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Amyloid-beta peptide affects viability and differentiation of embryonic and adult rat hippocampal progenitor cells

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Amyloid-beta peptide affects viability and differentiation of embryonic and adult rat hippocampal progenitor cells

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TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Alzheimer's Disease (AD) is a debilitating human neurodegenerative condition, the risk of which increases steadily with age. AD is characterized by a progressive neurological deterioration and corresponding loss of mental faculties. Eventually an AD patient becomes unable to care for him/herself and requires full-time assistance. The disease is presently terminal in all cases, and represents a forthcoming public health crisis as human lifespans increase and the large post-World War II generation enters the age group at highest risk for the disease.

Current therapies for AD are extremely limited, and provide only temporary, symptomatic relief. The handful of AD drugs on the market can slow the progression of the disease for a few months at best, but soon lose their efficacy (Eastley *et al.*, 2001). At present, no therapy exists which can halt or reverse the CNS deterioration that is characteristic of the disease.

Neural stem cells have recently generated much excitement in the medical community for their potential use as a source of new neurons to replace those lost to neurodegenerative diseases. However, the potential of these cells is largely still untapped, owing mainly to our limited understanding of their basic biology. Here, we investigate the biology of

neural stem cells in an *in vitro* model of AD to assess their potential for therapeutic use.

Thesis organization

The following thesis is composed of three main sections. The first section (Chapter 1) includes a general introduction and a literature review on Alzheimer's Disease, Beta-Amyloid peptide, stem and progenitor cells, and transplantation therapy.

Chapter 2: Amyloid-Beta Peptide Affects Viability and Differentiation of Embryonic and Adult Rat Hippocampal Progenitor Cells, *James N. Eucher, Etsuro Uemura, Donald S. Sakaguchi, and M. Heather West Greenlee*, is a manuscript which will be submitted for publication in Experimental Neurology. Eucher was the major contributor to the investigation, and author of the manuscript, under the supervision of Greenlee, Uemura, and Sakaguchi.

Chapter 3 is a general discussion of the results presented in Chapter 2, as well as discussion of results from additional, unpublished experiments. It also includes direction of future research. The appendix contains the unpublished results referred to in Chapter 3.

Literature Review

Alzheimer's Disease

Alzheimer's Disease (AD) was first recognized as a distinct clinical entity in the early 20th century. An autopsy was conducted on a 51-year-old female who had exhibited a variety of symptoms, including depression, hallucinations, and general cognitive impairment. The physician examining the brain (Dr. Alois Alzheimer, after whom the disease was later named) noted gross pathological changes including thinning of gyri and widening of sulci in the cerebral cortex, as well as an enlargement of the cerebral ventricles. On microscopic examination, extensive formations of dense extracellular plaques were noted, as were intracellular fibrillary tangles within many neurons (Alzheimer, 1907).

Over the next several decades, further characterization of the disease was accomplished. The extracellular plaques that Alzheimer had observed were determined to be composed primarily of a small peptide of consistent sequence known as amyloid. The intracellular neurofibrillary tangles (NFTs) were determined to be repeats of a hyperphosphorylated cytoskeletal component called Tau. Neurons containing NFTs or located near amyloid plaques tend to die, and this neuronal loss seems to be concentrated in specific areas of the brain including the hippocampus, frontal cortex, and limbic system. Neuronal death in these areas leads to specific functional

deficits characteristic of AD, such as difficulty in reasoning, emotional instability, and memory impairment.

A key molecular player in AD was identified as a small protein of about 700 amino acids called Amyloid Precursor Protein (APP). This protein is widely expressed throughout the body in many tissue types, and is highly expressed in the central nervous system (CNS). Despite much study, the exact function of APP in normal physiology is not clear. APP knockout animals survive and are generally healthy, if less active than wild-type animals (Zheng *et al.* 1995).

Within the benign APP protein, a potential threat resides. A short component sequence of APP, called amyloid, is an oligopeptide of 39-43 residues in size, and is located near the C terminus of APP (Mattson *et al.*, 1993). If liberated, amyloid is thought to be involved in a plethora of toxic effects on neurons (discussed in next section).

In the human brain, APP is initially cleaved by one of two proteases. The first, dubbed α-secretase, cleaves APP in the middle of the amyloid segment (Parvathy *et al.*, 1998), precluding the liberation of the toxic amyloid fragment. Alternatively, cleavage by ß-secretase cuts at a site closer to the N terminus of APP, lopping off a fragment of approximately 100 AA that contains the amyloid sequence (Vassar, 2004).

For some time, the enzyme responsible for paring down the 100 AA fragment into the 39-43 AA fragment known as amyloid

was not identified. Recent work by many groups, however, has established the identity of a polysubunit enzyme known as γ-secretase (for review see Kimberly and Wolfe, 2003). Cleavage by γ-secretase produces two prominent forms of amyloid, known as amyloid 1-40 or 1-42, which are, respectively, 40 or 42 AA in length (Wolfe *et al.*, 1999). These monomers tend to aggregate together, assuming a ß-pleated sheet conformation. An aggregation of many amyloid monomers in this conformation is known as amyloid-beta (Aß).

Although there is not complete consensus within the scientific community that Aß is the primary causal agent of AD, it is clear that a process of neuroinflammation is a major contributor to neuronal loss. Studies have shown that the presence of a wide variety of molecules, including interleukins, tumor necrosis factor, other cytokines, and Aß itself, can cause an increase in inflammation in the brain, and subsequent neuronal damage/death (for review see Wang and Shuaib, 2002, and Eikelenbloom *et al.*, 2002). Mice transgenic for a mutant APP gene (which leads to increased Aß production) show higher levels of the inflammatory mediator p38 MAP-kinase in their microglia, and increased microglial activation; when subjected to focal brain ischemia, they show significantly more neuronal damage then do controls, suggesting that the neuroinflammatory process renders neurons less resistant to additional insults (Koistinaho *et al.*, 2002). In another study, primary cultures of hippocampal neurons were engineered

to express mutations in presenilin-1 (one of the components of the γ-secretase) that lead to increased Aß liberation. Addition of a molecule that damages DNA, etoposide, caused a more vigorous inflammatory response, and more cellular damage, than was observed in controls (Chan *et al., 2002)*.

Several studies have attempted to determine the distribution of various proteins that are thought to be involved in the pathology of Alzheimer's Disease. Konishi *et al.*, 2003 utilized immunochemical techniques to show that frame-shift mutant forms of APP were present in the brains of AD patients, as well as those patients who at time of death did not have AD but showed more AD-like neuropathology than controls. This study also noted that complement proteins were seen only in diagnosed AD brains, suggesting that inflammatory events such as complement activation may not be observed when the disease is still at subclinical levels. Yan *et al.*, 2004 made use of γ-secretase inhibitors to map the distribution of γ-secretase in adult rat brain. Most γ-secretase was localized to the forebrain, cerebellum, and brainstem. Of these locations, only the forebrain is typically associated with much AD pathology – but interestingly, within these areas, γ-secretase seemed to be present at the highest levels in areas of most neuronal terminals – and by extension, in areas of most synaptic plasticity.

Imaging technologies that permit *in vivo* examination of the AD brain, allowing clinicians and researchers to observe

the progress of the disease, are still at a relatively primitive stage. However, some findings of interest have already been made. A study using MRI and labeled brain metabolites such as choline found that metabolite levels were lower in the left temporal lobe in patients with diagnosed AD, as well as patients with mild cognitive impairment (not enough to make a diagnosis of AD) relative to the levels in healthy controls (Chantal *et al.*, 2004). MRI and 3-D cortical mapping has been used to visualize the loss of gray matter in AD patients. When compared to healthy age-matched controls, AD patients began with less gray matter volume, and lost it at a faster rate, than did their control counterparts. Gray matter loss was greatest in the areas typically associated with worst AD pathology, and decreased volume of gray matter correlated very strongly with worsening cognitive status (Thompson *et al.*, 2003).

Amyloid-Beta

Several decades of study have resulted in a general consensus among AD researchers that Aß is a major contributor to neurodegeneration in the Alzheimer's brain. AD is a sufficiently complex disease that its actual etiology is likely to be multifactorial; thus one can examine the role of Aß in development of AD pathology without closing a door to the additional contribution of other molecules such as Tau.

The amino acid sequence of Aß is known and is consistent within a given species. In the human, Aß 1-40 and 1-42 are most prevalent, with 1-42 generally considered the more toxic form. As the disease progresses, the ratio of Aß 1-40 to Aß 1-42 typically shifts to favor the latter form (Kienlen-Campard *et al.*, 2002). The N-terminus of the peptide is hydrophilic, while the C-terminus is hydrophobic. The region 26-29 is a ß-turn and is necessary for amyloid monomers to aggregate. The 25-35 region of Aß is believed to be the neurotoxic site – amyloid segments of various lengths and configurations are not toxic if the 25-35 region is missing or incomplete; however the rest of the protein can add to its toxicity – for example, full length Aß 1-42 is more toxic than Aß 25-35 (Pike *et al.*, 1995).

A number of reviews have examined the various mechanisms proposed for Aß toxicity over the past decade. Mattson, 1997 focuses on the biochemistry of APP, concluding that cleavage by the α-secretase generates beneficial products, unlike β and γ-secretase cleavage which liberates Aß. Harkany, *et al.*, 2000 explore Aß toxicity in terms of what has been learned from various pharmacological studies, suggesting that Aß may act directly on the NMDA receptor. A fairly recent review, written since the identification of the γ-secretase, is Atwood *et al.* (2003), which points out that Aß may even be neurotrophic in certain situations, but that its oxidative properties are largely deleterious to the cell.

A number of toxic activities have been attributed to Aß in recent years. One theory is that Aß subjects the brain to a level of oxidative stress that exceeds its antioxidant capacity (Mattson, 1997). Most formulations of this theory include the idea that the oxidative damage affects not only neurons directly, but also indirectly by causing astrocytic dysfunction – some argue that Aß's indirect actions, via astrocytes, are ultimately the more damaging than its direct effects on neurons (Paradisi, *et al*, 2004). Aß 1-42 and 25-35 have been shown to decrease levels of the antioxidant glutathione in astrocytes, which are normally responsible for neutralizing reactive oxygen species (ROS) in the brain (Abramov *et al.*, 2003). Indeed, Aß has been shown to increase production of ROS in astrocytes, through activation of NADPH oxidase, which in turn makes neurons in the vicinity more vulnerable to ROS attack (Abramov *et al.*, 2004). Wyss-Coray *et al.*, 2003 showed that cultured astrocytes migrated toward Aß plaques in brain sections, and reduced the Aß load after arriving in areas with dense Aß accumulation. However, examination of post-mortem brains from AD victims has demonstrated that many small amyloid plaques are built around astrocytes containing Aß 1-42 as well as neuron-specific proteins like ChAT and nAChR, suggesting that while astrocytes may clean up destroyed neurons and synapses, the astrocytes eventually become overburdened, exceed their phagocytic capacity, lyse, and die, adding to the overall plaque load in

the brain (Nagele *et al.*, 2003). Paradisi, *et al.*, 2004, report that neurons exposed to Aß for several days do better than controls when healthy astrocytes are then added in culture. Conversely, neurons actually sustained more damage when they were grown in co-culture with astrocytes, and both cell types were exposed to Aß from the beginning – the implication being that healthy astrocytes can aid neurons, but unhealthy astrocytes may contribute to the pathology.

Several studies have shown direct effects on neurons by Aß. Treatment of neurons in primary culture with Aß 1-42 or 25-35 inhibits fast axonal transport within the cell (Hiruma *et al.*, 2003). Treatment of primary culture neurons with Aß 25-35 has also been shown to inhibit glucose uptake, possibly via a mechanism involving inhibition of GLUT3 vesicle fusion with the neuronal plasma membrane (Uemura and Greenlee, 2001). Neurons treated with Aß 1-42 show an inability to bind cholesterol, which would in turn result in a neuron's inability to repair or remodel its cell membrane (Yao and Papadopoulos, 2002).

In addition to deleterious effects on neurons and astrocytes, Xu *et al.*, 2001 were the first to demonstrate that Aß treatment caused decreased viability and increased cell death of oligodendrocytes in primary culture. Myelin loss from the brain's white matter is observed in a significant proportion of brains from AD victims (Tian *et al.*, 2004).

Much of the neuronal death that occurs in Alzheimer's Disease is thought to proceed via apoptosis. Several proteins that are players in the apoptotic cascade have been discovered to be significantly upregulated in the frontal cortex of patients with AD relative to controls (Engidawork *et al.*, 2001). Mattson, 2000, breaks the apoptosis pathway down into three distinct steps: initiation of apoptosis by influences such as oxidative stress, excitotoxicity, or growth factor deprivation; mitochondrial dysfunction leading to caspase activation; and enzyme activity which results in membrane blebbing, nuclear fragmentation, and death. Several studies have shown that Aß activates caspase-3 in cultured neurons or AD brain sections, and that activation of neuronal caspase-3 is a near-guarantee that a cell will undergo apoptotic death (Stadelmann *et al.* 1999; Harada *et al.* 1999; Uetsuki *et al.* 1999; Ioudina and Uemura, 2003). Uetsuki *et al.* 1999 also reported that inhibition of caspase-3 restored cell survival to nearly 100%. Some debate exists as to whether caspase-3 is the most important therapeutic target in relation to Aß toxicity. Troy *et al.* 2000 argue that caspase-2 is more important than is caspase-3 – using inhibitors to caspases 1, 2, or 3, they found that inhibiting caspase-2 returned cell survival to near 100%, whereas inhibiting the other two had minimal effect on survival. Another study drew yet another conclusion, finding that selectively inhibiting any one of caspases 2, 3, or 6 was significantly neuroprotective.

