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Behavioural and expressional phenotyping of nitric oxide synthase-I knockdown animals

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Summary The gaseous messenger nitric oxide (NO) has been implicated in a wide range of behaviors, including aggression, anxiety, depression, and cognitive functioning. To further elucidate the physiological role of NO and its down-stream mechanisms, we conducted behavioral and expressional phenotyping of mice lacking the neuronal isoform of nitric oxide synthase (NOS-I), the major source of NO in the central nervous system. No differences were observed in activity-related parameters; in contrast to the a priori hypothesis, derived from pharmacological treatments, depression-related tests (Forced Swim Test, Learned Helplessness) also yielded no significantly different results. A subtle anxiolytic phenotype however was present, with knockdown mice displaying a higher open arm time as compared to their respective wildtypes, yet all other investigated anxiety-related parameters were unchanged. The most prominent feature however was gender-independent cognitive impairment in spatial learning and memory, as assessed by the Water Maze test and an automatized holeboard paradigm. No significant dysregulation of monoamine transporters was evidenced by qRT PCR. To further examine the underlying molecular mechanisms, the transcriptome of knockdown animals was thus examined in the hippocampus, striatum and cerebellum by microarray analysis. A set of >120 differentially expressed genes was identified, whereat the hippocampus and the striatum showed similar expressional profiles as compared to the cerebellum in hierarchical clustering. Among the most significantly up-regulated genes were *Peroxiredoxon 3, Atonal homologue 1, Kcnj1, Kcnj8, CCAAT/enhancer binding protein (C/EBP), alpha*, 3 genes involved in GABA(B) signalling and, intriguingly, the glucocorticoid receptor *GR*. While GABAergic genes might underlie reduced anxiety, dysregulation of the glucocorticoid receptor can well contribute to a blunted stress response as found in *NOS1* knockdown mice. Furthermore, by CREB inhibition, glucocorticoid receptor up-regulation could at least partially explain cognitive deficits in these animals. Taken together, *NOS1* knockdown mice display a characteristic behavioural profile consisting of reduced anxiety and impaired learning and memory, paralleled by differential expression of the glucocorticoid receptor and GABAergic genes. Further research has to assess the value of these mice as animal models e.g. for Alzheimer's disease or attention deficit disorder, in order to clarify a possible pathophysiological role of NO therein.

Keywords: Knockout, mouse, NO, NOS-I, microarray, gene chip, ADHD, depression

Abbreviations

BDNF	brain derived neurotrophic factor
CREB	cyclic AMP response element-binding protein
DAT	dopamine transporter
GABA	γ-amino-butyric acid
GR	glucocorticoid receptor
5-HT	serotonin
5-HTT	serotonin transporter
LTP	long-term potentiation
NO	nitric oxide
NOS	nitric oxide synthase
PCP	phenylcyclidine
SSRI	selective serotonin reuptake inhibitor

Introduction

The gaseous messenger molecule nitric oxide (NO) is synthesized from its precursor L-arginine by a family of three NO synthases (NOS), designated as ''neuronal'' NOS-I, ''inducible'' NOS-II and ''endothelial'' NOS-III. In the adult brain, the inducible isoform NOS-II is present only at very low levels in microglia and immune cells, while ''endothelial'' NOS-III is expressed predominantly in the vasculature. Whether or not this isoform is also expressed in neural cells, is still a matter of debate but data arguing

Dedicated to Prof. Dr. Peter Riederer on occasion of his 65th birthday.

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for this are only sparse. The quantitatively major source for NO in the CNS thus is the ''neuronal'' isoform NOS-I present in approximately 1% of all neurons. Nitrinergic transmission is especially important in limbic structures, in the basal ganglia – where NO regulates striatal output – and in the cerebellum (Snyder and Ferris, 2000). NO exerts multiple actions in the CNS and from animal studies, it has been suggested that it is involved in behavioral processes such as learning and memory formation. Pathologies of the NO pathway have been implicated in almost every major neu-

Table 1. *Summarized behavioural phenotype of NOS1 knockdown and NOS3 knockout animals. Findings of the present study are printed in bold*

Test	NOS-I knockdown	NOS-III knockout
General		
Sensorimotor screening/ observation	Increased touch-escape reaction, body position, locomotion, elevation and reduced vocalization, increased grooming → ''anxiety like behaviour'' (Weitzdoerfer et al., 2004)	Increased forelimb strength, otherwise no difference (Demas et al., 1999)
Pole/plank test (balance/ coordination)	Nocturnal impairment (Kriegsfeld et al., 1999); no difference (not shown; (Nelson et al., 1995))	No difference (Demas et al., 1999; Dere et al., 2002)
Rotarod	No difference (Chiavegatto et al., 2001); no difference (Kirchner et al., 2004); **No difference**	Not published
Hotplate (pain sensitivity)	Increased sensitivity to pain ((Nelson et al., 2006), unpublished). **No difference**	No difference (Reif et al., 2004)
Activity & Novelty		
Open field	More active during the active cycle, more time spent in center of the open field (Bilbo et al., 2003); no difference (not shown; (Nelson et al., 1995)); more center crossings and more center entries (Weitzdoerfer et al., 2004); normal (Chiavegatto et al., 2001); Higher center time/crossings (Kirchner et al., 2004); No difference (Salchner et al., 2004); **No difference**	Reduced exploratory activity with no habituation; more time spent in center of open field (Dere et al., 2002); no difference (not shown; (Demas et al., 1999)); less activity, more time spent in corners (Frisch et al., 2000); no difference (Reif et al., 2004)
Novelty seeking	**No difference**	Not published
Emotionality & Depression		
Light-Dark-Box	**No difference**	No difference (Reif et al., 2004)
Porsolt	Reduced immobility time (Salchner et al., 2004); fewer depression-like responses ((Nelson et al., 2006), unpublished); **No difference**	No difference (Reif et al., 2004)
Learned helplessness	**No difference**	Better & faster learning (Reif et al., 2004)
Tail suspension	n.p.	Not published
Novel cage	**No difference**	No difference (Reif et al., 2004)
Elevated plus maze	No difference (Bilbo et al., 2003); more time spent in closed arm (Weitzdoerfer et al., 2004), more entries in closed arm (Kirchner et al., 2004); **Higher open arm time**	Less locomotion in open arms, otherwise no difference (Dere et al., 2002); no difference (Demas et al., 1999); less time in open arms, more time in closed arms (Frisch et al., 2000)
Mazes & Learning		
COGITAT/holeboards	**Impaired spatial learning**	Not published
8-Arm radial maze	n.p.	No difference (Dere et al., 2001)
Morris water maze	Worse performance (Weitzdoerfer et al., 2004); worse performance (Kirchner et al., 2004); **Impaired spatial learning**	Superior performance (Frisch et al., 2000)
T-Maze	Better performance (Weitzdoerfer et al., 2004)	Not published
Aggression & Maternality		
Resident-intruder aggression	Males – increase (Nelson et al., 1995), testosterone-dependent (Kriegsfeld et al., 1997); not in BL/6 back-crossed mice (Le Roy et al., 2000)	Male animals – no aggression (Demas et al., 1999)
Neutral aggression	Males – increase (Nelson et al., 1995), testosterone-dependent (Kriegsfeld et al., 1997); not in BL/6 back-crossed mice (Le Roy et al., 2000)	Male animals – no aggression (Demas et al., 1999)
Maternal aggression	Absent (Gammie and Nelson, 1999)	No difference (Gammie et al., 2000)
Maternal behavior	Otherwise no difference (Gammie and Nelson, 1999)	No difference (Gammie et al., 2000)
Stress		
Baseline corticosterone	Higher in knockout mice (Bilbo et al., 2003)	n.p.
Stress-induced increase	Dampened corticosterone response in knockout animals (Bilbo et al., 2003)	n.p.

ropsychiatric disorder including schizophrenia (Bernstein et al., 2005), affective disorders (van Amsterdam and Opperhuizen, 1999), alcoholism (Gerlach et al., 2001), Alzheimer's dementia (Law et al., 2001), Parkinson's and Huntington's disease (Hunot et al., 1996). For some of these disorders, NOS-I has also been identified as a risk gene in human case-control association studies (Galimberti et al., 2007; Reif et al., 2006a, b). The role of NO in the regulation of normal human brain functioning however is still unclear, although first genetic studies argue for a function of NOS-I in the regulation of impulsive behaviors.

Knockout animals are valuable tools to identify both the behavioral impact of a given gene, as well as subsequent changes of the transcriptome to correlate behavior to molecular pathways. With respect to NOS-I, two genetically modified mouse strains have been described in the literature. While in the recently generated KOex6 knockout, disruption of NOS-I exon 6 results in the complete absence of catalytically active NOS-I (Packer et al., 2003), previously generated animals harbor a targeted deletion of exon 1 (Nelson et al., 1995). The latter results in a loss of the PDZ binding domain and thus residual NOS-I expression of up to 7%, rendering these mice actually NOS-I knockdown animals. This situation may more closely mirror human genetic variation of NOS-I, since a complete disruption of the gene has not yet been described in man. There are several studies on the behavioral phenotype of these animals (as summarized in Table 1); however, they are in part contradictory and lack dedicated investigations of depression-like behaviors. Thus, we aimed to perform detailed behavioral phenotyping of *NOS1* knockdown animals with special emphasis on depression- and ADHD-relevant tests. To reveal molecular mechanisms underlying the behavioral phenotype, we also performed a microarray study using a custom made gene chip featuring almost 1.000 genes which have been a priori selected for their relevance to CNS functioning.

