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# Forum Original Research Communication

# Beta-Amyloid-Dependent Expression of NOS2 in Neurons: Prevention by an α2-Adrenergic Antagonist

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## ABSTRACT

**The neurotransmitter noradrenaline (NA) exerts important antiinflammatory effects on glial cells including suppression of the inducible form of nitric oxide synthase (NOS2). The authors examined the consequences of manipulating NA *in vivo* by treating adult rats with the neurotoxin DSP4, which selectively lesions noradrenergic neurons of the locus ceruleus (LC), and reduces cortical NA levels. Following LC lesion, intracortical injection of aggregated amyloid beta 1–42 (Aβ1–42) caused appearance of NOS2 within neurons, and increased neuronal damage assessed by staining for nonphosphorylated neurofilament proteins with antibody SMI-32. Co-treatment with a selective α2-adrenergic antagonist reduced neuronal NOS2 staining as well as SMI-32 staining. Neuronal damage was dependent on NOS2 expression since injection of Aβ1–42 into DSP4-treated NOS2-deficient mice did not result in neuronal damage. These results demonstrate that decrease of NA levels *in vivo* can exacerbate inflammatory responses and neuronal damage due to inflammatory stimuli such as Aβ. These findings suggest that α2-adrenergic antagonists could provide therapeutic benefit in neurological diseases such as AD or PD where LC loss is known to occur.** *Antioxid. Redox Signal.* 8, 873–883.

## INTRODUCTION

The activation of inflammatory responses in brain contributes to the initiation and progression of neurodegenerative diseases including multiple sclerosis (MS) and Alzheimer's disease (AD). These diseases are accompanied by both glial and neuronal activation, and include induction of proinflammatory cytokine expression and production of reactive oxygen and nitrogen species. Another hallmark of neurodegenerative diseases is the expression of the inducible form of nitric oxide synthase (NOS2), which occurs in MS and AD as well as several other neurological conditions including stroke and Parkinson's disease (15, 20). Although in some experimental conditions brain NOS2 expression can provide beneficial effects (6, 7, 19, 68), in most cases increased NO production and subsequent formation of reactive oxygen species is thought to be detrimental. NOS2 expression in brain has usually been described as being localized to glial or endothelial cells, however, in some cases NOS2 has been observed in neurons (21, 32, 63). In fact, one hallmark of AD is the appearance of neuronal NOS2 or of various indirect markers of NO such as nitrosylated tyrosine (47, 64, 70). The mechanisms that regulate NOS2 expression in brain, and in particular restrict expression to glial versus neuronal cells are not fully understood.

Numerous compounds have been shown *in vitro* to regulate NOS2 expression, ranging from growth factors, to antiinflammatory cytokines, prostaglandins, and several neurotransmitters. Previous work from our lab and others has shown that the endogenous transmitter noradrenaline (NA)

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can potently reduce glial NOS2 expression mediated by activation of β2-adrenergic receptor and elevation of intracellular cAMP (13, 17, 18). These findings led to the hypothesis that in brain, conditions in which endogenous NA levels, or NA signaling, were reduced might be conducive to increasing NOS2 expression. Our *in vivo* studies confirmed that if central NA levels were first reduced by treatment with the selective noradrenergic neurotoxin DSP4, then the cortical inflammatory responses were increased in response to injection of aggregated amyloid beta (33). These increases were reversed by co-injection of NA, and were accompanied by a decrease in levels of antiinflammatory molecules including the inhibitory IκB proteins (34). Since perturbation of central NA levels occurs in AD (as well as Parkinson's disease, PD) due to damage to noradrenergic neurons of the locus ceruleus (LC) (23, 28, 50, 51, 53), our findings suggest that AD and PD pathogenesis might be aggravated by such NA loss, and therefore that treatments to minimize or reverse the consequences of NA loss might prove therapeutic.

In the current study, we have assessed the ability of an α2-adrenergic antagonist (F14413) to reduce the consequences of NA loss (due to LC lesion) in adult rats that received an intraparenchymal injection of oligomeric Aβ1–42. The α2-adrenergic antagonists have neuroprotective effects *in vivo* in models of excitotoxicity (54) and cerebral ischemia (30), and improve functional recovery after brain injury and stroke (39). There are several mechanisms by which α2-adrenergic antagonists exert neuroprotection, including by increasing growth factor expression (11) and by reducing neuronal apoptosis (2). However, since these antagonists can also increase NA release (by preventing α2-adrenergic receptor mediated inhibition), α2-adrenergic antagonists might be able to partially counter the effects of LC loss. We show here that increases in neuronal NOS2 expression as well as neuronal damage due to Aβ1–42 are significantly reduced by intraperitoneal injection of an α2-adrenergic antagonist. Furthermore, using NOS2 null mice, we show that neuronal damage depends on NOS2 expression. These data suggest that α2-adrenergic antagonists could be useful for treatment of neurological disease or trauma where normal NA signaling is disturbed.

