The Effect of Ketamine and Glutamate on Proliferation, Differentiation and Migration of Neural Progenitor Cells Derived From the Subventricular Zone and Spinal Cord

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DEDICATION

This dissertation is dedicated to my beloved parents. Much love.

Abstract

During spinal cord injury (SCI), glutamate excitotoxicity and astrocytic scar formation can impede repair. In a preliminary study we found that ketamine, a N-methyl-D-aspartate (NMDA) receptor non-competitive antagonist, can contribute to functional recovery post SCI. Therefore, we investigated the cellular basis for this recovery with respect to neural progenitor cells using an *in vitro* cell culture model. We examined whether ketamine and glutamate influenced the proliferation, differentiation, and migration of differentiating endogenous neural progenitor cells (NPCs) found in the subventricular zone and spinal cord. Our study illustrates that the post functional recovery may have been due to ketamine's influence on delaying spinal cord NPCs derived astrocyte maturation and migration while increasing radial glial cell migration. These results are promising since ketamine administration may help alleviate some of the adverse affects glutamate has on the NPCs found in the spinal cord following SCI.

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LIST OF ABBREVIATIONS

βIIIT	β-III-tubulin
BLBP	Brain lipid binding protein
BrdU	5'-Bromo-2-deoxyuridine
AMPA receptor	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid receptor
BBB locomotor test	Basso, Beattie and Bresnahan locomotor rating scale
EGF	Epidermal growth factor
FGF-2	Fibroblast growth factor 2
GFAP	Glial fibrillary acidic protein
Kainate receptor	2-carboxy-3-carboxy-methyl-4-isopro-penylpyrrolidine receptor
NPC	Neural progenitor cells
NMDA receptor	N-methyl-D-aspartate receptor
PLGA biochannel	*Poly(lactic-co-glycolic acid biochannel*
SCI	Spinal cord injury
SGZ	Subgranular zone
SVZ	Subventricular zone
TUNEL assay	Terminal deoxynucleotidyl transferase dUTP nick end-labeling assay

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Chapter 1.0 - Introduction

1.1 Spinal Cord Injury

Spinal cord injury (SCI) can be a devastating neurological condition, disrupting ascending and descending axonal pathways resulting in a significant loss of function. The severity of the injury depends on the segment of the spinal cord that is injured. Currently, there are no fully restorative treatment options for SCI, which is worrisome considering the fact that the spinal cord is the main information pathway between the brain and the body.

1.1.1 Epidemiology and Impact of SCI

In developed countries, the annual incidence of traumatic SCI ranges from 15 to 40 cases per million population (Botterell et al., 1975; Sekhon & Fehlings, 2001). The incidence of SCI in the United States of America varies from 25 to 59 cases per million population, which means approximately 12, 400 new cases of SCI are reported annually (Devivo, 2012). The incidence in Canada was estimated to be approximately 37.2 cases per million population (Pickett, Simpson, Walker, & Brison, 2003) to 52.5 cases per million population (Dryden et al., 2003). Though incidence of this type of injury is relatively low, the mortality is between 48.3% and 79% (J. F. Kraus, Franti, Riggins, Richards, & Borhani, 1975; Sekhon & Fehlings, 2001).

In the United States of America, the prevalence, the number of persons presently living with SCI, is estimated to be about 721-1009 per million population (Cripps et al., 2011; DeVivo, Fine, Maetz, & Stover, 1980; Harvey, Rothschild, Asmann, & Stripling, 1990; Kurtzke, 1975; Lasfargues, Custis, Morrone, Carswell, & Nguyen, 1995; Stover & Fine, 1987). The prevalence of SCI in Canada is estimated at approximately 1173 per million population (Cripps et al., 2011). The incidence and prevalence of SCI varies from one country to another, and even within a given country there has been noticeable differences (Cripps et al., 2011; Pickett, Simpson, Walker, & Brison, 2003; Sekhon & Fehlings, 2001; M. Wyndaele & Wyndaele, 2006).

Typically, the majority of cases are among males (approximately 80%) compared to females (Devivo, 2012; Sekhon & Fehlings, 2001; M. Wyndaele & Wyndaele, 2006). It has also been reported that about two thirds of all new SCI occur in individuals less than 30 years of age (Sekhon & Fehlings, 2001; Stover & Fine, 1987).

In developed countries, over 50% of the reported cases of acute SCI are due to traffic accidents. The other common causes are accidents related to violence (10-25%), falls (20%), sports and recreation (10-25%) (Sekhon & Fehlings, 2001). In Canada, the leading cause of SCI is unintentional falls (43.2%), commonly among the elderly (Ackery, Tator, & Krassioukov, 2004; Furlan, Bracken, & Fehlings, 2010; A. V. Krassioukov, Furlan, & Fehlings, 2003) and transport injuries (42.8%), common among those aged less than 40 years (Pickett, Simpson, Walker, & Brison, 2003). In less-developed countries falls are the most common cause (Ackery, Tator, & Krassioukov, 2004).

This traumatic disorder does not only causes great personal hardship for the patient but it can have profound consequences for their families and society in general (M. Wyndaele & Wyndaele, 2006). Over the patient's lifetime, they will have to spend over $1 million in health care associated costs (McKinley, Jackson, Cardenas, & DeVivo, 1999).

Traumatic spinal injury can happen at all levels of the spinal cord. In Canada, cervical spine injury is the most common type, with an incidence of about 56.3% (Devivo, 2012; Sekhon & Fehlings, 2001). Following a SCI, the victim can lose controlled motion, sensation and autonomic control below the site of injury. There may be either complete or incomplete loss of function below the injury site. For example, an injury at the cervical level in the spinal cord can result in the victim becoming completely tetraplegic or tetraparetic. These conditions are generally permanent due to the very limited innate regenerative response of the central nervous system. Currently there is no clinically available treatment to regenerate or cure SCI and treatment has focused on stabilization and prevention of further injury and complications.

1.1.2 Behavioral assessment

A complete transection model is usually used to study possible regenerative mechanisms following SCI, however since this is a severe model of injury, there is very minimal locomotor recovery (Talac et al., 2004). The Basso, Beattie and Bresnahan (BBB) locomotor rating scale is widely used to test behavioral consequences of SCI in experimental rodents (Basso, Beattie, & Bresnahan, 1995; Scheff, Saucier, & Cain, 2002). The 21-point scale is used to assess the efficacy of various treatment options following SCI, as a significant increase in the BBB score would be an indicator of a beneficial treatment option. A

preliminary study from our lab found that the administration of 10 mg/kg of (*RS*)-2-(2-Chlorophenyl)-2-(methylamino) cyclohexanone hydrochloride, here on called ketamine, correlated with improved locomotor function in Sprague Dawley rats (Fig 1.1).

As seen in figure 1.1A, there is very minimal functional recovery following a complete transection at the eighth thoracic (T8) spinal level. In a preliminary study done at the lab, experimental animals were implanted with either a poly(lactic-co-glycolic acid biochannel (PLGA) or PLGA biochannel embedded with iron nanoparticles (PLGA-NP) following a complete T8 transection. The BBB functional test demonstrated a significant improvement in the PLGA-NP implanted rats compared with the PLGA implanted rats. It should be mentioned that the experimental animals that showed an increase in their locomotor ability also received ketamine administration, which means the improvement in locomotor function could be due to either the iron nanoparticles or the administration of ketamine. In another separate experiment, an experimental rat that was implanted with a pure PLGA biochannel, showed an improvement in hindlimb locomotor function when it received ketamine administration four weeks after a complete T8 transection. These results are important, since a BBB score of 5 corresponds to slight or extensive movement of the three-hindlimb joints (ankle, knee and hip), and a score of 8 corresponds to a sweeping/plantar placement of the paw with no weight support (Basso, Beattie, & Bresnahan, 1995). These results indicate that ketamine administration might facilitate improvement in hindlimb locomotor function as opposed to the iron nanoparticle channels. However, the cellular mechanism by which ketamine improves hindlimb locomotor function is not known.

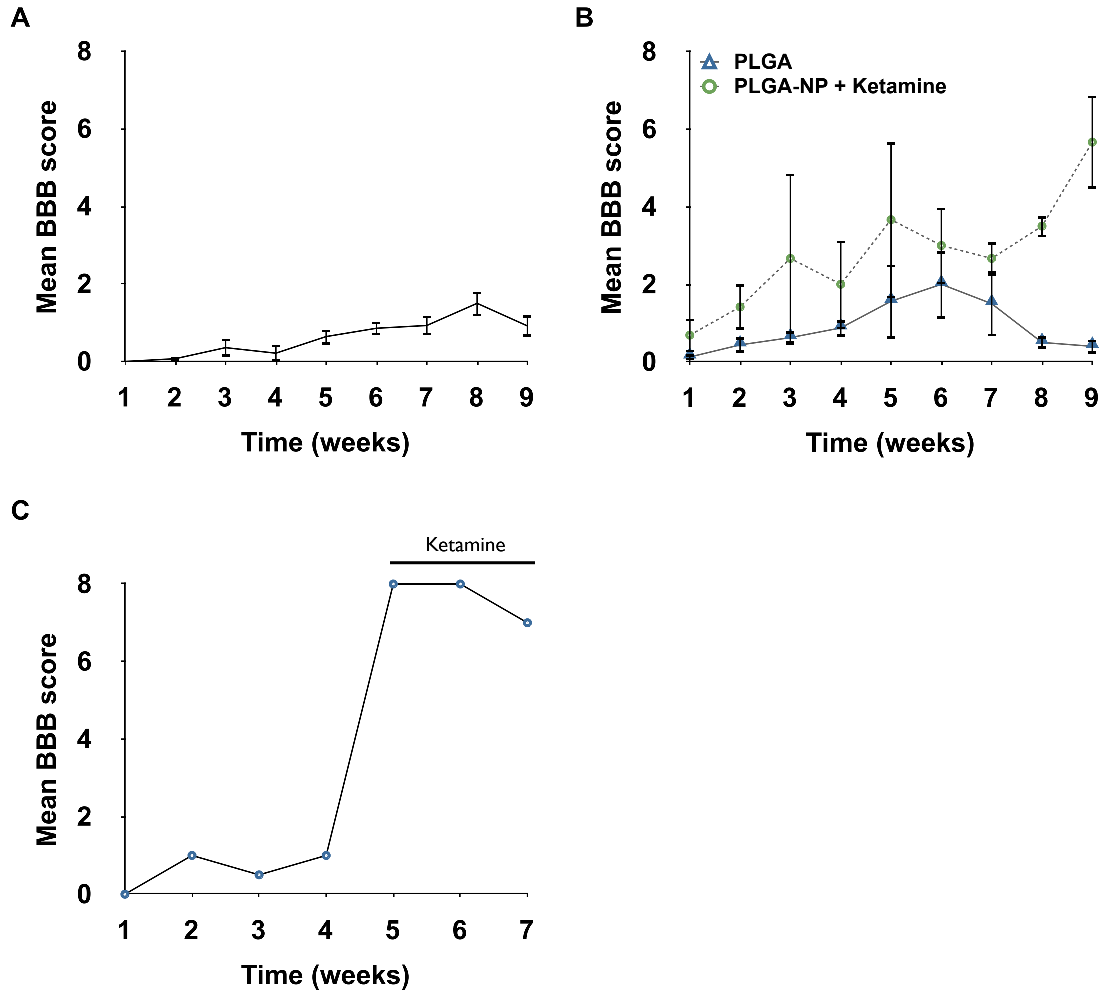

Figure 1.1 – Locomotor recovery in experimental rats following a complete transection at the eighth thoracic (T8) spinal level. Animals were assessed for hindlimb motor function weekly using the Basso, Beattie, and Bresnahan Locomotor Rating Scale. (A) Hindlimb functional recovery is minimal following a complete T8 transection (*n*=3) (B) Weekly ketamine administration promotes hindlimb functional recovery (*n*=3) (C) Short term weekly ketamine administration increases hindlimb functional recovery (n=1). Results presented as mean±standard error of the mean. PLGA, poly(lactic-co-glycolic acid biochannel*;* PLGA-NP, poly(lactic-co-glycolic acid biochannel imbedded with iron nanoparticles*.*

1.1.3 Pathophysiology of SCI

The complexity surrounding the treatment for SCI is due to the physiological environment created at the injury site; it is not permissive to spinal cord regeneration (Berry, Rees, Hall, Yiu, & Sievers, 1988; David & Aguayo, 1981). The events following a SCI can be grouped into two successive stages: primary and secondary injuries. Primary injury is the initial direct injury to the cord. Secondary injury exacerbates the physical damage and further limits restorative processes (Oyinbo, 2011; Sekhon & Fehlings, 2001; C. H. Tator & Fehlings, 1991).

