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Locus Coeruleus Degeneration Induces Forebrain Vascular Pathology in a Transgenic Rat Model of Alzheimer's Disease

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Abstract

Noradrenergic locus coeruleus (LC) neuron loss is a significant feature of mild cognitive impairment and Alzheimer's disease (AD). The LC is the primary source of norepinephrine in the forebrain, where it modulates attention and memory in vulnerable cognitive regions such as prefrontal cortex (PFC) and hippocampus. Furthermore, LC-mediated norepinephrine signaling is thought to play a role in blood-brain barrier (BBB) maintenance and neurovascular coupling, suggesting that LC degeneration may impact the high comorbidity of cerebrovascular disease and AD. However, the extent to which LC projection system degeneration influences vascular pathology is not fully understood. To address this question *in vivo*, we stereotactically lesioned LC projection neurons innervating the PFC of six-month-old Tg344–19 AD rats using the noradrenergic immunotoxin, dopamine- β -hydroxylase IgG-saporin (DBH-sap), or an untargeted control IgG-saporin (IgG-sap). DBH-sap-lesioned animals performed significantly worse than IgG-sap animals on the Barnes maze task in measures of both spatial and working memory. DBH-sap-lesioned rats also displayed increased amyloid and inflammation pathology compared to IgG-sap controls. However, we also discovered prominent parenchymal albumin extravasation with DBH-sap lesions indicative of BBB breakdown. Moreover, microvessel wall-to-lumen ratios were increased in the PFC of DBH-sap compared to IgG-sap rats, suggesting that LC deafferentation results in vascular remodeling. Finally, we noted an early emergence of amyloid angiopathy in the DBH-sap-lesioned Tg344–19 AD rats. Taken together, these data indicate that LC projection

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system degeneration is a nexus lesion that compromises both vascular and neuronal function in cognitive brain areas during the prodromal stages of AD.

Keywords

Alzheimer's disease; blood-brain barrier; cerebral amyloid angiopathy; locus coeruleus; vascular remodeling

INTRODUCTION

Alzheimer's disease (AD) is believed to have an extensive preclinical stage since older people with a clinical diagnosis of no cognitive impairment or mild cognitive impairment (MCI) consistently reveal pathological signatures similar to those with frank AD [1–3]. Moreover, the majority of MCI and AD cases also present with cerebrovascular pathology such as microinfarctions, microbleeds, and white matter lesions [4–9], and vascular comorbidities are significant risk factors for cognitive decline [10–14]. Vascular lesions impair the structure and function of the neurovascular unit, which alters cerebral blood flow regulation, disrupts blood-brain barrier (BBB) function, and reduces the brain's repair potential, supporting the notion that vascular pathology potentiates AD by reducing the threshold for cognitive impairment and accelerating the pace of dementia [15]. Hence, the identification of neurovascular pathologic events during the preclinical and prodromal stages of AD may provide a unified framework for understanding vascular contributions to AD and improving therapeutic target identification within a disease modifying window.

In this regard, we recently demonstrated that the locus coeruleus (LC) displays a ~30–35% loss of noradrenergic neurons during the transition from no cognitive impairment to amnesic MCI, and that decreases in LC neuron number were significantly associated with poorer performance on neuropsychological tests of episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability [16]. Taken together with previous reports that LC neurons are among the first to display neurofibrillary tangle pathology [17, 18] and that LC neuron loss correlates with Braak stage and clinical status [19, 20], our observations support the concept that LC projection system degeneration is a prominent feature of prodromal AD that contributes to cognitive impairment [21]. However, the mechanisms by which the loss of forebrain norepinephrine (NE) contributes to AD are unresolved. LC-NE signaling modulates attention, memory, executive function, and arousal [22], and *in vitro* and *in vivo* studies show that NE exerts a wide array of neuroprotective and neurotrophic effects that reduce inflammation, oxidative stress, and plaque and tangle pathology [23–28]. On the other hand, the LC also provides central regulation of the cerebral vascular system [29–33], yet the extent to which LC degeneration impacts cerebrovascular pathology during AD progression has received virtually no attention. To address this knowledge gap, we administered a dopamine- β -hydroxylase IgG-saporin (DBH-sap) immunotoxin [34] into the prefrontal cortex (PFC) of Tg344–19 AD rats [35], which effectively mimicked LC-NE deafferentation of a major LC projection zone [36]. DBH-sap and control IgG-saporin (IgG-sap)-treated rats were compared for cognitive performance, AD-like pathology including parenchymal and vascular amyloidosis, and cerebrovascular

pathology including markers for BBB function and vascular remodeling. As reported below, our results suggest that prodromal degeneration of LC projection neurons promotes forebrain vascular pathology, which may contribute to the onset of cognitive impairment during the progression of AD.

MATERIALS AND METHODS

Animals and timeline

We used male and female Tg344–19 AD rats on the Fischer 344 background, overexpressing both human amyloid- β precursor protein (A β PP) bearing the A β PP KM670/671NL Swedish mutation (*APP-swe*) and human presenilin-1 (PS1) bearing the exon 9 deletion mutation (*PS1 E9*) under the mouse prion protein gene promoter [35]. Tg344–19 AD rats manifest an age-dependent cerebral deposition of amyloid- β (A β) plaque-like pathology that precedes tauopathy, gliosis, apoptotic loss of neurons in the cerebral cortex and hippocampus, and cognitive disturbances [35, 37, 38]. Original breeding colonies were provided by the Rat Resource Center (St. Louis, MO; funded by NIH grant P40 OD011062). Rats were bred by backcrossing hemizygous transgene positive animals to wild type Fischer 344 littermates. All animals were pair-housed in 12 h:12 h reverse light-dark cycle conditions and *ad libitum* access to chow and water. The experimental timeline was as follows: 1) at six months of age, animals were administered IgG-sap or DBH-sap, 2) six weeks later, animals were behaviorally tested, and 3) following behavioral testing, the animals were sacrificed for postmortem studies. All procedures were conducted in accordance with guidelines set by the Institutional Animal Care and Use Committee of Michigan State University.

