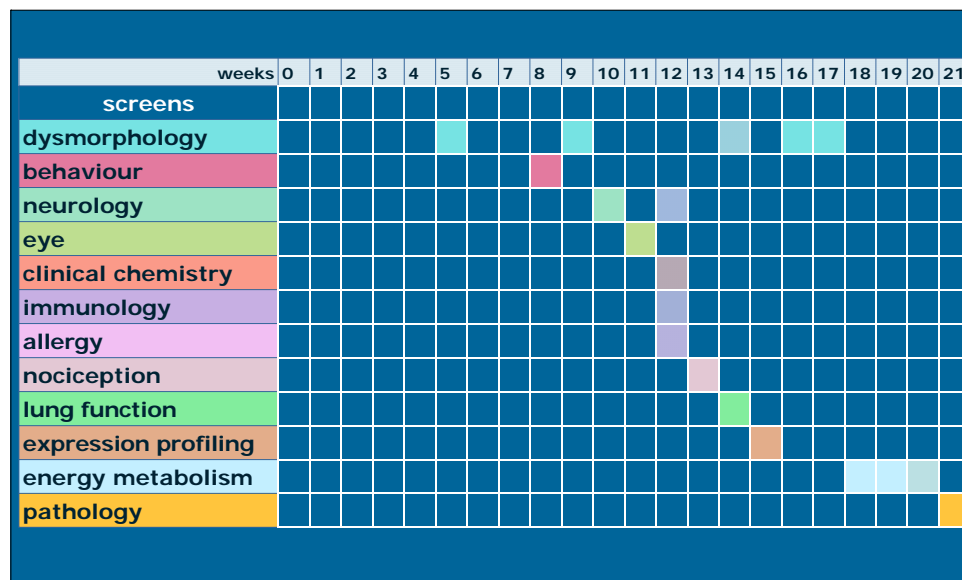



Figure 1: Workflow of the primary screen

Explanation below,  Analysis of blood-based parameters.

After the mice arrive at the GMC, they are acclimatized in the new environment for one week. The males then start in the Behavior Screen. There they stay for three weeks. Directly after the Behavior Tests, the anatomical inspection of the Dysmorphology Screen is performed. In the next week, the Neurology Screen is applied. One week later the mice go through the tests of the Eye Screen. When the mice were 12 weeks old, blood is taken, and samples are distributed to the blood-based screens for Clinical Chemistry, Immunology, Allergy and the Lactate test. One week later, the animals are tested in the Nociceptive Screen. Two weeks after testing of the first blood sample, a second sample is taken to confirm outliers, and to supply the Dysmorphology Screen with material for determination of blood-based bone-related parameters. In parallel, 10 mutant animals (five males / five females) and 10 controls (five males / five females) leave the animal facility for the Lung Function

Analysis, which for technical reasons is located elsewhere. These animals are, for hygienic reasons, not allowed to re-enter the German Mouse Clinic. The females go directly to Pathology. The males are used to freeze organs for future expression profiling on demand (remaining organs from those animals are analyzed by the Pathology). All other animals go through the bone and cartilage tests of the Dymorphology Screen, and then stay three weeks in the Metabolic Screen. After completion of the primary screen, all animals end up in the Pathology.

The screening of female animals starts one week later and follows the same workflow (with the exception of Expression Profiling sampling). Deviations from our Standard operation procedure (SOP) are listed below; please take the specific number of analyzed animals from the sections of the applied screen.

2.6.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the MCHR1 mutant mouse line. As the demanded number of 60 animals (15 mice per sex per genotype) could not be delivered, the workflow was adapted to the available number of animals. Some parameters measured in the blood based screens could not be determined in all animals, as it was not possible to get the needed amount of blood from these animals. Two animals died after blood withdrawal and another two mice had to be killed during the primary screen and thus could not be analyzed for all parameters (please see Table 1).

2.6.3 Quality Management

As a routine quality control, we take blood samples from all animals for serological tests of the sanitary status of all mice after completing the GMC primary screen. The serum is tested for MHV (BioDoc, Hannover). We chose MHV as a "sentinel" pathogen, as it is one of the most common viruses in mouse facilities worldwide and it is transmitted easily. To be open for collaboration for as many partners as possible, we allow MHV positive animals to enter our facility.

2.7 Statistical Analysis of Data

If not otherwise stated, data of males and females was analyzed separately comparing mutant and control data using a Student's t-test. Sex differences within the mutant or the control group also were determined with a t-test. Tables summarizing the data will show mean \pm standard error of the mean. Significant differences are indicated stepwise from 0.05, 0.02, 0.01, 0.001 to 0.0001.

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Abbreviations and wording

MCHR1	Melanin-Concentrating-Hormone-Receptor-1
<i>Mchr1</i>	gene coding for the Melanin-Concentrating-Hormone-Receptor-1
MCHR1 KO	mutant mouse line
GMC	German Mouse Clinic
IVC	individually ventilated cage
control	homozygous wild-type control littermate (<i>Mchr^{+/+}</i>)
mutant	homozygous mutant (<i>Mchr1^{-/-}</i>)
KO	knock out
FELASA	Federation of European Laboratory Animal Science Associations, 25 Shaftesbury Avenue, London W1D 7EG, UK, www.felasa.org

Table 2: Primary Screen at GMC

Screens	Goal	Methods
Dysmorphology, Bone and Cartilage	morphological analysis of body, skeleton, bone and cartilage	morphological observation, bone densitometry, X-ray, AVL analyzer, micro-computer tomography
Behavior	locomotor, exploratory, emotional and social behavior, object recognition memory	modified hole board
Neurology	assessment of muscle, spinocerebellar, sensory, and autonomic function	modified SHIRPA protocol
Eye	assessment of morphological and functional alterations of the eye	electroretinography, slit lamp biomicroscopy
Clinical Chemistry	determination of clinical-chemical and hematological parameters in blood	blood autoanalyzer, ABC-animal blood counter
Immunology	analysis of peripheral blood samples for immunological parameters	flow cytometry, ELISA
Allergy	analysis of total plasma IgE	ELISA
Nociception	detection of altered pain response	hot plate assay
Lung function	assessment of alterations in breathing patterns	whole body plethysmography (Buxco®)
Expression Profiling	RNA expression profiling	DNA-chip technology
Energy Metabolism	measurement of altered body weight regulation, body temperature and energy balance	bomb calorimetry
Pathology	microscopic and macroscopic examination	histology, immunochemistry

3 Specific part

3.1 Behavior Screen

3.1.1 Summary

Genetic studies in the mouse are important for the elucidation of molecular pathways underlying behavior. The goal of this endeavor is not only the identification of genes that control brain function and influence behavior, but also understanding of genetic factors involved in human psychiatric disorders (Tarrantino & Bucan, 2000; Bucan & Abel, 2002). These disorders are associated with quantitative phenotypes called “intermediate traits” or endophenotypes, some of which, in contrast to the full complex disorder, can readily be modeled in mice. These traits are risk factors which are considered to be closer to the genetic etiology than the full syndrome. Examples are anxiety in depression, prepulse inhibition and working memory deficits in schizophrenia, and social interaction deficits in autism and schizophrenia (Seong *et al.*, 2002; Gottesman & Gould, 2003; Inoue & Lupski, 2003).

In the attempt to efficiently screen for candidate endophenotypes within a limited time frame, we use the modified Hole Board (mHB) test as primary screen in the behavioral phenotyping module of the GMC. This test allows the comprehensive analysis of a range of parameters known to be indicative of behavioral dimensions such as locomotor activity, exploratory behavior, arousal, emotionality, memory and social affinity in a single short test (See Ohl *et al.*, 2001).

The *Mchr1*-mutant females demonstrated hyperactivity during the test, which was undertaken during the light phase. These changes might be due to a hyper-reactivity revealed by the novel environment test situation. In mutant males no phenotype could be detected.

3.1.2 Mice

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype. They entered the laboratory at the age of six weeks, were given two weeks for acclimatization and were tested at the age of eight weeks. Three days before testing, an object (metal cube) was placed into the home cage and removed one day before testing.

In this screen, 25 female mice (14 control, 11 mutants) and 27 male mice (12 control, 15 mutants) were available for analysis. For video-track analysis, 11 control and 10 mutant females and 11 control and 14 mutant males were available. Grey coat colored mice had to be excluded from this analysis as the system could not detect these mice against the background of the test box.

3.1.3 Material and Methods

The **modified hole board test** was carried out according to the procedures described by Ohl *et al.*, 2001. The test apparatus consisted of a test arena (100 x 50 cm), in the middle of which a board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus representing the central area of the test arena as an open field. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box (See Ohl *et al.*, 2001). Both box and board were made of dark grey PVC. All lids were closed before the start of a trial. For each trial, an unfamiliar object (a blue plastic tube lid, similar in size to the metal cube) and the familiar object (metal cube) were placed into the test arena with a distance of 2 cm between them. The illumination levels were set at approximately 150 lux in the corners and 200 lux in the middle of the test arena.

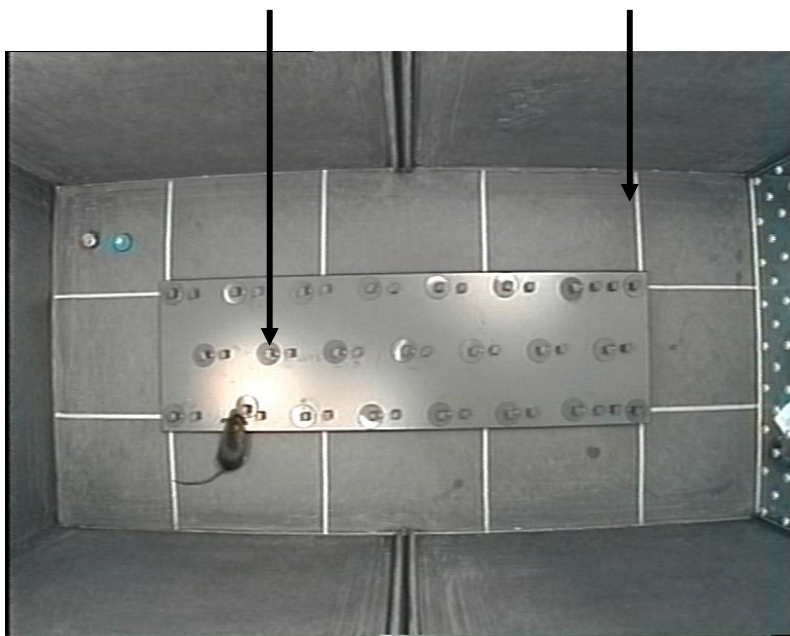
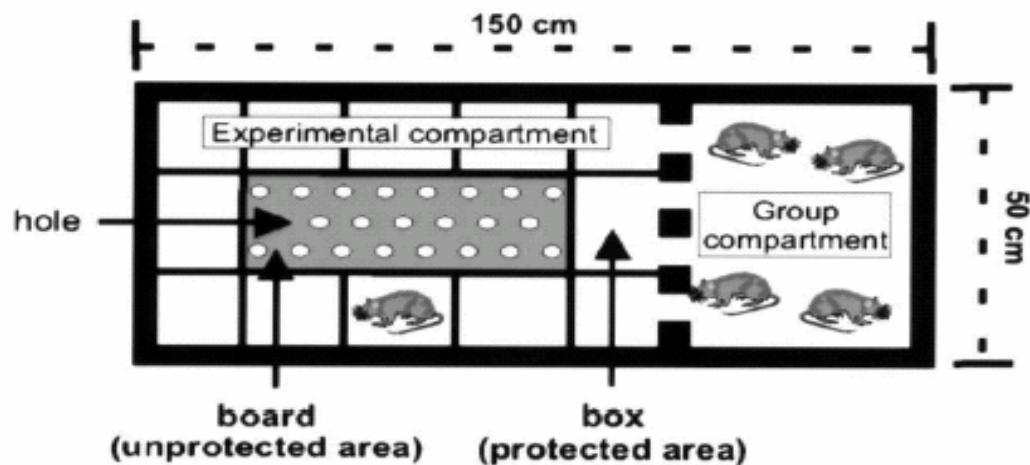


Figure 2: Test Arena for modified hole board test (Ohl *et al.*, 2001).

For testing, each animal was placed individually into the test arena and allowed to explore it freely for 5 min. The animals were always placed into the test arena in the same corner next to the partition, facing the board diagonally. The two objects were placed in the corner quadrant diametrical to the starting point. During the 5 min trial, the animal's behavior was recorded by a trained observer with a hand-held computer. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen). Additionally, a camera was mounted 1.20 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analyzed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). After each trial, the test arena was cleaned carefully with a disinfectant.

Data were statistically analyzed using SPSS software (SPSS Inc, Chicago, USA). The chosen level of significance was $p < 0.05$.

3.1.4 Results

Behavioral analysis of spontaneous activity in a novel environment, as measured by the modified Hole Board test (mHB), revealed increased forward locomotor activity in mutant females (line crossing, Table 4; total distance travelled, Table 5) and travelling with less changes in direction of movement per unit distance (meander, Table 5) as compared to wild-type control females. Additionally, mutant females showed reduced anxiety-related behaviours indicated by enhanced entries and time on board (Table 4). Concerning horizontal exploratory activity, mutant females explored the holes on board and the unknown object (Table 4) more often than control females did. No alteration in vertical exploration as indicated by rearings (Table 4) could be detected. In contrast to females, males did not show any altered behavioural parameters in the mHB.

3.1.5 Discussion

The behavioral observation in the modified Hole Board demonstrated a clear effect of the *Mchr1* mutation on forward locomotor activity in female and no effect on behavior in male mice. Mutant females travelled more and with less meandering, indicating hyperactivity. This assumption was supported by increased horizontal exploratory activity (hole and unknown object exploration). Concerning the anxiety-related behaviors, reduction of anxiety levels in mutant females is most cautiously explained as secondary to enhanced baseline activity. Whether the metabolic data showing higher rasping activity in mutant mice fit to the hyperactivity seen in the mHB in mutant females is unclear as both sexes exhibited altered rasping activity (3.11.5). However, the reduction in body weight in mutant females only is in line with their hyperactivity seen in the mHB.

Table 3: Evaluation of the behavioral phenotype	
Behaviors which are considered as affected in mutants due to the pattern of significantly altered parameters are marked in red.	
Behavior	Measured parameters
Forward locomotor activity	Line crossings, Total distance travelled
Vertical locomotor activity	Rearings in the box , Rearings on the board
Speed of movement	Mean and maximum velocity
Immobility	Time spent immobile
Risk assessment	Stretched attends
Anxiety-related behavior	Latency until first board entry, Time spent on board, Board entries
Exploratory behavior	Directed: Hole exploration, object exploration; Undirected: Rearings, activity levels
Grooming behavior	Latency to grooming, Time spent grooming, Number of groomings
Defecation	Latency to defecation, Number of boli
Social affinity	Group contacts, Time spent at partition
Familiar object exploration	Latency to obj. expl., Time spent in obj. expl., Number of obj. expl.
Unfamiliar object exploration	Latency to obj. expl., Time spent in obj. expl., Number of obj. expl.

It was known that *Mchr1*-mutant mice are hyperactive, but only during the dark phase (both sexes), and have an increased sympathetic tone modulation of dopaminergic system by MCH (Saito *et al.*, 1999), suggested to be the most important part of the mechanism of action (Marsh *et al.*, 2002). Our results extend these findings by showing hyperactivity in female mutants also during the light phase, which could be a hyper-reactivity revealed by the novel environment test situation.

As discussed with the mouse provider, MCHR1 mutants are currently bred in the behavioural screen and tested for learning and memory deficits. Results will be presented and discussed when the analysis is finished.

