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Mechanisms of Selective Vulnerability in
Alzheimer's Disease and Stress Disorders

By

Alexander James Ehrenberg

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Graduate Division

of the

University of California, Berkeley

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Abstract

Mechanisms of Selective Vulnerability in Alzheimer's Disease and Stress Disorders

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To date, neurodegenerative diseases remain with lackluster treatment options. Diseases such as Alzheimer's are defined based on proteinopathic inclusions, and these inclusions have been the targets of most therapeutic development. The premise that simply addressing these inclusions will be sufficient for therapeutics is poorly supported. Here, I attempt to build an evidence base using selective vulnerability as a substrate to work towards more biologically informed disease-modifying therapeutics. First, I examine the physiological underpinnings of a potent risk factor for dementia and other age-related diseases – social isolation. In humans, social isolation has well-established epidemiological associations with dementia, cardiovascular disease, immune system dysfunction, metabolic disorders, and mental health disorders. Mice exposed to long-term social isolation are studied across several organ systems. Associations with the stressor are detected in renal structure and blood-brain-barrier dysfunction; however, I find that mice are a poor model of social isolation.

Next, I examine a potential mechanism underlying why neurons with tau proteinopathic inclusions die. The best correlate of functional decline in neurodegeneration is neuron loss, so focusing on processes of neuron loss might confer better therapeutics. Here, I find that the Ras GTPase, Rhes, is dysregulated in neurons harboring tau proteinopathic inclusions in sporadic tauopathies. This study reinforces *in vitro* and *in vivo* work done in models of familial tauopathy demonstrating that a farnesyltransferase inhibitor may suppress neurodegeneration in tauopathies. I then approach selective vulnerability by focusing on the neuromodulatory subcortical system. Parts of the neuromodulatory subcortical system are affected early in Alzheimer's disease and feature a wide gradient of selective vulnerability and resilience. Here, I find pathways related to cholesterol homeostasis and stress hormone signaling to the noradrenergic locus coeruleus that differentiate it from the more resilient substantia nigra. I close by focusing on the epistemological frameworks used to study neurodegenerative disease and comment on how a focus on selective vulnerability may drive significant paradigm changes in the field. Overall, this dissertation provides evidence of pathophysiology that may drive neurodegenerative diseases and adverse outcomes of stress disorders.

Dedication

To my family members who have been gripped by neurodegeneration, both as patients and caregivers, and have remained steadfast supporters of my growth as a scientist.

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Acknowledgements

Selective vulnerability in the brain is a weird thing. In this dissertation, I have mainly focused on molecular mechanisms because I believe they are critical for therapeutic discovery. Looking even just to family members affected by neurodegeneration, the fact that some parts of the brain can die while others remain resilient in the face of disease is incontrovertible. My grandmother, Jean Ehrenberg, has Limbic-Predominant Age-Related TDP-43 Encephalopathy. Her near-absent hippocampus leaves her without memory of her meal an hour prior, but her resilient cortex allows her to continue to read, socialize, and tell stories. The facts of selective vulnerability are why she can sit with her favorite book every single day and, not remembering she finished it the day before, enjoy it like it is the first time through again and again.

My granddad, Jim Ellis, had a mix of vascular dementia and Alzheimer's disease. He convinced me to go to Berkeley for undergrad, ultimately setting me on the path to where I am today. His support for my academics throughout my life up until that point transitioned to motivation as I saw him fall to the grips of neurodegeneration. I saw his poise fall to the vulnerability of his basal ganglia, but his pride remains with his resilient anterior cingulate, insula, and medial prefrontal cortex. My last salient memory was from escorting him to one of his first neurology appointments at UCSF. He was lagging behind me and my mom and told us, "I'm sorry for being a burden." He was never a burden. By the time I started this PhD, he was too sick to know, and by the time I finished, he was gone. His impact on me continues and will forever influence my choices in work and life.

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Publications related to this work

This dissertation contains parts of or the entirety of work that has been previously published. These publications have been reformatted and/or modified to fit the format and structure of this dissertation:

Chapter 1

Ehrenberg AJ, et al. Priorities for research on neuromodulatory subcortical systems in Alzheimer's disease: Position paper from the Neuromodulatory Subcortical System PIA of ISTAART. *Alzheimers Dement*. 2023 May;19(5):2182-2196.

Ehrenberg AJ, et al. Relevance of biomarkers across different neurodegenerative diseases. *Alzheimers Res Ther*. 2020 May 13;12(1):56.

Chapter 3

Ehrenberg AJ, et al. Patterns of neuronal Rhes as a novel hallmark of tauopathies. *Acta Neuropathol*. 2021 May;141(5):651-666.

Chapter 1: Introduction

Despite only accounting for a minority of neurodegenerative disease cases, select familial mutations have served as archetypes of neurodegeneration to those probing molecular underpinnings in model systems. While benefiting from the predictability and intensity such mutations offer to those using model systems, the field has tended to over-generalize findings – presupposing that pathophysiology due to familial mutations is indicative of pathophysiology in the more common, sporadic neurodegeneration cases. Efforts to understand the pathophysiology underlying sporadic neurodegenerative diseases would benefit from (1) the use of multi-modal molecular profiling tools along with biomarkers to develop hypotheses of selective vulnerability mechanisms and (2) model-based work to understand how potent risk factors for neurodegeneration are pathogenic. Here, I first conducted a study of chronic social isolation in mice to uncover mechanisms underlying social isolation's classification as a risk factor for neurodegeneration and other disorders. Next, I examine how a specific protein mediates neurodegeneration in sporadic tauopathies. Finally, the neuromodulatory subcortical system is probed as a substrate to understand selective vulnerability to neurodegeneration and stress disorders, focusing on catecholaminergic members.

Section 1: Neurodegenerative diseases

This section contains modified text from Ehrenberg AJ, et al. Relevance of biomarkers across different neurodegenerative diseases. Alzheimers Res Ther. 2020 May 13;12(1):56.

Neurodegenerative diseases represent the confluence of a range of pathophysiologic cascades with associated clinical spectra. While typically defined by proteinopathic hallmarks, it is unclear whether these hallmarks are driving the disease or are themselves consequences of other underlying processes. Alzheimer's disease, frontotemporal lobar degeneration, and Parkinson's disease are the most common neurodegenerative diseases. Overlapping diseases, otherwise known as co-pathologies, are frequent and have complex contributions to clinical phenotypes (Robinson et al., 2018).

ALZHEIMER'S DISEASE

Alzheimer's disease is the most common cause of dementia, with an incidence of 1,700-2,900/100,000 individuals per year in the USA (Rajan et al., 2019). Alzheimer's disease is a dual proteinopathy characterized by the accumulation of tau neurofibrillary tangles and extracellular amyloid- β plaques (Montine et al., 2012). *Postmortem* studies indicate that Alzheimer's disease features a long preclinical phase where tau lesions and associated neuronal loss first appear in subcortical nuclei and begin to involve limbic regions with associated subjective cognitive decline and neuropsychiatric

symptoms (Braak et al., 2011; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Ehrenberg et al., 2018; Jack et al., 2018; Stratmann et al., 2016b). Cortical tau lesions appear in later stages (Braak et al., 2011), which correlates with the prototypical amnesic Alzheimer's clinical syndrome (Elahi & Miller, 2017; Jack et al., 2018).

Also in the preclinical phase, amyloid- β lesions initially appear in neocortical regions, followed by allocortical, subcortical, and cerebellar involvement (Thal et al., 2002). In contrast to tau lesions, the distribution of Amyloid- β is not significantly associated with symptoms (Giannakopoulos et al., 2003).

Besides age, risk factors include cerebrovascular diseases, diabetes, hypertension, obesity, dyslipidemia, and genetic factors such as the *APOE- ϵ 4* allele and *TREM2* mutations (Livingston et al., 2017; Yaffe, 2018). Approximately 5% of cases have an age of onset under 65 and are referred to as early-onset Alzheimer's disease cases. Familial Alzheimer's disease, accounting for about 20% of early-onset cases and less than 1% of all Alzheimer's disease cases, is mainly caused by rare, dominantly inherited *PSEN1*, *PSEN2*, or *APP* mutations (Table 1.1) (Bekris et al., 2010; Silva et al., 2019; Tanzi et al., 1987).

PARKINSON'S DISEASE

Synucleinopathies are the second most common neurodegenerative disease (Dorsey et al., 2007). The most common clinical presentation of a synucleinopathy, Parkinson's disease, has an estimated incidence of 10-18/100,000 individuals per year in the USA (Van Den Eeden et al.). Synucleinopathies feature the aggregation of α -synuclein in the form of Lewy bodies starting subcortically, spreading into limbic and neocortical regions (Braak et al., 2004). Generally, synucleinopathies present as Parkinson's disease, Parkinson's disease dementia, or dementia with Lewy bodies, which collectively feature varying degrees of behavioral, cognitive, and autonomous symptoms on top of motor dysfunction. Mutations in a number of genes (Table 1.1) have been linked to synucleinopathies (Elahi & Miller, 2017; Nussbaum, 2018), as has pesticide exposure and traumatic brain injury (Noyce et al., 2012).

FRONTOTEMPORAL LOBAR DEGENERATION

Frontotemporal lobar degeneration is nearly as prevalent as early-onset Alzheimer's disease in dementia cases below the age of 65, with an incidence of 1.6-4.1/100,000 individuals annually in the USA and Europe (Coyle-Gilchrist et al., 2016; Knopman & Roberts, 2011). Frontotemporal lobar degeneration describes a spectrum of diseases characterized by vacuolation, gliosis, and neuronal loss in external cortical layers of predominantly frontal and temporal neocortices and feature the accumulation of tau, TDP-43, or fused-in sarcoma, which define the frontotemporal lobar degeneration subtype (Cairns et al., 2007; Mackenzie, Neumann, Cairns, et al., 2011).

Frontotemporal lobar degeneration is associated with a spectrum of frontotemporal dementia syndromes ranging from behavioral, language, to motor variants. Amyotrophic Lateral Sclerosis and other motor neuron diseases can be considered part of the frontotemporal lobar degeneration spectrum due to common neuropathologic and

genetic features (Karch et al., 2018). Roughly one-third of all frontotemporal dementia cases are familial (Lashley et al., 2015).

Table 1.1: Genetic biomarkers for neurodegenerative diseases

Gene	Protein	Associated Syndrome(s)	Reference
<i>APP</i>	Amyloid precursor protein	EOAD (familial), CAA	Tanzi et al. (1987)
<i>PSEN1</i>	Presenilin 1	EOAD (familial)	Sherrington et al. (1995)
<i>PSEN2</i>	Presenilin 2	EOAD (familial)	Levy-Lahad et al. (1995)
<i>MAPT</i>	MAPT	bvFTD, PSP	Hutton et al. (1998); Poorkaj et al. (1998)
<i>C9orf72</i>	C9orf72	bvFTD, ALS	Renton et al. (2011)
<i>GRN</i>	Progranulin	bvFTD, CBS	Gass et al. (2006)
<i>VCP</i>	Valosin-containing protein	ALS	Johnson et al. (2010)
<i>TARDBP</i>	TDP-43	ALS	Sreedharan et al. (2008)
<i>SOD1</i>	Superoxide dismutase 1	ALS	Rosen et al. (1993)
<i>FUS</i>	Fused-in sarcoma	ALS	Kwiatkowski et al. (2009); Vance et al. (2009)
<i>HTT</i>	Huntingtin	HD	HDCRG (1993)
<i>SNCA</i>	α -synuclein	PD, DLB, MSA	Kruger et al. (1998)
<i>GBA</i>	β -glucocerebrosidase	PD, DLB, Gaucher	Sidransky and Lopez (2012)
<i>PRNP</i>	Prion protein	Prion	Liao et al. (1986); Kretschmar et al. (1986); Hsiao et al. (1989)
<i>ApoE</i> (ϵ 4 allele)	Apolipoprotein-E	AD (risk factor)	Corder et al. (1993); Saunders et al. (1993)
<i>TREM2</i>	TREM2	AD (risk factor)	Guerreiro et al. (2013); Jonsson et al. (2013)
<i>NOTCH3</i>	NOTCH3	CADASIL	Joutel et al. (1996)

Abbreviations: EOAD, Early-onset Alzheimer's disease; MAPT, Microtubule associated protein tau; CAA, Cerebral amyloid angiopathy; bvFTD, Behavioral-variant frontotemporal dementia; PSP, progressive supranuclear palsy; ALS, Amyotrophic Lateral Sclerosis; TDP-43, Transactive response DNA binding protein 43 kDa; FUS, Fused-in Sarcoma; CBS, corticobasal syndrome; HD, Huntington's disease; PD, Parkinson's disease; DLB, Dementia with Lewy bodies; MSA, multiple system atrophy; AD, Alzheimer's disease; TREM2, Triggering receptor expressed on myeloid cells 2; CADASIL, Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

HUNTINGTON'S DISEASE

Huntington's disease (HD) is a rare, autosomal dominant disease with an incidence of 0.16-0.94/100,000 individuals annually (Pringsheim et al., 2012). The age-of-onset of symptoms is typically in the mid-40's but can range from as young as 2 to over 80 and features chorea with behavioral and cognitive symptoms (Myers, 2004). While there is a correlation between CAG-repeat length and age-of-onset (Duyao et al., 1993; HDCRG, 1993), there is a high degree of variability, making the predictive power of CAG-repeat size poor, illustrating the complexity involved with treating HD. HD features progressive neuronal loss and astrogliosis in the striatum (Vonsattel et al., 1985) as well as significant degeneration of cortical regions, cerebellum, and brainstem nuclei (Rub, Seidel, et al., 2016). While the Huntingtin polyglutamate expansion leads to protein aggregation and transcriptional dysregulation, the mechanisms tying this to neuron loss are unclear.

PRION DISEASES

Prion diseases are characterized by spongiform encephalopathy with underlying neuronal loss, gliosis, and aggregation of the prion protein (PrP) encoded by the *PRNP* gene (Hsiao et al., 1989). PrP distinguishes prion diseases from other neurodegenerative diseases that may feature prion-like mechanisms, whereby misfolded amyloidogenic proteins spread throughout the brain via seeding aggregation processes. Several genetic, sporadic, and acquired/zoonotic forms of prion diseases exist with sporadic Creutzfeldt-Jakob disease, accounting for roughly 85% of cases (Mackenzie & Will, 2017). With a mortality rate of 1.5-2 individuals per million, sCJD is an extremely rare disease with an incidence rate that is difficult to ascertain (Mackenzie & Will, 2017). Relevant clinical features include rapid cognitive decline, myoclonus, akinetic mutism, visual and cerebellar difficulties, and pyramidal or extrapyramidal features. A definitive identification requires neuropathological confirmation with *postmortem* or biopsied tissue. Probable identification requires either an electroencephalogram, magnetic resonance imaging, or cerebrospinal fluid-based biomarkers with the use of real-time quaking-induced conversion preferred over 14-3-3 detection (Peckeu et al., 2017).

Section 2: The utility of biomarkers across different neurodegenerative diseases

This section contains modified text from Ehrenberg AJ, et al. Relevance of biomarkers across different neurodegenerative diseases. Alzheimers Res Ther. 2020 May 13;12(1):56.

Neuropathological investigations are critical for closing intellectual gaps regarding pathophysiologic mechanisms, as *postmortem* examination serves as a gold-standard and provides adequate resolution to observe the elements of pathophysiological cascades. However, they are limited to cross-sectional assessment. The use of *antemortem* biomarkers renders possible the detection of hallmarks longitudinally, throughout disease stages. Even in situations where conclusions drawn from *antemortem* biomarkers might differ from *postmortem* observations, *antemortem* biomarkers still offer utility. Here, we will illustrate ways that evidence can be weighed to understand neurodegenerative diseases holistically, focusing on methodology and the limitations and strengths of different modalities.

Despite sometimes indirect associations with lesions, neuropathologically validated biomarkers can be used to assess disease course, particularly as degeneration relates to clinical manifestations. For trials, biomarkers are of paramount importance. They are key for efficiently identifying and tracking cohorts by defining inclusion criteria and outcome variables (Jack et al., 2018). As shown in a follow up to an amyloid- β immunization trial (Nicoll et al., 2019), neuropathology can be informative for trials; however the timelines associated with *postmortem* donation hinder drug development. As treatments emerge, biomarkers will become even more valuable as diagnostic tools.

***IN VIVO* BIOMARKERS FOR THE STUDY OF PATHOPHYSIOLOGY AND ETIOLOGY**

Decades of neuropathological studies augmented by novel biomedical tools have revealed that many complex, interrelated mechanisms underlie neurodegenerative diseases. A principal limitation of the cross-sectional nature of neuropathology is difficulty in assessing temporal relationships. Neuropathological investigations typically only observe end-stages; however existing population-based cohorts can capture the full range of disease progression (Grinberg et al., 2007). Detecting and addressing early phases of pathophysiological cascades is likely key for successful therapeutics, thus a granular understanding of the early underlying biology and the identification of biomarkers representing these changes is paramount.

Given the clear etiology and sometimes predictable age of onset, several studies have focused on hereditary neurodegenerative diseases to investigate early disease stages. For example, positron emission tomography (PET) imaging studies of familial Alzheimer's disease cases have suggested that brain amyloid- β deposition begins up to 20 years before clinical symptoms, while cortical tau deposition arises roughly six years before onset (Bateman et al., 2012; Quiroz et al., 2018). Similarly, biomarker studies of frontotemporal lobar degeneration mutation carriers suggest that pathological changes start decades before symptoms (Jiskoot et al., 2019). These studies demonstrate that a latency period exists prior to clinical decline, which should be targeted therapeutically, confirming neuropathological observations. There is evidence that plasma Amyloid- $\beta_{42/40}$ is sensitive to pre-clinical amyloidosis, providing a more economical means than cerebrospinal fluid or PET to identify participants in trials focused on early stages (Schindler et al., 2019).

Curiously, conclusions from *in vivo* studies of the relative onset of amyloid- β and tau lesions in Alzheimer's disease often conflict with investigations using large *postmortem* case series that indicate that tau lesions occur prior to amyloid- β deposition in the brain (Braak et al., 2011; Ehrenberg et al., 2018). This may be due to biases in the design of event-based modeling approaches with biomarkers. Young and colleagues (Young et al., 2014) found that CSF levels of total tau and phosphorylated tau become abnormal prior to CSF levels of amyloid- β_{42} , concurring with *postmortem* studies. It was not until they isolated the cohort to those who were already amyloid- β +, APOE+, or amyloid- β + and APOE+, applying data-driven autopsy-validated cutoffs, that the sequence of biomarker changes recapitulated those typically found in biomarker studies. Further, differences between investigators regarding thresholds and characterization of what belongs to 'clinically meaningful' disease spectra will affect the conclusions drawn. To illustrate, Weigand and colleagues found that amyloid- β PET negative, tau PET positive individuals tend to present with subtle cognitive changes, which they interpret to represent early Alzheimer's disease-related changes (Weigand et al., 2020). That is to say that tau PET positive and amyloid- β PET negative individuals belong to an early phase of the disease spectra. This argument is strengthened by evidence that tau PET distribution predicts subsequent atrophy, whereas Amyloid- β PET does not (La Joie et al., 2020). At the same time, others would characterize tau PET positive and Amyloid- β PET negative individuals as having "Suspected non-Alzheimer disease

pathophysiology”, implying that they belong to a different disease spectrum instead of a, rather, early phase of Alzheimer’s disease that may later feature both lesions (Jack et al., 2018). As the field remains split regarding the precise, biological definition of Alzheimer’s disease stages, interdisciplinary efforts must harmonize observations by acknowledging their varied, but complementary strengths and limitations.

Information gleaned from longitudinal studies has been used to construct models of multiple biomarkers. The widely recognized ‘Jack curves’ (Jack et al., 2013), for example, were explicitly intended to provide a “model of [Alzheimer’s disease] biomarkers” to help stage cases *in vivo* (Jack et al., 2013), setting a precedent for the 2018 guidelines for an *in vivo* research definition of Alzheimer’s disease (Jack et al., 2018). However, the curves should not be interpreted as providing a comprehensive model of Alzheimer’s disease pathophysiology and are subject to revision as biomarkers become more advanced (Jack et al., 2013).

Challenges in interpreting pathobiology

When combining biomarkers in a study, it is also important to account for differences in how they represent respective disease hallmarks. Importantly, the biomarkers must have comparable target engagement with their respective hallmarks, or, at the least, empirical measures of target engagement to adjust for different sensitivities. For fluid biomarkers, the kinetics of how biomarkers appear in the fluid samples (i.e. how a protein might be digested, processed, or passed into CSF or blood) may differ and must be accounted for. While expensive and labor-intensive, kinetics can be measured through *in vivo* labeling techniques (Paterson et al., 2019) that track protein production and clearance. Significant technological gaps exist with the detection of soluble biomarkers *in vivo*. Of note, lipid peroxidation and metabolism has been implicated in neurodegenerative diseases (Reed, 2011), yet current tools limit the degree to which this can be monitored *in vivo*. Similarly, robust fluid biomarkers of blood-brain-barrier function have been lacking, with several under development.

Idiosyncratic properties of pathophysiological processes can manifest misleading results in biomarker-based studies of pathophysiology. For PET, there is heterogeneous uptake of different ligands throughout the brain and differences in this uptake, off-target binding, and imaging sensitivity should be accounted for. In Alzheimer’s disease, early tau lesions begin in brainstem nuclei followed by limbic involvement, whereas amyloid- β lesions begin in neocortical regions (Braak & Braak, 1991; Braak et al., 2011; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017). Several limitations with PET may lead to biases in detecting these two patterns. PET cameras are susceptible to partial volume effects and lack resolution to measure tau-ligand binding in subcortical regions, which are small but can have profound neurobiological and behavioral effects (Betts, Kirilina, et al., 2019; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Ehrenberg et al., 2018; Hoffman et al., 1979; Soret et al., 2007; Stratmann et al., 2016b; Theofilas et al., 2017b; Wang & Edison, 2019). Lowe and colleagues demonstrated that tau PET positivity denotes individuals who, at autopsy, are all already at Braak stage IV (Lowe et al., 2020), at which point many subcortical

structures and limbic regions would feature significant degeneration (Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Stratmann et al., 2016b; Theofilas et al., 2017b). Further, a tau PET imaging study (Schwarz et al., 2016), with relatively low SUVR values for positivity, estimated the prevalence of Braak stage 0 individuals as roughly 55% of their study population (n=161) while a population-based autopsy study (Ehrenberg et al., 2018) featured just 28% of the study population (n=455) as Braak stage 0. Depending on the thresholds used, tau PET recapitulates the histological progression of tau lesions seen at autopsy; however, the thresholds set may render tau PET “Braak stage” an underestimate of the true Braak stage at autopsy, particularly at early stages, as seen in these studies (Maass et al., 2017; Schwarz et al., 2016). Additionally, some of the first regions to feature protein aggregation in neurodegenerative diseases contain neuromelanin, which many PET ligands will non-specifically bind to (Lemoine et al., 2018; Scholl et al., 2019). By contrast, signal from ^{11}C -PiB and other ligands targeting amyloid- β can detect early, neocortical involvement of plaques (La Joie et al., 2019), making amyloid- β easier to detect.

Other biological factors, including the significant impact of low-abundance hallmarks such as mutant huntingtin, synaptic proteins, and neurogranin should also be considered, as their low abundance may lead to underestimation of the roles these hallmarks play using event-based modeling approaches. To illustrate, differences in relative abundances of tau versus amyloid- β lesions due to tau tangles accumulating within cells and amyloid- β occurring extracellularly, makes the relative determination of proteinopathy onset difficult *in vivo*. The strengths of amyloid- β biomarkers may outweigh the weaknesses of tau biomarkers, potentially overrepresenting the relative involvement of amyloid- β versus tau lesions at different Alzheimer’s disease stages, especially in early stages. Development of biomarkers and studies with multidisciplinary, postmortem observations, are needed to reconcile these issues.

Biomarkers are invaluable for elucidating pathophysiology with appropriate caution. Specific biological questions may call for different implementations of biomarkers and establishing universal guidelines for their research use would be presumptuous. Biomarker status may be sensitive and specific to certain neuropathological diagnoses; however, one should not assume that clinical utility implies broad-sweeping research utility or vice versa. *In vivo* labeling approaches and high-resolution biomarker-neuropathology correlations are critical for assessing the validity of individual biomarkers as they relate to underlying lesions (Paterson et al., 2019). Unless the extent of the factors connecting lesions to the detection of their corresponding biomarkers is elaborated, one must be cautious when employing biomarkers in studies of disease etiology, particularly when comparing biomarkers with varied biases.

BIOMARKERS FOR THE STUDY OF DISEASE INTERVENTION

Rigorous validation of biomarkers through methods such as kinetics quantification (Paterson et al., 2019) or postmortem correlation are necessary for selecting inclusion criteria and outcomes for clinical trials. Those trials that target underlying proteinopathy, such as the amyloid- β -modifying drugs for treatment of Alzheimer’s disease, require an

in vivo measurement of lesions to verify target engagement. By contrast, intervention studies that focus on specific symptoms of diseases, such as depression, may not require *in vivo* measurements of proteinopathy. Nevertheless, an operational, validated biomarker-based definition of different diseases is paramount for trials focused on disease-modifying therapies.

The 2018 NIA-AA guidelines for an *in vivo* research definition of Alzheimer's disease set an outline for clinical trials based on a biomarker definition of Alzheimer's disease (Jack et al., 2018). The authors argue that since amyloid- β and tau lesions, together with neurodegeneration, define Alzheimer's disease *postmortem*, participants should be identified *in vivo* based on these hallmarks. There remains disagreement regarding which biomarkers can be used to identify these hallmarks, though. Biomarkers for frontotemporal lobar degeneration, synucleinopathies, and other neurodegenerative diseases are underdeveloped or have not yet been fully validated so a similar biomarker definition of other neurodegenerative diseases has yet to be established.

Amyloid- β - and tau-PET imaging could serve as useful outcomes in clinical trials due to their ability to detect regional changes *in vivo* preceding significant atrophy (Ossenkoppele et al., 2013; Ten Kate et al., 2018). The implementation of tau-PET in tau-targeting therapies is particularly encouraged due to the target's associations with neurodegeneration and cognition (Harrison et al., 2019; La Joie et al., 2020). Further consideration regarding proteinopathy biomarker implementation in Alzheimer's disease trials is warranted, though, as therapeutic changes in Amyloid- β load have not associated with clinical changes or neurodegeneration (Nicoll et al., 2019). Fluid levels of neurofilament light chain are candidate outcomes for trials as they correlate with cognitive status and atrophy, however they are not specific to underlying proteinopathy and instead represent the presence of general neurodegeneration (Mattsson et al., 2017).

Similarly, therapeutic development for huntington's disease serves to benefit from biomarker development. Despite the existence of a highly specific and sensitive genetic marker (Duyao et al., 1993; HDLRG, 1993), it is difficult to predict age of onset (Myers, 2004). Furthermore, clinical trials cannot rely on genetic markers as they will not change following a therapeutic intervention. Decreased striatal uptake of phosphodiesterase-10 PET tracer (Russell et al., 2014), atrophy in magnetic resonance imaging (Tabrizi et al., 2013), and plasma levels of IL-8, TNF- α (Bjorkqvist et al., 2008), and neurofilament light chain may be valuable markers for Huntington's disease progression that could be used for evaluating the effectiveness of interventions.

Novel ultrasensitive immunoassays make blood biomarkers promising for the future as less invasive, cost-effective screening instruments (Rissin et al., 2010). In Alzheimer's disease, plasma amyloid- β 42 is decreased compared to controls (Janelidze et al., 2016; Nakamura et al., 2018), and plasma amyloid- β 42/40 is reduced in amyloid- β -PET positive individuals (Ovod et al., 2017; Verberk et al., 2018). Plasma neurofilament light chain, which correlates with cerebrospinal fluid neurofilament light chain protein, may be useful for tracking neurodegeneration and associates with cognitive decline and

atrophy (Ashton, Leuzy, et al., 2019; Mattsson et al., 2017). These require further validation but may serve as more accessible markers in the future (Blennow & Zetterberg, 2018). Factors such as how plasma amyloid- β levels change in response to disease-modifying therapies, exposure time to drugs, and harmonization of analytical methodology remains a challenge (Ashton et al., 2018; Blennow & Zetterberg, 2018). With well-characterized cohorts that now include amyloid- β -PET and cerebrospinal fluid measures, the opportunity to use an 'endophenotype' approach to discover peripheral markers of lesions is increasing. Pilot data from discovery mass spectrometry and large panel-based approaches suggest associations of many plasma proteins and metabolites with Alzheimer's disease (Ashton, Nevado-Holgado, et al., 2019; Burnham et al., 2016; Voyle et al., 2016). However, these data should be interpreted with caution, as they are derived from multimarker panels with the mechanistic understanding of the associations lacking.

DEVELOPING BIOMARKERS FOR CLINICAL USE

In individuals with suspected neurodegenerative diseases that lack known monogenic etiologies, biomarkers are currently used on an exclusionary basis to rule out other diseases and may be used to support a specific diagnosis of a neurodegenerative disease. Other biomarkers are in the pipeline for clinical validation and determination of clinical value.

For example, structural magnetic-resonance imaging can be used to exclude space-occupying lesions and assess patterns of atrophy that aid diagnoses made by neurological assessment. Functional MRI and ^{18}F -FDG PET can also be used to identify affected brain regions and networks (Elahi & Miller, 2017; Pereira et al., 2018; Seeley et al., 2009). Neurofilament light chain is the best-established fluid marker for measuring neurodegeneration but has poor specificity (Lewczuk et al., 2018). Following the discovery and validation phases of biomarker development, standardization and clinical utility are key steps for clinical implementation. In these phases of biomarker development, key questions regarding optimal procedures, reproducibility, and elucidation of the biological relevance can be assessed. Further, these phases will determine the sensitivity and specificity for each biomarker to a given disease. For example, plasma neurofilament light chain well represents underlying neurodegeneration (Ashton, Leuzy, et al., 2019), but the consistency between labs is unclear and a great deal of overlap between diagnostic groups exist (Lewczuk et al., 2018). Another consideration in the standardization phase is the establishment of cutoffs for diagnostic thresholds. This requires a large study to assess biomarker values across diverse healthy and disease populations, accounting for possible differences in sample collection and analysis.

A further criterion for establishing biomarkers in clinical practice, even when biologically meaningful, is the utility for patient management which can determine financial coverage for the test. An ongoing study in the USA is examining how amyloid- β -PET results influence patient management (Rabinovici et al., 2019). Preliminary results suggest that management is influenced by the use of amyloid- β -PET, however the effects on prognosis is not yet known (Rabinovici et al., 2019).

NEW FRONTIERS IN BIOMARKERS

In addition to the array of targets that can be detected by standard fluid and imaging biomarkers, other features of neurodegenerative diseases have been proposed as viable biomarkers of disease progression. One technique, the Multimer Detection System-Oligomeric Amyloid- β , examines the tendency of plasma proteins to oligomerize, circumventing issues in measuring concentrations of Amyloid- β itself (An et al., 2017). Another uses biophysical properties associated with the propensity of the amyloid protein to form β -sheets to measure blood levels with an immune-infrared sensor assay (Nabers et al., 2016; Nabers et al., 2018). *In vivo* measurement of an early-affected region, the locus coeruleus, is being explored as a viable early biomarker using specialized MRI sequences and analysis (Betts, Kirilina, et al., 2019). In line with this, pupillometry is being explored as a measure of noradrenergic activity and thus, locus coeruleus integrity (Betts, Kirilina, et al., 2019). Signal analysis from transcranial magnetic stimulation, electroencephalography, or magnetoencephalography has also shown promise as a biomarker of functional connectivity and may be sensitive to early changes (McMackin et al., 2019; Rodrigues Brazete et al., 2016; Stam, 2010). Other functional readouts, such as sleep polysomnography and app-based digital phenotyping assessments, have been proposed as early biomarkers as well (Dagum, 2018; Possin et al., 2018; Winer et al., 2019).

CONCLUSIONS

In combination with basic science and neuropathologic examination, biomarkers are invaluable for building a narrative on pathophysiology, clinical etiology, and strategies for interventions. As there are numerous ways biomarkers may be informative, caution must be exercised to ensure appropriate interpretation given the varied limitations of different modalities. Importantly, a significant gap remains for biomarker availability in non-Alzheimer's disease neurodegenerative diseases. As the field works towards therapies for dementias, there continues to be a grave need for novel biomarkers, improved development of existing tools, standardization, and improved accessibility for clinical and research communities.

See Appendix 1.1 for a full list of authors who contributed to this section's associated publication, funding, disclosures, and specific acknowledgments.

Section 3: Priorities for research on neuromodulatory subcortical systems in Alzheimer's disease

*This section is composed of modified text from Ehrenberg AJ, et al. Priorities for research on neuromodulatory subcortical systems in Alzheimer's disease: Position paper from the Neuromodulatory Subcortical System PIA of ISTAART. *Alzheimers Dement.* 2023 May;19(5):2182-2196.*

Despite making up fewer than 1% of neurons in the human brain (Azevedo et al., 2009), the neuromodulatory subcortical system nuclei serve as critical hubs for the processes underlying survival, hedonic tone, and homeostasis. Counter to ionotropic fast synaptic transmission, neuromodulation acts through slower, long-lasting signal transduction mechanisms to tune neuronal excitability. The neuromodulatory subcortical system is a phylogenetically conserved network of nuclei located in the brainstem's reticular formation, diencephalon, and basal forebrain. Historically, the neuromodulatory nuclei were considered to encompass only those producing either serotonin, dopamine, norepinephrine, or acetylcholine; however, more contemporary criteria consider whether nuclei produce and release molecules that act on neurons and glia by way of metabotropic receptors. These contemporary criteria expand the neuromodulatory subcortical system to include nuclei that produce histamine, orexin, melanin-concentrating hormone, and dozens of other neuropeptides (Bargmann & Marder, 2013; Marder, 2012).

Neuromodulatory subcortical system nuclei comprise polygonal, unspecialized neurons with overlapping and symmetrically extending dendritic fields and broad, poorly myelinated axonal outputs enabling neurotransmitter release to extensive projection areas. The shared properties of neuromodulatory subcortical system nuclei make them critical nodes for understanding, diagnosing, and treating neurodegenerative diseases. Neuromodulatory subcortical system nuclei are vulnerable to different neurodegenerative diseases and are believed to modulate several psychiatric illnesses. For decades, dysfunction and degeneration (including tau accumulation, decreased transmitter production, synapse loss, and neuron loss) of the neuromodulatory subcortical system have been well-documented in Alzheimer's disease (Alzheimer's disease), with particular focus on the cholinergic basal forebrain (Curcio & Kemper, 1984; Mann et al., 1980; Mufson et al., 1988; Ohm & Braak, 1989; Parvizi et al., 2001; Scinto et al., 1999; Whitehouse et al., 1982; Yamamoto & Hirano, 1985; Zweig et al., 1988). In fact, the prevailing treatment for Alzheimer's disease targets cholinergic system dysfunction (Birks, 2006). More recent work has provided a full appreciation of the early involvement, severity, and clinical consequences of neuromodulatory subcortical system dysfunction and degeneration in Alzheimer's disease, with bodies of literature growing around the locus coeruleus, dorsal raphe nucleus, hypothalamic nuclei, and others.

In the early 2000s, findings describing the extensive degeneration of lower brainstem nuclei preceding substantia nigra involvement in Lewy body disease (Braak et al., 2003) challenged early beliefs that parkinsonism was the earliest manifestation of Lewy body disease. Later studies confirmed the association between alpha-synuclein-driven degeneration of caudal brainstem nuclei and early non-motor features of synucleinopathies, such as REM sleep disorder and hyperalgesia (Grinberg et al.).

These findings in Lewy body disease inspired interest in re-examining the chronology of neuromodulatory subcortical system involvement in Alzheimer's disease. Independent studies showed that neuromodulatory subcortical system degeneration, including intraneuronal tau aggregation, diminished neuron viability, and cell and synaptic loss,

occurs early in Alzheimer's disease pathogenesis (Rub, Stratmann, et al., 2016). At least 15 neuromodulatory subcortical system components, including noradrenergic, serotonergic, cholinergic, and orexinergic nuclei, show abnormal tau neuronal inclusions at Braak stage 0 (i.e., preceding tau pathologic lesions in the entorhinal cortex) (Stratmann et al., 2016b). Like in Lewy body disease, neuromodulatory subcortical system dysfunction and degeneration manifest as non-pathognomonic symptoms in Alzheimer's disease. In the case of Alzheimer's disease, changes in sleep, vigilance, affect, and appetite precede memory decline and relate to underlying neuromodulatory subcortical system degeneration (Ehrenberg et al., 2018; Jacobs et al., 2019). The relevance of early neuromodulatory subcortical system dysfunction in Alzheimer's disease goes beyond just clinical manifestations. Experimental studies show systematic effects of Alzheimer's disease-related neuromodulatory subcortical system degeneration resulting in dysregulated neuroinflammation, cortical tau seeding, and exacerbated cortical plaque accumulation (Chalermpananupap et al., 2018; Ghosh et al., 2019; Heneka et al., 2006; Jandanhazi-Kurutz et al., 2010; Kalinin et al., 2007; Kang et al., 2020; Rey et al., 2012; Schmitz et al., 2020). The bi-directional relationships between neuromodulatory subcortical system vulnerability and cortical dysfunction and degeneration are underexplored but have important implications for clinical practice and pathogenesis.

The molecular basis of selective vulnerability of neuromodulatory subcortical system nuclei in Alzheimer's disease is poorly understood but likely linked to the shared properties of these nuclei. Neuromodulatory subcortical system neurons regulate cognition and behavior via long, unmyelinated projections from neurons with relatively high metabolic activity. Additionally, these nuclei require high gene and protein turnover rates to maintain the cytoskeletal integrity necessary to regulate axonal trafficking/transmission and multi-modal dendritic inputs (Arendt, 2000; Berridge & Waterhouse, 2003; Mattson & Magnus, 2006; Mesulam, 1999; Sara, 2009). Finally, the neuromodulatory subcortical system is positioned close to circumventricular organs of the blood-brain barrier, making them uniquely vulnerable to neurovascular contributors to neurodegeneration (Finley, 1936, 1939; Finley & Cobb, 1940).

Addressing Alzheimer's disease-driven neuromodulatory subcortical system dysfunction and degeneration is a promising strategy to identify, monitor, and modify Alzheimer's disease progression from what are currently considered presymptomatic stages (Figure 1.1). The need for early detection and intervention in Alzheimer's disease is widely accepted, but despite their early involvement, neuromodulatory subcortical system changes in Alzheimer's disease remain underrecognized, understudied, and undertreated. Here, Alzheimer's disease-tau-mediated neuromodulatory subcortical system degeneration is considered part of Alzheimer's disease neuropathological stages even in the absence of amyloid- β cortical plaques (Braak et al., 2011; Duyckaerts et al., 2015).

CLINICAL RESEARCH

Cohort design

By the time individuals with Alzheimer's disease pathology manifest episodic memory loss in typical amnesic Alzheimer's disease, neuromodulatory subcortical system atrophy and dysregulation have already reached moderate stages (Stratmann et al., 2016b). Thus, cohorts from population-based aging studies are more suitable than clinical dementia cohorts to inform on neuromodulatory subcortical system selective vulnerability since they will capture transition phases when dysfunction and degeneration of neuromodulatory subcortical system nuclei begin. Enriching these cohorts with participants presenting with subjective memory decline (Jessen et al., 2014) and mild behavioral impairment may be required to reach sufficient power to detect changes in the neuromodulatory subcortical system. Investigating Alzheimer's disease-mediated neuromodulatory subcortical system degeneration requires longitudinal observations and better tools to track granular neuropsychiatric symptoms, subtle cognitive changes, and responses to psychotropic medications. Such cohorts should incorporate existing 'ATN'-type biomarkers (Jack et al., 2018) and prioritize enrollment in autopsy studies. Inclusion criteria should prioritize phenotypes such as adult incidence of unexplained affective symptoms or changes in sleep, vigilance, and appetite.

