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Heat shock protein responses to aging and proteotoxicity in the olfactory bulb

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Abstract

The olfactory bulb is one of the most vulnerable brain regions in age-related proteinopathies. Proteinopathic stress is mitigated by the heat shock protein (Hsp) family of chaperones. Here we describe age-related decreases in Hsc70 in the olfactory bulb of the female rat and higher levels of Hsp70 and Hsp25 in middle and old age than at 2-4 months. In order to model proteotoxic and oxidative stress in the olfactory bulb, primary olfactory bulb cultures were treated with the proteasome inhibitors lactacystin and MG132 or the pro-oxidant paraquat. Toxin-induced increases were observed in Hsp70, Hsp25, and Hsp32. In order to determine the functional consequences of the increase in Hsp70, we attenuated Hsp70 activity with two mechanistically distinct inhibitors. The Hsp70 inhibitors greatly potentiated the toxicity of sublethal lactacystin or MG132 but not of paraquat. Although ubiquitinated protein levels were unchanged with aging *in vivo* or with sublethal MG132 *in vitro*, there was a large, synergistic increase in ubiquitinated proteins when proteasome and Hsp70 functions were simultaneously inhibited. Our study suggests that olfactory bulb cells rely heavily on Hsp70 chaperones to maintain homeostasis during mild proteotoxic, but not oxidative insults, and that Hsp70 prevents the accrual of ubiquitinated proteins in these cells.

Keywords

olfaction; proteostasis; Hsp60; GRP78; HO1; CHIP; Hop; Hsp90

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Introduction

Disruptions in the sense of smell adversely affect health and safety and are surprisingly common in a large number of age-related proteinopathies, such as Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies, frontotemporal dementias, corticobasal syndrome, progressive supranuclear palsy, multiple system atrophy, amyotrophic lateral sclerosis, and Huntington's disease (Attems *et al.* 2014). Furthermore, individuals who will go on to develop Parkinson's and Alzheimer' disease can become hyposmic or anosmic years before the onset of motor or cognitive deficits, suggesting that olfactory dysfunction has the potential to serve as an early biomarker of neurodegenerative disease (Ponsen *et al.* 2010, Morley & Duda 2010, Luzzi *et al.* 2007, Bohnen *et al.* 2010). The molecular basis of smell disruptions in the conditions listed above is likely due to the protein misfolding (i.e., proteotoxicity) that develops early in the olfactory system (Dickson 2009, Braak *et al.* 2003a, Daniel & Hawkes 1992, Pearce *et al.* 1995). For example, neurofibrillary tangles and Lewy pathology are thought to gain a foothold in the olfactory bulb and anterior olfactory nucleus long before similar protein aggregates appear deeper in the cerebral cortex or basal ganglia (Attems et al. 2014, Ohm & Braak 1987, Braak et al. 2003a, Daniel & Hawkes 1992, Pearce et al. 1995, Braak *et al.* 2003b, Hawkes *et al.* 2007). Furthermore, olfactory impairments in neurodegenerative disorders correlate with significant decreases in olfactory bulb volume (Brodoehl *et al.* 2012, ter Laak *et al.* 1994, Muller *et al.* 2002, Wang *et al.* 2011). With the recent appreciation of non-cardinal symptoms in Parkinson's and Alzheimer's disease, there is a growing interest to identify therapeutics against pathologies in understudied brain regions such as the olfactory bulb. However, there are few studies on the functional impact of proteinopathic stress on the olfactory bulb and whether it mounts any endogenous defenses that might be responsible for the delayed onset and slow progression of neurodegenerative disorders. Therefore, one goal of the present study was to determine whether olfactory bulb cells engage natural defenses against proteotoxic or oxidative stress.

Aging is the major risk factor for neurodegenerative disorders and is associated with proteotoxic and oxidative stress, which act in a positive feedback loop (Keller *et al.* 2000b, Cecarini *et al.* 2007). For example, aging is associated with an increase in protein inclusions in the olfactory bulb (Attems *et al.* 2005). One of the major endogenous defenses against the formation of such inclusions is the heat shock protein (Hsp) family of molecular chaperones. Hsps such as Hsp70 and heat shock cognate 70 (Hsc70) help refold misfolded proteins or guide irreparably damaged proteins to the proteasome or lysosome for clearance (Lanneau *et al.* 2010, Kalia *et al.* 2010). In addition, Hsp70 and a small Hsp, Hsp25, are both known to inhibit apoptosis (Stetler *et al.* 2009, Beere 2001). The impact of aging on Hsps has been reported primarily for regions affected in neurodegenerative disorders, such as the cortex, hippocampus, striatum, and ventral mesencephalon (Leak 2014). In contrast, the impact of aging on Hsps in the olfactory bulb is poorly understood. Thus, our second major goal was to characterize age-related changes in Hsps and co-chaperones in the olfactory bulb.

Chaperones and co-chaperones are both essential players in protein triage (Lanneau et al. 2010, Kalia et al. 2010, Hohfeld *et al.* 2001, Frydman & Hohfeld 1997). For example, Hsp70 proteins form complexes with Hip, Hop, Hsp40, and/or Hsp90 during substrate

refolding (Esser *et al.* 2004, Frydman & Hohfeld 1997). When proteins must be degraded, Hsp70 complexes with Hsp90 and the E3 ubiquitin ligase CHIP (Esser et al. 2004, Ballinger *et al.* 1999). Resident members of these complexes help regulate Hsp70 activity: Hsp40 activates the ATPase activity of Hsp70 (Bukau & Horwich 1998, Minami *et al.* 1996), and Hsp90 stabilizes, folds, and activates numerous client proteins downstream of Hsp70 (Pearl & Prodromou 2006). Hip, also known as Hsp70-interacting protein, prolongs ADP-Hsp70 substrate complexes (Frydman & Hohfeld 1997, Hohfeld *et al.* 1995), whereas Hop, the Hsp70-organizing protein, transfers client proteins from Hsp70 to Hsp90 (Scheufler *et al.* 2000). A characterization of the impact of aging on these chaperones is warranted because loss of effective protein triage is the major hallmark of age-related neurodegenerative disorders (Leak 2014).

Our third goal was to examine the functional role of Hsp70/Hsc70 under conditions of cellular stress in olfactory bulb neurons. To this end, we developed a primary culture model of the olfactory bulb and elicited cellular stress with the proteasome inhibitors lactacystin and MG132 and the redox-active agent paraquat. Proteasome activity is impaired in the course of aging (Keller *et al.* 2002) and proteasome activity is reduced in both Parkinson's and Alzheimer's disease (Keller *et al.* 2000a, McNaught *et al.* 2002), providing justification for the use of proteasome inhibitors. In addition, neurodegenerative disorders and aging are both strongly linked to oxidative stress (Butterfield *et al.* 2010, Lovell & Markesbery 2007), justifying the use of paraquat. Paraquat has been used as an herbicide and is associated with an increased risk for developing Parkinson's disease (Tanner *et al.* 2011). Here we tested the hypothesis that loss of Hsp70 activity would potentiate the loss of olfactory bulb neurons in response to mild cellular stress. Low concentrations of proteasome inhibitors and paraquat that did not lead to significant loss of neuron numbers on their own were applied so that we could resolve a synergistic potentiation of cell loss in response to Hsp70/Hsc70 inhibition. As these concentrations of toxins raised Hsp70 levels, we were able to determine if inhibition of Hsp70 activity would exacerbate mild proteotoxic and oxidative stress. If this hypothesis were supported, the results would reinforce the idea that olfactory bulb cells lean on this chaperone to help preserve homeostasis.