The primary injury is the physical consequence of the trauma, which manifests as transected axons and broken neural cell membranes. Also, there is immediate neurological dysfunction following the initial injury due to the loss of neurons and their connections. The secondary injury is the result of the cascade of biochemical and cellular events that occur in response to the initial physical damage. Mechanisms of secondary injury include but are not limited to hemorrhage, ischemia, excitotoxicity, calcium-mediated secondary injury, apoptosis, astroglial scar launch, inflammation, etc. (R. J. Dumont et al., 2001; Oyinbo, 2011). The secondary injury phase can further be divided into the acute phase, sub-acute phase, and chronic phase. The acute phase lasts up to 24 hours post injury. During this phase there is necrosis of cells and the first wave of apoptosis. The sub-acute phase is categorized as the period between 24 hours and 2 weeks post jury. During this phase, in addition to the second wave of apoptosis, there is also the formation of a glial scar, composed of reactive astrocytes, glial progenitors, microglia, and macrophages, and is usually oriented perpendicular to the neuraxis at the injury site (Jones, Yamaguchi, Stallcup, & Tuszynski, 2002; Jones, Margolis, & Tuszynski, 2003; G. Yiu & He, 2006). By the chronic phase there is an increase in the

injured area as there is a significant inflammatory response leading to apoptosis of surrounding cells and axons being stripped of their myelin sheaths, hindering their ability to function properly (Collins, 1983; Sandler & Tator, 1976; Sekhon & Fehlings, 2001; C. H. Tator & Fehlings, 1991). Moreover, the presence of secreted and transmembrane inhibitory molecules at and around the injury site prevents axon growth (Fawcett, 2006; Silver & Miller, 2004; G. Yiu & He, 2006). Therefore, replacing the lost neurons, remyelinating the nerve cells and controlling the proliferation and migration of glial cells are essential to treating SCI.

1.1.3.1 Excitotoxicity

During the secondary injury phase following SCI, there is an elevation in the levels of excitatory neurotransmitters (Farooque, Olsson, & Hillered, 1997), which can damage the surrounding spinal cord tissue (Faden, Lemke, Simon, & Noble, 1988; Faden & Simon, 1988; Panter, Yum, & Faden, 1990). Excitotoxicity describes the process whereby the excessive activation of glutamate receptors results in neuronal damage (Olney, 1994). Glutamate, one of the most abundant excitatory neurotransmitters in the mammalian central nervous system (CNS), is stored in synaptic vesicles and is found at the axon terminal in neurons (Curtis & Johnston, 1974; Fonnum, 1984). Following an action potential, these synaptic vesicles are released by exocytosis into the synaptic cleft. Glutamate then can activate fast-acting, excitatory ionotropic glutamate receptors (iGluR) and slower-acting metabotropic glutamate receptors (mGluR) found on the post-synaptic cell. There are three types of iGluR: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxy-methyl-4-isopro-penylpyrrolidine (kainate) receptors (von Euler, Li-Li, Whittemore, Seiger, & Sundstrom, 1997; Wrathall,

Teng, & Marriott, 1997). It has been suggested that the activation of NMDA, AMPA and kainate receptors may play a role in the production of ischemic damage (Guha & Tator, 1988).

The activation of glutamate receptors leads to the accumulation of intracellular sodium (D. W. Choi, 1987), and results in intracellular acidosis. There is also an accumulation of intracellular calcium (Agrawal & Fehlings, 1997), which also damages the cell. The activation of NMDA receptors by glutamate turns on a cascade of cellular events, resulting in reactive molecules that damage the cell and lead to neuronal death. Previous research suggest that neural cell death is caused by but not limited to inhibition of Na^+-K^+ ATPase activity, inactivation of membrane sodium channels, and oxidative modification of proteins (R. J. Dumont et al., 2001).

Initial studies have indicated that *in vivo* extracellular glutamate concentrations rise to 0.53 mM following traumatic impact to the spinal cord (D. Liu, Xu, Pan, & McAdoo, 1999; Xu, McAdoo, Hughes, Robak, & de Castro, 1998). It has also been suggested that glutamate levels at the site of injury are in the toxic range for around two hours following trauma (D. Liu, Thangnipon, & McAdoo, 1991; D. Liu, Xu, Pan, & McAdoo, 1999). Interestingly, this value is significantly higher than what has been proposed to be toxic to cultured cells *in vitro* [0.05 mM] (D. W. Choi, Maulucci-Gedde, & Kriegstein, 1987; Regan & Choi, 1991). A possible reason why low levels of glutamate *in vitro* resulted in toxicity could be due to the fact that the *in vitro* system is drastically different in terms of cell composition and intracellular/extracellular volume ratios from the *in vivo* system.

Previous research has shown that the administration of glutamate at concentrations close to that released following SCI causes functional impairment (Xu, Hughes, Zhang, Cain, & McAdoo, 2005) and causes the death of neurons and oligodendrocytes (D. Liu, Xu, Pan, & McAdoo, 1999). It has been shown that other NMDA receptor agonists significantly worsen post-traumatic tissue damage, whereas, the administration of MK-801, which is a non-competitive antagonist of the NMDA receptor, significantly reduced post-traumatic tissue damage (Faden, Lemke, Simon, & Noble, 1988; Faden & Simon, 1988).

1.2 Ketamine

Ketamine is a phencyclidine derivative and is a widely used anesthetic that is administered either intravenously or intramuscularly. It is thought to mainly work as a non-competitive antagonist of the NMDA receptor (Chizh, 2007; Persson, 2010). However, it has been reported that at high concentrations ketamine can interact with other receptors such as opioid, monoamine, cholinergic and adenosine (Chizh, 2007; F. Lois & De Kock, 2008; Persson, 2010).

1.2.1 Pharmacology and Mechanism of action

The ketamine molecule [(RS)-2-(O-chlorophenyl)- 2-methylamino cylohexanone] has a molecular weight of 238. Due to the presence of a chiral center, it has two optical isomers (enantiomers). Ketamine is water-soluble and also has high lipid solubility. When injected intramuscularly, the onset of action is approximately 5 minutes and the duration of action of ketamine is roughly 30 minutes to 2 hours. It is metabolized in the liver into norketamine and

dehydronorketamine and is excreted in the urine (Quibell, Prommer, Mihalyo, Twycross, & Wilcock, 2011).

Ketamine primary works by binding to the phencyclidine site on the NMDA-receptor channel (Oye, 1998). As a non-competitive inhibitor, ketamine does not compete with glutamate for binding at the active site. Ketamine binds in the NMDA receptor channel pore but the agonist, glutamate binds to the extracellular surface of the receptor. The NMDA receptor is both ligand and voltage dependent. At normal resting membrane potentials, the NMDA receptor is blocked by magnesium and is inactive. Following cell depolarization, the channel is unblocked and calcium and sodium ions enter the cell while potassium ions exists the cell (Mayer, Westbrook, & Guthrie, 1984). Ketamine has been shown to inhibit excitotoxic signaling, reduce neuronal apoptosis, and attenuate the systemic inflammatory response to tissue injury (Hudetz & Pagel, 2010). This indicates that ketamine may help alleviate some aspects of secondary injury following SCI.

1.2.2 Neuroprotection and Neurotoxicity

Previous research suggests that ketamine can play a neuroprotective role (Anand et al., 2007; Engelhard et al., 2003; Green & Cote, 2009; Himmelseher, Pfenninger, & Georgieff, 1996; Himmelseher, Pfenninger, Kochs, & Auchter, 2000; Himmelseher & Durieux, 2005; Hudetz & Pagel, 2010; Orser, Pennefather, & MacDonald, 1997; Shapira, Lam, Artru, Eng, & Soltow, 1993; D. H. Smith, Okiyama, Gennarelli, & McIntosh, 1993). Ketamine's neuroprotective role is suggested to work by reducing the interaction between NMDA receptors and their respective intracellular pathway (Himmelseher & Durieux, 2005).

On the other hand, ketamine has also been shown to induce neurotoxicity under certain experimental conditions (C. Wang et al., 2005; C. Wang et al., 2006). There was cell death when neurons from the rat forebrain were cultured for a prolonged period with ketamine at concentrations greater than 10 μM. However, when these cells were cultured with 0.1 or 1.0 μM of ketamine, there was no significant cell death. This indicates that neurotoxicity through ketamine exposure *in vitro* may be dose dependent. It has also been shown that ketamine administration induces neuroapoptosis in rodent and rhesus monkeys *in vivo* (Ikonomidou et al., 1999; W. Slikker Jr et al., 2007). However, roughly ten times the amount of ketamine that is normally used to sedate a human was used in this study (S. G. Soriano, 2012). This hints at the fact that neurotoxicity through ketamine exposure *in vivo* may be dose dependent. For these reason, it is essential that a range of ketamine concentrations be examined to fully understand the influence ketamine has on a certain cell population.

Furthermore, it has been found that when cell cultures are induced to glutamate injury, administration of ketamine increased neuronal and astroglial viability, preserved cellular morphology, and reduced cell swelling (Pfenninger & Himmelseher, 1997). In terms of SCI, research has demonstrated that the administration of ketamine prevented neurological injuries in a rabbit model of spinal cord ischemia (Q. J. Yu et al., 2008). In addition to its neuroprotective effects, ketamine also has an anti-inflammatory effect on astrocytes (Mazar et al., 2005; Wu, Chen, Ueng, & Chen, 2008). It has been found that ketamine when co-incubated with glutamate, reduced astrocytic swelling in a dose-dependent manner, suggesting ketamine is involved in protecting astrocytes from glutamate-induced swelling.

Moreover, Dong *et al.* found enhanced neuronal differentiation of rat cortical NPCs following exposure to ketamine (Dong, Rovnaghi, & Anand, 2012). Also, ketamine exposure has been shown to increase synaptogenesis (De Roo et al., 2009). These multi-faceted benefits make ketamine an ideal candidate for the treatment of SCI. However, the effects ketamine has on other cell types cannot be fully extrapolated to cells found in the spinal cord, because the cells originating from the spinal cord would have different biological characteristics compared to other cells types (Kulbatski & Tator, 2009; Shihabuddin, Ray, & Gage, 1997). Therefore, it is important to evaluate the effect ketamine and glutamate has on neural progenitor cells found in the spinal cord.

1.3 Neural progenitor cells

One potential therapy for SCI is the use of progenitor or stem cell based therapies such as embryonic stem cells, mesenchymal stem cells and fetal neural stem cells. Although these cell therapies show some promise they also have immense clinical limitations. These include the possibility of immuno-rejection of exogenous stem cells, a multitude of ethical problems surrounding the use of stem cells, as well as the poorly controlled proliferation and differentiation of transplanted stem cells (Bongso, Fong, & Gauthaman, 2008). The recent discovery of endogenous neural progenitor cells (NPC) within the adult central nervous system provides a more ideal alternative source of cells for the treatment of SCI, as endogenous NPCs eliminate the problems mentioned above. NPCs are undifferentiated self-renewing, multipotent cells that give rise to the three main cell types found in the nervous system: neurons, astrocytes, and oligodendrocytes (Fisher, 1997; Gage, 2000).

The adult CNS was regarded as not being able to regenerate and showed no signs of plasticity. Santiago Ramon y Cajal, who is recognized as the father of modern neuroscience, famously stated: “Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated” (Colucci-D'Amato, Bonavita, & di Porzio, 2006; Ramon y Cajal, 1913). This was the prevalent view in neurobiology until adequate techniques were developed to detect cell division in the adult brain (Colucci-D'Amato, Bonavita, & di Porzio, 2006; Messier, Leblond, & Smart, 1958) overturning the long-held dogma that neurogenesis is restricted to the embryonic phase.

