International Journal of Neuropsychopharmacology (2010), **13**, 461–473. Copyright © CINP 2009
doi:10.1017/S1461145709990241

ARTICLE

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Increased stress-evoked nitric oxide signalling in the Flinders sensitive line (FSL) rat: a genetic animal model of depression

Gregers Wegener[1], Brian H. Harvey[2], Birgit Bonefeld[1], Heidi K. Müller[1], Vallo Volke[3], David H. Overstreet[4] and Betina Elfving[1]

[1] *Centre for Psychiatric Research, Aarhus University Hospital, Risskov, Denmark*
[2] *Division of Pharmacology, School of Pharmacy, North West University, Potchefstroom, South Africa*
[3] *Department of Physiology, University of Tartu, Tartu, Estonia*
[4] *Bowles Center for Alcohol Studies, University of North Carolina, Chapel Hill, NC, USA*

Abstract

Stress engenders the precipitation and progression of affective disorders, while stress-related release of excitatory mediators is implicated in the degenerative pathology observed especially in the hippocampus of patients with severe depression. Nitric oxide (NO) release following stress-evoked *N*-methyl-D-aspartate (NMDA) receptor activation modulates neurotransmission, cellular memory and neuronal toxicity. We have investigated the Flinders rat (FSL/FRL), a genetic animal model of depression, regarding the response of the hippocampal nitrergic system following exposure to an escapable stress/inescapable stress (ES-IS) paradigm. Hippocampal tissue from naive FSL/FRL rats and those exposed to ES-IS were studied with respect to constitutive nitric oxide synthase (cNOS) activity and neuronal nitric oxide synthase (nNOS) protein levels, as well as transcript expression of upstream regulatory proteins in the NMDA–NO signalling pathway, including NMDAR1, nNOS, CAPON, PIN and PSD95. Within stress-naive animals, no differences in hippocampal cNOS activity and nNOS expression or PIN were evident in FSL and FRL rats, although transcripts for NMDAR1 and CAPON were increased in FSL rats. Within the group of ES-IS animals, we found an increase in total hippocampal cNOS activity, nNOS protein levels and mRNA expression in FSL *vs*. FRL rats, together with an increase in PSD95 transcripts, and a reduction in PIN. In conclusion, ES-IS enhanced hippocampal cNOS activity in FSL rats, but not FRL rats, confirming the NMDA–NO cascade as an important vulnerability factor in the depressive phenotype of the FSL rat.

Received 31 December 2008; Reviewed 26 January 2009; Revised 20 April 2009; Accepted 11 June 2009; First published online 23 July 2009

Key words: Animal model, depression, nitric oxide synthase, resilience, stress.

Introduction

Nitric oxide (NO) has been implicated in the regulation of various behavioural, cognitive, and emotional processes, e.g. learning, aggression, locomotion, anxiety, and mood (Bernstein *et al*. 1998, 2002, 2005; Dzoljic *et al*. 1997; Holscher, 1997; Oliveira *et al*. 2008; Suzuki *et al*. 2001; Xing *et al*. 2002), while it has also been implicated in glutamate-induced neurotoxicity (Dawson *et al*. 1991), suppression of brain-derived neurotrophic factor (BDNF) release and expression (Canossa *et al*. 2002) and inhibition of hippocampal neurogenesis (Cardenas *et al*. 2005; Park *et al*. 2001; Zhu *et al*. 2006).

Since depression can be causally linked to prior and ongoing psychosocial stress (Kendler *et al*. 2001), and repeated exposure to inescapable stressors increases hippocampal NOS activity and nitrogen oxide levels in animal studies (Harvey *et al*. 2004, 2005), this prompts the question whether increased NO levels in the hippocampus may be a major contributor to the development of behavioural (Masood *et al*. 2003) and structural (Park *et al*. 2001) changes following exposure to stressful conditions. Mechanistically this could be attributed to NO-mediated activation of soluble guanylate cyclase (sGC), nitrosylation of proteins and enzymes and modulation of neuronal excitability (Kiss, 2000; Prast & Philippu, 2001; Snyder & Ferris,

Address for correspondence: Dr G. Wegener, Centre for Psychiatric Research, Aarhus University Hospital, Risskov, DK-8240 Risskov, Denmark.
Tel.: +45 7789 3524 *Fax*: +45 7789 3549
Email: wegener@dadlnet.dk

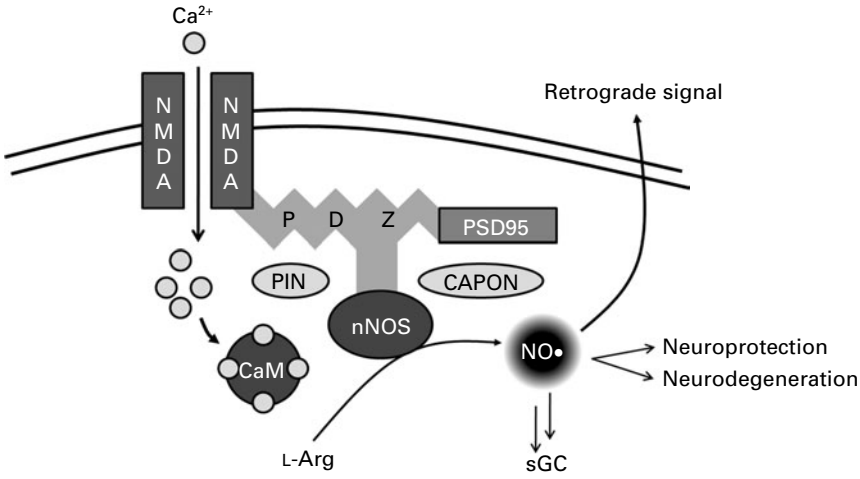

Fig. 1. The nitric oxide (NO) signalling pathway. Following activation of the NMDAR and the influx of Ca^{2+} and its association with Ca^{2+} calmodulin (CaM), neuronal nitric oxide synthase (nNOS) is activated to produce NO. nNOS is closely connected to the NMDAR complex via PDZ terminals, where PSD95 plays a major role. Endogenous inhibitors of nNOS are CAPON and PIN.

