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Effect of N-methyl-D-aspartate (NMDA) receptor antagonists on α-synuclein-evoked neuronal nitric oxide synthase activation in the rat brain

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Abstract:
α-Synuclein (ASN), a small presynaptic protein that is abundant in the brain, is implicated in the pathogenesis of neurodegenerative disorders including Parkinson's and Alzheimer's disease. The central domain of α-synuclein, the non-amyloid β component of the Alzheimer's disease amyloid (NAC) is probably responsible for its toxicity. However, the molecular mechanism of α-synuclein action remains largely elusive. The present study examined the effect of α-synuclein and the NAC peptide on nitric oxide synthase (NOS) activity in rat brain cortical and hippocampal slices using a radiochemical technique. Moreover, nitrite levels in brain slices incubated in the presence of α-synuclein were measured using the Griess reaction. ASN and the NAC stimulated NOS activity by about 70% and 40%, respectively. β-Synuclein, a homologous protein of ASN that lacks the NAC domain, had no effect on NOS activity. Under the same experimental conditions, α-synuclein increased nitrite levels by 27%. α-Synuclein and the NAC affected the activity of constitutive neuronal isoform of NOS, but had no impact on the endothelial or inducible NOS isoforms. The effect of α-synuclein and the NAC peptide on NOS activity was inhibited by MK-801 and APV, antagonists of the NMDA receptor. These results indicate that the NMDA receptor plays an important role in α-synuclein-evoked nitric oxide synthesis. We suggest that nitric oxide liberated by the over-activated neuronal isoform of NOS could react with superoxide to form peroxynitrite, which modulates the function of a variety of biomolecules including proteins, lipids, and DNA.

Key words:
α-synuclein, NAC peptide, nitric oxide synthase, NMDA receptor, MK-801, APV

Abbreviations: APV – ((2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate); ASN – α-synuclein; Aβ – amyloid β-peptide; BSN – β-synuclein; MK-801 – (dizocilpine: (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate); MPTP – (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); NAC – non-amyloid β component of Alzheimer's disease amyloid; NMDA – N-methyl-D-aspartate; NNLA – N^G-nitro-L-arginine; NO – nitric oxide; NOS – nitric oxide synthases (EC 1.14.13.39); $O_2^{\cdot-}$ – superoxide; $^{\cdot}OH$ – hydroxyl radical; $ONOO^-$ – peroxynitrite; PD – Parkinson's disease

Introduction

α-Synuclein (ASN), a small (140 amino acids) acidic synaptic protein, is recognized in various cell types, but is primarily present in the central nervous system and is especially abundant in presynaptic terminals [37]. The function of ASN under normal physiological conditions, as well as its role in neurodegenerative

diseases, depends on its concentration and assembly state [31]. At nanomolar concentrations, ASN protects neurons against oxidative stress and inhibits apoptosis; it also plays important roles in synaptic plasticity and the regulation of vesicle transport and acts as a chaperone protein [37]. Conversely, ASN overexpressed at micromolar concentration in the cell results in cytotoxicity [5, 14]. Because ASN is a classical cytosolic protein, it was generally assumed that either its protective or pathogenic effect was limited to the intracellular organelles and proteins.

However, recent data has emphasized the significance of extracellular ASN and its internal hydrophobic fragment, the non-amyloid β component of the Alzheimer's disease amyloid (NAC) [8, 23, 25, 27]. In humans, ASN is present in blood plasma and cerebrospinal fluid in both monomeric and oligomeric forms [10, 18, 26, 42]. Moreover, intravesicular ASN could be secreted from cells *via* exocytosis [24]. Our previous data indicated that ASN is liberated from synaptoneurosomes into extracellular space during oxidative stress evoked by $FeCl_2$/ascorbate, hydrogen peroxide, and the nitric oxide (NO) donor sodium nitroprusside [2]. Seo and co-workers demonstrated that extracellular ASN increased the level of Bax protein and decreased Bcl-xL in PC12 cells leading to mitochondrial failure, cytochrome c release, caspase cascade activation, and cell death [36]. Moreover, our previous results showed that ASN enhanced the release and toxicity of the amyloid β-peptide (Aβ) leading to NO-mediated irreversible mitochondrial dysfunction and caspase-dependent programmed cell death [23]. Extracellular ASN and the NAC were shown to potentiate Ca^{2+} influx in rat synaptoneurosomes [4] and inhibit dopamine uptake into rat striatal synaptosomes by altering NO-mediated dopamine transporter activity [3].

