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Neuroinflammation not associated with cholinergic degeneration in aged-impaired brain

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Abstract

Degeneration of the cholinergic neurons in the basal forebrain and elevation of inflammatory markers are well-established hallmarks of Alzheimer's disease; however, the interplay of these processes in normal aging is not extensively studied. Consequently, we conducted a neuroanatomical investigation to quantify cholinergic neurons and activated microglia in the medial septum/vertical diagonal band (MS/VDB) of young (6 months) and aged (28 months) Fisher 344 × Brown Norway F₁ rats. Aged rats in this study were impaired relative to the young animals in spatial learning ability as assessed in the Morris water maze. Stereological analysis revealed no difference between aged and young rats in the total numbers of cholinergic neurons, demonstrating that loss of cholinergic neurons is not a necessary condition to observe impaired spatial learning in aged rats. In this same region, the total number of activated microglia was substantially greater in aged rats relative to young rats. Jointly, these data demonstrate that aging is characterized by an increase in the basal inflammatory state within the MS/VDB, but this inflammation is not associated with cholinergic neuron death.

Keywords

aging; memory; acetylcholine; inflammation; microglia

1. Introduction

The loss of cholinergic neurons in the basal forebrain is a reliable trait of Alzheimer's disease (AD; Whitehouse et al., 1981). Additionally, the AD brain is characterized by activated microglia that release pro-inflammatory cytokines (reviewed in Schwab and McGeer, 2008), suggesting that neural inflammation and related glial responses may contribute to degeneration of cholinergic neurons (Willard et al., 1999). Aging remains the predominant risk factor for AD but the effect of chronological aging on the cholinergic and

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Disclosure

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microglial populations of the basal forebrain is not fully understood. To address this matter, we utilized rigorous stereological techniques to estimate the total numbers of cholinergic neurons and activated microglia in the medial septum/vertical diagonal band (MS/VDB), the subdivision of the basal forebrain that innervates the hippocampus (Amaral and Kurz, 1985). Fisher 344 $\times$ Brown Norway F₁ (F344 $\times$ BN) rats were used in this study as this is a particularly vigorous hybrid strain that ages robustly albeit exhibiting a pattern of memory impairment similar to other strains including Long-Evans and F344 rats (LaSarge and Nicolle, 2009). We hypothesized that aged rats with spatial learning impairment would exhibit a loss of cholinergic neurons and concurrent increases in activated microglia in the MS/VDB.

2. Methods and Materials

2.1. Subjects

Young (6 months, $n = 9$) and aged (28 months, $n = 6$) F344 $\times$ BN hybrid male rats were obtained from the colony maintained by Harlan Sprague Dawley, Inc. (Indianapolis, IN) from the National Institutes of Aging and housed at Wake Forest University School of Medicine in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained on 12 hour light/dark cycle with *ad libitum* access to food and water. The Institutional Animal Care and Use Committee approved all protocols described in this report.

2.2. Spatial reference memory in the Morris water maze

Rats were trained according to the procedure of Gallagher (1993) and data were collected and analyzed using EthoVision software (Noldus, Leesburg, VA). The primary measures used to assess spatial-learning performance were path length and average distance from platform (Maei et al., 2009). Average distance values obtained from the second, third and fourth probe trials administered during the protocol were summed to generate individual “proximity scores.” This score reflects the development of an efficient search pattern near the escape platform location; lower scores indicate better performance and higher scores reflect search farther from the platform. Visible-platform training administered at the end of the protocol provided an assessment of sensorimotor and motivational factors that might influence performance in the spatial learning task.