Experimental Procedures

Additional methods can be found under Supplementary Information.

Animals

Animal use was approved by the Duquesne University Institutional Animal Care and Use Committee (protocol number 1306-21) and was in compliance with the principles outlined in the *NIH Guide*. For the aging study, we used female Sprague Dawley rats (Charles River Labs, Wilmington, MA) that formed part of a breeding colony designed to generate rat pups for our postnatal cultures (see below). Dams were all sacrificed together at ages spanning 2-22 months, as the aged females did not survive well past the 22-month mark. All animals had *ad libitum* access to food and water and were housed in a 12:12 light-dark cycle.

Primary Olfactory Bulb Cultures

Olfactory bulb cells were harvested from day 1-2 postnatal rat pups with modifications to previously described methods (Posimo *et al.* 2013). Dissociated olfactory bulb cells were plated in Neurobasal-A medium supplemented with 2% v/v B27 (Life Technologies, Grand Island, NY) and 4 mM L-glutamine. Cultures were treated with the proteasome inhibitors MG132 and lactacystin and with the oxidative toxin paraquat (see Supplementary Methods) on day-*in-vitro* 5 (DIV5) and assayed on DIV7 with three independent viability assays.

Viability Assays and Immunocytochemistry

We used three assays for structure and function to quantify viability in a blind and unbiased manner: the infrared DRAQ5 stain for nuclei, infrared immunocytochemistry for the neuronal marker microtubule associated protein 2 (MAP2), and a luminescent assay for ATP levels, as previously described (Posimo et al. 2013, Posimo *et al.* 2014). For high-magnification visualization of neurons, synapses, and astrocytes, olfactory bulb cultures were immunostained with mouse anti-MAP2 in conjunction with rabbit anti-synaptophysin or rabbit anti-glial fibrillary acidic protein (GFAP). Antibodies are listed in **Supplementary Tables 1** and **2**. Antibody binding was visualized with fluorescent secondary goat/donkey anti-mouse IgGs (488 nm) and secondary goat anti-rabbit IgGs (555 nm). Nuclei were stained with the Hoechst reagent (Sigma-Aldrich). As a negative control for the immunostaining, primary antibodies were omitted in all assays to ensure loss of fluorescent signal. Two blinded observers counted $MAP2^+$ and $Hoechst^+$ profiles from images captured on an epifluorescent microscope (EVOS, Life Technologies).

Statistical Analyses

Data are presented as the mean ± SEM from 3-6 animals per age group for the *in vivo* arm of the experiments. The Grubb's outlier test was performed once on all the data. For the infrared Western blotting, protein bands with fluorescent lint or air bubbles during transfer were also excluded from further analysis. Statistical significance for the *in vivo* Western blotting data was determined by one-way ANOVA followed by the LSD *post hoc* correction (IBM SPSS Statistics, Version 20, Armonk, NY). The *in vitro* Western blotting data were analyzed by the two-tailed *t* test. The remaining *in vitro* viability data were analyzed by one-, two-, or three-way ANOVA followed by the Bonferroni *post hoc* correction. With the exception of immunoblotting, all *in vitro* experiments were performed in triplicate wells. Data from these triplicate wells was averaged to generate an “n” of 1. Each experiment was then repeated on at least 3 completely independent occasions. Differences were deemed significant only when $p \leq 0.05$.

Results

Age-related changes in chaperones and chaperone-associated proteins in the olfactory bulb

We assessed the impact of age on several Hsps and co-chaperones in olfactory bulb tissue from female rats (**Fig.1**). An increase in Hsp25 was observed when animals transitioned from early youth (2-4 months) to 4-6 months of age and high Hsp25 levels were sustained

into old age (19-22 months). A transient decrease in Hsp40 levels was observed in the 16-19 month group. A modest trend towards a rise in Hsp40 levels was also observed from 16-19 to 19-22 months of age ($p = 0.090$). In contrast, Hsp70 levels rose at 8-9 months and at 19-22 months relative to the youngest, 2-4 month-old group. Relative to the 2-4 month-old group, there was a significant decrease in Hsc70 from 16 months of age onwards. Glucose-regulated protein 78 (GRP78), an endoplasmic reticular Hsp70 family member (Otero *et al.* 2010), exhibited high levels of expression only in 2-4 and 8-9 month old animals.

No age-related changes were observed in the co-chaperone CHIP or the heat shock inducible protein heme oxygenase 1 (HO1; also known as Hsp32; see **Fig.S1**), a protein important for antioxidant defense (Motterlini & Foresti 2014). The chaperone-associated proteins Hip and Hop also remained unaffected by age, as were levels of Hsp60, which facilitates the folding of select proteins in eukaryotes (Horwich *et al.* 1993, Ryabova *et al.* 2013). However, there was a trend towards a decline in Hop levels between 4-6 and 19-22 months of age ($p = 0.062$; **Fig.S1**). Mitochondrial Hsp70 (mtHsp70) and Hsp90 remained unchanged with age, although a trend towards lower expression of mtHsp70 was evident in the oldest group relative to the 8-9 month old animals ($p = 0.077$).

Modeling proteotoxic and oxidative stresses in primary olfactory bulb cultures

We next developed primary cultures of the olfactory bulb to conduct mechanistic analyses of the Hsps in this model system. Blinded counts from two independent observers indicated that ~67% of Hoechst$^+$ cells in these cultures also expressed the neuronal phenotypic marker MAP2 (first observer: 66.9%; second observer: 67.1%; n = 3 independent experiments). The synaptic protein synaptophysin was also densely expressed in olfactory bulb cultures, especially in puncta abutted against the MAP2$^+$ postsynaptic dendrites (**Fig.2**). The remaining, MAP2$^-$ cells appeared to be largely astrocytic as many expressed the glial marker GFAP. These findings are consistent with our previous work on postnatal cultures (Posimo et al. 2013), and with the observation that astrocyte births peak neonatally (Bayer & Altman 1991, Miller & Gauthier 2007).

In order to model proteotoxic and oxidative stress, primary olfactory bulb cultures were treated with increasing concentrations of lactacystin, MG132, and paraquat for 48h and assayed by three independent, unbiased viability measures (**Fig.3**). The strengths and weaknesses of these relatively high-throughput viability assays have been discussed before (Posimo et al. 2013, Posimo et al. 2014, Unnithan *et al.* 2012). The In-Cell Western analysis of the neuronal marker MAP2 was the most sensitive of the three measures, consistent with previous studies (Posimo et al. 2013, Posimo et al. 2014). Notably, lethal concentrations of MG132 elicited greater loss of ATP than lethal concentrations of lactacystin. Thus, the two proteasome inhibitors elicited somewhat different biochemical responses, which may arise from distinct compensatory responses (Mu *et al.* 2008). These divergent patterns support the use of distinct assays to gain a more comprehensive view of cellular integrity as well as the use of two independent classes of proteasome inhibitors to induce proteotoxic stress.

To ensure that MAP2$^+$ neurons were killed, a blinded observer counted MAP2$^+$ cell numbers after toxin treatments (**Fig.S2**). A comparison of Fig.3 and Fig.S2 reveals that the In-Cell Western analyses exhibited slightly greater loss of signal than the cell count data in

response to all three compounds. The microscopic images showed that this difference was not attributable to a loss of MAP2 expression per cell, but possibly due to a loss of dendritic profiles in the treated groups (**Fig.S2B**). However, the differences between the two assays were slight and the loss of $MAP2^+$ signal in the In-Cell Western assay was largely attributable to a loss in neuron numbers, as expected.

