

The proliferation and differentiation of adult neural progenitors with a novel biomaterial

By

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Abstract:

Endogenous neural progenitor cells (NPCs) have the potential to repair the brain and spinal cord after injury. Using an *in vitro* neurosphere culture system we evaluated the ability of modifying adult rat spinal cord and subventricular zone progenitor cell differentiation into neurons, oligodendrocytes and astrocytes with retinoic acid, platelet derived growth factor and bone morphogenic protein-4, respectively. NPCs from both regions were exposed to varying concentrations of each factor. We found that SC and SVZ derived cells respond differently to these differentiation factors indicating that the therapeutic controls intended for one region may be different for the other. To assess delivery of potential therapeutic control, we evaluated a poly (lactide-co-glycolide) (PLGA) biomaterial designed to deliver these therapeutic agents to the injured central nervous system. The biocompatibility of PLGA for NPC proliferation, differentiation and survival was assessed using an *in vitro* neurosphere and differentiation assay. Our assessment of this biomaterial reveals that there were detrimental effects of PLGA degradation at later time points, suggesting, the need to control the degradation rate of this biomaterial, as its by-products -lactic acid and glycolic acid- may hinder the efficacy of delivered therapeutic factors to NPCs following injury.

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Abbreviations

B27 – B27 nutrient supplement mixture

bFGF – basic fibroblast growth factor

bHLH – basic-helix-loop-helix

BIIT – β III tubulin

BLBP – brain lipid binding protein

BMP-4 – bone morphogenic protein 4

BMPs – bone morphogenic proteins

BrdU - bromodeoxyuridine

BSA – bovine serum albumin

CC – central canal

CNS – central nervous system

CSPG – chondroitin sulphate proteoglycan

DIV – days in vitro

DKK - Dickkopf homologue 1 1

Dlx – neural stem cell marker

DMEM/F12 – dulbecco's modified eagle medium: nutrient mixture F-12

EGF – epidermal growth factor

ESCs – embryonic stem cells

fNSC – fetal neural stem cells

GFAP – glial fibrillary acidic protein

IN-1 – anti-Nogo-A antibody IN-1

MAP-2 – microtubule-associated protein 2

MCAO – middle cerebral artery occlusion

MEM- α – minimum essential media alpha

MSCs – mesenchymal stem cells

NB – neural basal medium

NeuN – neuronal nuclei
NPCs – neural progenitor cells
NSCs – neural stem cells
OCT – optimal cutting temperature
OPCs – oligodendrocyte progenitor cells
PBS – phosphate buffered saline
PCs – progenitor cells
PDGF – platelet derived growth factor
PFA - paraformaldehyde
PLGA – poly-lactic-co-glycolic-acid
PNS – peripheral nervous system
PSA-NCAM - polysialylated neural cell adhesion molecule
PVA – polyvinyl alcohol
RA – retinoic acid
RAR – retinoic acid receptor
RAS – rat sarcoma
RPM – revolutions per minute
SC – spinal cord
SCI – spinal cord injury
SEM – standard error of the mean
SGZ – subgranular zone
SHH – sonic hedgehog
SOX - SRY (sex determining region Y)-box
SVZ – subventricular zone
TGF- β – transforming growth factor beta
TUNEL – terminal deoxynucleotidyl dUTP nick end labelling

Chapter 1 - Introduction

Spinal cord injury – Prevalence

Spinal cord injury (SCI) is a devastating neurological condition for which there is still no cure. Currently, SCI has an annual incidence of 53 per million of a population in Canada (1). Additionally, the highest level of SCI, that of the cervical spinal cord, has been on the rise in recent years. Even more worrisome is that the fatality rates of these patients are reaching 48.3-79% (2). In addition to the emotional hardships of this debilitating disorder, patients and their families must also face very heavy economic hardships. Most tetraplegic patients will require \$3 million worth of health care services over their remaining lifespan (3). Finding a cure for spinal cord injury is thus a hope for many researchers as it will relieve the burden placed on these victims of spinal cord injury.

Repairing the damaged spinal cord

It is the nature of the central nervous system (CNS) response to injury which makes SCIs so difficult to treat. Unlike peripheral nervous system (PNS) neurons, who receive support from surrounding glia in the form of endoneurial tubes (4), CNS neurons are hindered from freely regenerating following injury. The supportive cells of the PNS provide a number of trophic factors and physical guidance cues which enable cut axons to return back to their original targets (5). Thus, it is the response of the CNS supportive cells that is thought to be problematic, as neurons which originated from within the CNS have been able to regenerate when cultured with PNS tissue (6, 7), and similarly, when PNS tissue is transplanted within the damaged CNS some regeneration can occur (8, 9). If given a permissive environment, these CNS axons may be

encouraged to regenerate past the inhibitory lesion restoring long tract connections between the brain and spinal cord circuitry distal to the injury level.

There are many barriers preventing recovery from traumatic SCI, traditionally categorized as primary and secondary injury, encompassing all the physical, chemical and biological factors of traumatic SCI (9). The primary injury results from direct physical forces leading to extensive cellular injury, neuronal death and glial death (10).

The secondary injury collectively encompasses many of the chemical and biological changes which occur after the direct physical insult, leading to a hostile, inhibitory environment for regeneration. This inhibitory environment following injury is responsible for a significant proportion of the total cell population lost following SCI, and collectively cell death from this inhibitory environment is referred to as the secondary injury (11). Many researchers are targeting secondary injury mechanisms as a potential treatment for SCI; for instance, clinical trials using pharmacological therapies like methylprednisolone treatments aim to limit the extensive inflammatory effects of the secondary injury (12). In the secondary injury, myelin debris, as well as oligodendrocytes, produce inhibitory molecules for growth cones attempting to regenerate (5). Directly adjacent to the lesion site astrocytes become reactive and infiltrate the site of injury and can contribute to an inhibition of axon regeneration (11). Together the inhibitory environment created by myelin debris, oligodendrocytes and reactive astrocytes prevent proximal axons from making any real gains toward their targets. Furthermore, both distal somatosensory and motor axons go through extensive Wallerian degeneration (13), characterized by neurons and their axons being severed from each other provoking degeneration of the axons since they no longer receive sustaining nutrients from their soma (14).

Researchers have approached SCI therapy by targeting means of limiting the cascade of cytotoxic biochemical signals involved in the secondary injury. There are a multitude of factors present in the damaged area including: excitotoxicity, ionic imbalance, increased free radical production and an inflammatory response which all lead to further cell loss (9). No one treatment has been found to reduce the effects of all these factors. For example, many antagonists have been developed to block the signalling mediated by many inhibitory molecules such as NOGO and other oligodendrocyte related molecules. The anti-Nogo-A antibody IN-1 (IN-1) developed by Martin Schwab's group (15) produced some of the most robust regeneration currently seen in the field. Others have attempted to degrade reactive astrocyte specific inhibitory extracellular matrix molecules, chondroitin sulphate proteoglycans (CSPG) by infusing a degradation enzyme chondroitinase into the spinal cord to reduce the influence of glial scar on regenerating axons (16). Neither approach nor any others (17), have been successful in promoting complete functional recovery. As well, these treatments do not focus on the replacement of lost cells. Therefore, to maximize recovery, established therapies should be used in combination with cellular regeneration therapies.

Cell transplantation therapies for spinal cord injury

Recently, a number of different stem cells have been identified for spinal cord regeneration; including but not limited to embryonic stem cells (ESCs), mesenchymal stem cells and fetal neural stem cells (fNSC). Limitations of these therapies include: ethical considerations surrounding the procurement of ESCs and fNSCs, the risk of immune rejection and finally low viability (18). It is argued that immune-rejection is not an issue for ESC transplantation as ESCs

tend not to harbour any of the immune targeted antigens. Although most transplantation studies still require immunosuppressant drugs to ensure ESC transplant survival (19). For these stem cells to be useful for transplantation therapies they require a considerable amount of pre-treatment which may influence reproducibility.

Furthermore, transplantation approaches have significant issues with cell survival. Researchers have shown that following transplantation only 10-20% of the introduced cells remain viable (20). Even when other supportive glia like peripheral Schwann cells and olfactory ensheathing cells (21) are present, these cells show limited migration from the point of transplantation and in turn support only modest recovery and regeneration. It is for the above reasons that the development of an alternative method for cellular replacement is paramount.

Endogenous neural progenitor cells in the adult central nervous system

Ramon y Cajal's states in his book; Degeneration and Regeneration of the Nervous System, that "in adult centres the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated." For a long time, this was the accepted dogma in neuroscience. Not until the study of song bird learning by Nottebohm and colleagues in the early 1990s did researchers find something that might disprove Cajal's classic theory. The study found that adult black-capped chickadees showed seasonal levels of neurogenesis and neuronal replacement (22) In addition, they found that this increase in cell proliferation was primarily seen in the hippocampus and may be linked with food caching behaviour. The neurogenesis was found to be linked with a bird's ability to recall the various locations of hidden food.

Around the same time Canadian researchers in Calgary discovered that neurogenesis also existed in rodent models. Not only did Reynolds and Weiss (1992) find that stem cells are located in the mammalian CNS within the striatum but they also found that they were able to induce these cells to proliferate *in vitro* through the application of epidermal growth factor. Additionally, Reynolds and Weiss found that these cells were progenitors and that they expressed GABA and substance P. They therefore concluded that cells of the adult mouse striatum have the capacity to divide and differentiate into neurons and astrocytes (23). Both of these studies were breakthroughs in the neuroscience field. For more than 60 years it was thought that neurogenesis did not exist in the adult brain. In this sense, these discoveries were revolutionary. Brent Reynolds said it best when he stated that “just because things don’t occur on their own, doesn’t mean we can’t make them occur.” (24) Adult neurogenesis could potentially lead to a variety of treatment options for the ever increasing patient population dealing with acquired and degenerative neurological disorders.

NPCs have been found in three specific areas in the adult CNS; the subventricular zone (SVZ) of the lateral ventricles, the subgranular zone (SGZ) of the dentate gyrus and finally the ependymal zone (central canal) of the spinal cord (25). NPCs migrate from the SVZ to the olfactory bulb differentiating into olfactory bulb interneurons (26). In the SGZ of the dentate gyrus they provide neural progenitors to the granular layer of the dentate gyrus (27). The function of the spinal cord progenitor cells within the spinal cord has not yet been determined; however, it is primarily thought that during injury they migrate to the damaged area and contribute to the influx of new glia cells within the glia scar (28-30).

The SVZ is located next to the ependyma, a thin layer of tissue that lines the lateral ventricle of the brain. It has been hypothesized that the cells found within the ependyma are

responsible for producing the NSCs needed for neurogenesis within the SVZ (31). This idea is still controversial as other studies have found these ependyma cells to be relatively dormant (32). Although it is still undecided, it is thought that the cells found within the subependyma of the SVZ are the main source of new neuroblasts which contribute to the long-term neurogenesis seen under normal conditions (26, 33). However, at times of injury the ependymal cells do demonstrate the ability to differentiate into SVZ cell types. During this process the ependymal region becomes exhausted indicating they do not possess true stem cell characteristics (34). There are three types of precursor cells within the SVZ: type B GFAP-positive progenitors, Dlx2 positive type C transit amplifying cells and PSA-NCAM positive type A migrating neuroblasts (26, 32). Recent studies using genetic fate mapping techniques further substantiate the observation that it is the Type-b astrocyte located in the subependyma which is responsible for the production of new neuroblasts (26, 33, 34) and to a lesser degree oligodendrocyte progenitors (35). There still are a number of questions to answer before the identity of the true NSC is revealed within the adult SVZ (27).

The extension of the ventricular system in the spinal cord, the central canal (CC), is also home to progenitors that can proliferate and give rise to all three types of cells *in vitro* (28). Instead of the three different cell types discussed above, the CC is home to just one defined progenitor type; a nestin positive ependymal cell. These ependymal cells remain relatively dormant during homeostatic conditions; however, once the spinal cord parenchyma is disturbed either through mechanical or chemical injury, these cells migrate from the ependyma and give rise to new glia (25, 29, 31, 36-38). When harvested and cultured using the neurosphere assay these cells can be cultivated over more than five passages and can give rise to all three neuronal

cell types (39-41). Unlike the SVZ, the CC does not show any evidence of being neurogenic *in vivo* (42).

Spinal cord progenitor cell response to injury

Progenitor cells (PCs) in the spinal cord have two important features which make them exceptional targets for spinal cord regeneration research: 1) they are immature cells which are able to migrate to the injury site and 2) they are multipotent cells that can differentiate into neurons (*in vitro*), oligodendrocytes and astrocytes. PCs migrate freely from the ependymal zone to the damaged area of the spinal cord (28, 29, 34, 36). Within the mammalian spinal cord there are three main cell types that proliferate after injury: ependymal derived PCs, NG2 positive oligodendrocyte progenitors and finally reactive astrocytes. Although these three main cell types proliferate and give rise to new cells following injury it is the ependymal cells which generate the most significant numbers of progeny rather than reactive astrocytes or OPCs (29, 43).

Upon arrival to the injury site ependymal cells become integrated into the glial scar (29). Two additional sources of the glial scar come from reactive astrocytes and pericytes, which create the fibrotic characteristics of the scar (43). Ependymal cells only generate astrocytes and immature oligodendrocytes following spinal cord injury (28, 29) *in vivo*. Examining, further detailed molecular markers it is possible to see that the ependymal cells that arrive and integrate into the glial scar do not express the expected proteoglycans (28). Ependymal derived astrocytes do not express CSPG - the inhibitory marker commonly associated with dense glial scars. Furthermore, sprouting axons found within the dense glial scar are mostly found directly adjacent to ependymal derived glia (28). It has yet to be revealed whether there is a true difference between these glial populations within the scar. However, if it can be shown that

astrocytes originating from the ependymal cells are more supportive of regeneration than both the reactive astrocyte and pericyte glia then perhaps therapies that harness ependymal cell proliferation and differentiation can be created to realize greater degrees of recovery following injury (30).

Controlling endogenous ependymal PCs after injury

In both *in vivo* and *in vitro* studies, endogenous ependymal PCs have been encouraged to proliferate when exposed to the mitogens - epidermal growth factor and basic fibroblast growth factor (25, 44). Although promising, these results indicate that these mitogens have very powerful influences on surrounding cells which can cause abnormal cell growth creating meningeal proliferative lesions (44). Therefore, further research is required to ensure that proliferation occurs in a more controlled manner so as not to inadvertently cause increased detriment to the patient.

To control the extent of proliferation and also to ensure that cells do not solely differentiate into unsupportive glia, mitogens can be added to other factors to encourage neuronal and supportive glia differentiation. For example, sonic hedgehog and retinoic acid have been shown to encourage the differentiation of mesenchymal cells into neuronal cells making them a candidate for controlling NSCs in a similar fashion (45). As well, platelet derived growth factor (PDGF) has been identified as an important peptide for controlling oligodendrocyte numbers in the developing CNS. The over expression of PDGF in a transgenic mouse model showed promise by causing enhanced oligodendrocyte differentiation (46). Interestingly, astrocyte differentiation is not detrimental in all cases. When CNS progenitors are treated with bone morphogenic protein 4 (BMP-4), they have been found to produce a certain subtype of astrocyte

that facilitates greater sensory recovery following spinal cord injury (47-49). Although it appears that there is the possibility of differentiating NPCs into cell types which support great extents of regeneration, the research presented above has yet to be extensively tested on adult PCs from the ependymal zone of the spinal cord. Therefore, a considerable amount of study still needs to be completed on these ependymal PCs before we can attempt to control them *in vivo*. For this reason, retinoic acid (RA), PDGF and BMP-4 will be evaluated in this study as to how they affect adult SC derived PC viability, proliferation and differentiation.

Retinoic acid's influence on NPC behaviour

Retinoic acid has two main roles during development. First, in the patterning of the nervous system and second in the differentiation of various types of neurons and glia. RA contributes to both the anterior-posterior polarity and the dorsal ventral polarity within the neural tube. Mutants who are deficient for retinoic acid's receptor (RAR) do not develop any ant-post polarity and have abnormal hindbrain morphology. As well, the neural tube fails to extend neurites out into the periphery (50).

RA is believed to produce its differential effects using gradients established over the developing CNS with the highest concentration found at the floorplate of the neural tube (51). RA is produced in the posterior mesoderm while the CYP26 enzymes which degrade it are produced in the anterior mesoderm. This results in a gradient that influences the patterning of the hindbrain and anterior spinal cord. RA patterning leads to the production of sensory neurons, interneurons and motor neurons (52). This action is carried out by activating various genes, including those that code for additional transcription factors, cell signalling proteins, and well as

structural proteins. For example, RA treatment results in an increased expression of microtubule-associated protein 2 (MAP-2) and the transcription factors, SRY (sex determining region Y)-box (SOX) 1 and 2 as well as neurogenin 1 (Ngn 1) (53).

RA also induces the Wnt inhibitor, Dickkopf homologue 1 (DKK1), which is necessary for the RA mediated differentiation of mouse ESCs. Without the blockade of the Wnt pathway within these ESCs they would remain in a pluripotent state. Furthermore when recombinant Dkk1 was applied alone to the ESCs the same action caused by RA is seen. Therefore there appears to be a direct link to Dkk1 depending on RA for its activation and the subsequent inhibition of the Wnt pathway (54). This finding is still controversial as many studies have found that Wnt, particularly Wnt7a is responsible for increasing neurogenesis within the hippocampus. Consequently, the result caused by the application of RA in this regard seems to be highly dependent on the area of application (55).

Many studies have explored the differentiation power of RA and have found that it too is a probable contender for directing stem cells to differentiate into neural lineages (45, 56). When RA is placed in culture with SVZ NSCs it has a similar function as SHH, producing close to 50% more neurons than control cultures. The total number of neurons can potentially be increased through encouraging greater numbers of true neural precursors to proliferate, which will allow differentiation factors like RA to support further increases in neuronal differentiation. What is exceptionally promising about RA is its ability to override the mitogenic effects of EGF and FGF. NSCs that were exposed to all three factors in a previous passage produced fewer BrdU positive cells in the next, indicating a reduced amount of proliferation (56).

When embryonic spinal cord NSCs were exposed to SHH and RA, there was an increased expression of the immature neuronal marker β -Tubulin III, whereas controls had very limited expression (57). These effects are not exclusive to NSCs; many other types of stem cells have been receptive to the influences of SHH and RA. For instance, mesenchymal stem cells (MSC) generally produce red and white blood cells; however, once exposed to SHH and RA they are seen to divert towards neural lineages. The differentiated cells show expression of MAP2, TUJ1 and NeuN, which are only found in terminally differentiated neurons (45). Therefore, the influence that SHH and RA have on NSCs is equally potent with these MSCs, making them great factors for influencing many different stem cell lines. Retinoic acid was selected over a combined SHH treatment because SHH substantially influences NPC proliferation (58) and may lead to added proliferation rather than differentiation.

Platelet derived growth factor's effect on NPC behaviour

Oligodendrocytes arise in a unique manner during central nervous system development. It is known that these cells develop from the ventral area of the brain and the spinal cord then migrate to their appropriate targets. Unlike the neural precursor cells affected by SHH and RA, oligodendrocytes arise from a unique group of O2-A precursor cells. The proliferation of these cells is controlled by PDGF. While there are a number of receptors for this factor within the CNS it is the PDGF- α receptor that mediates its action (46, 59, 60). The PDGF- α receptor is found specifically in the ventral regions of the spinal cord as this is where oligodendrocytes develop and unsurprisingly, the transcription factors Olig 1 and Olig 2 (exclusive to the O2-A progeny) are highly represented here as well (51).

Within the developing spinal cord, PDGF is produced and released by astrocytes. While there are a number of isoforms of PDGF (AA, BB, AB), astrocytes only produce the AA version of PDGF (61). Once it is released, it binds to the PDGF- α receptor causing the activation of a variety of growth related genes. The main hypothesized function of the receptor is based on its tyrosine phosphorylation domain, making it a likely candidate for mediating certain cell cycle regulators like cyclin dependent kinases (62). In fact, the differentiation of O2-A into oligodendrocytes is highly dependent on the accumulation of the cycle regulator, cyclin dependent kinase inhibitor p27. Once this inhibitor has accumulated in the cells, they are forced out of the cell cycle and differentiate into oligodendrocytes (63).

Although p27 has an integral role in limiting the perpetuation of O2-A proliferation it does not have the same extensive control of PDGF. Studies have shown that oligodendrocyte proliferation is highly dependent on the degree of stimulation provided by PDGF. When PDGF-AA is over expressed in mouse embryos, there is a large increase in the number of immature oligodendrocytes that develop (64), and, in contrast, PDGF knockout experiments show that when embryos develop with limited numbers of oligodendrocytes, they suffer from severe hypomyelination (65). In the embryos which over express PDGF the extra oligodendrocytes are removed from the CNS as it matures. This indicates that there are secondary signals involved following differentiation that encourage survival. These secondary signals are believed to come from neurons in the form of trophic factors and electrical stimulation. Therefore, even though PDGF is directly associated with O2-A proliferation, oligodendrocyte's survival is reliant on additional factors (64).

The mechanisms by which PDGF influences the differentiation of neural precursors is still unclear. However, the signal transduction is believed to be linked to affecting cell cycle

progression. Most of the experiments which employ PDGF require the use of NSCs, those either obtained from embryonic tissues (66) or from adult brain tissues (67). Rarely are pluripotent cells used, as the nature of signalling is exclusive to O2-A progenitors. If pluripotent stem cells are to be used they require a pre-treatment with a variety of transcription factors like Olig1/olig2, Sox 2 and Nkx6.2; as well they must be cultured with a variety of growth factors and BMP antagonists. Following this treatment process the cells are then screened to attain the derived O2-A cells. Therefore, the feasibility of this method for oligodendrocyte production seems limited as there is a number of transition tasks required (68).

A much more feasible option for producing oligodendrocytes comes from the proliferation of endogenous oligodendrocyte precursors cells (O2-A or OPCs). Various studies have shown that following a relatively simple exposure to PDGF, OPCs proliferate contributing to an end increase in immature and mature oligodendrocytes. When PDGF-AA (20ng/ml) is administered to a mix of cultured embryonic derived NSCs and OPCs, mature oligodendrocytes are produced. When control conditions (EGF and FGF) are compared with experimental conditions (PDGF), the number of positive cells for the PDGF receptor PDGF- α increases by 30%. However, when looking for immature oligodendrocyte markers, the treated group only show a 10% increase in their expression (66).

There is still a fair amount of optimization that must occur before *in vivo* differentiation of oligodendrocytes becomes a clinical treatment for neurological disorders. It is more plausible that endogenous neural progenitor cells will become the target of PDGF effects, as in the study presented earlier, where over expression of PDGF-AA creates a surplus of oligodendrocytes (64). Additionally, when PDGF-AA is administered *in vivo* to the CNS an increase in OPC proliferation is noted (60).

Bone morphogenic protein 4's effect on NPC behaviour

BMPs play a very important role in neural development especially in the dorsal-ventral patterning of the nervous system subsequently aiding with the determination of many dorsal cell types. BMPs are members of the transforming growth factor (TGF- β) protein family. TGF- β signalling is responsible for a number of different processes including: cell fate decisions, tissue patterning, cell proliferation, death and differentiation. The family TGF- β bind to a complex of type I and type II serine-threonine transmembrane kinase receptors. Each receptor binds to a different TGF- β protein. In the case of BMP, the receptors which bind to it are BMPRIA and BMPRII. BMPRIA and BMPRII form different complexes with BMPRII. Once BMP has bound to the BMPRII complex, it activates the BMPRIIs. These phosphorylated BMPRIIs then move to activate the Smad protein, R-Smad, which in turn forms a new complex with the mediator Smad, Co-smad. This new complex then enters the nucleus and begins to modulate the expression of various genes (69).

The signalling of BMPs can be easily blocked through the exposure to noggin. Noggin binds to the BMP itself removing the chance of it connecting with the targeted receptors. This action is especially important during development over acting BMP can cause many problems within the developing nervous system (69).

Initially, the inhibition of BMPs is important for the development of neural tissue for proliferation. Following the separation of neural and non-neural ectodermal tissue the inhibition of the BMP must cease in some regions. There exist two main gradients in the dorsal-ventral direction of the spinal cord, the first is SHH leading ventral to dorsal and the second is BMP leading dorsal to ventral (51). The gradient of BMP is just as important as SHH, as knockouts of

BMP show that without the expression of the BMP, normal neural differentiation does not occur in the dorsal spinal cord. There are three specific BMPs located in the roof plate which control the patterning of the spinal cord: BMP-2, BMP-4 and BMP-7 (70).

We have explored the roles of BMP in development but have not yet touched on how it may cause the differentiation of astrocytes versus other types of cells. Indeed, BMP has a number of actions, many of which may not involve the specific direct differentiation of astrocytes during development. However, if we look closer at the origins of the astrocyte's progenitors we see that they are located in close approximation to the roofplate of the neural tube (51). Therefore, since BMP is produced in the roofplate and the astrocyte progenitors are localized within the boundaries of the BMP signalling and outside the grasp of noggin's inhibition, researchers were able to hypothesize that perhaps BMP has a role in astrocyte determination.

One BMP family member that shows great potential in instructing astrocytic fate is BMP-4. When BMP-4 is transgenically over expressed in mice it effectively increases the degree of astrogliogenesis. The over expression of BMP-4 created a substantial 40% increase in the number of astrocytes throughout the brain, while reducing the overall number of oligodendrocytes by 26%. Interestingly, when these transgenic neurons were placed in co-culture with SVZ derived progenitors they caused an increase in astrogliogenesis as well. Treating these cultures with noggin effectively removed these effects, indicating that the increased astrocyte commitment was in fact caused by the over expression of BMP-4 (71). This effect of BMP-4 release from transfected neurons led researchers to conclude that perhaps BMP-4 could be used to mediate the differentiation of other progenitors.

We have selected the above differentiation factors for their ability to instruct NPSC behaviour in many different contexts. Furthermore, we intended to investigate these differentiation factors under various concentrations due to the importance gradients have on instructing differential effects on NPCs during development. A summary of the expected results can be seen in Table 1.

