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Age-related proteomic changes in the subventricular zone and its association with neural stem/progenitor cell proliferation

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Abstract

In the mammalian central nervous system, generation of new neurons persists in the subventricular zone throughout life. However, the capacity of neurogenesis in this region declines with aging. Recent studies have examined the degree of these age-related neurogenic declines and the changes of cytoarchitecture of the SVZ with aging. However, little is known about the molecular changes in the SVZ with aging. In this study, we dissected the SVZs from rats aged at postnatal day 28, 3 months and 24 months. The SVZ tissues were processed for 2-D gel electrophoresis to identify protein changes following aging. Protein spots were subsequently subjected to mass spectrometry analysis to compare age-related alterations in the SVZ proteome. We also examined the level of cell proliferation in the SVZ in animals of these three age groups using BrdU labeling. We found significant age-related changes in the expression of several proteins that play critical roles in the proliferation and survival of neural stem/progenitor cells in the SVZ. Among these proteins, glial fibrillary acidic protein (GFAP), ubiquitin carboxy terminal hydrolase 1 (UCHL-1), glutathione s-transferase omega (GSTO-1) and preproalbumin were increased with aging while collapsin response mediated protein 4 (CRMP-4), CRMP-5 and microsomal protease ER60 exhibited declines with aging. We have also observed a significant decline of neural stem/progenitor cell proliferation in the SVZ with aging. These alterations in protein expression in the SVZ with aging likely underlie the diminishing proliferative capacity of stem/progenitor cells in the aging brain.

Keywords

Subventricular zone; aging; neurogenesis; proteome

Introduction

The dramatic increase in numbers of the elderly population has precipitated a sense of urgency in our desire to understand the cellular and molecular basis of aging. This is particularly the case for our understanding of the biology of the aging brain. Both clinical and research studies have established that the elderly show declines in cognitive function (Selkoe, 1992; Della-Maggiore et al., 2002), have a greater susceptibility to neurodegenerative diseases (Hopkins, 1993) and a limited cognitive recovery following brain injury (Eiben, 1984). Nevertheless, promising therapeutic strategies aimed at facilitating healthy brain aging and repair have been proposed based on the recent

observation that neurogenesis persists within the CNS throughout life (Mattson et al., 2002; Parent, 2003; Hallbergson et al., 2003). In the mature CNS, populations of neural stem and progenitor cells (NS/NPCs) reside in the subventricular zone (SVZ) and the hippocampal dentate gyrus (Altman and Das, 1965; Lois and Alvarez-Buylla, 1993). NS/NPCs are highly migratory cells capable of functional integration into the existing neuronal circuitry of the brain (Doetsch and Alvarez-Buylla, 1996; Hastings and Gould, 1999; van Praag et al., 2002). They likely function in replenishing cells lost during normal CNS activity and play an important role in learning and memory processes (Kempermann, 2002). Increasing evidence indicates that these cells may also play regenerative and reparative roles in response to CNS insult or disease (Chirumamilla et al., 2002; Sun et al., 2007).

While neurogenesis continues throughout life, studies have shown that the neurogenic potential of NS/NPCs decreases with age in both the dentate gyrus and the SVZ (Kuhn et al., 1996; Seki and Arai, 1995; Luo et al., 2006; Walter et al., 2009). This age-related decline in the neurogenic capacity of these cells likely impairs the ability of these cells to function in normal tissue turnover and maintenance and may contribute to the progressive deterioration of cognitive skills associated with aging (Driscoll, 2005). Furthermore, this age-related reduction in the neurogenic potential of these cells has been linked to the high vulnerability of the aged brain to neurodegenerative diseases (Rodriguez et al., 2008 and 2009; Demars et al., 2010) and the reduced cognitive recovery capacity following brain insults (Limke and Rao, 2003). Such findings highlight the importance of endogenous NS/NPCs and underscore the need to elucidate the underlying mechanisms that mediate neurogenesis in the aging brain.

