Soluble Amyloid Precursor Protein Regulates Neurogenesis:

Implications for Brain Repair

BY

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THESIS

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DEDICATION

First I would like to dedicate this thesis to my parents and my brother. My parents taught us from a young age to value an education and that we could accomplish any goal that we put our full effort into. My brother is my role model for strength and perseverance as he has battled with epilepsy for the better part of his life. I would next like to dedicate this work to my beautiful domestic partner, Pamela. Throughout this process she has been a pillar of sanity in an otherwise hectic World. She makes me laugh when it's the last thing I want to do and makes it easy to remember that science does not have to be a lifestyle. Finally, I would like to thank my friends Adam, Archana and Benjamin who made coming to work a pleasurable experience. I know that these people are very proud of my accomplishment and I too am proud to call them my family and friends.

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LIST OF ABBREVIATIONS

APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
KPI	Kunitz Type Serine Protease Inhibitors
APLP	Amyloid Precursor Like Protein
HBD	Heparin Binding Domain
GFLD	Growth Factor Like Domain
CuBD	Copper Binding Domain
ZnBD	Zinc Binding Domain
AICD	Amyloid Precursor Protein Intracellular Domain
sAPP	Soluble Amyloid Precursor Protein
CTF	Carboxyl-terminal Fragment
BACE	Beta Site Amyloid Precursor Protein Cleaving Enzyme
PS	Presenilin
APH1	Anterior Pharynx Defective 1
PEN2	Presenilin Enhancer 2
ADAM	A Disintegrase and Metalloproteinase
NPC	Neural Progenitor Cell
NSC	Neural Stem Cell
DG	Dentate Gyrus
OB	Olfactory Bulb
BrdU	5-bromo-2'-deoxyuridine
NeuN	Neuronal Nuclei
SGL	Subgranular Layer
SVZ	Subventricular Zone
EGF	Epidermal Growth Factor
bFGF	Basic Fibroblast Growth Factor
EGFR	Epidermal Growth Factor Receptor
NCAM	Neural Cell Adhesion Molecular
RMS	Rostral Migratory Stream
GABA	Gamma-Amino Butyric Acid
NKCC1	Na+-K+-2CL-transporter
DNA	Deoxyribonucleic Acid
MAM	Methylazoxy-Methanol Acetate
NT-3	Neurotrophin 3
MBD1	Methyl-CpG Binding Protein
GFAP	Glial Fibrillary Acidic Protein
TK	Thymidine Kinase
SORLA	Sortillin Related Receptor with Type-A Repeats
GPI	Glycophosphatidylinositol
SHH	Sonic Hedgehog
PTCH1	Patched 1
SMO	Smoothened
APP-BP-1	Amyloid Precursor Protein Binding Protein 1
CNS	Central Nervous System

LTP	Long Term Potentiation
FAD	Familial Alzheimer's Disease
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
NMDA	N-Methyl-D-Aspartic Acid
IGF1	Insulin-like Growth Factor 1
VEGF	Vascular Endothelial Growth Factor
HB-EGF	Heparin Binding Epidermal Growth Factor
MAPK	Mitogen Activated Protein Kinase
AKT	Protein Kinase B
ERK	Extracellular Signal-regulated Kinase
DMEM	Dulbecco's Modified Eagle's Medium
MSC	Mesenchymal Stem Cells
HdpPSC	Human Decidua Parietalis Placental Stem Cells
MMP	Matrix-Metalloproteinase
TBS	Tris Buffered Saline
DCX	Doublecortin
RNA	Ribonucleic Acid
ICV	Intracerebroventricular
IP	Intraperitoneal
DAPI	4'6-Diamidino-2-Phenylindole
DIV	Days In Vitro
PI3K	Phosphoinositide 3-Kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DRM	Detergent Resistant Membrane

SUMMARY

In this study, several advances in the understanding of the physiological role of amyloid precursor protein (APP) metabolites are presented. APP is a type-I transmembrane glycoprotein that can undergo extensive, sequential cleavage to form a number of intra- and extra-cellular metabolites. One of these metabolites, soluble APP (sAPP), has been shown to regulate the proliferation of a number of cell types and bind to rapidly proliferating neural progenitor cells (NPC) in the adult brain. In the adult brain, there are two discrete regions where continuous neurogenesis is thought to occur throughout the lifespan in mammals, the subventricular zone (SVZ), lining the lateral ventricles, and the subgranular layer (SGL) at the border of the dentate gyrus and hilus in the hippocampal formation. The work presented here will examine the role of sAPP in the proliferation of NPC, the mechanism through which sAPP exerts this effect and the effect of alterations in APP metabolism on NPC proliferation in Alzheimer's disease (AD) and normal aging.

As previously mentioned, APP undergoes sequential cleavage. This occurs through two distinct pathways termed amyloidogenic, for its role in the formation of amyloid beta (Aβ) that is the core component of amyloid plaques characteristic of AD, and non-amyloidogenic. In the non-amyloidogenic pathway, APP is cleaved by a group of enzymes termed α-secretase to produce an extracellular metabolite, sAPPα. sAPPα has been shown to have trophic properties in numerous cell types. With this information in mind, the first portion of this study was designed to test the hypothesis that sAPPα could act as a proliferation factor for NPC of the adult brain.

Several approaches were implemented in order to examine the above hypothesis. The first was the characterization of APP, sAPP and α-secretase expression in the adult neurogenic regions. Through this analysis it was determined that not only were all of these proteins expressed in NPC but that the most abundant expression of sAPP and α-secretase correlated with the region of the most abundant neurogenesis, the SVZ. Utilizing a broad-spectrum matrix-metalloproteinase inhibitor that inhibits α-secretase enzymes, it was shown that α-secretase inhibition reduced NPC proliferation and that this could be reversed through treatment with sAPPα. These experiments were repeated in mesenchymal stem cells and human placental stem cells, providing strong evidence that sAPPα is a potent proliferation factor for stem cells of various lineage.

Previous work has suggested that due to heparin-binding domains on sAPP and the presence of binding sites for sAPP on EGF-responsive NPC, sAPP may act as a co-factor for epidermal growth factor (EGF) or other trophic factors. Additional evaluation of sAPPα signaling has suggested that stimulation of cells by sAPPα can induce intracellular signaling of at least two downstream pathways, the mitogen activated protein kinase (MAPK) and protein kinase B (Akt) pathways. This led to the examination of downstream signaling pathways of sAPPα in NPC proliferation. After removal of exogenous growth factor support from the NPC media, sAPPα retained the ability to stimulate proliferation. Additionally, stimulation by sAPPα resulted in increased phosphorylation of extracellular-signal regulated kinase (Erk), a part of the MAPK pathway, but no change in Akt phosphorylation. Furthermore, microarray analysis of gene targets for sAPPα signaling in NPC revealed alterations in genes regulating cell cycle, energy metabolism, neurogenesis and neurotransmission. Therefore, the initial

portion of this work demonstrates that sAPPα is a potent proliferation factor for NPC of the adult brain; exerting its proliferative affects through the MAPK pathway to modify gene expression of a number of functional categories.

In the AD brain there is a decline in neurogenesis and a reduction in proliferating NPC. Mutations that cause familial forms of AD lead to increased amyloidogenic processing of APP by β- or γ-secretase. In familial AD linked mutant mice, reductions in proliferation of NPC are seen prior to Aβ plaques or memory impairments and may intrinsically contribute to proliferation decline in NPC. Therefore, the next portion of this work examined the hypothesis that the soluble metabolite of the amyloidogenic pathway, sAPPβ, may not provide the potent trophic support of sAPPα. sAPPβ fails to rescue impaired proliferation of NPC induced by either α-secretase or β-secretase inhibition. Furthermore, increasing concentrations of sAPPβ may reduce NPC viability suggesting a potential toxic effect.

Neurogenesis and the number of proliferating NPC decline dramatically beginning at approximately 2 months of age in mice. While the nature of this decline remains controversial, there is evidence to suggest that environmental influences and trophic stimulation have the ability to ameliorate some of these deficits even in old age. The processing of APP may also be altered in the aging brain. Evidence suggests that in aging cells, APP may preferentially incorporate in lipid rafts where it is cleaved by the amyloidogenic pathway. In light of these studies, it was hypothesized that alterations in APP metabolism in aging would result in decreased sAPPα and declining proliferation. Indeed, sAPP expression does decline in the aging brain, correlating with aging-linked decline in proliferating NPC. *In vitro* analysis revealed that NPC derived from the aged

brain retain the ability to respond to sAPPα stimulation. Finally, infusion of sAPPα to the lateral ventricles stimulates proliferation of NPC in both the SVZ and SGL suggesting that altered metabolism of APP in aging could contribute to the decline in proliferating NPC in the aged brain.

In summary, the findings presented in this thesis provide important new evidence in the physiological role of sAPP and the physiological significance of APP metabolic pathways. Essentially, it is shown that sAPPα is a potent proliferation factor for NPC of the adult brain. An alteration in APP metabolism in either AD or aging leading to increased amyloidogenic APP processing could result in declining trophic support and could contribute to declining NPC proliferation. This is the most extensive evaluation of sAPPα activity with respect to proliferation and provides some of the first mechanistic insight into how altered APP metabolism could play a role in NPC decline.

CHAPTER 1:

INTRODUCTION

A. Amyloid precursor protein

1. Family, isoforms and homologues

Amyloid precursor protein (APP) is a type-I transmembrane glycoprotein whose function has yet to be fully elucidated. Evidence suggesting that Alzheimer's disease plaques were comprised of an APP metabolite, amyloid beta (Aβ) (Masters et al., 1985), led to the initial cloning of the gene and localization to chromosome 21 providing significance to the accumulation of Aβ plaques in Down's syndrome (Kang et al., 1987). The subsequent development of APP antibodies led to the discovery that APP was expressed in both neural and non-neural tissues (Selkoe et al., 1988). Alternative splicing of the *App* gene leads to at least 3 different isoforms encoding 695, 751 and 770 amino acid proteins; the latter two containing an N-terminal domain similar to Kunitz type serine protease inhibitors (KPI) (Ponte et al., 1988; Tanaka et al., 1988). In the CNS, APP_{695} is highly enriched in neurons while astrocytes and microglia express similar amounts of all 3 isoforms that appear to be mainly internalized to vesicles and more resistant to cleavage (Haass et al., 1991).

Homologues of the APP gene family are highly conserved throughout evolution with the presence of APP-like genes in species including human (Kang et al., 1987), non-human primate (Podlisny et al., 1991), mouse (Yamada et al., 1987), rat (Shivers et al., 1988), *Xenopus* (Okado and Okamoto, 1992), *Drosophila melanogaster* (Rosen et al., 1989) and *C. elegans* (Daigle and Li, 1993). *Dropsophila* APPL expression is restricted to the nervous system (Luo et al., 1990) while *C. elegans* APL-1 expression is more

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similar to the ubiquitous expression pattern of mammalian APP (Hornsten et al., 2007).

However, deletion of APL-1 in *C. elegans* leads to lethality that can be recovered by

neuronal expression of the C-terminal region alone (Hornsten et al., 2007).

In rodents and humans, amyloid precursor-like proteins (APLP) have been

discovered. APLP1 was discovered in both the mouse (Wasco et al., 1992) and human

(Paliga et al., 1997). The mouse APLP1 shares 42% sequence identity to APP and 64%

similarity at the amino acid level. The strongest sequence homology lies in the C-

terminal and regions of the extracellular domain. The N-terminal portion of the Aβ

region is not present in APLP1 (Wasco et al., 1992). APLP2 has been discovered in

human (Wasco et al., 1993), mouse (Slunt et al., 1994) and rat (Sandbrink et al., 1994).

Like APLP1, APLP2 also lacks the N-terminal portion of the Aβ region. In mice, this

751 amino acid protein contains a highly similar carboxyl-terminus and regions of very

high sequence homology in the extracellular domain including the KPI domain (Slunt et

al., 1994). The lack of an Aβ region in the APLPs precludes their metabolism into Aβ

and its subsequent aggregation.

2. Functional domains

The APP family of proteins was discovered based on the presence of several

distinct conserved domains. From N-terminus to C-terminus these domains are as

follows: heparin binding domain 1/growth factor like domain (HBD1/GFLD) (Small et

al., 1994; Rossjohn et al., 1999), copper binding domain (CuBD) (Hesse et al., 1994),

zinc binding domain (ZnBD) (Bush et al., 1994), KPI domain (Kitaguchi et al., 1988),

heparin binding domain 2 (HBD2) (Multhaup et al., 1994), growth promoting region (Jin

et al., 1994), collagen binding domain (Beher et al., 1996), Aβ, clathrin attachment site

(Nordstedt et al., 1993). Many of these domains are widely conserved such as the clathrin attachment site and ZnBD, which are conserved across the APP superfamily. Others such as Aβ are restricted to APP. Isoform differences also occur such as in the KPI domain, which is not present in APP_{695}. Differences in the conservation of these regions may confer variability in protein function of the APP family and APP isoforms. An example of APP isoforms conferring potentially differential function comes from a recent report that APP_{695} produces the transcriptionally active metabolite, APP intracellular domain (AICD), more readily than other isoforms and preferentially through the β-secretase pathway (Belyaev et al., 2010).

3. Metabolism and cellular localization

The metabolism of APP is a complex, sequential process that produces several intra- and extra- cellular metabolites. The sequential processing occurs through two mutually exclusive pathways that have been denoted "amyloidogenic", due to production of Aβ, and "non-amyloidogenic" based on processing which results in a non-aggregating peptide. In the amyloidogenic pathway, APP is cleaved first by β-secretase at the N-terminal of the Aβ region leading to the release of a ~100Kd metabolite (sAPPβ) and a membrane tethered C-terminal fragment (βCTF). This cleavage is mediated by beta-site APP cleaving enzyme 1 (BACE1) (Vassar et al., 1999). BACE1 has an acidic pH optimum and most of its activity is localized to acidic organelles such as the Golgi apparatus and endosomes (Haass and Selkoe, 1993; Knops et al., 1995). Subsequent cleavage occurs at the C-terminal portion of the Aβ region and releases Aβ to the lumen and an APP intracellular domain (AICD). This cleavage is mediated through an enzyme complex that has been named γ-secretase. The gamma-secretase complex is comprised

of four core component proteins; presenilin 1 or 2 (PS1, PS2)(Rogaev et al., 1995; Sherrington et al., 1995), anterior pharynx defective 1 (APH-1)(Francis et al., 2002), nicastrin (Yu et al., 2000) and presenilin enhancer 2 (Francis et al., 2002). Presenilin was discovered to be an aspartyl-protease that contains the catalytic core of the γ-secretase complex but cleavage can be regulated by other members of the complex either permitting complex stabilization or altering catalytic activity (Thinakaran et al., 1996b; Wolfe et al., 1999; Li et al., 2000; St George-Hyslop and Fraser, 2011). While APH-1, nicastrin and PEN2 do not contain the catalytic core of the enzyme, they each have been shown to be both necessary and sufficient for the proteolytic activity of γ-secretase (Francis et al., 2002; Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). Both BACE1 and γ-secretase are associated with lipid rafts (Cordy et al., 2003; Vetrivel et al., 2004). Depletion of cholesterol reduces the internalization of APP to the sorting endosomes where it would normally be in contact with BACE1 thus inhibiting the amyloidogenic pathway (Schneider et al., 2008). In the non-amyloidogenic pathway of APP metabolism, primary cleavage by α-secretase occurs between amino acid 16 and 17 of the Aβ region releasing sAPPα to the lumen and leaving a membrane tethered αCTF (Sisodia et al., 1990). There are a number of enzymes that have been shown to exhibit α-secretase activity including members of the a disintegrase and metalloproteinase (ADAM) family, ADAM10 (Lammich et al., 1999) and ADAM17 (Buxbaum et al., 1998; Asai et al., 2003; Postina et al., 2004), as well as BACE2 (Yan et al., 2001). Utilizing a conditional knockout of ADAM10 in NPC and NPC derived neurons and glia, it was shown that deletion resulted in perinatal lethality. Furthermore, neurons derived from embryos of ADAM10 cKO mice had dramatically reduced α-secretase cleavage of

APP. These results suggest that ADAM10 is the primary α-secretase of the brain in both development and in postmitotic neurons (Jorissen et al., 2010). The ADAMs are thought to cleave their substrates primarily on the plasma membrane (Lammich et al., 1999; Schlondorff et al., 2000). Cholesterol depletion and lipid raft disruption through administration of methyl-β-cyclodextrin increased sAPPα excretion and ADAM10 membrane localization (Kojro et al., 2001). This suggests that processing of APP by α-secretase occurs outside of lipid rafts. This differential compartmentalization of secretase cleavages may be a regulatory mechanism mediating the pattern of cleavage without changing concentration of enzyme or substrate. Subsequent cleavage by γ-secretase releases a non-amyloidogenic p3 peptide to the lumen (Haass et al., 1991; Kimberly et al., 2001) and an AICD to the cytoplasm.

B. Adult Neurogenesis

1. Death of a dogma

Santiago Ramon y Cajal, to many the father of modern neuroscience, once famously quipped, *"In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated"* (Ramón y Cajal and May, 1928). The concept that no regeneration of neurons was possible in the adult brain persisted for several decades until it was first challenged by Joseph Altman who was examining trauma induced glial proliferation in adults using tritiated thymidine labeling. Altman astutely noticed that not only had the thymidine incorporated in glial cells around the lesion, but indeed a population of neurons had incorporated it as well suggesting that they had undergone proliferation during the time of the thymidine administration (Altman, 1962). This work was largely ignored by the scientific community and the

previous dogma was reinforced when a group reported no postnatal neuronal proliferation in adult non-human primates (Rakic, 1974). Again the dogma was challenged in the late 1970's and early 1980's when Michael Kaplan showed tritiated thymidine incorporation in adult granule neurons of the dentate gyrus (DG) and olfactory bulb (OB) of rats one month after injection (Kaplan and Hinds, 1977; Kaplan, 1985). The reaction from the scientific community was to once again unequivocally support the dogmatic view that no new neurons were formed in adulthood. Rakic continued to show that in the adult primate brain, tritiated thymidine was not incorporated in what he interpreted as neuronal populations and thus he declared that neurogenesis in adults may be restricted to lesser mammalian species and therefore not relevant to humans (Rakic, 1985). This persistent denial and discrediting of Kaplan's findings ultimately led him to leave science entirely as he would detail in a candid *Trends in Neurosciences* forum (Kaplan, 2001). In the late 1980's, Fernando Nottebohm and colleagues found that new neurons were produced in the canary and that the production of new neurons peaked during song learning and maturation and waned once the song had been stabilized (Goldman and Nottebohm, 1983; Alvarez-Buylla et al., 1988). These experiments suggested that neurogenesis may play a role in learning or shaping the song and provided the first insight into a potential functional significance of neurogenesis in the adult brain. However, without any evidence of neurogenesis in non-human primates or humans the dogma persisted until a pivotal study in the late 1990's provided the first evidence for neurogenesis in the adult human brain. By injecting 5-bromo-2'-deoxyuridine (BrdU) into terminally ill cancer patients and analyzing the post-mortem tissue with new cell-type specific markers like NeuN, calbindin and neuron specific enolase, researchers were able to show cells that had

proliferated in the adult brain had differentiated into neurons in the DG (Eriksson et al., 1998). With the evidence of adult neurogenesis in humans, the dogma had finally been overturned and a wealth of studies began assessing neurogenesis in adults. We now know that neurogenesis persists throughout adulthood in at least two discrete regions of the adult mammalian brain; the subgranular layer (SGL) of the DG in the hippocampal formation and the SVZ lining the lateral ventricles (Cameron et al., 1993; Lois and Alvarez-Buylla, 1994; Kuhn et al., 1996; Kempermann et al., 1997b; Doetsch et al., 1999a). With the stigma of studying adult neurogenesis finally lifted, the race to determine both the underlying mechanisms governing neurogenesis and its functional significance in the adult would begin.

2. Subventricular zone

The SVZ is the most prominent neurogenic zone of the adult brain. Here, astrocyte-like type-B, or primary progenitor cells, that are slowly proliferating are nestled alongside type-C, transit amplifying cells, type-A, neuroblasts, and non-proliferating ependymal cells that line the lateral ventricular wall. The type-C cells are the rapidly proliferating direct progeny of type B cells (Doetsch et al., 1997; Kriegstein and Alvarez-Buylla, 2009). They are often in close proximity to microvasculature which may provide vital cues necessary to their proliferation (Tavazoie et al., 2008). Newly generated neuroblasts from the SVZ migrate in chains toward the olfactory bulb (Lois and Alvarez-Buylla, 1994; Lois et al., 1996) where they replace most of the inner granule layer and 50% of the superficial zone (Imayoshi et al., 2008). During this migration, neuroblasts are nestled in a glial sheath provided by type-B progenitors (Kaneko et al., 2010). The neurogenic niche is believed to be a tightly regulated center where a combination of

factors allows for the constant proliferation of NPC throughout the lifespan of an organism as displayed by the inability of SVZ NPC to proliferate and form neurons when injected into nonneurogenic regions (Gage, 2000; Lim et al., 2000).   It is believed that only a portion of those neuroblasts that reach the OB ultimately survive (Winner et al., 2002). Those that survive have been shown to integrate into the existing circuitry and form functional interneurons (Carlen et al., 2002; Carleton et al., 2003).

The NPC of the SVZ rely on a number of cues from the niche in order to sustain proliferation, differentiate, migrate and ultimately incorporate into existing functional circuits [Reviewed in (Ihrie and Alvarez-Buylla, 2011)]. NPC have been isolated from tissue of the ventricular walls and grown in culture. These NPC grow *in vitro* as "neurospheres" which is apparently a phenomenon conditional to their culture and not seen thus far *in vivo* (Reynolds and Weiss, 1992). In order to sustain the *in vitro* proliferation of NPC, culture systems generally employ media which contains epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or a combination of the two (Reynolds and Weiss, 1992; Vescovi et al., 1993; Scheffler et al., 2005). Immunostaining of SVZ progenitor populations suggests that cells responsive to these growth factors represent two distinct populations (Jackson et al., 2006). The epidermal growth factor receptor (EGFR) is primarily expressed in type-C, transit amplifying cells and a subset of type-B cells (Doetsch et al., 2002). *In vivo* infusion of EGF into the forebrain of adult mice for 6 consecutive days resulted in dramatic increases in proliferation of NPC (Craig et al., 1996). Furthermore, NPC in culture arrest their proliferation after the removal of EGF from the media (Vescovi et al., 1993). In contrast, fibroblast growth factor responsive cells remain in the SVZ following ablation of transit

amplifying cells and neuroblasts (Jackson et al., 2006). Furthermore, *fgf2* knockout mice show a reduced NPC with a no decrease in cell cycle length of transit amplifying cells. This reduction seems to stem from a decrease in the pool of type-B cells and FGF seems to play a pivotal role in self-renewal within the niche (Zheng et al., 2004).

In the SVZ-olfactory bulb system, the functional significance of neurogenesis has proven somewhat difficult to elucidate. Though NPC from the SVZ replace nearly all of the inner granule layer and 50% of the superficial zone of the OB (Imayoshi et al., 2008), ablation of neural cell adhesion molecule (NCAM) in mice does not impair olfactory discrimination learning and impaired migration through the (RMS) due to a knockout of Bax does not impair olfactory discrimination or memory (Schellinck et al., 2004; Kim et al., 2007). However, in another study of the NCAM-null model, it was shown that alterations in NPC migration led to a 40% reduction in inner granule interneurons and fine olfactory discrimination deficits but normal olfactory threshold and memory (Gheusi et al., 2000). More recent evidence has emerged that newly generated neurons are vital to paternal and maternal behaviors intimately associated with olfactory cues (Mak and Weiss, 2010; Sakamoto et al., 2011). Short-term olfactory memory improvement associated with olfactory enrichment depends on newborn neurons (Rochefort et al., 2002). In addition, more complex olfactory associated tasks such as olfactory perceptual learning seem to require on-going neurogenesis (Moreno et al., 2009) suggesting that newly formed cells may be specially adapted to handle fine or complex olfactory function.

There is also evidence that the SVZ may have the ability to respond following brain trauma and function to replace dying neurons. Following ischemic stroke, the NPC

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of the SVZ show enhanced proliferation and migration toward the lesion site. Several studies have shown that these NPC can differentiate into neurons and replace those that have died following trauma (Arvidsson et al., 2002; Parent et al., 2002; Zhang et al., 2004; Yamashita et al., 2006). Other forms of trauma such as traumatic brain injury have also been shown to increase the proliferation of NPC in the SVZ (Chirumamilla et al., 2002) suggesting that brain injury may trigger the proliferation of these endogenous progenitors as a means to facilitate repair.

