Neuromelanin Marks the Spot: Identifying a Locus Coeruleus Biomarker of Cognitive Reserve in Healthy Aging

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Abstract

Leading a mentally stimulating life may build up a reserve of neural and mental resources that preserve cognitive abilities in late life. Recent autopsy evidence links neuronal density in the locus coeruleus (LC), the brain's main source of norepinephrine (NE), to slower cognitive decline before death, inspiring the idea that the noradrenergic system is a key component of reserve (Robertson, I. H. (2013). A noradrenergic theory of cognitive reserve: implications for Alzheimer's disease. *Neurobiology of Aging. 34*, 298-308). Here, we tested this hypothesis using neuromelanin-sensitive magnetic resonance imaging to visualize and measure LC signal intensity in healthy younger and older adults. Established proxies of reserve, including education, occupational attainment, and verbal intelligence were linearly correlated with LC signal intensity in both age groups. Results indicated that LC signal intensity was significantly higher in older than younger adults and significantly lower in females than in males. Consistent with the LC-reserve hypothesis, both verbal intelligence and a composite reserve score were positively associated with LC signal intensity in older adults. LC signal intensity was also more strongly associated with attentional shifting ability in older adults with lower cognitive reserve. Together these findings link *in vivo* estimates of LC neuromelanin signal intensity to cognitive reserve in normal aging.

Introduction

Brain and cognitive reserve refer to an individual's capacity to cope with cognitive decline or pathology in late adulthood (Stern, 2002; Stern, 2006; Stern, 2009). The concept of reserve has generated considerable interest in the field of aging, particularly regarding Alzheimer's disease (AD), as factors believed to promote reserve not only predict lowered risk of dementia in older adults but can also mask the severity of underlying brain pathology (Valenzuela & Sachdev, 2006).

Reserve capacity is theorized to rely on both passive and active factors. The "passive" model, referred to as "brain reserve capacity," is characterized by the availability of physical neural resources (Stern, 2009). For instance, larger regional gray matter volumes, such as in the prefrontal and parietal cortices, are associated with higher cognitive reserve scores (e.g., (Bartrés-Faz et al., 2009). In contrast, the "active model," otherwise known as "cognitive reserve," refers to an individual's capacity to invoke compensatory abilities developed over a lifetime of mentally enriching experiences (e.g., education). Epidemiological studies characterize years of education (Bennett et al., 2003; Le Carret et al., 2003), occupational attainment (Richards & Sacker, 2003; Stern, Tang, Denaro, & Mayeux, 1995), and IQ (Alexander et al., 1997) as three of the most common elements of cognitive reserve (Steffener & Stern, 2012; Stern et al., 1994; Whalley, Deary, Appleton, & Starr, 2004).

A critical question is how and why these cognitive reserve factors can protect brain function in order to either stave off or compensate for neurodegeneration. One possibility is that cognitive reserve proxies do not causally affect cognition but are instead indicative of a shared genetic factor that shields certain individuals from age-related pathology. This does not seem to be the case, however, since identical twins with different levels of education (Gatz et al., 2007) or occupational complexity (Andel et al., 2005) exhibit different risk of dementia. Thus, cognitive reserve proxies more likely promote healthy cognitive aging by modulating underlying brain systems.

Supporting this view, active and passive factors of reserve are far from mutually exclusive; for instance, neuroplasticity represents a critical intersection of both factors, as greater availability of neuronal resources might not only enhance healthy cognition but also affords more opportunities for compensation to occur in response to pathology (see Bartrés-Faz & Arenaza-Urquijo, 2011). Thus, the ideal biomarker of overall reserve is one that captures their interaction, representing a quantifiable neuronal resource that is modified by mentally enriching experiences; in turn, this substrate should be associated with better cognitive outcomes, even in healthy older individuals (Robertson, 2013; 2014). Identifying a shared neuromechanism of active and passive reserve is important since it could: 1) quantify an individual's level of reserve and susceptibility to cognitive decline, 2) become the target of therapeutic, cognitive, or pharmacological intervention, and 3) serve as a biomarker of the efficacy of such interventions.

According to a hypothesis by Robertson (2013), the locus coeruleus-norepinephrine (LC-NE) system is a candidate neuromechanism of reserve based on its key role in recruiting mental resources to promote executive function (functional) and in regulating neuroplasticity (physical) throughout the brain (Berridge & Waterhouse, 2003; Sara, 2009). The LC is a small bilateral nucleus located in the dorsal pontine tegmentum and is the primary source of NE for the brain. Via its widespread release under arousal, NE enhances learning (Ahissar, Haidarliu, & Shulz, 1996; Chamberlain, Müller, Blackwell, Robbins, & Sahakian, 2006; Harley, 1987), regulates synaptic plasticity (Ahissar et al., 1996; Neuman & Harley, 1983; Salgado, Kohr, & Trevino, 2012), and optimizes higher-order cognitive processes, including working memory (Arnsten & Li, 2005; Wang et al., 2007). NE is also neuroprotective and can lower toxicity in neurons (Counts & Mufson, 2010) and buffer neurons from oxidative stress (Troadec et al., 2001). Aging is characterized by decreased LC volume and cell density (Chan-Palay & Asan, 1989; German et al., 1988; German et al., 1992; Lohr & Jeste, 1988; Manaye, McIntire, Mann, & German, 1995; Vijayashankar & Brody, 1979), suggesting that altered noradrenergic structure or signaling in late adulthood is likely to correspond with cognitive decline.

Indeed, new evidence reveals an important link between LC functional/neuronal integrity and cognitive function. For example, a human functional magnetic resonance imaging (fMRI) study found that greater functional connectivity between the LC and parahippocampal gyrus was associated with higher memory performance in healthy older adults, and this connectivity was lower in older adults with mild cognitive impairment (Jacobs et al., 2014). In another fMRI study, healthy older adults with higher levels of cognitive reserve showed reduced activity in an LC-related brainstem cluster while participants encoded scenes, which the authors interpreted as increased neuronal efficiency (Solé-Padullés et al., 2009). Perhaps most compellingly, a recent autopsy study found reduced postmortem neuronal density in the LC was associated with cognitive decline ~6 years prior to death, even after controlling for neuron density in other aminergic nuclei, including the dorsal raphe, ventral tegmental area and substantia nigra (Wilson et al., 2013). In the Wilson et al. (2013) study, controlling for LC neuronal density diminished the association between Lewy body pathology and cognitive decline, suggesting that, like cognitive reserve variables (Ritter, Freyer, Curio, & Villringer, 2008; Roe et al., 2008; Roe, Xiong, Miller, & Morris, 2007), LC structural integrity could account for discrepancies between the amount of brain pathology and apparent cognitive decline (Wilson et al., 2013). Thus, via its regulation of healthy cognition and central neuronal function, the LC-NE system may mediate the protective effects of reserve on cognitive aging processes (Robertson, 2013; Robertson, 2014).