Recent studies examining the age-related decline of neurogenesis in the SVZ have found that it is partly due to the lengthening of the progenitor cell cycle and increased apoptosis of neuroblasts, as well as changes in SVZ cytoarchitecture (Luo et al., 2006, Bouab et al., 2011). Declines in SVZ neurogenesis with aging are also associated with a reduction in EGFR signaling (Enwere et al., 2004), a reduction of gene expression of NSC markers and transcription factors which are important in development, and an increased expression of proteins linked to senescence (Molofshy et al., 2006; Ahlenius et al., 2009). Other factors associated with this age-related decline in SVZ neurogenesis include a decrease telomerase activity and increased levels of corticosteroid and inflammation (Cameron and McKay, 1989). However, it is not clear how changes in the neurogenic niche during aging affect the degree of neurogenesis. Here, we address this question by utilizing proteomic methodologies to generate protein expression profiles of the SVZ from rats at various ages. A comparison of protein profiles from these groups revealed a number of differentially expressed proteins that are known to play critical roles in the proliferation and viability of CNS cells. This information may provide valuable insight into the cellular and molecular processes that underlie aging associated declines in SVZ neurogenesis.

Material and Methods

Animals

Juvenile (P28 day), adult (6 month) and aged (24+ month) male Sprague Dawley rats (Harlan, IN) were used in the current study. Animals were housed in the animal facility, with a 12-hour light/dark cycle, water and food provided *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee in Virginia Commonwealth University.

Tissue preparation for Immunohistochemical and Proteomic Methods

In order to examine levels of cell proliferation, bromodeoxyuridine (BrdU) was used to label mitotically active cells. Due to the diminished level of cell proliferation in the aging SVZ (Maslov et al., 2004; Bouab et al., 2011), we used a 7- day BrdU injection paradigm in order to maximally label dividing cells in this region. Rats (n=3/ age group) were given single daily intraperitoneal injections of 300mg/kg BrdU (Sigma, St. Louis) for a period of 7 days. BrdU is a thymidine analog that incorporates into the DNA of dividing cells during S phase and can be detected immunohistochemically (Miller and Nowakowski, 1988). Twenty-four hours following the final BrdU injection, animals were euthanized with euthasol, transcardially perfused with phosphate-buffer saline (PBS) followed by 4% paraformaldehyde in PBS and the brains were dissected and postfixed overnight in 4% paraformaldehyde at 4°C. Brains were then cut coronally at 60µm with a vibratome throughout the rostro-caudal extent of the lateral ventricles and processed for BrdU immunohistochemistry (see below). For proteomic analysis of SVZ tissue, animals of all three age groups (n=6/ age group) were transcardially perfused with saline. The brains were dissected, placed in a brain mold and cut into 1mm serial coronal sections throughout the rostro-caudal extent of the SVZ. The sections were collected in petri dishes filled with PBS and the SVZs were visualized using an Olympus SZX9 dissecting microscope and subsequently dissected free from the surrounding tissue from both hemispheres. Nissl staining and nestin immunohistochemistry (see below) were performed on alternate coronal sections to determine the region of the lateral ventricular wall to be dissected. Extracted SVZ tissues were prepared for 2D gel electrophoresis by homogenization in an osmotic lysis buffer containing protease inhibitors and nucleases. All samples were quantified with a BCA protein assay to ensure that equal quantities of proteins (50µg) were loaded into each 2-D gel. To obtain an accurate picture of the protein profile found within each sample group, while accounting for slight variability between individual rats and in gel running, a total of three duplicate gel sets each from SVZ samples pooled from two animals for all three age groups were generated.

Immunohistochemistry

60µm free floating coronal sections were immunostained for BrdU according to our previously published protocol (Sun et al., 2007 and 2009). Mouse anti-BrdU (Dako, Carpinteria, CA) primary antibody was used at a dilution of 1:200. Nestin (mouse anti-nestin, 1:500; Chemicon, Temecula, CA) and GFAP (rabbit anti-GFAP, 1:1000; Dako) immunohistochemistry was also performed according to a previously published protocol (Sun et al., 2007 and 2009). Secondary antibodies used include peroxidase- conjugated goat anti-mouse IgG-HRP and goat anti-rabbit IgGHRP (Santa Cruz Biotech, CA), both of which were diluted to 1:200. Histochemical detection of peroxidase activity was performed with diaminobenzidine.

