

The

GERMAN MOUSE CLINIC

Report for MAG

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1 Summary

1.1 Primary Screen

In a primary screen 78 MAG mice have been analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Metabolism, Expression Profiling and a comprehensive Pathology. We found:

Dymorphology Screen: MAG^{-/-} mice showed a reduced response to the clickbox test. Furthermore, an abnormal struggling of MAG mutants was observed when suspended by the tail. MAG^{-/-} mice were shorter and lighter at the age of 16 weeks. In the DEXA analysis for bone density and body composition, male mutants had a decreased bone mineral content (BMC) and Lean mass.

Neurology Screen: The comparison of MAG knockout to control mice revealed a significant neurological phenotype in the MAG knockout mice. Male and female MAG knockout mice had an obvious tremor. Furthermore, we detected significant alterations in gait of male MAG knockout mice. Female MAG knockout mice indicated a slight tendency towards reduced spontaneous behavior.

Nociception Screen: We found significantly delayed pain response in MAG knockout male animals in hind paw licking. However, in jumping MAG mice showed significantly shorter latency. On the base of these data it is not possible to define the pain-related phenotype of this genotype; therefore we would suggest performing further pain-related studies.

Clinical Chemical Screen: We found lower inorganic phosphorus concentrations in plasma samples of MAG-deficient mice. Additionally we detected a lower creatine kinase level for MAG mutant male mice. However, all clinical chemical and haematological parameters were within the normal ranges.

Lung Screen: Comparing wild-type to mutant mice, mutant mice showed significantly lower breathing rates and consequently significantly higher inspiratory and expiratory timing compared to wild-type mice meaning mutant mice breathed slower. During rest, lower breathing rate was compensated by higher tidal volumes in the mutants resulting in comparable minute ventilations. This compensation was less sufficient during activity. The results suggest disturbances in the central respiratory control causing the observed alterations in the rhythmogenesis. It is unlikely that the detected changes are due to reduced chemosensitivity or altered lung structure.

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

1.2 Secondary Screen

Secondary screening is suggested from the Neurology, Clinical Chemistry and Nociceptive Screen. We would recommend analyzing:

Neurology Screen: Tremor as detected in MAG knockout animals is the most common type of all movement disorders. Considering that tremor can be a manifestation of a variety of neurological conditions, we recommend measuring balance and motor coordination of the MAG knockout mice by means of the Rotarod test. In addition, we would suggest assessing EMG since physiological tremor has a neurogenic basis and similar oscillations should be reflected in EMG signals of the affected muscles.

Clinical Chemical Screen: It would be interesting to confirm the findings of the primary screening and to place emphasis on bone and muscle relevant parameters, since findings of the Dymorphology Screen and Neurology Screen suggest changes in bone metabolism and muscle function of MAG-KO mice

Nociceptive Screen: More detailed pain related studies which include:

1. Base studies e.g.,
 - von Frey filament test to study the reaction of animals to mechanical pain,
 - acetic acid test to study the reaction to visceral inflammation.
2. Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.
3. Chronic pain tests:
 - Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals,
 - Carrageenan test to study the reaction to inflammation.

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

2 General Part

2.1 The Role of the Gene

The myelin-associated glycoprotein (MAG), a minor component of myelin in the central and peripheral nervous system, has been implicated in the formation and maintenance of myelin (for a review see Schachner and Bartsch, 2000).

2.2 Previously Known Phenotypes

CNS

- delayed myelination, hypomyelination, increased number of unmyelinated axons
- loss of oligodendrocytes; characteristic alterations of the periaxonal oligodendrocyte processes, consisting of intracytoplasmic deposition of vesicular material, multivesicular bodies, mitochondria, and lipofuscin granules, as well as granular or paracrystalline inclusions: „dying back oligodendro-gliopathy“; abnormal oligodendrocyte periaxonal cytoplasmic collar: either absent or reduced in length
- some doubly myelinated axons are present
- delayed maturation of nodal regions; diffuse or abnormal locations of Caspr and Kv1.1 (delayed rectifier potassium channel)
- presence of oligodendrocyte cytoplasm in compact myelin
- decreased phosphorylation of neuronal cytoskeletal elements (NF-H, NF-M, MAP1B) and their associated kinases (ERK1/2; cdk5)
- enhanced cholesterol level in lipid rafts and myelin derived from cerebellar membranes
- normal cholesterol level in liver and blood
- enhanced apoE level in lipid rafts derived from cerebellar membranes

PNS

- impaired maintenance of myelinated fibers: degeneration of myelin and axons
- dilated periaxonal space or reduced periaxonal spacing and a lack of the periaxonal collars in some fibers

All further findings we consider as new.

It is suggested that synthesis and transport of cholesterol and fatty acids are impaired.

2.3 Possible disease model

Dying back oligodendrogliopathy as described in diseases of toxic immune-mediated demyelination including multiple sclerosis (Lassmann *et al.*, 1997).

2.4 Mice

2.4.1 Number and kind of mice

As described by the owner, the mice analyzed were generated by a targeted mutation and backcrossed to C57BL/6J.

A small batch of MAG animals was sent to the GSF. The Colony was expanded by intercross of heterozygotes. Due to low fertility, the MAG mice entered the GMC primary screen in two batches:

Table 1: Composition of the MAG-mice batches arrived at the GMC				
Date	Mutant Female	Mutant Male	Wild Type Female	Wild Type Male
25.08.2003	8	10	25	16
10.11.2003	11	8	7	22
Total Number	19	18	32	38

2.4.2 Housing conditions

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

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

2.5 Workflow

2.5.1 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). Analyzed parameters are listed in Table 2.

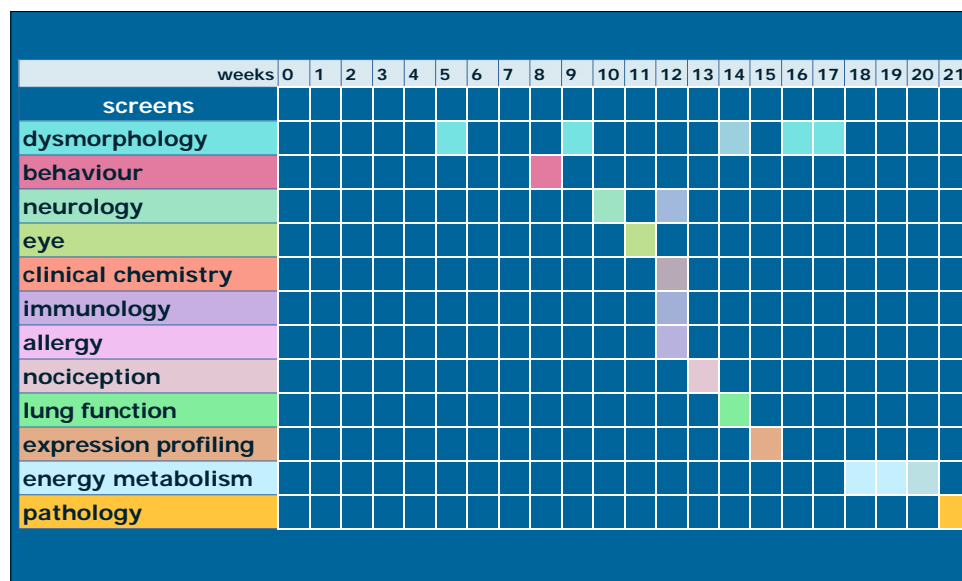


Fig. 1: Workflow of the primary screen
 Explanation below, =Analysis of blood-based parameters.

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 (5 males / 5 females) and 10 controls (5 males / 5 females) leave the animal facility for the Lung Function Analysis, which for technical reasons is located elsewhere. These animals are, for hygienic reasons, not allowed to re-enter the German Mouse Clinic. The females

go directly to Pathology. The males are used to freeze organs for future expression profiling on demand (remaining organs from those animals are analyzed by the Pathology). All other animals go through the bone and cartilage tests of the Dymorphology Screen, and then stay three weeks in the Metabolic Screen. After completion of the primary screen, all animals end up in the Pathology.

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

2.5.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the MAG mice. Additional wild-type mice were analyzed in the lung function screen. Some parameters from 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. A few animals died during the primary screen and thus they could not be analyzed for all parameters.

2.5.3 Quality Management

As a routine quality control, we take blood samples from for serological tests of the sanitary status of all mice after they went through the GMC primary screen. When indicated, 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)

2.6 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.

2.7 References

Brielmeier M., H. Fuchs, G. Przemeczek, V. Gailus-Durner, M. Hrabé de Angelis, J. Schmidt (2002) The GSF – Phenotype Analysis Center (German Mouse Clinic, GMC): A sentinel-based health-monitoring concept in a multi-user unit for standardized characterization of mouse mutants. In: J. Guenet and C. Herweg (Eds.) Laboratory Animals Science - Basis and Strategy for Animal Experimentation Vol. 11, Proceedings of the 8th FELASA Symposium, Laboratory Animals Ltd., Aachen, pp. 19-22.

Lassmann H., U. Bartsch, D. Montag and M. Schachner (1997): Dying-back oligodendrogliopathy: a late sequel of myelin-associated glycoprotein deficiency. *Glia* 19: 104-110

Schachner, M. and U. Bartsch (2000): Multiple functions of the myelin-associated glycoprotein MAG (siglec-4a) in formation and maintenance of myelin. *Glia* 29: 154-165

Abbreviations

MAG	Myelin associated glycoprotein
GMC	German Mouse Clinic
IVC	individually ventilated cage
+/+	homozygote wildtype
+/-	heterozygote mutant
-/-	homozygote mutant
KO	knockout
B6	C57BL/6
FELASA	Federation of E uropean L aboratory A nimal S cience A ssociations, 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 histological examination	histology, immunochemistry

3 Specific part

3.1 Behavior Screen

3.1.1 Summary

The modified hole board 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 (See Ohl *et al.*, 2001).

In the case of MAG-deficient mice the social parameter could not be evaluated, because not enough mice were group housed with at least three animals per cage. No phenotype could be detected in MAG-deficient mice in the primary screen.

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 (13 wt, 18 mutants) and 38 male mice (22 wt, 16 mutants) were available for analysis, because not all animals had been group housed.

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.