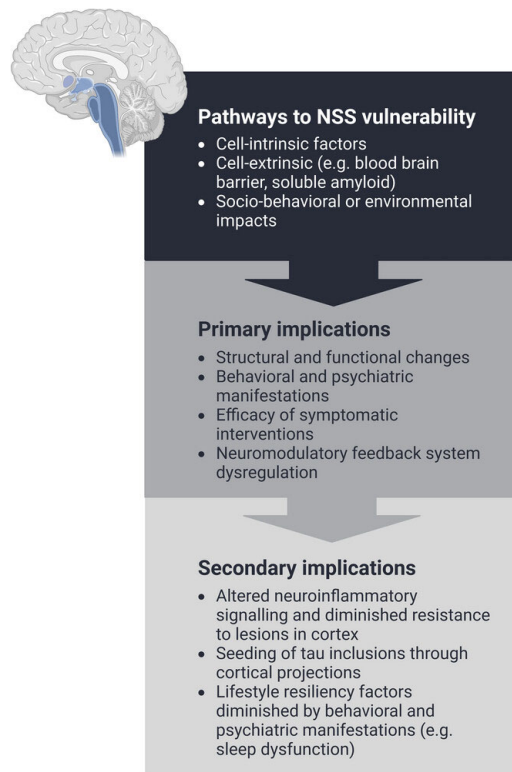


Figure 1.1: Investment in research on pathways to selective vulnerability in neuromodulatory subcortical system and how dysfunction and degeneration of neuromodulatory subcortical system contribute to pathogenesis and clinical consequences is needed. Myriad disciplines are positioned to generate knowledge within any of these research themes

Biomarkers

Neuroimaging has the potential to inform novel perspectives on the dynamic neuromodulatory subcortical system changes in aging and Alzheimer's disease and how they might underscore cognitive-behavioral aging trajectories. Recent years have featured a vast improvement in neuroimaging tools to detect the neuromelanin-containing locus coeruleus and substantia nigra in clinical settings in clinically feasible acquisition times (Betts, Kirilina, et al., 2019; Lee et al., 2022; Sulzer et al., 2018). Expanding the neuroimager's toolbox to include the detection of other neuromodulatory subcortical system nuclei is a needed but challenging goal because of the size and location of these nuclei. Emerging acquisition sequences and analytical pipelines utilizing deep learning to identify several hypothalamic subregions (Billot et al., 2020) and the basal forebrain (Fritz et al., 2019; Greve et al., 2021) based on standard structural neuroimaging are catalyzing this front, but constraints to spatial resolution jeopardize sensitive evaluation of neuromodulatory subcortical system. For example, even the novel pipelines to segment the hypothalamus can only resolve groupings of nuclei as hypothalamic subunits, rather than single subnuclei (e.g. the inferior tubular subunit which includes the infundibular nucleus, ventromedial nucleus, supraoptic nucleus, lateral tubular nucleus, and tuberomammillary nucleus) (Billot et al., 2020). Expanding adoption of 7T ultra-high-field magnetic resonance imaging and approaches to address noise, such as multi-echo functional sequences (Lynch et al., 2020; Puckett et al., 2018) and multiband imaging (Miletic et al., 2020; Risk et al., 2021), may offer solutions. PET ligands specific to neuromodulatory subcortical system and blood oxygenation level-dependent imaging (Munn et al., 2021) may provide further information about neuromodulatory subcortical system function and integrity (Doppler et al., 2021; Tiepolt et al., 2022). Novel neuroimaging methods must leverage effective data sharing and granular postmortem validation to improve standardization, reproducibility, and understanding of biological correlations.

Evolving analytical chemistry techniques make evaluating biofluids' neuromodulator and associated metabolite levels more feasible and affordable. Still, it has been challenging to interpret plasma and cerebrospinal fluid levels of neuromodulators and their metabolites. Cerebrospinal fluid neuromodulator levels may have an inverted U-shape in Alzheimer's disease because of compensatory overexpression or increased release during the early stages of Alzheimer's disease-related neuromodulatory subcortical system changes (Jacobs et al., 2019; Weinshenker, 2018). Moreover, several neuromodulators are produced in the peripheral nervous system. For example, norepinephrine detected in saliva and plasma likely reflects peripheral activity rather than locus coeruleus function. Thus, target detection in cerebrospinal fluid likely represents neuromodulatory subcortical system functional integrity best since blood-brain barrier dynamics and peripheral activity influence it less. However, it is not without influence from these variables or analytical differences.

Due to roles in upregulating sympathetic and downregulating parasympathetic activity, the integrity of neuromodulatory subcortical system structures like the locus coeruleus may also covary with physiological indices such as heart rate variability (Jacobs et al., 2020; Mather et al., 2017), pupil diameter changes (Alnaes et al., 2014; Clewett et al.,

2018; Grueschow et al., 2021; Murphy et al., 2014; Sterpenich et al., 2006; Strauch et al., 2022), skin conductance, salivary amylase, saccadic and smooth pursuit eye movements, and critical flicker fusion threshold. All of these indices have been explored as functional readouts of neuromodulatory subcortical system changes. Pupillometry has garnered much attention as a tool to evaluate locus coeruleus integrity and may aid Alzheimer's disease clinical stratification (Cazettes et al., 2021; Joshi & Gold, 2020; Reimer et al., 2016), but its utility remains unclear. Numerous neuromodulatory subcortical system nuclei, including the cholinergic system, modulate pupil dilation, making it more likely a marker of neuromodulatory subcortical system integrity as a whole than a specific locus coeruleus marker. Scalp electrophysiologic recordings may also serve as a useful biomarker in Alzheimer's disease (Babiloni et al., 2021; Babiloni et al., 2020) and, as an index of cortical arousal, should be explored as a measure of neuromodulatory subcortical system functional integrity. Systematic neuropathological studies addressing the human neuromodulatory subcortical system along Alzheimer's disease progression are critical to inform biomarker development and its integration with clinical studies or diagnostic paradigms.

Clinical assessment

An extensive functional neuroanatomic literature and mounting research specific to Alzheimer's disease over the past decade have highlighted the tight associations between Alzheimer's disease-related neuromodulatory subcortical system dysfunction and neuropsychological manifestations of Alzheimer's disease. Individuals with Alzheimer's disease pathology can experience a constellation of behavioral and psychiatric symptoms such as apathy, depression, anxiety, irritability, agitation and aggression, delusions, eating disorder, aberrant motor behavior, wandering and sundowning, hallucinations, sleep-wake cycle disorder, disinhibition, elation, and mania (Grand et al., 2011; Kwon & Lee, 2021) (Figure 1.2). Such symptoms negatively affect patients' quality of life and caregiver burden, leading to higher institutionalization rates (Okuda et al., 2019).

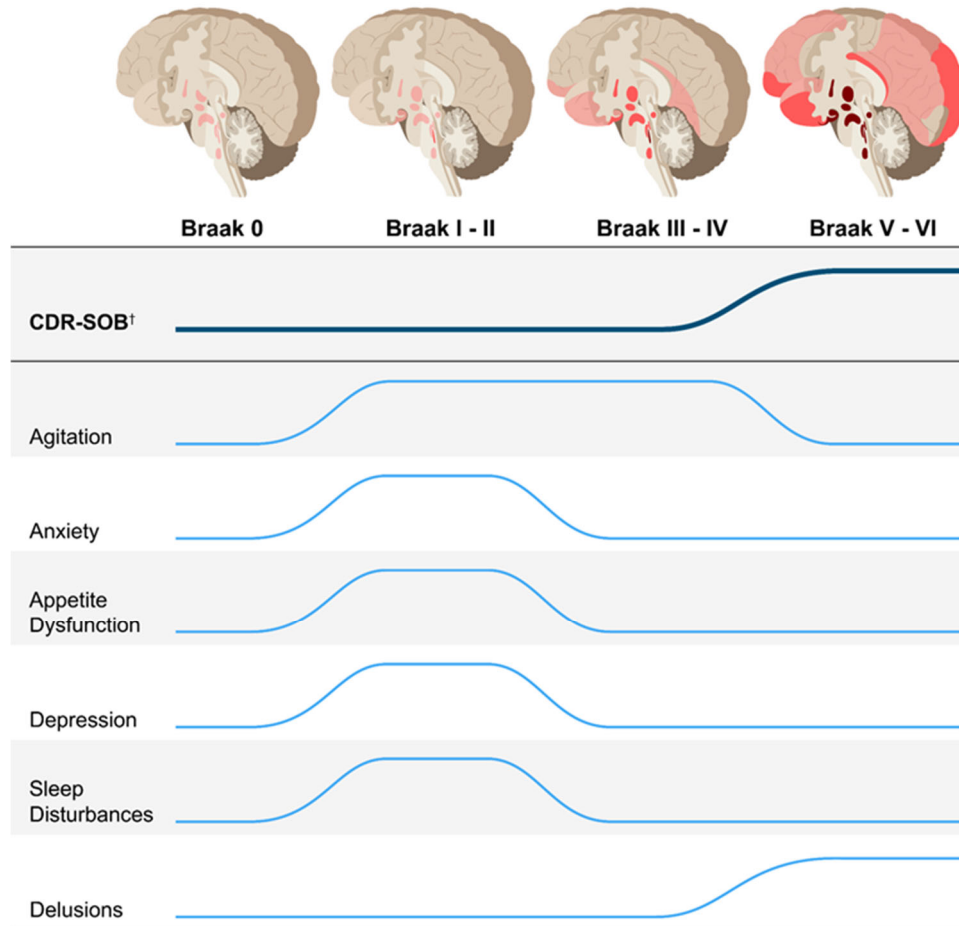


Figure 1.2: Neuropsychiatric symptoms manifest prior to the onset of cognitive decline throughout Alzheimer's disease neuropathologic stages.

Affective symptoms are the most prevalent neuropsychiatric manifestation among people with Alzheimer's disease-type dementia (Leung et al., 2021). Dysregulation of subcortical regions, such as the locus coeruleus and hypothalamic nuclei, are conventionally associated with anxiety (D. S. Charney et al., 1998; Charney & Redmond, 1983). While depression has historically been more closely related to the deterioration of the frontal-subcortical-limbic circuit, early Alzheimer's disease-related degeneration of the serotonergic dorsal raphe nucleus has been neglected in clinical and biomarker research and may contribute to depressive mood. Agitation and anxiety are related to Alzheimer's disease progression due to impaired emotional regulation networks (Rosenberg et al., 2015) and certain neurochemical disturbances, including potential compensatory overactivity of the noradrenergic system (Liu et al., 2018).

Diurnal and nocturnal polysomnographic studies in Alzheimer's disease patients report sleep fragmentation, diurnal sleepiness with increased napping, a prominent decrease in slow-wave (Liguori et al., 2020), and an average or modest decline in total sleep and REM sleep time (Liguori et al., 2014). A combination of early tau-associated dysregulation and degeneration of the noradrenergic, orexinergic, histaminergic, and basal forebrain cholinergic systems – all critical nodes in the ascending arousal network

that organizes the sleep-wake cycle – contributes to sleep disturbances in Alzheimer’s disease (Cassidy et al., 2022; Ehrenberg et al., 2018; Kjaerby et al., 2022; Lew et al., 2021; Oh et al., 2019; Scammell et al., 2017; Theofilas et al., 2017b; Van Egroo et al., 2022; Van Egroo et al., 2019; Van Egroo et al., 2021). Sleep disturbances precede memory decline in a significant portion of Alzheimer’s disease patients. Even during the early stages, these disturbances may also contribute to diminished sleep-dependent brain maintenance, which, in turn, exacerbates the propagation of Alzheimer’s disease protein hallmarks, creating a positive feedback loop (Van Egroo et al., 2019; Wang & Holtzman, 2020). Consequently, sleep dysfunction has emerged as a promising modifiable risk factor for delaying or slowing initial Alzheimer’s disease-related processes before the onset of dementia.

Beyond psychiatric and behavioral manifestations, the neuromodulatory subcortical system critically contributes to higher-order cognitive functions such as attention and memory (Cools & Arnsten, 2022; Dahl, Mather, & Werkle-Bergner, 2022; Mather et al., 2016; Thiele & Bellgrove, 2018). Degeneration of the neuromodulatory subcortical system has thus been proposed as significant determinants of cognitive decline (Braver & Barch, 2002; Li et al., 2001; Mather & Harley, 2016; Weinshenker, 2018). In line with this, correlative evidence links dopaminergic (Backman et al., 2010; Backman et al., 2006; Nyberg et al., 2016) and noradrenergic (Dahl et al., 2019; Elman et al., 2021; K. Y. Liu et al., 2020) neuromodulation to cognition.

Currently, psychometric assessment tools for individuals with Alzheimer’s disease, such as the Neuropsychiatric Inventory (Cummings et al., 1994), rely on retrospective subjective ratings by proxy, which are vulnerable to recall bias in quasi-experimental clinical settings. Digital instruments can assess affect, sleep, vigilance, agitation, social behavior, and psychiatric manifestations via objective and immediate measures in ecologically valid (e.g., in-home) settings (Berron et al., 2022; Ludtke et al., 2021). Furthermore, more sensitive, ecologically valid neuropsychiatric instruments may offer advantages for monitoring the success of disease-modifying therapeutics compared with evaluating cognitive changes alone. Validation of non-intrusive digital sensing systems to capture early cognitive, behavioral, and physiological impairments in preclinical and prodromal Alzheimer’s disease is ongoing in international multicohort studies.

Observational studies in aging populations should carefully track the numerous psychiatric and behavioral changes associated with dementia in a detailed manner. Furthermore, the accumulating evidence of clinical consequences associated with early neuromodulatory subcortical system degeneration in Alzheimer’s disease pathogenesis begs the question if, despite the lack of specificity for Alzheimer’s disease, syndromic definitions should be expanded to incorporate non-cognitive symptoms as early manifestations of Alzheimer’s disease-type neuropathologic lesions.

Neuropathology

Despite the exponential gain of knowledge about Alzheimer’s disease-related neuromodulatory subcortical system pathology in the past years, many gaps remain.

Although over a dozen neuromodulatory subcortical system nuclei develop Alzheimer's disease-tau inclusions preceding changes in the transentorhinal cortex (i.e., before Braak stage I) and others develop Alzheimer's disease-type inclusions at very early Braak stages (Attems, Thomas, et al., 2012c; Rub, Stratmann, et al., 2016; Stratmann et al., 2016b), systematic neuropathological investigations have mainly focused on the locus coeruleus, dorsal raphe nucleus, and cholinergic basal forebrain (Andres-Benito et al.; Arendt et al.; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rüb, et al.; Lyness et al.; Theofilas et al.). Moreover, the relationship and timing between the appearance of Alzheimer's disease-like neuropathologic inclusions (e.g., hyperphosphorylated tau) and the frank degeneration of neuromodulatory subcortical system nuclei (e.g., loss of axons or cell bodies) has rarely been rigorously characterized.

Existing human brain tissue sampling methods are not presently optimized for collecting neuromodulatory subcortical system nuclei, creating a significant roadblock for neuromodulatory subcortical system research. For instance, the NIA-AA guidelines (Montine et al., 2012) recommend sampling the basal ganglia at the level of the anterior commissure, pons, and midbrain to collect the basal nucleus of Meynert, locus coeruleus, and substantia nigra, respectively. This scheme is too coarse for capturing the precise anatomical levels, landmarks, and intranuclear gradients of vulnerability. The basal nucleus of Meynert includes several subnuclei (Grinberg & Heinsen; Mesulam & Geula) with varied vulnerability to Alzheimer's disease-type neurodegeneration throughout (Liu et al., 2015). Similarly, the locus coeruleus exhibits notable gradients of vulnerability, with the middle rostrocaudal third showing high susceptibility and the caudal third showing relative sparing (Betts, Cardenas-Blanco, et al., 2019; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al.; Theofilas et al., 2017b).

Furthermore, most brain sampling schemes for age-related neuropathology do not include the hypothalamus, which contains many neuromodulatory subcortical system nuclei showing early vulnerability for Alzheimer's disease. Despite its complicated cytoarchitectonics, the main hypothalamic structures of interest can be identified in coronal hypothalamic slides by Nissl staining and immunohistochemistry. The advent of *in situ* 'omics' and methods enabling RNA sequencing from formalin-fixed, paraffin-embedded brain tissue has expanded the opportunities for molecular studies in individual neuromodulatory subcortical system nuclei and subnuclei, which systematic sampling of the structures can enable.

Response analysis

Alzheimer's disease's behavioral and psychiatric symptoms begin in the early pathological stages (Ehrenberg et al., 2018). In many individuals with Alzheimer's disease, these symptoms are refractory to treatments by conventional anti-depressant and anxiolytic medications (Banerjee et al., 2011; Jones et al., 2016). Moreover, these and other psychotropic medications, including sedative drugs, sleep medication, and antipsychotics, often have harmful side effects in Alzheimer's disease (Wucherer et al., 2017). This observation is not surprising given the magnitude of degeneration, including

frank cell loss, in specific neuromodulatory subcortical system nuclei in Alzheimer's disease. In fact, the etiology of these symptoms in Alzheimer's disease may widely differ from that in the general population, necessitating research on appropriate disease- or individual-level management of symptoms. Nevertheless, tracking how patients respond to various psychiatric medications, including novel or repurposed agents, in a longitudinal design may facilitate earlier disease detection, subtyping, prognostic stratification, and improved understanding of functional neurobiological Alzheimer's disease changes.

MODEL SYSTEMS

A mechanistic understanding of neuromodulatory subcortical system vulnerability in Alzheimer's disease and neuromodulatory subcortical system contributions to disease progression remains limited. *In vivo* research in vertebrate animal models benefits from the phylogenetic conservation of neuromodulatory subcortical system nuclei. For example, the locus coeruleus has been identified in all Gnathostomata lineages, placing its evolutionary origins to at least 380 million years ago (Wang et al., 2022). As such, even aquatic models such as zebrafish (Ma, 1994) offer a platform for research on neuromodulatory subcortical system selective vulnerability.

Unlike their mouse counterparts, rat Alzheimer's disease models can develop endogenous hyperphosphorylated tau, even when not carrying a tauopathy transgene (Rorabaugh et al., 2017). The TgF344-AD rat, which carries a transgene expressing mutant human amyloid precursor protein and presenilin 1, (Cohen et al., 2013), show tau pathology in the locus coeruleus before the emergence of forebrain pathology (Rorabaugh et al., 2017), making it a good option for preclinical studies incorporating subcortical pathologic lesions in Alzheimer's disease. Other vulnerable neuromodulatory subcortical system nuclei, such as the serotonergic dorsal raphe nucleus, histaminergic tuberomammillary nucleus, orexin/hypocretin system, and cholinergic nuclei have yet to be characterized in this model. While the TgF344-AD rat has several advantages for studying neuromodulatory subcortical system, mouse models (e.g., APPsw/PS1, P301S, 3xTg-AD, 5xFAD) are often used due to the available genetic toolkits to manipulate their cell signaling networks. However, while some of these transgenic strains demonstrate pathology within brainstem regions, such as amyloid- β oligomers and hyperphosphorylated tau in the locus coeruleus (Chalermpananupap et al., 2018; Jankowsky & Zheng, 2017; Kelly et al., 2021; Yoshiyama et al., 2007), they do not recapitulate the temporal disease patterns observed in humans because the ubiquitous promoters used lead to simultaneous overexpression of amyloid- β or pathological tau throughout the brain. Also, mouse tau does not contain the full human complement of isoforms and is aggregation-resistant. Notably, most Alzheimer's disease cases are sporadic, and observations made in models of familial-based systems will not always be generalizable.

Although transgenic rodent models of familial tauopathy exist, their utility for examining neuromodulatory subcortical system vulnerability in Alzheimer's disease is limited. While some subcortical systems appear similarly affected in rare, autosomal dominant forms of Alzheimer's disease (Dahl, Mather, Werkle-Bergner, et al., 2022; Jacobs et al., 2022),

developing new preclinical models of sporadic Alzheimer's disease that induce susceptibility to pathologic lesions in neuromodulatory subcortical system is of utmost importance. It is also unclear whether neuromodulatory subcortical system nuclei have the same vulnerability profile in humans and animal models. Emerging mouse models of sporadic Alzheimer's disease from consortiums such as MODEL-Alzheimer's disease should be systematically examined for disease-related phenotypes in neuromodulatory subcortical system nuclei and existing phenotyping schemes.

Due to the small sizes of the neuromodulatory subcortical system nuclei, it remains challenging to analyze their molecular, cellular, and physiological properties *in vivo*. *In vitro* models could be particularly useful for defining the effects of Alzheimer's disease-type lesions on neuromodulatory subcortical system neurons. Induced pluripotent stem cell (iPSC) and fibroblast-based 2D and organoid differentiation protocols are also being developed to specify noradrenergic, serotonergic, and cholinergic neuron fates (Kang et al., 2019; Li et al., 2019; Pirhajati Mahabadi et al., 2015; Vadodaria et al., 2016). Unfortunately, the cells generated from existing protocols represent forebrain neuron populations rather than the isodendritic hindbrain neurons. This limitation is also the case for the SH-SY5Y line (Kovalevich & Langford, 2013; Rohm et al., 2013), which is often used for locus coeruleus research. Given the selective vulnerability of neuromodulatory subcortical system neurons to neurodegeneration, developing protocols to generate human cells that reflect hindbrain neuron populations is a high priority and would meaningfully complement animal models, biomarker findings, and neuropathology through high-throughput molecular genetic screens, drug screens, electrophysiological recording, or cell signaling assays.

An essential step towards generating valid *in vitro* differentiation protocols includes investment in research on the developmental origins of neuromodulatory subcortical system nuclei (Okaty et al., 2019). Understanding of specific developmental origins of neuromodulatory subcortical system beyond recognizing broad categories of transcription factors involved in hindbrain fate is deficient. For example, the locus coeruleus has been thought to hold a single developmental origin within dorsal rhombomere-1 (r1) (Aroca et al., 2006a; Schwarz & Luo, 2015). More recent genetic fate-mapping studies examining the expression of *Fgf8* and *Pax7* have disputed this perception by expanding the developmental origins of the locus coeruleus to include the isthmus and regions outside of the alar plate of the dorsal neural tube (Aroca et al., 2006a; Chambers et al., 2009; N. W. Plummer et al., 2017; Watson et al., 2017).

INTERVENTIONS

As attempts to reverse neurodegeneration are yet to be available, intervention should focus on attempting to reduce or prevent widespread neuronal loss. Targeting neuromodulatory subcortical system function may mitigate neuronal loss and behavioral and psychiatric manifestations of Alzheimer's disease. As Alzheimer's disease pathology in neuromodulatory subcortical system may be detectable in mid-life well before the typical age by which individuals with cognitive decline seek medical attention (Neth et al., 2019; Ritchie et al., 2015), interventions during this period are likely to have

a stronger impact on late-life brain health than interventions initiated during the cognitive stages of Alzheimer's disease (Crous-Bou et al., 2017; McKeown et al., 2021).

Symptomatic therapies

The currently available pharmacological therapies to treat cognitive symptoms in Alzheimer's disease-type dementia are based on cholinergic or glutaminergic neurotransmitter deficits, which play roles in distinct cognitive dysfunctions (Chamberlain & Robbins, 2013; Everitt & Robbins, 1997; Robbins & Everitt, 1987). Cholinesterase inhibitors are modestly effective and widely used for mitigating cognitive symptoms associated with degeneration of the basal forebrain (Whitehouse et al., 1981).

In addition to cholinesterase inhibitors, existing results from clinical trials suggest that other neuromodulatory subcortical system targeting treatments can be effective in Alzheimer's disease. In a phase III trial, the orexin receptor antagonist suvorexant improved sleep in participants with mild to moderate Alzheimer's disease patients (Herring et al., 2020). Atomoxetine, a clinically approved norepinephrine transporter inhibitor for attention-deficit/hyperactivity disorder treatment, showed improving levels of Alzheimer's disease biomarkers in participants with mild cognitive impairment due to Alzheimer's disease (Levey et al., 2021). Other neurotransmitter-based approaches currently under investigation include guanfacine, brexpiprazole, and escitalopram (Cummings et al., 2022). Still, symptomatic agents targeting cognitive enhancement or neuropsychiatric symptoms currently comprise less than a fifth of all ongoing Alzheimer's disease clinical trials.

Neuropsychiatric symptoms like sleep-wake dysfunction are both early symptoms of Alzheimer's disease and risk factors for disease progression; thus, whether through appropriate pharmacological or non-pharmacological approaches, improving some neuropsychiatric symptoms may facilitate improved brain maintenance and resistance to Alzheimer's disease progression (Van Egroo et al., 2019; Wang & Holtzman, 2020).

Not all approaches to neuropsychiatric symptoms are appropriate for Alzheimer's disease, though. Neuropsychiatric symptoms are controlled by multiple neurologic and endocrine systems and, thus, a neuropsychiatric disorder can have multiple etiologies. What might cause a neuropsychiatric symptom in the general population may not cause that same symptom in Alzheimer's disease. Because of this, therapeutic approaches may differ. For example, sedative medications like benzodiazepines are commonly prescribed for insomnia in the general population but have only a modest effect on mitigating sleep-wake dysfunction in Alzheimer's disease and are associated with severe side effects (DeFrancesco et al., 2015). Whereas comorbid psychiatric conditions often cause insomnia, stress, or poor sleep hygiene in the general population, the dysregulation of the ascending arousal network is a likely etiology for insomnia in Alzheimer's disease. The differences in the etiology of neuropsychiatric symptoms in Alzheimer's disease versus the general population may affect therapeutic efficacy for commonplace interventions.

Disease-modifying interventions

Based on neuropathological, biomarker, and clinical evidence of early neuromodulatory subcortical system dysfunction in Alzheimer's disease, protecting the neuromodulatory subcortical system from neurodegenerative changes may improve Alzheimer's disease outcomes through several pathways. Neuromodulatory subcortical system nuclei project widely throughout the brain as central nodes of nervous-system-wide modulatory activity. Experimental studies suggest that neuromodulatory subcortical system activity is neuroprotective against cortical Alzheimer's disease pathology via neurotransmitters' cell signaling and endocrine properties. For example, β -adrenoreceptor-mediated engagement of the cAMP receptor element-binding protein pathway by norepinephrine leads to upregulation of canonical brain-derived neurotrophic factor and nerve growth factor production loops (Counts & Mufson, 2010). Maintaining locus coeruleus-noradrenergic integrity may help stabilize such pathways, slowing cortical neurodegeneration. In animal models, locus coeruleus dysfunction exacerbates beta-amyloid plaque burden in the cortex (Heneka et al., 2006; Jandanhazi-Kurutz et al., 2010; Kalinin et al., 2007; Rey et al., 2012), potentially via microglia function (Heneka et al., 2010). The vast projections of the neuromodulatory subcortical system may additionally facilitate disease spread into the cortex, with preclinical evidence indicating the capacity of subcortical tau to seed forebrain pathology (Ghosh et al., 2019; Kang et al., 2020).

Cerebrovascular health

Cerebrovasculature is extensively innervated by subcortical noradrenergic, dopaminergic, serotonergic, and cholinergic neurons (Bekar et al., 2012; Bonvento et al., 1991; Cohen et al., 1997; Estrada et al., 1983; Hartman et al., 1972; Krimer et al., 1998). Several human postmortem and antemortem biomarker studies have established associations between neuromodulatory subcortical system degeneration and markers for cerebral amyloid angiopathy, blood-brain barrier breakdown, and neurovascular integrity (Giorgi et al., 2020; Jacobs et al., 2021; Priovoulos et al., 2018). In line with this, *in vivo* studies have shown that experimental lesioning of noradrenergic (Harik, 1986; Kelly et al., 2019) and cholinergic (Beach et al., 2000; Nizari et al., 2021) systems leads to deleterious changes in cerebrovascular structure and function (Nizari et al., 2021).

The neuromodulatory subcortical system nuclei are uniquely exposed to the brain's extensive vascular system. The locus coeruleus, substantia nigra, and several hypothalamic nuclei have some of the densest capillary beds in the brain (Finley, 1936, 1939; Finley & Cobb, 1940), raising questions about possible vascular contributions to their vulnerability. Furthermore, neuromodulatory subcortical system nuclei uniquely interface structurally with the cerebrovascular system (Felten & Crutcher, 1979). For instance, tanycytes, a specialized type of glia cell, are found within circumventricular organs at the third and fourth ventricles near hypothalamic and brainstem neuromodulatory subcortical system nuclei, including the locus coeruleus (Feng et al., 2011; Horstmann, 1954; Wingstrand, 1951). Tanycytes serve as a tri-directional interface between cerebrospinal fluid, blood, and these nuclei for hormone and neurotropic factor detection and release. Despite their functional importance, research

on tanycytes has been limited by methodological constraints since their discovery in the 1950s (Rodriguez et al., 2019). Recent development with single cell -omics technology is well-positioned to revive research on tanycytes, fill in critical gaps about neuromodulatory subcortical system neurovascular unit physiology, and uncover factors influencing vulnerability of the neuromodulatory subcortical system.

There are critical knowledge gaps in the reciprocal interactions between neuromodulatory subcortical system integrity and cerebrovascular health and the mechanisms by which the neuromodulatory subcortical system regulates vascular tone, perfusion, permeability, and neurovascular coupling; moderate or mediate risk factors for cerebrovascular disease; and contribute to the development of cerebrovascular lesions during the early course of Alzheimer's disease (Iturria-Medina et al., 2016). Research programs addressing these underexplored areas in human clinical series will be of critical importance for understanding the impact of neuromodulatory subcortical system degeneration on postsynaptic cerebrovascular cell dysregulation and how this relates to neuromodulatory subcortical system-mediated neuronal and glial dysfunction, Alzheimer's disease neuropathology, and alterations in functional connectivity. In model systems, work incorporating new technologies to stimulate or inhibit various afferent and efferent neuromodulatory subcortical system inputs, next-generation single-cell transcriptomics, and small animal neuroimaging approaches may offer exciting new possibilities to home in on the precise mechanisms underlying neuromodulatory subcortical system -associated phenomena in the cerebrovascular system within and outside of the context of Alzheimer's disease.

Non-pharmacological interventions

The non-pharmacologic interventions under evaluation for Alzheimer's disease span from 'electroceuticals' (i.e., transcutaneous vagal nerve stimulation), gene and stem-cell therapy, cognitive training, mindfulness, and relaxation, to diet and exercise (Bachman et al., 2022; Chen et al., 2020; Chen et al., 2021; Davis et al., 2021; Jacobs et al., 2015; Kivipelto et al., 2018; Ko & Chye, 2020; Toljan & Homolak, 2021). While many of these interventions do not target the neuromodulatory subcortical system in isolation, they do modulate the neuromodulatory subcortical system due to the fundamental neuromodulatory tone with which they engage.

Transcutaneous vagal nerve stimulation is being explored to improve memory function (Jacobs et al., 2015; Keute et al., 2021; Luckey et al., 2020; Mertens et al., 2020; Ricci et al., 2020). In principle, the delivery of electrical pulses to the cyma concha in the outer ear engages the innervating vagus nerve. Signals travel via synaptic endings at the nucleus tractus solitarius to several regions, including the locus coeruleus. Due to the locus coeruleus's widespread projections to the neocortex, allocortex, and other neuromodulatory subcortical system structures, transcutaneous vagal nerve stimulation may enable improved noradrenergic tone in Alzheimer's disease (Ludwig et al., 2021). Non-invasive stimulation of the greater occipital nerve – mediated by neuromodulatory subcortical system activation – has improved memory function in studies of healthy young humans and animal models.

Observational studies have shown that lifestyle-related risk factors, like alcohol consumption, smoking, diabetes, hypertension, cerebrovascular disease, stress, anxiety, depression, and sleep-wake dysfunction are statistical contributors to the emergence and severity of Alzheimer's disease-type dementia (Kivipelto et al., 2018; Ko & Chye, 2020; Livingston et al., 2020; Rosenberg et al., 2020). Neuromodulatory subcortical system integrity is tightly linked to many of these risk factors, including sleep-wake dysfunction (D. A. Lee et al., 2020; Mir & Jha, 2021; Monti et al., 2015; Swift et al., 2018), stress (Eck et al., 2020; George et al., 2013; Krystal et al., 2018), depression (Bernard et al., 2011; Klimek et al., 1997), drug abuse (Espana et al., 2016; Pomrenze et al., 2021), eating disorder (Ravichandran et al., 2021), and diabetes (Alba-Delgado et al., 2016; Figlewicz et al., 1996). Therefore, it is reasonable to expect that positive lifestyle changes may galvanize neuromodulatory subcortical system resilience to Alzheimer's disease. To answer these questions, studies must incorporate better tools to measure neuromodulatory subcortical system function and lifestyle factors, particularly regarding socio-economic status. The impact of lifestyle adaptations at the individual level is unknown and challenging to measure. While doctors should motivate their patients to improve their lifestyles, it is also important not to instill false hopes in the effectiveness of the individual case when necessary. Thus, while a healthy lifestyle certainly decreases the risk for Alzheimer's disease dementia on average (Livingston et al., 2020), the field should more deeply explore such interventions on a physiologic level, focusing on the neuromodulatory subcortical system, to predict individual benefits over time.

CONCLUSION

Interest in the role of neuromodulatory subcortical system vulnerability in Alzheimer's disease etiology and pathogenesis is nascent. While only several of the many nuclei affected in early Alzheimer's disease stages have been investigated, the appreciation for their roles has enabled novel perspectives on neurodegenerative motifs and clinical paradigms. Notably, the study of neuromodulatory subcortical system vulnerability has required the integration of numerous disciplines from throughout the Alzheimer's disease field (Table 1.2).

Table 1.2: Domain-specific recommendations from the ISTAART Neuromodulatory Subcortical Systems PIA.

Research domain	Recommendations
Clinical manifestations	• Develop and deploy more sensitive, granular neuropsychiatric instruments in longitudinally followed cohorts to better evaluate clinical manifestations of neuromodulatory subcortical system degeneration, especially for earlier Alzheimer's disease stages. • Follow representative population-based cohorts longitudinally with serial neuropsychiatric and cognitive instruments, biomarkers (including Alzheimer's disease biomarkers), and neuropathologic endpoints to capture age-related changes and the full range of Alzheimer's disease neuropathologic changes versus other neuropathologic entities. • Revise Alzheimer's disease syndromic definitions to include neuropsychiatric manifestations as supportive clinical features indicative of neuromodulatory

	subcortical system degeneration and incorporate such symptoms as endpoints in clinical trials.
Neuropathology and biomarkers	• Integrate Alzheimer's disease-specific and neuromodulatory subcortical system-specific biomarkers and neuropathologic endpoints in population-based cohorts to enable clinicopathological study of neuromodulatory subcortical system degeneration. • Revise autopsy guidelines to more systematically sample neuromodulatory subcortical system structures in neuropathologic evaluation and biobanking. • Harmonize brainstem neuropathologic work with cell and molecular biologic approaches to investigate mechanisms underlying selective vulnerability. • Develop a staging scheme for neuromodulatory subcortical system degeneration to understand the order by which structures are affected, including all neuromodulatory subcortical system structures. • Systematically evaluate antemortem biomarkers of neuromodulatory subcortical system structure integrity against gold-standard postmortem anatomical and neuropathological observations. • Assess all biomarker-based risk stratification frameworks in clinically and demographically diverse populations. • Develop and harmonize <i>in vivo</i> imaging tools and electroencephalography for evaluation of neuromodulatory subcortical system structural and functional integrity.
Pathophysiology	• Describe and select animal models carefully, considering how each incorporates effectors of degeneration and exhibits temporal involvement patterns in neuromodulatory subcortical system nuclei. • Guide the development of animal, organoid, and cell models with discovery-based 'omics' in human postmortem tissue to understand the strengths and limitations of experimental systems. • Pursue a better understanding of pathways underlying neuromodulatory subcortical system development to establish iPSC differentiation protocols into neuromodulatory subcortical system-type neurons.
Interventions	• Granularly document psychotropic medication use in well-characterized cohorts to enable biologically informed, hypothesis-driven response analyses. • Develop guidelines for treating neuropsychiatric symptoms in Alzheimer's disease versus the general population informed by possibly unique etiologies. • Identify the mechanisms underlying how non-pharmacological interventions mitigate neurodegenerative disease risk.

Additional investment in multidisciplinary approaches to understanding neuromodulatory subcortical system selective vulnerability and consequences of neuromodulatory subcortical system dysfunction and degeneration will accelerate the establishment of better diagnostic frameworks, identification of potent therapeutic targets, and understanding of core effectors of age-related brain diseases. A comprehensive understanding of neuromodulatory subcortical system degeneration requires studies on central themes in cell biology, systems neuroscience, bioinformatics, physiology, neuropathology, neuropsychology, medical physics, analytical chemistry, epidemiology, and public health.

See Appendix 1.2 for a full list of authors who contributed to this section's associated publication, funding, disclosures, and specific acknowledgements.

Section 4: The locus coeruleus

BACKGROUND

The locus coeruleus is a group of noradrenergic neurons in the pons found in nearly every vertebrate lineage with vast projections via the dorsal tegmental bundle to regions in the cerebellum, mesencephalon, diencephalon, and telencephalon, as well as the spinal cord (Butler & Hodos, 2005). In humans, the locus coeruleus is subject to excessive metabolic demand as it falls vastly under the predicted size relative to the high encephalization of the many regions it projects to, potentially making the locus coeruleus a vulnerable target for pathology (Sharma et al., 2010). Furthermore, the locus coeruleus is located very close to the area postrema, one of the regions of the brain with an inherently leaky blood-brain barrier, which may relate to risk factors for neurodegeneration such as inflammation (Miller & Leslie, 1994).

In large postmortem samples, the number of locus coeruleus neurons positive for hyperphosphorylated (phospho-Ser202) tau has been shown to significantly increase alongside significant decreases in locus coeruleus volume early on in Alzheimer's disease during the transition from Braak stage 0 to I (Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Theofilas et al., 2017a). This early pathology in the locus coeruleus may work as a seed for the propagation of tau pathology into the cortical regions it projects to. Additionally, there is evidence that early degeneration of the locus coeruleus may exacerbate global pathology and cognitive deficits in Alzheimer's disease due to norepinephrine dysfunction (Chalermpananupap et al., 2018; Heneka et al., 2006; Jardimhaz-Kurutz et al., 2010; Kalinin et al., 2007; Rey et al., 2012). Furthermore, there is potential to use locus coeruleus degeneration as a biomarker for early Alzheimer's disease using neuroimaging modalities (Betts et al., 2017; Priovoulos et al., 2017), pupillometry (Hämmerer et al., 2018), or indirectly with behavioral phenotypes (Ehrenberg et al., 2018).

Given the sporadic nature of tauopathies such as Alzheimer's disease and the complexities of its underlying biology, it seems prudent to consider how the manner in which the brain is built relates to its underlying vulnerability to neurodegenerative diseases. A conceptual framework for Alzheimer's disease research centered around the locus coeruleus may provide for more integrated and comprehensive investigations of the Alzheimer's disease pathobiological process with a means to translate work into biomarkers and tangible clinical outcomes.

COMPARATIVE ANATOMY AND DEVELOPMENT

The locus coeruleus (Figures 1.3-1.4), historically referred to as the nucleus dorsolateris tegmenti or nucleus pigmentosus, is a member of the isodendritic core and is the predominant source of norepinephrine for the central nervous system (Amaral & Sinnamon, 1977; Butler & Hodos, 2005; Chandler, 2016; Russell, 1955; Samuels & Szabadi, 2008). First described in 1809, it exists as two rostro-caudal columns (Figures 1.3-1.4) along the posterior portion of the pons, lateral to the anterior wall of the fourth ventricle, stretching between the caudal portion of the inferior colliculus, and ending

caudal to the motor nucleus of the trigeminal nerve (Amaral & Sinnamon, 1977; Olszewski & Baxter, 1982; Russell, 1955; Swanson, 1976).

Neurons of the locus coeruleus can be immunohistochemically identified by the presence of tyrosine hydroxylase and dopamine- β -hydroxylase (Pickel et al., 1975; Swanson & Hartman, 1975). In rodents, all of the 1400-1600 locus coeruleus cells express these markers of neurotransmitter production (Descarries & Saucier, 1972; Ross et al., 1975; Swanson, 1976). In humans, there is massive variation in the neuron population ranging between 13,000 to 125,000 neurons, not all of which stain positive for tyrosine hydroxylase (Sharma et al., 2010; Theofilas et al., 2017a).

