Published in final edited form as:

Neurobiol Aging. 2020 October ; 94: 243–249. doi:10.1016/j.neurobiolaging.2020.06.006.

Calbindin-D_{28K}, Parvalbumin and Calretinin in Young and Aged Human Locus Coeruleus

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Abstract

Certain neuronal populations, including the cholinergic neurons of the basal forebrain (BFCN) and the noradrenergic neurons of the locus coeruleus (LC), are selectively vulnerable to pathology and loss early in the course of aging and Alzheimer's disease (AD). We have shown that BFCN in the human brain show a substantial loss of the calcium binding protein, calbindin-D_{28K} (CB), over the course of normal aging, which is associated with formation of neurofibrillary tangles and loss of BFCN in AD. The purpose of this study was to determine if, similar to the BFCN, LC neurons contain CB or the other two ubiquitous calcium binding proteins (CBP) parvalbumin and calretinin, and if so, whether these proteins display an age-related loss from LC neurons. Immunostaining for CBPs and tyrosine hydroxylase (TH), a marker of catecholaminergic neurons, were used in sections from the LC of young and aged human brains. Parvalbumin and calretinin immunoreactivities were completely absent from human LC neurons. A subpopulation of LC neurons (~10%) contained CB immunoreactivity. Quantitative analysis using sections stained concurrently for visualization of CB and TH revealed no age-related loss of the former from the LC neurons. These findings indicate that, unlike the BFCN, age-related loss of CB does not figure prominently in the selective vulnerability of LC neurons to tangle formation and degeneration in AD.

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Conflict Disclosure

The authors have no conflicts to disclose.

Keywords

Alzheimer's Disease; Calcium Binding Proteins; Calbindin-D_{28k}; Locus Coeruleus; Noradrenergic

1. Introduction

Alzheimer's disease (AD) and other neurodegenerative disorders selectively target specific neuronal populations early in the course of disease (Zarow et. al, 2003). The cholinergic neurons of the basal forebrain (BFCN) are a neuronal population that is selectively vulnerable to degeneration in AD, and in a number of other disorders such Parkinson's Disease and Lewy body dementia (Ahmadian et. al, 2015; Geula et. al, 2003a; Geula et. al, 2008; Mesulam et. al, 2004; Perry et. al, 1993; Riascos et. al, 2011; Schliebs and Arendt, 2010). The BFCN display extensive neurofibrillary tangle formation (Geula et. al, 2008), accumulation of intra-neuronal β -amyloid (A β) peptide (Baker-Nigh et. al, 2015) and degeneration in aging and AD (Geula & Mesulam, 1999). There is a corresponding loss of cortical cholinergic axons originating from the BFCN in AD (Geula et. al, 2008; Mesulam et. al, 2004).

AD pathology, loss of BFCN, and degeneration of cortical cholinergic axons occurs relatively early and is initiated in the course of normal aging (Geula et. al, 2003a; Geula et. al, 2008; Mesulam et. al, 2004; Riascos et. al, 2011). We have shown that BFCN are vulnerable to progressive age-related accumulation of phosphorylated tau in neurofibrillary tangles (Geula et. al, 2008; Mesulam et. al, 2004). A similar age-related progression is seen in abnormalities displayed by cortical cholinergic axons (Geula et. al, 2008). These observations, along with the fact that age is the primary risk factor for AD, point to age-related changes as potential causative factors in the selective vulnerability of BFCN.

We have discovered a substantial age-related change in the BFCN, which can partially explain the selective vulnerability of these neurons in AD. BFCN in the normal young human and non-human primate brains, but not in the rodent, are rich in the calcium binding protein (CBP) calbindin-D_{28k} (CB) (Ahmadian et. al, 2015; Geula et. al, 1993; Geula et. al, 2003a; Riascos et. al, 2011). In a series of experiments, we have shown a substantial and significant age-related loss of CB from the human and non-human primate BFCN (Geula et. al, 2003b; Wu et. al, 1997). The age-related loss of CB is strongly related to tangle formation and degeneration of BFCN (Ahmadian et. al, 2015; Riascos et. al, 2011). Pre-tangles and tangles form almost exclusively in BFCN that have lost their CB, and these inclusions are nearly absent from CB-positive BFCN in aging and AD (Ahmadian et. al, 2015; Riascos et. al, 2011). Therefore, it is likely that the age-related loss of CB from the BFCN deprives these neurons from the calcium buffering capacity conferred by this protein, rendering them vulnerable to fluctuations of intracellular calcium and potential loss through calcium toxicity.

