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Epigenetic Regulation of Neurogenesis in Non-Demented Humans with Alzheimer's Disease Neuropathology

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Epigenetic Regulation of Neurogenesis in Non-Demented Humans with Alzheimer's Disease Neuropathology

by

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Dedication

In memory of all those who have volunteered their brain to medical science, that we might gain an understanding of Alzheimer's, and liberate others from suffering its declines.

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Epigenetic Regulation of Neurogenesis in Non-Demented Humans with Alzheimer's Disease Neuropathology

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This project was designed to investigate the role of neurogenesis and its epigenetic regulation by microRNA in the preservation of cognition against Alzheimer's disease. This was accomplished by comparing by immunohistochemistry the capacity for neurogenesis in the subgranular zone of the hippocampus in 4 distinct populations of human subject's tissue representing the full disease, intermediate cognitive decline, healthy controls, and a poorly characterized group who have all of the histopathological hallmarks of the full disease but are cognitively normal.

To investigate the microRNAs of interest in the correct context, as microRNA expression can vary significantly by region, the granular cell layer and the subgranular zone were microdissected from the same subjects investigated for neurogenesis. This was paired with an investigation of neurogenesis and the same microRNAs in a mouse model of metabolic syndrome, as metabolic syndrome is one of the major risk factors for the development of AD. These animals had previously demonstrated memory deficits when raised on a high-fat diet.

Cognitively impaired human subjects showed relative decreases in neurogenic capacity. Meanwhile those with histopathology but without cognitive decline demonstrated a significantly enhanced capacity for neurogenesis, in spite of the usually deleterious accumulations associated with the disease. These individuals also demonstrated a significant decrease of expression of all the microRNAs investigated; a trend that clearly opposed the direction of regulation seen in the mildly and significantly impaired individuals. In the animal model of metabolic disorder, no difference in neurogenesis was discovered but the direction of the microRNA expression was increased, consistent with that seen the cognitively impaired humans.

It is reasonable to conclude that preserved or expanded neurogenesis is one of the factors that contributes to the preserved cognitive capabilities in the non-demented humans with Alzheimer's Disease neuropathology, and that this change is in part due to deregulation of the miRNA activity in the neurogenic niche. Further, the investigated mouse model appears promising as an atypical model for Alzheimer's for its tendency toward recapitulating uncommon but features of the neurocognitive disease.

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List of Abbreviations

Aβ:	Amyloid beta
Aβo:	Amyloid beta oligomers
AD:	Alzheimer's Disease
AICD:	APP intracellular domain
APOE:	Apolipoprotein E
APP:	Amyloid precursor protein
BACE1:	beta-site amyloid precursor protein cleaving enzyme 1
CDR:	Clinical Dementia Ratio
CERAD:	Consortium for Establishing a Registry for Alzheimer Disease
DG:	Dentate gyrus
ENPP1:	Ectonucleotide pyrophosphatase/phosphodiesterase 1
GCL:	Granular cell layer
GWAS:	Genome-wide association study
HFD:	High-fat Diet
IACUC:	Institutional Animal Control and Use Council
IFC:	Immunofluorochemsitry
IRB:	Institutional Review Board
LCM:	Laser Capture Microdissection
LTP:	Long-term potentiation
MCI:	Mildly cognitively impaired
MMSE:	Mini-Mental State Examination
MWM:	Morris Water Maze
NDAN:	Non-demented with Alzheimer's disease neuropathology
NFκB:	Nuclear-factor kappa-light-chain enhancer of activated B cells
NMDA:	N-methyl-D-aspartate
sAPP(α,β):	soluble APP (N-terminal, extracellular domains)
qPCR:	real-time quantitative polymerase chain-reaction

CHAPTER 1: INTRODUCTION

A HISTORY OF AD

In 1907, Alois Alzheimer published a clinical case study entitled: "On an Unusual Illness of the Cerebral Cortex," describing the unusual case of Auguste Deter, a 51-year old woman who presented with dementia (Alzheimer 1907); (Alzheimer et al. 1995). The case was remarkable for her young age as well as the extent of her confusion and dementia. The account included a description of the histopathological features that he and his collaborators had observed, specifically that fibrillar aggregations had replaced the cells, minute focal densities were visible without staining and strongly resistant to dyeing, the "pathological metabolic substance" and how these features impacted a substantial portion of the cortex; all features which have continued to remain central to the diagnosis of Alzheimer's Disease (AD) at autopsy. Interestingly, he also reported atherosclerotic change of the vascular tissue, which suggested to some in later times that Auguste Deter was in fact a vascular dementia case, although the rediscovery of the original Deter samples in the 1990's did allow for a modern evaluation and determination that her dementia was in fact non-vascular in origin. His department chair, Emil Kraepelin would write a book on Psychiatry in 1910, in which he gave the clinical presentation of presenile dementia with its accompanying histopathology the name of Alzheimer's Disease (Small and Cappai 2006). In his 1911 paper, Alzheimer described a second case of presenile dementia, including an extensive description of the patient's clinical presentation and accompanied by simple yet characteristic sketches of the post-mortem histopathology (Möller and Graeber 1998); (Alzheimer 1911). However, with Dr. Alzheimer's premature death of heart

failure in 1915, his writings ceased. Alas, the work was not carried forward by his student Gaetano Perusini either, who was responsible for much of the histological work that defined the disease, because of his participation in WWI, during which he was fatally struck by shrapnel while assigned as a physician to the San Floriano del Collio first-aid station in 1915 (Lucci 1998).

Research into AD was sparse but steady until the end of the 1970's, after which the number of annual publications consistently increased year after year (Figure 1.1). The first formally published guidelines for diagnosing AD were established in 1984. These took into account the cognitive and behavioral deficits seen during life, and integrated them for diagnosis with the post-mortem histopathological features although it did acknowledge that the "criteria are not yet fully operational because of insufficient knowledge about the disease" (McKhann et al. 1984). Interestingly, it would be during 1984 that the amyloid beta (Aβ) peptide was isolated and sequenced by Glenner and Wong (Glenner and Wong 1984). It was determined to be the same peptide that accumulated in Down's Syndrome individuals (Glenner and Wong 1984, Masters et al. 1985) and also the main component of cerebral vascular amyloid disease (Wong et al. 1985). At that time, the proposal was that Aβ migrates from the vasculature into the brain to accumulate, although it is now widely accepted that pathological Aβ originates from the CNS and moves into the vasculature.

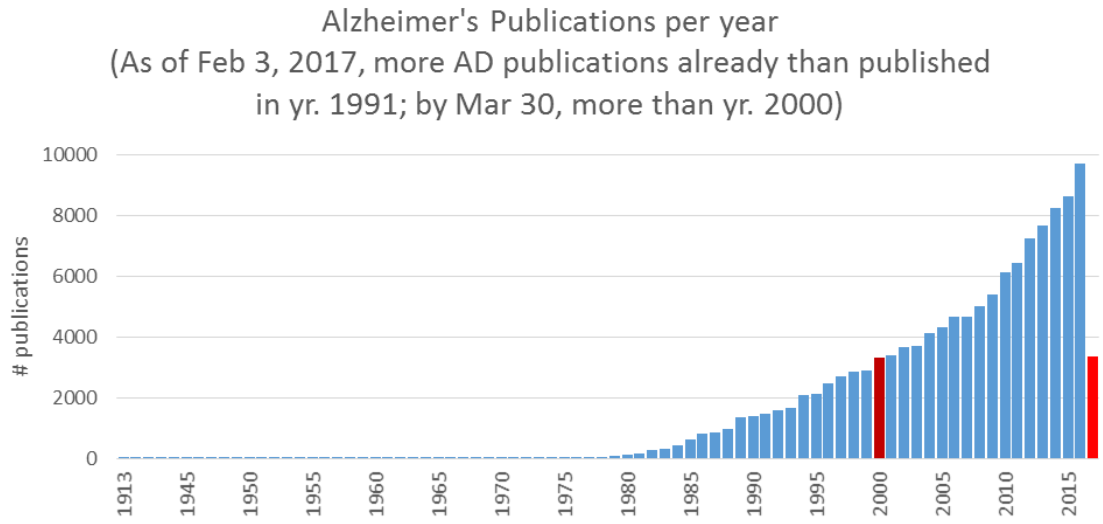

Figure 1.1: The number of Alzheimer's publications annually from 1913 through 2017, as registered in PubMed.

Over the last 30 years, the research efforts aimed at understanding and treating Alzheimer's disease have taken many directions, proposing various hypotheses based on the aberrant features of the disease. The data generated to investigate each has been valuable, but none has yet been sufficient to describe or predict the disease in its entirety.

EPIDEMIOLOGY AND ECONOMIC IMPACT

Alzheimer's Disease (AD) is an insidious, progressive neurodegenerative disease that slowly strips those it afflicts of their ability to function normally, particularly their executive functions, their short-term memory and their everyday life-skills. According to the 2016 Facts and Figures report produced by the Alzheimer's Association, over 5.4 million Americans are currently afflicted with AD with an estimated associated health care cost of $236 billion per year (Alz. Assoc., 2016). The number of afflicted individuals in the United States is projected to increase to around 13.8 million by 2050 (Hebert et al.

2013) with the cost of care projected to exceed $1 trillion per year (Alz. Assoc., 2015).

Many studies on AD have attempted to focus on a 'pure' disease state without complicating factors, by excluding co-morbidities (Watson et al. 2014) often including diabetes. But the reality is that the co-morbidity of non-psychiatric conditions in AD exceeds the rate of co-morbidities in age-matched non-demented populations (Duthie et al. 2011), and co-morbidities complicate primary care and the management of chronic conditions (Min et al. 2013). While these projections may appear to consider unrealistically long time frames, the current absence of clinically efficacious treatments for AD, paired with the very large cohort of people in the US that are about to reach the age affiliated with sporadic onset, a dark cloud truly looms over our future unless Alzheimer's disease can be understood, and true therapies developed.

DIAGNOSIS & ETIOLOGY

The first guideline for the diagnosis of AD was published in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Work Group (McKhann et al. 1984). By establishing AD as a diagnosis possible only "if there are no other systemic or brain diseases that could account for the progressive memory and other cognitive deficits," and requiring histopathological confirmation. At this point, the criteria only provided for "probable, possible, and definite" AD, with "definite" only being possible post-mortem (due to the histopathological confirmation). Presciently, the Work Group acknowledged that "the criteria are not yet fully operational because of insufficient knowledge about the disease. … These criteria must be regarded as tentative and subject

to change. Additional longitudinal studies, confirmed by autopsy, are necessary to establish the validity of these criteria."

Subsequent to the NINDS-ARDA publication of 1984, the criteria for AD diagnosis were next updated in 2011 to reflect and incorporate the great amount of knowledge that had been gained in the nearly 30 years of research conducted (Jack et al. 2011). The guideline was expanded to 3 full documents, setting recommendations for the pre-clinical diagnosis of AD (Sperling et al. 2011), the diagnosis of pre-Alzheimer's MCI (Albert et al. 2011), and for the neuropathological assessment post-mortem (Hyman et al. 2012).

Perhaps one of the most important changes in these official guidelines was the inclusion of "preclinical AD" as a formal diagnostic state. This has both recognized that research results have shown amyloid accumulation can begin two decades prior to the onset of symptoms, and has created a framework to enroll younger participants to try and track pre-disease and early disease states. Such work is also increasingly feasible thanks to intravital labeling compounds such as Pittsburgh B compound, (PiB) a thioflavin T analog molecule that is both BBB permeable and exhibits binding to fibrillar amyloid. While PiB was the first compound to be used in human trials, in 2004 (Sojkova and Resnick 2011), several additional agents are currently being tested[1]. These new agents are exciting as they have been able to demonstrate promising labeling by coregistering in multi-modal imaging techniques, normally involving comparative pre- and post-mortem labeling (Manook et al. 2012).

While imaging has the ability to indirectly observe the features of interest in the

[1] Some agents under investigation include [^{18}F]AV-1451 and [^{18}F]-THK-5351, both anti-tau PET agents, [^{11}C]-PBR-28 which binds to active microglia (neuroinflammatory response is also seen); recently approved florbetapir (a ^{11}C based radio-labeling agent) which was validated in hospice patients and supported by their post-mortem autopsies.

brain, it does also require capital and access to infrastructure that is not always available. As one of the simplest to administer, and therefore the most well established evaluation, the Mini-Mental State Examination (MMSE) is a 30 points test that assesses several executive, memory, and discriminatory and semantic abilities in patients (Folstein et al. 1975). It is an easy to implement examination requiring only about 15 minutes to execute, and with rather clear guidelines: individuals receiving scores of 27 and above are considered cognitively normal (O'Bryant et al. 2008), and scores below 17 reflect cognitive impairment, usually accompanying dementia (with the range between 18 and 26 being considered mild cognitively impaired (MCI) after accounting for the education level of the patient. The MMSE has gone through several refinements since it was first introduced in the late 60s and throughout the 70's (Hodkinson 1972), and it does have solid correlation with the progression of histopathological features of AD throughout the cortex (Nelson et al. 2009). By using cognitive screening measures like the MMSE, the more extensive Alzheimer's Disease Assessment Scale – Cognitive Subscale test (ADAS-COG), and the care-taker corroborated Clinical Dementia Rating scale (CDR), individuals with early and minor disruptions in cognition are able to be targeted toward attempted enrollment in imaging studies. The other benefit in the shift to focusing on earlier onset of the disease ostensibly comes from improved support for longitudinal studies that allow for far greater level of detail regarding progression to be developed.

While McKahnn et al., 1984 made clear that a diagnosis of definite Alzheimer's required histopathological evidence, the document doesn't define which features should be present, nor does it indicate which document should be used as the basis to determine histopathological diagnosis (McKhann et al. 1984). In 1991, Braak and Braak published

histopathological staging criteria that offered a correlation between post-mortem histopathology and pre-mortem cognitive ability (Braak and Braak 1991). Although it was not the first such staging criteria to be created (Ball 1976, Wilcock and Esiri 1982), it has been very reliable and has demonstrated a strong correlation with cognitive decline (Nelson et al. 2009). It is interesting to note that Braak staging for AD almost entirely ignores the accumulations of amyloid, focusing instead on the locations and density of the neurofibrillary tangles (NFTs). Strangely enough, while amyloid plaques are a necessary component for AD diagnosis, they have been shown not to correlate with cognitive ability (Murphy and LeVine 2010).

Unfortunately, the elements that are responsible for inducing Alzheimer's disease have not yet been determined. There is a long list of known risk-factors and relative-risk factors of diminishing strength, with some of the topmost factors being insulin resistance (metabolic syndrome), traumatic brain injury (TBI), including mild-traumatic brain injury (Gardner and Yaffe 2015), hypertension, and high mid-life BMI (Xu et al. 2015). A literature search for "Alzheimer's disease risk factor" returns an array of other links being drawn, a sampling of which include stress, hormones, smoking, diet, environmental toxin exposure, stroke, and "small inhaled particles." After nearly 40 years, the lack of a clear etiology for AD has inspired new approaches to understanding the disease. Complicating this for a variety of biological and cultural reasons, women appear to be at greater risk for developing AD with increased age in both low homogeneity samples (Miech et al. 2002) and the wider populace (Pike 2017). While these differences are many and do increase the complexity of understanding AD, separating them out does provide a great deal of nuance to understanding the disease (Mielke et al. 2014), and may even reveal differential effects

for treatment efficacy (Canevelli et al. 2017). Broad studies to determine biomarkers in serum and even CSF, predictive behavioral features and assays, as well as the development of imaging agents and techniques could aid in the diagnosis of dementia in general and AD in particular (Henry et al. 2013), aided also by a growing focus on longitudinal studies (Erten-Lyons et al. 2012). The field is branching out to make sense of this amorphous and inscrutable disease.

AN OVERVIEW OF THE BIOCHEMISTRY OF AD

The term "amyloid" was derived from the Latin to describe "starch-like" substances in the middle of the 19th Century[2]. While the term is general, and has been used to describe a variety of starch-like deposits in different organ systems, including liver, spleen, and vasculature, the name achieved a certain staying power in AD research. The histopathological presence of amyloid plaques (with neurofibrillary tangles of hyper-phosphorylated tau proteins) is critical for a diagnosis of AD. However, the primary component of those plaques became named the amyloid beta (Aβ) peptide, possibly because no biological functions have been readily determinable for it.

AMYLOID PRECURSOR PROTEIN

From the first sequencing of any amyloid proteins by Glenner et al in the 1970 (Glenner et al. 1970), several amyloid deposits were purified and sequenced up until the same group successfully sequenced the "cerebrovascular amyloid deposit in Alzheimer's

[2] The definition at Online Etymology Dictionary

disease" in 1984 (Glenner and Wong 1984). APP is a highly-conserved, membrane bound protein that is conserved between species and ubiquitously expressed in human tissue (Zheng and Koo 2011). In humans, APP has 10 isoforms ranging from 365 to 770 AA residues in length, with a 695 residue isoform showing expression limited to CNS neurons (Selkoe 1998, Zheng and Koo 2011). It is highly expressed in the olfactory bulb, frontal cortex and the hippocampus (Iwai et al. 1995), all of which are heavily impacted in AD.

