NEUROGENESIS IN THE ADULT BRAIN, GENE NETWORKS, AND ALZHEIMER'S DISEASE

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New neurons are generated throughout adulthood in two regions of the brain, the dentate gyrus of the hippocampus, which is important for memory formation and cognitive functions, and the sub-ventricular zone of the olfactory bulb, which is important for the sense of smell, and are incorporated into hippocampal network circuitry. Disruption of this process has been postulated to contribute to neurodegenerative disorders including Alzheimer's disease [1]. AD is the most common form of adult-onset dementia and the number of patients with AD escalates dramatically each year. The generation of new neurons in the dentate gyrus declines with age and in AD. Many of the molecular players in AD are also modulators of adult neurogenesis, but the genetic mechanisms influencing adult neurogenesis in AD are unclear. The overall goal of this project is to identify candidate genes and pathways that play a role in neurogenesis in the adult brain and to test the hypotheses that 1) hippocampal neurogenesis-related genes and pathways are significantly perturbed in AD and 2) neurogenesis-related pathways are significantly associated with hippocampal volume and other AD-related biomarker endophenotypes including brain deposition of amyloid-β and tau pathology. First, potential modulators of adult neurogenesis and their roles in neurodegenerative diseases were evaluated. Candidate genes that control the

turnover process of neural stem cells/precursors to new functional neurons during adult neurogenesis were manually curated using a pathway-based systems biology approach. Second, a targeted neurogenesis pathway-based gene analysis was performed resulting in the identification of *ADORA2A* as associated with hippocampal volume and memory performance in mild cognitive impairment and AD. Third, a genome-wide gene-set enrichment analysis was conducted to discover associations between hippocampal volume and AD-related endophenotypes and neurogenesis-related pathways. Within the discovered neurogenesis enriched pathways, a gene-based association analysis identified *TESC* and *ACVR1* as significantly associated with hippocampal volume and *APOE* and *PVLR2* as significantly associated with tau and amyloid beta levels in cerebrospinal fluid. This project identifies new genetic contributions to hippocampal neurogenesis with translational implications for novel therapeutic targets related to learning and memory and neuroprotection in AD.

Andrew J. Saykin, PsyD, Chair

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ACVR1	Activin A receptor type 1
AD	Alzheimer’s disease
ADORA2A	Adenosine 2a receptor
AHBA	Allen Human Brain Atlas
ALS	Amyotrophic lateral sclerosis
Ang	Angiopoietin
APOE	Apolipoprotein E
APP	Amyloid precursor protein
AV-45	^{18}F-florbetapir
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
BrdU	5-bromo-2'-deoxyuridine
CBF	Cerebrospinal fluid
CCR1	C-C chemokine receptor type 1
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5

CHRFAM7A	Cholinergic receptor nicotinic alpha 7
CN	Cognitively normal older individuals
CREB	cAMPresponse element binding
CSF	Cerebrospinal fluid
CXCR2	C-X-C chemokine receptor type 2
CXCR3	C-X-C chemokine receptor type 3
CXCR4	C-X-C chemokine receptor type 4
D2R	Depomine receptor 2
DCX	Doublecortin
DG	Dentate Gyrus
DISC1	Disrupted in Schizophrenia 1
DNTMs	DNA methyltransferases
DPP4	Dipeptidyl peptidase-4
EGF	Epidermal growth factor
EGFR	Epidermal growth factor
ENIGMA	Enhancing Neuro Imaging Genetics through Meta-Analysis
EphA	Ephrin type-A receptor
EphB	Ephrin type-B receptor
FGF	Fibroblast growth factor

FGF2	Fibroblast growth factor 2
FGFR	Fibroblast growth factor receptor
GABA	Gamma-Aminobutyric acid
Gadd45a	DNA-damage-inducible protein 45 alpha
GFAP	Glial fibrillary acidic protein
GSEA	Gene Set Enrichment Analysis
GSK-3β	Glycogen synthas kinase 3 beta
GWAS	Genome wide association study
HD	Huntington disease
HDAC3	Histone deacetylase 3
HDAC5	Histone deacetylase 3
HDAC7	Histone deacetylase 7
ICV	Intracranial volume
IGF-1	Insulin-like growth factor
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
KGG	Knowledge-based mining system for Genome-wide Genetic studies
LD	Linkage disequlibrium

MAF	Minor allele frequency
MANGO	Mammalian Adult Neurogenesis Go Ontology
MAPT	Microtubule-associated protein tau
MBD-1	Methyl-CpG-binding domain protein
MBDs	Methyl-CpG-binding domains
MCI	Mild Cognitive Impairment
MCP-1	Monocyte chemotactic protein 1
MeCP2	Methyl-CpG-binding protein 2
MRI	Magnetic resonance imaging
MSRB3	Methionine Sulfoxide Reductase B3
NCAM1	Neural cell adhesion molecule 1
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NHE1	Na(+)/H(+) exchanger
NMDA	N-methyl-D-aspartate
Nrg1	Neuregulin
NSCs	Neural stem cells
OB	Olfactory bulb
PD	Parkinson disease

PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PKC	Protein Kinase C (PKC)
PSEN1	Presenilin 1
PSEN2	Presenilin 2 (PSEN2)
PVLR2	Poliovirus Receptor-Related 2
RELN	Reelin
REST	RE1 silencing transcription factor
RMS	Rostral migratory stream
SDF-1	Stromal cell-derived factor 1
SGZ	Subgranular zone
Shh	Sonic hedgehog
SMC	Significant memory concern
Smo	Smoothen
SNP	Single nucleotide polymorphism
SPM8	Statistical Parametric Mapping 8
SVZ	Subventricular zone
TESC	Tescalcin
TNF-α	Tumor necrosis factor alpha

TrkB	Tyrosine receptor kinase B
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

I. Adult neurogenesis and neurodegenerative diseases: A systems biology perspective

A. Introduction

New neurons are generated throughout adulthood in two regions of the brain, the olfactory bulb and dentate gyrus of the hippocampus, and are incorporated into the hippocampal network circuitry; disruption of this process has been postulated to contribute to neurodegenerative diseases including Alzheimer's disease and Parkinson disease. Known modulators of adult neurogenesis include signal transduction pathways, the vascular and immune systems, metabolic factors, and epigenetic regulation. Multiple intrinsic and extrinsic factors such as neurotrophic factors, transcription factors, and cell cycle regulators control neural stem cell proliferation, maintenance in the adult neurogenic niche, and differentiation into mature neurons; these factors act in networks of signaling molecules that influence each other during construction and maintenance of neural circuits, and in turn contribute to learning and memory. The immune system and vascular system are necessary for neuronal formation and neural stem cell fate determination. Inflammatory cytokines regulate adult neurogenesis in response to immune system activation, while the vasculature regulates the neural stem cell niche. Vasculature, immune/support cell populations (microglia/astrocytes), adhesion molecules, growth factors, and the extracellular matrix also provide a homing environment for neural stem cells. Epigenetic changes during

hippocampal neurogenesis also impact memory and learning. Some genetic variations in neurogenesis related genes may play important roles in the alteration of neural stem cells differentiation into new born neurons during adult neurogenesis, with important therapeutic implications.

After the discovery of neurogenesis in the adult human brain, there were many studies of adult neurogenesis over two decades to identify the underlying genetic and environmental mechanisms. In 1998, the presence of adult-born neurons in the dentate gyrus of the human hippocampus had been identified by using cancer patients who had received the labelled 5-bromo-2'-deoxyuridine (BrdU) in hippocampal neurons [2]. By measuring the concentration of nuclear bomb-test-derived 14C in genomic DNA in the adult human brain, Spalding and colleagues found that neurons are added in the hippocampus per day corresponding to an annual turnover of 1.75% of the neurons within the renewing fraction, with a modest decline during aging [3]. Alterations in adult neurogenesis have been associated with neurological and psychiatric disorders. Enhanced understanding of the contribution of biological processes and genetic factors related to neurogenesis could lead to novel therapeutic strategies for neurodegenerative disease progression as well as many other conditions.

Numerous intrinsic and extrinsic factors affect the processes of adult neurogenesis, including the proliferation of neural progenitor cells, fate determination of neural progenitor cell progenies, and the differentiation,

migration and maturation of adult neurons. Following these processes, adult–born neurons integrate into the complex circuitry of the olfactory bulb and hippocampus [4, 5]. Since the hippocampus plays a crucial role in the formation of episodic and spatial memory and is associated with many neurodegenerative diseases [6-8], we focus on hippocampal adult neurogenesis in this review. We discuss mechanisms of and interactions between these modulators of adult neurogenesis, as well as implications for neurodegenerative disease and current therapeutic research.

i. Neurogenesis Role in Cognition

In the mammalian brain, adult neurogenesis occurs in two main regions: the dentate gyrus of the hippocampus, which is important for memory formation and cognitive functions, and the subventricular zone (SVZ) of the olfactory bulb, which is important for the sense of smell [9, 10]. Newborn neurons added to hippocampal circuitry during adult neurogenesis are important for the stimulation of spatial memory and learning [11, 12]. Spatial memory is defined as pattern separation, the ability to discriminate among similar experiences [13]. The dentate gyrus and CA3 regions of the hippocampus are implicated in spatial memory function and the capacity for pattern separation, and are associated with learning new information. These cognitive functions have all been shown to decrease with age. Integration of new neurons into the existing hippocampal neural circuitry and environmental and behavioral factors modulating adult

neurogenesis play important roles in hippocampal-dependent learning and memory [14].

Although there are a number of studies showing adult neurogenesis involvement in the formation of spatial memory and learning, the results are somewhat controversial. Genetic ablation of GFAP-expressing (GFAP+) neural progenitor cells did not impair a hippocampal-dependent learning or memory task, while it did inhibit contextual fear conditioning [15]. In contrast, rats treated with the DNA methylating agent methylazoxymethanol acetate (MAM) toxin for proliferating cells showed a reduction in the formation of newborn neurons in the dentate gyrus, which was associated with impaired hippocampal-dependent memory formation, but not contextual fear conditioning [16].

New neurons generated by adult neurogenesis in the granule layer of the dentate gyrus within the hippocampus play a crucial role in the development of memory and learning [17]. There is delayed maturation of the adult-generated granule cells in the dentate gyrus [18]. After one week of differentiation, newborn neurons' apical dendrites reach the molecular layer and their axonal projections reach to the CA3 region, and spines form 16 days after division. Dendritic spines express glutamate receptors and the TrkB receptor for BDNF on their surface to regulate the survival of the spines [19, 20]. Dendritic and axonal outgrowth accompany the maturation of the neurons. Spine density increases in the fourth week post-division. The hippocampal-dependent Morris water maze test showed

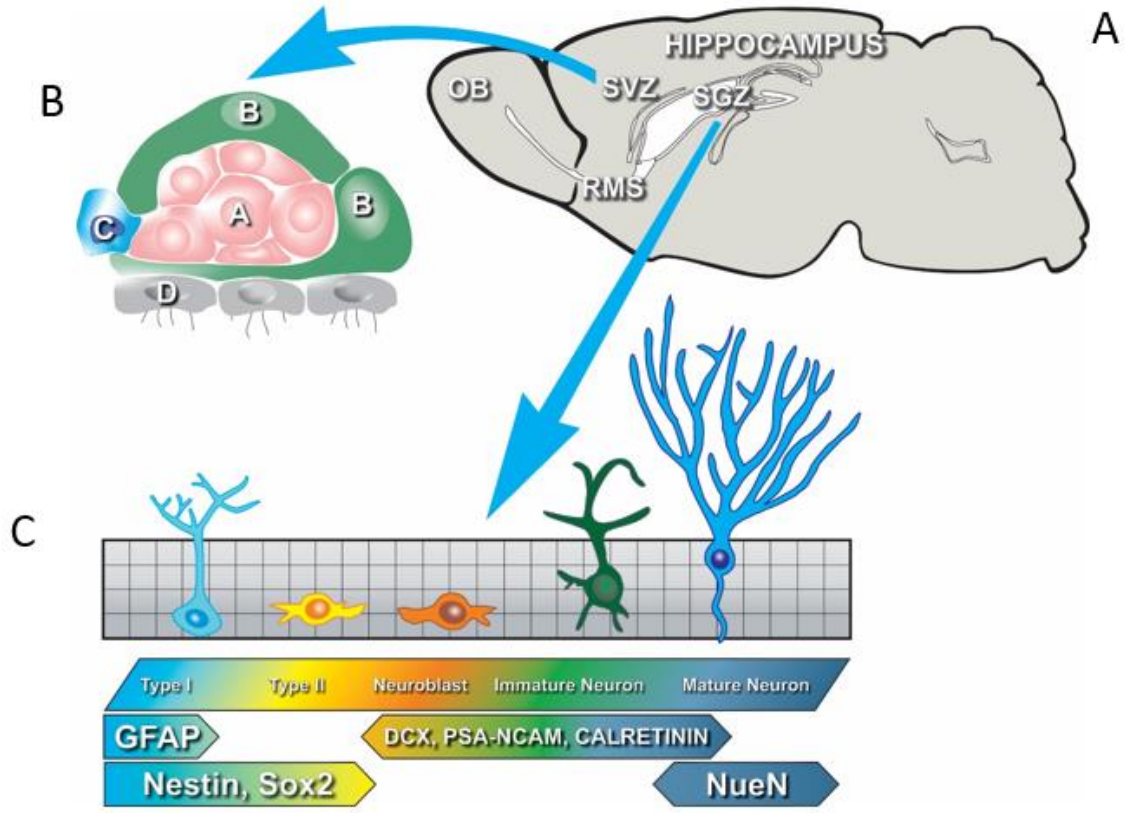
that 4-28 days old newborn neurons are required for the formation of long-term hippocampus-dependent spatial memory [21]. Differential synaptic connectivity of the hippocampus along the septo-temporal axis disrupts the septal and temporal connections of the hippocampus and negatively effects learning and memory [22]. By two months, structurally-modified highly dynamic and plastic spines are regulated by neuronal activity [23]. Synaptic connectivity and excitability of new neurons in the adult hippocampus are stimulated by bHLH transcription factors such as Neurod1 and Neurod2 during adult neurogenesis and memory formation. A recent study showed that Neurod1 overexpression in dividing neural stem cells (NSCs) leads to differentiation of the new neurons, and inhibits memory deficits and rescues memory impairment in APP/PS1 mice [24]. Adult hippocampal neurogenesis is highly involved in memory formation and learning which has important implications for neurodegenerative disease.

ii. Cell Types in Adult Neurogenesis

In the adult brain, two important regions where neural stem cells are retained are responsible for adult neurogenesis, the SVZ and the subgranular zone (SGZ) of the hippocampal dentate gyrus. In the SVZ, B1 cell residues line the border between the striatum and lateral ventricle; this subpopulation of cells possesses astroglial properties and acts as NSCs. B1 cells give rise to intermediate progenitors, which divide and generate the neurons destined for the olfactory bulb [25]. Unlike B1 cells in the SVZ, which lie in the ventricular wall and have

processes that contact the cerebrospinal fluid (CSF), radial astrocytes in the SGZ reside deeper in the brain parenchyma in the granule cell layer of the dentate gyrus, which does not have contact with the ventricular system, and act as NSCs, also known as type 1 cells. Radial astrocytes generate intermediate progenitors (type2a and type 2b cells), which differentiate into type 3 cells, or immature granule cells. Radial astrocytes have three main domains. The side of the radial astrocytes known as the **proximal domain** faces the hilus and has contact with blood vessels and lateral processes, which also contact the other radial astrocytes. The second domain is the **intermediate domain**, which includes the cell body and main shaft. The intermediate domain of the radial astrocytes interacts with intermediate progenitor cells and granule cells. This second domain is important for cell-cell interactions of NSCs with their progeny and for detection of local neural activity and signaling from granule cells. The third domain is the **distal domain,** which is highly branched and contacts with other granule cells, axons, and synapses in the molecular layer [26, 27]. Briefly, the primary progenitor cells are known as Type-I or B-type cells. They divide to produce intermediate stage progenitors (Type-II, Type-III, or D-type cells), which then undergo further rounds of cell division to generate post-mitotic immature granule neurons. These glutamatergic neurons then integrate into the dentate gyrus as functional components of the hippocampal circuitry [28-30]. Regulatory signals from the peripheral environment play a crucial role in identifying neural progenitor fate to become neurons or astrocytes [31] (Figure 1).

Figure 1. Adult neurogenesis in the brain. A) Adult neurogenesis occurs in two regions: the subgranular zone (SGZ) and the subventricular zone (SVZ). B) In the SVZ, neural progenitor cells (type B cells) give rise to type C cells, which differentiate to neuroblasts (type A cells). Type A cells migrate via the rostral migratory stream (RMS) and differentiate into neurons in the olfactory bulb (OB). Neuroblasts migrate via the RMS to the olfactory bulb and generate new neurons. C) In the SGZ, glial-like radial stem cells known as Type-I cells express glial fibrillary acidic protein (GFAP) and nestin. They divide to produce intermediate stage progenitors (Type-II cells), which then undergo further rounds of cell division to generate neuroblasts and post-mitotic immature granule neurons. Type II cells express Sox2, while neuroblast and immature neurons express doublecortin (DCX), PSA-NCAM, and calretinin. Mature neurons are defined by expression of NeuN.



Neural progenitor cells in the SVZ of the lateral ventricle migrate through the rostral migratory stream and become granule and perigranular neurons of the olfactory bulb, whereas the neural progenitor cells in the SGZ differentiate and integrate into the local neural network as granule cells of the hippocampus in the adult human brain. [2, 3, 7, 32]. NSCs in the SGZ and SVZ undergo self-renewal to provide a lifelong supply for the mature hippocampal dentate gyrus granule neurons and olfactory bulb respectively. In both regions, NSCs lead to neural progenitor cells, which are limited in proliferation and differentiate into neurons or glia [23].

Although there are many differences between adult SVZ and SGZ neurogenic niches, NSCs in both, like glial radial stem cells in the embryo, express GFAP, nestin, and Sox2, and they directly contact blood vessels. There is overlap between expression of these markers from different neural cell types and they might be responsible for the generation of the heterogeneous neural cell types [4, 33]. The NSC population in the SVZ generates neurons and oligodendrocytes, while the NSC population in the SGZ generates neurons and astrocytes. Adult progenitors line the hilar side of the granule cell layer of the dentate gyrus where SGZ astrocytes lead to intermediate progenitors. These progenitors mature into granule neurons of the dentate gyrus and send axonal projections to the CA3 region [34, 35]. GABA plays an important role in progenitor differentiation into newborn neurons and regulation of neuronal network activity and synaptic integration of proliferation in the SGZ; GABA-releasing neuroblast progeny of the SVZ stimulate differentiation through GABAergic synaptic input into progenitor cells. [36]. After these progenitors differentiate to immature neurons, the dendrites of the newborn dentate gyrus cells become more complex and spread deeper into the molecular cell layer [23, 37]

Adult hippocampal neurogenesis begins with the proliferation of neural progenitor cells in the SGZ zone. While a small population of neural progenitor cells differentiate into glia, most of the neural progenitor cells differentiate into dentate granule cells within the hippocampus and undergo the process of morphological and physiological maturation, [8, 23].

iii. Neurogenesis Role in the Hippocampus

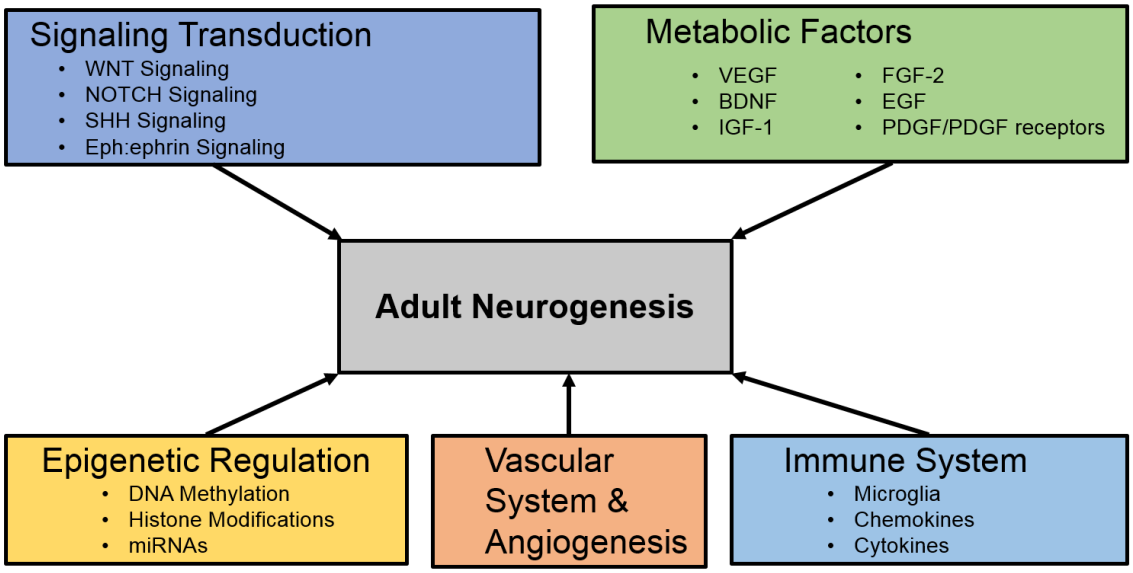
The hippocampus is the most important region for learning and episodic/spatial memory. Within the hippocampus, the dentate gyrus provides a steady level of neurogenesis throughout life. Intrinsic and extrinsic regulatory factors affect the proliferation, differentiation, maturation, fate determination, and survival of newborn cells in the dentate gyrus [38]. Pyramidal and granule cells comprise most of the cells in the hippocampus. The hippocampus receives a large number of sensory inputs uni-directionally from neocortical regions, which leads to the regulation of hippocampal formation. The granule cell population in the dentate gyrus is smaller than the pyramidal cell population in the CA3 region of the hippocampus, encodes the information from the entorhinal cortex, and projects to the CA3 region [39-41]. CA3 projects a signal to CA1 that changes the inputs in a more linear fashion from the entorhinal cortex to the CA3 region. CA1 subsequently projects the signal into the subiculum and entorhinal cortex [42]. This neuronal signaling flow plays an important role in the formation of memory and learning [43]. NSCs in the dentate gyrus also differentiate into the astrocytes between the granule cell layer and the hilus, which support hippocampal-dependent memory function [43].

B. Modulators in Adult Neurogenesis

Recent studies have highlighted five important modulators of neurogenesis, including signaling transduction pathways, the vascular and immune systems, metabolic factors, and epigenetic regulation (Figure 2) [44-46]. The alteration in these modulators during adult neurogenesis may be related to the development of neurodegenerative diseases. A more complete understanding of the role and function of each modulator in regulating NSC fate and integration as neurons in the SGZ and olfactory bulb may provide crucial insights leading to new therapies for neurological diseases in humans.

Figure 2. Schematic illustration of adult neurogenesis related pathways.

There are five crucial modulators controlling neural stem cell (NSC) proliferation, differentiation, migration, and maintenance during adult neurogenesis: Signaling transduction pathways, the vascular and immune systems, metabolic factors, and epigenetic regulation. These five modulators are composed of diverse molecules and biological pathways and mechanisms acting to control neurogenesis.



Modulators of Adult Neurogenesis

i. Signaling Transduction in Adult Neurogenesis

Several essential signaling transduction pathways regulate proliferation and differentiation of NSCs, as well as migration and survival of the new-born neurons and their integration into the neuronal circuitry network in the adult brain. In this section, we discuss key signaling pathways Wnt, Notch, Sonic hedgehog (Shh), and Eph:ephrin, which modulate different stages of adult neurogenesis.

Wnt Signaling regulates adult hippocampal neurogenesis on molecular, cellular, and behavioral levels. It has been shown that stem cells in the adult hippocampus express receptors and signaling molecules that correspond to Wnt proteins. Astrocytes and the hilar cells of the dentate gyrus express Wnt-3, and promote neuroblast proliferation and neuronal differentiation into hippocampal granule neurons through Wnt signaling-mediated activation of NeuroD1 [47, 48]. NeuroD1 is required for the survival and the maturation of adult neurons. NSC proliferation and cell fate determination is controlled by Wnt signaling in hippocampal neurogenesis [49]. In the Wnt signaling pathway, β-catenin is necessary for the expression of Wnt target genes. The expression level of β-catenin by NSCs in the dentate gyrus of the hippocampus affects proliferation of stem cells, as well as axonal and dendritic development. Wnt signaling protects neurons from injury by neurotoxins such as amyloid-β accumulation in AD. For

example, Wnt activation of Protein Kinase C (PKC) inhibits GSK-3β activity, which plays an important role in the phosphorylation and degradation of β–catenin and inhibits apoptosis of the progenitor cells [47, 50, 51]. Another Wnt signaling pathway component, survivin, increases adult neurogenesis in the dentate gyrus of the hippocampus during traumatic brain injury [52]. On the other hand, it has been shown that the Wnt/β-catenin signaling pathway promotes neuronal proliferation, but not differentiation, via Disrupted in Schizophrenia 1 (DISC1), which is an inhibitor of GSK-3β, in the adult hippocampus [53]. Yet another Wnt family member, Wnt-7A, is activated by the nuclear orphan receptor Tlx and enhances proliferation and self-renewal of NSCs via the canonical Wnt signaling pathway in adult neurogenesis [54]. To sum up, the canonical Wnt signaling pathway, including Wnt-3, Wnt-7A, GSK-3β, and Tlx, may induce progenitor cell proliferation and survival in the dentate gyrus of the hippocampus.

