Astrocyte-derived soluble factors promoting neuronal differentiation of adult neural progenitor cells

Jisun Oh
Iowa State University

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Astrocyte-derived soluble factors promoting neuronal differentiation of adult neural progenitor cells

by

Jisun Oh

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Neuroscience; Biomedical Science (Cell Biology)

Program of Study Committee:
Donald S. Sakaguchi, Major Professor
Jeffrey J. Essner
M. Heather West Greenlee
Surya K. Mallapragada
Michael A. McCloskey

Iowa State University
Ames, Iowa
2010

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ABSTRACT

Adult neurogenesis occurs in two regions of the brain: the subventricular zone of lateral ventricles and the subgranular zone of hippocampus. Neural progenitor cells found in these neurogenic regions are capable of self-renewing and generating mature neurons and glia. It is known that reciprocal interactions with the local environment can regulate cellular maintenance and plasticity of neural progenitor cells. Astrocytes in the microenvironment surrounding neural progenitor cells have a profound influence on a variety of inter- and intra-cellular processes during adult neurogenesis. Thus, we have investigated the roles of astrocyte-derived factors which influence neural progenitor cell differentiation in vitro. The neural progenitor cells used in our studies were hippocampal progenitor cells isolated from adult rat hippocampus (a gift from F. Gage, Salk Institute, La Jolla, CA).

Previous immunocytochemical results showed that co-culture with enriched neonatal astrocytes significantly and selectively increased neuronal differentiation of adult hippocampal progenitor cells. We proposed that the astrocytes present discrete modulators to the overlying adult hippocampal progenitor cells involving contact-mediated or release of soluble factors, or a combination of both. From the first investigation in this dissertation, we found that astrocyte-derived soluble factors specifically promote neuronal differentiation of neural progenitor cells. In the second investigation, we tested a hypothesis that a candidate neurogenic factor produced from astrocytes, interleukin-6, is a key regulator that stimulates functional differentiation of neural progenitor cells to a neuronal fate. Using immunocytochemical analysis and whole-cell recording, we have demonstrated that astrocyte-derived interleukin-6 enhances neuronal differentiation of AHPCs by presenting three main observations: (a) neuronal marker expression was increased, (b) the average
length of neurites from neuronal-restricted AHPCs was increased and (c) voltage-gated inward current density was increased with no significant differences in voltage-gated outward current density, apparent resting membrane potential, or cell capacitance. In the third investigation, we examined the influence of extracellular calcium and voltage-gated calcium channel activity on interleukin-6-mediated neuronal differentiation of neural progenitor cells. We observed that interleukin-6-enhanced neuronal differentiation was reduced when cultured in low calcium-containing culture medium or with L-type voltage-gated calcium channel antagonists. Interleukin-6 treatment also increased the fraction of neural progenitor cells immunoreactive for a neuronal marker and for cAMP response element binding protein.

Overall, we conclude that astrocyte-derived soluble factors promote neuronal differentiation of adult neural progenitor cells, and astrocyte-derived interleukin-6 is a neurogenic factor able to induce neural progenitor cells to differentiate into neurons. These findings may provide important insights into mechanisms for controlling neural progenitor cell differentiation and facilitate development of cell-based therapeutic strategies using adult neural progenitor cells.
CHAPTER 1.
GENERAL INTRODUCTION

Introduction

The adult mammalian central nervous system can generate new neurons and glial cells from neural progenitor cells in a process known as adult neurogenesis. Neural progenitor cells are known to reside in two regions in the brain: the subventricular zone along the wall of lateral ventricles, and the subgranular zone of the dentate gyrus of the hippocampus. A variety of factors derived from local environment of neural progenitor cells can influence different stages of neurogenesis in the adult brain in vivo. Astrocytes are a prevalent cell type surrounding neural progenitor cells and possess region-specific characteristics.

The following chapters discuss astrocyte-derived soluble factors influencing differentiation of adult neural progenitor cells. We have demonstrated that adult hippocampal progenitor cells favor differentiating into a neuronal lineage when cultured in the presence of soluble factors produced from hippocampal astrocytes (Chapter 2), and the cytokine interleukin-6 is an astrocyte-derived factor which specifically promotes neuronal differentiation of adult neural progenitor cells (Chapter 3). In addition, we investigated possible mechanisms of interleukin-6-mediated neuronal differentiation in adult neural progenitor cells (Chapter 4).
Dissertation Organization

This dissertation begins with a literature review (in Chapter 1) on adult neurogenesis, the neurogenic niche and neurogenesis-regulating factors.

The body of this dissertation is composed of three chapters (Chapter 2, 3 and 4) based on the following three manuscripts published or in preparation to be submitted to peer-reviewed international journals.


Chapter 4: **Effects of calcium influx on interleukin-6-mediated neuronal differentiation of adult neural progenitor cells**, by Jisun Oh, Michael A. McCloskey, Michael Z. Khan, Senyo S. Whyte, Pavel A. Brodskiy and Donald S. Sakaguchi, *in preparation*. 
Abbreviations

[Ca]ext, extracellular calcium concentration
[Ca]i, intracellular calcium concentration
AraC, arabinosylcytosine
Ba, barium
BDNF, brain-derived neurotrophic factor
bFGF, basic fibroblast growth factor or fibroblast growth factor-2
bHLH, basic helix-loop-helix
BMPs, bone morphogenetic proteins
BrdU, bromodeoxyuridine
Ca, calcium
Cav channels, voltage-gated calcium channels
CNS, central nervous system
ECM, extracellular matrix
EGF, epidermal growth factor
ERK, extracellular signal-regulated kinase; also known as MAPK
FBS, fetal bovine serum
FGF, fibroblast growth factor
Gadd45b, growth arrest and DNA-damage-inducible protein 45 beta
GFAP, glial fibrillary acidic protein
IL-6, interleukin-6
IL-6R, interleukin-6-specific receptor
JAKs, janus kinases
KCl, potassium chloride
MAP2ab, microtubule associated protein 2ab
MAPK, mitogen-activated protein kinase
MBP, myelin basic protein
MMP, matrix metalloproteases
NG2, chondroitin sulfate proteoglycan
NGF, nerve growth factor
Ngn2, neurogenin 2
NMDA, N-methyl-D-aspartic acid
NPC, neural progenitor cells
NT, neurotrophin
PSA-NCAM, poly-sialated neural cell adhesion molecule
RMS, rostral migratory stream
SGZ, subgranular zone
Shh, sonic hedgehog
Sox, SRY (sex determining region Y)-related HMG (high-motility group) box
STAT, signal transducer and activator of transcription
SVZ, subventricular zone
Tbr1, T-box transcription factor
VEGF, vascular endothelial growth factor
Wnt, wingless-related MMTV (mouse mammary tumor virus) integration site
Literature Review

Adult neurogenesis

Neurogenesis encompasses the whole process of generating new functional neurons from pluripotent stem cells or multipotent neural progenitor cells (NPCs). In general, neurogenesis includes proliferation, survival, fate determination, and differentiation of NPCs as well as migration, functional maturation and integration of the progeny. Neurogenesis was previously believed to occur only during embryonic stages. Adult mammalian brains were considered to be postmitotic structures which do not produce or incorporate newborn cells after the conclusion of development.

However, using a method for detecting cell proliferation (incorporation of $[H^3]$-thymidine and autoradiography), Smart (1961) reported for the first time the existence of actively dividing cells in the subependymal layer in the region of the lateral ventricles and their ability to migrate toward the periphery of the cortex in the neonatal mouse brain. In the late 1960s, Altman and colleagues demonstrated that proliferating and migrating cells can be found in the hippocampus and olfactory bulb of the neonatal rat brain, using $[H^3]$-thymidine labeling and histological analysis. In 1977, Kaplan and colleagues reported findings consistent with those of Altman and colleagues but derived from an additional technique – electron microscopy. Kaplan’s group found that new neurons were generated in the hippocampus and olfactory bulbs of the mature rodent (adult mouse and rat) brain and that some of newborn cells formed synapses. In the 1980’s, Nottebohm and colleagues demonstrated that new neurons were persistently added into the brain of adult songbirds during seasonal song learning. From a series of studies, they found that (a) generation of
new neurons in the adult songbird brain during seasonal song learning was regulated by seasonal changes in gonadal hormone levels, (b) only a subpopulation of newborn cells survived and integrated into the brain circuits, and (c) generation and integration of new neurons in the songbird brain were associated with learning new syllables. Based on these results, Nottebohm and colleagues suggested four criteria to address the generation of new neurons in the adult brain as ‘adult neurogenesis’: (1) presence of newborn neurons derived from proliferating primitive cells (incorporating [H³]-thymidine), (2) morphological and phenotypical characteristics (displaying the appropriate neurite arborization and expressing neuronal markers), (3) electrophysiological membrane properties, and (4) connections with neighboring cells (forming synapses).

In the 1990s, self-renewing and multipotent NPCs were isolated from the neurogenic regions of the mature rodent brain, propagated for various in vitro studies, and used for examining cell plasticity following transplantation¹⁴-¹⁶. Accumulating evidence from in vitro and in vivo studies have demonstrated that NPCs exist in the brain of adult mammals, and continuous neurogenesis occurs in two specific areas of the adult brain throughout life¹⁵,¹⁷-²², (i) the subventricular zone (SVZ) lining the lateral ventricles and (ii) the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. Adult neurogenesis is widely accepted as a definite biological event which occurs in the SVZ and SGZ of the intact adult mammalian brain²³-²⁵. The occurrence of adult neurogenesis in other brain regions, such as the amygdala, hypothalamus, substantia nigra, and striatum, remains disputed due to the lack of replicable evidence²⁶.

*Adult neural progenitor cells in the subventricular zone*
Adult NPCs can proliferate by generating more of themselves (self-renewal) and give rise to neural cells (i.e. neurons, oligodendrocytes, and astrocytes; multipotency)\textsuperscript{18,22,27}. The SVZ of the lateral ventricle wall is one of the neurogenic regions where NPCs are born in the adult rodent brain\textsuperscript{23,28}. Neuroblasts produced in the SVZ migrate toward the olfactory bulbs along the rostral migratory stream (RMS; in a unique way of ‘tangential chain migration’). Neuroblasts that migrated rostrally migrate further radially into the granule cell layer and glomerular layer in the olfactory bulbs\textsuperscript{23}. But almost all newborn cells in the olfactory bulbs differentiate into granule cells\textsuperscript{29,30}. Apical dendrites of newborn cells are extended and arborized, and form reciprocal synapses with the dendrites of mitral and tufted cells\textsuperscript{28}. Functionally, newborn cells in the olfactory bulbs are known to be involved in maintenance of the structure of existing neural circuitry as well as in discrimination of odors\textsuperscript{1,31}.

The presence of a human RMS in the adult human brain is still debated\textsuperscript{26,32}. However, several reports describe a population of proliferative cells exists in the SVZ\textsuperscript{33} and that migratory neuroblasts are present around a lateral ventricular extension reaching the olfactory bulb\textsuperscript{34,35}. Thus, it appears widely agreed that adult NPCs are present in the SVZ of the human brain, migratory neuroblasts are found in a rostral extension of the primary proliferative zone, and new neurons are generated in the adult human olfactory bulb.

During adult neurogenesis in the SVZ, there are three types of precursor cells found\textsuperscript{36,37}: (1) type B cells that are non-proliferative, and nestin- and GFAP-positive; (2) type C cells (transit amplifying cells) that are proliferative, and BrdU- and nestin-positive; and (3) type A cells (migrating neuroblasts) that are proliferative, and nestin- and doublecortin-positive. Type B cells have been defined as NPCs in the SVZ because of their ability to generate neurospheres and differentiated progeny \textit{in vitro}\textsuperscript{38}. Type B cells are a subpopulation
of radial glia-derived astrocytes\textsuperscript{39} or ventricle-contacting ciliated ependymal cells (which is a final fate of radial glial cells)\textsuperscript{38,40}. However, these ependymal cells are quiescent and lack apparent self-renewal capacity under normal conditions; i.e. they are consumed when stimulated to produce their progeny\textsuperscript{40,41}. Therefore, quiescent ependymal cells are distinguished from NPCs even though they are multipotent. Moreover, Mirzadeh et al. (2008) recently demonstrated that there are at least three types of ventricle-contacting cells along the ependyma according to their morphology\textsuperscript{36}: (i) type B cells, which possess a single, short primary cilium; (ii) ependymal cells, which contain multiple long cilia encircling B cells (so-called E1 cells); and (iii) cells which often have two long cilia and a relatively larger surface area of the basal body facing the lateral ventricles (referred to as E2 cells). In addition, these ventricle-contacting cells were all found to be quiescent \textit{in vivo} unless they were activated\textsuperscript{36}. Therefore, it remains to be elucidated what kind of cells in the adult SVZ actually function as NPCs; whether they are capable of self-renewing as well as whether SVZ-derived newly generated neural cells are originating from a common NPC or from distinct progenitors for each cell type.

\textit{Adult neural progenitor cells in the subgranular zone}

In the SGZ of the dentate gyrus in the hippocampus, two types of NPCs have been identified according to their morphology and molecular phenotypes\textsuperscript{42,43}: (1) type 1 cells, which have a radial process spanning the entire granule cell layer and ramifying in the inner molecular layer, and which are GFAP/nestin/Sox2-positive; (2) type 2 cells, which have multiple, short processes and are nestin/Sox2-positive but GFAP-negative. Suspicions that actively self-renewing type 2 cells originate from quiescent or infrequently dividing type 1
cells are based mainly upon research by Seri et al. (2004) and Ahn and Joyner (2005). In these studies, they reported that (a) treatment with anti-mitotic drugs, such as AraC and/or procarbazol to eliminate fast-dividing cells, decreased the number of newborn cells in the SGZ of adult mice, and (b) in AraC-treated mice, rarely dividing GFAP-positive (type 1) cells survived, produced fast-dividing cells and differentiated into PSA-NCAM-positive granule neurons in the dentate gyrus.

Recently, Suh et al. (2007) have studied different properties of two morphologically different mouse hippocampal NPCs using retrovirus-mediated fate-tracing. First, they found that Sox2-positive NPCs (including both types of hippocampal NPCs) were multipotent, able to give rise to granule neurons and astrocytes. Second, they showed that, after seven days of voluntary running (exercising is a mitotic signal), (a) the total number of Sox2-positive cells increased, (b) the number of Sox2-positive NPCs (which is a subpopulation of Sox2-positive cells in vivo) did not change, and (c) the number of doublecortin-positive neuronal precursors increased. These results demonstrated that hippocampal NPC pool size remains unchanged while certain cells acquire a potential to become proliferative or some cells in the pool differentiate into more mature cell types.

Taken together, these findings suggest that type 1 cells may play an important role in maintaining the number of NPCs and generating newborn granule cells in the adult mammalian hippocampus. However, since these studies were performed at the population level, rather than at a single-cell or clonal level, the relationship between type 1 and type 2 hippocampal NPCs remains to be clarified.
Newly generated neurons in the SGZ are noted to be involved in learning and memory processing, as the hippocampus plays a role in certain forms of learning and memory. In the past several years, various approaches have been taken to understand the function of newly generated hippocampal neurons in the adult brain. After van Praag et al. (1999) demonstrated that physical activity (voluntary running) increased hippocampal neurogenesis in the adult mouse brain, Farmer et al. (2004) and Kempermann et al. (2002) suggested that the exercise-induced increase in the adult hippocampal region is associated with enhanced spatial learning in the Morris water maze task. Moreover, some researchers attempted to block hippocampal neurogenesis by ablating actively dividing cells in the dentate gyrus using pharmacological agents (such as methylazoxymethanol acetate), irradiation, or genetic strategies. Observations from these studies indicate that a blockage of adult hippocampal neurogenesis affects some, but not all, of hippocampus-dependent tasks, such as spatial navigation in a Morris water maze and fear-conditioned eye-blink. Therefore, newly generated neurons in the hippocampus can be functionally integrated into the existing hippocampal circuitry and appear to make a distinct contribution to certain types of hippocampal functions. We do not yet fully understand the significance of hippocampal neurogenesis in the adult brain: whether it is for the expansion of memory-storage capacity to avoid catastrophic interference, for the temporary storage of hippocampus-dependent memories before those memories are stored in other parts of the brain, for the clearance of older memories by replacing old granule neurons with newborn cells, or for the refinement of the existing hippocampal neural circuits.

Neurogenesis-regulating factors
Cellular maintenance and plasticity of NPCs in the neurogenic regions are believed to be determined by endogenous (intrinsic) regulators and/or exogenous (extrinsic or environmental) signals. SRY-related HMG box containing gene 2 (Sox2) and basic helix-loop-helix (bHLH) transcription factors are examples of endogenous regulators. By epigenetic modifications, gene expression in NPCs can be controlled, which alters NPC fates as well as synaptic formation during the whole process of neurogenesis. On the other hand, extrinsic signals include a variety of factors from the local microenvironment of NPCs which is referred to as the ‘niche’ \textit{in vivo}. The niche builds up a neurogenic environment which can influence NPC maintenance, differentiation, maturation and functional integration of the progeny. Neighboring cells, such as neurons, glia, and vascular endothelial cells, and their secreting or contact-mediated factors as well as physiological or pathological stimuli are considered critical signals regulating different stages of adult neurogenesis.

\textit{Endogenous factors}

Sox2 is a transcription factor necessary for maintenance and proliferation of NPCs\textsuperscript{42,56}. Graham et al. (2003) reported that constitutive expression of Sox2 prevented neuronal differentiation of mouse embryonic NPCs and resulted in the retention of progenitor characteristics\textsuperscript{56}. In addition, inhibition of Sox2 signaling caused NPCs to delaminate from the SVZ and to exit the cell cycle\textsuperscript{56}. Furthermore, Ferri et al. (2004) examined adult neurogenesis in mutant mice in which the Sox2 expression level is about 30\% of that of wild-type mice\textsuperscript{57}. They showed that NPC proliferation was remarkably reduced in both the SVZ and SGZ\textsuperscript{57}. Recently, Cavallaro et al. (2008) demonstrated that NPCs isolated from Sox2 knockdown mutant mice failed to differentiate into mature neurons\textsuperscript{58}. However, an induction
of Sox2 overexpression at an early, but not later, stage of differentiation rescued the abnormal neuronal phenotype and morphology, and suppressed differentiation of the NPCs into glia\textsuperscript{58}. These results suggest that Sox2 plays important roles in the maintenance and proliferation of NPCs and also in the early stage of neuronal differentiation of NPCs.

bHLH transcription factors, called proneural proteins, are known to control the fate commitment of NPCs. In the SVZ, Ascl1 (also called Mash1) is expressed in type C cells during the differentiation into GABAergic neurons in the olfactory bulb\textsuperscript{59}. Berninger et al. (2007) and Brill et al. (2009) reported that forced expression of Neurogenin2 (Ngn2) and the T-box transcription factor (Tbr1) in the SVZ-derived NPCs induced the NPCs to acquire glutamatergic neuronal identity\textsuperscript{60,61}. In the SGZ, the bHLH transcription factors Ngn2 and NeuroD are transiently expressed in doublecortin-positive or PSA-NCAM-positive immature neurons\textsuperscript{62,63}. Ascl1 is also found in NPCs destined to become granular cells in the dentate gyrus\textsuperscript{59}. Recently, Jessberger et al. (2008) demonstrated that retrovirus-mediated overexpression of Ascl1 in the SGZ-derived NPCs led them to differentiate into NG2-positive and MBP-positive oligodendrocyte precursors, rather than into neurons\textsuperscript{64}. These results suggest that the expression of proneural proteins influence the fate determination of the adult NPCs.

Epigenetic modifications can also regulate the expression of genes that are associated with various steps of adult neurogenesis\textsuperscript{65}. Mice lacking methyl-CpG-binding protein 1 or 2, members of the methylated DNA-binding protein family, showed reduced production of immature hippocampal neurons, delayed dendritic development, and altered expression of synaptic proteins\textsuperscript{66-69}. Recently, Ma et al. (2009) demonstrated that Gadd45b (a stress response gene) is essential for DNA demethylation to express genes, such as BDNF and FGF,
that are critical for neural activity-induced neuronal differentiation of the adult hippocampus\textsuperscript{70,71}. Furthermore, inhibition of histone deacetylase induced neuronal differentiation and suppressed glial differentiation of adult hippocampal NPCs\textsuperscript{72}. These results suggest that epigenetic modification is one of the intrinsic mechanisms controlling adult neurogenesis.

\textit{Environmental control}

In addition to the endogenous factors, adult neurogenesis can be regulated by exogenous signals originating from the local microenvironment near multipotent NPCs which has a profound influence on the fate of the NPCs\textsuperscript{73-78}. Herrera et al. (1999) reported that adult mouse SVZ-derived NPCs were capable of differentiating into olfactory bulb interneurons when transplanted into the adult olfactory bulb, although the most of NPCs implanted into the neocortex differentiated into astrocytes\textsuperscript{79}. Suhonen et al. (1996) demonstrated that adult rat SGZ-derived NPCs were able to accommodate environmental information\textsuperscript{80}. As they transplanted the SGZ NPCs into neurogenic regions (hippocampus or rostral migratory stream) or into a non-neurogenic region (cerebellum), NPCs grafted into neurogenic (but not non-neurogenic) regions differentiated into neurons\textsuperscript{80}. Interestingly, the NPCs grafted in the RMS (then migrated into the olfactory bulb) differentiated into tyrosine hydroxylase-positive neurons, i.e. non-hippocampal neurons, while the NPCs implanted into the hippocampus became hippocampal neurons\textsuperscript{80}. These results suggest that local microenvironments of NPCs retain the ability to control the fate of the NPCs and that adult NPCs respond to environmental signals, and their vital properties can be altered by extrinsic cues.
The neurogenic niche contains several types of cells, diffusible factors, extracellular matrix (ECM) components, and topographical cues. A variety of extracellular signals provided from the niches can regulate cell survival, maintenance, differentiation and integration of NPCs during early neural development and adult neurogenesis. Endothelial cells, ependymal cells, astrocytes and mature neurons are the major cellular components of neurogenic niches. In the adult neurogenic regions, dividing NPCs are present in proximity to the capillaries. Shen et al. (2004) showed that the endothelial cells interacting with NPCs stimulated propagation of NPCs and enhanced neuron production. An angiogenic protein, VEGF, is a factor which can stimulate NPC proliferation in the neurogenic regions of the adult rat brain. It was also observed that the expression level of VEGF was increased in the hippocampus where neurogenesis was increased by an enriched environmental induction. In addition, blocking VEGF signaling affected enrichment-induced and exercise-induced hippocampal neurogenesis. These findings suggest that VEGF is a regulator that can create a proliferative environment in the neurogenic regions for self renewal of NPCs.