Under normal physiological conditions APP undergoes cleavage by a handful of secretases, so named for the soluble fragments that they produce after interacting with their substrate. In non-amyloidogenic processing, an alpha secretase (any one of the

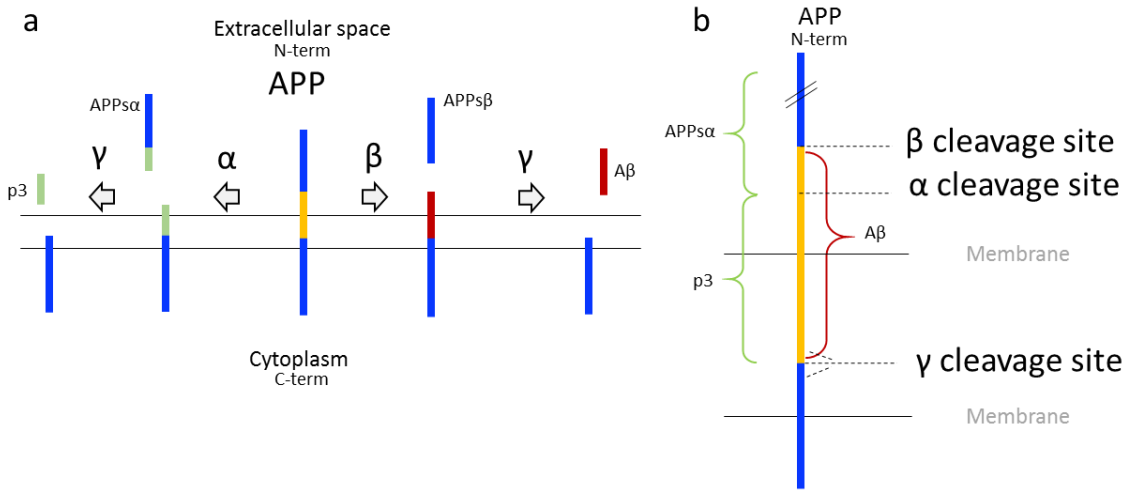

Figure 1.2: Biogenesis of Amyloid Beta.

a) Summary of APP cleavage in non-amyloidogenic (left, green) pathway, and amyloidogenic (right, red). b) Detail diagram of APP cleavage sites.

enzymes that proteolyzes the alpha site in APP) cleaves APP at residue 687, which is 16 residues into the amyloid beta segment (Selkoe 1998). This releases the vast majority of the mass of the N-terminal, extracellular side of APP as a soluble segment, sAPP-alpha, which has been demonstrated to have several beneficial roles, including increasing LTP, supporting neuritogenesis, and neuroprotection (summarized in review; (Chasseigneaux

and Allinquant 2012)). Subsequently, gamma secretase (itself in fact an enzymatic complex; (Kaether et al. 2006)) cleaves the remaining C83 stub to release the P3 peptide (again; the Aβ sequence less 16 residues on the N-Terminal side. The P3 peptide can also be represented as Aβ 17-40) as well as the Amyloid Intercellular Domain (AICD).

The amyloidogenic processing of APP sees the beta-secretase, the only one found so far being Beta-site Amyloid precursor protein Cleaving Enzyme 1 (BACE1), proteolyzing APP at residue 671, releasing sAPP-beta which has not been as closely investigated, but its impact appears generally less protective than its alpha-cleaved counterpart, though it does appear to support neuronal differentiation and retain a positive role for neurite outgrowth (Chasseigneaux and Allinquant 2012). Subsequently, when the gamma-secretase act on the remaining C99 fragment to act on its site within the membrane, the AICD is produced again in addition to a full-length amyloid beta (Aβ) between 39 and 43 residues in length (Figure 1.2). While APP alone has not been shown to play a critical role in neuronal function (as determined by knockout studies), it has been shown to be important for neurite outgrowth and branching, as well as synaptic adhesion (Müller and Zheng 2012).

AMYLOID BETA

Aβ is a 4kDA peptide between 39 and 43 residues in length, as the cleavage site depends on the isoforms of the proteins present in the gamma-secretase complex. The peptide is not a ligand for any known receptors, and does not seem to have any deliberate biological role as a monomer. When multimeric, Aβ adopts either a soluble oligomeric or an insoluble fibrillar conformation. It is comprised of a few beta strands that, when in the

oligomeric conformation, tend to form anti-parallel beta pleated sheets, unlike the fibrils which almost expressly demonstrate parallel beta pleated sheets (Breydo and Uversky 2015). The configuration of fibrillar Aβ had long been inferred by the fact that large aggregates of it consisted of regularly twisting strands (Kidd 1963), and its birefringence when stained with Congo Red. For many years the toxicity of Aβ was believed to be tied to its length, with many attempts to understand whether Aβ 1-40 or Aβ 1-42 was more toxic, and if there was a safe ratio of the two species (Jan et al. 2008). In the end the monomer of Aβ is not itself toxic (Podlisny et al. 1995), and the polymeric fibrillar form is largely considered harmless (He et al. 2012). Finally, it was discovered that Aβ was capable of adopting a soluble oligomeric form, and that these conformations were diffusible. This has led much of the field to now focus on investigating amyloid oligomer toxicity in an attempt to determine its role in AD histopathology. Oligomers do have a particularly high affinity for synapses and can disrupt their functionality (Lacor et al. 2007), which leads to inter-neuronal communication deficits which can in turn cause behavioral and cognitive impairments (He et al. 2012).

TAU

Tau is a microtubule-associated protein represented by 6 isoforms ranging from 45-65 kDa in the human brain (Buée et al. 2000). Its main role is to stabilize microtubules by direct, reversible binding controlled by phosphorylation. Unlike Aβ, tau dysfunction is not exclusive to AD. Abnormal accumulations of tau have been observed in several diseases, described together generally as tauopathies, including Pick's disease, progressive supranuclear palsy, and dementia pugilistica, among others (Williams 2006).

Neurofibrillary tangles, which are comprised of hyperphosphorylated tau, have been known to be fibrillar in nature since the 1960's, from early work using electron microscopy (Kidd 1963). Paired Helical Filaments (PHF) and Straight filaments were later determined to all be indisputably made of tau protein, the difference being its conformation in each (Crowther 1991). Like the early work with Aβ, fibrillar tau was long believed to be a toxic element in the brain of AD patients, although it was eventually demonstrated that it can also take on an oligomeric conformation, which has been shown to be neurotoxic in cultured cells (Kayed et al. 2003).

TREATMENTS

The goal of research towards any disease is to understand it sufficiently to be able to modify, lessen or eliminate it altogether, in order to improve the lives of patients (Salter 1986). Alzheimer's disease offers a significant challenge; while we understand much of it, we have yet to determine how or why it is initiated. The earliest modern theory of AD development was the cholinergic hypothesis, which arose from the realization in the early 1970's that Alzheimer's disease patients showed disruptions to the cholinergic systems, with the concurrent realization of the importance of acetylcholine (ACh) in learning and memory (Francis et al. 1999). While ACh was insufficient to explain how AD starts, the fact that it has a verifiable mechanistic explanation still gives it an important relevance today (Craig et al. 2011). Furthermore, as of Spring 2017 the FDA has still approved a total of 5 drugs for the treatment of AD, only 4 of which are still commercially available in the US. Of the four remaining drugs available, 3 (donepezil, rivastigmine, galantamine, as well as tacrine when it was being produced) are cholinesterase inhibitors of various specificities, and the remainder is the N-methyl-D-aspartate (NDMA) receptor non-competitive

antagonist memantine[3] (the production of tacrine under the trade name Cognex was discontinued by Pfizer in 2013 in response to safety concerns). All of these drugs enhance memory by increasing excitatory signaling at the synapse (Cummings et al. 2014). Unfortunately, all of these approved drugs have rather limited efficacy, and are only able to temporarily slow down cognitive deterioration, although data from studies longer than 3 years are scarce (Lanctot et al. 2009).

Throughout the 90's it came to be acknowledged that the cholinergic hypothesis, was a good mechanistic description of cognitive difficulties in AD but could not address the disease origins, its progression, nor could it truly solve Alzheimer's disease (Francis et al. 1999). This loss of favor coincided with the rise of two additional hypotheses for the progression of AD; the amyloid beta hypothesis and the tau hypothesis.

The amyloid hypothesis arose organically at the beginning of the 90's with the discovery of APP, the mutations seen in familial AD, the similarity of dementia and amyloid accumulations in trisomy-21 individuals, and the recognition of different levels of activity by the enzymes that process Aβ (Hardy and Selkoe 2002). Armed with the new-found genetic understanding of AD, as well as a hypothesis to work within, animal models of the disease began to be published in 1996, and made widely available. Countless models of AD have now been produced, usually based on some combination of a familial APP mutation, PSen1 or/and tau (Elder et al. 2010). Many of the models have successfully recapitulated the histopathology and some of the behavior with varying degrees of success and their own strengths and weaknesses (Schaeffer et al. 2011) (Webster et al. 2014), and

[3] FDA approved drugs for AD treatment, and corresponding year: donepezil, 1996; rivastigmine, 2000; galantamine, 2001; memantine, 2003; donepezil with memantine, 2014.
http://www.alz.org/research/science/alzheimers_disease_treatments.asp

have a wide array of research published using them. In the ongoing attempt to produce increasingly accurate models of age-related dementia, the National Institute on Aging has recently awarded a major Center grant to laboratory animal supplier Jackson Laboratories in conjunction with Dr. Bruce Lamb of Indiana University with the express goal of producing late-onset animal models.

However, the poor record of amyloid-hypothesis based therapeutics in translation has led to sufficient push-back in the field, in addition to the normally existing dissenting opinions. Some criticisms of the theory that Aβ is the primary driver of the disease include that it has little more than acute toxicity, that it cannot be solely responsible for the cascading problems observed in AD, and that its accumulation begins too many years prior to the onset of symptoms. This is not to say that Aβ isn't involved in AD, but that emphasizing the role of Aβ to the detriment of pursuing other hypotheses is not supported by the continuing experimental and observational data (Klein 2002, Castello and Soriano 2014, Herrup 2015). Fortunately, the field is not without other promising avenues.

NDAN

To study the underlying pathology of AD, it is necessary to be able to define and investigate the differences between cognitively normal individuals and those who suffer from dementia. This has caused a conundrum however: if one is cognitively normal, they do not to seek treatment because they possess no deficit that disrupts their daily life. Presumably this normal cognitive state is without notable histological features, and is considered healthy. In demented individuals, we have a state that needs to be resolved, and we have found histopathological accumulations that correspond with that state. Generally,

it has been assumed that these correlate; however, this system can cause a crucial confirmation bias to arise regarding 'normal' tissue.

Autopsy investigation for features that are affiliated with a disease state usually only occur when a disease state is believed to be present; if there was no disruption of daily life, there's no need to assume someone was anything but normal. This confirmation bias, as well as the variety and prevalence of non-conforming conditions that have arisen in AD research are addressed in the comprehensive review by Nelson et al. (Nelson et al. 2009). To combat this bias and gain perspective on the development of the disease, research studies attempt to enroll 'control' individuals, so that we have a comprehensive understanding of the difference between the pathological and normative states. Within AD studies, control individuals are assumed to be devoid of lesions, however the absence of one type of histopathology in a cognitively normal individual does not mean that they are devoid of accumulations, as was demonstrated by the European brain bank that found extensive histopathological inclusions in 16 of 17 "control" individuals (Nolan et al. 2015).

There exist certain individuals who remain cognitively intact up through their natural death at advanced ages who have extensive histopathological inclusions in their tissue, consistent with full-fledged Alzheimer's disease. These individuals appear to have retained their cognitive abilities while also possessing the histopathological features that are affiliated with extensive cognitive impairment, meaning either that these features are in fact not toxic, or these individuals are somehow resilient to that toxicity. While these individuals do not appear to be frequent in the population, they have been observed and identified consistently in papers for at least 3 decades so far (Katzman et al. 1988).

There is a growing interest in the AD field to investigate this understudied group

of individuals, with the authors making the observation in the 2011 histopathological guidelines that "It is important to recognize that the recommended use of NFTs, parenchymal Aβ deposits, and neuritic plaques as the defining histopathologic lesions of AD neuropathologic change according to the criteria proposed here does not preclude the possibility that other processes or lesions may be critical contributors to the pathophysiology of AD." (Hyman et al. 2012) While such a statement may not herald a departure from the classical guidelines for AD post-mortem diagnostic criteria, that it is even included in a guiding document signals a critical acknowledgment that new approaches and markers must not be ruled out, and that AD research over all must be allowed to follow promising and innovative paths.

As certain species of Aβ and tau have each been demonstrated to be explicitly neurotoxic, to understand how these individuals evade injury we must investigate how they differ from both the normal disease state as well as Control individuals; those who are cognitively normal and also devoid of AD affiliated histopathology. We refer to this intriguing subset of individuals as non-demented with Alzheimer's disease neuropathology, abbreviated to NDAN. These remarkable individuals demonstrate the classically-incompatible statuses of possessing extensive histopathological accumulations – pathology consistent with Braak staging V/VI – and high cognitive function as evaluated by the MMSE - scores of 26 or greater, i.e. cognitively normal.

The Taglialatela lab has been contributing to the investigation of the NDAN phenotype to determine how they differ from prodromal stages of AD, specifically mildly cognitively impaired (MCI) individuals, the explicit disease state of full AD, and even from the age matched cognitively normal controls who do not demonstrate pathology. Our

collaborator at Oregon Health and Science University, from whom we receive the human tissue samples, has shown that these NDAN retain brain and hippocampal volume in spite of the extensive histopathological features (Erten-Lyons et al. 2009). Our lab has further contributed the determination that NDAN individuals have phospho-CaMKII distribution that is consistent with control (Reese et al. 2011) and unpublished data), have brain Aβ levels that are consistent with fully symptomatic AD but are devoid of Aβ when the synaptic fraction is isolated and observed (Bjorklund et al. 2012), and have shown inferentially that insulin signaling is enhanced via increased insulin pathway maker levels compared to AD (unpublished). Most recently we have shown that neurogenesis appears to be preserved or expanded in the NDAN, that the capacity for it is certainly enhanced over AD and MCI individuals (Chapter 3 of this dissertation).

NEUROGENESIS

One of the late-stage hallmarks of AD that can be readily observed even by MRI is extensive cortical shrinkage and ventricular enlargement due to neuronal loss (McDonald et al. 2009). This atrophy and loss of tissue volume is able to be detected as early as MCI when focusing on the hippocampus, one of the known neurogenic niches in the brain (Schuff et al. 2009). However the lack of atrophy observed in the NDAN individuals (Erten-Lyons et al. 2009) raises questions of what mechanisms may be at play to preserve both hippocampal and cortical volume; besides avoiding cellular death, we investigated and demonstrated that neurogenic capacity is greater in the hippocampus of NDAN than AD (data that I will expand on in Chapter 3).

For the idea that neurogenesis occurs beyond in utero development to be accepted

as fact is a relatively recent phenomenon that runs counter to the dogma that dominated for more than a century, that regardless of what the glia were doing, new neurons were never produced (Gross 2000). Further, although the first solid evidence that neurogenesis did occur in 3-month post-natal rat studies by Altman and Das in 1965 (Altman and Das 1965), and even though it was well supported by several others, the evidence was largely handled dismissively or ignored, and mammalian adult neurogenesis wasn't really accepted until the 1990's (Gross 2000). Adult neurogenesis was demonstrated in humans by Eriksson et al in 1998 by analyzing tissue from a cancer study where patients had volunteered to receive BrdU injections (Eriksson et al. 1998). Currently adult neurogenesis is understood to be limited to a few neurogenic niches in the brain, including the olfactory bulb (Lepousez et al. 2013), the sub-ventricular zone (Alvarez-Buylla and Garcia-Verdugo 2002), and sub-granular zone of the hippocampus (Kempermann et al. 2015).

Hippocampal neurogenesis has been shown to originate from Sox2-expressing Type-1 radial glia-like cells expressing Sox2 in the subgranular zone- the daughter cells that follow a neuronal fate migrate toward the granular-cell layer itself as immature neurons (Goncalves et al. 2016). This intermediate state, Type-2, gives the cells the identifier "transiently amplifying progenitor cell," and begins the expression of doublecortin (DCX), and sees the loss of Sox2 expression by the time they cells are considered Type-3. As the cells become post-mitotic and are considered to be young, maturing neurons, DCX expression is lost and NeuN expression begins (an antibody named for the fact that it stained neuronal nuclei, but which specifically targets the transcription factor RbFoxO3; (Kempermann 2015)). While a handful of neuronal maturity/differentiation-state markers have been found to be expressed across these delineations (nestin, PSA-NCAM), the

markers discussed above, specifically Sox2, DCX, NeuN, do not overlap expression in rodents.

Critically, neurogenesis in the hippocampus has been robustly correlated with memory (Cameron and Glover 2015), both by the demonstration that activities affiliated with increasing memory (e.g. environmental enrichment, access to treadmills for running) in mice and rats (van Praag 2008), as well as via the selective ablation of neurogenesis resulting in impaired memory (Saxe et al. 2006). It is not only the animal studies that have shown the activity-related memory effect to be resolved in spatial learning tasks (Marlatt et al. 2012). Impairments in cognition are a known and troubling side-effect of radiation therapy for various head cancers (Greene-Schloesser et al. 2012), which has been linked to loss of neural stem cell populations in both rodents (Monje et al. 2002) and human postmortem samples (Monje et al. 2007). Although a loss in hippocampal neurogenesis alone may be insufficient to explain many neurocognitive diseases, having the process be alive and well-functioning may serve indirect benefits to cognitive and emotional well-being (DeCarolis and Eisch 2010).

OTHER HYPOTHESES

Other factors beyond those described above have been proposed to be involved in AD, including neuro-inflammation, the possibility of infectious agents catalyzing the cascades (Honjo et al. 2009), environmental toxicological hypotheses (Chin-Chan et al. 2015), calcineurin dysregulation (Taglialatela et al. 2015), mitochondrial dysfunction (Moreira et al. 2010), and other lines of non-canonical investigation. There is growing acknowledgement by the field that AD is not able to be explained simply, and that it is a

multi-factorial disease (Iqbal and Grundke-Iqbal 2010).

PROJECT AIMS

Given the above information, the central hypothesis of this project is to test whether a sustained or expanded rate of neurogenesis and neuronal differentiation correlate with overcoming the cognitive declines that result from the progression of histopathological features observed in AD. In order to test this, the project was developed along two Aims:

Specific Aim 1: The aim of this project is to understand the impact of neurogenesis on cognitive reserve, via cognitive function, in human subjects, and to compare this with neurogenesis in a unique memory-impaired animal model of insulin resistance, that may be able to serve as an AD-diabetes comorbid animal model.

Specific Aim 2: Determine the extent of neurogenesis in a mouse model of metabolic syndrome that recapitulates some critical CNS features of AD. The working hypothesis is that neurogenesis is decreased in metabolic syndrome by altered expression of miRNAs associated with progenitor proliferation. A mouse model of peripheral metabolic syndrome will elucidate the impact of DG neurogenesis through progenitor cell proliferation, fate mapping, and epigenetic regulation. Timed treatment with BrdU will provide the high level of temporal resolution for in vivo cell proliferation required to understand the fates of these cells and complement the interpretation of the results from Specific Aim 1.