Another layer of complexity is added by the interaction between caspases and calpains (calcium-dependent protein kinases), which are thought to be involved in both apoptotic and necrotic cell death (Denecker *et al.*, 2001). Neumar *et al.*, 2003, report that when calpains were inhibited with calpastatin in human neuroblastoma cells, and the pro-apoptotic molecule staurosporine was then added, caspase activity spiked sharply, and the caspases degraded calpastatin. Subsequently, calpain activity also rose, and apoptosis increased substantially. Taken together, the results of these studies on Aß and cell survival indicate that any therapeutic approach aimed solely at a single one or single group of pro-apoptotic enzymes such as caspases or calpains will likely be insufficient.

Within the last five years, the idea of recruiting the immune system to clear Aß from the brain has received much attention (Weiner and Selkoe, 2002). Immunization experiments in rodents showed that immunization with Aß led to a reduction in Aß deposits in the brain (Schenk *et al.*, 1999; Weiner *et al.*, 2000). However, a human trial, in which AD patients were immunized with synthetic Aß 1-42 and the adjuvant QS21, was discontinued in Phase II clinical trials when several patients experienced a severe aseptic meningoencephalitis (review by Broytman and Malter, 2002).

A fundamental, and unanswered, question regarding Aß is its effective concentration *in vivo*. While several

researchers have offered estimates ranging from the nanomolar (Abe and Kimura, 1996; Kato *et al.*, 1997) to micromolar (Behl *et al.*, 1994; Dore *et al.*, 1997) concentrations, these represent an "educated guess" as no method presently exists for quantifying Aß load in the living brain. A recent study identified a series of styrylbenzoxazole derivatives that bind Aß with high affinity, easily cross the blood-brain barrier, and do not cause side effects in rodents (Okamura *et al.* 2004). These compounds may be useful in conjunction with PET or CT imaging to image Aß plaques (although not quantify their concentrations) *in vivo*.

Stem Cells and Transplantation Therapy

Because the neurological deficits in AD are ultimately due to damage and death of neurons (and perhaps glia) in affected regions, stem cell transplantation therapy has been proposed as a possible therapeutic approach (Suguya, 2003). The general concept behind transplantation therapy is to introduce neural stem cells into the brain of an AD patient. These stem cells may either respond to cues from the damaged area, or be pre-treated prior to transplantation, such that they differentiate into new cells that can replace those lost to the disease, ameliorating the functional deficits that had been present.

To some extent, the concept of a "stem cell" is intuitive to the anatomy of a complex multicellular animal, when one

considers that all the billions of cells comprising an adult must originate from a single zygote. Current definitions of a stem cell all include two main characteristics. The cell must first be multipotent – have the capacity to give rise to several kinds of differentiated cells; and second, stem cells must be self-renewing – capable of replicating more undifferentiated cells identical to itself (Gage, 2000).

The first cells of an organism that meet the aforementioned criteria, and thus can be considered true stem cells, are those of the inner cell mass of the blastocyst – the so-called "embryonic stem cells". These cells have the capacity for self-renewal – placed in appropriate tissue culture conditions, they will freely replicate themselves. They also have a very broad capacity for differentiation – a single embryonic stem cell (ESC), depending on the signals it receives, should be capable of differentiation into nearly any cell type in the body (Gage, 2000).

A cell's differentiation ability becomes noticeably less broad at the level of the multipotent stem cell. Multipotent stem cells can self-renew, and generally have the ability to form all the cell types in a given tissue. However, their self-renewal capacity may be limited – after a certain number of divisions, they may enter a senescent state and be capable of no further divisions. They are usually referred to in reference to the tissue type they give rise to (ex: neural stem cells, hematopoietic stem cells, etc). Multipotent stem

cells have long been recognized in the embryo or fetus, and in certain adult tissues that frequently replace their cells, such as epithelium. However, until recently, adult stem cells had not been identified in tissues with minimal adult regenerative capacity, such as those of the nervous system. Only within the last decade have neural stem cells been positively identified in the adult animal (Lois and Alvarez-Buylla, 1993; Kuhn *et al.*, 1996; Palmer *et al.*, 1997).

For most stem-like cells, there is some measure of ambiguity as to when they leave the category of multipotent stem cells, and enter the next – that of progenitor cells. Typically, progenitor cells are defined as cells which can give rise to all the cell types in a particular structure or region (ex: hippocampal progenitor cells, retinal progenitor cells, etc). Significantly, progenitor cells may exhibit a lower limit on their self-renewal capacity compared to multipotent stem cells. They also may be more restricted in terms of the cell types they can generate – for example, a hippocampal progenitor cell may not be able to give rise to neuronal types not seen in the hippocampus, such as Purkinje cells.

While a progenitor cell can give rise to all the cell types in a particular structure, a committed progenitor cell is limited to giving rise only to the cells of a distinct developmental lineage within that structure (ex: a glial precursor cell can give rise to astrocytes and

oligodendrocytes but not neurons). Committed progenitors are sometimes limited in the number of divisions they can undergo; at this point the cells may also begin expressing molecular markers, and/or showing morphological features, characteristic of the differentiated cell types they can generate. Important to note is that while committed progenitor cells may share some phenotypic characteristics with the mature cell types they can generate, they lack the functional characteristics of those cells – for example, a neuronal progenitor cell may look something like a neuron, but it is not capable of producing action potentials (Zhang, 2001).

At the opposite end of the spectrum from the ESC, we find the fully differentiated cell. These are functional cells that no longer have any degree of multipotency – they are fully committed to a specific cell type, and possess all the appropriate phenotypic and functional characteristics. In the central nervous system, the three primary types of differentiated cells are neurons, astrocytes, and oligodendrocytes.

Although adult neurogenesis is thought to be limited to two regions, the hippocampal dentate gyrus and the subventricular zone beneath the lateral ventricles (van der Kooy, 2002), neural stem cells have been isolated from both neurogenic and non-neurogenic regions of the nervous system. Areas from which NSCs have been isolated include hippocampus (Palmer *et al.* 1997), white matter (Nunes *et al.* 2003),

olfactory bulb (Pagano *et al.* 2000), spinal cord (Rao *et al.* 1998), and hypothalamus (Markakis *et al.* 2004).

Several recent studies have explored the control of proliferation and differentiation of neural stem cells. The Wingless (Wnt) signaling pathway appears to be involved in the switch from proliferation to differentiation. Pharmacological stimulation of the Wnt pathway causes cells to remain proliferative; conversely, inhibition of Wnt gene products induces cells to stop proliferating and begin differentiation (Sato *et al.*, 2004). The gene *Notch1* has also been linked to control of proliferation. Attenuation of *Notch* signaling in neural stem cells increased differentiation of both neurons and glia, while increased activation of *Notch1* led to more proliferation and less differentiation (Hitoshi *et al.*, 2002). The protein Sonic hedgehog (*Shh*) causes increases in both proliferation and differentiation of all lineages in neural stem cells (Lai *et al.* 2003). Son *et al.*, 2001 report that dexamethasone, a synthetic glucocorticoid, has an inhibitory effect on the differentiation of hippocampal progenitor cells, and that this effect could be blocked by application of RU486, a glucocorticoid receptor antagonist. Chronic high levels of interleukin-6 (IL-6, a proinflammatory molecule) have been shown to significantly reduce proliferation, differentiation, and survival of neural progenitor cells in the adult mouse, which may help explain why diseases with substantial neuronal

loss often include an inflammatory component (Vallieres *et al.*, 2002).

Several proteins have been identified which seem to bias neural stem cells toward the adoption of a particular lineage fate. Bone morphogenic proteins were identified over a decade ago as important players in embryonic development, and have been shown to direct NSCs to adopt a predominantly glial fate (Shah *et al.*, 1996). Ueki *et al* 2003 identified a protein, which they named Neurogenesin-1, that exerted antagonistic effects on the BMPs and thereby enhanced neurogenesis. Turnley *et al* 2002 show that knockouts of the protein SOCS2 (suppressor of cytokine signaling 2) produce substantially more glia than wild-types, while overexpression of SOCS2 yields more neurons than normal. Amouroux *et al.*, 2000, found that NSCs cultured in the presence of the neural cell adhesion molecule (NCAM) tended to differentiate along a neuronal lineage.

Progenitor cells of the hippocampus have received significant attention in the literature over the last several years. The hippocampus is a particularly intriguing site for progenitor cell isolation for two reasons. First, a number of factors are known to be particularly injurious to the hippocampus in disease states, such as ischemia, oxidative stress, and high levels of excitatory neurotransmitters (Yagita *et al.*, 2001; Dash *et al.*, 2001). Second, the dentate

gyrus of the hippocampus represents one of only two known regions of adult neurogenesis (Gage, 2000).

The first detailed isolation and characterization of neural progenitor cells from the hippocampus was done by Palmer, *et al.*, 1997. They isolated EGF and bFGF-responsive cells from the hippocampus of adult rats. In culture, these cells (termed AHPCs – adult hippocampal progenitor cells) showed the ability to self-renew without chromosomal or other abnormalities for at least 35 population doublings. The cells could be induced to differentiate by withdrawal of growth factors, and generated neurons, astrocytes, and oligodendrocytes under *in vitro* differentiation conditions.

Effect of various growth factors on HPCs was one of the first, and still major, topics of interest for these cells. Kuhn *et al.*, 1997 investigated the effects of EGF and bFGF on neural progenitors of the hippocampus and subventricular zone *in vivo*. Intracerebroventricular infusion of EGF and bFGF suggested that neither affected *in vivo* proliferation of HPCs, but that EGF increased gliogenesis and decreased neurogenesis. Another intriguing finding was that progenitor cells isolated from non-neurogenic regions of the adult CNS, such as cortex and optic nerve, will generate neurons *in vitro* after exposure to bFGF, just as HPCs do (Palmer, 1999). The implication is that fully capable neural progenitor cells may exist throughout the adult CNS, but in most regions they produce

only glia because they are lacking certain environmental signals to encourage or permit neurogenesis.

Shetty and Turner, 1998 investigated the effects of brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial-derived neurotrophic factor (GDNF) on HPCs in culture. Cells treated with BDNF showed increased survival and differentiation along a neuronal lineage; NT-3 and GDNF showed no effect.

Another growth factor thought to affect HPCs is insulin-like growth factor 1 (IGF-1). Aberg *et al.* 2000 have demonstrated that peripheral (subcutaneous) administration of IGF-1 to adult rats leads to increased progenitor cell proliferation and neurogenesis in the hippocampus, with no effects on gliogenesis. A later *in vitro* study by this group concluded that the IGF-1 effects are mediated by the mitogen-activated protein kinase (MAPK) pathway. This study also demonstrated an additive effect of bFGF and IGF-1 in stimulating the proliferation of the HPCs. Significantly, bFGF treatment increased the expression of the IGF-1 receptor, suggesting a mechanism for their synergistic action (Aberg *et al.* 2003).

Toda *et al.*, 2003 used a variety of cDNA and RNA screening methods to identify a survival factor secreted by adult HPCs themselves. Stem cell-derived neural stem/progenitor cell supporting factor (SDNSF) is expressed in the rat hippocampus throughout life, and is upregulated in

response to ischemic injury. HPCs treated with SDNSF after withdrawal of bFGF maintained their self-renewal potential. Based on their experiments, the authors suggest that SDNSF acts in an autocrine and/or paracrine manner. Interestingly, SDNSF was not detected outside of the hippocampus, and was conspicuously absent from the subventricular zone, another known area of adult neurogenesis.

The AHPC line characterized by Palmer, 1997, has been of some interest for use in transplantation to the retina. Takahashi *et al.*, 1998 were the first to do so, grafting AHPCs into the vitreous of newborn and adult rats. The transplanted cells integrated well, particularly in the newborn eyes. Many cells took on morphologies consistent with retinal cell types such as Müller glia, bipolar cells, and photoreceptors, although the cells did not express all the immunological markers consistent with these cell types. Young *et al.* 2000 injected GFP-transfected AHPCs into the vitreous of RCS rats (a particular strain frequently employed as a model of retinal degeneration). AHPCs survived in the retina for as long as 18 weeks. The cells' progeny could be identified by GFP expression; progeny expressed a number of mature neuronal markers and appeared to migrate to appropriate layers within the retina. A later study by Suzuki *et al.* 2003 injected BDNF along with the AHPCs; when BDNF was included as a component of the cell injections, more AHPCs were found to express neuronal markers (such as MAP2) and fewer were seen to express neural

stem cell markers (such as Nestin) when examined several weeks after transplantation.