## METHODS AND MATERIALS

### *Materials*

The α2-adrenergic antagonist F14413 was synthesized by P. Mayer and J. L. Maurel, Centre de Recherche Pierre Fabre, Castres, France. The Aβ was from Global Peptide (Fort Collins, Colorado). Primary antibodies were mouse mAb anti-SMI-32, 1:1,000 (Sternberger Monoclonals Inc., Lutherville, MD), mouse mAb anti-NOS2, 1:200 (BD Biosciences Pharmingen, San Diego, CA), and mouse mAb anti-NeuN, 1:100 (Chemicon Int'l., Temecula, CA). Secondary antibodies were from Jackson ImmunoResearch Inc. (West Grove, PA) and mouse on mouse kit (Vector Labs, Burlingame, CA).

### *Preparation of Aβ*

Oligomeric Aβ1–42 was prepared according to the method described (9). Briefly, Aβ1–42 peptide was resuspended in 1,1,1,3,3,3-hexafluoro-propanol (HFIP, Sigma–Aldrich, St. Louis, MO), then HFIP was allowed to evaporate. Immediately prior to use, the HFIP-treated aliquots were resuspended in anhydrous dimethyl sulfoxide (DMSO, Sigma) followed by bath sonication for 10 min. Aβ1–42 oligomers were prepared by diluting Aβ to 100 μ*M* in phenol red-free Ham's F-12 (Biosource International, Camarillo, CA), vortexing for 30 s, and incubating at 37°C for 24 h.

### *Animals*

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 gm, were housed in groups of four under standard conditions at 22°C and a 12 h light–dark cycle with free access to food and water.

### *Pretreatment with DSP4, and injection of immunostimulants and inhibitors*

After acclimatization (7–10 days), rats received two intraperitoneal injections (one week apart) of either *N*-(2-chloroethyl)-*N*-ethyl-2 bromobenzylamine (DSP4, 5 mg/kg) dissolved in PBS or PBS alone (Fig. 1A). Four weeks after the second treatment, the animals were anesthetized with pentobarbital (50 mg/kg i.p.), and placed in a stereotaxic frame (Stoelting, Wood Dale, IL) on a heating blanket. Body temperature was maintained at 37 ± 0.5°C for the time of surgery. After exposure of the skull, holes were drilled bilaterally at the injection sites and 2 μl of a mixture containing $A\beta_{1-42}$ oligomers (0.5 μg/μl) were injected over a period of 120 s into each cortical hemisphere using a 2 μl Hamilton syringe. Injections were at AP +2.0, L ±2.5, and V 3.0 mm relative to Bregma (57). Controls received 2 μl of PBS.

On the day of the Aβ injections, rats received three i.p. injections of the α2-adrenergic antagonist F14413 (0.16 mg/kg each time) or saline vehicle separated by 4 h intervals, starting 30 min before the Aβ injections. One day after surgery, the animals were killed by an overdose of pentobarbital and brains removed. Half the brain had the injection site excised in a 2 mm coronal slice and further trimmed to approxi-

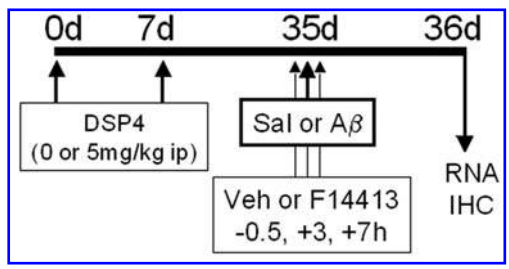


**FIG. 1. Schematic to illustrate treatment paradigm.** Adult male rats were treated on days 0 and 7 with an i.p. injection of vehicle (saline) or DSP4 (5 mg/kg) to lesion locus ceruleus noradrenergic neurons. On day 35, animals were given an intraparenchymal injection of saline or oligomeric Aβ1–42 into the frontal cortex. On the same day, animals were give three i.p. injections of vehicle (saline) or the α2AR antagonist F14413 (0.16 mg/kg). One day later, animals were sacrificed and brains samples prepared for immunohistochemistry and RNA analysis.

mately 2 × 2 × 4 mm encompassing the site of injection. This piece was sonicated in Trizol reagent (Invitrogen Inc., Carlsbad, CA) for RNA isolation and PCR analysis. The comparable region from the left hemisphere was prepared for immunohistochemical staining. All experiments were carried out in accordance with the local animal welfare guidelines and were approved by the local animal care and use committee.

### *Tissue preparation and sectioning*

Rat brain regions were removed 24 h after Aβ or vehicle injection, and placed in 3.7% formaldehyde, 10% acetic acid, 80% methanol fixative overnight. Dehydration of 2 mm coronal sections encompassing the site of injection was done in a series of 80% and 95% ethanol 1 h each, followed by 100% ethanol overnight. Two 100% xylene washes were done for 1 h each and then for 1 h in 60°C Paraplast Plus (Tyco/Healthcare, Mansfield, MA). After a change of Paraplast Plus, tissue was placed in a 60°C vacuum oven for 2 h prior to placing in molds to cool and solidify.

Coronal sections (8 μ*M* thick) located 0, 200, and 400 μ*M* rostral and caudal to the injection site were prepared from paraffin embedded tissue, floated onto slides, dried 2 h, heated to 54°C for 2 h on a slide warmer, and stored at 22°C. Slides were deparaffinized with xylene and ethanol. Antigen unmasking was done with 10 m*M* Na citrate pH 6.0, heated in a microwave, kept at 100°C for 10 min, and then cooled to room temperature for 20 min. Sections were immediately processed for immunohistochemistry.