3.1.6 Reference

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Table 4: Results of behavioral observation in the modified hole board testData are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=12)	(n=14)	<i>p - value</i>	(n=15)	(n=11)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Line crossing [frequency]	105.33 \pm 4.81	79.71 \pm 6.22	N.A.	89.67 \pm 6.06	110.18 \pm 7.51	N.A.	n.s.	p<0.01
Line crossing [latency]	1.09 \pm 0.3	1.09 \pm 0.19	N.A.	1.45 \pm 0.4	0.86 \pm 0.16	N.A.	n.s.	n.s.
Rearings in box [frequency]	18.08 \pm 1.85	12 \pm 3.12	N.A.	14.6 \pm 1.86	18.36 \pm 2.36	N.A.	n.s.	n.s.
Rearings in box [latency]	38.83 \pm 2.98	68.01 \pm 17.11	N.A.	55.48 \pm 18.02	42.14 \pm 7.5	N.A.	n.s.	n.s.
Hole exploration [frequency]	17.5 \pm 2.24	12.36 \pm 2.64	N.A.	19.07 \pm 3.29	21.36 \pm 3.21	N.A.	n.s.	p<0.05
Hole exploration [latency]	58.13 \pm 16.08	97.88 \pm 31.21	N.A.	55.37 \pm 15.85	56.94 \pm 18.85	N.A.	n.s.	n.s.
Hole visit [frequency]	0 \pm 0	0 \pm 0	N.A.	0 \pm 0	0 \pm 0	N.A.	n.s.	n.s.
Hole visit [latency]	300 \pm 0	300 \pm 0	N.A.	300 \pm 0	300 \pm 0	N.A.	n.s.	n.s.
Board entry [frequency]	5.33 \pm 0.87	3.71 \pm 0.85	N.A.	5.53 \pm 0.98	7.73 \pm 1.39	N.A.	n.s.	p<0.05
Board entry [latency]	87.26 \pm 15.68	152.14 \pm 30.58	N.A.	124.43 \pm 25.03	99.45 \pm 26.54	N.A.	n.s.	n.s.
Board entry [total duration %]	8.57 \pm 1.68	5.7 \pm 1.18	N.A.	8.9 \pm 1.68	11.05 \pm 2.1	N.A.	n.s.	p<0.05
Rearing on board [frequency]	0.08 \pm 0.08	0.07 \pm 0.07	N.A.	0.2 \pm 0.11	0.09 \pm 0.09	N.A.	n.s.	n.s.
Rearing on board [latency]	296.76 \pm 3.24	298.57 \pm 1.43	N.A.	286.43 \pm 8.24	296.81 \pm 3.19	N.A.	n.s.	n.s.

Risk assessment [frequency]	0 ± 0	0.21 ± 0.15	N.A.	0.4 ± 0.4	0.27 ± 0.27	N.A.	n.s.	n.s.
Risk assessment [latency]	300 ± 0	270.79 ± 21.6	N.A.	282.93 ± 17.07	274.08 ± 25.92	N.A.	n.s.	n.s.
Group contact [frequency]	10.83 ± 0.59	10.07 ± 0.86	N.A.	10.13 ± 0.58	9.18 ± 1.07	N.A.	n.s.	n.s.
Group contact [latency]	15.43 ± 3.14	26.48 ± 3.25	N.A.	13.16 ± 3.01	18.65 ± 5	N.A.	n.s.	n.s.
Group contact [total duration %]	31.12 ± 3.42	26.28 ± 3.22	N.A.	26.29 ± 2.68	23.94 ± 3.22	N.A.	n.s.	n.s.
Grooming [frequency]	0.92 ± 0.26	1.57 ± 0.39	N.A.	1.67 ± 0.54	1.55 ± 0.31	N.A.	n.s.	n.s.
Grooming [latency]	224.78 ± 23.85	200.93 ± 25.66	N.A.	188.78 ± 0	175.16 ± 25.05	N.A.	n.s.	n.s.
Grooming [total duration %]	3.68 ± 1.52	5.13 ± 2.02	N.A.	6.74 ± 1.89	3.18 ± 0.93	N.A.	n.s.	n.s.
Defecation [frequency]	0.58 ± 0.26	0.36 ± 0.13	N.A.	0.53 ± 0.24	0.36 ± 0.2	N.A.	n.s.	n.s.
Defecation [latency]	233.46 ± 29.64	238.41 ± 30.57	N.A.	242.17 ± 24.47	237.26 ± 34.24	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	4.33 ± 0.41	2.86 ± 0.4	N.A.	3.6 ± 0.34	4.36 ± 0.41	N.A.	n.s.	p<0.05
Familiar object exploration [frequency]	5.5 ± 0.8	4.5 ± 0.55	N.A.	4.87 ± 0.32	5 ± 0.45	N.A.	n.s.	n.s.
Unfamiliar object exploration [latency]	25.68 ± 5.97	55.84 ± 19.69	N.A.	47.89 ± 16.51	42.58 ± 13.46	N.A.	n.s.	n.s.
Familiar object exploration [latency]	21.18 ± 6.36	42.62 ± 10.25	N.A.	14.06 ± 1.5	26.25 ± 10	N.A.	n.s.	n.s.

Unfamiliar object exploration [total duration %]	0.86 ± 0.12	0.68 ± 0.09	N.A.	0.83 ± 0.09	1.06 ± 0.08	N.A.	n.s.	p<0.01
Familiar object exploration [total duration %]	1.09 ± 0.2	1.15 ± 0.22	N.A.	1.03 ± 0.14	1.05 ± 0.12	N.A.	n.s.	n.s.
Object Index	-0.11 ± 0.06	-0.21 ± 0.08	N.A.	-0.09 ± 0.07	0.02 ± 0.05	N.A.	n.s.	n.s.

Table 5: Video-tracking results regarding locomotor behavior

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=11)	<i>p</i> -value	(n=14)	(n=10)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Total Distance Moved [cm]	2826.66 \pm 106.14	2173.62 \pm 200.66	N.A.	2576.39 \pm 174.73	3003.91 \pm 162.86	N.A.	n.s.	p<0.01
Mean Velocity [cm/sec]	18.47 \pm 0.21	17.06 \pm 0.75	N.A.	18.41 \pm 0.53	18.56 \pm 0.89	N.A.	n.s.	n.s.
Maximum velocity [cm/sec]	61.51 \pm 2.7	61.74 \pm 2.9	N.A.	65.74 \pm 1.92	61.76 \pm 5.21	N.A.	n.s.	n.s.
Turns [Frequency]	1512.36 \pm 50.22	1176.64 \pm 88.65	N.A.	1347.79 \pm 78.66	1605.5 \pm 49.45	N.A.	n.s.	p<0.001
Mean Turn Angle [degrees]	197.41 \pm 10.77	184.87 \pm 17.55	N.A.	208.2 \pm 12.82	160.37 \pm 8.09	N.A.	n.s.	n.s.
Angular Velocity [degrees/sec.]	20.95 \pm 0.88	21.56 \pm 1.23	N.A.	21.81 \pm 1.24	16.79 \pm 0.75	N.A.	n.s.	p<0.01
Absolute Meander [degrees/sec.]	9.27 \pm 1.25	6.01 \pm 1.58	N.A.	7.83 \pm 1.5	8.76 \pm 1.53	N.A.	n.s.	n.s.
Distance to Wall [cm]	6.84 \pm 0.33	4.84 \pm 0.6	N.A.	6.08 \pm 0.45	7.13 \pm 0.46	N.A.	n.s.	p<0.01
Distance to Board [cm]	9.42 \pm 0.23	11.27 \pm 0.57	N.A.	10.13 \pm 0.38	9.24 \pm 0.31	N.A.	n.s.	p<0.01

3.2 Dysmorphology, Bone and Cartilage

3.2.1 Summary

In the Dysmorphology, Bone and Cartilage Screen of the German Mouse Clinic mice are analyzed for morphological abnormalities in different organ systems with special focus on bone and cartilage development and homeostasis. We adapted the successful dysmorphology screening protocol from the Munich ENU-Mutagenesis Screen (Hrabé de Angelis *et al.*, 2000) for use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the Mammalian Phenotype Ontology wording (www.informatics.jax.org/searches/MP_form.shtml). Further tests for defects in bone development and homeostasis were taken over from human diagnosis, and were adapted for the use in mice analysis. Such tests include: X-ray analysis, bone densitometry and, in a limited number of animals, micro-computer tomography.

A total of 54 animals of *Mchr1* mutant mouse line were analyzed in the Dysmorphology, Bone, and Cartilage module of the German Mouse Clinic. The *Mchr1*-mutant mice did not show any genotype-associated phenotypes in the anatomical observation and in the X-ray analysis. DXA analysis showed decreased bone mineral density (BMD) and bone mineral content as well as differences in the body composition of the mutants. We expect that at least part of the differences in the bone related parameters BMD, pBMD, and BMC are due to body weight differences.

Mice

Twenty-nine male (14 controls, 15 mutants) and 25 female (14 controls, 11 mutants) mice were analyzed by morphological inspection at the age of 9 weeks. Blood was taken at the age of 14 weeks for determination of ionic calcium from 20 mutant and 23 littermate control animals, and 16-week-old mutants (15 animals) and controls (18 animals) entered the bone density and X-ray analysis.

3.2.2 Material and Methods

The Dysmorphology, Bone and Cartilage module of the German Mouse Clinic analyzed the mice in different phases:

1. At the age of 5 weeks, i.e. when the mice entered the facility, the general physical condition and health were checked;
2. At the age of 9 weeks, a morphological observation as a whole-body checkup was performed;
3. The ionized fraction of calcium in blood was analyzed in 14-week-old mice, and
4. At the age of 16 to 17 weeks, X-ray analysis and bone densitometry were performed.

Morphological Observation

The animals were screened using the protocol for morphological analysis from Fuchs *et al.* (2000) as adapted for the German Mouse Clinic.

Using a clickbox (supplied by the MRC Institute of Hearing Research, Nottingham, UK) we tested the mice's ability to hear a sound of 20 kHz. The reaction of the animals was classified into six categories (0=no reaction at all, 1=no Preyer reflex, 2= retarded reaction, 3= normal reaction, 4= strong reaction, 5= particularly strong reaction).

Ionized calcium Analysis

Equipment: AVL 9180 Electrolyte Analyzer (distributed by Roche Diagnostics GmbH, Mannheim, Germany)

cleaning solution and conditioning solution (Roche),

ISEtrol Quality Control Solutions (Roche),

lithium-heparin polypropylen tubes,

glass capillary (0.8 mm diameter, 32 mm length, without heparin; special product of Laborteam K+K, Munich).

Quality control: Calibration of the system and quality control were performed at intervals recommended and with solutions provided by the manufacturer. The results from the quality control were recorded by the system. Before blood measurement, daily cleaning, conditioning and calibration of the analyzer were performed.

Procedure: Blood (100 µl) was collected from anesthetized mice in lithium heparin tubes and transferred directly to the analyzer. Values were transferred directly to the database.

X-ray Images

Equipment: Faxitron X-ray Model MX-20 (Specimen Radiography System, Illinois, USA),

NTB Digital X-ray Scanner EZ 40 (NTB GmbH, Diepholz, Germany),

Quality control: Calibration of the system is done in monthly intervals,

Settings: Voltage 25 kV, integration time 40 ms,

Procedure: The anesthetized mouse was fixed on an X-ray-permeable plate and placed in the machine. Using iX-Pect software supplied by the manufacturer of the X-ray scanner, the image was taken and analyzed. Analysis was done qualitatively by visual inspection of the images as well as quantitatively by using the ruler tool of iX-Pect software.

Bone density analysis

Equipment: pDEXA Sabre X-ray Bone Densitometer (Norland Medical Systems. Inc., Basingstoke, Hampshire, UK; distributed by Stratec Medizintechnik GmbH, Pforzheim, Germany),

Quality control: Calibration of the system was done in daily intervals using the QC and the QA phantoms delivered by the manufacturer. Results from the quality control were recorded by the system.

Settings: Scan speed 20 mm/s, Resolution 0.5 mm x 1.0 mm, HAW 0.020

Procedure: After anesthesia, the weight and length of the mouse were recorded, and the mouse was placed in the analyzer. After a scout scan, the area of interest was optimized and the measure scan started.

Data-analysis: For analysis of the data, regions have to be defined. The standard analysis comprises a whole body analysis as well as a whole body analysis excluding the skull.

Statistical analysis of data

Analysis of quantitative data sets was carried out using StatView software package (SAS Corporation).

3.2.3 Parameters

<p>Morphological inspection</p> <p><i>Growth/weight/body size:</i> abnormality <i>Eye:</i> dysmorphology, corneal or lens defect <i>Coat:</i> hair growth defects, hair texture defects, color anomalies, hair follicle, structure/orientation anomalies <i>Skin:</i> pigmentation anomalies, texture/condition, anomalies <i>Vibrissae:</i> dysmorphology <i>Extremities:</i> limb dysmorphology, digit dysmorphology, tail dysmorphology <i>Teeth:</i> tooth dysmorphology <i>Ears:</i> auditory defects/deafness, dysmorphology <i>Musculature:</i> muscle dysmorphology <i>Skeletal:</i> osteogenesis/developmental anomalies, axial defects, extremities defects, craniofacial defects <i>Neurological / behavioral:</i> seizures/epilepsy, motor capabilities / coordination / movement anomalies, feeding / drinking anomalies <i>Respiratory system:</i> dysmorphology <i>Reproductive system:</i> dysmorphology <i>Other aberrant phenotype</i></p>
<p>X-ray analysis</p> <p>Skull shape, mandibles, maxilla, teeth, orbit, number of vertebrae (cervical, thoracic, lumbar, pelvic, sacral), vertebrae shape, number of ribs, rib shape, scapulas, clavicle, pelvis, femur diameter, femur shape, tibia, fibula, humerus, ulna, radius, number of digits, completeness of digits, subcutaneous fat, joints</p>
<p>Dual energy X-ray absorption</p> <p>Bone mineral density (BMD), partial bone mineral density (pBMD, whole body excluding skull), specific bone mineral density (sBMD), bone mineral content (BMC), lean mass, fat mass, bone content, lean content, fat content</p>
<p>AVL analyzer</p> <p>Free ionic calcium</p>
<p>Computer tomography</p> <p>3D-visualization of whole skeleton, 2D-examination of inner organs and soft tissue, high-resolution analysis of regions of interest</p>

3.2.4 Results

No striking gross anatomical abnormalities were detected in all mutant mice. We screened for coat color, syndactyly, eye, digit, cramping, hearing, tail, teeth and limb defects, to name only a few observations as listed above (Tables 6-7). In the X-ray analysis, no additional phenotypes were detected (Table 8). We focused our analysis (more tightly) on the body, femur and digit lengths, vertebral height and length, skull shape, and various muscle spans (data not shown).

In several quantitative bone and weight related parameters (Table 9) significant differences between genotypes homozygous and control littermates were uncovered and exhibited a particular inclination toward females (e.g. pBMD, BMC, lean and fat mass, weight).

3.2.5 Discussion

The body weight of female mutants was significantly decreased, the same tendency was observed for male mutants. Body length was slightly decreased in male mutants, no difference was found in female mutants. We confirm the results of Marsh *et al.* (2002) and Chen *et al.* (2002) showing a significant and dramatic decrease in fat mass in females (the same tendency was observed in male mutants) and in fat content among both males and females. As expected, lean mass was increased significantly and dramatically in the female *Mchr1*-mutant mice while we measured only a slight increase in the male mutant mice. The lean content was significantly increased in both female and male mutants; however, male mutant mice displayed a more borderline boundary of significance and a less substantial increase in lean content. As an aside, the differential effects of the disrupted *Mchr1* locus may reflect the higher baseline fat content for females as opposed to males, as *Mchr1* is expressed in adipocytes and provides a feasible functional role.