Seaberg and van der Kooy, 2002, reported that the stem-like cells found in the dentate gyrus were neuronally-restricted progenitors rather than true neural stem cells. They used microdissection techniques to remove very small samples of tissue from hippocampal DG and subventricular zone. After culturing the cells and processing them with immunocytochemistry, they concluded that the cells isolated from the DG were not multipotent – they could form neurons, but not glia. Conversely, they argued that the cells of the SVZ exhibited true multipotency; thus the SVZ contained true neural stem cells while the DG did not. Seaberg and van der Kooy additionally believed that the multipotent AHPCs were not true DG residents but instead were an artifact of SVZ cells contaminating samples obtained from the DG. Aberg *et al.*, 2003 took issue with these claims, responding that the dissection technique used by Seaberg and van der Kooy was also flawed, allowing DG contamination of their SVZ section. They also noted that Alvarez-Buylla and others have used the van der Kooy dissection technique to successfully isolate multipotent stem cells from the DG (unpublished data). This dispute has not yet been fully resolved, but to date additional studies supporting the viewpoint of Seaberg and van der Kooy have failed to emerge. Much less controversial are findings reported by a number of groups that the neuronal

progeny of hippocampal progenitor cells are able to develop properties of mature, functional CNS neurons. Song, *et al.* 2002 examined AHPCs and their progeny *in vitro*, observing that the new neurons showed electrical activity, morphology, and immunochemical labeling consistent with forming functional synapses. Van Praag *et al.*, 2002 conducted a similar study *in vivo*, using GFP and a retroviral vector to label HPCs and newly formed cells. The new neurons expressed neuronal markers TUJ1 and NeuN, exhibited appropriate synaptic morphology, and elicited action potentials. Mistry, *et al.* 2002 examined E16 HPCs *in vitro*. After 21 days in differentiation conditions, the newly formed neurons expressed neurotransmitters, neurotransmitter receptors, and synaptic proteins. They formed elaborate networks with each other and spontaneously generated action potentials.

A very recent study has examined the electrophysiological characteristics of AHPCs as they undergo the differentiation process. As the cells acquired a differentiated neuronal phenotype, they became excitable, and began expressing voltage-sensitive, tetrodotoxin-sensitive Na+ channels, low and high voltage-sensitive Ca++ channels, and delayed-rectifier K+ channels. The authors maintain that the appearance of these several types of ion channels represents one of the most important steps in the stem cell to neuron transition (Hogg *et al.* 2004).

A fundamental question of relevance to stem cell transplantation is whether allogenic cells will be recognized as foreign by the host immune system. Klassen *et al.* 2003 examined rat hippocampal progenitor cells in culture and found only minimal MHC Class I expression, and no detectable Class II expression. The rat HPCs did not elicit a response from human monocytes or spleen cells. Hori *et al.* 2003 transplanted neural progenitor cells beneath the kidney capsule (an immunologically non-privileged site) of adult mice. The transplanted cells survived, did not sensitize the host, and failed to express either MHC Class I or Class II antigens. The brain is an immunologically privileged site, and transplants there also fail to elicit an immune response (Wenkel *et al.*, 2000).

A second question of relevance is whether the site into which stem cells are transplanted affects the types of stem cell progeny that can be generated. Neural stem and progenitor cells have been transplanted into several sites in adult animals, including spinal cord (Cao *et al.* 2001), striatum (Dziewczapolski *et al.* 2003), retina (Suzuki *et al.* 2003), and hippocampal dentate gyrus (Shihabuddin *et al.* 2000). A common finding is that neural stem cells transplanted to locations of adult neurogenesis display the ability to generate substantial numbers of both neurons and glia, whereas those transplanted to non-neurogenic regions typically form only glia, or mostly glia and only very few

neurons. Some studies have tried to prime stem cells with factors favoring neurogenesis, or utilize neuronal restricted progenitors, to allow neurons to be generated in non-neurogenic regions; these approaches have been somewhat successful (Suzuki *et al.* 2003, Han *et al.* 2002).

A third issue germane to transplantation is whether the newly generated cells can functionally integrate into the existing synaptic networks. Studies using hippocampal slice culture have suggested that embryonic stem cell-derived neurons (Benninger *et al.*, 2003) and astrocytes (Scheffler *et al.*, 2003) can functionally integrate into the surrounding tissue. Another study found functional integration of adult-born neurons into the olfactory bulb (Carlen *et al.* 2002), lending further support to the idea that even in the adult brain, newly formed neurons are capable of inserting themselves properly into a pre-existing synaptic network.

Despite the fact that neural stem cells have been transplanted into animal models of diseases such as Parkinson's Disease (Dziewczapolski *et al.* 2003) and ischemic stroke (Hoehn *et al.* 2002), transplantation studies using an APP-mutant mouse or other animal model of AD are lacking. Since the AD brain contains the neurotoxic compound Aß, *in vitro* studies exposing NSCs to Aß can therefore be useful in predicting what may happen to the cells in an *in vivo AD* model.

Prior studies examining the effects of amyloid-beta on neural stem cells are limited to three published works. Haughey *et al.*, 2002a show that Aß injected into the lateral ventricle of adult mice caused a decrease in migration, proliferation, and neurogenesis from neural stem cells of the subventricular zone. They also demonstrated a decrease in proliferation and differentiation, and an increase in apoptosis, when Aß was applied *in vitro* to cultured neurospheres from human embryonic cortex. Haughey *et al.*, 2002b showed similar decreases in neural stem cell proliferation and differentiation in the brains of mice expressing an APP mutation that increased brain Aß levels. Application of Aß to cultured mouse and human NSCs caused increased apoptotic cell death, and upregulation of caspases and calpains. Lopez-Toledano and Shelanski, 2004, exposed NSCs from rat striatum and mouse hippocampus to Aß *in vitro*, noting that oligomeric Aß 1-42 had a neurogenic effect – a directly contradictory conclusion to the work of Haughey *et al.*, who maintain that Aß inhibits neurogenesis.

Although these prior studies represent a starting point for further investigation, there are many issues that remain to be resolved. Further information on the effects of Aß on neural stem cells is needed before NSCs can be seriously considered for use in transplantation therapy for AD. Therefore, we have examined the effects of Aß on adult and embryonic neural stem cells from the same species (rat) and

same anatomical region (hippocampus). This permits a comparison between NSCs from two very different stages of development. We examined the effects of Aß on the viability, proliferation, and differentiation of the NSCs, since these are basic characteristics of the cells that may determine, in large part, the cells' suitability (or lack thereof) for transplantation into an AD brain environment. Our results are of interest both in terms of basic NSC biology, as well as when assessing the challenges that might be posed when introducing these cells into an *in vivo* environment containing substantial levels of Aß.

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CHAPTER 2: AMYLOID-BETA PEPTIDE AFFECTS VIABILITY AND DIFFERENTIATION OF EMBRYONIC AND ADULT RAT HIPPOCAMPAL PROGENITOR CELLS

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Abstract

Neural stem cells (NSCs) have received recent attention due to their potential use in transplantation therapy for a number of neurological diseases. The Alzheimer's Disease (AD) brain is a uniquely challenging environment for transplanted cells due to the presence of amyloid-beta (Aß) peptide, which is known to be toxic to neurons. The effects of Aß on NSCs have just recently begun to be investigated. Here, we examine the effect of Aß 25-35 on hippocampal progenitor cells (HPCs) from embryonic and adult rat. We also examine the effect of the tripeptide Gly-Pro-Arg (GPR) which has been reported to rescue primary culture neurons from Aß-induced toxicity. HPCs exposed to Aß in culture showed decreased viability as measured by MTT mitochondrial dehydrogenase assay; GPR was not protective against Aß-induced viability loss. Aß affected differentiation of adult, but not embryonic HPCs; adult HPCs exposed to Aß expressed the neural stem cell marker Nestin at

higher levels than did controls. Aß did not affect proliferation of embryonic HPCs, although GPR did suppress embryonic HPC proliferation. Aß also did not show differential effects on proliferating neurons or glia, as determined by colabeling of BrdU and the neuronal marker MAP2 or the astrocytic marker GFAP. Our findings suggest that Aß decreases viability and affects differentiation of neural progenitor cells; however specific effects upon neurogenesis or NPC proliferation were not seen.

Introduction

The neurological deficits that are characteristic of Alzheimer's Disease (AD) are ultimately a result of neuronal loss in distinct anatomical regions of the brain. For this reason, neural stem cell (NSC) transplantation therapy has been proposed as a possible therapeutic approach (for review, see Suguya, 2003). The AD brain represents a particularly inhospitable environment for transplanted cells, as it contains beta-amyloid (Aß), a small protein of 39-43 amino acids in length (Yankner, 1996). The toxic effects of Aß on neurons have been demonstrated *in vitro* as well as *in vivo*, on several cell types of the brain including neurons (Pike, 1995; Walsh, 2002), astrocytes (Abramov, 2003; Nagele, 2003), and oligodendrocytes (Xu, 2001). Data concerning the effects of Aß on NSCs, however, is still sparse. A study that examined cultured human and mouse NSCs (Haughey, 2002) suggested that

Aß disrupted neurogenesis and impaired NSC proliferation. A different study using cultured rat and mouse NSCs reached the opposite conclusion, suggesting that Aß's effects on NSCs were neurogenic, and did not impair proliferation (Lopez-Toledano, 2004). Comparisons between these earlier studies are complicated by the use of NSCs from three different species, obtained at a number of different developmental stages, and taken from several different anatomical regions within the brain. To further clarify how Aß may affect NSCs, we have examined its effects on NSCs isolated from one species (rat), from the same location (hippocampus), at two different time points (embryonic day 17 and adulthood). We also examined the effect of the tripeptide Gly-Pro-Arg (GPR) on NSCs; GPR has recently been reported to rescue cultured hippocampal neurons from Aß-induced toxicity (Ioudina and Uemura, 2003). Our data suggest that Aß affects viability and differentiation of NSCs from rat hippocampus, and that these effects differ based on the developmental stage from which the NSCs are obtained. However, our data does not indicate any particular effect of Aß upon neurogenesis, nor does Aß appear to affect NSC proliferation. GPR does not appear to rescue NSCs from viability loss due to Aß; in addition, GPR may have a moderate degree of effects on NSC differentiation.

Materials and Methods

Hippocampal progenitor cell isolation and culture

Adult HPCs (AHPCs; Palmer *et al.*, 1997) were a gift from Dr. Fred Gage, The Salk Institute, and were typically obtained at approximately seven passages. Embryonic HPCs (EHPCs) were isolated at Iowa State University. 17-day timed-pregnant HSD rats were anesthetized with halothane and the uteri were removed. Fetuses were removed and their hippocampi were dissected out. Tissues were digested in 0.25% trypsin for 15 min at room temperature, followed by 5 minutes in 2 mg/ml trypsin inhibitor. Neurobasal/B27 media was added and tissues were dispersed by trituration with a fire-polished glass pipette. EHPCs were then placed in tissue culture flasks. Each flask was filled with 15 ml of EHPCs suspended in culture media.

Both cell types were maintained in growth media consisting of Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12, Invitrogen) supplemented with EGF (Invitrogen) at 40 ng/ml and bFGF (Promega) at 20 ng/ml. Every two days, fresh media was added to the flasks. Cells were passaged weekly and were typically used for experiments between P8-P12 (AHPCs) or P3-P7 (EHPCs).

For experiments, cells were plated onto coverslips (for ICC) or directly into 24-well plates (for MTT) coated with polyethyleneimine (PEI, Sigma). Differentiation media used consisted of Neurobasal media with B27 supplement

(Invitrogen), and contained bFGF at 5 ng/ml. Cells were allowed 24 hours after plating to adhere to the PEI substrate before any treatments were added. Cells were allowed to differentiate for 3 or 7 days before processing. In the case of 7-day groups, 1/2 of the media in each well was replaced, and cells were re-treated, three days after the first treatment.

Cell treatments

Cells listed as receiving amyloid were treated with 1 or 10 μM Aß 25-35 (Bachem), which had been allowed to pre-aggregate at 37°C for a minimum of one week (Pike *et al.*, 1995). Cells listed as receiving GPR were treated with synthetically produced Gly-Pro-Arg tripeptide (Bachem) at a concentration of 50 μM. Cells labeled with Bromodeoxyuridine (BrdU) were treated with BrdU at a concentration of 10 μM.

MTT Viability Assay

After 3 or 7 days, cells were processed for the MTT mitochondrial reduction assay. This assay is based on the ability of active mitochondrial dehydrogenases to convert the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to water-insoluble purple formazan crystals. The degree of conversion of yellow MTT to purple product is indicative of a cell's mitochondrial activity, and provides an estimate of the cell's overall

viability (Abe, 1999). Cells were incubated with MTT (Sigma) for 4 hours at 37°C. The crystals were then dissolved in isopropanol; the solution was aspirated and transferred to a 96-well plate, and absorbance was measured on a Molecular Devices SpectraMax 190 spectrophotometer, at a wavelength of 570 nm, with background subtracted at 630 nm.