Mild proteotoxic and oxidative stress increase Hsp levels in primary olfactory bulb cells

Next, we measured the levels of various Hsps by immunoblotting following mild proteotoxic and oxidative stress (**Fig.4** and **Fig.S3**). To accomplish this goal, we chose concentrations of lactacystin (1 μM), MG132 (0.0625 μM), and paraquat (6.25 μM) that were half of the lowest concentration to elicit loss of MAP2 in the curves from Figure 3. HO1 and Hsp70 levels were increased by all three compounds, whereas Hsc70 was unaffected. Hsp25, Hop, and GRP78 were significantly increased by low concentrations of the two proteasome inhibitors but were not significantly affected by paraquat treatment. Hsp40 and Hsp60 levels were only increased by MG132. CHIP was only significantly increased by lactacystin. MG132 and paraquat both increased Hsp90 levels. Hip and mtHsp70 were not detectable in the *in vitro* model. These findings indicate toxin-dependent adaptive changes in Hsps and chaperone cofactor levels in the olfactory bulb culture model.

Inhibition of Hsp70 exacerbates proteotoxicity in primary olfactory bulb cultures challenged with proteasome inhibitors

Our Western blotting data confirmed that Hsp70 was increased *in vitro* by low concentrations of MG132, lactacystin, and paraquat. If Hsp70 was responding to these insults in a compensatory fashion, inhibition of Hsp70 under mild proteotoxic and oxidative conditions should exacerbate cell loss. In order to test this hypothesis, we treated olfactory bulb cultures with low concentrations of lactacystin, MG132, and paraquat in conjunction with the previously characterized Hsp70/Hsc70 inhibitor VER155008 (Schlecht *et al.* 2013, Massey *et al.* 2010, Saykally *et al.* 2012, Chatterjee *et al.* 2013, Williamson *et al.* 2009, Macias *et al.* 2011). As expected, the toxicity of low concentrations of lactacystin and MG132 was potentiated when Hsp70/Hsc70 activity were inhibited with VER155008 (**Fig. 5A-H**). That is, the DRAQ5 and MAP2 assays were in agreement that both proteasome inhibitors were significantly more toxic when Hsp70/Hsc70 activity was reduced. However, the cells were more sensitive to MG132 than lactacystin. VER155008 was also slightly toxic by itself, as revealed by the ATP and MAP2 assays. Furthermore, VER155008 only potentiated ATP loss in the MG132-treated cells, suggesting again that olfactory bulb cells were more sensitive to MG132 than lactacystin when Hsp70 activity was inhibited.

As with Hsp70, the Western blotting data had shown that HO1 levels were increased by all three compounds. Therefore, we also applied the HO1 inhibitor tin protoporphyrin (SnPPx) (Drummond & Kappas 1981) to olfactory bulb cells in conjunction with low concentrations of lactacystin and MG132 (**Fig.5A-H**). SnPPx failed to modify the toxicity of either proteasome inhibitor, although it was significantly toxic under basal conditions and elicited ATP loss. These experiments support the view that HO1 induction by MG132 and lactacystin is not as protective as Hsp70 induction in olfactory bulb cells.

We next delivered the selective Hsp70/Hsc70 inhibitor MAL3-101 in conjunction with two low concentrations of MG132 in order to validate the VER155008 findings. MAL3-101 inhibits Hsp40-stimulated ATP hydrolysis, thereby interfering with Hsp70/Hsc70 chaperone activity (Huryn *et al.* 2011, Braunstein *et al.* 2011, Hatic *et al.* 2012, Kilpatrick *et al.* 2013, Adam *et al.* 2014, Fewell *et al.* 2004). In contrast, because VER15508 binds to the ATP binding site in the chaperone, it will inhibit all Hsp70/Hsc70-mediated activities. This includes ATP binding and hydrolysis, peptide binding, and possibly the interaction with Hsp40 co-chaperones and chaperone-specific nucleotide exchange factors. In contrast, some chaperone activities specifically require the action of the Hsp40 co-chaperones, which are able to bind to select polypeptide substrates and activate Hsp70/Hsc70 ATPase activity (Kampinga & Craig 2010). Therefore, if MAL3-101 exerts a similar effect as VER15508, one may assume that it is this more specific activity of Hsp70/Hsc70 that is disabled and consequently leads to cellular toxicity. As expected, MAL3-101 potentiated MG132 toxicity according to all three viability assays (**Fig.5I-M**). Similar to VER155008, MAL3-101 was also toxic on its own.

In order to test the hypothesis that the paraquat-triggered increase in Hsp70 also protects olfactory bulb cells against oxidative damage, we delivered paraquat in conjunction with the two Hsp70/Hsc70 inhibitors. Neither VER155008 nor MAL3-101 significantly increased the toxicity of paraquat (**Fig.S4**). Paraquat toxicity was also unchanged in the presence of the HO1 inhibitor SnPPx. These data suggest that the paraquat-induced increases in Hsp70 or HO1 do not significantly prevent loss of homeostasis, or that the increase in Hsps is overwhelmed by other cytotoxic effects of this electrophilic compound.

Hsp70 inhibition potentiates an increase in ubiquitinated proteins during mild proteotoxic stress

Under conditions of severe proteotoxic stress that overwhelm the proteasome, the levels of ubiquitinated proteins will rise because they cannot be degraded efficiently. However, if the proteotoxic stress is mild, compensatory increases in chaperones may help refold damaged proteins or accelerate their removal (for example, see Mu et al. 2008). Thus, under mildly stressed, homeostatic conditions one would not expect a net increase in ubiquitinated proteins. To examine which of these scenarios was evident in our *in vivo* and *in vitro* models, we visualized ubiquitinated protein levels in olfactory bulb tissue from aged female rats and in olfactory bulb cultures treated with low concentrations of lactacystin, MG132, and paraquat (**Fig.6A-B**). No net change in ubiquitinated proteins was observed, confirming that protein homeostasis was not severely compromised.

We showed above that Hsp70 inhibition potentiated the toxicity of lactacystin and MG132. The magnitude of the effect was higher when the Hsp70/Hsc70 inhibitor was co-administered with MG132, as opposed to lactacystin. Therefore, we tested the hypothesis that Hsp70 inhibition in MG132-treated cells would elicit a net increase in ubiquitinated proteins due to severely compromised homeostasis. In support of this hypothesis, ubiquitinated protein levels greatly increased when MG132-treated cells were also exposed to VER155008 (**Fig.6C,D**). Two different ubiquitin antibodies confirmed the robustness of these effects. The first antibody recognizes pan-ubiquitinated proteins (**Fig.6C**), and the

second recognizes only K48-linked ubiquitin (**Fig.6D**), which specifically targets proteins for proteasomal degradation (Sadowski & Sarcevic 2010). These findings suggest that there is sufficient protein misfolding to overwhelm the proteasome when Hsp70/Hsc70 activity is inhibited in olfactory bulb cultures.

Taken together, our studies reveal the natural resilience of olfactory bulb cells to proteotoxic insults, but also indicate that the magnitude and type of a specific stress can overwhelm cellular homeostasis and lead to cell death.

