

The

GERMAN MOUSE CLINIC

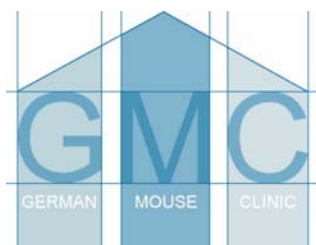
at the Helmholtz Zentrum München
German Research Center for
Environmental Health (GmbH)

Report for Gsk3 α -flox

Confidential Data

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The German Mouse Clinic



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

In the GMC, experts from various fields of mouse genetics, physiology and pathology in close collaboration with clinicians work side by side at one location. We offer a primary phenotypic analysis of mouse mutants (more than 320 parameters/mouse line) in the areas of allergology, behavior, bone and cartilage, cardiovascular diseases, clinical chemistry, energy metabolism, eye development and vision, immunology, lung function, molecular phenotyping, neurology, nociception, pathology, and steroid metabolism. Additional screens for host-pathogen interaction can be performed at the HZI Braunschweig. Secondary and tertiary screening for in-depth analysis is offered by the different screens and is available on request.

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1 Summary

1.1 Primary Screening

In a primary screen 60 *Gsk3 α* -flox mice have been analyzed in the German Mouse Clinic (GMC) in the screens Dymorphology, Behavior, Neurology, Eye, Nociception, Energy Metabolism, Clinical Chemistry, Immunology, Allergy, Steroid Metabolism, Cardiovascular Function, Lung Function, Molecular Phenotyping, and Pathology. The screening started on October 22nd, 2007. Additional experiments were performed by the Screens Behavior, Dymorphology, Immunology and Pathology.

1.2 Results by Screen

The comparison of the two knockdown lines *Gsk3 α* and *Gsk3 β* is outlined in more detail in the respective chapters.

Dymorphology, Bone and Cartilage: In the dual-energy X-ray absorptiometry (DXA) sBMD (BMD related to the body weight) was significantly increased in male mutants with the same tendency in females. The differences in bone parameters might be due to the smaller body size and reduced body weight of male mutants.

Behavior: The analysis revealed an increase in anxiety-related behavior in a novel environment. Genotypic differences in body weight in this mouse line may have contributed to the behavioral outcome in all tests. Additional tests have been performed already; results are presented in Chapter 3.2.

Energy Metabolism: *Gsk3 α* mutants were significantly lighter than wild-type controls both under ad libitum and food restricted conditions. Body temperature was also reduced under ad libitum conditions. Especially female mutants did not cope with the fasting challenge diagnosed by extreme body temperature reduction. Therefore this test had to be stopped after one day only.

Clinical Chemistry and Hematology: We detected differences in hematological and in blood lipid parameters. These findings may indicate an influence of the *Gsk3 α* genotype on hematopoiesis and possibly on fat/energy metabolism. In addition, the physiological sex-related differences were found.

Immunology: The analysis of *Gsk3 α* mutant mice in the primary Immunology Screen revealed a statistically significant higher frequency of T-cells and the tendency of a lower frequency of B-cells in female mutant mice compared to wild-type controls. Furthermore, there was higher expression of CD62L and a lower expression of CD44 in T-cells of female mutant mice. However, analyses of the immune cells in other compartments (spleen, thymus) did not confirm the findings of the primary screen.

Molecular Phenotyping: Brain and muscle were selected for the *Gsk3 α* knockdown mouse line due to strong expression of the gene in these organs. Additionally, the selection of brain based on phenotypical alteration identified in the behavior screen. The data analysis and various statistical methods detected a limited number of genes differentially regulated between knockdown and reference tissues in brain and muscle. Genes significantly regulated in muscle are associated with blood circulation and gas exchange and protein phosphorylation.

Pathology: Luxol fast blue staining of the brain was performed. Some mutant mice, predominantly females, exhibit reduced myelin density in some structures of the brain (e.g. the corpus callosum and the mammillothalamic tract), which is suspicious of the presence of demyelination similar to the *Gsk3 β* knockdown mice.

In the screens **Neurology, Nociception, Allergy, Steroid Metabolism, and Lung Function**, no genotype-specific differences could be found.

Cardiovascular: Data from the cardiovascular screening will be submitted later.

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

2 General Part

2.1 The Role of the Gene

Glycogen synthase kinase 3 (Gsk3) is involved in many important pathways which are listed in the summary given by the provider.

2.2 Known Phenotypes

The provider noted a reduction in body size and body weight. All further findings which will be shown in this report we consider as new.

2.3 Expected Phenotypes

Since Gsk3 plays a role in many pathways, multiple additional phenotypes are possible.

2.4 Suggested Human Disease Model

These mice are supposed to be a model especially for mood disorders, because Lithium chloride, one of the primary drugs to treat bipolar mood disorders inhibits Gsk3.

2.5 Mice

2.5.1 Number and kind of mice

Table 1: Gsk3α mice provided for analysis. Numbers in brackets indicate animals which were kept in reserve.	
Genotype / Sex	Number of Animals
Mutant female	15 (4 died*)
Mutant male	16 (4 died*)
Control female	14 (1 died*)
Control male	15 (1 died*)

*One mouse died after IpGTT and 9 after blood withdrawal.

In this knockdown mouse line Gsk3 α is downregulated by means of siRNA. Although it should be neuronal and glia cell precursor-specific (due to the activation of downregulation by means of Nestin-promotor-coupled Cre-excision),

the provider detected Cre-activity in many organs besides neuronal cells. Therefore, multiple effects are possible.

2.5.2 Housing conditions

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

Outbred 8-week-old male SPF Swiss mice are used as sentinels and kept on a mixture of new bedding and aliquots of soiled bedding (50:50) from all cages of the IVC rack. In addition, the sentinels were also exposed to soiled air from all “upstream” cages of the IVC rack. Health monitoring is carried out by on-site examination of the sentinel mice by certified laboratories according to FELASA recommendations (www.felasa.org).

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

2.6 Workflow

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

Mouse mutants entering the GMC are examined in a primary screen according to the following standard workflow (Fig. 1; modified after Gailus-Durner, Fuchs *et al.*, 2005; Brown *et al.*, 2005). Analyzed parameters are listed in Table 2. After the mice arrive at the GMC, they are acclimatized in the new environment for four weeks and divided into two groups to enter the GMC in two pipelines (Fig. 1).

The mice of the **first pipeline** are subjected to a morphological whole-body checkup in the Dysmorphology Screen and are passed to the Cardiovascular Screen. After the blood pressure is taken, the energy metabolism is analyzed by calorimetry. One week later a simplified IpGTT is performed by the Clinical Chemical Screen. In the next week, the mice re-enter the Dysmorphology Screen for X-ray and DXA analysis. After the determination of eye size parameters, 12 mutant animals (six males / six females) and 12 controls (six males / six females) leave the animal facility for the Lung Function Analy-

sis, which for technical reasons is located elsewhere. The males are used to freeze organs for future molecular phenotyping on request.

The **second pipeline** starts in the Behavior Screen. The initial screening of the naïve mice includes also neurological tests and lasts three weeks. One week later, the animals are tested in the Nociceptive Screen. On the following week the mice go through the tests of the Eye Screen. When the mice are 14 weeks old, blood is taken, and samples are distributed to the blood-based screens for Clinical Chemistry, Immunology, Cardiovascular, and Allergy. One week later the mice were passed to the Cardiovascular Screen wherein the mice stay two weeks. ANP level is determined in the plasma samples, whereas ECG or Echo analysis is performed on request. Three weeks after testing of the first blood sample, a second sample is taken to confirm the findings and analyze steroid levels. After completion of the primary screen all animals of the second pipeline are analyzed macro- and microscopically in the Pathology.

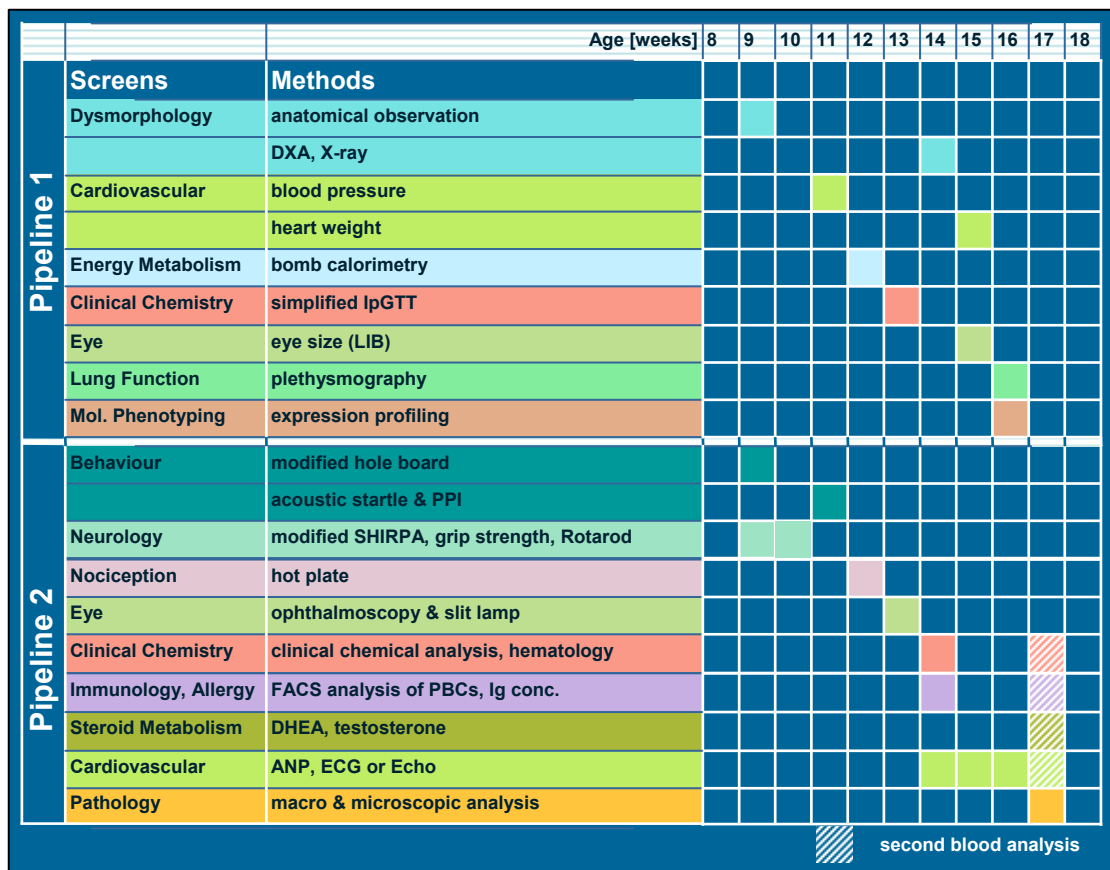


Figure 1: Workflow of the primary screen
 Explanation below, Analysis of blood-based parameters.

Deviations from our Standard operation procedure (SOP) are listed below; please take the specific number of analyzed animals from the sections of the applied screen.

2.6.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the Gsk3 α -floxed mice. As the demanded number of 80 animals (20 mice per sex per genotype) could not be delivered, the workflow was adapted to the available number of animals. Some parameters measured in the blood based screens could not be determined in all animals, as it was not possible to get the needed amount of blood from these animals. A few animals died during the primary screen and thus could not be analyzed for all parameters. The mice were about 20 weeks older compared to the regular workflow. The Behavior Screen included additional secondary tests which are outlined in more detail in Chapter 3.2. Additional experiments were also performed by the Screens Dysmorphology, Immunology and Pathology. Results are presented in the respective chapters.

2.6.3 Quality Assurance

The Quality Assurance as part of the Quality Management at the GMC consists of the following elements: standardized analyses via Standard Operating Procedures (SOP) and validation of analysis protocols by different institutions within the EUMORPHIA program, standardized data and project management supported by the central database system MausDB (Maier *et al.*, 2008) and the GMC coordination tool CoordDB as well as Quality Control and continuous training of the staff.

Coordination of the GMC's operations

The GMC management team (Core Facility) coordinates the scientific issues, logistics and administration of the GMC. The coordination software tool CoordDB supports the GMC management team in handling the incoming phenotyping requests and managing the complex phenotyping workflow of the primary and secondary screening. Besides the operational business activities the management team organizes the expansion of the screening services in collaboration with its partners. Additionally, the management arranges regular training of the staff members and the clinic's quality assurance.

Standardized Operation Procedures (SOP) and Validation of Protocols

The GMC developed a set of SOPs which cover all steps from mouse import and handling to phenotyping and data analysis. These SOPs are strictly followed during the whole screening process in the GMC and all procedures are documented.

The GMC is one of the major partners of the EUMODIC consortium that emerged from the EUMORPHIA program (Brown *et al.*, 2005), a consortium for the selection, establishment, and standardization of phenotyping protocols for mice as models for human diseases and for mouse husbandry. Cross-validation of protocols by EUMORPHIA is performed by the different institutions. A collection of the protocols (EMPreSS) is posted on the EUMORPHIA web site at (<http://www.eumorphia.org/EMPreSS/> Mallon *et al.*, 2008).

Central Database System

Another tool for quality assessment is the central database system which ensures full traceability of samples and documentation of all data. All mouse data is entered into the system (e.g. date of birth, sex, cage) and all screening results linked to the corresponding SOP as well as any changes of the mouse conditions are immediately put in.

Quality Control

In addition to routinely screen-specific quality control tests, control animals of selected strains (e.g. C57BL/6 and C3HeB/FeJ) are analyzed through the standard protocol for all phenotypes at regular intervals. This data is reviewed by the coordination team.

A tissue archive has been established for the storage of tail and blood plasma samples taken from all mice that have ever been analyzed in the GMC. The tail clips can be used for post-hoc genotyping in case of doubtful genotype information. The sanitary status of every mouse completing the screening can be tested by means of these plasma samples.

Continuous Training

Regularly specific training courses are held at the GMC. Specialists are invited to give lectures and to offer practical training at special days. Staff training is documented and maintained by the management team.

2.7 Statistical Analysis of Data

If not otherwise stated, data was analyzed by ANOVA or Student's t-test (In the t-test, data of males and females was analyzed separately). 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. Raw data are available in Excel sheets on request.

2.8 References

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Abbreviations and Wording

Gsk3 α	Glycogen synthase kinase 3 α
GMC	German Mouse Clinic
IVC	individually ventilated cage
KD	knock down
FELASA	F ederation 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
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 grip strength Rotarod
Eye	assessment of morphological alterations of the eye	funduscopy laser interference biometry slit lamp biomicroscopy
Nociception	detection of altered pain response	hot plate assay
Energy Metabolism	measurement of altered body weight regulation, body temperature and energy balance	bomb calorimetry
Clinical Chemistry and Hematology	determination of clinical-chemical and hematological parameters in blood glucose tolerance	blood autoanalyzer, ABC-animal blood counter simplified IpGTT
Steroid Metabolism	analysis of steroid hormones in blood plasma: testosterone and DHEA	ELISA
Immunology	analysis of peripheral blood samples for immunological parameters	flow cytometry, Multiplex Bead Array
Allergy	analysis of total plasma IgE	ELISA
Cardiovascular	assessment of functional cardio-vascular parameters analysis of plasma ANP	non-invasive tail-cuff blood pressure measurement, surface limb ECG / Echo, heart weight, ELISA
Lung Function	assessment of alterations in breathing patterns	whole body plethysmography (Buxco®)
Molecular Phenotyping	RNA expression profiling	DNA-chip technology
Pathology	microscopic and macroscopic examination	histology, immunochemistry

3 Specific part

3.1 Dysmorphology, Bone and Cartilage

3.1.1 Introduction

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. The aim of the screen is to establish mouse models for human skeletal diseases like osteoporosis (McLean & Olsen, 2001; Rosen *et al.*, 2001), scoliosis (Giampietro *et al.*, 2003), limb defects (Mariani & Martin, 2003), osteogenesis imperfecta (Rauch & Glorieux, 2004; Chipman *et al.*, 1993) or osteoarthritis (Abe *et al.*, 2006). We adapted the successful dysmorphological screening protocol from the Munich ENU-Mutagenesis Screen (Hrabé de Angelis *et al.* 2000) for use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the Mammalian Phenotype Ontology wording (www.informatics.jax.org/searches/MP_form.shtml). Further tests for defects in bone development and homeostasis were taken over from human diagnosis, and were adapted for the use in mice analysis. Such tests include: X-ray analysis, bone densitometry, and, in special cases, micro computed tomography. Detailed protocols for screening for bone and cartilage phenotypes in mice are described in Fuchs *et al.* (2006).

3.1.2 Summary

In the morphological investigation via visual inspection and X-ray analysis no genotype-specific differences were found. In the dual-energy X-ray absorptiometry (DXA) sBMD (BMD related to the body weight) was significantly increased in male mutants with the same tendency in females. We confirmed reduced body weight and body length in male mutants. The differences in bone parameters might be due to the smaller body size and reduced body weight of male mutants.

3.1.3 Mice

Fifteen male (7 controls, 8 mutants) and 14 female (7 controls, 7 mutants) mice were analyzed by morphological inspection at the age of 29-30 weeks. 34-35-week-old mutants (13 animals) and controls (14 animals) entered the bone density and X-ray analysis.

3.1.4 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 five weeks, i.e. when the mice entered the facility, the general physical condition and health were checked,
2. at the age of nine weeks, a morphological observation as a whole-body checkup was performed; and

- at the age of 14 weeks, X-ray analysis and bone densitometry were performed.

Morphological Observation

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

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

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).

3.1.5 Results

In the morphological investigation via visual inspection and X-ray analysis no genotype-specific differences were found (Tables 3 and 4). In the Clickbox test (Table 5) to test the hearing ability of the mice, we observed a retarded or

no reaction in mutants and controls. This is not an abnormal phenotype because the C57BL/6 strain exhibits severe sensorineural hearing loss at an early age (Turner *et al.*, 2005; The Jackson Laboratory: <http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml>). In the bone densitometry using DXA analysis (Table 6), sBMD (BMD related to the body weight) was significantly increased in male mutants with the same tendency in females. We confirmed reduced body weight and body length in male mutants. Lean mass was significantly decreased in male mutants.

Compared to the Gsk3 β mutant mouse line the observed differences were similar, but seemed to be less pronounced as we observed no significant differences between female Gsk3 α mutants and controls. We have to keep in mind, that the number of female Gsk3 α mutants (n=5 per group) for DXA analysis was very small which makes it difficult to reach significance.

3.1.6 Discussion

Gsk3 is a key molecule of the canonical Wnt/ β -catenin pathway. The canonical Wnt/ β -catenin pathway plays an important role in bone metabolism. Signaling through the canonical Wnt/ β -catenin pathway increases bone mass through a number of mechanisms including renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis (Krishnan *et al.*, 2006). By increasing the ratio of osteoprotegerin (OPG) to RANKL, β -catenin represses osteoclastogenesis. Liu *et al.* (2007) revealed recently the genetic requirement for Gsk3 β in midline development. Conditional Gsk3 β mutants showed cleft palate, incomplete fusion of the ribs at the midline and bifid sternum as well as delayed sternal ossification. In the literature it has been described that inhibition of Gsk3 α/β with lithium chloride or a specific synthesized dual inhibitor increased bone formation and bone mass (Clement-Lacroix *et al.*, 2005; Kulkarni *et al.*, 2006). In the overview it was mentioned that the used Nestin-cre mice express Cre recombinase in neuronal and glia cell precursors under the control of the rat Nestin (Nes) promoter and enhancer (Tronche *et al.*, 1999). The mice show strong expression of Cre recombinase in the central and peripheral nervous system and occasional positive cells in heart and kidney. The expression is present from embryonic day 11. In adult mice strong Cre activity was detected in the muscle tissue comparable to that in the brain. In heart and kidney there was some activity detected to a lesser content. *It is not clear if Gsk3 α was also silenced in bone of mutant mice.* The differences in bone parameters might be due to the smaller body size and reduced body weight of mutants. The sex differences we observed are common in many mouse strains, and thus are not abnormal (unpublished data).

3.1.7 Suggestions

For further analysis of the observed differences in bone parameters additional data should be generated by pQCT. The pQCT technology enables real volumetric bone density measurements in g/cm³ and it separates cortical and trabecular bone compartments and thus can monitor metabolic changes very quickly and precisely.

3.1.8 References

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Abbreviations

BMC	bone mineral content (excluding skull)
BMD	bone mineral density (excluding skull)
DXA	dual-energy X-ray absorptiometry
μ CT	micro computed tomography
pQCT	peripheral quantitative computed tomography
sBMD	specific bone mineral density (excluding skull)

Table 3: Results from the morphological inspection (29-30-week old mice)				
Parameter	Male		Female	
	Control	Mutant	Control	Mutant
Body appearance				
normal	7	8	7	7
Craniofacial / head morphology				
normal	7	8	7	7
Limbs				
normal	7	8	7	7
Digits				
normal	7	8	7	7
Tail				
normal	7	8	7	7
Eyes				
normal	7	8	7	7
Ears				
normal	7	8	7	7
Teeth				
normal	7	8	7	7
Vibrissae				
normal	7	8	7	7
Coat appearance				
normal	7	8	7	7
Coat / hair growth				
normal	7	8	7	7
Coat / hair texture				
normal	7	8	7	7
Hair follicle structure / orientation				
normal	7	8	7	7
Skin pigmentation				
normal	7	8	7	7
Skin condition / texture				
normal	7	8	7	7
Muscle morphology				
normal	7	8	7	7
Seizures / epilepsy				
no	7	8	7	7
Motor capabilities / coordination				
normal	7	8	7	7
Movement				
normal	7	8	7	7
Eating / drinking behavior				
normal	7	8	7	7
Respiratory system				

normal	7	8	7	7
Reproductive / urinary system				
normal	7	8	7	7
Other abnormalities				
no	7	8	7	7
Animals analyzed	7	8	7	7

Table 4: Results from the X-ray analysis (34-35-week old mice)

Parameter	Male		Female	
	Control	Mutant	Control	Mutant
Skull				
normal	7	8	7	5
Mandibles				
normal	7	8	7	5
Maxilla				
normal	7	8	7	5
Teeth				
normal	7	8	7	5
Orbit				
normal	7	8	7	5
Spine				
normal	7	8	7	5
Number of cervical vertebrae				
normal (7)	7	8	7	5
Number of thoracic vertebrae				
normal (13)	7	8	7	5
Number of lumbar vertebrae				
normal (6)	7	8	7	5
Number of sacral vertebrae				
normal (4)	7	8	7	5
Number of caudal vertebrae				
normal	7	8	7	5
Vertebrae				
normal	7	8	7	5
Ribs				
normal (26)	6	8	6	5
27	1	-	1	-
Scapulas				
normal	7	8	7	5
Clavicle				
normal	7	8	7	5
Pelvis				
normal	7	7	7	5
partial deformed	-	1	-	-

Femur				
normal	7	8	7	5
Tibia				
normal	7	8	7	5
Fibula				
normal	7	8	7	5
Humerus				
normal	7	8	7	5
Ulna				
normal	7	8	7	5
Radius				
normal	7	8	7	5
Digits				
normal (20)	7	8	7	5
Joints				
normal	7	8	7	5
Animals analyzed	7	8	7	5

Table 5: Results from clickbox test (hearing test; 29-30-week old mice)

Phenotype	Male		Female	
	Control	Mutant	Control	Mutant
0	-	3	2	4
1	2	4	3	-
2	1	-	-	1
3	4	1	2	2
4	-	-	-	-
5	-	-	-	-
Mean Score	2.29	0.88	1.29	1.14

Kruskal-Wallis ANOVA on Ranks: n.s.