Single-label immunocytochemistry

All staining procedures were performed at room temperature. For single-label ICC, cells were fixed with 4% paraformaldehyde for 20 min and then rinsed 3 times (10 min each) in 50 mM KPBS (Potassium Phosphate Buffered Saline; per 1 L double-distilled water, contains NaCl 9.00 g/L, K_2HPO_4 5.85 g/L, KH_2PO_4 2.25 g/L). Cells were incubated in blocking solution (consisting of KPBS as solvent; per 1 L KPBS, contains normal donkey serum (NDS, Jackson) at 0.9 g/L, bovine serum albumin at 10.0 g/L, Triton-X 100 at 4.28 g/L) for 2 hrs. Primary antibodies were then added (Mouse anti-Nestin, MAP2, GFAP, RIP) and cells were incubated for 20 hrs. The following day cells were rinsed 3 times (10 min each) in 50 mM KPBS containing Triton-X 100 at .214 g/L (KPBS-TrX). Secondary antibody was then added (Biotinylated Donkey anti-Mouse, 1:600, Jackson) and cells incubated for 2 hrs. Cells were rinsed 3 times (10 min each) in KPBS-TrX, and then incubated in KPBS-TrX containing Cy3-Avidin (Jackson) at 1:5000 concentration for 30 min. Cells were washed 3 times in

KPBS (each 10 min), then once (for 5 min) in KPBS containing 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) at 1:333 concentration to label cell nuclei. After three more washes in KPBS (5 min each) coverslips were mounted to glass slides using VectaShield mounting media (Vector Laboratories). Primary antibody concentrations were as follows (all are mouse monoclonals): α-Nestin clone 401, 1:10 (Developmental Studies Hybridoma Bank, University of Iowa); α-MAP2ab, 1:750 (Sigma); α-GFAP, 1:2400 (Sigma); α-RIP, 1:125 (Developmental Studies Hybridoma Bank, University of Iowa).

Double-Label BrdU Immunocytochemistry

All staining procedures were performed at room temperature. For double-label ICC, cells were fixed with 70% ethanol for 30 min, and then rinsed twice (5 min each) in 50 mM KPBS. Cells were incubated in 2.0 N HCl for 15 min, and then in 0.1 M sodium borate for 5 minutes. Cells were rinsed twice (5 min each) in KPBS and incubated in blocking solution containing normal goat serum (NGS, Jackson) for 2 hrs. Primary antibody was then added (rat anti-BrdU) and cells were incubated for 20 hrs. The following day cells were rinsed 3 times (10 min each) in KPBS-TrX. Secondary antibody (Biotinylated Goat anti-Rat, 1:200, Jackson) was applied and cells incubated for 2 hrs. Cells were rinsed 3 times (10 min each) in KPBS-TrX, and then incubated in KPBS-TrX containing Cy2-Avidin (Jackson) at 1:750 concentration for 30 min. Cells

were rinsed 3 times (10 min each) in KPBS, and then incubated in a blocking solution containing normal donkey serum (NDS) for 2 hrs. Primary antibody was then added (Mouse anti-Nestin, MAP2, GFAP) and cells were incubated for 20 hrs. The third day, cells were rinsed 3 times (10 min each) in KPBS-TrX. Secondary antibody (Cy3-conjugated Donkey anti-Mouse, 1:250, Jackson) was applied and cells incubated for 2 hrs. Cells were washed 3 times in KPBS-TrX (each 10 min), then once (for 5 min) in KPBS containing 4',6-diamidino-2-phenylindole (DAPI) at 1:333 concentration to label cell nuclei. After three more washes in KPBS (5 min each) coverslips were mounted to glass slides using VectaShield mounting media. Primary antibody concentrations were as follows: Polyclonal rat α-BrdU, 1:200 (Accurate Chemical and Scientific Co.); monoclonal mouse α-Nestin clone 401, 1:10 (Developmental Studies Hybridoma Bank, University of Iowa); monoclonal mouse α-MAP2ab, 1:75 (Sigma); monoclonal mouse α-GFAP, 1:240 (Sigma).

Fluorescent Imaging / Cell Quantification

Cells were visualized using a Nikon Eclipse E800 fluorescent microscope. For each coverslip, cells in 16 random fields under 40x oil-immersion objective were counted. Data collected for each coverslip included total number of cells (based on DAPI staining), number of cells immunopositive for each marker, and percentage of cells immunopositive for each marker. Images were obtained using a Q-Imaging Retiga

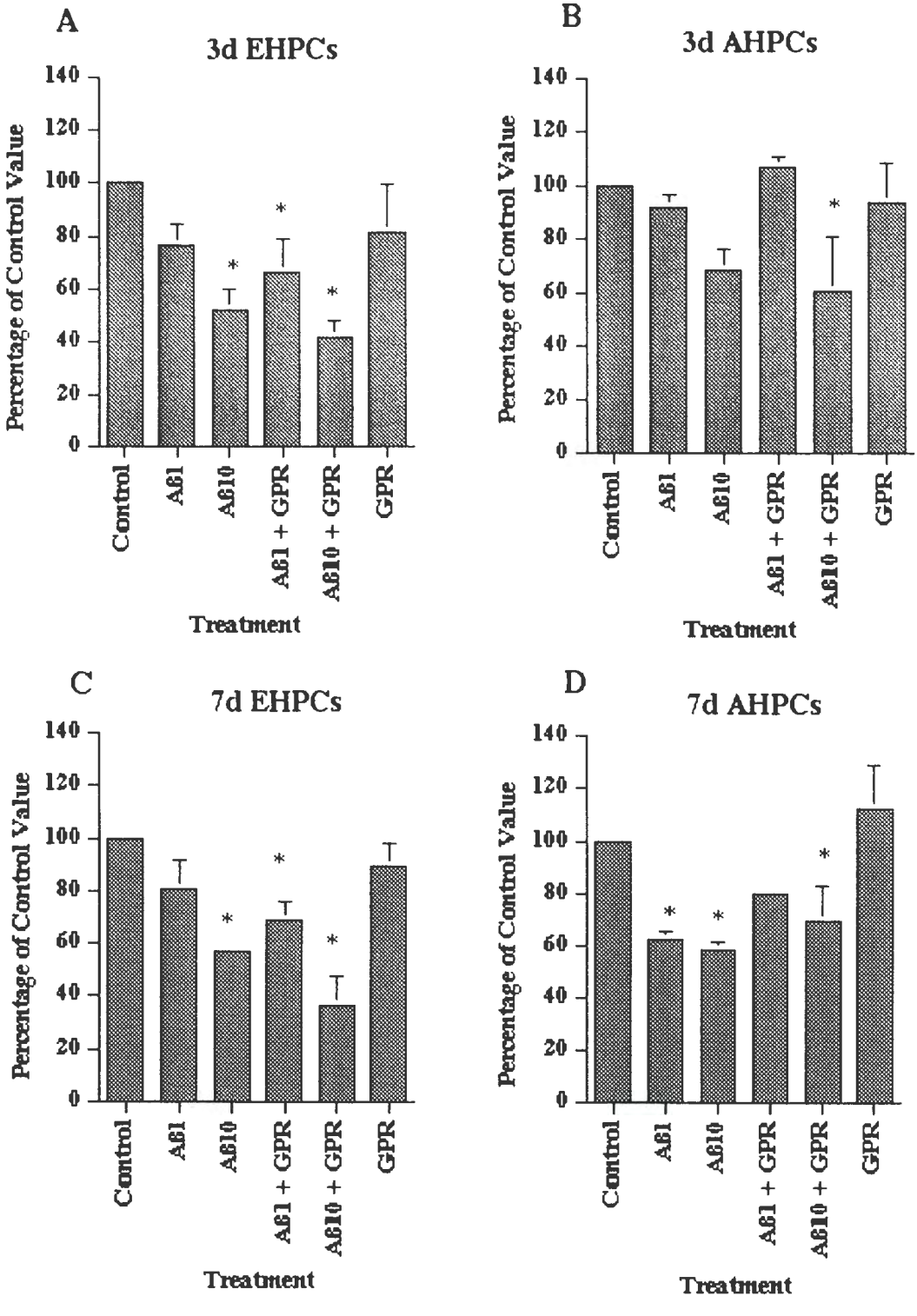
1300 digital camera, and processed on a Macintosh G4 computer using OpenLab 3.1.3 (Improvision), Photoshop 8.0 (Adobe), and Freehand MX 11.0 (Macromedia).

RESULTS

Amyloid-beta 25-35 causes decreased viability in HPCs.

We tested the effect of treatment with Aß 25-35, as well as treatment with the GPR peptide, on HPC viability, as measured by the MTT mitochondrial dehydrogenase assay. This assay allows assessment of mitochondrial dehydrogenase activity; a decrease in activity suggests a loss of cellular viability (Abe, 1999). Cultured AHPCs or EHPCs were treated with 1 or 10 μM Aß, with or without 50 μM GPR peptide. Viability was assayed via the MTT assay at either 3 or 7 days after the first treatment. EHPCs showed significantly decreased viability relative to controls at 3d when treated with 10 μM Aß ($P<.0034$), 1 μM Aß + 50 μM GPR ($P<.016$), or 10 μM Aß + 50 μM GPR ($P<.002$) (Fig. 1A). At 7d, EHPC viability in the same treatment groups was also significantly decreased (P values respectively are $P<.0034$, $P<.0194$, $P<.002$) relative to controls (Fig. 1B). AHPCs showed significantly decreased viability relative to controls at 3d when treated with 10 μM Aß + 50 μM GPR ($P<.0205$) (Fig. 1C). At 7d, AHPC viability was decreased relative to controls when treated with 1 μM Aß ($P<.0039$), 10 μM Aß ($P<.002$), or 10 μM Aß + 50 μM GPR ($P<.0111$) (Fig. 1D). For all MTT experiments, MTT values for untreated controls were set at 100%. Also, in all experiments an additional control, consisting of treatment with 50 μM GPR, but no Aß, was included. In no case was viability significantly decreased by 50 μM GPR alone. These data

Figure 1: MTT viability data for 3d and 7d EHPCs (A, C), and 3d and 7d AHPCs (B, D). Results are expressed at percentage of control value, which is set at 100%. EHPCs show significantly decreased viability after treatment with 10 μM Aß, 1 μM Aß + 50 μM GPR, or 10 μM Aß + 50 μM GPR, and this effect is similar at both 3d and 7d time points. AHPCs show significantly decreased viability at 3d after treatment with 10 μM Aß + 50 μM GPR; at 7d, significant effects are also seen in 1 μM Aß and 10 μM Aß groups. Treatment means (n=3) are compared to control means; * = $P<.05$.


A
3d EHPCs
Percentage of Control Value
140
120
100
80
60
40
20
0
Control
A61
A610
A61 + GPR
A610 + GPR
GPR
Treatment
B
3d AHPCs
Percentage of Control Value
Control
A61
A610
A61 + GPR
A610 + GPR
GPR
Treatment
C
7d EHPCs
Percentage of Control Value
Control
A61
A610
A61 + GPR
A610 + GPR
GPR
Treatment
D
7d AHPCs
Percentage of Control Value
Control
A61
A610
A61 + GPR
A610 + GPR
GPR
Treatment

suggest that Aß significantly decreases EHPC viability by 3d, and that the GPR peptide is not protective against this effect, and may partially contribute to it when combined with Aß (Aß 1 μM alone did not lead to significantly decreased viability, but Aß 1 μM + 50 μM GPR did). These effects persist when EHPC viability is assayed at 7d. The data suggest that Aß may take longer to affect AHPC viability – at 3d, only Aß 1 μM + 50 μM GPR led to significantly decreased viability relative to controls. At 7d, AHPCs treated with 1 μM Aß, 10 μM Aß, or 10 μM Aß + 50 μM GPR showed significantly decreased viability relative to controls. The effect of the GPR peptide, in combination with Aß, on AHPC viability is less clear. At 3d, AHPCs receiving 10 μM Aß + 50 μM GPR had significantly decreased viability, while those receiving 10 μM Aß alone did not. However at 7d, AHPCs receiving 1 μM Aß alone had significantly decreased viability, while those receiving 1 μM Aß + 50 μM GPR did not. Thus, for both cell types, Aß most commonly decreases viability, while the GPR peptide has no effect alone, but may contribute to decreased viability in combination with Aß.

Amyloid-beta 25-35 affects differentiation of adult, but not embryonic, HPCs.

To determine whether differentiation profiles of HPCs are affected by Aß 25-35 or GPR peptide, single-label immunocytochemistry was performed either 3d or 7d after

Figure 2: Representative single-label ICC images for EHPCs at 7 days in differentiation conditions. Bar = 20 μm in each image. Columns show treatment applied (control, 10 μM Aß alone, both 10 μM Aß and 50 μM GPR, 50 μM GPR alone. Rows show antibody labeling for Nestin (neural stem cells), MAP2 (neurons), GFAP (astrocytes), and RIP (oligodendrocytes).

