

Decreased Levels of PSD95 and Two Associated Proteins and Increased Levels of BCl₂ and Caspase 3 in Hippocampus from Subjects with Amnestic Mild Cognitive Impairment: Insights into Their Potential Roles for Loss of Synapses and Memory, Accumulation of A β , and Neurodegeneration in a Prodromal Stage of Alzheimer's Disease

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Alzheimer's disease (AD) is the most common form of dementia and is pathologically characterized by senile plaques, neurofibrillary tangles, synaptic disruption and loss, and progressive neuronal deficits. The exact mechanism(s) of AD pathogenesis largely remain unknown. With advances in technology diagnosis of a pre-AD stage referred to as amnestic mild cognitive impairment (MCI) has become possible. Amnestic MCI is characterized clinically by memory deficit, but normal activities of daily living and no dementia. In the present study, compared to controls, we observed in hippocampus from subjects with MCI a significantly decreased level of PSD95, a key synaptic protein, and also decreased levels of two proteins associated with PSD95, the N-methyl-D-aspartate receptor, subunit 2A (NR2A) and the low-density lipoprotein receptor-1 (LRP1). PSD95 and NR2A are involved in long-term potentiation, a key component of memory formation, and LRP1 is involved in efflux of amyloid beta-peptide (1-42). A β (1-42) conceivably is critical to the pathogenesis of MCI and AD, including the oxidative stress under which brain in both conditions exist. The data obtained from the current study suggest a possible involvement of these proteins in synaptic alterations, apoptosis and consequent decrements in learning and memory associated with the progression of MCI to AD. © 2009 Wiley-Liss, Inc.

Key words: Alzheimer's disease; mild cognitive impairment; hippocampus; post synaptic density protein 95 (PSD95); low density lipoprotein receptor (LRP1); N-methyl D-aspartate receptor-2A (NR2A)

Alzheimer's disease (AD) is the most common form of dementia in the elderly. Pathologically AD is characterized by senile plaques (SP), neurofibrillary tangles (NFT), synapse loss, and progressive neuronal deficits (Hardy and Selkoe 2002; Wenk 2006). In addition to the formation of SP and NFT, gliosis, chronic inflammatory reactions, excitotoxic damage and oxidative stress all appear to contribute to the progression of AD (Butterfield and Stadtman 1997; Butterfield et al., 2001; 2006a; Lovell et al., 2001; Dalle-Donne et al., 2006; Jacob et al., 2007; Aisen 2008). There is an abundance of evidence suggesting that oxidative stress and N-methyl-D-aspartate (NMDA) receptor-associated Ca²⁺-mediated excitotoxicity are involved in the pathogenesis of AD; however, the exact mechanisms operative in these effects largely remain unknown. Amnestic mild cognitive impairment (MCI) is a pre-AD stage, the study

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TABLE I. Demographic information of the MCI and age-matched controls used in the present study

Samples (n=6)	Age (Yr) (Mean \pm SD)	Gender (M/F)	APOE genotype	Post Mortem Interval (h) (Mean \pm SD)	Braak stage	Brain weight (g)
Control	81 \pm 6.40	2/4	3/3 (5), 3/4 (1)	2.87 \pm 0.52	I-II	1260 \pm 120
MCI	88.0 \pm 3.8	2/4	2/3 (1), 3/3 (4), 3/4 (1)	3.12 \pm 0.40	III-V	1120 \pm 61

of which conceivably can provide insights into progression of MCI to AD.

MCI individuals are broadly grouped into 2 subtypes referred to as: amnesic (memory-affecting) MCI or non-amnesic MCI (no change in memory) (Petersen 2004; Portet et al., 2005). Pathologically, MCI brain shows mild degradation of the hippocampus, sulci, and gyri using magnetic resonance imaging technology (Jack et al., 1999; Fleisher et al., 2008). The rate of amnesic MCI conversion to AD is roughly 10–15% per year; however, in some cases MCI individuals can revert to normal (Petersen 2000).

