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REGULAR ARTICLE

Impact of aging on heat shock protein expression in the substantia nigra and striatum of the female rat

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Abstract Many heat shock proteins are chaperones that help refold or degrade misfolded proteins and battle apoptosis. Because of their capacity to protect against protein misfolding, they may help keep diseases of aging at bay. A few reports have examined heat shock proteins (eg. Hsp25, Hsp60, Hsp70, and heat shock cognate 70 or Hsc70) as a function of age in the striatum and nigra. In the present study, we examined the impact of aging on Hsp25, heme oxygenase 1 (HO1 or Hsp32), Hsp40, Hsp60, Hsc70, Hsc/Hsp70 interacting protein (Hip), 78 kDa glucose-regulated protein (GRP78), Hsp90, and ubiquitinated proteins in the nigra and striatum of the female rat by infrared immunoblotting. Female animals are not typically examined in aging studies, adding further to the novelty of our study. Striatal HO1 and Hsp40 were both higher in middle-aged females than in the oldest group. Hsp60 levels were also highest in middle age in the nigra, but were highest in the oldest animals in the striatum. Striatal levels of Hsc70 and the co-chaperone Hip were lower in the oldest group relative to the youngest animals. In contrast, Hsp25 rose with advancing age in both regions. Hsp25 was also colocalized with tyrosine hydroxylase in nigral neurons. Ubiquitinated proteins exhibited a trend to rise in the oldest animals in both regions, and K48 linkage-specific ubiquitin rose significantly from 4–6 to 16–19 months in the striatum. Our study reveals a complex array of age-related changes in heat shock proteins. Furthermore, the age-related rises in some proteins, such as Hsp25, may reflect endogenous adaptations to cellular stress.

Keywords Hormesis · U-shaped · Adaptation · Basal ganglia · Parkinson's disease

Introduction

Neurodegenerative diseases are primarily diseases of protein misfolding. For this reason, they are often called proteinopathies (Morimoto 2008; Dickson 2009; Uversky 2009). Excessive protein misfolding is known as proteotoxicity and can lead to oxidative stress, protein aggregations, and cell death. However, cells have evolved an extensive network of chaperone proteins to blunt proteotoxic stress. The best-studied examples of chaperones are the heat shock proteins, which are expressed constitutively or induced by protein denaturation (Mayer and Bukau 2005; Morimoto 2008; Lanneau et al. 2010). In addition to their well-studied role in protein folding, chaperones also escort irreparably damaged proteins to the proteasome and lysosome for removal, or activate the unfolded protein response in the endoplasmic reticulum (Szegezdi et al. 2006; Kalia et al. 2010; Lanneau et al. 2010; Aridon et al. 2011; Gorbatyuk and Gorbatyuk 2013). For example, heat shock cognate 70 (Hsc70) recognizes and guides damaged proteins with a consensus motif to the lysosome for degradation by chaperone-mediated autophagy (Arias and Cuervo 2011). In addition, some heat shock proteins, such as heat shock protein 70 (Hsp70) and Hsp25 (Hsp27 in humans), are known to reduce caspase-mediated apoptosis (Arya et al. 2007; Acunzo et al. 2012). Hsp70 is highly inducible upon protein denaturation and forms one of several complexes with other chaperones such as Hsp90, which has numerous client proteins that it stabilizes, folds, activates, and assembles (Pearl and Prodromou 2006; Johnson and Brown 2009; Aridon et al. 2011). Hsp40 stimulates the ATPase and refolding activities of Hsc70 (Hohfeld 1998; Ballinger et al. 1999), and cooperates with α-synuclein to promote neurotransmitter release (Witt 2013). The

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Hsp60 chaperonin helps convert partially folded proteins into native proteins, but mostly acts as a 'loner' with few interacting partners (Clare and Saibil 2013; Saibil 2013).

Chaperones often work in conjunction with co-chaperones that help with protein triage decisions, such as the Hsc/Hsp70-interacting protein Hip (Mayer and Bukau 2005; Morimoto 2008; Kalia et al. 2010; Lanneau et al. 2010; Aridon et al. 2011). Hip stabilizes the ADP-bound state of Hsc/Hsp70, thereby promoting chaperone/substrate interactions (Mayer and Bukau 2005). Chaperones in the endoplasmic reticulum, such as glucose-regulated protein 78 (GRP78), are also integral to protein homeostasis. In unstressed cells, GRP78 associates with protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). Following endoplasmic reticulum stress, GRP78 dissociates from these proteins, leading to the activation of the unfolded protein response (Szegezdi et al. 2006; Walter and Ron 2011). The heat shock protein heme oxygenase 1 (HO1 or Hsp32) is also induced by cellular stress and breaks down toxic heme into the antioxidant biliverdin and the prosurvival molecule carbon monoxide (Schipper 2000; Schipper et al. 2009; Aztatzi-Santillan et al. 2010; Wu et al. 2011; Grochot-Przeczek et al. 2012). Thus, some stress-induced heat shock proteins are also important for defense against oxidative stress, which can propel and propagate proteotoxicity if left unchecked.