At least two other neuronal populations are known to display early vulnerability to tangle formation and degeneration in AD. One is layer II stellate cell islands of entorhinal/peri-rhinal cortex, which have received extensive experimental attention due to their major role in funneling information from multimodal association cortex to the hippocampus, ensuring

successful memory formation (Braak et. al, 2011; Eichenbaum et. al, 2007; Roy et. al, 2017). The second is the noradrenergic neurons of the locus coeruleus (LC) (Braak et. al, 2011; Braak and Tredici, 2011; Chalermpananupap et. al, 2017; Ehrenberg et. al, 2017; Eser et. al, 2018; Grudzien et. al, 2007; Haglund et. al, 2006; Liu et. al, 2019; Lyness et. al, 2003; Samuels and Szabadi, 2008b; Stratmann et. al, 2016; Theofilas et. al, 2017; Tomlinson et. al, 1981). The LC is located in the rostral pons, extending from the level of the inferior colliculus to the fourth ventricle (Bhagwandin et. al, 2013; Dordevic et. al, 2017; German et. al, 1988). It consists of a column of magnocellular, neuromelanin-containing, noradrenergic neurons (Chalermpananupap et. al, 2017; German et. al, 1988; Grudzien et. al, 2007; Liu et. al, 2019). These neurons are the nearly exclusive source of cortical noradrenergic input (Chalermpananupap et. al, 2017; Dordevic et. al, 2017; Samuels and Szabadi, 2008a; Sara, 2009). LC neurons can be visualized by the presence of neuromelanin, a waste product of catecholamine metabolism that accumulates in dark brown granules within the cell body (Dordevic et. al, 2017; Tomlinson et. al, 1981). They can also be visualized by immunohistochemical staining for the noradrenergic synthetic enzyme dopamine- β hydroxylase, or tyrosine hydroxylase (TH), an enzyme involved in the synthesis of monoamines, including noradrenaline, that is present in all LC neurons (Eser et. al, 2018; Iversen et. al, 1983; Pearson et. al, 1983). Iversen et. al (1983) demonstrated that counts of neuromelanin pigmented neurons or TH immunoreactive neurons in the LC produced nearly identical results.

Among a number of functions, LC neurons are involved in promoting cortical arousal and attention, processes crucial for memory formation and retrieval (Aston-Jones and Bloom, 1981; Samuels and Szabadi, 2008a; Sara, 2009; Szabadi, 2013). Like the BFCN, LC neurons are vulnerable to tau pathology as early as the third decade of life (Braak and Tredici, 2011; Chalermpananupap et. al, 2017; Ehrenberg et. al, 2017; Grudzien et. al, 2007; Stratmann et. al, 2016). In AD, tangle formation in the LC precedes eventual neuronal loss (Theofilas et. al, 2017). In normal individuals, the number of LC neurons may decrease with age (Liu et. al, 2019; Samuels and Szabadi, 2008b; Tomlinson et. al, 1981) but LC neuronal loss is prominent in AD (Chalermpananupap et. al, 2017; Eser et. al, 2018; Haglund et. al, 2006; Lyness et. al, 2003; Samuels and Szabadi, 2008b; Theofilas et. al, 2017; Tomlinson et. al, 1981). LC neuron loss is also seen in Parkinson's disease (PD) (Zarow et. al, 2003).