Materials and methods

Animals

For behavioural experiments, wildtype control (+/+) and homozygous NOS-I knockdown (−/−) mice aged between 2 and 6 months were used. In all experiments, the respective controls were wildtype littermates. For the micorarray study, an additional set of 7 knockdown (−/−) and 7 wildtype controls (+/+) were examined, which were also littermates. All animals had the same genetic background (C57BL/6, for review see Huang et al., 1995) and were housed under identical conditions. Genotype was confirmed in each animal by PCR, and also immunohistochemistry showed complete loss of NOS-I protein in the hippocampus, striatum and the cortex (data not shown). All animal protocols have been reviewed and approved by the review board of the Government of Lower Franconia and the University of Würzburg and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

Behavioural analyses

All experiments were preceded by an acclimatization period of approximately 30 min (Forced Swim Test, Hotplate, Learned Helplessness, Novel Cage, Rotarod) or 24 h (Elevated Plus Maze, Open Field, Water Maze, COGITAT holeboard) to the experimental room. During all experiments, the experimenter was blind to the genotype.

Barrier test

The Barrier test was performed in a type II macrolon cage, which was divided into two sections by a 1 cm high hurdle. Observation was conducted with red light illumination assessing the latency to cross the barrier within a maximum duration of 300 sec.

Open field test

The open field consisted of a PVC plastic box ($82 \times 82 \times 25$ cm).

Activity monitoring was conducted using the computer-based video tracking software VideoMot 2 (TSE, Bad Homburg). Illumination at floor level was 200 lux. The area of the open field was divided into a 70×70 cm central zone and the surrounding border zone. Mice were individually placed in a corner of the arena. The time spent in the central zone, the number of entries into the central zone and the overall distance travelled by the mice were recorded during a period of 5 min.

Novel cage test

The novel cage test is used to investigate exploratory behavior in a new environment by measuring vertical activity. Animals were placed into a new standard macrolon cage and rearings were counted for 5 min.

Light–Dark Box

The Light–Dark Box consisted of a square box divided into a black and a white compartment, connected by a small tunnel; the white compartment was brightly illuminated with a 600 lux light source. Light intensity in the black compartment, covered by a black top, was 1 lux. Latency to first exit, total number of exits, and time in the light compartment were recorded for 5 min.

Porsolt's Forced swim test

Mice were placed twice, at 24 h interval, into a glass cylinder (23 cm height, 13 cm diameter) which was filled with water (23°C) up to a height of 10 cm, which prevented the mice from touching the bottom of the beaker with their paws or the tail. Mice were tested for 5 min and their behavioural activity was scored by a well-trained observer. The times spent on climbing, swimming, and immobility were recorded to determine active vs. passive stress-coping performance. Mice were considered immobile when floating passively in the water, performing only those movements required to keep their heads above the water level (Cryan et al., 2002). In addition, duration of immobility was automatically assessed using the ''mobility'' feature of the Noldus software, EthoVison 1.96 (Noldus Information Technology, Wagingen, NL).

Morris water maze

The water maze consisted of a dark-gray circular basin (120 cm diameter) filled with water (24–26°C, 31 cm deep) made opaque by the addition of

non-toxic white tempera paint. A circular platform (8 cm diameter) was placed 1 cm below the water surface in the centre of the goal quadrant, 30 cm from the wall of the pool. Distant visual cues for navigation were provided by the environment of the laboratory; proximal visual cues consisted of four different black and white posters placed on the inside walls of the pool. Animals were transferred from their cages to the pool in an opaque cup and were released from eight symmetrically placed positions on the pool perimeter in a predetermined but not sequential order. Mice were allowed to swim until they found the platform or until 180 sec had elapsed. In this last case, animals were guided to the platform and allowed to rest for 20 sec. The animals were submitted to six trials per day for five days using a hidden platform at a fixed position (south-east) during the first three days (18 trials, acquisition phase) and in the opposite quadrant (north-west) for the last two days (12 trials, reversal phase). Trials 19 and 20 were defined as probe trials to analyze the precision of spatial learning.

Elevated plus maze

A plus-shaped maze made of grey PVC plastic was used. The device comprised two opposing open arms (30×5 cm) and two opposing closed arms (30×5 cm) that had 15 cm high, nontransparent walls. The four arms were connected by a central platform (5×5 cm). The maze was elevated 500 mm above the floor. The open arms were illuminated with an intensity of 200 lux, the central area with 150 lux and the closed arms with 100 lux. Mice were initially placed in the centre of the maze facing one of the open arms and then were allowed to investigate the area for 5 min. Their behaviour was recorded by video-tracking (VideoMot2, TSE Systems, Bad Homburg, Germany). Entry into an arm was defined when the mouse placed its four paws into the arm. The time spent in, and the number of entries made into the open and closed arms as well as the centre time were measured, and the total distance travelled during the test session was recorded.

Hot plate test

Each mouse was placed on a metal surface maintained at 53.0 ± 0.2°C (ATLab, Montpellier, France). The response to the heat stimulus was measured by assessing the latency to first reaction, i.e. hindpaw lick or jump, which are considered as typical nociceptive responses (Hammond and Ruda, 1989). Animals were removed from the plate immediately after responding or after a maximum of 45 s (cut-off) to prevent tissue damage.

Learned helplessness

This experiment was performed as previously described (Chourbaji et al., 2005; Reif et al., 2004; Ridder et al., 2005). Briefly, animals were exposed to a transparent plexiglass shock chamber equipped with a stainless steel grid floor (Coulborn precision regulated animal shocker, Coulborn Instruments, Düsseldorf, Germany), through which they received 360 footshocks (0.150 mA) on two consecutive days. Footshocks were unpredictable with varying shock- and interval-episodes (1–12 s), up to a total duration of 52 min. 24 h after the second day of the shock procedure, learned helplessness was assessed by testing shuttle box performance (Graphic State Notation, Coulborn Instruments, Düsseldorf, Germany). The shuttle box consisted of two equal-sized compartments, separated by a gate, and was equipped with a grid floor, through which the current was applied. Spontaneous initial shuttles were counted during the first two minutes by infrared beams. Performance was analyzed during 30 shuttle escape trials (light stimulus: 5 s, footshock: 10 s, intertrial interval: 30 s). Avoidance was defined as the adequate reaction to a cuing light stimulus by changing to the other compartment; escapes were defined as shuttling to the other compartment as reaction to the electric shock; when no attempt to escape was made, a failure was denoted. Total time of testing for helplessness was about 20 min depending on the animals' individual performance.

Modified holeboard paradigm

To assess attention and spatial memory, a modified 5×5 holeboard system equipped with 3-level infrared beams was used (COGITAT, Cogitron, Goettingen, Germany), which was connected to a videotracking software system (VideoMot 2, TSE Systems, Bad Homburg, Germany) as described by Wultsch et al. (submitted). During this test, animals were trained to learn a pattern of baited holes, in which odor-free pellets were hidden. The ground below the feeding plate and the cylindrical tubes were covered with vanilla powder to prevent animals from working out the pattern of the distribution of the pellets by using olfactory stimuli. By the combined use of the IR grid and the videotracking software, a number of measures including erroneous visited holes, eaten pellets, time to learn the pattern, total activity and distance travelled was taken.

Microarray studies

Hippocampus, striatum and cerebellum of 7 wildtype (+/+) and 7 NOS-I knockdown (−/−) mice were prepared. Total RNA was thereafter isolated using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) and the RNase-free DNase Set (Qiagen). Mean RNA concentration was 154 ng/ml. RNA samples of each structure have been randomly assigned to 3 pools for both wildtypes as well as knockdown animals. 3 µg of each RNA sample were labeled, wildtype total RNA in Cyanine 3 and knockout total RNA in Cyanine 5. The incubation took place over night at 42°C. Purification of the samples was performed with a QIAGEN kit (Protocol GP4). At this step, purified samples were labeled blue (Cy5) or pink (Cy3). Each eluate was quantified on a Nanodrop ND-1000 device (PeqLab, Erlangen, Germany) to determine cDNA quantity. Samples were pooled two by two, but some volumes were readjusted to obtain the same quantity of cDNA. Purified samples were evaporated in a Speedvacuum (for 30 min), and then resuspended in the specific hybridization mix. Thereafter, 9 slides were hybridized over night at 42°C with a total of 18 samples (3 structures, 2 phenotypes); for each sample, 15 µl were used. The chips have been washed with decreasing stringency bathes and scanned on a Scanarray Scanner. Signal quantification was performed with Imagene 4.1 (BioDiscovery, Inc., El Segundo, CA) and data were normalized with VARAN (http://www.bionet.espci.fr/; Golfier et al., 2004). Three hybridization types have been statistically analyzed and compared (cerebellum, striatum and hippocampus experiments) using the SAM software (http://www-stat.stanford.edu/~tibs/SAM/). The purpose of this tool is to allow the selection of genes associated to significant variations between the conditions analyzed based on biologically independent experimental replicates. SAM is based on the computation of a statistic called d (for difference). A gene will be selected as significantly differentially expressed if the observed d is significantly higher than an expected value, computed using the whole set of experimental data. A threshold (δ-value) is defined by the user and corresponds to the minimum absolute value of δ(observed)- δ(expected) that will be associated to a significant variations. This value can be defined regarding measurements provided by SAM such as false discovery rate, the number of selected significant genes and the number of false positives. For determination of global knockout effects, δ has been set such as the number of false positive is lower than one. For all other analyses, δ has been set in order to select a number of significant genes similar to the number selected for the whole analysis of the 9 experiments.