2000) (see Fig. 1). Important in this context, is the NO interaction with other classical transmitters involved in mood regulation, particularly the monoamines, as well as glutamate and GABA (Segovia *et al*. 1994; Trabace & Kendrick, 2000; Wegener *et al*. 2000). Also of note is that preclinical studies have shown that conventional antidepressants can modulate NO synthase (NOS) signalling *in vitro* (Finkel *et al*. 1996; Wegener *et al*. 2003), and *in vivo* following local administration (Wegener *et al*. 2003).

Neuronal NOS (nNOS) activity is dependent on glutamate *N*-methyl-D-aspartate (NMDA) receptor activation (Dawson *et al*. 1991), and the involvement of the NMDA receptor (NMDAR) is well described in depression (Nowak *et al*. 1995; Nudmamud-Thanoi & Reynolds, 2004), while antidepressant treatments elicit changes in the NMDAR ion channel (Harvey *et al*. 2002; Paul, 2001; Skolnick, 1999; Stewart & Reid, 2002). Consequently, changes in the expression of the regulatory components of this pathway may significantly change the activity of the NMDA–NOS system, with profound effects on neuronal integrity and function. In the central nervous system, NO synthesis is regulated by post-synaptic density protein 95 (PSD95), protein inhibitor of nNOS (PIN) and the carboxy-terminal PDZ (PSD95-DlgA-zo-1) ligand of nNOS (CAPON) (see Fig. 1). PSD95 is a scaffold protein in the post-synaptic density required for the efficient coupling of nNOS to the glutamate NMDAR (Sattler *et al*. 1999), CAPON is a cytoplasmic protein that competes with PSD95 for binding to nNOS and as such interferes with NMDAR–NOS coupling (Jaffrey *et al*. 1998), while PIN is a cytoskeletal transport protein that inhibits nNOS activity (Jaffrey & Snyder, 1996).

The Flinders sensitive line (FSL) rat has been widely described and highly validated as a genetic animal model of depression (Overstreet *et al*. 2005). These animals present with exaggerated immobility in the forced swim test (FST), the protypical screening procedure on depressed-like phenotype in rodents (Porsolt, 1979; Porsolt *et al*. 1979). As an animal model, FSL animals present with several of the measurable characteristic features of 'clinical' depression, as well as increased stress responsiveness, while they also respond to chronic but not acute treatment with antidepressants when examined in the FST (Overstreet *et al*. 2005; Yadid *et al*. 2000). At the neurobiological level, the FSL rat displays multiple abnormalities consistent with proposed theories of depression, in particular altered serotonergic and cholinergic function (Yadid *et al*. 2000). That serotonergic (Linthorst *et al*. 2002) and cholinergic dysfunction (Janowsky *et al*. 1972) are strongly implicated in the neurobiology of depression, and with both serotonergic (Chanrion *et al*. 2007; Harvey *et al*. 2006*a*) and cholinergic function (Brink *et al*. 2008) known to interact with the NO cascade, supports a basis for NO involvement in mood disorders. However, a causal role for the glutamate–NO pathway in the depressogenic nature of the FSL rat has not been studied.

Considering the prominent sensitivity of the hippocampus to the deleterious effects of stress (McEwen, 2007) as well as evidence of hippocampal shrinkage in patients with depression (Duman, 2004; MacQueen

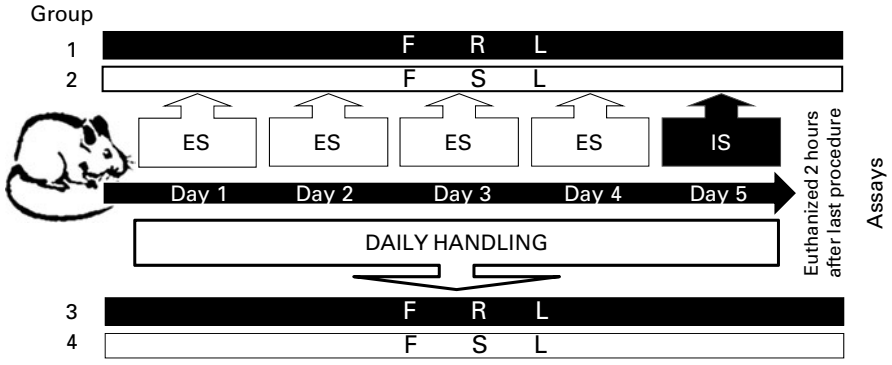

Fig. 2. Experimental design. Animals (FSL and FRL) were each divided into two groups. Two of the four groups were left undisturbed, except for daily handling, whereas the other two groups were exposed to 4-d escapable stress (ES) and 1-d inescapable stress (IS) (see text for further description). The animals were euthanized 2 h after the last experimental procedure. The non-behaviourally tested animals were euthanized within the same daily time window.

et al. 2003), the aims of the present study were to investigate responses in the hippocampal nitrergic system under basal conditions as well as following exposure to a mild, subacute stress paradigm in the FSL rat compared to their control, the Flinders resistant line (FRL) rat. Specifically, looking at changes in total constitutive NOS (cNOS) activity and nNOS protein levels were studied. Thereafter the expression of selected transcripts of upstream regulatory messengers of the NMDA–NOS cascade, i.e. NMDAR1, nNOS, PIN, CAPON and PSD95 under the above-mentioned conditions.