CHAPTER 2: METHODS

HUMAN SUBJECTS AND AUTOPSY BRAIN TISSUES

Post-mortem brain tissue was obtained from the Oregon Brain Bank at Oregon Health and Science University (OHSU), in Portland, OR. Donor subjects were enrolled and clinically evaluated in studies at the NIH-sponsored Layton Aging and AD Center (ADC) at OHSU, in accordance with protocols that were approved by the OHSU Institutional Review Board (IRB). Informed written consent was obtained from all participants prior to their enrollment in the studies at the ADC. Donor subject samples were de-identified by ADC prior to being provided to UTMB, so that no approval was required from the UTMB Institutional Review Board under CFR §46.101(a)(1). As participants in brain aging studies at the ADC, subjects received annual neurological and neuropsychological evaluations, administered by an experienced clinician. Participants were classified as Alzheimer's disease (AD) when possessing a National Institute for Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorder Association diagnostic criteria for clinical AD (CDR) including a mini-mental state exam (MMSE) score below 10. Control participants performed normally in cognitive examinations (MMSE of 28-30). Mild cognitive impairment (MCI) cases had some cognitive impairment with MMSE scores in the mid-20s. Non-demented with AD pathology (NDAN) cases displayed little to no cognitive impairment (MMSE 26-30) while having extensive amyloid plaques and neurofibrillary tangles comparable to fully symptomatic AD. At autopsy, a neuropathological assessment was performed in compliance with IRB-approved protocols. A neuropathologist scored autopsy brain tissue for amyloid plaques and neurofibrillary tangles, according to standardized Consortium to

Establish a Registry for Alzheimer's Disease criteria (CERAD) and Braak staging (Braak, 1991). The diagnosis, age, sex, Braak stage, MMSE score and post-mortem interval (PMI) for all subjects used is included in Table 2.1.

Table 2.1 - Human subjects biographical data

Diagnosis	Subject #	Age (yrs.)	Sex	Braak stage	MMSE	PMI (hr.)
Ctrl	1104	86	F	2	29	16
Ctrl	1229	>89	F	2	30	12
Ctrl	1563	80	M	1	30	2
Ctrl	1731	74	F	2	29	7.5
MCI	781	89	F	3	22	20
MCI	811	>89	F	5	20	12
MCI	975	>89	F	2	25	4
AD	995	81	F	6	0	12
AD	1770	82	F	6	15	6.5
AD	1678	76	F	6	1	25
AD	1774	>89	M	6	2	3.3
AD	1776	>89	F	6	6	6.3
AD	1777	67	F	6	9	20.5
NDAN	1016	>89	F	6	26	8
NDAN	1317	>89	F	6	27	4.5
NDAN	1362	>89	F	4	27	48
NDAN	697	>89	M	5	29	5

HUMAN TISSUE PROCESSING AND IMMUNOFLUOROCHEMISTRY

Fresh frozen hippocampal tissue blocks were removed from storage at -80 ºC, embedded in O.C.T. compound (Tissue-Tek; Tokyo, Japan) and sectioned at 10 μm onto Superfrost/Plus slides (Fisherbrand; Fisher Scientific, U.S.A.). Slides were stored at -80 ºC until use. Slides were removed from -80 ºC and fixed in 4% paraformaldehyde in 0.1M PBS, pH 7.4 for 30 minutes at room temperature, permeabilized with 5% normal goat serum (NGS)/0.3%Triton X-100/0.05% Tween-20, and incubated with primary antibodies diluted in PBS containing 1.5% NGS overnight at room temperature. Primary antibodies used were rabbit anti-SOX2 (1:200; Cell Signaling, Danvers, MA) and mouse anti-NeuN

(1:1000; Millipore, Billerica, MA). Slides were washed in PBS before incubation with Alexa-conjugated secondary antibodies (donkey-anti rabbit and donkey anti-goat; 1:400; Life Technologies, USA) in PBS containing 1.5% NGS for 1 hour at room temperature. Finally, slides were washed in PBS, treated with 0.3% Sudan Black B (in 70% EtOH), washed again, and coverslipped using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, US).

IMMUNOFLUORESCENCE IMAGE ANALYSIS AND CELL COUNTS

The slides were imaged using a confocal scanning module (Bio-Rad Radiance 2000 with LaserSharp software, Hercules, CA, USA) and a 20X/0.75NA objective. Fluorescent images were acquired using constant settings for laser power, detector gain and offset. For each subject, 3 sections were used for quantification. For each section, three images were taken along the granular cell layer (GCL) and subgranular zone (SGZ; defined as an area adjacent to and equal in thickness to the GCL) of the dentate gyrus region. Image analysis was performed using ImageJ. An independent evaluator blind to the experimental groups counted the number of $SOX2^+$ and $NeuN^+$ cells in each of the 3 fields of view per sections. DAPI (4',6-diamidino-2-phenylindole) was used to counterstain all nuclei. The number of cells positive for SOX2 only, NeuN only or both SOX2 and NeuN was calculated and expressed as percentage of the total number of DAPI-positive nuclei in the DG. The number of cells in each field was assessed by counting nuclei stained with DAPI. No significant differences were found in the total number of $DAPI^+$ nuclei both within each experimental group and between the experimental groups.

Statistical analysis was performed using Sigmaplot. One-way ANOVA between diagnoses (i.e. Ctrl, MCI, AD or NDAN) on ratio's described above for all images from each individual within a group, with Holm-Sidak pairwise multiple-comparison analysis post hoc test to determine differences when significance was detected. If the data failed the normality test (Shapiro-Wilk), a Bonferroni correction was made before statistical analysis.

LASER CAPTURE MICRODISSECTION OF HUMAN TISSUE

Human subjects' hippocampal sections (10μm thick) were stained using cresyl violet and dehydrated in graded alcohol solutions under RNAse-free conditions. Specifically, tissue was subjected to 75% ethanol (1 minute), ddH2O (1 min), Cresyl violet (~20-30 seconds), 3x ddH2O (1 min), 95% ethanol and 100% ethanol (30 seconds each), and finally 2x Xylenes (3 minutes). Slides were mounted into the laser capture system. The dentate gyrus (DG) including the granular cell layer (GCL) and subgranular zone (SGZ; defined functionally here as an area adjacent to and equal in thickness to hilar side of the GCL), were captured and collected onto CapSure MacroLCM caps, using an ArcturusXT LCM system (Applied BioSciences/ThermoFisher). Tissue from a total of ten sections were used per subject. The captured cells were placed into lysis buffer from the RNAqueous-Micro Total RNA Isolation Kit (Ambion/ThermoFisher) and stored at -80 °C until RNA preparation.

QUANTITATIVE RT-PCR OF HUMAN MIRNAS

Total RNA was prepared using the protocol provided with the RNAqueous kit from Ambion (Life Technologies/ThermoFisher; USA) according to the manufacturer's

instructions. For the optimal preparation of mature miRNAs, the HiSpec buffer was used, per manufacturer instructions. Reverse transcription was performed utilizing the reagents and protocols provided with the miScriptII RT kit (Qiagen), using 10ng total RNA for the RT reaction. Sequences for qPCR primers used are found in Table 2.2. Primers were purchased from the miScript Primer Assays products (Qiagen), and used according to the manufacturer's instructions with the miScript SYBR Green PCR kit (Qiagen). Briefly, for reverse-transcription: thaw RNA and kit reagents on ice. Determine RNA concentration for each sample, using Nanodrop (ThermoFisher), and calculate the volume required of each sample to obtain 10ng RNA (this is the lower limit of feasibility, however this was required because the RNA yield from the laser microdissection was minimal). To prepare each 20 µl reverse transcription reaction, add to a tube the reagents, the volume of RNA required for 10 ng, and (12 µl less the volume of RNA added) of RNAse-free water. Run reaction in thermocycler per instructions. For qPCR, briefly: the minimum input

Table 2.2 – miRNA primer sequences

primer	Sequence
miR-9-5p	5'UCUUUGGUUAUCUAGCUGUAUGA
miR-25-3p	5'CAUUGCACUUGUCUCGGUCUGA
miR-29a-3p	5'UAGCACCAUCUGAAAUCGGUUA
miR-124-3p	5'UAAGGCACGCGGUGAAUGCC
miR-132-3p	5'UAACAGUCUACAGCCAUGGUCG
miR-137	5'UUAUUGCUUAAGAAUACGCGUAG
miR-184	5'UGGACGGAGAACUGAUAAGGGU

(miScript II line of products available from Qiagen)

requirement was 20 pg of cDNA; based on the input RNA to the RT reaction and assuming an approximate 1:1 conversion efficiency, there should be a known 50 pg of cDNA per µl in the RT product. A master mix was prepared with the SYBR mix, universal primer, and the DNAse-free water, mixed and loaded into the PCR plate. The 10x miScript primer and

sample cDNAs were loaded into appropriate wells, the plate sealed and spun down, and the samples run.

ANIMAL EXPERIMENTS

All research and handling of the animals was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (UTMB, Galveston, TX, USA) and was performed according to the guidelines of the National Institutes of Health Guidelines on the use of laboratory animals.

Wild-type C57BL/6 background strain and AtENPP1 transgenic strain, described in Chapter 4) were maintained in the UTMB vivarium with a 12 hr. light/dark cycle, food and water ad libitum. Within both strains of animal, two conditions existed: cage food was the standard laboratory mouse diet (nutritional composition: 4% calories from fat; Teklad 7001; Teklad, Madison, WI, USA) or a high-fat diet (nutritional composition: 60% calories from fat, 37.1% saturated fats; Research Diets D12492, New Brunswick, NJ, USA). Prior to the current study, animals were assigned at weaning to be raised on either RC or HF diets. Our collaborator (Dr. Jonathan Hommel, experiments performed by Dr. James Kasper) assessed all animals using a Morris Water Maze assay. All animals were between 7 and 9 months of age when I received them for this study.

Upon receipt, animals were randomly assigned to the 1-day or 28-day post-injection condition. All mice were weighed and injected 3 days serially IP with 100mg/kg BrdU (10mg BrdU per milliliter of 0.9% sterile saline). Based on assignment into experimental groups, subjects were euthanized either at 24 hours or on the 28th day subsequent to the final injection (Figure 2.1). The distribution of animals can be found in Table 2.3.

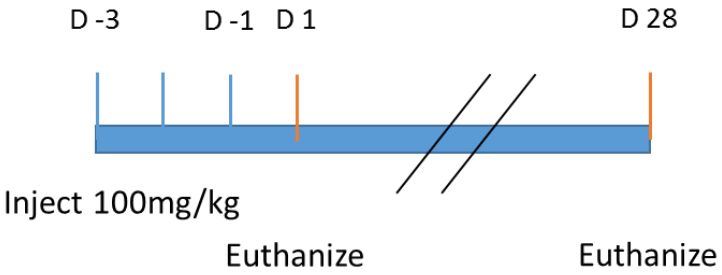

Figure 2.1: Overview of BrdU injection paradigm

At the experimentally-determined end-point, brains were collected and hemisected, with one hemisection being post-fixed in 4% PFA for 24 hours followed by sucrose sinking at 20% and 30% for all animals. For female animals, the second hemisphere was flash-frozen in liquid nitrogen and stored at -80C. Male tissues were not available for fresh-frozen hemisections.

Table 2.3 – Number of animals assigned per group.

	1-Day post-injection				28-Day post-injection			
	Regular Chow		HFD		Regular Chow		HFD	
	M	F	M	F	M	F	M	F
Wt	4	4	4	4	4	1 (+5)	3	3
Tg	4	4	4	3	3	4	5	6
Totals:	8	8	8	7	7	5 (10)	8	9

ANIMAL TISSUE IMMUNOFLUOROCHEMSITRY

Tissue sections were prepared coronally onto Fisherbrand Superfrost Plus slides using a Thermo Scientific Microm HM252 cryo-microtome (Thermo Scientific). Prepared slides were stored at −80 °C until use. Slides were removed from −80 °C, warmed briefly to ambient temperature and fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4 for 30 minutes at room temperature, and treated with 2N HCl for 30 minutes at 37C.

Subsequently, slides were incubated in 100mM sodium tetraborate for 2x 5 minutes, and permeabilized with 5% normal goat serum (NGS)/0.3% Triton X-100/0.05% Tween-20 for 30 minutes. Primary antibodies were applied with 1.5% NGS and 0.05% Tween-20 overnight (at least 16 hours) at 4C. Primaries used were rat-anti-BrdU (1:500; BioRad, part#: OBT0030CX), and rabbit anti-Sox2 (1:1000; Millipore, part#: AB5603), and for 1 month animals rabbit anti-NeuN (1:1000; Millipore, part#: 7260) and chicken anti-GFAP (1:1000; Aves Labs Inc., part#: GFAP). Slides were washed 2x 5 minutes in PBS and incubated with secondary antibodies and 1.5% NGS in PBS for 1 hour at room temp. Slides were washed 1x 5 minutes with PBS and mounted using Fluoromount-G with DAPI (SouthernBiotech).

ANALYSIS OF IMMUNOFLUOROCHEMSITRY

Images were acquired by confocal microscopy using a 40x (NA 1.05) water-immersion objective, using image tiling so as to acquire the entire dentate gyrus (from medial to lateral, superior and inferior blade of the granular cell layer) in a single image. The number of BrdU positive cells for each DG were counted blindly by myself, and classified by their geography as being in the GCL, SGZ (which approximately included 2 cell-bodies widths into the SGZ side and 1-2 cell bodies into the GCL), or hilus (which included the entire area between the blades of the GCL that was not a part of the SGZ).

The number of cells labeled with BrdU, Sox2 (24-hour animals; only counted where labeling was in the SGZ), and NeuN+ cells that were also colocalized with BrdU (28-day animals), were evaluated per region, as well as being summed in order to evaluate the "whole DG". Within the animal's sex, One-Way ANOVA were run against the combined

genotype/feed category, giving 4 categories to be analyzed. Animals were also evaluated by Three-Way ANOVA with the levels for analysis being Sex (male vs. female), Genotype (wildtype "Wt" vs. transgenic "Tg"), and Diet (regular chow "RC" vs. high-fat chow "HF"). Post-hoc analysis Holm-Sidak was used to determine significant differences between groups where applicable.

RNA ISOLATION FROM MURINE TISSUE

All mouse tissues were reduced to the area of interest using a clean razor blade, and then stored at -80C until RNA processing. Tissue from each animal was held in its own tube, and prepared using the TRIzol manufacturer's protocol, with samples being held on ice during incubations and between steps. In brief, the protocol was as follows: To each tube, add 1mL of TRIzol reagent, homogenize and incubate 5 minutes. Add 200 µl of chloroform, shake for 15 seconds incubate for 2 minutes. Centrifuge for 15 minutes at 12,000 g under 4C. Carefully transfer clear supernatant to clean tube and add 500 µl of isopropanol, incubate 10 minutes. Centrifuge for 10 minutes at 12,000g under 4C; decant the supernatant. Add 1mL 75% EtOH and invert tubes for 20 seconds, then centrifuge for 5 minutes at 7,500 under 4C. Decant supernatant, invert tubes over a Kim-wipe and allow to dry under a chemical hood for 1 to 3 hours, until dry. Resuspend the pellet in 50 µl (or less if the pellet is small and concentration is a concern) of RNase-free water by pipetting several times. Incubate at 60C for 10 minutes. Use a Nanodrop 2000c (FisherScientific) to establish nucleotide concentration, and dilute samples to 400 ng/µl as necessary using RNAse-free water.

REALTIME PCR

A panel of miRNA were investigated, including miR-9, miR-25, miR-29a, miR-124, miR-132, miR-137, and miR-184. For the optimal preparation of cDNA of mature miRNAs, the HiSpec buffer was used, per manufacturer instructions. Reverse transcription was performed utilizing the reagents and protocols provided with the miScriptII RT kit (Qiagen), using 200ng total RNA for the RT reaction. Sequences for qPCR primers used are found in Table 2.2. The primer sequences are provided in Table 2.2; each sequence was checked and found to have 100% homology with the probes used against human miRNA, so the same probes were re-used. The RT and qPCR protocols used were described above (same as for human miRNA).

A panel of messenger RNAs known to be targeted by the above miRNA were selected to investigate whether changes in miRNA had an impact on mRNA levels. The mRNA selected are published to interact directly with the miRNAs investigated (as determined using miRTarBase; http://mirtarbase.mbc.nctu.edu.tw/), and are also relevant to AD and neurogenesis. The targets selected were FOXO1, ROCK2, BACE1, AKT2, NFκB, and BCL2, with both GAPDH and Beta-Actin as housekeeping genes. Primer sequences for targets were selected from Primer-Bank (https://pga.mgh.harvard.edu/primerbank/) by selecting the forward/reverse pairing with transcript lengths closest to 120 bases. Primers were purchased via custom sequence synthesis from Sigma-Aldrich (using the following parameters: 0.025 μmole, desalt purification, dry primer). Sequences used are found in Table 2.4. cDNA was prepared for mRNA was prepared using amfiRivert Platinum RT kit (GeneDepot). Real-time PCR probe kit used was SYBR FAST qPCR Universal Master Mix (Kappa Biosystems).

Protocols for RT and qPCR used were consistent with manufacturer's instructions. Briefly, for RT PCR, RNA was pre-diluted to around 400 ng/µl and concentration determined. RNA within a genotype/feed/timepoint homogenous group was pooled, summing to 800 ng RNA per pool, which in a 20 µl reaction with an assumed 1:1 conversion will give 40 ng per µl final concentration. Master mix was prepared and added to the pool for each group and placed in thermocycler. For qPCR, the master mix was prepared and loaded into the reaction plate, and the fixed volume of cDNA was added. The plate was spun, the thermocycler run.

Table 2.4 – murine mRNA primer sequences

Primer		Sequence
β-actin	for:	5' GGCTGTATTCCCCTCCATCG
	rev:	5' CCAGTTGGTAACAATGCCATGT
GAPDH	for:	5' AGGTCGGTGTGAACGGATTTG
	rev:	5' TGTAGACCATGTAGTTGAGGTCA
AKT2	for:	5' ACGTGGTGAATACATCAAGACC
	rev:	5' GCTACAGAGAAATTGTTCAGGGG
BACE1	for:	5' GGAACCCATCTCGGCATCC
	rev:	5' TCCGATTCCTCGTCGGTCTC
BCL2	for:	5' ATGCCTTTGTGGAACTATATGGC
	rev:	5' GGTATGCACCCAGAGTGATGC
FOXO1	for:	5' CCCAGGCCGGAGTTTAACC
	rev:	5' GTTGCTCATAAAGTCGGTGCT
NFκB	for:	5' ATGGCAGACGATGATCCCTAC
	rev:	5' TGTTGACAGTGGTATTTCTGGTG
ROCK2	for:	5' TTGGTTCGTCATAAGGCATCAC
	rev:	5' TGTTGGCAAAGGCCATAATATCT

(Sequences obtained from PrimerBank. Synthesized by Sigma-Aldrich: 0.025 µmole, desalt purification, dry primer)

cDNA was prepared by pooling animals' RNA experimental category and by their time-point of sacrifice after the last BrdU injection. Real-time PCR was performed on samples against each miRNA or messengerRNA target of interest in triplicate (technical replicates to support each Ct). qPCR were performed on a Mastercycler4 (Eppendorf)

thermal cycler. Delta-Ct method of analysis was calculated for each primer against the C_t of that pool's housekeeping (U6 for miRNA, β-actin and GAPDH were each used for mRNA). Because there was no additional manipulation of 28-day animals, their C_ts could be pooled with each groups' 1-day C_ts, creating biological duplicates for the analysis. delta-delta C_t and fold-change were calculated against the Wild-type Regular chow, except where indicated. Significance was evaluated by Two-Way ANOVA on genotype (Wt or Tg) and feed (RC or HF) on all technical replicates of a pool including both pools per group (n of 7 or 8 animals in two pools for each group). For graphical representation of analysis, please see Figure 2.2.

