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Towards functional regeneration of the central nervous system: glial calcium signaling in reactive gliosis and the therapeutic potential of bone marrow-derived mesenchymal stem cells for retinal degenerative diseases

A dissertation submitted in partial satisfaction of the

requirements for the Degree Doctor of Philosophy

in

Bioengineering

by

Diana Xuan Yu

Committee in charge:

Professor Gabriel Silva, Chair
Professor Shu Chien
Professor Marla Feller
Professor Wayne Giles
Professor William Kristan

2008

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2008

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ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the guidance of my committee members, professors Gabriel Silva, Shu Chien, William Kirsten, Wayne Giles and Marla Feller. I want to thank Dr. Silva for the opportunity to carry out my Ph.D. research work in his laboratory as well as my thesis committee who took time from their busy schedule to provide me with valuable assistance. During my graduate studies at UCSD, I met many bright and fascinating colleagues with whom I shared great companionships. In particular, I want to thank: Mai Ho for her collaboration on the stem cell work; Marius Buibas for his collaboration in implementing and using the dynamic signaling model to map the calcium networks; Jacky, who was a most dedicated undergraduate assistant and a pleasure to mentor through his master thesis; and finally Marie, who managed the lab and kept it in good working order for our experiments.

I also want to express my gratitude to my family for a lifetime of motivation and support, for instilling in me that the road of a scholar is "ten years of frosted window." While I had the good fortune of passing part of that journey in sunny San Diego, it was the unwavering and unconditional faith of my husband that was my comfort and strength.

Chapter 1 is a reprint of the material as it appears in Stem Cell Sources and Therapeutic Approaches for Central Nervous System and Neural Retinal Disorders, Yu, Diana; Silva, Gabriel, Neurosurgical Focus, vol. 24, 2008. Chapter 2 is a reprint of the material as it appears in Comparison of Standard Surface Chemistries for Culturing Mesenchymal Stem Cells Prior to Neural Differentiation, Ho, Mai*; Yu, Diana*; Davidson, Marie; Silva, Gabriel, Biomaterials, vol. 24, 2006. * *contributed equally.*

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Peer-Reviewed Publications

- Yu D, Buibas M, Chow SK, Silva GA. Quantitative analysis of intercellular calcium signaling in glial networks using deterministic mapping of functional complex network structures with single node resolution. (in preparation)
- Yu D*, Chow SK*, Lee I, Silva GA. The associated effects of Alzheimer's amyloid-β-peptide and disrupted calcium homeostasis on astrogliosis. (in preparation)
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- Yu D, Buibas M, Chow SK, Lee I, Silva GA. Characterization of calcium-mediated intracellular and intercellular signaling in the rMC-1 Müller glial cell line. Experimental Biology and Medicine, 2008. (in revision)

- Buibas M, Yu D, Chiao K, Silva GA. Tracking functional signaling in neurons and glia by mapping vector fields of calcium changes using optical flow. J. Neuroscience Methods. 2008. (in revision)

- MacDonald C, Yu D, Silva GA. Diffusion model of ATP signaling in astrocyte networks suggests two distinct mechanisms for intercellular calcium waves. Front. Neuroeng. 2008. (in press)

- Yu D, Silva GA. Stem cell sources and therapeutic approaches for central nervous system and neural retinal disorders. Neurosurgical Focus 2008; 24 (3-4): E11.

- Yu D*, Ho M*, Davidson MC, Silva GA. Comparison of standard surface chemistries for culturing mesenchymal stem cells prior to neural differentiation. Biomaterials 2006 Aug; 27(24): 4333-9.*contributed equally

- Kozak I, Kayikcioglu OR, Cheng L, Falkenstein I, Silva GA, Yu D, Freemen WR. The effect of recombinant human hyaluronidase on dexamethasone penetration into the posterior segment of the eye after sub-tenon's injection. J Ocul Pharmacol Ther. 2006 Oct; 22(5): 362-9.

- Yu D, Volponi J, Chhabra S, Brinker S, Mulchandani A, Singh A. Aqueous sol-gel encapsulation of genetically engineered Moraxella spp. cells for the detection of organophosphates. Biosensors & Bioelectronics 2005; 20: 1433-7.

Books, Book Chapters, Multimedia

- Pathak S, Yu D, and GA Silva. Nanoengineering approaches for the regeneration and neuroprotection of the central nervous system. In: "Nanotechnology for Medicine and Biology". GA Silva, Editor. Springer Life Sciences, New York, New York. (in press)

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ABSTRACT OF THE DISSERTATION

Towards functional regeneration of the central nerous system: glial calcium signaling in reactive gliosis and the therapeutic potential of bone marrow-derived mesenchymal stem cells for retinal degenerative diseases

by

Diana Xuan Yu

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2008

Professor Gabriel Silva, Chair

While great strides have been made in medicine and technology in the recent decades, satisfactory treatment of injuries and diseases in the central nervous system remain largely elusive. Neurodegenerative disorders such as Alzheimer's and Parkinson's disease as well as degenerative retinal disorders such as Age-related macular degeneration and Retinitis pigmentosa are devastating illnesses that severely affect the quality of life of patients and current therapies for them are at best symptomatic treatments. It is no doubt that effective therapeutic strategy for these diseases will require thorough knowledge of the pathogenesis of the diseases as well as development of the central nervous system to not only halt the progression of the degeneration but also repair the loss or dysfunctional tissue in order to restore cognitive functions.

Stem cells have roused great enthusiasm in recent years due to their therapeutic potential to restore function by replacing the lost or dysfunctional cell types. Bone marrow-derived mesenchymal stem cells (BMSCs) are of particular interest as somatic

(adult) stem cells because of its relative ease of isolation and the minimal ethical concerns associated with its use. Numerous studies have suggested the potential of bone marrow-derived mesenchymal stem cells (BMSCs) to differentiate into cell types of all three germ layer. However, as is common with most fast growing research fields, its rapid progress to yield clinical treatments leaves many fundamental questions unanswered. Amongst them, large variations in experimental protocols make comparison of findings between different studies particularly difficult. In the studies presented here, we compare the viability and differentiation potential of BMSCs on standard surface chemistries and report the optimum growth conditions of these cells for neuronal differentiation as well as induction of neural progenitor marker in BMSCs.

In addition, recent studies in neurobiology have revealed significant functional roles of glial cells in regulating synaptic activity and information processing that were previously unappreciated. Specifically, calcium signaling has been highlighted as a potential mechanism of intra- and intercellular communication in networks of neurons and glia that is not only essential in normal physiology but may also play crucial roles in progression of degeneration in disease via processes mediated by the glia. In the studies presented here, we investigate the role of glial calcium signaling in reactive gliosis, an endogenous inflammatory response of the CNS to injury and disease that often aggravates the loss function and hampers effectively recovery. We introduce the rMC-1 Müller glia cell line as a model for studying calcium signaling and present evidence for complex signaling dynamics in intercellular calcium transient propagation within glial networks that may be altered under pathological conditions. In addition, we show that

disruption of glial calcium homeostasis via altered calcium signaling dynamics directly correlates with known hallmarks of reactive astrogliosis and may play an important role in pathogenesis of neurodegenerative disease.

Chapter I

Stem cell sources and therapeutic approaches for central nervous system and neural retinal disorders

Abstract

In the past decades, stem cell biology has made a profound impact on our views of mammalian development as well as opened new avenues in regenerative medicine. Their potential to differentiate into various cell types of the body is the principle reason they are being explored in treatments for diseases where there may be dysfunctional cells and/or lost of healthy cells due to pathology. In addition, other properties unique to stem cells; its endogenous trophic support, ability to home to sites of pathology, and stability in culture to allow genetic manipulation, are also being utilized to formulate stem cell-based therapy for CNS disorders.

In this chapter, we will review key characteristics of embryonic and somatic (adult) stem cells, consider therapeutic strategies employed in stem cell therapy, and discuss the recent advances made in stem cell-based therapy for a number of progressive neurodegenerative diseases in the CNS as well as neuronal degeneration secondary to other abnormalities and injuries. While a great deal of progress has been made in our knowledge of stem cells and it's utility in treating CNS disorders, much still needs to be elucidated regarding the biology of the stem cells as well as the pathogenesis of targeted CNS disease in order to maximize therapeutic benefits.

Introduction

In a broad sense, by definition stem cells are a population of cells capable of indefinite self-renewal that give rise to "daughter" cells committed to specific differentiation lineages through asymmetric cell division. Their ability to control proliferation, differentiation, and apoptosis distinguishes them from neoplastic cells. Embryonic stem cells are primordial cells of the developing blastula capable of generating an entire organism while somatic stem cells reside within individual organs and usually only give rise to cell types specific to that tissue. The normal function of stem cells include the maintenance of homeostasis mediated by providing trophic support, as well as serving as a reservoir for replacing dysfunctional and senescent cells throughout the lifetime of the organism (Thomas, Thomson et al. 2006). The fact that stem cells have the potential to differentiate into various cell types and tissues is the principle reason they are being explored in treatments for diseases where there may be dysfunctional cells and/or lost of healthy cells due to pathology.

The central nervous system (CNS), traditionally believed to have limited regenerative capabilities, does retain a limited number of stem cells in adulthood, particularly in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) that replenishes olfactory bulb neurons (Singec, Jandial et al. 2007; Zietlow, Lane et al. 2007). However, these cells have little capability to participate in CNS repair following degenerative diseases or traumatic injury (Ninomiya, Yamashita et al. 2006), and also present limitations as a cell source for transplantation therapies. This implies that new potential sources of stems cells for CNS therapies need to be considered. Ultimately, the

goals are to replace lost neurons, re-establish functional neural circuitry, and restore neurological function. As such, the investigation and development of stem cell based therapies aimed at the CNS have received a tremendous amount of attention(Ourednik, Ourednik et al. 2000; Pluchino, Zanotti et al. 2005; Miller 2006).

In this chapter, we start by first reviewing some fundamental characteristics of embryonic stem cells (ESCs) and somatic (adult) stem cells, using the neural stem cell (NSC) and bone marrow-derived mesenchymal stem cell (BMSC) as examples. We then consider in more detail three main approaches to neurological stem cell therapies: cell transplantation, neuroprotective strategies, and gene therapy approaches. Finally, we will discuss in depth the recent advances made in stem cell-based therapies for treatment of progressive neurodegenerative diseases in the brain and the neural sensory retina, as well as neuronal degeneration that is secondary to other pathology.

A. Stem cell sources

A wide range of stem cells from various sources is currently being investigated for their potential in stem cell-based therapies for CNS disorders. Although a detailed discussion of their properties and characteristics is beyond the scope of this review, we present here a broad introduction and provide extensive references for the interested reader.

A.1. Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells isolated from the inner cell mass of day 5 to day 8 blastocysts with indefinite self-renewal capabilities as well as the ability to differentiate into all cell types derived from the three embryonic germ layers. This was demonstrated with mouse ESCs (mESCs), first isolated in 1981, where *in vitro* cultures of mESCs could be propagated indefinitely without telomerase mediated cell senescence, and were able to give rise to all cell types of the body both *in vitro* and *in vivo* (Evans and Kaufman 1981; Martin 1981; Nagy, Rossant et al. 1993).

Successful isolation of human ESCs (hESCs) was achieved in 1998, and they were shown to share similar self-renewal and differentiation properties to mESCs(Thomson, Itskovitz-Eldor et al. 1998). Since then, hESCs work has relied heavily on previous studies done on mESCs and embryonic carcinomas (EC), cells derived from testicular teratocarcinomas(Draper, Pigott et al. 2002; Draper and Fox 2003). While identifying precise markers that define undifferentiated ESCs is still a very active area of investigation, it is generally accepted that the transcription factors Oct-4, Sox-2, Nanog, Rex-1 and myc are important for maintaining the pluripotency and self-renewal properties of mouse and human ESCs(Boyer, Lee et al. 2005). However, several lines of evidence caution against over extrapolating mESCs results to hESCs. For example, unlike mESCs which express stage-specific embryonic antigen 1 (SSEA-1), hESCs express SSEA-3 and SSEA-4(Henderson, Draper et al. 2002). In addition, surface antigens associated with matrix keratin sulphate/chondroitin sulphates (TRA-1-60, TRA-1-81, GCTM-2, TG-30, and TG-343) are expressed by hESCs but not mESCs(Laslett, Filipczyk et al. 2003) . And finally, in contrast to mESCs, hESCs have been shown to

express CD9, Thy1, and class 1 major histocompatibility complex (MHC-1) (Henderson, Draper et al. 2002). .

Other research is focusing on identifying signaling pathways necessary for sustaining stem cell pluoripotency so that undifferentiated hESC can be maintained and expanded *in vitro* without potential trans-species contamination associated with xenogeneic feeder layers (Xu, Inokuma et al. 2001; Amit, Margulets et al. 2003)or animal-derived reagents (Stojkovic, Lako et al. 2004). It has been shown that while leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP4) inhibit neural differentiation of mESCs and promote self-renewal under feeder-free conditions (Smith, Heath et al. 1988; Ying, Nichols et al. 2003), it is not sufficient to support undifferentiated hESCs (Reubinoff, Pera et al. 2000; Amit and Itskovitz-Eldor 2002; Daheron, Opitz et al. 2004; Humphrey, Beattie et al. 2004). There is evidence that inhibition of BMP and fibroblast growth factor 2 (FGF-2) signaling are important to maintain undifferentiated hESCs without feeder layer (Xu, Peck et al. 2005).

To date, hESCs have been shown to differentiate into various derivatives of the ectoderm including neural precursors(Reubinoff, Itsykson et al. 2001; Schuldiner, Eiges et al. 2001; Zhang, Wernig et al. 2001), dopamine and motor neurons(Perrier, Tabar et al. 2004; Park, Minn et al. 2005; Yan, Yang et al. 2005; Martinat, Bacci et al. 2006), retinal cells (Banin, Obolensky et al. 2006), keratinocytes (Green, Easley et al. 2003; Iuchi, Dabelsteen et al. 2006), and melanocytes(Zhang, Wernig et al. 2001; Fang, Leishear et al. 2006). In addition, they have also been differentiated into connective and structural tissue cell types as well as cell subpopulations from blood and cardiovascular tissues normally

derived from the mesoderm(Kaufman, Hanson et al. 2001; Levenberg, Golub et al. 2002; Sottile, Thomson et al. 2003; Barberi, Willis et al. 2005; Pilat, Carotta et al. 2005; Wang, Menendez et al. 2005; Karp, Ferreira et al. 2006; Passier, Denning et al. 2006). And finally, they have also been shown to give rise to cell types from the endoderm including pancreatic, hepatocytes and lung epithelium(Assady, Maor et al. 2001; Shirahashi, Wu et al. 2004; Samadikuchaksaraei, Cohen et al. 2006). However, it should be appreciated that most of these differentiated cell types were assessed morphologically using immunohistological methods only, so that their functional characterization and therefore physiological potential remain to be determined.

In addition to the challenges introduced above for using ESCs in transplantation therapies as well as its associated ethical concerns, there are a number of other obstacles that need to be overcome before ESCs can be considered for clinical use. First, the most widely used method today for differentiating ESC involves culturing them as free floating embryoid bodies which inevitably give rise to cell types of different lineages derived from all three germ layers (Schuldiner, Yanuka et al. 2000). This approach however does not allow efficient production of mature, functional, and homogenous cultures of the desired cell type with reliable precision and accuracy. Again, this highlights the need for better isolation, identification, and maintenance of undifferentiated hESCs as well as the development of better differentiation protocols. Secondly, since MHC-1 is up-regulated during differentiation (Drukker 2004), clinical applications of hESCs could lead to host immune rejection in the absence of immunosuppressive therapy. To address this issue, therapeutic cloning via somatic cell nuclear transfer (SCNT), where the nuclear genome

of the host is introduced into an enucleated oocyte to generate hESC lines that are histocompatible with the patient, is currently being explored (Barberi, Klivenyi et al. 2003; Hochedlinger and Jaenisch 2006). And finally, as demonstrated by the formation of teratocarcinomas following injection of mESCs into the striatum of Parkinsonian rats (Bjorklund, Sanchez-Pernaute et al. 2002), unregulated cell growth of undifferentiated cells *in vivo* pose major challenges for the safety of using ESCs in any kind of transplantation therapy.

A.2. Adult (Somatic) stem cells

The discovery of somatic stem cells, sources of multipotent stem cells in adult animals with the capacity to differentiate into tissue-specific cell types, have caused much excitement because they represent a potential source of autologous cells for transplantation therapies that eliminates immunological complications associated with allogeneic donor cells as well as bypasses ethical concerns associated with ESCs. The realization that a cell source must exist in the adult animal for cell replacement in normal physiology and following injury was first was recognized in the hematopoetic system with the development of bone marrow transplantations. Hematopoetic stem cells (HSCs) in the bone marrow transplanted to lethally irradiated subjects have the capability to reconstitute all the lineages of the blood system (erythroid, lymphoid, myeloid, and megakaryocytic)(McCulloch and Till 1971; Yoon, Chung et al. 2007). Interestingly, in the wake of HSCs, the discovery of unexpected plasticity and regenerative capabilities in the adult CNS made it the first solid organ system shown to possess somatic stem cells,

later termed neural stem cells (Snyder, Deitcher et al. 1992). While it may not be surprising to find stem cells in the bone marrow that would support the rapid turnover of blood cells in the hematopoetic system, the discovery of stem cells in the brain, an organ that was previously thought to be largely immutable following embryogenesis, revolutionized traditional principles of adult organogenesis and cell replacement and opened new avenues of investigation in regenerative medicine.

Stimulated by work in the CNS and blood, multipotent somatic stem cells were soon discovered in other tissues and organ systems including adult mammalian testis, epidermis, gut, heart, pancreas, lung, retina, vasculature, and breast (da Silva Meirelles, Chagastelles et al. 2006; Molyneux, Regan et al. 2007; Sohn, Jain et al. 2007). Unlike ESCs that are able to generate all cell types of the body, somatic stem cells are largely believed to be only capable of differentiating into cells types from their tissue of origin, although recent work on "trans-differentiation" of bone marrow-derived mesenchymal stem cells (BMSCs) is challenging such notions (Bossolasco, Cova et al. 2005; Choi, Shin et al. 2005; Rice and Scolding 2007). To date, the general consensus is that these somatic stem cells originate from pluripotent ESCs during development and are stored in specific stem cell niches to participate in organogenesis and support cell turnover throughout the lifetime of the organism. Since a lot of the current somatic stem cell work on CNS regeneration involves the use of neural stem cells (NSCs) and bone marrow-derived mesenchymal stem cells (BMSCs), these two cell types will discussed in more detail in the following sections as examples of somatic stem cells.

A.3. Neural stem cell (NSC)

As introduced above, the concept that the adult mammalian CNS contains NSCs was first inferred from evidence of neuronal turnover in the olfactory bulb and hippocampus in the adult organism (Altman and Das 1965; Altman and Das 1966). Since then, NSCs and neural progenitor cells (NPCs), cells with more restricted neural differentiation capabilities committed to specific subpopulation lineages, have been generated from hESCs (Conti, Pollard et al. 2005) or directly isolated from neurogenic regions of fetal and adult CNS, such as the subventricular zone (SVZ) that provides neuroblasts to replenish inhibitory interneurons in the olfactory bulb (Snyder, Deitcher et al. 1992; Flax, Aurora et al. 1998; Shihabuddin, Palmer et al. 1999; Rietze and Reynolds 2006). The multipotency of NSCs were demonstrated *in vitro* in the 1990s by their ability to differentiate into neurons, astrocytes, and oligodentrocytes as well as various forms of neural precursors (Weiss, Dunne et al. 1996; Palmer, Takahashi et al. 1997; Flax, Aurora et al. 1998; Takahashi, Palmer et al. 1999; Gage 2000). In addition, *in vivo* delivery of these cells to animal models of neurodegenerative diseases demonstrated varying degrees of functional recovery (discussed below)(Ourednik, Ourednik et al. 2002).

Currently, there is still no set of markers or protein expression profiles that precisely define and fully characterize undifferentiated NSCs. To date, they are primarily isolated and propagated *in vitro* as cells that form free-floating neurospheres when cultured in serum-free medium on non-adherent surfaces in the presence of mitogentic factors such as basic fibroblasts growth factor (bFGF or FGF-2) and epidermal growth factor (EGF) (Snyder, Deitcher et al. 1992; Kitchens, Snyder et al. 1994; Gritti, Parati et

al. 1996; Reynolds and Rietze 2005), although there have also been reports of monolayer cultures (Johe, Hazel et al. 1996; Gage 2000). However, the neurospheres are by no means homogenous. They consist of heterogeneous cells expressing a continuum of surface markers and transcription factors that change during long-term culture. Furthermore, the clonality of individual neurospheres is not absolute; time-lapse video microscopy has captured merging and exchanging of cells between neurospheres (Singec, Knoth et al. 2006; Singec, Jandial et al. 2007). While it has been shown that undifferentiated fetal NSCs can be maintained *in vitro* for up to 6 month (Carpenter, Cui et al. 1999) and that the derived neurons preferentially differentiate down the GABAergic lineage (Jain, Armstrong et al. 2003), there is evidence that the fraction of neuron to glia generation from NSCs declines with extended culture time(Jain, Armstrong et al. 2003; Kelly, Tyers et al. 2005; Wright, Prowse et al. 2006). Adult derived NSCs show similar properties *in vitro* to fetal NSCs but with more limited expansion potential. The data and results to date suggest that the development of a fully validated protocol to characterize and extensively propagate undifferentiated NSCs is still much needed before there is any hope of their clinical use.

Transplantation of both fetal and adult NSCs *in vivo* have so far showed little tumorgeneic complications, however, there has also been limited differentiation of tissue-specific mature neurons (also further discussed below). The underlying problem is believed to be the short time window following neural induction during which the cells can be directed toward specific neuronal lineages (Bouhon, Kato et al. 2005). Functional recovery following NSC transplantation is likely due to neuroprotective effects of the

grafted cells rather than the replacement of lost neurons. Nevertheless, because they can potentially be an autologous stem cell source for transplantation with minimal risk of tumor formation, the therapeutic use of NSCs is actively being investigated.

A.4. Bone marrow-derived mesenchymal stem cells (BMSCs)

Bone marrow-derived mesenchymal stem cells (BMSCs), also termed bone marrow stromal cells, are another example of somatic stem cell being studied for its therapeutic potential in the CNS and in other tissues. They are a population of adherent cells in the bone marrow, independent of the hematopoietic system, that normally give rise to osteoblasts, chondrocytes, and adipocytes (Abdallah and Kassem 2007). In comparison to NSCs, an additional attractive quality of BMSCs is the relative ease of clinically viable cell isolation. They can then be further expanded and differentiated *in vitro* using various media formulations and culture surface conditions in order to direct them to different cell lineages (Ho, Yu et al. 2006). Like other stem cells, BMSCs have been shown to be able to migrate to areas of injury, even crossing the blood brain barrier (Akiyama, Radtke et al. 2002; Tang, Yasuhara et al. 2007). Although the reproducibility and efficiency of BMSC therapies need to be carefully examined, these early experiments suggest that BMSCs can potentially be administered intravenously to CNS targets, although much work remains.

Despite the relative ease of isolation and migration potential of BMSCs, there is considerable debate in the literature regarding the true plasticity of these cells and therefore their clinical utility. First, BMSCs are normally obtained from the bone marrow

by plastic adhesion assay but there has been no characterization of the full spectrum of adherent cells that reside in the bone marrow. While some studies use fluorescent-activated cell sorting (FACS) to obtain a more homogenous population of cells, there is no consensus on the set of surface markers that characterize undifferentiated BMSCs, making it difficult to directly compare studies. In fact, one current school of thought in the field is that the bone marrow may serve as a reserve for a wide variety of stem cells, such that BMSCs obtained by plastic adherence assay alone may actually include heterogeneous subpopulations of multipotent stem cells with differing preferences for varying cellular lineages(Rice and Scolding 2007). Secondly, original reports of *in vivo* BMSC differentiation into neurons and glia (Brazelton, Rossi et al. 2000; Wislet-Gendebien, Wautier et al. 2005), termed "trans-differentiation" since BMSCs do not normally differentiate down neural lineages (Song and Tuan 2004), were challenged by confounding results claiming trans-differentiation to be the result of cell fusion (Terada, Hamazaki et al. 2002). However, recent evidence seems to show that BMSCs can generate neurotransmitter-responsive cells with electrophysiological properties similar to neurons (Wislet-Gendebien, Hans et al. 2005). In addition, there are reports of the co-expression of markers from different neural phenotypes in BMSCs following putative trans-differentiation events, including GABA and tyrosine hydroxlase (Suon, Yang et al. 2006), nestin and glial fibrillary acidic protein (GFAP)(Wislet-Gendebien, Leprince et al. 2003), nestin and ß-tubulin (Tropel, Platet et al. 2006), GFAP and Gal-C (Tsai, Hwang et al. 2006). Still, most of these co-expression patterns have not been observed in primary neural cells nor in cells derived from NSCs, therefore, whether these observations

actually support the neurogenetic plasticity of BMSCs remains to be determined. Finally, an additional potential therapeutic application of BMSCs is in neuroprotection. These cells have been shown to release a variety of neurotrophic growth factors that can enhance cell survival in injured CNS regions (Pisati, Bossolasco et al. 2007), although again the effectiveness of any neuroprotective applications need to be further investigated and ultimately assessed *in vivo*.

B. *In vivo* stem cell transplantation therapeutic strategies

To date, there are three major classes of applications being explored for functional restoration of the CNS that in part make use of stem cells, namely, cell replacement strategies, neuroprotection, and gene therapy. Each of these takes advantage of the unique properties and capabilities offered by stem and progenitor cells, and they are often used in combination to maximize therapeutic effects.

B.1. Cell replacement: Restoring lost neurons and glia

Arguably the most desired outcome of stem cell-based transplantation therapies is to provide injured areas of the CNS a population of cells, potentially engineered, that can use local biochemical and mechanical cues to differentiate into cell types that have been lost or altered by disease mechanisms and ultimately restore neurological function. Snyder *et al.* demonstrated the potential use of stem cells in cell replacement therapies in 1997 when they showed that grafting C17.2 cells, a NSC cell line derived from the external germinal layer of neonatal mouse cerebellum and immortalized with the *v-myc*

gene (Snyder, Deitcher et al. 1992; Kitchens, Snyder et al. 1994), into areas of photolysis-induced apoptotic pyramidal neurons in the adult mouse neocortex led to integration into regions of cell death, differentiation into pyramidal neuron-like cells, and extension of neurites that established appropriate afferent synaptic contacts(Snyder, Yoon et al. 1997). These results suggest that there may be local environmental cues during injury in the adult neocortex that can direct the differentiation of NSCs to regenerate areas of damage even though the normal developmental window has passed. The sensitivity to differentiation cues was emphasized in this study by the preferential differentiation of NSCs into glia in areas outside the photolytic lesion. This in itself raises technical issues related to how does one control the proliferation and differentiation of transplanted cells outside target areas. One potential strategy may be to use local cues induced by injury mechanisms or other secondary mechanisms that regulate proliferation and differentiation processes under the control of specific inducible promoters, although this is in itself is challenging task.

Since this early work, many other studies have focused on demonstrating and optimizing stem cell based methods for the replacement of CNS cells lost to injury or disease. We discuss several of these below in the next section. However, a number of challenges still need to be addressed before stem cell transplantation for cell replacement can be successfully translated to the clinical setting. With increasing knowledge of stem cell biology, there need to be developed ways to more reliably characterize the differentiation and cell cycle states of stem cells prior to transplantation in order to better evaluate and control clinical outcomes. Also, there is evidence that differing

transplantation techniques (e.g. implanting multiple micrografts versus a single large graft) may have a significant effect on the outcome and success of the procedure(Nikkhah, Bentlage et al. 1994). In addition, for grafts with allogeneic donor cells, establishing a post-operative immunosuppression regimen to avoid acute and chronic rejection of the graft is also essential(Lindvall and Bjorklund 2004).

B.2. Neuroprotection: Secretion of neurotrophic factors

The notion that stem cells may also play a neuroprotective role *in vivo* has been suggested by several studies that documented functional improvement following transplantation stem cells in the absence of any significant differentiation and re-establishment of functional synaptic connections. Ourednik *et al.* first showed that undifferentiated NSC C17.2 produced glial derived neurotrophic factor (GDNF) *in vitro* which may have contributed to the observed ”rescuing” of dopamine neurons in regions of NSC transplantation in aged mouse brains *in vivo*(Ourednik, Ourednik et al. 2002). hNSCs isolated from fetal spinal cord expressed and released GDNF as well brain-derived neurotrophic factor (BDNF) when implanted into a transgenic rat model of amyotophic lateral sclerosis (ALS), leading to delayed onset of motor dysfunction and extended life spans(Xu, Yan et al. 2006). NSCs graphed into rats models of spinal cord injury have also been shown to express nerve growth factor (NGF), neurotrophin-3 (NT-3), and glial growth factor receptors such as ErbB-2; and PASK, the mammalian homologue of the fray gene that is involved in axon ensheathment(Lu, Jones et al. 2003; Yan, Welsh et al. 2004). This list is by no means all inclusive, and it is conceivable that

there may be additional unidentified neurotrophic factors that we are not yet aware of released by stem cells under stress of pathological conditions that may contribute to the neuroprotective effect. In addition to NSCs, this effect has also been found in stem cells from other organ systems. hMSCs have been shown to secrete NT-3 in co-culture with neonatal cortical brain slices(Pisati, Bossolasco et al. 2007). The same neurotrophin expression profile was observed at 45 days after transplantation of hMSCs in nude mice (Pisati, Bossolasco et al. 2007).

B.3. Vehicles for gene therapy: Delivery of factors to delay, arrest, and reverse injury

Contrary to localized and acute traumatic insults, in a number of degenerative disorders, multiple subpopulations of cells and anatomical regions can be affected due to dysfunctional proteins (e.g. lysosomal storage diseases) or inadequate synthesis of key factors (e.g. dopamine in Parkinson's disease). Stem cells possess several characteristics that make them promising delivery vehicles for gene therapy approaches aimed at reversing genetic mutations. Most can be propagated for numerous passages *in vitro* to allow genetic manipulation prior to transplantation, there is evidence that at least some stem and progenitor cells tend to selectively target regions of injury or disease, they seem to display intrinsic neurotrophic properties, and may have the potential to differentiate into cell types endogenous to the targeted regions. It has been shown, at least in NSCs, a variety of molecules released during inflammation (e.g. SDF-1α) and chronic diseases

can act as chemo-attractants to guide stem cells to the site of injury (Imitola, Raddassi et al. 2004).

C. Stem cell therapies for CNS disorders

In this final section, we summarize recent progress in stem cell therapies aimed at six major CNS disorders and illustrate how some of the methods and strategies discussed above are being utilized to formulate clinically viable treatments.

C.1 Parkinson's Disease

Parkinson's disease is a neurodegenerative disorder caused by decreased stimulation of the motor cortex due to progressive degeneration of the nigral dopaminergic neurons of the substantia nigra, leading to motor function deficiencies characterized by muscle rigidity, tremor, bradykinesia, and akinesia(Kandel, Schwartz et al. 2000). To date, the main stem cell therapy strategy for Parkinson's disease is cell replacement aimed at the restoration of dopaminergic neurotransmission in the striatum. Animal model studies suggest that cellular transplantation may improve striatal function by normalizing dopamine receptor sensitivity, regulating dopamine receptor activity, and reconstructing both afferent and efferent connections in the striatum(Forni, Brundin et al. 1989; Bjorklund and Lindvall 2000). Functional improvements have been reported following the transplantation of fetal mesencephalic grafts into Parkinson's disease patients. Clinical improvements have been observed to develop gradually over 6-24 months after the procedure, and to last 5-10 years (Lindvall and Bjorklund 2004).

However, issues with fetal tissue availability, complications with optimizing treatment conditions, and variations in functional recovery following the procedure limit this approach from developing into a standardized therapy.

ESCs and somatic NSCs may provide a reliable and more plentiful source of dopamine neurons needed for transplantation(Wagner, Akerud et al. 1999; Kim, Auerbach et al. 2002; Perrier, Tabar et al. 2004). *In vitro* protocols have been shown to be able to differentiate mouse, monkey, and human ESCs into dopamine neuron-like cells expressing specific transcription actors (e.g. Pax2, Pax5, En1) indicative of dopamine synthesis(Barberi, Klivenyi et al. 2003; Perrier, Tabar et al. 2004; Yan, Yang et al. 2005; Martinat, Bacci et al. 2006) and electrophysiological properties of midbrain dopamine neurons(Kim, Auerbach et al. 2002; Takagi, Takahashi et al. 2005; Kim, Kim et al. 2007). A recent study transplanting human ESC-derived dopaminergic neurons into the neostriata of 6-hyoxydopamine-lesioned rat model of Parkinson's disease showed long term restoration of motor function (Roy, Cleren et al. 2006). However, the grafts also showed expanding cores of undifferentiated mitotic neuroepithelial cells that could potentially become tumorgenic.

While a great deal of progress has been made in this area, a number of challenges still remain to be addressed before stem cell-based therapies can be used as standardized treatments for Parkinson's disease. First, differentiation protocols needs to be further optimized to reliably produce highly purified and homogeneous populations of desired cell types. Secondly, the ability of transplanted stem cell-derived dopamine producing neurons to survive, re-innervate, and restore dopaminerigc neurotransmission in the

striatum still needs to be fully assessed *in vivo* in order to ensure the long term efficacy and safety of the procedure. Finally, treatment conditions such as patient selection, postoperative rehabilitation, and immuno-suppression also need to be optimized to maximize therapeutic benefits.

C.2. Huntington's Disease

Huntington's disease or Huntington's chorea is an inherited autosomal dominant disorder caused by a repeating CAG mutation in the Huntingtin gene (chromosome 4) leading to abnormal processing of the defective protein, accumulation of its fragments in cellular compartments, and eventual loss of GABAergic medium spiny neurons in the striatum as well as neurons in the cortex (Kandel, Schwartz et al. 2000). In Huntington's disease patients, progressive loss of these neurons disrupts functions in the cortico-striatal-pallidal circuit through dis-inhibition of pallidal signaling output, leading to severe physical and cognitive impairments as well as psychopathological symptoms. Currently, there is no treatment for Huntington's disease. The stem cell therapy strategy most explored for this disease has been cellular transplantation of somatic stem cells of adult and fetal origins in an attempt to restore the inhibitory connections. This is based on promising results obtained with animal studies (McBride, Behrstock et al. 2004; Visnyei, Tatsukawa et al. 2006), and subsequent phase I clinical trials (Philpott, Kopyov et al. 1997; Bachoud-Levi, Gaura et al. 2006) of intra-striatal implantation of fetal striatal primodium from the forebrain.

Huntington's disease is uniquely suitable for fetal striatal neural grafts because the treatment uses population of cells that are just entering active developmental growth and maturation into the exact cell type that needs to be replaced. As shown using animal models, varying levels of restoration, from the establishment of efferent and afferent connectivity to the development of complex fronto-striatal neural circuitry, result in different degrees of functional improvement. These include reduced chorea to recovery of complex motor/cognitive abilities (Dunnett and Rosser 2007) and even restoration of habit learning capabilities (Brasted, Watts et al. 1999). In a phase I clinical trials, three of the five Huntington's disease patients that received fetal neural transplantations had metabolically active grafts (demonstrated using fluorodeoxyglucose PET scans) as well as clinical improvements in motor and cognitive functions for up to six years following the procedure (Bachoud-Levi, Gaura et al. 2006). A post-mortem examination of a Huntington's disease transplant patient who died 18 months after grafting from unrelated causes showed that the implanted neural grafts had successfully integrated into the host tissue, had extended neurites and had innervated dopaminerigc fibers of the host tissue (Freeman, Hauser et al. 2000).

Though still under investigation, it is thought that the native population of NPCs in fetal tissues is the critical element responsible for the observed improvements. Therefore, it is presumable that NSCs may offer a treatment option to stop or even reverse the progress of Huntington's disease by providing a reliable source of homogenous NPCs for transplantation. There are numerous studies on stem cell grafts transplanted into different animal models of Huntington's disease, most of them using

neural progenitor cells (NPCs) expanded as neurospheres and differentiated *in vitro* prior to transplantation. A recent study injecting adult NPCs isolated from the subventricular zone into a rodent quisqualic acid lesion model of Huntington's disease showed good graft viability with extensive cell migration at 8 weeks with presence of differentiated cells positive for DARPP-32 and GAD67 (markers for GABAergic spiny striatal neuron) as wells as reduced functional impairment determined by apomorphine-induced rotational asymmetry and spontaneous exploratory forelimb use (Vazey, Chen et al. 2006). Studies done with fetal human and rodent NPCs have also demonstrated good graft survival and integration into host tissue with varying degrees of differentiation(McBride, Behrstock et al. 2004; Visnyei, Tatsukawa et al. 2006), but further assessment of functional recovery is needed. In addition, transplantation of grafts with stem cells of mesenchymal orgin (i.e. bone marrow and human umbilical cord blood cells) showed longer subject survival (Ende and Chen 2001) as well as improved motor function (Chen and Ende 2000) and working memory (Lescaudron, Unni et al. 2003). In comparison to NPCs, fewer studies were done using ESCs. Recent work by Dihne *e.t al.* demonstrated > 75% ESC differentiation into β-tubulin(+) immature neurons *in vitro* prior to transplantation with no tumor growth following the procedure (Dihne, Bernreuther et al. 2006). However, no functional assessments were done in these studies.

While tremendous progress has been made, there are still challenges to be addressed before stem cells can replace fetal neural tissue as cell transplantation therapy for Huntington's disease. While a GABAergic phenotype appears to be the default differentiation pathways of NPCs (Jain, Armstrong et al. 2003), protocols need to be

developed that can more precisely direct differentiation into the striatal medium spiny projection neurons and interneurons needed for transplantation. Also, despite evidence that differentiated cells from grafts sprout neurites and make synaptic contacts with host tissue, observations of extensive axonal outgrowth have been limited and there is no conclusive evidence for reconstruction of neural circuits in damaged areas (Dunnett and Rosser 2007). Since restoration of the corticostriatal circuit is crucial for long term functional recovery, more work is needed to optimize graft-host connectivity as well as the development of more rigorous tests to accurately assess motor and cognitive functions.

C.3. Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive neurodegenerative disease caused by degeneration of motor neurons in the cortex and ventral grey matter of the spinal cord, leading to muscular atrophy throughout the body and eventually death(Kandel, Schwartz et al. 2000). Unlike Parkinson's disease and Huntington's disease, ALS has a more rapid clinical progression in which glial cells may play a key role (Julien 2007). In fact, experimental evidence of wild-type non-neuronal cells ameliorating degeneration of SOD1 mutant-expressing motor neurons in a chimeric mouse model of ALS composed of a mixture of normal and mutant cells suggests that neurotrophic support by stem cells could provide an effective treatment even without neuronal differentiation(Clement, Nguyen et al. 2003). As a result, stem cell therapeutic strategies for ALS involve transplantation aimed at neurotrophic support and neuroprotection in addition to the replacement of lost motor neurons. In animal studies of stem cell therapies for ALS, transplantation of genetically modified

human fetal NSCs into the spinal cord of a mouse ALS model have been found to survive and secret GDNF at the 11-week end point of the study with some differentiation into astrocytes(Klein, Behrstock et al. 2005). However, the cells were not able to completely halt neurodegeneration and the rapidly progressing etiology. Similar decreases in the rate of disease progression was observed in a recent study transplanting NSCs that had been first differentiated *in vitro* into cholingeric motor neuron-like cells and transplanted into the spinal cord of the same animal model(Corti, Locatelli et al. 2007).

In addition to neural stem cells, reconstitution of wild-type BMSCs following irradiation of the SOD-1 transgenic mouse model of ALS showed delayed disease onset and increased life span(Corti, Locatelli et al. 2004). Similar results have been obtained by intravenous infusion of human umbilical cord blood cells(Garbuzova-Davis, Willing et al. 2003). While direct delivery of cells into the CNS may circumvent complications with peripheral injections, the more widely distributed and extensive pathology associated with ALS presents a unique surgical challenge in terms of cellular transplant delivery. Instead, the disease may benefit from a therapeutic strategy that utilizes the intrinsic capability of stem cells to migrate to areas of damage in order to provide neurotrophic support.

It should also be noted that since potential causes for ALS could have a hereditary component, it is unclear whether autologous stem cells could provide therapeutic benefits. Despite the fact that many treatment parameters still await elucidation, clinical trials are underway based on the transplantation of autologous BMSCs into spinal cords of ALS patients(Mazzini, Fagioli et al. 2003). Cells isolated and expanded for 32 days *in vitro*

were transplanted into seven rapidly progressing ALS patients. No major complications were reported following the procedure and no abnormal cell proliferation was observed in MRI at 4 years post surgery. Four of the seven patients demonstrated significant reduction in the rate of decline of respiratory function and on the ALS-functional rating score(Mazzini, Mareschi et al. 2007). However, one noted problem with using autologous stem cells is the variation in expansion potential between different patients dependent largely on age, and may significantly affect the efficacy and outcomes of the treatment.