### *Immunohistochemistry*

Slides were washed for 5 min with PBS and blocked with 5% normal donkey serum in PBS at room temperature for 30 min. Primary antibodies were diluted in 1% normal donkey serum in PBS. Sections were incubated with primary antibodies (mouse mAb anti-SMI-32, 1:1,000 dilution; or mouse mAb anti-NOS2, 1:200 dilution) at 37°C for 1 h. Sections were washed three times with PBS. Secondary antibodies were preabsorbed to minimize cross reactivity (Jackson ImmunoResearch), and were donkey anti-mouse Rhodamine Red-X (RRX) conjugated and donkey anti-goat FITC conjugated. Sections were incubated with secondary antibodies (diluted 1:200 in PBS with 1% normal donkey serum) for 1 h at 37°C. Slides were washed three times for 5 min with PBS and postfixed in 3.7% formaldehyde in PBS for 20 min. Autofluorescence was quenched with 50 m*M* $NH_4Cl$ in PBS for 15 min. Nuclei were stained using DAPI (400 ng/ml in PBS for 3 min). Vectashield mounting fluid (Vector Laboratories Inc., Burlingame, CA) was used. Images were obtained on an Axioplan2 fluorescence microscope equipped with an Axiocam MRm digital camera and Axiovision 4.2 imaging software (Carl Zeiss International, Oberkochen, Germany).

### *Real-time polymerase chain reaction (PCR)*

Total cytoplasmic RNA from tissues was prepared from cells using TRIzol reagent according to manufacturer procedures (Invitrogen/GIBCO); aliquots were converted to cDNA using random hexamer primers, and mRNA levels were estimated by quantitative touchdown PCR (QPCR). Conditions were 35 cycles of denaturation at 94°C for 10 s, annealing at 58°–64°C for 15 s, and extension at 72°C for 20 s on a Corbett Rotorgene Real-Time PCR unit (Corbett, Australia). The PCR mastermix contained SYBR Green (1 μl diluted 1:10,000 SybrGreen I 10,000X concentrate, Molecular Probes, Eugene, OR). Relative mRNA concentrations for IκBα and tyrosine hydroxylase (TH) were calculated from the takeoff point of reactions using manufacturer's software. Melting curve analysis and agarose gel electrophoresis were performed to ensure production of single and corrected size products. Relative mRNA values were normalized to housekeeping genes, either α-tubulin (α-Tub), β-actin (β-Act) or glyceraldehyde-3-phosphate dehydrogenase (GDH). The primers used for QPCR (all listed 5′ to 3′) were:

| | |
|---|---|
| IκBα forward | GCCTGGCCAGTGTAGCAGTCTT |
| IκBα reverse | CAGCACCCAAACTCACCAAGTG |
| TH forward | GGGCTTCTCTGACCAGGTGTA |
| TH reverse | GGCAGGCATGGGTAGCATAG |
| α-Tub forward | CCCTCGCCATGGTAAATACAT |
| α-Tub reverse | ACTGGATGGTACGCTTGGTCT |
| GDH forward | GCCAAGTATGATGACATCAAGAAG |
| GDH reverse | TCCAGGGGTTTCTTACTCCTTGGA |
| β-Act forward | CCTGAAGTACCCCATTGAACA |
| β-Act reverse | CACACGCAGCTCATTGTAGAA |

### *Noradrenaline release in cortex as measured by microdialysis and HPLC*

Rats were surgically prepared as described previously (67) and were implanted under stereotaxic control with a microdialysis probe (CMA/12, 3 mm membrane length; CMA/Microdialysis AB, Stockholm) terminating in the medial prefrontal cortex (3.0 mm anterior to bregma, 1.5 mm lateral to the midline suture, 4.5 mm ventral from the dura, at a medially-directed vertical angle of 14°) (57). Dialysis probes were anchored to the skull, and following surgery the animals were placed in bowl-shaped Plexiglass cages with free access to food and water. Probes were immediately connected to a microliter syringe pump and perfused with Ringer's solution (NaCl, 147 m*M*; KCl, 4 m*M*; $CaCl_2$, 1.3 m*M*) at a flow rate of 0.2–0.4 μl/min during an overnight recovery period. The following morning, the infusion syringes were refilled with fresh filtered and degassed Ringer's solution, and the pump infusion rate was increased to 2.0 μl/min. After a 2.5 h equilibration period, three 20-min dialysate samples were collected in glass microtubes containing 10 μl of 1 m*M* $Na_2EDTA$ in 0.05 *M* HCl, for baseline measurements of endogenous NA. Animals then received an i.p. injection of vehicle (saline) or F14413 (0.16 mg/kg), and dialysate samples were continuously collected for 5 h. At the end of the experiment, rats were decapitated and the brains removed and fixed in 4% formalin solution. Coronal sections (500 μm thick) of fixed brain were prepared using a Vibroslice oscillating microtome (Campden Instruments, London, UK), and probe placement was assessed by visual inspection of the probe tract in the tissue.