Multiple lines of evidence indicate that increased NO synthesis leads to macromolecular oxidation as well as mitochondrial and DNA damage, which are common pathogenic mechanisms involved in Parkinson's disease (PD) and other neurodegenerative disorders. Pathologic studies in *post-mortem* PD brains and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice suggest that NO indeed plays an important role in PD [12, 43]. It was reported that neuronal NOS (nNOS) overexpression enhances the susceptibility of SH-SY5Y neuroblastoma cells to rotenone treatment [6]. In addition to the studies on PD-related neurotoxins, it has been demonstrated that NO evokes structural changes in the ASN protein. Giasson detected an accumulation of nitrated ASN in Lewy bodies of the PD brain [19]. Thus, nitration of ASN might enhance the formation of Lewy bodies in PD patients. Other studies show that parkin can be S-nitrosylated by NO, and this modification reduces parkin's E3 ligase activity [13, 45]. Taken together, it has been documented that NO, when converted to peroxynitrite ($ONOO^-$), becomes a powerful detrimental oxidant with direct neuropathological consequences.

Our previous results indicated that ASN and the NAC peptide stimulated the generation of NO and free radicals [3]. However, it is still far from clear how these toxic proteins contribute to NO overproduction. In the present work we have focused our attention on the role of particular NOS isoforms in extracellular ASN- and NAC-evoked enhancement of NO synthesis in the rat brain slices. Moreover, the study reports that the uncompetitive and competitive NMDA-receptor antagonists MK-801 and APV, respectively, elicit a protective effect against ASN- and NAC-evoked NO over-production.

Materials and Methods

All experiments on animals were accepted by the Polish National Ethics Committee, and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Materials

ASN and β-synuclein (BSN) were obtained from rPeptide (Bogart, GA, USA). Protease inhibitor cocktails were from Roche (Mannheim, Germany). [^{14}C(U)]L-arginine (360 mCi/mmol), NAC peptide, and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

Preparation of ASN and evaluation of its oligomerization

ASN protein was dissolved in filtered, deionized, 18.2-MΩ water at a stock solution of 100 μM. For evaluation of ASN oligomerization, 3.6 μl of the stock solution, containing 5 μg of protein, was mixed with 2X SDS sample buffer and boiled for 5 min at

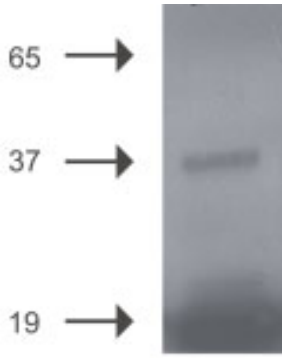

Fig. 1. Western blot analysis of ASN conformation. ASN was prepared and analyzed as described in Materials and Methods section. The western blot analysis with anti-ASN antibody indicates that ASN used in experiments is in monomeric (ca. 19 kDa)/ oligomeric (ca. 37 kDa) form

100°C. The electrophoretic mobility of ASN analyzed in this study was determined by SDS-polyacrylamide gel electrophoresis using 15% acrylamide concentrations. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes that were then blocked for 1 h in a solution of 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.3% Tween-20 (PBS-T). The membrane was then incubated overnight at 4°C with ASN antibody (1:1000) in 1% BSA solution in 0.1% PBS-T. After treatment for 1 h with anti-rabbit horseradish peroxidase-coupled secondary antibody (1:8000), the protein bands were visualized with enhanced chemiluminescence (GE Healthcare, UK) and light emission was detected by using Hyperfilm ECL (Kodak). The western blot analysis indicated that ASN used in these experiments is in monomeric (ca. 19 kDa)/oligomeric (ca. 37 kDa) form (Fig 1).

