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ORIGINAL PAPER

Reaching Out to Send a Message: Proteins Associated with Neurite Outgrowth and Neurotransmission are Altered with Age in the Long-Lived Naked Mole-Rat

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Abstract Aging is the greatest risk factor for developing neurodegenerative diseases, which are associated with diminished neurotransmission as well as neuronal structure and function. However, several traits seemingly evolved to avert or delay age-related deterioration in the brain of the longest-lived rodent, the naked mole-rat (NMR). The NMR remarkably also exhibits negligible senescence, maintaining an extended healthspan for ~75 % of its life span. Using a proteomic approach, statistically significant changes with age in expression and/or phosphorylation levels of proteins associated with neurite outgrowth and neurotransmission were identified in the brain of the NMR and include: cofilin-1; collapsin response mediator protein 2; actin depolymerizing factor; spectrin alpha chain; septin-7; syntaxin-binding protein 1; synapsin-2 isoform IIB; and dynamin 1. We hypothesize that such changes may contribute to the extended lifespan and healthspan of the NMR.

Keywords Naked mole-rat · Proteomics · Phosphoproteomics · Aging · Neurite outgrowth · Neurotransmission

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Introduction

The naked mole-rat (NMR) is a subterranean rodent indigenous to the sub-Saharan region of North East Africa. Living in large, eusocial colonies in an underground and thermally stable ecological niche, NMRs have evolved several remarkable traits that have made this rodent an intriguing animal research model to investigate, some of which include: extremely long lifespan [9], sustained heathspan [18], negligible senescence [8], resistance to cancer [34, 49], protein stability [45], high tolerance to hypoxia [32] and oxidative stress [1], tightly regulated metabolism [28], and efficient removal of cellular detritus [64], among others [20, 33, 43, 48]. This current study focuses on brain proteins and underlying mechanisms that may promote neurite outgrowth and neurotransmission in the NMR with age.

Mitochondrial dysfunction and resultant decreased ATP production is implicated in various neuronal degenerative diseases and leads to decreased neuroplasticity and neurite outgrowth [13]. Specifically, in Alzheimer disease (AD), up to 50 % of synapses, which are associated with learning and memory, are lost throughout the brain [38]. Further in AD, more than 50 % of neurons can become degenerative, correlating to disease duration and severity [22]. Uncovering proteins and related mechanism that prevent such catastrophic neuronal loss may conceivably identify

potential therapeutic targets to halt or even ameliorate neurodegenerative damage.

Ways of making new synaptic connections in the brain is dependent upon neurite outgrowth and neuronal pathfinding, processes in which the neuronal outgrowth is extended to target neurons by the growth cone. The growth cone is a highly motile, actin-based structure located at the tip of neuronal processes that contain lamellipodia and filopodia projections that respond to surrounding environmental cues to direct growth cone movement [15, 36]. Once the growth cone has finalized a pre-synaptic terminal formation neurotransmitters can be repeatedly released, triggering enlargement of axonal spines. In order to maintain rapid neurotransmitter activity, efficient priming, releasing and recycling of synaptic vesicles is essential.

We used a proteomic and phosphoproteomic approach in the brain of NMRs of various ages to assess if subtle changes in the proteome may be occurring and if these changes could play a key role in averting the age-related decline in brain function commonly observed in other mammals. In this comprehensive study, we identified several significant changes in the NMR brain proteome and phosphoproteome with age. Previously, we have reported on changes of metabolic proteins [57, 58] and on proteins associated with the proteostasis network [57, 58] with age in the NMR. In this report, we describe significant changes with age in neuroplastic-related brain proteins and phosphoproteins in the NMR and their contribution to underlying mechanisms that may contribute to the unusually long and salubrious lifespan of this rodent.

Materials and Methods

Materials

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Criterion precast polyacrylamide gels, ReadyStrip IPG strips, TGS and MOPS electrophoresis running buffers, mineral oil, Precision Plus Protein All Blue Standards, SYPRO Ruby protein stain, biolytes, urea, dithiothreitol (DTT), iodoacetamide (IA), and nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA). Pro-Q Diamond phosphoprotein gel stain, anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies were purchased from Invitrogen (Grand Island, NY, USA). Protein A/G beads Amersham ECL IgG horseradish peroxidase-linked secondary antibodies, and ECL Plus Western blotting detection reagents were procured from GE Healthcare (Pittsburgh, PA, USA). C_{18} ZipTips and Re-Blot Plus Strong stripping solution were obtained from Millipore (Billerica, MA, USA). Modified trypsin solution was purchased from Promega (Madison, WI, USA). Pierce BCA protein assay reagents A & B were purchased from Thermo Scientific (Waltham, MA, USA). Anti-septin7, anti-CRMP2 and anti-cofilin1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Animals