NA in the dialysates was derivatized and analyzed by HPLC with fluorescence detection (77). Derivatized samples were

loaded into a refrigerated HPLC system (Model 2690 Alliance Separation Module, Waters, St-Quentin-en-Yvelines, France) for automated injection and analysis. Chromatographic conditions consisted of a $C_{18}$ reverse-phase column (Waters Symmetry Shield, 2.1 × 150 mm, 5 μm particle size) maintained at 30°C, and a mobile phase (flow rate 0.4 ml/min) composed of sodium acetate (15 m*M*), octane sulfonic acid (4 m*M*), and acetonitrile (25% vol/vol), final pH 4.5. Fluorescent derivatives were detected with a Waters Model 474 scanning fluorescence detector ($\lambda_{ex}$ = 345 nm, $\lambda_{em}$ = 480 nm). Quantification of the chromatographic peak of NA was based on the comparison with calibration curves derived from authentic standards of NA (0.1–10 pg/μl). NA levels in dialysates were expressed as a percentage of the average amount of NA in the three baseline samples preceding drug or vehicle injection. For the comparison of drug and vehicle treatments between different subjects at corresponding time points, Kruskal–Wallis ANOVA followed by Mann–Whitney U-test was used.

### *Confirmation NOS2 null genotype by PCR*

NOS2-deficient mice [Nos2tm1Lau (44)] were obtained from Jackson Laboratories. The absence of a functional NOS2 gene (lacking the calmodulin binding domain) was confirmed by PCR analysis. Total cytoplasmic RNA was prepared from brain tissue using TRIzol reagent (Invitrogen). The primers used for NOS2 detection were NOS2F: CTG TCA CGG AGA TCA ATG TGG corresponding to bases 1415–1435 of mouse NOS2; and NOS2R: AAG GCG TAG CTG AAC AAG GTG corresponding to bases 1848–1868 of mouse NOS2 mRNA, and which yield a 454 bp product when used with the samples from wild-type mice, but no PCR product using samples from NOS2 null mice in which the exon containing the calmodulin-binding domain of NOS2 is replaced by the neomycin resistance gene. PCR conditions were 35 cycles at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s, followed by 5 min at 72°C in an Eppendorf Thermoreactor (Hamburg, Germany). PCR products were separated by electrophoresis through 1.5% agarose gels containing 0.1 μg/ml ethidium bromide.

### *Data analysis*

Quantitative analysis of NOS2 positively staining cells was performed as previously described (33). In brief, NOS2 positive containing neurons having large cell bodies were counted in five sections having a defined distance (0, 200, and 400 μ*M* rostral and caudal) relative to the level of cortical injection, in brain sections from at least 3 or 4 animals per group. The number of cells within the respective fields was determined using a counting grid, and cells within the needle tract were not counted. Quantification of SMI-32 immunoreactivity was performed using Zeiss Axiovision software (Carl Zeiss International). The area corresponding to cells with positive staining (over a background threshold value that was determined from images where primary antibody was omitted) under 10X magnification images was measured and the ratio of area stained to total area was calculated. The results are the average of four measurements of images corresponding to the same area of slides.

## RESULTS

Adult rat Sprague Dawley rats were treated with the selective neurotoxin DSP4 to lesion locus ceruleus (LC) noradrenergic neurons and deplete cortical NA levels as previously described (33). Analysis of brain samples showed a significant decrease (57% versus control levels, $p < 0.05$, Fig. 2A) in tyrosine hydroxylase (TH) mRNA levels in the LC as expected since this is the primary site of DSP4-induced neuronal loss. The decrease in TH mRNA has previously been shown to be associated with a reduction in cortical NA levels (32). There was a slight, albeit nonsignificant decrease (25% reduction) in cortical TH mRNA levels ($p > 0.05$; Fig. 2B), but a significant decrease (45% versus control values, $p < 0.01$, Fig. 2C) in cortical antiinflammatory IκBα mRNA levels that mediates the

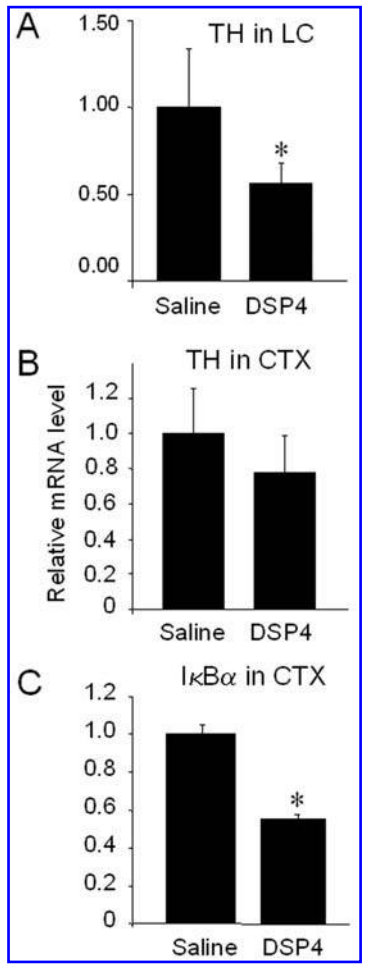


**FIG. 2. Effects of DSP4 on TH and IkBα mRNA levels.** RNA samples from control or DSP4-treated rats ($n$ = 4 each) were converted to cDNA and analyzed by QPCR for relative levels of (**A**) tyrosine hydroxylase (TH) in the locus ceruleus (LC); (**B**) TH in the frontal cortex around the Aβ injection site; and (**C**) the inhibitor of κB alpha (IκBα) mRNAs. DSP4 significantly reduced LC TH (by 43%) and cortical IκBα mRNA levels (by 55%) versus control values ($n = 4$, $p < 0.01$).

subsequent exacerbated responses to inflammatory stimuli (5) (Fig. 2).

