

# Effects of nitric oxide synthase deficiency on a disintegrin and metalloproteinase domain-containing protein 12 expression in mouse brain samples

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**Abstract.** A disintegrin and metalloproteinase domain-containing protein 12 (ADAM12) belongs to the ADAM family of transmembrane proteins. Via proteolysis, cell adhesion, cell-cell fusion, cell-matrix interaction and membrane protein shedding, ADAM proteins are involved in normal brain development, and also in cancer genesis and progression, and in inflammation. Therefore, neurobiological research focusing on this protein is increasing. Nitric oxide (NO), which is endogenously produced by NO synthases (NOS), is associated with glial tumors. However, knock-out of NOS produces only limited antitumor effects. The present study analyzed the expression of ADAM12 in the cortex and hippocampus of C57/BL6 wild-type mice, and endothelial NOS-, neuronal NOS- (nNOS) or inducible NOS (iNOS)-deficient (<sup>-/-</sup>) mice, at different stages of development. Expression of ADAM12 was quantified using immunoblot analysis of cortical and hippocampal tissue samples from fetal, neonatal (5 days postnatal), adult (12 weeks old) or >1 year old mice. Using reverse transcription-quantitative polymerase chain reaction, ADAM12 expression was analyzed in cultured N9, OLN93, C6 and PC12 cells, representing the four main cell types in the brain, following NOS inhibition. ADAM12 expression was low in all mouse genotypes and regions of the brain, and in fetal and neonatal mice, an increase in expression was observed with increasing age. The highest levels of expression were observed in the cortex of adult mice, iNOS<sup>-/-</sup> mice of >1 year and wild-type mice, and in the hippocampus of adult and iNOS<sup>-/-</sup> mice of >1 year. By contrast, ADAM12

expression was lowest in adult nNOS<sup>-/-</sup> mice. Inhibition of NOS using N<sup>ω</sup>-Nitro-L-arginine methyl ester hydrochloride, induced ADAM12 mRNA expression in N9 and PC12 cell lines. Inhibition of NOS using L-N<sup>6</sup>-(1-Iminoethyl)lysine dihydrochloride, induced ADAM12 mRNA expression in N9 and C6 cell lines. No change in ADAM12 expression was observed in OLN93 cells following NOS inhibition. ADAM12 expression in mouse hippocampus and cortex samples demonstrated considerable variation during development, with a marked increase observed in adult and >1 year old mice, compared with that in fetal and neonatal mice.

## Introduction

Nitric oxide (NO) is an important signaling molecule found in animals, including humans. Reduced NO production is associated with important cardiovascular risk factors, such as hyperlipidemia (1,2), diabetes, hypertension, smoking, atherosclerosis and aging (3). The bioavailability of NO in cells and tissues decreases with age. This may be a result of a decrease in the expression of the constitutive isoforms [endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS)], with age (4). Inducible NOS (iNOS)-mediated NO formation has been shown to affect longevity. In mice, iNOS overexpression may lead to increased mortality, which is associated with cardiac hypertrophy and sudden cardiac death as a result of bradyarrhythmias (5). iNOS expression is typically not observed in the brains of young (1-3 months old) animals and its expression increases with age (6). Furthermore, it has been shown that in the brain superoxides combine with NO to form peroxynitrite, thereby reducing the bioavailability of NO (7). Age-associated impairment of macrophage function is associated with a substantial decrease in iNOS levels in the immune system (8).

In the healthy nervous system, NO contributes, with other molecules, to learning and memory; synaptic activity; neural plasticity, including neurogenesis and cell survival; and cell differentiation. NO is associated with neurodegeneration, neuroinflammation and pathophysiological conditions, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis, Huntington's disease (9,10) and brain tumors.

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NO activity has been extensively studied in tumor biology (11-28). It appears to be involved in all phases of carcinogenesis (12). Tumorcidal and tumor-promoting effects of NO have been observed, which depend on the type of cancer and the availability of NO (13). eNOS-derived NO has been shown to enhance angiogenesis in gliomas (14). Low grade gliomas demonstrate constitutive expression of eNOS in vessel endothelial cells and astrocytes, whereas malignant gliomas demonstrate overexpression of eNOS in aberrant vessels (15). Furthermore, studies have demonstrated that eNOS-deficient (eNOS<sup>-/-</sup>) mice may be resistant to chemical carcinogenesis (16) and platelet-derived growth factor-induced gliomagenesis (17). eNOS inhibition was shown to lead to a decrease in tumor neovascularization, vascular permeability and tumor growth, in a number of carcinoma models (18,19). Furthermore, increased nNOS expression has been observed in glial neoplasms. The highest nNOS values were observed in world health organization (WHO) grade III and IV tumors, and in carcinoma and melanoma metastatic tissues, whereas no or low nNOS expression was observed in WHO grade I or II tumors, and in meningiomas (20,21). iNOS expression is induced in different types of human brain tumors, including gliomas (22), where it is known to promote glioma stem cell proliferation and tumor growth (23). Therefore, the inhibition of iNOS expression may be a useful therapeutic approach for the suppression of local cancer growth (11,24).

Studies have suggested that NO donors may exhibit antiglioma (25) or antileukemic (26) effects. Evidence from previous studies indicates that NOS knockout may induce tumor development and progression (27,28). NO is referred to as a 'double-edged sword' for a number of reasons: The three NOS isoforms show different spatial and temporal expression patterns; the levels of NO production are variable, depending on its (sub-)cellular source; NO may cause either cell death or proliferation; and, depending on its concentration, NO may exhibit anti- or pro-inflammatory activity.

As with NOS isoforms, studies have shown that members of the matrix metalloproteinase (MMP) family and a disintegrin and metalloproteinase domain-containing proteins (ADAMs) are overexpressed in high-grade glioma. Glioma-associated proteins include MMP-1, MMP-11, MMP-19 (29,30) and ADAM12 (31). These extracellular matrix degrading enzymes are associated with the fatal invasive capacity of high grade gliomas (32,33). Furthermore, ADAM12 determines the localization, and promotes the activation, of MMP-14 (34).

