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Morphological and functional studies of cells derived from post-natal neural stem cells

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Morphological and functional studies of cells derived from post-natal neural stem cells

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Abstract

This dissertation investigates the morphological and functional characteristics of cells that have been differentiated from post-natal neural stem cells. The population of neural stem cells that were investigated in these experiments are oligodendrocytic precursor cells (OPC). Previous studies have shown that OPC that are sequentially exposed to bone morphogenetic protein and basic fibroblast growth factor convert into a cell that can give rise to oligodendrocytes, astrocytes, and neurons. However, none of these previous studies investigated the functional properties of the differentiated cells.

The second chapter of this work describes both the morphological and functional characteristics of the differentiated cells. We show that a large percentage of these cells differentiate into functional neurons that have voltage gated calcium channels, ligand gated calcium channels, and are able to release glutamate in response to ATP (adenosine triphosphate) stimulation. Furthermore, we show that these differentiated neurons have morphological markers consistent with neuronal differentiation.

The third chapter of this dissertation demonstrates that astrocytes influence receptor expression in the differentiated neurons. The receptor that was investigated was N-methyl-D-aspartate (NMDA) which is a subtype of glutamate receptor and has been shown to be important in many processes critical to neuronal function.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

In the last decade interest in the field of stem cell research has intensified with most of this interest being generated by the potential use of stem cells to treat many human diseases. The potentials for stem cells to be used as therapeutic agents are varied and include providing replacements for lost cells, activation of endogenous stem cell populations, or transplantation of modified stem cells to release specific trophic factors (Cao *et al.,* 2002). Before any of this can become reality we must understand the basic potentials that stem cells possess, the functionality of the differentiated cells, the cell's ability to integrate into the damaged tissues, and the role that the recipient environment plays in all of this. The role of the local environment in which cells are grafted has been shown to have great influence on the fate of the transplanted cells (Gage, 2000). In the case of the central nervous system, it has been shown that glial cells have a large influence on the recipient environment. A better understanding of how glia influence the recipient environment and what effects this has on both stem cell differentiation and the functional characteristics of these cells is crucial to further research in the area of stem cell transplantation and manipulation.

The focus of this dissertation is to further assess the morphological and functional characteristics of neurons derived from multipotent neural stem cells and to determine how astrocytes influence some of these characteristics.

Dissertation Organization

This dissertation is composed of two journal papers. In the first paper (Chapter 2), the functional characteristics of neurons derived from multipotent stem cells of the central nervous system are assessed. This paper has already been published electronically in *Stem Cells Express* and it will also be published in the September issue of *Stem Cells*. All of the material has been reproduced with permission from the publisher. The second paper (Chapter 3) investigates the role that astrocytes have on the functional properties of neurons that are derived from multipotent neural stem cells and it has been submitted for publication in *Glia*.

Each journal article comprises a single chapter of this dissertation. The articles follow a general introduction (Chapter 1) containing a literature review. Chapter 4 of this dissertation consists of a general discussion section and summary. The references are listed at the end of the chapter in which they are cited.

Literature Review

Stem cells

In the last decade, interest in the field of stem cell research has intensified. This increased interest has led to the identification and isolation of adult stem cells from a variety of organs including the central nervous system, bone marrow, liver, intestine, retina, skeletal muscle, pancreas, cornea, and skin (Kirschstein and Skirboll, 2001). The potential therapeutic uses of these cells to ease human suffering and provide cures for human disease have been the driving forces for most of the interest in stem cell research. Although stem cell

research is in its infancy, it is hoped that someday stem cells can be used to cure many diseases such as Parkinson's, amyotrophic lateral sclerosis (Lou Gehrig's disease), Huntington's and diabetes mellitus.

Stem cells fall into two categories, somatic (adult) and embryonic. All stem cells are undifferentiated, capable of self-renewal and, when given the proper cues, can give rise to fully differentiated mature cell types (Gage, 2000). Even though adult and embryonic stem cells share basic characteristics, there are also very important differences. One of the differences between adult and embryonic stem cells is their origin. Embryonic stem cells are derived from the inner cell mass of an embryonic blastocyst while adult stem cells are derived from differentiated tissues (Gage, 2000). The different origins of these two types of stem cells is an important social issue. The acquisition of embryonic stem cells results in the loss of a developing embryo, while adult stem cells can be harvested from donors without negatively affecting the person or animal. Mature mammals have small populations of adult stem cell distributed throughout the body and these cells are thought to play a role in the repair of damaged organs and tissues (Rossi and Cattaneo, 2002). One enticing prospect of adult stem cells is their potential to be used as autographs, which would eliminate the rejection of transplanted cells (Roskams and Tetzlaff, 2005). Another therapeutic potential for adult stem cells is selective *in situ* activation allowing for repair of damaged tissues and organs without cell transplantation (Roskams and Tetzlaff, 2005).

The other major difference between embryonic and adult cells is their plasticity. Adult stem cells are thought to be much more limited, with respect to what they can differentiate into, when compared to embryonic stem cells. All cells of multicellular organisms are derived from a single cell called the zygote. As this single cell undergoes

division the daughter cells slowly become restricted by 'turning' genes on and off to generate all of the specialized cells found in multicellular organisms (Clarke *et al.,* 2000). Even though every cell of the body has the same genetic make up, the functional and morphological characteristics of the cell are dictated by the genes that are expressed. Embryonic stem cells are pluripotent and are capable of differentiating into nearly every cell type of the body. This is due to the fact that these cells are collected from the inner cell mass of the blastocyst and very little cellular specialization has occurred, whereas, adult stem cells have varying degrees of plasticity because they are harvested from tissues (Gage, 2000; Kirschstein and Skirboll, 2001). Some types of adult stem cells, mesenchymal stem cells for example, are much more plastic than was originally thought and can differentiate into cell types very different from their tissue of origin (Lovell-Badge, 2001).

Neural stem cells

It has long been believed that all neurogenesis was confined to the *in utero* and early postnatal periods of development and no new neurons could be generated in the adult brain. This assumption was based on the fact that neurons present in the adult brain did not have mitotic figures and a lack of neural proliferation following brain injury (Altman and Das, 1964). The first evidence that a population of stem cells existed in the central nervous system (CNS) came from Altman and Das in the 1960's. They demonstrated the presence of dividing cells in the murine hippocampus and olfactory bulb that became neurons. Despite this fact, the presence of a stem cell population giving rise to neurons in the adult mammalian CNS was not widely accepted until the 1990's. In 1992, Reynolds and Weiss were the first to isolate neural stem cells (NSC) from the adult mammalian CNS. They found a population

of cells isolated from the striatum of the adult mouse brain that could differentiate into neurons and astrocytes after they were exposed to epidermal growth factor. Furthermore, they demonstrated that the differentiated neurons were immunoreactive for substance P and γ-aminobutyric acid indicating that the cells might be functional. Since this initial description of NSCs they have been identified in several regions within the adult CNS including the hippocampus, subventricular zone, olfactory bulb, and spinal cord (Gage, 2000; Temple, 2001). The first description that NSC existed in humans occurred in 1998 when Ericksson and colleagues demonstrated that neurogenesis occurred in the adult human hippocampus. The study consisted of five cancer patients that were injected with BrdU and their brains were examined post-mortem. They found a small population of cells that co-labeled for both BrdU and several neuronal markers indicating that the co-labeled cells were created during BrdU exposure.

Neural stem cells are an adult type of stem cell because they are derived from differentiated tissue and divide to become other NSC, neuronal progenitor or glial progenitor cells. It is believed that neuronal progenitor cells are destined to become neurons and glial progenitor cells will give rise to astrocytes and oligodendrocytes (Gage, 2000; Clarke *et al.*, 2000; Seaberg *et al.*, 2003). It has been demonstrated that NSCs can generate glial and neuronal cells *in vitro* when they are exposed to certain trophic factors (Ahmed *et al.*, 1995; Koblar *et al.*, 1998; Zigova *et al.*, 1998), and *in vivo* after transplantation (Campbell *et al.*, 1995; Craig *et al.*, 1996; Carpenter *et al.*, 1997; Winkler *et al.*, 1998). However, this neural restriction can be over come if NSCs are transplanted into a chick blastocyst where they can contribute to all three germ layers (Seaberg *et al.*, 2003). This helps elucidate the importance of the external cellular environment in maintaining cellular restriction. Additional work

demonstrates that glial progenitor cells that are given the proper cues are capable of becoming more than there progenitor restriction would suggest and can differentiate into neurons (Clarke *et al.,* 2000; Gage, 2000; Kondo and Raff, 2000; Seaberg *et al.*, 2003).

Oligodendrocyte precursor cells

Noble, Raff, and others have shown that the adult optic nerve retains an active population of glial progenitors termed oligodendrocyte precursor cells (OPCs) (Raff *et al.*, 1984, 1987; Wolswijk and Noble, 1989). OPCs are also called O-2A progenitor cells because initial work demonstrated that they could not only differentiate into oligodendrocytes, but also astrocytes. The astrocyte that was derived from O-2A progenitors was called a type 2 astrocyte to indicate that it came from a separate linage (Franklin and Blakemore, 1995). The demonstration of type 2 astrocytes *in vivo* has proven to be very difficult and there is some question to their existence (Franklin and Blakemore, 1995). However, recent research demonstrates that OPCs that are exposed to bone morphogenetic proteins (BMP) promotes type 2 astrocyte differentiation and if followed by basic fibroblast growth factor (bFGF) these cells revert to a state in which they can not only self-renew but are capable of differentiating into oligodendrocytes, astrocytes, and neurons (Kondo and Raff, 2000). Furthermore, if these reverted cells are kept in bFGF for 30 days, approximately 62% (± 5%) of them will differentiate into neurons (Kondo and Raff, 2000). This is an example of a glial restricted progenitor cell taking on the characteristics of a neural stem cell and these cells have been termed multipotent CNS stem cells or neural stem cell like cells

(Kondo & Raff, 2000, 2004a,b). The possibility of neural progenitor contamination from other neurogenic regions is remote because the optic nerve was harvested rostral to the optic chiasm eliminating concerns of any contamination.

In work done by Nunes *et al.* (2003) they found that human OPCs have the same potential to convert into a multipotent CNS stem if first exposed to fetal bovine serum, which contains BMPs. They did not observe the conversion to the type 2 astrocyte, but the BMP in the serum are still believed to be the inducing factor. As stated previously, the conversion of OPCs into a type 2 astrocyte is under question. There is no doubt that these cells express glial fibrillary acidic protein (GFAP), which is considered to be an astrocytic marker, but GFAP expression does not necessarily indicate functional astrocyte differentiation (Goldman, 2003; Garcia *et al.,* 2004). Many cells, in addition to astrocytes, express GFAP and include cells found in the liver, kidney, and gut. This indicates that GFAP immunoreactivity is not an exclusive astrocyte marker and it also has no bearing on the functional properties of the cells (Garcia *et al.,* 2004). That being said, GFAP expression has been confirmed in other NSC populations and some evidence is beginning to suggest that radial glia act as neural stem cells (Imura *et al.*, 2003; Garcia *et al.,* 2004).

One of the important genetic steps for conversion of OPCs into multipotent NSC is the induction of *Sox2* gene expression (Kondo and Raff, 2004a). The *Sox2* gene belongs to the SOXB1 group of transcription factors which is made up of 3 closely related genes including *Sox1*, *Sox2,* and *Sox3* (Pevny and Lovell-Badge, 1997; Graham *et al.,* 2003). The SOXB1 genes are widely expressed in vertebrate neural stem cells throughout the developing and adult brain (Graham *et al.,* 2003). In fact, *Sox2* is one of the first transcription factors expressed in the developing neural tube (Kondo and Raff, 2004a). The SOXB1 factors that

are expressed in NSC have been shown to be important for the maintenance and self-renewal of these cells (Episkopou, 2005; Graham *et al.,* 2003). As NSC differentiate and exit the cell cycle expression of *Sox2* decreases. In work done by Graham *et al.* (2003) they found that NSC that over expressed *Sox2* failed to differentiate and cells in which *Sox2* expression was blocked exited the cell cycle and differentiated into neurons.