Of relevance to the LC-reserve hypothesis, LC neurodegeneration has received increasing attention as a possible contributor to the pathophysiology of AD (Braak & Del Tredici, 2011; Braak, Thal, Ghebremedhin, & Del Tredici, 2011; Chalermpalanupap et al., 2013; Grudzien et al., 2007; Mravec, Lejavova, & Cubinkova, 2014). LC tau pathology is also visible in early adulthood, and there are some indications that it might even precede one of the hallmark signs of AD—amyloid pathology — in the medial temporal lobe (Braak et al., 2011). The Braak

et al. (2011) findings also indicate that pathological tau processes are not exclusive to older age but instead are present at low levels even among younger adults, with the extent of pathology progressing across the lifespan at different rates for different individuals. Consistent with this observation, LC neurofibrillary tangles and abnormal tau show an age-MCI-AD continuum, such that higher levels of these cytopathologies are associated with greater cognitive impairment (Grudzien et al., 2007). From the perspective that both LC cytopathology and the transition from normal cognitive aging to clinical dementia occur along a continuum, it might be possible to link estimates of LC structure to reserve *within* a healthy population.

The aim of this neuroimaging study was to link variations in LC neuromelanin signal intensity, or contrast-to-noise ratio, to proxies of cognitive reserve in normal aging. To this end, we used neuromelanin-sensitive weighted magnetic resonance imaging (MRI) to visualize and measure the mean signal intensity of the LC in healthy younger and older adults. Until recently, imaging the human LC was notoriously difficult due to its small size (~2-15mm; (Chan-Palay & Asan, 1989) and low MR signal in conventional T1-weighted anatomical images. However, a growing number of studies have taken advantage of the fact that - unlike most other structures in the brainstem - the LC contains neuromelanin, a byproduct of NE metabolism (Sasaki et al., 2006). Using MR sequences sensitive to neuromelanin, the LC can be visualized effectively (Shibata et al., 2006). For instance, 3T fast spin-echo (FSE) T1-weighted MRI sequences can enhance MR signal contrast between the LC and neighboring brainstem tissue (Keren, Lozar, Harris, Morgan, & Eckert, 2009; Sasaki et al., 2006; Shibata et al., 2006; Takahashi et al., 2014). Estimating neuromelanin signal intensity is therefore a promising *in vivo* biomarker of LC structure in humans.

To test the LC-reserve hypothesis, we examined the association between LC signal intensity and three proxies of cognitive reserve: years of education, occupational attainment scores, and verbal intelligence. Human neuroimaging studies have also identified that gray matter volume in several brain regions, including the prefrontal and parietal cortices, are

associated with a composite measure of cognitive reserve (Bartrés-Faz et al., 2009). Thus, to determine the specificity of the predicted LC-reserve association, we also used voxel-based morphometry (VBM) to examine whether proxies of cognitive reserve were related to regional variations in brain structure more generally.

We hypothesized that education, occupational complexity, and verbal intelligence – and, in particular, an additional cognitive reserve factor score capturing shared variance among these three variables – would be positively associated with LC signal intensity in older adults. Additionally, we examined age and sex differences in LC signal intensity based on evidence that human LC neuromelanin concentration changes across the lifespan (Shibata et al., 2006) and that LC volume differs by sex in rodents (Babstock, Malsbury, & Harley, 1997). As suggested by Christensen et al., (2007), the most meaningful test of the reserve hypothesis examines how the relationship between biomarkers and cognitive measures vary as a function of reserve level. Thus, to test this possibility, we performed a moderation analysis to determine whether cognitive reserve factor scores moderated the relationship between LC signal intensity and the "Shifting" subscale of the Attentional Control Scale (Derryberry & Reed, 2002) in healthy older adults.

Methods

2.1 Participant characteristics

Twenty-eight healthy older and 35 healthy younger adults were recruited to participate in this study. All participants had normal or corrected-to-normal vision and hearing, and self-reported no history of chronic illness or cognitive impairment. One male older adult participant

was taking a beta-blocker.[1] All participants provided written informed consent approved by the University of Southern California Institutional Review Board. For all analyses, 5 older adults (2 F) were excluded: 3 participants had extreme motion artifacts in their FSE images, 1 participant had an incidental finding on a separate radiological MRI scan, and an FSE scan was not collected for 1 participant. Two younger males were excluded because they did not have FSE scans collected. Thus, the LC signal intensities of 23 healthy older (9 F; age: *M* = 67, *SD* = 5; range = 58-75) and 33 healthy younger adults (14 F; age: *M* = 24, *SD* = 5; range = 18-34) were analyzed in this study (Table 1).

Table 1: Demographic and Cognitive Performance Data

Measures	Young Adults	Older Adults
***n* (females)**	33 (14)	23 (9)
Age (*SD*)	24 (5)	67 (5)
Education (*SD*)	16 (3)	17 (2)
WTAR (*SD*)	43 (6)	41 (7)
Occupation (*SD*)	N/A	81 (11)
Attention Control Scale (ACS)	N/A	60 (6)
ACS – Shifting (*SD*)	N/A	14 (3)
ACS – Focusing (*SD*)	N/A	23 (2)
State Anxiety (STAI; *SD*)	39 (11)	27 (6)
Trait Anxiety (STAI; *SD*)	36 (10)	28 (7)

Notes: WTAR = Wechsler Test of Adult Reading. STAI = State-Trait Anxiety Inventory. Not all sample sizes for the behavioral/demographic variables are equal – see text in section 2.2 for specific exclusions and scoring procedures. Most young adults were full-time students, so they do not have an occupational attainment score. Age and education were measured in years.

2.2 Cognitive reserve variables

[1]After removing the older adult on atenolol, the results of all analyses in this study not only remained the same but also – in some instances – became more statistically significant. For instance, in Section 3.1, the new mixed ANOVA revealed larger main effects of Age, $F(1,51) = 5.69$, $p = .021$, and Sex, $F(1,51) = 4.29$, $p = .043$, on LC signal intensity. Furthermore, whereas the LC-Education, LC-Occupation, and LC-Shifting correlations in Section 3.2 remained non-significant, the relationship between LC signal intensity and composite reserve score became stronger both when not controlling for, $\beta(16) = .57$, $p = .0067$, and controlling for the influence of sex and age, $\beta(13) = .51$, $p = .02$. In the original LC-reserve analysis, controlling for the effects of sex and age made this relationship non-significant ($p = .077$; see Section 3.2). Finally, removing this older adult also increased the strength of the moderation effect of cognitive reserve reported in Section 3.4, $\Delta R^2 = .31$, $F(1,10) = 6.96$, $p = .025$.