DSP4-treated rats were injected intracortically with oligomeric Aβ1–42 and analyzed for NOS2 expression (Fig. 3). NOS2 immunoreactivity was occasionally observed in control (non-DSP4 treated, saline-injected) rat brain (not shown); and in scattered microglial cells following Aβ1–42 injection into control (non-DSP4 treated) rats, as previously observed (4). NOS2 was detected in some neurons after saline injection into DSP4-treated rats (Fig. 3A, top panel). Aβ1–42 injection into DSP4-treated rats (Fig. 3A, middle panel) significantly increased NOS2 expression, primarily in large cells having characteristic neuronal morphology, and which we showed could be co-stained for the neuronal specific marker NeuN (4).

Intraperitoneal injection of the selective α2-adrenergic antagonist F14413 increased NA release in frontal cortex of normal adult rats (Fig. 4). Co-treatment of DSP4 treated rats with F14413 significantly decreased neuronal NOS2 immunoreactivity due to Aβ1–42 intraparenchymal injection (Fig. 3A, bottom panel and Fig. 3B) although residual staining was observed in some smaller elongated cells (Fig. 3A, bottom panel). PCR analysis of cortical RNA samples showed that treatment with F14413 also increased (22% *vs.* non-treated, $p < 0.05$) cortical IκBα mRNA levels (Fig. 5B), although cortical TH levels were not increased (Fig. 5A).

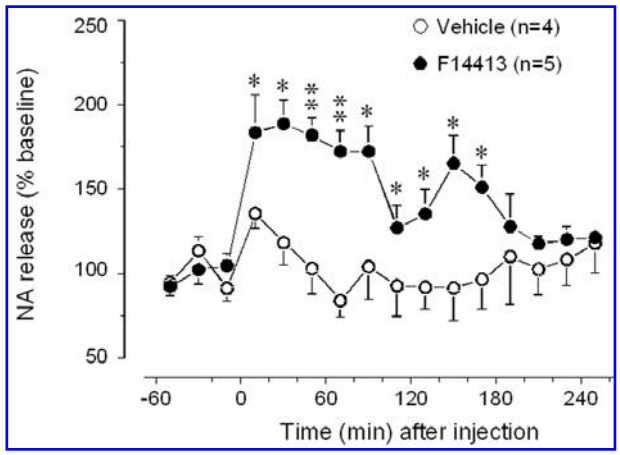


**FIG. 4. Effect of F 14413 on cortical NA release.** NA release in rat frontal cortex was measured by microdialysis in conscious freely-moving animals. Rats received a single i.p. injection of F14413 (0.16 mg/kg) or saline vehicle at $t = 0$ min. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* corresponding sample in vehicle control animals, Kruskal–Wallis ANOVA and Mann–Whitney U-test, $n$ = number of rats per group.

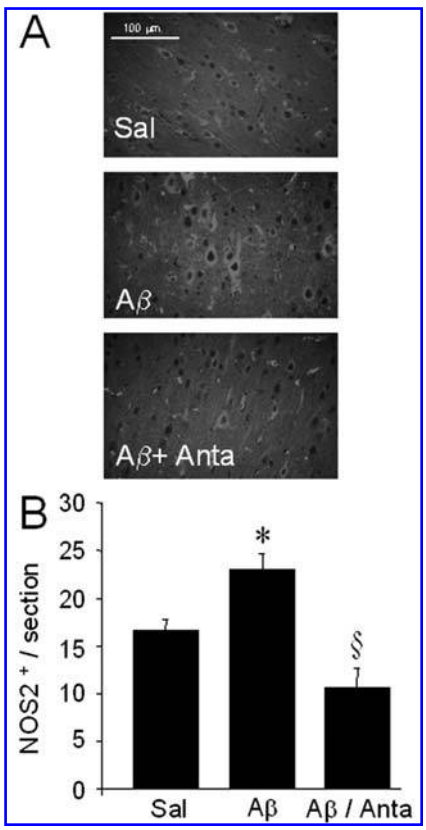


**FIG. 3. NOS2 expression in DSP4-treated rat brain.** (**A**) Representative coronal sections prepared from DSP4-treated rats, injected intraparenchymally with saline (Sal), Aβ alone (Aβ), or Aβ together with i.p. α2AR antagonist F14413 (Aβ + Anta). Sections were stained for NOS2. (**B**) Quantification of number of large, round NOS2 staining cells in 4 animals per group. $^{*}p < 0.05$ versus saline; § $p < 0.05$ versus Aβ alone,

The induction of neuronal NOS2 was associated with an increase in the immunostaining for the nonphosphorylated form of neurofilament H (NP NF) utilizing antibody SMI-32 (Fig. 6), a marker of neuronal damage, and this staining was significantly reduced by treatment with F14413, suggesting a causative role for NOS2 in neuronal damage. To test this possibility, we examined the effects of Aβ1–42 injection into NOS2 deficient mice (Fig. 7). In DSP4-treated wild-type mice, Aβ1–42 injection increased neuronal NOS2 staining, similar to what was observed in DSP4-treated rats, and likewise increased SMI-32 staining (Fig. 8). In contrast, injection