Notch Signaling is important for the maintenance and differentiation of NSCs in adult neurogenesis [46, 55]. Notch signaling activates the expression of bHLH transcription factors such as Hes1, Hes3, and Hes5, which suppresses proneuronal gene expression and promotes maintenance of NSCs by inhibiting differentiation of NSCs in the SVZ [56]. Likewise, Notch1 is necessary for the self-renewal and maintenance of neural stem and progenitor cells in the dentate gyrus of the hippocampus [57]. Notch signaling and EGFR signaling regulate the balanced interaction between NSCs' and progenitor cells' number and self-renewal in the SVZ region [58]. Notch signaling is necessary for the maintenance

of undifferentiated cells, while EGFR promotes the proliferation and migration of progenitor cells [59]. Since Notch1 and EGFR are substrates of ADAM 10, which prevents amyloid-β production, the well-known hallmark of AD, by proteolysis of the alpha site of the amyloid precursor protein (APP), there is strong link between Notch signaling and AD pathology as well as hippocampal neurogenesis [60-62].

There are also connections between Wnt signaling and Notch signaling during neurogenesis. FGF2 causes accumulation of β-catenin by inhibiting GSK-3β. β–catenin accumulation leads to proliferation and maintenance of NSCs by activating LEF/TCF transcription factors. Then, β-catenin and Notch1 make a complex with anti-neurogenic Hes1 and enhance Hes1 expression, which inhibits the differentiation of the progenitor cell population [63].

Sequential signaling through Notch1 and erbB receptors plays an important role in glial cell differentiation [64]. ErbB4 and neuregulin-1 and -2 receptors are expressed by immature neuroblasts as well as astrocytes and ependymal cells. When ErbB4 binds to neuregulin ligands, it drives progenitor cells proliferation and migration of neuroblasts in the SVZ. However, ErbB4 controls the formation and maintenance of glial cells and differentiation of glial cells via activation of Notch1 signaling from granule cells in the astroglia [64]. As a result, Notch signaling is involved in the regulation of NSC proliferation, maintenance, and cell fate determination.

Sonic Hedgehog Signaling (Shh) plays a crucial role in differentiation of cell types and the formation of neurons during adult neurogenesis [65]. Shh receptor Patch is expressed by the progenitor cells of the dentate gyrus as well as pyramidal cells in the CA1 through CA3, and drives hippocampal formation during adult neurogenesis. Shh controls the proliferation of progenitor cells in the dentate gyrus during hippocampal neurogenesis [66, 67]. Smoothen (Smo), the other Shh receptor, is expressed in the adult hippocampus [68]. Smo knockout mice show a significant reduction of proliferation of NSCs and progenitor cells in the dentate gyrus of the hippocampus and SVZ [69, 70]. Both Patch1 and Smo are located on the primary cilia on the GFAP+ stem/progenitor cells, and primary cilia control the proliferation of Type2a progenitor cells without affecting the amplification of Type 1 radial NSCs in adult hippocampal neurogenesis, which is important for spatial learning [71].

Eph:ephrin signaling, especially via receptor EphB1-B3 and ligand Ephrin-B1-B3, plays an important role in the migration of neuroblasts to the olfactory bulb and increases the proliferation of NSCs in the SVZ in adult neurogenesis [72] EphB1 is expressed in NSCs in the SGZ and controls proliferation, migration, and polarity of the neural/progenitor cells during hippocampal neurogenesis [73]. A knockout mice study showed that Ephrin-A2 and Ephrin-A3 inhibit progenitor cell proliferation by activating Ephrin-A7-mediated signaling in the SVZ and SGZ regions [74]. In addition, Ephrin-B2 is expressed in astrocytes, while corresponding EphB4 receptors are expressed in NSCs, and Ephrin-B2:EphB4

signaling activates β-catenin independently of Wnt signaling and increases the regulation of proneuronal transcription factors as well as inducing neuronal differentiation of progenitor cells in the dentate gyrus of the SGZ [75, 76] . EphB receptors are expressed in dendrites and play roles in the maturation and plasticity of synapses, regulating spine and synapse formation [44, 77]. Epha1 has also been identified as a risk gene for Alzheimer's disease (AD) [78]. Alteration in EphA4 and EphB2 have been shown to cause memory impairment in an AD mouse model [79], highlighting the importance of this signaling pathway in neurogenesis processes and its relevance to neurodegenerative disease. Several signaling pathways including Wnt, Notch, Shh, and Eph:ephrin, regulate self-renewal, proliferation, and differentiation of NSCs, as well as migration of neuroblasts and integration of newborn neurons in the adult brain. Addressing the roles of these crucial signaling mechanisms in NSC regulation and integration into hippocampal network circuitry may lead to targeted new therapies for currently intractable human neurological disorders.

ii. Vascular System and Angiogenesis

The vasculature plays an important role in stem cell niche regulation and maintenance in neurogenesis. Angiogenesis, the development of new vascular networks with existing vessels, is linked with neurogenesis since angiogenesis is stimulated by angiogenic genes expressed in neural progenitor cells [80]. NSCs are deliberately distributed around blood cells in the brain to facilitate access to circulating signaling molecules, growth factors and nutrients. The vascular beds of the SVZ and SGZ support adult neurogenesis. While a three-dimensional niche including NSC and transit-amplifying cells resides close to SVZ blood vessels and increases the vascular contact to other cells in the SVZ, radial astrocyte residues in areas near blood vessels and endothelial cells promote neural progenitor stem cell proliferation, neuronal differentiation, and survival by expressing pro-angiogenic factors such as FGF, VEGF, IGF-1, which enhance neurogenesis and neuroprotection in the SGZ. [81-83]. Angiogenesis and neurogenesis are combined in the hippocampus; up to 37% of the cells in the SGZ are endothelial precursors. Neural progenitors and angioblasts proliferate together in the clusters associated with the microvasculature of the SGZ. The cells in this cluster express VEGF receptors, while the tissue surrounding this cluster expresses VEGF. Furthermore, newly generated capillaries express BDNF, which promotes the recruitment and migration of newborn neurons [84]. Exercise induces angiogenesis as well as neurogenesis in the hippocampus by increasing the expression of NGF and BDNF [85]. However, more research is

needed to elucidate the factors modulating endogenous stem cell mobilization out of the stem cell niche. Some studies show that activated microglia and astrocytes express cytokines such as MCP-1, SCF, CXCL12, and VEGFA to attract NSC migration; NSCs express the compatible receptors CCR2, SCF receptor c-kit, CXCR4, and VEGFR, respectively, during disease processes such as stroke and brain tumor [86-89]. Additionally, microvessel entorhinal cortex cytokine expression profiles show that many growth factors, chemokines, adhesion molecules and extracellular matrix proteins such as PDGF-BB, RANTES, I-TAC, NAP-2, GROα, Ang-2, and M-CSF are secreted by the entorhinal cortex to promote chemo-attraction of NSCs [90]. Many vascular and angiogenic factors regulate neurogenesis in the adult brain; better understanding of the vascular compartment of the NSC niche may provide therapeutic insights for neuronal diseases**.**

iii. Metabolic Factors and Their Role in Adult Neurogenesis

Adult neurogenesis is regulated by metabolic growth factors such as VEGF, BDNF, IGF-1, FGF2, IGF and PDGF. As it is mentioned below, these growth factors play important roles in neural stem/progenitor cell proliferation, migration, cell fate determination and maturation into new neurons.

VEGF signaling plays an important role in NSC proliferation, survival, and neural progenitor migration and maturation [91]. VEGF is a glycoprotein, and is necessary for angiogenesis and vascularization. There are four isoforms belonging to the VEGF gene: VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGFA is expressed by endothelial cells and the choroid plexus, and regulates NSC renewal and progenitor cell migration by binding to the VEGF receptors (VEGFR1 and VEGFR2/Flk-1) secreted by the neural stem cell niche [92-94]. VEGFA expressed by astrocytes diffuses to the lateral ventricle and has a trophic effect on neural progenitor cells, which promotes their survival as well as increasing neurogenesis after cerebral ischemia [95]. Therefore, VEGFA/VEGFR signaling modulates vascular permeability and angiogenesis [91, 96]. Lastly, VEGF-C/VEGFR3 signaling is required for NSC renewal and regulates adult neurogenesis. Inhibition of VEGR3 from NSCs causes reduction of neurogenesis [97].

BDNF is secreted by endothelial cells to promote neural progenitor cell proliferation, differentiation, and survival in adult neurogenesis [98]. NSCs express the neurotrophin receptor TrkB, which binds to BDNF and has multiple roles in NSC survival and neuronal plasticity [99]. In the SVZ, neuroblasts secrete GABA, which induces TrkB expression in astrocytes to catch extracellular BDNF, which in turn stimulates neuroblast migration in the rostral migratory stream [100]. Besides BDNF, multiple neurotrophic factors such as FGF and EGF determine the NSC transition between proliferation and differentiation [101, 102]. BDNF/TrkB signaling regulates differentiation and survival of neurons and synaptic plasticity by activating Ras/MAP kinase, Phospholipase C, and PI3K pathways. BDNF/TrkB signaling also enhances hyperphosphorylation of the tau protein, which contributes to AD pathology, while BDNF gene expression is elevated in transgenic AD mice via environmental enrichment, demonstrating the importance of this neurotrophic factor in neurodegenerative disease processes as well as neurogenesis [103].

IGF-1 upregulation in the bloodstream, induced by exercise, promotes BDNF expression in the dentate gyrus, which modulates synaptic plasticity and cognitive enhancement [104]. IGF-1 induces the proliferation and differentiation of NSCs via MEK/ERK pathway signaling and the PI3K/Akt pathway signaling in the SGZ and SVZ, respectively [105].

FGF-2 is the other important endothelial-derived effector of adult neurogenesis. FGF-2 modulates NSC renewal and proliferation of the granule cell precursors in the dentate gyrus of the hippocampus and the SVZ [106]. While GFAP+ cells, astrocytes, and GFAP+ radial glia stem cells express FGF-2 in the SVZ, astrocytes express FGF-2 to promote neurogenesis by inducing neural progenitor proliferation in the SGZ during neurodegenerative disorders such as AD, Parkinson disease (PD) and traumatic brain injury [107, 108]. In addition to FGF-2, its receptor FGFR1 is expressed in NSCs in the SVZ and dentate gyrus of the hippocampus to enhance NSC proliferation [106].

EGF is expressed by the microvascular entorhinal cortex, while EGF receptors are expressed by type C transit-amplifying cells in the SVZ region. SDF-1 (CXCL12) stimulates EGFR to increase TypeA neuroblast mobility to migrate from the SVZ to the olfactory bulb [109]. Another study showed the association between BDNF and EGF; BDNF treatment of EGF-induced cultured human stem cells in the SVZ promoted cell proliferation and migration [110]. The combination of EGF and FGF-2 neurotrophic factors induced progenitor cell proliferation in both the dentate gyrus of the hippocampus and the SVZ region in ischemia-induced rat brains [111].

PDGF and PDGF receptors are important for the maintenance of neural progenitor cells in adult neurogenesis [112]. GFAP+ B cells in the SVZ express PDGFRA, which is necessary for oligodendrocyte formation, though not for

neurogenesis. PDGF alone has mitogenic effects on B cells, but inhibits neuroblast production. PDGF/PDGFRA signaling modulates the balance between oligodendrogenesis and neurogenesis [113].

As a result, neurotrophic factors mentioned above promote neural stem/progenitor cell proliferation, differentiation, and migration through different signaling cascades as well as the inhibition of neurodegenerative mechanisms for neuronal protection [114, 115].

iv. Immune System and Neurogenesis

Immune mechanisms modulate neural plasticity and behavioral processes in the human brain. Physiological and psychological factors are crucial to promote immune mechanisms. The alterations of signals between immune, nervous, and hormonal systems such as elevation of adrenaline and norepinephrine levels in blood modulate synaptic plasticity and neurogenesis [116, 117]. The immune system controls communication between the environment and the neurogenic niche [31]. Activated microglia and cytokine release as an immune response affects adult hippocampal neurogenesis as well as learning and memory [31].

The hippocampus and the immune system are interconnected during injury. Non-neuronal glial cells such as astrocytes and microglia and the cells around the choroid plexus such as T cells and B cells play important roles in immune-derived remodeling by controlling interactions with the environment, such as

exchange of nutrients and other compounds, between the brain and the rest of the body, and modulate neural progenitor proliferation and differentiation in the adult hippocampus [31]. Microglia cell populations are distributed in the dentate hilus and granule cell layer and regulate the apoptosis of newborn cells via phagocytosis during hippocampal neurogenesis [118, 119]. Besides non-neuronal cells and immune cells, identification of the MHC class I molecules in uninjured neural cell population confirmed that these molecules play significant roles in neurogenesis such as neuronal differentiation and synaptic plasticity [116, 120].

It has been shown in an immune-deficient AD mouse model that T cells are involved in the regeneration of neural precursor cells in the mature hippocampus and maintenance of neuronal plasticity [121]. In contrast, during early stages of human development, maternal infections change immune activation by intensely increasing inflammatory cytokine levels in the fetus, which leads to behavioral abnormalities such as autism, schizophrenia and depression in adulthood [122, 123]. Furthermore, environmental disruption has been shown to affect immune modulators and lead to hippocampal dysfunction by the activation of microglia and astrocytes, the accumulation of peripheral leukocytes (i.e. T cells) into injured brain region(s), and secretion of cytokines, chemokines, and prostaglandins to the site of brain pathology [43, 116, 124, 125]. Inflammatory and immune molecules such as cytokines and chemokines have important roles in hippocampal neurogenesis and synaptic plasticity throughout the human

lifespan [126, 127], as well as an having an important impact on neurological disease processes, and further study of this modulator of adult neurogenesis may provide important future direction to therapeutic efforts.

The Role of Chemokines in Adult Neurogenesis

Chemokines modulate brain plasticity and vulnerability in the hippocampus [43]. There are various central nervous system cells such as microglia, astrocytes, endothelial cells, oligodendrocytes, perivascular macrophages and neurons which produce cytokines and chemokines under different circumstances [128-130]. Chemokines induce chemotaxis, which facilitates the migration of leukocytes to injured areas [131, 132]. During injury, endothelial cells, astrocytes, and microglia cells secrete chemokines across the blood-brain-barrier (BBB), which facilitates neuroimmune signaling and recruits immune cells to the injury area [133, 134].

Chemokines play significant roles in the migration and development of progenitor cells, which express chemokine receptors such as CCR1, CCR2, CCR5, CXCR2, CXCR3 and CXCR4 in the dentate gyrus of the hippocampus in the postnatal brain [135]. In CXCR4 receptor knockout mice, the number of dividing cells in the migratory stream of the hippocampus and the dentate gyrus was dramatically reduced because CXCR4 is a receptor for CXCL12 chemokine, which is expressed in dividing progenitor cells, and CXCR4/CXCL12 signaling is

necessary for progenitor cell population proliferation and movement via the migratory stream [136]. In addition, as a neurotransmitter, CXCL12 promotes GABAergic transmission by a postsynaptic mechanism. GABAergic transmission depends on the synergic release of CXCL12 from neural progenitors in the dentate gyrus. As a result, neural progenitor cells receive GABAergic inputs, and CXCL12 is necessary for transmission of the GABAergic signal to the dividing progenitors [126]. CX(3)CR1 and CX(3)CL1 regulate microglial responses modulating glutamate toxicity for neuroprotection and glutamatergic synaptic input (transmission) on the neurons in the hippocampus [137]. Chemokines are important modulators in hippocampal signaling and synaptic plasticity, which is important for learning and memory [138, 139].

The Role of Inflammatory Cytokines in Adult Neurogenesis

Inflammation adversely affects hippocampal neurogenesis and proper function of the hippocampus. IL-1β, IL-6, and TNF-α are the most important pro-inflammatory cytokines, which play key roles in hippocampal neurogenesis as well as memory function.

IL-1β, expressed in many immune cells and glia cells as well as neurons, impairs proliferation and differentiation of neural precursor cells and decreases NSC survival rate in hippocampal neurogenesis. IL-1β is the predominant pro-inflammatory cytokine in the brain, and negatively correlates with the proliferation

of NSCs in the dentate gyrus of the hippocampus [140]. The nuclear factor kappa B (NFκB) cascades and mitogen-activated protein kinase (MAPK) as well as several transcription factors in the nucleus induce the activation of IL-1β/ IL-1β receptor signaling [141, 142]. Serum level of IL-1β increases with depressive symptoms and impairs learning and memory function [143, 144].

IL-6 plays an important role in progenitor cell survival in the dentate gyrus of the hippocampus [43]. Overexpression of IL-6 from astrocytes reduces neurogenesis in the dentate gyrus of the hippocampus by influencing proliferation, survival, and differentiation of progenitor cells [140].

TNF-α inhibits neural progenitor cell proliferation during brain injury, while a normal level of TNF-α increases neurogenesis by affecting expression of neurotrophic factors such as NGF and BDNF [145-147]. Since ischemic stroke causes neuronal death in the striatum and the cerebral cortex, neurogenesis increases after this insult in the dentate gyrus of the SGZ and the rostral SVZ by migration of neuroblasts originating from the SVZ into the damaged striatum [148, 149]. In response to ischemia, TNF-α produced by the microglia, astrocytes, and choroid plexus ependymal cells is upregulated. Similarly, TNF receptors such as TNF1 and TNF2 are expressed by glial and neuronal cells in response to ischemia. Depending on the TNF receptors, cell death or cell proliferation is activated [150, 151]. Proinflammatory cytokines such as IL-1β, IL-6, and TNF-α alter hippocampal structural plasticity and neuronal structure via

alteration in morphology of the granule cells differentiated from NSCs in the dentate gyrus and CA1 during inflammation.

v. Epigenetic Modulators

Epigenetics refers to changes in gene activity not resulting from changes in DNA sequence. Changes in DNA methylation, histone modifications and regulation by non-coding RNAs have important effects on different neuronal phenotypes [152, 153]. Proteins with methyl-CpG-binding domains (MBDs) bind methylated DNA and regulate gene expression by blocking the binding site for transcription factors, while DNA methyltransferases (DNTMs) are directly responsible for methylating DNA and silencing gene expression. MBD-1 has been shown to play a crucial role in NSC differentiation [154]. The specific binding of MBD1 to the FGF2 promoter decreases FGF2 expression and inhibits the differentiation of proliferative neural stem cells during adult neurogenesis [155]. Similarly, overexpression of DNA methyltransferases DNMT1 and DNMT3a decreases the differentiation and migration of NSCs. Methyl-CpG-binding protein 2 (MeCP2) regulates gene expression similarly to MBD1. MeCP2 binds to GFAP and inhibits its expression, thus controlling neuronal differentiation and maturation, as well as cell fate [153, 156]. DNA-damage-inducible protein 45 alpha (Gadd45a) plays a role in DNA demethylation and DNA repair during neurogenesis [157]. Gadd45a demethylates neurotrophic factors such as BDNF and FGF-1, which affects self-

renewal and proliferation of NSCs and promotes neuronal maturation and dendritic growth [158-160].

Histone acetylation is a crucial process for the proliferation and differentiation of neural stem cells. HDAC3, HDAC5, and HDAC7 interact with the orphan nuclear receptor homologue of the Drosophila tailless gene (Tlx or NR2E1) and manipulate NSC self-renewal and proliferation [161]. Other epigenetic mechanisms involve non-coding RNAs such as microRNAs. MicroRNAs such as Let-7b, miR-9, miR-34a, and miR-184 regulate proliferation of NSCs and neuronal differentiation. MiR-137 and miR-132 also regulate synaptogenesis and the neuronal network, while miR-34a and miR-125b regulate dendritogenesis and spine morphology [162, 163]. All of these epigenetic mechanisms highlight the importance of looking beyond the genome to understand the biological underpinnings of neurogenesis, which will be crucial to advance the state of research in therapeutic efforts to address neurogenesis in neurodegenerative disease. Epigenetic changes during neurogenesis have an important impact on memory and learning, and can play significant roles in neuropsychiatric disorders as well such as depression and schizophrenia [164-166].

C. Role of Genetic Variation in Adult Neurogenesis

Many gene expression level changes have been observed during adult neurogenesis, as presented in the previous sections; these changes affect NSC and progenitor proliferation, maintenance in the adult neurogenic niche, and differentiation into mature neurons. Although most of the studies focused on the alteration of gene expression during adult neurogenesis, some studies showed that genetic variations in adult neurogenesis-related genes affect hippocampal structure and memory impairment. For instance, the REST gene, a known transcriptional repressor, negatively regulates neuronal differentiation during neurogenesis, and nonsynonymous variation in this gene is associated with less hippocampal loss and greater cortical thickness in individuals who carry at least one minor allele [167-169]. Another important gene related to adult neurogenesis is G-coupled protein receptor adenosine receptor A2A (ADORA2A) which plays a role in neurite growth. Alteration of the expression level of ADORA2A during adult neurogenesis affected neuronal differentiation, migration and maturation of new neurons [170, 171]. Variants in the ADORA2A gene differentially influence the transfer of information into working memory in homozygous rare genotype groups due to alteration of glutamatergicneural transmission [172, 173]. Moreover, it has been shown that an ADORA2A antagonist reduced cognitive decline and resulted in a protective effect on memory formation in Parkinson's disease, Huntington's disease and Alzheimer's disease models. [174, 175]. An additional Schizophrenia susceptibility gene, DISC-1, regulates neuronal

integration of new neurons from neural progenitors into the adult brain and promotes structural plasticity [176]. DISC-1 missense variation leads to a reduction of the proliferation of progenitor cells, which alters the balance between quiescent and proliferative neural stem cells in a transgenic mouse model [177]. A missense mutation in the DISC-1 gene is related to alteration of the hippocampal structure by reducing gray matter volume and increases the risk for Schizophrenia [178]. As previously discussed, BDNF plays an important role in neural progenitor cell proliferation, differentiation and survival; additionally, overexpression of BDNF enhances adult neurogenesis by increasing dendritic spine density on granule cells. BDNF polymorphism Val66Met modulates integration of neurons *in vivo* and regulates episodic memory and hippocampal physiological activation in humans [179, 180]. Moreover, genetic variation in BDNF associated with hippocampal atrophy and cognitive decline have been identified using neuroimaging-genetics methods [181]. Pro-inflammatory cytokine IL-6 plays an important role in the formation of new neurons and glial cells from neural progenitor cells during adult neurogenesis, and IL-6 variations have been associated with AD, multiple sclerosis, and severe traumatic brain injury [182-185]. A single nucleotide polymorphism (SNP) within the GRIN2B gene, which is an *N*-Methyl-*D*-Aspartate (NMDA) glutamate receptor and enhances synapse maturation and survival of new-born neurons, is strongly associated with temporal lobe volume in patients with AD and mild cognitive impairment (MCI) [186, 187]. Finally, variation within genes strongly related to adult neurogenesis processes in AD such as CHRFAM7A, REST, RELN, BCHE, NCAM1 and

ADORA2A have been identified by our colleagues in our laboratory using neuroimaging-genetic methods [168, 188-192].

To sum up, both expression differences and allelic variations in neurogenesis-related genes in the human genome may have compensatory advantages or confer impairment of biological processes during adult neurogenesis.

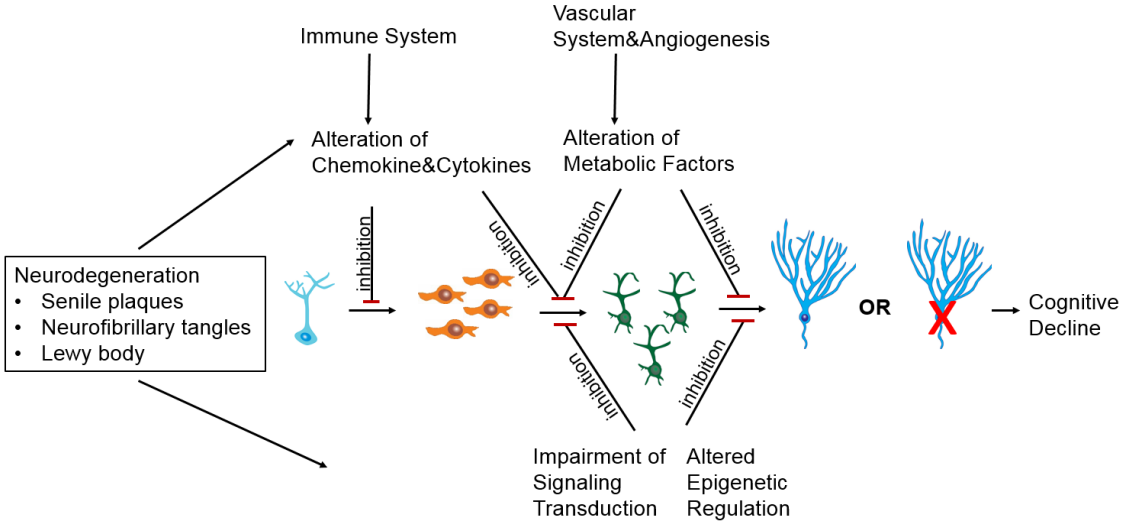
D. Adult Neurogenesis and Neurodegenerative Diseases

Over fifty years ago, it was reported for the first time that new neurons are generated in the dentate gyrus and SVZ of several species during the adult period, and this observation launched a new era of research to understand the mechanism of adult neurogenesis in humans [193]. The important feature of adult neurogenesis is that there are many modulators playing roles during NSC proliferation, differentiation, migration and survival including genetic, transcriptional, and epigenetic factors as well as environmental factors and disease status [194]. Neurogenesis is a very important process for synaptic transmission and is associated with axonal and dendritic formation. Impaired adult neurogenesis in neurodegenerative diseases results in loss of existing neurons and reduced capacity for NSC renewal; the putative function of new neurons eventually is compromised or lost (Figure 3). The crucial question is how neurodegenerative diseases affect adult neurogenesis and in turn how alterations in neurogenesis impact pathophysiological mechanisms of neurodegenerative disease. We consider five diseases that display symptoms

related to hippocampal and olfactory dysfunction, the two main brain regions where adult neurogenesis occurs. Parkinson's disease (PD), AD, Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) are neurodegenerative diseases, while schizophrenia is a condition with both neurodevelopmental and neurodegenerative aspects.