Recently, Bohlooly *et al.* (2004) described that *Mchr1*-inactivated mice (*Mchr1*^{-/-}) developed osteoporosis, caused by a reduction in the cortical bone mass, while the amount of trabecular bone was unaffected. The reduction in cortical bone mass was due to decreased cortical thickness measured by pQCT. Serum levels of c-telopeptide, a marker of bone resorption, was increased in *Mchr1*^{-/-} mice, indicating that these *Mchr1*^{-/-} mice had a high bone turnover osteoporosis. They demonstrated by DXA analysis that areal BMD of excised tibia and femur was reduced in four-month-old male *Mchr1*^{-/-} mice compared with wild-type mice. This is in accordance with our data. We detected a significant decrease of BMD in male mutants, pBMD and BMC values showed the same tendency. In female mutants pBMD and BMC were significantly decreased and BMD values showed the same tendency.

In summary, for the noted differences throughout the primary screen, a particular sex tendency was observed for female mutant mice while the respective observations were only partially found in the male population (please compare to the results of the Energy Metabolism Screen, 3.11.5).

For the most part, *Mchr1*-mutant mice were lighter in weight owing to shifts in the aforementioned lean and fat mass and content calculations.

Raw data will be available on demand.

3.2.6 References

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Abbreviations

BMC	bone mineral content
BMD	bone mineral density
pBMD	partial bone mineral density (excluding skull)
sBMD	specific bone mineral density

Table 6: Results from clickbox test				
Score	Male		Female	
	control	mutant	control	mutant
1	1	4	2	1
2	7	8	8	6
3	6	3	4	4
Animals analyzed	14	15	14	11

0: no reaction at all
1: very slow reaction
2: retarded reaction
3: normal reaction
4: strong reaction

Table 7: Results from the morphological inspection				
Phenotype	Male		Female	
	control	mutant	control	mutant
Pigmentation defects	2	5	2	1
Smaller		1		
Coat color abnormalities	2	2	1	
Animals analyzed	14	15	14	11

Table 8: Results from the X-Ray analysis				
Phenotype	Male		Female	
	control	mutant	control	mutant
13 th rib on X-ray image not visible	1	1	1	
Second lumbar vertebra looks longer and brighter		1		
Animals analyzed	10	9	6	8

Table 9: Bone- and weight-related quantitative parameters

(Data presented as mean ± standard error of mean)

Parameter	Control (A)		Mutant (B)		A~B	A~B	ANOVA		
	Male	Female	Male	Female	Male	Female	p – value genotype	p – value sex	p – value interaction
	(n=10)	(n=8)	(n=9)	(n=6)	p – value	p – value			
BMD [mg/cm²]	60.40 ± 1.61	72.88 ± 2.22	55.44 ± 1.29	66.17 ± 2.60	< 0.05	n.s.	< 0.01	< 0.0001	n.s.
pBMD [mg/cm²]	50.80 ± 1.89	62.00 ± 2.04	46.00 ± 1.48	54.00 ± 2.11	n.s.	< 0.05	< 0.01	< 0.0001	n.s.
sBMD [10⁻³ x cm⁻²]	1.89 ± 0.03	2.47 ± 0.01	1.89 ± 0.04	2.62 ± 0.07	n.s.	n.s.	n.s.	< 0.0001	n.s.
BMC [mg]	829 ± 68.73	951 ± 79.11	664 ± 35.93	665 ± 57.39	n.s.	< 0.05	< 0.01	n.s.	n.s.
Body Length [cm]	10.55 ± 0.09	10.13 ± 0.08	10.28 ± 0.09	10.17 ± 0.11	< 0.05	n.s.	n.s.	< 0.01	n.s.
Body Weight [g]	32.07 ± 1.19	29.73 ± 0.69	29.49 ↓ ± 0.98	25.25 ↓ ± 0.89	n.s.	< 0.01	< 0.01	< 0.01	n.s.
Lean mass [g]	22.52 ± 1.23	11.99 ± 1.15	24.21 ↑ ± 0.58	16.57 ↑ ± 0.89	n.s.	< 0.05	< 0.01	< 0.0001	n.s.
Fat mass [g]	5.96 ± 1.99	13.78 ± 1.57	2.04 ↓ ± 0.46	5.23 ↓ ± 1.27	n.s.	< 0.01	< 0.001	< 0.01	n.s.
Bone Content [%]	2.56 ± 0.01	3.18 ± 0.22	2.24 ± 0.06	2.63 ± 0.19	< 0.05	n.s.	n.s.	< 0.05	n.s.
Lean Content [%]	71.43 ± 0.05	40.89 ± 0.05	82.34 ↑ ± 1.31	66.01 ↑ ± 4.29	< 0.05	< 0.01	< 0.001	< 0.0001	n.s.
Fat Content [%]	17.32 ± 4.60	45.81 ± 4.52	6.64 ↓ ± 0.01	20.19 ↓ ± 4.42	< 0.05	< 0.01	< 0.001	< 0.0001	n.s.
	Male	Female	Male	Female	A~B Male	A~B Female	ANOVA		
	(n=15)	(n=8)	(n=14)	(n=6)	p – value	p – value	p – value genotype	p – value sex	p – value interaction
Ionized Calcium [mmol/l]	1.12 ± 0.02	1.26 ± 0.03	1.12 ± 0.03	1.20 ± 0.04	n.s.	n.s.	n.s.	< 0.001	n.s.

3.3 Neurology Screen

3.3.1 Summary

In the primary neurological screen, 26 *Mchr1*-mutant mice (15 males/ 11 females) and 28 control mice (14 males/ 14 females) were screened. These animals were analyzed according to our modified SHIRPA protocol, which includes a battery of behavioral tests. This primary observation screen is a modification of the Irwin procedure (Irwin, 1968) and is proposed as a rapid, comprehensive and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in a mouse (Rogers *et al.*, 1997).

We carried out 23 test parameters to detect phenotypic differences between mutant and control mice (see web page: http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html). Each test parameter contributes to an overall assessment of muscle and lower motor neuron function, spinocerebellar function and sensory and autonomic function. The primary neurological screen is thereby focused on the investigation of neurological reflexes to determine the overall neurological functioning of a mouse. In addition, we examined lactate in the blood of mice to draw conclusions about energy metabolism.

Female mutant mice were lighter than the controls and showed significantly altered coat appearance. All other SHIRPA test parameters were without pathological findings.

3.3.2 Mice

Fifteen 10-week-old male mutant and fourteen 10-week-old male control mice entered the neurological screen at the beginning of the 29th calendar week. Fourteen female mutant and 11 control mice of the same age entered the neurological laboratory one week later. All animals were fed *ad libitum* for a period of one week during their stay in the neurological screen.

3.3.3 Material and Methods

Assessment of each animal at age 10 weeks began with observation of undisturbed behavior (*Viewing Jar Behavior*) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. Locomotor activity and motor behavior within this area was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes (*Behavior recorded on or above the arena*). Measurements were completed with the recording of provoked biting, and body weight. The last part of the primary screen also involved the analysis of righting reflex, and contact righting reflex. A glass cylinder (35 mm diameter, 135 mm length) was used for testing of the contact righting reflex. Throughout the entire procedure, abnormal behavior, and vocalization were recorded. Between testing of each mouse, fecal pellets and urination were removed from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing.

Values for body length, body weight and locomotor activity are presented as means \pm SEM. Kruskal-Wallis-test (S-PLUS, Insightful) was used to test for effects of genotype and sex factors on these parameters. The Chi-Squared test was applied for all other parameters.

3.3.4 Parameters

Muscle/lower motor neuron function
Body position, gait, Positional passivity, tail elevation, abdominal tone, grip strength, urination, defecation
Spinocerebellar function
Body position, gait, righting reflex, tail elevation, abdominal tone, grip strength
Sensory function
Transfer arousal, touch escape, gait, pinna reflex, righting reflex
Autonomic function
Palpebral closure, urination, defecation
Neurological reflexes
Righting reflex (pons), contact righting reflex, pinna reflex (hearing test)
General appearance
Body weight, body position, transfer arousal, touch escape, irritability, vocalization, positional passivity, aggression, spontaneous activity, locomotor activity, skin color

3.3.5 Results

Female mutants showed a significantly reduced mean body weight when compared to their control littermates (Table 9). Another parameter with significant finding for female mutant mice was coat appearance (Table 11). All other SHIRPA parameters were without significant findings. Additionally, mean blood lactate levels did not differ significantly between the four groups (Table 14).

Raw data for each individual are available on demand in Excel sheets.

3.3.6 Discussion

In our neurological screening, male and female mutant mice showed no altered neurological behavior that was obvious from the initial screening. In the primary screen (SHIRPA), the only distinctive feature was a reduced body weight and an altered coat appearance in female mutant mice.

Since female mutant mice have a lower body weight compared to controls under our feeding conditions, body weight changes in these mice might be due to an altered energy metabolism or to the fact that mutant mice are hyperactive during the dark phase (see also results of the screens Behavior, 3.1.5, Dymorphology, 3.2.5, and Energy Metabolism, 3.11.5).

Locomotor activity as measured in our screen was slightly but not significantly elevated that may also contribute to the low body weight. The significant difference in the coat appearance of the female mutant mice could be an indicator of underlying illness (Suckow *et al.*, 2001).

3.3.7 References

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Suckow MA, Dannemann P and Brayton C. (2001): *The Laboratory Mouse*. A volume in the Laboratory Animal Pocket Reference Serie.

Abbreviations

SHIRPA **S**mithKline Beecham Pharmaceuticals, **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit, **I**mperial College School of Medicine at St Mary's **R**oyal London Hospital, St Bartholomew's and the Royal London School of Medicine **P**henotype **A**ssessment
http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html

Table 10: Recording of body length and body weightData are presented as mean \pm standard error of mean.

Parameter	Male			Female		
	Control (n=14)	Mutant (n=15)	<i>p-value</i>	Control (n=14)	Mutant (n=11)	<i>p-value</i>
Body Weight [g]	29.79 \pm 0.6	28.61 \pm 0.6	<i>n.s.</i>	24.31 \pm 0.52	21.95 \pm 0.03	0.005

Table 11: Behavior recorded in viewing jarData shown represents the results of test parameters from major tests where a behavioral response was observed. Test parameters which did not elicit any response were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female		
	Control (n=14)	Mutant (n=15)	<i>p-value</i>	Control (n=14)	Mutant (n=11)	<i>p-value</i>
Body Position						
Inactive	1	0	<i>n.s.</i>	14	11	<i>n.s.</i>
Active	13	15		0	0	
Excessive Activity	0	0		0	0	
Tremor						
Absent	14	14	<i>n.s.</i>	13	11	<i>n.s.</i>
Present	0	1		1	0	
Palpebral closure						
Eyes open	13	15	<i>n.s.</i>	14	11	<i>n.s.</i>
Eyes closed	1	0		0	0	
Coat Appearance						
Tidy and well groomed	12	13	<i>n.s.</i>	13	4	0.01
Irregularities	2	2		1	7	
Whiskers						
Present	12	14	<i>n.s.</i>	14	11	<i>n.s.</i>
Absent	2	1		0	0	
Lacrimation						
Absent	14	15	<i>n.s.</i>	12	9	<i>n.s.</i>
Present	0	0		2	2	
Defecation						
Present	10	10	<i>n.s.</i>	14	11	<i>n.s.</i>
Absent	4	5		0	0	

Table 12: Recording of locomotor activity and behavior in the arena

Locomotor activity data are shown as mean (\pm SEM). Data from behavior recorded in the Arena represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female		
	Control (n=14)	Mutant (n=15)	<i>p</i> -value	Control (n=14)	Mutant (n=11)	<i>p</i> -value
Transfer Arousal						
Extended freeze	3	1	<i>n.s.</i>	1	1	<i>n.s.</i>
Brief freeze	11	14		13	10	
Immediate movement	0	0		0	0	
Locomotor Activity	17.36 \pm 1.8	19.33 \pm 0.8	<i>n.s.</i>	15.29 \pm 2.1	20.18 \pm 2.6	<i>n.s.</i>
Gait						
Fluid movement	11	12	<i>n.s.</i>	1	2	<i>n.s.</i>
Lack Fluidity	3	3		13	9	
Tail Elevation						
Dragging	1	0	<i>n.s.</i>	14	11	<i>n.s.</i>
Horizontally extension	13	15		0	0	
Elevated/Straub tail	0	0		0	0	
Touch Escape						
No response	1	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Response to touch	13	15		14	11	
Flees prior to touch	0	0		0	0	
Positional Passivity						
Struggles when held by tail	13	14	<i>n.s.</i>	13	11	<i>n.s.</i>
Struggles when hold by neck	1	1		1	0	
Struggles when laid supine	0	0		0	0	
No struggle	0	0		0	0	

Table 13: Behavior recorded in or above the arena

Data shown represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female		
	Control (n=14)	Mutant (n=15)	<i>p-value</i>	Control (n=14)	Mutant (n=11)	<i>p-value</i>
Skin Color						
Blanched	0	0		0	0	
Pink	14	15		14	11	
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Trunk Curl						
Absent	14	15		14	10	
Present	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Limb Grasping						
Absent	14	15		14	11	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Pinna Reflex						
Present	14	15		14	11	
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Corneal Reflex						
Present	14	15		14	11	
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Righting Reflex						
Rights itself	13	15		13	11	
Fails to right when released	1	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Contact Righting						
Present	14	15		13	11	
Absent	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Evidence of Biting						
None	14	15		14	11	
Biting in response to handling	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Vocalization						
None	14	14		14	11	
Vocal	0	1	<i>n.s.</i>	0	0	<i>n.s.</i>

Table 14: Lactate levels						
Data shown represent the results of the mean blood lactate concentrations, value (\pm SEM)						
	Male			Female		
	Control (n=12)	Mutant (n=12)	<i>p-value</i>	Control (n=14)	Mutant (n=11)	<i>p-value</i>
Lactate [mmo/l]	5.7 \pm 0.2	5.04 \pm 0.3	<i>n.s.</i>	6.05 \pm 0.4	5.6 \pm 0.2	<i>n.s.</i>

3.4 Eye Screen

3.4.1 Summary

In the Eye Screen, a high throughput electroretinography method (ERG) was employed to examine mice for retinal impairment (Dalke *et al.*, 2004). Furthermore, mice were examined for anterior segment abnormalities by slit lamp biomicroscopy (Favor, 1983).

In humans blindness is caused by several different ocular diseases. Among these, the cataracts are responsible for half of all cases (Johnson and Foster, 2003). The retinal disorders cover a broad variety of clinical symptoms and many different genes are involved in the corresponding pathological conditions in humans. The two most important groups are retinitis pigmentosa (RP) and age-related-macular-degeneration (ARMD; for recent reviews, see Rivolta *et al.*, 2002 and Stone *et al.*, 2001). Mouse models are appropriate tools to understand the genetic and biochemical mechanisms of ocular disorders. There is a rapid increasing number of mouse mutants available suffering from various types of eye diseases (for recent reviews see Graw, 2003 and Dalke & Graw, 2005).

No genotype-specific differences were detected between *Mchr1*-mutant mice and their wild-type control littermates.

3.4.2 Mice

Twenty-nine control (15 male, 14 female) and 25 mutant mice (14 male, 11 female) entered the Eye Screen at the age of 11 weeks. Mice were first examined by slitlamp biomicroscopy and on the following day, an ERG was performed. Mice were kept under standard laboratory conditions with food and water *ad libitum*.