Stereotactic surgeries

A total of 27 age matched transgenic rats were used for this study, as follows: DBH-sap, male ($n = 7$); DBH-sap, female ($n = 6$); IgG-sap, male ($n = 8$); IgG-sap, female ($n = 6$). Male and female rats were randomized using a randomization table, anesthetized with equithesin (~3 ml/kg, i.p.), and placed in a stereotaxic frame. DBH-sap or control IgG-sap (Advanced Targeting Systems, San Diego, CA, 2.5 μ g/injection) were administered to the PFC bilaterally at coordinates (relative to bregma; from dura) AP + 1.2, $\pm$ 2.0, DV –3.0 using a Hamilton syringe (26 s/2" needle; Hamilton, Reno, NV) [39]. The needle was lowered to the site and immunotoxin injection began immediately at a rate of 0.5 μ l/min and remained in place after the injection for an additional 5 min before being slowly retracted. Following surgeries, rats were administered buprenorphine (1.2 mg/kg), placed on a heating pad, and returned to a clean home cage once they started to ambulate.

Barnes maze

Rats were evaluated behaviorally at six weeks after stereotactic surgeries. The investigator was blinded to rat treatment group by a randomization table prior to testing and by coding the videos for all testing outcomes. All experimental sessions were recorded by a video camera placed above the apparatus and analyzed with video-tracking AnyMaze software (Stoelting, Wood Dale, IL). The software detected the center of the animal body and recorded distance moved and time active based on color differences between animal coat color and testing apparatus recorded by the camera. The short-protocol Barnes Maze for

spatial and working memory function [40] was adapted from Attar and colleagues [41]. Briefly, during the habituation session, the animals were slowly pulled to the escape hole in a clear cylinder and then given 120 s to freely enter the escape hole. Afterwards, the animals were submitted to a set of two daily training sessions, with at least two trials. The first training session was performed 24 h after the habituation session. All trials lasted 120 s or until the animals reached the escape box. However, if the rats did not reach the target hole, the experimenter gently guided the animal towards it at the end of the trial using a clear cylinder. After reaching the escape box, animals remained inside for at least 60 s before being returned to their home cages. The escape box was always located in the same place during training. Animals were tested in groups of four, in order that all trials averaged 20 min between each trial per animal. Retrieval of spatial learning was evaluated in the probe session, which was conducted after the rest day, 48 h after the last training day. The procedure was similar to the training trials, but the escape box was removed and rats were evaluated for 120 s. At the beginning of each session, the animals were placed in an opaque container at the center of the maze. The container was then pulled up, and the animal was released to explore the maze. Parameters analyzed in these experiments included time spent in target quadrant and latency to target hole entry (measures of spatial learning and memory) and incorrect revisits to holes already investigated (a measure of working or procedural memory). Incorrect revisits were defined as searching the same hole twice within a trial when the revisit occurred after the inspection of other holes [42].

Open field test

Locomotor activity was evaluated using a standard open field test [43]. The open field apparatus (Stoelting) consisted of an open topped, 2 times 2 acrylic black box measuring $40.6 \times 40.6 \times 38$ cm. The box placed at table height and all experimental sessions were recorded by a video camera placed above the apparatus and analyzed with the video-tracking software. All movements were automatically recorded and time mobile and distance traveled were plotted and the data were used to measure locomotor activity. On the day of testing, rats in their home cages were brought into the experimental room. Rats remained in the experimental room for 30 min, after which each rat was placed into the center of the observation box and recording began immediately. Movement was recorded in 5-min bins for 30 min.

Elevated plus maze

The open field test can also be used to measure anxiety [44]. However, to ensure the DBH-sap lesion did not produce any anxiolytic or anxiogenic effects that might impact behavioral outcomes [45], we also tested the rats on the elevated plus maze [44]. The maze consisted of four arms arranged in a plus shape, elevated 50 cm off the floor, with two arms opened as extend platforms away from the maze (open arms) and the other two arms as platforms with walls (closed arms). Rats were put in the center of the platform facing the same open arm and were allowed to explore the maze for 5 min. Time spent in open compared to closed arms was analyzed as a measure of relative fear/anxiety.

Tissue preparation

Twenty-four hours after completing the final behavior trials, rats were deeply anesthetized (pentobarbital, 60 mg/kg, i.p.) and perfused intracardially with 0.9% saline containing 10,000 USP/L heparin. Rat brains were immediately removed and hemisected in the sagittal plane at midline using a brain block. One hemisphere was post-fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M phosphate buffer (pH 7.2) for 24–48 h and processed for immunohistochemistry, whereas the other hemisphere was flash frozen and processed for biochemical analyses.

Immunohistochemistry

Post-fixed brain hemispheres were transferred to 15% sucrose in 0.1 M phosphate buffer until saturated, then 30% sucrose in 0.1 M phosphate buffer until saturated. Brains were frozen on dry ice and sectioned at a 40 μ m thickness in 1:12 series in the coronal plane using a freezing-sliding microtome (American Optical, Buffalo, NY). Serial sections were processed for immunohistochemistry (IHC) using the free-floating method [16, 46, 47]. All antibodies and dilutions used in these experiments are listed in Table 1. Control experiments including primary antibody deletions were performed for each antibody.