C.4. Retinal Degenerative Diseases

Another area where stem cell-based therapies are being actively pursued as a potential therapeutic alternative is in the treatment of retinal degenerative diseases. In comparison to other cell based translational CNS targets, the retina offers unique advantages that render it considerably more favorable for development of transplantation and stem cell therapy. The retina is a unique structure of the CNS in that all of the essential cell types and neuronal circuitry necessary for function are contained within its five primary layers spanning approximately 200 µm in thickness(Kandel, Schwartz et al. 2000). This indicates that, with the exception of replacement of retinal ganglion cells (RGCs) for optic nerve degeneration (i.e. glaucoma), functional neuronal synapses that need to be established between host and transplanted cells are local and short range connections with minimal need for guidance. In addition, information flow is essentially unidirectional towards the RGCs and many of the diverse functional cell types in the mammalian retina have been identified morphologically and physiologically(Masland

2001; Masland 2004). Furthermore, the retina is the most accessible part of the CNS which facilitates surgical delivery of cells and grafts via transplantation or injection. It can be assessed structurally and functionally using noninvasive imaging tools such as the fundus scope and optical coherence tomography (OCT), as well as standard electroretinogram (ERG) techniques.

With the exception of glaucoma, which primarily affects retinal ganglion cells, and some forms of vitreoretinal degeneration which also first affect the inner retina, most retinal degenerative diseases such as age-related macular degeneration (AMD), retinitis pigmentosa (RP), and the numerous forms of retinopathy are all characterized by progressive loss of retinal photoreceptors, resulting in remodeling of neural retinal circuitry and eventually vision loss. Stem and progenitor cells isolated from a number of sources including embryonic tissue, adult brain, adult bone marrow, and even the retina itself are been explored for their potential as stem cell-based therapies for these diverse classes of disorders(Lund, Wang et al. 2006; MacLaren, Pearson et al. 2006; Gamm, Wang et al. 2007; Lund, Wang et al. 2007).

Of the numerous studies being done, there have been two general approaches to the transplantation of stem cells or stem cell-derived cells to rescue the degenerating retina: Photoreceptor survival promoted by restoring the supportive functions of the retinal pigment epithelial (RPE) cells through subretinal grafts of stem cell-derived RPE-like cells; and directly replacing lost photoreceptors with transplanted stem cells and retinal precursor cells coaxed to differentiate and integrate into the outer nuclear layer of the degenerating retina. In contrast to Huntington's disease and ALS, which have already

entered clinical trials, and Parkinson's disease, for which graft viability and host integration have been investigated and verified, the potential of these cell-based therapies for retinal degenerative disorders are still being assessed in animal models.

A recent study of subretinal injections of hESC-derived RPE into the Royal College of Surgeon (RCS) rat model of retinal degeneration (caused by inhibition of RPE phagocytosis of photoreceptor outer segments due to mutation in the receptor tyrosine kinase *mertk* gene (D'Cruz, Yasumura et al. 2000)) reported rescue of visual response and acuity assessed using electroretinogram, optomotor acuity thresholds, and luminance threshold recordings in the superior colliculus (Lund, Wang et al. 2006). However, there is no direct evidence showing that the observed functional improvements were due to rescue of RPE phagocytosis rather than a general neuroprotective effect brought on by the transplanted cells. Indeed, a variety of cell types have been shown to provide temporary rescuing effects through secretion of neurotrophic factors when transplanted into the retina (Gamm, Wang et al. 2007; Lund, Wang et al. 2007) and the hESC-derived RPE cells have been shown to produce PEDF (Klimanskaya, Hipp et al. 2004), a trophic factor with protective and morphogeneic effects on photoreceptors (Aymerich, Alberdi et al. 2001; Barnstable and Tombran-Tink 2004; Imai, Yoneya et al. 2005). Furthermore, the grafted cells were observed to aggregate at the injection site instead of integrating into the host retina with poor long term cell retention that parallels the decline in rescue effects at 4 weeks after transplantation (Lund, Wang et al. 2006).

Attempting to rescue the degenerating retina by cellular replacement of lost photoreceptor neurons presents its own unique set of challenges. In cases of severe retinal

degeneration, the retina may have already undergone substantial post-receptor remodeling (Fisher, Lewis et al. 2005) (in the early stages of these diseases the retina beyond the photoreceptor layer remains relatively anatomically and functionally intact). Therefore, grafted stem cells would need to differentiated into functional photoreceptor neurons and establish the intricate functional connections with bipolar and horizontal cells in order to restore the neural retinal circuitry. A recent study of subretinal injections of photoreceptor precursor cells (isolated from the retina during rod photoreceptor genesis) into mouse models of retinal degeneration demonstrated cell integration into the retina, expression of the synapse protein bassoon, formation of synaptic contacts with bipolar cells identified by immunostaining with phosphokinase C (PKC), positive rod photoreceptor differentiation based on immunostaining for proteins involved in the phototransduction pathway (phosducin and rhodopsin), and improved visual responses assessed with extracellular field potentials of the retinal ganglion cell layer and papillary reflex to light stimulation(MacLaren, Pearson et al. 2006). Progenitor cells harvested at earlier and later developmental stages were shown unable to achieve these results, which the authors attributed to a lack of *Nrl* transcription factor expression generally only observed in post mitotic immature rod precursors(MacLaren, Pearson et al. 2006). However, despite the promising results of this study, the availability human fetal photoreceptor precursors (which would need to be harvested in the second trimester of fetal development) limit the feasibility of translating such a treatment into the clinical setting. A better understanding of the developmental processes that guide embryonic and adult stem cells towards photoreceptor differentiation will allow optimization of

protocols to reliably direct stem cell differentiation for photoreceptor replacement therapies. And advances in therapeutic cloning could lead to successful generation of stem cells from adult somatic cells to facilitate transplantation of autologous cells for treatment of RD patients.

In addition to treating pathologies involving photoreceptors of the outer retina, stem cell therapy is also being investigated for treating degeneration of the inner retina, namely of diseases that cause deterioration of the optic nerve and retinal ganglion cells (i.e. glaucoma, Leber's hereditary optic neuropathy, ischemic optic neuropathy), where there is currently no treatment to improve vision once its is compromised(Bull and Martin 2007). Regeneration of the optic nerve after injury or disease is hindered by a few major obstacles. Optic nerve degeneration is usually accompanied with RGC apoptosis, whether it is due to elevated intraocular pressure as in glaucoma or excitotoxicity as in retinal ischemia and reperfusion injuries(Dreyer, Zurakowski et al. 1996; Dreyer and Grosskreutz 1997). Furthermore, RGCs in the adult mammalian retina does not have the ability to initiate axonal elongation after injury despite having this capacity during development(Chierzi and Fawcett 2001). Finally, similar to other areas of the CNS, reactive gliosis and up-regulation of factors such as myelin-associated proteins (i.e. Nogo) in the local environment in the retina following injury inhibit re-growth of the axon to establish synaptic connections with the LGN(Chierzi and Fawcett 2001). Overcoming these challenges using stem cell therapy would require a combination of the transplantation approach to replace lost RGCs with neuroprotective strategies to optimize viability and function of the host as well as transplanted cells by producing a more

permissive environment for axonal regeneration and regulating the reactive gliosis response.

Embryonic stem cells have been attractive as a potential cell source for cell replacement therapy, despite its teratogenic tendencies when transplanted *in vivo*. Mouse ESCs have been reported to generate eye-like structures consisting of lens, neural retina, and retinal pigment epithelium cells when co-cultured with PA6 cells and induced with bFGF, dexamethasone, and cholera toxin(Hirano, Yamamoto et al. 2003). Furthermore, these structures have also been found to harbor retinal progenitor cells that could be differentiated into specific retinal cells types(Aoki, Hara et al. 2006). A recent study that co-cultured these ESC-derived eye-like structures with both normal adult mouse retinal tissue and retinas with NMDA-induced excitotoxicity damage developed Tuj1-postive neurons that migrated to the ganglion cell layer. Most of these neurons in NMDA-treated retina went on to express ganglion cell-specific markers, Hu and Bm3b(Aoki, Hara et al. 2007). However, functional assessment in a more physiologically relevant *in vivo* model remains to be done.

Finally, the adult retina offers another potential source of transplantable cells. Although residual proliferative activity have been detected in the ciliary marginal zone of the mouse and human retina(Tropepe, Coles et al. 2000; Coles, Angenieux et al. 2004), recent reports suggest that the adult mammalian neural retina may contain another small population of regenerative cells of glial origin, possibly a subpopulation of Müller cells, that are normally quiescent *in vivo*, but can be induced to de-differentiate into a more progenitor cell-like phenotype following injury(Kohno, Sakai et al. 2006). It has been

recently reported that the Sonic hedgehog (Shh) signaling protein can induce Müller glia de-differentiation to a more progenitor-like state with expression of Pax6, Sox2, and nestin both *in vitro* and *in vivo*(Wan, Zheng et al. 2007). Furthermore, these Müller-derived progenitor-like cells were shown to differentiate into rhodopsin-positive rod photoreceptor-like in a rat model of pharmacologically induced photoreceptor apoptosis(Wan, Zheng et al. 2007). While extremely fascinating, these results still need further morphological and functional assessment and evaluate the potential of these cells to replace RGCs in optic nerve degeneration, but the identification of endogenous proliferative cells within the adult retina suggests an exciting new avenue for therapeutic research.

C.4. Ischemia

Ischemia is due to inadequate oxygen supply and leads to activation of metabolic cascades that ultimately result in apoptosis of cells in the affected region (Choi 1988). In the brain, the effects of ischemia- the size of the lesion as well as the affected neuronal and glial cell types- can vary depending on the ischemic location and duration. Transplantation of naïve and genetically manipulated stem cells are being explored as both a cell replacement strategy for lost cells and as a neuroprotective strategy. Transplantation of mESC-derived neural precursors into an ischemic rat model of endothelian-induced middle cerebral artery occlusion showed graft survival up to 12 weeks, differentiation of neural precursors into immunohistochemically mature neurons and glia, as well as electrophysiological function (Buhnemann, Scholz et al. 2006).

Similarly, delivery of fetal hNSCs and hNPCs into focal ischemic lesions yielded differentiated cells that expressed neuronal and glial markers (MAP-2, NeuN, GFAP), synaptic connections between graft-derived neurons and host tissue, and improvements in neurological functions (Ishibashi, Sakaguchi et al. 2004). Functional improvements were also observed with intravenously administered hNSCs in rat ischemic models that demonstrated the ability of these cells to migrate to lesion sites in the hippocampus(Chu, Kim et al. 2003; Chu, Kim et al. 2004). However, despite promising results in the rodent models, differentiation of grafted stem cells and limited migration to injury sites have been observed to a much lesser extend in primates models (Roitberg, Khan et al. 2003).

It was shown recently that the period of neurogenesis following stroke needs to extend to at least 4 month post injury (Thored, Arvidsson et al. 2006). NSC from the subventricular zone form neuroblasts that migrate to lesion sites but are unable to repair damaged neural connections due to poor survival rates (Ninomiya, Yamashita et al. 2006). The observation that infusion of caspase inhibitors can prevent apoptosis of these neuroblasts caused by local inflammation suggests that stem cells may also be used for their neurotrophic properties to maximize the native regenerative potential of the CNS (Thored, Arvidsson et al. 2006). Growth factors and cytokines secreted by stem cells such as NSCs, BMSCs, and umbilical cord blood cells (UCBC) may aid in the survival of neurons and endogenous precursors by increasing proliferation and controlling inflammation(Borlongan, Hadman et al. 2004; Xiao, Nan et al. 2005; Park, Himes et al. 2006). Intravenous delivery of UCBCs into a rat stroke model conferred short-term therapeutic benefits that were independent of stem cell migration to lesion sites

(Borlongan, Hadman et al. 2004). These trophic effects were further enhanced by the over-expression of growth factors secreted by transplanted stem cells. Transplantation of BMSCs over-expressing BDNF and GDNF (Kurozumi, Nakamura et al. 2005), hepatocyte growth factor (Zhao, Nonoguchi et al. 2006), and bFGF/FGF-2 (Ikeda, Nonoguchi et al. 2005) have been showed to improve functional recovery. In addition, it has been reported that while transplantation of NSCs into brains of postnatal mice subjected to unilateral hypoxic-ischemic injury showed targeted homing and integration into ischemic areas with ~5% differentiation into neuron-like cells, over-expressing NT-3 in these NSCs by retrovirus transduction enhanced these regenerative effects by increasing the fraction of NSC-derived neurons to 20% in infarct regions. These NSC-derived neurons reportedly encompassed a variety of neuronal subtypes appropriate to the cortex; including cholinergic, GABAergic, and glutamatergic neurons, with little glial differentiation and reduced astroglial scarring (Park, Himes et al. 2006). Furthermore, grafting of hNSCs genetically modified to over-express VEGF into mice cerebral cortex overlying intracerebral hemorrhage induced increased angiogenesis and behavioral recovery in animal models (Lee, Kim et al. 2007).

C.5. Brain Tumors

Stem cells are also being used to improve the treatment of brain neoplasms, such as glioblastoma multiforme (GBM), by improving understanding of the disease etiology through studying stem cell biology; as well as by providing a therapeutic tool for targeted delivery of chemotherapeutic agents. Currently, the median survival rate of GBM patients

is only 12-14 months following diagnosis despite aggressive treatment with radiation and chemotherapy and surgical resection (Kandel, Schwartz et al. 2000). The primary difficulty in treating GBM is the highly invasive nature of malignant cells, leading to diffuse and widespread distribution of tumor growth throughout the brain. In addition, limited knowledge regarding the initiating cells and subsequent carcinogenesis of the tumor limit our ability to accurately characterize the tumor and formulate effective therapeutic strategies.

Experimental data showing similar migratory, self-renewal, and molecular characteristics between NSCs and brain neoplasias suggest that the abnormal cells responsible for initiating tumorgenic growth may be stem-like cells that have lost proper control over proliferation and differentiation (Donnenberg, Luketich et al. 2006; Yip, Sabetrasekh et al. 2006). In fact, the presence of stem-like cells with uncontrolled growth and differentiation have been shown to correlate with malignancy of the tumor and its prognosis (Glinsky, Berezovska et al. 2005). Therefore, it is conceivable that deciphering the differences between a normal stem cell and its tumorgenic counterpart may provide insights into the processes responsible for initiating the formation of brain tumors.

In addition to providing a better understanding of the etiology of the disease, stem cells are also being employed as therapeutic tools to treat GBM. Aboody *et. al.* showed that murine and human NSCs grafted to different locations (intratumoral, contralateral hemispheres, intraventricular) with respect to the tumor as well as delivered intravenously showed remarkable abilities for targeting tumor sites, tracking down even individual cancer cells, and providing trophic support(Aboody, Brown et al. 2000).

Factors secreted by tumor cells for growth and angiogenesis as well as cytokines released by damaged cells are believed to be responsible for attracting the NSCs to the neoplastic lesions. Specifically, chemokine receptor CXCR4 and its ligand SDF-1α have been shown to contribute to this targeting, along with stem cell factor (SCF), monocyte chemoattractant protein-1 (MCP1), and vascular endothelial growth factor (VEGF) (Yip, Sabetrasekh et al. 2006). Furthermore, grafting of NSCs modified to express cytosine deaminase allowed targeted delivery of chemotherapeutic drug through tumor-site specific conversion of pro-drug to 5-flurouracil, resulting in dramatically reduced tumor mass. A variety of stem cell-based brain tumor therapeutics involving genetically modified and naïve NSCs are currently under investigation(Yip, Sabetrasekh et al. 2006; Lin, Najbauer et al. 2007).

D. Conclusion

A variety of stem cells, embryonic and somatic, are being investigated for their potential to treat CNS disorders. Many studies are yielding promising results at the experimental level using animal models. However, clinical trials of stem cell transplantation for a number of CNS disorders have resulted in only moderate outcomes and have yet to demonstrate their ability to fully replace degenerating neuronal and glial cells in the CNS. Translational strategies must be based on a robust understanding of the fundamental principles of stem cell biology and CNS pathology in order to safely and effectively develop stem cell-based therapeutics for CNS disorders, and this will undoubtedly take time. Much still needs to be elucidated regarding the biology of the

stem cells as well as the pathogenesis of targeted CNS diseases in order to maximize therapeutic benefits. Still, stem cell-based therapies hold extraordinary promise for a wide range of neurodegenerative diseases.

Acknowledgement

Chapter 1 is a reprint of the material as it appears in Stem Cell Sources and Therapeutic Approaches for Central Nervous System and Neural Retinal Disorders, Yu, Diana; Silva, Gabriel, Neurosurgical Focus, vol. 24, 2008.

Chapter II

Comparison of morphology and protein expression of bone marrow-derived mesenchymal stem cells cultured on standard surface chemistries prior to neural differentiation

Abstract

A critical element of any stem cell differentiation protocol is the ability to compare its effects relative to an undifferentiated population of the same cells, In an attempt to standardize pre-differentaiton conditions of adult bone marrow-derived mesenchymal stem cells (BMSCs) prior to neural induction experiments, we asked what is the simplest chemical surface that will support the growth and maintenance of these cells in a predifferentiated state. Adult rat BMSCs were expanded in vitro on Permanox Lab-tek tissue culture treated plastic (TCP), poly-D-lysine (PDL) coated glass, PDL-laminin-1 coated glass, and untreated glass. TCP provided the best surface for maintaining morphologies generally considered to be undifferentiated, PDL coated glass and uncoated glass provided the least suitable surfaces. Expansion of BMSCs on PDL-lamin-1 coated glass resulted in expression of nestin, a marker associated with neuronal and other progenitor cells, and therefore may confound experimental results if used as a pre-differentiation surface.

Introduction

Adult bone marrow-derived mesenchymal stem cells (BMSCs) are precursor cells that may present an ideal source of stem cells for cellular replacement therapies because of their relative ease of isolation, high *in vitro* expansion rate, and demonstrated multipotency. These properties allow for an autologous cell source which in some cases circumvents host immune response issues. Under specialized induction conditions, BMSCs have been shown to give rise to cell types derived from all three of germ layers (Jiang, Jahagirdar et al. 2002; Kotobuki, Hirose et al. 2004; Song and Tuan 2004). In addition to differentiating into mesenchymal cell types such as osteoblasts, chondrocytes, and adipocytes, recent evidence also suggests their potential for neural differentiation both *in vitro* (Sanchez-Ramos, Song et al. 2000; Woodbury, Schwarz et al. 2000; Deng, Obrocka et al. 2001; Woodbury, Reynolds et al. 2002; Hermann, Gastl et al. 2004; Tondreau, Lagneaux et al. 2004) and *in vivo* (Kopen, Prockop et al. 1999; Zhao, Duan et al. 2002) . Murine BMSCs implanted into neonatal mouse brains have been shown to develop into astrocytes (Kopen, Prockop et al. 1999) and cultured BMSCs have been shown to display neuron-like morphologies and express neuronal protein markers following induction with a wide variety of signaling factors (Altshuler, Lo Turco et al. 1993; Jaiswal, Haynesworth et al. 1997; Prockop 1997; Azizi, Stokes et al. 1998; Mackay, Beck et al. 1998; Kopen, Prockop et al. 1999; Pittenger, Mackay et al. 1999; Sanchez-Ramos, Song et al. 2000; Woodbury, Schwarz et al. 2000; Deng, Obrocka et al. 2001; Kohyama, Abe et al. 2001; Jiang, Jahagirdar et al. 2002; Alhadlaq and Mao 2004; Tondreau, Lagneaux et al. 2004; Tropel, Noel et al. 2004). Transplanted BMSCs into the

In a broad sense, by definition stem cells are a population of cells capable of indefinite self-renewal that give rise to "daughter" cells committed to specific differentiation lineages through asymmetric cell division. Their ability to control proliferation, differentiation, and apoptosis distinguishes them from neoplastic cells. Embryonic stem cells are primordial cells of the developing blastula capable of generating an entire organism while somatic stem cells reside within individual organs and usually only give rise to cell types specific to that tissue. The normal function of stem cells include the maintenance of homeostasis mediated by providing trophic support, as well as serving as a reservoir for replacing dysfunctional and senescent cells throughout the lifetime of the organism (Thomas, Thomson et al. 2006). The fact that stem cells have the potential to differentiate into various cell types and tissues is the principle reason they are being explored in treatments for diseases where there may be neural retina of a rat model of retinal degeneration have been shown to integrate with host tissue and provide regenerative support to injured photoreceptor neurons (Prockop 1997).

As a precursor to induced neural differentiation and transplantation experiments with these cells, we asked the fundamental question what is the simplest and most reproducible surface that would support and sustain the growth and proliferation of undifferentiated BMSCs prior to differentiation. Different published methods for the expansion and growth of BMSCs can produce varying results, with some resulting in early neural protein expression that may confound induced differentiation conditions. Furthermore, it is difficult to compare and draw conclusions from experimental protocols which make use of different surfaces. In an attempt to standardize pre-differentiation

conditions of adult derived mesenchymal stem cells prior to neural induction experiments, we directly compared four simple chemical surfaces for their ability to support the growth and maintenance of these cells in a pre-differentiation state. Specifically, we cultured BMSCs on tissue culture treated plastic (TCP), poly-D-lysine (PDL) coated glass, PDL-laminin-1 coated glass, and untreated glass. During cell expansions we tracked changes in BMSC morphology, protein expression, and growth. Specifically, we measured the expression of CD90, a membrane protein specific for undifferentiated BMSCs (Kicic, Shen et al. 2003; Tondreau, Lagneaux et al. 2004); glial fibrillary acidic protein (GFAP), an intermediate filament protein specific for mature macroglial cells(Altshuler, Lo Turco et al. 1993; Libby, Hunter et al. 1996; Qian and Saltzman 2004); and nestin, an intermediate filament protein specific for neuronal progenitor cells (Silva, Feeney et al. 1998; Sockanathan and Jessell 1998). We conclude that for the purpose of expanding and maintaining multipotent undifferentiated BMSC populations aimed at neural differentiation experiments, simple tissue culture treated plastic provides the most consistent and reliable surface, allowing the comparison of differentiation parameters without confounding experimental variablese Therefore, attempting to expand BMSC's on these routinely used cell culture surfaces (specifically, untreated glass, PDL-glass, and PDL-laminin-1 on glass) prior to controlled experimental differentiation conditions may result in unintended and subtle early phenotypic and/or morphological conditions that may confound the analysis and interpretation of experimental results. The only surface chemistry that, in our hands, reliably and reproducibly resulted in truly undifferentiated BMSCs after multiple passages was tissue culture treated plastic.

Materials and Methods

A. Cell culture

Rat BMSCs were obtained from the Tulane Center for Gene Therapy at Tulane University. Primary cultures were harvested and expanded based on a modification of published protocols [30]. For all experiments only passage 8 BMSCs were used. BMSCs were cultured on Permanox Lab-Tek® chamber slides (Nalgene Nunc International, Rochester, NY), poly-D-lysine (PDL) coated culture slides (Becton Dickinson, Franklin Lakes, NJ), PDL-laminin-1 coated culture slides (Becton Dickinson, Franklin Lakes, NJ), and untreated glass culture slides (Becton Dickinson, Franklin Lakes, NJ). BMSCs were seeded into wells at 1000 cells/cm^2 in culture media consisting of α minimal essential media (αMEM), 20% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA), 4mM L-glutamine, and antibiotic-antimycotic (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B as Fungizone in 0.85% saline), which were all obtained from Invitrogen (Carlsbad, CA) unless otherwise specified. Media changes, in which all the media was removed, were performed every other day.

B. Immunocytochemistry

Immunocytochemistry (ICC) was performed on day 6 of culture. MSCs were fixed at room temperature in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 minutes. After fixing, the cells were washed twice with PBS (Invitrogen, Carlsbad, CA) and some cultures permeabilized in 0.2% Triton X-100 (Fisher Scientific International, Hampton, NH) for 30 minutes. The remaining cultures were not permeabilized and instead incubated in PBS for 30 minutes. Cultures were incubated at room temperature for 2 hours with either one of the following primary antibodies: $CD90^{+}$ (1:160; Pharmingen, San Diego, CA), glial fibrillary acidic protein (GFAP; 1:1000; Sigma, St. Louis, MO), or nestin (1:1000; Pharmingen, San Diego, CA) with 10% FBS in PBS. Cultures not permeabilized were stained using $CD90^{+}$, since this protein is expressed on the cell membrane surface. All other proteins were intracellular and therefore required permeablization in order to allow the antibodies access to their epitopes. Routine negative controls for all conditions included the omission of the primary antibody and incubation with 10% FBS in PBS during the primary incubation step in order to control for background fluorescence of the secondary antibodies. For secondary antibody staining cells were incubated with tetramethylrhodamine isothiocyanate (TRITC) conjugated anti-mouse IgG (1:200; Sigma, St. Louis, MO). Following ICC, all slides were mounted using Molecular Probes Prolong® Gold antifade reagent with DAPI (Eugene, Oregon).

C. Phase contrast and fluorescent microscopy

All images were acquired using an Olympus IX81 inverted fluorescent confocal microscope (Olympus Optical, Tokyo, Japan) that included epifluoresence, confocal, phase, brightfield, and Hoffman differential interference contrast (DIC) modalities. Our microscope was equipped with a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and Image-Pro Plus data acquisition and morphometric software (version 5.1.0.20, Media Cybernetics, Inc., Silver Spring, MD).

D. Morphology counts

Morphology cell counts were conducted using ImageJ (version 1.34j)/Cell Counter (v2), an application that permits counting of multiple character types in an image. This software enabled the selected labeling and counting of cells from each morphology category on each image. To avoid bias, for each of the four chemical surfaces five randomly selected fields of view (670um by 670um) were used for counting for each of the 6 days in culture. Cells were categorized under three morphology classifications: 1) fibroblastic, 2) flat, and 3) other morphologies (see Results for details). After each image was counted, the results were recorded in Microsoft Excel (2002) and the average fraction of total cells for each classification per chemical surface per day was computed, along with the standard deviation and standard error of the mean. For consistency in cell classification all counts were done by one of the co-authors.

E. Fluorescence counts

Using an Olympus IX81 inverted fluorescent confocal microscope fluorescence counts for BMSCs cultured on PDL-laminin-1 were conducted by scanning over the entire area ($0.7cm^2$) of the culture wells through a field of view of 170um by 170um. DAPI fluorescence was used to quantify total cell count in the well and cells positive for both DAPI and TRITC were used to quantify counts for the corresponding antibody labeling. Counts were recorded in Microsoft Excel and the percent positive antibody labeling was computed.

Results

A. Tissue culture treated plastic provided the most consistent and robust culture surface

We expanded and characterized the morphology of BMSCs over the course of six days on four different culture surfaces with distinct functional chemistries: Permanox Lab-Tek® tissue culture treated plastic (TCP), poly-D-lysine (PDL) coated glass, PDL-laminin-1 coated glass, and untreated glass. TCP and PDL promote cell adhesion through non-specific electrostatic interactions (Silva, Feeney et al. 1998; Qian and Saltzman 2004), while PDL-laminin-1 presented a bioactive surface with laminin being a strong inducer of neuronal differentiation in neural progenitor cells (Fan, Lin et al. 1996; Silva, Feeney et al. 1998; Mehler and Kessler 1999; Pittenger, Mackay et al. 1999; Okano 2002). This condition was tested to see if laminin-1 alone could induce some degree of neural differentiation in untreated BMSCs, or if the cells would remain undifferentiated. If they remained undifferentiated, growing BMSCs on a neuronal promoting surface

would provide a supportive environment for neural differentiation in conjunction with other differentiation manipulations. If not, then we wanted to avoid potential confounding results between this surface and experimental induction parameters. We classified cell morphologies as: 1) fibroblastic, exhibiting elongated cell body with directional

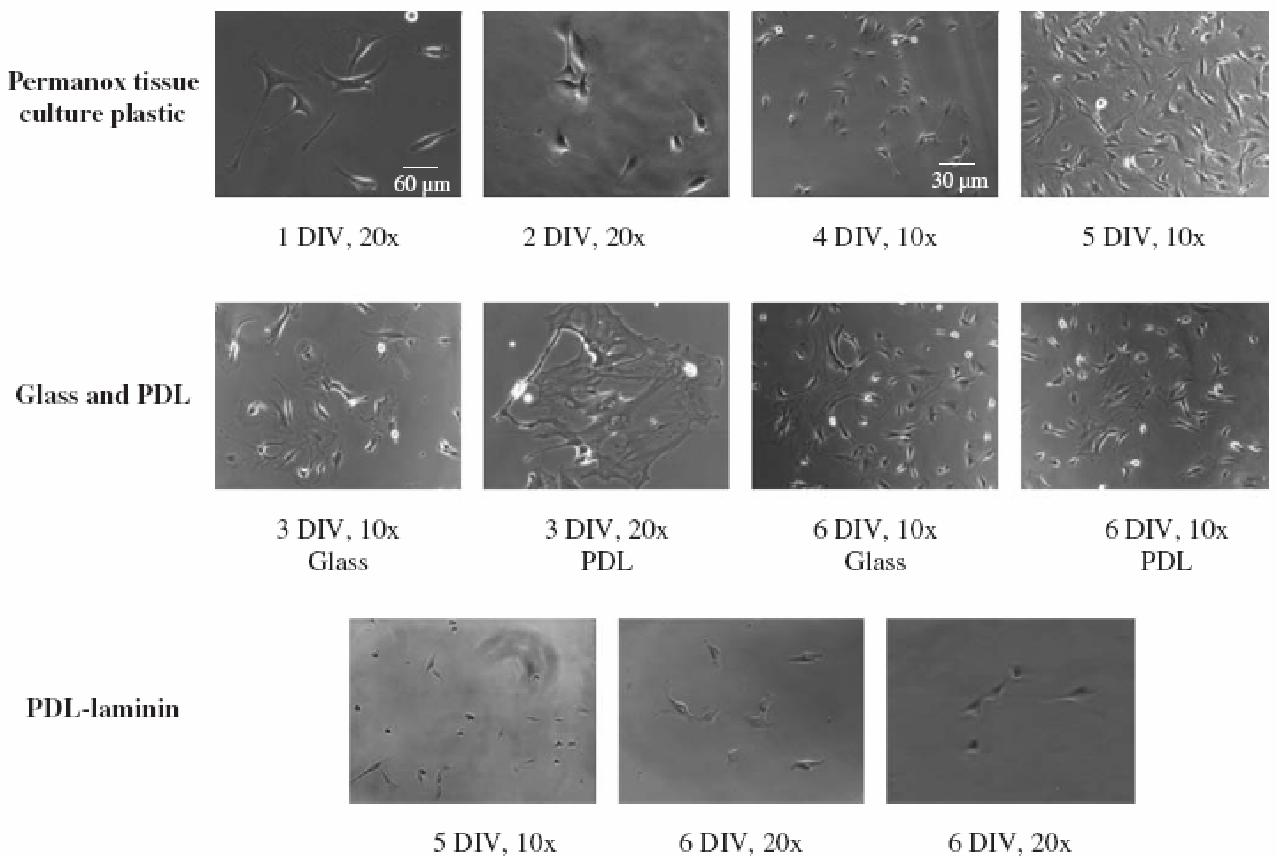


Fig. 2.1 Representative fields showing BMSC morphologies for different culture surfaces (see text for detail). Morphologies were classified as 1) fibroblast, exhibiting elongated cell body with directional lamellopodia and pseudopodia; 2) flat, having indistinct cell body extensive spread of lamellopodia; 3) other morphologies, which includes subpopulations of pod-shaped cells with extensions of lamellopodia along one edge of the cell body, rounded cells with round bodies and circumferential extension of lamellopodia, or globular cells with rounded cell bodies and little processes.

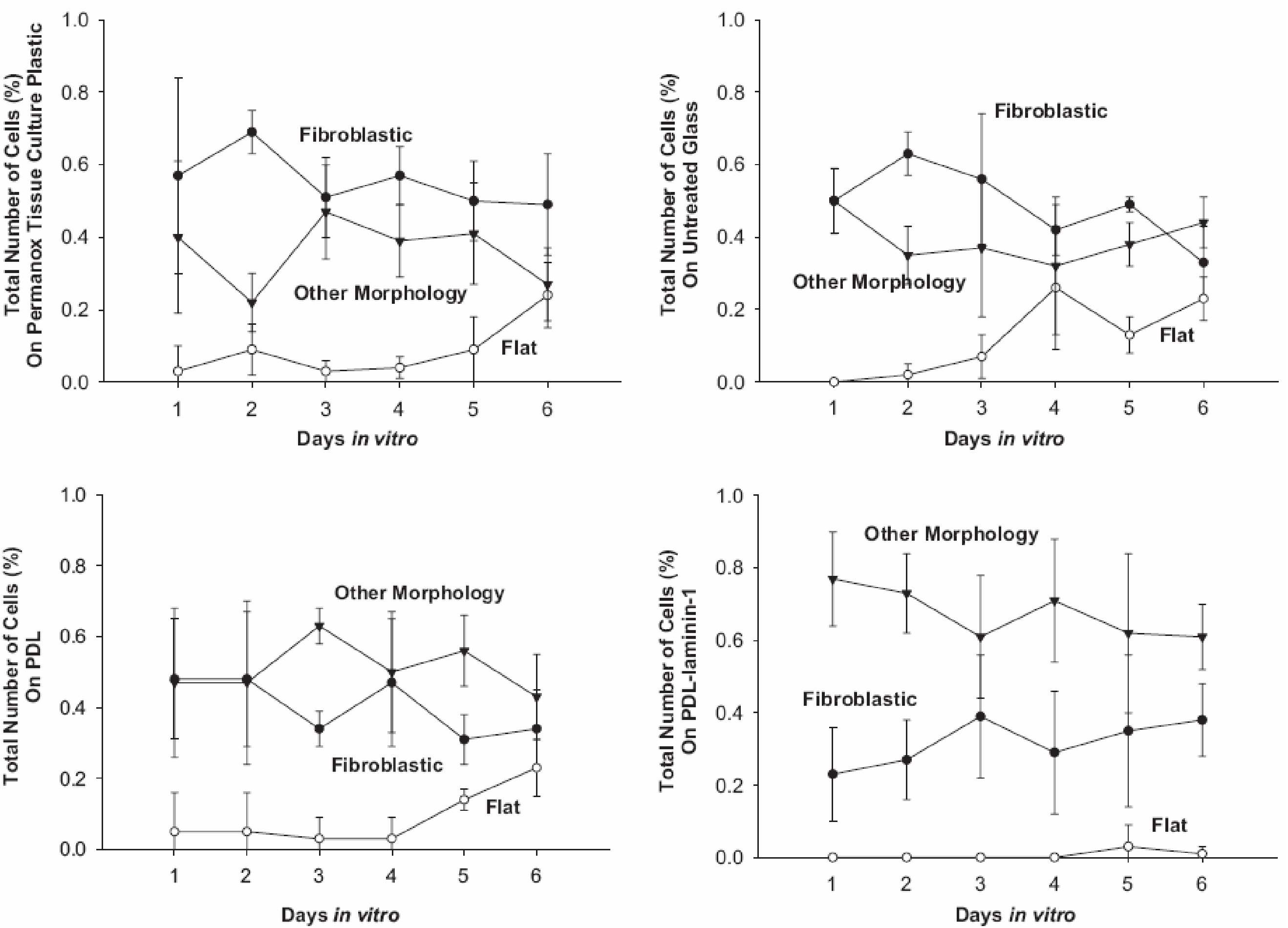


Fig. 2.2 Summary data for cell counts of morphological changes as a function of the culture surface. The different morphologies were classified as described in Fig. 2.1 and the text. Error bars represent standard deviations.

lamellopodia and pseudopodia; 2) flat, having indistinct cell body with extensive spread of lamellopodia; and 3) other morphologies, which included subpopulations of pod-shaped cells with extensions of lamellopodia along one edge of the cell body, rounded cells with round bodies and circumferential extension of lamellopodia, or globular cells with rounded cell bodies and little processes (Fig. 2.1). A CD90 +/GFAP -/nestin - fibroblastic morphology (as defined above) was interpreted as BMSCs in a completely undifferentiated pluripotent state (Kicic, Shen et al. 2003), while the positive expression of either GFAP and/or nestin, which is associated with committed neural lineages (Sotelo, Toh et al. 1980; Madigan, Penfold et al. 1994; Lendahl 1997; Eng, Ghirnikar et al. 2000; Wiese, Rolletschek et al. 2004), or a flat morphology, which associated with osteogenic lineages (Sotelo, Toh et al. 1980; Madigan, Penfold et al. 1994), were interpreted as BMSCs that were not in a completely undifferentiated state.

There were some common morphological patterns but also noticeable differences in the way cells developed on the different surfaces over the culture period we investigated (Figs. 2.1-2.2). With the exception of cells cultured on PDL-laminin-1, most cells initially displayed the fibroblastic morphology typically associated with undifferentiated BMSCs (Okano 2002). As a function of time in culture, there were evident changes in cell shape (i.e. >10% in the cell counts) that suggested a transition from the initial state, through morphologies listed in the ‘other’ category, into the flat morphology that has been previously characterized by others to be indicative of dedifferentiation, senescence, and loss of multipotency (Javazon, Colter et al. 2001; Lange, Schroeder et al. 2005). These cells were demonostrated to have differentiation

capability limited to the osteogenic pathway intrinsically taken by BMSCs and to sometimes undertake such differentiation spontaneously (Digirolamo, Stokes et al. 1999; Muraglia, Cancedda et al. 2000).

During 1-4 days *in vitro* (DIV), the morphology of BMSCs cultured on tissue culture treated plastic remained relatively stable (Fig. 1, top panels). About 55% of the cells appeared fibroblastic with directional lamellopodia along one edge of the elongated cell body and pseudopodia extending in other direction. About 9% of the BMSCs were flat with a significantly large spread of lamellopodia and the remaining 36% appeared small with distinct pod-shaped or rounded cell bodies (Fig. 2.2). The percentage of fibroblastic cells remained constant for the initial 4 days in culture, but by 6 DIV ~24% of cells on tissue culture plastic adopted the flat morphology (Fig. 1, top panels), suggesting of commitment to osteogenic differentiation.

BMSCs cultured on PDL coated glass and untreated glass showed similar morphologies across all time points. On 1 and 2 DIV, there were 40-50% fibroblastic and 10-15% flat cells (Fig. 2.2). However, the transition to the flat morphology was observed earlier (as early as 3 days *in vitro*) on these two surfaces in comparison with tissue culture plastic. In addition to cells with large circumferential spread of lamellopodia, a few flat cells appeared to have large loop-shaped cell bodies with branching pseudopodia. By 6 DIV, only ~33% of the cells on PDL and untreated glass still displayed the fibroblastic morphology (compared to ~49% on tissue culture treated plastic) (Fig. 2.1, middle panels).

The most significant morphological differences were observed for BMSCs cultured on PDL-laminin-1 coated glass. Throughout the 6 day culture period, MSCs on PDL-laminin-1 exhibited sub-confluent cultures due to a reduced proliferation rate in comparison to cells grown on the other surfaces. While 32% cells remained fibroblastic and only 1% appeared flat, an overwhelming majority of BMSCs (67%) were noticeably small and globular in shape for DIV 1-4 (Fig. 2.1, bottom panels). Some cell spreading was observed during later days in culture, but in general each cell occupied significantly less surface area than BMSCs grown on other culture surfaces.

B. Laminin-1 alone was able to induce neural progenitor cell characteristics

Immunocytochemistry was done to detect the expression of CD90, nestin, and GFAP in all experimental conditions at 6 DIV. As expected, cells on PDL-laminin labeled positive for CD90 in upwards of 80%, indicating that most of the BMSCs were to some degree in an undifferentiated state (Fig. 2.3, left column of panels). GFAP labeled negative for all four experimental conditions (Fig. 2.3, third column of panels). There have been conflicting reports in the literature regarding the presence of GFAP in cultured BMSCs (Prockop 1997; Azizi, Stokes et al. 1998; Libby, Champliaud et al. 2000) and our results suggested that BMSCs cultured on standard surface chemistries under basic conditions that only sustain growth do not inherently express GFAP. Nestin stained negative on TCP, untreated glass, and PDL coated glass (Fig. 2.3, second column of panels). However, there was strong positive nestin labeling in PDL-laminin-1 cultures. The presence of this neuronal progenitor specific protein suggests a bioactive signaling

role for laminin-1 in the induction of BMSCs towards the neuronal lineage. Of the total number of cells counted ($32 \leq n \leq 100$ between different experiments), 84% of MSCs cultured on PDL-laminin-1 expressed CD90, while about half of them (53%) expressed nestin. In all cases, negative controls with omitted primary antibodies were done to assess levels of background fluorescence and verify specific staining (data not shown).