Brain from subjects with MCI, like AD, contains pathological hallmarks of AD, including SP and NFT and also like AD, has elevated indices of oxidative stress (Keller et al., 2005; Butterfield et al., 2006c; 2007a; 2007b; Small et al., 2006). The main component of SP is amyloid beta-peptide (1–42) [A β (1–42)]. Under both *in vitro* and *in vivo* conditions A β (1–42) is known to cause increased oxidative stress (Yatin et al., 1999; Boyd-Kimball et al., 2005; Opii et al., 2008). This observation is consistent with the notion that A β (1–42) plays a causal role in the development and progression of AD (Selkoe 2000). Further, a number of studies suggest that the small oligomers of A β are the actual toxic species of this peptide (Aksenov et al., 2001; Lambert et al., 2001; Drake et al., 2003; Murphy et al., 2007). Many MCI patients showed low levels of A β in cerebrospinal fluid, in contrast to increased levels of A β deposits in the brain (Andreasen et al., 1999; Andreasen and Blennow 2005), suggesting a diminished clearance mechanism of A β from brain. Current research identified a number of proteins that could be potentially involved in the efflux of A β from brain, including low-density lipoprotein-related receptor-1 (LRP1) (Ito et al., 2007; Jaeger and Pietrzik 2008), a protein that is investigated in this current study.

Recent reports have shown that protein synthesis in MCI brain may also be decreased in response to oxidative damage to mRNA, which could lead to decreased levels of important proteins that could be involved in the progression of disease (Ding et al., 2005; Cenini et al., 2007; Sultana et al., 2008). Hence, in hippocampus obtained from MCI and control subjects at a short postmortem interval, we tested the hypothesis that levels of PSD95, a key synaptic protein, and two additional proteins associated with PSD95, NR2A and LRP1, had decreased levels. In addition, we tested the hypothesis that hippocampal levels of Bcl₂ and Caspase-3 were elevated. PSD95 and NR2A are involved in learning and memory via Ca²⁺ influx into neurons and as noted

above LRP1 is involved with clearance from brain of A β (1–42). The data obtained from the current study suggest a possible involvement of these proteins in this prodromal stage of AD.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) with exceptions of nitrocellulose membranes (Bio-Rad, Hercules, CA), electrophoretic transfer system (Trans-blot Semi-dry Transfer Cell; Bio-Rad).

Control and MCI brains

The Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease clinical (UK ADC) provided frozen hippocampus samples from six each of MCI and age-matched controls for the present study. All subjects came from the longitudinally followed normal control group that had annual neuropsychological testing and neurological and physical examinations every 2 years. Control subjects had no cognitive complaints, normal cognitive test scores, especially objective memory test scores, and normal neurological examinations. Patients with amnesic MCI met the criteria described by Petersen (Petersen, 2004), which include the following: A memory complaint corroborated by an informant; objective memory test impairment (age and education adjusted); general normal global intellectual function; Clinical Dementia Rating score of 0.0–0.5 (no dementia); and a clinical evaluation that revealed no other cause for memory decline. The post mortem intervals of the samples used in the present study were extremely short, i.e., 2.9 \pm 0.5 hr for controls and 3.1 \pm 0.4 hr for MCI (Table I).

Sample Preparation

The brain tissues (hippocampus) from control or MCI subjects were homogenized in a lysis buffer (10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 0.6 mM MgSO₄) containing the protease inhibitors [leupeptin (0.5 mg/mL), pepstatin (0.7 mg/mL), trypsin inhibitor (0.5 mg/mL), and PMSF (40 mg/mL)]. Homogenates were centrifuged at 15,800 \times g for 10 min to remove debris. The supernatant was used to determine the total protein concentration by the BCA method (Pierce, Rockford, IL).