Astrocytes are prevailing glial cells in the central nervous system. Astrocytes interacting with NPCs in neurogenic regions are capable of promoting neuronal differentiation of the NPCs. Lim and Alvarez-Buylla (1999) reported that SVZ astrocytes co-cultured with type C and A cells in contact promoted (a) proliferation of type C cells and (b) production and migration of type A cells. Moreover, Song et al. (2002) demonstrated that adult NPCs preferred to adopt a neuronal fate when they were cultured onto a hippocampal astrocyte feeder layer and that the NPC-derived neurons in the presence of the astrocytes originating from the neonatal or adult hippocampus displayed
functional neuronal characteristics, including the electrophysiological membrane properties and synaptic formation typical of newborn cells. These results suggest that the astrocytes in neurogenic regions retain the potential to generate an instructive environment for neuronal differentiation of adult NPCs.

Wnt3 is one of the soluble factors that are produced from the neurogenic astrocytes and enhances neuronal differentiation of NPCs. Lie et al. (2005) demonstrated that hippocampal astrocytes expressed Wnt3 and adult hippocampal NPCs expressed receptors for Wnt protein and intracellular signaling molecules for Wnt/β-catenin pathway. They also observed that overexpression of Wnt increased NPC proliferation and that interference of Wnt signaling resulted in a lack of doublecortin-positive or MAP2ab-positive neurons in the dentate gyrus. These observations suggest that neurogenic astrocyte-derived factors are actively involved in generating new neurons from NPCs in the niches. In addition to Wnt3, numerous factors are proposed to contribute to the neurogenic niches. Morphogenic factors (such as BMPs, Shh), growth factors (such as EGF, bFGF), neurotrophins, neurotransmitters, neural excitation, and glial cell-derived cytokines were demonstrated to induce neuronal differentiation of NPCs. Moreover, matrix metalloprotease-9, retinoic acid, neurogenesin-1, interleukin-1β and interleukin-6 (further discussed below) were recently reported as factors derived from neurogenic astrocytes that can induce neuronal differentiation of NPCs or oligodendrocyte fate-restricted progenitor cells. These findings strongly suggest that a neurogenic local microenvironment provides molecular controls to regulate neuronal differentiation of NPCs.

The ECM components contain a complex set of molecules, such as collagens, fibronectins, laminins, tanascins, and proteoglycans. These ECM molecules interact
with integrin receptors (heteromeric complexes of 18 α and 8 β subunits) on the cell surface, and thereby the ECM function as a scaffold for cell adhesion\textsuperscript{110,112}. In addition, the interaction between the ECM and integrin triggers intracellular signaling pathways which control cell survival, migration, differentiation, neurite outgrowth, and synaptic formation of neural precursors and developing neurons\textsuperscript{110,112,113}. Several studies have showed that the ECM molecules interacting with integrin receptors influenced differentiation of embryonic stem cells\textsuperscript{114-116} or adult hippocampal NPCs\textsuperscript{117,118}.

Matrix metalloproteases (MMPs) are able to degrade all the components of the ECM and involved in cell migration by matrix remodeling which can regulate cell-cell and cell-ECM interaction\textsuperscript{119}. MMPs were reported to regulate differentiation of adult mesenchymal stem cells into various lineages\textsuperscript{120}. Recently, Barkho et al. (2008) demonstrated that MMP-3 and MMP-9 produced from adult SVZ-derived NPCs promoted differentiation of the NPCs into migratory progeny\textsuperscript{107}. These results suggest that the ECM molecules are an important element in the neurogenic niche not only for cellular support but also for relaying signals from extracellular to intracellular environment.

In concert with other niche signals, topographical cues can also influence stem cell adhesion, survival as well as their fates and migration\textsuperscript{121}. Several studies using various kinds of stem or progenitor cells, such as umbilical cord-derived hematopoietic stem cells\textsuperscript{122}, bone marrow-derived osteoprogenitors\textsuperscript{123}, mesenchymal stem cells\textsuperscript{124}, showed that the distinct topographical features (created by various methods for surface functionalization/fabrication) had effects on the fates and behaviors of stem or progenitor cells. Especially, a study by Recknor et al. (2006) using adult hippocampal NPCs demonstrated that (a) the grooved surface on which the NPCs were cultured increased an alignment in a direction of the groove
and (b) in co-cultures with astrocytes, neuronal differentiation of hippocampal NPCs was dramatically enhanced on the grooved surface, compared to the non-grooved surface. In addition, Christopherson et al. (2009) reported that hippocampal NPCs cultured on the surface of electrospun nanofibers preferred to differentiate into a neuronal lineage, rather than a glial lineage. These findings suggest that topographical cues play a role in regulating NPC growth and differentiation and may be developed as an instructive tool to control fate specification or directional growth of NPCs.

**Interleukin-6**

The cytokine IL-6 is best known as a regulator of hematopoiesis and supports the proliferation and survival of bone marrow progenitor cells. In addition, IL-6 is found in the nervous system and in conjunction with its receptor complex can trigger cellular responses mediating inflammation, neurogenesis, gliogenesis, cell growth and survival, and myelination/demyelination. In the brain, IL-6 is normally expressed at relatively low levels. However, during progression of inflammation or brain injury, IL-6 protein levels are elevated in the cerebral spinal fluid and brain homogenates. Moreover, chronic over-expression of IL-6 in transgenic mice caused neuroanatomical and neurophysiological alterations reminiscent of various neurological diseases. Interestingly, neurotrophins such as NGF are co-expressed with IL-6 at sites of nerve injury. IL-6 in combination with its specific receptor, IL-6R, has been reported to regulate a local supply of neurotrophins that are critical for neuronal survival and protection. These reports suggest that IL-6 expression is up-regulated under diverse conditions.
pathological and physiological conditions in the CNS, and also contributes to the production and function of neurotrophins at the injured site of the CNS.

IL-6 is recognized by the high-affinity IL-6R which is expressed on target cells \(^{130,136-138}\). There are two forms of the IL-6R, one transmembrane form and one soluble form. The latter is believed to be more important for neural signaling \(^{139}\). The IL-6/IL-6R complex interacts with gp130, which is a general receptor for the IL-6-type cytokines including IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1 \(^{137,140,141}\). Gp130, a 130-kD non-ligand-binding membrane glycoprotein, does not bind directly to IL-6 \(^{141-143}\). The association of IL-6/IL-6R complex with gp130 causes autophosphorylation of the gp130 dimer followed by activation of the JAKs \(^{137}\). The activation of JAK/STAT, Ras/ERK or other signal transduction pathways results in the modulation of various genes that control neural development \(^{129,135,144-146}\).

Through the JAK/STAT pathway, IL-6 has been implicated in induction of glial differentiation from NPCs in the mammalian brain \(^{145,147,148}\). Recent studies examining inflammatory responses mediated in part by cytokines, including IL-6, on hippocampal neurogenesis showed that IL-6 production from activated microglia interferes with the generation of new neurons in the hippocampus of the adult brain \(^{149-151}\). Thus far, the role of IL-6 in the neurogenic niche has been suggested to be inhibition of neurogenesis via a decrease in NPC proliferation or an increase in glial differentiation, both depleting the pool of NPCs. However, a number of studies have shown that IL-6, in conjunction with its specific receptor, can also exert neurotrophic effects \(^{128,152-154}\). By stimulating region-specific astrocytes to provide local supplements of neurotrophins, IL-6 facilitates neuronal survival \(^{152}\). In addition, IL-6 with IL-6R (especially the soluble form) induces neuronal-like
differentiation of PC12 cells and contributes to the regeneration of axotomized mature sensory or hippocampal neurons. Therefore the effects of IL-6 on NPC differentiation or survival appear to be context dependent.

Astrocytes are an important source of IL-6 in the CNS and the expression levels vary for different brain regions. Barkho et al. (2006) demonstrated different gene expression profiles of astrocytes originating from neurogenic regions (newborn hippocampus, adult hippocampus, and newborn spinal cord) known to promote neuronal differentiation of adult hippocampal neural progenitors with cells from non-neurogenic regions (adult spinal cord astrocytes and adult skin fibroblasts). They found that IL-6 mRNA is expressed at a relatively higher level in newborn hippocampal astrocytes than in non-neurogenic astrocytes. When adult hippocampus-derived neural progenitor cells were cultured in the presence of IL-6, neuronal promoter activity and neuronal marker-immunoreactivity were increased.

**Extracellular calcium influx**

In resting cells, generally, intracellular calcium (Ca) concentration, [Ca]i, is maintained at very low level (~100 nM) by Ca pumps present in the plasma membrane (such as plasma membrane Ca-ATPases or Na/Ca exchangers) as well as intracellular Ca stores (Sarcoendoplasmic reticulum Ca-ATPases). [Ca]i can be increased by extracellular Ca entry or Ca release from internal Ca stores. There are several types of channels through which extracellular Ca ions enter across plasma membrane; for example, Cav channels, ligand-gated channels (such as NMDA receptors), store-operated Ca channels (such as Orai1), and transient receptor protein channels. From internal Ca stores, Ca ions can be released through inositol-1,4,5-triphosphate receptors or ryanodine receptors.
In NPCs of young or adult rodent brains, an elevation of \([Ca]_{i}\) is an important event for membrane depolarization-dependent neuronal differentiation\(^{103,159-162}\). The elevation of \([Ca]_{i}\) in NPCs is known to promote neuronal differentiation and neurite development\(^{103,159-161}\). Deisseroth et al. (2004) reported that membrane depolarization enhanced adult neurogenesis\(^{160}\). They cultured rat hippocampal NPCs onto primary hippocampal neurons and glia or fixed hippocampal cellular substrate under differentiation conditions (excluding mitogens and including 2% FBS, 0.5 \(\mu\)M all-trans retinoic acid, 10 \(\mu\)M forskolin and 20 ng/ml NT3) for 2 weeks. When they applied excitatory stimuli (application of 20 mM KCl or 50 \(\mu\)M glutamate) to the NPC cultures, they observed an increase in the fraction of NPC progeny that expressed a neuronal marker, MAP2ab. In addition, the excitatory stimuli were sensed by L-type Cav channels (especially Cav1.2 and Cav1.3) and NMDA receptors for neuronal differentiation of the NPCs. Furthermore, they observed that membrane depolarization caused changes in gene expression in 6 hours; glial fate gene (Hes1 and Id2) expression was down-regulated, and neuronal gene (NeuroD) expression was up-regulated. Similarly, D’Ascenzo et al. (2006) reported that membrane depolarization promotes neuronal differentiation of cortical NPCs\(^{159}\). They cultured neonatal mouse cortex-derived NPCs in the presence of 1% FBS up to 12 days, and applied 50 mM KCl to elicit membrane depolarization. At 0, 3, 6, 9 and 12 days after induction of differentiation, they examined phenotypic marker expression, changes in \([Ca]_{i}\) and Ba current density. They observed that, over the culture period, membrane depolarization increased the proportion of cells which expressed neuronal markers, increased the amplitude of Ca signal, increased the density of high voltage-activated Ba currents which were blocked by nifedipine (5 \(\mu\)M) and increased
Cav1.2 expression. These findings suggest that Ca influx through L-type Cav channels is important for induction of neuronal differentiation of NPCs.

Following chronic exposure to IL-6, [Ca]i in developing cerebellar granule cells (isolated from ~1 week-old rats) is increased due to excessive activation of NMDA receptors, and ends up increasing susceptibility to excitotoxic insults leading to neuronal loss\textsuperscript{163-166}. Other groups claim that treatment of neonatal rat cerebellar granule neurons with exogenous IL-6 prevents neuronal cell death by reducing apoptosis and keeps intracellular Ca from being overloaded\textsuperscript{167,168}. In addition, some groups showed a neuroprotective effect of IL-6 on hippocampal neurons\textsuperscript{126,169-171}. Considering the variable results obtained from multiple studies\textsuperscript{165,172,173}, the effects of IL-6 are likely to depend upon neuronal cell type examined, the varying times/duration of IL-6 treatment, and concentrations of IL-6 applied.

We have examined the effects of various concentrations of exogenous IL-6 (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml) on adult NPC differentiation \textit{in vitro}\textsuperscript{106}. Adult NPC cultures treated with IL-6 greater than 1 ng/ml for 6 days had significantly higher fractions of cells expressing a neuronal marker, TuJ1, and possessed significantly longer neurite lengths, compared to the cells cultured with no IL-6\textsuperscript{106}. In addition, from all culture conditions of different IL-6 concentrations, we observed no considerable changes in the total number of cells. These observations from our previous study suggest that IL-6 treatment provides neurogenic effects on NPC differentiation (IL-6 enhanced neuronal differentiation of adult NPCs), rather than cytotoxic effects.

Qiu et al. (1995) reported that IL-6 treatment caused cerebellar granule neurons to be more sensitive to NMDA application and increased [Ca]i when NMDA receptors were
agonized\textsuperscript{164}. Moreover, NMDA-induced [Ca]i increase in IL-6-treated cells was significantly decreased when [Ca]ext was reduced and intracellular Ca stores were depleted\textsuperscript{164}. They further demonstrated that both calcium release from intracellular stores\textsuperscript{163} and Ca influx through L-, N- and P/Q-type Cav channels\textsuperscript{166} may contribute to [Ca]i rise in IL-6-treated neurons. These results suggest that IL-6 renders the developing neurons more sensitive to membrane depolarization stimuli by increasing [Ca]i.

In our culture system, we examined the effects of L-type Cav channel antagonists on NPC neuronal differentiation in the presence of exogenous IL-6 (see Chapter 4). Interestingly, when adult hippocampal NPCs were cultured with L-type Cav channel antagonists, IL-6 treatment failed to enhance neuronal differentiation of NPCs. Thus it is presumed that, in our culture system, L-type Cav channels play a critical role in IL-6-mediated neuronal differentiation of adult NPCs.
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CHAPTER 2.

SOLUBLE FACTORS FROM NEOCORTICAL ASTROCYTES ENHANCE NEURONAL DIFFERENTIATION OF NEURAL PROGENITOR CELLS FROM ADULT RAT HIPPOCAMPUS ON MICROPATTERNED POLYMER SUBSTRATES

A paper published in *Journal of Biomedical Materials Research Part A*

Jisun Oh\(^{1,2,4,†}\), Jennifer B. Recknor\(^{2,3,‡,§}\), Justin C. Recknor\(^\#\), Surya K. Mallapragada\(^{2,3,*}\) and Donald S. Sakaguchi\(^{1,2,4,*}\)

1 Department of Biomedical Sciences, 2 Neuroscience Program, 3 Department of Chemical and Biological Engineering, 4 Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011

† These authors contributed equally to the work described in this manuscript

* Corresponding authors

‡ Current address: National Institute of Standards and Technology, Boulder, CO 80305

§ Current address: Eli Lilly and Company, Greenfield, IN 46140

Abstract

Rat adult hippocampal progenitor cells (AHPCs) are self-renewing, multipotent neural progenitors that have the ability to differentiate into neurons and glia. Previously, we demonstrated that co-culture of AHPCs with postnatal day two, type I cortical astrocytes on laminin-coated micropatterned polymer substrates facilitates selective neuronal differentiation of the AHPCs. Under this condition, multi-dimensional cell-cell and/or cell-extracellular matrix interactions, as well as possible soluble factors released from astrocytes provided spatial and temporal control selectively enhancing neuronal differentiation and neurite alignment on topographically different regions of the same substrate. To investigate the potential role of astrocyte-derived soluble factors as cues involved in neuronal differentiation, a non-contact co-culture system was used. Under control conditions, approximately 14% of the AHPCs were immunoreactive (IR) for the neuronal marker, class
III β-tubulin (TUJ1-IR). When co-cultured in physical contact with astrocytes, neuronal differentiation increased significantly to about 25%, consistent with our previous results. Moreover, under non-contact co-culture conditions using Transwell insert cultures, neuronal differentiation was dramatically increased to approximately 64%. Furthermore, neurite outgrowth from neuronal cell bodies was considerably greater on the patterned substrate, compared to the non-patterned planar substrate under non-contact co-culture conditions. Taken together, our results demonstrate that astrocyte-derived soluble factors provide cues for specific neuronal differentiation of AHPCs cultured on micropatterned substrates. In addition, a suppressive influence on neuronal differentiation appears to be mediated by contact with co-cultured astrocytes. These results provide important insights into mechanisms for controlling neural progenitor/stem cell differentiation and facilitate development of strategies for CNS repair.

**Introduction**

During development of the central nervous system (CNS), reciprocal interactions between neural stem cells and their milieu are important for regulating and coordinating a variety of inter- and intra-cellular processes, such as proliferation, differentiation, migration, and cell survival. These developmental events also impact tissue organization and matrix remodeling. The local microenvironment of multipotent neural stem/progenitor cells (NPCs), referred to as the neural stem cell ‘niche’, have a profound influence on the fate of the NPCs. Extracellular matrix (ECM) components have been known to regulate the differentiation of NPCs. Moreover, numerous studies have demonstrated that
astrocytes surrounding NPCs play a critical role in mediating neurogenesis. In addition, astrocyte-derived signals have been reported to regulate the structural formation and functional plasticity of synapses in developing and adult CNS. Our previous results suggested that the enriched astrocytes enhance neuronal differentiation of NPCs isolated from adult rat hippocampus (adult hippocampal progenitor cells, AHPCs). In addition, the synergistic combination of spatial control from three-dimensional (3-D) micropatterned polystyrene substrates coated with the ECM molecule, laminin, along with the biological influence of the astrocytes aligned in the direction of the patterned substrate provided guidance cues for promoting neuronal differentiation of the AHPCs. Based on these results we proposed that, in the multi-dimensional environment, the astrocytes present discrete cues to the overlying AHPCs involving contact-mediated or release of soluble factors, or a combination of both. These factors may include specific molecules known to mediate cellular mechanisms that regulate cell growth, development, maturation and communication among the cells. The soluble factors released by astrocytes may be spatially restricted due to the topography of the micropatterned polystyrene substrate as well as the physically aligned astrocytes within the microgrooves. The astrocyte-derived factors may be locally constrained in the microgrooves and could potentially influence AHPC differentiation. It is possible that the stem cell niche has been mimicked in vitro through the presentation of an optimal combination of signals necessary for neuronal differentiation of the AHPCs.

In the present study, we have investigated the factors responsible for selective neuronal differentiation of AHPCs within the micropatterned multi-dimensional environment. Non-contact co-cultures were established using Transwell® semi-porous membrane inserts to separate the astrocytes from AHPCs cultured on micropatterned substrates but in the same
well. In this culture system, the membrane inserts prohibit the physical interaction between the two types of cells but permit exchange of soluble factors between the cells. In an effort to identify the optimal combination of signals creating biological and spatial control over AHPC differentiation, we examined whether the factors responsible for the selective differentiation in the co-cultures were contact-mediated or soluble (or both) and possible roles of the factors.

**Materials and Methods**

**Micropatterned substrate fabrication**

Micropatterned polystyrene (PS) substrates were prepared as described in the previous study \(^1,\)\(^2,\)\(^5\). The pattern dimensions used were 16/13/4 µm [groove width/groove spacing (or mesa width)/groove depth] and substrate thickness was approximately 50-70 µm. The micropatterned/non-patterned PS substrates were washed in deionized water, sterilized with 70% ethanol and used to construct cell growth chambers as described previously \(^25\). The PS substrates were coated with poly-L-lysine (PLL, 100 µg/ml; Sigma, St. Louis, MO) solution and mouse-derived laminin (LAM, 10 µg/ml; R&D systems, Inc., Minneapolis, MN) diluted in Earle’s Balanced Salt Solution (EBSS; Gibco, Grand Island, NY) before plating cells.

**Astroglial cell isolation and purification**

All animal procedures were conducted in accordance with and had the approval of the Iowa State University Committee on Animal Care. Astrocytes were obtained from cerebral
cortex of two day old Sprague-Dawley rats as previously described. Dissected and
dissociated cells were grown in modified minimal essential culture medium (MMEM; Gibco)
containing minimum essential medium (MEM; Gibco) supplemented with 40 mM glucose, 2
mM L-glutamine, 1 mM sodium pyruvate and 14 mM sodium bicarbonate, penicillin (100
IU/ml) and streptomycin (100 µg/ml) with 10% v/v fetal bovine serum (FBS; HyClone,
Logan, UT), pH 7.35. The cells were cultured in an incubator (37°C, 5% CO₂/95%
humidified air atmosphere) until being confluent in 25 cm² tissue culture flasks (T-25;
Falcon), and screened with an anti-GFAP antibody (see Immunocytochemistry and
Antibodies below) before use to ensure purification of an enriched population of type-1
astrocytes. Greater than 95% of the cells in these astrocyte cultures were immunoreactive for
the GFAP antibody and no immunostaining for oligodendrocyte (RIP immunoreactivity) or
neuronal (TUJ1 immunoreactivity) cell-types were observed. The cultures were not passaged
more than 5 times.

Adult hippocampal progenitor cell culture

Adult hippocampal progenitor cells (AHPCs; a gift from Dr. F. H. Gage, La Jolla,
CA) were originally isolated from the brains of adult Fischer 344 rats and the expanded
cultures from single clones were infected with retrovirus to express enhanced green
fluorescent protein (GFP) as reported by Palmer and colleagues. The AHPCs were
cultured as described previously. Briefly, AHPCs were maintained in 75 cm² tissue
culture flasks (T-75; Fisher Scientific, Pittsburgh, PA) coated with 10 µg/ml of poly-L-
ornithine (Sigma) and 5 µg/ml of LAM. The AHPCs were propagated in complete medium
containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/ml basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and 2.5 mM L-glutamine (Gibco BRL, Gaithersburg, MD). For in vitro analysis, the AHPCs were detached using 0.05% Trypsin-EDTA (Gibco BRL, Gaithersburg, MD), harvested, and plated onto the micropatterned/non-micropatterned PS substrates coated with PLL (100 µg/ml) and LAM (10 µg/ml in EBSS) (PS-LAM substrates) and maintained in appropriate culture media.

Co-culture of astrocytes and AHPCs

Astrocyte-AHPC co-cultures were established as described previously. Briefly, 1.5 x 10⁴ cells/cm² of AHPCs were plated on top of an astrocyte monolayer and the co-cultures were maintained in a mixed medium (referred to as co-culture medium, CCM) that consisted of astrocyte MMEM without FBS in a 1:1 mixture with AHPC differentiation medium (AHPC complete medium excluding bFGF). Contact co-cultures, as well as control cultures (AHPCs alone and astrocytes alone), were grown for 6 days and then fixed in 4% paraformaldehyde in 0.1 M PO₄ buffer, pH 7.4 for immunocytochemical analysis.