0: no reaction at all,
1: very slow reaction,
2: retarded reaction,
3: normal reaction,
4: strong reaction,
5: extremely excited

Table 6: Bone- and weight-related quantitative parameters

(data presented as mean \pm standard error of mean)

34-35-week old mice	Control		Mutant		C ~ M		ANOVA		
	Male	Female	Male	Female	Male	Female	<i>p</i> – value genotype	<i>p</i> – value sex	<i>p</i> – value interaction
	(n=7)	(n=7)	(n=8)	(n=5)	<i>p</i> – value	<i>p</i> – value			
BMD [mg/cm ²]	56 \pm 1	49 \pm 2	52 \pm 2	50 \pm 3	n.a.	n.a.	n.s.	< 0.05	n.s.
sBMD [10 ⁻³ x cm ⁻²]	1.74 \pm 0.07	2.17 \pm 0.08	2.03 \uparrow \pm 0.06	2.34 \pm 0.15	< 0.01	n.s.	< 0.05	< 0.001	n.s.
BMC [mg]	416 \pm 67	308 \pm 46	255 \pm 35	257 \pm 64	n.a.	n.a.	n.s.	n.s.	n.s.
Bone Content [%]	1.25 \pm 0.17	1.36 \pm 0.19	0.99 \pm 0.13	1.16 \pm 0.24	n.a.	n.a.	n.s.	n.s.	n.s.
Body Length [cm]	10.21 \pm 0.15	9.57 \pm 0.07	9.63 \downarrow \pm 0.08	9.30 \pm 0.12	< 0.01	n.s.	< 0.001	< 0.001	n.s.
Body Weight [g]	32.70 \pm 1.18	22.54 \pm 0.75	25.50 \downarrow \pm 1.03	21.42 \pm 1.02	< 0.001	n.s.	< 0.001	< 0.0001	n.s.
Fat mass [units]	7.93 \pm 1.40	3.56 \pm 0.86	4.63 \pm 0.87	3.56 \pm 0.86	n.a.	n.a.	n.s.	< 0.05	n.s.
Fat Content [units x 100/g]	23.58 \pm 3.56	15.39 \pm 3.48	17.69 \pm 3.16	14.67 \pm 3.74	n.a.	n.a.	n.s.	n.s.	n.s.
Lean mass [units]	18.14 \pm 0.64	13.48 \pm 0.67	15.22 \downarrow \pm 0.78	13.02 \pm 0.34	< 0.05	n.s.	< 0.05	< 0.0001	n.s.
Lean Content [units x 100/g]	56.14 \pm 3.40	60.14 \pm 3.25	60.01 \pm 2.83	61.40 \pm 3.69	n.a.	n.a.	n.s.	n.s.	n.s.

3.2 Behavior Screen

3.2.1 Introduction

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

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

As Gsk3 mutants may serve as models for mood disorders since Lithium chloride, one of the primary drugs to treat bipolar mood disorders, inhibits Gsk3 (Hashimoto *et al.*, 2003), we previously analysed Gsk3 β x Nestin-Cre kd mice additionally for their stress response in the **Forced Swim Test**. This test is considered to assess depression-like behaviour (Porsolt *et al.*, 1977; for review see Petit-Demouliere *et al.*, 2005). To allow for a comparison of the behavioural phenotypes of Gsk3 β x Nestin-Cre kd and Gsk3 α x Nestin-Cre kd mice, we also analysed the Gsk3 α x Nestin-Cre kd mice in the Forced Swim Test as a secondary screen.

3.2.2 Summary

The analysis revealed an increase in anxiety-related behavior in a novel environment. Genotypic differences in body weight in this mouse line may have contributed to the behavioral outcome in all tests.

3.2.3 Mice

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype. Three days before the mHB test, an object (metal cube) was placed into the home cage and removed one day before testing.

In this screen, 29 female mice (14 control, 15 mutants) and 31 male mice (15 control, 16 mutants) were available for analysis. The mHB was performed at the age of 27-29 weeks, and the Forced Swim Test was performed at the age of 30-32 weeks. Due to time constraints because of the GMC work-

flow, the Forced Swim Test could only be performed on 31 animals (16 mutants, 15 controls).

3.2.4 Material and Methods

The modified Hole Board test (Ohl *et al.*, 2001) was carried out as previously described (Kallnik *et al.*, 2007). The test apparatus consisted of a test arena (100 x 50 cm), in the middle of which a board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus representing the central area of the test arena as an open field. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box (See Ohl *et al.*, 2001). Both box and board were made of dark grey PVC. All lids were closed before the start of a trial. For each trial, an unfamiliar object (a blue plastic tube lid, similar in size to the metal cube) and the familiar object (metal cube) were placed into the test arena with a distance of 2 cm between them. The illumination levels were set at approximately 150 lux in the corners and 200 lux in the middle of the test arena.

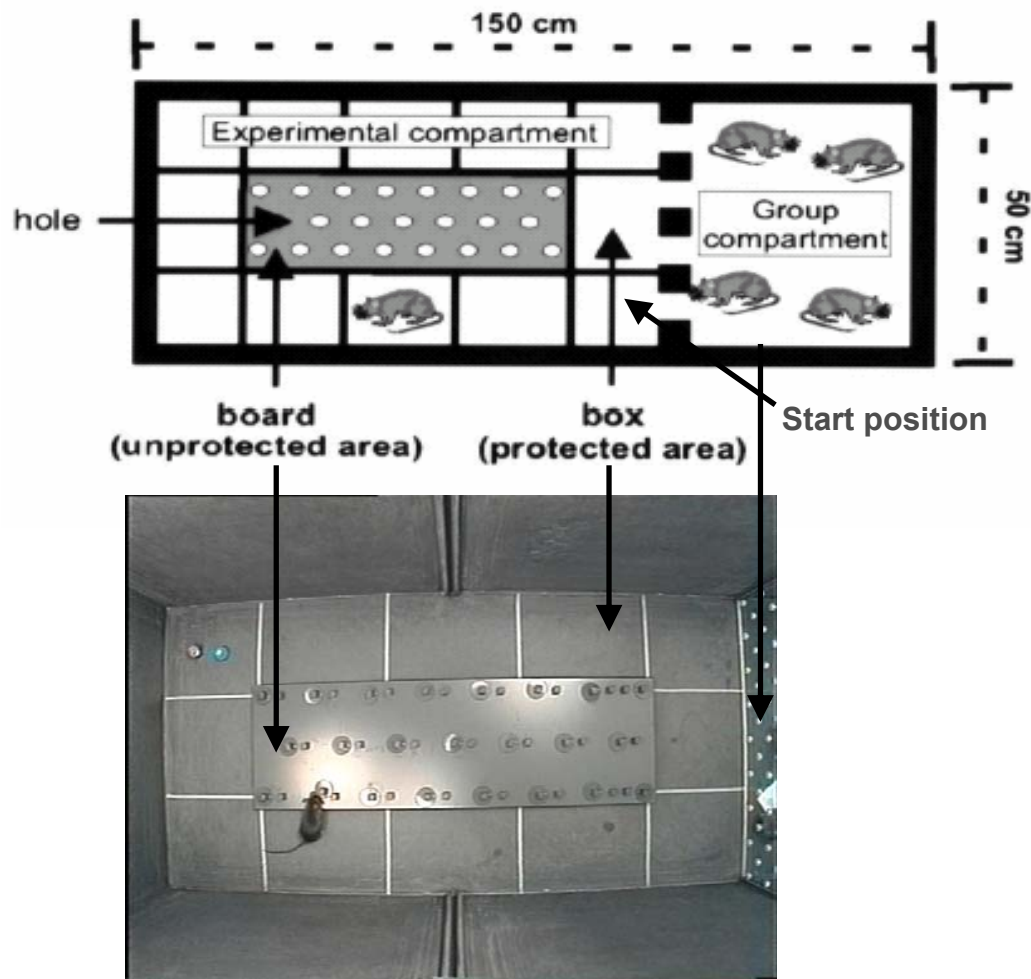


Figure 2: Test arena for modified Hole Board test.

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.

Forced Swimming

The forced swimming procedure was adapted from Ebner *et al.* (2002). The forced swimming apparatus consisted of a cylindrical 10 l glass tank (24.5 cm in diameter), filled to a depth of 20 cm with water (25 ± 1 °C). A trained observer recorded the animals behavior in moderate lighting conditions (ca. 30 lx) for 6 min with a hand-held computer, monitoring the following behaviors: (1) struggling, defined as movements during which the forelimbs broke the water's surface; (2) swimming, defined as movement of the animal induced by movements of the fore and hind limbs without breaking the water surface, and (3) floating, defined as the behavior during which the animal used limb movement just to keep its equilibrium without any movement of the trunk. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen). After each trial, firstly, the mouse tested was dried with a tissue and put in a new cage, secondly, the water was renewed before testing was continued.

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

3.2.5 Results

Modified Hole Board

Behavioral analysis of spontaneous activity in a novel environment, as measured by the modified Hole Board test, revealed several behavioral alterations in mutants. Mutants of both sexes moved with a higher mean velocity, and showed a reduction in angular velocity, mean turn angles and meandering, suggesting a straighter path shape (Table 9).

Mutants started exploration of the novel environment earlier indicated by reduced line crossing latency, and reared more in the experimental arena. Mutants explored the unprotected, exposed area of the board less, kept closer to the wall of the experimental compartment, spent more time in group contact and groomed more frequently (Table 8).

The pattern of behavioural alterations is summarized in Table 7.

Table 7: Evaluation of the behavioral phenotype
Behaviors which are considered affected in mutants due to the pattern of significantly altered parameters are marked in red.

Behavior	Parameters
forward locomotion	distance moved, line crossings (latency, frequency) , path shape
speed of movement	velocity (mean, maximum, angular)
exploration	vertical: rearings (box, board; latency, frequency) horizontal: holes (latency, frequency) , objects (novel, familiar; latency, frequency, duration)
risk assessment	stretched attends (latency, frequency)
anxiety-related	board entries (latency, frequency, duration, maximum duration) ; distance to the wall
grooming	grooming (latency, frequency , duration)
defecation	boli (latency, frequency)
social affinity	exploration of the partition (latency, frequency, duration)
object memory	object recognition index

Forced Swim Test

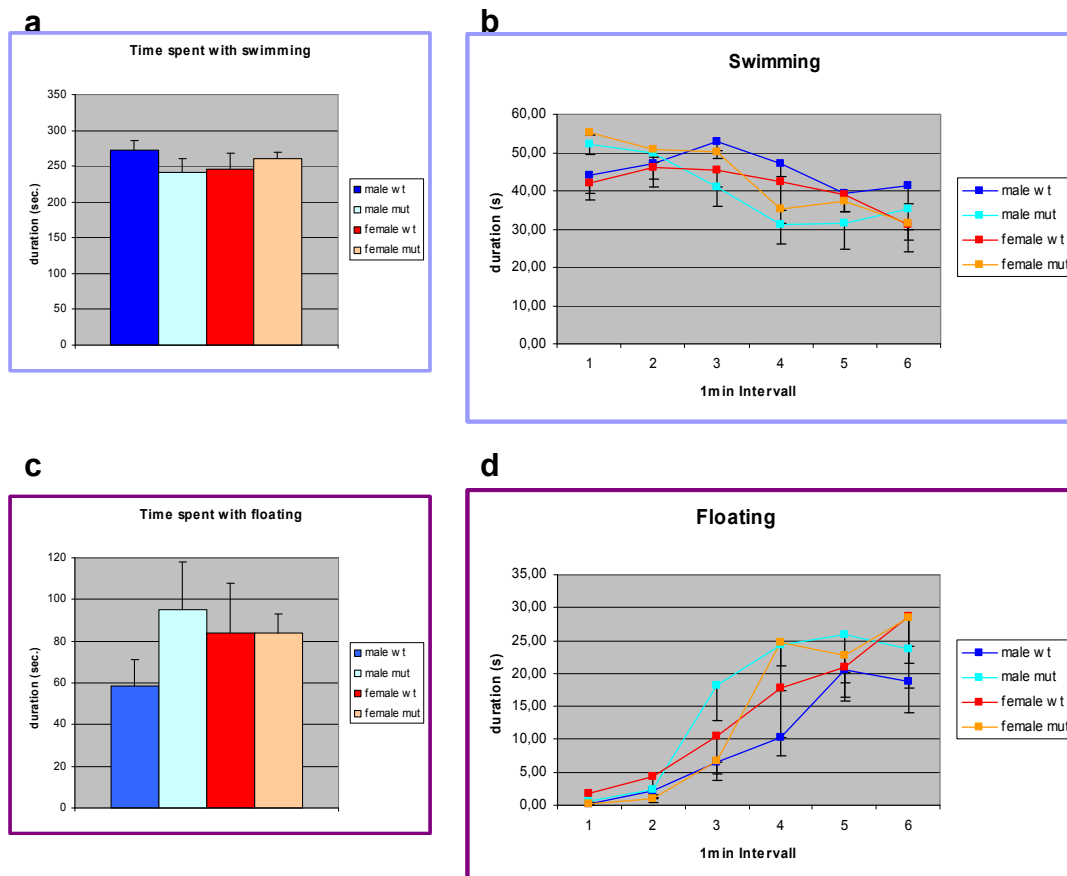


Figure 3: Forced Swimming in Gsk3 α x Nestin-Cre knock down mice.
Time spent swimming and floating in total (a, c) and in 1-min intervals (b,d) in Gsk3 α x Nestin-Cre control males (male wt) and females (female wt) as well as Gsk3 α x Nestin-Cre kd males (male mut) and females (female mut).

Mutants of both sexes spent the same time swimming and floating as controls did. This was evident in total (swimming, Fig. 3a; floating, Fig. 3c) as well as in the time course of swimming (Fig. 3b) and floating (Fig. 3d). There were no significant genotype effects in any of the parameters observed in this test.

3.2.6 Discussion

The **primary behavioral observation** in the modified Hole Board demonstrated alterations in mutants of both sexes in forward locomotion, velocity, path shape, grooming, social affinity and anxiety-related behavior. This pattern most likely reflects an increased anxiety in the novel environment, reflected by (1) reduced entry and exploration of the unprotected, exposed area, the board, (2) closer proximity to the protected area, i.e. the wall, (3) more group contact and (4) increased grooming frequency. The increased mean velocity and straighter path shape might well be a consequence of increased anxiety, since more anxious animals move faster and take less time for careful exploration of the environment, which reduces the level of meandering.

It should be taken into account that differences in body size and weight (mutants were smaller) could have influenced the behavioral outcome. Compared to the behavioural phenotype of Gsk3 β x Nestin-Cre kd mice, the Gsk3 α x Nestin-Cre kd mice presented with a similar, but stronger phenotype, showing a clear increase in anxiety-related behaviour compared to a subtle one in the Gsk3 β x Nestin-Cre kd mice.

Concerning the stress response measured in the Forced Swim Test, no differences were found in mutants of either sex. This is in contrast to the Gsk3 β x Nestin-Cre kd mice, in which the females reduced swimming and increased floating behavior with time, but only half the number of animals could be tested in forced swimming (31) than in Gsk3 β mice (60). If anything, there might be a trend towards reduced swimming and increased floating behaviour with time in **male** Gsk3 α x Nestin-Cre kd mice.

Taken together, the analysis revealed an anxiety-related phenotype in Gsk3 α x Nestin-Cre kd mice, but could not reveal a conclusive depression-related phenotype. Since genotypic differences in body weight in this mouse line may have influenced the behavioral outcome in all tests, the behavioral data should be interpreted with caution.

3.2.7 References

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Table 8: Results of behavioral observation in the modified Hole Board test

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

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=15)	(n=14)	(n=16)	(n=15)	(n=29)	(n=31)			
Line crossing [frequency]	103.13 \pm 5.73	118.36 \pm 4.61	119.94 \pm 4.97	119.93 \pm 5.93	110.48 \pm 3.91	119.94 \pm 3.78	ns	ns	ns
Line crossing [latency]	3.24 \pm 0.97	1.59 \pm 0.5	1.14 \pm 0.08	0.91 \pm 0.05	2.44 \pm 0.57	1.03 \pm 0.05	ns	p<0.05	ns
Rearings in box [frequency]	22.67 \pm 1.47	24.64 \pm 1.72	28.25 \pm 1.59	24.93 \pm 1.07	23.62 \pm 1.12	26.65 \pm 1	ns	p=0.05	ns
Rearings in box [latency]	31.35 \pm 4.96	26.21 \pm 4.57	22.41 \pm 3.79	30.69 \pm 3.24	28.87 \pm 3.36	26.41 \pm 2.58	ns	ns	ns
Hole exploration [frequency]	26.07 \pm 2.87	25.21 \pm 2.2	19.19 \pm 2.55	15.27 \pm 1.92	25.66 \pm 1.8	17.29 \pm 1.62	ns	p<0.01	ns
Hole exploration [latency]	26.11 \pm 5.7	23.66 \pm 5.69	50.05 \pm 13.42	29.87 \pm 5.25	24.93 \pm 3.96	40.28 \pm 7.49	ns	ns	ns
Hole visit [frequency]	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	ns	ns	ns
Hole visit [latency]	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	ns	ns	ns
Board entry [frequency]	3.93 \pm 0.67	3.07 \pm 0.46	2.25 \pm 0.45	2 \pm 0.45	3.52 \pm 0.41	2.13 \pm 0.31	ns	p<0.01	ns
Board entry [latency]	98.33 \pm 23.44	102.56 \pm 20.72	179.55 \pm 24.16	142.13 \pm 22.91	100.37 \pm 15.44	161.44 \pm 16.76	ns	p<0.05	ns

Table 8: Results of behavioral observation in the modified Hole Board testData are presented as mean \pm standard error of the mean.

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=15)	(n=14)	(n=16)	(n=15)	(n=29)	(n=31)			
Board entry [total duration %]	5.94 \pm 1.1	5.37 \pm 0.96	3.66 \pm 0.68	2.91 \pm 0.62	5.66 \pm 0.72	3.3 \pm 0.46	ns	p<0.01	ns
Rearing on board [frequency]	0 \pm 0	0.29 \pm 0.13	0.13 \pm 0.13	0 \pm 0	0.14 \pm 0.07	0.06 \pm 0.06	ns	ns	p<0.05
Rearing on board [latency]	300 \pm 0	270.66 \pm 13.54	295.47 \pm 4.53	300 \pm 0	285.83 \pm 6.98	297.66 \pm 2.34	p=0.07	p=0.07	p<0.05
Risk assessment [frequency]	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	ns	ns	ns
Risk assessment [latency]	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	ns	ns	ns
Group contact [frequency]	18.47 \pm 1.33	18.36 \pm 1.87	18.94 \pm 1.29	22.2 \pm 1.56	18.41 \pm 1.11	20.52 \pm 1.03	ns	ns	ns
Group contact [latency]	15.59 \pm 4.02	14.12 \pm 1.83	15.59 \pm 2.86	17.79 \pm 2.15	14.88 \pm 2.23	16.66 \pm 1.79	ns	ns	ns
Group contact [total duration %]	12.42 \pm 1.57	13.19 \pm 1.59	14.1 \pm 0.86	16.81 \pm 0.76	12.79 \pm 1.1	15.41 \pm 0.62	ns	p<0.05	ns
Grooming [frequency]	2 \pm 0.48	2.43 \pm 0.44	2.81 \pm 0.57	4.33 \pm 0.84	2.21 \pm 0.32	3.55 \pm 0.51	ns	p<0.05	ns
Grooming [latency]	195.83 \pm 23.89	185.73 \pm 18.45	153.15 \pm 15.64	173.15 \pm 16.58	190.96 \pm 15	162.83 \pm 11.33	ns	ns	ns

Table 8: Results of behavioral observation in the modified Hole Board testData are presented as mean \pm standard error of the mean.

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=15)	(n=14)	(n=16)	(n=15)	(n=29)	(n=31)			
Grooming [total duration %]	1.68 \pm 0.41	1.7 \pm 0.36	2.33 \pm 0.32	2.23 \pm 0.32	1.69 \pm 0.27	2.28 \pm 0.22	ns	ns	ns
Defecation [frequency]	0.27 \pm 0.15	0.21 \pm 0.11	0.06 \pm 0.06	0.13 \pm 0.09	0.24 \pm 0.09	0.1 \pm 0.05	ns	ns	ns
Defecation [latency]	253.45 \pm 27.04	265.63 \pm 22.24	282.46 \pm 17.54	261.41 \pm 26.3	259.33 \pm 17.36	272.27 \pm 15.47	ns	ns	ns
Unfamiliar object exploration [frequency]	7.6 \pm 0.76	5.93 \pm 0.47	6.5 \pm 0.61	6.4 \pm 0.65	6.79 \pm 0.47	6.45 \pm 0.44	ns	ns	ns
Familiar object exploration [frequency]	6.07 \pm 0.64	6.93 \pm 0.62	7.31 \pm 0.72	5.87 \pm 0.58	6.48 \pm 0.44	6.61 \pm 0.48	ns	ns	ns
Unfamiliar object exploration [latency]	23.08 \pm 4.53	38.19 \pm 14.5	22.04 \pm 3.69	37.12 \pm 10.97	30.38 \pm 7.38	29.34 \pm 5.71	ns	ns	ns
Familiar object exploration [latency]	33.19 \pm 9.36	32.02 \pm 12.4	20.78 \pm 5.91	36.13 \pm 9.42	32.63 \pm 7.56	28.2 \pm 5.57	ns	ns	ns
Unfamiliar object exploration [total duration %]	1.51 \pm 0.48	1.32 \pm 0.24	1 \pm 0.09	0.96 \pm 0.1	1.42 \pm 0.27	0.98 \pm 0.07	ns	ns	ns

Table 8: Results of behavioral observation in the modified Hole Board testData are presented as mean \pm standard error of the mean.