The noradrenergic neurons of LC share a number of characteristics with the BFCN. Both neuronal populations project diffusely to the entire cortical mantle and represent the primary source of corresponding specific neurotransmitters for the cerebral cortex (Geula et. al, 2008; Mesulam et. al, 1983; Samuels and Szabadi, 2008a). These similarities raise the possibility that the two neuronal populations share similar mechanisms of vulnerability in AD, and that similar to the BFCN, age-related loss of CB or other CBPs also occurs in LC. CB, parvalbumin (PV) and calretinin (CRT) are CBPs that are expressed ubiquitously in distinct neuronal populations throughout the nervous system. Parvizi and Damasio (2003) have shown that a sparse population of LC neurons in the rhesus macaque monkey display CB immunoreactivity. Presence and age-related changes in these calcium-binding proteins in the human LC neurons have not received experimental attention. The purpose of the present study was to determine if LC neurons contain the above CBPs, and whether there are age-related alterations in these proteins. We found that LC neurons are completely free of PV or

CRT immunoreactivities. However, a sparse population of human LC neurons contain CB immunoreactivity. There was no significant age-related change in the percentage of CB-positive LC neurons.

2. Materials and Methods

2.1 Participant information and tissue processing

Brainstem blocks containing the LC from five young (ages 20–63) and five old (ages 70–77) normal participants with no neurological or psychiatric abnormalities were used in these experiments. Postmortem human tissue was acquired in accordance with Institutional Review Board approved protocols. Tissue blocks were fixed in 4% paraformaldehyde for 30–36 hours and taken through increasing sucrose gradients (10%–40%) for cryoprotection. Blocks were cut at a thickness of 40 μ m on a freezing microtome and 1 in 24 series of sections were stored in 0.1 M phosphate buffer until use. The characteristics of each subject are presented in Table 1. One series of sections from each brain was stained for Nissl using the Cresyl violet stain and used for delineation of anatomical boundaries and identification of neuromelanin-containing LC neurons.

2.2 Immunohistochemistry

Full series from each case were immunohistochemically processed using the avidin-biotin-peroxidase (ABC) method as described elsewhere (Hsu et. al, 1981; Geula et. al, 1998) employing the vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Antibodies against the monoaminergic synthetic enzyme tyrosine hydroxylase (TH, Sigma Immunochemicals, mouse monoclonal, 1/1000, catalogue # AB152), and the CBPs CB (Swant, mouse monoclonal, 1/1000, catalogue # 300), PV (Swant, mouse monoclonal, 1/1000, catalogue # 235), and CRT (Swant, mouse monoclonal, 1/1000, catalogue # CR 7697) were used. Tissue sections were incubated in primary antibodies for 18–20 hours at 4°C.

For concurrent visualization of a CBPs and TH, the double-immunohistochemical method described by Levey et al. (1986) was used. A separate TH antibody was used for the double-immunohistochemistry procedure, (Millipore Sigma, Rabbit, Polyclonal 1/1000, catalogue # T8700), to ensure that the two antibodies used were produced in different species. Tissue sections were first processed for TH using diaminobenzidine (DAB) as chromogen, resulting in a brown reaction product. The tissue sections were then processed for a CBP using benzidine dihydrochloride as a second chromogen, resulting in a granular blue / black reaction product. Sections without staining were used to detect the naturally occurring neuromelanin in the LC neurons.

2.3 Quantitative Analysis

All stained sections were subjected to microscopic examination. Noradrenergic LC

neurons were identified by the presence of TH immunoreactivity and/or the presence of granular neuromelanin within the cell. Qualitatively, CB, PV and CRT positive LC neurons were identified by colocalization of immunoreactivity and neuromelanin in the same cell.

Since neuromelanin accumulates with age (Dordevic et. al, 2017), and this study used brainstem blocks from both young (under 65 years) and old (over 65 years) groups, TH-immunoreactivity was used to obtain LC neuronal counts in each section. Initial quantitative estimates were obtained using adjacent series of sections stained for TH or each CBP. Complete quantitative analysis was carried out using double stained sections. Counting was conducted utilizing live images at 40X magnification using a counting box (325×218 μm) placed in the eyepiece of a ZEISS Axio Imager M2 microscope. The total number of immunoreactive neurons in each section were counted. An average of 7 sections were used for counting per case. Counts were expressed as the number or percentage of neurons per section. The counter was blind to the age of each case.