The locus coeruleus arises early in development; its generation is modulated by the expression of bone morphogenetic proteins, *Mash1*, and the homeobox genes *Phox2a* and *Phox2b*, followed by TH and DBH in dorsal rhombomere 1 (r1) (Guillemot et al., 1993; Hirsch et al., 1998; Lo et al., 1998; Marshall et al., 1991; Morin et al., 1997; Pattyn et al., 2000; Pattyn et al., 1999; Pickel et al., 1975; Swanson & Hartman, 1975; Vogel-Hopker & Rohrer, 2002). The locus coeruleus has historically been perceived to hold a single developmental origin following these pathways within r1 (Aroca et al., 2006b; Schwarz & Luo, 2015). More recent fate-mapping studies examining the expression of *Fgf8* and *Pax7* have disputed this perception by expanding the developmental origins of the locus coeruleus to include the isthmus as well as regions outside of the alar plate of the dorsal neural tube (Aroca et al., 2006b; Chambers et al., 2009; Nicholas W. Plummer et al., 2017; Watson et al., 2017). These diverse developmental origins are certainly key to understand selective neuronal vulnerability to neurodegenerative proteinopathies.

This early generation of the locus coeruleus allows for innervation to the vast cortical and neocortical regions prior to the establishment of synapses. Poorly myelinated projections from the locus coeruleus travel to the cerebellum, mesencephalon, diencephalon, and telencephalon via the dorsal tegmental bundle, as well as to other regions in the brainstem and the spinal cord (Butler & Hodos, 2005; Pickel et al., 1974). Within the locus coeruleus, neuron subpopulations are organized based on the regions they project to (Loughlin et al., 1986; Samuels & Szabadi, 2008). Roughly, the rostral third of the locus coeruleus projects to allocortical areas, the middle third to neocortical areas, and the caudal third to other brainstem and spinal cord areas (Loughlin et al., 1986; Samuels & Szabadi, 2008; Westlund & Coulter, 1980).

The locus coeruleus is ubiquitous in vertebrates. To date, a locus coeruleus has been identified along the ventromedial wall of the fourth ventricle in every vertebrate lineage with the possible exception of Agnatha (hagfish and lamprey) (Butler & Hodos, 2005). Interestingly, certain lineages such as Actinopterygii exhibit an inverse relationship between neuron populations of serotonergic raphe nuclei and the noradrenergic locus coeruleus (Nieuwenhuys & Pouwels, 1983). Within Aves, the rostral half of what appears to be the locus coeruleus cytoarchitecturally does not have tyrosine hydroxylase activity and thus only produces dopamine (Dube & Parent, 1981). This highlights an important role the locus coeruleus also plays in dopamine production. In

fact, dopamine production in the locus coeruleus has been implicated in spatial learning and memory in the dorsal hippocampus in mice (Kempadoo et al., 2016). There is mounting evidence that in humans, the locus coeruleus does not scale allometrically with the high encephalization of the many regions it projects to. For example, a study comparing humans and nonhuman primates found that the number of locus coeruleus neurons is best predicted by allometric scaling with the medulla size instead of total brain size. In fact, the number of locus coeruleus neurons is much lower than would be predicted based on scaling rules between neocortical size and locus coeruleus neuron population size in primates (Sharma et al., 2010).

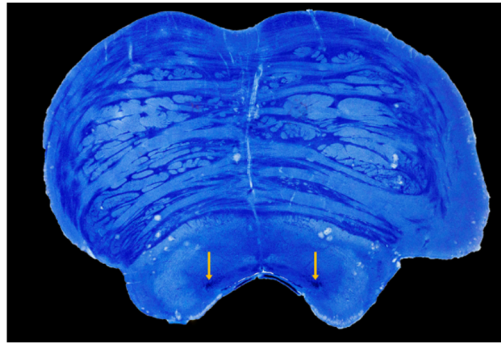


Figure 1.3: A nissl-stained section through the human pons displaying the bilateral locus coeruleus (gold arrows).

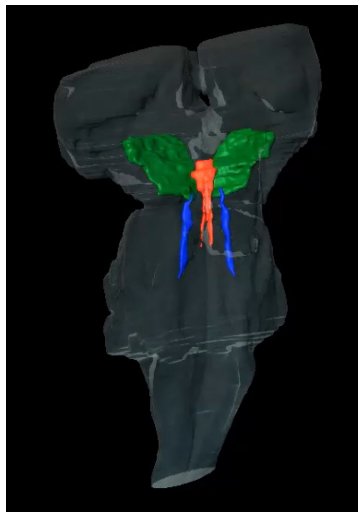


Figure 1.4: A 3D reconstruction of a human brainstem highlighting the locus coeruleus (blue), dorsal raphe nucleus (red), and substantia nigra (green).

In comparison to rodents, the mouse brain contains about 75 million neurons with about 16,000 belonging to the locus coeruleus, meanwhile the human brain contains about 86 billion neurons with roughly 50,000 neurons belonging to the locus coeruleus (Azevedo et al., 2009; Descarries & Saucier, 1972; Ross et al., 1975; Sharma et al., 2010; Swanson, 1976; Williams, 2000). Accordingly, as a proportion of total neuron population,

humans have a locus coeruleus roughly 370x smaller than a mouse does. This disparity in humans may underlie the role of the locus coeruleus in several pathologies.

LOCUS COERULEUS IN NEURODEGENERATIVE DISEASES

The locus coeruleus is involved in several neurodegenerative diseases, including synucleinopathies such as Parkinson's disease and dementia with Lewy bodies, and tauopathies such as Alzheimer's disease (Figures 1.5-1.6), progressive supranuclear palsy, and corticobasal degeneration (Andres-Benito et al.; Attems, Thal, et al.; Attems, Thomas, et al.; Braak & Del Tredici; Braak et al.; Buddhala et al., 2015; Del Tredici & Braak, 2013; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Eser et al., 2018; German et al.; Grinberg et al.; Grinberg et al.; Parvizi et al.; Rub et al.; Stratmann et al.; Theofilas et al., 2017a; Vermeiren & De Deyn, 2017; Zweig et al.). It also shows exacerbated degeneration in cases with concomitant vascular dementia (Brunnstrom et al., 2011).

In Alzheimer's disease, neuropathological studies in humans have identified progressive degeneration of the locus coeruleus beginning very early in disease stages, with some suggesting that Alzheimer's disease may begin in the locus coeruleus (Frosch, 2017).

Between healthy controls and the first Braak stage for neurofibrillary tangle pathology, there is a nearly two-fold increase in the number of neurons with hyperphosphorylated tau pathology (Andres-Benito et al., 2017; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017).

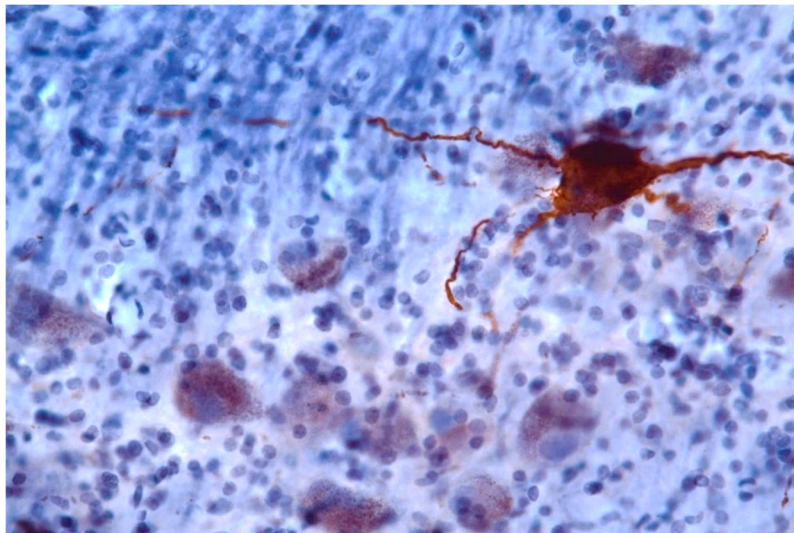


Figure 1.5: A CP-13 stained locus coeruleus testing positive for hyperphosphorylated tau in a human case at Braak stage I. Nissl counterstained, note the neuromelanin inclusions within hyperphosphorylated tau-negative neurons.

This increasing tau burden is accompanied by about 8% lower locus coeruleus volume in Braak stage I compared to controls, with this trend continuing across subsequent Braak stages (Theofilas et al., 2017a). Interestingly, the neuron population size of the

locus coeruleus appears to stay stable until the transition from Braak stage II to III (Theofilas et al., 2017a). It was also found that the middle and rostral thirds are the most affected portions of the locus coeruleus, while the caudal third is more spared (Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Theofilas et al., 2017a).

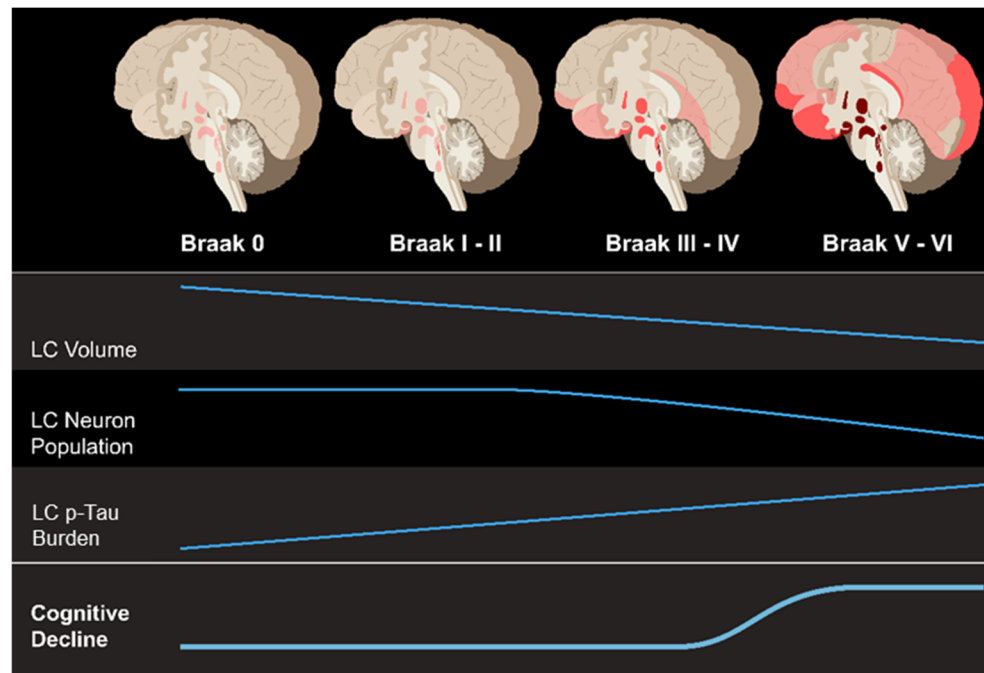


Figure 1.6: Subsequent pathologic trajectories in the locus coeruleus against cognitive decline in Alzheimer's disease. Note the early accumulation of phosphorylated tau inclusions and volumetric decrease along a stable neuron population and cognitive status. "p-Tau Burden" represents the proportion of neurons present harboring hyperphosphorylated tau neuronal cytoplasmic inclusions.

In parallel with the effects of Alzheimer's disease progression within the locus coeruleus, there is mounting evidence that locus coeruleus degeneration may also exacerbate global Alzheimer's disease pathology. Two studies in 2006 and 2007 demonstrated that ablation of the locus coeruleus with the selective neurotoxin N-(2-chloroethyl)-N-ethyl-bromo-benzylamine (DSP-4) in amyloid precursor protein transgenic mice significantly exacerbated β -amyloid pathology and glial inflammation (Heneka et al., 2006; Kalinin et al., 2007). Other studies in 2010 and 2012 in more comprehensive animal models of Alzheimer's disease (APP/PS1 transgenic mice) corroborated these findings and also demonstrated that locus coeruleus degeneration has downstream cognitive effects (Jardanhazi-Kurutz et al., 2010; Rey et al., 2012). Another study in 2018 showed that locus coeruleus ablation increases global tau burden, exacerbates cognitive deficits, and increases lethality in a mouse model of tauopathy (Chalermpanupap et al., 2018). A 2017 study presented evidence that restoration of locus coeruleus norepinephrine dysfunction may rescue reversal learning capabilities in Alzheimer's disease, indicating that the locus coeruleus may be a valuable therapeutic target (Rorabaugh et al., 2017).

CLINICAL STUDIES AND DIAGNOSTICS

Given the early degeneration of the locus coeruleus in Alzheimer's disease, there is a great deal of interest in using it as a biomarker for preclinical or prodromal stages. It is likely that effective therapeutics will address these stages, in which neurons are still viable and cognitive decline is minimal (Korczyn, 2012).

Behaviorally, assessing locus coeruleus degeneration is very challenging. The locus coeruleus is not directly involved in executive functioning – the most striking clinical phenotype of Alzheimer's disease. There is increasing evidence, however, that locus coeruleus degeneration may relate to neuropsychiatric phenotypes of Alzheimer's disease and other proteinopathies. Symptoms such as depression, sleep dysfunction, and anxiety have typically been regarded as risk factors or epiphenomena, but the recent evidence indicates that they may be early manifestations of the pathophysiology and as such, are part of the disease phenotype.

A recent study demonstrated in a large population-based sample of healthy controls and 'pure' Alzheimer's disease cases that increased odds for agitation, anxiety, depression, appetite disturbances, and sleep dysfunction correlate with Braak stages I-II when pathology is restricted to subcortical regions and parts of the limbic system (Ehrenberg et al., In review). Agitation and anxiety have long been associated with locus coeruleus degeneration (Dennis S. Charney et al., 1998; Charney & Redmond, 1983; Geerts et al., 2013; Rosenberg et al., 2015), including several lesion studies in rats and cats (Beleslin et al., 1981; Nakamura & Thoenen, 1972; Sorenson & Gordon, 1975). Similarly, depression has been associated with locus coeruleus degeneration, in line with the monoamine hypothesis of depression (Belmaker & Agam, 2008; Förstl et al., 1992; Krishnan & Nestler, 2008; Moret & Briley, 2011). Along with the ventromedial and lateral hypothalamic nuclei, norepinephrine produced by the locus coeruleus plays an important role in appetite regulation (Ahima & Antwi, 2008; Bresch et al., 2017; Neary et al., 2004; Talakoub et al., 2017; Vettor et al., 2002; Vilberg & Beatty, 1975). Finally, sleep dysfunction has been strongly associated with norepinephrine deficiency and locus coeruleus degeneration (Eser et al., 2018; Khanday et al., 2016; Takahashi et al., 2010; Wilson et al., 2013). Further research in humans would be needed to identify the subtypes of various neuropsychiatric symptoms and their relation to the pathological burden of Alzheimer's disease in the locus coeruleus. However, in combination with other clinical markers, neuropsychiatric symptoms may be valuable for early diagnosis of Alzheimer's disease.

Pupillometry and neuroimaging are other techniques actively being explored to measure locus coeruleus degeneration. Strong data are indicating that pupil diameter and P3 event-related potential are closely correlated to locus coeruleus-noradrenergic activity (Granholm et al., 2017; Murphy et al., 2011). As such, pupillometry has the potential to be a valuable window into early degeneration. Several studies have evaluated the viability of pupillometry as a biomarker for early Alzheimer's disease and show that it does, in fact, hold predictive power with regard to the conversion from mild cognitive impairment to dementia (Granholm et al., 2017; Hämmerer et al., 2018). Larger

population-based studies would need to be used to evaluate its predictive power in early neuropathologic stages prior to mild cognitive impairment.

Humans accumulate iron in the locus coeruleus, which binds to neuromelanin with increased age (Figure 1.6) (Zecca et al., 2004; Zecca, Zucca, Costi, et al., 2003; Zecca, Zucca, Wilms, et al., 2003; Zucca et al., 2006). Neuromelanin accumulation has been associated with oxidation of dopamine (Wakamatsu et al., 2012). Due to the paramagnetic properties of iron, magnetic resonance imaging has been utilized to measure locus coeruleus volume *in vivo* (Figure 1.7).

Many existing attempts to image the locus coeruleus using magnetic resonance imaging are within the context of Parkinson's disease; however, several promising studies have applied the technique to Alzheimer's disease (Betts et al., 2017; Priovoulos et al., 2017; Takahashi et al., 2015). There are concerns regarding the viability of this technique mostly centered on the lack of resolution to changes in regions as small as the locus coeruleus. Several studies are presently investigating the use of 7T magnetic resonance imaging in both postmortem brain samples and *in vivo*. Different contrasting parameters, such as QSM, T1, and T2*, have also been examined. A 2015 study of the substantia nigra highlighted the usefulness of combining contrasts; however, differences between T1 and T2* were noted (Langley et al., 2015). Templates used to identify the locus coeruleus in magnetic resonance imaging have also been developed (Betts et al., 2017). From all these studies, it is clear that complete histological validation of the entire locus coeruleus 3D structure will be critical to interpret magnetic resonance image signal properly.

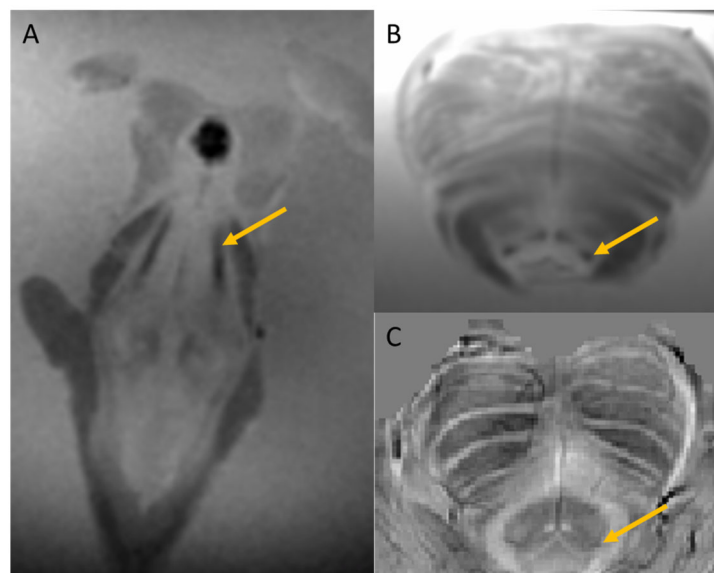


Figure 1.7: The locus coeruleus in magnetic resonance imaging (left locus coeruleus denoted with yellow arrow). A, Coronal slice of a post-mortem fixed brainstem. B, Axial slice of a post-mortem fixed brainstem. C, QSM image of a 22-year-old male. Note the lack of neuromelanin due to young age.

Section 5: Social isolation, loneliness, PTSD, and dementia

Social isolation and loneliness are increasingly recognized as significant public health issues. A 2023 U.S. Surgeon General's advisory was issued regarding the health risks of loneliness (U.S. Surgeon General, 2023). Social isolation and loneliness are related but distinct phenomena. Social isolation is an objective quality of having few social relationships and infrequent social interaction with others, whereas loneliness is a subjective experience resulting from the perception of isolation. Both social isolation and loneliness have myriad adverse health effects, costing Medicare an estimated \$6.7B annually (Flowers et al., 2017). Well over a hundred longitudinal studies of social isolation have demonstrated significant effects on mortality with social connection increasing odds of survival by 50% in some populations (Holt-Lunstad et al., 2010).

Adverse outcomes range from increased cardiovascular disease (Courtin & Knapp, 2017; Crystal et al., 2022; Grant et al., 2009; Leigh-Hunt et al., 2017), poor mental health (Courtin & Knapp, 2017; Leigh-Hunt et al., 2017; Loades et al., 2020; Mann et al., 2022; Sepúlveda-Loyola et al., 2020), musculoskeletal disorders (Hämmig, 2019), frailty (Mehrabi & Béland, 2020), and dementia (Lazzari & Rabottini, 2022; Penninkilampi et al., 2018). In older adults, social isolation and loneliness have been linked to up to a 50% increased risk of developing dementia (Lazzari & Rabottini, 2022). Over 12 years, cognitive abilities in older adults declined 20% faster in lonely individuals (Donovan et al., 2017).

People with a diagnosis of post-traumatic stress disorder (PTSD) also have a higher risk of developing dementias, with hazard ratios as high as four (Günak et al., 2020; Rafferty et al., 2018; Ritchie et al., 2019; Yaffe et al., 2010). PTSD is associated with several dementia phenotypes, including Alzheimer's disease, vascular dementia, and, notably, semantic frontotemporal dementia (Bonanni et al., 2018). A study from the Alzheimer's Disease Neuroimaging Initiative has demonstrated that PTSD does not correlate with increased PiB-PET signal (Weiner et al., 2017), while another study in veterans suggests that the increase in dementia risk occurs via a tauopathy-mediated etiology (Mohamed et al., 2019). It is not clear that the kind of dementia PTSD is driving is due to Alzheimer's disease or if this risk is representing, rather, episodic memory issues tied to hippocampal degeneration (Bremner et al., 1995; Schuff et al., 2001). While the epidemiological evidence for adverse outcomes due to social isolation, loneliness, and dementia are clear, the mechanisms underlying its pathogenicity are not well established.

Chapter 2: Social isolation effects in laboratory mice

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Abstract

Social isolation and loneliness are well-established public health crises in developed countries. Exposure to prolonged social isolation is associated with myriad adverse outcomes ranging from mental health to cardiovascular diseases, metabolic disorders, and neurological diseases. However, mechanisms underlying social isolation's contributions to these health outcomes are unclear. An improved appreciation for pathways underlying their pathogenicity could inform interventions for stress-driven diseases. Mice were exposed to chronic single housing for 13 months – markedly longer than typical mouse social isolation studies. There was evidence of sex-dependent activation of the hypothalamic-pituitary adrenal (HPA) axis, but results related to adverse health outcomes were mixed. Together with existing literature, we find that rodents may be a lackluster model for capturing the underlying biology of social isolation and loneliness in humans.

Introduction

Social isolation and loneliness are increasingly recognized as significant public health issues. A 2023 U.S. Surgeon General's advisory was issued regarding the health risks of loneliness (U.S. Surgeon General, 2023). Social isolation and loneliness have myriad adverse health effects, costing Medicare an estimated \$6.7B annually (Flowers et al., 2017). Adverse outcomes range from increased mortality (Holt-Lunstad et al., 2015; Leigh-Hunt et al., 2017; Timothy et al., 2021) to increased cardiovascular disease (Courtin & Knapp, 2017; Crystal et al., 2022; Grant et al., 2009; Leigh-Hunt et al., 2017), poor mental health (Courtin & Knapp, 2017; Leigh-Hunt et al., 2017; Loades et al., 2020; Mann et al., 2022; Sepúlveda-Loyola et al., 2020), musculoskeletal disorders (Hämmig, 2019), frailty (Mehrabi & Béland, 2020), and dementia (Lazzari & Rabottini, 2022; Penninkilampi et al., 2018). While the epidemiological evidence for adverse

outcomes due to social isolation and loneliness is clear, the mechanisms underlying its pathogenicity are not well established.

Chronically increased levels of circulating cortisol have been proposed as a driver of adverse health outcomes of social isolation. Indeed, in conditions like Cushing's disease, where a pituitary adenoma chronically over-activates the HPA axis, myriad health outcomes are present. Patients with Cushing's disease experience, among other complications, metabolic disorders (Barbot et al., 2020; Pivonello et al., 2016; Reznik et al., 2013), cardiovascular disease (Pivonello et al., 2016; Varlamov et al., 2021), musculoskeletal disorders (Barbot et al., 2020; Hasenmajer et al., 2020; Pivonello et al., 2016), mental health and cognitive disorders (Barbot et al., 2020; Braun et al., 2001; Feelders et al., 2012; Pivonello et al., 2016), and immune system disorders (Hasenmajer et al., 2020; Pivonello et al., 2016; Schernthaner-Reiter et al., 2021; Varlamov et al., 2021). However, there is mixed evidence of chronically elevated cortisol levels due to social isolation. Increased cortisol awakening response and cortisol output throughout wake periods have been described due to social isolation (Grant et al., 2009; Levine, 1993). However, the levels observed in these studies (Grant et al., 2009), especially late at night, fall far below what is seen in Cushing's disease (Raff et al., 1998).

To probe mechanisms underlying chronic social isolation's adverse outcomes, we utilized a colony of male and female C57BL/6 mice kept in single or group housing. Mice are social animals, and numerous studies have evaluated the effects of housing conditions on several experimental outcomes. Single-housed mice exhibit hyperactivation, depression-like behavior (N. Liu et al., 2020; Voikar et al., 2005), and, depending on the behavioral assay used, increased anxiety (Bartolomucci et al., 2003; Voikar et al., 2005). There is additional evidence of cognitive impairment in these models (Buckinx et al., 2021; N. Liu et al., 2020; Voikar et al., 2005). There is evidence of altered serotonergic signaling associated with depressive states (Kallioikoski et al., 2014) and decreased markers of neuroplasticity (N. Liu et al., 2020). Increased food intake and associated visceral fat have been documented in single-housed conditions (Bartolomucci et al., 2009; Schipper et al., 2018). Notably, many of these studies span only several weeks of single housing. In our model, we decided to expose the mice to single-housing for 13 months to assess how extended social isolation can affect age-related outcomes.

Methods

ANIMAL COHORT

C57BL/6J (JAX# 000664) mice were raised in group housing until five months old. At five months, mice either remained in group housing or were split into single housing, where they remained for 13 months. Mice were kept at a 14-10 light-dark cycle with unrestricted food (LabDiet® 5K52 formulation) and water access. Animal health monitoring and regular cage changes were completed by animal facility technicians for the Office of Laboratory Animal Care (OLAC) at the University of California, Berkeley.

Group-housed mice, which were littermates, were kept in cages of four mice except for one cage of female mice, which had three. All animal procedures were approved by the UC Berkeley Animal Care and Use Committee (AUP-2020-11-13859-1). The final cohort consisted of 12 group-housed males, 11 group-housed females, 10 single-housed males, and nine single-housed females.

At 18 months old, the mice were weighed and then euthanized using a lethal dose (300mg/kg, i.p.) of sodium pentobarbital. The mouse was photographed with a color calibration card and ruler. Once a lack of pedal reflex was determined, the mouse was decapitated. The brain was extracted from the skull and dissected. A coronal slice was made approximately 1.5mm posterior to the caudal end of the longitudinal fissure. This posterior block was placed into 4% paraformaldehyde in a tissue cassette. The portion of the brain anterior to this block was sliced sagittal along the longitudinal fissure with the left half fixed and the right half snap frozen in liquid nitrogen. The lower digestive tract was removed and dissected. Fecal pellets from the colon, luminal scrapings from the small intestine, colon, and cecum, and the small intestine, colon, and cecum were individually frozen in liquid nitrogen. From the abdomen and thorax, the medial lobe of the liver and the right kidney were snap-frozen, and the left lobe of the liver, left kidney, and heart were fixed in formaldehyde. A dorsal strip of skin and hair from the neck to the tail (~2cm wide) was removed and frozen at -20C.

HAIR HORMONE ANALYSIS

Hair corticosterone and testosterone levels were measured by trimming hair from the dorsal skin patch. The hair was washed using 5x 5ml isopropanol in a 100µm cell strainer. The hair was left to dry in a fume hood overnight. 10-15mg of the cleaned hair was weighed into a Precellys MK28-R (P000917) tube. The hair was pulverized in the MK28-R tube using a Precellys tissue homogenizer with liquid nitrogen cooling. 1 ml of HPLC-grade methanol was pipetted into each tube and incubated overnight under pulsating rotation using a tube rotator at room temperature. Tubes were centrifuged at 14,000 rpm for 15 minutes at room temperature. 0.8 ml of the supernatant was transferred to a clean tube, which was centrifuged again at 14,000 rpm for 15 minutes to ensure the supernatant was debris-free. All tubes were left open in a desiccator under a slight vacuum overnight to evaporate the methanol. Samples were reconstituted in ELISA buffer, as directed by the Corticosterone sandwich ELISA kit from Cayman Chemical (501320). Tubes were left to reconstitute under pulsating rotation overnight. Corticosterone and testosterone concentrations were measured in triplicate using instructions and reagents provided by the respective ELISA kits (501320 and 582701, Cayman Chemical).

MORPHOMETRIC AND HAIR COLOR ANALYSIS

Body mass used in analyses was collected on the date of necropsy. Dorsal and ventral photos of the mice were collected using a Sony a7 II digital camera with a 35mm lens affixed to a copy stand. Photos included a color calibration card and a ruler. Mouse surface area, excluding the tail, was measured from each mouse's dorsal and ventral photos. Considering that lean mice would be smaller relative to their overall body

weight, the average of the dorsal and ventral surface area, in combination with body mass, was used here as an approximation of body composition.

TRANSCRIPTOMIC ANALYSIS

Frozen samples of hippocampus, hypothalamus, liver, and kidney from a subset of 24 mice were sent to a vendor (Novogene Inc., Davis, CA) for RNA extraction and sequencing. The cohort subset included, for each sex, four group-housed mice, the four single-housed mice with the highest corticosterone concentrations in hair, and the four single-housed mice with the lowest corticosterone concentrations in hair. Extraction was performed in TRIzol using the phase separation method from cell debris. An Agilent bioanalyzer 2100 was used to measure RNA quality and concentration. At this stage, the liver sample from a single-housed male, the hypothalamus sample from a male group, and the hippocampus from a female group were excluded due to either RNA quantity or quality. Library preparation with poly A enrichment was done with the NEBNext Ultra II RNA Library Prep Kit for Illumina. Sequencing was performed with the Illumina NovaSeq 6000 to generate 150bp paired-end (PE150) reads.

The quality of sequence files was assessed using the FastQC package (Andrews, 2010) before and after trimming steps. Trimmomatic (Bolger et al., 2014) (ILLUMINACLIP: TruSeq3-PE.fa:2:30:10:2:True, LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4:15, and MINLEN: 36) was used to remove adapter sequences and any sequences with low mean quality scores. Sequences were aligned to GRCm39 using STAR alignment (Dobin et al., 2013) and count matrices were generated using featureCounts (Liao et al., 2014). The count matrices were converted to counts per million (CPM) using the edgeR package.

Principal component analyses (PCA) were performed using the RunPCA command from the Seurat package in R with 20 principal components computed. Differential expression analyses were performed using the edgeR package (Robinson et al., 2010). Only genes with at least five counts in at least 25% of samples were included in differential expression analyses. Multiple comparison correction was done using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995), and the upper bound of the expected false discovery rate (FDR) is reported in analyses as the FDR. Differential expression analyses set cutoffs for hits at 5% FDR and +/- 0.5 log-fold change (logFC). The design matrix for differential expression analyses is formed from an additive model formula, including the case. The design matrix formula did not include an interaction term as we sought to assess the average effect of nucleus identity in our cohort rather than the effect of nucleus identity for a given case.

Gene set enrichment analysis (GSEA) was done using the fgsea package (Korotkevich et al., 2021) in R. The Hallmark gene set collection was downloaded from the GSEA Molecular Signatures Database (Liberzon et al., 2015; Mootha et al., 2003; Subramanian et al., 2005). The gene set files were filtered to contain only genes in the differential expression table and then reformatted to the specifications required by the fgsea package. The differentially expressed genes (DEGs) were ranked based on the negative log(p-value) such that the genes with the smallest p-values were ranked at the

top of the ranking list. The fgsea function was run on the filtered gene set files with the ranked genes using a minimum gene set size of 10 and a maximum of 500.

HISTOLOGY, IMMUNOHISTOCHEMISTRY, AND MICROSCOPY

Tissue fixed in 4% paraformaldehyde was embedded into paraffin blocks before slicing at 8 μ m. All sections were stained using hematoxylin and eosin and scanned. With the hypothesis that chronic stress may affect blood-brain-barrier dynamics, multiplex immunohistochemistry was used to examine how astrocytic TGF- β signaling may differ between groups. After deparaffinization of sagittal cut brain sections, tissue underwent antigen retrieval in 5% citrate buffer with 0.05% tween at 95°C. Slides underwent blocking with 2% normal goat serum (NGS) for 30 minutes, then incubated overnight in primary antibodies diluted in 2% NGS. Antibodies included phospho-SMAD2 (AB3849-I; 1:2500), GFAP (ab4674; 1:1000), and NeuN (266 014; 1:600). After PBST washing, the slides were incubated in a cocktail of secondary antibodies at 1:400 – goat anti-rabbit Alexa Fluor 488 (A11008), goat anti-chicken Alexa Fluor 546 (A11040), and goat anti-guinea pig Alexa Fluor 647 (A21450) – diluted in PBST (0.05% tween). Nuclei were labeled using DAPI at 1:1000, followed by lipofuscin blocking by Sudan Black-B for 40 minutes. After washing excess Sudan black in 70% ethanol, slides were coverslipped using ProLong Glass Antifade Mountant.

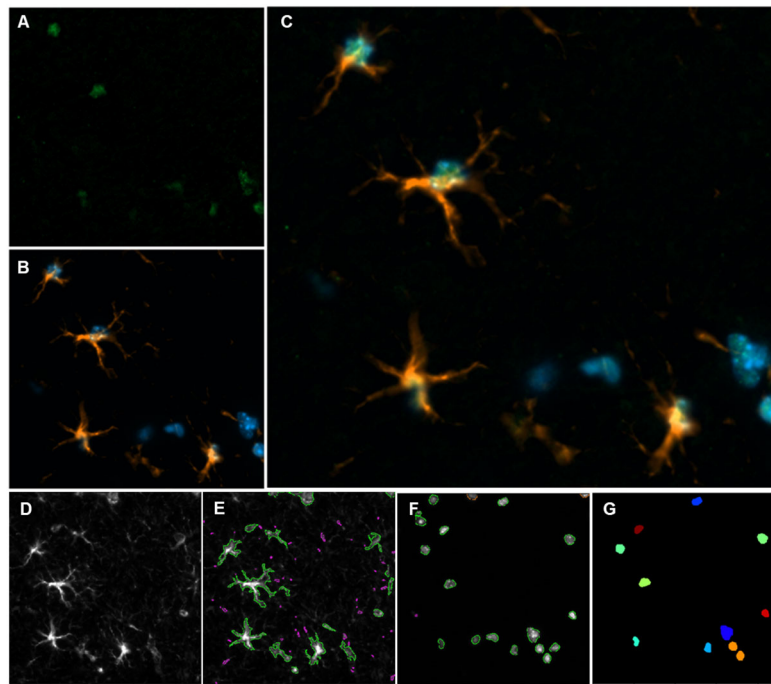


Figure 2.1: Astrocytic TGF- β activation was analyzed using multiplex IHC staining and a CellProfiler pipeline. pSMAD2 (A) and GFAP (B) were multiplex (C) stained using immunofluorescence with DAPI counterstaining (blue). Regions of interest (D) were first analyzed to identify astrocytes on the basis of GFAP staining (E) and selected as primary objects. Nuclei were identified (F) as additional primary objects on the basis of DAPI labeling. pSMAD2 intensity within nuclei colocalizing with astrocytes (G) was measured as the primary outcome for each ROI.

All micrographs were collected using a Zeiss Axioscan.A1 slide scanner. To analyze astrocytic TGF- β signaling, regions of interest were selected from cornu Ammonis 1 (150x150 μ m), cornu Ammonis 2 (150x150 μ m), cornu Ammonis 3 (100x100 μ m), and the polymorph layer of the dentate gyrus ("hilus" 100x100 μ m). An image analysis pipeline was established using CellProfiler (Carpenter et al., 2006) (Figure 2.1).

The number of glomeruli was counted from three 1000x1000 μ m ROIs from photomicrographs of the hematoxylin and eosin-stained mouse kidneys. ROIs were set near the cortical layer of the kidney, with one at the rostral portion, one at the caudal portion, and one at the lateral portion. A single investigator counted the glomeruli and recorded the total number per kidney from the three ROIs.

Results

HPA-AXIS ACTIVATION

To investigate the effect of 13-month housing conditions on HPA-axis activation, we examined differences between groups in the hair concentrations of corticosterone stratified by sex. Statistically significant differences were detected within males but not in females (Figure 2.2A). Differences in testosterone concentration were also examined between housing conditions, and no statistically significant differences were detected between groups (Figure 2.2B). The relationship between corticosterone concentration and testosterone concentration was also examined. There was no significant correlation between corticosterone and testosterone in males ($r=0.302$, $p=0.171$), but there was a significant positive correlation in females ($r=0.58$, $p=0.007$).

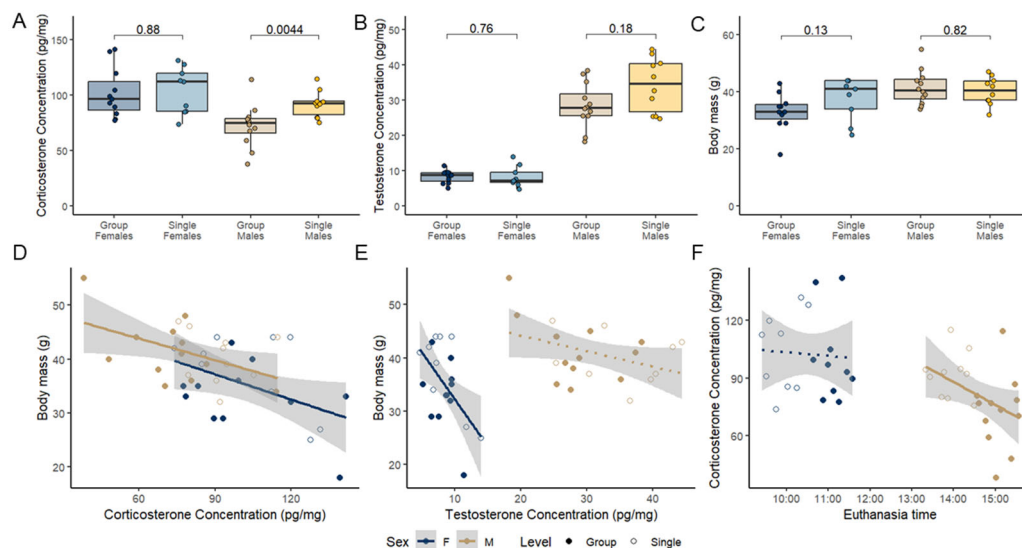


Figure 2.2: (A) There is a statistically significant increase (Wilcoxon $p=0.0044$) in the concentration of corticosterone in single-housed males relative to group-housed males, but there is no difference between group- and single-housed females. (B) There is no statistically significant (measured by Wilcoxon rank-sum-test) difference between group- and single-housed mice in testosterone levels for males or females. (C) There is a lack of statistically significant difference in body mass between groups, however (D) there are significant negative correlations between body mass and corticosterone concentration (Males: $r=-$

0.464, $p=0.0295$; Females: -0.462, $p=0.0403$). There is also a significant correlation between testosterone concentration and body mass in females ($r=-0.559$, $p=0.009$), but not in males ($r=-0.397$, $p=0.067$) (E). A statistically significant negative correlation between corticosterone concentration and time of day was detected in males ($r=0.433$, $p=0.044$), but not females (F).

Associations between group and body mass were examined, and no statistically significant differences were detected (Figure 2.2C). However, when associations between hormone concentrations and body mass were examined, significant associations were detected between corticosterone and body mass in both sexes (Figure 2.2D). Testosterone and body mass associations were detected only in females (Figure 2.2E). Approximations of body composition did not differ between groups (Supplementary Figure 2.1). Despite hair concentrations of corticosterone being, in principle, more resistant to transient spikes, the time of day the mouse was euthanized was examined in relation to corticosterone concentration. A statistically significant ($p < 0.05$) negative correlation between corticosterone concentration and time of day was detected in males ($r=0.433$, $p=0.044$) but not in females (Figure 2.2F).

TRANSCRIPTOMIC ANALYSIS OF THE HIPPOCAMPUS AND HYPOTHALAMUS

We examined two cortical regions associated with regulation of stress response using bulk mRNA sequencing – the hippocampus and hypothalamus. The housing condition did not segregate along any variance in gene expression in the hypothalamus or hippocampus through principal components 1-3, which collectively explains 76.7% of the total variance in our sample. We did not identify any genes that were significantly ($FDR < 0.05$) up- or down-regulated in the hippocampus or hypothalamus in males or females (Figure 2.3).

A set of 444 curated genes (Appendix 2.1) associating with blood-brain-barrier function or structure were specifically analyzed using a cutoff of $p < 0.01$ instead of an $FDR < 0.05$. Differentially expressed genes related to blood-brain-barrier function or structure were identified in the hypothalamus and hippocampus among female mice based on housing condition (Figure 2.3E & 2.3G). Two differentially expressed genes related to blood-brain-barrier function were identified in the hypothalamus of males but not the hippocampus (Figure 2.3F).