Table 1 – Summary of the predicted effect of each factor on NPCs differentiation

Factor	Model	Concentration	Reference	Hypothesis
PDGF	Adult rat spinal cord PCs	20ng/ml	Kulbatski et al., 2009	Increase in O4 / Oligo
BMP-4	Mouse NSC	50ng/ml	Gross et al., 1996	Increase in GFAP / Astrocytes
Retinoic Acid	Adult mouse NSPCs	300ng/ml	Wang et al., 2005	Increase in BIIT / Neurons

Biomaterial delivery of growth factors into the damaged CNS

Biomaterials have been proposed as a potential tool that can be used to encourage endogenous repair of the nervous system following injury. Recent work has been completed that attempts to support endogenous capabilities namely axon regeneration and progenitor activation following SCI (72-74). Certain materials have already been developed that encourage tissue bridge formation and in turn axon regeneration (8, 75). These biomaterials can offer structural support by limiting muscle infiltration, removing pressure on regenerating tissue. In addition, they can offer a means for delivering certain therapeutic factors to the injury site encouraging neuroprotection(76), mitigation of the inhibitory environment (77), and recruitment of local progenitors (74) for repair.

One such biomaterial that has been used extensively in regeneration approaches has been poly-lactide-co-glycolide acid (PLGA) (78-80). This material has a great potential to act as structural support in its channel form as well as a means of delivering therapeutic factors to the injury site. PLGA can be fabricated into a number of different forms making it a very adaptable material. In other applications, PLGA has been made into microspheres, fabricated in such a way as to encapsulate various factors for local delivery (74). PLGA microspheres have been used previously as a source of sustained drug release in many different applications. Many studies have been completed to assess the biocompatibility of these materials within a 2 week period only (81, 82), leaving the long term effects will be past these two week time periods.

In these feasibility studies, drug release patterns and acute exposure are generally assessed with cell culture models. Few *in vitro* studies that aim to assess the effect of the degradation of the material on the cell models have been completed. Initial biocompatibility is an important assessment for tissue scaffolding, as cell seeding becomes the main concern (72). However, it is also exceptionally important to assess the effect that the degradation components could potentially have on these cells.

The main goal of creating a biomaterial delivery system is to allow the implant to degrade *in vivo* following the application of the therapy therefore limiting the need for follow up procedures to remove the implant. Consequently, the local environment where the implant is placed will encounter a prolonged exposure to not only the implant in its original form but also to its degradation by-products. For this reason, we wished to assess the prolonged effect of PLGA as a layered channel and as microspheres on neural progenitors.

We chose NPCs from both the SVZ and CC of the SC for our assessment of the effect of PLGA degradation on proliferation and differentiation. PLGA degradation occurs through the hydrolysis of its ester linkages (83). This hydrolysis begins immediately once the material is exposed to aqueous surroundings: however, it is only following a three week period when the material begins to release its monomers (lactic acid and glycolic acid) into the surroundings quantifiably (84). Therefore, we assessed the effect of PLGA degradation on proliferation and differentiation of NPCs over a 28 day *in vitro* (DIV) period. At 14, 21 and 28 div cells were assessed for their proliferation capacity as well as the effect that the exposure to PLGA had on differentiation.

Neurosphere culture of adult neural progenitors

The main experimental technique to be used in this study to evaluate NPC behaviours is the neurosphere culture technique. First developed by Reynolds and Weiss in 1992, the neurosphere assay continues to be the method of choice for many research groups interested in evaluating NSPCs in various contexts (85). Using a fairly crude dissection method of any area within the CNS that is considered to be a NPC niche, single cell suspensions can be harvested and plated in serum free media conditions with the mitogens EGF and FGFb in order to obtain cell aggregates known as neurospheres (86). These neurospheres can then be plated without mitogens with or without serum to investigate their differentiation potential.

Generally, to investigate NSC behaviour the neurospheres must first be generated under clonal conditions where cell densities are kept below 10 cells/ul. This ensures the neurospheres that develop are from single cell proliferation rather than cell aggregation. An additional step is required to evaluate stem cells with the neurosphere assay; the spheres must be passaged into

single cell suspensions at least 5 times to ensure all spheres are of stem cell origin rather than from proliferating progenitors (85).

In our experimental model, we chose to evaluate both SC and SVZ derived NPC behaviour using a primary neurosphere model generated under high density conditions. For this reason, that we will refer to our cells as progenitors rather than stem cells throughout this thesis. Many advantages come from generating neurospheres under high density conditions. One of the main reasons for investigating non-clonally derived spheres is due to the impracticality of instructing purely stem cell behaviour *in vivo*. Using primary neural progenitors generated under non-clonal conditions will ensure that *in vitro* manipulations can be transferred to *in vivo* manipulations in the future. Clonal cultures produce very few spheres at each progressive passage. Thus, it is thought that evaluating primary progenitors *in vitro* will most closely model the progenitor population found to proliferate after a SCI.

Rationale for the current research

Three aspects require improved understanding before *in vivo* NPC control can be realized. First, the proliferation and differentiation ability of NPCs require phenotyping. To truly be able to control spinal cord progenitors we must first understand the factors that can regulate their proliferation *in vitro* so as to aid in the design of later *in vivo* applications. As well deciding when to stop *in vivo* expansion of the NPC may also be dependent on their differentiation profile after extended periods of exposure to EGF and bFGF. Adult NPC from the SVZ have already been profiled extensively, therefore using these cells as a point of comparison may aid in developing a greater understanding of the behaviour of SC derived PCs. This will in turn aid in understanding how these cells may be influenced at times of injury.

The second area which requires further study is the evaluation of the factors which instruct progenitor cell differentiation. Many studies have been completed over the last decade which aimed to control stem cell differentiation into neural lineages. Using knowledge gained from these stem cell differentiation studies factors were selected that would facilitate differentiation along neural, oligodendrocytic and astrocytic lineages (45, 56, 67, 87). Following this study the most successful factors can be selected for later *in vivo* evaluation. Again since SVZ progenitor differentiation has been evaluated extensively, their behaviour in response to these selected factors will act as a comparison to SC derived NPCs and may aid in a better characterisation of the SC progenitor cells.

The final area of study which remains to be assessed is the non-invasive methods of growth factor release. Current methods involve either osmotic pump infusions (44). This method increases the risk for damage from further secondary injury as catheters and needles will produce new sources for compression. A probable circumvention of these issues may come from the utilization of biodegradable materials that can be fabricated to incorporate growth factors which would in turn be released as the material degrades.

Objectives:

The objectives of this study are as follows:

1. Phenotype the proliferation and differentiation of primary spinal cord PCs.
2. Evaluate the utilization of selected factors to influence NPC differentiation into all three neural lineages
3. Investigate the use of biomaterials as a drug delivery system for instructing neural progenitor behaviour.

Chapter 2 - Material and Methods

Primary cell culture:

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and approved protocols from the Animal Care Committee of the Ottawa Hospital Research Institute, Ottawa, Ontario, Canada. Neural progenitors were harvested from adult female Sprague Dawley rats (~150g). Rats were deeply anesthetised with isoflurane and decapitated. Using sterile surgical tools the skull and spinal column were removed and placed in chilled dissection media composed of: 87% MEM- α (Gibco-Invitrogen; Burlington, ON, Canada, 12492-013), 10% fetal bovine serum (Gibco-Invitrogen, 12483-020), 1% L-Glutamine (Gibco-Invitrogen, 25030-081) and 2% Penicillin & Streptomycin (Gibco-Invitrogen, 15140-122).

Dissection of the Sub-ventricular zone

The skull was removed from solution and placed in a sterile petri dish (Fisher scientific, 08-757-9B; Mississauga, Ontario, Canada) containing chilled dissection media. The occipital, temporal, and parietal bones were excised to expose both cerebral hemispheres. Using a sterile pair of curved forceps and by sliding under the ventral aspect of the entire brain including the cerebellum the brain was gently removed and placed into a new petri dish containing new chilled dissection media. Then using an adult rat brain matrix and sterile stainless steel razor blades, 1mm slices of the brain were prepared. Only slices containing the lateral ventricles were placed in a new petri dish for further dissection. The entire ependymal and subependymal regions of the

NPC Harvesting

Sub-ventricular Zone & Sub-ependymal Zone
from Adult Sprague Dawley rats (~150g)

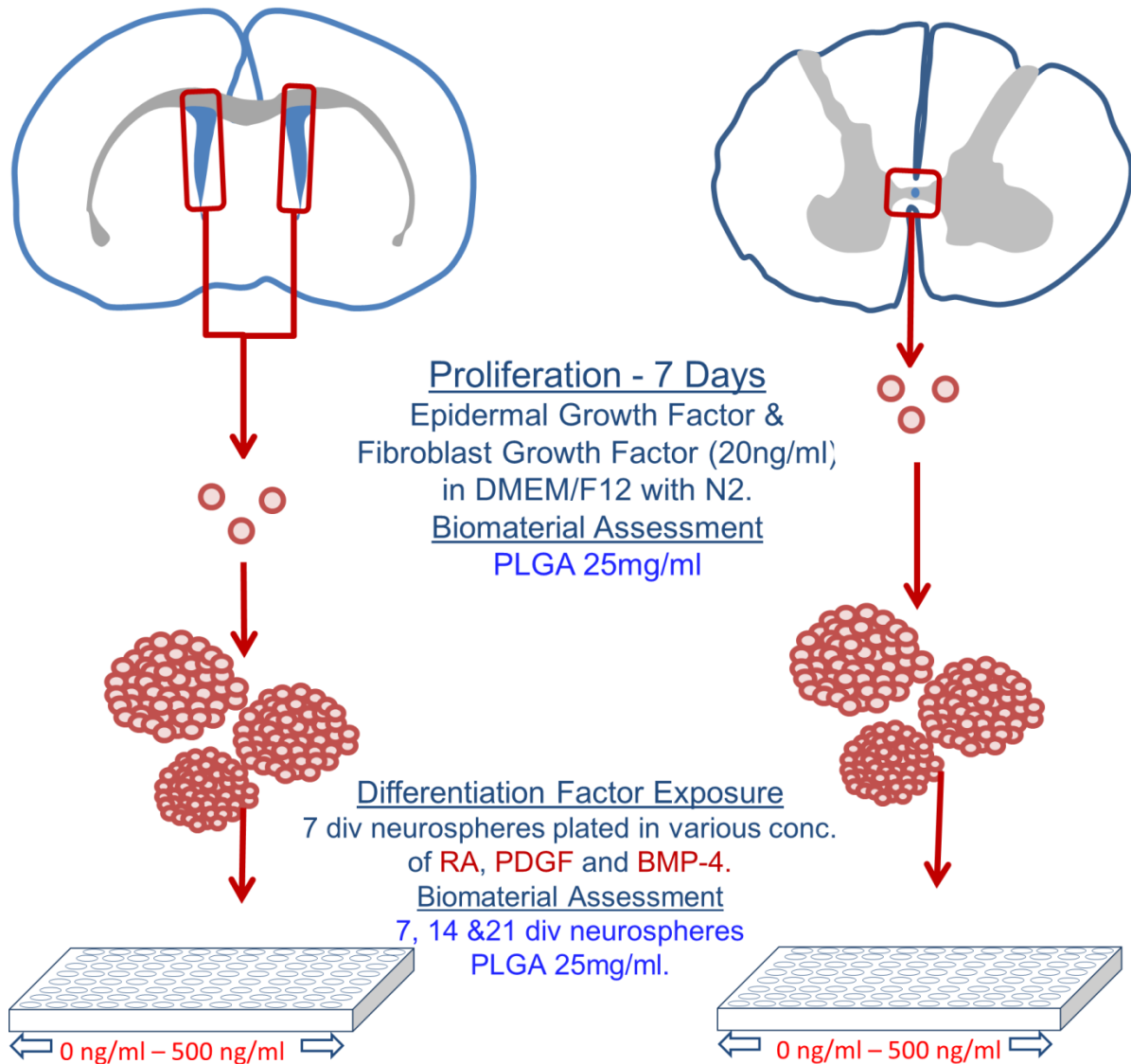


Figure 2.1 – *In vitro* technique used to study the effects of differentiation factors on adult NPCs from sub-ventricular zone and sub-ependymal zone. As we wished to model the effects of differentiation factors on NPCs after spinal cord trauma, cells were not passaged since they would not be passaged *in vivo* after trauma. Tissue harvesting and plating method used for both biomaterial assessments and differentiation factor assessments.

lateral ventricle were excised using a dissection microscope and Moria ultrafine tipped forceps (Fisher Scientific, NC9100523). The tissue was cut into 1 mm³ pieces and placed in a 15 ml centrifuge tube (Fisher Scientific, 50819798) containing new chilled dissection media.

Dissection of the Ependymal Zone of the Central Canal

The spinal column was removed from the dissection media and placed in a sterile petri dish containing chilled dissection media. Using 6 mm serrated malleus nippers (Storz, N1430) a laminectomy was performed from C1 to S1 revealing a dorsal view of the spinal cord. Using small scissors the spinal roots were cut removing any remaining connection between the spinal cord and the remaining spinal column. The entire spinal cord from cervical to lumbar was removed using blunt forceps ensuring no compression of the cord. The tissue was placed in a new petri dish containing chilled dissection media. The spinal cord from cervical to thoracic, ending right before the lumbar enlargement, was then sliced into 5 equal parts approximately 1 cm in length. One at a time, the dura was removed from the spinal cord by pulling it apart with fine tipped forceps. The ventral medial line of the spinal cord was located. Using fine tipped forceps the ventral portion of the spinal cord was cut in a rostral-caudal direction. Following this the spinal cord could then be opened revealing with grey matter and central canal. Fine tipped forceps were then used to remove the central canal and grey matter from the white matter using the same rostral-caudal cutting technique. Tissue segments were ensured to be no more than 1 mm³ in volume and were placed in a 15ml centrifuge tube with chilled dissection media.

Under sterile conditions both spinal (SC) and SVZ tissues were triturated 20 times using a fire polished glass cotton plugged Pasteur pipette (Fisher Scientific, 13-678-8B) in their

respective 15 ml tubes. The cell suspensions were centrifuged at 400rpm for 4 minutes. The supernatant was removed carefully so not to disturb the undesired pellet and placed into a new 15 ml centrifuge tube. Two millilitres of new dissection media was added to each 15 ml tube containing the pellet. This cycle of trituration, spinning and supernatant removal was repeated 2 additional times. The new centrifuge tubes containing the cell suspensions were then centrifuged for 5 min at 1500 rpm. The supernatant containing the dissection media was discarded without disturbing the pellet. Then the culture media was added containing: 98% DMEM/F12 (Gibco–Invitrogen, 10565-042), 1% L-glutamine (Gibco–Invitrogen, 25030-081), 1% N₂ Supplement (containing: 10000 mg/L human transferrin, 500 mg/L insulin recombinant full chain, 0.63 mg/L progesterone, 1611 mg/L putrescine and 0.52 mg/L selenite) (Gibco–Invitrogen, 17502-048). Cells were suspended in the media and passed through a 40µm cell sieve (BD Biosciences) to capture any large tissue segments which remained. The media was supplemented additionally with epidermal growth factor 20ng/ml (Peprotech, Rocky Hill, New Jersey, 100-44) prepared in 0.1% BSA (Fisher Sci, PI-77110) PBS and fibroblast growth factor 20ng/ml (Peprotech, 100-18B) prepared in 5 mM Tris buffered saline (Sigma Aldrich, 154563) with a pH of 7.6. Cells were seeded (~ 1.5 x 10⁶/ml) in a 6 well low attachment culture plate (Fisher Sci., 07-200-601) for each the SVZ and SC cells which were maintained at 37°C in an incubator with 95% humidity and 5% CO₂. To encourage primary neurosphere formation EGF and FGF-2 were supplemented every 2 days by removing half of the media and replacing it with new media containing the mitogens at a 2 x (40 ng/ml) concentration. Neurospheres first appeared 5 days post isolation and were agitated on a daily basis to prevent aggregate formation and attachment to the plate substrate.

Long-term culture of Neural Progenitor Cells:

For both undifferentiated neurosphere cryosectioning experiments and biomaterial assessments, neurospheres were grown in suspension culture for extended periods. In these instances neurospheres were collected at 7 days post isolation and transferred from high density conditions to low density - 3 neurospheres per 150 ul - conditions. This low density approach prevented neurosphere aggregates from forming, where two spheres in close proximity to each other can merge together forming a larger neurosphere cluster. For both the cryosectioning and biomaterial assessments neurospheres were placed 3 per well in low attachment culture plates and both media and mitogens were supplemented every two days.

Neurosphere sectioning:

Neurospheres from both the SC and SVZ derived cultures were collected at the end of each proliferation period (7, 14 & 21 div) and fixed in 4% paraformaldehyde (PFA) for 20 minutes. Neurospheres were centrifuged at 400 rpm for 4 minutes. The supernatant was discarded and 10 mM PBS was used to complete three successive washes of the newly fixed spheres. Neurospheres were cryopreserved in 15% (W/V) sucrose in 10mM PBS overnight. Neurospheres were then placed in 2 cm petri-dishes and using a Nikon dissection microscope 4 neurospheres were placed in 1 cm cryomolds filled with Tissue-Tek Optimal Cutting Temperature (OCT). Molds were then flash frozen by using a shallow placement in liquid nitrogen. Molds were stored in -80°C until required. All molds were brought to -20°C prior to sectioning. Using a cryostat (Leica) 10 µm sections were taken of each sphere and placed on

superfrost slides (Fisher). Slides were placed on a slide warmer (Fisher) set to 45°C overnight to ensure proper adhesion to the slide's substrate. Neurospheres were then stored at -80°C until immunocytochemistry was performed.

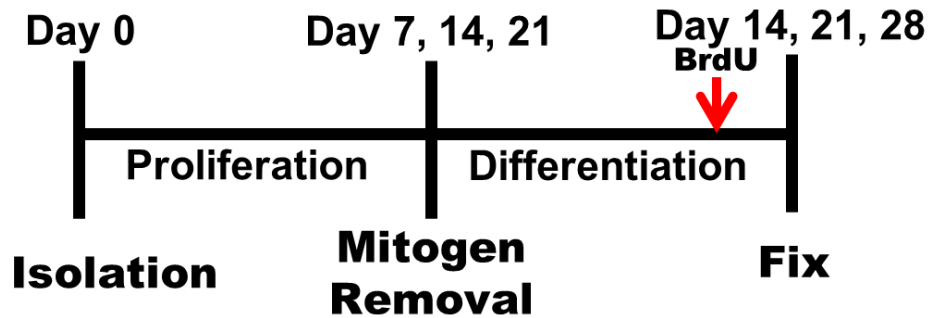


Figure 2.2 – Cell culture experimental timeline indicating proliferation and differentiation periods. NPCs were allowed to proliferate for 7, 14 or 21 days *in vitro*. Following this proliferation period neurospheres were collected and plated in differentiation conditions which lacked mitogens and placed in 96 well plates treated with poly-l-ornithine. NPCs were then exposed to BrdU for 24hrs prior to fixation.

Differentiation factor preparation:

A range of 15ng/ml to 500ng/ml was selected based on previous studies of differentiation factors effect on NSPCs *in vitro* (45, 67, 87). All differentiation factors were prepared in a similar fashion. Retinoic acid (Sigma) was prepared with special care to restrict its decomposition. Aliquots of its crystal were prepared using a dry-box which contained a nitrogen atmosphere thus enabling further long term storage once the original packaging was opened. Retinoic acid was weighed and stored in small air tight dark containers until dilutions could be created. Retinoic acid was dissolved in 100% ethanol to a concentration of 2.7 mg/ml which was diluted further with 0.1% BSA in 10mM PBS to obtain a stock concentration of 10 µg/ml. Thus,

the final ethanol concentration was far less than 0.1%, which has been documented as being not detrimental for reported cell models (88).

Platelet derived growth factor isoform AA (Biovision) and bone morphogenic protein – 4 (Peprotech) were dissolved in 0.1% BSA 10mM PBS to prevent protein adhesion to centrifuge tubes and other plastic apparatus. All differentiation factors were filter sterilized using a 0.2 μ m syringe pressure filter (VWR). Differentiation factors were made into small aliquots and kept at -80°C until required. Appropriate dilutions were obtained through serial dilution of the stock 500ng/ml concentration using fresh culture media. 8 wells of a 96 well plate were used for each concentration assessment of differentiation, proliferation and toxicity. Seven day old neurospheres from both the SC and SVZ derived cultures were obtained and plated at low density (3-4 neurospheres/condition) in poly-l-ornithine treated plates. Following the 7 day exposure to each differentiation factor NPCs were fixed with 4% PFA for 20 minutes and washed three times with 10 mM PBS. Cells were stored in 10mM PBS at 4°C until immunocytochemistry could be performed.

PLGA Channels Fabrication:

All PLGA fabrication was kindly carried out by Sagedeh Sadat Shahabi from Dr. Xudong Cao's lab at the University of Ottawa. Poly (D, L-lactide-co-glycolide acid) pellets of D, L-lactide/glycolide ratio of 50/50 with an Inherent Viscosity range (I.V. range) of 0.76 – 0.94 dL/g. were obtained from LACTEL Absorbable Polymers. Chloroform was purchased from VWR International. Dry CO₂ was supplied by BOC (Ottawa, ON) and glass rods with outer diameter 3 mm were purchased from Pegasus Industrial Specialties Inc (Cambridge, ON). Polymer was

dissolved in chloroform to obtain 20 % (W/V) solution and the glass rod was dipped in to the solution and dried for 3 hours horizontally using BDC 2002 digital stirrer at a speed 250 rpm to avoid formation of bubbles, prior to the coating of another layer on top. This process was repeated 5 times in order to achieve 5 layer channels with a channel wall diameter 0.1 mm. Following dip coating process, the polymeric coated glass rods were dried for 5 days in a vacuum chamber, at a gauge pressure of -24 kPa and room temperature. Later on, the dried channels were placed into cylindrical Teflon molds and capped at both ends very well to ensure the final accurate dimensions for the channels. At the end, the loaded molds were soaked in sub critical CO₂ foaming at a pressure of 5.6 MPa at room temperature (25°C) for 6 hours.

PLGA Microsphere Fabrication:

Using an emulsion process three solutions were prepared, including PVA (0.1%), PVA (1%) and PLGA (0.05%). Next, the PVA (1%) solution was added to PLGA solution and the mixture was sonicated for 5 minutes. The sonicated solution was then poured in to the filtered PVA (0.1%) and stirred for approximately for 4 hours. The whole procedure was done under sterile conditions in a Biosafety Cabinet. Once the stirring procedure was completed samples were washed 5 times with DDH₂O, and refrigerated for 5-6 hours before the freeze drying step. To achieve total sterilization, after 48 hours of freeze drying, microspheres were spread on a glass board and left under ultraviolet (282 $\mu\text{W}/\text{cm}^2$) exposure for almost 12 hours.

PLGA preparation for Cell Culture:

PLGA channels were sliced to weigh 3.7mg per pieces then soaked in providone iodine (Betadine) solution for 24 hrs. The iodine solution was aspirated and channels were rinsed in 70% ethanol for a total of three washes. Following the final ethanol rinse the channel pieces were washed three times with sterile Dulbecco's PBS (Gibco–Invitrogen) for 30 minutes. Channels were then placed in a 100x antibiotic (penicillin/streptomycin) solution overnight. The antibiotic solution was aspirated and channels segments were washed a final three times in sterile PBS. Two mm pieces of PLGA were then placed into 150 μ l of NPC culture media. PLGA microspheres were fabricated under sterile conditions and did not require an iodine/ethanol wash. Briefly, PLGA microspheres were diluted with culture media to a concentration of 25mg/ml. 150 μ l of the final dilution was added to each well of a 96 well plate. The final mass of PLGA microspheres in each well was 3.7mg.

PLGA culture supplementation:

Supplementation was carried out by removing half of the culture media from each well and replacing it with the same volume of new or conditioned (containing PLGA channels or microspheres) media containing new mitogens for the proliferation period of the experiments and no mitogens for the differentiation period of the experiments. Cells were maintained in proliferation conditions for 7, 14 and 21 days. Following the completion of these proliferation periods primary neurospheres were transferred from their low attachment culture plates to poly-ornithine coated 96 well plates and allowed to differentiate in mitogen free conditions for an additional 7 days. Therefore, our longest time-point for PLGA exposure is 28 days *in vitro*

(28DIV). On the sixth day of the differentiation experiments, NPCs were pulsed for 24hrs with 20 µg/ml of 5-bromo-2'-deoxyuridine (BrdU).

Table 2: Primary Antibodies and targeted antigens for the different cell types assessed.

Catalog # : Company	Targeted Antigens	Clone	Isotype	Species Specificity	Working Dilution
Primary : NEURONS					
01409: SCT	Neuronal Class III b-tubulin	TUJ1	Mouse IgG2a	Mouse, rat, human	1/500
Secondary					
A-11037: Invitrogen	Alexa Fluor 594	-	Goat IgG (H+L)	Mouse Ig G	1/300
Primary : ASTROCYTES					
AB5804: Millipore	Glial Fibrillary Acidic Protein (GFAP)	-	Rabbit IgG1	Mouse, Rat, Human	1/200
Secondary					
A-11008: Invitrogen	Alexa Fluor 488	-	Goat IgG (H+L)	Rabbit Ig G	1/300
Primary : OLIGODENDROCYTES					
MAB1326: R & D	Oligodendrocyte Marker O4	O4	Mouse IgM	Mouse, Rat, human	1/150
Secondary					
A-11032: Invitrogen	Alexa Fluor 594	-	Goat Poly.	Mouse Ig M	1/300
Primary: Radial Glia					
Ab32423	Brain Lipid binding protein (BLBP)	-	Rabbit Polyclonal	Mouse, Rat	1/300
Primary: PROGENITORS					
MAB353: Millipore	Nestin	-	Mouse IgG	Mouse, rat, human	1/200
Secondary					
A-11037: Invitrogen	Alexa Fluor 594	-	Goat IgG (H+L).	Mouse IgG	1/300
Primary					
Ab97959: AbCAM	SOX2	Polyclonal	Rabbit IgG	Mouse, Rat, Chicken	1/200
Secondary					
A-11008: Invitrogen	Alexa Fluor 488	-	Goat IgG (H+L)	Rabbit, IgG	1/200
Primary					
AB5320: Millipore	NG2	Polyclonal	Rabbit IgG	Mouse, Rat, Human	1/100
Secondary					
A-11008: Invitrogen	Alexa Fluor 488	-	Goat IgG (H+L)	Rabbit, IgG	1/200
Primary					
MAB3424	BrdU	AH4H7-1	Mouse IgG	N/A	1/200
Secondary					
A-11037: Invitrogen	Alexa Fluor 594	-	Goat IgG (H+L).	Mouse IgG	1/300

Assessment of pH change:

To assess the effect of PLGA degradation on the pH of the culture media, culture media was collected at each final time point (14, 21 and 28 div) and compared to its respective control. In order to determine the pH of the media a bench top pH reader was used.