The exposure of OPCs to BMP is important for the induction of multipotent neural stem cells. Bone morphogenetic proteins are a subclass of the transforming growth factor β superfamily and are known to be important in many developmental processes including differentiation, mophogenesis, lineage commitment, cell survival and apoptosis (Mehler *et al.*, 1997). In addition to their role in development, they have been shown to be important in maintaining pluripotency in both mouse embryonic stem cells and NSCs (Nakashima *et al.*, 2001). Nakashima *et al.*, 2001 showed that mouse telencepahlic neural progenitor cells were able to differentiate into astrocytes after exposure to both BMP and leukemia inhibitory factor. After brief exposure to BMP, OPCs are able to convert to a type 2 astrocyte and into a multipotent CNS stem cell (Mabie *et al.,* 1997; Kondo and Raff, 2000, 2004a,b). The exact mechanism by which this happens in OPCs is not fully understood but in mouse neural progenitor cells BMP exposure increases the expression of inhibitor of differentiation genes I*d* 1 and I*d* 3 (Nakashima *et al.*, 2001).

BMP are present in the adult and developing nervous system and yet there is no evidence that OPCs are able to become multipotent CNS stem cells *in vivo* (Kondo and Raff 2004b). This can partly be explained by Noggin which is a BMP antagonist. Noggin is expressed by oligodendrocytes and type 1 astrocytes and it interferes with the binding of BMP to their receptors (Kondo and Raff 2004b). Kondo and Raff (2004b) demonstrated

that over expression of Noggin by OPCs decreased the number of cells that were able to become multipotent stem cells, and interference of Noggin production by siRNA increased the ability of conversion. This helps demonstrate the importance of environmental cues in maintaining cell restrictions.

The effect of exogenous factors on the plasticity of OPCs is important in understanding not only the basic biology of these cells, but also their potential use in therapies. Oligodendrocyte precursor cells are widely distributed in the CNS and constitute a major cycling population in the parenchyma of the brain and spinal cord (Dawson *et al.,* 2003). In some regions of the adult rat CNS, as many as 70% of the cells that are dividing have been found to be OPCs (Dawson *et al.,* 2003). In humans, OPCs comprise 3% of the cells in the subcortical white mater (Nunes *et al.*, 2003). The widespread distribution of OPCs and their potential to revert into a cell that can give rise to all three major cell types of the CNS makes them very appealing therapeutic targets (Kondo and Raff, 2004a).

Stem cells as therapeutic candidates

Successful repair of any injured tissue involves the removal of the damaged cells followed by replacement of those cells allowing a return to its original structure and function (Rossi and Cattaneo, 2002). The potentials for stem cells to be used as therapeutic agents are varied and include providing replacements for lost cells, activation of endogenous stem cell populations, or transplantation of modified stem cells to release specific trophic factors (Cao *et al.,* 2002). There are many obstacles to overcome before stem cells can be used effectively as neuronal replacements in the treatment of diseases associated with the CNS. One of them is what is considered a functional neuron? In order for a stem cell derived neuron to be

considered functional it must be stably differentiated, polarized showing a single axon and multiple dendrites, capable of generating an action potential, and not only able to release neurotransmitters but also possess receptors for them (Reh, 2002). Another problem in the CNS is the integration of the transplanted neurons. Even though you have a functionally differentiated cell the proper integration of replacement neurons is very difficult due to the complex connections that these cells make and the distances over which some of the axons must travel to make proper connections (Rossi and Cattaneo, 2002).

Regardless of the avenue that is chosen for stem cell therapies certain key factors must be met, and these depend on the successful engraftment into recipient tissue which depends on: 1) Survival of the stem cell in the recipient tissue 2) stable phenotypic expression of the differentiated stem cell and 3) proper integration of the cell into recipient tissue (Rossi and Cattaneo, 2002). Stated another way, successful engraftment depends on the intrinsic potential of the stem cell and multiple environmental factors of the recipient tissues. The role of the local environment in which cells are grafted has been shown to have great influence on the fate of transplanted cells (Gage, 2000). In the case of the CNS, it has been shown that glial cells have a large influence on the recipient environment. A better understanding of how the glia influence the recipient environment and what effects this has on cell receptor expression and differentiation is crucial to further research in the area of stem cell transplantation and manipulation.

Glia

'*What is the function of glia cells in neural centers? The answer is still not known, and the problem is even more serious because it may remain unsolved for many years to come until physiologists find direct methods to attack it.*'-Santiago Ramón y Cajal 1911 (Ransom, 2003).

This statement made by Cajal in 1911 has proven to be a very accurate assessment of our ability to determine the role glial cells play in the CNS. The problems associated with studying the function of glial cells resides in the fact that these cells act in extremely subtle ways when compared to the action potentials of neurons (Ransom, 2003). It is only with new research technologies that we are beginning to learn about the importance of glial cells.

The first recorded description of glia in the CNS occurred in 1846 by the German pathologist Rudolf Virchow (Gomes and Rehen, 2004). He used the word Nervenkitt (nerve glue) to describe the connective tissue or non-neuronal component of the brain. This initial description of the glial portion of the CNS is still reflected in its name. The word glia comes from the Greek word *gliok* which means 'glue' or 'slime', and until recently these cells were thought of as supportive elements for neurons (Svendsen, 2002; Haydon, 2001, Nedergaard *et al.*, 2003). Glial cells constitute about 90% of the cell population in the central nervous system and include the astrocyte, oligodendrocyte, microglia, and ependymal cells.

The astrocyte and oligodendrocyte are often referred to as macroglia due to their size. Oligodendrocytes are known to produce and maintain the myelin of the CNS (Nedergaard and Dirnagl 2005). The myelin sheaths that surround axons allow for the salutatory conduction of action potentials. Astrocytes are known to have many functions and are

discussed in the next section. Ependymal cells line the ventricles of the vertebrate brain and central canal of the spinal cord (Reichenbach and Robinson 1995), and their primary function is thought to be protection of the CNS from potentially harmful substances in the cerebral spinal fluid (Hauwel *et al.,* 2005). The microglial cells represent about 20% of the glial population in the CNS and are responsible for providing immune surveillance and are the macrophage equivalent with in the nervous system (Streit, 1995; Nedergaard and Dirnagl 2005; Panickar and Norenberg, 2005). They function to protect the CNS from infectious agents, remove cellular debris secondary to injury and are the main phagocytic cell of the CNS (Hauwel *et al.,* 2005). Since the microglial cell is related to macrophage they are the only cell type found in the CNS that is not of ectodermal origin. New research is starting to show that all of the glial cells have many more functions than simply providing support for neurons.

The astrocyte

Astrocytes are the most common cell type of the CNS and account for nearly 50% of the cells present in the adult brain (Song *et al.* 2002b). The traditionally accepted roles for the astrocyte has been one of support and maintenance including modulation of the perineural environment, providing both structural and metabolic support to the neuron, axonal guidance during development and maintenance of the blood brain barrier (Haydon, 2001; Svendsen, 2002). This is no longer believed to be the limit of astrocyte function.

Astrocytes are important for proper synaptic communication within the CNS. Glutamate is the most abundant, and perhaps the most important, excitatory neurotransmitter in the mammalian CNS and it is important in synaptic plasticity, learning and memory, and

development (Maragakis *et al.,* 2005). The rapid removal of glutamate from the synapse is crucial for synaptic function and the astrocyte is responsible for most of the glutamate transport in the CNS (Schlüter *et al.,* 2002; Hertz & Zielke, 2004; Maragakis *et al.*, 2005). Glutamate uptake is accomplished by high affinity Na^+ dependent transports and astrocytes have two types of transporters known as GLAST (glutamate /aspartate transporter) and GLT-1 (glutamate transporter 1) (Gegelashvili and Schousboe, 1997; Swanson *et* al., 1997; Schlüter *et al.*, 2002). Astrocytes are very efficient at sequestering glutamate and they have intracellular glutamate concentrations 3,000-10,000 times greater than the extracellular space (Panickar and Norenberg, 2005).

The removal of glutamate from the extracellular space is not only crucial for synaptic function but it is also important for neuronal protection. Following ischemic injury astrocytic glutamate transporter function is compromised and the increased levels of glutamate in the extracellular space are thought to play a role in neuronal loss (Panickar and Norenberg, 2005). Some research has centered on administering N-methyl-D-aspartate (NMDA) receptor antagonists to diminish the effects of glutamate induced neurotoxicity (Dingledine *et al.,* 1999). NMDA receptors are an ionotropic sub-type of glutamate receptor that are present in neurons and they have been shown to be important in development, learning and memory, synaptic plasticity and motor and sensory functions (Maragakis *et al.,* 2005; Xin *et al.,* 2005). Several neurodegenerative diseases are starting to be linked to reduced glutamate transport function and include Alzheimer's disease, amyotrophic lateral sclerosis, and Huntingon's disease (Gegelashvili and Schousboe, 1997; Schlüter *et al.,* 2002).

Within the astrocyte glutamate is metabolized to glutamine which is than released and transferred to the neuron where it is converted back to glutamate (McKenna *et* al., 1996;

Hertz & Zielke, 2004). This glutamate-glutamine-neuron-astrocyte cycling is required because neurons lack the ability to produce the building blocks required for glutamate production. Glutamate is unable to pass the blood brain barrier and all of the glutamate in the CNS must be produced *in situ* (Hertz & Zielke, 2004). The astrocyte is able to compensate for glutamate degradation by producing new glutamate from glucose (Hertz & Zielke, 2004). Without the astrocyte, glutamate would not be an effective neurotransmitter.

Other studies indicate that astrocytes can interact with and modulate neuronal function. Astrocytes are capable of propagating calcium waves in response to glutamate and these calcium waves can spread to other functionally connected astrocytes (Haydon, 2001; Fumagalli *et al.,* 2003; Newman 2003). It is also known that astrocytes release excitatory amino acids (Purpura *et al.,* 1994; Jeftinija *et al.,* 1996; Jermic *et al.*, 2001) and ATP (Guthrie *et al.*, 1999; Newman 2003) in response to intracellular calcium increases. This raises the possibility that the astrocyte may be an active participant in modulating synaptic activity (Araque *et al.,* 1999; Haydon 2001; Newman 2003; Perea and Araque 2005). Recent research by Perea and Araque (2005) suggests that astrocytes are capable of discriminating between the activity of different synapses that belong to different axons. Furthermore they showed that Ca^{2+} increases that occurred within the astrocytes, secondary to synaptic activity, were capable of eliciting NMDA receptor currents in other neurons. The astrocytic synaptic modulation that could occur is even more significant when we look at the number of synapses that one astrocyte could be associated with. It has been estimated that each astrocyte occupies a volume of roughly 66,000 μM^3 of nervous tissue and in the CA1 region of the rat brain it has been estimated that every 100 μM^3 of tissue contains approximately 213 synapses (Bushong *et al.*, 2002). This means that one astrocyte could be involved with as

many as 140,000 synapses (Bushong *et al.*, 2002). The shear number of synapses that one astrocyte can be associated with, and their ability to propagate calcium waves to other astrocytes could result in wide spread synaptic modulation. All of this data raises the possibility that astrocytes could be involved in information processing in the CNS (Perea and Araque, 2005).