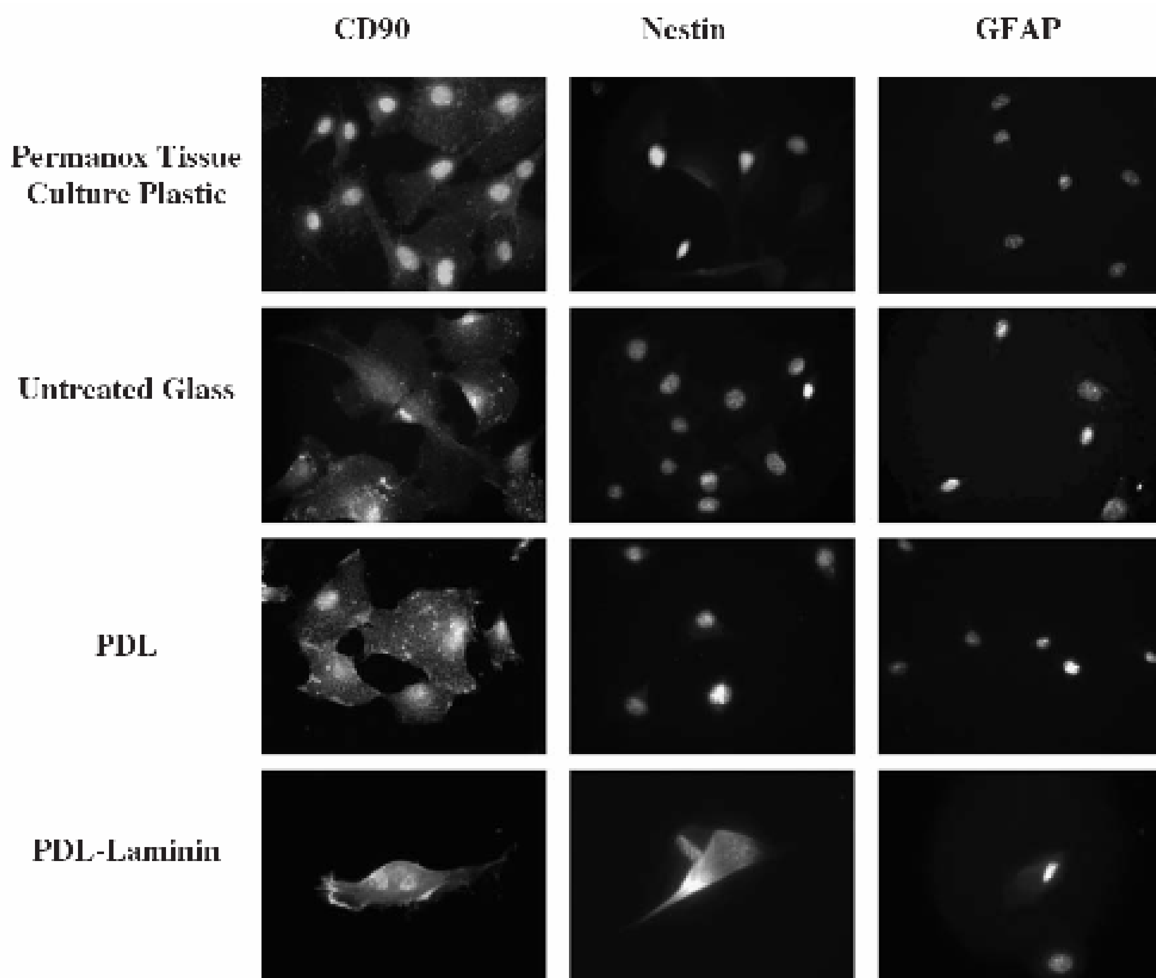


Fig. 2.3 Immunocytochemical characterization of BMSCs cultured on different surfaces for anti-CD90, anti-nestin, and anti-GFAP antibodies, specific markers for undifferentiated multipotent BMSCs, neural progenitor cells, and mature macroglial cells, respectively. The nuclei were counter-stanied using Dapi.

Discussion

In order to identify the simplest culture surface that supports BMSC expansion in a pre-differentiated state in order to avoid experimentally confounding results in subsequent differentiation experiments, we cultured adult rat BMSCs on four different representative standard surface chemistries: Permanox Lab-Tek® tissue culture treated plastic (TCP), poly-D-lysine (PDL) coated glass, PDL-laminin-1 coated glass, and untreated glass. Over the course of 6 days we observed morphological differences on the different culture surfaces. Our results indicate that TCP provided the most reliable and consistent surface for the growth and long term maintenance of undifferentiated BMSC's, consistent with other reports (Colter, Sekiya et al. 2001; Sekiya, Larson et al. 2002; Neuhuber, Gallo et al. 2004; Qian and Saltzman 2004; Rodriguez, Gonzalez et al. 2004). This is most likely due to the enhanced wetability and cell attachment properties of this surface brought about by surface treatment of oxygen plasma. Since oxygen plasma is the most prevalent technique used to functionalize polystyrene for tissue culture, we believe that the result we observed on our TCP surface will be representative of most commercially available polystyrene tissue cultureware.

In comparison to cells grown on TCP, BMSC's on PDL and untreated glass generally displayed a lower percentage of the fibroblastic morphology, the characteristic morphology of undifferentiated BMSCs, and promoted an earlier transition to the flat morphology typical of cell senescence and restriction to the osteogenic lineage (Jaiswal, Haynesworth et al. 1997; Digirolamo, Stokes et al. 1999; Muraglia, Cancedda et al. 2000; Lange, Schroeder et al. 2005). These results are consistent with other investigations (Qian

and Saltzman 2004), indicating that coating surfaces with PDL alone, a suitable surface for culturing other cell types, does not significantly enhance or support BMSC attachment. This reduced cell attachment could putatively be a factor that promotes commitment to osteogenic lineage in BMSC's as indicated by an earlier transition and higher percentage of the flat morphology on these surfaces.

PDL-laminin-1 coated glass can itself constitute a neural differentiation condition for various cell types as shown by us and others (Bellamkonda, Ranieri et al. 1995; Powell, Rao et al. 2000; Mehes, Czirok et al. 2002; Silva, Czeisler et al. 2004). Our results here indicate that this surface combination is unsuitable for the purpose of maintaining pre-differentiation conditions of BMSC during cell expansion and passaging. Our results suggest that this surface chemistry does not support BMSC growth particularly well, while at the same time is sufficient to induce nestin expression in undifferentiated cells. Laminins are large multi-domain trimeric proteins that exist in various isotypes due to alternative splicing and post-translational modifications (Luckenbill-Edds 1997; Tunggal, Smyth et al. 2000). The expression of different laminin isoforms varies spatially and temporally between tissues as well as throughout development and adulthood, playing key roles in signaling and coordinating specific cell events. Laminin-1 consists of a 400 kDa $\alpha 1$ chain, a 200 kDa $\beta 1$ chain, and $\gamma 1$ chain and is the most studied of the twelve known laminin isoforms. Laminin-1 directly participates in the development of the central nervous system while laminin-$\beta 2$ is known to promote photoreceptor proliferation and development in the neural retina and sub-retinal space (Libby, Hunter et al. 1996; Libby, Champliaud et al. 2000; Powell, Rao et al. 2000).

BMSCs have been shown to express integrin receptors under various conditions during normal development, the principle receptor for laminins (Muller, Wang et al. 1997; Ekholm, Hankenson et al. 2002; Klees, Salasznyk et al. 2005). It is conceivable that if laminin-1 is able to induce early neural specific protein expression in undifferentiated BMSCs, other extracellular matrix derived proteins may be able to do the same, making them unsuitable for simple cell expansion. Ultimately, in a direct comparison of these four basic surfaces, simple tissue culture treated plastic provided the most consistent and reliable surface for maintaining and expanding BMSCs in a pre-differentiation condition and is the most suitable surface for avoiding confounding experimental results that may result from the choice of surface chemistry during an experiment.

Conclusions

We demonstrate that simple tissue culture treated plastic provides the most consistent and reliable surface for the purpose of expanding and maintaining multipotent undifferentiated BMSC populations prior to differentiation experiments. These results suggest that tissue culture treated plastic can be used to expand BMSC's without the danger of unintended differentiation effects that may confound experimental variables in subsequent differentiation experiments. The other surfaces we tested, untreated glass, PDL-glass, and PDL-laminin-1 on glass, resulted in reduced cell growth and minimal proliferation, morphologies associated with early committed phenotypes, and/or the positive expression of proteins associated with early committed phenotypes.

Acknowledgement

Chapter 2 is a reprint of the material as it appears in Comparison of Standard Surface Chemistries for Culturing Mesenchymal Stem Cells Prior to Neural Differentiation, Ho, Mai*; Yu, Diana*; Davidson, Marie; Silva, Gabriel, Biomaterials, vol. 24, 2006. * *contributed equally.*

CHAPTER III

Glial Calcium Signaling in Health and Disease

Introduction

Glia, similar to other eukaryotic cells, relies in calcium ions to display a large variety of signaling capabilities inside the cell and among its neighbors. These calcium events are key to regulate not only the growth and survival of glial cells but also its ability to respond to cues from the extracellular microenvironment as well as communicate with the local neuron population. Since glial cells are not electrically excitable, calcium signaling is particularly important to understanding how glia regulate synaptic activity and CNS function. Calcium signaling is largely based on the existence of a strong calcium gradient between the extracellular and intracellular environments. The tight regulation of this gradient is key to ensuring the efficacy and reliability of the signal. The way glial cells achieve this control is similar to many other cell types and denotes a high degree of conservancy among nonexcitable cell types. This section will discuss the details of this type of signaling, its role in neurophysiology and pathology, the different underlying mechanisms that contributes this signaling behavior, and finally, the experimental tools used to visualize, quantify, and study its spatial and temporal dynamics.

A. Role of glial calcium signaling in neurophysiology

The cytosolic calcium concentration in glial cells, such as astrocytes, is normally maintained at approximately 50-200 nM as compared with ~1 mM in the extracellular space. Upon stimulation, rapid and transient increases in the cytosolic calcium can reach levels as high as 1 μM or more. The functional roles of macroglia (i.e. astrocytes and oligodendricytes in the CNS and Müller cells in the retina) may extend beyond their traditional roles as homeostatic support cells to include extensive intercellular signaling that directly contributes to information processing in the CNS (Dani, Chernjavsky et al. 1992; Porter and McCarthy 1996; Kang, Jiang et al. 1998; Biedermann, Bringmann et al. 2004; Newman 2004; Schipke and Kettenmann 2004; Newman 2005). Specifically, changes in the intracellular calcium concentration of both Müller cells and astrocytes have been implicated as a key mechanism of intra- and intercellular signaling in networks of these cells (Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991; Porter and McCarthy 1996; Araque, Martin et al. 2002; Newman 2005). Asynchronous changes in intracellular calcium and synchronous propagation of calcium transient mediated signaling have been documented in networks of hippocampus and spinal cord astrocytes in response to neurotransmitters (glutamate, γ-amino butyric acid (GABA), and acetylcholine) released by neurons (Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991; Cornell-Bell and Finkbeiner 1991; Porter and McCarthy 1996; Pasti, Volterra et al. 1997; Kang, Jiang et al. 1998; Araque, Martin et al. 2002; Schipke and Kettenmann 2004; Newman 2005; Haas, Schipke et al. 2006), the activation of ionotropic and metabotropic purinergic receptors (Franke, Krugel et al. 1999), and mechanical deformations of the plasma membrane (Charles, Merrill et al. 1991). Astrocytes in the

hippocampal and cortical brain slices are capable of responding to stimulated neuronal activity with calcium oscillations that are modulated by the frequency of the neuronal stimulations, although these oscillations were found to involve activation of metabotropic glutamate receptors (Pasti, Volterra et al. 1997). Furthermore, these astrocyte calcium oscillations are accompanied by similar oscillations in adjacent neurons depending on the activation ionotropic glutamate receptors, supporting the notion that astrocytes and neurons can establish a bi-directional form of communication based on their specific patterns of calcium oscillations. Finally, spontaneous calcium oscillations have been observed in thalamic and hippocampal astrocytes in *in situ* (Nett, Oloff et al. 2002; Parri and Crunelli 2003). Similar to the neuron-induced oscillations, these spontaneous oscillations require release from IP3-sensitive intracellular stores and depend on store refilling via plasma membrane calcium channels.

Similar responses have been reported for astrocytes and Müller cells in explant retina preparations (Newman and Reichenbach 1996; Newman 2001) following the application of light stimuli (Newman 2005), neurotransmitters (GABA, adenosine triphosphate (ATP), and glycine) (Biedermann, Bringmann et al. 2002; Biedermann, Bringmann et al. 2004; Newman 2005), and mechanical stimulation (Newman and Zahs 1997). Increases in intracellular calcium levels in Müller cells is believed to modulate synaptic transmission in the retina by regulating local glutamate, glycine, and potassium concentrations (Newman 1987; Keirstead and Miller 1997; Gadea, Lopez et al. 2002; Schopf, Ruge et al. 2004; Bringmann, Pannicke et al. 2006). In addition, it has been associated with the opening of K^+ channels on retinal ganglion cells in response to

activation of A_1 receptors by adenosine converted from extracellular ATP (Newman and Zahs 1998; Newman 2003; Newman 2004).

While calcium signaling in forms of intracellular calcium oscillations and intercellular waves have been extensively studied in *in vitro* cultures and in *in situ* tissue samples, it is still unclear their involvement in neurophysiology. It is speculated that intracellular oscillations can encode information by specific frequency of the events. This has been shown in detail in cells of the immune system, in which particular frequencies of calcium activity promoted both qualitative and quantitative differences in gene expression compared with a steady level of calcium (Li and Carter 1998). However, an important aspect of frequency-coded information is the need for cellular decoders to translate the message into a cellular response. Protein kinase C (PKC) has been thought to be involved, specifically in astrocytes, by responding to the oscillating levels of DAG and calcium concentrations (Codazzi, Teruel et al. 2001). As for intracellular calcium waves, it is still an open question whether they are indeed initiated under physiological conditions. Since induction of calcium waves usually requires strong stimulation, it is though that this type of signaling might relate more to pathoophysiological events rather than normal neurophysiology. In fact, calcium wave in astrocytes do share several features with the phenomenon of spreading depression that is associated with stroke migraine, and head trauma and will be discussed in more detail in the following section.

B. Molecular mechanisms of calcium signaling in glia

Studies on the ability of various extracellular signals to elicit calcium transients in macroglia has indicated heterogeneity in the expression of different receptors (i.e. glutamate receptors like NMDA, AMPA, as well as various subtypes of metabotropic and ionotropic purinergic receptors) among glia derived from different region of the CNS. However, the primary mechanisms of cytosolic calcium increase in glial cells is release from intracellular stores, specifically from the endoplasmic reticulum (ER), where calcium concentrations are kept in the high micromolar range. It is thought that activation of the G-protein-coupled metabotropic purinergic receptors (P2YR) on the plasma membrane by extracellular ATP activate phospholipase C (PLC) and stimulate the generation of the second messenger inositol 1,4,5-triphosphate (IP3) (as well as diacylglycerol (DAG)) by PLC-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2). Binding of IP3 to IP3 receptors on the ER will open these channels and allow the efflux of calcium into the cytosol. (Munshi, Pang et al. 1991; Cunha, Milusheva et al. 1994; Frohlich, Boehm et al. 1996; Schubert, Ogata et al. 1997; Newman and Zahs 1998; Jimenez, Castro et al. 2000; Brambilla, Cottini et al. 2003; Fumagalli, Brambilla et al. 2003; Newman 2003; Nobile, Monaldi et al. 2003; Newman 2004). In addition, the intracellular diffusion of the second messenger inositol-1,4,5-trisphosphate (IP_3) throughout the glial syncitium via Cx43 gap junctions have also been implicated in the intercellular propagation of calcium transients to adjacent neighboring cells (Berridge and Galione 1988; Berridge 1990; Charles, Merrill et al. 1991; Zahs and Newman 1997; Ceelen, Lockridge et al. 2001) (Suadicani, Flores et al. 2004).

Astrocytes possess an array of receptors linked to the mobilization of intracellular calcium stores (Deitmer, Verkhratsky et al. 1998). There are three isoforms known for the IP3 receptor family, however, which isoforms are involved in glial intracellular calcium release is still unclear. Immunohistochemical studies in intact tissue have so far been inconclusive, showing either the presence of IP3R3 but not type 1 or 2 (Yamamoto-Hino, Miyawaki et al. 1998) or predominately IP3R2 (Sharp, Nucifora et al. 1999). The latter group also reported the expression IP3R2 in cultured astrocytes, yet RT-PCR studies on similar cultures found mRNA for only IP3R1 (Pearce and Murphy 1993). It is generally believed that IP3 is then subjected to dephosphorylation by 5-phosphatase, thus terminating its effect. However, its also a substrate for the enzyme IP3-3-kinase, which yields inositol 1,3,4,5-tetrakisphosphate. Two isoforms of the 3-kinase (A and B) are know to exist where the B isoform is shown to exist in astrocytes and is regulated by PKC and Ca-calmodulin-dependent kinase II (Communi, Dewaste et al. 1999). The function of 1,2,4,5-IP4 is still unclear. Some possibilities include: protection of IP3 from 5-phosphatase hydrolysis, and thus enhancing calcium mobilization; regulation of calcium influx in response to store depletion; inhibition of the IP3Rs contribution to the generation of calcium oscillations; a precursor for more highly phosphorylated messenger IPs.

While the initial calcium response is determined by the expression and location of purinergic receptors and IP3 receptors in the glial cells, two additional mechanisms have been identified that lead to subsequent amplification of the initial calcium signal. Initial release of calcium from ER from IP3 channels can be followed by further release,

mediated by the ryanodine receptors (RyRs) via calcium-induced calcium release (CICR). CICR has been previously reported in cultured hippocampal astrocytes, but the extent of this calcium activity in astrocytes needs to be further investigated (Berridge, Lipp et al. 2000). In addition, ER calcium release can be followed by calcium entry across the plasma membrane through store-operated calcium channels which are activated when the ER calcium stores are depleted. This process, termed capacitative calcium entry (CCE), provides a more sustained calcium increase in to the cytosol as long as the ER calcium store remains empty. CCE have been demonstrated in cortical and cerebellar astrocytes upon depletion of ER store with thapsigargin, which inhibit the sarco-endoplasmic reticulum calcium adenosine triphosphatase (SERCAs) and preventing its function to refill the ER (Wu, Bungard et al. 2001).

In order to maintain tight control of intracellular calcium concentration, extrusion channels, buffering protein, and intracellular organelles are activated by the transient increase in cytosolic calcium to immediately reverse the change and return to homeostatic state. High affinity calcium binding proteins such as troponin C, parvalbumin, calcineurin, and calmodulin in the cytosol rapidly work to chelate the excess calcium ions while calcium ATPase pumps on the ER (SERCAs) and plasma membrane (PMCAs) as well as Na/Ca exchangers quickly siphons calcium ions into the ER or the extracellular space. In addition, mitochondira are also activated to uptake the excess calcium using a low-affinity uniporter. Although we have a good understanding of the key molecular mechanisms involved in glial cell signaling, essentially nothing is known about the network properties of these macroglial networks, and it is not understood how they may

be participating and contributing to the modulation of information processing in the retina and rest of the CNS.

C. Role of glial calcium signaling in pathophysiology

Calcium signaling in glial cells has been implicated in facilitating and attenuating various pathological conditions in the CNS. Studies done on cultured and acute slices of hippocampus and neocortex suggested interactions between Ca^{2+} waves within the astrocyte population and the phenomenon of spreading depression (SD) among neurons (Martins-Ferreira, Nedergaard et al. 2000) (Nedergaard, Cooper et al. 1995; Theis, Jauch et al. 2003) (Basarsky, Duffy et al. 1998) (Kunkler and Kraig 1998), a slowly propagating wave of neuronal depolarization that has been recognized to play an important role in the aura of migraine and progression of cortical infarct volume after stroke (Gorji 2001; Gorji, Scheller et al. 2001) (Hadjikhani, Sanchez Del Rio et al. 2001) (Back, Ginsberg et al. 1996). SD propagates with a velocity of 15-35 µm/s and has been shown to be preceded by a wave of astrocyte calcium increases which spreads with the same velocity but is ahead of the neuronal depolarization by 6-16 seconds (Neadergaard and Hansen, 1993). Furthermore, cortical SD initiated in neocortex slices by local delivery of KCl has been shown to increase the propagation speed of the associated astrocyte Ca^{2+} wave (Peters, Schipke et al. 2003).

In addition, there is also evidence of elevated calcium signaling among retinal Müller cells during reactive gliosis, a neuroflammation response of the CNS to disease (i.e. retinal degenerative diseases) characterized by proliferation and infiltration of glial

cells into the injury sites. In animal models of retinal detachment, Müller cells in the detached area as well as close-by attached regions showed enhanced responsiveness to extracellular adenosine 5'-triphosphate (ATP) (Iandiev, Uckermann et al. 2006) (Uhlmann, Bringmann et al. 2003) (Bringmann, Pannicke et al. 2001) (Francke, Weick et al. 2002) as evidenced by higher number of cells exhibiting increases in intracellular calcium, due most likely to the upregulation of P2Y receptors during Müller cell reactive gliosis (Bringmann, Francke et al. 1999; Bringmann, Pannicke et al. 2001; Francke, Weick et al. 2002; Francke, Uhlmann et al. 2003; Uckermann, Uhlmann et al. 2003). In addition, it has been proposed that a potential mechanism for the enhanced calcium responsiveness in gliotic Müller cell is the active resensitization of P2Y receptors by PDGF, EGF, and NGF (Weick, Wiedemann et al. 2005), growth factors that have been crucially implicated in gliotic responses and proliferation during retinal detachment and PVR (Esser, Weller et al. 1992; Wiedemann 1992) (Charteris 1995; Faude, Francke et al. 2001). Furthermore, elevations in intracellular calcium via P2Y receptors, in conjunction with transactivation of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptor tyrosine kinases, have been shown to promote DNA synthesis (Moll, Weick et al. 2002) and extracellular signal-regulated kinase (ERK1/2) activity (Milenkovic, Weick et al. 2003) through calcium-dependent big conductance K^+ channels (BK channels) (Kodal, Weick et al. 2000; Moll, Weick et al. 2002; Weick, Wiedemann et al. 2005). In addition, activation and upregulation of ionotropic P2X receptors on Müller cells have been shown to contribute to intracellular calcium transients via influx of extracellular calcium in proliferative vitreoretinopathy (PVR), thus enhancing the

mitrogenic activity of BK channels (Sugioka, Zhou et al. 1999; Sugioka, Zhou et al. 1999; Bringmann, Pannicke et al. 2001).

D. Imaging calcium fluctuations using fluorescent calcium indicators

Intracellular calcium oscillations and intercellular propagation of calcium transients in networks of glial cells are visualized using fluorescent calcium indicator dyes. The commonly available fluorescent indicators for Ca^{2+} fall into two classes: single-wavelength intensity-modulating dyes and dual-wavelength ratiometric dyes. For single-wavelength indicators, changes in calcium concentrations bring about changes in the intensity of their fluorescence excitation and emission spectra where as the spectral maxima remain essentially unchanged. The excitation wavelength of Fluo-4, a single-wavelength indicator, is at 488nm, and produces >100-fold increase in intensity of emission (516nm) upon binding to calcium (K_d = 345nM)(Gee, Brown et al. 2000) (Fig. 3.1). The free Ca^{2+} concentration is given by:

$$[Ca^{2+}] = K_d\ (F-F_{min})/(F_{max}-F)$$

where F_{min} and F_{max} are fluorescence intensities of indicator when it is Ca^{2+} free and Ca^{2+} bound, respectively. F is the measured fluorescence intensity elicited by a corresponding value of $[Ca^{2+}]$.

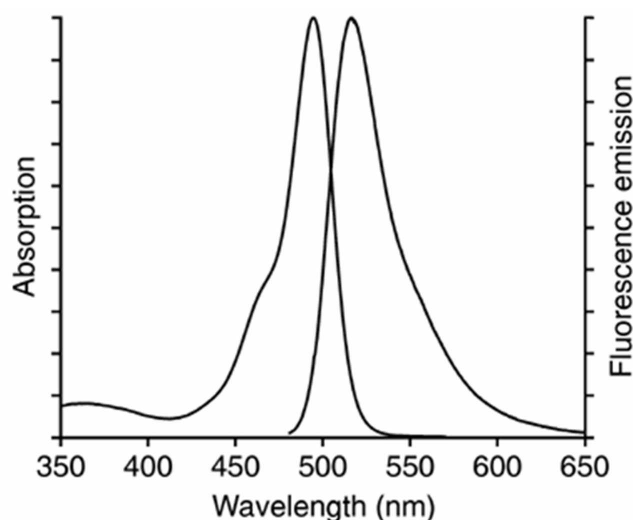


Fig.3.1 Characteristic absorbance and emission spectrum of Fluo-4 AM.

The Fluo class of calcium indicators possess a BAPTA-like calcium chelator component covalently linked to a fluorogenic, fluorescein-like component that undergo large fluorescence enhancement upon the binding of calcium. However, the common fluorescent Ca^{2+} indicators are polycarboxylate anions that cannot cross lipid bilayer membranes and therefore are not cell permeate. In the negatively charged form, the indicators can only be delivered into the cells by microinjection or by permeabilization procedures. To facilitate intracellular loading of the indicator dye, an acetoxymethyl (AM) ester group is linked to the carboxyl group via an ester bound to mask the negative charges, thus creating an uncharged and hydrophobic moiety that can freely diffuse across the cell membrane (Fig. 3.2).

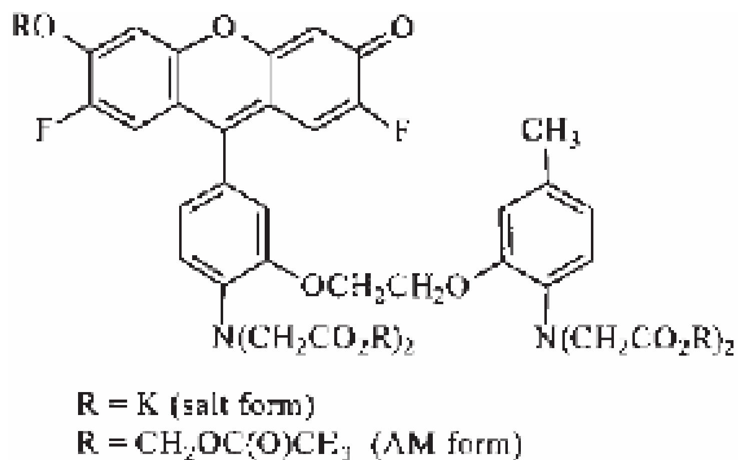


Fig. 3.2 The chemical structure of Fluo-4 calcium indicator dye and its modified cell-permeate form using acetoxymethyl ester.

Once the indicator is in the cytoplasm, the AM group is removed by esterases via enzymatic hydrolysis and the Ca^{2+}-sensitive polycarboxylate form of the indicator becomes trapped inside the cell (Fig. 3.3).

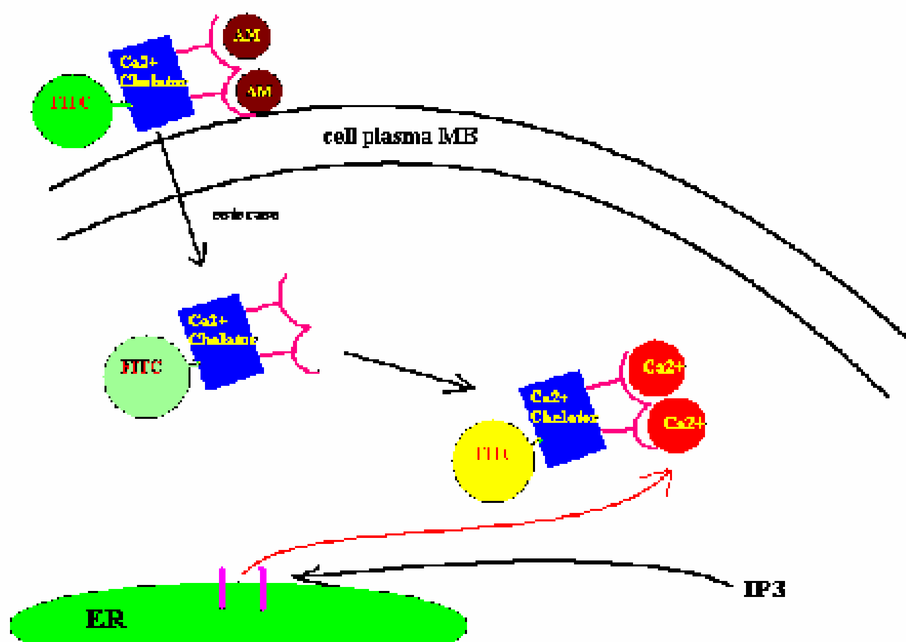


Fig. 3.3 Schematic of fluo-4 AM mechanism upon intracellular de-esterification.

E. Graph theory basics and applications to biological networks

This section will give a brief introduction to network theory and its basic mathematical principles. Interactions between basic elements of a complex network can be described using a set of theoretical rules that is independent of the physical nature of the network. Therefore, network topologies of neural network may be characterized and explored in a similar way as for metabolic networks. There are various types of network structures that has been developed (i.e. random, small-world, scale-free) each displaying different behaviors and properties and determined by the underlying mathematical rules that describe them. The scale-free network will be discussed here as it has shown to be most relevant to biological applications. As in all networks, the fundamental element of the network is termed a "node," and different nodes can be assigned a number, k, to uniquely identify it. The existence of a connection between two nodes can be described using a probability function, $P(k)$. For example, $P(k) = 1/k$ for a random network since each node would have the same chance of being connected to another node in the network(Evans 2004). The scale-free network is characterized by a probability function that is proportional to the inverse of the total number of nodes in the network:

$$P(k) \sim k^{-\gamma}$$

where gamma is a constant (Strogatz 2001). As such, two main features emerge from these networks: 1) structured growth (scale free networks tend to grow in an organized way), and 2) preferential attachment (new nodes will preferentially link to existing nodes with high number of existing attachments). A direct consequence of this arrangement is that a few nodes in the network will tend to have an un-proportionately high number of

links, while most nodes will have smaller, more constant number of links. One can intuitively appreciate the difference between a scale-free versus a random network, where the probability of any two nodes being connected with each other is the same for all nodes. Mathematically, preferential attachment is achieved by defining the probability that a new node connects to an existing node *i* as:

$$\Pi(k_i) = k_i / \Sigma_j k_j$$

The probability of a new node connecting to node *i* is equal to the ratio of the connectivity of *i*, denoted by k_i, and the total number of connections *j* that exist in the network(Albert and Barabasi 2002). It is important to appreciate that the underlying mathematics that describes the scale free network topology (or any other topology) is independent of the physical details of the networks, making it is applicable to complex glial networks in the CNS.

F. Algebraic Description of Dynamic Signaling Model

We have previously introduced experimental methods and computational algorithms to map the functional activation states and connectivity topologies of glial networks with single cells resolution in order to describe the spatiotemporal evolution of functional calcium signaling in these networks (Buibas et. al.). Briefly, we begin by considering a series of growing volumes that define a signaling envelope emanating from a newly activated node (Fig. 3.4), referred to as a vertex in the language of graph and network theory, with a tolerance $\Delta\gamma$ such that if the envelope crosses another vertex that vertex becomes activated, assuming it is not refractory. For symmetry we assume signals

spread from vertices as growing circles in two dimensional networks and spheres in three dimensions with $\Delta\gamma$ as the thickness of the expanding circle in two dimensions or shell in three dimensions. This model is valid for any network where vertices are connected by edges where physical dimensions represent the shortest Euclidian distance between them, a condition that covers essentially all complex networks with a few exceptions. The growth rate (i.e. rate of change) of signaling volumes represents the signaling speed of the network, which can be a constant value, or a scalar or vector valued function. A functional connection between two vertices, also called an edge, is established if a vertex goes from being inactive to active at the same moment a signaling volume reaches it from a previously activated vertex (Fig. 3.5).

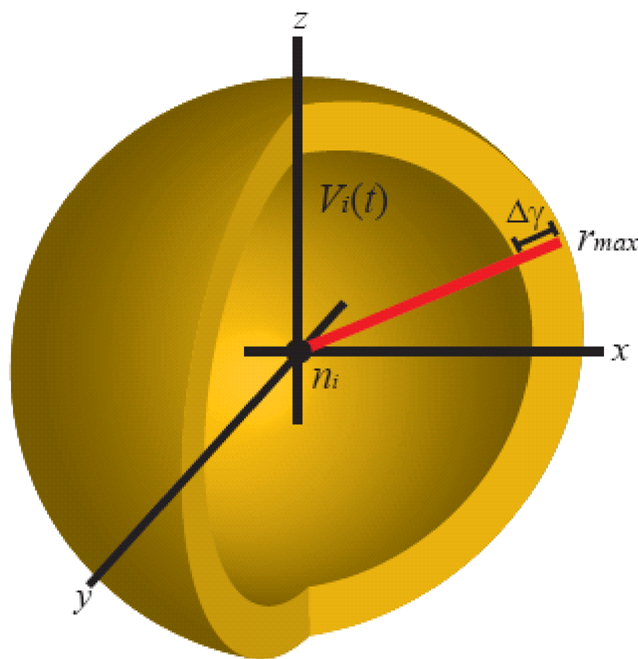


Fig. 3.4 Schematic of the Dynamic Signaling Model. The signaling volume extending from an activated node with parameters for signaling velocity, tolerance, and maximum radius of signaling.

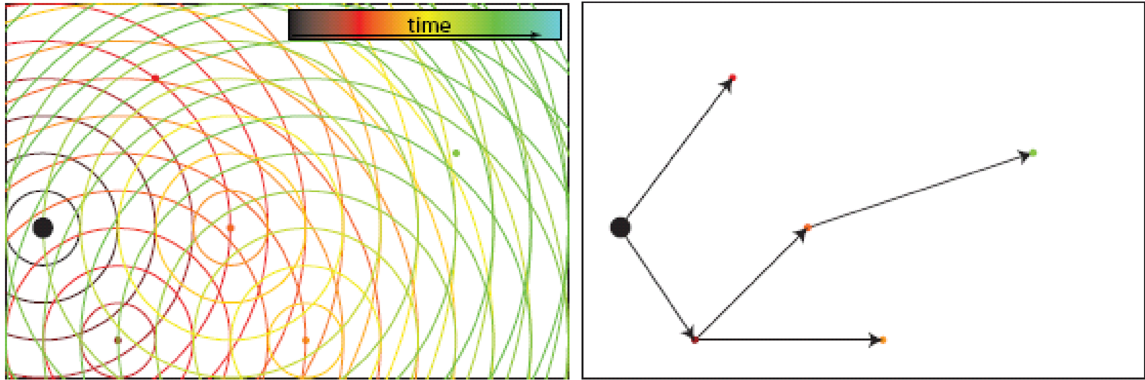


Fig. 3.5 Deterministic mapping of a simple network based on its dynamics. Node action time and expanding signaling circles are color coded with respect to time.

Mathematically the objective is to define a unique condition whereby an edge e_{ij} connects two vertices p_i and p_j, where p_i is the position of vertex i in space (e.g. $p_i = (x_i, y_i, z_i)$). An expanding volume, where the surface represents the region of active signaling, begins at the moment of vertex i activation ${}^{na}t_i$, and is defined as

$$V_i(\tau) = p_i + S(\tau)\begin{bmatrix} cos\theta \cdot cos\phi \\ sin\theta \cdot cos\phi \\ sin\phi \end{bmatrix}; \qquad 0 \le \theta < 2\pi, -\pi \le \phi \le \pi$$

where $\tau = t - {}^{na}t_i$. The signaling volume $V_i(\tau)$ is bounded for values of t so that it is positive and limited to a maximum radius r_{max} for all vertices. r_{max} is the maximum length an edge can take between any two vertices, a known or measurable network specific parameter (e.g. the maximum diffusion distance of a signaling molecule between cells, or the effective distance between routers in the internet). $S(\tau)$ is the effective radius of the expanding volume that takes into account the tolerance $\Delta\gamma$ and is defined as

$$S(t) = s \in \mathrm{R}; \qquad r(t) - \frac{\Delta\gamma}{2} < s < r(t) + \frac{\Delta\gamma}{2}$$

$r(t)$ is the positive time dependent expanding radius of the volume (i.e. $r(t) \leq r_{max}$) given by

$$r(t) = \int_{\tau}^{\tau+T} f(t)dt$$
$$\frac{dr(t)}{dt} = f(t)$$

where $f(t)$ is the speed of the expanding radius and therefore the expanding signaling volume. If a delay time D is assumed, defined as the time delay between the arrival of a signal at a vertex and its subsequent propagation to other vertices, $r(t)$ takes on the form

$$r(t) = \int_{(\tau-D)}^{(\tau-D)+T} f(t)dt$$

As strictly written, $r(t)$ implies a signaling speed $f(t)$ that is the same function for all vertices p_i. However, depending on the details of the network under consideration the signaling speed could be a vertex dependent function $f_i(t)$, resulting in a set of inhomogeneous vertex dependent signaling volumes $V_i(\tau)$ given by $r_i(t)$. Or the signaling speed could be a scalar constant such that $dr(t)/dt = F$. Given these definitions then, an edge e_{ij} is mapped if

$$p_j, ({}^{na}t_j - {}^{na}t_i) \in V_i(\tau)$$

If a vertex p_j which activates at time ${}^{na}t_j$ is in the set of points describing the expanding shell $V_i(\tau)$, then the edge e_{ij} is mapped.

Two simplifying assumptions are made. First, the vertex locations (the set of all $\mathbf{p}_i = \mathbf{P}$) are fixed in space. In other words, we assume that the spatial arrangement of vertices do not change over the period during which the functional structure of the network is being mapped, which in general is true for all networks. Secondly, we assume simple linear non-computational dynamics for the vertices themselves, in the sense that if a vertex receives a signal, it waits a specified time (i.e. the delay period D) before faithfully passing that signal on to connected vertices. There are no nonlinearities via input signal summations or activation functions in individual vertices that determine whether to pass on a signal. If a vertex is signaled it signals in turn. This puts the focus on the dynamics of the edges and allows their deterministic identification independent of any dynamics associated with the vertices. Although beyond the scope of this work, there are many rich and interesting questions that need to be explored combining more sophisticated vertex models with the dynamic signaling model we propose here. This approach would potentially contribute to a wide range of fields where network dynamics are important. Furthermore, our dynamic signaling model compliments statistical approaches such as Bayesian nets and optimization theories that can be used for estimating network topology. Finally, another parameter that the model can take into account depending on the specifics of the network being modeled is a vertex refractory period following an activation event during which a vertex cannot be re-activated. The refractory period can have significant effects on the kinetics of an evolving network by controlling ${}^{na}t_i$.

Chapter IV

Characterization of calcium-mediated intracellular and intercellular signaling in the rMC-1 glial cell line

Abstract

Retinal Müller glial cells, in addition to providing homeostatic support to the retinal neurons, have been shown to engage in modulation of neuronal activity and regulate vasomotor responses in the retina. Furthermore, calcium transient mediated signaling in Müller cells has been implicated to play a significant role in the intracellular and intercellular interactions necessary to carry out these functions. Although the basic molecular mechanisms of calcium signaling in Müller cells have been described, the dynamics of calcium responses in Müller cells have not been fully explored. Here, we provide a quantitative characterization of calcium signaling in an *in vitro* model using the rMC-1 cell line, a well established cell line developed from rat Müller cells. We show that rMC-1 cells displayed robust intracellular calcium transients and the capacity to support calcium transient mediated intercellular calcium waves with signaling dynamics similar to that of Müller cells in *in situ* retinal preparations. Furthermore, pharmacological perturbation of intracellular calcium transients with Thapsigargin as well as intercellular calcium waves using purinergic receptor antagonist and gap junction blocker (PPADS and FFA, respectively) suggest that the molecular mechanism underlie calcium signaling in rMC-1 cells is consistent with that previously described for Müller cells. In conclusion, this model provides a robust *in vitro* system for investigating

specific mechanistic hypotheses of intra and intercellular calcium signaling in Müller cell networks in a highly controllable environment.