Brains from NMRs, aged 2–24 years, were acquired from the well-characterized colonies [7] of Dr. Rochelle Buffenstein at the University of Texas Health Science Center, San Antonio. The fabricated burrow systems, which housed the NMRs, mimicked conditions of their natural habitat and were maintained at 30 °C with 30–50 % relative humidity. The NMR diet consisted of fresh fruits and vegetables (fed ad libitum), supplemented with a high protein and vitamin enriched feed (Pronutro, South Africa). NMRs of different ages were anesthetized with isoflurane and euthanized by cardiac exsanguination. Brains were immediately harvested and flash frozen in liquid nitrogen. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio, TX. Experimental animal groups consisted of 5–9 individual brains from both male and female individuals of both subordinate and breeding status. NMRs were divided into the following four age groups for analysis: 2–3 year-olds (age group 1; young), 4–6 year-olds (age group 2; intermediate), 7–12 year-olds (age group 3; old) and 15–24 year-olds (age group 4; oldest).

Sample Preparation

NMR brains were homogenized using a Wheaton glass homogenizer (~40 passes) with ice-cold isolation buffer [0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES, 0.2 μg/mL PMSF, 5 μg/mL aprotinin, 4 μg/mL leupeptin, 4 μg/mL pepstatin, and 10 μg/mL phosphatase inhibitor cocktail 2] and sonicated for 10 s on ice. Protein concentrations of homogenates were determined by the Pierce BCA method (Rockford, IL, USA) [51].

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

Isoelectric Focusing (IEF)

2-D PAGE experiments were performed as previously described [53]. Briefly, 200 μg of each sample was suspended in 200 μL of rehydration buffer [8 M urea, 2 M thiourea, 50 mM DTT, 2.0 % (w/v) CHAPS, 0.2 % Biolytes, 0.01 % bromophenol Blue], applied to IPG strips (pH 3–10), actively rehydrated and isoelectrically focused.

After completion of the run, IPG strips were immediately stored at −80 °C.

SDS PAGE

IPG strips were thawed and equilibrated in DTT and IA-containing buffers. Strips were rinsed in Tris/Glycine/SDS (TGS) running buffer and placed into Criterion precast polyacrylamide gels (8–16 % Tris–HCl). Precision Plus Protein All Blue molecular standards and samples were run at a constant voltage of 200 V for approximately 65 min at 22 °C in TGS running buffer.

SYPRO Ruby and Pro-Q Diamond Staining

After completion of 2D-PAGE, gels were stained according to manufacturer's directions and as described previously (16). In brief, gels were fixed [10 % (v/v) acetic acid, 50 % (v/v) methanol] and stained with 60 mL of Pro-Q Diamond for exactly 90 min. Gels were destained four times by destaining solution [20 % acetonitrile (ACN), 50 mM sodium acetate, pH 4] (100 mL, 30 min each). The gels were scanned at 580 nm using ChemiDoc XRS+ imaging system (Bio-Rad). Next, gels were incubated overnight (15 h) in 50 mL of SYPRO Ruby protein gel stain. Gels were imaged at 450 nm and stored in DI water in covered containers at 4 °C until protein spot extraction.

Image Analysis

Expression Proteomics

Spot intensities from SYPRO Ruby-stained gel images were quantified according to total spot density and normalized to total gel density using PDQuest analysis software (Bio-Rad). Normalized spot densities of the four age groups were compared and only spots with statistically significant differences between the age groups ($p < 0.05$) were considered for in-gel trypsin digestion and protein identification by MS/MS.

Phosphoproteomics

Spot intensities from Pro-Q Diamond-stained gel images were quantified and matched as described previously (16). A high match analysis between the SYPRO Ruby and Pro-Q Diamond-stained gels was conducted. Phosphoprotein spot densities were normalized to SYPRO Ruby spot densities in order to differentiate between a lightly phosphorylated protein that is highly abundant and a protein of low abundance that is highly phosphorylated. The normalized spot densities were compared between the four age groups, and spots that were statistically significant ($p < 0.05$) were considered for in-gel trypsin digestion and protein identification by MS/MS.