3.4.3 Materials and Methods

Electroretinography (ERG) was used to examine the retinal function as described (Dalke *et al.*, 2004). Mice were dark-adapted for at least 12 hours and anaesthetized with 137 mg Ketamine and 6.6 mg Xylazine per kg body weight. After pupil dilation (1 drop Atropine 1%), individual mice were fixed on a sled with Velcro straps. Gold wires (as active electrodes) were placed on the cornea; care was taken not to obstruct the pupillary opening. The ground electrode was a subcutaneous needle in the tail; a reference electrode was placed subcutaneously between the eyes. The mice were introduced into an ESPION ColorBurst Handheld Ganzfeld LED stimulator (Diagnosys LLC, Littleton, MA, USA) on a rail to guide the sled (High-Throughput Mouse-ERG, STZ for Biomedical Optics and Function Testing, Tübingen, Germany). To minimize temperature influences on the ERG, body temperature was kept at 37°C using a warming plate. 10 ms light pulses were delivered at a frequency of 0.48 Hz in two steps at 500 and 12,500 cd/m². Bandpass filter was set ranging from 0.15 to 1000 Hz. Responses were recorded simultaneously from both eyes with an ESPION Console (Diagnosys LLC, Littleton, MA, USA) and stored for offline analysis after averaging 10-40 individual measurements at each step.

Slit Lamp Biomicroscopy: Mice were examined biomicroscopically for eye abnormalities as previously described (Favor, 1983). Briefly, pupils were dilated with a 1% atropine solution applied to the eyes at least 10 min prior to examination. Both eyes of the mice were examined by slit lamp biomicroscopy (Zeiss SLM30) at 48x magnification with a narrow beam slit lamp illumination at 25-30° angle from the direction of observation. Observed phenotypic variants of the eyes were carefully documented.

Statistical Analysis: ERG data were statistically analyzed using MS-Excel. Differences between mouse groups were evaluated with the Student's t-test. Statistical significance was set at $p < 0.05$. Data are presented as mean values \pm standard error of the mean (SEM).

3.4.4 Parameters

Electroretinography (ERG)
a/b-wave, left/right eye at 500/12.50 cd/m ²
Slit lamp biomicroscopy
(qualitative) abnormalities of lens and cornea like opacity and development disorders
Histology
(qualitative) retinal lamination and morphology of cell layers and lens
Morphology
(qualitative) like size and degree of closure

3.4.5 Results and Discussion

ERG responses were recorded from the groups of MCHR1 (control – mutant) mice with light pulses at two different light intensities. These two luminance levels were chosen because at 500 cd/m² a well discernable b-wave amplitude (nearly no a-wave) mainly stemming from the rod system is induced, while light pulses at 12,500 cd/m² induce a maximally developed b-wave response and an a-wave, coming presumably from rods and cones (Dalke *et al.*, 2004).

At first, a comparison of the left and right eyes for each group was performed on the amplitudes of a- and b-wave for both luminance intensities (data not shown). Since no differences were observed between the left and right eye, ERG amplitudes of both eyes were averaged for further evaluation. The mean value and standard error was calculated for each group of mice, male and female, mutant and control (Table 15). The comparison of a- and b-wave amplitudes of males and females revealed significant differences for some parameters, presenting the non-pathological sex differences. Between the groups of mutant and control mice, no significant differences were found, neither in the male nor in the female group.

In the **histological analysis** normal retinas were observed in mutant and control mice (Fig. 3).

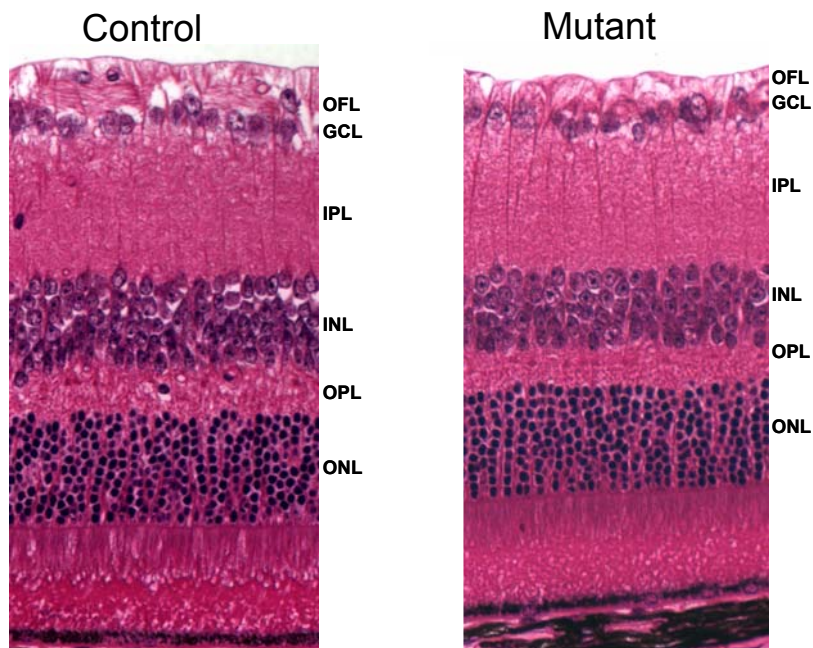


Figure 3: Comparison of retina structure of mutant and control mice

OFL: outer fibre layer
 GCL: ganglion cell layer
 IPL: inner plexiform layer
 INL: inner nuclear layer
 OPL: outer plexiform layer
 ONL: outer nuclear layer

A total of 54 mice were examined ophthalmologically by **slit lamp biomicroscopy**. All mice (mutant and control) showed slight nuclear opacity, but no association with genotype and the occurrence of anterior segment abnormalities were found.

3.4.6 References

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Abbreviations

cd/m ²	candela per square meter
ERG	electroretinography
Hz	hertz
n.s.	not significant
NAD	no abnormality detected

Table 15: Comparison of ERG-responses at illumination levels of 500 and 12,500 cd/m².
Mean ± standard error is calculated for a- and b-wave amplitudes.

Parameter	Control (A)			Mutant (B)			A~B	
	Male	Female		Male	Female		Male	Female
	(n=15)	(n=14)	<i>p</i> -value	(n=14)	(n=11)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
a-wave 500 cd/m ²	-9 ± 1.2	-6 ± 1.0	n.s.	-11 ± 1.1	-7 ± 1.8	n.s.	n.s.	n.s.
b-wave 500 cd/m ²	196 ± 10.0	227 ± 59.1	n.s.	213 ± 9.4	159 ± 9.1	<0.001	n.s.	n.s.
a-wave 12,500 cd/m ²	-52 ± 2.4	-32 ± 1.9	<0.001	-50 ± 3.2	-32 ± 2.6	<0.001	n.s.	n.s.
b-wave 12,500 cd/m ²	288 ± 10.7	230 ± 11.0	<0.001	275 ± 11.9	228 ± 13.3	<0.02	n.s.	n.s.

3.5 Clinical-Chemical Screen

3.5.1 Summary

The aim of the Clinical-Chemical Screen is the detection of hematological changes, defects of various organ systems, and changes in metabolic pathways and electrolyte homeostasis by means of suitable laboratory diagnostic tools. Since most inherited metabolic disorders are known to lead directly or indirectly, via altered organ function, to changes in the parameters investigated, this screening process provides a comprehensive investigation of clinical phenotypes with counterparts in humans and animal species (Rathkolb *et al.*, 2000). The methods used are routine procedures, allowing the appropriate screen of large numbers of mice for a broad spectrum of clinical-chemical and hematological parameters (Champy *et al.*, 2004; Hough *et al.*, 2002).

In the primary clinical chemical screen, twenty-eight (14 males/14 females) control mice and twenty-five (14 males /11 females) mutant mice were analyzed. Twenty different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured eight basic hematological parameters.

All parameters of both mutant and control mice were within the normal ranges usually found in mice at the age of three month. In our investigations the most striking findings were the decreased triglyceride and cholesterol concentrations in *Mchr1*-mutant animals. These results go with the finding of a lower body weight of mutant animals compared to the controls. The hematological parameters did not show any significant difference between mutants and control animals.

3.5.2 Mice

Fourteen 12-week-old control and fourteen 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 31st calendar week. Fourteen control and 11 mutant females entered the screen one week later.

3.5.3 Materials and Methods

Blood Withdrawal and Storage

The Clinical-chemical Screen of the German Mouse Clinic routinely analyzed 12-week-old mice. A blood sample was taken from an ether-anesthetized mouse by puncturing the retro-orbital sinus with a non-heparinized capillary (0.8 mm in diameter; Laborteam K&K; Munich, Germany; Art.No. 1.28.13.1.2). The time for sample taking was recorded in a work list. Blood was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). An additional smaller sample was collected (using the same capillary) in an EDTA-coated tube (KABE, Art.No 078035). Each tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant.

After removal of 40 µl blood for the Neurology Screen, the Li-heparin-coated tubes were stored in a rack at room temperature for two hours. Afterwards, cells and plasma were separated by a centrifugation step (10 min,

4656 x g; Biofuge, Heraeus; Hanau, Germany). Plasma was distributed between the Immunology Screen (30 µl), the Allergy Screen (30 µl), the Clinical Chemical Screen (130 µl) and the Steroid Screen (residual), while the cell pellet was given to the Immunology Screen for FACS-analysis. The plasma sample for the clinical chemical analysis was transferred into an Eppendorf tube and diluted 1:2 with aqua dest. The solution was mixed for a few seconds (Vortex genie, Scientific Industries, New York, America) to prevent clotting and then centrifuged again for 10 min at 4656 x g. Additionally the Clinical Chemical Screen received the EDTA-blood sample for hematological investigations.

Clinical Chemistry

The screen was performed using an Olympus AU 400 autoanalyzer and adapted reagents from Olympus (Hamburg, Germany) and Roche (Mannheim, Germany). In the primary screen, 20 different parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes.

Hematology

A volume of 50 µl EDTA-blood was used to measure basic hematological parameters with a blood analyzer, which has been carefully validated for the analysis of mouse blood (ABC-Blutbild-Analyzer, Scil Animal Care Company GmbH, Viernheim). Red blood cells, white blood cells, and platelets are measured by electrical impedance and hemoglobin by spectrophotometry. Mean corpuscular volume (MCV) is calculated directly from the cell volume measurements, the hematocrit (HCT) from $MCV \times \text{red blood cell count}$. Mean corpuscular hemoglobin (MCH) and mean concentration of corpuscular hemoglobin (MCHC) are calculated from hemoglobin/red blood cells count (MCH) and hemoglobin/hematocrit (MCHC).

Analysis of Data

Data were statistically analyzed using Excel and Sigma Stat 2.0 with the level of significance set at $p < 0.05$.

3.5.4 Parameters

Proteins and plasma enzyme activities
Alkaline phosphatase (EC 3.1.3.1), α -Amylase (EC 3.2.1.1), Creatine kinase (EC 2.7.3.2), Aspartate-aminotransferase (AST/GOT; EC 2.6.1.1), Alanine-aminotransferase (ALT/GPT; EC 2.6.1.2), Ferritin, Transferrin, Lipase (EC 3.1.1.3), Total protein
Plasma concentrations of specific substrates
Glucose, Cholesterol, Triglycerides, Uric acid, Urea, Creatinine
Plasma concentrations of electrolytes
Potassium, Sodium, Chloride, Calcium, Inorganic phosphate
Basic hematology
White blood cell count (WBC), Red blood cell count (RBC) Hematocrit (HCT), Hemoglobin (HGB), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), and Platelet count (PLT)

3.5.5 Results

Clinical Chemistry

Most values obtained for the clinical chemical parameters were within the normal ranges usually found in mice at the age of three months as supported by previously published data (Hough *et al.*, 2002; Quimby, 1999; Table 16). Sex differences were detected for many clinical chemical parameters in the control animals as well as in the mutant mice.

Differences between mutants and controls were seen in following parameters: Male mutant mice showed significantly decreased serum concentration of triglycerides compared to the wild-type control animals. Additionally, a lower mean ferritin level was detected for mutant male mice. In the female mutants the cholesterol concentration was decreased and the alkaline phosphatase level was increased compared to the control animals. A similar tendency for cholesterol and triglycerides but no significant difference was visible also in the males.

Hematology

In the primary screen for hematological parameters all results of both mutants and controls were within normal ranges (Table 17) with significant differences between mutant females and control females only for white blood cell count.

Raw data for each individual are available on demand in Excel sheets.

3.5.6 Discussion

In contrast to the results published by Marsh *et al.*, (2002) we saw significant differences in triglyceride concentration. But Marsh and coworkers analyzed 5 to 7-month-old animals which were fasted for 4 h prior to blood collection. In our investigations the most striking findings were the **decreased triglyceride** and **cholesterol concentrations** in mutant animals. These results are consistent with the finding of a lower body weight of mutant animals compared to the controls (please compare to the results of the screens Neurology, Dysmorphology, Energy Metabolism, and Pathology)

However, most values of mutant and control animals for all parameters were within the normal ranges typical for a mixed background of 129/Sv and C57BL/6 mice. Therefore all clinical chemical and hematological parameters were without pathological findings.

3.5.7 Reference

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Table 16: Clinical-chemical parameters.Data are presented as mean \pm standard error of mean.

Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=14)	(n=11)	<i>p</i> -value	(n=14)	(n=14)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Sodium [mmol/l]	160 \pm 0.74	156 \pm 0.59	<0.001	161 \pm 0.44	155 \pm 0.35	<0.001	n.s	n.s.
Potassium [mmol/l]	4.1 \pm 0.10	3.7 \pm 0.08	<0.01	4.0 \pm 0.08	3.7 \pm 0.06	<0.01	n.s	n.s.
Calcium [mmol/l]	2.1 \pm 0.03	1.8 \pm 0.02	<0.001	2.0 \pm 0.02	1.8 \pm 0.01	<0.001	n.s	n.s.
Chloride [mmol/l]	112.2 \pm 0.31	113.4 \pm 0.48	<0.05	113.2 \pm 0.64	113.8 \pm 0.23	n.s	n.s	n.s.
Inorganic Phosphorus [mmol/l]	1.6 \pm 0.09	1.4 \pm 0.07	n.s	1.5 \pm 0.06	1.4 \pm 0.08	n.s	n.s	n.s.
Total Protein [g/dl]	5.0 \pm 0.11	5.2 \pm 0.14	n.s	4.8 \pm 0.06	5.4 \pm 0.10	<0.001	n.s	n.s.
Creatinine [mg/dl]	0.336 \pm 0.01	0.373 \pm 0.01	<0.001	0.323 \pm 0.01	0.379 \pm 0.00	<0.001	n.s	n.s.
Urea [mg/dl]	55.2 \pm 2.79	68.2 \pm 3.94	<0.02	53.1 \pm 3.03	70.1 \pm 2.87	<0.001	n.s	n.s.
Uric acid [mg/dl]	1.6 \pm 0.17	1.6 \pm 0.19	n.s	2.0 \pm 0.33	1.3 \pm 0.16	n.s	n.s	n.s.
Cholesterol [mg/dl]	85.1 \pm 4.37	78.4 \pm 5.76	n.s	96.6 \pm 4.39	100.6 \pm 3.27	n.s	n.s	<0.01
Triglyceride [mg/dl]	107.1 \pm 6.70	123.7 \pm 15.00	n.s	157.1 \pm 15.20	151.7 \pm 9.38	n.s	<0.01	n.s.
Creatine Kinase [U/l]	44 \pm 12.04	87 \pm 24.32	n.s	58 \pm 15.79	93 \pm 22.53	n.s	n.s	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	26 \pm 4.91	18 \pm 2.01	n.s	20 \pm 2.02	19 \pm 1.47	n.s	n.s	n.s.
Aspartate-Amino-transferase (AST,GOT) [U/l]	26 \pm 3.25	27 \pm 2.54	n.s	25 \pm 3.95	28 \pm 2.02	n.s	n.s	n.s.
Alkaline Phosphatase [U/l]	70 \pm 5.32	85 \pm 4.59	<0.05	63 \pm 4.24	73 \pm 2.46	n.s	n.s	<0.05
α -Amylase [U/l]	2417 \pm 108.95	2342 \pm 112.88	n.s	2692 \pm 120.60	2495 \pm 119.6	n.s	n.s	n.s.
Glucose [mg/dl]	157.7 \pm 7.98	157.1 \pm 6.70	n.s	169.5 \pm 8.54	160.8 \pm 8.00	n.s	n.s	n.s.
Ferritin [ng/ml]	37.2 \pm 2.18	48.2 \pm 4.08	<0.05	49.6 \pm 2.11	53.1 \pm 2.66	n.s	<0.001	n.s.
Transferrin [mg/dl]	154.4 \pm 3.76	155.4 \pm 3.45	n.s	153.5 \pm 2.70	156.3 \pm 1.76	n.s	n.s	n.s.
Lipase [U/l]	32.5 \pm 2.14	67.8 \pm 3.03	<0.001	36.4 \pm 2.35	66.7 \pm 3.27	<0.001	n.s	n.s.

