

1 Summary

In a primary screen, 58 NOX-KO mice (28 mutants, 30 control litter mates) 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, Expression Profiling, Metabolism, and Pathology.

Behavior Screen: Analysis of the observed behavioral parameters revealed a prolonged latency to unfamiliar (novel) object exploration in male mutant mice.

Neurology Screen: We found that female mutant mice displayed an altered tail elevation and were more irritable as compared to female control mice. The significantly altered tail elevation of the female mutant mice suggests a change in the muscular tone of these animals. However, we did not detect any variation in other muscle parameters that would point towards a muscular dysfunction. Irritability is a varying test parameter, which alone cannot offer any conclusive information about a neurological phenotype.

Clinical Chemistry: The only genotype-related significant difference we detected, was an increase of the transferrin concentration in the female mutant mice and a slightly higher mean red blood cell count and hematocrit in the male mutant mice. However, the findings were limited to one sex and the values detected were situated within the normal range of mice. Therefore we believe them to be an effect of coincidence and no real genotype-related phenotype.

In the screens **Dymorphology, Eye, Immunology, Allergy, Nociception, Lung Function, Energy Metabolism** and **Pathology**, no genotype-specific differences were found.

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

2 General Part

2.1 The Role of the Gene

In bacteria, NADH-oxidases function in a two component system called alkyl hydroperoxide reductase (AhpR). The system is composed of the flavoprotein AhpF, a NADH consuming oxidoreductase and the colorless component AhpC. In concert with AhpC, AhpF can reduce “damaging” hydroperoxides to “harmless” alcohols. A human *AhpF* homologue (*NOX*) was cloned from an expression cloning approach. A cDNA derived from HELA cells was found to preserve Burkitt lymphoma (BL) cells from apoptosis at low cell density (Brielmeier *et al.* 2001). Therefore *NOX* was termed an antiapoptotic gene. Apoptosis in BL cells is mediated by oxidative stress due to insufficient cystin import. Cystin is a component of the tripeptide glutathione (GSH) that is a key player in cellular oxidative stress response.

Recently two papers have been published describing cDNAs identical to the one we have cloned (Wu *et al.*, 2002; Ohiro *et al.*, 2002) which assign the gene pro-apoptotic functions. These authors detected a different subcellular localization and a deviating overall expression pattern. Brielmeier *et al.* (2001) found *NOX* as an antiapoptotic gene in their assay, showed expression in heart, liver, kidney and testis and found co-localization with the ER. The conflicting data might be due to differences in cell lines or assays used.

2.2 Known Phenotypes

No obvious phenotypic abnormality has been detected so far.

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

2.3 Mice

2.3.1 Number and kind of mice

Table 1: NOX-KO mice provided for analysis.	
Genotype / Sex	Number of Animals
Mutant female	15, one animal died
Mutant male	13
Control female	16
Control male	14

As described by the owner, the mice have been generated from the ES cell line E14 (129/SV) as a lacZ knock-in replacing the NADH-binding domain thereby removing Exons 4 and 5 of the long form and the first exon of the short form. The mutant mouse line is maintained on a C57BL/6J background. The animals were in backcross generation 2.

2.3.2 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 $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$, 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 carried 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.

2.4 Workflow

2.4.1 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.

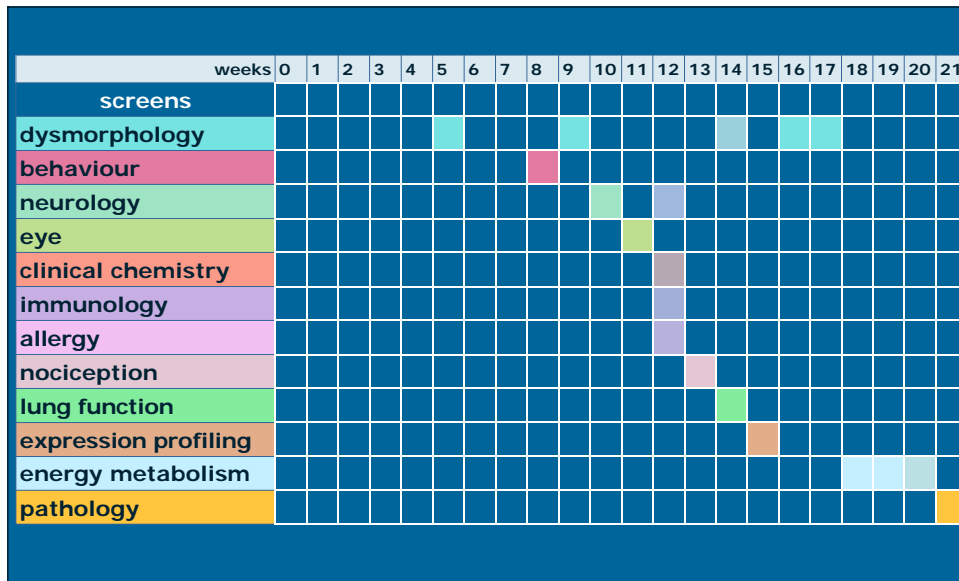



Figure 1: Workflow of the primary screen

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 Dysmorphology 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.

2.4.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the 58 NOX-KO mice. 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. One animal died during the primary screen and thus could not be analyzed for all parameters (Table 1).

2.4.3 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.

Microgranulomas in the liver are observed commonly in mice on a C57BL/6 genetic background. In those cases the results of the MHV tests are used to exclude MHV as one possible reason for these infiltrates (See chapter 3.12 Pathology Screen)

2.5 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.

2.6 References

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Abbreviations and wording

NOX	NADH-oxidase (AMID, PRG3), protein
NOX	gene coding for NOX
NOX-KO	mutant mouse line deficient in NOX
GMC	German Mouse Clinic
IVC	individually ventilated cage
control	homozygous wild-type littermates control, $NOX^{+/+}$
mutant	homozygous mutant, $NOX^{-/-}$
KO	knockout
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

The modified hole board (mHB) test is used as primary screen in the behavioral phenotyping module of the GMC, because it allows the comprehensive analysis of a range of behavioral 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 (Ohl *et al.*, 2001).

In the case of NOX-KO mice, the social parameter could not be evaluated, because not all mice were group housed with at least three animals per cage. Using this test, we could not detect any particular behavioral phenotype in mutant mice. Therefore, further behavioral analysis with these mice is not indicated at present.

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 31 female mice (16 controls, 15 mutants) and 27 male mice (14 controls, 13 mutants) were available for analysis.

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.

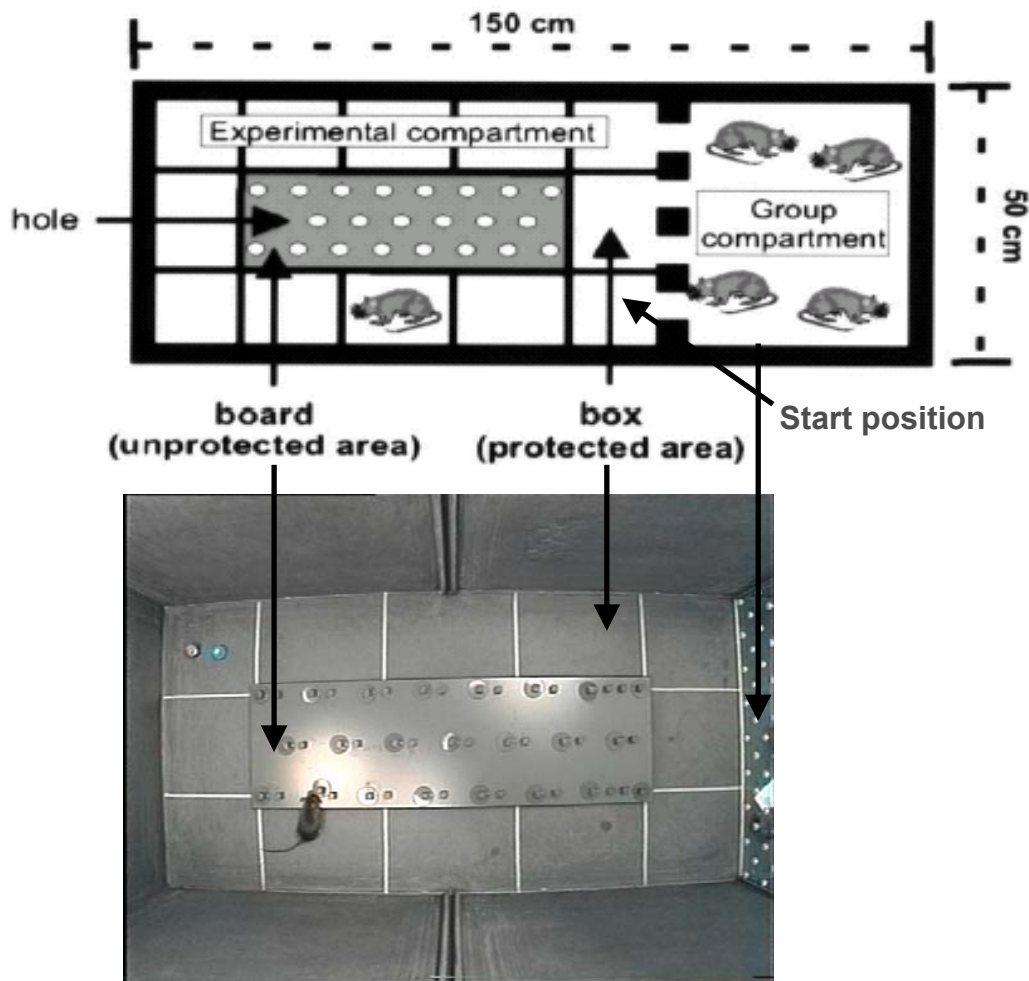


Figure 2: Test arena for modified Hole Board test (Ohl *et al.*, 2001).

