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Research Article

Genetic functional inactivation of neuronal nitric oxide synthase affects stress-related Fos expression in specific brain regions

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Abstract. To identify neuronal substrates involved in NO/stress interactions we used Fos expression as a marker and examined the pattern of neuronal activation in response to swim stress in nNOS knock-out (nNOS–/–) and wild-type (WT) mice. Forced swimming enhanced Fos expression in WT and nNOS–/– mice in several brain regions, including cortical, limbic and hypothalamic regions. Differences in the Fos response between the two groups were observed in a limited set (6 out of 42) of these brain areas only: nNOS–/– mice displayed increased stressor-induced Fos expression in the medial amygdala, periventricular hypothalamic nucleus, supraoptic nucleus, CA1 field of the hippocampus, dentate gyrus and infralimbic cortex. No differences were observed in regions including the septum, central amygdala, periaqueductal grey and locus coeruleus. During forced swimming, nNOS–/– mice displayed reduced immobility duration, while no differences in general locomotor activity were observed between the groups in the home cage and during the open field test. The findings indicate that deletion of nNOS alters stress-coping ability during forced swimming and leads to an altered pattern of neuronal activation in response to this stressor in specific parts of the limbic system, hypothalamus and the medial prefrontal cortex.

Key words. Neuronal nitric oxide synthase; knock-out; c-Fos; forced swimming; NADPH-diaphorase; plasma corticosterone; open field.

Within the central nervous system (CNS), the gaseous signalling molecule nitric oxide (NO) is produced by constitutively expressed NO synthases (NOS) that are located either in the vascular endothelium (endothelial NOS; eNOS) or in distinct neuronal populations (neuronal NOS, nNOS). While NO originating from eNOS regulates local blood flow, the activity of nNOS has additionally been directly linked to information processing and interneuronal communication [for review see ref. 1]. Of the three major nNOS splice isoforms (nNOS-α, nNOS-β and nNOS-γ), nNOS-α produces approximately 95% of the NO in neurons [2, 3]. The NO system has been described as modulating synaptic transmission and is thought to be involved in a number of physiological neuronal functions including learning and memory [for review see ref. 1].

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There is evidence to suggest that NO also modulates the stress response (for review see ref. 4] and might be involved in the pathogenesis of stress-related disorders such as depression and anxiety [5, 6; for review see ref. 7]. Along these lines, NOS inhibitors have been found to alter stress-induced behavioural responses in experimental models of depression and anxiety [8–16]. Although NO modulators have been shown to influence the neuronal activity and the release of a variety of neurotransmitters in several brain areas known to be sensitive to stressful stimuli [for reviews see ref. 1, 4], the neuronal correlates and mechanisms involved in NO-stress interactions are not well defined.

To identify neuronal substrates involved in NO-stress interactions we used nNOS knock-out (nNOS–/–) mice and investigated whether and in which brain region(s) the neuronal responsiveness to stress is affected by genetic inactivation of nNOS and subsequent lack of NO production. For this purpose, the pattern of Fos expression, which is used as an index of neuronal activity in the CNS [17, 18] was investigated in nNOS–/– and wild-type (WT) mice exposed to an acute stress challenge, forced swimming.

Materials and methods

Animals

Protocols of experiments were approved by the Bundesministerium fuer Bildung, Wissenschaft und Kultur, Kommission fuer Tierversuchsangelegenheiten, Austria, and Sachsen-Anhalt, Germany (G/1/99). Male mutant mice with targeted disruption of the nNOS gene (nNOS–/–), showing >95% loss of NO production in the CNS [19] and WT animals C57B6J, which share >99.9% genetic similarity with the transgenic mice [20] were used. The foundation stock of these animals was initially established at the Massachusetts General Hospital (Boston, Mass). The nNOS gene mutation was generated by homologous recombination [19]. The genetic background is on a combination of the 129X1/SvJ and C57BL/6J strains with a predominance of C57BL/6J, because mice were backcrossed for three generations into C57BL/6J and then intercrossed to obtain knock-out mice and WT littermates.

Animals from these littermates were bred separately and housed in groups (10–15 animals) under controlled conditions of temperature, lighting (lights on from 7:00 to 19:00 h) and humidity with food and water available ad libitum**.** All animals were 4–5 months of age at the time of testing. Mice were allowed to habituate to the experimental room for at least 24 h before the experiment. All tests were carried out between 10:00 h and 15:00 h.