Brightfield and fluorescence IHC

Tissue sections (1:12 series/experiment) were rinsed in Tris-buffered saline (TBS; pH 7.4) and nonfluorescence sections were quenched in 0.3% H_2O_2 for 1 h at room temperature. Tissue was then permeabilized with TBS+0.5% Triton X-100 (TBS-TX) and blocked in TBS-TX/10% normal goat serum for 1 h, followed by overnight incubation in primary antibody in TBS-TX/1% goat serum at 4°C under constant agitation. Following primary antibody incubation, sections were rinsed with TBS-TX and incubated in biotinylated goat anti-rabbit or anti-mouse IgG (1:500 dilution for both secondary antisera; Vector Labs, Burlingame, CA) or fluorescent goat anti-rabbit (1:500; Alexa Fluor 488; Thermo Scientific/Molecular Probes, Eugene, OR) or goat anti-mouse (1:500; Alexa Fluor 594) IgG for 2 h at room temperature, followed by TBS-TX washes. Brightfield IHC was further processed with the Vector Labs ABC detection kit for 1 h at room temperature and antibody labeling was visualized by exposure to 0.5 mg/ml 3,3' diaminobenzidine (DAB) and 0.03% H_2O_2 in TBS or to DAB+2.5 mg/mL nickel ammonium sulfate and 0.03% H_2O_2 in TBS. Sections were mounted on subbed slides, dehydrated via ascending ethanol washes, cleared with xylenes, and cover-slipped with Cytoseal (ThermoFisher, Waltham, MA). Images were taken on a Nikon Eclipse 90i microscope with a Nikon DS-Ri1 camera.

LI-COR infrared IHC and imaging

Serial tissue sections (1:12) were rinsed in TBS-TX and blocked in TBS-TX/10% normal goat serum for 1 h. Sections were then incubated in primary antisera (Table 1) overnight at 4°C under constant agitation. Following primary incubation, sections were rinsed with TBS-TX then incubated with LI-COR near-infrared secondary antibodies for 2 h at room temperature in the dark. Antibodies used were IRDye 800 conjugated goat anti-rabbit (1:500; LI-COR Biosciences, Lincoln, NE) and IRDye 680 conjugated goat anti-mouse IgG (1:500). Sections were then rinsed in TBS, mounted on subbed slides, dehydrated via

ascending ethanol washes, cleared with xylenes, and cover-slipped with Cytoseal. Slides were left to dry for 48 h in the dark at room temperature. Slides were then scanned on a LI-COR Odyssey imaging station to determine DBH, A β , and albumin signal intensity (Table 1). For DBH signal intensity, boundaries were drawn around the entire PFC using the LI-COR Image Studio 3.1 software to obtain an average signal strength. Tracings began with sections at +5 mm from bregma and were terminated at bregma (~8–10 sections/animal) [39]. Reported integrated intensity measurements of DBH expression rostrocaudally across the sections were collected using the 800 nm channel and were normalized to background levels obtained from a 100-pixel sampling area in the basal ganglia of each brain. Data were represented as the mean cortical DBH integrated intensity measurement per brain. DBH staining intensity data showed that DBH signal loss was also found in cortical regions beyond PFC (Fig. 3), most likely due to the highly bifurcated morphology of corticopetal LC axons [48]. Therefore, to measure A β signal intensity, boundaries were drawn around the entire cortex and hippocampus to obtain an averaged signal strength normalized to area. For albumin signal intensity, boundaries were drawn around the entire cortex. Reported integrated intensity measurements of albumin or A β expression rostrocaudally across the sections were collected using the 800 nm channel and were normalized to background levels obtained in the intermediate reticulate nucleus (caudal to the LC) of each brain. Investigators were blinded to rat treatment group during all procedures. For CAA analysis, the same tissue sections and boundaries used to quantify A β load in each animal were analyzed in a blinded manner for MOAB-2-positive vascular profiles. Profiles were counted for 100% of the area using a Nikon Eclipse 90i microscope outfitted with a near-infrared filter cube (Chroma Tech, Bellows Falls, VT).

Western blot analyses

The flash frozen hemisphere of the brain (stored at -80°C) was placed on a modified cold plate (Teca, Chicago, IL) at -18°C for 1 h in a Leica CM3050 S cryostat chamber before being microdissected with a small cortical tissue punch (1.5 mm diameter) at the level of the PFC rostral to the injection site. Frozen dissected structures were placed in pre-chilled microcentrifuge tubes and stored at -80°C until analysis.

Samples for western blot analyses were homogenized at 4°C for 2 h using the RIPA Lysis Buffer System (Santa Cruz, Dallas, TX). Total protein concentration was determined by the Pierce BCA Protein Assay (ThermoFisher). The western blot protocol was performed as previously described [49, 50]. Protein lysates (20 $\mu\text{g}/\text{sample}$) were electrophoresed in duplicate using SDS-PAGE Criterion gels (BIO-RAD, Hercules, CA) and transferred to Immobilon-FL membranes (Millipore, Bedford, MA). Membranes were incubated in primary antisera to albumin (as a measure of BBB leakage) or glial fibrillary acidic protein (GFAP; as an index of astrogliosis/inflammation) overnight. A monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was co-incubated with the albumin and GFAP antisera as a loading control (see Table 1). Blots were rinsed and then incubated using the LI-COR IRDye800-conjugated goat anti-rabbit and IRDye680-conjugated goat anti-mouse IgG (1:20,000) secondary antibodies. All antibody dilutions were made in TBS/2% nonfat dairy milk. Multiplexed signal intensities were imaged with both 700 and 800 nm channels in a single scan with a resolution of 169 μm using the LI-COR Odyssey

image system. Albumin and GFAP immunoreactive signals were normalized to GAPDH for quantitative analysis using LI-COR Image Studio software.