Figure 3. Impaired neurogenesis in neurodegenerative diseases.

Neurodegeneration negatively affects the adult neurogenesis process due to alteration of chemokines and cytokines, metabolic factors, and epigenetic regulation, as well as impairment of signaling transduction. Alteration of chemokines and cytokines impairs neuronal stem cell self-renewal and differentiation. Alteration of metabolic factors and epigenetic regulators, and defects in signaling transduction molecules, inhibits the proliferation of progenitor cells and their differentiation into neuroblasts. The inhibition of newborn neuron formation may contribute to cognitive decline.



Parkinson's disease

PD is a movement disorder with frequent psychiatric complications as well as a high prevalence of cognitive impairment [195]. The accumulation of α-synuclein as the major component of Lewy bodies and Lewy neurites is the pathological hallmark of PD [196]. Even though there are some conflicting findings regarding how adult neurogenesis affects PD processes, some studies in humans show that there is reduced proliferation of NSC progenitors in the SVZ [197, 198]. Postmortem human PD brain studies and transgenic PD animal models showed that the number of EGF and EGF2 receptor positive cells decrease in the adult SVZ, causing impairment of olfactory bulb neurogenesis associated with dopaminergic deafferentation [199]. In addition, transgenic PD animal model studies revealed that stimulation with neurotrophic factors such as EGF and FGF-2 cause massive proliferation and migration of neuroblasts into the SVZ region [200-202]. α-synuclein accumulation in neurogenic regions where adult neurogenesis occurs impairs olfactory bulb formation and hippocampal neurogenesis. Selective serotonin uptake inhibitor fluoxetine treatment of PD increases levels of neurotrophic factors such as BDNF and GDNF, and rescues impaired hippocampal neurogenesis in transgenic PD mice [203].

Alzheimer's disease

AD is the most common form of adult-onset dementia. Patients display hippocampal atrophy, memory impairment, and other cognitive and olfactory deficits. There are two crucial hallmarks of AD: neurofibrillary tangles caused by hyperphosphorylation of the tau protein and amyloid-□ plaque deposition [204]. Currently, there is no approved disease modifying treatment for AD. It has been shown in numerous genetic studies that APP, presenilin 1 (PSEN1) and PSEN2 missense mutations cause familial AD [205]. These mutations result in increased amyloid-β production and intracellular and extracellular accumulation. Presenilin is a catalytic component of γ-secretase, which cuts APP, and also regulates Notch and Wnt signaling mechanisms by sequentially cleaving the Notch receptor to generate the Notch Intracellular Domain (NICD) [206, 207]. α-secretase (ADAM protease) cleaves APP to make sAPPα, and also cleaves Notch-1 and components of EGFR, which, as previously discussed, play important roles in adult neurogenesis [60]. Many of the molecular players in AD are also modulators of adult neurogenesis. The most important of these, PSEN1, modulates NSC differentiation in the adult brain, while sAPPα regulates the proliferation of NSCs [208, 209]. BDNF/TrkB signaling enhances dephosphorylation of the tau protein in AD pathology, while BDNF gene expression is elevated in transgenic AD mice via environmental enrichment.
It is not completely understood how hippocampal neurogenesis is affected by AD pathology. However, alterations in the early stage of AD progression such as

amyloid-β deposition and inflammation impair the maturation of newborn neurons and inhibit hippocampal neurogenesis [210]. Since abnormal accumulation of amyloid-β activates microglia and astrocytes to secrete more inflammatory cytokines such as IL-1β, IL-6, and TNF-α, which regulate the inflammatory response, AD has been proposed as a chronic inflammatory disorder of the central nervous system [211-213]. Meta-analysis of proinflammatory cytokine levels in cerebrospinal fluid and peripheral serum have shown significant elevation of TNF-α and IL-1β in AD patients; this elevation might reflect activated neural progenitor cell proliferation as a compensatory mechanism during neurodegeneration [214]. Additional cytokine signaling processes have also been linked to neurodegenerative processes; for example, CXCL12/CXCR4 signaling causes astrocytes in the CA1 to release glutamate neurotransmitter, which regulates synaptic integration and neuronal excitability and decreases amyloid-β deposition [215]. Another signaling process involves MCP-1 and CCR2, which are both strongly upregulated in AD patients. Expression of MCP-1 secreted by macrophages, monocytes, and astrocytes is induced by amyloid-β; MCP-1 is known to regulate the migration of neural cells in the dentate gyrus toward damaged areas in the brain, suggesting an important function in neurodegenerative disease [216, 217]. Furthermore, amyloid-β accumulation in the brain increases IL-1β, IL-6, and TNF-α as well as transforming growth factor (TGF)-β secretion, which has a negative effect on NSC proliferation and survival and inhibits hippocampal neurogenesis, emphasizing the importance of cytokines as a modulator of neurogenesis in neurodegenerative disease [145, 218-220].

Huntington's disease

HD is an autosomal dominant neurodegenerative disorder caused by tri-nucleotide repeat expansion within the Huntington gene [221, 222]. There is no reported dysfunction of hippocampal neurogenesis in HD patients. Although there is potential migration of neural stem cells progenitors to the degenerating striatum, they do not differentiate to mature neurons, indicating a significant downstream effect of this process [222-224]. Transgenic HD mouse model studies showed that reduction of the hippocampal progenitor cells was associated with diminished CREB signaling and elevated TGF-β1 signaling [225]. Moreover, it has been found that a D2R antagonist partially enhance adult neurogenesis by increasing the proliferation of the neural precursor cells and immature neurons in the SGZ region of the HD mice model while a D2R antagonist did not have any effect on motor performance [226]. The other study showed that mutant HD mice had decreased NeuroD1 in neural progenitors in the dentate gyrus of the hippocampus and doublecortin and calretinin expression in newborn neurons, as well as impairment of spatial memory. NeuroD1 has a crucial effect on proliferation, differentiation and maturation of the neural progenitor cells; HD pathology adversely affects the function of NeuroD1 [227, 228]. Alterations of proteins in HD pathology may impair effective adult neurogenesis and cause cognitive impairment.

Amyotrophic lateral sclerosis

ALS is a progressive neurodegenerative disorder caused by the degeneration of motor neurons, leading to muscle wasting, paralysis, and difficulty breathing [229]. Neural stem cell culture from transgenic ALS mice showed that late stage ALS microenvironment impairs the functional capacity of NSCs [230]. There reduction in number of proliferative GFAP+ cells in SOD-1 transgenic mice, causing dominant form of ALS, in the olfactory bulb of the SVZ, and dentate gyrus of the hippocampus. However, in early stages of ALS, neurogenesis was preserved and there were no alterations of the NSCs [231]. ALS onset and progression promotes *de novo* neurogenesis with NSC proliferation and migration to the spinal cord increasing concomitantly with motor neuron degeneration. During disease onset and progression, an ALS-like mouse model showed increased expression of CXCR4, which is directly associated with NSC migration into the spinal cord, compared to age-matched control mice [232]. In addition to several ALS studies, immunohistochemical and histological studies of FTLD-ALS patients showed that the number of proliferative neural cells in the SVZ increased corresponding to disease progression as a compensatory mechanism for neurodegeneration [233].

Schizophrenia

Schizophrenia is a complex mental disorder with a strong genetic background. Schizophrenia is associated with impairment of adult neurogenesis by disrupting NSC proliferation and migration to the SGZ and SVZ [234, 235]. Cognitive impairment in schizophrenia might be related to impairment of adult neurogenesis [236]. Several candidate genes have been suggested to play roles in adult neurogenesis as well as schizophrenia. Neuregulin (Nrg1)-ERBB signaling, altered in schizophrenia, normally promotes the maintenance of radial glial cells and their migration to the cerebral cortex by movement of cerebellar granule cells, which express neuregulin, along radial glial fibers, which express ErbB4 [237-239]. The other important gene significantly associated with schizophrenia as well as adult neurogenesis is the disrupted-in schizophrenia 1 (DISC1) gene [240, 241]. DISC1 knockout rats showed aberrant positioning and impaired morphogenesis of newborn neurons in the dentate gyrus of the hippocampus [241]. Some genes, such as Wnt, GSK-3β, and Reelin, play roles in neuronal development and proliferation, cell fate determination, cell adhesion and migration of NSCs, and are also known to have abnormal function in schizophrenia, which suggests that there is a strong link between expression of developmental genes with abnormal mechanisms of neurogenesis in schizophrenia [242-245].

Summary of Adult Neurogenesis and Neurodegenerative Diseases

Each neurodegenerative condition has different effects on NSC fate during adult neurogenesis by controlling synaptic plasticity, spine morphology, and axonal pathology. Newly generated neurons play critical roles in brain development and maintenance in the adult brain. Several genes including PSEN1, MAPT, α-synuclein, SOD-1, and Huntingtin, are the main players in modulating synaptic plasticity and brain morphology. Alterations in these genes are linked to neurodegenerative diseases and changes in neurogenesis in specific areas such as the dentate gyrus of the hippocampus and the SVZ in early stages of neurodegenerative diseases. Discovery of the genetic mechanisms underlying adult neurogenesis and how neurodegenerative disorders affect new neuron formation could significantly inform therapeutic approaches to treat neurodegenerative diseases in early stages, when intervention has the most potential to prevent long-term dysfunction.

E. Therapeutic Research Focused on Adult Neurogenesis

Adult neurogenesis is defined by the formation of new functional neurons from NSCs and their integration into the neuronal circuitry to promote structural plasticity. Even though the complete mechanism underlying this process is not yet known, hippocampal neurogenesis appears to be critical for the formation and maintenance of hippocampal structure as well as memory and learning. Since

modulators including signaling transduction, the vascular and immune systems, and epigenetic regulation enhance structural and synaptic plasticity during maturation of the newly generated neurons from their progenitors, many recent studies focus on novel pharmacologic strategies targeting adult neurogenesis and identification of biomarkers for human neurogenesis.

One novel therapeutic approach targets inflammatory molecules such as chemokines and cytokines known to be related to adult neurogenesis. Activated microglia and cytokines released as an immune response enhance neural progenitor proliferation, differentiation, and maturation into new neurons during adult hippocampal neurogenesis as well as facilitating memory formation and learning [31]. Moreover, some cytokines such as IL-6, IL-β, and TNF-α modulate neural progenitor cell fate and contribute to neural repair mechanisms during neuroinflammation [246]. The development of anti-inflammatory drugs targeting inflammatory molecules to preserve adult neurogenesis during chronic inflammation may provide novel insights into early stages of neurodegenerative diseases. Since neuroinflammation both a key component of AD and other neurodegenerative disorders and known to affect adult neurogenesis, it has been the focus of several therapeutic approaches. Studies have focused on anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoid steroids, which target expression of proinflammatory cytokines and their complimentary molecules, as treatments for AD patients [247, 248]. These approaches have not been successful to date although it is suspected that this

may reflect their introduction in later stages of disease after substantial degenerative changes have already occurred.

Another therapeutic approach to modulate neurogenesis targets neurotrophic factors in AD pathology since there is an imbalance between neurotrophic factors in the AD brain. For example, the level of neurotrophic factors such as FGF-2 and NGF increases to compensate for increasing AD pathology, while the level of BDNF decreases in the hippocampus [249, 250]. NGF gene therapy causes NGF secretion from autologous fibroblasts into the basal forebrain, leading to expression of the neurotrophin receptors by cholinergic neurons and cholinergic axonal sprouting in the patients with AD for 10 years after gene transfer [251]. Also, it has been shown that neuroproliferation of the immature neurons in the dentate gyrus of the SGZ increases in AD patients, though it is not clear whether these immature neurons are differentiating into mature neurons [252]. VEGF levels significantly increase in cerebrospinal fluid while there is decrease of them in serum level in AD patients. [253, 254]. Keilhoff and colleagues found that antipsychotics haloperidol and risperidone increased neuronal precursor cell proliferation and survival of the new granule cells via VEGF and MMP2 in adult schizophrenic rat brain [255]. These factors play crucial roles in the determination of cell fate and new neuron formation, and an imbalance of these factors affects neurogenesis. The environmental changes in the NSC niche due to the altered levels of neurotrophic and neuroinflammatory factors in the hippocampus may adversely affect the differentiation of progenitor cells into mature neurons.

Further studies are needed to address these factors with regard to possible therapeutic strategies.

F. Discussion

Adult neurogenesis is important for structural plasticity of the brain through turnover of neural stem cells/precursors to new functional neurons. Even though the molecular mechanisms underlying this process remain unclear, adult hippocampal neurogenesis plays a significant role in learning and memory formation, and it is affected by environmental changes and disease conditions [256]. There are multiple modulators that affect the formation of newborn neurons such as neurotrophic factors, cytokines and chemokines, epigenetic factors, and signaling pathways. Each modulator drives NSC proliferation, differentiation, migration and survival in different ways. The alteration of these modulators under disease conditions negatively affects cognition and hippocampal structure and function. Future studies should also focus on identification of genetic variation contributing to neurogenesis in healthy adults as well as in neurodegenerative disease; such research has strong translational potential to identify novel therapeutic targets. It is crucial for future research to continue to investigate the functional role of adult neurogenesis in the normal human brain as well as alterations in neurodegenerative diseases. Loss of NSC populations and impairment of neuron formation are common hallmarks in neurodegenerative diseases such as AD, PD, HD and ALS. Future therapeutic strategies hold

promise for stimulation of neuronal plasticity and maintenance of newborn neurons in early stages of neurodegenerative diseases, potentially halting or reversing clinical symptoms in these common, devastating diseases.

II. Targeted neurogenesis pathway-based gene analysis identifies *ADORA2A* associated with hippocampal volume in mild cognitive impairment and Alzheimer's disease

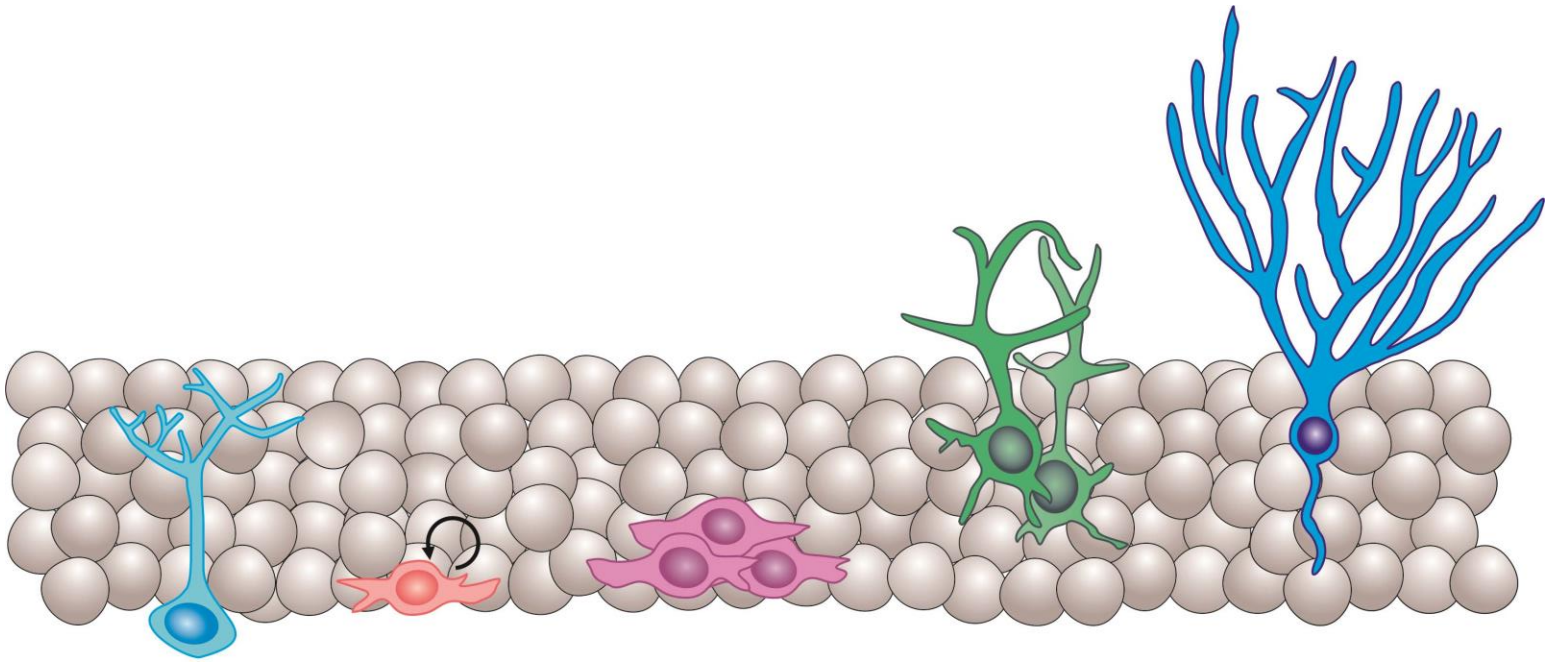
A. Introduction

Adult neurogenesis occurs throughout life in specific regions of the brain in humans. In rodents, neural stem cells differentiate to new neurons in several regions of the brain, but studies show that adult neurogenesis is limited to the dentate gyrus (DG) of the hippocampus and the sub-ventricular zone of the olfactory bulb in humans [2, 3, 257]. The hippocampus is the most important region of the brain for new learning and episodic/spatial memory. The new neurons generated during adult neurogenesis are incorporated into hippocampal network circuitry during construction and maintenance of neural circuits and contribute to learning and memory [222, 258]. The progenitor cells in DG divide periodically, and DG experiences stability in neurogenesis throughout life [259, 260]. In rodents, neural stem cells (NSCs) in the DG make approximately 8,000 to 10,000 new neurons per day. However, the proportion of hippocampal neurogenesis decline in human is smaller than mice with aging [3, 261]. In 1998, the presence of adult-born neurons in the dentate gyrus of the human hippocampus had been identified by using cancer patients who had received the labelled 5-bromo-2'-deoxyuridine (BrdU) in hippocampal neurons [2]. In adults, the annual turnover of stem cells into neurons is 1.75% with a modest decline

during aging [3]. By contrast, the estimated annualized hippocampal atrophy rate is 1.41% per year for cognitively normal older people and 4.66% for patients with AD pathology [262]. Disruption of adult neurogenesis process has been postulated to contribute to neurodegenerative diseases including AD. Alterations in hippocampal neurogenesis in AD could either provide protection by proliferation of neural progenitor cells or cause accelerated neural degeneration due to impairment of neuronal networks and synaptic plasticity. Several studies in mice have combined structural MRI and histological approaches to investigate newborn neurons and neural stem/progenitor cells in neurogenesis-related brain regions and found that neurogenesis was associated with increased gray matter volume [263]. The relationship between hippocampal volume and adult neurogenesis in the human brain has not been studied yet.

Many molecular mechanisms and pathways play a role in the hippocampal neurogenesis process , including the proliferation of neural progenitor cells, the differentiation, migration, and maturation of adult neurons [260, 264]. Known modulators of adult neurogenesis include signaling transduction, vascular and immune systems, metabolic factors, and epigenetic regulation [260, 264, 265]. In particular, multiple factors such as neurotrophic factors, transcription factors, and cell cycle regulators control NSC proliferation, maintenance in neurogenic niche, and differentiation into mature neurons; these factors play role in networks of signaling molecules that influence each other during construction of neural circuits, and contribute to learning and memory (Figure 4).

Figure 4. Genes playing roles in stem cells proliferation, differentiation, migration, and survival to new neurons during adult neurogenesis process. Glial-like radial stem cells (light blue); progenitors (pink); neuroblasts (purple); immature neurons (green); mature neurons (blue).



Self-Renewal/Proliferation

ADAM10	ESR1	NOS3
ADRA2A	FGF1	PLCG1
APBB1	GRIN2B	PRNP
APP	GRN	PSEN1
BCL2	GSK3B	PTGS2
BDNF	HTR2A	S100B
CCL2	IGF1	SORL1
CHRNA7	IGF1R	TET1
CHRNB2	IL1B	TLR4
CNTF	LRRK2	TNF
CXCL1	NGF	VEGFA
DKK1	NOS1	

Differentiation/Migration

ADAM10	IFNG
APBB1	IGF1
APOE	IGF1R
BCHE	MEF2C
CAV1	MIF
CDK5	NEUROD1
CDK5R1	OLIG2
CXCL12	RELN
DYRK1A	S100B
FAS	SIRT1
HIF1A	

Maturation/Survival

ACHE	CHRNB2
ADORA2A	IL6
BCHE	MEF2C
CASP3	NOS1
CAV1	NPY
CCL2	S100B
CDK5	SLC6A4
CHAT	THRA

Disruption of the neurogenesis process has been postulated to contribute to neurodegenerative diseases including AD [266]. However, the mechanisms by which AD pathology affects neurogenesis are not completely understood. Alterations in the early stages of AD, such as amyloid-β deposition and inflammation, may impair the maturation of newborn neurons and inhibit hippocampal neurogenesis [210]. Genetic changes in neurogenesis-related pathways and genes may also play important roles in the alteration of NSCs maturation into newborn neurons [260]. Pathway- or gene-based association analysis has been used to study a number of complex neurodegenerative diseases, including AD, using a wide variety of phenotypes, including cerebrospinal fluid Aβ1-42 peptide level [267, 268], cerebral amyloid deposition [269], brain glutamate levels [270], and episodic memory [192]. However, no study to date has evaluated the association between candidate neurogenesis-related genes and hippocampal volume. Thus, the goal of the present study was to perform a gene-based association analysis of neurogenesis pathway-related candidate genes in cognitively normal and impaired participants from ADNI cohort. Identification of genes that play a role in both hippocampal neurogenesis and AD may hold great promise for better understanding the role of neurogenesis in AD, as well as to aid in discovery of novel therapeutic targets for AD.

We used well-characterized participants from extensively studied cohort Alzheimer's Disease Neuroimaging Initiative (ADNI), which uniquely have GWAS data sets on the same participants as well as multi-modal structural and

functional neuroimaging (MRI, PET) data. A quantitative phenotype approach to genetic association studies provides the advantage of increased power sizes to detect significant genetic effects as compared to a traditional case-control design. We used hippocampal volume as a quantitative phenotype measured by MRI imaging, metabolic activity and amyloidosis in the hippocampus measured by PET imaging, and composite memory scores as quantitative traits to investigate that adult hippocampal neurogenesis-related genes and pathways are significantly associated with AD-related endophenotypes.

Highlights:

- Candidate pathways and genes which play a role in neurogenesis in the adult brain are manually-curated.
- ADORA2A is significantly associated with hippocampal volume
- A SNP (rs9608282) upstream of *ADORA2A* is associated with larger hippocampal volume and better memory performance.
- rs9608282 may have a protective effect on brain structure and function in neurogenesis-related brain regions.
- There is a significant interaction effect of NMDA-receptor antagonist use and the *ADORA2A* rs9608282-T on memory performance.

B. Materials and Methods

Study Participants

We used the participants of the Alzheimer's Disease Neuroimaging Initiative Phase 1 (ADNI-1) and its subsequent extensions (ADNI-GO/2) for this study. ADNI was launched in 2004 by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, the Food and Drug Administration (FDA), private pharmaceutical companies, and nonprofit organizations as a public-private partnership. The aim of ADNI has been to identify whether serial MRI, positron emission tomography (PET), sensitive and specific other markers, and clinical and neuropsychological assessments could be combined to measure the progression of mild cognitive impairment (MCI) and early AD. Participants aged 55-90 in ADNI cohort include approximately 400 cognitively normal older individuals (CN), 100 individuals with significant memory concerns (SMC), 800 individuals diagnosed with MCI, and 300 individuals diagnosed with AD. Clinical and neuroimaging procedures and the other information about the ADNI cohort can be found at http://www.adni-info.org/. After the initial analysis, a meta-analysis was conducted with ADNI [271, 272] and two independent datasets, including the AddNeuroMed study (N=218; 66 CN, 77 MCI, 76 AD) [271, 273], and the Indiana Memory and Aging Study (IMAS) study (N=59; 29 CN, 24 MCI, 6 AD) [271].

Written informed consent was obtained from each participant and all protocols were approved by each participating study and site's Institutional Review Board.

Subject selection

Only non-Hispanic Caucasian participants were selected for this analysis by genetic clustering with CEU (Utah residents with Northern and Western European ancestry from the CEPH collection) and TSI (Tuscans in Italy) populations using HapMap 3 genotype data and multidimensional scaling (MDS) analysis after performing standard quality control (QC) procedures for genetic markers and participants [190]. Overall, 1,563 non-Hispanic Caucasian participants were included, as their genome-wide association study (GWAS) data passed the above population stratification and all other standard QC procedures [268]. Demographic information is shown in Table 1 for these participants.