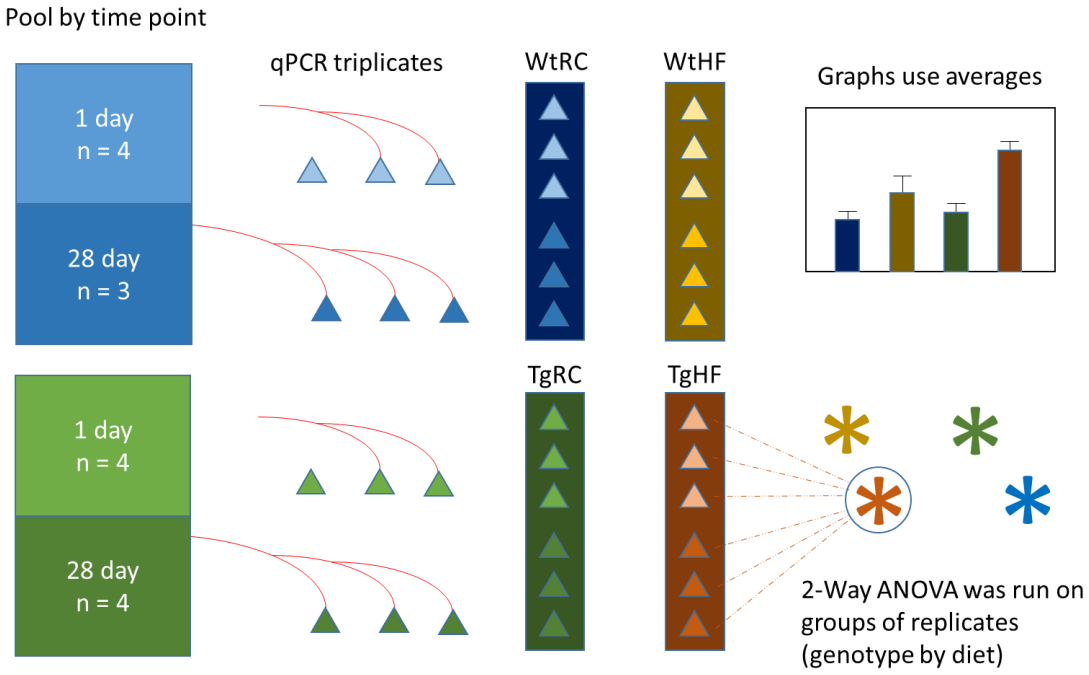

Figure 2.2: Schematic summarizing how animal RNA replicates were pooled for analyses and presentation.

CHAPTER 3: PRESERVED NEUROGENESIS IN NON-DEMENTED INDIVIDUALS WITH AD NEUROPATHOLOGY.

Adapted from Briley, Ghirardi, Woltjer, Renck, Zolochevska, Taglialatela, & Micci, 2016

"Preserved neurogenesis in non-demented individuals with AD neuropathology"

Published June, 2016 in Scientific Reports, DOI: 10.1038/srep27812

INTRODUCTION

Despite extensive research effort on Alzheimer's disease (AD) now being into its third decade (Mandelkow and Mandelkow 2012), exactly how AD starts, how the disease progresses, and how to stop or to slow its progression are still all unresolved questions (Mayeux and Stern 2012). A handful of pharmacological interventions are available and approved as palliative treatments, but these show only limited efficacy in a small population of patients, and for only a limited time (Salomone et al. 2012).

In recent years several reports have described rare individuals who remain cognitively intact despite the presence of neuropathological features usually associated with a fully symptomatic stage of AD (Lue et al. 1999, Riudavets et al. 2007, Iacono et al. 2008, Erten-Lyons et al. 2009, Kramer et al. 2011, Bjorklund et al. 2012). The existence of these unusual cases, herein referred to as Non-Demented with Alzheimer's disease Neuropathology (NDAN), suggests that there is a natural way for the human brain to resist (or significantly delay) the neurotoxic events that normally lead to cognitive impairment

in AD. Understanding the mechanisms involved in such cognitive resilience may suggest conceptually novel treatment strategies for AD centered on promoting in affected individuals endogenous resistance to disease-driven cognitive decline.

The discovery that new neurons are continuously generated in the hippocampus, an area of the brain that plays a critical role in learning and memory and is most affected by AD (Squire 1992), suggests that plasticity of the central nervous system could provide an endogenous protective mechanism to sustain cognitive functions. Indeed a growing body of literature has emerged demonstrating a strong correlation between neurogenesis, memory and cognitive function in animal models. Notably, promotion of adult hippocampal neurogenesis is associated with improved spatial memory, while a decline in neurogenesis underlies cognitive impairments reportedly associated with aging, trauma and various neurodegenerative disorders including AD (Clelland et al. 2009, Lazarov and Marr 2010, Sahay et al. 2011, Stone et al. 2011, Shetty et al. 2013).

The process of neurogenesis in the hippocampus consists of several phases, each one corresponding to different stages of maturation of the developing cells. Type 1 neural stem cells (NSCs) generate neuroblasts and immature granule cells, which in turn differentiate into mature granule cells. SOX2 is a transcription factor that has been shown to play a critical role in the maintenance of stem cell pluripotency and is commonly used as a marker of NSCs in the dentate gyrus of the hippocampus (Komitova and Eriksson 2004, Episkopou 2005, Suh et al. 2007, Shimozaki et al. 2012).

Adult hippocampal neurogenesis is modulated by a variety of genetic and epigenetic factors (Li and Zhao 2008, Ma et al. 2010). In addition, because the differentiation and maturation of newborn neurons involves the concerted action of

multiple genes, micro-RNAs (miRNA), short non-coding RNA sequences that bind to mRNA targets and inhibit their translation, have been recently identified as important regulators of neurogenesis (Luikart et al. 2012, Schouten et al. 2012).

To ask whether neurogenesis is linked to preserved cognitive ability in humans with AD neuropathology, in this study we evaluated the expression of SOX2 and of the mature neuronal marker NeuN in post-mortem human tissues from NDAN, mild cognitively impaired (MCI) and AD individuals in comparison to age-matched healthy subjects. In order to begin investigating the mechanisms involved in the regulation of neurogenesis in demented and non-demented individuals, we further analyzed the expression of selected microRNAs in laser-captured dentate gyrus samples from autopsy specimens of NDAN, MCI, AD and age matched healthy subjects.

RESULTS

SOX2 and NeuN are co-expressed in the human hippocampus dentate gyrus.

SOX2 immunoreactivity was observed in both the granular cell layer (GCL) and subgranular zone (SGZ) of the dentate gyrus (DG) in all autopsy human hippocampus specimens analyzed. In some cells, the expression of SOX2 co-localized with the neuronal marker NeuN (Figure 3.1). This was surprising because, in the rodent DG, SOX2 expression is limited to undifferentiated NSCs and is not observed in $NeuN^+$ granule cells (Suh et al. 2007). We therefore tested the specificity of our antibody by performing immunofluorescence staining of the murine hippocampus (Figure 3.1b) and by Western blot analysis of human hippocampus total protein lysate (Figure 3.1c). The results confirmed that the antibody used specifically recognizes SOX2 and that, contrary to the

human DG, in the murine DG, SOX2 did not co-localize with NeuN^{+} nuclei. Same results were obtained when nuclei extracted from frozen human or mouse hippocampus were

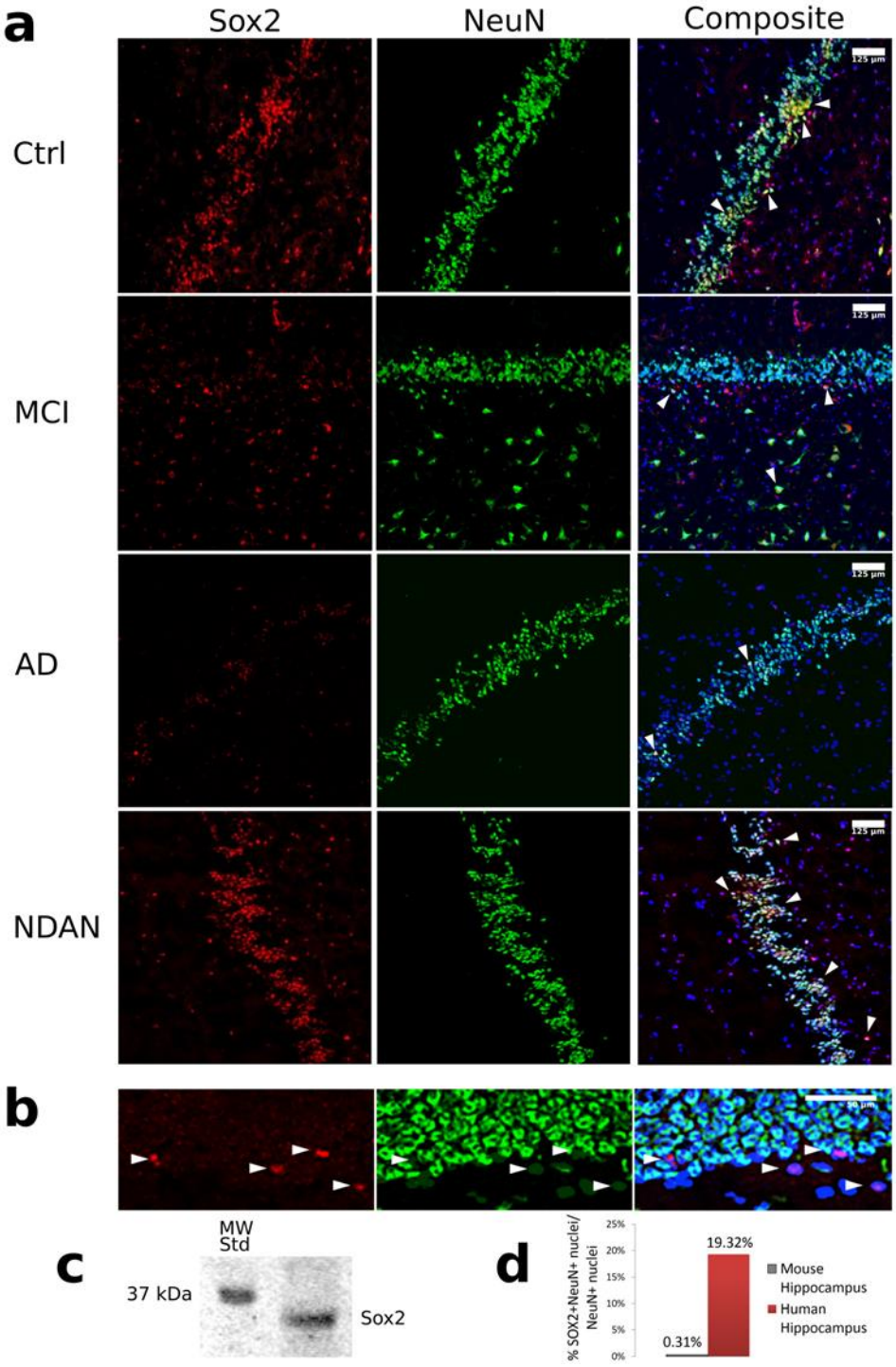

Figure 3.1: SOX2 and NeuN are co-expressed in the human dentate gyrus.

a) Representative images of human hippocampus sections showing immunofluorescence staining for SOX2 (red) and NeuN (green) in the dentate gyrus. Nuclei are counterstained blue with DAPI. Arrowheads indicate cells that co-express SOX2 and NeuN. b) Immunofluorescent staining of SOX2 (red) and NeuN (green) in wild type mouse hippocampus dentate gyrus. Nuclei are stained blue with DAPI. c) Representative Western blot showing human hippocampus lysate probed with the same anti-SOX2 antibody used in A and B. A band corresponding to the predicted molecular weight for SOX2 (34 kDa) is detected. d) Flow cytometry analysis of NeuN and SOX2 expression in nuclei isolated from mouse and human hippocampus. Data is expressed as percentage of nuclei co-expressing SOX2 and NeuN versus total neuronal nuclei (expressing NeuN).

analyzed by flow cytometry, according to methods published earlier (Portier et al. 2006), after co-staining with SOX2 and NeuN. Nuclei from human hippocampus showed a substantial co-localization of SOX2 and NeuN (19.32% of the NeuN-expressing nuclei), whereas nuclei from mouse hippocampus showed virtually none (Figure 3.1d). Thus, the observed co-localization of SOX2 and NeuN in the human DG appears to be specific. An attractive possibility is that such SOX2/NeuN co-localization in the human DG may reflect newly formed neurons where SOX2 expression has not yet been turned off.

$SOX2^+$ cells are increased in the hippocampus of NDAN individuals.

The total number of $SOX2^+$ cells in the DG was significantly increased in NDAN individuals as compared to both MCI and AD patients (Figure 3.2A). There was also a trend of a higher number of $SOX2^+$ cells in NDAN as compared to age-matched controls that however did not reach statistical significance. When we quantified both the number of $SOX2^+/NeuN^+$ cells and of $SOX2^+/NeuN^-$ cells, the percentage of $SOX2^+$ cells also expressing NeuN was increased in NDAN individuals while it was decreased in both MCI and AD patients as compared to age-matched healthy controls (Figure 3.2b). On the other

hand, the percentage of cells expressing only SOX2 ($SOX2^+/NeuN^-$) was increased in both NDAN and MCI subjects, while it was decreased in AD patients as compared to age-matched healthy controls (Figure 3.2c).

In order to determine whether the number of granular neurons differed between demented and non-demented individuals with AD pathology, we quantified the number of cells expressing only NeuN. The total number of $NeuN^+$ cells was not significantly

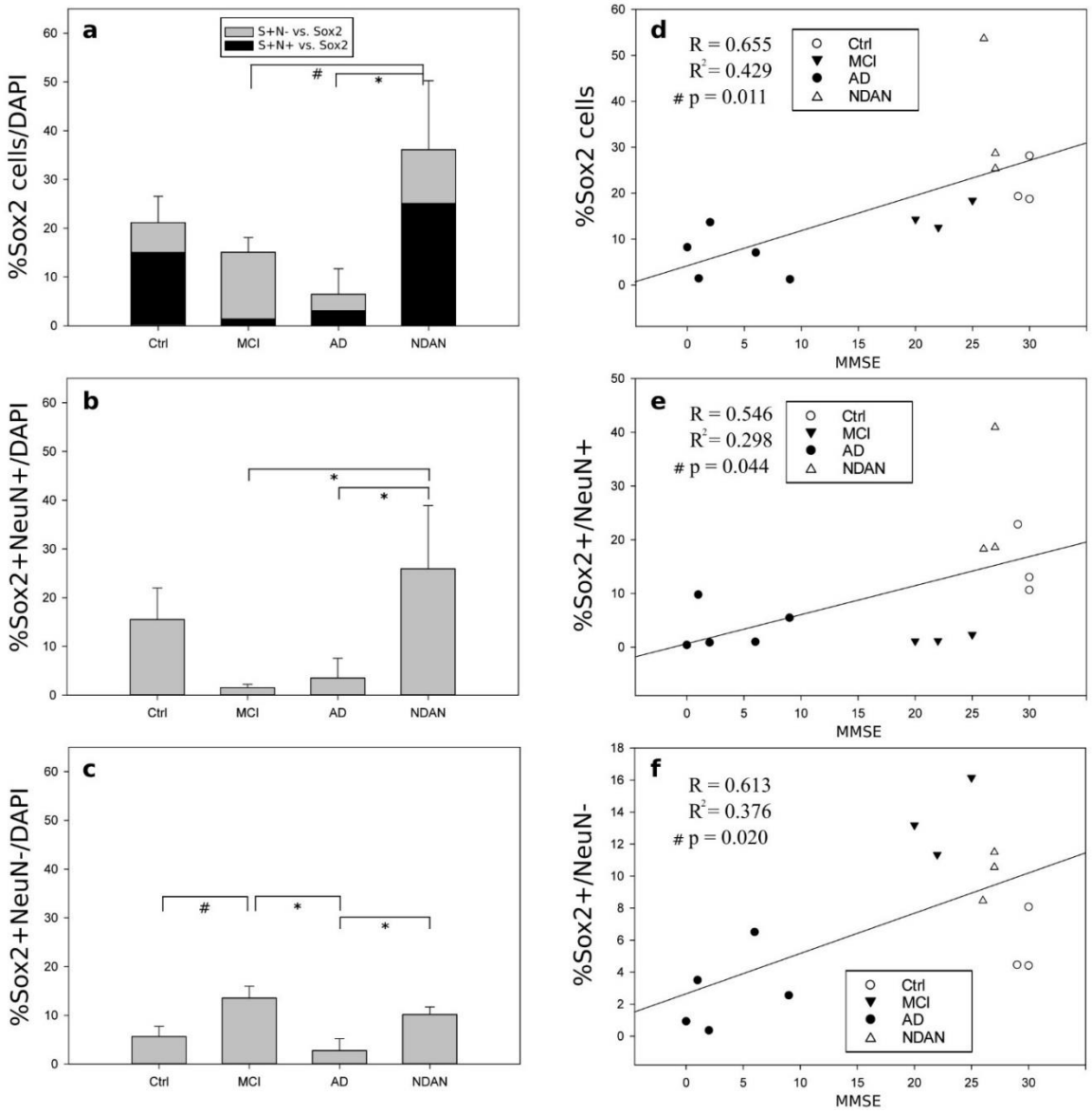

Figure 3.2: Quantification of SOX2 expression in the human dentate gyrus.