Forced-swim test

Mice were individually placed in an open cylindrical container (diameter 15 cm, height 25 cm) containing 15 cm of water mantained at 24–25 °C and were allowed to swim for 10 min while their activity was videotaped. The water in the cylinder was changed before each trial. After the swim test, mice were gently dried and put into a fresh cage under a warming lamp for 30 min. The videotaped behaviour was analyzed by a rater blind to the mouse genotypes. The behaviour of the animals was rated during the last 8 min of the 10 min test period as one of the following three categories: (1) immobility – floating in the water without struggling and making only those movements necessary to keep the head above water; (2) swimming – making active swimming motions, more than necessary to merely keep the head above water; (3) climbing – making active movements with the forepaws in and out of the water, usually directed against the walls. Animals assigned to the control group remained undisturbed in their home cages.

Open field test

The open field test consisted of a plastic box (41 × 41 × 41 cm) equipped with an automated activity monitoring system (Tru Scan; Coulbourn Instruments, Allentown, Pa.). Illumination at floor level was 150 lux. The area of the open field was divided into a 28 × 28 cm central zone and the surrounding border zone. Mice were individually placed into the centre of the open field and their behaviour was tracked with the activity-monitoring system. The time spent in the central zone, the number of entries into the central zone and the overall distance travelled by the mice were monitored during an interval of 10 min.

Homecage locomotor activity

Mice (WT and nNOS–/– n=8 each) were individually placed in plastic cages (36 × 20 × 15 cm) and were allowed to acclimate for 6 h. Locomotor activity was recorded for 24 h starting at 19:00 h by a computer-based automatic system (Inframot; TSE, Bad Homburg, Germany). Behavioural data were taken in 1-min bins and averaged over 3-h periods.

Fos immunohistochemistry

Two hours after the onset of forced swimming, mice were deeply anaesthetized with an overdose of sodium pentobarbital and transcardially perfused with 20 ml of 0.9% saline followed by 20 ml of 4% paraformaldehyde. Previous experiments showed that within a 2-h time window, reliable results concerning the selective Fos protein synthesis are obtained [17, 21; Engelmann, unpublished results] and that maximal induction of Fos protein expression in stressor-sensitive neurons in different parts of the brain appears 2 h after swim stress (22–24). Non-stressed control mice were sacrificed immediately after removal

from their home cages. Brains were then removed and post-fixed at 4 °C overnight in 4% paraformaldehyde. Coronal sections (100 µm) were cut using a Vibratome (St. Louis, Mo., USA) and collected in 0.1 M phosphate buffer. The sections were processed for Fos-like immunoreactivity (Fos-LI) as described previously [25]. Briefly, sections were incubated for 48 h in a polyclonal primary antibody (Sigma-Genosys, Cambridge, UK) diluted (1:2000) in immunobuffer (pH 7.4) comprising 0.1 M NaCl, 5 mM KCl, 8 mM Na_2HPO_4, 15 mM NaH_2-PO_4, 10 mM Tris-HCl, 0.3% Triton X-100 and 0.04% thimerosal. The primary antibody was raised in sheep against residues 2–16 of the N-terminal region of the Fos molecule, and it recognizes Fos and related antigens. The sections were then rinsed and placed in a biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories, Burlingame, Calif.) for 24 h. An avidin-biotin-horseradish peroxidase procedure with 3,3-diaminobenzidine as the chromogen was used to visualize the immunoreactivity. Cells containing a nuclear brown-black reaction product were considered positive for Fos-LI and are referred to hereafter as Fos-positive cells. The anatomical localization of Fos-positive cells was aided by using the illustrations in a stereotaxic atlas [26]. The AP levels of sections included for detailed analysis [26] and associated structures were: AP +1.78, cingulate cortex, infralimbic cortex, prelimbic cortex, primary motor cortex, secondary motor cortex; AP +1.42, nucleus accumbens; AP +0.62, caudate-putamen, intermediate lateral septal nucleus, ventral lateral septal nucleus, medial septal nucleus, nucleus of the horizontal limb of the diagonal band; AP +0.14, bed nucleus of stria terminalis, medial preoptic area; AP –0.82, paraventricular hypothalamic nucleus, periventricular hypothalamic nucleus, lateral hypothalamic area, anterior hypothalamic area, supraoptic nucleus, paraventricular thalamic nucleus, basal nucleus of Meynert; AP –1.46, lateral habenular nucleus, CA1 field of hippocampus, CA3 field of hippocampus, dentate gyrus, central nucleus of the amygdala, medial amygdala, basolateral amygdala, dorsomedial hypothalamic nucleus; AP –1.82, posterior hypothalamic area; AP –3.64, dorsomedial and lateral part of the periaqueductal grey, ventral tegmental area, substantia nigra, superior colliculus; AP –4.24, median raphe nucleus, pontine nuclei; AP –4.84, dorsolateral and ventrolateral part of the periaqueductal grey, dorsal raphe nucleus, cuneiform nucleus; AP –5.20, inferior colliculus; AP –5.40, locus coeruleus. The sections were viewed at 100× magnification and the number of Fos-positive cells within a defined area (0.01 mm^2) in each region of interest was quantified bilaterally by an observer blind to the experimental groups.