Table 17: Hematological parameters.

Data are presented as mean \pm standard error of mean.

Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=14)	(n=11)	<i>p</i> - value	(n=15)	(n=14)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [10 ³ /μl]	6.93± 0.66	3.84± 0.21	<0.001	7.29± 0.47	4.80± 0.20	<0.001	n.s	<0.01
Red blood cell count [10 ⁶ /μl]	9.86± 0.15	10.51± 0.11	<0.01	10.23± 0.16	10.71± 0.10	<0.02	n.s	n.s.
Hemoglobin [g/dl]	15.61± 0.23	16.11± 0.22	<0.001	15.96± 0.13	16.53± 0.18	<0.02	n.s	n.s.
Hematocrit [%]	46± 0.74	48± 0.81	<0.05	47± 0.61	50± 0.61	<0.01	n.s	n.s.
Mean corpuscular volume [fl]	46.21± 0.42	45.82± 0.57	n.s	46.07± 0.41	46.36± 0.48	n.s	n.s	n.s.
Mean corpuscular hemoglobin [pg]	15.84± 0.13	15.33± 0.17	<0.05	15.64± 0.20	15.45± 0.11	n.s	n.s	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	34.29± 0.16	33.43± 0.30	<0.05	33.99± 0.23	33.35± 0.18	<0.05	n.s	n.s.
Platelet count [10 ³ /μl]	745± 21.22	602± 27.02	n.s	764± 15.70	597± 39.48	<0.01	n.s	n.s.

3.6 Immunology Screen

3.6.1 Summary

Mouse models have been a primary source of information for understanding the intricate mechanisms of the immune system (Blüethmann and Ohashi, 1994; Mak *et al.*, 2001; Fischer 2002; Rogner and Avner, 2003). The Immunology Screen at the GMC was set up to conduct a broad immunological phenotyping of mouse mutant lines with the intention of identifying distinct gene functions, which play key roles in the immune defenses of the organism through a complex network of cellular and soluble components (Janeway *et al.*, 2004).

According to the data summary presented to the GMC, no immunological phenotype was known for the MCHR1 KO mutant line. Its analysis in the Immunology Screen revealed minor differences between the mutants and their littermate controls, i.e., the frequencies of NK (Natural Killer) cells, which tended to be higher in male mutant mice. In addition, the level of IgA was significantly lower in mutant female mice.

3.6.2 Mice

We analyzed 25 mutant animals (11 females and 14 males) and 29 age- and sex-matched control littermates (14 females and 15 males).

3.6.3 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500 μ l blood by erythrocyte lysis with NH_4Cl (0.17M) - Tris buffer (pH 7.45) directly in 96-well microtiter plates. After subsequent washing with FACS staining buffer (PBS, 0.5% BSA, 0.02% sodium azide, pH 7.45), PBLs were incubated for 20 min with 1 μ M ethidium monazide bromide (EMA, Molecular Probes, The Netherlands) and Fc block (clone 2.4G2, PharMingen, San Diego, USA). EMA bound to the DNA of dead cells was photocrosslinked by brief light exposure. Cells were then stained with fluorescence-conjugated monoclonal antibodies (PharMingen).

The following main cell populations were analyzed: B cells (CD19⁺ clone 1D3), B1 B cells (CD19⁺CD5⁺, clone 53-7.3), B2 B cells (CD19⁺CD5⁻), T cells (CD3⁺, clone 145-2C11), CD4⁺ T cells (clone RM4-5), CD8⁺ T cells (CD8 α , clone 53-6.7; CD8 β , clone H35-17.2), γ/δ T cells (clone GL3), granulocytes (Gr-1⁺, clone RB6-8C5), and NK cells (CD49b⁺, clone DX5). We also analyzed additional subpopulations based on the following surface antigens: IgD (clone 11-26c.2a), B220 (clone RA3-6B2), CD11b (clone M1/70), CD103 (clone 2E7), CD25 (clone PC61), CD62L (clone MEL-14), CD45RA (clone 14.8), Ly-6C (clone AL-21), and CD44 (clone IM7). Data were acquired on a FACS Calibur (Becton Dickinson, San Diego, USA) and were analyzed using FlowJo software (TreeStar Inc, USA). All samples were acquired until a total number of 25,000 cells was reached.

The plasma levels of IgM, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgA were determined by standard sandwich ELISAs using goat anti-mouse immunoglobulin antibodies and alkaline phosphatase (AP) conjugates (SouthernBiotech, Birmingham, USA). The presence of rheumatoid factor and anti-DNA antibodies

was evaluated by indirect ELISA with rabbit IgG (Sigma-Aldrich, Steinheim, Germany) and calf thymus DNA (Sigma-Aldrich), respectively, as antigens and AP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). Serum samples from MRL/MpJ-Tnfrsf6^{lpr} mice (Jackson Laboratory, Bar Harbor, USA) were used as positive controls in the autoantibody assays.

3.6.4 Parameters

Flow cytometry
B cells (CD19 ⁺), B1 B cells (CD19 ⁺ CD5 ⁺), B2 B cells (CD19 ⁺ CD5 ⁻), T cells (CD3 ⁺), CD4 ⁺ T cells, CD8 ⁺ T cells, γ/δ T cells, granulocytes (Gr-1 ⁺), and NK cells (CD49b ⁺). Furthermore, all potential subpopulations which can be identified by co-staining for other surface markers (IgD, B220, CD11b, MHC II, I-A ^K , CD25, CD8 β , CD62L, CD45RA, Ly-6C, CD44) using 6 parameter/5 color flow cytometry were analyzed.
ELISA
IgM, IgG ₁ , IgG _{2a} , IgG _{2b} , IgG ₃ , IgA; anti-DNA antibodies, rheumatoid factor

3.6.5 Results and Discussion

The analysis of the MCHR1 KO mutant mouse line in the primary Immunology Screen did not reveal profound alterations in the tested parameters. However, we were able to detect some minor, but statistically significant differences in the frequencies of NK cells, which tended to be higher in male mutant mice. In addition, the level of IgA was significantly lower in mutant female mice.

Although we were able to detect these slight alterations, most likely they do not represent a major phenotype affecting the immune system, and are probably caused by physiological variation.

3.6.6 References

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Table 18: Basic parameters analyzed in the immunology screen.

Data are presented as mean ± standard error of mean.

Parameter	Mutants (A)			Control (B)			A ~ B	
	Male	Female	p - value	Male	Female	p - value	Male	Female
	(n=14)	(n=11)		(n=15)	(n=14)		p - value	p - value
CD19 ⁺ [%]	36.5±3.4	28.2±3.0	n.s.	38.0±2.7	27.8±1.2	<0.001	n.s.	n.s.
CD19 ⁺ CD5 ⁻ [%]	84.4±1.3	86.6±1.0	n.s.	85.4±1.3	88.5±0.8	n.s.	n.s.	n.s.
CD19 ⁺ CD5 ⁺ [%]	15.6±1.3	13.4±1.0	n.s.	14.5±1.3	11.4±0.8	n.s.	n.s.	n.s.
CD3 ⁺ [%]	34.2±2.0	37.4±4.0	n.s.	37.9±2.1	35.6±2.0	n.s.	n.s.	n.s.
γ/δ TCR ⁺ [%]	0.7±0.06	0.5±0.07	n.s.	0.6±0.05	0.3±0.03	<0.01	n.s.	n.s.
Gr-1 ⁺ [%]	12.2±2.5	4.9±0.8	<0.05	10.3±1.4	4.4±0.5	<0.01	n.s.	n.s.
CD49b ⁺ [%]	28.8±1.4	25.3±1.7	n.s.	22.6±1.6	22.9±0.8	n.s.	<0.02	n.s.
CD4 ⁺ [%]	27.3±1.5	25.4±2.0	n.s.	25.7±1.3	25.7±1.5	n.s.	n.s.	n.s.
CD8β ⁺ [%]	14.2±0.7	14.0±1.3	n.s.	15.8±0.8	15.1±1.1	n.s.	n.s.	n.s.
IgG ₁ [μg/ml]	216.6±25.6	172.7±25.6	n.s.	234.0±24.1	145.2±18.6	<0.02	n.s.	n.s.
IgG _{2a} [μg/ml]	286.3±41.4	217±38.1	n.s.	281.6±47.5	262.8±57.1	n.s.	n.s.	n.s.
IgG _{2b} [μg/ml]	229.6±9.1	175.5±21.3	n.s.	219.5±11.7	217.5±23.5	<0.02	n.s.	n.s.
IgG ₃ [μg/ml]	474.2±89.9	165.2±22.0	<0.01	352.2±42.0	212.0±35.6	<0.05	n.s.	n.s.
IgM [μg/ml]	107.5±13.8	54.8±3.8	<0.01	96.9±10.1	57.2±2.8	<0.01	n.s.	n.s.
IgA [μg/ml]	31.3±4.5	13.9±1.6	<0.01	33.2±6.1	26.1±5.1	n.s.	n.s.	<0.01
Anti-DNA Ab [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.
Rheumatoid factor [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.

Raw data will be available on demand.

3.7 Allergy Screen

3.7.1 Summary

The goal of the Allergy screen within the German Mouse Clinic (GMC) is to search for IgE mutants in order to establish mouse models for allergic diseases and to find new strategies for antiallergic therapy. The increased production of IgE in response to common environmental antigens is the hallmark of atopic diseases in man (Hamelmann *et al.* 1999). Mouse mutants with phenotypic alterations in IgE production represent a valuable tool to study and characterize the molecular mechanisms of IgE-mediated allergic hypersensitivity (Zhang *et al.* 1997).

In the primary Allergy screen of the MCHR1 KO mutant mouse line, 27 control and 29 mutant animals were screened. Their analysis did not reveal any profound differences between mutant and control mice.

3.7.2 Mice

An age- and sex-matched group of 27 control (12 females, 15 males) and 29 mutant (14 females, 15 males) mice aged 12 weeks was analyzed in the Allergy Screen.

3.7.3 Material and Methods

Twelve-week-old male and female mice were screened for alterations in plasma total IgE concentrations. Blood samples were taken from animals by puncturing the retroorbital plexus under ether anesthesia. Plasma IgE concentrations were measured by isotype-specific sandwich ELISA technique with a lower detection limit of 1 ng/ml. briefly, microtiter plates were coated with the IgG fraction of sheep anti-mouse IgE in sodium bicarbonate buffer (pH 9.6). After incubation, plates were washed with Tris buffer (pH 7.4) and blocked with 3% (w/v) bovine serum albumin at room temperature. Diluted plasma samples and standard were added to the plates. After overnight incubation biotinylated rat anti-mouse IgE was added and plates were incubated at room temperature for 2 h. Then plates were incubated in the presence of peroxidase-labeled streptavidin. After washing, tetramethylbenzidine (TMB) substrate solution was added and after an appropriate incubation time the stop solution (sulphuric acid, 2M) was added. The plates were read in a standard microplate reader at a wavelength of 450 nm. Total murine IgE data are reported in ng/ml, based on a standard curve of purified murine IgE (Alessandrini *et al.*, 2001).

3.7.4 Results and Discussion

No statistically significant difference between *Mchr1*-mutant and control mice was found. In both mutant and control animals, the mean concentration of total IgE was significantly higher in females than in males (Table 19) which is known for many inbred strains (Alessandrini *et al.*, 2000; Corteling *et al.*, 2004; Seymour *et al.*, 2002).

Taken together, under standard screening conditions for primary Allergy screen, *Mchr1*-mutant mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

Raw data will be available on demand.

Table 19: Total plasma IgE in <i>Mchr1</i> mice								
Data are presented as mean ± standard error of mean.								
	Control (A)			Mutant (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=12)	(n=15)	<i>p</i> - value	(n=14)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Total IgE [ng/ml]	106± 21.1	56± 9.2	<0.05	112± 16.9	62± 8.3	<0.05	n.s.	n.s.

3.7.5 Reference

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3.8 Nociceptive Screen

3.8.1 Summary

Pain is the perception of an aversive or unpleasant sensation that originates from a specific region of the body. The highly subjective nature of pain is one of the factors that make it difficult to define and to treat clinically. Pain is more than a conspicuous sensory experience that warns of danger.

Nociceptors are activated by tissue injury but also by mechanical, thermal, or chemical stimuli. Harmful stimuli applied to the skin or to subcutaneous tissue, activate nociceptors, the peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root or in the trigeminal ganglia.

A noxious stimulus activates the nociceptor by depolarizing the membrane of the sensory ending. When peripheral tissues are damaged, the sensation of pain in response to subsequent stimuli is enhanced. This phenomenon termed hyperalgesia, may involve a lowering of threshold of the nociceptors or an increase in the magnitude of pain evoked by suprathreshold stimuli. Hyperalgesia can occur both at the site of tissue damage (primary hyperalgesia) and in the surrounding undamaged areas (secondary hyperalgesia; Wall and Melzak, 1984). By means of different inbred mouse strains it could be demonstrated that rodents display large and heritable differences in both nociceptive and analgesic sensitivity (Mogil, 1999; Mogil *et al.*, 1999)

In the Primary Screen the responsiveness of the intact somatosensory system to thermal pain was tested in the *Mchr1* mutant mouse line by means of the hot plate test (nociceptive pain). We did not find any significant differences in pain reactivity between the control and mutant animals.

3.8.2 Mice

Twenty-five mutant mice (14 male, 11 female), and 28 control animals (15 male, 13 female) were tested in our first screen.

3.8.3 Material and Methods

Hot plate test

The mice were placed on a metal surface maintained at $52 \pm 0.2^\circ\text{C}$ (Hot plate system was made by TSE GMBH, Germany; Eddy and Leimbach, 1953). Locomotion of the mouse on the hot plate was constrained by 20 cm high Plexiglas wall to a circular area with a diameter of 28 cm (Fig. 4). Mice remained on the plate until they performed one of three behaviors regarded as indicative of nociception: hind paw lick (h.p. licking), hind paw shake/flutter (h.p. shaking) or jumping.

We evaluated only hind paw but not the front paw responses, because fore paw licking and lifting are components of normal grooming behavior. Each mouse was tested only once since repeated testing leads to profound changes in response latencies. The latency was recorded to the nearest 0.1 s. To avoid tissue injury 60 s cut-off time was used. The data values are given in seconds.

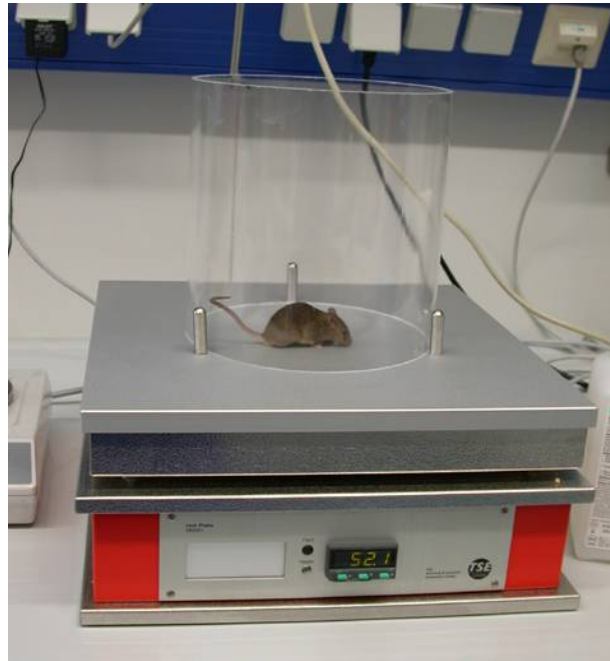


Figure 4: Hot plate system

Statistical analysis

Statistical analysis was performed using a statistical package Statgraphics® (Statistical Graphics Corporation, Rockville, MD). The differences between the groups were compared with ANOVA; LSD test was used as *post hoc*. Statistical significance was assumed at $p < 0.05$.