To co-culture AHPCs with the astrocytes without physical contact, purified astrocytes were seeded onto 0.4 µm semi-porous polyester membrane of Transwell® inserts inside 6-well plates (Corning, Inc., Corning, NY) at the same initial plating density, and cultured in MMEM including 10% FBS. After 2 days, the MMEM inside the inserts was replaced with CCM following rinses with EBSS and incubated for 4 hours at 37°C. Meanwhile, AHPCs
were prepared separately. 1.5 x 10^4 cells/cm^2 of AHPCs were plated on PS-LAM substrates and allowed to attach for 1 hour before removing the o-ring. The PS-LAM substrate on which AHPCs were plated was placed at the bottom of each well and additional CCM was added to the wells. Astrocytes on the insert membrane were then placed inside the well. AHPC-astrocyte non-contact co-cultures were terminated after 6 days and fixed in 4% paraformaldehyde for further analysis.

To obtain astrocyte-conditioned CCM, purified astrocytes were cultured in T-25 flasks in MMEM including 10% FBS at similar seeding densities to those plated on the insert membrane. After 2 days, the MMEM was replaced by CCM after rinses with EBSS. After 2 days of further growth, the CCM conditioned by astrocytes was collected, centrifuged to remove any debris and then used to feed AHPC alone cultures without astrocytes. The cultures were fed every 24 hours.

Immunocytochemistry and Antibodies

AHPCs cultured on PS-LAM substrates were processed for immunocytochemistry as described previously. Briefly, fixed cells were incubated in blocking solution containing 5% normal donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% Triton X-100 (Fisher Scientific), followed by incubation with primary antibodies overnight at 4°C. After rinsing in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), cells were incubated in the appropriate biotinylated secondary antibodies, followed by incubation with streptavidin-conjugated Cy3 (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI), diluted at 1:2000 in PBS. Preparations were then mounted
onto microscope slides using an antifade mounting medium (GelMount; Biomeda Corp., Foster City, CA).

To identify differentiated neurons, antibodies against class III β-tubulin (TUJ1, mouse monoclonal IgG; R&D systems, Inc.) or against microtubule-associated protein 2ab (MAP2ab, mouse monoclonal IgG; Sigma) were used at a dilution of 1:750. To identify differentiated glial cells, anti-receptor interacting protein (RIP, mouse monoclonal IgG; Developmental Studies Hybridoma Bank) diluted at 1:1,600 for oligodendrocytes and anti-glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; ICN, Costa Mesa, CA) diluted at 1:500 for astrocytes were used. Biotinylated donkey anti-mouse secondary antibody (Jackson ImmunoResearch) and Cy3-conjugated streptavidin were diluted at 1:500 and 1:15,000 in PBS, respectively. Negative controls were performed in parallel by omission of the primary and/or secondary antibodies. No antibody labeling was observed in these controls.

Quantitative analysis of immunocytochemistry

Following immunocytochemical procedures, the preparations were imaged using a Nikon Eclipse (Nikon Corp., Melville, NY) inverted microscope equipped with standard epifluorescence illumination and digital camera controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA). A total of 12 microscopic fields (0.24 mm²/field) from each micropatterned/non-patterned PS-LAM substrate, 6 fields from micropatterned half and 6 fields from non-patterned half, were randomly taken. To calculate the percentage of AHPCs immunoreactive (IR) for anti-TUJ1, MAP2ab, RIP or GFAP, the
number of GFP-expressing and phenotypic marker-IR AHPCs were divided by the total number of cells (DAPI-stained nuclei).

Neurite outgrowth assay

The images (400 X magnification) of AHPCs co-cultured and immunolabeled against TUJ1 were analyzed. From the images, 21 to 25 TUJ1-IR AHPCs per condition were analyzed using MetaMorph software. The length of major neurites emanating from each cell body was measured. To calculate the average neurite length per cell, total length of the major neurites of each cell, measuring from the edge of the cell body to the tip of the growth cone, was divided by the number of primary neurites. Statistical analysis was performed using GraphPad PRISM (ver. 3.0). All tests were two-tailed tests and p-values less than an alpha of 0.05 were considered significantly different.

Statistical analyses

Statistical analyses were performed on the percentage of the AHPCs IR for each antibody of interest. Since each field was a sub-sample of that half of the substrate, the means of the 6 fields were calculated for use in the analysis so that each field would receive equal weight. The experiment followed a split plot design since the treatment (co-culture or control) was applied to the entire substrate and both micropatterned and non-patterned halves were within one substrate. Due to this design, there were two random effects in the model, (1) for the whole plot effect, consisting of the error term for the treatment and (2) for the split plot effect including the pattern effect and the interaction between the treatment and pattern.
The whole plot experimental unit was the entire PS-LAM substrate (approximately 1 cm² in area) while the split plot experimental unit was the half of the substrate that was either patterned or non-patterned. For each primary antibody (anti-TUJ1, MAP2ab and RIP), N=12 for the whole plot analysis and N=24 for the split plot analysis. Mixed model analysis was performed on the means using the PROC MIXED procedure in SAS. All tests performed were two-sided tests and p-values less than an alpha value of 0.05 were considered significant. Analysis of the residuals was performed and there was no evidence of assumption violations.

Results

To delineate between contact-mediated and soluble neuronal inducing activities associated with the astrocytes, the AHPCs were differentiated in parallel under four different culture conditions: (1) AHPCs cultured alone in CCM (AHPCs alone), (2) AHPCs co-cultured in physical contact with astrocytes (contact co-culture), (3) AHPCs cultured alone but in astrocyte-conditioned CCM (conditioned CCM), and (4) AHPCs co-cultured with astrocytes in non-contact co-culture conditions (non-contact co-culture).

When AHPCs were cultured alone, approximately 14% of the AHPCs were TUJ1-IR, 13% MAP2ab-IR, 17% RIP-IR and 4% GFAP-IR averaging across the entire substrate (Fig. 1 A). In addition, comparison between patterned and non-patterned surfaces of PS-LAM substrates revealed no statistically significant differences in phenotypic differentiation of AHPCs cultured alone (Fig. 1 B1-B4). When co-cultured in physical contact with astrocytes, approximately 25% of the AHPCs were TUJ1-IR, 21% MAP2ab-IR and 22% RIP-IR (Fig. 1
A). Under these contact co-culture conditions, significantly more AHPCs were immunoreactive for the TUJ1 antibody compared to the AHPCs cultured alone (AHPC alone: 14% versus contact co-culture: 25%; Fig. 1 A). Furthermore, under the contact co-culture conditions, significantly more AHPCs were immunolabeled with the TUJ1 antibodies when co-cultured on the patterned side of the substrate compared to the non-patterned side (patterned surface: 31% versus non-patterned surface: 19%; α = 0.05; p < 0.0001) (Fig. 1 B1). However, no statistically significant differences in AHPC differentiation were observed between the patterned and non-patterned sides of the substrates when performing our analysis for the other phenotypic markers (MAP2ab, RIP and GFAP shown in Fig. 1 B2, B3 and B4, respectively). These results were consistent with our previous study. When AHPCs were cultured alone, but in astrocyte-conditioned CCM, we observed a significant increase in the percentage of AHPCs immunolabeled with the TUJ1 antibody (39% TUJ1-IR) in comparison to AHPCs alone and AHPCs in contact co-culture on the non-patterned surfaces (α = 0.05; p ≤ 0.0006) (Fig. 1 B1). These results suggest that the astrocytes secrete soluble factors that induce neuronal differentiation of the AHPCs. However, this activity appeared to be neurogenic since there was no significant change in the percentage of RIP-IR in the AHPCs when compared to the contact co-cultured AHPCs (Fig. 1 B3). To further explore this neurogenic activity, AHPCs were co-cultured with astrocytes under non-contact co-culture conditions. Under these conditions, we observed a dramatic increase in the percentage of AHPCs immunolabeled with the TUJ1 antibody (64%), illustrating an even greater neuronal induction activity for the soluble astrocyte-derived factor (Fig. 1 A). This level of TUJ1 differentiation was significantly greater compared to TUJ1 expression in the other three parallel culture conditions (α = 0.05; p ≤ 0.001). However,
no statistically significant differences in AHPC differentiation were observed between the patterned and non-patterned sides of the substrate in astrocyte-conditioned CCM or non-contact co-cultures for any of the phenotypic markers (TUJ1, MAP2ab, RIP or GFAP; Fig. 1 B1-B4). These results provide strong evidence that astrocytes secrete soluble factors that facilitate the differentiation of the AHPCs towards a neuronal fate. This differentiating activity of the soluble factor(s) secreted from the astrocytes appears to be specifically neurogenic, since no significant effect was observed on the differentiation of RIP- or GFAP-IR cells. In addition, the soluble factor(s) might be short-lived, since the proportion of TUJ1-IR cells cultured in the astrocyte-conditioned CCM (39%) was lower than the proportion of TUJ1-IR cells cultured under non-contact co-culture conditions (64%).

Morphologically, TUJ1-IR AHPCs were observed to possess longer processes under non-contact co-culture condition compared to those in the contact co-cultures. To investigate this possible morphological difference, we performed a quantitative analysis measuring the length of primary major neurites emanating from the cell bodies of TUJ1-IR AHPCs. Under both co-culture conditions, the average length of primary processes oriented in the direction of the grooves were significantly longer on the micropatterned side compared to that on the non-patterned side [Fig. 2; 68.25 μm on patterned surface vs. 40.73 μm on non-patterned surface under non-contact co-culture (α = 0.05; p < 0.0001); 52.60 μm on patterned surface vs. 39.53 μm on non-patterned surface under contact co-culture (α = 0.05; p = 0.0043)].

When comparing the morphology of the AHPCs cultured on the patterned sides under both co-culture conditions, the average length of neurites of the AHPCs in non-contact co-cultures was significantly longer than that in contact co-cultures [Fig. 2; non-contact co-culture vs. contact co-culture (α = 0.05; p = 0.006)] or in the AHPCs alone cultures (data not shown).
On the patterned substrates, RIP-IR cells elaborated extensive processes weaving intricately inside the grooves and along the mesas of the substrate (Fig. 3 G-I). On the non-patterned substrates, extensive radial outgrowth from RIP-IR cells was consistently observed (Fig. 4 G-I). GFAP-IR cells differentiating on the patterned side of the PS-LAM substrate often elaborated filamentous processes oriented in the direction of the pattern (Fig. 3 J-L). GFAP-IR cells differentiating on the non-patterned side of the substrate usually displayed flattened morphologies with large nuclei (Fig. 4 J-L).

**Discussion**

There are multiple factors in the microenvironment directly surrounding cells that can induce and maintain their functional stability. Elucidating which factors are involved and how they interact with cells is extremely helpful in understanding the intra- or inter-cellular mechanisms occurring with the *in vivo* microenvironment. Micropatterned 3-D constructs have been used to study the effects of the microenvironment on cell growth and differentiation. Microfabrication technology has been applied to the design of substrates having specific architectures for such purposes. Culture environments incorporating these substrates are designed to encourage isolated cells to function as they would in their *in vivo* microenvironments or niches. Our previous study demonstrated an enhancement of selective neuronal differentiation of AHPCs co-cultured with astrocytes on PS-LAM substrates. These results provided evidence that physical, chemical and biological cues supplied by the surrounding microenvironment could influence the differentiation of the AHPCs by providing spatial and temporal control through soluble factors and/or contact-mediated
cellular mechanisms which can influence CNS development and function\textsuperscript{12,22}. In this study, we investigated the factors which play an important role in selective neuronal differentiation and the directed outgrowth of AHPCs using a non-contact co-culture system on micropatterned PS-LAM substrates.

\textit{Effect of guidance cues on AHPC differentiation and outgrowth}

In contact co-cultures, we observed a significantly greater percentage of TUJ1-IR cells on the patterned side of the substrate compared to the non-patterned side, supporting our previous findings that 3-D micropatterned PS substrates can enhance neuronal differentiation of AHPCs\textsuperscript{1-3}. In addition, on the patterned side of the substrate, the neurites of TUJ1-IR AHPCs were oriented along the direction of the pattern provided by the 3-D environment and they were significantly longer than neurites on the non-patterned side. Under the non-contact co-culture condition, elongation of the neurites oriented in the direction of the grooves were also enhanced on the patterned surfaces, although there was no significant difference in TUJ1 immunoreactivity when cultured on the patterned compared to the non-patterned surfaces. These findings demonstrate that the astrocyte-derived factors in combination with 3-D micropatterned construct coated with purified ECM (laminin) play a role in promoting elongation of the neurites emanating from the differentiated AHPCs. Interestingly, between the two co-culture systems, in the non-contact co-culture, about 2.5 times greater proportion of the AHPCs were TUJ1-IR compared to that in contact co-cultures while there were no significant differences in the percentage of RIP- or GFAP-IR cells. This result provides strong evidence in support of soluble factor(s) being released from astrocytes co-cultured
with AHPCs and that the factor(s) specifically promotes neuronal differentiation. In addition, via a contact-mediated mechanism, astrocyte-associated factors likely provide an activity that suppresses neuronal differentiation of AHPCs. To confirm that astrocyte-derived soluble factors enhance neuronal differentiation of AHPCs, we cultured the AHPCs alone (in the absence of astrocytes) but in astrocyte-conditioned CCM which presumably includes the factors released from astrocytes. When the AHPCs were cultured in the astrocyte-conditioned CCM, TUJ1 immunoreactivity was increased approximately 3-fold compared to when cultured in normal CCM. Furthermore, there were significantly more TUJ1-IR AHPCs, on the non-patterned side of the substrate, in astrocyte-conditioned CCM compared to the contact co-culture condition. These results strongly support our hypothesis that astrocyte-derived soluble factors selectively stimulate neuronal differentiation of AHPCs which can be modulated by a contact-mediated suppressive mechanism.

During in vitro differentiation, no significant differences in the percentage of MAP2ab-IR AHPCs were observed among the different culture conditions. It is possible that astrocyte-derived factors selectively affect axonogenesis and outgrowth of AHPCs, rather than dendritic development, since the TUJ1 (class III β-tubulin) antibody is commonly used as an axonal marker in young neurons and the MAP2ab (microtubule-associated protein) antibody as a dendritic marker in more mature neurons. During neuronal development, multiple environmental cues can influence the formation and arborization of neuronal processes. Although we observed no significant differences in MAP2ab-IR across the different conditions, our results revealed a dramatic increase in TUJ1-IR under co-culture conditions and in astrocyte-conditioned CCM in comparison to the AHPC alone cultures.
These results suggest that the astrocyte-derived soluble factors may play a critical role in neurite extension and axonal development during early neuronal differentiation.

Microenvironmental regulation of AHPC outgrowth and differentiation

Our results have demonstrated that the 3-D microenvironment can provide physical and/or molecular cues to the micropatterned PS-LAM substrate in the co-culture conditions and, thus, influence neuronal differentiation of AHPCs. However, in the non-contact co-culture condition, as well as in the astrocyte-conditioned CCM, no significant difference of TUJ1 immunoreactivity was observed between the patterned and non-patterned surfaces. It is possible that the AHPCs are in competition with the astrocytes for the released soluble factors. These factors, secreted from astrocytes, in contact co-culture condition, may concentrate within the grooves where astrocytes are aligned, but they may presumably be taken up by the AHPCs that are proximal to the astrocytes or could influence the astrocytes themselves through an autocrine signaling system. Since many of the factors are short-lived, it may be unlikely that the factors would have time to accumulate or diffuse across the substrate. However, in the non-contact co-culture, there may be no direct competition among AHPCs and astrocytes for the released factors. Thus it is possible that the AHPCs are directly and immediately affected by the soluble cues originating from the astrocytes. In contact co-culture, on the patterned surface, simple diffusion of the soluble factors may be less obstructed by the aligned astrocytes than on the non-patterned surface. Thus, a greater proportion of the AHPCs on the patterned surface might be able to differentiate into neuronal cells. However, in the non-contact co-culture condition, diffusion may not be constrained by co-cultured astrocytes because the factors secreted from astrocytes would be released directly
into the media and be presented to the AHPCs on both patterned and non-patterned surfaces immediately upon release. The astrocyte-derived factors, thus, are uniformly presented to all AHPCs on the substrates, whether or not the surface is micropatterned. These factors appear to be critical cues for maximum effect on neuronal differentiation and outgrowth of AHPCs.

Extracellular transport processes play critical roles in cellular morphogenesis. During development, diffusion of morphogens or substances that assign different cell fates or their localization at different concentrations specifies many patterns of cell and tissue organization. Morphogen transport from a localized site forms microgradients through unknown mechanisms which might be simple diffusion or more elaborate mechanisms. It is not yet known how diffusion may be controlling outgrowth and differentiation in our co-culture systems and which factors are released from astrocytes to modulate the neuronal differentiation of the neural progenitor cells. Further studies need to be undertaken to distinctly characterize the mechanism(s) behind this enhanced effect and to elucidate the specific soluble factor(s) involved. The controlled *in vitro* biological model introduced in this study can potentially provide new insights into diffusion mechanisms that govern cell fates *in vivo* during development.

**Conclusion**

The results of this study have demonstrated that astrocyte-derived soluble factors specifically promote neuronal differentiation of adult hippocampal neural progenitor cells (AHPCs). A non-contact co-culture system using semi-porous polyester membrane inserts was established to study a potential role of soluble factors derived from neonatal cortical
astrocytes in selective neuronal differentiation of AHPCs. In comparison to control cultures (AHPCs cultured alone and AHPCs co-cultured in physical contact with astrocytes), neuronal differentiation was dramatically increased when AHPCs were co-cultured with astrocytes under non-contact co-culture conditions. These results suggest that astrocyte-derived soluble factors provide cues for enhancing neuronal differentiation of AHPCs cultured on PS-LAM substrates. Furthermore, under non-contact co-culture conditions, neurite length was significantly greater when compared with contact co-culture conditions. In addition, neurite outgrowth on the patterned side of the substrates was considerably greater compared to the non-patterned, planar surface of the PS-LAM substrates. Our results demonstrate that astrocyte-derived soluble factors facilitate neuronal differentiation of the AHPCs, in combination with multiple microenvironmental cues, such as 3-D micropatterned PS substrate and purified ECM molecules (laminin). These results have important implications for developing strategies to promote neuronal differentiation from NPCs and for stem cell-mediated repair of the CNS.

Acknowledgments

Financial support provided by The Glaucoma Foundation, New York to DSS and from the National Institutes of Health to SKM and DSS are gratefully acknowledged (NIGMS 1 RO1 GM072005). The authors would like to thank Dr. Fred H. Gage at the Salk Institute for the gift of the AHPCs. The authors are also grateful to Dr. Robert T. Doyle at the Roy J. Carver Laboratory for Ultrahigh Resolution Biological Microscopy, Department of Genetics, Development & Cell Biology at Iowa State University (ISU) for his helpful
advice and suggestions. The authors would also like to thank Christopher C. Blong for his help with substrate preparation and Drs. H. Levine, M. Nilsen-Hamilton and M. Smiley for helpful discussions.
References


Figure legends

**Figure 1.** Differentiation of AHPCs under the four different culture conditions - AHPCs alone, contact co-culture, conditioned CCM and non-contact co-culture. (A) Average percentages ± SEM of phenotypic marker-IR AHPCs, TUJ1 and MAP2ab for neurons and RIP and GFAP for oligodendrocytes and astrocytes, respectively. All phenotypic marker-IR cells were averaged over both patterned and non-patterned surfaces. (B) Quantification of phenotypic marker-IR AHPCs on patterned versus non-patterned substrates. The percentages of TUJ1-IR (B1; asterisks, *, statistically significant difference when \( p \leq 0.001 \); NS, no statistically significant differences), MAP2ab-IR (B2), RIP-IR (B3) and GFAP-IR (B4) AHPCs on patterned and non-patterned substrates in different conditions. \( N \) (number of parallel experiments) = 3 or 4. For the other phenotypic markers (MAP2ab and RIP), under all culture conditions, no statistically significant differences in AHPC differentiation were observed between the patterned and non-patterned sides of the substrates. A statistical analysis was not performed on GFAP-IR cells as the data were obtained from two independent experiments and little differences were observed in the average number of GFAP-IR cells for the various experimental conditions.

**Figure 2.** Morphological differences between TUJ1-IR AHPCs cultured with astrocytes with physical contact or without contact, on non-patterned (NP) or patterned (P) PS-LAM substrates. (A) Representative fluorescence images of TUJ1-IR AHPCs cultured in non-contact co-culture conditions or contact co-culture conditions on the NP or P side of the PS-LAM substrates. These are merged images created by the superimposition of TUJ1-IR (red),
GFP-expression (green) and DAPI nuclear counterstain (blue) fluorescent images. Dotted white lines indicate the location of a groove on the micropatterned substrate. Scale bar = 50 μm. (B) Line drawing reconstructions of individual TUJ1-IR AHPCs under the different culture conditions illustrating the major primary neurites emanating from the cell body. (B1) Reconstructions of TUJ1-IR AHPCs in (A) for each condition. (B2, B3) Two additional examples of line drawing reconstructions of TUJ1-IR cells. (C) Average primary neurite lengths for TUJ1-IR AHPCs growing under the respective culture conditions. Values are the averaged neurite lengths of TUJ1-IR GFP-expressing AHPCs, mean ± SEM. Asterisks indicate statistical difference ($p < 0.05$, $t$ test).