Astrocytes not only modulate synaptic activity but they also promote the formation of functional synapses and are vital in the maintenance of synapse function (Mauch *et al.,* 2001; Song *et al.,* 2002a; Slezak & Pfrieger, 2003). In the rodent brain, astrocytes increase in number around the time of birth, which coincides with increased synaptogenesis that will continue for 2-3 weeks after birth (Slezak and Pfrieger, 2003). In pure cultures of retinal ganglion cells few functional synapses form unless they are cultured in the presences of glial conditioned media or co-cultured with astrocytes (Barres and Smith 2001; Ullian *et al.,* 2001). The soluble astrocytic factors that are responsible for synaptogenesis are lipoprotein ApoE and cholesterol (Mauch *et al.,* 2001). Astrocytes also appear to be important in helping stabilize the synapse once it is formed (Mauch *et al.,* 2001; Ullian *et al* 2001; Pfrieger 2003). Astrocytes also influence the functional characteristics of synapses by increasing synaptic efficacy (Ullian *et al.,* 2001; Song *et al.,* 2002a), influencing receptor expression (Liu *et al.,* 1997; Ullian *et al.,* 2001) and modulating voltage gated calcium channel function (Mazzanti and Haydon, 2003). The influence of astrocytes on synaptic properties also extends to NSC derived neurons. Imaging studies also show that NSC derived neurons, grown in the presence of astrocytes, have both an increase in the number and functionality of synapses (Song *et al.,* 2002a). NSC grown in co-culture with astrocytes were found to generate neurons that had active recycling of vesicles in response to depolarization and in voltage

clamp experiments about 40% of the differentiated neurons showed spontaneous synaptic currents (Song *et al.,* 2002a).

Additional research suggests that astrocytes in different regions of the CNS help form specialized niches that influence stem cell differentiation. Within these niches, astrocytes release factors that influence the resident progenitor and stem cells into gliogenic or neurogenic types of differentiation (Horner & Palmer, 2003). Work done by Song *et al.* (2002b) showed that neural stem cells were six times more likely to become neurons if they were grown in the presence of embryonic astrocytes from the hippocampus. Furthermore, they demonstrated that adult astrocytes from the same region were only half as effective at inducing neuronal differentiation. To determine if this effect was unique to astrocytes of the hippocampus they conducted the same types of experiments using astrocytes from the spinal cord. This showed that only the embryonic spinal cord astrocyte would help promote a neuronal differentiation, but to a lesser degree when compared to the hippocampal population. These observations are consistent with *in vivo* findings where adult neurogenesis occurs in the hippocampus and not in the spinal cord (Palmer *et al.,* 1997; Gage 2000, Tempel 2001). In fact, most of the astrocytes found in the brain are not supportive of neuronal differentiation (Goldman, 2003). This also demonstrates that astrocytes are not a homogeneous population of cells, and that astrocytes from different brain regions can have very different and unique functional properties (Panickar and Norenberg, 2005).

In a broader sense, if the astrocyte is considered from a phylogenic perspective some interesting things can be noted in the proportion of astrocytes to neurons in relation to brain complexity. Astrocytes are found in higher proportions in more complex nervous systems. In the leech ganglion there is only one astrocyte per 25-30 neurons, and in rats and mice there

is 1 astrocyte per 3 neurons. In the human cortex there are 1.4 astrocytes per neuron (Nedergaard *et al.,* 2003). One explanation proposed for this increase of astrocytes in animals with more complex brains is that more sophisticated neural networks require greater modulation (Nedergaard *et al.,* 2003). Another argument could be that the increase in astrocytes may be responsible for complex thought, learning and memory. With all of this new research evidence, the astrocyte is being unveiled as a central player in many events critical to the function of the nervous system.

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CHAPTER 2. DEVELOPMENT OF FUNCTIONAL NEURONS FROM POSTNATAL STEM CELLS *IN VITRO*

A paper published in Stem Cells and Stem Cells Express[1]

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Abstract

In order for stem cells to fulfill their clinical promise we must understand their developmental transitions, and it must be possible to control the differentiation of stem cells into specific cell fates. To understand the mechanism of the sequential restriction and multipotency of stem cells, we have established culture conditions that allow the differentiation of multipotential neural stem cell from postnatal stem cells. We used immunocytochemistry, fluorescence microscopy, and calcium imaging to demonstrate that progeny of adult rat neural stem cells develop into functional neurons that release excitatory neurotransmitters. We also found that the nontoxic fragment heavy chain (H_C) of tetanus toxin, a toxin which targets neurons with high specificity, retained the specificity towards neural stem cell-derived neurons. These studies show that neural stem cells derived from adult tissues retain the potential to differentiate into functional neurons with morphological and functional properties of mature CNS neurons.

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Introduction

In the last decade there has been an explosion of research related to stem cells. This has led to the identification and isolation of adult stem cells from many organs including the brain, spinal cord, bone marrow, liver, intestine, retina, skeletal muscle, pancreas, cornea, and skin [1]. Adult and embryonic stem cells differ from each other in certain aspects. Embryonic stem cells are derived from the inner cell mass of a blastocyst and are pluripotent, while adult stem cells are derived from differentiated organs and have varying degrees of plasticity [2]. Adult stem cells, like embryonic stem cells, are capable of self-renewal and they can give rise to fully differentiated mature cell types [2].

Neural stem cells (NSC) can generate cells of glial and neuronal lineages and have been identified in the hippocampus, subventricular zone, olfactory bulb and spinal cord of adult mammals [2,3]. They are the object of increasing attention for their potential use in therapies of central nervous system disorders [3-5]. Recent research demonstrates that FBS promotes differentiation of optic nerve oligodendrocyte precursor cells (OPCs) to a type 2 astrocyte, and when followed by exposure to basic fibroblast growth factor (bFGF) these cells revert to a state in which they can not only self-renew but are capable of differentiating into oligodendrocytes, astrocytes, and neurons [6]. The possibility of neural progenitor contamination from other neurogenic regions is remote because the optic nerve was harvested rostral to the optic chiasm eliminating concerns of any neuronal contamination.

OPCs are widely distributed in the CNS and constitute a major cycling population in the brain and spinal cord [7]. In some regions of the adult rat CNS 70% of the dividing cells have been found to be OPCs [7]. In humans OPCs comprise 3% of the cells in the

subcortical white mater [8]. The widespread distribution of these cells and their potential to revert into a cell type that can give rise to all three major cell types of the CNS makes them very appealing therapeutic candidates [9].

Before stem cells can be used successfully as replacement therapies for neurodegenerative disorders we must understand the functional properties of these cells. For a stem cell derived neuron to be considered functional it must be 1) stably differentiated, 2) polarized showing a single axon and multiple dendrites, 3) capable of generating an action potential, and 4) not only be able to release neurotransmitters but also possess receptors for them [10]. In this paper we examine whether cells that are differentiated from OPCs and display morphological characteristics of neurons also have functional properties similar to neurons from other brain regions in culture. Specifically, we demonstrate that NSC-derived neurons release glutamate in a calcium dependent manner and express a set of glutamate receptors.

Methods

Stem Cell Cultures

As described previously [11-13], optic nerves were harvested rostral to the optic chiasm from Sprague-Dawley rats 21 days post-natal. The tissues were minced and digested in a solution of papain (0.01%) and DNase (0.01%) dissolved in EBSS for 50-60 minutes at 37 ° C. Cell and tissue fragments were washed three times with DMEM containing 10% fetal bovine serum (FBS). The tissues were disassociated with a glass pipette and maintained for 24 hours in high glucose DMEM/F12 supplemented with 2.5 ml L-glutamine and 10% FBS in a tissue culture flask kept at 37° C and 5% CO_2. After 24 hours the medium was changed to a growing medium made up of DMEM/F-12 and 20 ng/ml bFGF. To compensate for instability of growth factors, the freshly prepared growing medium was changed every 2 days. The cells were split by gently washing the flask. Cells were seeded to poly-L-lysine coated coverslips for 3-5 days before being used in experiments. In experiments where the NSC were differentiated the medium consisted of DMEM/F-12, 1%FBS, all trans-retinoic acid (100 ng/ml), bFGF (1 ng/ml), and 0.5 ml of penicillin and streptomycin per 50 ml. All of the cells that were used in experiments were in culture for at least 3 weeks.

Intracellular calcium imaging

Intracellular calcium concentrations $[Ca^{2+}]_i$ were evaluated by ratiometric imaging techniques. Cells were loaded with Fura 2-AM for 40-60 minutes at room temperature. The loading solution contained 1μl of 25% (w/w) Pluronic F-127 and 4 nM of Fura 2-AM diluted in 1 ml of HEPES buffer. The loading solution was removed and the culture was incubated

another 10 min in HEPES buffered solution to allow for de-esterification of Fura 2-AM. The coverslips were then placed onto a perfusion chamber and connected to a micro pump with a flow rate of 200 µl/minute. The test substances were placed in syringes on a six valve manifold and applied into the perfusion chamber by the same perfusion system. As result of spatial distance between the syringe and the culture in the chamber there was a time delay between the turning on the valve and onset of the response.

All image processing and analysis was performed using an Attoflour system with an inverted Zeiss microscope. Wavelengths of 340 and 380nm were used to excite the Fura-2 and the emitted light was collected at 520 nm. Background subtraction and ratio images were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 Grynkiewitz et al [14].

Release methods and HPLC

The release of excitatory amino acids was determined using the procedure previously described [15]. Briefly, the coverslips with stem cell cultures were mounted into a 50 µl perfusion chamber and perfused at 200µl/min with normal HEPES buffer (in mM: NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, glucose 5 and HEPES 10 ; pH 7.4) for a period of 30 to 40 min to allow equilibration. After equilibration, 200 µl samples were collected every minute. Four control samples were collected for determination of basal concentrations of amino acids. Test substances were dissolved in the recording solution and delivered in known concentrations to the cultures. The amino acid content in the samples was determined by high-performance liquid chromatography (HPLC) with fluorescence detection. Prior to injection, aliquots of the samples were derivatized with o-phthalaldehyde (OPA) 2-mercaptoethanol reagent (Pierce). Chromatography was performed on a 15cm Microsorb-

MV HPLC column (Rainin Instrument Co.) using a pH 5.9 sodium acetate methanol gradient. Basal rates of amino acids released were determined as the mean of the amino acids in 4 samples collected just prior to stimulation.

Immunocytochemistry

Immunocytochemistry was performed using monoclonal antibodies raised against glial fibrilary acidic protein (GFAP; 1:5000; ICN Immunobiological) and microtubule-associated protein MAP-2 (1:2000; Boehringer), and polyclonal antibodies raised against synaptotegmin (Sigma 1:5000). Inactivation of endogenous hydrogen peroxidase was achieved by rinsing the culture for 15 min in 0.3% H_2O_2 in 50mM KPBS. Normal horse serum (1:1000; Vector Lab, for monoclonal antibodies) and normal goat serum (1:1000; Vector Lab, for polyclonal antibodies) were used to block endogenous antibodies. Primary antisera were diluted at above dilution and incubated overnight at room temperature. Secondary antibodies and ABC kit were applied at room temperature using dilutions recommended by the supplier (Vectastain, Vector Lab). Negative controls were processed by omitting the specific antiserum.

Results

Morphological characterization of NSCs

To determine whether there were progenitors in the optic nerve with a latent ability to generate neurons, the optic nerve was harvested, dissociated, and fractionated. As described by Kondo and Raff [6] short term exposure of acutely dissociated OPCs to FBS followed by culturing in serum free medium with bFGF predictably resulted in cells expressing neuronal markers. In contrast, less than 5% of cells cultured in platelet-derived growth factor or FBS for a month or longer were microtubule-associated protein 2 (MAP2) positive [6]. These results demonstrate that OPCs cultured sequentially in FBS and bFGF will generate NSC as a result of treatment rather than the effect of time in culture. Using this protocol, but in the tissue culture flasks without poly-L-lysine coating, the cells proliferated in bFGF and created neurosphere bodies for many weeks and months. After several weeks in culture, a small but significant minority of cells were MAP2 positive neurons when induced to differentiate. Neurons were often found in small clusters, suggesting a clonal derivation. Figure 1A. illustrates MAP2 antibody stained cells that were exposed to differentiation media (Fig 1A), indicating that the cells were neurons and that few if any non-neuronal cells were present. The high percentage of MAP2 positive cells grown under these conditions is constant with the findings of Kondo and Raff [6]. In addition to expressing neuronal microtubule marker most of the cells (over 90%) reacted with secretory protein synaptotegmin antibody (Fig 1B). Most of the synaptotegmin immunoproduct labeling was confined to the cell bodies of the

differentiated cells which is a characteristic of young neurons. The fact that these neurons were generated from cells isolated from the optic nerve dispels any concerns of contamination from other known neurogenic zones within the central nervous system.