It is hypothesized that ADAM12 and NO may act synergistically. NO suppresses MMP-9 expression by destabilizing its mRNA in rat mesangial cells (35), whereas inhibition of NOS has been found to promote cytokine-induced MMP-9 expression in aortic smooth muscle cells (36) and exotoxin-mediated MMP expression in iNOS<sup>-/-</sup> mice (37). Furthermore, NO may inhibit hypoxia-induced expression of ADAM10 (38). Therefore, it is hypothesized that NO may affect the expression of other ADAMs, including ADAM12. The present study analyzed ADAM12 expression levels in the cortex and hippocampus of wild-type mice, and in eNOS<sup>-/-</sup>, nNOS<sup>-/-</sup> and iNOS<sup>-/-</sup> mice, at different stages of development. For NO production, an age dependency is clear, however concerning ADAM12 expression in aged animals, available data are rare and limited to the musculature only (39).

ADAM12 belongs to a subgroup of the ADAM family, consisting of ADAM8, ADAM9, ADAM10, ADAM17 and ADAM19. They are cell-surface glycoproteins, containing metalloprotease, disintegrin (Arg-Gly-Asp-binding motif), cysteine-rich and epidermal growth factor (EGF)-like domains, and they are responsible for the release of the extracellular parts of membrane-bound proteins (shedding). Two splice variants of ADAM12 occur in humans: Membrane-anchored (ADAM12L) and cytoplasmic secreted (ADAM12S) (40). ADAM12 is proteolytically active *in vitro* and *in vivo*. ADAM12 may cause the shedding of pro heparin-binding-EGF (proHB-EGF), insulin-like growth factor-binding protein 3 (IGFBP-3), IGFBP-5 and oxytocinase (41). Recently, ADAM12 has been associated with ectodomain shedding of endothelial proteins in tumor vasculature (42). A consensus sequence that is required for, or facilitates, ADAM12 cleavage remains to be identified (43). The cytoplasmic tail of ADAM12 is one of the longest observed among the ADAMs (179 amino acids). It appears to be involved in the regulation of ADAM12 cellular localization (44). The cytoplasmic domain of ADAM12 contains a number of proline-rich motifs that are putative Src homology 3 (SH3) binding sites, which enable the recruitment of adapter molecules and subsequent activation of cellular signaling pathways. In addition, the cytoplasmic domain may contain one potential tyrosine phosphorylation site (SH2-binding site) and several serine/threonine phosphorylation sites (40). The following cytoplasmic binding partners have been identified for ADAM12: Src, proto-oncogene tyrosine-protein kinase Yes, growth factor receptor-bound protein 2, phosphoinositide 3-kinase,  $\alpha$ -actinin-1,  $\alpha$ -actinin-2, protein kinase C  $\Delta$ , fluorescence *in situ* hybridization and protein kinase C, and casein kinase substrate in neurons protein 3 (40,41). Cytoplasmic binding sites may influence the maturation, trafficking, membrane (raft) localization and proteolytic activity of ADAMs.

In the early stages of mouse development, ADAM12 mRNA expression is prominent in mesenchymal cells, which develop into skeletal muscle, bone and visceral organs (45). ADAM12 mRNA may be detected at >10.5 DNA protein cross-link levels. It was originally reported to exhibit a restricted expression pattern in adult tissues, and the highest levels of ADAM12 expression have been observed in bone tissue samples (46). More recently, it has been shown that ADAM12 is ubiquitously expressed in adult mice. Studies have demonstrated that ADAM12 expression in mouse brain is predominantly, yet not exclusively, observed in oligodendrocytes (47,48).

## Materials and methods

**Mice.** Experiments were performed in accordance with the recommendations of and was approved by the Commission for Animal Care of the State of Saxony-Anhalt (Dessau, Germany) and German law of the Protection of Animals (permit number: 42502-2783 UniMD). The present study was conducted with laboratory-bred mice. Mice (C57BL/6 and wild-type control strains) were obtained from the homozygous institute breeding colony. nNOS<sup>-/-</sup> mice were provided by the Hunag Lab (49,50), eNOS<sup>-/-</sup> mice were provided by the Gödecke Lab (51), iNOS<sup>-/-</sup> mice were obtained from Charles River Laboratories (Sulzfeld, Germany) as developed in the

C57BL/6 strain (Charles River Laboratories) and their wild-type control strains. Animals were housed at 21°C, exposed to a 12 h light and darkness cycle with access to food and water, *ad libitum*. Tissue from fetal, neonatal (5 days postnatal), adult (12 weeks old) and >1 year old mice was used, whereby the >1 year group consisted of animals aged between 12 and 18 months old. (n=3 per age group).

**Tissue preparation.** In order to minimize circadian influences on the expression of hypothalamic peptides, mice were sacrificed between 09:00-09:30 h. Mice were anesthetized using isoflurane (Baxter GmbH Deutschland, Unterschleißheim, Germany). Subsequently, the mice were subjected to perfusion through the left ventricle for 30 sec, with 0.1 M phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich, Heidelberg, Germany) and then at 15 ml/min with 250 ml 4% buffered paraformaldehyde (pH 7.4; PFA; Sigma-Aldrich). Brains were removed and post-fixed in 4% buffered PFA overnight at room temperature. The brains were subjected to cryoprotection for 2 days using 20% sucrose in 0.4% buffered PFA (pH 7.4) and embedded in paraffin. Brain coronal sections (10 µm) were prepared using a sliding microtome (SM2010 R, Leica, Bensheim, Germany). Tissue samples were obtained from mice (n=10 per age group) that had not been subjected to perfusion and were directly frozen in liquid nitrogen (-80°C), in order to conduct reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunoblot analyses.