The number of cells immunoreactive for SOX2 and NeuN in human dentate gyrus specimens was quantified by an independent investigator who was blinded to the experimental groups and normalized to the number of $DAPI^+$ nuclei. Colocalization of SOX2 and NeuN was assessed using ImageJ plugins. Values are represented as the mean percentage of positive cells/total nuclei ± SEM. a) The percentage of all $SOX2^+$ cells (inclusive of $NeuN^+$ and $NeuN^-$ cells) is significantly increased in NDAN as compared to both MCI and AD. Within each column the percentage of $SOX2^+$ cells that are negative for NeuN (grey) or positive for NeuN (black) is shown. b) The percentage of cells that express SOX2 but are negative for NeuN ($SOX2^+/NeuN^-$) are significantly increased in MCI as compared to AD and control and significantly increased in NDAN as compared to AD. c) The percentage of cells that are positive for both SOX2 and NeuN ($SOX2^+/NeuN^+$) is significantly greater in NDAN as compared to MCI and AD. Statistical significance was determined by ANOVA followed by multiple comparison procedures using the Holm-Sidak method. # $p < 0.05$; * $p < 0.01$
Linear regression analysis was performed to examine the relation between the percentage of $SOX2^+$ cells (d), $SOX2^+/NeuN^-$ cells (e), $SOX2^+/NeuN^+$ cells (f) and cognitive function (MMSE score). Positive and significant correlations were found for all the regression performed, $p<0.05$.

different between NDAN, MCI, AD and age-matched control subjects (Figure 3.3a). Similarly, no differences were found in the percentage of cells expressing NeuN but not SOX2 ($SOX2^-/NeuN^+$), and presumed to be mature neurons (Figure 3.3b)

The number of $SOX2^+$ cells in the DG correlates with cognitive function.

A linear regression analysis was performed to correlate the mini mental state examination (MMSE) scores of each individual examined with the proportion of cells expressing SOX2 and NeuN. A positive correlation was found between MMSE scores and the number of $SOX2^+$ cells (Figure 3.2d). Similarly, a positive correlation was found between MMSE scores and the number of $SOX2^+/NeuN^+$ cells (Figure 3.2e), and the number of $SOX2^+/NeuN^-$ cells (Figure 3.2f). Conversely, MMSE scores did not correlate with the total number of cells expressing NeuN (Figure 3.3c) and a negative correlation was found between the number of $NeuN^+/SOX2^-$ cells and MMSE scores (Figure 3.3d).

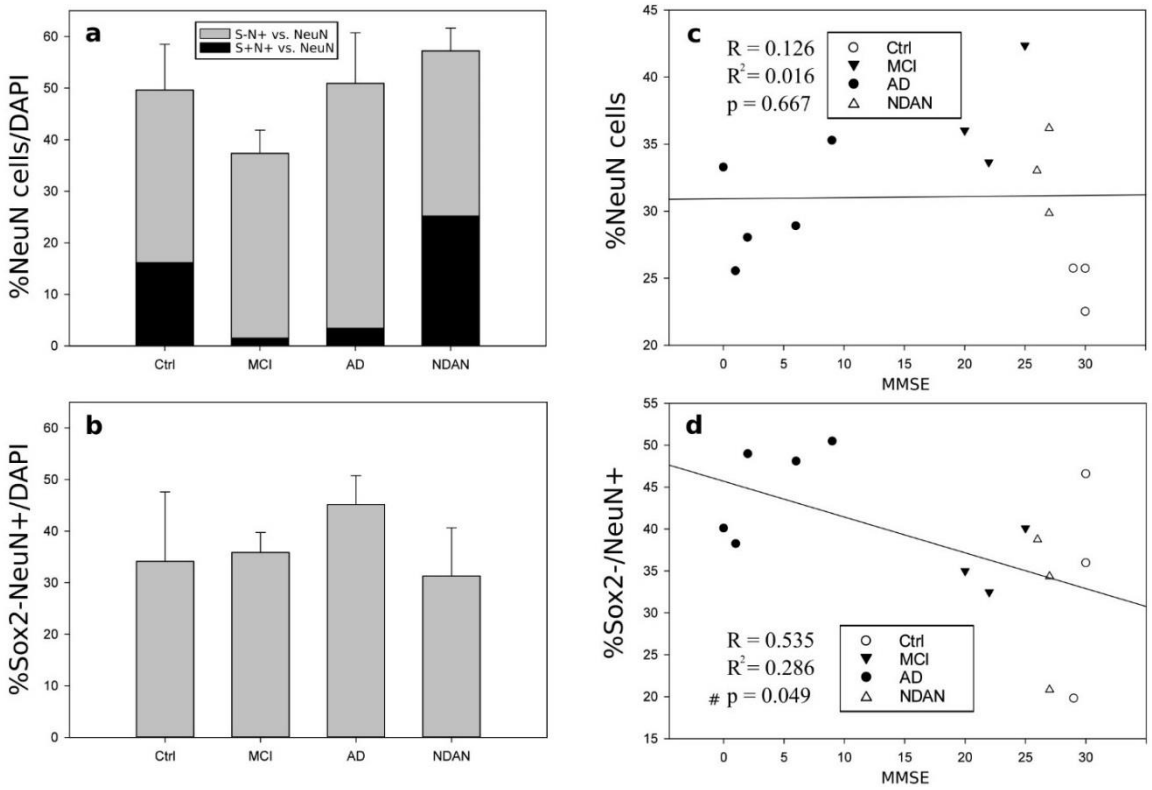

Figure 3.3: Quantification of NeuN expression in the human dentate gyrus.

a) No significant differences between the groups are observed in the percentage of NeuN positive cells as a proportion of DAPI-positive cells. Within each column, the percentage of $NeuN^+$ cells that are negative for SOX2 (grey) or positive for SOX2 (black) is shown. b) No differences between the groups are observed in the percentages of cells expressing NeuN but not SOX2 ($NeuN^+/SOX2^-$). c) Linear regression analysis show no correlation between the percentage of total $NeuN^+$ cells and cognitive function (MMSE score), while d) it showed a significant negative correlation between the percentage of $NeuN^+/SOX2^-$ cells and MMSE, $p<0.05$.

In order to control for possible confounding effects on protein expression due to variability in tissue collection time, we performed a correlation analysis between the postmortem interval of tissue collection and SOX2 or NeuN expression. The results showed that the postmortem interval did not correlate with any of the measurements made (Figure 3.4).

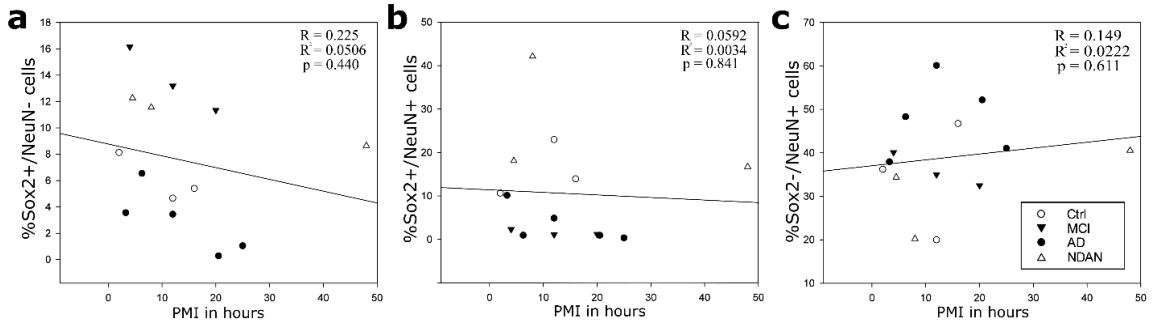

Figure 3.4: PMI does not correlate with the staining of any of the markers investigated.

Linear regression analyses found no correlation between the percentage of total a) Sox2+/NeuN-, b) Sox2+NeuN+ co-positive, nor c) Sox2-NeuN+ cells, and the length of the post-mortem interval, $P > 0.05$. This demonstrates that the PMI did not interfere with any of the parameters we investigate here.

Levels of miRNAs modulating neurogenesis differ between non-demented (NDAN) and demented (MCI and AD) individuals.

We used qPCR to measure the levels of selected miRNAs known to regulate all stages of neurogenesis (miR-9, miR-25, miR-29a, miR-124, miR-132, miR-137) (Li et al. 2008, Jobe et al. 2012, Schouten et al. 2012) in laser-captured DG samples from the NDAN, MCI, AD and healthy controls (Figure 3.5a). We found that the levels of miR-9, miR-25 and miR-124 were significantly lower in NDAN subjects as compared to MCI individuals and miR-25 and miR-124 lower as compared to AD patients (Figure 3.5b). The same pattern of lower levels in NDAN subjects was observed for miR-29, miR-132 and miR137, which however did not reach statistical significance (Figure 3.5b). Figure 3.5b also shows that, regardless of individual statistical significance, the levels of all miRNAs assayed followed a pattern of reduction in NDAN, whereas there was a consistent pattern of increased miRNAs levels in MCI or AD. When we considered all different miRNAs in aggregate for each patient group, we found that there was a highly significant difference

between NDAN subjects and both MCI and AD patients, confirming a significant pattern of decreased levels for all measured miRNAs in NDAN contrasted by a pattern of increased levels in MCI or AD patients. These data strongly suggest that, in NDAN, there may be an epigenetic regulation of neurogenesis driven by lower levels of modulating miRNAs.

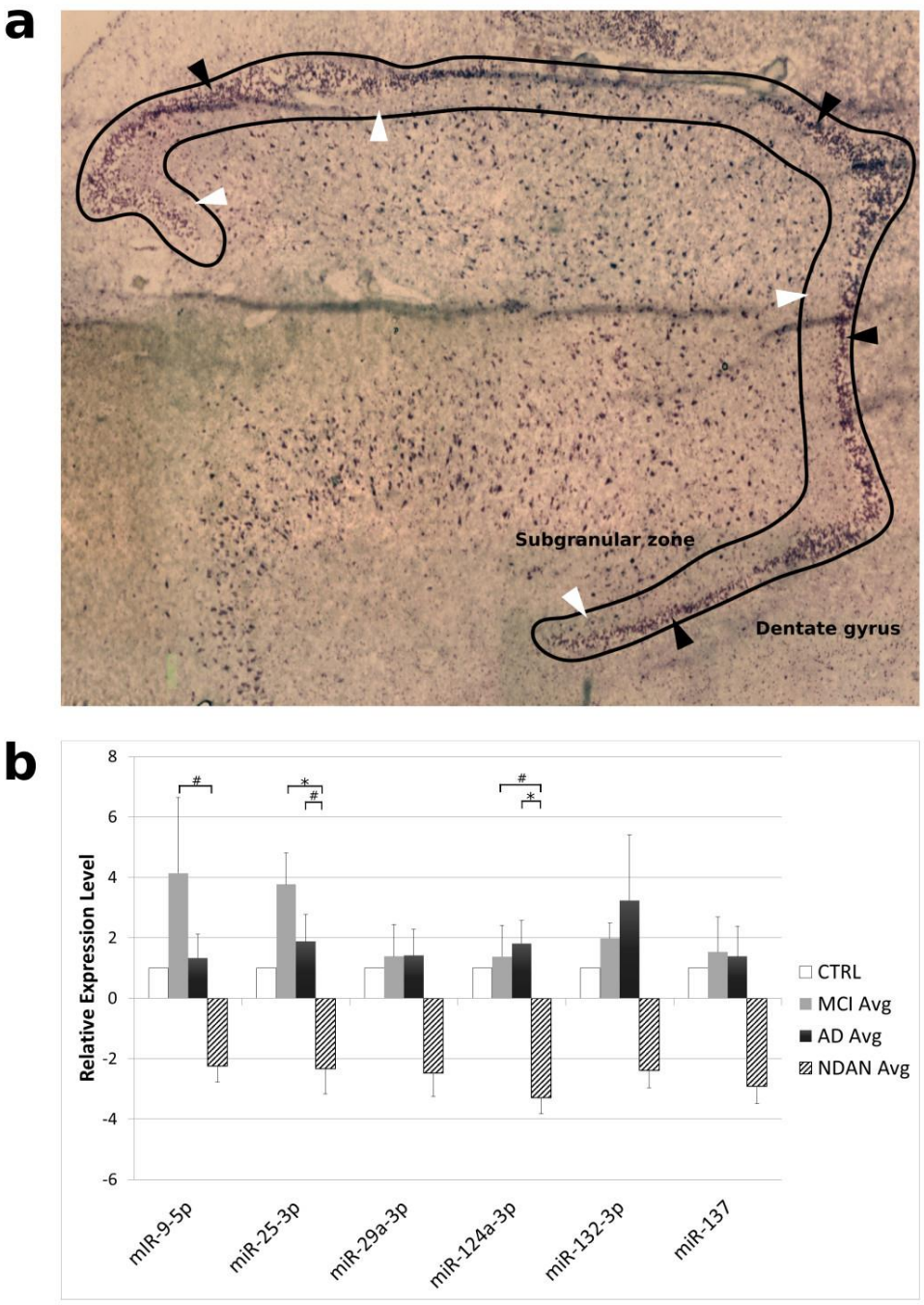

Figure 3.5: The expression of selected miRNAs in the DG is significantly different between non-demented and demented individuals with AD pathology.

a) Representative section of human DG stained with cresyl violet. The black outline indicates the region collected by laser capture microdissection (LCM). Black arrowheads indicate the granular cell layer (GCL). White arrowheads indicate the subgranular zone (SGZ). b) The relative level of expression of six miRNAs, known to play a role in the regulation of neurogenesis, was determined in the DG by qPCR and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Age-matched healthy controls were used as calibrator, with its fold change taken as 1. The relative expression of all evaluated miRNAs between groups was found to be significantly less in NDAN than both MCI ($Q=1.507$, $p<0.001$) and AD patients ($Q=5.247$, $p<0.001$; One Way ANOVA). For each miRNA, statistical analysis was performed by one-way ANOVA followed by pairwise multiple comparison procedures using the Holm-Sidak method, # = $p<0.05$, * = $p<0.01$.

DISCUSSION

NDAN individuals are characterized by normal cognition at the time of their death, despite the presence of amyloid plaques and neurofibrillary tangles characteristic of a fully developed disease. The aim of this work was to study neurogenesis in the hippocampus DG of NDAN and to compare it to MCI subjects, AD patients and healthy age-matched control individuals. Because it is not possible to measure neurogenesis as a dynamic process in the human brain, due to the practical inability to track newborn cells and follow their differentiation over time, we used the expression of SOX2, a key regulator of neural stem cells (Episkopou 2005, Suh et al. 2007), as an indicator of neurogenic potential in human hippocampus samples.

Firstly, we found that in the human DG, the expression of SOX2 overlaps with the neuronal marker NeuN in a subset of cells. This was surprising because in the rodent DG the expression of SOX2 is restricted to undifferentiated NSCs (Suh et al. 2007, Suh et al. 2009). Indeed, using the same antibodies utilized in the human brain samples, we confirmed that SOX2 and NeuN are not co-expressed in the murine DG. Furthermore, flow

cytometry analysis of nuclei isolated from human or mouse hippocampi also confirmed co-localization of SOX2 and NeuN in human but not in mouse specimens. We conclude therefore that in the human DG, SOX2 expression persists for a longer period of time during the differentiation of neural progenitor cells, and that $SOX2^+/NeuN^+$ cells could represent newly formed neuronal cells.

Consistently with previous reports (Boekhoorn et al. 2006), we found fewer $SOX2^+$ cells in the DG of AD patients as compared to healthy controls. On the other hand, in NDAN individuals, $SOX2^+$ cells were significantly more abundant in the DG as compared to both AD and MCI subjects and even higher than healthy subjects.

Interestingly, when we analyzed the proportion of cells co-expressing SOX2 and NeuN, we found that these were significantly more abundant in NDAN DG as compared to both MCI and AD subjects, whereas, on the other hand, cells that exclusively express SOX2 ($NeuN^-$) were significantly more abundant in both NDAN and MCI DG as compared to AD and healthy controls. These data suggest that not only NDAN individuals have a greater number of $SOX2^+$ cells in the DG, but also that these cells possess a greater capacity to generate new neurons. On the other hand, $SOX2^+$ cells in MCI individuals remain mostly in the undifferentiated state with the majority of cells not expressing NeuN. Linear regression analysis confirmed that SOX2 expression, whether co-expressed with NeuN or alone, positively correlates with preserved cognitive function. Specifically, higher percentages of both $Sox2+/NeuN^-$ cells, corresponding to undifferentiated NSC, and $SOX2^+/NeuN^+$ cells, likely representing newly formed neurons, significantly correlate with higher MMSE scores.

The changes in the percentages of $SOX2^+$ cells in the DG cannot be explained by a reduced number of granular neurons because the overall expression of NeuN showed no significant differences among the groups, indicating that the total number of granular neurons in the DG remain constant. Interestingly, a negative correlation was found between cells expressing only NeuN ($Sox2^-$) and MMSE scores, thus suggesting that these cells might represent an older population of neurons and that having more of these older neurons is associated with poor cognitive performance.

Recent evidence has identified small non-coding RNAs (miRNAs) as important regulators of neurogenesis (Schouten et al. 2012). Here, we measured the expression of six miRNAs known to modulate neurogenesis in the DG, and found that the levels of all miRNAs assayed were approximately two-fold lower than control subjects in NDAN, whereas they were consistently higher than control in MCI and AD DG. Although, when analyzed individually, only miR-9, miR-25 and miR-124 reached statistical significance, when considered as a group, the level of all miRNAs analyzed was highly significantly different between NDAN and both MCI and AD, showing a consistent pattern of decrease in the former one and of increase in the latter two. Because miRNAs can affect a wide array of targets, it is difficult to determine the influence of each individual miRNA analyzed in our study on human DG neurogenesis (including proliferation, migration and differentiation of NSCs). However, our data demonstrate that the levels of such miRNAs in aggregate show a distinctive pattern that sets NDAN apart from both cognitively affected groups (MCI and AD), further supporting our results showing both increased number of NSCs in NDAN and of their neurogenic potential. Thus, in NDAN a unique epigenetic

regulation mediated by miRNAs could be responsible for sustained levels of NSCs ($SOX2^+/NeuN^-$) and their ability to generate new neurons ($SOX2^+/NeuN^+$) in the DG.

Interestingly, we found that both the number of SOX2+ cells and the level of regulating microRNAs (specifically miR9 and miR25) were much higher in the DG of MCI subjects as compared to both AD and healthy individuals. This suggests that in the early phases preceding a fully symptomatic AD, increased proliferation of NSC in the DG might represent an attempt of the brain to counteract the progression of the disease. It is interesting to note however that, while the number of SOX2+ cells is increased in MCI individuals, the number of SOX2+ cells that co-express NeuN is decreased. This observation, along with the concomitant increased levels of specific regulating microRNAs, suggests that the neurogenic potential of NSC is impaired even at an early stage of AD progression.