To further assess the morphological characteristics of neurons derived from NSC we exposed these cells to the heavy chain (H_C) component of tetanus toxin. The C-terminal portion of the H_C is responsible for the neurospecificity of tetanus toxin. Recent studies demonstrated that the H_C of the tetanus toxin is a multivalent oligosaccharide-binding protein and the sugar-binding site in the C-terminal domain insures specific binding of tetanus toxin to carbohydrate-containing receptors on the neuronal membrane [for review see 16]. Differentiated NSC were exposed to the heavy chain component of tetanus toxin that was labeled with fluorescein isothiocyanate (FITC) (Fig. 1C). The cultures were then examined using confocal microscopy and over 90% of the cells in culture were FITC labeled. This demonstrates that the tetanus toxin has a high affinity for the differentiated NSC neurons and further illustrates that these cells are morphologically similar to neurons.

Functional Identification of NSC

Several independent criteria were used to functionally identify NSC as cells that possess functional properties similar to that of functional neurons. These included the presence of voltage gated calcium channels, ligand gated calcium channels, and ionotropic glutamate AMPA (α amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. Coverslips containing NSC were mounted onto a fast rate exchange perfusion chamber for

imaging experiments. Fura –2 calcium imaging was used to examine the stimulatory effects of a nonselective depolarizing stimulus, 50mM K^+, and two neurotransmitters, ATP and AMPA.

In order to determine the presence of voltage gated calcium channels in cultured cells 50mM K^+ was used. Elevated potassium concentrations are commonly used to functionally identify neurons [17]. In resting conditions, the cytoplasmic calcium level of NSC was 91 $\pm$ 2 nM (n=201). Brief perfusion application of 50mM K^+ (2min) produced an increase in the level of calcium in 97% of the cells studied (n=194; Fig. 2). This increase of intracellular calcium reached the peak level of 207 $\pm$ 7 nM (n=189; Fig. 2) about 80 seconds (mechanical delay) after the initiation of 50mM K^+ application and was sustained for several minutes. Removal of external calcium from the bathing medium abolished the potassium induced calcium transients in NSC-derived neurons (Fig. 3), indicating that the increase in intracellular calcium was dependent on external calcium sources. It has been well documented that perfusion application of 50mM K^+ on astrocytes was without effect on intracellular calcium concentration [15, 17]. These data demonstrate that NSC-derived neurons express voltage dependent calcium channels similar to that of functional neurons.

Activation of purinoceptors leads to an increase in intracellular calcium in a variety of excitable and unexcitable cells [18]. Two mechanisms are involved in ATP-evoked intracellular calcium increase. First, ATP can activate ATP-gated cation channels [19]. This first mechanism is consistent with the P2X sub-type of receptor [20]. Second, extracellular ATP can stimulate the breakdown of inositol phospholipids and the resulting increase in inositol 1,4,5,-triphosphate (IP_3) is responsible for the elevation of cytosolic Ca^{2+} [15]. This mechanism is coupled to the P2Y type of receptor. Astrocytes have a relatively small

population P2X receptors, but a large population P2Y receptors that mediate increases in intracellular calcium [15, 20]. Neurons also display both the P2X and P2Y receptor, but the P2Y receptor mediates slow changes in membrane potential [20]. This evidence prompted us to use ATP in combination with low extracellular calcium to functionally discriminate astrocyte-like from neuron-like cells.

Figure 2 illustrates a typical response of differentiated NSC cultures to perfusion application of 50mM K^+ followed by perfusion application of 100μM ATP for 1 min. Application of ATP induced an increase in calcium level in 83% of cells (180 of 216 cells, in 8 experiments), and this effect was completely abolished in 87% of the cells bathed in low calcium (121 of 139 cells, 6 independent cultures). This finding strongly suggests that the differentiated cells have a P2X type of purinergic receptor which is consistent with neurons, and that few non-neuronal cells are present.

To further assess the functional characteristics of differentiated cells we investigated whether these cells were capable of releasing glutamate in response to ATP stimulation. The release of glutamate from NSC cultures was assayed using HPLC on the superfusate. The basal release of glutamate in to the superfusate was 19 ± 2 nM ($p<0.01$). Addition of 100 μM ATP caused an increase in release of glutamate from differentiated NSC cultures to 30 ± 7 nM ($p<0.01$) (Fig. 2B).

To confirm that optic nerve derived stem cells were mainly of neuronal phenotype, we plated NSC onto established cortical astrocyte cultures and stimulated them with potassium and ATP. As it can be seen in Figure 3 there were two kinds of cells. One group of cells responded to potassium and ATP in normal calcium but failed to respond to either in low calcium (Fig. 3, Ca^{2+} transients of cells in upper set of tracings). The second group of

cells did not respond to potassium but responded to ATP in both normal (2mM) calcium and low (26nM) calcium HEPES buffer (Fig. 3; lower set of tracings). For functional identification the cells in upper tracings were identified as neuron like cells and lower tracing cells were identified as astrocytes.

Fast excitatory transmission between the neurons of the central nervous system occurs when glutamate directly activates AMPA and kainate receptors. AMPA receptors lacking the GluR2 sub-unit are permeable to Ca^{2+} [8]. To further functionally characterize NSC, we applied 10 μM AMPA for 1 minute. Figure 4 illustrates the typical response of differentiated NSC to applications of potassium and AMPA. Stimulatory effects of AMPA were abolished in low calcium, indicating that AMPA receptors in NSC are permeable to extracellular Ca^{2+}. Perfusion application of potassium and 10 μM AMPA were without effect on enriched cortical astrocyte cultures (Fig. 4 B).

Discussion

Our data demonstrates that rat oligodendrocyte precursor cells grown under the proper conditions are capable of differentiating into cells that have both morphological and functional characteristics similar to neurons. The morphological findings are consistent with the observations of Kondo and Raff [6] and Nunes et al. [8]. Our findings that differentiated cells respond to both depolarizing stimuli (50mM K) and glutamate receptor agonist (AMPA) are consistent with the previously described work by showing that a large percentage of NSCs have voltage dependent Ca^{2+} channels and widespread presence of glutamate receptors [8]. In addition, we demonstrated that the differentiated cells have ATP receptors that are

ligand gated Ca^{2+} channels as indicated by the lack of increase in $[Ca^{2+}]_i$ in the presence of a low Ca^{2+} extracellular environment.

Functional differentiation of neurons is a critical first step in assessing their potential for therapeutic use. Part of the criteria used to assess neuronal functional differentiation includes not only the ability to respond to neurotransmitters but also the ability to release them [10]. To do this we demonstrated that these cells have immunoreactivity to synaptotegmin, which indicates that at least part of the machinery necessary for synaptic release is present. The ability of differentiated cells to release glutamate in response to ATP exposure was confirmed by measuring the glutamate levels in the superperfusate using HPLC analysis. This further demonstrates that these differentiated cells have functional capabilities similar to that of neurons. To our knowledge this is the first demonstration that neurons derived from OPCs are capable of releasing glutamate.

This is an example of a glial restricted progenitor cell taking on the characteristics of a neural stem cell. This raises the question of cell plasticity and transdifferentiation [21, 22]. In theory, this is a population of somewhat restricted cells, but with the proper external cues they are capable of breaking out of their glial progenitor niche and generate cells with both the morphological and functional characteristics of neurons. These data suggests that environmental cues are important in maintaining and determining the fate of progenitor cells and it is not solely do to the lineage commitment of the cell [22-24]. It is known that cells in culture for periods of time have the potential for mutation, which would allow for more plasticity *in vitro* than *in vivo* [25]. However, it has been shown that progenitor cells acutely

isolated from non-neurogenic regions can generate neurons [25]. These results could also be explained by the isolation of another, yet unidentified, neural stem cell that were co-isolated with the OPC.

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Figure 1. Neuronal marker expression in optic nerve derived NSC cultured for 6 months. **A.** 90 % of the cells grown in differentiation media show immunoreactivity for MAP2 antibody. Both individual and cell clusters were stained. **B.** In addition to expressing MAP2 over 90% of the cells reacted with secretory protein synaptotagmin. Note the higher concentration of immunoproduct in the cell body, which is characteristic of young neurons. Arrows indicate cells outside the cluster that expressed immunoproduct in bodies only. **C.** FITC labeled heavy chain tetanus toxin has the same tropism for the NSC differentiated neurons as it does for neurons. Scale bar is 20μm.

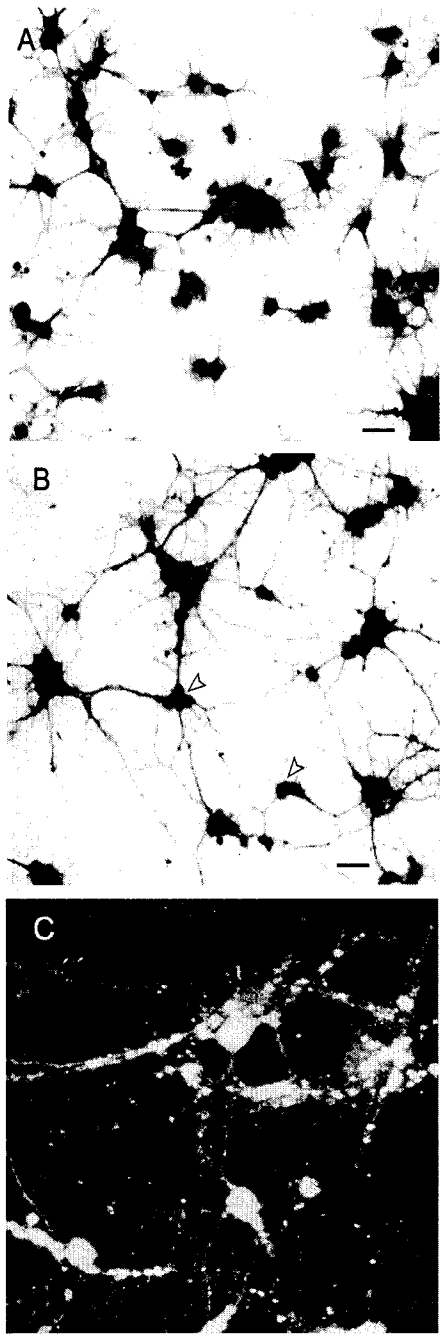
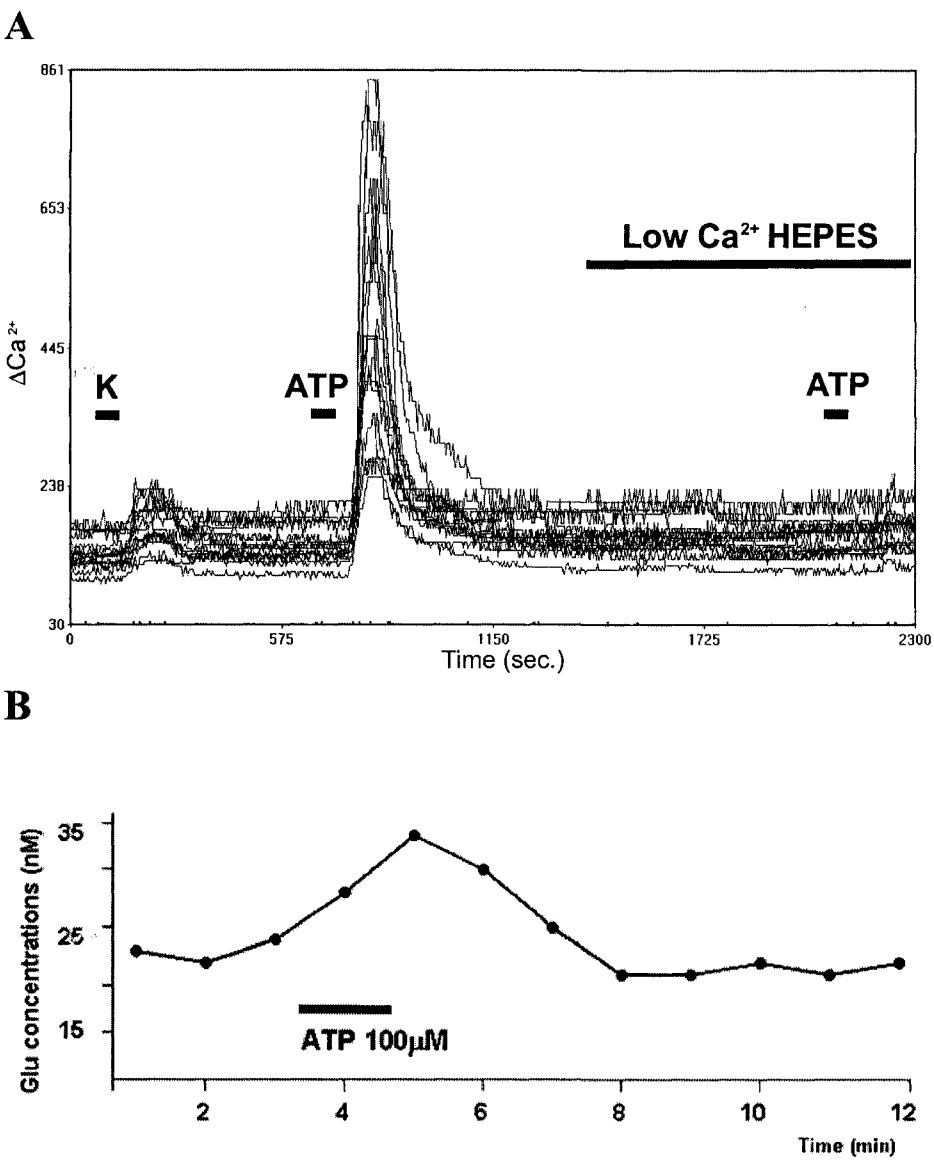


