Different Populations of Human Locus Ceruleus Neurons Contain Heavy Metals or Hyperphosphorylated Tau: Implications for Amyloid-β and Tau Pathology in Alzheimer’s Disease

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Abstract. A marked loss of locus ceruleus (LC) neurons is a striking pathological feature of Alzheimer’s disease (AD). LC neurons are particularly prone to taking up circulating toxicants such as heavy metals, and hyperphosphorylated tau (tauHYP) appears early in these neurons. In an attempt to find out if both heavy metals and tauHYP could be damaging LC neurons, we looked in the LC neurons of 21 sporadic AD patients and 43 non-demented controls for the heavy metals mercury, bismuth, and silver using autometallography, and for tauHYP using AT8 immunostaining. Heavy metals or tauHYP were usually seen in separate LC neurons, and rarely co-existed within the same neuron. The number of heavy metal-containing LC neurons did not correlate with the number containing tauHYP. Heavy metals therefore appear to occupy a mostly different population of LC neurons to those containing tauHYP, indicating that the LC in AD is vulnerable to two different assaults. Reduced brain noradrenaline from LC damage is linked to amyloid-β deposition, and tauHYP in the LC may seed neurofibrillary tangles in other neurons. A model is described, incorporating the present findings, that proposes that the LC plays a part in both the amyloid-β and tau pathologies of AD.

Keywords: Alzheimer’s disease, amyloid, disease model, environmental toxicant, heavy metals, locus ceruleus, locus coeruleus, mercury, noradrenaline, tau

INTRODUCTION

A striking feature of Alzheimer’s disease (AD) is a marked loss of neurons in the locus ceruleus (LC), the collection of cells in the brain stem that supplies noradrenaline to the brain [1, 2]. Hyperphosphorylated tau ( tauHYP), the major component of neurofibrillary tangles, is found extensively in the LC of AD patients, and the number of LC neurons containing tauHYP correlates with the degree of cognitive deterioration [3]. TauHYP in the LC has now been found in individuals as young as 6 years of age [4], so the LC appears to be the first region of the brain to be affected by the widespread tau changes that are seen later in AD [5, 6]. The possibility has been raised that cell-to-cell transfer of tauHYP from LC neurons into cortical neurons could be responsible for the widespread neurofibrillary tangles seen in AD [7]. It has also been suggested that...
there are two distinct phases in the development of AD, an early hyperphosphorylation of tau and a later deposition of amyloid-beta (Aβ), that follow different assaults to the nervous system [4]. The nature of these assaults remains, however, unknown [8].

AD is likely to be a complex disorder with both genetic and environmental components [9]. Toxic substances in the environment mostly generated by human activity, termed "toxicants" to distinguish them from biological toxins, have long been considered to play a part in AD, and arguments have been put forward implicating heavy metals such as mercury as triggers for the disease [10]. It has been difficult, however, to find convincing evidence that occupational or domestic exposure to environmental toxicants comprises a significant risk factor for AD [11], and toxicants such as mercury do not appear to localize to those parts of the brain that are predominantly involved in the disease [12]. A toxic attack on all of the many parts of the brain affected by AD therefore appears unlikely to play a major part in the pathogenesis of this dementia.

A link between the tau abnormalities found in the LC and the concept of toxicant-induced AD could be the recent finding that the human LC seems to be uniquely predisposed to take up circulating toxicants. This was first noted in a man who injected himself with metallic mercury, in whom mercury uptake was markedly higher in the LC than in any other part of the brain [13]. Furthermore, uptake of heavy metals primarily by the LC appears to be fairly common in humans, since it can be seen both in patients with motor neuron disease and in individuals without neurological disease [14]. It has been postulated that this propensity to take up circulating toxicants is because LC neurons inter- vate virtually all microvessels in the brain [15]. In an attempt to uncover what role the uptake of toxicants into the LC could be playing in dementia, we therefore looked for both metal toxicants and tauHYP in the LC neurons of AD patients and controls.