Quantification of BrdU-positive cells

The total number of BrdU-positive cells in the SVZ of both hemispheres was determined using unbiased stereological methods and the Olympus CAST Stereology program following our previously published protocols (Sun et al., 2009 and 2010). Every fourth 60µm coronal section throughout the rostro-caudal extent of the left lateral ventricle was examined using the optical fractionator method of quantification. Briefly, BrdU-positive cells within a known fraction of the SVZ were quantified and the total number of BrdU-positive cells was then extrapolated by multiplying the number of cells counted by the reciprocal of the fraction of tissue that was quantified. All values are presented as mean $\pm$ SEM and the statistical significance of the cell counts was determined using a one-way analysis of variance (ANOVA) and Tukey's W-procedure. Differences were deemed significant with $p < 0.05$.

2-D gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed (by Kendrick Labs) according to the method of O'Farrell (O'Farrell, 1975) and as previously published by our lab (Colello, et al., 2002). 2-D gel electrophoresis separates proteins based on their charge in the first dimension and according to their molecular weight in the second dimension. Standard IEF-2D-PAGE was performed on 10% acrylamide slab gels to resolve proteins ranging from 15 – 200kD in molecular weight, with isoelectric points in the pH range 3.5–10. A total of 50 µg of sample protein was loaded in each gel run. Additionally, 50ng of purified tropomyosin standard (MW 33,000; pI 5.2) was added to each sample to act as a reference marker protein. Gels were stained with a special silver stain according to the Vorum method (Mortz et al., 2001).

Image analysis – manual and computerized comparisons of 2-D gels

All gels from a given experimental condition were digitally scanned using a GS-800 densitometer (BioRad) and analyzed using the Discovery Series PDQuest 2-D Analysis software (BioRad). A comparative analysis between gel sets of the juvenile, adult, and aged SVZ was performed in order to reveal differences in protein expression levels. The method of computerized comparisons of 2-D gels included automatic spot finding and quantification, automatic background subtraction (lowest on spot boundary) and automatic spot matching in conjunction with detailed manual checking of the spot finding and matching functions. While the gel densitometry analyses were performed for all three age groups, only those spots representing at least a 3-fold change in protein expression were considered proteins of interest and were subjected to the follow-up mass spectrometry analysis to determine the amino acid sequence of proteins. These proteins spots with over 3-fold changes were between the juvenile and aged animals. A modest difference less than 3-fold in the intensity/size of these same gel spots were observed between the adult and aged animals.

Immunoblotting

2-D gels were transblotted onto PVDF membranes and the section of the PVDF membrane corresponding to the molecular weight and isoelectric point of proteins of interest were excised using a scalpel. Immunoblotting for nestin (MW- 220kDa; pI- 5.4) and CRMP-4 (MW- 62kDa; pI- 6.04) was performed according to a previous published protocol (Colello et al., 2002) and protein levels were visualized using an advanced chemiluminescence detection kit (Amersham, UK). Primary antibodies used include mouse anti-nestin (Chemicon, Temecula, CA) and rabbit anti-TOAD-64 (recognizes CRMP-4; BD Pharmingen, San Diego, CA) at a dilutions of 1:5000 and 1:10,000, respectively. Immunoblotting was also performed on the section of the PVDF membrane corresponding to the molecular weight and isoelectric point of actin in order to verify that the proteins properly transferred to the PVDF membranes.