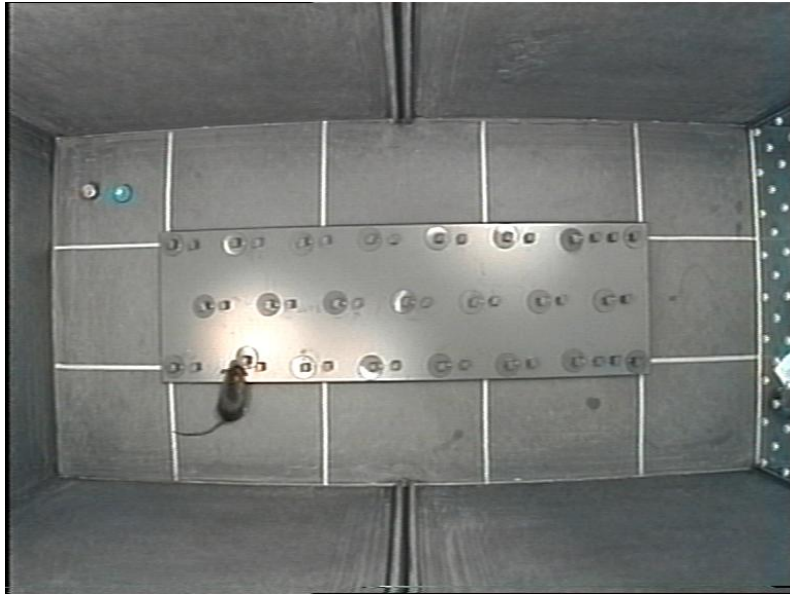


Fig. 2: Test Arena for modified hole board test.

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 and Discussion

Analysis of both the behavioral observation data (see Table 3) and the video-track data (see Table 4) did not reveal any genotype effect of MAG deficiency in the parameters assessed here.

The relatively subtle phenotypic alterations found in the Dymorphology, Neurology and Nociceptive screen did not lead to general behavioral alterations detectable in the primary Behavioral screen.

3.1.6 Reference

Ohl, F., Sillaber, I., Binder, E., Keck, M.E. & Holsboer, F. (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 TestData are presented as mean \pm standard error of mean.

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=22)	(n=13)	<i>p</i> - value	(n=16)	(n=18)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Line crossing [frequency]	109.09 \pm 6.99	137.08 \pm 10.23	N.A.	107 \pm 7.38	127.44 \pm 5.25	N.A.	n.s.	n.s.
Line crossing [latency]	9.29 \pm 1.42	6.12 \pm 1.72	N.A.	13.59 \pm 2.96	7.02 \pm 1.56	N.A.	n.s.	n.s.
Rearings in box [frequency]	26.59 \pm 1.91	26 \pm 2.2	N.A.	26 \pm 2.85	25.61 \pm 3.01	N.A.	n.s.	n.s.
Rearings in box [latency]	50.68 \pm 6.21	42.15 \pm 4.69	N.A.	58.82 \pm 9.89	62.92 \pm 10.65	N.A.	n.s.	n.s.
Hole exploration [frequency]	18.23 \pm 1.71	2.67 \pm 2.67	N.A.	19.5 \pm 2.23	24.06 \pm 2.06	N.A.	n.s.	n.s.
Hole exploration [latency]	60.26 \pm 8.64	64.27 \pm 8.5	N.A.	82.16 \pm 12.62	75.3 \pm 11.78	N.A.	n.s.	n.s.
Hole visit [frequency]	0 \pm 0	0 \pm 0	N.A.	0 \pm 0	0 \pm 0	N.A.	n.s.	n.s.
Hole visit [latency]	300 \pm 0	300 \pm 0	N.A.	300 \pm 0	300 \pm 0	N.A.	n.s.	n.s.
Board entry [frequency]	4.77 \pm 0.5	7.31 \pm 1.22	N.A.	4.69 \pm 0.71	7.5 \pm 1.03	N.A.	n.s.	n.s.
Board entry [latency]	125.75 \pm 13.99	118.12 \pm 21.61	N.A.	151.33 \pm 18.53	111.52 \pm 12.95	N.A.	n.s.	n.s.
Board entry [total duration %]	3.63 \pm 0.37	6.04 \pm 0.94	N.A.	4.86 \pm 0.69	6.1 \pm 0.77	N.A.	n.s.	n.s.
Rearing on board [frequency]	0 \pm 0	0.23 \pm 0.23	N.A.	0.13 \pm 0.09	0.11 \pm 0.08	N.A.	n.s.	n.s.
Rearing on board [latency]	300 \pm 0	298.35 \pm 1.65	N.A.	296.36 \pm 2.6	293.41 \pm 4.52	N.A.	n.s.	n.s.
Risk assessment [frequency]	0.55 \pm 0.21	0.31 \pm 0.17	N.A.	0.25 \pm 0.14	0.67 \pm 0.21	N.A.	n.s.	n.s.

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
Group contact [frequency]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Group contact [latency]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Group contact [total duration %]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Grooming [frequency]	1.27 ± 0.18	1 ± 0.2	N.A.	1.06 ± 0.19	0.89 ± 0.14	N.A.	n.s.	n.s.
Grooming [latency]	152 ± 15.03	148.31 ± 20.53	N.A.	168.53 ± 0	186.93 ± 19.12	N.A.	n.s.	n.s.
Grooming [total duration %]	1.46 ± 0.24	0.91 ± 0.2	N.A.	1.47 ± 0.25	0.78 ± 0.13	N.A.	n.s.	n.s.
Defecation [frequency]	0.18 ± 0.18	0.08 ± 0.08	N.A.	0.19 ± 0.1	0 ± 0	N.A.	n.s.	n.s.
Defecation [latency]	286.69 ± 13.31	280.82 ± 19.18	N.A.	250.99 ± 26.74	300 ± 0	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	5.59 ± 0.48	6.08 ± 0.65	N.A.	5.31 ± 0.49	6.06 ± 0.59	N.A.	n.s.	n.s.
Familiar object exploration [frequency]	5.77 ± 0.54	7.15 ± 0.52	N.A.	5.75 ± 0.61	6.56 ± 0.59	N.A.	n.s.	n.s.
Unfamiliar object exploration [latency]	52.47 ± 8.77	44.14 ± 8.25	N.A.	60.95 ± 10.36	46.5 ± 12.33	N.A.	n.s.	n.s.
Familiar object exploration [latency]	55.15 ± 7.61	56.67 ± 8.46	N.A.	80.68 ± 14.56	69.47 ± 9.52	N.A.	n.s.	n.s.
Unfamiliar object exploration [total duration %]	1.24 ± 0.16	1.56 ± 0.15	N.A.	1.04 ± 0.11	1.24 ± 0.12	N.A.	n.s.	n.s.
Familiar object exploration [total duration %]	1.22 ± 0.14	1.45 ± 0.18	N.A.	1.03 ± 0.1	1.28 ± 0.12	N.A.	n.s.	n.s.
Object Index	0.08 ± 0.05	0.11 ± 0.04	N.A.	0.07 ± 0.07	0.04 ± 0.06	N.A.	n.s.	n.s.

Table 4: Video-Tracking Results Regarding Locomotor BehaviorData are presented as mean \pm standard error of mean.

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=22)	(n=13)	<i>p</i> -value	(n=16)	(n=18)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Distance moved [cm]	2994.01 \pm 166.32	3559.54 \pm 198.55	N.A.	2940.05 \pm 168.28	3406.19 \pm 131.53	N.A.	n.s.	n.s.
Mean velocity [cm/sec])	17.5 \pm 0.66	19.28 \pm 0.9	N.A.	17.31 \pm 0.71	18.59 \pm 0.57	N.A.	n.s.	n.s.
Maximum velocity [cm/sec]	63.95 \pm 2.73	54.14 \pm 3.05	N.A.	59.48 \pm 3.49	63.4 \pm 3.65	N.A.	n.s.	n.s.
Turns [Frequency]	1670.95 \pm 60.13	1912.77 \pm 57.71	N.A.	1651.94 \pm 66.7	1865.28 \pm 48.81	N.A.	n.s.	n.s.
Board entry [max. duration, sec.]	4 \pm 0.47	5.42 \pm 1.28	N.A.	5.89 \pm 1.2	5.31 \pm 0.47	N.A.	n.s.	n.s.
Mean distance to wall [cm]	5.97 \pm 0.17	6.56 \pm 0.25	N.A.	6.51 \pm 0.24	6.45 \pm 0.28	N.A.	n.s.	n.s.
Mean distance to board [cm]	9.58 \pm 0.16	9.06 \pm 0.19	N.A.	9.11 \pm 0.23	9.2 \pm 0.22	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 the use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the “Phenoslim” wording (<http://www.informatics.jax.org/userdocs/phenoslim.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: X-ray analysis, bone densitometry and, in a limited number of animals, micro-computer tomography.

A total of 65 animals of MAG mice have been analyzed in the Dysmorphology, Bone and Cartilage module of the German Mouse Clinic. MAG^{-/-} mice showed a reduced response to the clickbox test. Furthermore, an abnormal struggling of MAG mutants was observed when suspended by the tail. MAG^{-/-} mice were shorter and lighter at the age of 16 weeks. In the DEXA analysis for bone density and body composition, male mutants had a decreased bone mineral content (BMC) and Lean mass.

3.2.2 Mice

Thirty-three male (17 +/+, 16 -/-) and 32 female (14 +/+, 18 -/-) 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 MAG knockout and control animals, and 16-week-old KOs (23 animals) and controls (19 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, when the mice entered the facility, the general physical condition and health were checked;
2. At the age of 9 weeks, a morphological whole-body checkup was carried out;
3. The ionized fraction of calcium in blood was analyzed in 14-week-old mice, and
4. At the age of 16 weeks the animals are analyzed by taking X-ray images and by DEXA analysis for bone densitometry and body composition

Morphological test

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

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

The most interesting finding in the first part of the Dysmorphology screen was the weaker response of the MAG^{-/-} animals in the **Clickbox test** (Table 6), which gives information about the ability of the animals to hear. MAG^{-/-} mice reached a mean score of 1.9, whereas the wild types responded with a mean score of 3.2. This result can only be taken as a first hint, because the C57BL/6 genetic background has strong influence on the clickbox results (Zettel et al., 1997).

In addition, we observed **abnormal struggling behavior** while hung by the tail in more than 50% of MAG^{-/-} mice (Table 5). A similar phenotype was observed in a mutant with motor neuron degeneration (Hafezparast *et al.* 2003). In Table 5 further observations are shown, which have been detected only in single animals, and which can be considered as minor findings which are not associated with the MAG knock out.

In the qualitative analysis of the **X-ray** images of the mice, no genotype specific observations could be detected. One MAG^{-/-} male mouse displayed a kinky tail and in two MAG^{-/-} animals the ulna appeared to be thinner.