Methods

Animals

Male Flinders line rats (FSL and FRL; age 10–12 wk), from the colony maintained at University of Aarhus (originally derived from the colony at the University of North Carolina, USA), weighing 280–350 g were cage-housed in pairs (Cage 1291H Eurostandard Type III H, $425 \times 266 \times 185$ mm, Techniplast, Italy) at 20 ± 2 °C on a 12-h light/dark cycle (lights on 07:00 hours). Tap water and chow pellets were available *ad libitum*. The animal colony was protected from outside noise, and all experimental procedures were performed in specially equipped rooms within the animal house.

All animal procedures were approved by the Danish National Committee for Ethics in Animal Experimentation (2007/561–1378).

Stress responsiveness of FSL vs. *FRL rats in the FST*

In order to confirm the increased stress responsiveness of FSL rats over their control (FRL) in our colony, and using separate groups of animals, we performed a behavioural evaluation of FSL and FRL rats in the FST, as previously described, using a modified version of the original protocol defined by Porsolt *et al*. (1977, 1978). On the first day, the animals were placed into a perspex cylinder (height 60 cm and diameter 24 cm) filled with water (25 °C) to a height of 40 cm. The animals spent 15 min in the cylinder. On the following day, the animals were placed for 5 min in the cylinder and subsequently dried and returned to their home cages. The behaviours during the 5 min on day 2 were blindly assessed according to previously described methods (Cryan *et al*. 2002).

Study design

Two identical sets of FSL and FRL animals were selected from our colony, and were either left in their home cage with daily handling, or subjected to the stress paradigm described below (Fig. 2).

For methodological reasons, as we wanted the lowest possible analytical variance, parts of the study relating to mRNA expression and Western blotting were designed primarily to investigate the effect of no-stress/ES-IS (escapable stress/inescapable stress) within the FSL/FRL pair, i.e. whether carrying a genetical vulnerability (FSL *vs*. FRL) would be reflected in a difference in response in the NMDA–NO system following ES-IS.

Stress paradigm: ES-IS

ES-IS is a combination of two forms of swim stress based on the animal's aversion to water and its drive to escape. The animals were exposed to an escapable swim stress (ES) procedure performed daily for 4 d, followed by an inescapable swim stress (IS) procedure on day 5 (see Fig. 2). The ES procedure was designed

closely around the typical swimming-learning protocol used in the Morris water maze protocol (Morris, 1981). In this way, the stressor (swimming in the pool) promotes resilience due to adaptive learning (learning to escape the water) in the face of an applied stressor, which may be construed as a controllable subacute mild stressor (Engelmann *et al*. 2006; Francis *et al*. 1995). However, this response may be compromised in vulnerable individuals, such as the FSL rat strain, and will influence subsequent responding to an IS applied on day 5 in which FSL rats already are known to demonstrate increased stress responsiveness compared to their FRL controls (Overstreet *et al*. 2005). Both Morris water maze swimming (Engelmann *et al*. 2006; Francis *et al*. 1995) and forced swimming (Connor *et al*. 1997; Linthorst *et al*. 2002; Yarom *et al*. 2008) are known to evoke stress in animals, while swim-related stressors have been found to activate hippocampal NOS activity (Harvey *et al*. 2004, 2005).

ES procedure

The test animals were subjected to six training sessions per day repeated over 4 d (Fig. 2). The animals were trained to locate a submerged platform (diameter 15 cm) in a circular swimming pool (diameter 1.8 m) using spatial cues surrounding the pool. Briefly, animals were placed tail first into the pool facing the pool wall and allowed to navigate the pool for a maximum period of 120 s. If the animal had located the platform in that time, it was allowed to remain on the platform for 10 s for orientation before being gently lifted from the platform, dried and placed in its home cage until the next training session. If the animal failed to locate the platform within the designated period, it was guided to the platform and allowed to orientate for 10 s before being dried and placed into its home cage.

IS procedure

The test animals were subjected to one 5-min swim session on day 5 (Fig. 2) in a perspex cylinder (height 50 cm and diameter 24 cm) which was filled with water (25 °C) to a height of 30 cm. After 5 min swimming, the animals were dried and returned to their home cages. The short swim duration of 5 min, as well as maintaining the depth of water to allow the tail of the animal to always be in contact with the base of the cylinder, were closely controlled to avoid a sensation of drowning or panic in the animal.

Tissue preparation

All animals were euthanized within the same time window each day (11:00–13:00 hours). Stress animals were euthanized exactly 2 h following ES-IS, as previous studies have reported that conventional brain neurotransmission normalizes at this time following forced swimming (Connor *et al*. 1997; Linthorst *et al*. 2002). Following decapitation and brain removal, the hippocampi were rapidly dissected, and immediately frozen in dry-ice powder. The hippocampi were weighed and stored at −80 °C until further analysis.

cNOS activity

The cNOS activity analytical method was based on the radiometric conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as described previously (Volke *et al*. 1998). Briefly, brain samples of the hippocampus were homogenized separately (1:10 w/v) in ice-cold Tris–HCl (pH 7.4) buffer containing EDTA with a mixer-mill (Retsch; twice for 1 min at 30 Hz/s). After centrifugation the supernatants were removed and used immediately to measure NOS activity. The aliquots of supernatant were added to reaction buffer and incubated for 15 min at 37 °C. The blank samples received buffer without $CaCl_2$ and NADPH. The reaction was stopped by addition of 1 ml ice-cold Hepes buffer containing EDTA and subsequently transferred to ice. [^{3}H]L-citrulline and [^{3}H]L-arginine were separated using a Packard Radiomatic 150 (Packard Instruments Ltd, USA) radio HPLC system (Volke *et al*. 2006). Protein concentrations were measured according to the method of Lowry using bovine serum albumin as standard (Lowry *et al*. 1951).