2.4 Statistical Analysis

Statistical analysis was carried out using the Prism software (version 6). A Shapiro-Wilk test of normality was carried out to determine if the data was normally distributed. A correlation analysis between age and number of TH+ and CB+ neurons per section and percentage of LC neurons that contain CB per section was performed. A correlation analysis was also performed between post-mortem interval (PMI) and the above measures. The average numbers of TH-positive and CBP-positive neurons per section were compared across young and aged groups using the t-test to determine significant differences. The average number of CBP-positive LC neurons per section was divided by the average number of LC noradrenergic neurons per section to determine the average percentage of CBP-positive neurons per section. The percent of LC neurons positive for each CBP in each age group was compared using the same method. The probability for significant effects was set at $p < 0.05$.

3. Results

3.1 Identification of LC Neurons

Antibodies to TH, CB, CRT and PV were used to visualize noradrenergic LC neurons and to determine the presence of CBPs within these neurons. Black neuromelanin granules were present within the perikarya of LC neurons (Figure 1A–B). Neuromelanin containing LC neurons were magnocellular in appearance with respect to surrounding neurons and were multipolar in shape. Robust TH-immunoreactivity was present in LC neurons of all cases (Figure 1C–D). Virtually all neuromelanin containing neurons were TH-positive as determined by comparison between unstained and TH stained adjacent sections.

3.2 Presence of CBPs in LC neurons

Presence of CBPs in LC neurons was determined by comparison of TH stained sections and neuromelanin containing LC neurons in unstained sections to CBP stained sections. LC neurons were identified by shape and the presence of granular neuromelanin within the neuron in the CBP stained sections. PV and CRT immunoreactivities were not detected in any LC neurons in any case (Figure 2A, C). Lack of PV and CRT immunoreactivities in LC neurons was not due to methodological issues as abundant staining was present in other neurons and fiber bundles within the same sections. PV immunoreactivity was present in a number of cell groups, including the central gray adjacent to the fourth ventricle, raphe nuclei, pontine nuclei and neurons and fibers of the medial longitudinal fasciculus (Figure

2B). CRT immunoreactivity was also present in a number of neuronal populations and axonal bundles, including those listed for PV (Figure 2D). In contrast to PV and CRT, CB immunoreactivity was detected in a relatively sparse population of LC neurons (Figure 3A–B).

3.3 Qualitative analysis of CB immunoreactivity in LC neurons

No apparent morphological difference was observed between LC neurons that were CB+ compared to those that were not. There was no apparent age-related difference in the size of LC neurons. In order to make a more accurate assessment of the presence of CB in the noradrenergic LC neurons, a double chromogen immunohistochemical approach was used. Qualitative microscopic analysis revealed colocalization of TH and CB in some but not all LC neurons (Figure 3C–D). No age-related difference in the density of CB-positive LC neurons was apparent when young cases were compared to the old.

3.4 Quantitative Analysis

The number of LC neurons that displayed concurrent immunoreactivity for TH and CB, or TH immunoreactivity only were determined in double stained sections. All CB-positive neurons observed in the region in which LC neurons are located were TH-positive, i.e. were noradrenergic neurons. Counts obtained were expressed as average neuronal counts per section in each case (Table 1). A Shapiro-Wilk test indicated the counts were normally distributed. Neuron counts were then compared by age group. The average number of TH-positive LC neurons per section, (young = 58.0, old = 48.8), CB-positive LC neurons per section (young = 6.2, old = 5.2) and the average percentage of CB positive LC neurons per section (young = 9.8, old = 11.5) in each age group were compared using parametric two-tailed t-tests. No significant differences were observed in any of these measures between the young and old groups ($p = 0.53$, $p = 0.61$, $p = 0.57$ respectively; Figure 4). Correlation analysis showed no significant relationships between age and the percentage of CB-positive LC neurons per section ($r = 0.19$, $p = 0.59$), TH+ neurons per section ($r = 0.11$, $p = 0.76$) or CB+ neurons per section ($r = 0.06$, $p = 0.87$). Additionally, correlation analysis showed no significant relationships between PMI and the percentage of CB-positive LC neurons per section ($r = -0.24$, $p = 0.51$), TH+ neurons per section ($r = -0.04$, $p = 0.91$) or CB+ neurons per section ($r = -0.22$, $p = 0.53$). Thus, while a relatively small proportion of LC neurons contain CB immunoreactivity, there does not appear to be any age-related changes in the number of CB-positive LC neurons.

