Oxidative and Nitrosative Modifications of Biliverdin Reductase-A in the Brain of Subjects with Alzheimer’s Disease and Amnestic Mild Cognitive Impairment

Eugenio Barone a, b, *, Fabio Di Domenico a, c, *, Giovanna Cenini a, Rukhsana Sultana a, Raffaella Coccia a, Paolo Preziosi b, Marzia Perluigi b, Cesare Mancuso b and D. Allan Butterfield a, *

a Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, USA
b Institute of Pharmacology, Catholic University School of Medicine, Roma, Italy
c Department of Biochemical Sciences, Sapienza University of Rome, Rome, Italy

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Abstract. Biliverdin reductase-A (BVR-A) is a pleiotropic enzyme and plays pivotal role in the antioxidant defense against free radicals as well as in cell homeostasis. Together with heme oxygenase, BVR-A forms a powerful system involved in the cell stress response during neurodegenerative disorders including Alzheimer’s disease (AD), whereas due to the serine/threonine/tyrosine kinase activity the enzyme regulates glucose metabolism and cell proliferation. In this paper, we report results that demonstrate BVR-A undergoes post-translational oxidative and nitrosative modifications in the hippocampus, but not cerebellum, of subjects with AD and amnestic mild cognitive impairment (MCI). A significant increase of nitrated BVR-A was demonstrated only in AD and MCI hippocampi, whereas no significant modifications were found in cerebellar tissue. In addition, a significant reduction in protein carbonyl-derivatives of BVR-A was found in both AD and MCI hippocampi (15% and 18%, respectively). Biliverdin reductase-bound 4-hydroxynonenals were not modified in hippocampi and cerebella from AD and MCI subjects. These results supported the hypothesis of a prevalence of nitrosative stress-induced modifications on BVR-A structure, and this evidence was confirmed by a significant upregulation of inducible nitric oxide synthase in hippocampal tissue of subjects with AD and MCI that was not present in cerebellum. In conclusion, nitrosative stress-induced modifications on hippocampal BVR-A are an early event in the pathogenesis of AD since they appear also in MCI subjects and could contribute to the antioxidant and metabolic derangement characteristic of these neurodegenerative disorders.

Keywords: Alzheimer’s disease, biliverdin reductase, heme oxygenase, mild cognitive impairment, neurodegenerative disorders, oxidative stress

INTRODUCTION

Heme oxygenase (HO) catalyzes the oxidation of the alpha-meso-carbon bridge of heme moieties, resulting in equimolar amount of ferrous iron, carbon monoxide, and biliverdin-IX-alpha (thereafter BV). This latter is further reduced by the cytosolic enzyme biliverdin reductase (BVR) into bilirubin (BR), the
Heme oxygenase exists in two main isoforms, HO-1 and HO-2. Heme oxygenase-1 (HO-1), also known as heat shock protein (Hsp)-32, is induced by various stimuli, including reactive oxygen and nitrogen species (ROS and RNS, respectively), ischemia, heat shock, bacterial lipopolysaccharide (LPS), hemin, and the neuroprotective agent tetramethylammonium (Neotrofin) [2–4], whereas HO-2, the constitutive isoform, is responsive to developmental factors and adrenal glucocorticoids [2, 4]. The central nervous system (CNS) is characterized by measurable HO activity under basal conditions. Most can be attributed to HO-2, which is expressed in neuronal populations in almost all brain areas [2], whereas the inducible isoform is present at low levels in scattered groups of neurons, including the ventromedial and paraventricular nuclei of the hypothalamus [2, 4]. HO-1 is also found in glial cells where its expression can be induced by oxidative stress [5]. Similarly, two isoforms of BVR were described and named BVR-A and BVR-B [6–8]. Both these enzymes generate bilirubin (BR), but only BVR-A reduces BV into the powerful antioxidant molecule BR-IX-alpha (hereafter BR), whereas BVR-B prefers the other BV isoforms, such as BV-beta, BV-gamma, and BV-delta [6–8]. Biliverdin reductase is co-expressed with HO-1 and/or HO-2 in cells of the rat brain that express these enzymes under normal conditions. It is also found in regions and cell types that can express heat shock-inducible HO-1 [9].