Western blot

To confirm the nNOS data obtained from the mRNA expression (see below) and activity analyses (see above), Western blotting was conducted with the same samples also used for total NOS activity measures. Supernatants were mixed with 1 volume of Tris buffer (50 mM Tris–HCl, 150 mM NaCl) containing 2% Triton X-100 and Complete™ protease inhibitors (Roche Applied Science, USA) and incubated on ice for 30 min. The samples were centrifuged at 15000 *g* for 10 min and the supernatants were incubated with SDS sample buffer [125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% Bromphenol Blue, and 125 mM dithiothreitol] and incubated at 50 °C for 30 min. Samples were analysed by SDS–PAGE using 10% precast NuPAGE gels (Invitrogen, USA) with a MOPS buffer system. Proteins were transferred onto nitrocellulose membranes using the iBlot dry blotting system (Invitrogen) and membranes were blocked with 5% dry milk in TBS-T [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.5% Tween-20] for 1 h at room

temperature. The membranes were probed overnight at 4 °C with the primary antibodies: rabbit polyclonal anti-nNOS (1:500, sc-546; Santa Cruz, USA) and mouse monoclonal anti-β-actin (1:2000, A 5316; Sigma, USA) followed by incubation with the appropriate peroxidase-con jugated secondary antibody: goat anti-rabbit antibody (1:25.000, no. 1 858 415; Pierce, USA) and goat anti-mouse antibody (1:2000, no. 1 858 413; Pierce) for 1 h at room temperature. Immunoreactive bands were visualized using ECL Advance Western Blotting detection Reagent (GE Healthcare, USA) and the chemiluminescent signals were captured on a Kodak image station 440 and relative intensities were quantified with the Kodak 1D3.6 image analysis software.

Measurements of mRNA transcripts with real-time quantitative polymerase chain reaction (real-time qPCR)

Tissue homogenization, RNA extraction, RNA characterization, cDNA synthesis, and real-time qPCR were carried out as described previously (Elfving *et al*. 2008).

Briefly, hippocampi from the opposite site of the activity/blot measures were homogenized in Lysis buffer (Applied Biosystems, USA) with a mixer-mill (Retsch; twice for 1 min at 30 Hz/s). Total RNA was isolated using the ABI Prism™ 6100 nucleic acid prepstation (Applied Biosystems) according to the manufacturer's instructions, where 13 mg homogenized tissue was loaded per well. Aliquots of the RNA solution were taken for both RNA quantification and qualification.

The integrity of RNA and the RNA concentration were determined with RNA StdSens microfluidic chips using the Experion automated electrophoresis system (Bio-Rad, USA). The RNA purity and the RNA concentration were determined by spectrophotometer (UV1650PC Shimadzu, Japan). To assure the same RNA basal properties in the groups, data on quality, concentration, and purity of the extracted RNA from the FRL and FSL groups were compared with Student's *t* test. Afterwards RNA was reversely transcribed using random primers and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions and with a RNA concentration per reaction of 28 ng/μl. The cDNA synthesis was repeated three times. Afterwards, each cDNA synthesis was tested, and the ones responding properly were pooled and stored undiluted at −80 °C until real-time qPCR analysis. The cDNA samples were diluted 1:30 with DEPC water before being used as a qPCR template.

Real-time qPCR

The real-time qPCR reactions were carried out in 96-well PCR plates using Mx3000P (Stratagene, USA) and SYBR Green. The gene expression of NMDAR1, nNOS, PSD95, PIN and CAPON and eight different reference genes [18s subunit ribosomal RNA (*18s rRNA*), β-actin (*ActB*), Cyclophilin A (*CycA*), Glyceraldehyde-3-phosphate dehydrogenase (*Gapd*), Hydroxy-methylbilane synthase (*Hmbs*), Hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*), Ribosomal protein L13A (*Rpl13A*), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*Ywhaz*) were investigated. The reference genes were selected as previously described (Bonefeld *et al*. 2008). The primers were designed and tested prior to use as in our previous description (Elfving *et al*. 2008).

The following forward and reverse primers were used:

NMDAR1 – forward: AACCTGCAGAACCGCAAG, reverse: GCTTGATGAGCAGGTCTATGC (344 bp);
nNos – forward: ACCCCGTCCTTTGAATACCA, reverse: ACGCTGTTGAATCGGACCTT (455 bp);
PSD95 – forward: CAAGAAATACCGCTACCAAG, reverse: CCTCAGGGTCAATGACGAC (361 bp);
PIN – forward: GATCAAAAATGCAGACATGTC, reverse: GTGTTTGGTCTCGTGTGTC (196 bp);
CAPON – forward: CTGGTGATGCAGGACCCTAT, reverse: CCCACTGTCCGTACGATTCT (167 bp);
18s rRNA – forward: ACGGACCAGAGCGAAAGCAT, reverse: TGTCAATCCTGTCCGTGTCC (310 bp);
ActB – forward: TGTCACCAACTGGGACGATA, reverse: GGGGTGTTGAAGGTCTCAAA (165 bp);
CycA – forward: AGCACTGGGGAGAAAGATT, reverse: AGCCACTCAGTCTTGGCAGT (248 bp);
Gapd – forward: TCACCACCATGGAGAAGGC, reverse: GCTAAGCAGTTGGTGGTGCA (168 bp);
Hmbs – forward: TCCTGGCTTTACCATTGGAG, reverse: TGAATTCCAGGTGAGGGAAC (176 bp);
Hprt 1 – forward: GCAGACTTTGCTTTCCTTGG, reverse: CGAGAGGTCC TTTTCACCAG (81 bp);
Rpl13A – forward: ACAAGAAAAAGCGGATGGTG, reverse: TTCCGGTAATGGATCTTTGC (167 bp);
Ywhaz – forward: TTGAGCAGAAGACGGAAGGT; reverse: GAAGCATTGGGGATCAAGAA (136 bp).