Vessel wall-to-lumen measurements

Wall-to-lumen ratios (WLR) of PFC parenchymal arterioles were measured based on previous methods as an index of cerebral vessel remodeling, where increased wall: lumen measurements indicate stenosis, increased myogenic tone, and reduced vasoreactivity [51, 52]. Briefly, slides of α -actin 2 (smooth muscle actin)-labeled tissue (see Table 1) were randomized, the rater was blinded, and the first six α -actin 2-immunopositive PFC parenchymal arterioles coursing perpendicular to the visual plane as identified using Meander Scan (MBF Bioscience, Williston, VT) were measured. Since the majority of arterioles sampled were smaller than 50 μ m in diameter and larger arterioles were not equally found in-plane between the two groups of rats, intergroup comparison of arteriole profile was based only on arterioles with an external diameter < 50 μ m. Measurements were made on a Nikon Eclipse 90i microscope with a Nikon DS-Ri1 camera using Nikon Elements AR analysis software. WLR was calculated as follows [53]:

$$WLR = \frac{1}{2} \times \frac{\text{vessel outer diameter} - \text{vessel luminal diameter}}{\text{vessel luminal diameter}}$$

Sample size and power

A total of 27 age matched transgenic rats were used for this study, as follows: DBH-sap, male ($n = 7$); DBH-sap, female ($n = 6$); IgG-sap, male ($n = 8$); IgG-sap, female ($n = 6$). Power analyses using results from preliminary behavioral experiments indicated an $n = 5$ /group would have 90% power to detect 1.25 standard deviations between DBH and IgG-sap-lesioned animals. The prospective experimental design required that DBH-sap-lesioned animals exhibit > 30% DBH signal loss compared to mean IgG-sap signal to be included in the study. No sex differences were observed in the animals in behavior or pathology (data not shown), consistent with previous observations in Tg344–19 rats [35, 38], so both sexes were evaluated together as DBH-sap or IgG-sap groups.

Statistics

Data analysis and graph creation were performed using GraphPad Prism software (GraphPad, version 7; La Jolla, CA). All data sets were verified for normality using D'Agostino and Pearson omnibus testing. To compare DBH-sap versus IgG-sap rats, Student's t -tests were used. The level of statistical significance was set at $p < 0.05$ (two-tailed). Linear regression was performed to assess the relationships among the cognitive and pathological outcome variables.

RESULTS

DBH-sap lesioned animals exhibit spatial and working memory deficits

DBH-sap and IgG-sap-treated Tg344–19 AD rats were evaluated on the Barnes Maze at six weeks after surgery [40, 41]. Animals with the DBH-sap lesion spent significantly less time in the target quadrant during the probe trial (Fig. 1A; $p = 0.0083$) and were significantly

slower to find the target hole (Fig. 1B; $p = 0.0002$) than IgG-sap rats. Both of these measures indicate a deficit in spatial memory. Interestingly, the DBH-sap animals also showed a greater number of revisits to holes that they had already investigated during the probe trial (Fig. 1C; $p = 0.0003$) suggesting working memory deficits [54]. By contrast, there were no significant differences between DBH-sap or IgG-sap-lesioned animals during the open field test, which included measurements of distance traveled (Fig. 1D; $p = 0.85$) and time mobile (Fig. 1E; $p = 0.35$). Although DBH-sap lesions were administered in PFC, LC axons projecting into the telencephalon through the dorsal tegmental bundle are highly bifurcated, with lateral branches providing an immense, reciprocal innervation of the amygdala [55, 56]. To ensure that there were no amygdala-related anxiogenic or anxiolytic effects of the lesions that might impact cognitive or motor behavior, we evaluated the animals using the elevated plus maze [57]. There were no differences in the time spent in the open arms during this task (Fig. 1F; $p = 0.8$).

The DBH-sap lesion results in noradrenergic deafferentation of PFC

DBH-sap has been previously validated as a NE-specific immunotoxin in intraventricular surgeries [34, 58, 59]. DBH IHC revealed a striking loss of fibers within PFC, as well as in adjacent cortical and subcortical regions, in DBH-sap (Fig. 2B) compared to IgG-sap-lesioned animals (Fig. 2A). LC fiber loss beyond the PFC injection site likely reflected degeneration of LC axon bifurcations [45, 48]. Optical densitometry showed a 50% reduction in DBH-immunopositive profiles in the PFC of DBH-sap compared to IgG-sap animals (Fig. 2E; $p < 0.0001$). Notably, IHC of brainstem tissue from the same animals revealed a loss of DBH-immunoreactive profiles in the rostral LC, suggesting a concomitant loss of forebrain-projecting LC noradrenergic neurons in DBH-sap (Fig. 2D) compared to IgG-sap-lesioned animals (Fig. 2C).

DBH-sap lesioned Tg344 rats exhibit increased amyloid pathology and microgliosis in the cortex and hippocampus

We investigated the extent to which DBH-sap lesions result in increased amyloid pathology in the PFC target lesion field, which would replicate the effects of intraperitoneal injections of the neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) on exacerbating amyloidosis in transgenic mouse models of AD [26, 60–63]. We performed IHC using the MOAB-2 antibody, which recognizes human A β residues 1–4 but not A β PP [64]. DBH-sap-lesioned Tg34419 AD rats exhibited increased numbers and size of MOAB-2-labeled plaques in the cortex and hippocampus (Fig. 3B) compared to IgG-sap animals (Fig. 3A). Quantitative analysis of MOAB-2 label density revealed a significant ~30% increase in amyloid burden in the cortex (Fig. 3C; $p < 0.001$) and a significant ~20% increase in amyloid pathology in the hippocampus (Fig. 3D; $p < 0.05$) of DBH-sap compared to IgG-sap-lesioned animals at six weeks post-surgery. Strikingly, we also found evidence that DBH-sap lesions resulted in the emergence of amyloid deposition within PFC microvessels (Fig. 3B, F,G) suggesting the onset of cerebral amyloid angiopathy (CAA) months before it is typically observed in these animals at 16 months of age [35]. There was a significant increase in the number of MOAB-2-labeled, CAA-like vascular profiles in the cortex and hippocampus of DBH-sap-treated (9.9 ± 2.8 [mean $\pm$ SD]) compared to IgG-sap-treated (0.8 ± 1.3) animals (Fig. 3H). Finally, using the tau antibodies AT8 and CP-13, which are

directed against phosphoepitopes associated with neurofibrillary tangles in AD [65, 66] (Table 1), we did not detect any immunohistochemical evidence for tau pathology in these animals (data not shown).