Western Blot Analysis

For Western blot analysis, 50 μ g of hippocampus brain sample from controls and MCI were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of the proteins to nitrocellulose membranes at 160 mA/gel for 2 hr. The nitrocellulose membranes were blocked for 1 hr at room temperature in fresh blocking

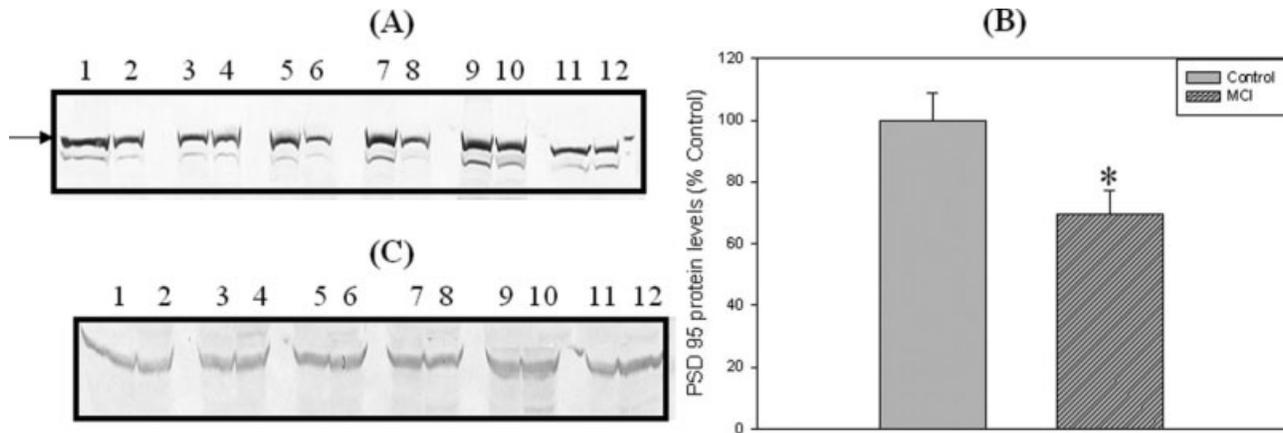


Fig. 1. **A:** A representative Western blot of hippocampal PSD95 protein levels in MCI and age-matched controls. **B:** The bar graph of the PSD95 Western blot results. An arrow indicate the position of PSD 95 on the blot. Control and MCI hippocampal samples were loaded in adjacent lanes. Lanes 1, 3, 5, 7, 9, 11 represent control

samples, and lanes 2, 4, 6, 8, 10, 12 represent MCI samples. See text for methods and further details. Data are shown as Mean \pm S.D. $n=6$, * $p < 0.05$. **C:** actin levels in Control and MCI samples, and analysis shows no significant difference in actin in MCI hippocampus compared to control hippocampus. See text.

buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, pH 7.4, containing 5% BSA). This procedure was then followed by incubation of the membrane with primary antibodies against anti-PSD95 [1:1000] (Cell signaling, Danvers, MA), anti-LRP1 [1:1000] (Santa Cruz Biotech, CA), anti-NR2A [1:1000] (Sigma-Aldrich, St. Louis, MO), anti-BCI2 [1:1000] (Calbiochem, NJ), or anti-caspase-3 [1:1000] (Calbiochem, NJ) prepared fresh in wash blot (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, pH 7.4) with 3% BSA. Following three washes with wash blot, the blots were incubated with secondary antibody conjugated with horse radish peroxidase in wash blot for 1 hr at room temperature. The blots were washed again three times in wash blot, and the bands were visualized by enhanced chemiluminescence (ECL; Amersham, Life Sciences Inc.).

Image Analysis

After immunodetection of proteins of interest, the membranes were completely dried at room temperature and were then scanned using a Microtek Scanmaker 4900 scanner. Images were saved as tiff files and the intensity of the proteins were quantified using scion image analysis software.

Statistical analysis

The results are presented as means \pm SD. Statistical evaluation was performed using a Student's *t*-test. Differences were considered to be significant at $p < 0.05$.