METHODS

Alzheimer’s disease patients and controls

Paraffin tissue blocks from individuals classified as having either AD (n = 21) or being non-demented controls (n = 43) were obtained from the New South Wales Tissue Resource Centre at the University of Sydney. The AD patients (9 male, 12 female, mean age 82.5 y, SD 7.9 y, range 63–98 y) and the control group were cardiac (n = 11), respiratory (n = 6), infection (n = 2), renal (n = 1), and stroke (n = 1), and in the control group were cardiac (n = 32), respiratory (n = 6), cancer (n = 4), and suicide (n = 12). The project was approved by the Human Ethics Review Committee (RPAH Zone) of the Sydney Local Health District (X14-0029).

Tissue preparation

At the time of postmortem examination, the brain was either hemisected in the sagittal plane, with one half fixed in formalin and the other half frozen (53 individuals), or all fixed in formalin (11 individuals). No tissue was subjected to prolonged formalin fixation. Brain stems were dissected in the transverse plane into 5 mm blocks. Single paraffin-embedded blocks of rostral pons that included the LC, and of the midbrain that contained the substantia nigra, were examined.

Autometallography for heavy metals

7μm paraffin sections were stained with silver nitrate autometallography, a silver amplification method which under routine conditions stains the sulphides or selenides of mercury, silver, and bismuth [17, 18]. These three metals are referred to here as “heavy metals”. The silver-coated deposits of these metals in tissues are seen histologically as black-staining grains. Briefly, paraffin sections were placed in physical developer containing 50% gum arabic, citrate buffer, hydroquinone, and silver nitrate at 26°C for 80 min in the dark, then washed in 5% sodium thiosulphate to remove unbound silver. Sections were counterstained with mercury-free Improved Harris hematoxylin and viewed under bright-field microscopic illumination. In each staining run, a positive control section was included of mouse spinal cord in which the motor neuron cell bodies contained mercury following an intraperitoneal injection of 2 μg/g mercuric chloride [19].

To quantitate the number of LC neurons staining with AMG, a 10 × 10 grid, with right and lower exclusion margins, viewed at 400x magnification, was stepped sequentially through the LC to calculate the percentage of neurons containing heavy metals within the LC nucleus [14] (the term “nucleus” refers here to the collection of neurons within the pons, rather than the nucleus within the cell body). In the 11 samples...
where both LC nuclei were present, a random side was chosen for quantitation. Neurons counted were those enclosed by an area bordered by neuromelanin-containing (“pigmented”) neurons. To be included for quantitation, a “heavy metal LC neuron” was defined as any neuron with a minimum greatest diameter of 26 μm (the length of one grid square) that contained 10 or more autometallography grains. The quantifier (RP) was blinded to the diagnosis by having the label on each slide obscured. One slide counted on 10 different occasions gave a coefficient of error of less than 5%.

Hyperphosphorylated tau immunostaining

7 μm paraffin sections were treated in 1% hydrogen peroxide in methanol followed by 10% normal horse serum and incubated in the monoclonal antibody AT8 AntiHuman Phos PHF Tau pSer202/Thr 205 (Thermo MN1020) at 1:1000 for 60 min at 37 °C. Slides were incubated with Envision Dual Link HRP (Dako K4061) at room temperature for 30 min and visualized with 3,3 diaminobenzidine tetrahydrochloride (MP Biomedicals AF312215). Sections were counterstained with Improved Harris hematoxylin and viewed under bright-field microscopic illumination. The same grid-based system as above was used to count the number of LC neuronal cell bodies that contained tauHYP.

Combined autometallography and hyperphosphorylated tau immunostaining

For combined staining to examine the relationship between tauHYP and heavy metals within individual LC neurons, sections were immunostained with AT8 after autometallography. To detect AMG grains within darkly-stained tauHYP-containing neurons, the microscope illumination was set to high and contrast was increased by reducing the aperture of the substage condenser. Using the combined stain, quantitation of LC neurons containing heavy metals and/or tauHYP was undertaken in 10 individuals who had had both pathologies detected on previous separate staining.