NADPH-diaphorase enzyme histochemistry

Alternate slices were stained histochemically for NADPH-diaphorase (NADPH-d) activity. Vibratome sections were incubated for 6 h in 0.1 M phosphate buffer (pH 7.4) containing 1 mg/ml β-NADPH, reduced form, and 0.1 mg/ml nitroblue tetrazolium. Following numerous rinses in 50 mM Tris-HCl, sections were mounted onto slides and dried overnight. Mounted sections were cleared in 100% ethanol and Histoclear (National Diagnostics, Atlanta, Ga.) and coverslipped in Eukitt (Kindler, Freiburg, Germany). NADPH-d-reactive neurons were readily recognized by the blue reaction product in cell bodies and processes.

Blood sampling and corticosterone measurement

Mice were separated 24 h before the experiment. On the day of the experiment, mice were either left in their home cage (controls) or forced to swim, gently towel dried and returned to their home cages. Immediately after removal from their home cage (controls) or 15 min after the onset of forced swimming, the animals were transferred to the adjacent room and immediately decapitated (<15 s after removal from the cage) using scissors. Trunk blood was collected into tubes containing ice-chilled EDTA (100 µl per tube). The blood was centrifuged at 2500 g for 5 min. Plasma was collected and stored at – 20 °C until radioimmunoassay measurement. Plasma corticosterone concentrations were determined using a commercially available radioimmunoassay (ICN Biomedials, Costa Mesa, Calif.). The sensitivity for the assay was 10 ng/ml. All samples were run in the same assay.

Data and statistical analysis

Results are expressed as means±SEM. Statistical analysis of the number of Fos-positive cells was performed using one-way ANOVA (Kruskal-Wallis test) followed by a Mann-Whitney U test to assess significant differences between the groups. Behavioural data were analysed using the Mann-Whitney U test. Correlation analysis between behaviour and swim stress-induced Fos expression was performed using the Spearman rank correlation test. Plasma corticosterone levels were compared using completely randomized two-way ANOVA followed by the Tukey-HSD test.

Results

Gross brain anatomical and histological inspections failed to provide differences between WT and nNOS–/– animals, thereby confirming the original report of Huang and co-workers [19]. Similar observations were also been made by other researchers (e.g. F. Rothe, University of Magdeburg, personal communication).

NADPH-diaphorase enzyme histochemistry

In the amygdaloid complex, particularly in the medial amygdala, WT animals exhibited intense NADPH-d staining, which was virtually absent in nNOS–/– animals

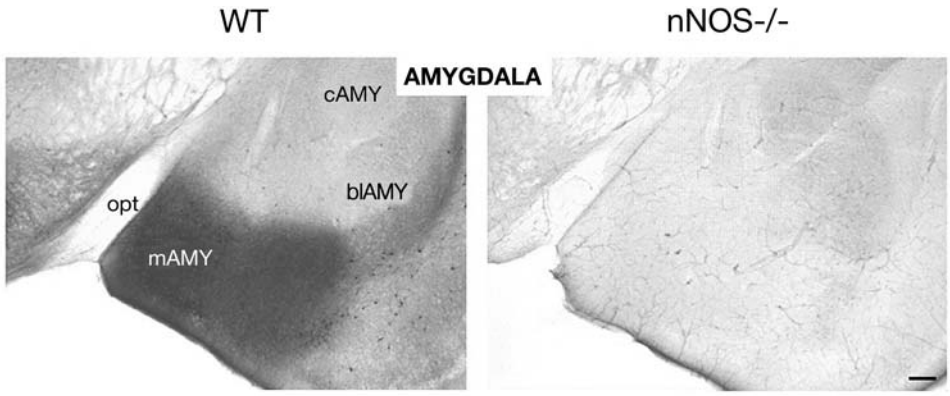

Figure 1. Photomicrographs showing NADPH-d staining in the amygdala of WT and nNOS–/– mice. In contrast to WT mice, neurons displaying NADPH-d activity are virtually absent in nNOS–/– mice. Scale bar = 200 μm. mAMY, medial amygdala; blAMY, basolateral amygdala; cAMY, central nucleus of the amygdala; opt, optic tract.