Figure 1.

Figure 2. Kinetic changes of $[Ca^{2+}]_i$ and glutamate release in differentiated NSC in response to perfusion application of 50 mM K^+ and ATP.

A. Perfusion application of 50mM K^+ and 100μM ATP in the presence of low Ca^{2+} was without effect.

B. The increase in intracellular calcium induced by perfusion application of ATP coincided with the increase in the release of glutamate from differentiated NSC.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4
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8
10
12
Time (min)

Figure 2.

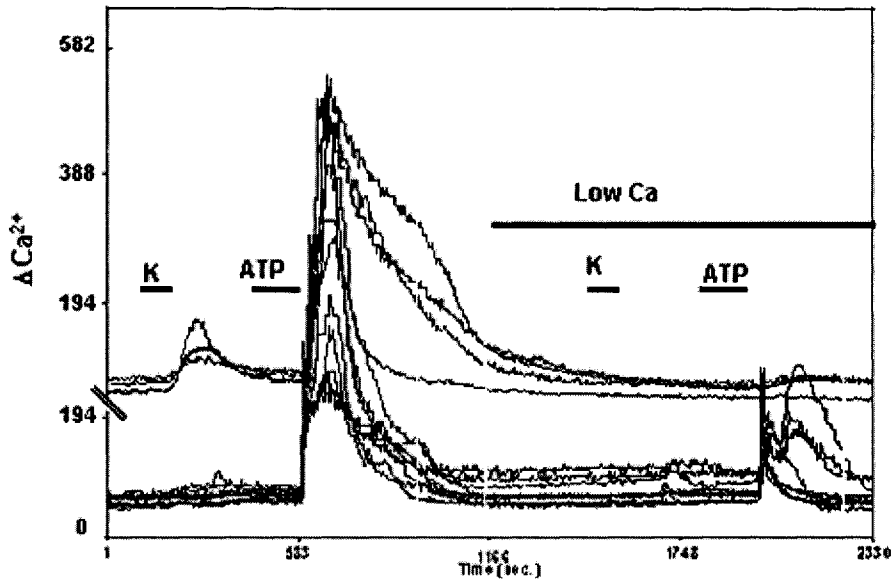
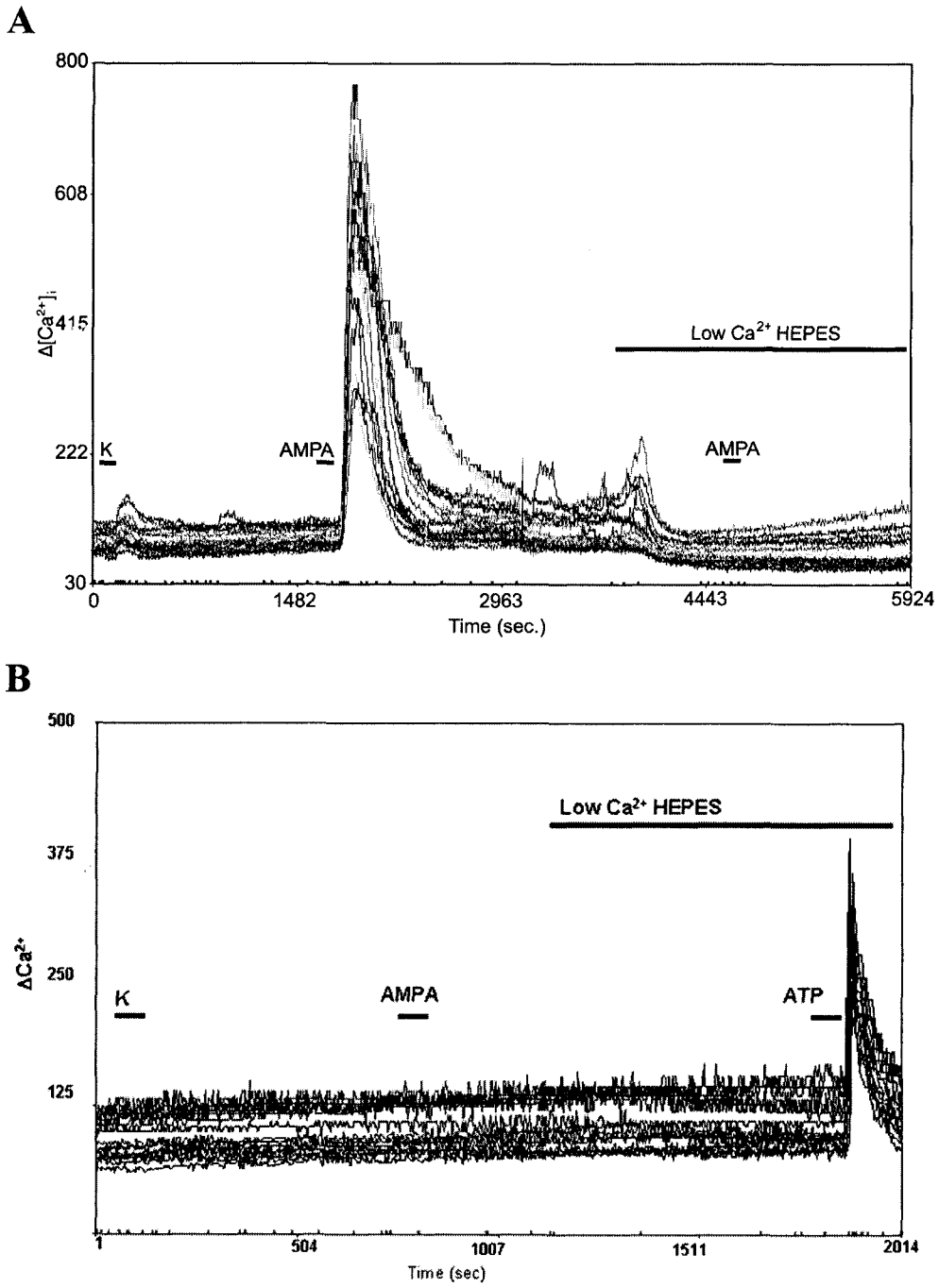


Figure 3. Kinetic changes of $[Ca^{2+}]_i$ in a mixed NSC astrocyte culture in response to perfusion application of 50 mM K^+ and 100 μM ATP. The group of cells in the upper set of graphs responded to both K^+ and ATP under normal conditions but failed to respond in the presence of a low extracellular calcium solution. This type of response is characteristic of neurons. The cell kinetic tracing in the lower set of graphs failed to respond to 50mM K^+ under both conditions but responded to ATP which is characteristic of an astrocyte. The separation of the graphs is done artificially using software in order to make the effect more obvious.

Figure 4. Kinetic changes of $[Ca^{2+}]_i$ in a NSC culture and enriched astrocyte culture in response to perfusion application of 50 mM K^+ and 10 μM AMPA.

A. The AMPA response is abolished in low calcium HEPES indicating that the increase in $[Ca^{2+}]_i$ is dependent on extra-cellular calcium.

B. Both 50mM K and AMPA failed to induce calcium increase in enriched astrocyte culture, while cells responded to application of ATP in low Ca.


A
800
608
415
222
30
Δ[Ca2+]i
K
AMPA
AMPA
Low Ca2+ HEPES
0
1482
2963
4443
5924
Time (sec.)
B
500
375
250
125
ΔCa2+
Low Ca2+ HEPES
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AMPA
ATP
1
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1511
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Time (sec)

Figure 4.

CHAPTER 3. ASTROCYTES INFLUENCE NMDA RECEPTOR EXPRESSION IN POSTNATAL NEURAL STEM CELLS

A paper submitted for publication in *Glia*

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Abstract

During an investigation into the role of astrocytes in fate specification of adult neural stem cells (NSC) we discovered that astrocytes influence the neuronal response to N-methyl-D-aspartate (NMDA). Using a combined morphological and calcium imaging approach, we showed that the presence of an NMDA response in postnatal neural stem cells was dependent on the close proximity of GFAP positive astrocytes. This finding, together with recent reports that astrocytes modulate synaptic transmission and synapse formation in both embryonic neurons and neural stem cells contributes to the notion that astrocytes play an active role in development and function of the central nervous system.

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[2] Primary researcher and author
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[4] Assisted with data acquisition, analysis, and cell culture
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Introduction

The first evidence that neurons could be generated in the adult central nervous system (CNS) came from Altman and Das in the 1960's. Despite this fact the presence of a stem cell population giving rise to neurons in the adult mammalian CNS was not widely accepted until the 1990's when Reynolds and Weiss (1992) isolated cells from the adult mouse striatum that could give rise to both neurons and glia. Since then neural stem cells (NSC) have been identified in the hippocampus, subventricular zone, olfactory bulb and spinal cord of adult mammals (Gage, 2000; Kirschstein and Skirboll, 2001). It has been demonstrated that NSCs can generate cells of both glial and neuronal lineages *in vitro* when they are exposed to certain neurotrophic factors (Ahmed *et al.*, 1995; Koblar *et al.*, 1998; Zigova *et al.*, 1998), and *in vivo* after transplantation (Campbell *et al.*, 1995; Craig *et al.*, 1996; Carpenter *et al.*, 1997; Winkler *et al.*, 1998).

Recent research has demonstrates that cultured oligodendrocyte precursor cells that are first exposed to bone morphogenetic proteins followed by basic fibroblastic growth factor exhibit characteristics very similar to NSC and are capable of both glial and neuronal differentiation (Kondo and Raff 2000, Nunes *et al.,* 2003, Kondo and Raff 2004 a,b). Not only do these cells possess morphological markers that are consistent with neuronal differentiation but they also have functional characteristics similar to neurons (Rowe *et al.,* 2005). The widespread distribution of these cells (Dawson *et al.,* 2003; Nunes *et al.*, 2003) coupled with their potential for both neuronal and glial differentiation could make them a very enticing therapeutic candidate.