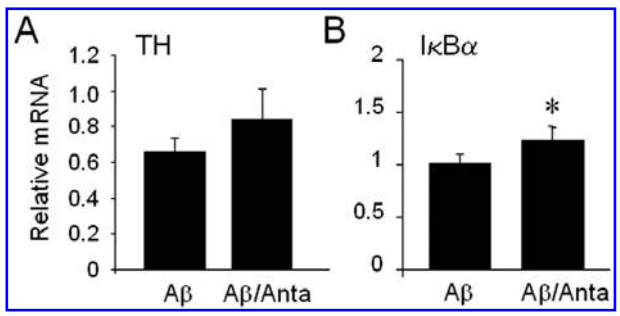


**FIG. 5. Effects of α2AR antagonist on TH and IκBα mRNA levels.** Cortical RNA samples were obtained 24 h after injection of Aβ1–42 (alone or with i.p. F14413) into DSP4-treated rats ($n = 4$), were converted to cDNA and analyzed by QPCR for relative levels of (**A**) tyrosine hydroxylase (TH) and (**B**) IκBα mRNA. F14413 had no effect on TH levels but significantly increased IκBα mRNA levels (22% increase versus Aβ1–42 alone, $p < 0.01$).

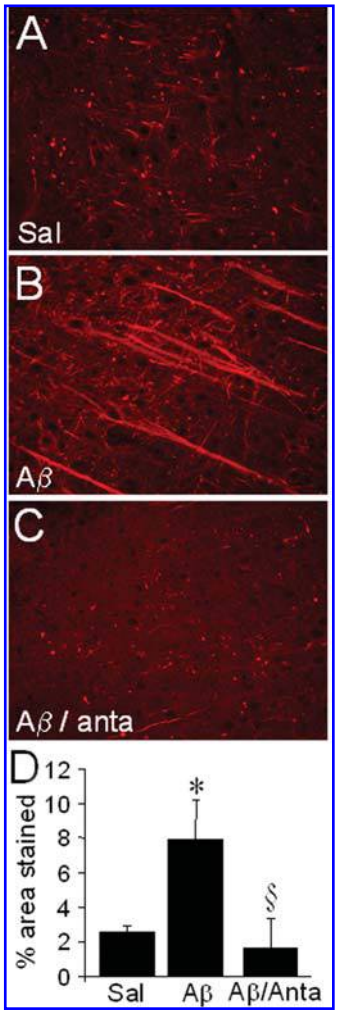


**FIG. 6. Effects of DSP4 on SMI-32 immunoreactivity.** Representative coronal sections prepared from DSP4-treated rats, injected intraparenchymally with (**A**) saline; (**B**) Aβ alone, or (**C**) Aβ together with i.p. α2AR antagonist F14413. Sections were stained for SMI-32. (**D**) Quantification of SMI-32 immunoreactivity, in four animals per group. *$p < 0.01$ versus saline; §$p < 0.01$ versus Aβ alone.

of Aβ1–42 into DSP4-treated NOS2-deficient mice did not cause a significant increase in SMI-32 immunoreactivity. Together, these findings suggest that NOS2 expression (and activity) is an important factor in the development of neuronal damage due to Aβ.

## DISCUSSION

The current results confirm and extend previous findings that cortical neurons will express NOS2 in response to Aβ if basal NA levels are first reduced by lesion of LC noradrenergic neurons, which extend projections to frontal cortex (25, 61). In our previous study (33), we showed that induction of neuronal NOS2 in DSP4-treated rats was attenuated by co-injection into the cortex of exogenous NA. In the current

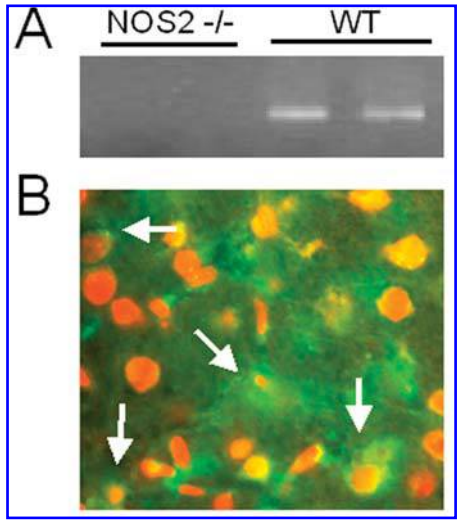


**FIG. 7. NOS2 induction in wild-type mice.** (**A**) Genotyping to confirm identity of NOS2 null and wild-type littermates. (**B**) Wild-type mice were treated with DSP4 as described for rats, and injected with oligomeric Aβ1–42. One day later brain sections were analyzed for NOS2 (*green*) and NeuN (*red*) staining. *White arrows* indicate neurons labeled with both antibodies.