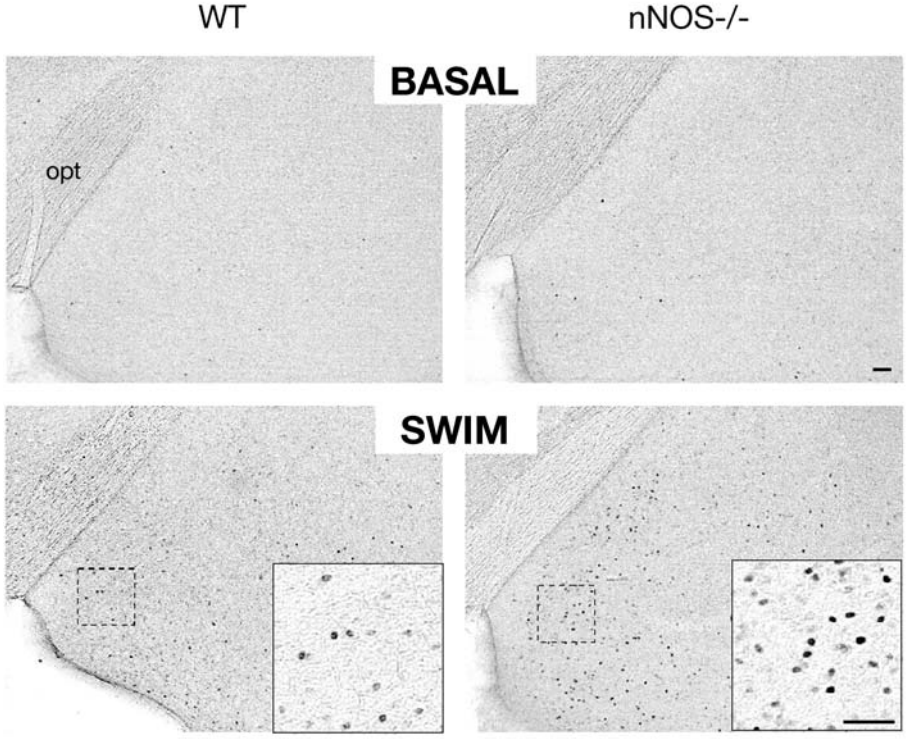

Figure 2. Representative photomicrographs illustrating the induction of Fos expression following the forced swim test in WT and nNOS–/– mice in the medial amygdala. Boxed areas are shown on high-magnification photographs in the insets. Scale bars, 50 μm. opt, optic tract.

and only spared in some blood vessels at the amygdaloid level (fig. 1). Similarly, NADPH-d staining was absent or greatly reduced at all remaining brain levels investigated [not shown; see also ref. 19 for cerebellum and hippocampus]).

Analysis of Fos expression

Non-stressed animals displayed little or no Fos expression in the regions quantified, with the exception of the paraventricular thalamic nucleus and the dorsomedial hypothalamic nucleus, which displayed moderate Fos expression under basal conditions. There were no statistically significant differences between non-stressed WT and nNOS–/– mice in any of the brain regions investigated (table 1).

Exposure to the forced swim test for 10 min increased Fos expression in both WT and nNOS–/– mice in a variety of brain regions throughout the brain axis, including cortical, limbic and hypothalamic areas (table 1). Pronounced effects were particularly noted in the paraventricular thal-

Table 1. Fos expression following the forced swim test in WT and nNOS–/– mice. Values are mean (± SEM) number of Fos positive cells/0.01 mm^2; n = 5 (basal groups), n = 8 (swim groups). * $p < 0.05$, ** $p < 0.01$ vs. corresponding basal group; + $p < 0.06$, ♦ $p < 0.05$, ♦♦ $p < 0.01$ vs. WT-swim.