RESULTS

Loss of synapses is one of the key pathological hallmarks of AD. In the current study, we measured the protein levels of some key synaptic proteins in the hippocampal region from subjects with MCI and from age-matched controls. Western blot analyses of hippocampal samples probed with anti-PSD95 showed a

significant ($p < 0.05$) decrease in the levels of this protein in MCI hippocampus compared to the age-matched controls. The PSD 95 blot was stripped with stripping buffer (Sigma-Aldrich, St. Louis, MO) and probed with anti-actin antibody (1:1000) as a loading control (Figure 1C). No significant difference in actin levels was observed between the control and MCI hippocampus. Further, we also measured the levels of post synaptic associated proteins, i.e., NR2A and LRP-1, using anti-NR2A or anti-LRP-1 antibodies, respectively, and both these proteins showed significant decreased levels ($p < 0.05$) in MCI brain compared to control brain (Fig 2 and Fig 3, respectively). Proteins sometimes used as markers of the cell death, such as BCl₂ and caspase-3, showed a significantly increased level ($p < 0.05$) in MCI hippocampus compared to age-matched control (Fig 4 and 5, respectively). Table II shows the percent control of PSD95, NR2A, LRP-1, BCl₂ and caspase-3 protein levels in MCI hippocampus relative to normal control values.

DISCUSSION

In the present study we measured the protein levels of PSD95, LRP-1, NR2A, caspase-3, and Bcl₂ in MCI and control hippocampus mainly for two reasons: First, hippocampus is one of the regions of the brain that is involved in learning and memory and is known to be significantly involved in AD and amnesic MCI; second, these proteins are directly or indirectly involved in learning and memory processes, which are severely affected in amnesic MCI and AD. Synaptic communications are important in the process of learning and memory; however, in AD there is clear evidence of loss of synaptic connections in the neocortex and hippocampus, and this loss strongly correlated with the cognitive

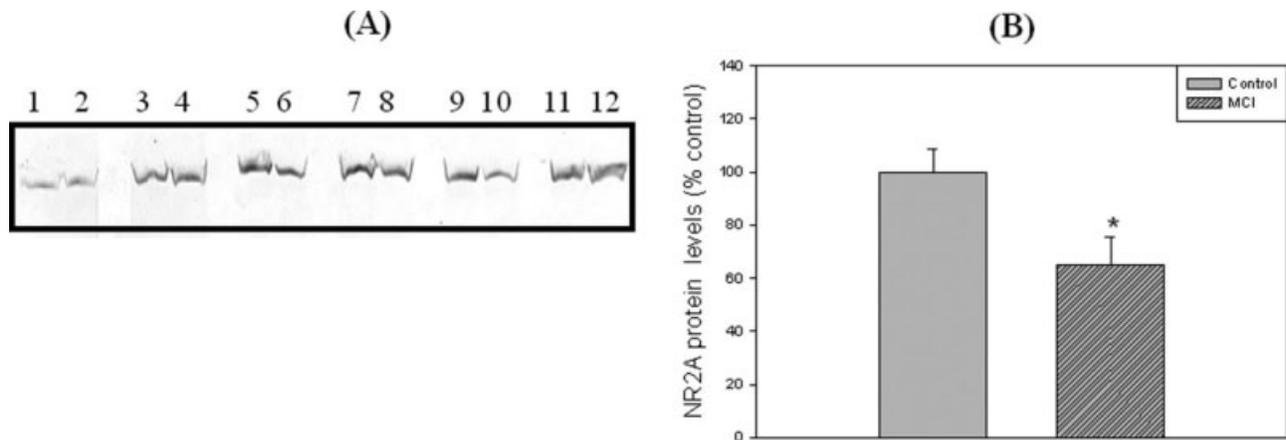


Fig. 2. **A:** a representative Western blot of hippocampal NR2A protein levels in MCI and age-matched controls. **B:** the bar graph of the NR2A Western blot results. Control and MCI hippocampal samples were loaded in adjacent lanes. Lanes 1, 3, 5, 7, 9, 11 represent control samples, and lanes 2, 4, 6, 8, 10, 12 represent MCI samples. See text for details. Data are shown as Mean \pm S.D. n=6, * p < 0.05.