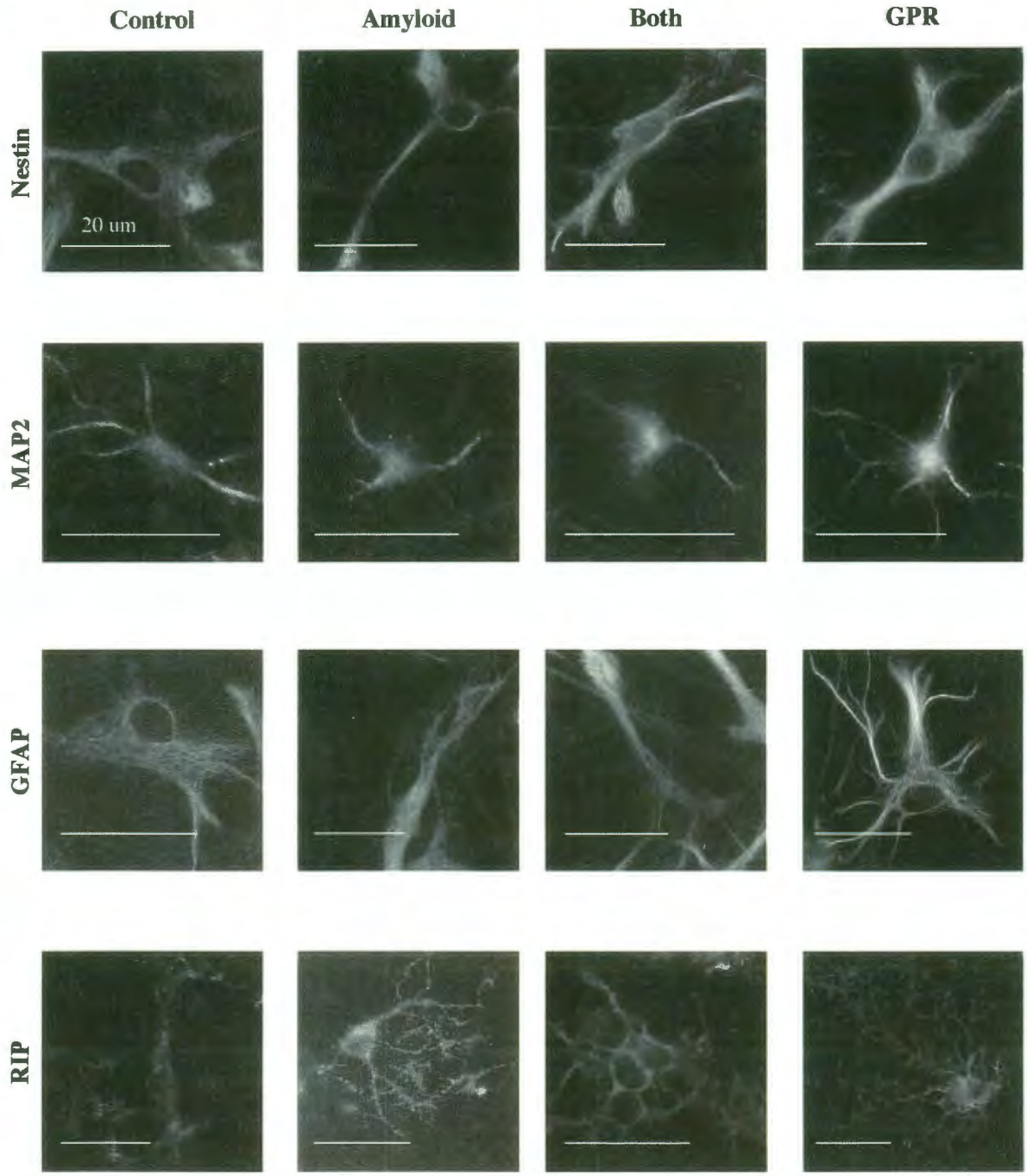
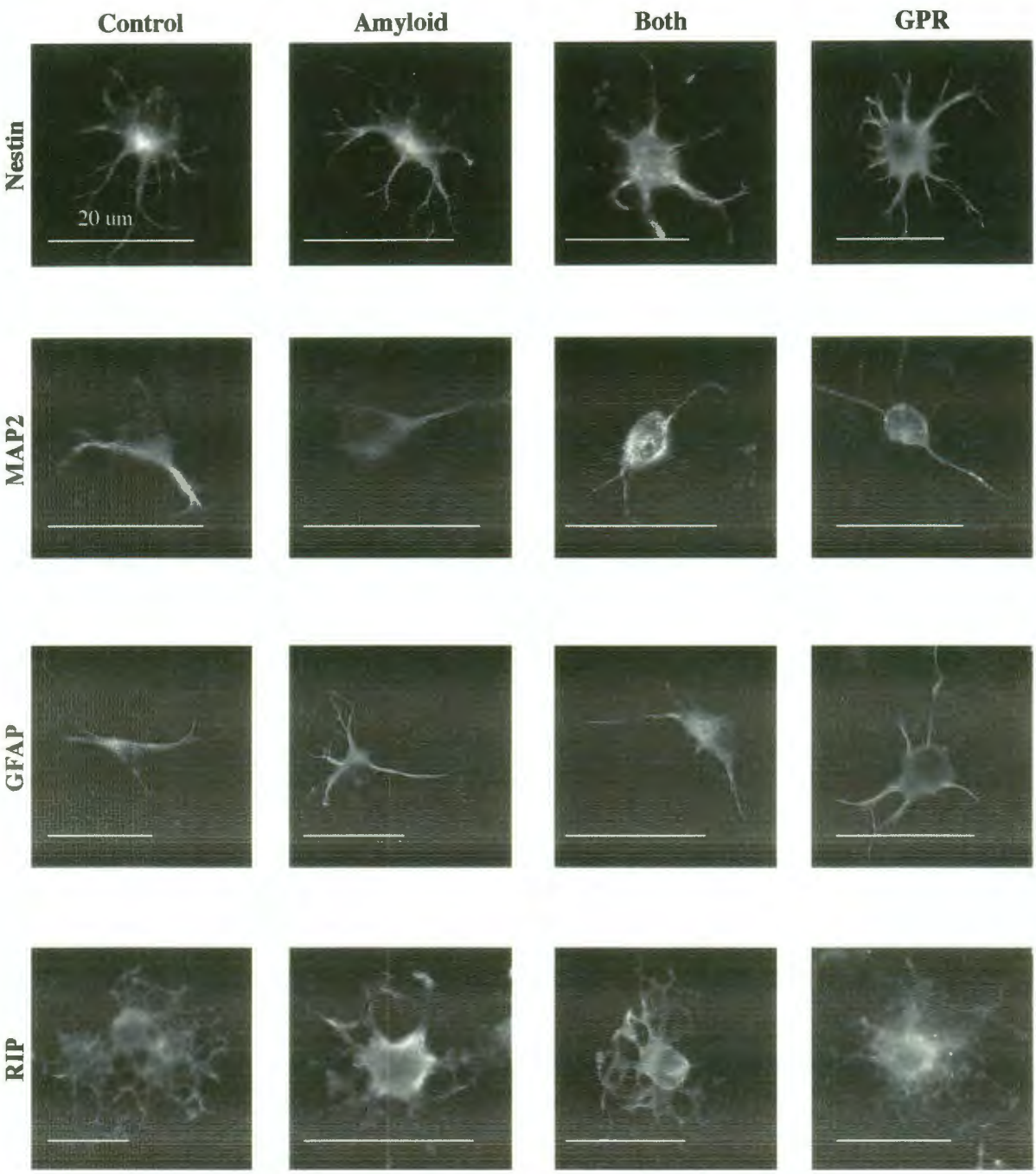

Control
Amyloid
Both
GPR
Nestin
MAP2
GFAP
RIP
20 um

Fig. 2, continued: Representative single-label ICC images for AHPCs at 7 days in differentiation conditions. Bar = 20 μm in each image. Columns show treatment applied (control, 10 μM Aß alone, both 10 μM Aß and 50 μM GPR, 50 μM GPR alone. Rows show antibody labeling for Nestin (neural stem cells), MAP2 (neurons), GFAP (astrocytes), and RIP (oligodendrocytes).

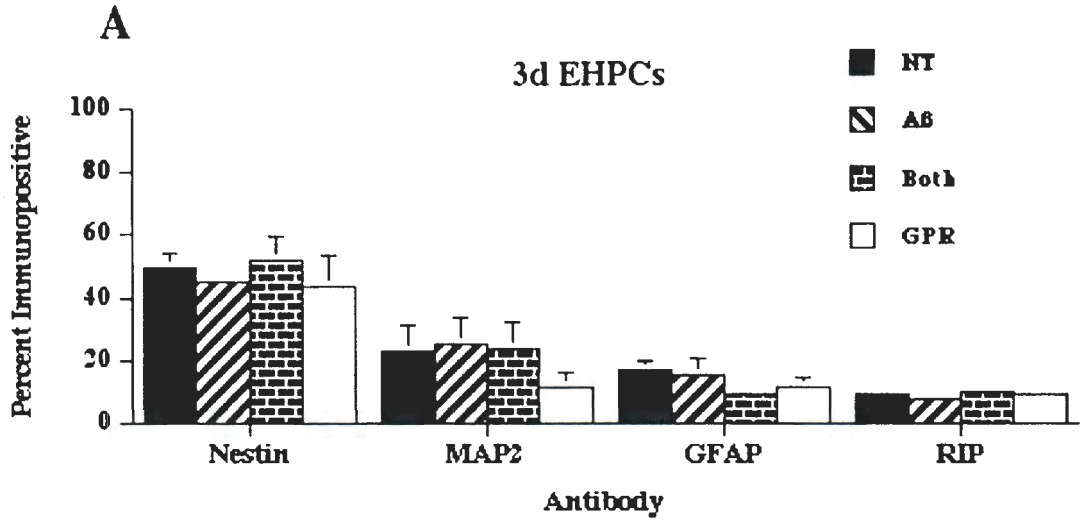

Control
Amyloid
Both
GPR
Nestin
MAP2
GFAP
RIP
20 um

initial treatment with 10 μM Aß, 50 μM GPR, or 10 μM Aß + 50 μM GPR. Representative ICC images are shown in Fig. 2A for EHPCs; in Fig. 2B for AHPCs. Cells were stained with cell-type specific antibodies against Nestin (neural stem cells), MAP2 (neurons), GFAP (astrocytes), and RIP (oligodendrocytes) (Fig. 2A, EHPCs; Fig. 2B, AHPCs).

Control percentages for 3d EHPCs were Nestin 49.28 +/- 4.57%; MAP2 22.85 +/- 8.65%; GFAP 16.63 +/- 2.94%; RIP 8.89 +/- 1.45%. Control percentages for 3d AHPCs were Nestin 72.40 +/- 7.68%; MAP2 6.88 +/- 2.87%; GFAP 0.22 +/- 0.22%; RIP 19.50 +/- 7.90%. At 3d, no effect was observed in either cell type, for any antibody, among all treatment groups.

Control percentages for 7d EHPCs were Nestin 58.48 +/- 4.35%; MAP2 32.54 +/- 7.70%; GFAP 21.33 +/- 2.19%; RIP 4.93 +/- 1.84%. Control percentages for 7d AHPCs were Nestin 48.17 +/- 3.50%; MAP2 0.71 +/- 0.28%; GFAP 4.07 +/- 3.77%; RIP 51.72 +/- 6.87%. At 7d, EHPCs treated with 50 μM GPR showed significantly fewer MAP2-positive cells (11.38 +/- 3.06%; $P<.0085$) relative to controls. None of the EHPCs receiving treatment with 10 μM Aß or 10 μM Aß + 50 μM GPR showed significant differences relative to controls (Fig. 3B). At 7d, AHPCs treated with 10 μM Aß or 10 μM Aß + 50 μM GPR showed significantly more Nestin-positive cells (respectively, 69.05 +/- 2.52%, $P<.0138$; 66.46 +/- 9.10%, $P<.0269$) than did controls. AHPCs treated with 10 μM Aß + 50 μM GPR also showed significantly more MAP2-positive cells (15.22 +/- 6.02%,

Figure 3: Single-label immunocytochemistry data for 3d and 7d EHPCs (A, B). Comparisons were made between treatment means and control mean (n=4 or n=5) within each antibody. For 3d EHPCs, treatment means were not significantly different from control means for any antibody. For 7d EHPCs, cells treated with 50 μM GPR showed significant less MAP2 expression than did controls; * = $P<.05$. Treatment means were not significantly different from control means for other antibodies at 7d.