In 1992, Reynolds and Weiss definitively isolated NPCs from the striatal tissue of adult mice brain. They were able to induce proliferation *in vitro* using epidermal growth factor and fibroblast growth factor and showed that the cultured cells were able to differentiate into neurons and astrocytes (Reynolds & Weiss, 1992; Reynolds, Tetzlaff, & Weiss, 1992; Reynolds & Weiss, 1996). To date, NPCs have been identified and isolated from embryonic, fetal and adult mammalian brain and spinal cord (Adrian & Walker, 1962; Gage, 2000; Horner et al., 2000; Johansson et al., 1999; Kehl, Fairbanks, Laughlin, & Wilcox, 1997; Kulbatski et al., 2007; Morshead et al., 1994; Palmer, Ray, & Gage, 1995; Palmer, Takahashi, & Gage, 1997; Rietze, Poulin, & Weiss, 2000; Weiss et al., 1996).

NPCs have been found in three specific areas throughout the CNS: the subgranular zone (SGZ) of the hippocampal dentate gyrus, which gives rise to new granule cells (Altman & Das, 1965; Cameron, Woolley, McEwen, & Gould, 1993; Kornack & Rakic, 1999); the subventricular zone (SVZ) of the lateral ventricles, which generates new interneurons that

migrate to the olfactory bulb (Altman, 1969; Alvarez-Buylla & Lim, 2004; C. Lois & Alvarez-Buylla, 1993) and the ependymal zone of the central canal in the spinal cord (SC) (Kulbatski, Mothe, Nomura, & Tator, 2005; Martens, Seaberg, & van der Kooy, 2002; Meletis et al., 2008). This study focuses predominantly on the NPCs derived from the SC, although references will be made to the SVZ derived NPCs. SC and SVZ NPCs will be examined in this study since it is important to identify differences in the way the two cell populations will respond to the therapeutic intervention in question. Any differences we find will have an impact on future therapeutic administration methods because if there are differences, optimized therapeutic administration may require targeted focal delivery.

1.3.1 Subventricular zone NPCs

In the adult mammalian brain, the SVZ is located next to the ependyma, which lines the lateral ventricle (Alvarez-Buylla & Garcia-Verdugo, 2002). This region is composed of quiescent ependymal cells, and the subependymal cells which includes type B GFAP-positive progenitors, type C transit-amplifying progenitors, type A migrating neuroblasts, tanycytes, microglia, and blood vessels (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; Hamilton, Truong, Bednarczyk, Aumont, & Fernandes, 2009). Postnatally, NPCs are thought to be direct descendants of the embryonic ventricular zone (Marshall & Goldman, 2002). It is believed that the subependymal type B GFAP-positive progenitors are the true neural stem cells in this region (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; Hamilton, Truong, Bednarczyk, Aumont, & Fernandes, 2009; Imura, Kornblum, & Sofroniew, 2003; Morshead, Garcia, Sofroniew, & van Der Kooy, 2003).

The GFAP-positive progenitors give rise to transit-amplifying progenitors, which in turn produce migrating neuroblasts (Alvarez-Buylla & Garcia-Verdugo, 2002; Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997; Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999). These neuroblasts then begin to mature as they migrate to the olfactory bulb (Carleton, Petreanu, Lansford, Alvarez-Buylla, & Lledo, 2003; Menezes, Smith, Nelson, & Luskin, 1995), where they differentiate into granule and periglomerular neurons (Alvarez-Buylla & Garcia-Verdugo, 2002; Biebl, Cooper, Winkler, & Kuhn, 2000; Carleton, Petreanu, Lansford, Alvarez-Buylla, & Lledo, 2003). Previous studies have found that the differentiating ability of SVZ cells is limited and relies on environmental information (Merkle, Mirzadeh, & Alvarez-Buylla, 2007).

It has been shown that when the SVZ derived NPCs are harvested and cultured under the appropriate conditions in the presence of mitogens such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2), these cells proliferate producing neurospheres, which are three-dimensional, free floating clusters of undifferentiated cells (Gritti et al., 1996; Gritti, Vescovi, & Galli, 2002; Vescovi & Snyder, 1999; Weiss et al., 1996). Upon the removal of the mitogens, the cells differentiate into the three main neural cell types found in the CNS (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; Kilpatrick & Bartlett, 1993; Vescovi, Reynolds, Fraser, & Weiss, 1993; Weiss et al., 1996; Weiss & van der Kooy, 1998).

1.3.2 SC NPCs

The central canal in the spinal cord is another region in the adult mammalian CNS where NPCs can be isolated (Martens, Seaberg, & van der Kooy, 2002). In 1962, Adrian and

Walker used ^{3}H-thymidine to label a population of dividing cells in a non-injured adult rat spinal cord (Adrian & Walker, 1962). It has since been shown that cells residing near the central canal are able to proliferate in the normal and injured spinal cord (Mothe & Tator, 2005; Namiki & Tator, 1999). However, in adult mammals, under normal conditions the ependymal region exhibits limited proliferative ability (Adrian & Walker, 1962; Horner et al., 2000), while cell proliferation has been shown to be enhanced following different forms of SCI (Johansson et al., 1999; Namiki & Tator, 1999). The discovery of NPCs within the spinal cord has opened the door to a wide array of research in the field of regenerative medicine, since SCI results in the loss of neurons and glial cells (Lindvall & Kokaia, 2006). Therefore, the ability to regenerate these lost cells using NPCs in future cell-based therapies would be very beneficial.

Research in mammals has shown that in response to a traumatic event these precursor cells, which reside near the central canal, are able to proliferate and migrate towards the site of injury. However, it has been shown that following SCI the NPCs from the central canal proliferate and differentiate predominantly into glial cells such as astrocytes and oligodendrocytes, thereby further strengthening the glial scar and in turn playing a role in inhibiting axon regeneration (Attar, Kaptanoglu, Aydin, Ayten, & Sargon, 2005; Dervan & Roberts, 2003; Frisen, Johansson, Torok, Risling, & Lendahl, 1995; Kojima & Tator, 2000; Kulbatski et al., 2007; Meletis et al., 2008; Mothe & Tator, 2005; Namiki & Tator, 1999; Parr et al., 2008). Similar to SVZ derived NPCs, isolated NPCs from the spinal cord have the ability to proliferate and differentiate into three main cell lineages in the CNS: neurons, astrocytes and oligodendrocytes *in vitro* (Kulbatski et al., 2007; Shihabuddin, Ray, & Gage, 1997; Weiss et al., 1996).

1.3.3 Cell proliferation

Previous studies have shown that the activation of the NMDA receptor influences the proliferation rate of neural progenitor cells derived from the brain (Dong, Rovnaghi, & Anand, 2012; Joo et al., 2007; Kitayama, Yoneyama, & Yoneda, 2003; Kitayama, Yoneyama, Tamaki, & Yoneda, 2004; Luk, Kennedy, & Sadikot, 2003; Luk & Sadikot, 2004; Mochizuki et al., 2007; Sah, Ray, & Gage, 1997; M. Suzuki et al., 2006; Yoneyama et al., 2008). However, the involvement of NMDA receptor agonist and antagonist in cell proliferation remains controversial (Kitayama, Yoneyama, & Yoneda, 2003; Luk, Kennedy, & Sadikot, 2003; Nacher, Rosell, Alonso-Llosa, & McEwen, 2001).

On one hand it has been shown that exposure to NMDA antagonists promote cell proliferation in certain areas of the central nervous system (Cameron, McEwen, & Gould, 1995; Gould, Cameron, & McEwen, 1994; Hirasawa, Wada, Kohsaka, & Uchino, 2003; Kitayama, Yoneyama, & Yoneda, 2003; Nacher, Rosell, Alonso-Llosa, & McEwen, 2001; Okuyama, Takagi, Kawai, Miyake-Takagi, & Takeo, 2004), suggesting that glutamate would decrease cell proliferation. On the other hand, it has also been shown that NMDA receptor activation through factors such as glutamate can increase cell proliferation (Haydar, Wang, Schwartz, & Rakic, 2000; Joo et al., 2007; Luk, Kennedy, & Sadikot, 2003; Luk & Sadikot, 2004; Mochizuki et al., 2007; Sah, Ray, & Gage, 1997; M. Suzuki et al., 2006). A plausible reason for this discrepancy is the differential influence NDMA receptor agonists and antagonists may have on certain cell populations. Hayder *et al.* (2000) found that when glutamate is exogenously administered, it induces proliferation of cells found in the embryonic rat ventricular zone, yet it inhibits proliferation of the cells found in the embryonic rat SVZ (Haydar, Wang, Schwartz, & Rakic, 2000). Brazel *et al.* (2005) have

shown that glutamate increases adult SVZ derived cell proliferation (Brazel, Nunez, Yang, & Levison, 2005). Whereas it has been shown that, MK-801, a non-competitive NMDA receptor antagonist, has no affect on adult SVZ derived cell proliferation. These results indicate that the NMDA receptor's regulatory role at least in terms of cell proliferation will differ depending on the source of neural progenitor cells.

One aspect of this study will examine the effect ketamine and glutamate has on SC and SVZ derived NPC proliferation. Based on previous studies, we believe that glutamate will increase cell proliferation and ketamine will have no affect on cell proliferation, whereas, the combination of both glutamate and ketamine will result in a decrease in cell proliferation.

1.3.4 Cell differentiation

As mentioned above, previous studies have demonstrated that NPCs derived from the SC and SVZ differentiate predominately into glial cells (Brazelton, Rossi, Keshet, & Blau, 2000; Cao et al., 2001; Gritti et al., 1996; Kulbatski, Mothe, Nomura, & Tator, 2005; Kulbatski et al., 2007; Kulbatski & Tator, 2009; Palmer, Markakis, Willhoite, Safar, & Gage, 1999). The differentiation potential of SC and SVZ derived NPCs following exposure to NMDA receptor agonist and antagonist has not been extensively studied. Studies looking at the influence of the NMDA receptor signalling on cell differentiation focused mainly on the SGZ cells in the dentate gyrus of the hippocampus. It has been shown that glutamate increases neuronal differentiation of cells derived from the adult hippocampus (Bengzon et al., 1997; Deisseroth et al., 2004; Parent et al., 1997). Moreover, it has been shown that MK-801 did not influence gliogenesis in the SGZ of the hippocampus (Petrus et al., 2009). These results suggest that the NMDA receptor plays a more prominent role in neuronal

differentiation compared to glial differentiation. However, recently glutamate has been shown to increase oligodendrocyte differentiation (Cavaliere, Urra, Alberdi, & Matute, 2012), while MK-801 does not affect gliogenesis of cells derived from the SVZ (Petrus et al., 2009).

The second aspect of this study will examine the effect ketamine and glutamate has on SC and SVZ derived NPC differentiation. Based on previous studies, we believe that glutamate will increase cell differentiation and ketamine will have no affect on cell differentiation, whereas, the combination of both glutamate and ketamine will result in a decrease in cell differentiation.

1.3.5 Cell migration

Studies looking at the involvement of NMDA receptors in cell migration are scarce and many studies have focused on neuronal migration only. Also, much of the research on the NMDA receptor's role in cellular migration has been conducted in SGZ cells. It has been shown that glutamate enhances neuronal migration (Komuro & Rakic, 1993). Behar et al. found that glutamate increased neuronal migration of cells derived from the SVZ (Behar et al., 1999). The third aspect of this study will examine the effect ketamine and glutamate has on SC and SVZ derived NPC migration. Based on previous studies, we believe that glutamate will increase cell migration and ketamine will have no affect on cell migration, whereas, the combination of both glutamate and ketamine will result in a decrease in cell migration.

Most of the studies presented above examine the effects of NMDA receptor agonist and antagonist on cell proliferation, differentiation and migration using an *in vivo* method. Even though, evidence does point towards NDMA receptor agonists and antagonists as regulating cell proliferation, differentiation and migration, this has not been examined in a pure NPCs population. When looking at the influence of these factors on NPCs *in vivo*, it is inevitable that the cell population in question is growing among other differentiated cells. Therefore, so far it is likely that we have been observing a wide general effect that is influenced by the cell's environment; it is not a direct response since the agonist and antagonist may be acting on other cells in the environment. It is highly likely that mature cells in the environment, who are either activated or inactivated by these factors, may be influencing proliferation, differentiation and migration rate of nearby NPCs. Thus, it would be beneficial to study the influence NMDA receptor agonist and antagonist would have on NPCs cultured from the SC and SVZ.