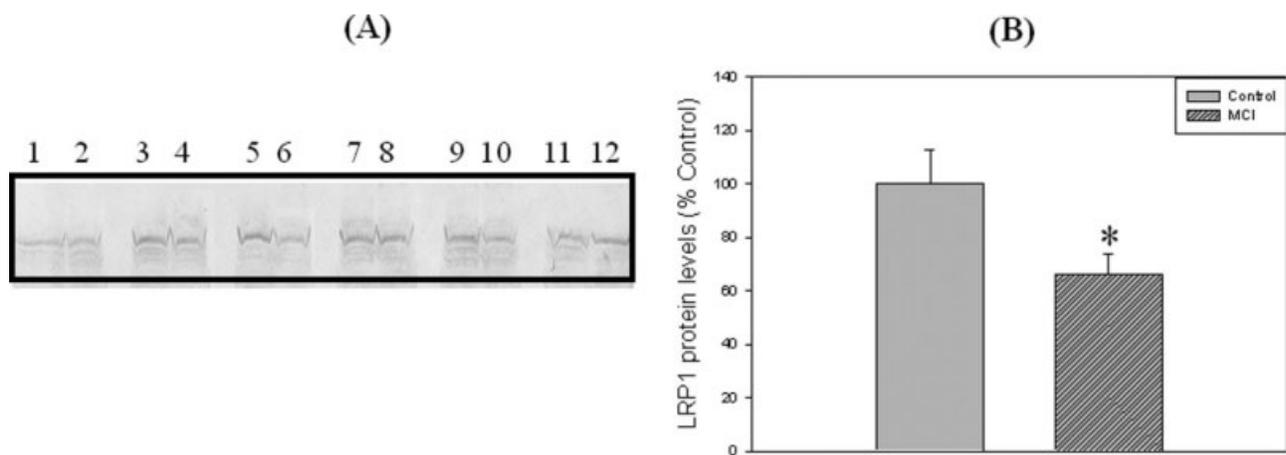


Fig. 3. **A:** is a representative Western blot of hippocampal LRP1 protein levels in MCI and age-matched controls. **B:** represents the histogram of the LRP1 Western blot results. Control and MCI hippocampal samples were loaded in adjacent lanes. Lanes 1, 3, 5, 7, 9, 11 represent control samples, and lanes 2, 4, 6, 8, 10, 12 represents MCI samples. See text for details. Data are shown as Mean \pm S.D. n=6, * p < 0.05.

decline observed in AD patients (Masliah et al., 1994; Scheff and Price 2003). PSD95, NR2A, and LRP1 proteins are localized at synapses, and the maintenance of the levels of these proteins is important for structure and function of brain cells (Niethammer et al., 1996; Martin et al., 2008). PSD95 is a neuronal scaffolding protein that contains several domains, including three PDZ/DHR (PSD-95, Dlg, ZO-1/Dlg-homologous region) domains, an SH3 (Src-homology-3) domain and a guanylate kinase (GK)-homology domain. PSD95 domains participate in protein-protein interactions; for example, among other proteins, these PSD95 domains are reported to bind to the C-terminus of NR2 subunits

(Niethammer et al., 1996) and LRP1 protein (Martin et al., 2008). Hence, PSD95 is important in regulating the functions of other proteins. In the present study, we observed a significant decrease in the level of PSD95 in MCI hippocampus compared to that of control. These results are consistent with the two previous reports of decreased levels of PSD95 in AD brain (Gyllys et al., 2004; Love et al., 2006). In contrast, recently another group reported an increased level of PSD95 in AD (Leuba et al., 2008).

In vivo studies in mice reported decreased PSD95 levels could lead to learning and memory impairments, in addition to locomotive impairment (Migaud et al.,