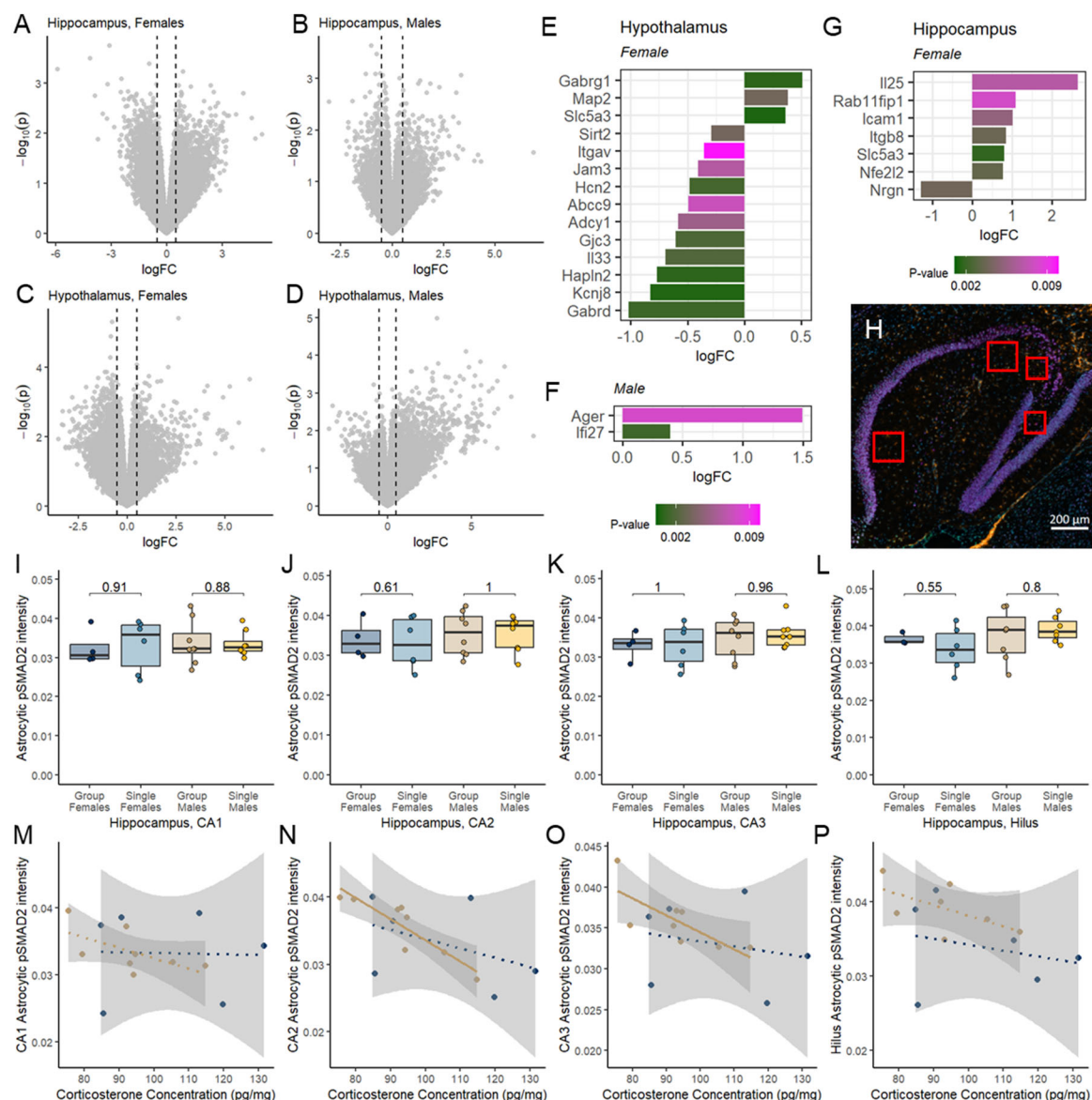


Figure 2.3: (A-D) We did not identify any differentially expressed (FDR < 0.05) genes at a bulk transcriptomic level between group and single-housed mice. A list of 444 genes associated with blood-brain-barrier structure and/or function were specifically examined using a p-value cutoff of 0.01. In the hypothalamus, females (E) 14 genes were significantly different between the housing conditions, three upregulated among single-housed mice relative to group-housed, and 11 downregulated. Two genes were upregulated in single-housed mice among males (F). Seven genes were differentially expressed in the hippocampus of females with six upregulated in single-housed mice relative to group-housed mice, with one downregulated (G). No blood-brain-barrier-related genes were differentially expressed between housing conditions in the hippocampus of males. Astrocytic pSMAD2 activation was examined in four regions of interest in the hippocampus (H). Housing conditions also did not significantly (Wilcoxon test) affect astrocytic pSMAD2 intensity in cornu Ammonis 1 (I), cornu Ammonis 2 (J), cornu Ammonis 3 (K), or the Hilus (L). Correlation between corticosterone concentration and astrocytic pSMAD2 intensity was examined among single housed mice (M-P). Males color coded as yellow and females as blue. Solid lines denote statistical significance ($p < 0.05$). A significant negative correlation was found among single housed males between corticosterone concentration and astrocytic pSMAD2 intensity in cornu Ammonis 2 ($p = 0.003$) and cornu Ammonis 3 ($p = 0.030$). No significant correlation was found in males or females in the cornu Ammonis 1 (K) or Hilus (N).

ASTROCYTIC TGFB ACTIVATION

Given evidence of blood-brain-barrier and associated neuroinflammatory changes in response to stress, we examined a marker of TGF β activation, pSMAD2, in astrocytes. We did not identify any statistically significant (Wilcoxon test, $p < 0.05$) differences between housing conditions in males or females in the intensity of pSMAD2 in astrocytes in cornu Ammonis 1, cornu Ammonis 2, cornu Ammonis 3, or the Hilus (Figure 2.3G-J). When we examined the correlation between astrocytic pSMAD2 intensity and corticosterone concentrations in single-housed mice, we identified a significant negative correlation in males' cornu Ammonis 2 and cornu Ammonis 3. These correlations were not apparent in females in any hippocampal subfield examined or cornu Ammonis 1 or the Hilus in males.

SEX-SPECIFIC STRESS EFFECTS IN THE KIDNEY

Bulk transcriptomic analysis of the kidney revealed 1450 differentially expressed genes in females between group- and single-housed conditions, but only ten differentially expressed genes in males (Figure 2.4). Of the differentially expressed male genes, only one, *Lars2*, is protein-coding. Two, *Rn18s* and *Rn7sk*, are ribosomal proteins, and one, *Mir6236*, is a nuclear microRNA. The remaining six genes are all pseudogenes. Gene set enrichment of the differentially expressed genes in females examining the Hallmark pathways identified 20 gene sets significantly enriched in our dataset ranging from metabolic pathways to DNA repair mechanisms and hormone signaling. We next examined counts of the number of glomeruli in each kidney. There was a statistically significant difference in the number of glomeruli between group- and single-housed females but no difference between housing conditions in males. These differences did not correlate with corticosterone concentrations.

DIFFERENCES IN CIRCADIAN RHYTHM GENES IN THE LIVER

mRNA extracted from the liver was examined using differential expression analysis, broken down by males and females (Figure 2.5). No differentially expressed genes were detected in males, but ten were detected in females. Of those ten, two were transcriptional regulators – *Per3* and *Dbp*. Two, *A930033H14Rik* and *Gm26684* were predicted genes. Because of the identification of circadian genes differing between groups, we investigated the possibility that the time of day of euthanasia (specifically, the recorded time the brain was extracted from the skull) could explain the result. There was a statistically significant correlation between *Per3* expression in the liver in both males and females. *Dbp* correlated with euthanasia time only in females. Because circadian rhythms can affect other genes' expression, we also examined the other eight hits. Except for the pseudogene *Gm26684*, all differentially expressed genes correlated with euthanasia time, at least in females.

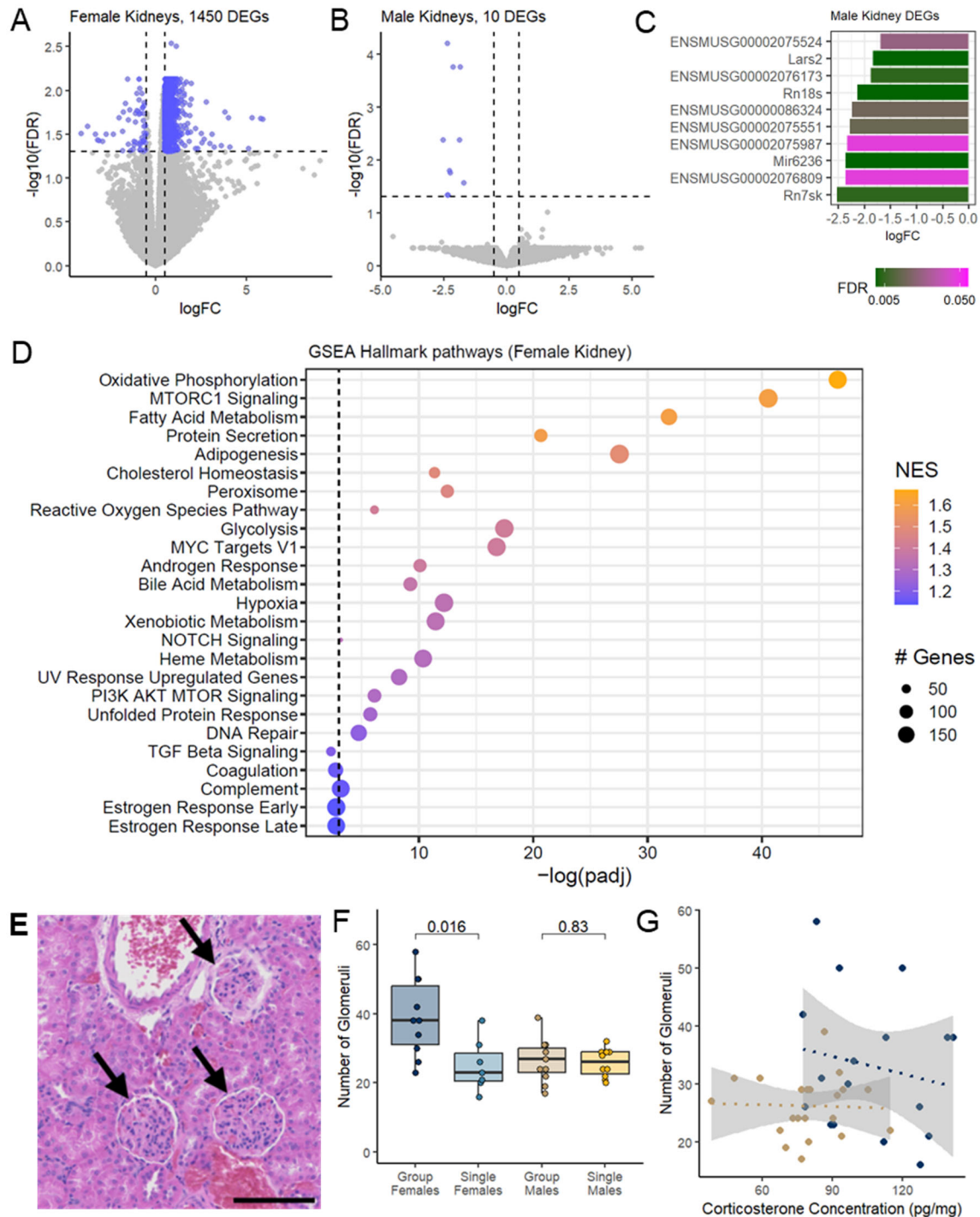


Figure 2.4: Differential expression analysis of bulk RNA extracted from kidneys identified 1393 genes that were upregulated and 57 genes that were downregulated ($\text{FDR} < 0.05$) in single-housed female mice relative to group-housed female mice (A). In males, there were only 10 downregulated genes (B-C). Among the 1450 DEGs, 20 Hallmark pathways were found to be enriched ($p\text{-adj} > 0.05$) (D). Glomeruli (E) were counted in each kidney and significant differences (Wilcoxon $p < 0.05$) were identified between housing conditions in females, but not males (F). Corticosterone concentration did not correlate with the number of glomeruli in males (gold) or females (blue) (G).

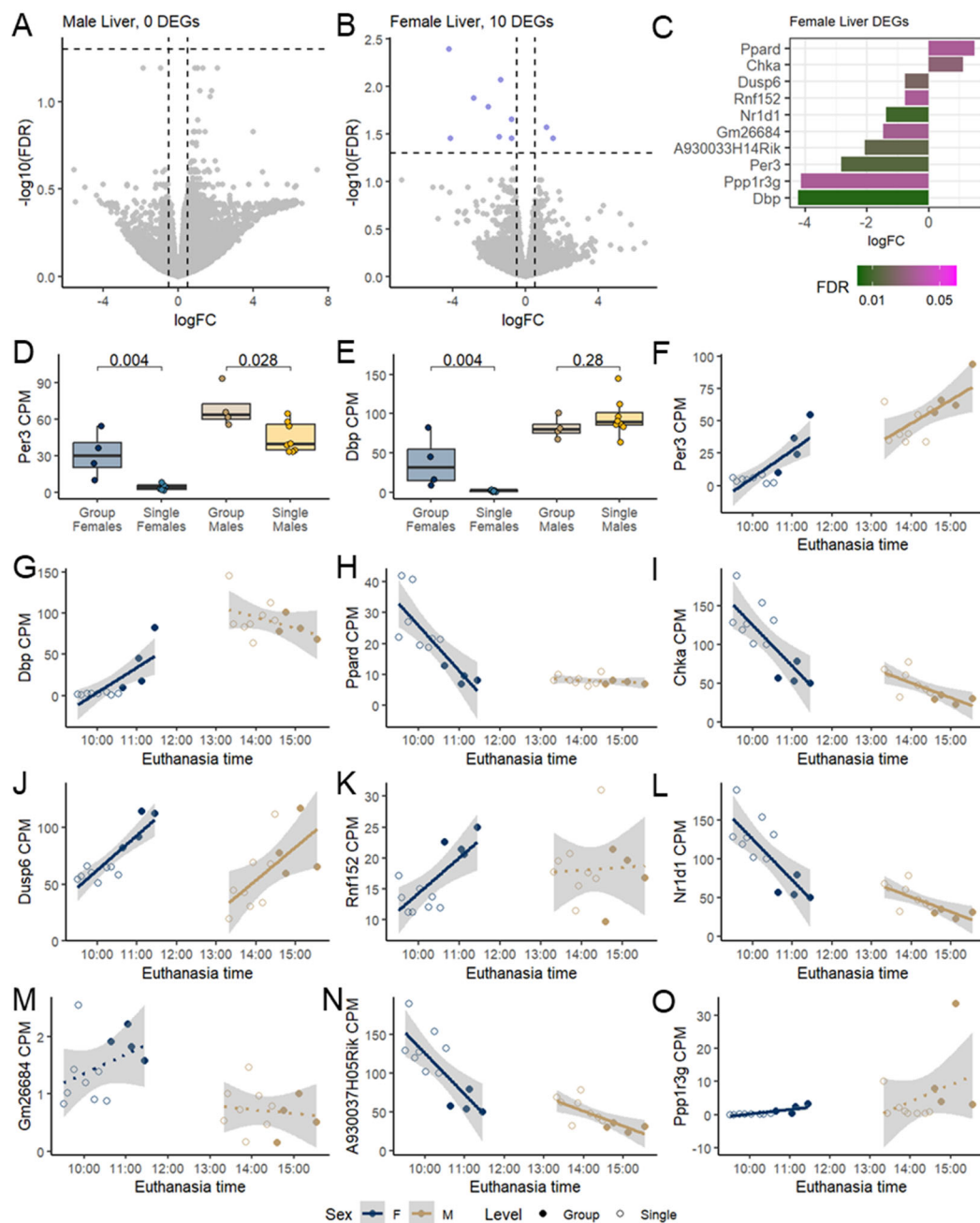


Figure 2.5: (A) No differentially expressed genes were identified in the liver of males, but 10 (B) were identified in females. Two were upregulated in single-housed mice ($\text{FDR} < 0.05$; $\log_{2}(\text{FC}) > 0.5$) and eight were downregulated ($\text{FDR} < 0.05$; $\log_{2}(\text{FC}) < -0.5$) (C). Two of these genes, *Per3* (D) and *Dbp* (E) are circadian regulators so we sought to examine if there was an association between time of euthanasia and their levels. *Per3* (F) tightly correlates with time of day in females ($r=0.809$; $p=0.001$) and males ($r=0.683$; $p=0.014$). *Dbp* (G) correlated with time of day in females ($r=0.770$; $p=0.003$) but not males ($r=-0.423$; $p=0.170$). The other differentially expressed genes were analyzed (I-O) to examine possible associations between their liver expression and time of day, stratified by sex. Solid lines indicate a statistically significant ($p < 0.05$) correlation, and dotted lines indicate that the correlation failed to reach statistical significance.

Discussion

Here, a group of C57BL/6 mice were aged in single- or group-housing conditions to investigate the effects of social isolation stress on age-related health outcomes. Mice were sacrificed at an age when markers of senescence become consistently present, but survival remains high (Flurkey et al., 2007). We sampled several tissues to appreciate the organism-wide consequences of persistent single-housing.

Examining the stress hormone corticosterone from hair samples revealed statistically significant differences between housing conditions in males, not females. Group-housed males have lower levels of corticosterone than single-housed males. Hair corticosterone levels have been proposed to represent an average of corticosterone levels over periods lasting weeks to months in rodents; however, tests of this method suggest that it represents hours- to days-long time scales (Colding-Jorgensen et al., 2023). Nevertheless, it is robust against transient spikes such as could be found during handling for euthanasia. The difference in housing conditions among males could reflect increased HPA-axis activation by persistent single-housing. Persistent HPA-axis activation is predictive of depression (Karten et al., 1999; van Donkelaar et al., 2014), but we did not collect any data regarding depression-like behaviors in our rodents. Alternatively, our data could suggest that the repeated social defeat group-housed male mice can be subject to (Kappel et al., 2017) may blunt HPA-axis activation after extended periods. If this were the case, though, we would expect a positive correlation between males' corticosterone levels and body mass. We do observe significant differences in corticosterone levels based on the time of euthanasia. The changes we see, however, are opposite to the increase in corticosterone occurring in the afternoon in mice (Gong et al., 2015). Considering this and the hair's resistance to transient spikes, we conclude that the association with euthanasia time is driven by the range of time each study group was euthanized. Taken together, we conclude that single-housed males, but not females, have significantly more corticosterone levels than group-housed males.

We found no differentially expressed genes at the initial cutoffs for our bulk transcriptomic analysis of the hypothalamus and hippocampus (Welcome & Mastorakis, 2020). Given known associations between stress and blood-brain-barrier dynamics, we sought to examine a curated set of 444 genes with known roles in blood-brain-barrier structure and function. Using a more liberal threshold than the bulk transcriptomic analysis in the hypothalamus RNAseq dataset, 14 genes were differentially expressed in females and 2 in males. Seven blood-brain-barrier genes were differentially expressed in the hippocampus in females but none in males. Only one gene, *Slc5a3*, was differentially expressed in the hippocampus and hypothalamus.

In the hippocampus, we histologically examined the effects on blood-brain-barrier dysfunction and astrocytic pSMAD2 signaling. There were no statistically significant differences between housing conditions in the astrocytic intensity of pSMAD2. In single-housed males, we detected a significant negative correlation between hair corticosterone concentration and pSMAD2 intensity in cornu Ammonis 2 and cornu

Ammonis 3. There are two main explanations for this finding. First, increased corticosterone may inhibit astrocytic TGF β activation, decreasing pSMAD2 levels. Second, variation in blood-brain-barrier dysfunction in the hippocampus as the mice aged may affect its negative regulation of stress-induced HPA-axis activation (Herman et al., 2005).

Examination of peripheral tissues, specifically the kidney and liver, suggest increased health effects of housing conditions on females rather than males. In females, 1450 differentially expressed genes were found between housing conditions in the kidney, whereas only ten were found in males. Of the ten found in males, only one was protein-coding. GSEA of Hallmark pathways for the differentially expressed genes in the female kidneys feature numerous enriched pathways. Redox-related biology, oxidative phosphorylation, fatty acid metabolism, and cellular repair pathways were all featured. A histological readout of kidney function, the number of glomeruli, featured a significant difference between housing conditions in females but not in males. Curiously, mice from both housing conditions in males had similar numbers of glomeruli to the single-housed females. This finding could mean that glomeruli numbers were unaffected by housing status in males and that males have, at baseline, lower numbers of glomeruli. This possibility is supported by early histological work on renal structure in mice, which found that males have fewer glomeruli (Messow et al., 1980). Another possibility is that male mice in both housing conditions experienced stressors associated with decreased glomeruli numbers.

Analysis of the liver found only differentially expressed genes in females but not males. Among the genes found to be differentially expressed between housing conditions in females, two were circadian regulators. We sought to examine if this could have been affected by the time of necropsy. There is evidence of an effect by the time of day on the gene expression of *Per3* and *Dbp*. Because circadian signaling can affect other genes' expression, we examined the other eight genes differentially expressed in the liver. All but one showed significant correlations in females, and four showed significant correlations with males. Because mouse groups were euthanized in a particular order (e.g., single-housed females, then group-housed females), the finding of differential expression is also reflected by statistically significant correlations with euthanasia time. Due to this, we cannot draw conclusions about the effect of housing condition on the expression of these genes versus the euthanasia time.

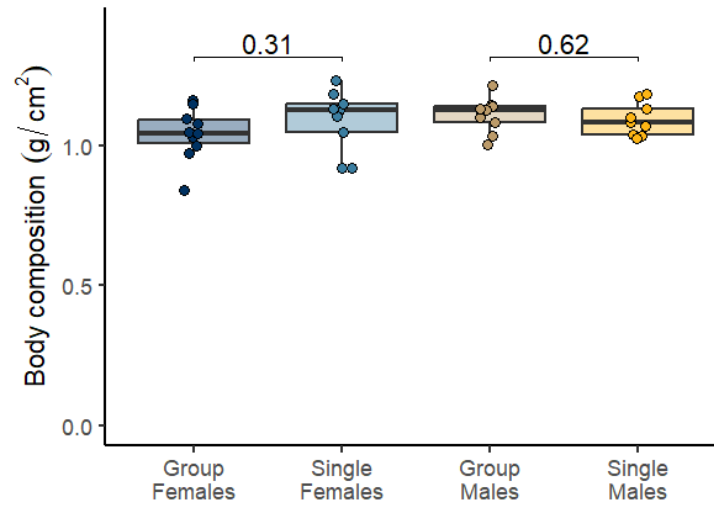
Our study is limited in several ways. The cross-sectional nature of the analysis and lack of behavioral data limits our ability to draw certain conclusions. Our conclusions are purely correlational because we only manipulated housing conditions and none of the underlying biological processes we were evaluating. Notably, there are challenges brought on by group-housing males over a study as long as ours that are difficult to account for. Specifically, within-cage social dynamics can create comparable stressors to single-housing for males (Kappel et al., 2017). As such, the control group for the males is lackluster. Finally, using bulk transcriptomics instead of single-cell or spatial transcriptomics hampers our ability to investigate complex tissues like the brain. Slight

regional variation in sampling can introduce significant variation that can be difficult to deconvolute, particularly for regions like the hypothalamus.

Aside from evidence of decreased renal capacity in females, we cannot conclude that long-term (greater than one year) single housing significantly affects adverse age-related health outcomes. This question has mixed literature, largely from studies with shorter durations. Some have found that single housing can affect memory, affect, thermal regulation, immune function, and metabolism (Bartolomucci et al., 2003; Hebda-Bauer et al., 2019; Kalliokoski et al., 2014; Kuleshkaya et al., 2011; N. Liu et al., 2020; Schipper et al., 2018; Skop et al., 2021; Voikar et al., 2005), while others have found no effect. Policies for the use of laboratory animals, as provided by The Guide in the United States (Institute of Laboratory Animal Resources (U.S.). Committee on Care and Use of Laboratory Animals.), specify that as a social species, mice are to be group-housed unless otherwise justified. Our data here suggest that, while social, male mice may fare similarly well in both housing conditions. This finding aligns with other literature suggesting that male mice can readily adapt to single-housing conditions (Melotti et al., 2019). Critically, these findings should not be applied directly to humans. Numerous lines of evidence describe adverse outcomes of social isolation and loneliness in humans (Courtin & Knapp, 2017; Crystal et al., 2022; Donovan et al., 2017; Hämmig, 2019; Holt-Lunstad & Steptoe, 2021; Holt-Lunstad et al., 2015; Leigh-Hunt et al., 2017; Loades et al., 2020; Mehrabi & Béland, 2020; Sepúlveda-Loyola et al., 2020; Timothy et al., 2021). Given this, we conclude that mice may not be a suitable model to capture the complexity of human social isolation and loneliness and facilitate a better mechanistic understanding of their adverse health outcomes.

Human postmortem tissue can serve as an additional resource to probe the pathophysiology of age-related diseases. At present, there lack well-characterized cohorts of brains that have been deeply characterized for psychosocial phenotypes and, thus, could be used to study the effects of social isolation. Instead, in chapter 3, we turn to a focus on neurodegenerative disease pathophysiology.

Supplementary Figures



Supplementary Figure 2.1: To investigate if body composition, rather than weight, associated with housing condition, the body mass was divided by the average of the cross-sectional area taken from the dorsal and ventral photos of the mice. In this analysis, one group-housed female, three group-housed males, and one single-housed females were excluded due to the absence of photos. No statistically significant differences were detected.

Chapter 3: Patterns of neuronal Rhes as a novel hallmark of tauopathies

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Abstract

The farnesyltransferase inhibitor, Lonafarnib, reduces tau inclusions and associated atrophy in familial tauopathy models through activation of autophagy, mediated by the inhibition of farnesylation of the Ras GTPase, Rhes. While hinting at a role of Rhes in tau aggregation, it is unclear how translatable these results are for sporadic forms of tauopathy. We examined histological slides of allocortex and neocortex from multiple postmortem cases in five different tauopathies, frontotemporal lobar degeneration-TDP, and healthy controls using immunofluorescence for Rhes, several tau post-translational modifications, and phospho-TDP-43. Single nucleus RNA data suggest that Rhes is found in all cortical neuron subpopulations but not in glia. Histologic investigation showed that nearly all neurons in control brains display a pattern of diffuse cytoplasmic Rhes positivity. However, in the presence of abnormal tau, but not abnormal TDP-43, the patterns of neuronal cytoplasmic Rhes tend to present as either punctiform or entirely absent. This observation reinforces the relevance of findings that link Rhes changes and tau pathology from the *in vivo* and *in vitro* models of tauopathy. The results here support a potential clinical application of Lonafarnib to tauopathies.

Introduction

Despite being the most frequent underlying cause of dementia, tauopathies remain without effective treatments and are increasing in prevalence (Hebert et al., 2013; Onyike & Diehl-Schmid, 2013). Tauopathies encompass many distinct neuropathological entities that feature the accumulation of abnormally folded tau protein followed by associated neuron and synapse loss. Alzheimer's disease is the most common tauopathy and features both 3- and 4-repeat tau inclusions. Among the sporadic tauopathies, progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain disease show 4-repeat tau inclusions predominantly while Pick's disease features 3-repeat tau inclusions. Although the overwhelming majority of tauopathies occur sporadically, more than 50 mutations in *MAPT* lead to abnormal tau accumulation and different expression patterns of frontotemporal lobar degeneration (FTLD) in an autosomal dominant pattern of inheritance (Munoz et al., 2003; Tacik et al., 2016; Takada, 2015). These mutations are the basis for biomedical research in tauopathies, as they can be incorporated into transgenic cell lines or *in vivo* systems that model tauopathies; however, it is unclear how generalizable findings from these familial models are for the more common sporadic tauopathies.

Immunotherapies have been the most common class of drugs brought to clinical trials for tauopathies; however, they have, thus far, produced lackluster results (Hochgrafe et al., 2015; VandeVrede et al.). Other approaches have attempted to modulate post-translational modifications of tau (Mead et al., 2016; Min et al., 2015; VandeVrede et al.; Zhang et al., 2013) or reduce *MAPT* mRNA expression using antisense oligonucleotides (DeVos et al., 2017). The well-documented dysfunction in autophagy across tauopathies (Caballero et al.; Theofilas et al.) establishes autophagy modulation as an attractive strategy to enhance clearance of aggregated tau across the broad spectrum of tauopathies (Caballero et al., 2018; Lopez et al., 2017). These efforts have shown some promise in preclinical settings and may become effective treatment strategies for tauopathies.

Farnesyltransferase inhibitors were initially developed for cancer treatment by acting on farnesyl protein transferase, a necessary protein CaaX farnesylation catalyst, to inhibit Ras oncoproteins (Kohl et al., 1993). A pharmacological class effect of farnesyltransferase inhibitors is enhanced autophagy (Pan et al., 2009). The farnesyltransferase inhibitor, Lonafarnib, was successfully used *in vitro* and in mouse models of familial tauopathy to reduce the burden of tau inclusions by enhancing autophagic activity (Hernandez et al., 2019). Specifically, Lonafarnib acts through the Ras guanosine triphosphatase, Ras homolog enriched in striatum (Rhes) to activate autophagic degradation of tau aggregates. Rhes may suppress autophagy by binding to and activating the mammalian target of rapamycin (mTOR) (Subramaniam et al., 2011). Through sequestration of Beclin-1 from inhibitory binding to Bcl-2, Rhes can also activate autophagy independent of mTOR (Mealer et al., 2014). When farnesylation is blocked by an inhibitor such as Lonafarnib, Rhes dissociates from membranes, allowing it to sequester Beclin-1 and upregulate autophagy (Hernandez et al., 2019).

Although promising, these model-based observations' translatability to treating tauopathies using a farnesyltransferase inhibitor, particularly in the case of sporadic tauopathies, is unclear. Furthermore, little is known about the pattern of Rhes expression in human neurons, particularly outside the striatum and in the context of neurodegenerative conditions.

Rhes is encoded by the gene *RASD2* and was thought to be predominantly expressed in the striatum (Hernandez et al., 2019; Vitucci et al., 2016). Some studies suggest that Rhes expression in specific neuronal subpopulations is the basis of selective vulnerability to Huntington's disease, a genetic neurodegenerative condition causing profound striatal and mild cortical neuronal loss (Heinsen et al.). However, tauopathies show only mild striatal involvement, with significant involvement of cortical and other subcortical structures. The evidence for Rhes expression in human cortical neurons is scant with just one study, to our knowledge, showing scattered expression of *RASD2* mRNA throughout the cortices of humans and mouse models using autoradiography (Vitucci et al., 2016).

An in-depth understanding of Rhes expression, localization within neurons, and disease-related changes in cortical regions vulnerable to tauopathies, if any, is a necessary step to inform whether LonaFarnib should be explored as a treatment for tauopathies. To fill this gap, we used multiplex immunofluorescence and single nucleus RNA sequencing (snRNA-seq) in human brain tissue from healthy controls and a broad range of sporadic tauopathies to characterize and measure phenotype changes in Rhes and its relationship to the accumulation of tau aggregates.

Materials and Methods

PARTICIPANTS AND NEUROPATHOLOGICAL ASSESSMENT

Cases were sourced from the Neurodegenerative Disease Brain Bank at the University of California, San Francisco Memory and Aging Center. Consent for brain donation was obtained from subjects or next of kin following a protocol approved by the Institutional Review Board of the University of California, San Francisco.

Upon autopsy, the brains were slabbed fresh into coronal slabs and fixed in 4% neutral buffered formalin or frozen within 24 hours of death. Slabs undergoing formalin fixation were transferred to phosphate buffered saline-azide after 72-hours of fixation and stored at 4°C. A standardized set of 26 tissue blocks from neurodegenerative regions of interest are dissected from these slabs and embedded into paraffin blocks.

Immunohistochemical and basic histological stains were used to assess neurodegenerative lesions for diagnoses using antibodies for phospho-Ser202 Tau (CP13, Mouse, 1:250, courtesy of Peter Davies), TDP-43 (Rabbit, 1:2000, 10782-2-AP, Proteintech, Chicago, IL), amyloid- β (Mouse, 1:500, MAB5206, Millipore, Billerica, MA), and α -Synuclein (Mouse, 1:5000, LB509, courtesy of J. Trojanowski and V. Lee). A final neuropathologic diagnosis was obtained after consensus (LTG, SS, and WWS) using

currently accepted guidelines (Mackenzie, Neumann, Baborie, et al., 2011; McKeith et al., 2005; Montine et al., 2012).

Table 3.1: Demographics and case characteristics for analysis of tauopathies.

Diagnosis	n	Age (mean, sd)	Female (n, %)	PMI (mean, sd)	CDR (median, IQR)	ABC Score
HC ^a	5	78, 10	5, 100%	8.2, 2.4	0, 0	A0B1C0 (1) A1B1C0 (2) A1B1C1 (1) A2B0C0 (1)
AD	5	66, 5	2, 40%	8.6, 0.9	3, 0	A3B3C3 (5)
AGD	5	84, 12	1, 20%	8.7, 4.1	0.5, 0	A?B1C1 (1) A?B2C1 (1) A0B2C0 (1) A1B2C0 (2)
CBD	5	66, 4	2, 40%	9.3, 2.7	1, 2.5	A1B1C0 (5)
PiD	5	68, 8	2, 40%	10.2, 3.9	3, 0	A0B0C0 (1) A1B0C0 (1) A1B1C0 (2) A1B0C1 (1)
PSP	5	76, 10	3, 60%	11.1, 4.9	0.5, 0	A0B0C0 (1) A0B1C0 (3) A1B1C0 (1)
Total	30	73, 10	15, 50%	9.3, 3.3	0.75, 2.5	A0B0C0 (2) A0B1C0 (4) A0B2C0 (1) A1B0C0 (1) A1B0C1 (1) A1B1C0 (10) A1B1C1 (1) A1B2C0 (2) A2B0C0 (1) A3B3C3 (5) A?B1C1 (1) A?B2C1 (1)

^aOne of the HC cases had inclusions of α -synuclein in the form of Lewy bodies in the brain stem; however, none noted in the cortex.

Abbreviations: HC, Healthy Controls; AD, Alzheimer's disease; AGD, Argyrophilic Grain Disease; CBD, Corticobasal Degeneration; PiD, Pick's disease; PSP, Progressive Supranuclear Palsy; PMI, Postmortem Interval; CDR, Clinical Dementia Rating

For the immunohistochemical component of this study, we selected 37 cases encompassing five healthy control cases, 25 sporadic tauopathy (Alzheimer's disease, progressive supranuclear palsy, corticobasal degeneration, Pick's disease, and argyrophilic grain disease) cases, and seven cases of frontotemporal lobar degeneration with TDP-43 inclusions (frontotemporal lobar degeneration-TDP Type A and Type B). Half of the cases for the tauopathy analysis were female, albeit with an

uneven distribution of sexes between groups, with a mean (sd) age of death of 73 (10) years (Table 3.1).

A majority (71%) of cases for the TDP-43 analysis were female, with a mean (sd) age of death of 11.8 (7.4) years (Table 3.2). Inclusion criteria included: lack of more than one primary or contributing neuropathological diagnosis, lack of an Axis I psychiatric disorder, lack of non-dementia neurological disorder, lack of gross non-degenerative structural neuropathology, and a postmortem interval below 24 hours. Healthy control cases were free of any clinical record of cognitive decline, neurological, or a non-incidental neuropathological diagnosis.

Table 3.2: Demographics and case characteristics for analysis of TDP-43.

Diagnosis	n	Age (mean, sd)	Female (n, %)	PMI (mean, sd)	Clinical syndrome	ABC Score
FTLD-TDP Type A	3	63, 3.6	3, 100%	12.4, 4.1	CBS (2) bvFTD (1)	A0B0C0 (1) A1B1C0 (1) A1B1C1 (1)
FTLD-TDP Type B	4	59, 3.7	2, 50%	11.5, 9.8	bvFTD (2) bvFTD-ALS (1) nfvPPA-ALS (1)	A0B0C0 (3) A0B1C0 (1)
Total	7	60, 4	5, 71%	11.8, 7.4	bvFTD (3) CBS (2) bvFTD-ALS (1) nfvPPA-ALS (1)	A0B0C0 (4) A0B1C0 (1) A1B1C0 (1) A1B1C1 (1)

Abbreviations: FTLD, Frontotemporal lobar degeneration; TDP, TAR DNA-binding protein; PMI, Postmortem Interval; CBS, Corticobasal syndrome; bvFTD, Behavioral variant frontotemporal dementia; ALS, Amyotrophic Lateral Sclerosis; nfvPPA, non-fluent variant primary progressive aphasia

TISSUE PROCESSING, IMMUNOHISTOCHEMISTRY, AND MICROSCOPY

Analysis of Rhes cytoplasmic neuronal distribution in healthy controls and tauopathies

Thirty cases encompassing healthy controls and tauopathies were used in this analysis. The paraffin block containing the hippocampal formation at the level of the lateral genicular body was sectioned at 8 μ m for all 30 cases. The paraffin block containing the superior frontal gyrus was also sectioned at 8 μ m for all cases, excluding the five argyrophilic grain disease cases (as argyrophilic grain disease lacks inclusions in the superior frontal gyrus) for a final n=25 sections. Multiplex immunofluorescence staining included antibodies for phospho-Ser396/404 tau (PHF-1), Rhes, and NeuN (a pan-neuronal marker). Supplementary Methods 3.1 describe the staining protocol in detail.

To establish criteria for analyzing Rhes in cortical neurons, we qualitatively examined the cytoplasmic neuronal distribution of Rhes signal. Based on consensus (AJE and LTG), we defined three different intraneuronal Rhes distribution patterns: Diffuse, Punctiform, and Absent. To further understand the intraneuronal location of Rhes, we used multiplex immunofluorescence against MAP2, Rhes, and NeuN (See Supplementary Methods 3.2) in sections cut from the hippocampal formation cut at the

level of the genicular body from two tauopathy cases (one 64-year-old female with Alzheimer's disease and one 69-year-old female with Pick's disease). Next, we quantified digital images of our sections in ImageJ, classifying and counting neurons based on the pattern of Rhes cytoplasmic distribution and the presence of PHF-1 tau inclusions. Methodological details are described in Supplementary Methods 3.1.

Analysis of Rhes cytoplasmic neuronal distribution in frontotemporal lobar degeneration-TDP

We selected cortical areas representing regions with a moderate-to-severe pathological burden in frontotemporal lobar degeneration-TDP. Paraffin blocks representing the inferior frontal gyrus (IFG) were cut at 8 μ m from three frontotemporal lobar degeneration-TDP Type A cases. Similarly, we cut sections from paraffin blocks representing the middle frontal gyrus of four frontotemporal lobar degeneration-TDP Type B cases. Multiplex immunofluorescence staining included antibodies against phospho-TDP-43, Rhes, and NeuN (see Supplementary Methods 3.3). Next, neurons were quantified based on the Rhes pattern and the presence of phospho-TDP-43 inclusions (see Supplementary Methods 3.3). We did not include frontotemporal lobar degeneration-TDP Type C cases because this subtype rarely shows cytoplasmic neuronal inclusions in neocortical areas.

Analysis of the association between Rhes neuronal patterns association and tau post-translational modifications

To examine if the association between tau inclusions and changes in Rhes neuronal patterns vary among inclusions featuring different post-translational modifications, in addition to the analysis using PHF-1 labeled phospho-tau, we sectioned paraffin blocks containing the hippocampal formation at the level of the lateral genicular body from two Alzheimer's disease cases (one 59-year-old male, one 64-year-old female). These sections were immunostained with antibodies against Rhes, NeuN, and a variety of abnormal tau forms: AT100 (phospho-Thr212/Ser214 tau), MC1 (conformational tau changes), 1f3c (acetyl-Lys274 tau), and Tau-C3 (Δ Asp421 Tau). As in previous analyses, neurons were quantified based on Rhes subtype and the presence of tau inclusions. Supplementary Method 3.4 describe the staining, imaging, and quantification protocols in detail.