1.4 Purpose and Hypothesis

In light of a previous study from our lab showing improved locomotor ability following ketamine administration, the purpose of this project was to investigate the cellular basis for this improvement. More specifically, we were interested in looking at whether progenitor cells found in the spinal cord were influenced by ketamine. This study will examine whether ketamine and glutamate can regulate the fundamental properties of NPC derived from the SC and SVZ. The objectives of this study is to:

1. Phenotype the proliferation, differentiation and migration of differentiating neural progenitor cells following exposure to ketamine and glutamate *in vitro*

2. Determine whether a common anesthetic, ketamine, will counter the influence of glutamate on spinal cord and subventricular zone derived neural progenitor cells.

Based on previous studies, we hypothesize that glutamate will increase cell proliferation, differentiation and migration and ketamine will have no affect on cell proliferation, differentiation and migration whereas, the combination of both glutamate and ketamine will result in a decrease in cell proliferation, differentiation and migration.

CHAPTER 2.0 – MATERIALS AND METHODS

2.1 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. Endogenous neural progenitor cells isolation procedures were based on the protocol developed by Kulbastiki *et al.* (Kulbatski et al., 2007). Tissues were harvested from adult female Sprague Dawley rats weighing between 150 and 200 grams. All surgical instruments were autoclaved to ensure sterility. Experimental animals were deeply anesthetized with isofluorane and 2% oxygen, and then decapitated with a rodent guillotine. Using sterile surgical tools the skulls and spinal columns were dissected out using scissors, sprayed with 70% ethanol and stored in a 50 millilitre (mL) centrifuge tube containing cold dissection media composed of: 87% MEM-α (Gibco-Invitrogen; Burlington, Ontario, Canada, 12483-020), 10% fetal bovine serum (Gibco-Invitrogen, 12483-020), 1% L-glutamine (Gibco-Invitrogen, 25030-081) and 2% penicillin & streptomycin (Gibco-Invitrogen, 15140-122). Cells were isolated from the adult subventricular area in the brain and the central canal of the spinal cord by dissecting away the area of interest from the surrounding tissue.

2.1.1 Dissection of the subventricular zone of the brain

The skull was transferred from the 50 mL centrifuge tube and placed in a sterile petri dish

(Fisher Scientific, 08-757-9B; Mississauga, Ontario, Canada) containing cold dissection media. The occipital, temporal, and parietal bones were excised to expose both cerebral hemispheres. Using a sterile pair of curved forceps, the brain was gently removed and placed into a new petri dish containing new cold dissection media.

Then using an adult rat brain matrix and sterile stainless steel razor blades, two millimetre (mm) slices of the brain were prepared. Only slices containing the lateral ventricles were placed in a new petri dish containing cold dissection media, for further dissection. The entire ependymal and subventricular regions of the lateral ventricle were excised using a dissection microscope (Nikon) and Moria ultrafine tipped forceps (Fisher Scientific, NC9100523). The dissected tissue was placed in a 15 mL centrifuge tube (Fisher Scientific, 50819798) containing fresh dissection media that was sitting on ice.

2.1.2 Dissection of the central canal in the spinal cord

The spinal column was transferred from the 50 mL centrifuge tube and placed in a sterile petri dish containing cold dissection media. Using sterile instruments the spinal cord was isolated. The spinal cord was removed from the vertebrae after bilateral laminectomy was performed using a six mm serrated malleus nippers (Storz, N1430) along the length of the spinal column from the cervical to the sacral spinal level to reveal the dorsal side of the spinal cord. Using micro-scissors the nerve roots were cut removing any remaining connection between the spinal cord and the remaining spinal column. The entire spinal cord from cervical to sacral region was removed using blunt forceps ensuring no compression of the cord. The tissue was placed in a new petri dish containing cold dissection media. The spinal cord was transected with a razor blade one mm rostral to the conus medullaris, and

then sliced into approximately one-centimeter (cm) long sections using a razor blade.

One at a time, the dura was removed from the spinal cord sections by pulling it apart with micro ultra fine tipped forceps (Fisher Scientific, NC9100523). The dorsal medial line of the spinal cord was located. Using fine forceps the dorsal portion of the spinal cord was cut in a rostral-caudal direction. Following this the spinal cord could then be opened revealing the grey matter and central canal. The tissue surrounding the central canal was micro-dissected using micro-ultra fine tipped forceps. Tissue segments were ensured to be no more than one mm^3 in volume and were placed in a 15 mL centrifuge tube with cold dissection media.

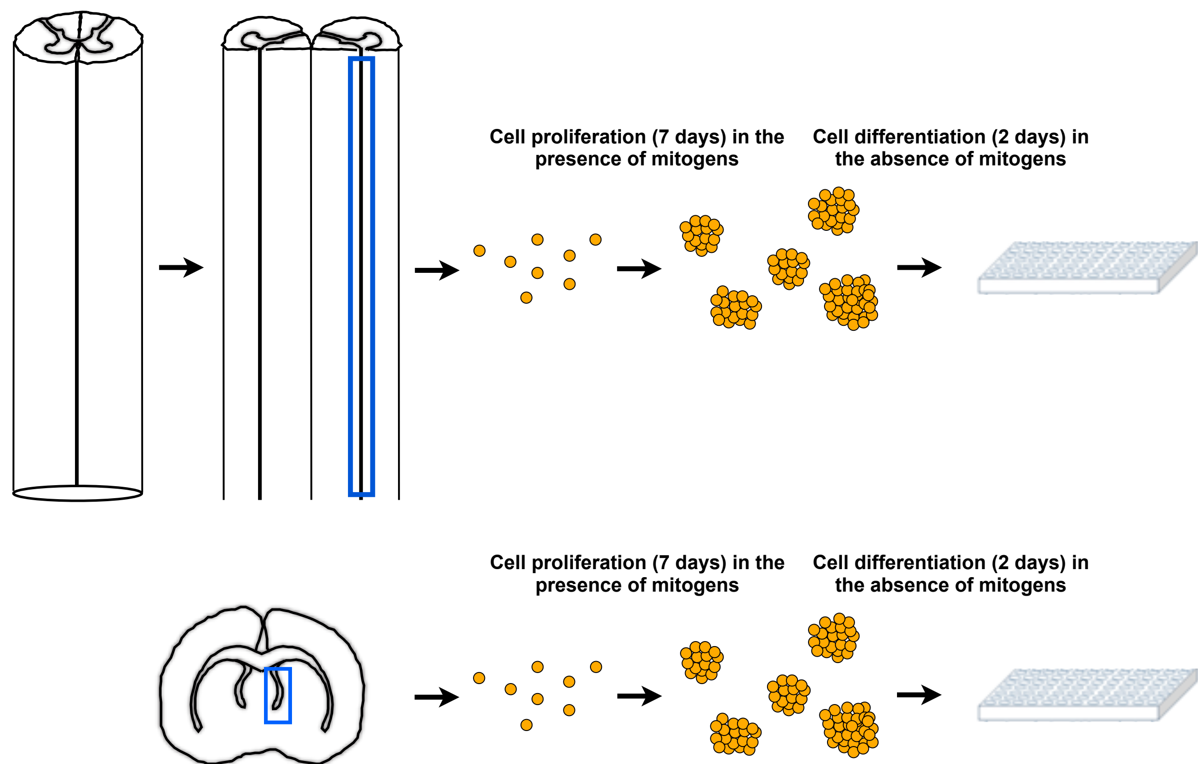

Figure 2.1 – *In vitro* experimental technique used to study the influence of glutamate and ketamine on adult endogenous neural progenitor cells from the spinal cord and the brain.

2.2 Culturing endogenous neural progenitor cells

Under sterile conditions tissues harvested from both the SVZ and SC were triturated 20 times using a fire polished glass cotton plugged pasteur pipette (Fisher Scientific, 13-678-8B) in their respective 15 mL centrifuge tubes. The cell suspensions were then centrifuged at 400 rotations per minute (rpm) for 4 minutes. This cycle of trituration and centrifugation was repeated three additional times. Following each centrifugation step the supernatant was collected in a new 15 mL centrifuge tube. Special care was taken to avoid disturbing the settled pellet to prevent additional contamination of the desired cell suspension. The pellet was mixed with fresh dissection media for each trituration step.

The centrifuge tubes containing the supernatant were then centrifuged at 1500 rpm for five minutes. The supernatant was discarded without disturbing the pellet. The pellets were then washed with culture media containing: 98% Dulbecco's modified eagle medium: nutrient mixture F12 (DMEM/F12) (Gibco-Invitrogen, 10565-042), 1% L-glutamine, 1% N-2 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). The cell suspensions were then centrifuged at 1500 rpm for one minute following which the supernatant was discarded and fresh culture media was added to the pellet. The cell suspensions were mixed using fire polished glass cotton plugged pasteur pipette and then filtered through a 40 µm cell sieve (BD Biosciences). The filtered single cell suspensions were supplemented additionally with the following mitogens: 20 ng/mL of epidermal growth factor (EGF) (Peprotech, Rocky Hill, New Jersey, 100-44) and 20 ng/mL of fibroblast growth factor 2 (FGF-2) (Peprotech, 100-18B). EGF was prepared in 0.1% bovine serum albumin (BSA) (Fisher Scientific, PI-77110) phosphate buffered saline (PBS)

to prevent absorptive loss. FGF-2 was prepared in 5 mM Tris buffered saline (Sigma Aldrich, 154563) with a pH of 7.6.

The SC and SVZ cell suspensions were separately seeded in a sterile 6 six well low attachment culture plate (Fisher Scientific, 07-200-601) and maintained at 37°C in an incubator with 95% humidity and 5% carbon dioxide (CO_2). In order to remove debris and encourage the formation of primary neurospheres, for each plate, half of the culture medium was replaced every two days and re-supplemented with fresh 20 ng/mL of EGF and FGF-2. Under these conditions, cells readily proliferated producing neurospheres, which are free floating clonally derived clusters of undifferentiated cells.

2.3 Neurosphere differentiation

The affect of ketamine and glutamate on NPCs from the SVZ and SC was studied over two days. Appropriate dilutions of ketamine (100 mg/mL, Vetalar, Bioniche) were obtained through serial dilution of the stock, using culture media. A range of 0.05 μM to 500 μM ketamine was selected based on previous *in vitro* studies of ketamine's effect on cultured cells from the brain (Dong, Rovnaghi, & Anand, 2012; Hudetz & Pagel, 2010; C. Wang et al., 2005; C. Wang et al., 2006). 50 μM glutamate condition was selected based on previous studies that looked at glutamate toxicity *in vitro* (D. W. Choi, Maulucci-Gedde, & Kriegstein, 1987; D. Liu, Thangnipon, & McAdoo, 1991; Regan & Choi, 1991). The benefit of using an *in vitro* neurosphere system is that it allows us to study the full potential of the cells since the cells have been taken out of their normal environment. Furthermore, this

system allows us to easily manipulate and control the extrinsic cues the cells will be exposed to, since we can add precise amounts of extrinsic factors to the media.

Following a seven-day proliferation period, neurospheres were isolated and plated at low density (3-4 neurospheres/condition) onto poly-l-ornithine coated sterile 96 well culture plates. A two-day assessment period was chosen to ensure that there was no direct interaction between the cells migrating from two distinct neurospheres. Following two day exposure to 0-500 μM ketamine, 50 μM glutamate, or 50 μM glutamate + 0-500 μM ketamine, treated cell cultures were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS (pH 7.4) for 25 minutes at room temperature. The PFA was then removed and three washes in 10 mM PBS for five minutes each was done. The effect on differentiating NPCs proliferative ability was assessed using 5'-Bromo-2-deoxyuridine (BrdU). 24-hours post-plating, differentiating NPCs were pulsed for 24 hours with 20 μg/mL of BrdU. BrdU is an exogenous proliferation marker. It is a thymidine analogue that competes with endogenous thymidine during Deoxyribonucleic acid (DNA) synthesis in proliferating cells. Fixed experimental cells were stored in 10 mM PBS at 4°C for a week before immunocytochemistry was performed.