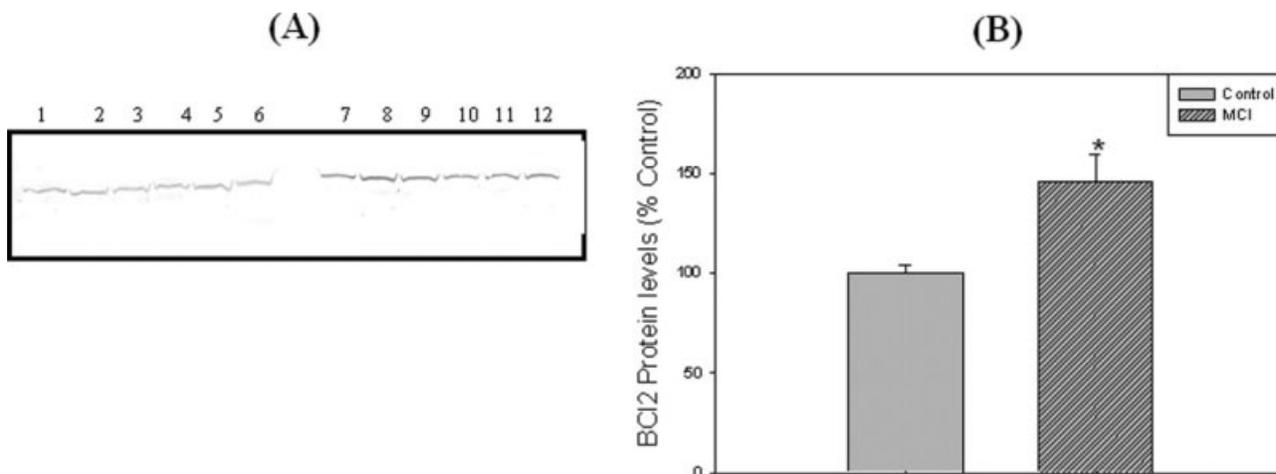


Fig. 4. **A:** represents Western blot of hippocampal Bcl₂ protein levels in MCI and age-matched controls. **B:** represents histogram of Bcl₂ blot. Lanes 1-6 represent control hippocampus, and lanes 7-12 represent MCI hippocampus. See text for details. Data are shown as Mean ± S.D. n=6, * p < 0.05.

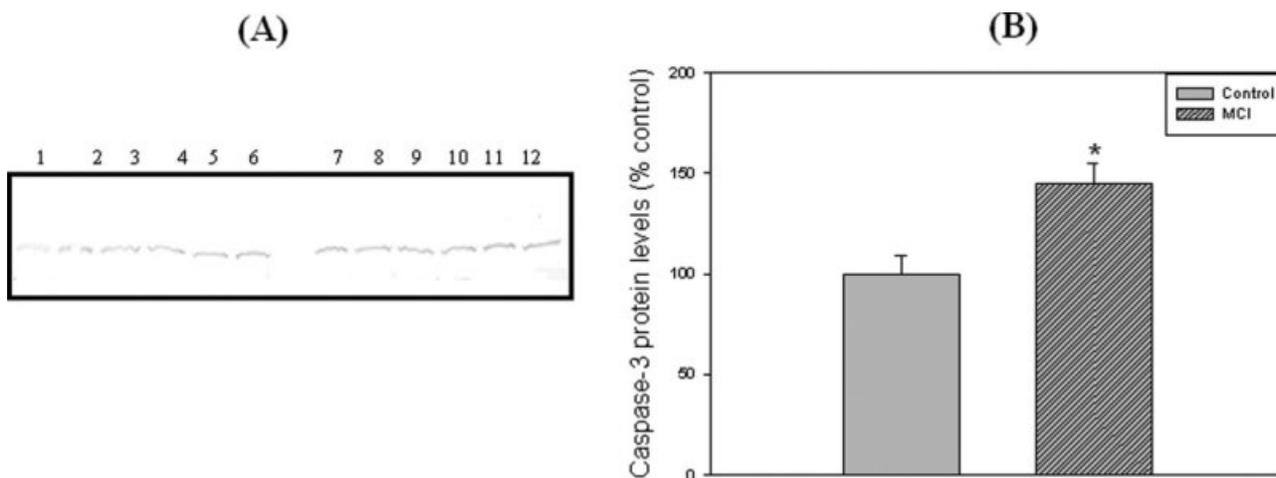


Fig. 5. **A:** Western blot of hippocampal caspase-3 protein levels in MCI and age-matched controls. **B:** the bar graph of the caspase-3 blot. Lanes 1-6 represent control hippocampus, and lanes 7-12 represent MCI hippocampus. See text for details. Data are shown as Mean ± S.D. n=6, * p < 0.05.