3. Subgranular Layer

The second region of persistent neurogenesis in the adult brain is the SGL that lies at the inner potion of the DG bordering the hilus in the hippocampal formation. In the SGL, type-I cells are radial glia-like cells which are similar to type-B cells of the SVZ (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Type-II cells are more rapidly proliferating and similar in nature to the type-C, transit amplifying cells, of the SVZ (Suh et al., 2007). The progenitors of the SGL give rise almost exclusively to neuronal populations (Duan et al., 2008). Neuroblasts from the SGL migrate only a short distance to populate the inner third of the granular layer of the DG (van Praag et al., 2002). Within days these immature neurons begin sending projections through the hilus toward the CA3 region of the hippocampus (Zhao et al., 2006). These immature neurons respond to tonic gamma-aminobutyric acid (GABA) release. Using shRNA directed toward the Na^+-K^+-$2Cl^-$ transporter, NKCC1, Ge et al showed that transient expression of NKCC1 leads to an abundance of chlorine inside of the cell and thus a depolarizing effect of tonic GABA. By knocking down the NKCC1 expression, the authors showed that this tonic GABA signaling was important to the maturation of the immature neurons,

including their synaptogenesis and formation of dendrites (Ge et al., 2006). Following

this period of tonic GABA stimulation, cells receive initial innervation from GABAergic

synaptic inputs followed by glutamatergic inputs (Esposito et al., 2005; Overstreet-

Wadiche and Westbrook, 2006).

The functional role of neurogenesis in the adult hippocampus has yet to be fully

elucidated. Due to the subpopulation of NPC's incorporating into the dentate gyrus of

the hippocampus, a region associated with memory, much of the focus of the biological

function of neurogenesis has been on a potential role in learning and memory. The initial

evidence for neurogenic involvement in memory formation came from the seasonal

changes in songbird neurogenesis. It was shown that the newly born cells responded to

songs and that neurogenic increases could be correlated with seasonal and hormonal

changes associated with song learning (Nottebohm, 2004). Studies in mammals using

different strains of mice (Kempermann et al., 1997a), environmental enrichment

(Kempermann et al., 1997b; van Praag et al., 1999) and running (van Praag et al., 1999)

have each shown a correlation between increases in neurogenesis and enhanced

performance on a spatial memory task. Conversely, experiments using different aged rats

(Bizon and Gallagher, 2003), stress paradigms (Lemaire et al., 2000), irradiation (Madsen

et al., 2003; Raber et al., 2004; Rola et al., 2004) and the DNA methylating agent

methylazoxy-methanol acetate (MAM) (Shors et al., 2001; Shors et al., 2002) have each

shown correlations between a decrease in neurogenesis and impairment on hippocampal

dependent memory tasks though studies have provided conflicting results as to which

memory paradigms are affected. Genetic manipulations have provided further evidence

for the role of neurogenic processes in memory formation. Deletion of either

neurotrophin 3 (NT-3) (Shimazu et al., 2006) or methyl-CpG binging protein 1 (MBD1), a methylated DNA binding protein (Zhao et al., 2003), produced a decrease in neurogenesis as well as an impairment on the Morris water maze task. A study using both irradiation and glial fibrillary acidic protein (GFAP)-thymidine kinase (TK) transgenic mice that express a herpes virus TK in progenitor cells allowing for ablation of these cells through ganciclovir administration showed that spatial memory was retained while contextual fear conditioning was impaired. Long term potentiation in the dentate gyrus was also impaired in both models of neurogenic ablation (Saxe et al., 2006). Also of note, a recent study showed the preferential recruitment of new neurons into spatial memory networks following water-maze learning using the expression of c-fos, an immediate early gene, to examine neuronal excitation (Kee et al., 2007). Furthermore, an inducible deletion of TLX from the adult mouse brain drastically reduced neurogenesis and impaired spatial learning (Zhang et al., 2008). Emerging evidence also suggests that neurogenesis is crucial to pattern separation in the DG (Clelland et al., 2009; Sahay et al., 2011).

4. Amyloid precursor protein metabolites and proliferation of neural progenitor cells

The first studies to draw a correlation between APP and neurogenic processes showed that embryonic expression of APP peaked during the height of neuronal differentiation and neurite outgrowth (Hung et al., 1992; Salbaum and Ruddle, 1994). Evidence soon emerged that the cleavage of Notch1, an integral protein involved in the maintenance of neural stem cell pools by enhancing symmetric division (Alexson et al., 2006), and APP undergo strikingly similar proteolytic cleavage patterns including cleavage by some of

the same enzymes (ADAM10, PS1 etc.) (De Strooper et al., 1999; LaVoie and Selkoe, 2003; van Tetering et al., 2009). The following is a brief guide to what we currently understand about the role of various APP metabolites in proliferation of NPC.

Under non-pathological circumstances, the non-amyloidogenic cleavage pathway of APP dominates through cleavage by alpha-secretase and release of the sAPPα metabolite. A crystal structure of sAPP suggests that the growth factor like domain is similar to cysteine-rich growth factors (Rossjohn et al., 1999). In light of the appearance of a growth factor like domain, it is not surprising that addition of sAPPα stimulates the proliferation of a number of different cell types including fibroblasts (Saitoh et al., 1989), thyroid epithelial cells (Pietrzik et al., 1998), embryonic stem cells (Ohsawa et al., 1999) and carcinoma cells (Ko et al., 2004). Caillé et al showed that rapidly proliferating, EGF-responsive type-C cells of the adult SVZ had saturable binding sites for sAPPα and infusion of sAPPα into the lateral ventricles of adult mice was sufficient to increase NPC numbers (Caille et al., 2004). Furthermore, studies on a mouse model exhibiting a knockout of the sortillin related receptor with type-a repeats (SORLA), a sorting receptor that inhibits the processing of APP to form both sAPP and Aβ, showed both increased APP metabolites as well as enhanced proliferation of NPC and neurogenesis in adult mice (Rohe et al., 2008). Following cerebral stroke, an increase in ADAM17 proteolytic activity and APP expression leads to increased proliferation in the SVZ (Katakowski et al., 2007).

The AICD is formed following cleavage of APP by either alpha- or beta-secretase and subsequent RIP through the activity of the gamma-secretase complex (Haass and Selkoe, 1993; Kimberly et al., 2001). It has been shown to contain a

YENPTY domain that is a binding site for phosphotyrosine binding domain containing adapter proteins (Kavanaugh and Williams, 1994). The AICD itself can also undergo modification through phosphorylation events that may underlie docking of some of its many adapter proteins (Tarr et al., 2002). The AICD has been shown to form a transcriptionally active complex with Fe65 and Tip60, a histone acetyltransferase, that translocates to the nucleus and regulates transcription of a number of genes [(Cao and Sudhof, 2001; Gao and Pimplikar, 2001), for review (Muller et al., 2008)]. Much interest has evolved in the relationship between AICD and neurogenesis due to the finding that the cleavage pattern, formation of an ICD and its translocation to the nucleus mimic the integral neurogenesis protein Notch 1 [for review (Ebinu and Yankner, 2002)].

Conversely to sAPP function, AICD has been shown to be a negative regulator of proliferation in NPC. First, evidence suggests that AICD negatively regulates the transcription of the EGFR, a receptor that drives NPC proliferation (Zhang et al., 2007b; Ayuso-Sacido et al., 2010). Ma et. al. showed that the glycophosphatidylinositol (GPI)-linked recognition protein, TAG1, extracellularly interacts with APP to increase AICD release. Using a TAG1 null mouse model with aberrantly high proliferation of embryonic NPC, they showed that either administration of recombinant TAG1 or transfection with AICD59 *in vitro* could ameliorate enhanced neurogenesis. However, transfection of AICD with a mutated Fe65 binding site had no effect (Ma et al., 2008). This suggests that AICD is a negative regulator of NPC proliferation and that the binding to Fe65 is necessary for its action. Additionally, in APPKO mice expressing AICD there is a reduction in proliferation of NPC in the adult DG suggested to be due, at least in part, to neuroinflamation in light of the fact that ibuprofen or naproxen treatment could reverse

these alterations (Ghosal et al., 2010). In trisomic NPCs, enhanced AICD binding to the sonic hedgehog (shh) receptor, patched1(ptch1), promoter was sufficient to decrease proliferation. Shh binding to ptch1 results in the suppression of second receptor smoothened (Smo) and repressed signaling (Goodrich et al., 1999; Taipale et al., 2002; Trazzi et al., 2010). Interestingly, in post-mitotic neurons it has been suggested that the interaction of the intracellular domain of APP with APP binding protein 1 (APP-BP1) results in re-entry to the cell cycle and ultimately apoptosis (Chen et al., 2003).

The Aβ peptide is produced from the sequential cleavage of APP by beta- and gamma-secretase and is most well known for its aggregation to form amyloid plaques in AD. Studies on the effect of Aβ with respect to NPC proliferation have generally met with conflicting results. Haughey et al showed that treatment with Aβ in human cortical NPC culture *in vitro* and following intraventricular infusion of Aβ *in vivo*, NPC proliferation was drastically reduced (Haughey et al., 2002a; He and Shen, 2009). He et al observed that Aβ could reduce β-catenin signaling and propose this as a mechanism for Aβ action. These findings were in agreement with the observation that Aβ restricted the formation of colonies from human NPC (Mazur-Kolecka and Frackowiak, 2006). However, other studies have shown that Aβ could stimulate the proliferation of adult murine SVZ derived NPC (Lopez-Toledano and Shelanski, 2004) and that this action may be through the p75 neurotrophin receptor (Sotthibundhu et al., 2009). This contradiction may be due to the use of different isoforms of Aβ or the use of various cell culture systems and potentially different aggregation states of Aβ. Thus the physiological role of Aβ with respect to proliferation of NPC remains unclear.

The above discussion of the affects of APP metabolites in relation to proliferation, suggests great complexity in potential signaling. The complexity of cleavage and the number of metabolites produced confer great convolution in the study of APP function. By attempting to understand the physiological role of each individual metabolite, we are able to better understand the signaling pathways associated. Some of these complexities have been gleaned from the study of mouse models of the APP family. These models not only highlight the complexity in APP signaling but new models may provide vital insight into the role of APP metabolites.

5. Amyloid precursor protein family mouse models and neurogenesis

In order to determine a physiological role for APP, the first logical step was to create an APP-null mouse model and to infer from any impairment what the function of APP may be. Surprisingly, the APP knockout mouse was viable and fertile, giving the first clue that APP homologues may possess functional redundancy with APP. The mice weighed 15-20% less than their wild-type littermates and showed impaired locomotor activity and decreased forelimb grip strength. In the CNS, the only gross alteration was a reactive gliosis in some mice (Zheng et al., 1995; Zheng et al., 1996). Further investigation revealed that APP null mice did exhibit reduced brain weight, reduced size of forebrain commisures and an increased frequency of neuroanatomical abnormalities such as callosal agenisis but these effects were mouse background specific (Magara et al., 1999). APP-null mice also showed aging linked impairments in dendritic length and synaptic plasticity along with both decreased long term potentiation and impaired GABAergic post-synaptic currents. These mice also display aging linked impairments learning and memory (Dawson et al., 1999; Seabrook et al., 1999). Another model of

APP deficiency that utilizes a truncated mutant lacking amino acids 20-75 and expressed at 5% of normal levels (APPΔ/Δ) also shows abnormalities such as callosal agenesis, reduced brain weight and decreased locomotor activity (Muller et al., 1994; Magara et al., 1999). The APPΔ/Δ mice also showed cognitive impairments associated with swimming navigation during the Morris water maze task and an apparent decline in motivation to avoid a noxious stimuli (Muller et al., 1994). Interestingly, mice tested at early postnatal stages (pd 3-10) did not show impairments while major impairments only became evident at later postnatal stages (pd 11-19) and while reduced grip strength persisted into adulthood, deficits in spatial learning, contrary to phenotypic expectation, could not be shown (Tremml et al., 1998). Further investigation revealed that handling during these early periods resulted in rescued impairment in spatial learning as tested by the Morris water maze but failed to rescue impaired activity in an open field test (Tremml et al., 2002). This result suggests that impairments caused by APP deficiency do not completely disrupt plasticity or the ability of environmental enrichment to enhance cognitive function. In fact, studies on NPC derived from E14 APP-null mice showed aberrantly increased neurogenesis (Ma et al., 2008). While the direct affect of APP disruption on neurogenesis *in vivo* in the adult has not been reported, studies on a PDGF-APP$_{WT}$ mouse line overexpressing human APP have shown that the overexpression results in decreased proliferation of NPC in the adult hippocampus, an increased survival rate of these NPC and impaired maturation of NPC to either neuronal or glial fate (Naumann et al., 2010). However, these animals have also been shown to display high levels of Aβ$_{42}$, synaptic dysfunction, neuronal degeneration and alterations in isoform expression patterns (Rockenstein et al., 1995; Mucke et al., 2000; Simon et al., 2009).

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Deletion of APP homologues, APLP1 and APLP2, has also been attempted. Like APP-null mice, both APLP1 and APLP2 knockout mice are viable and fertile (von Koch et al., 1997; Heber et al., 2000). APLP2 knockout mice present with normal size and weight, no gross tissue abnormalities, normal grip strength, normal posture and no apparent cognitive deficits (von Koch et al., 1997). This is in contrast to APP-null mice which exhibit reduced weight, forelimb grip strength and aging linked cognitive deficits. Mice exhibiting a knockout of APLP1 show approximately 10% reduction in body weight but are absent of most other abnormalities associated with APP-null mice including grip strength, locomotor activity, callosal agenesis and spatial learning (Heber et al., 2000). The generation of mouse lines designed to either ablate or overexpress APP and APP homologues present a number of problems with respect to the study of APP function. First, the APP family has been shown to confer much of its activity through its extensive proteolytic cleavage and release of various metabolites. Many of these metabolites have been shown to have potential synergistic and/or antagonistic activities on a number of different cellular processes. The regulation of APP function may not be solely driven by the expression of APP, but through the activity of proteases and formation of metabolites. Simply ablating APP or APP homologues may be valuable in discerning the dominant pathway but does not necessarily represent the most physiological pathway of APP signaling. Second, a number of processes have been shown to affect neurogenesis including neuronal excitability, learning, inflammation, physical activity, enriched environment and stress among others [Reviewed in (Ming and Song, 2005)]. None of the knockout models described above confer spatial or temporal specificity. As such, all systems, cell types and regions are void of APP or APP homologues. Lack of cell type

specificity could lead to alterations in mature neuronal or glial function that could alter neurogenic processes. Impaired LTP (Seabrook et al., 1999), decreased locomotor activity or reactive gliosis (Zheng et al., 1995) as seen in APP-null mice could compromise the study of neurogenesis in these mice. Lack of temporal regulation could also compromise the study of APP function by allowing for compensatory alterations during development that may mask some of the functions of the protein. Finally, functional redundancy between the homologues could hinder the study of single-knockouts by masking the role of a single homologue. For example, APP-null mice express both APLP1 and APLP2 and were shown to be viable and fertile despite the belief that APP plays a major role in development.

To overcome the issue of redundancy APP/APLP1 knockout, APP/APLP2 knockout, APLP1/APLP2 knockout and APP/APLP1/APLP2 triple knockout mice were generated (Heber et al., 2000; Herms et al., 2004). APP/APLP2 and APLP1/APLP2 knockout mice proved to be postnatally lethal, while APP/APLP1 knockout mice were viable and fertile (von Koch et al., 1997; Heber et al., 2000). These results further suggest a functional redundancy between various APP homologues and imply that various homologues may be involved in differential functions. At first there seemed to be few gross abnormalities in double knockouts, even in mice that were postnatally lethal. However, further investigation has revealed that APP/APLP2 double knockout mice have defects at neuromuscular synaptic junctions including increased nerve terminal sprouting, reduced presynaptic vesicles, deficits in neurotransmitter release and a higher incidence of synaptic failure (Wang et al., 2005b). This phenomenon was also seen in interneuronal synapses at the submandibular ganglion (Yang et al., 2005a). In neurons

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derived from APP/APLP2 knockout embryonic stem cells, a decrease in vGLUT2 was correlated with a reduction in field excitatory post synaptic potentials signaling further impairments in neuronal function (Schrenk-Siemens et al., 2008). Keratinocyes derived from APP/APLP2 double knockout mice also show impaired proliferation *in vivo* and *in vitro* that can be rescued by exogenous addition of sAPPα (Siemes et al., 2006). APP and APLP2 have been implicated in lipoprotein and cholesterol metabolism in the brain as well through modulation of the expression of LRP1, a major lipoprotein receptor. APP/APLP2 knockout mice show increased expression of LRP1 that is directly attributable to the lack of AICD as AICD was shown to bind to the LRP1 promoter and repress transcription (Liu et al., 2007). Insulin and glucose homeostasis was shown to be impaired in APP/APLP2 double knockout even further than that of single knockouts, which grow to hypoglycemic adults (Needham et al., 2008). The APP/APLP1/APLP2 triple knockout appears to have the most severe phenotype with cranial abnormalities and cortical dysplasia resembling type 2 lissencephaly where neuroblasts have migrated through the basal lamina and pial membrane (Herms et al., 2004). This phenotype would suggest a critical role for the APP family in migration and adhesion. However, embryonic stem cells derived from triple knockouts were reported to show normal migration, polarity and to form functional synapses (Bergmans et al., 2010). While these models addressed the downfall of previous models in so much as they accounted for functional redundancy, they nonetheless failed to address which metabolite could be ascribed to each phenotypic change in these mice. Ring et al addressed this question by developing a pair of mutant mice, one a sAPPα knock-in and the other an APP knock-in with a truncated C-terminus lacking the YENPTY motif, to understand if sAPPα could

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rescue impairments associated with the APP-null mouse. Indeed, both mutants either attenuated or completely reversed phenotypic alterations in APP-null mice including brain and body weight, locomotor deficits, grip strength impairment, exploratory behavior, long term potentiation and spatial learning deficits (Ring et al., 2007). Studies using similar knock-in models crossed with an APLP2 knockout mutant showed mixed results with the addition of sAPP recovering postnatal lethality in one of the models but unable to ameliorate postnatal lethality in the other (Li et al., 2010; Weyer et al., 2011). Both studies showed that impairments in neuromuscular synapses persisted in sAPPα knock-in double mutants. Further, Weyer et al went on to show that while sAPPα knock-in double mutants had normal CNS morphology, they displayed hippocampal dysfunction, spatial learning deficits and impaired long term potentiation (Weyer et al., 2011). Analysis of differential mRNA expression in the prefrontal cortex of adult wild type, APP-null, APLP2 knockout and sAPPα knock-in mice showed that APP-null and sAPPα knock-in mice had very similar profiles suggesting a potentially dominant role of AICD in transcriptional regulation (Aydin et al., 2011). Recently, the development of APP and APLP2 conditional knockout mice has been accomplished with the hope that they may be crossed with viable single knockouts to allow for postnatal and adult study of the effect of these ablations. The APP conditional knockout mice showed similar deficits in brain weight, body weight and grip strength to the classic APP-null mouse model (Mallm et al., 2010).

C. Alzheimer's disease

1. History

With the dramatic rise in lifespan seen in the 20th century, from approximately 50 years of age to around 75 years of age, the appearance of neurodegenerative conditions linked to aging became increasingly more prominent. One of the most profound and most common of these disorders is Alzheimer's disease. The disease is named for the clinician who was the first to describe the pathological hallmarks of this dementia in 1906, Alois Alzheimer. Clinically he would describe progressive cognitive decline, memory impairment, aphasia, delusions, paranoia and at the latest stages of progression, a decline in motor function. Macroscopic investigations of the brain upon autopsy revealed a decrease in the size of gyri in the brain and an increase in sulci size. Further microscopic evaluation would show the appearance of large dense plaques most abundantly in the frontal, parietal and temporal lobes and the appearance of intracellular fibrils (Alzheimer et al., 1995). Alzheimer was able to distinguish this dementia from typical senile dementia due to the early onset in the described cases, occurring in the patients 40's and 50's. It would take decades before the nature of the pathological hallmarks of AD would be characterized due to the unavailability of methods suitable for such a purpose. The Aβ peptide was first reported to be associated with meningovascular plaques from AD brains (Glenner and Wong, 1984). Subsequent investigations found that this amyloid peptide comprised the amyloid plaques of both AD patients and down syndrome patients showing that they were the derivative of APP which is located on chromosome 21 (Masters et al., 1985; Kang et al., 1987). Around the same time, immunohistocemical manipulations allowed for the discovery that the other hallmark of

AD, neurofibrillary tangles, was composed of the microtubule associated protein, tau. It also became apparent that in AD tau was hyperphosphorylated and dissociated from microtubules (Iqbal et al., 1986; Kosik et al., 1986). By this time it was already apparent that Alzheimer's disease was prevalent in some families and was inherited in an autosomal dominant fashion. However, the underlying cause of these pathological hallmarks remained elusive. In the early 1990's researchers began to describe what would be the first genetic mutations directly associated with AD. The first was a missense mutation in APP at AA 717 which substituted Val→Ile at the carboxyl-terminus of the Aβ region (Goate et al., 1991). Subsequent mutations were found at the N-terminal of the Aβ region of APP (Citron et al., 1992), in PS1 (Sherrington et al., 1995) and PS2 (Levy-Lahad et al., 1995). While the mutations were only found to underlie approximately 5% of Alzheimer's disease cases, termed familial Alzheimer's disease (FAD), they would provide a key understanding of the mechanisms underlying the formation of Alzheimer's disease plaques and neurofibrillary tangles.

2. Mechanisms

Shortly after the discovery of mutations on APP, PS1 and PS2, researchers began to unravel the functional consequence of these anomalies. In the case of APP, missense mutations each occur at or near the proteolytic cleavage sites for β- or γ-secretase. Study of these mutations in cell lines and transgenic animals have revealed that they all increase the amyloidogenic processing of APP, specifically producing larger quantities of the more readily aggregating form of Aβ, $Aβ_{42}$ (Citron et al., 1992; Haass et al., 1994; Suzuki et al., 1994; Scheuner et al., 1996). FAD-linked mutations in PS1 and PS2 are numerous

and not generally localized to one region of the proteins. However, analysis of transgenic mice expressing PS1 mutations shows increased levels of $A\beta_{42}$ in the brain (Duff et al., 1996; Thinakaran et al., 1996b; Citron et al., 1997). In human patients with PS1 mutations, the density of $A\beta_{42}$-containing plaques was higher than that of patients with sporadic forms of AD (Lemere et al., 1996; Mann et al., 1996). Taken together, these results provided evidence that FAD-linked mutations lead to enhanced amyloidogenic processing of APP and increased $A\beta_{42}$.

Due to these insights, the amyloid cascade hypothesis was derived in an attempt to explain a causative link between amyloid plaques and neuronal loss or cognitive decline seen in AD patients. Essentially, this theory stated that mutations in FAD caused increased amyloidogenic processing of APP, leading to accumulation and plaque formation in distinct brain regions. The formation of plaques led to a neuroinflammatory response characterized by activation of microglia and astrocytes (Itagaki et al., 1989). Inflammation and $A\beta$ aggregation were thought to induce free radicals and peroxidative injury (Behl et al., 1994; Harris et al., 1995) and lead to alterations in metabolic process' such as calcium homeostasis (Mattson et al., 1992) that ultimately led to neuronal death. This theory sought to explain neurofibrillary tangle formation as well. Researchers showed that altered kinase activity in AD neurons led to increased tau phosphorylation (Patrick et al., 1999). Tau mutations in humans are able to induce severe neurodegeneration (Spillantini and Goedert, 1998). For quite a number of years, the amyloid cascade hypothesis and the notion that amyloid plaques were essential to neurodegeneration held firm as the dogma in the field.

However, this idea began to change when it was discovered that not only is significant amyloid neuropathology often seen in cognitively normal patients upon autopsy (Dickson et al., 1992), but the degree of soluble $A\beta_{42}$ in the brain is a better predictor of cognitive decline than amyloid plaque density (Nordberg, 2008). More recent evidence suggests that $A\beta$ in soluble forms, particularly oligomeric, may represent the most harmful and detrimental species. There is evidence that soluble $A\beta$ can impair synaptic function by reducing glutamatergic synapse strength and plasticity (Hsia et al., 1999; Walsh et al., 2002). Oligomeric $A\beta$ not only impairs long term potentiation in hippocampal slice preparations, but when injected into the brain can impair memory for a complex learned behavior. Researchers went on to show that dendritic spine density was reduced and that this reduction could be blocked by administration of $A\beta$ antibodies (Selkoe, 2008). These results provided a direct relationship between oligomeric $A\beta$ and memory impairments, the prominent symptom of AD. The collapse of glutamatergic dendritic spines and reduction in synaptic strength has been directly attributed to $A\beta$ induced reductions in AMPA and NMDA receptor expression on the membranes (Hsieh et al., 2006; Shankar et al., 2007). While the complexity of $A\beta$ aggregation states and APP proteolysis makes unraveling the exact mechanisms that drive memory impairments and cognitive decline in AD difficult, research continues to develop new methods that may provide key insights in the future such as imaging techniques for $A\beta$ evaluation in the human brain. It is likely that AD pathogenesis is a complex and multifaceted process that will not be explained by one mechanism. Here we have provided one particularly intriguing mechanistic possibility but the reader should note that the understanding of the

effects Aβ and tau pathology are to this day going through extensive scrutiny and there remains no clear consensus as to the cause of particular deficits in the AD brain.