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 Parameters

Manually recorded behavior
Line crossings, rearings, board entries, hole explorations, hole visits, stretched attends (risk assessment), partition (group contact), grooming, defecation, unfamiliar and familiar object exploration
Video-track analysis
Total distance moved, mean velocity, maximum velocity

3.1.5 Results

Analysis of the observed behavioral parameters only revealed a prolonged latency to unfamiliar (novel) object exploration in male mutant mice. This was the only statistically significant genotype effect (Table 3).

3.1.6 Discussion

In general, an increased avoidance of a novel object can indicate neophobia. However, since this was only reflected in an increased latency towards unfamiliar object exploration, but not in the duration of the unfamiliar object exploration, and not in any other parameter either, these mutants do not present a strong case for a neophobia phenotype. Because otherwise mutant mice appeared to be behaviorally unobtrusive, no further behavioral analysis is indicated at present.

3.1.7 Reference

Ohl, F., Sillaber, I., Binder, E., Keck, M.E. and F. Holsboer (2001): Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test. *J. Psychiatr. Res.* 35: 147-154.

Table 3: Results of behavioral observation in the modified hole board test

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=14)	(n=16)	<i>p - value</i>	(n=13)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Line crossing [frequency]	124.14 \pm 8.33	110.69 \pm 7.68	N.A.	127.38 \pm 11.66	121 \pm 8.03	N.A.	n.s.	n.s.
Line crossing [latency]	3.48 \pm 0.93	6.03 \pm 1.59	N.A.	3.39 \pm 0.94	3.19 \pm 0.69	N.A.	n.s.	n.s.
Immobility [Total duration %]	0 \pm 0	0.4 \pm 0.4	N.A.	0 \pm 0	0 \pm 0	N.A.	NA	NA
Rearings in box [frequency]	23.21 \pm 2.3	18.88 \pm 2.65	N.A.	28 \pm 4.56	23.4 \pm 2.85	N.A.	n.s.	n.s.
Rearings in box [latency]	58.33 \pm 10.44	63.94 \pm 16.8	N.A.	42.12 \pm 9.29	38.07 \pm 5.16	N.A.	n.s.	n.s.
Hole exploration [frequency]	29.29 \pm 3.68	24.44 \pm 2.6	N.A.	30.15 \pm 3.14	26.67 \pm 2.95	N.A.	n.s.	n.s.
Hole exploration [latency]	29.52 \pm 4.23	56.06 \pm 16.91	N.A.	32.99 \pm 6.8	28.27 \pm 4.51	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.	NA	NA
Hole visit [latency]	300 \pm 0	300 \pm 0	N.A.	300 \pm 0	300 \pm 0	N.A.	NA	NA
Board entry [frequency]	11.86 \pm 1.76	8.63 \pm 1	N.A.	10.85 \pm 1.21	8.67 \pm 1.32	N.A.	n.s.	n.s.
Board entry [latency]	52.42 \pm 7.55	79.03 \pm 17.79	N.A.	51.2 \pm 7.6	75.25 \pm 10.17	N.A.	n.s.	n.s.

Board entry [total duration %]	13.41 ± 2.34	10.09 ± 1.41	N.A.	12.43 ± 1.2	10.66 ± 1.78	N.A.	n.s.	n.s.
Rearing on board [frequency]	0.14 ± 0.1	0.38 ± 0.18	N.A.	0.69 ± 0.41	0.6 ± 0.29	N.A.	n.s.	n.s.
Rearing on board [latency]	285.49 ± 9.9	277.03 ± 14.01	N.A.	279.72 ± 11.46	289.28 ± 4.99	N.A.	n.s.	n.s.
Risk assessment [frequency]	0 ± 0	0.13 ± 0.13	N.A.	0 ± 0	0 ± 0	N.A.	NA	NA
Risk assessment [latency]	300 ± 0	287.38 ± 12.62	N.A.	300 ± 0	300 ± 0	N.A.	NA	NA
Group partition [frequency]	N.A.	NA	N.A.	N.A.	NA	N.A.	NA	NA
Group partition [latency]	N.A.	NA	N.A.	N.A.	NA	N.A.	NA	NA
Group contact [total duration %]	N.A.	NA	N.A.	N.A.	NA	N.A.	NA	NA
Grooming [frequency]	1.93 ± 0.22	1.25 ± 0.34	N.A.	1.38 ± 0.14	1.33 ± 0.21	N.A.	p=0.05	n.s.
Grooming [latency]	109.12 ± 9.02	178.65 ± 25.06	N.A.	129.13 ± 12.1	121.98 ± 19.07	N.A.	n.s.	n.s.
Grooming [total duration %]	2.1 ± 0.28	1.38 ± 0.52	N.A.	1.6 ± 0.24	1.73 ± 0.41	N.A.	n.s.	n.s.
Defecation [frequency]	0.93 ± 0.29	0.75 ± 0.27	N.A.	2.08 ± 0.68	0.4 ± 0.21	N.A.	n.s.	n.s.
Defecation [latency]	180.7 ± 33.7	197.46 ± 31.28	N.A.	140.15 ± 39.41	245.4 ± 27.55	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	6.64 ± 0.62	4.5 ± 0.67	N.A.	5.46 ± 0.57	4.93 ± 0.52	N.A.	n.s.	n.s.

Familiar object exploration [frequency]	5.36 ± 0.49	5.38 ± 0.6	N.A.	4.77 ± 0.46	5.2 ± 0.42	N.A.	n.s.	n.s.
Unfamiliar object exploration [latency]	32.28 ± 4.86	79.63 ± 22.44	N.A.	61.95 ± 9.89	42.04 ± 8.59	N.A.	p<0.05	n.s.
Familiar object exploration [latency]	65.88 ± 18.36	62.71 ± 16.77	N.A.	39.31 ± 7.51	54.71 ± 8.3	N.A.	n.s.	n.s.
Unfamiliar object exploration [total duration %]	1.26 ± 0.08	1.53 ± 0.3	N.A.	1.25 ± 0.1	1.69 ± 0.31	N.A.	n.s.	n.s.
Familiar object exploration [total duration %]	0.91 ± 0.09	0.95 ± 0.1	N.A.	0.77 ± 0.08	0.93 ± 0.07	N.A.	n.s.	n.s.
Object Index	0.18 ± 0.05	0.13 ± 0.08	N.A.	0.23 ± 0.05	0.22 ± 0.05	N.A.	n.s.	n.s.

Table 4: 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=13)	(n=16)	<i>p - value</i>	(n=12)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Total Distance Moved [cm]	3520.17 \pm 203.51	3067.34 \pm 177.41	N.A.	3377.5 \pm 245.5	3331.78 \pm 214.42	N.A.	n.s.	n.s.
Mean Velocity [cm/sec]	19.12 \pm 0.73	17.46 \pm 0.75	N.A.	18.89 \pm 1.17	18.54 \pm 0.75	N.A.	n.s.	n.s.
Maximum velocity [cm/sec]	59.85 \pm 6.74	52.54 \pm 2.23	N.A.	63.75 \pm 4.1	55.54 \pm 1.44	N.A.	n.s.	n.s.
Turns [Frequency]	1887.69 \pm 59.85	1740 \pm 77.71	N.A.	1811.83 \pm 59.96	1821.14 \pm 80.47	N.A.	n.s.	n.s.
Mean Turn Angle [degrees]	24.94 \pm 1.77	23.13 \pm 0.94	N.A.	24.81 \pm 0.79	23.77 \pm 1.12	N.A.	n.s.	n.s.
Angular Velocity [degrees/sec.]	175.23 \pm 20.5	146.18 \pm 4.25	N.A.	165.76 \pm 6.41	153.81 \pm 5.15	N.A.	n.s.	n.s.
Absolute Meander [degrees/sec.]	17.12 \pm 0.87	16.83 \pm 0.8	N.A.	17.66 \pm 0.62	17.1 \pm 0.94	N.A.	n.s.	n.s.
Distance to Wall [cm]	8.3 \pm 0.49	7 \pm 0.38	N.A.	7.7 \pm 0.33	7.39 \pm 0.35	N.A.	n.s.	n.s.
Distance to Board [cm]	7.92 \pm 0.27	8.87 \pm 0.29	N.A.	8.37 \pm 0.25	8.56 \pm 0.26	N.A.	n.s.	n.s.

