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Hippocampal calbindin-1 immunoreactivity correlate of recognition memory performance in aged mice

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ABSTRACT

Aging-related dysregulation of neuronal calcium metabolism, which not only involves the control of calcium fluxes but also the cytosolic calcium buffering system such as calbindin-1 (Calb1), may disturb synaptic plasticity and thereby memory functioning. Calb1 expression has been shown to affect hippocampal long-term potentiation and learning and to play a neuroprotective role in animal models of ischemic brain injury and neurodegenerative disorders. We hypothesize that memory performance in aged mice correlates with neuronal Calb1 protein expression in the hippocampal formation. We studied a set of 18 aged and 22 young male C57BL/6N mice, in which the aged group performed poorer than the young in single-trial novel object recognition testing (two-tailed $p = 0.005$, U test). Apparent decreases in the Calb1 immunoreactivity (measured by quantitative immunohistochemistry) in aged mice compared to that in young mice were not statistically significant either in the hippocampal CA1 subfield or dentate gyrus. In the aged mouse group, levels of Calb1 immunoreactivity both in the CA1 subfield and dentate gyrus correlated directly with the measure of recognition memory performance (Spearman rank correlation $r_s = 0.47$ and 0.48, two-tailed $p = 0.047$ and 0.044, respectively). Our results suggest that hippocampal Calb1 expression affects memory performance in aged mice probably via its role in maintaining neuronal calcium homeostasis. Alternatively, our finding of lower Calb1 immunoreactivity with poorer memory performance in aged mice might be attributed to saturation of Calb1 protein by higher levels of intracellular calcium, due to aging-related dysregulation of neuronal calcium fluxes.

1. Introduction

Dysregulation of intracellular calcium-mediated signaling is suggested to play a role in the pathophysiology of cognitive decline in old age [31]. Brain aging may involve alterations in the ability of neurons to control calcium fluxes and to recover from calcium loads [19], including the intracellular calcium buffering capacity. In the aged rodent brain, changes in neuronal calcium fluxes in the hippocampus led to a decrease in activity-dependent synaptic strength [9]. Among members of the high-affinity cytosolic EF-hand family of intracellular calcium-binding proteins found in the mammalian brain, calbindin-1 (Calb1, also designated as calbindin-D28k), calretinin and parvalbumin, unlike *ubiquitous* calmodulin, are expressed selectively in well-defined subpopulations of principal neurons and in mostly non-overlapping populations of GABAergic interneurons with species-dependent variability in their distribution [1].

Calb1 was shown to alleviate cell degeneration in different toxic conditions by buffering elevated intracellular calcium levels in PC12 cells [21] and cultured embryonic rat hippocampal neurons [20]. In rat models of ischemic brain injury, Calb1 overexpression or pretreatment led to an improvement in neuronal survival and a decrease in infarct volume [8,34]. The involvement of Calb1 in learning and memory, as well as neurodegenerative disorders, has also been explored in animal models. Calb1-deficient antisense transgenic mice exhibited spatial learning impairments [23]. Over-expression of Calb1 selectively in dentate granule cells of rats disrupted mossy-fiber presynaptic function, reduced long-term potentiation (LTP), and impaired spatial memory [6]. In the presenilin-1 (PS1-M146V) transgenic mouse model of Alzheimer's disease, Odero et al. [25] found increased protein expression of Calb1 and enhanced LTP in the hippocampal CA1 subfield, as well as alterations in some parameters of hippocampus-dependent spatial memory by the age of 6 months. Loss of Calb1 immunoreactivity in the dentate gyrus was reported in the amyloid-precursor-protein/presenilin-1 transgenic mice [26].

Abbreviations: Calb1, calbindin-1; LTP, long-term potentiation; DR, discrimination ratio; IRn, immunoreactivity normalized to neuroanatomic area measured; AOI, area of interest; DAB, diaminobenzidine; IQR, interquartile range.

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In animal models of MPTP-induced parkinsonism, subpopulations of midbrain dopaminergic neurons relatively spared from degeneration were immunoreactive for Calb1 [10]. Aging-related reductions in Calb1 expression were found, although not consistently across different studies [4,12,17], in selective brain regions in a species-dependent manner; however, the correlative analyses with behavioral or neuropsychological data were still lacking.