Discussion

This report represents the first characterization of Hsps- and chaperone-associated proteins in the olfactory bulb of female rats as a function of age and in response to specific stressors. We discovered that Hsp25 and Hsp70 protein levels are higher at middle and old age than at 2-4 months, and that aging decreases the levels of constitutive Hsc70 and transiently decreases the co-chaperone Hsp40. GRP78 levels were higher in young and middle-aged animals, perhaps magnified by endoplasmic reticulum stress during development and at the peak of middle age. Indeed, endoplasmic reticulum stress is known to occur as a natural outcome during specific developmental stages (Rutkowski & Hegde 2010). No other age-related changes in Hsps were observed *in vivo*. Next, to examine the functional role of Hsp70 in olfactory bulb cells, we developed a primary culture model from dissociated tissue. By applying low concentrations of lactacystin, MG132, and paraquat, we discovered that Hsp70 and HO1 levels were both significantly increased by mild proteotoxic and oxidative stress in olfactory bulb cells. Furthermore, Hsp25, CHIP, Hsp40, Hsp60, Hop, and GRP78 levels increased when proteotoxic, but not oxidative stress was triggered, whereas no change in Hsc70 was observed after treatment with any of our tested compounds. These findings show an imperfect overlap between the stress of aging *in vivo* and proteotoxic and oxidative stress *in vitro*. On the other hand, the *in vitro* model may mimic aspects of age-related proteinopathies, as several of the Hsps whose levels rose *in vitro* (e.g., HO1, Hsp70, Hsp25) are similarly increased in postmortem tissue from Alzheimer's and/or Parkinson's patients (Hauser *et al.* 2005, Renkawek *et al.* 1999, Lee *et al.* 2008, Di Domenico *et al.* 2010, Hoozemans *et al.* 2005, Braak *et al.* 2001, Schipper *et al.* 2006).

In our *in vitro* model, synergistic exacerbations were evident when mild proteotoxic stressors (i.e., low concentrations of MG132 or lactacystin) and Hsp70/Hsc70 inhibitors were combined. These data strongly suggest that the chaperones mitigate toxic effects when select stress conditions are applied. Because constitutive Hsc70 protein levels were unaffected, the chaperone inhibitors most likely potentiate cell loss *via* inhibition of Hsp70. In addition, the level of K48-linked ubiquitinated proteins was increased by low concentrations of MG132 only when Hsp70 activity was simultaneously inhibited. These data suggest further that Hsp70 prevents a toxic buildup of misfolded proteins under conditions of mild injury and are consistent with Hsp70 being one of many contributors to protein homeostasis, or “proteostasis” (Balch *et al.* 2008). If these findings are generalized to the *in vivo* condition, they support the idea that Hsp70 may preserve proteostasis in the olfactory bulb, perhaps even at middle and old age. We also speculate that disease-related increases in this protein may delay the onset of cell loss in neurodegenerative disorders.

Accordingly, this model may explain the slow, progressive nature of many neurodegenerative disorders. Future studies to modulate Hsp activity or levels *in vivo* and determine the effects of Hsp70 on age- or disease-related neurodegeneration and loss of olfaction are warranted.

The literature on Hsp expression in aging and in neurodegenerative conditions is mixed and the effects depend upon the Hsp in question, the brain region, and the underlying condition, among other factors (Leak 2014). For example, Hsp90 is decreased in the temporal cortex in Alzheimer's disease (Yokota *et al.* 2006) but increased in the cingulate cortex in Parkinson's disease (Uryu *et al.* 2006). GRP78 is reportedly increased in Alzheimer's disease in the temporal cortex (Hoozemans et al. 2005) and the substantia nigra with aging (Alladi *et al.* 2010). However, other studies have shown a decrease in GRP78 in the cortex, striatum, and hippocampus with aging (Arumugam *et al.* 2010, Paz Gavilan *et al.* 2006, Hussain & Ramaiah 2007). In contrast, Hsp27 expression is consistently increased in neurodegenerative conditions and with aging (Zhang *et al.* 2005, Renkawek et al. 1999, Shinohara *et al.* 1993, Dickey *et al.* 2009, Gupte *et al.* 2010). Although we did not observe any change in several Hsps, including Hsp90, the present study did show similar stress-induced increases in Hsp25 as in the abovementioned literature.

The present study reveals that Hsp70 expression is higher in olfactory bulb cells mildly stressed with lactacystin, MG132, and paraquat. Hsp70 levels were also higher in 8-9- and 19-22-month old animals than in the youngest group. Some investigators have reported increases in Hsp70 expression with age in the cortex, striatum, hippocampus, and cerebellum (Calabrese *et al.* 2004), whereas others have reported losses in Hsp70 with aging in the cortex and striatum (Arumugam et al. 2010), in olfactory neurons (Getchell *et al.* 1995), and in the inferior colliculus (Helfert *et al.* 2002). An increase in the expression of the major genes that encode Hsp70 proteins (HSPA1A and HSPA1B) has been found in the substantia nigra in patients with Parkinson's disease, progressive supranuclear palsy, and frontotemporal dementia with Parkinsonism (Hauser et al. 2005). Furthermore, Hsp70 is more abundant in the inferior parietal lobule of patients with mild cognitive impairment, a potential precursor to Alzheimer's dementia (Di Domenico et al. 2010). Notably, Hsp70/Hsc70 colocalizes with protein inclusions in both Alzheimer's and Parkinson's diseases, perhaps in an endogenous attempt to ameliorate these protein aggregations (Sherman & Goldberg 2001, Kalia et al. 2010). Furthermore, Hsp70 decreases β-amyloid and MPTP toxicity and is protective in numerous experimental models of neurodegeneration (Kumar *et al.* 2007, Muchowski & Wacker 2005, Magrane *et al.* 2004, Dong *et al.* 2005). Our data are consistent with these findings and agree with other reports showing that Hsp70 induction is a “common response to mitigate the toxic effects of misfolded protein” (Hauser et al. 2005).

We previously found an age-related loss in Hsc70 in the striatum of female rats (Gleixner *et al.* 2014), consistent with the present study. Hsc70 levels have also been reported to fall in the temporal cortex in Alzheimer's patients (Yoo *et al.* 2001), particularly in the entorhinal cortex and hippocampus (Silva *et al.* 2014), and in the substantia nigra in Parkinson's patients (Alvarez-Erviti *et al.* 2010, Chu *et al.* 2009, Mandel *et al.* 2005). Unlike Hsp70, Hsc70 levels were unaffected by low concentrations of MG132, lactacystin, or paraquat in the present study. Furthermore, aged animals had higher Hsp70 levels but lower Hsc70

levels than the very youngest group. These findings suggest that our *in vitro* model does not capture all the sequelae of aging or development *in vivo*, or that the concentrations of these reagents were too low to elicit loss of Hsc70. The contrast between Hsp70 and Hsc70 in the present study may reflect the classic distinction between stress-inducible (Hsp70) and constitutively expressed (Hsc70) proteins. On the other hand, it must be noted that the Hsc70 response to stress is quite variable; some studies have shown *increases* in Hsc70 with age and proteinopathic disease states. For example, Hsc70 mRNA levels are higher in blood samples from Parkinson's and Alzheimer's patients than from control subjects (Molochnikov *et al.* 2012). Furthermore, studies in male rats indicate an increase in the basal level of Hsc70 in the pons, medulla, striatum, and thalamus (Unno *et al.* 2000). Others have also reported an age-related increase in Hsc70 in the striatum and substantia nigra of male rats (Calabrese et al. 2004). Finally, Hsc70 mRNA-expressing neurons become denser in the human hippocampus with aging (Tohgi *et al.* 1995). These discrepancies could reflect differences in gender, rodent strain, cell type, brain region, and/or species.