1998; Nyffeler et al., 2007). A previous study showed that mice with a mutated form of PSD95, lacking the last PDZ domain, had impaired learning and LTP (Migaud et al., 1998; Mattar et al., 2005).

Learning and memory impairment reported previously can be explained based on the alterations in synaptic signaling possibly through NMDA receptors or through interaction with dopamine receptors or perhaps by altering the interplay of glutamatergic and dopaminergic axons on postsynaptic dendritic spines. NMDA receptors and PSD-95 co-cluster in cotransfected cells (Kim et al., 1996). Furthermore, PSD95 and NMDA receptors were reported to be co-localized in primary neurons (Kornau et al., 1995). The molecules involved

in regulation of the receptor function appear to be recruited to the proximity of the receptor at synapses by PSD95 (Shiraishi et al., 2003).

TABLE II. PSD95, NR2A, LRP-1, Bcl₂ and Caspase-3 Protein Levels Obtained from Both Control and MCI Hippocampus are Shown as Percent Control (The Latter Taken to be 100%)

Proteins	Controls (Mean ± S.D)	MCI (Mean ± S.D)
PSD95	100 ± 21.3	69.55 ± 19.2
NR2A	100 ± 19.3	64.9 ± 24.1
LRP1	100 ± 31.5	66.2 ± 25.0
Bcl ₂	100 ± 10.3	155 ± 33.1
Caspase-3	100 ± 21.7	145 ± 24.2

In the present study, we also found that the levels of NR2A are decreased in amnesic MCI hippocampus compared to that of control. As mentioned previously, PSD95 interacts with the NR2A subunit of the NMDA receptor, a heteromeric complex that is composed of the NR1 subunit combined with various types of NR2 subunits, *e.g.*, NR2A, NR2B, NR2C, and NR2D. Consistent with the interaction of PSD95 with NR2A, an *in vitro* study in fibroblasts showed that PSD-95 may be required for the localization of NMDA receptors to synapses (Buller and Monaghan 1997; Hoe et al., 2006).

The decreased levels of NR2A protein together with decreased levels of PSD95 could possibly make the NMDA receptor less functional with consequent reduced LTP associated with the NMDA receptors. Previous studies showed conflicting results regarding the levels of these various NMDA receptors in AD brain, and the binding sites for NMDA receptors have been shown to be either reduced or stable (Scheuer et al., 1996; Sze et al., 2001; Mishizen-Eberz et al., 2004). The mRNA levels are decreased in the AD brain (Hynd et al., 2003). One study showed reduction of NR1/NR2B receptor expression levels with the progression of AD (Mishizen-Eberz et al., 2004). An *in vivo* study showed reduced number of cell surface NMDA receptors in mice carrying the familial Swedish APP mutation, suggesting the involvement of A β (Snyder et al., 2005). Based on the above-mentioned reasons, we are tempted to speculate that the decreased levels of NR2A found in brain of subjects with amnesic MCI in the current study could be due to reduced levels of PSD95. This suggestion could result in keeping NR2A at the cell surface, which could lead to activation of the NMDA channel, with consequent elevated intracellular Ca²⁺ and increased oxidative stress and excitotoxicity. Other explanations for lower levels of NR2A in MCI brain could include truncation of the C-terminus of NR2A with consequent lack of recognition of this subunit by the anti-NR2A antibody (Sultana and Babu, 2003). Some findings demonstrate reduce synaptic plasticity and glutamatergic transmission is associated with the non-toxic amounts of secreted A β peptide. However, the exact role of NMDA receptor activation in AD pathogenesis is still not fully elucidated.