In-Gel Trypsin Digestion/Peptide Extraction

Significantly differential protein spots were excised from 2D-gels and transferred to individual Eppendorf microcentrifuge tubes for trypsin digestion as previously described [55]. In brief, DTT and IA were used to break and cap disulfide bonds and the excised gel plugs were incubated overnight (17 h) in modified trypsin solution with shaking at 37 °C. Salts and contaminants were removed from the tryptic peptide solutions using C_{18} ZipTips. Tryptic peptide solutions were reconstituted in 10 µL of a 5 % ACN/0.1 % formic acid (FA) solution and stored at −80 °C until MS/MS analysis.

NanoLC-MS with Data Dependent Scan

Reconstituted tryptic peptide solutions were analyzed by a nanoAcquity (Waters, Milford, MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) platform using a data dependent scan mode and separated by a capillary column (0.1 × 130 mm column packed in-house with 3.6 µm, 200 Å XB-C18) with a gradient using 0.1 % FA and ACN/0.1 % FA at 200 nL/min. Spectra obtained by MS were measured by the orbitrap at 30,000 resolution; and the MS/MS spectra of the six most intense parent ions were acquired by the orbitrap at 7500 resolution. Swiss-Prot database by SEQUEST (Proteome Discoverer v1.4, Thermo Scientific) was used to interrogate the data files for sample identification. Proteins were identified by at least two high-confidence peptide matched sequences with a false discovery rate <1 %. Proteins matched with the same peptides were reported as one protein group. Tabular data reported from these analyses includes: the SwissProt accession number, the percentage of the protein sequence identified by matching peptides, the number of peptide sequences sequenced in the MS/MS analysis, the confidence score of the protein, the protein's expected molecular weight (MW) and isoelectric point (pI).

Immunoprecipitation and Western Blotting

Immunoprecipitation (IP)

Brain homogenates (250 µg) were suspended individually in 500 µL of IP buffer [0.05 % NP-40, aproprotin 5 µg/mL, leupeptin 4 µg/mL, pepstatin 4 µg/mL, and phosphatase inhibitor cocktail 10 µg/mL] in a phosphate buffer solution, pH 8 [8 M NaCl, 0.2 M KCl, 1.44 M Na_2HPO_4, and 0.24 M KH_2PO_4]. Samples were incubated with Protein A/G agarose beads in 500 mL of IP buffer for 1.5 h at

4 °C. Each sample was incubated overnight with anti-cofilin1 antibody (1:50 dilution) at 4 °C. The next day, samples were incubated with Protein A/G agarose beads for 1.5 h at 4 °C and washed with IP buffer (500 mL, 5 times), preserving the protein-linked beads for a 1D-PAGE experiment.

One-Dimensional Polyacrylamide Gel Electrophoresis (1D-PAGE)

Sample homogenates (50 μg) or beads from immunoprecipitation were suspended in 4X sample loading buffer [0.5 M Tris, pH 6.8, 40 % glycerol, 8 % SDS, 20 % β-mercaptoethanol, 0.01 % Bromophenol Blue] (diluted to 1X with DI water). Samples were heated at 95 °C for 5 min, cooled on ice and loaded into Criterion precast 18 well polyacrylamide gels (4–12 % Bis–Tris). Using MOPS running buffer, gels were run at 80 V for 15 min and then at 120 V for approximately 100 min.

1D-Western Blotting

In-gel proteins were transferred to nitrocellulose membranes (0.2 nm) using a Trans-Blot Turbo Blotting System (Bio-Rad) at 25 V for 30 min. Membranes were blocked in solution [3 % bovine serum albumin (BSA) in TBS-T (8 M NaCl, 2.4 M Tris, and 0.1 % (v/v) Tween 20)] for 1.5 h. The membranes were then separately incubated with primary antibodies: CRMP2 and septin7 (1:3000 dilution), tubulin (1:5000), and phosphoserine, phosphothreonine and phosphotyrosine antibodies (1:6000) for 2 h. The blots were washed with TBS-T (3 times, 5 min each), incubated (1 h) with a horseradish peroxidase secondary antibody in TBS-T (1:5000), and washed again in TBS-T (3 times, 10 min each). Western blots were developed using chemiluminesence (in dark, 5 min) with Clarity Western ECL substrate, scanned with the ChemiDoc and quantified using Image Lab software (Bio-Rad). Blots were stripped up to two times with Re-Blot Plus Strong solution (15 min each) for further probing.