**Figure 3.** Differentiation of AHPCs co-cultured with astrocytes on patterned substrates in the non-contact condition. Fluorescence images of TUJ1-IR (A), MAP2ab-IR (D) RIP-IR (G) and GFAP-IR (J) GFP-expressing AHPCs (B, E, H and K, respectively) were merged with DAPI nuclei counterstaining (C, F, I and L, respectively). In non-contact co-cultures, on the patterned surfaces, directed neuritic extension from the neuronal cell body was observed with prominently longer and elaborated processes in the direction of the grooves than on the non-patterned surfaces (TUJ1-IR, A-C and MAP2ab, D-F). RIP-IR cells elaborated extensive processes weaving intricately inside the grooves and along the mesas of the substrate (G-I). GFAP-IR cells displayed flattened morphologies with large nuclei and in many cases, processes strongly immunoreactive for GFAP (J-L). Dotted white lines indicate the location of a groove on the micropatterned substrate. Scale bar = 20 μm.
**Figure 4.** Differentiation of AHPCs co-cultured with astrocytes on non-patterned, planar substrates in the non-contact condition. Fluorescence images of TUJ1-IR (A, B), MAP2ab-IR (D, E), RIP-IR (G, H) and GFAP-IR (J, K) GFP-expressing AHPCs were merged with DAPI counterstaining (C, F, I, and L, respectively). In general, immunoreactive AHPCs displayed radially directed processes when cultured on non-patterned, planar substrates. Scale bar = 20 µm.
Figure 1. Differentiation of AHPCs under the four different culture conditions - AHPCs alone, contact co-culture, conditioned CCM and non-contact co-culture.
Figure 2. Morphological differences between TUJ1-IR AHPCs cultured with astrocytes with physical contact or without contact, on non-patterned (NP) or patterned (P) PS-LAM substrates.
Figure 3. Differentiation of AHPCs co-cultured with astrocytes on patterned substrates in the non-contact condition.
Figure 4. Differentiation of AHPCs co-cultured with astrocytes on non-patterned, planar substrates in the non-contact condition.
CHAPTER 3.

ASTROCYTE-DERIVED INTERLEUKIN-6 PROMOTES SPECIFIC NEURONAL DIFFERENTIATION OF NEURAL PROGENITOR CELLS FROM ADULT HIPPOCAMPUS

A paper published in Journal of Neuroscience Research

Jisun Oh, Michael A. McCloskey, Christopher C. Blong, Lee Bendickson, Marit Nilsen-Hamilton and Donald S. Sakaguchi

1 Neuroscience Program, 2 Department of Genetics, Development and Cell Biology, 3 Department of Biomedical Sciences, 4 Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011

# Corresponding author

Abstract

The purpose of this study was to investigate the ability of astrocyte-derived factors to influence neural progenitor cell differentiation. We previously demonstrated that rat adult hippocampal progenitor cells (AHPCs) immunoreactive for the neuronal marker, class III β-tubulin (TUJ1) were significantly increased in the presence of astrocyte-derived soluble factors under non-contact co-culture conditions. Using whole cell patch clamp analysis, we observed that the co-cultured AHPCs displayed two prominent voltage-gated conductances - tetraethyl ammonium (TEA)-sensitive outward currents and fast transient inward currents. The outward and inward current densities of the co-cultured AHPCs were approximately 2.5-fold and 1.7-fold greater, respectively, than those of cells cultured alone. These results suggest that astrocyte-derived soluble factors induce neuronal commitment of AHPCs. To further investigate the activity of a candidate neurogenic factor on AHPC differentiation, we cultured AHPCs in the presence or absence of purified rat recombinant interleukin-6 (IL-6).
We also confirmed that the astrocytes used in this study produced IL-6 by ELISA and RT-qPCR. When AHPCs were cultured with IL-6 for 6-7 days, the TUJ1-immunoreactive AHPCs and the average length of TUJ1-immunoreactive neurites were significantly increased, compared to the cells cultured without IL-6. Moreover, IL-6 increased the inward current density to a comparable extent as did co-culture with astrocytes, with no significant differences in the outward current density, apparent resting potential, or cell capacitance. These results suggest that astrocyte-derived IL-6 may facilitate AHPC neuronal differentiation. Our findings have important implications for understanding injury-induced neurogenesis and developing cell-based therapeutic strategies using neural progenitors.
Abbreviation

AHPC, adult hippocampal progenitor cell
bFGF, basic fibroblast growth factor
Brain-Astro, astrocytes isolated from whole cerebral hemispheres of the brain
CCM, co-culture medium
CNS, central nervous system
CTX-Astro, astrocytes isolated from cortical region of the brain
DIV, days in vitro
GFAP, glial fibrillary acidic protein
HC-Astro, astrocytes isolated from hippocampal region of the brain
IL-6, interleukin-6
MEM, minimal essential medium
MMEM, modified MEM
NCCC, non-contact co-culture
NPC, neural progenitor cell
NGF, nerve growth factor
NT, neurotrophin
PSA-NCAM, polysialic acid neural cell adhesion molecule
RIP, receptor interacting protein
TEA, tetraethyl ammonium
TUJ1, class III β-tubulin
TTX, tetrodotoxin
Introduction

The cytokine interleukin-6 (IL-6) is best known as a regulator of hematopoiesis (Eglitis and Mezey 1997). In addition, IL-6 is found in the nervous system and in conjunction with its receptor complex can trigger cellular responses mediating inflammation, neurogenesis, gliogenesis, cell growth and survival, and myelination/demyelination (Gradient and Otten 1997; Gruol and Nelson 1997; Van Snick 1990; Van Wagoner and Benveniste 1999). In the brain, IL-6 is normally expressed at relatively low levels (Gradient and Otten 1997). However, during progression of inflammation or brain injury, IL-6 protein levels are elevated in the cerebral spinal fluid and brain homogenates (Gruol and Nelson 1997; Van Wagoner and Benveniste 1999). Chronic over-expression of IL-6 in transgenic mice caused neuroanatomical and neurophysiological alterations reminiscent of various neurological diseases (Campbell et al. 1993; Gruol and Nelson 1997; Vallieres et al. 2002). Interestingly, neurotrophins (NTs) such as nerve growth factor (NGF), are co-expressed with IL-6 at sites of nerve injury (Otten et al. 2000). IL-6 in combination with its specific receptor, IL-6R, has been reported to regulate a local supply of NTs that are critical for neuronal survival and protection (Gradient and Otten 1994; Gradient and Otten 1997; Otten et al. 2000). These reports suggest that IL-6 expression is up-regulated under diverse pathological and physiological conditions in the CNS, and also contributes to the production and function of NTs at the injured site of the CNS.

Astrocytes are an important source of IL-6 in the CNS (Gruol and Nelson 1997) and the expression levels vary for different brain regions. Barkho et al. (2006) demonstrated that astrocytes originating from neurogenic regions (newborn hippocampus, adult hippocampus, and newborn spinal cord) compared with cells from non-neurogenic regions (adult spinal
cord astrocytes and adult skin fibroblasts) displayed different gene expression profiles (Barkho et al. 2006; Song et al. 2002). IL-6 mRNA was expressed at relatively higher levels in newborn hippocampal astrocytes than in non-neurogenic astrocytes. Furthermore, when adult hippocampus-derived neural progenitor cells were cultured in the presence of IL-6, neuronal promoter activity and neuronal marker immunoreactivity were increased (Barkho et al. 2006). Our previous results confirmed that neuronal differentiation of adult hippocampal progenitor cells (AHPCs) was enhanced by soluble factors derived from neonatal astrocytes (Oh et al. 2009; Recknor et al. 2006). However, it was not known if astrocyte-derived IL-6 influenced NPC differentiation into functional neurons.

In the present study, we tested the hypothesis that IL-6 produced from neurogenic astrocytes is a key regulator in our culture system that stimulates functional differentiation of AHPCs to a neuronal fate. Using immunocytochemical analysis and whole-cell recording, in the presence of exogenous IL-6, we observed that (1) neuronal marker expression was increased, (2) the average length of neurites from neuronal-restricted AHPCs was increased and (3) voltage-gated inward current density was increased with no significant differences in voltage-gated outward current density, apparent resting membrane potential or cell capacitance. These results demonstrate that astrocyte-derived IL-6 enhances neuronal differentiation of AHPCs.

Materials and Methods

Cell culture

All animal procedures were conducted in accordance with and had the approval of the
Astrocytes were isolated from cerebral cortices and hippocampi of postnatal day two Sprague-Dawley rat brains as described previously (Recknor et al. 2004). Adult hippocampal progenitor cells (AHPCs from F. Gage, Salk Institute, La Jolla, CA), isolated from the brains of adult Fischer 344 rats and infected with retrovirus to express enhanced green fluorescent protein (GFP) (Gage et al. 1995; Ray et al. 1995) were maintained as described previously (Oh et al. 2009) (for details, see Supplementary Materials and Methods online).

**Astrocyte-AHPC co-culture**

Transwell® inserts (Corning, Inc., Corning, NY) were used to establish co-cultures of astrocytes and AHPCs growing together in the absence of physical contact (referred to as non-contact co-culture, NCCC) as described previously (Oh et al. 2009) (for details, see Supplementary Materials and Methods online).

**IL-6 treatment**

To examine the effect of IL-6 on AHPC differentiation, purified rat recombinant IL-6 (20 ng/ml; R&D Systems) and/or neutralizing antibody against IL-6 (anti-IL-6) produced in goat (10 ng/ml; R&D Systems) were added to the culture media. A concentration-response analysis was performed to determine optimal concentrations of purified IL-6 (Supplementary Figure 1). Based on this analysis and from the literature (Barkho et al. 2006; Hakkoum et al. 2007), an IL-6 concentration of 20 ng/ml was used in this study. The anti-IL-6 neutralizing antibody was tested at various concentrations (10, 100 and 1000 ng/ml in NCCCs of astrocyte-AHPC; 10 and 1000 ng/ml in AHPC alone cultures with 20 ng/ml of purified IL-6).
There were no significant effects of these concentrations of blocking antibody on the percentages of TUJ1- or RIP-immunoreactive AHPCs (see ‘Immunocytochemistry’ section below) when the cells were cultured with the antibody in the absence of IL-6 (data not shown). Thus, the anti-IL-6 neutralizing antibody was used at 10 ng/ml in this study. As a control for the neutralizing antibody, a purified preimmune goat immunoglobulin G (goat IgG; Sigma-Aldrich) was used at the same concentration.

*Immunocytochemistry*

AHPCs cultured on laminin-coated substrates were processed for immunocytochemistry according to standard protocols described previously (Oh et al. 2009). Fixed AHPCs were labeled with antibodies against phenotypic markers, class III β-tubulin (TUJ1, mouse monoclonal IgG; R&D Systems), receptor interacting protein (RIP in concentrated form supplied by vendor, mouse monoclonal IgG; Developmental Studies Hybridoma Bank) and glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; Lab Vision Corp., Fremont, CA) Biotinylated donkey anti-mouse secondary antibody and Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) were used for visualizing the primary antibody labeling. Nuclei were counterstained with 1 μM 4’, 6-diamidino-2-phenylindole, dilactate (DAPI). Preparations were then mounted onto microscope slides using GelMount antifade mounting medium (Biomeda Corp., Foster City, CA) (for details, see Supplementary Materials and Methods online).

*Neurite outgrowth assay*
AHPCs were cultured in the absence or presence of purified IL-6 at different concentrations (from 0.00001 to 100 ng/ml; Figure 3 C) for 6 days. After fixation, cultured AHPCs were immunolabeled for TUJ1 and imaged using a 40X objective. From the images, 17 to 22 TUJ1-immunoreactive AHPCs per condition were analyzed for the length of neurites emanating from the cell body using ImageJ, image processing and analysis program (developed at the National Institutes of Health, http://rsbweb.nih.gov/ij/) (for details, see Supplementary Materials and Methods online).

Whole cell patch clamping

AHPCs were plated on a laminin-coated polystyrene substrate that was attached to the bottom of a recording chamber made of a 25-mm circular coverglass (Cat # 12-545-102, Fisher Scientific) with an attached silicone O-ring (Cat # 9396K22, McMaster Carr Supply, Atlanta, GA). After the cells were cultured in the recording chamber for a designated culture period, the chamber with cells was placed on the stage of a Nikon Diaphot inverted microscope (Tokyo, Japan). The cultures were bathed in an external solution containing (in mM): 145 NaCl, 3 KCl, 10 HEPES, 8 glucose, 2 MgCl₂, 3 CaCl₂ (pH 7.4, adjusted with NaOH). Patch pipettes were pulled from thick-walled borosilicate glass capillaries (Cat # PG52165-4, World Precision Instrument, Inc., Sarasota, FL) and fire-polished to resistances of 3 - 5 MΩ when filled with internal solution containing (in mM): 136.5 K-gluconate, 17.5 KCl, 9 NaCl, 10 HEPES, 8 glucose, 0.2 EGTA and 1 MgCl₂ (pH 7.2, adjusted with KOH). For the cultures maintained in the presence of basic fibroblast growth factor (bFGF), cells were randomly chosen for recording. For the cells cultured under differentiation condition, cells that
possessed neuronal-like morphology typical of TUJ1-immunoreactive cells were selected (Ray et al. 1993; Sah et al. 1997).

Conventional whole cell recording was performed at room temperature using an Axopatch 200B patch clamp amplifier (Molecular Devices, Sunnyvale, CA) and IT-16 interface (Instrutech, Inc.) or an EPC9 (HEKA Instruments, Inc., Bellmore, NY). Current was filtered at 1 kHz and digitized at 5 kHz. Stimulation and acquisition were controlled with PULSE software (v8.78, HEKA Instruments, Inc.).

A liquid junction potential of 14.3 mV was estimated using JPCalc software (P. H. Barry, University of New South Wales). Membrane voltages were corrected after the experiment by subtraction of the liquid junction potential; the apparent resting potential was estimated 10 min after break-in to the whole cell mode as the zero current potential during current-clamp recording and corrected for the liquid junction potential. Ionic currents were measured by application of 100 ms depolarizing steps in 10 mV increments from the holding potential (V_H) of -100 mV. The interpulse interval was 5 s. Current densities were calculated by normalizing the peak currents to the membrane capacitance (Cm). Input resistance (R_in) was estimated from the reciprocal of the slope of voltage ramps at subthreshold potentials in the whole cell mode; because the values of R_in estimated this way were similar to seal resistances, we could only place a lower limit on the true R_in.

Pharmacological blockade of inward and outward currents was performed with 300 nM tetrodotoxin (TTX; Sigma-Aldrich) for voltage-gated sodium (Na^+) channels, or 5 mM tetraethylammonium (TEA; Sigma-Aldrich) for voltage-gated potassium (K^+) channels, respectively.
Statistical analysis

Statistical analysis was performed using GraphPad PRISM (ver. 3.0). All tests were two-tailed tests and p-values less than an alpha of 0.05 were considered significantly different.

Results

Astrocyte-derived soluble factors enhance AHPC neuronal differentiation

Our previous results demonstrated that astrocyte-derived soluble factors induced neuronal differentiation of AHPCs (Oh et al. 2009). When AHPCs were co-cultured with astrocytes (isolated from neonatal rat brain, cerebral hemispheres) in the absence of physical contact (referred to as non-contact co-culture, NCCC) using Transwell® inserts, neuronal differentiation of AHPCs was significantly augmented compared to AHPCs cultured alone (without astrocytes) (Figure 1; 63.7% in NCCC vs. 13.1% in alone culture). To further delineate the source of neurogenic activity, we isolated separately hippocampal and cortical astrocytes. Immunocytochemical analysis revealed that under NCCC conditions, using either hippocampal or cortical astrocytes, the percentage of TUJ1-immunoreactive (IR) AHPCs was significantly higher compared to that when AHPCs were cultured alone (Figure 1). In addition, the percentage of TUJ1-IR cells was significantly greater for AHPCs when co-cultured with hippocampal astrocytes (NCCC with HC-Astro) than with cortical astrocytes (NCCC with CTX-Astro) (Figure 1; 54.4% in NCCC with HC-Astro vs. 34.2% in NCCC with CTX-Astro). These results suggest that the astrocyte-derived soluble factors induce neuronal differentiation of AHPCs, which is consistent with our previous results (Oh et al.)
Moreover, on a cell per cell basis, hippocampal astrocytes appear to possess significantly greater neurogenic activity, compared to cortical astrocytes.

The astrocyte-derived factors appeared specific for inducing AHPC neuronal differentiation, because no effect was observed on astroglial differentiation (Figure 1; RIP and GFAP immunoreactivities). Under NCCC using astrocytes from whole cerebral hemispheres (referred to as brain astrocytes, Brain-Astro), the percentage of oligodendrocytes (RIP-IR AHPCs) was greater than when AHPCs were cultured alone (Figure 1; 26.8% in NCCC with Brain-Astro vs. 13.0% in alone culture). However, under NCCC using either cortical astrocytes or hippocampal astrocytes, there was no significant difference in RIP immunoreactivity compared to the AHPCs cultured alone (Figure 1; 15.4% in NCCC with HC-Astro vs. 19.1% in NCCC with CTX-Astro vs. 13.0% in alone culture). This result demonstrates that the factors from cortical astrocytes or from hippocampal astrocytes have little effect on oligodendrocytic differentiation of AHPCs. However, whole brain astrocytes have a small incremental effect on oligodendrocytic differentiation of AHPCs.

To examine whether the AHPCs with neuronal morphology possessed membrane characteristics consistent with neuronal differentiation, patch clamp analysis in conventional whole cell mode was performed. AHPCs were cultured in the absence or presence of the astrocytes for 6-7 days or 9-10 days. AHPCs from both conditions had similar capacitance (Cm) values (Table 1) and input resistance (Rin, > 2 GΩ) (data not shown). The apparent resting potential was more hyperpolarized under differentiation conditions compared to the proliferation conditions (Table 1). AHPCs at 6-7 DIV in co-culture with brain-derived astrocytes showed significantly greater current densities for both TEA-sensitive sustained
outward currents (voltage-gated K\(^+\) channel-mediated) and transient inward currents (voltage-gated Na\(^+\) channel-mediated) in response to the voltage-step stimuli compared to the AHPCs cultured alone (Figure 2 A2 and B2; Table 1). These results demonstrate that astrocyte-derived neurogenic factors promoted neuronal differentiation with respect to the electrical membrane properties of AHPCs, as well as in cell morphology.

**IL-6 enhances neuronal differentiation of AHPCs**

IL-6 has been suggested as a candidate astrocyte-derived factor that can induce AHPC differentiation (Barkho et al. 2006). To investigate the molecular mechanism(s) by which astrocytes promote neurogenesis, a neutralizing antibody against rat IL-6 (referred to as anti-IL-6) was added to the AHPC cultures. As a control, equal concentrations of affinity-purified goat IgG were added to parallel cultures. Addition of anti-IL-6 to the hippocampal NCCC resulted in a significant decrease in neuronal differentiation of the AHPCs (percentage of TUJ1-IR AHPCs: 54.4% without anti-IL-6 vs. 27.7% with anti-IL-6) (Figure 3 A). In contrast, the blocking antibody had no effect on TUJ1 immunoreactivity in cortical NCCC (34.2% without anti-IL-6 vs. 35.2% with anti-IL-6) (Figure 3 A). These results suggest that IL-6 derived from neurogenic hippocampal astrocytes may play a critical role in neuronal differentiation of AHPCs.

To examine further whether IL-6 itself can directly influence AHPC differentiation, we cultured AHPCs (alone, in the absence of astrocytes) with or without rat recombinant IL-6 for 6 days. Incubation with purified IL-6 specifically increased the percentage of TUJ1-IR AHPCs (38.5% with IL-6 vs. 13.1% without IL-6) with no effect on the percentages of RIP- or GFAP-IR AHPCs (RIP immunoreactivity: 13.6% with IL-6 vs. 13.0% without IL-6;
GFAP immunoreactivity: 1.5% with IL-6 vs. 1.6% without IL-6 (Figure 3 B). Moreover, the IL-6-mediated augmentation of TUJ1 immunoreactivity was eliminated by addition of anti-IL-6 neutralizing antibody (Figure 3 B). These results suggest that IL-6 is a key molecule that can induce neuronal differentiation of AHPCs.

In addition, IL-6 influences neurite outgrowth of AHPCs. When AHPCs were cultured in the presence of various concentrations of purified IL-6, the average length of TUJ1-IR processes extending from the soma of TUJ1-IR AHPCs was positively correlated with the concentration of IL-6 (Figure 3 C). At concentrations greater than 1 ng/ml of IL-6, the average process length per cell was significantly longer than that of the control cultures (AHPCs alone with no IL-6). The average length of neurites analyzed from the cells cultured in the presence of IL-6 was approximately 40 µm, which is comparable to the process length of AHPCs co-cultured with enriched postnatal astrocytes as demonstrated previously (Oh et al. 2009). However, there was no significant difference observed in the total length of processes per cell with increasing IL-6 concentrations (Supplementary Figure 2). These data indicate that IL-6 increases the length of TUJ1-expressing neurites from AHPCs undergoing neuronal differentiation.

To investigate the influence of IL-6 on electrical membrane properties, AHPCs were cultured under differentiation conditions in the absence or presence of IL-6. The AHPCs cultured in the presence of IL-6 showed significantly greater transient inward currents compared to cells cultured in the absence of IL-6 (Figure 4; Table 2). In three out of three IL-6-treated AHPCs for which it was tested, 300 nM TTX inhibited the transient inward currents, which suggests mediation by TTX-sensitive Na\(^+\) channels. Augmentation of the transient inward current in the AHPCs cultured in the presence of IL-6 was comparable to
that seen with the AHPCs co-cultured with astrocytes. The effect appears to be selective for Na\(^+\) current, as no enhancement of a presumed K\(^+\) current (TEA-sensitive current) was apparent (Table 2). The observed changes in Cm and apparent resting potential due to exogenous IL-6 were statistically insignificant (Table 2). These results suggest that IL-6 can facilitate expression of voltage-gated Na\(^+\) channels during AHPC differentiation into neurons.

**IL-6 is produced from the co-cultured astrocytes**

To quantify the amount of IL-6 protein secreted into the culture media, we performed enzyme-linked immunosorbent assays (ELISA) (Supplementary Figure 3 A). These results demonstrate that (a) the major source of IL-6 production in the non-contact co-culture system was the astrocytes, rather than AHPCs, and (b) the IL-6 production capability of astrocytes appeared to be independent of the AHPCs co-cultured with them. The expression levels of IL-6, its specific receptor, IL-6R\(\alpha\) (transmembrane and soluble) and gp130 in AHPCs and astrocytes were analyzed by RT-qPCR (Supplementary Figure 3 B, C) (for details, see Supplementary Results and Discussion online).

**Discussion**

The local microenvironment is crucial in regulating the survival, proliferation and differentiation of NPCs (Conover and Notti 2008; Garcion et al. 2004; Soen et al. 2006; Watt and Hogan 2000; Wurmser et al. 2004). In addition to the extracellular matrix (ECM), astrocytes surrounding NPCs can provide various biological cues for regulating neurogenesis *in vivo* (Lim and Alvarez-Buylla 1999; Recknor et al. 2006; Song et al. 2002). Our previous
results demonstrated that astrocyte-derived soluble factors promote neuronal differentiation of AHPCs, in combination with three-dimensional spatial control (Oh et al. 2009). In the present study, we identified a candidate molecule, IL-6, which specifically induces neuronal differentiation of adult NPCs by demonstrating that (1) newborn hippocampal astrocytes produced more IL-6 protein than non-neurogenic skin-derived fibroblasts and (2) IL-6 treatment increased the fraction of TUJ1-immunoreactive cells. These results support the earlier findings by Barkho and colleagues (Barkho et al. 2006). Furthermore, in our study we reported for the first time electrophysiological properties of AHPCs undergoing differentiation in the presence of IL-6. Our findings demonstrate that IL-6 derived from neurogenic astrocytes promotes functional differentiation of AHPCs and accelerates neurite outgrowth.