Evidence for BBB disruption in DBH-sap-lesioned Tg344–19 AD rats

Dual-label immunofluorescence studies showed a dense network of DBH-positive fibers apposed to parenchymal microvessels labeled with SMI71[67], which recognizes a vascular/BBB epitope (Fig. 4A). To investigate the impact of the DBH-sap lesion on BBB integrity, we analyzed albumin immunoreactivity in PFC tissue of DBH-sap or IgG-sap-lesioned Tg344–19 AD rats. A striking increase in albumin staining was observed in the DBH-sap animals (Fig. 4 C, E) compared to IgG-sap animals (Fig. 4B, D), suggesting BBB breakdown and leakage and/or increased microvascular permeability upon LC deafferentation. This observation was confirmed using optical densitometry of albumin immunoreactivity in adjacent fixed tissue sections (Fig. 4 G; $p = 0.0242$) and quantitative western blotting of frozen tissue from the opposite hemisphere of the same animals (Fig. 4 H; $p = 0.0052$). Interestingly, further dual-label immunohistochemical investigations revealed a substantial colocalization of albumin with the reactive astrocytic marker GFAP in the DBH-sap-lesioned animals (Fig. 4F). Quantitative immunoblotting of frozen cortical tissue containing PFC revealed a 50% increase in GFAP protein in DBH-sap compared to IgG-sap-lesioned animals (Fig. 4I; $p = 0.011$). Taken together, these data show an astroglial immune response following LC deafferentation, similar to that observed in other LC lesion paradigms [26]; however, this response may be due at least in part due to cerebrovascular damage and BBB leakage.

Evidence for small vessel remodeling in DBH-sap-lesioned Tg344–19 AD rats

The wall-to-lumen ratio is an important parameter in vascular medicine as it indicates the extent of vascular wall stenosis and vessel remodeling [52, 68,69]. PFC parenchymal arterioles that were perpendicular to the plane of view were evaluated for this ratio in DBH- and IgG-sap-lesioned animals (Fig. 5A). Quantitative analysis revealed that arterioles in DBH-sap-lesioned animals displayed an ~45% increase in wall-to-lumen ratio compared to IgG-sap animals (Fig. 5B; $p < 0.0001$) indicating a compensatory remodeling of the vessel structure with presumably pathological stenosis as a result of the loss of LC-NE signaling.

Cortical albumin load and vascular wall-to-lumen ratios predict cognitive impairment in Tg344–19 AD rats

Regression analysis was performed to test for relationships among the cognitive and pathological outcome variables measured six weeks after the IgG-sap or DBH-sap lesions. Notably, lower DBH-immunoreactive fiber intensity in the cortex predicted increased amyloid load ($p = 0.02$, $r^2 = 0.43$) but not GFAP levels as measured by western blotting ($p = 0.1$, $r^2 = 0.24$). By contrast, reductions in DBH signal predicted all measures of vascular pathology, including albumin load ($p = 0.0004$, $r^2 = 0.73$), albumin protein levels as measured by western blotting ($p = 0.004$, $r^2 = 0.58$), and wall-to-lumen ratio ($p = 0.002$ $r^2 = 0.62$). Predictors for the three Barnes maze cognitive outcomes are shown in Table 2. With respect to spatial memory tasks, the time animals spent in the target quadrant was related to DBH fiber intensity, albumin load, albumin levels, and wall-to-lumen ratio, whereas the

latency to find the target hole was influenced only by albumin load, albumin levels, and wall-to-lumen ratio. By contrast, lower DBH fiber intensity, higher albumin levels, and increased wall-to-lumen ratio predicted a greater number of incorrect revisits made by the rats, suggesting that these factors influenced working memory performance. Notably, amyloid load and GFAP levels did not reveal significant associations with cognition on any of the Barnes maze tasks.

DISCUSSION

In the early 1970 s, several groups noted DBH-containing fibers in close association with parenchymal arterioles and capillaries in monkey and rat forebrain (Fig. 4A), which persisted following superior cervical ganglionectomy [70, 71]. Subsequent studies using electrophysiological and pharmacologic stimulation of the LC demonstrated central regulation of cerebral blood flow and vascular permeability by this nucleus [29, 31]. More recently, it was reported that LC stimulation in rats increased cerebral perfusion to stimulate cortical neuronal activity [33]. In light of these data, we hypothesized that LC degeneration negatively impacts cerebrovascular function in target fields and hastens the progression of AD. However, to our knowledge, the extent to which this phenomenon occurs has not been examined experimentally. In the present study, we investigated the effects of lesioning the noradrenergic LC forebrain projection system on vascular pathology in AD by injecting a central noradrenergic immunotoxin into the PFC of six-month-old Tg344–19 AD rats. Transgenic and genome-edited rats may offer some advantages over mice for lesion studies, including more defined motor and cognitive behaviors, greater synaptic complexity, genetics and pharmacokinetics more similar to humans, and a larger brain and body size [38, 72]. The DBH-sap lesion resulted not only in LC fiber loss but also in LC neurodegeneration compared to the IgG-sap group. DBH-sap-mediated degeneration of LC forebrain input resulted in deficits in working and spatial memory, increased parenchymal and vascular amyloidosis, astroglial activation, as well as evidence for BBB breakdown and leakage and vascular remodeling consistent with decreased vasoreactivity. Therefore, this *in vivo* paradigm is well-suited as a model of preclinical coeruleo-forebrain disconnection and its role as a nexus lesion in promoting both neural and vascular dysfunction during the onset of AD.