In-gel digestion of proteins and LC ESI-MS/MS analysis

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) was used to determine the amino acid sequence of the proteins of interest. For LC ESI-MS/MS, spots of interest were excised using varying sized tissue punchers in an attempt to minimize the volume of the background gel, chopped into 1 mm × 1 mm cubes and transferred into separate low retention/siliconized microcentrifuge tubes. The gel particles were washed with 100 µl of HPLC water for 5 minutes and spun down. Following the removal of the liquid, 100 µl of acetonitrile (ACN) was added and placed on a mixer for 15 minutes until the gel particles were shrunken together. The particles were spun down, the liquid was removed and the samples were dried by vacuum centrifugation. The gel particles

were then reduced by incubating in 10 mM dithiothreitol/100 mM ammonium bicarbonate for 30 minutes at 56° C. After spinning the gel pieces down and removing the liquid, they were again shrunk with 100 µl of ACN for 15 minutes. Upon ACN removal, gel pieces were alkylated with 55 mM Iodoacetamide/100 mM ammonium bicarbonate and incubated for 20 minutes in the dark at room temperature. Following spinning and removal of the 55 mM iodoacetamide the gel pieces were again shrunk with 100 µl of ACN, spun down, and dried by vacuum centrifugation.

For tryptic digestion of proteins, the gel particles were rehydrated with digestion buffer (50 mM ammonium bicarbonate/5 mM calcium chloride/12.5 ng/µl sequencing grade modified trypsin) and placed in an ice bucket (4° C) for 30 minutes to allow for absorption. The remaining supernatant was removed and replaced by 25 µl of buffer without sequencing-grade modified trypsin and incubated overnight at 37° C. In order to extract the tryptically digested peptides from the gel particle, 15 µl of 25 mM ammonium bicarbonate was added and incubated at 37° C for 15 minutes with shaking. The particles were then spun down and 100 µl of ACN was added and incubated at 37° C for 15 minutes with shaking. The gel particles were again spun down and the supernatant was collected and set aside. Fifty microliters of 5% formic acid was added to the gel particles and vortexed for 15 minutes at 37° C. The gel particles were spun down, 100 µl of ACN was added for 15 minutes with shaking, and spun down again. The gel particles were removed and discarded, and the extract was pooled together with the previous supernatant, dried down in a vacuum centrifuge, and frozen at -20° C until mass spectrometric analysis. Frozen, extracted, unfractionated tryptic peptides were resuspended in a solution containing 1% formic acid in ACN and subjected to LC ESI-MS/MS. Ten microliters of the resuspended peptide fragment solution was picked up by the auto-sampler and fed to the nano-HPLC which was connected directly to a ThermoFinnigan LCQ Deca XP Plus (ThermoFinnigan, San Jose, CA) utilizing a fully automated coupling to the mass spectrometer. The ThermoFinnigan Bioworks 3.0 software controls the LC Packing nano-HPLC and all experiments. A Data-Dependent™ acquisition cycle begins with a full MS scan; followed by a full MS/MS scan of the most intense ions selected from the preceding MS spectrum. Proteins were identified by submitting the MS and MS/MS spectra to a MASCOT database search.

RESULTS

Changes in the level of cell proliferation in the SVZ with aging

In order to assess the extent to which the proliferative capacity of SVZ neural stem/progenitor cells changes throughout the lifespan of an animal, P28, 6 month and 24 month old rats received daily single dose of BrdU injection for 7 days. The total number of BrdU-labeled cells in the SVZs was quantified. Similar to what has been reported before (Luo et al., 2006; Bouab et al., 2011), we found a significant reduction of BrdU-labeled cells in the SVZ with aging, with a particularly sharp decline occurring between juveniles and 6 month old adult (fig. 1; juvenile, 180,841±6,470; adult, 79,451±4,048; aged, 57,929±1,242; *p<0.05.). To assess the age-related changes of neural stem/progenitor cell pools in the SVZ, parallel sections from the same animals were immunostained for nestin, an intermediate filament protein expressed by neural stem/progenitor cells (Lendahl et al, 1990). Nestin immunohistochemistry revealed a similar labeling pattern to that of BrdU in that the juvenile SVZ contained a considerably greater number of nestin-positive cells when compared to adult and aged counterparts (fig. 2.).