Real-time PCR

After the preparation of the striatum and the brainstem, containing the raphe nuclei, total RNA was isolated as described above. 0.5 µg of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit® (BIO-RAD, München, Germany). Real-time PCR was performed using an iCycler iQ™ Real-Time Detection System (BIO-RAD Laboratories, Hercules, USA) in the presence of SYBR-green. The optimization of the real-time PCR re-

action was performed according to the manufacturer's instructions but scaled down to 25 µl per reaction. Standard PCR conditions were used following the manufacturers protocol (serotonin transporter, 5-HTT: iQTM SYBR® Green Supermix protocol, BIO-RAD; dopamine transporter, DAT: QuantiTectTM SYBR® Green PCR protocol, Qiagen, Hilden, Germany). Three series of experiments were performed with similar results; PCR reactions of each series were run in duplicate. Ribosomal 18 s and GAPDH were used to normalize each template using the GeNorm normalization program (Vandesompele et al., 2002). Standard curves for each amplification product were generated from 10-fold dilutions of pooled cDNA amplicons isolated from Agrarose gel electrophoresis. The primer sequences were as follows: 5-HTT forward, 5′ – GAC AGC CAC CTT CCC TTA CA – 3′; 5-HTT reverse, 5′ – CTA GCA AAC GCC AGG AGA AC – 3′; GAPDH forward, 5′ – AAC GAC CCC TTC ATT GAC – 3′; GAPDH reverse, 5′ – TCC ACG ACA TAC TCA GCA C – 3′; 18S forward, 5′ – GAA ACT GCG AAT GGC TCA TTA AA – 3′; and 18S reverse, 5′ – CCA CAG TTA TCC AAG TAG GAG AGG A – 3′.

Results

Behavioral assessment

As a number of pharmacological studies argue for an involvement of NOS in the pathophysiology of depression and anxiety, we examined whether *NOS1* knockdown mice display behavioral traits related to such phenotypes. However, no significant differences could be observed in the Forced Swim Test (Fig. 1A) or any of the most relevant parameters of the Learned Helplessness paradigm (Fig. 1B, C), strongly arguing against a depression-like phenotype of *NOS1* knockdown animals. In the Hotplate test there were no changes in pain sensitivity, which could have interacted with the unpleasing stress procedures, supporting the results obtained in the Learned Helplessness procedure (Fig. 1D). Further potential influences by alterations of activity-, or motoric-dependent traits could also be excluded, since no significant alterations were detected in the Novel Cage (Fig. 1E), the Rotarod (Fig. 1F), Barrier- (data not shown), or Open Field test (see below). In the Elevated Plus Maze, mutant mice spent significantly more time in the open arm of the Plus Maze (Fig. 2A, B), indicating less anxiety. In another anxiety-related paradigm, i.e. the Light–Dark Box, no significant differences however emerged (data not shown). Differences in the Elevated Plus Maze could not

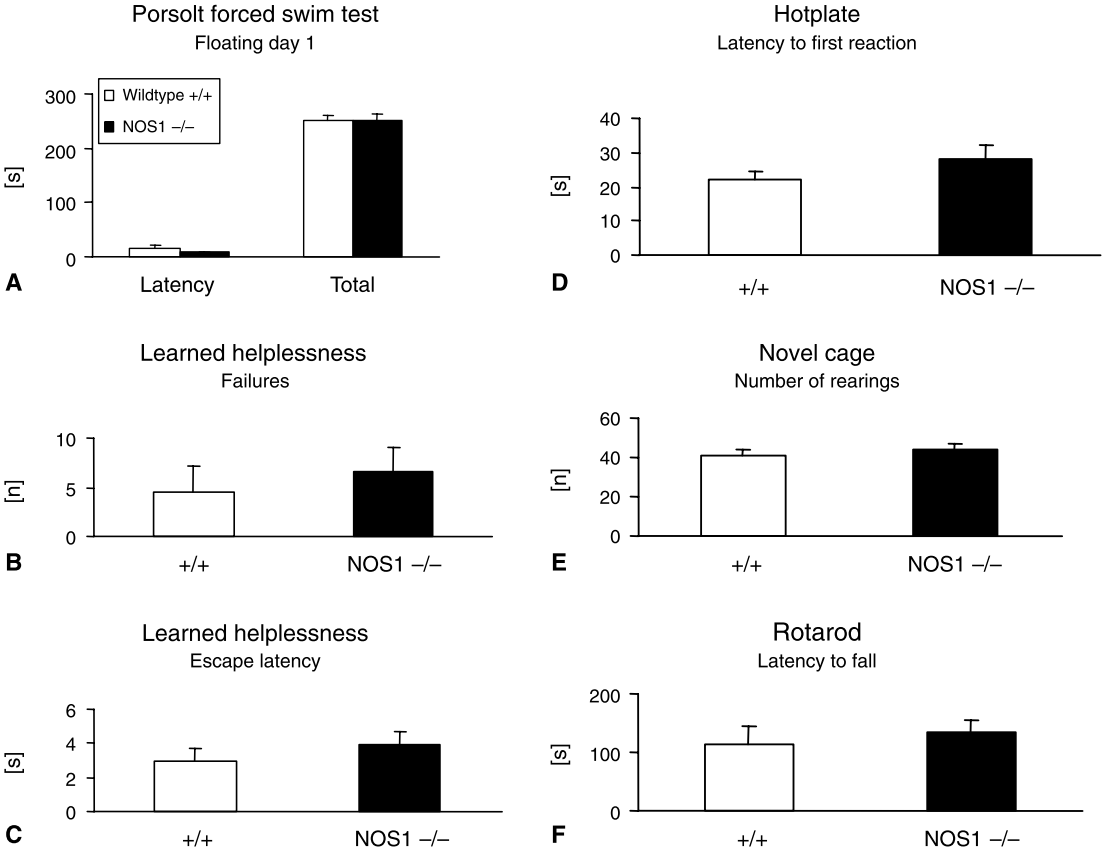


Fig. 1. Depression-related behaviors of male *NOS1* knockdown mice. Neither in the Forced Swim Test (**A**), nor in the Learned Helplessness Paradigm (**B**, **C**), *NOS1* knockdown mice displayed depressive-like behavior. Also in control experiments testing for pain sensitivity (**D**), novelty-related emotionality (**E**) and overall locomotor activity (**F**), *NOS1* (−/−) mice showed no significantly different behavior. Closed bars, knockdown mice (n = 13); open bars, wildtype littermates (n = 13)

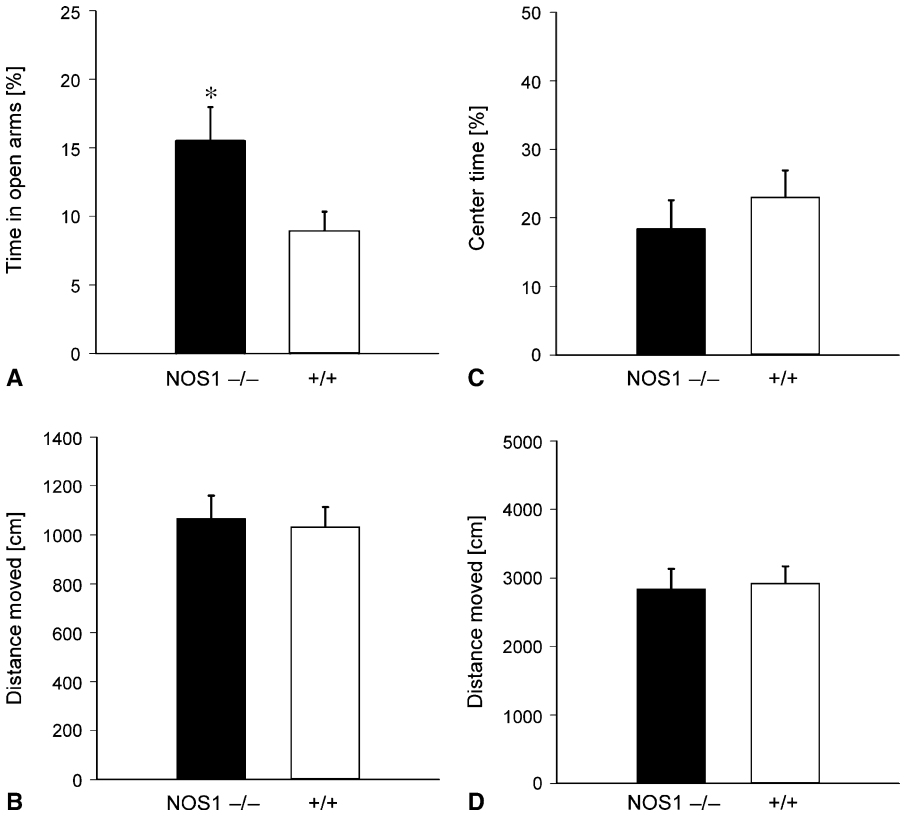


Fig. 2. Anxiety- and activity-related behaviors of male *NOS1* knockdown mice. While *NOS1* knockdown (−/−) mice had a higher open arm time in the Elevated Plus Maze as compared to wildtype (+/+) animals (**A**, **B**), arguing for reduced anxiety, activity-related parameters in the Open Field (**C**, **D**) were not significantly different. Closed bars, knockdown mice (n = 10); open bars, wildtype littermates (n = 10); *, significant difference ($p > 0.05$, Student's *t*-test)