**Cell cultures.** The following cell lines, representing the four main cell types in the brain, were used in the experiments: N9 mouse microglia cell line, [Ricciardi-Castagnoli lab (52)], C6 rat astroglia [CCL-107<sup>TM</sup>; American Type Culture Collection (ATCC), Manassas, VA, USA], OLN-93 rat oligodendroglial [Richter-Landsberg lab (53)] and rat neuronal PC12 (CRL-1721<sup>TM</sup>; ATCC). Cryopreserved cells were defrosted, resuspended in an RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 1% L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies GmbH, Darmstadt, Germany), and transferred to culture flasks (Sarstedt, Nümbrecht, Germany). Following 3 days of incubation, cells were removed from the flasks by gentle agitation and incubated on poly-D-lysine coated Ø35-mm petri dishes (Sarstedt; ~50,000 cells/dish), for 6 days. Subsequently, the respective experiments were performed, using the following NOS inhibitors: L-N<sup>6</sup>-(1-Iminoethyl)lysine dihydrochloride (L-NIL; 0.5 mM; specific inhibitor of iNOS), N<sup>ω</sup>-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.5 mM; inhibitor of nNOS and eNOS, to lesser extent also of iNOS) and asymmetric dimethylarginine (ADMA; 10 µM; specific inhibitor of eNOS) all for 24 h, all from Sigma-Aldrich. Cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere and the medium was changed every other day.

**Immunoblot analysis of ADAM12.** Frozen tissue samples were pulverized in liquid nitrogen and subsequently homogenized in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 10% glycerol, 10 mM K<sub>2</sub>HPO<sub>4</sub> and 0.5% NP-40; all from Sigma-Aldrich), containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany), 1 mM sodium vanadate, 0.5% deoxycholate, 0.1 mM

phenylmethanesulfonylfluoride, 20 mM NaF, and 20 mM glycerol 2-phosphate (Sigma-Aldrich). Tissue homogenates were centrifuged at 15,000 x g for 15 min and the resulting supernatant was stored at -20°C.

Extracted proteins (30 µg per lane) were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell BioScience GmbH, Dassel, Germany). Membranes were blocked using 1 x Roti-block solution (Carl Roth, Karlsruhe, Germany) and then incubated with a primary antibody against ADAM12 cytoplasmic domain (AB19032; 1:1,000 dilution, affinity isolated, polyclonal, anti-rabbit; Merck Millipore, Darmstadt, Germany) diluted in PBS (0.1%) buffer with Tween-20<sup>®</sup> in bovine serum albumin (BSA; 5%; Sigma-Aldrich, Hofheim, Germany). Subsequently, the blots were washed three times in PBS (0.3%) with Tween-20 and then polyclonal horseradish peroxidase-conjugated anti-rabbit antibodies (#7074; 1:2,000 dilution with 1 x Roti-Block; Cell Signaling Technology, Frankfurt, Germany) were applied. The SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) was used in order to detect chemiluminescence. In order to compare groups, densitometric quantification was performed on equally processed blots that had been exposed to the same X-ray film [CEA (Deutschland) GmbH, Hamburg, Germany].

**RNA extraction and RT-qPCR analysis of ADAM12 expression.** Total RNA from cortical and hippocampal tissue samples was extracted using a two-step protocol using a TRIzol<sup>®</sup> extraction (Invitrogen Life Technologies, Carlsbad, CA, USA) and the RNeasy kit<sup>TM</sup> (Qiagen, Hilden, Germany) according to a previous study (54). Tissue samples were frozen in liquid nitrogen and subsequently stored in 0.5 ml TRIzol at -80°C. Tissue samples were then homogenized using DSTROY-S pistils (Biozym Scientific GmbH, Oldendorf, Germany) by subjecting the sample to 3-5 freezing (using liquid nitrogen; -195°C) and thawing (using an ice bath; 0°C) cycles. After complete homogenization, 0.2 ml chloroform was added, and the mixture was extensively vortexed and centrifuged using a microcentrifuge (12,000 x g, 4°C) for 15 min. The supernatant was incubated with isopropanol (volume ratio 1:1) at room temperature for 10 min, and the precipitated RNA was obtained by centrifugation (12,000 x g, 4°C, 10 min). The RNA pellet was resolved in 100 µl RNase-free water and subsequently purified using the RNeasy kit, according to the manufacturer's instructions. Finally, the RNA was eluted in 50 µl RNase-free water, confirmed using gel electrophoresis, and 5 µl of RNA was used to measure the concentration of the RNA samples via ultraviolet spectroscopy (NanoDrop 2000c; Thermo Fisher Scientific, Wilmington, DE, USA).

For the cell lines, RNA was isolated using the innuPREP RNA isolation kit (Analytik Jena, Jena, Germany), as described previously (55).

Total RNA (1 µg) was transcribed to a final volume of 40 µl using 20 units of avian myeloblastosis virus reverse transcriptase (Promega GmbH, Mannheim, Germany), containing 1 x reaction buffer, 0.5 mM dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 10 mM random hexanucleotides and 50 units of placenta RNase inhibitor (Promega GmbH). The samples were incubated at 42°C for 1 h. Subsequently enzymes



were inactivated at 95°C for 10 min and the reaction mixture was frozen at -80°C, prior to enzymatic amplification.