3. Alzheimer's disease and neural progenitor cell proliferation

The idea that ongoing neurogenesis occurred throughout the lifespan in mammals gave researchers new hope for neurodegenerative conditions such as AD. Could the brain repair damage that was being incurred? Perhaps the first link between AD and neurogenesis was the observation that one of the earliest signs of the disease was anosmia, or a decline in olfactory function (Warner et al., 1986; Serby, 1987; Wilson et al., 2009). The olfactory system is the direct recipient of neuroblasts from the adult SVZ. The second neurogenic region in the adult, the SGL, lies in the hippocampal formation, long noted for its link to several types of learning and memory. Taken together, this evidence might suggest that neurogenesis is not only failing to ameliorate neurodegenerative conditions, but may be impaired in these conditions as well. Thus alterations in neurogenesis may exacerbate AD symptoms. The first transgenic models to investigate neurogenesis in AD were FAD-linked mutants that possess either mutations in APP or PS1, most times causing a shift in cleavage patterns toward the amyloidogenic pathway, up-regulating beta- or gamma- secretase cleavage and producing higher levels of Aβ [For Review (Selkoe, 2001)]. The affect of these mutations on neurogenic processes has been reviewed extensively (Lazarov and Marr, 2010). Briefly, APP mutants have been shown to have both decreased (Haughey et al., 2002a; Haughey et al., 2002b; Donovan et al., 2006) and increased (Lopez-Toledano and Shelanski, 2007; Kolecki et al., 2008) proliferation of NPC. In mice exhibiting mutations in both APP and PS1, studies have for the most part shown impaired proliferation (Lopez-Toledano and

Shelanski, 2007; Zhang et al., 2007a; Kolecki et al., 2008). These studies have all shown

impairments in mice after amyloid deposition and plaque formation which can induce

inflammation and may impact neurogenesis directly. As we first described in the

APPSwe/PS1ΔE9 mouse, impairments in proliferation and neurogenesis occur before

memory impairment or pathological alterations in these mice (Demars et al., 2010b).

Furthermore, when NPC are cultured from the SVZ of 2 month old APPSwe/PS1ΔE9

mice, they show impairments in proliferation *in vitro* suggesting that mutations may

cause intrinsic impairments in NPC proliferation irrespective of environmental or niche

cues. This supports the notion that neurogenic impairments could exacerbate the

symptoms of AD. In human AD patients, it was first reported that the expression of

immature neuronal markers were increased (Jin et al., 2004). However, this report was

later challenged (Boekhoorn et al., 2006) and subsequent investigations revealed

decreased proliferation of NPC in the AD brain (Brinton and Wang, 2006). The

mechanism through which FAD mutations or AD pathology affect NPC proliferation and

neurogensis is not fully established. As previously mentioned, studies on the direct effect

of Aβ with respect to NPC proliferation have generally met with conflicting results.

Haughey et al showed that treatment with Aβ in human cortical NPC culture *in vitro* and

following intraventricular infusion of Aβ *in vivo*, NPC proliferation was drastically

reduced (Haughey et al., 2002a; He and Shen, 2009). He et al observed that Aβ could

reduce β-catenin signaling and propose this as a mechanism for Aβ action. These

findings were in agreement with the observation that Aβ restricted the formation of

colonies from human NPC (Mazur-Kolecka et al., 2006). However, other studies have

shown that Aβ could stimulate the proliferation of adult murine SVZ derived NPC

(Lopez-Toledano and Shelanski, 2004) and that this action may be through the p75

neurotrophin receptor (Sotthibundhu et al., 2009). This contradiction may be due to the

use of different isoforms of Aβ or the use of various cell culture systems and potentially

different aggregation states of Aβ. Thus the physiological role of Aβ with respect to

proliferation of NPC remains unclear. However, the previous discussion of APP

metabolism and AD conveys the complexity of potential neurogenic signaling with

respect to APP metabolites. It is likely that the alteration in the metabolism and a shift in

the dynamics of metabolites, rather than a single metabolite, are responsible for

neurogenic alterations in AD.

4. Neurogenesis in Normal Aging

The greatest risk factor for the generation of AD is aging. To date there is no

known cause for the sporadic, late-onset form of AD. However, insight gleaned from

normal aging may provide a key to understanding what goes wrong in AD. With respect

to neurogenesis, both neurogenic niches of the adult brain, SVZ and SGL, exhibit aging

linked decline in neurogenesis (Bernal and Peterson, 2004). This decline occurs in a

nonlinear pattern, with neurogenesis peaking around 6-8 weeks in the SGL of mice and

declining sharply thereafter (Cameron and McKay, 1999; Kronenberg et al., 2006). This

decline has been shown to manifest in a decline in olfactory function and hippocampal-

dependent spatial learning tasks (Bizon et al., 2004; Enwere et al., 2004; Dupret et al.,

2008). There is much debate in the field as to the cause of decline in neurogenesis with

aging. Some studies have shown that proliferation and maturation of NPC declines with

age (Kuhn et al., 1996; Rao et al., 2005). Further investigations have shown that the

newly formed neurons that do mature properly and survive form functional synapses and

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appear to be no different from neurons born during early adulthood (Toni et al., 2008).

However, other groups have argued that the rate of proliferation does not decline with

aging, but the cells may exhibit increased quiescence that may be attributed to a decline

in the vascular niche (Hattiangady and Shetty, 2008). While this debate has not yet been

settled, it is apparent that changes do occur in the neurogenic niche that could underlie

alterations in neurogenic signaling. It is well established that many of the growth factors

and receptors that are integral to neurogenesis in the adult peak during development and

decline thereafter [For review see (Klempin and Kempermann, 2007)]. These factors

include FGF2, IGF-1, VEGF and FGFR-2 (Shetty et al., 2005; Chadashvili and Peterson,

2006). Supporting the theory of waning trophic support in the neurogenic niche, NPC

derived from the brains of aged mice show no impairments in proliferation *in vitro* in the

presence of exogenous growth factor support (Ahlenius et al., 2009).

While there remains some controversy over what drives neurogenic decline in the

aging brain, it has become increasingly apparent that the impairments do not intrinsically

prevent NPC from proliferating in response to stimuli. Housing mice in cages with

running wheels can partially rescue some of the aging-linked impairments in

neurogenesis (Kronenberg et al., 2006). Running is thought to be the key component to

environmental enrichment induced neurogenic increases (van Praag, 2008). Likewise,

the infusion of exogenous factors into the neurogenic niche such as FGF-2 and HB-EGF

were sufficient to restore some NPC proliferation in both the SVZ and SGL of 20 month

old mice (Jin et al., 2003). These data provide key evidence that NPC of the aging brain

retain the ability to proliferate in response to stimuli and thus may be susceptible to

pharmacological or therapeutic interventions.

In conclusion, this introduction has focused on the main concepts that underlie the questions addressed in this thesis. It has covered much of the basic background as well as some more in depth concepts concerning APP, neurogenesis in the adult brain, neurogenesis and AD and neurogenesis in normal aging. By understanding the basic concepts surrounding APP metabolism and neurogenesis these studies have been shaped around the following idea: the metabolism of APP to form sAPPα provides trophic support for NPC of the adult brain. Alternate cleavage to form sAPPβ does not stimulate proliferation to the same extent. Thus, alterations in the cleavage pattern or expression of APP during normal aging or AD leads to reduced NPC trophic support and decreased proliferation. Aging NPC retain the ability to respond to sAPPα stimulation and therefore sAPPα could provide significant therapeutic value.

CHAPTER 2:

SPECIFIC AIMS

Rationale: APP undergoes complex metabolic cleavage by at least three different enzymatic activities termed α-, β-, and γ-secretase. These cleavage events produce a number of intra- and extracellular metabolites. In the non-amyloidogenic pathway of APP cleavage, α-secretase cleavage produces the extracellular metabolite, sAPPα. sAPP is thought to have the structure of a growth factor and has been shown to enhance proliferation in a number of different cell types. Of particular interest is the observation that, EGFR-expressing rapidly proliferating NPC in the adult SVZ have binding sites for sAPP. In both FAD and normal aging, proliferation of NPC in the adult brain is dramatically reduced. Studies have shown that intrinsic mutations in FAD may alter NPC proliferation. In aging, one theory suggests that waning trophic support in the niche microenvironment leads to reduced proliferation. However, the mechanisms underlying these changes are poorly understood. In this proposal, we hypothesize that *sAPPα is a proliferation factor for stem cells of various origins and that a decline in the metabolism of APP/sAPPα during aging and in Alzheimer's disease contributes to the senescence of stem cells.* This hypothesis was tested using the following aims:

Aim 1 - *To determine the role of sAPPα in self-renewal and proliferation of NPC in the adult brain.*

Aim 2 - *To elucidate the molecular mechanism(s) underlying sAPPα regulation of neural progenitor cell proliferation in the adult brain*

Aim 3 - *To determine the role of sAPP in aging- and FAD-linked deficits in neurogenesis*

CHAPTER 3:

EXPERIMENTAL DESIGN

In order to test the hypothesis that sAPPα is a growth factor for NPC that may have altered expression in FAD and aging, an in-depth analysis of sAPPα function with respect to NPC was carried out. The following is the experimental design that corresponds to each of the major questions asked in this work:

1. *Does sAPPα promote the proliferation of NPC derived from the adult SVZ?* To accomplish this, we derived NPC from the SVZ of 2 month old C57/Bl6 mice and used a broad-spectrum matrix metalloproteinase inhibitor (GM6001) to examine the effect of α-secretase inhibition. Further, we tried to rescue deficits in proliferation with either conditioned media containing sAPPα or a recombinant sAPPα. We then examined the nature of sAPPα induced alterations by utilizing immunocytochemistry to examine alterations in cell-type specific proliferation. We also used *in vivo* immunhistochemistry and Western blotting to examine the nature of sAPP and α-secretase, ADAM10, in the neurogenic regions of the brain.

2. *Is sAPPα a ubiquitous stem cell growth factor?* As APP is ubiquitously expressed, we utilized mesenchymal and human placental stem cell cultures to determine if the trophic properties of sAPPα were indeed ubiquitous to stem cells of various lineages. We again treated cells with matrix metalloproteinase inhibitors and recombinant sAPPα then assessed total cell numbers following 3 days *in vitro*.

3. *What are the molecular pathways that drive sAPPα signaling in NPC?* To begin to unravel the basis of sAPPα trophic properties in NPC we first examined the possibility of its action as a co-factor by examining proliferation in a clonogenic

assay without exogenous growth factor support. We also used phosphorylation sensitive Western blotting to examine the downstream effects of sAPPα on the mitogen activated protein kinase (MAPK) and protein kinase B (Akt) pathways. Both are known to respond to sAPPα and are integral to NPC proliferation. Finally, we used microarray analysis to provide insight into potential gene targets of sAPPα signaling.

4. *Do the amyloidogenic pathway of APP cleavage and sAPPβ confer the same trophic properties to NPC as sAPPα?* To examine the signaling of sAPPβ with respect to NPC, we utilized a β-secretase inhibitor, recombinant sAPPβ and clonogenic analysis. We also examined the potential for toxicity of sAPPβ through the use of MTT and active caspase-3 analysis.

5. *Do changes occur in APP metabolism with aging and can sAPPα ameliorate aging-linked decline in neurogenesis?* To determine if APP metabolism changes during aging in NPC, we performed Western blot analysis of NPC and conditioned media of NPC derived from 2 month and 7-9 month old C57/Bl6 mice. We also examined basal levels of neurogenesis by immunohistochemistry and stereological analysis in the SVZ and SGL of 2, 7-9 and 20 month old mice. Finally, in order to determine the effect of sAPPα and sAPPβ on proliferation in the aged brain, we performed intracerebroventricular injections of sAPP's in 7-9 month old mice, followed by BrdU injection, immunohistochemical analysis and stereology.

CHAPTER 4:

MATERIALS AND METHODS

A. Animals

The APP knockout [APP (-/-)] model has been described previously

(Zheng et al., 1995; Zheng et al., 1996). Briefly, the authors generated the mice through

homologous recombination in embryonic stem cells. Mice heterozygous for APP

expression where cross-mated and APP (+/+) (APP wild type), APP (+/-) and APP (-/-),

resulted from this breeding. Wild type mice 2 months, 7-9 months and 20 months of age

are on a C57Bl/6 background and were maintained in our colony. The 7-9 month old

animals were purchased as retired breeders from Jackson Laboratories (Bar Harbor,

Maine). Our colony is maintained via group housing (<5 mice per cage) in a barrier

facility under a 14:10 light:dark cycle with free access to food and water. Animal care

and procedures were conducted according to the National Institutes of Health Guide for

the Care and Use of Laboratory Animals.

B. Primary neurosphere culture

Two-month-old C57/Bl6 wild-type mice were euthanized and their brains were

removed and placed into sterile Dulbecco's modified Eagle's medium/F12 (DMEM/F12).

A coronal slice (approximately 1 mm) was dissected starting 1 to 2 mm posterior to the

olfactory bulb. The region occupying the lateral wall and anterior horn of the lateral

ventricles was removed with the aid of a dissecting microscope and diced with a sterile

scalpel. Neurosphere culture was prepared as previously described (Demars et al.,

2010b). Briefly, tissue pieces were collected in a mixture of Papain and DNase in Earl's

balanced salt solution and incubated at 37°C for 40 minutes. Then, tissue pieces were

pelleted by centrifugation and dissociated to a single-cell suspension, and cells were plated in complete medium-water, DMEM/F12 (Gibco, now part of Invitrogen Corporation, Carlsbad, CA, USA), glucose (Sigma-Aldrich, St. Louis, MO, USA), NaHCO3 (Sigma-Aldrich), HEPES (Sigma-Aldrich), L-glutamine (Invitrogen Corporation), penicillin/streptomycin (Invitrogen Corporation), putrescine (9.6 μg/mL; Sigma-Aldrich), apotransferrin (0.1 mg/mL; Sigma-Aldrich), insulin (0.025 mg/mL; Roche, Indianapolis, IN, USA), selenium (5.2 ng/mL; Sigma-Aldrich), progesterone (6.3 ng/mL; Sigma-Aldrich), bovine serum albumin (BSA) (2 mg/mL; Sigma-Aldrich), heparin (4μg/mL; Sigma-Aldrich), EGF (20 ng/mL; PeproTech Rocky Hill, NJ, USA), and bFGF (10 ng/mL; Pepro-Tech)-and passaged after 10 days.

C. **Isolation of mesenchymal stem cells**

After euthanasia, the bone marrow contents of the femurs and tibia of donor Balb/C mice were flushed through a 40-μm filter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) into a 50-mL tube (Corning, Corning, NY, USA) containing MSC media: 40% alpha-modified Eagle's medium (Invitrogen Corporation, Rockville, MD, USA), 40% F-12 nutrient mixture (Invitrogen Corporation), 10% fetal bovine serum (Valley Biomedical, Winchester, VA, USA), and 1% antibiotic-antimycotic solution (Invitrogen Corporation). Bone marrow cells were plated at a density of 20×10^6 per 9.6 cm^2 in MSC media at 37°C in 5% CO2 as previously described (Polchert et al., 2008). The non-adherent population was removed after 72 hours, and the adherent cells were washed with fresh media and cultured for 7 days. The resulting adherent cells were harvested by incubating with 0.25% trypsin (Invitrogen Corporation) followed by gentle scraping. By means of negative selection via immunomagnetic column (Miltenyi Biotec,

Auburn, CA, USA), cells negative for CD11b (eBioscience, San Diego, CA, USA) and CD45 (eBioscience) were placed back into culture. A homogenous cell phenotype was confirmed on the basis of the expression of CD29, CD44, and Sca1 and the absence of hematopoietic (CD45, CD14, and CD11b) markers. Prior to use, cells had been passaged from one to four times.

D. **Human decidua parietalis placental stem cells**

All studies were approved by the Institutional Review Board of the University of Illinois. hdpPSCs were isolated from the decidua parietalis dissected from placental membranes after normal vaginal delivery at term, as previously described in detail (Strakova et al., 2008). Human placenta tissue was obtained from the Human Female Reproductive Tissue bank in the Center for Women's Health and Reproduction at the University of Illinois at Chicago. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated and charcoal-stripped fetal bovine serum, 0.1 mM sodium pyruvate, and 1% penicillin/streptomycin. At confluence, cells were trypsinized, propagated, and used for experiments in passage numbers three to five.

E. **Neurosphere formation (clonogenic) assay**

Briefly, neurospheres were singly dissociated by mechanical dissociation and plated at 1,000 cells per well onto 96-well plates. For matrix-metalloproteinase (MMP) inhibitor experiments, cells were then treated with the indicated molar concentration of GM6001 or GM6001 negative control (Millipore Corporation) and the indicated molar concentrations of sAPP or conditioned media. If not otherwise indicated, 1 μM GM6001 and negative control inactive inhibitor (NC) were used. Cells were treated every 72 hours for 10 days. After 10 days in culture, neurospheres were

counted under an inverted light microscope, and the average neurosphere diameter was calculated from 25 randomly assigned squares of the grid by using a Zeiss AX10 microscope (Carl Zeiss Ltd., Hertfordshire, UK) and StereoInvestigator software (MBF Bioscience, Williston, VT, USA). After sphere size determination, cells were singly dissociated with a p200 pipette and counted with a hemocytometer. The remaining cells were placed onto Matrigel-coated chamber slides for 30 minutes and then fixed in 4% paraformaldehyde for 30 minutes for immunocytochemistry. Briefly, cells were washed four times in Tris-buffered saline (TBS) and then placed into blocking solution (5% normal donkey serum, 0.25% Triton-X 100 in TBS) for 30 minutes at room temperature. Next, cells were incubated in primary antibodies-mouse anti-nestin (1:100; Millipore Corporation) and goat anti-Sox2 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)-in TBS containing 0.25% Triton-X 100 for 1 hour at room temperature. After primary antibodies, cells were again incubated in blocking solution for 30 minutes at room temperature before secondary antibody incubation-anti-goat cy5 (1:250; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and antimouse cy3 (1:500; Jackson ImmunoResearch Laboratories, Inc.) in TBS with 0.25% Triton-X 100 for 30 minutes at room temperature in the dark. Cells were then washed four times in TBS and incubated for 5 minutes with DAPI (4'-6-diamidino-2-phenylindole) (1:50,000; Invitrogen Corporation) at room temperature in the dark. Cells were then washed three times and mounted with polyvinyl alcohol-DABCO mounting solution. Cell counts were made by means of StereoInvestigator software version 8 (MBF Bioscience).

F. BrdU incorporation assay

NPC were dissociated into single cells and were plated into 96-well plates (Becton Dickinson tissue culture treated round bottom) (12,500 cells/well). Cells were treated with GM6001 or GM6001 negative control (Chemicon, Billerica, MA) and conditioned media if applicable. Following 4 hours, each well was treated with 5 µM BrdU and incubated for 72 hours at 37°C, 5% CO_2. Cells were fixed with 70% ethanol/0.1N NaOH for 30 minutes at room temperature, blocked with 5% donkey serum and then incubated in mouse mAb BrdU (1:300, Novocastra, Newcastle, UK) for 1 hour at room temperature. Cells were rinsed in 1XTBS three times and incubated in secondary antibody rabbit anti-mouse HRP (1:5000, Pierce) for 30 minutes at room temperature, followed by the addition of tetramethylbenzidine (TMB) substrate solution (Invitrogen) in the dark for 15 minutes. The reaction was terminated by adding 2.5N sulfuric acid into each well. Absorbance was measured using a DTX-880 Multimode Detector (Beckman Coulter, Brea, CA) at dual wavelength of 450-595 nm. Each experimental group includes 5 replicates (N=5).

G. Self renewal assay

Neurospheres were dissociated mechanically and plated 1,000 cells/well in a 96 well plate with 100µl media and treated every 72 hrs with GM6001 negative control (NC) GM6001 (GM) or GM6001 + recombinant sAPPα (GM+rec sAPP). Following 8 DIV, the total number of neurospheres was counted and cells were removed by mechanical pipetting, spun and recounted before again plating at 1,000 cells/well. Cells were again treated every 72 hours and the secondary neurosphere number was counted after 8 DIV. The steps were repeated as above for the tertiary neurospheres. N=6.

H. Conditioned media

Neurosphere media was conditioned by plating 3×10^5 NPCs in each well of a 12-well plate in 500 μL of complete media. After 1 hour, media was removed and spun at 1,000g for 10 minutes to remove any cells or debris. For depletion of sAPP, conditioned media was precleared with protein A-agarose beads (Pierce, Rockford, IL, USA) and then incubated overnight at 4°C with 22C11 antibodies against the N-terminus of APP or IgG antibodies (Millipore Corporation, Billerica, MA, USA) as a control. Protein A-agarose beads were added for 30 minutes, the mixture was spun at 4,000 revolutions per minute (rpm) for 3 minutes, and the supernatant was used as depleted media. Regular conditioned media was subjected to the same process without antibody incubation as a control. All media was filtered through a 0.22-mm filter prior to addition. For sAPPα vs. sAPPβ enriched conditioned media experiments, N2a cells that express either WT APP$_{695}$ or FAD-linked mutant APPswe were used. The "Swedish" mutation enhances β-secretase activity and thus produces a conditioned media enriched in sAPPβ (Thinakaran et al., 1996a).

I. Recombinant sAPPα and sAPPβ

sAPPα (Sigma-Aldrich) was used at 10 nM concentrations unless otherwise indicated (dissolved in phosphate-buffered saline, or PBS). sAPPβ (Sigma-Aldrich) was also dissolved in PBS and is used in the indicated concentrations.

J. Detection of sAPP

For the detection of soluble APP from brain lysates, protein was extracted in immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris-Cl, 5 mM

ethylenediaminetetraacetic acid (EDTA), 1% Triton-X 100, 0.5% sodium deoxycholate, protease inhibitor cocktail, and 250 μM phenylmethylsulfonyl fluoride. To remove full-length APP, protein samples were immunodepleted by using 369 antibodies against the C-terminus of APP (a gift from Sangram S Sisodia, The University of Chicago). Briefly, samples were precleared with 50 μL of immobilized protein A-agarose beads (Pierce) at 4°C for 30 minutes. Samples were centrifuged at 4,000 rpm for 3 minutes, and the pellet was discarded. To the supernatant, 5 μL of 369 antibody was added and incubated overnight at 4°C. The next morning, 50 μL of immobilized protein A was again added for 30 minutes at 4°C and spun at 4,000 rpm for 3 minutes. The pellet contains the full-length APP-369 antibody complex, and the supernatant was probed for sAPP by using 22C11 antibodies raised against the N-terminus of APP (Millipore Corporation).

K. <u>Brain tissue processing</u>

For in vivo immunohistochemical staining, all mice were anesthetized with a mixture of ketamine and xylazine and transcardially perfused with 100 mL of ice-cold PBS. The brains were then removed and halved in the sagittal plane. The left half was immediately placed into 4% paraformaldehyde on ice. From the right half of the brain, the following regions were dissected for biochemical analysis and immediately placed into Eppendorf tubes on dry ice: SVZ, hippocampus, olfactory bulb, frontal cortex, and cerebellum.

L. <u>Immunohistochemistry</u>

Left hemibrains from PBS-perfused mice were post-fixed in 4% paraformaldehyde for 3 days and stored in 30% sucrose at 4°C. Hemibrains were sectioned sagittally at

50 μm by using a microtome and placed into cryopreservent (47.6% PBS, 28.57% ethylene glycol, and 25% glycerin vol/vol). Sections were blocked by using a solution containing 0.25% vol/vol Triton-X 100 (Sigma-Aldrich) and 5% vol/vol Normal Donkey Serum (Jackson ImmunoResearch Laboratories, Inc.) in TBS. The following antibodies were used: BrdU (1:400; Accurate Chemicals, Westbury, New York), Dlx-2 (1:200; Millipore Corporation), nestin (1:100; Millipore Corporation), doublecortin (1:400; Santa Cruz Biotechnology, Inc.), GFAP (1:500 Millipore Corporation), NeuN (1:400; Millipore Corporation) Sox2 (1:100; Santa Cruz Biotechnology, Inc.), ADAM10 (1:200; Millipore Corporation), and APP (22C11; Millipore Corporation and A8717; Sigma-Aldrich). Floating sections were incubated in primary antibodies for 72 hours at 4°C before continuing with blocking, biotin conjugation (Jackson ImmunoResearch Laboratories, Inc.), and secondary antibody incubation (cy2 Streptavidin, anti-mouse cy3, anti-goat cy5, and anti-rabbit cy5; Jackson ImmunoResearch Laboratories, Inc.).

M. <u>Stereological analysis</u>

The number of positively stained cells in sagittal brain sections was quantified using design-based stereology (StereoInvestigator version 8, MBF Bioscience, Williston, VT, USA). For the analysis, every sixth section of brain tissue was quantified by applying Nv x VRef method. The following parameters were used; for SVZ, sections were traced using a Zeiss AX10 microscope (Carl Zeiss Ltd., Hertfordshire, England) in low magnification (5X) and counting was performed at high magnification (63X), counting frame= 100μmX100μm, grid size 200μmX300μm and all sections were counted using 15μm top and bottom guard zones. For the dentate gyrus the counting frame was set equal to the grid size (140μm X140μm) in order to count the entirety of the DG due to

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the relative paucity of cells. All other parameters remained the same. All stereological

experiments were done with an N ≥ 4).