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=15)	(n=14)	(n=16)	(n=15)	(n=29)	(n=31)			
Familiar object exploration [total duration %]	0.67 \pm 0.05	0.77 \pm 0.08	0.79 \pm 0.07	0.77 \pm 0.11	0.72 \pm 0.05	0.78 \pm 0.06	ns	ns	ns
Object Index	0.23 \pm 0.07	0.18 \pm 0.07	0.11 \pm 0.05	0.1 \pm 0.09	0.21 \pm 0.05	0.1 \pm 0.05	ns	ns	ns

Table 9: Video-tracking results regarding locomotor behavior

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

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=15)	(n=14)	(n=16)	(n=15)	(n=29)	(n=31)			
Total Distance Moved [cm]	2523.85 \pm 123.01	2823.84 \pm 96.64	2787.57 \pm 105.49	2773.52 \pm 128.79	2668.67 \pm 82.52	2780.77 \pm 81.36	ns	ns	ns
Mean Velocity [cm/sec]	16.41 \pm 0.6	17.92 \pm 0.52	18.32 \pm 0.45	18.25 \pm 0.49	17.14 \pm 0.42	18.29 \pm 0.32	ns	p<0.05	ns
Maximum Velocity [cm/sec]	48.34 \pm 2.41	51 \pm 2.25	52.21 \pm 1.79	52.13 \pm 1.55	49.62 \pm 1.64	52.17 \pm 1.17	ns	ns	ns
Turns [Frequency]	1503.4 \pm 44.91	1620.29 \pm 35.48	1561.25 \pm 43.67	1561.33 \pm 54.4	1559.83 \pm 30.43	1561.29 \pm 34.06	ns	ns	ns
Mean Turn Angle [degrees]	25.34 \pm 0.82	23.15 \pm 0.58	23.15 \pm 0.54	21.67 \pm 0.62	24.28 \pm 0.54	22.43 \pm 0.42	p<0.01	p<0.01	ns
Angular Velocity [degrees/sec.]	154.49 \pm 3.08	149.28 \pm 3.76	144.16 \pm 2.01	135.51 \pm 2	151.97 \pm 2.42	139.97 \pm 1.6	p<0.05	p<0.001	ns
Absolute Meander [degrees/sec.]	19.09 \pm 0.71	17.15 \pm 0.48	17.12 \pm 0.45	16.15 \pm 0.56	18.15 \pm 0.46	16.65 \pm 0.36	p<0.05	p<0.05	ns
Board entry [maximum duration. sec.]	7.29 \pm 1.15	8.85 \pm 1.76	6.15 \pm 1.07	4.91 \pm 0.91	8.04 \pm 1.03	5.55 \pm 0.7	ns	p<0.05	ns
Mean distance to wall [cm]	6.6 \pm 0.25	6.35 \pm 0.24	5.79 \pm 0.25	5.39 \pm 0.17	6.48 \pm 0.17	5.6 \pm 0.15	ns	p<0.001	ns
Mean distance to board [cm]	8.73 \pm 0.2	8.89 \pm 0.23	9.32 \pm 0.22	9.77 \pm 0.16	8.8 \pm 0.15	9.54 \pm 0.14	ns	p<0.001	ns

3.3 Neurology Screen

3.3.1 Introduction

Neurological dysfunction results in a wide variety of disorders ranging from impaired movement to severe mental illness. Studying the neurobehavioral phenotype of mutant mice is a powerful tool to understand the neural basis of behavior and the pathophysiology of neurological disorders. Comparison of the mouse and human brain transcriptomes shows a good correlation for highly expressed genes in both transcript identity and abundance (Fougerousse *et al.*, 2000). Therefore, screening of mice with respect to neurological disorders potentially offers an understanding of etiology and pathogenesis of the human nervous system (Hafezparast *et al.*, 2002).

The 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.*, 1997). Dependent upon results of this primary screen and due to specific questions, additional tests can be carried out for further assessment of neurological functions in a hierarchical way (Schneider *et al.*, 2006).

3.3.2 Summary

We examined the mice using designed 23 SHIRPA test parameters (See web page: http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html) to detect phenotypic differences between knockout and control mice. The test parameters contribute to an overall assessment of muscle, motor neuron, spinocerebellar, sensory and autonomic functions. The primary neurological screen is focused on investigating neurological signs to determine the neurological functioning of a mouse. We also examine forelimb grip force for the assessment of muscular function and test the mice with the accelerating rotarod for the evaluation of motor coordination.

The comparison of *Gsk3 α* mutant mice to controls showed no altered SHIRPA test parameters. Body weight was significantly reduced in the mutants. There were no genotype-related differences in grip strength or rotarod performance.

3.3.3 Mice

Eight *Gsk3 α* mutant and eight male control mice as well as 7 female *Gsk3 β* mutant and 8 female control mice entered the neurological laboratory in October 2007 at the age of 30 weeks. All animals were fed *ad libitum* for a period of two weeks during their stay in the neurological screen.

3.3.4 Material and Methods

Primary screening 1: modified SHIRPA protocol

Assessment of each animal at age 10 weeks began with observation of undisturbed behavior (*Viewing Jar Behavior*) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex

box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. Locomotor activity and motor behavior within this area was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes (*Behavior recorded on or above the arena*). Measurements were completed with the recording of body weight. The last part of the primary screen also involved the analysis of righting reflex, and contact righting reflex. A glass cylinder (35 mm diameter, 135 mm length) was used for testing of the contact righting reflex. Throughout the entire procedure, abnormal behavior, biting, defecation, and vocalization were recorded. Between testing of each mouse, fecal pellets and urination were removed from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing (Schneider *et al.*, 2006).

Primary screening 2: grip strength

The grip strength meter system determines the grip strength of the limbs, i.e. muscle strength of a mouse. The device exploits the tendency of a mouse to grasp a horizontal metal grid while being pulled by its tail. During the trial set-up, the mouse grasps a special adjustable grid mounted on a force sensor. The mouse is allowed to catch the grid with either 2 or 4 paws. Three trials were undertaken for each mouse and measurement within one minute. The mean values are used to represent the grip strength of a mouse.

All experimental equipment was thoroughly cleaned with Pursept-A and dried prior subsequent tests. Values were presented as means \pm standard error of mean (SEM).

Statistical analysis of the grip strength trial results. Grip strength trial results are compared between genotypes, controlling for the effects of sex and weight, by fitting linear mixed effect models (Pinheiro and Bates, 2000). A linear mixed effect model is a modified analysis of variance/covariance approach allowing for dependencies in the data. In our case, dependencies arise from repeated trials within each mouse. Genotype, sex and weight are modeled as fixed effects. Mouse-specific intercepts are modeled by including the intercept as random effect. Interaction effects are tested for and included in the model if they show a significant contribution. A serial dependency on the trial number can be tested by including the trial number as random effect with an autoregressive correlation structure. Model fitting is performed by the nlme-Package in the open-source statistical software R, a close relative of S-PLUS (The R Project for Statistical Computing, 2004). The p-value for the genotype effect within the specific model found for the data indicates the significance of the statistical test of interest; a confidence interval for the genotype effect can also be extracted.

Primary Screening: Rotarod test

The TSE-RotaRod 3375 apparatus (Accelerating Model, TSE, Bad Homburg) was used to measure fore limb and hind limb motor coordination, balance and motor learning ability (Jones and Roberts, 1968). The machine was set up in an environment with minimal stimuli such as noise and movement.



Figure 4: The rotarod apparatus

The rotarod device is equipped with a computer controlled motor-driven rotating rod. The unit consists of a rotating spindle and five individual lanes for each mouse (Fig. 4). The software allows pre-programming of session protocols with varying rotational speeds. Infrared beams are used to detect when a mouse falls onto the grids beneath the rotarod.

In general, the mouse is placed perpendicular to the axis of rotation, with head facing the direction of the rotation. All mice were placed on the Rotarod at an accelerating speed from 4 to 40 rpm for 300 sec with 20 min between each trial. In motor coordination testing, mice were given four trials at the accelerating speed at one day. The mean latency to fall off the Rotarod during the trials was recorded and used in subsequent analysis. Before the start of the first trial, mice were weighed.

Statistical analysis of the Rotarod performance results: The Rotarod data contain dependencies, which are more complex than the grip strength data. Repeated measurements arise from four different trials with a break in between. To compare the performance results between genotypes, linear mixed-effect models are fitted, that allow for the dependencies of genotype and trial and for the effects of sex and weight. The latter are modeled as fixed effects. Interaction effects are considered and included in the model, if necessary.

In each model, the parameter of interest is the coefficient of the genotype effect. A significance test or a confidence interval for this coefficient can be extracted from the model fitted.

3.3.5 Parameters

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

3.3.6 Results

The only significant finding of Gsk3 α mice was the very big difference in body weight. All **SHIRPA parameters** were without significant findings (Tables 10-13).

Since we are especially interested in mitochondrial functions we also measure serum lactate levels in collaboration with the Clinical-Chemistry screen. There were no alterations detected within these mice either (Table 14).

Comparing the **grip strength** revealed no genotype-related differences in mutants of both sexes in both 2- and 4-paw measurement (Fig. 5).

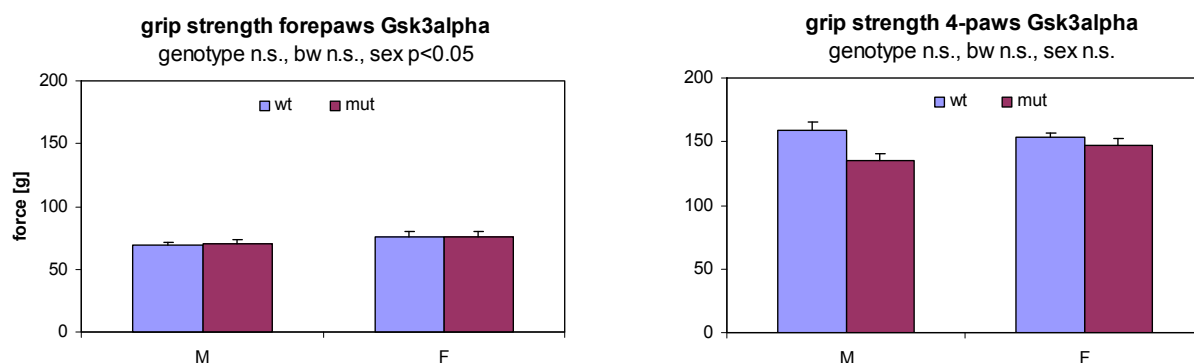


Figure 5: Results from grip strength testing

In addition, the *Gsk3α* mice were tested for differences in motor coordination/balance with the **accelerating rotarod** (Fig. 6).

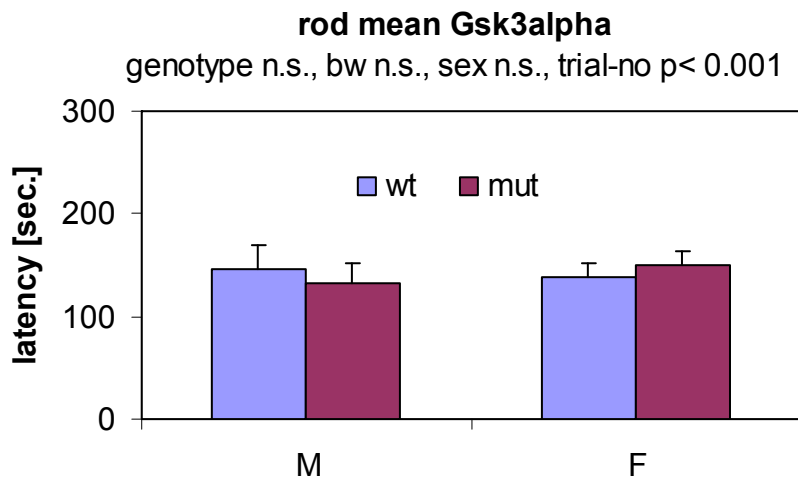


Figure 6: Results from rotarod testing: mean values.

Here were no differences detected with this test either. The only significance arose from trial number indicating the usual improvement of performance over trials (Fig. 7) that was not different between the groups.

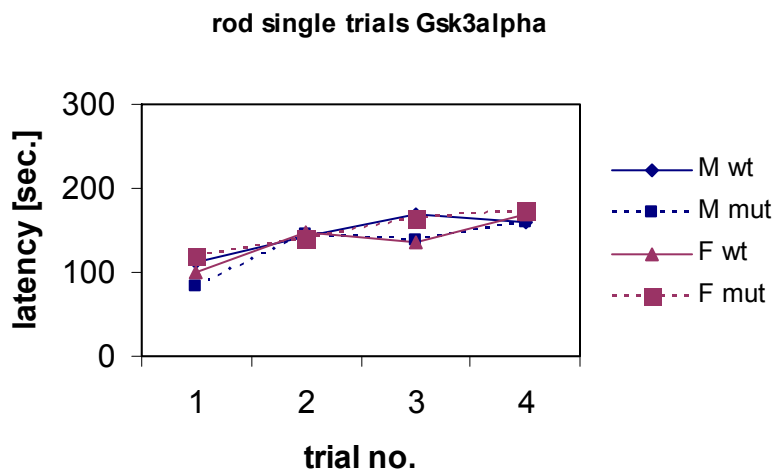


Figure 7: Depiction of rotarod single trial results.

3.3.7 Discussion

In our neurological screening, male and female *Gsk3α* knockdown mice showed no alterations in the modified SHIRPA protocol. Basic neurological functions seem not to be affected in general by the absence/reduction of the *Gsk3α* protein, at least under normal conditions.

In contrast to the *Gsk3β* mice, we also did not find a reduction of grip force in mutants. Body weight reduction was less pronounced in the mice ana-

lyzed here, too, so the influence of weight on grip and rotarod performance was also lower.

In the mutants analyzed, the protein should be downregulated in neuronal tissue as well as in muscle, heart and kidney. *Gsk3 α* and *Gsk3 β* had been described to play a role in many regulatory processes. *Gsk3 α* knockout mice published in 2007 displayed enhanced glucose and insulin sensitivity accompanied by reduced fat mass and are suggested as a model for type II diabetes (MacAulay *et al.*, 2007).

The knockdown mice analyzed here are thought to be a model for mood disorders since Gsk3 can be inhibited by lithium chloride (provider info) and *Gsk3 α* has been shown to be involved in Alzheimer's disease (AD) in phosphorylation of Tau protein (Phiel *et al.*, 2003).

However, in these mice no phenotype could be detected in the neurological analysis. Some of the functions of the protein might be compensated by the other protein isoform or the critical threshold for malfunction is beyond the downregulation of the protein here. In addition, more complex behavioural alterations affected by AD would not necessarily show up in the analysis of basic neurological functions.

3.3.8 References

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Abbreviations

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

Table 10: Recording of body weight

Data are presented as mean ± standard error of mean.

Parameter	Male			Female			both
	Control (n=8)	Mutant (n=8)	<i>p</i> -value	Control (n=7)	Mutant (n=8)	<i>p</i> -value	<i>p</i> -value
Body Weight [g]	27.4±1.2	23.1±2.2	<i>n.s.</i>	27.0±2.0	21.2±0.9	<0.05	<0.01

Table 11: Behavior recorded in viewing jarStatistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female			both
	Control (n=8)	Mutant (n=8)	<i>p</i> -value	Control (n=7)	Mutant (n=8)	<i>p</i> -value	<i>p</i> -value
Body Position							
Inactive	0	0		0	0		
Active	8	8		7	8		
Excessive Activity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Tremor							
Absent	8	8		7	8		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Palpebral closure							
Eyes open	8	8		7	8		
Eyes closed	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Coat Appearance							
Tidy and well groomed	7	7		6	8		
Irregularities	1	1	<i>n.s.</i>	1	0	<i>n.s.</i>	<i>n.s.</i>
Whiskers							
Present	8	8		6	5		
Absent	0	0	<i>n.s.</i>	1	3	<i>n.s.</i>	<i>n.s.</i>
Lacrimation							
Absent	8	8		7	8		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Defecation							
Present	8	8		7	8		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

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

Statistical analysis: chi-squared test; significance $p < 0.05$. Locomotor activity data are shown as mean (\pm SEM)

Parameter	Male			Female			both
	Control (n=8)	Mutant (n=8)	<i>p</i> -value	Control (n=7)	Mutant (n=8)	<i>p</i> -value	<i>p</i> -value
Transfer arousal							
Extended freeze	0	0		0	0		
Brief freeze	8	8		7	8		
Immediate movement	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Locomotor activity	20.4 \pm 2.6	19.2 \pm 2.4	<i>n.s.</i>	16.4 \pm 2.5	19.0 \pm 2.9	<i>n.s.</i>	<i>n.s.</i>
Gait							
Fluid movement	8	8		7	8		
Lack Fluidity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Tail Elevation							
Dragging							
Horizontally extension	0 3	0 4		0 3	0 3		
Elevated/Straub tail	5	4	<i>n.s.</i>	3	3	<i>n.s.</i>	<i>n.s.</i>
Touch Escape							
No response	0	0		0	0		
Response to touch	8	8		7	8		
Flees prior to touch	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Positional Passivity							
Struggles when held by tail	8	8		7	8		
No struggle	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Startle Response							
None	2	3		1	0		
Flick of the pinnae	6	5		6	8		
Jumping	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

Table 13: Behavior recorded in or above the arenaStatistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female			both
	Control (n=8)	Mutant (n=8)	<i>p-value</i>	Control (n=7)	Mutant (n=8)	<i>p-value</i>	<i>p-value</i>
Skin color							
Blanched	0	0		0	0		
Pink	8	8		7	8		
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Trunk curl							
Absent	8	8		7	8		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Limb Grasping							
Absent	8	8		7	8		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Pinna Reflex							
Present	8	8		7	8		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Corneal Reflex							
Present	8	8		7	8		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Urination							
Present	7	6		2	6		
Absent	1	2	<i>n.s.</i>	5	2	<i>n.s.</i>	<i>n.s.</i>
Contact Righting							
Present	7	5		7	8		
Absent	1	3	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Evidence of biting							
None	7	8		6	8		
Biting in response to handling	1	0	<i>n.s.</i>	1	0	<i>n.s.</i>	<i>n.s.</i>
Vocalisation							
None	5	5		6	3		
Vocal	3	3	<i>n.s.</i>	1	5	<i>n.s.</i>	<i>n.s.</i>

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

	Male			Female			both
	Control (n=8)	Mutant (n=8)	<i>p-value</i>	Control (n=7)	Mutant (n=8)	<i>p-value</i>	<i>p-value</i>
Lactate (mmo/l)	11.9 \pm 0.4	11.7 \pm 0.4	<i>n.s.</i>	11.7 \pm 0.3	13.0 \pm 0.7	<i>n.s.</i>	<i>n.s.</i>

3.4 Eye Screen

3.4.1 Introduction

In the primary screen, different methods were employed to analyze the eyes of mutant mouse line in comparison to their control littermates. Mice were examined for anterior segment abnormalities by slit lamp biomicroscopy (Favor, 1983), as well as for posterior segment abnormalities by funduscopy. The axial eye length was measured by laser interference biometry (LIB; Puk *et al.*, 2006). If required, the retinal function can be tested with a high throughput electroretinography (ERG; Dalke *et al.*, 2004) in a secondary screen.

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

3.4.2 Summary

Subtle genotype-specific differences between wild-type control and mutant *Gsk3 α* mice were detected by Laser Interference Biometry. However, the difference in the axial eye length between control and mutant males is considered as a secondary or subtle effect.

3.4.3 Mice

Fifteen *Gsk3 α* control (8 male, 7 female) and 16 *Gsk3 α* knockdown mice (8 male, 8 female) entered the Eye Screen at the age of 13 weeks for Slit Lamp Biomicroscopy and Ophthalmoscopy. Another batch of 26 *Gsk3 α* control (7 male, 7 female) and 12 *Gsk3 α* knockdown mice (7 male, 5 female) entered the Eye Screen at the age of 15 weeks for Laser Interference Biometry. Mice were first examined by slitlamp biomicroscopy and funduscopy, on the following day the laser interference biometry was performed. Mice were kept under standard laboratory conditions with food and water *ad libitum*. When the mice were killed for pathological examinations the eyes of some mice were fixed for histological analysis in the eye screen.

3.4.4 Materials and Methods

Funduscopy (Ophthalmoscopy): The posterior parts of both eyes were examined by funduscopy. After pupil dilation with one drop of atropine (1%), the mouse is grasped firmly in one hand and clinically evaluated using a head-

worn indirect ophthalmoscope (Sigma 150 K, Heine Optotechnik, Herrsching, Germany) in conjunction with a condensing lens (90D lens, Volk, Mentor, OH, USA) mounted between the ophthalmoscope and the eye.

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.

Laser Interference Biometry (LIB) was performed using the “AC Master” (Meditec, Carl Zeiss, Jena, Germany) equipped with a new technique, optical low coherence interferometry (OLCI), adapted for short measurement distances (Schmucker and Schaeffel, 2004). Mice were anaesthetized with 137 mg Ketamine and 6.6 mg Xylazine per kg body weight and placed in front of the ACMaster.

Histology: Eyes were fixed 24 hours in Davidson solution, dehydrated and embedded in plastic medium. Transverse 2 µm sections were cut with an ultramicrotome, stained with methylene blue and basic fuchsin and evaluated with a light microscope.

Statistical Analysis: Laser interference biometry 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).

3.4.5 Parameters

Funduscopy
(qualitative) abnormalities of the retinal fundus and optic disc, vessel alterations and development disorders
Slit lamp biomicroscopy
(qualitative) abnormalities of lens and cornea like opacity and development disorders
Laser Interference Biometry (LIB)
axial eye length abnormalities
Histology
(qualitative) retinal lamination and morphology of cell layers and lens
Morphology
(qualitative) like size and degree of closure

3.4.6 Results and Discussion

Laser Interference Biometry data were recorded from the groups of control and *Gsk3 α* mutant mice. A comparison of the axial eye length of left and right

eye was performed for each group. Since no differences were observed between the left and right eye, data of both eyes were averaged for further evaluation. The mean value and standard error was calculated for each group of mice, male and female, wild type control and mutant (Table 15). Sex-specific differences were found for the axial length in the female group and after normalisation in both groups. Comparing *Gsk3 α* mutant mice with their littermate controls significant difference was found for males, but not for females. In addition, the body length and the weight were significantly different between control and mutant males (data not shown). Therefore, the difference in the axial eye length between control and mutant males is considered as a secondary or subtle effect.

All *Gsk3 α* mice were examined by **funduscopy**. No abnormalities associated with the *Gsk3 α* mutation were detected (Table 16).

A total of 31 mice were examined ophthalmologically by **slit lamp biomicroscopy** (Table 17). **No** anterior segment phenotype was shown to be associated with the *Gsk3 α* mutation. All animals expressed nuclear and zonular opacity which may be due to strain 129 background.

To conclude, only subtle genotype-specific differences were detected between wild-type control and knockdown *Gsk3 α* mice in the eye screen. These data are in accordance with the results of the *Gsk3 β* mouse line.

3.4.7 References

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Abbreviations

n.s.	not significant
NAD	no abnormality detected

Table 15: Axial eye length

Mean ± standard error

Parameter	Control (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=7)	(n=7)	<i>p</i> -value	(n=7)	(n=7)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Axial length [mm]	3.761 ± 0.013	3.668 ± 0.024	<0.02	3.685 ± 0.009	3.663 ± 0.008	n.s.	<0.001	n.s.
Axial length / body length	0.038 ± 0.0004	0.039 ± 0.0006	<0.05	0.039 ± 0.0003	0.040 ± 0.0003	<0.05	<0.01	n.s.

Table 16: Results from Funduscopy

Genotype	NAD	white dots, unilateral	Pigment patches and vessel alterations	Vessel alterations
male control (n=8)	8			
female control (n=7)	7			
male kd (n=8)	7	1		
female kd (n=8)	7	1		

Table 17: Results from Slit Lamp Biomicroscopy

Genotype	NAD	Nuclear and zonular opacity	Posterior capsule opacity	Microphthalmia/ Anophthalmia
male control (n=8)		8		
female control (n=7)		7		
male kd (n=8)		8		
female kd (n=8)		8		

3.5 Nociceptive Screen

3.5.1 Introduction

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

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

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

3.5.2 Summary

In the first screen we tested the responsiveness of the intact somatosensory system to the thermal pain of the *Gsk3 α* knockdown mouse line. The hot plate test was used as the primary screen in the nociception phenotyping module of the GMC.