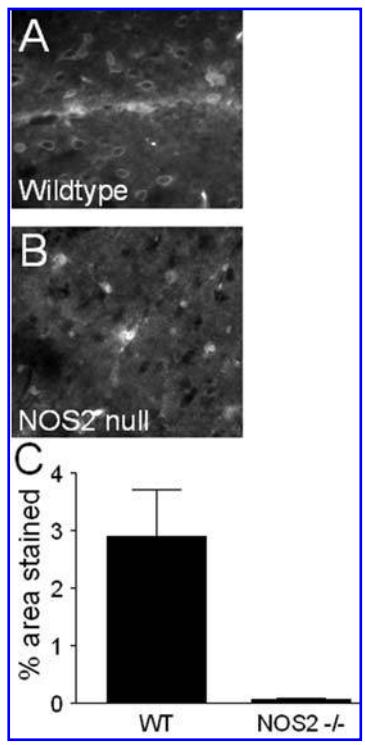


**FIG. 8. Effects of Aβ on SMI-32 staining.** Wild-type and NOS2 null mice were treated with two i.p. injections of DSP4 as described for rats (see Fig. 1), and injected with oligomeric Aβ1–42 4 weeks after the second DSP4 injection. One day later brain sections were prepared from the mice, and sections from wild-type (**A**) and NOS2 null mice (**B**) were stained for SMI-32. (**C**) Quantification of SMI-32 immunoreactivity in three animals per group. *$p < 0.01$ versus wild-type mice.

study, we show that NOS2 induction can also be reduced by i.p. administration of an α2-adrenergic antagonist (F14413), which increases cortical NA levels by inhibiting α2-AR activation. Moreover, the expression of neuronal NOS2 was accompanied by an increase in neuronal damage, which was similarly reduced by the antagonist. Together with our findings that levels of the inhibitory IκBα mRNA were also increased by this antagonist, our results suggest that neuronal inflammatory activation and associated damage due to Aβ can be reduced by antagonism of α2-ARs and induction of antiinflammatory gene expression. The ability to administer a centrally active α2-adrenergic antagonist peripherally rather than via invasive intracerebral injection provides better options for possible therapeutic application.

In this study we used F14413 ([+]-[S]-2-[5-fluoro-2-methoxy-benzo(1–4)dioxan-2-yl]-4,5-dihydro-1H-imidazolium chloride), a newly-discovered, selective and centrally-active α2-adrenergic antagonist from a benzodioxan imidazoline structural family (56). F14413 is approximately four times more potent in comparison to dexefaroxan, an α2-adrenergic antagonist often used in studying α2-AR function *in vivo* (53). F14413 also has greater efficacy as an α2-adrenergic antagonist than dexefaroxan in several *in vivo* and *in vitro* tests, which may be related to its negligible intrinsic (agonist-like) activity (56). The dose and route of administration used here for F14413 produces optimal central α2-AR blocking activity and increases cortical NA release in rats and mice *in vivo* (Fig. 4). A repeated injection treatment protocol was employed since F14413 has a relatively short duration of central α2-AR blocking effect in rodents (2.5–3.0 h) (55). Our findings indicate that F14413 administration just prior to and following within several hours of Aβ injection significantly reduced neuronal NOS2 expression, and accompanying neuronal damage. The present microdialysis experiments confirm a 2–3 h long elevation in cortical dialysate levels of NA following a single i.p. injection of F 14413, an effect consistent with established operational characteristics of presynaptic inhibitory α2-autoreceptors on LC-noradrenergic afferents, the blockade of which facilitates cortical NA release *in vivo* (14, 69).

Whether other administration paradigms with F14413 would be more or less effective in this model is not yet known, nor is the duration of the effect yet established. However, since increased NA is a potent inhibitor of NOS2 expression *in vitro* (26), these data suggest that increases in NA release induced by F14413 might be a basis for inhibiting neuronal NOS2 levels. We have also shown that F14413 increased cortical IκBα mRNA levels, consistent with previous findings that IκBα is increased by NA (27), and thus provides a mechanism to explain the antiinflammatory effects on NOS2 expression.

Our current data confirms previous findings that NOS2 can be expressed in cortical neurons under certain conditions (32). Neuronal NOS2 expression has been reported several times, in response to different inflammatory including bacterial endotoxin lipopolysaccharide (LPS), stimulatory cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), or tumor necrosis factor-α (TNF- α), or following various forms of stress (32). Our data demonstrate that Aβ can also induce neuronal NOS2 expression; however it is likely that this induction involves glial derived factors since Aβ can potently stimulate glial cell activation to release proinflammatory substances (3, 4).

Our results indicate that oligomeric Aβ1–42 not only induces an inflammatory response, but also leads to axonal damage as soon as 24 h after injection as assessed by increases in immunostaining for nonphosphorylated neurofilament proteins (NP NF) using antibody SMI-32. Increases in the levels of NP NFs can occur following a variety of trauma conditions, including those due to excitotoxic injury (24); brain contusion (62), and as a consequence of demyelination in animal models of multiple sclerosis (29, 43, 49) and in MS brain (74). Thus, an increase in SMI-32 immunostaining provides an indirect index of axonal damage. The lack of an increase in SMI-32 staining in NOS2-deficient mice suggests that a key factor underlying Aβ induced neuronal damage is NOS2-derived NO, consistent with numerous reports showing that NO induces neurotoxicity. However, since the NOS2 null mice lack NOS2 in all cell types, we cannot conclude whether neuronal NOS2 is more or less important than glial NOS2 in causing this damage