Behavioral testing of DBH-sap and IgG-saplesioned animals at six weeks post-surgery revealed impairment in working and spatial memory on the Barnes maze (Fig. 1) as a consequence of the sustained loss of LC innervation of PFC and hippocampus (Fig. 2). These effects were reminiscent of previous studies showing that LC lesions with the neurotoxin DSP-4 [73] disrupts spatial memory in transgenic mouse models of AD [26, 60–63] and that LC stimulation or pharmacological increases in NE levels enhance cognitive performance [38, 74,75]. Although we specifically targeted PFC with the immunotoxin, the highly bifurcated nature of long, poorly myelinated LC axons likely led to deafferentation of adjacent cortical structures and hippocampus, as well [48]. Cognitive function depends greatly on LC-NE system integrity [22, 76–81]. In this regard, both β_1 [82] and β_2 [83] receptor signaling are critically required for spatial learning in animal models. Moreover, LC innervation of the cortical mantle modulates PFC activity in the context of attentional function [22, 79] and selective impairment in NE transmission within the PFC leads to

disrupted working memory [84, 85]. In a similar vein, we demonstrate that DBH-sap lesioned animals displayed a higher number of incorrect visits on the Barnes maze, which has been proposed to represent deficits in prefrontal-mediated working memory and attentional function in rodents [42]. This result is reminiscent of findings by Janitzky and colleagues, who reported deficits in the attentional set-shifting paradigm following optogenetic silencing of the LC in mice [86]. Taken together with recent clinical pathologic studies in well-characterized autopsy cohorts [16, 19, 20], these results support the prevailing concept that LC degeneration contributes to cognitive impairment in AD.

Upon postmortem evaluation, we observed increased A β deposition and astroglial activation in the cortex and hippocampus of our lesioned animals (Figs. 3 and 4), similar to previous findings [26, 60–63]. However, it is notable that amyloid load in Tg344–19 AD rats lesioned at six months of age resembled the degree of amyloid accrual observed in these animals at ~16 months of age but without experimental LC degeneration [35]. Furthermore, we also noted the emergence of CAA in parenchymal vessels in the LC-lesioned animals, which was virtually absent at this age in unlesioned rats and has not been previously noted prior to 16 months of age (Fig. 3) [35]. The mechanism for this accelerated pace of vascular amyloid deposition is unclear, but may be related to impaired amyloid clearance if NE depletion results in vascular hypertrophy and increased myogenic tone, as suggested by the observed DBH-sap-induced increases in arteriole wall-to-lumen ratio (Fig. 5, see below).

Alternatively, recent computational data suggest that vascular smooth muscle cells provide the motive force for intramural periarterial drainage (IPAD) of brain solutes including A β [87, 88]. Given data that VSMCs express adrenergic receptors [89], LC deafferentation during AD may impair the motive forces for IPAD, thus contributing to reduced A β clearance and increased CAA.

Tg344–19 AD rats reportedly display age-dependent endogenous hyperphosphorylated tau and argyrophilic tangles in the hippocampus and cortex by 16 months [35, 38] and even an early accrual of tau pathology within the LC at 6 months [38]. When examining the brains of our control or lesioned animals for tau pathology (Table 1) at 6–8 months, including a detailed analysis of pontine tissue, we were unable to detect an immunopositive signal above background denoting the presence of tau pathological epitopes. This may be due to methodological differences (e.g., free-floating versus paraffin-embedded tissue [38]) or even age-related phenotypic differences between our colony and others. Nonetheless, future studies will re-examine the accrual of tau pathologic epitopes following DBH- or IgG-sap lesions in older cohorts and different tissue preparation protocols.

While these observations of impaired cognition and exacerbated amyloid-related pathology were consistent with previous *in vivo* studies [26, 60–63], the primary goal of the present study was to evaluate the extent to which LC deafferentation impacts cerebrovascular function in target fields. In this regard, we noted prominent extravasation of the blood protein albumin within the PFC of DBH-sap-lesioned animals. As all rats were equally saline-perfused at sacrifice, parenchymal albumin deposition is likely the result of impaired BBB integrity specific to the DBH-sap lesion. Perivascular accumulation of serum albumin, plasma proteins, and immunoglobulins have been detected in microvascular segments associated with senile plaques and CAA in AD brains [90,91], while increased cerebrospinal

fluid/serum ratio of albumin observed in AD patients has been long used as a proxy for BBB disruption [92–94]. From a translational perspective, these results suggest that LC degeneration during the preclinical and prodromal stages of AD [16, 19, 20] contributes to the BBB pathology observed during AD [95–97], driving disease pathophysiology by increasing BBB permeability and allowing blood-derived molecules and microbes to enter the brain. This insult may in turn trigger multiple neurodegenerative pathways such as inflammation and oxidative stress, as well as impairing neural connectivity via reduced perfusion and dysregulated neurovascular coupling [98, 99]. In support of this hypothesis, Kalinin and colleagues noted a loss of forebrain tight junction protein expression following DSP-4 lesions in wild type rats [100]. Furthermore, Rorabaugh and colleagues found that designer receptor exclusively activated by designer drugs (DREADD)-induced LC activation restored normal reversal learning to aged Tg344-AD rats, indicating that enhancing LC tone can improve cognition, even in the presence of AD pathology [38]. We posit that this may be the result of increased cerebrovascular perfusion from LC activation [33], though this would need to be tested directly. Future studies will determine the extent to which cerebral blood flow and hemodynamic responsiveness along forebrain neural circuits are impaired following LC projection system deafferentation.