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.

Fifty-eight mice were analyzed in the Dysmorphology module of the GMC. No significant differences were found between mutant mice and their control littermates for any quantitative, bone related parameter. Sex difference was significant in mutant and control for lean mass only. In the X-ray analysis, no correlation of phenotypes with a genotype was detected.

3.2.2 Mice

Twenty-seven male (14 control, 13 mutant) and 31 female (14 control, 16 mutant) 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 17 mutant and 20 control animals, and 16-week-old mutants (17 animals) and controls (20 animals) entered the bone density and X-ray analysis.

3.2.3 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).

3.2.4 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>

3.2.5 Results and Discussion

In the morphological analysis via visual inspection and X-ray analysis a few minor phenotypes were found in single mutant mice and wild-type control littermates with no genotype specific differences (Tables 5 and 6). In the bone densitometry using DEXA analysis (Table 7) no genotype-specific differences were detected. The sex differences we observed are common in many mouse strains and thus are not abnormal

To summarize, we analyzed the NOX-KO mutant mouse line for anatomical anomalies, ionized calcium (Table 8), bone mineral density and body composition, but we did not find any genotype-specific differences.

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 5: Results from the morphological inspection male mice		
Phenotype	Male	
	Control	mutant
Twisted toes	2	
Crippled 2 nd and 4 th toe in hind limb	1	

Table 6: Results from the morphological inspection female mice		
Phenotype	Female	
	Control	mutant
Digit bent	1	
Digit missing		1
Slow reaction to clickbox		1
Smaller		1

Table 7: Bone-related quantitative parametersData are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			Male A~B	Female A~B
	Male (n=9)	Female (n=11)	p-value	Male (n=8)	Female (n=9)	p-value	p-value	p-value
BMD [mg/ cm ²]	61 ± 1	61 ± 1	n.s.	63 ± 3	59 ± 2	n.s.	n.s.	n.s.
pBMD [mg/ cm ²]	52 ± 1	49 ± 1	n.s.	52 ± 2	48 ± 2	n.s.	n.s.	n.s.
sBMD [10 ⁻³ x cm ⁻²]	1.80 ± 0.05	2.35 ± 0.18	< 0.02	1.95 ± 0.09	2.15 ± 0.19	n.s.	n.s.	n.s.
BMC [mg]	0.814 ± 0.035	0.666 ± 0.073	n.s.	0.692 ± 0.057	0.732 ± 0.104	n.s.	n.s.	n.s.
Lean mass [g]	22.30 ± 0.88	17.10 ± 0.61	< 0.0001	22.87 ± 0.78	17.00 ± 1.19	< 0.02	n.s.	n.s.
Fat mass [g]	8.57 ± 0.91	8.16 ± 3.76	n.s.	5.99 ± 1.85	10.57 ± 5.14	n.s.	n.s.	n.s.
Body Length [cm]	8.9 ± 0.1	8.6 ± 0.1	< 0.001	8.7 ± 0.1	8.7 ± 0.1	n.s.	< 0.05	n.s.
Weight [g]	34.05 ± 1.17	28.98 ± 4.08	n.s.	32.41 ± 1.28	30.63 ± 4.26	n.s.	n.s.	n.s.
Bone Content [%]	2.39 ± 0.05	2.38 ± 0.09	n.s.	2.13 ± 0.14	2.38 ± 0.07	n.s.	n.s.	n.s.
Lean Content [%]	65.7 ± 2.2	66.9 ± 5.8	n.s.	71.5 ± 3.9	64.5 ± 8.2	n.s.	n.s.	n.s.
Fat Content [%]	24.9 ± 2.2	20.2 ± 6.0	n.s.	17.4 ± 4.7	24.8 ± 8.9	n.s.	n.s.	n.s.

Table 8: Concentration of ionic Calcium in bloodData are presented as mean \pm standard error of mean.

	Control (A)			Mutant (B)			A~B Male	A~B Female
	Male (n=9)	Female (n=11)	p - value	Male (n=8)	Female (n=9)	p - value	p - value	p - value
Ionized Calcium [mM]	1.15 ± 0.03	1.18 ± 0.03	n.s.	1.17 ± 0.03	1.19 ± 0.03	n.s.	n.s.	n.s.

3.3 Neurology Screen

3.3.1 Summary

Animals were analyzed according to our modified SHIRPA protocol where a battery of behavioral tests is carried out. This primary observation screen is a modification of the Irwin procedure (Irwin, 1968) and was proposed as a rapid, comprehensive and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in a mouse strain (Rogers *et al.*, 1994). We carried out 37 of 40 designed test parameters (See web page: http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html) to detect phenotypic differences between mutant and control mice. Each test parameter contributes to an overall assessment in muscle, lower motor neuron, spinocerebellar, sensory and autonomic function. The primary neurological screening is focused on the investigation of neurological reflexes to determine the neurological functioning of a mouse. Additionally, we examine lactate in blood of mice to draw conclusions about energy metabolism.

Female mutant mice were significantly more irritable during supine restraint and their tail elevation differed significantly when compared to the female control mice. We did not detect any pathological findings for male mutant mice. In general, there were no hints for a neurological phenotype in this mutant mouse line.

3.3.2 Mice

Twenty-seven 10-week-old male (13 mutants and 14 controls) mice entered the neurological screen at the beginning of the 22nd calendar week in 2003. The females (15 mutants and 16 controls) mice 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

At the age of 10 weeks assessment of each animal started 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. In this arena, locomotor activity and motor behavior was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes, grip strength, toe pinch and wire maneuver (*Behavior recorded on or above the arena*). For the wire maneuver test, a rigid horizontal wire (3 mm in diameter) is secured across the rear right corner of the arena. For grip strength testing, a grid (270 x 275 mm) is secured across the width of the arena. In the last part of the observation (*Behavior recorded during Supine Restraint*), the animals were restrained in a supine position to record autonomic responses such as salivation. Measurements were completed with the recording of limb tone, provoked biting, and body length. The last part of the primary screen also involves the analysis of righting reflex, negative geotaxis and contact righting reflex. A glass cylinder

(35 mm diameter, 135 mm length) is used for testing the contact-righting reflex. Throughout the entire procedure, abnormal behavior, irritability, fear, aggression and vocalization were recorded. Between testing of each mouse, faecal pellets and urination were removed from the viewing jar and arena. All experimental equipment is 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 gender 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, wire maneuver, tail elevation, limb tone, body tone, abdominal tone, grip strength, urination, defecation
Spinocerebellar function
Body position, gait, righting reflex, tail elevation, visual placing, limb tone, body tone, abdominal tone, grip strength
Sensory function
Transfer arousal, touch escape, gait, visual placing, toe pinch, pinna reflex, righting reflex
Autonomic function
Palpebral closure, urination, salivation, respiration rate, defecation
Neurological reflexes
Righting reflex (pons), contact righting reflex, visual placing, toe pinch/flexion reflex (cerebellar/spinal cord), negative geotaxis, corneal reflex (medulla), pinna reflex (hearing test)
Physiological parameters
Body weight, body length
General appearance
Body weight, body length, body position, transfer arousal, fear, touch escape, irritability, vocalization, positional passivity, aggression, spontaneous activity, locomotor activity, skin color

3.3.5 Results

We only found two parameters with significant pathological findings for female mutant mice: female mutant mice were more irritable (Table 13); additionally, observation of tail elevation revealed that all female mutant mice showed a horizontally extended tail as compared to female controls (Table 11). These differences were not detected for the male mice. All other SHIRPA test parameters were without pathological findings. Lactate screening revealed no significant differences (Table 14).

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

3.3.6 Discussion

The knockout of *NOX* did not lead to a visible neurological phenotype in homozygous mutant mice. In our neurological screen, we only found that female mutant mice displayed an altered tail elevation and were more irritable as compared to female control mice. The significantly altered tail elevation of the female mutant mice suggests a change in the muscular tone of these animals. However, we did not detect any variation in other muscle parameters that would point towards a muscular dysfunction. Irritability is a varying test parameter, which alone cannot offer any conclusive information about a neuro-

logical phenotype. Therefore, no major pathological findings in male and female mutant mice were found.

