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Regional selectivity of rab5 and rab7 protein up regulation in mild cognitive impairment and Alzheimer's disease

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Abstract

Endocytic alterations are one of the earliest changes to occur in Alzheimer's disease (AD), and are hypothesized to be involved in the selective vulnerability of specific neuronal populations during the progression of AD. Previous microarray and real-time quantitative PCR (qPCR) experiments revealed an up regulation of the early endosomal effector rab5 and the late endosome constituent rab7 in the hippocampus of people with mild cognitive impairment (MCI) and AD. To assess whether these select rab GTPase gene expression changes are reflected in protein levels within selectively vulnerable brain regions (basal forebrain, frontal cortex, and hippocampus) and relatively spared areas (cerebellum and striatum), we performed immunoblot analysis using antibodies directed against rab5 and rab7 on postmortem human brain tissue harvested from cases with a premortem clinical diagnosis of no cognitive impairment (NCI), MCI and AD. Results indicate selective up regulation of both rab5 and rab7 levels within basal forebrain, frontal cortex, and hippocampus in MCI and AD, which also correlated with Braak staging. In contrast, no differences in protein levels were found in the less vulnerable cerebellum and striatum. These regional immunoblot assays are consistent with single cell gene expression data, and provide protein-based evidence for endosomal markers contributing to the vulnerability of cell types within selective brain regions during the progression of AD.

Keywords

basal forebrain; cerebellum; endosome; hippocampus; frontal cortex; mild cognitive impairment; rab GTPase; selective vulnerability; striatum

Introduction

In neurons, the endosomal-lysosomal pathway performs a multiplicity of integral functions including internalizing nutrients and neurotrophic factors, degrading and recycling receptors, and integrating signaling information to relevant intracellular pathways [1-3]. Endocytosis enables neurons to modify or degrade molecules from the cell surface into intracellular compartments. A family of small ras-related GTPase (rab) proteins highly regulate trafficking of vesicles from early to late endosomes and other organelles along endosomal-lysosomal pathways [4-8]. Endosomes also play a critical role in neuronal development and homeostasis and normal synaptic transmission [9-12], as well as neuronal dysfunction during the progression of Alzheimer's disease (AD) [13-15]. In addition, signaling endosomes contain rab GTPases and neurotrophin receptor signaling complexes, which are responsible for growth factor signal transduction from synaptic sites to the nucleus [10,16,17]. Specific rab GTPases, including the early endosome effector rab5 and late endosome constituent rab7, have been implicated in the regulation of nerve growth factor (NGF) signaling *in vitro* [16-19], and we have demonstrated that up regulation of rab5 down regulates the brain-derived neurotrophic receptor (BDNF) receptor TrkB [20].

Endosomal-lysosomal system dysfunction is one of the earliest disturbances observed in AD [2,15,21], and may be one of the fundamental mechanisms underlying neurodegenerative changes during the progression of AD. Increases in rab5, an effector molecule that promotes early endosome fusion, a positive mediator of endocytosis, regulates early endosomal enlargement [6,17]. Enlargement of rab5-positive endosomes is a pathological feature that precedes cerebral and vascular amyloid-beta peptide (A β) deposition, neurofibrillary tangle (NFT) formation, and is selective for AD [21-23]. Many vulnerable cell types within the forebrain demonstrate enlarged endosomes and increased rab5 immunoreactivity in human AD as well as in animal models of AD that display endosomal disturbances [21,22,24]. rab5 overexpression affects several vulnerable cellular phenotypes including cholinergic basal forebrain neurons, hippocampal pyramidal neurons, and neocortical pyramidal neurons [2,21,24-26]. Up regulation of rab5 also reproduces key aspects of the early endosomal phenotype found in AD, and may have downstream effects in other compartments including late endosomes [27,28].

Microarray analysis has demonstrated significant up regulation of select rab GTPases within vulnerable CA1 hippocampal pyramidal neurons harvested from people who died with a clinical diagnosis of mild cognitive impairment (MCI) and AD, including *rab5* and the late endosome constituent *rab7* [20]. Notably, up regulation of *rab5* and *rab7* in CA1 neurons also correlates with cognitive decline in the same cohort used for microarray analysis [20]. Regional real-time quantitative PCR (qPCR) analysis and immunoblot analysis demonstrated up regulation of both rab5 and rab7 in the hippocampus [20], further indicating that early and late endosome dysfunction occurs in one of the most pathologically vulnerable forebrain regions affected in MCI and AD [29,30].