Brain region	Basal		Swim	
	WT	nNOS–/–	WT	nNOS–/–
Forebrain areas				
Cingulate cortex	0.6 ± 0.2	1.7 ± 1.1	5.5 ± 0.6**	7.3 ± 0.6**+
Infralimbic cortex	1.5 ± 0.4	2.7 ± 1.4	6.4 ± 0.8**	8.8 ± 0.5** ♦
Prelimbic cortex	1.1 ± 0.5	2.2 ± 1.1	6.4 ± 0.6**	9.1 ± 0.9**+
Primary motor cortex	0.1 ± 0.1	0.3 ± 0.3	0.9 ± 0.2*	1.5 ± 0.4*
Secondary motor cortex	0.3 ± 0.2	1.2 ± 1.1	5.3 ± 0.8**	5.9 ± 0.5**
Nucleus accumbens	1.2 ± 0.8	1.5 ± 0.8	11.4 ± 0.9**	8.4 ± 1.4**
Caudate-putamen	0.2 ± 0.1	0.5 ± 0.4	6.3 ± 0.8**	7.9 ± 1.6**
Bed nucleus of stria terminalis	0.8 ± 0.3	0.9 ± 0.6	6.2 ± 0.5**	7.8 ± 0.9**
Lateral septal n., ventral	5.3 ± 2.8	2.9 ± 1.7	18.4 ± 1.2**	18.6 ± 1.4**
Lateral septal n., intermediate	2.1 ± 0.7	2.3 ± 1.4	5.6 ± 0.5**	7.1 ± 0.9*
Medial septal n.	0.6 ± 0.2	0.1 ± 0.1	1.4 ± 0.3*	1.1 ± 0.2**
Horizontal limb diagonal band n.	0.1 ± 0.1	0.1 ± 0.1	0.7 ± 0.2*	0.9 ± 0.3*
Basal n. of Meynert	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Thalamus				
Paraventricular thalamic n.	7.4 ± 0.7	7.5 ± 1.5	22.8 ± 1.4**	20.3 ± 1.4**
Lateral habenular n.	1.7 ± 0.8	1.4 ± 0.6	7.3 ± 0.9**	5.8 ± 1.1**
Hypothalamus				
Paraventricular hypothalamic n.	0.6 ± 0.3	1.1 ± 0.8	10.6 ± 1.8**	12.3 ± 3.7*
Periventricular hypothalamic n.	0.8 ± 0.2	1.6 ± 0.4	5.6 ± 0.7**	9.4 ± 1.1** ♦♦
Supraoptic n.	0.7 ± 0.4	1.0 ± 0.7	4.3 ± 0.5**	6.5 ± 0.6** ♦
Medial preoptic area	3.7 ± 1.2	3.6 ± 1.5	14.7 ± 1.1**	14.8 ± 1.2**
Anterior hypothalamic area	3.1 ± 1.3	3.3 ± 1.3	18.7 ± 1.7**	21.8 ± 1.6**
Lateral hypothalamic area	0.6 ± 0.3	0.9 ± 0.8	5.0 ± 0.5**	3.7 ± 0.5*
Dorsomedial hypothalamic n.	7.3 ± 4.1	8.4 ± 3.3	17.8 ± 1.3*	17.9 ± 1.9*
Posterior hypothalamic area	2.0 ± 0.7	2.0 ± 0.4	6.4 ± 0.5**	6.6 ± 0.6**
Amygdala				
Central nucleus of the amygdala	0.4 ± 0.2	0.4 ± 0.4	4.0 ± 1.0**	3.1 ± 0.6**
Medial amygdala	1.6 ± 0.7	1.8 ± 0.8	10.6 ± 1.1**	14.0 ± 1.0** ♦
Basolateral amygdala	1.0 ± 0.5	0.9 ± 0.6	6.5 ± 0.5**	6.8 ± 0.4**
Hippocampal formation				
CA1	0.4 ± 0.1	0.1 ± 0.1	0.9 ± 0.1*	1.5 ± 0.2** ♦
CA3	0.1 ± 0.1	0.1 ± 0.1	3.8 ± 0.6**	5.3 ± 0.4**
Dentate gyrus	3.0 ± 0.7	3.4 ± 0.5	6.6 ± 0.7**	9.4 ± 0.6** ♦
Midbrain, pons				
Periaqueductal grey				
Dorsomedial	4.7 ± 0.7	3.7 ± 1.3	9.4 ± 0.5**	9.3 ± 0.8*
Dorsolateral	3.4 ± 0.5	3.6 ± 1.0	12.7 ± 0.9**	12.4 ± 0.9**
Ventrolateral	1.6 ± 0.9	2.2 ± 0.8	7.8 ± 0.5**	6.6 ± 0.5**
Lateral	2.3 ± 0.9	1.6 ± 0.7	14.7 ± 0.9**	15.1 ± 0.7**
Ventral tegmental area	0.1 ± 0.1	0.3 ± 0.1	1.5 ± 0.6	1.1 ± 0.4
Substantia nigra, pars reticulata	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Dorsal raphe n.	0.1 ± 0.1	0.1 ± 0.1	3.1 ± 0.3**	4.1 ± 0.4**
Median raphe n.	0.8 ± 0.6	0.2 ± 0.2	7.1 ± 1.1**	5.3 ± 1.3**
Pontine nuclei	1.9 ± 0.7	1.4 ± 0.5	5.6 ± 1.2*	6.1 ± 0.9**
Cuneiform nucleus	0.9 ± 0.7	0.3 ± 0.2	2.9 ± 0.2**	2.4 ± 0.2**
Superior colliculus, deep grey layer	1.8 ± 0.6	2.4 ± 0.8	4.1 ± 0.6*	5.5 ± 0.5*
Inferior colliculus, external cortex	4.8 ± 0.2	4.9 ± 0.5	9.3 ± 1.1*	11.6 ± 0.7**
Locus coeruleus	2.6 ± 1.1	1.8 ± 0.5	17.4 ± 1.3**	19.0 ± 0.9**