Astrocyte-derived IL-6 promoting neuronal differentiation of AHPCs

Previously we demonstrated that the percentage of TUJ1-IR AHPCs was dramatically increased when AHPCs were co-cultured with astrocytes compared to the cells cultured alone (Oh et al. 2009). This result suggested that astrocyte-derived soluble factors enhance neuronal differentiation of AHPCs. To identify the source of neurogenic activity of the astrocytes, we separated the hippocampi, a neurogenic region of the brain, from the surrounding cerebral cortex, thus generating two distinct populations of astrocytes. A significantly higher percentage of AHPCs differentiated into neurons (TUJ1 immunoreactivity) when co-cultured with hippocampal astrocytes compared to those co-cultured with cortical astrocytes. This suggests that the soluble factors derived from hippocampal astrocytes are in part responsible for the increase in neuronal differentiation of
AHPCs, which agrees with the previous finding that astrocytes from adult hippocampus can instruct the NPCs to adopt a neuronal fate (Song et al. 2002).

The increased neuronal differentiation of AHPCs co-cultured with hippocampal astrocytes was diminished in the presence of neutralizing antibody against IL-6. However, the neutralizing antibody had no effect on the TUJ1 immunoreactivity of AHPCs co-cultured with cortical astrocytes. These results might be explained by the combined action of multiple factors produced by astrocytes and/or AHPCs. For example, hippocampal astrocytes may produce additional neurogenic factors that promote neuronal differentiation of AHPCs. Recently, metalloprotease-9, all-trans retinoic acid, Wnt3 and neurogenesin-1 were reported as factors derived from neurogenic astrocytes that can induce neuronal differentiation of NPCs or oligodendrocyte fate-restricted progenitor cells (Barkho et al. 2008; Barkho et al. 2006; Gaughwin et al. 2006; Kornyei et al. 2007; Lie et al. 2005; Ueki et al. 2003). Furthermore, cortical astrocytes appear to express developmental signals, such as Notch, Noggin, sonic hedgehog and thrombospondin-1 which can participate in generating a neurogenic microenvironment surrounding NPCs (Lu and Kipnis; Taupin 2006). Factors derived from cortical astrocytes may also suppress neuronal differentiation of AHPCs. For instance, insulin-like growth factor (IGF)-1 is known to be important for early neural development as well as maintenance of the adult CNS (Russo et al. 2005). In the adult CNS, IGF-1, which is expressed both in neurons and astrocytes, binds to IGF binding proteins (IGFBPs) that are mainly produced by astrocytes (Chesik et al. 2007; Feldman et al. 1997; Russo et al. 2005; Ye et al. 2004). IGFBP-6, in particular, was reported to be expressed at much higher levels in non-neurogenic astrocytes than in neurogenic-astrocytes, so it is thought to inhibit neuronal differentiation of NPCs (Barkho et al. 2006). Bone morphogenic
proteins (BMPs), having multiple roles in embryonic brain development, are also known to influence glial and neuronal differentiation of NPCs (Gross et al. 1996; Taga and Fukuda 2005; Zhang and Li 2005). Therefore, the neurogenic effect of IL-6 released from cortical astrocytes may be prevented by other cortical astrocyte-derived factors. Taken together, reduced neuronal differentiation by neutralizing antibody against IL-6 demonstrates that an astrocyte-derived soluble factor, IL-6, is a critical soluble factor that can enhance neuronal differentiation of AHPCs. Furthermore, astrocytes isolated from the hippocampal region, a neurogenic niche, may be more potent in inducing neuronal differentiation of AHPCs than the general astrocyte population.

Direct application of IL-6 enhanced AHPC neuronal but not glial differentiation. Moreover, IL-6-mediated neuronal differentiation was neutralized by anti-IL-6 function-blocking antibody. This result suggests that AHPCs are capable of responding with neuronal differentiation to exogenous IL-6. RIP immunoreactivity of AHPCs under co-culture conditions tended to be slightly higher compared to that of AHPCs cultured alone. This result is not surprising because numerous soluble factors derived from the astrocytes may influence NPC fate specification during CNS development or inflammation response (Martino and Pluchino 2006; Taupin 2008). For example, sonic hedgehog secreted from reactive astrocytes can induce NPCs to differentiate into oligodendrocytes as well as neurons (Martino and Pluchino 2006). Platelet-derived growth factor produced by astrocytes has been known to stimulate the proliferation of oligodendrocyte precursors in the CNS (Abe 2000; Martino and Pluchino 2006). Interestingly, oligodendrocyte differentiation (assessed by RIP immunoreactivity) from AHPCs co-cultured with astrocytes from the whole cerebral hemispheres (including the hippocampus) was significantly higher than that under control
conditions (AHPCs cultured alone). However, under co-culture conditions with either
cortical or hippocampal astrocytes, the RIP immunoreactivity was not significantly different
compared to that under control conditions. Region-specific factors may be produced only
from the cortical or hippocampal astrocytes that potentially promote oligodendrocyte
differentiation of AHPCs. The oligodendrocyte differentiation-promoting activity of those
factors may be reinforced by juxtacrine or paracrine stimulation when the cortical and
hippocampal astrocytes are in close proximity.

*Neurite extension in the presence of IL-6*

IL-6 application specifically influenced TUJ1 immunoreactivity of AHPCs in a
concentration-dependent manner, with no effect on RIP immunoreactivity. Above 1 ng/ml of
IL-6, the percentage of TUJ1-IR AHPCs appeared to have reached saturation. In addition,
the average length of processes extended from the TUJ1-IR AHPCs was significantly longer
than that for the cells in the control condition (0 ng/ml of IL-6), although neither the average
number of processes per cell (Supplementary Table 1) nor the total length of processes
(Supplementary Figure 2) was significantly different.

Marz et al. (1999) reported that the application of the IL-6/IL-6R fusion protein
(covalently linked by an intermediate peptide(Fischer et al. 1997)) increased expression
levels of neurotrophins (NTs) in brain-derived astrocytes(Marz et al. 1999). Both
hippocampal and cortical astrocytes express NGF at high levels following IL-6
treatment(Marz et al. 1999). NGF can suppress the activation of STAT3 which itself inhibits
neurite outgrowth of PC12 cells(Ihara et al. 1997). Moreover, hippocampal and cortical
astrocytes exclusively express NT-3 and NT-4/5, respectively(Marz et al. 1999). NT-3
facilitates neuronal differentiation and neurite outgrowth of mouse NPCs through the MAPK-ERK pathway (Lim et al. 2007). Thus, the astrocyte-derived IL-6 may stimulate neurogenic astrocytes that express IL-6R to produce NTs, such as NGF and NT-3. Such NTs might then inhibit STAT3 activation and induce ERK activation following MAPKK activation. Therefore, the IL-6 released from the astrocytes may be a key molecule that influences neuronal differentiation and neurite outgrowth of AHPCs.

### Electrophysiological properties of AHPCs

For whole cell patch clamp analysis, only cells possessing a neuronal morphology were selected for recording. In this analysis, we examined resting potential, capacitance and current density. AHPCs cultured in the absence or presence of IL-6 showed similar apparent resting potentials, -79 ~ -82 mV on average. Schmidt-Hieber et al. (2004) reported that the resting membrane potentials for PSA-NCAM-expressing newborn hippocampal granule cells were about -75 mV which was similar to that for PSA-NCAM immunonegative mature cells (about -81 mV) (Schmidt-Hieber et al. 2004). Resting potentials of AHPCs cultured under differentiation condition for 1 week were comparable to the values for the granule cells in the hippocampus. AHPCs cultured without or with IL-6 also showed similar membrane capacitance values in the range of 10 ~ 15 pF. These values are comparable to those reported for rat hippocampus-derived NPCs cultured for less than 2 weeks in vitro with various factors (Sah et al. 1997). Considering that cell capacitance is proportional to cell surface area, more mature dentate granule cells extending longer dendrites, as expected, have greater cell capacitance than do their immature counterparts (Schmidt-Hieber et al. 2004; van Praag et al. 2002). In brain slices, 4 week-old hippocampal neurons displaying complex morphologies
with highly branched dendritic arborizations exhibit 3.5-times higher capacitance values (van Praag et al. 2002) than do AHPCs cultured for 6 days. From our result TUJ1-IR AHPCs cultured for 6 days in the presence of exogenous IL-6 displayed a total length of presumed axonal processes per cell of approximately 150 µm regardless of IL-6 concentrations. This insignificant difference in the total length of processes between cells cultured without and with IL-6 is consistent with the result of no significant difference in cell capacitance values between the two conditions.

Interestingly, the average length of TUJ1-IR processes for AHPCs cultured with IL-6 was about 1.5-fold greater than that for cells in control condition without IL-6. However total length of TUJ1-IR processes and the membrane capacitance were similar between the two conditions. In the presence of IL-6, it is possible that some TUJ1-IR neurites that may be presumptive axons were extended while others that were destined to become dendrites were retracted compared to the control condition (Ferreira and Caceres 1992; Laferriere et al. 1997; Winckler 2007). This result suggests that IL-6 may be involved in neurite specification during neuronal development. The remodeling of neurites during neuronal polarization (formation of axons and dendrites) is an important aspect of neuronal differentiation (Craig and Banker 1994; Da Silva et al. 2005). Axons can be distinguished from the dendrites by the expression of distinct molecules, such as plasma membrane ganglioside sialidase (PMGS) found in the axon of mature neurons (Da Silva et al. 2005) and higher glycogen synthase kinase (GSK)-3β activity in dendrites than in axons of polarized hippocampal neurons (Jiang et al. 2005). Immunocytochemically TUJ1 (class III β-tubulin) is commonly used as an axonal marker (Joshi and Cleveland 1989; Lee et al. 1990). Our result demonstrating longer average length of TUJ1-IR neurites of AHPCs cultured with IL-6 than
without IL-6, with no effect on total TUJ-IR neurite length, suggests that IL-6 presumably facilitated axonal specification. However it remains to be elucidated how IL-6 influences the selection of neurites to be remodeled in conjunction with microtubule reorganization (Cao et al. 2006; Hakkoum et al. 2007; Zhang et al. 2007).

AHPCs differentiating along a neuronal path exhibited two kinds of macroscopic conductances - sustained outward currents (sensitive to TEA, which blocks delayed rectifying potassium channels) and transient inward currents. Our electrophysiological data demonstrate that the presumed Na\(^+\) current density of AHPCs cultured in the presence of IL-6 was 2-fold greater than that in the absence of IL-6, whereas there was no significant difference in K\(^+\) current density between the two conditions; co-culture with astrocytes at 6-7 DIV, in contrast, significantly amplified K\(^+\) current density 2.5-fold and appeared to increase Na\(^+\) current density 1.7-fold although this difference was not statistically significant. This result suggests that IL-6 application may selectively enhance expression of voltage-gated Na\(^+\) currents and that other astrocyte products may enhance the K\(^+\) currents.

During rapid current clamp recording, depolarizing current steps failed to trigger action potentials, although slow regenerative events (maximal rate of depolarizing phase, < 12 V/s) with modest amplitude were elicited. The maximal slope of the upswing is set by the Na\(^+\) current density, which was about 10-fold lower in AHPCs than in mature granule cells of the dentate gyrus (van Praag et al. 2002), which are thought to arise from AHPCs in vivo. Steady-state inactivation of the Na\(^+\) current is also sufficiently depolarized to limit availability of Na\(^+\) current from the resting potential in some cells including glia (Barres et al. 1989). Detailed biophysical and molecular characterization of Na\(^+\) channels induced by IL-6 awaits further study.
AHPCs are multipotent NPCs in the hippocampus thought to give rise to mainly granule cells and possibly glial cells in the dentate gyrus (Gage et al. 1998; Namba et al. 2005). In rodent hippocampi, NPCs expressing PSA-NCAM and GFP driven by a Nestin promoter undergo biphasic changes in input resistance upon commitment and maturation along the neuronal lineage; neural progenitors in the subgranular zone have a low Rin (< 100 MΩ), restricted progenitors and immature granule cells a high Rin (~ 4 GΩ) and mature granule cells a 20-fold lower Rin of ~ 200 MΩ (Overstreet-Wadiche and Westbrook 2006; Schmidt-Hieber et al. 2004). AHPCs cultured under proliferation or differentiation conditions, without or with IL-6, had Rin values > 2 GΩ. By this criterion AHPCs are consistent either with cells that have committed to a neuronal lineage or with lineage-restricted progenitors.

AHPCs that have differentiated in response to IL-6 may also be at an ‘intermediate’ stage of neuronal and glial differentiation (as bipotent ‘neuro-glial’ precursors). Past studies have reported that O2A glial precursors (Barres et al. 1990), type-1 and type-2 astrocytes (Barres et al. 1989) and immature hippocampal astrocytes (Sontheimer et al. 1991) express sodium currents that are comparable to those in neurons. However, based on our immunocytochemical results and the striking morphological differences between neuronal and glial cells, it is unlikely that recordings were made from AHPCs differentiating into astrocytes because only a few cells express GFAP in our culture system (< 2%) and their morphology is clearly distinguishable from the other cell types. In addition, it is not likely that the AHPCs examined in this study were oligodendrocytes because the capacitance that is normally measured in oligodendrocytes is much greater than 10 pF (Barres et al. 1990).
Conclusion

Soluble factors secreted from rat hippocampal astrocytes promoted neuronal differentiation of multipotent AHPCs. Under non-contact co-culture conditions, the percentage of AHPCs expressing a neuronal marker, TUJ1, was dramatically increased compared to that when cultured alone without astrocytes. In addition to the neuronal morphology, AHPCs under non-contact co-culture conditions developed membrane properties that are consistent with those of differentiated neurons. When the AHPCs were cultured in the presence of exogenous IL-6, a candidate for a neurogenic astrocyte-derived soluble factor, the percentage of TUJ1-IR AHPCs was significantly greater than that in the absence of IL-6. Moreover, AHPCs cultured with IL-6 developed a subset of neuronal-like membrane properties at an early time point. These results suggest that IL-6 is an astrocyte-derived cytokine that can induce neuronal differentiation of the AHPCs.

Acknowledgments

Financial support provided by the NIH (NIGMS RO1 GM072005-01), the Stem Cell Research Fund, and the Department of Genetics, Development and Cell Biology at Iowa State University are gratefully acknowledged. Authors would like to thank Dr. Fred H. Gage at the Salk Institute for the gift of AHPCs and S.K. Mallapragada, M.L. Kohut, Y.J. Sim, C. Ciraci, R. Steele, A. Tentinger, R. Doyle and C.J. Jeon for technical assistance and advice.
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Table 1. Passive membrane properties of AHPCs cultured alone or under non-contact co-culture conditions

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>N (cells)</th>
<th>Apparent RMP (mV)</th>
<th>Cm (pF)</th>
<th>Outward current density # (pA/pF)</th>
<th>Inward current density (pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHPCs alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation (3-4 DIV)</td>
<td>14</td>
<td>-71.1 ± 4.5</td>
<td>12.6 ± 1.3</td>
<td>42.5 ± 7.9 ^a,b</td>
<td>-3.7 ± 1.0 ^d,e</td>
</tr>
<tr>
<td>Differentiation (6-7 DIV)</td>
<td>22</td>
<td>-79.6 ± 5.0</td>
<td>10.9 ± 0.7</td>
<td>47.6 ± 6.0 ^c</td>
<td>-6.6 ± 1.5</td>
</tr>
<tr>
<td>AHPCs in NCCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation (6-7 DIV)</td>
<td>19</td>
<td>9.9 ± 0.8</td>
<td></td>
<td>120.9 ± 18.1 ^a,c</td>
<td>-10.9 ± 2.7 ^d</td>
</tr>
<tr>
<td>Differentiation (9-10 DIV)</td>
<td>11</td>
<td>-85.8 ± 5.1</td>
<td>9.9 ± 1.0</td>
<td>92.2 ± 26.0 ^b</td>
<td>-17.0 ± 9.0 ^e</td>
</tr>
</tbody>
</table>

DIV, days in vitro; RMP, resting membrane potential; Cm, cell capacitance; mV, millivolt; pF, picofarad; pA, picoampere

#, Current densities were calculated by normalizing the peak currents to the membrane capacitance.

*, Maximum outward currents were measured at +60 mV-step depolarization for 100 milliseconds in voltage-clamp mode (holding potential, V_H = -100mV).

^a, ^b, ^c, ^d, ^e and ^f, Statistically significant difference (p < 0.05, t test)
Table 2. Passive membrane properties of AHPCs cultured in the absence or presence of IL-6 (1 ng/ml)

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>N (cells)</th>
<th>Apparent RMP (mV)</th>
<th>Cm (pF)</th>
<th>Outward current density (pA/pF)</th>
<th>Inward current density (pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation (6-7 DIV)</td>
<td>− IL-6</td>
<td>22</td>
<td>-79.6 ± 5.0</td>
<td>10.9 ± 0.7</td>
<td>47.6 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>+ IL-6</td>
<td>21</td>
<td>-82.1 ± 4.3</td>
<td>12.9 ± 1.8</td>
<td>47.8 ± 6.0</td>
</tr>
</tbody>
</table>

\(^a\), Statistically significant difference (\(p < 0.05\), \(t\) test)
Figure Legends

**Figure 1.** Differentiation of AHPCs under non-contact co-culture conditions (NCCC).

AHPCs were cultured under four different culture conditions: (1) AHPCs cultured alone without astrocytes (AHPCs alone), (2) non-contact co-culture with astrocytes isolated from cerebral hemispheres of neonatal rat brain (NCCC with Brain-Astro), (3) non-contact co-culture with cortical astrocytes (NCCC with CTX-Astro) and (4) non-contact co-culture with hippocampal astrocytes (NCCC with HC-Astro). Differentiation of AHPCs was characterized using cell-type specific antibodies: TUJ1 for young neurons, RIP for oligodendrocytes and GFAP for astrocytes. Values represent the average percentage of AHPCs immunolabeled by each antibody. N (number of independent experiments) = 6 for AHPCs alone and N = 3 for each NCCC. Error bars represent standard error of the mean (SEM). Asterisks represent significant difference from the control culture of AHPCs alone ($t$ test; *, $p < 0.05$; **, $p < 0.0001$). TUJ1 immunoreactivity of AHPCs under all NCCC conditions was significantly greater compared to AHPCs cultured alone. The average percentage of TUJ1-IR AHPCs co-cultured with hippocampal astrocytes was significantly greater than that of the AHPCs co-cultured with cortical astrocytes. The RIP-IR AHPCs were significantly increased only when the AHPCs were co-cultured with Brain-Astro, compared to the AHPCs cultured alone or co-cultured with either CTX-Astro or HC-Astro. The percentage of GFAP-IR AHPCs did not vary significantly under these conditions.

**Figure 2.** Comparison of electrophysiological properties of AHPCs cultured alone or co-cultured with neonatal astrocytes. AHPCs with neuronal-like morphologies were selected for
whole cell recording in voltage-clamp mode. (A) AHPCs cultured in the absence of astrocytes under differentiation condition. (A1) A superimposed image of TUJ1-IR shown in red, GFP in green and DAPI-stained nuclei in blue. (A2) A representative whole cell recording trace showing sustained outward currents and small transient inward currents elicited by a family of depolarizing voltage steps (inset, magnified inward currents). (B) AHPCs co-cultured with astrocytes. (B1) A superimposed image of TUJ1-IR AHPCs. (B2) A representative trace displaying sustained outward currents and substantial transient inward currents. Scale bars in (A1) and (B1) represent 20 µm.

Figure 3. Interleukin (IL)-6 enhances expression of neuronal marker protein in AHPCs. (A) Average percentages (± SEM) of cell-type specific antibody labeling of AHPCs under three different NCCCs in the presence of goat anti-IL-6 neutralizing antibody (Anti-IL-6) or non-specific IgG produced from goat (goat IgG) as a control. Goat IgG was added at an equal concentration of Anti-IL-6 added (10 ng/ml). With addition of Anti-IL-6 to the hippocampal NCCC, TUJ1-IR AHPCs were significantly decreased (54.4% in control and 51.5% with goat IgG vs. 27.7% with Anti-IL-6). The blocking antibody had no effect on TUJ1 immunoreactivity in cortical NCCC (34.2% in control and 34.4% with goat IgG vs. 35.2% with Anti-IL-6). N (number of independent experimental sessions) = 3 for each co-culture session. Asterisks represent statistical difference at p < 0.05. NS, no significant difference.

(B) Recombinant rat IL-6 (20 ng/ml) specifically induced neuronal differentiation of AHPCs (increased percentage of TUJ1 immunoreactivity). N = 6. The double asterisks represent a significant difference from the control culture (AHPCs alone) at p < 0.0001. The percentage of TUJ-IR AHPCs was significantly greater when cultured in the presence of purified IL-6,
compared to that in the absence of IL-6 (38.5% with IL-6 vs. 13.1% without IL-6). Addition of Anti-IL-6 blocking antibody dramatically reduced the percentage of TUJ1-IR AHPCs when cultured with IL-6. (C) IL-6 enhanced primary neurite growth. AHPCs were cultured under differentiation condition for 6 days in the presence of various concentrations of purified IL-6 from 0 to 100 ng/ml (see ‘IL-6 treatment’ in ‘Methods and Materials’ section). The average length of primary neurites emanating from a TUJ1-IR AHPC was measured. From each condition of an experimental set, 17 to 23 TUJ1-IR AHPCs were analyzed. N = 3 independent experiments. Values are mean ± SEM. Asterisks indicate statistical difference (t test; *, p < 0.05; **, p < 0.0001).