In the present study, we examined the relationship between hippocampal Calb1 expression and memory performance in aged mice assessed by single-trial novel object recognition testing. This behavioral task is a relatively simple test of recognition memory that does not require aversive learning (e.g., spatial navigation to escape aversive stimuli) or food restriction and has relatively low physical demands that may introduce potential confounds in aged mice [5]. This task has been widely used in rodent models of aging among other studies [5]. We performed quantitative immunohistochemistry to measure Calb1 protein expression. We hypothesize that memory performance in aged mice correlates with neuronal Calb1 protein expression in the hippocampal formation.

2. Materials and methods

2.1. Animals and behavioral testing

Two groups of male C57BL/6N mice, young (6-month-old, $n = 22$) mice purchased from Charles River Laboratories (Wilmington, MA, USA) and aged (26-month-old, $n = 18$) mice from the U.S. National Institute on Aging stock located in Charles River Laboratories, were used for single-trial novel object recognition testing as previously described [29]. Following the object recognition test, the animals also underwent other behavioral tests including prepulse inhibition, attentional-set-shifting, and fear conditioning [35,36]. The animals were killed at least 7 days after the completion of all the behavioral tests (60 days after the object recognition test). All procedures were in accordance with the Principles of Laboratory Animal Care (National Institutes of Health [NIH] publication No. 86-23, revised 1985) and approved by the University of California, San Diego Animal Care Committee. The young and aged mouse groups did not significantly differ in the overall locomotor activity (i.e., the total travel distance, velocity, and zone entries) during the habituation phase or in the total amount of time spent exploring both identical objects during the training (sample) phase [29]. The ratio of the time spent exploring the novel object over the total amount of time spent exploring both novel and familiar objects during the retention (choice) phase of the novel object recognition test (i.e., [novel object exploration time]/[novel object + familiar object exploration time]), referred to as the discrimination ratio (DR) [3], was used to measure the recognition memory performance (i.e., the higher DR, the better memory performance).

2.2. Immunohistochemistry

Five μm-thick formalin-fixed paraffin-embedded parasagittal sections of all the right hemi-brains revealed neuroanatomic landmarks consistent with the levels from 1250 to 1750 μm from the midline [32]. No significant histopathologic changes were observed in any of the sections stained with hematoxylin and eosin. For immunohistochemistry with anti-Calb1 antibody (rabbit polyclonal, #AB1778, Millipore, Billerica, MA, USA, 1:300 dilution), antigen retrieval was performed by autoclaving at 121 °C for 20 min with 10 mM Tris/1 mM EDTA-2Na/0.05% Tween 20 buffer (pH 9). Immunohistochemical signals were developed using the ImmPRESS™ anti-rabbit IgG (peroxidase) polymer detection kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (ImmPACT™ DAB peroxidase substrate, Vector Laboratories), as described [29]. All the sections were processed in the same batch. For the negative reagent control, the primary antibody was omitted.

2.3. Quantification of immunohistochemical reactivity

The immunoreactivity signals were measured by means of two-dimensional computer-assisted image analysis. Briefly, the entire hemi-brain sections immunostained with DAB were digitally scanned using a microscope slide scanner (Aperio ScanScope® GL, Vista, CA, USA) equipped with a 20× objective lens (yielding the resolution of 0.5 μm per pixel). Using the ImageScope™ software (Aperio), a square of 3000 μm × 3000 μm covering the entire dorsal hippocampal formation was extracted from each hemi-brain.

On each of the hippocampal-formation images having the same size and resolution, the outline of area of interest (AOI, i.e., the combined CA1 strata oriens, pyramidale, radiatum and lacunosum-moleculare, and the dentate stratum granulosum) was digitally delineated with the Image-Pro Analyzer software (version 6.3, Media Cybernetics, Bethesda, MD, USA), as described [29]. In measurement of immunoreactivity levels within AOI using the Image-Pro Analyzer software, the same setting of histogram-based RGB color segmentation was applied to all the hemi-brains. The values of immunoreactivity normalized to the AOI (IRn), calculated as described [29], were used for statistical analysis.

2.4. Statistical analysis

We chose to use non-parametric methods because they were less influenced by outlier data compared to parametric methods; additionally, in the present study the number of animals in each group was fewer than 24 [24]. The Mann–Whitney U test was used to compare continuous variables between two independent groups. The Spearman rank correlation (r_s) test was employed to evaluate the linear relationship between two continuous variables in a given group. The GraphPad InStat 3 (GraphPad Software, La Jolla, CA, USA) was used to perform all statistical analysis. All p values were two-tailed and considered statistically significant at a threshold of $p < 0.05$.