Statistical Analysis

An initial conservative analysis was carried out on PDQuest data using both a two-tailed Student's *t* test and a Mann–Whitney U statistical test, independently comparing each age group to the youngest age group. Protein spots were considered significant if $p < 0.05$ in both tests. Significant differences ($p < 0.05$) between the age groups for PDQuest data were determined using one-way ANOVA with post hoc Bonferroni correction analyses. Fold-change values of proteins were calculated by dividing the average, normalized spot intensity of older age group by the average, normalized spot intensity of the younger age group in the comparison. For Western blot data, a one-way ANOVA with a post hoc Tukey multiple comparisons test was conducted using GraphPad Prism (version 6.02). All data are presented as mean + SEM. Proteins identified by the SEQUEST search algorithm were considered statistically significant if $p < 0.01$. At least two peptide sequences were used to identify each protein and a visual comparison was made between the expected MW and pI of the identified protein to the spot of the extracted 2-D gel plug.

Results

Age-Related Changes in Neuroplasticity-Related Proteins

Neuroplasticity-related proteins with statistically significant alterations in protein and/or phosphorylation levels in the four age cohorts are labeled in the 2-D gel images of Figs. 1 and 2. PDQuest analyses of gels from all age groups identified 9 proteins related to neurite outgrowth and neurotransmission with significant changes in the NMR brain as a function of age (Table 1). These proteins were: cofilin-1; isoform 2 of dihydropyrimidinase-related protein 2, aka collapsin response mediator protein 2; destrin, aka actin depolymerizing factor; isoform 3 of spectrin alpha chain; septin-7; syntaxin-binding protein 1; synapsin-2 isoform IIB; and both isoform 3 and 4 of dynamin1.

Immunoprecipitation and Western Blotting Validations

Immunoprecipitation and Western blot experiments were carried out on selected proteins to confirm MS/MS results and provides confidence of all proteomic or phosphoproteomic identifications. Western blot analysis of CRMP2 (Fig. 3a) confirmed a significant increase in CRMP2 levels in the brain of the oldest age group compared to the two younger age groups ($p = 0.008$) and ($p = 0.011$), respectively. The results of the Western blot analyses of the levels of septin-7 (Fig. 3b) verified a significant increase in the old age group ($p = 0.038$) compared to the youngest age group, while also showing increased levels of septin-7 in the intermediate age group ($p = 0.034$) also compared to the youngest age group. Analyses of the immunoprecipitation of cofilin-1 with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies confirmed a significant decrease in the phosphorylation of cofilin-1 in the brain of the NMR for the intermediate age group ($p = 0.039$), old age group ($p = 0.037$), and oldest age