It is important to study heat shock proteins in the brain because they may offer considerable protection against proteinopathies and cellular stress in general (Muchowski and Wacker 2005). Indeed, levels of some heat shock proteins, including ones involved in antioxidant defense, are raised in neurodegenerative conditions, perhaps reflecting an endogenous adaptation against proteotoxic and oxidative damage. For example, levels of HO1 are increased in hippocampal and cortical neurons and astrocytes in Alzheimer's victims (Schipper 2000; Schipper et al. 2006). HO1 is also increased in astrocytes in Parkinson's disease and is present in Lewy bodies (Schipper et al. 1998). Some have argued that these changes are pathogenic because HO1 also breaks down heme into ferrous iron, increasing the risk for Fenton chemistry and iron toxicity (Schipper 2011). Although heme-derived iron and carbon monoxide may increase oxidative stress (Zhang and Piantadosi 1992; Desmard et al. 2007), co-induction of apoferritin synthesis is thought to limit HO1 toxicity (Dennery 2000; Ryter and Tyrrell 2000). Furthermore, the vast majority of experimental studies of HO1 manipulation demonstrate that HO1 is protective (Calabrese et al. 2009; Jazwa and Cuadrado 2010; Zhang et al. 2013). Similar to the increases in HO1, patients with mild cognitive impairment exhibit increased Hsp70 and Hsp27 levels in the inferior parietal lobule (Di Domenico et al. 2010). Hsp27 levels are also raised in the nigrostriatal pathway of Parkinson's victims (Zhang et al. 2005). Similarly, Hsp90 levels are higher in the brain in Parkinson's disease (Uryu et al. 2006), and Hsp27, Hsp40, Hsp60, Hsp70, and Hsp90 are all major components of Lewy bodies (Auluck et al. 2002; McLean et al. 2002; Klucken et al. 2004; Kalia et al. 2010). One might speculate that at least some of these endogenous increases in heat shock proteins help delay the onset of neurodegenerative diseases or slow their progression once they have commenced. However, such defenses may collapse with aging in end-stage disease states. Furthermore, other heat shock proteins are actually decreased in neurodegenerative disorders. For example, it has been demonstrated that Hsc70 levels are reduced within nigral neurons in Parkinson's disease (Chu et al. 2009). In short, the heat shock protein response to neurodegenerative disorders is complex and likely to vary with the age of the individual, disease stage, oxidative stress levels, and many other variables.

Aging is the major risk factor for neurodegenerative diseases and is thought to lead to increased proteotoxic and oxidative stress. For example, the activity of the ubiquitin–proteasome and autophagic systems both decline with aging (Conconi et al. 1996; Keller et al. 2000b, c, 2002; Cuervo et al. 2005; Zeng et al. 2005). Furthermore, aging increases lipofuscin content in the brain, suggestive of increased autophagic stress (Goyal 1982b, a; Sulzer et al. 2008). Signs of oxidative damage are especially abundant in aged tissue and have helped inspire the free radical theory of aging (Sohal and Allen 1990; Harman 2006; Muller et al. 2007). Furthermore, aging is thought to exacerbate the damage associated with proteinopathies. Relative to age-matched controls, Alzheimer's and Parkinson's patients exhibit reductions in proteasomal activity in select brain regions (Keller et al. 2000a; McNaught and Jenner 2001; McNaught et al. 2003) and other signs of increased proteotoxicity such as hallmark protein inclusions (Braak and Braak 1995; Braak et al. 2003a, b; Thal et al. 2004). Parkinson's and Alzheimer's diseases are also strongly associated with oxidative stress (Alam et al. 1997; Gabbita et al. 1998; Jenner 2003; Zhu et al. 2005; Lovell and Markesbery 2007; Butterfield et al. 2010).

Although it is not clear why the aged brain exhibits increased proteotoxic and oxidative stress, heat shock proteins may be able to slow down age-related toxicity. If heat shock proteins that are reduced with aging can be identified, such molecules would be rational targets for gene therapies against age-related neurodegenerative disorders. Alternatively, if heat shock proteins are raised in some brain regions with aging, this may reflect endogenous defenses against proteotoxic stress that should be boosted by gene- or pharmacotherapies. The goal of the present study was to study the impact of aging on heat shock proteins in the ventral mesencephalon and striatum, because the dense dopaminergic projection from the substantia nigra to the caudoputamen is known to degenerate in Parkinson's disease (Damier et al. 1999). We examined multiple heat shock proteins and co-chaperones in female

rats ranging in age from 2 to 22 months. Female rats are not often used for aging studies, adding to the novelty of the present study. In our assessments, we relied on infrared immunoblotting, as this technique is highly sensitive and offers much higher resolution than X-ray film (Mathews et al. 2009; Aguilar et al. 2010; Jinwal and Dickey 2011). The infrared Odyssey imager has a bit-depth of 16 (2^{16} or 65,536 shades of infrared) and a 4.5 log dynamic range. In contrast, X-ray film can only resolve 150 shades of gray. Noise is also low in the infrared wavelengths, thereby leading to higher signal-to-noise ratios (Patonay and Antoine 1991).

Methods

Tissue collection Animal use was approved by the Duquesne University Institutional Animal Care and Use Committee and is in compliance with the principles outlined in the *NIH Guide for the Care and Use of Laboratory Animals*. We used female Sprague Dawley rats (Charles River, Wilmington, MA, USA) that were part of an in-house breeding colony to generate rat pup tissue for primary neuron cultures. As they did not survive well past the 22-month mark, all rats were sacrificed by this age. Animals were anesthetized with 5 % isoflurane immediately prior to decapitation and tissue microdissection. The boundaries of the substantia nigra and the striatum were defined according to the Paxinos rat atlas (Paxinos and Watson 1998). All animals were sacrificed within 48 h of each other and processed together. In addition, three young female rats (2–4 months old) were perfused with 4 % paraformaldehyde for immunofluorescent staining of heat shock proteins. Fixed brains were cryoprotected in 30 % sucrose in phosphate-buffered saline and cut at 40 μm on a freezing microtome (Microm HM450; Thermo Scientific, Pittsburgh, PA, USA).