Immunocytochemistry:

Once cells were fixed at room temperature in 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes cells were washed with 10mM PBS and stained using standard immunofluorescence techniques. Cells were blocked and permeablized with 10% normal goat serum (Genotec) and 0.1% Triton X for 45min if they were to be stained for beta-III-tubulin (Stem Cell Technologies, Vancouver BC), glial fibrillary acidic protein (GFAP), brain lipid binding protein (BLBP), nestin, BrdU. For Oligodendrocyte maker, O4, cells were blocked for the total 45 minutes but permeablized for only 15 minutes. Neurosphere sections stained for NG2 were permeablized and blocked with 5% NGS in 0.1% Triton-X for 45 minutes. Cells were then washed three times with 10 mM PBS to remove all of the permeablizing agent. After the third wash, cells were incubated with their respective antibodies overnight at 4°C. Antibody dilutions are available in Table 1. Primary antibodies were removed the following day and the cells/sections were washed three times with 10mM PBS. Following the third wash, cells were incubated in the dark at 25°C with anti-mouse and/or anti-rabbit Alexa Fluor 488 and/or 594 conjugated secondary antibodies (1/200; Invitrogen, ON, Canada). After the secondary incubation cells were washed an additional three times with 10mM PBS. After the final wash, nuclei were labelled with Hoechst (1 µg/ml, Invitrogen) for 15-30 minutes and then quickly washed with 10mM PBS. Monolayer cells were

maintained in 10mM PBS while being visualized while neurosphere sections were covered with coverslips mounted using Vectashield mounting media (Vector Labs). Culture plates were maintained in 4°C until visualized and neurosphere sections were stored at -20°C until visualized.

TUNEL assay and BrdU co-labelling:

To assess DNA fragmentation cells were labelled using the Terminal deoxynucleotidyl transferase dUTP nick end labelling assay (TUNEL). Briefly, cells were permeabilized with ice cold 0.1% Triton X in sodium citrate buffer (Sigma) and using the *In Situ* Cell Death Kit from Roche DNA fragmentation was labelled with a fluorescein conjugated dUTP. Following permeabilization cells were washed and treated with the Roche Enzyme mixture containing the terminal deoxynucleotidyl transferase and the fluorescein conjugated dUTP. Cells were placed in humidified chamber at 37°C for 1 hour then washed with PBS. When cells or sections were to be co-labeled with BrdU further staining procedures occurred. First, a BrdU antigen retrieval was required. Cells were treated for 30 minutes at 37°C for 30 minutes in a humidified chamber. Cells were quickly washed three times with PBS and standard immunocytochemistry was used to label BrdU positive cells. Cells were counterstained with hoescht and maintained in PBS during visualization.

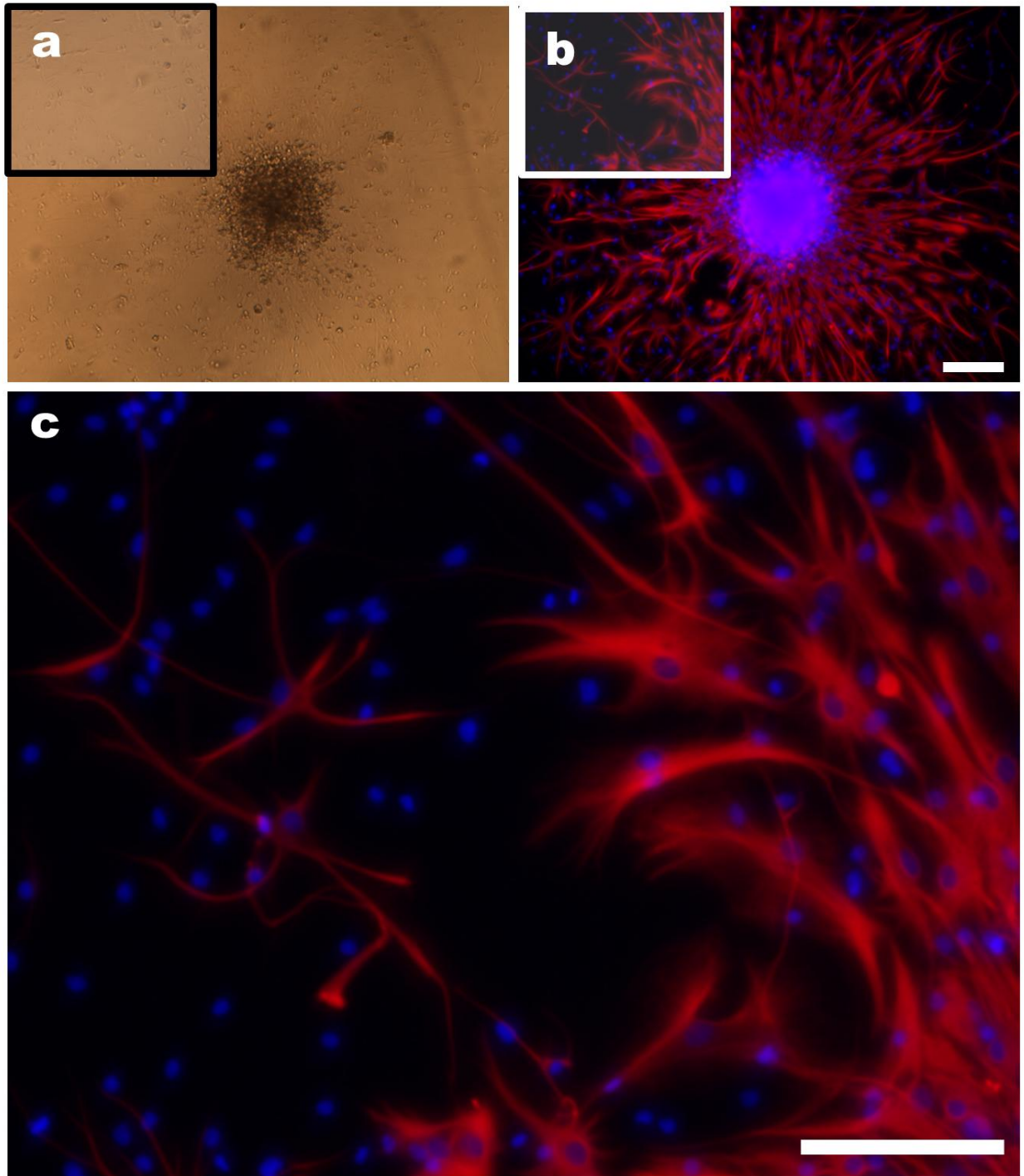


Figure 2.3 – Selection of field of view for quantification of NPC differentiation potential. Neurospheres were located with brightfield (a) and EPI fluorescence (b). 20x fields of view (c) were taken directly adjacent to the border of the densely Hoescht packed core of the spheres. Only cells which migrated outside of the neurosphere core were used in total cell counts and differentiation potential assessments. Scale bar = 150 μm .

Field of view selection:

All conditions were visualized with a Nikon Ti Eclipse epifluorescence or C1 laser scanning confocal microscope. Fields of view were selected by locating the neurosphere and taking a photo directly adjacent to each neurosphere as shown in Figure 2.3 (41, 89). Most conditions contained 3 neurospheres each, thus three fields of view from each well were taken, 1 adjacent to each neurosphere found within the well. In the earlier time points of 7 div proliferation and 7 div differentiation NPCs readily migrated out from the neurospheres forming dense monolayers. In these instances where neurosphere borders could not be easily identified fields of view were taken where the densest collection of Hoescht labelled nuclei could be found. This collection of Hoescht labeled nuclei was assumed to be cells originating from neurospheres.

Image analysis and statistical analysis:

Stained wells were visualized with a Nikon Ti eclipse fluorescent microscope. To obtain sufficient samples, bound neurospheres were located and one field of view was captured directly adjacent to each neurosphere for a total of 3 neurospheres per stain per trial. Images were merged and manually analyzed using Image J (NIH). The total number of immuno-positive cells and total number cells (Hoescht+) was captured. Percent averages were obtained by combining data of 3 fields of view from 3 biologically independent experiments. Therefore, a total of 9 fields of view were obtained. For neurosphere cryosections at least 8 spheres were used per stain and three sections from the middle portion of each sphere were imaged. Differentiation factor comparisons were conducted using pooled control data across three differentiation factor experiment. To determine statistical significance we used chi-squared analysis (neurosphere

size), a one-way or two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test was completed comparing each experimental group to their respective control group using SPSS (IBM). Data is presented as percent mean \pm standard error of the mean.

Chapter 3 – Results

Phenotyping of primary neural progenitors from the SVZ and SC: Neurosphere size.

Neurosphere size is a common observation used to assess the degree of proliferation and stemness of a colony of progenitors. We assessed our NPC cultures for neurosphere diameter over the proliferation periods of 7, 14 and 21 days *in vitro*. Figure 3.1 shows the distribution of both SC (Fig. 3.1, a & c) and SVZ (Fig. 3.1, b & c) derived neurospheres following 7 days of mitogen exposure. Using a chi-squared analysis we found that SC derived cultures produced predominately smaller (<100) neurospheres 80% that were $65 \pm 4.1 \mu\text{m}$ in diameter, while SVZ derived cultures produced a larger range of neurosphere sizes with 44.4 % being on average $124.6 \pm 1.2 \mu\text{m}$ in diameter. Additionally, SVZ derived cultures had 29.6 % of neurospheres much larger than the spinal cord derived cultures with an average neurosphere size of $195.7 \pm 8.5 \mu\text{m}$ ($\chi^2(1, N=930)=227.5, p<0.001$).

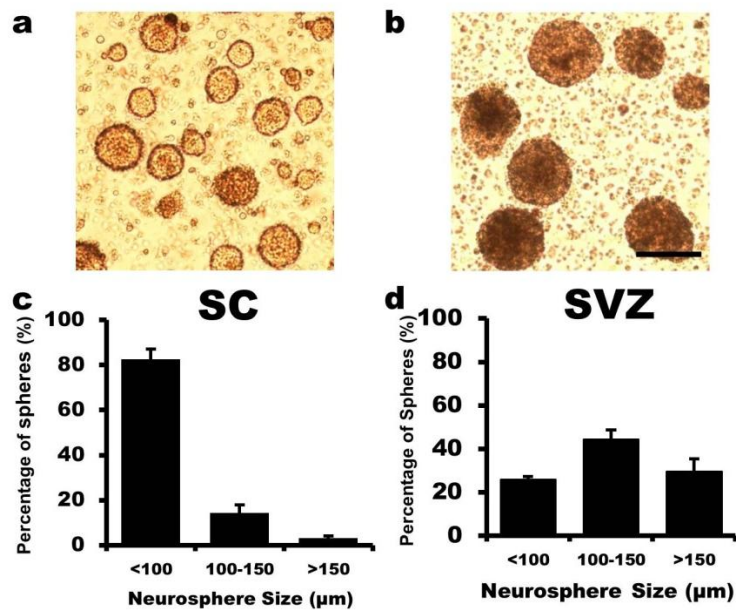


Figure 3.1 – Primary neurosphere size following a 7 div proliferation period. Spinal cord derived n=439 (a & c) and SVZ derived n=491 (b & d) neurospheres show distinct differences in size after 7 days *in vitro* assessed using a chi-squared analysis. Results presented as mean \pm SEM and are expressed as % total number of spheres found in each size range. Scale bar = 150 μm .

Once neurospheres became visible at the 7 div point they were transferred to low density conditions, as can be seen in the difference between the neurosphere backgrounds seen in Figure 3.1 a & b (high density) and Figure 3.2 a & b (low density). This transfer from high to low density ensured that sphere size increases were a result of true neurosphere growth and not neurosphere mergers which can commonly occur in high density conditions. Looking at the distribution of neurosphere size it is possible to see that SC and SVZ derived cultures continue to produce larger neurospheres. It is more evident in SC derived cultures with the distribution of neurosphere size showing a more equal distribution over all categorized ranges (Fig 3.2 c). In SC derived cultures at 14 div the majority - 38.3 % - of neurospheres were greater than 100 μm but less than 150 μm with an average neurosphere size of $123.2 \pm 1.1 \mu\text{m}$. SVZ derived cultures also showed a continued increase in neurosphere size, however, the majority of neurospheres were found in the greater than 150 μm range with an average neurosphere size of $206.4 \pm 1.9 \mu\text{m}$ (Fig 3.2 d). No statistical difference was noted between SC and SVZ neurosphere sizes ($\chi^2 (1, N=1010) = 3.7, p=0.053$).

Following an additional 7 days in culture both SC and SVZ derived neurospheres continued to grow larger. SC derived cultures produced many more larger neurospheres with 68.2 % found to be larger than 150 μm . The average spinal cord derived neurosphere size after 21 div was $252.2 \pm 9.8 \mu\text{m}$ (Fig 3.3 a & c). SVZ derived neurospheres also were predominately larger than 150 μm with 50.4% of spheres having an average diameter of $231.3 \pm 17.8 \mu\text{m}$ (Fig 3.3 b & d). A final chi-squared analysis was performed showing that both SC and SVZ derived spheres produced larger (>100) spheres ($\chi^2 (1, N=532) = 2.98, p=0.84$).

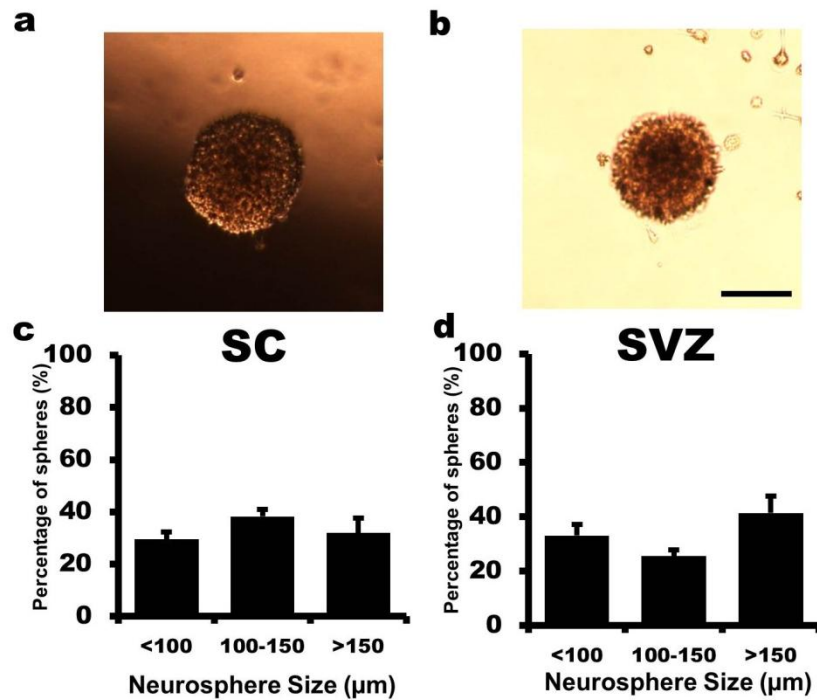


Figure 3.2 – Primary neurosphere size following a 14 div proliferation period. Spinal cord derived n=517 (a & c) and SVZ derived n=493 (b & d) neurospheres show no differences (chi-squared) in size after 14 days *in vitro*. Results presented as mean \pm SEM and are expressed as % total number of spheres found in each size range. Scale bar = 150 μm .

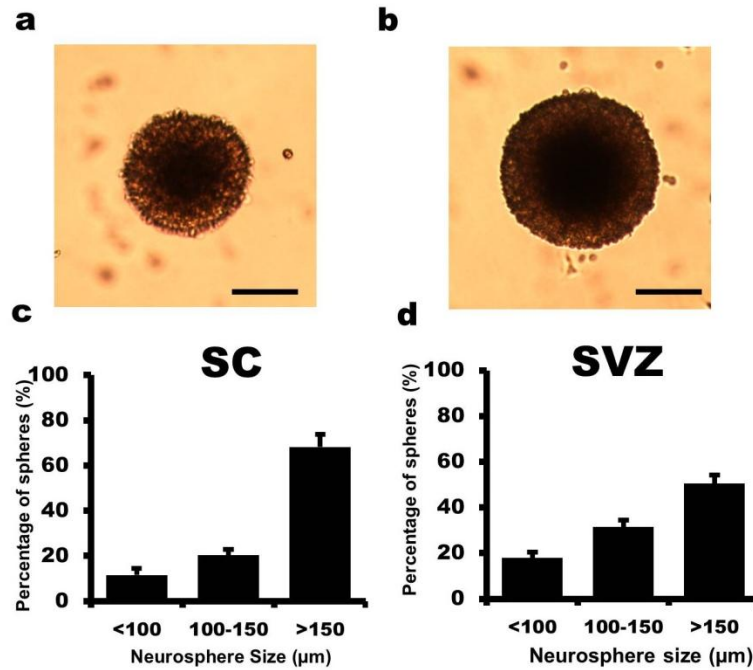


Figure 3.3 – Primary neurosphere size following 21 div proliferation period. Spinal cord derived n=312 (a & c) and SVZ derived n=220 (b & d) neurospheres show no differences in size after 21 days *in vitro* following a chi squared analysis. Results presented as mean \pm SEM and are expressed as % total number of spheres found in each size range. Scale bar = 150 μ m.

Progenitor proliferation and toxicity:

Neurospheres from each proliferation time point were collected and sectioned. Immunofluorescence revealed the effect of time in culture on the proliferation and cell viability of the NPCs within the primary neurospheres. Both SC and SVZ derived NPCs had similar percentages of BrdU positive cells at all-time points 7, 14 and 21 div (Fig 3.4). At 7 div SC neurospheres had 34.6% positive cells for BrdU while SVZ derived neurospheres had 31%. Following an additional 7 days in culture we found that SVZ derived neurospheres had a significant decrease in the percentage of NPCs positive for BrdU. Interestingly, BrdU positive progenitors are still found through the core of the neurosphere section. Therefore, even cells found within the deep centre of the neurosphere in both cell types are still proliferating (Fig.3.5). A further 7 days in culture we see that NPC proliferation does not change from the 14 div percentages with proliferation remaining close to 20% positive for BrdU (Fig 3.4). Thus, there was a significant decrease in the proliferation of neurospheres as time progressed in culture ($F(2, 164) = 10.8, p < 0.001$), There was no significant interaction between time in culture and cell type ($F(2, 164) = 1.6, p = .209$). No significant changes in cell viability were noted between each cell type or each time point.

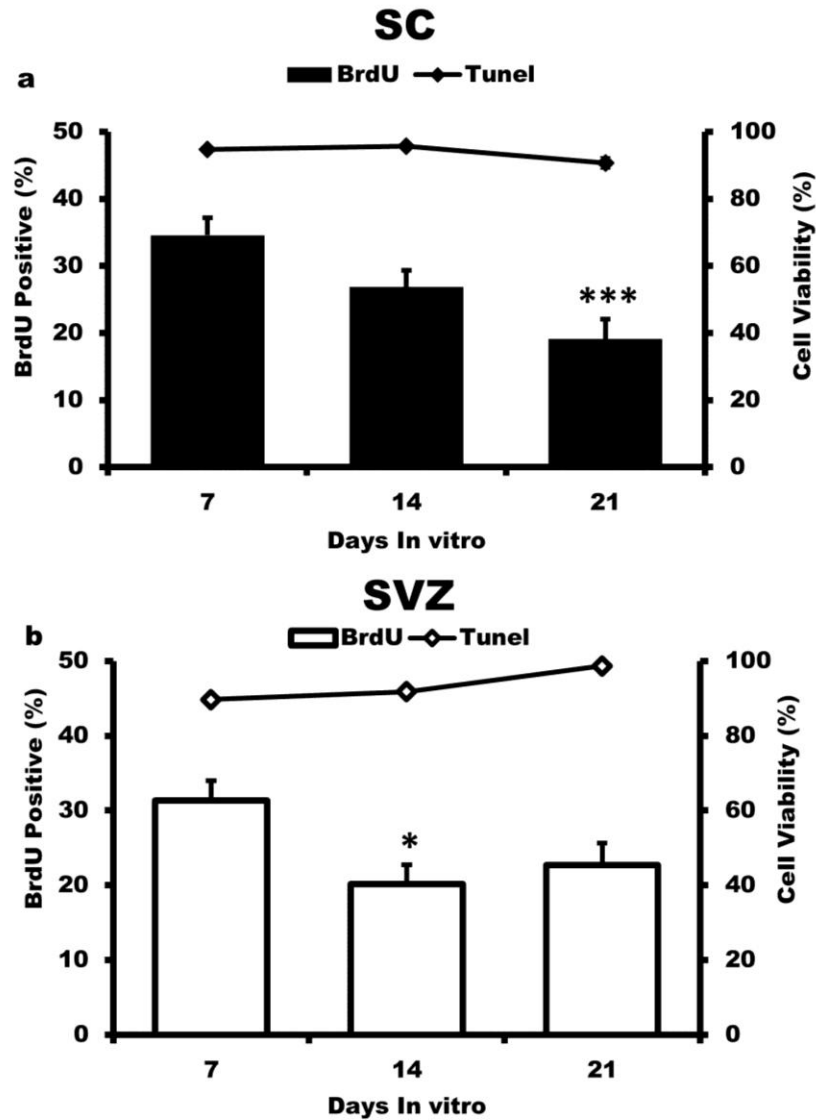


Figure 3.4 – Time in culture’s effect on whole sphere cell viability and proliferation. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for BrdU or TUNEL and Hoescht. A) SC derived NPC proliferation and cell viability. B) SVZ derived NPC proliferation and cell viability. Statistical significance assessed with a two-way ANOVA and shown with * ($p < 0.05$) and *** ($p < 0.001$).

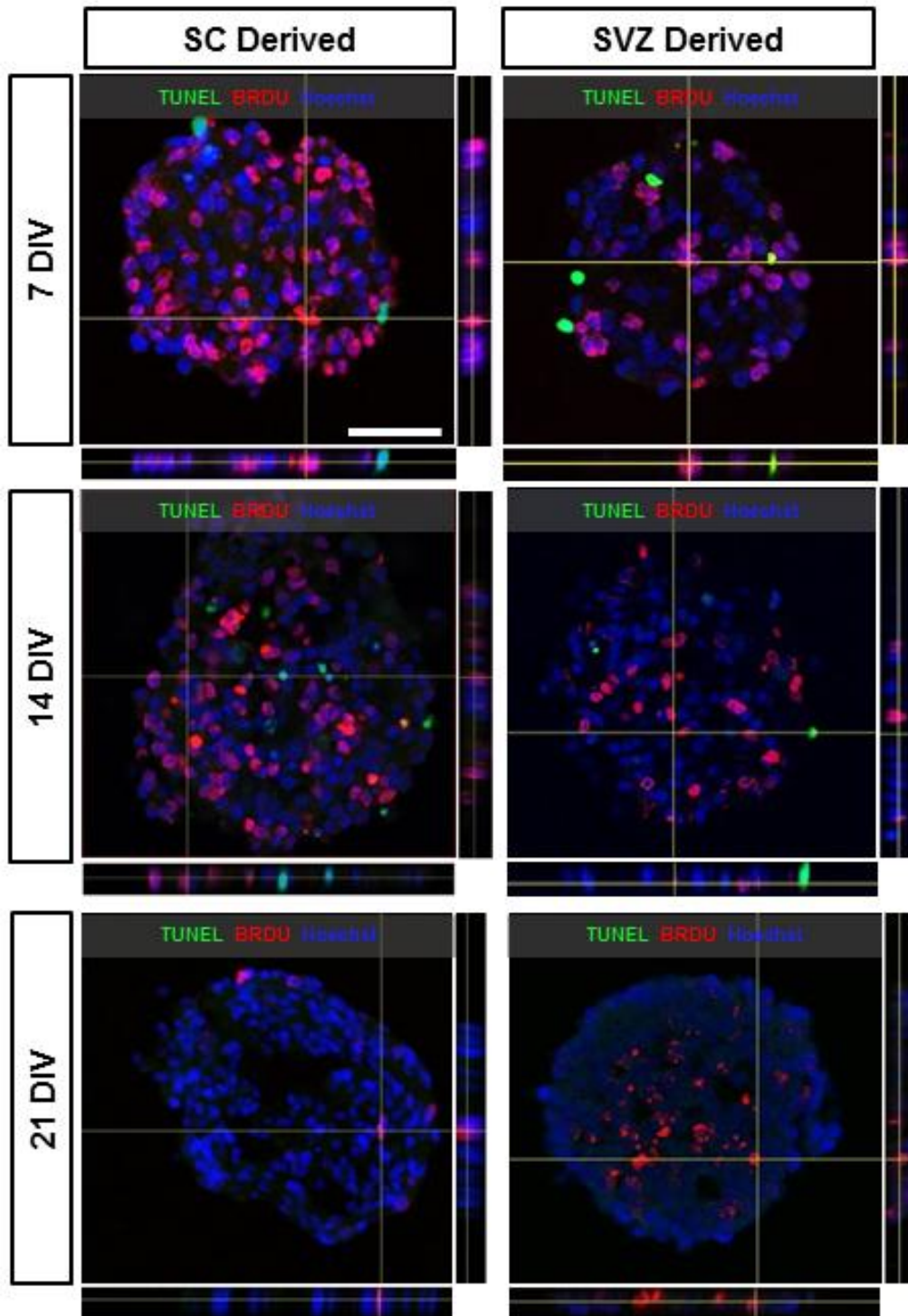


Figure 3.5 – Primary neurosphere proliferation and viability following extended time in culture. Both SC derived and SVZ derived NPCs show BrdU labeling in the core of whole spheres with limited TUNEL labeling. Indicating that time in culture has limited effects on the position of viable and proliferating cells. Scale bar = 50 μ m.