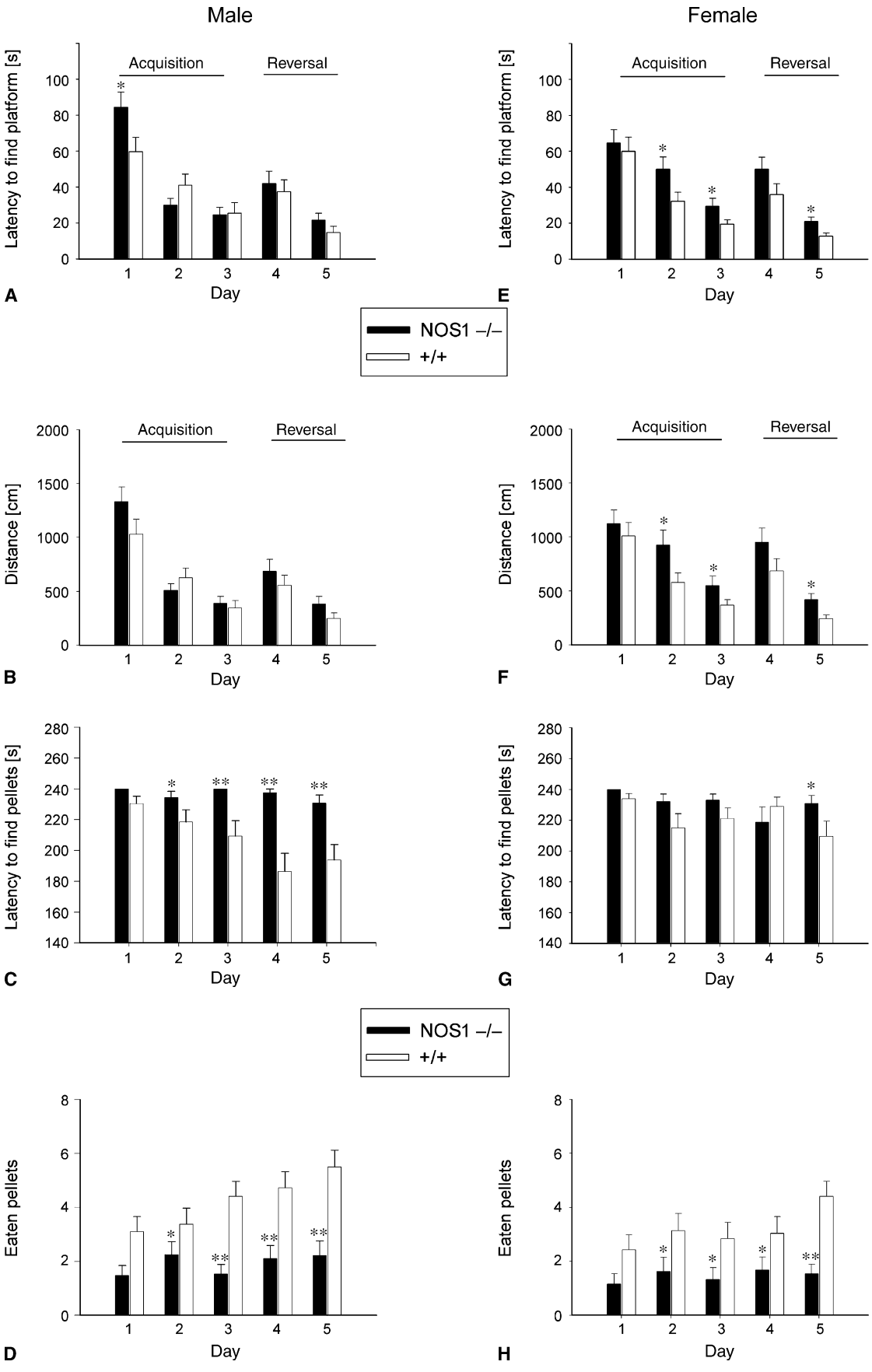
be attributed to alterations in locomotor activity, as performance in the Rotarod test (Fig. 1F) and all measured parameters in the Open Field test (center time, distance to walls, total distance moved, velocity; Fig. 2C, D) were unaltered in knockdown (−/−) animals.

In a second series of experiments, we investigated whether *NOS1* knockdown animals have cognitive deficits. Therefore, two different paradigms were employed: the Morris Water Maze, the standard test for hippocampus-dependent spatial memory, and a modified holeboard paradigm. In the Morris Water Maze, knockdown (−/−) mice had a higher latency to find the hidden platform (Fig. 3A) in acquisition, but not reversal trials. Correspondingly, in the holeboard test, knockdown (−/−) animals did not show a decreasing latency to find pellets during the trials, indicating that spatial learning was impaired (Fig. 3C). Furthermore, the number of found pellets was significantly lower in knockdown (−/−) as compared to wildtype (+/+) mice (Fig. 3D). As cognitive deficits were the most pronounced behavioral phenotype, we also tested a cohort of female animals to examine whether this is a gender-specific effect. As depicted in Fig. 3E–H, also females were cognitively impaired in both paradigms with the Water Maze results being even more pronounced in females, as they were impaired both in acquisition as well as in reversal trials.

Quantitative PCR of 5-HTT and DAT transcripts

Because tight interactions of NO and both the serotonergic as well as the dopaminergic systems have been suggested, we speculated that disruption of the *NOS1* might lead to a modified expression of the DAT or the 5-HTT, which are key molecules in the regulation of serotonin (5-HT) and dopamine circuitries. For 5-HTT, RNA was extracted from the brainstem containing the raphe nuclei, where 5-HTT RNA is almost exclusively detectable. However, no significant differences in 5-HTT expression were observed (Fig. 4A). Quantification of DAT transcripts in the

Fig. 3. *NOS1* knockdown mice are cognitively impaired. Male *NOS1* knockdown mice had a higher latency to find the platform in the Water Maze in acquisition, but not in reversal trials (**A**) while the total distance moved was not different in males (**B**), but females (**F**). In the COGITAT holeboard test, knockdown animals had a longer latency to find the hidden pellets (**C**) and found less pellets (**D**). Comparable results were obtained with female animals (**E–H**). Closed bars, knockdown mice (n = 10 males and 11 females); open bars, wildtype littermates (n = 10 males and 9 females); *, significant difference ($p > 0.05$, ANOVA for repeated measures. Significant differences between genotypes were identified with the Holm-Sidak method). Together, these results indicate that both male as well as female *NOS1* knockdown mice are cognitively impaired


Male
Female
Acquisition
Reversal
Latency to find platform [s]
Distance [cm]
Latency to find pellets [s]
Eaten pellets
Day
NOS1 –/–
+/+
A
B
C
D
E
F
G
H

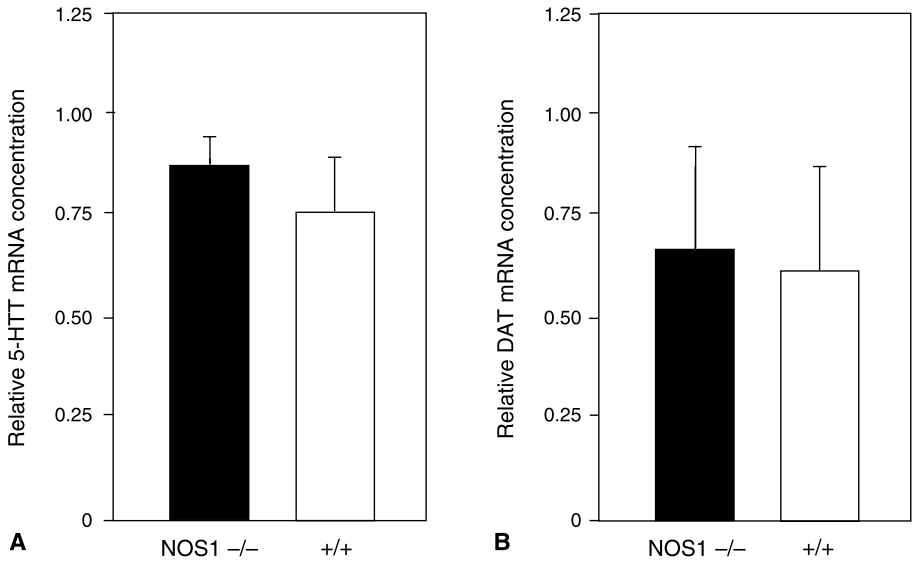


Fig. 4. Serotonin (5-HTT) and dopamine transporter (DAT) mRNA levels in *NOS1* knockdown mice. By means of qRT PCR, no significant differences in the expression of 5-HTT (brainstem, containing the raphe nuclei; **A**) or DAT (striatum; **B**) were observed in *NOS1* knockdown mice as compared to their wildtype littermates (Student's *t*-test, $p > 0.1$). Absolute cDNA values have been normalized against the housekeeping genes 18S and GAPDH. Closed bars, *NOS1* knockdown mice; open bars, wildtype controls. Data are given as means ± SEM; 7 animals have been investigated in each group

striatum (n = 7 knockdown and 7 wildtype control animals) by quantitative real-time PCR also revealed no significant changes in its relative expression (Fig. 4B).