3.3.7 References

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Rogers D. C., E.M. Fisher, S.D. Brown, J. Peters, A.J. Hunter, J.E. Martin (1997): Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome*. 8(10): 711-713.

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

s.a. Sub-maxillary area

Table 9: Recording of body length and body weightData are presented as mean \pm standard error of mean.

Parameter	Male			Female		
	Control (n=14)	Mutant (n=13)	<i>p-value</i>	Control (n=16)	Mutant (n=15)	<i>p-value</i>
Body Length [g]	8.7 ± 0.09	8.4 ± 0.08	<i>n.s.</i>	8.3 ± 0.07	8.4 ± 0.07	<i>n.s.</i>
Body Weight [g]	27.6 ± 0.6	27.4 ± 0.42	<i>n.s.</i>	24.26 ± 1.6	24.4 ± 1.3	<i>n.s.</i>

Table 10: 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=13)	<i>p-value</i>	Control (n=16)	Mutant (n=15)	<i>p-value</i>
Body Position						
Sitting or standing						
Rearing on hind legs	10	6		15	11	
Repeating vertical leaping	3	7		1	3	
	1	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Spontaneous Behavior						
Vigorous stretch, groom, moderate movement	11	13		13	12	
Vigorous	0	0		3	2	
Extremely vigorous	3	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Respiration rate						
Normal	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Tremor						
None	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>

Table 11: 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=13)	<i>p</i> -value	Control (n=16)	Mutant (n=15)	<i>p</i> -value
Locomotor Activity	13 \pm 1.1	14.6 \pm 2.5	<i>n.s.</i>	14 \pm 0.37	16.2 \pm 0.37	<i>n.s.</i>
Transfer arousal						
Brief freeze	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Momentary freeze	0	0		1	0	
No freeze	14	13		15	14	
Palpebral Closure						
Eyes wide open	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Piloerection						
None	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Gait						
Normal	12	11	<i>n.s.</i>	16	14	<i>n.s.</i>
Fluid but abnormal	2	2		0	1	
Pelvic Elevation						
Markedly flattened	2	1	<i>n.s.</i>	1	0	<i>n.s.</i>
Barely touches	5	5		2	1	
Normal	6	6		8	13	
Elevated	1	1		5	1	
Tail Elevation						
Dragging	9	10	<i>n.s.</i>	10	15	<0.05
Horizontally extended	5	3		6	0	
Touch Escape						
None	0	0	<i>n.s.</i>	2	1	<i>n.s.</i>
Mild	2	1		4	2	
Moderate	7	8		6	7	
Vigorous	5	4		4	5	
Positional Passivity						
Struggles when held by tail	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>

Table 12: 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=13)	<i>p-value</i>	Control (n=16)	Mutant (n=15)	<i>p-value</i>
Trunk Curl						
Present	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Limb Grasping						
Absent	14	13		16	15	
Present	0	0	<i>n.s.</i>	0	2	<i>n.s.</i>
Visual Placing						
Upon nose contact	0	0		0	0	
Upon vibrassee contact	9	6		12	8	
Before vibrassee contact	4	7		4	6	
Early vigorous extension	1	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Grip strength						
Moderate grip	14	13		16	14	
Active grip	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Body Tone						
Flaccid, no return	0	0		0	1	
Slight resistance	14	13	<i>n.s.</i>	16	14	<i>n.s.</i>
Pinna reflex						
None	0	1		0	1	
Active retraction	14	12	<i>n.s.</i>	16	14	<i>n.s.</i>
Corneal Reflex						
Active single eye blink	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Toe Pinch						
None	2	1		6	6	
Slight withdrawal	6	5		4	3	
Moderate withdrawal	3	4		6	4	
Brisk	3	3	<i>n.s.</i>	0	2	<i>n.s.</i>
Wire maneuver						
Active grip	2	2		5	8	
Difficulty to grasp	8	10		6	4	
Unable to lift	1	0		2	2	
Falls immediately	3	1	<i>n.s.</i>	3	1	<i>n.s.</i>

Table 13: Behavior during supine restraint

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=13)	<i>p-value</i>	Control (n=16)	Mutant (n=15)	<i>p-value</i>
Skin Color						
Pink	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Limb Tone						
No resistance	14	13		16	14	
Moderate resistance	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Abdominal Tone						
Slight resistance	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Lacrimation						
None	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Salivation						
None	2	2		2	3	
Slight margin of s.a.	10	8		11	7	
Wet zone entire of s.a	2	3	<i>n.s.</i>	3	5	<i>n.s.</i>
Provoked biting						
Absent	11	13		16	14	
Present	3	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Righting reflex						
No impairment	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Contact righting reflex						
Absent	1	0		0	0	
Present	13	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Negative Geotaxis						
Turns and climb the grid	13	14		15	14	
Moves, but fails to turn	0	1		1	1	
Falls off	1	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Fear						
None	14	13	<i>n.s.</i>	16	15	<i>n.s.</i>
Irritability						
None	9	9		16	10	
Struggle during sup. res.	5	4	<i>n.s.</i>	0	5	0.04
Aggression						
None	12	13		16	15	
Provoked biting or attack	2	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Vocalization						
None	4	4	<i>n.s.</i>	5	5	
Provoked during handling	10	9		11	10	<i>n.s.</i>

Table 14: Lactate levels

Data shown represent the results of the mean blood lactate concentrations, value (\pm SEM). Statistical analysis: Chi-squared test; significance $p < 0.05$.

	Male			Female		
	Control (n=14)	Mutant (n=13)	<i>p-value</i>	Control (n=16)	Mutant (n=15)	<i>p-value</i>
Lactate [mmo/l]	4.4 ± 0.29	4.5 ± 0.3	<i>n.s.</i>	4.72 ± 0.3	4.78 ± 0.4	<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).

There was no association with genotype and the occurrence of either anterior segment or ERG response abnormalities.

3.4.2 Mice

Thirty control (14 male, 16 female) and 28 mutant mice (13 male, 14 female) entered the Eye Screen at the age of 11 weeks. Mice were first examined by slit lamp 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

ERG responses were recorded from the groups of NOX-KO (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. 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). Due to the fact that no significant differences (T-Test: $p < 0.05$) 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). No significant differences were observed between mutant and control animals for most values.

A total of 58 mice (14 control and 13 mutant males; 16 control and 15 mutant females) were examined by **slit lamp biomicroscopy**. There was no association with genotype and the occurrence of anterior segment abnormalities.

3.4.6 Discussion

ERG screening is a quick, robust and reproducible *in-vivo* method to detect functional retinal impairment in mice. For the analysis of ERG data the average of amplitudes from left and right eye is used, because no major differences were obtained in the ERG response. The comparison of a- and b-wave amplitudes of males and females, control and mutant revealed no consistent differences between the groups. Most of the p-values (T-test) calculated in Table 15 are not significant

3.4.7 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	A-B
	Male	Female		Male	Female		Male	Female
	(n=14)	(n=16)	<i>p - value</i>	(n=13)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
a-wave 500 cd/m ²	-8 ± 1.0	-9 ± 1.0	n.s.	-8 ± 1.7	-9 ± 1.3	n.s.	n.s.	n.s.
b-wave 500 cd/m ²	161 ± 7.6	165 ± 7.8	n.s.	157 ± 8.9	161 ± 7.8	n.s.	n.s.	n.s.
a-wave 12.500 cd/m ²	-29 ± 1.7	-37 ± 2.2	<0.01	-27 ± 2.1	-37 ± 2.9	<0.02	n.s.	n.s.
b-wave 12.500 cd/m ²	188 ± 7.8	217 ± 13.9	n.s.	185 ± 6.9	195 ± 10.9	n.s.	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, thirty (14 males/16 females) control mice and twenty-seven (13 males/14 females) mutant mice were analyzed. Nineteen different clinical-chemical parameters are measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured eight basic hematological parameters. The only genotype related significant difference, we detected, was an increase of the transferrin concentration in the female mutant mice and a slightly higher mean red blood cell count and hematocrit in the male mutant mice. However, the findings were limited to one sex and the values detected were situated within the normal range of mice. Therefore we believe them to be an effect of coincidence and no real genotype related phenotype.

3.5.2 Mice

Fourteen 12-week-old male control and 13 mutant mice entered the clinical-chemical screen at the beginning of the 24th calendar week in 2003. The females (16 controls and 14 mutants) 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 EDTA-coated tubes (KABE, Art.No 078035). The tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant.

After removal of 40 μ l 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 μ l), the Allergy Screen (30 μ l), the Clinical Chemical Screen (130 μ l) 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 high-throughput of the screen was insured by the use of 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

The values obtained for the clinical chemical parameters were within the normal ranges usually found in mice of this strain at the age of three months and were supported by previously published data (Table 16; Suckow *et al*, 2001; Quimby, 1999 and publications cited therein).