N. <u>Western blotting</u>

Protein extraction from brain tissue was performed in lysis buffer containing 1X

TNE, 50 mM Tris, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail (Sigma-

Aldrich), and 100 mM PMSF. Quantification of protein was performed by using the

bicinchoninic acid (BCA) method (Pierce), and equal amounts of protein were subjected

to direct immunoblotting. For the extraction of protein from neurosphere, mesenchymal,

and Human decidua parietalis placenta stem cells cultures, a lysis buffer containing 150

mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 1% Triton-X 100, 0.5% sodium deoxycholate,

protease inhibitor cocktail, and 250 μM PMSF was used. For quantification, at least three

cultures were used.

O. <u>Erk and Akt signaling</u>

To assay phosphorylation of Erk and Akt, neurospheres were singly dissociated

and plated at 5×10^5 cells per well in a six-well plate in Earle's balanced salt solution

(Sigma-Aldrich) and treated immediately with 1 μM GM6001 or GM6001 negative

control. After 1-hour incubation at 37°C, one of the GM6001-treated groups was

subsequently treated with 10 nM recombinant sAPPα (Sigma-Aldrich) for 15 minutes

before all groups were lysed in ROLB buffer: 10 mM HEPES, pH 7.4, 0.5% Triton X-

100, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate,

100 nM staurosporine, 100 nM K252a, 50 nM okadaic acid, 50 nM microcystin,

mammalian protease inhibitor cocktail (Sigma-Aldrich), and phosphatase inhibitor

cocktail II (Calbiochem, now part of EMD Biosciences, Inc., San Diego, CA, USA) in water. After lysis, protein quantification was performed by using the BCA method (Pierce), and equal amounts of protein were run on Tris-glycine gels and transferred to nitrocellulose membranes. For blocking and antibodies, we employed a solution of 0.05% vol/vol Tween, 10% wt/vol milk, and 0.1% wt/vol BSA (Sigma-Aldrich) in TBS. The following antibodies were used: pErk (1:500; Santa Cruz Biotechnology, Inc.), Erk (1:1,000; Santa Cruz Biotechnology, Inc.), pAkt (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), and Akt (1:500; Cell Signaling Technology, Inc.). (N = 3 for Erk and Akt Western blot quantification).

P. **Microarray analysis**

Microarray analysis was done based on three different neurosphere preparations and samples were prepared in triplicates. Specifically, 500,000 NPC were plated and treated with either 1μM GM6001, 1μM GM6001 + 10nM rec sAPP or 1μM inactive GM6001 inhibitor control compound. Following 20 hours of treatment, total cellular RNA was isolated with the use of RNeasy columns (Qiagen) according to the manufacturer's protocol. RNA quality control was performed with the use of automated electrophoresis system Experion (BioRad). Labeling reactions and hybridizations were carried out according to the standard GeneChip® WTsense target labeling protocol (Affymetrix Mouse Gene 1.0 ST array). A 100 ng of total cellular RNA per sample was used for each labeling reaction. Hybridizations were followed by binding to streptavidin-conjugated fluorescent marker. Detection of bound probe was achieved following laser excitation of the fluorescent marker and scanning of the resultant emission spectra using a scanning confocal laser microscope (Affymetrix). Data acquisition was performed

using Affymetrix AGCC suite. Hybridization images were subjected to quality control with the use of Expression Console analysis tool (Affymetrix). Data analysis was performed as follows: Signal normalization, background correction, additional quality control and all statistical analysis were performed with the use of Partek Genomics Suite (Partek). Robust Multi-array Average (RMA) method was used to background correct and normalize hybridization signal intensities across all collected samples. ANOVA test was used to calculate significance of the differential expression. Raw p-values were corrected for False Discovery Rate (FDR) according to step-up or Benjamini-Hochberg (BH) procedure. Differentially expressed transcripts were annotated according to the latest current release of Affymetrix NetAffx Analysis Center. We performed KEGG analysis using WebGestalt2 (**WEB**-based **GE**ne **SeT AnaL**ysis **T**oolkit). We partitioned the data into those genes up- or down-regulated significantly in either GM+sAPP v. Control or GM v. Control groups and ran a KEGG analysis using the mouse genome as a reference set. We used hypergeometric statistics with a Benjamini-Hochberg (BH) multiple test adjustment and a significance level of 0.05.

Q. <u>Intracerebroventricular sAPP injection</u>

A PBS vehicle or recombinant sAPPα or sAPPβ (Sigma-Aldrich) at a concentration of 1μM (1μl/mouse; 0.25μl/min.) were stereotaxically injected into the left lateral ventricle of 7-9 month old C57BL/6 mice using the following coordinates: (anteroposterior, 0 mm; mediolateral, -0.8 mm; dorsoventral, -2.0mm from bregma). Mice were anesthetized using a mixture of ketamine and xylazine. The heads of the mice were then shaved and wiped with 70% ethanol. Animals were placed into a stereotaxic frame and a one-inch incision was made in the midline of the scalp to reveal the bregma.

The scalp was rinsed with 30% hydrogenperoxide and a small hole was drilled at the coordinate site as measured from bregma according to the mouse atlas (Franklin and Paxinos, 2008). Animals then received unilateral injection of sAPP to the lateral ventricle. The sAPP was delivered through a 5µl Hamilton syringe connected to a hydraulic injection system set to inject at a rate of 0.25µl/min. The injection needle was then left in place for an additional minute to ensure distribution on the solution. The needle was slowly removed and the incision closed using EZ-clips from Stoelting. Following 6 hrs of recovery, mice were given a single IP dose of 100mg/kg BrdU solution. 24 hours after the BrdU injection, mice were transcardially perfused and the brains processed for immunohistochemistry as previously described.

R. <u>Statistical analysis</u>

The results within are presented as the mean ± standard error. Statistical significance was analyzed by Student's t-test or ANOVA with post-hoc analysis where applicable.

CHAPTER 5:

EXPERIMENTAL RESULTS

1. Soluble amyloid precursor protein alpha promotes proliferation of neural progenitor cells derived from the adult subventricular zone.

Proteolytic cleavage of amyloid precursor protein (APP) by α-secretase to produce soluble APP-alpha (sAPPα) is the first step in the sequential processing of the non-amyloidogenic pathway of APP metabolism. sAPP contains a cysteine-rich growth factor-like domain and has been shown to stimulate the proliferation of a variety of cell types. Saturable binding sites for sAPPα are localized to type-C, EGF-responsive, neural progenitor cells (NPC) in the adult subventricular zone (SVZ). However, the direct effect of sAPPα on NPC proliferation has not been addressed. Figure 1 shows the expression of APP in the SVZ with respect to NPC populations. The top panels depict APP expression surrounding the lateral ventricle in APP(+/+) (left) and APP (-/-) (right). APP expression is particularly high in the SVZ immediately surrounding the ventricle. The lower panels represent APP co-localization with NPC markers Sox-2, nestin and Dlx-2. While Sox-2 and nestin are expressed in both neural stem cells (NSC) and NPC, Dlx-2 expression is restricted to rapidly proliferating NPC. APP is co-localized with all three markers suggesting it is expressed in NPC and potentially in NSC as well.

In order to produce sAPPα, APP must be cleaved inside the amyloid beta (Aβ) region by α-secretase. Potentially the most active α-secretase enzyme in the brain is a member of the a disintegrase and metalloproteinase (ADAM) family of enzymes, ADAM10. Figure 2A shows the expression of ADAM10 in the SVZ of the adult brain. Like APP, ADAM10 staining is particularly high in the SVZ immediately surrounding
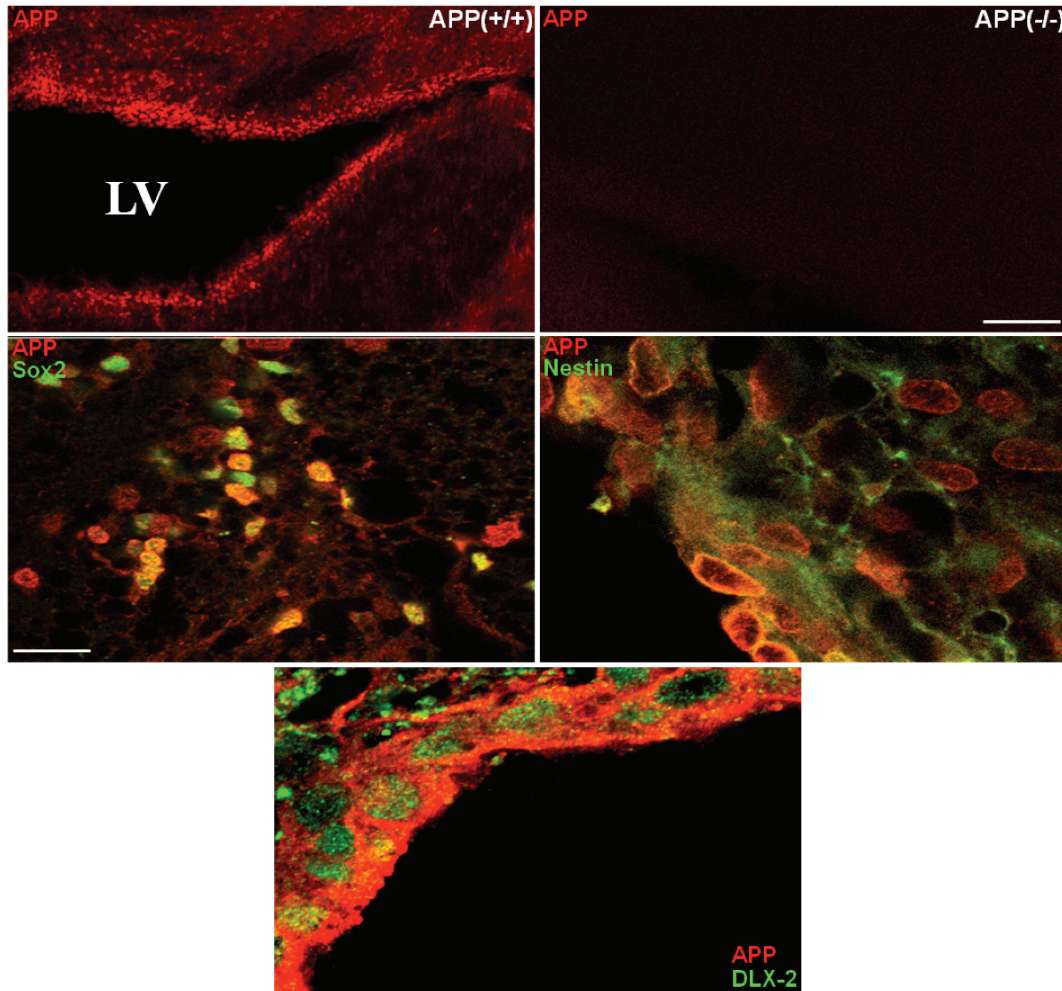
Figure 1. Amyloid precursor protein is expressed in neural stem and progenitor cells in the adult subventricular zone. To establish that APP is expressed in NPC of the subventricular zone, we performed immunohistochemical labeling of tissue with APP antibodies (22C11 and A8717 Sigma), Sox2 and nestin for neural progenitor and stem cells and Dlx-2 for transit amplifying type-C cells. APP expression was shown to be co-localized with each of these markers suggesting it is expressed in NPC and stem cells of the adult SVZ (Figure 1).
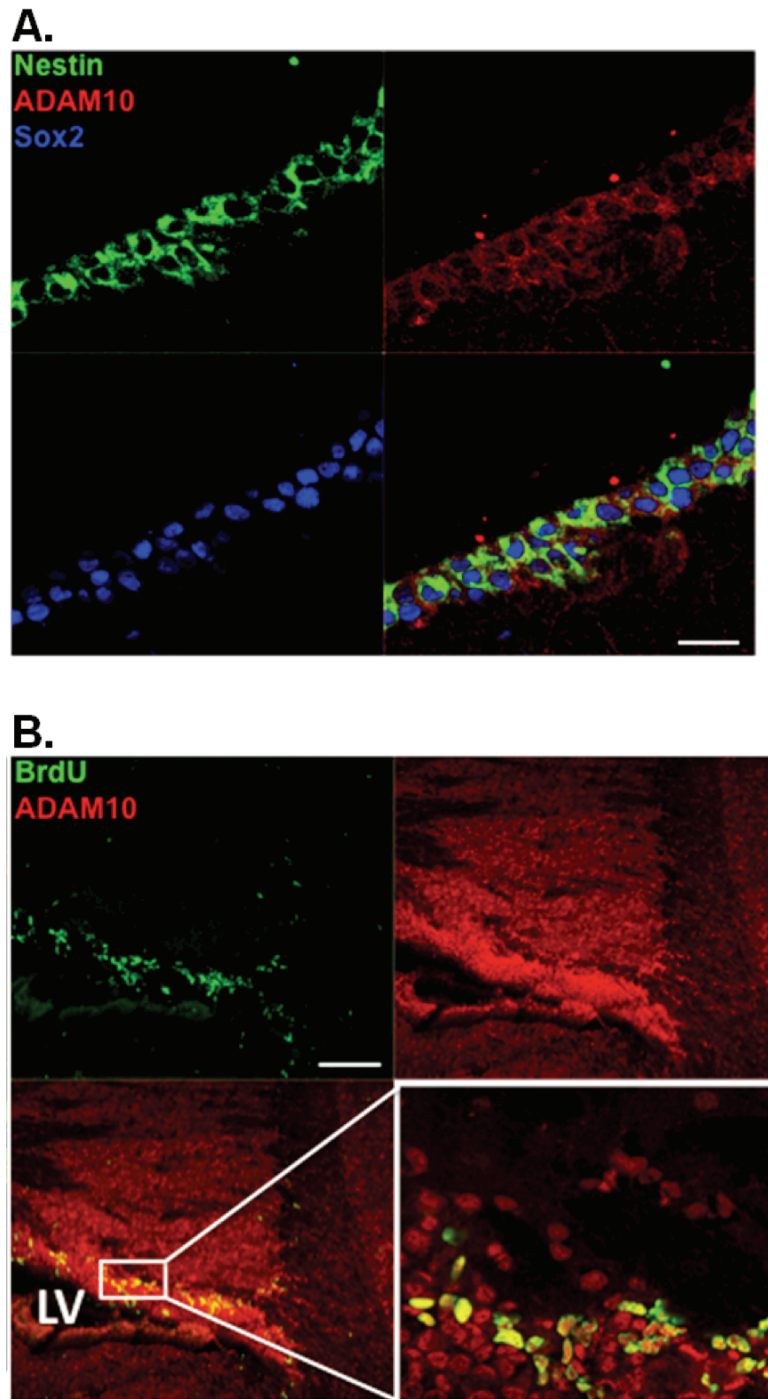
Figure 2. ADAM10 is expressed in neural progenitor cells of the adult SVZ. (A)
Staining for ADAM10 and NPC markers, Sox2 and nestin, show strong co-localization in
the adult mouse SVZ. (B) Mice were injected with BrdU 24 hours prior to sacrifice and
the expression of ADAM10 in proliferative cells was examined by co-localization of
ADAM10 with BrdU in the SVZ.

the ventricles. ADAM10 also co-localizes with markers of both NPC and NSC. For analysis of ADAM10 expression in proliferating populations in the adult SVZ, we injected 5-bromo-2'-deoxyuridine (BrdU) into mice 24 hours prior to sacrifice. Staining for ADAM10 and BrdU clearly shows strong co-localization in the adult SVZ (Figure 2B). These results suggest that both APP and ADAM10 are expressed in NPC and NSC of the adult brain.

To examine the level of expression of APP, sAPP and ADAM10 in the neurogenic regions of the brain, we micro-dissected the SVZ, hippocampus and cerebellum and subjected the protein lysates to Western blot analysis. Figure 3A depicts Western blot analysis of APP (+/+), APP (+/-) and APP (-/-) SVZ, hippocampus and cerebellum. This blot was probed with 22C11 antibodies which recognize the N-terminal portion of APP and thus represents both sAPP and full-length APP (fl-APP). As expected, APP(+/+) animals show greater expression than the APP(+/-), which only possess one allele of APP. APP(-/-) samples show no reactivity. The SVZ, highly enriched in NPC, shows a slight elevation of APP when compared with the less enriched hippocampus or non-neurogenic cerebellum. In order to investigate the expression of sAPP, we first immunodepleted fl-APP from our samples by using 369 or CT-15 antibodies directed at the C-terminal region of APP. We then probed the depleted fractions with 22C11 antibodies for sAPP detection. sAPP expression is markedly elevated in SVZ when compared with either the hippocampus or cerebellum (Figure 3B). This is difference is represented graphically by quantification of n=4 Western blots (Figure 3D, E). In order to understand if enhanced sAPP expression was the result of increased cleavage of APP, we performed Western blots for ADAM10. Levels of mature
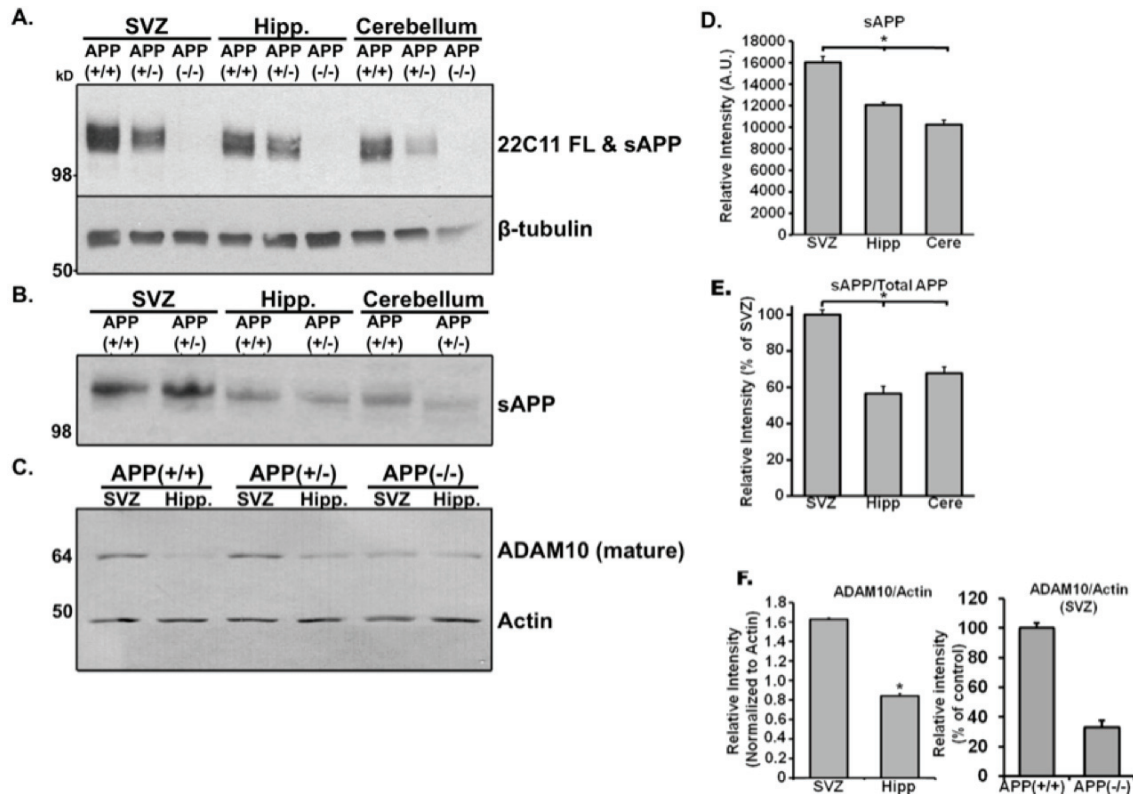
Figure 3. sAPP and ADAM10 expression is higher in the SVZ than the hippocampus.
(A) Western blot analysis shows higher expression levels of APP in the SVZ of APP(+/+)
and APP (+/-) mice compared with the hippocampus or cerebellum. (B) Levels of sAPP
are dramatically higher in the SVZ of APP(+/+) and APP(+/-) mice compared with the
hippocampus and cerebellum. (C) Expression of the α-secretase, ADAM10, is high in
the SVZ compared with the hippocampus of APP(+/+) and APP(+/-) mice, suggesting a
correlation with sAPP levels. (D, E, F) Graphic representation of expression levels in
Western blotting of total APP (D), sAPP/total APP (E) and ADAM10 (F). Error bars
represent the mean ± SEM (* $p < 0.009$ D; * $p < 0.01$ E;
* $p < 0.001$ F, Student's t-test).

ADAM10 are significantly higher in the SVZ when compared with the hippocampus (Figure 3C, F). This suggests that increased sAPP expression in the SVZ is likely the result of increased enzymatic activity. Finally, we compared the expression of ADAM10 in the APP(+/+) and APP(-/-) SVZ. Here, we show that mature ADAM10 expression is significantly higher in the SVZ of APP(+/+) mice (Figure 3F, right panel). This could implicate APP in the regulation of ADAM10 activity but warrants further investigation.

Next, we addressed whether inhibition of α-secretase had an impact on the proliferation of NPC derived from the adult SVZ. To this end, we employed a broad-spectrum matrix metalloproteinase inhibitor (GM6001). NPC were derived from SVZ of 2 month old mice and treated with either GM6001 or its inactive control (NC). Figure 4A shows Western blot analysis of sAPP in conditioned media following 2 hour treatment of NPC's with GM6001 or NC. GM6001 dramatically reduces metabolism of APP to form sAPP. Graphic representation of the difference in sAPP is shown in figure 4B. We next assessed the ability of GM6001 to impact NPC proliferation in a dose-dependent manner. We used a clonogenic assay to examine proliferation. Briefly, 1000 cells/well were plated and treated with either NC, GM6001 or GM6001+10nM recombinant sAPPα for 8-10 DIV and the diameter of the spheres formed as well as sphere number and total number of cells from dissociated spheres were assessed. We show that GM6001 reduced proliferation in a dose-dependent manner with the most potent inhibition occurring at 1μM (Figure 4C). Addition of recombinant sAPPα was able to completely ameliorate this deficit (Figure 4C, D, E). The number of neurospheres remained unchanged across conditions (Figure 4F). We further examined the dose-response profile of sAPPα following 1μM GM6001 treatment. sAPPα enhanced

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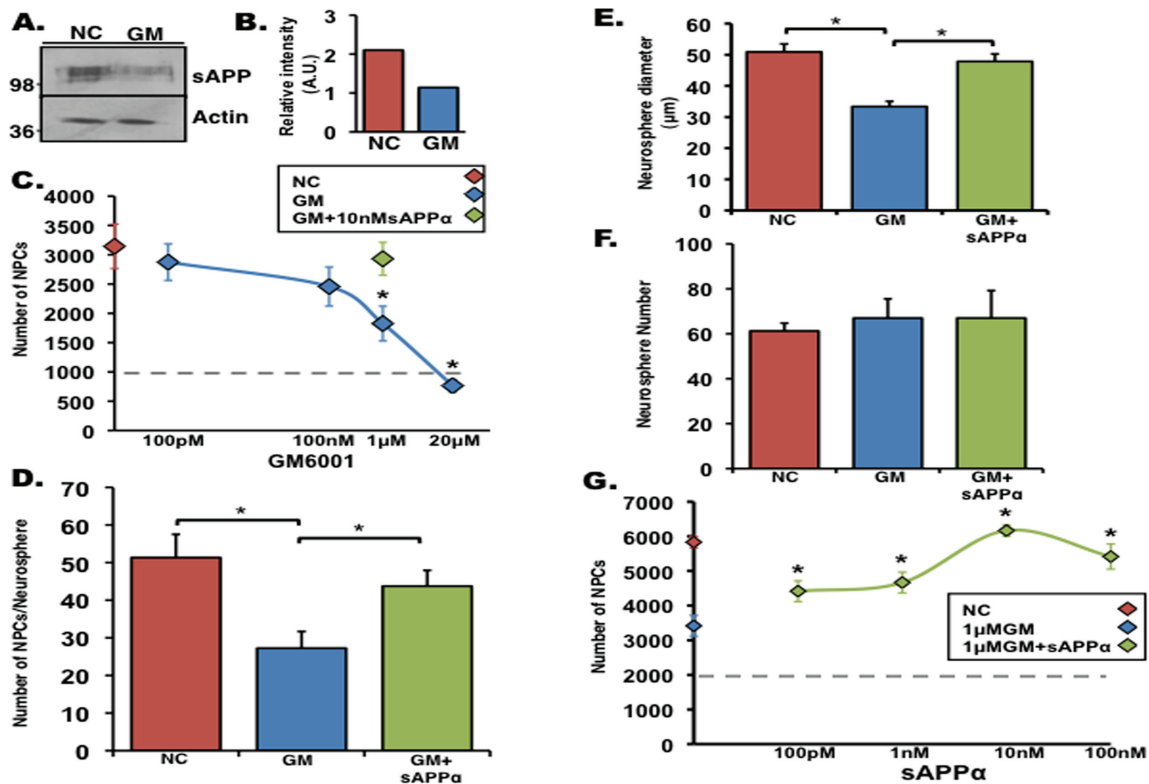
Figure 4. Matrixmetalloproteinase inhibition reduces proliferation of NPC but can be ameliorated in a dose dependent manner by sAPPα. sAPPα ameliorates matrix-metalloproteinase inhibitor induced proliferation deficits in neural progenitor cells

Western blot analysis shows that levels of secreted sAPP are dramatically reduced in NPCs following treatment with MMP inhibitor GM6001 (GM) when compared with NPC treated with an inactive inhibitor (NC). (B) Optical density of expression intensity of sAPP normalized to actin. (C) NPC proliferation as assayed by a clonogenic assay. Total number of NPCs is reduced by GM6001 in a dose dependent manner (dotted line indicates the number of cells originally plated). (D) Recombinant sAPPα can recover GM6001-induced deficits in NPC proliferation as shown by reduction in the number of NPCs counted per neurosphere. (E) Measure of diameter of neurospheres formed following in a clonogenic assay (F) The number of neurospheres formed in a clonogenic assay (G) Dose response of sAPPα effect on NPC proliferation following inhibition with 1μM GM6001 in a clonogenic assay (Red diamond 1μM NC, blue diamonds GM 6001, green diamonds recombinant sAPPα). Error bars represent ± SEM *p < 0.05, ANOVA with post-hoc analysis.

proliferation in a dose-dependent manner with the most potent trophic activity at a concentration of 10nM (Figure 4F).