Microarray experiments

To examine whether changes in the transcriptome of *NOS1* knockdown animals parallel behavioral changes, a gene chip microarray study was conducted. The hippocampus, being the prime region responsible for spatial learning, was investigated along with the NOS-I rich structures striatum and cerebellum. For each of these structures, 7 knockdown $(-/-)$ and 7 wildtype $(+/+)$ mice were examined; pooled structures were hybridized in triplicate. For the global knockout effect, each experiment has been considered as identical in order to reveal knockout induced global or

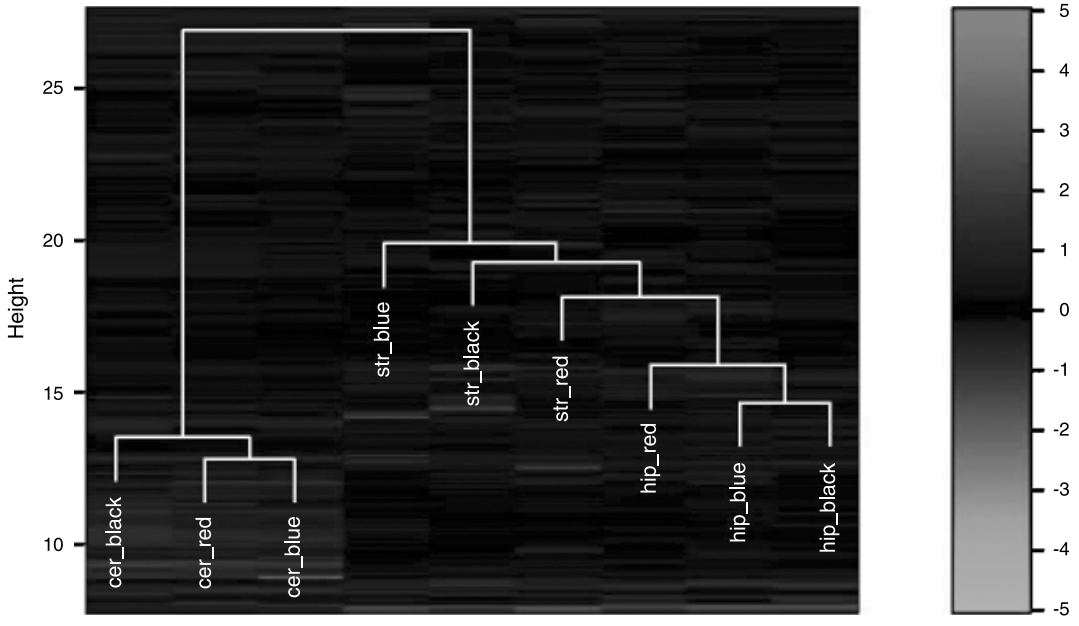


Fig. 5. Hierarchical clustering of hybridization profiles. The white dendrogram tree summarizes the results of the experiments clustering. Each column of the matrices corresponds to an experiment and each row to a probe (gene). The color scale ranges from wildtype over-expressed genes to *NOS1* knockdown over-expressed genes and is given in the Scaled_fold value as computed by VARAN. Genes have been clusterized based on their similarity expression profiles among the experiments. Probes associated to Scaled_fold ranging from −1 to 1 are localized in the VARAN error areas, where a differentially expressed gene cannot be distinguished from an invariant gene at the single hybridization level due to experimental variability. The dendrogram shows that the hybridization profile of the hippocampus and the striatum are very similar yet different to the profile of the cerebellum. Colour figure available on request from the communicating author

Table 2. *Significantly and meaningfully (>two-fold) up-regulated genes. Region denotes the structure, in which a significantly up-regulated gene was detected: either the striatum, the hippocampus, the cerebellum, both the hippocampus and the striatum combined, or a global knockdown effect, i.e. each experiment has been considered as identical in order to reveal knockdown induced global or common effects between all cerebral structures. d, the observed d absolute-value as computed by the SAM software (see Material and Methods). A gene will be selected as significantly differentially expressed if it is significantly higher than an expected value, computed using the whole set of experimental data. SD, standard deviation of d; p, the p-value of the classical t-test; q, the q-value of the statistical test performed by SAM, which is a modified t-test. Both p and q values correspond to the probability of an error when a gene is selected as significantly differentially expressed between the conditions knockout and wildtype. Fold-change denotes the degree of up-regulation (e.g., fold-change of 2 corresponds to a doubled expression). Name, official gene symbol; Full name, official gene full name. Rows of identical genes are shaded for the sake of clarity. Genes which are >3-fold up-regulated are printed in bold*

Region	*d*	SD	p	q	Fold-change	Name	Full name
Hippocampus	8.5489	0.0809	0.0001	0.1435	6.3038	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Global	5.9819	0.3065	<0.0001	0.0023	4.3674	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Striatum	6.2980	0.2169	0.0007	0.1649	5.0813	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Str. and Hip.	9.3724	0.1587	0.0001	0.0188	6.5111	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Cerebellum	1.4162	0.0732	0.0010	0.0985	2.2376	Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha
Hippocampus	4.1162	0.3313	0.0018	0.1435	4.9580	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Global	6.4689	0.2675	<0.0001	0.0022	4.1344	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Striatum	5.2895	0.3497	0.0013	0.1649	6.3739	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Str. and Hip.	6.9230	0.2302	0.0002	0.0188	5.6226	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Hippocampus	4.3416	0.2068	0.0017	0.1435	3.7209	Gabbr2	**gamma-aminobutyric acid (GABA) B receptor 2**
Global	4.9821	0.2468	0.0001	0.0025	2.7777	Gabbr2	gamma-aminobutyric acid (GABA) B receptor 2
Striatum	4.8852	0.2460	0.0019	0.1649	3.8945	Gabbr2	**gamma-aminobutyric acid (GABA) B receptor 2**
Str. and Hip.	7.0349	0.1445	0.0001	0.0188	3.8075	Gabbr2	**gamma-aminobutyric acid (GABA) B receptor 2**
Hippocampus	2.2189	0.2371	0.0093	0.1453	2.0506	Nr3c1	glucocorticoid receptor 1
Striatum	4.8402	0.1037	0.0021	0.1649	2.3857	Nr3c1	glucocorticoid receptor 1
Str. and Hip.	4.4865	0.1256	0.0004	0.0188	2.2120	Nr3c1	glucocorticoid receptor 1
Striatum	4.4538	0.1587	0.0025	0.1649	2.6379	Hes7	hairy and enhancer of split 7 (Drosophila)
Striatum	4.8394	0.0585	0.0022	0.1649	2.0499	Hgf	hepatocyte growth factor
Hippocampus	2.2442	0.3419	0.0089	0.1453	2.4333	Hoxb7	homeo box B7
Global	5.6997	0.1690	<0.0001	0.0023	2.3658	Hoxb7	homeo box B7
Striatum	3.0286	0.3456	0.0053	0.1649	2.8634	Hoxb7	homeo box B7
Str. and Hip.	3.9634	0.2237	0.0005	0.0188	2.6399	Hoxb7	homeo box B7
Cerebellum	1.5900	0.0171	0.0003	0.0985	2.3222	Hoxb9	homeo box B9
Hippocampus	8.2754	0.0867	0.0003	0.1435	6.1435	Hoxb9	**homeo box B9**
Global	7.7041	0.2192	<0.0001	0.0022	4.1888	Hoxb9	**homeo box B9**
Striatum	9.4005	0.0962	0.0004	0.1649	5.1558	Hoxb9	**homeo box B9**
Str. and Hip.	11.8297	0.0811	<0.0001	0.0188	5.6299	Hoxb9	**homeo box B9**
Cerebellum	1.3302	0.3164	0.0018	0.0985	2.6664	Hoxc6	homeo box C6
Hippocampus	3.8073	0.1879	0.0024	0.1435	3.0111	Hoxc6	**homeo box C6**
Global	8.2376	0.1426	<0.0001	0.0022	2.9856	Hoxc6	homeo box C6
Striatum	3.8905	0.2891	0.0029	0.1649	3.3169	Hoxc6	**homeo box C6**
Str. and Hip.	5.7845	0.1573	0.0002	0.0188	3.1607	Hoxc6	**homeo box C6**
Hippocampus	3.4955	0.1932	0.0026	0.1435	2.7868	Hoxd13	homeo box D13
Str. and Hip.	4.7135	0.2061	0.0003	0.0188	2.9957	Hoxd13	homeo box D13
Global	6.1236	0.1741	<0.0001	0.0023	2.5773	Hoxd13	homeo box D13
Striatum	3.0077	0.4053	0.0055	0.1649	3.2194	Hoxd13	**homeo box D13**
Hippocampus	3.0883	0.1630	0.0035	0.1435	2.3184	DrosophilaIrx1	iroquois related homeobox 1 (Drosophila)
Str. and Hip.	3.8519	0.1438	0.0005	0.0188	2.0757	DrosophilaIrx1	iroquois related homeobox 1 (Drosophila)
Hippocampus	2.7801	0.1711	0.0046	0.1435	2.1654	DrosophilaIrx5	iroquois related homeobox 5 (Drosophila)
Cerebellum	1.3750	0.1484	0.0011	0.0985	2.3482	Junb	jun-B oncogene
Cerebellum	1.0937	0.6522	0.0062	0.0990	2.8891	Lamr1	laminin receptor-like 1/ribosomal protein SA
Hippocampus	2.9641	0.1879	0.0039	0.1435	2.3590	Lhx1	LIM homeobox protein 1
Striatum	5.2604	0.0448	0.0014	0.1649	2.0759	Lhx1	LIM homeobox protein 1
Str. and Hip.	5.0831	0.0958	0.0003	0.0188	2.2131	Lhx1	LIM homeobox protein 1
Hippocampus	5.9673	0.0928	0.0010	0.1435	3.7976	Lyl1	**lymphoblastomic leukemia**
Global	4.8840	0.2247	0.0001	0.0026	2.5255	Lyl1	lymphoblastomic leukemia
Striatum	4.5387	0.1917	0.0024	0.1649	2.9812	Lyl1	lymphoblastomic leukemia
Str. and Hip.	6.9224	0.1232	0.0002	0.0188	3.3655	Lyl1	**lymphoblastomic leukemia**
Cerebellum	1.4702	0.4801	0.0008	0.0985	3.4930	Prdx3	**peroxiredoxin 3**
Hippocampus	4.8584	0.3102	0.0013	0.1435	6.1614	Prdx3	**peroxiredoxin 3**
Global	8.2297	0.2159	<0.0001	0.0022	4.5309	Prdx3	**peroxiredoxin 3**