Up regulation of *rab5* and *rab7* expression within selectively vulnerable hippocampal neurons occurs during the progression of AD [20], suggesting that dysregulation of a select rab GTPase phenotype is a molecular pathogenic marker for neuronal dysfunction in other highly vulnerable regions of the brain early in the disease process. Therefore, we hypothesize that regions with neuronal cell types vulnerable to AD neurodegeneration will display select rab GTPase up regulation, whereas relatively spared regions will show little or no rab5 and/or rab7 dysregulation. To this end, a survey of several vulnerable regions (including basal forebrain, frontal cortex, and hippocampus) and relatively spared regions (including cerebellum and striatum) was performed via immunoblot analysis for rab5 and rab7 to assess whether differential expression of these two rab GTPases is a selective event

in vulnerable regions in the MCI and/or AD brain, or conversely, that up regulation of these discrete endosomal markers is a global event during AD progression.

Materials and Methods

Brain tissue collection

This study was performed under the auspices of IRB guidelines administrated by the Nathan Kline Institute/New York University Langone Medical Center. Immunoblot analysis using antibodies directed against rab5 and rab7 was performed using brain samples obtained from a total of 82 postmortem human subjects. Cases were clinically categorized premortem with no cognitive impairment (NCI; $n = 27$), MCI insufficient to meet criteria for dementia ($n = 17$), and AD ($n = 27$). The MCI population was defined as persons with impaired cognitive testing who were not found to have frank dementia by a neurologist [31,32], commensurate with the current consensus criteria for the clinical classification of MCI [33,34]. Only cases with age at death > 65 years and postmortem interval (PMI) ≤ 36 hours were included in the study. Frozen brain tissues were obtained from the Rush Religious Orders Study ($n = 45$; <http://www.rush.edu/rumc/page-R12394.html>), the University of Pennsylvania Brain Bank ($n = 24$; Center for Neurodegenerative Disease Research; <http://www.pennadc.org/>), the Harvard Brain Bank ($n = 10$; Harvard Brain Tissue Resource Center; <http://www.brainbank.mclean.org/>) and the Emory Brain Bank ($n = 3$; Center for Neurodegenerative Disease; <http://neurology.emory.edu/ENNCF/neuropathology/resources.php>). Samples from each case were collected from the substantia innominata of the basal forebrain, cerebellum, frontal cortex {Brodmann area (BA) BA9 and BA10}, hippocampus, and striatum. However, tissue was not always available for each case (see Table I). Demographic information of the 82 cases is presented in Table II. Antemortem cognitive assessments collected within one year prior to death using the Mini Mental State Exam (MMSE) were available for 44 participants of the Religious Orders Study [35,36], 15 people from the University of Pennsylvania Brain Bank, and 1 case from the Emory Brain Bank. Exclusion criteria included argyrophilic grain disease, frontotemporal dementia, Lewy body disease, mixed dementias, Parkinson's disease, and stroke. A board certified neuropathologist blinded to the clinical diagnosis performed a neuropathological diagnosis. Neuropathological designations were based on established criteria [37-39]. Tissue handling practices across brains banks are similar, and immunoblotting findings using frozen brain specimens from these repositories have been reported previously [20,40]. Brain samples were stored at -80°C until processed for immunoblot analysis.

Immunoblot analysis

Frozen regional brain samples were homogenized in a 20 mM Tris-HCl (pH 7.4) buffer containing 10% (w/v) sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis (β -aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA), 2 mg/ml of the following: (aprotinin, leupeptin, and chymostatin), 1 mg/ml of the following: {pepstatin A, antipain, benzamidin, and phenylmethylsulfonyl fluoride (PMSF)}, 100 $\mu\text{g/ml}$ of the following: {soybean trypsin inhibitor, N α -p-tosyl-L-lysine chloromethyl ketone (TLCK), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)}, 1 mM of the following: (sodium fluoride and sodium orthovanadate) and centrifuged as described previously [40-42]. All protease inhibitors were purchased from Sigma (St. Louis, MO). Identical amounts of homogenates (10 μg) were loaded into a gel electrophoresis apparatus, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4-15% gradient acrylamide gels; Bio-Rad, Hercules, CA), and transferred to nitrocellulose by electroblotting (Mini Transblot, Bio-Rad). Nitrocellulose membranes were blocked in blocking buffer (LiCor, Lincoln, NE) for 1 hour at 4°C prior to being incubated with antibodies directed