Several published reports have shown a correlation between the rate of neurogenesis and brain function in rodents (Zhao et al. 2008). Specifically, decreased proliferation of NSC in the DG results in impairments in learning and memory, while on the other hand, external factors known to increase NSCs proliferation and neurogenesis, like exercise and environmental enrichment, result in improved learning and memory (Zhao et al. 2008, Blaiss et al. 2011, Hill et al. 2015). It is therefore tempting to speculate that sustained neurogenesis in the DG of NDAN subjects is an important factor mediating their ability to evade dementia in spite of the presence of a degree of neuropathology (plaques and tangles) usually associated with clinically manifest AD.

In conclusion, our data strongly suggest that NDAN individuals have increased neurogenesis in the DG, likely driven by unique changes in the levels of modulating

miRNAs, and further support the notion that NDAN represents a condition distinct from both MCI and AD. While the present results provide new evidence linking sustained neurogenesis to cognitive competency in humans, further studies are needed to fully characterize the involved mechanisms and evaluate their possible clinical significance.

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CHAPTER 4: IMPAIRED MEMORY IN HIGH-FAT FED ATENPP1 MICE IS NOT MEDIATED BY PERTURBED NEUROGENESIS

INTRODUCTION

Globally, two major public health crises are on the rise- Type 2 Diabetes, and dementia in aging individuals. Both can be linked to increasing industrialization, which improves access to highly-processed and high-calorie diets while simultaneously reducing the degree of physical activity otherwise required in a non-industrialized society, and the increase in longevity as a result of sanitation and medical advancements (Lunenfeld and Stratton 2013). Research suggests however there may be more that links insulin resistance and dementia (Mittal and Katare 2016), and understanding these links will provide a wealth of new tools to combat global increases in both Type 2 Diabetes and Alzheimer's disease (AD).

Type II diabetes has been found to increase the risk of developing AD by 60% (Chatterjee et al. 2016), with some authors finding the risk to increase to 50-100% (Biessels et al. 2006). Alzheimer's disease itself is a primarily idiopathic neurodegenerative disease that irreversibly robs its patients of their memory, and is currently costing $236 billion in direct costs, with an estimated additional $220 billion worth of unpaid caretaking by family and friends annually (Alzheimer's Association 2016). Except for hereditary cases of AD, which represent only up to 5% of diagnosed cases, the etiology of AD is not yet known. With continuing evolution in the amyloid hypothesis (Castello and Soriano 2014) and tau hypothesis seeking validation, the evidence is growing that AD is likely a multi-factorial disorder that will require many avenues of attack to reduce its prevalence (Iqbal and Grundke-Iqbal 2010). Towards this end, unraveling the mechanisms that underlie the risk

factors of AD, such as smoking, traumatic brain injury and especially diabetes, is increasingly important.

Recently, Fan et al. have published a retrospective cohort study of more than 51,000 participants showing that Diabetes Mellitus and hypertension are significant risk factors for the development of dementia (Fan et al. 2017). Global insulin resistance being the underlying pathology in metabolic syndrome (Lann and LeRoith 2007) is unsurprising given the large glucose uptake by skeletal muscle and adipocytes depend on the insulin-responsive GLUT4 glucose transporter (Huang and Czech 2007), and how widely damaging to organs hyperglycemia is. While the CNS was once considered to be insulin insensitive, partly because circulating insulin didn't impact CNS insulin levels, partly due to the predominance of insulin-independent GLUT3 in neurons, this perspective is rapidly changing (Blazquez et al. 2014). While neurons do not require insulin to take up glucose, they do possess insulin receptors, and it has been shown that insulin has a role in modulating synapse regulation (Chiu et al. 2008), in LTP (Zhao et al. 2010), and even in memory function in humans (Benedict et al. 2011). Furthermore, it has been demonstrated that central nervous system insulin resistance is common in AD because oligomeric Aβ is able to activate TNF-α, which in turn decreases the signaling efficiency of insulin receptor (De Felice et al. 2014). The investigation of insulin sensitizers, for example via PPar gamma agonism, have shown promise in rodent models of AD though not yet in clinical trials (Sodhi et al. 2011), and recent clinical trials have even shown efficacy for the direct nasal administration of insulin in improving cognitive performance (Hölscher 2014).

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is a membrane-bound enzyme that inhibits insulin receptor autophosphorylation by depleting the available

ATP in the vicinity (referred to as PC-1; (Maddux and Goldfine 2000), or by direct interaction with the alpha subunit (Sortica et al. 2011) inhibiting downstream insulin signal transduction. The expression of ENPP1 has been shown to be elevated in the adipose tissues of obese human patients (Pan et al. 2011) and polymorphisms of ENPP1 gene have been linked to differing susceptibility to the development of Type 2 Diabetes. To better understand the role of ENPP1 in diabetes, Dr. Abate and his collaborators designed a transgenic mouse model with over-expression of the human ENPP1 to the adipose tissue (AtENPP1-Tg mouse; Pan, Ciociola et al. 2011). Because prior work was unable to determine where the insulin resistance in ENPP1 overexpressing animals originated, they have developed these AtENPP1 animals to investigate the impact of the overexpression of ENPP1 in adipose tissue, as well as its effects on systemic metabolism.

The overexpression of ENPP1 inhibits preadipocyte maturation(Liang et al. 2007) reducing the ability of the body to engage in the appropriate storage of fatty acids, and in turn leading to insulin resistance(Abate 2012). To make the overexpression of the ENPP1 gene specific to adipose tissue, Dr. Abate et al. placed the transgene under the aP2 promoter (adipocyte protein 2) expressed in maturing preadipocytes. Under normal metabolic balance, preadipocytes remain in immature states, and the number of adipocytes itself stays static. Depending on how acute or sustained the positive energy balance is, adipocytes normally mature to take up excess lipids, the precursors divide and produce additional preadipocytes to increase the available pool of adipocytes, or both (Sarjeant and Stephens 2012). When exposed to a normal laboratory mouse diet AtENPP1 animals are virtually indistinguishable from wildtype animals, but when it is exposed to a high-fat diet requiring mature adipocytes, the overexpression of ENPP1 prohibits normal insulin signaling and

maturation, leading to an increased number of immature adipose cells (Pan et al. 2011). The inability of the adipose tissue to normally store excess energy under a high-fat diet leads to ectopic fat deposition, in addition to insulin resistance (Pan et al. 2011). Recently Dr. Abate's group in collaboration with Dr. Taglialatela's group have published that the AtENPP1 model additionally demonstrates central insulin resistance, despite the biologically limited expression of its transgene, when exposed to a high-fat diet (Sallam et al. 2015). This work also showed that synaptic communication in the hippocampus, measured via field excitatory post-synaptic potentials (fEPSP), was suppressed by the high-fat diet (HF) and that this effect was exacerbated the transgene on HF, though the transgene alone did not impact fEPSPs. Further collaborative work has shown that these disruptions seen in hippocampal communication under the HF diet in fact propagate to disruptions in memory behavior (Kasper et al. 2017).

The hippocampus, important for spatial and semantic memory (Bird and Burgess 2008), is one of most heavily impacted structures in AD. The hippocampus also contains a site of neurogenesis in the subgranular zone (SGZ) of the granular cell layer (GCL) of the dentate gyrus (DG). Neurogenesis in the SGZ undergoes an asymptotic decrease in rate with increasing age (Knoth et al. 2010). While the literature can appear to show mixed results based on methods and time-points of observations, there is a general consensus that the impairment of neurogenesis in the hippocampus is positively correlated with reduced performance in spatial memory tasks in rodents (Cameron and Glover 2015), and our recent work has shown that AD, and even mildly cognitively impaired individuals, have decreased neurogenic potential compared to non-demented individuals with AD neuropathology (Briley et al. 2016). In the AtENPP1 transgenic animals, the HF diet significantly impaired

memory, leading us to the question of whether this effect on memory correlated with a reduction in hippocampal neurogenesis.

In this study, I tested the hypothesis that a decrease in neurogenesis is responsible for memory deficits seen in the AtENPP1 transgenic mice when fed a high-fat diet. Further, I tested a panel of miRNA known to modulate neurogenesis, as well as mRNA targeted by these miRNA, to determine whether AtENPP1 overexpression, coupled to high fat diet, impacted these neurogenesis regulatory elements.

RESULTS

NSC proliferation in the hippocampus decreases with increasing age

5-Bromo-2-deoxyuridine (BrdU) is a brominated thymidine analogue which becomes integrated into DNA during the S-phase, and which has a limited bioavailable half-life. When applied to a population of cells, it allows for the direct determination of which cells were duplicating DNA during the period that the BrdU was available. *In vivo*, by limiting the area of consideration to the known neurogenic niche of the SGZ, the number of cells labeled for BrdU gives us a metric for the rate of neural stem cell proliferation.

Neurogenesis studies are often performed on young animals that demonstrate strong BrdU uptake in the SGZ due to their basal rate of neurogenesis. To investigate the rate of neurogenesis in our study animals, 6 wild type fed with regular chow (WtRC) animals between 45 and 80 days of age and 9 animals between 250 and 300 days of age were given BrdU (100 mg/kg, intraperitoneally daily for 3 days), and euthanized 24 hours after the last injection. The number of dividing (BrdU+) cells in the SGZ of the old animals

(>250 days of age) was significantly decreased as compared to that seen in young animals (< 90 days of age; Student's t-test, $p < 0.05$). (Figure 4.1).

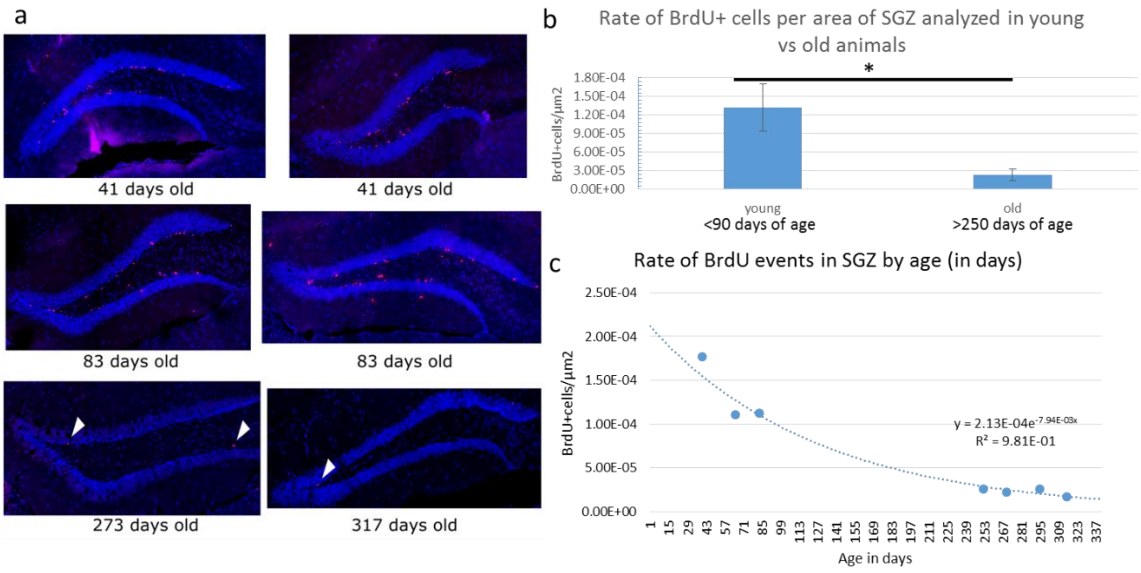


Figure 4.1: Neurogenesis decreases sharply with age.

a) Representative images of BrdU staining (red) in the DG of WtRC mice of different ages (41, 83, 273 and 317 days old). Arrowheads indicate positive signal where staining is sparse. Nuclei are counterstained in blue with DAPI. b) The number of BrdU+ cells in the SGZ is significantly greater in animals under 90 days of age (n=6) compared with those over 250 days of age (n=9) ($p < 0.001$, Student's t-test.) c) BrdU+ cells shown in relation to age (n=2 per time point).

Neural stem cells proliferation is not altered by AtENPP1 or by diet.

Subsequent to the final BrdU injection, one set of animals was euthanized after 24 hours. At this early time point, cells that were in the S phase during the past 3 days will have incorporated BrdU into their DNA. Neuronal differentiation of newborn neural stem cells in the SGZ is known to take 3-4 weeks (Goncalves et al. 2016), and with at most only 3 days between labeling (provided the cell was in S-phase during the first injection) and

euthanasia, BrdU labeled cells would not have had the time to differentiate into neurons. Therefore, evaluating the number of BrdU+ cells in the neurogenic niche of the DG, the SGZ, gives insight into the number of proliferating neural stem cells in each animal and experimental condition analyzed.

In order to test whether the number of proliferating NSCs in the DG is altered in response to ENNP1 overexpression in the adipose tissue and/or high fat diet, I injected BrdU (using the same paradigm described above) in wild type (Wt) and transgenic (Tg) AtENNP1 mice that had been raised on regular lab chow (RC) or a high-fat diet (HF), and counted the number of proliferating (BrdU+) NSC in the hippocampus DG. Moreover, I used immunofluorescence and a specific antibody against Sox2 (a NSC marker) to visualize and quantify the total number of NSCs in the SGZ.

Fields of view were acquired using a 40x objective on a confocal microscope to capture the entire DG in a single image. This enabled the evaluation of three distinct regions of the DG simultaneously- the GCL, the SGZ, and the remaining area between the superior and inferior blade, labeled here entirely as the hilus (Figure 4.2a). The number of positive counts (BrdU+, Sox2+) were marked in their respective channels for individual counts, then the marks that were spatially related were individually investigated to determine colocalization (Figure 4.2b).

When male and female mice were considered together using Three-Way ANOVA (sex vs genotype vs. diet), the number of BrdU+ cells in the entire DG was greater in males than in females (Figure 4.2c, $P < 0.05$), but no differences existed between groups in the neurogenic niche, the SGZ (Figure 4.2d) nor any other region analyzed (GCL, hilus; not shown). Overall, females did have more Sox2+ cells in the SGZ compared to males (Figure

4.2e; $p < 0.05$), possibly indicating a greater neurogenic pool. However, the number of cells co-expressing Sox2 and BrdU did not show any differences among groups nor between sexes (Figure 4.2f; $p > 0.05$).

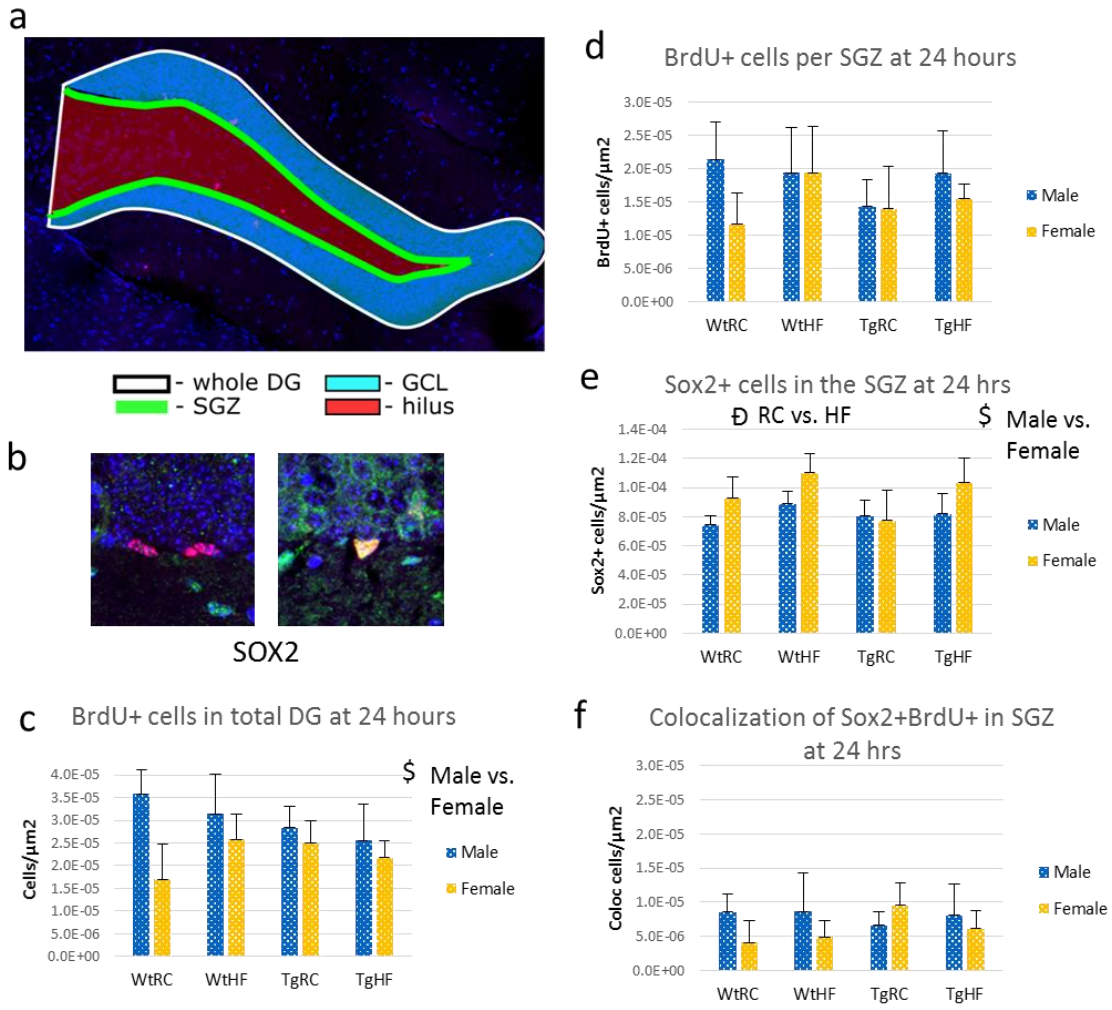


Figure 4.2: Neurogenesis is not altered by AtENPP1 or by diet.

a) Representation of counting area. White border gives area, and describes "whole DG" (incorporating SGZ, GCL and hilus). No significant difference in the total of DG area analyzed between the groups. b) Representative fluorescent images of BrdU+ labeled cells in the SGZ that are not expressing Sox2, left, and that are co-expressing Sox2, right. c) The number of BrdU+ cells in the whole DG were increased in males $ $p < 0.05$. d) BrdU+ cells in the SGZ did not differ between groups, $p>0.05$. e) The number of SOX2+ cells in the SGZ is significantly higher in mice on HF diet as compared to RC, Ð < 0.05, and in female mice vs male mice, $ $p< 0.05$ by Three-Way ANOVA. f) Number of BrdU+/Sox2+ cells in the SGZ at 24 hours, no differences between the groups; $p>0.05$ by Three-Way ANOVA. n = 27 animals.