A
3d EHPCs
NT
Aß
Both
GPR
Percent Immunopositive
100
80
60
40
20
0
Nestin
MAP2
GFAP
RIP
Antibody

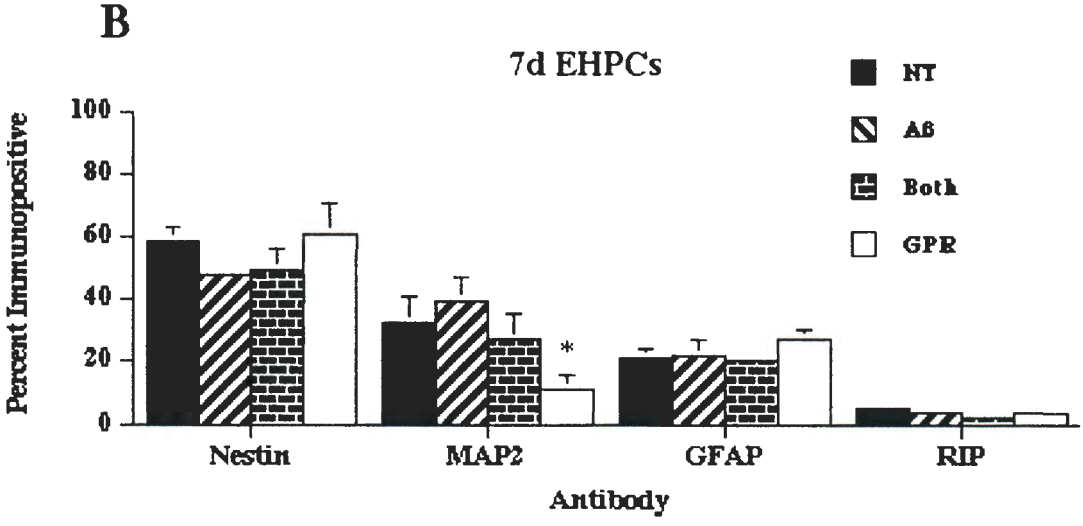
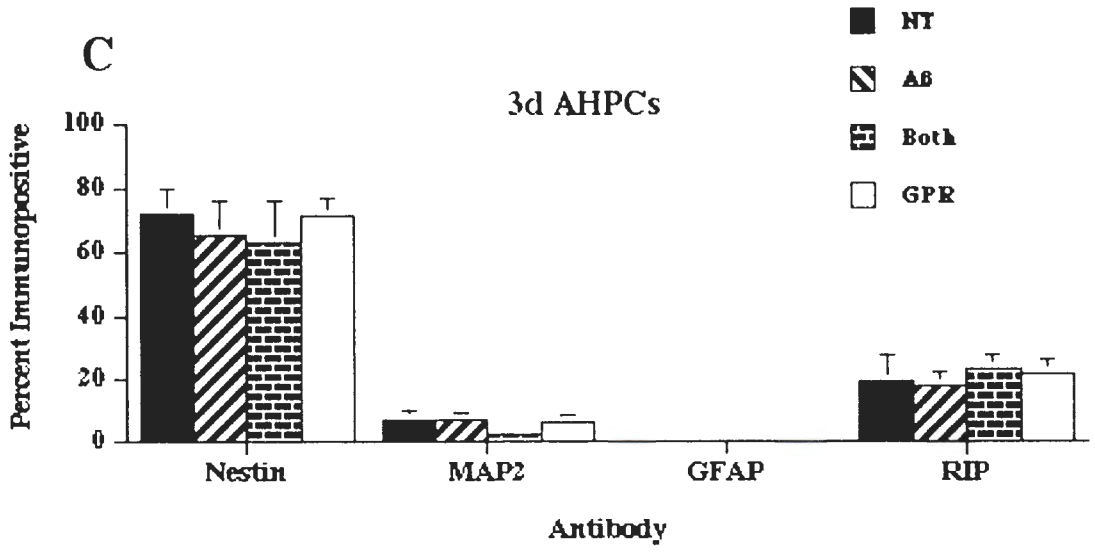
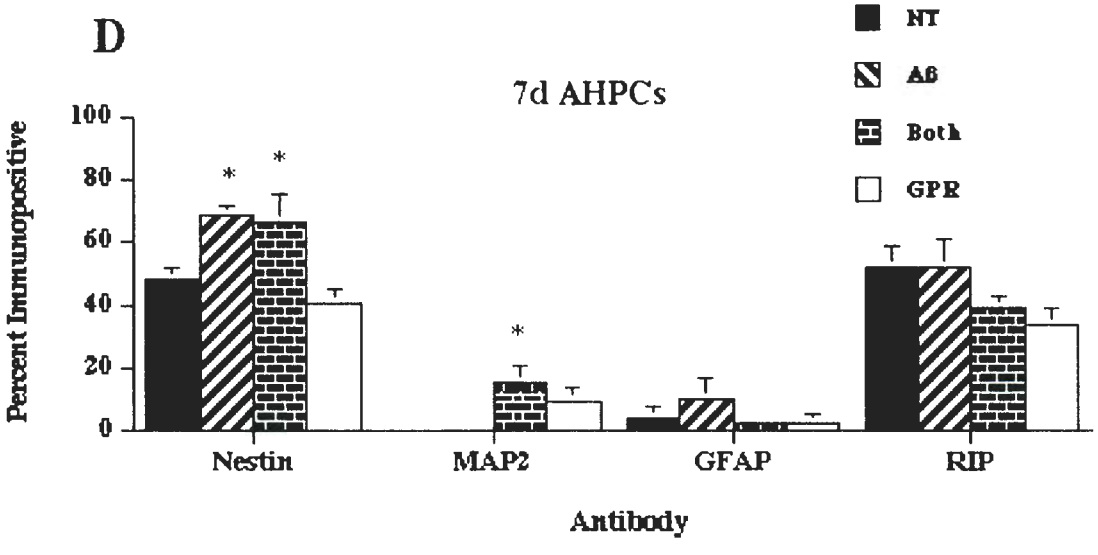

B
7d EHPCs
NT
Aß
Both
GPR
Percent Immunopositive
100
80
60
40
20
0
*
Nestin
MAP2
GFAP
RIP
Antibody

Figure 3, continued: Single-label immunocytochemistry data for 3d and 7d AHPCs (C, D). Comparisons were made between treatment means and control mean (n=4 or n=5) within each antibody. At 3d, treatment means were not significantly different from control means for any antibody. At 7d, cells treated with 10 μM Aß or 10 μM Aß + 50 μM GPR showed significantly more Nestin expression than controls. Cells treated with 10 μM Aß + 50 μM GPR showed significantly more MAP2 expression than controls. * = $P<.05$.


C
3d AHPCs
NT
Aß
Both
GPR
Percent Immunopositive
100
80
60
40
20
0
Nestin
MAP2
GFAP
RIP
Antibody

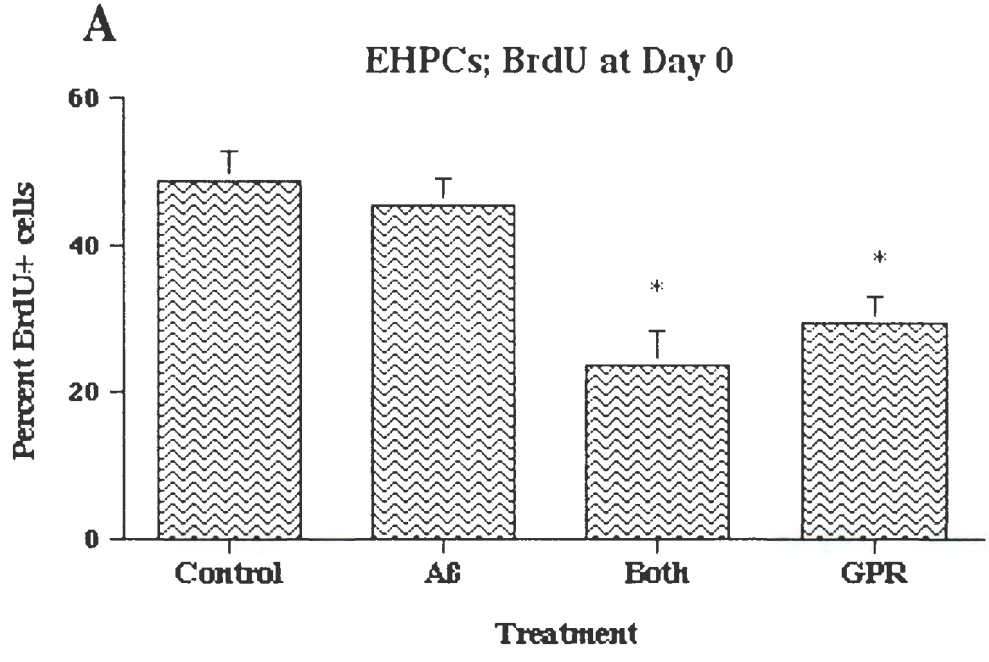

D
7d AHPCs
NT
Aß
Both
GPR
Percent Immunopositive
100
80
60
40
20
0
*
*
*
Nestin
MAP2
GFAP
RIP
Antibody

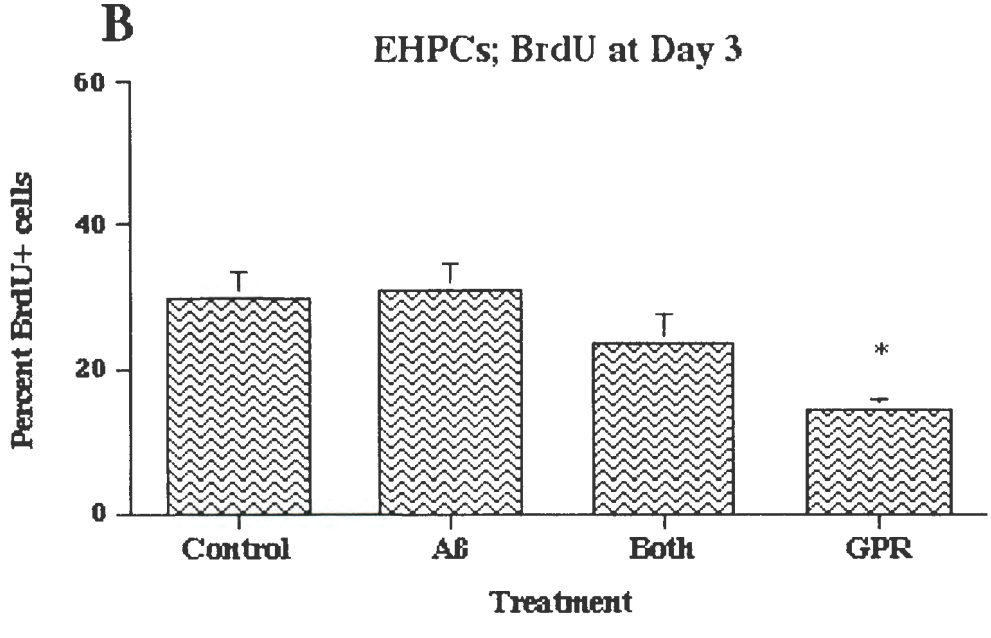
P<.0254) than did controls. None of the AHPCs receiving treatment with 50 μM GPR alone showed significant differences relative to controls (Fig. 3D). Taken together, our results indicate that Aß does not affect EHPC or AHPC differentiation when assayed 3d after treatment. At 7d after treatment, EHPC differentiation is not affected by Aß, while AHPC differentiation is affected by Aß, and the effect is not rescued by GPR.

Amyloid 25-35 does not affect EHPC proliferation, while GPR decreases EHPC proliferation.

To ascertain whether Aß or GPR affects cell proliferation, BrdU labeling of cell proliferation ICC was performed on EHPCs 7d after initial treatment with 10 μM Aß, 10 μM Aß + 50 μM GPR, or 50 μM GPR. BrdU was added with either the first treatment, at Day 0, or the re-treatment, at Day 3. ICC with an antibody directed against BrdU was done after culture termination on day 7. ICC on EHPCs which received BrdU at Day 0 (Fig. 4A) revealed a significant decrease in cell proliferation (as measured by percentage of total cells positive for BrdU) in groups treated with 10 μM Aß + 50 μM GPR (P<.0001), or 50 μM GPR alone (P<.0011), relative to controls. ICC on EHPCs which received BrdU at Day 3 (Fig. 4B) revealed a significant decrease in cell proliferation only in the group treated with 50 μM GPR (P<.0018). The group treated with 10 μM Aß + 50 μM GPR showed a mean percentage

Figure 4: BrdU incorporation data for EHPCs; BrdU ICC was performed 7d after initial treatment with Aß and/or GPR; cells were labeled with BrdU at Day 0 (A) or Day 3 (B). For each set of experiments, treatment means (n=4 or n=5) are compared to control means. For cells receiving BrdU at Day 0, treatment groups receiving 10 μM Aß + 50 μM GPR, or 50μM GPR alone, showed significantly lower percentage of cells immunoreactive for BrdU than did controls. For cells receiving BrdU at Day 3, the treatment group receiving 50 μM GPR showed significantly lower percentage of cells immunoreactive for BrdU than did controls. * = $p<.05$.