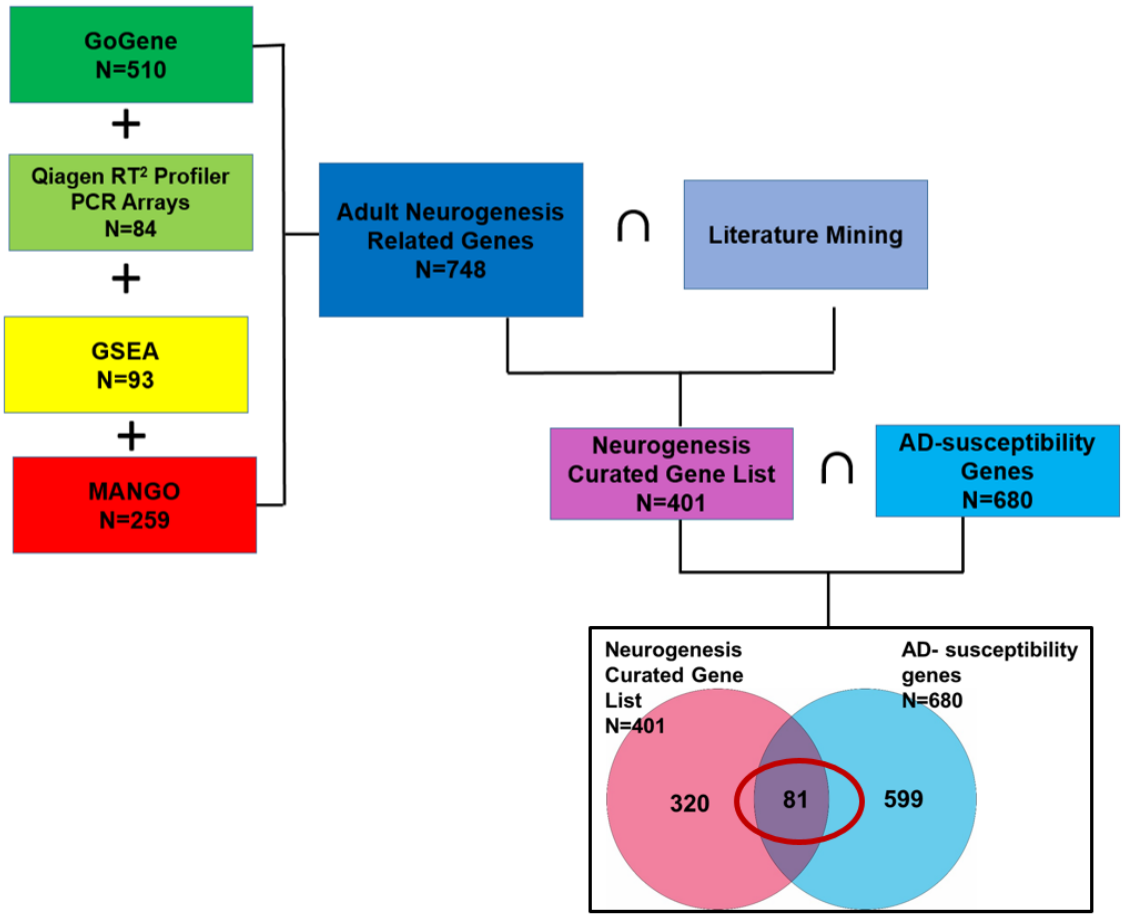
Table 1. Demographic and clinical characteristics of ADNI participants

	CN	SMC	EMCI	LMCI	AD
N	367	94	280	512	310
Age	74.59 (5.57)	71.77 (5.65)	71.14 (7.26)	73.52 (7.65)	74.65 (7.79)
Gender (M/F)	192/175	38/56	158/122	318/194	176/134
Education	16.32 (2.68)	16.81 (2.57)	16.08 (2.67)	15.97 (2.91)	15.23 (2.97)
APOE (ε4–/ε4+)	267/99	62/32	160/119	232/280	104/206

Identification of candidate genes

Candidate genes which control the turnover process of neural stem cells/precursors to new functional neurons during adult neurogenesis were manually curated using a pathway-based systems biology approach (Figure 4). Genes from known modulators of adult neurogenesis include those involved in signaling transduction, vascular and immune system function, metabolic factors, and epigenetic regulation [112, 264, 265, 271, 274]. Pathway-based approaches were used to manually curate these hippocampal neurogenesis-related genes through a review of existing databases and literature mining, resulting in a final gene list (Figure 5). Specifically, we identified hippocampal neurogenesis-related genes using four publicly available databases (GoGene, Qiagen RT^2 Profiler PCR Arrays, Gene Ontology (GO) with Gene Set Enrichment Analysis (GSEA), and Mammalian Adult Neurogenesis Gene Ontology (MANGO)) and literature mining.

Figure 5. Venn diagram of adult neurogenesis-related genes and AD-susceptibility genes. Manually curated neurogenesis and AD related gene. {(GoGene) U (Mango) U (Qiagen) U (GSEA)} ∩ {Pubmed Mining} = 401. AD-susceptibility genes (N=680) from AlzGene database and large-scale GWAS results. Eighty-one common genes were identified associated with both neurogenesis and AD. U: Union; ∩: Intersection.



GoGene contains high-quality manual annotation with high-throughput text mining from literature using ontology terms. This database includes associations between genes and gene-related terms for ten model organisms extracted from more than 18,000,000 Pubmed entries that cover process, function, location of genes and their relationship with diseases and compounds (http://gopubmed.org/gogene) [275]. We performed a search for GoGene term “adult neurogenesis” to identify neurogenesis-related genes.

Qiagen RT^2 Profiler PCR Arrays are the one of the most reliable tools to analyze the expression of genes related to specific pathways. The human Neurogenesis RT² Profiler™ PCR Array contains 84 genes, manually curated using literature mining, which are highly related to the process of neurogenesis, such as neural stem/progenitor cells proliferation, differentiation, migration, and maturation into newborn neurons (www.qiagen.com). Growth factors, inflammatory cytokines, cell adhesion molecules, and cell signaling genes involved in the neurogenesis process were also represented in this profiling array. We included all genes from this Neurogenesis array in our gene list.

DAVID (the Database for Annotation, Visualization, and Integrated Discovery) is a publicly available functional tool which includes annotations from Gene Ontology (GO). Gene Set Enrichment Analysis (GSEA) pathway annotations were downloaded from Molecular Signatures Database version 5.0 (http://software.broadinstitute.org/gsea/msigdb). This annotation data comprised

a collection of GO. A GO annotation contains a GO term associated with a specific reference which expresses the target or analysis associated with a specific gene product. Each GO term belongs to molecular function [276], cellular component (CC), or biological process (BP). “Neurogenesis” was used as the GO term in this analysis to identify neurogenesis related gene sets from the GSEA database.

We also used MANGO, which consists of 259 genes designed and curated by Overall et al. [277]. In MANGO, all genes are classified by their positive, negative, or neutral effect on hippocampal neurogenesis and thusly annotated. We used recently updated MANGO version 3.1 to annotate genes.
We identified 510 genes from GoGene, 84 genes from Neurogenesis RT² Profiler™ PCR Array, 259 genes from MANGO database, and 93 genes from GSEA/Molecular Signatures Database. We combined all genes related to neurogenesis from the four databases (N=748). Then, each gene related to hippocampal neurogenesis was annotated from relevant literature. Initial Pubmed search using the keywords “adult hippocampal neurogenesis” for papers published until 9/2016 included 2,717 articles. We used Pubmed to identify if all 748 genes are related to hippocampal neurogenesis from 2,717 articles. After literature mining, among 748 genes, only 401 genes were related to adult hippocampal neurogenesis.

Finally, since a key goal was to identify candidate genes playing a role in both adult hippocampal neurogenesis and AD pathology, we focused on hippocampal

neurogenesis-related genes which are also implicated in AD. For this purpose, we identified AD-associated susceptibility genes using the AlzGene database (http://www.alzgene.org/), which provides a comprehensive meta-analysis of genes previously identified in various AD association studies, and large-scale AD GWAS results (N=680) (Lambert, Ibrahim-Verbaas et al. 2013). The AlzGene database consists of a comprehensive, unbiased, publicly available catalog of all genetic association studies in the field of AD, which was identified from published papers by pubMed search using keywords “alzheimer* AND (genet* OR associat*)”. The gene list in AlzGene represents a summary of promising AD candidate genes. We then compared this gene list to the 401 genes previously identified as involved in adult hippocampal neurogenesis to filter the lists to 81 common genes related to both hippocampal neurogenesis and AD. These 81 genes were used in the association analysis.

Endophenotypes

Pre-processed baseline 1.5T and 3T MRI scans from 1,563 participants were downloaded from ADNI public website (http://adni.loni.usc.edu). FreeSurfer version 5.1 was used to extract total hippocampal and hippocampal subfield volumes, as well as total intracranial volume (ICV) [278-281]. Total hippocampal volume, as well as selected adult neurogenesis-related subfield volumes (CA1, CA23, CA4, and DG) (N=1,563), were used as endophenotypes for the association analysis. In addition, we used a composite score of episodic memory

(N=1,563) [282] and CSF total tau levels (N=1,112) as endophenotypes to further characterize neurodegeneration [283, 284].

Genotyping and Quality Control

ADNI samples were genotyped using Human 610-Quad, HumanOmni Express, and HumanOmni 2.5M BeadChips. Sample and SNP quality control procedures of GWAS data such as SNP call rate < 95%, Hardy-Weinberg equilibrium test $p < 1 \times 10^{-6}$, and frequency filtering (MAF ≥ 5%) were performed [190, 285-287]. Imputation of un-genotyped SNPs was performed using MaCH (Markov Chain Haplotyping) software based on the 1000 Genomes Project as a reference panel [288].

Association Analysis and Meta-Analysis

SNPs from the 81 candidate genes were located in untranslated regions [44], 3′ UTR, 5′ UTR, coding regions, intronic regions, and regulatory regions (±20 kb of upstream and downstream regions). A gene-based association analysis of hippocampal neurogenesis pathway-related candidate genes was performed in an additive genetic model using a set-based test in Plink v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) [286]. After frequency and genotyping pruning, 18407 SNPs remained from 81 genes. [286]. After frequency and genotyping pruning, 18407 SNPs remained from 81 genes. A mean test

statistic for each SNP within a gene was computed to determine other SNPs in linkage disequilibrium (LD; i.e., r^2>0.5). A quantitative trait analysis (QT) was then performed with each SNP. For each gene (set), the top independent SNPs (i.e., not in LD; maximum of 5) were selected if their p-values were less than 0.05 in the QT analysis. The SNP with the smallest p-value was selected first; subsequent independent SNPs were selected in order of decreasing statistical significance. From these subsets of SNPs, the statistic for each set was calculated as the mean of these single SNP statistics. The analysis was performed to estimate the additive effect of the selected SNP minor allele on the phenotypic mean [269, 286]. Covariates included gender, age, years of education, ICV, MRI field strength (1.5T vs 3T) and diagnosis status. An empirical p-value (20,000 permutations) was reported for each gene. In the discovery sample (ADNI-1/GO/2), a conservative significance threshold ($p < 0.00061$) was used based on Bonferroni correction for 81 genes. We subsequently performed a meta-analysis for genes and SNPs associated with hippocampal volume using data from the ADNI-1/GO/2, AddNeuroMed, and IMAS cohorts. The gene-based meta-analysis was performed using the weighted z statistic test (Stouffer's weighted z statistic) as implemented in R, with weight accounting for the sample size of each cohort. For SNP-based meta-analysis, METAL was used with a fixed-effect inverse variance model [289]. For meta-analysis, effect sizes were provided by standardized β coefficients from linear regression.

We also evaluated the effect of the minor allele rs9608282-T on hippocampal volume and composite memory score after the participants, which included only those diagnosed with MCI or AD, were classified as either a Memantine user (NMDA (+)) or a Memantine non-user (NMDA (-)) using two-way analysis of covariance (ANCOVA) for continuous variables and a chi-square for categorical variables implemented in SPSS 23.0. In addition, two-way analysis of covariance (ANCOVA) was used to examine the effect of the minor allele (rs9608282-T) on hippocampal volume in both amyloid-negative and amyloid-positive participants (classified as positive or negative by either baseline [^{18}F]Florbetapir PET scans and/or CSF Aβ1-42 level).

Image analysis

Non-Hispanic Caucasian participants from the Alzheimer's Disease Neuroimaging Initiative GO/2 (ADNIGO/2) cohort with arterial spin labeling (ASL) perfusion imaging (N=220) and genotype data were used. Statistical Parametric Mapping 8 (Wellcome Trust Centre for Neuroimaging) with a cluster threshold of 25 voxels was used to perform voxel-wise analysis of the effect of rs9608282-T variant in the *ADORA2A* gene on CBF. Age, sex and diagnosis were used as covariates.

Gene expression

We examined mRNA expression profiles of the 81 manually-curated adult neurogenesis genes in normal brain tissues from the Allen Human Brain Atlas (Allen Institute for Brain Science, Seattle, WA; available from http://www.brain-map.org/). The Allen Human Brain Atlas (AHBA) provides publicly available online data sets including extensive gene expression information for the human brain. The AHBA gene expression data contains information for approximately 62,000 gene probes with 93% of known genes represented by at least 2 probes for each gene (~30,000 genes) from ~500 brain samples from each hemisphere representing cortical, subcortical, cerebellar, and brainstem regions. We examined the identified adult neurogenesis-related genes for their expression levels in five brain regions related to hippocampal neurogenesis, including CA1, CA2, CA3, CA4 and DG. We used a heat map tool to visualize the microarray data for the selected neurogenesis-related genes and brain regions across tissue samples.

C. Results

Gene-based and SNP-based analysis of mean volumes of hippocampus and hippocampal sub-regions

The manual gene/pathway curation for hippocampal neurogenesis yielded 18407 SNPs in 81 genes (Figure 5). In the discovery sample, the gene-based association analysis showed that *APOE* and *ADORA2A* were significantly associated with hippocampal volume after Bonferroni correction (p-value=$5x10^{-5}$, respectively; Table 2).

Table 2. 81 genes of gene-based association results in the discovery sample for hippocampal volume using common variants (MAF ≥ 0.05) where empirical p-values were calculated using 20,000 permutations in PLINK.

Gene	Number of SNPs in gene	Number of significant SNPs (p<0.05, r2<0.5)	Empirical gene-based *p*-value	List of significant SNP
ADORA2A	55	1	5×10^{-5}	rs9608282
APOE	85	5	5×10^{-5}	rs429358|rs7259620| rs34095326|rs4803770| rs157580
TLR4	58	2	1.5×10^{-2}	rs11789302|rs10759930
BCHE	201	4	2.8×10^{-2}	rs2686409|rs1355538| rs12107166|rs6807910
CXCL10	42	1	4.8×10^{-2}	rs4256246
S100B	220	1	6.0×10^{-2}	rs118078026
CXCL12	306	4	6.2×10^{-2}	rs11238990|rs11238991| rs1144472|rs17659345
TET1	387	3	0.06593	rs113716271|rs12776586|rs12221107
BCL2	368	5	0.07493	rs9957149|rs28564323| rs7236090|rs6567334| rs11872403
PTGS2	52	2	0.08492	rs7547677|rs2206593
ACHE	51	2	0.08791	rs13245899|rs73714210
SORL1	233	2	0.09091	rs9665907|rs643010
SLC6A4	63	1	0.09191	rs16965628
GRIN2B	1054	5	0.0999	rs34870448|rs11612709| rs12582848|rs11611667| rs2300256
NGFR	86	5	0.0999	rs584589|rs11466150| rs2072444|rs535717| rs2537710
MEF2C	232	1	0.1019	rs1065861
CXCL1	39	1	0.1039	rs2968710
NR3C1	238	5	0.1209	rs4912912|rs17209237| rs10050756|rs7719514| rs12653301

DKK1	40	2	0.1249	rs11001581|rs7100461
GRN	37	1	0.1319	rs114641762
CHAT	189	5	0.1379	rs885834|rs11101179| rs1720367|rs4615945| rs74981858
CHRNB2	39	5	0.1499	rs9616|rs9427094| rs12072348|rs67860750| rs4845653
SYN3	1388	5	0.1718	rs2157188|rs9609643| rs2710348|rs180958069| rs5749521
CCL2	43	1	0.1758	rs111843487
IGF1	89	4	0.1888	rs1549593|rs10860862| rs12821878|rs80280982
VEGFA	59	3	0.1908	rs9381248|rs3025006| rs699946
NGF	135	2	0.1978	rs6537860|rs4320778
MIF	77	1	0.2008	rs738807
IL6	77	2	0.2338	rs2069840|rs62449498
CASP3	94	2	0.2378	rs4647634|rs2696059
MMP9	57	2	0.2537	rs73112805|rs3918253
PRNP	94	2	0.2747	rs6052766|rs67017873
SNCA	444	1	0.2927	rs187644542
CDK5	53	2	0.3147	rs4148853|rs34403003
IL1B	42	1	0.3147	rs3917381
ADAM10	232	1	0.3197	rs544282
NOS3	71	1	0.3197	rs12666075
NRG1	2784	5	0.3227	rs147179882|rs2466068| rs7829383|rs2347071| rs11998153
APBB1	119	1	0.3487	rs11040880
LRRK2	360	3	0.3526	rs189800607| rs10878411|rs11564173
OLIG2	123	2	0.3626	rs17632819|rs76708155
NOS1	559	5	0.3826	rs67313272|rs4767542| rs816284|rs12228022| rs10850829
HIF1A	82	1	0.4186	rs12891737
FGF1	221	5	0.4326	rs1808258|rs2070715| rs249925|rs10041541| rs13179022
TGFB1	131	1	0.5135	rs4803459
IGF1R	520	5	0.5325	rs11631965|rs4966039| rs3743254|rs7166348| rs2272037

FAS	175	1	0.5774	rs12767306
APP	554	5	0.6214	rs2829960|rs6516705| rs13046930|rs6516715| rs117104544
NTRK2	605	4	0.6264	rs17087710|rs28580203| rs1047896|rs1006446
DYRK1A	380	1	0.6593	rs28550863
ESR2	412	4	0.6983	rs1152576|rs4986938| rs10146107|rs12587140
RELN	1552	5	0.7932	rs2299373|rs3819491| rs39377|rs1476446| rs694894
ESR1	810	1	0.7972	rs55650062
ABCA2	61	0	1	N/A
ADRA2A	27	0	1	N/A
BDNF	122	0	1	N/A
CAV1	129	0	1	N/A
CDK5R1	24	0	1	N/A
CHRNA7	2	0	1	N/A
CNTF	29	0	1	N/A
FOS	48	0	1	N/A
GSK3B	329	0	1	N/A
HTR2A	203	0	1	N/A
NEUROD1	54	0	1	N/A
NPY	131	0	1	N/A
PLCG1	60	0	1	N/A
PSEN1	154	0	1	N/A
SIRT1	98	0	1	N/A
THRA	82	0	1	N/A
TNF	40	0	1	N/A
TNFR1A	4	0	1	N/A
TNFR1B	54	0	1	N/A
DLD	24	0	1	N/A
GLP1R	98	0	1	N/A
IFNG	19	0	1	N/A
PLAU	30	0	1	N/A
PSEN2	19	0	1	N/A
SOD2	5	0	1	N/A
TP53	5	0	1	N/A
CST3	92	0	1	N/A
CHRM1	47	0	1	N/A

One SNP (rs9608282) upstream of *ADORA2A* was most significantly associated with total hippocampal volume and sum of the hippocampal sub-region volumes (p = 1.14x10^{-5} and 2.5x10^{-4}, respectively; Table 3). Specifically, participants with no copies of the minor allele (N=1,317; GG genotype) had a smaller mean hippocampal volume compared to participants with one copy of the minor allele (N=236; TG genotype) or participants with two copies of the minor allele (N=10; TT genotype).

Table 3. Association of rs9608282 in *ADORA2A* with neuroimaging phenotypes and memory composite scores with and without diagnosis (DX) adjustment. SNP-based association results (p-values) in the discovery sample for hippocampal volume, neurogenesis-related hippocampal sub-regions, memory performance, and CSF total tau level.

rs9608282	*p*-value after adjusting for DX	*p*-value before adjusting for DX
Hippocampal Volume	3.29 x 10^{-4}	1.14 x 10^{-5}
Neurogenesis-Related Hippocampal Sub-regions	4.55 x 10^{-3}	2.50 x 10^{-4}
Memory Composite Score	0.218	7.45 x 10^{-3}
CSF Total Tau	0.2	2.3 x 10^{-2}

For replication of our major significant SNP finding, we analyzed independent samples from the ADNI, AddNeuroMed IMAS cohorts. SNP-based meta-analysis of *ADORA2A* in three independent cohorts (ADNI1/GO/2, AddNeuroMed, and IMAS) identified that rs9608282-T in *ADORA2A* are significantly associated with hippocampal volume (p = 0.000043, N=1,840, Table 4; $p = 7.88 \times 10^{-6}$, N= 1,840, Table 5, respectively). In ADNI1/GO/2, AddNeuroMed cohorts except IMAS, rs9608282-T exhibited a positive direction of effect on hippocampal volume.

Table 4. Meta-analysis of *ADORA2A* with hippocampal volume in three independent cohorts: ADNI, AddNeuroMed and IMAS.

	ADNI *p*-value	AddNeuroMed *p*-value	IMAS *p*-value	Meta-analysis *p*-value
ADORA2A	$5x10^{-5}$	2.1×10^{-1}	2.95×10^{-1}	4.3×10^{-5}

Table 5. Meta-analysis of rs9608282 with hippocampal volume in three independent cohorts: ADNI, AddNeuroMed and IMAS.

rs9608282	N	Effect of rs9608282 (T) (β value)	*p*-value
ADNI	1563	146.9	1.14×10^{-5}
AddNeuroMed	218	362.4	2.344×10^{-2}
IMAS	59	-263.2	4.59×10^{-2}
Meta-analysis	1840		**7.88×10^{-6}**

Following the SNP-based association analysis, we performed a post-hoc analysis to measure the interaction effect of APOE and the ADORA2A SNP on hippocampal volume. There was no evidence of epistasis, modeled as interaction between *APOE* ε4 status and the minor allele of rs9608282 ($p = 0.54$). However, *ADORA2A* rs9608282-T and *APOE* ε4 exhibited independent but opposite effects on hippocampal volume (Figure 6A, 6B), with a comparable effect sizes between the APOE ε4 allele and the presence of at least one copy of the minor allele at rs9608282. Participants carrying at least one copy of the minor allele of the ADORA2A SNP have larger hippocampal volumes than those without the minor allele, even in participants with APOE ε4 ($p = 0.001$; Fig. 3C). The positive effect of the ADORA2A rs9608282-T allele on hippocampal volume was seen in both amyloid-negative and amyloid-positive participants (classified as positive or negative by either baseline [^{18}F]Florbetapir PET scans and/or CSF Aβ1-42 level). Specifically, rs9608282-T was significantly associated with larger hippocampal volumes in Aβ negative (p-value = 0.027) and Aβ positive participants (p-value = 0.015, Figure 7). In addition, the association of the rs9608282-T allele with hippocampal volume and neurogenesis-related to sub-regions independent of diagnosis suggests that this effect might be a global phenomenon.

Figure 6. *APOE* ε4 and rs9608282 (*ADORA2A*) appear to exhibit independent, but opposite effect on hippocampal volume. Baseline hippocampal volume (adjusted for age, gender, ICV, MRI field strength) ± standard errors are shown based on (a) rs9608282 in *ADORA2A* (A2aR) across genotype groups. Presence of at least one copy of the minor allele (T) of rs9608282 was significantly associated with increased hippocampal volume (p = 0.002). Baseline hippocampal volume is also shown by (b) the number of *APOE* ε4 allele copies. Presence of at least one copy of the ε4 allele was significantly associated with decreased hippocampal volume (p < 0.0001). (c) For participants having *APOE* ε4 allele copies, participants carrying minor allele of rs9608282 had larger hippocampal volume than those who did not carry minor allele of the *ADORA2A* rs9608282 (p = 0.001).