Neural progenitor migration, but not their differentiation, is altered in AtENNP1 Tg mice.

To study the migration and differentiation of NSC in the hippocampus DG, I injected BrdU (using the same paradigm described above) and euthanized the animals 28 days after the last injection. By this time, BrdU incorporating cells in the SGZ have had the opportunity to divide, migrate into the GCL and to differentiate into young granular neurons expressing the mature neuronal marker, NeuN. By evaluating BrdU labeled cells at 28 days for the colocalization of the BrdU and NeuN, I could determine whether the experimental conditions impacted the survival, migration and neuronal differentiation of NSCs from the SGZ.

Among the female animals, there was no difference in NeuN+/BrdU+ colocalizing in the SGZ, GCL, hilus, or total DG area taken all together (One-Way ANOVA, $p > 0.05$) suggesting no differences in their ability to survive and differentiate. Among male animals (by One-Way ANOVA), the TgHF showed a significant increase in NeuN+/BrdU+ cells in the hilus compared with the Wt animals, including both WtRC and WtHF. When all animals were considered together (Three-Way ANOVA, genotype by diet by sex), the

number of BrdU+ cells (Figure 4.3b), as well as the NeuN+/BrdU+ colocalized cells were increased in the entire DG in HF fed animals (Figure 4.3c). Although the number of BrdU+ cells was not altered in the SGZ (not shown) nor in the GCL (Figure 4.3d), they were significantly increased in the hilus in Tg animals (Figure 4.3e).

This finding was surprising because new neurons do not normally migrate into the hilus from the SGZ. Two potential explanations for this phenomenon are that the BrdU positive cells observed in the hilus originated elsewhere (Eriksson et al. 1998) did find BrdU+ cells in the human hilus, but with the time post-labeling ranging from 16-780 days they likely did not originate in the SGZ, and it is also possible that they may have been glial in nature), or that they represent an ectopic migration, which has been shown to occur in response to other injuries, such as TBI and induced seizures (Robinson et al. 2016). The population of cells that did not follow a neuronal fate, those that were NeuN- but BrdU+, was significantly increased in female animals in the GCL (Figure 4.4b). However, no other region showed significant differences in the number of non-neuronal BrdU+ cells. (Figure 4.4a, c, d).

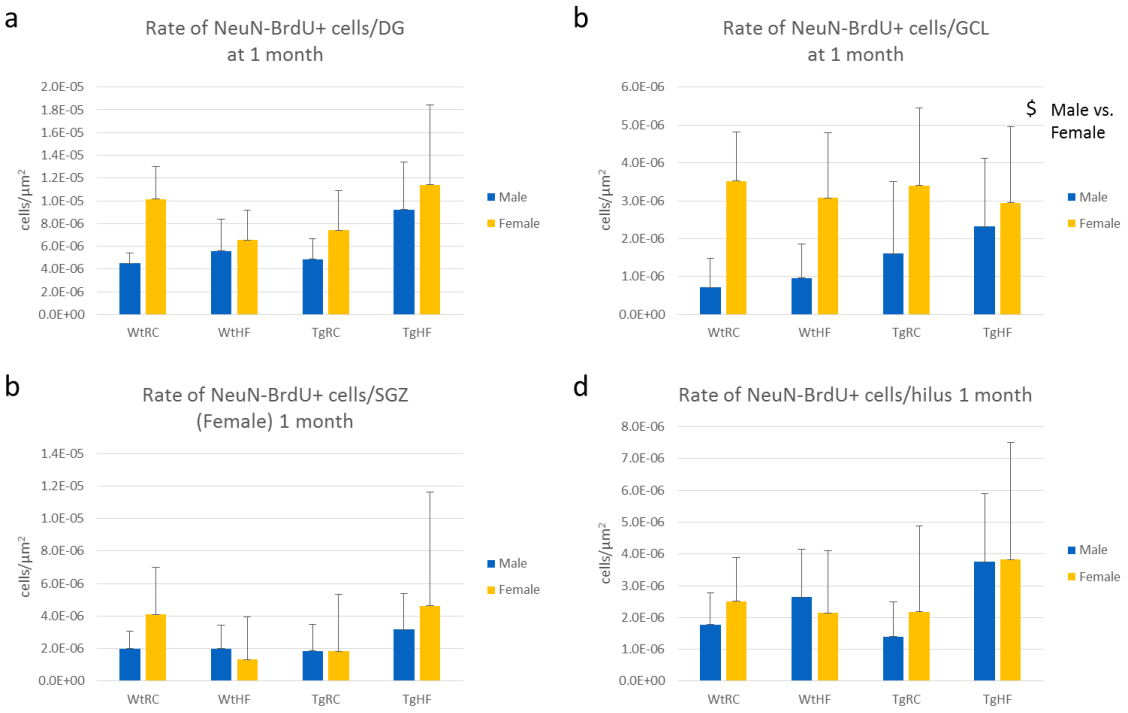


Figure 4.4: Non-neuronal BrdU+ cells in the DG at 1 month

a) BrdU+ NeuN- in the whole DG ($p > 0.05$, Three-Way ANOVA) b) BrdU+NeuN- in the GCL were greater in females than males ($\$ = p < 0.05$, Three-Way ANOVA) c) BrdU+ NeuN- in the SGZ ($p > 0.05$, Three-Way ANOVA) d) BrdU+ NeuN- in the hilus ($p > 0.05$, Three-Way ANOVA)

High fat diet and ENPP1 expression affects the expression of regulatory miRNAs in the hippocampus.

Despite their small size, microRNAs (miRNAs) are being increasingly recognized as important modulators of translation, in part because of the ability of one miRNA to have many target mRNAs (Bartel 2004). A panel of miRNAs known to have roles in modulating neurogenesis and neuronal differentiation were selected for investigation, including miR-9, miR-25, miR-29a, miR-124, miR-124, miR-137, and miR-184 (Schouten et al. 2012). To focus on the miRNA expression in the hippocampus, I coarsely dissected fresh-frozen hemispheres to remove anterior, posterior, dorsal, ventral, and lateral material, leaving the

hippocampus and some other tissues, including many of the thalamus structures. To analyze the real-time quantitative PCR, threshold cycles (C_t) were pooled for animals by treatment group, regardless of their post-injection survival time (1 vs 28 days). Two-Way ANOVA were run on the C_ts from these groups, using genotype and diet as the levels of analysis (all animals analyzed were female). No difference was found in the expression of miR-25, nor miR-132. The HF diet increased the expression of miR-9, miR-124, and miR-137 relative to the RC diet. The AtENPP1 animals showed an increased expression of miR-184 relative to the Wt animals. miR-29a was found to change based on both genotype and diet, but exhibited a strange response whereby the strongest increase in expression was seen in the WtHF. All of these results are summarized as fold change relative to WtRC expression in Figure 4.5.

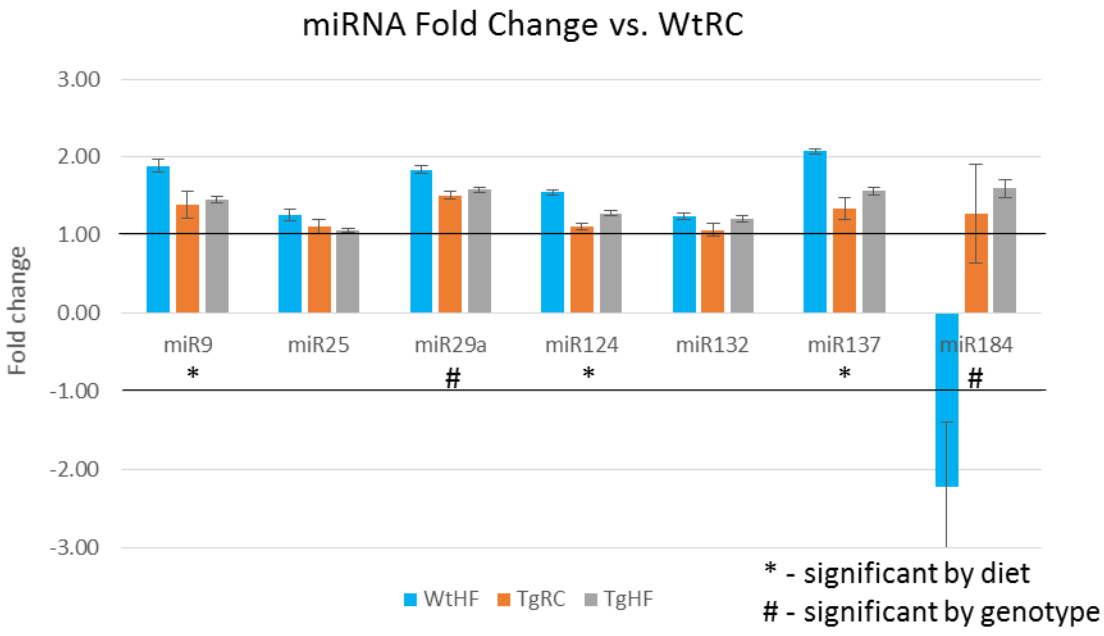


Figure 4.5: miRNA expression in females.

Fold-change in miRNA in relation to WtRC animals. Diet was linked to a significant change in expression for miR-9, -29a, -124, and -137, while miR184 showed a significant change due to genotype (2-Way ANOVA; Holm-Sidak, $p < 0.05$). n = 30

ROCK2 expression is decreased by HF diet

Knowing that the expression of miRNA can have profound impacts on the amount of its target mRNA in the cell, I also investigated the expression of six mRNA important in AD that are also regulated by the miRNA investigated. These mRNA are AKT2, BACE1, BCL2, FOXO1, NFκB, and ROCK2. Only ROCK2, a confirmed target of miR-29a, miR-124 and miR-137 demonstrated a simple relationship, where the high-fat diet decreased expression. BACE1 and BCL2 expression also both were significantly impacted, but only within the RC was it found the Tg and Wt differed; for BACE1, Tg animals had a significant decrease in expression, while for BCL2 they had a significant increase (Figure 4.6).

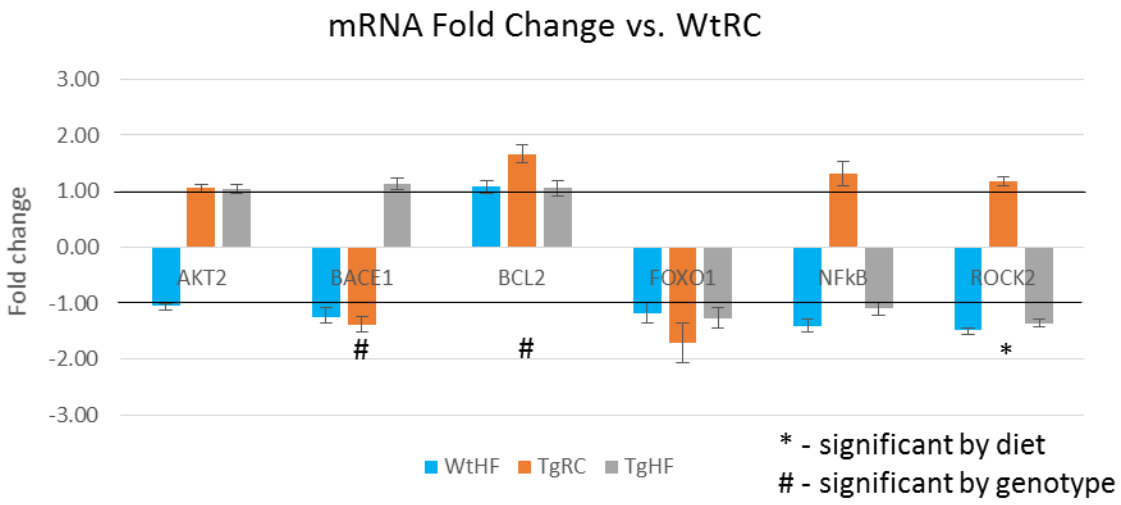
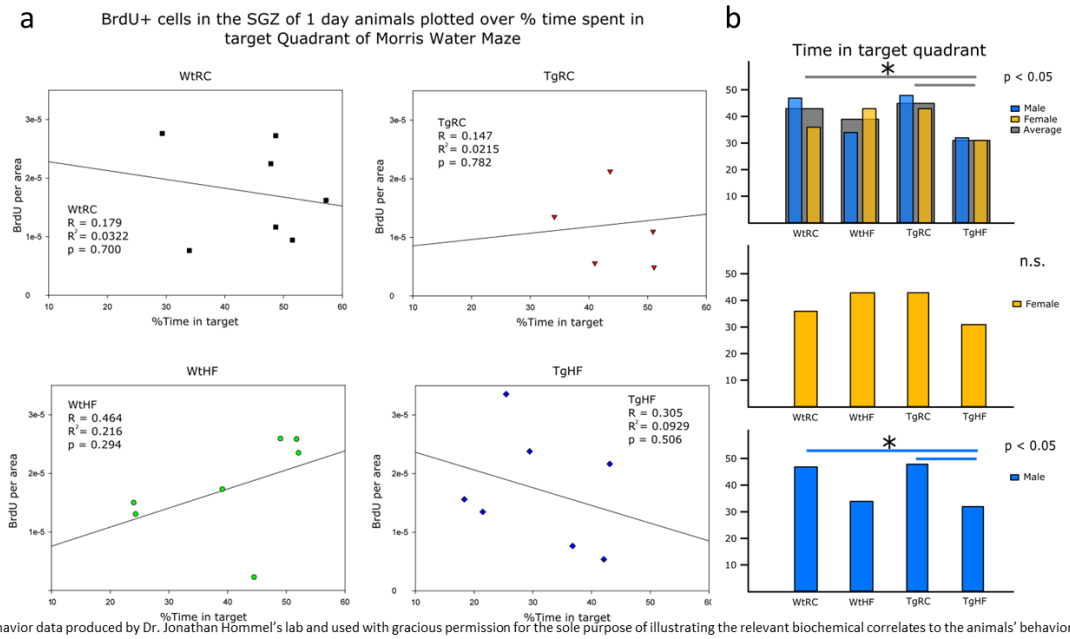


Figure 4.6: mRNA expression in females.

mRNA for BCL2 and ROCK2 changed significantly by feed (2-Way ANOVA; Holm-Sidak, $p < 0.05$). BACE1 showed an interaction effect where genotype caused difference depending on diet ($p < 0.05$). miRNA previously confirmed by other researchers to interact with these mRNA targets are displayed above target mRNA. n = 30

Neurogenesis did not correlate with Water Maze performance

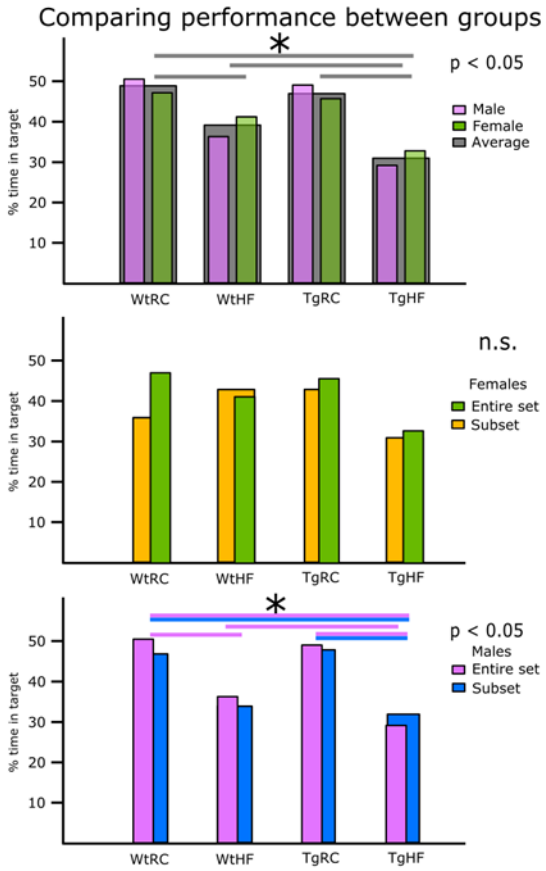
The Morris Water Maze (MWM) is a behavioral test that evaluates hippocampal-mediated spatial memory (Vorhees and Williams 2006). Briefly, for this task (performed by Dr. Jonathan Hommel's group) animals are trained to find a hidden platform as a reward stimulus to escape an aversive environment; the cold water it is forced to swim in. For the trial, the platform is removed, and the animal's performance is evaluated as the proportion of the total length of time that it spends swimming in the four quadrants of the pool. Animals with better memory performance are those who spend the most time swimming in the quadrant where they were trained to find the platform. To investigate whether the number of proliferating NSC in the hippocampus DG correlate with memory performance in the MWM , each of the 24-hour animal's BrdU+ cells/SGZ area were graphed over the percent time in the target quadrant. None of the regressions attained significance (Figure 4.7a).



(Behavior data produced by Dr. Jonathan Hommel's lab and used with gracious permission for the sole purpose of illustrating the relevant biochemical correlates to the animals' behavior.)

Figure 4.7: No strong significant describe neurogenesis and behavior relationship. a) Regression comparing animal's rate of BrdU staining per total DG against their memory performance (%time in target quadrant of Morris Water Maze; higher value percentage shows greater memory) b) Summary of animals performance per group and by sex (n of all = 60). TgHF animals demonstrated decreased memory compared with TgRC and WtRC animals as a result of the males (One-Way ANOVA; $p < 0.05$).

Of the 104 animals that performed the MWM task, I received a subset of 60 of those animals for our study (Figure 4.7b). The memory performance of the animals received was generally reflective of the trends of the whole group (Figure 4.8), with the notable exception of the female WtRC animals. Upon inspection, the total group of female WtRC animals had a non-normal distribution (Table 4.1). The female WtRC animals exhibited closer to a bimodal distribution, with 5 of the 12 animals performing above 50% of their time in the target quadrant, while the remaining 7 of 12 animals spent less than 40% of their time in the target quadrant. The subset of female WtRC animals that I received (n = 5) included 4 of the low performers, with only the lowest performer from the high-performance group, giving a non-representative performance of the females in our WtRC group compared with the average.



(Behavior data produced by Dr. Jonathan Hommel's lab and used with gracious permission for the sole purpose of illustrating the relevant biochemical correlates to the animals' behavior.)

Figure 4.8 Comparison of animal behaviors.