Statistical analyses

GraphPad Prism 5.0f was used for statistical analyses. D’Agostino and Pearson omnibus normality tests were used to distinguish parametric from non-parametric distributions. Unpaired t-tests compared parametric distributions of two unmatched groups. Non-parametric tests used were Mann-Whitney to compare the distributions of two unmatched groups and Spearman correlations to compare continuous variables. 2 × 2 contingency tables with Fisher’s exact tests were used to compare categorical variables. Significance was assessed at the 0.05 level.

RESULTS

The level of sampling of the pons was not the same in each individual, so accurate comparisons of LC neuronal numbers could not be made. However, when sections were available at the equivalent horizontal levels of the pons in AD patients and controls, numbers of LC neurons were usually seen to be greatly reduced in AD patients (Fig. 1). The number of LC neurons of AD patients available for inspection (mean 37 neurons, SD 27 neurons, range 7–95 neurons) was fewer than the number of control LC neurons (mean 64 neurons, SD 23 neurons, range 16–111 neurons) (t-test p = 0.0001).

Fig. 1. Neuronal numbers in AD and control LC nuclei. Sections taken at the same transverse level of the pons show a decreased number and density of LC neurons in a patient with AD (A), compared to a non-demented control individual (B). Bars = 300 μm. Autometallography + hematoxylin.
Fig. 2. Range of heavy metal staining in the LC. No heavy metal staining: in pigmented (open arrows) or non-pigmented (asterisked) neurons. Sparse heavy metal staining: small numbers of small black grains, in pigmented neurons (thin arrows). Dense heavy metal staining: large numbers of large black grains (thick arrows) that often obscure the background cytoplasm. Bar = 25 μm. Autometallography + hematoxylin.

The amount of heavy metal staining by autometallography ranged from a few black grains scattered in the pigmented or non-pigmented component of the cytoplasm of LC neurons, to dense clusters of grains that obscured the background of the cell (Fig. 2). No heavy metal staining was seen in any raphe neurons. In addition, no heavy metal staining was seen in any substantia nigra neurons, even when numerous heavy metal LC neurons were present in the same individual.

No significant correlation was found between age and the percentage of neurons in the LC that contained heavy metals, when AD patients and controls were analyzed together (Spearman rho $-0.04$, $p = 0.73$). Because controls were younger on average than AD patients, a subgroup of controls and AD patients whose ages overlapped (between 63–85 y) was compared; in this analysis, age correlated slightly positively with the heavy metal content of the LC (Fig. 3), but this was not statistically significant (Spearman rho $0.15$, $p = 0.37$).

Taking all AD and control individuals together, the proportion of males and females having LC nuclei with heavy metal LC neurons did not differ significantly (Fisher exact $p = 0.20$), and no significant correlations were found between the percentage of heavy metal LC neurons and either brain weight (Spearman rho $0.10$, $p = 0.44$) or postmortem delay (Spearman rho $0.09$, $p = 0.49$).

The percentage of LC neurons containing heavy metals was higher in the AD group than in controls, but this was not statistically significant (AD median 16%, range 0–100%; control median 5%, range 0–71%; Mann-Whitney $p = 0.34$). To compare groups within the same age ranges, a subgroup of AD patients and controls between 63–85 years of age underwent the same analysis. This analysis showed a stronger trend for patients with AD to have more LC neurons with heavy metals (Fig. 4), though this also did not reach statistical significance (AD median 43%, range 0–100%; control median 5%, range 0–64%; Mann-Whitney $p = 0.07$).