Introduction

Müller cells are the primary macroglia of the neural sensory retina and have diverse functions in both health and disease. The classical role of these cells is providing homeostatic support to retinal neurons (Bringmann, Pannicke et al. 2006), although a number of other functions including modulating neuronal activity via bi-directional communication with neurons in the inner nuclear layer (Newman 2003; Biedermann, Bringmann et al. 2004; Newman 2004; Newman 2005; Bringmann, Pannicke et al. 2006), regulating vasomotor responses in the retina (Stevens, Esguerra et al. 2003; Metea and Newman 2006; Gustafson, Stevens et al. 2007), and contributing to degenerative retinal pathologies through reactive gliosis have been proposed (Iandiev, Uckermann et al. 2006; Bringmann, Iandiev et al. 2007). Underlying the bi-directional signaling of Müller glia with neurons and vascular regulation are calcium changes that mediate intra- and intercellular signaling processes (Metea and Newman 2006). Müller cells in *in situ* retinal and eye cup preparations have been shown to generate transient increases in intracellular calcium both spontaneously (Rogers, Stinnakre et al. 2005; Agulhon, Platel et al. 2007) and in response to light stimulation (Newman 2005), with frequencies and durations comparable to those observed from astrocytes in brain slices (Nett, Oloff et al. 2002; Parri and Crunelli 2003) and *in vivo* (Hirase, Qian et al. 2004). Activation of metabotropic purinergic P2Y receptors by extracellular adenosine 5'-triphosphate (ATP)

leading to formation of inosital triphosphate (IP_3) by phospholipase C (PLC), and IP_3-dependent calcium release from intracellular stores have been implicated as key steps in the generation of calcium transients *in vitro* (Keirstead and Miller 1995; Lopez-Colome and Lee 1996) and *in situ* (Lopez-Colome and Lee 1996; Li, Holtzclaw et al. 2001; Newman 2001; Uckermann, Grosche et al. 2002; Newman 2005) (Fig. 4.1), although there is also evidence for activation of ionotropic purinergic receptors (i.e. $P2X_7$) that augment the calcium increase via influx from the extracellular milieu (Pannicke, Fischer et al. 2000; Bringmann, Pannicke et al. 2001). Stimulation of *in situ* preparations with 488 nm light flashes has been shown to increase neuronal activity that correlates with an increased frequency of calcium transients in Müller cells (Newman 2005). Light-evoked calcium responses have been shown to be blocked by suramin, a purinergic antagonist, and apyrase, which hydrolyzes ATP, providing support for an ATP dependent mechanism. Interestingly, tetrodotoxin (TTX) is also able to block light induced calcium responses in Müller cells, suggesting that amacrine and ganglion cells (retinal neurons that generate action potentials) may be necessary for light evoked signaling to Müller cells (Newman 2005). Conversely, signaling from Müller cells to neurons may also be mediated by calcium changes in the glial cells. It is suggested that calcium increases in Müller cells mediate the release of ATP that, upon being hydrolyzed to adenosine by ecto-nucleotidases, induces hyperpolarization in retinal ganglions cells by activating A_1 adenosine receptors that open potassium channels (Newman 2003). However, the details of this calcium-dependent ATP release require further investigation.

Calcium signaling in Müller cells has also been implicated to play an important role in pathology. Specifically, upregulated intracellular calcium responses have been associated with gliosis of Müller cells in retinal detachment and proliferative vitreoretinopathy (PVR) (Uckermann, Uhlmann et al. 2003; Iandiev, Uckermann et al. 2006; Bringmann, Iandiev et al. 2007). Müller cell endfeet in acutely isolated porcine retinal wholemounts display increased calcium response to ATP stimulation 1 to 3 days following experimental rhegmatogenous detachment along with increased expression of $P2Y_1$ and $P2Y_2$ receptors. Furthermore, the increase in calcium sensitivity extended to Müller cells beyond the region of detachment; potentially caused by alterations in the functional state of P2 receptors or resensitization of receptors by soluble growth factors released during pathology (Weick, Wiedemann et al. 2005; Iandiev, Uckermann et al. 2006). Intracellular calcium increases via activation of metabotropic and ionotropic P2 receptors by extracellular ATP (as observed in retinal pathology) have also been shown to stimulate DNA synthesis and cell proliferation in primary Müller cells (Pannicke, Fischer et al. 2000; Moll, Weick et al. 2002; Milenkovic, Weick et al. 2003) and are implicated as a potential mechanism for increased glial mitogenic activity in PVR (Bringmann, Pannicke et al. 2001; Iandiev, Uckermann et al. 2006). Finally, adenosine, the degradation product of ATP, has also been found at elevated concentrations under pathological conditions such as retinal hypoxia (Ribelayga and Mangel 2005), and has been shown to potentiate increased Müller calcium transients in response to light stimulation (Newman 2005).

An intriguing but controversial observation made in *in situ* and *in vitro* experiments is the capability of Müller cells to support intercellular calcium waves mediated by intracellular calcium transients (Newman and Zahs 1997; Newman 2001; Newman 2005; Agulhon, Platel et al. 2007). Stimulation of Müller cells and retinal astrocytes in acutely isolated retina and eye cups using ATP ejection, mechanical, or electrical stimulation evoke calcium transients that propagate outward to adjacent glial cells as intercellular calcium waves (Newman and Zahs 1997; Newman 2001). Intercellular signaling between Müller cells occurs via the release of ATP from the initially stimulated cells and activation of purinergic receptors on adjacent cells, since waves have been shown to be blocked by the purinergic receptor antagonist, suramin (Newman 2001). In addition, gap junctions channels formed via homotypic and heterotypic coupling of hemichannels involving connexin 43 have also been implicated in the calcium transient-mediated intercellular signaling, presumably via allowing exchange of small secondary messengers (i.e. Ca and IP3) (Nedergaard, Cooper et al. 1995; Zahs, Kofuji et al. 2003; Suadicani, Flores et al. 2004; Kihara, Mantovani de Castro et al. 2006; Iacobas, Suadicani et al. 2007). Although experimentally evoked calcium waves have been shown to participate in the modulation of neuronal activity and vasomotor responses (Metea and Newman 2006), these observations are controversial because intercellular calcium waves in Müller cells have not been observed under physiological conditions *in vivo*, raising the issue of whether the *in vitro* and *in situ* observations may be an experimental artifact (Newman 2005). Calcium transients observed in individual Müller cells of acutely isolated eye cups in response to 488 nm light stimulation were not seen to

propagate through networks (Newman 2005), although neither the physiological range of luminous stimuli nor physiologically realistic stimulation patterns have been fully explored, thus necessitating more detailed follow up studies. However, the same paper did report intercellular calcium signaling between Müller cells under conditions that mimic neuropathology (Newman 2005). In the presence of elevated adenosine, light stimulation sometimes produced a delayed calcium response in Müller cells that propagated into neighboring cells (Newman 2005), suggesting a potential contribution of intercellular calcium waves to disease states in the neural retina.

Although the basic molecular mechanisms of calcium signaling in Müller cells have been described, the dynamics of calcium responses in Müller cells have not been fully explored. This is critical for investigating any physiological or pathophysiological roles calcium signaling may be playing. Here we provide a quantitative characterization of calcium signaling in an *in vitro* model using the rMC-1 cell line, an well established line developed from rat Müller cells (Sarthy, Brodjian et al. 1998) that has been used in numerous studies to investigate the cell biology of Müller cells and their contributions to pathology (Sarthy, Brodjian et al. 1998; Kannan, Bao et al. 1999; Lu, Bao et al. 1999; Du, Smith et al. 2002; Du, Miller et al. 2003; Du, Sarthy et al. 2004; Shelton, Kern et al. 2007). The dynamics of individual intracellular calcium transients and intercellular calcium waves were analyzed in individual and networks of rMC-1-cells. Very similar to data from primary Müller cells *in vitro* and *in situ*, rMC-1 cells displayed robust intracellular calcium transients and the capacity to support calcium transient mediated intercellular calcium waves. Furthermore, pharmacological experiments suggest that the

molecular mechanism underlie calcium signaling in rMC-1 cells are dependent on activation of purinergic receptors by extracellular ATP and, to a lesser extent, IP_3 mediated gap junction signaling, as has been described for Müller cells. Lastly, the dynamics of this calcium signaling in rMC-1 cells are quantitatively very similar to that of *in situ* Müller cells of intact retinal preparations with preserved local cytoarchitecture. Although *in vitro* systems are simplified representations of physiological conditions, the calcium signaling mechanisms in rMC-1 cells seem to have been conserved with respect to the known physiological mechanisms in Müller cells. And although rMC-1 cells differ significantly from their relatives in the retina in some respects (e.g. morphology, functional polarization, etc.), the results we provide in the present work as well as the data from others (Sarthy, Brodjian et al. 1998; Kannan, Bao et al. 1999; Lu, Bao et al. 1999; Du, Smith et al. 2002; Du, Miller et al. 2003; Du, Sarthy et al. 2004; Shelton, Kern et al. 2007) suggest that, on a molecular and cellular level, rMC-1 cells are a good model of Müller cells, and can provide an opportunity to study these fundamental processes under controlled experimental conditions where complexity of the physiology or pathophysiology may confound direct measurements.

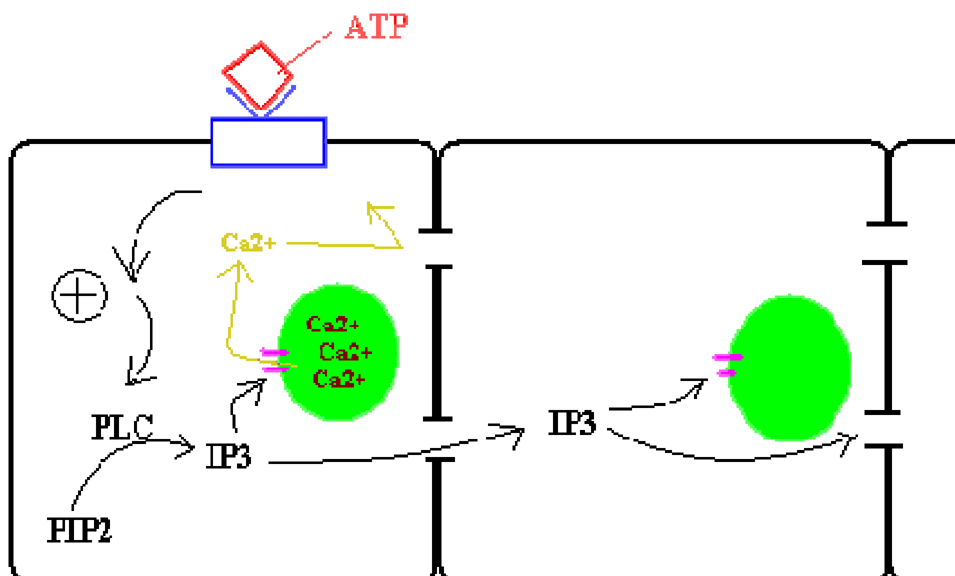


Fig. 4.1 Schematic of the molecular mechanism of calcium signaling in glial cells involving activation of metabotropic purinergic (P2Y) receptors and immobilization of calcium from intracellular stores.

Material and Methods

A. Reagents and Cell Cultures

rMC-1 Müller cells (originally obtained courtesy of Dr. Vijay Sarthy, Northwestern University, Chicago, Illinois) were passaged four to five times to expand them from frozen stocks. All experiments were performed one day after recovered cells were seeded on P35 glass-bottom Petri dishes (MatTek Corp., Ashland MA) at ~200,000 cells/cm^2 incubated in culture media (high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, and 1% (v/v) Pen/Strep) at 37ºC and 5% CO_2. Cell cultures reached workable confluency (> 80%) overnight. Media

changes, in which all media was replaced, were performed every two days. Unless otherwise stated, all reagents were obtained from Sigma (St. Louis MO).

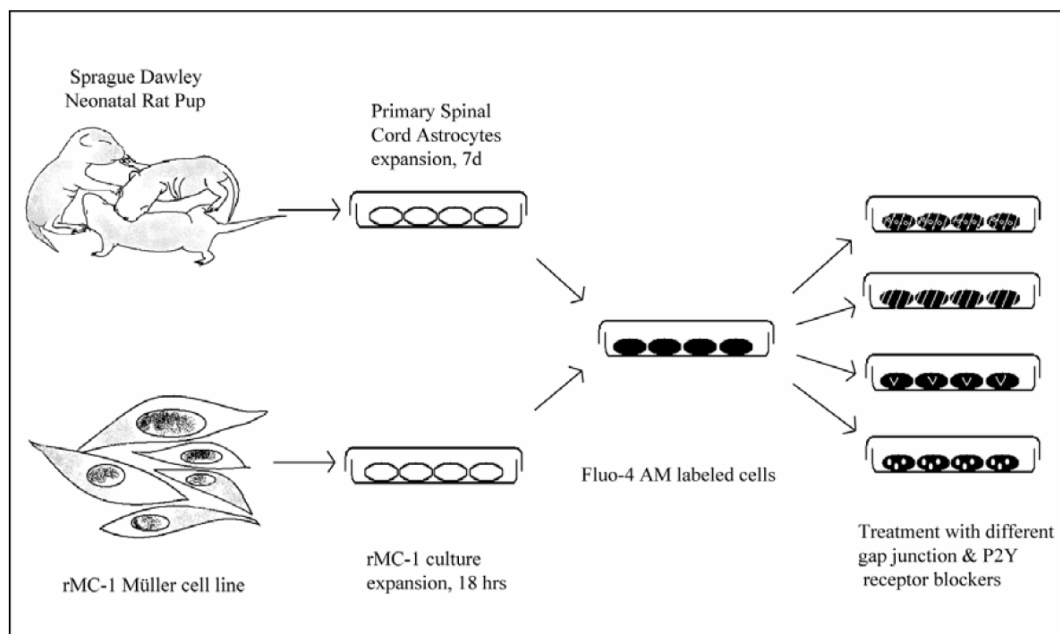
B. Calcium Imaging

rMC-1 cultures of ~80% confluency were washed twice with Kreb-HEPES buffer (KHB) solution (10 mM HEPES, 4.2 mM $NaHCO_3$, 10 mM glucose, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 4.69 mM KCL, 118 mM NaCl, 1.29 mM $CaCl_2$, pH 7.4) and incubated with 5 μM Fluo-4AM in KHB for 1 hr at room temperature. Excess dye was removed by washing twice with KHB and an additional incubation of 30 min at room temperature was done to equilibrate intracellular dye concentration and ensure complete intracellular esterification. Intracellular calcium transients were induced by treatment with Adenosine 5'-triphosphate (ATP; 50 μM) and imaged following the initial simultaneous intracellular calcium increase. Treatment of rMC-1 cultures with Thapsigargin (1 μM) were done at room temperature for 20 min prior to addition of ATP. Intercellular calcium waves were initiated by a single mechanical stimulation delivered to a localized region of 1-3 cells using a 0.5μm i.d. micropipette tip (WPI Inc., Sarasota FL) mounted on a M325 Micrometer Slide Micromanipulator (WPI Inc., Sarasota FL) (Fig. 4.2A). Comparable data were obtained using pharmacological ATP stimulation, although it was harder to ensure that only a localized region was initially stimulated; therefore, only data and results for mechanical stimulation are presented. Treatment of rMC-1 culture with pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; 100 μM), flufenamic acid (FFA; 100 μM), MRS2179 (100 μM), and apyrase (50 U/ml) is done by incubation with the respective compounds during the de-esterification phase (Fig.

3.2B). We analyzed seven FFA treated cultures; three PPADS treated cultures; three MRS2179 treated cultures; three apyrase treated cultures; and compared them to the five untreated rMC-1 cultures.

Visualization of calcium indicator dye fluorescence was done using a 488 nm (FITC) filter on an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Tokyo, Japan) that included epifluoresence, confocal, phase, brightfield, and Hoffman differential interference contrast (DIC) modalities. Real-time movie recordings of intracellular calcium transients were acquired at 5 Hz for 500s while intercellular calcium transient propagation were acquired at 16.3 Hz until dissipation of wave using a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and Image-Pro Plus data acquisition and morphometric software (version 5.1.0.20, Media Cybernetics, Inc., Silver Spring, MD).

(A)



(B)

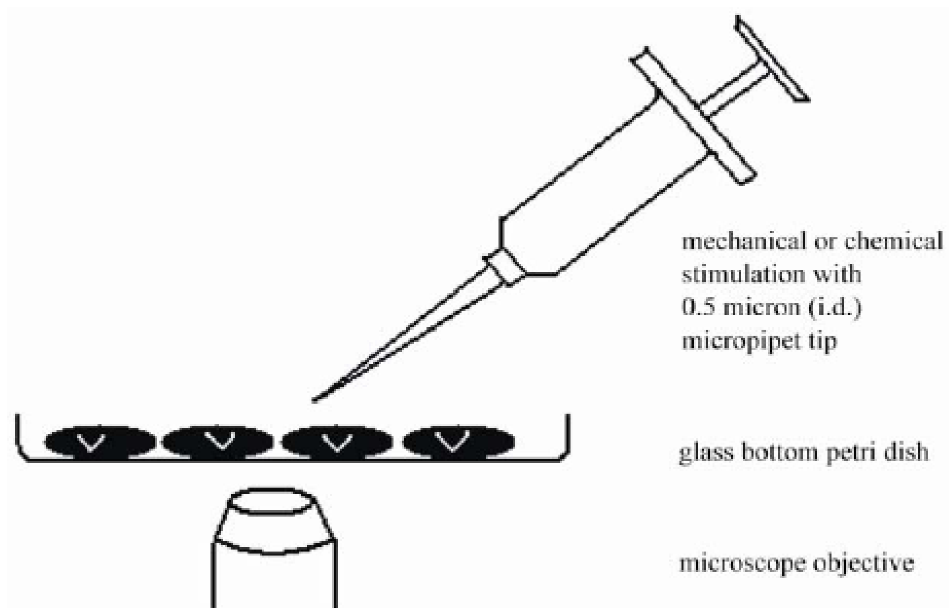


Fig. 4.2 Schematic of *in vitro* experimental approach. **(A)** Preparation of in vitro cell cultures for calcium sensitive fluorescence imaging **(B)** Mecahnical stimulation of cells in vitro with 0.5 µm needled tip.

Using ImageJ, an NIH funded open source morphometric application, the circle-select tool was modified to allow manual selection of individual cells on the *xy*-plane of each movie using circles of 4 pixels (~5 μm) in diameter. Each cell was considered as an individual region of interest (ROIs). In building the ROI list for each movie we traced cells in the frame in which they appeared brightest as a result of an activation event. By going through all the frames in a movie, we were able to catalog every cell in the field of view that participated in the propagation of signaling waves in the network for a given movie. An ImageJ plugin was used to calculate the average intensity for each ROI in each frame as well as the *x-y* coordinates of its area centroids. All of this data was organized in matrix format for post-processing analyses. Since the fluorescence intensity of Fluo-4 AM is proportional to calcium concentration, changes in cytosolic calcium concentrations can be inferred from the fluorescence profile of individual cells.

For analysis of individual intracellular calcium transients, the data is processed to identify periods of sustained increase in fluorescence intensity. Due to the highly dynamic and cyclic nature of the intracellular calcium transients, an averaging filter of 15 frames was applied to reduce noise and a first-derivative filter was then used to identify significant and sustained increases in calcium (i.e. 15 or more consecutive frames with positive derivative values). We established those portions of the signal as the rise phase of the intracellular calcium transients. For analysis of intercellular calcium waves, an averaging filter of 5 frames was applied to the data to reduce noise in the fluorescence signals. The change in fluorescence intensity normalized to the level of baseline fluorescence ($\Delta F/F$) was taken to indicate the magnitude of calcium changes within

Müller cells. $\Delta F/F$ greater than two standard deviations from baseline and a decrease of fluorescence intensity to 10% of its peak value were used as criteria for fluorescence profiles of completed calcium transients (i.e. that had experienced both full activation and deactivation). Real-time recordings of calcium signaling in response to mechanical stimulations were assessed at both the network and individual cell level. All calculations and graphs were done using Matlab (Mathworks, Natick, MA).

D. Immunocytochemistry

Immunocytochemistry (ICC) was performed on rMC-1 cultures prepared identically to those used for calcium imaging. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min and washed twice with physiologically buffered saline (PBS; Invitrogen, Carlsbad, CA). Cultures labeled for glial fibrillary acidic protein (GFAP) were permeabilized in 1% FBS and 0.1% Triton X-100 (Fisher Scientific International, Hampton, NH) for 30 min before incubating with anti-GFAP primary antibody (1:50; Sigma, St. Louis, MO) while cultures labeled for $P2Y_1$ receptor, $P2X_7$ receptor, and Connexin 43 were incubated with the respective primary antibodies at 1:25 (Invitrogen, San Francisco, CA), 1:10 (Sigma, Saint Louis, MI), and 1:50 (Chemicon, Temecula, CA) dilutions, respectively, with 10% FBS in PBS for 2 hrs. Routine negative controls for all conditions included the omission of the primary antibody and incubation with 10% FBS in PBS during the primary incubation step. For secondary antibody labeling, cells labeled for GFAP were incubated with tetramethylrhodamine isothiocyanate (TRITC) conjugated anti-mouse IgG (1:50; Sigma, St. Louis, MO) while cells labeled for $P2Y_1R$, $P2X_7R$, and Cx43 were incubated with

fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG (1:50; Sigma St. Louis, MO). Following the ICC, all slides were mounted using Molecular Probes Prolong® Gold antifade reagent with DAPI (Eugene, OR).

RESULTS

A. rMC-1 cells express specific markers for glial cells and metabotropic purinergic receptors

Qualitatively, the rMC-1 cells show expression patterns for key proteins both in our hands and as previously described . Immunocytochemical characterization of glial fibrillary acidic protein (GFAP) showed low levels of baseline expression (Fig. 4.3A, C, E), consistent with *in vivo* Müller cells which only express high levels of GFAP when they become reactive following injury (36, 37). Antibody labeling of $P2Y_1R$, a metabotropic purinergic receptor that is G-protein coupled to induce release of calcium from intracellular stores through the formation of IP_3 by PLC, displayed a strong expression profile (Fig. 4.3B, D, F). In addition, we also obtained positive labeling for the presence of $P2X_7$ receptors, demonstrating the presence of both ionotropic and metabotropic forms of purinergic receptors on the rMC-1 cells as is consistent with known mechanisms for the role of ATP in calcium signaling by Müller cells (Pannicke, Fischer et al. 2000; Bringmann, Pannicke et al. 2001). Finally, we provide evidence for the presence of gap junctions for this cell line by positive labeling for CX43, a subtype of connexin protein commonly expressed on primary Müller cells to form gap junction channels (Zahs, Kofuji et al. 2003; Kihara, Mantovani de Castro et al. 2006).

B. rMC-1 cells exhibited both spontaneous and ATP induced intracellular calcium transients

Using real-time calcium-sensitive fluorescence imaging, we recorded spontaneous intracellular calcium transients in rMC-1 cells at a low frequency. Five 500-second real-time recordings were analyzed and showed that approximately 2% of the cells in culture produced intracellular calcium transients in the imaging buffer in the absence of stimulation; an average of 23.3 ± 3.0 calcium transients were observed in each movie at a rate of 1.67 ± 0.71 transients/signaling cell/500 seconds, with calcium elevations averaging 5.9 ± 0.5 seconds in duration. Application of 50 μM ATP significantly increased the number of intracellular calcium transients in rMC-1 cells to 482.8 ± 241.5 per movie ($p < 0.01$, Fig. 4.4A). Furthermore, our results show that ATP-induced increase in calcium signaling is due to significant increase in the number of cells exhibiting intracellular calcium transients (from 2% to 31%; $p < 0.01$, Fig. 4.4B) rather than an increase in the frequency of calcium transients per signaling cell; which are 1.67 ± 0.71 and 1.74 ± 0.25 transients/signaling cell/500 seconds under control and 50 μM ATP, respectively, with no significant differences detected via the Student-t test. Interestingly, along with the increase in the number of intracellular calcium transients in ATP treated cultures, we also observed a significant decrease in the average duration of the calcium elevations to 3.4 ± 0.6 seconds (Fig. 4.4C) following application of ATP.

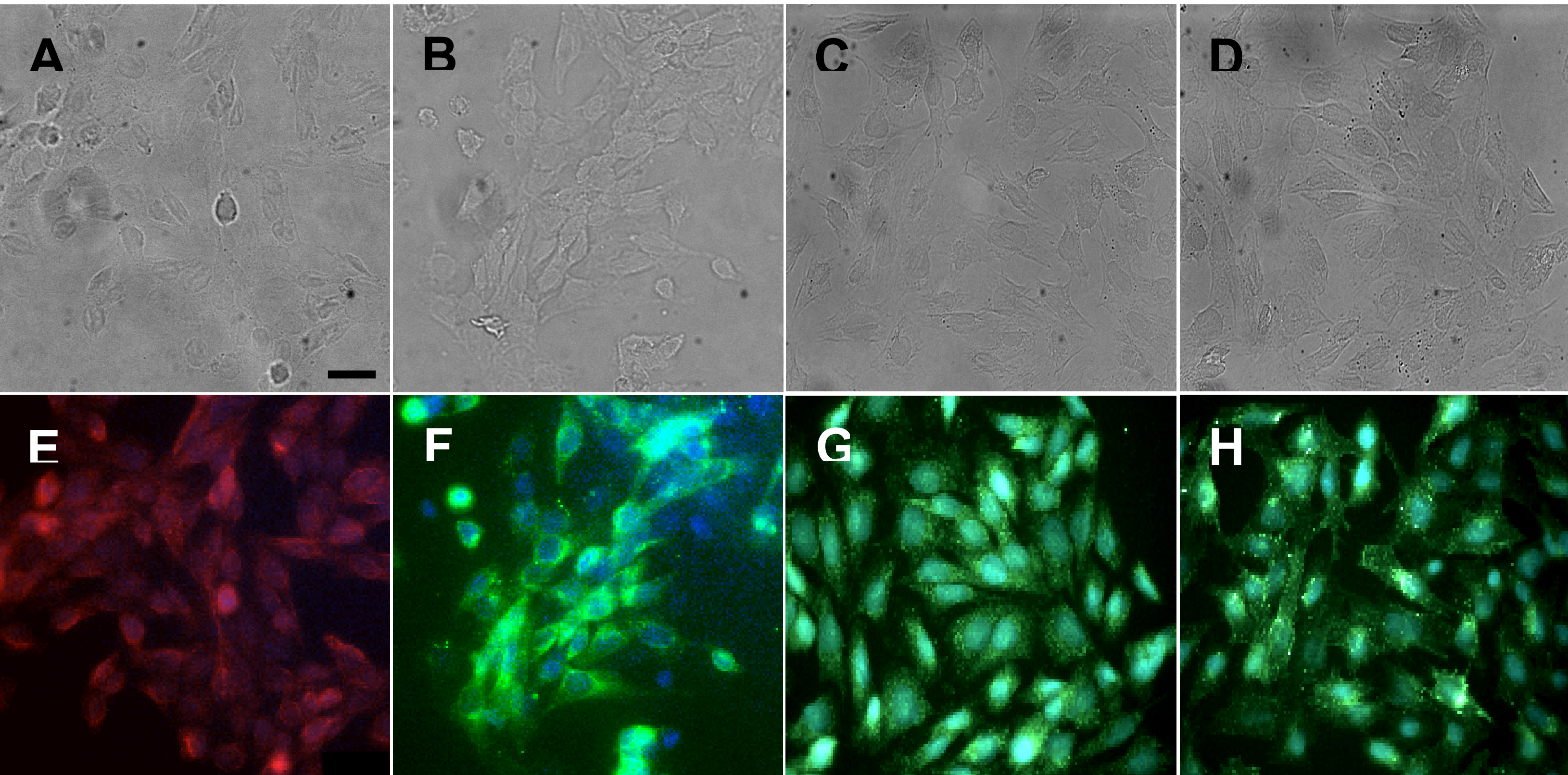


Fig. 4.3 Immunocytochemistry of cultured rMC-1 Müller cells for GFAP, $P2Y_1R$, $P2X_7R$, and Cx43. **(A-D)** Phase-contrast micrographs of fluorescence images in panels E-H, respectively. **(E-H)** GFAP (panel E), $P2Y_1R$ (panel F), $P2X_7R$ (panel G), and Cx43 (panel H) immunoreactivity. All images were taken at 400X, scale bar = 25 µm.

Quantification of the amplitude change (ΔF/F) of spontaneous and ATP stimulated Ca transients shows an amplitude change of 31.8 ± 5.7% for control cultures. Corresponding to the shorter duration of calcium elevations with the application of ATP, we observed a decrease in the average amplitude of calcium transients in comparison to controls (14.3 ± 2.9%, $p<0.01$). Taken together, these results indicate that elevated extracellular ATP induced a higher number and faster calcium transients with smaller amplitudes versus that of the control condition.

To probe the molecular mechanisms responsible for spontaneous and ATP induced intracellular calcium transients in rMC-1 cells, we analyzed the effects of applying thapsigargin, a drug that depletes intracellular calcium stores (Thastrup, Cullen et al. 1990). Thapsigargin applied at a concentration of 1 μM effectively blocked the increase in the number of intracellular calcium transients previously observed with the application of 50 μM extracellular ATP and rendered it comparable to that of control cultures (Fig. 4.4A). As before, our results indicate that this decreased incidence of calcium transients in the presence of elevated ATP (to 25.2 ± 10.0 transients per movie) was due to significantly lower number of signaling rMC-1 cells in thapsigargin treated cultures rather than changes in the frequency of transients per siganling per cell (which are 1.51 ± 0.39, 1.74 ± 0.25, and 1.51± 0.57 transients/signaling cell/500 seconds under 1 μM thapsigargin, 50 μM ATP, and 50 μM ATP plus 1 μM thapsigargin, respectively, with no significant differences detected via ANOVA). Furthermore, in the presence of thapsigargin, the amplitude and duration of calcium elevation in ATP stimulated calcium transients are comparable to that of the control cultures (23.7 ± 8.0% and 5.15 ± 1.1

seconds). Interestingly, 1 μM thapsigargin alone did not significantly affect the frequency of spontaneous calcium transients or the number of cells that displayed them (Fig. 4.4A-C). As the primary pharmacological effect of thapsigargin is the discharging of intracellular stores via inhibition of calcium ATPase pump on the ER (Thastrup, Cullen et al. 1990), we suggest that the spontaneous calcium transients observed in the presence of thapsigargin may involve extracellular source of calcium. This is further collaborated with our observation that no spontaneous calcium transients were seen under zero extracellular calcium condition where the imaging buffer is modified to exclude $CaCl_2$ and include 1.5mM BAPTA. Spontaneous calcium transients ceased within seconds of switching to the calcium-free buffer, but were observed again in these cultures upon returning to the original imaging buffer with calcium.

Finally, to explore the possible ionotropic as well as the metabotropic components of the purinergic receptors response, we also investigated the ATP induced calcium transients in the presence of zero extracellular calcium as well as by using specific inhibitors. In our hands, rMC-1 demonstrated no intracellular calcium oscillations to ATP stimulation in the presence of zero-extracellular calcium, thus also implicating a role for extracellular calcium, potentially via ionotropic purinergic receptor signaling. Returning to calcium-containing imaging buffer restores the ATP response. To further substantiate the role of metabotropic purinergic receptor signaling, specifically of the $P2Y_1$ receptor, in the ATP potentiated intracellular calcium transients, we applied MRS2179 to specifically block the receptor. We report that 100μM MRS2179 reduced the percentage of signaling cells from 31% to 11% in the presence of ATP as well as decrease the

number of intracellular calcium transients detected by approximately 50% (to 246.2 ± 182.8) with no significant changes to the duration and amplitude of oscillations. Finally, we report that application of 5mM PPADS, a broad spectrum P2Y receptor antagonist, is able to completely, but reversibly, inhibit the ATP stimulated calcium transients. The removal of PPADS from these cultures restores the response of these cells to extracellular ATP, thus further substantiating the role of metabotropic purinergic receptors signaling in these intracellular calcium oscillations.

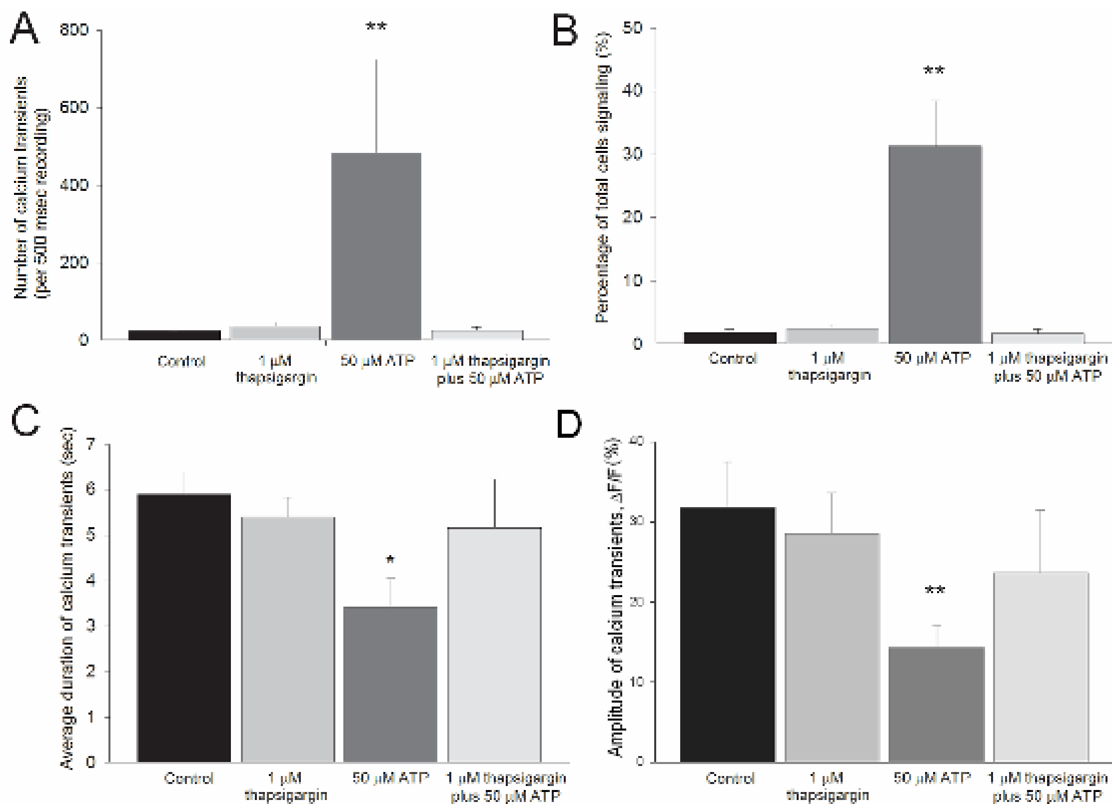


Fig. 4.4 Intracellular calcium transients in untreated rMC-1 versus cells treated with 50 µM ATP, 1 µM Thapsigargin, and 50 µM ATP with 1 µM Thapsigargin. Two-tailed student t-tests were used to test for statistical significance. **(A)** The total number of calcium transients recorded during 500 second movies. **(B)** Percentage of cells that exhibited intracellular calcium transients during 500 second movies. **(C)** The average duration of calcium transients. **(D)** The average amplitude change of calcium transients calculated as ΔF/F. * $p < 0.01$, ** $p < 0.001$, $n = 7$.

C. Intercellular calcium waves are mediated by ATP and IP_3 and display specific signaling dynamics

Localized stimulation of the rMC-1 cells induced a propagating wave of calcium transients that spread radially outward from the initial point of activation (Fig. 4.5A-B). For each recording, all cells participating in calcium waves were individually analyzed to quantitatively study the spatial and temporal properties of calcium transient propagation. In order to characterize the dynamics of intracellular calcium transient responses that underlie the calcium wave, the following parameters were measured: 1) activation time, the rise time from 10% to 90% of the peak amplitude, 2) deactivation time, the decay time from 90% to 10% of the peak amplitude, and 3) $\Delta F/F$, the percent change of maximum Fluo-4 fluorescence intensity with respect to the baseline resting state (Fig. 4.6). Combining five data sets (100 ± 20 cells were analyzed per recording), the averaged values for activation and deactivation times were 3.87 ± 0.62 seconds and 18.91 ± 2.60 seconds, respectively. The average change in the amplitude of the fluorescence signal of the calcium indicator ($\Delta F/F$) was 67.4 ± 16.5 %. It is interesting to note that intracellular calcium transients in the context of a calcium wave were significantly longer in duration than spontaneous or ATP induced transients in individual cells.

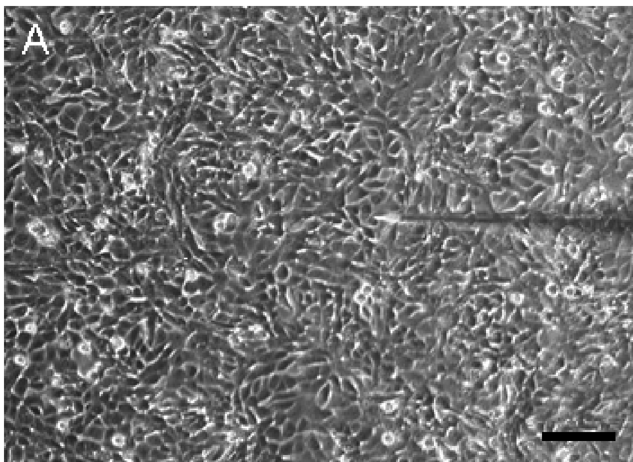


Fig. 4.5A Mechanical stimulation of rMC-1 Müller cells with 0.5 µm needle tip. Phase-contrast light at 100X showing a typical field of view and the needle tip. Scalebar = 100 µm

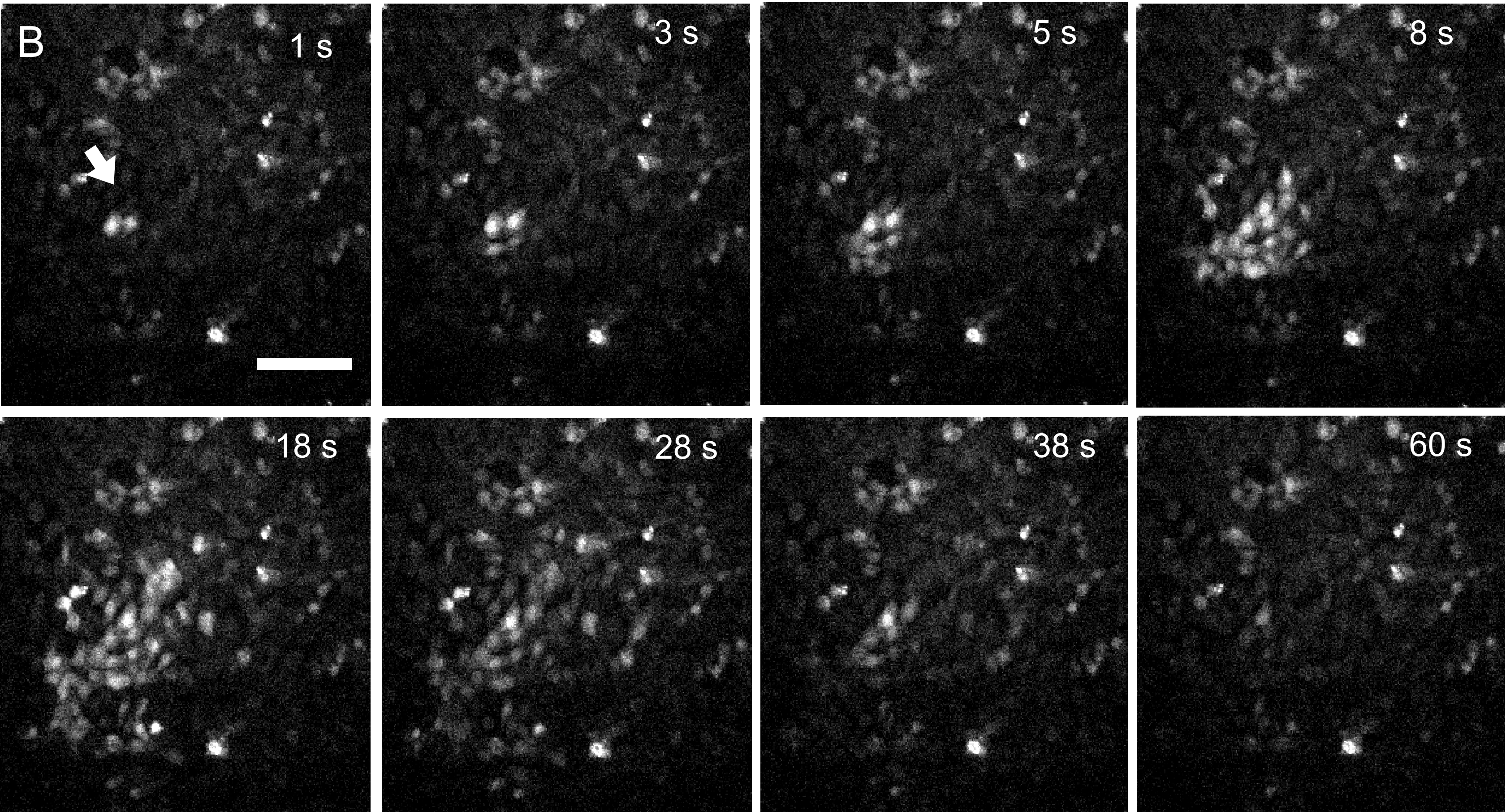


Fig. 4.5B Time-lapse FITC fluorescence micrographs of calcium transients induced by mechanical stimulation visualized with Fluo-4AM at 10X for a representative signaling wave. Scalebar = 100 µm

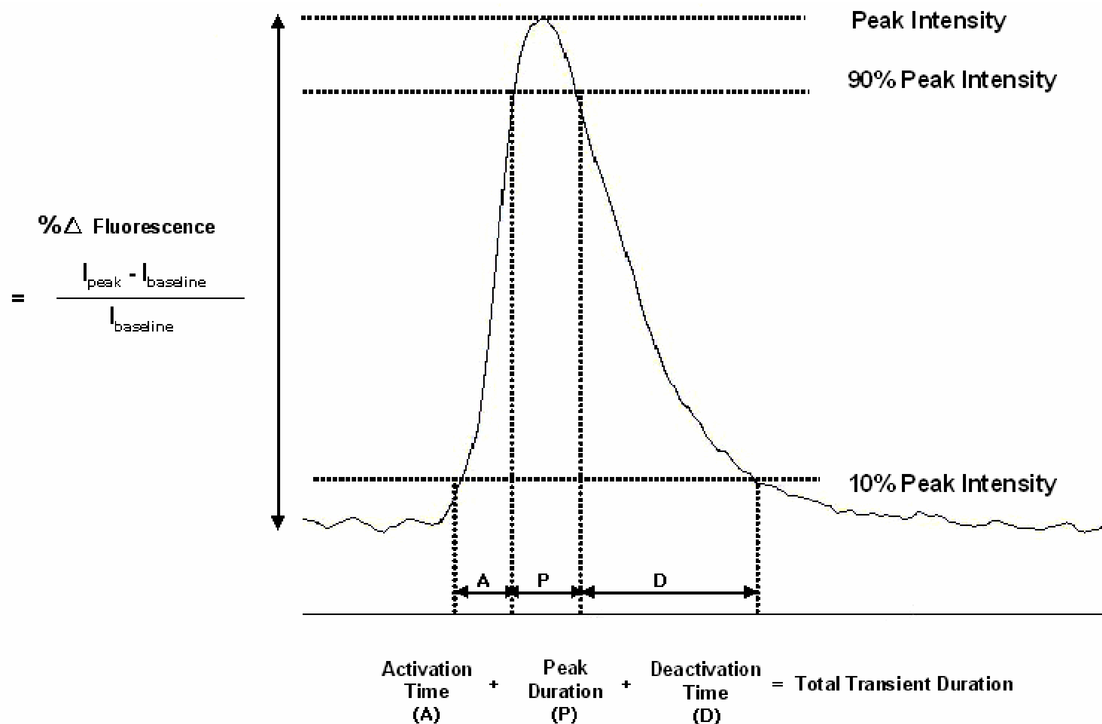


Fig. 4.6 Schematic of characterization parameters defined for the analyses of calcium transients.