Prior to scanning, participants completed a demographic questionnaire measuring age, sex, and two environmental factors that have been previously linked to cognitive reserve: education and occupational complexity (Table 1). For occupation, participants provided their job title along with a detailed description of their profession. This information was converted into an "occupational attainment" variable using a scale reported in Nam and Boyd (2004), which scores a wide range of occupations based on their intellectual complexity and difficulty (Nam & Boyd, 2004). Occupational attainment was scored by two separate raters (D.C. and A.P.: ICC = .85) and then averaged together. Occupational attainment scores were only determined for older adults since most of the younger adults were students.

Participants also completed the Wechsler Test of Adult Reading (Wechsler, 2001), which served as a proxy for verbal IQ (Armstrong et al., 2012; Leeson et al., 2011). On the WTAR, participants were prompted by an experimenter to pronounce 50 irregularly spelled words. WTAR performance was measured as the number of correct word pronunciations out of a total of 50. Independent samples *t*-tests revealed no significant age difference in WTAR scores or years of education between young and older adults (*p*s > .1).

To test for a relationship between LC signal intensity and reserve, each of these cognitive reserve variables was individually correlated with LC signal intensity in each age group, separately. Robust correlations were performed using a Theil-Sen estimator, a robust linear correlation technique that is less sensitive to outliers than parametric regressions (Wilcox, 2004). One additional male older adult missed over 12 consecutive words on the WTAR, resulting in the test being discontinued; therefore, this participant was excluded. Additionally, WTAR data was not collected for 3 other older adults, yielding a total *n* of 19 for older adult LC-WTAR correlation analyses. WTAR data was not collected for two young adults (1F), because they were non-native English speakers. Thus, a total *n* of 31 young adults was analyzed in the LC-WTAR correlation analyses.

2.3 Cognitive reserve factor analysis in older adults

The idea of cognitive reserve refers to an amalgamation of mentally stimulating factors that confer resilience to cognitive impairment. Thus a factor analysis was performed in the older adults to identify shared variance among education, occupational attainment, and WTAR scores. We performed a principal components analysis of a correlation matrix with an unrotated factor solution. Factor extraction was constrained to eigenvalues greater than 1. This analysis produced a single composite "cognitive reserve" score that was then linearly correlated with LC signal intensity using a Theil-Sen estimator. A total of 19 older adults were analyzed based on the criteria of having all three reserve measures. The factor loadings were high for all three variables, indicating they were highly inter-correlated: WTAR (.85), occupational attainment (.84), and education (.91).

2.4 Executive attention measures

To examine the relationship between LC structure and cognitive ability in older adults, we administered the Attentional Control Scale (ACS; Table 1). The ACS is a 20-item self-report measure assessing an individual's ability to switch attention between tasks and focus attention. Although this cognitive outcome measure is based on self-report rather than performance, previous studies demonstrate that the ACS is highly correlated with performance on a Go/No-Go task (Judah, Grant, Mills, & Lechner, 2014).

Consistent with Derryberry and Reed's (2002) proposal, one large behavioral studies validated and confirmed that ACS data has a two-factor structure, such that the item responses can be dissociated into separate aspects of attention: Focusing and Shifting (Judah et al., 2014). In a validation factor analysis, the two-factor solution included 7 items loading on the Focusing factor and 5 items loading on the Shifting factor. The item with the highest loading score for the Focusing subscale was, "When I need to concentrate and solve a problem, I have trouble focusing my attention," whereas the item that had the largest loading for the Shifting subscale was, "It is easy for me to alternate between two different tasks."

The same items identified in this factor analysis were used to calculate a Focusing and Shifting score for each of the older adults in our dataset. One older adult was not administered the ACS, so was excluded from the moderation analysis (resulting in an n = 18). Based on much evidence that the noradrenergic system regulates set-switching and cognitive flexibility via its modulation of the prefrontal cortex (Robbins & Arnsten, 2009), we expected that Shifting – which is also associated with working memory performance (Derryberry & Reed, 2002) - would be significantly associated with LC signal intensity in the older adults.

Additionally, to control for the confounding effects of state anxiety on ACS attentional Shifting (Judah et al., 2013) we also administered the 40-question State-Trait Anxiety Inventory (STAI; (Spielberger, 1983) to the participants before their MRI scan so that we could model state anxiety (20 of the questions) as a nuisance covariate in the moderation analysis (see Table 1 and Section 2.5). In the absence of brain damage in healthy older individuals, the brain reserve model posits that neural reserve estimates should be directly associated with better cognitive outcomes. To test for this, ACS Shifting scores were linearly correlated with LC signal intensity using a Theil-Sen estimator. One female older adult had missing values on her ACS questionnaire, so her incomplete data was excluded from this analysis (n = 22).

2.5 Cognitive reserve moderation analysis

It was recently suggested that a fundamental test of the cognitive reserve hypothesis tests whether or not reserve scores moderate the strength of the relationship between brain structure and healthy cognitive outcomes (Christensen et al., 2007). Thus, to determine whether older adults' cognitive reserve scores moderated the strength of the association between LC signal intensity and ACS Shifting scores, we performed a multiple linear regression.

In a stepwise regression, continuous values for age, state anxiety, cognitive reserve loading score, LC signal intensity and sex (only categorical variable) were mean-centered and modeled as predictors of Shifting in the first step of the regression. An interaction term for LC signal intensities and cognitive reserve factor loading scores was computed by multiplying their

centered values together. This moderation term was then modeled in the second step of the regression and the reserve moderation effect was tested by examining whether this interaction term in step two made a significant change to the Adjusted R^2 with a nominal $\alpha = .05$.

2.6 *MRI data acquisition*

Neuroimaging data were acquired with a 3T Siemens MAGNETOM Trio scanner at the USC Dana & David Dornsife Cognitive Neuroscience Imaging Center. One neuromelanin-sensitive weighted MRI scan was collected using a T1-weighted fast spin echo (FSE) imaging sequence (TR = 750 ms, TE = 12 ms, flip angle = 120°, 1 average to increase SNR, 11 axial slices, FOV = 220 mm, bandwidth = 220 Hz/Px, slice thickness = 2.5 mm, slice gap = 3.5mm; in-plane resolution = 0.429 x 0.429 mm^2, scan duration: 1 minute and 53 seconds). To examine the relationship between regional gray matter volumes and cognitive reserve scores, we also collected a high-resolution T1-weighted anatomical image for each participant (TR = 2300 ms, TE = 2.26 ms, TI = 1060 ms, flip angle = 9°, 176 sagittal slices, FOV = 256 mm, bandwidth = 200 Hz/Px, voxel resolution = 1 mm^3 isotropic, scan duration: 4 minutes and 44 seconds).