2.3. Tissue processing and immunohistochemical staining

Rats were deeply anesthetized with ketamine/xylazine and perfused transcardially with phosphate buffered saline (pH 7.4) followed by 4% paraformaldehyde in phosphate buffer. Brains were extracted and processed as described in Schindler et al (2008). Tissue sections containing the MS/VDB were sequentially immunolabelled with anti-choline acetyltransferase (ChAT; a marker of cholinergic neurons; Chemicon; 1:150) and anti-CD68 (ED1 clone; a marker of activated microglia; AbD Serotec; 1:500). Labeling was detected with the appropriate biotinylated anti-IgG (Vector; 1:300) and peroxidase conjugated avidin-biotin complex (ABC; Vector) using Vector SG substrate, forming a grey/black reaction product (ChAT), or diaminobenzidine, forming a brown reaction product (CD68). Sections were then counterstained with the nuclear binding dye 40,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Aldrich). Sections were mounted on charged slides, dehydrated, defatted and cover-slipped with Cytoseal mounting medium (Richard Allan Scientific).

2.4. Quantitative analyses

A modified optical dissector technique was employed to estimate total cell numbers (Kempermann et al., 1998). ChAT⁺ cells were counted in every 8th section and CD68⁺ cells in every 16th section of the basal forebrain in a systematically random series beginning rostrally at the genu of the corpus callosum and ending caudally at the decussation of the anterior commissure. Using an Olympus microscope equipped with a motorized stage controlled by a PC running the NeuroLucida software program (MicroBrightField), the MS/VDB was defined at low magnification according to (Paxinos and Watson, 1998); see Supplementary Figure S1A). Immunopositive cells were observed through a 40× plan-apo objective lens, 0.75 N. A., and counted exhaustively within each section. In accordance with previously applied counting procedures, only ChAT⁺ cells with visible nuclei were counted (Baskerville et al., 2006) and CD68⁺ cells in the top focal plane were excluded to avoid overestimation (Schindler et al., 2008; see Supplementary Figure S1B and C). The sum of profiles counted was multiplied by the inverse of the section sampling fraction to achieve an estimate of final cell number.

2.5. Statistical analyses

Training trial performance was analyzed by repeated measures ANOVA with trial block as a within-subjects factor and age as a between-subjects factor. The proximity score and the estimates of total cell numbers were compared between young and aged rats by means of independent-samples t-tests. Cell density measures were analyzed by repeated measures ANOVA with section as a within-subjects factor and age as a between-subjects factor. Significant effects were subsequently tested using a paired-samples t-test.

3. Results

3.1. Morris water maze performance

Aged rats demonstrated impairment relative to young rats during the course of the training protocol ($F_{(1,13)} = 15.45, p = 0.002$; Figure 1A). Similarly, aged rats were characterized by significantly higher proximity scores, reflecting a poor spatial bias for the platform location ($t_{(13)} = 6.82, p < 0.001$; Figure 1B). Despite the observed performance deficits on the hidden platform version of the task, no differences were observed between young and aged rats in finding a visible platform, indicating that performance deficits were not due to sensorimotor or motivational differences ($t_{(13)} = 1.17, p = 0.27$ NS; Figure 1C).

3.2. Quantitative measures of cholinergic neurons and activated microglia in MS/VDB

The *total number* of cholinergic neurons (ChAT⁺ cells) did not differ between young and aged rats ($t_{(13)} = -0.12, p = 0.991$ N.S.; Figure 2A). Similarly, there were no age-related differences in *density* of neurons observed when considering the rostro-caudal extent of sections counted ($F_{(1,6)} = 0.591, p = 0.736$ N.S.; Figure S2). In contrast, there was a robust increase in the *total number* of activated microglia (CD68⁺ cells) in aged relative to young ($t_{(13)} = 18.29, p < 0.001$; Figure 2B), indicating an increased basal inflammatory state in the aged MS/VDB. In aged rats, there was a greater *density* of activated microglia in the rostral MS/VDB compared to the medial and caudal divisions, suggesting that the neuroinflammatory response is not homogeneous throughout this nucleus ($F_{(2,10)} = 7.262, p = 0.011$; Figure 2C).