ADAM12 transcript levels were determined using RT-qPCR with an iCycler (Bio-Rad, Munich, Germany) and the SensiMix™ dT kit (Bioline GmbH, Luckenwalde, Germany). The PCR reaction mixture (25 µl) consisted of 12.5 µl of 2 x concentrated master mix (SensiMix™; Bioline GmbH), 2 µl RT-reaction and 0.25 µM reverse and forward primers for ADAM12, large ribosomal protein p0 (RPLP0) or 60S ribosomal protein L13a (RPLP13a). The latter two primers were used for standardization. The following PCR protocol was performed: Initial denaturation and activation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 20 sec and elongation at 72°C for 30 sec. Fluorescence intensity of the double-stranded specific SYBR-Green I, reflecting the quantity of PCR-product, was measured (CFX96 Thermocycler; Bio-Rad) at the end of each elongation step. Correlation coefficients of all three standard curves were >0.95. Final results are expressed as artificial units. RPLP0 and RPLP13a expression levels were used to normalize the cDNA contents. In order to verify the size of the PCR product, samples were separated on 1.8% agarose gels and stained with ethidium bromide. The following primers, which were designed using the Invitrogen OligoPerfect Designer and obtained from Invitrogen Life Technologies were used for the RT-qPCR analysis: Forward: 5'-GCACTCTCgCTT TCTGGAGGGTGT-3' and reverse: 5'-TGACTTGGTTGC TTTGGCGGGATT-3' for mouse RPLP0 (344 bp), forward: 5'-CTGGTACTTCCACCCGACCTC-3' and reverse: 5'-GGA TCCCTCCACCCTATGACA-3' for rat RPLP13a (131 bp), forward: 5'-CAGCAACTCCTGTGACCTCC-3' and reverse: 5'-GTACCAATGACAGGTCGGCT-3' for mouse ADAM12 (333 bp) and forward: 5'-GCTTGCAGGAACCAAGTG TG-3' and reverse: 5'-CTGGTTATCTGCTTGCCGGA-3' for rat ADAM12 (226 bp).

**Immunohistochemistry.** ADAM12 immunoreactive material was immunolocalized using a rabbit polyclonal antiserum against the appropriate peptide sequence (1:200 dilution; SA-378; Biomol GmbH, Hamburg, Germany) and a nickel-amplified avidin-biotin technique, as previously described (47). The sections were incubated with methanol and H<sub>2</sub>O<sub>2</sub> in order to suppress endogenous peroxidases. Subsequently, they were repeatedly washed with PBS and the primary antiserum was then applied. The immunohistochemical protocol used an avidin-biotin method and a vectastain-peroxidase kit (Cameron, Wiesbaden, Germany), using 3,3'-diaminobenzidine as chromogen. The color reaction was enhanced by adding 2 ml of a 0.5% nickel ammonium sulfate solution, which yields a dark purplish-blue reaction product. Specific antibodies were replaced with buffer solution or normal rabbit serum (both from Sigma-Aldrich) for the control samples. Pre-absorption of the antiserum was performed using a peptide in order to produce the antiserum, according to the methods described in a previous study (47).

**Statistical analysis.** Data from brain tissue experiments were analyzed using Microcal Origin™ version 6.0 (OriginLab Corporation; Northampton, MA, USA). A Mann-Whitney test was used for the evaluation of ADAM12 expression.

For statistical analysis of the culture experiments, GraphPad Prism® 6.0 program package (Graphpad Software, Inc., La Jolla, CA, USA) was used. A Kolmogorov-Smirnov-Test was conducted in order to check that the results were normally distributed, and a one-way analysis of variance was performed in order to compare groups. Significant interactions were investigated using Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Localizations of ADAM12 expression.** In the wild-type mouse brain samples, ADAM12 immunoreactivity was observed almost exclusively in oligodendrocytes (Fig. 1A). Oligodendrocytes were also the predominant cell type to express ADAM12 in eNOS<sup>-/-</sup> and nNOS<sup>-/-</sup> mouse brain samples (Fig. 1B and C; P>0.05 vs. wild-type). In addition to oligodendroglial cells, a number of neurons were observed to be ADAM12 immunoreactive in eNOS<sup>-/-</sup> (Fig. 1D) and nNOS<sup>-/-</sup> (Fig. 1E) mouse brain samples. By contrast, ADAM12 immunopositivity was observed less frequently in neurons (P<0.001 vs. nNOS<sup>-/-</sup>; P<0.002 vs. eNOS<sup>-/-</sup>), astrocytes and blood vessel endothelial cells of wild type mice (Fig. 1F).

**ADAM12 mRNA expression in the cortex and hippocampus of wild type mice.** RT-qPCR analysis was conducted in order to investigate the expression levels of ADAM12 in the cortex and hippocampus of wild-type mice. An increase in ADAM12 mRNA expression was observed with increasing age. The highest levels of ADAM12 mRNA expression were observed in the hippocampus of adult mice (Fig. 2).

**Expression of ADAM12 in the cortex of wild-type and NOS<sup>-/-</sup> mice.** In order to verify the age-associated changes in ADAM12 mRNA expression levels, immunoblot analyses were performed. Mature (90 kDa) ADAM12 was detected in the cortex of wild-type C57/BL6 mice, at all ages. In accordance with the mRNA expression data, protein expression was low in the cortex of fetal and neonatal mice. By contrast, a markedly increased level of ADAM12 expression was observed in adult and >1 year old mice. A similar increase in ADAM12 expression with age was observed in the cortex of nNOS<sup>-/-</sup>, iNOS<sup>-/-</sup> and eNOS<sup>-/-</sup> mice. However, in adult mice of the latter genotype, a tendency for ADAM12 expression levels to remain below those of wild-type mice was observed (P>0.05). Likewise, iNOS<sup>-/-</sup> mice >1 year old exhibited a tendency towards reduced levels of ADAM12 expression compared with wild-type mice (P<0.1) (Fig. 3).

**Expression of ADAM12 in the hippocampus of wild-type and NOS<sup>-/-</sup> mice.** As with ADAM12 expression levels in the cortex, mature (90 kDa) ADAM12 expression was observed in the hippocampus of mice of all ages and genotypes. A marked increase in ADAM12 expression was observed in adult and mice >1 year old, compared with that in fetal and neonatal mice. There was a tendency towards increased levels of mature ADAM12 in iNOS<sup>-/-</sup> mice and reduced expression in eNOS<sup>-/-</sup> and nNOS<sup>-/-</sup> mice when compared with wild-type mice (P<0.1; Fig. 4).