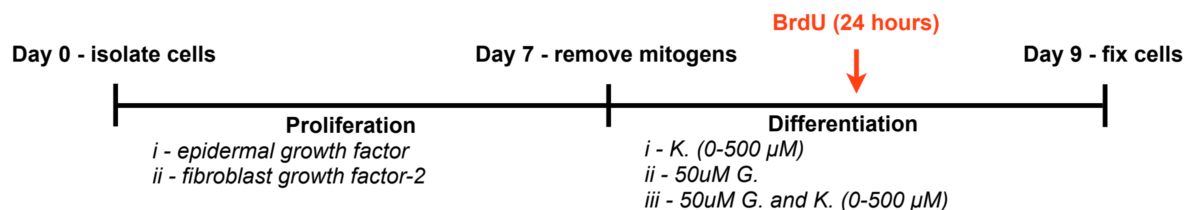

Figure 2.2 – Experimental timeline. Isolated NPCs were allowed to proliferate for

seven days in the presence of growth factors to form neurospheres. These neurospheres were then plated on 96-well plates coated with poly-l-ornithine, and exposed to various experimental conditions for two days. Differentiating cells were exposed to BrdU for 24 hours prior to fixation.

2.4 Immunocytochemistry

Single-label and double-label immunocytochemical staining were carried out to allow for the identification and quantification of the specific cell-types present. Prior to beginning immunocytochemical procedures all fixed cells were equilibrated with 10 mM PBS at room temperature for 20 minutes. Controls were run to test the protocol and the specificity of the antibody used. As a negative control, cells were incubated without the primary antibody and ensured that no staining was present.

2.4.1 TUNEL assay and BrdU co-labelling

Cells that have undergone DNA fragmentation were identified using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. Cells were permeabilized with 0.1% sodium citrate (w/v) with 0.1% Triton-X-100 for five minutes at 4°C, following which, three washes in 10 mM PBS for five minutes each was performed. The cells were then exposed to Roche Enzyme mixture, from the *In Situ* Cell Death Kit (Roche), containing terminal deoxynucleotidyl transferase and the fluorescein conjugated dUTP label for two hours at room temperature. Following the incubation period, the Roche Enzyme mixture was removed and the cells were washed three times with 10 mM PBS for five minutes each.

Cell proliferation was assessed by the cell's ability to incorporate BrdU. For BrdU staining, the cell's DNA was denatured using 0.2 N hydrochloric acid at room temperature for 30 minutes. The acidic media was carefully removed and two quick washes with 10 mM PBS were performed. Cells were blocked with 10% normal goat serum (NGS) (Genotec) to prevent non-specific binding of antibody, and the cell membranes were permeabilized with 0.1% Triton-X 100 (Sigma) in 10 mM PBS for 45 minutes at room temperature. The cells were then washed with 10 mM PBS to remove all of the permeabilizing agent. After the third wash, cells were incubated with mouse IgG anti-BrdU (Millipore, MAB3424), diluted in PBS containing 10% NGS overnight at 4°C. The following day, three washes in 10 mM PBS for 5 min each was done and incubated with the mouse IgG Alexa Fluor 594 secondary antibody (Invitrogen, A-11037) diluted in 10 mM PBS containing 2% NGS for two hours at room temperature in the dark. Followed by three washes in 10 mM PBS for 5 min each and the nucleus was labelled with 1 μg/mL Hoescht 33258 (Invitrogen). Cells were maintained in 10 mM PBS at 4°C.

2.4.2 Cell phenotype

Nestin was used as a marker to identify progenitor cells. β-III-tubulin (βIIIT) was used as a marker to identify differentiated immature neurons. βIIIT labels the cell bodies, dendrites, and axons of young neurons. Oligodendrocyte marker O4 was used as a marker to identify cells of the oligodendrocyte lineage. O4 is a surface marker of oligodendrocyte progenitors. Glial fibrillary acidic protein (GFAP) was used as a marker to identify mature astrocytes. GFAP is an intermediate filament found in differentiated astrocytes. Brain lipid binding

protein (BLBP) was used as a marker to identify radial glial cells (Kulbatski et al., 2007; White, McTigue, & Jakeman, 2010).

Cells were blocked with 10% normal goat serum (NGS) (Genotec) to prevent non-specific binding of antibody, and the cell membranes were permeabilized with 0.1% Triton-X 100 (Sigma) in 10 mM PBS for 45 minutes at room temperature (except for cells stained with the O4 antibody). For O4 staining, cell membranes were not permeabilized, instead cells were blocked with 10% NGS in 10 mM PBS for 45 minutes at room temperature. The media was carefully aspirated using a fine polished glass pasteur pipette and the cells were washed three times with 10 mM PBS with careful assurance not to disrupt the cell's adherence to the bottom of the well, to remove all of the permeabilizing agent.

Cells were incubated overnight with primary antibodies (see Table 2.1) diluted in blocking buffer solution (10% NGS and 10 mM PBS) at 4°C. The primary antibodies were rabbit IgG anti-nestin (Sigma, N5413), mouse IgG2a anti-neuronal class III β –tubulin (Stem Cell Technologies, 01409), mouse IgG1anti-glial fibrillary acidic protein (Millipore, AB5804), mouse IgM anti-O4 monoclonal (R&D, MAB1326) and rabbit polyclonal anti-brain lipid binding protein (Millipore, AB32423). Following the incubation period, the primary antibody was removed and the cells were washed three times with 10 mM PBS. Secondary antibody incubation was carried out using Alexa fluorescent-conjugated secondary antibody diluted in blocking buffer solution (2% NGS and 10 mM PBS) for one hour at room temperature in the dark. The secondary antibodies used were goat anti-mouse IgG Alexa Fluor 594 (Invitrogen, A-11037), goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, A-11008), and anti-mouse IgM Alexa Fluor 594 (Invitrogen, A-11032). Another set of three washes in PBS was carried out. Nuclei were counterstained with 1 μg/mL Hoescht 33258

(Invitrogen) for ten minutes at room temperature following which one wash in 10 mM PBS for five minutes was performed. Cells were maintained in 10 mM PBS at 4°C.

Table 2.1 – Primary and secondary antibodies

Primary Antibody	Isotype	Dilution Rates	Secondary Antibody	Dilution Rates
anti-BrdU	Mouse IgG	1:300	Alexa Fluor 594	1:300
anti-Nestin	Rabbit IgG	1:200	Alexa Fluor 594	1:300
anti-βIIIT	Mouse IgG2a	1:500	Alexa Fluor 594	1:300
anti-GFAP	Mouse IgG1	1:200	Alexa Fluor 594	1:300
anti-Oligodendrocyte Marker O4	Mouse IgM	1:150	Alexa Fluor 594	1:300
anti-BLBP	Rabbit polyclonal	1:500	Alexa Fluor 488	1:300

BrdU, bromo-deoxyuridine; βIIIT, Neuronal Class III β-tubulin, GFAP, glial fibrillary acidic protein; BLBP, brain lipid binding protein

2.5 Image and statistical analysis

Immunostained cells were examined using a Nikon Ti Eclipse fluorescent microscope and images were taken with a digital camera (Nikon, DS-Fil). Bound neurospheres were imaged for a total of three to five neurospheres per stain per trial. Figure 2.3 shows how the fields of view were taken for image analysis. Neurospheres were located and either the whole neurosphere was imaged for cell migration analysis or four fields of view per neurosphere were imaged for cell proliferation, survival and differentiation analysis. Under certain experimental conditions, differentiating NPCs migrated at a fast rate resulting in the formation of a monolayer. Since, the neurosphere border could not be easily identified under these conditions, images were taken around where there was a dense collection of Hoechst-positive cells. It was assumed that the cells found around the periphery originated from the dense collection of Hoechst-positive cells. Images were merged and manually analyzed using

Image J analysis program (National Institutes of Health; Bethesda, MD).

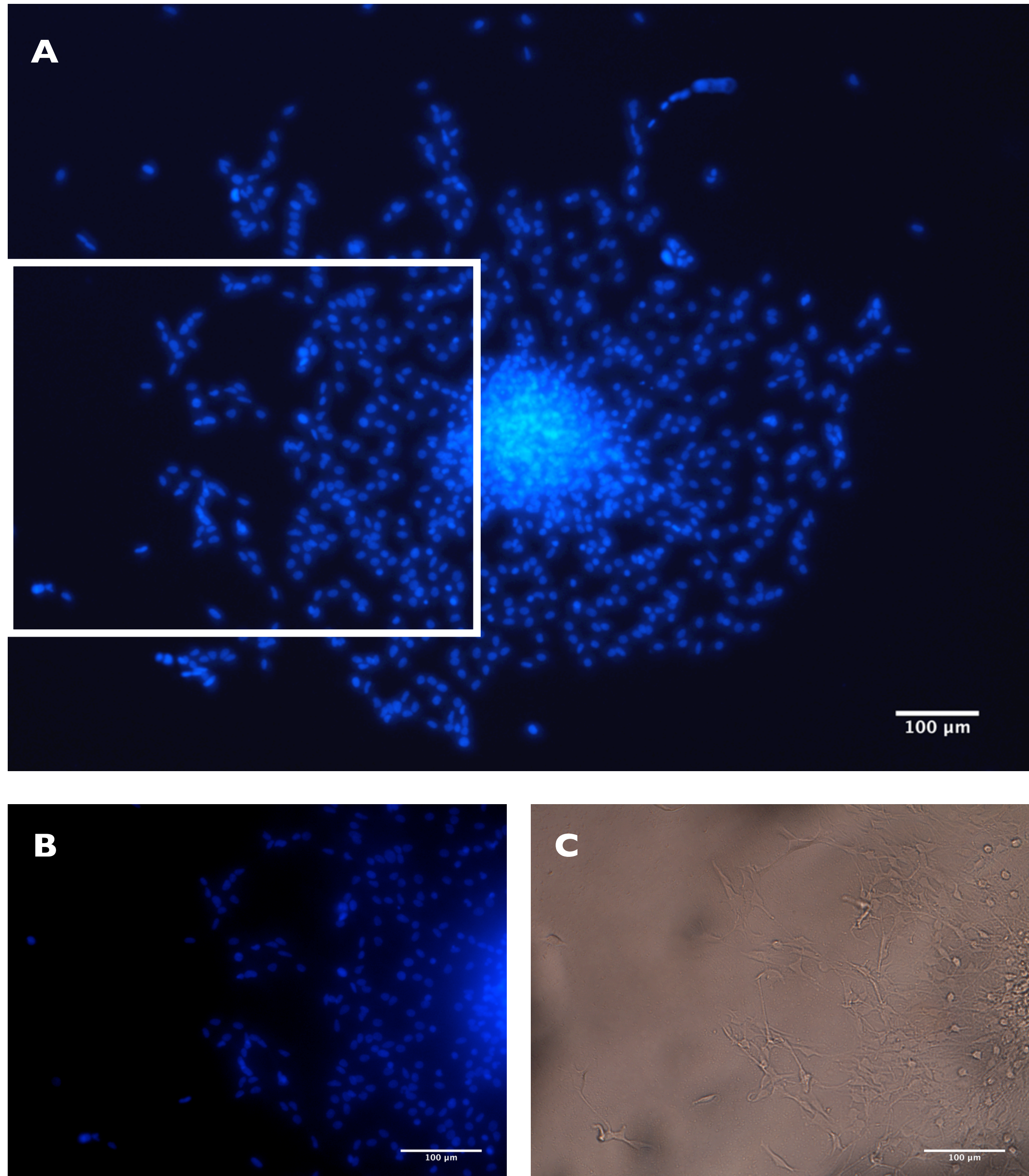

Figure 2.3 – Sample field of view fluorescent images used to quantify differentiated NPC proliferation, cell survival, differentiation and migration. (A) To quantify cell

migration, bound neurospheres were imaged at either 10x or 20x depending on the distance the farthest cell migrated. (B, C) To quantify cell proliferation, survival and differentiation, four fields of view (left, bottom, right, and top) adjacent to the edge of the neurosphere were taken. Only the cells that have migrated out of the neurosphere were used for quantification of cell proportions. Scale bar = 100 μm.

Proliferation, cell death, and differentiation were calculated as the percentage of immunopositive cells among the total number of cells counterstained with Hoechst. Figure 2.4 shows sample fluorescent images of differentiated cell types. To determine the effects on migratory potential, the distance from edge of neurosphere to the nucleus of the cell type in question was measured. Data from three independent experiments were averaged. The edge of the sphere was identified as the area where there was a dense collection of Hoechst-positive cells Figure 2.5 shows sample fluorescent images of GFAP-positive cells migrating out of a neurosphere.

Data management was completed with Excel (Microsoft) and statistical analysis was completed using data analysis programs found in Excel. Statistical significance between experimental groups and their respective control group was evaluated with a single factor one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Two-way ANOVA was used to examine the influence of two factors (cell location and drug concentration) on cell proliferation, differentiation, and migration. In all analyses, $p<0.05$ was considered statistically significant. Data is presented as mean±standard error of the mean.