4. Discussion

4.1. No loss of cholinergic neurons in aged-impaired MS/VDB

Our findings demonstrate that spatial learning impairment in aged F344×BN rats is not accompanied by loss of cholinergic neurons that project to the hippocampus. These results are in agreement with a recent stereological study in aged F344 rats and a non-stereological study in aged mice showing no changes in total numbers of MS/VDB cholinergic neurons (Ypsilanti et al., 2008; Hornberger et al., 1985). Our estimate of the total number of cholinergic neurons in the MS/VDB in F344×BN rats is similar to those reported in F344 rats by Ypsilanti and colleagues (2008) and also provides evidence indicating the stability of this neuronal population despite spatial learning impairment. These findings are in contrast to previous non-stereological studies in aged rats that describe a loss of septohippocampal cholinergic neurons in rats with cognitive impairment. This pattern of loss occurs across a number of rat strains including Sprague-Dawley (Fischer et al., 1992), Fisher 344 (Armstrong et al., 1993), Dark Agouti (Greferath et al., 2000) and Long-Evans rats (Baskerville et al., 2006); but see also (Stemmelin et al., 2000). It is possible that caveats such as exact age, precise anatomical localization of the counting area or counting method contributes to the apparent discrepancies in the literature. Importantly, stereological evaluations, such as ours, yield precise estimates of total number that may be readily compared across studies, unlike profile-based counts that may be presented as a variety of statistics with or without correction factors.

4.2. Numbers of activated microglia are increased in aged MS/VDB

Importantly, a substantial increase in the number of activated microglia was observed with aging, indicating a considerable increase in the local inflammatory state. Previously, it was assumed that cholinergic neurons were uniquely susceptible to inflammatory insults. The inflammatory agent lipopolysaccharide stimulates microglial activation and proliferation and subsequently induces degeneration of cholinergic neurons when infused intracerebrally into the basal forebrain (Willard et al., 1999) or applied to mixed neuronal/glia cultures (McMillian et al., 1995). In contrast, our data show that cholinergic neurons persist in spite of an age-dependent increase in microglial activity. Notably, a greater density of activated microglia was observed in the rostral MS/VDB of aged rats relative to medial and caudal divisions. The rostral MS/VDB mainly contains the ventral division of the cholinergic basal forebrain that preferentially innervates the septal, or dorsal, hippocampus (Amaral and Kurz, 1985). As the dorsal hippocampus is critical for spatial learning performance (Moser and Moser, 1998), these findings may be suggestive of biochemical alterations that explain observed memory impairments. While a link between neuroinflammation and cognitive impairment is far from definitive, the release of inflammatory cytokines by activated microglia alter a number of neurophysiological parameters including neurotransmitter release, maintenance of long-term potentiation and neurite outgrowth (reviewed in Godbout and Johnson, 2009).

4.3. Conclusions

In summary, we propose that outright loss of cholinergic neurons comprising the septohippocampal system is not a requisite feature of cognitive aging. In the absence of overt neuron loss, the increased number of activated microglia indicates that the aged basal forebrain is characterized by increased inflammation, although the relationship between neuroinflammation and memory loss in older rats remains unclear. Although previous experiments have demonstrated that administration of proinflammatory agents activate microglia and subsequently trigger death of cholinergic neurons in young rats and in cell culture, our findings indicate that a naturally occurring, proinflammatory environment in the MS/VDB of aged rats does not result in a loss of cholinergic neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Morris water maze performance in young and aged rats

Aged rats were impaired relative to young in a place-learning task in the Morris water maze, as evidenced by greater path length measures throughout the training protocol (A) and greater composite proximity scores derived from probe trials (B). The solid line denotes group mean. Aged rats were not impaired on a visible-platform version of the task (C).

Figure 2. Quantitative measures of cholinergic neurons and activated microglia in young and aged rats within the MS/VDB

No difference between stereological estimates of total number of ChAT⁺ cells (cholinergic neurons) in young and aged rats (A). Total number of CD68⁺ cells (activated microglia) was substantially increased in aged relative to young (B). When considering only aged rats, a greater *density* of activated microglia was observed in the rostral MS/VDB of aged rats (C).