The primers were obtained from DNA Technology A/S, Denmark. Each SYBR Green reaction (20 μl total volume) contained 1 × SYBR Green master mix (Bio-Rad), 0.5 μM primer pairs, and 6 μl diluted cDNA. The mixture was heated initially to 95 °C for 3 min in order to activate hot-start iTaq DNA polymerase and then 40 cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s were

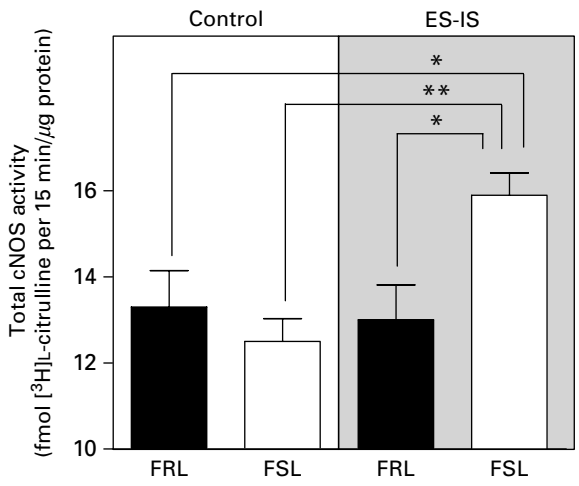

Fig. 3. Hippocampal constitutive nitric oxide synthase (cNOS) activity data under basal conditions [FSL ($n=8$), FRL ($n=7$)], and following escapable stress/inescapable stress (ES-IS) [FSL ($n=8$), FRL ($n=7$)]. Following ES-IS, cNOS activity is significantly elevated in FSL rats compared to unstressed FSL controls (** $p<0.005$) and *vs.* pre- and post-stress FRL animals (* $p<0.05$). Pre- and post-stress activity levels for FRL rats did not differ from one another. Values shown are means ± S.E.M.

applied. To verify that only one PCR product was detected the samples were subjected to a heat dissociation protocol; after the final cycle of the PCR, the reactions were heat-denatured as the temperature was increased from 60 °C to 95 °C. All samples were run in duplicate. A standard curve, performed in duplicate, was generated on each plate.

Data analysis and statistics

Gene expression normalization was done according to our recently published work (Bonefeld *et al*. 2008). Briefly, we first measured mRNA levels for the eight reference genes. Stability comparison of the expression of the reference genes was then conducted with Normfinder software (Andersen *et al*. 2004). Values for each individual test gene were subsequently normalized with the optimal reference genes, based on the Normfinder mathematical algorithm (Andersen *et al*. 2004).

Statistical analyses of the data were analysed by comparing the differential response to stress/no stress within the FSL or FRL strain using Student's *t* test (GraphPad Prism 5.0; GraphPad Software Inc., USA). In the cNOS activity assay, the across-group data were analysed using two-way ANOVA, followed by Bonferroni's *post-hoc* test (Systat 12; Systat Inc., USA). Differences were considered statistically significant when p was <0.05. All data in the figures are ± standard error of the mean (S.E.M.). The numbers of animals in each group are given in the figure legends.

Results

Behavioural validation of FSL/FRL rats in the FST

In the population of animals used for this study, a 5-min FST session found a consistent increase in immobility time (seconds) in the FSL (125.3 ± 10.76, $n=16$) *vs.* the FRL (77.14 ± 16.07, $n=7$, $p<0.05$, $t=2.479$, d.f. $=21$) animals. The basal home cage locomotion in the animals was not different (results not shown).

Normal and ES-IS evoked changes in hippocampal cNOS activity in FSL vs. *FRL rats (Fig. 3)*

The cNOS activity was clearly affected by ES-IS. Using two-way ANOVA, we found no effect of strain, but an effect of ES-IS [$F(1,26)=5.26$, $p<0.03$], and an effect of strain × ES-IS [$F(1,26)=7.49$, $p<0.01$]. Bonferroni's *post-hoc* analysis revealed that the cNOS activity in FSL animals was profoundly influenced by ES-IS ($p<0.005$), but was unaffected by stress in the FRL strain (Fig. 3).

Within the FSL and FRL pair not exposed to ES-IS, we found no marked differences in hippocampal cNOS activity (Fig. 3*a*, left-hand histograms). However, in the FSL/FRL pair subjected to ES-IS, we observed a significant increase in cNOS activity in FSL rats compared to FRL rats ($p<0.05$, Fig. 3, right-hand histograms).

Normal and ES-IS evoked changes in hippocampal nNOS protein expression in FSL vs. *FRL rats (Fig. 4)*

In much the same manner as that described for hippocampal cNOS activity, the hippocampal nNOS protein expression in stress-naive FSL and FRL rats was similar, with no significant differences observed (Fig. 4*a*). However, following exposure to ES-IS we found a significant increase in hippocampal nNOS protein in FSL animals compared to the healthy FRL control rats ($p<0.001$, $t=5.508$, d.f. $=13$; Fig. 4*b*).