GFAP/Nestin + Nestin positive progenitors:

Neurospheres were sectioned to examine the phenotype of the progenitor cells found within the core of the spheres. We were interested in discovering whether progenitor identity changed as time in culture progressed.

Both SC and SVZ derived NPCs produced GFAP/nestin and nestin positive cells (Fig 3.6 a & b; Fig 3.7). SC derived NPCs were found to have GFAP negative / nestin positive ($25.7 \pm 3.1\%$) and also GFAP/nestin positive ($16.8 \pm 2.8\%$). SVZ derived NPCs were also GFAP/nestin positive ($24.8 \pm 2.0\%$) and GFAP negative / nestin positive ($22.86 \pm 1.73\%$). Interestingly, as time progressed in culture a significant reduction in the percentage of both nestin and GFAP/nestin positive progenitors was found. No significant reduction in the number of cells was seen as time progressed in culture. SC derived NPCs saw an overall reduction in nestin expression from $42.6 \pm 2.9\%$ at 7 div to $15.5 \pm 1.6\%$ at 14 div and $21.6 \pm 2.2\%$ at 21 div. SVZ derived NPCs showed a similar reduction in the percentage of nestin positive progenitors at both 14 and 21 div with nestin values dropping from $47.7 \pm 2.0\%$ at 7 div to $19.0 \pm 2.8\%$ and $21.4 \pm 3.4\%$ for 14 and 21 div, respectively. SVZ progenitors were positive for both GFAP and nestin at both 14 ($16.4 \pm 2.8\%$) and 21 ($16.9 \pm 3.9\%$) div. While SC derived NPCs were also GFAP/nestin positive (14div : $9.34 \pm 1.4\%$; 21 div: $11.0 \pm 1.3\%$) and GFAP negative / nestin positive (14div: $6.8 \pm 1.2\%$; 21div: $13.2 \pm 2.4\%$) at both 14 and 21 div. There was a significant decrease in the percentage of GFAP/nestin + Nestin positive cells as time progressed in culture ($F(2, 161) = 68.1, p < 0.001$), However, there was no significant interaction between time in culture and cell type ($F(2,161) = 0.583, p = .210$). Furthermore, the position of the nestin

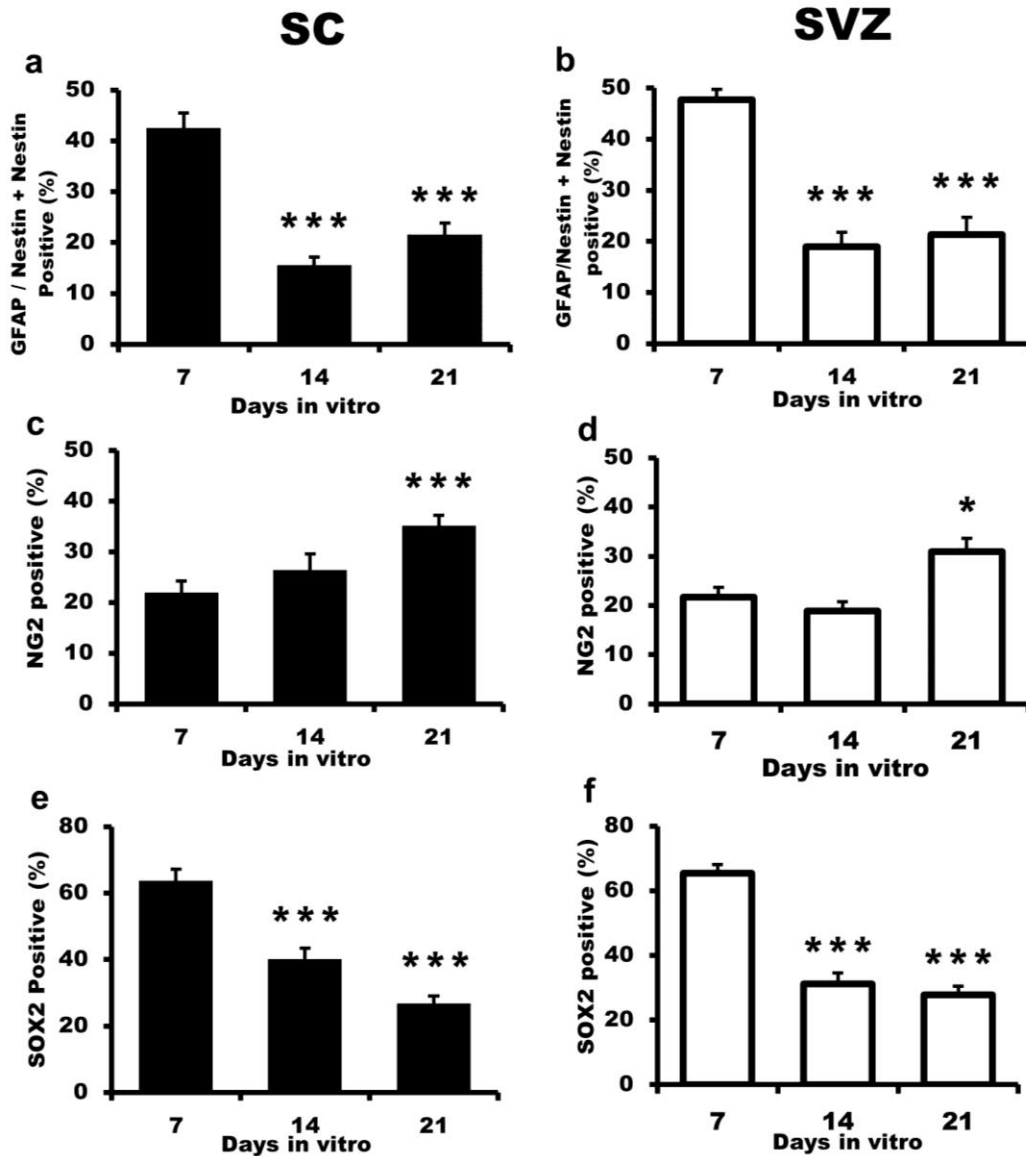


Figure 3.6 – Time in culture’s effect on neural progenitor characteristics. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for Nestin (a & b) or NG2 (c & d) or Sox2 (e & f) and Hoescht. Both SC derived (a) and SVZ derived (b) NPCs showed significant reductions in Nestin and Sox2 positive cells with an increase in NG2 positive NPCs with extended time in culture.. Statistical significance found with a two way ANOVA and Tukey’s post-hoc test results shown here with * ($p < 0.05$) and *** ($p < 0.001$).

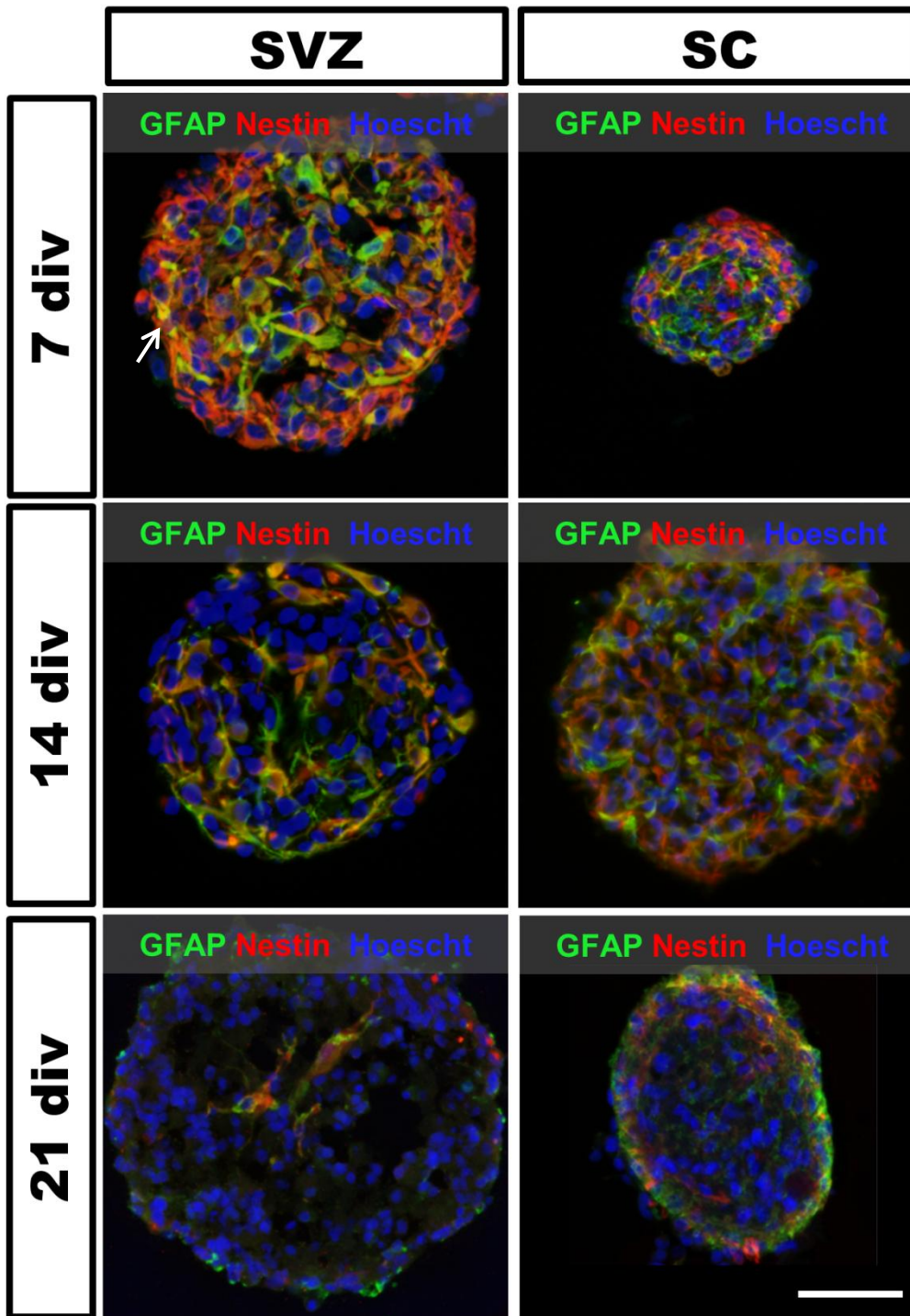


Figure 3.7 – Primary neurospheres derived from both the SVZ and SC co-express GFAP and nestin. An example of the co-expression is indicated by the arrow. Following extended time in culture the percentage of GFAP/nestin and nestin positive cells decreases. Scale bar = 50 μ m.

positive cells changed with extended time in culture. At 7 days *in vitro* nestin positive cells can be found throughout the section without localizing to any particular aspect of the neurosphere. At 21 days *in vitro* we can see that these GFAP/nestin positive cells have localized to the perimeter of the SC spheres rather than throughout the center of the section (Fig. 3.7).

Sox2 positive progenitors:

We chose to use Sox2 as a measure of the proliferative and multipotency capacity of these primary neural progenitors (90). As is evident from Figure 3.8 primary neurospheres have progenitors that are positive for Sox2 at all proliferative time points assessed. However, significant differences can be seen in the percentages of progenitors found within each sphere at each time point. Both SC and SVZ derived NPCs were found to be predominately Sox2 positive with $63 \pm 3\%$ and $65.4 \pm 2.4\%$ positive nuclei being found in each, respectively. As time progressed in culture neurospheres were maintained in proliferative conditions; however, a significant reduction in Sox2 labeling still occurred. At both 14 and 21 div we see a substantial decrease in the percentage of Sox2 positive cells found in both SC and SVZ derived neurospheres Figure 3.6 (e &f). Indicating that both SC and SVZ derived NPCs do not maintain Sox2 expression as they are maintained in large cell aggregates. There was a significant decrease in the percentage of Sox2 positive cells as time progressed in culture ($F(2, 160) = 88.1$, $p < 0.001$), However, there was no significant interaction between time in culture and cell type ($F(2,160) = 1.9$, $p = 0.147$).

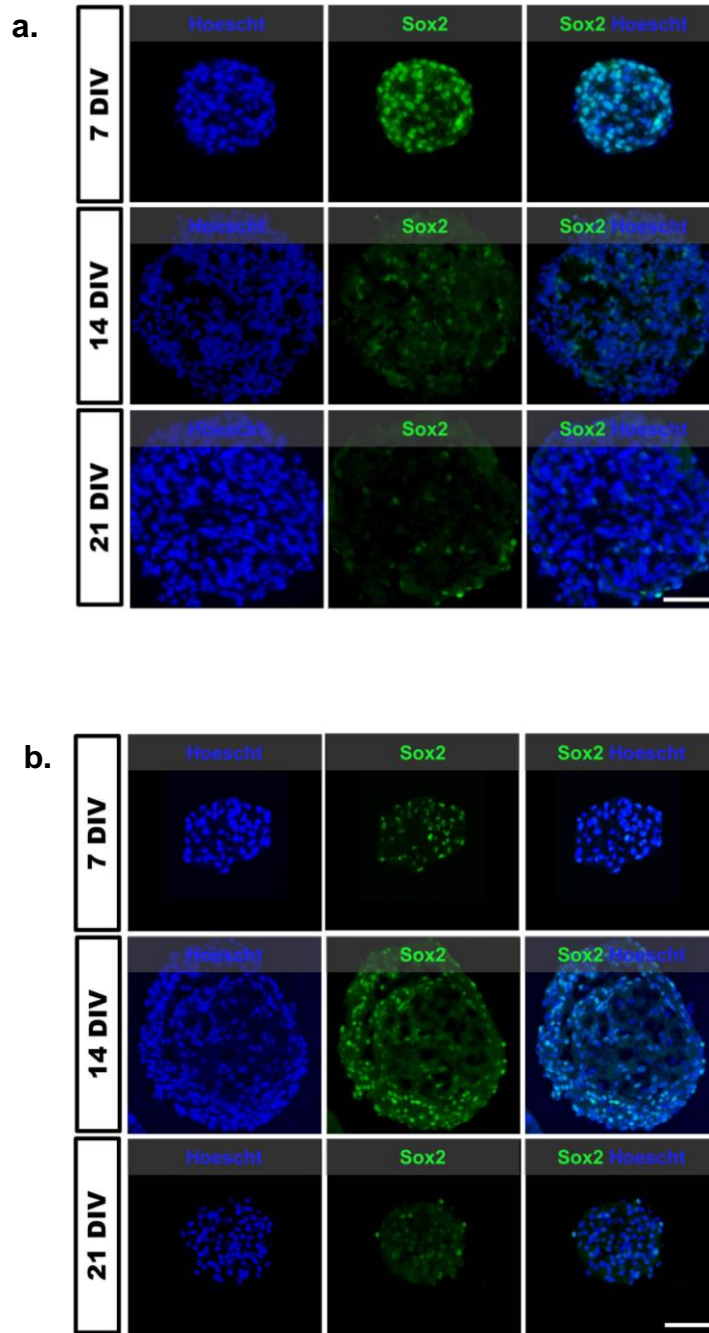


Figure 3.8 – NPC derived from SC and SVZ show reduction in Sox2 labeling following extended time in culture. SC (a) & SVZ (b) derived NPCs showed extensive Sox2 labeling at 7 days *in vitro* and subsequently a decrease in the percentage of Sox2 positive cells in older neurospheres. Scale bar = 50 μ m.

Ng2 positive progenitors:

The final progenitor phenotype assessed was that of NG2 positive glial progenitors. NG2 progenitors are thought to primarily support glial lineages when they differentiate they primarily give rise to astrocytes and oligodendrocytes (28, 29). We looked at the expression of NG2 within the neurospheres at 7, 14 and 21 div and found surprisingly that the number of NG2 positive progenitors does change with time in culture. Both SC and SVZ derived primary neurospheres started with close to the same percentage of NG2 positive progenitors with $21.9 \pm 2.2\%$ and $21.7 \pm 1.9\%$, respectively (Figure 3.6 c & d). At 14 div no significant increases in the percentage of NG2 positive progenitors occurred within both SC and SVZ derived progenitors. While after 21 div we saw the average percentage of NG2 positive cells found within the SC and SVZ neurospheres to be substantially increased to $35.1 \pm 2.1\%$ and $30.9 \pm 2.7\%$, respectively. There was a significant increase in the percentage of NG2 positive cells as time progressed in culture ($F(2, 149) = 13.7, p < 0.001$). However, there was no significant interaction between time in culture and cell type ($F(2,149) = 1.1, p = 0.335$). SC and SVZ derived neurosphere did differ in the percentage of NG2 positive cells found at later time points ($F(2,149) = 4.025, p = 0.47$).

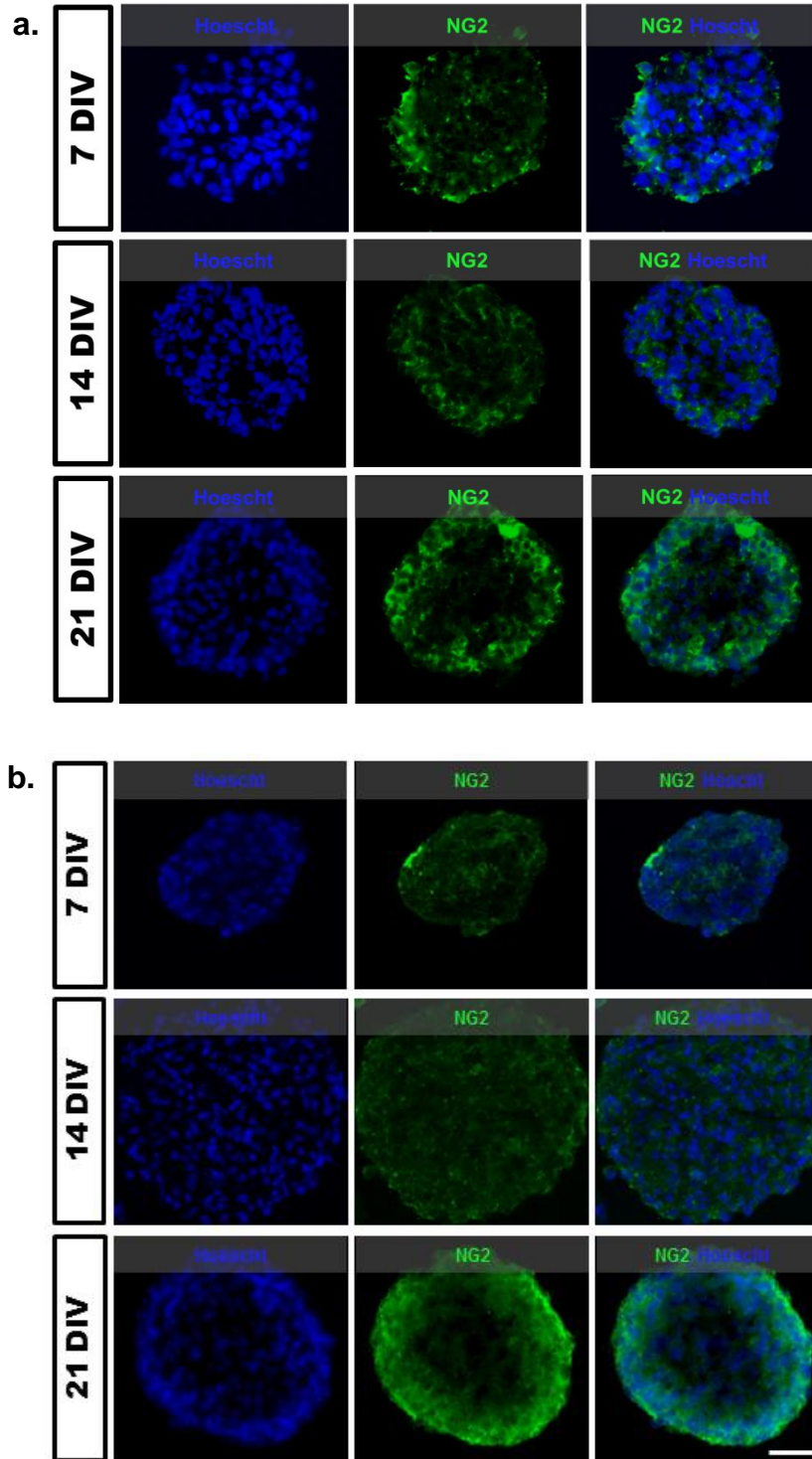


Figure 3.9 – NPC derived from SC and SVZ show an increase in NG2 labeling following extended time in culture. SC (a) and SVZ (b) derived NPCs showed little NG2 labeling following 7 days in vitro. After 14 and 21 days in vitro the percentage of NG2 positive cells increased. Scale bar = 50 μ m.

The effect of time in culture on NPC proliferation following a 7 day differentiation period:

Following the proliferation period neurospheres were transferred to poly-l-ornithine coated plates and allowed to differentiate in mitogen minus conditions. Following 7 days of differentiation the proliferation and toxicity was assessed using a 24 hour pulse of BrdU and TUNEL labeling for apoptotic cells. Both SC and SVZ derived NPCs showed similar degrees of proliferation at the end of each differentiation period. SC derived NPCs had $20.4 \pm 0.9\%$ of cells still proliferating while SVZ derived NPCs had $18.8 \pm 2.5\%$ of cells still proliferating. As time in culture progressed from 7 div to the later time points of 14 and 21 div it was evident that NPC proliferation remained relatively unaffected. SC derived NPCs continued to show extensive proliferation with the percentage of cells still proliferating at 14 and 21 div reaching $14.1 \pm 1.4\%$ and 13.8 ± 3.3 , respectively. SVZ derived NPCs had similar ranges of proliferation with the percentage of cells proliferating remaining at $22.2 \pm 2.8 \%$ and $17.8 \pm 3.1 \%$ for 14 div and 21 div groups, respectively (Figure 3.10).

Both cell types showed no changes in viability from 7 div through 14 and 21 div. SC and SVZ derived cultures had minimal TUNEL positive cells with viability remaining well above 95% viable at each time point.

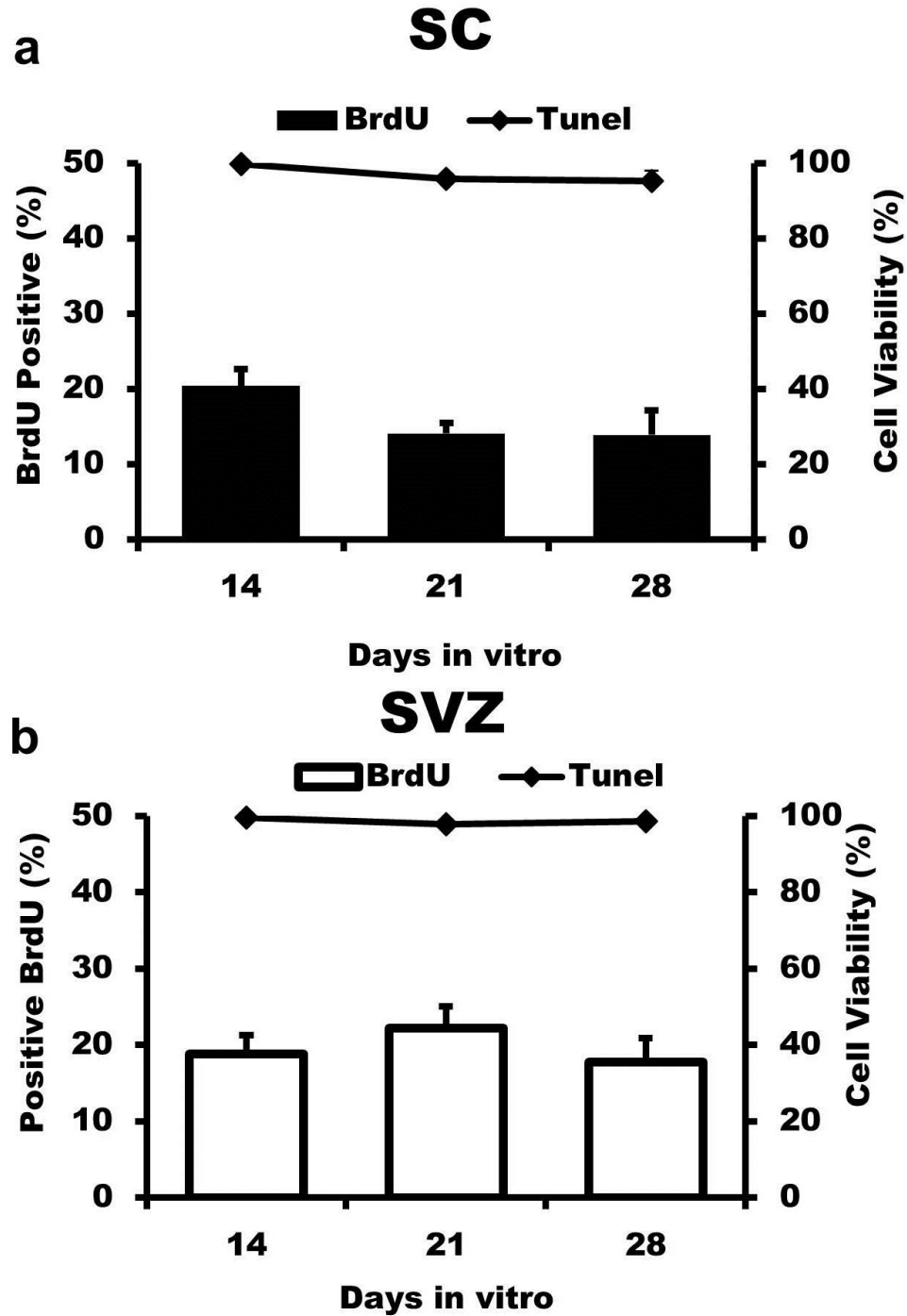


Figure 3.10 – Seven day old neurospheres derived from both the SC and SVZ showed no differences in proliferation and viability following a 7 day differentiation period after 7, 14 and 21 days in vitro. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for BrdU (a) or TUNEL (b). Both cell types were compared with an ANOVA single factor analysis. No statistical significance was found between cell types or across time points.