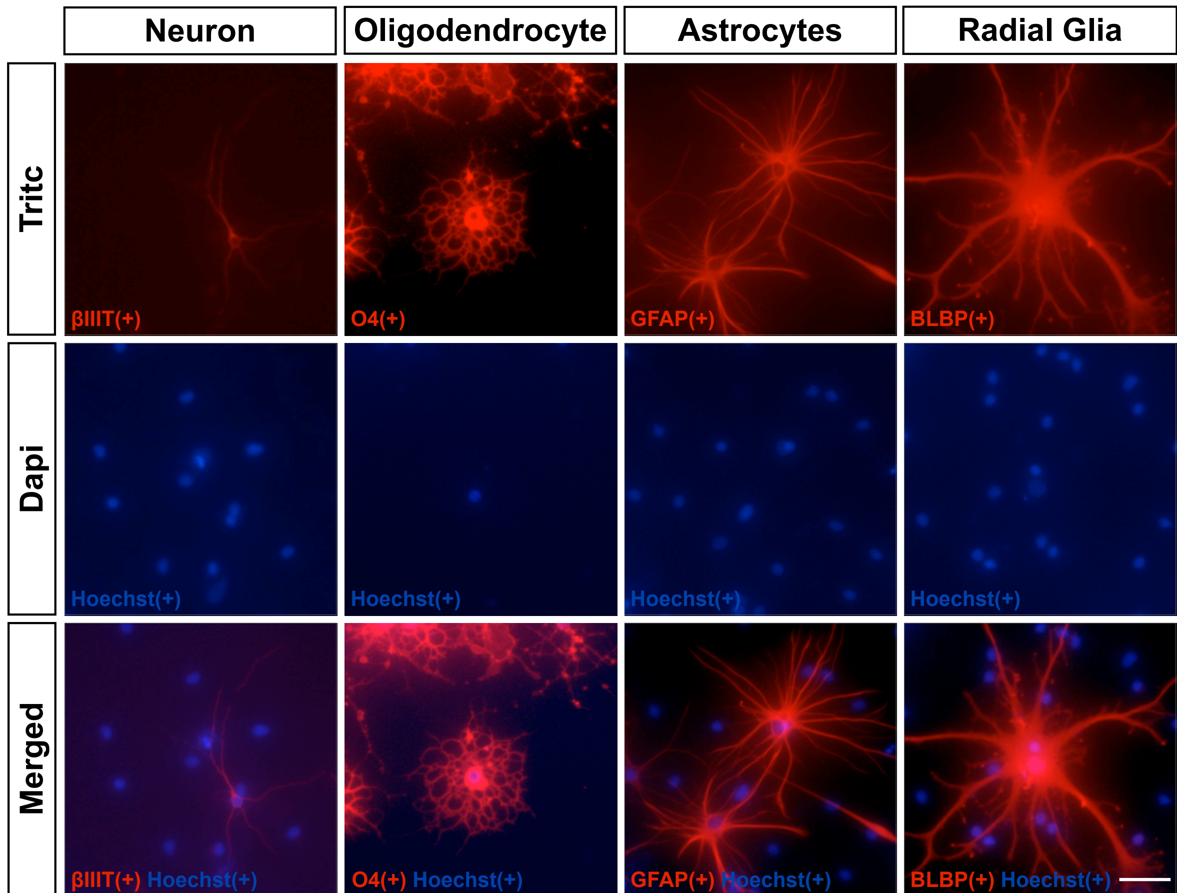

Figure 2.4 – Sample fluorescent images of differentiated cell types considered immunopositive for cell count. Only the cells that have migrated out of the neurosphere were used for quantification of cell proportions. Scale bar = 10 μm. βIIIT, Neuronal Class III β-tubulin, GFAP, glial fibrillary acidic protein; BLBP, brain lipid binding protein

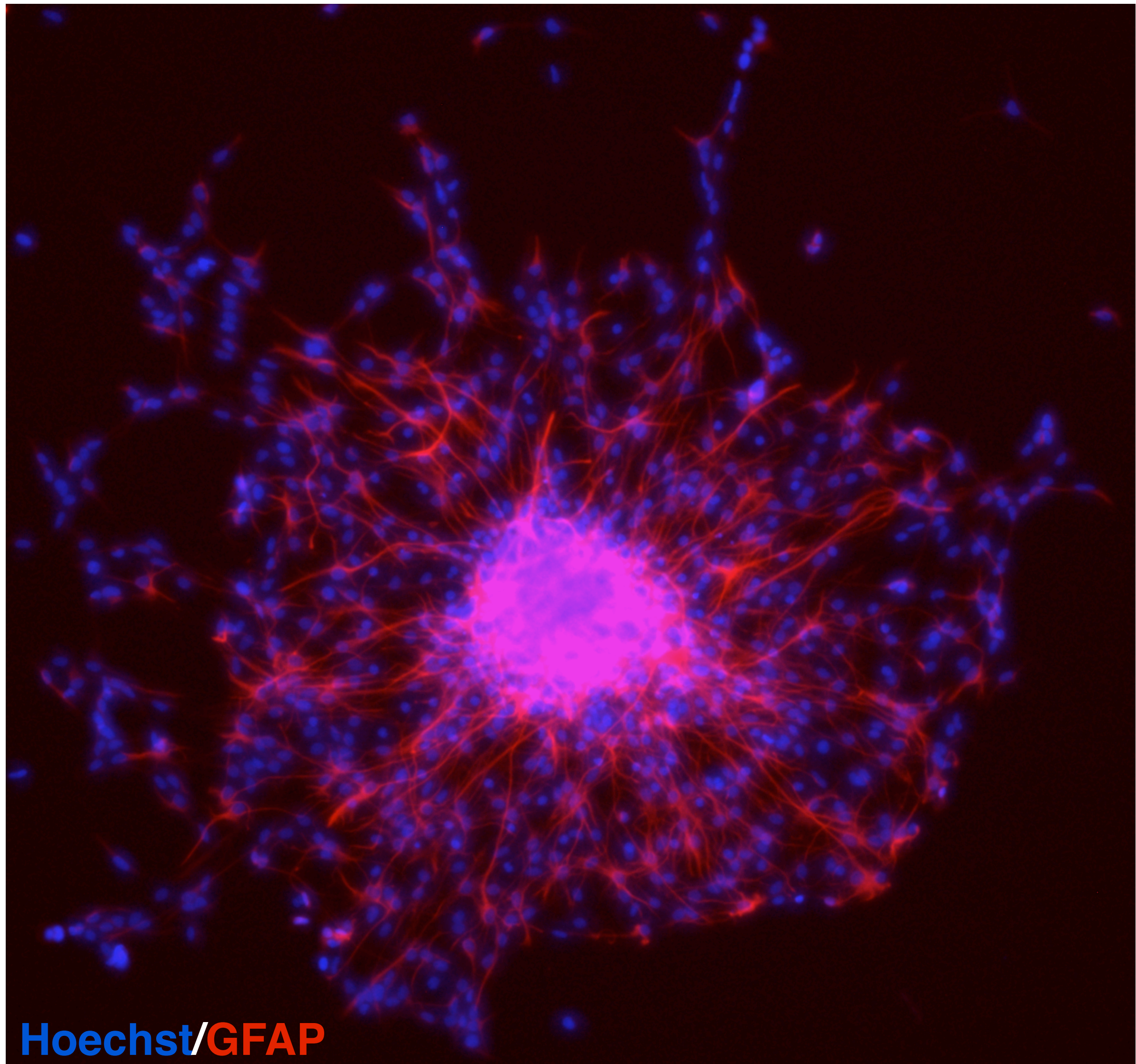

Figure 2.5 – Sample fluorescent image of migrating GFAP-positive cells. Note the white dash line surrounding the dense collection of Hoechst-positive cells that denotes the edge of the neurosphere. Migratory potential of a cell type was determined by measuring the distance from the edge of neurosphere to the cell body (solid white line). GFAP, glial fibrillary acidic protein.

CHAPTER 3.0 - RESULTS

3.1 *In vitro*: differentiating NPCs' proliferation and survival

The effect of ketamine on the proliferative activity of differentiating NPCs derived from the SC and SVZ was assessed through BrdU incorporation, which labels dividing cells in the S phase of the cell cycle. Both cell populations were exposed to a selected range of concentrations of ketamine (0-500 μM) to assess whether ketamine influenced cell proliferation and/or there was a dose response to the selected ketamine concentrations during cell differentiation. Cultures were incubated with BrdU for 24 hours before fixation. Proliferating cells were determined by counting the number of BrdU-positive cells among total number of Hoechst-positive cells that had migrated out of the neurosphere. It was found that the proliferative ability of the NPCs cultured from the SC and SVZ was not affected by the presence of ketamine (Fig. 3.1). The proportion of BrdU-positive cells was not significantly different between the experimental conditions and control condition (ANOVA, $P>0.05$). Though it is not statistically significant, it is interesting to note the increase in cell proliferation when the NPCs were exposed to 5 μM ketamine compared to control conditions. The influence ketamine has on cell survival of NPCs was assessed using an apoptosis cell death kit. It was found that these cells were able to survive in the presence of ketamine, as shown by the lower proportion of TUNEL-positive cells (Fig. 3.1). Following a 48-hour exposure to ketamine only 3-10% of the cells were TUNEL-positive.

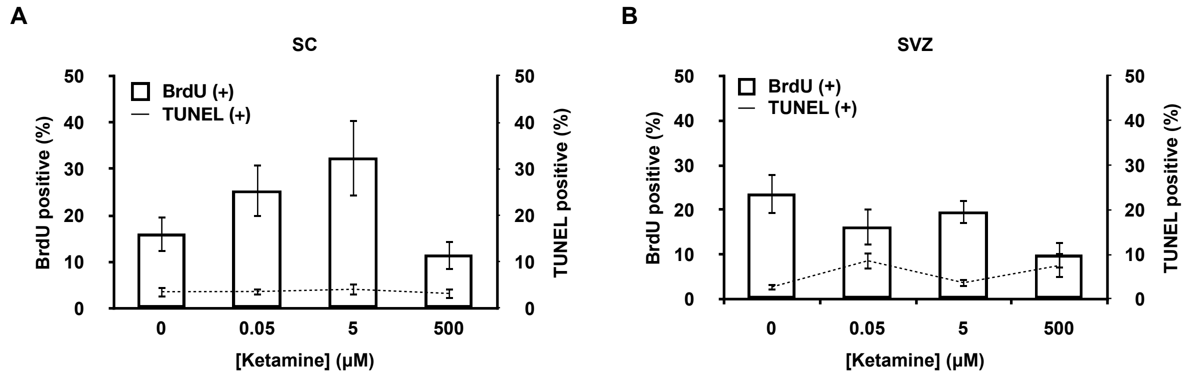

Figure 3.1 - Ketamine does not affect differentiating NPCs proliferation and survival. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's proliferation and survival. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of nuclei positive for BrdU or TUNEL. Each cell type was compared with a one-way ANOVA single factor analysis (p>0.05).

As shown in figure 3.2 it was interesting that glutamate differentially affects SC and SVZ derived NPC's proliferative ability. SC and SVZ NPC derived neurospheres were exposed to 50 μM glutamate for 48 hours, following a seven-day proliferation period. There was no significant difference in proliferative ability when SC derived NPCs were exposed to 50 μM glutamate compared to 0 μM glutamate condition (Fig 3.2A). Contrarily, as seen in figure 3.2B, there was a higher proportion of BrdU-positive cells among NPCs derived from the SVZ, following exposure to 50 μM glutamate condition (23.4±2.4%) compared to 0 μM glutamate condition (11.8±1.4%). 50 μM glutamate does not significantly affect SC and SVZ NPCs' survival. Post glutamate exposure, roughly 97% of the SC and SVZ derived cells'

survived, which is similar to control conditions. These results raise the question of how the NPCs would respond if they were exposed to both glutamate and ketamine at the same time.