Significant differences between mutants and controls were detected for transferrin concentration only in female mice. The following parameters demonstrated sex-dependent differences in the NOX-KO mice: potassium, chlorid, uric acid, alkaline phosphatase, amylase, and transferrin concentrations. In the control animals, we saw sex-related differences in the parameters sodium, potassium, calcium, uric acid, alkaline phosphatase, and amylase.

Hematology

The primary screen for hematological parameters revealed significant genotype-dependent differences in red blood cell count and hematocrit concentration only in males (Table 17). We detected gender-related differences in mutants in white blood cell count. In the control animals, we saw sex-specific differences in hemoglobin and hematocrit concentrations.

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

3.5.6 Discussion

Clinical chemistry.

We detected no differences between mutant and control animals, except for **transferrin**. We found a higher mean transferrin level in the mutant mice compared to the control mice. Synthesis of transferrin occurs in hepatocytes, in the mucosa of the small intestine, and in the bone marrow. Transferrin is a protein responsible for binding and transport of iron. An increase of transferrin is associated with iron deficiency. However, the transferrin values detected were situated within the normal range of mice. Therefore, this result is likely to be a finding of coincidence rather than representing a genotype-related phenotype.

Haematology

The screening of the haematological parameters did not reveal any pathological deviation in the mutant mice. There was a significant difference for the red blood cell count and hematocrit in the male mice. However, as mentioned before concerning the transferrin, the values detected were situated within the normal range.

Comparison to baseline data

All parameters of both mutant and control mice were within physiological ranges. The sex differences detected are normal for most strains of mice (Kile, 2003).

3.5.7 References

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Table 16: Clinical-chemical parameters								
Data are presented as mean ± standard error of mean.								
Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=13)	(n=14)	<i>p</i> -value	(n=14)	(n=16)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Sodium [mmol/l]	156 ±0.51	156 ±0.51	n.s.	157 ±0.46	154 ±0.76	<0.01	n.s.	n.s.
Potassium [mmol/l]	4.2 ±0.05	3.9 ±0.08	<0.02	4.2 ±0.10	3.9 ±0.09	<0.02	n.s.	n.s.
Calcium [mmol/l]	2.0 ±0.03	2.0 ±0.02	n.s.	1.9 ±0.03	2.0 ±0.03	<0.05	n.s.	n.s.
Chloride [mmol/l]	110.1 ±0.64	114.2 ±0.56	<0.001	111.6 ±0.58	112.9 ±0.63	n.s.	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.4 ±0.07	1.5 ±0.07	n.s.	1.5 ±0.06	1.4 ±0.08	n.s.	n.s.	n.s.
Total Protein [g/dl]	5.0 ±0.08	5.1 ±0.05	n.s.	5.0 ±0.06	5.1 ±0.08	n.s.	n.s.	n.s.
Creatinine [mg/dl]	0.322 ±0.01	0.315 ±0.00	n.s.	0.314 ±0.00	0.319 ±0.01	n.s.	n.s.	n.s.
Urea [mg/dl]	68.5 ±3.36	9.5 ±2.61	n.s.	65.8 ±2.10	65.6 ±2.5	n.s.	n.s.	n.s.
Uric acid [mg/dl]	1.4 ±0.32	2.9 ±0.29	<0.01	1.6 ±0.3	3.7 ±0.40	<0.001	n.s.	n.s.
Cholesterol [mg/dl]	110.4 ±8.51	100.1 ±7.33	n.s.	104.0 ±7.9	95.4 ±7.0	n.s.	n.s.	n.s.
Triglyceride [mg/dl]	181.1 ±19.91	145.3 ±12.02	n.s.	142.5 ±11.6	139.1 ±14.0	n.s.	n.s.	n.s.
Creatine Kinase [U/l]	40 ±6.05	26 ±5.96	n.s.	70 ±18.00	36 ±5.00	n.s.	n.s.	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	17 ±1.31	15 ±2.05	n.s.	21 ±3.00	17 ±2.00	n.s.	n.s.	n.s.
Aspartate-Amino-transferase (AST,GOT) [U/l]	26 ±1.74	27 ±1.37	n.s.	31 ±3.00	29 ±2.00	n.s.	n.s.	n.s.
Alkaline Phosphatase [U/l]	153 ±5.30	228 ±10.66	<0.001	148 ±8.00	216 ±11	<0.001	n.s.	n.s.
α-Amylase [U/l]	3068 ±147.87	2535 ±155.79	<0.05	2890 ±118.0	2353 ±86.00	<0.01	n.s.	n.s.
Glucose [mg/dl]	172.8 ±7.19	174.5 ±10.74	n.s.	170.2 ±6.0	165.1 ±8.0	n.s.	n.s.	n.s.
Ferritin [ng/ml]	26.3 ±1.55	25 ±1.26	n.s.	25.3 ±0.90	26.5 ±1.4	n.s.	n.s.	n.s.
Transferrin [mg/dl]	145.4 ±5.15	162.0 ±2.21	<0.01	147.3 ±2.1	151.8 ±2.30	n.s.	n.s.	<0.01

Table 17: Hematological parametersData 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=13)	(n=14)	<i>p - value</i>	(n=14)	(n=16)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
White blood cell count [10 ³ /μl]	4.64± 0.54	6.19± 0.43	<0.05	6.06± 0.76	6.71± 0.38	n.s.	n.s.	n.s.
Red blood cell count [10 ³ /μl]	10.44± 0.26	10.11± 0.14	n.s.	9.36± 0.43	10.26± 0.15	n.s.	<0.05	n.s.
Hemoglobin [g/dl]	15.19± 0.66	15.72± 0.22	n.s.	14.04± 0.62	15.68± 0.18	<0.05	n.s.	n.s.
Hematocrit [%]	48.73± 1.03	48.24± 0.62	n.s.	43.67± 2.02	48.75± 0.60	<0.05	<0.05	n.s.
Mean corpuscular volume [fl]	48.85± 0.58	47.86± 0.38	n.s.	46.71± 0.40	47.50± 0.45	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin [pg]	15.12± 0.28	15.57± 0.18	n.s.	15.03± 0.18	15.31± 0.16	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	32.29± 0.42	32.56± 0.21	n.s.	32.23± 0.22	32.18± 0.22	n.s.	n.s.	n.s.
Platelet count [10 ³ /μl]	732± 27.04	761± 31.09	n.s.	744± 47.99	747± 28.72	n.s.	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 of what is already known about the mutant mouse line presented to the GMC by the mouse provider, no immunological phenotype was known in the NOX-KO mutant mouse line. Its analysis in the Immunology Screen could not reveal profound differences between mutants and their littermate controls.

3.6.2 Mice

We analyzed 27 mutant animals (14 females and 13 males) and the 30 age- and sex-matched littermate controls (16 females and 14 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 standard immunological parameters measured in the primary screen (Table 18) could not reveal significant differences between *NOX*-deficient mice and their littermate controls. Similarly to what we found analyzing “normal” inbred strains of mice, we were able to detect some sex-specific differences in both mutant and control mice. No significant differences could be established with regard to the other cell surface antigens included in the screen.

Under standard screen conditions, this mutant mouse line did not show profound changes in immunological parameters.

3.6.6 References

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Table 18: Basic parameters analyzed in the Immunology Screen.