Western blot analyses Tissue samples were weighed and sonicated in 1× Cell Lysis Buffer (20 μL/mg tissue; Cat. no. 9803; Cell Signaling, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Cat. no. P8340; Sigma-Aldrich, St. Louis, MO, USA) and 10 mM sodium fluoride (Leak et al. 2010). Laemmli buffer was added to this solution prior to heating. The final lysis buffer contained 1 % Triton and 0.7 % sodium dodecyl sulfate (SDS). Equal amounts of protein were separated by SDS-PAGE electrophoresis and transferred to Immobilon-FL polyvinylidene fluoride membranes or nitrocellulose membranes (EMD Millipore). Membranes were blocked with 5 % nonfat dry milk in Tris-buffered saline (TBS) or with Odyssey Block (Cat. no. 927-40000; LI-COR Bioscience, Lincoln, NE, USA) diluted 1:1 with phosphate buffered saline (PBS). Primary and secondary antibodies were prepared in 5 % bovine serum albumin (Cat. no. A30075; Research Products International, Mt. Prospect, IL, USA) in TBS with 0.1 % Tween 20 (Cat. no. BP337; Fisher Scientific, Pittsburgh, PA, USA) or Odyssey Block and PBS (1:1 ratio), also supplemented with 0.1 % Tween 20. Immunolabeled blots were visualized on an infrared Odyssey Imager and quantified with Odyssey Software (v.3; LI-COR Bioscience). All primary and secondary antibodies are listed in Tables 1 and 2. Depending upon the species of primary antibody and molecular weight of the heat shock protein, β-actin, α-tubulin, or GAPDH were used as protein loading controls. Several blots were probed for more than one protein; the original protein loading control was used for normalization in these instances.

Immunofluorescence Free-floating coronal sections through the midbrain and striatum were stained for Hsp25, Hsp60, and HO1 in conjunction with the dopaminergic phenotypic marker tyrosine hydroxylase. Briefly, all sections were blocked in a 50 % solution of Odyssey Block diluted in PBS for 1 h. Sections were then incubated in primary antibodies overnight at 4 °C. The following day, sections were incubated in fluorescent secondary antibodies for 1 h at room temperature after a series of washes in PBS. Sections were then washed again and mounted on glass slides, coverslipped with Fluoromount-G (Cat. no. 0100-01; Southern Biotech, Birmingham, AL, USA), and viewed with confocal microscopy (Leica TCS SP2; Wetzlar, Germany). All primary and secondary antibodies used for immunofluorescence are listed in Tables 1 and 2. For each antibody, we tested multiple dilutions in order to optimize the signal-to-noise ratio, centered around dilutions recommended by the manufacturer. For every immunostaining experiment, we included the following controls: (1) exposure to all solutions except both primary antibodies and (2) exposure to one primary antibody at a time, followed by exposure to both secondary antibodies. The latter control allowed us to determine whether there was cross-reaction between the secondary and primary antibodies.

Statistical analyses Data are presented as the mean ± standard error of the mean from 3–6 animals per age group. Animals were excluded from analysis when air bubbles during the transfer or highly fluorescent lint interfered with the western blot signal. Statistical significance was determined by one-way ANOVA followed by the least significant difference (LSD) post hoc correction (IBM SPSS Statistics, v.20; Armonk, NY, USA) when $p \leq 0.05$. Trends toward statistical significance were defined as $0.05 < p \leq 0.10$.

Results

Striatum

Hsp25 levels were higher in 19- to 22-month-old animals than at 2–4 months of age. There was also a trend for Hsp25 levels