In order to understand the nature of the cells stimulated by sAPPα, we dissociated spheres following a clonogenic assay and plated them as single cells on a matrigel matrix for 30 min before fixation and immunocytochemistry. We stained the cells with nuclear marker, 4',6-diamidino-2-phenylindole (DAPI), Sox-2 and nestin. The total number of cells was increased in the clonogenic assay and resulted in increased DAPI+ cells (Figure 5A, representative images of clonogenic before sphere dissociation E). The number of cells positive for sox-2 and sox-2/nestin were also increased (Figure 5B, C, representative images in E). This suggests that the increased cell number is the result of increased proliferation of populations of NPC and NSC. Aside from the rate of proliferation, an alternative means through which sAPPα could mediate enhanced cell number would be through an increase in the self-renewal of NSC. We assessed the ability of a single cell/well in a 96 well plate to form a clone. From those cells that formed clones, we then dissociated the clones and again plated a single cell/well and assessed the formation of secondary spheres. Treatment of NPC with GM6001 decreased their self-renewal capacity. However, sAPPα was unable to ameliorate this deficit (Figure 5D).

Together, these experiments are the first to show the direct proliferation effect of sAPPα on NPC of the adult brain. Furthermore, we show that the expression of sAPP is higher in the SVZ when compared with the hippocampus, a region less enriched in NPC. Finally, we show sAPPα acts to increase NPC numbers by driving the
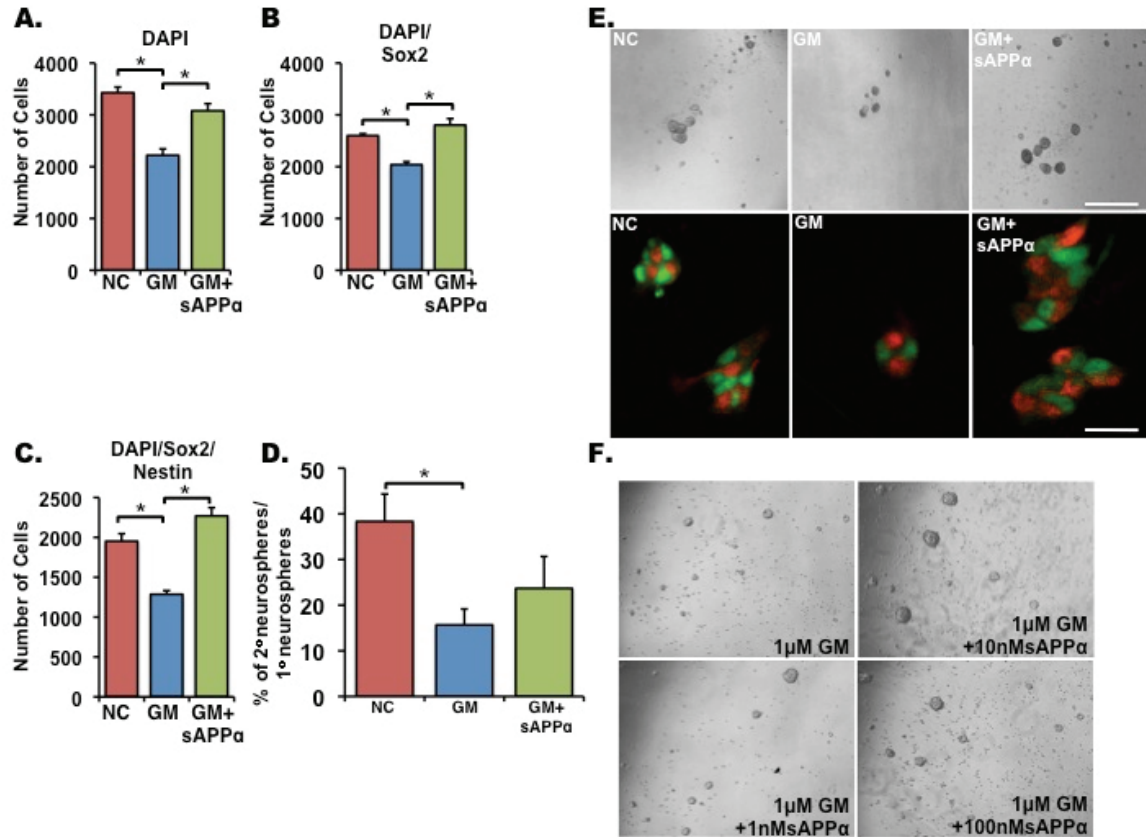
Figure 5. sAPPα acts on both neural stem cells (NSCs) and NPCs to enhance self-renewal and proliferation. Single dissociated cells derived from neurosphere formation assay were immunostained for DAPI (A), Sox2 (B) and Sox2/Nestin (C). GM6001 not only reduced total cell number, but significantly reduced populations of Sox2 and Sox2/nestin -expressing cells. sAPPα rescued the total cell number by enhancing the proliferation of these populations. (E) Representative images of Sox2/Nestin double labeling. (D) The effect of GM6001 on NSC self-renewal. Primary neurospheres were dissociated and a single cell/well was cultured in a 96 well plate in the presence of NC, GM or GM+sAPPα. The percentage of single cells that formed secondary neurospheres is shown. Scale bar=85μ (upper panel, 50μ (lower panel) (F) Representative images of neurosphere formation assay showing the effect of varying concentrations of sAPPα on the size of GM6001-treated neurospheres. Error bars represent ± SEM *$p < 0.05$, ANOVA with post-hoc analysis.

proliferation of rapidly proliferating cells but seemingly does not affect self-renewal of NSC.

2. Soluble amyloid precursor protein alpha acts as a proliferation factor for stem cells of various lineage.

The previous experiments clearly establish that sAPPα conveys trophic properties with respect to the proliferation of NPC derived from the adult brain. However, APP is a ubiquitously expressed protein and sAPP has been shown to have trophic effects on non-neural cell types. Therefore, we examined the effect of α-secretase inhibition and sAPPα stimulation on the proliferation of mesenchymal (MSC) and human deciduas parietalis placental (hdpPSC) stem cells.

In figure 6A,B we examined the ability of GM6001 to inhibit the metabolism of APP by α-secretase in MSC. As in NPC, we show marked reduction of sAPP in conditioned media of GM6001 treated MSC. Next we examined the effect of α-secretase inhibition on proliferation by assessing total cell number following 3 days in culture. Treatment of MSC with GM6001 reduced proliferation in a dose-dependent manner. Furthermore, 10nM sAPPα administration was able to reverse this impairment (Figure 6 C,D). Finally, we tested the dose-response profile of sAPPα with respect to proliferation. As in NPC, sAPPα stimulates proliferation of MSC in a dose-dependent manner with a peak activity around 10nM (Figure 6E).

Next we performed identical experiments in hdpPSC. GM6001 treatment severely reduced sAPP formation in hdpPSC (Figure 7A,B). Treatment with GM6001 also reduced proliferation as assayed by total cell number after 3 DIV in a dose-dependent manner (Figure 7C). This effect could be reversed with concomitant sAPPα

administration (Figure 7 C,D). Finally, sAPPα reversed GM6001 induced proliferation deficits in a dose-dependent manner with peak activity around 10nM (Figure 7E). These results suggest that the proliferative function of sAPPα is not exclusive to NPC. Importantly, this evidence provides functional significance to the ubiquitous expression of APP in the regulation of proliferative populations throughout the body.

3. **Soluble amyloid precursor protein acts independently of exogenous growth factors through the mitogen activated protein kinase pathway.**

While the above experiments provide evidence that sAPPα is a potent proliferation factor, the possibility of co-factor binding and the down-stream signaling involved in its activity have not been addressed. Due to the presence of heparin binding domains in sAPP and the localization of sAPP binding sites to EGF-responsive NPC, it has been postulated that sAPP may act as a co-factor to promote cellular proliferation. To address this issue we examined the effect of α-secretase inhibition and sAPPα administration on proliferation in NPC cultures devoid of exogenous growth factors, EGF and bFGF. Removal of exogenous growth factor stimulation greatly reduces sphere diameter in otherwise naïve NPC. However, treatment with GM6001 retained the ability to further reduce the diameter of neurospheres formed in a clonogenic assay. Likewise, sAPPα reversed this deficit in the absence of EGF and bFGF suggesting that sAPPα acts independently of EGF and bFGF in promoting proliferation (Figure 8 A). To determine whether sAPPα operates synergistically to these growth factors in regulation of neurosphere proliferation, we quantified the effect of sAPPα in medium devoid of EGF/bFGF and supplemented with GM6001 as well as the effect of EGF/bFGF in a medium supplemented with GM6001, and compared it to the effect of EGF/bFGF in
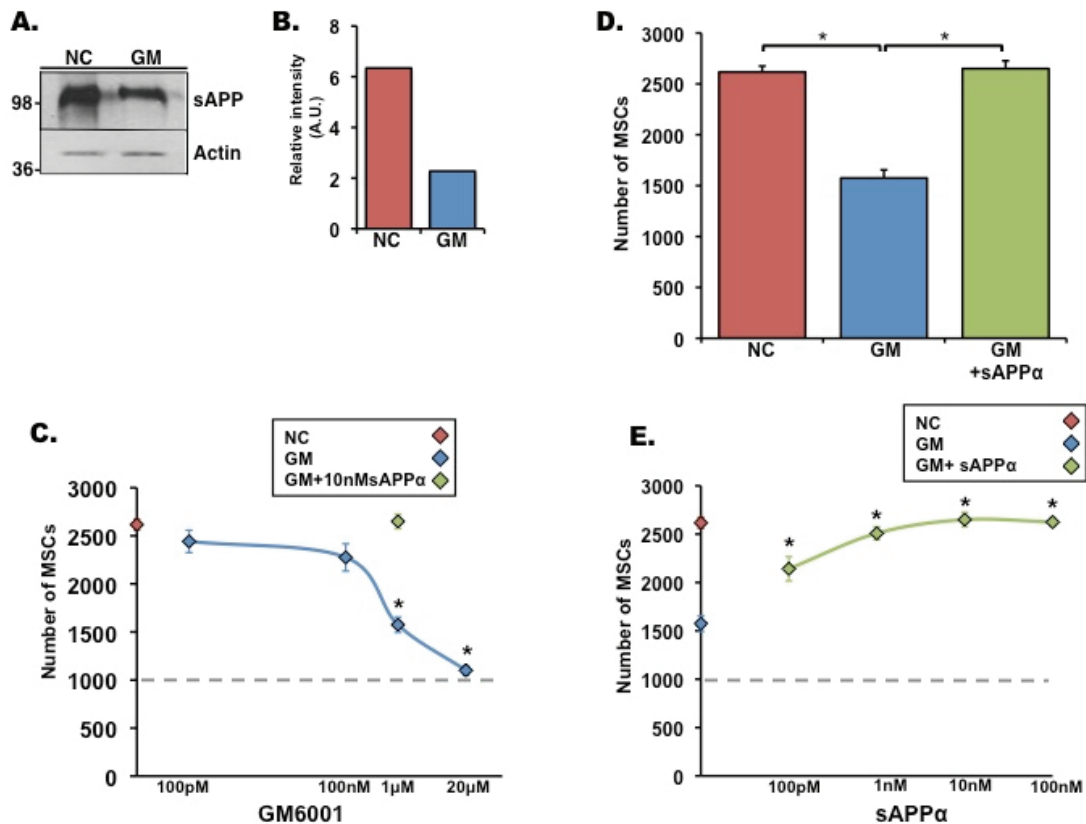
Figure 6. sAPPα can recover matrix-metalloproteinase inhibitor induced deficits in mesenchymal stem cell proliferation. (A) Western blot analysis shows that levels of secreted sAPP are dramatically reduced in MSCs following treatment with MMP inhibitor GM6001 (GM) when compared with MSC treated with an inactive inhibitor (NC). (B) Optical density of expression intensity of sAPP (A) normalized to actin. (C) MSC proliferation as assayed by total cell number following 3 days in culture is significantly reduced by GM6001 in a dose dependent manner. (orange diamond 1µM NC, blue diamonds GM6001, green diamond 1µM GM6001+10nM sAPPα; dotted line indicates the number of cells originally plated). (D) The number of MSC counted following 3 days of 1µM NC, 1µM GM6001 or 1µMGM6001+10nM sAPPα treatment after original plating of 1000 cells/well. (E) Dose response of sAPPα effect on proliferation of MSC following 1µM GM6001 addition (orange diamond 1µM NC, blue diamond 1µM GM6001, green diamonds recombinant sAPPα). Error bars represent ± SEM *p < 0.01, ANOVA with post-hoc analysis.
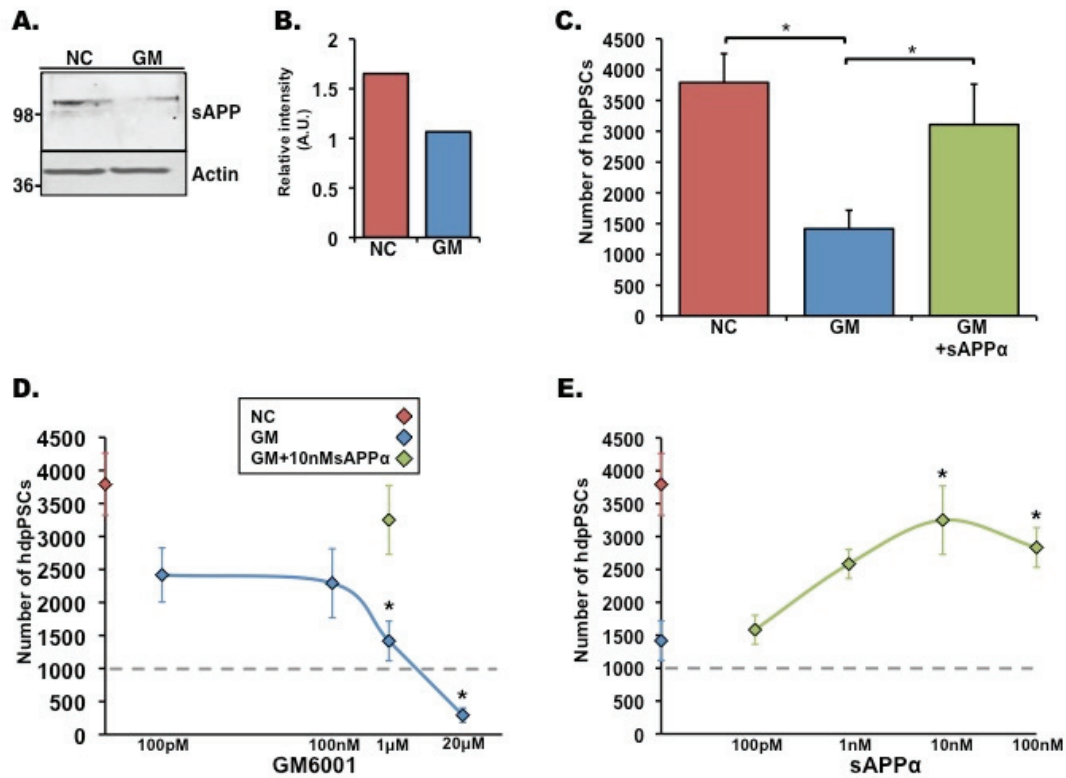
Figure 7. sAPP can ameliorate proliferation deficits in human placental stem cells induced by GM6001. (A) Western blot analysis shows that hdpSCs treated with GM6001 secrete significantly less sAPP compared with hdpSCs treated with an inactive control (NC). Actin serves as a protein loading control (B) Optical density representation of expression intensity of sAPP in Western blot shown in (A). (C) The number of hdpSC counted following 3 days of 1μM NC, 1μM GM6001 or 1μMGM6001+10nM sAPPα treatment after original plating of 1000 cells/well. (D) hdpSC proliferation as assayed by total cell number following 3 days in culture is reduced by GM6001 in a dose dependent manner. (orange diamond 1μM NC, blue diamonds GM6001, green diamond 1μM GM6001+10nM sAPPα; dotted line indicates the number of cells originally plated). (E) Dose response of sAPPα effect on proliferation of hdpSC following 1μM GM6001 addition (orange diamond 1μM NC, blue diamond 1μM GM6001, green diamonds recombinant sAPPα). Error bars represent ± SEM *$p < 0.05$, ANOVA with post-hoc analysis.

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medium supplemented with the negative control for GM6001. We observed that the

additive effect of sAPPα in medium devoid of EGF/bFGF and supplemented with

GM6001 and the effect of EGF/bFGF in a medium supplemented with GM is greater than

the effect of EGF/bFGF in medium supplemented with the negative control for GM,

suggesting a synergistic effect of sAPPα, EGF and bFGF (Figure 8B).

In order to elucidate downstream pathways of sAPPα signaling in NPC, we

examined two integral NPC proliferation pathways that have been previously reported to

be activated in response to sAPPα. These pathways are the extracellular signal-regulated

kinase (ERK)/MAP kinase and protein kinase B (Akt)/phosphoinositide 3-kinase (PI3K)

pathways. We examined both pathways through phosphorylation sensitive Western

blotting of NPC lysates following 15 min treatment with either NC, GM6001 or

GM6001+sAPPα. Treatment of NPC with GM6001 reduces ERK phosphorylation. The

reduction can be reversed via administration of sAPPα (Figure 9A,B). Neither treatment

with GM6001 or sAPPα imposed alterations in Akt phosphorylation (Figure 9 C,D).

These results imply that sAPPα may stimulate proliferation of NPC through the

ERK/MAPK pathway.

We next wanted to address the potential gene targets of sAPPα signaling in

NPC. To identify molecular targets of sAPPα and to gain an insight into the mechanism

by which sAPPα exerts its effect on NPC proliferation, we employed microarray analysis

and compared gene expression in GM6001-treated neurospheres with and without

sAPPα. When compared with the negative control treated NPC, those treated with

GM6001 showed alterations in 484 total genes. Cells treated with GM6001 and sAPP

displayed significant alteration in 574 genes when compared with inactive GM6001-

treated NPC. A total of 153 genes (30 upregulated, 123 downregulated when compared

with NC) were altered significantly in NPC that were treated with GM6001 and sAPP but

not in those simply treated with GM6001 (Figure 10). Alternatively, in NPC treated with

GM6001 only, 137 genes (46 up-regulated and 91 downregualted) were significantly

changed from control levels (Figure 10). The genes altered in GM6001 treatment but not

changed significantly upon addition of sAPPα to the treatment represent gene expression

that is returned to baseline levels by sAPPα even in the presence of MMP inhibitor.

Those that are altered only in combined GM6001 and sAPPα treatment are changed

significantly from control levels following sAPPα addition but are seemingly unaltered

by the inhibition of MMP activity. Of these genes, those that are upregulated from

control levels fall into several noteworthy functional categories, many of which relate

specifically to cell proliferation (Table 1). Intriguingly, sAPPα enhanced the expression

of *CyclinB1*, a key component in the G2/M phase transition (Jackman et al., 2003). Also

up-regulated were mRNA for *Sly*, a gene involved in spermatogenesis (Cocquet et al.,

2009), *Vrk2*, a kinase thought to play a role in cellular proliferation (Blanco et al., 2006),

Foxd4, a winged helix transcription factor (Freyaldenhoven et al., 2002), and *P2ry6,* a G-

protein coupled receptor up-regulated in radiation induced bone tumor development

(Daino K 2009). In addition to genes regulating cell cycle, notable functional categories

that were found to be up-regulated by sAPPα treatment include genes that play a role in

neurogenesis (e.g., *Gabbr1, Pgap1, Emx1*), neurotransmission (e.g., *Gabbr1, Chrna10*),

gene expression (e.g., *Hist1h3g, Smarca5*), cellular energy metabolism (e.g., *Ckm,*

Adipoq, Atp6v0b) and cellular homeostasis (e.g., *Rgn, Ttbk2, Abca16, Hexb*) (Table 1).

These genes simply represent potential downstream molecular targets of sAPP signaling

and imply that enhancement of proliferation may involve alterations in cell cycle and increase in cellular metabolism. The results here provide the initial framework to the understanding of sAPPα signaling in NPC. We show that sAPPα acts independently of other growth factors to stimulate the ERK/MAPK pathway and potentially drive transcriptional alterations. Further elucidation of these mechanisms could provide for the formation of therapeutics involving sAPP.

4. **Soluble amyloid precursor protein beta and familial Alzheimer's disease linked "Swedish" variant fail to recover proliferation deficits in NPC induced by α-secretase inhibition.**

Production of sAPPβ occurs through the amyloidogenic pathway of APP metabolism. Many FAD-linked mutations in APP result in enhanced β-site cleavage of APP, promoting this pathway and the formation of Aβ. Though sAPPβ lacks only 16 C-terminal amino acids compared with sAPPα, several investigations have shown that sAPPβ may be less potent or, in some cases, have divergent functions from its slightly longer counterpart [For review see (Chasseigneaux and Allinquant, 2011)]. Therefore, we examined the effect of sAPPβ or sAPPβ$_{Swe}$ on the recovery of proliferation following α-secretase inhibition. First, we compared the sphere diameter following treatment with GM6001+sAPPα or sAPPβ. We show that sAPPβ does not reverse proliferation deficits following GM6001 (Figure 11 A,C). Next, we compared the effect of sAPPα on NPC proliferation with that of sAPPβ$_{swe}$, a shorter soluble form that is an FAD-linked product of Bace1 cleavage of mutant APP. For this purpose, we examined formation of neurospheres in a culture of singly- dissociated NPC treated with GM6001 inhibitor and supplemented with conditioned media enriched with either sAPPα or sAPPβ$_{swe.}$ We

observed that sAPPα- enriched conditioned media was able to rescue much of the proliferative deficit incurred by treatment of NPC with GM6001. However, treatment of cells with sAPPβ$_{swe}$- enriched conditioned media was entirely unable to rescue GM6001-induced proliferation deficits (Figure 11B). This result suggests that the C-terminal portion of sAPPα is vital for its activity. Furthermore, these results indicate that α- and β-secretase cleavage pathways of APP may have divergent functions. Alterations in the dynamics of APP cleavage, such as those seen in FAD, could have a significant impact on proliferation of NPC.