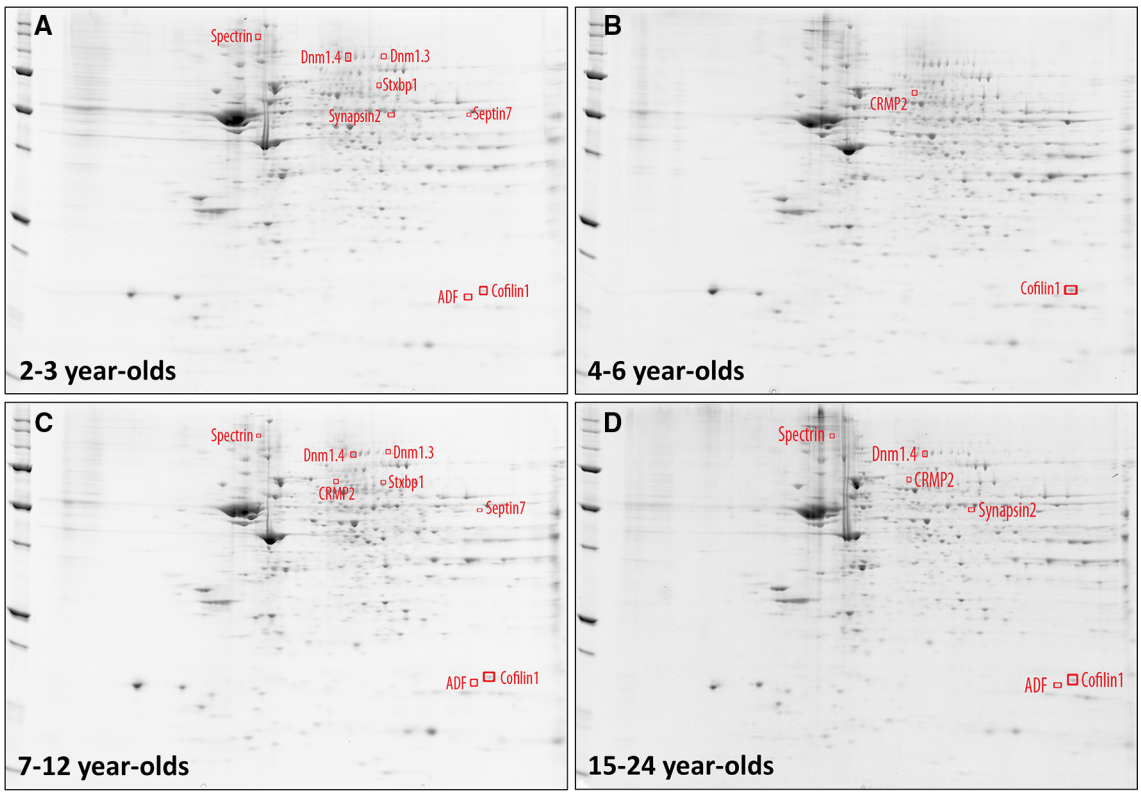

Fig. 1 Representative Sypro Ruby-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2–3 years (**a**), 4–6 years (**b**), 7–12 years (**c**), and 15–24 years (**d**). Proteins whose expression and/or phosphorylation state was significantly altered ($p < 0.05$) in the particular age group are labeled in the images

group ($p = 0.037$) all relative to the youngest age group (Fig. 3c).

Discussion

Evaluation of the proteins and phosphoproteins that significantly change with age in the brain of the NMR, whose functions are associated with neuroplasticity, fall into two distinct yet connected processes: neurite outgrowth and neurotransmission.

Neurite Outgrowth

Rapid assembly and disassembly of the actin cytoskeleton at the leading edge of the cone is required for motility of the growth cone [35, 41]. Actin depolymerizing factor (ADF; destrin) and cofilin-1, members of the ADF/cofilin family, modulate actin dynamics in the growth cone by binding and depolymerizing F-actin [25, 31] and by regulating the rate at which these monomers separate from the actin filament (Maciver and Hussey [37]. In the current study, NMRs exhibited increased ADF/cofilin-1 levels with significant elevation of ADF levels in the two oldest age cohorts compared to the youngest age group and increased cofilin-1 levels in the old age group. The increase in the expressed levels of these two proteins with age in the NMR brain is an interesting finding as overexpression of cofilin has been reported to increase neurite outgrowth [40] and neurite extension by the growth cone [19]. Further, phosphorylation of ADF was decreased in the oldest age group, while cofilin-1 phosphorylation was decreased in all age groups as compared to the youngest. Phosphorylation is an important regulatory mechanism as phosphorylation at Ser-3 reportedly inactivates ADF/cofilin, while dephosphorylation activates actin depolymerization Toshima [19, 56]. Moreover, it has been reported that an increased F-actin turnover rate stimulates longevity, while a decreased rate may trigger cell death [24]. Taken together, the increased levels and activity of ADF/cofilin suggest a possible contributing factor promoting the increase in dendrite length and complexity that is reported to occur with age in the NMR [44].

Collapsin response mediator protein 2 (CRMP2) is a pleiotropic protein involved in regulating growth cone dynamics (e.g., organization of the dendritic field, guidance and collapse of the growth cone, neurite outgrowth) as well as synaptic assembly, neurotransmitter release, endocytosis,