Normal and ES-IS evoked expression patterns of NMDAR1, nNOS, PIN, CAPON and PSD95 transcripts in the hippocampus of FSL vs. *FRL rats (Figs 5 and 6)*

In the analysis of non-stress pairs, *Ywhaz* and *Hmbs* were found to be the most stably expressed reference genes, whereas we found *Hmbs* and *Gapd* to be most stably expressed in the ES-IS pairs. Using this

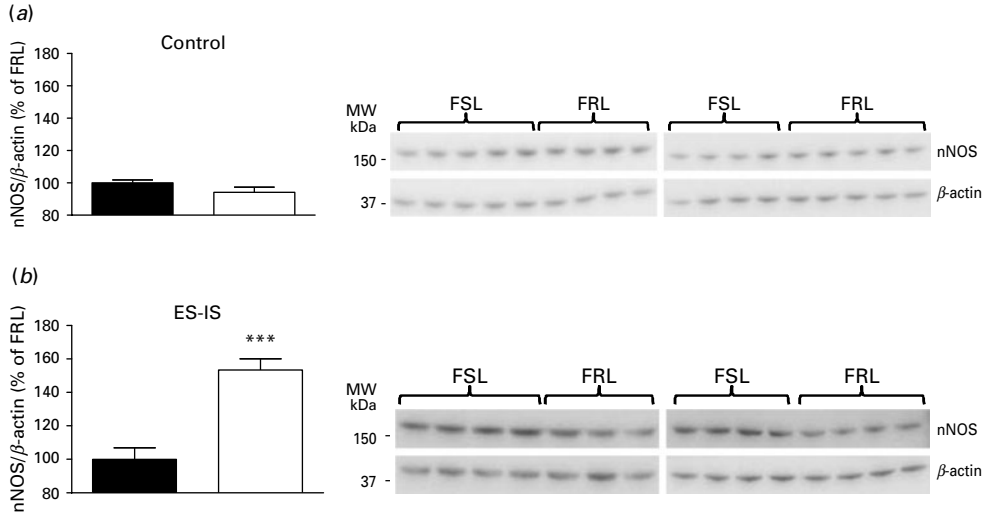

Fig. 4. Hippocampal Western blot of neuronal NOS (nNOS) under (*a*) ambient conditions [FSL ($n=8$), FRL ($n=7$)], and (*b*) following escapable stress/inescapable stress (ES-IS) [FSL ($n=8$), FRL ($n=7$)]. The nNOS is significantly up-regulated in FSL (□) compared to FRL (■) animals following stress (* $p<0.001$), but unchanged under non-stressful conditions. The relative intensities of nNOS immunoreactive bands (nNOS/β-actin) are converted to percentage of control mean values within each blot and combined to express data as percentage ± S.E.M. of control FRL rats.

mathematical approach for normalization we found that in FSL animals not experiencing ES-IS, a significant increase in expression of NMDAR1 subunit was observed ($p<0.01$, $t=2.393$, d.f.$=12$; Fig. 5*a*) as well as a highly significant increase in CAPON expression ($p<0.001$, $t=6.051$, d.f.$=16$; Fig. 5*d*), compared to the FRL strain. No difference in the expression patterns of nNOS, PIN, or PSD 95 was found (Fig. 5*b*, *c*, *e*).

Interestingly, following ES-IS exposure, the mRNA expression patterns showed pronounced changes. When comparing the FSL strain with the FRL strain, we observed a significantly increased expression of nNOS ($p<0.05$, $t=2.796$, d.f.$=14$; Fig. 6*e*) and PSD95 ($p<0.01$, $t=3.403$, d.f.$=13$; Fig. 6*b*) in the stress-sensitive FSL strain, together with a significant decrease in PIN expression ($p<0.05$, $t=2.870$, d.f.$=14$; Fig. 6*c*). Contrary to that observed in the non-stressed cohort, the expression patterns of NMDAR1 and CAPON in FSL and FRL rats were now no longer different from one another (Fig. 6*a*, *d*).

Discussion

Dysregulation of the nitrergic system has been well documented in depression (Bernstein *et al*. 1998, 2002, 2005; Chrapko *et al*. 2004; Kim *et al*. 2006; Lee *et al*. 2006; Oliveira *et al*. 2008; Selley, 2004; Suzuki *et al*. 2001; Xing *et al*. 2002). However, previous studies have only emphasized the unequivocal nature of NO changes in depression and do not agree on whether a hypoactive or hyperactive nitrergic system is involved, or what mediates these changes at the molecular level.

The main finding in the present paper is that, following a mild stress regime, animals that are genetically 'vulnerable' to stress (FSL rats), display significant changes within the NMDA–NOS signalling cascade in the hippocampus compared to FRL control rats. When the same comparison is made in unstressed FSL and FRL rats, these changes are either absent or reversed. Finally, FRL rats showed no change in cNOS activity pre- *vs*. post-stress, while stress evoked a significant increase in cNOS activity in FSL rats. These findings conclude that animals genetically vulnerable to stress may be more prone to excessive NO formation in the hippocampus following stress, thus consistent with an important role for NO in the stress axis and, as such, with the NO hypothesis of depression (Harvey, 1996).

Exposure of rats to water can be defined as being either an escapable or inescapable swim stress depending on the particular aversive event, e.g. navigating a swimming pool with a *known or learned escape mechanism* in place, as opposed to the presence of a life-threatening event, e.g. underwater stress that is inescapable. The latter forms the basis for animal models of post-traumatic stress disorder (Brand *et al*. 2008; Harvey *et al*. 2003, 2006*a*; Uys *et al*. 2003). In contrast, the ES-IS paradigm has attempted to model the prodromal aversive events that predate and predict the later development of a mood and/or anxiety