In the **DEXA analysis** significant differences between the mutants and controls were detected for Bone mineral content (BMC), Lean mass, Body length, and Weight in males only (Table 7). Female mutants did not show any differences compared to the control group. The differences in male mutants in Bone mineral content and Lean mass disappeared when the data was related to the weight of the animals (Bone content, Lean content). This would indicate that the observed differences in Bone mineral content and Lean mass are only due to the significant differences in body weight. On the other hand, data obtained in the Clinical Chemical screen give hints for alterations in bone metabolism. Thus, secondary screening for bone parameters would depend on the results from the secondary tests in the Clinical Chemical screen.

Raw data will be available on demand.

3.2.6 References

Fuchs H, Schughart K, Wolf E, Balling R, Hrabé de Angelis M. (2000): Screening for dysmorphological abnormalities - a powerful tool to isolate new mouse mutants. *Mammalian Genome* 11(7): 528-30.

Hafezparast et al. (2003): Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science* 300: 808-812.

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Zettel M.L., R. D. Frisina, S. Haider and W. E. O'Neill (1997) Age-related changes in calbindin D-28k and calretinin immunoreactivity in the inferior colliculus of CBA/CaJ and C57Bl/6 mice. *J. Comp. Neurol.* 386: 92-110

Abbreviations

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

Table 5: Results from the Morphological Inspection				
Phenotype	Male		Female	
	wt	KO	wt	KO
Abnormal struggling		7	1	9
Body size small	-	-	-	2
Trembling slightly	1	4	-	1
Long snout	-	-	1	-
Long teeth	-	-	1	-
Kinked tail	-	2	-	1
Thin tail	-	-	-	1
Eye abnormality	-	1	1	1
Animals analyzed	17	16	14	18

Table 6: Results from the Clickbox Test for the Ability to Hear				
Score	Male		Female	
	wt	KO	wt	KO
0 (no reaction at all)	-	1	-	-
1 (nearly no reaction to clickbox sound)	1	7	-	9
2 (reduced reaction)	-	2	3	4
3 (normal)	7	2	4	4
4 (stronger response)	9	4	1	-
5 (very strong response)	-	-	-	-

Table 7: Bone-Related Quantitative ParametersData are presented as mean \pm standard error of mean.

Parameter	Wild Type (A)			Mutant (B)			Male A~B	Female A~B
	Male (n=12)	Female (n=7)	p-value	Male (n=12)	Female (n=11)	p-value	p-value	p-value
BMD [mg/ cm ²]	57 ± 1	59 ± 3	n.s.	56 ± 2	59 ± 2	n.s.	n.s.	n.s.
pBMD [mg/ cm ²]	46 ± 1	46 ± 2	n.s.	44 ± 1	46 ± 1	n.s.	n.s.	n.s.
sBMD [10 ⁻³ x cm ⁻²]	2.01 ± 0.06	2.81 ± 0.12	< 0.001	2.19 ± 0.07	2.91 ± 0.13	< 0.001	n.s.	n.s.
BMC [mg]	622 ± 27	469 ± 32	< 0.01	504 ± 39	455 ± 35	n.s.	< 0.05	n.s.
Lean mass [g]	22.42 ± 0.68	16.43 ± 0.61	< 0.001	20.35 ± 0.70	16.08 ± 0.40	< 0.001	< 0.05	n.s.
Fat mass [g]	3.43 ± 0.50	1.96 ± 0.20	n.s.	2.28 ± 0.35	2.02 ± 0.50	n.s.	n.s.	n.s.
Body Length [cm]	8.62 ± 0.06	8.14 ± 0.09	< 0.001	8.33 ± 0.07	8.13 ± 0.07	< 0.05	< 0.01	n.s.
Weight [g]	28.48 ± 0.79	21.09 ± 0.64	< 0.001	25.55 ± 0.61	20.78 ± 0.65	< 0.001	< 0.01	n.s.
Bone Content [%]	2.17 ± 0.06	2.23 ± 0.15	n.s.	1.97 ± 0.14	2.16 ± 0.11	n.s.	n.s.	n.s.
Lean Content [%]	78.94 ± 1.96	77.88 ± 1.65	n.s.	79.49 ± 1.51	77.81 ± 2.14	n.s.	n.s.	n.s.
Fat Content [%]	11.77 ± 1.54	9.27 ± 0.89	n.s.	8.98 ± 1.32	9.19 ± 2.10	n.s.	n.s.	n.s.
Subcutaneous fat span [mm]	3.3 ± 0.1	3.1 ± 0.2	n.s.	3.3 ± 0.2	3.3 ± 0.2	n.s.	n.s.	n.s.

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

	Wild Type (A)			Mutant (B)			Male A~B	Female A~B
	Male (n=17)	Female (n=6)	p - value	Male (n=16)	Female (n=10)	p - value	p - value	p - value
Ionized Calcium [mM]	1.12 ± 0.03	1.13 ± 0.02	n.s.	1.17 ± 0.02	1.19 ± 0.03	n.s.	n.s.	n.s.

3.3 Neurology Screen

3.3.1 Summary

In the primary neurological screen, 37 MAG knockout mice (19 males/18 females) and 32 control mice (18 males/14 females) were screened. 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/mutabase/shirpa_summary.html) to detect phenotypic differences between MAG knockout and control mice. Each test parameter contributes to an overall assessment in muscle, lower motor neuron, spinocerebellar, sensory and autonomic function. The primary neurological screen is focused on investigating neurological reflexes to determine the neurological functioning of a mouse. We also examine lactate levels in the blood of mice to draw conclusions about energy metabolism.

The comparison of MAG knockout to control mice revealed a significant neurological phenotype in the MAG knockout mice. Male and female MAG knockout mice had an obvious tremor. Furthermore, we detected significant alterations in gait of male MAG knockout mice. Female MAG knockout mice indicated a slight tendency towards reduced spontaneous behavior.

3.3.2 Mice

Eighteen 10-week-old male MAG-knockout and eighteen 10-week-old male control mice entered the neurological screen at the beginning of the 38st and 49th calendar week 2003. Eighteen 10-week-old female MAG-knockout and fourteen 10-week-old female control 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 manoeuvre (*Behavior recorded on or above the arena*). For the wire manoeuvre 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. Measure-

ments 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

Male and female MAG knockout mice showed an obvious tremor as compared to controls (Table 8). Another parameter with significant finding for male MAG knockout mice was reduced body weight (Table 7). In addition, the gait of the male MAG knockout mice was fluid but exhibited an abnormality (ataxy) (Table 9). Observation of spontaneous behavior revealed that in contrast to controls, female MAG knockout mice showed a tendency towards slower

movement (Table 7). All other SHIRPA test parameters were without significant pathological findings. Blood lactate screening showed that both male and female MAG knockout mice had no significant changes in their blood lactate level as compared to control mice (Table 11).

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

3.3.6 Discussion

The knockout of the myelin-associated glycoprotein (MAG) leads to a visible phenotype in homozygous MAG knockout mice. In our neurological screen, male and female MAG knockout mice displayed distinct defects (also see 3.2.5, Dymorphology Screen). Especially noticeable was the atactic gait of the male MAG knockout mice. This points towards a defect in cerebellum or in spinocerebellar pathways.

Tremor as detected in MAG knockout animals is the most common type of all movement disorders. Considering that tremor can be a manifestation of a variety of neurological conditions, we recommend **secondary screening** with the Rotarod test which would accurately measure balance and motor coordination of the MAG knockout mice. In addition, we would suggest measuring EMG since physiological tremor has a neurogenic basis and similar oscillations should be reflected in EMG signals of the affected muscles.

3.3.7 References

Irwin S. (1968) Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia*. 13(3): 222-257.

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 Pharmazeuticals; **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; **I**mperial Collegeschool of Medicine at St. Mary`s; **R**oyal London Hospital, **P**henotype **A**ssessment
http://www.mgu.har.mrc.ac.uk/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		
	Wild Type (n=18)	Mutant (n=18)	p-value	Wild Type (n=14)	Mutant (n=18)	p-value
Body Length [g]	8.51 \pm 0.02	8.38 \pm 0.03	<i>n.s.</i>	8 \pm 0.03	7.9 \pm 0.02	<i>n.s.</i>
Body Weight [g]	26.06 \pm 0.1	23.98 \pm 0.2	0.008	19.1 \pm 0.1	19.5 \pm 0.09	<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		
	Wild Type (n=18)	Mutant (n=18)	p-value	Wild Type (n=14)	Mutant (n=18)	p-value
Body Position						
Sitting or standing	15	16		12	18	
Rearing on hind legs	3	2	<i>n.s.</i>	2	0	<i>n.s.</i>
Spontaneous Behavior						
Slow movement	3	4		0	4	
Moderate movement	13	13		12	14	
Vigorous	2	1	<i>n.s.</i>	2	0	0.05
Tremor						
None	18	13		14	11	
mild	0	5	0.03	0	7	0.027

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		
	Wild Type (n=18)	Mutant (n=18)	<i>p-value</i>	Wild Type (n=14)	Mutant (n=18)	<i>p-value</i>
Locomotor Activity	12.17 \pm 0.4	11.68 \pm 0.2	<i>n.s.</i>	16.43 \pm 0.8	11.78 \pm 0.2	<i>n.s.</i>
Transfer arousal						
Brief freeze	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Momentary freeze	9	13		5	12	
No freeze	9	5		8	6	
Palpebral Closure						
Eyes wide open	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Piloerection						
None	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Gait						
Normal	18	7	<i>0.0004</i> <i>8</i>	13	13	<i>n.s.</i>
Fluid but abnormal	0	11		1	5	
Pelvic Elevation						
Markedly flattened	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Barely touches	2	4		0	1	
Normal	16	14		11	16	
Elevated	0	0		2	1	
Tail Elevation						
Horizontally extended	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Touch Escape						
None	0	0	<i>n.s.</i>	1	1	<i>n.s.</i>
Mild	0	2		0	0	
Moderate	11	14		12	14	
Vigorous	7	2		1	3	
Positional Passivity						
Struggles when held by tail	18	18	<i>n.s.</i>	14	18	<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		
	Wild Type (n=18)	Mutant (n=18)	<i>p-value</i>	Wild Type (n=14)	Mutant (n=18)	<i>p-value</i>
Trunk Curl						
Absent	8	8		6	11	
Present	10	10	<i>n.s.</i>	8	7	<i>n.s.</i>
Limb Grasping						
Absent	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Visual Placing						
Upon nose contact	2	2		3	3	
Upon vibrassee contact	14	14		8	12	
Before vibrassee contact	2	2		2	3	
Early vigorous extension	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Grip strength						
Slight	1	0		0	0	
Moderate	7	8		7	11	
Active grip	10	10		7	6	
Unusually effective	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Body Tone						
Slight resistance	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Pinna reflex						
Active retraction	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Corneal Reflex						
Active single eye blink	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Toe Pinch						
None	1	1		0	2	
Slight withdrawal	5	5	<i>n.s.</i>	5	9	<i>n.s.</i>
Moderate withdrawal	5	7		4	6	
Brisk	7	5		5	1	
Wire maneuver						
Active grip	2	4		7	2	
Difficulty to grasp	10	8	<i>n.s.</i>	7	11	<i>n.s.</i>
Unable to grasp	4	6		0	3	
Falls immediately	2	0		0	2	