The effect of time in culture on NPC differentiation:

The differentiation potential of NPCs from the SC and SVZ was assessed following the 7 day differentiation period after each timepoint, 7, 14 and 21 div. Initially, the SC and SVZ derived NPCs showed different differentiation potentials with SC NPCs – $23.3 \pm 4.2\%$ - producing fewer GFAP positive astrocytes than SVZ neurospheres – $36.4 \pm 3.8\%$, ($F(1,48) = 12.8$, $p=0.001$) (Figure 3.11). SC derived NPCs were observed to differentiate into predominately O4 positive oligodendrocyte progenitors – $48.7 \pm 3.6\%$ while SVZ derived NPCs produced fewer O4 positive oligodendrocyte progenitors – $38.6 \pm 5.5\%$. However, following a two-factor ANOVA no statistical significance difference was found between the O4 differentiation potential of SC and SVZ derived NPCs at the 14 div time point. Both SC and SVZ derived NPCs showed modest degrees of BIIT positive neuron differentiation with SC NPCs producing $5.21 \pm 0.89\%$ and SVZ NPCs producing $6.96 \pm 1.23\%$.

As time in culture progressed SC and SVZ derived NPCs began to show more similarities in their differentiation potentials. SVZ derived NPCs at 21 div increased in the percentage of O4 positive progenitors. Although, an increase in O4 differentiation can be seen at both 21 and 28 div in SVZ derived cultures no statistical significant difference was noted (Figure 3.11).

SC derived cultures showed a significant increase in neuronal differentiation at extended times in culture. The neuronal differentiation potential of SC derived NPCs increased from 5.21

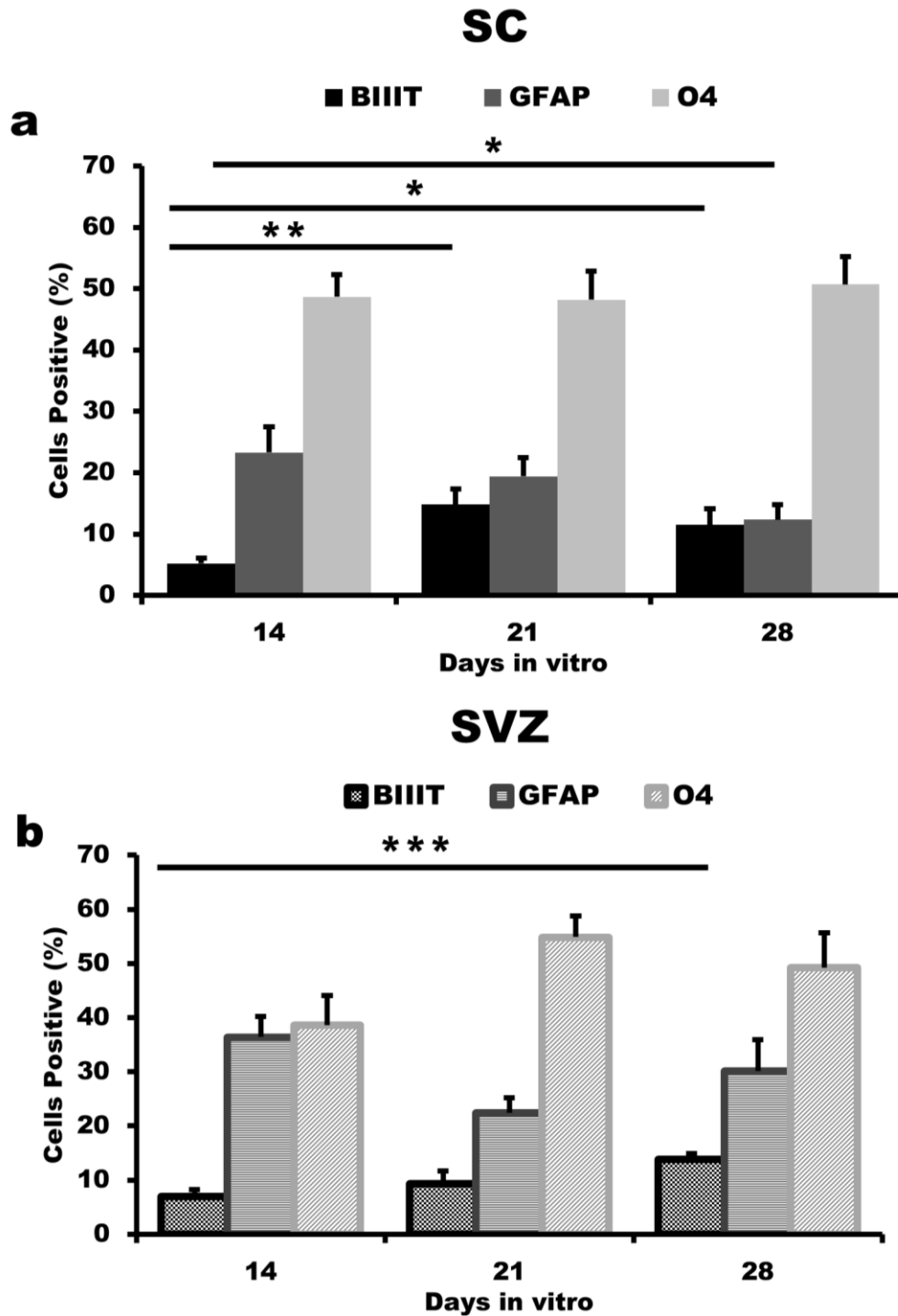


Figure 3.11 – The effect of time in culture on NPC differentiation. Results presented as mean \pm SEM and are expressed as % total number of cells positive for BIIT (neurons), GFAP (Astrocytes) and O4 (oligodendrocytes) (a & b). Both cell types were compared with a two factor ANOVA. Statistical significance found with a Tukey's post hoc test shown with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to 14 div.

$\pm 0.89\%$ to $14.79 \pm 2.5\%$ at 21 div and $11.5 \pm 2.6\%$ at 28 div ($F(2,48) = 6.2$, $p=0.04$) (Figure 3.11a). SVZ derived NPCs also increased their neuronal differentiation potential at extended time points. Only 28 div BIIT percentages were found to be statistically significant from 14 div differentiation percentages (Figure 3.11b).

NPCs from both the SC and SVZ were also assessed for their differentiation into radial glia and for the continued labeling of the progenitor marker nestin. As was found with the whole neurosphere assessment pre differentiation condition, SVZ derived cultures showed a continued reduction in nestin expression as time in culture progressed (Figure 3.12) ($F(2,24) = 4.77$, $p<0.05$). Also, SC derived neurospheres also showed a reduction in nestin expression at later time points, although this reduction was not statistically significant. BLBP was used as a marker for radial glia, interestingly time in culture did not seem to effect its expression in GFAP positive astrocytes (Figure 3.12 a & b). Indicating that SVZ derived neurosphere astrocytes may be losing their progenitor identity but still continuously express BLBP maintaining their radial glia identity. SC derived NPCs show reductions in both nestin and BLBP following time in culture which is complementary to the reduction seen in GFAP positive cells at later time points since it is most common to see nestin and BLBP expression co-expressed in GFAP positive astrocytes.

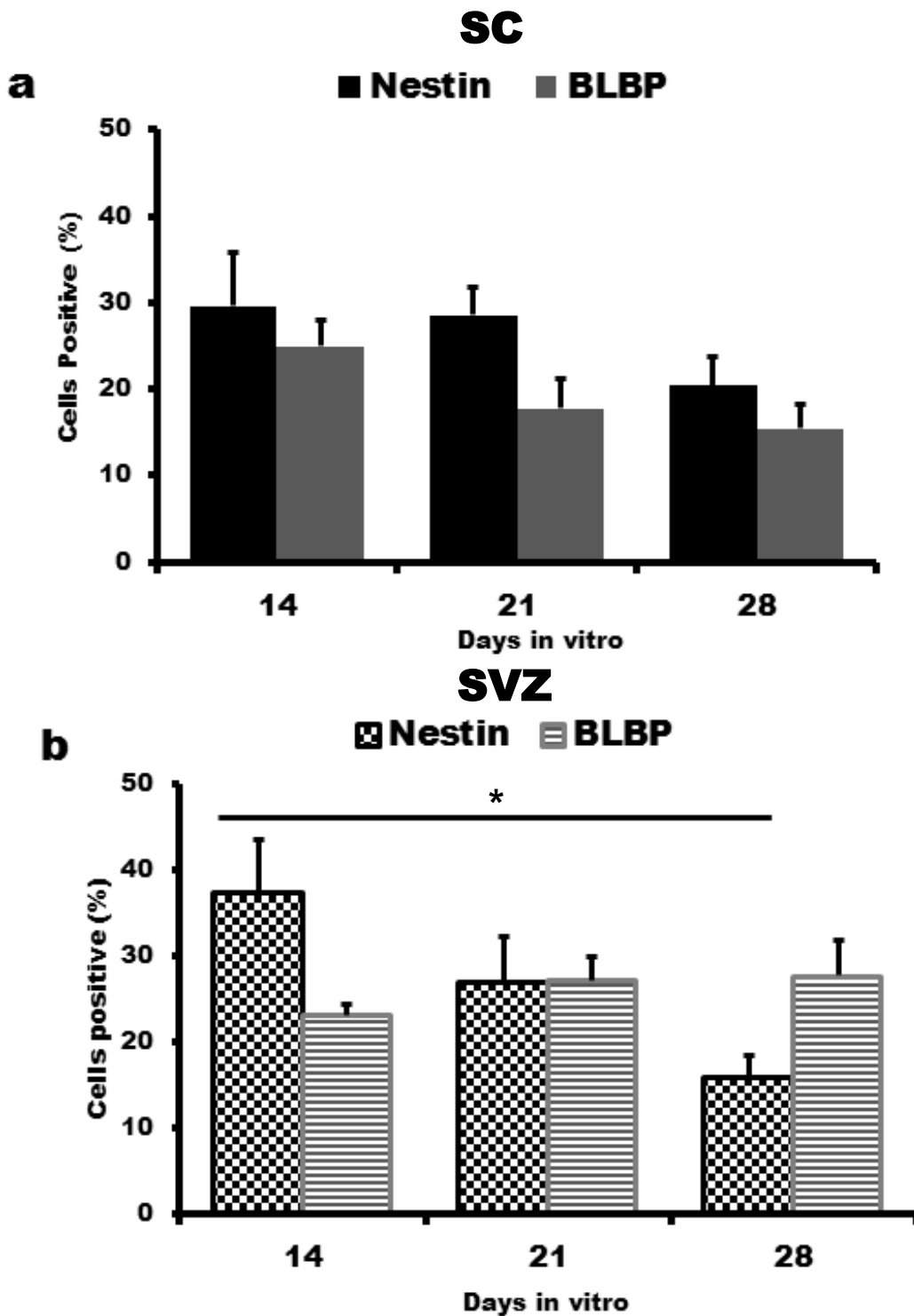


Figure 3.12 – The effect of time in culture on NPC differentiation. Results presented as mean \pm SEM and are expressed as % total number of cells positive for nestin (progenitors), BLBP (radial glia) (a & b). Statistical analysis between 14 day and later time points was completed with a one way ANOVA followed by Tukey's post hoc test. Statistical significance shown with * $p < 0.05$ when compared to 14 div.

Controlled differentiation of adult NPCs from the SVZ and SC

Retinoic acid as a differentiation factor for adult derived neural progenitor cells:

Our first assessment of RA's effect on NPCs was to determine its usefulness in driving differentiation rather than proliferation. Figure 3.13 reveals that in both SC and SVZ derived cultures higher concentrations of RA reduced the amount of BrdU positive cells. Although a reduction was noted in both cell types no significant decrease in the percentage of cells that were positive for BrdU was noted after a one-way ANOVA analysis. No differences in TUNEL labeling were observed at all concentrations within both cell types.

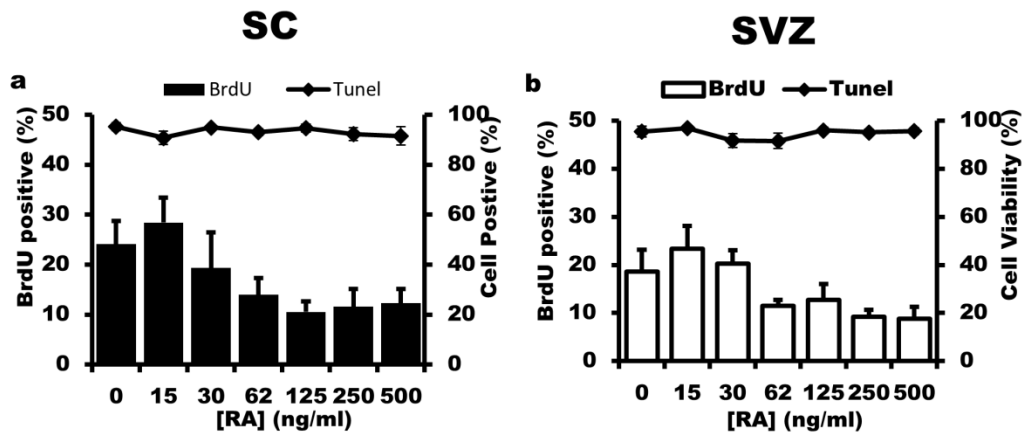


Figure 3.13 – Retinoic Acid's effect on cell viability and proliferation. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for BrdU or TUNEL and Hoechst. Only SC derived (a) NPCs showed significant reductions in proliferation at higher concentrations. Each concentration (15-500ng/ml) was compared to control (0ng/ml) conditions with an ANOVA single factor analysis. Although there was a trend towards a decrease in BrdU with increased RA, this was not statistically significant.

RA was chosen as a differentiation factor for its ability to encourage neuronal differentiation from a number of cell types (45, 54, 56). Here we find that RA was successful in increasing neuronal differentiation in these adult NPCs. However, SVZ derived NPCs were found to be more responsive than SC derived NPCs. Figure 3.14 a & b shows that concentrations of greater than 62 ng/ml of RA were able to support increases in neuronal differentiation of SVZ

derived NPCs. Neuronal differentiation was increased from $4.8 \pm 0.8\%$ to a maximum percentage of β IIIIT positive cells of $20.2 \pm 4.2\%$ at 250ng/ml. statistical significance was noted with a one-way ANOVA, $F(6,56) = 4.7, p=0.001$).

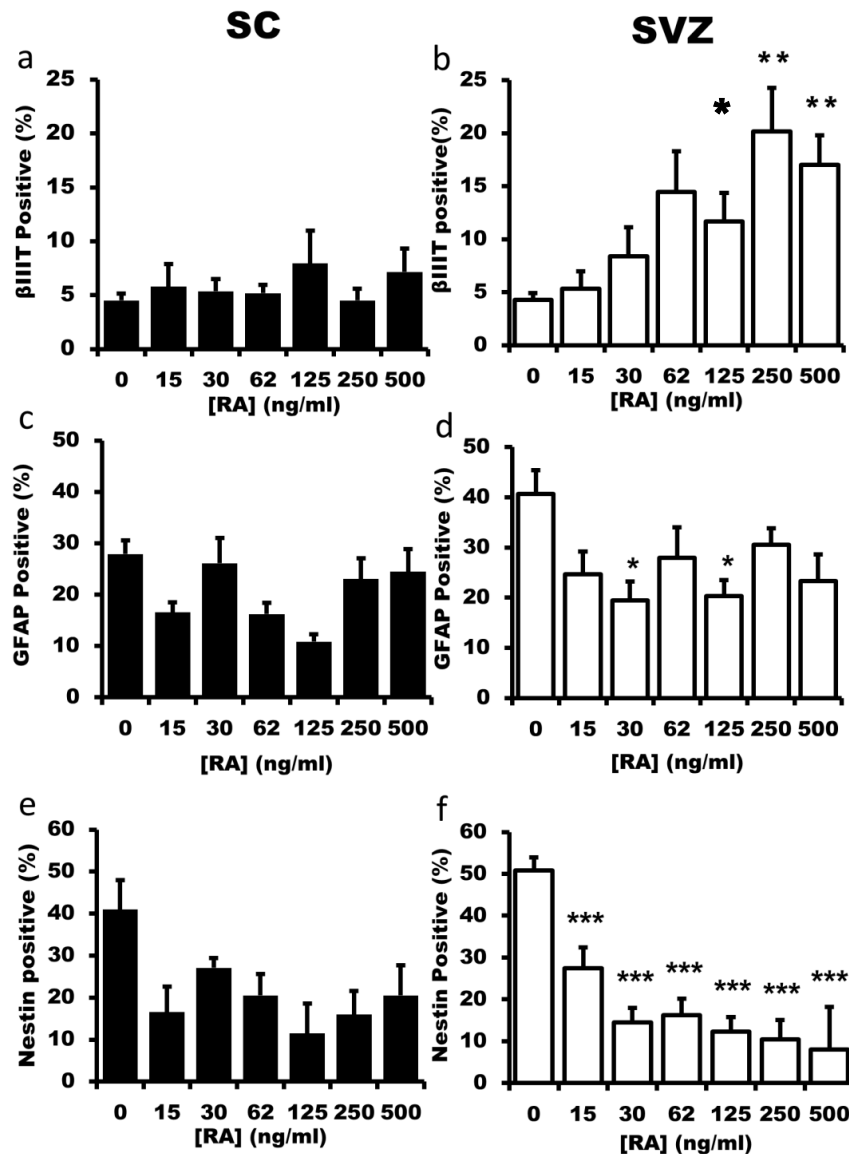


Figure 3.14 – Retinoic acid’s effect on NPC differentiation. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for β IIIIT (a & b), GFAP (c & d), Nestin (e & f) and Hoescht. Only SVZ derived (b) NPCs showed significant gains in β IIIIT positive cells at higher concentrations. Additionally, SVZ derived NPCs showed a reduction in both GFAP (d) and Nestin (f). Each concentration (15-500ng/ml) were compared to control (0ng/ml) conditions with an one way ANOVA. Statistical significance found with Tukey’s post hoc test shown here * ($p<0.05$), ** ($p<0.01$), ***($p<0.001$).

SC derived cultures showed a trend to an increase percentage of BIIT when compared to 0ng/ml, however, no statistically significant increase was noted.

The assessment of the glia cell markers revealed that RA had a negative impact on progenitor cell labeling. In SVZ derived cultures we found a significant decrease in the percentage of cells that were positive for GFAP, ($F(6,56) = 3.1, p=0.01$)(Figure 3.14d). Furthermore, we see a significant decrease in the percentage of cells that were positive for nestin (Figure 3.14f), $F(6, 56) = 16.7, p<0.001$). SC derived NPCs were found to be minimally affected by retinoic acid in terms of its effect on differentiation with no increases in neuronal differentiation noted; as well, no effect on GFAP and nestin labelling was seen either. Both O4 positive cells in both cell types remained at their control levels of approximately 40% for SVZ derived NPCs and ~57% for SC derived NPCs.

PDGF as a differentiation factor for adult derived neural progenitors:

Platelet derived growth factor was selected for its ability to promote oligodendrocyte differentiation. We first sought to assess the effect that a range of concentrations of PDGF (0-500ng/ml) may have on NPC proliferation and toxicity. Interestingly, we found that SVZ NPCs are quite susceptible to PDGF at high concentrations (>250ng/ml). A significant increase in BrdU incorporation was found at and above this concentration (Figure 3.15b), ($F(6,56) = 4.162, p=0.002$) . No changes in TUNEL labeling were observed at any of the concentrations assessed. SC derived NPCs showed no significant increases in BrdU labeling at higher concentrations, nor was there any detrimental effects seen on cell viability (Figure 3.15a).

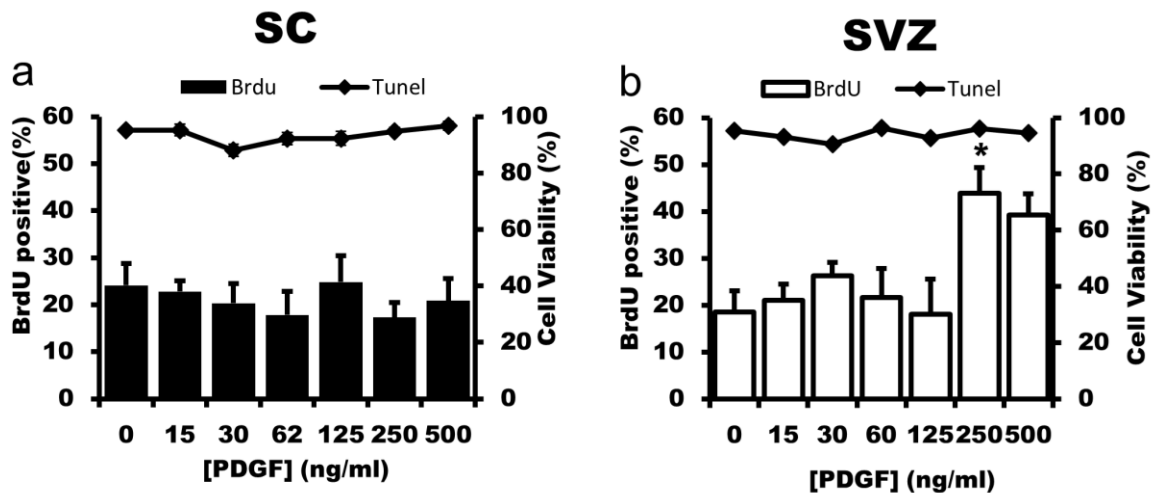


Figure 3.15 – Platelet derived growth factor’s effect on cell viability and proliferation. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for BrdU or TUNEL and Hoechst. SVZ derived (b) NPCs showed significant increases in proliferation at higher concentrations of PDGF. Each concentration (15-500ng/ml) was compared to control (0ng/ml) conditions with an one way ANOVA analysis. Statistical significance found with Tukey’s post hoc test shown with * ($p < 0.05$).

Following the assessment of PDGF effect on proliferation and toxicity we also assessed its effect on NPC differentiation. SC derived NPCs already differentiated primarily into oligodendrocytes with an average of $57 \pm 3.72\%$. With the addition of PDGF into culture conditions during the 7 day differentiation period we did not see any effects on O4 differentiation. The differentiation of oligodendrocytes remained constant throughout each concentration of PDGF (Figure 3.16a). SVZ derived NPCs; however, did show to be more susceptible to PDGF as a differentiation factor. Increases in O4 labeling were seen starting at 62 ng/ml with the percentage of O4 positive cells reaching a maximum of $55.62 \pm 7.68\%$ at 125 ng/ml (Figure 3.16). No statistically significant increases in differentiation of O4 positive cells were noted at 125 ng/ml and 500 ng/ml.

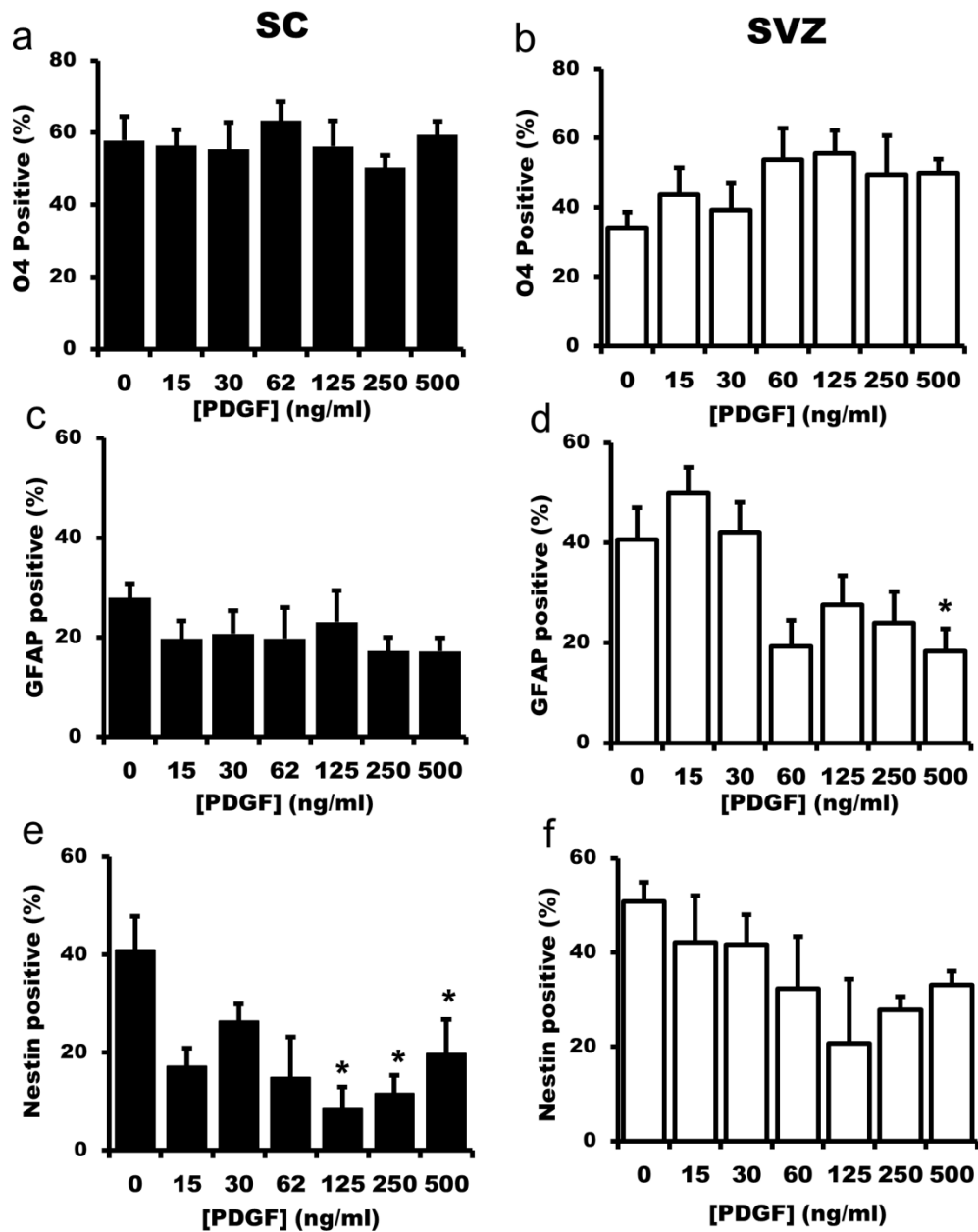


Figure 3.16 – Platelet derived growth factor’s effect on glial differentiation. Results presented as mean \pm SEM and are expressed as % total number of cells positive for O4 (a & b) or GFAP (c & d) or Nestin (e & f). SVZ derived (b) NPCs showed significant increases in O4 labeling at higher concentrations and significant reductions in both GFAP (d) and Nestin (f) positive cell bodies. No significant differences in SC derived NPC differentiation were noted. Each concentration (15-500ng/ml) was compared to control (0ng/ml) conditions with an ANOVA single factor analysis. Statistical significance found with Tukey’s post hoc test and shown with * ($p < 0.05$).