Only few studies to date have examined Hsps in the olfactory system. A proteomics study of the olfactory bulb demonstrated an increase in age-related protein oxidation in this structure and a 2.3-fold increase in Hsc70 levels in the olfactory bulb of aged male mice (Vaishnav *et al.* 2007). Again, the difference between our *in vivo* experiments and the previous study might reflect gender differences. A subset of olfactory receptor neurons expresses high levels of Hsp70/Hsc70 (Carr *et al.* 1994, Carr *et al.* 1999). Furthermore, Getchell and colleagues reported a down-regulation of Hsp70/Hsc70 in olfactory sensory neurons in older individuals and in patients with Alzheimer's disease (Getchell et al. 1995). As might be expected for a stress-inducible protein, Hsp70 is increased in olfactory bulb tissue in response to hypothermia, heat shock, and water deprivation (Frenkel *et al.* 2008, Kaneko & Kibayashi 2012). Following damage to olfactory receptor neurons, Hsp25 is highly expressed in astrocytes of olfactory bulb glomeruli (Hirata *et al.* 2008). As in the present study, these reports reveal dynamic changes in Hsps in the stressed olfactory system. However, our study is the first mechanistic exploration of the functional consequences of this stress response in cells from this structure.

A number of caveats in the interpretation of our collective data are worth noting. First, the *in vitro* cultures are mostly neuronal, whereas astrocytes are the major cell type in the brain (Sofroniew & Vinters 2010). However, some astrocytes remained in our postnatal cultures, perhaps better reflecting the *in vivo* milieu than pure, neuronal embryonic cultures. Second, the *in vitro* model involves dissociated, neonatal cells and can never fully represent an adult or aged intact brain. Postnatal neurons are acutely injured and do not suffer from a lifetime of accumulated insults. Third, some of the changes elicited with MG132 were more robust than with lactacystin. MG132 can inhibit cellular cathepsins and calpains in addition to its effect on the chymotrypsin-like activity of the proteasome (Lee & Goldberg 1998), and different effects on proteostasis have been observed with these compounds (Mu et al. 2008). Although lactacystin is generally held to be more specific, it can also inhibit cathepsin A (Aikawa *et al.* 2006, Kisselev & Goldberg 2001). One might add that autophagic stress, through interference with cathepsins, may be viewed as desirable when modeling proteinopathic diseases (Nixon & Yang 2012). Fourth, Hsp70 ATPase activity could not be

measured directly in our model because numerous ATPases are present in cellular lysates. On the other hand, VER155008 and MAL3-101 are well-characterized inhibitors of Hsp70/Hsc70. VER155008 is an adenosine-derived ATP-competitive inhibitor that arrests the nucleotide-binding domain of Hsp70/Hsc70 in a half-open conformation (Schlecht et al. 2013). The downstream effect of Hsp70/Hsc70 inhibition with VER155008 includes autophagy inhibition, caspase-dependent and independent apoptosis, and Hsp90 client protein degradation (Massey et al. 2010, Budina-Kolomets *et al.* 2013). MAL3-101 is a pyrimidinone that inhibits Hsp40-stimulated Hsp70/Hsc70 ATPase activity (Fewell et al. 2004). Our findings on the impact of MAL3-101 on MG132 toxicity are consistent with previous reports that MAL3-101 increases α-synuclein aggregation in neuroglioma cells (Kilpatrick et al. 2013).

Despite a number of limitations, the strengths of the present study include the validation of multiple findings with more than one technique: 1) use of three independent, unbiased viability assays, 2) use of microscopy to verify the MAP2 In-Cell Western data, 3) two independent classes of proteasome inhibitors with unique modes of action, 4) two mechanistically independent Hsp70/Hsc70 inhibitors, 5) measurements of both pan-ubiquitinated proteins as well as K48-linked ubiquitinated proteins, and 6) two independent observers to determine the neuronal purity of olfactory bulb cultures. It is also worth mentioning that although all pharmacological inhibitors exert some non-specific effects, it is very unlikely that two inhibitors with unique modes of action have precisely the same non-specific effects. Therefore, if both inhibitors show the same effects (i.e. both potentiate proteotoxic stress), it is likely that these effects can indeed be attributed to the one mechanism that they share in common: Hsp70/Hsc70 inhibition. Additional strengths of the present study include verification of the specificity of the Hsp70 effects on proteotoxicity by including paraquat as an oxidative toxin as well as an inhibitor of an entirely different class of Hsps, the HO1 inhibitor SnPP. Paraquat toxicity was not exacerbated with the Hsp70/Hsc70 inhibitors and SnPP was not as effective as the Hsp70/Hsc70 inhibitors in olfactory bulb cells.

In conclusion, our study identifies a protective role for Hsp70 in olfactory bulb cells, in agreement with the notion that this chaperone is a natural defense against loss of proteostasis. These findings support attempts to boost levels of Hsp70 in elderly patients at risk for neurodegeneration with pharmacological tools or lifestyle interventions, such as daily exercise (Noble & Shen 2012). Future studies to measure the impact of Hsp70 inhibition on olfactory bulb structure and function *in vivo* and to examine age-related changes in the expression of Hsp70 within the layers of the olfactory bulb are justified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

RKL designed the experiments, generated some figures, and wrote the paper. TSC, AMG, and DMM collected and analyzed the data and generated the figures. JMP conducted some *in vitro* experiments. MTB and SDH performed cell counts. PW and JLB provided the Hsp70 inhibitor MAL3-101 and provided feedback on the manuscript. We

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Abbreviations

CHIP	C-terminus of Hsc70-interacting protein
GFAP	glial fibrillary acidic protein
GRP78	glucose regulated protein
Hsc70	heat shock cognate 70
Hsp	heat shock protein
HO1	heme oxygenase 1
MAP2	microtubule associated protein 2
mtHsp70	mitochondrial Hsp70
SnPPx	tin protoporphyrin

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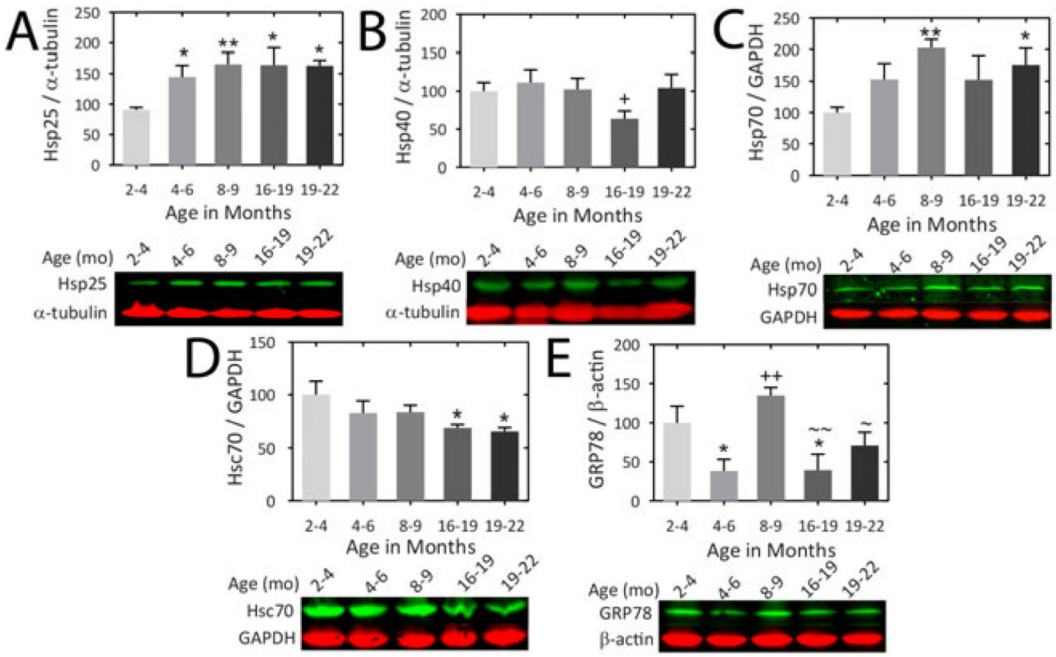