We then investigated the population dynamics of calcium wave events in intact networks by plotting the calcium transient parameters described above as a function of radial distance from the stimulation source for all individual cells that contributed to a wave (Fig. 4.5B). We were interested in assessing statistical trends associated with changes in individual calcium transients as a function of radial distance. The box plots show the spatiotemporal data binned in 50 μm radial sections (Fig. 4.7). The centers of the boxes denote the median value while the upper and lower edges are the 75th and 25th percentiles, respectively. The whiskers show the range of the data and extreme outliers (+; defined by >1.5 of inter-quartile distance).

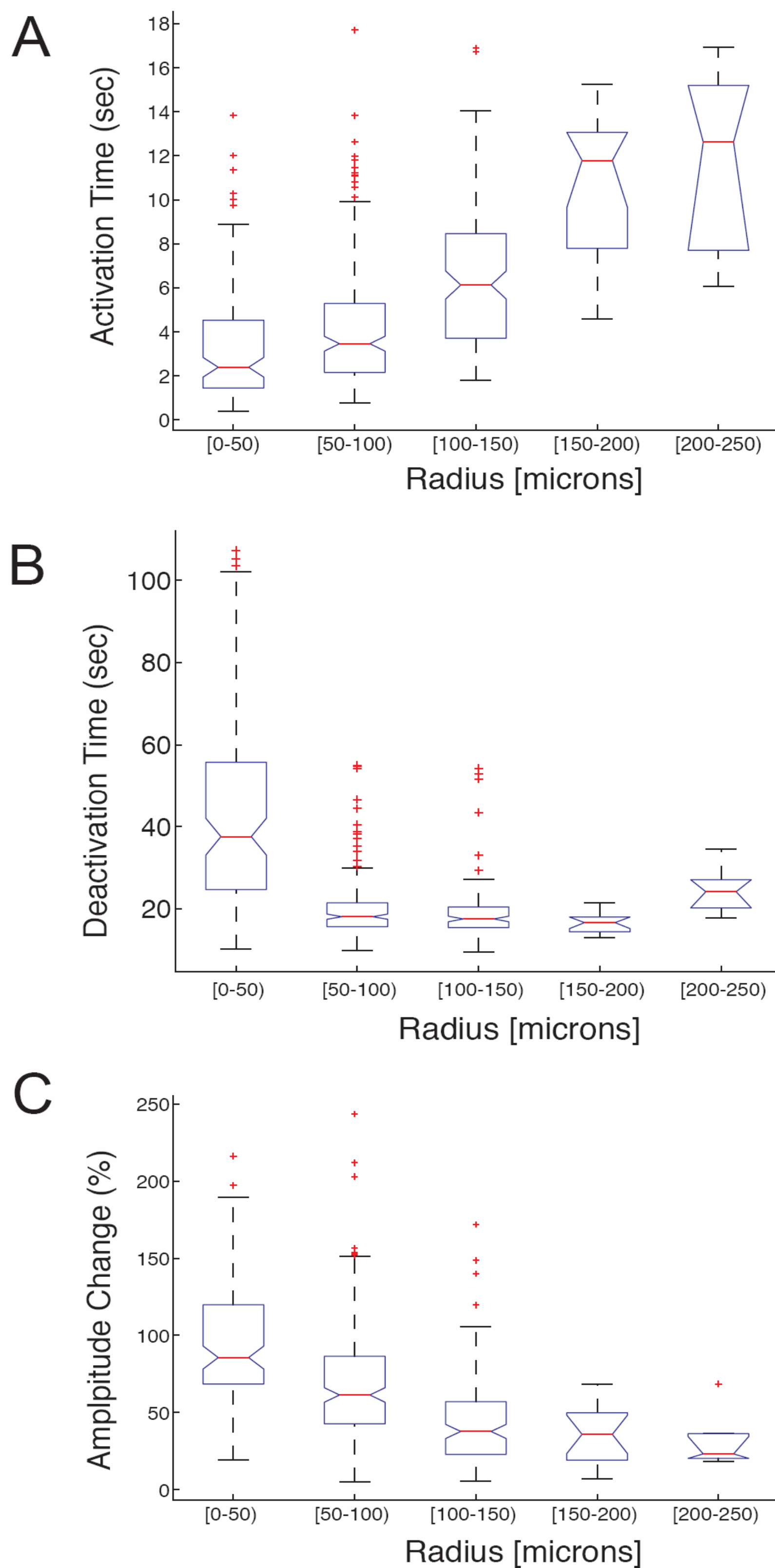


Fig 4.7 Box plots of descriptive calcium transient parameters versus radial distance from the site of stimulation. **(A)** Activation time **(B)** Deactivation time **(C)** Amplitude ($\Delta F/F$).

The plots showed that cells located closer to the stimulation site preferentially displayed shorter activation times (Fig. 4.7A; ~2 seconds at radius, r < 50 µm, and > 6 seconds at r > 150 µm), longer deactivation times (Fig. 4.7B; ~37 seconds at r < 50 µm, and < 25 seconds at r > 150 µm), and larger calcium transient amplitudes (Fig. 4.7C; $\Delta F/F$ = 80% at r < 50 µm, and $\Delta F/F$ < 30% at r > 150 µm). We note that these observations may be due to higher extracellular ATP concentrations near the center of a calcium wave (Newman 2001) possibly due to the clustering of the initially activated cells following mechanical stimulation. Furthermore, cells located closer to the stimulation site exhibited a larger range of amplitude changes (i.e. 20-200% at r < 50 µm, and 10-75% at r > 150 µm) and deactivation times (i.e. 10-100 sec at r < 50 µm, and 15-35 sec at r > 150 µm), while a large range of activation times were observed in cells irrespective of their location (e.g. 0.5-14 seconds at r < 50 µm, and 4.7-17 sec at r > 150 µm).

Finally, we calculated the velocity of signal propagation between rMC-1 cells, by plotting the radial distance of each activated cell from the stimulation site against its time to activation (defined at 10% of its measured peak amplitude) from the time of simulation. The distance of signal propagation as a function of time followed a logarithmic trend that could be fitted well with a power function (Fig. 4.8, $R^2 = 0.86 \pm 0.06$). To obtain a sense of the signaling speed of the wave, the calculated average propagation velocities at 0.2, 0.4, 0.6, 0.8, and 1.0 seconds following stimulations were 23.8 ± 3.7, 18.8 ± 2.2, 16.8 ± 1.5, 15.5 ± 1.0, 14.6 ± 0.8 µm/s, respectively. The average signaling speed decreased to10.5 ± 3.3 after 5 seconds, and 7.8 ±1.0 um/s after 10 seconds. This data suggests that

decrease in the signaling speed and transient amplitude along the radial direction may be associated with mechanisms responsible for the cessation of signal propagation, perhaps by reducing the regenerative component of the calcium wave.

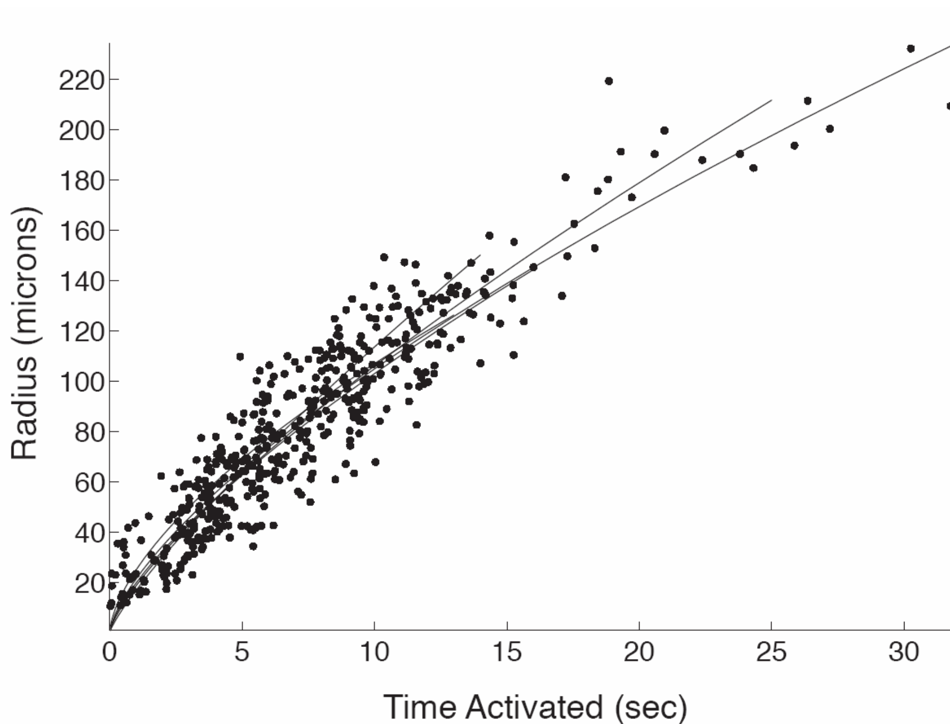


Fig. 4.8. Plots of the relative spatial locations of activated rMC-1 cells as function of activation time. Each data set (n = 5) fitted with a power function, $0.77 < R^2 < 0.91$.

In order to examine the molecular mechanisms underlying intercellular calcium waves, we pharmacologically perturbed calcium-mediate signaling by applying the purinergic receptor antagonist, PPADS (Liu and Wakakura 1998), and the gap junction blocker, FFA (Rana and Dringen 2007). Cultures treated with FFA and PPADS resulted in a 70% and 86% decrease in the number of responsive cells, respectively, as compared

to control cultures (Fig. 4.9A). PPADS also affected the duration of individual responses. There was a significant increase in the average duration of intracellular calcium transients in cultures treated with PPADS (86.7.1 ± 46.6s, mean ± s.e.) as compared to FFA (29.1 ± 3.3s, mean ± s.e) or non-treated controls (31.1 ± 3.1s, mean ± s.e; Fig. 4.9B). These results suggest that the underlying molecular mechanisms responsible for intercellular calcium waves in rMC-1 cells are similar to those described for Müller cells and astrocytes (Newman 2001). Finally, we examined the role of ATP as the extracellular signaling factor in this phenomenon using apyrase and obtained that application of the ATPase at 50U/ml reliably inhibited propagation of calcium transients from initially stimulated cells to additional neighboring cells. Application of apyrase at 10U/ml also substantially reduced calcium transient propagation to neighboring cells, although calcium transient is sometimes observed to travel to an adjacent cell in immediate cell-cell contact with the initially simulated cell. These results provide further evidence for the involvement of ATP in the molecular mechanism underlying intercellular calcium waves in these rMC-1 cells.

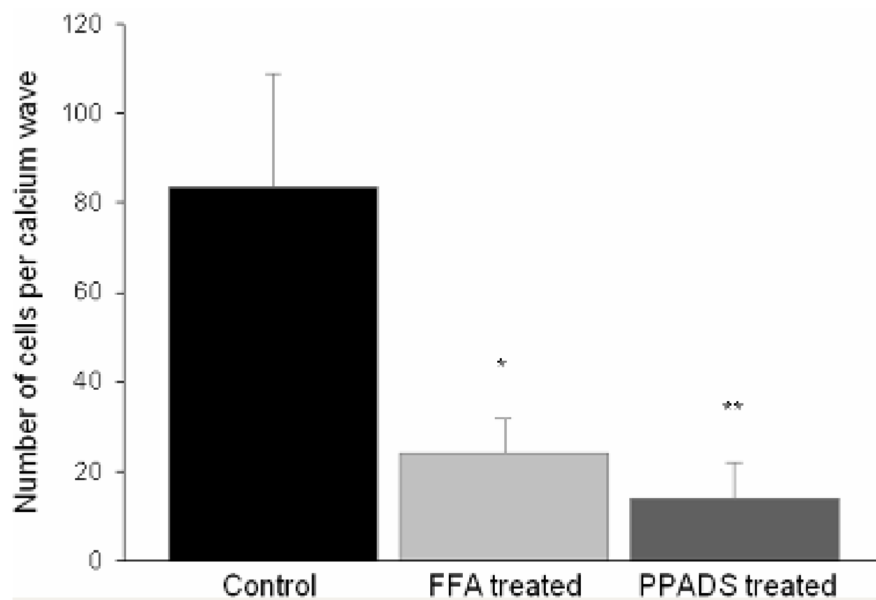


Fig. 3.9A Average number of cells per calcium wave in control versus FFA and PPADS treated rMC-1 cultures * $p < 0.01$, $n = 17$; ** $p < 0.01$, $n = 12$

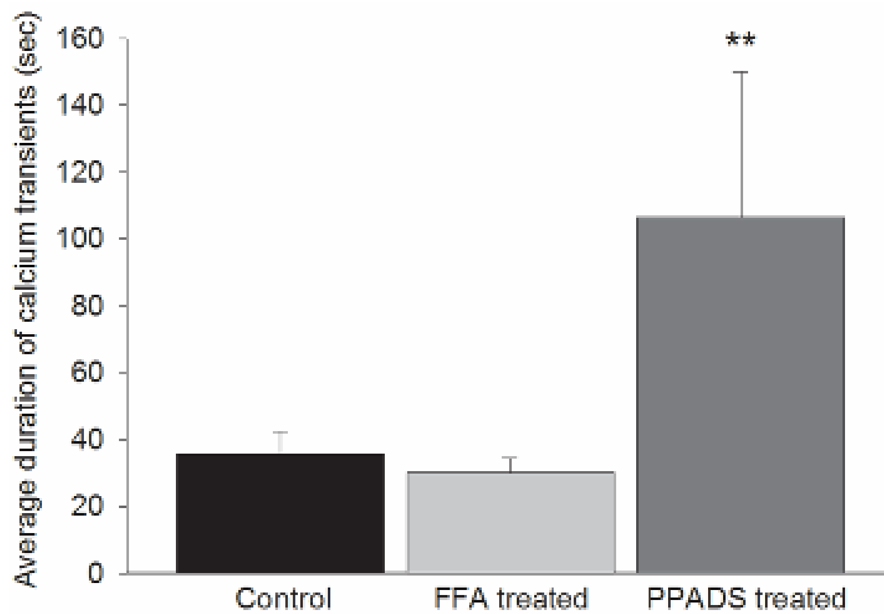


Fig. 4.9B Average duration of calcium transients for control versus FFA and PPADS treated rMC-1. ** $p<0.01$, $n = 12$.

Discussion

We introduce and characterize the dynamics of an *in vitro* model for studying intracellular and intercellular calcium signaling using the rMC-1 cell line derived from primary rat Müller cells (Sarthy, Brodjian et al. 1998). Although *in vitro* systems are simplified representations of physiological conditions, culture systems provide an opportunity to manipulate and investigate molecular and cellular processes in isolation. If the fundamental molecular mechanisms under investigation in the *in vitro* system are conserved with respect to known physiological processes, then the former provides an opportunity to study elements of these processes at a fundamental level under controlled experimental conditions. The molecular mechanisms that underlie calcium signaling in rMC-1 cells and the dynamics of intracellular calcium transients and intercellular calcium waves are similar to those reported for primary Müller cells and *in situ* retinal preparations, and thereby provide a molecular model of calcium signaling in Müller cells.

Immunocytochemically, rMC-1 cells exhibited low baseline levels of GFAP, a specific marker for astrocytic and related macroglial cells (Trimmer, Reier et al. 1982; Eisenfeld, Bunt-Milam et al. 1984), similar to non-reactive Müller cells. In addition, they presented positive expression of $P2Y_1R$, a G-protein-coupled metabotropic purinergic receptor involved in calcium mobilization from intracellular stores (Li, Holtzclaw et al. 2001; Uckermann, Grosche et al. 2002; Metea and Newman 2006) as well as $P2X_7$ ionotropic purinergic receptor and connexin 43 which have been respectively show to augment the cytosolic calcium increase via influx of extracellular calcium in response to ATP (Pannicke, Fischer et al. 2000; Bringmann, Pannicke et al. 2001) and coordinate

intercellular coupling of calcium transients via formation of gap junction channels(Suadicani, Flores et al. 2004; Iacobas, Suadicani et al. 2007). Functionally, rMC-1 cells exhibited intracellular calcium transients that significantly increased in the presence of ATP, suggesting that these cells express functionally intact purinergic receptors. The effect of ATP was blocked by the application of thapsigargin, which has been previously shown to deplete intracellular calcium stores by specifically inhibiting endoplansmic reticulum calcium-ATPases (Thastrup, Cullen et al. 1990). Our results suggest that ATP-evoked calcium transients in rMC-1 cells are initiated by the release of calcium from intracellular stores similar to the mechanism reported for primary Müller cells (Moll, Weick et al. 2002). To further elucidate signaling via metabotropic purinergic receptors, we applied 2'-Deoxy-N^6-methyladenosine-3',5'-bisphosphate (MRS2179), a specific antagonist of the $P2Y_1$ receptor (Camaioni, Boyer et al. 1998; von Kugelgen 2006), and show that at it significantly reduced the percentage of signaling cells as well as decreased the number of intracellular calcium transients in the presence of ATP by approximately 50%. The inability of MRS2179 to completely abolish the ATP-induced increase in intracellular calcium transients as observed with thapsigargin suggest the possibility of additional ATP sensitive metabotropic purinergic receptors subtypes that have been identified on primary Müller glia (i.e. $P2Y_2$, $P2Y_4$, $P2Y_6$) (Reifel Saltzberg, Garvey et al. 2003; Fries, Goczalik et al. 2005). We report that application of pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), a non-specific P2Y receptor antagonist that blocks intracellular calcium immobilization via inhibition of IP_3 channels (Vigne, Pacaud et al. 1996) and have been used against $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{13}$

(Charlton, Brown et al. 1996; Charlton, Brown et al. 1996; Robaye, Boeynaems et al. 1997; Suarez-Huerta, Pouillon et al. 2001; Marteau, Le Poul et al. 2003; von Kugelgen 2006) as well as the $P2Y_1$ receptor (Schachter, Li et al. 1996; Brown, Charlton et al. 1997; Marcet, Chappe et al. 2004), is able to completely, but reversibly, inhibit the ATP stimulated calcium transients. In addition, our results indicate potential involvement of ionotropic purinergic receptors as removal of extracellular calcium impede the ability to these cells to exhibit calcium transients in the presence of ATP, however, additional pharmacological characterization is needed to further elucidate.

The presence of spontaneous calcium transients have been reported in Müller cells in retinal explants under dark adapted conditions using bioluminescence imaging of Müller cells transfected to express a GFP-aequorin calcium reporter (Agulhon, Platel et al. 2007). Spontaneous calcium transient rates in these Müller cells (an average 1.31 ± 0.29, up to 4.72 events/active cell/1000s) (Agulhon, Platel et al. 2007) were comparable to those measured in rMC-1 cells in the absence of applied ATP (average 1.67 ± 0.71 events/active cell/500s). Mechanistically, these intracellular calcium oscillations in the rMC-1 were not affected by discharging of intracellular calcium stores using Thapsigargin but were abolished with the removal of extracellular calcium, indicating that the latter might be the primary source of calcium for these signaling events. The average duration of both spontaneous and ATP-induced intracellular calcium transients in the rMC-1 cells, 5.9 ± 0.5 s and 3.4 ± 0.6 s respectively, were within the range of calcium transient durations reported for Müller cells in retinal preparations, which were measured to be between 2.5 to 6 s (Newman 2005). Furthermore, it has been suggested that light

induces Müller cell calcium transients in the retina via the release of ATP by amacrine and/or retinal ganglion cells (Newman 2005). The exceptional similarity between the duration of calcium transients in the ATP-stimulated rMC-1 cells (3.4 ± 0.6 s) and that previously reported for Müller cells in light-stimulated retinal preparations (3.84 ± 0.82 s) further supports that the rMC-1 cell line retains the principle mechanisms associated with physiological intracellular calcium mobilization and signaling pathways in Müller cells.

Since *in situ* experiments have also showed the ability of Müller cells to support intercellular calcium waves (Newman and Zahs 1997) and similar events have been observed under conditions that mimic pathology (Newman 2005), we also characterized the dynamics of calcium waves in rMC-1 cultures and tested whether their propagation necessitated an ATP and/or IP_3-dependent signaling mechanism. The ability of rMC-1 cell networks to support intercellular calcium waves was demonstrated by the radial propagation of signal following a single mechanical stimulation. Published studies by several groups have implicated extracellular ATP as the primary facilitator of calcium waves in Müller cell networks of healthy and diseased retina (Uckermann, Grosche et al. 2002; Uckermann, Uhlmann et al. 2003; Bringmann, Pannicke et al. 2006; Metea and Newman 2006). There is evidence for a similar mechanism in rMC-1 networks based on measurements of calcium waves following the application of the purinergic receptor antagonist PPADS (Liu and Wakakura 1998) which resulted in a significant (86%) reduction in the size of the calcium wave as measured based on the number of participating cells. Apyrase, an ATP diphosphohydrolase, inhibited the propagation of calcium transients to secondary cells adjacent to the stimulation site, thus further

verifying the role of extracellular ATP in the intercellular calcium waves observed in the rMC-1 networks. However, there were also some differences between rMC-1 cells and Müller cells. Specifically the inhibition of gap junctions using flufenamic acid (FFA), a pharmacological agent that has been shown to reduce Cx32, Cx43, Cx46, and Cx40 currents by 85 to 95% (Srinivas and Spray 2003; Rana and Dringen 2007), caused a decrease in intercellular calcium signaling (by 70%) in rMC-1 cells, which has only been previously reported as the primary mechanism for intercellular calcium waves between retinal astrocytes(Newman 2001). There was also a significant increase in the averaged duration of the intracellular calcium transients in cultures treated with PPADS.

Our calculated value of relative calcium transient amplitudes ($\Delta F/F$), activation, and deactivation times (67.4 ± 16.5 %, 3.87 ± 0.62 s, and 18.91 ± 2.60 s, respectively) for intercellular calcium waves in rMC-1 cell networks were comparable to published results for Müller calcium transients in mechanically stimulated calcium waves *in situ* (Newman and Zahs 1997) (Newman 2001). The calculated speed of the initial signal propagation in rMC-1 networks was 23.8 ± 3.7μm/second along the radial direction which was similar to the reported value of 23.1 ± 6.7μm/second for mechanically stimulated calcium waves *in situ*. Combining the results of our kinetics analysis and pharmacological studies, we conclude that intercellular calcium transient propagation in rMC-1 network is characteristically similar to that reported for Müller cells *in situ* retinal preparations.

Conclusion

Müller cells are the primary macroglia of the neural sensory retina with diverse functions in both health and disease that relie on sensitive and intricate interactions with the extracellular environment as well as communication with surrounding neuron populations. Several line of evidence have implicated changes in intracellular calcium levels in these cells, in the form of calcium transients, to mediate the intra- and intercellular signaling process involved in the bi-directional communication between Müller glia and its microenvironment. In this study, we provided a quantitative characterization of the calcium signaling dynamics of Müller cells in an *in vitro* model using the rMC-1 cell line and showed that they displayed robust intracellular calcium transients as well as the capacity to support intercellular calcium waves with properties similar to that of Müller cells in *in situ* retinal preparations. In addition, assessment with pharmacological agents indicated that the molecular mechanisms of calcium signaling in rMC-1 cells is consistent with that previously described for primary Müller cells. Taken together, our study show that the rMC-1 provides a robust *in vitro* model for investigating specific mechanistic hypotheses of intra and intercellular calcium signaling in Müller cell networks in a highly controllable environment.

Chapter V

Quantitative analysis of pharmacological perturbations to intercellular Ca^{2+} transient propagation using mapped functional topology in glial networks

Abstract

Complex cellular networks underlie the functional foundation of the mammalian central nervous system (CNS). In this study, we tested the application previously introduced mapping algorithm based on the dynamic signaling model to biological data of mechanically stimulated intercellular propagation of calcium transients and explored its sensitivity to detecting changes in complex network topologies induced by pharmacological compounds that selectively disrupt the principle known intercellular signaling mechanisms. This is important if the algorithms are to be used to detect and differentiate between physiologically normal versus pathological cell signaling. Calcium indicator dye (Fluo4-AM) is used with fluorescence microcopy to provide real-time image sequences documenting intercellular propagation of calcium transients and antagonist of metabotropic purinergic receptors (PPADS) and gap junction blocker (FFA) were used to perturb the signaling network under controlled conditions. Our analysis allows us to quantitatively assess differences between normal and pharmacologically perturbed intercellular calcium signaling as well as tease apart in the roles played by the two molecular mechanisms proposed for calcium transient propagation. Our results suggest that, for rMC-1 cells, the activation of metabotropic purinergic receptors by extracellular soluble signals (i.e. ATP) may function as the primary mechanism in longer

distance signaling and may play a more prominent role in determining the connectivity and topology of the signaling network, while diffusion of second messenger (i.e. IP_3) through gap junctions may serve as a secondary mechanism for local amplification of the signal. The analysis we present in this study also provides evidence for the ability of the dynamic signaling model to quantitatively investigate the spatiotemporal dynamic properties of cellular networks.

Introduction

Complex cellular networks underlie the functional foundation of the mammalian central nervous system (CNS). Paracrine and autocrine signaling are examples of cell-cell communication in large complex networks of neuons and glia are responsible for emergent system level phenomena such as the processing and representation of complex visual information (Schummers, Marino et al. 2004) and associative learning (Fanselow and Poulos 2005). Changes in the functional structure of these networks underlie the development of multidimensional CNS disorders such as epilepsy (Jefferys 2003) and addiction (Kelley 2004). Understanding the physiological dynamics of these networks in health and disease not only adds to our knowledge of neural and glial information processing on the single-cell level, but also contributes to developing more effective treatments for multidimensional CNS disorders where the topology and connectivity of these networks are significantly altered.

We have previously shown that localized activation of cultured rat retinal Müller cells (rMC-1) and primary rat spinal cord astrocytes using mechanical stimulation established spontaneously forming glial signaling networks *in vitro* that demonstrate intercellular propagation of calcium transients (a.k.a. calcium waves) via non-trivial dynamics and connectivity. To better investigate these functional signaling networks and potential alterations in connectivity under pathophysioloy, we previously developed computational algorithms to map the activation states and connectivity topologies of glial networks with single cells resolution in order to describe the spatiotemporal evolution of functional calcium signaling in these networks. Here, we use this tool to quantitatively assess specific perturbations to intercellular calcium wave dynamics by characterizing alterations in the signaling network topology introduced by antagonists of purinergic receptors and gap junctions. These experiments simulate remodeling of the network structure associated with changes in the signaling mechanism under carefully controlled conditions. Analysis of alterations in the network topology under perturbed signaling conditions (i.e. application of P2YR antagonists and gap junction blockers) is the first step towards relating the observed network properties with underlying biological processes. It will facilitate 1) further fine-tuning of the mapping algorithms to detect changes in network properties that reflect changes in biology, and 2) insights into the role and relationship between the two key signaling mechanisms involved in the propagation of calcium transients and provide a molecular basis for understanding alterations in calcium signaling observed under diseased conditions.

Materials and Methods

A. Cell culture

rMC-1 Müller cells (originally obtained courtesy of Dr. Vijay Sarthy, Northwestern University, Chicago, Illinois) were passaged four to five times to expand them from frozen stocks. All experiments were performed one day after recovered cells were seeded on PDL-coated P35 glass-bottom Petri dishes (MatTek Corp., Ashland MA) at ~200,000 cells/cm^2 incubated in culture media (high glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% (v/v) Pen/Strep) at 37ºC and 5% CO_2. Cell cultures reached workable confluency (> 80%) overnight. Media changes, in which all media was replaced, were performed every two days.

B. Calcium Imaging with Fluorescence Microscopy

Cell cultures of ~80% confluency were washed twice with Kreb-HEPES buffer (KHB) solution (10 mM HEPES, 4.2 mM $NaHCO_3$, 10 mM glucose, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 4.69 mM KCL, 118 mM NaCl, 1.29 mM $CaCl_2$, pH 7.4) and incubated for 30 min at room temperature in a solution of 5 µM Fluo-4AM, 0.01% (w/v) Pluronic F-127, and 0.002% (v/v) Cremophor EL, the latter two being detergents that minimize clumping of Fluo-4AM and facilitate even dye loading. Excess dye was removed with two washes in KHB w/ 100 µM Sulfinpyrazone, an anion transporter blocker used to minimize dye extrusion. An additional incubation of 30min was done in solution of pharmacological agent prepared in KHB w/ 100 µM Sulfinpyrazone to allow drug effect, equilibration of intracellular dye concentration, and complete intracellular

esterification. The P2Y receptor antagonists used was Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 100 µM), and the gap junction blocker used was Flufenamic Acid (FFA, 50 µM). Intercellular calcium waves were initiated by mechanical stimulation of a single cell using a 0.5µm i.d. micropipette tip. Visualization of calcium indicator dye fluorescence was done using a 488 nm (FITC) filter on an Olympus IX81 inverted fluorescence confocal microscope. Real-time recordings of calcium transient propagation were acquired at 10-15 Hz using a Hamamatsu ORCA-ER digital camera and Image-Pro Plus data acquisition and morphometric software

C. Implementation of the dynamic signaling model to map network structure

This algorithm is developed and implemented by Marius Buibas of the Silva lab. Briefly, a graph $G = (V,E)$ is defined where V is the set of n vertices and E is the set of edges connecting the node of V with elements $e = (a,b)$ denoting a directed edge from a to b. E is stored as a $n \times n$ array where e_{ij} is 1 if and only if there is an edge in G, and 0 otherwise. A vertex activation matrix F is defined where F_{vt} with $v = 1,\ldots,n$ and $t = 1,\ldots,m$ describes the state of the vertex v at time t as determined by observing the network. T is defined as the timing matrix with elements t_{ij} that correspond to all possible edges in the network and contains the times that a signal would take to go form one vertex to another based on the signaling model. For each frame k in F, the set of newly active vertices is denoted as ${}^{na}V_k$ and contains the set of vertices that have just activated at frame k in a recorded movie. ${}^{na}V_s$ and ${}^{na}V_e$ are defined as the sets of newly activated vertices at frames s and e, where $s = 1,\ldots,m$, $e = \min(s+\delta, m)$, and $\delta = 1,\ldots$ *viewframe*

where *viewframe* is the timing matrix equivalent of r_{max}, the maximum signaling radius of a vertex. Frame *s* is the starting or reference frame where the signal of a given vertex originates, while *e* is the ending frame that starts at *s* + 1 and goes out *viewframe* number of frames. *Viewframes* defines the maximum length of an edge, and each set of ${}^{na}V_e$ from *s* to *viewframes* keeps track of newly activated vertices in frame e. δ is the time difference between *s* and *e*. *s* is iterated from 1 to one less than the total number of frames in the image sequence and for each value of *s*, *e* is iterated from *s*+1 to *s*+*viewframe* or end of the movie. The observed timing matrix ${}^{o}T$, with elements ${}^{o}t_{ij}$ that is defined as δ for all combinations of ${}^{na}V_s$ and ${}^{na}V_e$ or 0 otherwise with *i* being the vertex index contained in ${}^{na}V_s$ and j the vertex index contained in ${}^{na}V_e$. By comparing the set of all vertices in the network activated as given by ${}^{o}T$ with the calculated timing matrix T that describes when vertices must be activated and iterating over the entire observable temporally dynamics network, the functional links connecting the vertices can be computed.

Results

A. PPADS and FFA significantly reduced intercellular calcium waves in rMC-1 Müller glia

We mapped the structure of intracellular calcium-mediated intercellular calcium waves in spontaneously forming networks of rMC-1 Müller glia. Using epiflourecence microscopy we recorded the calcium wave in response to a single localized mechanical stimulation and derived the temporal and spatial evolution of the signaling events in the resultant network. Calcium waves in macroglial networks are not uniformly propagating

waves, but rather are inhomogeneous events that show the potential for complex signaling. Consequently, the signaling edges derived using the dynamic signaling model formed complex and intricate patterns between cells. rMC-1 cultures individually treated with PPADS or FFA showed reduced calcium signaling. Qualitatively, fewer cells participated in the signaling event, the calcium transients exhibited shorter durations, and potentially different spatial patterns. Specifically, cultures treated with PPADS, an antagonist of P_2Y receptors, showed activation of cells localized to the site of stimulation and propagation of calcium wave is restricted to adjacent cells with cell-cell contact, thus resulting in decreased range of calcium signal (Fig. 5.1A).

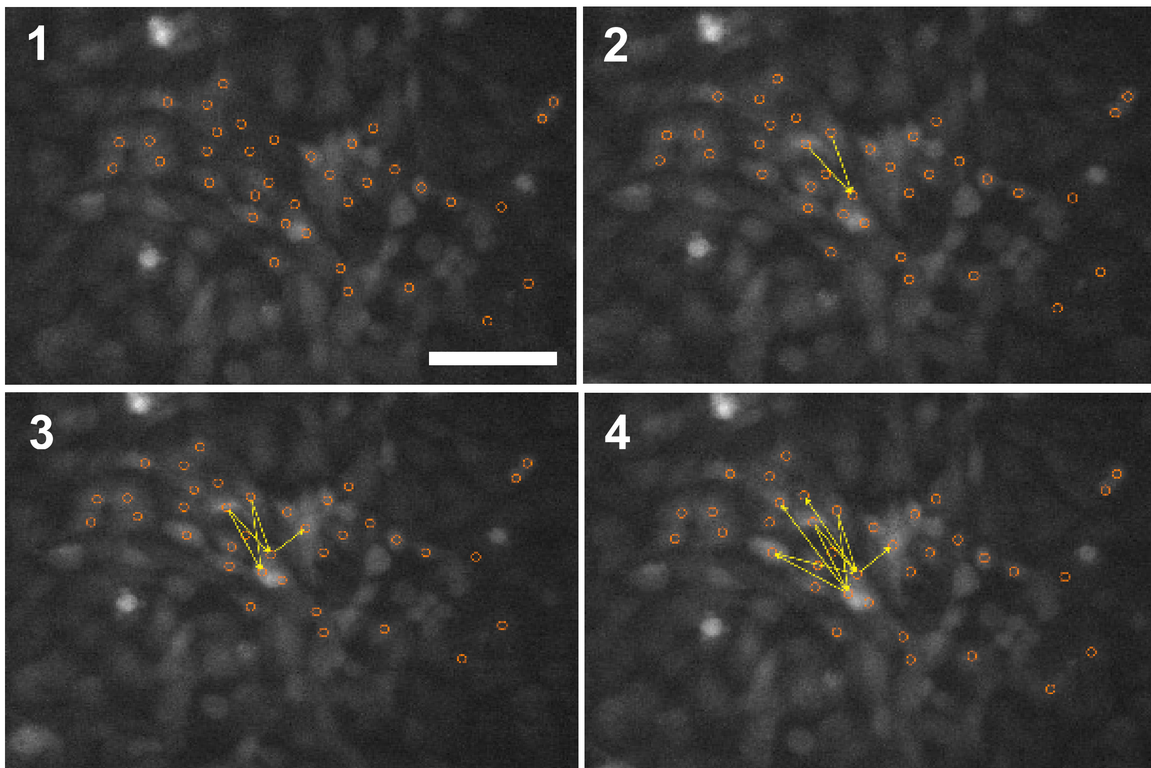


Fig. 5.1A Select frames from a mapped rMC-1 calcium signaling network after treatment with PPADS (100 μM). The last panel shows the fully evolved network. Scale Bar = 100 μm.

On the other hand, cultures treated with FFA, a gap junction blocker, showed activation of cells located far away from the site of stimulation with minimal propagation of calcium transients to adjacent cells. Consequently, while fewer cells are involved in the signaling after treatment of FFA, there is not a noticeable reduction in the distance of the signal propagation from the site of stimulation (Fig. 5.1B).

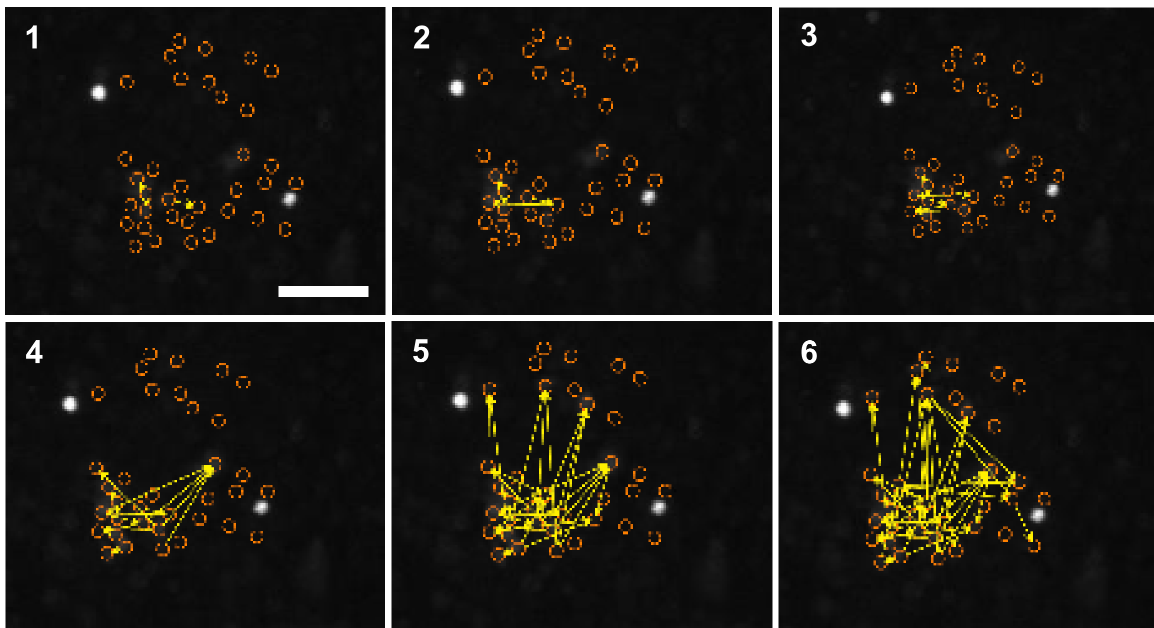


Fig. 5.1B Select frames from a mapped rMC-1 calcium signaling network after treatment with FFA (50 μM). The last panel shows the fully evolved network. Scale Bar = 100 μm.

B. Reduced mapped nodes and edges reflect pharmacologically induced attenuation of intercellular calcium signaling

After using the mapping algorithm to determine the connectivity of the signaling networks, five metrics were adapted from graph theory to quantitatively describe the topology of the functional networks (Table 1). In addition to the number of mapped nodes and edges, we calculated average degree, path length, and diameter of the networks. In accordance with the qualitative observations, the decrease in the number of activated cells and their intercellular signaling are reflected in the significant decrease in the number of mapped nodes and edges in glial networks treated with FFA or PPADS as compared to controls (Fig. 5.2).