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		
	Wild Type (n=18)	Mutant (n=18)	<i>p-value</i>	Wild Type (n=14)	Mutant (n=18)	<i>p-value</i>
Skin Colour						
Pink	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Limb Tone						
No resistance	10	10		6	7	
Slight resistance	8	8		8	11	
Moderate resistance	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Abdominal Tone						
Slight resistance	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Lacrimation						
None	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Salivation						
None	0	0		0	1	
Slight margin of s.a.	17	15		13	15	
Wet zone entire of s.a	1	3	<i>n.s.</i>	1	2	<i>n.s.</i>
Provoked biting						
Absent	16	17		14	15	
Present	2	1	<i>n.s.</i>	0	3	<i>n.s.</i>
Righting reflex						
No impairment	18	18	<i>n.s.</i>	14	18	<i>n.s.</i>
Contact righting reflex						
Absent	3	3		0	0	
Present	15	15	<i>n.s.</i>	14	18	<i>n.s.</i>
Negative Geotaxis						
Turns and climbs the grid	11	15		9	15	
Turns but then freezes	4	3		1	0	
Moves, but fails to turn	2	0		4	3	
Falls to turn	1	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Irritability						
None	12	15		13	17	
Struggle during sup. res.	6	3	<i>n.s.</i>	1	1	<i>n.s.</i>
Aggression						
None	16	17	<i>n.s.</i>	14	18	<i>n.s.</i>
Provoked biting or attack	2	1		0	0	
Vocalization						
None	4	2	<i>n.s.</i>	2	0	<i>n.s.</i>
Provoked during handling	14	16		12	18	

Table 14: Lactate LevelsData shown represent the results of the mean blood lactate concentrations, value (\pm SEM)

	Male			Female		
	Wild Type (n=18)	Mutant (n=18)	<i>p-value</i>	Wild Type (n=14)	Mutant (n=18)	<i>p-value</i>
Lactate [mmo/l]	5.3 \pm 0.56	5.3 \pm 0.4	<i>n.s.</i>	4.05 \pm 0.6	4.06 \pm 0.1	<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 a recent review see Graw, 2003).

No genotype-specific differences between wild-type and mutant NCAM mice were detected.

3.4.2 Mice

Thirty-three MAG wild-type (18 male, 15 female) and 36 MAG mutant (18 male, 18 female) mice 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 MAG (wild type – 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). The mean value and standard error was calculated for each group of mice, male and female, wild type and mutant (Table 13).

A total of 69 mice were ophthalmologically examined by **slit lamp microscopy**. No eye phenotype was shown to be associated with the MAG mutation (Table 14).

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 amplitudes from left and right eyes was used, as no major differences

between the eyes were detected in the ERG response. The comparison of a- and b-wave amplitudes of males and females revealed some significant differences, however, all ERG amplitudes varied in normal, non-pathologic ranges. Between the groups of mutant and wild-type MAG mice no consistent differences were found, neither in the male nor in the female group. No line-specific effect of the MAG knockout mice could be observed in ERG.

Results from slit lamp biomicroscopy indicated that there was no effect on eye phenotype due to loss of function of the MAG gene.

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Abbreviations

cd/m ²	candela per square meter
ERG	electroretinography
Hz	hertz

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	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=18)	(n=15)	<i>p - value</i>	(n=18)	(n=18)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
a-wave 500 cd/m ²	-7 ± 1.5	-14 ± 1.2	<0.001	-11 ± 1.3	-9 ± 1.5	n.s.	n.s.	<0.02
b-wave 500 cd/m ²	170 ± 5.6	182 ± 7.1	n.s.	168 ± 6.1	178 ± 5.9	n.s.	n.s.	n.s.
a-wave 12,500 cd/m ²	-34 ± 2.2	-38 ± 2.4	n.s.	-30 ± 2.4	-37 ± 2.0	<0.05	n.s.	n.s.
b-wave 12,500 cd/m ²	197 ± 7.8	191 ± 6.7	n.s.	169 ± 4.8	194 ± 5.4	<0.02	<0.01	n.s.

Table 16: Results from Slit Lamp Biomicroscopy

Genotype	Normal	Nuclear flecks	Corneal erosions	Post Capsule	Microphthalmia
-/-	27	4	1	2	2
+/+	21	8	2	2	

3.5 Clinical-Chemical Screen

3.5.1 Summary

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

In the primary clinical chemical screen, twenty-nine (17 males/12 females) control mice and thirty-three (15 males /18 females) MAG-KO mice were analyzed. Twenty different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured eight basic hematological parameters.

All clinical chemical and haematological parameters were within the normal ranges. In the primary screen, we found a lower inorganic phosphorus concentration in MAG mice. Additionally we detected a lower creatine kinase level for MAG-deficient male mice. In a secondary screen, it would be interesting to confirm these findings and to place emphasis on bone and muscle relevant parameters, since findings of the Dymorphology Screen and Neurology Screen suggest changes in bone metabolism and muscle function of MAG-KO mice.

3.5.2 Mice

The MAG mice were delivered in two batches:

- The first group entered the Clinical-Chemical Screen at the beginning of the 40th (males) and 41st (females) calendar week in 2003.
- The second group started at the beginning of the 51st calendar week.

Out of these, ten (six male, four female) MAG-KO mice and eleven (seven male, four female) control mice were analysed a second time for 13 energy metabolism related parameters after food restriction at the age of 20 weeks.

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.

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 (130 µl) 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. Blood samples were distributed to Clinical Chemical, Immunology and Neurology (Lactate) Screens, whereas the Immunology, Allergy, and Clinical Chemical Screens received plasma samples .

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

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)
Plasma concentrations of specific substrates
Glucose, Cholesterol, Triglycerides, Total protein, 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

Results are shown in Table 17. In the control animals, we detected sex-related differences in potassium, chloride, cholesterol, triglyceride, amylase and ferritin concentrations. In the MAG mice, sex-related differences were visible in sodium, potassium, chloride, creatinine, cholesterol, triglyceride, aspartate-aminotransferase, alkaline phosphatase, amylase and ferritin concentrations. In males, differences between mutants and controls were seen in inorganic phosphorus, creatine kinase, aspartate-aminotransferase, glucose and lipase concentrations. While in females, differences were found in inorganic phosphorus and cholesterol levels (Table 17). Most values obtained for the clinical chemical parameters were within the normal ranges usually found in C57BL/6 mice at the age of three months and were supported by previously published data (Suckow *et al.*, 2001; Quimby, 1999 and publications cited therein).

Hematology

Significant differences between mutants and controls were seen in white blood cell count only in males (Table 18). All parameters of both wild type and mutants were in normal ranges.

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

3.5.6 Discussion

All clinical chemical and haematological parameters were within the normal ranges. In the primary screen, we found a lower inorganic phosphorus concentration in MAG mice. Reduced serum levels are associated with renal dis-

orders, diseases of the endocrine regulation of the calcium and phosphate metabolism as well as in case of altered skeletal metabolism. Additionally we detected a lower creatine kinase level for MAG-deficient male mice. At the age of 12 weeks, analysis of the blood samples revealed differences in cholesterol levels (females), also in glucose and lipase concentrations (males) between MAG-KO mice and wild-type animals.

In a **secondary screen**, it would be interesting to confirm these findings and to place emphasis on bone and muscle relevant parameters, since findings of the Dysmorphology Screen and Neurology Screen suggest changes in bone metabolism and muscle function of MAG-KO mice.

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Table 17: Clinical-Chemical Parameters.								
Data are presented as mean \pm standard error of mean.								
Parameter	Mutant (A)			Wild Type (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=15)	(n=18)	<i>p- value</i>	(n=17)	(n=12)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
Sodium [mmol/l]	155 ± 0.36	153 ± 0.54	<0.001	155 ± 0.66	154 ± 0.63	n.s.	n.s.	n.s.
Potassium [mmol/l]	3.9 ± 0.06	3.6 ± 0.10	<0.001	3.8 ± 0.09	3.4 ± 0.09	<0.01	n.s.	n.s.
Calcium [mmol/l]	2.0 ± 0.03	1.9 ± 0.03	n.s.	1.9 ± 0.02	1.9 ± 0.05	n.s.	n.s.	n.s.
Chloride [mmol/l]	111.5 ± 0.40	113.6 ± 0.47	<0.01	110.7 ± 0.63	113.4 ± 0.64	<0.01	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.4 ± 0.09	1.4 ± 0.08	n.s.	1.7 ± 0.09	1.7 ± 0.10	n.s.	<0.05	<0.05
Total Protein [g/dl]	4.9 ± 0.05	5.0 ± 0.06	n.s.	4.7 ± 0.06	5.0 ± 0.13	n.s.	n.s.	n.s.
Creatinine [mg/dl]	0.353 ± 0.01	0.320 ± 0.01	<0.01	0.347 ± 0.01	0.333 ± 0.01	n.s.	n.s.	n.s.
Urea [mg/dl]	73.7 ± 3.38	67.6 ± 2.00	n.s.	68.5 ± 2.0	64.5 ± 3.0	n.s.	n.s.	n.s.
Uric acid [mg/dl]	2.6 ± 0.37	2.2 ± 0.26	n.s.	2.2 ± 0.3	2.3 ± 0.3	n.s.	n.s.	n.s.
Cholesterol [mg/dl]	98.9 ± 3.44	65.3 ± 2.76	<0.001	99.2 ± 5.5	82.5 ± 4.2	<0.05	n.s.	<0.01
Triglyceride [mg/dl]	149.4 ± 11.64	84.6 ± 4.94	<0.001	140.8 ± 10.8	97.5 ± 10.6	<0.01	n.s.	n.s.
Creatine Kinase [U/l]	45 ± 6.55	65 ± 12.01	n.s.	115 ± 29	132 ± 55	n.s.	<0.05	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	20 ± 1.32	20 ± 1.75	n.s.	21 ± 1.00	18 ± 2.00	n.s.	n.s.	n.s.
Aspartate-Amino-transferase (AST,GOT) [U/l]	30 ± 1.38	25 ± 1.60	<0.05	38 ± 3.00	40 ± 6.00	n.s.	<0.05	n.s.
Alkaline Phosphatase [U/l]	113 ± 7.65	147 ± 10.53	<0.02	117 ± 9.00	164 ± 21.00	n.s.	n.s.	n.s.
α-Amylase [U/l]	2903 ± 103.36	2328 ± 76.43	<0.001	2837 ± 118	2373 ± 100	<0.01	n.s.	n.s.
Glucose [mg/dl]	166.6 ± 6.47	168.2 ± 7.13	n.s.	187.3 ± 6.8	171.7 ± 10.0	n.s.	<0.05	n.s.
Ferritin [ng/ml]	24.5 ± 2.42	32.4 ± 1.47	<0.02	28.3 ± 2.0	34.0 ± 1.6	<0.05	n.s.	n.s.
Transferrin [mg/dl]	153.7 ± 4.80	156.2 ± 3.67	n.s.	149.7 ± 5.2	155.5 ± 4.9	n.s.	n.s.	n.s.
Lipase [U/l]	68.3 ± 4.34	56.9 ± 4.13	n.s.	54.3 ± 2.3	62.7 ± 8.5	n.s.	<0.05	n.s.