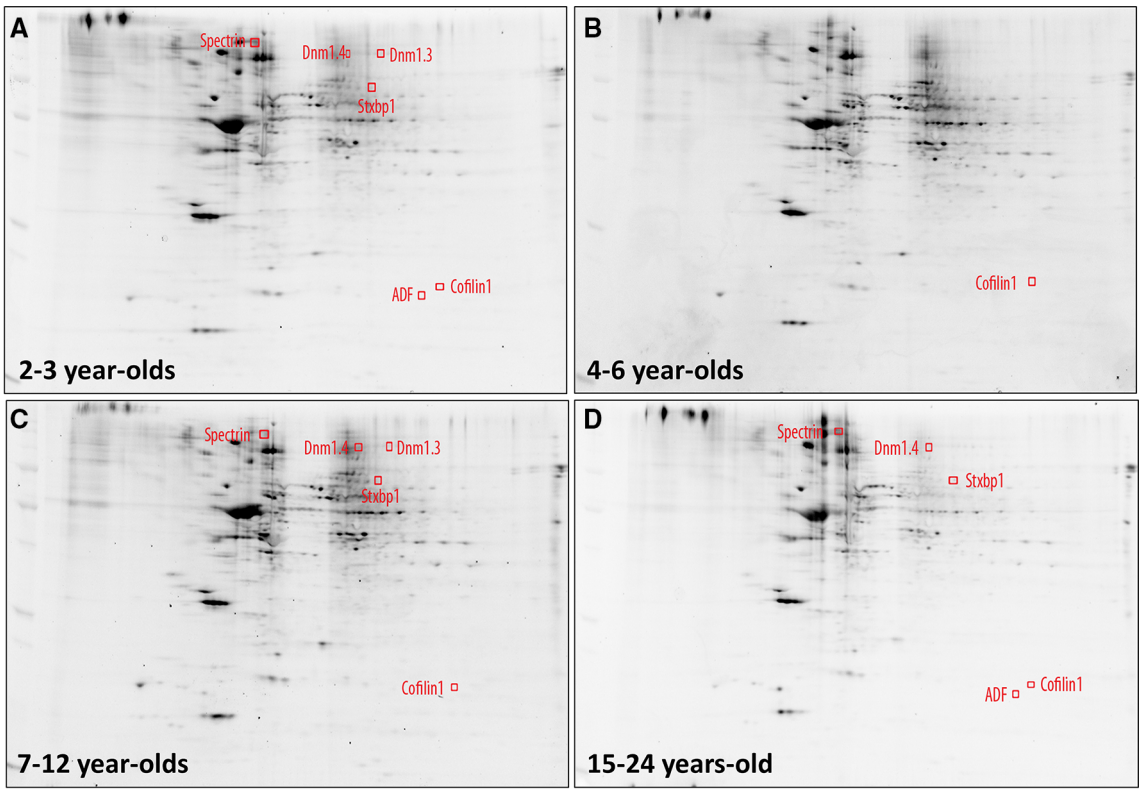

Fig. 2 Representative Pro-Q Diamond-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2–3 years (**a**), 4–6 years (**b**), 7–12 years (**c**), and 15–24 years (**d**). Proteins with significantly altered phosphorylation levels ($p < 0.05$) in the particular age group are labeled in the images

and Ca^{2+} homeostasis [5, 10, 23, 27, 29, 59]. In previous studies, we have shown that CRMP2 is oxidatively modified in brain of subjects with AD [11] and that phosphorylation levels of CRMP2 are increased in the hippocampus of brains from AD brain [17]. Further, it has been shown that CRMP2 levels decrease in the central nervous system as we age [12]. Conversely, in this current study, levels of CRMP2 were significantly increased in the oldest NMRs compared to both the intermediate and old age groups. This increase in CRMP2 suggests that older NMR brains have increased neuronal plasticity and provides another mechanism by which the NMR is able to ward away cognitive decline with age.

We found evidence for the differential regulation of another family of neuroplasticity-related proteins in NMR brains, the septins. Members of the septin family are highly conserved cytoskeletal GTPases involved in various cellular functions, including: dendritic field maturation, spine dynamics, synaptic transmission, vesicle trafficking, DNA response to cytoskeletal damage, protein scaffolding, membrane compartmentalization, cell division, and apoptosis [4], Kremer et al. [26, 30, 52, 54, 60]. Septin7, in particular, is reportedly essential in the regulation of dendritic branching and spine morphology [60]. In this study of the NMR brain, expression of septin7 was significantly increased in the old age group with respect to the youngest age group. The increase in structural plasticity, as noted by others [44] and as supported by the increase in expression and/or activity in the above identified proteins, provides a neuronal correlation to a plausible mechanism promoting longevity in the NMR.

Neurotransmission

Rapid release and recycling of synaptic vesicles is one facet required for efficient neurotransmission. In the brain of the NMR, four proteins involved in this process were altered with age: syntaxin-binding protein 1 (stxbp1), two isoforms of dynamin-1 (dnm1), and spectrin.

Stxbp1 plays a role in both neurite extension and neurotransmission. Not only is stxbp1 reported to regulate the filopodia of the growth cone to modulate plasticity [6], but stxbp1 also can bind to syntaxin and modulate the formation of the SNARE complex and subsequent neurotransmitter release [42, 62]. In the current study, the expression of stxbp1 was increased in the oldest age group compared to the youngest, and phosphorylation levels of stxbp1 were decreased in the two oldest groups relative to the youngest.