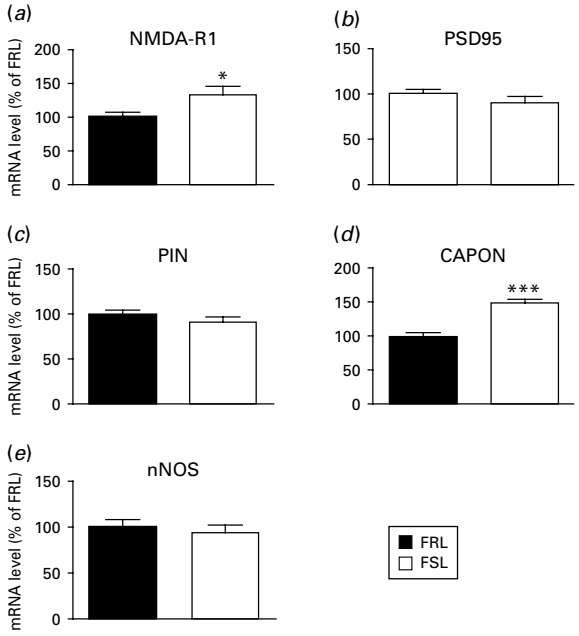

Fig. 5. qPCR data showing the FSL/FRL expression patterns under basal conditions of (*a*) NMDAR1 [FSL ($n=7$), FRL ($n=7$)], (*b*) PSD95 [FSL ($n=9$), FRL ($n=9$)], (*c*) PIN [FSL ($n=9$), FRL ($n=9$)], (*d*) CAPON [FSL ($n=9$), FRL ($n=9$)], and (*e*) nNOS [FSL ($n=9$), FRL ($n=9$)] transcripts. Expression of NMDAR1 and CAPON transcripts are significantly increased (* $p<0.05$, *** $p<0.001$). Values shown are means ± S.E.M. of control FRL rats.

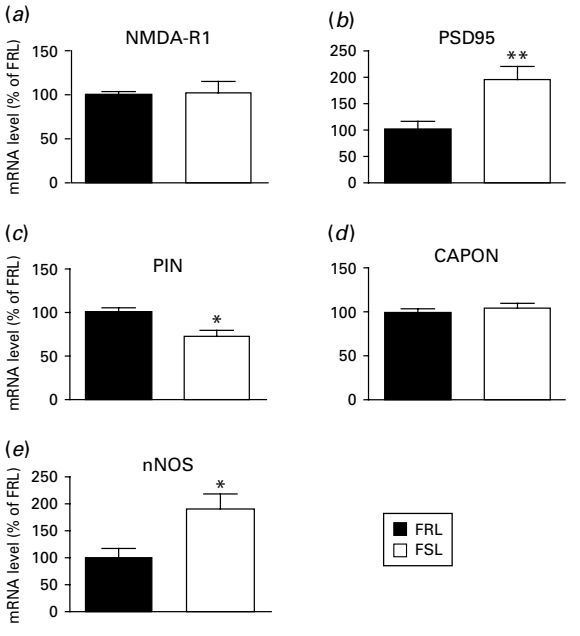

Fig. 6. qPCR data showing the FRL/FSL expression patterns following escapable stress/inescapable stress (ES-IS) exposure of (*a*) NMDAR1 [FSL ($n=8$), FRL ($n=8$)], (*b*) PSD95 [FSL ($n=7$), FRL ($n=8$)], (*c*) PIN [FSL ($n=8$), FRL ($n=8$)], (*d*) CAPON [FSL ($n=7$), FRL ($n=8$)], and (*e*) neuronal NOS (nNOS) [FSL ($n=8$), FRL ($n=8$)] transcripts. Expression of nNOS and PSD95 transcripts are significantly increased, whereas PIN is significantly decreased following stress (* $p<0.05$, ** $p<0.01$). Values shown are means ± S.E.M. of control FRL rats.

disorder in a susceptible individual. We anticipated that the aversive events should not immediately be perceived by the animal as being life threatening, in line with the assumption that water at ambient (25 °C) temperature could be considered a mild stressor, much similar to the normal rodent wild environment (Maier, 1989).

A number of pre-clinical studies have confirmed excessive NOS activation and NO release in the cortex and hippocampus following a protracted stressful event (Harvey *et al*. 2004, 2005; Madrigal *et al*. 2001), as well as concomitant changes in hippocampal NMDAR density (Harvey *et al*. 2004). Chronic stress is a recognized mediator of depressive illness in susceptible individuals (Kendler *et al*. 2001), while clinical observations in post-mortem tissue from patients with bipolar disorder and major depression have suggested changes in the NMDAR complex, particularly in the frontal cortex (Beneyto & Meador-Woodruff, 2008; Feyissa *et al*. 2008; Nowak *et al*. 1995) and hippocampus (Nudmamud-Thanoi & Reynolds, 2004). These latter studies thus affirm the approach of studying glutamate NMDA–NOS signalling as an important protagonist in the psychopathology of major depression, and also in the present study as suggested by evidence of elevated cNOS sensitivity in stress-sensitive FSL rats. This observation was extended through concomitant analysis of upstream messengers of the NMDA–NOS cascade. In unstressed FSL *vs*. unstressed FRL rats, we observed an increase in NMDAR1 transcripts, although without changes in nNOS (Fig. 5*e*). As previous studies with the selective knockout of the *NMDA-NR1* gene in mice have been associated with impaired normal subcellular targeting of NMDA channels (Fukaya *et al*. 2003), so events associated with altered glutamate subunit expression can lead to changes in how the receptor couples to intracellular processes, which could be reflected in the observed increase in CAPON (148% of FRL, Fig. 5*d*). CAPON competes with PSD95 for binding to nNOS, and plays an important role in trafficking, membrane targeting and internalization of NMDAR complexes. Under basal conditions, CAPON could therefore interfere with the NMDA–NOS response (Jaffrey *et al*. 1998), with the observed increase in CAPON mRNA being instrumental in toning down the effects of

PSD95 (Fig. 5*b*) in the signalling cascade, and thereby restraining nNOS transcripts (Fig. 5*e*), protein level (Fig. 4*a*) and cNOS activity (Fig. 3) in unstressed FSL *vs*. FRL rats. With no associated increase in PIN mRNA expression being evident (Fig. 5*c*), the increase in CAPON mRNA could represent a compensatory mechanism preventing excessive NO release in the face of raised NMDA activity (as can be expected following a memory-provoking event as in MWM), and possibly explaining the NMDA–NOS paradox observed here. Further work is needed to confirm these suggestions.