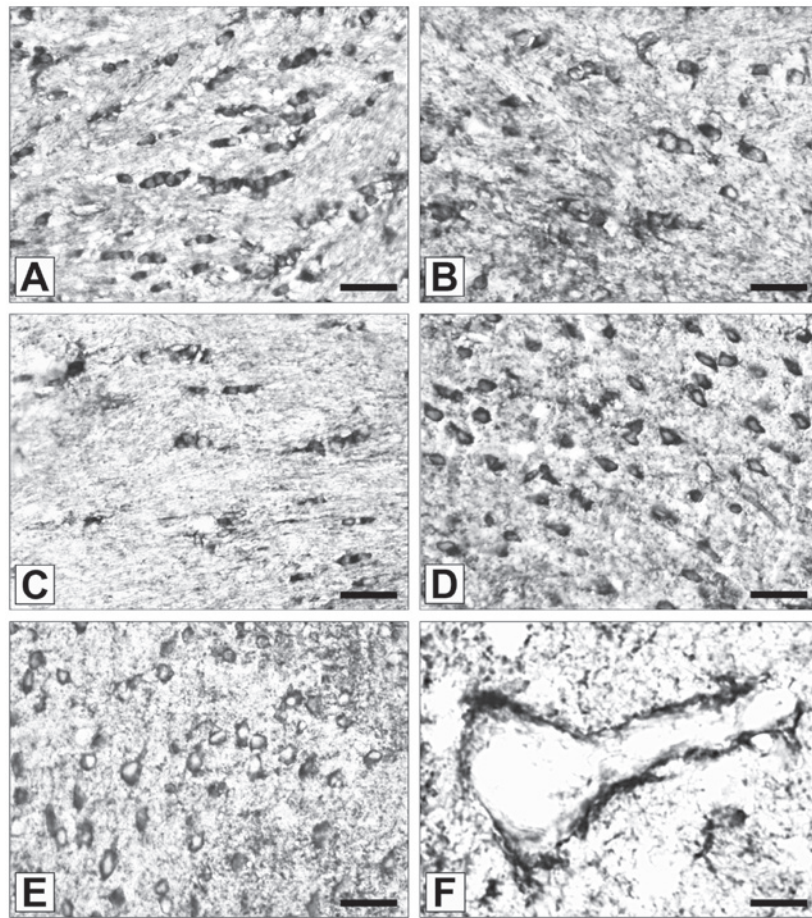


Figure 1. Cellular expression of ADAM12 in brain samples from representative wild-type, nNOS<sup>-/-</sup> and eNOS<sup>-/-</sup> mice. (A) Wild-type mouse. A number of parafascicular oligodendrocytes exhibit ADAM12 immunopositivity. Bar=20  $\mu$ m. (B) ADAM12-expressing parafascicular oligodendrocytes in an eNOS<sup>-/-</sup> mouse brain sample. Bar=15  $\mu$ m. (C) ADAM12-expressing parafascicular oligodendrocytes in an nNOS<sup>-/-</sup> mouse brain sample. In the upper left corner an ADAM12 immunoreactive astrocyte is visible. Bar=20  $\mu$ m. (D) Cluster of immunostained neurons in an eNOS<sup>-/-</sup> mouse brain sample. Bar=30  $\mu$ m. (E) Cluster of immunostained neurons in the brain of an nNOS<sup>-/-</sup> mouse brain sample. Bar=30  $\mu$ m. (F) A cerebral microvessel surrounded by ADAM12 immunoreactive astrocytic end-feet and an ADAM12-expressing astrocyte in a wild-type mouse brain sample. iNOS<sup>-/-</sup> was not included in this figure as the structure preservation was unsatisfactory. ADAM12 immunoreactive neurons were rarely observed in wild-type mice. Bar=15  $\mu$ m. ADAM12, a disintegrin and metalloproteinase domain-containing protein 12; nNOS<sup>-/-</sup>, neuronal nitric oxide synthase-deficient; eNOS<sup>-/-</sup>, endothelial nitric oxide synthase-deficient.

**Effects of pharmacological inhibition of NOS on ADAM12 expression in glial cell lines.** The N9 microglial cell line was exposed to the following NOS inhibitors: L-NIL, L-NAME and ADMA for 24 h. Subsequently, ADAM12 mRNA expression in N9 cells was determined using RT-qPCR. NOS inhibition using L-NIL and L-NAME (0.5 mM) led to a significant increase in ADAM12 mRNA expression levels compared with the controls ( $300 \pm 50\%$ ,  $P < 0.05$  and  $400 \pm 50\%$ ,  $P < 0.03$ , respectively; Fig. 5A). NOS inhibition using ADMA at 10  $\mu$ M led to a significant, 2-fold increase in ADAM12 mRNA expression in N9 cells, compared with that in the control cells ( $P < 0.02$ ).

The effects of pharmacological NOS inhibition on ADAM12 expression in the PC12 neuronal cell line was then investigated. In the PC12 cell line, inhibition of NOS using L-NAME, significantly increased ADAM12 mRNA expression, compared with that of the controls ( $173 \pm 25\%$ ,  $P < 0.05$ ). By contrast, no differences were observed in ADAM12 mRNA expression levels between PC12 cells treated with L-NIL or ADMA, and those in the control cells (Fig. 5B).