Table 1 All primary antibodies used in the present study

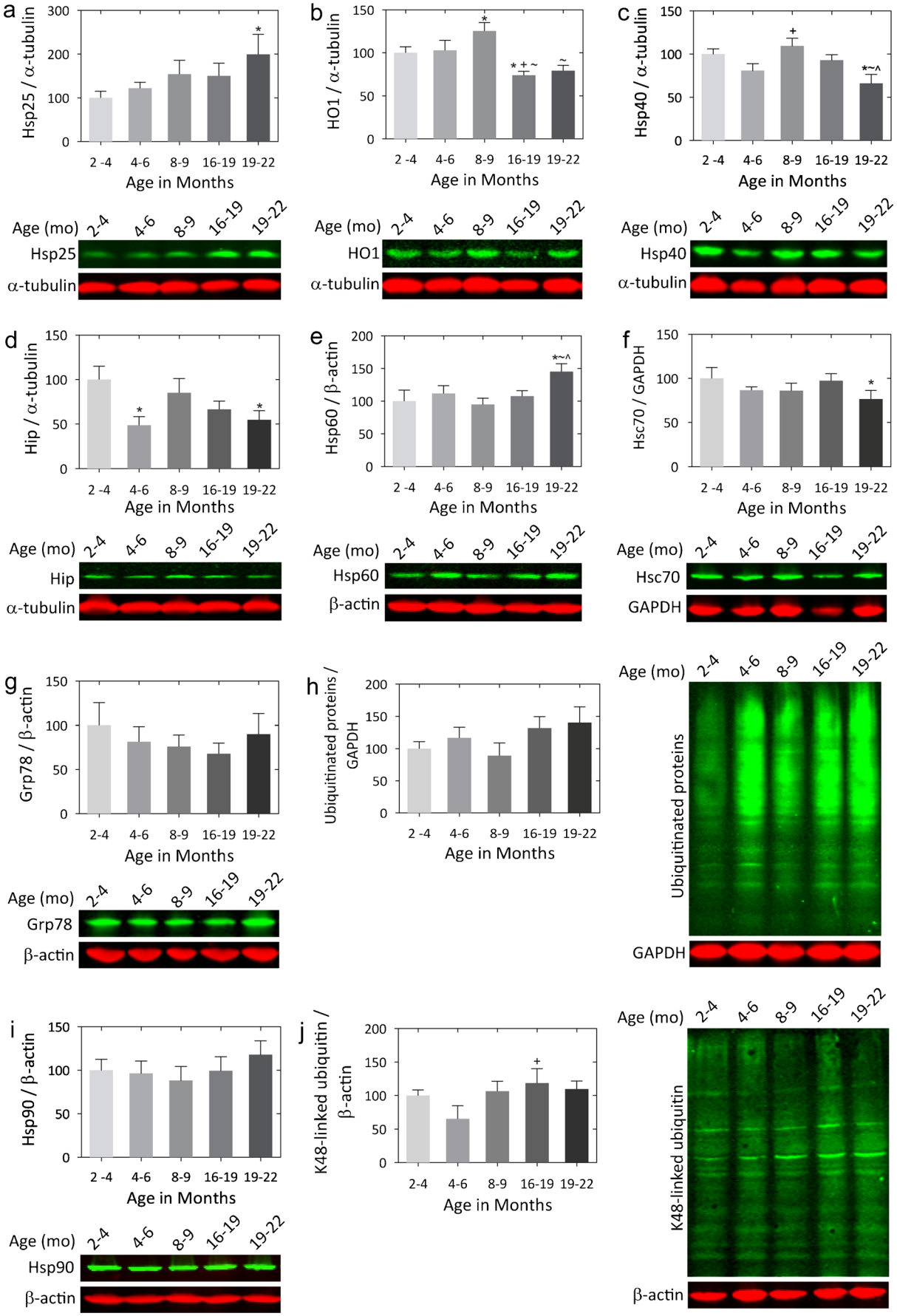
Antibody	Source	Company	Catalog #	Lot #	Western blot	Immunofluorescence
α-tubulin	Mouse	Sigma-Aldrich	T5168	078K4781	Quantified	
β-actin	Mouse	Sigma-Aldrich	A5441	030M4788	Quantified	
CHOP	Mouse	Thermo Fisher	MA1-250	OE184158	Nonspecific	
GAPDH	Rabbit	Cell Signaling Technology	21185	Ref: 09/2012 Lot: 8	Quantified	
Grp78	Rabbit	Enzo Life Sciences	ADI-SPA-826	08021219	Undetectable	
Grp78	Rabbit	Cell Signaling Technology	3177	Ref: 11/2012 Lot: 5	Nonspecific	
Grp78	Rabbit	Abcam	ab21685	GR127425-1	Quantified	
Hip	Rabbit	Cell Signaling Technology	2723	Ref: 10/2012 Lot: 1	Quantified	
Hop	Rabbit	Cell Signaling Technology	4464	Ref: 09/2012 Lot: 1	Undetectable	
Hsc70	Rabbit	Enzo Life Sciences	ADI-SPA-815		Quantified	
Hsp25	Goat	Santa Cruz	sc-1048	DO312	Quantified	Photographed (Fig. 3)
HO1 (Hsp32)	Rabbit	Sigma-Aldrich	H4535	081M1122	Quantified	Low signal
HO1 (Hsp32)	Mouse	Enzo Life Sciences	ADI-OSA-110	06051244		Low signal
Hsp40	Rabbit	Cell Signaling Technology	4868	Ref: 11/2012 Lot: 2	Quantified	
Hsp60	Rabbit	Cell Signaling Technology	4870	Ref: 10/2012 Lot: 2	Quantified	Low signal
Hsp70	Rabbit	Cell Signaling Technology	4876	Ref:10/2012 Lot:2	Undetectable	
Hsp70	Mouse	Calbiochem	386032	D00141952	Undetectable	
Hsp90	Rabbit	Cell Signaling Technology	4877	Ref: 08/2012 Lot: 3	Quantified	
PERK	Rabbit	Sigma-Aldrich	P0074	080M4876	Nonspecific	
PERK	Goat	Santa Cruz	sc-9477	B0163	Undetectable	
Phospho-PERK	Rabbit	Cell Signaling Technology	3179	Ref: 07/2013 Lot: 13	Undetectable	
Tyrosine Hydroxylase	Mouse	Millipore	MAB318	NG1723972		Photographed (Fig. 3)
Tyrosine Hydroxylase	Sheep	Millipore	AB1542	NG1721744		Photographed (not shown)
Ubiquitinated proteins	Mouse	Santa Cruz Biotechnology	sc=8017	D0412	Quantified	
K48-linked ubiquitinated proteins	Rabbit	Cell Signaling Technology	42895	Ref: 10/2013 Lot: 1	Only showed single band	
K48-linked ubiquitinated proteins	Rabbit	Millipore	05-1307	2299608	Quantified	

Table 2 All secondary antibodies used in the present study

Antibody	Company	Catalog #	Lot #
Donkey anti-Rabbit 680	LI-COR	926-68073	C20925-03
Donkey anti-Mouse 680	LI-COR	926-68072	C20919-01
Donkey anti-Mouse 800	LI-COR	926-32210	C30820-01
Donkey anti-Goat 800	LI-COR	926-32214	C00621-02
Goat anti- Rabbit 800	LI-COR	926-32211	C10406-03
Donkey anti-Mouse 488	Life Technologies	A21202	1423052
Donkey anti-Sheep 488	Life Technologies	A11015	687630
Donkey anti-Goat 546	Life Technologies	A11056	784578
Goat anti-Mouse 555	Life Technologies	A21424	1141876
Goat anti-Rabbit 555	Life Technologies	A21429	872656

to be higher in 19- to 22-month-old rats than at 4–6 months of age (Fig. 1a; $p=0.072$). HO1 levels were higher in 8- to 9-month-old middle-aged animals than at 2–4 months of age and were significantly lower in 16- to 19- and 19- to 22-month-old animals than at middle age (Fig. 1b). HO1 levels were also lower in the 16- to 19-month-old animals than in the 2- to 4- and 4- 6-month-old animals. Hsp40 levels in 8- to 9-month-