2.7 Locus coeruleus neuromelanin signal intensity analysis

To measure LC neuromelanin signal intensity, left and right LC regions-of-interest (ROIs) were hand-drawn by two separate researchers (D.C. and E.M.) on each participant's FSE T1-weighted images using the FSLVIEW tool in FSL version 5.0.4 (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/). Both researchers were blind to all other participant information. The intra-class coefficient (ICC) ranged from .81-.88, indicating high inter-rater reliability in estimating the location and mean signal intensity of the LC. For an overview of the anatomical tracing protocol, see Figure 1.

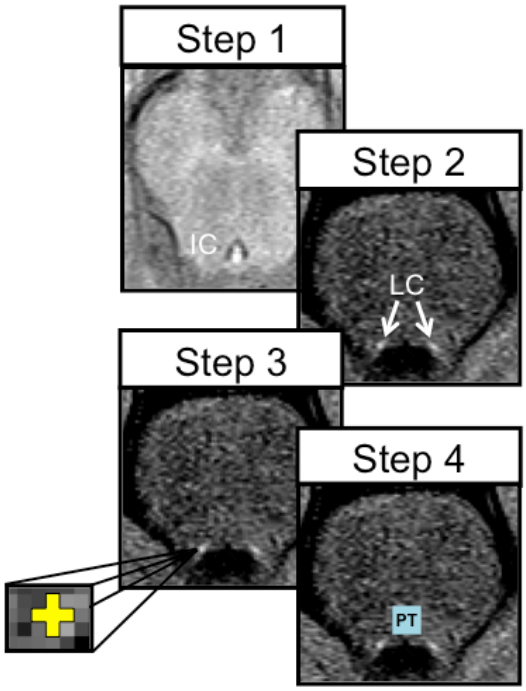

Figure 1. Locus coeruleus (LC) anatomical tracing protocol. Step 1) In the axial plane, the inferiormost slice of the inferior colliculus (IC) was located; we then moved down 7 mm (2 slices) into a slice where LC signal intensities were most apparent. Step 2) Left and right LC regions-of-interest (ROIs) appeared as high (bright) signal intensities neighboring the corners of the fourth ventricle. Step 3) A small cross (3 x 3 voxels; yellow) of approximately the width of the LC (~1-2 mm) was placed on the voxels with peak signal intensity. Step 4: A dorsal pontine tegmentum (PT) reference ROI (light blue) was defined as a 10 x 10 voxel square located 6 voxels above the more ventral (higher in the MRI axial image) of the two LCs and equidistantly between them.

Consistent with earlier LC-tracing procedures, LC ROIs were defined in the axial slice located 7 mm below the inferior boundary of the inferior colliculus where signal consistent with the neuroanatomical location of the LC is most evident (Shibata et al., 2006). Within this axial slice, two LC ROIs were manually delineated as a cross that was ~1.29 mm wide and ~1.29 mm high (i.e., 3 x 3 voxels; see Figure 1) to approximate the 1-2 mm distribution of LC neurons in this slice (German et al., 1988). These ROIs were centered on the left and right voxels with the highest signal intensities in locations that were anatomically consistent with the LC. In some instances, the peak voxel was located immediately adjacent to the fourth ventricle. Thus, to

avoid low signal value in the ventricle (i.e., partial volume effects), the center of the ROI was placed one voxel further away from the ventricle; that way, the LC ROI still captured the peak voxel while avoiding under-estimating overall signal intensity. The mean signal intensities from the left and right LC ROIs were then extracted and averaged to increase the signal-to-noise ratio of the intensity estimates.

To control for noise variability in the FSE images, a reference ROI was drawn in the dorsal pontine tegmentum (PT) using a 10 x 10 voxel square (18.4 mm^2). This size was chosen in order to avoid capturing low signal intensities in the ventral tip of the fourth ventricle and the darker medial leminiscus/pons. The dorsal boundary of the PT ROI was determined by moving 6 voxels (2.57 mm) from the center of the more ventral of the two LC ROIs towards the pons. The PT was drawn equidistantly between the left and right LC ROIs. If there was an odd number of voxels between the left and right LC ROIs, the PT square was drawn 1 voxel closer to the left LC ROI (right side in MNI space; see Figure 1).

LC contrast-to-noise ratios (CNR) were calculated based on the mean LC signal intensity relative to the reference PT signal intensity using the following formula: $LC_{CNR} = (LC_{intensity} – PT_{intensity})/PT_{intensity}$ (Sasaki et al., 2006; Shibata et al., 2006). The two raters' estimates of LC CNR were averaged together to increase the signal-to-noise ratio. A 2 x 2 Analysis of Variance (ANOVA) was performed with Age (young vs. old) and Sex (female vs. male) modeled as between-subjects factors and LC CNR – henceforth simply referred to in the text as LC signal intensity – as the dependent variable. Follow-up Bonferonni-corrected independent samples *t*-tests were performed to test for main effects of Sex within each age group, separately.

2.8 Voxel-based morphometry analysis

Next, to determine whether cognitive reserve was specifically associated with LC signal intensity or whether it was related to brain structural variability more broadly, we performed a voxel-based morphometry (VBM) analysis. FSLVBM (http://www.fmrib.ox.ac.uk/fsl/fslvbm) was

used to identify brain regions that showed a significant correlation between regional brain volume and cognitive reserve factor loading scores (Good et al., 2002). First, the high-resolution T1-weighted anatomical images were brain extracted. These images were then prepared for VBM by: 1) segmenting the unsmoothed volumes into partial-volume probabilistic cerebrospinal fluid, white matter, and gray matter masks; 2) transforming these native-space masks into MNI space using affine and subsequent non-linear transformations and averaging these images together to create a study-specific gray matter template; 3) modulating MNI-space gray matter masks for each older participant using the Jacobian of the warp field; and 4) smoothing these images with a Gaussian kernel with a sigma = 3 mm.