The local environment in which stem cells are found influences both the differentiation and functional characteristics of the differentiated cells (Gage, 2000; Tsai McKay, 2000). Astrocytes account for nearly half of the cell population in the adult brain and are known to have great influence on the CNS environment. Astrocytes help clear neurotransmitters, buffer extracellular ions, provide metabolic and structural support to neurons, and function in axonal guidance (Haydon, 2001; Svendsen, 2002). In addition to these functions astrocytes have been shown to promote synaptogenesis (Barres and Smith 2001; Mauch *et al.,* 2001; Ullian *et al.* 2001), influence the functional properties of the synapse (Newman and Zahs, 1998; Araque *et* al., 1999; Ullian *et al.,* 2001; Song *et al.,* 2002a; Perea and Araque 2005) and effect the differentiation of NSC (Lim and Alvarez-Buylla 1999; Song *et al.,* 2002b; Horner & Palmer, 2003).

A better understanding of how astrocytes influence the differentiation of NSC and what effects this has on both the differentiation and the functional characteristics of differentiated cells is crucial to further research in the area of stem cell transplantation and manipulation. In this paper we investigate the role that astrocytes play in glutamate receptor expression.

Methods

Cell Culture

Neural stem cell cultures were prepared as previously described Rowe *et al.,* 2005. Briefly, optic nerves were harvested rostral to the optic chiasm from 21 day old Sprague-Dawley rats. The tissues were digested in a solution of papain (0.01%) and DNase (0.01%) dissolved in EBSS for 50-60 minutes at 37 ° C. Tissue fragments were washed three times with DMEM containing 10% fetal bovine serum (FBS) and disassociated with a glass pipette. The cells were maintained for 24 hours in high glucose DMEM/F12 supplemented with 2.5 ml L-glutamine and 10% FBS in a tissue culture flask incubated at 37° C and 5% CO_2. After 24 hours in culture the medium was completely replaced to a growing medium which consists of DMEM/F-12 and 20 ng/ml bFGF. The growing medium was partially replaced every 2-3 days. The cells were split by gently washing the flask. For experimentation the cells were seeded to poly-L-lysine coated coverslips 3-5 days before being used. Differentiation medium consisted of DMEM/F-12, 1% FBS, all trans-retinoic acid (100 ng/ml), bFGF (1 ng/ml), and 0.5 ml of penicillin and streptomycin per 50 ml. All cells that were used in experiments were in growing conditions for at least 3 weeks.

Primary astrocyte cultures from embryonic day 17 rat cerebral cortex were established as previously described (Jeftinija et al, 1996). Briefly, the cortex was freshly dissected and enzymatically (papain 20 IU/ml; 1hour at 37°C) and mechanically dissociated and plated onto the flasks. After cultures reached confluence (9-12 days), the flasks were "preshaken" (260 rpm) for 90 min and the medium replaced to remove microglia and dividing type I astrocytes. The cultures were shaken overnight (12-18 hours) at 260 rpm at

37°C. Cultures enriched in type I astrocytes were obtained by trypsinizing (0.25%) the attached cells for 3 min. Trypsin was inactivated by adding 3 ml of Eagle's αMEM supplemented with 10% heat-inactivated FBS. The disassociated astrocytes were plated on poly-L-lysine coated dishes (10 mg/ml). All experiments were conducted on cells in culture for 3-5 days after re-plating.

Intracellular calcium imaging

Ratiometric imaging techniques were used to evaluate intracellular calcium concentrations. The cells were placed in a loading solution of 1μl of 25% (w/w) Pluronic F-127 and 4 nM of Fura 2-AM diluted in 1 ml of HEPES buffer and kept at room temperature for 40-60 minutes. The loading solution was removed and the cells were incubated for an additional 10 min in HEPES buffered solution at room temperature to allow for de-esterification of Fura 2-AM. The coverslips with Fura-2 loaded cells were transferred to a perfusion chamber and connected to a micro pump with a flow rate of 200 μl/minute. All test substances were placed in syringes on an inline six valve manifold and delivered to the perfusion chamber by the pump. As result of the distance between the syringe and the chamber there was a mechanical time delay between turning on the valve and the substance reaching the perfusion chamber. All image processing and analysis was performed using an Attoflour system with an inverted Zeiss microscope. Wavelengths of 340 and 380nm were used to excite the Fura-2 and the emitted light was collected at 520 nm. Background subtraction and ratio images were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 Grynkiewitz et al (1985).

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde for 30 min at room temperature. Following fixation the cells were rinsed three times with KPBS. Inactivation of endogenous hydrogen peroxidase was achieved by rinsing the culture for 15 min in 0.3% H_2O_2 in 50mM KPBS and permeabilized in 0.4% Triton X. Normal horse serum (1:1000; Vector Lab, for monoclonal antibodies) and normal goat serum (1:1000; Vector Lab, for polyclonal antibodies) were used to block endogenous antigens. Primary antisera were diluted to 1:5000 for against glial fibrilary acidic protein (GFAP; ICN Immunobiological) and 1:2000 for microtubule-associated protein (MAP-2; Boehringer) and incubated overnight at room temperature. Secondary antibodies and ABC kit were applied at room temperature using dilutions recommended by the supplier (Vectastain, Vector Lab). Negative controls were processed by omitting the specific antiserum.

Results

After developing reliable tools to functionally and morphologically identify neurons and astrocytes in NSC differentiated cultures, we began to search for an N-methyl-D-aspartate (NMDA) response (Rowe *et al.*, 2005). The presence of a response to NMDA application was determined as an increase in intracellular calcium, detected by a calcium imaging system, and used as an indicator for the presence of functional NMDA receptors (NMDAR). To study the role of astrocytes in the expression of NMDAR by NSC, we maintained NSC in differentiation medium, which supports both neuronal and astrocyte differentiation. MAP2- staining revealed that a majority of the cells were neurons (Fig. 1A).

Immunocytochemistry (ICC) for GFAP revealed the presence of a low number of stellated GFAP-immunoreactive (GFAP-IR) astrocytes that were confined to clusters in the NSC cultures (Fig. 1B).

As the morphological identification of astrocytes was done on fixed cells we needed a tool to functionally identify astrocytes in living cultures. Here we have taken advantage of the previously reported fact that astrocytes do, but neurons do not, express metabotropic P2Y receptors and therefore respond to ATP in low Ca^{2+} (ATP/low Ca^{2+}) (Jeremic *et al.,* 2001; Rowe *et al.,* 2005). Functional identification of neurons was achieved by application of 50mM K^+. Neurons respond with an increase in $[Ca^{2+}]_i$ in response to the depolarization induced by 50mM K^+ while non neuronal brain cells fail to respond to application of 50mM K^+ (Morton *et al.,* 1992; Jeftinija *et al.,* 1996; Pasti *et al.,* 1997; Bezzi *et al.,* 1998; Jeremic *et al.,* 2001; Rowe *et al.,* 2005).

Figure 2 illustrates a typical response of differentiated NSC cultures to 50mM K^+, NMDA (10μM), α amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA 100μM) and ATP (100μM). A majority of cells responded to high K^+, AMPA, and ATP and a small proportion of cells responded to NMDA, suggesting a neuronal phenotype. We have previously demonstrated a small fraction of differentiated NSC respond to ATP in low Ca^{2+} (ATP/low Ca^{2+}), suggesting a functional astrocyte phenotype (Rowe *et al.,* 2005). Figure 3 illustrates a typical arrangement of cells within clusters responding to ATP/low Ca^{2+}. However, the response of neurons to NMDA depended on the presence of cells responding to ATP/low Ca^{2+} that were in close proximity (Fig. 3A). Follow-up ICC revealed that cells responding to ATP/low Ca^{2+} were GFAP positive astrocytes (Fig. 3 and 4). In about 25% of the clusters responding to high K^+ we were not able to obtain a response to either NMDA or

ATP/low Ca^{2+} (Fig. 3). ICC on these same clusters indicated the cells were MAP2 positive and GFAP negative.

These initial results suggested that astrocytes may play a determining role in neuron-like cells responsiveness to NMDA. To test whether astrocytes determine NSC response to NMDA we proposed that low density NSC cultures grown on pre-established cortical astrocyte cultures would respond to NMDA. NSC were plated on an established cortical astrocyte feeder layer (developed using procedure described in methodology section) and maintained for 5-7 days. As shown in figure 4 cultures prepared in this way were of low density which allowed us to do $[Ca^{2+}]_i$ imaging experiments in combination with GFAP-ICC to demonstrate that the NSC NMDA response depended on the close proximity to astrocytes. Figure 4 illustrates a typical example of low density NSC-derived neurons seeded on a low density astrocyte feeder layer, in which a proportion of the cells responded to 50mM K^+, a proportion responded to ATP/low Ca^{2+}, and some cells did not responded to either stimulus. Of the cells that responded to 50mM K^+, only a certain number responded to NMDA. All of the cells that responded to NMDA responded to 50mM K^+ and none of the cells that responded to ATP/low Ca^{2+} responded to 50mM K^+. ICC confirmed that the cells responding to ATP/ low Ca^{2+} stained for GFAP and GFAP positive cells or their processes were in close proximity to the cells responding to NMDA (Fig. 4). Plating the NSC on the top of astrocytes at low density resulted in some NSC-derived neurons attaching outside the astrocyte feeder layer and none of those cells responded to either NMDA or ATP/low Ca^{2+}.

Discussion

NMDA receptors are an ionotropic type of glutamate receptor and they have been demonstrated to be important in learning and memory, synaptic plasticity, motor and sensory function, and development (Maragakis *et al.,* 2005; Xin *et al.,* 2005). Our work shows that the presence of an astrocyte with in a field of study greatly increases the likelihood of a NSC differentiated neuron having a response to NMDA. The presence of an astrocyte was confirmed by both GFAP immunoreactivity and functional analysis. Furthermore, only a few of the cells with in a field of study were capable of responding to NMDA indicating that intimate contact between the astrocyte and neuron is most likely required. In previous work, astrocytes have been shown to influence the neuronal response to L-glutamate (Liu et al., 1997; Blondel *et al.,* 2000; Ullian *et* al., 2001; Beattie *et al.,* 2002). Ullian et al., (2001) showed that retinal ganglion cells that were cultured with astrocytes had a threefold increase in inward current secondary to L-glutamate exposure as opposed to RGC that were not grown with glia. Liu and colleagues (1997) demonstrated that E19 hippocampal neurons that were co-cultured on a monolayer of astrocytes expressed significantly greater densities of NMDA receptor currents when compared to neurons grown on either poly-D-lysine or fibroblast coated dishes.

One possible explanation for this increase in NMDAR function could be astrocytic derived activity dependent neurotrophic factor (ADNF). Blondel *et al.*, (2000) showed that ADNF increased the expression of both the NR2A and NR2B subunits of the NMDA receptor in E16 hippocampal neurons. Functional NMDARs require both the NR1 and NR2 subunits (Dingledine *et al.*, 1999). Tumor necrosis factor α (TNFα) is a glial derived

cytokine that has been shown to effect L-glutamate receptor expression. Postnatal rat hippocampal neurons that were exposed to TNFα for 15 minutes showed a twofold increase in the levels AMPAR displayed in the cell membrane (Beattie *et al.,* 2003). The speed at which TNFα is able to induce these changes suggests that it may be acting in a modulatory role (Slezak and Pfrieger, 2003). Astrocytic contact has been shown to increase N-type calcium currents in cultured neurons and one possible explanation for this is the astrocyte modulates the calcium channel (Mazzanti and Haydon, 2003).