Low-density lipoprotein receptor-related protein 1 (LRP1) is a large, endocytic receptor that mediates the cellular uptake of a great variety of ligands, among which are ApoE-containing lipoproteins, lipoprotein lipase, complexes of proteinases-proteinase inhibitors, and hormones and growth factors such as insulin and platelet-derived growth factor (PDGF) (Croy et al., 2004; May et al., 2005; Martin et al., 2008). LRP1 is also involved in phagocytosis and signaling pathways in addition to many other functions that are still to be discovered (Zilberberg et al., 2004). LRP1 is present both on glial and neuronal cells, and an *in vitro* study suggests involvement of LRP1 in different aspects of neuronal

metabolism (May et al., 2004). Further, LRP1 has been shown to interact with amyloid precursor protein (APP) and also is involved in the regulation of proteolytic processing and the production of the A β peptide (Goto and Tanzi 2002; Shi et al., 2006). In addition, LRP1 has been reported to be as one of the mechanism(s) of A β clearance from brain, and down-regulation of LRP1 has been reported in human AD brain and endothelial cells (Pritchard et al., 2005).

The down regulation of LRP1 in brain of subjects with amnesic MCI reported here could lead to accumulation of A β in the brain resulting in increased SP formation and eventually to the progression to AD pathology. Further, the increased levels of A β could also increase the degradation of LRP1 by enhancing the proteasome degradation of LRP1. Since LRP1 is involved in APP processing and the clearance of A β , decreased levels of LRP1, as observed in MCI brain in the current study compared to the control, suggest that LRP1 may be involved in the development of SP in AD and other secondary consequences (Lovell et al., 2001; Hardy and Selkoe 2002; Butterfield et al., 2006a; Wenk 2006; Aisen 2008). LRP1 and the NMDA receptor have been assumed from immunohistochemical and co-immunoprecipitation experiments to interact at synapses (Martin et al., 2008). Moreover, LRP1 has been found to regulate calcium influx into neurons after stimulation with the glutamate receptor agonist NMDA (Martin et al., 2008). Since LRP1 has been shown to interact with the postsynaptic density protein PSD-95 and NMDA receptor either directly or indirectly, alteration in the levels of all these three proteins may be able to modulate the conductance of Ca²⁺ through NMDA channel. Hence, decreased levels of these proteins observed in the present study could have consequent detrimental physiological and pathological effects in MCI and could be related to synapse and memory loss, A β (1-42) accumulation, and neurodegeneration.

Brains from subjects with MCI show morphological changes that are indicative of cell loss; however, to the present the exact mechanism(s) of cell loss is not clear, but a role of oxidative stress and altered levels of calcium homeostasis have been proposed (Mattson et al., 1993; Mattson and Chan, 2001). One of the proteins that is involved in calcium homeostasis is Bcl₂, a member of the Bcl₂ family that plays an important role as an anti-apoptotic and cell survival protein. Alterations in the levels of Bcl₂ together with NMDA receptors could lead to altered calcium homeostasis and consequently to activation of apoptotic proteins like caspases-3. Previous studies showed an upregulation of Bcl₂ protein levels in the hippocampus and entorhinal cortex of AD brains (Satou et al., 1995; Su et al., 1996). Consistent with and confirming our previous finding of the increased levels of Bcl₂ in the inferior parietal lobule of subjects with amnesic MCI (Bader Lange et al., 2008), we observed an increased level of Bcl₂ in hippocampus of amnesic MCI subjects. Further, we also observed the upregulation of caspase-3 in MCI subjects, suggesting that

apoptosis might be initiated in MCI hippocampus. The upregulation of the Bcl₂ conceivably can be considered as a cellular compensatory mechanism to protect against cell loss.

In conclusion, the hippocampus is one of the areas of the brain that was reported previously to show pronounced neuronal and synaptic loss in AD. Our current findings, suggesting decreased levels of PSD95, NR2A, and LRP-1, with elevated levels of caspase-3 and Bcl₂ proteins, may reflect or contribute to neuronal and synaptic loss in the MCI hippocampus. Since PSD95 has been reported as an intracellular linker between LRP1 and the NMDA receptor and all of these proteins are localized at synapses, their decreased levels may alter neuronal functions, consequently leading to the synaptic loss and neurodegeneration. Further molecular dissection of these proteins may be helpful in unraveling the mechanism of conversion of MCI to AD, and may help in finding a therapeutic approach to delay or prevent this devastating disease.

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