Preparation of brain slices

Four-month-old male Wistar rats (250–300 g) were decapitated and their brains were rapidly removed. For each rat, the cerebral cortex, including the hippocampus, was dissected and chopped into 0.35-mm sections in both the sagittal and coronal planes using a tissue chopper. Slices were placed in ice-cold calcium-free KRBS buffer (in mM: NaCl, 120; KCl, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 10) equilibrated with 5% CO_2 in 95% O_2 to maintain a pH of 7.4. Slices were then washed by decantation and stabilized in KRBS buffer under 5% CO_2 in 95% O_2 atmosphere at 37°C for 90 min.

Determination of the effects of ASN, BSN, and NAC on NOS activity

After stabilization, slices were washed twice with pre-warmed, magnesium-free KRBS containing 2 mM calcium and 1 μM glycine, and pre-incubated in the presence of 10 μM ASN, BSN, or NAC for 30 min at 37°C. In experiments analyzing response to a NMDA receptor antagonist (MK-801 [10 μM] or APV [10 μM]) [7, 40], an additional 5 min pre-incubation was performed.

At this point, slices were centrifuged at 1,731 × g for 10 min at 4°C and homogenized in 2 ml of ice-cold lysis buffer, pH 7.2 (in mM: Tris-HCl, 50; saccharose, 320; EDTA, 1; DTT, 1; protease inhibitor cocktail). NOS activity in the lysate was assayed by measuring the conversion of radiolabeled arginine to citrulline using ion-exchange separation as described by Czapski et al. [15]. Lysate (ca. 300 μg of protein) was incubated in a solution of 50 mM Tris-HCl pH 7.4, 100 μM [^{14}C]L-arginine (0.1 μCi), 2 mM $CaCl_2$, 1 μM calmodulin, 15 μM FAD, 10 μM tetrahydrobiopterin, 1 mM NADPH, 1 mM EDTA, and 1 mM DTT in a final volume of 300 μl. A separate set of experiments was designed to evaluate the activity of each NOS isoform. After pre-incubation with ASN or NAC, a NOS assay was performed for each sample in conditions inhibiting activity of particular NOS isoforms: A) in the absence of any inhibitor for determination of total NOS activity; B) in the presence of 10 μM 7-nitroindazole (7-NI) for inhibition of nNOS activity; C) in the absence of Ca^{2+} and in the presence of 2 mM EGTA and 100 μM N-nitro-L-arginine (NNLA) for inhibition of constitutive NOS activity (Tab. 1). These inhibitors of NOS were added 5 min before incubation. The mixture was incubated for 20 min at 37°C. Reactions were stopped by adding 1 ml 100 mM Tris-HCl buffer, pH 5.5, containing 10 mM EDTA. After centrifugation at 3,000 × g for 10 min, 0.5 ml of supernatant was passed through 0.5 ml of Dowex™ 50WX-8 (Serva, Germany) column (Na^+ form) and [^{14}C]L-citrulline was eluted with 2 × 1 ml H_2O. The radioactivity of eluted and [^{14}C]L-citrulline was determined using Bray's scintillation solution in a Wallac 1409 counter (Wallac Oy, Finland).

Determination of the effect of ASN on nitrite level

Nitrite levels in brain slices were determined using Griess reagent [20]. Slices from the brain cortex in-

Tab. 1. Calculation of activity of separate NOS isoforms in rat brain slices

	Inhibitors	Description	Control	ASN	NAC
			NOS activity [pmol × mg protein^{-1} × min^{-1}]		
A	–	total NOS	11.51 ± 1.49	18.88 ± 1.36**	15.59 ± 0.63*
B	7NI	nNOS-devoid	2.21 ± 0.32	2.91 ± 0.17	2.59 ± 0.19
C	NNLA + EGTA	iNOS	0.45 ± 0.23	0.44 ± 0.20	1.00 ± 0.45

Activities of separate NOS isoforms were calculated according to formulas: nNOS act. = A – B; iNOS act. = C; eNOS act. = A – nNOS act. – iNOS act., respectively. Data represent the mean value ± SEM for 3 separate experiments, each carried out in triplicate. Significance of data was determined with one-way ANOVA followed by Bonferoni *post-hoc* test, *, **p < 0.05, 0.01 *vs.* control

cluding hippocampus were prepared for this determination in the same manner as for the NOS activity assay. After a 30-min pre-incubation in the presence of 10 μM ASN or BSN, slices were homogenized and centrifuged at 21,000 × g for 20 min. Supernatant (100 μl) or serial dilution of $NaNO_2$ standard (linear range 0–100 μM) were applied to a microtiter plate well. The total nitrite in each sample was then determined by adding 50 μl Griess reagent 1 (1% sulfanilamide in 1M HCl), followed by Griess reagent 2 (0.1% N-(1-napthyl)-ethylenediamine). The mixture was incubated for 10 min at room temperature in the dark. Optical density was measured spectrophotometrically at 540 nm. Nitrite level was expressed as nanomoles per mg of tissue protein.