Proteomic changes in the SVZ following aging

Based on our speculations that these age-related changes in the proliferative capacity of NS/NPCs are reflective of changes occurring at the proteomic level, we utilized 2D gel

electrophoresis to generate proteomic maps of SVZ tissue derived from juvenile, adult and aged rats (fig. 3). Densitometry analyses for 2D gels were performed for all three age groups, a comparison of protein profiles generated from the three age groups revealed a number of differentially expressed proteins undergoing an elevation or decline in expression level with aging in a consistent manner (i.e. juvenile<adult<aged, or juvenile>adult>aged). For example, several proteins are strongly expressed in the juvenile SVZ and are down-regulated with aging, as shown by distinct protein spots in the juvenile proteomic map and a decreased intensity or absence of corresponding spots in adult and aged counterparts (fig. 3; **yellow arrows**). Conversely, several proteins appear to be up-regulated in the SVZ as a result of aging (fig. 3; **red arrows**). Following both manual and computer automated analysis/ comparisons of 2D gels generated from the three age groups, approximately 900 to 980 distinct protein spots could be detected on each gel, 31 of which exhibited age-associated changes in expression levels of twofold or greater. Thirteen of those proteins (representing ~1.4% of total proteins detected) showed declines in expression levels with aging; whereas eighteen proteins (representing ~1.9% of total proteins detected) exhibited an increase in expression with age. Those proteins/gel spots which underwent 3-fold or greater changes (increase or decrease) in expression with aging were observed only between juvenile and aged, and there was only a modest difference in the intensity/size of these gel spots between the adult and aged animals.

In order to confirm the ability of our 2D gel experimental paradigm to resolve NS/NPCs-associated proteins and to accurately reveal age-related changes in the SVZ proteome, proteins from duplicate gels of those shown in fig. 3 were transferred to PVDF membranes and immunoblotted for the NS/NPCs protein nestin. Using this methodology, a comparison of nestin levels within the SVZ of juvenile and aged rats revealed a substantial decline in nestin expression in this region with aging (fig.2). This finding is likely the result of the diminishing pool of proliferating NS/NPCs residing in the SVZ during the aging process (figs. 1 and 2) and demonstrates the resolving power and accuracy of our 2D gel proteomic maps.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) was then utilized to obtain amino acid sequence information for the proteins that exhibited the greatest change in expression levels as a function of aging (3 fold or higher) (fig. 4). Using this amino acid sequence information, MASCOT database searches were performed in order to determine the identity of these differentially expressed proteins (fig. 5). Proteins that underwent an increase in expression levels with aging include glial fibrillary acidic protein (GFAP), ubiquitin carboxy terminal hydrolase 1 (UCHL-1), glutathione s-transferase omega (GSTO-1) and preproalbumin. Conversely, collapsin response mediated protein 4 (CRMP-4, also known as TOAD-64), CRMP-5 and microsomal protease ER60 exhibited declines in expression as a function of aging. Immunohistochemistry and immunoblotting were performed to confirm the identity and expression patterns of selected proteins that were identified using mass spectrometric methods (fig. 6). For example, the age-related increase in the expression of GFAP observed in our proteomic analysis was confirmed immunohistochemically on coronal sections of the juvenile and aged rat SVZ. Similarly, the age-associated decline in CRMP-4 levels observed in our gels was verified with immunoblots to 2D gels of SVZ tissue extracted from the three age-groups.

DISCUSSION

The current study investigated the age-related changes in cell proliferative capacity in the SVZ and the protein profiles in the SVZ niche. We observed a significant reduction in the level of cell proliferation as demonstrated by BrdU labeling, and a decline in the expression

of the neural stem cell protein-nestin with aging in this region. This is in agreement with previous findings (Seki and Arai, 1995; Kuhn et al., 1996; Luo et al., 2006; Bouab et al., 2011). The age-related decline in cell proliferation and stem cell protein expression may due to the quiescence of neural stem cell, their decrease of cell division, the survival of their progeny or the loss of neural stem cells through apoptosis. In order to better understand the underlying mechanisms modulating this age-related change in the SVZ, we examined the protein expression profiles of this region during the aging process using proteomic approach. Our proteomic results revealed changes of a number of proteins that play critical roles in cell proliferation and survival. Proteins that have shown at least a 3-fold increase in expression level with aging include GFAP, GSTO-1, UCHL-1 and Preproalbumin; whereas those exhibiting a significant decrease with aging include CRMP-4, CRMP-5 and Microsomal protease ER64.