The up-regulation of the HO/BVR-A system is considered an antioxidant response because: (i) the intracellular levels of pro-oxidant heme are reduced [4, 10], (ii) CO, the gaseous compound formed during the two-step reaction, has antinflammatory and antiapoptotic effects in some tissues [10–18], and (iii) bilirubin is an efficient scavenger of ROS, RNS, and nitric oxide [19–26]. Indeed, the sustained activation of the HO-1 could result in a cytotoxic response since it could originate a significant increase in both carbon monoxide and ferrous ion deposition which triggers lipid and protein oxidation through the Fenton reaction or by altering the ferrous/ferric iron balance [27, 28]. In support of this dual effect of HO-1 on neurodegenerative disorders, Schipper and colleagues demonstrated that post-translational modification in the BVR-A structure, such as the autophosphorylation of selected serine-residues, regulates its own reductase activity [6, 38–40]. Very recently, our group reported on changes in BVR activity secondary to differential phosphorylation on serine/hemoprotein/tyrosine residues in brains of subjects with AD or MCI [41]. These findings prompted us to explore the possibility that oxidative and nitrosative modifications could alter BVR-A function in AD. This hypothesis is further corroborated by several reports which demonstrated that many proteins involved in the regulation of important cellular functions became dysfunctional in AD brain due to oxidative and nitrosative modifications [42–50].

The aim of this study was to investigate the post-translational oxidative and nitrosative modifications of BVR-A in both hippocampal and cerebellar samples of subjects affected by AD or arguably its earliest form, MCI, the latter being the transitional phase between normal aging and early AD [51]. MCI shares with AD evidence demonstrate the activation of the HO/BVR-A axis in AD, its pathophysiological and clinical significance is still under debate. An intriguing aspect is to understand whether or not the pro-oxidant conditions in AD could modify the structure of members of the HO-1/BVR-A system thus affecting the antioxidant potential. Early studies by Maines and coworkers demonstrated that post-translational modification in the BVR-A system affects the antioxidative agent leteprinim potassium (Neotrofin) [2–4], whereas 3-nitrotyrosine (3-NT) was evaluated as a marker of nitrosative stress. Whereas 3-nitrotyrosine (3-NT) was evaluated as a marker of nitrosative stress. Therefore, BVR-A overexpression is a useful mechanism for the brain tissue to counteract the oxidative/nitrosative damage that is a common feature of neurodegenerative disorders, including AD [31–36]. The activation of the HO/BVR-A system was then demonstrated in the inferior parietal lobule, plasma and lymphocytes of AD patients [33]. In addition, Kimpara and colleagues showed that AD subjects have an increased concentration of BR in cerebrospinal fluid with respect to control [37]. Although these lines of evidence demonstrate the activation of the HO/BVR-A axis in AD, its pathophysiological and clinical significance is still under debate. An intriguing aspect is to understand whether or not the pro-oxidant conditions in AD could modify the structure of members of the HO-1/BVR-A system thus affecting the antioxidant potential. Early studies by Maines and coworkers demonstrated that post-translational modification in the BVR-A system affects the antioxidative agent leteprinim potassium (Neotrofin) [2–4], whereas 3-nitrotyrosine (3-NT) was evaluated as a marker of nitrosative stress. Therefore, BVR-A overexpression is a useful mechanism for the brain tissue to counteract the oxidative/nitrosative damage that is a common feature of neurodegenerative disorders, including AD [31–36].

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obtained from Bio-Rad (Hercules, CA, USA). Anti-
mouse and anti-rabbit IgG horseradish peroxidase
conjugate secondary antibody and ECL plus Western
detecion reagents were obtained from GE Health-
care Bio-Sciences corp. (Piscataway, NJ, USA).