Figure 1. Impact of aging on Hsps and co-chaperones in the olfactory bulb of the female rat

(**A-E**) Infrared Western immunoblots are shown for the indicated Hsps and co-chaperones. Olfactory bulb tissue was harvested from female rats at 2.0-3.9 months, 4.0-6.0 months, 8.0-9.0 months, 16.0-18.9 months, and 19.0-22.0 months of age. GAPDH, α-tubulin, or β-actin was used as a protein loading control, depending on the species of the primary antibodies and the expected molecular weights. n = 3-6 rats per group. * $p \leq 0.05$, ** $p \leq 0.01$, versus 2-4 month old, + $p \leq 0.05$, ++ $p \leq 0.01$ versus 4-6 month old, ~ $p \leq 0.05$, ~~ $p \leq 0.01$ versus 8-9 month old, LSD *post hoc* following one-way ANOVA.

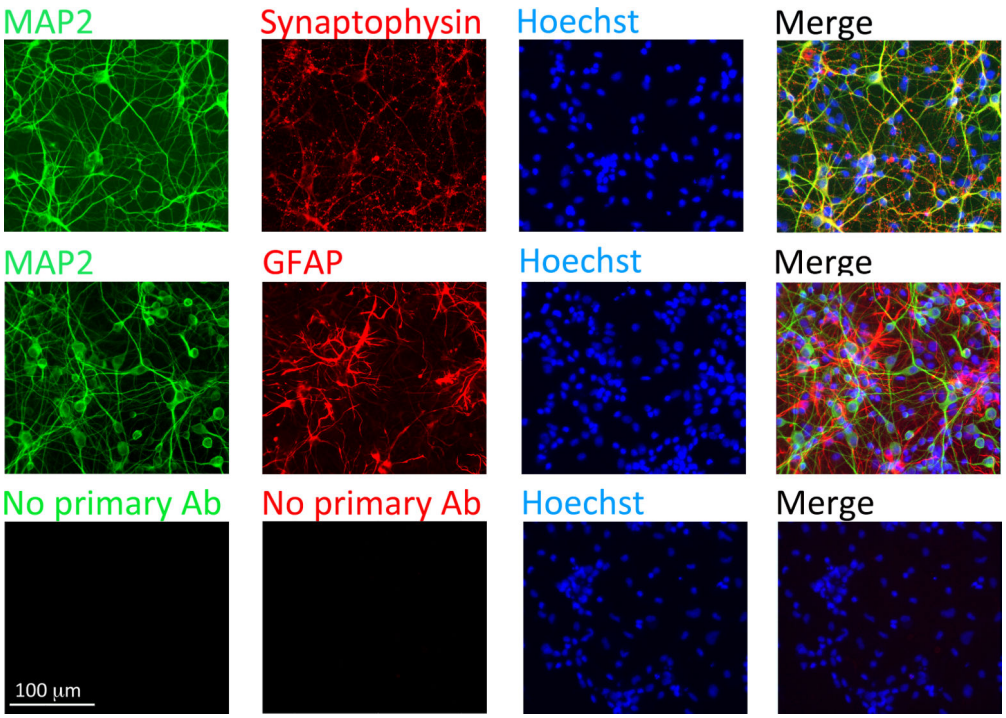

Figure 2. Primary postnatal cultures of the olfactory bulb

Dissociated primary olfactory bulb cells from day 1-2 neonatal rat pups were stained on day-*in-vitro* 5 (DIV5) for the neuronal marker MAP2, the synaptic protein synaptophysin, the astrocyte marker GFAP, and the Hoechst nuclear stain. Control wells (bottom panels) were exposed to all solutions except the primary antibodies.

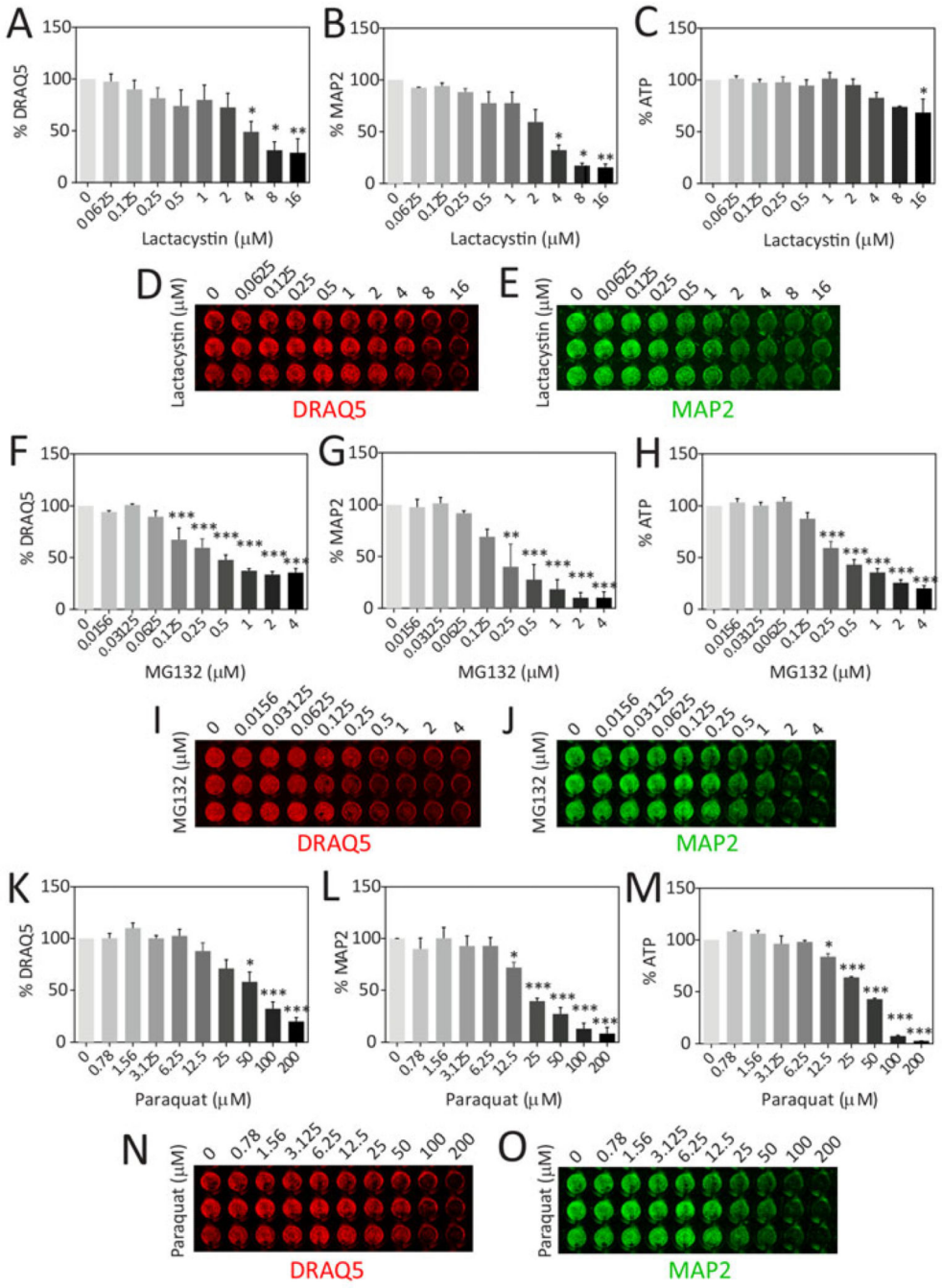

Figure 3. In vitro model of proteotoxic and oxidative stress in olfactory bulb cultures