ADAM12 mRNA expression in rat OLN93 cells was not affected by L-NAME, L-NIL nor ADMA treatment, compared

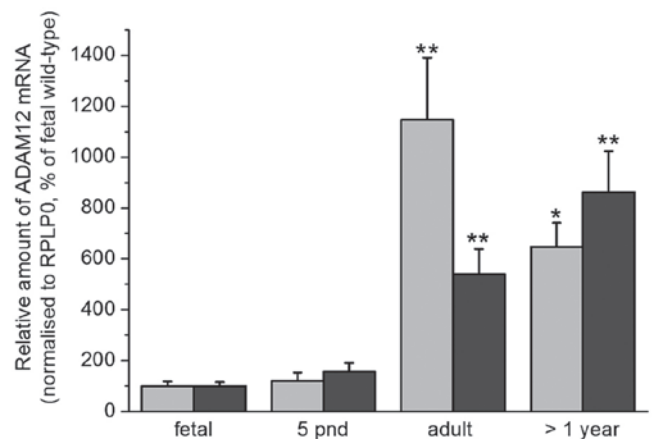


Figure 2. ADAM12 mRNA expression in brain samples from wild-type mice increases with age. Hippocampal (grey) and cortical (black) ADAM12 mRNA expression levels were assessed using reverse transcription-quantitative polymerase chain reaction in fetal, neonatal, adult and >1 year old mice. Expression levels were normalized to large ribosomal protein p0 (mouse) mRNA. Relative values with fetal expression levels set to 100% (mean  $\pm$  standard error, n=8) \* $P < 0.05$  and \*\* $P < 0.01$  vs. fetal group. ADAM12, a disintegrin and metalloproteinase domain-containing protein 12; pnd, postnatal days.

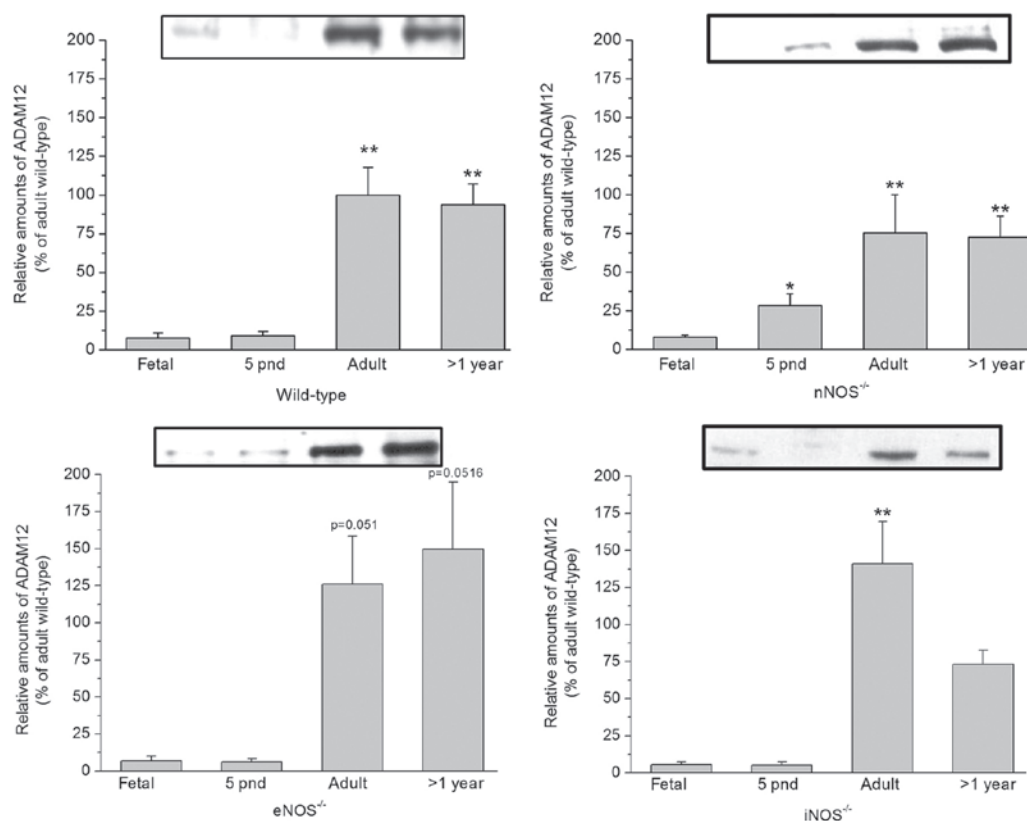


Figure 3. ADAM12 expression in the cortex of wild-type (top left), nNOS<sup>-/-</sup> (top right), eNOS<sup>-/-</sup> (bottom left) and iNOS<sup>-/-</sup> (bottom right) mice at different ages, assessed using immunoblot analysis. Data are presented relative to that of adult wild-type mice, standardized to 100% (mean  $\pm$  standard error,  $n \geq 6$ ). \*P<0.05 and \*\*P<0.01 vs. fetal). ADAM12, a disintegrin and metalloproteinase domain-containing protein 12; nNOS<sup>-/-</sup>, neuronal nitric oxide synthase deficient; iNOS<sup>-/-</sup>, inducible nitric oxide synthase deficient; eNOS<sup>-/-</sup>, endothelial nitric oxide synthase deficient; pnd, postnatal days.