The observed age-related increase of GFAP expression in the SVZ is in agreement with other proteomic studies revealing increased astrocytic reactivity and heightened GFAP expression in various regions of the brain as a function of normal aging (Lee et al., 2000; Goss et al., 1991; Wu et al., 2005). Studies have demonstrated that interactions between GFAP-expressing astrocytes and NS/NPCs are crucial for neural stem cell function and that astrocytes residing in the neurogenic regions play a critical role in regulating neural progenitor cell proliferation and neurogenesis, both *in vitro* and *in vivo* (Lim and Alvarez-Buylla, 1999; Alonso, 2001). It is known that GFAP is expressed by NS/NPCs derived from radial glial cells and niche astrocytes in the SVZ, since the number of NS/NPCs decreases with aging, we posit that the increased levels of GFAP expression detected in the current study were likely due to the increased activity of niche astrocytes. This is in agreement with findings showing that increased levels of GFAP with aging in the SVZ and the dentate gyrus correlated to age-related declines in neurogenesis observed in these regions (Larsson et al., 2004; Luo et al., 2006). Furthermore, a recent study has demonstrated that in the hippocampus age-related decline of neurogenesis is due to the differentiation of neural stem cells to mature hippocampal astrocytes (Encinas et al., 2011), which further confirmed our observation showing increased GFAP expression in the SVZ coupling with decreased neural stem cell pools in normal aging.

We found increased levels of GSTO-1 in the SVZ with aging. GSTO-1, a member of the glutathione S transferase family, is a small stress response protein that has been shown to be involved in cellular redox homeostasis. GSTO-1 is expressed in both neural and glial cells and its capacity to respond to oxidative stress has been demonstrated in a wide variety of tissues, including the CNS (Townsend and Tew, 2003). While brain aging has been associated with increased levels of oxidative stress (Mattson et al., 2002), it is likely that the expression level and activity of antioxidant proteins such as GSTO-1 would increase in an effort to combat this oxidative stress. Moreover, in contrast to other CNS cell types, NS/NPCs exhibit relatively enhanced resistance to reactive oxygen species and oxidative stress (Madhavan et al., 2005). The ability of these cells to respond to and strictly regulate oxidative stress may be due a heightened capacity for redox homeostasis maintenance. However, it is yet to be established whether this protein is being expressed by NS/NPCs in the SVZ or by other cell types residing in this region.

We also observed an increase expression of UCHL-1 in the SVZ with aging. UCHL-1 is an abundantly expressed neuron-specific deubiquitinating enzyme expressed by newly generated neurons in the olfactory bulb (Lombardino et al., 2005). UCHL-1 plays a critical role in the proteasomal protein degradation system and has been shown to influence cellular survival and/or apoptosis through its ability to regulate free ubiquitin levels (Kim et al., 2003; Wilkinson et al., 1997; Harada et al., 2004). For example, retinal neurons in UCHL-1-null (*gad*) mice display heightened anti-apoptotic properties compared to wildtypes following

ischemic retinal injury (Harada et al., 2004). The proposed mechanism behind this increased survival is that diminished levels of UCHL-1 result in reduced free ubiquitin, thereby sparing the degradation of normal ubiquitin targets during a stress response (i.e. anti-apoptotic proteins Bcl-2 and XIAP and pro-survival protein BDNF). The basal levels of neurogenesis observed in the SVZ result from a balance between proliferation, survival, differentiation and apoptosis. Thus, it is possible that the lower levels of UCHL-1 seen in the juvenile SVZ contribute to the robust levels of neurogenesis observed in this age group by promoting the survival of newly generated cells. UCHL-1 expression has also been linked to various forms of cancer and has been demonstrated to exert an anti-proliferative effect on tumor cells (Liu et al., 2003). Thus, the increased UCHL-1 levels in the aged SVZ may contribute to the declines in NSC/NPC proliferation with aging.