against rab5 (rab5A; rabbit polyclonal sc-309; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000 dilution), rab7 (rabbit polyclonal sc-10767; Santa Cruz Biotechnology 1:1,000 dilution), or β -tubulin (TUBB; monoclonal antibody T5293; Sigma, 1:1,000 dilution) in blocking buffer overnight at 4 °C. Specificity for these well-characterized antibodies has been demonstrated previously by the manufacturer {rab5 (<http://datasheets.scbt.com/sc-309.pdf>), rab7 (<http://datasheets.scbt.com/sc-10767.pdf>), TUBB (<http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Datasheet/t5293dat.Par.0001.File.tmp/t5293dat.pdf>)} and within individual research reports [43-46]. Moreover, rab5 [21,28,43], rab7 [28,47], and TUBB [20,40,48] antibodies have been used previously by our group and independent laboratories for human brain analyses. Membranes were developed using affinity-purified secondary antibodies conjugated to IRDye 800 (Rockland, Gilbertsville, PA) and visualized using an infrared detection system (Odyssey, LiCor) [41]. Individual samples were assayed 2-4 times per antibody.

Data analysis

Immunoblots were quantified by densitometric software supplied with the instrument. Signal intensity of immunoreactive bands was normalized to TUBB immunoreactivity for each assay as described previously [40-42]. TUBB expression has been well established as a suitable protein for normalization in AD by numerous independent research groups. However, this may not be absolute, as microtubules have been shown to decrease in AD and aging [49]. Alterations in immunoreactive band intensity as well as demographic and clinical/neuropathological variables (age, gender, educational level, MMSE, and Braak scores) were compared across clinical diagnostic groups using Kruskal-Wallis test or Fisher's exact test with Bonferroni correction for pairwise comparisons [48,50,51], as appropriate. The association between rab5 and rab7 levels and Braak scores were assessed by Spearman rank correlation, as were the correlation of rab5 or rab7 levels between and within brain regions [40,52]. The level of statistical significance was set at ($p < 0.05$).

Results

rab5 immunoblot analysis

Quantitative immunoblotting was performed using homogenates prepared from brain tissue obtained from the basal forebrain, cerebellum, frontal cortex, hippocampus, and striatum from NCI, MCI, and AD cases using a well-characterized rab5 antibody. Immunoblotting with the rab5 antibody identified an ~27 kilodalton (kDa) band, consistent with our previous evaluation of this antibody [20]. Quantitative analysis of rab5 protein levels (normalized to TUBB) demonstrated regionally selective differential regulation. Specifically, rab5 was significantly up regulated in the frontal cortex ($p < 0.0001$) and hippocampus ($p < 0.0001$) in MCI and AD (Fig. 1), indicating a prodromal increase of this early endosome effector during the progression of dementia (Table III). rab5 protein levels were up regulated in MCI and further up regulated in the AD basal forebrain ($p < 0.003$); only the comparison between NCI and AD reached statistical significance, in part due to the relatively small number of basal forebrain MCI samples. In contrast, no differential regulation of rab5 levels was observed in either the cerebellum or striatum (Table III). In addition, increased levels of rab5 in the basal forebrain, frontal cortex, and hippocampus correlated with Braak staging ($r = 0.56$; $p < 0.001$), suggesting an association between rab5 levels and the development of NFT pathology as well as cognitive decline during the progression of AD.

rab7 immunoblot analysis

Immunoblot analysis of regional tissue homogenates using a well-characterized rab7 antibody identified an ~25 kDa band that demonstrated regionally selective differential

regulation similar to that seen for rab5, though slightly less in magnitude. Specifically, significant up regulation of rab7 was observed in the frontal cortex ($p < 0.0001$) and hippocampus ($p < 0.003$) in MCI and AD (Fig. 1), demonstrating increased expression of this late endosome constituent during the progression of dementia (Table IV). As with the rab5 immunoblot analysis, rab7 expression was also elevated in MCI and further up regulated in the AD basal forebrain, although this comparison did not reach statistical significance. rab7 protein levels were stable within the cerebellum and striatum across all three groups examined. Up regulation of rab7 protein levels also correlated with Braak stage in the frontal cortex ($r = 0.55$; $p < 0.001$). Unlike rab5 protein levels, only a weak correlation was found between rab7 protein levels and Braak stage in the basal forebrain and hippocampus.