Table 18: Hematological Parameters.Data are presented as mean \pm standard error of mean.

Parameter	Mutant (A)			Wild Type (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=18)	(n=18)	<i>p</i> - value	(n=18)	(n=14)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [10 ³ /μl]	5.78 ±0.39	4.44 ±0.36	<0.02	4.24 ±0.43	3.79 ±0.37	n.s.	<0.02	n.s.
Red blood cell count [10 ³ /μl]	10.39 ±0.12	10.00 ±0.12	<0.05	9.99 ±0.39	10.27 ±0.16	n.s.	n.s.	n.s.
Hemoglobin [g/dl]	15.81 ±0.12	15.43 ±0.22	n.s.	15.39 ±0.57	15.80 ±0.25	n.s.	n.s.	n.s.
Hematocrit [%]	50 ±0.53	48 ±0.65	n.s.	48 ±1.86	49 ±0.79	n.s.	n.s.	n.s.
Mean corpuscular volume [fl]	47.89 ±0.18	48.17 ±0.15	n.s.	48.06 ±0.19	47.71 ±0.24	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin [pg]	15.23 ±0.12	15.43 ±0.07	n.s.	15.42 ±0.11	15.40 ±0.08	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	31.86 ±0.21	32.04 ±0.10	n.s.	32.08 ±0.20	32.28 ±0.12	n.s.	n.s.	n.s.
Platelet count [10 ³ /μl]	689 ±16.17	643 ±23.50	n.s.	651 ±25.83	651 ±20.87	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 (Bluethmann and Ohashi, 1994; Mak *et al.*, 2001; Fischer 2002; Rogner and Avner, 2003). The Immunology Screen at the GMC was set up to conduct a broad immunological phenotyping of mouse mutant lines with the intention of identifying distinct gene functions, which play key roles in the immune defenses of the organism through a complex network of cellular and soluble components (Janeway *et al.*, 2004).

According to the data summary presented to the GMC, no immunological phenotype was known in MAG mutant mice. Their analysis in the Immunology Screen revealed minor differences in the level of IgA between female mutants and their littermate controls.

3.6.2 Mice

We analyzed 33 mutant animals (18 females and 15 males) and 31 age- and sex-matched littermate controls (14 females and 17 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 (BectonDickinson, 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

The analysis of standard immunological parameters measured in the primary screen (Table 19) revealed significantly higher IgA levels in female MAG mutant mice as compared to their littermate controls.

3.6.6 Discussion

Under standard screening conditions all immunological parameters measured in MAG mice were within the normal ranges for the background strain (C57BL/6J). Although we established significantly higher IgA levels in mutant females, the observed values were not outside the expected values for this immunoglobulin. This finding is most likely due to natural variation, or differences between the tested groups of animals (the MAG line was screened in 2 independent experiments due to breeding problems). Without any evidence for further dysregulation of immune function in MAG mice, no additional immunological investigations are recommended.

3.6.7 References

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Table 19: Basic Parameters Analyzed in the Immunology Screen.Data are presented as mean \pm standard error of mean.

Parameter	Mutants (A)			Wild Type (B)			A - B	
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=15)	(n=18)		(n=17)	(n=14)		<i>p</i> - value	<i>p</i> - value
CD19 ⁺ [%]	46.5 \pm 2.0	41.6 \pm 4.6	n.s.	46.5 \pm 1.6	36.3 \pm 3.4	<0.05	n.s.	n.s.
CD19 ⁺ CD5 ⁻ [%]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
CD19 ⁺ CD5 ⁺ [%]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
CD3 ⁺ [%]	30.5 \pm 1.4	41.1 \pm 5.0	<0.05	33.5 \pm 0.9	40.5 \pm 3.3	<0.05	n.s.	n.s.
γ/δ TCR ⁺ [%]	0.07 \pm 0.01	0.02 \pm 0.01	<0.05	0.06 \pm 0.01	0.12 \pm 0.06	n.s.	n.s.	n.s.
Gr-1 ⁺ [%]	19.9 \pm 1.9	17.9 \pm 2.0	n.s.	19.5 \pm 2.3	12.9 \pm 1.4	<0.05	n.s.	n.s.
CD49b ⁺ [%]	25.3 \pm 2.4	17.9 \pm 2.1	<0.05	28.0 \pm 1.9	21.9 \pm 9.4	n.s.	n.s.	n.s.
CD4 ⁺ [%]	19.2 \pm 1.0	26.3 \pm 1.6	<0.01	21.3 \pm 0.5	25.4 \pm 1.2	<0.01	n.s.	n.s.
CD8 β ⁺ [%]	12.2 \pm 0.6	15.9 \pm 0.7	<0.01	11.7 \pm 0.6	14.6 \pm 0.2	<0.01	n.s.	n.s.
IgG ₁ [μ g/ml]	581.4 \pm 100	409.0 \pm 26.6	n.s.	551.7 \pm 63.4	430.1 \pm 43.5	n.s.	n.s.	n.s.
IgG _{2a} [μ g/ml]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IgG _{2b} [μ g/ml]	247.2 \pm 3.7	259.4 \pm 32.1	n.s.	227.9 \pm 22.7	210.1 \pm 69.7	n.s.	n.s.	n.s.
IgG ₃ [μ g/ml]	289.8 \pm 43.5	272.7 \pm 41.4	n.s.	239.2 \pm 22.1	376.3 \pm 89.7	n.s.	n.s.	n.s.
IgM [μ g/ml]	155.8 \pm 13.7	178.6 \pm 12.8	n.s.	134.4 \pm 14.0	203.5 \pm 24.2	<0.05	n.s.	n.s.
IgA [μ g/ml]	49.5 \pm 10.1	99.2 \pm 12.8	<0.05	64.2 \pm 17.1	51.0 \pm 5.5	n.s.	n.s.	<0.01
Anti-DNA Ab [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.
Rheumatoid factor [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.

Raw data will be available on demand.

3.7 Allergy Screen

3.7.1 Summary

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

In the primary Allergy screen 34 MAG-deficient and 31 wild-type animals were screened. The analysis of MAG-deficient mice in Allergy screen did not reveal any profound differences between knockout and wild-type mice.

3.7.2 Mice

An age- and sex-matched group of 31 wild-type (14 females, 17 males) and 34 knockout (18 females, 16 males) mice aged 12 weeks was analyzed in the Allergy Screen.

3.7.3 Material and Methods

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

3.7.4 Results and Discussion

The analysis of total IgE levels in plasma of MAG-deficient mice and their sex- and age-matched wild-type littermates revealed higher mean IgE concentrations in female mutant and wild-type animals. This sex-difference was statistically significant in knockout mice (Table 20).

Table 20: Total plasma IgE in MAG mice								
Data are presented as mean \pm standard error of mean.								
	Wild Type (A)			Mutant (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=14)	(n=17)	<i>p</i> -value	(n=18)	(n=16)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Total IgE [ng/ml]	77.9 \pm 18.9	51.8 \pm 11	n.s.	89.5 \pm 19.2	36.6 \pm 6.6	<0.05	n.s.	n.s.

No statistically significant difference between MAG-deficient and wild-type mice was found. In both MAG-deficient and wild-type animals, the mean concentration of total IgE was higher in females than in males, the elevation was significant in knockout animals.

Nevertheless, the mean concentration of total plasma IgE of both MAG-knockout and wild-type females as well as males was close to the normal value for total IgE in C57BL/6 mice established in our laboratory (females 87.6 \pm 20.12 ng/ml vs. males 30.3 \pm 4.41 ng/ml).

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

Raw data will be available on demand.

3.7.5 Reference

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3.8 Nociceptive Screen

3.8.1 Summary

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

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

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

In the Primary Screen the responsiveness of the intact somatosensory system to thermal pain was tested in the MAG mice by means of the hot plate test (nociceptive pain). We found significantly delayed pain response in MAG knockout male animals in hind paw licking, which is not the first sign of pain. However, in jumping MAG mice showed significantly shorter latency compared to the wild-type mice. On the base of these data it is not possible to define the pain-related phenotype of this genotype; therefore we would suggest performing further pain-related studies.

3.8.2 Mice

Thirty-three MAG knockout mice (16 male, 17 female), and 28 control animals (17 male, 11 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 Results

The first nociceptive response observed in these mice was **hind paw shaking**; the reaction times did not differ between wild-type and knockout animals. Both strains showed **hind paw licking**, another typical nociceptive response. This behavior was observed in wild-type animals earlier than in knock-out animals. The third examined response was **jumping of animals**; MAG-knock-out animals jumped significantly earlier than their wild-type littermates (Table 21). There was a significant sex difference in knock out animals in hind paw licking only (Table 21).

Raw data will be available on demand.

3.8.5 Discussion

We could not clearly determine the type of pain reactivity of this strain on the base of the hot plate test. We would suggest making further pain-related studies to specify the pain sensitivity of this mutant line.

More detailed pain related studies would include:

1. Base studies e.g.,
 - von Frey filament test to study the reaction of animals to mechanical pain,
 - acetic acid test to study the reaction to visceral inflammation.
2. Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.
3. Chronic pain tests:
 - Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals,
 - Carrageenan test to study the reaction to inflammation.

The results of the whole set of experiments will provide a complete picture about the pain reactivity of this mutant line.