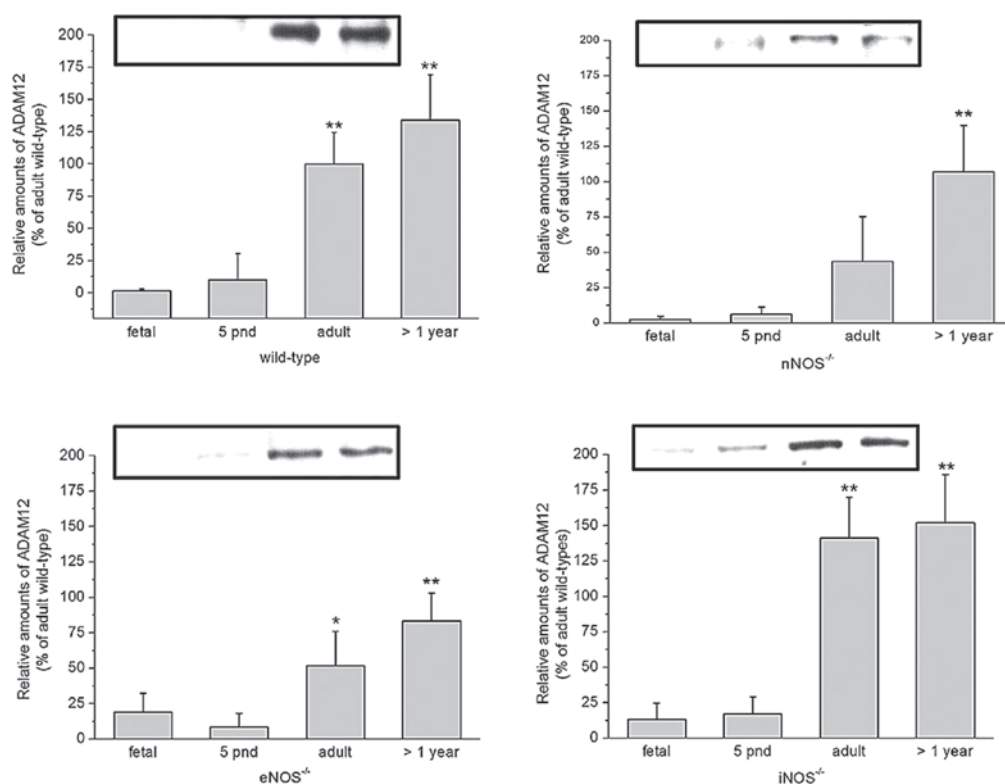


Figure 4. ADAM12 expression in the hippocampus of wild-type (top left), nNOS<sup>-/-</sup> (top right), eNOS<sup>-/-</sup> (bottom left) and iNOS<sup>-/-</sup> (bottom right) mice at different age, as assessed using immunoblot analysis. Data are presented relative to that of adult wild-type mice, standardized to 100% (mean  $\pm$  standard error,  $n \geq 6$ , \*P<0.05 and \*\*P<0.01 vs. fetal). ADAM12, a disintegrin and metalloproteinase domain-containing protein 12; nNOS<sup>-/-</sup>, neuronal nitric oxide synthase deficient; iNOS<sup>-/-</sup>, inducible nitric oxide synthase deficient; endothelial nitric oxide synthase deficient, eNOS<sup>-/-</sup>; pnd, postnatal days.



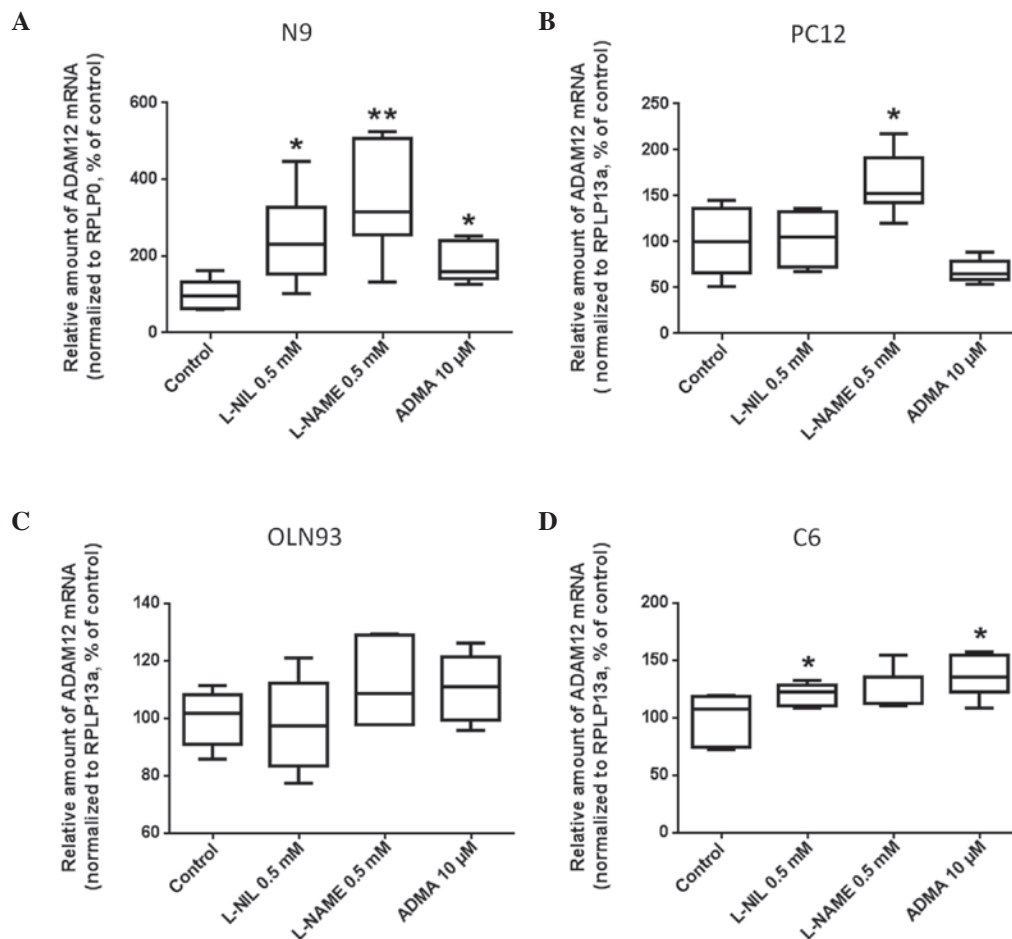


Figure 5. Effect of NOS-inhibition on ADAM12 mRNA expression in (A) N9 cells, (B) PC12 cells, (C) OLN93 cells and (D) C6 cells. Cells were exposed to NOS inhibitors at different concentrations for 24 h and ADAM12 mRNA levels were assessed using reverse transcription-quantitative polymerase chain reaction. Expression levels were normalized to that of large ribosomal protein p0 (mouse) or 60S ribosomal protein L13a (rat) mRNA levels, and the relative expression of control cells was set to 100%. All data are provided as medians; boxes represent the first and third quartiles and whiskers represent the ranges. A Mann-Whitney-U test for unpaired group comparison was performed in order to determine any differences between the groups. (n=4; \*P<0.05 and \*\*P<0.01 vs. control). ADAM12, a disintegrin and metalloproteinase domain-containing protein 12; L-NIL, L-N6-(1-Iminoethyl)lysine dihydrochloride; L-NAME, N<sup>o</sup>-Nitro-L-arginine methyl ester hydrochloride; ADMA, asymmetric dimethylarginine.

with expression the control cells (Fig. 5C). Rat C6 glia cells exhibited increased ADAM12 mRNA expression in response to L-NIL and ADMA treatment, although not in response to L-NAME treatment (Fig. 5D). The efficacy of the NOS inhibitors was confirmed in pilot experiments by measuring the alterations in NO concentration using an NO-sensitive electrode (ISO-NO-METER; World Precision Instruments, Berlin, Germany) as described in (56).