**Subjects**

Frozen hippocampal and cerebellar samples from
well-characterized subjects with AD, MCI and respec-
tive age-matched controls (n = 6 for each group, see
also Table 1) were obtained from the University of
Kentucky Rapid Autopsy Program of the Alzheimer's
Disease Clinical Center (UK ADC) with a postmortem
interval within the range 1.75–5.75 h for AD and
MCI subjects and relative control subjects. All the
subjects were longitudinally followed and underwent
annual neuropsychological testing, and neurological
and physical examinations. Control subjects were
without history of dementia or other neurological dis-
orders and with intact activities of daily living (ADLs),
and they underwent annual mental status testing and
semi-annual physical and neurological exams as part
of the UK ADC normal volunteer longitudinal aging
study. The control subjects showed no significant
histopathological alterations and the Braak score was
within the range I-II for the MCI age-matched controls,
and I-III for the AD age-matched controls. Patients suf-
ferring from MCI met the criteria described by Petersen
[51], which include: a memory complaint supported
by an informant, objective memory test impairment
(age- and education-adjusted), general normal global
intellectual function, intact ADLs, Clinical Dementia
Rating score of 0.0 to 0.5, no dementia, and a clini-
cal evaluation that revealed no other cause for memory
decline. AD patients diagnosis was made according to
criteria developed by the National Institute of Neu-
rological and Communicative Disorders and Stroke
(NINCDS) and the Alzheimer’s disease and Related
Disorders Association (ADRDA) [53]. All AD patients
displayed progressive intellectual decline. The Braak
scores were within the range 3–5 and 5–6 for MCI and
AD patients, respectively.

**Sample preparation**

Brain tissues samples (hippocampus and cere-
bellum) from control, MCI and AD subjects were
sonicated in Media 1 lysis buffer (pH 7.4) con-
taining 320 mM Sucrose, 1% of 990 mM Tris-HCl
(pH = 8.8), 0.098 mM MgCl2, 0.076 mM EDTA,
the proteinase inhibitors leupeptin (0.5 mg/mL),
pepstatin (0.7 μg/mL), aprotinin (0.5 mg/mL), and
PMSF (40 μg/mL) and phosphatase inhibitor cocktail
(Sigma-Aldrich). Homogenates were centrifuged
at 14,000 × g for 10 min to remove debris. Protein
concentration in the supernatant was determined by
the Pierce BCA method (Pierce, Rockford, IL, USA).

**Immunoprecipitation**

The immunoprecipitation procedure was performed
as previously described [50] with modifications.
Briefly, 150 µg of protein extracts were dissolved in
500 µL of RIPA buffer (10 mM Tris, pH 7.6; 140 mM
NaCl; 0.5% NP40 including protease inhibitors) and
then incubated with 1 µg of anti-BVR-A antibody at
4 °C overnight. Immunocomplexes were collected by
using protein A/G beads suspension for 2 h at 4 °C
and washed five times with immunoprecipitation buffer.
Immunoprecipitated biliverdin reductase was recov-
ered by resuspending the pellets in reducing SDS
buffers and subjected to electrophoresis on 12% gels
followed by Western blot analysis to identify BVR-A’s
protein carbonyls, HNE or 3-NT adducts (see below).
In these experiments, total BVR-A was used as loading
control [38, 40] and it was detected on the same mem-
branes after a stripping/reprobing procedure by using
a specific anti-BVR-A antibody (see below).

**Western blot analysis**

For Western blot analyses, 50 µg of protein were
denatured in sample buffer for 5 min at 100 °C, and
proteins separated on 12% precast Criterion gels
(Bio-Rad) by electrophoresis at 100 mA for 2 h in

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**Table 1**

Demographic information of AD and amnestic mild cognitive
impairment (MCI) subjects and their respective age-matched con-
trols. The values reported are the average of 6 samples