Coordinate protein level analysis within and between the five brain regions

Within each of the five brain regions, levels of rab5 and rab7 were most strongly correlated in the frontal cortex and hippocampus ($r = 0.59$, $r = 0.68$; $p < 0.001$). On the other hand, correlation analysis between regions showed that, despite the small sample sizes, levels of rab5 in hippocampus displayed a strong correlation with levels in the basal forebrain ($n = 6$ with measures of rab5 available in both regions, $r = 0.94$; $p < 0.05$) and frontal cortex ($n = 11$, $r = 0.90$; $p < 0.0002$). We also found a correlation between rab5 levels in basal forebrain and frontal cortex ($n = 15$, $r = 0.62$; $p < 0.01$) as well as in cerebellum and striatum ($n = 13$, $r = 0.66$; $p < 0.01$). These correlative findings are consistent with the observed select up regulation of rab5 in the highly vulnerable basal forebrain, frontal cortex, and hippocampus and lack of rab5 regulation in the spared cerebellum and striatum in the early stages of dementia. In contrast, no significant coordinate expression of rab7 levels were observed between regions.

Discussion

Molecular and cellular evidence exists for endosomal abnormalities in AD, relevant animal models of neurodegeneration, and *in vitro* [11,15,17,19,²¹,24,28,53,54]. Notably, expression levels of genes regulating early endosomes (including *rab5*) and late endosomes (including *rab7*) were selectively up regulated in homogeneous populations of CA1 neurons from individuals with a clinical diagnosis of MCI and AD [20]. Importantly, levels of these genes were selectively increased among individuals with cognitive decline [20]. Based on these expression findings, a regional assessment of protein levels for select rab GTPases was performed using postmortem brains samples from regions that are selectively vulnerable to AD pathology compared to areas relatively spared by the disease. The present results indicate regionally selective up regulation of both rab5 and rab7, which correlate with cognitive decline and Braak NFT staging. Notably, we found that distinct rab GTPase protein levels are increased in regions of the neocortex, limbic system and basal forebrain that show a predilection for the early development of AD pathology [37,55-57]. These findings underscore the importance of evaluating changes in early and late endosomal pathways during the progression of AD in human postmortem brain tissues as well as within appropriate animal and cellular models.

Early endosomes receive their contents via endocytosis and designate specific cargo for vesicular transport to late endosomes en route to lysosomes, deliver certain cargoes to the Golgi apparatus via the retromer, or recycle elements back to the plasma membrane [10,58]. From early endosomes, materials are transported to endosome carrier vesicles or multivesicular bodies, which are responsible for transferring cargo from early endosomes to late endosomes [1,59]. Late endosomes obtain enzymes for degradation, including acid hydrolases such as the cathepsin family of proteases, from the trans Golgi network or through fusion with lysosomes [60,61]. Intracellular vesicular trafficking between

compartments is regulated via specific rab GTPases [6,8]. Specifically, rab GTPases contribute to vesicle formation, motility, docking, and fusion, and are considered regulatory switches of protein trafficking, transport, and degradation [4]. Distinct rab GTPases are associated with discrete organelles and/or compartments where they are functionally active. For example, rab5 regulates early endosome uptake and fusion, whereas rab7 mediates the fusion of late endosomes. Both rab5 and rab7 were differentially regulated as assessed via microarray, qPCR, and immunoblotting approaches in CA1 neurons [20], and by regional immunoblot analysis herein. Other rab GTPases, including *rab3* (synaptic localization), *rab4* (early endosome localization), and *rab24* (presumed trafficking compartment localization) also display differential regulation in AD [20], and await further regional assessment.