Statistical analysis

Analyses among multigroup data were conducted using one-way analysis of variance (ANOVA), followed by a Bonferoni *post-hoc* test. Differences among groups were considered significant if the probability of error was less than 5%. The data represent the mean ± SEM. Results presented as percent of control were calculated as crude data, statistically analyzed and then re-calculated and expressed as % of control, considering value of control group as 100%.

Results

This study analyzed the effects of extracellular monomeric/oligomeric ASN and NAC peptides on NOS activity in rat brain slices. As shown in Figure 2, 10 μM ASN or the toxic NAC fragment significantly

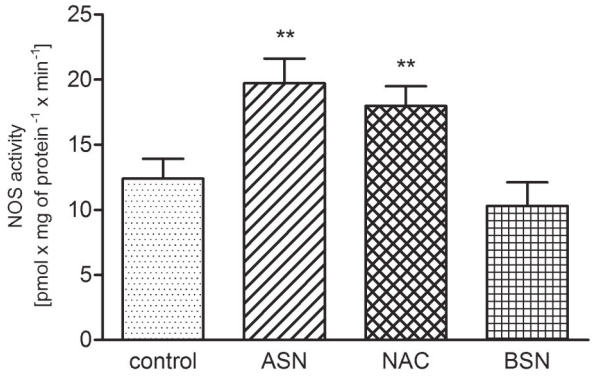

Fig. 2. The effects of ASN, BSN, and NAC on NOS activity in rat brain slices. NOS activity was measured in rat brain slices after 30-min incubation in the absence (control) and presence of 10 μM ASN, BSN, or NAC. Data represent the mean value ± SEM for 5 separate experiments, each carried out in triplicate. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test, ** p < 0.01 *vs.* control

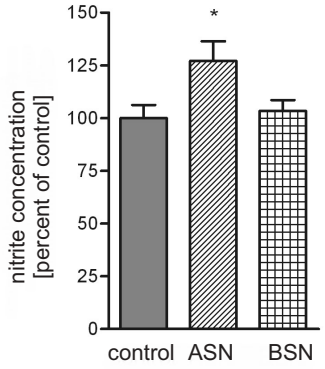

Fig. 3. ASN increases nitrite formation in rat brain slices. Nitrite level was measured in rat brain cortex including hippocampus after 30-min pre-incubation in the absence (control) and presence of 10 μM ASN or BSN. Data represent the mean values ± SEM, expressed as % of control value, for 2–8 determinations. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test, * p < 0.05 *vs.* control

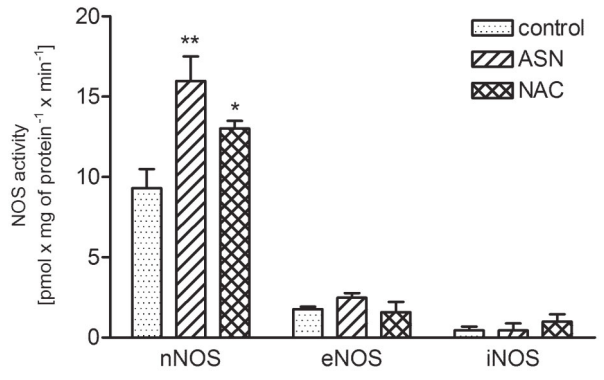

Fig. 4. The effects of ASN and NAC on the activity of separate NOS isoforms in rat brain slices. NOS activity was measured in rat brain slices after 30-min incubation in the absence (control) and presence of 10 μM ASN or NAC. Data represent the mean value ± SEM for 3 separate experiments, each carried out in triplicate. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test; *, ** $p < 0.05$, 0.01 *vs.* control

stimulated total NOS activity during the 30-min pre-incubation period. Treatment of brain slices with 10 μM BSN that lacks the NAC region did not change NOS activity, indicating the effect to be specific to the isoform of synuclein (Fig. 2). Under the same experimental conditions, ASN increased the level of nitrite in the brain slices by 27%, whereas BSN had no effect