3.8.6 References

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Abbreviations

h.p. hind paw
m male
f female

Table 21: Nociceptive Screen									
Data are presented as mean ± standard error of mean.									
Parameter	Mutant (A)			Wild Type (B)			A~B	A~B	AN-NOVA
	Female	Male		Female	Male		Female	Male	
	(n=17)	(n=16)	<i>p</i> -value	(n=11)	(n=17)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
H.p. licking	17.7± 2.1	28.1± 2.2	0,001	19.9± 2.6	18.0± 2.1	n.s.	n.s.	0.001	0,0088 9
H.p. shaking	14.5± 1.1	14.2± 1.1	n.s.	16.2± 1.1	16.8± 1.3	n.s.	n.s.	n.s.	0,9103 4
Jumping	50.3± 2.4	49.1± 2.5	n.s.	57.8± 3.0	59.3± 2.4	n.s.	0.058	0.0045	0,5896 2

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). 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 16 week old male and female MAG and wild-type mice. Concerning the absolute parameters of the spontaneous breathing pattern, statistically relevant sex differences were not pronounced in wild-type mice, neither in mutants. For the specific values, which take the body weight into account, typical sex differences were found. Females demonstrated higher specific tidal volumes and, thus, higher specific minute ventilation than males in both wild-type and MAG mice. These differences are due to the differences in body weight between male and female mice.

Comparing wild-type to mutant mice, mutant mice showed significantly lower breathing rates and consequently significantly higher inspiratory and expiratory timing compared to wild-type mice meaning mutant mice breathed slower. During rest, lower breathing rate was compensated by higher tidal volumes in the mutants resulting in comparable minute ventilations. This compensation was less sufficient during activity.

3.9.2 Mice

Wild-type and mutant mice of both sexes were studied at the age of 15 weeks. Mean body weights of male mice were 28.6 ± 0.8 g (wild type, $n = 4$) and 27.2 ± 0.9 g (mutants, $n = 5$; n.s.), respectively. As to be expected, body weight in female mice was significantly lower, mean values for female mice being 21.3 ± 0.5 g (wild type, $n = 8$) and 21.8 ± 0.5 g (mutant, $n = 6$, n.s.), respectively (Table 22).

3.9.3 Material and Methods

Whole Body Plethysmography

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



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

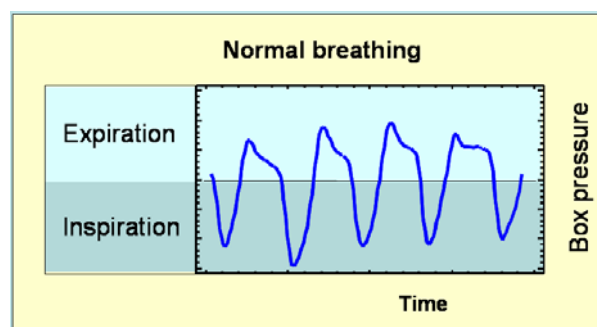


Fig. 4: Recorded data used to calculate the breathing parameters.

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

sMV) were calculated by relating the absolute values to the body weight of the animal. Furthermore, the mean of all breathing frequencies (mean_f) 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 and Discussion

Table 23 summarizes the results obtained for spontaneous breathing under resting and active conditions. Raw data are available on demand.

Sex differences: No significant sex differences were observed for the absolute values. During rest and activity females demonstrated higher specific tidal volumes and, thus, higher specific minute ventilation than males in both wild type and MAG mice. Since these specific parameters take the body weight into account and the absolute values primarily are not affected, the observed sex differences are likely due to the significantly higher body weight in males compared to females. Additionally, it was found that wild type females displayed significantly higher breathing rates and, thus, smaller expiratory timing during activity phase. This tendency was also seen in MAG females. In conclusion, these sex differences are common observations within various mouse inbred strains.

Differences between wild-type and MAG mice: In general, mutant mice showed significantly lower breathing rates and consequently significantly higher inspiratory and expiratory timing compared to WT mice meaning mutant mice breathed slower. During rest, the lower breathing rate was compensated by higher tidal volumes in the mutants resulting in comparable minute ventilations. This compensation was less sufficient during activity.

The results suggest disturbances in the central respiratory control causing the observed alterations in the rhythmogenesis. It is unlikely that the detected changes are due to reduced chemosensitivity or altered lung structure.

3.9.6 References

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Abbreviations

bw	body weight (g)
mean_f	mean of all respiratory rates (1/min)
f	respiratory rate (1/min)
TV	tidal volume (ml)
sTV	specific tidal volume (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 22: Characterization of Studied Mice

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

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=5)	(n=8)	p - value	(n=4)	(n=6)	p - value	p - value	p - value
bw	28.6 \pm 0.8	21.3 \pm 0.5	< 0.001	27.2 \pm 0.9	21.8 \pm 0.5	< 0.001	n.s.	n.s.
mean_f	423.4 \pm 15.7	474.7 \pm 26.6	n.s.	337.7 \pm 19.8	376.1 \pm 15.9	n.s.	<0.001	<0.02

Table 23: Spontaneous Breathing Pattern during Rest and ActivityData are presented as mean \pm standard error of mean.

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=5)	(n=8)	p - value	(n=4)	(n=6)	p - value	p - value	p - value
Rest								
f	358.6 \pm 5.8	390.9 \pm 10.9	0.0514	284.2 \pm 10.8	285.6 \pm 5.8	n.s.	< 0.001	< 0.001
TV	0.26 \pm 0.02	0.22 \pm 0.05	n.s.	0.31 \pm 0.02	0.30 \pm 0.009	n.s.	0.15124	< 0.01
sTV	9.1 \pm 0.5	10.5 \pm 0.3	n.s.	11.4 \pm 0.6	13.9 \pm 0.5	< 0.02	< 0.05	< 0.001
MV	91.1 \pm 7.1	85.6 \pm 1.8	n.s.	85.7 \pm 3.1	83.1 \pm 2.2	n.s.	n.s.	n.s.
sMV	3.2 \pm 0.2	4.0 \pm 0.1	< 0.001	3.2 \pm 0.06	3.8 \pm 0.1	< 0.01	n.s.	n.s.
Ti	48.7 \pm 1.0	46.0 \pm 1.4	n.s.	60.2 \pm 2.5	60.8 \pm 4.0	n.s.	< 0.01	< 0.01
Te	118.7 \pm 1.9	108.4 \pm 3.4	n.s.	151.8 \pm 5.6	149.8 \pm 3.4	n.s.	< 0.001	< 0.001
Ti/TT	0.29 \pm 0.003	0.30 \pm 0.006	n.s.	0.28 \pm 0.006	0.29 \pm 0.02	n.s.	n.s.	n.s.
PIF	9.5 \pm 0.6	8.7 \pm 0.3	n.s.	8.9 \pm 0.2	8.8 \pm 0.3	n.s.	n.s.	n.s.
PEF	5.7 \pm 0.4	5.0 \pm 0.3	n.s.	5.9 \pm 0.6	5.4 \pm 0.2	n.s.	n.s.	n.s.
MIF	5.4 \pm 0.4	4.9 \pm 0.1	n.s.	5.2 \pm 0.2	5.0 \pm 0.2	n.s.	n.s.	n.s.
MEF	2.2 \pm 0.2	2.1 \pm 0.05	n.s.	2.0 \pm 0.06	2.0 \pm 0.08	n.s.	n.s.	n.s.
Activity								
f	511.5 \pm 2.5	542.5 \pm 5.8	< 0.01	426.1 \pm 14.6	438.4 \pm 2.9	n.s.	< 0.001	< 0.001
TV	0.28 \pm 0.02	0.25 \pm 0.008	n.s.	0.31 \pm 0.02	0.28 \pm 0.008	n.s.	n.s.	n.s.
sTV	9.9 \pm 0.4	11.7 \pm 0.4	< 0.01	11.3 \pm 0.5	13.1 \pm 0.6	n.s.	n.s.	n.s.
MV	143.6 \pm 9.6	133.7 \pm 4.4	n.s.	128.0 \pm 4.7	122.5 \pm 4.1	n.s.	n.s.	n.s.
sMV	5.0 \pm 0.2	6.3 \pm 0.2	< 0.01	4.7 \pm 0.1	5.6 \pm 0.3	< 0.05	n.s.	n.s.
Ti	39.1 \pm 0.3	38.1 \pm 0.6	n.s.	45.8 \pm 1.3	43.2 \pm 0.5	0.06415	< 0.001	< 0.001
Te	78.3 \pm 0.7	72.5 \pm 1.0	< 0.01	95.5 \pm 3.3	93.7 \pm 1.0	n.s.	< 0.001	< 0.001
Ti/TT	0.33 \pm 0.003	0.34 \pm 0.005	n.s.	0.32 \pm 0.003	0.32 \pm 0.004	n.s.	0.0771	< 0.001
PIF	12.4 \pm 0.7	11.3 \pm 0.4	n.s.	11.4 \pm 0.4	11.1 \pm 0.3	n.s.	n.s.	n.s.
PEF	8.4 \pm 0.7	7.4 \pm 0.3	n.s.	8.1 \pm 0.8	7.3 \pm 0.3	n.s.	n.s.	n.s.
MIF	7.3 \pm 0.4	6.5 \pm 0.2	n.s.	6.7 \pm 0.3	6.6 \pm 0.2	n.s.	n.s.	n.s.
MEF	3.6 \pm 0.3	3.4 \pm 0.1	n.s.	3.2 \pm 0.1	3.0 \pm 0.1	n.s.	n.s.	n.s.

3.10 Expression Profiling

3.10.1 Summary

In this report, we describe the results of using close to genome-wide 21K cDNA microarrays for the RNA expression profiling of brain and bone tissue of four animals of the MAG mutant mouse line.

The data analysis and various statistical methods detected no genes differentially regulated between mutant and wild-type tissues in all experiments.

3.10.2 Mice

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Nine male mice of the MAG strain were provided to the molecular phenotyping screen (Table 24).

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 24: MAG-deficient and Wild-Type Mice Stored for Expression Profiling.

Mouse ID	Strain and Batch	Sex	Date of Birth	Genotype	Date of Collection
30013206	MAG2	m	15.09.2003	-/-	14.01.2004
30013248	MAG2	m	18.09.2003	-/-	14.01.2004
30013258	MAG2	m	18.09.2003	-/-	14.01.2004
30013268	MAG2	m	18.09.2003	-/-	14.01.2004
30013205	MAG2	m	15.09.2003	+/+	14.01.2004
30013208	MAG2	m	15.09.2003	+/+	14.01.2004
30013213	MAG2	m	17.09.2003	+/+	14.01.2004
30013214	MAG2	m	17.09.2003	+/+	14.01.2004
30013226	MAG2	m	17.09.2003	+/+	14.01.2004

3.10.3 Material and Methods

Isolation of Total RNA

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

Chip Design

We use a glass-surface DNA-chip containing ≈ 21,000 probes. About 20,200 of these probes are from the commercial Lion mouse array-TAG clone set, which is mostly derived from 3'UTRs. All Lion probes have been sequenced. The remaining probes were isolated in a subtractive screen for differentially expressed genes in the mesoderm of Delta/Notch pathway deficient mouse embryos. Mouse array-TAG clones have the general ID MG-VW-XYZ and the Delta/Notch specific probes are named rda-X.