Endosomes are integral mediators of metabolism and cellular communication through trafficking and signaling functions, and are linked to a variety of pathways implicated in the pathogenesis of AD, including amyloidogenic amyloid- β precursor protein (APP) processing and neurotrophin signaling [10,11,28,54,62,63]. Early endosomes are the first major sorting station on the endosomal-lysosomal pathway and the site of internalization and initial processing of proteins relevant to AD pathogenesis including APP, apolipoprotein E (ApoE), low-density lipoprotein, and low-density lipoprotein receptor-related protein, among others [15,64]. Moreover, early endosomes contain APP secretases and/or secretase activities, demonstrating a relationship between endosomal-lysosomal pathway activity, APP processing, A β generation, and β -carboxyl-terminal fragment (β CTF) production [27,65-67]. The morphological appearance of abnormal endosomes coincides with A β accumulation within neuronal endocytic compartments [26,68]. Overexpression of rab5 *in vitro* increases A β and β CTF production [27,69]. Recent studies implicate APP and β CTF, and exclude A β and α CTF, as the cause of endocytic pathway dysfunction in AD and Down syndrome (DS) [54]. Specifically, endosome defects in DS fibroblasts depend on the overexpression of APP and β CTF production. Knockdown of either APP or the β -secretase BACE1 via RNA interference restored both normal endosome function and morphology. Conversely, overexpressing APP or β CTF in normal human fibroblasts induced endosome pathology [54]. APP binds to a protein complex which includes rab5 [70]. Moreover, rab5 is markedly up regulated in DS fibroblasts, and altering rab5 expression creates a similar endosomal phenotype as manipulating β CTFs [28]. Further characterization of the role(s) of rab5 (and rab7) on APP processing and A β regulation, as well as understanding the endosome/neurotrophic signaling axis, is crucial to provide mechanistic data to aid in understanding the pathobiology of selectively vulnerability of basal forebrain, cortical, and hippocampal neurons during the progression of AD.

Our findings of increased rab5 and rab7 expression in MCI and AD support the concept that endocytic pathway abnormalities early in AD onset and during the progression of AD reflect an over activation of the endocytic pathway. The present data describing up regulation of select rab GTPases in selectively vulnerable brain regions to the disease process illustrates the importance of the endosomal pathway in the pathogenesis of AD and lends support to emerging genetic evidence implicating a growing number of genes which influence endocytosis as putative risk factors for AD [71-74]. In summary, up regulation of rab5 and rab7 protein levels was found within the basal forebrain, frontal cortex, and hippocampus across the progression of dementia, whereas no differences in expression levels were found in the less vulnerable cerebellum and striatum, confirming our gene expression findings in CA1 neurons [20], and further arguing that these endosomal markers contribute to the vulnerability of specific regions and cell types during the onset of the AD. The role that rab GTPases play in the development of the major pathological hallmarks of AD (including NFTs and amyloid plaques) continues to be an exciting area of future basic and translational research.

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Figure 1.

Representative immunoblots illustrating rab5 (left panel) and rab7 (right panel) protein levels in tissue homogenates derived from the cerebellum, frontal cortex, and hippocampus during the progression of AD. Up regulation of rab5 and rab7 was observed in frontal cortex and hippocampus in MCI and AD as compared to NCI, whereas no differences were observed in cerebellum. TUBB is depicted as a loading control for the frontal cortex homogenates.

Table 1

Summary of tissue samples obtained from each source

	ROS	UPenn	Harvard	Emory	Total
Basal forebrain	5	12	6	3	26
Cerebellum	16	8	0	0	24
Frontal cortex	27	16	4	0	47
Hippocampus	20	11	6	0	37
Striatum	11	7	5	0	23

Abbreviations: ROS, Religious Orders Study; UPenn, University of Pennsylvania

Table II

Clinical and neuropathologic demographics.