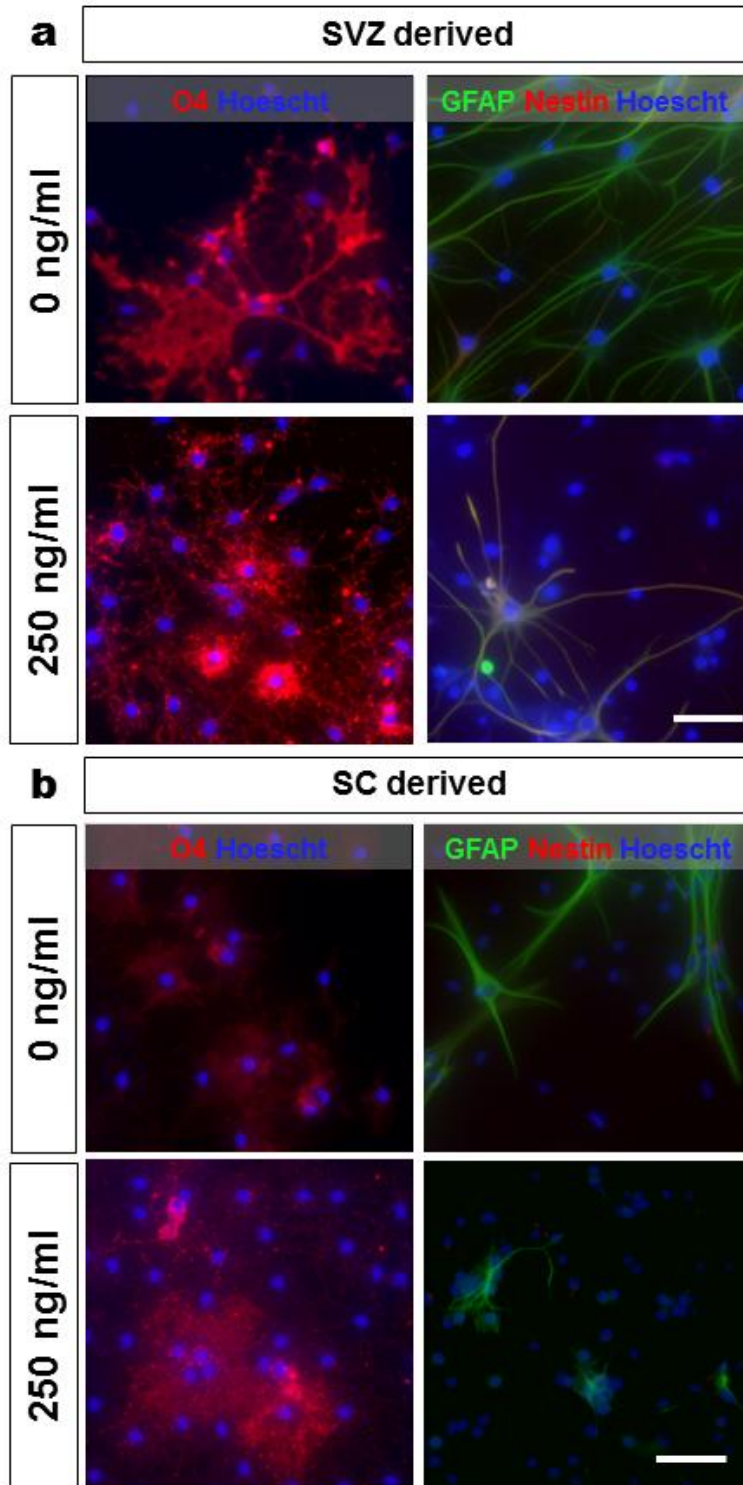


Figure 3.17 – Platelet derived growth factor's effect on SVZ (a) and SC (b) derived NPC differentiation. NPCs were grown for 7 days under proliferation conditions and then transferred to differentiation conditions with PDGF ranging in concentrations (0-500ng/ml). Select images shown illustrate the increase in O4 differentiation in SVZ derived NPCs and the decrease in GFAP/nestin positive glia with increased concentration of PDGF. Scale bar =50 μ m.

In addition to the modest gains in oligodendrocyte differentiation, SVZ derived NPCs also showed a reduction in GFAP and nestin positive cells starting at mid-range concentrations. The percentage of GFAP positive cells changed from $40.6 \pm 4.5\%$ at 0 ng/ml to $18.3 \pm 6.3\%$ at 500 ng/ml (Figure 3.16d). However, no significant difference between control and factor conditions existed. SC derived GFAP levels remained relatively constant with each concentration remaining close to 20% (Figure 3.16c). Following 7 days differentiation SVZ derived astrocytes have a high co-expression of nestin. We see that the co-expression of GFAP and nestin remains as the reduction in GFAP is paired with a reduction in nestin expression (Figure 3.16f). Only SC derived neurospheres showed significant reduction in nestin expression ($F(6,45) = 4.4, p=0.001$). Figure 3.17, reveals the increase in O4 expression and reduction of GFAP/nestin positive cells in SVZ derived NPC cultures.

BMP-4 as a differentiation factor for adult derived neural progenitors:

Following the exposure of BMP-4 to both SC and SVZ derived NPCs similar responses were seen when proliferation and toxicity were assessed. Both SC and SVZ derived NPCs showed a significant reduction in BrdU incorporation following a 24 hr pulse. The percentage of SC NPCs proliferating following a 7 day exposure to BMP-4 dropped from 24% at 0 ng/ml to 3.2% at 500 ng/ml (Figure 3.18 a) ($F(6,38) = 5.797, p<0.001$). Similarly, SVZ derived NPCs showed similar reductions in BrdU incorporation at concentrations greater than 150 ng/ml (Figure 3.18b) ($F(6,52) = 5.5, p<0.001$).

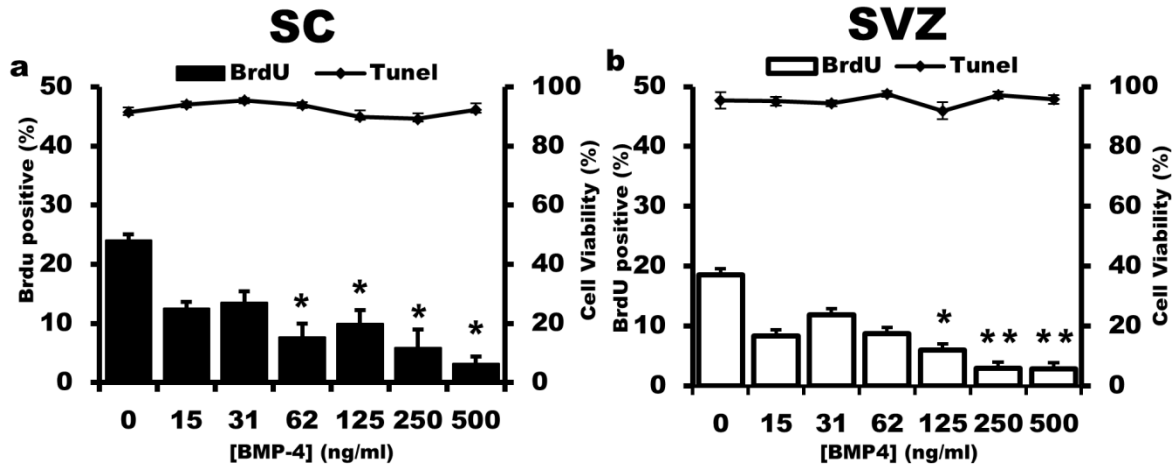


Figure 3.18 – Bone morphogenic protein 4 effect on cell viability and proliferation. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for BrdU or TUNEL and Hoescht. Both SC derived (a) and SVZ derived (b) NPCs showed significant reductions in proliferation at higher concentrations of BMP-4. Each concentration (15-500ng/ml) was compared to control (0ng/ml) conditions with an ANOVA single factor analysis. Statistical significance found using Tukey's post hoc test and shown with * ($p < 0.05$) and ** ($p < 0.01$).

The response of both SVZ and SC derived NPCs to BMP-4 was very interesting. BMP-4 was selected due to its ability to be a differentiation factor that promotes astrocyte formation. Interestingly, in SVZ derived cultures BMP-4 did as predicted; however, in SC derived cultures the response of NPCs was unexpected.

SVZ derived NPCs showed an initial reduction in GFAP positive cells from low to mid-range concentrations. At concentrations above 250 ng/ml we see a slight increase in the percentage of GFAP positive cells (Figure 3.19b). No statistically significant differences could be found following an ANOVA single factor assessment. Interestingly, at all concentrations of BMP-4, SVZ derived NPCs showed significant decrease in nestin expression with $p < 0.01$ at all assessed concentrations (Figure 3.19b) ($F(6,56) = 6.4$, $p < 0.001$). SC derived cultures did not show any observable changes in the percentage of GFAP positive cells; however, at higher concentration - >125 ng/ml- we found a substantial decrease in nestin expression (Figure 3.19c).

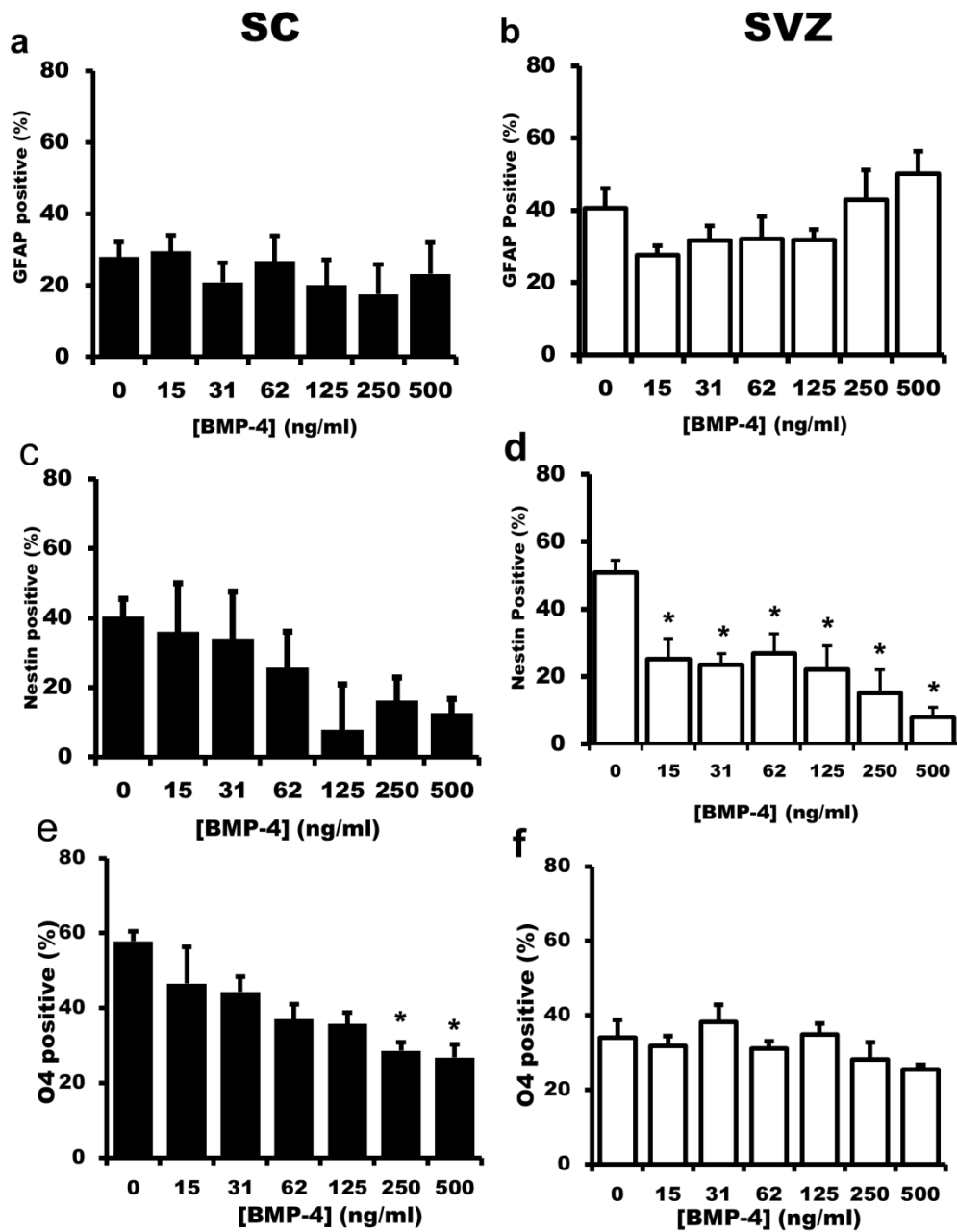


Figure 3.19 – Bone morphogenic protein 4 effect on glial differentiation. Results presented as mean \pm SEM and are expressed as % total number of cells positive for GFAP (a & b) or Nestin (c & d) and Hoescht. Both SC derived (a) and SVZ derived (b) NPCs showed no significant increases in GFAP positive cells. Each concentration (15-500ng/ml) was compared to control (0 ng/ml) conditions with an ANOVA single factor analysis. Statistical significance found using Tukey's post hoc test shown with * ($p < 0.05$).

Oligodendrocyte differentiation was also assessed at all concentrations of BMP-4. SVZ derived NPCs showed no changes in the percentage of O4 positive cells while SC derived NPCs showed substantial reductions in the percentage of O4 positive oligodendrocyte progenitors ($F(6,56) = 3.2, p=0.008$). We can see from Figure 3.19 e, that O4 percentages drop from 57% at 0 ng/ml to 26.7% the lowest observed percentage of O4 positive oligodendrocyte progenitors ever noted in the experiments carried out in this study on SC derived NPCs.

The final cell marker that was assessed was β IIIIT for immature neurons. SVZ derived NPCs showed modest gains in immature neuron differentiation at the relatively low concentrations of 31 and 62 ng/ml. SC derived NPCs responded to all concentrations of BMP-4 by increasing the percentage of immature neurons from 4.51% to a steady 18-28% (Figure 3.20a) ($F(6,56) = 5.374, p<0.001$). Distinct differences are also apparent in the morphology of neurons between SVZ and SC derived. SVZ NPCs seemed to produce neurons with multiple arborisations while SC derived NPCs produced more bipolar morphologies with intense staining remaining within the cell body of the cell and process' extending in two directions never branching (Figure 3.21 a & b).

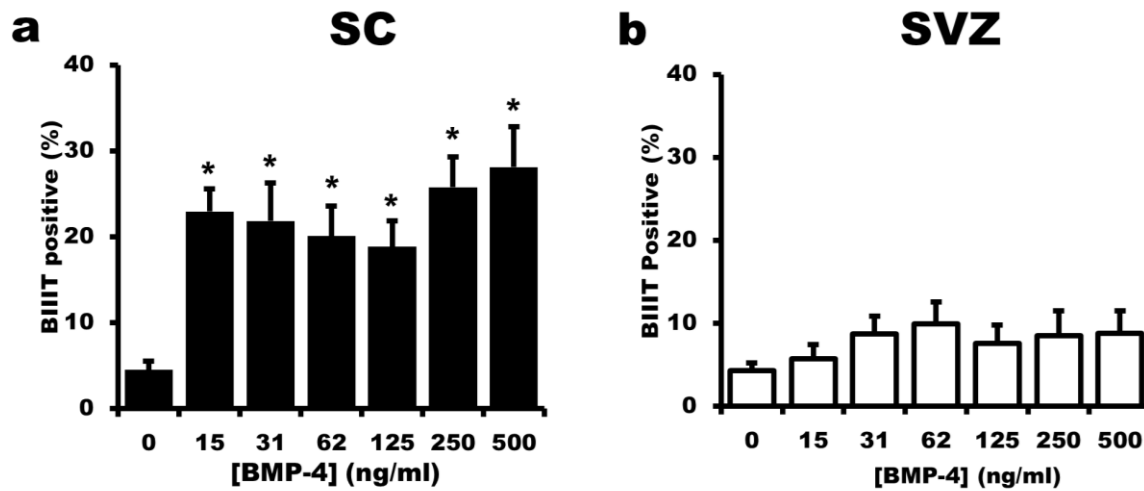


Figure 3.20 – Bone morphogenic protein 4 effect on neuronal differentiation. Results presented as mean \pm SEM and are expressed as % total number of cells positive for BIIT and Hoescht. SC derived (a) NPCs showed a significant increase in BIIT positive cells while SVZ derived NPCs showed no significant gains in BIIT positive cells. Each concentration (15-500 ng/ml) was compared to control (0 ng/ml) conditions with an ANOVA single factor analysis. Statistical significance found with Tukey’s post hoc test and shown with * ($p < 0.05$).

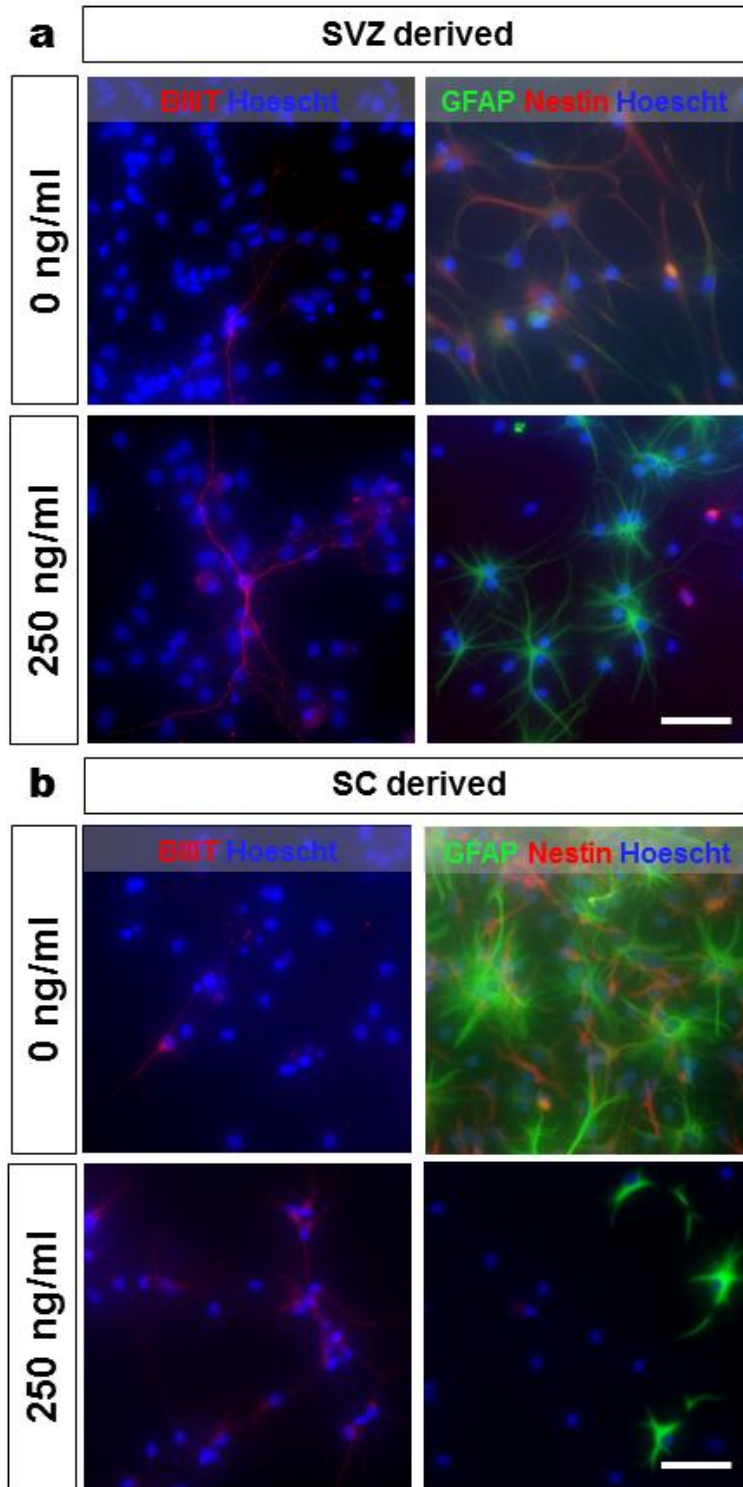


Figure 3.21– Bone morphogenic protein-4’s effect on SVZ (a) and SC (b) derived NPC differentiation. NPCs were grown for 7 days under proliferation conditions and then transferred to differentiation conditions with BMP-4 ranging in concentrations (0-500 ng/ml). Select images shown, illustrating the increase in β III-T differentiation in SC derived NPCs and the decrease in GFAP/nestin positive glia in both SC and SVZ derived. Scale bar =50 μ m.

The effect of biomaterial degradation on adult NPC behaviour

PLGA degradation:

PLGA degradation was assessed *in vitro* with a table top pH reader. It is understood that as PLGA degrades through hydrolysis either as its layered form or microsphere form that it will release both lactic acid and glycolic acid monomers (83). We have yet to see an assessment on the effect these monomers have on cell culture conditions and this is the reason why we decided to assess pH at longer time points. We found that initially both PLGA layered and PLGA microspheres degraded significantly to change the pH of culture media after 14 div. Figure 3.22 clearly illustrates this decrease in pH from 7.6 to 7.0. Later time points revealed that PLGA layered channels degraded at an accelerated rate changing the pH from 7.4 (control conditions) to a much more acidic environment of a pH of 5. PLGA microspheres did not degrade as rapidly, however, they still degraded substantially and changed the pH from 7.4 to 6.95 at 21 div and 6.67 at 28 div.

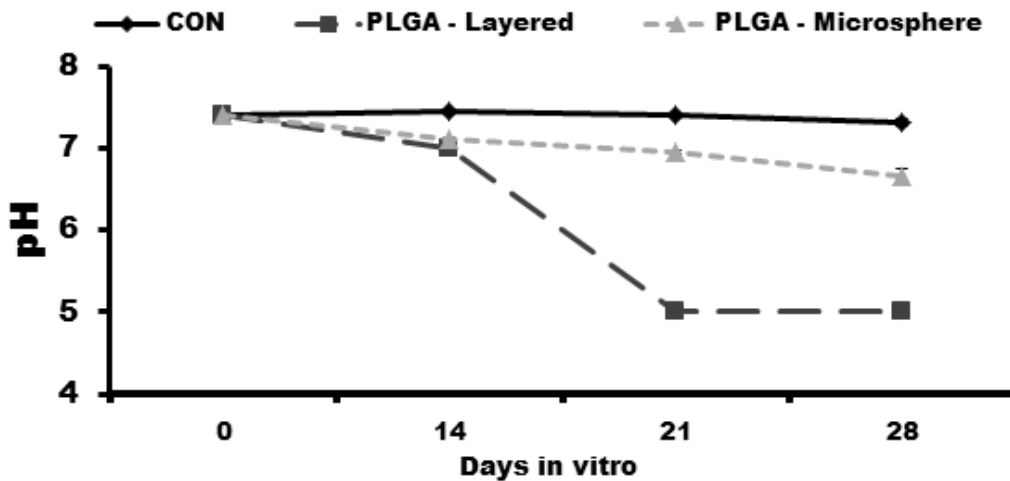


Figure 3.22 – PLGA degradation causes significant pH change after prolonged time in culture. Buffered media remains at a constant pH throughout the experiment (black diamonds). Media exposed to PLGA-microspheres showed a moderate decrease in pH throughout the length of the experiment. Media containing PLGA-layered segments showed an extensive decrease in pH following 21 and 28 days in vitro.

PLGA's effect on NPC survival:

Using TUNEL as an assessment of apoptosis we found a significant difference between control conditions and PLGA exposed conditions in both SC derived and SVZ derived NPCs. Both PLGA microsphere and layered channels showed significant increases in the number of TUNEL positive cells at both 14 and 21 days *in vitro* (Fig. 3.24 & 3.25). Furthermore, consistent with the accelerated change in pH seen in PLGA layered exposed cultures, cells in these conditions showed a significantly greater number of TUNEL positive cells at 28 div in both SC ($F(2,17) = 622.10, p < 0.001$) and SVZ ($F(2,18) = 210, p < 0.001$) derived NPCs (Fig. 3.24, 3.25). Also observed in PLGA layered conditions at 28 div that was not observed in any other group was chromatin condensation as is apparent by regions of intense Hoechst labeling within nuclei of both SC and SVZ derived progenitors (Fig. 3.23). Thus, a substantial decrease in cell viability was found following an extended exposure to both PLGA –layered channel segments and as well to a lower extent with PLGA – microspheres.

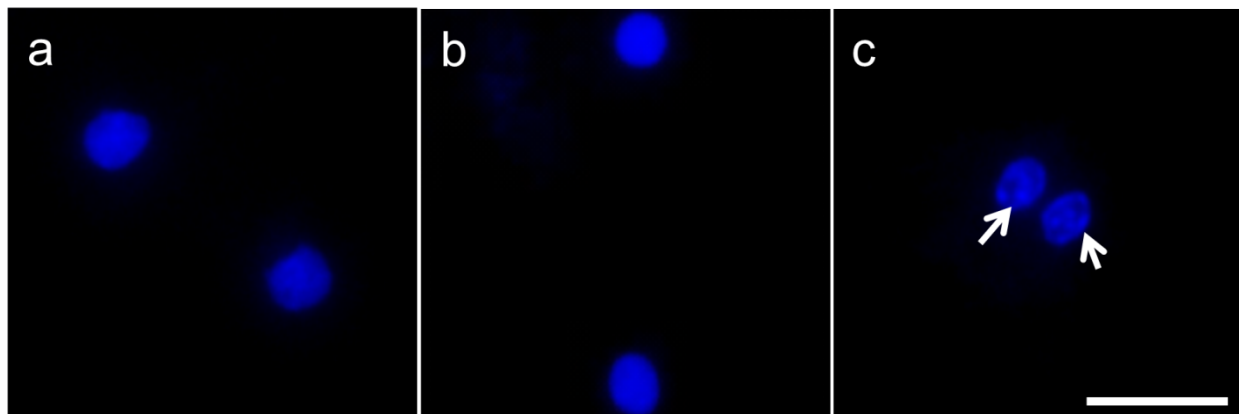


Figure 3.23 – NPCs exposed to PLGA layered channels showed chromatin condensation. Representative images of nuclei morphology of control (a), PLGA-Microparticle (b), and PLGA layered (c) exposed cells. Arrows show dense Hoechst staining indicative of chromatin condensation. Scale bar = 25 μm .