DNA Microarrays

PCR products with 5'-aminogroup were amplified from the mouse arrayTAG library from Lion Bioscience comprising approximately 20.200 clones (Heidelberg, Germany). PCR products were dissolved in 3-fold SSC buffer and spotted on aldehyde-coated slides (Telechem, USA) using a Microgrid TAS II spotter (Biorobotics) with 48 Stealth™ SMP3 pins (Telechem). Spotted slides were rehydrated overnight in a humid chamber containing 50-70% aqueous solution of glycerol. Rehydrated slides were immersed in blocking solution (0.1 M sodium borohydride in 0.75fold PBS with 25% ethanol) for 5 minutes, oiled in water for 2 minutes, briefly immersed in 100% ethanol and air-dried. Slides were pre-hybridised for 1 hour in pre-hybridisation buffer (6-fold SSC, 1%BSA, 0.5%SDS) rinsed in water, dried and hybridised the same day. (Seltmann et al, in press)

Reverse Transcription and Fluorescent Labelling

For labelling 20µg of total RNA were used for reverse transcription and indirectly labelled with Cy3 or Cy5 fluorescent dye according the TIGR protocol (http://pga.tigr.org/sop/M004_1a.pdf). Labelled cDNA was dissolved in 30µl hybridisation buffer (6x SSC, 0.5% SDS 5fold Denhardt's solution and 50% formamide) and mixed with 30 µl of reference cDNA solution (pool from 5 wt animals) labelled with the second dye. This hybridisation mixture was placed on a pre-hybridised microarray, under a cover slip, placed into a hybridisation chamber (Genetix) and immersed in a thermostatic bath at 42°C for at least 16 hours. After hybridisation slides were washed in 40 ml of 3x SSC, 40 ml of 1x SSC and 40 l of 0.25x SSC at room temperature. For drying slides were placed in an empty 50 ml Falcon tube (Becton Dickinson, USA) and centrifuged at 4000 m/s². Dried slides were scanned with a GenePix 4000A microarray scanner and the images were analysed using the GenePix Pro3.0 image processing software (Axon Instruments, USA). All data were normal-

ised by adjusting the median of log-ratios of Cy5 to Cy3 intensities to 0. For data analysis in-house produced LabView based software was used (Drobyshev et al, in press).

Chip Hybridisation

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

3.10.4 Results

Amount of RNA obtained from different organs

Brain and bone were selected as organs for expression profiling analysis based on data from other GMC-screens. We isolated total RNA of brain and bone of four MAG mutant mice and five wild-type individuals.

Table 25: Amount of total RNA isolated from brain and bone of each individual		
Mouse ID	µg total RNA bone	µg total RNA brain
30013206	112	288
30013248	89	260
30013258	71	295
30013268	97	338
30013205	125	265
30013208	142	380
30013213	96	350
30013214	105	384
30013226	98	294

Chip hybridisation

Four chip hybridisations were performed with RNA from brain of each individual mutant mouse. Each chip hybridisation was performed against the identical pool of wt RNAs (reference RNA pool). For each individual the chip experiments included two colour-flip experiments. Fifteen chip hybridisations

were used for data analysis. Excluded was one experiment of individual 263 due to hybridisation artefacts.

Due to degradation of the isolated RNA from bone, we have not performed chip experiments of this tissue.

Data analysis

In all experiments, signals of MAG spots were below detection thresholds, in knockout and in wild-type tissue, too.

Genes which showed signals in all 15 chip-hybridizations were determined. The selected genes were ranked according to the lowest of 15 ratios of reproducibility in those chip-hybridisations (“minimum of maximum”; Table 26). In this ranking all genes were either consistently up- or down-regulated. For the different selection of genes the estimated minimal number of false positive, non-differentially expressed (NDE) genes is given for the probabilities $p < 0.05$ und $p < 0.1$.

Table 26: Pattern analysis of gene expression data.				
Number of Genes	Non-uniform Patterns	NDE (False Positives)		Range of Fold Induction (Minimum of 12Chips)
		$p < 0.05$	$p < 0.01$	
5	4	5	5	1,46 – 1,12
10	9	10	10	1,12 – 1,09
25	24	25	25	1,09 – 1,07

Among the top 10 genes all genes are not reproducibly expressed and for the probability $p < 0.1$ ten genes are false positive (NDE).

3.10.5 Discussion

The data analysis by means of various statistical methods did not reveal any genes differentially regulated in MAG mutant tissue in all experiments.

We could not detect any expression of the MAG gene, neither in knockout nor in wild-type tissue. One possible reason maybe that the MAG gene is not expressed in brain tissue of adult mice. Otherwise, we would have expected a signal in the wild-type samples. Based on our expression profiling data the effect of the MAG mutant allele on the global expression pattern is weak. One reason for this could be that the effect of the allele is localised to particular regions of the brain (cerebellum?) or a specific subset of cells throughout the brain. Alternatively, the mutant allele may have an effect on gene expression at an earlier stage either during embryonic development or early post-partum development. It should also be considered that MAG en-

codes a structural protein, the myelin-associated glycoprotein, rather than a component of a signal transduction pathway or a transcription factor. In particular, in the brain it may be expected that a rather strong (behavioural) phenotype could result merely from epigenetic (structural or similar) causes that do not require significant changes in global gene expression. This notion would be supported by our gene expression data.

3.10.6 References

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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 focused 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 fifteen (9 male/6 female) wild-type mice and nineteen (9 male/10 female) MAG KO mice were analyzed. They were first fed under *ad libitum* conditions for two weeks, followed by one week of food restriction to 60% of *ad libitum*.

No genotype-specific differences could be found in any metabolic parameter, e.g. body weight and food intake. Due to similar body weights, no energetic differences could be found in body weight related parameters like energy uptake or ratio of metabolised energy per unit body weight.

3.11.2 Mice

Two batches of control and MAG-deficient mice entered the metabolic screen, in total 15 wild-type (9 males and 6 females) and 19 mutant mice (9 males and 11 females). 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 (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 comparison. 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 and Discussion

Prior to the metabolic screening of MAG-deficient mice, no information about metabolic parameters was available. Males of both genotypes showed higher body weights than females, but no differences between the two genotypes could be measured (Table 27). Additional sex differences could be found in food and energy uptake, even considering body weight (calculating energy uptake and ratio of metabolised energy per unit body weight). However, no differences were observed between the two genotypes.

To conclude, the parameters investigated in the primary metabolic screen could not confirm any metabolic phenotype.

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

3.11.6 Reference

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Abbreviations

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

Table 27: Metabolic Parameters Recorded in the Primary ScreenData are presented as mean \pm standard error of mean.

Parameter	Wild Type (A)					Mutant (B)					A~B	
	<i>ad libitum</i>		<i>p</i> -value	food reduction, 7 days to 60%		<i>ad libitum</i>		<i>p</i> -value	food reduction, 7 days to 60%		<i>p</i> -value	<i>p</i> -value
	Male	Female		Male	Female	Male	Female		Male	Female		
(n=9)	(n=6)	(n=9)	(n=6)	(n=9)	(n=10)	(n=9)	(n=10)	(n=9)	(n=10)			
Body weight [g]	28.1 \pm 0.69	21.6 \pm 0.84	< 0.001	22.9 \pm 0.87	18.3 \pm 0.82	26.4 \pm 0.59	20.9 \pm 0.64	< 0.001	22.1 \pm 0.5	18.0 \pm 0.6	n.s.	n.s.
Rectal body temperature [°C]	36.4 \pm 0.18	36.7 \pm 0.19	n.s.	34.6 \pm 0.2	33.3 \pm 0.5	36.4 \pm 0.17	36.8 \pm 0.12	n.s.	34.7 \pm 0.22	34.6 \pm 0.47	n.s.	n.s.
Food consumption [g day⁻¹]	3.58 \pm 0.13	3.2 \pm 0.08	< 0.05	60% of <i>ad libitum</i>		3.64 \pm 0.09	3.17 \pm 0.15	< 0.05	60% of <i>ad libitum</i>		n.s.	n.s.
Energy uptake [kJ day⁻¹]	66.4 \pm 2.41	59.2 \pm 1.45	< 0.05	39.8 \pm 1.44	35.5 \pm 0.87	67.3 \pm 1.65	58.7 \pm 2.64	< 0.05	40.3 \pm 0.99	35.2 \pm 1.7	n.s.	n.s.
Energy uptake BW⁻¹ [kJ g⁻¹ day⁻¹]	2.36 \pm 0.07	2.76 \pm 0.12	< 0.02	1.74 \pm 0.05	1.96 \pm 0.09	2.55 \pm 0.06	2.79 \pm 0.1	n.s.	1.83 \pm 0.06	1.96 \pm 0.09	n.s.	n.s.
Feces production [g day⁻¹]	0.65 \pm 0.02	0.55 \pm 0.02	< 0.02	0.38 \pm 0.02	0.35 \pm 0.02	0.69 \pm 0.02	0.58 \pm 0.03	< 0.01	0.39 \pm 0.01	0.35 \pm 0.02	n.s.	n.s.
Energy content feces [kJ g⁻¹]	16.3 \pm 0.04	16.1 \pm 0.08	n.s.	16.02 \pm 0.11	15.97 \pm 0.09	16.3 \pm 0.08	16.05 \pm 0.08	n.s.	16.25 \pm 0.08	15.85 \pm 0.07	n.s.	n.s.
Metabolized energy [kJ day⁻¹]	56.1 \pm 2.11	50.5 \pm 1.35	n.s.	33.8 \pm 1.13	30.1 \pm 0.66	56.2 \pm 1.42	49.6 \pm 2.44	< 0.05	34.2 \pm 0.85	29.7 \pm 1.45	n.s.	n.s.
Metabolized energy [kJ g⁻¹ day⁻¹]	1.99 \pm 0.06	2.35 \pm 0.11	< 0.02	1.48 \pm 0.04	1.66 \pm 0.08	2.13 \pm 0.06	2.36 \pm 0.11	< 0.05	1.55 \pm 0.05	1.65 \pm 0.08	n.s.	n.s.
Food assimilation coefficient [%]	84.5 \pm 0.37	85.3 \pm 0.39	n.s.	85.0 \pm 0.35	84.7 \pm 0.41	83.5 \pm 0.28	84.5 \pm 0.4	n.s.	84.8 \pm 0.38	84.4 \pm 0.4	n.s.	n.s.