(*continued*)

Table 2 (*continued*)

Region	*d*	SD	p	q	Fold-change	Name	Full name
Striatum	5.2374	0.2479	0.0015	0.1649	4.3255	Prdx3	**peroxiredoxin 3**
Str. and Hip.	6.9488	0.2111	0.0002	0.0188	5.1635	Prdx3	**peroxiredoxin 3**
Cerebellum	1.5877	0.0957	0.0004	0.0985	2.5287	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Hippocampus	4.0897	0.0872	0.0019	0.1435	2.4561	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Global	10.3064	0.0853	<0.0001	0.0022	2.6098	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Striatum	3.8589	0.2380	0.0032	0.1649	2.8648	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Str. and Hip.	5.5545	0.1237	0.0002	0.0188	2.6530	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Hippocampus	3.5613	0.3241	0.0025	0.1435	3.9246	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Global	5.1275	0.2924	0.0001	0.0025	3.3650	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Striatum	3.5077	0.5326	0.0037	0.1649	5.3279	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Str. and Hip.	5.1555	0.2957	0.0003	0.0188	4.5735	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Hippocampus	4.6003	0.3238	0.0015	0.1435	5.8424	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Global	4.8389	0.3619	0.0001	0.0026	3.9680	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Striatum	11.7943	0.0833	0.0003	0.1649	7.0450	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Str. and Hip.	9.2194	0.1612	0.0001	0.0188	6.4167	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Hippocampus	1.9702	0.4041	0.0119	0.1461	2.3765	Slc6a12	slc6 (neurotransmitter transporter, betaine/GABA) 12
Hippocampus	5.3764	0.0655	0.0011	0.1435	3.0057	Tle2	**transducin-like enhancer of split 2, hom. of Drosophila E(spl)**
Global	3.2615	0.2949	0.0006	0.0083	2.1759	Tle2	transducin-like enhancer of split 2, hom. of Drosophila E(spl)
Striatum	5.5345	0.1709	0.0008	0.1649	3.4983	Tle2	**transducin-like enhancer of split 2, hom. of Drosophila E(spl)**
Str. and Hip.	7.5427	0.0954	0.0001	0.0188	3.2434	Tle2	**transducin-like enhancer of split 2, hom. of Drosophila E(spl)**
Hippocampus	7.6808	0.1550	0.0004	0.1435	7.7578	Vax2	**ventral anterior homeobox containing gene 2**
Global	5.5139	0.3774	<0.0001	0.0025	5.1017	Vax2	**ventral anterior homeobox containing gene 2**
Striatum	13.9215	0.0759	0.0001	0.1649	9.3307	Vax2	**ventral anterior homeobox containing gene 2**
Str. and Hip.	13.6041	0.0974	<0.0001	0.0188	8.5103	Vax2	**ventral anterior homeobox containing gene 2**
Cerebellum	1.2929	0.0400	0.0022	0.0985	2.0250	IkarosZnfn1a1	zinc finger protein, subfamily 1A, 1 (Ikaros)
Striatum	3.2333	0.1762	0.0047	0.1649	2.1031	IkarosZnfn1a1	zinc finger protein, subfamily 1A, 1 (Ikaros)

common effects between all the cerebral structures. By doing so, 54 genes were found to be significantly over-expressed, while 12 genes were under-expressed. Hierarchical clustering (Fig. 5) revealed that the set of differentially regulated genes were closer together for the striatum and the hippocampus as compared to the cerebellum, suggesting that the mechanisms of expressional control due to NOS-I are similar for the further two structures. Accord-

Table 3. *Significantly and meaningfully (<0.5-fold) down-regulated genes. For further explanations see legend to Table 2*

Region	*d*	SD	p	q	Fold-change	Name	Full name
Cerebellum	−1.2559	0.0661	0.0025	0.0985	0.4926	OxyR	cold shock domain protein A
Cerebellum	−1.5239	0.2859	0.0006	0.0985	0.3358	Gna14	guanine nucleotide binding protein, alpha 14
Cerebellum	−1.1987	0.0679	0.0037	0.0985	0.5080	Grid1	glutamate receptor, ionotropic, delta 1
Cerebellum	−1.3333	0.0433	0.0017	0.0985	0.4816	Mef2b	myocyte enhancer factor 2B
Cerebellum	−1.0405	0.7473	0.0087	0.0990	0.3403	Ppox	protoporphyrinogen oxidase
Cerebellum	−1.1994	0.0805	0.0036	0.0985	0.5025	v-reloncogenerelatedB(Relb)	avian reticuloendotheliosis viral (v-rel) oncogene related B
Cerebellum	−1.2255	0.1186	0.0029	0.0985	0.4792	Sstr4	somatostatin receptor 4
Cerebellum	−1.6838	0.1020	0.0001	0.0985	0.3711	Lamr1	laminin receptor-like 1/ribosomal protein SA
Global	−5.6208	0.1723	<0.0001	0.0025	0.4223	Lamr1	laminin receptor-like 1/ribosomal protein SA
Str. and Hip.	−3.9597	0.2290	0.0005	0.0188	0.3736	Lamr1	laminin receptor-like 1/ribosomal protein SA
Cerebellum	−1.3209	0.1710	0.0021	0.0985	0.4314	Chrna6	cholinergic receptor, nicotinic, alpha polypeptide 6
Cerebellum	−1.3429	0.0653	0.0015	0.0985	0.4694	DrosophilaNkx2-4	NK2 transcription factor related, locus 2 (Drosophila)
Cerebellum	−1.2559	0.0661	0.0025	0.0985	0.4926	OxyR	cold shock domain protein A
Cerebellum	−1.5239	0.2859	0.0006	0.0985	0.3358	Gna14	guanine nucleotide binding protein, alpha 14

ingly, hybridization profiles were very similar for the striatum and the hippocampus with 53 over- and 13 under-expressed genes in both structures combined. In the striatum alone, only 25 over-expressed genes were detected, while in the hippocampus alone, 64 genes were up- and one gene was down-regulated. In the cerebellum on the other hand, 13 genes were over- and 53 genes were under-expressed. When the cerebellum was compared against the striatum plus the hippocampus, 65 genes were found to be over-expressed in the striatum and hippocampus of knockdown (−/−) animals but not modulated or under-expressed in the cerebellum. Table 2 presents all genes which were significant and up-regulated at least two-fold. Printed in bold are the most meaningful genes (up-regulation >3-fold); those include *Peroxiredoxin 3, Atonal homolog 1, CCAAT/enhancer binding protein, Kcnj8, Vax2, HoxB9, GABA-B receptor 2* and *Similar to GABA-B receptor subunit 2 precursor.* In Table 3, all significant genes which were down-regulated by at least 50% are denoted.