CELL TYPE SPECIFICITY FOR RHES

Rhes has been extensively described in human striatal neurons, but its presence in other neuron groups or glia cells has been less explored. To close this gap, we first analyzed snRNA-seq data generated from an additional cohort of ten postmortem brain samples representing HC and individuals across the neuropathological spectrum of Alzheimer's disease, initially published in Leng et al. (2021). This dataset is also available on Synapse (ID: syn21788402). This dataset represents single-cell gene expression information and metadata from the entorhinal cortex, labeled as "EC_allcells.rds", and superior frontal gyrus (Brodmann area 8), labeled as "SFG_allcells.rds". Although the dataset includes different cases than those used in our immunohistochemical analyses, the brain regions examined are similar. The log-scaled CPM of *RASD2* transcripts in cells from the cell type subpopulations identified in the

entorhinal cortex and superior frontal gyrus of Braak stage 0 cases were used to assess expression patterns. Next, the mean log CPM of *RASD2* transcripts within groups 2 and 4 of excitatory entorhinal cortex neurons (Leng et al., 2021) was taken for each case (n=10) and compared between Braak stages 0, II, and VI (Braak & Braak, 1991) using a one-way ANOVA. These findings were validated in independent datasets containing other brain areas using an online tool (brainrnaseq.org) (Zhang et al., 2016).

Cell-type specificity for Rhes was further studied histologically, focusing on neurons and astrocytes. To illustrate the lack of Rhes in astrocytes, we immunostained the hippocampal formation cut at the level of the genicular body of an 82-year-old HC female. Supplementary Methods 3.5 describes this staining protocol in detail.

STATISTICAL ANALYSIS

To examine differences in the relative proportions of neuron populations belonging to each phenotype between diagnoses, the proportion of neurons belonging to each Rhes pattern was computed as the number of neurons featuring each of the three phenotypes divided by the total number of neurons counted per case. Wilcoxon-Mann-Whitney tests were used to test the null hypothesis that no significant difference exists in the proportion of total neurons counted that present as Diffuse Rhes between healthy controls and each disease in pairwise comparisons.

The proportions of neurons positive and negative for PHF-1 inclusions per Rhes phenotype were computed for each region of interest (entorhinal cortex, Cornu Ammonis 1, and superior frontal gyrus) to test for differences in PHF-1 tau inclusion burden within each neuronal Rhes phenotype population. Kruskal-Wallis rank-sum tests were used to test the null hypothesis that there was no significant difference in the burden of PHF-1 tau inclusions between the three neuronal Rhes phenotypes in each disease. At a group-level, combining all cases, one-way ANOVA's were used to test the null hypothesis that there was no significant difference in the burden of PHF-1 tau inclusions between the three neuronal Rhes phenotypes in the entorhinal cortex, Cornu Ammonis 1, or superior frontal gyrus. For both Kruskal-Wallis rank-sum tests and one-way ANOVA's, cases without a computed proportion (i.e., those with a denominator of 0) were omitted from the analysis. We repeated the same analysis for the frontotemporal lobar degeneration-TDP cases, classifying inclusions based on labeling for phospho-TDP-43 instead of PHF-1 positive tau.

The analyses involving other post-translational modifications of tau did not involve statistical tests as they were exploratory. Instead, the percentage of neurons positive for each post-translational modification classified as each Rhes phenotype was computed and averaged between the two cases.

The alpha level for all analyses was set to 0.05. Statistical analysis was conducted in the software R, and graphs were produced with the R package ggplot2.

Results

RHES IS WIDESPREAD IN CORTICAL NEURONS OF HEALTHY CONTROLS, PRESENTING IN A DIFFUSE GRANULAR CYTOPLASMIC DISTRIBUTION

Several studies have suggested that Rhes expression is mostly limited to the striatum, thus the name Rhes. We detected Rhes signal in neurons throughout the allocortex (Figure 3.1a, Supplementary Figure 3.1) and one neocortical area using immunofluorescence. In healthy controls, we found Rhes signal in over 95% of entorhinal cortex and over 99% of cornu Ammonis 1 and superior frontal gyrus neurons. In these healthy controls, most neurons present Rhes as a cytoplasmic granular distribution extending into the dendrite (Figure 3.1b-i).

To corroborate the findings, we analyzed snRNA-seq data from the entorhinal cortex (an allocortical region) and superior frontal gyrus (a neocortical region) from a different cohort (Leng et al., 2021). Within the Braak stage 0 cases (n=3), we detected *RASD2* transcripts in all excitatory and inhibitory neuronal populations of these two regions (Figure 3.1j-k). Moreover, these results were validated in independent datasets using the online tool at brainrnaseq.org (Zhang et al., 2016) and immunohistologically (Supplementary Figure 3.2). Both neuropathological and snRNA-seq data indicate that glia lack Rhes (Figure 3.2, Supplementary Figure 3.2).

IN TAUOPATHIES, NEURONS PRESENT AS THREE PHENOTYPES OF RHES CYTOPLASMIC DISTRIBUTION

We qualitatively examined neurons in the entorhinal cortex, cornu Ammonis 1, and superior frontal gyrus of all 30 healthy control and tauopathy cases (Table 3.1) and defined three distinct phenotypes of neuronal cytoplasmic Rhes distribution. The 'Diffuse' type features granular Rhes staining in the cell body extending into the dendrite (Figure 2a). The 'Punctiform' type has large cytoplasmic Rhes+ puncta with a variable granular background, extending into the dendrite (Figure 3.2b). The 'Absent' type features an absence of Rhes staining in the cell body and variable positivity in the dendrite (Figure 3.2c).

To confirm that Rhes is expressed in the dendrites, histological slides of the human hippocampal formation from Alzheimer's disease and Pick's disease cases underwent multiplex immunostaining with Rhes, MAP2, and NeuN. We observed that Rhes overlaps with MAP2 signal in the neuronal processes of all three subtypes, indicating that it is present in dendrites (Figure 3.3, Supplementary Figure 3.3).

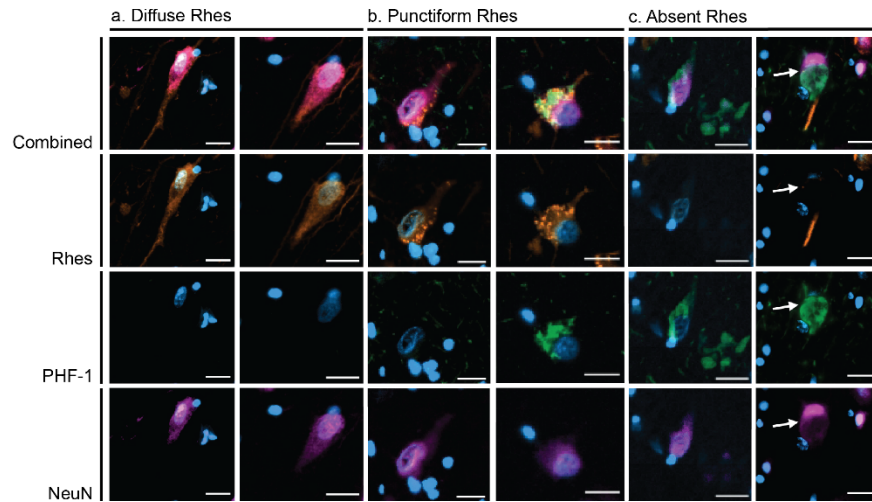


Figure 3.2: Diffuse Phenotype (orange) neurons (a) feature diffuse Rhes staining in the cell body extending into the apical dendrite. Punctiform phenotype neurons (c) feature punctiform Rhes signal in the cell body and often co-occur with PHF-1 (pink) positive tau inclusions. Absent phenotype neurons (c) feature clearance of Rhes from the cell body and often co-occur with PHF-1 positive tau inclusions. Blue, DAPI; Orange, Rhes; Green, PHF-1; Pink, NeuN. All scale bars correspond to 20 μ m.

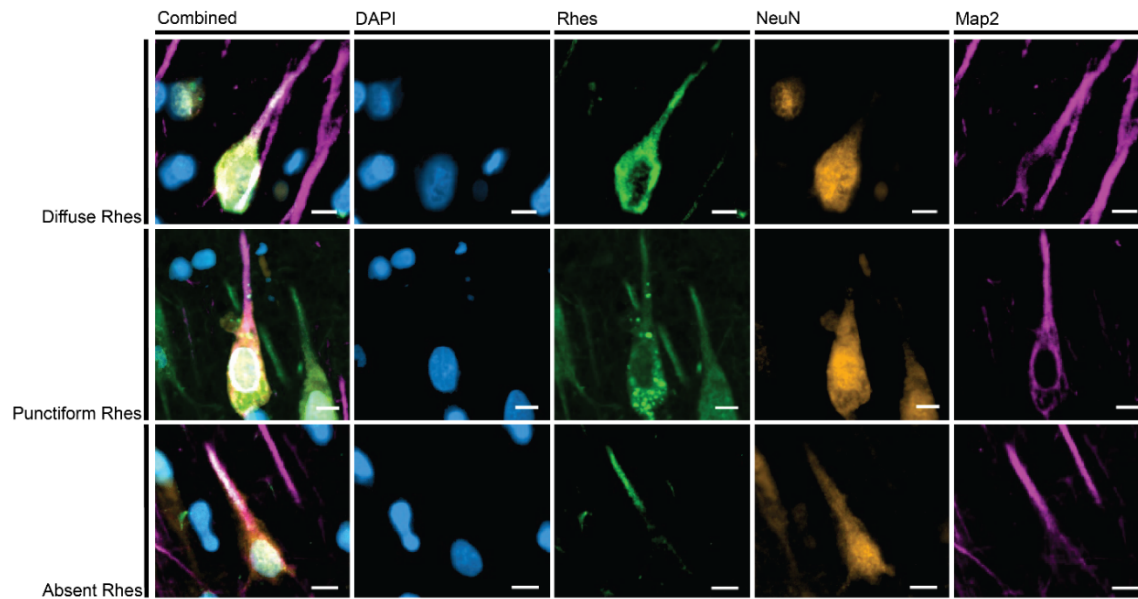


Figure 3.3: In all three Rhes Phenotypes, projections of Rhes (green) overlap with MAP2 (pink). This indicates that Rhes occurs in dendrites. Examples here from cornu Ammonis 1 of a 64-year-old female with Alzheimer's disease (A3B3C3). Scale bars correspond to 10 μ m.

THE RELATIVE ABUNDANCE OF RHES NEURONAL PHENOTYPES IN ALZHEIMER'S DISEASE AND OTHER TAUOPATHIES

In healthy controls, 94.4% of neurons in the entorhinal cortex, 97.6% of neurons in cornu Ammonis 1, and 99.8% of neurons in the superior frontal gyrus showed a diffuse Rhes phenotype, whereas tauopathy cases had a higher representation of Punctiform and Absent phenotypes (Figure 3.4). A one-way ANOVA showed that the proportion of neurons with a Diffuse phenotype was significantly different between each of the tauopathies and healthy controls in the entorhinal cortex ($p = 0.002$), cornu Ammonis 1 ($p < 0.001$), and the superior frontal gyrus ($p < 0.001$). This finding suggests that in tauopathies, intraneuronal Rhes assume a punctiform presentation or is cleared from the neuronal cytoplasm in affected neurons.

Alzheimer's disease and Pick's disease showed the highest proportion of neurons with Punctiform or Absent Rhes phenotypes (Figure 3.4a, c, d, Table 3.3). Notably, in argyrophilic grain disease, a four-repeat tauopathy featuring scarce to moderate numbers of cytoplasmic neuronal inclusions in the entorhinal cortex and cornu Ammonis 1, the proportion of neurons with diffuse Rhes phenotypes did not show a significant change compared to healthy controls. Similarly, corticobasal degeneration and progressive supranuclear palsy, 4-repeat tauopathies with mild neuronal pathology, showed no significant differences in the proportion of neurons with Diffuse Rhes phenotypes compared to healthy controls in the entorhinal cortex, cornu Ammonis 1, and superior frontal gyrus.

CHANGES IN RHES SUBTYPES CORRELATE WITH THE PRESENCE OF INTRANEURONAL TAU AGGREGATES

Seeing that there is a possible correlation between abnormal tau accumulation in neurons and Rhes cytoplasmic distribution changes, we examined how well the presence of PHF-1 positive tau inclusions associated with Rhes phenotype.

In slides immunostained for Rhes, PHF-1, and NeuN, we counted a total 26,357 neurons (8,121 in the entorhinal cortex, 5,636 in cornu Ammonis 1, and 12,600 in the superior frontal gyrus). Only a mean (sd) of 4.3% (0.7%) Diffuse Type neurons in the entorhinal cortex, 7.4% (17.1%) in cornu Ammonis 1, and 2.8% (3.8%) in the superior frontal gyrus were PHF-1 positive. Conversely, a mean (sd) of 91.9% (18.8%) entorhinal cortex, 93.6% (14.9%) cornu Ammonis 1, and 85.1% (35.7%) Absent Type superior frontal gyrus neurons harbored PHF-1 positive inclusions. Punctiform type neurons showed a more balanced distribution of PFH-1 positivity with a mean (sd) 72.6% (32.3%) in the entorhinal cortex, 84.1% (25.0%) in cornu Ammonis 1, and 56.7% (39.5%) in the superior frontal gyrus (Figure 3.5, Table 3.4).

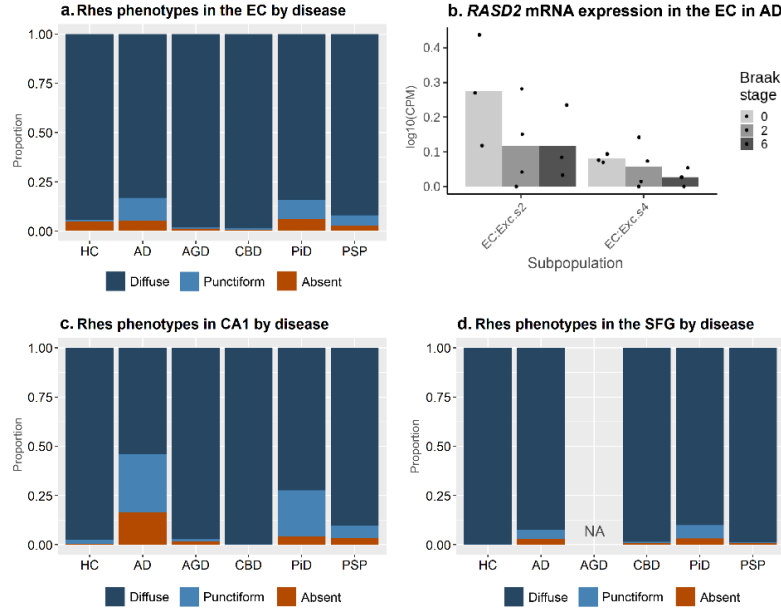


Figure 3.4: The relative proportions of Rhes phenotypes across tauopathies in the entorhinal cortex (A), cornu Ammonis 1 (C), and superior frontal gyrus (D). Diffuse Phenotype is the dominant phenotype, and its relative proportion decreases in tauopathies, most notably in Alzheimer's disease, Pick's disease, and progressive supranuclear palsy. Single-nucleus RNA expression data show a negative trend in the quantity of RASD2 with increasing Braak stages transcripts within two selectively vulnerable excitatory neuron populations, but the groups do not statistically significantly differ from one another (C).

Table 3.3: Proportion (mean, sd) of counted neuron population classified as each Rhes phenotype and with PHF-1 inclusions by region.

	Diagnosis	Diffuse Rhes	Punctiform Rhes	Absent Rhes	PHF-1 +
a) Entorhinal Cortex	Total (n=30)	0.918, 0.086	0.048, 0.062	0.034, 0.048	0.108, 0.12
	HC (n=5)	0.944, 0.103	0.008, 0.010	0.048, 0.094	0.061, 0.106
	AD (n=5)	0.832, 0.068	0.117, 0.061	0.051, 0.033	0.195, 0.075
	AGD (n=5)	0.983, 0.019	0.004, 0.006	0.013, 0.018	0.013, 0.017
	CBD (n=5)	0.984, 0.009	0.010, 0.011	0.006, 0.004	0.015, 0.006
	PiD (n=5)	0.843, 0.065	0.096, 0.053	0.061, 0.050	0.277, 0.107
	PSP (n=5)	0.920, 0.075	0.053, 0.079	0.027, 0.034	0.084, 0.071
b) Cornu Ammonis 1	Total (n=30)	0.852, 0.195	0.104, 0.136	0.044, 0.067	0.178, 0.24
	HC (n=5)	0.976, 0.029	0.020, 0.028	0.004, 0.006	0.027, 0.041
	AD (n=5)	0.540, 0.082	0.295, 0.077	0.165, 0.016	0.441, 0.089
	AGD (n=5)	0.972, 0.027	0.010, 0.007	0.017, 0.022	0.025, 0.026
	CBD (n=5)	0.998, 0.003	0.002, 0.003	0, 0	0.013, 0.013
	PiD (n=5)	0.723, 0.139	0.236, 0.112	0.040, 0.044	0.489, 0.287
	PSP (n=5)	0.904, 0.191	0.061, 0.114	0.035, 0.078	0.071, 0.112
c) Superior Frontal Gyrus	Total (n=25)	0.959, 0.05	0.025, 0.036	0.016, 0.018	0.061, 0.08
	HC (n=5)	0.998, 0.001	0.002, 0.002	0, 0.001	0, 0
	AD (n=5)	0.923, 0.056	0.046, 0.046	0.031, 0.02	0.118, 0.091
	CBD (n=5)	0.985, 0.009	0.008, 0.004	0.007, 0.005	0.022, 0.018
	PiD (n=5)	0.901, 0.046	0.068, 0.03	0.031, 0.019	0.148, 0.084
	PSP (n=5)	0.988, 0.005	0.003, 0.002	0.009, 0.007	0.016, 0.006

Abbreviations: HC, Healthy Controls; Alzheimer's disease, Alzheimer's disease; AGD, Argyrophilic Grain Disease; CBD, Corticobasal Degeneration; PiD, Pick's disease; PSP, Progressive Supranuclear Palsy

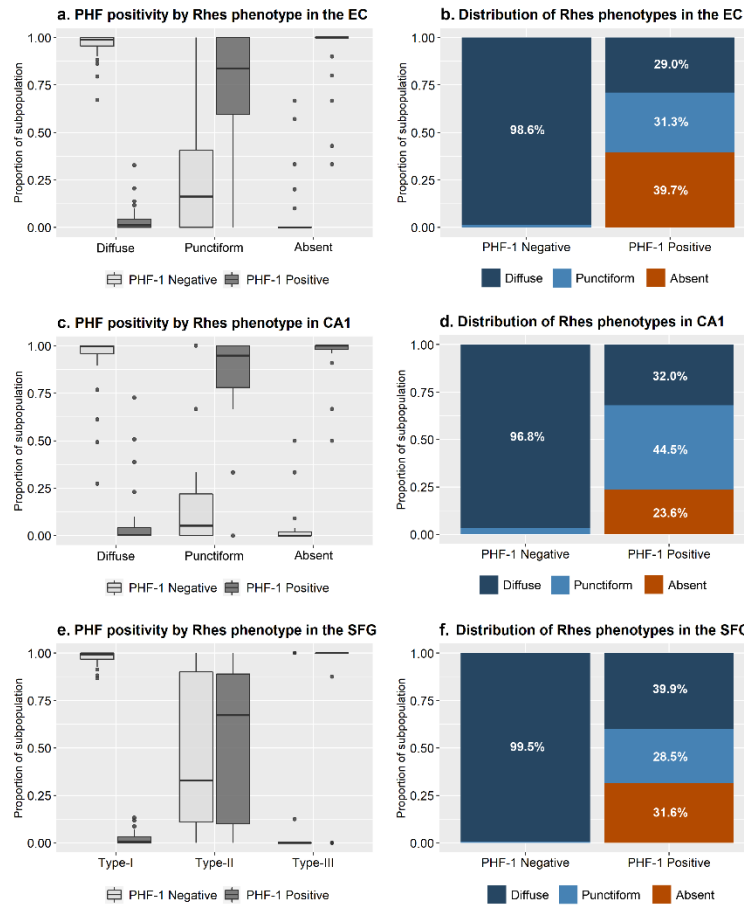


Figure 3.5: Entorhinal cortex neurons with Diffuse Rhes Phenotype are predominantly negative for p-tau (PHF-1), while neurons with Absent Rhes Phenotype are predominantly PHF-1 positive (a). While neurons with Punctiform Rhes Phenotype appear to be roughly split between PHF-1 positive and negative neurons in the entorhinal cortex, they make up roughly a third of the PHF-1 positive neurons, indicating a tendency to co-occur with tau inclusions (b). The majority of the PHF-1 positive neurons in the entorhinal cortex, either have Punctiform or Absent Rhes Phenotype. A similar correlation occurs in the cornu Ammonis 1 and the superior frontal gyrus (c-f).

The proportion of neurons with PHF-1 positive tau inclusions significantly differ between the three neuronal Rhes phenotypes ($p < 0.0001$ for EC, cornu Ammonis 1, and SFG). Kruskal-Wallis tests showed that this pattern is statistically significant ($p < 0.05$) in the entorhinal cortex for Alzheimer's disease, corticobasal degeneration, Pick's disease, and progressive supranuclear palsy individually; all tauopathies individually for cornu Ammonis 1; and Alzheimer's disease, Pick's disease, and progressive supranuclear palsy for the superior frontal gyrus. When not statistically significant for an individual tauopathy, the patterns generally followed the same trend.

Table 3.4: Proportions (mean, sd) of each Rhes phenotype with PHF-1+ tau inclusions by region.

		Diffuse Rhes		Punctiform Rhes		Absent Rhes		p-value ^a
	Dx	PHF-1+	PHF-1-	PHF-1+	PHF-1-	PHF-1+	PHF-1-	
a)	Total (n=30)	0.043, 0.073	0.957, 0.073	0.726, 0.323	0.274, 0.323	0.919, 0.188	0.081, 0.188	< 0.0001 ^b
Entorhinal cortex	HC (n=5)	0.01, 0.011	0.99, 0.011	0.556, 0.509	0.444, 0.509	0.889, 0.192	0.111, 0.192	0.0737
	AD (n=5)	0.074, 0.036	0.926, 0.036	0.688, 0.187	0.312, 0.187	0.96, 0.089	0.04, 0.089	0.0024
	AGD (n=5)	0.006, 0.009	0.994, 0.009	0.444, 0.509	0.556, 0.509	0.587, 0.361	0.413, 0.361	0.0693
	CBD (n=5)	0.004, 0.006	0.996, 0.006	0.675, 0.395	0.325, 0.395	1, 0	0, 0	0.0061
	PiD (n=5)	0.154, 0.118	0.846, 0.118	0.913, 0.092	0.087, 0.092	0.98, 0.045	0.02, 0.045	0.0049
	PSP (n=5)	0.008, 0.009	0.992, 0.009	0.927, 0.12	0.073, 0.12	1, 0	0, 0	0.0103
b)	Total (n=30)	0.074, 0.171	0.926, 0.171	0.841, 0.25	0.159, 0.25	0.936, 0.149	0.064, 0.149	< 0.0001 ^b
Cornu Ammonis 1	HC (n=5)	0.01, 0.018	0.99, 0.018	0.508, 0.45	0.492, 0.45	1, 0	0, 0	0.0586
	AD (n=5)	0.067, 0.102	0.933, 0.102	0.833, 0.078	0.167, 0.078	0.992, 0.017	0.008, 0.017	0.0016
	AGD (n=5)	0.001, 0.003	0.999, 0.003	0.917, 0.167	0.083, 0.167	0.833, 0.289	0.167, 0.289	0.0107
	CBD (n=5)	0.011, 0.013	0.989, 0.013	1, 0	0, 0	NA ^c	NA ^c	0.0486
	PiD (n=5)	0.345, 0.294	0.655, 0.294	0.965, 0.052	0.035, 0.052	0.894, 0.157	0.106, 0.157	0.0145
	PSP (n=5)	0.011, 0.018	0.989, 0.018	0.778, 0.385	0.222, 0.385	1, 0	0, 0	0.0392
c)	Total (n=25)	0.028, 0.038	0.972, 0.038	0.567, 0.395	0.433, 0.395	0.851, 0.357	0.149, 0.357	< 0.0001 ^b
Superior frontal gyrus	HC (n=5)	0, 0	1, 0	0, 0	1, 0	0, 0	1, 0	NA ^d
	AD (n=5)	0.056, 0.047	0.944, 0.047	0.764, 0.12	0.236, 0.12	0.8, 0.447	0.2, 0.447	0.0365
	CBD (n=5)	0.014, 0.013	0.986, 0.013	0.413, 0.433	0.587, 0.433	0.775, 0.437	0.225, 0.437	0.1765
	PiD (n=5)	0.065, 0.043	0.935, 0.043	0.816, 0.215	0.184, 0.215	1, 0	0, 0	0.0023
	PSP (n=5)	0.005, 0.003	0.995, 0.003	0.625, 0.479	0.375, 0.479	1, 0	0, 0	0.0163

^ap-value computed by a Kruskal-Wallis rank sum test comparing the PHF-1+ proportions in each Rhes phenotype for each disease.

^bp-value for all diseases combined computed using a one-way ANOVA comparing the PHF-1+ proportions in each Rhes phenotype

^cNo Cornu Ammonis 1 neurons with the absent Rhes phenotype were detected in CBD cases

^dThe Kruskal-Wallis rank sum test cannot estimate the significance of data lacking variance

Abbreviations: HC, Healthy Controls; Alzheimer's disease, Alzheimer's disease; AGD, Argrophilic Grain Disease; CBD, Corticobasal Degeneration; PiD, Pick's disease; PSP, Progressive Supranuclear Palsy

The cornu Ammonis 1 sector of Alzheimer's disease cases showed the highest proportion of Punctiform and Absent Rhes phenotypes. To examine if the correlation between neuronal Rhes phenotypes and tau inclusions differ across various tau post-translational modifications, we immunostained cornu Ammonis 1 of two Alzheimer's disease cases (59-year-old male and 64-year-old female) against Rhes, NeuN, and several post-translational modifications of tau (Supplementary Figure 4): phospho-Thr212/Ser214 Tau (AT100), acetyl-Lys274 Tau (1f3c), truncated Δ Asp421 Tau (Tau-C3), or conformationally-changed tau (MC1). Overall, the correlation between these different tau post-translational modifications and Rhes phenotypes followed the same patterns seen for PHF-1. We sought to identify tau post-translational modifications that better discriminated Rhes types than PHF-1 but did not identify such a post-translational modification (Table 3.5).

TDP-43 PATHOLOGICAL CHANGE IS NOT ASSOCIATED WITH RHES NEURONAL PHENOTYPE

To examine if abnormal Rhes phenotype also associates with phospho-TDP-43 inclusions, we examined cortical sections with moderate or severe TDP-43 pathological changes from three frontotemporal lobar degeneration-TDP Type A cases and four frontotemporal lobar degeneration-TDP Type B cases. We failed to identify any neurons with Punctiform or Absent Rhes phenotypes (Table 3.6, Figure 3.6).

Table 3.5: Proportions of tau inclusion positive cornu Ammonis 1 neurons classified as each Rhes phenotype for different post-translational modifications of tau.

Antibody	Epitope	Diffuse Rhes	Punctiform Rhes	Absent Rhes
PHF-1 ^a	phospho-Ser396, Ser404 Tau	5.99%	61.08%	32.93%
1f3c	acetyl-Lys274 Tau	10.42%	29.17%	60.42%
AT100	phospho-Thr212, Ser214 Tau	8.46%	55.38%	36.15%
Tau-C3	ΔAsp421 Tau	4.11%	35.62%	60.27%
MC1	Conformational tau	5.69%	60.98%	33.33%

^aWhile calculated from the same two Alzheimer's disease cases as the other tau post-translational modifications, PHF-1 was calculated from one large 1x1mm counting frame instead of the three 650x650μm counting frames used for the other post-translational modifications.

Table 3.6: TDP-43: Proportions (mean, sd) of counted neuron population classified as each Rhes phenotype and with pTDP-43 inclusions.

Diagnosis	Diffuse Rhes	Punctiform Rhes	Absent Rhes	pTDP-43 +
FTLD-TDP Type A (IFG, n=3)	1, 0	0, 0	0, 0	0.025, 0.027
FTLD-TDP Type B (MFG, n=4)	1, 0	0, 0	0, 0	0.032, 0.015
Total (n=7)	1, 0	0, 0	0, 0	0.028, 0.021

TDP-43, TAR DNA-binding protein 43; FTLD, Frontotemporal lobar degeneration; IFG, Inferior Frontal Gyrus; MFG, Middle Frontal Gyrus

RASD2 MRNA LEVELS IN SELECTIVELY VULNERABLE EXCITATORY NEURONS OF THE ENTORHINAL CORTEX

Recent work using snRNA-seq (Leng et al., 2021) has identified two subpopulations of excitatory neurons in the EC that show a higher vulnerability to Alzheimer's disease changes than neighboring excitatory neuron populations. Here, we used this same dataset, featuring three cases at Braak stage 0, four at Braak stage II, and three at Braak stage VI, to test if *RASD2* mRNA levels change in these two vulnerable neuronal populations across Alzheimer's disease progression. We computed the mean log₁₀(CPM) transcript levels for each case. For each of the two subpopulations, the mean log₁₀(CPM) of *RASD2* transcripts was low (< 0.5). We failed to detect significant differences in *RASD2* transcript levels among the three Braak stage groups for both subpopulations (EC:Exc.s2 at p=0.286 and EC:Exc.s4 at p=0.404); however, we noted a negative trend with increasing Braak stages (Figure 3.4b).

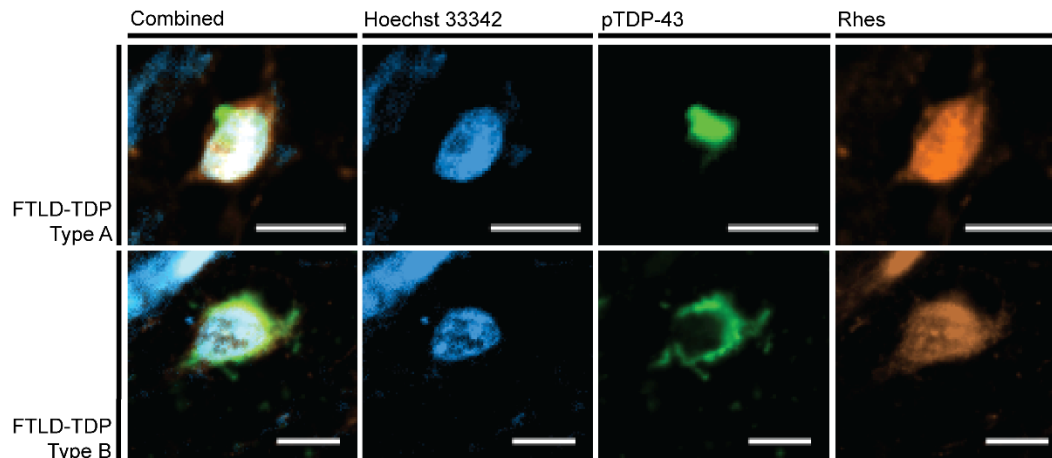


Figure 3.6: Neurons positive for pTDP-43 (green) proteinopathy display a Diffuse Rhes (orange) phenotype. The frontotemporal lobar degeneration-TDP Type A images come from the inferior frontal gyrus of a 66-year-old female with behavioral variant frontotemporal dementia. The frontotemporal lobar degeneration-TDP Type B images come from the middle frontal gyrus of a 57-year-old male with behavioral variant frontotemporal dementia and amyotrophic lateral sclerosis. Scale bar represents 10 μ m.

Discussion

As an abbreviation for Ras homolog enriched in striatum, Rhes has been thought to be a striatal-predominant protein. Here, we showed that Rhes is expressed throughout the allocortex and neocortex, confirming other studies suggesting that Rhes expression extends beyond striatal neurons (Hernandez et al., 2019; Vitucci et al., 2016). Rhes is widespread in cortical neurons and typically presents with a diffuse cytoplasmic distribution (referred to as the Diffuse type) in over 94% of the entorhinal cortex, cornu Ammonis 1, and superior frontal gyrus neurons in healthy controls, suggesting that such distribution represents the standard (i.e., healthy) neuronal phenotype. Noticeably, sporadic tauopathies feature an increased representation of neurons with a cytoplasmic distribution of Rhes presenting as punctiform in cell bodies (Punctiform type) or entirely without Rhes in cell bodies (Absent type) in the three areas examined (the entorhinal cortex, cornu Ammonis 1, and superior frontal gyrus).

We found a direct relationship between PHF-1 positive tau inclusions and changes in Rhes phenotype in all tauopathies. Neurons with Punctiform and Absent phenotypes were more likely to harbor tau inclusions than neurons with Diffuse Rhes. This observation suggests that the population-level decreases in the proportions of neurons with Diffuse Rhes in tauopathies are associated with either the tau inclusion itself or an upstream process affecting both tau aggregation and Rhes expression. While neurons with Diffuse Rhes feature few PHF-1 positive tau inclusions, Absent Rhes neurons are predominantly PHF-1 positive, suggesting that abnormal (i.e., diseased) neurons are cleared of Rhes in the soma. Meanwhile, punctiform Rhes appears to be an

intermediate phenotype (Figure 3.7). Similar patterns were observed for other tau post-translational modifications in Alzheimer's disease cases.

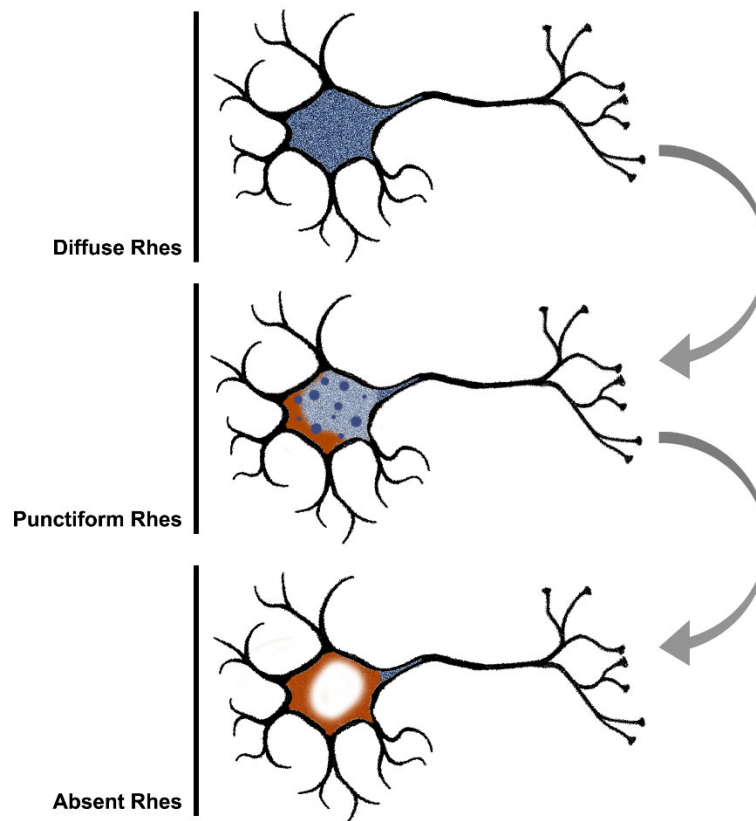


Figure 3.7: We propose an ordered manner by which the Rhes (blue) phenotypes relate to each other based on the collective evidence from this investigation. At baseline, Rhes is expressed throughout the cytoplasm extending into neuronal processes. As neuropathologic changes (orange) emerge, Rhes expression becomes dysregulated and appears punctiform within the cell body. Finally, Rhes is no longer expressed in the cell body of those neurons most affected by neuropathologic changes with some signal persisting in neuronal processes.

Given that the Absent phenotype is more common in Alzheimer's disease than HC, we examined whether *RASD2* mRNA expression levels decreased in the two excitatory neuronal subpopulations of the EC thought to be more vulnerable to tau inclusions and neuron loss than other excitatory neuronal subpopulations in Alzheimer's disease (Leng et al., 2021). Although we did not detect statistically significant changes in transcript levels of *RASD2* between Braak stages, a negative trend emerged. Further work with larger RNA-sequencing datasets should be done to understand how the expression of *RASD2* changes with disease progression in tauopathies.

This postmortem study in humans, together with *in vivo* and *in vitro* work done in familial tauopathy models (Hernandez et al., 2019), suggests the presence of a causal relationship between Rhes clearance and tau proteinopathic lesions. The lack of abnormal Rhes phenotypes (i.e., Punctiform or Absent types) associated with frontotemporal lobar degeneration-TDP reinforces a unique mechanistic link between

Rhes changes and accumulation of abnormal tau inclusions. Impaired autophagy is a well-established hallmark of neurodegenerative diseases, including tauopathies (Caballero et al., 2018; Nixon, 2013; Piras et al., 2016; Theofilas et al., 2018). Rhes has an established role in autophagy modulation (Hernandez et al., 2019; Mealer et al., 2014; Pan et al., 2009), including its role in forming tunneling nanotube-like protrusions (Subramaniam, 2020). These protrusions are thought to facilitate the selective transfer of membrane vesicles and organelles, including lysosomes and endosomes, between cells. Taking our data together with the previous work on Rhes, the high co-incidence of Absent type neurons with PHF-1 positive inclusions may be explained by the established role of Rhes in autophagy modulation (Hernandez et al., 2019; Mealer et al., 2014; Pan et al., 2009).

While our findings suggest a causal relationship between Rhes clearance and tau proteinopathic lesions, our study design precludes us from determining the direction of the association. Hernandez and colleagues (Hernandez et al., 2019) demonstrated that pathogenic *MAPT* mutations initially suppress age-related increases in Rhes, indicating that tau inclusions may drive neurons towards the Absent phenotype. Conversely, the abundance of Punctiform type neurons without PHF-1 positive tau inclusions suggests that changes in Rhes may precede tau accumulation.

Our observations provide further explanation for findings in Hernandez et al. (Hernandez et al., 2019). They demonstrated that Lonafarnib effectively prevented the accumulation of tau proteinopathic lesions in model systems but was not effective in clearing existing tau aggregations. If end-stage neurons feature an absence of Rhes, they would lack the druggable target for Lonafarnib to act. A notable proportion of PHF-1 positive neurons (23.6-39.7%) had an absent Rhes phenotype in our sample, possibly explaining why Lonafarnib did not promote significant clearance of tau aggregations in model systems.

The strong association between neuronal tau inclusions and abnormal (i.e., Punctiform and Absent) Rhes phenotypes may explain why corticobasal degeneration and progressive supranuclear palsy showed only a minimal number of neurons with Rhes phenotypic changes. Most cortical tau aggregation in corticobasal degeneration and progressive supranuclear palsy occurs in glia (Coughlin et al.), where we found little evidence of Rhes expression (Figure 1, Supplementary Figure 2). Despite this, the few cortical neurons with PHF-1 positive tau inclusions in progressive supranuclear palsy and corticobasal degeneration predominantly displayed either a Punctiform or Absent Rhes phenotype, as seen in Alzheimer's disease and Pick's disease. Similarly, argyrophilic grain disease showed minimal changes in neuronal Rhes; however, argyrophilic grain disease is thought to be a relatively benign tauopathy, with most tau deposits occurring in dendritic buttons and oligodendroglia (Ferrer et al., 2008; Rodriguez et al., 2016).