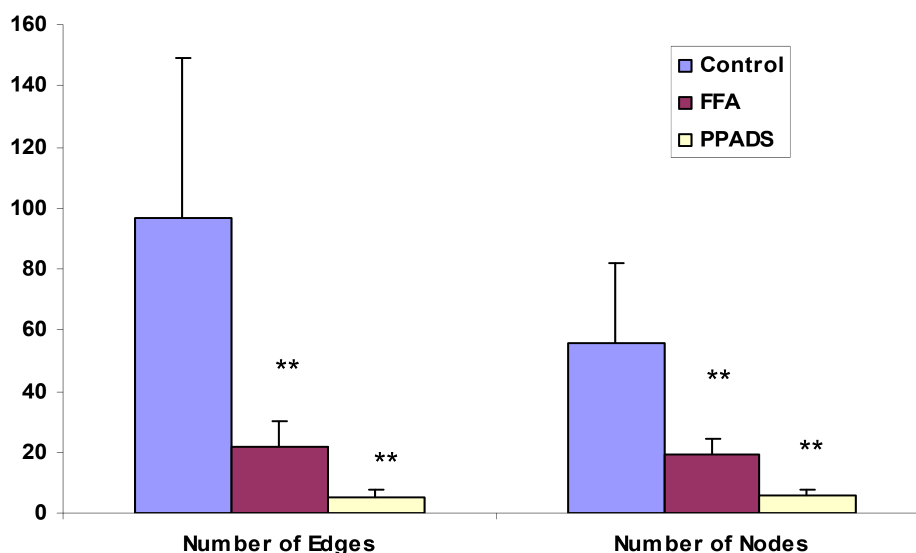


Fig. 5.2 Number of edges and nodes derived from using the dynamics signaling model to map the structure of the signaling network. The mapping showed a significant difference in the number of edges and nodes due to the attenuation in calcium signaling by FFA and PPADS. (** $p < 0.01$)

C. Differences in average degree, path length, and diameter reflect pharmacologically altered network topology

The marked reduction in intercellular connectivity between treated and untreated cultures is also demonstrated by the significant decrease in the average degree, or the average number of edges mapped to each node in a network (Fig. 5.3). In addition, qualitative observation of the differences in the signaling range between cultures treated with the two different drugs is quantitatively characterized by the significant difference in the average length, the average number of edges connecting between two nodes, and diameter, the highest number of edges connecting between two nodes, of the networks. Specifically, the graph theory metrics quantified the observation that while blocking gap junctions with FFA significantly reduces the local connectivity between cells in the signaling network (as evidenced by significant decrease in the average degree), the range of the signal propagation is not significantly altered as evidenced by no significant difference between FFA and control in the diameter of the mapped network. On the other hand, blocking the P_2Y receptors with PPADS significantly disrupted the connectivity of signaling networks, resulting in severely reduced number of signaling cells as well as distance of signal propagation. Our analysis allows us to tease apart and assess differences in the roles played by the two molecular mechanisms proposed for calcium transient propagation. Interestingly, no significant difference is detected in the simulated signaling speed (data not shown). This is understandable considering the signaling speed is a feature of the dynamic signaling model describing the diffusion of the signal (extracellular ATP and intracellular IP_3) from each *activated* cell and therefore, should not be affected by application of pharmacological agents that affects the activation of cells.

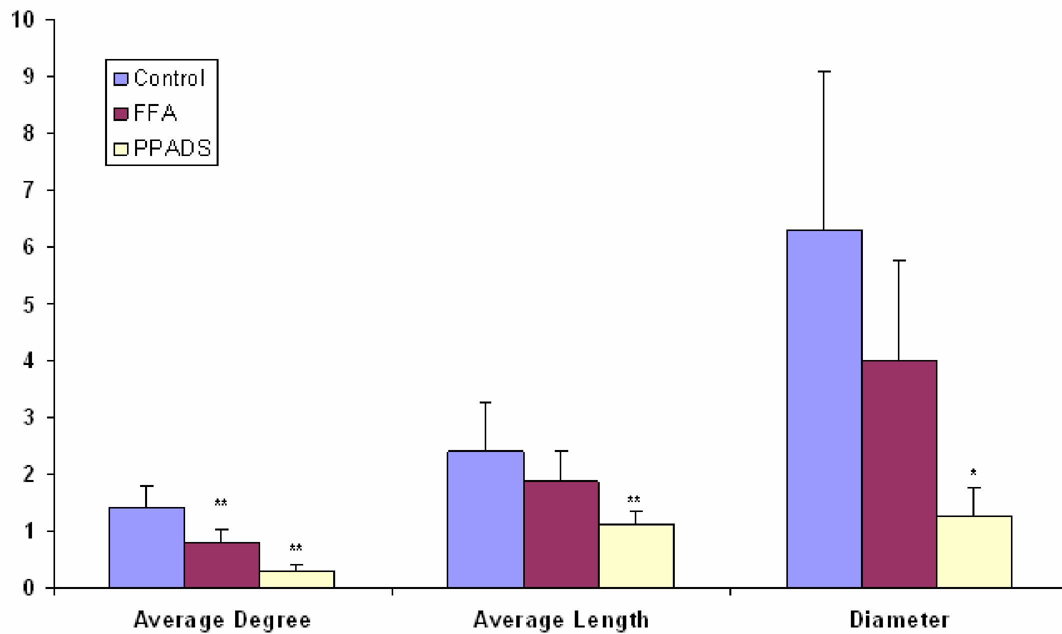


Fig. 5.3 Average degree, path length, and diameter calculated from functional signaling network structure derived using the dynamics signaling model. The graph theory metrics quantified reduced network connectivity by FFA and PPADS in comparison to control (significantly lower average degree) as well as distinguished the loss of local connection by FFA versus long range signaling by PPADS (significantly lower path length and diameter in PPADS treated cultures) * $p < 0.05$, ** $p < 0.01$.

Table 1. Summary of calculated complex network parameters from mapped networks

	n (number of movies analyzed)	Number of Edges	Number of Nodes	Average Degree	Average Length	Diameter
Control cultures	8	98.1±33.0	55.9±14.9	1.47±0.42	2.11±0.37	5.43±1.72
FFA treated cultures	8	22±8.0**$^{++}$	19.4±4.9**$^{++}$	0.80±0.52**$^{+}$	1.9±0.52^{++}	4.0±1.78^{++}
PPADS treated cultures	3	5.0±2.7**$^{++}$	6.0±1.4**$^{++}$	0.3±0.1**$^{+}$	1.1±0.24*$^{+}$	1.25±0.5**$^{+}$

* -- two-tail two-sample unequal variance T-Test p < 0.05 comparison with controls
** -- two-tail two-sample unequal variance T-Test p < 0.01 comparison with controls
$^{+}$ -- two-tail two-sample unequal variance T-Test p < 0.05 comparison FFA & PPADS
$^{++}$ -- two-tail two-sample unequal variance T-Test p < 0.05 comparison FFA & PPADS

Discussion

High throughput computationally intensive measurements of intercellular signaling and the extraction of statistically significant quantitative information about their underlying network structure and topology is not possible given current techniques. To begin to address this, we have previously developed a set of algorithms based on a novel dynamic signaling model that deterministically map the functional signaling topology of qualitatively observable intercellular networks with single cell resolution. By quantitatively analyzing the mapped topology of functional cell networks as illustrated in this study, their contribution to normal physiology and remodeling under pathophyisological conditions can begin to be understood at a systems level. In this study, we tested the application of the algorithm to biological data using mechanically stimulated intercellular propagation of calcium transients and explored its sensitivity to

detecting changes in network topologies induced by pharmacological compounds that selectively disrupt the principle known intercellular signaling mechanisms. This is important if the algorithms are to be used to detect and differentiate between physiologically normal versus pathological cell signaling. Treatment of rMC-1 Müller glia with pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) or flufenamic acid (FFA) resulted in a qualitatively observable attenuation of the calcium signal propagation through the networks. PPADS is a broad spectrum antagonist of metabotropic purinergic receptors while FFA is a gap junction blocker (Vigne, Pacaud et al. 1996; Srinivas and Spray 2003). For the various conditions tested, the algorithm detected quantifiable differences between pharmacologicall treated versus controls based on extracted networks metrics derived by the dynamic signaling model. For rMC-1 Müller glia, the algorithm detected statistically different changes number of mapped nodes and edges as well as in average degree for both PPADS and FFA treated cultures versus controls. There was also a significant difference in the average length and diameter of the network between FFA and PPADS treated cultures, reflecting the different alterations in network topology between blocking local signaling via gap junctions and longer range signaling via purinergic receptors. Our results suggest that, for rMC-1 cells, the activation of metabotropic purinergic receptors by extracellular soluble signals (i.e. ATP) may function as the primary mechanism in longer distance signaling and may play a more prominent role in determining the connectivity and topology of the signaling network, while diffusion of second messenger (i.e. IP_3) through gap junctions may serve as a secondary mechanism for local amplification of the signal.

A number of recent studies have reflected the trend in neuroscience to move beyond static and isolated descriptions of dynamical processes to one of understanding complex cellular interactions at a systems level (Schummers, Sharma et al. 2005; Gobel, Kampa et al. 2007). Recent advances in imaging of functional intercellular network signaling and computational methods for extracting information about individual cells in large networks are making significant strides towards overcoming the significant challenges and experimental limitations associated with studying and understanding complex cellular networks (Schummers, Sharma et al. 2005; Yaksi and Friedrich 2006). The analysis we present in this study compliments these approaches and provides evidence for the ability of the dynamic signaling model to quantitatively investigate the spatiotemporal dynamic properties of cellular networks.

Conclusion

We applied mapping algorithm based on the dynamic signaling model to biological data of mechanically stimulated intercellular propagation of calcium transients and explored its sensitivity to detecting changes in complex network topologies induced by antagonist of metabotropic purinergic receptors (PPADS) and gap junction blocker (FFA) under controlled conditions. Our analysis allows us to quantitatively assess differences between normal and pharmacologically perturbed intercellular calcium signaling as well as tease apart in the roles played by the two molecular mechanisms proposed for calcium transient propagation. Our results suggest that activation of

metabotropic purinergic receptors may function as the primary mechanism for longer distance signaling and may play a more prominent role in determining the connectivity and topology of the signaling network in rMC-1 Muller glia, while diffusion of second messenger (i.e. IP_3) through gap junctions may serve as a secondary mechanism for local amplification of the signal. The analysis we present in this study also provides evidence for the ability of the dynamic signaling model to quantitatively investigate the spatiotemporal dynamic properties of cellular networks.

Chapter VI

Associative effects of calcium signaling and Amyloid-beta peptide on reactive astrogliosis of Alzhermer's Disease

Abstract

Alzheimer's Disease (AD) is the most common form of age-related dementia affecting more than 26 million people worldwide. Its neuropathological hallmarks include loss of neurons, deposition of neuritic plaques, and reactive astrogliosis in affected brain regions. Amyloid-β-peptide (Aβ) and neuronal calcium dysregulation have been investigated as possible causes for AD, little is known on the effects of Aβ on astrocyte calcium signaling in AD. Intracellular calcium oscillations and intercellular calcium wave are critical modes of signaling in neural networks and have been suggested to contribute to the early and underlying pathophysiology of broad classes of neurodegenerative disorders. Here, we explored the effects of Aβ at pathophysiological concentrations on primary cortical astrocytes. We show that Aβ enhances intracellular oscillations characterized by statistically significant increases in the number of signaling cells and frequency of calcium oscillations. More importantly, we find that Aβ induces robust and reproducible spontaneous intracellular calcium waves in astrocytic networks. The waves travel in unique patterns of activation, displaying radial versus unidirectional propagation. We also examined associated effects of Alzheimer's Aβ and disrupted calcium homeostasis on reactive astrogliosis, an essential feature of AD. The protein expression levels of two astrogliosis markers, S100B and GFAP, were scrutinized in a

time-dependent manner. We report that $[Ca^{2+}]_i$ modulates Aβ's effect on increasing S100B and GFAP expression in astrocytes, and S100B loses its inhibitory effects on GFAP polymerization at high [S100]. These findings illustrate the promixal interactions between Aβ, calcium homeostasis, and reactive astrocytosis in AD. Although these observations require further investigation, they suggest that Aβ induced dysregulation of calcium homeostasis plays a critical role in reactive astrogliosis observed association with pathological features of AD; and the different modes of astrocytic calcium signaling may coordinate intracellular as well as long-range intercellular signaling that contribute to the pathogenesis of the disease.

Introduction

Alzheimer's Disease (AD) is the most devastating age-related neurodegenerative disease affecting more than 26 million people worldwide in 2006 with the number of cases estimated to double every 20 years (Ferri, Prince et al. 2005). Neuropathological hallmarks of AD include loss of neurons, deposition of neuritic plaques, neurofibrillary tangles, and reactive astrogliosis in the affected brain regions, particularly the hippocampus and neocortex (Jorgensen, Brooksbank et al. 1990; Yankner 1996). Accumulation of amyloid-β-peptide (Aβ) fragments and sustained disruption of intracellular calcium homeostasis are prevailing hypotheses for pathogenesis associated with AD (Khachaturian 1994; Hardy and Selkoe 2002; LaFerla 2002), influencing both neurons and astrocytes (Jorgensen, Brooksbank et al. 1990; Mark, Hensley et al. 1995; Fiacco and McCarthy 2006). Relevant studies in astrocytes have also revealed significant

insights to the integral role of calcium in the bidirectional communication between neurons and astrocytes (Nedergaard 1994; Pasti, Volterra et al. 1997) as well as the consequences in disrupting these regulatory interactions on neuron modulation in AD (Pasti, Volterra et al. 1997; Pekny and Nilsson 2005).

Intracellular calcium oscillations and intercellular calcium wave are modes of calcium signaling in astrocytes with important roles in modulating neuronal activity and survival (Charles, Merrill et al. 1991; Verkhratsky and Kettenmann 1996; Bezzi, Domercq et al. 2001; Fiacco and McCarthy 2006). Astrocytic calcium oscillations are a highly plastic, bidirectional form of communication in neuron-astrocyte interaction (Nedergaard 1994; Pasti, Volterra et al. 1997), and dysregulation of intracellular calcium affects glial and neuronal functions (Haughey and Mattson 2003; Perea and Araque 2005) and plays a pathological role in neurodegeneration associated with AD (Smith, Green et al. 2005). Reactive astrogliosis in AD, characterized by upregulation of glial fibrillary acidic protein (GFAP) and S100B expressions (Crols, Saerens et al. 1986; Ma, Ye et al. 1988; Griffin, Stanley et al. 1989; Sheng, Mrak et al. 1994; Casas, Sergeant et al. 2004), is highly associated with Tau-2 positive Aβ plaques in the molecular layer of cerebral cortex as well as the abnormal growth of imperfect neurites, both being key elements in formation of neuritic plaque and neurofibrillary tangles (Dickson, Farlo et al. 1988; Mandybur and Chuirazzi 1990; Wisniewski and Wegiel 1991; Sheng, Mrak et al. 1994; Casas, Sergeant et al. 2004). Previous studies have stressed the significance of intracellular calcium and the effects of Aβ on neurons in AD (LaFerla 2002), but little is known regarding potentially coupled effects of the two on astrocytes and reactive gliosis.

The aim of the current study was to explore the effects of increased levels of Aβ on astrocytic calcium transients, and the associated effects of Aβ and disrupted calcium homeostasis on reactive astrogliosis in AD.

Materials and Methods

A. Cortical Astrocyte Cell Culture

Cortical cultures were prepared from postnatal day 1 Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) using methods modified from those described previously (Haughey and Mattson, 2003). Briefly, 1-day-old Sprague-Dawley rat pups were euthansized by anesthesia overdose. Their brains were removed, with the cerebral cortices dissected in sterile Krebs Hepes Buffer (KHB) (10mM HEPES, 4.2mM $NaHCO_3$, 10mM dextrose, 1.18 mM $MgSO_4 \cdot 2H_2O$, 1.18mM KH_2PO_4, 4.69mM KCl, 118mM NaCl, 1.29mM $CaCl_2$; pH 7.3). Cells were mechanically dissociated in KHB through a 70µm cell strainer (BD Biosciences, San Jose, CA) to remove viable neurons, and plated in uncoated culture dish containing Modified Eagle Medium (Cellgro/Fisher, Herndon, VA) with 10% heat-inactivated horse serum (Omega Scientific, Tarzana, CA), 1% Penicillin-Streptomycin (Gibco/Invitrogen, Carlsbad, CA), and 1% Glut-Max (Gibco/Invitrogen, Carlsbad, CA). Type 1 astrocytes were purified by the mechanical removal of less adherent cells. Culture medium was changed twice per week, and cultures were used within 2 passages. Astrocytes were removed from dishes by trypsinization and plated onto poly-D Lysine (PDL) coated 35mm glass bottom dishes (MakTek, Ashland, MA). Cells were allowed to grow at 37ºC and 5% CO_2 to confluency (>80%) prior to performing calcium imaging studies and immunocytochemistry.

B. Glial Calcium Imaging

Cortical astrocyte cultures of ~90% confluency were washed twice with KHB and incubated with 8μM Fluo-4AM (Molecular Probes/Invitrogen, Carlsbad, CA) in KHB for 40 minutes at room temperature. Excess dye was removed by washing twice with KHB and an additional incubation of 20 minutes at room temperature was performed to equilibrate intracellular dye concentration and ensure complete intracellular esterification. In the intracellular calcium modulating experiments, pretreatment for 30 minutes of thapsigargin (Sigma, T9033) or BAPTA-AM (Sigma, A1076) with a concentration of 10μM was executed. A Ca^{2+} free KHB with 1.5mM BAPTA (Sigma, A4926) was used to eliminate extracellular calcium in one of the experimental conditions. 5μM of Amyloid-β-peptide fragment 1-40 (Sigma, A1075), an amyloid fragment known to be neurotoxic *in vivo* and *in vitro*, was used in the experiment (Emre et al., 1992; Kowall et al., 1992). The time point of Aβ dosing was regarded as time 0 minute, at which the first calcium oscillation movie was taken. Real-time movie recording of calcium transient was acquired at 5 Hz using a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Each movie was 4.5 minutes long and was taken at every 15 minute intervals for 30 minutes. Visualization of Ca^{2+} indicator dye fluorescence was done using a 488 nm (FITC) filter on an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Tokyo, Japan). Images were acquired with a customized LabVIEW program developed in the Silva Lab and processed in Image J and Matlab (Mathworks, Natick, MA).

C. Quantification of Ca^{2+} Transients

Using ImageJ, an NIH funded open source morphometric application, the circle-select tool was modified to allow manual selection of individual cells on the *xy*-plane of each movie using circles of 7 pixels in diameter. Each cell was considered as an individual region of interest (ROIs). We traced all the cells from the frames in which they appeared brightest as a result of an oscillation event. The entire population of cells was traced so that the percentage of cells participating in calcium oscillation per condition per unit time could be investigated. Calcium imaging with fixed coordinate allows $[Ca^{2+}]_i$ tracing on the same group of cells within the designated experimental time. An ImageJ plugin was used to calculate the average intensity for each ROI in each frame as well as the *x-y* coordinates of its area centroids. All of this data was organized in matrix format for post-processing analyses. Since the fluorescence intensity of Fluo-4 AM is proportional to calcium concentration, changes in cytosolic calcium concentrations can be inferred from the fluorescence profile of individual cells. A running-average filter of 3 frames and a low pass filter were applied using a custom written Matlab routine to the data to reduce noise in the fluorescence signals. For analysis of individual intracellular calcium transients, the data is processed to identify periods of sustained increase in fluorescence intensity. Due to the highly dynamic and cyclic nature of the intracellular calcium transients, an averaging filter of 10 frames was applied to reduce noise and a first-derivative filter was then used to identify significant and sustained increases in calcium (i.e. 10 or more consecutive frames with positive derivative values). We established those portions of the signal as the rise phase of the intracellular calcium

transients. The percentage of activated cells and oscillation frequency per activated cell per unit time were computed. All calculations and graphs were done using Matlab (Mathworks, Natick, MA) and SigmaPlot (Systat, San Jose, CA).

D. GFAP and S100B Immunocytochemistry

Cultures grown on PDL-coated dishes were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes. After washing two times with PBS (5 mins each), cells were incubated in PBS containing 0.2% (v/v) Triton X-100. The cells were then washed twice again with PBS (5 mins each) before being incubated for 2 hrs with mouse monoclonal anti-S100B antibody (Sigma, S2657) or mouse anti-GFAP cocktail (BD Biosciences, 556330), diluted 1:100 and 1:400 respectively in PBS containing 10% FBS. After a double wash with PBS (5 mins each), goat anti-mouse IgG (whole molecule)-FITC (Sigma, F0257) was applied and incubated for 1 hr. The secondary antibodies were diluted 1:100 in PBS with 5% FBS prior to addition. Cells were finally washed two times with PBS and mounted with a cover slip in a drop of ProLong Gold antifade reagent counterstained with DAPI (Molecular Probes/Invitrogen, Carlsbad, CA). The cells were analyzed using an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Tokyo, Japan) using filter settings for FITC, TRITC, and DAPI. Images were taken with a customized LabVIEW program developed in the Silva Lab. 3 sets of experiment were conducted per condition per time point, and 20 images were taken per set. Images were then analyzed by Image J employing the built-in Threshold Function. Images of negative control without primary

antibody treatment were used to subtract the fluorescence background. The fluorescence intensity of each ICC picture was then measured in Image J, with the area of fluorescence normalized to the area occupied by the cell in the field of view to eliminate the effect of various cell numbers between samples.

RESULTS

A. Aβ increases intracellular calcium oscillation and signaling cortical astrocytes

Intracellular calcium oscillation frequency measured for the cortical astrocytes were all within the previously reported physiological range of 10 to 110 mHz (Cornell-Bell, Finkbeiner et al. 1990). Addition of Aβ immediately increased the calcium oscillation frequency in astrocytes by ~58% (from 33.15 ± 1.5 mHz to 52.30 ± 2.95 mHz), and application of intracellular calcium chelator, BAPTA-AM, to sequester excess intracellular calcium fluctuation abolished the disruption in calcium homeostasis due to Aβ. Furthermore, the Aβ induced calcium oscillations was shown to be affected by both intra- and extracellular sources of calcium (Fig. 6.1A). Depleting intracellular calcium stores using thapsigargin, an inhibitor of calcium-ATPase pump on the endoplasmic reticulum (ER) (Lytton, Westlin et al. 1991), effectively reduced the oscillation frequency enhanced by Aβ. Removal of extracellular calcium ($[Ca^{2+}]_e$) was also effective in attenuating the effects of Aβ on calcium oscillation frequency. In addition to the increase in oscillation frequency, the number of recruited cells displaying intracellular calcium oscillations followed similar trends under Aβ treatment (Fig. 6.1B). Perturbation of intracellular and extracellular calcium sources also significantly reduced the percentage of activated cells from 91.56 ± 3.52% under Aβ condition, to 38.76 ± 14.8% and 32.24 ±

55.84% in the presence of Aβ with thapsigargin and zero extracellular calcium, respectively. Interestingly, in the absence of $[Ca^{2+}]_e$, the percentage of activated cells dropped sharply from 98.70 ± 1.62% immediately following Aβ application to 1.52 ± 0.9% after 15 min ($p<0.0001$, n=4), suggesting a role for $[Ca^{2+}]_e$ in maintaining the Aβ induced intracellular calcium oscillations in astrocytes.

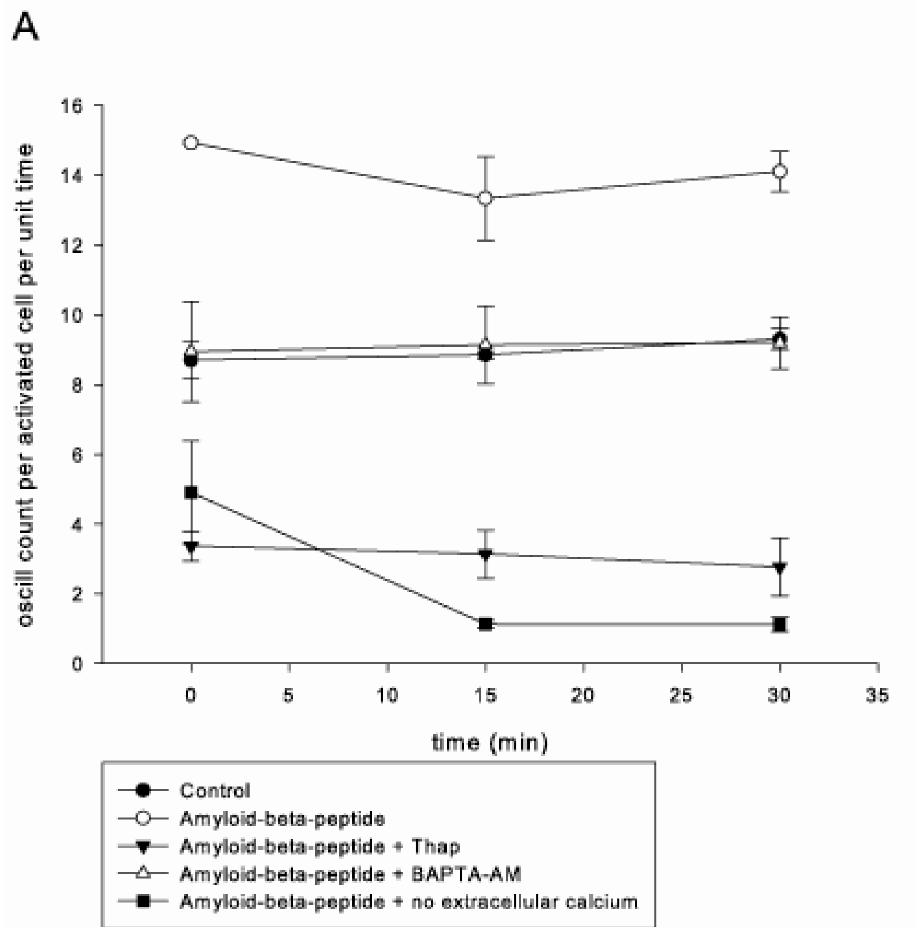


Fig. 6.1A Intracellular calcium oscillation frequency of astrocytes in response to Aβ and manipulation of intracellular and extracellular calcium content. Aβ enhances oscillation frequency in astrocytes and operates via intracellular and extracellular calcium sources.

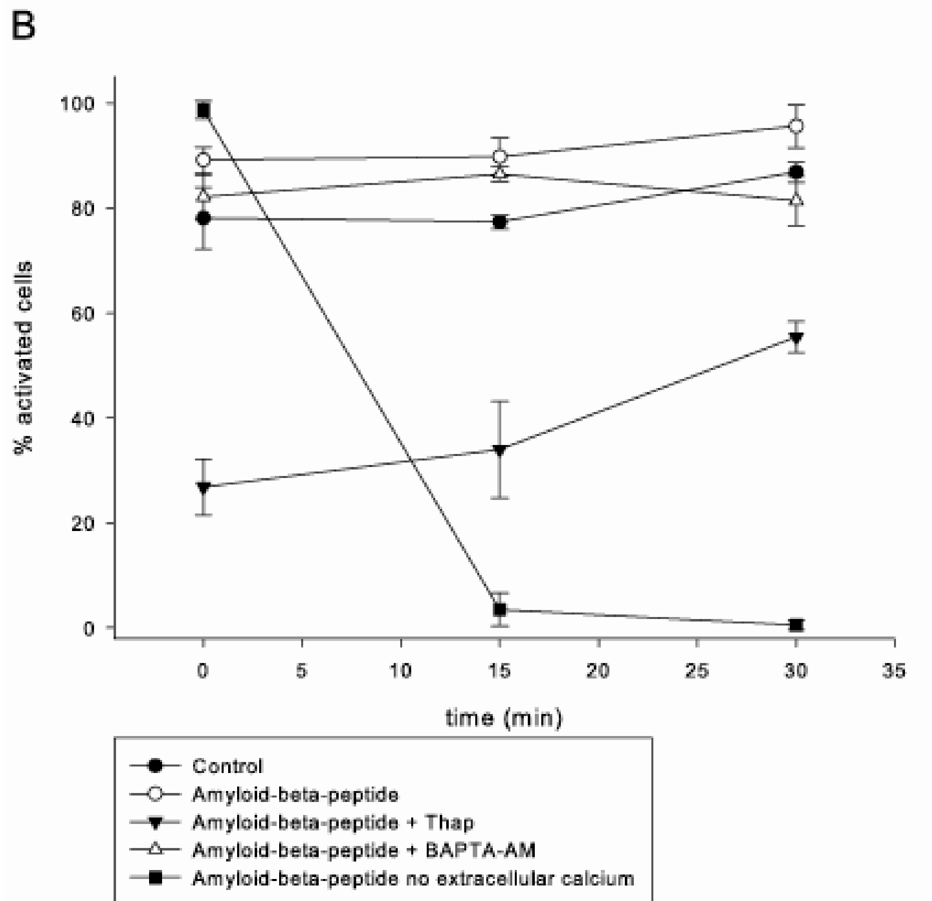


Fig. 6.1B. Percentage of activated signaling astrocytes in response to Aβ and manipulation of intracellular and extracellular calcium content. Aβ increased the percent of signaling astrocytes, but is attenuated by depletion of intracellular and extracellular calcium sources.

B. Aβ induced spontaneous intercellular calcium waves in astrocytes

In addition to affecting astrocytic intracellular calcium oscillations, Aβ induced spontaneous intercellular calcium waves in astrocytes networks. The calcium waves showed unique patterns of activation in cultures treated with Aβ, displaying radial versus unidirectional propagations (Fig. 6.2A, B). The traveling distances of radial and

unidirectional waves were 141.1 ± 30.9 μm and 160.9 ± 22.5 μm, respectively, with corresponding velocities of 6.03 ± 1.60 μm/sec and 4.66 ± 0.48 μm/sec. Aβ-induced intercellular calcium waves occurred as early as 30 minutes after dosing with Aβ, indicating that the waves formed spontaneously rather than being induced by unintentional mechanical stimulation during the dosing process. The two forms of intercellular calcium waves we observed took highly specific and tortuous paths instead of translating via the shortest distance between cells. Some cells in the path of the wave front were skipped and not recruited to the coupled astrocytic signaling network, resulting in spatially heterogeneous activation patterns. These findings support the existence of plastic functional circuits between astrocytes, as calcium elevations may spread to some but not all adjacent astrocytes (Sul, Orosz et al. 2004). Both types of the wave forms are observed in individual experiments, although there was no switching from one type to another. The preference on the type of propagation was not correlated with the age and density of cultures.

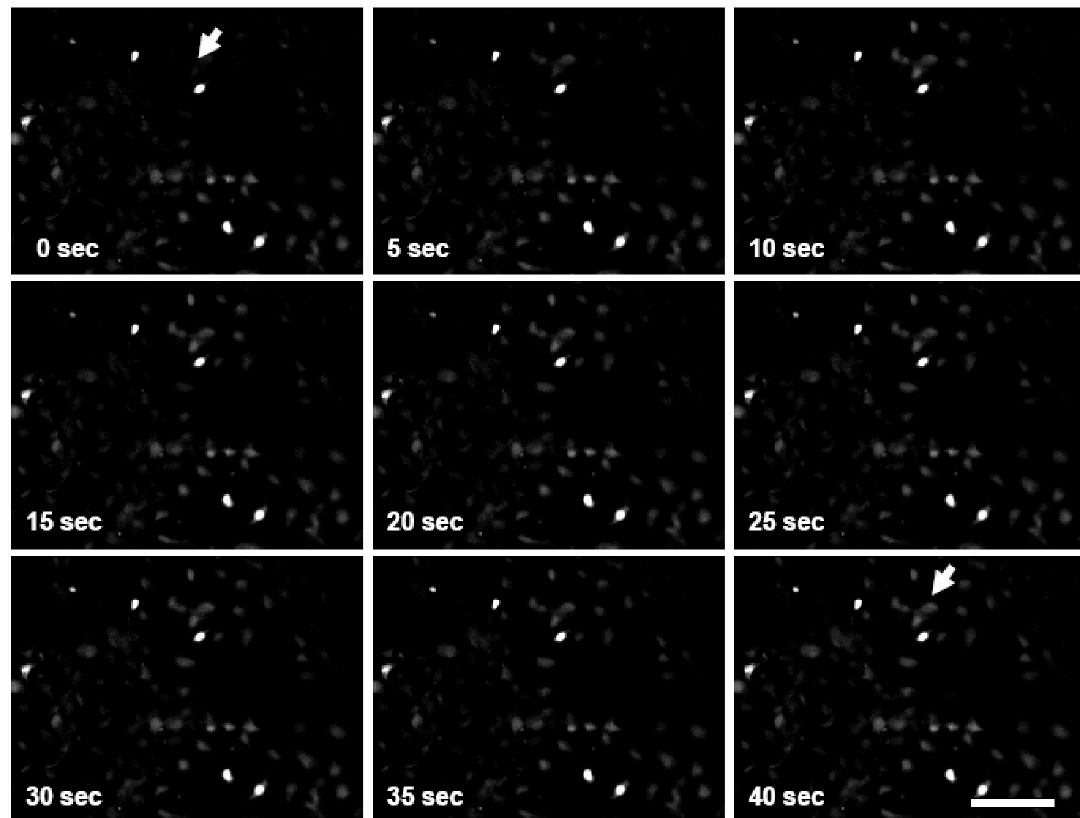


Fig. 6.2A. Spontaneous intercellular calcium waves observed in Aβ-treated astrocytes cultures, propagating in radial direction. Select frames of recorded intercellular calcium wave in 5 second intervals. The traveling distances of radial is 141.1 ± 30.9 μm with calculated velocity of 6.03 ± 1.60 μm/sec. White arrow indicates the initiation site of the calcium wave; red arrow indicates traveling direction. Scale bar, 100μm.

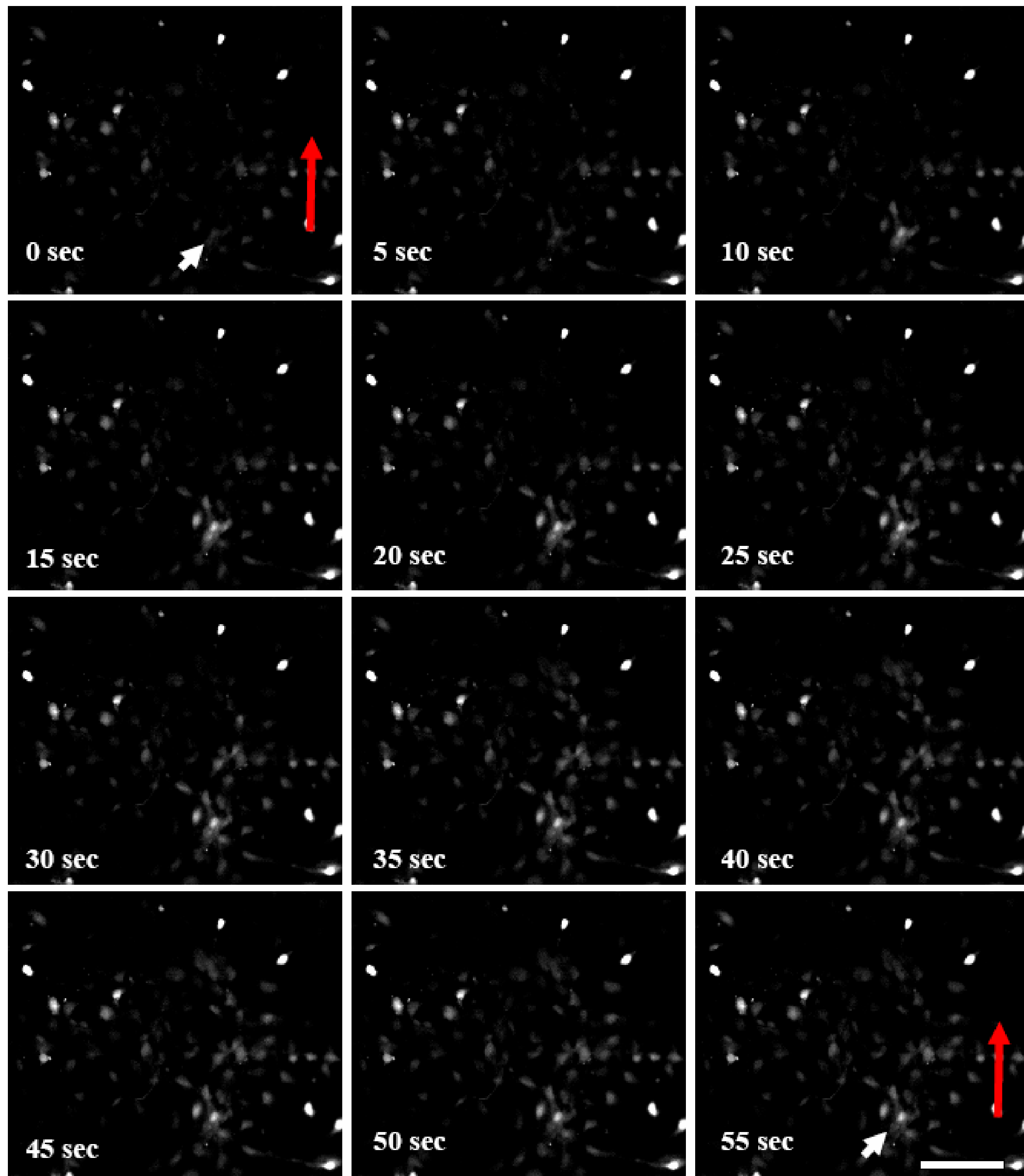


Fig. 6.2B Spontaneous intercellular calcium waves in Aβ-treated astrocyte cultures propagating in unidirectional pattern. Select frames of recorded intercellular calcium wave in 5 second intervals. The traveling distance is 160.9 ± 22.5 µm with calculated velocity 4.66 ± 0.48 µm/sec. White arrow indicates the initiation site; red arrow indicates traveling direction. Scale bar, 100µm.

C. $[Ca^{2+}]_i$ modulates Aβ's effect on increasing S100B and GFAP expression in astrocytes

Aβ significantly increased GFAP and S100B expression as early as 12 hrs after treating with Aβ. The increase in protein level was observed within the first 12 hrs following Aβ treatment and remained fairly stable throughout the rest of the 48 hrs experimental period. However, when the effect of Aβ on intracellular calcium homeostasis was suppressed as described in previous sections by thapsigargin and BAPTA-AM, the protein expression levels were significantly altered depending on the mode of $[Ca^{2+}]_i$ modulation (Fig. 6.3A-B). Finally, application of thapsigargin or BAPTA-AM alone without Aβ induced no change in GFAP and S100B expression, indicating Aβ is the primary component of reactive astrogliosis with its effects modulated by $[Ca^{2+}]_i$.

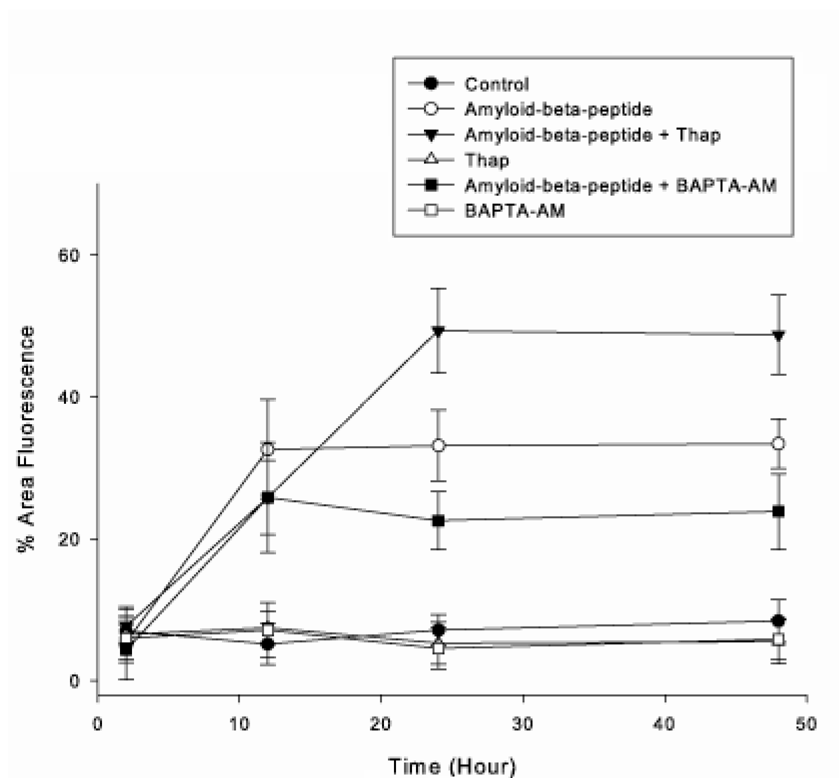


Fig. 6.3A Quantification of S100B level in cortical astrotcytes treated with Aβ, Aβ+thapsigargin, and Aβ+BAPTA-AM compared to control conditions (no Abeta, thapsigargin, BAPTA-AM) at 2, 12, 24, and 48 hr time points. Interestingly, application of thapsigargin to increase intracellular calcium induced higher levels of S100B at the 24 and 48hr time points, while intracellular calcium chelator (BAPTA-AM) moderately decreased S100B levels in comparison to Aβ treated cultures.