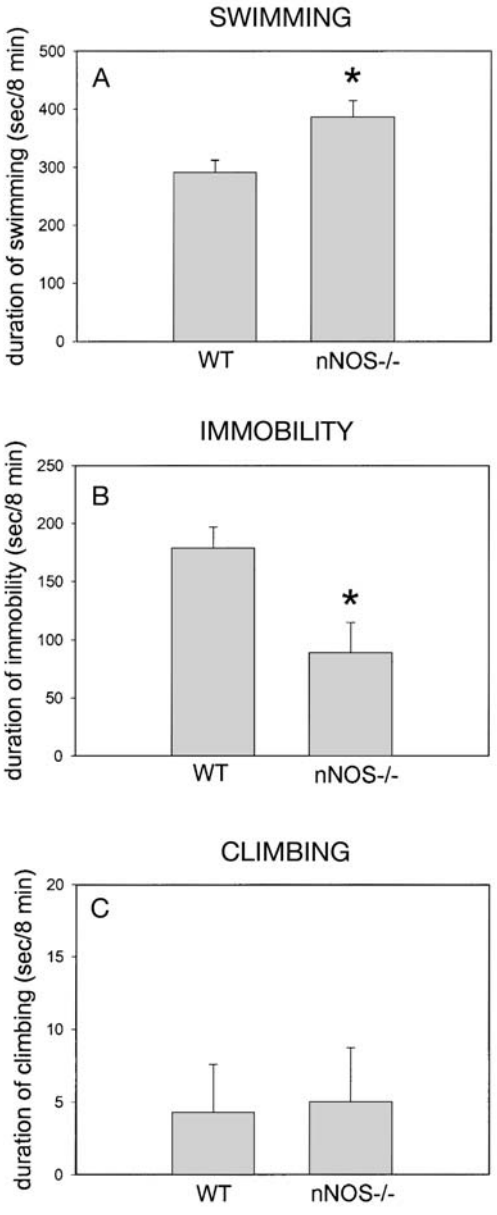

Figure 3. Swimming time (*A*), immobility time (*B*) and struggling time (*C*) during the forced swim test in WT (n = 7) and nNOS–/– (n = 7) mice. * $p < 0.05$ compared with WT.

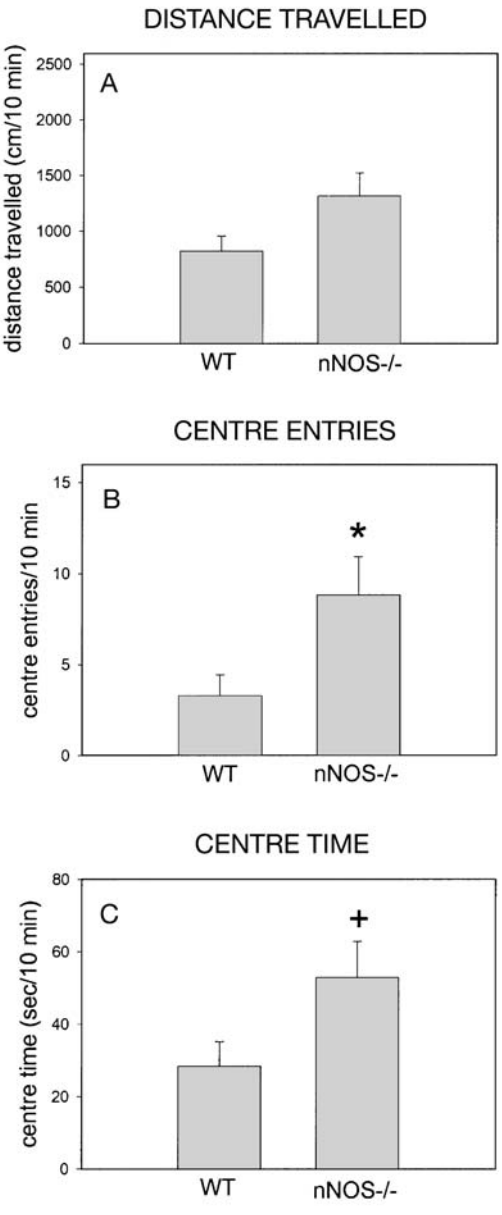

Figure 4. Overall distance travelled (*A*), number of entries into the central zone (*B*) and the time spent in the central zone (*C*) during the open field test in WT (n = 14) and nNOS–/– (n = 12) mice. +$p < 0.06$, * $p < 0.05$ compared with WT.