<table>
<thead>
<tr>
<th>A) Subjects demographics data</th>
<th>Control (MCI)</th>
<th>MCI</th>
</tr>
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<tbody>
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<tr>
<td>Gender</td>
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<td>2M, 4F</td>
</tr>
<tr>
<td>Age (years)</td>
<td>82 (78-89)</td>
<td>89 (82-99)</td>
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<tr>
<td>Brain weight (g)</td>
<td>1204 (1080-1315)</td>
<td>1102 (930-1200)</td>
</tr>
<tr>
<td>PMI (hours)</td>
<td>1.75–4.00</td>
<td>2.00–5.00</td>
</tr>
<tr>
<td>Braak stage</td>
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</table>

<table>
<thead>
<tr>
<th>B) Subjects demographics data</th>
<th>Control (AD)</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
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<td>6</td>
</tr>
<tr>
<td>Gender</td>
<td>5M, 1F</td>
<td>2M, 4F</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>85 (80-92)</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>1219 (1020-1410)</td>
<td>1104 (835-1260)</td>
</tr>
<tr>
<td>PMI (hours)</td>
<td>2.00–3.75</td>
<td>2.00–5.75</td>
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<tr>
<td>Braak stage</td>
<td>I-III</td>
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MOPS buffer (Bio-Rad) into the Bio-Rad apparatus. The proteins from the gels were then transferred to nitrocellulose membrane using the Transblot-Blot SD Semi-Dry Transfer Cell at 20mA for 2h. Subsequently, the membranes were blocked at 4°C for 1h with fresh blocking buffer made of 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST). The membranes were incubated at room temperature in PBST for 2h with the following primary antibodies as separate experiments: polyclonal anti-rabbit BVR-A (Sigma-Aldrich, dilution 1:1000) which recognizes a single band at 35 KDa; polyclonal anti-rabbit nitrotyrosine antibody (3-NT) (Sigma-Aldrich dilution 1:100 in wash blot); polyclonal anti-rabbit DNP protein adducts antibody (Millipore, Billerica, MA, USA, dilution 1:100); polyclonal anti-rabbit HNE antibody (Alpha Diagnostic International, San Antonio, TX, USA, dilution 1:100); polyclonal anti-rabbit iNOS antibody (Alpha Diagnostic International, San Antonio, TX, USA, dilution 1:500), and polyclonal anti-rabbit β-actin (Sigma-Aldrich, dilution 1:2000).

The respective membranes were then washed three times for 5 min with PBST followed by incubation with anti-mouse alkaline phosphatase or horseradish peroxidase conjugate secondary antibody (1:3000) in PBST for 2h at room temperature. Membranes were then washed three times in PBST for 5 min and developed using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) color developing reagent for alkaline phosphatase secondary antibody or ECL plus WB detection reagents for horseradish peroxidase conjugate secondary antibody. Blots were dried, scanned in TIF format using Adobe Photoshop on a Canoscan 8800F (Canon) or STORM UV transilluminator (λex = 470 nm, λem = 618 nm, Molecular Dynamics, Sunnyvale, CA, USA) for chemiluminescence. The images were quantified with Image Quant TL 1D version 7.0 software (GE Healthcare). The optical density of bands was calculated as volume (optical density × area) adjusted for the background.

Post-derivatization of protein

Samples were post-derivatized with DNPH on the membrane and probed with anti-DNPH antibody to identify the carbonylated proteins. The nitrocellulose membranes where equilibrated in solution A (20% (v/v) methanol, 80% (v/v) wash blot buffer) for 5 min, followed by incubation of membranes in 2N HCl for 5min. The proteins on blots were then derivatized in solution B (0.5 mM DNPH in 2N HCl) for exactly 10min as described by Conrad et al. [54]. The membranes were washed three times in 2N HCl for 5 min each and then five times with 50% methanol and two times with wash blot each for 5 min. The 2,4-dinitrophenylhydrazone (DNP) adducts of the carbonyls of the brain proteins were detected immunochromatically as described above.

Statistical analysis

All statistical analysis was performed using a two-tailed Student's t-test. P < 0.05 was considered as being significantly different from control.