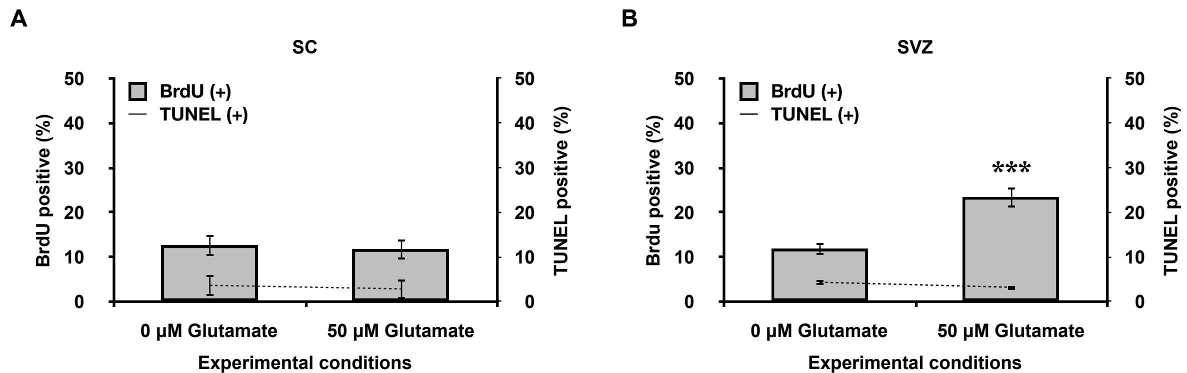

Figure 3.2 – Glutamate increases SVZ derived NPC proliferation. Glutamate differentially affects differentiating SC (A) and SVZ (B) derived NPCs' proliferative ability. Exposure to 50 μM glutamate significantly increases differentiating SVZ cells' proliferative ability. Glutamate does not significantly affect differentiating SC and SVZ NPCs' survival. Cells were treated with or without medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of nuclei positive for BrdU or TUNEL. Experimental condition was compared to control (0 μM glutamate) condition with a one-way ANOVA single factor analysis. Statistical significance shown with *** $p<0.0001$.

Figure 3.3 shows the proportion of proliferative and apoptotic cells when NPCs are cultured with both 50 μM glutamate and selected concentrations of ketamine. TUNEL analysis, performed to examine cell death, revealed that exposure to 50 μM glutamate and the selected concentration range of ketamine did not affect the survival of NPCs. As expected, SC

derived NPCs proliferative ability was not affected by the presence of both glutamate and ketamine. The level of BrdU expression in the SVZ NPCs that were exposed to 50 μM glutamate and either 0.05 μM or 5 μM ketamine, were similar to the 50 μM glutamate only condition. When SVZ derived NPCs were exposed to 50 μM glutamate and 500 μM of ketamine, there was a significant decrease in the proportion of BrdU-positive cells compared to when the cells were exposed to 50 μM glutamate alone. Furthermore, there was significantly less BrdU-positive cells in the 50 μM glutamate and 500 μM of ketamine condition compared to the control condition (0 μM glutamate and 0 μM ketamine).

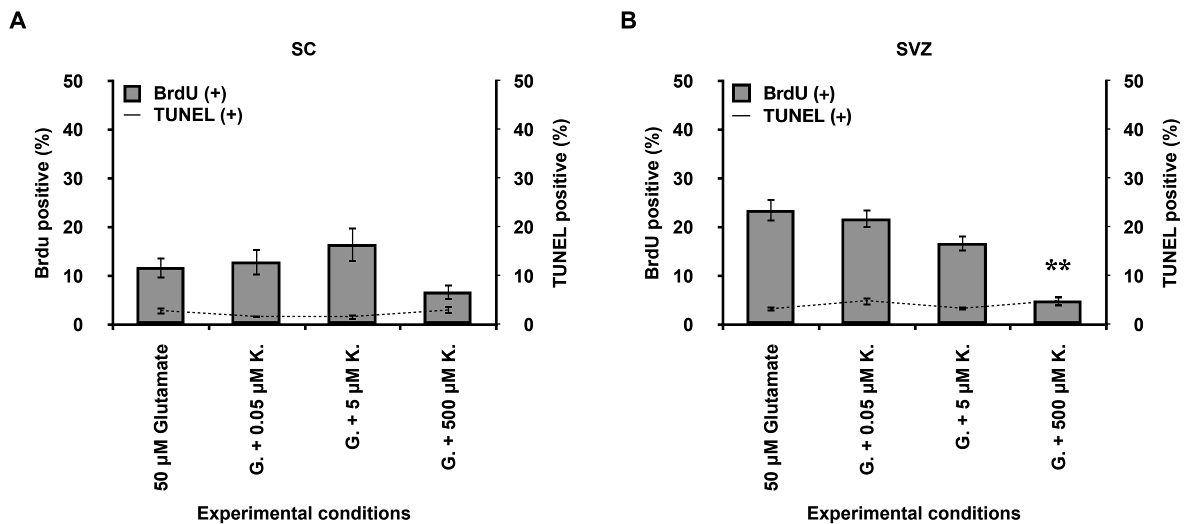

Figure 3.3 – Ketamine can overcome glutamate's influence on SVZ derived NPC proliferation. In the presence of glutamate, ketamine differentially affects SC (A) and SVZ (B) zone derived NPCs' proliferative ability. Ketamine does not significantly affect differentiating SC derived NPCs' proliferative ability. In the presence of glutamate, exposure to 500 μM ketamine reduces differentiating SVZ derived NPCs' proliferative ability. Differentiating SC and SVZ NPCs' survival is not significantly

affected. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of nuclei positive for BrdU or TUNEL. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.0001$). Statistical significance shown with ** $p<0.01$. G. = Glutamate; K. = Ketamine.

The changes in the proliferative ability of NPCs derived from the SVZ following exposure to ketamine and glutamate led us to investigate the influence these factors would have on the proportion of nestin-positive progenitor cells (Fig. 3.4). The proportion of nestin-positive progenitors from SC derived NPCs was not affected by the presence of neither glutamate nor ketamine. However, there was a significant increase in the proportion of nestin-positive progenitor cells when NPCs derived from the SVZ were exposed to 50 μM glutamate condition compared to 0 μM glutamate condition. On the other hand, when these cell types were exposed to 50 μM glutamate and 500 μM of ketamine, there was a significant decrease in the proportion of nestin-positive progenitors compared to 50 μM glutamate condition.

In summary, SC and SVZ derived NPCs respond differently to glutamate and ketamine. Compared to control conditions, glutamate and ketamine does not significantly influence SC derived NPC proliferation, apoptosis, and proportion of nestin-positive progenitors. However, glutamate increases SVZ derived NPC proliferation and the proportion of nestin-positive progenitor cells compared to control conditions. Contrarily, 500 μM of ketamine in the presence of 50 μM glutamate decreases SVZ derived NPC proliferation compared to control condition. Interestingly, simultaneous exposure to 50 μM glutamate and 500 μM

ketamine restores the proportion of nestin-positive progenitor cells to values comparable to control conditions (Table 3.1).

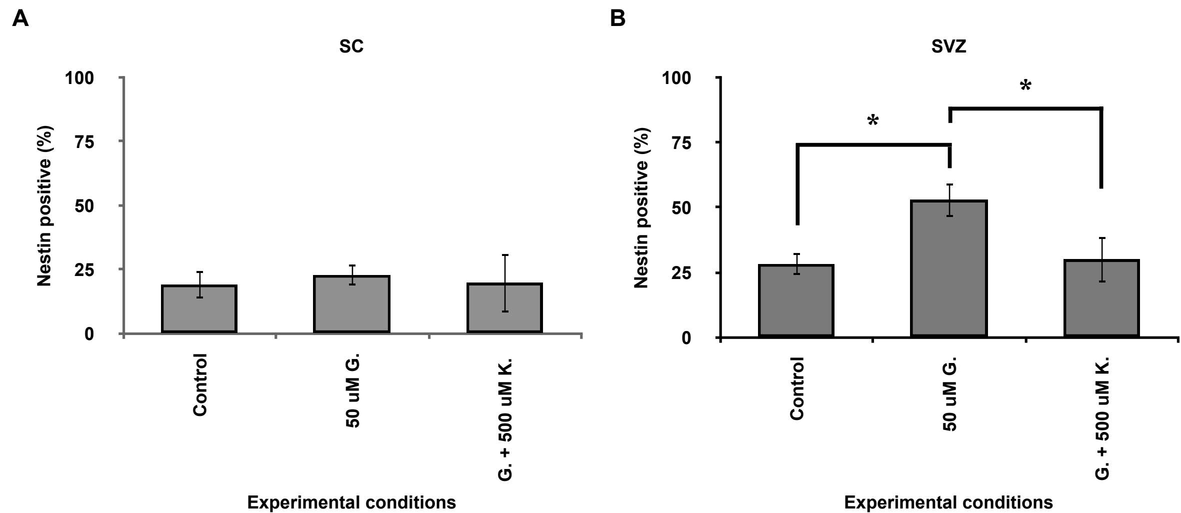

Figure 3.4 – Glutamate increases the proportion of nestin-positive cells. In the presence of glutamate, ketamine differentially affects the proportion of nestin-positve cells derived from the SC (A) and SVZ (B) NPCs. The proportion of SC NPCs derived progenitors is not significantly affected by the experimental factors. Glutamate increases the proportion of SVZ NPCs derived progenitors, while 500 μM ketamine significantly decreases the proportion of SVZ NPCs derived progenitors in the presence of glutamate. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with 500 μM of ketamine for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for nestin. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.006810$). Statistical significance shown with * $p<0.05$. G. = Glutamate; K. = Ketamine

Table 3.1 – Summary of the influence of glutamate and ketamine on SC and SVZ NPCs proliferation, apoptosis and proportion of progenitors compared to baseline values[1]. Ketamine can overcome glutamate's influence on differentiated SVZ derived NPC proliferation and the proportion of progenitors. G. = Glutamate; K. = Ketamine.

	SC		SVZ	
	50 μM G.	50 μM G. + 500 μM K.	50 μM G.	50 μM G. + 500 μM K.
Proliferation[a]	-	-	↑	↓
Apoptosis[b]	-	-	-	-
Progenitors[c]	-	-	↑	-

[1] control condition = 0 μM glutamate + 0 μM ketamine
[a] % total number of cells positive for BrdU
[b] % total number of cells positive for TUNEL.
[c] % total number of cells positive for nestin.

3.2 *In vitro*: differentiating NPCs' phenotype

Previous research has shown that neural progenitor cells often differentiate into glial cells (Brazelton, Rossi, Keshet, & Blau, 2000; Cao et al., 2001; Gritti et al., 1996; Kulbatski et al., 2007; Palmer, Markakis, Willhoite, Safar, & Gage, 1999). Therefore, it is necessary to study the affect ketamine and glutamate would have on the NPCs' differentiation potential. The total proportion of cells was determined by counting the number of immunopositive cells (βIIIT, O4, GFAP, BLBP, and GFAP+BLBP) among the total number of Hoechst-positive cells per neurosphere. Seven-day-old NPC derived neurospheres were exposed to selected concentrations of ketamine (0-500 μM) to assess whether ketamine influenced differentiated cell proportions and/or there was a dose response to the selected ketamine concentration range. Following a two-day differentiation period there were no βIIIT-positive cells derived

from both the SC and SVZ neurospheres following exposure to 0-500 μM ketamine, 50 μM glutamate, and 50 μM glutamate + 0-500 μM ketamine.

Figure 3.5, shows the change in O4-positive oligodendrocyte cell proportions when NPCs from the SC and SVZ were exposed to the selected concentrations of ketamine for 48 hours. There was a significant decrease in the proportion of O4-positive oligodendrocytes when NPCs were exposed to 500 μM of ketamine compared to control conditions (0 μM ketamine). It should be noted that there was no significant difference in O4-positive cell proportion when NPCs were exposed to concentrations of ketamine less than 500 μM.