A
EHPCs; BrdU at Day 0
Percent BrdU+ cells
60
40
20
0
Control
Aß
Both
GPR
Treatment

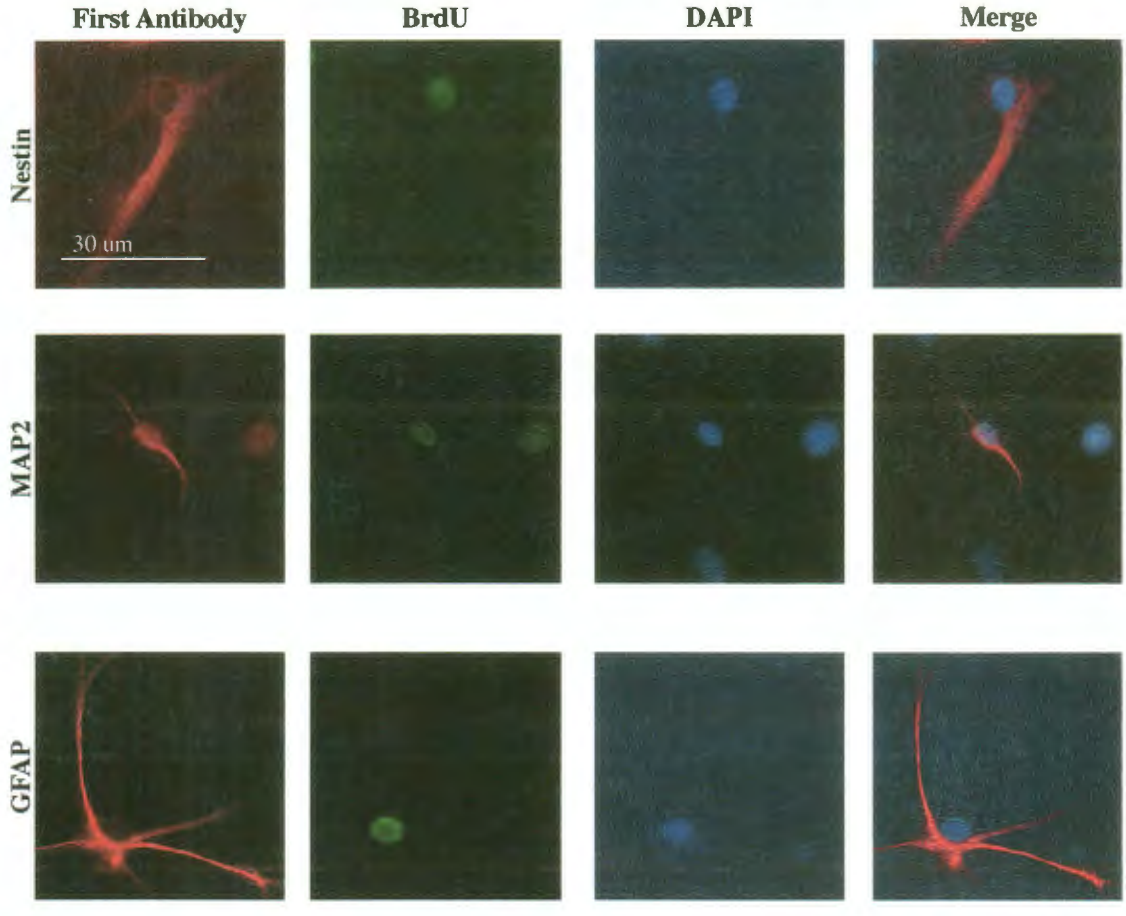

B
EHPCs; BrdU at Day 3
Percent BrdU+ cells
60
40
20
0
Control
Aß
Both
GPR
Treatment

BrdU-positive cells that was intermediate between values observed for control and 50 μM GPR groups, but this value was not significantly decreased relative to the control group. These data suggest that when EHPC proliferation is measured during the timeframe immediately following initial treatment, cells receiving both Aß 10 μM + 50 μM GPR, as well as 50 μM GPR alone, show significantly less proliferation as measured by BrdU incorporation. When EHPC proliferation is measured during the timeframe immediately following the 3d re-treatment, cells treated with 50 μM GPR alone show significantly less proliferation relative to controls. These results indicate that Aß does not effect EHPC proliferation in differentiation conditions.

Amyloid 25-35 does not affect proportion of proliferating cells that colabel with neuronal or astroglial markers

To determine what cell phenotypes constituted the cells that took up BrdU in culture, double-label immunocytochemistry was performed on 7d EHPCs. BrdU was applied at either Day 0 or Day 3; at Day 7 cells were double-stained with antibodies to BrdU and one of the following: Nestin, MAP2, or GFAP (Fig. 5). Comparisons were made between time points and treatments for each of the three antibodies. Values are expressed as the percentage of BrdU-positive cells also positive for Nestin, MAP2, or GFAP.

Figure 5: Representative double-label ICC images for EHPCs at 7d. Images are from control cells with BrdU added at Day 0. Columns show cells labeled with first antibody (red), BrdU (green), DAPI (blue), and all three (merged images). Rows show cells immunoreactive for the following three first antibodies: Nestin (neural stem cells), MAP2 (neurons), GFAP (astrocytes).


First Antibody
BrdU
DAPI
Merge
Nestin
MAP2
GFAP
30 um

Comparison data for Nestin is shown in Fig. 6A. For the group given BrdU at Day 0, cells treated with 10 μM Aß + 50 μM GPR showed significantly increased Nestin/BrdU colabeling ($P<.0262$) relative to controls. For the group given BrdU at Day 3, none of the treated groups shows significantly different Nestin/BrdU colabeling relative to controls, suggesting that by 3 days after the initial treatment, Nestin-positive cells represent a similar portion of BrdU-positive cells for all treatment groups.

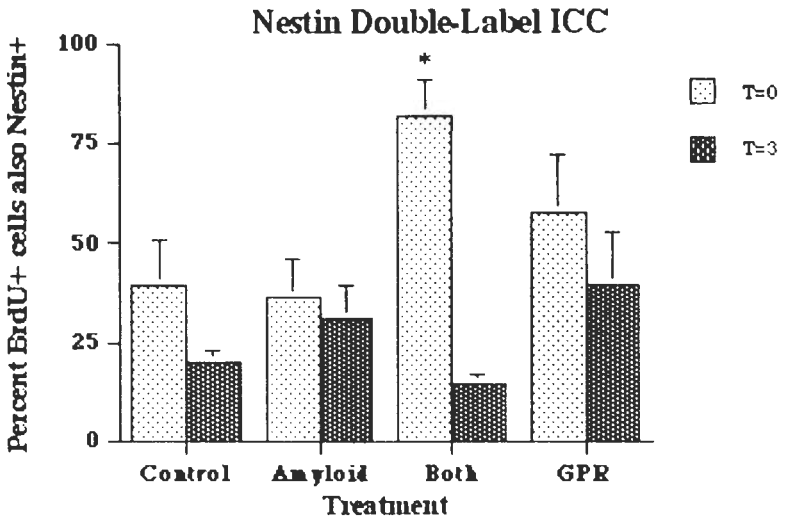
Comparison data for MAP2 is shown in Fig. 6B. For the group given BrdU at Day 0, none of the treated groups shows significantly different MAP2/BrdU colabeling relative to controls. For the group given BrdU at Day 3, the cells treated with 50 μM GPR alone show significantly decreased MAP2/BrdU colabeling ($P<.0304$) relative to controls. This suggests that by 3 days after initial treatment, GPR-treated cells have a smaller proportion of BrdU-positive cells that colabel with MAP2, relative to all other treatment groups.

Comparison data for GFAP is shown in Fig. 6C. At both BrdU timepoints, none of the various treatments induces significantly different amount of GFAP/BrdU colabeling relative to controls.

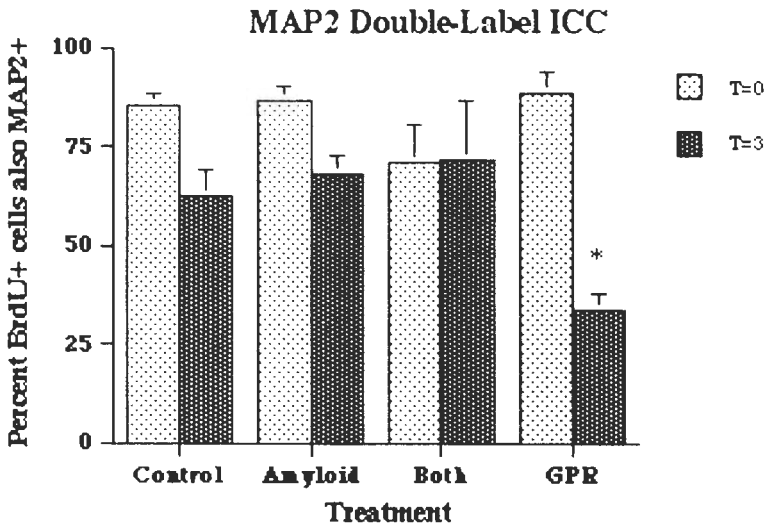
As a group, these experiments demonstrate that Aß does not effect the percentage of BrdU-positive, proliferative cells that also express either the neuronal marker MAP2 or the astroglial marker GFAP. GPR affected only division of cells

Figure 6: Double-label immunocytochemistry data for Nestin/BrdU (A), MAP2/BrdU (B), and GFAP/BrdU (C). Data are expressed as percent of BrdU+ cells also Nestin+, MAP2+, or GFAP+. Data from groups given BrdU at Day 0 and Day 3 are presented side-by-side to allow for visual comparison. Cells treated with 10 μM Aß + 50 μM GPR showed significantly more Nestin/BrdU colabeling relative to control cells, among Day 0 BrdU group (Fig 6A). Cells treated with 50 μM GPR showed significantly less MAP2/BrdU colabeling relative to control cells, among Day 3 BrdU group (Fig 6B). All significance calculations are made within Day 0 or Day 3 groups; amyloid, amyloid + GPR, and GPR treatments are compared against untreated controls (n=4 or n=5). * = $P<.05$.

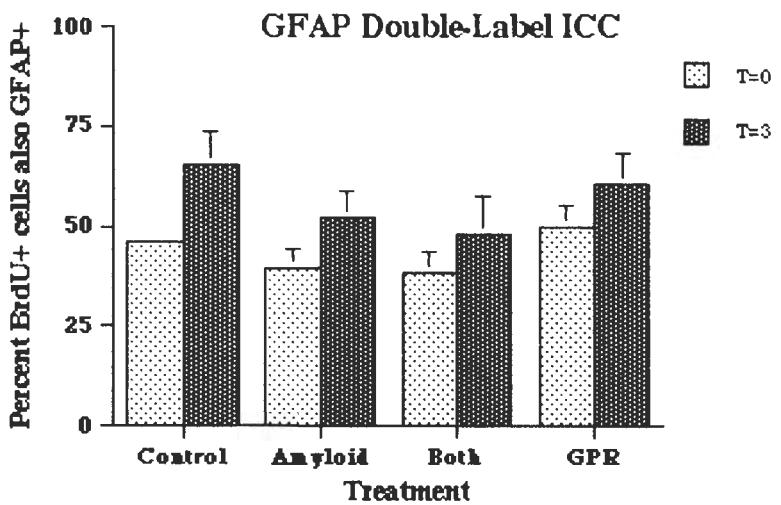
A


Nestin Double-Label ICC
Percent BrdU+ cells also Nestin+
100
75
50
25
0
*
T=0
T=3
Control
Amyloid
Both
GPR
Treatment

B


MAP2 Double-Label ICC
Percent BrdU+ cells also MAP2+
100
75
50
25
0
*
T=0
T=3
Control
Amyloid
Both
GPR
Treatment

C


GFAP Double-Label ICC
Percent BrdU+ cells also GFAP+
100
75
50
25
0
T=0
T=3
Control
Amyloid
Both
GPR
Treatment

expressing one marker (MAP2) at one BrdU time point (Day 3), and only when applied alone, but not when applied with Aß. The combination of Aß + GPR affects Nestin/BrdU colabeling, but only at the earlier BrdU time point (Day 0); this effect seems not to be present at the later time point (Day 3).

In summary, Aß does not affect the proportion of proliferating cells (as measured by BrdU incorporation) that colabel with the neuronal marker MAP2 or the astroglial marker GFAP.

Discussion

The two prior studies examining amyloid effect on neural stem cells have reached essentially opposite conclusions. Haughey *et al.*, 2002a,b (considered together as one connected study) report that amyloid impairs NSC neurogenesis and proliferation. Conversely, Lopez-Toledano and Shelanski, 2004 report that amyloid has the opposite effects. Our results largely fall in between these extremes. It is important to note that of the three groups that have investigated this topic, each has utilized different cell populations, isolated from different species, anatomical regions, and developmental stages.

Our data from the MTT viability assay suggests that Aß causes some loss of viability in NSCs, but is not fatal to them – the MTT value for dead cells is zero. With both EHPCs and AHPCs, a consistent finding was that exposure to Aß caused MTT levels to drop significantly; however, very rarely did they go below 60% of control values within the timeframe of the longest experiments (7d exposure to Aß). Haughey et al., 2002b shows a threefold increase in apoptosis in NSCs exposed to Aß relative to controls. Lopez-Toledano and Shelanski, 2004, report no significant increase in apoptosis after Aß treatment. Although our experiments did not include direct measurements of cell death, prior studies have been done in our lab using the lactate dehydrogenase (LDH) cell death assay to examine neonatal murine brain progenitor cells. This

murine NPC line shows no increase in LDH levels when treated with Aß (Uemura *et al.*, unpublished observation). Conversely, treatment of primary culture neurons with Aß is characterized by rapid and substantial increase in LDH levels due to loss of membrane integrity and death (Ioudina and Uemura, 2003). These observations suggest a physiological difference exists between NSCs and mature neurons, such that NSCs are not fully susceptible to the effects of Aß that are so deleterious to neurons.