		Clinical Diagnosis		
		NCI (n = 27)	MCI (n = 17)	AD (n = 38)
Age at death (years), mean $\pm$ SD (range)		80.8 $\pm$ 6.8 (69-92)	86.5 $\pm$ 5.9 (79-97)	84.6 $\pm$ 5.8 (67-97)
Number (%) of males		15 (56%)	7 (41%)	14 (37%)
Educational level (years), mean $\pm$ SD (range) *		17.8 $\pm$ 3.9 (8-25)	19.0 $\pm$ 2.3 (15-23)	16.4 $\pm$ 3.4 (11-24)
MMSE, mean $\pm$ SD (range) *		27.6 $\pm$ 1.2 (25-29)	26.5 $\pm$ 2.9 (20-30)	12.0 $\pm$ 7.8 (0-24)
Brain weight (grams), mean $\pm$ SD (range) *		1258 $\pm$ 156 (1000-1625)	1246 $\pm$ 222 (990-1600)	1149 $\pm$ 154 (815-1460)
Distribution of Braak scores *	0	1	0	0
	I/II	11	2	0
	III/IV	13	12	4
	V/VI	1	1	31
Brain Bank	ROS	17	15	13
	UPenn	9	0	15
	Harvard	1	2	7
	Emory	0	0	3

* Education level was not available for 8 NCI, 2 MCI, and 11 AD cases. MMSE was not available for 9 NCI, 2 MCI, and 11 AD cases. Brain weight was not available for 2 MCI and 1 AD cases. Braak score was not available for 1 NCI, 2 MCI, and 3 AD cases.

Abbreviations: SD, standard deviation; ROS, Religious Orders Study; UPenn, University of Pennsylvania

rab5 levels (normalized to TUBB; mean $\pm$ standard deviation) in the five studied regions, by clinical diagnostic group.

Table III

Region	Clinical Diagnosis			Comparison by clinical diagnosis ^a	Pair-wise comparison ^a
	NCI	MCI	AD		
Basal forebrain	0.76 $\pm$ 0.15 (n = 8)	0.97 $\pm$ 0.19 (n = 4)	1.12 $\pm$ 0.18 (n = 11)	p < 0.003	NCI < AD ^b
Cerebellum	0.95 $\pm$ 0.09 (n = 8)	0.99 $\pm$ 0.12 (n = 5)	0.96 $\pm$ 0.06 (n = 9)	p = 0.9	-
Frontal cortex	0.81 $\pm$ 0.12 (n = 16)	1.11 $\pm$ 0.15 (n = 11)	1.19 $\pm$ 0.19 (n = 19)	p < 0.0001	NCI < (MCI, AD)
Hippocampus	1.01 $\pm$ 0.08 (n = 13)	1.14 $\pm$ 0.10 (n = 8)	1.23 $\pm$ 0.13 (n = 16)	p < 0.0001	NCI < (MCI, AD)
Striatum	0.98 $\pm$ 0.05 (n = 7)	0.98 $\pm$ 0.12 (n = 5)	1.02 $\pm$ 0.17 (n = 11)	p = 0.9	-

Not all cases had tissue available in all regions.

^aKruskal-Wallis test with Bonferroni correction for pair-wise comparisons.

^bThe comparison between NCI and MCI did not reach statistical significance, possibly due to the small number of MCI cases.

rab5 levels (normalized to TUBB; mean $\pm$ standard deviation) in the five studied regions, by clinical diagnostic group.

Table IV

Region	Clinical Diagnosis			Comparison by clinical diagnosis ^a	Pair-wise comparison ^a
	NCI	MCI	AD		
Basal forebrain	0.94 $\pm$ 0.14 (n = 6)	0.97 $\pm$ 0.19 (n = 4)	1.07 $\pm$ 0.14 (n = 14)	p < 0.2	-
Cerebellum	0.92 $\pm$ 0.07 (n = 9)	0.99 $\pm$ 0.12 (n = 5)	0.94 $\pm$ 0.08 (n = 8)	p = 0.7	-
Frontal cortex	0.92 $\pm$ 0.09 (n = 17)	1.11 $\pm$ 0.15 (n = 11)	1.16 $\pm$ 0.18 (n = 19)	p < 0.0001	NCI < (MCI, AD)
Hippocampus	0.99 $\pm$ 0.09 (n = 13)	1.14 $\pm$ 0.10 (n = 8)	1.13 $\pm$ 0.10 (n = 16)	p < 0.003	NCI < (MCI, AD)
Striatum	0.96 $\pm$ 0.07 (n = 7)	0.98 $\pm$ 0.12 (n = 5)	0.97 $\pm$ 0.07 (n = 11)	p = 0.6	-

^a Kruskal-Wallis test with Bonferroni correction for pair-wise comparisons.