Fig. 1 Impact of aging on heat shock proteins and ubiquitinated proteins in the caudoputamen. Whole tissue lysates of striatal tissue from female Sprague–Dawley rats were probed by infrared Western blots for the indicated proteins. GAPDH, α-tubulin, or β-actin was used as a protein loading control. Infrared signal was pseudocolored *red* or *green*. *$p\leq0.05$ versus 2–4 months old, + $p\leq0.05$ versus 4–6 months old, ~ $p\leq0.05$ versus 8–9 months old, ^ $p\leq0.05$ versus 16–19 months old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Hsp25 / α-tubulin
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α-tubulin
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Hsp40 / α-tubulin
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Hsp60 / β-actin
Hsp60
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Grp78 / β-actin
Grp78
h
Ubiquitinated proteins / GAPDH
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Hsp90 / β-actin
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K48-linked ubiquitin / β-actin
K48-linked ubiquitin

old animals were higher than at 4–6 months of age and striatal Hsp40 expression in 19- to 22-month-old animals was lower than in all other groups except the 4- to 6-month-old rats (Fig. 1c). Hip fell from 2–4 to 4–6 months of age and showed a trend to rise in 8- to 9-month-old animals relative to the 4- to 6-month group (Fig. 1d; $p=0.059$). There was also a trend towards a decrease in Hip levels from 2–4 to 16–19 months of age ($p=0.083$) and Hip levels in the oldest group were significantly lower than the 2–- to 4-month-group. Hsp60 levels in 19- to 22-month-old animals were higher than all age groups except for the 4- to 6-month-old rats (Fig. 1e). Hsc70 levels were lower in 19- to 22-month-old animals than in the youngest group (Fig. 1f). Grp78 (Fig. 1g) and Hsp90 (Fig. 1i) levels were not affected by aging in this model. Ubiquitinated proteins exhibited a trend to rise in 19- to 22-month-old animals relative to the 8- to 9-month-group (Fig. 1h; $p=0.058$). This antibody recognizes all ubiquitinated proteins, including ones not destined for proteasomal removal. A second antibody that specifically recognizes ubiquitin linked at Lys48 was used to measure levels of proteins targeted for the proteasomal barrel (Ciechanover and Brundin 2003; Elsasser and Finley 2005). K48 linkage-specific ubiquitinated protein levels were significantly higher at 16–19 months of age relative to the 4- to 6-month-old group. In addition, there was a statistical trend towards a difference between the 4- to 6- and 19- to 22-month-old groups (Fig. 1j; $p=0.10$).

Substantia nigra

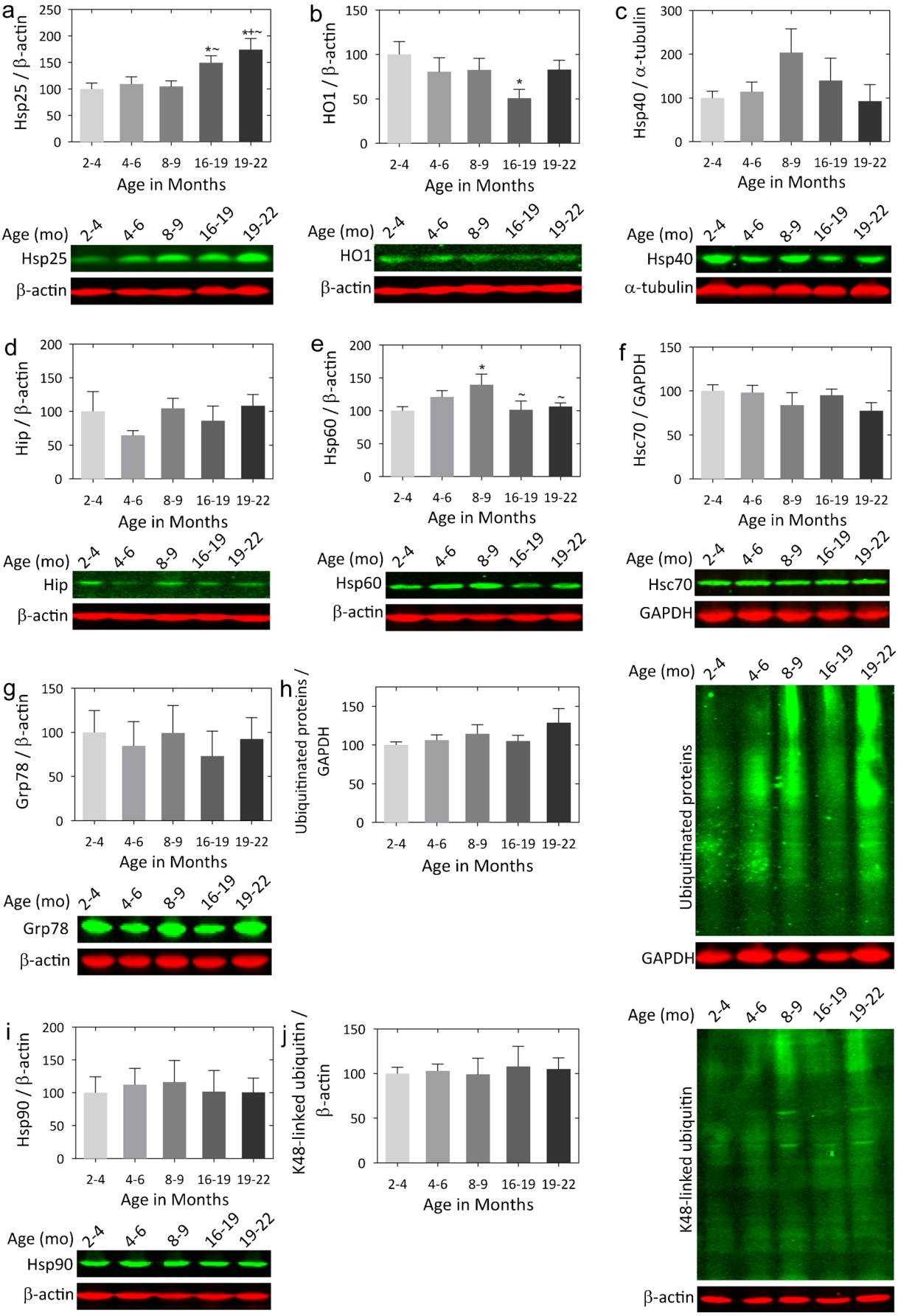
In the substantia nigra, Hsp25 levels were higher in the 16- to 19- and 19- to 22-month old animals than in the youngest group and the 8- to 9-month-old animals (Fig. 2a). In addition, Hsp25 levels in 19- to 22-month-old animals were higher than at 4–6 months of age. There was only a trend towards a difference in Hsp25 levels between 4- to 6-month-old animals and 16- to 19-month-old animals ($p=0.074$). HO1 levels exhibited a transient drop at 16–19 months of age relative to the youngest group (Fig. 2b). Hsp40 exhibited trends towards a rise in 8- to 9-month-old animals relative to the youngest group (Fig. 2c; $p=0.082$) and relative to the oldest group ($p=0.054$). Hip did not change significantly with aging (Fig. 2d). Hsp60 showed a rise in 8- to 9-month-old animals relative to the youngest group, and Hsp60 levels at 16–19 months and 19–22 months were lower than at 8–9 months (Fig. 2e). Hsc70 (Fig. 2f), GRP78 (Fig. 2g), and Hsp90 (Fig. 2i) levels were not impacted by aging in the nigra. Ubiquitinated proteins exhibited a trend to rise in 19- to 22-month-old animals relative to the 2- to 4-month group (Fig. 2h; $p=0.078$). However, K48 linkage-specific ubiquitinated proteins were not affected by aging in the nigra (Fig. 2j).