We found no significant difference in pain reactivity between the wild-type control and knockdown animals. There was no significant sex difference either. We do not suggest making further and more detailed pain-related studies in *Gsk3 α* knockdown mice.

3.5.3 Mice

Sixteen *Gsk3 α* knockdown mice (8 male, 8 female), and 15 wild-type control animals (8 male, 7 female) were tested in our first screen.

3.5.4 Material and Methods

Hot plate test

The mice were placed on a metal surface maintained at $52\pm 0.2^{\circ}\text{C}$ (Hot plate system was made by TSE GMBH, Germany; Eddy and Leimbach, 1953). Locomotion of the mouse on the hot plate was constrained by 20 cm high Plexiglas wall to a circular area with a diameter of 28 cm (Fig. 8). Mice remained on the plate until they performed one of three behaviors regarded as indica-

tive of nociception: hind paw lick (h.p. licking), hind paw shake/flutter (h.p. shaking) or jumping.

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

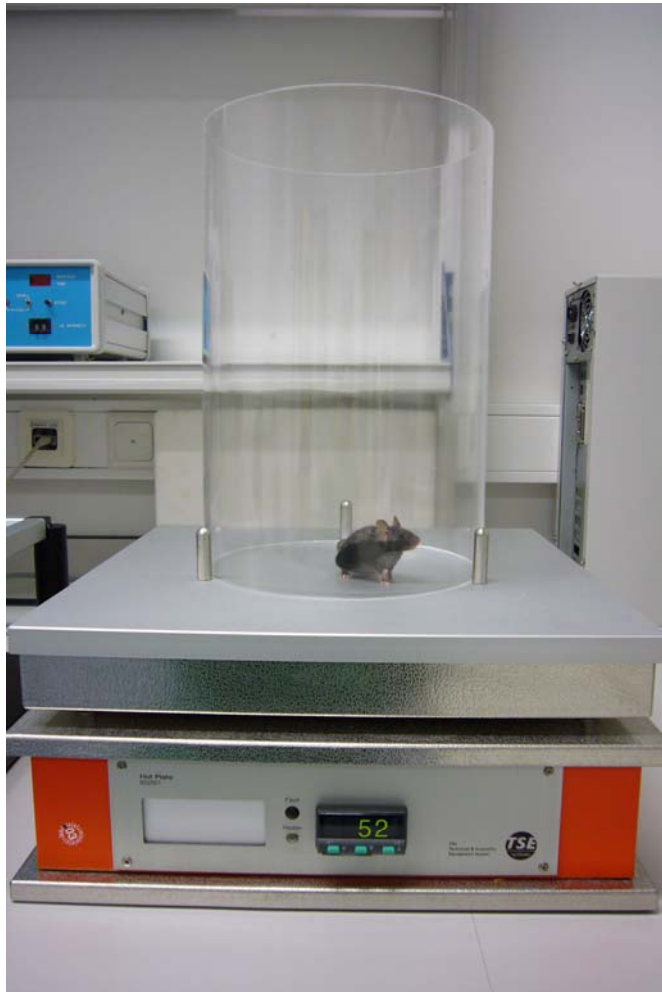


Figure 8: Hot plate system

Statistical analysis

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

3.5.5 Parameters

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

3.5.6 Results and Discussion

The first nociceptive response observed in these mice was "hind paw shaking", the second one "hind paw licking". Both groups of wild-type control and knockdown animals showed these reactions at the same mean reaction time. The third response was "jumping". We could not detect any difference between the control and the mutant animals in this reaction either. We found no significant difference between the sexes.

Knockdown of *Gsk3 α* does not result in a nociceptive phenotype. Therefore we do not suggest making more pain-related studies in these mice.

3.5.7 References

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Abbreviations

h.p. hind paw

Table 18: Nociceptive Screen									
Data are presented as mean ± standard error of mean.									
						ANOVA			
						genotype		sex*genotype	
Parameter Latency [s]	Control (B)			Mutant (A)			A~B	A~B	ANOVA
	Male	Female		Male	Female		Male	Female	
	(n=8)	(n=7)	<i>p - value</i>	(n=8)	(n=8)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
h.p.shaking	12.5± 2.28	15.4± 2.43	n.s.	15.14± 2.28	11.2± 2.3	n.s.	n.s.	n.s.	n.s.
h.p.licking	16.15± 1.22	14.23± 1.3	n.s.	17.19± 1.22	13.7± 1.2	0.055	n.s.	n.s.	n.s.
jumping	50.5± 3.44	55.5± 3.68	n.s.	47.19± 3.43	52.78± 3.44	n.s.	n.s.	n.s.	n.s.

ANOVA:

SHAKING: genotype; p = 0.741, sex; p = 0.822, genotype*sex; p = 0.154

LICKING: genotype; p = 0.831, sex p = 0.039, genotype*sex; p = 0.540

JUMPING: genotype; p = 0.398, sex p = 0.139, genotype*sex; p = 0.938

3.6 Metabolic Screen

3.6.1 Introduction

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

3.6.2 Summary

Mice were fed under *ad libitum* conditions for two weeks followed by one day of acute fasting. The primary metabolic screen focuses on the investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature. *Gsk3 α* mutants were significantly lighter than wild-type controls both under *ad libitum* and food restricted conditions. Body temperature was also reduced under *ad libitum* conditions. Especially female mutants did not cope with the fasting challenge diagnosed by extreme body temperature reduction. Therefore, this test had to be stopped after one day only.

3.6.3 Mice

Seven adult *Gsk3 α* mutant males and seven adult control litter mates entered the Metabolic Screen at the beginning of calendar week 46 in 2007. The females (seven controls and seven mutant animals) entered the metabolic laboratory one week later. The mice were single caged on grid panels (0.5 cm grid hole diameter). They were fed *ad libitum* for a period of 14 days, followed by one day of acute fasting to analyze adaptive responses of metabolism.

3.6.4 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 strain and sex (ANCOVA with body mass as covariate). The Fisher test was applied for post hoc multiple comparisons. The Mann-Whitney-Test for paired samples was used to analyze the effect of nutritional status on parameters of energy metabolism.

3.6.5 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})

3.6.6 Results and Discussion

Gsk3 α mutants were significantly lighter than wild-type controls (males –23%, females –5%) both under *ad libitum* and food restricted conditions. Body temperature was slightly but also significantly decreased in mutants. During fasting female mutants did not compensate energy shortage but reduced body temperature to very low levels. Therefore, the fasting challenge had to be interrupted for animal welfare reasons after one day only. No other significant effects of the mutation could be detected.

Prior to the metabolic screening, body mass and body size differences between mutant and control mice were expected from previous studies. We could confirm this phenotype even though only males showed the strong reduction by about 20%. Mutant females strongly reduced body temperature during fasting. Seemingly, they were not able to compensate for acute energy shortage. Therefore, the duration of the challenge test was reduced to only one day. In *Gsk3 β* mutants, a **similar** metabolic phenotype could be described. These mice also showed a strong response to fasting indicating that the mobilization of endogenous energy reserves could be impaired.

3.6.7 Suggestions for Secondary Screening

Further secondary phenotyping is recommended to investigate the metabolic phenotype in detail. We suggest conducting a fasting test combined with indirect calorimetry and monitoring of body temperature and locomotor activity to investigate the regulation of energy homeostasis in this mutant line during acute energy shortage.

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Abbreviations

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

Table 19: Metabolic parameters recorded in the primary screen											
Data are presented as mean \pm standard error of mean.											
Parameter	Control				Mutant				2 – Way - ANOVA		
	<i>ad libitum</i>		<i>1 days acute fasting</i>		<i>ad libitum</i>		<i>1 days acute fasting</i>		<i>p</i> <i>genotype</i>	<i>p</i> <i>sex</i>	<i>p</i> <i>interaction</i>
	male (n=7)	female (n=7)	male (n=7)	female (n=7)	male (n=7)	female (n=7)	male	female	<i>ad libitum</i> <i>food reduced</i>	<i>ad libitum</i> <i>food reduced</i>	
Body weight [g]	33.5 \pm 1.1	21.3 \pm 0.8	29.5 \pm 1.0	18.3 \pm 0.7	25.7 \pm 0.6	20.2 \pm 1.7	22.4 \pm 0.6	17.6 \pm 1.4	< 0.001 < 0.001	<0.001 <0.001	<0.01 <0.01
Rectal body temperature [°C]	36.5 \pm 0.2	37.0 \pm 0.1	35.3 \pm 0.2	33.2 \pm 1.0	36.3 \pm 0.1	36.4 \pm 0.2	35.1 \pm 0.3	31.1 \pm 1.3	< 0.05 n.s.	n.s. < 0.001	n.s. n.s.
Food consumption [g day ⁻¹]	3.4 \pm 0.1	2.8 \pm 0.1	no food for one day		3.0 \pm 0.1	2.6 \pm 0.2	no food for one day		< 0.05	< 0.01	n.s.
Energy uptake [kJ day ⁻¹]	56.72 \pm 2.09	47.02 \pm 1.60			50.21 \pm 1.50	43.05 \pm 3.98			n.a.	n.a.	n.a.
Feces production [g day ⁻¹]	1.00 \pm 0.04	0.77 \pm 0.03			0.85 \pm 0.03	0.70 \pm 0.07			n.a.	n.a.	n.a.
Energy content feces [kJ g ⁻¹]	15.44 \pm 0.11	15.34 \pm 0.14			15.46 \pm 0.06	15.35 \pm 0.13			n.s.	n.s.	n.s.
Metabolized energy [kJ day ⁻¹]	41.10 \pm 1.67	34.56 \pm 1.14			36.36 \pm 1.26	31.69 \pm 2.97			n.s.	< 0.01	n.s.
Metabolized energy [kJ g ⁻¹ day ⁻¹]	1.24 \pm 0.07	1.63 \pm 0.07			1.42 \pm 0.06	1.56 \pm 0.05			n.s.	< 0.001	n.s.
Food assimilation coefficient [%]	72.4 \pm 0.5	73.5 \pm 0.5			72.4 \pm 0.6	73.5 \pm 0.6			n.s.	n.s.	n.s.

3.7 Clinical Chemistry and Hematology

3.7.1 Introduction

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 functions, 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).

3.7.2 Summary

Twenty-one different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we determined ten basic hematological parameters and tested the animals for glucose tolerance.

We detected differences in hematological and in blood lipid parameters. These findings may indicate an influence of the *Gsk3 α* genotype on hematopoiesis and possibly on fat/energy metabolism. In addition, the physiological sex-related differences were found.

3.7.3 Mice

Eight control males and eight *Gsk3 α* mutant males of about 34 weeks of age entered the clinical-chemical screen in the 48th calendar week of 2007. Seven control females and eight *Gsk3 α* mutant females of about 34 weeks of age entered the clinical-chemical screen in the same week. The IpGTT had been performed one week before in the 47th calendar week of 2007.

3.7.4 Materials and Methods

Intraperitoneal Glucose-Tolerance-Test

The mice used for the intraperitoneal glucose tolerance test were fasted for 16 to 18 hours overnight. In the beginning of the test the body weight of mice was determined. For the determination of the baseline blood glucose level of the fasted mouse, the tip of the tail was scored using a sterilized scalpel blade and a small drop of blood was analyzed with the Accu-Chek Aviva glucose analyzer (Roche/ Mannheim). Thereafter mice were injected intraperitoneally with 2 g of glucose/kg body weight using a 20% glucose solution, a 25 gauge needle and a 1 ml syringe. 15, 30, 60, 90 and 120 minutes after glucose injection, additional blood samples (one drop each) were collected and used to determine blood glucose levels as described before. Repeated bleeding was induced by removing the clot from the first incision and massaging the tail of

the mouse. After the experiment was finished, mice were placed in a cage with plentiful supply of water and food.

Blood Withdrawal and Storage

For the analysis of blood based parameters blood samples were taken from isoflurane-anesthetized mice by puncturing the retro-orbital sinus with non-heparinized capillaries (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. Each blood sample was divided into two portions. The major portion was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). The smaller portion was collected (using the same capillary) in an EDTA-coated tube (KABE, Art.No 078035). Each tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant.

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, 9503 x g; Biofuge, Heraeus; Hanau, Germany). Plasma was distributed between the Immunology Screen (30 µl), the Allergy Screen (30 µl), the Clinical-Chemical Screen (130 µl) and the Cardiovascular Screen (residual), while the cell pellet was given to the Immunology Screen for FACS-analysis. The plasma sample for the clinical chemical analysis was transferred into an Eppendorf tube and diluted 1:2 with aqua dest. The solution was mixed for a few seconds (Vortex genie, Scientific Industries, New York, America) to prevent clotting and then centrifuged again for 10 min at 9503 x g. Additionally the Clinical-Chemical Screen received the EDTA-blood sample for hematological investigations.

Clinical Chemistry

The screen was performed using an Olympus AU 400 autoanalyzer and adapted reagents from Olympus (Hamburg, Germany). Creatinine was measured using a Kit from Biomed (Oberschleißheim, Germany). In the primary screen, 21 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, Germany) and the C57BL/6mouse-Chipcard for this analyser. Number and size of red blood cells, white blood cells, and platelets are measured by electrical impedance and hemoglobin by spectrophotometry. Mean corpuscular volume (MCV), mean platelet volume (MPV) and red blood cell distribution width (RDW) are calculated directly from the cell volume measurements. The hematocrit (HCT) is assessed by multiplying the MCV with the red blood cell count. Mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentrations (MCHC) are calculated from hemoglobin/ red blood cells count (MCH) and hemoglobin/ hematocrit (MCHC), respectively.

Second sample analysis

A second sample was collected from 12 female animals and 11 male animals three weeks later in the 51st week of 2007. A subset of clinical-chemical parameter and all hematological parameters were retested to check the reproducibility of the first results.

Analysis of Data

Data were statistically analyzed using Excel and Sigma Stat 3.1 with the level of significance set at $p < 0.05$, by an ANOVA test on the influence of genotype and sex and subsequent pairwise comparisons of the means by T-test.

3.7.5 Parameters

Proteins and plasma enzyme activities
Alkaline phosphatase (EC 3.1.3.1), α -Amylase (EC 3.2.1.1), Creatine kinase (EC 2.7.3.2), Aspartate-aminotransferase (ASAT/ GOT; EC 2.6.1.1), Alanine-aminotransferase (ALAT/ GPT; EC 2.6.1.2), Ferritin, Transferrin, Total protein, Albumin
Plasma concentrations of specific substrates
Glucose, Cholesterol, Triglycerides, Urea, Creatinine, Non-esterified fatty acid (NEFA)
Plasma concentrations of electrolytes
Potassium, Sodium, Chloride, Calcium, Inorganic phosphate, Iron
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), Red blood cell distribution width (RDW), Platelet count (PLT) and Mean platelet volume (MPV)
Intraperitoneal Glucose-Tolerance-Test (IpGTT)
Blood glucose concentration after 16 to 18 hours fasting (time 0) as well as 15 min, 30 min, 60 min, 90 min and 120 min after glucose administration

3.7.6 Results

Intraperitoneal Glucose Tolerance Test

The IpGTT showed no significant differences between *Gsk3 α* male and female mutants and their respective control animals (Figure 9-12). However, female *Gsk3 α* mice showed interesting data since two animals displayed a markedly reduced glucose tolerance compared to the rest of the group (Figure 11).

Clinical Chemistry

In the first test, ANOVA revealed a significant influence of the genotype on the values/activities of potassium, total protein, cholesterol, triglycerides, NEFA and LDH (Table 20). While potassium values and LDH activities in *Gsk3 α* mutants were significantly increased as compared to controls, total protein, cholesterol, triglycerides and NEFA values were decreased in mutant mice as compared to control mice. An influence of the genotype on ferritin values was almost significant ($p=0.052$). Mutant mice showed a tendency for lower ferritin values than control mice. A significant interaction of genotype and sex was found for sodium. Sodium was increased in female mutants and decreased in male mutants as compared to the respective control groups.

In the second tests the results from the first test could not be confirmed (Table 21). However cholesterol and triglyceride values of female mutant mice were also significantly decreased as compared to female controls. Sex-related differences were found for many parameters.

Hematology

In the first test, ANOVA showed a significant influence of the genotype on the RDW (Table 22). Compared to the control mice, mutant mice showed significantly increased RDW values. The influence of the *Gsk3 α* genotype on the WBC was almost significant ($p=0.05$). Mutant mice of both sexes showed a tendency for a higher WBC.

In the second test, the influence of the genotype (ANOVA) on the RDW was confirmed (Table 23): Mutant mice showed a significantly increased RDW as compared to control mice. In addition, a significant influence of the genotype was found for the RBC, the MCV and the MCH. While the RBC was significantly decreased in mutant mice as compared to controls, the MCV and MCH were significantly increased in *Gsk3 α* mutants. In addition, mutant mice showed a tendency for a lowered hematocrit ($p=0.07$). A further tendency was detected for the interaction of genotype and sex of the MPV ($p=0.054$). While male mutants showed a lower MPV mean value than male controls, female mutants showed a higher MPV mean value than female controls.

Sex-related differences were found for many parameters.

3.7.7 Discussion

Intraperitoneal Glucose Tolerance Test

As no significant difference in glucose tolerance between *Gsk3 α* mutant and control animals was observed, an influence of the genotype on glucose metabolism seems to be unlikely. The two female outliers may be due to the ~87% C57BL/6J genetic background. C57BL/6J mice can carry a naturally occurring deletion in the nicotinamide nucleotide transhydrogenase (*Nnt*) exons 7-11. This deletion results in the absence of the NNT protein, and is associated with impaired glucose homeostasis control and reduced insulin secretion (Toye *et al.*, 2005).

Clinical Chemistry

The results of the first clinical-chemical test were not reproducible in the second clinical-chemical test. At least, the same tendencies were seen in the second test for cholesterol and triglyceride values in female mutants. The significantly **decreased blood lipid values** (cholesterol, triglycerides, NEFA) of mutant mice might be related to the confirmed phenotype of *Gsk3 α* mutants which show a significantly reduced body weight and body temperature which theoretically could result from an altered energy metabolism (see Energy Metabolism Screen, Chapter 3.6).

Hematology

In both haematological tests, mutants display a significantly **increased anisocytosis** (RDW; Figure 17). In the second test, tendencies of the first test became significant (Figures 13-15), i.e. mutants showed a significantly decreased RBC and a significantly increased MCV and MCH. This macrocytic blood cell count may indicate difficulties of *Gsk3 α* mutants to cope with the challenge of the first blood withdrawal.

Comparison to baseline data

Sex-related differences were detected for many parameters, reflecting the physiological differences, often observed in various mouse strains (Kile *et al.*, 2003). All values for all the clinical-chemical parameters were situated within the normal variation range of C57BL/6 mice as supported by previously published data (Hough *et al.*, 2002; Loeb and Quimby, 1999; Rathkolb *et al.*, 2000; Kile *et al.*, 2003; own unpublished results).

A secondary screen is not recommended.

3.7.8 References

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Table 20: Clinical-chemical parameters, 1st sample.

Data are presented as mean ± standard error of mean.

Parameter	Control (A)		Mutant (B)		A~B	A~B	ANOVA		
	Male	Female	Male	Female	Male	Female	genotype	sex	inter.
	(n=8)	(n=7)	(n=8)	(n=8)	p-value	p-value	p-value	p-value	p-value
Sodium [mmol/l]	153 ± 0.38	150 ± 0.62	150.8 ± 1.31	151.3 ± 0.53	n.s.	n.s.	n.s.	n.s.	p<0.05
Potassium [mmol/l]	4.2 ± 0.07	4.31 ± 0.04	4.38 ± 0.12	4.68 ± 0.11	n.s.	p<0.05	p<0.01	p<0.01	n.s.
Calcium [mmol/l]	2.23 ± 0.02	2.22 ± 0.03	2.18 ± 0.03	2.2 ± 0.01	n.s.	n.s.	n.s.	n.s.	n.s.
Chloride [mmol/l]	111.1 ± 0.36	109.7 ± 0.36	110.5 ± 1.29	110.9 ± 0.42	n.s.	p<0.05	n.s.	n.s.	n.s.
inorg. Phosphorus [mmol/l]	1.08 ± 0.08	1.03 ± 0.09	1.13 ± 0.04	1.03 ± 0.08	n.s.	n.s.	n.s.	n.s.	n.s.
Total Protein [g/dl]	5.75 ± 0.05	5.66 ± 0.11	5.45 ± 0.07	5.43 ± 0.1	p<0.01	n.s.	p<0.01	n.s.	n.s.
Creatinine [mg/dl]	0.221 ± 0.006	0.239 ± 0.01	0.24 ± 0.005	0.247 ± 0.007	p<0.05	n.s.	n.s.	n.s.	n.s.
Urea [mg/dl]	75.8 ± 1.85	62 ± 4.2	73.9 ± 4.41	74.7 ± 5.01	n.s.	n.s.	n.s.	n.s.	n.s.
Albumin [g/dl]	2.73 ± 0.04	2.8 ± 0.04	2.63 ± 0.05	2.75 ± 0.05	n.s.	n.s.	n.s.	p<0.05	n.s.
Cholesterol [mg/dl]	102.3 ± 4.85	87.3 ± 6.31	85.5 ± 4.96	71.3 ± 4.58	p<0.05	n.s.	p<0.01	p<0.01	n.s.
Triglycerides [mg/dl]	134 ± 12.8	86 ± 9.2	98 ± 12.3	71 ± 7.8	n.s.	n.s.	p<0.05	p<0.01	n.s.
NEFA [mg/dl]	0.92 ± 0.043	1.04 ± 0.051	0.85 ± 0.048	0.84 ± 0.044	n.s.	p<0.05	p<0.01	n.s.	n.s.
Amylase [U/l]	851 ± 49.04	642.2 ± 29.91	755.6 ± 32.92	674.6 ± 30.81	n.s.	n.s.	n.s.	p<0.001	n.s.
ALAT [U/l]	28.5 ± 2.13	30 ± 4.09	37 ± 5.73	30 ± 2.24	n.s.	n.s.	n.s.	n.s.	n.s.
ASAT [U/l]	56 ± 1.56	66 ± 7.56	68.8 ± 5.54	68.3 ± 4.41	n.s.	n.s.	n.s.	n.s.	n.s.
AP [U/l]	76.8 ± 2.64	106.9 ± 4.84	110.8 ± 23.72	109 ± 5.03	n.s.	n.s.	n.s.	n.s.	n.s.
LDH [U/l]	214 ± 12.5	292 ± 20.1	270 ± 19.7	321 ± 24	p<0.05	n.s.	p<0.05	p<0.01	n.s.
Glucose [mg/dl]	127.7 ± 9.5	128.8 ± 11.6	111.3 ± 8.3	114.7 ± 8.4	n.s.	n.s.	n.s.	n.s.	n.s.
Ferritin [ng/ml]	42.8 ± 4.1	54.4 ± 13.57	33.8 ± 1.91	35.9 ± 3.33	n.s.	n.s.	p=0.052	n.s.	n.s.
Transferrin [mg/dl]	145.9 ± 1.59	153.9 ± 2.4	145.2 ± 2.23	154 ± 1.77	n.s.	n.s.	n.s.	p<0.001	n.s.
Iron [µg/dl]	104 ± 3.88	118.4 ± 7.66	116.9 ± 5.88	123.8 ± 8.87	n.s.	n.s.	n.s.	n.s.	n.s.

Table 21: Clinical-chemical parameters, 2nd sample.