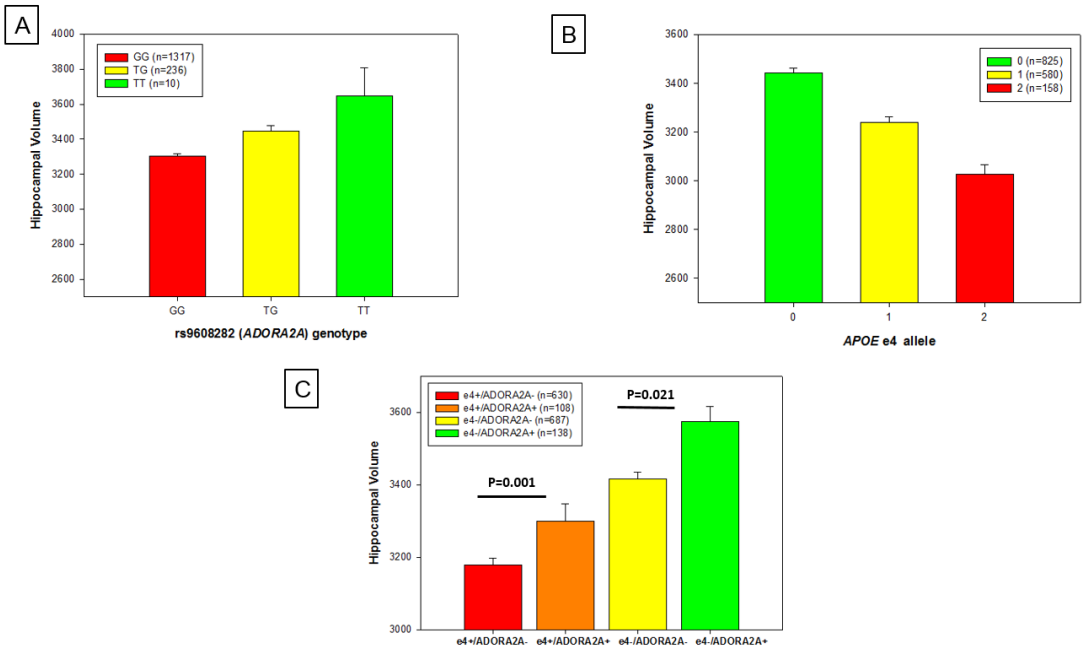
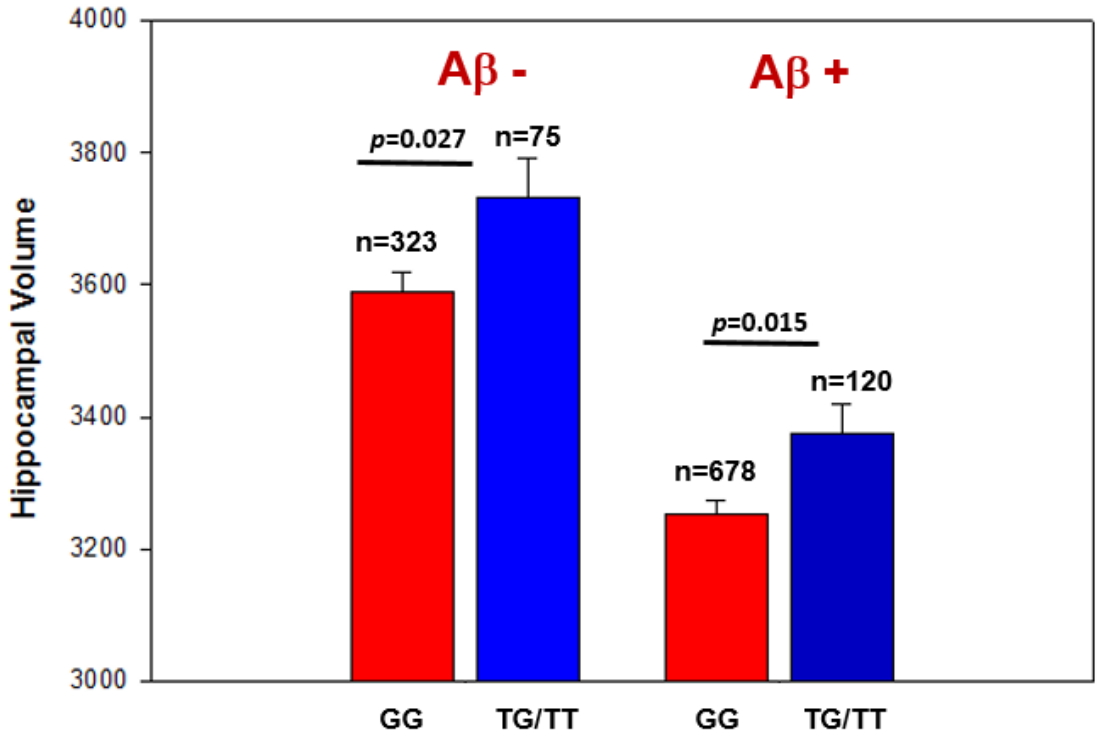
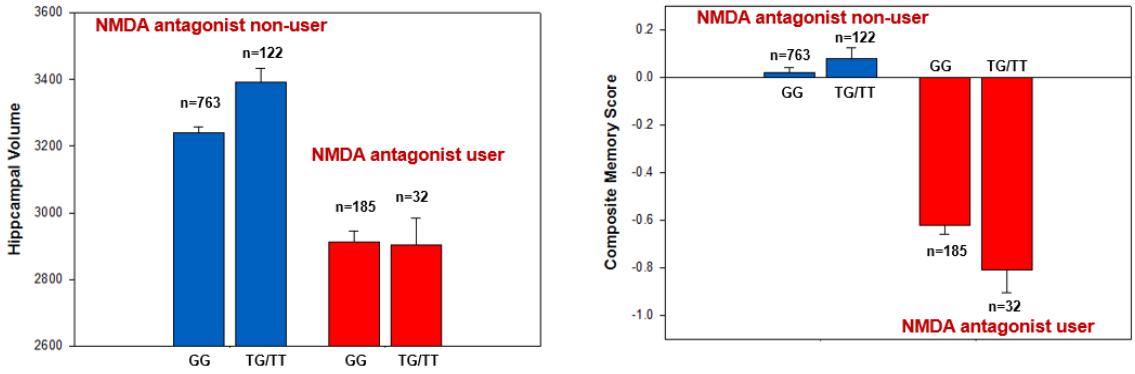


Figure 7. *ADORA2A* rs9608282 is associated with larger hippocampal volume in amyloid-positive participants (classified by PET scan and/or CSF-amyloid beta level). For statistical analysis, each participant was classified by their amyloid status (positive versus negative) at the baseline visit (determined by standard cutoffs on [^{18}F]Florbetapir PET scan and/or CSF amyloid level). The effect of the T allele on hippocampal volume was present in both amyloid-negative (left column) and amyloid-positive (right column) participants. Upon statistical analysis, rs9608282 is significantly associated with hippocampal volume in amyloid-negative and even amyloid-positive participants (p = 0.027, p = 0.015, respectively).



Since previous studies have suggested that *ADORA2A* plays an important role controlling NMDA-dependent synaptic toxicity and memory impairment [290-292], we examined the interaction of taking Memantine, a NMDA-receptor antagonist, and *ADORA2A* rs9608282 on hippocampal volume and memory performance. Participants diagnosed with MCI or AD were classified as either a Memantine user (NMDA+) or a Memantine non-user (NMDA-). We found that NMDA- participants carrying at least one copy of the minor allele (T) of the *ADORA2A* rs9608282 had a larger mean hippocampal volume ($p<0.001$; Figure 8A). There was a significant interaction effect of NMDA-receptor antagonist use and *ADORA2A* rs9608282 on memory performance ($p = 0.009$). NMDA+ participants carrying two copies of the major allele (G) of the *ADORA2A* rs9608282 had better memory performance (Figure 8B).

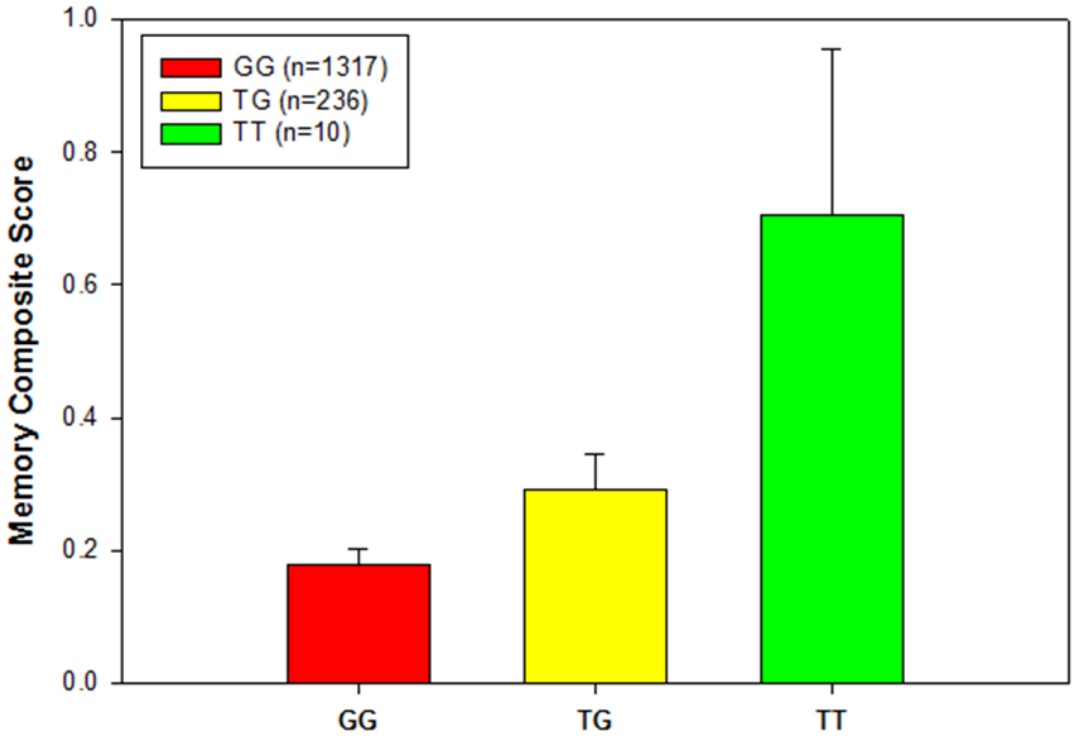
Figure 8. *ADORA2A* rs9608282 is associated with larger hippocampal volume in Memantine non-users (NMDA (-)) and poorer memory performance in Memantine users (NMDA (+)). For statistical analysis, cognitively impaired participants were classified as either a Memantine user (NMDA (+)) or a Memantine non-user (NMDA (-)). (A) The rs9608282 T allele was associated with a larger mean hippocampal volume in Memantine non-user participants ($p < 0.001$). (B) Participants carrying at least one copy of minor allele (T) of the ADORA2A rs9608282 variant and using a NMDA-receptor antagonist had poorer memory performance.



Association of rs9608282 with episodic memory and CSF level of total tau

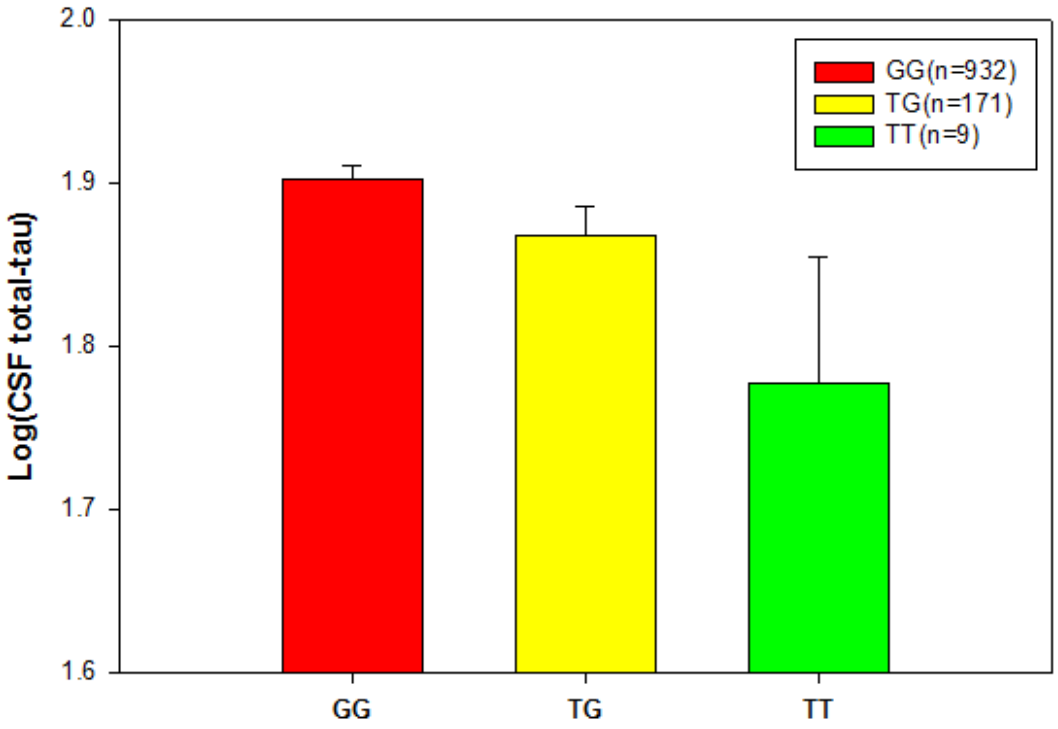
Given the association of *ADORA2A* rs9608282-T with larger hippocampal volume, we hypothesized that *ADORA2A* would also associated with episodic memory scores as they are highly related to hippocampal structure. As might be hypothesized, rs9608282-T was significantly associated with a better composite memory score ($\beta = 0.065$; $p = 0.015$) after controlling for age, gender, and years of education (Figure 9).

Figure 9. Association of memory composite score with rs9608282 in *ADORA2A* across genotype. Baseline memory composite score (adjusted for age, gender and education) ± standard errors are displayed based on rs9608282 genotype. Individuals with a TT genotype at the rs9608282 variant showed a 5% increase in memory performance relative to those with a GG genotype.



A previous study suggested that hyperphosphorylated tau decreases adult neurogenesis in mouse model [293]. Therefore, we also assessed the effect of the rs9608282-T minor allele on CSF total tau level. As we hypothesized, rs9608282-T carriers showed decreased CSF total tau levels relative to non-carriers (β = -0.061; p = 0.039), after controlling for age and gender (Figure 10). However, there is no correlations of rs9608282 with CSF Aβ and phospho-tau levels.

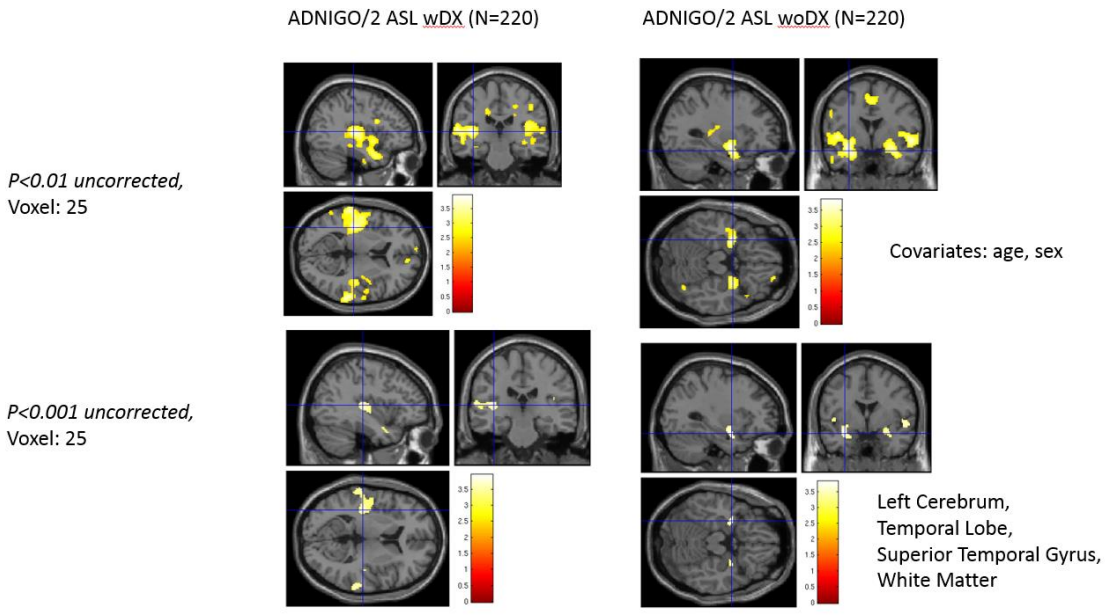
Figure 10. Association of CSF tau level with rs9608282 in *ADORA2A* across genotype. CSF total tau level (adjusted for age and gender) ± standard errors are displayed based on rs9608282 genotype. Individuals with a TT genotype at the rs9608282 variant showed significant decrease in CSF tau level relative to those with a GG genotype.



Association of rs9608282 with cerebral blood flow

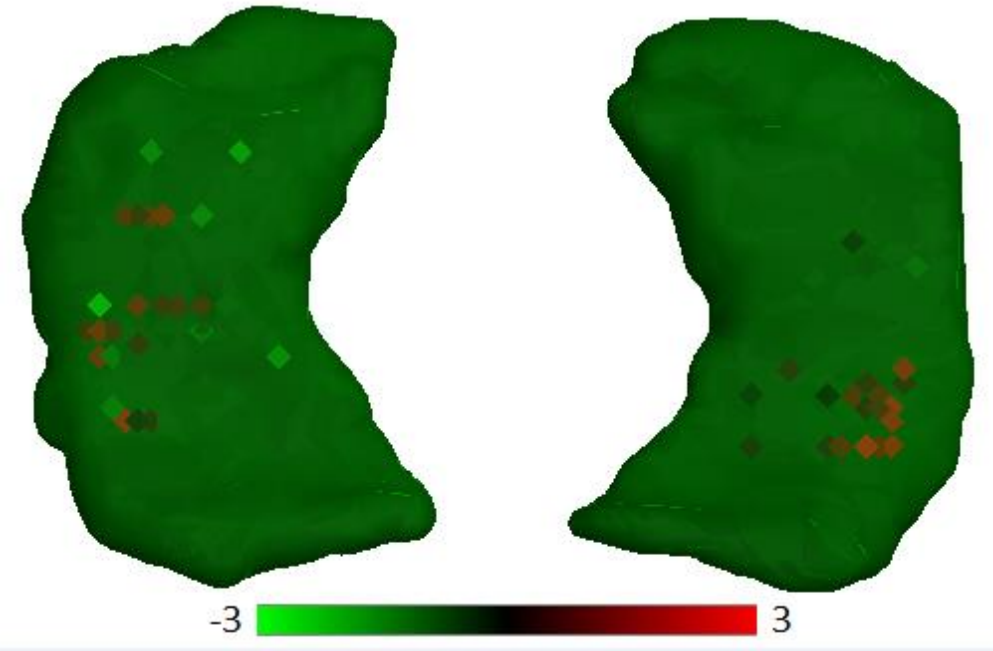
Since selective blockade of the adenosine A2A receptor reduced the pial vasodilation in certain situations, we tested the hypothesis that rs9608282 in ADORA2A is significantly associated with cerebral blood flow (CBF). Voxel-wise analysis of ASL perfusion demonstrated significantly decreased regional CBF especially in the left cerebrum, temporal lobe, and superior temporal gyrus in individuals with at least one minor alleles (T) of rs9608282 compared to those with no minor allele (Figure 11).

Figure 11. Individuals with at least one minor alleles (T) of rs9608282 associated with perfusion decrease



Finally, we used gene expression data from the Allen Human Brain Atlas to evaluate if *ADORA2A* was expressed in neurogenesis-related regions in normal brains. *ADORA2A* was in fact highly expressed across the major adult neurogenesis related regions of the brain (Figure 12) and was especially highly expressed in CA1 and CA2.

Figure 12. *ADORA2A* expression profiles across the hippocampus region. The square dot indicates the tissue sample location for human brain. In each square, heat map color represents the z-score over a probe ranging from green (z-score of -3 and below) through black to red (z-score of +3 and above). Red squares represents overexpression of the *ADORA2A* in specific locations, most especially in CA1 and CA2.



D. Discussion

We used well-characterized participants from the extensively studied cohort Alzheimer's Disease Neuroimaging Initiative (ADNI), which uniquely includes participants with cognitively normal older individuals (CN), significant memory concerns (SMC), mild cognitive impairment (MCI), and Alzheimer's Disease (AD). Using targeted neurogenesis pathway-based gene analysis, we discovered a significant association of *ADORA2A* rs9608282-T with larger mean hippocampal volumes and volumes of neurogenesis-related hippocampal sub-regions, better episodic memory performance, and reduced CSF total tau. These findings suggested a protective effect of this SNP on brain structure and function in neurogenesis-related brain regions.

ADORA2A (Adenosine Receptor Subtype A2a) is a G-protein-coupled adenosine receptor that is involved with controlling synaptic plasticity in glutamatergic synapses [294, 295]. Previous work had indicated a physical and functional interaction of ADORA2A with dopamine D_2 receptors [296]. However, A1R–A2AR heteromer controls the affinity of agonist binding to A2a receptors in the striatum and localizes in glutamatergic nerve terminals to control glutamate release [297]. In addition to its abundance in the striatum, ADORA2A also plays an important role in hippocampus, particularly in neurogenesis in the CA3 region. A previous study demonstrated that inhibition of the A2a receptor induced synaptic damage in rat hippocampal nerve terminals [298].

A reduction of A2a receptors in mice with traumatic brain injury has also been shown to decrease cognitive impairment [299]. In fact, the adenosine 2a receptor localizes in microglial cells and may be a regulation of microglial function in response to brain damage [294]. Since neuroinflammatory blockade is thought to enhance neural stem/progenitor cells activity and promote adult neurogenesis, A2a receptor-mediated control of neuroinflammation might be a vital mechanism in neurodegenerative diseases [218]. Inhibition of the A2a receptor also prevents early Aβ-induced synaptotoxicity and memory dysfunction through a p38 MAPK-dependent pathway [300], potentially suggesting additional roles for this receptor in AD. In fact, *ADORA2A* blockade prevented memory decline secondary to amyloid-beta accumulation, which is a major pathological hallmark in AD [301]. Another important role of ADORA2A is to modulate brain-derived neurotrophic factor (BDNF). Administration of an ADORA2A antagonist inhibits the actions of BDNF on GABA and glutamate release from the hippocampal nerve terminals [302]. In addition, the A2a receptor is involved with control of N-methyl-D-aspartate (NMDA) receptor function by co-localizing with metabotropic glutamate 5 receptors (Glu5R) in hippocampal synapses [292]. The synaptic localization of A2a receptors plays a key role controlling NMDA-dependent synaptic transmission in the hippocampus [303]. In fact, glutamate release is dependent on the activation of adenosine A2AR by endogenous adenosine [300, 302]. Previous studies showed the relationship between NMDA receptor and Adenosine Receptor Subtype A2a, which supports our finding of a significant

interaction effect of NMDA-receptor antagonist use and the *ADORA2A* rs9608282-T on memory performance.

rs9608282 is located upstream of *ADORA2A* (UCSC Genome Browser (GRCh37/hg19)) and is characterized by occurring during read-through transcription of two neighbor genes, *SPECC1L* (sperm antigen with calponin homology and coiled-coil domains 1-like) and *ADORA2A* (adenosine A2a receptor) on chromosome 22. This read-through transcription is a candidate for nonsense-mediated mRNA decay (NMD), which leads to no protein production. The inhibition of *ADORA2A* has been shown to enhance spatial memory and hippocampal plasticity through adult neurogenesis [304]. In the present study, rs9608282-T was associated with better memory and a larger hippocampal volume, suggesting that this variation may inhibit protein production of *ADORA2A*. Animal models or cell culture studies are needed to more completely characterize the function of this variation on brain structure and adult neurogenesis.

Based on gene expression data from postmortem human brains, the A2A receptor is highly expressed in neurogenesis-related regions (CA1, CA2, CA3 and dentate gyrus) of the hippocampus in the adult human brain. Since the dentate gyrus and CA3 regions are important for memory formation and pattern separation processes, as well as for learning new information, we believe the observed effect of the rs9608282-T variation may be protective for memory

performance by altering neurogenesis in these regions. In addition, the association of the rs9608282-T allele with hippocampal volume and neurogenesis-related sub-regions independent of diagnosis suggests that this effect might be a global rather than AD-specific phenomenon. Consistent with protective effect of this variant, decreased CSF total-tau protein levels were also observed in participants with at least one minor allele (T) of rs9608282. Interestingly, *ADORA2A* rs9608282-T and *APOE* ε4 exhibit an independent but opposite effect on hippocampal volume. In sum, we observed a significant protective effect of a variant (rs9608282) in the neurogenesis-related *ADORA2A* gene on brain structure and function, including increased hippocampal volume, better memory performance, and reduced CSF tau. This finding suggests that the adenosine A2a receptor warrants further investigation as a potential target for future therapeutics to treat neurodegenerative disease and cognitive decline.

The eQTL analysis using the BRAINEAC brain tissue microarray-based gene expression database (http://www.braineac.org/) revealed that rs9608282 in *ADORA2A* is marginally associated with *ADORA2A* gene expression level in the hippocampus (p-value = 0.172). Individuals carrying minor allele rs9608282-T have decreased expression levels in the hippocampus, showing a potential protective effect same as our SNP-based association results with hippocampal volume and memory.

The limitation of the present report is that even though we used three independent publicly available databases to identify a curated gene list related to adult neurogenesis, it is possible that we may have missed other neurogenesis related genes not represented in these databases. The other limitation is that even though a few studies combined MRI-based hippocampal volume with immunochemistry to reveal that there is a significant hippocampal atrophy and the reduction of hippocampal neurogenesis in animal models, it is still not clear if hippocampal atrophy is related to adult neurogenesis in humans due to lack of data sources. Another limitation for this study is the lack of replication in the gene-based analysis. In the AddNeuroMed and IMAS, *ADORA2A* did not show a significant association with hippocampal volume but showed a trend. After combining three independent cohorts, the meta-analysis result was significant due to the increased detection power. In addition, future studies are needed to identify functional evidence to validate this SNP in *ADORA2A*. However, the present findings support that the *ADORA2A* gene plays a role in adult neurogenesis. AD is associated with hippocampal volume loss the observed effects indicates the potential importance of further investigation of this gene in independent cohorts.

III. Genome-wide association analysis of hippocampal volume identifies enrichment of neurogenesis-related pathways

A. Introduction

New neurons are generated throughout adulthood in two regions of the brain, the dentate gyrus of the hippocampus and olfactory bulb, and are incorporated into the hippocampal network. The pathways of adult neurogenesis include signaling transduction, the proliferation of neural progenitor cells, the fate determination of neural progenitor cell progenies, and the differentiation, migration, and maturation of adult neurons [4, 5]. In particular, multiple factors such as neurotrophic factors, transcription factors, and cell cycle regulators control neural stem cell (NSC) proliferation, maintenance in the adult neurogenic niche, and differentiation into mature neurons. The estimated annualized hippocampal atrophy rate is 1.41% for cognitively normal older adults and in adults, new neurons are added in each hippocampus daily via adult neurogenesis with an annual turnover of 1.75% and a modest decline during aging [3, 305]. A few studies focused on structural MRI and histological approaches to investigate newborn neurons and neural stem/progenitor cells in neurogenesis-related brain regions in mice, and it was found that neurogenesis is associated with increased hippocampal gray matter volume in mice [306]. Other studies combined MRI-based hippocampal volume with immunochemistry to reveal that there is a significant hippocampal atrophy and the reduction of hippocampal neurogenesis

in adult rats who were exposed to oxygen deprivation during birth [307]. Molecular pathways and genes affect the induction of neurogenic niche and neural/progenitor cells turnover to newborn neurons for the formation of the hippocampal structure during hippocampal neurogenesis.