The expression levels of CRMP-4 (previously known as TOAD-64) and CRMP-5 declined in the SVZ with aging. Members of the CRMP family are highly expressed in the developing brain where they have been implicated in several aspects of developmental neurogenesis, including neuronal differentiation and axonal outgrowth (Quinn et al., 2003). CRMP-4 increases in abundance over the period of corticogenesis and subsequently decreases to relatively low levels in the adult brain (Minturn et al., 1995). While neural progenitor cells do not express CRMP-4, this protein is one of the earliest markers for postmitotic cells that have made a commitment to a neuronal phenotype (Minturn et al., 1995). Various CRMP family members, including CRMP-4 and CRMP-5, are expressed in newly generated neurons during development and in selected regions of the adult brain (Veyrac et al., 2005; Nacher et al., 2000; Seki, 2002). We and others have previously shown that many BrdU-labeled proliferative cells in the SVZ and in the dentate gyrus are CRMP-4 positive immature neurons (Seki, 2002; Sun et al., 2010). Our finding that CRMP-5 levels decline with aging is in agreement with a previous study investigating age-related protein expression patterns of various CRMP members in the rodent olfactory bulb (Veyrac et al., 2005). With regard to CRMP-4, our findings are in line with findings revealing decreased CRMP-4 levels in the dentate gyrus with aging and are likely a reflection of the diminishing levels of neurogenesis observed with aging in both of these neurogenic regions (Seki, 2002).

Microsomal protease ER60 is another protein that displays an age-associated decline in the SVZ. ER60 is an endoplasmic reticulum luminal protein that has been implicated in numerous cellular functions, including protein assembly, folding and biosynthesis (Morrice, 1998; Hughes, 1998; Celli, 2003). While ER60 is expressed in all tissues, it displays comparatively reduced expression in brain, heart and muscle tissues that exhibit relatively impaired regenerative capacity and low levels of cellular proliferation (Celli, 2003). Our finding suggests a link between the low levels of ER60 expression observed in the aged SVZ and the diminished mitotic capacity seen in this age group. Interestingly, increased ER60 expression has been demonstrated in highly proliferative tumorigenic tissue (Celli, 2003) as well as in cells that have been exposed to mitogens (Goldfien, 1991), further substantiating the association between this protein and cell proliferation.

The age-related decline in nestin expression levels observed in this study was not surprising in light of evidence showing age-related declines in SVZ cell proliferation and neurogenesis (Jin, 2003). However, it might seem surprising that our proteomic experimental paradigm did not lead to the identification of age-related changes in the expression of several other known NS/NPC-associated proteins i.e. musashi-1, hes-1, etc. Furthermore, based on previous knowledge of NS/NPC mitogens and their expression patterns in the SVZ, it would also seem likely that our proteomic experimental paradigm should have led to the identification of known growth factors such as EGF, their receptors and other proteins involved in mitogenic signaling cascades. Our study utilized standard 2-D gel analysis which resolves proteins in the molecular weight (MW) range of 15–200 kDa, with an

isoelectric point (pI) ranging from 3.5–10. Consequently, nestin, with a MW of 200–220 kDa, was not detected by the standard 2-D gels but by immunoblotting. Additionally, the MWs of known mitogens EGF, bFGF and IGF range from 6–14 kDa and fall on the cusp or completely outside of the detection limits. Likewise, NS/NPC-associated proteins musashi-1 and hes-1 have pIs in the high 9 to 10 range, which also fall right on the cusp or outside of the detection range. Thus, while the data presented in the current study provides a valuable initial survey of age-associated changes that occur in the SVZ proteome, future studies will aim to further resolve and identify a wider range of proteins by altering the MW and pI range of gels. Additionally, various prefractionation and tissue preparation techniques can be used to select for specific protein types (i.e. membrane proteins, low abundance proteins, etc.) or specific post-translational modifications (Gorg et al., 2004; Lubec et al., 2003; Taylor and Pfeiffer, 2003; Babu et al., 2004).