Discussion

The animal model investigated in this study harbors a targeted deletion in exon 1 of the *NOS1* gene resulting in a loss of the PDZ binding domain. Thus, residual NOS-I enzyme and activity (up to 7% of the wildtype) has been reported in these animals which therefore actually constitute knockdown mice. In contrast, complete knockout animals have been engineered in which exon 6, coding for a part of the catalytic center of the enzyme, has been disrupted (Packer et al., 2003). Unfortunately no behavioral data exist for these animals, and likewise, behavioral studies are lacking for *NOS1*-overexpression transgenics (Packer et al., 2005). The *NOS1* knockdown used in the present (and all other, except for the aforementioned Packer et al. study; Packer et al., 2003) investigations might however more closely resemble human genetic variation in *NOS1*, as this likely will result in dysregulation of the gene but not in a complete knockout. In our hands, these animals are thus still highly valuable tools in NOS research.

The present investigation attempted to correlate the behavioral phenotype of *NOS1* knockdown mice to their gene expression profile as assessed by a custom made chip encompassing >1.000 brain-specific genes. *NOS1* knockdown (−/−) animals displayed a specific behavioral phenotype with cognitive deficits and decreased anxiety, while no depression-related behavior was evident. This was paralleled by a set of up-regulated genes, while only one gene (laminin-receptor like 1) was meaningfully down-regulated.

Cognitive deficits in NOS1 knockdown mice

The most consisting finding in the present study was that *NOS1* knockdown animals were cognitively impaired in two different tasks (the Morris Water Maze and the Cogitat Holeboard). The latter allows scrutinizing spatial learning and re-learning parallel to activity measures. In the Water Maze, knockdown animals had a higher latency to find the hidden platform in acquisition and, in females, in reversal trials. This was paralleled by the holeboard results: knockdowns had a higher latency to find all hidden pellets, and accordingly ate less food pellets. No significant activity changes related to these data ensuring that indeed a disturbance of spatial learning, a hippocampus-dependent task, was observed. Likewise, a previous set of studies also demonstrated an increased latency in finding the hidden platform (Kirchner et al., 2004). However, in this set of experiments, learning in the multiple T-maze was not negatively affected. As this is considered a less stressful task as compared to the Water Maze, it was argued that NOS-I inhibition selectively impairs learning under stressful, aversive conditions. The underlying connections between stressful learning conditions and the differential effects of nitric oxide knockdown are however unclear. Involved mechanisms might include dysregulation of the hypothalamus-pituitary-adrenal axis, as *NOS1* knockdowns feature higher baseline corticosterone levels and a dampened stress-induced corticosterone response (Bilbo et al., 2003) and, on the other hand, stressful situations activate nitrinergic neurons (Beijamini and Guimaraes, 2006). Nevertheless, in addition to the Water Maze, we used a less stressful learning paradigm and thereby replicated spatial learning deficits of the *NOS1* knockdown mice. Yet not only hippocampus-dependent learning tasks, but also amygdala-dependent fear conditioning requires nitric oxide signalling (Schafe et al., 2005) arguing that NO-mediated retrograde messaging is a prerequisite for long-term potentiation(LTP)-dependent learning mechanisms. Taken together, decreased nitrinergic tone as found in *NOS1* knockdown animals as well as in animals treated with NOS inhibitors (Koylu et al., 2005; Majlessi et al., 2003; Prendergast et al., 1997) clearly impairs spatial learning.

Phenylcyclidine (PCP) administration represents an accepted rodent model of schizophrenia (Javitt and Zukin, 1991) as it mimics several key symptoms of schizophrenia such as impairment of prepulse inhibition, deficits in social behavior and cognitive dysfunctioning. A functional NO system has however to be present to obtain these effects (Bird et al., 2001). Most interestingly, spatial learning in the Water Maze was shown to be impaired upon PCP ad-

ministration, and this was normalized upon NOS inhibition (Wass et al., 2006a, b). Administration of the inhibitor alone however had no effect on Water Maze performance. This, together with our study, suggests that NO-mediated learning processes are fine-tuned and that either decreased (knockdown) as well as increased (PCP administration) nitrinergic tone leads to impaired learning mechanisms. Given that NO in the PCP model acts as the second messenger of the NDMA receptor, and that it thereby acts as a retrograde messenger mediating LTP, increased levels of NO may result in neuronal noise leading to dysfunctional memory traces and impaired learning and memory. Furthermore, highly elevated NO concentrations are neurotoxic, which can further contribute to cognitive dysfunctioning. Grossly decreased NO production on the other hand, as found in *NOS1* knockdown animals, most likely will result in impaired LTP (Hawkins et al., 1998) and consecutive behavioral abnormalities. Thus, the effect of NO on cognitive functioning seems to follow an U-shaped curve with either too less or too much NO causing cognitive impairment.

NOS1 knockdown mice and anxiety and depression-like behavior

The role of NO in the regulation of mood and anxiety is less clear than its involvement in cognition. From pharmacological experiments, there is numerous data from rats and mice, which however are highly inconsistent, pointing towards a complex role of NO in these behaviors. Several studies using systemic administration argued for an anxiogenic effect of NOS inhibition (Czech et al., 2003; De Oliveira et al., 1997; Pokk and Vali, 2002b; Vale et al., 1998), which was also the case when the inhibitor L-NAME was injected directly into the amygdala or the hippocampus (Monzon et al., 2001). At the same time, NOS inhibition, either intra-amygdalar (Forestiero et al., 2006) or systemical (Del Bel et al., 2005; Dunn et al., 1998; Faria et al., 1997; Pokk and Vali, 2002a, b; Volke et al., 1995, 1997, 2003; Yildiz et al., 2000), resulted in anxiolytic effects in several paradigms. These discrepant results were obtained using similar experimental protocols, comparable animal strains, similar inhibitor compounds (in most cases, N^{ω}-nitro-L-Arginine, L-NAME oder 7-nitroindazole) and partially by the same working group, so that these differences cannot be readily explained. Again, the baseline stress level of the animals and the according set of the hypothalamus-pituitary-adrenal axis might be crucial, as NOS inhibition was shown to counteract anxiolytic effects of corticotrophin (Reddy and Kulkarni, 1998). Furthermore, it was shown that NOS inhibition does not follow a linear cause – effect relationship but rather an inverse U-shaped curve with respect to anxiolytic behaviors (Volke et al., 1995). Considering that, in some paradigms, NOS inhibition also accomplished decreased locomotor activity especially in higher doses (Del Bel et al., 2005; Yildiz et al., 2000), motor side effects of NOS inhibitors might affect anxiety measures in several tests (like the elevated plus maze and the Light–Dark Box) as well, which again highlights the necessity of comprehensive behavioral phenotyping.

Finally, with respect to anxiety-related parameters, *NOS1* knockdown animals (Table 1) displayed a unchanged (Bilbo et al., 2003; Kirchner et al., 2004) or more anxious (Weitzdoerfer et al., 2004) phenotype in previous studies. The latter finding however is doubtful, as the same group in a parallel paper failed to replicate this data (Kirchner et al., 2004), and in our set of experiments, NOS knockdown clearly resulted in a less anxious phenotype independent of locomotor impairment. The lux value is a crucial factor which has to be taken into account due to the anxiogenic effect of bright light, but, however, is not given in these papers. Taken all this data together, the effect of NO on anxiety-related behaviors seems to be complex and state-dependent; most of the studies published to date however argue, if at all, to a anxiogenic effect of NO.

The role of NO in depression-like behaviors is equally controversial. Treatment with NOS inhibitors results in a reduced immobility time in the Forced Swim Test to the same extend as imipramine (either systemically; Harkin et al., 1999; Karolewicz et al., 2001; Volke et al., 2003; or by direct application into the hippocampus; Joca and Guimaraes, 2006), i.e., NOS inhibitors can be regarded as antidepressant-like in these tests. This effect however occurs only in lower, but not high doses of the inhibitors (da Silva et al., 2000; Ergun and Ergun, 2007) so that again a U-shaped curve was suggested. Paradoxically, not only NOS inhibitors, but also its substrate L-arginine has biphasic anti- as well as prodepressant properties (da Silva et al., 2000; Ergun and Ergun, 2007; Inan et al., 2004). However, only the pro-, but not the antidepressant effect of L-arginine seems to be due to NO mediated pathways (Ergun and Ergun, 2007).