The gray matter volumes for older adults with a reserve loading score (N = 19) were analyzed voxelwise using permutation-based nonparametric testing (i.e., using FSL's randomise tool) with 5000 permutations and threshold-free cluster enhancement to correct for multiple comparisons (Smith & Nichols, 2009). The association between regional gray matter volume and cognitive reserve scores was tested using a general linear model (GLM) with demeaned composite reserve scores as the covariate of interest. Demeaned values for age, sex, and total intracranial volume were also included in the GLM as nuisance variables. In the resulting regression maps, clusters of "activation" would signify regions that had a significantly positive or negative partial correlation with cognitive reserve values.

Results

3.1. The effects of age and sex on LC signal intensity

A 2 x 2 between-subjects ANOVA was used to determine the effects of Age and Sex on mean LC signal intensity (Figure 2). The results indicated a significant main effect of Age such that older adults exhibited higher mean LC signal intensity than younger adults, $F(1,52) = 5.47$, $p = .023$, $\eta^2 = .095$ (Old: $M = .18$, $SEM = .009$; Young: $M = .15$, $SEM = .007$). Females also showed significantly attenuated LC signal intensity compared with males, $F(1,52) = 4.08$, $p =$

.049, η^2 = .073 (Females: *M* = .15, *SEM* = .009; Males: *M* = .18, *SEM* = .007). There was no significant Age x Sex interaction effect or any main effect of Sex within either age group (*p*s > .1). Follow-up Bonferroni-corrected independent samples *t*-tests revealed that this sex difference in LC signal intensity was not significant within either age group alone (*p*s > .1).

To acquire a consistent comparison between the LC signal intensities analyzed in the between-subjects ANOVA and their linear associations with the demographic/behavioral data, we performed a follow-up analysis using only the participants that had complete data for both, YA: *n* = 31 (13 F); OA: *n* = 19 (9 F). Across this subsample, the main effects of Age and Sex were even more significant, Age: $F(1,46) = 8.42$, $p = .006$, $\eta^2 = .16$; Sex: $F(1,46) = 5.72$, $p = .021$, $\eta^2 = .11$. While there was still no significant Age x Sex interaction effect ($p > .1$), two independent samples *t*-tests with Bonferonni correction revealed a marginally significant effect of Sex on LC signal intensity in the older adult subgroup (males > females; $p = .057$), but not in the young adult subgroup (males > females; $p = .23$).

Overall brainstem signal intensity differed across age groups, as older adults showed significantly lower PT signal intensity than younger adults, $F(1,52) = 24.04$, $p < .001$, $\eta^2 = .32$, as well as significantly lower raw (non-normalized) LC signal intensity, $F(1,52) = 12.09$, $p = .001$, $\eta^2 = .19$. But, for our purposes, the critical factor was the degree to which LC has greater contrast than the PT control regions, which is a reference for idiosyncrasies in within-participant image noise variability.

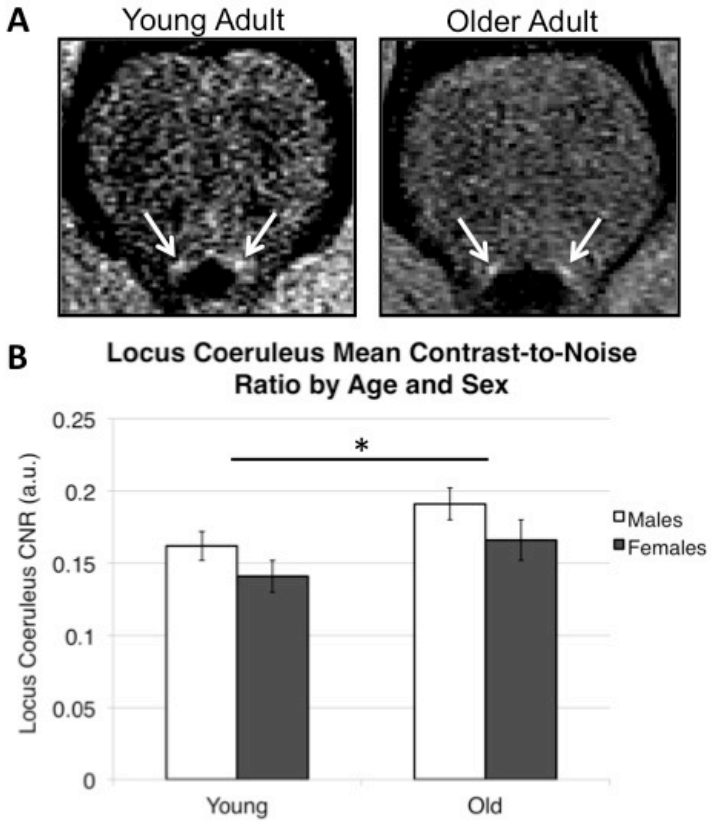

Figure 2. Age and sex differences in locus coeruleus (LC) signal intensity. (A) An example of a left and right LC in the neuromelanin-sensitive T1-weighted fast spin echo images of one younger and one older adult. For illustrative purposes only, the intensity thresholds have been adjusted to maximize the visual contrast between the LC and adjacent brainstem tissue, because the mean LC signal intensities differ significantly between age groups. Since each participant's FSE image is normalized relative to the noise in his/her image, such adjustments are only meant to demonstrate that the LC is clearly delineable in both age groups. (B) Age and sex differences in LC signal intensity are displayed as bars (means) for each subgroup. Bars reflect standard errors of the means. *$p < .05$.

3.2. Relationship between LC signal intensity and cognitive reserve variables by age group

To test our main hypothesis that LC signal intensity is positively associated with cognitive reserve, we performed robust linear correlations in each age group, separately (Figure 3). Among the three cognitive reserve proxies, verbal intelligence was the only variable significantly positively associated with LC signal intensity in older adults, $\beta(17) = 0.56$, $p =$

.0033. Thus, older adults with higher LC signal intensity also exhibited better verbal performance on the WTAR. This LC-WTAR relationship was still significant after controlling for the effects of age and sex, $\beta(14) = 0.56$, $p = .013$. Neither of the reserve measures collected in younger adults (education and WTAR) was significantly associated with LC signal intensity (*p*s > .05).

As predicted, LC signal intensity was positively associated with the composite cognitive reserve score in older adults, $\beta(17) = 0.54$, $p = .03$, which was calculated based on the shared variance amongst years of education, occupational attainment, and WTAR scores. However, the strength of this LC-reserve relationship diminished after controlling for the effects of age and sex, $\beta(14) = 0.43$, $p = .077$.