Primary olfactory bulb cultures were treated with the proteasome inhibitors lactacystin (**A-E**) and MG132 (**F-J**), and the redox-active herbicide paraquat (**K-O**) on DIV5 and assayed by three independent assays for cell viability on DIV7: the infrared DRAQ5 stain for nuclei (**A, D, F, I, K, N**), infrared In-Cell Western analyses for the neuronal marker MAP2 (**B, E, G, J, L, O**), and the Cell Titer Glo assay for ATP (**C, H, M**). Representative DRAQ5 and MAP2 images were pseudocolored red and green, respectively. Shown are the mean and S.E.M of 3 independent experiments, each performed in triplicate. * $p \leq 0.05$, ** $p \leq 0.01$,

*** $p \leq 0.001$ versus vehicle-treated groups, Bonferroni *post hoc* following one-way ANOVA.

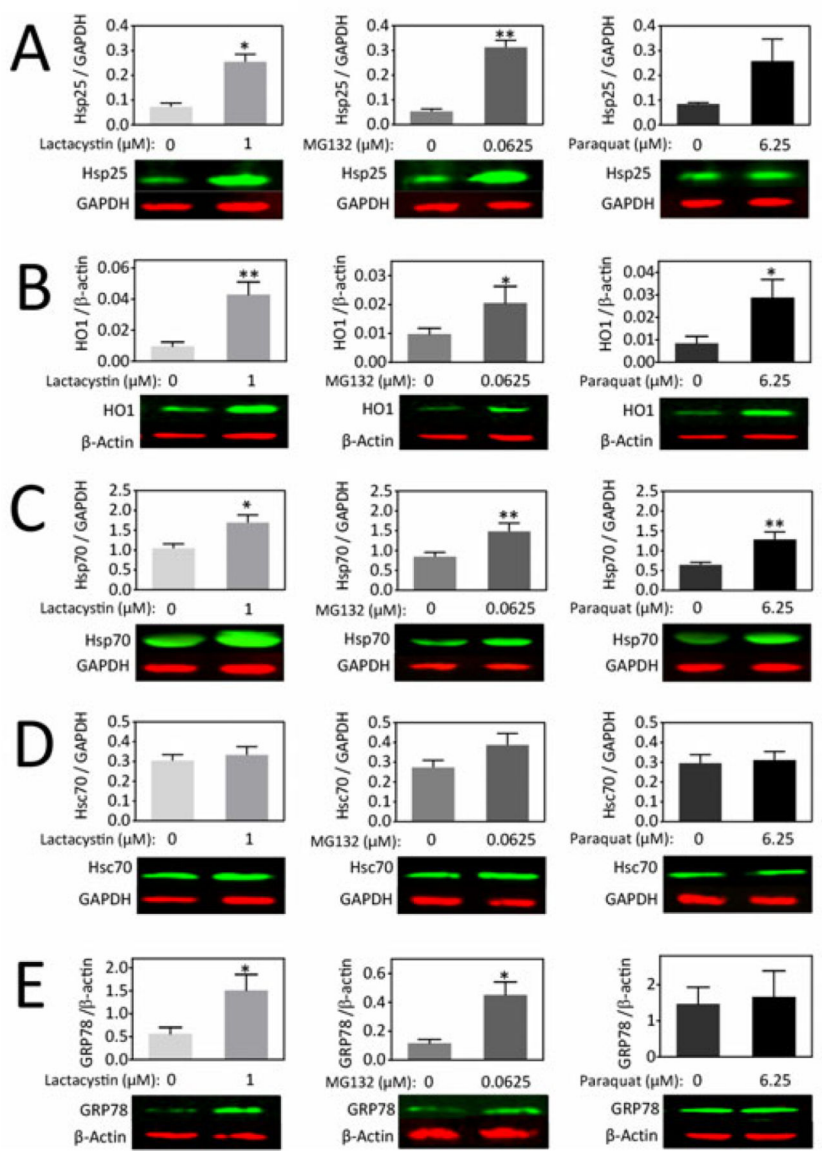

Figure 4. Mild proteotoxic and oxidative stress increases Hsps in olfactory bulb cultures

(**A-E**) Primary olfactory bulb cultures were treated with low concentrations of lactacystin, MG132, and paraquat on DIV5 and harvested for measurements of Hsps on DIV6 by infrared Western blotting. GAPDH or β-actin was used as a protein loading control. Shown are the mean and S.E.M of 3-6 independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus vehicle-treated groups, two-tailed *t-test.*