Interestingly, we also demonstrated an overlap between reactive astroglial profiles and albumin suggesting that a proportion of astrogliosis in AD and LC-lesioned animal models involves uptake of albumin and other blood-derived proteins in response to BBB breakdown [101]. As astrocytic end feet serve as a first line of defense against the infiltration of potentially neurotoxic blood products from the cerebrovasculature [102] and astrocytes are recruited to wall off damaged areas and restore BBB integrity [101, 103], this has been traditionally viewed as a neuroprotective response [104]. However, studies from the stroke and epilepsy fields have demonstrated that albumin uptake by astrocytes reduces astrocytic expression of inward-rectifying potassium channels, which may compromise their ability to buffer extracellular K^+ [105, 106] and may form a mechanistic basis for network hyperexcitability [107]. Astrocytic uptake of albumin also reduces aquaporin 4 expression, which likely impacts vascular permeability [108]. Furthermore, albumin has been shown to induce astrocytic release of the pro-inflammatory ligands interleukin-1 β [109] and monocyte chemoattractant protein-1 [110]. Hence, astrocyte activation and uptake of albumin in response to BBB breakdown may ultimately compromise neuronal function and protection within the neurovascular unit where LC innervation has been lost.

Perhaps even more strikingly, we found that DBH-sap-lesioned animals display increased cerebrovascular wall-to-lumen ratios in PFC target fields compared to IgG-sap rats. This measure is widely viewed as indicative of vascular remodeling and hypertrophy, increased myogenic tone, and blunted vasoreactivity [52, 68, 69, 111]. Vascular hypertrophy is of particular physiologic importance since it has been hypothesized to be the result of a remodeling process [53, 112–115] that limits blood flow during maximal vasodilatation and increases vascular responsiveness to constrictor stimuli [53]. Future physiological studies will identify specific changes in tone, constriction, and dilation in LC-denervated parenchymal arterioles [116]. Notably, Joo and colleagues detected dystrophic mural cell morphology and altered hypercapnic responses indicative of vascular remodeling in Tg344–19 AD rats compared to nontransgenic littermates at 9 months of age, suggesting that

amyloid accrual itself has the potential to compromise blood flow [117]. The present findings complement and expand upon this observation to show that a loss of LC-NE input to blood vessels likely further exacerbates blood flow to areas of higher order cognition [32, 33, 45, 118]. Moreover, our demonstration that markers of vascular dysfunction arising from DBH-sap lesions, but not parenchymal amyloid load or astrogliosis (Table 2), predicts cognitive outcomes in these animals underscore the importance of LC-mediated vascular tone in regulating cognition in health and disease.

In summary, we provide translational evidence that degeneration of the vulnerable LC noradrenergic projection system impairs forebrain cerebrovascular function during the preclinical and prodromal stages of AD [16–20], thus providing an additional mechanism for the therapeutic benefit of targeting NE-mediated neuronal activity [119–121]. A key caveat to this interpretation is that LC neurons also release other neurochemical transmitters along with NE such as galanin, neuropeptide Y, dopamine, and even brain-derived neurotrophic factor, albeit at lower levels [122–125]. Future directions will use this animal model to test the extent to which NE replacement therapies such as the NE precursor L-threo-3,4-dihydroxyphenylserine (L-DOPS) [126] or genetic approaches to manipulate the levels of NE or non-NE LC transmitters ameliorate forebrain vascular pathology induced by LC projection system degeneration. Moreover, this model is well-suited to determine the extent to which other neurochemical projection systems regulating neurovascular function, such as the cholinergic nucleus basalis or the serotonergic raphe [127, 128], might undergo compensatory cerebrovascular innervation patterns in response to LC deafferentation. Finally, this model can also be used to explore potential neurotoxic mechanisms of BBB leakage of proteins such as albumin or fibrinogen [99, 129–131] or the preclinical value of using blood proteins such as platelet-derived growth factor receptor- β as a diagnostic and prognostic biomarker of disease progression [132]. Altogether, the present findings point to the continuing need to consider noradrenergic system pathophysiology as a key and early component associated with the progression of AD and posit that strategies aimed at LC neuroprotection or NE replacement remain viable therapeutic options.

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Fig. 1.

DBH-sap-lesioned Tg344–19 AD rats are impaired in measures of spatial and working memory at six weeks after surgery. Animals were evaluated on the Barnes Maze six weeks after stereotactic PFC administration of DBH IgG- or control IgG-saporin. Data are displayed as scatter plots with mean $\pm$ SD. A) DBH-sap-lesioned animals spent significantly less time in the target quadrant during the probe trial ($p = 0.0083$), suggesting LC-mediated deficits in spatial memory. B) DBH-sap animals were also significantly slower to find the target hole during probe trial ($p = 0.0002$), which is another measure of spatial memory. C) DBH-sap-lesioned rats made more revisits to holes already investigated ($p = 0.0003$), suggesting an LC-mediated deficit in working memory compared to IgG-sap rats. By contrast, open field testing showed no significant differences between DBH-sap or IgG-sap-lesioned animals in (D) distance traveled ($p = 0.85$) or (E) time mobile ($p = 0.35$), indicating no locomotor or anxiogenic effects of the lesion. F) Elevated plus maze testing as an additional measure of fear or anxiety-related behaviors revealed no differences in the time spent in the open arms ($p = 0.8$). ** $p < 0.01$; *** $p < 0.001$ via Student's t test.

Fig. 2.

Evidence for LC system degeneration following the DBH-sap lesion in Tg344-19 AD rats. Representative DBH immunostaining in control IgG-sap lesioned cortex (A) and DBH-sap lesioned cortex (B). Note the loss of noradrenergic fibers following DBH-sap lesions. DBH-sap lesions also resulted in a loss of DBH-immunoreactive neuron density within the LC (D) compared to IgG-sap lesions (C). E) Quantitative optical densitometry measurements revealed a ~60% loss of cortical DBH immunoreactivity in DBH-sap compared to IgGsap-lesioned rats. Data are displayed as a scatter plot with mean $\pm$ SD. **** $p < 0.0001$ via Student's t test. Panels A and B scale bars: large images = 1000 μ m; inset images = 50 μ m.

Fig. 3.