Aß's influence on NSC differentiation was dissimilar between the two cell populations. Differentiation of EHPCs was not affected by Aß at any point studied. Conversely, AHPC differentiation was unaffected at 3d, but by 7d cells treated with Aß showed increased Nestin expression relative to controls, while cells treated with Aß and GPR in combination showed increased Nestin expression and increased MAP2 expression. One possible explanation for these results is that AHPCs exposed to Aß compensate by retaining Nestin expression for a longer time. Nestin is typically used as a marker for undifferentiated neural stem cells (Duittoz, 2001), although several authors have recently suggested that Nestin expression may continue even after a cell begins to express proteins such as GFAP that are thought of as markers of more differentiated cells (Seri *et al.*, 2001; Fillippov *et al.*, 2003; Steiner *et al.*, 2004). It is possible that retention of Nestin is associated with retention of other undifferentiated

characteristics, some of which may reduce a cell's vulnerability to Aß-induced toxicity. If so, it might suggest the existence of a "survival mode" for stem cells exposed to a difficult environment such as one with high levels of Aß. Because Aß did not affect EHPC differentiation at all, it is possible that these cells have not yet developed the cellular machinery through which Aß acts to affect differentiation, while the same machinery may be present, at least to some extent, in AHPCs.

Our experiments with EHPCs suggest that Aß does not affect NPC proliferation, which is consistent with the observations of Lopez-Toledano and Shelanski, 2004. Exposure to GPR alone, however, did cause a significant decrease in proliferation when BrdU was present in the media at Day 0 or Day 3. The combination of Aß + GPR also caused significant decrease in proliferation at Day 0, but this effect was no longer present at Day 3. This is a somewhat unexpected observation in light of the fact that the GPR tripeptide is biochemically very similar to another tripeptide, GPE, that is a product of IGF-1 (Ioudina and Uemura, 2003). IGF-1, in turn, has shown to be necessary for the proliferation of neural stem cells (Arsenijivic *et al.*, 2001). Further studies might elucidate the mechanism through which GPR appears to inhibit proliferation of NSCs.

Double-label immunocytochemistry was performed to examine the possibility that Aß and/or GPR might selectively affect

the proliferation of either neurons or glial cells. Cells were labeled with BrdU and either Nestin, MAP2, or GFAP. Aß failed to affect the percentage of cells co-expressing either BrdU and MAP2 or BrdU and GFAP. GPR alone caused a significant decrease in coexpression of MAP2 and BrdU, but only at the Day 3 BrdU time point. In total, the effects of Aß or GPR on the coexpression of MAP2 or GFAP with BrdU were very minimal, suggesting that there is not a differential effect on the proliferation of either neuronal or glial cells. This is in contrast to the findings of Haughey *et al.*, 2002b, who reported that exposure to Aß selectively decreases the population of NSCs that colabel with BrdU and E-NCAM, another neuronal marker.

In summary, the results of our study are very distinct from those reported previously by Haughey *et al.*, 2002a,b as well as Lopez-Toledano and Shelanski, 2004. Our results highlight the differences that exist between neural stem cells isolated from dissimilar stages of development, even if taken from the same species and anatomical region. These results also underscore the heterogeneity of the large number of cell types collectively conceptualized as "neural stem cells". It is likely that each distinct NSC line/population may have a unique degree of tolerance for Aß. This would argue for the necessity of *in vitro* characterization studies, in the presence of Aß, for any neural stem cell line under investigation as a candidate for AD transplantation therapy.

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CHAPTER 3: GENERAL CONCLUSIONS

General Discussion

We are one of the first three groups to examine the effects of Aß on neural stem cells *in vitro*. A group from Johns Hopkins University conducted the first experiments in 2002, producing two closely related publications (Haughey *et al.*, 2002a; Haughey *et al.*, 2002b). This group examined cultured progenitor cells from human fetal cortex, mouse embryonic brain, and mouse cerebellum. In summary, they concluded that Aß decreased neurogenesis and inhibited proliferation. A group from Columbia University published their findings earlier this year (Lopez-Toledano and Shelanski, 2004). They examined cultured progenitor cells from embryonic rat striatum and neonatal mouse hippocampus. This group reported a neurogenic effect of Aß on their NPCs, with no effect on proliferation. Our results represent a third set of conclusions. Examining hippocampal progenitor cells, from embryonic and adult rat, we found that although Aß did affect viability and differentiation, it did not specifically affect neurogenesis, nor did it affect NPC proliferation.

The data collected from our study, considered in conjunction with the two prior works, permits some general overarching conclusions to be drawn. The first is that NPCs in culture appear able to tolerate Aß in their environment. This stands in sharp contrast to prior studies examining the

effect of Aß on cultured neurons, which uniformly demonstrate that neurons find Aß very toxic and are killed by Aß at high nanomolar to moderate micromolar concentrations (Yankner *et al.*, 1989; Loo *et al.*, 1993; Ueda *et al.*, 1994; Ioudina and Uemura, 2003). Our data suggests that although exposure to Aß causes decreased HPC viability, as measured by MTT reduction, the viability rarely drops below 60% of controls after one week of Aß exposure; conversely, cultured neurons undergo substantial degeneration and death after only 24 hours exposure to Aß (Loo *et al.*, 1993). Although Haughey *et al.*, 2002a and 2002b report an increase in apoptosis among NPCs exposed to Aß in culture, the cells that express markers of apoptosis still represent only a fraction of their total cells. Our group has exposed murine NPCs to Aß for up to one week; this exposure causes no increase in cell death as measured by lactate dehydrogenase (LDH) assay (Uemura *et al.*, unpublished observation). In a general sense, then, although NPCs tend to show some indicators of physiological stress when exposed to Aß, they survive in its presence at concentrations which are lethal to neurons.

A second general conclusion is that NPCs will generally yield at least some neurons when differentiated in an environment containing Aß. All three groups which have conducted experiments of this type thus far have obtained differentiated cells which have gross morphological features consistent with neurons and that express neuronal marker

antigens such as TUJ1, MAP2, and NeuN. This is of obvious importance in terms of the feasibility of NSC transplants for Alzheimer's Disease, since the primary goal of this approach is to induce the transplanted NSCs to produce new neurons to replace endogenous neurons that are lost to the disease (Suguya, 2003; Rice *et al.*, 2003; Limke and Rao, 2003). Had these early studies instead suggested that NSCs are incapable of generating new neurons in an environment containing Aß, it would have represented a substantial roadblock to the idea of transplantation therapy for AD. However, further studies need to be made to ascertain that these putative neurons possess the proper physiological characteristics, such as ability to generate action potentials. Obviously, cells that have neuron-like morphology but cannot produce action potentials will not be of therapeutic benefit.

A third general conclusion that can be made is that different lines of stem cells respond to Aß in different ways – that is, that the effect of Aß is far from uniform on all neural stem cells. This is not altogether surprising, given that the three studies have utilized neural stem/progenitor cells from different species, developmental stages, and anatomical regions. Prior experiments have shown that the differentiation patterns of progenitor cells from the same species, developmental stage, and anatomical region (rat embryonic hippocampus) can be drastically altered by changing the differentiation media utilized (Eucher *et al.*, unpublished

data; see Appendix). One consistent theme that has emerged from the literature over the past several years is that what are broadly categorized as "neural stem cells" represent an incredibly heterogeneous group of cells, with behavior that can vary widely based on the innate biology of each cell line, as well as influences such as culture media, presence of growth factors, cell density, growth substrate, etc.

Owing to the substantial diversity of neural stem cells, it is desirable that any candidate line for transplantation therapy should first be examined in an *in vitro* system to assess its response to Aß. Further studies of NSCs transplanted into *in vivo* AD model systems then represent the next logical step. Much work remains to be done before transplantation of NSCs into an actual AD patient can be attempted. Our study, as well as the aforementioned other early studies, represents a first step in the direction of this ultimate goal. The ability of neural stem cells to survive and differentiate *in vitro*, despite the presence of Aß in their environment, confirms their therapeutic potential for the treatment of Alzheimer's Disease.

Recommendations for Future Research

A number of further experiments can be suggested based upon questions raised by our results. Because "neural stem cells" are a very heterogeneous group, with wide differences between cell lines, any NSC line under study as a candidate for transplantation therapy should first be thoroughly characterized *in vitro*, both in the absence and presence of Aß, preferably at a range of Aß concentrations since the exact physiological concentration of amyloid is not known and is likely to be widely variable.

NSCs such as our EHPCs and AHPCs that have been characterized *in vitro* should be transplanted into an established *in vivo* AD model organism such as APP-overexpressing mouse. Survival, differentiation, integration, and physiological function of the transplanted NSCs and their progeny should be assessed. Threats to the safety of the transplant recipient, such as tumor formation, should be searched for carefully.

NSCs that show promise in an *in vivo* AD model system should be transplanted into more complex species such as the geriatric cat or monkey. Continued success and safety in these systems would eventually suggest that transplantation to the human AD brain should be attempted. A quality-of-life improvement in an AD patient receiving transplanted neural stem cells represents the ultimate realization of their therapeutic potential.

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APPENDIX

Media Comparison

As a first step in our experiments, it was necessary to decide on a particular formulation for the media used to differentiate the HPCs. Two different formulas had been in general use in our lab prior to the beginning of the experiments described in Chapter 2.

The first formula utilized Gibco Dulbecco's Modified Eagle Medium with F12 Supplement (DMEM/F12). Also present in this media was glucose, fetal bovine serum, nystatin, N2 supplement, L-glutamine, penicillin, and streptomycin.

The second formula utilized Gibco Neurobasal media. Also present in this media was B27 supplement, L-glutamine, L-glutamate, and a small concentration of basic fibroblast growth factor (bFGF).

In order to make a meaningful comparison between the two media formulas, a single HPC type was used. E17 hippocampal progenitors (EHPCs) were utilized because they were plentiful in the culture laboratory at the time of these experiments.

EHPCs were isolated and kept in growth media for several passages (typically between 3 and 6). Between the third and sixth passage, EHPCs were plated onto polyethyleneimine (PEI)-coated coverslips, into 24-well plates; each well contained 500 μl of either Neurobasal or DMEM/F12 differentiation media. Cells were given 24 hours to adhere to the PEI substrate; time 0 of day 0 (the point at which the experimental time

officially was begun) was set as 24 hours after plating. Cells were incubated in the differentiation media for three days; at day 3 50% of the media was removed from each well and replaced with fresh differentiation media. Cells were returned to the incubator for a further 4 days. At day 7 the cells were removed from the incubator and processed for immunocytochemistry by the same procedure used for single-label immunocytochemistry in Chapter 2. Cells were stained with antibodies against Nestin (neural stem cells), MAP2 (neurons), GFAP (astrocytes), and RIP (oligodendrocytes) at concentrations identical to those used in Chapter 2. For each 24-well plate, approximately five coverslips were allocated to staining with each antibody. Three replicates (three full 24-well plates) were generated for both DMEM/F12 and Neurobasal media formulations.

	DMEM Mean	DMEM SEM	Neuro Mean	Neuro SEM
Nestin *	50.80	2.92	31.15	3.93
MAP2 **	12.78	2.95	69.64	3.30
GFAP **	68.96	2.32	17.59	1.34
RIP	10.28	4.05	14.61	1.70

Table 1: Media comparison data. Data given are the mean and SEM of n=3 experiments for each media type. DMEM = DMEM/F12 media; Neuro = Neurobasal media. Comparisons are made between DMEM mean and Neuro mean for each antibody using one-way ANOVA and Fisher's Protected LSD. * = significant difference at $P<.05$; ** = significant difference at $P<.01$.

As indicated in Table 1, significant differences were observed between cells differentiated in the DMEM/F12 and Neurobasal media. A significantly higher ($P<.001$) percentage of cells differentiated in DMEM/F12 media expressed the astrocytic marker GFAP (68.96% +/- 2.32%) than did cells in Neurobasal (17.59% +/- 1.34%). A significantly higher ($P<.0159$) percentage of cells in DMEM/F12 also expressed the stem cell marker Nestin (50.80% +/- 2.92%) than did cells in Neurobasal (31.15% +/- 3.93%). Conversely, a significantly lower ($P<.0002$) percentage of cells in DMEM/F12 expressed the neuronal marker MAP2 (12.78% +/- 2.95%) than did their counterparts in Neurobasal (69.64% +/- 3.30%). Percentage of cells expressing the oligodendrocyte marker RIP did not differ significantly between the two media groups; approximately 12% of cells expressed RIP in both groups.

Our data indicate that the type of differentiation media used can exert a substantial effect on the expression of cell-type specific markers. EHPCs in DMEM/F12 media appeared to differentiate predominantly into astrocytes (approximately 70% expressing GFAP), while EHPCs in Neurobasal media appeared to become largely neuronal (approximately 70% expressing MAP2). Because the primary goal of stem cell transplantation therapy is to replace neurons lost to degenerative disease processes, we selected the Neurobasal media to use for our further experiments, the results of which are reported in Chapter 2.