amic nucleus, lateral septum, various hypothalamic nuclei, the medial amygdala, parts of the periaqueductal grey and the locus coeruleus. In the hippocampal formation, Fos expression in response to swim stress was almost exclusively noted in granular cells of the dentate gyrus and pyramidal cells of the CA1 and CA3 region. Quantitative comparison between the two groups revealed that nNOS–/– mice displayed a higher level of swim stress-induced Fos expression compared to WT mice in a limited number (6 out of 42) of brain areas. These regions were the medial amygdala, periventricular hypothalamic nucleus, supraoptic nucleus, CA1 field of the hippocampus, dentate gyrus and infralimbic cortex (fig. 2; table 1). Amongst these regions, correlation analysis revealed that there was a significant negative correlation ($p<0.05$) between immobility time and the number of Fos-positive cells in the medial amygdala, periventricular hypothalamic nucleus and dentate gyrus, while the duration of swimming correlated positively ($p<0.05$) with Fos expression in the medial amygdala, periventricular hypothalamic nucleus, dentate gyrus and infralimbic cortex. There was a tendency ($p<0.06$) for enhanced swim stress-induced Fos expression in nNOS–/– versus WT mice in the cingulate cortex and the prelimbic

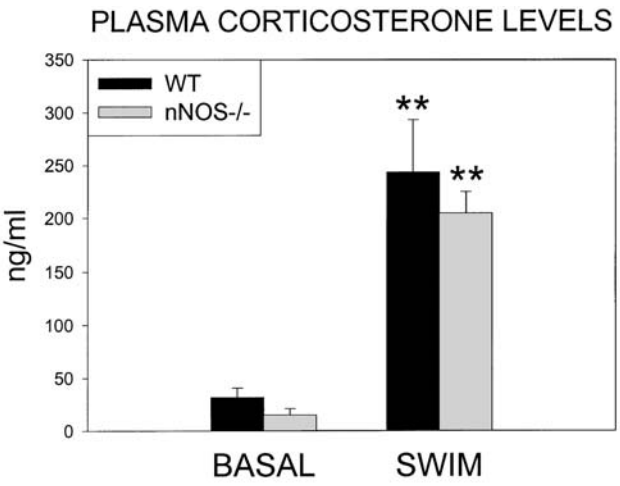

Figure 5. Plasma corticosterone levels of WT and nNOS–/– mice (both genotypes: basal n = 4, forced swimming n = 5) under resting conditions (basal) and 15 min after onset of a 10-min forced-swimming session (SWIM). ** $p < 0.01$ compared with corresponding basal group.

cortex (table 1). No differences in the magnitude of swim stress-induced Fos expression were observed in the remaining brain structures investigated (table 1).

Behaviour in the forced-swim test

In the forced-swim test, nNOS–/– mice displayed a considerably lower level of immobility (–50%) than WT mice. The duration of swimming behaviour was enhanced by 33% in nNOS–/– compared to WT mice. No difference in the duration of climbing behaviour was observed between the two genotypes (fig. 3).

Locomotor activity

During the dark phase of the light/dark cycle, no significant differences in locomotor activity were detected between nNOS–/– and WT mice. During the light phase, nNOS–/– mice displayed higher activity scores compared to WT mice solely during the first 3-h period (7:00–10:00: nNOS–/– 161±48 cm/min, WT 63±34 cm/min, $p<0.05$) while no differences in locomotor activity were detected during the remaining 3-h intervals, in which the behavioural tests were conducted (10:00–13:00: nNOS–/– 172±14 cm/min, WT 127±27 cm/min; 13:00–16:00: nNOS–/– 93±26 cm/min, WT 106±22 cm/min).

Behaviour in the open field test

In the open field test, no differences in general locomotor activity were detected between nNOS–/– and WT mice, since the total distance travelled did not differ between the groups (fig. 4). The number of entries into the central zone was increased in nNOS–/– compared to WT mice. nNOS–/– mice also displayed a tendency to spend more time in the central zone than WT mice; however, this difference failed to reach statistical significance ($p<0.06$) (fig. 4).

Plasma sampling and corticosterone measurement

Statistical analysis revealed that forced swimming caused a dramatic and significant rise in plasma corticosterone (fig. 5) without showing differences between the genotypes.

Discussion

In the present study, we demonstrated that functional inactivation of the gene encoding nNOS leads to an altered pattern of neuronal activation in response to swim stress in specific parts of the limbic system, hypothalamus and the medial prefrontal cortex. Plasma corticosterone levels increased in response to forced swimming and did not differ between WT and nNOS–/–. Furthermore, the results suggest that nNOS–/– mice display an altered behavioural response in the forced swim test, indicative of a more active stress-coping strategy.