Table 1 PDQuest and MS/MS results of NMR brain proteins related to plasticity, structure and neurotransmission with significant altered expression and/or phosphorylation states as a function of age

Spot	Protein identified	Accession #	Coverage (%)	# of Peptides	Score	MW (kDa)	pI	Age groups	*p* value expression	Fold change expression	*p* value phosphorylation	Fold change phosphorylation
8004	Cofilin-1	P23528	43.37	7	29.67	18.5	8.09	1 v 2	0.0398	6.46	0.0001	0.0082
								1 v 3	0.0103	7.48	0.0001	0.0059
								1 v 4	–	–	0.0005	0.166
4710	Collapsin response mediated protein 2, Isoform 2	Q16555–2	24.25	9	61.28	58.1	6.15	2 v 4	0.0387	2.98	–	–
								3 v 4	0.0160	3.93	–	–
8002	Destrin	F6RFD5	21.48	3	18.75	15.4	8.59	1 v 3	0.0371	63.3	–	–
								1 v 4	–	–	0.0466	0.172
2907	Spectrin alpha chain, Isoform 3	Q13813–3	8.77	19	89.26	282	5.34	1 v 3	0.0330	6.60	0.0003	0.121
								1 v 4	–	–	0.0004	0.180
8502	Septin-7	H0Y3Y4	9.65	3	3.91	43.0	7.78	1 v 3	0.0381	17.2	–	–
6704	Syntaxin-binding protein 1	P61764	26.43	13	66.50	67.5	6.96	1 v 3	0.0159	6.63	0.0339	0.0203
								1 v 4	–	–	0.0277	0.125
6506	Synapsin-2, Isoform IIB	Q92777–2	16.95	8	50.75	52.1	7.72	1 v 3	0.0498	44.8	–	–
6803	Dynamin1 Isoform3	Q05193–3	14.62	13	53.89	96.0	7.01	1 v 3	–	–	0.0372	0.104
5802	Dynamin1 Isoform 4	Q05193–4	26.87	25	116.9	96.3	7.17	1 v 3	–	–	0.0046	0.317
								1 v 4	–	–	0.0014	0.246

Significance was determined via one-way ANOVA with post hoc Bonferroni correction analysis. *Comparison is of the older age group to the younger age group. Age group 1 consists of 2–3 year-olds, 2 has 4–6 year-olds, 3 has 7–12 year-olds, and 4 has 15–24 year-olds (n = 4–6 for each age group)

Protein kinase C phosphorylation on Ser-306 and Ser-313 of stxbp1 reportedly modulates neurotransmission by increasing rapid vesicle cycling and vesicle release (Barclay et al. [3, 14]. However, since PhosphoSite lists over 30 residues of stxbp1 that can be phosphorylated, it is conceivable that the global phosphorylation events measured here may be responsible for modulation of other cellular activities, such as changing affinity to binding partners or cellular localization or other activities. Furthermore, based on the NMR's reputation for maintaining a long healthspan as well as the implications of other proteins identified in this study, it may not be likely that the decreased phosphorylation of stxbp1 seen here would decrease synaptic activity. Further investigations into decreases of phosphorylation at particular phosphorylation sites are warranted.

Dnm1 is a brain-specific GTP-dependent motor protein that is abundant in the post-synaptic synapse [47]. Furthermore, Dnm1 phosphorylation plays a key role in regulating synaptic vesicle endocytosis (reviewed in Smillie and Cousin [50]). Dnm1 is activated by the dephosphorylation that occurs upon depolarization of the axon terminal, and then is deactivated by phosphorylation upon repolarization. We speculate that upregulation of the activity of this protein observed in the current study among the oldest age group compared to the youngest age group may be the result of the brain's defensive mechanism to ameliorate reduced neurotransmission, which normally occurs as we age (McGeer and McGeer [39], by increasing the uptake of excitatory neurotransmitters into the post-synaptic synapse.

Spectrin is an α-β heterodimer that makes up to 2–3 % of all proteins in the brain and is responsible for cross-linking F-actin, membrane lipids, and proteins to form a resilient 3-D cellular matrix to increase the stability of the cytoskeleton and transmembrane proteins [2, 61, 63]. Spectrin is found at greater concentrations at the