4. Discussion

Similar to the BFCN, LC neurons display selective and early vulnerability to pathology and degeneration in AD (Braak et. al, 2011; Braak and Tredici, 2011; Chalermpananupap et. al, 2017; Ehrenberg et. al, 2017; Eser et. al, 2018; Grudzien et. al, 2007; Haglund et. al, 2006; Liu et. al, 2019; Lyness et. al, 2003; Samuels and Szabadi, 2008b; Stratmann et. al, 2016; Theofilas et. al, 2017; Tomlinson et. al, 1981). We have shown that the human BFCN are rich in CB and display significant age-related loss of this protein (Geula et. al, 2003b; Wu et. al, 1997), and that the age-related loss of CB is associated with the degeneration of these neurons (Ahmadian et. al, 2015; Riascos et. al, 2011). The goal of the present study was to

investigate whether the major CBPs PV, CRT and CB are present in LC neurons, and whether they show a similar age-related loss.

The CBPs, PV and CRT, were absent from the human LC neurons, visualized by the presence of neuromelanin or TH immunoreactivity. The absence of these calcium binding proteins in LC neurons was not due to staining problems as robust PV and CRT immunoreactivities were present in other regions of the brainstem within the same sections.

In contrast to PV and CRT, a subpopulation of LC neurons contained CB immunoreactivity. Quantitative analysis of TH and CB double-stained sections demonstrated that 5.28% - 14.09% of LC neurons in young brains contain this CBP. In contrast, over 70% of the human BFCN in young brains contain CB immunoreactivity. Thus, while a proportion of LC neurons contain CB, this proportion is considerably smaller when compared with the BFCN. CB immunoreactivity is also present in a large proportion of BFCN in non-human primates. However, BFCN in the rodent brain are devoid of CB immunoreactivity (Geula et. al, 1993). In a study of the brainstem in the Macaque monkey, qualitative observations indicated that a small proportion of LC neurons in this non-human primate species also contain CB (Parvizi & Damasio, 2003). However, the presence of CB in the rodent LC remains unknown.

Quantitative analysis failed to reveal any age-related changes in the number or percentage of TH immunoreactive LC neurons which contained CB. This is in contrast to the BFCN, over 60% of which lose their CB in individuals over age 65, and 75% lose their CB in the oldest old (Geula et. al, 2003a; Wu et. al, 1997).

Age-related loss of CB in the BFCN is associated with tangle formation and degeneration in AD. The normal age-related loss of CB in the BFCN is accelerated in AD (Ahmadian et. al, 2015; Riascos et. al, 2011). Our findings indicate that within the LC of healthy individuals, CB is present in a subpopulation of noradrenergic neurons, but does not show any age-related loss. This finding suggests that the selective vulnerability of LC neurons seen in Alzheimer's disease is not directly related to age-related alterations of CB in these neurons. Age-related loss of CB is likely to lead to calcium dysregulation that contributes to degeneration in the BFCN in AD. However, this does not appear to be the case in LC neurons. While age-related change in CB does not appear to contribute to the selective vulnerability of the LC in AD, this finding does not exclude the contribution of age-related changes in other mechanisms that may result in calcium dysregulation, to the vulnerability of these neurons. First, CB is a member of a very large family of CBPs (Yáñez et. al, 2012), and presence or age-related changes in other CBPs may contribute to LC vulnerability. Second, CBPs are only one of several mechanisms through which neurons regulate levels of intracellular calcium. Calcium pumps / channels, and calcium stores also make a major contribution to intraneuronal calcium regulation (Villereal and Palfery, 1989) and any age-related changes in these mechanisms can potentially result in LC vulnerability through calcium dysregulation. Further research is needed to determine the role, if any, of intraneuronal calcium dysregulation in the early vulnerability of LC neurons in AD. The status of CB in LC neurons in AD remains to be investigated.