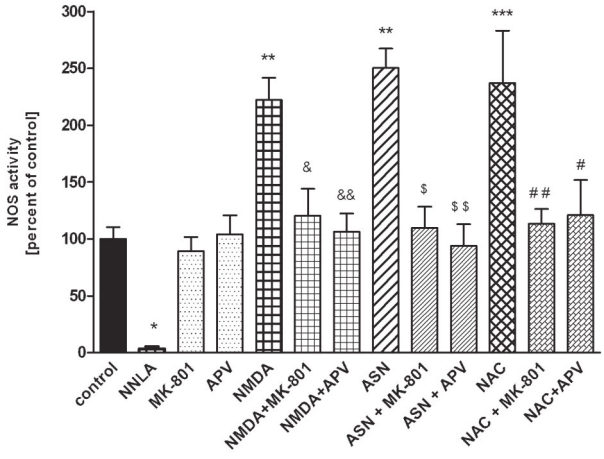

Fig. 5. Involvement of NMDA receptor in ASN and NAC-evoked NOS activation. NOS activity was measured in rat hippocampal slices. Data represent the mean value ± SEM (expressed as % of control value) for 3–8 separate experiments, each carried out in triplicate. NNLA was used as a positive control. Significance of the data was determined with one-way ANOVA followed by the Bonferoni *post-hoc* test; *, **, *** $p < 0.05$, 0.01 and 0.001 *vs.* control, $^{\$}$, $^{\$\$}$ $p < 0.05$ and 0.01 *vs.* ASN $^{\#}$, $^{\#\#}$ $p < 0.05$ and 0.01 *vs.* NAC $^{\&}$, $^{\&\&}$ $p < 0.05$ and 0.01 *vs.* NMDA

(Fig. 3). To analyze which NOS isoform was activated by ASN and NAC peptides, the NOS assays were performed in conditions enabling calculation of the activity of each isoform, as presented in Table 1. As shown in Figure 4, the investigated proteins exclusively affected the constitutively active neuronal isoform of NOS (nNOS) without affecting the endothelial or inducible NOS isoforms. ASN and the NAC stimulated nNOS by about 70% and 40%, respectively (Fig. 4). Because the primary pool of nNOS is physically and functionally connected to the NMDA receptor, we further investigated the involvement of this receptor in nNOS stimulation evoked by ASN and the NAC. Both NMDA receptor antagonists, competitive APV (10 μM) and uncompetitive MK-801 (10 μM) prevented NOS stimulation evoked by ASN and the NAC, suggesting that activation of NMDA receptor may be responsible for this peptide-evoked NOS activation (Fig. 5).

Discussion

ASN is a small presynaptic protein that is released by neurons into extracellular space, both as a part of its normal cellular processing [18] and under oxidative/nitrosative stress and neurodegeneration [2, 41]. In this study, we have shown for the first time that extracellular ASN in monomeric/oligomeric form activates NMDA receptor-mediated NO synthesis in rat brain slices. Moreover, BSN, which is characterized by the lack of the NAC domain, had no effect on NOS activity. Prolonged action of ASN on cells in culture (8–24 h) leads to increase of nNOS mRNA expression without effect on the endothelial or inducible protein isoforms (data not shown). Our findings are consistent with previous data showing that the action of ASN is related to its internal NAC domain [3, 4, 17]. Similar effects of nNOS activation in the cerebral cortex and hippocampus were observed in the presence of amyloid β-peptide (Aβ), which is an amyloidogenic protein and may interact with ASN [32, 39]. Overactivation of NMDA receptors may trigger cytotoxicity and cell death [9]. The increased intracellular Ca^{2+} concentration leads to activation of many Ca^{2+} dependent enzymes, including nNOS. The main product of NOS-catalyzed reactions is NO, but in some conditions (low levels of the substrate arginine or its

co-factor, tetrahydrobiopterin) the superoxide radical (O_2^-) and H_2O_2 can also be produced by NOS. NO alone is a rather low-reactivity radical; it can diffuse freely in tissue, but may react with O_2^- to form $ONOO^-$, a very potent oxidizing agent. It is now well documented that NO and its toxic metabolite, $ONOO^-$, can inhibit components of the mitochondrial respiratory chain leading to a cellular energy deficiency [16]. Our previous results indicated that the NAC peptide activated generation of free radicals and caused DNA fragmentation in rat brain slices; enhanced $ONOO^-$ production might be responsible for this DNA damage [1].