Whether NOS2 activity is toxic or beneficial is a source of ongoing debate. Several studies have provided convincing evidence suggesting a direct role for NOS2-derived NO in eliciting neurotoxic effects. *In vitro* studies have demonstrated that NOS2-derived NO creates oxidative stress, lipid peroxidation, DNA damage, impairment of mitochondrial function, and eventually cell death (10), while *in vivo* studies have demonstrated toxic effects of NOS2-derived NO in PD (15), ischemia (36), and other neurological conditions (22, 66). The involvement of NOS2 in these conditions is implicated by use of NOS2-selective inhibitors, as well as studies using NOS2-deficient mice (44, 48, 72). Disruption of the NOS2 gene has shown to be protective against neurological insults including ischemia (37), the psychostimulant methamphetamine (38), the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (12), and experimental bacterial meningitis (76).

However, there are also reports in which inhibition of NOS2 or studies in NOS2-deficient mice have pointed to a protective role for this enzyme. NO can protect neurons against damage due to NGF deprivation (68), reduce cerebellar granule cell apoptotic cell death (7), protect PC12 neurons from death due to trophic factor deprivation (16), and reduce excitotoxicity by nitrosylating NMDA receptor subunits (6). In autoimmune diseases such as MS and its animal model EAE, NOS2 plays a beneficial role by restricting proliferation of activated T cells and thus limiting further proinflammatory cytokine production (19, 75) as well as by inducing Th2-type cytokines (40). Our results indicate that, at least under conditions of acute NOS2 activation due to a strong inflammatory stimulus, the NOS2-derived NO appears to be primarily deleterious.

Although NOS2 expression has previously been described in AD brain (31, 70, 71), in brains of patients with dementia with Lewy bodies (42), in transgenic mouse models of AD (46. 60), and following intraparenchymal injection or infusion of Aβ (73), its consequences are largely unknown. In the current study, we show that upregulation of neuronal NOS2 protein in rat brain is associated with an increase in neuronal damage as assessed by staining with antibody SMI-32 for NP

NFs, while in mice, the same treatment led to increased SMI-32R staining as well as for another marker of neuronal damage (fluorojade B, not shown). Although we did not test the effects of injecting a selective NOS2 inhibitor on these markers of neuronal damage, our results using NOS2-deficient mice are consistent with the conclusion that NOS2 is necessary to induce neuronal damage. However, since NOS2 null mice lack NOS2 in all cell types, we cannot conclude whether the damaging effects of NOS2 are due to glial as opposed to neuronal expression. Regardless, our data suggest that antiinflammatory approaches to reduce cytokine as well as NOS2 expression could be beneficial in AD.

DSP4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine) has been used for many years to investigate the consequences of LC neuronal loss and NA depletion on brain function (1, 11, 41, 59) and pathology (8, 52, 65). However, the exact mechanism by which DSP4 causes LC damage is still unclear. The damage induced by DSP4 is thought to be due to hydrolysis of the molecule to a quaternary aziridium ion (a highly reactive alkylating species and the active toxic compound) that strongly reacts (irreversibly and covalently) with nucleophilic moieties such as thiols and amines. The azyridinium ion can in turn be further metabolized by monoamine oxidase B and taken up by NA terminals. The selectivity of the toxin for noradrenergic neurons is likely due to its ability to selectively bind to and irreversibly inhibit the NA transporter.

Loss of LC noradrenergic neurons in AD (5, 50, 58) and PD (23, 51) has been known for almost 30 years, yet the exact consequences of that loss remain unclear, but recent data suggests a contribution to neuronal damage (28, 53). Our initial studies showed that LC loss and central depletion of NA could exacerbate cortical inflammatory responses, increasing the duration and magnitude of Aβ-dependent increases in cytokine and NOS2 expression. Moreover, DSP4 treatment shifted NOS2 localization from being primarily glial to being neuronal. The basis for this cellular switch is not yet understood, but *in vitro* studies carried out with primary neuronal cultures suggest that microglial derived factors, other than IL-1β or TNFα, are required to induce neuronal NOS2 (Madrigal and Feinstein, unpublished observations). Since neuronal NOS2 expression has been described in AD brain (20, 31, 35, 45, 47, 70, 71), our results suggest that experimentally induced LC loss may replicate features of AD pathology that are not currently being considered in existing models of AD.

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## ABBREVIATIONS

Aβ, amyloid beta; AD, Alzheimer's disease; α2-AR, alpha 2 adrenergic receptor; β2AR, beta2 adrenergic receptor; cAMP, cyclic AMP; DSP4, *N*-(2-chloroethyl)-*N*-ethyl-2 bromobenzylamine; F14413, [+]-[S]-2-[5-fluoro-2-methoxy-benzo(1–4) dioxan-2-yl]-4,5-dihydro-1H-imidazolium chloride; GDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of NFκB; LC, locus ceruleus; LPS, lipopolysaccharide; MS, multiple sclerosis; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NA, noradrenaline; NFκB, nuclear factor κ beta; NO, nitric oxide; NOS2, the inducible form of NO synthase; NPNF, nonphosphorylated neurofilament proteins; PD, Parkinson's disease; QPCR, quantitative PCR; ROS, reactive oxygen species; TH, tyrosine hydroxylase; Tub, tubulin.

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