Taup was visible on AT8 immunostaining in the cell bodies of scattered LC neurons, where it often occupied most of the perikaryon. The consistency of AT8-stained taup was either uniform, fibrillar, or a mixture of the two. Neuromelanin stained a similar brown color as tau, and a uniform brown background between neuromelanin granules was taken as evidence of taup. Tau often extended into

Fig. 3. Age in relation to heavy metal content of LC neurons. Increasing age (within the 63–85 y subgroup) tended to correlate positively with the proportion of LC neurons containing heavy metals, when AD patients (closed circles), and controls (open circles) were considered together, but this did not reach statistical significance.
neuronal processes, either in axons or dendrites in continuity with the neuronal cell body, or in neurites in the neuropil. A variable number of tauHYP-positive raphe neuronal cell bodies and neurites were seen. Significantly greater proportions of tauHYP LC neurons were present in AD patients (median 14.5%, range 0–100%) compared to controls (median 0.0%, range 0–24%) (Mann-Whitney $p < 0.0001$).

Combined AMG and AT8 staining in 394 LC neurons of 10 AD patients showed that the majority of neurons contained neither heavy metals nor tauHYP (58%), while roughly equal proportions contained either heavy metals (19%) or tauHYP (21%) (Fig. 5, Table 1). Only 3% of LC neurons contained both heavy metals and tauHYP (Fig. 5E). The proportion of LC neurons in these four groups varied widely between individuals (Table 1).

The number of LC neurons containing heavy metals (in all AD patients and controls) showed no significant correlation with the number of neurons staining for tauHYP (Spearman rho 0.12, $p = 0.35$; Fig. 6).

**DISCUSSION**

Our findings do not suggest that heavy metal uptake later in life causes hyperphosphorylation of tau in LC neurons, since very few LC neurons in our study were found with both heavy metal and tauHYP staining, the numbers of LC neurons containing heavy metals did not correlate with those containing tauHYP, and some individuals had dense heavy metal staining of their LC neurons without tauHYP accumulation. The reason behind heavy metals and tauHYP being located in different LC neurons is not known, but could be because LC neurons have a wide range of synaptic connections to blood vessels, neurons, and glia in different parts of the brain and spinal cord. There is, however, no detailed knowledge of the connections and functions of subsets of LC neurons in the human brain.

The heavy metal seen with autometallography in these neurons is likely to be mercury, since mercury is the only one of the autometallographically-demonstrable heavy metals to which humans are commonly exposed. Autometallography reveals inorganic mercury in the brain, and it is becoming clear that all types of mercury (elemental, organic, and inorganic) are stored long-term in the brain in the inorganic form [20]. Hence any mercury we demonstrated in LC neurons could have arisen from various sources of mercury pollution. The fact that the amount of heavy metal staining in the LC did not increase with increasing age suggests that in our cohort single or a few exposures to heavy metals are more likely to have occurred than a slow accumulation over a period of years.

Neurons containing tauHYP can live for a long time [21], and only a proportion of LC neurons in our AD patients contained tauHYP. Furthermore, dorsal raphe neurons in our study often contained as much tauHYP as those in the LC, but the loss of dorsal raphe neurons in AD is less marked than the loss of LC neurons [22]. No dorsal raphe neurons in our cohort contained heavy metals. This suggests a second insult, such as the uptake of circulating toxicants, rather than tauHYP alone, accounts for the marked loss of LC neurons in AD.

Numerous environmental toxicants have been implicated in AD, so it is serendipitous that one of the few toxicants we could detect, mercury, is one that has arguably the most evidence associating it with AD [10, 23]. Mercury in the LC could well damage tau, since inorganic mercury increases tau phosphorylation [24] and aggregation [25]. Furthermore, the APOE ε4 allele interacts with mercury [26], and differences in the metallothionein family of genes could render people more susceptible to the actions of mercury, since low levels of some metallothionines have been reported in AD [27]. Of particular interest have been recent studies showing how mercury cycles between the soil, the atmosphere, and the ocean [28] and the global extent of environmental pollution with mercury [29]. The fact that mercury is widely dispersed across the globe when it is deposited in water and air could explain a conundrum that has puzzled epidemiologists for some time, i.e., that most toxicants implicated in AD have a limited distribution between and within populations