**Figure 4.** IL-6 promotes the development of sodium currents in AHPCs undergoing differentiation. AHPCs were cultured alone without astrocytes under differentiation condition in the absence (A) or presence (B) of IL-6. AHPCs cultured in the presence of IL-6 developed membrane properties consistent with those of functional neurons. The AHPCs possessing neuronal morphologies were analyzed using whole cell patch clamping. (A) AHPCs cultured without IL-6. (A1) A superimposed image of TUJ1-IR AHPCs shown in red, GFP in green and DAPI-stained nuclei in blue. (A2) A representative family of traces showing TEA-sensitive sustained outward currents and very small transient inward currents elicited by depolarizing voltage steps (inset, magnified inward currents). (B) AHPCs cultured with IL-6. (B1) A superimposed image of TUJ1-IR AHPCs. (B2) A representative traces showing TEA-sensitive outward currents and larger TTX-sensitive, transient inward currents. (C) IV-plot showing the relationship between stimulating voltage and current density. (Upper) Recording showing TEA-sensitive outward currents (N = 5). (Lower)
Recording showing TTX-sensitive inward currents (N = 3). Scale bars in (A1) and (B1) represent 20 µm.
Figure 1. Differentiation of AHPCs under non-contact co-culture conditions (NCCC).
Figure 2. Comparison of electrophysiological properties of AHPCs cultured alone or co-cultured with neonatal astrocytes.
Figure 3. Interleukin (IL)-6 enhances expression of neuronal marker protein in AHPCs.
Figure 4. IL-6 promotes the development of sodium currents in AHPCs undergoing differentiation.
Supporting information

Supplementary Materials and Methods

Cell culture

All animal procedures were conducted in accordance with and had the approval of the Iowa State University Committee on Animal Care. Astrocytes were isolated from cerebral cortices and hippocampi of postnatal day two Sprague-Dawley rat brains as described previously (Parpura et al. 1994; Parpura et al. 1993; Recknor et al. 2004). Briefly, rats were deeply anesthetized with halothane. Cerebral hemispheres were dissected and hippocampal regions were separated from the cerebral cortex. Cortical and hippocampal tissues were placed in separate conical tubes and enzymatically-treated using papain (20 IU/ml; Sigma-Aldrich, Saint Louis, MO) in Earle’s Balanced Salt Solution (EBSS; Gibco BRL, Gaithersburg, MD) for 1 hour in a culture incubator (37°C, 5% CO2 / 95% humidified air atmosphere). The digested tissues were rinsed in EBSS and then mechanically dissociated in astrocyte maintenance medium (see below) using a 5-ml serological pipet. Dissociated cells were grown to confluence in 25 cm² tissue culture flasks (T-25; Falcon) in a culture incubator. Astrocytes were maintained in modified minimal essential medium (modified MEM) composed of MEM (Gibco), 40 mM glucose (Sigma), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich), 14 mM sodium bicarbonate (Fisher Scientific), penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively; Sigma-Aldrich), and 10% v/v, pH 7.35 (Parpura et al. 1994; Parpura et al. 1993). To ensure purification of an enriched population of type-1 astrocytes, cultures were screened with an anti-glial fibrillary acidic protein (GFAP) antibody (see ‘Immunocytochemistry’ section below). Astrocyte
populations used in this study were greater than 95% immunoreactive for GFAP and no immunostainings for oligodendrocytic (RIP immunoreactivity) or neuronal (TUJ1 immunoreactivity) cell-types were observed. The astrocytes were passaged up to 5 times.

Adult hippocampal progenitor cells (AHPCs; a gift from F. Gage, Salk Institute, La Jolla, CA), originally isolated from the brains of adult Fischer 344 rats and infected with retrovirus to express enhanced green fluorescent protein (GFP) (Gage et al. 1995; Ray et al. 1995) were maintained as described previously (Oh et al. 2009; Recknor et al. 2006). Briefly, AHPCs were maintained in 75 cm² tissue culture flasks (T-75; Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 µg/ml; Sigma-Aldrich) and purified mouse laminin I (5 µg/ml; R&D Systems, Inc., Minneapolis, MN). The maintenance medium for AHPCs included Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1; Omega Scientific, Tarzana, CA), 2.5 mM L-glutamine, 1x N2 supplement (containing 100 mg/L human transferrin, 5 mg/L recombinant insulin, 6.3 µg/L progesterone, 16.11 mg/L putrescine and 5.2 µg/L selenite; Gibco BRL), and 20 ng/ml basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI). For in vitro analysis, the AHPCs were detached from the T-75 flask using 0.05% Trypsin-EDTA (Gibco BRL), harvested by centrifugation at 1000g for 5 minutes, and resuspended in differentiation medium (i.e. maintenance medium without bFGF). AHPCs (95 cells/mm²) were plated on polystyrene substrates coated first with poly-L-lysine (100 µg/ml) and then purified laminin I (10 µg/ml) and maintained in appropriate culture media. Culture media were replenished every other day by removing one-half the volume of media and adding an equal volume of fresh media.
As a cellular control, fibroblasts were isolated from 0.5 cm² of skin biopsies from postnatal day 2 rats (including epidermal and dermal layers), which were first rinsed in EBSS then minced with forceps. The resulting skin tissue fragments were digested in 0.1% trypsin (from bovine pancreas; Sigma-Aldrich) in EBSS. After 15 min incubation at 37°C, tissues were rinsed in EBSS and mechanically dissociated by triturating in modified MEM with 10% FBS. Cells derived from a piece of skin were grown in a monolayer in a T-25 flask. Cells were fed every 4 days by refreshing 70% v/v of media and maintained in the culture incubator. Before being used in experiments, cells were passaged up to 2 times.

Astrocyte-AHPC co-culture

Transwell® inserts (Corning, Inc., Corning, NY) were used to establish co-cultures of astrocytes and AHPCs growing together in the absence of physical contact (referred to as non-contact co-culture, NCCC) as described previously (Oh et al. 2009). Briefly, astrocytes were plated on the semi-porous polyester membrane inserts inside the wells of 6-well plates (Corning, Inc.) at an initial cell density of 95 cells/mm² and cultured in astrocyte maintenance medium [modified minimal essential medium (MMEM) including 10% fetal bovine serum (FBS; Atlanta Biologicals, Inc., Lawrenceville, GA)]. After 2 days, the MMEM was replaced with co-culture media, a 1:1 mixture of MMEM without FBS and AHPC differentiation media, following rinses with EBSS. The AHPCs at the same cell density as the astrocytes were plated on polystyrene substrates coated first with poly-L-lysine (100 µg/ml; Sigma-Aldrich) and then purified laminin I (10 µg/ml; R&D Systems, Inc., Minneapolis, MN), and allowed to attach onto the substrates for 1 h. The AHPCs seeded on the substrates were then placed at the bottom of each well, and the inserts with astrocytes
were placed inside the well, above the AHPCs. Under these conditions, AHPCs are separated from direct contact with astrocytes but soluble factors released from astrocytes or AHPCs can diffuse through the 0.4 μm pores in the membrane insert. After 6 days, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for immunocytochemistry.

**Immunocytochemistry**

AHPCs cultured on laminin-coated substrates were processed for immunocytochemistry according to standard protocols described previously (Oh et al. 2009; Recknor et al. 2006). Fixed AHPCs were labeled with antibodies against phenotypic markers, class III β-tubulin (TUJ1, mouse monoclonal IgG; R&D Systems) diluted at 1:750 to a final concentration of 1.3 μg/ml, receptor interacting protein [RIP in concentrated form supplied by vendor, mouse monoclonal IgG; developed by Susan Hockfield; obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Iowa City, IA)] diluted at 1:1,500 and glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; Lab Vision Corp., Fremont, CA) diluted at 1:2,000 to a final concentration of 0.5 μg/ml. Biotinylated donkey anti-mouse secondary antibody and Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) were used at final concentrations of 4 μg/ml and 120 ng/ml, respectively, for visualizing the primary antibody labeling. Nuclei were counterstained with 1 μM 4’ ,6-diamidino-2-phenylindole, dilactate (DAPI). Preparations were then mounted onto microscope slides using GelMount antifade mounting medium (Biomedia Corp., Foster City, CA).

The preparations were examined and photographed using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled
by QCapture software (QImaging, Surrey, BC, Canada). For image analysis, six to eight microscope fields from each substrate were taken randomly using a 20X objective. Each field represents 0.17 mm$^2$ ($476 \mu$m x 357 $\mu$m). To calculate the percentage of immunoreactive AHPCs on each substrate, the number of cells immunoreactive for each antibody was divided by the total number of cells (DAPI-stained nuclei).

*Neurite outgrowth assay*

AHPCs were cultured in the absence or presence of purified IL-6 at different concentrations (from 0.00001 to 100 ng/ml; Figure 3 C) for 6 days. After fixation, cultured AHPCs were immunolabeled for TUJ1 and imaged using a 40X objective. From the images, 17 to 22 TUJ1-immunoreactive AHPCs per condition were analyzed for the length of neurites emanating from the cell body using ImageJ, image processing and analysis program (developed at the National Institutes of Health, [http://rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/)). Cells we classified as TUJ1-immunoreactive possessed some neurites that were and others that were not immunoreactive for TUJ1; only TUJ1-immunoreactive neurites were examined. TUJ1 is found in neurites committed to become axons in young neurons (Joshi and Cleveland 1989; Lee et al. 1990). To calculate the average neurite length per cell, total length of TUJ1-immunoreactive neurites of each cell, measured from the edge of the cell body to the tip of the TUJ1-immunoreactive neurite, was divided by the number of the TUJ1-immunoreactive neurites. Secondary neurites branching from the primary neurites were not included in this analysis. Neurites that appeared to originate from neighboring cells, although touching the target cell, were also excluded from the measurement. Three independent experiments were analyzed for each condition.
Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Nucleospin® RNA II Total RNA Isolation kit (Macherey-Nagel, Düren, Germany), treated with RNase-free rDNase (Macherey-Nagel) for 15 min at 23˚C then reverse transcribed by Superscript III™ reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting samples were prepared for qPCR in a 25 µl volume containing 25 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.1 mM dNTP, 12.5U/ml iTaq DNA polymerase, 1.5 mM MgCl₂, iQ™ SYBR® Green I, 5 nM fluorescein, stabilizers (Cat # 170-8880, Bio-Rad Laboratories, Inc., Hercules, CA), the appropriate primers at 0.5 µM each, and cDNA template as defined by the experiment. The qPCR was performed using an Opticon thermocycler (MJ Research, Inc., Waltham, MA, model CFD-0200) using a 95˚C for 5 min premelt followed by 39 cycles of 95˚C 10 sec, 60˚C 30 sec and including a final determination of melting curves from 65˚C to 90˚C. The primer sequences for the genes examined were as follows: IL-6, AATCTGCTCTGGTCTTCTGGAGT, TTG- CCGAGTAGACCTCATAAG TGA; IL-6Rα, CGAGGGAAGGAGGAGTTTGA, GATGATGAAAGGAGGAGGAGGAGTTTGA, GATGATGAAGACACAGAGAGCAAT; sIL-6Rα, TCCCTCTCCAACCACGAA, ACACCTTGTCCACCCTCCA; gp130, ATTAAGGGAATGGGAAAGGG, TACATAGCGGTATTGCTGGAG; Cyclophilin A, TGGCTTCTCGACATCACGGCTGAT, GGACTTGCCACCAGCCATT A. The amount of each mRNA was quantified by using a standard curve evaluated in the same qPCR run using a series of known dilutions of amplified fragments of the target gene. Values that were above or below the range of the standards were not included for the analysis and the affected samples were reanalyzed at dilutions to ensure that the values were within the range of the standard curve.
**Enzyme-linked immunosorbent assay (ELISA)**

AHPCs were cultured alone or co-cultured with either astrocytes or skin-derived fibroblasts without physical contact. All cultures were maintained in co-culture media (see ‘Astrocyte-AHPC co-culture’ section above). At 2 and 4 days after culture establishment, co-culture media in which all cultures were maintained (referred to as conditioned co-culture media) were collected for ELISA. The concentrations of IL-6 protein secreted into the conditioned co-culture media were determined using an ELISA kit (Quantikine Rat IL-6 Immunoassay; R&D Systems) according to the manufacturer’s protocol. Each condition was analyzed in duplicate. The concentration of secreted IL-6 was calculated by extrapolation from the standard curves generated using rat recombinant IL-6 contained in the assay kit. Three independent experiments were performed for the cultures of AHPCs alone and co-cultures of AHPCs and astrocytes. Two independent experiments were analyzed for co-cultures of AHPCs and fibroblasts.

**Supplementary Results and Discussion**

**Versatile roles of IL-6 in the central nervous system**

IL-6 is recognized by the high-affinity receptor (IL-6R) which is expressed on target cells(Scheller and Rose-John 2006; Van Snick 1990). There are two forms of the IL-6R, one transmembrane and the other (sIL-6R) being soluble. The latter is believed to be more important for neural signaling(Marz et al. 1999b). Both bind to IL-6 and the IL-6/IL-6R complex interact with gp130, which is a general receptor for the IL-6 family cytokines(Jones
et al. 2005; Taga and Kishimoto 1997). Gp130, a 130 kD non-ligand-binding membrane glycoprotein, does not bind directly to IL-6(Hibi et al. 1990; Taga et al. 1989). The association of IL-6/IL-6R complex with gp130 causes auto-phosphorylation of the gp130 dimer followed by activation of the Janus kinases (JAKs)(Jones et al. 2005). The activation of JAK/STAT, Ras/ERK or other signal transduction pathways results in the modulation of various genes that control neural development(Gadient and Otten 1994; Gruol and Nelson 1997; Heinrich et al. 2003; Marz et al. 1997; Taga and Fukuda 2005).

Through the JAK/STAT pathway, IL-6 has been implicated in induction of glial differentiation from NPCs in the mammalian brain(Bonni et al. 1997; Islam et al. 2008; Taga and Fukuda 2005). Recent studies examining inflammatory responses mediated in part by cytokines, including IL-6, on hippocampal neurogenesis showed that IL-6 production from activated microglia interferes with the generation of new neurons in the hippocampus of the adult brain(Ekdahl et al. 2003; Monje et al. 2002; Monje et al. 2003). Thus far, the role of IL-6 in the neurogenic niche has been suggested to be inhibition of neurogenesis via a decrease in NPC proliferation or an increase in glial differentiation, both depleting the pool of NPCs. However, a number of studies have shown that IL-6, in conjunction with IL-6R, can also exert neurotrophic effects(Gadient and Otten 1997; Marz et al. 1999a; Schafer et al. 1999; Thier et al. 1999). By stimulating region-specific astrocytes to provide local supplements of neurotrophins (NTs), IL-6 facilitates neuronal survival(Marz et al. 1999a). In addition, IL-6 with IL-6R (especially the soluble form) induces neuronal-like differentiation of PC12 cells(Marz et al. 1997) and contributes to the regeneration of axotomized mature sensory or hippocampal neurons(Cao et al. 2006; Hakkoum et al. 2007; Hirota et al. 1996).
Therefore the effects of IL-6 on NPC differentiation or survival appear to be context-dependent.

*IL-6 is produced from the co-cultured astrocytes*

To quantify the amount of IL-6 protein secreted into the culture media, we performed enzyme-linked immunosorbent assays (ELISA). Five different culture conditions were established: (1) AHPCs cultured alone, (2) cortical astrocytes cultured alone, (3) co-culture of AHPCs and cortical astrocytes, (4) hippocampal astrocytes cultured alone, and (5) co-culture of AHPCs and hippocampal astrocytes. In addition, as a cell control, skin-derived fibroblasts were cultured alone as well as co-cultured with AHPCs in parallel with the other cultures. All seven culture conditions were maintained for 4 days. Conditioned co-culture media from the seven cultures were collected at 2 and 4 DIV. The conditioned medium from each culture condition was examined for the amount of IL-6 secreted from the cultured cells.

Supplementary Figure 3 A demonstrates that, when cultured alone, the AHPCs secreted relatively low levels of IL-6 (average of 2.6 pg/ml) while both cortical and hippocampal astrocytes produce approximately 88 pg/ml and 70 pg/ml, respectively. In addition, a comparable level of IL-6 was detected from both co-cultures of AHPC-cortical astrocytes and AHPC-hippocampal astrocytes (Supplementary Figure 3 A). Statistical analysis showed that there were no significant differences in the amounts of IL-6 in the medium of cortical or hippocampal astrocytes cultured alone or in co-cultures with AHPCs. These results demonstrate that (a) the major source of IL-6 production in the non-contact co-culture system is the astrocytes, rather than AHPCs, and (b) the IL-6 production capability of astrocytes appears to be independent of the AHPCs co-cultured with them.
Recently, it was reported that rat hippocampal and cortical astrocytes displayed distinct gene expression profiles (Barkho et al. 2006). Moreover, IL-6 mRNA was shown to be highly expressed in hippocampal astrocytes and exogenous application promoted neuronal differentiation of NPCs (Barkho et al. 2006). Our results are consistent with these and our ELISA results further demonstrated that both hippocampal and cortical astrocytes produce over 2.5-times more IL-6 than the AHPCs. Moreover, the amount of IL-6 present in the media from co-cultures (AHPC-cortical astrocyte or AHPC-hippocampal astrocyte) was comparable to that in medium from the astrocytes cultured alone. There was no significant difference in terms of the secreted IL-6 level between the astrocyte alone cultures and the co-cultures of AHPCs with hippocampal or cortical astrocytes. These results suggest that (a) the astrocytes used for this study are capable of expressing and releasing IL-6 and (b) the extracellular IL-6 protein is not likely to be regulated by the co-cultured AHPCs. However, the level of IL-6 mRNA was considerably increased in hippocampal astrocytes that were co-cultured with AHPCs. It is possible that the hippocampal astrocytes produce more IL-6 when co-cultured with AHPCs, but as part of their response to the IL-6, the AHPCs bind and take it up by way of the IL-6R and gp130 receptor complex leaving approximately the same amount of IL-6 in the medium as seen for astrocytes cultured alone.

To examine the expression levels of IL-6, its specific receptor, IL-6Rα (transmembrane and soluble) and gp130 in AHPCs and astrocytes, all seven different culture conditions [see above, culture number (1) through (5) and fibroblast cultures (alone and in co-culture)] were established. The cultures were maintained in co-culture media for 5 days. The IL-6 and IL-6Rα mRNA levels in AHPCs and astrocytes or fibroblasts were analyzed by RT-qPCR. Under all conditions, AHPCs expressed IL-6 mRNA at a much lower level than
did astrocytes; cortical and hippocampal astrocytes expressed 260- and 280-fold more, respectively, than did AHPCs (Supplementary Figure 3 B). This result is consistent with the observed IL-6 protein levels in the medium of these cell types. By comparison with IL-6 mRNA levels, IL-6Rα and gp130 mRNA expression levels did not vary greatly between cell types (Supplementary Figure 3 C).

When in co-culture with AHPCs, the level of IL-6 mRNA was higher in cortical and hippocampal astrocytes (approximately 4- and 7-fold, respectively) than when they were cultured alone. Higher IL-6 mRNA levels were not observed in fibroblasts or AHPCs when co-cultured with astrocytes compared with when they were cultured alone. Thus, it appears that - of these cell types - astrocytes are uniquely responsive to secretions of AHPCs to increase the levels of IL-6 gene expression. This result demonstrates that astrocytes synthesize and secrete IL-6 and that both astrocytes and AHPCs express the genes encoding the receptors necessary for responding to IL-6.

Acknowledgments

In addition to the funding sources, authors are deeply grateful to Dr. Surya K. Mallapragada (Department of Chemical and Biological Engineering, Iowa State University) for the supply of polystyrene substrates and Dr. Robert Doyle (Roy J. Carver Center for Ultrahigh Resolution Biological Microscopy; Department of Genetics, Development and Cell Biology, Iowa State University) for his advice and suggestions. Authors would like to thank Dr. Marian L. Kohut and Dr. Young-Je Sim (Department of Kinesiology, Iowa State University) for their technical support in ELISA analysis, Ceren Ciraci (Department of
Biochemistry, Biophysics and Molecular Biology, Iowa State University) for designing primers for IL-6 and IL-6R and for optimizing RT-qPCR conditions, and Amy Tentinger (Department of Statistics, Iowa State University) for helpful discussion in statistical analyses. Authors also thank Ryan Steele (NSF-REU summer intern, 2008) for assistance in the neurite length analysis and Dr. Chang-Jin Jeon (Department of Biology at Kyungpook National University, Daegu, South Korea) for helpful discussions.

References


Supplementary Table 1. Average number of TUJ1-IR processes emanating from TUJ-IR AHPCs cultured with various concentrations of exogenous IL-6

<table>
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<th>Conc of IL-6 (ng/ml)</th>
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<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Average</td>
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<td>4.08</td>
<td>4.11</td>
<td>4.26</td>
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Supplementary Figure Legends

**Supplementary Figure 1.** Dose-dependent effect of IL-6 on AHPC neuronal differentiation. AHPCs were cultured under differentiation conditions for 6 days in the presence of different concentrations of purified IL-6 (0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml). The percentages of TUJ1-IR or RIP-IR AHPCs were quantitatively analyzed and averaged over the total number of cells. N = 3 independent experimental culturing sessions. Error bars represent standard error of the mean (SEM). Asterisks represent a significant difference from the controls at 0 ng/ml (p < 0.05, t test). The percentages of TUJ1-IR AHPCs cultured in the presence of concentrations of IL-6 greater than 0.1 ng/ml were significantly increased, compared to the control cultures (0 ng/ml of IL-6). No significant differences in RIP immunoreactivity were observed at any IL-6 concentrations.

**Supplementary Figure 2.** Total length of processes per a TUJ1-IR AHPC. AHPCs were cultured in the presence of various concentrations of IL-6 from 0 to 100 ng/ml (0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml conditions). For each TUJ1-IR cells, the length of every process was measured and total length of all primary processes from the cell was calculated. In each condition, 17 to 23 TUJ1-IR AHPCs were analyzed. The total length of all processes was averaged and three independent experimental sets were analyzed (N = 3). Values are mean ± SEM. There was no significant difference among the conditions.