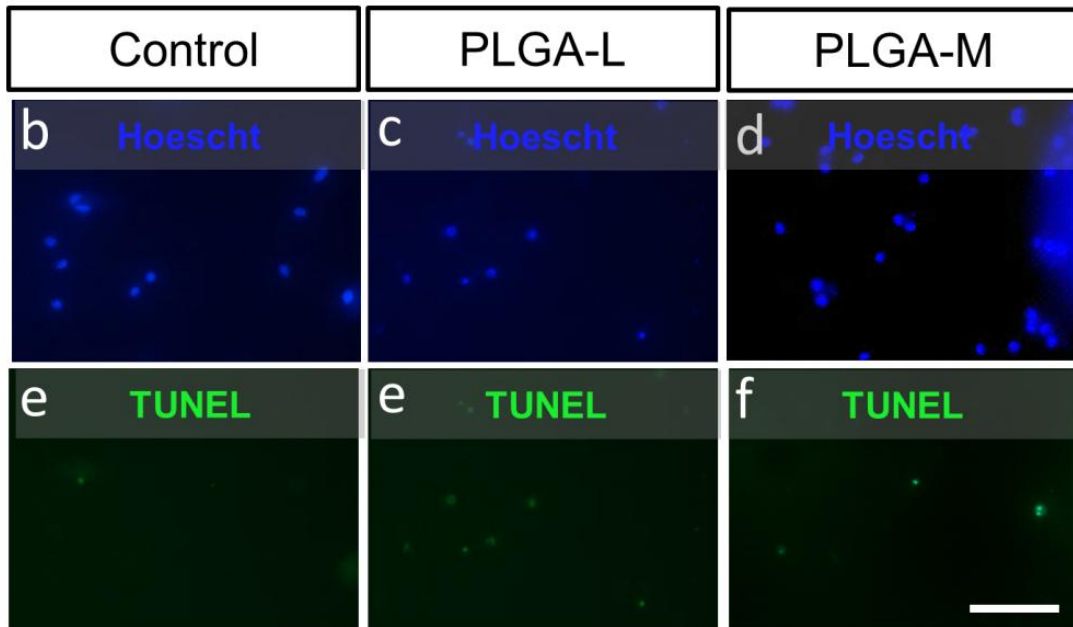
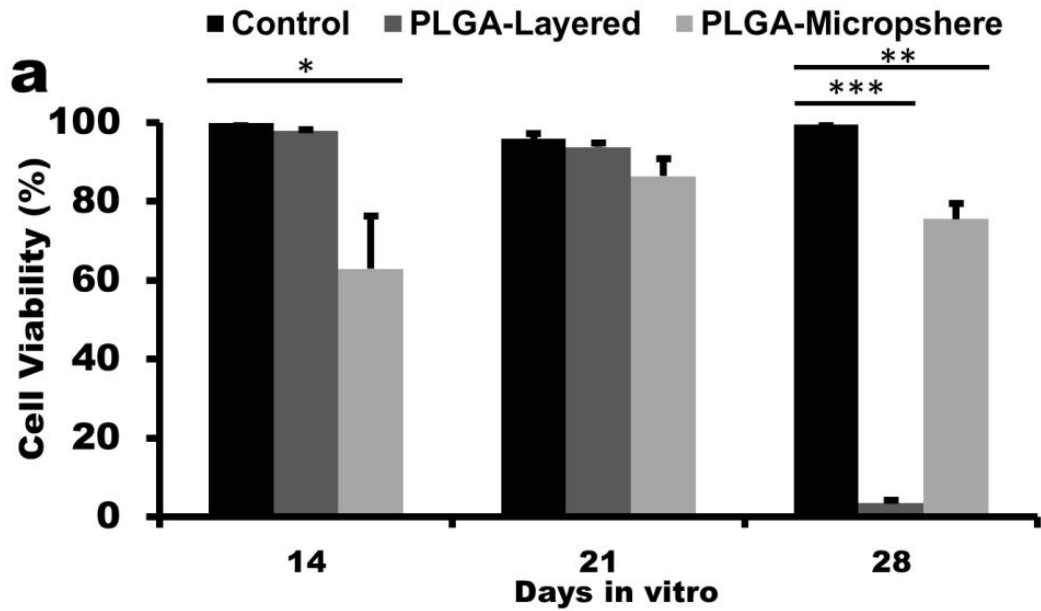


Figure 3.24 - Prolonged exposure to PLGA significantly reduces cell viability. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for TUNEL and Hoescht (a) SC derived NPC viability is significantly reduced following exposure to both PLGA-layered and PLGA-microspheres, statistical significance assessed with one way anova and Tukey's post hoc test shown here with $p < 0.05 = *$, $p < 0.01 = **$ and $p < 0.001 = ***$. (b-f) Sample SC derived NPC epifluorescent images used to assess cell viability. (Scale bar = 100um).

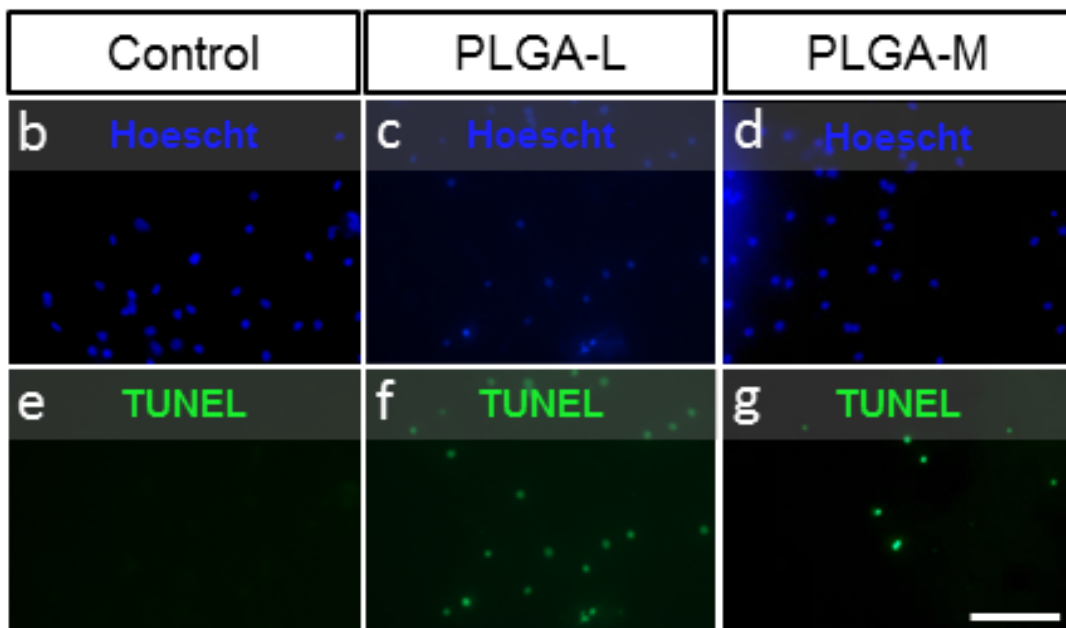
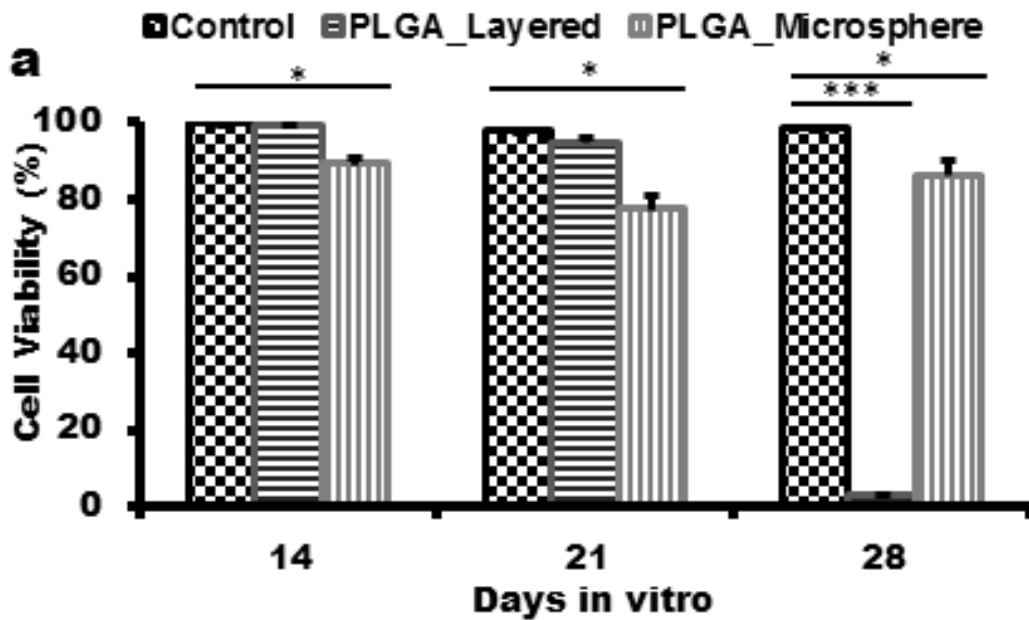


Figure 3.25 - Prolonged exposure to PLGA significantly reduces cell viability. Results presented as mean \pm SEM and are expressed as % total number of nuclei positive for TUNEL and Hoescht (a) SVZ derived NPC viability is significantly reduced following exposure to both PLGA-layered and PLGA-microsphere statistical significance assessed with one way anova and Tukey's post hoc test shown here with $p < 0.05 = *$, and $p < 0.001 = ***$. s. (b-f) Sample SVZ derived NPC epifluorescent images (b-f) used to assess cell viability. (Scale bar = 100um).

PLGA's effect on NPC proliferation:

Assessing the effect of both materials on proliferation revealed significant detriments. SVZ and SC derived NPCs showed consistent decreases in BrdU incorporation at 28 div (Fig. 3.26a & b). Even though SC derived NPCs showed reduced BrdU labeling in control conditions across all time points PLGA layered and microspheres exposed groups showed a further significant reduction at 28 div. Both SC ($F(2,21) = 8.5, p < 0.01$) and SVZ ($F(2,21) = 18.8, p < 0.001$) derived neural progenitors showed a complete loss of proliferation after 28 days *in vitro* with PLGA-layered. However, both SC and SVZ derived progenitors showed a modest (<5%) degree of proliferation following a 28 div exposure to PLGA-microspheres.

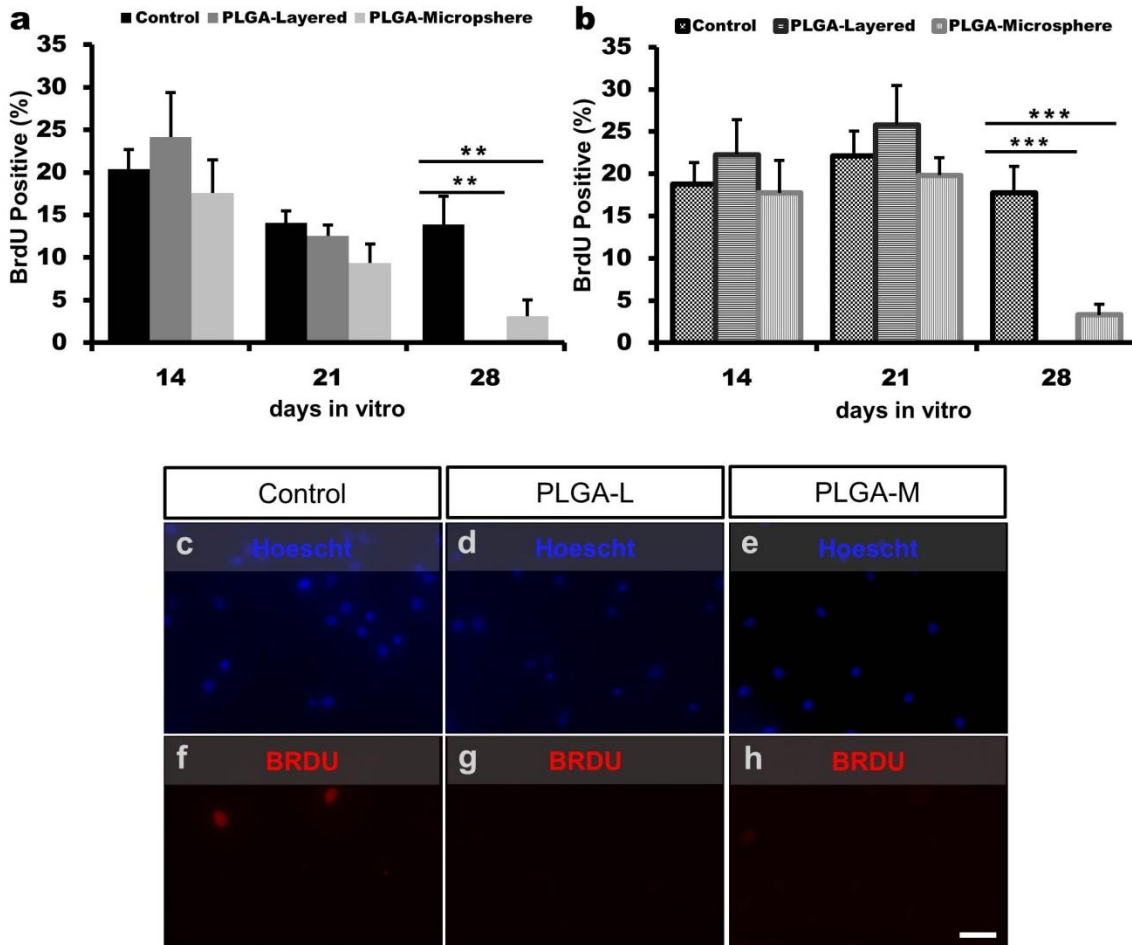


Figure 3.26 - Prolonged exposure to both PLGA layered and PLGA microspheres significantly reduced cell proliferation. (a) SC derived NPC proliferation is significantly reduced following exposure to both PLGA-layered and PLGA-Microspheres. (b) SVZ derived NPC proliferation is significantly reduced following exposure to both PLGA-layered and PLGA-microspheres. Sample SVZ derived NPC immunofluorescent images used to assess cell proliferation (c-h) (Scale bar = 50um). Statistical significance assessed with one way anova and Tukey's post hoc test shown here with ** for p<0.01 and *** for p<0.001.

PLGA's effect on NPC differentiation:

Adult neural progenitors have been well documented for their ability to differentiate into all three major subtypes of the CNS making them excellent targets for neural repair strategies. We assessed the effect that these biomaterials had on NPC differentiation over the entire culture period. No difference was seen between NPCs exposed to PLGA-layered, PLGA-microspheres and control conditions at the initial time point of 14 div. In all three conditions SC derived progenitors differentiated primarily into oligodendrocytes (>50%) (Fig. 3.27a, 3.28a & 3.29a), while SVZ derived progenitors differentiated into a more equal distribution of GFAP positive astrocytes and O4 positive oligodendrocytes (Fig. 3.27b). Continuing to the middle time point of 21 div we see that no significant changes to differentiation had occurred between control and PLGA groups. However, SVZ derived NPCs show a significant increase in the percentage of O4 positive oligodendrocytes from 14 div (Figure 3.28b).

Prolonged exposure to both materials led to significant differences in differentiation capacity. After 28div with PLGA-layered and PLGA-microspheres, NPCs from both the SC ($F(2,23) = 23.6, p < 0.001$) and SVZ ($F(2,24) = 19.2, p < 0.001$) showed significant decreases in both BIIT. Also, we noted significant decreases in GFAP labeling in SC ($F(2,24) = 9.2, p < 0.001$) as well as in the SVZ ($F(2,24) = 10.5, p = 0.001$) (Fig. 3.28 a & b). There were very few cells found attached to the culture plate in the PLGA-layered conditions. Those cells that were attached in the SVZ derived NPCs were predominately found to be O4 positive oligodendrocytes (Fig. 3b).

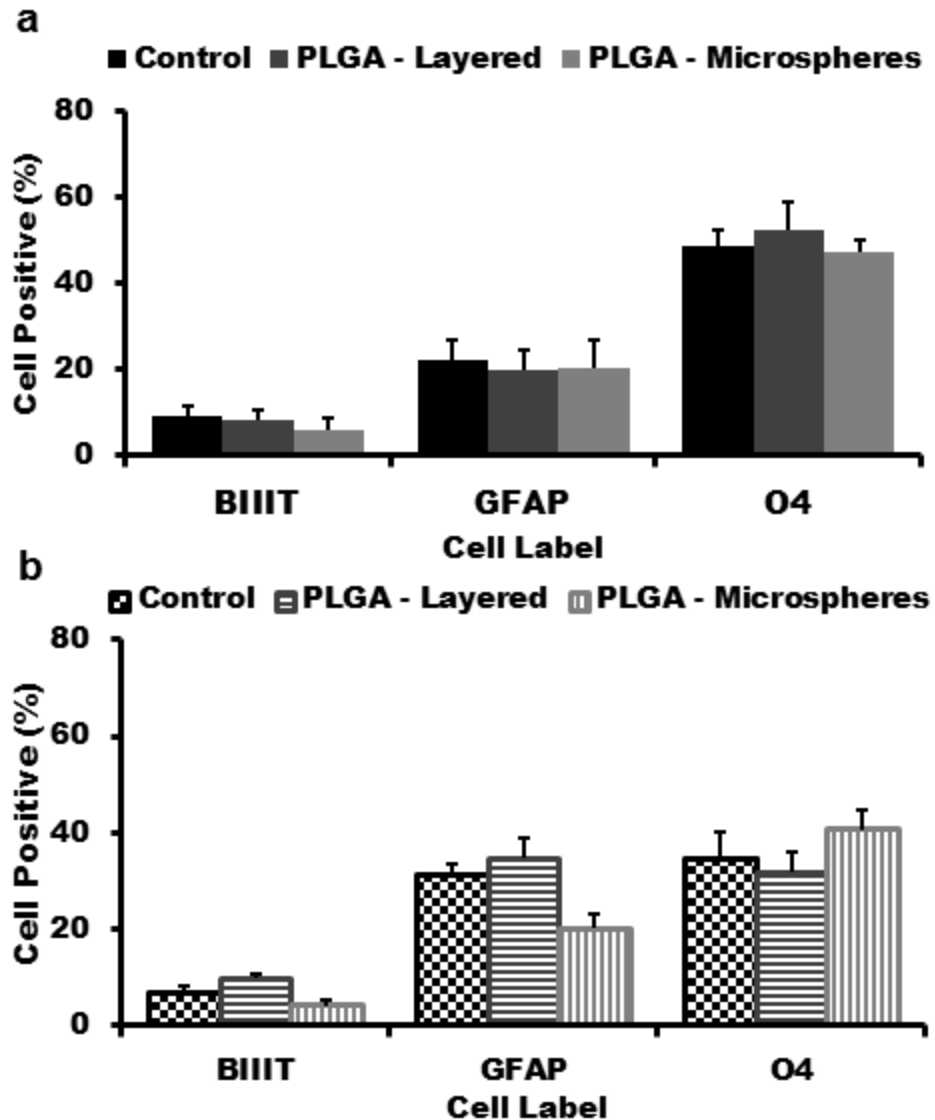


Figure 3.27 – PLGA degradation does not affect NPC differentiation following 14 days in vitro. Results presented as mean \pm SEM and are expressed as % total number of cells positive for O4, GFAP & Nestin. (a) SC derived NPC differentiation at 14 div. (b) SVZ derived NPC differentiation at 14 div. SC derived NPCs predominately differentiated into O4 positive oligodendrocytes while SVZ derived NPCs more equally differentiated into GFAP positive astrocytes and O4 positive oligodendrocytes. No differences were found between control and both PLGA groups.

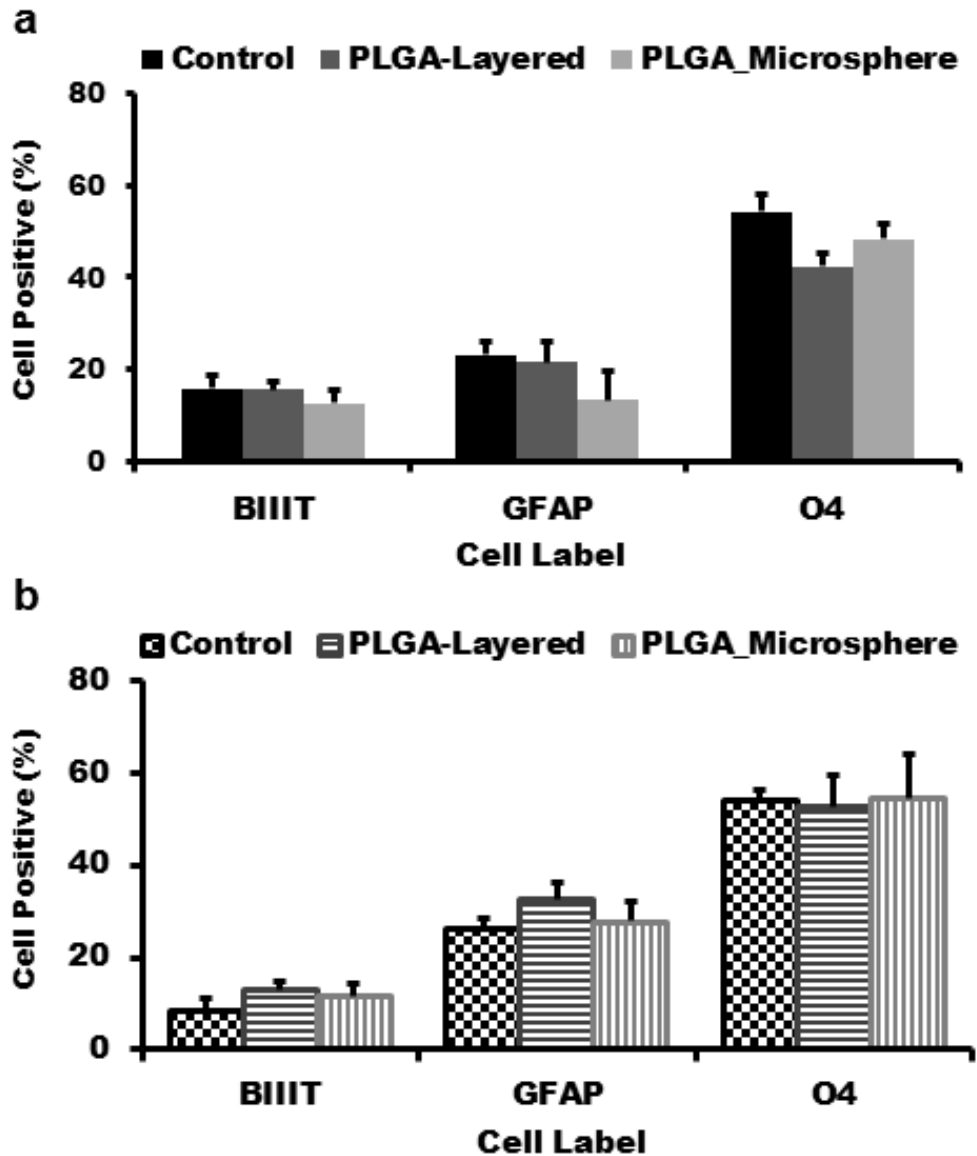


Figure 3.28 - PLGA degradation does not affect NPC differentiation after 21 days in vitro. Results presented as mean \pm SEM and are expressed as % total number of cells positive for O4, GFAP & BIIT. (a) SC derived NPC differentiation at 21 div. (b) SVZ derived NPC differentiation at 21 div. Both PLGA layered and PLGA microsphere groups were compared to control conditions using a one way ANOVA.

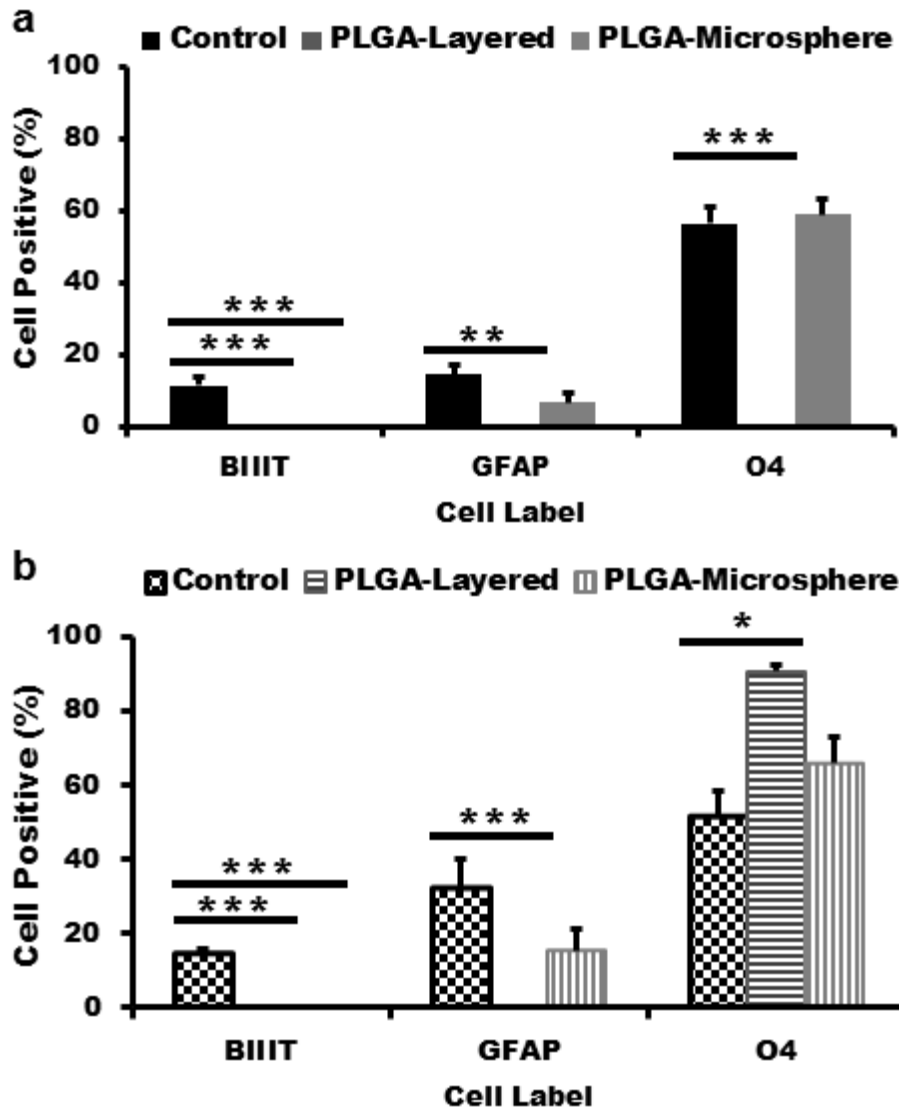


Figure 3.29 - PLGA layered and PLGA microspheres reduce neuronal and astrocyte differentiation. Results presented as mean \pm SEM and are expressed as % total number of cells positive for O4, GFAP & BIIT. (a) SC derived NPC differentiation at 28 div.(b) SVZ derived NPC differentiation at 28 div. Both PLGA layered and PLGA microsphere groups were compared to control conditions using a one way ANOVA and a Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Neurosphere viability and proliferation

Neurosphere size is an essential aspect of the neurosphere assay. A number of studies have used neurosphere size as one of the main measurements for assessing the stemness of a neurosphere, with a relationship between size and number of stem cells (85, 91). One main caveat is that neurospheres are not static entities and that if given the chance to form contacts with each other they will merge generating larger neurospheres (92, 93).