3. Results and discussion

We found a characteristic laminar pattern of the Calb1 immunoreactivity in the dorsal hippocampal formation (Fig. 1A–C). The immunoreactivity signals were variably intense in the soma (both cytoplasm and nuclei) of CA1 pyramidal neurons, particularly those superficial neurons toward the stratum radiatum, and were weak in the CA1 strata oriens, radiatum (with relative enhancement of the apical dendrites), and lacunosum-moleculare. The dentate granule cells were intensely immunoreactive for Calb1 in their soma (both cytoplasm and nuclei), with moderate immunoreactivity seen in the stratum molecular and mossy fiber bundle. Scattered Calb1-immunoreactive interneurons were present. Pyramidal neurons in the CA3 subfield were non-reactive for Calb1. In addition, moderate to intense Calb1 immunoreactivity was observed in the cerebral cortical layer II and III neurons, scattered interneurons in lower cerebral cortical layers, striatal and thalamic neurons, superior-collicular neurons, and cerebellar Purkinje's cells with weak Calb1 immunoreactivity in the cerebellar molecular layer. Collectively, the Calb1 immunoreactivity pattern seen in our study was consistent with that pattern reported previously in rodents [1,7,28]. We observed no difference in the laminar pattern of Calb1 immunoreactivity between young and aged mice or among different levels of memory performance.

In our study, apparent decreases in the Calb1 IRn in aged mice compared to that in young mice were not statistically significant either in the CA1 subfield (median = 2.82 and 3.73, interquartile

Fig. 1. Calbindin-1 (Calb1) immunoreactivity in the dorsal hippocampal formation of mice. The immunoreactivity signals are present in principal neurons of the CA1 subfield (arrows in [A], a young mouse) and dentate gyrus (arrowheads in [A]), mossy fibers, and scattered interneurons; no Calb1 immunoreactivity observed in principal neurons of the CA3 subfield. The Calb1 immunoreactivity appears more intense in an aged mouse with relatively good memory performance (B) than in an aged mouse with relatively poor memory performance (C) in novel object recognition testing (the discrimination ratio [i.e., the ratio of the time spent exploring the novel object over the total amount of time spent exploring both familiar and novel objects during the retention phase] = 0.68 and 0.41, respectively).

range [IQR] = 3.69 and 2.99, respectively; $p = 0.82$, U test) or dentate gyrus (median = 25.42 and 27.39, IQR = 19.73 and 31.06, respectively; $p = 0.21$, U test). In previous studies, Dutar et al. [7] found a variation in Calb1 immunoreactivity in the CA1 stratum pyramidale sections from aged rats, which appeared decreased compared to that immunoreactivity in young-adult rats, while Iacopino and Christakos [12] showed aging-related reductions in Calb1 mRNA and protein levels in the striatum and cerebellum but not in the hippocampus or cerebral cortex of rats. No behavioral data were available in these two reports.

In the present study, the DR (a measure of recognition memory performance) distribution in aged mice was lower than that in young mice (median = 0.54 and 0.67, IQR = 0.11 and 0.19, respectively; $p = 0.005$, U test, Fig. 2). In aged mice the Calb1 IRn both in the CA1 subfield and dentate gyrus showed direct linear correlation with DR ($r_s = 0.47$ and 0.48, $p = 0.047$ and 0.044, respectively, Fig. 3A), while in young mice the linear correlation between the Calb1 IRn and DR was not statistically significant either in the CA1 subfield or dentate gyrus ($r_s = 0.25$ and 0.19, $p = 0.26$ and 0.40, respectively, Fig. 3B). The relationship between recognition memory performance and hippocampal Calb1 expression in aged mice can be considered biologically relevant, as evidence has suggested that the hippocampus, as well as the perirhinal cortex, is essential for object recognition memory in rodents [30]. Kruger et al. [16] reported that 4-month-old rats exposed to prolonged (8-day) subordination stress and killed immediately after testing exhibited physical signs of stress and increased Calb1 immunoreactivity specifically in the CA1 stratum pyramidale without neurodegenerative changes. In contrast to this stress-associated change in Calb1 immunoreactivity [16], our present study demonstrated that the Calb1 IRn in the dentate gyrus showed strong direct correlation with that in the CA1 subfield in each of the young ($r_s = 0.82$, $p < 0.0001$) and aged ($r_s = 0.82$, $p < 0.0001$) mouse groups. In our behavioral experiments, we allowed a recovery period of at least 7 days following the completion of the test battery before the animals were killed. Accordingly, it is unlikely that levels of hippocampal