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Fig. 4. Distribution of the percentage of heavy metal LC neurons in AD patients and controls (within the 63–85 y subgroup). The median percentage of neurons containing heavy metals was slightly (but non-significantly) higher in AD patients than controls.
Fig. 5. Heavy metals and tauHYP in LC neurons visualized with combined AMG and AT8 staining. A) A tauHYP LC pigmented neuron (arrowhead) lies adjacent to a dense heavy metal LC neuron (arrow). A nearby pigmented LC neuron (open arrow) shows neither heavy metals nor tauHYP. Bar = 40 μm. B) Two LC neurons show dense heavy metal staining (arrows), but no tauHYP. TauHYP neurites (asterisks) are present in the neuropil. Bar = 40 μm. C) A tauHYP LC neuronal cell body contains patchy neuromelanin, but no heavy metals. Bar = 25 μm. D) A tauHYP LC neuronal cell body with no visible neuromelanin contains no heavy metals. Bar = 25 μm. E) A rare LC neuron (arrowhead) contains both small black AMG grains, indicating the presence of heavy metals, as well as immunostaining for tauHYP. Below this are neurons containing either sparse heavy metal staining (arrow) or tauHYP-only staining (open arrow). This image was manipulated digitally with increased brightness and contrast to simulate the microscopic conditions used to visualize the cells. Bar = 30 μm. Combined autometallography/AT8 + hematoxylin.
Table 1

<table>
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<th>TauHYP only n (%)</th>
<th>HM + tauHYP n (%)</th>
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<th>Total N</th>
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<td>1 (4)</td>
<td>6 (32)</td>
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</tr>
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<td>14 (54)</td>
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<tr>
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<td>81 (21)</td>
<td>10 (3)</td>
<td>227 (58)</td>
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Fig. 6. Correlation of numbers of LC neurons containing either heavy metals or tauHYP. When all AD patients (closed circles) and controls (open circles) were analyzed together, no significant correlation was found between the numbers of LC neurons containing heavy metals and those staining for tauHYP.

Fig. 7. Correlation of numbers of LC neurons containing either heavy metals or tauHYP. When all AD patients (closed circles) and controls (open circles) were analyzed together, no significant correlation was found between the numbers of LC neurons containing heavy metals and those staining for tauHYP.

[11], but no marked variations exist in the geographical prevalence of age-specific dementia [30]. This places mercury, with its global distribution, as a prime candidate for an environmental agent that could trigger AD.

A model of the potential consequences of our findings for AD is presented in Fig. 7. The upper panel in the figure illustrates the previous suggestion that the tauHYP that forms early on in life in LC neurons spreads via cell-to-cell transfer to other neurons where it forms neurofibrillary tangles [7]. The finding of changes in tau protein in LC neurons of individuals younger than 10 years of age [4] is consistent with the concept that neurodegenerative diseases such as AD may be initiated by environmental exposures early in life [31]. The formation of this early tauHYP could be triggered by environmental toxicants entering the LC, or from physiological stressors that are able, usually reversibly, to phosphorylate tau [32]. It is possible that in genetically predisposed individuals, tau protein could remain hyperphosphorylated in LC neurons and later spread within and between cells.

The lower panel in Fig. 7 illustrates the possibility that a double hit of tauHYP and toxicants within LC neurons could result in the deposition of Aβ in the brain. Damage to the LC with a consequent decrease in its output of noradrenaline has often been implicated in the pathogenesis of AD [33], and a considerable body of evidence now indicates that a decrease of noradrenaline promotes the formation of Aβ plaques [34–36], probably because of breakdown of the blood-brain barrier [37–39]. In our model, toxicants that enter the LC later in life are at first neutralized by neuromelanin, but as this pigment degenerates with aging, the toxicants are released into the cytoplasm [40]. Another factor that could increase the toxicity of mercury in aging LC neurons is a lack of selenium, since mercury is detoxified in cells largely by binding to selenium, and low selenium levels have been implicated in AD [41]. Eventually, these intracellular toxicants, together with the presence of LC neurons partially damaged by tauHYP, decrease the output of noradrenaline, which damages to blood-brain barrier and initiates the deposition of Aβ.