All of this demonstrates that NMDAR function is influenced by astrocytes and our data suggests that NMDAR function in NSC differentiated neurons requires the presence of an astrocyte. The exact mechanism by which this occurs has not been determined, but the astrocyte may be providing some type of support to the neuron allowing for receptor expression or modifying the functional properties of the receptor. Lim *et al.*, 1999 proposed that astrocytes may present specialized domains at their cell surface that influences neuronal differentiation and the same could be true for receptor expression. Cell-cell interactions have been shown to be important in determining the morphological differentiation of stem cells (Lim and Alvarez-Buyllia, 1999; Tsai and McKay, 2000; Song *et al.*, 2002). Work done by Song *et al.* (2002b) showed that neural stem cells were six times more likely to become neurons if they were grown in the presence of hippocampal embryonic astrocytes. These observations are consistent with *in vivo* findings where adult neurogenesis occurs in the hippocampus (Palmer *et al.,* 1997; Gage 2000, Tempel 2001).

One potential route for stem cell therapies is one of neuronal replacement. Before this type of therapy can be pursued the functional characteristics of the NSC derived neuron needs to be assessed. The criteria that are used for functional differentiation of neurons

derived from stem cells are 1) the cell must be stably differentiated, 2) polarized showing a single axon and multiple dendrites, 3) capable of generating an action potential, and 4) not only be able to release neurotransmitters but also possess receptors for them (Reh, 2002). Our results, coupled with all of the other research, highlight the importance of the astrocyte in determining the functional properties displayed by a neuron. They also raise a question of validity when evaluating neuronal functional characteristics in culture conditions devoid of astrocytes.

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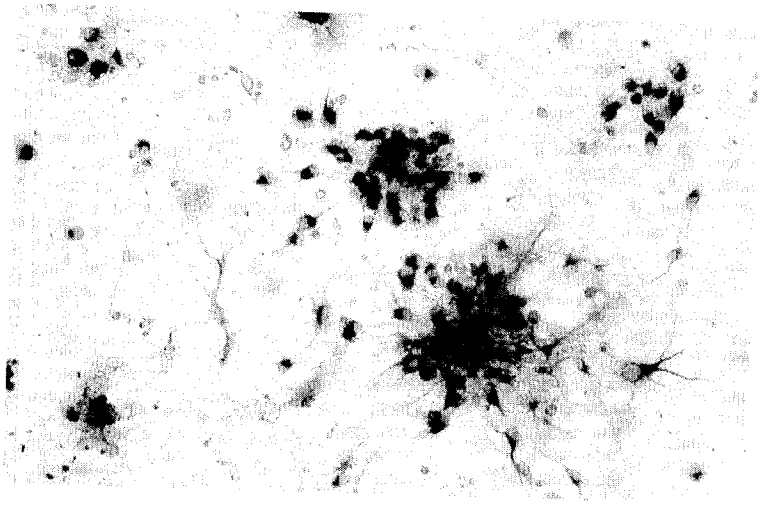
Figure 1. Neuronal and astrocyte marker expression in optic nerve derived NSC culture. **A.** The majority of the cells grown in differentiation media show immunoreactivity for MAP2 antibody indicating a predominately neuronal differentiation. Both individual and cell clusters were stained.

B. A small number of cells in the clusters stained for GFAP. Note that not all of the clusters contained GFAP positive cells (lower cluster). In no instance were GFAP positive cells detected outside the clusters. Scale bar is 20μm.

A



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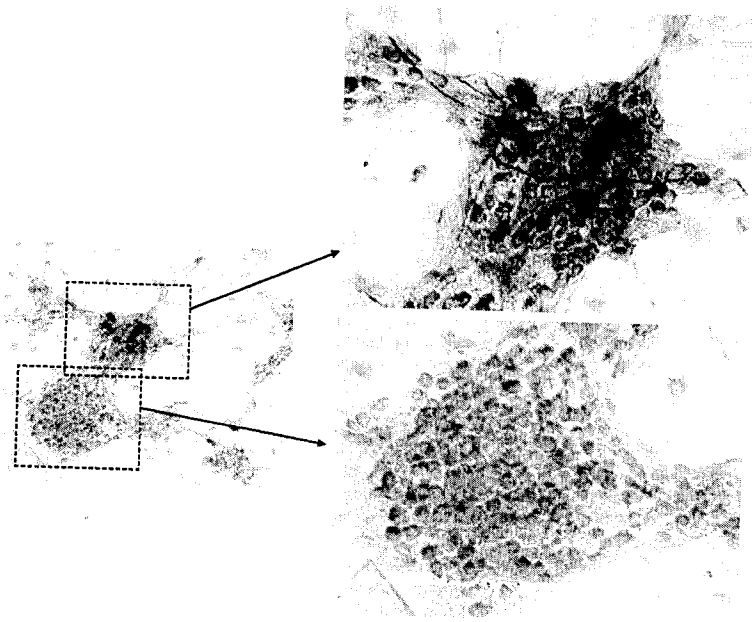

Figure 1

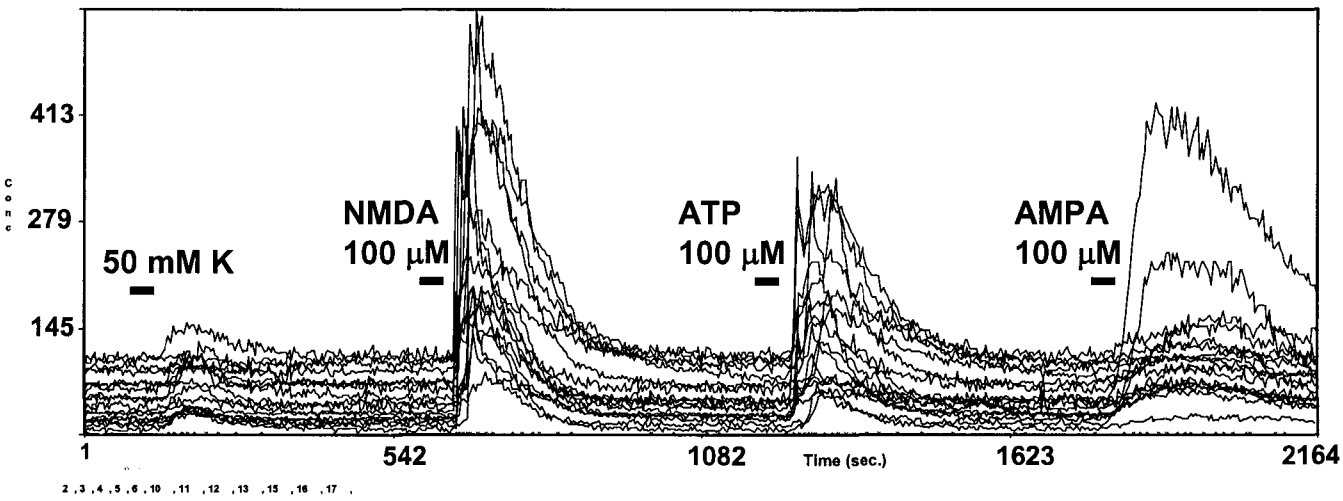

Figure 2. Kinetic changes of $[Ca^{2+}]_i$ in differentiated NSC in response to perfusion application of 50 mM K^+, NMDA, ATP and AMPA. This demonstrates that a majority of the differentiated NSC have functional characters of neurons as indicated by the presence of voltage gated calcium channels and ligand gated ionotropic calcium channels for NMDA, ATP, and AMPA.

Figure 3. Pseudocolor images (**A**) and kinetic changes (**B**) of $[Ca^{2+}]_i$ in a NSC culture in response to perfusion application of 50 mM K^+ , 100 μM NMDA and 100 μM ATPin low Ca^{2+}. The group of four cells in the upper set of graphs (tagged as n in far left panel of pseudocolor images) responded to both K^+ and NMDA but failed to respond to ATP in the presence of low extracellular calcium. This type of response is characteristic of neurons. The two cells kinetic tracing in the lower set of graphs (tagged with * in far left panel) failed to respond to 50mM K^+ and NMDA but responded to ATP which is characteristic of astrocytes. The separation of the graphs is done artificially using software in order to make the effect more obvious.

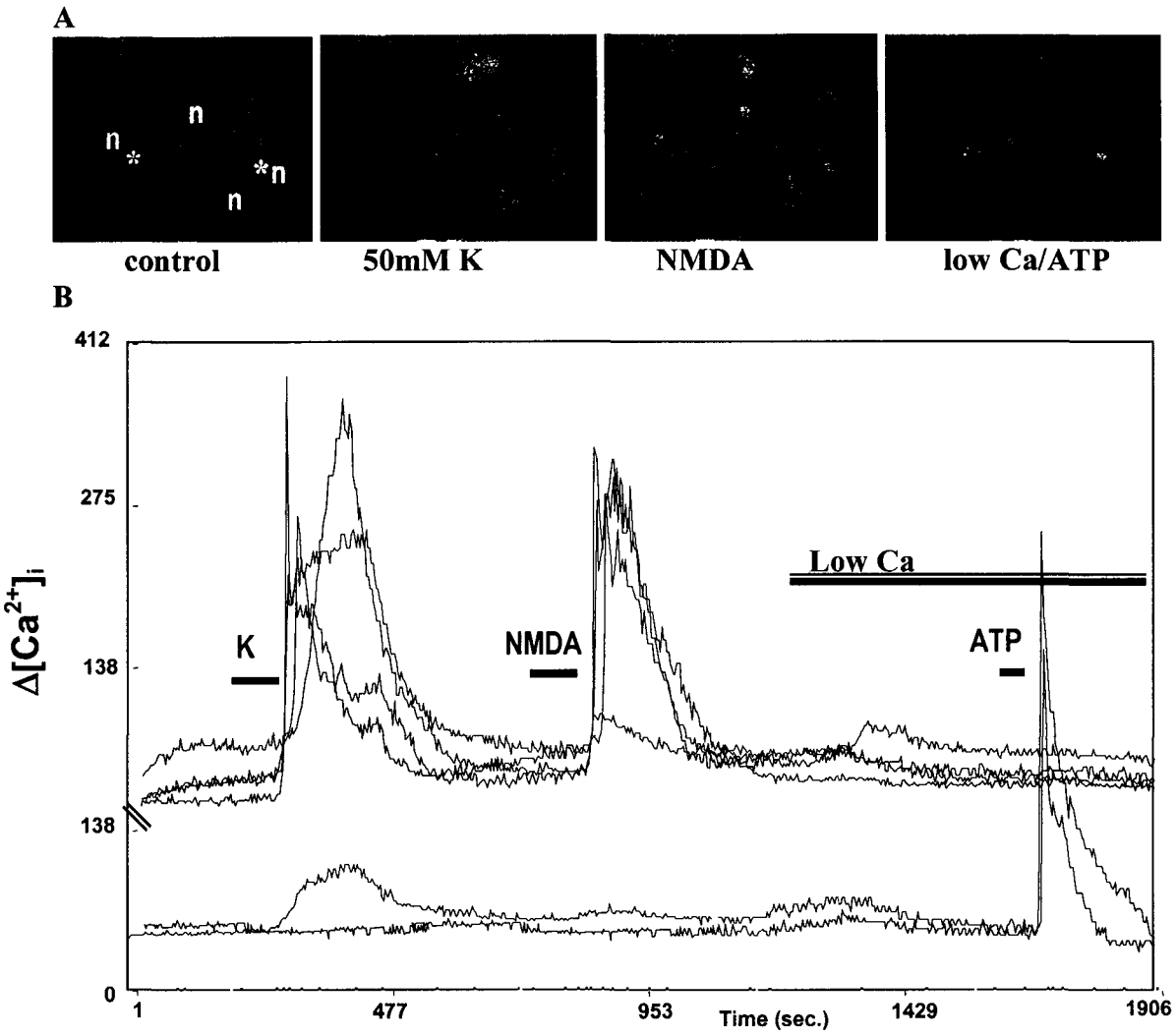
A
control
50mM K
NMDA
low Ca/ATP
B
412
275
138
138
0
$\Delta[Ca^{2+}]_i$
K
NMDA
Low Ca
ATP
1
477
953
Time (sec.)
1429
1906

Figure 3

Figure 4. Kinetic changes of $[Ca^{2+}]_i$ in a mixed NSC astrocyte culture in response to perfusion application of 50 mM K^+, 100 μM NMDA and 100 μM ATP in low $[Ca^{2+}]_e$. The cells in the upper set of graphs responded to both K^+ and NMDA (100 μM) under normal conditions but failed to respond to ATP (100 μM) in the presence of a low extracellular calcium. This type of response is characteristic of neurons. The cell kinetic tracing in the lower set of graphs failed to respond to 50mM K^+ but responded to ATP in low calcium which is characteristic of an astrocyte. The separation of the graphs is done using software in order to make the effect more obvious. The deflection in the baseline $[Ca^{2+}]_i$ of a few nM detected in the lower set of recordings during application of 50mM K^+ and NMDA and the upper set during ATP application are the result of NSC derived neurons growth on the sublayer of astrocytes (see inset) and change in the fluorescence is diffusing into adjacent cell region. The flattened images in the inset are astrocytes while brighter spheres with processes are neuron-like cells.