To our knowledge, there is no study directly associating adult neurogenesis related pathways with hippocampal volume. In this study, we investigated whether neurogenesis-related pathways are enriched for hippocampal volume using a large-scale human neuroimaging genetics meta-analysis summary statistics (N~13,000). Although neurogenesis is an important contributor to the formation of the hippocampus in mice, it has not been studied fully in human brain whether adult neurogenesis is related to hippocampal volume yet. Genetic variation in neurogenesis-related genes in the human genome may have compensatory advantages or confer vulnerability to biological processes during adult neurogenesis but studies are needed to identify actual mechanism by which genetic variants affect neural stem cells differentiation, proliferation, and their maturation to new neurons in human brain.

To better understand hippocampal volume and hippocampal atrophy are significantly associated with neurogenesis pathway, we performed a pathway enrichment analysis on a genome-wide association study (GWAS) from the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium and Alzheimer's Disease Neuroimaging Initiative data sets to identify

functional pathways associated with hippocampal volume and hippocampal atrophy. For further analysis, weighted gene co-expression network analysis (WGCNA) analysis (WGCNA) was performed using human brain gene expression data of cognitively normal individuals to identify clusters of highly correlated genes and investigate which each cluster is related to neurogenesis-related pathways.

B. Materials and Method

Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA)

The Enhancing NeuroImaging Genetics through Meta-Analysis Consortium (ENIGMA) was initiated in December 2009. The research group involved in neuroimaging and genetics worked together on a range of large-scale studies that integrated data from 70 institutions worldwide. The goal of ENIGMA was to merge neuroimaging data with genomic data and identify common variants that might affect the brain structure. The first project of ENIGMA was focused on identifying common variants associated with hippocampal volume or intracranial volume (ICV) [308]. The aim of ENIGMA2, follow-on study of ENIGMA1, was to perform genome-wide association analysis (GWAS) using the volumes of all subcortical structures. ENIGMA includes magnetic resonance images (MRI) of 30,717 individuals from 50 cohorts [212]. GWAS was conducted using mean hippocampal volume as a phenotype controlling for age, gender, four

multidimensional scaling components, ICV, and diagnosis. The hippocampal volume was obtained from structural MRI data. MRI scans and genetic imputation were processed and examined by following standardized protocols freely available online (http://enigma.ini.usc.edu/protocols/imaging-protocols/). In this study, we used a meta-analysis GWAS summary statistics in the discovery sample of 13,163 subjects of European ancestry from the ENIGMA consortium (Hibar et al. 2015). 21% of the discovery participants have anxiety, Alzheimer's disease, attention-deficit/hyperactivity disorder, bipolar disorder, epilepsy, major depressive disorder or schizophrenia. 9,339 out of 13,163 individuals are cognitively normal subjects.

Alzheimer's Disease Neuroimaging Initiative (ADNI)

ADNI was launched in 2003 by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, the Food and Drug Administration (FDA), private pharmaceutical companies, and nonprofit organizations as a public-private partnership and recruited from 59 sites across the U.S. and Canada. ADNI includes over 1700 subjects consisting of cognitively normal older individuals (CN), significant memory concerns (SMC), mild cognitive impairment (MCI) and Alzheimer's Disease (AD) aged 55-90 (http://www.adni-info.org/). Subjects in ADNI have structural MRI scans and functional and psychological test data. The goal of ADNI is to identify whether serial MRI, positron emission tomography (PET), sensitive and specific other markers, and clinical and

neuropsychological assessments could be merged to measure the progression of mild cognitive impairment (MCI) and early AD. Participants for this study included 367 CN, 94 SMC, 280 early MCI, 512 late MCI and 310 AD. Clinical and neuroimaging procedures and the other information about the ADNI cohort can be found at http://www.adni-info.org/. Table 6 shows selected demographic and clinical characteristics of these participants at the MRI scan time.

Table 6. Demographic and clinical characteristics of ADNI participants

	CN	SMC	EMCI	LMCI	AD
N	367	94	280	512	310
Age	74.59 (5.57)	71.77 (5.65)	71.14 (7.26)	73.52 (7.65)	74.65 (7.79)
Gender (M/F)	192/175	38/56	158/122	318/194	176/134
Education	16.32 (2.68)	16.81 (2.57)	16.08 (2.67)	15.97 (2.91)	15.23 (2.97)
APOE (ε4–/ε4+)	267/99	62/32	160/119	232/280	104/206

Genotyping data and quality control

The genotyping data of ADNI participants were collected using the Illumina Human 610-Quad, HumanOmni Express, and HumanOmni 2.5M BeadChips. Standard quality control procedures of GWAS data for genetic markers and subjects were performed using PLINK v1.07 (pngu.mgh.harvard.edu/~purcell/plink). Sample and SNP quality control procedures were excluded with the criteria as SNP call rate < 95%, Hardy-Weinberg equilibrium test $p < 1 \times 10^{-6}$, and frequency filtering (MAF ≥ 5%), participant call rate < 95%, sex check and identity check for related relatives [190, 285, 286, 309]. Non-Hispanic Caucasian participants were selected using HapMap 3 genotype data and the multidimensional scaling (MDS) analysis after performing standard quality control procedures for genetic markers and subjects. For imputation of un-genotyped SNPs, MaCH (Markov Chain Haplotyping) software based on the 1000 Genomes Project as a reference panel was used [271, 288].

Gene-set enrichment analysis

Gene-set enrichment analysis of GWAS summary statistics was performed to identify pathways and functional gene sets with significant association to the hippocampal volume. All SNPs (n=6,571,356) and subjects with European ancestry were included in this study. Pathway annotations were downloaded

from Molecular Signatures Database version 5.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp/). This annotation data comprised a collection of GO Ontology. GO gene sets comprise of publically available 1,454 pathways. 825 gene sets belong to GO biological process, 233 gene sets belong to GO cellular components, and 396 gene sets belong to GO molecular function. The GSA-SNP software [310] uses the p-value of each SNP from GWAS to test if a pathway-phenotype association is significantly different from all other pathway-phenotype associations. In GSA-SNP, '–log' on each the kth best SNP-level p-value for each gene is taken. To avoid spurious conservative predictions, the second best SNP (k=2) was chosen for each gene as a default option instead of the best SNP. Each pathway (gene sets) was assessed by z-statistics for the identification of the enriched pathways with phenotype [311]. Analysis of these pathways was restricted to those containing between 10 and 200 genes. The FDR (Benjamini-Hochberg) method was applied for multiple comparison correction to the p-values generated by the enrichment algorithm [312]. We identified as enriched pathways with hippocampal volume if FDR-corrected p-value < 0.05 for each pathway.

Genetic association analysis

The KGG (Knowledge-based mining system for Genome-wide Genetic studies) software was used to perform a genome-wide gene-based analysis using GWAS p-values. KGG uses HYST (hybrid set-based test) to analyze the overall

association significance in a set of SNPs at the gene level. HYST is the combination of the gene-based association test using extended Simes procedure (GATES) and the scaled chi-square test [313, 314]. First, SNPs in each gene were divided into different LD blocks depending on pairwise LD coefficients r^2 for all SNPs. Second, for each block, block-based p-value for association was calculated, and the key SNP was derived and marked. Next, the block-based p-values were combined accounting for LD between the key SNPs using the scaled chi-square [315].

A targeted gene-based association analysis of enriched neurogenesis pathway-related candidate genes for hippocampal volume was performed using a set-based test in Plink v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) [286]. The SNPs with $p<0.05$ for each gene were chosen. A mean test statistic for each SNP within a gene was computed to determine with which other SNPs it is in linkage disequilibrium (LD); i.e., if the correlation coefficient between them was $r^2>0.5$. A quantitative trait analysis (QT) was then performed with each SNP. For each gene (set), the top independent SNPs (i.e., not in LD; maximum of 5) are selected if their p-values are less than 0.05. The SNP with the smallest p-value is selected first; subsequent independent SNPs are selected in order of decreasing statistical significance. From these subsets of SNPs, the statistic for each gene is calculated as the mean of these single SNP statistics. The analysis was performed using an additive model or in other words, the additive effect of the minor allele on the phenotypic mean was estimated [269, 286]. Covariates

included gender, age, years of education, and diagnosis status. An empirical *p*-value (20,000 permutations) was reported for each gene for multiple comparison adjustment.

Gene expression correlation analysis

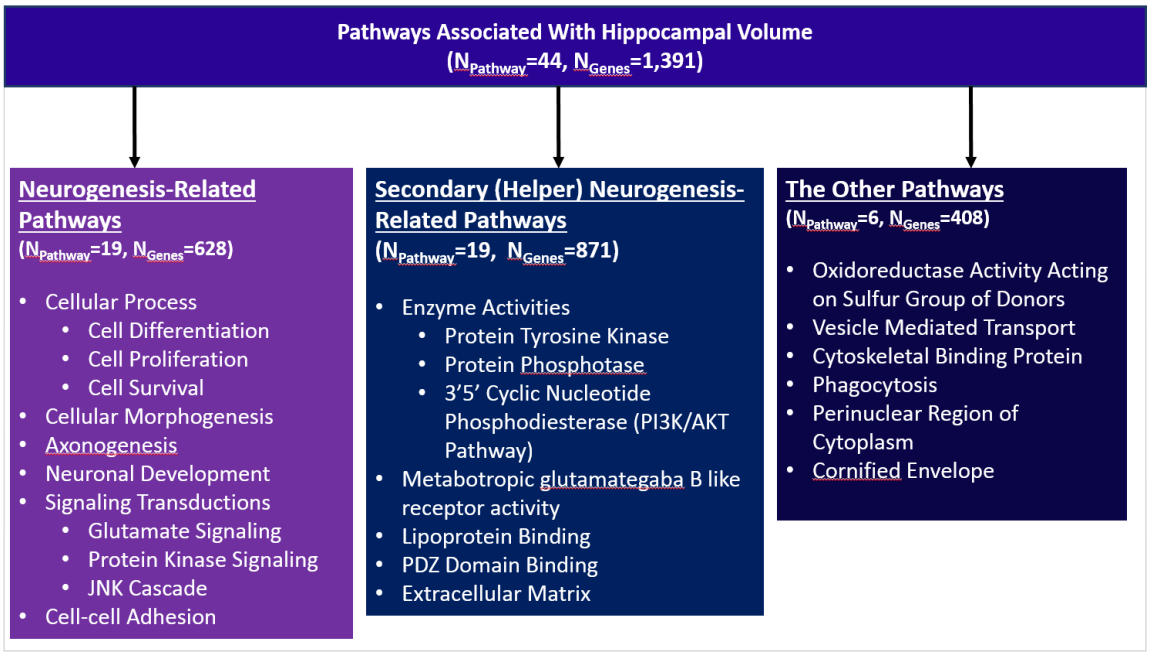
We used gene expression data for 32 cognitively normal samples generated using CA1 and CA3 regions of hippocampal tissues in NCBI's Gene Expression Omnibus (GEO). The Illumina HumanHT-12 v3 Expression BeadChip (48,803 probes) was used to measure expression of over 25,000 annotated genes. We processed data and removed the outliers as previously described [316]. We excluded probes if they present in three or fewer samples or if they do not correspond to any gene symbol annotations. Lastly we removed duplicate probes for a gene and just kept the highest expression level probe. After all data cleaning process, 15,037 genes remained. We performed weighted gene correlation network analysis (WGCNA) using processed expression data to identify clusters of highly correlated genes expressed in specific brain regions (CA1 and CA3) as modules. Pearson correlations between each gene pair were calculated. This matrix was transformed into signed adjacency matrix by using a power function. Then, topological overlap (TO) was calculated by using the components of this matrix. Thus, we measured the biological gene similarity depending on co-expression relationships of two genes with all other genes in the network. Genes were clustered hierarchically by the distance measure, 1-TO,

and the dynamic tree algorithm determined initial module assignments [317]**.** Gene module membership between each gene and each module eigengene was calculated. We tested these modules for enrichment of neurogenesis-related pathways.

C. Results

Gene-set enrichment analysis, using 6,571,356 SNPs from the ENIGMA GWAS summary statistics, identified 44 significantly enriched biological pathways (FDR-corrected p-value < 0.05) (Table 7) including 38 pathways related to neurogenesis processes. We classified neurogenesis related pathways as primary neurogenesis related pathways (N=19) and secondary (helper) neurogenesis related pathways (N=19) by using existing knowledge [318] and literature mining (Figure 13).

Figure 13. Conceptual classification of 44 pathways enriched for the hippocampal volume.



Nineteen primary pathways were related to cellular processes such as neuronal proliferation, differentiation and survival, cellular morphogenesis, axonogenesis, neuronal development, and signal transduction as well as cell-cell adhesion. Secondary neurogenesis pathways were consisted of enzyme activities related to neurogenesis, metabotropic receptor activity, lipoprotein binding and extracellular matrix. Six pathways were not related to any neurogenesis process such as oxidoreductase activity, phagocytosis, perinuclear region of cytoplasm and cornified envelope.

Table 7. Molecular Signatures Database (MSigDB) GO Ontology pathways enriched for hippocampal volume.

Pathway (N=44)	# of genes/set size	Corrected *p*-value
OXIDOREDUCTASE ACTIVITY ACTING ON SULFUR GROUP OF DONORS	10/10	4.68×10^{-4}
NEURON DIFFERENTIATION	73/76	1.18×10^{-3}
CELL PROJECTION	105/108	1.18×10^{-3}
MICROVILLUS	11/11	1.48×10^{-3}
NEURITE DEVELOPMENT	51/53	3.12×10^{-3}
CELL RECOGNITION	18/19	3.12×10^{-3}
GENERATION OF NEURONS	80/83	3.12×10^{-3}
TRANSMEMBRANE RECEPTOR PROTEIN KINASE ACTIVITY	50/51	3.12×10^{-3}
PROTEIN DOMAIN SPECIFIC BINDING	71/72	3.12×10^{-3}
NEURON DEVELOPMENT	59/61	3.24×10^{-3}
AXONOGENESIS	41/43	3.24×10^{-3}
CELLULAR MORPHOGENESIS DURING DIFFERENTIATION	47/49	4.27×10^{-3}
NEUROGENESIS	90/93	5.65×10^{-3}
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE ACTIVITY	42/43	5.90×10^{-3}
VESICLE MEDIATED TRANSPORT	188/194	1.18×10^{-2}
GLUTAMATE RECEPTOR ACTIVITY	20/20	1.18×10^{-2}
CYTOSKELETAL PROTEIN BINDING	153/159	1.18×10^{-2}
JNK CASCADE	45/47	1.19×10^{-2}
STRESS ACTIVATED PROTEIN KINASE SIGNALING PATHWAY	47/49	1.30×10^{-2}
METABOTROPIC GLUTAMATEGABA B LIKE RECEPTOR ACTIVITY	10/10	1.6×10^{-2}
PHAGOCYTOSIS	16/17	1.83×10^{-2}
REGULATION OF AXONOGENESIS	10/10	1.83×10^{-2}
REGULATION OF ANATOMICAL STRUCTURE MORPHOGENESIS	24/25	1.83×10^{-2}
PERINUCLEAR REGION OF CYTOPLASM	51/54	1.88×10^{-2}
GLUTAMATE SIGNALING PATHWAY	16/17	2.13×10^{-2}
CORNIFIED ENVELOPE	12/13	2.32×10^{-2}
LIPOPROTEIN BINDING	18/18	2.46×10^{-2}
PDZ DOMAIN BINDING	14/14	2.54×10^{-2}

PROTEIN TYROSINE KINASE ACTIVITY	62/63	2.7×10^{-2}
3 5 CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY	13/13	2.7×10^{-2}
NEGATIVE REGULATION OF CELL PROLIFERATION	148/156	2.87×10^{-2}
PROTEIN OLIGOMERIZATION	35/40	2.87×10^{-2}
EXOPEPTIDASE ACTIVITY	29/32	2.87×10^{-2}
EXTRACELLULAR MATRIX	95/100	3.02×10^{-2}
CELL CELL ADHESION	83/86	3.02×10^{-2}
PROTEINACEOUS EXTRACELLULAR MATRIX	93/98	3.02×10^{-2}
MAINTENANCE OF PROTEIN LOCALIZATION	12/13	3.02×10^{-2}
MAINTENANCE OF CELLULAR PROTEIN LOCALIZATION	11/11	3.02×10^{-2}
TRANSMEMBRANE RECEPTOR PROTEIN PHOSPHATASE ACTIVITY	19/19	3.02×10^{-2}
CELL PROJECTION BIOGENESIS	23/25	3.04×10^{-2}
CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY	14/14	3.08×10^{-2}
CENTRAL NERVOUS SYSTEM DEVELOPMENT	110/123	3.08×10^{-2}
PROTEIN TYROSINE PHOSPHATASE ACTIVITY	52/53	3.08×10^{-2}
ACTIVE TRANSMEMBRANE TRANSPORTER ACTIVITY	113/122	4.10×10^{-2}

Since the inhibition of neurogenesis could be relevant to the hippocampal atrophy [319], we also examined if neurogenesis related pathways were enriched with hippocampal atrophy over two years with cognitively normal (CN) individuals with negative amyloid β pathology ([^{18}F]Florbetapir PET scan or CSF amyloid β measurement) (N=112) in ADNI. Seven pathways related to neurogenesis process were significantly enriched with hippocampal atrophy (FDR-corrected p-value < 0.05) in cognitively normal adults. These pathways were related cellular differentiation, cellular morphogenesis during development, neurite development, axonogenesis, cell cell adhesion and neuron development (Table 8).

Table 8. Molecular Signatures Database (MSigDB) GO Ontology pathways enriched with 2 years hippocampal atrophy.

Pathway (N=7)	**# of genes/set size**	**Corrected *p*-value**
CELLULAR MORPHOGENESIS DURING DIFFERENTIATION	33/49	8.2×10^{-3}
REGULATION OF ANATOMICAL STRUCTURE MORPHOGENESIS	18/25	8.2×10^{-3}
NEURITE DEVELOPMENT	34/53	8.2×10^{-3}
AXONOGENESIS	30/43	1.3×10^{-2}
CELL CELL ADHESION	54/86	1.3×10^{-2}
NEURON DEVELOPMENT	40/61	5.0×10^{-2}
TRANSMEMBRANE RECEPTOR PROTEIN PHOSPHATASE ACTIVITY	15/19	5.0×10^{-2}

Furthermore, we performed a targeted gene-based association analysis of hippocampal neurogenesis-pathway associated candidate genes using the ENIGMA GWAS summary [313]. The gene-based analysis showed that 4 genes (*MSRB3, TESC, DPP4, ACVR1*) were significantly associated with hippocampal volume (*corrected p-value* < 0.05, Table 9).

Table 9. Gene-based association results (*p*-value) of four significant genes for hippocampal volume using common variants (MAF ≥ 0.05), where *p*-values were calculated using GATES software.

Gene	Corrected *p*-value
MSRB3	3.4×10^{-6}
TESC	1.3×10^{-2}
DPP4	3.7×10^{-2}
ACVR1	4.8×10^{-2}

Since hippocampal volume is correlated with memory performance, we performed a gene based association analysis of these four genes (with 682 SNPs) with composite memory scores. The gene-based association analysis showed that *TESC* gene is significantly associated with composite memory score after Bonferroni correction in ADNI dataset (*p*-value = 5.7×10^{-3}, Table 10).

Table 10. Gene-based association results (*p*-values) of four genes for composite memory scores using common variants (MAF ≥ 0.05), where empirical p-values were calculated using 20,000 permutations in PLINK.

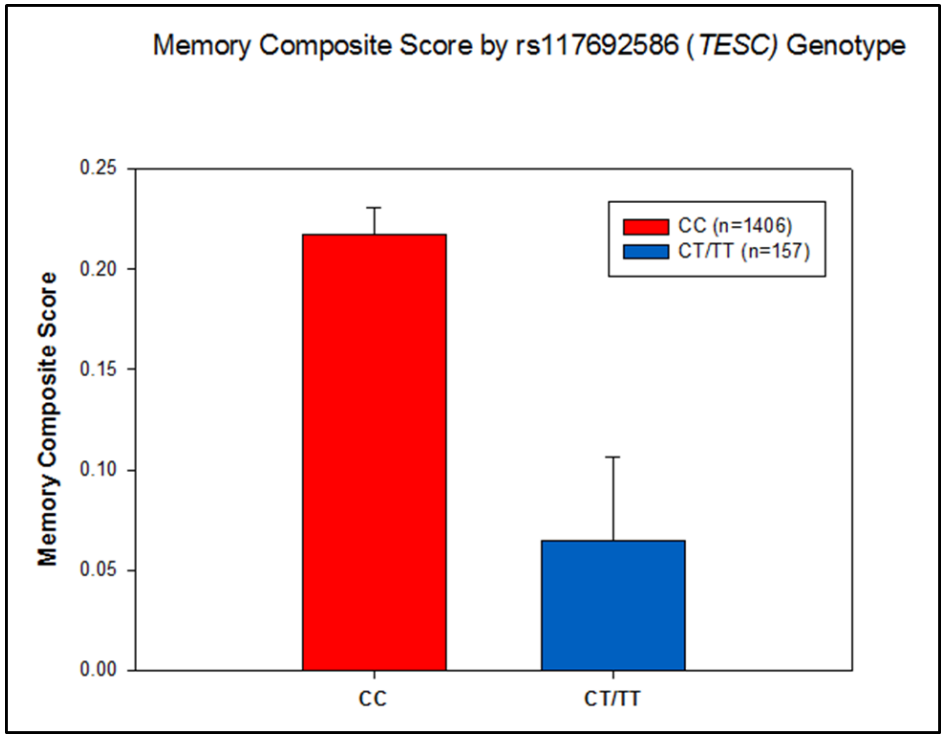
Gene	ADNI (N=1,563)	
	***p*-value**	**Significant independent SNP**
MSRB3	0.26	rs7294862|rs6581626
TESC	5.7×10^{-3}	rs117692586|rs12302906
DPP4	0.26	rs35635667|rs3788979
ACVR1	1	N/A

One SNP (rs117692586) upstream of TESC was most associated with composite memory score and Rey Auditory Verbal Learning Test (RAVLT) scores (*p*-value = $4.3x10^{-4}$ and $6.8x10^{-4}$, respectively; Table 11). rs117692586-T is associated with poorer memory performance (Figure 14).

Table 11. SNP-based analysis results for memory scores in ADNI.

rs117692586 (*TESC*)	**ADNI (N=1,563)**	
	β	***p*-value**
Memory Composite Score	-0.1487	4.3×10^{-4}
RAVLT-Total	-2.486	6.8×10^{-4}

Figure 14. *TESC* (rs117692586-T) is significantly associated with poorer composite memory score: Presence of at least one copy of the minor allele (T) of rs117692586 was significantly associated with poorer memory score (*p*-value ≤ 0.001)



Finally, we used gene expression data from Gene Expression Omnibus (GEO) to evaluate if the neurogenesis pathways were enriched for CA1 and CA3 regions of the hippocampus in normal brain tissues. A weighted gene correlation network analysis (WGCNA) yielded 20 modules of co-expressed genes in these data. These 20 modules were tested for enrichment of neurogenesis pathways. Six modules were found to be significantly enriched with neurogenesis pathways after correcting for multiple testing. The six significantly enriched modules are all related to neurogenesis pathways such as neuronal proliferation and differentiation as well as cellular process (Table 12).

Table 12: GSEA pathway analysis result showed that six pathways are related to neurogenesis after Bonferroni correction.

Neurogenesis Pathway	Corrected *p*-value
Module 1	5.2×10^{-84}
Module 2	1.0×10^{-21}
Module 3	3.8×10^{-17}
Module 4	4.4×10^{-11}
Module 5	3.0×10^{-7}
Module 6	7.6×10^{-4}

D. Discussion

Using a large-scale genome-wide association study (GWAS) summary statistics associated with hippocampal volume in the discovery sample of 13,163 subjects of European ancestry from the ENIGMA consortium, we performed a genome-wide gene-set enrichment analysis and identified 44 GO Ontology pathways with enrichment for hippocampal volume. These enriched pathways showed that hippocampal volume is related to neurogenesis and cellular processes including neuronal cell proliferation, differentiation and maturation as well as cell adhesion. In addition, the evidence for the enrichment of neurogenesis related pathways in the hippocampus was obtained by performing WGCNA on gene expression data from 32 healthy hippocampal brain regions (CA1 and CA2).

At a cellular level, the enriched pathways showed significant relationships between neurogenesis and hippocampal volume/atrophy. Since several studies showed the neurogenesis occurs in the dentate gyrus of the hippocampus [318], it is not surprising hippocampal volume is significantly related to neurogenesis-related pathways. In particular, we observed significant enrichment of pathways related to cell proliferation, neuron differentiation, neuron generation, neurite development, neuronal development, cell recognition, neurogenesis and axonogenesis. The neural progenitor cells in the subgranular zone of the hippocampus differentiate and incorporate into neural network circuitry as mature neurons in the adult human brain [3]. In addition these newborn neurons

enhance the formation of the hippocampus during neurogenesis and many genes involve these processes [12, 258]. Moreover, our pathway enrichment analysis for hippocampal volume revealed that hippocampal volume is significantly related to signal transductions such as glutamate signaling, protein kinase signaling and JNK cascade. We identified five neurogenesis related pathways in our previous review paper and signal transduction pathways were one of the important pathways in adult neurogenesis process [318]. During adult neurogenesis, functional granule cells in the dentate gyrus of the adult hippocampus release glutamate, project to target cells in CA3 region, and receive glutamatergic and γ-aminobutyric acid GABAergic inputs to control their spiking activity in neuronal network which supports the formation of the memory and learning [43, 320]. Phosphoinositide 3-kinase (PI3K)/protein kinase pathways enhance neuronal differentiation and inhibit the apoptosis of the progenitor cells [50, 321]. In addition, studies showed that JNK1 in JNK cascade plays a role in neuronal differentiation, neuronal and axonal maturation [322-324]. Also, it has been showed that absence of JNK1 enhances hippocampal neurogenesis and reduces anxiety-related phenotype in mice model [322].