## Discussion

ADAMs exhibit a multi-domain structure and ectopeptidase activity, and are involved in the regulation of cell-matrix interactions and cell-cell communication. ADAM expression and activity has been linked to important functions, such as cell differentiation, proliferation, development and inflammation, and to tumor cell growth, invasion and metastasis. ADAM12 is a proteolytically active ADAM, and two isoforms are produced as a results of alternative splicing: The membrane-bound, ADAM12-L, and the shorter soluble form, ADAM12-S (57). Increased expression levels of ADAM12-L and ADAM12-S, have been observed in several types of cancer,

including carcinoma of the breast, bladder and lung, and in glioblastoma (58). Urinary concentrations of ADAM12 have been shown to correlate with cancer stage, making it a potential biomarker with which to monitor tumor progression (59-61). ADAM12 facilitates tumor progression by stimulating cell proliferation and survival pathways (62-64). This is achieved by the proteolytic activation and release (ectodomain shedding) of membrane-bound growth factors, including HB-EGF, erythroblastic leukemia viral oncogene homolog 4, tumor necrosis factor  $\alpha$  and tumor growth factor  $\alpha$  (40,65).

In the present study, an increase in the expression of ADAM12 in the cortex and hippocampus samples from mice was observed as increasing age. In addition, ADAM12 expression levels were significantly higher in eNOS<sup>-/-</sup>, nNOS<sup>-/-</sup> and iNOS<sup>-/-</sup> adult, and mice >1 year old, compared with those in wild-type mice. The results of the present study suggest there may be an association between NOS activity and ADAM12 expression levels. In support of this view, an increased expression of ADAM12 was observed in response to NOS inhibition in murine N9 microglia, rat C6 astroglia and, to a lesser extent, in rat PC12 neuronal cells. By contrast, no induction of ADAM12 expression was observed in OLN93

rat oligodendrocytes. Consistent with the predominant and apparently high constitutive expression of ADAM12 in oligodendrocytes (47,48), it may be hypothesized that ADAM12 expression may not be subject to significant regulation, at least by NO, in oligodendrocytes. The association between ADAM12 and NOS isotype expression observed in the current study together with the known effects of NO on neural cell development (65), suggest that ADAM12 and NO are interdependently associated with neuronal development and function.

At the dosages applied in the present study, the NOS inhibitor L-NIL would be expected to inhibit iNOS activity, whilst eNOS activity remains largely unaffected. L-NAME would be expected to inhibit nNOS, eNOS and, to a lesser extent, iNOS activity. By contrast, ADMA (10  $\mu$ M) would be expected to inhibit eNOS activity, and not iNOS activity (66).

NO affects glutamatergic neurotransmission and is associated with the storage, uptake and/or release of a number of neurotransmitters in the central nervous system, such as acetylcholine, dopamine, noradrenaline,  $\gamma$ -aminobutyric acid, taurine and glycine (64,65,67), as well as certain neuropeptides (68). Since NO is a highly diffusible molecule, it is capable of mediating synaptic and non-synaptic communication processes. Furthermore, NO is a free radical due to its unpaired electron ( $\bullet$ NO). Therefore, it exhibits toxic effects at higher concentrations (69-72). NO toxicity is accentuated in the presence of oxidative radicals such as  $O_2^{\cdot-}$ , which may be generated by NOS when L-arginine substrate concentrations are low (73,74). The aging process is also associated with increased nitrosative and oxidative damage (75). NO is involved in brain development by influencing synaptic plasticity and mediating the change from cell proliferation to differentiation during neurogenesis (76).

ADAM12 has been shown to cause shedding of the  $\Delta$ -like protein 1 ectodomain, thereby facilitating the activation of Notch signaling (77). In human umbilical vein endothelial (HUVEC) cells, the activation of Notch induces the expression of the NO receptor, soluble guanylyl cyclase heterodimer, guanylate cyclase 1 soluble  $\alpha$ 3 (GUCY1 $\alpha$ 3) and GUCY1 $\beta$ 3 (78). Phosphatidylinositol-3 kinase/protein kinase B-dependent phosphorylation/activation of eNOS was observed in HUVEC cells. Therefore, Notch signaling may induce NO production and NO-receptor expression in HUVEC cells. By contrast, downregulation of Notch1 has been shown to induce iNOS expression in a model of myocardial ischemia/reperfusion (79). iNOS-mediated NO production, together with the induction of the subunit of NADPH oxidase, gp91phox, and the resulting increase in superoxide anion production, is likely to contribute to increased infarct size and fibrosis via the enhanced formation of peroxynitrite (79).

It has recently been established that Notch signaling regulates the expression of ADAM12 (80). Therefore, it is hypothesized that there is an association between ADAM12 expression, Notch signaling and NOS/NO. The induction of ADAM12 in response to NOS-inhibition that was observed in the present study is in accordance with this hypothesis. However, the role of Notch signaling for ADAM12 expression requires further investigation.

In conclusion, the results of the present study demonstrate that cortical and hippocampal ADAM12 expression increases with mouse development and age. NOS-deficiency leads to

increased ADAM12 expression in adult and 1-year old mice, compared with that in fetal and neonatal mice. However, the physiological and pathophysiological mechanisms underlying these patterns require further investigations.

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