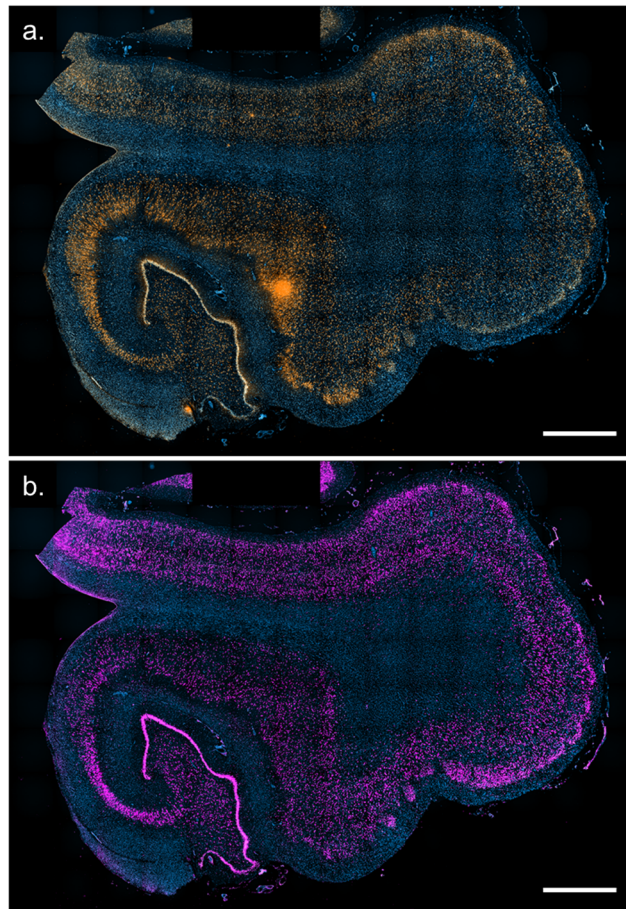
Caution should be taken in interpreting the results, as our investigation has limitations. Postmortem studies are cross-sectional in nature. As such, we cannot assess longitudinal changes in neuronal Rhes phenotypes in tauopathies and precisely how

they mechanistically relate to tau. For this reason, our study should be interpreted with others using model systems (Hernandez et al., 2019). Additionally, we have not yet identified sub-cellular structures that colocalize with the puncta observed in Punctiform Rhes, nor have we resolved the pathophysiologic changes that accompany the PHF-1 negative Punctiform neurons. Gaining a better mechanistic understanding of the Rhes neuronal phenotypes in the manner of Figure 3.7 would be essential for the precise manipulation of Rhes as a therapeutic target. Finally, although we counted thousands of neurons, they only represented a fraction of the entorhinal cortex, cornu Ammonis 1, and superior frontal gyrus and did not inform on other cortical regions affected by tauopathies. The primary outcomes here were agnostic to the neuropathological diagnosis. They focused on the relationship between tau proteinopathic inclusions and changes in neuronal Rhes phenotype in tauopathies as a whole class of diseases, to which we are sufficiently powered to make conclusions. However, we lacked the power to conduct an extensive investigation comparing each tauopathy.

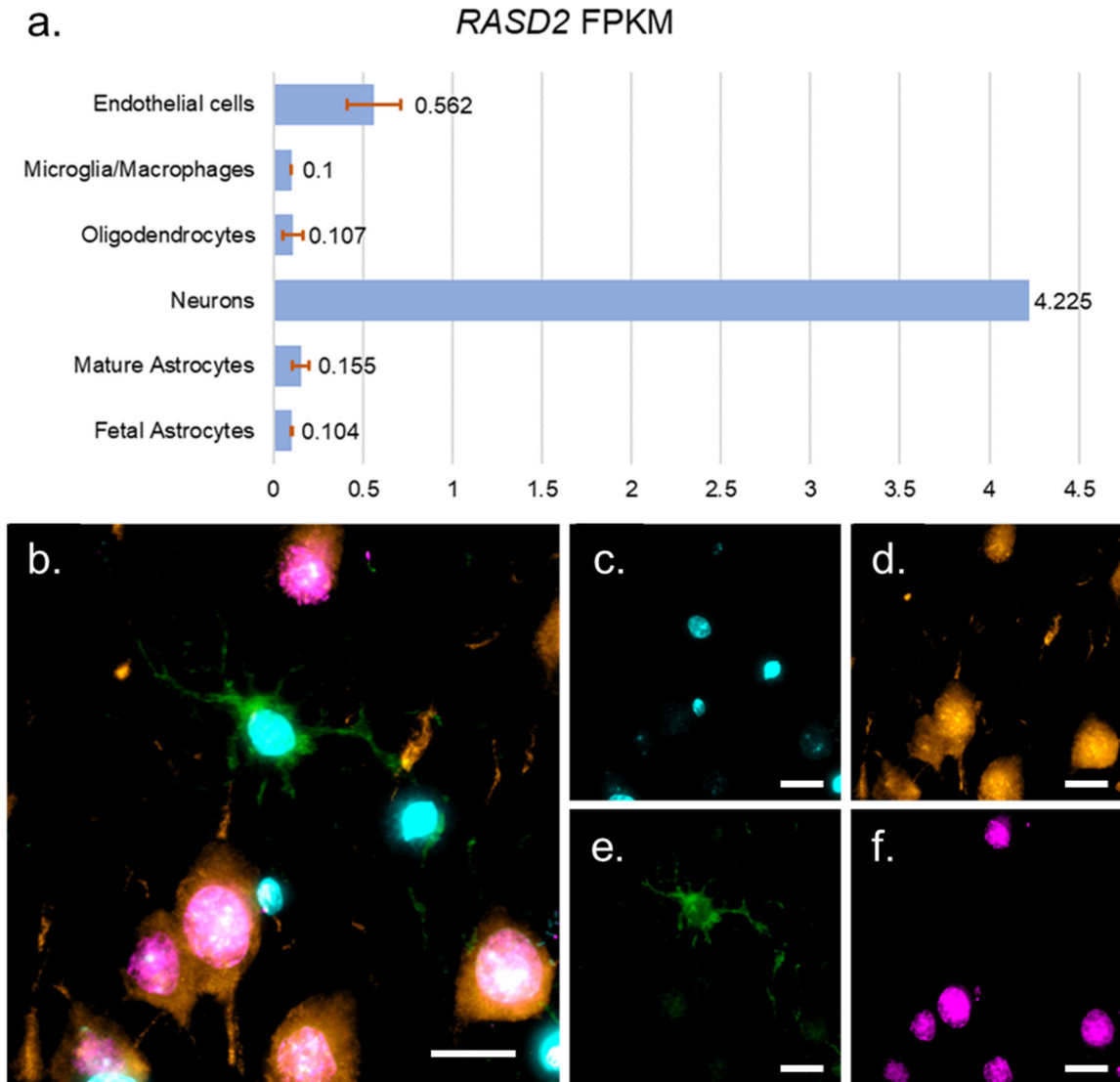
This study also has several strengths. First, we used multiple independent datasets of RNA-seq data to validate our immunohistochemical findings. Additionally, we investigated well-characterized postmortem brain tissue of individuals with a range of sporadic tauopathies, making us well-positioned to examine the translatability of results drawn from model systems based on familial tauopathies. One limitation of *in vivo* and *in vitro* tauopathy models is the use of *MAPT* mutations. While also featuring tau aggregations, the specific pathophysiology and etiology of rare familial tauopathies have differences with the more common sporadic tauopathies studied here. It is infeasible to completely model the entire molecular and physiological milieu surrounding neurodegeneration in model systems. As such, it is unclear how generalizable the results of interventions in these model systems are. It is imperative to examine the conclusions of model systems in the context of human cases, as we have done here. Lonafernib is a well-tolerated drug with which there is existing clinical experience (Gordon et al., 2012; Gordon et al., 2014; Kieran et al., 2007; Yust-Katz et al., 2013). Here, we show that the expression of a target of Lonafernib, Rhes, is altered in tauopathies, reinforcing the conclusions drawn from model systems. Our findings here, in combination with the mechanistic work done by Hernandez and colleagues (Hernandez et al., 2019), lend support to the establishment of a clinical study of Lonafernib for early intervention in tauopathies, including Alzheimer's disease.

Selective vulnerability can also be examined without specific relation to proteinopathic lesions. Instead, a focus on why certain groups of neurons die may confer more clinically effective therapeutics. Unfortunately, studying neurons after they die brings epistemological constraints. Specifically, neurons cannot be studied once they are dead. Instead, evaluating regions that reliably degenerate prior to degenerative stages can offer clues. In chapter 4, this is done with a focus on an early affected brainstem nucleus.

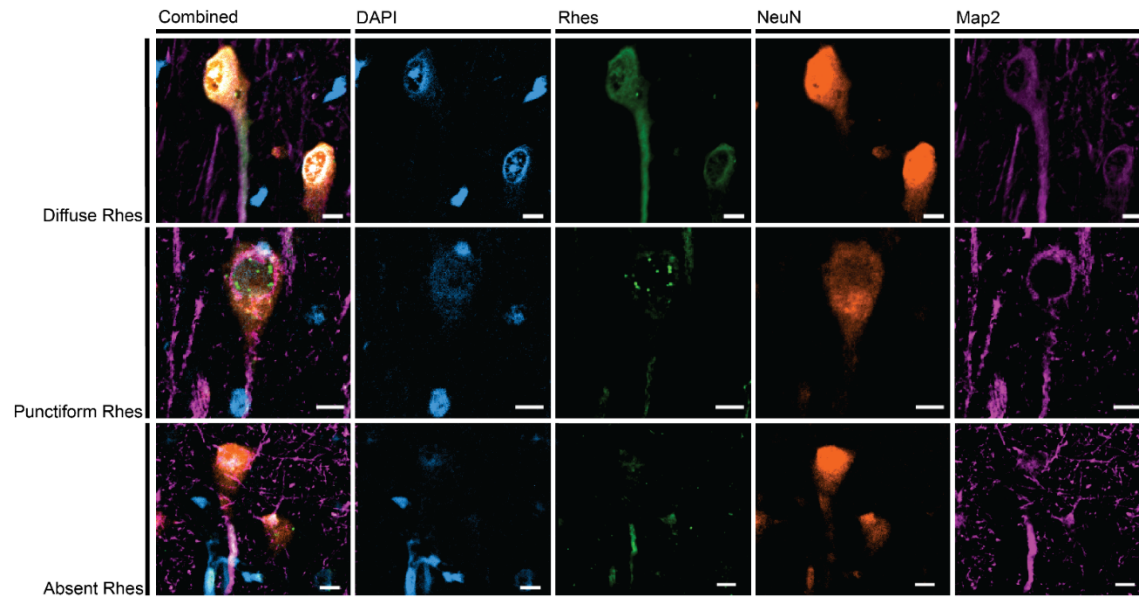
Supplementary Figures



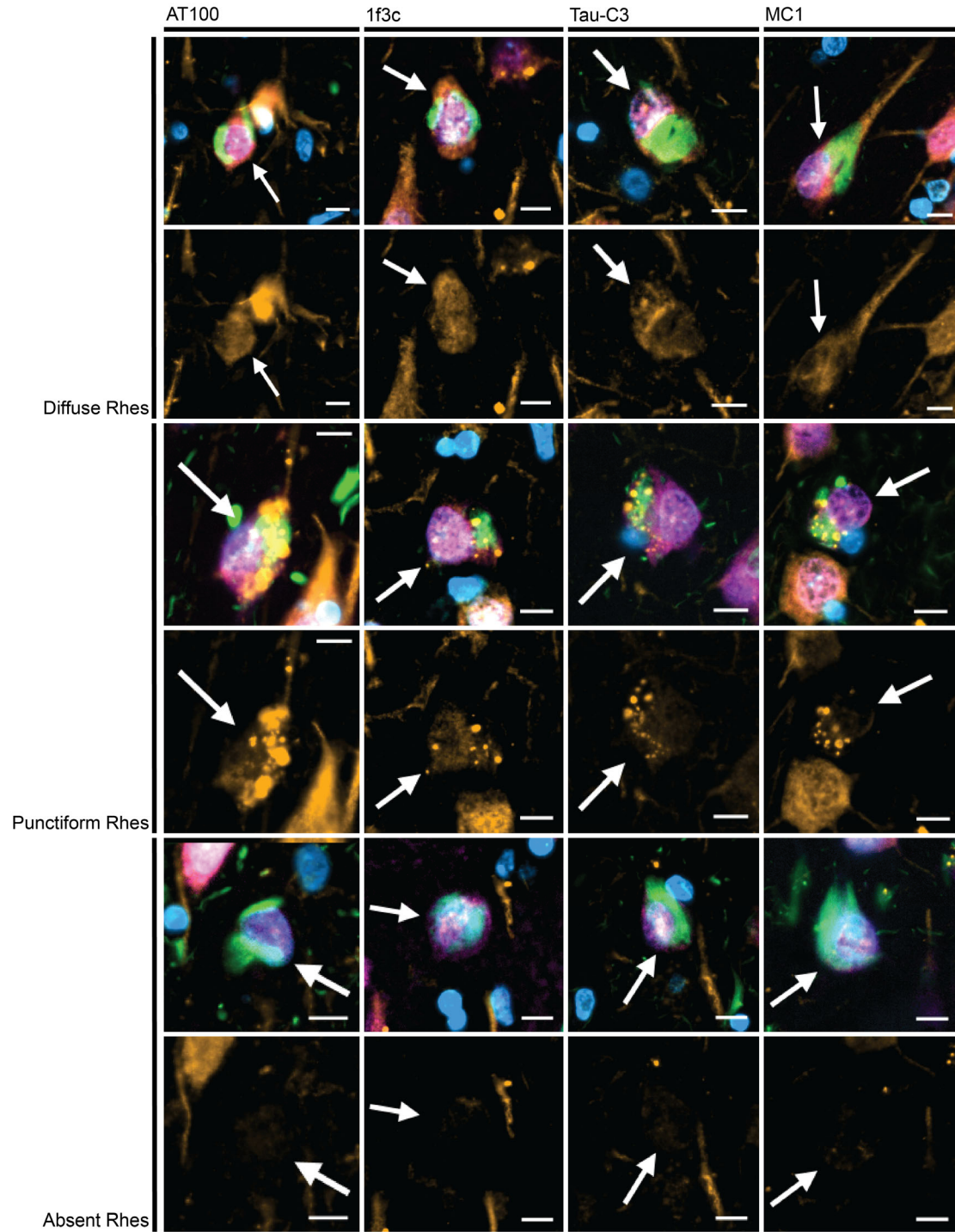
Supplementary Figure 3.1: Rhes (orange) is ubiquitously expressed throughout the neurons (pink) of the human hippocampal formation from an 82-year-old female free of any neuropathological diagnosis. Scale bar corresponds to 2 mm.



Supplementary Figure 3.2: According to the online tool brainrnaseq.org, *RASD2* mRNA is primarily expressed in Neurons, with negligible expression in glia (a). The protein form, Rhes, is only seen in neurons, as seen when doing a triple stain with GFAP (b-f). Here, blue (c) is Hoechst 33342, orange (d) is Rhes, green (e) is GFAP, and pink (f) is NeuN. Scale bar corresponds to 10 μ m.



Supplementary Figure 3.3: In all three Rhes neuronal presentations, projections of Rhes (green) overlap with Map2 (pink). This indicates that Rhes occurs in dendrites. Examples here from cornu Ammonis 1 of a 69-year-old female with PiD (A0B0C0). Scale bars correspond to 10 μ m.



Supplementary Figure 3.4: Examples of each Rhes phenotype with different post-translational modifications of tau. Combined images with Hoechst 33342 (blue), the tau post-translational modification (green), Rhes (orange), and NeuN (pink) are shown in the top row of each set of Rhes phenotypes. Images of the Rhes (orange) channel alone are shown in the bottom row of each set of Rhes phenotypes. Scale bars correspond to 10 μ m.

Supplementary Methods

SUPPLEMENTARY METHOD 3.1

Staining and analysis of sections used to evaluate relationship between phospho-tau accumulation and Rhes (30 cases with hippocampal formation, 24 of which also with superior frontal gyrus).

Staining protocol

1. Deparaffinization and rehydration: 3x 10 minutes in xylene, 2x 2 minutes in 100% ethanol, 2 minutes in 96% ethanol, 2 minutes in 80% ethanol, 2 minutes in distilled water
2. Antigen retrieval: Sections immersed in 0.1M citrate buffer (pH 6.0) with 0.05% tween-20 and cycled through an autoclave at 121°C at the five-minute setting
3. Sections left to cool to room temperature for 1 hour
4. Sections washed 3x 2 minutes in a 1x solution of phosphate-buffered saline with 0.05% tween-20 (PBST)
5. Sections incubated in 5% milk dissolved in PBST for 30 minutes
6. Primary antibody incubation for 18 hours with 1:600 α -NeuN (266 004, Synaptic Systems), 1:200 α -Rhes (GTx85428, GeneTex), and 1:600 PHF-1 (phospho-Ser396/Ser404 Tau, Gift from Peter Davies) diluted in 5% milk/PBST.
7. Wash with PBST 3x 2 minutes.
8. Secondary antibody incubation for 90 minutes with 1:400 Goat anti-Mouse IgG (H+L) Alexa Fluor 488 (A-11001, Invitrogen), 1:400 Goat anti-Rabbit IgG (H+L) Alexa Fluor 546 (A-11010, Invitrogen), and 1:400 Goat anti-Guinea Pig IgG (H+L) Alexa Fluor 647 (A-21450, Invitrogen) in PBST
9. Wash with PBST 3x 2 minutes
10. 1x 2-minute wash in 70% ethanol
11. Submerged in Sudan black B solution for 40 minutes
12. 2x 10-second wash in 70% ethanol
13. 1-minute wash in distilled water
14. Sections incubated in 1:50,000 DAPI*
15. 3x 2-minute wash in PBS
16. Sections cover-slipped with Prolong (P36980, Invitrogen)

**Some sections did not get incubated in DAPI and were instead cover slipped with Prolong with NucBlue (P36981, Invitrogen).*

Imaging and analysis

1. Sections were scanned using a Zeiss Axio Scan.Z1 slide scanner (Molecular Imaging Center, University of California, Berkeley) with a Plan-Apo 10X/0.45 NA objective using DAPI (Zeiss Filter Set 49), eGFP (FS 38 HE), Cy3 (FS 43 HE), and Cy5 (FS 50) filters.
2. One 1x1mm frame was delineated in cornu Ammonis 1 and the entorhinal cortex of the hippocampal formation. This counting frame was not systematically placed in any subregion of either the cornu Ammonis 1 and the entorhinal cortex, however the placement was most representative of superficial neuronal layers. Two 1x1mm frames were delineated in the superior frontal gyrus, one bordering cortical layer one, the other bordering cortical layer six. All neurons in these

regions were counted, classifying them based on Rhes subtype and PHF-1 inclusion.

SUPPLEMENTARY METHOD 3.2

Staining and analysis to examine the intraneuronal distribution of Rhes.

Staining protocol

1. Deparaffinization and rehydration: 3x 10 minutes in xylene, 2x 2 minutes in 100% ethanol, 2 minutes in 96% ethanol, 2 minutes in 80% ethanol, 2 minutes in distilled water
2. Antigen retrieval: Sections immersed in 0.1M citrate buffer (pH 6.0) with 0.05% tween-20 and cycled through an autoclave at 121°C at the five-minute setting
3. Sections left to cool to room temperature for 1 hour
4. Sections washed 3x 2 minutes in a 1x solution of phosphate-buffered saline with 0.05% tween-20 (PBST)
5. Sections incubated in 5% milk dissolved in PBST for 30 minutes
6. Primary antibody incubation for 18 hours with 1:600 α -NeuN (266 004, Synaptic Systems), 1:200 α -Rhes (GTX85428, GeneTex), and 1:900 MAP-2 (NB300-213, Novus) diluted in 5% milk/PBST.
7. Wash with PBST 3x 2 minutes
8. Secondary antibody incubation for 90 minutes with 1:400 Goat anti-Rabbit IgG (H+L) Alexa Fluor 488 (A-11008, Invitrogen), 1:400 Goat anti-Guinea Pig IgG (H+L) Alexa Fluor 546 (A-11074, Invitrogen), and 1:400 Goat anti-Chicken IgY (H+L) Alexa Fluor 647 (A-32933, Invitrogen) in PBST
9. Wash with PBST 3x 2 minutes
10. 1x 2-minute wash in 70% ethanol
11. Submerged in Sudan black B solution for 40 minutes
12. 2x 10 second wash in 70% ethanol
13. 1 minute wash in distilled water
14. 3x 2-minute wash in PBS
15. Sections cover-slipped with Prolong with NucBlue (P36981, Invitrogen)

Imaging and analysis

1. Sections were scanned using a Zeiss Axio Scan.Z1 slide scanner (Molecular Imaging Center, University of California, Berkeley) with a Plan-Apo 20X/0.8 NA objective using DAPI (Zeiss Filter Set 49), eGFP (FS 38 HE), Cy3 (FS 43 HE), and Cy5 (FS 50) filters.
2. An example of each Rhes subtype in a neuron with MAP2 processes present was identified to generate a figure. No Rhes+ processes were identified that were also not positive for MAP-2.

SUPPLEMENTARY METHOD 3.3

Staining and analysis of sections used to evaluate relationship between phospho-TDP-43 inclusions and Rhes (7 cortical sections).

Staining protocol

1. Deparaffinization and rehydration: 3x 10 minutes in xylene, 2x 2 minutes in 100% ethanol, 2 minutes in 96% ethanol, 2 minutes in 80% ethanol, 2 minutes in distilled water
2. Antigen retrieval: Sections immersed in 0.1M citrate buffer (pH 6.0) with 0.05% tween-20 and cycled through an autoclave at 121°C at the five-minute setting
3. Sections left to cool to room temperature for 1 hour
4. Sections washed 3x 2 minutes in a 1x solution of phosphate-buffered saline with 0.05% tween-20 (PBST)
5. Sections incubated in 5% milk dissolved in PBST for 30 minutes
6. Primary antibody incubation for 18 hours with 1:600 α -NeuN (266 004, Synaptic Systems), 1:200 α -Rhes (GTX85428, GeneTex), and 1:200 anti-phospho-S409/410 TDP 43 (1:200, Rat, TAR5P, gift of Manuela Neumann, Munich, Germany) diluted in 5% milk/PBST.
7. Wash with PBST 3x 2 minutes
8. Secondary antibody incubation for 90 minutes with 1:400 Goat-anti-rat IgG (H+L), HRP conjugate (R-05075-500, advansta), 1:400 Goat anti-Rabbit IgG (H+L) Alexa Fluor 546 (A-11010, Invitrogen), and 1:400 Goat anti-Guinea Pig IgG (H+L) Alexa Fluor 647 (A-21450, Invitrogen) in PBST
9. Wash with PBST 3x 2 minutes
10. 15-minute tyramide signal amplification with a solution of 1:100 AF488 Tyramide (B40953, Thermo Fisher) and 1:100 of 100x H₂O₂ in 1x tris buffered saline
11. Wash with PBST 3x 2 minutes
12. 1x 2-minute wash in 70% ethanol
13. Submerged in Sudan black B solution for 40 minutes
14. 2x 10 second wash in 70% ethanol
15. 1 minute wash in distilled water
16. Sections incubated in 1:50,000 DAPI
17. 3x 2-minute wash in PBS
18. Sections cover-slipped with Prolong (P36980, Invitrogen)

Imaging and analysis

1. Sections were scanned using a Zeiss Axio Scan.Z1 slide scanner (Molecular Imaging Center, University of California, Berkeley) with a Plan-Apo 10X/0.45 NA objective using DAPI (Zeiss Filter Set 49), eGFP (FS 38 HE), Cy3 (FS 43 HE), and Cy5 (FS 50) filters.
3. 1x1mm frames were delineated from each section, one bordering cortical layer one, the other bordering cortical layer six. All neurons in these regions were counted, classifying them based on Rhes subtype and phospho-TDP-43 inclusion.

SUPPLEMENTARY METHOD 3.4

Staining and analysis to examine the relationship between Rhes and different post-translational modifications of Tau.

Staining protocol

1. Deparaffinization and rehydration: 3x 10 minutes in xylene, 2x 2 minutes in 100% ethanol, 2 minutes in 96% ethanol, 2 minutes in 80% ethanol, 2 minutes in distilled water
2. Antigen retrieval: Sections immersed in 0.1M citrate buffer (pH 6.0) with 0.05% tween-20 and cycled through an autoclave at 121°C at the five minute setting
3. Sections left to cool to room temperature for 1 hour
4. Sections washed 3x 2 minutes in a 1x solution of phosphate-buffered saline with 0.05% tween-20 (PBST)
5. Sections incubated in 5% milk dissolved in PBST for 30 minutes
6. Primary antibody incubation for 18 hours with 1:600 α -NeuN (266 004, Synaptic Systems), 1:200 α -Rhes (GTX85428, GeneTex), and one of:
 - AT100 (phospho-Thr212/Ser214 Tau, 1:800, MN1060, Invitrogen)
 - 1f3c (acetyl-Lys274 Tau, 1:1000, Gift from Li Gan)
 - Tau-C3 (Δ Asp421 Tau, 1:300, AHB0061, Invitrogen)
 - MC1 (Conformational Tau, 1:500, Gift from Peter Davies)
 diluted in 5% milk/PBST.
7. Wash with PBST 3x 2 minutes
8. Secondary antibody incubation for 90 minutes with 1:400 Goat anti-Mouse IgG (H+L) Alexa Fluor 488 (A-11001, Invitrogen), 1:400 Goat anti-Rabbit IgG (H+L) Alexa Fluor 546 (A-11010, Invitrogen), and 1:400 Goat anti-Guinea Pig IgG (H+L) Alexa Fluor 647 (A-21450, Invitrogen) in PBST
9. Wash with PBST 3x 2 minutes
10. 1x 2 minute wash in 70% ethanol
11. Submerged in Sudan black B solution for 40 minutes
12. 2x 10 second wash in 70% ethanol
13. 1 minute wash in distilled water
14. 3x 2 minute wash in PBS
15. Sections cover-slipped with Prolong with NucBlue (P36981, Invitrogen)

Imaging and analysis

1. Sections were scanned using a Zeiss Axio Scan.Z1 slide scanner (Molecular Imaging Center, University of California, Berkeley) with a Plan-Apo 20X/0.8 NA objective using DAPI (Zeiss Filter Set 49), eGFP (FS 38 HE), Cy3 (FS 43 HE), and Cy5 (FS 50) filters.
2. Three 650x650um frames were delineated in cornu Ammonis 1 of the hippocampal formation. All neurons in these regions were counted, classifying them based on Rhes subtype and tau inclusion.

SUPPLEMENTARY METHOD 3.5

Staining and imaging of sections used to look at possible astrocyte inclusions of Rhes.

Staining protocol

1. Deparaffinization and rehydration: 3x 10 minutes in xylene, 2x 2 minutes in 100% ethanol, 2 minutes in 96% ethanol, 2 minutes in 80% ethanol, 2 minutes in distilled water
2. Antigen retrieval: Sections immersed in 0.1M citrate buffer (pH 6.0) with 0.05% tween-20 and cycled through an autoclave at 121°C at the five minute setting

3. Sections left to cool to room temperature for 1 hour
4. Sections washed 3x 2 minutes in a 1x solution of phosphate-buffered saline with 0.05% tween-20 (PBST)
5. Sections incubated in 5% milk dissolved in PBST for 30 minutes
6. Primary antibody incubation for 18 hours with 1:600 α -NeuN (266 004, Synaptic Systems), 1:200 α -Rhes (GTX85428, GeneTex), and 1:900 GFAP (ab4674, Abcam) diluted in 5% milk/PBST.
7. Wash with PBST 3x 2 minutes
8. Secondary antibody incubation for 90 minutes with 1:400 Goat anti-Chicken IgY (H+L) Alexa Fluor 488 (A-11039, Invitrogen), 1:400 Goat anti-Rabbit IgG (H+L) Alexa Fluor 546 (A-11010, Invitrogen), and 1:400 Goat anti-Guinea Pig IgG (H+L) Alexa Fluor 647 (A-21450, Invitrogen) in PBST
9. Wash with PBST 3x 2 minutes
10. 1x 2 minute wash in 70% ethanol
11. Submerged in Sudan black B solution for 40 minutes
12. 2x 10 second wash in 70% ethanol
13. 1 minute wash in distilled water
14. 3x 2 minute wash in PBS
15. Sections cover-slipped with Prolong with NucBlue (P36981, Invitrogen)

Imaging and analysis

1. Sections were scanned using a Zeiss AxioImager.A2 microscope equipped with a Zeiss Colibri 7:Type FRR[G/Y]CBV-UC 7-channel fluorescence light source. NucBlue (Hoechst 33342) was visualized with a DAPI filter set, AF488 visualized with a GFP filter set, AF546 with a DsRed filter set, and AF647 with a Cy5 filter set.

Chapter 4: Pathways underlying selective vulnerability to Alzheimer's disease in aminergic brainstem nuclei

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Abstract

The neuromodulatory subcortical system consists of nuclei exhibiting early vulnerability to tauopathies, including Alzheimer's disease. Within the neuromodulatory subcortical system, there is a spectrum of vulnerability that becomes apparent in the earliest stages of Alzheimer's disease, offering a chance to probe factors underlying vulnerability to Alzheimer's disease. In this study, we applied transcriptomics in well-characterized postmortem human tissue to understand why this susceptibility gradient exists by examining two nuclei with very similar neurons but differing in their vulnerability to Alzheimer's disease the locus coeruleus and substantia nigra. By sampling both nucleus from the same individual cases, we identify differences and at progressive Alzheimer's disease stages aiming to identify vulnerability-related pathway. Differences in cholesterol homeostasis and antioxidant pathways emerge as important candidates.

Introduction

Alzheimer's disease features a stereotypical progression of amyloid- β plaques and tau positive neurofibrillary tangles in the brain. The neuromodulatory subcortical system nuclei – those nuclei that produce neuromodulatory transmitters – are affected by lesions across a range of different neurodegenerative diseases. Beginning with research in the 1970's (Davies & Maloney, 1976), one of the neuromodulatory subcortical nuclei, the cholinergic basal forebrain, garnered significant focus in the Alzheimer's disease research field. Even decades later, the prevailing treatment for

cognitive impairment in Alzheimer's disease is based on observations from this early research (Birks, 2006). Many other neuromodulatory nuclei are affected in Alzheimer's disease too, though. In fact, neurodegenerative lesions have been documented in over a dozen subcortical nuclei in stages preceding involvement of the transentorhinal cortex (i.e. Braak stage I) (Attems, Thomas, et al., 2012a; Ehrenberg et al., 2023; Rub, Stratmann, et al., 2016; Stratmann et al., 2016b).

In recent years, the locus coeruleus has earned much attention as an early affected region in Alzheimer's disease. The locus coeruleus is the primary noradrenergic nucleus for the central nervous system. It exists as two columns flanking the fourth ventricle in the dorsal pons and projects widely throughout the brain. Evolving at least 400 million years ago, it functions to regulate many behaviors in humans including arousal and hedonic tone. Prior to Braak stage I in the Alzheimer's disease pathological process, there is already a significant burden of phospho-tau neuronal cytoplasmic inclusions (Andres-Benito et al., 2017; Braak & Del Tredici, 2011b, 2015; Braak et al., 2011; Ehrenberg, Nguy, Theofilas, Dunlop, Suemoto, Di Lorenzo Alho, Leite, Diehl Rodriguez, Mejia, Rub, et al., 2017; Jacobs et al., 2021; Jacobs et al., 2022) which increases with Braak stage. Even between Braak stages 0 and I, there is already a measurable decrease of about 8% in the volume of the locus coeruleus, with decreases in the estimated neuron population observed starting at Braak stage III (Theofilas et al., 2017b). These early changes have made the locus coeruleus an appealing biomarker for Alzheimer's disease detection at early stages (Betts, Kirilina, et al., 2019). While these incipient degenerative changes in the locus coeruleus associate with disease stages preceding Alzheimer's disease-type cognitive decline, they are thought to contribute to many of the concomitant neuropsychiatric symptoms observed in Alzheimer's disease (Ehrenberg et al., 2018; Jacobs et al., 2019; Oh et al., 2019).

Another deeply conserved neuromodulatory nucleus, the substantia nigra has been extensively studied in the context of Lewy body disease. As a major producer of dopamine in the brain, the early degeneration of the substantia nigra contributes significantly to the pathognomonic bradykinesia, tremors, and rigidity in conditions such as Parkinson's disease. While clearly vulnerable to synucleinopathies like Lewy body disease and tauopathies such as progressive supranuclear palsy and corticobasal degeneration, the substantia nigra remains mostly resistant to Alzheimer's disease-type neurodegeneration (i.e. neuronal loss) until late disease stages (Parvizi et al., 2001). Curiously, despite the differences in vulnerability, the substantia nigra and locus coeruleus are remarkably similar. Both are members of the isodendritic core, meaning they host large neurons with long and poorly myelinated neurons and the ascending reticular activating system (Grofova et al., 1982; Ramon-Moliner & Nauta, 1966) and, thus, are cytoarchitecturally similar. Both also feature a conspicuous cytoplasmic pigment, neuromelanin.

Just as how distinct patterns of cortical degeneration underscore the classical clinical differences between dementias, there is a striking specificity to the pattern of vulnerability observed in the neuromodulatory subcortical system in different neurodegenerative diseases (Parvizi et al., 2001). This pattern presents unique

opportunities to probe causative mechanisms of selective vulnerability. Classical neuropathologic research is epistemologically constrained in its ability to probe neurodegenerative processes by its cross-sectional nature and enrichment for cases at end-stage disease. Inter-individual variability can contribute to massive heterogeneity between groups in a study design that becomes even more apparent when utilizing modern “-omics” techniques. Further, classifying molecular signatures as vulnerability factors, secondary reactions to degeneration, compensatory factors, or simply just those retained by cells spared by the neurodegenerative processes is challenging at end stages.

Given the robust consistency in the vulnerability pattern of neuromodulatory nuclei, early-stage cases can be used to study baseline factors that contribute to pathogenesis. Leveraging this approach may help overcome constraints to typical neuropathologic research. Here, we utilize the pattern of vulnerability in the neuromodulatory subcortical system to seek out factors underlying neuronal vulnerability and resistance by comparing gene expression in the locus coeruleus and substantia nigra.

Materials and Methods

PARTICIPANT SELECTION AND NEUROPATHOLOGIC ASSESSMENT

Cases were sourced from the Human Brain Bank of the Brazilian Aging Brain Study Group at the University of São Paulo Faculty of Medicine and the Neurodegenerative Disease Brain Bank at the University of California, San Francisco Memory and Aging Center. Consent for brain donation was obtained from subjects or next of kin following the site-specific protocol approved by the relevant Institutional Review Board and the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

At autopsy, coronal brain slabs were cut down the midline with one hemisphere fixed in 4% neutral buffered formalin and the other frozen. The brainstem was similarly cut down the midline and stored as two complete halves extending from the rostral extent of the midbrain to the caudal extent of the medulla oblongata. One half was fixed in 4% formalin and the other half was frozen on dry ice then stored at -80°C. Slabs undergoing formalin fixation were transferred to PBS-Azide after 72-hours of fixation and stored at 4°C. A standardized set of 26 tissue blocks were sampled from the fixed hemispheres and embedded in paraffin blocks for neuropathological staging. Basic histological and immunohistochemical stains with the antibodies for phospho-Ser202 Tau (CP13, 1:250, courtesy of P. Davies), TDP-43 (1:2000, 10782-2-AP, Proteintech), amyloid- β (1:500, MAB5206, Millipore), α -Synuclein (1:5000, LB509, courtesy of J. Trojanowsky and V. Lee). Final neuropathologic diagnosis was made by a licensed and accredited neuropathologist (LTG, SS, WWS, VRP) using currently accepted guidelines (Mackenzie, Neumann, Baborie, et al., 2011; McKee et al., 2016; McKeith et al., 2005; Montine et al., 2012).

For this study, we included cases from Braak stage 0-III. Cases for this study were selected to exclude any with the presence of Lewy body inclusions, TDP-43 lesions, a primary or contributing diagnosis of chronic traumatic encephalopathy, an Axis I psychiatric disorder, and gross non-neurodegenerative structural neuropathology.

SAMPLE COLLECTION AND PROCESSING

The frozen half of the brainstem for selected cases was kept on dry ice during the dissection. A scalpel was used to shave down the midbrain until the pigmented substantia nigra was exposed. An additional 3-5mm of midbrain was shaved down around the rostral portion of the substantia nigra, with borders defined by the pigmented area. The protruding portion of the substantia nigra was sliced off of the shaved down face of the midbrain and put into RNAlater (AM7020, Invitrogen) to protect RNA in case of thawing during transport. The sample in RNAlater was frozen down to -80C and transported on dry ice.

The locus coeruleus was isolated by excising a tissue block approximately 5-10mm in length along the rostrocaudal axis and about 10mm in depth from the fourth ventricle near the medial eminence. After removal, the pigmented area of the locus coeruleus was identified. Tissue outside the pigmented locus coeruleus border was then discarded. The isolated locus coeruleus tissue was subsequently preserved in RNAlater and frozen.

The locus coeruleus and substantia nigra samples, frozen in RNAlater, were sent to a vendor (Novogene Inc., Davis, CA) for RNA extraction and sequencing. Tissue homogenization and cell lysis was performed in TRIzol. After cell lysis, impurities removal, and inhibition of RNase activity, total RNA was extracted by using phase separation method from cell debris. An Agilent bioanalyzer 2100 was used to measure RNA quality and concentration. Samples with an RNA integrity number (RIN) greater than 4 and at least 0.1µg of RNA available was passed to library preparation and sequencing. Library preparation with poly A enrichment was done with the NEBNext Ultra II RNA Library Prep Kit for Illumina. Sequencing was performed with the Illumina NovaSeq 6000 to generate 150bp paired end (PE150) reads for each sample.

TRANSCRIPTOMIC ANALYSIS

The quality of sequence files was assessed using the FastQC package (Andrews, 2010) before and after trimming steps. Trimmomatic (Bolger et al., 2014) (ILLUMINACLIP: TruSeq3-PE.fa:2:30:10:2:True, LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4:15, and MINLEN: 36) was used to remove adapter sequences and any sequences with low mean quality scores. Sequences were aligned to GRCh38 using STAR alignment (Dobin et al., 2013) and count matrices were generated using featureCounts (Liao et al., 2014). The count matrices were converted to CPM using the edgeR package. Expression levels of *DBH*, *Slc6A2*, and *Slc6A3* were checked to confirm accurate sampling. Cases with less than 100 CPM of *DBH* or 10 CPM of *Slc6A2* in the locus coeruleus, or less than 100 CPM or *Slc6A3* in the substantia nigra were excluded from subsequent analyses.

PCA were performed using the RunPCA command from the Seurat package in R with 20 principal components computed. Differential expression analyses were performed using the edgeR package (Robinson et al., 2010). Only genes with at least 5 counts present in at least 25% of samples were included in differential expression analyses. Multiple comparison correction was done using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995) and the upper bound of the expected FDR is reported in analyses as the FDR. Differential expression analyses set cutoffs for hits at 5% FDR and ± 0.5 logFC. The design matrix for differential expression analyses is formed from an additive model formula including the case. The design matrix formula did not include an interaction term as we sought to assess the average effect of nucleus identity in our cohort rather than the effect of nucleus identity for a given case.

GSEA was done using the fgsea package (Korotkevich et al., 2021) in R. The Hallmark gene set collection was downloaded from the GSEA Molecular Signatures Database (Liberzon et al., 2015; Mootha et al., 2003; Subramanian et al., 2005). The gene set files were filtered to contain only genes present in the differential expression table and then reformatted to the specifications required by the fgsea package. The DEGs were ranked based on the negative log(p-value) such that the genes with the smallest p-values were ranked at the very top of the ranking list. The fgsea function was run on the filtered gene set files with the ranked genes using a minimum gene set size of 10 and maximum of 500.

Results

NUCLEUS SAMPLING

RNA sequencing data for both the locus coeruleus and substantia nigra were available for 23 cases spanning Braak stages 0-III. We analyzed the CPM of *DBH*, *Slc6A2*, and *Slc6A3* and identified one case for exclusion because the CPM values of *DBH* and *Slc6A2* were lower than what is typical for a correctly sampled locus coeruleus (see Figure 4.1).

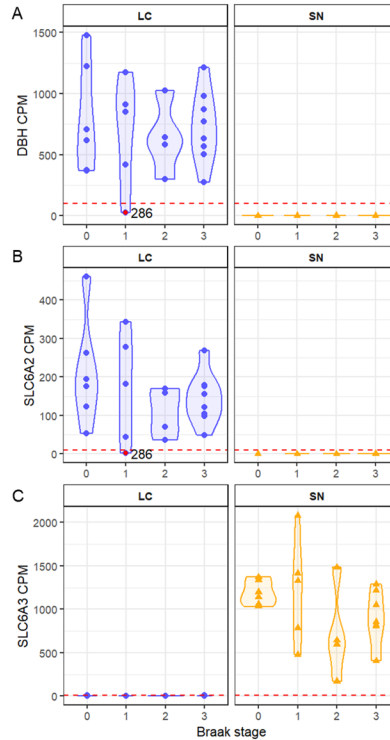


Figure 4.1: The CPM of DBH, Slc6A2, and Slc6A3 are used to assess sampling accuracy of the locus coeruleus and substantia nigra. While variation of each of these markers within the nuclei are expected, we set cutoffs to require >100 CPM of DBH in the locus coeruleus (A), >10 CPM of Slc6A2 in the locus coeruleus (B), and >100 CPM of Slc6A3 in the substantia nigra (C) for inclusion in this study. One case (#286) does not meet these cutoffs.