Unlike the mean LC CNR analysis, PT signal intensity did not significantly correlate with continuous age values in either age group (*p*s > .1). Thus, underlying differences in the reference ROI did not confound these LC-reserve relationships. As further validation, we examined whether raw LC signal intensities (i.e., mean values not normalized by PT intensity) also showed the same associations. Indeed, the LC-WTAR and LC-reserve correlations were still significant within the older adult group, indicating that these relationships were not artifacts of variability in PT intensity reference values.

A core postulate of the brain reserve hypothesis is that, in healthy individuals, brain reserve estimates should be correlated with better cognitive outcomes. Our LC-Shifting correlation analysis did not completely validate this prediction, with higher LC signal intensity trending towards a positive association with ACS Shifting scores, $\beta(20) = 0.31$, $p = .13$. Accounting for the effects of age, sex, and state anxiety had little effect on this relationship, $\beta(17) = 0.33$, $p = .18$. The same LC-Shifting correlations were also not significant in the smaller group of older participants with both cognitive reserve loading and ACS scores available (*n*= 18; *p*s > .1).

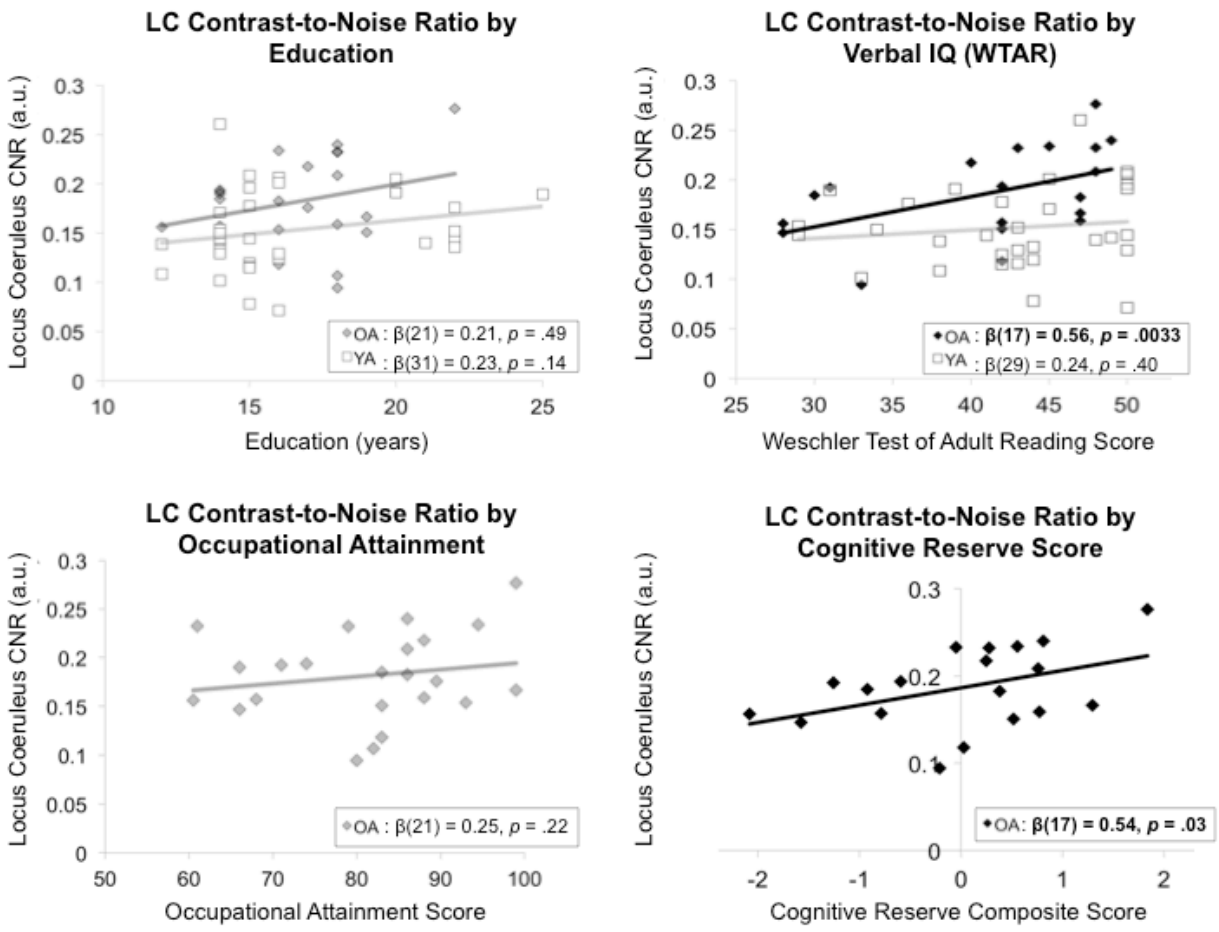

Figure 3. Correlations between education, WTAR (index of verbal intelligence), and occupational attainment displayed for each age subgroup, separately. In older adults, a cognitive reserve composite score – calculated as the shared variance among the three reserve variables – was also correlated with LC signal intensity. Darker bars indicate statistically significant correlations.

3.3. Voxel-based morphometry results

In the older adults, there were no brain regions where ICV-normalized gray matter volume was significantly correlated with cognitive reserve factor scores or LC signal intensity when controlling for sex and age. Thus, within our dataset, cognitive reserve scores were only significantly associated with LC signal intensity.

3.4 Cognitive reserve moderation analysis results

Although the main effect of LC signal intensity on attentional shifting was marginally significant, the idea of reserve is more readily invoked by the strength of this relationship

differing as a function of cognitive reserve levels. Indeed, consistent with our main prediction that reserve factor loadings would moderate the LC-Shifting relationship, the cognitive reserve x LC signal intensity interaction term explained a significant increase in variance in Shifting scores above and beyond the influence of any individual predictor, $\Delta R^2 = .26$, $F\ (1,11) = 5.17$, $p = .044$. The standardized regression coefficient for the moderation term was negative, $\beta = -.59$, indicating that, as cognitive reserve levels linearly decreased, the relationship between LC signal intensity and attentional shifting became stronger (Figure 4).