$ONOO^-$ and other reactive nitrogen species can modify protein functions by nitration and nitrosylation, which may result in cell damage and death. Schulz et al. [35] have shown that systemic administration of 7-nitroindazole attenuates lesions produced by striatal malonate injections or systemic treatment with 3-nitropropionic acid or MPTP. Furthermore, 7-nitroindazole attenuates increases in lactate production and hydroxyl radical (•OH) and 3-nitrotyrosine generation *in vivo,* which may be a consequence of $ONOO^-$ formation.

ASN stimulates NOS activity in isolated mitochondria and increases the intramitochondrial calcium concentration $[Ca^{2+}]_m$ [33]. However, until now, the detailed mechanism of how ASN induces NOS stimulation and NO liberation has been unknown. In the present study we identified that ASN and NAC exclusively affect nNOS. Our previous findings indicated that ASN induces Ca^{2+} influx in rat synaptoneurosomes [4]. The present data demonstrate that NMDA receptor inactivation by specific antagonists MK-801 and APV prevents NOS stimulation evoked by ASN and the NAC.

It has been suggested that accumulation of the Aβ peptide, which displays many similarities to ASN, is partly responsible for triggering neurodegeneration *via* glutamate-mediated excitotoxicity in AD brain. This is supported by observations that toxicity induced by Aβ in cultured neurons and in the adult rat brain is mediated by activation of NMDA receptors. Harkany and co-workers indicated that NMDA receptor antagonist MK-801 protected against the neurotoxic events evoked by administration of Aβ 1–42 into the magnocellular nucleus basalis of rats [21]. Additionally, recent clinical studies have shown that memantine, an uncompetitive NMDA receptor antagonist, can significantly improve cognitive functions in some Alzheimer's disease patients [30]. The last data demonstrated that Aβ (1–42)-induced toxicity in rat primary cortical cultured neurons is accompanied by increased extracellular and decreased intracellular glutamate levels. Memantine treatment significantly protected cultured neurons against Aβ-induced toxicity by attenuating phosphorylation and its associated signaling mechanisms [38]. Moreover, nitromemantine, a second-generation memantine derivative, is highly neuroprotective in both *in vitro* and *in vivo* animal models [29].

The previous investigations showed that ASN, through the NAC region, interacts with membrane proteins and through this interaction can modulate the function of synaptic terminals [44]. The detailed mechanism of how ASN activates NMDA receptor-mediated NO synthesis remains to be elucidated. However, on the basis of the present data we suggest that extracellular ASN in monomeric/oligomeric form interacts with plasma membranes, probably through the NAC domain, and activates NMDA channels, leading to an increase in the intracellular Ca^{2+} concentration and nNOS activation. Since both competitive and uncompetitive antagonists of the NMDA receptor prevented ASN-evoked NOS activation, we suggest that this protein could interact with lipid bilayers and consequently affect the NMDA receptor structure leading to its activation. Similar non-specific activation of glutamatergic receptors was recently observed for prefibrillar aggregates of Aβ [34]. Moreover, it was presented recently that Aβ up-regulates metabotropic glutamate receptors [11]. Another possibility is that ASN may affect extracellular glutamate levels. As was recently demonstrated, Aβ can increase glutamate release and inhibit glutamate uptake [22, 28].

In summary, our findings indicate that ASN and its fragment NAC stimulate nNOS activity *via* the NMDA receptor-mediated pathway. NO liberated by over-activated nNOS could react with O_2^- to form $ONOO^-$, which modulates the function of a variety of biomolecules including proteins, lipids, and DNA. Chronic stimulation of NMDA receptor-dependent signaling by ASN could contribute to the development of neurodegenerative processes.

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