Although we probed for Hsp70 with multiple antibodies (see Table 1), levels of this protein were undetectable in the striatum and nigra. As a positive control, we ran cortical lysates for Hsp70, and found detectable levels in this brain region. As a result, we do not report any data on Hsp70. We also probed for the co-chaperone Hop and for several proteins related to the unfolded protein response. However, Hop was undetectable in striatum and nigra (but detectable in cortex), and PERK and phospho-PERK antibodies either led to many non-specific bands or exhibited lack of binding. Similarly, an antibody for the endoplasmic stress indicator C/EBP homologous protein (CHOP) was highly non-specific. A recent review of methods to detect the unfolded protein response also reports non-specificity in many antibodies for CHOP (Cawley et al. 2011). Indeed, the authors make the case that antibodies against unfolded protein response-related proteins are difficult to work with because of non-specificity and low abundance of these proteins, especially IRE1 and ATF6. GRP78 is more abundantly expressed and, after purchasing three separate antibodies, we finally discovered one antibody to be specific and are therefore able to report GRP78 levels (see above).

Fig. 2 Impact of aging on heat shock proteins and ubiquitinated proteins in the ventral midbrain. Whole tissue lysates of substantia nigra tissue from female Sprague–Dawley rats were probed by infrared Western blots for the indicated proteins. GAPDH, α-tubulin, or β-actin was used as a protein loading control. Infrared signal was pseudocolored *red* or *green*. *$p\leq0.05$ versus 2–4 months old, + $p\leq0.05$ versus 4–6 months old, ~ $p\leq0.05$ versus 8–9 months old, ^ $p\leq0.05$ versus 16–19 months old

Dual immunofluorescence

Using dual-label immunofluorescence and confocal microscopy, we attempted to stain tyrosine hydroxylase$^+$ dopamine neurons with markers against the three heat shock proteins (Hsp25, Hsp60, and HO1) that were affected by aging in the substantia nigra. Hsp60 and HO1 levels in the nigra and striatum were below the threshold for immunofluorescent detection in fixed tissue. Hsp25 immunostaining was absent from the striatum but was present at low levels in the ventral mesencephalon. Figure 3a–c illustrates Hsp25$^+$ signal within tyrosine hydroxylase$^+$ dopamine neurons in the substantia nigra, pars compacta of a young female rat (see arrows for examples). Omission of the primary antibodies for tyrosine hydroxylase and Hsp25, either simultaneously or one at a time, led to loss of signal. The omission of one primary antibody at a time, followed by incubation in both secondary antibodies, helped ensure that there was no cross-reaction between the primary and secondary antibodies. For example, Fig. 3d–f shows a negative control nigral section incubated only in the mouse anti-tyrosine hydroxylase primary antibody, followed by incubation in both donkey anti-mouse 488 nm and donkey anti-goat 546 nm secondary antibodies. Although it tripled the amount of work, these were essential controls because we initially used sheep and goat primary antibodies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K48-linked ubiquitin
β-actin