RESULTS

BVR-A's protein carbonyls, HNE and 3-NT levels in brain of subjects with AD and MCI

Biliverdin reductase-A was immunodetected in the hippocampus and cerebellum of control and AD or MCI subjects [41]. BVR-A expression levels were markedly increased in hippocampi from subjects with AD and MCI (+45% and 44%, respectively, compared to control), whereas non-significant changes in levels of BVR-A were observed in cerebellum samples of AD and MCI subjects [41].

In the current study, following immunoprecipitation with specific anti-BVR-A antibody, the levels of protein carbonyls, HNE, and 3-NT on BVR-A were measured by Western Blot analyses. As shown in Figure 1, BVR-A's protein carbonyls (Panel A) were reduced by about 15%, whereas no significant changes were found for HNE levels (Panel B) in the hippocampus of AD subjects. On the contrary, AD subjects showed a significant increase (about 95%) in the levels of 3-NT-modified BVR-A (Panel C). Similarly, immunoprecipitated BVR-A from hippocampal samples of MCI subjects was characterized by a significant decrease (about 20%), in the levels of protein carbonyls and HNE (Figure 2, Panel A), whereas HNE levels did not change. With regard to 3-NT, levels were found increased by about 30%, compared with the matched controls (Panel C). To evaluate whether or not oxidative and nitrosative modifications were specific for hippocampal tissue, the same experiments were performed in the cerebellum of AD and MCI subjects. As shown in Figure 3, no changes were observed for protein carbonyls or 3-NT levels in the cerebella samples from AD or MCI subjects.
Fig. 1. Biliverdin reductase-A (BVR-A) protein carbonyls (PC), HNE and 3-NT levels in hippocampus of Alzheimer’s disease (AD) subjects. Brain samples of hippocampus of subjects with AD were immunoprecipitated with anti-BVR-A antibody and probed with anti-DNP protein adducts polyclonal antibody (Panel A), anti-HNE polyclonal antibody (Panel B) and anti-nitrotyrosine polyclonal antibody (Panel C) by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of each marker normalized per total BVR-A as loading control. In panels A-C representative gels are shown. Data are expressed as mean ± SD of six individual samples per group. *p < 0.05 and ***p < 0.001 versus control (Student’s t-test).

iNOS expression in brain of subjects with AD and MCI

The elevated levels of 3-NT on BVR-A in the hippocampus samples was paralleled by a significant increase in the expression of the inducible isof orm of nitric oxide synthase (iNOS). As shown in Figure 4, a significant induction of iNOS was found in the hippocampus from both AD (Panel A) and MCI (Panel B) subjects, whereas no significant changes were observed in cerebella (Panels C-D).

DISCUSSION

In this paper, the first evidence of the post-translational oxidative and nitrosative modifications of BVR-A in the hippocampus, and lack thereof in cerebellum of AD and MCI subjects, has been provided. The reason why we decided to focus the attention on these two selected brain areas depended on their differential selectivity to free radical-induced injury and histopathological alterations [55]. Indeed, hippocampus is broadly recognized as a main target of neurodegenerative damage during AD progression, presenting increased levels of oxidative stress, neuronal loss and marked atrophy in respect to whole brain [55–63]. Conversely, the cerebellum is largely devoid of pathology and oxidative stress [64–66], consistent with normal BVR levels, activity and phosphorylation state [41]. The first novel finding provided by this paper is the increased level of nitrated BVR-A and the decreased formation of protein carbonyls in the hippocampi of AD and MCI subjects.
both AD and MCI subjects. These results are consistent with the significant reduction in BVR activity coincident with an increased protein BVR-A expression in hippocampi of subjects with AD and MCI [41]. The rationale for loss of activity of BVR while protein levels were elevated in AD and MCI hippocampus was, at least in part, the reduced level of phosphorylation of serine/threonine residues which made BVR-A dysfunctional [6, 38, 41]. In accordance with these previous results are those provided in the current study, which showed a marked increase in the nitration of BVR-A’s tyrosine residues. It is well known that the nitration of tyrosine residues in proteins results in a marked decrease in their function, and this was corroborated by the significant reduction in BVR activity in hippocampi from subjects with AD and MCI [41]. Taken together, these findings underline the importance of post-translational modifications, such as phosphorylation and nitration, on BVR-A functions. Keeping this in mind, it is no longer correct to link only overexpression of total BVR-A, with the production of the antioxidant and anti-nitrosative molecule BR [20, 41] and, therefore, a direct neuroprotective effect.