Cortical and hippocampal amyloidosis is accelerated in DBH-sap-lesioned Tg344-19 AD rats. Representative images showing increased MOAB-2 immunostaining in both the cortex and hippocampus of (A) IgG-sap- and (B) DBH-sap-lesioned animals. Modest vascular MOAB2 deposition is observed in large vessels the DBH-sap lesioned cortex (arrows and panel B inset). Optical densitometry measurements revealed (C) a ~30 increase in cortical amyloid ($p = 0.0008$) and (D) a ~20% increase in hippocampal amyloid ($p = 0.015$) at 6 weeks following DBH-sap lesions compared to IgG-sap. Dual-label fluorescence microscopy with MOAB-2 (red) and the vascular marker SMI71 (green) revealed additional evidence for emerging CAA following DBH-sap (F) compared to IgG-sap (E) lesions. G) Brightfield microscopy in MOAB-2 immunostained tissue revealed additional evidence for emergent CAA in the vicinity of amyloid plaques in the PFC of DBH-sap-treated animals. H) A semi-quantitative counting procedure of MOAB-2 labeled tissue sections showed a

ten-fold increase in CAA-like vascular profiles in DBH-sap compared to IgG-sap rats. $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$ via Student's t test. Panels A and B scale bars = 1000 μm . Panel G scale bar = 50 μm .

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Fig. 4.

Cortical intraparenchymal albumin levels are increased in DBH-sap-lesioned Tg344-19 AD rats with evidence for albumin uptake by astrocytes. A) Dual-label fluorescence microscopy with DBH (red) and SMI71 (green) reveal dense apposition of LC noradrenergic fibers with parenchymal microvessels. B, C) Brightfield photomicrographs of PFC albumin immunoreactivity in IgG-sap- (B) or DBH-sap-lesioned rats (C) indicated an increase in parenchymal albumin deposition in with DBH-sap treatments. D, E) Dual-label fluorescence IHC with albumin (red) and SMI71 (green) further demonstrate prominent albumin extravasation within PFC in DBH-sap (E) compared to IgG-sap (D) animals. F) Dual-label fluorescence IHC with albumin (red) and GFAP (green) demonstrate albumin uptake by astrocytes within the PFC of DBH-sap-lesioned rats. G) Semi-quantitative analysis using LICOR infrared tissue immunoreactivity revealed a ~40% increase in cortical albumin load in DBH-sap lesioned animals ($p = 0.024$). H, I) Quantitative western blot analyses of cortical tissue demonstrated a significant ~60% increase in albumin levels ($p = 0.005$) and a

significant~50% increase in GFAP levels ($p = 0.011$). Data are displayed as scatter plots with mean $\pm$ SD. Representative western blots are shown beneath the scatterplots. $*p < 0.05$; $**p < 0.01$ via Student's t test. Panel A scale bar = 100 μm . Panel B scale bar = 50 μm (applies to panels C-F).

Fig. 5.

Wall-to-lumen ratio of parenchymal arterioles is increased in DBH-sap lesioned Tg344-19 rats. A) Representative brightfield photomicrograph of an arteriole labeled with smooth muscle actin (ACTA2). Outside wall diameter measurement is compared to luminal diameter to produce the wall-to-lumen ratio value. B) Quantitative analysis revealed a significant ~75% increase vascular wall-to-lumen ratio in the cortex of DBH-sap- compared to IgG-sap-lesioned animals. Data are displayed as a scatter plot with mean $\pm$ SD. **** $p < 0.0001$ via Student's t test.

Table 1

Antibodies used in the present study

Antibody	Source	Dilution (application)
DBH rabbit polyclonal antiserum	Immunostar #22806	1:4000 (IHC)
albumin rabbit polyclonal antiserum	ProteinTech #16475-1-AP	1:500 (IHC) 1:10,000 (WB)
smooth muscle actin (ACTA2) rabbit polyclonal antiserum	ProteinTech #23081-1-AP	1:1000 (IHC)
MOAB-2 (A β 1–4) mouse IgG2b monoclonal antibody	Gift from Dr. Nicholas Kanaan, Michigan State University	1:4000 (IHC)
GFAP rabbit polyclonal antiserum	Abeam #ab7260	1:10,000 (IHC) 1:20,000 (WB)
SMI71 mouse IgM monoclonal antibody	Biolegend #836804	1:1000 (IHC)
AT8 (tau phospho-serine 202, phospho-threonine 205) mouse IgG1 mouse monoclonal antibody	Thermo Fisher #MN1020	1:100–1:5000 (IHC)
CP-13 (tau phospho-serine 202) mouse IgG2b monoclonal antibody	Gift from Dr. Peter Davies, Northwell Feinstein Institute	1:100–1:5000 (IHC)

Table 2
Pathologic predictors of cognitive impairment in IgG-sap and DBH-sap-lesioned Tg344–19 AD rats

Pathologic index	Time in target quadrant	Latency to target hole	Incorrect visits
DBH fiber intensity	$p = 0.02, r^2 = 0.43$	$p = 0.08, r^2 = 0.28$	$p = 0.03, r^2 = 0.4$
amyloid load	$p = 0.28, r^2 = 0.11$	$p = 0.4, r^2 = 0.07$	$p = 0.08, r^2 = 0.27$
GFAP (western)	$p = 0.06, r^2 = 0.31$	$p = 0.26, r^2 = 0.12$	$p = 0.35, r^2 = 0.09$
albumin load	$p = 0.04, r^2 = 0.35$	$p = 0.005, r^2 = 0.57$	$p = 0.13, r^2 = 0.2$
albumin (western)	$p = 0.04, r^2 = 0.35$	$p = 0.05, r^2 = 0.34$	$p = 0.0008, r^2 = 0.7$
wall-to-lumen ratio	$p = 0.002, r^2 = 0.62$	$p = 0.02, r^2 = 0.44$	$p = 0.03, r^2 = 0.4$