3.8.4 Parameters

Hind paw licking
Reaction with licking of hind paw to the thermal pain
Hind paw shaking
Reaction with shaking of hind paw to the thermal pain
Jumping
Jumping reaction to the thermal pain

3.8.5 Results and Discussion

Typically, the first nociceptive response observed in mice is hind paw shaking (Table 20). Both genotypes showed hind paw licking, another typical nociceptive response. The third examined response was jumping of animals.

There was no significant difference in the pain reactivity (= latencies) between the wild-type controls and mutant mice or between male and female mice. Therefore we cannot address a pain phenotype to the knockout of the *Mchr1* gene.

Raw data will be available on demand.

3.8.6 References

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Abbreviations

h.p. hind paw

Table 20: Nociceptive Screen									
Data are presented as mean \pm standard error of mean.									
							ANOVA		
							genotype		sex*geno- type
Parameter Latency [s]	Mutant (A)			Control (B)			A~B	A~B	ANOVA
	Female	Male		Female	Male		Female	Male	
	(n=11)	(n=14)	<i>p - value</i>	(n=13)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
H.p. licking	40.5 \pm 4.35	41.6 \pm 3.86	n.s.	28.9 \pm 4.0	37.1 \pm 3.72	n.s.	n.s.	n.s.	n.s.
H.p. shaking	28.2 \pm 3.72	25.3 \pm 3.29	n.s.	27.1 \pm 3.42	26.2 \pm 3.18	n.s.	n.s.	n.s.	n.s.
Jumping	60	56.1 \pm 2.17	n.s.	59.0 \pm 2.24	53.9 \pm 2.09	n.s.	n.s.	n.s.	n.s.

3.9 Lung Function Screen

3.9.1 Summary

Neural and mechanical processes that control breathing frequency have been investigated in man for a long time (Mead, 1960; Otis *et al.*, 1959), but only with the availability of mouse inbred strains the contribution of genetic determinants to differential baseline breathing patterns could be elucidated (Tankersley *et al.*, 1997; Tankersley, 1999; Reinhard *et al.*, 2002; Reinhard *et al.*, 2005). By use of genetically engineered mice, candidate genes for human developmental disorders of breathing have been identified (Katz, 2003).

Spontaneous breathing pattern was studied in male and female *Mchr1*-mutant and wild-type control mice. Typical sex-specific differences, e.g., higher body weight-specific values for tidal volume and minute ventilation in females, were observed mainly during sleeping phase among both control and mutant mice but were more pronounced among control animals.

Between male control and mutant mice, no significant differences were found. Concerning female mice, the control animals showed significantly higher breathing rates accompanied by higher absolute and specific tidal volumes and minute ventilations, as well as higher flow rates compared to mutant mice. These differences were mainly pronounced during sleeping phase. The results suggest a **sex-specific phenotype**. However, female control mice revealed a somewhat atypical finding: tidal volumes were remarkably high during sleep and did not change with increasing levels of activity. Thus, with respect to this parameter, differences between control and mutant mice have to be interpreted with precaution. Therefore, we recommend the study of additional mice to verify the present findings.

3.9.2 Mice

The workflow of the screen provides male and female mice with a mean age of 15 weeks. Body weights were comparable between male control (bw = 31.9 g \pm 1.9) and male mutants (bw = 29.3 g \pm 1.8) and also between female control (bw = 25.2 \pm 2.1) and female mutant mice (bw = 22.3 \pm 0.4). Five animals were analyzed per group.

3.9.3 Material and Methods

Whole Body Plethysmography

A commercially available system from Buxco[®] Electronics (Sharon, Connecticut) was used to assess breathing patterns in unrestrained animals according to the principle described by Drorbaugh and Fenn (1955). It measures the pressure changes which arise from inspiratory and expiratory temperature and humidity fluctuations during breathing (Figs. 5 and 6).

Calibration of the system allows to transform these pressure swings into flow and volume signals so that automated data analysis provides tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow

rates (PIF, PEF). These data were stored online as mean values at 10 s intervals.



Figure 5: System used at GMC to assess breathing patterns.

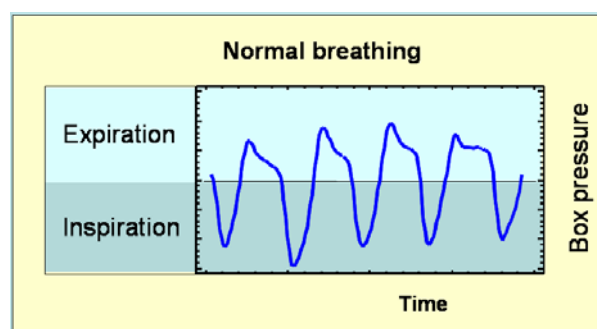


Figure 6: Recorded data used to calculate the breathing parameters.

Measurements were always performed between 8 a.m. and 11 a.m. to account for potential diurnal variations in breathing. The system was set up in a quiet room where temperature and humidity were kept constant throughout the measurements. Before each measurement, the system was calibrated and the actual barometric pressure, temperature, and humidity were supplied to warrant adequate calculations of flow rates and volumes. After placing the animals into the chamber, data recording was immediately started and was continued for 40 min. Mice underwent typical phases during the measuring period. Primarily, the animals were stressed so that the respiratory rate was highest at the beginning. Usually after 5 min. the animals became calmer, they slightly reduced their respiratory rate, and began to explore the chamber and start cleaning themselves – *phase of activity*. Later activity was more and more interrupted by phases of rest or even short periods of snoozing – *resting phase*. Some of the animals even went to *phases of sleep*, which resulted in a further marked decrease in respiratory rate. The frequency histogram of the respiratory rates was determined for each individual, and breathing was analyzed for the above mentioned parameters during the phases of activity and rest. In addition to the directly recorded parameters, mean inspiratory and expiratory flow rates (MEF, MIF) were calculated offline from the ratio of tidal volume and the respective time interval. The relative duration of inspiration (T_i/TT) was determined from the ratio of inspiratory time to total time required for the breathing cycle. Specific tidal volumes and minute ventilations (sTV, sMV) were calculated by relating the absolute values to the body weight of the animal. Furthermore, the mean of all breathing frequencies (mean_f) meas-

ured during the 40-minute-period was calculated as a rough and ready parameter to assess whether the duration of rest and activity was similar in all mouse strains.

Statistical Analysis of Data

Statistical analyses were performed using a commercially available statistics package (Statgraphics®, Statistical Graphics Corporation, Rockville, MD). Differences between strains were evaluated by Students t-test. Statistical significance was assumed at $p < 0.05$. Data are presented as mean values \pm standard error of the mean (SEM).

3.9.4 Parameters

Directly recorded data
Tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF).
Calculated data
mean inspiratory flow rates (MEF), expiratory flow rates (MIF), relative duration of inspiration (Ti/TT), specific tidal volumes (sTV), minute ventilations (sMV), mean of all breathing frequencies (mean_f)

3.9.5 Results and Discussion

Table 21 summarizes the results obtained for spontaneous breathing under sleeping, resting, and active conditions.

Genotype-specific differences. Breathing parameters of male control and mutant mice were comparable at all levels of activity. In contrast, female control mice showed higher breathing rates, higher absolute and specific tidal volumes and minute ventilations, shorter expiratory timing, and higher flow rates compared to female mutants during sleep. These differences were less pronounced during rest, and completely abolished during activity. Thus, it appears that with increasing oxygen demand and ventilatory drive, ventilation became similar in female mutant and control mice. However, a critical examination of tidal volumes measured in female control mice revealed a somewhat atypical finding: volumes were remarkably high during sleep and did not change with increasing levels of activity. Thus, with respect to this parameter differences between mutant and control mice have to be interpreted with precaution. A possible explanation for the present results could be the mixed background of the animals consisting of 129/Sv and C57BL/6, which are known to differ in their spontaneous breathing patterns (Reinhard *et al.*, 2002; Schulz *et al.*, 2002).

Sex-specific difference. Between both control and mutant mice sex differences were found during sleeping phase. The female mice showed higher breathing rates with higher absolute and specific tidal volumes, shorter expiratory timing, and higher flow rates. These differences were more pronounced among control type mice. During rest and activity breathing was comparable

among male and female animals; only body weight related values for tidal volume (sTV) and minute ventilation (sMV) were significantly higher in female control mice compared to male control mice.

In conclusion, the results suggest a **sex-specific phenotype affecting female** mice. However, due to the high tidal volumes measured in control females, interpretation of the data requires some precaution. Therefore, we recommend the study of a second batch of mice to verify the present findings.

3.9.6 References

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Abbreviations

bw	body weight (g)
mean_f	mean of all respiratory rates (1/min)
f	respiratory rate (1/min)
TV	tidal volume (ml)
sTV	specific tidal volume ($\mu\text{l/g}$)
MV	minute ventilation (ml/min)
sMV	specific ventilation (ml/min/g)
Ti	inspiratory time (ms)
Te	expiratory time (ms)
Ti/TT	relative duration of inspiration
PIF	peak inspiratory flow rate (ml/s)
PEF	peak expiratory flow rate (ml/s)
MIF	mean inspiratory flow rate (ml/s)
MEF	mean expiratory flow rate (ml/s).

Table 21: Spontaneous breathing pattern during sleep, rest, and activity

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=5)	(n=5)		(n=5)	(n=5)		<i>p</i> - value	<i>p</i> - value
Sleep	n = 3	n = 5		n = 4	n = 5			
f [1/min]	146.6 \pm 12.7	202.0 \pm 4.3	< 0.01	142.4 \pm 3.5	157.0 \pm 2.5	n.s.	n.s.	< 0.001
TV [ml]	0.22 \pm 0.01	0.28 \pm 0.01	< 0.02	0.21 \pm 0.01	0.22 \pm 0.002	n.s.	n.s.	< 0.01
sTV [μl/g]	7.4 \pm 0.6	11.5 \pm 0.7	< 0.01	7.6 \pm 0.8	9.9 \pm 0.3	< 0.02	n.s.	n.s.
MV [ml/min]	33.3 \pm 3.3	57.3 \pm 3.5	< 0.01	31.0 \pm 2.4	34.7 \pm 0.6	n.s.	n.s.	< 0.001
sMV [ml/min/g]	1.1 \pm 0.2	2.3 \pm 0.1	< 0.001	1.1 \pm 0.1	1.6 \pm 0.0	< 0.01	n.s.	< 0.001
Ti [ms]	101.1 \pm 6.4	90.5 \pm 4.7	n.s.	104.8 \pm 3.1	87.9 \pm 1.5	< 0.01	n.s.	n.s.
Te [ms]	314.6 \pm 36.9	207.0 \pm 7.7	< 0.01	317.4 \pm 13.1	294.6 \pm 5.4	n.s.	n.s.	< 0.001
Ti/TT	0.25 \pm 0.02	0.30 \pm 0.01	n.s.	0.25 \pm 0.01	0.23 \pm 0.003	n.s.	n.s.	< 0.01
PIF [ml/s]	3.7 \pm 0.7	5.3 \pm 0.2	< 0.01	3.5 \pm 0.2	4.3 \pm 0.1	< 0.001	n.s.	< 0.01
PEF [ml/s]	3.0 \pm 0.3	3.6 \pm 0.4	n.s.	2.7 \pm 0.3	2.8 \pm 0.2	n.s.	n.s.	n.s.
MIF [ml/s]	2.2 \pm 0.1	3.2 \pm 0.1	< 0.01	2.1 \pm 0.1	2.5 \pm 0.1	< 0.01	n.s.	< 0.01
MEF [ml/s]	0.7 \pm 0.1	1.4 \pm 0.1	< 0.01	0.7 \pm 0.1	0.8 \pm 0.1	n.s.	n.s.	< 0.001
Rest								
f [1/min]	285.4 \pm 14.5	276.3 \pm 6.8	n.s.	266.9 \pm 24.0	269.6 \pm 15.4	n.s.	n.s.	n.s.
TV [ml]	0.24 \pm 0.01	0.28 \pm 0.01	n.s.	0.25 \pm 0.02	0.23 \pm 0.01	n.s.	n.s.	< 0.01
sTV [μl/g]	7.7 \pm 0.7	11.4 \pm 0.6	< 0.01	8.7 \pm 0.7	10.4 \pm 0.5	n.s.	n.s.	n.s.
MV [ml/min]	67.6 \pm 5.3	76.3 \pm 4.0	n.s.	66.0 \pm 6.7	61.4 \pm 2.8	n.s.	n.s.	n.s.
sMV [ml/min/g]	2.1 \pm 0.2	3.1 \pm 0.2	< 0.02	2.2 \pm 0.1	2.8 \pm 0.1	n.s.	n.s.	n.s.
Ti [ms]	66.4 \pm 3.3	71.6 \pm 4.0	n.s.	71.3 \pm 6.3	63.4 \pm 3.7	n.s.	n.s.	n.s.
Te [ms]	146.2 \pm 8.9	146.1 \pm 3.4	n.s.	160.3 \pm 12.8	162.1 \pm 9.4	n.s.	n.s.	n.s.
Ti/TT	0.31 \pm 0.01	0.33 \pm 0.01	n.s.	0.31 \pm 0.007	0.28 \pm 0.004	n.s.	n.s.	< 0.01
PIF [ml/s]	6.4 \pm 0.6	6.8 \pm 0.3	n.s.	6.3 \pm 0.5	6.5 \pm 0.3	n.s.	n.s.	n.s.
PEF [ml/s]	4.4 \pm 0.4	4.1 \pm 0.3	n.s.	3.9 \pm 0.3	3.9 \pm 0.3	n.s.	n.s.	n.s.
MIF [ml/s]	3.7 \pm 0.3	4.0 \pm 0.2	n.s.	3.6 \pm 0.3	3.7 \pm 0.2	n.s.	n.s.	n.s.
MEF [ml/s]	1.7 \pm 0.1	1.9 \pm 0.1	n.s.	1.6 \pm 0.2	1.4 \pm 0.1	n.s.	n.s.	< 0.01

Activity								
f [1/min]	433.1 ± 11.4	431.3 ± 2.5	n.s.	415.6 ± 20.4	414.1 ± 9.8	n.s.	n.s.	n.s.
TV [ml]	0.25 ± 0.01	0.26 ± 0.01	n.s.	0.26 ± 0.02	0.23 ± 0.01	n.s.	n.s.	n.s.
sTV [µl/g]	7.8 ± 0.7	10.7 ± 0.7	< 0.02	9.0 ± 0.8	10.3 ± 0.5	n.s.	n.s.	n.s.
MV [ml/min]	104.7 ± 6.9	111.7 ± 5.8	n.s.	106.7 ± 10.4	93.8 ± 4.5	n.s.	n.s.	n.s.
sMV [ml/min/g]	3.3 ± 0.3	4.5 ± 0.3	< 0.02	3.6 ± 0.2	4.2 ± 0.2	n.s.	n.s.	n.s.
Ti [ms]	46.5 ± 0.8	48.1 ± 1.1	n.s.	47.8 ± 1.6	46.7 ± 0.9	n.s.	n.s.	n.s.
Te [ms]	92.5 ± 3.7	91.1 ± 1.6	n.s.	98.0 ± 5.9	98.6 ± 2.6	n.s.	n.s.	n.s.
Ti/TT	0.34 ± 0.01	0.35 ± 0.01	n.s.	0.33 ± 0.01	0.32 ± 0.004	n.s.	n.s.	n.s.
PIF [ml/s]	8.9 ± 0.7	9.4 ± 0.4	n.s.	9.2 ± 0.5	8.4 ± 0.4	n.s.	n.s.	n.s.
PEF [ml/s]	6.2 ± 0.6	6.2 ± 0.5	n.s.	6.4 ± 0.7	5.6 ± 0.5	n.s.	n.s.	n.s.
MIF [ml/s]	5.3 ± 0.4	5.5 ± 0.2	n.s.	5.4 ± 0.3	4.9 ± 0.2	n.s.	n.s.	n.s.
MEF [ml/s]	2.7 ± 0.2	2.9 ± 0.2	n.s.	2.7 ± 0.3	2.3 ± 0.1	n.s.	n.s.	n.s.