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

Parameter	Control (A)		Mutant (B)		A~B	A~B	ANOVA		
	Male	Female	Male	Female	Male	Female	genotype	sex	inter.
	(n=7)	(n=6)	(n=4)	(n=6)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
Sodium [mmol/l]	149.4 \pm 0.57	147.7 \pm 0.61	149 \pm 0.58	148 \pm 0	n.s.	n.s.	n.s.	p<0.05	n.s.
Potassium [mmol/l]	3.6 \pm 0.09	4.43 \pm 0.1	3.65 \pm 0.24	4.33 \pm 0.1	n.s.	n.s.	n.s.	p<0.001	n.s.
Chloride [mmol/l]	110.6 \pm 0.43	109.3 \pm 0.6	111.2 \pm 1.44	109.8 \pm 0.55	n.s.	n.s.	n.s.	n.s.	n.s.
Total Protein [g/dl]	5.06 \pm 0.06	5.27 \pm 0.12	5.1 \pm 0.13	4.93 \pm 0.08	n.s.	n.s.	n.s.	n.s.	n.s.
Albumin [g/dl]	2.429 \pm 0.052	2.6 \pm 0.052	2.6 \pm 0.082	2.467 \pm 0.067	n.s.	n.s.	n.s.	n.s.	p<0.05
Creatinine [mg/dl]	0.6 \pm 0.078	0.29 \pm 0.013	0.69 \pm 0.15	0.34 \pm 0.022	n.s.	n.s.	n.s.	p<0.001	n.s.
Urea [mg/dl]	68.1 \pm 1.71	58.2 \pm 3.44	73.8 \pm 1.8	63.5 \pm 3.9	n.s.	n.s.	n.s.	p<0.01	n.s.
Cholesterol [mg/dl]	94.3 \pm 5.91	80.5 \pm 2.29	93.7 \pm 6.39	64.1 \pm 5.19	n.s.	p<0.05	n.s.	p<0.001	n.s.
Triglycerides [mg/dl]	165 \pm 24.4	70 \pm 3.9	139 \pm 27.7	51 \pm 4.7	n.s.	p<0.05	n.s.	p<0.001	n.s.
NEFA [mmol/l]	4.6 \pm 0.35	0.8 \pm 0.03	5.3 \pm 0.21	0.7 \pm 0.05	n.s.	n.s.	n.s.	p<0.001	n.s.

Table 22: Hematological parameters, 1st sample.

Data are presented as mean ± standard error of mean.

Parameter	Control (A)		Mutant (B)		A~B	A~B	ANOVA		
	Male	Female	Male	Female	Male	Female	geno- type	sex	inter.
	(n=10)	(n=10)	<i>p- value</i>	(n=10)	(n=10)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
WBC [$10^3/\mu\text{l}$]	8.7 ± 0.93	5.6 ± 0.49	9.2 ± 0.59	8 ± 0.79	n.s.	p<0.05	p=0.05	p<0.01	n.s.
RBC [$10^6/\mu\text{l}$]	10.2 ± 0.14	10.2 ± 0.15	10 ± 0.13	9.9 ± 0.21	n.s.	n.s.	n.s.	n.s.	n.s.
PLT [$10^3/\mu\text{l}$]	978 ± 42.1	845 ± 36.4	962 ± 51.8	863 ± 47.8	n.s.	n.s.	n.s.	p<0.05	n.s.
Hemoglobin [g/dl]	14.2 ± 0.29	15 ± 0.31	14 ± 0.27	14.1 ± 0.32	n.s.	n.s.	n.s.	n.s.	n.s.
Hematocrit [%]	45.8 ± 0.77	46.9 ± 0.95	45.6 ± 0.66	45.2 ± 1	n.s.	n.s.	n.s.	n.s.	n.s.
MCV [fl]	45.3 ± 0.53	45.7 ± 0.36	45.8 ± 0.25	45.6 ± 0.26	n.s.	n.s.	n.s.	n.s.	n.s.
MCH [pg]	14 ± 0.16	14.6 ± 0.14	14.1 ± 0.16	14.3 ± 0.13	n.s.	n.s.	n.s.	p<0.05	n.s.
MCHC [g/dl]	31 ± 0.32	31.9 ± 0.14	30.7 ± 0.34	31.2 ± 0.15	n.s.	p<0.01	n.s.	p<0.05	n.s.
RDW [% of MCV]	14.4 ± 0.08	14.2 ± 0.12	14.7 ± 0.18	14.8 ± 0.25	n.s.	p<0.05	p<0.05	n.s.	n.s.
MPV [fl]	5.03 ± 0.1	5.2 ± 0.05	5.04 ± 0.06	5.4 ± 0.07	n.s.	p<0.05	n.s.	p<0.01	n.s.

Table 23: Hematological parameters, 2nd sample.

Data are presented as mean ± standard error of mean.

Parameter	Control (A)		Mutant (B)		A~B	A~B	ANOVA		
	Male	Female	Male	Female	Male	Female	geno- type	sex	inter.
	(n=10)	(n=10)	<i>p- value</i>	(n=10)	(n=10)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
WBC [$10^3/\mu\text{l}$]	9.5 ± 1.17	7.2 ± 0.45	9.7 ± 1.08	8.4 ± 0.98	n.s.	n.s.	n.s.	n.s.	n.s.
RBC [$10^6/\mu\text{l}$]	9.6 ± 0.12	10.1 ± 0.11	8.8 ± 0.38	9.4 ± 0.25	n.s.	n.s.	p<0.01	p<0.05	n.s.
PLT [$10^3/\mu\text{l}$]	1129 ± 37.9	985 ± 69.6	1176 ± 112	903 ± 68.9	n.s.	n.s.	n.s.	p<0.01	n.s.
Hemoglobin [g/dl]	14 ± 0.23	15 ± 0.26	13.7 ± 0.61	14.3 ± 0.26	n.s.	n.s.	n.s.	p<0.05	n.s.
Hematocrit [%]	43.8 ± 0.71	46.1 ± 0.55	42.2 ± 1.84	44 ± 0.83	n.s.	n.s.	p=0.07	p<0.05	n.s.
MCV [fl]	45.4 ± 0.69	45.7 ± 0.61	48 ± 0.41	46.8 ± 0.4	p<0.05	n.s.	p<0.01	n.s.	n.s.
MCH [pg]	14.6 ± 0.27	15 ± 0.33	15.6 ± 0.14	15.2 ± 0.15	p<0.05	n.s.	p<0.05	n.s.	n.s.
MCHC [g/dl]	32.1 ± 0.16	32.6 ± 0.3	32.5 ± 0.07	32.5 ± 0.12	p<0.05	n.s.	n.s.	n.s.	n.s.
RDW [% of MCV]	16.3 ± 0.19	15.9 ± 0.07	16.8 ± 0.26	16.9 ± 0.28	n.s.	p<0.05	p<0.01	n.s.	n.s.
MPV [fl]	5.06 ± 0.09	5.12 ± 0.05	5.03 ± 0.07	5.51 ± 0.14	n.s.	p<0.05	n.s.	p<0.05	p=0.05 4

Figure 9: IpGTT: Time course of glucose levels in male mice.

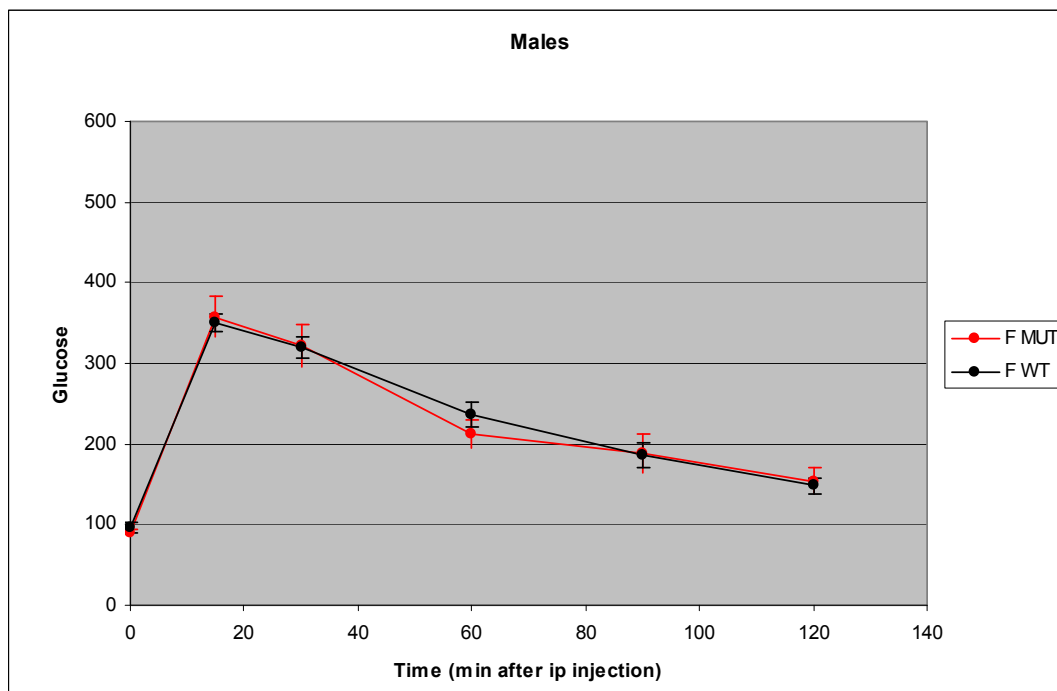
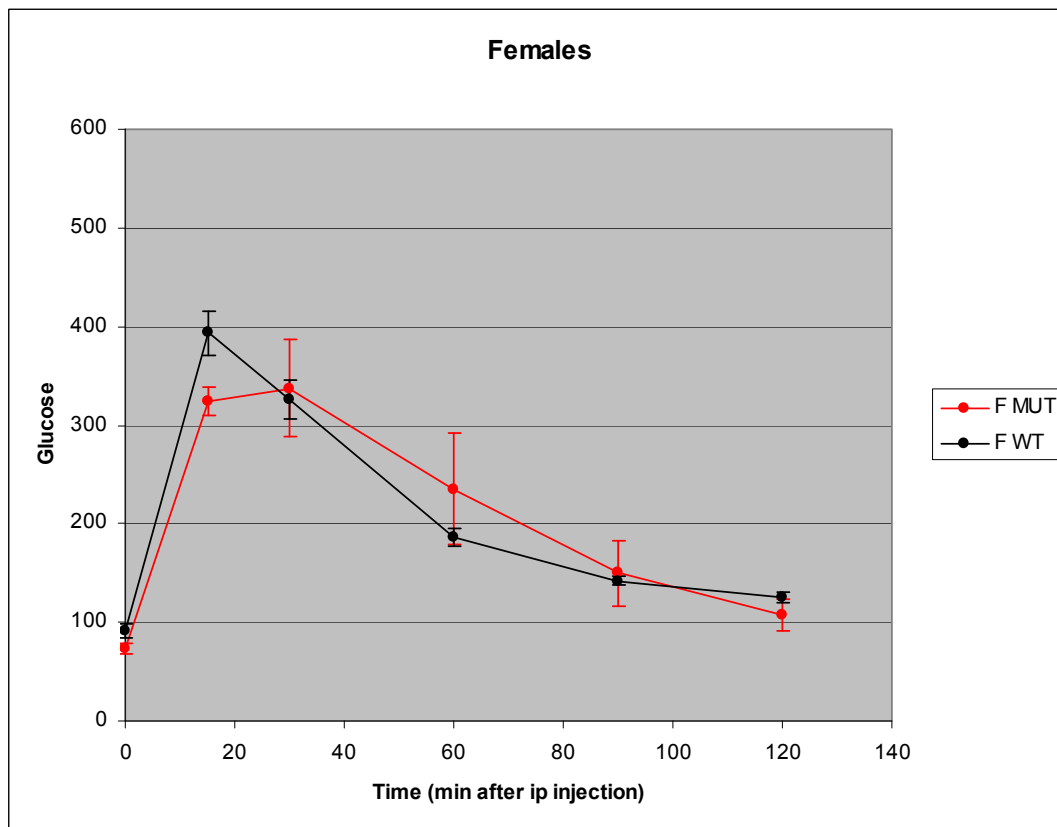


Figure 10: IpGTT: Time course of glucose levels in female mice.



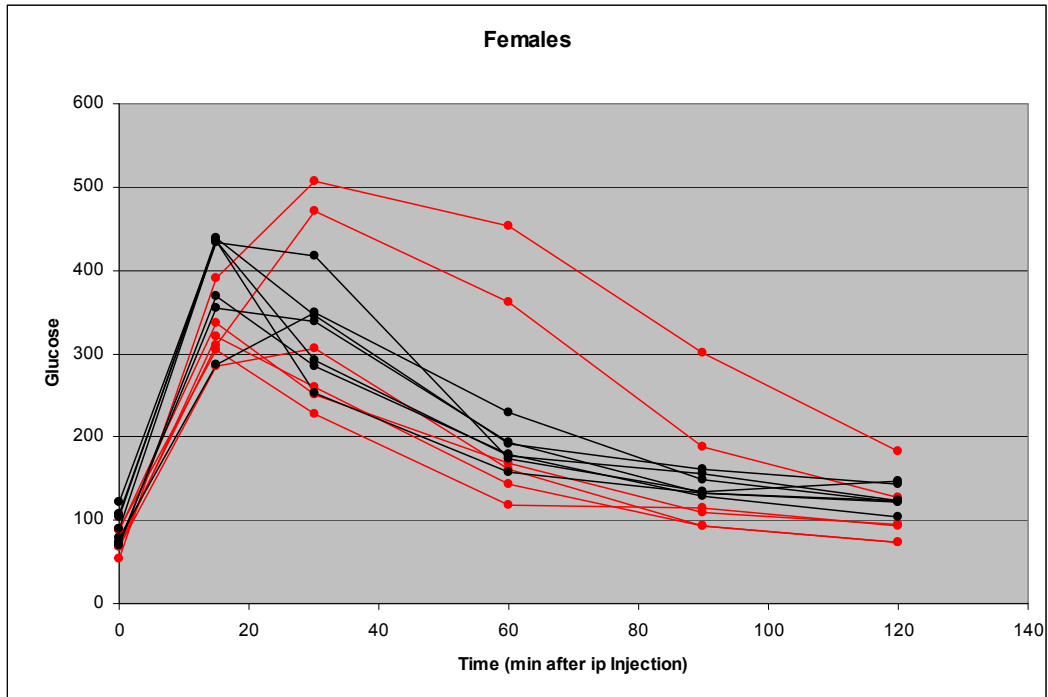


Figure 11: IpGTT: Data from individual animals

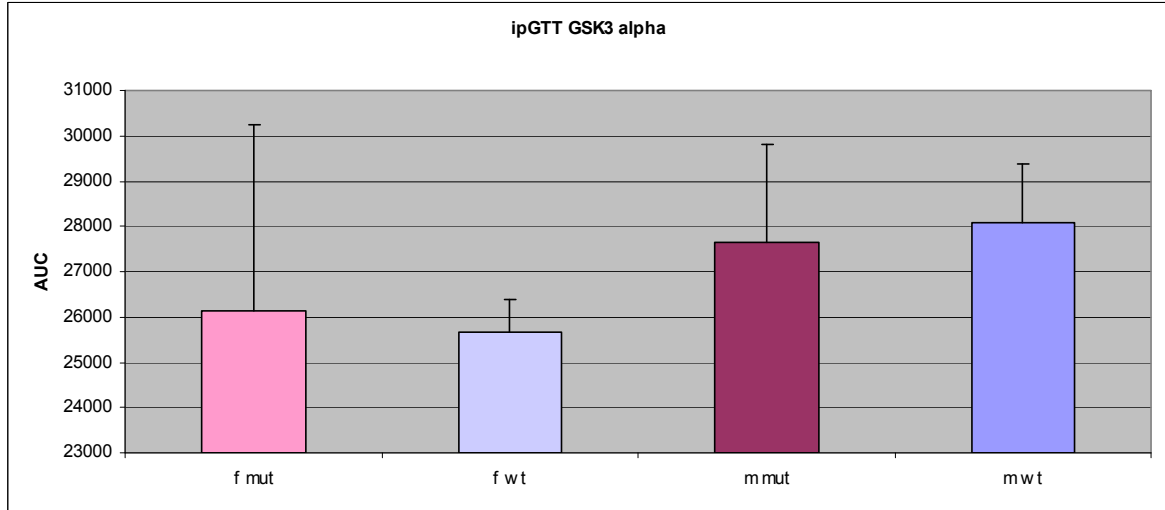


Figure 12: IpGTT: Area under the curve
 Approximated AUC of Figures 10 and 11, calculated using the trapezoidal rule.

Hematological parameters, comparison of levels of the first and second sample

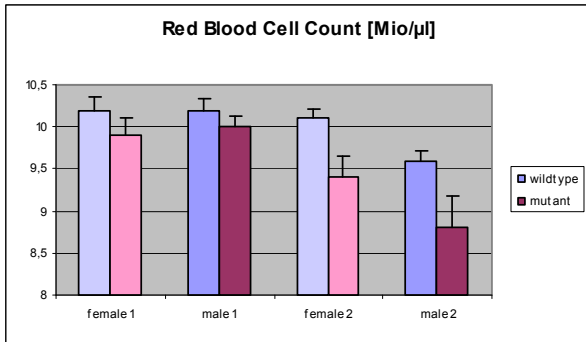


Figure 13: Red Blood Cell Count (RBC)

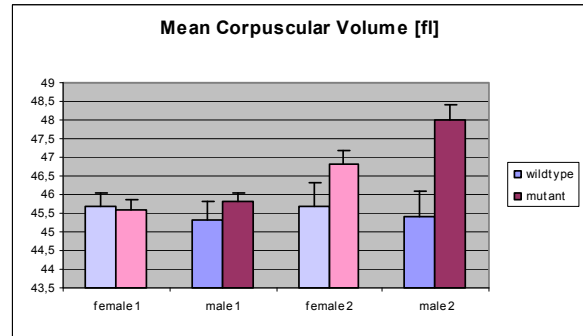


Figure 14: Mean Corpuscular Volume (MCV)

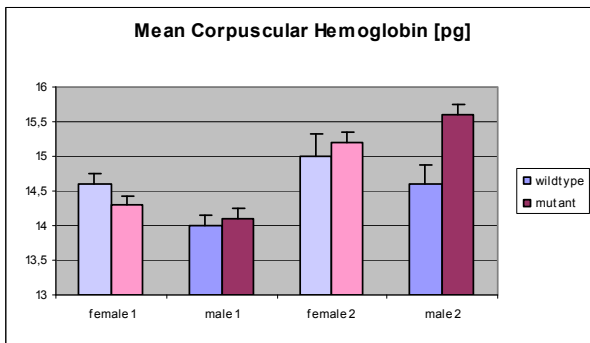


Figure 15: Mean Corpuscular Hemoglobin (MCH)

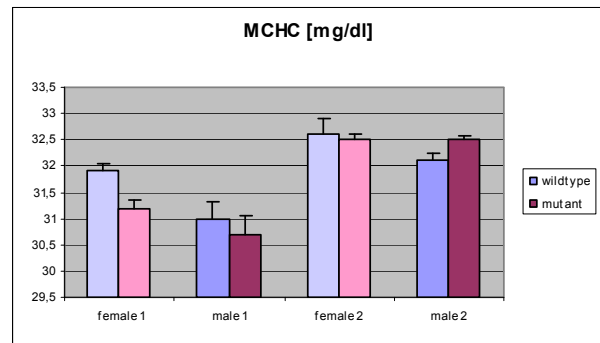


Figure 16: Mean Corpuscular Hemoglobin Content (MCHC)

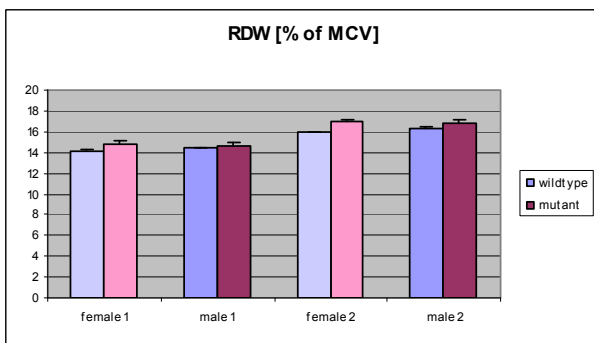


Figure 17: Red Cell Distribution Width (RDW)

A reduced red blood cell count with increased MCV and anisocytosis (RDW) indicates an impaired hematopoiesis in the mutant animals, especially after being challenged by the first bleeding.

3.8 Immunology Screen

3.8.1 Introduction

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). In primary screen we measure leukocyte populations in peripheral blood and immunoglobulin levels in blood plasma.

The proportions of leukocyte populations in peripheral blood are genetically regulated (eg. Mice: Chen and Harrison 2002; Men: Hall, *et al.*, 2000,). As a consequence, inbred strains differ in the frequency of leukocyte subsets in the lymphoid organs and in peripheral blood. Moreover, several CD antigens are restricted to specific mouse strains (eg. Carlyle *et al.*, 2006) or interstrain differences occur concerning the level of expression of certain CD antigens (eg. Haegel and Ceredig 2005). Strain specific differences in the immune response are further reflected in different susceptibilities to infectious agents (eg. Medina *et al.*, 2001).

In individual mice, the number of circulating leukocytes and the proportions of subpopulations show daily rhythmic variations (Yellon and Tran 1992) and depend further on homeostatic proliferation and/or retraction (Freitas and Rocha 2000), as well as on activation through environmental and/or microbial factors (eg. Grewal *et al.*, 1997), which might be related to subtle behavioral characteristics (eg. Kim *et al.* 1999). Furthermore, sex-dependent factors are documented to influence the immune status (Krzych *et al.*, 1978) and have an impact on infection susceptibility (Pasche *et al.*, 2005).

The levels of Ig classes and IgG isotypes are characteristic of a special inbred mouse strains and seem to underlie genetic control mechanisms (Sant'Anna *et al.*, 1985). The profile of Ig subclasses interests further, because it reflects the direction of T-cell help; e.g. Th2-directed switching goes along with preferred generation of IgG1 and IgE. In mice, antibodies of the IgG2a subclass is the predominant isotype produced to viral infections (e.g., Coutelier *et al.*, 1987) and in cytokine-induced Th1-type responses (e.g., Snapper and Paul, 1987; Finkelman *et al.*, 1988; Stevens *et al.*, 1988).

3.8.2 Summary

The analysis of *Gsk3 α* mutant mice in the primary Immunology Screen revealed a statistically significant higher frequency of T-cells and the tendency of a lower frequency of B-cells in female mutant mice compared to wild-type controls. Furthermore, there was higher expression of CD62L and a lower expression of CD44 in T-cells of female mutant mice.