**Supplementary Figure 3.** IL-6 proteins are secreted from the astrocytes. AHPCs were cultured alone or co-cultured with either cortical or hippocampal astrocytes without physical
contact. (A) The amount of secreted IL-6 proteins into the culture medium was assayed using an ELISA assay. Seven different culture conditions were established in parallel: (1) AHPCs cultured alone, (2) cortical astrocytes cultured alone, (3) co-culture of AHPCs and cortical astrocytes, (4) hippocampal astrocytes cultured alone, (5) co-culture of AHPCs and hippocampal astrocytes, (6) skin-derived fibroblasts cultured alone, and (7) co-culture of AHPCs and skin-derived fibroblasts. Conditioned culture media from every culture condition were collected. When AHPCs were cultured alone, approximately 2.6 pg/ml of IL-6 was measured from the conditioned culture medium. Cortical and hippocampal astrocytes produced approximately 88 pg/ml and 70 pg/ml, respectively. From both co-cultures of AHPC-cortical astrocyte and AHPC-hippocampal astrocyte, approximately 73 pg/ml and 72 pg/ml of IL-6 was detected, respectively. From the cultures of fibroblasts cultured alone and co-cultured with AHPC, less than 20 pg/ml of IL-6 was secreted (average amount: 15.5 pg/ml from fibroblasts alone and 9.1 pg/ml from AHPC-fibroblast co-culture). N = 3 for all culture conditions, except the conditions using skin-derived fibroblasts (N = 2). Values are mean ± SEM. Asterisks indicate statistical difference (t test; p < 0.05). (B, C) The mRNA expression levels for IL-6, IL-6Rα and gp130 were normalized to the cyclophilin A mRNA level for each sample. The normalized values were then calibrated within each experiment to the value for cortical astrocytes alone to obtain relative mRNA expression levels. Average relative expression levels (Y-axis) are shown for two experiments in which two and four biological replicates were analyzed with two technical replicates per sample. (B) IL-6 mRNA, (C) IL-6Rα and gp130 mRNA.
**Supplementary Figure 1.** Dose-dependent effect of IL-6 on AHPC neuronal differentiation.
Supplementary Figure 2. Total length of processes per a TUJ1-IR AHPC.
Supplementary Figure 3. IL-6 proteins are secreted from the astrocytes.
CHAPTER 4.

EFFECTS OF CALCIUM INFLUX ON INTERLEUKIN-6-MEDIATED NEURONAL DIFFERENTIATION OF ADULT NEURAL PROGENITOR CELLS

A manuscript in preparation

Jisun Oh\textsuperscript{1,2,3}, Michael A. McCloskey\textsuperscript{2}, Michael Z. Khan\textsuperscript{3}, Senyo S. Whyte\textsuperscript{4}, Pavel A. Brodskiy\textsuperscript{5} and Donald S. Sakaguchi\textsuperscript{1,2,3,4,5,\#}

\textsuperscript{1}Neuroscience Program, \textsuperscript{2}Department of Genetics, Development and Cell Biology, \textsuperscript{3}Department of Biomedical Sciences, \textsuperscript{4}College of Liberal Arts and Sciences, \textsuperscript{5}College of Agriculture and Life Sciences, \textsuperscript{5}Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

\# Corresponding author

Abstract

The purpose of this study was to investigate possible molecular mechanisms through which interleukin-6 (IL-6) induces neuronal differentiation of adult hippocampal neural progenitor cells (AHPCs). We previously reported that astroglial cells from neonatal rat brain produce soluble factors which enhance neuronal differentiation of AHPCs, and that IL-6 is an astrocyte-derived factor which specifically promotes neuronal differentiation of AHPCs\textsuperscript{1,2}. In the present study, we examined the influence of extracellular calcium and voltage-gated calcium channel antagonists on IL-6-induced neuronal differentiation of AHPCs. AHPCs were cultured in various concentrations of external calcium or with L-type voltage-gated calcium channel blockers. Our results demonstrate that extracellular calcium was critical for neuronal differentiation of IL-6-treated AHPCs, that a blockage of L-type Cav channels inhibited IL-6-enhanced neuronal differentiation of AHPCs, and that exogenous IL-6 treatment increased the percentage of AHPCs immunoreactive for an
activated from of CREB (phosphorylated CREB). These results suggest that IL-6 may induce AHPCs to commit to a neuronal lineage by promoting extracellular calcium influx which leads to CREB activation. Our findings may provide insight into understanding injury-induced neurogenesis and have implications for developing cell-based therapeutic strategies using adult neural progenitor cells.

Introduction

Depending on the interplay between neural progenitor cells (NPCs) and their microenvironment, NPCs can proliferate as well as give rise to neurons or glial cells\textsuperscript{3-5}. We previously reported that astroglial cells from newborn rat brain produce soluble factors which enhance neuronal differentiation of adult NPCs\textsuperscript{1}. In addition, using immunocytochemical and electrophysiological approaches, we found that interleukin-6 (IL-6) may serve as an astrocyte-derived factor and can enhance neuronal differentiation of adult NPCs \textit{in vitro}\textsuperscript{2}.

IL-6 is a cytokine that induces cell growth and differentiation in the hematopoietic and immune systems\textsuperscript{6,7}. IL-6 is also found in the cerebrospinal fluid and plays a role in mediating inflammatory responses in the injured brain\textsuperscript{8-10}. Astrocytes are a major source of IL-6 in the brain\textsuperscript{6,9,11}. In the normal brain, IL-6 is expressed at a basal level, but its production levels are elevated as the neural cells are infected or damaged\textsuperscript{9,12}. In addition, IL-6 can function as a neurogenesis-promoting factor to promote neuronal differentiation of adult NPCs \textit{in vitro}\textsuperscript{2,11}. However little is known about how IL-6 stimulates adult NPCs to commit to a neuronal lineage.
In NPCs of young or adult rodent brains, an increase in intracellular calcium concentration is important for membrane depolarization-dependent neuronal differentiation\textsuperscript{13-17}. L-type Cav channels and N-methyl-D-aspartic acid (NMDA) receptors have been reported to modulate NPC neuronal differentiation by sensing membrane-depolarizing stimuli\textsuperscript{14,15,18}. To investigate possible mechanisms of which IL-6 promotes neuronal differentiation of NPCs, we examined an involvement of voltage-gated calcium (Cav) channels in IL-6-mediated neuronal differentiation of adult NPCs. In this study, self-renewing, multipotent NPCs isolated from adult rat hippocampus (referred as to adult hippocampal progenitor cells, AHPCs) were cultured in the absence or presence of exogenous IL-6. To antagonize Cav channels, the dihydropyridines nifedipine or isradipine were used. Neuronal or glial differentiation of AHPCs and the activation of a transcription factor (cAMP response element binding protein, CREB) induced by external calcium influx were examined by immunocytochemical analyses.

\section*{Materials and Methods}

\textit{Neural progenitor cell culture}

Adult hippocampal progenitor cells (AHPCs from F. Gage, Salk Institute, La Jolla, CA), isolated from the brains of adult Fischer 344 rats and infected with retrovirus to express enhanced green fluorescent protein (GFP)\textsuperscript{19-21}, were maintained as described previously\textsuperscript{1,2}. Briefly, AHPCs were maintained in 75 cm\textsuperscript{2} tissue culture flasks (T-75; Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 µg/ml; Sigma-Aldrich) and purified mouse laminin I (5 µg/ml; R&D Systems, Inc., Minneapolis, MN). The maintenance medium for
AHPCs included Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1 mixture, which contains 1.045 mM CaCl$_2$; Omega Scientific, Tarzana, CA), 2.5 mM $L$-glutamine, 1x N2 supplement (containing 100 mg/L human transferrin, 5 mg/L recombinant insulin, 6.3 µg/L progesterone, 16.11 mg/L putrescine and 5.2 µg/L selenite; Gibco BRL), and 20 ng/ml basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI). To prepare DMEM/F-12 media that contained varying concentrations of calcium, calcium-free DMEM and F-12 nutrient mixture containing 0.299 mM of CaCl$_2$ (both obtained from Gibco BRL) were mixed at an 1:1 ratio. The final concentration of calcium in culture medium was adjusted with 1 M CaCl$_2$. Divalent cationic imbalance due to lowering calcium concentration in the culture medium was balanced by supplementing 1M MgCl$_2$.

For *in vitro* analyses, the AHPCs were detached from the T-75 flask using 0.05% Trypsin-EDTA (Gibco BRL), harvested by centrifugation at 1000 g for 5 minutes, and resuspended in differentiation medium (i.e. maintenance medium without bFGF). AHPCs (100 cells/mm$^2$) were plated on 12-mm diameter circular cover glass (Fisher Scientific) coated first with poly-L-ornithine (50 µg/ml) and then purified laminin I (10 µg/ml) and maintained in differentiation media. To induce neuronal differentiation, AHPCs were treated with purified rat recombinant IL-6 (1 ng/ml; R&D Systems)$^2$. To neutralize IL-6, anti-IL-6 function blocking antibody was used at 10 ng/ml. To antagonize L-type Cav cannels, two dihydropyridines, nifedipine and isradipine (dissolved in dimethyl sulfoxide, DMSO) were used at a concentration of 1 µM and 0.5 µM, respectively (see Supplementary Figure 1 for concentration-dependent effects on AHPC differentiation). Culture media were replenished
every other day by removing one-half the volume of media and adding an equal volume of fresh media including the drugs freshly made.

**Immunocytochemistry**

AHPCs cultured on laminin-coated substrates were processed for immunocytochemistry according to standard protocols described previously\(^1\). Fixed AHPCs were labeled with antibodies against phenotypic markers, class III \(\beta\)-tubulin (TuJ1, mouse monoclonal IgG; R&D Systems), receptor interacting protein (RIP in concentrated form supplied by vendor, mouse monoclonal IgG; Developmental Studies Hybridoma Bank), and glial fibrillary acidic protein (GFAP; mouse monoclonal IgG; Lab Vision Corp., Fremont, CA). Donkey anti-mouse secondary antibody, Cy3-conjugated (Jackson ImmunoResearch, West Grove, PA) was used for visualizing the primary antibody labeling. For immunocytochemical analysis of CREB and phosphorylated CREB (pCREB) in AHPCs, primary antibodies against CREB or pCREB (rabbit monoclonal IgG) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Donkey anti-rabbit secondary antibody, Cy5-conjugated (Jackson ImmunoResearch) was used for visualizing CREB or pCREB. Nuclei were counterstained with 1 \(\mu\)M 4’, 6-diamidino-2-phenylindole, dilactate (DAPI). Preparations were then mounted onto microscope slides using GelMount antifade mounting medium (Biomeda Corp., Foster City, CA).

The preparations were examined and photographed using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, BC, Canada). For image analysis, eight to ten microscope fields from each substrate were taken randomly using a 20X objective. Each
field represents 0.17 mm\(^2\) (476 µm x 357 µm). To calculate the percentage of immunoreactive AHPCs on each substrate, the number of cells immunoreactive for each antibody was divided by the total number of cells (DAPI-stained nuclei). The number of independent experiments performed was 6 for the culture of AHPCs alone and 3 for each non-contact co-culture.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM (ver. 3.0). All tests were two-tailed tests and p-values less than an alpha of 0.05 were considered significantly different.

Results

Extracellular calcium is required for IL-6-enhanced neuronal differentiation

To examine whether extracellular calcium is necessary for neuronal differentiation of AHPCs in the presence of IL-6, AHPCs were cultured in differentiation medium containing various concentrations of extracellular calcium ([Ca]\(_{\text{ext}}\)). Normal AHPC culture medium, DMEM/F-12, contains 1.045 mM of CaCl\(_2\). To lower the [Ca]\(_{\text{ext}}\), Ca-free DMEM and 0.299 mM [Ca]-containing F-12 nutrient mixture were mixed at an 1:1 ratio. [Ca]\(_{\text{ext}}\) in the mixed medium became 0.15 mM, which was the lowest [Ca]\(_{\text{ext}}\) examined in this experiment. By supplementing the mixed medium with 1 M of CaCl\(_2\), the [Ca]\(_{\text{ext}}\) was adjusted to be 0.25, 0.5, 1.045 (as a control), 2 and 5 mM. For in vitro analyses, AHPCs were cultured in the varying [Ca]\(_{\text{ext}}\)-containing media with or without application of IL-6 (rat recombinant IL-6,
1 ng/ml). After 6 days, cells were fixed and immunolabeled. AHPC neuronal differentiation was accessed by phenotypic marker expression, TuJ1 in an immunocytochemical analysis.

When cultured in lower concentrations of \([\text{Ca}^{2+}]_{\text{ext}} \leq 0.5 \text{ mM}\), exogenous IL-6 application failed to increase the proportion of AHPCs differentiating toward a neuronal fate, based on TuJ1 immunoreactivity (Figure 1 A). In contrast, when AHPCs were cultured in the medium which contained a normal \([\text{Ca}^{2+}]_{\text{ext}} 1.045 \text{ mM}\), IL-6 application increased the fraction of TuJ1-immunoreactive (IR) AHPCs. This result suggests that extracellular calcium is essential for IL-6-mediated neuronal differentiation of AHPCs. In addition, we observed that the average number of DAPI-stained AHPCs was significantly lower in 0.15 mM or 0.25 mM [Ca]ext-containing media, compared to the control cultures (containing a normal [Ca]ext) (Figure 1 B). From propidium idodide (PI) test, we additionally observed that considerably more AHPCs were stained for PI when cultured in 0.15 mM [Ca]ext-containing medium, compared to the other conditions which contained \(> 0.25 \text{ mM}\) of [Ca]ext (Supplementary Figure 1). In the presence of 5 mM of [Ca]ext, the highest [Ca]ext tested in this study, none of cells survived (data not shown). These results suggest that extracellular calcium is important for AHPC survival as well as cell adhesion onto the purified extracellular matrix, laminin.

**L-type Cav channel antagonists reduce IL-6-enhanced neuronal differentiation**

To examine whether IL-6 treatment induces extracellular Ca influx, AHPCs were cultured with IL-6 as well as nifedipine or isradipine, which are both L-type Cav channel antagonists. Based on the results of concentration-dependent tests (see Supplementary Figure 2), nifedipine and isradipine were used at 1 µM and 0.5 µM, respectively.
IL-6 treatment specifically increased the percentage of cells that were TuJ1-IR (Figure 2), which is consistent with our previous results. Moreover, the application of nifedipine or isradipine to the IL-6-treated cultures resulted in diminishing the fraction of TuJ1-IR cells, with no significant changes in the fraction of RIP-IR cells. These findings suggest that Ca influx through L-type Cav channels plays a critical role to promote neuronal differentiation (determined by TuJ1 expression) of AHPCs in the presence of IL-6.

**IL-6 treatment augments the fraction of CREB-IR and TuJ1-IR AHPCs**

An increase in intracellular calcium concentration ([Ca]i) by extracellular Ca entry results in rapid and acute expression of immediate early genes (such as c-fos and Jun family). These genes are known to encode transcription factors that control cellular responses to synaptic stimulation during neuritogenesis and synaptic formation of neuronal precursors. CREB is one of the transcription factors activated when [Ca]i is elevated. To investigate possible pathways through which IL-6 induces neuronal differentiation of AHPCs, we examined and quantified phosphorylated CREB (pCREB) in AHPCs after culturing with or without IL-6. CREB or pCREB in AHPCs was detected by monoclonal antibodies after termination of the culture session.

We observed that almost all AHPCs (approximately 95%) were CREB-IR regardless of IL-6 application and that in the presence of IL-6, the percentage of CREB- and TuJ1-IR AHPCs was about 3-fold greater than that in the absence of IL-6 after 3 days in culture (Figure 3 A). In addition, with IL-6 application, the fraction of pCREB-IR AHPCs was about 3-fold increased compared to the control culture condition (without IL-6). Moreover, when AHPCs were cultured with exogenous IL-6, the fraction of pCREB- and TuJ1-IR
AHPCs was about 9-fold increased compared to control culture. Interestingly, when AHPCs were treated with IL-6 as well as either nifedipine or IL-6 neutralizing antibody (anti-IL-6, IL-6 function blocking antibody), neither the fraction of only TuJ1-IR nor the fraction of pCREB- and TuJ1-IR AHPCs was increased. We also examined CREB activation in AHPCs expressing an oligodendrocytic marker, RIP, but found no significant differences in RIP- and/or pCREB-IR in the comparison of IL-6-treated cultures to the control (Supplementary Figure 3).

Furthermore, ratios of the percentages of CREB or pCREB-IR AHPCs versus the percentages of TuJ1-IR AHPCs in the absence or presence of IL-6 were calculated (Figure 3B). Results from this analysis are as follow: first, almost all AHPCs expressed CREB regardless of culture condition and all TuJ1-expressing AHPCs were expressing CREB. Second, there were about 2.4 times more TuJ1-positive AHPCs observed among all CREB-positive AHPCs in the IL-6-treated cultures compared to the control. Third, of TuJ1-positive AHPCs, about 3.3 times more pCREB-positive cells were observed in the IL-6-treated cultures compared to the non-IL-6-treated cultures. Fourth, of pCREB-positive AHPCs, there were about 2.2 times more TuJ1-positive cells found in the IL-6-treated cultured compared to the control cultures. Last but most important, although AHPCs were cultured with IL-6, when the cells were treated with nifedipine or anti-IL-6, the fractions of AHPCs immunoreactive only for TuJ1 as well as for both TuJ1 and pCREB were not increased.

These results suggest that (1) IL-6 treatment induces AHPCs to commit to a neuronal lineage, (2) L-type Cav channels are involved in IL-6-induced neuronal differentiation of AHPCs and (3) IL-6 application facilitates CREB activation in AHPCs.
Discussion

We previously demonstrated that IL-6 is an astrocyte-derived factor that promotes neuronal differentiation of AHPCs in vitro\(^2\), and in this study report a possible mechanism of IL-6-enhanced neuronal differentiation of the AHPCs. Our immunocytochemical results showed that extracellular calcium is essential for AHPC neuronal differentiation in the presence of IL-6, L-type Cav channel antagonists inhibit IL-6-mediated neuronal differentiation, and IL-6 treatment activates CREB in AHPCs.

*IL-6 and intracellular calcium rise*

IL-6 and its receptor are expressed in postnatal rat brains (especially the hippocampus and cerebellum\(^27\)) and their transcripts appear to be developmentally regulated in a region-specific manner\(^28\). However, roles of IL-6 remain controversial (i.e. neuroprotective vs neurotoxic\(^6\)). For instance, in developing cerebellar granule cells isolated from 6~8-day old rats, chronic exposure to IL-6 is reported to increase [Ca]\(_i\) due to excessive activation of NMDA receptors and ends up increasing susceptibility to excitotoxic insults leading to neuronal loss\(^29\)\(^{-32}\). Other groups claim that treatment of neonatal rat cerebellar granule neurons with exogenous IL-6 prevents neuronal cell death by reducing apoptosis and keeps intracellular Ca from being overloaded\(^33, 34\). In addition, some groups showed a neuroprotective effect of IL-6 on hippocampal neurons\(^35\)\(^{-38}\). Considering the variable results obtained from multiple studies\(^31, 39, 40\), the effects of IL-6 may be dependent upon neuronal cell type examined, the varying times/duration of IL-6 treatment, and concentrations of IL-6 applied. Previously, we have reported the effects of various concentrations of exogenous IL-
6 (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml) on AHPC differentiation in vitro. From the concentration-dependent test, we observed that AHPC neuronal differentiation (assessed by TuJ1 immunoreactivity) was significantly increased at the concentrations of IL-6 greater than 0.1 ng/ml compared to the control (no IL-6 added). In addition, AHPCs treated with purified IL-6 which concentration was greater than 1 ng/ml developed significantly longer processes (measured from TuJ-IR AHPCs). Moreover, from all culture conditions of different IL-6 concentrations, we observed no considerable changes in the total number of cells. Thus, our previous and present results suggest that, in our culture system, purified IL-6 (used at a concentration of 1 ng/ml) provides neurogenic effects on AHPC differentiation, rather than cytotoxic effects.

Qiu et al. (1995) reported that IL-6 treatment caused cerebellar granule neurons to be more sensitive to NMDA application and increased [Ca]i when NMDA receptors were agonized. Moreover, NMDA-induced [Ca]i increase in IL-6-treated cells was significantly decreased when [Ca]ext was reduced and intracellular Ca stores were depleted. They further demonstrated that both calcium release from intracellular stores and Ca influx through L-, N- and P/Q-type Cav channels may contribute to [Ca]i rise in IL-6-treated neurons. These results suggest that IL-6 renders the developing neurons more sensitive to membrane depolarization stimuli by increasing [Ca]i.

**NPC differentiation and calcium influx**

In the adult NPCs, [Ca]i can be increased by external Ca entry through Cav channels or NMDA receptors or by Ca release from internal Ca stores after external Ca influx. An increase in [Ca]i is known to promote neuronal differentiation of NPCs in young and adult
rodent brains. Deisseroth et al. (2004) reported that membrane depolarization through L-type Cav channels and NMDA receptors could enhance neuronal differentiation of adult NPCs. They cultured rat hippocampal NPCs onto primary hippocampal neurons and glia or fixed hippocampal cellular substrates under differentiation conditions (excluding mitogens and including 2% fetal bovine serum (FBS), 0.5 μM all-trans retinoic acid, 10 μM forskolin and 20 ng/ml NT3) for 2 weeks. Excitatory stimuli (application of 20 mM potassium chloride (KCl) or 50 μM glutamate) were then applied to the cultures. They found that the membrane-depolarizing stimuli increased the fraction of NPC progeny which expressed the neuronal marker (MAP2ab). In addition, the excitatory stimuli were sensed by L-type Cav channels (especially Cav1.2 and Cav1.3) and NMDA receptors for neuronal differentiation of the NPCs. Furthermore, they observed that membrane depolarization caused changes in gene expression in 6 hours; glial fate gene (Hes1 and Id2) expression was down-regulated, and neuronal gene (NeuroD) expression was up-regulated. Similarly, D’Ascenzo et al. reported that membrane depolarization promotes neuronal differentiation of cortical NPCs. They cultured neonatal mouse cortex-derived NPCs in the presence of 1% FBS up to 12 days, and applied 50 mM KCl to elicit membrane depolarization. At 0, 3, 6, 9 and 12 days after induction of differentiation, they examined phenotypic marker expression, changes in [Ca]i and barium (Ba) current density. They observed that, over the culture period, membrane depolarization increased the proportion of cells which expressed neuronal markers, increased the amplitude of Ca signal, increased the density of high voltage-activated Ba currents which were blocked by nifedipine (5 μM) and increased Cav1.2 expression. These findings suggest that Ca influx through L-type Cav channels is important for induction of neuronal differentiation of NPCs.
Our results in this study demonstrate that IL-6-induced neuronal differentiation of AHPCs is modulated through L-type Cav channels. We showed that nifedipine or isradipine application reduced the percentage of IL-6-induced TuJ1-immunoreactive cells. Moreover, in the culture medium which contained insufficient extracellular calcium, exogenous IL-6 was unable to enhance neuronal differentiation of AHPCs. From these results, it is presumed that the calcium influx through L-type Cav channels is, at least in part, critical for IL-6-mediated neuronal differentiation of AHPCs.