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

Parameter	Mutants (A)			Control (B)			A - B	
	Male	Female		Male	Female		Male	Female
	(n=13)	(n=14)	<i>p</i> - value	(n=14)	(n=16)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
CD19 ⁺ [%]	36.2 \pm 3.4	36.6 \pm 2.1	n.s.	31.4 \pm 2.9	35.7 \pm 1.9	n.s.	n.s.	n.s.
CD19 ⁺ CD5 ⁻ [%]	92.2 \pm 0.6	97.1 \pm 0.4	<0.001	92.1 \pm 0.8	96.5 \pm 0.4	<0.001	n.s.	n.s.
CD19 ⁺ CD5 ⁺ [%]	7.8 \pm 0.6	2.8 \pm 0.4	<0.001	7.9 \pm 0.8	3.5 \pm 0.4	<0.001	n.s.	n.s.
CD3 ⁺ [%]	34.7 \pm 2.9	38.4 \pm 2.2	n.s.	36.4 \pm 2.6	38.1 \pm 2.1	n.s.	n.s.	n.s.
γ/δ TCR ⁺ [%]	0.1 \pm 0.02	0.1 \pm 0.02	n.s.	0.1 \pm 0.02	0.2 \pm 0.04	n.s.	n.s.	n.s.
Gr-1 ⁺ [%]	18.4 \pm 1.5	17.9 \pm 1.3	n.s.	21.8 \pm 4.4	18.1 \pm 0.8	n.s.	n.s.	n.s.
CD49b ⁺ [%]	36.0 \pm 3.5	19.6 \pm 1.8	<0.001	31.7 \pm 2.6	19.3 \pm 1.5	<0.001	n.s.	n.s.
CD4 ⁺ [%]	18.9 \pm 2.3	24.4 \pm 1.7	n.s.	16.9 \pm 1.5	24.3 \pm 1.4	<0.01	n.s.	n.s.
CD8 β ⁺ [%]	12.7 \pm 1.1	14.5 \pm 0.8	n.s.	13.7 \pm 1.1	15.8 \pm 0.9	n.s.	n.s.	n.s.
IgG ₁ [μ g/ml]	42.2 \pm 12.2	40.0 \pm 9.2	n.s.	51.1 \pm 19.0	39.3 \pm 9.5	n.s.	n.s.	n.s.
IgG _{2a} [μ g/ml]	144.9 \pm 55.8	143.7 \pm 40.5	n.s.	43.0 \pm 4.0	171.4 \pm 32.0	<0.001	n.s.	n.s.
IgG _{2b} [μ g/ml]	113.8 \pm 14.6	138.1 \pm 32.4	n.s.	103.8 \pm 16.1	118.3 \pm 16.6	n.s.	n.s.	n.s.
IgG ₃ [μ g/ml]	692.1 \pm 150.3	358.1 \pm 128.6	n.s.	687.7 \pm 105.3	320.1 \pm 101.2	<0.02	n.s.	n.s.
IgM [μ g/ml]	252.2 \pm 64.4	164.4 \pm 43.3	n.s.	128.8 \pm 15.2	131.2 \pm 50.9	n.s.	n.s.	n.s.
IgA [μ g/ml]	67.4 \pm 9.6	29.6 \pm 4.6	<0.01	38.6 \pm 10.3	31.7 \pm 4.7	n.s.	n.s.	n.s.
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).

The analysis of the NOX-KO mutant mouse line in the Allergy Screen did not reveal profound differences between mutants and controls.

3.7.2 Mice

In the primary Allergy Screen, 30 control (16 females, 14 males) and 26 mutant (13 females, 13 males) mice were screened.

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 NOX-mutant and control mice was found. In NOX-control animals, the mean concentration of total IgE in females was higher than in males. This difference was statistically significant. Nevertheless, the mean concentrations of total IgE in both groups were in normal range.

Taken together, under standard screening conditions NOX-KO mutant mice did not show changes in total plasma IgE levels that would reveal an allergy phenotype.

Raw data will be available on demand.

Table 19: Total plasma IgE in NOX-KO mice Data are presented as mean \pm standard error of mean.								
	Control (A)			Mutant (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=16)	(n=14)	<i>p</i> - <i>value</i>	(n=13)	(n=13)	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>
Total IgE [ng/ml]	105 \pm 18.3	43 \pm 10.8	<0.01	52 \pm 9.6	52 \pm 13.4	n.s.	<0.02	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 supra-threshold 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)

We found no significant difference in pain reactivity between mutant and control animals in the NOX-KO mutant mouse line.

3.8.2 Mice

Twenty-seven mutant mice (13 male, 14 female), and 30 control animals (14 male, 16 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. 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.

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

We found no significant difference in pain reactivity between the mutant and control animals in this mouse mutant line. There was a significant sex difference only for hind paw licking but proven to be within the range of normal deviations after multiple comparisons. Therefore secondary screening of this mutant mouse line is not suggested.

Table 20: Nociceptive Screen									
Data are presented as mean \pm standard error of mean.									
Parameter	Mutant (A)			Control (B)			A~B	A~B	AN-NOVA
	Fe-male	Male		Female	Male		Fe-male	Male	
	(n=14)	(n=13)	<i>p</i> -value	(n=16)	(n=14)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
H.p. licking	12.9 ± 2.52	21.7 ± 2.62	0.0190	12.8 ± 2.36	13.9 ± 2.52	n.s.	n.s.	0.036	0.0150
H.p. shaking	10.6 ± 1.22	13.7 ± 1.27	n.s.	9.8 ± 1.14	13.5 ± 1.22	0.031	n.s.	n.s.	0.025
Jumping	56.9 ± 2.13	53.2 ± 2.21	n.s.	57.9 ± 1.99	57.2 ± 2.13	n.s.	n.s.	n.s.	n.s.

Raw data will be available on demand.

3.8.6 References

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Abbreviations

h.p. hind paw

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 patterns during rest and activity were studied in 15-week-old male and female NOX-KO mice by whole body plethysmography. Neither during sleep nor during rest nor during activity, measurements revealed any physiologically significant differences between groups.

3.9.2 Mice

Female mutant and control mice were studied at the age of 15 weeks. Mean body weights did not differ significantly (Table 21).

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 (Fig. 3 and 4).

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. 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 (Fig. 3), data recording was immediately started and was continued for 40 min.



Figure 3: System used at GMC to assess breathing patterns.

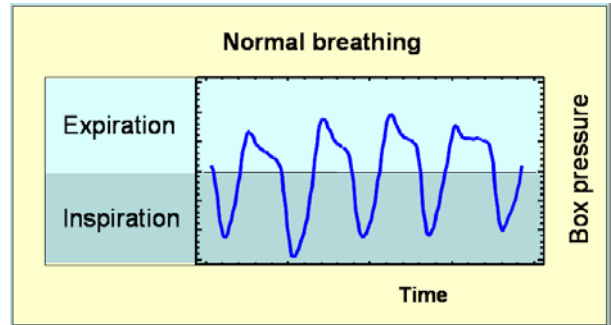


Figure 4: Recorded data used to calculate the breathing parameters.

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) measured 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

Tables 21 and 22 summarize the results obtained for spontaneous breathing under sleeping, resting, and active conditions.

At the time of measurement, all animals were 15 weeks old and had comparable body weights. During all different phases of activity, the 12 measured parameters characterising the spontaneous breathing pattern were similar in both groups. One animal of the five mutants did not show a sleeping phase assessed by the respiratory rate.

3.9.6 Discussion

The comparison of 12 different parameters describing the spontaneous breathing pattern did not reveal any significant differences between mutant and control mice. The changes in breathing pattern observed between the different levels of activity are typical for mice. All values are comparable between and within groups, which proves the high reproducibility of the measurements. The fact that ventilation and specific ventilation are comparable in all groups suggests that oxygen demand is similar and that oxygen uptake in the lungs is not affected by the mutation. The Metabolic Screen can provide a detailed picture on oxygen consumption.

The fact that one animal of the mutants did not have a sleeping phase is very likely to be related to individual differences in activity, a phenomena, which has also been observed in other inbred strains or mutant mice. Hence, it is most unlikely that it is related to the mutation.

Raw data are available on demand.

3.9.7 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 (μ 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 and rest of female mice
Data are presented as mean \pm standard error of mean.

Parameter	Control (A)	Mutant (B)	A~B
	(n=5)	(n=5)	<i>p</i> - value
Bw [g]	23.6 \pm 1.7	24.2 \pm 2.4	n.s.
Sleep		n = 4	
f [1/min]	181.3 \pm 7.5	191.6 \pm 12.2	n.s.
TV [ml]	0.26 \pm 0.005	0.26 \pm 0.02	n.s.
sTV [μ l/g]	11.4 \pm 0.7	11.1 \pm 1.3	n.s.
MV [ml/min]	48.3 \pm 2.9	50.6 \pm 4.7	n.s.
sMV [ml/min/g]	2.1 \pm 0.2	2.1 \pm 0.1	n.s.
Ti [ms]	95.3 \pm 3.4	88.6 \pm 6.9	n.s.
Te [ms]	237.9 \pm 16.4	228.3 \pm 23.6	n.s.
Ti/TT	0.29 \pm 0.02	0.28 \pm 0.04	n.s.
PIF [ml/s]	4.7 \pm 0.1	5.1 \pm 0.5	n.s.
PEF [ml/s]	4.3 \pm 0.2	4.0 \pm 0.3	n.s.
MIF [ml/s]	2.8 \pm 0.07	3.0 \pm 0.3	n.s.
MEF [ml/s]	1.1 \pm 0.1	1.2 \pm 0.2	n.s.
Rest	(n=5)	(n = 5)	
f [1/min]	325.2 \pm 3.5	327.6 \pm 5.4	n.s.
TV [ml]	0.27 \pm 0.01	0.26 \pm 0.02	n.s.
sTV [μ l/g]	11.6 \pm 0.9	10.9 \pm 0.8	n.s.
MV [ml/min]	85.7 \pm 4.1	82.9 \pm 6.8	n.s.
sMV [ml/min/g]	3.7 \pm 0.3	3.5 \pm 0.3	n.s.
Ti [ms]	60.2 \pm 2.3	58.2 \pm 4.8	n.s.
Te [ms]	124.4 \pm 1.7	125.2 \pm 2.7	n.s.
Ti/TT	0.33 \pm 0.01	0.32 \pm 0.02	n.s.
PIF [ml/s]	8.0 \pm 0.3	7.8 \pm 0.5	n.s.
PEF [ml/s]	5.8 \pm 0.4	5.6 \pm 0.5	n.s.
MIF [ml/s]	4.5 \pm 0.2	4.5 \pm 0.3	n.s.
MEF [ml/s]	2.2 \pm 0.1	2.1 \pm 0.2	n.s.