3.12 Pathology Screen

3.12.1 Summary

The Pathology screen performed a complete morphological analysis with standard stains. We did not find any genotype-specific morphological differences between the mutant and the knockout mice. Although we discussed interesting secondary results we can conclude that MAG mice do not show a specific morphological phenotype.

3.12.2 Mice

Sixty-one mice, 33 knockout mice (16 males, 17 females) and 28 control animals (17 males, 11 females) were analyzed. Due to the workflow in the GMC, mice of different ages were received from different screens (Table 28).

Table 28: MAG-deficient mice and their control littermates analyzed.						
	Wild Type		Mutant		Number of Animals	Age (days)
	Males	Females	Males	Females		
Lung Screen	0	4	0	6	10	16
Expression Profiling	5	0	4	0	9	16
Dysmorphology Screen	3	0	2	0	5	20-22
Metabolic Screen	9	7	10	11	37	20-22
Total Number of Animals	17	11	16	17	61	

3.12.3 Materials and Methods

Mice received in the laboratory of pathology were sacrificed with CO₂. The animals were analyzed macroscopically and weighed. The thymus and left lobe of the liver were measured. Blood samples were taken, centrifuged and the serum was saved at -20°C. Tails were preserved at -70°C for further genetic analysis. Following a complete dissection, an x-ray of the complete bone structure was taken, when indicated (Hewlett Packard, Cabinet X-Ray System Faxitron Series). All organs were fixed in 4% buffered formalin and embedded in paraffin for histological examination. Two- μ m-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.

3.12.4 Results

Line-specific results

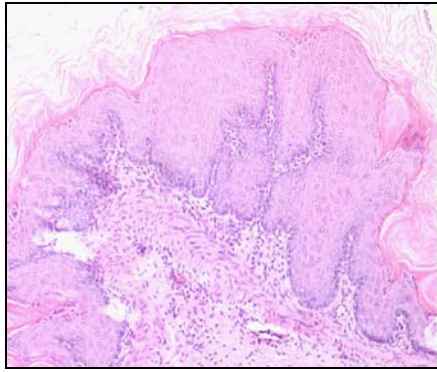
The MAG mice do not show a specific morphological phenotype (Table 28).

Table 29: MAG-mice. Genotype-specific morphological alterations.					
Organ	Skin	Musculoskeletal System	Eyes	Brain	Cerebellum
Alteration	no	no	no	no	no
Organ	Heart	Trachea	Lung	Teeth	Salivary glands
Alteration	no	no	no	no	no
Organ	Esophagus	Stomach	Small Intestine	Large Intestine	Liver
Alteration	no	no	no	no	no
Organ	Pancreas	Cervical Lymph Nodes	Thymus	Spleen	Thyroid
Alteration	no	no	no	no	no
Organ	Parathyroid	Adrenal Gland	Kidneys	Urinary Bladder	Testes
Alteration	no	no	no	no	no
Organ	Epididymis	Funiculus spermaticus	Ovaries	Uterus	Vagina
Alteration	no	no	no	no	no

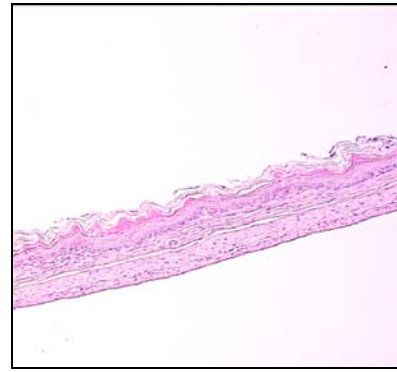
3.12.5 Secondary results

We did not find any genotype-specific alterations; however, we detected two types of lesions, which usually do not occur in B6 mouse strain.

Stomach: Proliferative lesions of surface forstomach epithelium. Focal or diffuse squamous cell hyperplasia of the forstomach was observed in 17 mice (10 knockouts and seven wild types)



H&E 25x B6 wild type

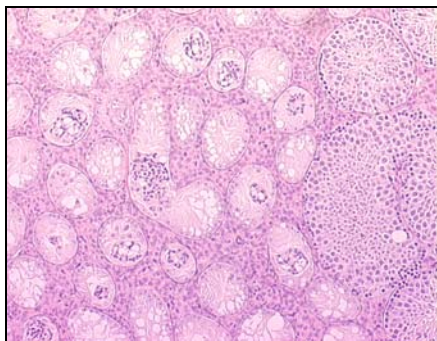


H&E 25x B6 wild type

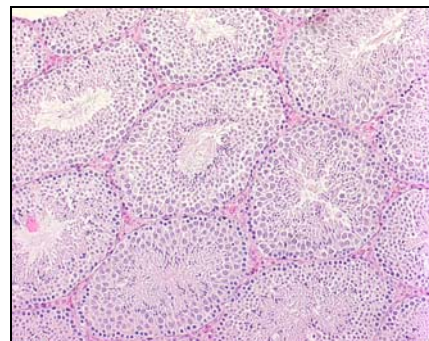
Fig. 5: Sections of forstomach.

On the left side squamous cell hyperplasia is depicted, compared with a normal forstomach on the right side

Testis: Several males (14 of 32) eight knockouts and six wild types revealed testicular tubular atrophy. In testicular atrophy the seminiferous tubules are vacuolated or necrotic and have reduced numbers of germinal cells. Two knockout and one wild-type mice had severe atrophy. The tubules atrophy occupied 40-60% of the testis. Eleven mice revealed mild partial unilateral atrophy and the degenerate tubules occupied 3 - 7% of the testis (not significant, Fisher's exact test, $p > 0.05$)



H&E 200x MAG mice



H&E 200x B6 wild type

Fig. 6: Sections of testis.

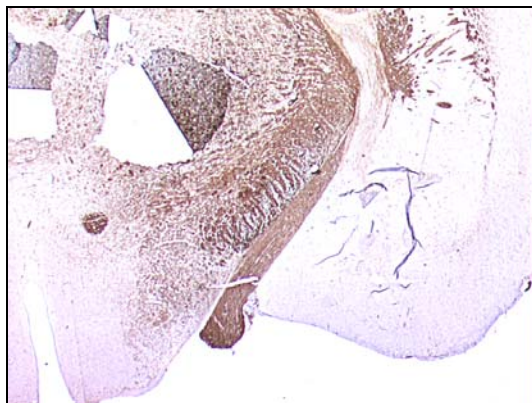
On the left side, severe testis atrophy is shown. The seminiferous tubules had reduced to complete absence of spermatogenesis, and are replaced with fat vacuoles. The right side shows a representative section of a normal testis.

Liver: several animals (15 of 61; nine knockouts and six wild type) developed a micro-vesicular steatosis of the liver, the steatosis appears as fatty clear microvacuoles within the hepatocytes. In 20 of 61 mice (nine knockouts and 11 wild type) non-specific infiltrates, and one to five microgranulomas were observed.

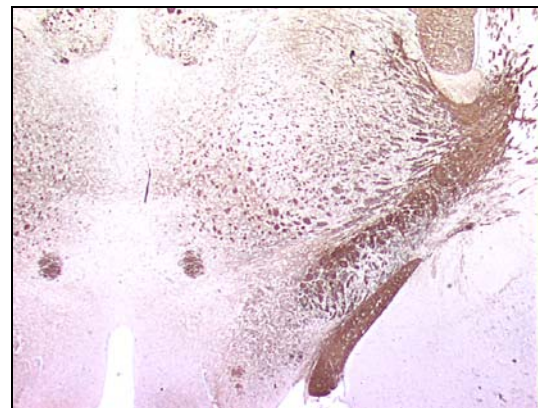
Brain: We could not see delayed myelination in coronal sections from brain at the level of the optical tract stained with luxol fast blue, a special stain for myelin, or with IHC with anti-neurofilament protein antibody. (This antibody labels only the myelinated axons of neurons). We could not see any difference in the myelin labels between MAG-mice or wild-type mice



IHC anti-neurofilament protein 150x, wild type



IHC anti-neurofilament protein 200x
MAG knockout



IHC anti-neurofilament protein 200x
wild type

Fig. 7: Coronal sections of brain.

The upper picture is representative coronal section of brain in wild-type animals. Note in this level the rostral section of the hippocampal formation and optic tract.

The two lower pictures are a higher magnification of the optic tract of MAG knockout and control mouse. Note that there are no mayor differences between the two.

3.12.6 Discussion

Proliferative lesions as hyperplasia of the forestomach are often observed in aging B6 mice and in toxicological studies. In this context this alteration is most probable non-specific, and associated to food intake. Both controls and knockouts showed the same changes. Therefore, the stomach phenotype found here is primarily due to the genetic background.

Partial testicular tubular atrophy with more than 60% degenerated tubules is a frequent finding in old mice. However due to the age of the mice, this finding is unusual. Unilateral testicular degeneration can be secondary to full or partial torsion of the testis. These changes were not documented to any of the mice.

Of note is that despite the relative frequent finding of testicular atrophy, no problems with fertility were observed. Since the atrophic changes were observed in both control and MAG knockout mice, this finding seems not to be line-specific. Interestingly testicular atrophy at this age in B6 mice is an extremely infrequent finding.

The infiltration of the liver with non-specific infiltrates, and microgranulomas confirm earlier results of C57BL/6J screening. Mice of this background seem to be more susceptible to develop non-specific inflammatory changes. To rule out the possibility of an MHV infection, we investigated the presence of MHV. All animals were serological negative. Concerning the steatosis of the liver we have observed this change in different strains and lines. This fatty change in the liver may occur spontaneously and is usually reversible. It is our opinion that this finding is more related to the type and amount of food that a mouse ingests than to some pathological feature

Acknowledgements

A large team consisting of scientists, technicians and animal caretakers all contribute to the success of the German Mouse Clinic. We want to thank Reinhard Seeliger, Elfi Holupirek, Christine Fürmann, Kerstin Kutzner, Mareike Maurer, Susanne Sommer, Rose Austin, Florian Schleicher, Gregor Pahnke, Susanne Wittich, Martin Taube, Claudia Zeller, Sandra Schädler, Elenore Samson, Nadine Kink, Jaqueline Müller, Sabine Holthaus, and Claudia Kloss for expert technical help and Daniela Kißling, Monika Katzbach, Uwe Drescher, Heiko Engelniederhammer, Manuela Krug, Tina Kohler, Petra Thalmeier, Daniela Elvert, Aline Weingärtner and Sven Korb for the care of the mice.

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Addresses of screeners and modules

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Allergy Screen

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