An interesting aspect of the current results is the reduced formation of BVR-A’s protein carbonyls together with the lack of production of HNE-adducts with respect to 3-NT. This finding was not surprising and resembles the results from previous studies, which showed a differential degree of protein oxidation and the prevalence of nitration over carbonyl...
Fig. 3. Biliverdin reductase-A (BVR-A) protein carbonyls (PC) and 3-NT levels in cerebellum of Alzheimer disease (AD) (Panel A and C) or amnestic mild cognitive impairment (MCI) (Panel B and D) subjects. In order to evaluate whether or not oxidative and nitrosative modifications were specific for hippocampal tissue, brain samples of cerebellum of subjects with AD or MCI were immunoprecipitated with anti-BVR-A antibody and probed with anti-DNP protein adducts polyclonal antibody (Panel A and C), or anti-nitrotyrosine polyclonal antibody (Panel B and D) by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of each marker normalized per total BVR-A as loading control. In panels A-D representative gels are shown. Data are expressed as mean ± SD of six individual samples per group.

formation in many dysfunctional proteins in AD [67, 68]. This hypothesis is also supported by the evidence of a significant induction of iNOS (Fig. 4) and NO formation, with consequent increase of nitrosative stress in the brain, plasma and lymphocytes of AD and MCI subjects (Fig. 4) [33, 46, 48, 49]. Considering that peroxynitrite formation occurs via the reaction between NO and superoxide anion (or sometimes hydrogen peroxide with the formation of intermediate nitrosonium) [69] and that this reaction proceeds very fast, it is plausible to conclude that in the presence of NO the formation of peroxynitrite prevails on ROS formation and the consequent protein oxidation [70, 71].

Another consequence of this study is the differential pattern of BVR-A’s carbonyls and 3-NT formation in the hippocampus and cerebellum. A possible explanation for the lack of oxidative damage in cerebellum characteristic of AD subjects is the abundance of BVR-A and BR in this brain area [9, 72]. Our results demonstrate that there is no evidence for an increased degree of BVR-A oxidation or nitration in the cerebellum of AD or MCI subjects with respect to controls. These data are in good agreement with previous studies showing a lack of pathology and oxidative stress in cerebellum [55, 64–66]. That BVR-A did not undergo oxidative or nitrosative modifications in the cerebellum of AD or MCI subjects with respect to controls is consistent with the notion that the levels of bilirubin formed are still high, and this may contribute to the lack of oxidative stress in this brain region.

The HO-1/BVR-A system is not the only one involved in the cell stress response. Two other members of the heat shock protein family, such as Hsp60 and Hsp70, were shown to be involved in the adaptive stress response in AD. However, data from the litera-
Fig. 4. Inducible nitric oxide synthase (iNOS) expression in hippocampus and cerebellum of Alzheimer’s disease (AD) and amnestic mild cognitive impairment (MCI) subjects. Brain samples of hippocampus (Panel A and B) and cerebellum (Panel C and D) of subjects with AD and MCI were assayed for iNOS by Western Blot as described under Materials and Methods. Densitometric values shown in the histograms are given as percentage of control, set as 100%, and are the product of the band value of the levels of iNOS normalized per β-actin as loading control. In panels A and B representative gels are shown. Data are expressed as mean ± SD of six individual samples per group. *p < 0.05 and **p < 0.01 versus control (Student’s t-test).

In conclusion, this study represents the first report of oxidative and nitrosative differences in BVR-A in the hippocampus of subjects with AD and MCI. The evidence that BVR-A nitration occurred also in the hippocampus of MCI subjects, suggests that any modification in terms of cell stress response is an early event in the pathogenesis and progression of AD. The implications of these results for the adaptive responses to stress in AD are profound, and research on these subjects is ongoing in our laboratory.

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