3.8.3 Mice

We analyzed 16 mutant (8 females and 8 males) and fifteen (seven females and eight males) of age- and sex-matched littermate controls. Analysis of a second blood sample from 10 mutant (six females and four males) and 13 controls (six females and seven males) from the same cohort of mice was performed

3.8.4 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500 μ l blood by erythrocyte lysis with NH₄Cl (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 Fc block (clone 2.4G2, PharMingen, San Diego, USA). Cells were then stained with fluorescence-conjugated monoclonal antibodies (PharMingen). After the antibody incubation, propidium iodide was added for the identification of dying/dead cells (Zamei *et al.*, 1996), which might bind antibodies unspecifically, and/or loose specific antigens upon apoptosis (Diaz *et al.*, 2004). Samples were acquired automatically from 96 well plates with an HTS on a LSRII flow cytometer (Becton Dickinson, USA) until a total number of 30000 living CD45+ cells is reached for each well. For analysis, intact cells are first identified by their FSC/SSC profile. These cells were gated on the basis of their propidium iodide/PE signal (compensated parameters), allowing the dead cells to be gated out. Living cells were then gated using their SSC/CD45 signal, gating out remaining erythrocytes, thrombocytes and debris (Weaver and Broud 2002). CD45+ cells are subsequently analyzed by software based analysis (Flowjo, TreeStar Inc, USA). In former experiments, FMO (Fluorescence minus one) controls from wt mice have been used to define 'positive' and 'negative' regions (Baumgarth and Roederer, 2000).

The plasma levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were determined simultaneously in the same sample using a bead-based assay (Fulton *et al.*, 1997) with monoclonal anti-mouse antibodies conjugated to beads of different regions (Biorad, USA), and acquired on a Bioplex reader (Biorad). 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-Tnfrsf6lpr mice (Jackson Laboratory, Bar Harbor, USA) were used as positive controls in the autoantibody assays.

3.8.5 Parameter

Flow cytometry
measured parameters and defined cell populations
Main lineages: T-cells (CD3+), CD4+ T-cells, CD8+ T-cells, $\gamma\delta$ T-cells, T reg cells (CD4+CD25+) B-cells (CD19+, resp. B220+), B1 B-cells (/CD5+), mature B-cells (/IgD+), granulocytes (CD11b+Gr1++), NK cells (NK1.1+ , resp. NKp46 (secondary screen) CD5-) and NK T-cells (NK1.1+ CD5+)
Subpopulations: Further subpopulations are identified by bi-variate gating with the following markers: T-cells: CD25, CD62L, Ly-6C, CD44. (CD103 - secondary screen) CD19+ cells: IgD, B220, CD11b, MHC-II(I-A, I-E), CD5, Gr1; CD19-cells: Gr1, B220, CD5, MHCII, CD11b.
Bioplex/ELISA
IgM, IgG ₁ , IgG _{2b} , IgG _{2a} IgG ₃ , IgA; anti-DNA antibodies, rheumatoid factor

3.8.6 Results

The analysis of *Gsk3 α* mutant mice in the primary Immunology Screen revealed a statistically significant higher frequency of T-cells and a lower frequency of B-cells in female mutant mice compared to controls (I). Furthermore a lower proportion of CD44 expressing cells and a higher tendency of CD62L expression within the CD8 T-cell cluster was found significantly different in female mice. These findings were confirmed in a second bleeding. No statistically significant changes were found in males; however, the expression pattern of CD44 and CD62L in T-cells showed a similar tendency as in females (II). The analysis of a second batch of mice (secondary screen) did not confirm the differences in females. There, in male mice, a significant higher proportion of CD62L expressing T-cells was found (Tables 24, 25 &27).

Analysis of leukocyte populations of spleen and thymus (secondary screen) of female mice did not show significant differences between mutant and controls mice (data not shown).

2.) Analysis of the plasma immune globulin levels revealed no differences between control and mutant mice (Table 26).

3.8.7 Discussion

Ad I.). We have found sex specific differences in *Gsk3 α* mice. The proportions of leukocyte subsets in the peripheral blood under baseline conditions remain relatively stable. Sex-dependent differences in the pattern of leukocyte subsets in peripheral blood occur frequently in wild-type mice and have been described (Pasche *et al.*, 2005). They might be related to effects of sex-hormones (Ahmed *et al.*, 1985). However, these differences in the leukocyte proportions do not show always the same patterns and might partly be inter-

puted as secondary effects. Often we observe a higher proportion of T-cells in female mice. The analysis of *Gsk3 α* mice, revealed a more pronounced sex dependent difference concerning leukocyte proportions in blood than in corresponding control mice. This goes together with higher sex dependent differences in body weight, which also account for changes in immunological parameters (Dorshkind *et al.* 2003).

Ad II) In both sexes, the tendency of a higher proportion of CD62L and a lower proportion of CD44 expressing T-cells is found. CD62L, L-selectin, is an essential molecule for the homing of T-cells to lymph nodes (Xu *et al.*, 1996) and is highly expressed on naïve T-cells. It is down-regulated upon activation, whereas CD44 is a marker for activation (Ponta *et al.*, 2003). CD62L is also expressed on a subset of memory T-cells, which can be recognized by their high expression of CD44. As a conclusion, T-cells of *Gsk3 α* mutant mice seem to be in a more activated state.

In our screen the increased or decreased expression of CD62L on T-cells is a frequently occurring phenotype in mutant mice. In wild-type mice from different strains we observe a positive correlation of T-cell frequency and CD62L expression within the T-cell compartment, which reflects, that the number of T-cells in the periphery under baseline conditions depends on the production of naïve T-cells in the thymus. High CD62L expression in peripheral blood from humans has been further correlated to panic disorder (Manfro *et al.* 2000) and nephropathy (De March *et al.* 1999).

3.8.8 Secondary Screening and Final Conclusion

In order to follow up blood-phenotype of mutant female *Gsk3 α* mice we analyzed the immune cells in other compartments (spleen, thymus), comparing three control and three mutant mice. There were no indications for differences in the proportions of leukocytes in these organs; however, we did not confirm the findings (blood) of the primary screen in this cohort of mice (Table 27). Although the latter does not speak for a direct effect of the *Gsk3 α* mutation on the immune system, we cannot exclude the occurrence of a *Gsk3 α* depend phenotype under challenge conditions (immunization, infection).

Compared to *Gsk3 β* mice, we did not see an obvious common phenotype. In both, we detected differences in the frequency of B-cells (primary screen of *Gsk3 α* and *Gsk3 β*), on one hand, a reduced frequency of B cells in female *Gsk3 α* went together with a higher proportion of T-cells, on the other hand, a higher proportion of B-cells in *Gsk3 β* mice correlated with a lower proportion of granulocytes compared to the corresponding controls.

3.8.9 References

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Table 24: Basic parameters analyzed in the Immunology Screen by flow cytometry
 Frequencies of main leukocyte subsets in blood of male and female *Gsk3α* mutant and control mice.
 [% of CD45+ viable leukocytes, respectively of parent gate].

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female	p - value	Male	Female	p - value	Male	Female
	(n=8)	(n=5)		(n=10)	(n=7)		p - value	p - value
3+	19.5 ± 1.79	18.7 ± 1.3	n.s.	18.4 ± 0.86	25.1 ± 1	p<0.001	n.s.	p<0.01
4+3e+	8.66 ± 0.85	7.11 ± 0.45	n.s.	8.3 ± 0.46	10.33 ± 0.66	p<0.05	n.s.	p<0.01
8a+3e+	9.01 ± 0.71	8.68 ± 0.7	n.s.	8.78 ± 0.5	11.53 ± 0.48	p<0.01	n.s.	p<0.01
4+3e+/25+	5 ± 0.52	4.8 ± 0.37	n.s.	4.8 ± 0.46	4.6 ± 0.31	n.s.	n.s.	n.s.
gd+3e+	0.34 ± 0.03	0.35 ± 0.03	n.s.	0.37 ± 0.04	0.55 ± 0.03	p<0.01	n.s.	p<0.001
4+/CD62L+	50.13 ± 7.56	41.79 ± 7.51	n.s.	54.08 ± 5.93	61.2 ± 2.66	n.s.	n.s.	p<0.05
4+/44+	74.475 ± 0.822	72.714 ± 0.827	n.s.	75.238 ± 1.54	76.075 ± 1.711	n.s.	n.s.	n.s.
8a+/CD62L+	59.7 ± 8.42	54.3 ± 8.61	n.s.	63.9 ± 6.39	78.5 ± 2.72	n.s.	n.s.	p<0.05
8a+/CD44+	63.81 ± 0.99	65.63 ± 1.85	n.s.	61.79 ± 0.95	59.85 ± 1.65	n.s.	n.s.	p<0.05
Gr1+11b+	14.4 ± 1.12	19.4 ± 1.44	p<0.05	13.8 ± 0.89	16.7 ± 1.63	n.s.	n.s.	n.s.
nonGra, nonNK 45+/11b+	3 ± 0.3	4 ± 0.4	p<0.05	3 ± 0.4	5 ± 0.5	n.s.	n.s.	n.s.
NK+5+	0.2 ± 0.02	0.1 ± 0.01	p<0.01	0.2 ± 0.02	0.3 ± 0.03	n.s.	n.s.	p<0.001
NK+5-	2.6 ± 0.13	2.4 ± 0.2	n.s.	3.5 ± 0.3	4.1 ± 0.25	n.s.	p<0.05	p<0.001
19+	54.4 ± 1.96	48.1 ± 2.6	n.s.	55 ± 2.12	38.8 ± 2.52	p<0.001	n.s.	p<0.05
19+/Igd+	94.5 ± 0.41	91.1 ± 1.09	p<0.05	93.7 ± 0.57	91.1 ± 1.14	n.s.	n.s.	n.s.
19+/5+	1 ± 0.2	1 ± 0.1	n.s.	1 ± 0.1	2 ± 0.1	p<0.05	n.s.	p<0.01
19+/MHC II+220+	90.6 ± 0.4	90.1 ± 0.7	n.s.	89.8 ± 0.8	91.4 ± 0.7	n.s.	n.s.	n.s.
NK+/CD11b+	10.3 ± 1.1	24.6 ± 3.3	p<0.01	17.6 ± 2.51	26.4 ± 3.63	n.s.	p<0.05	n.s.

Table 25: Second bleeding: Basic parameters analyzed in the Immunology Screen by flow cytometry.

Frequencies of main leukocyte subsets in blood of male and female *Gsk3α* mutant and control mice [% of CD45+ viable leukocytes, respectively of parent gate].

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female	p - value	Male	Female	p - value	Male	Female
	(n=8)	(n=5)		(n=10)	(n=7)		p - value	p - value
3+	18.1 ± 1.37	22.6 ± 3.01	n.s.	20.2 ± 2.35	30.5 ± 1.41	p<0.05	n.s.	p<0.05
4+3e+	7.95 ± 0.81	7.85 ± 0.77	n.s.	8.77 ± 1.29	11.72 ± 0.77	n.s.	n.s.	p<0.01
8a+3e+	10.49 ± 1.67	8.55 ± 0.93	n.s.	10.01 ± 1.18	12.45 ± 0.5	n.s.	n.s.	p<0.01
4+3e+/25+	6.7 ± 0.63	6.3 ± 0.6	n.s.	5.6 ± 0.57	4.4 ± 0.18	n.s.	n.s.	p<0.05
gd+3e+	0.53 ± 0.1	0.82 ± 0.12	n.s.	0.57 ± 0.08	1.22 ± 0.09	p<0.001	n.s.	p<0.05
4+/CD62L+	36.49 ± 6.86	51.57 ± 10.04	n.s.	52.33 ± 9.57	69.1 ± 1.7	n.s.	n.s.	n.s.
4+/44+	73.2 ± 1.303	74.967 ± 1.016	n.s.	76.6 ± 2.481	76.733 ± 2.071	n.s.	n.s.	n.s.
8a+/CD62L+	52.4 ± 9.22	66.8 ± 10.53	n.s.	65.8 ± 8.35	84.9 ± 1.47	n.s.	n.s.	n.s.
8a+/CD44+	66.21 ± 2.84	66.7 ± 1.5	n.s.	63.08 ± 1.29	59.5 ± 1.69	n.s.	n.s.	p<0.01
Gr1+11b+	17.2 ± 1.64	19.2 ± 1.92	n.s.	13.5 ± 2.5	14.7 ± 0.42	n.s.	n.s.	n.s.
nonGra nonNK 45+/11b+	3 ± 0.2	5 ± 0.5	p<0.05	3 ± 0.3	5 ± 0.4	p<0.05	n.s.	n.s.
NK+5+	0.2 ± 0.02	0.1 ± 0.02	p<0.05	0.2 ± 0.02	0.2 ± 0.02	n.s.	n.s.	n.s.
IK+5-	3.6 ± 0.24	2.3 ± 0.32	p<0.01	3.4 ± 0.32	2.9 ± 0.2	n.s.	n.s.	n.s.
19+	51.3 ± 2.6	49.7 ± 1.28	n.s.	52.6 ± 3.98	43.5 ± 2.45	n.s.	n.s.	n.s.
19+/lgD+	94.4 ± 0.39	93.7 ± 1.02	n.s.	94.4 ± 0.76	95 ± 0.43	n.s.	n.s.	n.s.
19+/5+	1 ± 0.1	1 ± 0.1	n.s.	1 ± 0.2	1 ± 0.2	n.s.	n.s.	n.s.
19+/MHC II+220+	88 ± 0.8	95.6 ± 0.6	p<0.001	89.5 ± 0.4	96.9 ± 0.4	p<0.001	n.s.	n.s.
NK+/CD11b+	12 ± 2.03	32.9 ± 3.26	p<0.001	15.4 ± 2.33	36.2 ± 4.53	p<0.01	n.s.	n.s.

Table 26: Basic parameters analyzed in the Immunology Screen by Bioplex/ELISA
 Concentration [$\mu\text{g/ml}$] of antibodies of different isotypes in blood plasma from mutant and control mice.

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=8)	(n=7)		(n=8)	(n=8)		<i>p</i> - value	<i>p</i> - value
IgG1 mean	543.1 \pm 90.32	337.5 \pm 38.36	n.s.	412.1 \pm 63.9	535 \pm 178.25	n.s.	n.s.	n.s.
IgG2b mean	507.4 \pm 41.98	717.73 \pm 58.98	p<0.05	651.14 \pm 93.88	672.52 \pm 48.25	n.s.	n.s.	n.s.
IgG3 mean	362.1 \pm 94.1	670.5 \pm 180.7	n.s.	274.4 \pm 65.95	446.6 \pm 122.67	n.s.	n.s.	n.s.
IgM mean	665.55 \pm 55.92	853.94 \pm 206.21	n.s.	718.84 \pm 84.61	691.64 \pm 86.66	n.s.	n.s.	n.s.
IgA mean	2279.39 \pm 802.92	777.73 \pm 138.55	n.s.	1393.43 \pm 316.84	1516.2 \pm 425.17	n.s.	n.s.	n.s.
anti-DNA	0.443 \pm 0.049	0.419 \pm 0.032	n.s.	0.366 \pm 0.037	0.399 \pm 0.038	n.s.	n.s.	n.s.
Rheuma	0.3 \pm 0.04	0.3 \pm 0.02	n.s.	0.3 \pm 0.03	0.3 \pm 0.02	n.s.	n.s.	n.s.
No autoantibodies above ,zero level' in mutant and control have been measured								

Table 27: Secondary screen (new cohort of mice): Basic parameters measured by flow cytometry.

Frequencies of main leukocyte subsets in blood of male and female *Gsk3α* mutant and control mice [% of CD45+ viable leukocytes, respectively of parent gate].

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female		Male	Female		Male	Female
	(n=16)	(n=15)	<i>p</i> - value	(n=3)	(n=6)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Tcells(CD3+)	21.6 ± 1.21	27 ± 1.66	p<0.05	25 ± 2.68	27.8 ± 1.72	n.s.	n.s.	n.s.
8+Tcells	9.22 ± 0.62	12.76 ± 0.86	p<0.01	11.47 ± 1.28	12.24 ± 1.34	n.s.	n.s.	n.s.
4+Tcells	9.91 ± 0.73	12.86 ± 0.87	p<0.05	10.61 ± 0.9	13.12 ± 0.62	n.s.	n.s.	n.s.
CD3+/103+	4.5 ± 0.42	7.6 ± 0.57	p<0.001	6.5 ± 0.3	8.4 ± 0.46	p<0.05	p<0.01	n.s.
4+/Tregs	4.52 ± 0.21	4.19 ± 0.21	n.s.	5.67 ± 0.14	4.42 ± 0.3	p<0.01	p<0.001	n.s.
4+/44+	73.42 ± 1.11	74.97 ± 1	n.s.	75.3 ± 1.5	74.72 ± 2.28	n.s.	n.s.	n.s.
4+/62L+	48.73 ± 4.8	53.64 ± 2.4	n.s.	64.17 ± 1.4	56.97 ± 2	p<0.05	p<0.01	n.s.
4+/6C+	12.4 ± 1.08	8.1 ± 0.45	p<0.01	9.8 ± 1.49	7.2 ± 0.74	n.s.	n.s.	n.s.
8+/44+	64.03 ± 1.38	62.51 ± 0.79	n.s.	59.63 ± 1.46	59.05 ± 1.94	n.s.	n.s.	n.s.
8+/62L+	64.7 ± 4.49	67.7 ± 2.7	n.s.	78.6 ± 0.87	71.4 ± 1.08	p<0.01	p<0.01	n.s.
8+/6C+	35 ± 2.2	24 ± 1.4	p<0.001	43 ± 2.6	28 ± 3.8	p<0.05	n.s.	n.s.
Bcells (CD19+)	54.1 ± 1.99	51.6 ± 1.43	n.s.	52.4 ± 3.52	49.1 ± 1.78	n.s.	n.s.	n.s.
19+/IgD	92.62 ± 0.63	94.27 ± 0.32	p<0.05	92.93 ± 0.09	92.92 ± 0.42	n.s.	n.s.	p<0.05
Granulocytes	11.05 ± 0.79	8.73 ± 0.9	n.s.	9.71 ± 0.69	9.01 ± 0.85	n.s.	n.s.	n.s.
NKTcells	0.1 ± 0.04	0.1 ± 0.01	n.s.	0.1 ± 0.02	0.1 ± 0.01	n.s.	n.s.	n.s.
NK	1.9 ± 0.54	1.81 ± 0.21	n.s.	1.62 ± 0.43	2.29 ± 0.56	n.s.	n.s.	n.s.
19+/11b+	5.609 ± 1.58	4.603 ± 0.35	n.s.	4.987 ± 0.771	4.773 ± 0.452	n.s.	n.s.	n.s.
19+/B220+	92.3 ± 3.89	95.7 ± 1.08	n.s.	97 ± 0.44	97.2 ± 0.23	n.s.	n.s.	n.s.
19+/IAk	40.74 ± 2.32	43.56 ± 1.53	n.s.	40.73 ± 1.83	42.1 ± 2.3	n.s.	n.s.	n.s.
19+/5+	1.9 ± 0.14	1.8 ± 0.08	n.s.	2.2 ± 0.18	1.8 ± 0.17	n.s.	n.s.	n.s.
NK+/11b+	62 ± 2.8	74 ± 3	p<0.01	81 ± 2	84 ± 2.6	n.s.	p<0.001	p<0.05

3.9 Allergy Screen

3.9.1 Introduction

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. IgE-mediated atopic disorders such as allergic asthma, allergic rhinitis, and atopic dermatitis are now considered as environmentally and exposure driven immune disorders leading to the expression of various clinical phenotypes in individuals with defined genetic risk profiles (Arruda, *et al.*, 2005; Kabesch 2006). Genome screens with classical linkage and fine mapping approaches suggest that susceptibility to asthma is determined by multiple genes with each gene having a moderate dose effect (Wjst, *et al.*, 1999). In this respect, the development of phenotypically and genotypically defined animal models will be an important step (Bochner and Busse 2005). To detect allergy prone mouse mutants in systematic screening efforts, total plasma IgE was established as a powerful screening parameter (Alessandrini, *et al.*, 2001; Jakob, *et al.*, 2007).

3.9.2 Summary

The analysis did not reveal profound differences in plasma IgE between knockdown and control mice.

3.9.3 Mice

Age- and sex-matched group of 15 controls (7 females, 8 males) and 16 mutants (8 females, 8 males) mice aged 14 weeks were analyzed in the Allergy Screen.

3.9.4 Material and Methods

Fourteen-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 isoflurane anesthesia.

Plasma was analyzed for total IgE, using a classical immunoassay isotype-specific sandwich ELISA. In brief, microtiter plates (96-well) were coated with 10 µg/ml anti-mouse-IgE rat monoclonal IgG (clone-PC284, The Binding Site) to detect total IgE. Serum samples were diluted 1:10 and standards for murine IgE (Mouse IgE, k clone C38-2 BD Pharmingen™) were appropriately diluted. As secondary antibodies, biotinylated rat anti-mouse IgE (clone R35-118, BD Pharmingen™) were used followed by incubation with BD OptEIA Reagent Set B (Cat. No. 550534 BD Pharmingen™) Plates were analyzed using a standard micro well ELISA reader at 450 nm. Total murine IgE data are reported in ng/ml, based on a standard curve of purified murine IgE (Alessandrini *et al.*, 2001).

3.9.5 Results and Discussion

The analysis of total IgE levels in plasma (Median \pm Stdev) of Gsk3 α mice revealed no statistically significant differences between the groups (Table 28). In both mutant and control animals, the median concentration of total IgE was higher in females than in males but no significant difference between groups were found. This is a common finding for many mouse inbred strains (Alessandrini *et al.*, 2000; Corteling *et al.*, 2004; Seymour *et al.*, 2002; Melgert *et al.*, 2005). A slight tendency of higher IgE levels was detected in knockdown mice but both controls and knockdown mice displayed elevated levels of IgE that are not usually found in wild type C57BL/6.

Taken together, under standard screening conditions for the primary allergy screen, Gsk3 α knockdown mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

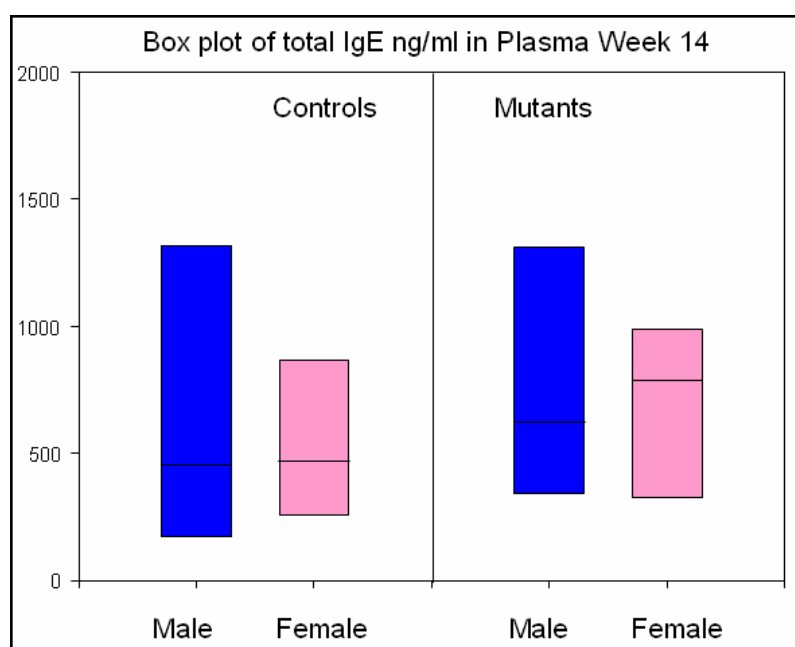


Figure 18: Total plasma IgE in Gsk3 α mice ng/ml

Table 28: Total plasma IgE in Gsk3α mice (14 weeks old)								
Data are presented as median values \pm standard deviation.								
	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=8)	(n=7)	<i>p</i> - value	(n=8)	(n=8)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Total IgE [ng/ml]	451 \pm 605	468 \pm 367	n.s.	621 \pm 549	789 \pm 614	n.s.	n.s.	n.s.