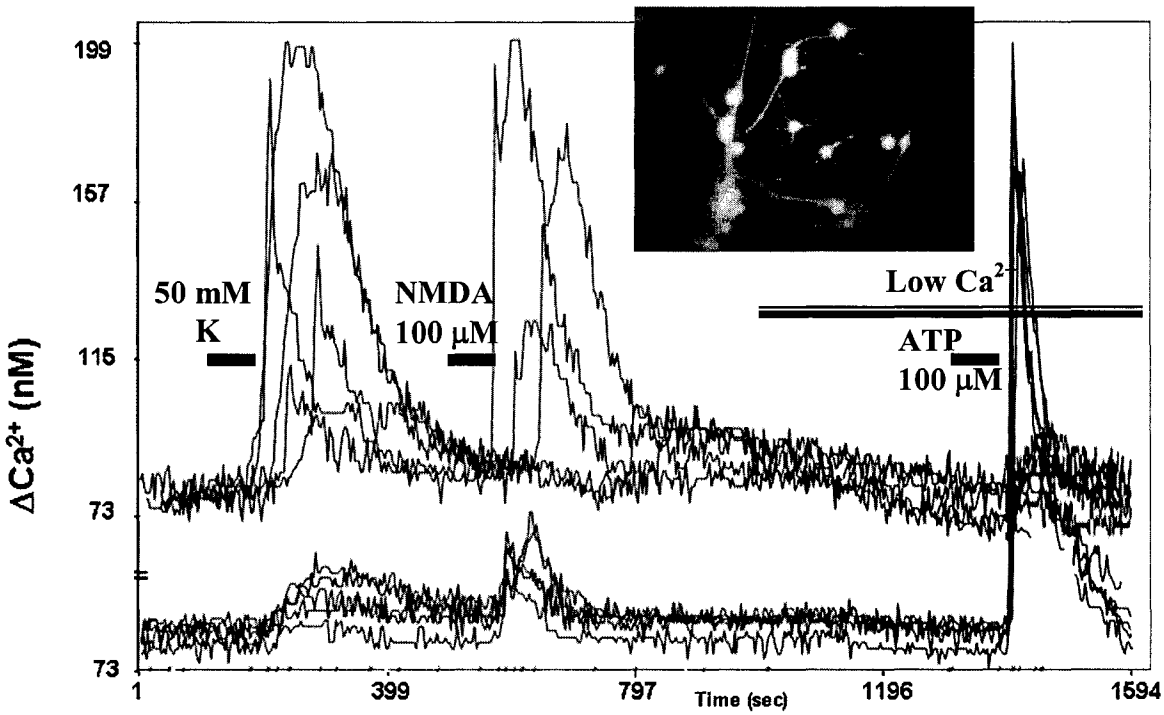
199
157
115
73
73
ΔCa2+ (nM)
50 mM
K
NMDA
100 μM
Low Ca2+
ATP
100 μM
1
399
797
1196
1594
Time (sec)

Figure 4

CHAPTER 4. GENERAL CONCLUSIONS

This dissertation presents the results of an investigation into the morphological and functional properties of neurons that are derived from multipotent neural stem cells and how astrocytes influence these properties. In this work we have demonstrated that these NSC are capable of differentiating into cells with both morphological and functional characteristics of neurons and astrocytes. Furthermore, we have shown that astrocytes influence functional NMDA receptor expression by neurons. Understanding the role that astrocytes play in the functional determination of NSC derived neurons is crucial to using stem cells for neuronal replacement since nearly half of all of the cells found in the CNS are astrocytes. This body of work furthers our understanding on both the functional characteristics that NSC derived neurons posses and how astrocytes influence these properties.

Summary

In the first study, both the morphological and functional characteristics of neurons derived from multipotent neural stem cells were investigated. Immuocytochemistry (ICC) using antibody against glial fibrillary acidic protein (GFAP) and microtubule-associated protein (MAP-2) confirmed the presence of a stem cell population that was capable of both neuronal and glial differentiation. Differentiated neurons were also exposed to fluorescein isothiocyanate (FITC) labeled heavy chain component of the tetanus toxin. Over 90% of the cells that were exposed to this construct were labeled with FITC. This demonstrates that the heavy chain component of tetanus toxin has a high affinity for the differentiated neurons and further illustrates that these cells are morphologically similar to neurons. ICC also revealed

that over 90% of the cells reacted with secretory protein synaptotegmin antibody indicating that at least part of the machinery necessary for synaptic release is present. The ability of differentiated cells to release glutamate in response to ATP exposure was confirmed by measuring the glutamate levels in the superperfusate using HPLC analysis. The basal release of glutamate from differentiated cells was 19 ± 2 nM ($p<0.01$) and exposure to 100 μM ATP caused glutamate levels to increase to 30 ± 7 nM ($p<0.01$). Calcium imaging experiments were used to further assess the functional properties of the differentiated cells and the cells were found to have voltage gated calcium channels, ligand gated calcium channels, and ionotropic glutamate AMPA (α amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. To functionally discriminate between cells of neuronal and glial differentiation their response to ATP was assessed. Our results demonstrate that NSC differentiated neurons are not only able to respond to many neurotransmitters but they also have the ability to release them.

In the second study we showed that the presence of an astrocyte in a field of study increased the likelihood of a differentiated neuron possessing functional receptors to N-methyl-D-aspartate (NMDA). This was accomplished by both functionally and morphologically identifying an astrocyte in a field of study. Functional identification was carried out using calcium imaging and observing 2 criteria 1) absence of a response to 50mM K^+ indicating that no voltage gated calcium channels were present and 2) response of the cells to ATP in the presence of a low calcium external environment which is consistent with a P2Y type of purinergic receptor. In addition to this we confirmed both the morphological and functional differentiation of these cells.

Discussion

Multipotent neural stem cells represent a very unique population of adult stem cells with in the central nervous system (CNS) that are derived from oligodendrocyte precursor cells (OPC). It has been shown that OPC that are first exposed to bone morphogenetic proteins (BMP) followed by basic fibroblast growth factor are able to differentiate into oligodendrocytes, astrocytes, and neurons (Kondo and Raff, 2000). OPCs are widely distributed and in some regions of the adult rat CNS, as many as 70% of the cells that are dividing are OPCs (Dawson *et al.,* 2003). In humans OPCs comprise 3% of the cells in the subcortical white mater (Nunes *et al.*, 2003). The widespread distribution of these cells coupled with their potential to transform into a state that can give rise to all three major cell types of the CNS makes them very appealing therapeutic targets (Kondo and Raff, 2004a).

All current research indicates that OPCs do not become neural stem cells *in vivo,* despite the widespread presence of BMPs. This can in part be explained by Noggin, a BMP antagonist, which is expressed by both oligodendrocytes and type 1 astrocytes (Kondo and Raff 2004b). Kondo and Raff (2004b) demonstrated that over expression of Noggin by OPCs decreased the number of cells that were able to become multipotent stem cells. Conversely they showed that interference of Noggin production by siRNA increased the ability of conversion. This helps to underscore the importance of cell-cell interaction in the maintenance and determination of cell fate.

The local cellular environment is known to influence both stem cell differentiation (Tsai and McKay, 2000; Song *et al.,* 2002) and the functional properties displayed by these cells (Song *et al.,* 2002). Astrocytes constitute roughly 50% of all of the cells that are found

in the CNS and they are known to influences the local cellular environment. The astrocyte has long been considered a general housekeeper of the CNS, in particular to neurons, and functions to provide both metabolic and structural support to the neuron, regulate the extracellular environment by maintaining ionic balance and sequestering neurotransmitters, release growth factors and participate in maintenance of the blood brain barrier (Haydon, 2001; Svendsen, 2002). New research indicates that the astrocyte is important in synaptogenesis (Mauch *et al.,* 2001; Ullian *et al.*, 2001; Pfrieger 2003), capable of modulating synaptic activity (Araque *et al.,* 1999; Haydon 2001; Newman 2003; Perea and Araque 2005), influencing receptor expression (Liu *et al.,* 1997; Ullian *et al.,* 2001) and modulating voltage gated calcium channel function (Mazzanti and Haydon, 2003). In chapter 3 of this dissertation we showed that astrocytes influence functional N-methyl-D-aspartate (NMDA) receptor expression in differentiated NSC derived neurons. NMDA receptors are an ionotropic type of glutamate receptor and they have been demonstrated to be important in learning and memory, synaptic plasticity, motor and sensory function, and development (Maragakis *et al.,* 2005; Xin *et al.,* 2005).

A lot of the excitement related to stem cells lies in the potential use of these cells to cure many human diseases. The potentials for stem cells to be used as therapeutic agents are varied and include providing replacements for lost cells, activation of endogenous stem cell populations, or transplantation of modified stem cells to release specific trophic factors (Cao *et al.,* 2002). If neuronal replacement is desired, than the differentiated neuron must be functional. The criteria that are used to consider a neuron functionally differentiated include 1) a polarized cell showing a single axon and multiple dendrites, 2) the neuron should be stably differentiated, 3) capable of generating an action potential, and 4) not only possess

receptors for neurotransmitters but also be able to release them (Reh, 2002). In chapter 2 of this dissertation we show that differentiated neurons possess ligand gated Ca^{2+} channels and they are able to respond to several neurotransmitters including ATP and AMPA, which is a selective glutamate agonist. Furthermore, we show for the first time that neurons derived from multipotent neural stem cells are capable of releasing glutamate in response to ATP stimulation. The differentiated cells appeared to be stably differentiated *in vitro* and showed the polarization typical of a neuron. All of this answers the first, second and fourth points of the criteria suggested by Reh (2002), and other work has shown that differentiated neurons are capable of generating action potentials. Song *et al.*, (2002) showed that 40% of NSC derived neurons that were grown in co-culture with astrocytes had spontaneous synaptic currents.

All of this demonstrates that we are able to produce functional neurons from stem cells, but the use of stem cells to replace lost neurons is still years away. One of the largest obstacles to overcome is getting NSC differentiated neurons to properly integrate into established neural networks. Embryonic stem cell derived neurons have recently been shown to functionally integrate into the developing rat brain (Wernig *et al.*, 2004). By injecting the cells into a developing brain the researchers were able to take advantage of two things 1) they avoided rejection because the cells were transplanted before the fetus was immunocompetent and 2) the transplanted cells were placed into an environment that was rich in factors promoting neurological development. In contrast, the neurological conditions which require neuronal replacement therapy these advantages are not present and the recipient tissue is destroying neurons making survival and integration an even larger obstacle to overcome. Before stem cell derived neurons can be used in replacement therapies we must understand

the disease process, the biology of the stem cell, and the interaction between the transplanted cell and the recipient environment.

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