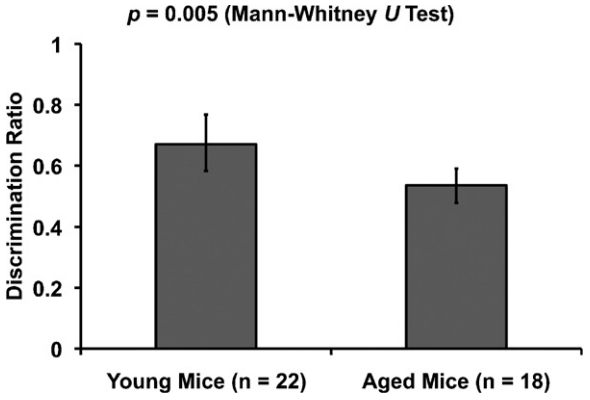

Fig. 2. Comparison of recognition memory performance between young and aged mice. The discrimination ratio (i.e., the ratio of the time spent exploring the novel object over the total amount of time spent exploring both familiar and novel objects during the retention phase of the novel object recognition test) distribution in aged mice is significantly lower than that in young mice (shown in median and interquartile range values). The p value is two-tailed.

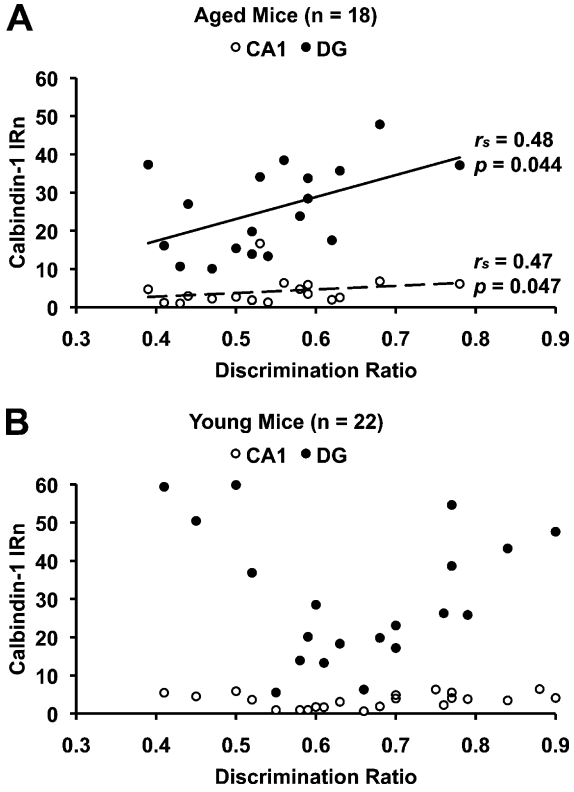

Fig. 3. The relationship between hippocampal calbindin-1 (Calb1) immunoreactivity and recognition memory performance. (A) In aged mice, scatter graphs with their trend lines show significant direct linear correlations between the discrimination ratio (i.e., the ratio of the time spent exploring the novel object over the total amount of time spent exploring both familiar and novel objects during the retention phase of the novel object recognition test) and the immunoreactivity normalized to the neuroanatomic area measured (IRn) for Calb1 in the CA1 subfield and dentate gyrus (DG) [Spearman rank correlation; the p values are two-tailed]. (B) In young mice, no significant linear correlation between the discrimination ratio and the Calb1 IRn is observed either in the CA1 subfield or DG ($r_s = 0.25$ and 0.19, two-tailed $p = 0.26$ and 0.40, respectively, Spearman rank correlation).

Calb1 immunoreactivity observed in our study were influenced by task-associated stress.

Our findings suggest that hippocampal Calb1 expression (measured by quantitative immunohistochemistry) affects memory performance in aged mice probably via its role in the maintenance of neuronal calcium homeostasis, a critical factor in synaptic transmission and plasticity [19]. In support of our results, an electrophysiological study by Jouvenceau et al. [15] using hippocampal slices of Calb1-deficient antisense transgenic mice (with intact NMDA-receptor and calcium-channel properties) showed that LTP induced by tetanic stimulation in the CA1 subfield was impaired due to the increased intracellular concentration of free calcium.