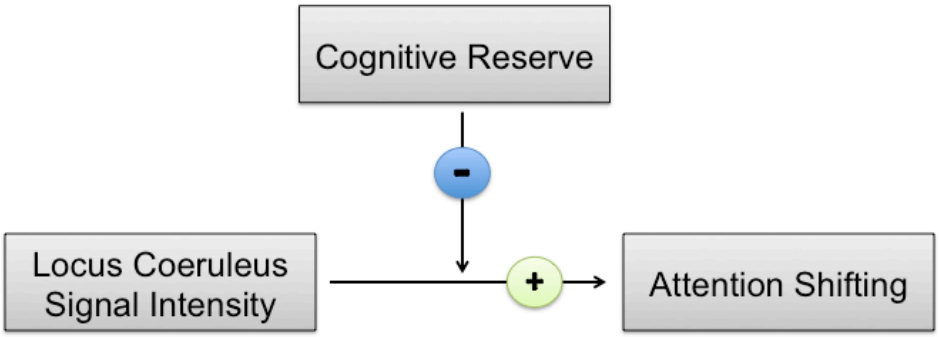

Figure 4. A schematic of cognitive reserve's moderating effect on the LC-attention association in healthy older adults. Continuous values for cognitive reserve factor scores moderated the strength of the positive relationship between LC signal intensity and attentional shifting. The negative standardized regression coefficient of the reserve x LC intensity interaction term indicated that, as reserve levels linearly decreased, the association between LC signal intensity and attentional shifting strengthened significantly.

Discussion

In the present study, we tested the recent proposal that the LC-NE system is an important component of neural reserve (Robertson, 2013). Our results supported this hypothesis by providing the first *in vivo* evidence that variations in human LC neuromelanin signal intensity were associated with established proxies of cognitive reserve in healthy older adults. Cognitive reserve loading scores moderated the strength of the relationship between LC

signal intensity and a self-report measure of attention shifting, such that this association became stronger in individuals with lower levels of cognitive reserve. This finding suggests that low-reserve older adults who may be more vulnerable to cognitive decline might also rely more heavily on normal noradrenergic system function to promote or maintain executive function. Together, our results support a growing literature implicating the noradrenergic system in cognitive aging.

Consistent with postmortem (Zecca et al., 2004; Zucca et al., 2006) and neuroimaging studies of LC structure in humans (Shibata et al., 2006), we found that older adults had higher LC CNR than younger adults. This finding accords with evidence that LC neuromelanin accrues in an inverted-U pattern across the lifespan, with peak concentrations occurring around the age of 60 before rapidly decreasing (Manaye et al., 1995; Shibata et al., 2006). It is noteworthy, however, that the age-related increase in LC CNR we observed appeared to be an artifact of a substantial age-related decrease (~9.6%) in raw pontine tegmentum (PT) signal intensity. This finding is consistent with an earlier human neuromelanin MRI study at 3T that found significant age-related decreases in PT signal intensity (Keren et al., 2009). Unfortunately, age differences in PT reference intensity were not reported in Shibata et al. (2006), so it is unclear whether their estimates of LC CNR were similarly inflated by age-related decreases in the reference signal.

Notably, there are some mixed findings concerning LC neuronal changes with age. In some human histological studies, there were no significant age-related changes in the quantity of neuromelanin-containing LC neurons (Mouton, Pakkenberg, Gundersen, & Price, 1994; Ohm, Busch, & Bohl, 1997), whereas in other studies, LC neuron count was lower in older than younger adults (Manaye et al., 1995). Combined with our current finding of reduced PT signal intensity with age, we conclude that age-related changes in LC CNR should be interpreted with caution. Nonetheless, fast spin echo MRI remains a highly effective method for localizing and quantifying neuromelanin-containing LC neurons *in vivo* (Keren et al., 2009; Keren et al., 2015).

Importantly, our main goal of identifying LC-reserve associations was not confounded

by issues with baseline PT intensity, since these relationships did not change when we used raw LC signal intensities. Our key finding was that, in older adults, cognitive reserve – as operationalized as shared variance among education, occupational complexity, and verbal intelligence - was positively correlated with LC intensity. Among the three individual reserve variables examined in this study, verbal intelligence (WTAR performance) was the only variable significantly correlated with LC signal intensity in older adults. Human research concerning the relationship between cognitive reserve variables and LC-NE system function is sparse, but there are some indications that pupil dilation, an index of LC activity (Murphy, O'Connell, O'Sullivan, Robertson, & Balsters, 2014), relates to IQ level. Individuals with higher than average IQ show greater pupil dilation to difficult analogies than individuals with average IQ (Bornemann et al., 2010; Van Der Meer et al., 2010). The cognitive-enhancing effects of modafinil administration, which alters LC activity (Minzenberg, Watrous, Yoon, Ursu, & Carter, 2008), also appear to vary as a function of IQ level, with lower IQ individuals' performance benefiting more from its administration (Randall, Shneerson, & File, 2005).

Our finding that low-reserve older adults showed a tighter LC-Shifting relationship than older adults with higher cognitive reserve supports this notion that the noradrenergic system's influence on cognition varies according to cognitive reserve capacity. A previous behavioral study demonstrated that the shifting subscale of the ACS is also correlated with working memory performance, as measured by letter-number sequencing performance (Judah et al., 2014). Thus, our findings accord with the well-established role of the LC-NE system in regulating executive function, particularly during effortful cognition and attention (Arnsten & Li, 2005; Bouret & Richmond, 2015; Murphy, Robertson, Balsters, & O'Connell, 2011).

In contrast, there were no significant LC-reserve relationships in the young adults. One potentially contributing factor is that younger adults have yet to accumulate their full neuromelanin levels. In the LC, neuromelanin pigment is a by-product of autophagic degradation of oxidized NE and NE metabolites (Wakamatsu et al., 2015). Thus older adults'

neuromelanin reflects a longer span of exposure to NE than does that of younger adults. In addition, healthy younger adults have yet to cope with age-related neuronal degeneration, so the compensatory function of the LC-NE system might not be as important and/or as apparent as in older adults. As our moderation analysis revealed, such compensation by the LC-NE system might only manifest in low-reserve older adults. In essence, LC neuromelanin signal intensity was more strongly associated with self-assessed impairments in cognitive ability in the context of lower cognitive reserve capacity. With respect to the other individual reserve proxies, most of the younger adults in this study were undergraduate college students who have not finished their education or have fulltime occupations; therefore, they have yet to build up their full reserve, which is accumulated over a lifetime of mentally stimulating experiences (Richards & Sacker, 2003).

Another key finding was a significant sex difference in LC signal intensity, such that, on average, females showed ~20% lower LC signal intensity than males. To our knowledge, this is the first *in vivo* evidence of sex differences in LC structure in humans. A similar finding was reported in Sprague-Dawley rates wherein dorsal LC neuronal volume was denser in males than in females (Babstock et al., 1997; Bangasser, Zhang, Garachh, Hanhauser, & Valentino, 2011). Lower LC signal intensity in females is intriguing given that various pathologies that are more prevalent in females have also been linked to the LC-NE system. For example, women have an increased risk of AD compared with men (Andersen et al., 1999), and female versus male carriers of the APOE4 allele, the strongest genetic risk factor for sporadic AD, are more likely to develop AD (Altmann, Tian, Henderson, & Greicius, 2014). Moreover, one large-scale histological study demonstrated that increased brain amyloid pathology, which also encompassed measurements in the lower brainstem, was more tightly correlated with age in females than in males (Braak et al., 2011). We examined a cognitively healthy population, however, so we can only speculate on this relationship.