3.9.6 References

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3.10 Steroid Metabolism Screen

3.10.1 Introduction

Steroids control differentiation and proliferation processes of cells and tissues. They participate in the regulation of apoptosis (Bansal *et al.*, 1991), bone remodeling (Jerome, 2004) and neuroregeneration (Chowen *et al.*, 2000). Severe diseases are caused by monogenic mutations with loss of function of steroid pathway proteins. But defects in steroid metabolism contribute as well to the pathogenesis of many different multifactorial diseases like cancer, diseases of cartilage and bone or neurological diseases (Mindnich *et al.*, 2007; Möller *et al.*, 2006; Prehn *et al.*, 2007). The main focus of the Steroid Screen is the identification of new animal models for human steroid-related diseases therewith supporting the development of their future medical treatment. For primary screening the key steroids dehydroepiandrosterone (DHEA) and testosterone (Labrie *et al.*, 1995) are extracted from plasma and quantified by ELISA.

These two steroids were chosen for the tests because of the: 1) robustness of the assay, 2) applicability to mouse model, 3) lack of circadian or other rhythm, 4) indicative value for steroid biosynthesis pathways. Due to low concentrations of plasma steroids, concentration differences can only be considered as significant when they differ in order of magnitude. Generally there are only limited published reference concentrations for the steroids in the rodents. For DHEA in rat was reported to be 1 ng/mL, and for mouse testosterone 5 ng/mL (Kimura *et al.*, 1998, Erben *et al.*, 2002).

3.10.2 Summary

The analysis did reveal profound differences in DHEA and testosterone levels between mutants and control female mice.

3.10.3 Mice

Two age- and sex-matched groups of 11 control (6 males, 5 females) and 10 mutant (4 males, 6 females) mice aged 17 weeks have been analyzed in the Steroid Metabolism Screen.

3.10.4 Material and Methods

Male and female mice were screened for alterations in plasma concentrations of DHEA and testosterone. Blood was collected retro-bulbar from 12 weeks old narcotized mice (isoflurane) and plasma was prepared by centrifugation and stored at -20°C.

Since no steroid ELISA kits are available for mouse samples, human ELISA kits have to be used, but analysis is disturbed by the mouse plasma matrix. Therefore, the steroids have to be extracted from the matrix by liquid/liquid-extraction. 40 µl of plasma were extracted three times in each case with 400 µl *tert*-butylmethylether (TBME). The combined organic extracts were evaporated, dissolved *de novo* in TBME, subdivided for the two ELISA

tests (15:25 respectively for DHEA and testosterone) and evaporated again. The material was reconstituted for the respective kit, DHEA in assay puffer, testosterone in steroid free serum.

The steroids are quantified by competitive ELISA according to the manufacturer's protocols. The plates were read in a standard microplate reader at a wavelength of 405 nm (DHEA) and 450 nm (testosterone). The concentrations are reported in pg/ml (DHEA) and ng/ml (testosterone) based on the respective kit standard curve. The sensitivity of the tests is 2.9 pg/ml for DHEA and 0.083 ng/ml for testosterone respectively.

We used the following ELISA kits:

Testosterone ELISA: DRG Instruments GmbH, Catalog No. EIA-1559

DHEA ELISA: AssayDesigns, Catalog No. 901-093

3.10.5 Results and Discussion

At the age of 17 weeks, the analysis of DHEA and testosterone concentrations in plasma of *Gsk3α* mice revealed statistically significant differences between mutant and control female mice (Table 29). Additionally, in both groups of mutant and control animals, the testosterone levels were significantly higher in males than in females. These are the typical sex-specific differences in the concentration of testosterone. In case of DHEA we have sex-specific differences in control mice, too.

Taken together, genotype-specific differences have been found in the primary screening of *Gsk3α* female mice. However, we do not recommend a secondary screening at this point because only a few animals could be analysed. Therefore, we suggest first repeating the DHEA assay with more animals to verify the results.

Table 29: Plasma levels of DHEA and testosterone of <i>Gsk3α</i> mice (17 weeks old)								
Data are presented as median (25 %/75 % - interquartile range)								
	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=6)	(n=5)	<i>p</i> - value	(n=4)	(n=6)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
DHEA [pg/ml]	47.2 (41.8 / 126.1)	31.3 (17.2 / 53.3)	n.s.	100.1 (81.7 / 157.9)	7.4 (<2.9 / 9.5)	0.010	n.s.	0.017
Testosterone [ng/ml]	0.300 (0.249 / 0.997)	0.092 (<0.083 / 0.181)	0.004	0.354 (0.194 / 3.269)	0.176 (<0.083 / 0.307)	0.352	n.s.	n.s.

3.10.6 References

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3.11 Lung Function Screen

3.11.1 Introduction

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

3.11.2 Summary

Spontaneous breathing patterns during rest and activity were studied in the Gsk3 α mutant mouse line. Breathing pattern in Gsk3 α mice exhibited typical sex-specific differences as well as typical changes from rest to activity. No significant differences were detectable between mutant and control Gsk3 α mice suggesting that the mutation does not affect the respiratory system.

3.11.3 Mice

Female and male mutant and control mice were studied at the age of 37 weeks (Table 30). Control males (33.0 ± 1.7 g) were 29% heavier (significant) than mutant males (25.6 ± 0.8 g). Control female (22.0 ± 0.7 g) were slightly heavier (not significant) than mutant female (20.6 ± 1.0 g).

3.11.4 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. 19 and 20).

Calibration of the system allows to transform these pressure swings into flow and volume signals so that automated data analysis provides tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF). These data were stored online as mean values at 10 s intervals.

Measurements were always performed between 8 a.m. and 11 a.m. to account for potential diurnal variations in breathing. The system was set up in a quiet room where temperature and humidity were kept constant throughout the measurements. Before each measurement, the system was calibrated and the actual barometric pressure, temperature, and humidity were supplied to warrant adequate calculations of flow rates and volumes. After placing the

animals into the chamber, data recording was immediately started and was continued for 40 min.



Figure 19: System used at GMC to assess breathing patterns.

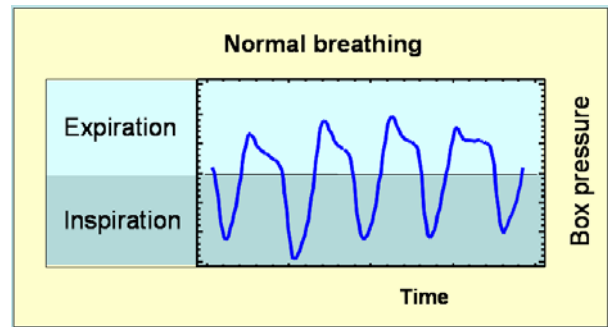


Figure 20: Recorded data used to calculate the breathing parameters.

Mice underwent typical phases during the measuring period. Primarily, the animals were stressed so that the respiratory rate was highest at the beginning. Usually after 5 min. the animals became calmer, they slightly reduced their respiratory rate, and began to explore the chamber and start cleaning themselves – *phase of activity*. Later activity was more and more interrupted by phases of rest or even short periods of snoozing – *resting phase*. Some of the animals even went to *phases of sleep*, which resulted in a further marked decrease in respiratory rate. The frequency histogram of the respiratory rates was determined for each individual, and breathing was analyzed for the above mentioned parameters during the phases of activity and rest. In addition to the directly recorded parameters, mean inspiratory and expiratory flow rates (MEF, MIF) were calculated offline from the ratio of tidal volume and the respective time interval. The relative duration of inspiration (T_i/TT) was determined from the ratio of inspiratory time to total time required for the breathing cycle. Specific tidal volumes and minute ventilations (sTV, sMV) were calculated by relating the absolute values to the body weight of the animal. Furthermore, the mean of all breathing frequencies (mean_f) measured during the 40-minute-period was calculated as a rough and ready parameter to assess whether the duration of rest and activity was similar in all mouse strains.

Statistical Analysis of Data

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

3.11.5 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)

3.11.6 Results and Discussion

Tables 30 - 33 summarize the results obtained for spontaneous breathing under sleeping (only females), resting, and active conditions. We found no significant differences in *Gsk3 α* knockdown mice compared to control mice. Breathing pattern in *Gsk3 α* mice exhibited typical sex specific differences as well as typical changes from rest to activity. Therefore, we suggest that the silencing of the gene does not affect the respiratory system.

A comparable situation was observed in *Gsk3 β* knockdown mice. *Gsk3 β* knockdown mice showed differences in tidal volume, minute ventilation, and flow rates, but the specific values for tidal volume and minute ventilation were not affected. This indicates that these differences are caused by the profound differences in body weight observed between *Gsk3 β* knockdown and control mice (33% in male and 26% in female) and are not related to the knockdown.

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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 30: Characterization of studied mice

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

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=6)	(n=7)		(n=6)	(n=5)		<i>p</i> - value	<i>p</i> - value
Bw [g]	33.0 \pm 1.7	22.0 \pm 0.7	< 0.001	25.6 \pm 0.8	20.6 \pm 1.0	< 0.01	< 0.01	n.s.
Age [d]	258.7 \pm 0.8	255.0 \pm 1.7		256.7 \pm 0.7	257.4 \pm 2.2			
Mean_f [1/min]	413.4 \pm 11.0	290.4 \pm 15.2	n.s.	413.1 \pm 23.2	361.3 \pm 28.9	n.s.	n.s.	n.s.

Table 31: Respiratory rate and timing at sleep rest and activityData are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=6)	(n=7)	<i>p</i> - value	(n=6)	(n=5)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Sleep		(n=6)			(n=3)			
f [1/min]		168.5 \pm 6.3			145.5 \pm 8.9			n.s.
Ti [ms]		82.8 \pm 3.4			95.8 \pm 4.8			n.s.
Te [ms]		275.6 \pm 9.9			319.9 \pm 23.3			n.s.
Ti/TT		0.23 \pm 0.00			0.23 \pm 0.01			n.s.
Rest	(n=5)	(n=7)		(n=5)	(n=5)			
f [1/min]	323.6 \pm 4.1	311.0 \pm 4.0	n.s.	332.6 \pm 18.2	322.4 \pm 7.3	n.s.	n.s.	n.s.
Ti [ms]	52.6 \pm 1.8	53.8 \pm 0.9	n.s.	52.6 \pm 4.2	51.1 \pm 1.7	n.s.	n.s.	n.s.
Te [ms]	133.0 \pm 2.8	139.3 \pm 2.5	n.s.	130.0 \pm 6.0	135.4 \pm 3.4	n.s.	n.s.	n.s.
Ti/TT	0.28 \pm 0.01	0.28 \pm 0.01	n.s.	0.29 \pm 0.01	0.27 \pm 0.01	n.s.	n.s.	n.s.
Activity	(n=6)			(n=6)				
f [1/min]	462.8 \pm 7.4	472.9 \pm 8.1	n.s.	458.0 \pm 13.3	463.1 \pm 5.2	n.s.	n.s.	n.s.
Ti [ms]	43.9 \pm 0.8	41.8 \pm 0.5	n.s.	43.7 \pm 0.9	41.3 \pm 0.9	n.s.	n.s.	n.s.
Te [ms]	85.9 \pm 1.7	85.3 \pm 1.9	n.s.	87.9 \pm 2.9	88.3 \pm 1.8	n.s.	n.s.	n.s.
Ti/TT	0.34 \pm 0.01	0.33 \pm 0.00	n.s.	0.33 \pm 0.01	0.32 \pm 0.01	n.s.	n.s.	n.s.

Table 32: Tidal volume and flow rates at sleep, rest and activity

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

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=6)	(n=7)	<i>p</i> - value	(n=6)	(n=5)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Sleep		(n=6)			(n=3)			
TV [ml]		0.24 \pm 0.01			0.24 \pm 0.02			n.s.
PIF [ml/s]		5.0 \pm 0.4			4.4 \pm 0.2			n.s.
PEF [ml/s]		2.5 \pm 0.2			2.2 \pm 0.2			n.s.
MIF [ml/s]		2.9 \pm 0.2			2.5 \pm 0.1			n.s.
MEF [ml/s]		0.89 \pm 0.08			0.75 \pm 0.01			n.s.
Rest	(n=5)	(n=7)		(n=5)	(n=5)			
TV [ml]	0.26 \pm 0.02	0.24 \pm 0.02	n.s.	0.23 \pm 0.01	0.21 \pm 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	8.3 \pm 0.5	7.6 \pm 0.5	n.s.	7.6 \pm 0.5	7.0 \pm 0.3	n.s.	n.s.	n.s.
PEF [ml/s]	4.6 \pm 0.2	4.0 \pm 0.3	n.s.	3.9 \pm 0.3	3.6 \pm 0.2	n.s.	n.s.	n.s.
MIF [ml/s]	4.9 \pm 0.3	4.4 \pm 0.3	n.s.	4.5 \pm 0.3	4.0 \pm 0.2	n.s.	n.s.	n.s.
MEF [ml/s]	2.0 \pm 0.2	1.7 \pm 0.1	n.s.	1.8 \pm 0.1	1.5 \pm 0.1	n.s.	n.s.	n.s.
Activity	(n=6)			(n=6)				
TV [ml]	0.28 \pm 0.02	0.23 \pm 0.01	n.s.	0.25 \pm 0.01	0.22 \pm 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	10.3 \pm 0.5	9.4 \pm 0.5	n.s.	9.2 \pm 0.4	8.7 \pm 0.4	n.s.	n.s.	n.s.
PEF [ml/s]	6.9 \pm 0.3	6.0 \pm 0.4	n.s.	5.9 \pm 0.5	5.4 \pm 0.4	n.s.	n.s.	n.s.
MIF [ml/s]	6.3 \pm 0.3	5.6 \pm 0.3	n.s.	5.6 \pm 0.3	5.3 \pm 0.3	n.s.	n.s.	n.s.
MEF [ml/s]	3.2 \pm 0.2	2.7 \pm 0.2	n.s.	2.8 \pm 0.2	2.5 \pm 0.2	n.s.	n.s.	n.s.

Table 33: Minute ventilation and body size/weight related parameters at sleep, rest and activity
 Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=6)	(n=6)	<i>p</i> - value	(n=6)	(n=6)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Sleep		(n=6)			(n=3)			
sTV [μ l/g]		11.2 \pm 0.8			12.2 \pm 0.5			n.s.
MV [ml/min]		40.7 \pm 3.6			34.2 \pm 0.4			n.s.
sMV [ml/min/g]		1.9 \pm 0.2			1.7 \pm 0.0			n.s.
Rest	(n=5)	(n=7)		(n=5)	(n=5)			
sTV [μ l/g]	8.0 \pm 0.5	10.8 \pm 0.7	< 0.02	9.1 \pm 0.4	10.0 \pm 0.4	n.s.	n.s.	n.s.
MV [ml/min]	82.5 \pm 7.5	71.4 \pm 5.4	n.s.	76.0 \pm 4.4	64.3 \pm 3.4	n.s.	n.s.	n.s.
sMV [ml/min/g]	2.5 \pm 0.2	3.3 \pm 0.2	n.s.	3.0 \pm 0.3	3.1 \pm 0.1	n.s.	n.s.	n.s.
Activity	(n=6)			(n=6)				
sTV [μ l/g]	8.5 \pm 0.4	10.7 \pm 0.5	< 0.01	9.7 \pm 0.7	10.7 \pm 0.5	n.s.	n.s.	n.s.
MV [ml/min]	127.9 \pm 6.6	109.3 \pm 6.0	n.s.	112.5 \pm 7.2	101.6 \pm 7.0	n.s.	n.s.	n.s.
sMV [ml/min/g]	3.9 \pm 0.2	5.0 \pm 0.2	< 0.01	4.5 \pm 0.4	4.9 \pm 0.3	n.s.	n.s.	n.s.

3.12 Molecular Phenotyping

3.12.1 Introduction

Comparative genome-wide expression profiling is a powerful tool in the effort to annotate the mouse genome with biological function. The analysis of RNA expression data of mouse lines might support the understanding of the molecular biology of such mutants and provide new insights into mammalian gene function. We demonstrated the feasibility to detect transcriptional affected organs employing RNA expression profiling as a tool for molecular phenotyping (Seltmann *et al.*, 2005). Within the German Mouse Clinic (GMC) we demonstrated the efficiency of systematic genome-wide expression profiling for the detection of molecular phenotypes in organs of a mammalian model organism (Horsch *et al.*, 2008).

Brain and muscle were selected for the *Gsk3 α* knockdown mouse line due to strong expression of the mutated gene in these organs. Additionally, the selection of brain based on phenotypical alteration identified in the behavior screen. In this report, we describe the results of using close to genome-wide 21K cDNA microarrays for the RNA expression profiling of the selected organs of six animals of the *Gsk3 α* mutant mouse line. In total 24 chip hybridizations including biological and experimental replicates were performed.

The data analysis and various statistical methods detected a limited number of genes differentially regulated between mutant and reference tissues in brain and muscle. Gene significantly regulated in muscle are associated with blood circulation and gas exchange and protein phosphorylation.

3.12.2 Methods and Materials

Organ Collection

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Twelve male mice (6 mutant and 6 reference animals) of the *Gsk3 α* mutant mouse line were provided to the molecular phenotyping screen.

Organs were collected at the age of 37 weeks. To minimize the influence of circadian rhythm on gene expression, mice were killed between 9 am and 12 am by carbon dioxide asphyxiation. The following ten organs were collected and archived in liquid nitrogen following our established SOPs (Standard operation protocols): spleen, kidney, testis, liver, heart, lung, thymus, skin/cartilage (outer ear), skeletal muscle and brain. Organs were immediately frozen and stored in liquid nitrogen until isolation of total RNA. The 100 organ samples collected either may be used for further expression profiling analysis in the GMC or, alternatively may be transferred to the collaborator.

Isolation of total RNA

Total RNA was isolated just before processing for expression profiling. For preparation of total RNA individual organs were thawed in buffer containing

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

Chip Hybridization

Two chip hybridizations were performed with RNA from all selected organs of each individual mutant mouse. Each chip hybridization was performed against the identical pool of the same organ of reference RNAs (reference RNA pool). For each individual mutant mouse the chip experiments include a color-flip experiment (in total 12 hybridizations/organ).

Reverse Transcription and Fluorescent Labeling

For labeling 15 µg of total RNA were used for reverse transcription and indirectly labeled with Cy3 or Cy5 fluorescent dye according the TIGR protocol (Hegde *et al.*, 2000, Horsch *et al.*, 2008). Labeled cDNA was dissolved in 50 µl hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardt's solution and 50% formamide) and mixed with 50 µl of reference cDNA solution (pool from six control animals) labeled with the second dye. This hybridization mixture was injected on a pre-hybridized microarray in a HS4800 Hybstation (Tecan) and incubated at 42°C for 16 hours. After hybridization slides were washed with 3x SSC, 1x SSC, 0.5xSSC and 0.1x SSC at room temperature. Slides were dried with nitrogen. Dried slides were scanned with a GenePix 4000A microarray scanner and the images were analyzed using the GenePix Pro6.1 image processing software (Axon Instruments, USA) (Drobyshev *et al.*, 2003; Beckers *et al.*, 2005; Seltmann *et al.*, 2005; Greenwood *et al.*, 2005; Mijalski *et al.*, 2005, Frey *et al.*, 2007).

Significance of Regulation

TIGR software package for Microarray analysis (TM4; Chu *et al.*, 2002, Saeed *et al.*, 2003) was used for normalization (MIDAS: Microarray Data Analysis System; Quackenbush 2002) and identification of genes with significant differential regulation (SAM, Significance Analysis of Microarrays; Tusher *et al.*, 2001). Expression data were normalized performing a total intensity normalization to transform the mean log₂ ratio to zero. To eliminate low-quality array elements several filtering methods were applied. They include: Background checking for both channel with a signal/noise threshold of 2.0, one bad tolerance policy parameter and flip dye consistency checking (Yang *et al.*, 2002).

SAM was used to identify genes with statistically significant changes in expression. Genes were ranked according to their relative difference value $d(i)$, a score assigned to each gene on the basis of change in gene expression levels relative to the standard deviation. Genes with $d(i)$ values greater than a threshold were selected as significantly differentially expressed. The percentage of such genes identified by chance is the false discovery rate (FDR). To estimate the FDR, nonsense genes were identified by calculating permutations of the measurements. The selection of the top differentially expressed

genes with reproducible up- or down-regulation includes less than 10% false positives (FDR).

Panther Classification system

To select pathways relevant for the selected genes of an organ we used the Panther Classification system (Thomas *et al.*, 2003). Significant regulated genes were mapped to molecular function and biological process categories, as well as biological pathways.

3.12.3 Results

Selection of Organs and RNA isolation

Brain and muscle were selected as organs for expression profiling analysis. We isolated total RNA of these organs of six mutant mice and six wild-type control animals (Table 34).

Table 34: Amount of total RNA [μg] isolated from brain and muscle			
Mouse ID	Genotype	Muscle	Brain
30066344	wt	124	305
30066345	wt	124	342
30066346	wt	40	348
30066347	wt	90	310
30066354	mut	50	310
30066355	mut	76	322
30066356	mut	54	153
30066356	wt	103	306
30066358	wt	121	268
30066359	mut	141	240
30066360	mut	65	261
30066362	mut	83	298

Chip experiments

Twelve hybridizations were performed for brain and muscle (in total 24 chip experiments).

Gene regulation in brain

Table 35 summarizes the results of 10 chip hybridisations performed with RNA from brain. Inspection of expression data from individual mice revealed a stronger correlation of regulated genes in samples #354, 355, 359, 360 and 362. Expression pattern of sample #356 does not correlate to the differentially expressed genes in the other five mice. Therefore, the data of mouse #356 were excluded from further analysis. Statistical analysis identified only one significantly regulated gene. The estimated number of falsely significant genes was calculated for 1000 permutations, yielding a FDR of 0% for this data set including 10 chip experiments.

Table 35: Heat plots of gene expression profiles from 10 DNA microarray experiments of *Gsk3α*-mutant versus control mice.

One dye-flip pair represents two experimental replicates of each analyzed animal. One Array TAG ID is the unique probe identifier from the Lion Bioscience clone set. Official gene symbols are given. The scale bar encodes the log ratio of the fold induction; 0% of the elements are above the upper limit of the colour range selection (red is up-regulated and green down-regulation in mutant mice).

Mean log2 ratio	ArrayTAG ID	Gene symbol	Comment
0.77	MG-16-146d16	H2-Q9	histocompatibility 2, Q region locus 9

Gene regulation in muscle

Table 36 summarizes the results of 10 chip hybridisations performed with RNA from muscle. Inspection of expression data from individual mice revealed a stronger correlation of regulated genes in samples #354, 355, 356, 360 and 362. Expression pattern of sample #359 does not correlate to the differentially expressed genes in the other five mice. Therefore, the data of mouse #359 were excluded from further analysis. Statistical analysis identified 13 significantly regulated genes in 10 chip experiments of five mice. All genes showed decreased expression levels. The estimated number of falsely significant genes was calculated for 1000 permutations, yielding a FDR of 2.6% for this data set including all chip experiments.