Upon exposure of the FSL and FRL animals to stress (ES-IS), CAPON mRNA was no longer altered (Fig. 6*d*) while that for PIN was now significantly decreased (70% of FRL, Fig. 6*c*). Contrary to that described above, ES-IS engendered an increase in nNOS mRNA (195% of FRL, Fig. 6*e*), followed by the observed increases in nNOS protein levels (160% of FRL, Fig. 4*b*) and cNOS activity (125% of FRL, Fig. 3*b*). A simultaneous increase in PSD95 mRNA (195% of FRL, Fig. 6*b*) is supportive of the suggestion that increased PSD95–nNOS coupling was stimulated by the applied stress paradigm. That the prevailing subcellular signalling components mediating NOS activity are different in FSL and FRL rats is clearly demonstrated by the fact that pre- and post-stress cNOS activity levels were the same in FRL rats, while in FSL rats post-stress cNOS activity was significantly increased compared to pre-stress levels (Fig. 3). At this juncture, however, it should be stated that the cNOS activity assay used in the present study cannot exclude a contribution from endothelial NOS (eNOS) in the total activity measured. Based on the smaller increase in cNOS activity compared to the larger increase evident in the nNOS Western blot assay (i.e. 125% *vs*. 160%), we believe that a contribution from eNOS is possible and that the measurable contribution of nNOS to total cNOS activity following stress is diluted by eNOS. Further studies, e.g. by separating membrane and cytosolic fractions of the homogenate, may assist in identifying the relative contributions from eNOS and nNOS.

Interestingly, we found that NMDA-NR1 transcripts following stress were unchanged in stressed FSL *vs*. stressed FRL rats, which was a reversal of the picture observed in unstressed FSL/FRL rats. Earlier animal studies have brought to light that a fully functional glutamatergic system is needed to evoke a normal stress response (Miyamoto *et al*. 2002). Since the NMDA-NR1 transcripts were unchanged following stress, although with a markedly elevated activity of the NOS system, implies that a disturbance in normal NMDA–NOS signalling could exist in FSL rats following environmental adversity. In support of this, we had earlier found that repeated stress-induced increases in hippocampal nitrogen oxides is reversed by a nNOS inhibitor but not an NMDAR antagonist (Harvey *et al*. 2005). The lack of any noteworthy difference in expression of NMDA-NR1 subunit transcripts in FSL rats *vs*. FRL controls following the ES-IS paradigm could also be hypothesized to be a negative feedback inhibition triggered by the increased NO produced. Indeed, NO inhibits glutamatergic function at various levels, including glutamate release (Segieth *et al*. 1995) and glutamate binding (Fujimori & Pan-Hou, 1991).

The FSL rat is a well-validated model of depression/vulnerability, having important differences with respect to serotonergic and cholinergic signalling (Yadid *et al*. 2000), both of which are implicated in the neurobiology of depression (Janowsky *et al*. 1972; Linthorst *et al*. 2002). Moreover, serotonin and acetylcholine share an interactive association with NO (Brink *et al*. 2008; Chanrion *et al*. 2007; Harvey *et al*. 2006*a*), such that these animals constitute an attractive model with which to study the dynamics of the NO cascade in depression. The observed changes in the NMDA–NOS signalling cascade therefore need to be interpreted in the light of the co-existing anomalies in these animals. The association between cholinergic and serotonergic neurotransmission (Overstreet *et al*. 1998), between cholinergic transmission and cGMP synthesis (Brink *et al*. 2008), and the hypercholinergic state of FSL rats, together suggest that altered serotonergic/cholinergic transmission in FSL rats could account for the changes in NOS signalling observed in these animals during stress. Since FSL rats present with a supersensitive hypothermic response to the 5-HT_{1A} receptor agonist, 8-OH-DPAT (Overstreet *et al*. 1998), reduced serotonergic transmission and an up-regulation of post-synaptic 5-HT receptors can also be suggested. As hippocampal NOS activity may be under tonic inhibition by serotonergic neurons, which may be lost following states of serotonergic depletion, a depressive-like phenotype in animals or depression in humans may be expected (Harvey *et al*. 2006*b*). Indeed, attenuated serotonergic function has been found to be associated with increased NOS activity (Tagliaferro *et al*. 2001), which in the present study could account for the increased NO signalling following exposure of FSL rats to ES-IS. However, further studies on the exact basis for NO activation in FSL rats are needed.

Certain limitations in the current study design need to be considered. Due to our methodological

approach, only cNOS across-group comparisons were possible, which in effect may limit the overall conclusions on causality of the NMDA–NOS markers relating to changes within the NO system in FSL rats. Nevertheless, we believe that the present study confirms that not only do FSL and FRL rats demonstrate important differences with respect to activity of the glutamate NMDA–NO cascade under ambient conditions, but that FSL rats demonstrate heightened responsiveness of NOS activation under conditions of subacute stress (ES-IS), together with associated changes in upstream modulators of NMDA–NOS signalling.

We therefore conclude that the NMDA–NO pathway may play an important role in the increased stress sensitivity that characterizes the FSL/FRL rat, a genetic animal model of depression, and as such provides novel evidence supportive of the involvement of NMDA–NOS signalling in depression.

Acknowledgements

G.W. and B.E. were supported by grants from The Danish Medical Research Council (grants 271-05-218 and 22-04-0566), the Lundbeck Foundation (158/02) and the Augustinus Foundation (no. 06-3280). V.V. was supported by grants from the Estonian Science Foundation (6081). B.H.H. is supported by the South African Medical Research Council and the National Research Foundation under grant number 2053203. We thank Nadia Knudsen and Helle Nygaard Buch for skilful technical assistance.

Statement of Interest

None.

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