Table 22: Spontaneous breathing pattern during activity of female mice			
Data are presented as mean \pm standard error of mean.			
Parameter	Control (A)	Mutant (B)	A~B
Activity	(n=5)	(n = 5)	<i>p</i> - value
f [1/min]	508.0 \pm 3.9	509.2 \pm 5.2	n.s.
TV [ml]	0.27 \pm 0.006	0.27 \pm 0.02	n.s.
sTV [μl/g]	11.7 \pm 0.6	11.3 \pm 0.9	n.s.
MV [ml/min]	137.2 \pm 2.5	134.5 \pm 7.6	n.s.
sMV [ml/min/g]	5.9 \pm 0.3	5.7 \pm 0.4	n.s.
Ti [ms]	40.6 \pm 0.8	40.0 \pm 1.1	n.s.
Te [ms]	77.5 \pm 1.3	77.9 \pm 1.1	n.s.
Ti/TT	0.34 \pm 0.008	0.34 \pm 0.008	n.s.
PIF [ml/s]	12.0 \pm 0.4	11.5 \pm 0.5	n.s.
PEF [ml/s]	8.7 \pm 0.3	8.4 \pm 0.6	n.s.
MIF [ml/s]	6.7 \pm 0.2	6.7 \pm 0.3	n.s.
MEF [ml/s]	3.5 \pm 0.08	3.4 \pm 0.2	n.s.

3.10 Expression Profiling

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Ten male mice of the NOX-KO mutant mouse line were provided to the molecular phenotyping screen.

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 23: Organs of NOX-KO mice stored for expression profiling				
Mouse ID	Strain	Sex	Genotype	Date of Collection
30009036	NADH	m	-/-	29.06.2003
30009077	NADH	m	+/+	09.02.2001
30009079	NADH	m	-/-	09.02.2001
30009080	NADH	m	-/-	09.02.2001
30009088	NADH	m	+/+	09.02.2001
30009030	NADH	m	-/-	09.02.2001
30009073	NADH	m	-/-	09.02.2001
30009058	NADH	m	+/+	09.02.2001
30009050	NADH	m	+/+	09.02.2001
30009042	NADH	m	+/+	09.02.2001

In a first discussion no organ was selected for analysis. When further examination is considered necessary, expression profiling analysis can be performed using our DNA-chip containing 21,000 probes. Please contact Johannes Beckers, (beckers@gsf.de) to discuss this option.

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 14 (seven male/ seven female) control mice and 13 (seven male/ six female) mutant mice were analysed. They are first fed under *ad libitum* conditions for two weeks, followed by one week of food restriction to 60%. The primary metabolic screen focuses on the investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature and adaptive capacity of metabolic processes. No significant genotype-specific differences were found.

3.11.2 Mice

Sven adult control and seven mutant males entered the Metabolic Screen at the beginning of calendar week 30 in 2003. 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_{fec}), 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 gender. 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

No significant differences between mutant and control mice were found. Some sex-specific differences could be found: females of both genotypes were lighter than the males. Concerning body temperature neither genotype- nor sex-specific differences were found, but all animals showed a tendency of hypothermia during the period of food restriction. The parameters metabolized energy per unit body weight and energy uptake per unit body weight were higher in females than in males, but this difference was significant only in control mice. In both genotypes, food assimilation was lower in females than in males.

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

3.11.6 Discussion

No information about metabolic parameters were available prior the metabolic screening of NOX-KO mice. As a metabolic adaptation to the period of food restriction, mutant and control mice showed a decrease of feces production. Only mutant males showed another adaptation to food reduction - they decreased the energy value of the feces. Altogether, only mutant females were able to increase the food assimilation coefficient while all other mice showed a slightly decreased food assimilation coefficient. This coefficient gives a hint to the ability of extracting energy from food chow. The higher this coefficient is the better is the energy utilization.

Due to the low number of backcrosses to C57BL/6J (two generations) the genetic background subtle metabolic changes might not have been uncovered.

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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 24: 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=7)	(n=6)	<i>p</i> - <i>value</i>	(n=7)	(n=6)	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>
Body weight [g]	33.16 \pm 1.19	23.23 \pm 0.50	<0.001	28.23 \pm 0.96	20.07 \pm 0.50	31.51 \pm 1.44	24.10 \pm 0.82	<0.02	26.30 \pm 1.87	19.73 \pm 0.42	n.s.	n.s.
Rectal body temperature [°C]	37.19 \pm 0.09	37.06 \pm 0.12	n.s.	35.23 \pm 0.24	34.82 \pm 0.34	37.11 \pm 0.18	37.21 \pm 0.10	n.s.	35.02 \pm 0.41	34.91 \pm 0.59	n.s.	n.s.
Food consumption [g day⁻¹]	3.95 \pm 0.14	3.11 \pm 0.13	<0.001	60% of <i>ad libitum</i>		3.99 \pm 0.16	3.28 \pm 0.19	<0.02	60% of <i>ad libitum</i>		n.s.	n.s.
Energy uptake [kJ day⁻¹]	73.66 \pm 2.66	57.90 \pm 2.39	<0.001	44.20 \pm 1.60	34.74 \pm 1.43	74.35 \pm 2.90	61.10 \pm 3.49	<0.02	44.61 \pm 1.74	36.66 \pm 2.10	n.s.	n.s.
Energy uptake BW⁻¹ [kJ g⁻¹ day⁻¹]	2.22 \pm 0.04	2.49 \pm 0.06	<0.02	1.57 \pm 0.02	1.73 \pm 0.03	2.37 \pm 0.08	2.53 \pm 0.12	n.s.	1.73 \pm 0.10	1.85 \pm 0.08	n.s.	n.s.
Feces production [g day⁻¹]	0.63 \pm 0.02	0.55 \pm 0.02	<0.02	0.43 \pm 0.01	0.35 \pm 0.01	0.64 \pm 0.03	0.60 \pm 0.03	n.s.	0.41 \pm 0.02	0.32 \pm 0.03	n.s.	n.s.
Energy content feces [kJ g⁻¹]	15.91 \pm 0.11	15.81 \pm 0.06	n.s.	16.05 \pm 0.11	15.95 \pm 0.06	15.97 \pm 0.05	15.74 \pm 0.08	<0.05	15.45 \pm 0.15	15.75 \pm 0.08	n.s.	n.s.
Metabolized energy [kJ day⁻¹]	63.82 \pm 2.41	49.33 \pm 2.11	<0.001	37.42 \pm 1.52	29.33 \pm 1.22	64.38 \pm 2.57	51.93 \pm 3.21	<0.02	38.34 \pm 1.57	31.73 \pm 1.87	n.s.	n.s.
Metabolized energy [kJ g⁻¹ day⁻¹]	1.93 \pm 0.04	2.12 \pm 0.06	<0.02	1.32 \pm 0.02	1.46 \pm 0.03	2.05 \pm 0.07	2.14 \pm 0.11	n.s.	1.49 \pm 0.10	1.60 \pm 0.07	n.s.	n.s.
Food assimilation coefficient [%]	86.61 \pm 0.27	85.17 \pm 0.30	<0.02	84.59 \pm 0.56	84.44 \pm 0.20	86.58 \pm 0.39	84.86 \pm 0.64	<0.05	85.91 \pm 0.52	86.51 \pm 0.81	n.s.	n.s.

3.12 Pathology Screen

3.12.1 Summary

The Pathology screen performed a complete morphological analysis with standard stains. NOX-KO mutant mice show no morphological phenotype when compared to their control littermates.

However, non-genotype-specific, secondary results were found. Four females (two controls, two mutants), housed together in one cage, weighted 53 to 61 g and had a severe steatosis of the liver. Additionally, mice of this batch showed inflammatory changes of several organs like lung, liver, pancreas, and kidney. Serum analysis for mouse hepatitis virus (MHV) antibodies revealed 10 positive animals of 27 tested (37%).

Mice

A total of 58 mice, 28 NADH mice (15 females, 13 males) and 30 control animals (16 females, 14 males) were analyzed. Due to the workflow in the GMC, mice of different ages were received from different screens (Table 25). The term "Other Screens" is used for any other screen of the GMC which is not specifically indicated.