3.10 Expression Profiling

3.10.1 Summary

In this report, we describe the results of using close to genome-wide 20K cDNA microarrays for the RNA expression profiling of brain of five animals of the MCHR1 KO mutant mouse line. In total 19 chip hybridizations were performed. The data analysis and various statistical methods detected no genes differentially regulated between mutant and reference tissues in brain experiments.

3.10.2 Mice

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Five male mice of each genotype were provided to the molecular phenotyping screen (Table 22).

Organs were collected at the age of 105-110 days. To minimize the influence of circadian rhythm on gene expression, mice were killed between 9 a.m. and 12 a.m. by carbon dioxide asphyxiation. The following 17 organs were collected and archived in liquid nitrogen following our established standard operating protocols: bulbourethral gland, spleen, kidney, seminal vesicles, testis, white fat, liver, heart, lung, thymus, skin/cartilage (outer ear), bone (femur), skeletal muscle, salivary gland, brain, brown fat, and eye.

Table 22: *Mchr1*-mutant and wild-type control mice stored for expression profiling.

Mouse ID	Line	Sex	Date of Birth	Genotype	Date of Collection
30019675	MCHR1 KO	m	29.04.2004	-/-	11.08.2004
30019676	MCHR1 KO	m	29.04.2004	-/-	11.08.2004
30019677	MCHR1 KO	m	29.04.2004	-/-	11.08.2004
30019698	MCHR1 KO	m	30.04.2004	-/-	11.08.2004
30019708	MCHR1 KO	m	30.04.2004	-/-	11.08.2004
30019669	MCHR1 KO	m	29.04.2004	+/+	11.08.2004
30019673	MCHR1 KO	m	29.04.2004	+/+	11.08.2004
30019686	MCHR1 KO	m	29.04.2004	+/+	11.08.2004
30019692	MCHR1 KO	m	29.04.2004	+/+	11.08.2004
30019694	MCHR1 KO	m	29.04.2004	+/+	11.08.2004

3.10.3 Material and Methods

Isolation of total RNA

Total RNA was isolated just before processing for expression profiling. For preparation of total RNA individual organs were thawed in buffer containing chaotropic salt (RLT buffer, Qiagen) and homogenized using a Polytron homogenizer. Total RNA from individual samples was obtained according to manufacturer's protocols using RNeasy Midi kits (Qiagen). 2 µg RNA aliquots were run on a formaldehyde agarose gel to check for RNA integrity and the concentration was calculated from OD_{260/280} measurement. The RNA was stored at -80°C in RNase free water (Qiagen).

Chip design

We use a glass-surface DNA-chip containing ≈ 21,000 probes. About 20,200 of these probes are from the commercial Lion mouse array-TAG clone set, which is mostly derived from 3'UTRs. All Lion probes have been sequenced. The remaining probes are genes associated with immune response. Mouse array-TAG clones have the general ID MG-VW-XYZ (e.g. MG-3-1a5, MG-12-190m5,...) and the other probes are named s0-geneID (e.g. s0-birk, s0-mark1...).

DNA Microarrays

PCR products with 5'-aminogroup were amplified from the mouse arrayTAG library from Lion Bioscience comprising approximately 20,200 clones (Heidelberg, Germany). PCR products were dissolved in 3x SSC buffer and spotted on aldehyde-coated slides (Telechem, USA) using a Microgrid TAS II spotter (Biorobotics) with 48 Stealth™ SMP3 pins (Telechem). Spotted slides were rehydrated overnight in a humid chamber containing 50-70% aqueous solution of glycerol. Rehydrated slides were immersed in blocking solution (0.1 M sodium borohydride in 0.75x PBS with 25% ethanol) for 5 minutes, boiled in water for 2 minutes, briefly immersed in 100% ethanol and air-dried. Slides were pre-hybridized for 1 hour in pre-hybridization buffer (6x SSC, 1% BSA, 0.5% SDS) rinsed in water, dried and hybridized the same day (Seltmann *et al*, 2005).

Reverse Transcription and Fluorescent Labeling

For labeling 20µg of total RNA were used for reverse transcription and indirectly labeled with Cy3 or Cy5 fluorescent dye according the TIGR protocol (http://pga.tigr.org/sop/M004_1a.pdf). Labeled cDNA was dissolved in 30µl hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardt's solution and 50% formamide) and mixed with 30 µl of reference cDNA solution (pool from five control animals) labeled with the second dye. This hybridization mixture was placed on a pre-hybridized microarray, under a cover slip, placed into a hybridization chamber (Genetix) and immersed in a thermostatic bath at 42°C for at least 16 hours. After hybridization slides were washed in 40 ml of 3x SSC, 40 ml of 1x SSC and 40 ml of 0.25x SSC at room temperature. For drying slides were placed in an empty 50 ml Falcon tube (Becton Dickinson, USA) and centrifuged at 4000 m/s². Dried slides were scanned with a GenePix 4000A microarray scanner and the images were analyzed using the

GenePix Pro3.0 image processing software (Axon Instruments, USA). All data were normalized by adjusting the median of log-ratios of Cy5 to Cy3 intensities to 0. For data analysis Pattern Analysis of Microarrays = PAM (http://www.gsf.de/ieg/groups/exppro_cpt.html#PAM) was used.

Chip Hybridization

Depending on the amount of RNA available for hybridization, in general four chip hybridizations were performed with RNA from all organs of each four individual mutant mice (in total 16 hybridizations). Each chip hybridization was performed against the identical pool of each organ of wt RNAs (reference RNA pool; wt). For each individual the chip experiments included two color-flip experiments.

3.10.4 Results

Selected Organs and Isolated RNA

Brain was selected as organ for expression profiling analysis based on high expression of *Mchr1* in this organ. We isolated total RNA of brain of five *Mchr1*-mutant mice and five control individuals (Table 23).

Table 23: Amount of total RNA [μg] isolated from brain.	
Mouse ID	Brain
30019675	387
30019676	296
30019677	370
30019698	288
30019708	300
30019669	361
30019673	341
30019686	400
30019692	342
30019694	304

Analysis of Gene Expression in Brain

Table 24: Chip hybridization of brain RNA: labeling and number of detected spots		
Chip ID	Cy5/Cy3	Detected Spots
#1	675/ ref	11809
#2	675/ ref	11754
#3	ref/ 675	11645
#4	676/ ref	9846
#5	676/ ref	9149
#6	ref/ 676	7106
#7	ref/ 676	9437
#8	677/ ref	9591
#9	677/ ref	9148
#10	ref/ 677	7995
#11	ref/ 677	7289
#12	698/ ref	9917
#13	698/ ref	9802
#14	ref/ 698	14228
#15	ref/ 698	17217
#16	708/ ref	10445
#17	708/ ref	11778
#18	ref/ 708	9876
#19	ref/ 708	12623
		4294 overlap

These genes were evaluated for the significance of differential gene expression. Genes were ranked according to the lowest absolute ratio of signal intensities in 19 microarray experiments (mutant versus ref). This ranking is independent of the reproducibility in terms of up- and down-regulation. The number of genes with non-reproducible up- or down-regulation („non-uniform patterns“) is given for different selections of genes in the ranking („ranked genes“). The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns was calculated for significance levels $p < 0.05$.

For example, the selection of the top 20 ranked genes contains 18 genes with non-reproducible chip data. The remaining two genes with reproducible up- or down-regulation contain one or more non-differentially expressed genes (NDE) with the significance level $p < 5\%$. The minimal ratios of expression for this selection ranged from 4.26 to 1.05 fold induction/repression.

Table 25: Chip hybridization of brain RNA (mutant vs. ref): Evaluation of data

Ranked Genes (According Lowest of 19 Ratios)	Non-uniform Patterns	NDE (False Positives) p<0.05	Fold Induction (Minimum of 19 Chips)
1 - 20	18	≥ 1	4.26 – 1.05
1 - 40	38	≥ 1	4.26 – 1.04
1 - 60	58	≥ 1	4.26 – 1.03

According to 90% non-reproducible chip data of the top 20 ranked genes, no gene with differential expression in brain of mutant mice was observed in all experiments.

3.10.5 Discussion

Using the selection criteria described above, we could not identify any gene differentially expressed in brain tissue of *Mchr1*-mutant mice.

3.10.6 References

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Seltmann M., M. Horsch, A. Drobyshev, Y. Chen, M. Hrabé de Angelis and J. Beckers (2005): Assessment of a systematic expression profiling approach in ENU-induced mouse mutant lines. *Mammalian Genome* 16: 1-10

3.11 Metabolic Screen

3.11.1 Summary

The metabolic screening provides a comparative analysis of bioenergetic parameters in mice. Mechanisms which lead to disturbances in body weight regulation and energy metabolism are determined. Hence, the basal energetic demands are monitored during *ad libitum* feeding and under food restricted conditions. In humans unbalanced energy uptake and energy expenditure cause the development of obesity (Spiegelman and Flier, 2001) or anorexia nervosa with severe weight loss (Hebebrand *et al.*, 2003). Some rodent and other species tend to increase activity upon food restriction leading to weight loss when given access to an activity wheel (Exner *et al.*, 2000). Several studies described that fasting in mice results in transient depression of metabolic rate, heart rate, body temperature and locomotor activity (Duffy *et al.*, 1990; Williams *et al.*, 2002). Therefore the primary Metabolic Screening focuses on the determination of food and energy uptake under *ad libitum* conditions and metabolic adaptations during food restriction and serves as the origin for further investigations in the Secondary and Tertiary screening which go into details of energy expenditure and energy storage.

In the primary metabolic screen, 12 mutant mice (six males and females) and 14 controls (seven males and females) were analyzed. The mice were first fed under *ad libitum* conditions for two weeks, followed by a period of food restriction to 60% of *ad libitum* for seven days to analyze adaptive responses of metabolism. The primary metabolic screen focuses on investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature and adaptive capacity of metabolic processes. Common sex-specific differences were found indicating males heavier than females. Genotype-specific differences could be determined in females showing mutant females lighter than the controls. Besides this genotype-specific difference, no further differences indicating a metabolic phenotype could be found.

3.11.2 Mice

Seven adult control males and six adult mutant males entered the Metabolic Screen at the beginning of calendar week 39 in 2004. The females (seven controls and six mutants) entered the metabolic laboratory one week later. The mice were single caged on grid panels (0.5°cm grid hole diameter). They were fed *ad libitum* for a period of 14 days, followed by a period of food restriction to 60% of *ad libitum* for seven days to analyze adaptive responses of metabolism.

3.11.3 Material and Methods

Recorded Data

During the different feeding regimes body weight, food consumption (F_{con}), rectal temperature (T_{re}), daily feces production (F_{ec}), energy uptake (E_{up}),

energy content of the feces (E_{fec}), metabolizable energy (E_{met}) and the food assimilation coefficient (F_{ass}) were recorded.

Analysis of Feces

The separation of mice in single cages allowed collection of feces in three day intervals. Samples of lab chow and feces (~1 g) were dried at 60°C for two days, homogenized in a coffee grinder and squeezed to a pill for determination of energy content in a bomb calorimeter (IKA Calorimeter C7000) based on dry measurement principle. Energy uptake is determined as the product of food consumed and the caloric value of the food. To obtain metabolizable energy (E_{met}) the energy content of feces and urine (2% of E_{up} ; Drozd 1975) were subtracted from energy uptake.

Statistical Analysis

All values are presented as means \pm SEM. Two-way-ANOVA (SigmaStat, Jandel Scientific) was used to test for effects of the factors genotype and sex. The Tukey test was applied for post hoc multiple comparisons. The Mann-Whitney-Test for paired samples was used to analyze the effect of nutritional status on parameters of energy metabolism.

3.11.4 Parameters

Recorded Data during the different feeding regimes
--

body weight, food consumption (F_{con}), rectal temperature (T_{re}), daily feces production (Fec), energy uptake (E_{up}), energy content of the feces (E_{fec}), metabolizable energy (E_{met}), food assimilation coefficient (F_{ass})
--

3.11.5 Results

Common sex-related differences in almost all parameters could be found in both genotypes (Table 26). Males were significantly higher in body weight, consumed more food and had higher ratios of energy uptake and assimilation. Comparing body weight between the genotypes, only females showed significantly lower body weight values in mutant mice. All other parameters were not statistically different between mutant and wild-type control mice.

Raw data for each individual are available on demand in Excel sheets.

3.11.6 Discussion

There are several publications about *Mchr1*-knockout mice which discuss a metabolic phenotype. All *Mchr1*-mutant lines are based on a mixed background of 129Sv/J and C57BL/6 mice but in different backcross generations:

- Marsh *et al.* (2002) described their knockout line as lean, but hyperphagic with increased metabolic rate. The lean phenotype might be caused by observed hyperactivity which also made them less susceptible to diet induced obesity (DIO).

- Chen *et al.* (2002) focused the hypermetabolic and hyperphagic phenotype mainly on males.
- This could be confirmed by Astrand *et al.* (2004) showing even increased heart rate during dark (activity) phase. Fasting experiments revealed that wild-type control mice rapidly down-regulated heart rate and body temperature, while this depression was delayed in *Mchr1*-knockout mice. They assumed that the inactivation of *Mchr1* counterbalances obesity which might be induced by a high-fat diet (resistance to DIO).

Results of the primary metabolic screening could not confirm any metabolic phenotype on **this** *Mchr1*-knockout line. In case of the body weight, only mutant females showed a significantly reduced body weight compared to control females. Both mutant sexes showed a tendency of elevated food consumption, but this could not be determined as statistically significant. Feces production and energy content of feces did not show any deviations between mutant and control mice. Hence, food assimilation was not different either, indicating no metabolic phenotype in the investigated mice.

Due to the metabolic characteristics reported in the literature, we enlarged the analysis for determination of **rasping activity**. Because the mice were housed on grid panels, it was possible to distinguish between feces produced and food which was rasped daily and might be measured as food consumed accidentally. Mutant females showed a 49.2% higher rasping activity than the wild-type controls, mutant males even 56.3%. The present data for food consumption and energy uptake, respectively, took the rasping activity into account. The studies described above by Chen *et al.* (2002), Marsh *et al.* (2002) and Astrand *et al.* (2004) did not mention that the mice were housed on grid panels to separate food remainings and feces.

Hence, it might be possible that the “hyperphagia” is due to the measurement of food consumption without subtraction of the rasped food. It is therefore strongly recommended to analyze basal metabolic rate (BMR), core body temperature and telemetrical activity pattern to confirm metabolic characteristics described in the literature.