Pathways related to enzyme activities such as protein tyrosine kinase, protein tyrosine phosphatase and 3’5’ cyclic nucleotide phosphodiesterase were enriched for hippocampal volume. Studies showed that three subfamilies, Tyro3, Axl and Mertk (TAM), of receptor protein tyrosine kinases plays a crucial role in adult neurogenesis. TAM receptors have different impacts on proliferation and

differentiation of neural stem cells to immature neurons by controlling overproduction of pro-inflammatory cytokines [325]. Protein tyrosine phosphatase controls the differentiation of the neural stem cells during neurogenesis [326].

Our enrichment analysis results suggest the impact of neurogenesis-related genetic variation on hippocampal structure. We examined genes that were highly-represented across the neurogenesis-related pathways and showed that two genes, tescalcin (*TESC*) and activin receptor 1 (*ACVR1*), were significantly related to hippocampal volume. In addition, in ADNI, *TESC* was significantly associated with memory performance. Previous structural imaging studies showed the *TESC*-regulating polymorphism is significantly associated with hippocampal volume and hippocampal gray matter structure [308, 327]. *TESC* cooperates with the plasma membrane Na(+)/H(+) exchanger NHE1 which catalyzes electroneutral influx of extracellular Na(+) and efflux of intracellular H(+) and establishes intracellular pH level as well as cellular hemostasis [328, 329]. *TESC* was expressed in the various tissues such as heart and brain and plays an important role during embryonic development [329]. *TESC* plays a crucial role in controlling cell proliferation and differentiation for the formation of the hippocampal structure during brain development [327]. In addition, *ACVR1*, member of a protein family called as bone morphogenetic protein (BMP) type I receptors, regulates dentate gyrus stem cells during neurogenesis [330]. In addition, gene co-expression results showed that *TESC* and *ACVR1* were co-expressed together in the neurogenesis related module.

There are some limitations in the present report: 1) in this study we used GO Ontology pathways from MSigDB. For pathway enrichment analysis design, there is no gold standard. There are many tools and strategies for pathway enrichment analysis and alternate databases and algorithms for pathway enrichment analysis can affect the analytic results [192, 331]; 2) another limitation is the lack of replication in the gene-set enrichment analysis. We could not find any large data sets with imaging and genetics data to replicate our results. Further study would be warranted using independent large data sets to replicate our pathway enrichment.

In summary, our results provide the evidence that neurogenesis-related pathways are enriched for hippocampal volume, suggesting that hippocampal volume may serve as a potential phenotype for the investigation of neurogenesis.

IV. Genome-wide association analysis of AD pathology-related phenotypes identifies enrichment of neurogenesis-related pathways

A. Introduction:

Alzheimer's disease (AD) is the most common form of dementia and characterized by two hallmarks, amyloid-β accumulation and neurofibrillary tangles. Tau, a microtubule associated protein (MAP), promotes microtubules assembly and stabilization in mature neuron [332]. Tau plays a crucial role in brain development. Absence of tau inhibits neuronal migration, normal morphology of radial cells, and synaptic maturation of newborn hippocampal granule neurons [333, 334] . The pathological overexpression of tau protein causes tau-related neurological diseases known as tauopathies [335]. AD is the most common form of tauopathy and is associated with hyperphosphorylation of tau protein leading to the formation of neurofibrillary tangles in the brain [333]. The protein tau is the main component of neurofibrillary tangles (NFT) leading to neuronal death in the hippocampus of AD patients. In contrast, tau plays a crucial role for neurogenesis and synaptic maturation of newborn hippocampal granule neurons, and the absence of tau can result in retarded neurogenesis and neuronal differentiation [334]. Total tau (t-tau), and tau phosphorylated at the threonine 181 (p-tau181p), measured in cerebrospinal fluid (CSF) samples are used as diagnostic biomarkers for AD patients.

In addition to tau pathology in AD, amyloid-β deposition in the brain is the other crucial hallmark for AD. Amyloid-β 1-42 peptide (Aβ1-42) in CSF is an established diagnostic biomarker for AD [268]. Amyloid precursor protein (APP) plays a significant role for new born neuron survival and maturation. Even though Aβ level decreases in the hAPP mouse model, overexpression of the human APP leads to impairment of the adult neurogenesis due to the reduction of the neural stem/precursor cells [336]. However, Aβ accumulation in the brain impairs neurogenesis in the subgranular layer of the dentate gyrus [337]. On the other hand, since impaired presynaptic GABAergic input leads to decrease in the maturation of the newborn neurons and GABAergic signaling normalizes neurogenesis in *APOE4* knock-in mice, the imbalance between GABAergic and glutamatergic neurotransmission has a negative effect on neurogenesis [338, 339].

While amyloid-β accumulation has a negative effect on adult neurogenesis, amyloid-β precursor protein (APP) plays an important role in neuronal survival and maturation. Interestingly, neurogenesis occurs in the dentate gyrus of the hippocampus during adulthood that is also a prominent site of NFT accumulation. To test whether tau tangles and amyloid-β deposition are significantly associated with neurogenesis-related pathways, we performed gene-set enrichment analysis on a genome-wide association study (GWAS) from the Alzheimer's Disease Neuroimaging Initiative (ADNI), which uniquely has GWAS data sets on the same participants as well as multi-modal structural and functional neuroimaging (MRI,

PET) data as well as CSF data. We used [^{18}F]Flortaucipir PET scans for the measurement of the tau pathology and [^{18}F]Florbetapir PET scans for the global cortical amyloid-β deposition as quantitative traits to investigate whether adult hippocampal neurogenesis-related genes and pathways are significantly associated with AD pathology-related endophenotypes. In addition, set –based pathway enrichment analysis was performed from three CSF biomarkers (Aβ1-42, t-tau, and p-tau181p) in ADNI.

B. Materials and Method

Alzheimer's Disease Neuroimaging Initiative (ADNI)

Data used in this study were obtained from the ADNI database (adni.loni.usc.edu). ADNI was launched in 2003 by a public-private partnership and initiated by Michael W. Weiner. Participants were recruited from more than 50 sites across the United States and Canada. ADNI participants consist of cognitively normal older individuals (CN), significant memory concerns (SMC), mild cognitive impairment (MCI) and AD aged 55-90 (http://www.adni-info.org/). ADNI dataset include structural MRI and PET scans, longitudinal CSF markers, and performance on neuropsychological and clinical assessments. In addition, All ADNI samples have *APOE* and genome-wide genotyping data.In this study, we used Amyloid-β 1-42 peptide ($A\beta_{1-42}$), total tau, and tau phosphorylated at the threonine 181 (p-tau181p) measured in CSF samples and [^{18}F]AV-1451

Flortaucipir PET. 1,154 (AD = 220, MCI = 597, SMC = 84, CN = 253) non-Hispanic Caucasian participants have CSF total tau and p-tau levels. 975 of the participants (AD = 213, MCI = 517, SMC = 60, CN = 185) have CSF amyloid beta level. 800 participants (AD = 131, MCI = 422, SMC = 90, CN = 157) have [^{18}F] Florbetapir PET scans and 99 of the ADNI participants (AD = 10, MCI = 44, SMC = 11, CN = 34) have [^{18}F]AV-1451 Flortaucipir PET scans.

CSF measurements and quality control

All data were downloaded from the LONI website as “UPENN CSF Biomarkers Elecsys”. The complete descriptions of the collection and process protocols are provided in the ADNI procedural manual at www.adni-info.org.

[^{18}F]Florbetapir and [^{18}F]Flortaucipir PET scans

^{18}F-labeled Aβ tracer florbetapir was used for PET imaging from the ADNIGO/2 participants. First, 370 MBq florbetapir was injected to participants. After 50 minutes, cranial PET scans were initiated. Florbetapir PET scans and processing were provided as online (http://adni.loni.usc.edu/wp-content/uploads/2010/05/ADNI2_PET_Tech_Manual_0142011.pdf; http://adni.loni.usc.edu/methods/pet-analysis/pre-processing/). Briefly, all of the florbetapir scans were examined for quality control. Images were averaged and aligned into a standard 160 × 160 × 96 voxel image grid with 1.5 mm cubic

voxels, and smoothed to a uniform isotropic resolution of 8 mm full width at half maximum and normalized to an atlas-based cerebellar reference region. These scans were downloaded ADNI website (http://adni.loni.ucla.edu/) for 802 participants. In the Statistical Parametric Mapping 8 (SPM8) software (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/), MarsBaR toolbox were used to extract mean regional SUVR values for the frontal, parietal, temporal, limbic, and occipital lobes that represent Aβ accumulation in the global cortical area. We used a global cortical measurement as a quantitative phenotype for GWAS. After quality control of data, 800 individuals were used for analysis.

Pre-processed 80-100 minute [^{18}F]Flortaucipir scans were downloaded from LONI and processed using standard techniques. Prior to download, [^{18}F]Flortaucipir images were averaged, aligned to a standard space, re-sampled to a standard image and voxel size, smoothed to a uniform resolution and intensity normalized as previously described [340]. After downloading, scans were co-registered to the closest time point structural MRI, normalized to Montreal Neurologic Institute (MNI) space, and intensity normalized to mean cerebellar crus uptake to create SUVR images. Regional [^{18}F]Flortaucipir from the global cortex was extracted from subject-specific regions of interest generated from Freesurfer version 5.1. After quality control of the data, 99 individuals were used for analysis.

Genotyping Data and Quality Control

Genomic DNA was extracted using the QIAamp DNA Blood Maxi Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol using 7 mL of blood in EDTA-containing Vacutainer tubes from all participants. 50 ng of genomic DNA from each sample was examined for quality control on a 1% Tris-acetate-EDTA agarose gel to check for degradation. Degraded DNA samples were excluded from further analysis [268]. ADNI samples were genotyped according to manufacturer's protocol (Illumina, Inc., San Diego, CA) using the Human610-Quad BeadChip (for subjects initially enrolled during ADNI-1) or the Human OmniExpress BeadChip (for subjects initially enrolled in ADNI-GO or ADNI-2). In addition, two SNPs that characterize APOE ε2/ε3/ε4 allele status (rs429358 and rs7412) were genotyped using DNA extracted by Cogenics from a 3 mL aliquot of EDTA blood [341]. These SNPs were genotyped by PCR amplification using HhaI restriction enzyme digestion and Metaphor Gel [342].

SNP quality control procedures of GWAS data such as SNP call rate < 95%, Hardy-Weinberg equilibrium test $p < 1 \times 10^{-6}$, and frequency filtering (MAF ≥ 5%) were performed using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/), version 1.07 [190, 285-287]. For sample quality procedures, due to the limitation of the population stratification, only non-Hispanic Caucasian participants were selected for this analysis by genetic clustering with CEU (Utah residents with Northern and Western European ancestry from the CEPH collection) and TSI

(Tuscans in Italy) populations using HapMap 3 genotype data and multidimensional scaling (MDS) analysis after performing standard quality control (QC) procedures for genetic markers and participants [190, 343].

Prior to imputation, all genotyped markers were verified using NCBI build 37 coordinates reference panel genome. Monomorphic variants from reference genome were excluded. Imputation of un-genotyped SNPs was performed using MaCH (Markov Chain Haplotyping) and minimac software based on the 1000 Genomes Project as a reference panel [288]. Following imputation, SNPs with less correlation ($r^2 < 0.5$) between imputed and assayed genotypes were removed [308]. The independently-imputed data sets were then merged to generate a common set of more than 10 million SNPs for the full ADNI sample. Following quality control (SNP call rate < 95%, Hardy-Weinberg $p < 1 \times 10^{-6}$) and frequency filtering (MAF < 5%), 6,112,217 SNPs were included in the GWAS.

Genome-wide association analysis

To generate input data for gene-set enrichment analysis, GWAS for AD-related phenotypes such as CSF biomarkers (Aβ1-42, t-tau and p-tau181) and PET measurements (tau and amyloid beta deposition) was performed using additive model with a linear association analysis for quantitative traits in Plink. Age, sex and diagnosis were used as covariates. In addition, *APOE* ε4 allele status (presence vs. absence) was also used as a covariate in the GWAS to account for

the largest known genetic effect on AD-related phenotypes. A p-value for each SNP was generated from GWAS using Plink.

Gene-set enrichment analysis

Pathway annotations were downloaded from Molecular Signatures Database version 5.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp/). This annotation data comprised a collection of GO Ontology. GO gene sets comprise of publically available 1,454 pathways. 825 gene sets belong to GO biological process, 233 gene sets belong to GO cellular components, 396 gene sets belong to GO molecular function. The GSA-SNP software [310] was used for a gene-set enrichment analysis. GSA-SNP is a JAVA-based stand-alone software and uses the p-values of each marker from genome-wide association results to test if a pathway-phenotype association is significantly different from all other pathway-phenotype association. In GSA-SNP, '–log' on the kth best SNP-level p-value for each gene is taken. According to author suggestion toavoid spurious conservative predictions, the second best SNP (k=2) was chosen for each gene as a default option instead of the best SNP. Each pathway (gene sets) was assessed by z-statistics for the identification of the enriched pathways with phenotype [311]. Analysis of these pathways was restricted to those containing between 10 and 200 genes. The FDR (Benjamini-Hochberg) method was applied for multiple comparison correction to the p-values generated by the enrichment algorithm [312]. We identified pathways as significantly enriched if FDR-corrected

p-value < 0.05 for each pathway.

Gene-based association analysis

The KGG (Knowledge-based mining system for Genome-wide Genetic studies) software was used to perform a genome-wide gene-based analysis using GWAS p-values for AD pathology-related endophenotypes such as CSF biomarkers (Aβ1-42, t-tau and p-tau181) and tau and amyloid beta deposition measured from PET scans. Hybrid set-based test, the combination of the set of genome wide association signals from all SNPs was used for gene-based association analysis [313, 314]. KGG procedures can be found in chapter III in detail.

C. Results

GWAS was performed from five different AD-related phenotypes: CSF biomarkers ($A\beta_{1-42}$, t-tau and p-tau181) and tau and amyloid beta deposition measured from PET scans. The p-values from the GWAS results were used as input for gene-set enrichment pathway analysis. We identified 47 pathways related to CSF t-tau levels, 54 pathways related to CSF p-tau levels and 43 pathways related to tau PET. 45 of the pathways were common in t-tau and p-tau levels in CSF. After we examined the enriched pathways deeply for tau pathology, we discovered that all of the 45 pathways are related to neurogenesis process, generation of neurons, axonogenesis, cell-cell adhesion, glutamate

transmission, signal transduction, cellular process and synaptic transduction (Table 13). 12 out of these 45 pathways were also common in enriched pathways for tau accumulation in the global cortical region in the brain (Table 13). We classified pathways associated with CSF t-tau and p-tau levels as primary neurogenesis pathways (N=25) and secondary (helper) pathways (N=20).

Table 13: Molecular Signatures Database (MSigDB) GO Ontology pathways enriched for CSF total tau and p-tau levels and tau PET.

Pathway N=45	Corrected *p*-value (CSF t-tau)	Corrected *p*-value (CSF *p*-tau)	Corrected *p*-value (tau PET)
CDC42 PROTEIN SIGNAL TRANSDUCTION	$< 10^{-11}$	$< 10^{-12}$	N/A
REGULATION OF AXONOGENESIS	2.27×10^{-11}	$< 10^{-12}$	2.4×10^{-2}
LEARNING AND OR MEMORY	2.14×10^{-8}	$< 10^{-12}$	N/A
REGULATION OF SYNAPSE STRUCTURE AND ACTIVITY	2.41×10^{-8}	$< 10^{-12}$	N/A
REGULATION OF NEUROGENESIS	1.06×10^{-7}	1.79×10^{-12}	1.5×10^{-2}
PROTEIN TETRAMERIZATION	1.59×10^{-6}	1.39×10^{-10}	N/A
HOMOPHILIC CELL ADHESION	1.59×10^{-6}	5.19×10^{-10}	N/A
LIPID HOMEOSTASIS	3.73×10^{-6}	5.90×10^{-9}	N/A
REGULATION OF ANATOMICAL STRUCTURE MORPHOGENESIS	4.03×10^{-6}	4.46×10^{-10}	N/A
NEURON DIFFERENTIATION	8.31×10^{-6}	1.47×10^{-7}	1.6×10^{-3}
NEURON DEVELOPMENT	8.31×10^{-6}	5.40×10^{-8}	1.2×10^{-2}
NEURITE DEVELOPMENT	8.31×10^{-6}	4.86×10^{-8}	4.5×10^{-3}
SYNAPTIC TRANSMISSION	2.73×10^{-5}	4.79×10^{-7}	N/A
PROTEIN DIMERIZATION ACTIVITY	2.73×10^{-5}	6.29×10^{-9}	N/A
CELL CELL ADHESION	2.83×10^{-5}	2.52×10^{-7}	N/A
CELLULAR MORPHOGENESIS DURING DIFFERENTIATION	2.83×10^{-5}	7.75×10^{-7}	6.6×10^{-3}
RHO PROTEIN SIGNAL TRANSDUCTION	2.93×10^{-5}	3.55×10^{-7}	N/A
GENERATION OF NEURONS	3.14×10^{-5}	1.41×10^{-6}	5.0×10^{-3}
AXONOGENESIS	6.17×10^{-5}	1.03×10^{-6}	1.16×10^{-2}
PROTEIN OLIGOMERIZATION	1.59×10^{-4}	6.72×10^{-6}	N/A
PROTEIN HOMODIMERIZATION ACTIVITY	1.84×10^{-4}	3.55×10^{-7}	N/A
NEUROGENESIS	1.85×10^{-4}	2.02×10^{-5}	4.5×10^{-3}
TRANSMISSION OF NERVE IMPULSE	2.15×10^{-4}	1.03×10^{-5}	N/A
RAS PROTEIN SIGNAL TRANSDUCTION	1.39×10^{-3}	1.88×10^{-4}	N/A

PROTEIN HETERODIMERIZATION ACTIVITY	1.40×10^{-3}	9.43×10^{-6}	N/A
LIPID TRANSPORT	3.05×10^{-3}	7.59×10^{-5}	N/A
GLUTAMATE RECEPTOR ACTIVITY	3.55×10^{-3}	2.74×10^{-3}	N/A
TRANSMEMBRANE RECEPTOR PROTEIN PHOSPHATASE ACTIVITY	3.76×10^{-3}	1.05×10^{-2}	5.9×10^{-4}
SMALL GTPASE MEDIATED SIGNAL TRANSDUCTION	4.07×10^{-3}	5.18×10^{-4}	N/A
POSITIVE REGULATION OF TRANSCRIPTION	5.31×10^{-3}	3.40×10^{-2}	N/A
CYTOSKELETAL PROTEIN BINDING	5.31×10^{-3}	2.60×10^{-4}	N/A
POSITIVE REGULATION OF TRANSCRIPTION DNA DEPENDENT	8.63×10^{-3}	3.40×10^{-2}	N/A
POSITIVE REGULATION OF RNA METABOLIC PROCESS	1.16×10^{-2}	4.28×10^{-2}	N/A
POSITIVE REGULATION OF NUCLEOBASE NUCLEOSIDE NUCLEOTIDE AND NUCLEIC ACID METABOLIC PROCESS	1.23×10^{-2}	4.75×10^{-2}	N/A
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE ACTIVITY	1.52×10^{-2}	1.09×10^{-2}	4.6×10^{-4}
PHOSPHOLIPID BINDING	1.88×10^{-2}	1.94×10^{-4}	N/A
3 5 CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY	2.31×10^{-2}	6.94×10^{-3}	9.97×10^{-3}
LIPID TRANSPORTER ACTIVITY	2.31×10^{-2}	4.60×10^{-3}	N/A
REGULATION OF RAS GTPASE ACTIVITY	2.64×10^{-2}	1.58×10^{-2}	N/A
NUCLEAR MATRIX	3.38×10^{-2}	1.58×10^{-2}	N/A
REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS	3.80×10^{-2}	8.66×10^{-3}	N/A
DEPHOSPHORYLATION	3.90×10^{-2}	4.30×10^{-2}	N/A
CENTRAL NERVOUS SYSTEM DEVELOPMENT	4.02×10^{-2}	4.28×10^{-2}	N/A
PROTEIN AMINO ACID DEPHOSPHORYLATION	4.0×10^{-2}	3.40×10^{-2}	N/A
PROTEIN TYROSINE PHOSPHATASE ACTIVITY	4.0×10^{-2}	4.75×10^{-2}	N/A

Primary pathways are related to cell differentiation, proliferation, survival, cellular morphogenesis, synaptic transduction, axonogenesis, neuronal development, regulation of cellular organization, glutamate signaling, cell-cell adhesion, lipid homeostasis, and memory and learning. Secondary neurogenesis pathways are related to enzyme activity, protein dimerization, phospholipid binding, lipoprotein binding, and extracellular matrix.

Next, 1,251 genes highly-represented across 45 enriched pathways common with CSF t-tau and p-tau levels were identified. Gene-based association analysis using KGG showed that three genes (*APOE, PVRL2* and *APOC4*) are significantly associated with CSF t-tau and p-tau levels after multiple comparison adjustments (Table 14).

Table 14. Gene-based association analysis of genes linked to the enriched neurogenesis-related pathways. Three genes are associated with CSF t-tau levels and p-tau levels after multiple comparison adjustments.

Gene N=1,251	Corrected *p*-value (CSF t-tau)	Corrected *p*-value (CSF p-tau)
APOE	1.16×10^{-10}	5.27×10^{-15}
PVRL2	4.19×10^{-7}	2.51×10^{-10}
APOC4	3.50×10^{-3}	3.92×10^{-5}

When we used APOE ε4 allele status as a covariate, only two pathways were related to CSF t-tau levels and eleven pathways were related to CSF p-tau in the gene-set enrichment analysis. After adjustment of APOE ε4 allele status, the most of the neurogenesis pathways were not enriched for CSF t-tau levels.

We performed pathway enrichment analysis using GWAS results from CSF $A\beta_{1-42}$ and amyloid beta deposition (measured from PET) in the cortical regions in the brain. Gene-set enrichment analysis results showed that 40 pathways were enriched with CSF $A\beta_{1-42}$, while 54 pathways enriched for amyloid β deposition. 36 common pathways enriched for CSF $A\beta_{1-42}$ and [^{18}F]Florbetapir PET measurement (Table 15).

Table 15. Molecular Signatures Database (MSigDB) GO Ontology pathways enriched for amyloid-β in CSF and Amyloid PET.