In our experiments at 7 div SVZ derived NPCs produced spheres with a statistically significant larger size than the SC derived spheres (Figure 3.1 a & b). The initial differences between SVZ and SC derived neurospheres were likely due to the differences in the cell population within the sphere, given their different origins. When the SVZ region was dissected out during the primary both the ependymal and sub-ependymal regions are captured. Unless special care is taken to exclude the ependymal region a mixed population of cells were found in the single cells plated. Few groups studying the SVZ region take the additional measures to exclude the ependymal region from their culture harvests. The spheres in most SVZ studies are initially comprised of a cell population generated from both ependymal cells and sub-ependymal cells. When these two distinct layers of the SVZ are separated and cultured under clonal conditions, distinct differences are seen between the sizes of the spheres (94). Cells derived from the septal layer – solely ependymal in origin - of the adult rat brain produce neurospheres that are <100 μm in diameter while sub-ependymal derived spheres are found to produce the >100 μm neurospheres (94).

On examination of the sphere size we noted comparative results with these previous studies (29, 94). SVZ derived neurospheres were heterogenous in sphere size, where both large and small spheres were initially produced; conversely SC derived spheres were generally more homogeneously smaller in size. The difference in sphere size are shown and quantified in Figure 3.1. These size differences correspond with the unique neural stem/progenitor identities in each region, where highly proliferative cells can form more larger spheres (94) The SC central canal niche is comprised of ependymal derived progenitor cells (28-30, 42) while the SVZ niche is more complex and made up of both ependymal and Type-B astrocytes which are thought to be the true stem cell in this environment (26, 33, 34).

As time progressed in culture we noted that the sphere size differences between both groups, SC ependymal derived spheres and the SVZ ependymal and Type-B astrocyte derived spheres were no longer different from each other. Both SC and SVZ derived spheres were found to be heterogenous in sizes. As time progressed further to the last time point we saw that both SC and SVZ derived spheres were found to be on average $252.2 \pm 9.8 \mu\text{m}$ and $231.3 \pm 17.8 \mu\text{m}$, respectively. The transition to similar size characteristics at later time points suggested that the initial differences in sphere size between the two cell origins and perhaps identity no longer existed. In previous studies SVZ spheres were found to reach diameters close to 2 mm using a commercialized neurosphere assay product which places spheres in a collagen matrix that facilitates giant sphere growth (4). The suspension culture system used in this study was not sufficient enough to encourage neurosphere sizes larger than $400 \mu\text{m}$ from both the SC and SVZ, but despite the size differences to commercial products, the further time points demonstrated similar progenitor characteristics.

Examination of the identity of each phenotypes of each progenitor in the neurospheres was possible by sectioning each type of sphere at all proliferation time points assessed. We observed that as time progressed in culture that the proliferation of NPCs was reduced. We attributed the plateau in sphere size past 14 div, to the hindering of proliferation from large sphere size, due to a lack of mitogen penetration as described in previous neurosphere studies (95). It is thought that as a sphere becomes larger, less mitogens are reaching the core of the sphere and thus only cells found on the exterior of the spheres are receiving the mitogens and proliferating. Both SC and SVZ derived spheres at 7 and 14 div show extensive BrdU labelling throughout the sphere. The 21 day old spheres do show less BrdU positive cells overall; however, BrdU positive cells can be seen both on the exterior of the sphere and in the core or centre most areas of the sphere as well. Furthermore, the lack of differences in the number of TUNEL positive cells within the neurospheres reveal that sphere size has very little effect on cell viability. Previous reports have shown that extended time in culture has limited effect on the number of TUNEL positive cells, with apoptotic cells rarely found within neurospheres of varying sizes (96), which is in accordance with our observations. With sphere sizes not increasing much after 14 and 21 div and significant decreases in BrdU incorporation observed, but no differences in TUNEL labeling as sphere sizes increase over time.

SVZ and SC progenitor phenotypes

We sought to assess the progenitor phenotype by using three main labels used for assessing neural progenitor identity, previously well established in the literature. First was the ratio of GFAP/nestin as a marker for multipotent neural progenitors second was Sox 2 a common maker for neural progenitors throughout development and adulthood (97, 98) and third was NG2 a common *in vitro* and *in vivo* marker for oligodendrocyte precursor cells (OPCs)((99, 100).

Both SVZ and SC derived spheres showed extensive labeling of both GFAP and Nestin positive cells at 7 days. These cells are scattered throughout the sphere and were not localized to any one region. However, SVZ spheres were found to produce more GFAP/nestin positive cells while SC derived spheres produced significantly greater numbers of GFAP negative/ nestin positive. The initial differences seen here at 7 div is in agreement with the observations of the two particular niches (42). The SVZ is comprised of both nestin positive ependymal cells and GFAP/nestin positive Type B astrocytes while the SC central canal niche was found to be predominately nestin positive ependymal cells, with no GFAP/nestin positive cells associated with the central canal region. Nestin positive ependymal cells are still being debated as to where they lie on the stem – progenitor cell continuum. After ischemic injury such as a severe middle cerebral artery occlusion, they are found to differentiate into GFAP/nestin positive astrocytes which ultimately commit to generating new neuroblasts which migrate to the olfactory bulb becoming new interneurons (34). Thus, in our culture conditions, it is possible that the GFAP/nestin positive cells found within the SC derived neurospheres may ultimately be of ependymal origin. Therefore, the initial differences in the percentage of nestin to GFAP/nestin positive cells seen between SC and SVZ derived spheres may be due to the fact that SC ependymal cells have not totally differentiated into GFAP/nestin positive cells. A further point is that ependymal cells are argued to be not the true stem cell within the SVZ niche because in times of injury when they differentiate into GFAP/nestin positive cells they do not replace themselves, instead the ependymal region becomes exhausted and no longer surrounds the ventricles following a severe MCAO (34). One shortfall of this theory is the exhaustion of the ependymal lining; if they were true stem cells they should be able to replenish the lost ependymal cells while contributing new cells to the SVZ (26). Similar observations have been

made in the CC where after spinal cord injury the ependymal region collapses and is lost as most ependymal cells migrate towards the site of injury failing to enclose the central canal (29, 30).

The reduction in nestin labeling that occurred over time in culture is further supported by the reduction in the percentage of cells that were positive for Sox2. We found that both SC and SVZ derived spheres were both positive for Sox2 at 7 div. As time in culture progressed we saw that the percentage of cells that remained positive for Sox2 decreased substantially. Sox2 within these spheres is an important measure for both the proliferation and differentiation potential of these spheres (98). Sox2 is a member of the high-mobility group of transcription factors which is found to be expressed early on in development in the neuroepithelium and continually in both the CC (100) and SVZ (101) throughout adulthood. Two main conclusions can be taken from the changes observed in the expression of Sox2. First, it is clear from both BrdU incorporation assessments and also now with Sox2 expression that these progenitors are no longer in the optimal proliferative conditions. Even with the constant exchange of the mitogens, we saw a significant decrease in the number of BrdU positive cells found in both SC and SVZ derived spheres. This leads us to conclude that the cells within the neurosphere are no longer predominately proliferating and must therefore be differentiating, as there was no noticeable increase in the number of TUNEL positive cells as well.

Our assessment of the percentage of cells that expressed NG2 also revealed that the NPCs from both the SC and SVZ were differentiating away from GFAP/nestin, nestin positive cells into more restricted lineages. We saw a significant increase in the percentage of cells that expressed NG2 after extended time in culture. NG2 and GFAP expression has been found to be both mutually exclusive where GFAP/nestin positive and NG2 positive cells are rarely observed in murine neurosphere cultures (90), suggesting that extended exposure to the mitogens EGF and

bFGF support the particular selection of certain progenitor phenotypes. In fact, the mitogens which cells are exposed to during the proliferation period of this neurosphere suspension culture have a large role to play on progenitor identity.

There have been a number of studies completed which have shown that EGF causes SVZ Type b astrocytes to preferentially produce oligodendrocyte precursor cells (102). When EGF is infused into the ventricular system there is a 300-400% increase in the number of myelinating oligodendrocytes which migrate from the SVZ originating from Type-b astrocytes (103). This occurs in a dose dependent manner with higher concentrations of EGF encouraging a greater differentiation and migration of OPCs from the SVZ (104). When mouse SVZ derived neurospheres are grown under clonal conditions for multiple passages with only bFGF we see that the number of GFAP positive cells remains the predominant phenotype (98, 105) while combining EGF and bFGF over time leads to SVZ derived spheres differentiating into mostly oligodendrocytes (41). Therefore, the increase in NG2 following extended time in culture may be due to the presence of EGF as a mitogen, preferentially supporting Type-b astrocyte differentiation into OPCs rather than maintaining them in a more multipotent state.

SVZ and SC progenitor differentiation

The differentiation of the neurospheres into monolayers comprised of all three main cell types of the CNS: neurons, astrocytes, and oligodendrocytes involved plating the spheres onto poly-l-ornithine coated plates and removing the mitogens from the culture media. Initially we saw that SC derived neurospheres preferentially produced O4 positive oligodendrocytes and SVZ derived neurospheres equally differentiated into GFAP/Nestin positive astrocytes and O4

positive oligodendrocytes. Time in culture reduced the number of astrocytes which migrated from SVZ derived spheres supporting the findings of the neurosphere section assessments. Further influences on differentiation can be found in the media composition. This study used a culture media composition that used N2 rather the more common B27 nutrient composition which may have influenced the degree of glial differentiation. N2 supplement has been used as a method of promoting glial differentiation in other NPCs neurosphere studies (89). However, reports using B27 have also been found to substantially support glial differentiation as well (41). Therefore, the supplementation used during the proliferation portion of the suspension culture may not have been the most essential component. Optimal media composition has been investigated thoroughly before with the preferred media being neurobasal media with B27 supplementation. When compared to DMEM/F12/N2 composition researchers found NB/B27 to better support rapid expansion of neurospheres enabling greater fold increases in NPC numbers. However, the effect of each supplement on differentiation produced inconclusive results with no significant differences being noted between all media compositions assessed (106). This further supports the possibility that mitogen selection may in fact be the main contributor to progenitor selection over extended time in culture.

Interestingly, the differentiation studies also revealed a substantial increase in the number of BIIT positive neurons as time in culture progressed. It is felt that rather than supporting the selection of progenitors that preferentially differentiate into neurons our increases in NPC differentiation into neurons is due to the fact that the neuronal differentiation marker used in this study is often seen much later than the glial differentiation markers used. Additionally, many studies have revealed that neuronal differentiation increases as time *in vitro* increases (98), indicating that neuronal markers may require greater differentiation time to develop.

Control of NPC differentiation

Retinoic acid

Retinoic acid was selected for its ability to promote NSC differentiation into neurons. We observed that SVZ derived NPCs were responsive to RA leading to an overall reduction in proliferation and GFAP/nestin positive cells as well as an increase in BIIT positive cells. However, in the SC derived NPCs no noticeable effects on differentiation were observed. Retinoic acid did influence the rate of proliferation of SC derived NPCs. Earlier reports of the effect of RA on SVZ derived NPCs showed that it could substantially increase the number of BIIT cells positive for BrdU. Interestingly, this increase in BrdU labeling occurred following a 2 hour pulse prior to fixation (56). With this short BrdU pulse paradigm it is difficult to conclude that RA increases the number of BIIT/BrdU positive cells as BIIT is a marker for neurons that have already terminally differentiated into the neuronal lineage and are no longer considered capable of proliferating. It is therefore difficult to understand how using a 2 hour pulse of BrdU was sufficient to capture these BIIT positive cells. We used a 24 hour pulse of BrdU prior to fixation and failed to see any increase in BrdU positive cells following a seven day exposure to RA under differentiation conditions. The only variation between this study and ours could be that in our study spheres are actively washed to ensure that no mitogens are present within the differentiation media. Perhaps the methods used by Wang et al., did not wash away mitogens, this detail remains relatively unclear within their methods section. Their effect of RA on glial differentiation is consistent with our results which caused an overall reduction in GFAP and nestin positive cells.

The difference in the response of the SC derived NPCs to the RA from the SVZ response is interesting. The effect of RA on SC ependymal cells has been assessed with SC ependymal cells responding in a similar fashion by increased proliferation. However, unlike with the SVZ NPCs, primary ependymal cells proliferated creating an 8.1 fold increase in the number of ependymal cells found both surrounding the CC *in vivo* and the number of cells *in vitro* (107). As was stated above RA has many different genetic targets. One of its targets PPAR β/δ promotes survival and proliferation rather than differentiation (108) while in cortical neuron development it is found to be important for neuronal maturation and differentiation (109). PPAR β/δ is also found to be expressed highly in the SC ependymal region (110). RA exerts its effects with a number of complement proteins and thus a closer examination of the differences in the RA associated genes expressed in SVZ NPCs and SC NPCs will enable a better understanding of the responses seen. Since RA has a multitude of possible targets it is difficult to anticipate that just one of its targets will be involved with mediating its effects. It is interesting that SC NPCs derived from CC ependymal cells in this study did not respond to RA by proliferating, rather a reduction in NPC proliferation was noted. The Pfenninger (107) study used primary ependymal cells isolated through cell sorting and then plated directly into experimental conditions. Our study first cultivated our primary cells as spheres and then removed them from proliferation conditions for differentiation factor assessments. Seeing as the culture conditions were so different it could explain why our results differed. As cells are grown in culture their identity will no doubt change and the soluble factors found *in vivo* within their cellular niche may not be the same. Therefore, special consideration for what physiological conditions each cell is exposed to must be taken before detailed comparisons can be drawn across each study. In our experiments both SVZ and SC derived spheres were plated following primary sphere formation. The

differences seen between SC and SVZ derived responses may also be due to a difference in the soluble factors available to each progenitor within the two different types of spheres.

Platelet Derived Growth Factor

Both SC and SVZ derived NPCs showed extensive oligodendrocyte differentiation under our culture conditions. We chose PDGF to attempt to further promote this oligodendrocyte differentiation. SC derived NPCs did not show any response to all concentrations of PDGF assessed *in vitro* while SVZ derived NPCs showed modest increases in oligodendrocyte differentiation with also an increase in SVZ proliferation at the highest concentrations of PDGF assessed.

PDGF has been shown to influence SVZ Type-B astrocyte behaviour quite profoundly. *In vivo* studies have revealed that 240 ng/ml infusions of PDGF into the lateral ventricles of the adult mouse led to extensive proliferation of Type-B astrocytes. This increased proliferation led to the formation of hyperplasias with similar features to gliomas (60). When PDGF was removed these cells preferentially differentiated into mature oligodendrocytes. The researchers also explored the effectiveness of low dose PDGF (40 ng/ml) to see whether it could cause the same gains in proliferation. Only high dose infusions of PDGF were effective in eliciting a proliferation response of Type-B astrocytes. These results are in agreement with our *in vitro* experiments, where low dose PDGF was insufficient in causing gains in BrdU labeling, however, high doses of PDGF (>250 ng/ml) caused significant increases in BrdU incorporation. Therefore, PDGF cannot be considered simply as a differentiation factor but also as a potent mitogen.

PDGF α receptor is found to be expressed in both the SC ependymal (28) and SVZ ependymal and sub-ependyma (60). Why the SC derived NPCs did not respond to PDGF in a

similar fashion to SVZ derived NPCs is unknown. Previous reports have shown that PDGF's production of oligodendrocytes is best seen when accompanied with mitogens, either during a pretreatment proliferation phase (67) or during the differentiation phase (66). This is an important contrast to the previous report discussed above. In these pretreatment and combined factor exposure experiments a low dose of PDGF (20ng/ml) was used. Thus, for low dose PDGF to enable greater amounts of oligodendrocyte differentiation it must be paired with EGF or FGFb. Oddly, studies have revealed at least for the Type-B SVZ astrocytes that PDGF and EGF receptors do not overlap. Therefore, these factors may be encouraging oligodendrocyte selection from two distinct cell types which together produce greater degrees of oligodendrocyte production. Future work to categorize PDGFR α and EGFR positive cells in both niches and phenotype their response to each factor separately, would help clarify mechanisms to promote oligodendrocyte production.

Bone morphogenic protein – 4

BMP-4 is a potent differentiation factor involved in the promotion of both neural and glial differentiation. In development it exerts its effects mainly through establishing gradients. Therefore, there is a difference in how high and low doses of BMP-4 affect neural progenitors. We saw two very different phenotypes from SC and SVZ derived NPCs in response to BMP-4. SVZ derived NPCs produced significantly greater amounts of GFAP positive / nestin negative astrocytes while SC derived NPCs predominantly differentiated into BIIT positive immature neurons. Interestingly, BMP-4 was the only factor assessed which was able to significantly alter SC derived NPC differentiation into oligodendrocytes. BMP-4 at higher concentrations significantly decreased the number of O4 positive progenitors in the SC derived cultures.

It is the gradients of BMP-4 established during development which see it involved in so many different types of cell specification. These gradients enable BMP-4 to influence cell death and proliferation during embryogenesis (111) while also influencing both neuronal and glial differentiation (59, 112). We found that SVZ derived NPCs showed a significant decrease in nestin expression after high dose exposure (>125ng/ml) indicating that GFAP astrocytes were maturing and losing the progenitor characteristics. Previous reports have shown that high dose BMP-4 promotes astrocyte differentiation over oligodendrocytes while lower doses instruct neuronal differentiation. We did not see any noticeable increases in neuronal differentiation within the SVZ derived NPCs. Interestingly, we found in the ependymal derived SC NPCs that neuronal differentiation was the primary phenotype. No noticeable differences in the astrocyte differentiation were noted, however significant reductions in nestin expression were observed. The differences in the response of SC and SVZ derived cultures may be due to the number of spheres which have originated from ependymal cells. We have established that the SC produces more ependymal derived spheres than the SVZ culture. One of the main regulators of BMPs in the CNS is noggin, which is found to be produced by ependymal cells (113). Noggin secretion plays a very important role in modulating BMP-4's signalling. Thus, one possible reason why more neuronal differentiation is noted in the SC derived cultures is because ependymal derived spheres are producing more Noggin than the SVZ derived spheres and therefore less modulation of BMP-4's signalling is occurring in the SVZ cultures. Furthermore, Noggin gradients in the spinal cord are incredibly important for establishing the borders of neuronal differentiation and glial differentiation (69).

In addition to the effects on neuronal differentiation we also noticed a significant effect on oligodendrocyte differentiation in the SC derived cultures. This is a very interesting

observation because BMP-4 signalling has recently been attributed to preferentially support neuronal and astrocyte differentiation at the cost of oligodendrocyte differentiation. Smad4 one of the main molecular targets of the BMP-4 signalling pathway and has been implicated in restricting oligodendrocyte differentiation. After the application of BMP-4 to adult NSCs researchers found an up regulation of Smad4 and a down regulation of the transcription factor Olig2 leading to a reduction in oligodendrocyte differentiation (114). Therefore, the observations seen within the SC derived cultures in this experiment show that neuronal differentiation occurs over oligodendrocyte differentiation possibly due to activation of the Smad4 transcription factor down regulating the Olig2 transcription factor.

The effect of biomaterial degradation on NPCs

Since the progenitors from each region within the CNS are so different specific drug delivery systems must be developed to instruct certain cell types over others. One of the main strategies that should be used is to isolate the drug delivery system to one specific area rather than utilizing a systemic delivery system which may instruct unwanted responses from other progenitor regions. We sought to develop one such system using a PLGA delivery system. PLGA channels would enable us to isolate our drug delivery to one select area while PLGA microspheres are essential for ensuring a continued release of specific factors over a designed time.

This study revealed the detrimental effects that PLGA degradation can have on adult neural progenitor cells in culture. Both PLGA channel segments and microspheres degraded over a 4 week period releasing their monomers into culture creating an acidic environment. This caused a reduction in cell viability and proliferation as well as changed the differentiation

potential of NPCs. Previous reports on the use of PLGA with NPCs have only assessed its effects in the short term. Here, we have shown that as PLGA degrades over time its effect on NPC survival, proliferation and differentiation becomes more detrimental. A number of factors may be able to explain this effect, however, one of the most profound observations that this study found was the sustained reduction in pH. Previous studies have found similar PLGA degradation rates and effects on cell survival (115), furthermore the effect that PLGA degradation will have on local environment pH is well accepted (116).

The observed effect on pH in culture is robust. In this study we replaced the media every two days replenishing the supplements and mitogens. Interestingly, a significant difference was seen between the pH of PLGA-layered cultures and the pH of PLGA-microsphere cultures. PLGA microspheres decreased pH at a much slower rate than PLGA-layered for the same quantity of material. This decelerated degradation supports why PLGA-microspheres exposed NPCs had fewer TUNEL positive cells at 28 div than PLGA layered. Also, the slightly acidic environment of PLGA-microsphere cultures allowed for decreased amounts of proliferation of both SC and SVZ derived NPCs at 28 div. One possible reason for the reduction in proliferation seen between control and the PLGA-microsphere group could be due to the effect that pH change had on potentially denaturing newly introduced mitogens. It is possible that the acidic environment may have negatively affected the bioactivity of both EGF and bFGF through biochemical denaturation, thus, reducing their ability to stimulate cell proliferation (117, 118).

Extended exposure to PLGA degradation altered both SC and SVZ derived NPC differentiation. The reduction in both neuronal and astrocyte differentiation showed that extended exposure to PLGA and its monomers can alter NPC differentiation, which has yet to be shown in other biocompatibility studies (119). In control conditions a significant increase in

neuronal differentiation was seen from 7 div to both 14 and 21 div, which is consistent with other studies on adult NPC differentiation (67), however, in both PLGA groups we saw an absence of neuronal differentiation at 28 div. Furthermore, the increase in oligodendrocyte differentiation that is seen in SVZ derived NPCs exposed to PLGA-layered should be considered carefully seeing as very few cells were viable at this time point in this group. PLGA's effect on *in vitro* cell differentiation has been assessed before (81), and cell viability and proliferation was studied for a maximum of 14 div. Clearly, PLGA as a non-degraded material can support cell seeding, proliferation and differentiation (46-48, 120-122). It is important to note that very few studies have shown the effect that its degradation has on differentiation at extended time points. Therefore, the results from this study clearly show the importance of monitoring the effect of PLGA's degradation on the local environment while pursuing its further use *in vivo*.

Chapter 5 - Conclusion

The neurosphere protocol used in this study revealed one of the main difficulties of targeting NPC *in vivo* following a neural insult. There are a number of different *progenitors in vivo*: Type-B GFAP positive progenitors, nestin positive progenitors and also NG2 glia restricted progenitors. Our study reveals that careful selection of appropriate factors and factor combinations must be explored in order to instruct the appropriate progenitor populations. For instance, to develop a remyelination therapy NG2 positive OPCs should be selected for rather than attempting to instruct earlier progenitors to proliferate and differentiate into mature oligodendrocytes. Thus, a traditional EGF exposure may be adequate in preferentially instructing OPC proliferation over other NPC populations. Furthermore, if neural phenotypes are required than careful mitogen selection/combinations should be explored to ensure a maintenance of neural differentiation throughout the proliferation period.

This study reveals a very important point when considering *in vivo* manipulations; NPC response to differentiation factors will be highly dependent on both the specific cell type targeted as well as the contributing factors found within the niche. It is difficult to believe that simple exogenous differentiation factor additions will be sufficient in overcoming pre-existing niche environment conditions. Instead a more sophisticated method must be considered. Pairing of specific differentiation factors with both an understanding of local pre-existing niche factors and other exogenous factors will enable us to target specific cell types and responses. As our knowledge of each progenitor niche expands we will be able to identify contributing and competing factors present during homeostasis and insults which in turn, will help researchers select the most appropriate factors for each targeted cell.

We sought to investigate a delivery method that would prevent further injury following an SCI as well providing specific and localized delivery. The current investigation found that PLGA-layered channels and PLGA microspheres degradation over a 4 week period led to detrimental effects on NPC proliferation, survival and differentiation. This study illustrates that a possible cause of the observation may be due to the acidic environment that the PLGA monomers create. Future studies should attempt to more readily control the degradation rates of PLGA and in turn limit the degree at which the local environment's pH changes.

Taken together this project further demonstrates the inherent potential of using adult spinal cord derived progenitors for repair following injury. Although there are significant obstacles to overcome we have shown that these progenitors can be controlled with exogenous factors and are resilient to long term non-clonal expansions protocols. With further advancement in progenitor differentiation and factor delivery methods we will be able to realize CNS repair through the use of endogenous progenitor sources.

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