3.11.7 References

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Abbreviations

F_{con}	Food consumption
T_{re}	rectal temperature
F_{ec}	daily feces production
E_{up}	energy uptake
E_{fec}	energy content of the feces
E_{met}	metabolizable energy
F_{ass}	food assimilation coefficient

Table 26: Metabolic Parameters Recorded in the Primary Screen

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)					Mutant (B)					A~B	
	<i>ad libitum</i>			food reduction, 7 days to 60%		<i>ad libitum</i>			food reduction, 7 days to 60%			
	Male	Female		Male	Female	Male	Female		Male	Female	Male	Female
	(n=7)	(n=7)	<i>p</i> - <i>value</i>	(n=7)	(n=7)	(n=6)	(n=6)	<i>p</i> - <i>value</i>	(n=6)	(n=6)	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>
Body weight [g]	31.0 \pm 0.92	27.8 \pm 0.6	< 0.01	25.2 \pm 1.09	21.6 \pm 0.21	32.8 \pm 1.79	24.3 \pm 0.81	< 0.001	25.7 \pm 1.9	19.7 \pm 0.34	n.s.	< 0.01
Rectal body temperature [°C]	37.4 \pm 0.2	37.4 \pm 0.06	n.s.	35.4 \pm 0.36	35.4 \pm 0.14	37.4 \pm 0.19	37.6 \pm 0.08	n.s.	35.7 \pm 0.51	35.7 \pm 0.4	n.s.	n.s.
Food consumption [g day⁻¹]	3.68 \pm 0.17	2.36 \pm 0.24	< 0.001	60% of ad libi- tum		3.92 \pm 0.2	2.72 \pm 0.23	< 0.01	60% of ad libi- tum		n.s.	n.s.
Energy uptake [kJ day⁻¹]	67.7 \pm 3.2	47.9 \pm 5.82	< 0.001	40.6 \pm 1.92	28.7 \pm 3.49	72.2 \pm 3.65	50.1 \pm 4.17	< 0.01	43.3 \pm 2.19	30.0 \pm 2.5	n.s.	n.s.
Energy uptake BW⁻¹ [kJ g⁻¹ day⁻¹]	2.18 \pm 0.1	1.56 \pm 0.16	< 0.01	1.63 \pm 0.12	1.11 \pm 0.13	2.22 \pm 0.14	2.08 \pm 0.21	n.s.	1.73 \pm 0.15	1.53 \pm 0.13	n.s.	n.s.
Feces production [g day⁻¹]	0.71 \pm 0.03	0.48 \pm 0.05	< 0.01	0.45 \pm 0.02	0.23 \pm 0.01	0.72 \pm 0.02	0.52 \pm 0.03	< 0.001	0.44 \pm 0.03	0.29 \pm 0.02	n.s.	n.s.
Energy content feces [kJ g⁻¹]	16.4 \pm 0.06	16.2 \pm 0.11	n.s.	16.0 \pm 0.11	16.0 \pm 0.1	16.48 \pm 0.07	16.18 \pm 0.09	< 0.05	16.1 \pm 0.13	15.9 \pm 0.08	n.s.	n.s.
Metabolized energy [kJ day⁻¹]	54.7 \pm 2.77	34.8 \pm 3.51	< 0.001	32.6 \pm 1.8	19.9 \pm 1.83	58.9 \pm 3.38	40.7 \pm 3.74	< 0.01	35.3 \pm 2.18	25.2 \pm 2.75	n.s.	n.s.
Metabolized energy [kJ g⁻¹ day⁻¹]	1.76 \pm 0.09	1.25 \pm 0.11	< 0.01	1.3 \pm 0.09	0.92 \pm 0.08	1.81 \pm 0.13	1.69 \pm 0.19	n.s.	1.42 \pm 0.14	1.27 \pm 0.12	n.s.	n.s.
Food assimilation coefficient [%]	80.7 \pm 0.4	80.1 \pm 0.4	n.s.	80.1 \pm 1.02	82.2 \pm 1.32	81.5 \pm 0.65	81.0 \pm 0.7	n.s.	81.4 \pm 1.65	82.8 \pm 0.7	n.s.	n.s.

3.12 Pathology Screen

3.12.1 Summary

The Pathology Screen performed a complete morphological analysis with standard stains. The knockout animals showed lower weight than the control littermates. In addition, although both groups developed dermatitis with abscess formation, this was more frequently found in the mutant male mice, and the difference was statistically significant ($p=0.05$). Skeletal malformations have been found in both, mutant mice and their control littermates. However, these changes were strain related and not genotype-specific. In conclusion, we found a specific pathological phenotype consisting of low weight and skin lesions in mutant males, most probably secondary to the more aggressive behavior or hyperactivity of this mouse line.

3.12.2 Mice

A total of 40 mice, 19 mutant mice (eight males, 11 females) and 21 control mice (nine males, 12 females) were analyzed. Due to the workflow in the GMC, mice of different ages were received from different screens (Table 27). In one case, the flow was interrupted due to severe dermatitis.

Table 27: <i>Mchr1</i>-mutant mice and their control littermates analyzed						
Origin	Control		Mutant		Number of Animals	Age [weeks]
	Male	Female	Male	Female		
Lung Screen	0	5	0	5	10	15
Dysmorphology Screen	2	2	3	0	7	20- 21
Metabolic Screen	6	5	5	6	22	20 - 21
Other Screens	1	0	0	0	1	8
Total Number of Animals	9	12	8	11	40	

3.12.3 Materials and Methods

Mice received in the laboratory of pathology were sacrificed with CO₂. The animals were analyzed macroscopically and weighed (www.eulep.org/Necropsy_of_the_Mouse/index_2004.php). The thymus and left lobe of the liver were measured. Blood samples were taken, centrifuged and the serum was saved at -20°C. Tails were preserved at -70°C for further genetic analysis. Following a complete dissection, an x-ray of the complete bone structure was taken, when indicated (Hewlett Packard, Cabinet X-Ray System Faxitron Series). All organs were fixed in 4% buffered formalin and embedded in paraffin for histological examination. Two μm thick sections from skin, heart, lung, brain, cerebellum, thymus, spleen, cervical lymph nodes, thy-

roid, parathyroid, adrenal gland, stomach, intestine, liver, pancreas, kidney, reproductive organs, and urinary bladder were cut and stained with hematoxylin and eosin (H&E). For statistic analysis the Fisher's exact test was used. A p-value of < 0.05 was interpreted as significant.

3.12.4 Genotype-specific Results

Overview

Table 28: Morphological alterations of <i>Mchr1</i>-mutant mice compared to their control littermates.			
Organ	Alteration	Organ	Alteration
Skin	Yes	Pancreas	No
Musculoskeletal system	No	Cervical lymph node	No
Eyes	No	Thymus	No
Brain	No	Spleen	No
Cerebellum	No	Thyroid gland	No
Heart	No	Parathyroid	No
Trachea	No	Adrenal gland	No
Lung	No	Kidneys	No
Teeth	No	Urinary bladder	No
Salivary glands	No	Testes	No
Esophagus	No	Epididymis	No
Stomach	No	Funiculus spermaticus	No
Small intestine	No	Ovaries	No
Large intestine	No	Uterus	No
Liver	No	Vagina	No
Mama	No	Weight	Yes

Skin lesions

In the mutant mice and their control littermates several lesions were identified. Inflammatory changes of the skin were observed only in males of both, control and mutant mice. Although it was found in both groups, it was more frequent in the mutant mice, and the difference was statistically significant ($p = 0.05$ measured by Fisher's exact test; Table 29).

Table 29: Skin lesions in *Mchr1* mice

Control + Mutant			Males		
Finding	Female	Male	Finding	Control	Mutant
Abscess / dermatitis	0	8	Abscess / dermatitis	3	5
Without lesions	23	9	Without lesions	8	1
Fisher test p=0.0003			Fisher test p=0.05		

Microscopically, acute inflammatory infiltrates were observed in the epidermis, dermis and subcutaneous tissue, in some cases with real abscess formation. In one case, the flow was interrupted due to severe dermatitis at the age of eight weeks. In five of eight cases of dermatitis, the mice had a systemic infection with foci of increased granulopoiesis in the spleen and in the liver (3/3 control; 2/5 mutant; Fig. 6).

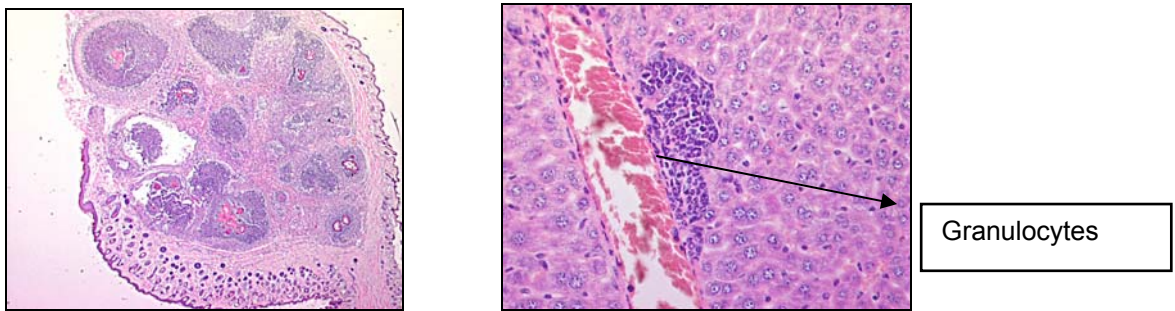


Figure 7: Acute inflammatory infiltrates.

The left panel shows an acute abscess in the dermis and subcutaneous tissue (100x H&E). The right picture demonstrates the severe granulopoiesis in the liver due to the infection (250x H&E).

Body Weight

The body weight of *Mchr1*-mutant mice was lower compared to the controls (see Table 30 for more details).

Table 30: Mean (range) body weight [g] of *Mchr1*-mutant mice and their control littermates.

Origin	Control		Mutant		Age [week]
	Female	Male	Female	Male	
Lung Screen	22.0 (19-25)	--	21.2 (20-22)	--	15
Dysmorphology Screen	30.0 (30-30)	29.5 (29-30)	--	25.5 (25-26)	22
Metabolic Screen	26.4 (26-27)	31.0 (27-33)	24.1 (22-25)	27.0 (25-30)	22

X-rays results

We performed a comprehensive radiological analysis of the carcasses. We observed skeletal malformations as illustrated in Figures 7 and 8 that consist of absence of one lumbar vertebra (in four of 21 control mice and in one of 19 mutant mice.), and short last rib in two of 21 control mice and in one of 19 mutants.

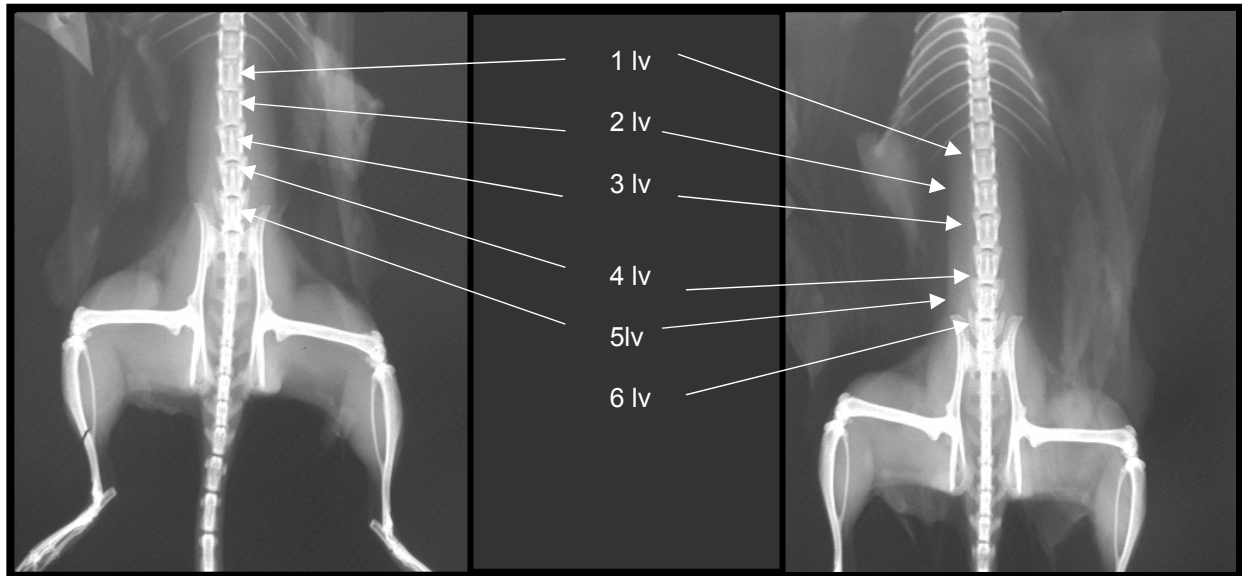


Figure 8: Skeletal malformations I.

Note the absence of the sixth lumbar vertebra on the left panel, compared to the right panel showing complete number of vertebrae.

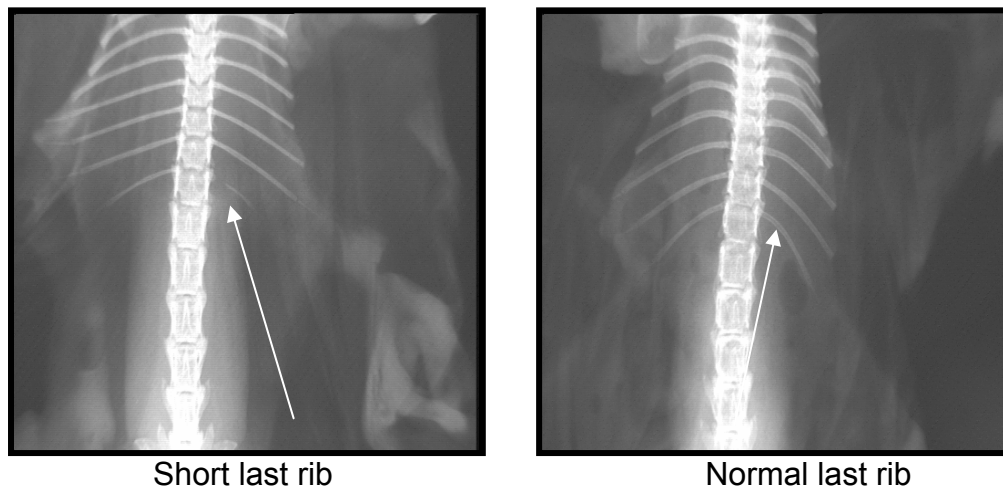


Figure 9: Skeletal malformations II.

The left panel demonstrates the short last rib, compared to a normal last rib on the right panel.

3.12.5 Discussion

In this batch of animals, skin inflammatory lesions were relatively frequently identified, in both mutant and control mice. Nevertheless, the lesions were more often in mutant mice, and the difference was statistically significant. Interestingly, these lesions were identified only in male mice, and could be a manifestation of the more aggressive behavior that the male animals showed during their stay in the Behaviour Screen. In the Behavior Screen some of the males were separated because they injured the other males of the same cage. Unfortunately, it was not possible to track down which animals were in the same cage to be able to corroborate this impression.

All the animals have been carefully examined radiologically. The analysis was performed with the carcasses (without viscera and muscles which might shadow subtle malformations). However, abnormalities like osteoporosis that have been described previously by Bohlooly *et al.* (2004) could not be confirmed by conventional radiological examination. Despite the relative frequent finding of ribs and vertebrae changes, these findings were observed more in control than in mutant mice, lacking any genotype-related significance. These bone alterations seem to be sporadic findings in different mouse strains.

In general the mutant mice were lighter than their control littermates; whether this phenotype is secondary to the known hyperactivity of these mice has to be determined.

In conclusion, the *Mchr1*-mutant animals showed lower weight than the control littermates. In addition, although both groups developed dermatitis with abscess formation, this was more frequently found in the knockout male mice, and the difference was statistically significant ($p=0.05$). Skeletal malformations have been found in both, knockout mice and their control littermates. However, these changes seem to be related to the mouse strain, and therefore, are not genotype-specific.

3.12.6 References

Bohlooly-Y M., Mahlapuu M., Andersén, H. *et al.* (2004): Osteoporosis in MCHR1-deficient mice. *BBRC* 318: 964-969

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