A number of pharmacological studies have assessed the connection between the serotonergic system, its pharmacology and the NO pathway. Indeed it was shown that selective 5-HT reuptake inhibitors (SSRIs) as well as tricyclic anti-depressants decrease the activity of hippocampal NOS (Wegener et al., 2003). On the other hand, NOS inhibitors exerted their anti-depressant effect only in the presence of 5-HT, as 5-HT depletion abolished it completely (Harkin

et al., 2003). These compounds decrease 5-HT turnover in frontal cortex, similar to imipramine, and in low doses cause an increase in frontal cortical 5-HT concentrations (Karolewicz et al., 2001). This is similar to findings in *NOS1* knockdown mice, where 5-HT turnover in the frontal cortex is reduced along with a concomitant increase in frontal 5-HT as well as 5-HT_{1A} and 5-HT_{1B} receptor hypofunctioning (Chiavegatto et al., 2001). Furthermore, either local or systemic administration of NOS inhibitors increased the extracellular concentration of 5-HT and dopamine in the hippocampus, while L-arginine had the opposite effect (Wegener et al., 2000). One possible mechanism might be the direct nitrosylation of the monoamine reuptake transporters (Kiss and Vizi, 2001). Endogenous NO thereby is able to stimulate monoamine reuptake (Kilic et al., 2003), which is prevented by NOS inhibition. Accordingly, treatment with NOS inhibitors significantly enhanced the antidepressant properties of SSRIs (Harkin et al., 2004), while L-arginine treatment counteracted it (Inan et al., 2004).

Given the above considerations, we expected to find an antidepressive phenotype in *NOS1* knockdown animals which however was not the case. Though neither in the Forces Swim Test nor the Learned Helplessness paradigm, the animals behaved different to their littermates strongly arguing against an ''affective'' phenotype of these mice. This is however in conflict with two previous studies, one of which is only presented in a review, demonstrating reduced immobility time in the Forced Swim Test (Nelson et al., 2006; Salchner et al., 2004). The reasons for this are unclear, it has however to be noted that the highly aggressive phenotype described earlier in *NOS1* knockdown animals (Chiavegatto et al., 2001; Chiavegatto and Nelson, 2003; Nelson et al., 1995) was also not observed in our animals. As it was shown that backcrossing of the mice onto a C57BL/6J background, as it was done in our strain, results in a less aggressive phenotype (Le Roy et al., 2000), genetic background effects might well account for these discrepancies. The unexpected lack of an antidepressant phenotype in the *NOS1* knockdown mice however might be due to developmental effects of the knockdown. Likewise, 5-HTT knockout mice, initially reasoned to mirror the effects of SSRIs, do not display an according behavioral phenotype (Holmes et al., 2002); the development of conditional knockout models thus is a desiderate.

The expressional profile of NOS1 knockdown mice

As NO directly nitrosylates monoamine transporters, we hypothesized that this also feeds back to the expressional levels of these molecules. This however was not the case, as both 5-HTT as well as DAT mRNA levels were unchanged in knockdown mice. We therefore chose to apply a less hypothesis-driven approach by conducting a genechip microarray study, which yielded a set of dysregulated genes (Tables 2 and 3). Some of the significantly and meaningfully up-regulated genes deserve a further look.

Peroxiredoxin 3

Peroxiredoxin 3 (*Prdx3*) was up-regulated in all examined structures by at least four-fold. It is localized in the mitochondria and considered an important intracellular antioxidant, regulating the level of H_2O_2 (Nonn et al., 2003). Prx-3 protects against reactive oxidative species and especially protein nitration, thereby protecting hippocampal neurons from excitotoxic cell death (Hattori et al., 2003), and it is down-regulated upon chlorpromazine treatment (La et al., 2006) and in spinal motor neurons of patients with motor neuron disease (Wood-Allum et al., 2006). As *NOS1* is up-regulated in the latter condition (Anneser et al., 2001), this further argues for a connection between the expressional control of both enzymes. The up-regulation of *Prdx3* might be a counter-regulatory detoxifying mechanism, as *NOS1* knockdown results in elevated xanthine oxidoreductase activity leading to a significant increase in superoxide production (Khan et al., 2004).

Atonal homolog 1

The transcription factor atonal homolog 1 (*Atoh1*; synonymous: *Math1*) is crucial for axial guidance and neuronal development, especially for cerebellar granule cells (Ben-Arie et al., 1997), where *NOS1* has an important developmental role as well (Schilling et al., 1994). Thus, *Atoh1* might a potential mediator of the neurodevelopmental roles of nitric oxide. The differential regulation of homeo box genes with largely unknown functions in tissue development (*HoxA3, B4, B7, B9, C6, D11, D13, iroquois related hox* genes as well as further homeodomain transcription factors) and *CCAAT/enhancer binding protein (C/EBP), alpha* (which however has not yet been convincingly been shown to affect neuronal development) further point to the important function of NO in the developing organism. The latter protein however interestingly binds to the promoter and subsequently regulates the L-arginine synthesizing enzyme arginosuccinate lyase (Chiang et al., 2007). The NOS1 promoter itself also harbors C/EBP binding sites.

Ion channels

While the potassium channel *Kcnj8* seems to have a predominant role in the cardiac conduction system, with a knockout of the gene resulting in Prinzmetal angina, a role in the central nervous system has not yet been established. Likewise, *Kcnj1* is involved in renal potassium absorption and, at least in some cases, in the pathogenesis of Bartter's syndrome, yet no function in excitable tissue has been described.

Glucocorticoid receptor 1

Most notably, the *glucocorticoid receptor 1* (*GR*) was up-regulated two-fold both in the striatum and in the hippocampus. At the same time *NOS1* knockdown mice also demonstrate higher baseline corticosterone levels and a blunted stress response (Bilbo et al., 2003), which is counter-intuitive to up-regulated *GR* levels. However, the up-regulation of *GR* may be compensatory to a primary dysregulated hypothalamus-pituitary-adrenal axis in *NOS1* knockdown mice. Intriguingly, mice which over-express *GR* are less susceptible to develop depressive-like behaviour, i.e., they feature an ''antidepressive'' phenotype (Ridder et al., 2005) along with increases in hippocampal brain derived neurotrophic factor (BDNF) content (Schulte-Herbruggen et al., 2006). Thus, an increase in *GR* expression might also underlie the antidepressive phenotype of *NOS1* knockdown animals in previous studies (Nelson et al., 2006; Salchner et al., 2004). As in our mice no increase in neither of BDNF mRNA nor protein was observed (Fritzen et al., in press), a differential effect of the BDNF response might explain the aforementioned discrepancies in their behavioural profile. Chronic GR activation was shown to inhibit the transcriptional activity of cyclic AMP response element-binding protein (CREB, Focking et al., 2003). As this condition might be mimicked by GR over-expression in *NOS1* knockdown mice, this mechanism might contribute to the observed learning and memory defects as CREB has a crucial role therein (Silva et al., 1998).

GABAergic genes

A whole set of genes impacting on the GABAergic systems were identified to be up-regulated in *NOS1* knockdown mice. Those include the GABA transporter *GAT2* (*Slc6a12*), *GABA-B receptor 2* and the gene *Similar to Gamma-aminobutyric acid type B receptor, subunit 2 precursor.* The heteromeric GABA(B) receptor complex also acts as a heteroreceptor at hippocampal glutamatergic neurons, and was shown to be implicated in anxiety and depression (Cryan and Kaupmann, 2005). In the hippocampus, all NOS-I-positive non-pyramidal cells are GABAergic local circuit neurons (Valtschanoff et al., 1993a, b), as it is the case in the prefrontal cortex (Gabbott and Bacon, 1995). Also in the striatum, GABAergic interneurons were shown to be positive for NOS-I (Kubota et al., 1993); there, NO negatively regulates extracellular GABA (Semba et al., 1995) arguing for a close connection between both systems as previously suggested (Fedele et al., 1997a). It is well established that NO is a downstream mediator of the behavioural effects of benzodiazepines and $GABA_A$ receptor agonists (Elfline et al., 2004), also arguing for a role of NO in the regulation of anxiety. Whether or not it is also involved in $GABA_B$ signalling is less clear, however, it was demonstrated that $GABA_B$ inhibitors increase hippocampal NO production (Fedele et al., 1997b). Most interestingly however, the $GABA_B$ antagonist baclofen caused marked memory deficits which were reversed upon treatment with a NO donor (Pitsikas et al., 2003) suggesting that $GABA_B$ has a role in learning and memory, which is mediated by NOS. Thus, $GABA_B$ up-regulation might be counter-regulatory; the $GABA_B$ – NO signalling cascade therefore might be implicated in the cognitive deficits of *NOS1* knockdown animals.

Conclusions

Taken together, in this study we demonstrate that *NOS1* knockdown mice feature a distinct behavioral phenotype including reduced anxiety and cognitive impairment. This was not paralleled by expressional changes in DAT or 5-HTT, but by a set of differentially regulated genes in the hippocampus and striatum. These genes included, amongst others, $GABA_B$ receptor subunits and the glucocorticoid receptor who may also be implicated in cognitive (dys-)functioning. These findings aid in the identification of nitrinergic signalling cascades and their role in memory formation; furthermore, *NOS1* knockdown animals might therefore be considered as rodent models of Alzheimer's dementia and/or attention deficit disorder, warranting further investigations.

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