Our results showed that elevation in intracellular calcium correlated with the upregulation of S100B protein in Aβ treated cortical astrocytes. While dosing with Aβ significantly increased the expression of S100B in comparison to controls (Fig. 6.3A, $p<0.0001$, $n=46$), the application of Aβ with thapsigargin, which sustained the Aβ

induced intracellular calcium elevation by inhibiting uptake of excess calcium into the ER (Thastrup, Cullen et al. 1990; Lytton, Westlin et al. 1991), resulted in still elevated levels of S100B at 24 and 48 hrs compared to Aβ alone (Fig. 6.3A, $p<0.0001$, n=46). Conversely, application of the intracellular calcium chelator, BAPTA-AM, together with Aβ reduced the increased S100B expression associated with Aβ treatment (Fig. 6.3A, $p<0.001$ at 24 hrs, $p<0.05$ at 48 hrs, n=46). The moderate effects of BAPTA-AM on S100B level in Aβ treated cultures was moderate at 12 hrs maybe be indicative of additional cellular events triggered initially by Aβ leading to upregulated S100B expression that were not abolished by intracellular BAPTA. Similar to S100B, decreased $[Ca^{2+}]_i$ in Aβ+BAPTA-AM condition resulted in significantly lower levels of GFAP compared to Aβ treated astrocytes (Fig. 6.3B).

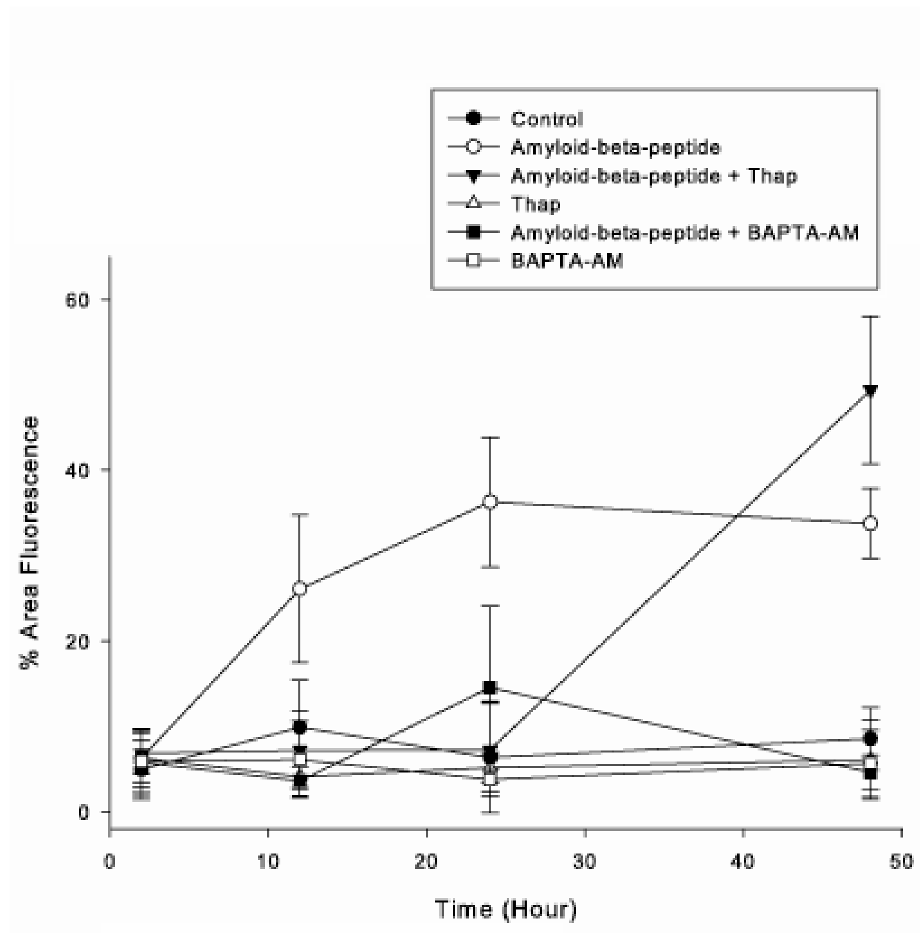


Fig. 6.3B Quantification of GFAP levels in cortical astorcytes treated with Aβ, Aβ+thapsigargin, and Aβ+BAPTA-AM compared to control conditions (no Abeta, thapsigargin, BAPTA-AM) at 2, 12, 24, and 48 hr time points. Application of BAPTA-AM to reduce intracellular calcium blocked the Aβ-induced increase in GFAP. Interestingly, increased levels of GFAP were observed in and Aβ+thapsigargin treated cultures at the 48 hr time point.

Interestingly, we observed a significant increase in GFAP level at 48 hrs in Aβ+thapsigargin treated cultures that were not seen at earlier time points. Based on previous studies that reported trophic effects and inhibition of GFAP monomer

phosphorylation by S100B at nanomolar cellular concentrations versus cytoxic and pro-inflammatory effects at the micromolar range (Selinfreund, Barger et al. 1991; Ziegler, Innocente et al. 1998; Wang, Rosengren et al. 1999; Li, Barger et al. 2000), the low GFAP expression observed at the earlier time points in Aβ+thapsigargin treated cultures may be due to the inhibitory effects of S100B on GFAP polymerization. Furthermore, when we applied a supplementary dose of thapsigargin to the Aβ+Thapsigargin treated cultures at 24 hrs, higher levels of both S100B and GFAP were observed ($p<0.001$ in S100B and GFAP, Aβ+thapsigargin compared to Aβ+thapsigargin supplement, $n=22$) (Fig. 6.3C). These findings are in keeping with previous reports that S100B exert inhibitory effects on GFAP only in low concentrations and may enhance its assembly into intermediate filaments at higher concentrations, although further investigation would be needed to decipher this regulatory mechanism.

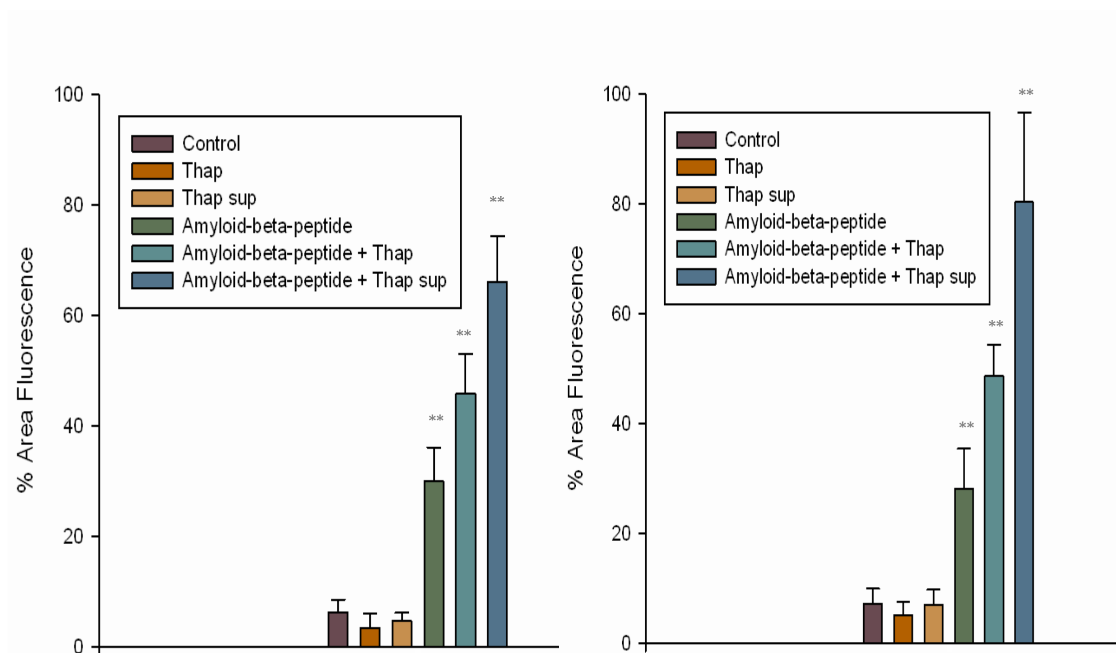


Fig. 6.3C Quantification of S100B and GFAP levels of cortical astrocytes treated with Aβ, Aβ+thapsigargin, Aβ+thapsigargin supplement (additional dose of thapsigargin at 24 hr time point). Prolonged elevation in intracellular calcium by thapsigargin supplement further increased S100B and GFAP levels. $p < 0.001$ (2-tailed *t*-test).

Discussion

The present study was designed to determine the effects of Aβ on astrocytic intracellular calcium oscillations, a key signaling mode for nonexcitatory glial cells, and the subsequent modulation of reactive astrogliosis associated with AD pathology by the calcium signaling. We found that Aβ increases oscillation frequency and number of signaling cortical astrocytes. More importantly, we report for the first time that Aβ induces spontaneous intercellular calcium waves in cortical astrocytic networks that propagate in unidirectional and radial directions with high degree of specificity. In

addition, we examined the coupled effects of Aβ and intracellular calcium homeostasis on reactive astrocytosis, one of the distinct features in AD. We found that $[Ca^{2+}]_i$ modulates the effects of Aβ on increasing levels S100B and GFAP in reactive astrocytes. Furthermore, S100B may interact with GFAP polymerization in a concentration-dependent manner. Our study revealed spontaneous intercellular calcium waves as a potential mode of extraneuronal long range signaling in reactive astrocytic networks under pathological conditions, and established the proximal interactions between Aβ, calcium homeostasis, and reactive astrogliosis in AD.

Intracellular calcium oscillations are observed in astrocytes from different regions of the CNS (Pasti, Volterra et al. 1997; Aguado, Espinosa-Parrilla et al. 2002; Nett, Oloff et al. 2002) and is believed to take part in the development neural network and modulation of synaptic activity by releasing glutamate on adjacent neuronal processes, thereby manipulating the excitability of the neighboring cells and synaptic transmission (Kang, Jiang et al. 1998; Nett, Oloff et al. 2002; Zhang, Pangrsic et al. 2004). We show that accumulation of the amyloid-beta peptide disrupts calcium homeostasis in astrocytic networks resulting in significantly increased calcium oscillation frequency as well as the number of signaling astrocytes. Sensitivity of the Aβ induced calcium signaling to zero-extracellular calcium condition and thapsigargin, which depletes endogenous intracellular calcium stores by inhibition of SERCA pumps(Thastrup, Cullen et al. 1990; Lytton, Westlin et al. 1991), indicate involvement of both intracellular and extracellular calcium sources as is consistent with previous reports on spontaneous and Aβ induced calcium

transients in neural networks (Stix and Reiser 1998; Aguado, Espinosa-Parrilla et al. 2002).

Previous studies have also reported that Aβ increased the traveling velocity and propagation distance of mechanically stimulated calcium waves, suggesting it enhances intercellular communication in astrocytic network mediated by extracellular signaling factors and gap junction coupling between cells (Haughey and Mattson 2003). Here we reported the first observation of spontaneously-formed intercellular calcium waves induced by Aβ that travel in unidirectional and radial directions with high degree of specificity. The propagation distances, 160.9 ± 22.5 and 141.1 ± 30.9 μm, and velocities, 4.66 ± 0.48 μm/sec and 6.03 ± 1.60 μm/sec, of these waves in unidirectional and radial directions, respectively, are in keeping with previous reports that described these astrocytic calcium waves as several hundred micrometers in distance and less than 50 μm/sec in velocity (Cornell-Bell, Finkbeiner et al. 1990; Haydon 2001). Furthermore, the specificity of the wave front support the presence of plastic functional circuits between astrocytes previously proposed in the hippocampus (Sul, Orosz et al. 2004)

Evidence in literature suggest that the changes we observed in calcium signaling from intracellular oscillations in controls to a hybrid of calcium waves and oscillations in Aβ-treated cultures may also be mediated by release of "gliotransmitters," such as glutamate and ATP, from the astrocytes (Cornell-Bell, Finkbeiner et al. 1990; Angulo, Kozlov et al. 2004; Hamilton, Vayro et al. 2008; Kumaria, Tolias et al. 2008). Glutamate has been known as a key molecule in bidirectional signaling between neurons and astrocytes where the release of glutamate by astrocyte is triggered by increased $[Ca^{2+}]_i$

(Cornell-Bell, Finkbeiner et al. 1990; Angulo, Kozlov et al. 2004). Glutamate at lower concentrations (below 1μM) causes asynchronous and localized intracellular oscillation in astrocytes while at higher concentrations (10-100 μM) induces intercellular calcium waves that can propagate through upwards of 60 astrocytes (Cornell-Bell, Finkbeiner et al. 1990). Aβ has been shown to increase the sensitivity of astrocytes to glutamate and calcium ionophore neurotoxicity (Mattson and Rydel 1992; Stix and Reiser 1998). Taken together, the transformation of calcium signaling modes can potentially be attributed to release of glutamate from astrocytes due to Aβ induced $[Ca^{2+}]_i$ elevation together with enhanced sensitivity to glutamate caused by Aβ. In addition, Aβ induced reactive astrocytes have been reported to produce nitric oxide (NO) and proinflammatory cytokines, i.e. TNF-α, IL-1β, and IL-6 which may also affect calcium homeostasis and enhance the release of gliotransmitters (Gitter, Cox et al. 1995; Rossi and Bianchini 1996; Akama, Albanese et al. 1998; Johnstone, Gearing et al. 1999; Meda, Baron et al. 2001; Ida, Hara et al. 2008).

Finally, elevated levels of GFAP and S100B have been found in astrocytes associated with neuritic plaques of AD (Simpson, Ince et al. 2008) and Aβ has also been reported to upregulate these proteins in cultured astrocytes after 72 hours (Meske, Hamker et al. 1998). However, our studies show that Aβ increases GFAP and S100B as early as 12 hrs after exposure to the pathogenic peptide, indicating a much faster response of glia activation and is suggestive of their involvement in the initial stages of AD pathogeneisis. Currently there is limited knowledge on the metabolic function of GFAP, but S100B, in contrast, plays versatile intracellular and extracellular roles (Donato 1999;

Donato 2003). S100B interacts with a large variety of target proteins by inhibiting their phosphorylation, including GFAP (Bianchi, Giambanco et al. 1993; Bianchi, Garbuglia et al. 1996). In addition to the concentration of S100B, S100-binding proteins, such as annexin VI and TRTK-12, can block S100-GFAP interactions thereby further modulating regulation of S100B on GFAP intermediate filaments (Bianchi, Garbuglia et al. 1996; Garbuglia, Verzini et al. 1998). Taken together, interactions between S100B and GFAP assembly are intricately controlled by a combination of factors, the complexity of which is reflected from our experimental results. From our experiments, reactive astrogliosis is more than a phenomenon that accompanies AD; it affects different modes of calcium signaling, resulting in a coordinated long range signaling system involved in neural injury of AD. Exploring the interactions between Aβ, calcium homeostasis, and reactive astrocytosis reveals the astrocyte-specific pathologies in AD thereby favoring the development of potential new therapeutic avenues.

Future Work

In the present studies, we compared the viability and differentiation potential of BMSCs on standard surface chemistries to determine the optimum growth conditions of these cells for neuronal differentiation as well as induction of neural progenitor marker in BMSCs. However, additional studies will be needed to fully assess the differentiation potential of these stem cells to become functional retinal neurons for treatment of retinal degenerative diseases. While one previously study have reported the differentiation of BMSCs into photoreceptor-like cells expressing rhodopsin (Kicic, Shen et al. 2003) using soluble factors *in vitro*, we and other groups have yet to replicate these results. In addition, it is likely that the complex array of spatial and temporal cues associated with the development of photoreceptors can be recapitulated in the *in vitro* system using limited soluble factors. To this end, ongoing efforts for the project include transplantation of naïve and induced GFP+ BMSCs into healthy and diseased animal models such as the homozygous *rd10/rd10* (a.k.a. $Pde6b^{rd10}$) retinal degenerate mice that is caused by a missense mutation in exon 13 of the beta subunit of the rod phosphodiesterase gene, a mutation also found in patients of retinitis pigmentosa.

In addition, we introduced here the rMC-1 Müller glia cell line as a model for studying calcium signaling and present evidence for complex signaling dynamics in intercellular calcium transient propagation within glial networks that may be altered under pathological conditions. Furthermore, we show that disruption of glial calcium homeostasis via altered calcium signaling dynamics directly correlates with known hallmarks of reactive astrogliosis and may play an important role in pathogenesis of

neurodegenerative disease. Additional studies are underway to further explore potential heterogeneity in glial networks at the individual cellular level that may be responsible for the complex signaling dynamics observed. A current opinion is that there may be additional subclasses of astrocytes that are specific to different regulatory functions (i.e. release of glutamate to regulate synaptic function and release of D-serine to regulate vasomotor responses). If that is the case, calcium signaling may also be a manifestation of their differences. Finally, additional investigation is needed to further elucidate the intracellular mechanisms for the regulatory role of calcium in astrogliosis. As an example, over expression of calcium-dependent phosphatase, calcineurin, have been shown to trigger astrocyte activation in aging and Alzheimer's disease (Norris, Kadish et al. 2005). Future work will investigate additional members in these intracellular pathways and how pathological changes in intracellular calcium dynamics lead to genomic and phenotypic profiles of reactive gliosis.

References

Abdallah, B. M. and M. Kassem (2007). "Human mesenchymal stem cells: from basic biology to clinical applications." Gene Ther.

Aboody, K. S., A. Brown, et al. (2000). "Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas." Proc Natl Acad Sci U S A 97(23): 12846-51.

Aguado, F., J. F. Espinosa-Parrilla, et al. (2002). "Neuronal activity regulates correlated network properties of spontaneous calcium transients in astrocytes in situ." J Neurosci 22(21): 9430-44.

Agulhon, C., J. C. Platel, et al. (2007). "Bioluminescent imaging of Ca2+ activity reveals spatiotemporal dynamics in glial networks of dark-adapted mouse retina." J Physiol 583(Pt 3): 945-58.

Akama, K. T., C. Albanese, et al. (1998). "Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism." Proc Natl Acad Sci U S A 95(10): 5795-800.

Akiyama, Y., C. Radtke, et al. (2002). "Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells." J Neurosci 22(15): 6623-30.

Albert, R. and A. Barabasi (2002). "Statistical mechanics of complex networks." Rev Mod Phy 74: 47-97.

Alhadlaq, A. and J. J. Mao (2004). "Mesenchymal stem cells: isolation and therapeutics." Stem Cells Dev 13(4): 436-48.

Altman, J. and G. D. Das (1965). "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." J Comp Neurol 124(3): 319-35.

Altman, J. and G. D. Das (1966). "Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions." J Comp Neurol 126(3): 337-89.

Altshuler, D., J. J. Lo Turco, et al. (1993). "Taurine promotes the differentiation of a vertebrate retinal cell type in vitro." Development 119(4): 1317-28.

Amit, M. and J. Itskovitz-Eldor (2002). "Derivation and spontaneous differentiation of human embryonic stem cells." J Anat 200(Pt 3): 225-32.

Amit, M., V. Margulets, et al. (2003). "Human feeder layers for human embryonic stem cells." Biol Reprod 68(6): 2150-6.

Angulo, M. C., A. S. Kozlov, et al. (2004). "Glutamate released from glial cells synchronizes neuronal activity in the hippocampus." J Neurosci 24(31): 6920-7.

Aoki, H., A. Hara, et al. (2006). "Embryonic stem cells that differentiate into RPE cell precursors in vitro develop into RPE cell monolayers in vivo." Exp Eye Res 82(2): 265-74.

Aoki, H., A. Hara, et al. (2007). "An in vitro mouse model for retinal ganglion cell replacement therapy using eye-like structures differentiated from ES cells." Exp Eye Res 84(5): 868-75.

Araque, A., E. D. Martin, et al. (2002). "Synaptically released acetylcholine evokes Ca2+ elevations in astrocytes in hippocampal slices." J Neurosci 22(7): 2443-50.

Assady, S., G. Maor, et al. (2001). "Insulin production by human embryonic stem cells." Diabetes 50(8): 1691-7.

Aymerich, M. S., E. M. Alberdi, et al. (2001). "Evidence for pigment epithelium-derived factor receptors in the neural retina." Invest Ophthalmol Vis Sci 42(13): 3287-93.

Azizi, S. A., D. Stokes, et al. (1998). "Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts." Proc Natl Acad Sci U S A 95(7): 3908-13.

Bachoud-Levi, A. C., V. Gaura, et al. (2006). "Effect of fetal neural transplants in patients with Huntington's disease 6 years after surgery: a long-term follow-up study." Lancet Neurol 5(4): 303-9.

Back, T., M. D. Ginsberg, et al. (1996). "Induction of spreading depression in the ischemic hemisphere following experimental middle cerebral artery occlusion: effect on infarct morphology." J Cereb Blood Flow Metab 16(2): 202-13.

Banin, E., A. Obolensky, et al. (2006). "Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells." Stem Cells 24(2): 246-57.

Barberi, T., P. Klivenyi, et al. (2003). "Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice." Nat Biotechnol 21(10): 1200-7.

Barberi, T., L. M. Willis, et al. (2005). "Derivation of multipotent mesenchymal precursors from human embryonic stem cells." PLoS Med 2(6): e161.

Barnstable, C. J. and J. Tombran-Tink (2004). "Neuroprotective and antiangiogenic actions of PEDF in the eye: molecular targets and therapeutic potential." Prog Retin Eye Res 23(5): 561-77.

Basarsky, T. A., S. N. Duffy, et al. (1998). "Imaging spreading depression and associated intracellular calcium waves in brain slices." J Neurosci 18(18): 7189-99.

Bellamkonda, R., J. P. Ranieri, et al. (1995). "Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension in vitro." J Neurosci Res 41(4): 501-9.

Berridge, M. J. (1990). "Calcium oscillations." J Biol Chem 265(17): 9583-6.

Berridge, M. J. and A. Galione (1988). "Cytosolic calcium oscillators." Faseb J 2(15): 3074-82.

Berridge, M. J., P. Lipp, et al. (2000). "Signal transduction. The calcium entry pas de deux." Science 287(5458): 1604-5.

Bezzi, P., M. Domercq, et al. (2001). "Neuron-astrocyte cross-talk during synaptic transmission: physiological and neuropathological implications." Prog Brain Res 132: 255-65.

Bianchi, R., M. Garbuglia, et al. (1996). "S-100 (alpha and beta) binding peptide (TRTK-12) blocks S-100/GFAP interaction: identification of a putative S-100 target epitope within the head domain of GFAP." Biochim Biophys Acta 1313(3): 258-67.

Bianchi, R., I. Giambanco, et al. (1993). "S-100 protein, but not calmodulin, binds to the glial fibrillary acidic protein and inhibits its polymerization in a Ca(2+)-dependent manner." J Biol Chem 268(17): 12669-74.

Biedermann, B., A. Bringmann, et al. (2004). "GABA(A) receptors in Muller glial cells of the human retina." Glia 46(3): 302-10.

Biedermann, B., A. Bringmann, et al. (2002). "High-affinity GABA uptake in retinal glial (Muller) cells of the guinea pig: electrophysiological characterization, immunohistochemical localization, and modeling of efficiency." Glia 39(3): 217-28.

Bjorklund, A. and O. Lindvall (2000). "Cell replacement therapies for central nervous system disorders." Nat Neurosci 3(6): 537-44.

Bjorklund, L. M., R. Sanchez-Pernaute, et al. (2002). "Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model." Proc Natl Acad Sci U S A 99(4): 2344-9.

Borlongan, C. V., M. Hadman, et al. (2004). "Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke." Stroke 35(10): 2385-9.

Bossolasco, P., L. Cova, et al. (2005). "Neuro-glial differentiation of human bone marrow stem cells in vitro." Exp Neurol 193(2): 312-25.

Bouhon, I. A., H. Kato, et al. (2005). "Neural differentiation of mouse embryonic stem cells in chemically defined medium." Brain Res Bull 68(1-2): 62-75.

Boyer, L. A., T. I. Lee, et al. (2005). "Core transcriptional regulatory circuitry in human embryonic stem cells." Cell 122(6): 947-56.

Brambilla, R., L. Cottini, et al. (2003). "Blockade of A2A adenosine receptors prevents basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes." Glia 43(2): 190-4.

Brasted, P. J., C. Watts, et al. (1999). "Associative plasticity in striatal transplants." Proc Natl Acad Sci U S A 96(18): 10524-9.

Brazelton, T. R., F. M. Rossi, et al. (2000). "From marrow to brain: expression of neuronal phenotypes in adult mice." Science 290(5497): 1775-9.

Bringmann, A., M. Francke, et al. (1999). "Human Muller glial cells: altered potassium channel activity in proliferative vitreoretinopathy." Invest Ophthalmol Vis Sci 40(13): 3316-23.

Bringmann, A., I. Iandiev, et al. (2007). "Porcine Muller glial cells increase expression of BKCa channels in retinal detachment." Curr Eye Res 32(2): 143-51.

Bringmann, A., T. Pannicke, et al. (2006). "Muller cells in the healthy and diseased retina." Prog Retin Eye Res 25(4): 397-424.

Bringmann, A., T. Pannicke, et al. (2001). "Upregulation of P2X(7) receptor currents in Muller glial cells during proliferative vitreoretinopathy." Invest Ophthalmol Vis Sci 42(3): 860-7.

Brown, C. A., S. J. Charlton, et al. (1997). "Enhancement of the response to purinergic agonists in P2Y1 transfected 1321N1 cells by antagonists suramin and PPADS." Br J Pharmacol 120(6): 1049-52.

Buhnemann, C., A. Scholz, et al. (2006). "Neuronal differentiation of transplanted embryonic stem cell-derived precursors in stroke lesions of adult rats." Brain 129(Pt 12): 3238-48.

Bull, N. D. and K. R. Martin (2007). "Optic nerve restoration: new perspectives." J Glaucoma 16(5): 506-11.

Camaioni, E., J. L. Boyer, et al. (1998). "Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors." J Med Chem 41(2): 183-90.

Carpenter, M. K., X. Cui, et al. (1999). "In vitro expansion of a multipotent population of human neural progenitor cells." Exp Neurol 158(2): 265-78.

Casas, C., N. Sergeant, et al. (2004). "Massive CA1/2 neuronal loss with intraneuronal and N-terminal truncated Abeta42 accumulation in a novel Alzheimer transgenic model." Am J Pathol 165(4): 1289-300.

Ceelen, P. W., A. Lockridge, et al. (2001). "Electrical coupling between glial cells in the rat retina." Glia 35(1): 1-13.

Charles, A. C., J. E. Merrill, et al. (1991). "Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate." Neuron 6(6): 983-92.

Charlton, S. J., C. A. Brown, et al. (1996). "Cloned and transfected P2Y4 receptors: characterization of a suramin and PPADS-insensitive response to UTP." Br J Pharmacol 119(7): 1301-3.

Charlton, S. J., C. A. Brown, et al. (1996). "PPADS and suramin as antagonists at cloned P2Y- and P2U-purinoceptors." Br J Pharmacol 118(3): 704-10.

Charteris, D. G. (1995). "Proliferative vitreoretinopathy: pathobiology, surgical management, and adjunctive treatment." Br J Ophthalmol 79(10): 953-60.

Chen, R. and N. Ende (2000). "The potential for the use of mononuclear cells from human umbilical cord blood in the treatment of amyotrophic lateral sclerosis in SOD1 mice." J Med 31(1-2): 21-30.

Chierzi, S. and J. W. Fawcett (2001). "Regeneration in the mammalian optic nerve." Restor Neurol Neurosci 19(1-2): 109-18.

Choi, D. W. (1988). "Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage." Trends Neurosci 11(10): 465-9.

Choi, K. S., J. S. Shin, et al. (2005). "In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract." Biochem Biophys Res Commun 330(4): 1299-305.

Chu, K., M. Kim, et al. (2003). "Human neural stem cells can migrate, differentiate, and integrate after intravenous transplantation in adult rats with transient forebrain ischemia." Neurosci Lett 343(2): 129-33.

Chu, K., M. Kim, et al. (2004). Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. Brain Res. 1016: 145-53.

Clement, A. M., M. D. Nguyen, et al. (2003). "Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice." Science 302(5642): 113-7.

Codazzi, F., M. N. Teruel, et al. (2001). "Control of astrocyte Ca(2+) oscillations and waves by oscillating translocation and activation of protein kinase C." Curr Biol 11(14): 1089-97.

Coles, B. L., B. Angenieux, et al. (2004). "Facile isolation and the characterization of human retinal stem cells." Proc Natl Acad Sci U S A 101(44): 15772-7.

Colter, D. C., I. Sekiya, et al. (2001). "Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells." Proc Natl Acad Sci U S A 98(14): 7841-5.

Communi, D., V. Dewaste, et al. (1999). "Calcium-calmodulin-dependent protein kinase II and protein kinase C-mediated phosphorylation and activation of D-myo-inositol 1,4, 5-trisphosphate 3-kinase B in astrocytes." J Biol Chem 274(21): 14734-42.

Conti, L., S. M. Pollard, et al. (2005). "Niche-independent symmetrical self-renewal of a mammalian tissue stem cell." PLoS Biol 3(9): e283.

Cornell-Bell, A. H. and S. M. Finkbeiner (1991). "Ca2+ waves in astrocytes." Cell Calcium 12(2-3): 185-204.

Cornell-Bell, A. H., S. M. Finkbeiner, et al. (1990). "Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling." Science 247(4941): 470-3.

Corti, S., F. Locatelli, et al. (2004). "Wild-type bone marrow cells ameliorate the phenotype of SOD1-G93A ALS mice and contribute to CNS, heart and skeletal muscle tissues." Brain 127(Pt 11): 2518-32.

Corti, S., F. Locatelli, et al. (2007). "Neural stem cells LewisX+ CXCR4+ modify disease progression in an amyotrophic lateral sclerosis model." Brain 130(Pt 5): 1289-305.

Crols, R., J. Saerens, et al. (1986). "Increased GFAp levels in CSF as a marker of organicity in patients with Alzheimer's disease and other types of irreversible chronic organic brain syndrome." J Neurol 233(3): 157-60.

Cunha, R. A., E. Milusheva, et al. (1994). "Excitatory and inhibitory effects of A1 and A2A adenosine receptor activation on the electrically evoked [3H]acetylcholine release from different areas of the rat hippocampus." J Neurochem 63(1): 207-14.

da Silva Meirelles, L., P. C. Chagastelles, et al. (2006). "Mesenchymal stem cells reside in virtually all post-natal organs and tissues." J Cell Sci 119(Pt 11): 2204-13.

Daheron, L., S. L. Opitz, et al. (2004). "LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells." Stem Cells 22(5): 770-8.

Dani, J. W., A. Chernjavsky, et al. (1992). "Neuronal activity triggers calcium waves in hippocampal astrocyte networks." Neuron 8(3): 429-40.

D'Cruz, P. M., D. Yasumura, et al. (2000). "Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat." Hum Mol Genet 9(4): 645-51.

Deitmer, J. W., A. J. Verkhratsky, et al. (1998). "Calcium signalling in glial cells." Cell Calcium 24(5-6): 405-16.

Deng, W., M. Obrocka, et al. (2001). "In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP." Biochem Biophys Res Commun 282(1): 148-52.

Dickson, D. W., J. Farlo, et al. (1988). "Alzheimer's disease. A double-labeling immunohistochemical study of senile plaques." Am J Pathol 132(1): 86-101.

Digirolamo, C. M., D. Stokes, et al. (1999). "Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate." Br J Haematol 107(2): 275-81.

Dihne, M., C. Bernreuther, et al. (2006). "Embryonic stem cell-derived neuronally committed precursor cells with reduced teratoma formation after transplantation into the lesioned adult mouse brain." Stem Cells 24(6): 1458-66.

Donato, R. (1999). "Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type." Biochim Biophys Acta 1450(3): 191-231.

Donato, R. (2003). "Intracellular and extracellular roles of S100 proteins." Microsc Res Tech 60(6): 540-51.

Donnenberg, V. S., J. D. Luketich, et al. (2006). "Tumorigenic epithelial stem cells and their normal counterparts." Ernst Schering Found Symp Proc 5: 245-63.

Draper, J. S. and V. Fox (2003). "Human embryonic stem cells: multilineage differentiation and mechanisms of self-renewal." Arch Med Res 34(6): 558-64.

Draper, J. S., C. Pigott, et al. (2002). "Surface antigens of human embryonic stem cells: changes upon differentiation in culture." J Anat 200(Pt 3): 249-58.

Dreyer, E. B. and C. L. Grosskreutz (1997). "Excitatory mechanisms in retinal ganglion cell death in primary open angle glaucoma (POAG)." Clin Neurosci 4(5): 270-3.

Dreyer, E. B., D. Zurakowski, et al. (1996). "Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma." Arch Ophthalmol 114(3): 299-305.

Drukker, M. (2004). "Immunogenicity of human embryonic stem cells: can we achieve tolerance?" Springer Semin Immunopathol 26(1-2): 201-13.

Du, Y., C. M. Miller, et al. (2003). "Hyperglycemia increases mitochondrial superoxide in retina and retinal cells." Free Radic Biol Med 35(11): 1491-9.

Du, Y., V. P. Sarthy, et al. (2004). "Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats." Am J Physiol Regul Integr Comp Physiol 287(4): R735-41.

Du, Y., M. A. Smith, et al. (2002). "Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine." J Neurochem 80(5): 771-9.

Dunnett, S. B. and A. E. Rosser (2007). "Stem cell transplantation for Huntington's disease." Exp Neurol 203(2): 279-92.

Eisenfeld, A. J., A. H. Bunt-Milam, et al. (1984). "Muller cell expression of glial fibrillary acidic protein after genetic and experimental photoreceptor degeneration in the rat retina." Invest Ophthalmol Vis Sci 25(11): 1321-8.

Ekholm, E., K. D. Hankenson, et al. (2002). "Diminished callus size and cartilage synthesis in alpha 1 beta 1 integrin-deficient mice during bone fracture healing." Am J Pathol 160(5): 1779-85.

Ende, N. and R. Chen (2001). "Human umbilical cord blood cells ameliorate Huntington's disease in transgenic mice." J Med 32(3-4): 231-40.

Eng, L. F., R. S. Ghirnikar, et al. (2000). "Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000)." Neurochem Res 25(9-10): 1439-51.

Esser, P., M. Weller, et al. (1992). "The effects of basic fibroblast growth factor on bovine retinal pigment epithelium in vitro." Ger J Ophthalmol 1(1): 58-61.

Evans, M. and M. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature 292: 154-56.

Evans, T. S. (2004). "Complex Networks." Contemporary Physics 1: 1-31.

Fan, W., N. Lin, et al. (1996). "Muller and RPE cell response to photoreceptor cell degeneration in aging Fischer rats." Exp Eye Res 63(1): 9-18.

Fang, D., K. Leishear, et al. (2006). "Defining the conditions for the generation of melanocytes from human embryonic stem cells." Stem Cells 24(7): 1668-77.

Fanselow, M. S. and A. M. Poulos (2005). "The neuroscience of mammalian associative learning." Annu Rev Psychol 56: 207-34.

Faude, F., M. Francke, et al. (2001). "Experimental retinal detachment causes widespread and multilayered degeneration in rabbit retina." J Neurocytol 30(5): 379-90.

Ferri, C. P., M. Prince, et al. (2005). "Global prevalence of dementia: a Delphi consensus study." The Lancet 366(9503): 2112-2117.

Fiacco, T. A. and K. D. McCarthy (2006). "Astrocyte calcium elevations: properties, propagation, and effects on brain signaling." Glia 54(7): 676-90.

Fisher, S. K., G. P. Lewis, et al. (2005). "Cellular remodeling in mammalian retina: results from studies of experimental retinal detachment." Prog Retin Eye Res 24(3): 395-431.

Flax, J. D., S. Aurora, et al. (1998). "Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes." Nat Biotechnol 16(11): 1033-9.

Forni, C., P. Brundin, et al. (1989). "Time-course of recovery of dopamine neuron activity during reinnervation of the denervated striatum by fetal mesencephalic grafts as assessed by in vivo voltammetry." Exp Brain Res 76(1): 75-87.

Francke, M., S. Uhlmann, et al. (2003). "Experimental dispase-induced retinopathy causes up-regulation of P2Y receptor-mediated calcium responses in Muller glial cells." Ophthalmic Res 35(1): 30-41.

Francke, M., M. Weick, et al. (2002). "Upregulation of extracellular ATP-induced Muller cell responses in a dispase model of proliferative vitreoretinopathy." Invest Ophthalmol Vis Sci 43(3): 870-81.

Franke, H., U. Krugel, et al. (1999). "P2 receptor-mediated proliferative effects on astrocytes in vivo." Glia 28(3): 190-200.

Freeman, T. B., R. A. Hauser, et al. (2000). "Neural transplantation for the treatment of Huntington's disease." Prog Brain Res 127: 405-11.

Fries, J. E., I. M. Goczalik, et al. (2005). "Identification of P2Y receptor subtypes in human muller glial cells by physiology, single cell RT-PCR, and immunohistochemistry." Invest Ophthalmol Vis Sci 46(8): 3000-7.

Frohlich, R., S. Boehm, et al. (1996). "Pharmacological characterization of P2 purinoceptor types in rat locus coeruleus neurons." Eur J Pharmacol 315(3): 255-61.

Fumagalli, M., R. Brambilla, et al. (2003). "Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors." Glia 43(3): 218-03.

Gadea, A., E. Lopez, et al. (2002). "Role of Ca2+ and calmodulin-dependent enzymes in the regulation of glycine transport in Muller glia." J Neurochem 80(4): 634-45.

Gage, F. H. (2000). "Mammalian neural stem cells." Science 287(5457): 1433-8.

Gamm, D. M., S. Wang, et al. (2007). "Protection of visual functions by human neural progenitors in a rat model of retinal disease." PLoS ONE 2(3): e338.

Garbuglia, M., M. Verzini, et al. (1998). "Annexin VI binds S100A1 and S100B and blocks the ability of S100A1 and S100B to inhibit desmin and GFAP assemblies into intermediate filaments." Cell Calcium 24(3): 177-91.

Garbuzova-Davis, S., A. E. Willing, et al. (2003). "Intravenous administration of human umbilical cord blood cells in a mouse model of amyotrophic lateral sclerosis: distribution, migration, and differentiation." J Hematother Stem Cell Res 12(3): 255-70.

Gee, K. R., K. A. Brown, et al. (2000). "Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes." Cell Calcium 27(2): 97-106.

Gitter, B. D., L. M. Cox, et al. (1995). "Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells." Proc Natl Acad Sci U S A 92(23): 10738-41.

Glinsky, G. V., O. Berezovska, et al. (2005). "Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer." J Clin Invest 115(6): 1503-21.

Gobel, W., B. M. Kampa, et al. (2007). "Imaging cellular network dynamics in three dimensions using fast 3D laser scanning." Nat Methods 4(1): 73-9.

Gorji, A. (2001). "Spreading depression: a review of the clinical relevance." Brain Res Brain Res Rev 38(1-2): 33-60.

Gorji, A., D. Scheller, et al. (2001). "Spreading depression in human neocortical slices." Brain Res 906(1-2): 74-83.

Green, H., K. Easley, et al. (2003). "Marker succession during the development of keratinocytes from cultured human embryonic stem cells." Proc Natl Acad Sci U S A 100(26): 15625-30.

Griffin, W. S., L. C. Stanley, et al. (1989). "Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease." Proc Natl Acad Sci U S A 86(19): 7611-5.

Gritti, A., E. A. Parati, et al. (1996). "Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor." J Neurosci 16(3): 1091-100.

Gustafson, E. C., E. R. Stevens, et al. (2007). "Endogenous D-serine contributes to NMDA-receptor-mediated light-evoked responses in the vertebrate retina." J Neurophysiol 98(1): 122-30.

Haas, B., C. G. Schipke, et al. (2006). "Activity-dependent ATP-waves in the mouse neocortex are independent from astrocytic calcium waves." Cereb Cortex 16(2): 237-46.

Hadjikhani, N., M. Sanchez Del Rio, et al. (2001). "Mechanisms of migraine aura revealed by functional MRI in human visual cortex." Proc Natl Acad Sci U S A 98(8): 4687-92.

Hamilton, N., S. Vayro, et al. (2008). "Mechanisms of ATP- and glutamate-mediated calcium signaling in white matter astrocytes." Glia 56(7): 734-49.

Hardy, J. and D. J. Selkoe (2002). "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics." Science 297(5580): 353-6.

Haughey, N. J. and M. P. Mattson (2003). "Alzheimer's amyloid beta-peptide enhances ATP/gap junction-mediated calcium-wave propagation in astrocytes." Neuromolecular Med 3(3): 173-80.

Haydon, P. G. (2001). "GLIA: listening and talking to the synapse." Nat Rev Neurosci 2(3): 185-93.

Henderson, J. K., J. S. Draper, et al. (2002). "Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens." Stem Cells 20(4): 329-37.

Hermann, A., R. Gastl, et al. (2004). "Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells." J Cell Sci 117(Pt 19): 4411-22.

Hirano, M., A. Yamamoto, et al. (2003). "Generation of structures formed by lens and retinal cells differentiating from embryonic stem cells." Dev Dyn 228(4): 664-71.

Hirase, H., L. Qian, et al. (2004). "Calcium dynamics of cortical astrocytic networks in vivo." PLoS Biol 2(4): E96.

Ho, M., D. Yu, et al. (2006). "Comparison of standard surface chemistries for culturing mesenchymal stem cells prior to neural differentiation." Biomaterials 27(24): 4333-9.