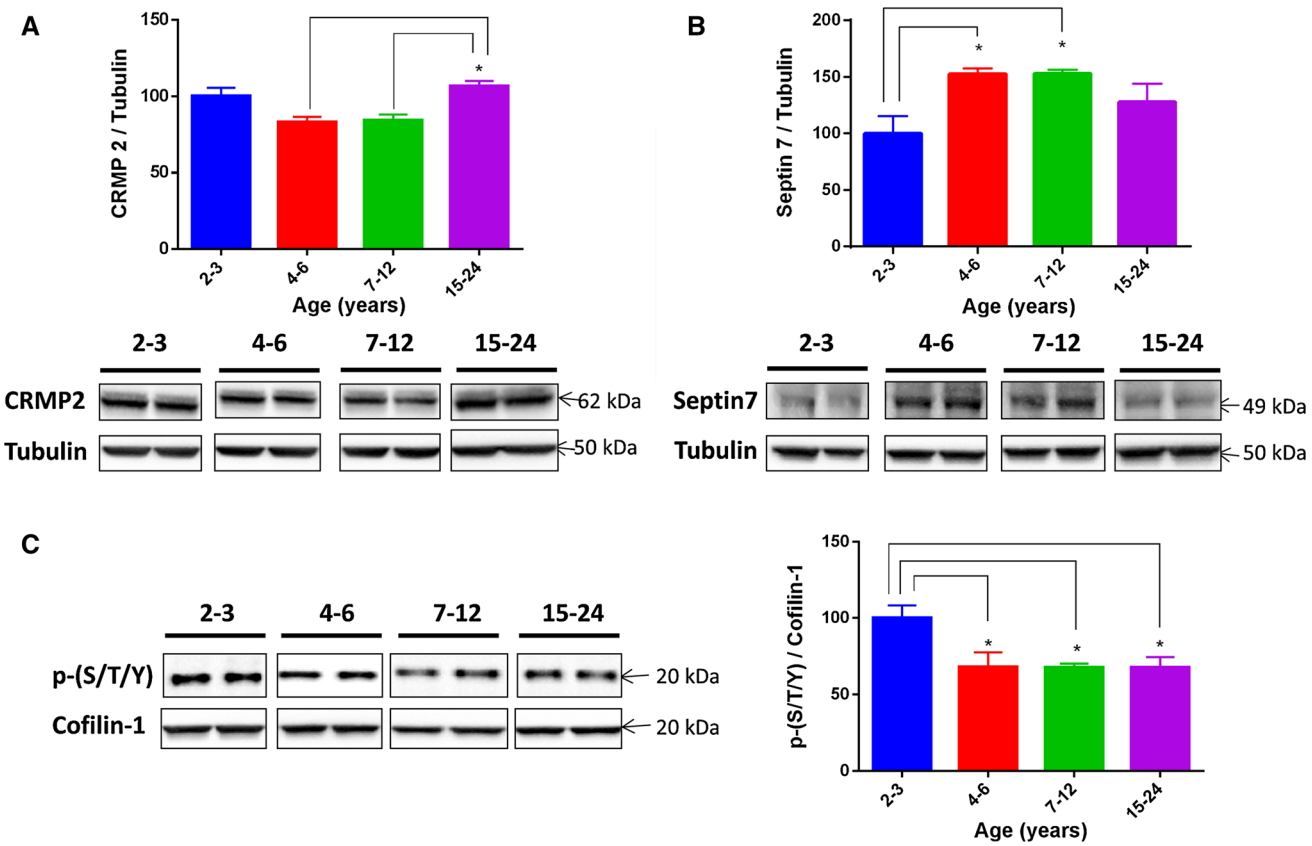

Fig. 3 Immunoprecipitation and Western blot analyses of (**a**) significant elevation of CRMP2 protein levels in oldest age group (**b**) increased septin-7 protein levels in the middle age groups, and (**c**) levels of phosphorylated Ser/Tyr/Thr residues normalized to total cofilin-1 protein levels after immunoprecipitation showing significant decrease ($*p < 0.05$) in the phosphorylation of cofilin-1 in the brains of NMRs (n = 4–6 for each age group). Immunoreactivity with specific antibodies was detected by chemiluminescence

presynaptic membrane and is thought play a role in synaptic transmission and organization, since spectrin mutations in *Drosophila* led to disrupted neurotransmission and aberrant synaptic protein localization [21]. Previously, we reported decreased expression of alpha-spectrin in the aged SAMP8 mouse brain [46]. Conversely, in the current study, expression of spectrin α-chain, isoform 3 is increased in the brain of old-aged NMRs relative to the youngest, suggesting a potential link to efficient neurotransmission in aged NMRs. In addition, the phosphorylation level of this protein is decreased in the brains of the two oldest NMR groups. Spectrin is known to be phosphorylated at numerous residues; however, the consequences of spectrin phosphorylation are not yet clear [2].

In summary, the NMR, with its many unique traits associated with salubrious aging, is an exceptional model organism in the study of proteins to target for therapeutic interventions in the aging process. The findings of the current age-related study of brains from NMRs identifies changes in protein expression and/or phosphorylation levels of key proteins involved in mechanisms that may be responsible for the increase in neuronal plasticity and the lack of senescence noted in the NMR.

Future Directions

Uncovering clues to the underlying mechanisms that promote successful aging in long-lived species may provide novel insights into the aging process and resistance to age-associated diseases. Further investigations into these proteomics-identified proteins in this current study may be warranted to identify targets for potential key therapies that conceivably may aid in delaying the onset and progression of aging in humans.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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