In the hippocampal formation, inhibitory GABAergic interneurons regulate the firing rate of principal neurons, modulate their spike timing, and synchronize their activity. In addition to principal neurons in the CA1 subfield and dentate gyrus, a subpopulation of hippocampal interneurons express Calb1 [28]. A specific subset of Calb1-immunoreactive interneurons that are localized in the CA1 stratum oriens and all layers of the CA3 subfield are somatostatin-immunoreactive H-MS cells, which project distantly to the medial septum to innervate mainly parvalbumin-immunoreactive GABAergic neurons and to a lesser extent cholinergic neurons [14]. The medial septum in turn provides rhythmic drive to the hippocampus, forming a reciprocal loop that plays a critical role in generating the hippocampal theta rhythm and synchronization. Accordingly, decreased Calb1 expression in hippocampal interneurons in aged animals [27,28] may alter the septo-hippocampal loop function and inhibitory regulation of excitability and synchronization of hippocampal principal neurons, thereby adversely affecting cognitive functioning. Nonetheless, our present study did not specifically measure Calb1 immunoreactivity in hippocampal interneurons.

As an alternative interpretation, our finding of lower Calb1 immunoreactivity with poorer memory performance in aged mice might be attributed to saturation of Calb1 protein by higher levels of intracellular calcium, due to aging-associated dysregulation of neuronal calcium fluxes. This Calb1 saturation might prevent the recognition of Calb1 protein by its antibody used in immunohistochemistry. This possibility was consistent with the findings in a study by Dutar et al. [7] using rat hippocampal slices with relatively short exposure to media containing different levels of calcium.

The importance of Calb1 in the pathophysiology of common neurodegenerative disorders has been examined in human brains. In Alzheimer's disease, reductions in the number of Calb1-containing neurons were observed in the hippocampal CA2 subfield [18] and various isocortical regions [13] in a layer-specific fashion [11]. In Parkinson's disease, Calb1-immunoreactive subpopulations of dopaminergic neurons residing in the dorsal substantia nigra pars compacta and ventral tegmental area were relatively spared from degeneration [10,33]. In a case-control association study, CALB1 rs1805874 was shown to be a susceptible gene for Parkinson's disease in the Japanese population [22].

The novel object recognition test measures spontaneous behavior of rodents and relies on their natural tendency to explore novelty. One challenge in memory research is the question of whether impairments detected reflect memory dysfunction or are merely due to non-specific effects of sensory, motor, or motivational systems [5]. Between the young and aged mouse groups, we found no significant difference in the overall locomotor activity during the habituation phase or in the total amount of object exploration time during the training (sample) phase. Accordingly, the aging-related difference in memory performance observed in our study likely indicated memory functioning rather than confounding effects.

4. Conclusion

Our present study found direct correlation between hippocampal Calb1 immunoreactivity and recognition memory performance in aged mice. The Calb1 immunoreactivity levels might directly reflect the levels of Calb1 protein expression. Alternatively, the lower levels of Calb1 immunoreactivity in aged mice exhibiting poorer memory performance might result from saturation of Calb1 protein by higher intracellular calcium levels [7], due to aging-related dysregulation of neuronal calcium fluxes. Nonetheless, the interpretation of our findings is limited by the correlative study approach. A more mechanistic study (e.g., direct pharmacological rescue) can be conducted in future experiments by treating aged mice with intracellular calcium chelator BAPTA-AM before behavioral testing, similar to a study of the relationship between intracellular calcium levels and ethanol-induced behavioral effects in mice [2]. Also, in situ hybridization for Calb1 mRNA may help determine whether there is an actual decrease in Calb1 gene expression in relation to memory impairment in aged mice, although the post-transcriptional regulatory mechanisms such as micro-RNA interference need to be considered as well.

Disclosure statement

The authors declare that they have no conflicts of interest. V.B. Risbrough and J.W. Young conducted animal behavioral testing. V. Soontornniyomkij managed literature search, optimized immunohistochemical protocols, undertook statistical analysis and data interpretation, and wrote the manuscript. B. Soontornniyomkij performed immunohistochemical experiments and image analysis. D.V. Jeste and C.L. Achim supervised the study design and interpretation of data. All authors contributed to and have approved the final article.

Role of funding sources

The funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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