5. Soluble amyloid precursor protein beta fails to rescue beta-secretase inhibitor induced proliferation deficits.

Previously we examined the affect of sAPPβ on proliferation following α-secretase inhibition. However, α-secretase inhibition leads to proliferation deficits due to sAPPα decline with endogenous sAPPβ levels likely unaffected. In order to examine the effect of reduction of endogenous sAPPβ levels on proliferation of NPC, we implemented a β-secretase inhibitor. We show that β-secretase inhibition induced proliferation deficits at 100nM but seemingly became toxic at higher concentration. However, addition of sAPPβ does not ameliorate this deficit (Figure 12 A,B representative images in C). Due to the fact that sAPPβ is unable to reverse proliferation deficits in β-secretase inhibited cells, we propose that the proliferation decline is likely mediated through an alternative pathway. It remains possibly that cross-inhibition of BACE2, an enzyme with α-secretase activity, could cause a reduction in sAPPα leading to proliferation decline. Next, we examined the effect of dual secretase inhibition on
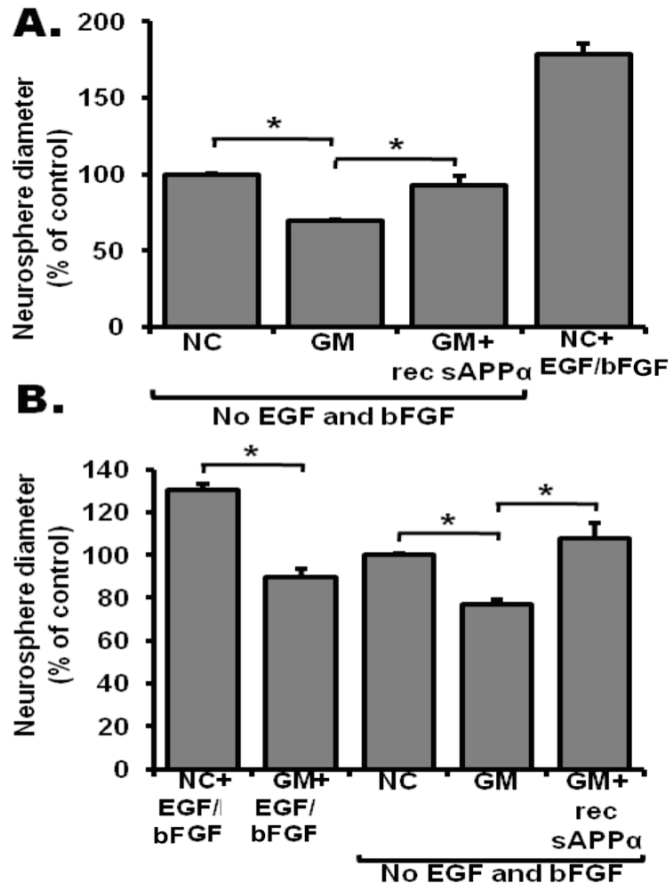
Figure 8. sAPPα enhances proliferation in an EGF/bFGF independent manner. (A) A
neurosphere formation assay was performed without growth factors (EGF and bFGF)
where NPC were treated with an inactive inhibitor (NC) or with GM6001 or
GM6001+recombinant sAPPα. Right column depicts sphere diameter of NPC treated
with NC in media containing growth factors. (B) The proliferative effect of sAPPα on
GM6001-treated neurosphere (GM) in medium devoid of EGF/bFGF , and the
proliferative effect of EGF/bFGF on GM6001-treated neurospheres is greater than the
effect of EGF/bFGF on neurospheres treated with NC, suggesting a synergistic effect of
sAPPα and EGF/bFGF. Error bars represent ± SEM *$p < 0.01$, ANOVA with post-hoc
analysis.
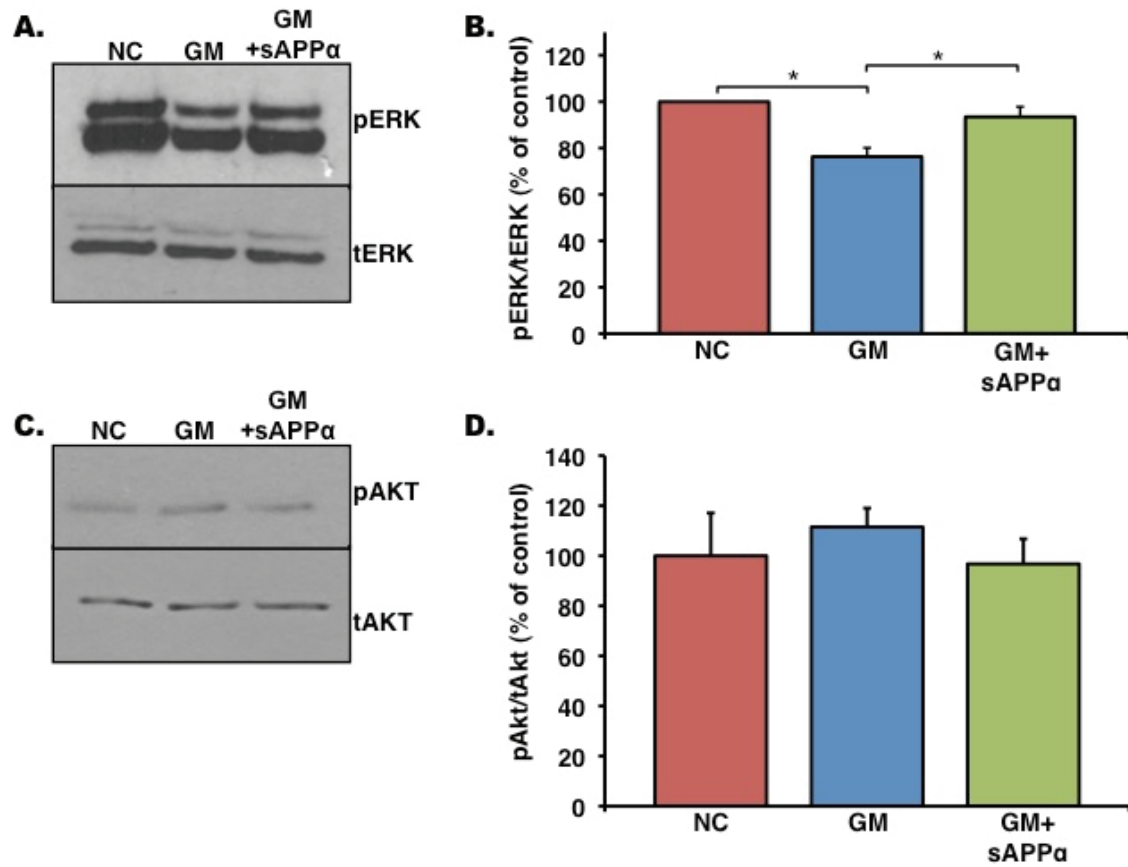
Figure 9. sAPP can recover GM6001 induced deficits in Erk phosphorylation.
(A) Representative Western blot of NPC treated with inactive inhibitor (NC), GM6001 or
GM6001 + recombinant sAPPα and probed for pErk (top) and total Erk (bottom). (B)
Quantification of perk/total Erk Western blots. (*= p<0.05 n = 4, ANOVA with post-hoc
analysis) (C) Representative Western blot of NPC treated as described above probed for
pAkt (top) and total Akt (bottom). (D) Quantification of pAkt/total Akt Western blots
(N=4).
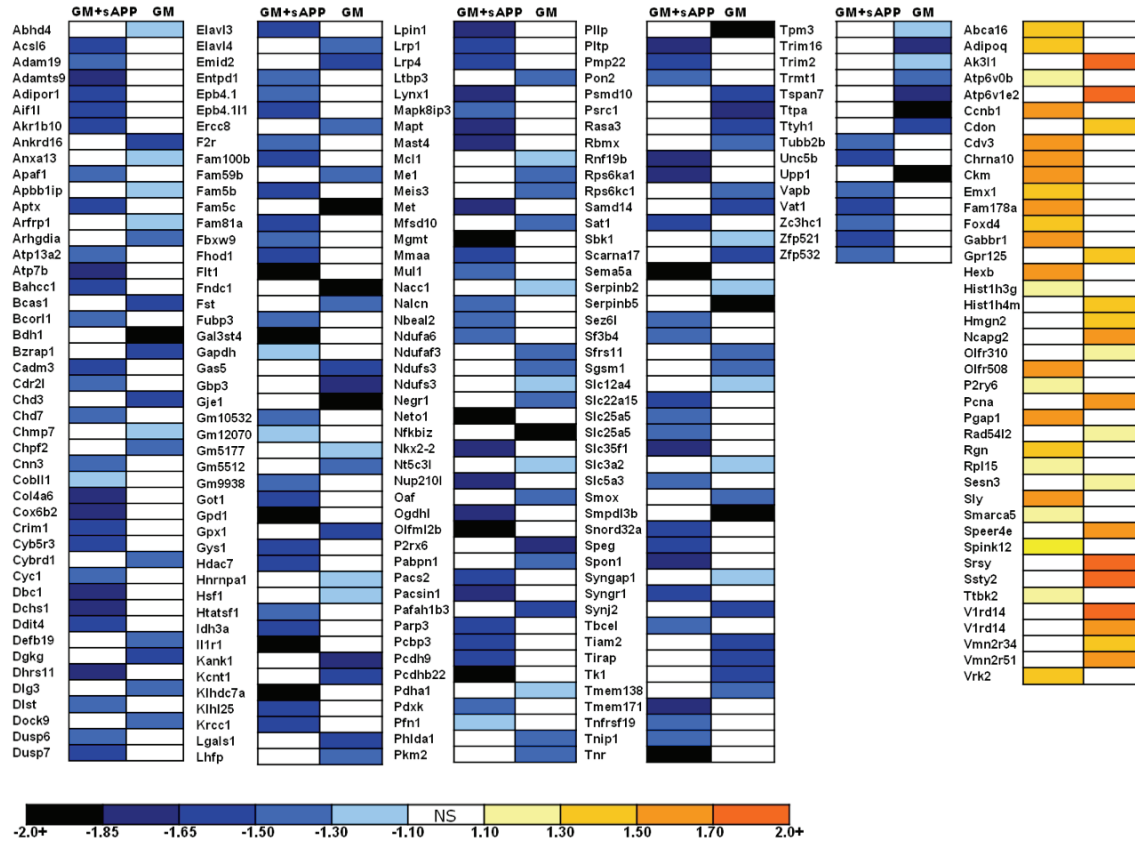
Figure 10. sAPPα upregulates genes implicated in cell cycle, transcription, neurogenesis, energy metabolism and cell homeostasis. Microarray analysis of GM6001 and GM6001+sAPPα vs. negative control 24 hours after treatment.
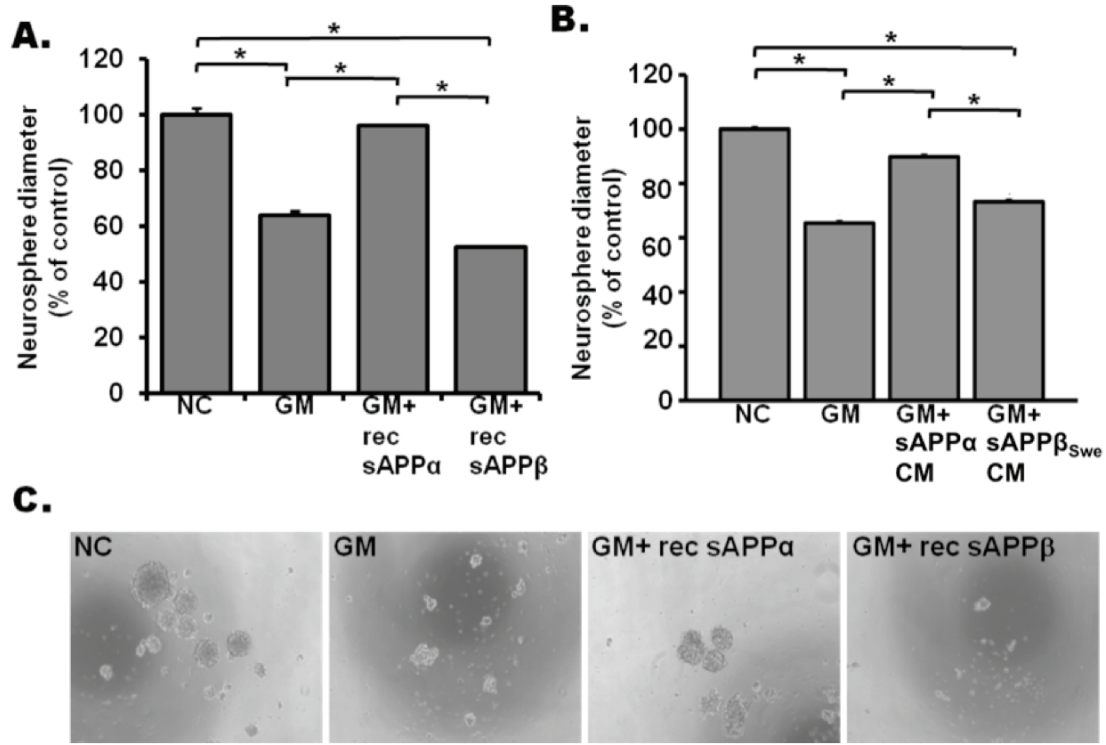
_Figure 11. sAPPβ and mutant sAPPβ__swe_ **_fail to recover neurosphere diameter in_**
GM6001 treated NPCs. (A) In a neurosphere formation assay, recombinant sAPPβ does
not recover deficits in proliferation caused by GM6001 treatment. (*=p<0.01) (B)
Conditioned media enriched with familial Alzheimer's disease-linked mutant sAPPβ$_{swe}$
fails to rescue GM6001-inhibited NPC proliferation, in contrast to media enriched with
sAPPα (* = p<0.04). (C) Representative images of neurospheres treated with negative
control, GM6001, GM6001+recombinant sAPPα and GM6001+ recombinant sAPPβ.

proliferation. Both α- and β-secretase inhibition lead to similar reductions in proliferation of NPC. Addition of both inhibitors concomitantly did not further reduce cell numbers or sphere diameter. Interestingly, sAPPα was able to fully recover proliferation deficits induced by dual secretase inhibition but sAPPβ showed no recovery (Figure 13A,B representative images D). These results suggest that sAPPβ does not act as a trophic factor in NPC.

6. **Soluble amyloid precursor protein beta reduces cell viability.**

In order to understand whether sAPPβ could actually promote toxicity of NPC at increasing concentrations, we employed an MTT assay to evaluate cell viability following sAPPβ treatment. At low concentration sAPPβ does not impact cell viability. However, as the concentration of sAPPβ was increased, the viability of cells was seemingly decreased (Figure 14). This would suggest that at high enough concentrations sAPPβ may be toxic to NPC. This result has major implications for the role of APP metabolism in AD and suggests that a decline in NPC could not only be the result of a decline in trophic sAPPα, but also the result of toxicity mediated by an increase in the concentration of sAPPβ.

7. **Proliferation and neurogenesis are reduced in the subventricular zone and subgranular layer of aged mice.**

Previous reports have suggested that there is an aging-linked decline in neurogenesis. In order to establish this finding in our own lab and characterize the nature of neurogenic deficits, we injected mice at 2 months of age and 20 months of age with BrdU and sacrificed 24 hours later. In both the SVZ and DG there is a dramatic decline in proliferating cells as indicated by BrdU labeling (Figure 15A, figure 16A). To further

examine the proliferation of neuroblasts and formation of immature neurons in the aged brains we performed co-staining of BrdU with DCX. The number of BrdU+DCX+ proliferating neuroblasts is severely reduced in the aged SVZ and DG (Figure 15B, 16B). This suggests that either neuroblast proliferation is altered or there is a reduction in the number of NPC becoming neuroblasts. Finally, we assessed the number of BrdU-DCX+ post-mitotic, immature neurons. Here we find that the number of immature neurons in the neurogenic regions of the aged brain is reduced as well (Figure 15C, Figure 16C). Together, these results suggest that the aged brain exhibits a decline in the number of proliferating NPC that likely manifests in reduced neuroblast formation and a reduced number of immature neurons.

8. **Amyloid precursor protein and its soluble metabolite are reduced in neural progenitor cells derived from the aged brain.**

One theory attempting to explain aging-linked decline in neurogenesis postulates that decreased trophic support in the neurogenic niche is responsible for declining proliferation of NPC. With the finding that sAPPα is a trophic factor for NPC of the adult brain, we hypothesized that sAPPα levels may decline during the aging process, thus contributing to NPC senescence. In order to test this theory, we derived NPC from the SVZ of 2 and 14 month old mice and performed Western blot analysis for sAPP from the conditioned media along with fl-APP and ADAM10 from the cell lysates. In figure 17 we show that steady-state levels of sAPP in the conditioned media are significantly reduced in the NPC derived from 14 month old animals. In order to
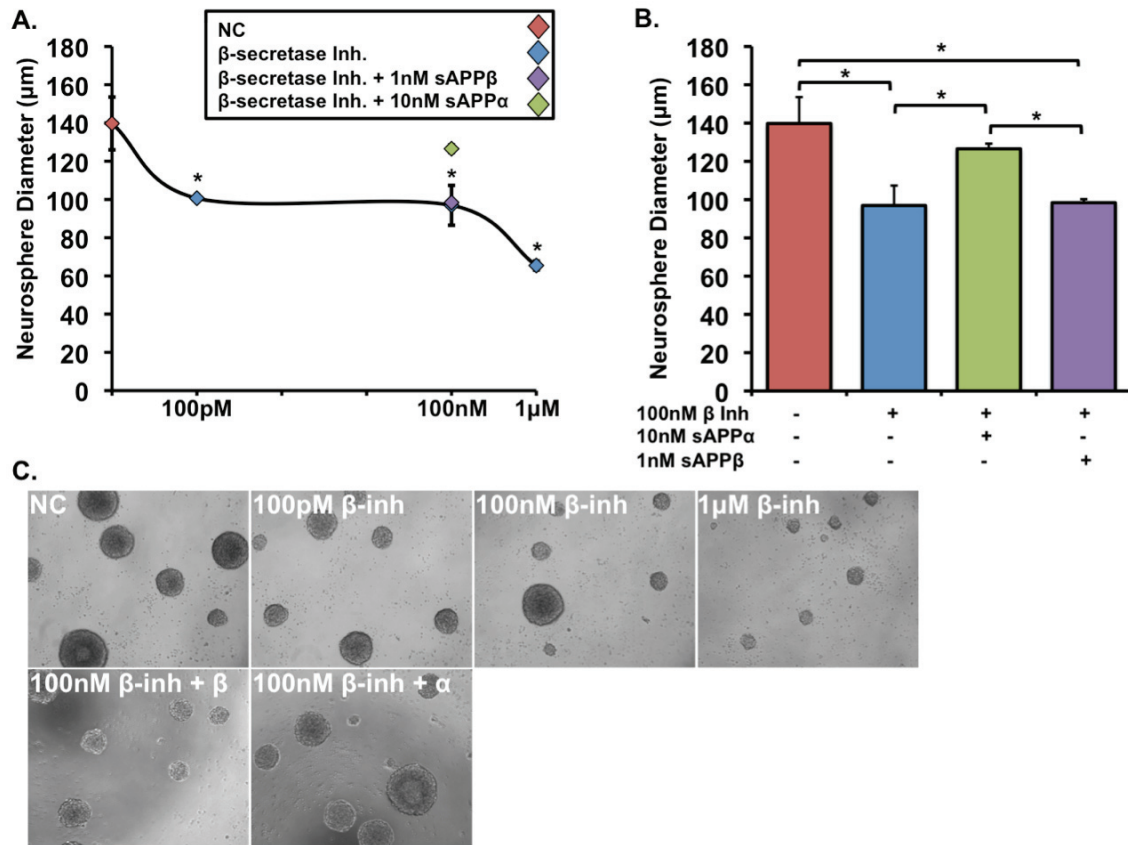
Figure 12. sAPPβ does not ameliorate beta secretase inhibitor induced reductions in proliferation of NPC. (A) β-secretase inhibitor reduces the size of neurospheres in a dose dependent manner but addition of sAPPβ fails to rescue this deficit. (B) sAPPβ is unable to rescue β-secretase induced neurosphere diameter deficits. However, sAPPα ameliorates these deficits (C) Representative images of neurospheres from all conditions tested. * $p < 0.05$ ANOVA with post-hoc analysis.
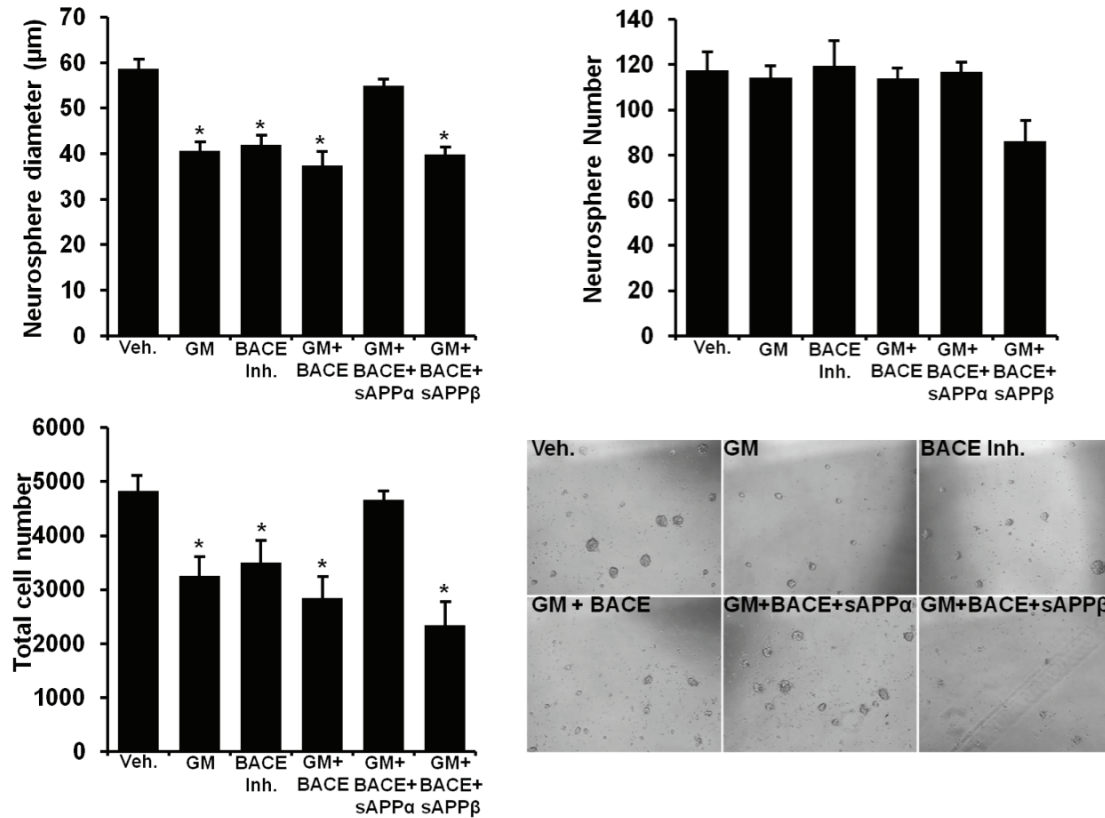
Figure 13. sAPPα, but not sAPPβ, ameliorates proliferation deficits imposed by double inhibition of matrix-metalloproteinases and β-secretase. (Top left) Neurosphere diameter is reduced to a similar extent following treatment with GM6001 or β-secretase inhibitor. No cumulative effect is apparent as double inhibition does not further reduce neurosphere diameter. sAPPα is able to reverse proliferation deficits but sAPPβ is unable to do so. (Top right) Neurosphere number is unaffected by inhibition but is reduced after sAPPβ treatment possibly owing to toxicity. (Bottom left) Total cell number of dissociated neurospheres reflects the neurosphere diameter affects of inhibitor and sAPP treatment. (Bottom right) Representative images of neurospheres derived from treated cells.