Few differences between Entire Set (n = 104) and the animals received for IFC Subset (n = 60)

Table 4.1: Normality test- Performance times per group

(All animals in full study; n = 104)

		W-Statistic	p value	Normality
female	WtRC:	0.852	P = 0.039	Failed
	WtHF:	0.908	P = 0.173	Passed
	TgRC:	0.957	P = 0.740	Passed
	TgHF:	0.882	P = 0.062	Passed
male	WtRC:	0.934	P = 0.350	Passed
	WtHF:	0.939	P = 0.448	Passed
	TgRC:	0.936	P = 0.476	Passed
	TgHF:	0.927	P = 0.244	Passed

DISCUSSION

In this work, I first demonstrated a geometric decline of NSC proliferation in the mouse hippocampus with increasing age, a phenomenon that has been described by other invesitgators in both rodent (Yang et al. 2015) and human hippocampus (Knoth et al. 2010)[4]. Confirming this reduction in neurogenesis set the context for the sparse BrdU+ staining observed in the aged animals. While no differences were found in the rate of division in the neurogenic niche at 1 day, I did find that the female mice possessed more Sox2+ cells in the SGZ than male mice. This was the only significant sex-linked change, although some of the relationships may show sex-linked differences if the number of animals per group were increased- such as the number of BrdU+ neurons (BrdU+NeuN+ copositive) in the entire DG at 28 days.

The only factor (among sex, genotype and diet) that was found to have an impact on the amount of BrdU found in the DG after differentiation (at 28 days) was diet, with the HF fed animals demonstrating greater BrdU labeling than those on RC, among both males and females. This had the effect of placing the amount of BrdU+ labeling post-differentiation in an apparent inverse relationship with behavioral performance- that is that the HF-fed animals, who had the worst memory performance, also demonstrated the greatest levels of BrdU+ cells at 28 days, although they were located in the hilus. Ectopic migration of new neurons into the hilus has been shown by others in traumatic brain injury models and induced seizures (Robinson et al. 2016). In the case of induced seizure, it has

[4] A similar pattern has also been shown in the lesser hedgehog tenrec Alpar, A., H. Kunzle, U. Gartner, Y. Popkova, U. Bauer, J. Grosche, A. Reichenbach and W. Hartig (2010). "Slow age-dependent decline of doublecortin expression and BrdU labeling in the forebrain from lesser hedgehog tenrecs." Brain Res **1330**: 9-19., and in canines Siwak-Tapp, C. T., E. Head, B. A. Muggenburg, N. W. Milgram and C. W. Cotman (2007). "Neurogenesis decreases with age in the canine hippocampus and correlates with cognitive function." Neurobiol Learn Mem **88**(2): 249-259..

been shown that this ectopic migration of new neurons was coincidental with a loss of Reelin signaling (Gong et al. 2007), where the expression of Reelin mRNA has been shown to be decreased in response to a high fat diet (Reichelt et al. 2015). In seizure models, these ectopic granular cell neurons have increased axosomatic connections or dendritic projections perpendicular to what is expected (depending on how far from the GCL they've migrated), and hyperpolarized membranes (Scharfman and Pierce 2012). Perhaps these ectopic cells represent a sufficient pressure on the DG function to disrupt spatial memory.

It's been shown that HF-diet decreases neurogenesis, however most of these studies are done in young animals (Park et al. 2010) – 5 wk; (Murata et al. 2017) – 4 wk). The effect becomes harder to observe the older that the animals get however (Ramos-Rodriguez et al. 2014). Critically however, Boitard et al have shown over a series of papers that high-fat diet impacts neurogenesis in an age-dependent fashion; mice beyond adolescence fed a high-fat diet do not exhibit a decrease in neurogenesis (Boitard et al. 2012), and rats that were raised on a high-fat diet exhibited impaired memory in MWM (Boitard et al. 2014), but those switched to regular food showed a normalization of behavior and neurogenesis (Boitard et al. 2016). Also of importance, in young rats it was shown that the same HF diet impaired neurogenesis in the GCL and SGZ at 2 weeks (as confirmed by BrdU+NeuN+ staining) in males but not in females (Lindqvist et al. 2006). While I did find a significant increase in males in BrdU+ cells at 1 day, that difference was only evident for the whole-DG, and not in the neurogenic niche, the SGZ. This not necessarily surprising based on the previous demonstration that HF diet does not impact neurogenesis beyond adolescence (Boitard et al. 2014). I also found an increase in Sox2+ cells in the SGZ in females, although this did not translate into a larger number of BrdU+ cells than males at 28 days.

To contrast with Boitard's findings in adult rats, I did find a small but significant increase in BrdU+ cells throughout the DG associated with the high-fat diet (Figure 4.3b). Based on the fact that others have shown behavioral impairment in adult mice is linked to neuroinflammation (Pistell et al. 2010), it would be interesting to explore the extent to which neuroinflammation differed in AtENPP1-Tg animals exposed to HF diet.

The analysis of 7 miRNA known to regulate either NSC proliferation or neuronal differentiation, and which we have previously shown to exhibit decreased expression in non-demented humans with AD pathology and increased in AD patients, were generally increased. Specifically miR-9, miR-124, and miR-137 were increased by the high-fat diet, while miR-29a and miR-184 were altered by genotype. For miR-29a, expression was increased in AtENPP1, but showed a sharper increase as a result of the HF diet in the RC animals. In miR-184, the relationship was much more direct- the transgene increased expression of miR-184 substantially. I found no change in the expression of miR-25 or miR-132. While only a few of the miRNA achieved significance by ANOVA, this trend of increasing expression levels in all miRNA is consistent with the direction of regulation that we observed previously in human tissue samples isolated from the GCL and SGZ of memory-impaired MCI and AD patients (Briley, 2016). Specifically, the high fat diet appeared to have the more pronounced effect on miRNA upregulation, but this effect was most dramatically seen in wild type mice, and appears to be suppressed in the Tg animals. This suggests that the effect of a high-fat diet on the normal functioning of the hippocampus is independent of additional risk factors, such as the insulin resistance given here by the transgene, but that a synergistic effect is working via another mechanism to impair memory.

The mRNA investigated were selected for their being confirmed targets of the miRNA investigated that were also involved in or related to processes implicated in AD. In particular, BACE1 (cleavage of APP) and BCL2 (an antiapoptotic pro-oncogene, the upregulation of which is affiliated with neuronal survival –Ahktar, 2004- and which is downregulated in response to Aβ; (Kudo et al. 2012)) have been directly implicated in AD, and both show significant changes due to the effects of the AtENPP1 genotype. Conversely, the transcription of ROCK2 was significantly decreased in response to the HF diet, where ROCK2 has been shown to be important for BACE1 activity by phosphorylating APP, as well as being elevated in AD and MCI humans (Herskowitz et al. 2013). While these mRNA did not demonstrate clear directional changes in expression, the transgenic animals tended to demonstrate the most pronounced changes.The transgene seems almost to have had the effect of modulating the impact of the high-fat diet towards baseline.

Finally, I did not find any significant correlations between the behavioral performance of the animals, and their degree of neurogenesis. It is interesting to note however that the behavioral performance of the female animals per group mirror the same relative levels that they exhibited in their BrdU+ cells in the SGZ at one day post injection. While a subtle effect, it may also be worth noting that the females as a group appeared less behaviorally impacted than the males by the HF diet, and also demonstrated significantly more Sox2+ cells in the SGZ than their male counterparts. Further, for each group the number of cells in the whole DG that did not follow a neuronal fate (those that were BrdU+ but NeuN- at 28 days) appears to have an inverse relationship with that group's behavioral performance- groups that performed less well had greater numbers of BrdU+NeuN- cells

throughout the DG. Coupled with the significantly enhanced labeling of BrdU+ cells in the hilus in the TgHF males, this may suggest that there is an important disruption of neuronal migration and differentiation that is induced by the the co-stress of insulin resistance and a high-fat diet that contributed to memory impairment.

CONCLUSION

We investigated whether differences in neurogenesis played a role in the previously observed changes in spatial memory demonstrated by AtENPP1 animals maintained on a high-fat diet. This study demonstrated that both diet, and sex have an impact on neurogenic potential, and yet not on change in neurogenesis. The HF diet did increase overall retention of new cells at 28 days, including the proportion of neurons. The AtENPP1 transgene increased the aberrant migration of newly generated neurons in the hilus. This combined with the stress induced by the HF diet may help explain the inferior behavior performance of the transgenic high-fat condition animals.

The AtENPP1 model represents an interesting approach to investigate the wide-reaching effects that even just peripheral insulin resistance can induce throughout the body. It has also shown behavior that make it intriguing as a model of diabetes-comorbid memory loss and potentially as for Alzheimer's itself. Not only does it demonstrate behavioral memory deficits in response to the high fat diet, a portion of a major risk factor for AD, but it has demonstrated epigenetic regulation that trends in the same direction as both mild cognitively impaired and AD human subject's tissues. Biologically, it should be noted that the trend of expression of the miRNA's investigated all moved in the same direction as in the MCI and AD humans relative to control (in this case WtRC; (Briley et al. 2016)). This

work has shown that the memory deficits previously observed in these animals do not appear to be tied to alterations in neurogenesis, although they may be affiliated with adult changes in neuronal differentiation in the hippocampus. Separately, this work also suggests that the high-fat diet alone is sufficient to alter epigenetic features that are relevant to neurogenesis, and that this process can be impinged upon by diet independent of any cognitive risk factors. This crucial observation ties back in to existing knowledge regarding risk factors of AD.

Further research into other mechanisms is warranted to attempt to discover what process is mediating the behavioral and central biochemical changes in this peripheral-insult animal model. Future research should seek to determine the role of inflammation, of blood-borne signaling (e.g. hormonal, exosomal), and of dysregulated fatty acids levels in disrupting neuronal function. One promising avenue may be to investigate the mechanism by which the peripheral ENPP1 expression is able to disrupt central insulin signaling, given how critical insulin signaling is to synaptic function. It also may be worthwhile to cross a traditional mouse model of AD with the AtENPP1 animals to determine if the peripheral ENPP1 phenotype interacts with and exacerbates the Aβ overproduction.

CHAPTER 5: CONCLUSION - EPIGENETIC CHANGES INCURRED BY RISK FACTORS MAY INFLUENCE COGNITIVE RESERVE

Alzheimer's disease is a neurodegenerative condition that is marked by cortical atrophy, and a substantial loss of volume in the hippocampus- a region crucial to memory- that occurs concomitant with confusion, memory loss, and declarative memory deficits. In addition to its roles in memory however, the hippocampus also happens to house one of the neurogenic niches that remains active across the mammalian life-span. The positive correlation between neurogenesis, and memory and cognition has been well established in rodents, and has increasing support from human data as well.

Among human participants in AD research, it has been observed that a small, but present, proportion of individuals demonstrate histopathology consistent with an advanced disease state, while remaining cognitively normal through the end of their life, usually at an advanced age (85 years or older). While some have argued that these individuals are "pre-clinical" AD patients, such a conclusion is beyond the scope of what is scientifically possible to conclude. Instead, we investigate them as a unique category that we believe represent a resistance to the neurodegenerative progression of Alzheimer's disease. Current evidence from ourselves and others indicate that these individuals differ from both healthy age-matched control, as well as from both the AD phenotype, and even MCI.

I wanted to determine whether neurogenesis was one of the features that differentiate these non-demented Alzheimer's neuropathology individuals from other categories of subjects. Because doing this in post-mortem tissue is difficult, I used the ratio of Sox2+ cells, which have neurogenic potential, in post-mortem human hippocampal sections. NeuN+ cells were intended to be act as a control, given that NeuN is a marker of

mature neurons. I discovered that in human tissue however that NeuN and Sox2 coexpress in hippocampal cells at a significant rate, potentially representing a novel developmental state for human neurons. Not only did the NDAN individuals exhibit substantially increased Sox2+ staining as a proportion of cells present compared with both AD and MCI individuals, but they also demonstrated coexpression of Sox2 with NeuN that were significantly increased compared with both AD and MCI individuals. Fittingly, both of these parameters were found to have significant regressions when staining rates were compared with cognitive function score, while the total proportion of NeuN+ cells was shown to have no correlation, and the proportion of Sox2-/NeuN+ cells was actually found to negatively relate to cognitive function. These results demonstrated explicitly for the first time that neurogenic capacity and cognition are positively correlated in human subjects, and that the proportion of mature (NeuN+ only) neurons is in fact negatively related to cognitive capacity.

The second question was whether differences exist in the epigenetic regulation of neurogenesis by a panel of miRNA known to influence neural stem cell division and maturation differed between these individuals with different diseases status (control, AD, MCI and NDAN). By isolating RNA explicitly from the neurogenic niche in the hippocampus of the human subjects investigated for neurogenesis, I was able to obtain a highly precise answer to our question. All 6 of the miRNA investigated showed some degree of increased expression in MCI and AD when compared with control subjects, while the NDAN showed a uniformly decreased expression. Rather than demonstrating changes in regulation in on or another miRNA toward a particular end, such as division or

maturation, the NDAN phenotype demonstrated an overall decrease in expression; a de-regulation that may be crucial in understanding their preserved cognitive status.

After aging, one of the strongest risk factors for developing AD is Type-II diabetes – a metabolic disorder underlined by its global insulin resistance. Individuals with AD are known to exhibit insulin resistance in the CNS, and our lab has previously published data demonstrating the protective role that insulin plays at the synapse against the binding of amyloid beta oligomers. Our lab further has evidence that insulin signaling pathways are more active in the NDAN individuals. With this in mind, I investigated a mouse model of insulin resistance that exhibits memory deficits when raised on a high-fat diet as a promising model for investigating AD with diabetes. I investigated these animals to determine whether neurogenesis was impaired in the animals that demonstrate impaired memory behavior. However, not only did these animals fail to exhibit differences in neurogenesis from their normal diet and non-transgenic counterparts, but the group that had performed most poorly in fact showed an increased rate of cells in the dentate gyrus having matured retaining the DNA labeling marker that I had introduced to them a month prior to collecting tissue.

I also investigated the epigenetic role of miRNA investigated in the humans in this animal model, from approximately the hippocampus and some surrounding tissue. Taking the wild-type, regular diet animals as control and investigating the high-fat and transgenic animals' miRNA expression against these, the most pronounced increase in the miRNA across all 7 targets was induced by the high-fat diet. In fact, the expression for nearly every case was increased- the same trend as seen for the expression of the miRNA in the cognitively impaired human subjects. This experiment lacked a non-demented equivalent

category (because the wild-type regular diet animals are most closely able to be compared with the control humans), but the trends in expression following in the same direction is promising, and suggests that indeed a regulatory influence from these miRNA on the neural stem cell population in the hippocampus may play a role in the improved memory performance.

This project has also led to the creation of additional questions for future research including, since not through impaired neurogenesis, by what mechanism is the peripheral transgene influencing memory? Can the induction of these miRNA alone decrease cognitive function or directly influence neurogenesis and maturation?

Together, the results of these projects have shown that neurogenesis, possibly coupled with a novel state of neuronal maturation, is important in the preservation of human cognition. Further, miRNAs that are known to influence neural stem cell division and maturation are correlated with decreased memory performance in both humans and mice. Although I did not find differences in neurogenesis between the groups of mice studied, the influence of the high fat diet was shown to be not just a risk, but an injurious influence in its own right, pushing up the regulation of miRNA's that were seen to be also increased in cognitively impaired humans. Such a conclusion may bode well for clinical Alzheimer's disease mitigation attempts, as altering a patient's diet to reduce overall fat intake is easily feasible, and apparently beneficial- regardless of other pre-existing risk factors that the individual may have no control over.

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Vita

David James Briley was born February 3, 1988 in Commerce Township, Oakland County, Michigan to Elaine Grace Gazan Briley & James 'Jim' Wallace Briley, Jr. Around two and a half years later, the family would move to Brucköbel, Germany where David began Kindergarten fully immersed in a German-speaking environment. After around three and a half years, they moved to the deep outskirts of London, residing in Stoke D'Abernon, where David would attend St. Andrew's Primary school in nearby Cobham, for a year and a half. David's younger sister, Emily, was born June 17, 1994. Upon returning to the U.S., the family spent approximately 7 months in Wheaton, Illinois where David was briefly enrolled in Wheaton Elementary school's 2nd year. They would relocate in the spring to Reading, Connecticut, where David finished 2nd and 3rd year in Reading Elementary school. David's younger brother Jeremy, was born April 30, 1996.

After Jim took another new position, the family relocated in 1997 to Charlotte, North Carolina. David began the 4th year at McKee Road Elementary school, but finished the school year at Carmel Christian School, where he stayed through 6th year. Fed up with the local schooling options, his mother decided to simply homeschool all three children for the next two years. While in Charlotte, Elaine gave David the opportunity to take courses in theatre performance, which sparked his love for both performance as well as technical production. Jim relocated the family again in early 2002, so that David began 9th year at the public McKinney High-School in McKinney, Texas. After completing his 11th year, the family relocated again to Fishers, Indiana, where David would complete 12th year and graduate from Hamilton Southeastern High School in 2006.

He completed his Bachelor of Arts in Psychology at nearby Butler University between 2006 and 2010, from which he graduate Cum Laude. While at Butler, David completed an Honor's Thesis project comprised of original research, titled "The Impact of Imagined Reactions on Feelings About Disclosing Stigmatized vs. Non-Stigmatized

Beliefs" under the mentorship and guidance of Dr. Kathryn Morris. In advance of his senior year at Butler, David's family moved to Hyderabad, India in summer 2009, where David was fortunate to be able to visit them for a month prior to the start of the school year. After graduation, throughout the fall and early winter of 2010, David spent a further 5 months living with his family in Hyderabad, including a month during which he lived alone in Bangalore at an unpaid internship with the small start-up cleanray (a Vignani Technologies company). At cleanray he was able to have first-hand experience with R&D, design, manufacturing and assembly, materials sourcing, and sales in an Indian market for industrial-scale LED lighting.

In February 2011, David was invited to interview at University of Texas Medical Branch, where he was offered a position in the Neuroscience Graduate Program with the addition of a Presidential Scholar scholarship. After the first year of classes, David joined the lab of Dr. Massoud Motamedi where he gained theoretical and hands-on experience with a wide array of imaging techniques, most extensively in confocal and non-linear optical microscopy techniques. In April 2014 David was able to join the lab of Dr. Giulio Taglialatela, where he undertook a project to evaluate the role of neurogenesis in nondemented individuals with Alzheimer's disease neuropathology, which would develop into this dissertation project.

His final oral defense and examination commenced May 03, 2017.

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