Age-related declines in proliferative and neurogenic behavior of NS/NPCs could be due to a loss of appropriate environmental signals in the stem cell niche or to an intrinsic reduction in NS/NPC capacity to respond to such signals. While a few of the proteins identified in this study have been previously shown to be associated with NS/NPCs (i.e. CRMP-4, CRMP-5) in the SVZ, our current findings report for the first time for age-associated changes of several proteins that play important role in regulating cell proliferation and neurogenesis in the SVZ niche. It is yet to be established, however, whether these are NS/NPC-associated proteins or proteins that are expressed by other cell types residing within the SVZ stem cell niche. While determining the specific cell types within the SVZ that express these proteins is beyond the scope of the current study, future investigations into the localization of these proteins may shed light onto their functional roles in the stem cell niche. In conclusion, this study establishes preliminary reference maps of the proteins expressed by SVZ NS/NPCs and the SVZ niche with aging and points to proteins that may underlie the diminishing proliferative capacity of stem/progenitor cells in the aging brain.

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Figure 1. Age-related decline in SVZ cell proliferation

Juvenile, adult, and aged rats were given daily i.p. injections of BrdU for a period of 7 days to label proliferating cells. 24 hours later animals were sacrificed and brains were coronally sectioned and immunostained for BrdU. Images of the SVZ of juvenile, adult and aged rats illustrate marked declines in BrdU-positive proliferating cells with age (scale bar = 0.5 mm). Using the optical fractionator method for stereological quantification, significant reductions in SVZ cell proliferation levels are observed with aging. All differences between age groups are statistically significant (* $p < 0.01 \pm \text{SEM}$, comparison between juvenile to adult and juvenile to aged; + $p < 0.05 \pm \text{SEM}$, comparison between adult and aged).

Figure 2. The expression of neural stem/progenitor cell-associated protein, nestin, is reduced with aging

Coronal sections through the SVZ of juvenile, adult and aged rats immunostained for nestin demonstrate an age-related decline in the expression of this protein. The lower panel shows immunoblots to 2-D gels of juvenile and aged SVZ protein samples demonstrating the age-induced reduction in nestin expression within the SVZ. Scale bar = 1mm.

Figure 3. Age-related alterations in the SVZ proteome

Silver-stained 2-D gels showing the proteome of the juvenile, adult and aged rat SVZ. Images represent a partial view of the gel and includes proteins that fall within the molecular weight range of 14–60kDa. Yellow arrows point to distinct proteins that are strongly expressed in the juvenile SVZ and are down-regulated with age. Red arrows point to proteins that undergo an increase in expression with aging. Arrowhead points to standard: Tropomyosin – MW 33kD, pI 5.2.

Figure 4. Proteomics experimental paradigm using 2-D gel electrophoresis and LC-ESI-MS/MS

Tissue derived from the rat SVZ (black line in Nissl stained section) is dissected free and homogenized. This area of the SVZ contains a large pool of neural stem/progenitor cells as evidenced by their staining with a nestin antibody. 2-D gels are generated from this tissue and protein expression patterns are analyzed both manually and using PDQuest 2D analysis software. Using trypsin as an enzymatic digest, proteins of interest are cleaved immediately downstream of the amino acids arginine and lysine, thereby generating peptide fragments of varying molecular weights. These peptide fragments are then subjected to LC-ESI-MS/MS to obtain amino acid sequence information, which is subsequently compared to theoretical mass spectra using a MASCOT database search in order to determine the identity of the protein.

Figure 5. Mass spectrometric identification of proteins displaying a three-fold or greater change in expression levels with aging

Proteins resolved on 2D gels that exhibit greater than a three-fold change in expression level with aging in the SVZ were identified using LC-ESI-MS/MS. To control for loading and running conditions between gels, 50ng of a purified tropomyosin standard was added to each sample preparation. The fold change in expression of a protein with aging was determined using PDQuest 2-D gel analysis software.

Figure 6. Immunohistochemistry and immunoblotting confirm the identity and expression pattern of selected proteins identified using mass spectrometric methods

GFAP-immunostained coronal sections of the juvenile and aged rat SVZ demonstrated an increased expression of this protein with aging (arrow). The lower panels represent a higher magnification view of the areas highlighted by black boxes (scale bar = 1mm). The bottom panel displays immunoblots to 2-D gels of juvenile, adult and aged SVZ protein samples that demonstrate the age-related reduction in the expression of the neuronal precursor-associated protein CRMP-4.