As detailed in Robertson's (2013) LC-reserve hypothesis, activating the LC-NE system

function via mentally stimulating experiences may improve cognitive outcomes by triggering a variety of neuroprotective and memory-enhancing effects. Much research indicates that central NE release protects target neurons throughout the brain, particularly those in regions that facilitate learning and memory. NE reduces neuroinflammation (Feinstein et al., 2002), helps ameliorate amyloid cellular toxicity (Counts & Mufson, 2010), and protects cultured neurons from metabolic (Madrigal, Leza, Polak, Kalinin, & Feinstein, 2009) and oxidative stress (Troadec et al., 2001). LC-NE activation can increase BDNF production (Jurič, Miklič, & Čarman-Kržan, 2006) and hippocampal neurogenesis (Kulkarni, Jha, & Vaidya, 2002; Masuda et al., 2012; Veyrac et al., 2009) – both of which promote healthy cognitive and memory function. Exploration of novel environments, one form of mental stimulation, activates the LC (Kitchigina, Vankov, Harley, & Sara, 1997; Sara, Vankov, & Hervé, 1994) and enhances memory via an adrenergic mechanism (Straube, Korz, Balschun, & Frey, 2003; Veyrac et al., 2009). Furthermore, activating β2-adrenergic receptors through environmental enrichment also prevents memory impairments induced by Aβ oligomers in the hippocampus (Li et al., 2013). Thus, cognitive reserve factors may protect cognitive abilities by enhancing neuroprotective and neuroplastic processes regulated by the LC.

Of key importance to the current study, neuromelanin also protects healthy LC function. LC neuromelanin chelates various metal oxidants, including mercury, lead, and iron (Zecca et al., 2008; Zecca et al., 2004; Zucca et al., 2006) and scavenges free radicals (Álvarez-Diduk & Galano, 2015). Such protection against cell damage is particularly important given the LC's widespread exposure to – and consequent regulation of - circulating toxicants in the bloodstream (Mann, 1983; Pamphlett, 2014; Pamphlett & Jew, 2013). This research has led to speculation that age-related disintegration of neuromelanin releases previously immobilized toxicants back into the cell, leading to impaired NE output (Pamphlett, 2014; Pamphlett & Kum Jew, 2014). Signal intensity might therefore be an effective biomarker of neuromelanin-mediated protection over LC neurons, because neuromelanin bound to iron and copper

generates the paramagnetic T1 effect exploited by FSE T1-imaging (Enochs, Petherick, Bogdanova, Mohr, & Weissleder, 1997; Tosk et al., 1992). It is noteworthy that local iron accumulation also affects signal estimates in T1 FSE images (Vymazal et al., 1999). However, unlike the substantia nigra, iron levels in the LC remain relatively stable across the lifespan (Zucca et al., 2006). Thus, we conclude that neuromelanin buffering rather than iron concentration most likely contributed to overall LC signal intensity measured in this study.

Taken together, our results are consistent with a rapidly growing literature implicating the noradrenergic system in healthy cognitive aging. However, there are several important limitations to address. Our sample sizes were modest, which limited our investigation of sex differences in the relationship between LC signal intensity and different reserve variables. Another important limitation is that we had a limited set of cognitive outcome measures. Although previous work shows that ACS Shifting is correlated with executive function, this is not a 1:1 relationship; thus, self-assessed cognitive ability might not fully approximate actual performance. Future investigations should include a battery of neuropsychological assessments to more thoroughly measure cognitive performance and confirm its relationship with LC signal intensity in older adults. Since this was a cross-sectional study, we were unable to determine whether the reserve variables causally increase LC neuromelanin concentration or vice versa, and whether or not variability in neuromelanin concentration causally relates to different cognitive outcomes. Thus additional longitudinal studies are needed to confirm whether cognitive changes over time correspond with the degree of LC neuromelanin signal attenuation in later adulthood. In particular, it would be useful to test whether LC neuromelanin still relates to cognitive reserve variables in MCI or Alzheimer's patients, which would provide additional support that the LC is key mediator of the protective influence of environmental enrichment on cognition.

Cognitive reserve is complex and is associated with an array of environmental factors, including time spent doing mentally engaging activities (e.g., puzzles), that share variance with

the variables used in our study. The WTAR, in particular, might not be an ideal measure of cognitive reserve since it relates to crystallized intelligence, which is relatively stable across the lifespan, rather than fluid intelligence, which is more closely related to cognitive flexibility and decreases with age (Schaie & Willis, 1993). Additionally, IQ is heavily determined by genetic factors, so WTAR itself may more likely be a proxy for other experience-dependent factors, such as openness to learning, more directly involved. Although the WTAR might fail to fully explain the variance in reserve, there are indications that WTAR performance can predict incidence of PD-related mild cognitive impairment above and beyond the influence of education (Armstrong et al., 2012). Nonetheless, the implications of the WTAR-LC association identified in this study should be interpreted with caution, as other mechanisms/proxies related to verbal intelligence may be at play.

Finally, combining fMRI measures of LC activity during a cognitive task with FSE imaging would help establish whether LC structure and proxies of cognitive reserve are also associated with increased neural efficiency in the LC. The role of the LC in cognitive reserve is also theorized to involve its modulation of a broader, right hemisphere-biased executive attention network (Robertson, 2014). Thus, fMRI would help elucidate whether this network's processing efficiency is differentially associated with the functional (BOLD) and structural (FSE) integrity of the noradrenergic system based on one's cognitive and brain reserve capacity.

Conclusion

Using neuromelanin-sensitive MRI in healthy older adults, we demonstrated that an *in vivo* measure of LC neuromelanin signal intensity is associated cognitive reserve, particularly verbal intelligence. We also found that older adults with lower levels of cognitive reserve showed a stronger association between LC signal intensity and attentional shifting, suggesting that – particularly for elders potentially vulnerable to cognitive decline – the integrity of the noradrenergic system helps support cognitive flexibility. Together, these findings support the idea that, by augmenting LC-NE system function, the same intellectually engaging experiences

that help enrich one's life might also protect cognitive health in later adulthood.

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Disclosure

The authors declare no conflicts of interest.

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