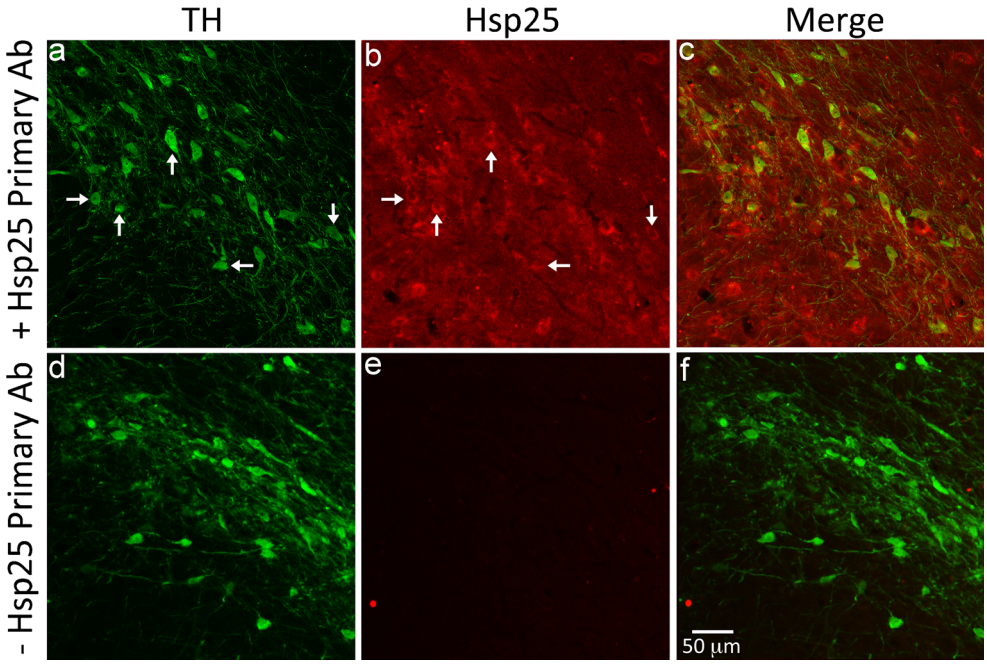

Fig. 3 Hsp25 expression in the ventral mesencephalon. Confocal analysis of Hsp25 (*red*) and TH (*green*) immunostaining in the substantia nigra, pars compacta of a young female rat (**a**–**c**). Examples of dual-labeled neurons are indicated by *white arrows*. The dual-labeled neurons do not appear yellow in the merged image on the *right* because the Hsp25 staining was weak and the images are not overly manipulated. **d**–**f** An adjacent midbrain section that was only incubated in primary antibodies against TH, followed by exposure to the same secondary antibody solutions as in (**a**–**c**). Omission of both primary antibodies at once, or one primary antibody at a time, led to loss of fluorescent signal

for tyrosine hydroxylase and Hsp25, respectively, and found that secondary staining in the red channel was still present even when we omitted primary antibodies against Hsp25 and that it looked exactly the same as the tyrosine hydroxylase staining in the green channel (see Tables 1, 2). This false positive appearance of 'dual-labeling' was the result of cross-reaction between the donkey anti-goat 546 secondary antibody with the sheep anti-tyrosine hydroxylase primary antibody, perhaps due to phylogenetic similarities between sheep and goat IgGs. Switching to mouse anti-TH antibodies eliminated the problem.

Discussion

Ours is the first report of changes in striatal and nigral heat shock proteins as a function of age in female rats. In the present study, we found that middle-aged animals expressed higher levels of HO1 and Hsp40 in the striatum than old animals, and higher levels of Hsp60 in the nigra. There was a trend for Hsp40 to rise with middle age in the nigra, reflecting an inverted U-shaped curve. With advanced aging, there was a loss in HO1, Hip, Hsp40, and Hsc70 in the striatum and a loss in HO1 and Hsp60 in the nigra. In both the nigra and the striatum, Hsp25 levels were highest in the oldest group sacrificed. Taken together, these results reveal dynamic changes in heat shock proteins in response to aging. Previous studies of changes in striatal and nigral heat shock proteins as a function of age are discussed below, as are experimental caveats of the present report.

We found a striking increase in nigral and striatal Hsp25 in the oldest animals examined. Because age-related changes in Hsp25 were apparent in both the striatum and nigra, we suspected that the changes in this protein occurred within dopamine neurons. Consistent with this interpretation, confocal analyses suggest that Hsp25 was expressed within dopamine neurons of the substantia nigra, pars compacta. In addition, Hsp25 was also expressed in non-dopaminergic cells of the ventral mesencephalon. Similar to our findings in female rats, previous studies have reported age-related increases in Hsp25 in both the striatum and substantia nigra of male Fischer 433/Brown Norway rats (Gupte et al. 2010) and in whole brain extracts from mice (Dickey et al. 2009). Dickey and colleagues examined male and female mice together when they reported age-related changes in Hsp70 and Hsp25 in whole brain extracts (Dickey et al. 2009). In primates, Hsp27 is known to rise in pallidonigral spheroids with aging (Schultz et al. 2001). The precise nature of the stimulus for this age-related rise in Hsp25/27 is not known, although we speculate that it reflects a compensatory response against cellular stress. Hsp25 is an important prosurvival protein as it enhances the catalytic activity of the proteasome particle and battles apoptosis (Lanneau et al. 2010; Acunzo et al. 2012). The natural rise in Hsp25 that we and others have observed may therefore slow down age-related declines in proteasomal function and blunt stress-induced proteotoxicity. Notably, Hsp27 levels are also raised in the nigrostriatal pathway in Parkinson's disease (Zhang et al. 2005). This response to the disease is therefore likely to be superimposed upon the age-related increase in Hsp25 that we and others have observed.

We found trends toward slight increases in ubiquitinated proteins in the striatum and nigra of aged female rats. These findings are consistent with several other reports. First, ubiquitin expression has been shown to rise in the nigra with aging

in Asian Indian populations (Alladi et al. 2010). Ubiquitin-positive degenerative dendrites are also known to rise with aging in the canine substantia nigra (Uchida et al. 2003). Further, ubiquitin is known to rise in pallidonigral spheroids of aged rhesus monkeys (Schultz et al. 2001). Finally, ubiquitin-positive axonal spheroids are common in the substantia nigra of normal elderly humans (Dickson et al. 1990). As the first antibody recognizes all ubiquitinated proteins, including ones not destined for proteasomal degradation, we also measured levels of K48 linkage-specific ubiquitin. Polyubiquitin chains linked through the Lys48 residue are most often associated with proteasomal degradation, whereas polyubiquitination through Lys63 regulates signal transduction, endocytosis, kinases, and tolerance of DNA damage (Ciechanover and Brundin 2003; Elsasser and Finley 2005, 2009; Sadowski and Sarcevic 2010). The results with the K48 linkage-specific antibody revealed no net change in the midbrain, but an increase in the striatum from 4–6 months to 16–19 months and a trend towards an increase in the oldest group. Although there seemed to be a decrease from 2–4 to 4–6 months in the striatum, this change was not significant. It is possible that levels of K48 linkage-specific ubiquitinated proteins were not lowest in the youngest group because of still-ongoing developmental events requiring proteasomal processing. Many previous studies have shown that proteasomal and autophagic activities decline with aging (Conconi et al. 1996; Keller et al. 2000b, c, 2002; Cuervo et al. 2005; Zeng et al. 2005) and that lipofuscin pigments, a sign of autophagic stress, increase in the aged brain (Goyal 1982b, a, Sulzer et al. 2008). Furthermore, there is also abundant evidence of enhanced oxidative stress with aging (Sohal and Allen 1990; Harman 2006; Muller et al. 2007). Taking into account this considerable body of literature and the well-established association of heat shock proteins with cellular stress, the present findings likely reflect age-related changes in proteotoxic and/or oxidative stress levels.