In conclusion, the results of this study indicate the absence of PV and CRT from the human LC neurons, while demonstrating the presence of CB immunoreactivity in a relatively small subpopulation of these neurons. Unlike the BFCN, we did not observe any age-related changes in CB immunoreactivity in the human LC neurons, indicating that age-related changes in this protein do not figure prominently in the vulnerability of these neurons in AD.

Sources of Funding

This work was supported in part by a Zenith Fellows Award (C.G.) from the Alzheimer's Association; grants from the National Institute on Aging (AG014706 and AG027141), National Alzheimer's Coordinating Center (NACC; U01 AG016976), and by an Alzheimer's Disease Center grant (AG013854).

Abbreviations

AD	Alzheimer's Disease
BFCN	basal forebrain cholinergic neurons
LC	locus coeruleus
Aβ	amyloid- β
CBP	calcium binding protein
CBP+	calcium binding protein positive
CB	calbindin-D _{28K}
CB+	calbindin positive
PV	parvalbumin
CRT	calretinin
TH	tyrosine hydroxylase
TH+	tyrosine hydroxylase positive

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Highlights

- A relatively small subpopulation of the human locus coeruleus noradrenergic neurons contain immunoreactivity for the calcium binding protein, calbindin-D_{28K}.
- There is no significant change in the number of calbindin-positive human locus coeruleus neurons over the course of normal aging.
- Human locus coeruleus neurons are devoid of parvalbumin and calretinin immunoreactivity.

Figure 1: Identification of LC Neurons

LC neurons in unstained sections (A-B) contain brown granules due to neuromelanin.

Robust TH immunoreactivity is present in LC neurons (C-D). Scale bars in A and C are 100 μm and in B and D 25 μm.

Figure 2: No PV and CRT Immunoreactivity in LC Neurons

PV immunoreactivity was not detected in LC neurons (A) but was present in the medial longitudinal fasciculus (B) in both fibers (arrowhead), and neurons (arrows). CRT immunoreactivity was not present in the LC neurons (C), but was detected in neurons within the pontine nuclei (D, arrows). Only neuromelanin brown granules are seen in LC neurons in sections immunostained for PV (A) and CRT (C). Scale bars in A and C are 25 μ m, and in C and D 100 μ m.

Figure 3: CB immunoreactivity in the LC neurons

Immunohistochemical staining for CB (A-B) showed reactivity (arrows) in the LC in some but not all (arrow head) neurons. Double staining for TH (brown) and CB (blue-black) (C-D) in LC neurons indicated the presence of CB immunoreactivity in a minority of neurons. Colocalization could be seen in both the cell body (arrows) and processes (arrow heads). All scale bars measure 25 μm .

Figure 4: Quantitative analysis of age-related differences in LC neurons

Parametric t-test showed no significant difference between the young and old age groups. A; average number of TH positive LC neurons per section in young (N=5) and old (N=5) age groups, $p=0.53$. B; average number of CB positive LC neurons per section in young and old age groups, $p=0.61$. C; CB positive LC neurons per section as percent of TH LC positive neurons per section in young and old age groups, $p=0.57$. Bars represent mean $\pm$ standard error.

Table 1.

Characteristics of Participants

Case #	Group	Age at Death	Sex	PMI (hours)	Average TH+ Neurons per Section	Average CB+ Neurons per Section
1	Young	20	F	12	27	2
2	Young	43	F	10	88	12
3	Young	56	M	5	53	7
4	Young	59	F	20	43	2
5	Young	63	M	8	78	8
6	Old	70	F	13	40	7
7	Old	73	M	16	26	3
8	Old	75	F	17	57	5
9	Old	76	F	13	68	6
10	Old	77	F	42	62	6

PMI, postmortem interval; F, female; M, male