Hochedlinger, K. and R. Jaenisch (2006). "Nuclear reprogramming and pluripotency." Nature 441(7097): 1061-7.

Humphrey, R. K., G. M. Beattie, et al. (2004). "Maintenance of pluripotency in human embryonic stem cells is STAT3 independent." Stem Cells 22(4): 522-30.

Iacobas, D. A., S. O. Suadicani, et al. (2007). "Gap junction and purinergic P2 receptor proteins as a functional unit: insights from transcriptomics." J Membr Biol 217(1-3): 83-91.

Iandiev, I., O. Uckermann, et al. (2006). "Glial cell reactivity in a porcine model of retinal detachment." Invest Ophthalmol Vis Sci 47(5): 2161-71.

Ida, T., M. Hara, et al. (2008). "Cytokine-induced enhancement of calcium-dependent glutamate release from astrocytes mediated by nitric oxide." Neuroscience Letters 432(3): 232-236.

Ikeda, N., N. Nonoguchi, et al. (2005). "Bone marrow stromal cells that enhanced fibroblast growth factor-2 secretion by herpes simplex virus vector improve neurological outcome after transient focal cerebral ischemia in rats." Stroke 36(12): 2725-30.

Imai, D., S. Yoneya, et al. (2005). "Intraocular gene transfer of pigment epithelium-derived factor rescues photoreceptors from light-induced cell death." J Cell Physiol 202(2): 570-8.

Imitola, J., K. Raddassi, et al. (2004). "Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway." Proc Natl Acad Sci U S A 101(52): 18117-22.

Ishibashi, S., M. Sakaguchi, et al. (2004). "Human neural stem/progenitor cells, expanded in long-term neurosphere culture, promote functional recovery after focal ischemia in Mongolian gerbils." J Neurosci Res 78(2): 215-23.

Iuchi, S., S. Dabelsteen, et al. (2006). "Immortalized keratinocyte lines derived from human embryonic stem cells." Proc Natl Acad Sci U S A 103(6): 1792-7.

Jain, M., R. J. Armstrong, et al. (2003). "Migration and differentiation of transplanted human neural precursor cells." Neuroreport 14(9): 1257-62.

Jain, M., R. J. Armstrong, et al. (2003). "GABAergic immunoreactivity is predominant in neurons derived from expanded human neural precursor cells in vitro." Exp Neurol 182(1): 113-23.

Jaiswal, N., S. E. Haynesworth, et al. (1997). "Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro." J Cell Biochem 64(2): 295-312.

Javazon, E. H., D. C. Colter, et al. (2001). "Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells." Stem Cells 19(3): 219-25.

Jefferys, J. G. (2003). "Models and mechanisms of experimental epilepsies." Epilepsia 44 Suppl 12: 44-50.

Jiang, Y., B. N. Jahagirdar, et al. (2002). "Pluripotency of mesenchymal stem cells derived from adult marrow." Nature 418(6893): 41-9.

Jimenez, A. I., E. Castro, et al. (2000). "Coexpression of several types of metabotropic nucleotide receptors in single cerebellar astrocytes." J Neurochem 75(5): 2071-9.

Johe, K. K., T. G. Hazel, et al. (1996). "Single factors direct the differentiation of stem cells from the fetal and adult central nervous system." Genes Dev 10(24): 3129-40.

Johnstone, M., A. J. Gearing, et al. (1999). "A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced." J Neuroimmunol 93(1-2): 182-93.

Jorgensen, O. S., B. W. Brooksbank, et al. (1990). "Neuronal plasticity and astrocytic reaction in Down syndrome and Alzheimer disease." J Neurol Sci 98(1): 63-79.

Julien, J. P. (2007). "ALS: astrocytes move in as deadly neighbors." Nat Neurosci 10(5): 535-7.

Kandel, E., J. Schwartz, et al. (2000). Principles of Neural Science, McGraw-Hill.

Kang, J., L. Jiang, et al. (1998). "Astrocyte-mediated potentiation of inhibitory synaptic transmission." Nat Neurosci 1(8): 683-92.

Kannan, R., Y. Bao, et al. (1999). "Protection from oxidant injury by sodium-dependent GSH uptake in retinal Muller cells." Exp Eye Res 68(5): 609-16.

Karp, J. M., L. S. Ferreira, et al. (2006). "Cultivation of human embryonic stem cells without the embryoid body step enhances osteogenesis in vitro." Stem Cells 24(4): 835-43.

Kaufman, D. S., E. T. Hanson, et al. (2001). "Hematopoietic colony-forming cells derived from human embryonic stem cells." Proc Natl Acad Sci U S A 98(19): 10716-21.

Keirstead, S. A. and R. F. Miller (1995). "Calcium waves in dissociated retinal glial (Muller) cells are evoked by release of calcium from intracellular stores." Glia 14(1): 14-22.

Keirstead, S. A. and R. F. Miller (1997). "Metabotropic glutamate receptor agonists evoke calcium waves in isolated Muller cells." Glia 21(2): 194-203.

Kelley, A. E. (2004). "Memory and addiction: shared neural circuitry and molecular mechanisms." Neuron 44(1): 161-79.

Kelly, C. M., P. Tyers, et al. (2005). "EGF and FGF-2 responsiveness of rat and mouse neural precursors derived from the embryonic CNS." Brain Res Bull 68(1-2): 83-94.

Khachaturian, Z. S. (1994). "Calcium hypothesis of Alzheimer's disease and brain aging." Ann N Y Acad Sci 747: 1-11.

Kicic, A., W. Y. Shen, et al. (2003). "Differentiation of marrow stromal cells into photoreceptors in the rat eye." J Neurosci 23(21): 7742-9.

Kihara, A. H., L. Mantovani de Castro, et al. (2006). "Expression of connexins 36, 43, and 45 during postnatal development of the mouse retina." J Neurobiol 66(13): 1397-410.

Kim, D. S., J. Y. Kim, et al. (2007). "Derivation of functional dopamine neurons from embryonic stem cells." Cell Transplant 16(2): 117-23.

Kim, J. H., J. M. Auerbach, et al. (2002). "Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease." Nature 418(6893): 50-6.

Kitchens, D. L., E. Y. Snyder, et al. (1994). "FGF and EGF are mitogens for immortalized neural progenitors." J Neurobiol 25(7): 797-807.

Klees, R. F., R. M. Salasznyk, et al. (2005). "Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway." Mol Biol Cell 16(2): 881-90.

Klein, S. M., S. Behrstock, et al. (2005). "GDNF delivery using human neural progenitor cells in a rat model of ALS." Hum Gene Ther 16(4): 509-21.

Klimanskaya, I., J. Hipp, et al. (2004). "Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics." Cloning Stem Cells 6(3): 217-45.

Kodal, H., M. Weick, et al. (2000). "Involvement of calcium-activated potassium channels in the regulation of DNA synthesis in cultured Muller glial cells." Invest Ophthalmol Vis Sci 41(13): 4262-7.

Kohno, H., T. Sakai, et al. (2006). "Induction of nestin, Ki-67, and cyclin D1 expression in Muller cells after laser injury in adult rat retina." Graefes Arch Clin Exp Ophthalmol 244(1): 90-5.

Kohyama, J., H. Abe, et al. (2001). "Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent." Differentiation 68(4-5): 235-44.

Kopen, G. C., D. J. Prockop, et al. (1999). "Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains." Proc Natl Acad Sci U S A 96(19): 10711-6.

Kotobuki, N., M. Hirose, et al. (2004). "Cultured autologous human cells for hard tissue regeneration: preparation and characterization of mesenchymal stem cells from bone marrow." Artif Organs 28(1): 33-9.

Kumaria, A., C. M. Tolias, et al. (2008). "ATP signalling in epilepsy." Purinergic Signal.

Kunkler, P. E. and R. P. Kraig (1998). "Calcium waves precede electrophysiological changes of spreading depression in hippocampal organ cultures." J Neurosci 18(9): 3416-25.

Kurozumi, K., K. Nakamura, et al. (2005). "Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model." Mol Ther 11(1): 96-104.

LaFerla, F. M. (2002). "Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease." Nat Rev Neurosci 3(11): 862-72.

Lange, C., J. Schroeder, et al. (2005). "High-potential human mesenchymal stem cells." Stem Cells Dev 14(1): 70-80.

Laslett, A. L., A. A. Filipczyk, et al. (2003). "Characterization and culture of human embryonic stem cells." Trends Cardiovasc Med 13(7): 295-301.

Lee, H. J., K. S. Kim, et al. (2007). "Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model." PLoS ONE 2(1): e156.

Lendahl, U. (1997). "Gene regulation in the formation of the central nervous system." Acta Paediatr Suppl 422: 8-11.

Lescaudron, L., D. Unni, et al. (2003). "Autologous adult bone marrow stem cell transplantation in an animal model of huntington's disease: behavioral and morphological outcomes." Int J Neurosci 113(7): 945-56.

Levenberg, S., J. S. Golub, et al. (2002). "Endothelial cells derived from human embryonic stem cells." Proc Natl Acad Sci U S A 99(7): 4391-6.

Li, X. and R. H. Carter (1998). "Convergence of CD19 and B cell antigen receptor signals at MEK1 in the ERK2 activation cascade." J Immunol 161(11): 5901-8.

Li, Y., S. W. Barger, et al. (2000). "S100beta induction of the proinflammatory cytokine interleukin-6 in neurons." J Neurochem 74(1): 143-50.

Li, Y., L. A. Holtzclaw, et al. (2001). "Muller cell Ca2+ waves evoked by purinergic receptor agonists in slices of rat retina." J Neurophysiol 85(2): 986-94.

Libby, R. T., M. F. Champliaud, et al. (2000). "Laminin expression in adult and developing retinae: evidence of two novel CNS laminins." J Neurosci 20(17): 6517-28.

Libby, R. T., D. D. Hunter, et al. (1996). "Developmental expression of laminin beta 2 in rat retina. Further support for a role in rod morphogenesis." Invest Ophthalmol Vis Sci 37(8): 1651-61.

Lin, D., J. Najbauer, et al. (2007). "Novel method for visualizing and modeling the spatial distribution of neural stem cells within intracranial glioma." Neuroimage 37 Suppl 1: S18-26.

Lindvall, O. and A. Bjorklund (2004). "Cell therapy in Parkinson's disease." NeuroRx 1(4): 382-93.

Liu, Y. and M. Wakakura (1998). "P1-/P2-purinergic receptors on cultured rabbit retinal Muller cells." Jpn J Ophthalmol 42(1): 33-40.

Lopez-Colome, A. M. and I. Lee (1996). "Pharmacological characterization of inositol-1,4,5,-trisphosphate binding to membranes from retina and retinal cultures." J Neurosci Res 44(2): 149-56.

Lu, P., L. L. Jones, et al. (2003). "Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury." Exp Neurol 181(2): 115-29.

Lu, S. C., Y. Bao, et al. (1999). "Regulation of gamma-glutamylcysteine synthetase subunit gene expression in retinal Muller cells by oxidative stress." Invest Ophthalmol Vis Sci 40(8): 1776-82.

Luckenbill-Edds, L. (1997). "Laminin and the mechanism of neuronal outgrowth." Brain Res Brain Res Rev 23(1-2): 1-27.

Lund, R. D., S. Wang, et al. (2006). "Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats." Cloning Stem Cells 8(3): 189-99.

Lund, R. D., S. Wang, et al. (2007). "Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease." Stem Cells 25(3): 602-11.

Lytton, J., M. Westlin, et al. (1991). "Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps." J Biol Chem 266(26): 17067-71.

Ma, K. C., Z. R. Ye, et al. (1988). "Glial fibrillary acidic protein immunohistochemical study of Alzheimer I & II astrogliosis in Wilson's disease." Acta Neurol Scand 78(4): 290-6.

Mackay, A. M., S. C. Beck, et al. (1998). "Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow." Tissue Eng 4(4): 415-28.

MacLaren, R. E., R. A. Pearson, et al. (2006). "Retinal repair by transplantation of photoreceptor precursors." Nature 444(7116): 203-7.

Madigan, M. C., P. L. Penfold, et al. (1994). "Intermediate filament expression in human retinal macroglia. Histopathologic changes associated with age-related macular degeneration." Retina 14(1): 65-74.

Mandybur, T. I. and C. C. Chuirazzi (1990). "Astrocytes and the plaques of Alzheimer's disease." Neurology 40(4): 635-9.

Marcet, B., V. Chappe, et al. (2004). "Pharmacological and signaling properties of endogenous P2Y1 receptors in cystic fibrosis transmembrane conductance regulator-expressing Chinese hamster ovary cells." J Pharmacol Exp Ther 309(2): 533-9.

Mark, R. J., K. Hensley, et al. (1995). "Amyloid beta-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca2+ homeostasis and cell death." J Neurosci 15(9): 6239-49.

Marteau, F., E. Le Poul, et al. (2003). "Pharmacological characterization of the human P2Y13 receptor." Mol Pharmacol 64(1): 104-12.

Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proc Natl Acad Sci U S A 78(12): 7634-8.

Martinat, C., J. J. Bacci, et al. (2006). "Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype." Proc Natl Acad Sci U S A 103(8): 2874-9.

Martins-Ferreira, H., M. Nedergaard, et al. (2000). "Perspectives on spreading depression." Brain Res Brain Res Rev 32(1): 215-34.

Masland, R. H. (2001). "Neuronal diversity in the retina." Curr Opin Neurobiol 11(4): 431-6.

Masland, R. H. (2004). "Neuronal cell types." Curr Biol 14(13): R497-500.

Mattson, M. P. and R. E. Rydel (1992). "beta-Amyloid precursor protein and Alzheimer's disease: the peptide plot thickens." Neurobiol Aging 13(5): 617-21.

Mazzini, L., F. Fagioli, et al. (2003). "Stem cell therapy in amyotrophic lateral sclerosis: a methodological approach in humans." Amyotroph Lateral Scler Other Motor Neuron Disord 4(3): 158-61.

Mazzini, L., K. Mareschi, et al. (2007). "Stem cell treatment in Amyotrophic Lateral Sclerosis." J Neurol Sci.

McBride, J. L., S. P. Behrstock, et al. (2004). "Human neural stem cell transplants improve motor function in a rat model of Huntington's disease." J Comp Neurol 475(2): 211-9.

McCulloch, E. A. and J. E. Till (1971). "Effects of short-term culture on populations of hemopoietic progenitor cells from mouse marrow." Cell Tissue Kinet 4(1): 11-20.

Meda, L., P. Baron, et al. (2001). "Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins." Neurobiol Aging 22(6): 885-93.

Mehes, E., A. Czirok, et al. (2002). "Laminin-1 increases motility, path-searching, and process dynamism of rat and mouse Muller glial cells in vitro: implication of relationship between cell behavior and formation of retinal morphology." Cell Motil Cytoskeleton 53(3): 203-13.

Mehler, M. F. and J. A. Kessler (1999). "Progenitor cell biology: implications for neural regeneration." Arch Neurol 56(7): 780-4.

Meske, V., U. Hamker, et al. (1998). "The effects of beta/A4-amyloid and its fragments on calcium homeostasis, glial fibrillary acidic protein and S100beta staining, morphology and survival of cultured hippocampal astrocytes." Neuroscience 85(4): 1151-60.

Metea, M. R. and E. A. Newman (2006). "Calcium signaling in specialized glial cells." Glia 54(7): 650-5.

Milenkovic, I., M. Weick, et al. (2003). "P2Y receptor-mediated stimulation of Muller glial cell DNA synthesis: dependence on EGF and PDGF receptor transactivation." Invest Ophthalmol Vis Sci 44(3): 1211-20.

Miller, R. H. (2006). "The promise of stem cells for neural repair." Brain Res 1091(1): 258-64.

Moll, V., M. Weick, et al. (2002). "P2Y receptor-mediated stimulation of Muller glial DNA synthesis." Invest Ophthalmol Vis Sci 43(3): 766-73.

Molyneux, G., J. Regan, et al. (2007). "Mammary stem cells and breast cancer." Cell Mol Life Sci.

Muller, U., D. Wang, et al. (1997). "Integrin alpha8beta1 is critically important for epithelial-mesenchymal interactions during kidney morphogenesis." Cell 88(5): 603-13.

Munshi, R., I. H. Pang, et al. (1991). "A1 adenosine receptors of bovine brain couple to guanine nucleotide-binding proteins Gi1, Gi2, and Go." J Biol Chem 266(33): 22285-9.

Muraglia, A., R. Cancedda, et al. (2000). "Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model." J Cell Sci 113 (Pt 7): 1161-6.

Nagy, A., J. Rossant, et al. (1993). "Derivation of completely cell culture-derived mice from early-passage embryonic stem cells." Proc Natl Acad Sci U S A 90(18): 8424-8.

Nedergaard, M. (1994). "Direct signaling from astrocytes to neurons in cultures of mammalian brain cells." Science 263(5154): 1768-71.

Nedergaard, M., A. J. Cooper, et al. (1995). "Gap junctions are required for the propagation of spreading depression." J Neurobiol 28(4): 433-44.

Nett, W. J., S. H. Oloff, et al. (2002). "Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity." J Neurophysiol 87(1): 528-37.

Neuhuber, B., G. Gallo, et al. (2004). "Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype." J Neurosci Res 77(2): 192-204.

Newman, E. and A. Reichenbach (1996). "The Muller cell: a functional element of the retina." Trends Neurosci 19(8): 307-12.

Newman, E. A. (1987). "Regulation of potassium levels by Muller cells in the vertebrate retina." Can J Physiol Pharmacol 65(5): 1028-32.

Newman, E. A. (2001). "Propagation of intercellular calcium waves in retinal astrocytes and Muller cells." J Neurosci 21(7): 2215-23.

Newman, E. A. (2003). "Glial cell inhibition of neurons by release of ATP." J Neurosci 23(5): 1659-66.

Newman, E. A. (2004). "Glial modulation of synaptic transmission in the retina." Glia 47(3): 268-74.

Newman, E. A. (2005). "Calcium increases in retinal glial cells evoked by light-induced neuronal activity." J Neurosci 25(23): 5502-10.

Newman, E. A. and K. R. Zahs (1997). "Calcium waves in retinal glial cells." Science 275(5301): 844-7.

Newman, E. A. and K. R. Zahs (1998). "Modulation of neuronal activity by glial cells in the retina." J Neurosci 18(11): 4022-8.

Nikkhah, G., C. Bentlage, et al. (1994). "Intranigral fetal dopamine grafts induce behavioral compensation in the rat Parkinson model." J Neurosci 14(6): 3449-61.

Ninomiya, M., T. Yamashita, et al. (2006). "Enhanced neurogenesis in the ischemic striatum following EGF-induced expansion of transit-amplifying cells in the subventricular zone." Neurosci Lett 403(1-2): 63-7.

Nobile, M., I. Monaldi, et al. (2003). "ATP-induced, sustained calcium signalling in cultured rat cortical astrocytes: evidence for a non-capacitative, P2X7-like-mediated calcium entry." FEBS Lett 538(1-3): 71-6.

Okano, H. (2002). "Stem cell biology of the central nervous system." J Neurosci Res 69(6): 698-707.

Ourednik, J., V. Ourednik, et al. (2002). "Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons." Nat Biotechnol 20(11): 1103-10.

Ourednik, V., J. Ourednik, et al. (2000). "Neural stem cells are uniquely suited for cell replacement and gene therapy in the CNS." Novartis Found Symp 231: 242-62; discussion 262-9, 302-6.

Palmer, T. D., J. Takahashi, et al. (1997). "The adult rat hippocampus contains primordial neural stem cells." Mol Cell Neurosci 8(6): 389-404.

Pannicke, T., W. Fischer, et al. (2000). "P2X7 receptors in Muller glial cells from the human retina." J Neurosci 20(16): 5965-72.

Park, C. H., Y. K. Minn, et al. (2005). "In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons." J Neurochem 92(5): 1265-76.

Park, K. I., B. T. Himes, et al. (2006). "Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: Evidence from engraftment of Neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury." Exp Neurol 199(1): 179-90.

Parri, H. R. and V. Crunelli (2003). "The role of Ca2+ in the generation of spontaneous astrocytic Ca2+ oscillations." Neuroscience 120(4): 979-92.

Passier, R., C. Denning, et al. (2006). "Cardiomyocytes from human embryonic stem cells." Handb Exp Pharmacol(174): 101-22.

Pasti, L., A. Volterra, et al. (1997). "Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ." J Neurosci 17(20): 7817-30.

Pearce, B. and S. Murphy (1993). "Protein kinase C down-regulation in astrocytes: differential effects on agonist-stimulated inositol phosphate accumulation." Neurochem Int 23(5): 407-12.

Pekny, M. and M. Nilsson (2005). "Astrocyte activation and reactive gliosis." Glia 50(4): 427-34.

Perea, G. and A. Araque (2005). "Glial calcium signaling and neuron-glia communication." Cell Calcium Frontiers in calcium signalling 38(3-4): 375-382.

Perrier, A. L., V. Tabar, et al. (2004). "Derivation of midbrain dopamine neurons from human embryonic stem cells." Proc Natl Acad Sci U S A 101(34): 12543-8.

Peters, O., C. G. Schipke, et al. (2003). "Different mechanisms promote astrocyte Ca2+ waves and spreading depression in the mouse neocortex." J Neurosci 23(30): 9888-96.

Philpott, L. M., O. V. Kopyov, et al. (1997). "Neuropsychological functioning following fetal striatal transplantation in Huntington's chorea: three case presentations." Cell Transplant 6(3): 203-12.

Pilat, S., S. Carotta, et al. (2005). "HOXB4 enforces equivalent fates of ES-cell-derived and adult hematopoietic cells." Proc Natl Acad Sci U S A 102(34): 12101-6.

Pisati, F., P. Bossolasco, et al. (2007). "Induction of neurotrophin expression via human adult mesenchymal stem cells: implication for cell therapy in neurodegenerative diseases." Cell Transplant 16(1): 41-55.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science 284(5411): 143-7.

Pluchino, S., L. Zanotti, et al. (2005). "Neural stem cells and their use as therapeutic tool in neurological disorders." Brain Res Brain Res Rev 48(2): 211-9.

Porter, J. T. and K. D. McCarthy (1996). "Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals." J Neurosci 16(16): 5073-81.

Powell, S. K., J. Rao, et al. (2000). "Neural cell response to multiple novel sites on laminin-1." J Neurosci Res 61(3): 302-12.

Prockop, D. J. (1997). "Marrow stromal cells as stem cells for nonhematopoietic tissues." Science 276(5309): 71-4.

Qian, L. and W. M. Saltzman (2004). "Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification." Biomaterials 25(7-8): 1331-7.

Rana, S. and R. Dringen (2007). "Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes." Neurosci Lett 415(1): 45-8.

Reifel Saltzberg, J. M., K. A. Garvey, et al. (2003). "Pharmacological characterization of P2Y receptor subtypes on isolated tiger salamander Muller cells." Glia 42(2): 149-59.

Reubinoff, B. E., P. Itsykson, et al. (2001). "Neural progenitors from human embryonic stem cells." Nat Biotechnol 19(12): 1134-40.

Reubinoff, B. E., M. F. Pera, et al. (2000). "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro." Nat Biotechnol 18(4): 399-404.

Reynolds, B. A. and R. L. Rietze (2005). "Neural stem cells and neurospheres--re-evaluating the relationship." Nat Methods 2(5): 333-6.

Ribelayga, C. and S. C. Mangel (2005). "A circadian clock and light/dark adaptation differentially regulate adenosine in the mammalian retina." J Neurosci 25(1): 215-22.

Rice, C. M. and N. J. Scolding (2007). "Autologous bone marrow stem cells - properties and advantages." J Neurol Sci.

Rietze, R. L. and B. A. Reynolds (2006). "Neural stem cell isolation and characterization." Methods Enzymol 419: 3-23.

Robaye, B., J. M. Boeynaems, et al. (1997). "Slow desensitization of the human P2Y6 receptor." Eur J Pharmacol 329(2-3): 231-6.

Rodriguez, J. P., M. Gonzalez, et al. (2004). "Cytoskeletal organization of human mesenchymal stem cells (MSC) changes during their osteogenic differentiation." J Cell Biochem 93(4): 721-31.

Rogers, K. L., J. Stinnakre, et al. (2005). "Visualization of local Ca2+ dynamics with genetically encoded bioluminescent reporters." Eur J Neurosci 21(3): 597-610.

Roitberg, B., N. Khan, et al. (2003). "Chronic ischemic stroke model in cynomolgus monkeys: behavioral, neuroimaging and anatomical study." Neurol Res 25(1): 68-78.

Rossi, F. and E. Bianchini (1996). "Synergistic induction of nitric oxide by beta-amyloid and cytokines in astrocytes." Biochem Biophys Res Commun 225(2): 474-8.

Roy, N. S., C. Cleren, et al. (2006). "Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes." Nat Med 12(11): 1259-68.

Samadikuchaksaraei, A., S. Cohen, et al. (2006). "Derivation of distal airway epithelium from human embryonic stem cells." Tissue Eng 12(4): 867-75.

Sanchez-Ramos, J., S. Song, et al. (2000). "Adult bone marrow stromal cells differentiate into neural cells in vitro." Exp Neurol 164(2): 247-56.

Sarthy, V. P., S. J. Brodjian, et al. (1998). "Establishment and characterization of a retinal Muller cell line." Invest Ophthalmol Vis Sci 39(1): 212-6.

Schachter, J. B., Q. Li, et al. (1996). "Second messenger cascade specificity and pharmacological selectivity of the human P2Y1-purinoceptor." Br J Pharmacol 118(1): 167-73.

Schipke, C. G. and H. Kettenmann (2004). "Astrocyte responses to neuronal activity." Glia 47(3): 226-32.

Schopf, S., H. Ruge, et al. (2004). "Switch of K+ buffering conditions in rabbit retinal Muller glial cells during postnatal development." Neurosci Lett 365(3): 167-70.

Schubert, P., T. Ogata, et al. (1997). "Protective mechanisms of adenosine in neurons and glial cells." Ann N Y Acad Sci 825: 1-10.

Schuldiner, M., R. Eiges, et al. (2001). "Induced neuronal differentiation of human embryonic stem cells." Brain Res 913(2): 201-5.

Schuldiner, M., O. Yanuka, et al. (2000). "Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells." Proc Natl Acad Sci U S A 97(21): 11307-12.

Schummers, J., J. Marino, et al. (2004). "Local networks in visual cortex and their influence on neuronal responses and dynamics." J Physiol Paris 98(4-6): 429-41.

Schummers, J., J. Sharma, et al. (2005). "Bottom-up and top-down dynamics in visual cortex." Prog Brain Res 149: 65-81.

Sekiya, I., B. L. Larson, et al. (2002). "Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality." Stem Cells 20(6): 530-41.

Selinfreund, R. H., S. W. Barger, et al. (1991). "Neurotrophic protein S100 beta stimulates glial cell proliferation." Proc Natl Acad Sci U S A 88(9): 3554-8.

Sharp, A. H., F. C. Nucifora, Jr., et al. (1999). "Differential cellular expression of isoforms of inositol 1,4,5-triphosphate receptors in neurons and glia in brain." J Comp Neurol 406(2): 207-20.

Shelton, M. D., T. S. Kern, et al. (2007). "Glutaredoxin regulates nuclear factor kappa-B and intercellular adhesion molecule in Muller cells: Model of diabetic retinopathy." J Biol Chem.

Sheng, J. G., R. E. Mrak, et al. (1994). "S100 beta protein expression in Alzheimer disease: potential role in the pathogenesis of neuritic plaques." J Neurosci Res 39(4): 398-404.

Shihabuddin, L. S., T. D. Palmer, et al. (1999). "The search for neural progenitor cells: prospects for the therapy of neurodegenerative disease." Mol Med Today 5(11): 474-80.

Shirahashi, H., J. Wu, et al. (2004). "Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage." Cell Transplant 13(3): 197-211.

Silva, G. A., C. Czeisler, et al. (2004). "Selective differentiation of neural progenitor cells by high-epitope density nanofibers." Science 303(5662): 1352-5.

Silva, G. A., C. Feeney, et al. (1998). "A novel and rapid method for culturing pure rat spinal cord astrocytes on untreated glass." J Neurosci Methods 80(1): 75-9.

Simpson, J. E., P. G. Ince, et al. (2008). "Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain." Neurobiol Aging.

Singec, I., R. Jandial, et al. (2007). "The leading edge of stem cell therapeutics." Annu Rev Med 58: 313-28.

Singec, I., R. Knoth, et al. (2006). "Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology." Nat Methods 3(10): 801-6.

Smith, A. G., J. K. Heath, et al. (1988). "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides." Nature 336(6200): 688-90.

Smith, I. F., K. N. Green, et al. (2005). "Calcium dysregulation in Alzheimer's disease: recent advances gained from genetically modified animals." Cell Calcium 38(3-4): 427-37.

Snyder, E. Y., D. L. Deitcher, et al. (1992). "Multipotent neural cell lines can engraft and participate in development of mouse cerebellum." Cell 68(1): 33-51.

Snyder, E. Y., C. Yoon, et al. (1997). "Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex." Proc Natl Acad Sci U S A 94(21): 11663-8.

Sockanathan, S. and T. M. Jessell (1998). "Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons." Cell 94(4): 503-14.

Sohn, R. L., M. Jain, et al. (2007). "Adult stem cells and heart regeneration." Expert Rev Cardiovasc Ther 5(3): 507-17.

Song, L. and R. S. Tuan (2004). "Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow." Faseb J 18(9): 980-2.

Sotelo, J., B. H. Toh, et al. (1980). "Cytoplasmic intermediate filaments in cultured glial cells." Neuropathol Appl Neurobiol 6(4): 291-98.

Sottile, V., A. Thomson, et al. (2003). "In vitro osteogenic differentiation of human ES cells." Cloning Stem Cells 5(2): 149-55.

Srinivas, M. and D. C. Spray (2003). "Closure of gap junction channels by arylaminobenzoates." Mol Pharmacol 63(6): 1389-97.

Stevens, E. R., M. Esguerra, et al. (2003). "D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors." Proc Natl Acad Sci U S A 100(11): 6789-94.

Stix, B. and G. Reiser (1998). "Beta-amyloid peptide 25-35 regulates basal and hormone-stimulated Ca2+ levels in cultured rat astrocytes." Neurosci Lett 243(1-3): 121-4.

Stojkovic, M., M. Lako, et al. (2004). "Derivation of human embryonic stem cells from day-8 blastocysts recovered after three-step in vitro culture." Stem Cells 22(5): 790-7.

Strogatz, S. H. (2001). "Exploring complex networks." Nature 410(6825): 268-76.

Suadicani, S. O., C. E. Flores, et al. (2004). "Gap junction channels coordinate the propagation of intercellular Ca2+ signals generated by P2Y receptor activation." Glia 48(3): 217-29.

Suarez-Huerta, N., V. Pouillon, et al. (2001). "Molecular cloning and characterization of the mouse P2Y4 nucleotide receptor." Eur J Pharmacol 416(3): 197-202.

Sugioka, M., W. L. Zhou, et al. (1999). "Ca2+ mobilization and capacitative Ca2+ entry regulate DNA synthesis in cultured chick retinal neuroepithelial cells." Int J Dev Neurosci 17(3): 163-72.

Sugioka, M., W. L. Zhou, et al. (1999). "Involvement of P2 purinoceptors in the regulation of DNA synthesis in the neural retina of chick embryo." Int J Dev Neurosci 17(2): 135-44.

Sul, J. Y., G. Orosz, et al. (2004). "Astrocytic Connectivity in the Hippocampus." Neuron Glia Biol 1(1): 3-11.

Suon, S., M. Yang, et al. (2006). "Adult human bone marrow stromal spheres express neuronal traits in vitro and in a rat model of Parkinson's disease." Brain Res 1106(1): 46-51.

Takagi, Y., J. Takahashi, et al. (2005). "Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model." J Clin Invest 115(1): 102-9.

Takahashi, J., T. D. Palmer, et al. (1999). "Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures." J Neurobiol 38(1): 65-81.

Tang, Y., T. Yasuhara, et al. (2007). "Transplantation of bone marrow-derived stem cells: a promising therapy for stroke." Cell Transplant 16(2): 159-69.

Terada, N., T. Hamazaki, et al. (2002). "Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion." Nature 416(6880): 542-5.

Thastrup, O., P. J. Cullen, et al. (1990). "Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase." Proc Natl Acad Sci U S A 87(7): 2466-70.

Theis, M., R. Jauch, et al. (2003). "Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin43." J Neurosci 23(3): 766-76.

Thomas, E., J. Thomson, et al. (2006). Essential of Stem Cell Biology, Elsevier Academic Press.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science 282(5391): 1145-7.

Thored, P., A. Arvidsson, et al. (2006). "Persistent production of neurons from adult brain stem cells during recovery after stroke." Stem Cells 24(3): 739-47.

Tondreau, T., L. Lagneaux, et al. (2004). "Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation." Differentiation 72(7): 319-26.

Trimmer, P. A., P. J. Reier, et al. (1982). "An ultrastructural and immunocytochemical study of astrocytic differentiation in vitro: changes in the composition and distribution of the cellular cytoskeleton." J Neuroimmunol 2(3-4): 235-60.

Tropel, P., D. Noel, et al. (2004). "Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow." Exp Cell Res 295(2): 395-406.

Tropel, P., N. Platet, et al. (2006). "Functional neuronal differentiation of bone marrow-derived mesenchymal stem cells." Stem Cells 24(12): 2868-76.

Tropepe, V., B. L. Coles, et al. (2000). "Retinal stem cells in the adult mammalian eye." Science 287(5460): 2032-6.

Tsai, M. S., S. M. Hwang, et al. (2006). "Clonal amniotic fluid-derived stem cells express characteristics of both mesenchymal and neural stem cells." Biol Reprod 74(3): 545-51.

Tunggal, P., N. Smyth, et al. (2000). "Laminins: structure and genetic regulation." Microsc Res Tech 51(3): 214-27.

Uckermann, O., J. Grosche, et al. (2002). "ATP-evoked calcium responses of radial glial (Muller) cells in the postnatal rabbit retina." J Neurosci Res 70(2): 209-18.

Uckermann, O., S. Uhlmann, et al. (2003). "Upregulation of purinergic P2Y receptor-mediated calcium responses in glial cells during experimental detachment of the rabbit retina." Neurosci Lett 338(2): 131-4.

Uhlmann, S., A. Bringmann, et al. (2003). "Early glial cell reactivity in experimental retinal detachment: effect of suramin." Invest Ophthalmol Vis Sci 44(9): 4114-22.

Vazey, E. M., K. Chen, et al. (2006). "Transplanted adult neural progenitor cells survive, differentiate and reduce motor function impairment in a rodent model of Huntington's disease." Exp Neurol 199(2): 384-96.

Verkhratsky, A. and H. Kettenmann (1996). "Calcium signalling in glial cells." Trends Neurosci 19(8): 346-52.

Vigne, P., P. Pacaud, et al. (1996). "The effect of PPADS as an antagonist of inositol (1,4,5)trisphosphate induced intracellular calcium mobilization." Br J Pharmacol 119(2): 360-4.

Visnyei, K., K. J. Tatsukawa, et al. (2006). "Neural progenitor implantation restores metabolic deficits in the brain following striatal quinolinic acid lesion." Exp Neurol 197(2): 465-74.

von Kugelgen, I. (2006). "Pharmacological profiles of cloned mammalian P2Y-receptor subtypes." Pharmacol Ther 110(3): 415-32.

Wagner, J., P. Akerud, et al. (1999). "Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes." Nat Biotechnol 17(7): 653-9.

Wan, J., H. Zheng, et al. (2007). "Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina." Biochem Biophys Res Commun 363(2): 347-54.

Wang, L., P. Menendez, et al. (2005). "Hematopoietic development from human embryonic stem cell lines." Exp Hematol 33(9): 987-96.

Wang, S., L. E. Rosengren, et al. (1999). "Bcl-2 expression regulates cell sensitivity to S100beta-mediated apoptosis." Brain Res Mol Brain Res 70(1): 167-76.

Weick, M., P. Wiedemann, et al. (2005). "Resensitization of P2Y receptors by growth factor-mediated activation of the phosphatidylinositol-3 kinase in retinal glial cells." Invest Ophthalmol Vis Sci 46(4): 1525-32.

Weiss, S., C. Dunne, et al. (1996). "Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis." J Neurosci 16(23): 7599-609.

Wiedemann, P. (1992). "Growth factors in retinal diseases: proliferative vitreoretinopathy, proliferative diabetic retinopathy, and retinal degeneration." Surv Ophthalmol 36(5): 373-84.

Wiese, C., A. Rolletschek, et al. (2004). "Nestin expression--a property of multi-lineage progenitor cells?" Cell Mol Life Sci 61(19-20): 2510-22.

Wislet-Gendebien, S., G. Hans, et al. (2005). "Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype." Stem Cells 23(3): 392-402.

Wislet-Gendebien, S., P. Leprince, et al. (2003). "Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells." J Cell Sci 116(Pt 16): 3295-302.

Wislet-Gendebien, S., F. Wautier, et al. (2005). "Astrocytic and neuronal fate of mesenchymal stem cells expressing nestin." Brain Res Bull 68(1-2): 95-102.

Wisniewski, H. M. and J. Wegiel (1991). "Spatial relationships between astrocytes and classical plaque components." Neurobiol Aging 12(5): 593-600.

Woodbury, D., K. Reynolds, et al. (2002). "Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis." J Neurosci Res 69(6): 908-17.

Woodbury, D., E. J. Schwarz, et al. (2000). "Adult rat and human bone marrow stromal cells differentiate into neurons." J Neurosci Res 61(4): 364-70.

Wright, L. S., K. R. Prowse, et al. (2006). "Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro." Exp Cell Res 312(11): 2107-20.

Wu, K. D., D. Bungard, et al. (2001). "Regulation of SERCA Ca2+ pump expression by cytoplasmic Ca2+ in vascular smooth muscle cells." Am J Physiol Cell Physiol 280(4): C843-51.

Xiao, J., Z. Nan, et al. (2005). "Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury." Stem Cells Dev 14(6): 722-33.

Xu, C., M. S. Inokuma, et al. (2001). "Feeder-free growth of undifferentiated human embryonic stem cells." Nat Biotechnol 19(10): 971-4.

Xu, L., J. Yan, et al. (2006). "Human neural stem cell grafts ameliorate motor neuron disease in SOD-1 transgenic rats." Transplantation 82(7): 865-75.

Xu, R. H., R. M. Peck, et al. (2005). "Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells." Nat Methods 2(3): 185-90.

Yaksi, E. and R. W. Friedrich (2006). "Reconstruction of firing rate changes across neuronal populations by temporally deconvolved Ca2+ imaging." Nat Methods 3(5): 377-83.

Yamamoto-Hino, M., A. Miyawaki, et al. (1998). "Apical vesicles bearing inositol 1,4,5-trisphosphate receptors in the Ca2+ initiation site of ductal epithelium of submandibular gland." J Cell Biol 141(1): 135-42.

Yan, J., A. M. Welsh, et al. (2004). "Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord." J Comp Neurol 480(1): 101-14.

Yan, Y., D. Yang, et al. (2005). "Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells." Stem Cells 23(6): 781-90.

Yankner, B. A. (1996). "Mechanisms of neuronal degeneration in Alzheimer's disease." Neuron 16(5): 921-32.

Ying, Q. L., J. Nichols, et al. (2003). "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3." Cell 115(3): 281-92.

Yip, S., R. Sabetrasekh, et al. (2006). "Neural stem cells as novel cancer therapeutic vehicles." Eur J Cancer 42(9): 1298-308.

Yoon, S. R., J. W. Chung, et al. (2007). "Development of natural killer cells from hematopoietic stem cells." Mol Cells 24(1): 1-8.

Zahs, K. R., P. Kofuji, et al. (2003). "Connexin immunoreactivity in glial cells of the rat retina." J Comp Neurol 455(4): 531-46.

Zahs, K. R. and E. A. Newman (1997). "Asymmetric gap junctional coupling between glial cells in the rat retina." Glia 20(1): 10-22.

Zhang, Q., T. Pangrsic, et al. (2004). "Fusion-related release of glutamate from astrocytes." J Biol Chem 279(13): 12724-33.

Zhang, S. C., M. Wernig, et al. (2001). "In vitro differentiation of transplantable neural precursors from human embryonic stem cells." Nat Biotechnol 19(12): 1129-33.

Zhao, L. R., W. M. Duan, et al. (2002). "Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats." Exp Neurol 174(1): 11-20.

Zhao, M. Z., N. Nonoguchi, et al. (2006). "Novel therapeutic strategy for stroke in rats by bone marrow stromal cells and ex vivo HGF gene transfer with HSV-1 vector." J Cereb Blood Flow Metab 26(9): 1176-88.

Ziegler, D. R., C. E. Innocente, et al. (1998). "The S100B protein inhibits phosphorylation of GFAP and vimentin in a cytoskeletal fraction from immature rat hippocampus." Neurochem Res 23(10): 1259-63.

Zietlow, R., E. L. Lane, et al. (2007). "Human stem cells for CNS repair." Cell Tissue Res.