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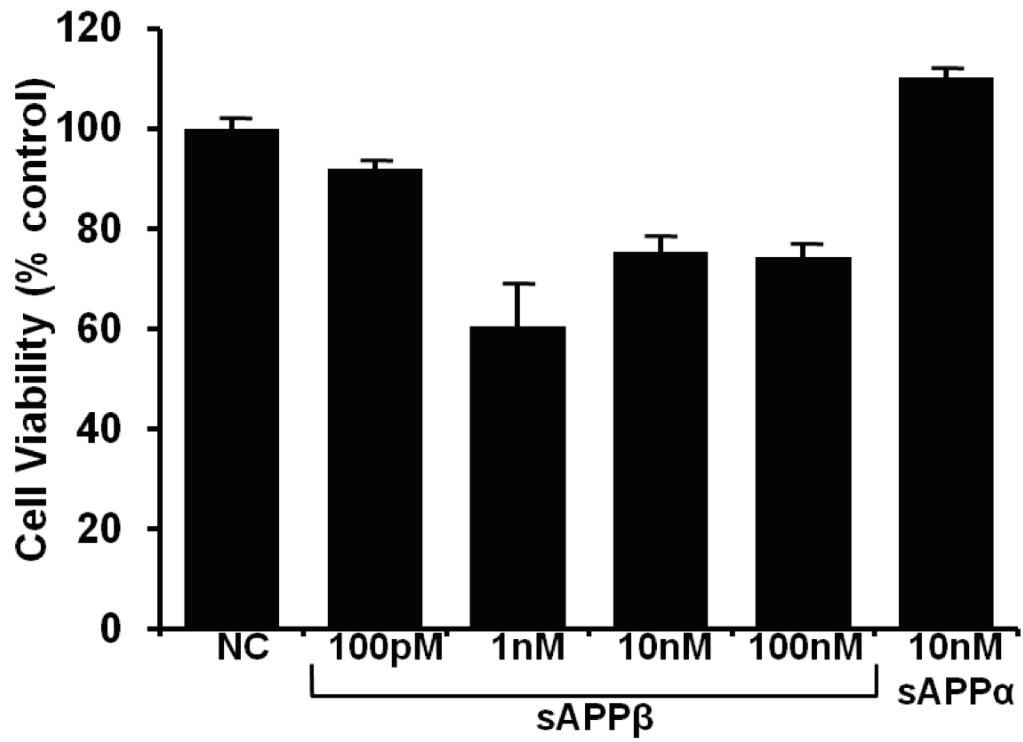
Figure 14. NPC viability is reduced in MTT assay following treatment with sAPPβ.
Treatment of single dissociated NPC with varying concentrations of sAPPβ impairs cell
viability at concentrations above 1nM. 10nM sAPPα is shown as a positive control.
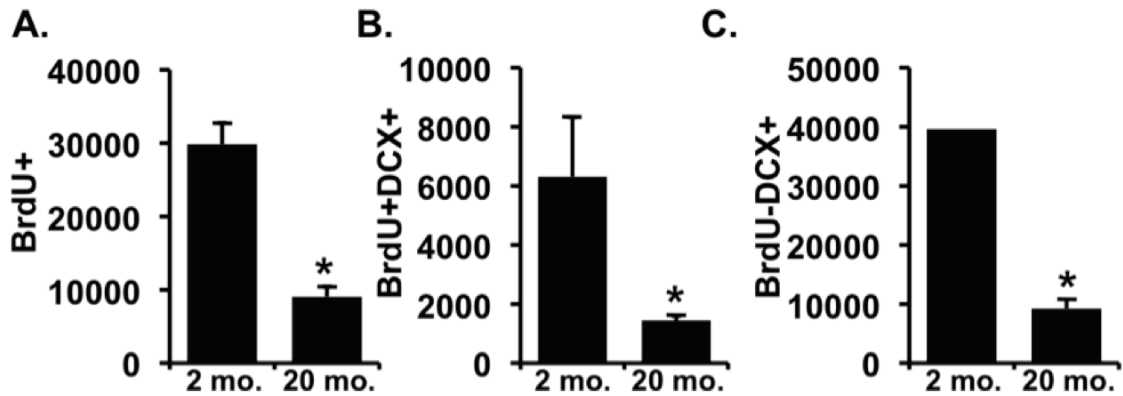
Figure 15. Proliferation and neurogenesis are reduced in the SVZ of aged mice.
Young (2 mo.) and aged (20 mo.) mice were injected with BrdU 24 hours prior to
sacrifice and labeled with BrdU and neuroblast/immature neuronal marker, doublecortin
(DCX). (A) The number of proliferating cells is drastically reduced in 20 month old mice.
(B) The number of proliferating neuroblasts, as assessed by double-labeling of BrdU and
DCX, is reduced in SVZ of aged mice. (C) The number of immature post-mitotic neurons
is also reduced in aged mice as determined by DCX+ cells that did not incorporate BrdU.
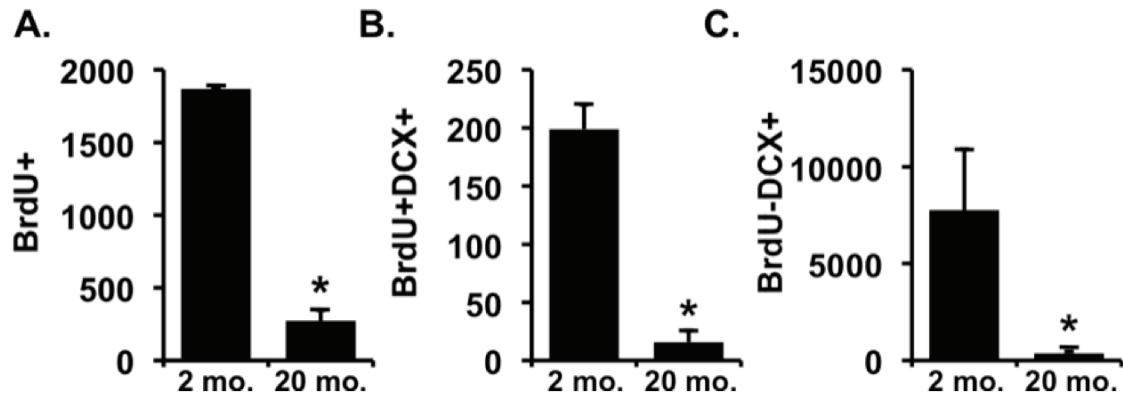
Figure 16. Proliferation and neurogenesis are reduced in the SGL of aged mice.
Young (2 mo.) and aged (20 mo.) mice were injected with BrdU 24 hours prior to
sacrifice and labeled with BrdU and neuroblast/immature neuronal marker, doublecortin
(DCX). (A) The number of proliferating cells is drastically reduced in 20 month old mice.
(B) The number of proliferating neuroblasts, as assessed by double-labeling of BrdU and
DCX, is reduced in SGL of aged mice. (C) The number of immature post-mitotic neurons
is reduced in aged mice as determined by DCX+ cells that did not incorporate BrdU.

understand whether this decline could be attributed to lower levels of substrate or enzyme we assessed the expression of fl-APP and ADAM10 in cell lysates. Evaluation of fl-APP and mature ADAM10 also show a significant aging-linked decline. This result suggests that reduction of APP expression and ADAM10 expression could both contribute to declining sAPP levels in the aged brain. The finding that ADAM10 decline correlates with a decline in APP is in agreement with our examination of ADAM10 in the APP(-/-) SVZ (Figure 3C) and further supports a potential role for APP in ADAM10 expression.

9. **Neural progenitor cells derived from the aged subventricular zone retain the ability to respond to soluble amyloid precursor protein alpha.**

In previous experiments we have shown that sAPPα is a trophic factor that declines with aging. Furthermore, we have shown that this decline correlated with the timing of neurogenic decline in the aging brain. However, it remains to be seen whether sAPPα retains its potency as a proliferation factor in NPC derived from the aged brain. To address this question, we used NPC derived from the SVZ of 2 month, 7-9 month and 20 month old mice. It has been shown that NPC derived from the aging brain show no deficits in proliferation *in vitro* (Ahlenius et al., 2009). It is theorized that the reason for this discrepancy is that exogenous growth factor support provided *in vitro* ameliorates what are *in vivo* deficiencies in niche trophic support. We first attempted to uncover proliferation deficits in NPC by examining neurosphere growth in experiments that used a media with limited exogenous growth factors. Even with limited exogenous growth factors we found no difference in neurosphere diameter or total cell number in NPC

derived from 7-9 month or 20 month old mice when compared with those derived from 2

month old mice (Figure 18 A, C). Next, we examined the ability of sAPPα to stimulate
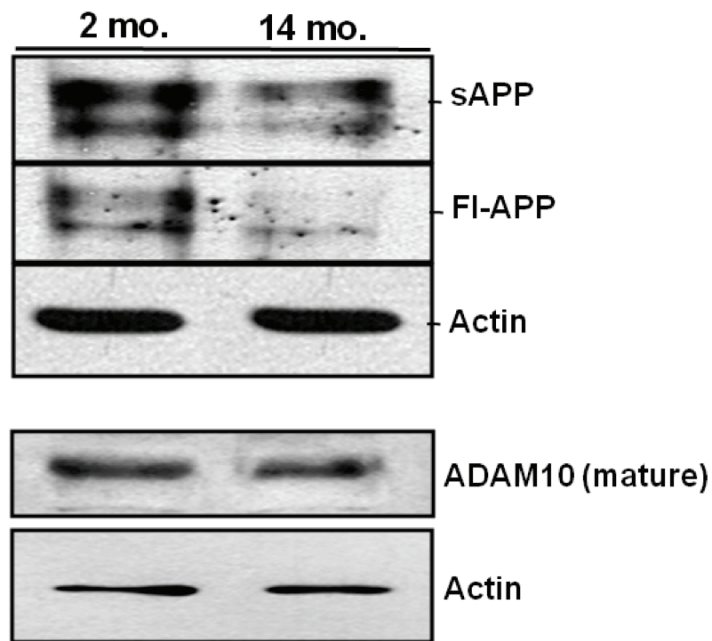
Figure 17. Steady state levels of sAPP and full-length APP are reduced in NPC derived from aging mice in vitro as well as in the aging neurogenic niches in vivo.
Western blot analysis of steady state sAPP levels in conditioned media of NPC derived from 2 and 14 month old mice shows aging linked decline. Analysis of fl-APP in cell lysates of NPC shows reduction suggesting decreased substrate for α-secretase cleavage. However, levels of mature ADAM10 are decreased with aging as well.
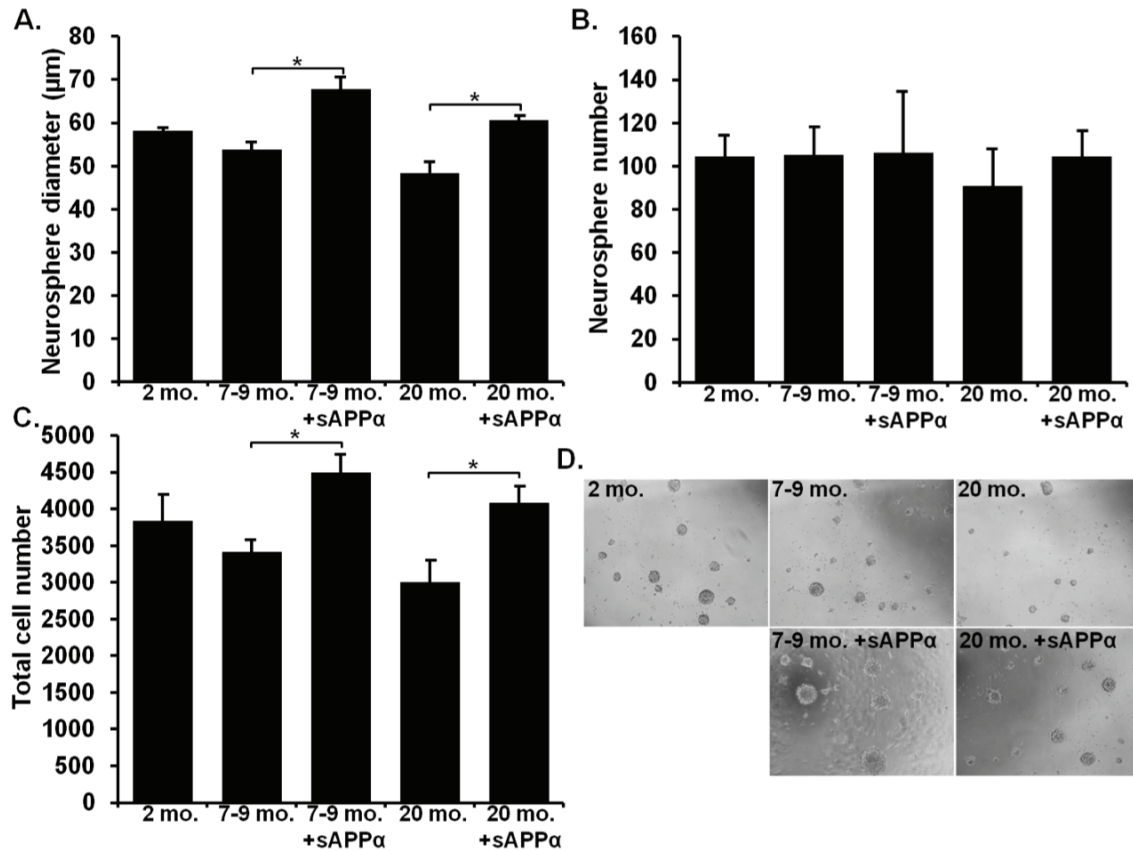
Figure 18. sAPPα is able to stimulate proliferation of NPC derived from aging mice.
NPC derived from 2 mo, 7-9 mo and 20 mo old mice were subjected to neurosphere formation assay in a limited growth factor media. (A) NPC derived from aging mice do not show significant alterations in sphere diameter but stimulation with sAPPα significantly increases the diameter of neurospheres. (B) No alterations were seen in the number of neurospheres across all groups. (C) The total number of cells from dissociated neurospheres shows again that aging NPC do not display significant impairment in proliferation but can be stimulated by sAPPα. (D) Representative images of neurospheres derived from single cells.

proliferation of NPC derived from aged brains. sAPPα increased sphere diameter and total cell number of NPC derived from both 7-9 month and 20 month mice (Figure 18A, C, representative images in D). This result suggests that NPC derived from aged brains retain the ability to be stimulated by sAPPα.

10. Soluble amyloid precursor protein alpha ameliorates aging-linked proliferation deficits in both the subventricular zone and subgranular layer.

To this point we have shown that sAPPα expression exhibits aging-linked decline that can be correlated with declining proliferation in the aged brain. Furthermore, we have shown that NPC derived from the aged brain retain the ability to proliferate in response to sAPPα treatment. Therefore, we postulate that sAPPα may have the potential to ameliorate aging-linked decline in neurogenesis. To test this we performed intracerebroventricular (ICV) infusion of sAPPα in 7-9 month old mice. After 6 hours, mice were injected with BrdU and sacrificed 24 hours later. Figure 19A shows that single ICV infusion of sAPPα approximately doubles the number of proliferating, BrdU+, cells in the SVZ when compared with mice infused with PBS. In order to further characterize these cells, we labeled them with progenitor cell markers nestin and sox-2. As NSC are postulated to proliferate only once every 20 hours according to cumulative BrdU injection (Zheng et al., 2004), the overwhelming majority of BrdU+ cells in our single injection paradigm is thought to be contained to faster proliferating type-C cells, neuroblasts and astroglial progenitors. In figure 19C we show that the proliferation increases can be attributed, at least in part, to an increased in the proliferation of type-C progenitors as assessed by BrdU+/Sox2+/Nestin+ staining. Furthermore, we show that
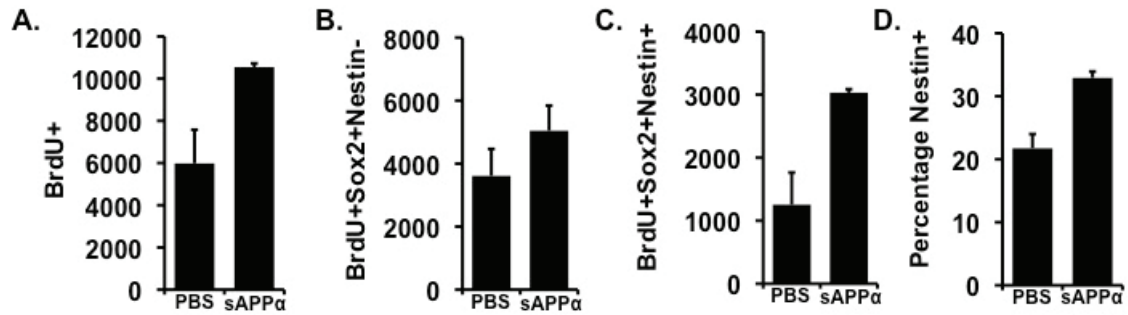
Figure 19. Intracerebroventricular injection of sAPPα stimulates proliferation in the SVZ of aged brains. sAPPα was injected directly into the ventricles of 7-9 month old mice followed 6 hours later by BrdU injection. Following 24 hours mice were sacrificed for immunohistochemical analysis. (A) sAPPα greatly increases proliferation in the SVZ as measured through BrdU incorporation. (B) BrdU+Sox2+Nestin- cells are not dramatically increased in the SVZ. (C) The number of proliferating type-C progenitors is enhanced by sAPPα injection. (D) The percentage of proliferating cells expressing nestin is increased following injection suggesting sAPPα action on fast proliferating NPC.
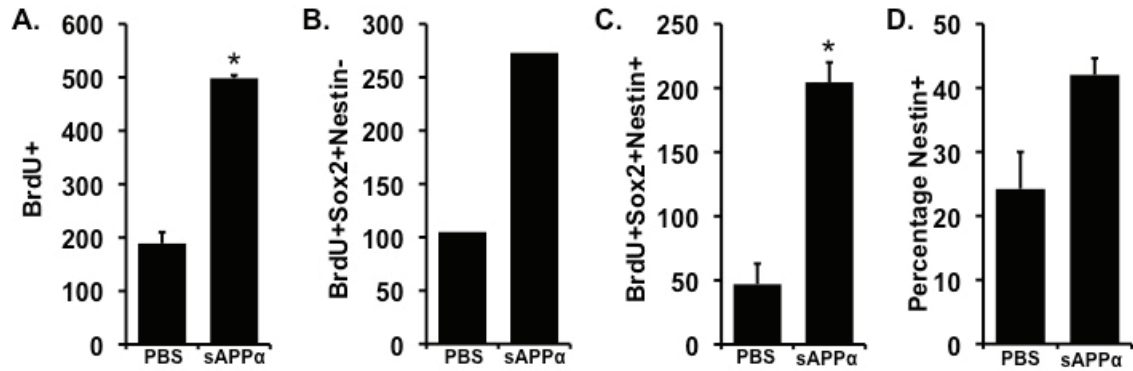
Figure 20. sAPPα stimulates proliferation of neural progenitors in the aged SGL.
Aged mice (7-9 months) were given a single intracerebroventricular injection of sAPPα
or PBS followed by BrdU administration 6 hours later. Following 24 hours the mice
were sacrificed for immunohistochemical analysis. (A) sAPPα greatly increases
proliferation in the SGL as measured through BrdU incorporation. (B)
BrdU+Sox2+Nestin- cells are dramatically increased in the SGL. (C) The number of
proliferating type-C progenitors is enhanced by sAPPα injection. (D) The percentage of
proliferating cells expressing nestin is increased following injection suggesting sAPPα
action on fast proliferating NPC.

the percentage of BrdU+ cells that are type-C progenitors is increased by sAPPα infusion (Figure 19D). These results are in agreement with the notion that binding sites for sAPPα are localized to type-C progenitors in the adult SVZ (Caille et al., 2004).

We further examined if single ICV infusion of sAPPα could ameliorate aging-linked NPC proliferation deficits in the SGL. Caille et al reported that NPC of the SGL had no apparent binding sites for sAPPα. However, we show that ICV infusion of sAPPα markedly enhanced proliferation in the SGL of 7-9 month old mice when compared with PBS infusion (Figure 20A). As in the SVZ, this enhanced proliferation can be attributed, at least in part, to an expansion of Sox2+Nestin+ type-II progenitors (Figure 20C,D). Taken together, these results suggest that declining trophic support to NPC by sAPPα in the aged brain could contribute to aging-linked NPC senescence. Furthermore, sAPPα may provide an attractive therapeutic target for the enhancement of proliferation.

CHAPTER 6:

DISCUSSION

In this study it has been shown that sAPPα is a potent proliferation factor for NPC signaling through the MAP kinase pathway to enhance the cell cycle. The study further shows that sAPPβ has limited capacity to stimulate NPC proliferation and can potentially prove toxic at increasing concentrations. Many mutations associated with FAD cause a shift in APP cleavage favoring the amyloidogenic pathway and β-secretase processing of APP. Furthermore, this study demonstrates that during normal aging, sAPP levels decline correlating with proliferation deficits. Infusion of sAPPα is able to ameliorate aging-linked proliferation deficits in the SVZ and SGL by stimulating proliferation of fast-proliferating, type-C or type-II cells. Taken together, these results suggest that reduced sAPPα due to decreased metabolism of APP in normal aging or FAD-linked mutations in APP could contribute to proliferation deficits.

Initially, we sought to characterize the expression of APP and α-secretase, ADAM10, in the neurogenic niches of the adult brain. Not surprisingly, due to the ubiquitous nature of APP expression, we show that APP and ADAM10 are expressed on NPC of the adult brain. However, the SVZ appears to express particularly high levels of sAPP and ADAM10 when compared with the hippocampus. While this may potentially suggest a differential role for sAPP in the neurogenic regions of the adult brain, it could also be due simply to the elevated proportion of NPC in the SVZ when compared with the whole hippocampal extract. ADAM10 is thought to be the most vital of the α-secretase enzymes in the adult brain (Jorissen et al., 2010). However, there are other enzymes that have the ability to cleave APP at the α site including ADAM17 (Buxbaum

et al., 1998), ADAM9 (Koike et al., 1999), ADAM19 (Tanabe et al., 2007) and BACE2 (Yan et al., 2001). Reports are conflicting as to the expression pattern of these enzymes in the neurogenic SVZ. One report found no ADAM10, ADAM17 and relatively low ADAM9 expression in the adult SVZ through immunohistochemical analysis (Yang et al., 2005b). However, a later study showed that ADAM17 and ADAM10 were present in the adult SVZ by laser capture microdissection and real time RT-PCR analysis as well as immunohistochemistry (Katakowski et al., 2007). In our studies, we also report that ADAM10 is present in the adult SVZ by both immunohistochemistry and Western blot analysis.

As the majority of these enzymes hail from the metalloproteinase family, we utilized a broad-spectrum matrix-metalloproteinase inhibitor to elucidate the affect on APP metabolism and proliferation in NPC derived from the adult brain. It is important to note that the ADAM's are a particularly promiscuous family of enzymes with a number of substrates [for review see (Deuss et al., 2008)]. We must also take into consideration that the N-terminal of APP is highly conserved in APLP1 & APLP2 (Wasco et al., 1992; Slunt et al., 1994). Studies have shown a potential functional redundancy of APP with the APLP's, particularly APLP2, through the use of combined knockout mice (Heber et al., 2000). By utilizing a broad spectrum inhibitor we are able to diminish cleavage at the α-secretase site by various enzymatic activities. The use of this inhibitor also allows for the inhibition of APLP1 & APLP2 cleavage and avoidance of functional redundancy issues. However, we are unable to rule out proliferation effects that are unrelated to diminished sAPPα. These enzymes are also key regulators of the metabolism of other integral neurogenesis proteins such as Notch 1 (Brou et al., 2000; Lieber et al., 2002),

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notch ligands Delta and Jagged (LaVoie and Selkoe, 2003; Dyczynska et al., 2007), EGF and heparin-binding EGF (Izumi et al., 1998; Sahin et al., 2004), N-cadherin (Reiss et al., 2005) and P75^{NTR} (Weskamp et al., 2004) among others. Therefore it is likely that proliferation deficits induced by GM6001 treatment are the result of decreased shedding of many substrates. The ability of conditioned media containing sAPPα and recombinant sAPPα to completely reverse these deficits speaks to the potency of sAPP as a trophic factor. However, the concentration of sAPPα in these experiments is probably higher than physiological concentrations and thus has the potential to mask deficits in other signaling pathways.

The physiological significance for the ubiquitous expression of APP remains to be fully elucidated. As discussed previously, the *drosophila* APP homologue, APPL, is limited in its expression to the nervous system (Luo et al., 1990). However, in mammals, sAPP has been shown to stimulate the proliferation of non-neural cell types including keratinocytes (Schmitz et al., 2002), fibroblasts (Saitoh et al., 1989) and thyroid epithelial cells (Pietrzik et al., 1998). In this study, we determined that sAPPα stimulates the proliferation of stem cells of various lineages as well; namely mesenchymal stem cells and human decidua parietalis placental stem cells. Together these studies suggest a potential physiological function to explain ubiquitous APP expression in mammals. While these experiments clearly demonstrate the ability of sAPPα to stimulate proliferation of these cell types, some question remains to the physiological contribution of sAPPα to proliferation *in vivo*. Further investigations will be necessary in order to determine the expression of APP in stem cells of various lineages and the effect of reduced sAPP on the proliferation of these cells.

The initial study suggesting that sAPP may play a role in neurogenesis showed that binding sites for sAPP were localized to fast-proliferating type-C cells in the adult SVZ (Caille et al., 2004). Our results are in agreement with this report as we determined that the up-regulation of proliferation following sAPPα administration was due to an increase in nestin+sox2+ type-C progenitors. This type of *in vitro* evaluation is limited in that we are only able to discern the effect of sAPP on the proliferation of undifferentiated cells. As neuroblasts and glial progenitors are also believed to have a capacity to proliferate transiently (Brown et al., 2003; Assanah et al., 2006), it remains to be seen whether sAPP can also stimulate these cells. Furthermore, analysis of sphere diameter and total cell number is likely to represent mostly the proliferation of type-C cells as the proportion of type-B NSCs in these cultures appears to be rather small (Doetsch et al., 1999b). The number of primary neurospheres formed from a known number of dissociated NPC does not seem to provide an accurate portrayal of self-renewal capacity of NSC in the culture. It is postulated that the rapidly proliferating progenitor cells have a limited capacity to form neurospheres in culture over passages [for review see (Pastrana et al., 2011)]. Therefore, we utilized a single-cell self-renewal assay in which a single cell was placed in a well of a 96 well plate and those that form primary spheres are calculated, dissociated and plated a second time as single cells/well to determine the number of secondary neurospheres produced per primary neurosphere. In this way, we are able to eliminate much of the sphere formation by progenitor cells and more readily address the self-renewal of NSC directly. However, there is some debate over the nature of these NSC. At least four different subpopulations of these type-B cells have been identified; many of which seem to have different abilities to self-renew

(Pastrana et al., 2011). *In vitro* assays also may not be suitable to distinguish affects on quiescent populations which may be prevalent *in vivo*. Thus, a combination of methods that determine self-renewal utilizing NSC markers for FACS sorting and *in vivo* examination of NSC may be necessary to more completely address the effect of sAPP on self-renewal of NSC.

In this work we further attempt to unravel the molecular signaling pathways involved in the stimulation of proliferation by sAPPα. First, we show that sAPPα acts in an EGF- and bFGF-independent manner to regulate proliferation as eliminating the exogenous EGF and bFGF from the media does not alter GM6001 inhibition or sAPP stimulation of proliferation. It has been suggested that, because of the presence of a heparin-binding domain adjacent to the putative growth factor domain in APP (Corrigan et al., 2011) and the presence of sAPP-binding sites on EGF-responsive NPCs (Caille et al., 2004), signaling of sAPP may involve other factors (such as EGF) which also can interact with heparin. Our results suggest that any interaction of sAPPα and EGF or bFGF is not necessary for the proliferation-related functions of the molecule.