Table 25: NOX-KO mice analyzed.						
Origin	Control		Mutant		Number of Animals	Age [weeks]
	Female	Male	Female	Male		
Lung Screen	5	-	5	-	10	14 - 15
Expression Screen	-	5	-	5	10	13 - 15
Dysmorphology Screen	4	2	3	1	10	18 - 22
Metabolic Screen	7	7	6	7	27	20 - 22
Other Screens	-	-	1	-	1	11
Total Number of Animals	16	14	15	13	58	

3.12.2 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 pre-

served 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, muscle, lung, brain, cerebellum, thymus, spleen, cervical lymph nodes, thyroid, parathyroid, adrenal gland, stomach, intestine, liver, pancreas, kidney, reproductive organs, and urinary bladder were cut and stained with haematoxylin and eosin (H&E). Prussian's Blue staining was performed when indicated. The presence of Mouse Hepatitis Virus (MHV) was serologically investigated using an immunofluorescence assay (1:20) by BioDoc (Hannover).

3.12.3 Genotype-specific Results

Overview

Table 26: Genotype-specific alterations of NOX-KO mice:			
Analyzed:	Alteration	Analyzed:	Alteration
Bodyweight	No	Pancreas	No
Skin	No	Cervical lymph node	No
Musculoskeletal system	No	Thymus	No
Eyes	No	Spleen	No
Cerebrum	No	Thyroid	No
Cerebellum	No	Parathyroid	No
Heart	No	Adrenal gland	No
Trachea	No	Kidneys	No
Lung	No	Urinary bladder	No
Teeth	No	Testes	No
Salivary gland	No	Epididymis	No
Esophagus	No	Funiculus spermaticus	No
Stomach	No	Ovaries	No
Small intestine	No	Uterus	No
Large intestine	No	Vagina	No
Liver	No	Mamma	No

Mutant mice show no genotype-specific morphological phenotype. However, secondary results (not genotype-specific) were observed.

Body weight

The body weight of NOX-KO mutant mice did not differ significantly ($p>0.1$) from the body weight of their control littermates (Table 27). Four female mice (two controls and two mutants), housed in the same cage, were heavier than all other mice (53, 57, 58, and 61 g). Due to the respectable difference in body weight, these mice were not included in the statistical analysis.

Table 27: Mean absolute body weight \pm standard deviation of NOX-KO.					
Origin	Control		Mutant		Age [weeks]
	Female	Male	Female	Male	
Lung Screen	23.6 \pm 3.3	-	24.2 \pm 4.7	-	14 - 15
Expression Screen	-	28.3 \pm 1.5	-	31.2 \pm 3.2	13 - 15
Dysmorphology Screen	26.5 \pm 2.5	34.0 \pm 1.0	23.0 \pm 0.0	29.0 \pm 0.0	18 - 22
Metabolic Screen	25.3 \pm 1.0	33.3 \pm 2.9	26.0 \pm 2.7	33.7 \pm 4.5	20 - 22

3.12.4 Secondary, non-genotype-specific Results

Inflammatory changes

A mild pneumonia was found in 20 of 58 mice analyzed (35%). Seven mice showed pyelonephritis, segmental nephritis or glomerulonephritis (12%). Abscesses or necrosis in the liver were present in three mice (5%), microgranulomas and small lymphocytic infiltrates were found in the liver in 32 of 58 mice (55%). Individual mice developed a mild pancreatitis, cholangitis, or gastritis. A positive result for serum antibodies against MHV was demonstrated in 10 of 27 mice (37%).

Fatty liver

Four female mice (two mutants, two controls) were obese and had a severe macro- and microvesicular steatosis of the liver (Fig. 5).

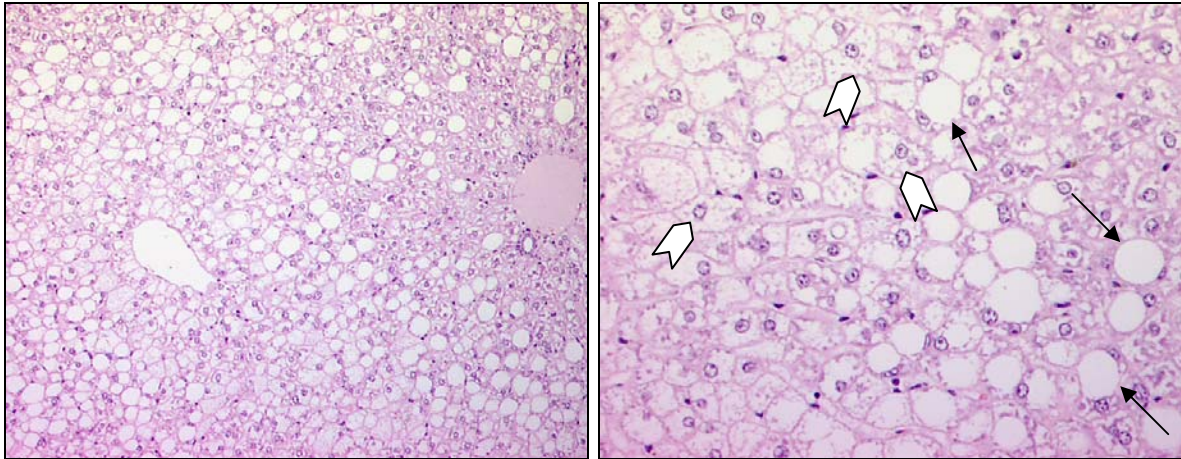


Figure 5: Mixed steatosis of the liver

Fatty changes of the liver. The cytoplasm of hepatocytes contains either one large fat vesicle (arrow, so called "macrovesicular steatosis") or multiple small fat vesicles (arrowhead, so called "microvesicular steatosis"). Note the displacement of the nucleus of hepatocytes which contain a large single fat vesicle (H&E, original magnification 100 and 320x).

3.12.5 Discussion

Although there was **no morphological phenotype** in the NOX-KO mutant mice, one cannot conclude that the loss of NADH does not lead to a phenotype. Subtle phenotypes may be detectable in a different background strain or just after some more backcrosses. Additionally, mice were not challenged to help to expose subtle phenotypes.

Mice of this batch had a **high incidence of inflammatory changes** when compared to other lines analyzed at the GMC. The reason for this is unknown but one has to take into account that the health certificate, handed by the provider mentions possible infections with *helicobacter spp.*, *pseudomonas spp.*, *mouse parvovirus*, and *mouse hepatitis virus*. Infection with MHV was proven in 37% of the mice. It is difficult to conclude whether these pathogens are the cause of the morphological changes detected.

Four females were extremely **obese** when compared to all other mice from the same batch. They were housed in the same cage, and most probably belong to the same litter. The body weight issue was discussed with the Behavior Screen, and interestingly, these four mice weighted already at the age of eight weeks at least 10 g more than all other mice. Unfortunately these mice were not examined in the Metabolic Screen, to exclude or confirm a metabolic disorder. Histologically a severe mixed **steatosis of the liver** was found. An absolute increase of stored fat in the liver can be due to many reasons, such as abnormal fat metabolism, intake of toxins, inherited metabolic disorders, or a couple of other systemic disorders. In human pathology to make the distinction between macro-, microvesicular and mixed steatosis is of bigger interest since it provides information about the possible origin of the disease. However, this information is not available in mice. In humans, macrovesicular steatosis is thought to be reversible and benign, whereas microvesicular steatosis is associated with serious conditions. A mixed pattern can be of diagnostic importance in alcoholic liver disease. Mice with steatosis analyzed in the Pathology Screen of

the GMC showed almost always a mixed pattern and at least, in rat it is suggested that the macrovesicular steatosis is a progressive form of the microvesicular steatosis (Sabesin *et al.*, 1977).

3.12.6 References

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www.eulep.org/Necropsy_of_the_Mouse/index_2004.php

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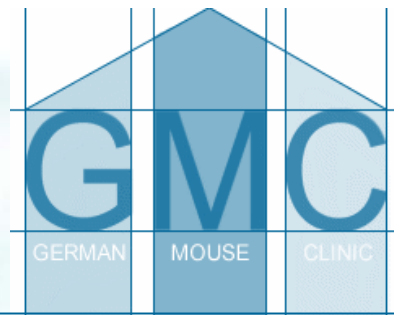
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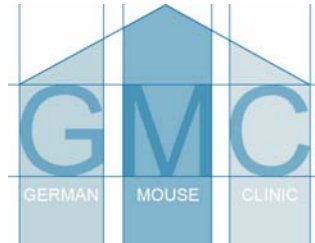
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GERMAN MOUSE CLINIC

Report for NOX-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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