Table 36: Heat plots of gene expression profiles from 10 DNA microarray experiments of *Gsk3α*-mutant versus control mice.

One dye-flip pair represents two experimental replicates of each analyzed animal. One Array TAG ID is the unique probe identifier from the Lion Bioscience clone set. Official gene symbols are given. The scale bar encodes the log ratio of the fold induction; 2.8% of the elements are above the upper limit of the colour range selection (red is up-regulated and green down-regulation in mutant mice).

Mean log2 ratio	ArrayTAG ID	Gene symbol	Comment
-0.84	MG-14-64n9	<i>Mark3</i>	MAP/microtubule affinity-regulating kinase 3
-0.70	MG-4-2e20	<i>Hba-a1</i>	Hemoglobin alpha, adult chain 1
-0.62	MG-4-145j12	<i>Hba-a1</i>	Hemoglobin alpha, adult chain 1
-0.62	MG-4-147o3	<i>Hba-a1</i>	Hemoglobin alpha, adult chain 1
-0.63	MG-4-146n10	<i>Plcl2</i>	Phospholipase C-like 2
-0.75	MG-8-54o6	<i>Rlf</i>	rearranged L-myc fusion sequence
-0.60	MG-4-5m17	<i>Hba-a2</i>	hemoglobin alpha, adult chain 2
-0.80	MG-4-3k8	<i>1300018I05Rik</i>	
-0.76	MG-4-146d10	<i>Tysnd1</i>	Trypsin domain containing 1
-0.65	MG-4-3k1	<i>Hba-a1</i>	Hemoglobin alpha, adult chain 1
-0.72	MG-15-226i17	<i>Ube1dc1</i>	Ubiquitin-activating enzyme E1-domain containing 1
-0.68	MG-4-3b2	<i>Hba-a2</i>	hemoglobin alpha, adult chain 2
-0.72	MG-8-86g2	<i>1110017I16Rik</i>	

Classification by molecular functions and biological processes

In a further analysis significantly regulated genes in muscle were classified by two categories of Gene Ontology Terms (Molecular Functions and Biological Processes) using the PANTHER classification system. Additionally, genes were mapped to biological pathways (Table 37).

Table 37: PANTHER classification				
Gene symbol	Molecular function	Biological processes	Pathway	Chr.
<i>1110017116Rik</i>	not classified	not classified		2
<i>1300018105Rik</i>	not classified	not classified		17
<i>Hba-a1</i>	Transfer/carrier protein	Transport; Blood circulation and gas exchange		11
<i>Mark3</i>	Non-motor microtubule binding protein	Protein phosphorylation; Cell structure		12
<i>Plcl2</i>	signaling molecule	not classified	Oxytocin receptor mediated signaling pathway	17
<i>Rlf</i>	Transcription factor; Nuclease	Nucleoside, nucleotide and nucleic acid metabolism		4
<i>Tysnd1</i>	not classified	not classified		10

3.12.4 Discussion

Significantly regulated genes could be identified in brain and muscle. In muscle all regulated genes show decreased expression levels. In brain only one significantly up-regulated gene was identified.

Inspection of expression data of brain and muscle from individual mice revealed non-correlation of the expression patterns in one of six samples for each organ. Maybe biological variability in gene expression oscillation or stress-responsive genes are potential reasons for non-correlation in the expression patterns between single mice. In addition, several recent publications have provided evidence for biological variability of expression levels for particular genes (Pritchard *et al.*, 2001; Churchill *et al.*, 2002; Oishi *et al.*, 2003).

The differential expression of *Hba-a1* was confirmed by four independent probes on the array in muscle. These different probes for the identical gene show similar regulation. The fact, that independent probes for the same gene show similar expression levels give additional confidence in these expression profiling data.

Changes of expression levels of several types of hemoglobin in muscle could be taken as a phenotypical alteration in the *Gsk3 α* mutant mouse line but it also could be caused by adherence of blood. During organ collection usually as much as possible blood is removed from the organs, nevertheless some blood still remains on the tissue.

Comparison with Gsk3beta

We compared gene expression patterns of GSK3 β of brain and muscle with those of GSK3 α . Three genes were significantly expressed in muscle of both mutant mouse lines: *Hba-a1*, *Hba-a2*, and *Tysnd1*. *Tysnd1*, a previously uncharacterized protein, is responsible both for the removal of the leader peptide from PTS2 (peroxisomal signal targeting sequences) proteins and for the specific processing of PTS1 proteins. All of the identified *Tysnd1* substrates catalyze peroxisomal beta-oxidation. *Tysnd1* itself undergoes processing through the removal of the presumably inhibitory N-terminal fragment. *Tysnd1* expression is induced by the proliferator-activated receptor alpha agonist bezafibrate, along with the increase in its substrates. A model is proposed where the *Tysnd1*-mediated processing of the peroxisomal enzymes promotes their assembly into a supramolecular complex to enhance the rate of beta-oxidation (PMID 17255948). No further overlap between the two analyzed MMLs was identified.

3.12.5 Conclusion

Using the statistical methods described above, a limited number of genes that are differentially expressed in brain and muscle in *Gsk3 α* -mutant mice were identified. The relevance of these genes in terms of the studied allele should be evaluated. This may be done by a detailed inspection of the functional annotations for each of these genes, as initiated here in the report. Please, contact us if you have questions concerning this analysis.

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3.13 Pathology Screen

3.13.1 Summary

The pathology primary screen performed a complete morphological analysis with standard stains. The *Gsk3 α* mutant mice lack the vacuolization of the brain, which was present in some of the *Gsk3 β* mutants in the H&E stained sections. However, Luxol fast blue staining was performed in a secondary screen. Interestingly, some mutant mice (4/6), mainly females (3/3), exhibit reduced myelin density in some structures of the brain (e.g. the corpus callosum and the mammillothalamic tract), which is suspicious of the presence of demyelination similar to the *Gsk3 β* mice (not statistically significant).

As a secondary result (not genotype-associated), half of the mice, predominantly the females showed mild dermatitis at thorax and shoulder (83% female controls, 63% female mutants, 40% male controls, and 25% male mutants).

3.13.2 Mice

A total of 31 mice were analyzed macroscopically. Histological analysis was performed in 28 mice, 16 mutant mice (8 females, 8 males) and 12 control mice (7 females, 5 males) at the age of 37 to 38 weeks. The analysis included seven mice, which died during the blood taking (6 mutants [2 females and 4 males] + 2 controls [one female and one male]) at the age of 33 to 37 weeks.

	Control		Mutant		Number of Animals	Age [weeks]
	Female	Male	Female	Male		
Number of Animals	7	5	8	8	31	33-38

3.13.3 Materials and Methods

Primary screening

Mice received in the laboratory of pathology were sacrificed with CO₂. The animals were analyzed macroscopically and weighed (http://eulep.anat.cam.ac.uk/Necropsy_of_the_Mouse/). 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, 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).

Secondary screening

Additionally, Luxol fast blue staining of the brain was performed in 12 mice (6 mutants [3 females and 3 males] + 6 controls [3 female and 3 male]).

3.13.4 Results

Overview

Table 39: Genotype-specific alterations of <i>Gsk3α</i> -knockdown mice:			
Analyzed:	Alteration	Analyzed:	Alteration
Bodyweight	Yes	Pancreas	No
Skin	No	Cervical lymph node	No
Musculoskeletal system	No	Thymus	No
Eyes	No	Spleen	No
Cerebrum	Yes	Thyroid	No
Cerebellum	Yes	Parathyroid	No
Heart	No	Adrenal gland	No
Trachea	No	Kidneys	No
Lung	No	Urinary bladder	No
Teeth	No	Testes	No
Salivary gland	No	Epididymis	No
Esophagus	No	Funiculus spermaticus	No
Stomach	No	Ovaries	No
Small intestine	No	Uterus	No
Large intestine	No	Vagina	No
Liver	No	Mamma	No

With the exception of a slightly reduced body weight (see results of other screens), the *Gsk3 α* mice showed no genotype-specific morphological phenotype in the primary screen. In a secondary screen, a mild to moderate demyelination of some structures of the brain was revealed in half of the mutants (4/6), mainly in females (3/3). Although only mutants exhibited this demyelination, the difference is not statistically significant (measured by Fisher's exact test; p-value > 0.05).

Brain

The brain of the *Gsk3 α* mutant mice did not show any histological abnormality (vacuolization) in the H&E stained sections (see Figure 21). However, the myelin-specific Luxol fast blue staining revealed a reduced myelin density in some structures of the brain of 4/6 mutants (3/3 females and 1/3 males), which can be diagnosed as early demyelination (see Table 40 and Figures 22 to 24 for more details). Especially the corpus callosum and the mammi-

thalamic tract are involved in this process. The alteration was graduated from mild to moderate. Severe demyelination could not be documented.

Table 40: Demyelination of <i>Gsk3α</i>-knockdown mice in detail			
Sex	Genotype	Sect. no. 07/ (Mouse-ID)	Demyelination
Female	Control	1396 (30066404)	No
Female	Control	1406 (30066392)	No
Female	Control	1407 (30066393)	No
Female	Mutant	1399 (30066383)	Moderate
Female	Mutant	1400 (30066384)	Mild
Female	Mutant	1404 (30066390)	Moderate
Male	Control	1375 (30066353)	No
Male	Control	1378 (30066369)	No
Male	Control	1379 (30066371)	No
Male	Mutant	1372 (30066350)	No
Male	Mutant	1374 (30066352)	Mild
Male	Mutant	1377 (30066366)	No

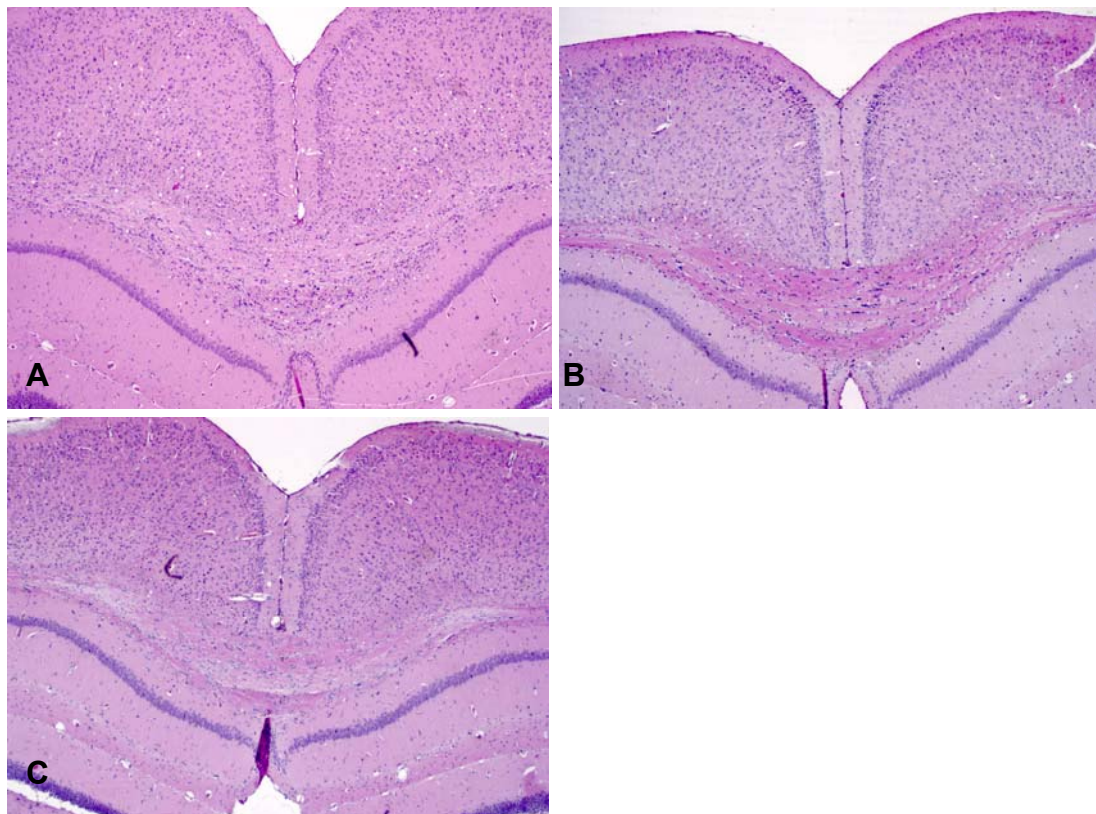


Figure 21: Brain: H&E stained sections

A. For comparative purposes, note the vacuolization and demyelination of the cerebral corpus callosum and the cingulum, which derived from a ***Gsk3β*** mouse. **B.** and **C.** (male ***Gsk3α*** mutants no. 07/1376 and no. 07/1374). In comparison, the ***Gsk3α*** mutant mice do not exhibit vacuolization. However, a slightly reduced density of the corpus callosum is suspicious of demyelination in picture **C.** (H&E, original magnification 40x).

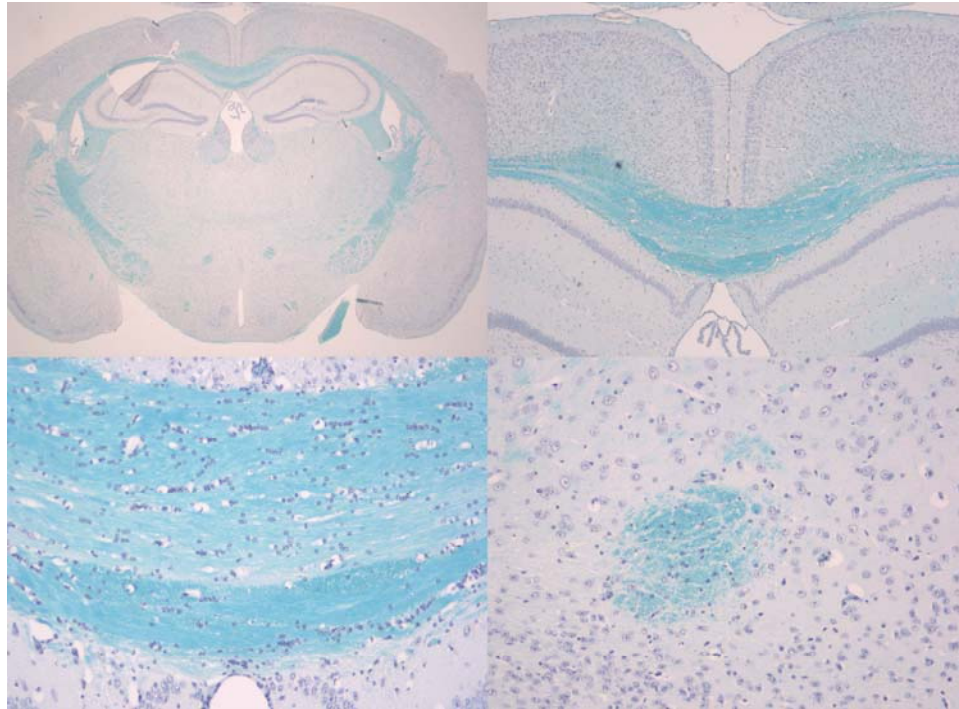


Figure 22: Brain: Luxol fast blue stained sections of a control mouse

This control mouse shows a very strong intensity of the myelin staining (control female # 07/1406). **Upper row left:** Cross section of the brain (original magnification 12.5x). **Upper row right:** The region of the corpus callosum (c.c.; original magnification 40x). **Lower row left:** Corpus callosum at higher magnification (160x). **Lower row right:** The mammillothalamic tract (mmt; original magnification 160x).

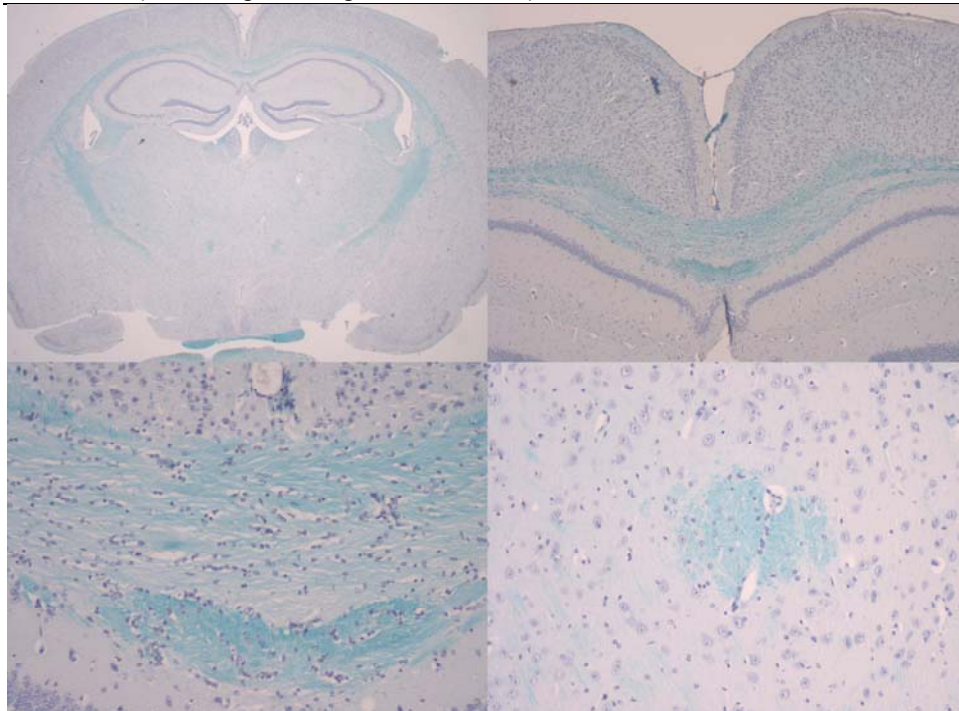


Figure 23: Brain: Luxol fast blue stained sections of a male mutant mouse

This male mutant mouse shows a slightly reduced intensity of the myelin staining, scored as mild demyelination (mutant male No. 07/1374). The demyelination is most obvious in the central area of the corpus callosum. **Upper row left:** Cross section of the brain (original magnification 12.5x). **Upper row right:** The region of the corpus callosum (c.c.; original magnification 40x). **Lower row left:** Corpus callosum at higher magnification (160x). **Lower row right:** The mammillothalamic tract. (mmt; original magnification 160x).

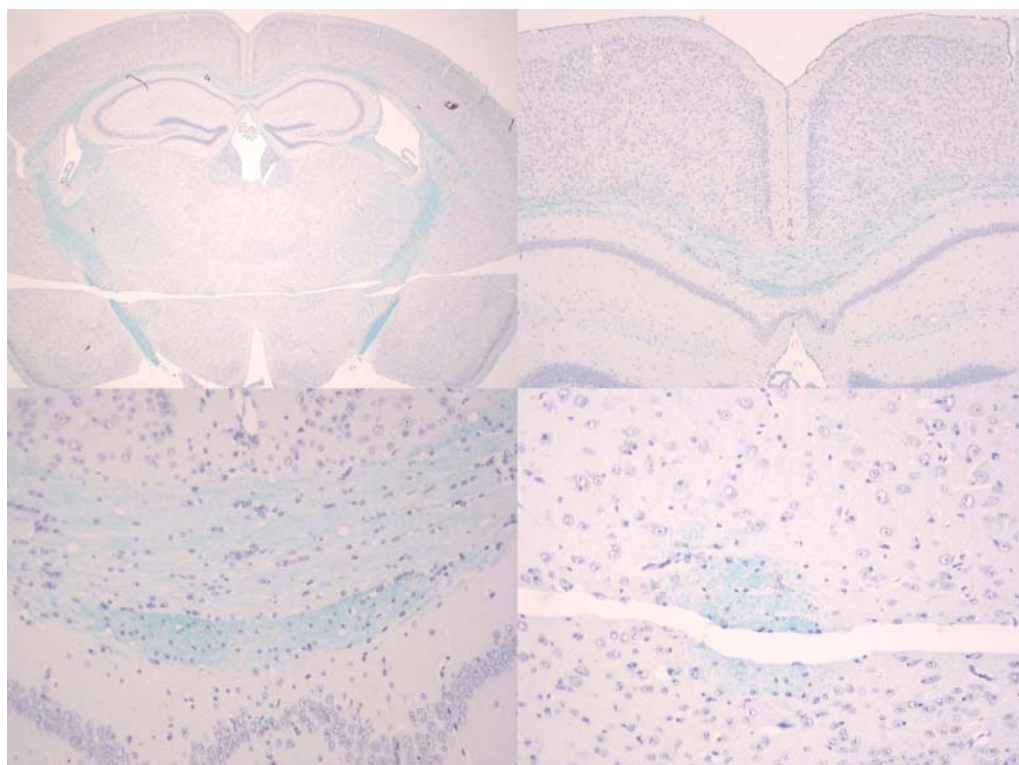


Figure 24: Brain: Luxol fast blue stained sections of a female mutant mouse

This female mutant mouse shows a reduced intensity of the myelin staining, scored as moderate demyelination (mutant female No. 07/1399). **Upper row left:** Cross section of the brain (original magnification 12.5x). **Upper row right:** The region of the corpus callosum (c.c.; original magnification 40x). **Lower row left:** Corpus callosum at higher magnification (160x). **Lower row right:** The mammillothalamic tract. (mmt; original magnification 160x).

3.13.5 Secondary, not Genotype-Specific Results

Inflammatory changes

Half of the mice, predominantly the females showed inflammatory changes of the skin. This mild dermatitis with mixed inflammatory cell infiltration and hyperkeratosis at thorax and shoulder was found in 83% (5/7) female controls, 63% (5/8) female mutants, 40% (2/5) male controls, and 25% (2/8) male mutants.

3.13.6 Discussion

The primary screen did not reveal a histopathological phenotype of the *Gsk3 α* knockdown mice; with the exception of a reduced body weight (see results of the other screens).

In the secondary screen, mild to moderate demyelination of some structures of the brain could be detected in 3/3 female and 1/3 male mutant mice (p-value > 0.05, n.s.), and in none of the six controls. In contrast to the *Gsk3 β* knockdown mice, the severe phenotype with vacuolization was not present in the analyzed mice. The lack of statistical significance (measured by Fisher's exact test) could be due to the low number of analyzed mice. Alternatively, a

reduced penetrance or an individual variability of the gene expression of the mutants could it explain. The demyelination was most prominent in the corpus callosum and the mammillothalamic tract. To establish a correlation between behavioral abnormalities and histopathological alterations, a detailed analysis of specific loci of the brain should be done.

As a secondary result (not genotype associated), half of the mice, predominantly the females showed mild dermatitis at the thorax (83% female controls, 63% female mutants, 40% male controls, and 25% male mutants). This inflammatory alteration should be taken into account when interpreting the hematological and immunological abnormalities.

See report of the *Gsk3 β* mutant mice for a more detailed discussion of the gene involved.

3.13.7 References

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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, Susanne Axtner, Miriam Backs, Christine Fürmann, Tamara Halex, Sabine Holthaus, Nadine Kink, Claudia Kloss, Regina Kneuttinger, Kerstin Kutzner, Maria Kugler, Jacqueline Müller, Elenore Samson, Sandra Schädler, Ann-Elisabeth Schwarz, Bettina Sperling, Susanne Wittich, and Claudia Zeller for expert technical help and Daniela Kißling, Manuela Huber, Petra Thalmeier, Sabine Schwarz, and Anica Miedl 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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