We found an age-related loss of Hsc70 in the striatum of female rats. In contrast to our observations, Calabrese and colleagues have found an age-related increase in Hsc70 in the striatum and nigra of male Wistar rats (Calabrese et al. 2004). Similarly, Unno and colleagues have found an increase in Hsc70 in the striatum in 24-month-old male Wistar rats (Unno et al. 2000). However, Gupte and colleagues reported that Hsc70 was not affected by age in either the striatum or nigra of male Fisher rats (Gupte et al. 2010). In addition, we found an age-related increase in striatal Hsp60 and a rise in nigral Hsp60 in middle-aged animals. These results also conflict with data from male rats collected by Gupte and colleageus, where no change in Hsp60 in either striatum or nigra was observed (Gupte et al. 2010). All these discrepancies may reflect differences in gender or rat strain.

Similar to our study of the striatum and nigra, Dickey and colleagues did not find an age-related increase in Hsp90 in whole-brain extracts (Dickey et al. 2009). However, we observed a rise in HO1 in the striatum of middle-aged animals and a loss in the striatum and nigra in the 16- to 19-month group. Previous studies have also shown age-related declines in HO1 in the substantia nigra of male rats (Ewing and Maines 2006). Our findings of a rise in striatal HO1 in middle age contrast with a previous study by Arumugam showing a drop in HO1 in middle-aged male mice (Arumugam et al. 2010). The discrepancies between the ventral midbrain and the striatum in our study suggest that the HO1 changes may not be occurring within dopamine neurons. Unfortunately, levels of HO1 were below the threshold for immunofluorescent detection in the ventral mesencephalon, as shown by two independent antibodies raised against HO1. Hsp60 was similarly undetectable by confocal analysis. Only the more sensitive infrared immunoblotting experiments were able to detect basal levels of these two proteins. These findings may reflect low levels of Hsp60 and HO1 in the nigrostriatal pathway under non-injured, physiological conditions or that the antibodies were not suited for antigen detection on fixed tissue.

Allawi and colleagues have shown that GRP78 protein levels rise with aging in the substantia nigra of Asian Indians (Alladi et al. 2010). The authors interpret this as evidence of endoplasmic reticulum stress. However, the authors did not distinguish between male and female subjects in their analyses. We did not observe any evidence of such changes in female rodents. Many heat shock proteins other than GRP78, such as Hsp40, Hsp25, Hsp60, Hsp70, and Hsc70, have also been shown to rise in the aged central nervous system (Lee et al. 2000; Lu et al. 2004), perhaps as a defensive measure against cumulative age-related damage. As far as we can tell, there are no previous reports of the impact of aging on striatal and nigral Hip, Hsp40, and Hsp90 in either male or female mammals. Hsp40 levels in the striatum rose in middle age relative to 4- to 6-month-old rats, and dropped in old age, in an inverted, roughly U-shaped pattern. The pattern in the nigra was similarly biphasic. Furthermore, Hsp60 levels rose in middle age in the nigra in a U-shaped pattern. Biphasic responses to stress are defined as hormesis, in which there are favorable reactions to low-level stress and negative reactions to high stress (Mattson 2008; Calabrese 2010). Given the stress-inducible nature of heat shock proteins, we interpret these natural rises in heat shock proteins in middle age to reflect mild stress on protein homeostasis. Although levels of proteotoxicity probably continue to rise into old age, Hsp40 and Hsp60 defenses may eventually fail because of overwhelming stress. This dualistic response to mild versus severe stress is the fundamental basis of U-shaped stress response curves (Mattson 2008; Calabrese 2010). Raising Hsp40 and Hsp60 with gene- or pharmacotherapies may therefore be useful to ameliorate age-related declines in proteasomal function. In contrast to these U-shaped patterns, Hsp25 continued to rise into old age. The reason for these

variations in heat shock protein responses to aging remain to be understood. It seems likely that the stimulus thresholds for their induction are different.

One weakness of the present set of experiments is that we did not ascertain protein activity levels. One can imagine that heat shock protein levels go up with aging in response to an age-related decline in activity. Another weakness is that, with the exception of Hsp25, we do not know the phenotype of the cells in which many of the heat shock proteins were expressed. The latter concern is actually a considerable weakness of all immunoblotting experiments, especially on heterogenous tissues such as the brain. Furthermore, the magnitude of changes within specific cell types can be masked by the presence of other abundant types of cells in which there is no change. Although one can usually identify the phenotype of cells by immunofluorescent staining, using this method on aged tissue is often confounded by the high autofluorescence of lipofuscin, a wear-and-tear pigment residue of failed lysosomal digestion (Brunk and Terman 2002; Sulzer et al. 2008). In addition, immunofluorescence on fixed tissue is anyway not a quantitative method anyway and is therefore not suitable for measuring age-related changes in heat shock proteins. Future studies using in situ hybridization for heat shock protein mRNA in phenotypically defined cellular populations are warranted to overcome some of these obstacles.

In conclusion, aging has a complex impact on heat shock protein defenses in female rats, leading to U-shaped response curves for some proteins, increases for some proteins, and declines in others. Future therapies against age-related proteotoxicity and oxidative stress should take endogenous changes in heat shock proteins into consideration.

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