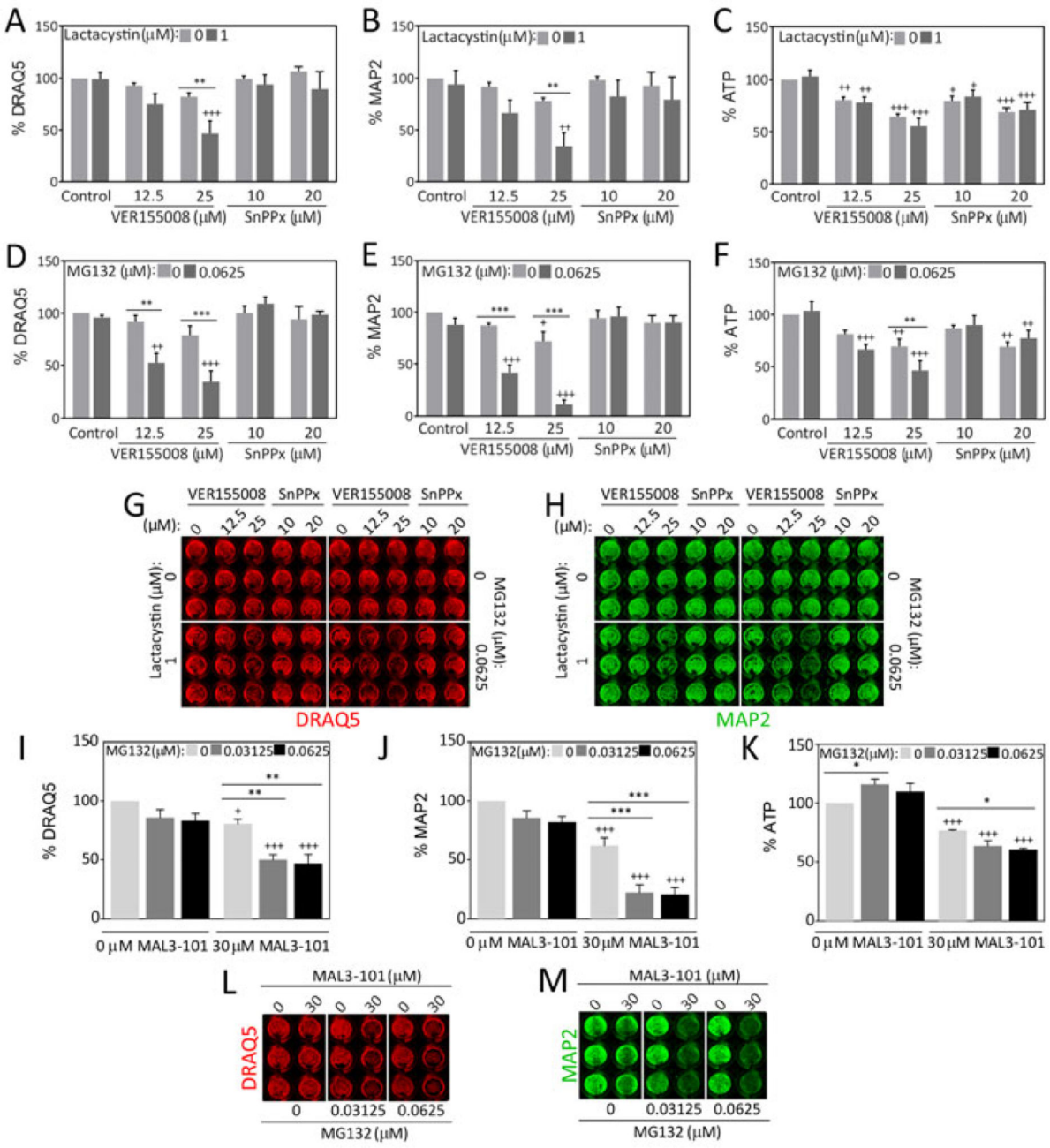

Figure 5. Effect of Hsp70/Hsc70 and HO1 inhibition on proteotoxic stress in olfactory bulb cultures

Primary olfactory bulb cultures were treated on DIV5 with low concentrations of lactacystin (**A-C**) or MG132 (**D-F**) in conjunction with the Hsp70/Hsc70 inhibitor VER155008 or the HO1 inhibitor tin protoporphyrin (SnPPx) and three independent viability assays were performed on DIV7. Representative infrared images are shown in G and H. (**I-M**) Primary olfactory bulb cultures were treated on DIV5 with low concentrations of MG132 in conjunction with the Hsp70/Hsc70 inhibitor MAL3-101 and three independent viability assays were performed on DIV7. Shown are the mean and S.E.M of 3-6 independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus 0 μM MG132 or lactacystin; + p

≤ 0.05, ++ $p \leq 0.01$, +++ $p \leq 0.001$ versus 0 μM VER155008, MAL3-101, or SnPPx, Bonferroni *post hoc* following two-way ANOVA.

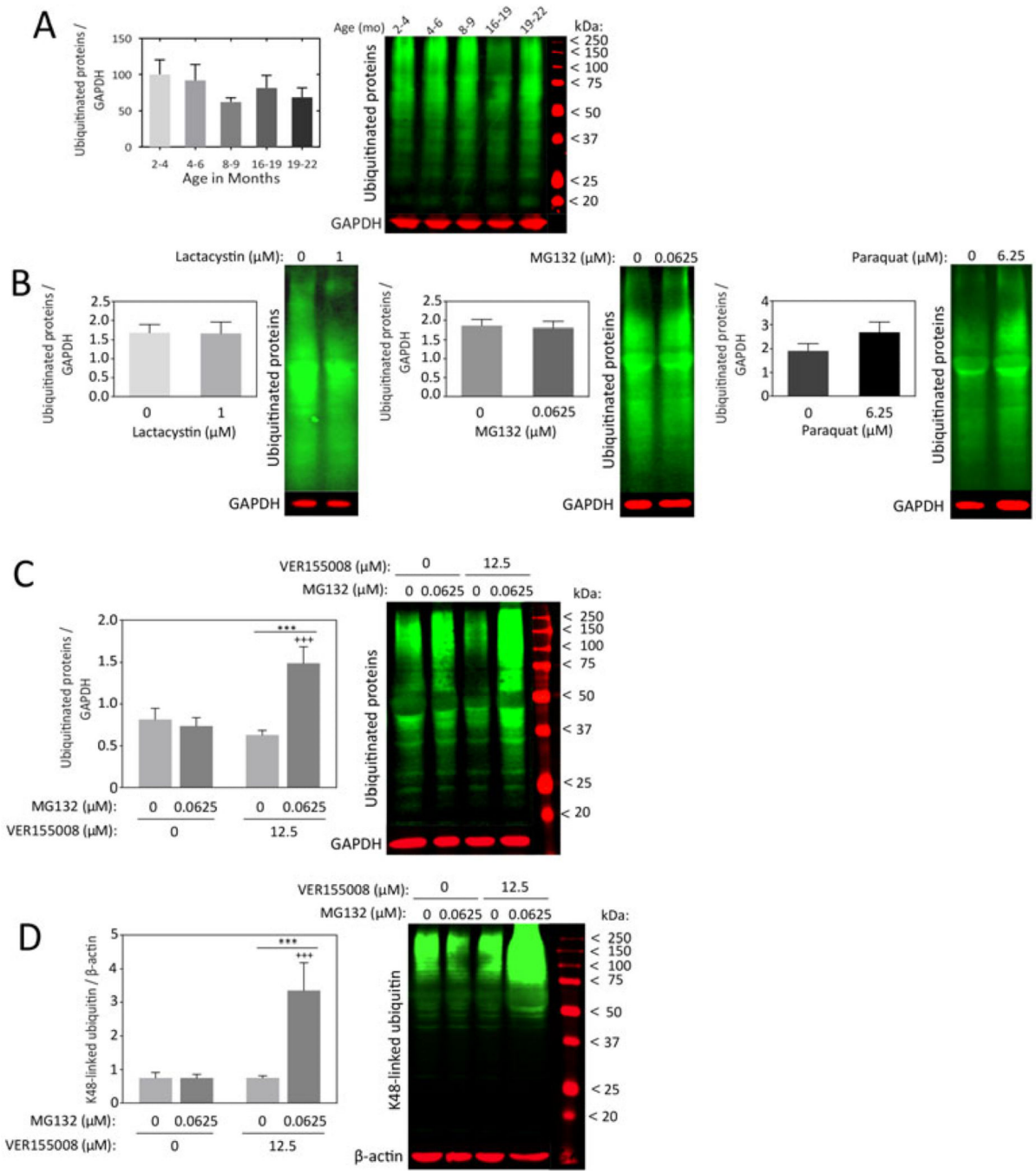

Figure 6. Impact of mild stress and Hsp70 activity on ubiquitinated protein levels in olfactory bulb cells

(**A**) Olfactory bulb tissue was harvested from female rats at 2.0-3.9 months, 4.0-6.0 months, 8.0-9.0 months, 16.0-18.9 months, and 19.0-22.0 months of age and probed for ubiquitin-conjugated proteins by infrared Western blotting. n = 3-6 rats per group. (**B**) Primary olfactory bulb cultures were treated with low concentrations of lactacystin, MG132, and paraquat on DIV5 and harvested for immunoblots of ubiquitinated proteins on DIV6. Shown are the mean and S.E.M from 5-6 independent experiments. (**C-D**) Primary olfactory bulb

cultures were treated on DIV5 with MG132 in the absence or presence of the Hsp70/Hsc70 inhibitor VER155008 and assayed for pan-ubiquitinated proteins (**C**) or K48-linked ubiquitin (**D**) on DIV6. GAPDH or β-actin was used as the loading control. Shown are the mean and S.E.M from 5-6 independent experiments. *** $p \leq 0.001$ versus 0 μM MG132; +++ $p \leq 0.001$ versus 0 μM VER155008, Bonferroni *post hoc* following two-way ANOVA.