Pathway N=36	Corrected *p*-value (CSF Aβ)	Corrected *p*-value (Amyloid PET)
REGULATION OF AXONOGENESIS	$< x 10^{-13}$	$< x 10^{-13}$
REGULATION OF SYNAPSE STRUCTURE AND ACTIVITY	$< x 10^{-13}$	$< x 10^{-13}$
CDC42 PROTEIN SIGNAL TRANSDUCTION	$< x 10^{-13}$	$< x 10^{-13}$
REGULATION OF NEUROGENESIS	$< x 10^{-13}$	$< x10^{-13}$
LEARNING AND OR MEMORY	$< x 10^{-13}$	$< x 10^{-13}$
PROTEIN TETRAMERIZATION	$< x 10^{-13}$	$< x 10^{-13}$
HOMOPHILIC CELL ADHESION	$< x 10^{-13}$	$< x 10^{-13}$
LIPID HOMEOSTASIS	$< x 10^{-13}$	$< x 10^{-13}$
REGULATION OF ANATOMICAL STRUCTURE MORPHOGENESIS	$< x 10^{-13}$	$< x 10^{-13}$
AXONOGENESIS	$< x 10^{-13}$	$1.84 x 10^{-10}$
NEURITE DEVELOPMENT	$1.52 x 10^{-13}$	$3.08 x 10^{-8}$
CELLULAR MORPHOGENESIS DURING DIFFERENTIATION	$3.95 x 10^{-13}$	$3.52 x 10^{-9}$
NEURON DIFFERENTIATION	$1.89 x 10^{-12}$	$6.88 x 10^{-7}$
NEURON DEVELOPMENT	$2.07 x 10^{-12}$	$8.41 x 10^{-7}$
PROTEIN HOMODIMERIZATION ACTIVITY	$6.33 x 10^{-12}$	$3.21 x 10^{-12}$
NEUROGENESIS	$1.68 x 10^{-11}$	$8.13 x 10^{-5}$
GENERATION OF NEURONS	$1.80 x 10^{-12}$	$1.94 x 10^{-6}$
PROTEIN OLIGOMERIZATION	$5.84 x 10^{-12}$	$1.36 x 10^{-12}$
LIPID TRANSPORT	$5.84 x 10^{-12}$	$3.46 x 10^{-11}$
PROTEIN DIMERIZATION ACTIVITY	$2.53 x 10^{-10}$	$1.28 x 10^{-10}$
RHO PROTEIN SIGNAL TRANSDUCTION	$4.25 x 10^{-10}$	$6.34 x 10^{-10}$
PHOSPHOLIPID BINDING	$5.81 x 10^{-9}$	$9.48 x 10^{-13}$
RAS PROTEIN SIGNAL TRANSDUCTION	$1.28 x 10^{-8}$	$6.85 x 10^{-5}$
CELL CELL ADHESION	$1.88 x 10^{-7}$	$3.22 x 10^{-10}$
SYNAPTIC TRANSMISSION	$2.66 x 10^{-7}$	$6.19 x 10^{-6}$
PROTEIN HETERODIMERIZATION ACTIVITY	$8.68 x 10^{-7}$	$4.53 x 10^{-6}$
TRANSMISSION OF NERVE IMPULSE	$1.15 x 10^{-6}$	$3.66 x 10^{-6}$
CYTOSKELETAL PROTEIN BINDING	$5.89 x 10^{-6}$	$3.10 x 10^{-3}$

SMALL GTPASE MEDIATED SIGNAL TRANSDUCTION	1.06×10^{-5}	1.67×10^{-4}
TRANSMEMBRANE RECEPTOR PROTEIN PHOSPHATASE ACTIVITY	5.43×10^{-5}	2.47×10^{-4}
PROTEIN COMPLEX ASSEMBLY	3.37×10^{-4}	3.23×10^{-4}
BEHAVIOR	3.57×10^{-4}	3.00×10^{-3}
LIPID BINDING	3.92×10^{-4}	1.11×10^{-5}
REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS	$1X10^{-3}$	3.32×10^{-2}
CYTOSKELETON ORGANIZATION AND BIOGENESIS	1.7×10^{-2}	2.51×10^{-2}
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE SIGNALING PATHWAY	3.9×10^{-2}	4.49×10^{-3}

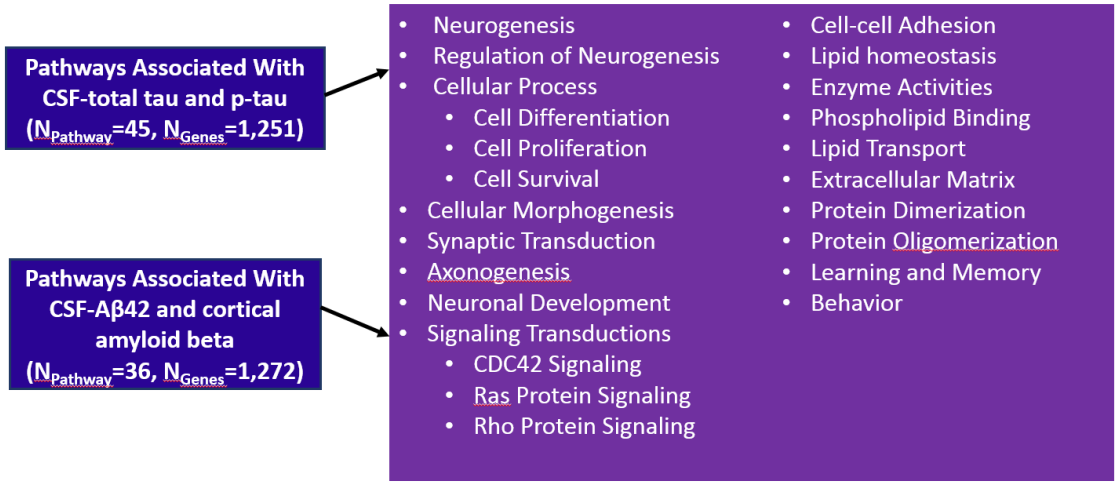
All the pathways were related to neurogenesis process, cellular process, neuronal development, neuronal differentiation, cell-cell adhesion, ras signaling pathway, rho signaling pathway and axonogenesis. Interestingly, all these 36 common pathways are primarily associated with neurogenesis processes or helpers playing a role in cellular processing during neurogenesis.1,272 genes were identified from 36 common pathways. Two genes, *APOE* and *PVLR2*, were significantly associated with CSF $A\beta_{1-42}$ and amyloid-β accumulation (PET) in the brain after multiple comparison adjustments (Table 16).

Table 16. Gene-based association analysis of genes linked to the enriched neurogenesis-related pathways

Gene N=1,272	Corrected *p*-value (CSF Aβ)	Corrected *p*-value (Amyloid PET)
APOE	6.07×10^{-27}	1.13×10^{-29}
PVRL2	1.07×10^{-16}	3.48×10^{-21}

36 pathways were common related to CSF t-tau, CSF ptau, CSF $A\beta_{1-42}$ and amyloid-β accumulation in the cortical region (Figure 15). Interestingly, common pathways were related to neuronal process, neurogenesis, cell-cell adhesion, signaling transduction, lipid homeostasis as well as learning and memory. Gene-based association analysis identified two novel genes, APOE and PVLR2, significantly associated with AD pathology.

Figure 15. Conceptual classification of pathways enriched in the AD pathology-related endophenotypes.



D. Discussion:

In this study, using AD pathology-related endophenotypes measured by CSF and PET scans as phenotypes in the sample of CN, SMC, MCI and AD patients in ADNI, we performed genome-wide pathway analysis and identified GO Ontology pathways with enrichment for AD-related phenotypes.

Tau protein enhances microtubule assembly and stabilization, and plays a crucial role in the establishment of the neuronal polarity and migration of the precursor neuronal cells in embryonic stage in axonal transport and in intracellular trafficking [333, 344-347]. The accumulation of the hyperphosphorylated microtubule-associated protein tau (MAPT) leads to tauopathies such as AD. Phosphorylation of the tau changes developmentally, and it is higher in fetal stage and reduces by aging [26, 348, 349]. In addition, p-tau is co-expressed temporally and spatially with DCX, a marker for neural precursor cells and immature cells, and NeuroD, differentiation marker, in the dentate gyrus of the hippocampus during postnatal development [293, 350, 351]. Adult neurons express tau with three microtubule-binding domains (tau-3R) and can be a new marker to identify new axons in SGZ [352, 353]. Tau-3R enables DCX expressed cells migration from the SGZ to upper layers and send axons into the CA3 regions via dynamic microtubule network [24]. These results may suggest that there is a significant association between p-tau and adult neurogenesis.

Spatial/temporal control of tau phosphorylation can be compensatory mechanism for the neuronal loss in neurological disorders, including AD. However, tau knockout mice have the impairment in the morphological and synaptic maturation of newborn hippocampal granule neurons under basal conditions [334]. Genetically modified human tau expressed mice model showed that there is the deduction of the adult neurogenesis in the brain by as early as 2 months of age because of the reduction of the proliferation of precursor cells, before the development of tau pathology. In addition, the altered phosphorylation of the tau protein with neurogenic precursors was found in mice with tauopathy during neurogenesis [293].

On the other hand, when adjusted by the *APOE* ε4 allele status for gene-set enrichment pathway analysis, most of the neurogenesis related pathways were not among enriched pathways for CSF tau levels. Neural stem/progenitor cells express APOE in the adult brain. APOE knockout mice showed approximately 60% lower adult neurogenesis in the dentate gyrus of the hippocampus than the wild type mice [339]. However, the maturation and the dendritic development of the newborn neurons in the hippocampus were significantly inhibited in the apoE4 knock-in mice because of the GABAergic interneuron dysfunction in the dentate gyrus while astrogenesis was stimulated in APOE4 knock in mice in the hippocampus. Under the stress, neurons expresses more APOE in the APOE4 knock in mice than wild type and generates neurotoxic fragments via proteolytic cleavage. These neurotoxic fragments may cause the detrimental effect on

GABAergic interneurons and this effect can be reduced by lowering the endogenous tau [354-356]. Interestingly, These APOE4 neurotoxic fragments can increase tau phosphorylation especially in the GABAergic neurons, one of the hallmarks of AD [339, 357]. Our result confirmed the relationships among *APOE* genotype status, amyloid-β load, and neurogenesis.

APP, PS1 and PS2 play crucial roles in neural stem cells proliferation, differentiation, maturation and survival while Aβ role is still unclear in adult neurogenesis. However, some studies showed that the reduction of the Aβ levels and amyloid deposition enhances adult neurogenesis in mice model [210, 358, 359].

As a result, the present results provide several new insights into key functional pathways associated with AD pathology-related phenotypes such as tau and amyloid-β in older adults with MCI or AD. Our gene-set enrichment analysis results highlight several candidates for further analysis of SNPs, genes, and gene sets underlying AD pathology and neurogenesis.

V. Conclusion and Future Directions

In this project, we evaluated the potential modulators of adult neurogenesis and their roles in neurodegenerative diseases. We highlighted five important modulators of neurogenesis including signaling transduction pathways, the vascular and immune systems, metabolic factors, and epigenetic regulation using a systems biology approach. The alteration in these modulators during adult neurogenesis may be related to the development of neurodegenerative diseases such as Alzheimer's disease. A more complete understanding of the role and function of each modulator in regulating NSC fate and integration of neurons in the SGZ and olfactory bulb may provide crucial insights leading to new therapies for neurological diseases in humans.

Disruption of adult neurogenesis processes contributes to neurodegenerative diseases including AD and many of the molecular players in AD are also modulators of adult neurogenesis. However, the genetic mechanisms underlying adult neurogenesis in AD have been under-explored. To address this gap, we performed a gene-based association analysis in cognitively normal and impaired participants using neurogenesis pathway-related candidate genes curated from existing databases, literature mining, and large-scale genome-wide association study findings. Using targeted neurogenesis pathway-based gene analysis, we discovered a significant association of *ADORA2A* rs9608282-T with larger hippocampal volumes and volumes of neurogenesis-related hippocampal sub-

regions, better episodic memory performance, and reduced CSF total tau levels. Our findings suggested a protective effect of this SNP on brain structure and function in neurogenesis-related brain regions. We also examined the interaction of treatment with Memantine, a NMDA-receptor antagonist, and *ADORA2A* rs9608282 on hippocampal volume and memory performance. Combining three independent cohorts in a meta-analysis to enhance statistical power, we found that hippocampal volume was significantly associated with *ADORA2A*. The eQTL analysis using the BRAINEAC brain tissue microarray-based gene expression database (http://www.braineac.org/) revealed that rs9608282 in *ADORA2A* is marginally associated with *ADORA2A* gene expression level in the hippocampus (p-value = 0.172). Individuals carrying minor allele rs9608282-T have decreased expression levels in the hippocampus, showing a potential protective effect similar to our SNP-based association results with hippocampal volume and memory. Increased ADORA2A levels lead to synaptic toxicity and memory impairment [289-291]. Functional studies are needed to identify the mechanistic role of the highlighted SNP. However, the present findings support the *ADORA2A* gene playing a role in adult neurogenesis and AD. The association with hippocampal volume indicates the potential importance of further investigation of this gene, especially within in independent cohorts.

Our gene-set enrichment analysis on genome-wide association studies (GWAS) from the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium and Alzheimer's Disease Neuroimaging Initiative (ADNI) showed that

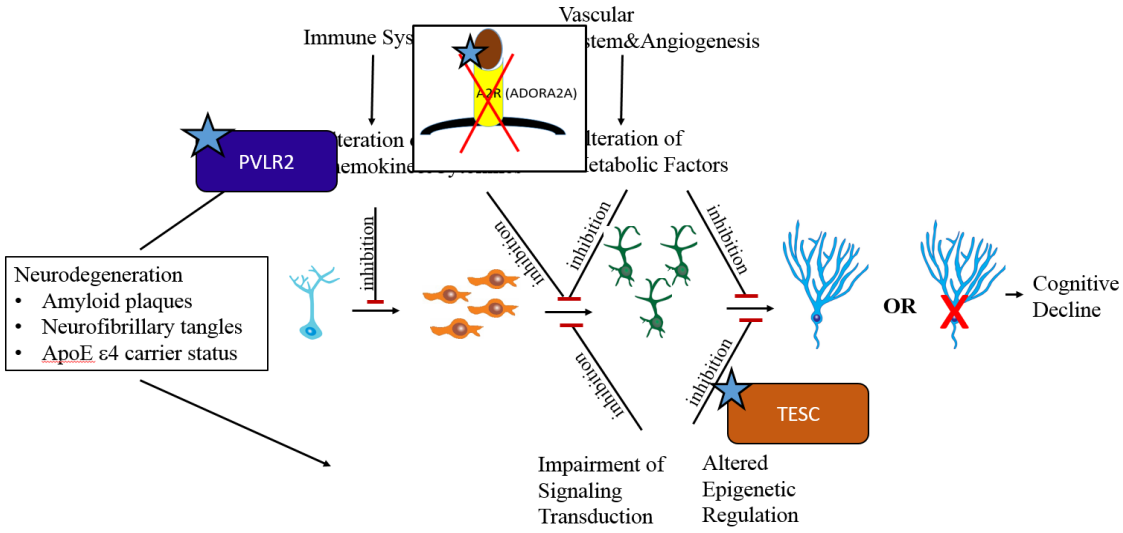
neurogenesis-related pathways were associated with hippocampal volume and hippocampal atrophy. Enriched biological processes included neurogenesis, generation of new neurons, neuronal development, neuronal migration, differentiation and development, glutamate receptor activity, cell-cell adhesion, and synaptic transmission. Among genes that were highly-represented in these enriched pathways, we found *TESC* and *ACVR1* were significantly associated with hippocampal volume after multiple comparison adjustment. In addition, using WGCNA, correlated gene expression analysis identified six significant network modules related to process of neurogenesis. We discovered that hippocampal volume is a potential phenotype for investigation of neurogenesis.

It is also noteworthy that neurogenesis-related pathways which drive neural stem cell proliferation, maintenance in the adult neurogenic niche, and differentiation into mature neurons, were significantly enriched for tau and amyloid-β deposition in the brain. Interestingly, we observed common neurogenesis-related pathways enriched with hippocampal volume, tau and Aβ burden. . Our results suggest that neurogenesis related genes and pathways affect the formation and maintenance of hippocampal structure and memory and may play a role in atrophy and memory impairment in AD.

In summary, we found three novel genes and variation related to adult neurogenesis as well as AD pathology. Since adenosine controls the pial vasodilation in the cerebral vasculature through binding of A2A receptors

secondary to endogenous production and the widening of blood vessels, ADORA2A plays an important role in vascular system. Also, that the adenosine 2a receptor localizes in microglial cells and may be a regulation of microglial function in response to brain damage highlights the potential importance of ADORA2A in immune response. The other novel gene *TESC* is a potential target of class I histone deacetylase, important for epigenetic regulation, and inhibitors in neurons [360]. The last novel finding gene *PVLR2*, cell-cell adhesion gene Nectin-2, controls T-cell signaling by binding the co-stimulatory receptor DNAM1 (CD226) and stimulates proliferation of T-cells and cytokines production [361]. The genetic variation in neurogenesis-related genes may play an important role in alteration of neural stem cell differentiation into new born neurons during adult neurogenesis with important therapeutic implications for AD (Figure 16).

Figure 16. Schematic illustration of three novel genes (*ADORA2A*, *TESC* and *PVLR2*) in impaired neurogenesis in neurodegenerative diseases



Recently, studies focused on understanding mechanism of hippocampal neurogenesis using stem cells and cell cultures/tissue cultures. Many intrinsic and extrinsic signals impact neural stem cells proliferation, differentiation, migration and survival of new born neuros and their integration into neuronal circuitry during adulthood. Neural stem cells might use the treatment of neurodegenerative diseases including Alzheimer's disease. Neurotrophins are growth factors and promote NSCs proliferation as well as differentiation [362]. Recently, Neurotrophin-3 (NT-3), important for survival, differentiation and migration, and gene transfection, promotes the in vitro proliferation and differentiation of bone marrow derived NSCs into cholinergic neurons [363]. Since NSCs are located specific regions in the brain such as subventricular region of dentate gyrus and subventricular zone of olfactory bulb during

adulthood, targeting these NSCs into new neurons might be potential treatment of AD. The other stem cell study showed that administration of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) into the hippocampus in AD mouse model reduced amyloid-β deposition and enhanced adult neurogenesis through secreted growth differentiation factor-15 (GDF-15) [364]. In addition, the inducement of 5-HT receptors in mouse model stimulates adult neurogenesis through differentiation of induced pluripotent stem (iPS) cells into NPCs through activation of PKA and CREB pathways. iPS cell therapy may be a valuable source of NPC production to enhance adult neurogenesis for the formation of the newborn neurons in adult brain. A 3D culture system of hippocampal neurogenesis from hippocampal tissue in a mouse model was established using air liquid interface (ALI) culture and Matrigel culture [365]. The 3D culture of hippocampus tissues might be a novel in vitro tool to understand the mechanism of neurodegenerative diseases and the process of hippocampal neurogenesis.

Most importantly, our project yielded a small list of confirmed candidate gene variants that can be brought forward for experimentally molecular and functional validation in model systems. Development of murine models that carry top candidate variants we identified in this study would help provide a better understanding of the effect of these variants. The resources of the IU/JAX Model Organism Development and Evaluation for Late-onset Alzheimer's disease (MODEL-AD), a recently established center to create novel animal models of AD

would be extremely helpful in advancing mechanistic follow-up studies. MODEL-AD is co-led by investigators at Indiana University and the Jackson Laboratory (JAX). The involvement of my mentors and committee members in the MODEL-AD center will further ensure a seamless transition and expansion of this project. Roles of specific variants and genes will be assessed by submitting novel candidates to MODEL-AD and JAX to develop new mouse models of late-onset AD.

In addition, we note that vascularization and neurotrophic factors are major players in adult neurogenesis that are crucial for the NSC proliferation, differentiation, and their cell fate. Association analysis of cerebral blood flow with neurotrophic growth factor genes and brain-connectivity related phenotypes extracted from the diffusion tensor imaging of the brain (DTI) with neurogenesis-related genes could be helpful for discovering additional novel candidate genes related to adult neurogenesis [366, 367]. We plan to perform an association analyses of brain-connectivity related phenotypes extracted from DTI with neurogenesis-related genes.

Hopefully these insights regarding the role of neurogenesis in Alzheimer's disease will contribute to a better understanding of AD and the quest for effective treatment and ultimately prevention of this important disease.

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CURRICULUM VITAE

Emrin Horgusluoglu

Education

2011-2017 **Ph.D. Medical and Molecular Genetics**

Indiana University, Indianapolis, IN

2006-2009 **Mater of Science Degree, Molecular Genetics**

Istanbul University, Health Sciences Institute, Istanbul, TURKEY

1999-2003 **Bachelor of Science Degree, Biology**

Istanbul University, Istanbul, TURKEY

Research Experience

2013-2017 **Indiana University, School of Medicine, Indianapolis**

Ph.D. Candidate (Advisor: Dr. Andrew J. Saykin, Psy.D.)

- Analysis and review of current status of pathways in adult neurogenesis
- Concepts and methods for biological pathway analysis of genomic data
- Targeted neurogenesis pathway-based gene analysis in Alzheimer's disease
- Targeted gene-based analysis of transcriptome guided neurogenesis pathway with hippocampal volume in Alzheimer's disease
- Biological pathway analysis of hippocampal volume in Alzheimer's disease
- Structural MRI study of hippocampal subfield volumes in Alzheimer's disease
- Process of genome-wide association data (GWAS)

- Next Generation Sequencing, SNP-set (Sequence) Kernel Association Test (SKAT), The gene-set analysis methods (GSA-SNP), Linux, R programming, Plink v1.07, SPSS SPM8

- Weighted gene correlation network analysis (WGCNA)

2011-2013 **Indiana University, School of Medicine, Indianapolis**

Ph.D. Student (Advisor: Dr. Simon Conway, Ph.D.)

- Investigation of genetic mechanism under lung development in bronchopulmonary dysplasia (BPD) in mice model

- Western blot, immunohistochemistry, cell culture

2010-2011 **Indiana University, School of Medicine, Indianapolis**

Center for Computational Biology and Bioinformatics

Research laboratory technologist (Advisor: Yunlong Liu)

- Manually curation of miRNA related to disease pathology

2006-2009 **Istanbul University, School of Medicine, Istanbul**

Institute for Experimental Medical Research

University College London (UCL), London

Master Student

(Advisor: Dr. Nihan Erginel and Dr. Steve Humphries)

- SNP analyzes with TaqMan Technology
- Perform association between cardiovascular diseases and metabolic syndrome with Apolipoprotein D gene variations
- RNA/DNA isolation, PCR, RT-PCR, AGE, PAGE, SSCP analyzes, SNP analyzes with TaqMan Technology, DHPLC, cell culture, nonradioactive probe & northern blot

2005-2006 **Istanbul University, School of Medicine, Istanbul**

Institute for Experimental Medical Research

Research laboratory technician

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- RNA/DNA isolation, PCR, RT-PCR, AGE, PAGE, SSCP analyzes, SNP analyzes with TaqMan Technology

2000 **Istanbul University, School of Medicine, Istanbul**

Institute for Experimental Medical Research

Genetic Department Internship (Supervisor: Ugur Ozbek)

- Identification of NAD(P)H:quinone oxidoreductase 1 null genotype in pediatric de novo acute leukemia.

Teaching Experience

Fall 2016 Indiana University School of Medicine

Q580 Basic Human Genetics

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Q580 Basic Human Genetics

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Honors, Awards and Services

2017	Alzheimer's Association International Conference Travel Fellowship Winner
2015-2017	Medical and Molecular Genetics Curriculum Committee
2016	Sigma Xi Graduate Student Biomedical Research Competition Winner, Indiana University School of Medicine
2016	CTSI 8th Annual Meeting Outstanding Poster Winner Indiana University School of Medicine
2016	Outstanding poster presentation at Eli Lilly and Company
2016	Indiana University School of Medicine Travel Grant Winner
2015-2016	Graduate Student Representative
2015	Alzheimer's Association International Conference Travel Fellowship Winner
2013	Indiana University School of Medicine Travel Grant Winner

Peer-Reviewed Publications

Manuscripts

- **Horgusluoglu E**, Nho K, Risacher SL, Kim S, Foroud T, Shaw LM, Trojanowski, JQ, Aisen PS, Petersen RC, Jack CR, Jr, Wiener, M, Saykin AJ; ADNI. Targeted neurogenesis pathway-based gene analysis identifies ADORA2A associated with hippocampal volume in mild cognitive impairment and Alzheimer's disease. Neurobiology of Aging. Manuscript number: NBA 16-996. 2017 (Accepted).
- Nho K, Kim S, **Horgusluoglu E**, Risacher SL, Shen L, Kim D, Lee S, Foroud T, Shaw LM, Trojanowski JQ, Aisen PS, Petersen RC, Jack CR, Weiner MW, Green RC, Toga AW, Saykin AJ; ADNI. Association analysis of rare variants near the APOE region with CSF and neuroimaging biomarkers of Alzheimer's disease. BMC Med Genomics. 2017 May 24;10(Suppl 1):29. doi: 10.1186/s12920-017-0267-0.
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- **Horgusluoglu E**, Nudelman K, Nho K, Saykin AJ. Adult Neurogenesis and Neurodegenerative Diseases: A Systems Biology Perspective. Am J Med Genet B Neuropsychiatr Genet. 2016 Feb 16. doi: 10.1002/ajmg.b.32429
- Nho K, Ramanan VK, **Horgusluoglu E**, Kim S, Inlow MH, Risacher SL, McDonald BC, Farlow MR, Foroud TM, Gao S, Callahan CM, Hendrie HC, Niculescu AB, and Saykin AJ. Comprehensive gene- and pathway-based analysis of depressive symptoms in older adults. J Alzheimers Dis. 2015 Jan 1;45(4):1197-206. doi: 10.3233/JAD-148009.
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Abstracts

- **Horgusluoglu E**, Nho K, Risacher SL, and Saykin AJ. Genome-wide association analysis of tau accumulation identifies enrichment of neurogenesis-related pathways. Alzheimer's Association International Conference 2017. London, UK. **Oral Presentation**
- **Horgusluoglu E,** Nho K, Risacher SL, Crane PK, Hibar DP, Paul M. Thompson PM, and Saykin AJ. Genome-Wide Association Analysis of Hippocampal Volume Identifies Enrichment of Neurogenesis-Related Pathways. Alzheimer's Association International Conference 2016. Toronto, Canada. **Oral Presentation**
- **Horgusluoglu E**, Nho K, Risacher SL, and Saykin AJ. VEGFA is associated with cerebral blood flow and white matter hyperintensity in mild cognitive impairment (MCI) and Alzheimer's disease. Alzheimer's Association International Conference 2017. London, UK. **Poster**
- **Horgusluoglu E**, Risacher SL, Saykin AJ and Nho K. ADORA2A polymorphism is associated with cerebral blood flow in mild cognitive impairment (MCI) and Alzheimer's disease. Alzheimer's Association International Conference 2017. London, UK. **Poster**
- Nho K, Kim S, **Horgusluoglu E**, Risacher SL, Saykin AJ. KLK8 as a modulator of Alzheimer's disease pathology: neuroimaging genetics. Alzheimer's Association International Conference 2017. London, UK. **Poster**

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Services

2016	Manuscript Reviewer in Brain Imaging and Behavior
2015-2016	Graduate Student Representative
2015-2017	Medical and Molecular Genetics Curriculum Committee Indiana University, School of Medicine
2014	Africa, Middle East and Central Asia at Eli Lilly
2014	Manuscript Reviewer in Brain Imaging and Behavior
2011-present	American Turkish Association of Indiana (ATA-IN)

Organizations and Memberships

2015-present	Alzheimer's Association International Conference (ISTAART)
2016-present	Society for Neuroscience (Sfn)
2015-216	The American Society of Human Genetics (ASHG)
2010-2013	The Rug and Textile Society of Indiana
2011-present	American Turkish Association of Indiana (ATA-IN)