During adult neurogenesis, newborn cells in the dentate gyrus express developmental stage-specific phenotypic markers and display electrophysiological characteristics at a given time throughout the whole process becoming functional mature neurons in vivo. At an early stage of neuronal differentiation (until ~1 week after cell birth), adult hippocampal NPCs are responding to GABA (as an excitatory input) but not to glutamate. GABA excitatory inputs cause an increase in [Ca]i and promote neuronal differentiation. Later on (during the third week after birth), NPC-derived newborn cells receive GABA inhibitory as well as glutamate excitatory inputs. Within the third week, NMDA receptor-dependent excitation is known to be important for selective survival/death and synaptogenesis of newborn neurons. Since AHPCs differentiated in the presence of exogenous IL-6 in our culture system are presumed to be undergoing an early stage of neuronal differentiation (based on the expression of an early neuronal marker and electrophysiological membrane properties), it is unlikely that NMDA receptors have a crucial influence in IL-6-induced neuronal differentiation during the time window we examined in this study. Further molecular and biophysical characterizations may help to underpin this presumption.
CREB activation and neuronal differentiation

Ca entry can be induced by a variety of stimuli mimicking neuronal activity, such as application of neurotransmitters or depolarization of plasma membrane\textsuperscript{43}. The elevation of \([\text{Ca}^{2+}]_i\) due to extracellular Ca entry then leads rapidly and transiently to transcription of immediate early genes\textsuperscript{22-24} which mostly encode transcription factors regulating cellular responses to synaptic stimulation during neuritogenesis and synaptic development\textsuperscript{24}. Three transcription factors – nuclear factor of activated T-cells (NFAT), and downstream regulatory element antagonistic modulator (DREAM) as well as CREB – have been extensively studied and are known to be activated though several conceptually different pathways upon \([\text{Ca}^{2+}]_i\) rise\textsuperscript{25,26}.

Recently, Jagasia et al. (2009) reported that, in newly generated neurons in adult mouse hippocampus, CREB phosphorylation is initiated in ~1-week old postmitotic cells after the expression of immature neuronal markers (doublecortin and NeuroD) is initiated\textsuperscript{44}. They also found that CREB phosphorylation declined ~3-weeks after birth when doublecortin or NeuroD expression decreased and the mature dentate granule cell marker (calbindin) expression increased\textsuperscript{44}. In addition, the peak of CREB phosphorylation in newborn granule cells was paralleled with the timing of membrane depolarization by activation of GABA\(_A\) receptors, which is essential for dendritic morphogenesis of newborn cells\textsuperscript{45}. These findings suggest that CREB activation may play a critical role in early morphological development and neuronal gene expression in young hippocampal neurons.

Considering the consistent observation that pCREB is highly enriched in immature hippocampal neurons\textsuperscript{44,46,47}, our findings showing the increased fraction of pCREB-
expressing AHPCs in IL-6-treated cultures suggest that IL-6 may accelerates CREB phosphorylation during an early stage of AHPC neuronal differentiation.

Conclusion

IL-6 is an astrocyte-derived factor which promotes neuronal differentiation of AHPCs in vitro. To investigate a possible mechanism of IL-6-mediated neuronal differentiation, we examined the influence of L-type Cav channel antagonists and a presence of extracellular calcium on IL-6-enhanced neuronal differentiation of AHPCs. Our immunocytochemical results demonstrated that (1) extracellular calcium was necessary for neuronal differentiation of IL-6-treated AHPCs, (2) L-type Cav channel antagonists inhibited an increase in the fraction of AHPCs expressing a neuronal marker, TuJ1, and (3) IL-6 treatment increased the fraction of AHPCs immunoreactive for an activated form of CREB (pCREB). Taken together, our results suggest that IL-6 may induce AHPC differentiation into a neuronal lineage by promoting CREB activation which is presumably caused by extracellular calcium influx.

Acknowledgments

Financial support provided by the NIH (NIGMS RO1 GM072005-01), the Stem Cell Research Fund, and the Department of Genetics, Development and Cell Biology at Iowa State University are gratefully acknowledged. We would like to thank Dr. Fred H. Gage at the Salk Institute for the gift of AHPCs. The anti-RIP antibody developed by Susan
Hockfield was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Iowa City, IA). We also thank Eun-Ah Ye (Neuroscience Program, Iowa State University) for helpful discussions, and Lavanya Singh (Undergraduate Biology Program, Iowa State University), Amanda Kite, Alicia Schiller-Holland (Research Experiences for Teachers program, RET, summer teacher interns, 2009), Peg Conlon, Becki Steinlage (RET summer teacher interns, 2010) for assistance in cell counting analyses.
References


Figure legends

Figure 1. Insufficient extracellular calcium prevented IL-6-enhanced neuronal differentiation of AHPCs. AHPCs were cultured in media containing varying concentrations of calcium (0.15, 0.25, 0.5 and 1.045 mM) in the presence or absence of IL-6 for 6 days and TuJ1-immunoreactivity was then analyzed. (A) An augmentation of the percentage of TuJ1-IR AHPCs was observed only in those cultures maintained in a normal calcium concentration (1.045 mM). In the presence of lower calcium concentrations (0.15, 0.25 and 0.5 mM), IL-6 application had no significant effect on increasing the percentage of TuJ1-IR AHPC. Results were obtained from three independent experiments (N = 3). (B) Average number of DAPI-stained cells counted per a unit area (mm²) for every culture condition presented in (A). A significantly smaller number of AHPCs were observed in the cultures containing 0.15 mM or 0.25 mM of external calcium, compared with the culture containing a normal concentration of calcium, 1.045 mM).

Figure 2. L-type Cav channel antagonists, nifedipine and isradipine, specifically inhibited neuronal differentiation of AHPCs induced by exogenous IL-6 application. AHPCs were cultured under differentiation conditions for 6 days in the absence or presence of purified rat recombinant IL-6 (1 ng/ml). Without IL-6 treatment, nifedipine or isradipine did not affect the fraction of AHPCs expressing TuJ1 nor RIP. However, in the cultures treated with exogenous IL-6, the percentage of TuJ1-IR AHPCs was increased and both nifedipine and isradipine reduced the TuJ1-IR fractions with no apparent effect on RIP-IR fractions. N = 4 for cultures with dihydropyridines and N = 6 for control cultures.
**Figure 3.** IL-6 treatment increased the fraction of TuJ1 and pCREB-immunoreactive AHPCs. (A) After 3-day culture session, AHPCs were immunolabeled with anti-CREB or anti-pCREB and/or anti-TuJ1. Approximately 95% AHPCs were CREB-positive regardless of IL-6 application. With IL-6 treatment (‘+ IL-6’ shown in black), the fraction of CREB and TUJ1-positive AHPCs was about 3-fold greater than that with no IL-6 added (‘- IL-6’, shown in white). In addition, with IL-6 treatment, the fraction of pCREB-positive AHPCs was about 3-fold increased compared to the control. Moreover, with IL-6 treatment, the fraction of pCREB and TuJ1-positive AHPCs was about 9-fold increased compared to the control. Furthermore, when AHPCs were treated with IL-6 and either nifedipine (‘Nif’, shown in gray) or IL-6 function blocking antibody (‘Anti-IL-6’, shown in green), IL-6-induced augmentation of TuJ1 and/or pCREB-positive fractions was not observed. N = 4 for ‘- IL-6’ and ‘+ IL-6’; N = 2 for ‘Nif’ and ‘Anti-IL-6’. (B) Percentage-wise results are presented in a ratio-based graph. Since all TuJ1-positive AHPCs were CREB-positive in all culture conditions setup for this experiment, the ratio of %CREB-positive over %TuJ1-positive (‘CREB+ of TuJ1+’) equals 1. Because Y-axis for the ratios was expanded to display the ratios lower than 0.5 evidently, bars in the graph for ‘CREB+ of TuJ1+’ were cut off. ‘TuJ1+ of CREB+’ represents a portion of AHPCs immunoreactive for TuJ1 within CREB-IR fraction. ‘pCREB+ of TuJ1+’ represents a portion of AHPCs immunoreactive for phosphorylated CREB within TuJ1-IR fraction. ‘TuJ1+ of pCREB+’ represent a portion of TuJ1-IR AHPCs within pCREB-IR fraction. (C) Representative immunofluorescence images from designated conditions - cultures with no IL-6 (a and a’) or with IL-6 (b and b’); immunolabeling for CREB (a and b) or pCREB (a’ and b’). Scale bars in a, a’, b and b’
represent 50 μm; ones shown in inset images within Figure a represent 20 μm, which is applicable for insets within a’, b and b’ as well.
Figure 1. Insufficient extracellular calcium prevented IL-6-enhanced neuronal differentiation of AHPCs.
Figure 2. L-type Cav channel antagonists, nifedipine and isradipine, specifically inhibited neuronal differentiation of AHPCs induced by exogenous IL-6 application.
Figure 3. IL-6 treatment increased the fraction of TuJ1 and pCREB-immunoreactive AHPCs.
**Supplementary Figure legends**

**Supplementary Figure 1.** Extracellular calcium appeared to be important for cell viability. After termination of 6-day culture session, AHPCs were stained with propidium iodide (PI) to visualize dead cells. With the lowest concentration of calcium present in culture medium, 0.15 mM, about one third cells were PI-positive. N = 2.

**Supplementary Figure 2.** IL-6-induced neuronal differentiation was inhibited by nifedipine or isradipine. AHPCs were cultured under differentiation conditions for 6 days in the presence of IL-6 (1 ng/ml) with varying concentrations of nifedipine (A) or isradipine (B). The percentage of TuJ1-IR AHPCs was about 2-fold increased with IL-6 treatment compared to the control (cultures without IL-6 application). With ≥ 1 µM of nifedipine (A) or ≥ 0.5 µM of isradipine (B), the fractions of TuJ1-IR AHPCs were significantly decreased compared to the control (IL-6-treated cultures without dihydropyridines). In comparison of the percentages of RIP-IR AHPCs, there were no significant changes observed across all culture conditions. N = 3.

**Supplementary Figure 3.** IL-6 treatment did not influence RIP and pCREB-immunoreactivities of AHPCs. After 3-day culture session, AHPCs were immunolabeled with anti-CREB or anti-pCREB and/or anti-RIP. There was no considerable difference in the average percentage of AHPCs immunoreactive for RIP between non-IL-6-treated and IL-6-treated cultures (RIP-positive fraction: ~11% in control vs ~12% in IL-6-treated condition). Almost all AHPCs (~96%) were CREB-positive regardless of IL-6 application. With and
without IL-6 treatment (‘+ IL-6’ presented in black and ‘- IL-6’ shown in white, respectively),
there was no difference in the percentage of AHPCs immunoreactive for both RIP and CREB.
In addition, the percentage of AHPCs immunoreactive for both RIP and pCREB in IL-6-
treated cultures was similar to that in non-treated cultures, although the percentage of AHPCs
immunoreactive only for pCREB was increased in IL-6-treated cultured compared to the
control cultures (pCREB-positive fraction: ~13% in control vs ~25% in IL-6-treated
condition). In IL-6-treated cultures, the fraction of pCREB-positive AHPCs was reduced by
nifedipine application. However, the fraction of AHPCs immunoreactive for both RIP and
pCREB was not affected by nifedipine application. N = 3 for ‘- IL-6’ and ‘+ IL-6’; N = 2 for
‘Nif’ and ‘Anti-IL-6’.
Supplementary Figure 1. Extracellular calcium appeared to be important for cell viability.
Supplementary Figure 2. IL-6-induced neuronal differentiation was inhibited by nifedipine or isradipine.
Supplementary Figure 3. IL-6 treatment did not influence RIP and pCREB-immunoreactivities of AHPCs.
CHAPTER 5.  
GENERAL CONCLUSIONS

The factors responsible for selective neuronal differentiation of adult neural progenitor cells were investigated in vitro. The results organized in this dissertation demonstrate that astrocyte-derived soluble factors specifically promote neuronal differentiation of adult hippocampal neural progenitor cells (AHPCs). In addition, interleukin-6 is a neurogenic factor derived from neurogenic astrocytes, and promotes neural progenitor cell differentiation into a neuronal lineage.

As described in the first research chapter (chapter 2), the influence of multiple environmental cues (such as, niche cell-derived soluble factors, cell-cell contact, and extracellular topography) on AHPC differentiation has been examined in a well-controlled in vitro culture system. Moreover, we have demonstrated that the presence of neonatal astrocytes increased AHPC neuronal differentiation and the removal of a physical contact between astrocytes and AHPCs further increased neuronal differentiation of AHPCs. Thus, we concluded that soluble factors produced from neonatal astrocytes act as neurogenesis-promoting cues for AHPCs to commit to a neuronal lineage. In addition, cellular contact-mediated interaction between astrocytes and AHPCs appeared to provide a suppressive influence on AHPC neuronal differentiation. Moreover, we observed that AHPCs emanated significantly longer neurites when cultured without physical contacts with astrocytes on the topographically grooved surface compared to those cultured with cellular contacts. This finding also supports a possibility that astrocytes have an activity which suppresses neuronal differentiation of AHPCs in a cell-cell contact-dependent manner. Further studies need to be...
undertaken to characterize possible mechanism(s) behind the contact-mediated suppression of neuronal differentiation.

In the second research chapter (chapter 3), we further investigated cellular responses of AHPCs to astrocyte-derived soluble factors promoting AHPC neuronal differentiation *in vitro*. To scrutinize a source of neurogenic effects of soluble factors produced from the postnatal brain-derived astrocytes. We have successfully isolated two populations of astrocytes from the same brain, hippocampus-derived and cerebral cortex-derived, and examined the influences of soluble factors from both astrocyte populations on AHPC differentiation by co-culturing AHPCs with astrocytes but without physical contact between both cell types. Our findings showed that soluble factors present in the cultures of hippocampus-derived astrocytes and AHPCs dramatically and specifically augmented neuronal differentiation of AHPCs. As well documented, the hippocampus is one of two regions where new cells are generated in the postnatal or adult brain and the most of newly generated cells are differentiated into hippocampal granule neurons. Considering that astrocytes are the most prevailing cell type in the niche, it was not so surprising that the molecules produced and released from hippocampal astrocytes had a profound effect on AHPC neuronal differentiation, compared to those from cerebral cortex-derived astrocytes did. Among the numerous molecules produced from neurogenic hippocampal astrocytes, we focused on interleukin-6 (IL-6), a neurogenesis-promoting factor. IL-6 is a pleiotropic cytokine which plays a role in both proinflammation and anti-inflammation. In addition, studies examining the role of IL-6 in the neurogenic niches in the brain have proposed to exert both neurotoxic and neurotrophic effects in neurogenesis. In our culture system, an exogenous application of IL-6 resulted in the enhancement of AHPC differentiation
preferentially into a neuronal lineage. Therefore, we concluded that IL-6 derived from neurogenic astrocytes is a soluble factor which induces neuronal differentiation of AHPCs. Understanding mutual interaction between neural progenitor cells and surrounding cellular components by diffusible molecules released in the niche is important to help to develop therapeutic strategies to control the fate, proliferation and differentiation of neural progenitor cells. To characterize not only additional astrocyte-derive factors but their combinatorial effects, further studies need to be carried out.

In the last research chapter (chapter 4), possible mechanism(s) by which IL-6 enhances AHPC neuronal differentiation were investigated. By culturing AHPCs in either low calcium-containing culture medium or voltage-gated calcium channel antagonist-containing medium in the presence of exogenous IL-6, we examined the influence of extracellular calcium on IL-6-enhanced AHPC differentiation. Our findings using immunocytochemical analysis indicate that a presence of extracellular calcium is necessary for IL-6 to increase AHPC neuronal differentiation and L-type calcium channel antagonists prevent the increase in the fraction of AHPCs which are expressing a neuronal marker. In addition, the fraction of phosphorylated CREB-positive AHPCs was significantly increased in IL-6-treated cultures, compared to non-treated cultures. Thus it is presumed that IL-6-enhanced neuronal differentiation of AHPCs is mediated by promoting CREB activation which is possibly caused by extracellular calcium entry though L-type calcium channels. Further molecular and electrophysiological characterizations may need to be performed to underpin this presumption.
In summary, our findings from the three investigations described in Chapter 2, 3 and 4 are as follow;

- AHPC neuronal differentiation (assessed by neuronal marker expression) was dramatically increased when AHPCs were co-cultured in physical contact with astrocytes.

- Neuronal differentiation was further increased when physical contact was removed from AHPC-astrocyte co-cultures (non-contact co-culture).

- Under non-contact co-culture conditions, neurite length was significantly greater when compared with control (AHPC alone culture) or contact co-culture conditions.

- AHPCs under non-contact co-culture conditions developed membrane properties that are consistent with those of differentiated neurons.

- A candidate for a neurogenic astrocyte-derived soluble factor, IL-6, was produced from neurogenic astrocytes.

- AHPC neuronal differentiation was significantly increased when AHPCs were cultured in the presence of exogenous IL-6 compared to the control (cultures without IL-6).

- AHPCs cultured with IL-6 developed a subset of neuronal-like membrane properties at an early time point.

- IL-6-enhanced neuronal differentiation was reduced when AHPCs were cultured in low calcium-containing culture medium or with L-type voltage-gated calcium channel antagonists.
• IL-6 treatment increased the fraction of AHPCs immunoreactive for a neuronal marker (TuJ1) and for pCREB.

Taken together, an overall conclusion is that astrocyte-derived soluble factors promote neuronal differentiation of adult neural progenitor cells and astrocyte-derived interleukin-6 is a neurogenic factor to instruct neural progenitor cells to commit to a neuronal lineage.
APPENDIX

Abstract of a manuscript in preparation which is not included in this dissertation

Characterization of Multipotent Adult Rat Hippocampal Progenitor Cells Maintained as Neurospheres: Differentiation \textit{in vitro} and \textit{in vivo} Following Transplantation

Jisun Oh\textsuperscript{1,2,3}, Gabrielle J. Daniels\textsuperscript{4}, Lawrence S. Chiou\textsuperscript{5}, Eun-Ah Ye\textsuperscript{1,2} and Donald S. Sakaguchi\textsuperscript{1,2,3,4,\#}

\textsuperscript{1} Neuroscience Program, \textsuperscript{2} Department of Genetics, Development and Cell Biology, \textsuperscript{3} Department of Biomedical Sciences, \textsuperscript{4} Biology Program, Iowa State University, Ames, IA 50011
\textsuperscript{5} Ames High School, Ames, IA 50010
\# Corresponding author

Abstract

Adult Hippocampal Progenitor Cells (AHPCs; a gift from Fred Gage, Salk Institute, La Jolla, CA) are generally maintained \textit{in vitro} as a dispersed monolayer population of multipotent neural progenitors. However little is known about whether cell-cell interactions influence their proliferation, differentiation, or migrational activities \textit{in vitro}, or \textit{in vivo} following transplantation. To investigate possible differences in phenotypic and behavioral characteristics, free-floating proliferating cellular aggregates of neurospheres were generated from the adherent monolayer population of AHPCs. Both populations of AHPCs (neurosphere and adherent) were cultured under differentiation conditions on laminin-coated glass coverslips, and their proliferation, phenotypic differentiation and migrational activities were compared. AHPC neurospheres displayed a greater percentage of glial marker-immunoreactive cells (RIP- or GFAP-positive) compared to adherent AHPCs, with no significant difference in the percentage of neuronal marker-immunoreactive cells (TuJ1-positive). In addition, AHPCs emigrating out from neurospheres migrated significantly faster
than AHPCs maintained as adherent cells. Our results from in vitro analyses demonstrate that AHPCs formed and maintained in neurospheres favored differentiation along a glial lineage and displayed greater migrational activity, compared to the adherent population of AHPCs. To examine the plasticity of AHPCs from both populations in the central nervous system (CNS), we transplanted the GFP-expressing AHPCs via intraocular injection into the eyes of postnatal day 1 Fischer 344 rats. Cell survival, morphological differentiation and integration were examined at 7, 14 and 28 days after transplantation. At each time point, similar numbers of transplanted cells were observed in the cryo-sectioned eyes that received either AHPC neurospheres or adherent AHPCs. At the later time points, considerably more cells were found integrated into the retina, often with highly branched processes. These findings demonstrate that both AHPC populations (neurosphere and adherent) are capable of surviving and integrating into the developing host CNS. Taken together, these results suggest that cell-cell interactions during AHPC maintenance may influence cell fate under differentiation conditions. Moreover, transplanted AHPCs display considerable plasticity in the developing CNS.
CURRICULUM VITAE

NAME OF AUTHOR
Jisun Oh

EDUCATIONAL EXPERIENCE
2004 – Present  Ph.D. candidate in Neuroscience and Biomedical Sciences (Cell Biology), Iowa State University, Ames, Iowa, USA
Supervisor: Donald Sakaguchi, Ph.D

2001 – 2003  M.S. in Animal Science and Biotechnology (Molecular Genetics), Kyungpook National University, Daegu, S. Korea
Supervisor: Jinkyu Lim, Ph.D

1996 – 2001  B.S. in Animal Science and Biotechnology, Kyungpook National University, Daegu, S. Korea

PROFESSIONAL EXPERIENCE
2007 – Present  Graduate mentor for honors students, Iowa State Univ

2006 – Present  Teaching Assistant (Human Anatomy and Physiology), Dept. of Genetics, Development and Cell Biology, Iowa State Univ
Instructor: Barbara Krumhardt, Ph.D

2005 – 2010  Research Assistant, Dept. of Genetics, Development and Cell Biology, Iowa State Univ

2004 – 2005  Research Assistant, Dept. of Biomedical Sciences, Iowa State Univ

2003 – 2004  Research Intern, Korea Science and Engineering Foundation, S. Korea

2001 – 2003  Research Assistant, Kyungpook National Univ

2000  Undergraduate research intern, Pohang Univ of Science and Technology, Gyeongbuk, S. Korea

AWARDS AND HONORS
2010  Nominee for Graduate Student Travel Awards from Ames, Iowa Chapter of the SfN

2007, 2009, 2010  Travel Award ($500) from Dept. of Genetics, Development and Cell Biology, Iowa State Univ

2002  Scholarship for highest honor graduate students, Kyungpook National Univ

1996 – 2000  Scholarship for highest honor undergraduate students, Kyungpook National Univ

PROFESSIONAL ACTIVITIES
2006 – Present  Student member, The Society for Neuroscience (SfN)

PUBLICATIONS
Oh, J., Daniels, G., Chiou, L.S., Ye, E-A. and Sakaguchi, D.S. Characterization of multipotent adult rat hippocampal progenitor cells maintained as neurospheres: Differentiation in vitro and in vivo following transplantation. (In preparation)


MEETING ABSTRACTS


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