DEMOGRAPHICS OF COHORT FOR TRANSCRIPTOMIC ANALYSIS

Bulk mRNA sequencing was performed for both the locus coeruleus and substantia nigra from the 22 cases meeting the abovementioned criteria. Eight (36%) of the cases were female. The average age of the cohort was 64 years (range 37-91). Six cases were Braak stage 0, four were Braak stage I, four were Braak stage II, and eight were Braak stage III (Table 4.1). The post-mortem interval was less than 24 hours across the final cohort.

Table 4.1: Demographics and case characteristics stratified by Braak stage for transcriptomic dataset. Race data was reported by an informant originally in Portuguese. The English translation displayed here corresponds to: Branca, White; Negra, Black; Parda, Brown; Amarela, Asian.

Case characteristics	
Braak 0	n=6, 33% female Mean age, 48y (range: 37-62) CDR-SOB > 0: 0 (0%) CERAD Absent: 6 (100%) Thal phase 0: 6 (100%)
Braak I	n=4, 50% female Mean age, 54y (range: 46-61) CDR-SOB > 0: 0 (0%) CERAD Absent: 2 (50%) Thal phase 0: 2 (50%)

Braak II	n=4, 25% female Mean age, 73y (range: 67-91) CDR-SOB > 0: 0 (0%) CERAD Absent: 1 (25%) Thal phase 0: 0 (0%)
Braak III	n=8, 38% female Mean age, 79y (range: 72-90) CDR-SOB > 0: 1 (13%) CERAD Absent: 4 (50%) Thal phase 0: 3 (38%)
All cases	n=22, 36% female Mean age, 65y (range: 37-91) CDR-SOB > 0: 1 (5%) CERAD Absent: 13 (59%) Thal phase 0: 11 (50%) Race: White (5, 23%), Brown (6, 27%), Black (9, 41%), Asian (1, 5%), Unk (1, 5%)

RNA AND SEQUENCING QUALITY EFFECTS ON COMPUTED GENE EXPRESSION

The median (range) RIN score was 5.8 (4.1-7.3). To examine the effects of RNA quality on results, we encoded RIN score data onto a PCA applied to all sequenced samples (Figure 4.2). RIN score, along with other inter-individual differences appear to account for some of the variation along PC-1. The top ten genes load on PC-1 are all mitochondrially encoded, underscoring the contributions of inter-individual variation in RNA quality to PC-1.

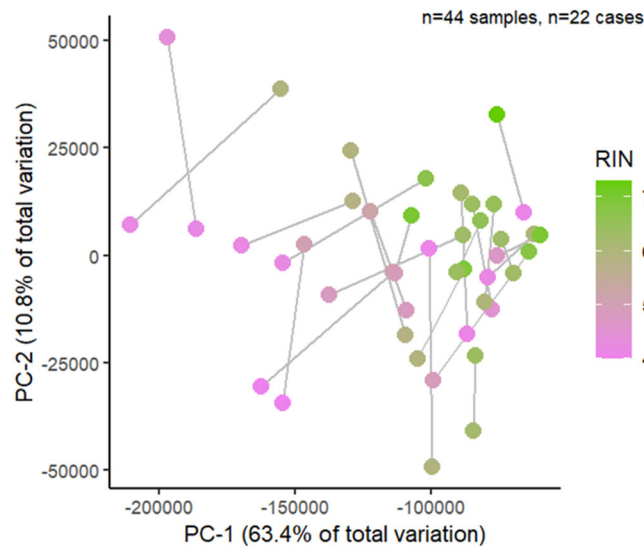


Figure 4.2: PC-1 and PC-2 are plotted for all matched samples (n=44, 50% locus coeruleus). While RIN appears to account for some of the variation along PC-1 (A), there also appear to be other inter-individual differences that account for some of the variation along PC-1 (B). PC-1 accounts for 63.4% of the variance in gene expression, and PC-2 accounts for 10.8% of the variance in gene expression.

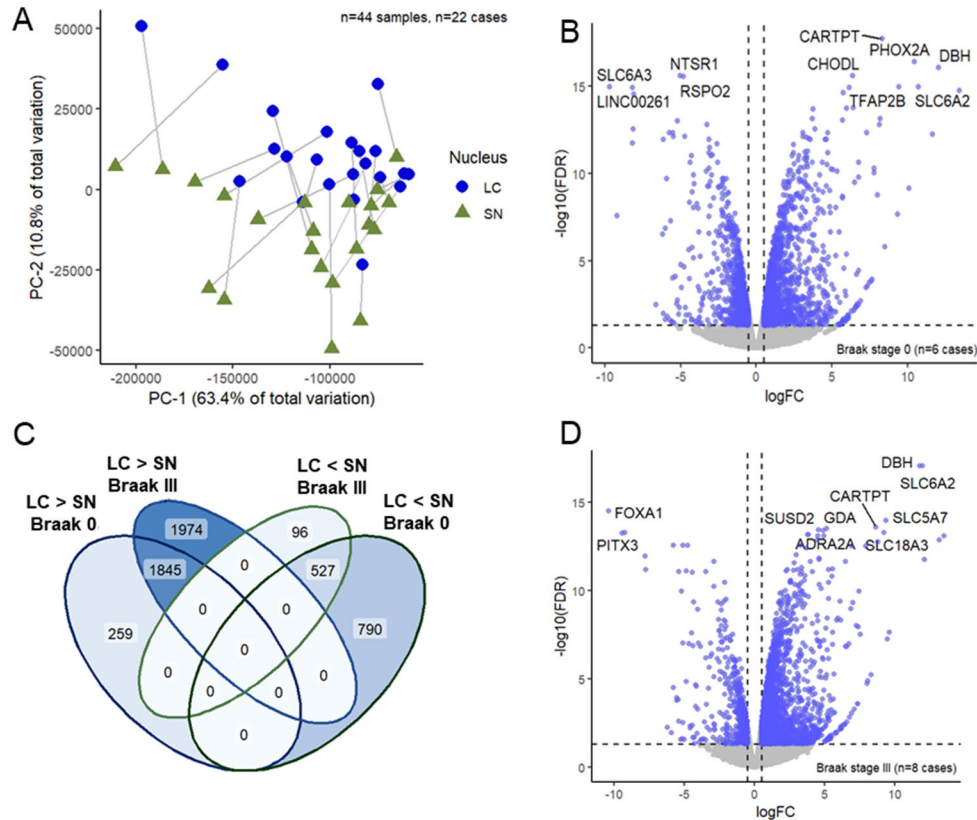


Figure 4.3: (A) The matched locus coeruleus and substantia nigra samples (n=22) segregate along PC-2 in a PCA of gene expression for all matched samples. Connecting lines between points denote the case that both samples were collected from. (B) There are 3421 DEGs between the locus coeruleus and substantia nigra in Braak stage 0 matched samples (n=6) with 2104 more expressed in the locus coeruleus than the substantia nigra and 1317 more expressed in the substantia nigra than the locus coeruleus. (D) There are 4442 DEGs between the locus coeruleus and substantia nigra in Braak stage 3 matched samples (n=8) with 3819 more expressed in the locus coeruleus than the substantia nigra and 623 more expressed in the substantia nigra than the locus coeruleus. (C) Of the 2104 genes more expressed in the locus coeruleus than the substantia nigra in Braak 0, 1845 remained more expressed in the locus coeruleus in Braak III. Of the 1317 genes more expressed in the substantia nigra than the locus coeruleus in Braak 0, 527 remained more expressed in the substantia nigra than the locus coeruleus in Braak III.

NUCLEUS IDENTITY

The transcriptomes of locus coeruleus and substantia nigra samples are compared to identify differences that may underlie the selective vulnerability of the locus coeruleus (Figure 4.3). In a PCA with all paired samples, locus coeruleus and substantia nigra distinctly separate along the second principal component (PC-2). We conducted differential expression analysis on paired samples for Braak stages 0 and 3, incorporating the participant identifier in the design formula to reflect the matched sample design. 3421 DEGs were identified between the locus coeruleus and substantia nigra among Braak stage 0 samples, 2635 DEGs between the locus coeruleus and substantia nigra in Braak stage I samples, 1662 DEGs between the locus coeruleus and substantia nigra in Braak stage II samples, and 4442 DEGs between the locus coeruleus and substantia nigra in Braak stage III samples (Supplementary Figure 4.1).

GSEA was used to analyze the DEGs identified between the locus coeruleus and substantia nigra in each Braak stage individually (Figure 4.4, Supplementary Figure 4.2). The 3421 DEGs between the locus coeruleus and substantia nigra in Braak stage 0 showed significant enrichment for 20 of the 50 gene sets from the Hallmark pathways collection (Liberzon et al., 2015) (Figure 4.4A). The 4442 DEGs between the locus coeruleus and substantia nigra in Braak stage 3 showed significant enrichment for 12 of the 50 gene sets from the Hallmark pathways collection (Figure 4.4B).

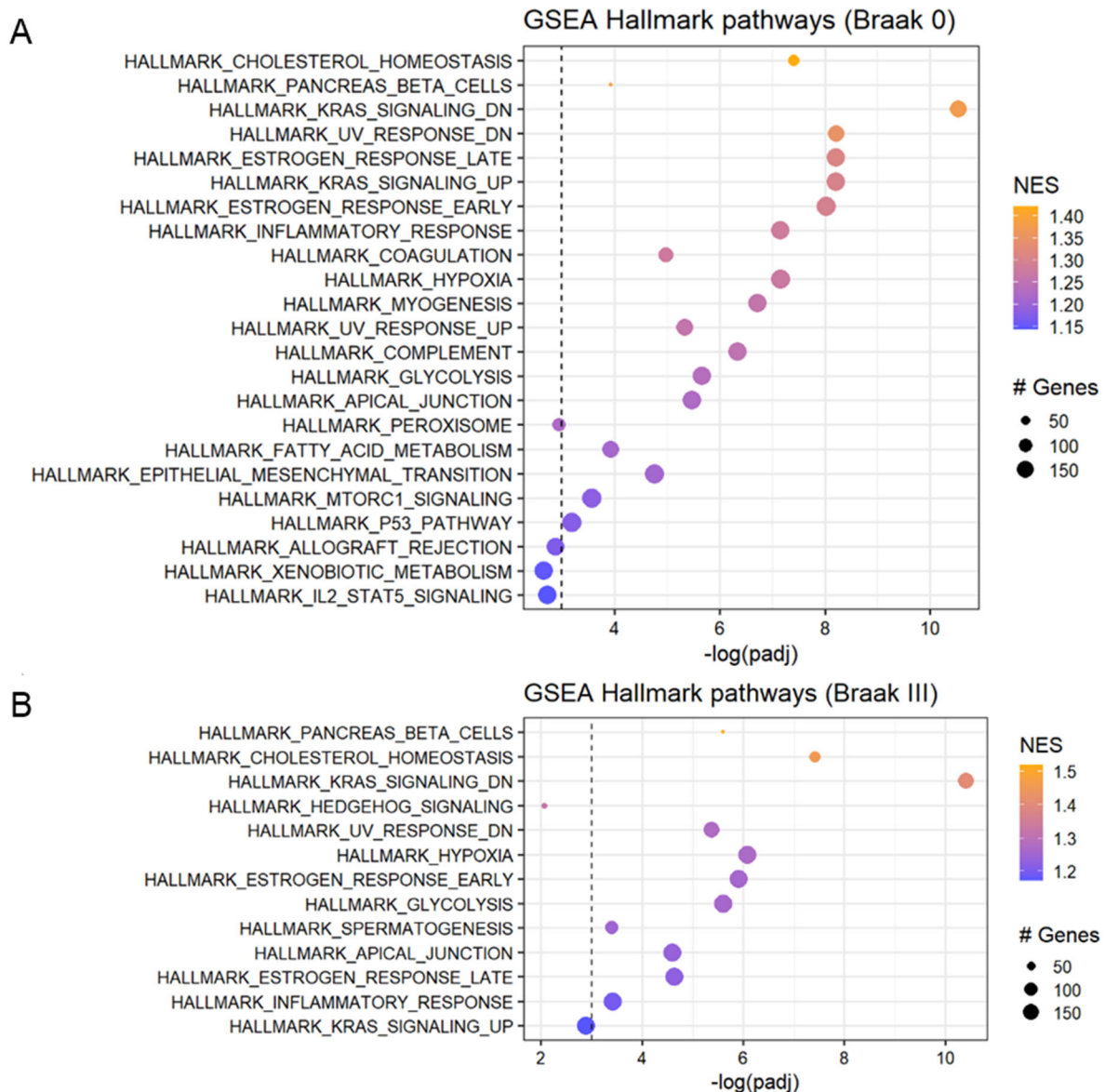


Figure 4.4: (A) In Braak stage 0, the DEGs between the locus coeruleus and substantia nigra show statistically significant ($p\text{-adj} < 0.05$) enrichment for 20 Hallmark pathways. (B) In Braak stage 3, statistically significant enrichment was found for 12 Hallmark pathways.

GLIA COMPOSITION DIFFERS BETWEEN THE LOCUS COERULEUS AND SUBSTANTIA NIGRA SAMPLES

Inflammatory pathways were among the top enriched pathways at both Braak stage 0 and Braak stage III differentiating the locus coeruleus and substantia nigra. Due to the nature of specimen collection, we considered the possibility that these results may be attributable in part to differences in cell type composition. To examine this possibility, we specifically looked at several genes previously identified (Olah et al., 2020) to differentiate clusters of microglial populations and several other non-neuronal cell types (Figure 4.5).

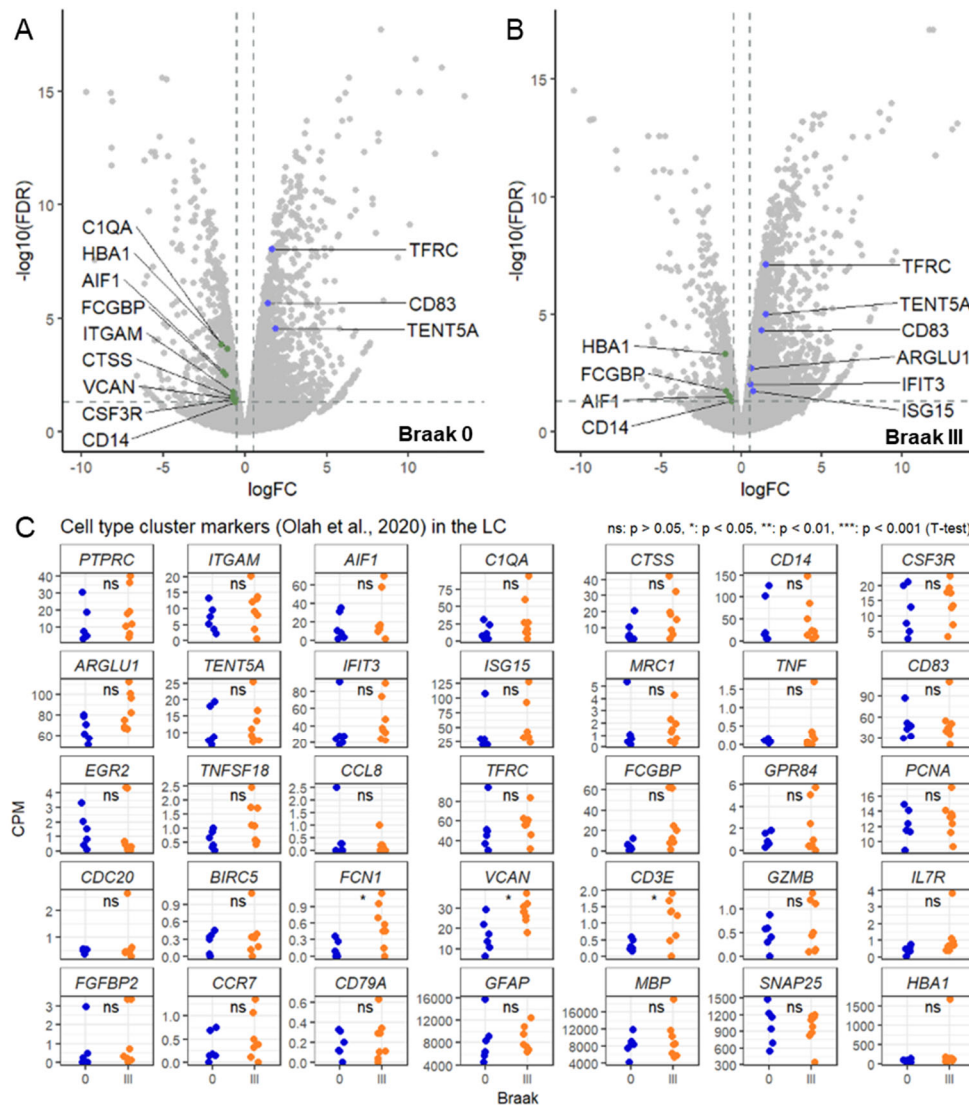


Figure 4.5: (A) Differential expression analysis of cases at Braak stage 0 with microglial subpopulation markers highlighted. Genes significantly more expressed ($\log FC < -0.5$; $FDR < 0.05$) in the substantia nigra are left-justified and green, and those more significantly expressed ($\log FC > 0.5$; $FDR < 0.05$) in the locus coeruleus are right-justified and blue. (B) There is a shift towards more microglial signatures in the locus coeruleus in Braak stage 3 cases, but key microglial markers such as AIF1 continue to be more expressed in the substantia nigra. (C) Three of the probed genes are significantly more expressed in the locus coeruleus in Braak stage III than 0 as measured by a T-test without multiple comparison correction.

Nine genes thought to be broad markers of the myeloid lineage are more expressed in the substantia nigra than the locus coeruleus at Braak stage 0 (Figure 4.5A). This suggests that the substantia nigra samples may have included a higher proportion of microglia than the locus coeruleus samples from the same cases. Three genes – *TENT5A*, *CD83*, and *TFRC* – were more expressed in the locus coeruleus than the substantia nigra in Braak stage 0 and III samples. These markers are associated with microglial clusters 3, 5, and 6 in previous work with single-cell mRNA sequencing (Olah et al., 2020). The same set of genes compared between Braak stages 0 and III locus coeruleus samples show an increase in the average transcript level of *FCN1*, *VCAN* (monocyte markers), and *CD3E* (T-cell marker) with the higher Braak stage.

ANTIOXIDANT COMPOSITION DIFFERS BETWEEN THE LOCUS COERULEUS AND SUBSTANTIA NIGRA

The DEGs between the locus coeruleus and substantia nigra at Braak stage 0 (Figure 4.6C) and III (Figure 4.6D) were examined to identify those appearing on a list of 63 antioxidant genes compiled by Gelain et al., (Gelain et al., 2009). At Braak stage 0, there were seven hits among the DEGs more expressed in the substantia nigra than the locus coeruleus. Of these seven genes, five were genes encoding metallothionein proteins. The remaining two included a peroxidase-encoding gene, *LPO*, and a superoxide dismutase, *SOD3*. The locus coeruleus at Braak stage 0 only had three DEGs related to antioxidant activity – two thioredoxin domain proteins and a peroxidase, *MPO*.

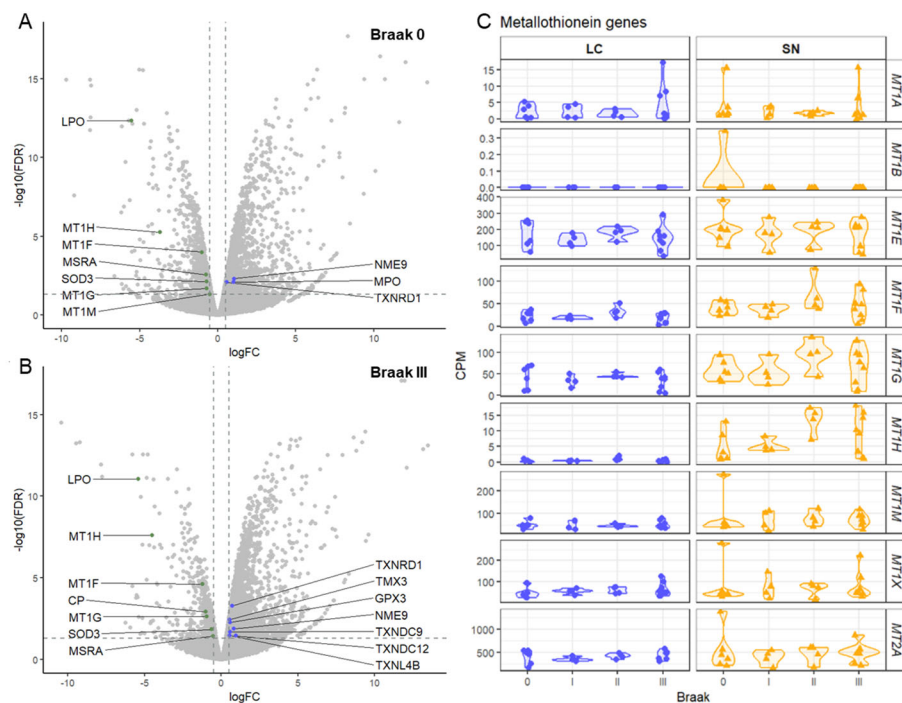


Figure 4.6: Differential expression of the locus coeruleus and substantia nigra ($\log_{2}(\text{FC}) > 0$ indicates greater expression of a gene in the locus coeruleus). Cutoffs for significance are a $\text{FDR} < 0.05$ and a $\log_{2}(\text{FC}) > 0.5$ or < -0.5 . (A-B) Significantly differentially expressed genes representing antioxidant enzymes

or antioxidant activity selected from Gelain et al., 2009 are labeled for analyses of cases at Braak stage 0 (A) and III (B). At Braak stage 0, 7 antioxidant genes are more expressed in the substantia nigra than the locus coeruleus and 3 are more expressed in the locus coeruleus than the substantia nigra. At Braak stage III, 7 antioxidant genes are more expressed in the substantia nigra than the locus coeruleus and 7 are more expressed in the locus coeruleus than the substantia nigra. (C) Metallothionein genes listed in Gelain et al., 2009 are expressed in both the locus coeruleus and substantia nigra, with some more expressed in the substantia nigra relative to the locus coeruleus.

Among DEGs more expressed in the substantia nigra than the locus coeruleus in Braak stage III cases relative to the analysis of Braak stage 0 cases, there was one fewer encoding a metallothionein protein with a gene encoding a peroxidase, *CP*, gained. Seven DEGs more expressed in the locus coeruleus than the substantia nigra in Braak stage III cases were identified including six thioredoxin domain proteins and one peroxidase, *GPX3*.

Of note, there were no genes encoding the metallothionein proteins examined here identified as DEGs more expressed in the locus coeruleus than the substantia nigra in either Braak stage. Similarly, there were no genes encoding the thioredoxin domain proteins examined here identified as more expressed DEGs in the substantia nigra than the locus coeruleus in either Braak stage. While the DEGs are functionally segregated in this regard, there is expression of metallothionein genes (Figure 4.6C) and thioredoxin domain genes in both nuclei (Figure 4.7).

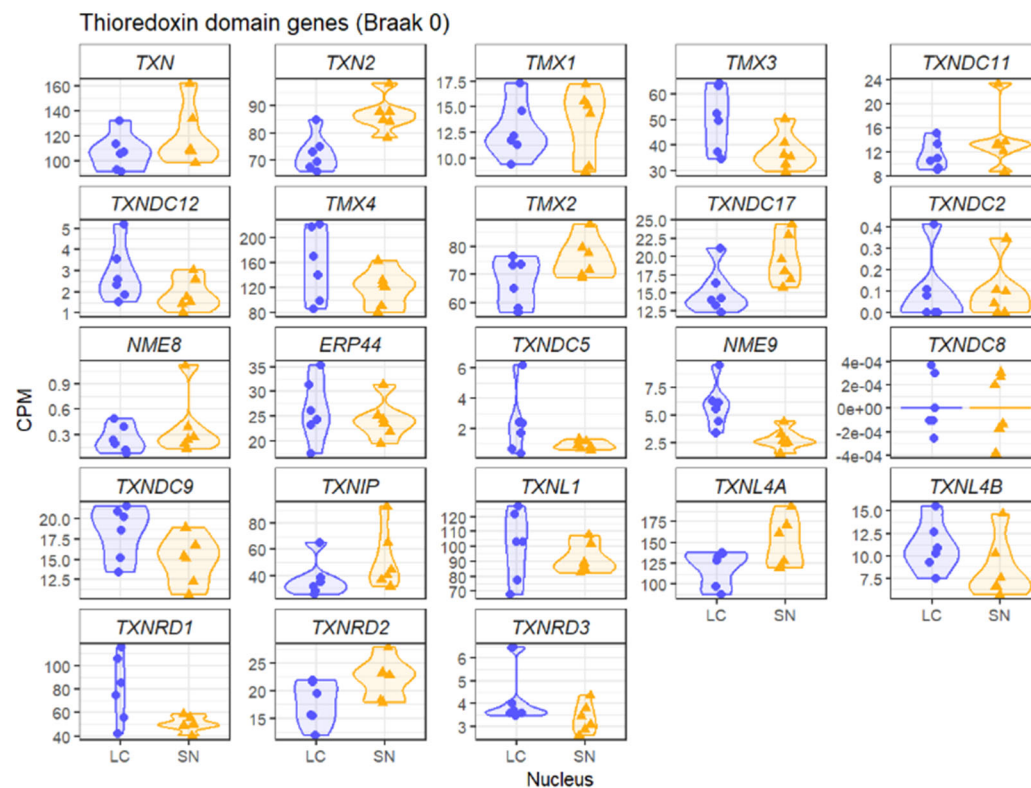


Figure 4.7: There is expression of thioredoxin domain protein genes in both the locus coeruleus and substantia nigra.

CHOLESTEROL REGULATION DIFFERS BETWEEN THE LOCUS COERULEUS AND SUBSTANTIA NIGRA AT BASELINE

Genes involved in cholesterol sensing and production were examined among Braak stage 0 cases. The selected genes included those coding proteins in the sterol regulatory element binding proteins (SREBP) and liver X receptor-retinoid X receptor (LXR/RXR) transcriptional axes, including the lanosterol and cholesterol producing pathways (Dietschy & Turley, 2004; S. H. Lee et al., 2020; Rawson, 2003; Varma et al., 2021; Yan et al., 2021). Of the 37 genes examined among Braak stage 0 cases, 6 had lower expression in the locus coeruleus relative to the substantia nigra, 21 were more highly expressed in the locus coeruleus than the substantia nigra, and 10 were not significantly differentially expressed (Figure 4.8A). The 21 genes with significantly more expression in the locus coeruleus than the substantia nigra were mostly involved in the SREBP-mediated processes of cholesterol production and influx. The 6 genes with lower expression in the locus coeruleus than the substantia nigra were mostly involved in cholesterol transport and efflux (Figure 4.8C), including *ApoE*.

STRESS HORMONE SIGNALING TO THE LOCUS COERULEUS

Because of the role of the locus coeruleus in the stress response, we examined our top hits for stress-signaling associated genes. *CRH* was significantly over-expressed in the locus coeruleus relative to the substantia nigra (FDR = 3.35e-10; logFC = 6.49) (Figure 4.9).

Discussion

Here, we used locus coeruleus and substantia nigra samples collected from a series of human postmortem cases enriched for early Alzheimer's disease-type neuropathologic stages to investigate pathways underlying the gradient of vulnerability in the neuromodulatory subcortical system in Alzheimer's disease. These nuclei were selected because they represent a vulnerable and resistant nucleus to Alzheimer's disease neuropathologic changes despite numerous similarities. Both are pigmented catecholaminergic nuclei in the reticular formation. They are cytoarchitecturally similar as isodendritic core nuclei with deep evolutionary origins in vertebrates. They are also, curiously, both similarly vulnerable to synucleinopathies. Yet, many differences were observed in gene expression even among the Braak stage 0 samples.

Certain genes represent the key differences between the locus coeruleus and substantia nigra – *DBH*, *Slc6A2* (encoding norepinephrine transporter), and *Slc6A3* (encoding dopamine transporter) – and high differential expression of these genes verifies the accuracy of dissection. Comparisons between locus coeruleus and substantia nigra show massive differences at baseline (Braak stage 0) and in intermediate stages of Alzheimer's disease progression (Braak stage III). Due to the large number of DEGs identified in these stages, GSEA was used to identify sets of pathways that differentiate the two nuclei.

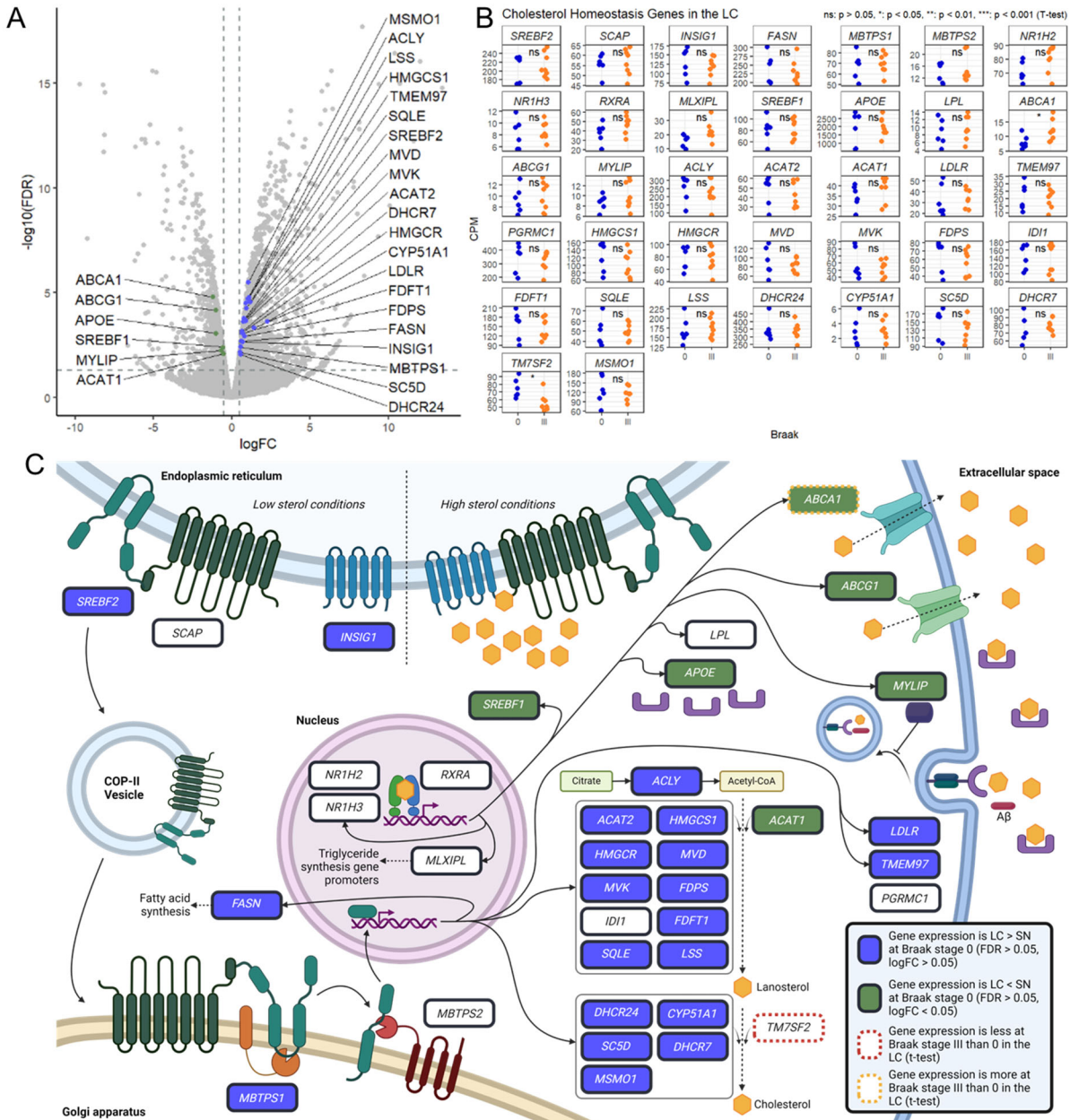


Figure 4.8: A list of 37 cholesterol-regulating or producing genes were examined among Braak stage 0 cases. (A) 6 genes (left-justified text, green dots) were less expressed in the locus coeruleus than the substantia nigra ($\logFC < -0.5$; $FDR < 0.05$) and 21 genes (right-justified text, blue dots) were more expressed in the locus coeruleus than the substantia nigra ($\logFC > 0.5$; $FDR < 0.05$). (B) Most of the examined genes did not have significantly different expression levels at Braak stage 0 versus III. At an uncorrected $p < 0.05$ (t-test), TM7SF2 was less expressed in the locus coeruleus at Braak stage III compared to Braak stage 0 and ABCA1 was more expressed in the locus coeruleus at Braak stage III compared to Braak stage 0. (C) The genes more expressed in the locus coeruleus than the substantia nigra at Braak stage 0 are mostly involved in SREBP-mediated production and influx of cholesterol. The genes less expressed in the locus coeruleus than the substantia nigra at Braak stage 0 are mostly involved in LXR/RXR-mediated transport and efflux of cholesterol.

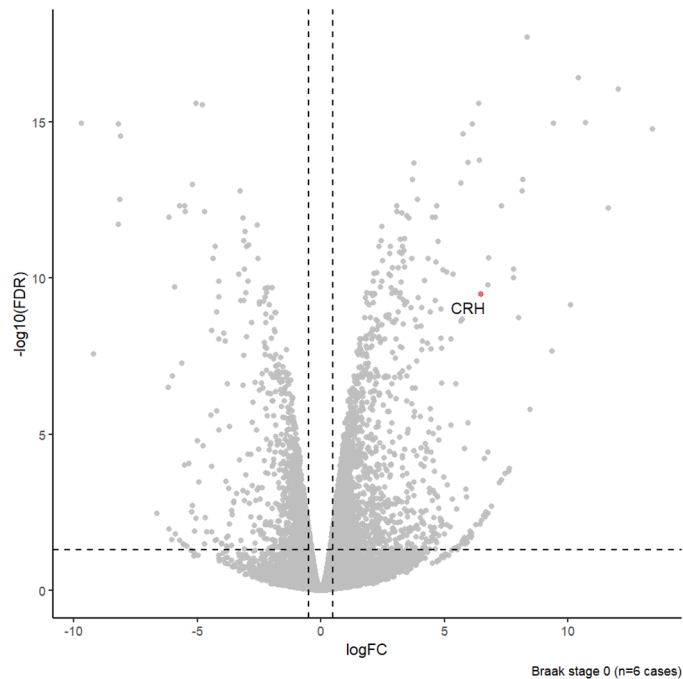


Figure 4.9: CRH is significantly over-expressed in the locus coeruleus versus the substantia nigra at baseline.

Inflammatory pathways were among the top enriched pathways for DEGs differentiating the locus coeruleus and substantia nigra. While we cannot rule out the possibility that there are differences in the baseline activation states of neuroinflammatory pathways between the locus coeruleus and substantia nigra, we are limited in our ability to conclude this versus the possibility of simple differences in cell composition. The substantia nigra appears to have a higher proportion of microglia than the locus coeruleus, except for three microglial clusters defined by previous single cell transcriptomic work, which had more signal in the locus coeruleus than the substantia nigra (Olah et al., 2020). From Braak stage 0 to III, there appears to be an increase in markers of microglia in the locus coeruleus.

We specifically chose to examine antioxidant-related genes to evaluate how differences in metabolism might relate to differential vulnerability of the two nuclei. Using a set of genes defined by Gelain et al., 2009 (Gelain et al., 2009), we found that, at baseline, the substantia nigra is enriched for genes encoding metallothionein proteins as well as a peroxidase and superoxide dismutase. The locus coeruleus had fewer DEGs associated with antioxidant activity at baseline, including two genes encoding thioredoxin domain proteins and a peroxidase. The DEGs related to antioxidant activity that were more expressed in the substantia nigra than the locus coeruleus were similar in Braak stage III cases as the baseline Braak stage 0 cases. Those DEGs related to antioxidant activity that were more expressed in the locus coeruleus than the substantia nigra were more numerous in Braak stage III cases than Braak stage 0 cases. This may suggest that antioxidant activity is increased in the locus coeruleus during early Alzheimer's disease progression but not in the substantia nigra. Our data do not

suggest that there are baseline differences in antioxidant activity in the locus coeruleus and substantia nigra, though.

Intriguingly, among the metallothionein and thioredoxin domain proteins examined in our dataset, there was segregation of DEGs by functional subtype among the two nuclei. The metallothionein protein DEGs were more expressed in the substantia nigra than the locus coeruleus and thioredoxin domain protein DEGs were more expressed in the locus coeruleus than the substantia nigra. Metallothionein proteins have a high cysteine content (Thirumoorthy et al., 2007). This allows them to function in roles beyond their antioxidant properties to include regulation of essential metals and protection of metal toxicity. Heavy metal accumulation is thought to contribute to neurodegeneration in both Alzheimer's disease and other neurodegenerative diseases such as Parkinson's (Huat et al., 2019; Montgomery, 1995). Both the locus coeruleus and substantia nigra have neuromelanin in humans, which makes them theoretically susceptible to the accumulation of high levels of heavy metals. This has been observed in both nuclei (Montgomery, 1995; Pamphlett et al., 2018) but curiously, there is some evidence to suggest that the locus coeruleus is more susceptible to heavy metal accumulation than the substantia nigra (Pamphlett & Kum Jew, 2013; Pamphlett et al., 2020). The lower expression of metallothionein transcript expression in the locus coeruleus than the substantia nigra may underlie the locus coeruleus's increased susceptibility to toxic metal accumulation.

Cholesterol synthesis and maintenance was a consistent factor differentiating the locus coeruleus and substantia nigra at the examined Braak stages. This was surprising given that locus coeruleus and substantia nigra have similarly large neuronal sizes and high degree of projections throughout the brain. About a fifth of the body's cholesterol is in the brain, most of which is in myelin sheaths (Bjorkhem & Meaney, 2004; Dietschy & Turley, 2004). The remaining brain cholesterol is mostly in astrocytic and neuronal plasma membranes to help maintain their structure and synaptic transmission. Because the brain is broadly isolated from peripheral cholesterol supply, it exhibits a very low turnover rate of cholesterol and produces its own to maintain sufficient supply. In contrast to the days-long half-life of circulating cholesterol in the body, the adult brain's cholesterol has a half-life on the scale of months to years (Andersson et al., 1990). Disruption of cholesterol maintenance and synthesis in the brain has numerous well-established links to Alzheimer's disease (Bjorkhem et al., 2006; Di Paolo & Kim, 2011; Feringa & van der Kant, 2021). Perhaps the most well-studied link between cholesterol biology and Alzheimer's disease has been the *ApoE* gene, with the minor allele, *ApoE4*, being the primary genetic risk factor for sporadic Alzheimer's disease (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). *ApoE* was among the DEGs between the locus coeruleus Alzheimer's disease substantia nigra at baseline (Braak stage 0) with less *ApoE* mRNA detected in the locus coeruleus than the substantia nigra.

The locus coeruleus exhibited a gene expression pattern at baseline that suggests that it has a higher demand for cholesterol than the substantia nigra. It appears to meet this by maintaining higher activation of the SREBP transcriptional axis, leading to more

production and influx of cholesterol, and suppression of the LXR/RXR transcriptional axis, leading to less efflux of cholesterol. Dysregulation or deterioration of the locus coeruleus's ability to produce and maintain sufficient levels of cholesterol could lead to its neuronal loss in Alzheimer's disease. This hypothesis is reinforced by work done by Varma et al. (2021) demonstrating reduced levels of cholesterol production in Alzheimer's disease (Varma et al., 2021). Curiously, Varma et al. did not observe these differences in Parkinson's disease cases. The locus coeruleus and substantia nigra are similarly vulnerable in Parkinson's disease but differentially vulnerable in Alzheimer's disease. As such, aberrant cholesterol regulation may underlie Alzheimer's disease-specific vulnerability of the locus coeruleus not underlying its vulnerability to synucleinopathies.