We next evaluated potential intracellular signaling cascades that might be responsible for sAPPα induced proliferation. In order to begin to elucidate these mechanisms we examined stimulation of two pathways, ERK/MAPK and Akt/PI3-K. Both of these pathways are stimulated downstream in response to sAPP (Cheng et al., 2002; Gakhar-Koppole et al., 2008). We show that sAPPα signaling in NPC is associated with ERK/MAP kinase activation. Increased phosphorylation of ERK is indicative of signaling through the MAP kinase pathway. In NPCs, ERK signaling is crucial for carbachol-induced increases in DNA synthesis (Li et al., 2001), inhibiting MAP kinase

signaling decreases BrdU incorporation after heparin-binding EGF activation (Jin et al., 2005), and MEK 1 and 2 inhibitor, U0126, inhibits proliferation after hypoxia/reoxygenation (Sung et al., 2007). All of these factors point to ERK/MAP kinase as a critical pathway in the proliferation of NPCs. Another critical pathway involved in proliferation of NPCs, Akt (Li et al., 2001; Wang et al., 2005a; Sung et al., 2007), was not activated by sAPPα *in vitro*. Previous investigations using pharmaceutical inhibitors of either the ERK/MAP kinase pathway or the Akt/PI3-K pathway suggest that PI3-K inhibition results in both self-renewal deficits and proliferation decline whereas MAP-kinase inhibition affected proliferation only (Torroglosa et al., 2007). Differential stimulation of these pathways in response to APP metabolites has been shown previously in a knockout of the sortillin-receptor with type-A repeats (SORLA) (Rohe et al., 2008). These mice present with increased levels of sAPPα and Aβ as well as increased neurogenesis and enhanced ERK/MAPK activation but not Akt/PI3-K stimulation in hippocampal neurons. Due to the fact that the receptor for sAPP remains unknown and direct inhibition of ERK activation would inherently inhibit proliferation of NPC, we are unable to rule out contributions to sAPPα signaling through alternative pathways. However, the stimulation of ERK by sAPPα and the evidence that ERK signaling stimulates proliferation of NPC directly, strongly suggests that his pathway plays a role in the sAPPα induced proliferation.

Finally, using microarray analysis, we identified molecular targets of sAPPα through which it exerts its proliferative effect in NPC. Functional classification of the target genes suggests upregulation of genes implicated in regulation of cell cycle, gene transcription, neurogenesis and cellular energy and homeostasis. Specifically, a

striking group of genes implicated in regulation of cell cycle was upregulated in GM6001-inhibited NPC following treatment with sAPPα, including *cyclin B1*, *Sycp3 like Y-linked protein* (*Sly*), The *vaccinia related kinase 2* (*VRK2*), *Forkhead box D4* (*Foxd4*) and *pyrimidinergic receptor P2Y, G-protein coupled, 6* (*P2ry6*). Cyclin B1 drives entry into mitosis along with its associated catalytically active partner Cdk1. Potent activation of Cyclin B1-Cdk1 prompts nuclear envelope breakdown and mitotic entry (Miyazaki and Arai, 2007). Examination of Sly in the testis suggests that it is transcribed after the first meiotic division in secondary spermatocytes and round spermatids and encodes two transcript variants, Sly_v1 and Sly_v2 (proteins referred to as SLY1 and SLY2). SLY1 interacts with the acrosomal protein DKKL1, the histone acetyltransferase KAT5 (also known as TIP60), and the microtubule-associated protein APPBP2 (Reynard et al., 2009). VRK2 protein belongs to a novel human putative serine/threonine protein kinase family (Nezu et al., 1997). Expression of VRK2 is widespread and elevated in highly proliferative tissues, such as fetal liver, testis and thymus and several cancer cell lines (Traktman et al., 1989; Kovacs et al., 2001). It is thought to be involved in cell-growth regulation, including control of the cell cycle, DNA replication and transcription, germ-cell development (Lu and Bishop, 2003) and embryonic development of hematopoiesis (Vega et al., 2003). Both VRK2 isoforms phosphorylate p53 resulting in its stabilization, suggesting that the VRK's might be components of a new signaling pathway that is likely to play a role in normal cell proliferation (Blanco et al., 2006). Foxd4 belongs to the family of Winged helix transcription factors thought to be critical regulators of embryonic development and tissue differentiation in numerous species (Ang and Rossant, 1994; Jacob et al., 1994; Xuan et al., 1995; Hatini et al., 1996).

Another important group of genes that are upregulated in NPC following treatment with sAPPα are implicated in neurogenesis, for example, *Gababr1*, *Pgap1* and *Emx1*. Numerous studies suggest that γ-aminobutyric acid receptors (GABAR) play a crucial role in the migration of neural stem and progenitor cells during development, leading to central nervous system (CNS) organization (Behar et al., 1998; Hatten, 1999; Lopez-Bendito et al., 2003). GABABR R1 subunits are preferentially localized to presynaptic and postsynaptic membranes in immature neurons from the developing human CNS while GABAAR β2 and γ3 subunits are predominantly expressed in young postnatal neurons (Laurie et al., 1992; Ma and Barker, 1995). Interestingly, imbalance and dysfunction of GABAergic transmission underlies, at least in part, impaired neurogenesis in Alzheimer's disease (Li et al., 2009; Sun et al., 2009). PGAP1 functions as a novel component of the Wnt pathway during mouse forebrain development (Tanaka et al., 2004; Ueda et al., 2007). Another member of this functional group is *Emx*, a divergent homeobox gene belonging to a large family of highly evolutionarily conserved transcription factors that are essential for development. Human EMX1 and EMX2 are essential to brain development (Kastury et al., 1994). The mouse homologs, Emx1 and Emx2, are also expressed in the developing brain (Simeone et al., 1992; Boncinelli et al., 1993). Taken together, these molecular targets suggest that sAPPα could exert its effects by enhancing cellular metabolism, neurotransmitter activity and cell cycle entry. The genetic alterations described here should provide a starting point from which we may further elucidate sAPPα signaling. While our findings implicate these pathways the reader should note that as with any high throughput analysis, further validation is warranted.

Several of the mutations in APP that are causative of FAD lie in close

proximity to the β-secretase cleavage site at the N-terminal portion of the Aβ region.

These mutations cause a shift in the metabolism of APP toward the amyloidogenic

pathway, increasing sAPPβ production at the expense of sAPPα (Thinakaran et al.,

1996a). FAD-linked transgenic mice display impaired proliferation of NPC prior to the

onset of pathological hallmarks or the presentation of memory deficits. *In vitro*, NPC

derived from these mice have impaired proliferation, suggesting a potential intrinsic

mechanism caused by the mutations (Demars et al., 2010a). In this work, we show that

sAPPβ and FAD-linked sAPPβ$_{Swe}$ are unable to ameliorate proliferation deficits incurred

through MMP inhibition. Further, sAPPβ does not show the same trophic potency as

sAPPα in reversing proliferation deficits caused by β-secretase inhibition or a dual

inhibition of α- and β-secretase. Increased concentration of sAPPβ also may have the

potential to produce toxicity in NPC. Prior to this investigation we had hypothesized that

β-secretase inhibition would enhance proliferation via a reduction of transcriptionally

active AICD. However, β-secretase inhibition led to a reduction in proliferation which

was not reversed by sAPPβ administration suggesting an alternative mode of action. We

propose that BACE1 may have other substrates that regulate proliferation or that cross

inhibition with BACE2 leads to a reduction in sAPPα and thus proliferation deficits. The

IC$_{50}$ for BACE2 with the beta-secretase inhibitor IV used in these studies is reported to

only be about 8 fold higher than for BACE1 in HEK293 cell lines (Stachel et al., 2004).

The divergence in activity of sAPPα and sAPPβ has been previously reported. In cultures

of hippocampal neurons subjected to glutamate or Aβ$_{25-35}$ in toxic concentrations,

recombinant sAPPα is highly protective across a range of concentrations beginning at

1nM and ranging into µM levels. However, sAPPβ is not neuroprotective at low concentrations. At higher levels sAPPβ shows some neuroprotective effects but remains less potent than similar concentrations of sAPPα (Furukawa et al., 1996). Both sAPPα and sAPPβ have also been shown to stimulate neurite outgrowth from embryonic derived primary cortical neurons. However, sAPPβ again shows a tendency to be less potent than its counterpart in axonal elongation and primary dendrite length (Chasseigneaux et al., 2011). Further investigations suggest that sAPPα is involved in hippocampal long term potentitation (LTP) and spatial memory. This study found that sAPPβ did not have a similar affect on these properties (Taylor et al., 2008). A recent report suggests that in peripheral neurons deprived of growth factor support and undergoing apoptosis, sAPPβ is released and binds to DR6 inducing neurodegeneration (Nikolaev et al., 2009). Additionally, in APP/APLP2 knockout mice that are normally perinatally lethal, knock-in of sAPPα rescues this lethality. In contrast, sAPPβ fails to rescue perinatal lethality in APP/APLP2 knockout mice (Li et al., 2010; Weyer et al., 2011). Together these results suggest that the two sAPPs may have divergent functions. The question remains how this divergent functionality is conveyed. sAPPα is 16 amino acids longer on its C-terminal extension. However, the putative trophic domains of sAPPα do not lie in this divergent region. Instead, they are located in regions shared between the two metabolites (Ninomiya et al., 1993; Ohsawa et al., 1999; Rossjohn et al., 1999). Peptides containing the C-terminal portion of sAPP alone were unable to promote proliferation in embryonic stem cells (Ohsawa et al., 1999). However, the divergent function suggests that the C-terminal of sAPPα is involved somehow in promoting its function. As these amino acids lie adjacent to the hydrophobic membrane region, it is possible that sAPPα could have an

altered conformation in order to shield these amino acids from the aqueous environment

and this conformational change could presumably increase access to trophic regions of

the molecule. Alternatively, this region could be involved in protein binding that may

either potentiate receptor activation or sequester sAPPα to prevent degradation. Neither

of these theories of divergent signaling with respect to the sAPPs have been examined

and thus there is a critical need to understand how sAPP signaling occurs as well as how

the process is modulated.

Adding further complexity to understanding the role of APP metabolites in

proliferation of NPC in FAD is the fact that AICD and Aβ may also play a role in NPC

proliferation. Mutations that cause an increase in β-secretase processing likely also

enhance production of AICD. The transcriptionally active AICD is preferentially

produced through the β-secretase cleavage and the amyloidogenic pathway (Belyaev et

al., 2010). The AICD has been implicated in the negative regulation of proliferation of

NPC from the adult brain. Studies utilizing APP knockout and APP knockout with

sAPPα knock-in suggest that AICD is the major regulator of transcription and thus may

be the dominate regulatory pathway (Aydin et al., 2011). Further supporting this notion,

APP overexpression results in a down-regulation of proliferation in the adult SGL

(Naumann et al., 2010). Using these findings as a model for APP metabolic regulation of

proliferation, we can speculate that the amyloidogenic and non-amyloidogenic pathways

oppose one another and potentially form a homeostatic balance for NPC proliferation in

the adult. One reason for this balance could be prevention of tumor formation as sAPPα

has been linked to glioblastoma cell proliferation (Culicchia et al., 2008). Mutations in

APP that cause a shift in this homeostatic balance, therefore, could alter proliferation of

NPC. This hypothesis has yet to be tested. In order to elucidate the full extent of APP

metabolism on NPC of the adult brain, we must examine the role and mechanism of each

metabolite individually before attempting to understand how a shift in the balance of

signaling can affect proliferation.

Aging remains the largest risk factor for sporadic forms of AD. However,

little has been shown with regards to APP expression and processing during normal

aging. One study of note did find that maturation and processing of APP decreased in

association with cellular aging in a human lung fibroblast cell line. This resulted in a

decline of sAPPα secretion from these cells (Kern et al., 2006). Here we show that in

NPC of the adult brain, there is an aging-linked decline in the expression of APP and

sAPP. These NPC are derived from the SVZ where we also show enhanced expression

of APP and sAPP at a young age and therefore the decrease in APP may not be indicative

of the expression in the brain as a whole but merely a tapering off of increased levels

during young adulthood. However, this decline does correlate strikingly with the timing

of decline in proliferative cells in both the SVZ and SGL. The question remains as to

what the cause of sAPP decline is. A potential explanation for this comes from the study

of detergent resistant membrane microdomains (DRM) or "lipid rafts". These DRM's

have a characteristically high level of cholesterol and provide a discrete membrane region

thought to be involved in the facilitation of cellular signaling (Pike, 2006). Some studies

have shown that in certain regions of the rodent brain, cholesterol content increases in an

age-dependent manner (Zhang et al., 1996). However, this view is a controversial one

with many groups showing no alterations or decreases in brain cholesterol (Soderberg et

al., 1990; Svennerholm et al., 1994). In human lung fibroblast cell lines, an increase in

cholesterol correlated highly with cellular age (Kern et al., 2006). Irrespective of total

cholesterol levels, changes have been shown in cholesterol and lipid distribution within

membranes during aging [for review see (Wood et al., 2002)]. Through manipulations of

DRM's via cholesterol depletion, it was shown that amyloidogenic cleavage of APP may

be sequestered in these domains (Simons et al., 1998; Schneider et al., 2008). APP,

BACE1 and presenilin1 have been shown to associate increasingly in DRM's with

increasing cellular age (Kang et al., 2006). Thus, it is possible that aging alters

membrane microdomains facilitating increased amyloidogenic processing in DRM's.

This increased amyloidogenic processing would result in a shift of the APP homeostatic

balance for proliferative signaling toward the inhibition of proliferation in turn leading to

reduced NPC in the aged brain. Therefore, we propose that both FAD-linked mutations

and membrane microdomain alterations in normal aging may act to shift metabolism of

APP toward the amyloidogenic, proliferation inhibiting, pathway.

Furthermore, single intracerebroventricular injection of sAPPα is able to

ameliorate these deficits in the SVZ of 7-9 month old mice. This increase was the result

of an expanded population of BrdU+nestin+ rapidly proliferating type-C cells. Due to

the short duration between sAPPα infusion and BrdU administration (6 hours) it is

unlikely that this increase is due to expanded progenitors formed from NSC proliferation.

Thus sAPPα seems to directly stimulate the proliferation of NPC. This result is in

agreement with the report that binding sites for sAPPα are localized to type-C cells of the

adult SVZ. It should be noted that the concentration of sAPPα injected in these

experiments was 1μM and it is assumed that this solution is diluted approximately 100

times in the cerebrospinal fluid (Caille et al., 2004). While the effective concentration of

sAPPα on proliferation of NPC *in vivo* is unknown, this should provide a concentration around 10nM, the optimal dose under our conditions *in vitro.* In the SGL we also observed a significant increase in proliferative type-II BrdU+nestin+ cells following ICV administration of sAPPα. This is in contrast the finding by Caillé et al that the SGL does not contain binding sites for sAPP (Caille et al., 2004). Though ICV injection can spread rapidly throughout a wide area of the brain, the SGL does not directly contact the lateral ventricles and there must remain some speculation that the increase in proliferating SGL NPC could be the result of migratory cells from the SVZ. Indeed, there remains controversy in the field as to whether the SGL actually contains the bona-fide NSC at all. Two studies have reported that multipotent self-renewing progenitors fail to grow from the adult SGL (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). However, again the short duration between sAPPα administration and BrdU injection supports the notion that whatever the origin of the progenitors in the SGL, they are stimulated in the SGL by ICV injection and not simply migrating there from the SVZ. Furthermore, no BrdU+ cells are seen in other brain regions including the site of needle insertion (data not shown). This further suggests that NPC from the SVZ are not migrating abnormally in response to the minor injury of a needle implantation. In a recent report, it was suggested that NPC of the SGL do indeed proliferate in response to sAPP *in vitro* (Baratchi et al., 2011).

In summary, the data presented in this work show that sAPPα is a ubiquitous stem cell proliferation factor. This proliferative effect occurs in NPC of the adult brain particularly on type-C rapidly proliferating progenitor cells. We further show that sAPPα acts independently of exogenous growth factor stimulation suggesting that

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sAPPα is not a co-factor for EGF signaling. Administration of sAPPα to NPC derived from the adult SVZ leads to stimulation of the Erk/MAP kinase pathway, an integral proliferation pathway for NPC, as well as increased mRNA expression of genes vital to cell cycle regulation, cellular metabolism and neurotransmission. In the FAD and aging brain, proliferation of NPC is decreased. We propose that under both conditions, a shift in the metabolism of APP from primarily non-amyloidogenic, α-secretase, processing to amyloidogenic, β-secretase, cleavage leads to reduced trophic sAPPα and transcriptionally active AICD that negatively regulates proliferation. We show a reduction in sAPPα in NPC derived from the aged brain which correlates with proliferative decline. Re-introduction of sAPPα to the aged brain via ICV injection is able to ameliorate proliferation deficits in both the SVZ and SGL, offering a potential therapeutic mechanism. This work provides an important basis to the understanding of APP metabolism with respect to NPC proliferation. Future research described in the following chapter will seek to expand upon this knowledge with the goal of eventual therapeutic intervention.

CHAPTER 7:

FUTURE DIRECTIONS

The previous chapters have focused on the role of soluble amyloid precursor protein alpha (sAPPα) as a proliferation factor for stem cells and, in particular, neural progenitor cells (NPC). This work has focused mainly on the mechanism through which this stimulation takes place as well as a potential role in proliferative deficits in both normal aging and familial Alzheimer's disease (FAD). The major impact of this work is not only the discovery of sAPPα as a novel stem cell proliferation factor but the idea that alterations in the metabolism of APP during normal aging and FAD could alter the regulation of NPC proliferation by APP. However, this work highlights some of the important questions that have yet to be addressed in the field.

Our data suggest a direct stimulation of NPC by sAPPα which corroborates the finding that NPC contain binding sites for the metabolite. We have also begun to unravel the intracellular signaling through which sAPPα exerts its effect. However, the potential receptor for sAPPα remains elusive. The discovery of a receptor would allow for more intricate evaluation of down-stream signaling pathways and gene expression effects of sAPPα. Furthermore, uncovering the receptor could lead to the development of therapeutic interventions aimed at ameliorating proliferation deficits in aging or FAD as a means to impede memory impairment. Our lab has designed experiments that will be at the forefront of this research and could provide the breakthrough that the field desperately yearns for.

While the discovery of a receptor for sAPPα could allow for the production of compounds designed to stimulate the receptor and enhance endogenous

proliferation of NPC, the metabolism of APP as a whole may contribute much more extensively to proliferation and neurogenic processes than is described in this work. Future work should focus on the individual role of each metabolite in not only proliferation but also in the migration, fate determination, survival and functional incorporation of neurons in the adult brain. While some of this work has begun in other labs, there is much that remains unknown. To this end, expression of individual metabolites should provide for key insights into how these processes are regulated both *in vitro* and *in vivo*. Additionally, a more extensive understanding of the enzymes that regulate APP metabolism in NPC is warranted along with insight into how these enzymes are regulated in the neurogenic niche. It is only when we understand these individual processes that we will be able to truly uncover how APP metabolism regulates neurogenic signaling.

We propose herein a potential mechanism whereby APP metabolism may be altered in both FAD and normal aging leading to proliferation deficits. However, this idea is merely speculation and warrants significant investigation in future studies. It has been established that FAD mutations can cause a shift in APP metabolism. However, the use of transgenic mouse models that over-express APP confounds these investigations. The use of knock-in models may be better suited to evaluate the intrinsic role of APP mutations in proliferation and neurogenic processes. With respect to normal aging, the idea that membrane microdomains may be altered causing increased APP localization to lipid rafts should be assayed. This could be a potential mechanism fueling altered APP metabolism. Understanding these mechanisms may hold the key to any type of

therapeutic intervention and may provide vital insight into potential dysfunction leading to sporadic forms of Alzheimer's disease.

However, any therapeutic intervention mimicking sAPPα or altering APP metabolism would most likely be given chronically. Thus the study of alterations in sAPPα expression or increased α-secretase processing would need to be studied extensively. Preliminary investigations have revealed a potential contribution of sAPPα to glial differentiation of NPC and thus chronic treatment may be able to enhance proliferation but may lead to increased astrocytes at the detriment of neurogenesis. Therefore, it may be imperative to strike a balance in APP metabolism for effective therapeutic intervention. Current interventions may not have the necessary characteristics for this type of regulation and thus new therapeutic approaches may be necessary. However, this would potentially occur far down the road. This brings us back to understanding the extensive nature of APP metabolism and how this metabolism regulates neurogenic processing, which remains the most vital future goal of research in this arena.

In conclusion, this study has shown that sAPPα regulates NPC proliferation in the adult brain. Alterations in sAPPα expression in FAD and normal aging, potentially caused by a shift in APP metabolism, lead to reduced proliferation that can be ameliorated by sAPPα infusion. This work represents a major step forward in understanding how APP metabolism regulates NPC biology. It will be of interest to observe what future discoveries in this line of investigation will provide as far as further understanding of how APP metabolism impacts neurogenic signaling and the development of therapeutic interventions designed to combat alterations in this process.

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Curriculum Vitae

Michael P Demars

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Research Experience:

2006-2011

Advisor: Orly Lazarov, Ph.D.
Projects: *Analysis of neurogenesis early in life in a familial Alzheimer's disease transgenic mouse model.*

The role of soluble amyloid precursor protein in proliferation of adult neural, mesenchymal and human placental stem cells

Soluble amyloid precursor protein in the aging brain: implications for aging-linked neurogenic decline

Analysis of neurogenesis in a fragile X mental retardation protein knockout mouse model

Experience Gained:

Basic laboratory experience: culture of cell lines, mouse mesenchymal stem cells, human placental stem cells and derivation and culture of mouse primary adult neural stem cells; *in vitro* assays including MTT, BrdU proliferation, clonogenic assay, neural stem cell differentiation and self renewal; dissection of mouse brain including dissection of hippocampus, subventricular zone, olfactory bulb, cerebellum, frontal cortex and pyriform cortex; membrane fractionation; density gradient ultracentrifugation; Western blot; phosphorylation sensitive Western blot; immunoprecipitation and immunodepletion; in vivo BrdU, CldU and Idu injection; stereotactic surgery; immunofluorescence; immunohistochemistry; immunocytochemistry; confocal, fluorescence, light and video microscopy; 3-D image reconstruction; image quantification; stereology; PCR; DNA, RNA, and protein extraction; colorometric multiwell plate assays; olfactory discrimination testing.

Writing and instructional experience: writing and publishing of scientific papers and review articles; preparation of posters and other presentations; oral presentation of academic material to varying audiences; review and revision of scientific writing; grant writing (NRSA); departmental instructor for introduction to stereology; instruction of junior lab members on various techniques.

Computer experience: Microsoft Power Point, Word and Excel; Abode Acrobat, Illustrator and Photoshop; GIMP; Image J; Carl Zeiss LSM Software; Beckmann Coulter Multimode Detection; Stereo Investigator; Endnote; Internet and NCBI research.

Certifications and membership: Completed Introduction to human embryonic stem cell course, WiCell, Madison, WI 2007.

Society for Neuroscience, student member *2008-present*

Presentations and Abstracts:

Soluble amyloid precursor protein is a proliferation factor for adult neural stem cells. Demars MP, Bartholomew A, Strakova Z and Lazarov O. (2011) Poster presented at the Stem Cell and Regenerative Medicine symposium, Chicago, IL.

Soluble amyloid precursor protein is a proliferation factor for adult neural stem cells. Demars MP, Bartholomew A, Strakova Z and Lazarov O. (2010) Oral presentation at Society for Neuroscience conference, San Diego, CA.

Soluble amyloid precursor protein promotes stem cell proliferation. Demars MP, Bartholomew A, Strakova Z and Lazarov O. (2009) Oral presentation at Society for Neuroscience conference, Chicago, IL.

The role of amyloid precursor protein and its metabolites in adult neurogenesis. Demars MP and Lazarov O (2009). Poster presented at the Chicago chapter of the Society for Neuroscience conference, Chicago, IL.

Amyloid precursor protein metabolites regulate self-renewal and proliferation of adult generated neural stem cells. Demars MP, Bongarzone ER and Lazarov O (2008). Oral Presentation at the Society for Neuroscience Conference, Washington D.C.

Familial Alzheimer's disease pathology differentially affects discrete neurogenic niches of the adult brain. Demars MP, Gadadhar A, Hu YS and Lazarov O (2008). Poster presented at the International Conference on Alzheimer's Disease (ICAD), Chicago, IL.

The cross-talk between famlial Alzheimer's disease and adult neurogenesis. Demars MP, Gadadhar A, Larson JR and Lazarov O (2008). Presented at the Chicago Society for Neuroscience conference, Chicago, IL.

Publications:
All in the family: How the APPs regulate adult neurogenesis (Review). Lazarov, O and Demars, MP. *In Prep 2012*.

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