Forced swimming-induced Fos expression

Exposure to the forced-swim test increased Fos expression in both WT and nNOS–/– mice in a variety of stress-related brain regions throughout the neuroaxis, including cortical, limbic and hypothalamic areas. The pattern of Fos expression and thus neuronal activation generally agrees with that found previously in mice [27] or rats [22–24, 28] subjected to forced swimming. Pronounced effects were particularly noted in limbic areas including the lateral septum, the paraventricular thalamic nucleus, various hypothalamic nuclei, parts of the periaqueductal grey and the locus coeruleus. In the amygdala, forced swimming elicited a response pattern in which medial amygdala activation predominated over central amygdala activation, a pattern shown to be characteristic for this type of ('psychological') challenge [29]. On the other hand, a difference in forced swimming-induced Fos expression between nNOS–/– and WT control mice was detected only in a limited number (6 out of 42) of these activated brain areas: nNOS–/– mice displayed a higher level of swim stress-induced Fos expression in the infralimbic cortex, medial amygdala, periventricular hypothalamic nucleus, supraoptic nucleus, CA1 field of the hippocampus and dentate gyrus. Since the enhanced Fos expression in nNOS–/– mice was accompanied to a certain extent by motor activation, i.e. longer swim intervals during the forced-swim test, the enhanced locomotor activity might have triggered the enhanced Fos response in these brain regions. However, this possibility seems unlikely, since no differences in the swim stress-induced Fos response were detected in areas known to be activated by

motor activity, for example the motor cortex, cuneiform nucleus or pontine nuclei [30, 31], suggesting that the enhanced Fos response in the nNOS–/– mice was not due to differences in motor activation. Furthermore, plasma corticosterone levels demonstrate that a 10-min forced swimming session provides a powerful stimulus for triggering the activity of the hypothalamic-pituitary-adrenal axis (fig. 5) [see ref. 32] the key neuroendocrine stress system, without showing differences between the genotypes. Thus, the endocrine data provide some evidence that both WT and nNOS–/– mice found the exposure to forced swimming similarly stressful, but chose different strategies to cope with this stressor (see below).

In many stressor-activated areas including those identified in this study, the presence of nNOS has been detected by immunohistochemical or in situ hybridization procedures, or by using NADPH-d histochemistry [see e.g. refs. 33–35]. This is particularly true for the medial amygdala (see fig. 1) and the supraoptic nucleus, which display very dense NOS labelling. nNOS has also been detected with lower abundance in the periventricular hypothalamic nucleus and parts of the hippocampus, including granular cells of the dentate gyrus and pyramidal cells of the CA1 field [see e.g. ref. 36].

The exact role of the NO system in stress mechanisms is not clear at present. Since both inhibitory and excitatory NO effects have been observed on different parts of the hypothalamic-pituitary-adrenal axis, current evidence suggests a complex role for NO in the modulation of the neuroendocrine and behavioural stress response [for review see ref. 4]. The modulatory role of NO in stress mechanisms is also supported by findings showing that the expression of nNOS increases in response to different stressors in specific stressor-sensitive brain areas, including the medial amygdala, supraoptic and paraventricular nucleus [35; for review see ref. 4]. The considerable overlap of these areas with those identified in the present study suggests that the differences in swim-induced Fos expression between nNOS–/– and WT mice are associated with differences in local NO production in these areas, although indirect effects via distant modulation of afferent projection neurons cannot be excluded.

Behaviour

During the forced-swim test, nNOS–/– mice displayed reduced immobility and longer swim duration compared to WT animals, indicating an altered stress-coping strategy in nNOS–/– mice [37]. nNOS–/– mice did not differ from their WT controls in spontaneous home cage locomotor activity during the time period when the forced-swim test was performed, which underscores the specificity of the behavioural differences observed during the forced-swim test. In line with this finding, nNOS–/– mice did not differ from WT mice in general locomotor activity in the open field, in accordance with previous findings [38]. In addition, nNOS–/– mice displayed an increased number of entries into the central zone of the open field, which is thought to indicate reduced anxiety [for review see ref. 39]. Previous studies reported that treatment with different NOS inhibitors induced behavioural effects in rodents similar to our observation. In particular, pharmacologic inhibition of NOS has been shown to decrease immobility in the forced-swim test [9–15] and induces a reduction in anxiety-related behaviour in several exploratory models of anxiety [e.g. 8, 15, 40]. Hence, although our findings have to be confirmed in additional tests of anxiety and depression, they point to the possibility that genetic inhibition of the nNOS in mice induces similar behavioural changes as the pharmacologic inhibition of NO synthesis. Among the brain areas identified here which show differences in Fos labelling between the two genotypes (table 1), the (medial) amygdala has been recently proposed to take part in the control of the behavioural response during acute forced swimming in rats [41]. Thus, further studies may particularly focus on this brain area to further characterize the impact of nNOS/NO in the generation of an appropriate stress-coping strategy.

Taken together, the results show that lack of NO produced by nNOS leads to an altered pattern of neuronal activation in response to swim stress in brain areas predominantly located in parts of the hypothalamus, amygdala, hippocampus and medial prefrontal cortex, suggesting that these brain regions play a significant role in the NO-mediated behavioral stress response.

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