We observed significantly less expression of *MYLIP* in the locus coeruleus than the substantia nigra with higher expression of *LDLR* and *TMEM97*. *LDLR* and *TMEM97*, together with *PGRMC1*, form the Sigma-2 receptor protein complex at the cell membrane and internalizes extracellular lipoproteins like LDL and ApoE. This complex can also internalize amyloid- β oligomers (Colom-Cadena et al., 2024; Riad et al., 2020). IDOL (encoded by *MYLIP*) is an E3-ubiquitin ligase that degrades *LDLR* and thus, negatively regulates the internalization of lipoproteins (Zelcer et al., 2009). The pattern of gene expression observed here suggests that even at Braak stage 0, the locus coeruleus has more capacity to internalize toxic amyloid- β oligomers than the substantia nigra. Taken together, dysfunction in cholesterol homeostasis and the negative pleiotropic (i.e., amyloid- β related) effects of the machinery used to internalize extracellular cholesterol may represent an Alzheimer's disease-specific substrate for selective vulnerability of the locus coeruleus.

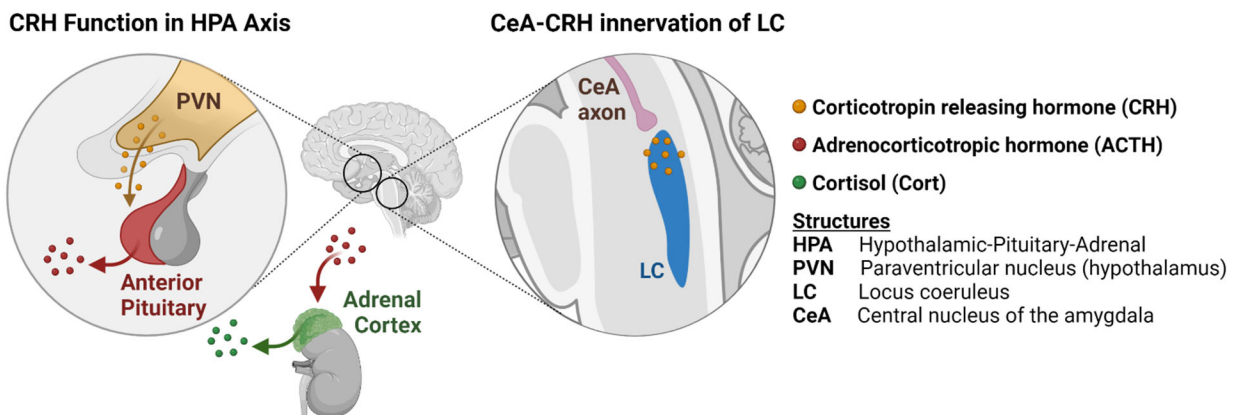


Figure 4.10: CRH mediates stress responsiveness through the HPA axis, as well as via central noradrenergic signaling.

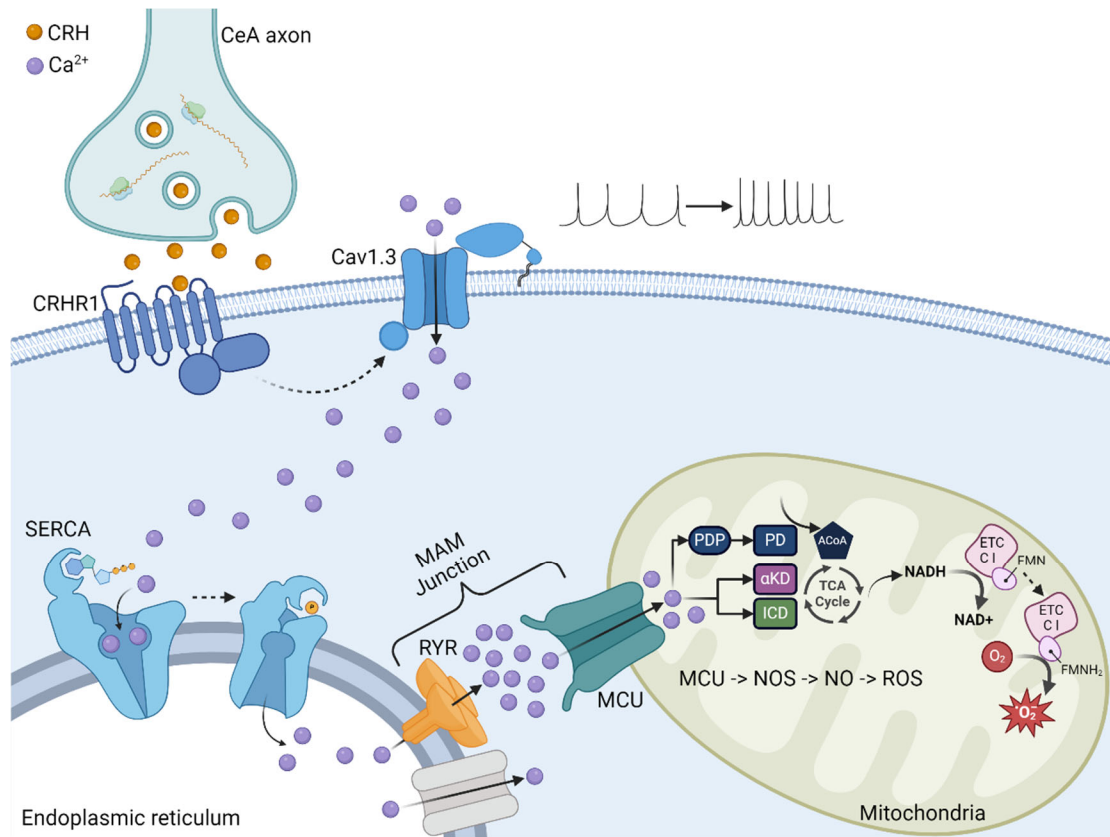


Figure 4.11: CRH signaling to the locus coeruleus may drive mitochondrial oxidative stress by way of increased tonic firing patterns. This hypothesis should be further tested in model systems.

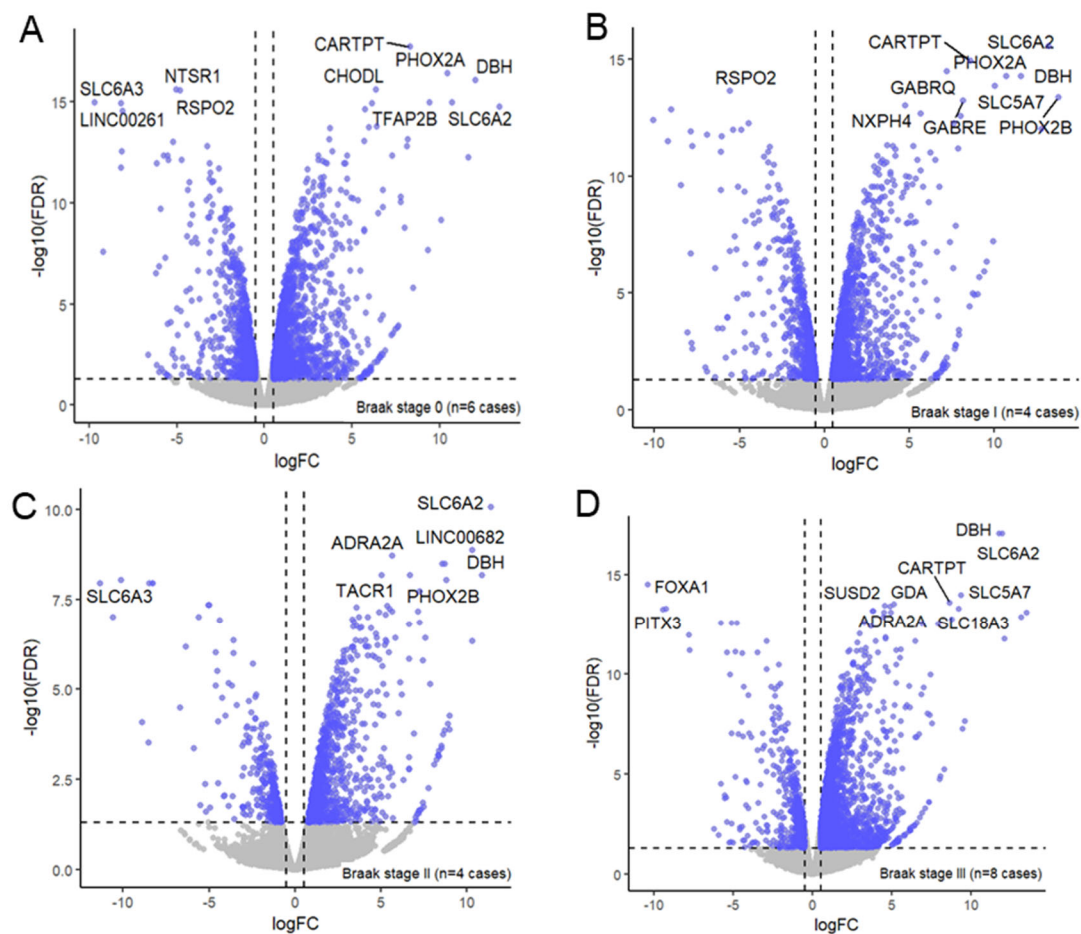
CRH was also a significantly upregulated hit in the locus coeruleus. CRH is typically studied in the context of the HPA-axis, but also has well-documented effects on the locus coeruleus by way of CRHR1 activity (Jedema & Grace, 2004; McCall et al., 2015) (Figure 4.10). The higher tonic spiking pattern induced by the CRH activity is likely to increase ROS activity by an L-type Cornu Ammonis 2⁺ channel altering NADH/NAD⁺ proportions in the mitochondria (Sanchez-Padilla et al., 2014). (Figure 4.11). Curiously, we detected high levels of *CRH* transcripts, suggesting that CRH is transcribed proximal to the synapse from projections originating at the central nucleus of the amygdala.

As with any human postmortem study, our study is limited by its cross-sectional nature. The application of Braak staging to represent disease progression requires one to make several assumptions about Alzheimer's disease pathophysiology that if not considered would require reinterpretation of some findings. Human postmortem tissue has massive heterogeneity due to both biological and technical artifacts that are unavoidable. Through PCA, we found that a significant amount of variation in gene expression can be attributed to RNA quality and other inter-individual factors not analyzed here. This emphasizes the value of using matched samples, as it minimizes the inter-individual variables that can affect measured gene expression such as post-mortem interval, biological variation (e.g., age, sex), and cause of death. We attempt to minimize the

effects of these limitations by focusing our study on the analysis of contrasting paired brain regions from individuals rather than modeling processes within a single region across individuals. This enables us to subtract out baseline differences between cases and more sensitively detect differences between regions. We also excluded cases with co-pathologies to reduce potential confounding factors. Furthermore, our study primarily interprets gene expression data at the bulk transcriptional level to include a larger number of participants, given the technical and financial limitations of single-cell 'omics' and proteomics technologies. We have refrained from making inferences based on patterns that might be explained by variations in cell composition, which ideally should be assessed using single-cell platforms as done by Weber et al. (2024) for the locus coeruleus.

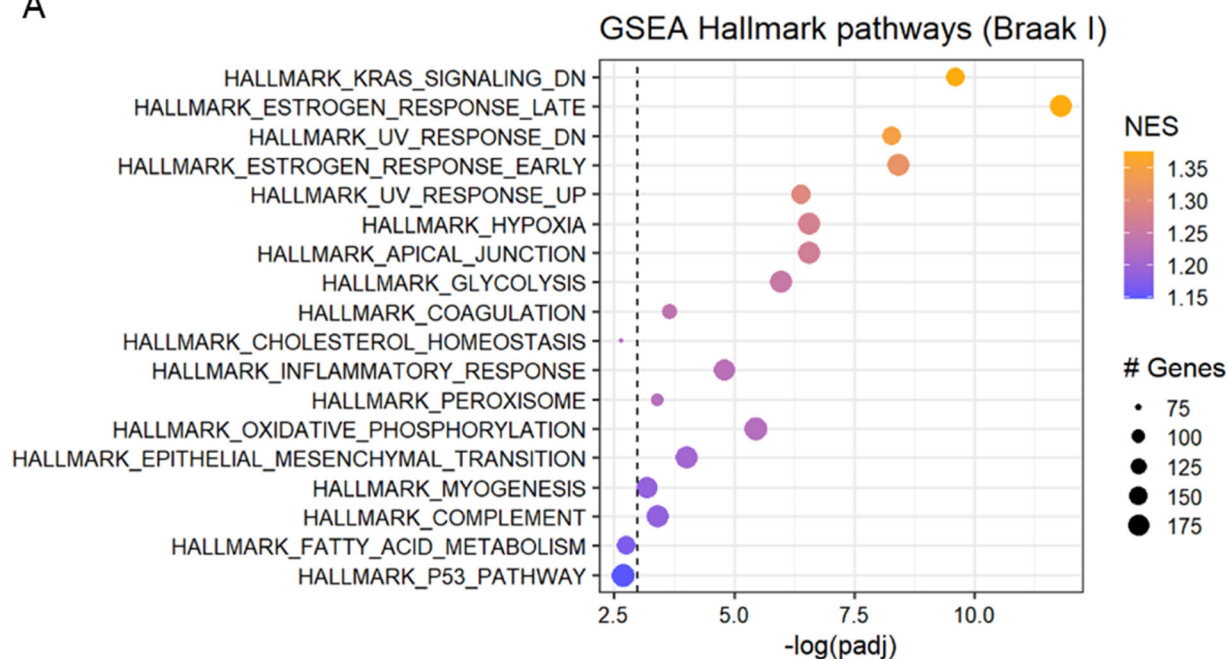
Overall, we have identified differences related to cholesterol homeostasis and synthesis, as well as heavy metal processing, that may underlie a baseline vulnerability of the locus coeruleus and substantia nigra to Alzheimer's disease. These differences may represent potential therapeutic targets after mechanistic verification. The ability to model processes in the locus coeruleus and substantia nigra has grown significantly in recent years. Transgenic mouse models allow inducible control of gene expression in dopaminergic and noradrenergic neurons (Abaira et al., 2017; Stubbusch et al., 2011). *In vitro* modeling of locus coeruleus and substantia nigra neurons are even now feasible using human pluripotent stem cells (Kim et al., 2021; Piao et al., 2021; Tao et al., 2023). These platforms enable thorough adjudication of hypotheses generated from datasets such as the one we present here. Gaining improved understanding of the causal factors driving degeneration of the locus coeruleus and capacity to modify its vulnerability may have an outsized effect in Alzheimer's disease and other neurodegenerative and stress disorders.

Supplementary Figures

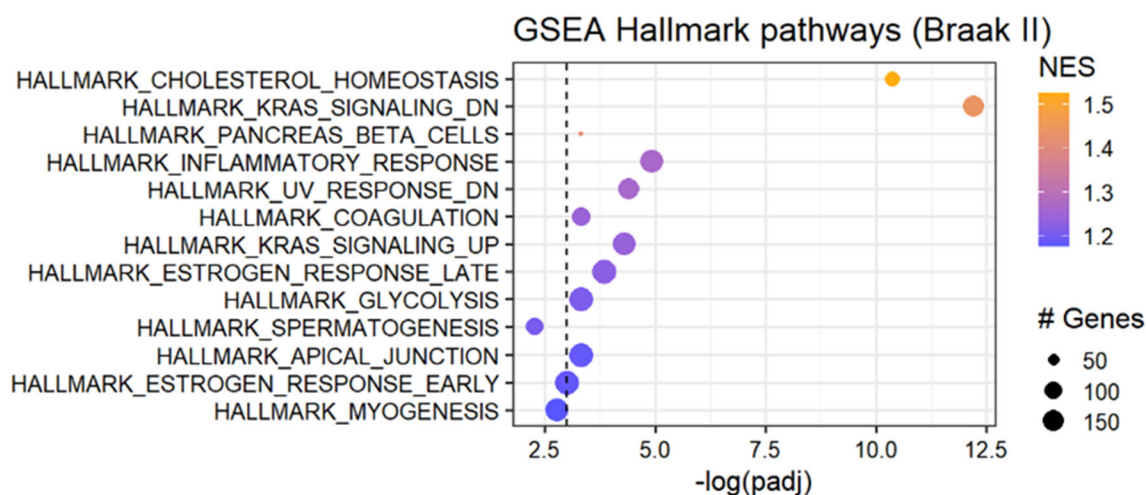


Supplementary Figure 4.1: Differentially expressed genes by Braak stage.

A



B



Supplementary Figure 4.2: GSEA of the Hallmark pathways in DEGs between the locus coeruleus and substantia nigra for Braak stage I and Braak stage II cases.

Chapter 5: Conclusion

Selective vulnerability driving a new research paradigm for neurodegeneration

Neurodegenerative diseases are growing in prevalence yet remain with lackluster treatment options. It is exceptionally challenging to fully capture the clinically-relevant patterns of neurodegeneration in model systems, but gain enough molecular resolution to observe disease processes in humans. Studies of psychiatric disorders like PTSD are challenged by many of the same problems. Selective vulnerability research as an academic framework promotes carefully-guided hypothesis generation based on unbiased, systematic observational work. Importantly, it facilitates translational efforts between model-based mechanistic work and clinical spaces (Figure 5.1).

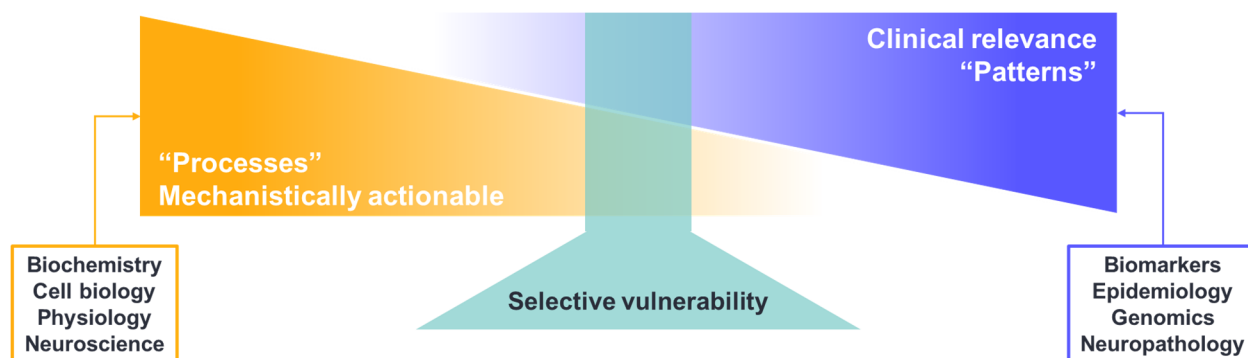


Figure 5.1: Selective vulnerability promotes translational work critical for neurodegenerative disease therapeutic development.

SCIENTIFIC REVOLUTIONS: HISTORICAL PRECEDENT

Throughout the 1970s, basic researchers supported by the National Cancer Act raced to elaborate the details of the world of what would be called retroviruses. Dozens of 'oncogenic RNA viruses' had been identified in animals (Green, 1970). Even so, their inner workings eluded the imagination of biochemists entrenched in the central dogma. Two serendipitous discoveries of reverse transcriptase (Baltimore, 1970; Temin & Mizutani, 1970) fortunately equipped biochemists with tools to assay for retroviruses in a convenient, sensitive way, giving rise to the discoveries of HTLV-1 and HTLV-2 (Kalyanaraman et al., 1982; Poiesz et al., 1980). The decades-long race, and accompanying methodological developments, surrounding retroviruses proved prescient to the emerging AIDS crisis in the 1980s.

Three years after the first clusters of *Pneumocystis pneumonia* and Kaposi's Sarcoma were reported, the discovery of Lymphadenopathy associated virus and HTLV-3, both later known as Human Immunodeficiency Virus (HIV), were nearly simultaneously announced as suspected pathogenic agents underlying what was at this point called

AIDS. Within a year, the Food and Drug Administration authorized a blood test for HIV drawing upon the tools developed for retrovirus research. The elucidation of the world of retroviruses, flying in the face of the central dogma, eventually led to effective treatments with the first reverse-transcriptase inhibitor approved in 2001.

While AIDS emerged against a background of exploration in the underlying biology, dementia research has struggled to gain its footing. Enough of the world of retroviruses had been understood such that solutions could be engineered to meet the needs of AIDS patients even in the mid-1980's. Since the dawn of the amyloid cascade hypothesis around the same time, though, the Alzheimer's research enterprise has largely tried – and failed – to engineer itself out of needing to deeply understand the world at its foundation.

On one side of the “valley of death”, clinical researchers have been compelled to apply the established frameworks for understanding the world of disease to attempt to describe neurodegeneration. Unfortunately, these established frameworks force the conceptual landscape of neurodegeneration into a linear, coarse model of pathophysiology. How can we possibly blame the field for this, though? The public is desperate for interventions, and it is sensible to respond to desperation with first principles – in this case, the classic pathophysiologic motifs engrained into the fabric of biomedical research. The other side of the valley has not yet provided clinical researchers with a useful elaboration of what the biological world of neurodegeneration and aging is like and clinical researchers are only working within the frameworks they know.

The Alzheimer's research enterprise needs to fundamentally rethink what the world of disease can be like with the best tools and biology available. Without being able to interrogate the supposed pathophysiologic models with the kinds of tools available to biochemists, cell biologists, and other biomedical scientists, clinical researchers are unable to meaningfully accept or, most importantly, refute any conceptual models. The enterprise is left without being able to distinguish between effectors and outcomes because the specialists trained with the fundamental tools to do so are out of sight across the valley.

Venturing across the valley into the world occupied by biologists, scientists are scrambling without a cogent vision for what is needed by patients. While they do have the tools to manipulate systems and elaborate out this strange pathophysiologic world, they are disincentivized from doing so. How else should they respond to a “biological definition of Alzheimer's disease” that conflates epistemological definitions of outcome and effector? Should a scientist elaborating complex molecular systems surrounding degeneration do so without referring to pathognomonic components of the ‘definition’ in their model, it is difficult to claim that their research program has any disease relevance. Models engineered to be relevant to disease definitions may lose biological relevance and fail to elaborate out fundamental processes. Even though pathognomonic features can only be created in models under fundamentally weird (e.g., transgenic) conditions, carrying biologically relevant findings that do not include pathognomonic features of

across the valley of death is somewhere between improbable and impossible. Potent therapeutic targets that could mitigate underlying disease vulnerability or key pathways that usefully index aging are likely to get lost across the valley. Aging and neurodegeneration seem to be emergent properties of complex systems. Accordingly, there probably is not a silver bullet like the identification of retroviruses that upturns *the* central dogma of biology. So how can the Alzheimer's research enterprise achieve comparable paradigm-shifting breakthroughs to usefully elaborate out the world of disease?

Summary of chapters

Here, I have sought a better understanding of neurodegenerative pathophysiology by parallel investigations of a potent risk factor, social isolation, and established patterns of selective vulnerability. In Chapter 2, I attempted to model long-term social isolation in a mouse model. While some associations were found in stress hormone signaling, blood-brain-barrier dysfunction, and differences in renal structure, I cannot draw vigorous conclusions about how social isolation influences age-associated pathophysiology. Due to social dynamics in laboratory mice, they are unlikely to be reliable organisms for studying the clinically relevant effects of social isolation.

In Chapter 3, I examined how a Ras-GTPase, Rhes, changes in relation to tau proteinopathic lesions in sporadic tauopathies. As was examined in models of familial tauopathies, I identified dysregulation and eventual clearance of the protein Rhes in Alzheimer's disease-affected neurons. Taking our data together with the previous work on Rhes, the high co-incidence of Absent type neurons with PHF-1 positive inclusions may be explained by the established role of Rhes in autophagy modulation (Hernandez et al., 2019; Mealer et al., 2014; Pan et al., 2009). This suggests that the application of a farnesyltransferase inhibitor such as Lonafarnib could effectively mitigate tau-associated neurodegeneration.

Finally, in Chapter 4, I identified possible mechanisms for selective vulnerability of early-affected structures in Alzheimer's disease. By comparing a known nucleus of vulnerability, the locus coeruleus, to a nucleus of resilience, the substantia nigra, in individuals free of neurodegenerative changes, I identified several pathways that may underlie selective vulnerability. Critically, differences in cholesterol homeostasis and differences in the signaling of CRH to the locus coeruleus and not the substantia nigra are potential drivers of pathogenesis.

Future directions

Given the obvious role of social isolation as a risk factor for neurodegeneration, Additional attempts to understand pathogenicity are warranted. As tools like spatial transcriptomics and single-cell omics become more available with more diverse postmortem cohorts including longitudinal psychosocial phenotyping, mechanisms could

be explored in humans. These pathways could also be modeled in more socially-bonded mammals, including rats, voles, and non-human primates.

Farnesyltransferase inhibitors such as Lonafarnib are safe and effective for other indications, like progeria. Given the evidence base built by model systems and human postmortem tissue across familial and sporadic tauopathies, a clinical trial of farnesyltransferase inhibitors in early (e.g., mild cognitive impairment) stages of biomarker-positive tauopathies is warranted.

The neuropathologic data featuring cholesterol homeostasis and stress-hormone signaling as substrates for selective vulnerability in early-affected regions presents several routes for possible investigation. A mouse model examining the hypothesis that CRH signaling to the locus coeruleus might affect stress-induced selective vulnerability is already underway. A mouse model containing a tamoxifen-inducible knockdown of CRHR1 in noradrenergic neurons has been generated. This model will be run through a chronic unpredictable stress model and evaluated with behavioral paradigms and postmortem. Investigations of cholesterol homeostasis pathways in the locus coeruleus are also warranted, which could be explored *in vivo* and *in vitro* using newly established cell culture protocols (Tao et al., 2023).

Taken together, this dissertation establishes that translational work focusing on risk factors and substrates for selective vulnerability may confer novel routes for therapeutic development for neurodegeneration. Conventional paradigms for investigating disease pathogenesis have largely failed to significantly improve outcomes of neurodegeneration. A new paradigm is warranted. Tools available to researchers – patient-derived cell lines, -omics in postmortem tissue, novel biomarkers – enable a paradigm shift in how an evidence base for therapeutic development can be generated. These tools, when deployed carefully, should drive hypothesis generation. Such hypotheses need to be evaluated multi-modally in a manner that supports improved outcomes for patients and their caretakers. Considering the larger historical scheme of scientific revolutions (Kuhn, 1962), we probably lack the conceptual framework to even structure meaningful questions about neurodegenerative disease. As we embrace emerging technologies, we will journey through swift tides of change in the neurodegenerative disease space, unveiling a world of better understanding of the brain and, most importantly, more hope for patients.

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Appendices

Appendix 1.1: Ehrenberg et al., 2020 authorship and acknowledgments

RELEVANCE OF BIOMARKERS ACROSS DIFFERENT NEURODEGENERATIVE DISEASES

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AUTHORS' CONTRIBUTIONS

AJE coordinated the writing of the review and was responsible for integrating the input from all authors. AK contributed to sections involving clinical trial implementation and fluid biomarkers. EC contributed to sections involving PET imaging. MJB contributed to sections involving MRI. FC contributed to sections involving epidemiology. EHT and MJ contributed to sections involving fluid biomarkers. KS contributed to sections involving biomarker implementation. TB contributed to sections involving novel tools for fluid biomarkers. PNEY contributed to sections involving neuroimaging. WJ, SFC, and JBP provided intellectual guidance on sections related to neuroimaging. TL and LTG provided intellectual guidance on sections related to neuropathology. NM, NJA, JH, and HZ provided intellectual guidance on sections related to fluid biomarkers. MS provided guidance on the coordination of authors and neuroimaging biomarkers. RWP provided guidance on the coordination of authors and served as the senior author. All authors reviewed and approved the final manuscript.

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Appendix 1.2: Ehrenberg et al., 2023 authorship and acknowledgments

PRIORITIES FOR RESEARCH ON NEUROMODULATORY SUBCORTICAL SYSTEMS IN ALZHEIMER'S DISEASE: POSITION PAPER FROM THE NEUROMODULATORY SUBCORTICAL SYSTEM PIA OF ISTAART

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Appendix 2.1: Blood-brain-barrier genes

<i>B2M</i>	<i>NR2F2</i>	<i>GABRA1</i>	<i>ITGA3</i>	<i>CCL7</i>	<i>IL6</i>	<i>ADM</i>	<i>RAB5B</i>
<i>TAPBP</i>	<i>NRP1</i>	<i>GABRA2</i>	<i>ITGAM</i>	<i>CCL8</i>	<i>IL6R</i>	<i>AGGF1</i>	<i>TFR2</i>
<i>CDH5</i>	<i>PACSIN2</i>	<i>GABRA3</i>	<i>ITGAV</i>	<i>CD248</i>	<i>IL6ST</i>	<i>CSPG4</i>	<i>TFRC</i>
<i>HMGB1</i>	<i>PANX1</i>	<i>GABRA4</i>	<i>ITGB1</i>	<i>CDC42EP4</i>	<i>IL7</i>	<i>GJA1</i>	<i>VLDLR</i>
<i>ICAM1</i>	<i>PANX2</i>	<i>GABRA5</i>	<i>ITGB5</i>	<i>CHI3L1</i>	<i>IL9</i>	<i>MMP9</i>	<i>ABCB1</i>
<i>NLRP3</i>	<i>PANX3</i>	<i>GABRA6</i>	<i>ITGB8</i>	<i>CSF1</i>	<i>KITLG</i>	<i>TIMP1</i>	<i>ABCC4</i>
<i>PECAM1</i>	<i>PARD3</i>	<i>GABRB1</i>	<i>LAMA1</i>	<i>CSF2</i>	<i>LIF</i>	<i>TIMP2</i>	<i>ABCC9</i>
<i>TEK</i>	<i>PDAP1</i>	<i>GABRB2</i>	<i>LAMA2</i>	<i>CSF3</i>	<i>LRRK2</i>	<i>TIMP4</i>	<i>ABCG2</i>
<i>VCAM1</i>	<i>PDCD10</i>	<i>GABRB3</i>	<i>LAMA3</i>	<i>CX3CL1</i>	<i>LTA</i>	<i>ACTA2</i>	<i>AGER</i>
<i>ADGRA2</i>	<i>PDGFB</i>	<i>GABRD</i>	<i>LAMA4</i>	<i>CXCL1</i>	<i>MIF</i>	<i>DES</i>	<i>APOA1</i>
<i>AKAP12</i>	<i>PLN</i>	<i>GABRE</i>	<i>LAMA5</i>	<i>CXCL10</i>	<i>NFKB1</i>	<i>PDGFRB</i>	<i>BIN1</i>
<i>AMHR2</i>	<i>PLXNA1</i>	<i>GABRG1</i>	<i>LAMB1</i>	<i>CXCL13</i>	<i>PPIA</i>	<i>ACTG2</i>	<i>CLTA</i>
<i>ANPEP</i>	<i>PTCH1</i>	<i>GABRG2</i>	<i>LAMB2</i>	<i>CXCL2</i>	<i>PTGES</i>	<i>ACVR1</i>	<i>CLTB</i>
<i>BMX</i>	<i>SEMA3G</i>	<i>GABRG3</i>	<i>LAMB3</i>	<i>CXCL5</i>	<i>PTGS2</i>	<i>ACVR1B</i>	<i>CLTC</i>
<i>CCM2</i>	<i>SERPINE1</i>	<i>GABRP</i>	<i>LAMB4</i>	<i>CXCL6</i>	<i>PTPRC</i>	<i>ACVR1C</i>	<i>CLTCL1</i>
<i>CD163</i>	<i>SERPINE3</i>	<i>GABRQ</i>	<i>LAMC1</i>	<i>CXCL8</i>	<i>PTX3</i>	<i>ACVR2A</i>	<i>DNM1</i>
<i>CD2AP</i>	<i>SHH</i>	<i>GABRR1</i>	<i>LAMC2</i>	<i>CXCL9</i>	<i>TBK1</i>	<i>ACVR2B</i>	<i>DNM2</i>
<i>CD40LG</i>	<i>SMO</i>	<i>GABRR2</i>	<i>LAMC3</i>	<i>EBI3</i>	<i>TGFB1</i>	<i>ACVRL1</i>	<i>DNM3</i>
<i>CDC42</i>	<i>SPP1</i>	<i>GABRR3</i>	<i>MMP1</i>	<i>FKBP4</i>	<i>TLR4</i>	<i>ALB</i>	<i>FABP4</i>
<i>CDH1</i>	<i>SRC</i>	<i>GNDF</i>	<i>MMP14</i>	<i>GRN</i>	<i>TNF</i>	<i>BMPR1A</i>	<i>HSPA8</i>
<i>CDH2</i>	<i>STMN3</i>	<i>GNG4</i>	<i>MMP15</i>	<i>IFNA2</i>	<i>TNFRSF1A</i>	<i>BMPR1B</i>	<i>LRP1</i>
<i>CLEC11A</i>	<i>TAGLN</i>	<i>GRIA1</i>	<i>MMP16</i>	<i>IFNB1</i>	<i>TNFRSF1B</i>	<i>BMPR2</i>	<i>LRP2</i>
<i>CNN1</i>	<i>TF</i>	<i>GRIA2</i>	<i>MMP17</i>	<i>IFNG</i>	<i>TNFSF10</i>	<i>ENG</i>	<i>LSR</i>
<i>CNN2</i>	<i>VEGFA</i>	<i>GRIA3</i>	<i>MMP2</i>	<i>IFNL2</i>	<i>TNFSF12</i>	<i>LTBP4</i>	<i>PPT1</i>
<i>DAB1</i>	<i>VEGFC</i>	<i>GRIA4</i>	<i>MMP3</i>	<i>IFNL3</i>	<i>TNFSF13</i>	<i>SMAD2</i>	<i>RAB11A</i>
<i>DAGLA</i>	<i>VTN</i>	<i>GRIK1</i>	<i>MYL9</i>	<i>IGKC</i>	<i>TNFSF13B</i>	<i>SMAD3</i>	<i>RAB11B</i>
<i>DAGLB</i>	<i>ESAM</i>	<i>GRIK2</i>	<i>NCAN</i>	<i>IL10</i>	<i>TNFSF14</i>	<i>SMAD4</i>	<i>RAB34</i>
<i>DLG4</i>	<i>ITGA1</i>	<i>GRIK3</i>	<i>NID1</i>	<i>IL11</i>	<i>TNFSF8</i>	<i>SNCG</i>	<i>RAB4A</i>
<i>EFNB2</i>	<i>ITGA6</i>	<i>GRIK4</i>	<i>NID2</i>	<i>IL12A</i>	<i>TREM2</i>	<i>TGFB2</i>	<i>RAB4B</i>
<i>EHD2</i>	<i>ADCY1</i>	<i>GRIK5</i>	<i>STAT3</i>	<i>IL12B</i>	<i>TSLP</i>	<i>TGFB3</i>	<i>RAB7A</i>
<i>FGF2</i>	<i>ALPL</i>	<i>GRIN1</i>	<i>TIMP3</i>	<i>IL13</i>	<i>CXCL12</i>	<i>TGFBR1</i>	<i>RAB7B</i>
<i>GJA3</i>	<i>APOE</i>	<i>GRIN2A</i>	<i>TNC</i>	<i>IL15</i>	<i>DUSP3</i>	<i>TGFBR2</i>	<i>RIN3</i>
<i>GJA4</i>	<i>APP</i>	<i>GRIN2B</i>	<i>TNR</i>	<i>IL16</i>	<i>IFI27</i>	<i>TGFBR3</i>	<i>SLC16A2</i>
<i>GJA5</i>	<i>CDKN1A</i>	<i>GRIN2C</i>	<i>VCAN</i>	<i>IL17A</i>	<i>IFNAR1</i>	<i>CGN</i>	<i>SLC22A1</i>
<i>GJB6</i>	<i>CDKN2A</i>	<i>GRIN2D</i>	<i>ALDOA</i>	<i>IL17F</i>	<i>IL10RB</i>	<i>CLDN1</i>	<i>SLC22A2</i>
<i>GJC2</i>	<i>GPX3</i>	<i>GRIN3A</i>	<i>LDHA</i>	<i>IL18</i>	<i>LY6E</i>	<i>CLDN12</i>	<i>SLC22A3</i>
<i>GJC3</i>	<i>MAPK14</i>	<i>GRIN3B</i>	<i>NOS3</i>	<i>IL19</i>	<i>VWF</i>	<i>CLDN3</i>	<i>SLC27A1</i>
<i>GJD2</i>	<i>MTOR</i>	<i>HCN2</i>	<i>PKM</i>	<i>IL1A</i>	<i>SPOCK2</i>	<i>CLDN5</i>	<i>SLC27A4</i>
<i>GKN2</i>	<i>NCF1</i>	<i>KCNJ8</i>	<i>CCL1</i>	<i>IL1B</i>	<i>VIM</i>	<i>F11R</i>	<i>SLC2A1</i>
<i>HEBP1</i>	<i>NCF2</i>	<i>KCNQ2</i>	<i>CCL11</i>	<i>IL1RN</i>	<i>CAT</i>	<i>JAM3</i>	<i>SLC2A13</i>
<i>HGF</i>	<i>NCF4</i>	<i>NRGN</i>	<i>CCL13</i>	<i>IL2</i>	<i>DNMT1</i>	<i>MARVELD2</i>	<i>SLC32A1</i>

<i>HTRA1</i>	<i>NFE2L2</i>	<i>SYN1</i>	<i>CCL15</i>	<i>IL20</i>	<i>DOT1L</i>	<i>OCN</i>	<i>SLC38A5</i>
<i>INS</i>	<i>OGG1</i>	<i>SYP</i>	<i>CCL17</i>	<i>IL21</i>	<i>GAD1</i>	<i>TJP1</i>	<i>SLC5A1</i>
<i>INSR</i>	<i>PPARGC1A</i>	<i>ADAMTS1</i>	<i>CCL19</i>	<i>IL22</i>	<i>HDAC1</i>	<i>TJP2</i>	<i>SLC5A3</i>
<i>KLF4</i>	<i>PRNP</i>	<i>BCAN</i>	<i>CCL2</i>	<i>IL23A</i>	<i>HDAC2</i>	<i>TJP3</i>	<i>SLC5A6</i>
<i>KRIT1</i>	<i>SIRT1</i>	<i>BGN</i>	<i>CCL20</i>	<i>IL25</i>	<i>KDM1A</i>	<i>CAV1</i>	<i>SLC7A5</i>
<i>L1CAM</i>	<i>SOD1</i>	<i>COL1A1</i>	<i>CCL21</i>	<i>IL26</i>	<i>OGT</i>	<i>CAV2</i>	<i>SMPD1</i>
<i>LEPR</i>	<i>SOD2</i>	<i>COL4A1</i>	<i>CCL22</i>	<i>IL27</i>	<i>PARP1</i>	<i>CAV3</i>	<i>TAP1</i>
<i>LRP8</i>	<i>TP53</i>	<i>COL4A2</i>	<i>CCL23</i>	<i>IL2RA</i>	<i>PRKAA1</i>	<i>CUBN</i>	<i>VAMP2</i>
<i>LYVE1</i>	<i>TXN</i>	<i>CRB1</i>	<i>CCL24</i>	<i>IL3</i>	<i>SESN1</i>	<i>MFSD2A</i>	<i>FLT1</i>
<i>MAP2</i>	<i>TXNRD1</i>	<i>CSPG5</i>	<i>CCL25</i>	<i>IL31</i>	<i>SESN2</i>	<i>PICALM</i>	<i>FLT4</i>
<i>MAPT</i>	<i>AVPR1A</i>	<i>CTNNB1</i>	<i>CCL26</i>	<i>IL32</i>	<i>SESN3</i>	<i>PLVAP</i>	<i>KDR</i>
<i>MARVELD3</i>	<i>BDNF</i>	<i>DAG1</i>	<i>CCL27</i>	<i>IL33</i>	<i>SIRT2</i>	<i>RAB11FIP1</i>	
<i>MGLL</i>	<i>BGLAP</i>	<i>HAPLN1</i>	<i>CCL3</i>	<i>IL34</i>	<i>SIRT6</i>	<i>RAB11FIP2</i>	
<i>MYLK</i>	<i>GABBR1</i>	<i>HAPLN2</i>	<i>CCL4</i>	<i>IL4</i>	<i>TET1</i>	<i>RAB11FIP3</i>	
<i>NGF</i>	<i>GABBR2</i>	<i>HSPG2</i>	<i>CCL5</i>	<i>IL5</i>	<i>UCP2</i>	<i>RAB5A</i>	