The "chicken and egg" debate has recently re-ignited as to whether in AD tauHYP leads to Aβ, Aβ leads to tauHYP, or whether they evolve separately. This followed reports that tauHYP may be found early in LC neurons, before the onset of Aβ deposition [5, 6]. Some have argued that the AT8 antibody does not differentiate between the different isoforms of tau, raising the possibility that young people with LC tauHYP may have preclinical non-AD tauopathies such as rarer forms...
Fig. 7. Possible consequences of hyperphosphorylated tau and heavy metals in LC neurons. **Top row.** Entry of circulating toxicants (red dots) into a LC neuron, or other stressors, cause hyperphosphorylated tau (orange) to form in the LC axon early in life. Over time, in genetically susceptible individuals, aggregated tau increases in LC neurons, and spreads via cell-to-cell transfer to other neurons, where it forms neurofibrillary tangles. **Bottom row.** Circulating toxicants that enter a LC neuron later in life are bound at first by neuromelanin (yellow dots). In individuals with a genetic susceptibility to the toxicant, toxicants that are freed into the cytoplasm when aging depletes neuromelanin decrease the output of noradrenaline (NA). Noradrenaline output is concomitantly decreased in other LC neurons that contain misfolded tau. This combined assault on noradrenaline output damages the blood-brain barrier and initiates amyloid deposition.

of early-onset dementia, or conditions such as head injury or cerebral infarction [42, 43], though counter-arguments to this have been put forward [44]. Of note, the possibility that non-AD conditions could be responsible for LC tauHYP was considered by Elobied et al. in their study of people aged 25 to 50 years of age
[6]; even though they included only people who died acutely without prolonged disease or intense pharmacological treatment, they found tauHYP in the LC of 33% of their subjects, a similar figure to those of Braak and Del Tredici whose study population included both acute and chronic medical conditions [4]. TauHYP may be found in the brain in the absence of Aβ [43], which argues against the suggestion that tauHYP in the LC is an automatic trigger for Aβ pathology [45]. In addition, the hypothesis that tauHYP in the LC is directly responsible for neuritic plaques is difficult to reconcile with the evidence that an altered blood-brain barrier is implicated in the deposition of Aβ [46]. Our finding of two pathologies in the LC goes some way toward reconciling these different views, since it takes into account both the concept of abnormal tau being transferred from the LC into other neurons to form neurofibrillary tangles, as well as evidence that blood-brain barrier abnormalities underlie the deposition of Aβ in the brain. The double-hit LC model supports the concept that there is neither a chicken nor an egg as regards tauHYP and Aβ in AD, but that both are essential to produce the disease [47].

Our study has a number of limitations: (1) The metal stained by autometallography is likely to be inorganic mercury, because as mentioned of the three metals that are demonstrable by this technique, mercury is the only one to which humans are commonly exposed [29]. Confirmation of this would however require elemental cell-based analyses of unfixed LC sections [47]. (2) Since we could detect only three heavy metals in this study, our findings may represent only the tip of the iceberg of various toxicants that can enter and damage the LC. (3) The remaining LC neurons in AD patients that did not contain toxicants, a “survivor” effect. We may therefore be underestimating the number of neurons that originally contained toxicants. (4) The prevalence of dementia is high in advanced age, and we have no way of knowing if the controls would have developed AD had they lived longer. (5) False positive staining with AMG can be seen after prolonged tissue fixation, points to the LC contributing to Aβ formation, points to the LC playing a role in both the major pathological changes of sporadic AD. Further research to identify the range of toxicants that reside within LC neurons of patients with AD could lead to public health measures to avoid these agents, and encourage future therapeutic measures such as reducing toxicant loads in the brain and raising brain noradrenaline levels.

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