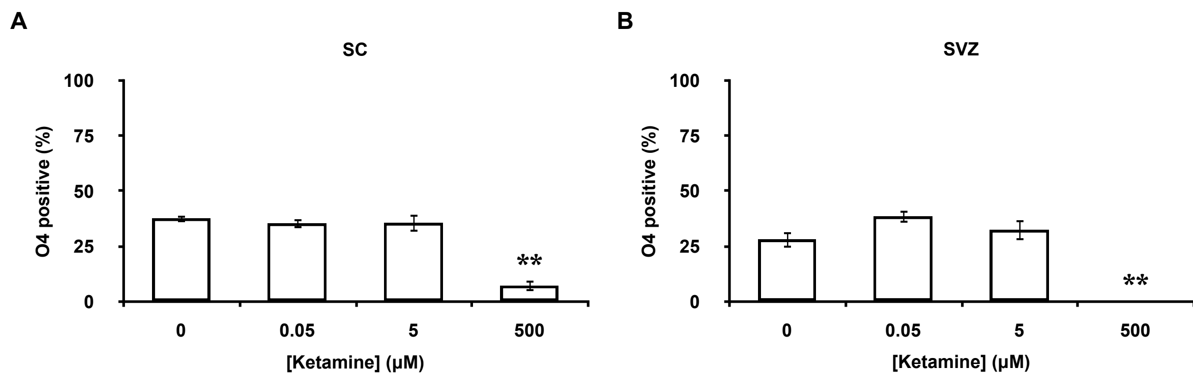

Figure 3.5 – 500 μM ketamine reduces the proportion of O4-positive oligodendrocyte. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's ability to differentiate into O4-positive oligodendrocytes. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for O4. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.001$). Statistical significance shown with ** $p<0.01$.

However, there was no significant difference in the proportion of SC and SVZ derived O4-positive cells following exposure to 50 μM glutamate compared to control (Fig 3.6). Although not significant there were higher proportion of O4-positive cells derived from SC NPCs compared to SVZ NPCs. These results raise the question about how the proportion of O4-positive cells would differ if they were exposed to both 50 μM glutamate and 0-500 μM of ketamine simultaneously.

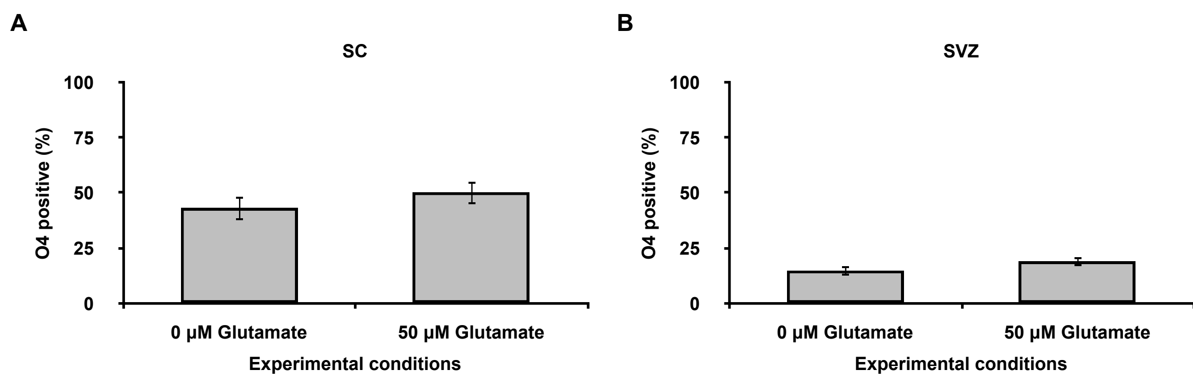

Figure 3.6 – Glutamate does not affect the proportion of O4-positive oligodendrocytes. Cells were treated with culture medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for O4. Each cell type was compared with a one-way ANOVA single factor analysis (P>0.05).

As seen in figure 3.7, the proportion of O4-positive oligodendrocytes differs based on the experimental condition. As quantified before, there is no significant difference in the proportion of SC and SVZ derived O4-positive cells following exposure to 50 μM glutamate compared to control. However, exposure to 500 μM ketamine in the presence of glutamate significantly reduces NPCs' ability to differentiate into O4-positive oligodendrocytes.

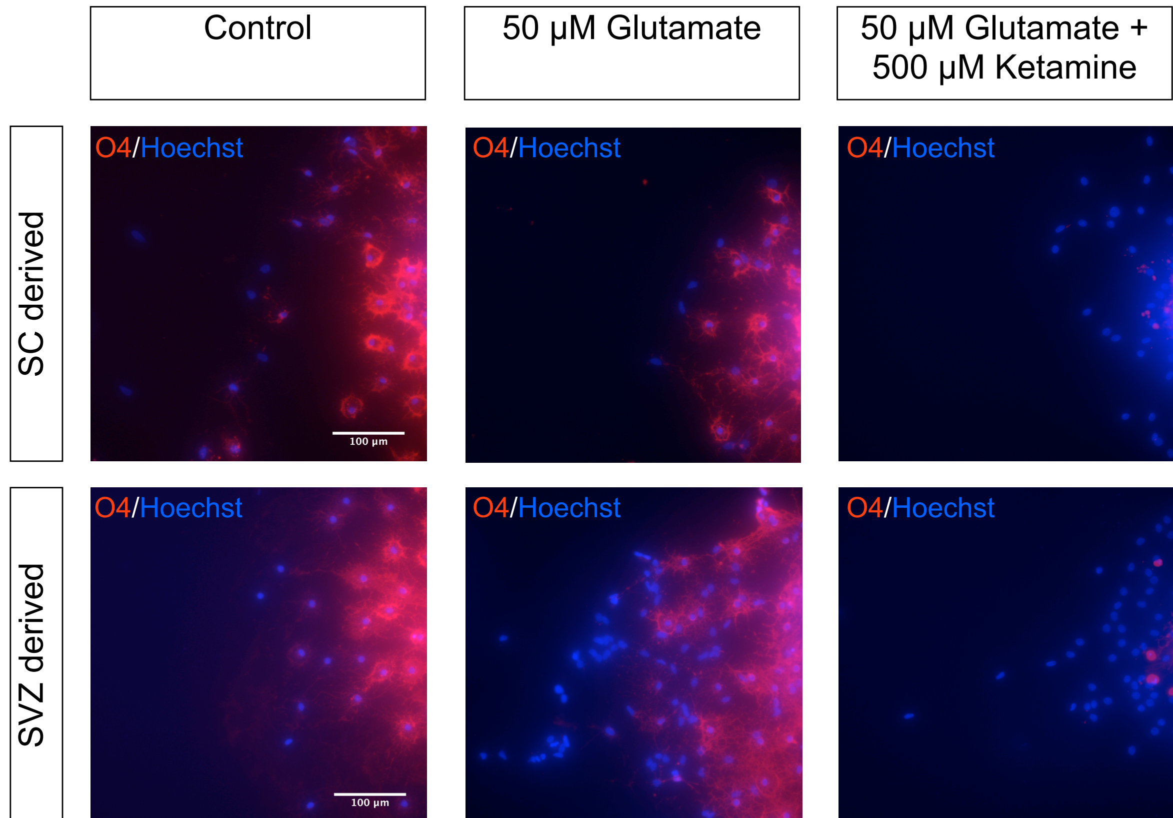

Figure 3.7 – Exposure to ketamine reduces NPCs' ability to differentiate into O4-positive oligodendrocytes. Sample SC and SVZ derived NPC fluorescent images used to assess the proportion of O4-positive cells. Scale bar = 100 μm

Figure 3.8 shows the changes in O4-positive cell proportions when the NPCs are cultured with both ketamine and glutamate simultaneously. Interestingly, the trend was similar to when the cells were exposed to ketamine alone. There was a significant decrease in the SVZ and SC derived O4-positive cell proportions when cells were incubated with both 50 μM glutamate and 500 μM of ketamine compared to when the neurospheres were exposed to 50 μM glutamate alone. Interestingly, the proportion of SVZ and SC NPC derived O4-positive cells when exposed to 50 μM glutamate with 500 μM of ketamine is significantly less than when exposed simultaneously to 0 μM glutamate and 0 μM of ketamine. Again, there was no

visible difference in the proportion of O4-positive cells following exposure to 50 μM glutamate and concentrations of ketamine less than 500 μM.

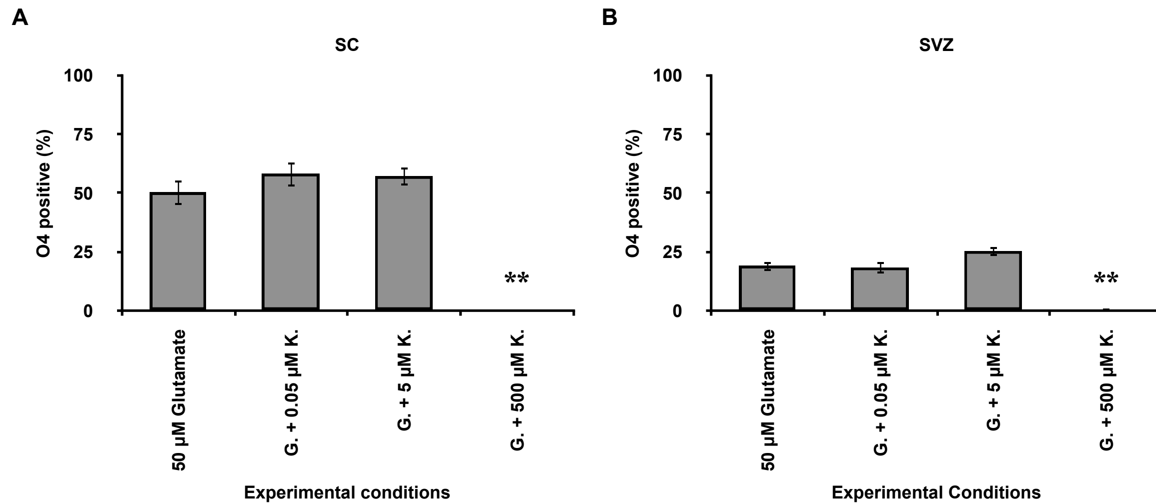

Figure 3.8 - Exposure to 50 μM glutamate+500 μM ketamine significantly reduces the proportion of O4-positive cells. The influence of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's ability to differentiate into O4-positive oligodendrocytes in the presence of glutamate. In the presence of glutamate, exposure to 500 μM ketamine reduces differentiating SC and SVZ derived NPCs' ability to differentiate into oligodendrocytes. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for O4. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.0001$). Statistical significance shown with ** $p<0.01$. G. = Glutamate, K. = Ketamine

The influence of ketamine on the proportion of NPCs that differentiated into GFAP-positive astrocytes was also investigated. As seen in figure 3.9, there was no significant difference in astrocytic proportions across the different ketamine concentrations. Roughly, 20-25% of the cells differentiated into GFAP-positive cells. The proportion of GFAP-positive cells derived from the SC was similar to the proportion of GPAP-positive cells derived from the SVZ.

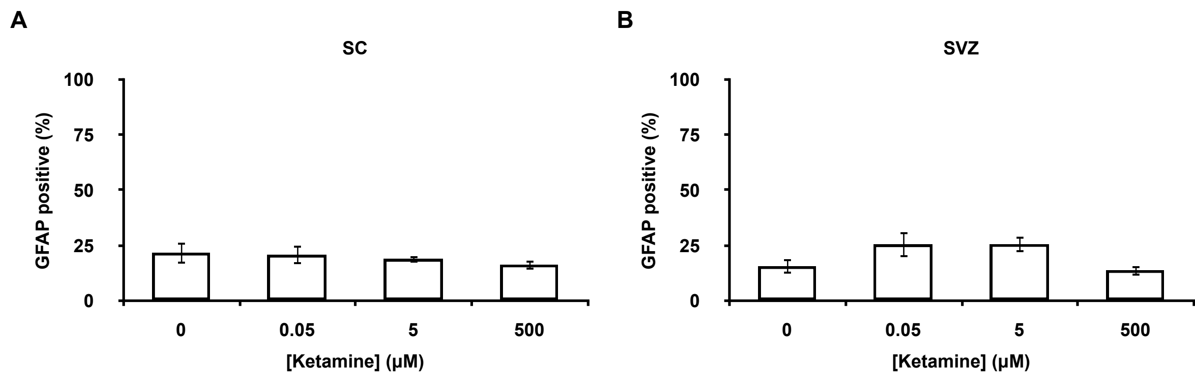

Figure 3.9 - The proportion of GFAP-positive cells is not affected by the presence of ketamine. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's ability to differentiate into GFAP-positive astrocytes. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for GFAP. Each cell type was compared with a one-way ANOVA single factor analysis ($p>0.05$).

Figure 3.10 shows the proportion of GFAP-positive cells when NPCs from the SC and SVZ are exposed to 50 μM glutamate. Interestingly, there was a significant decrease in the proportion of GFAP-positive cells when SC NPCs were exposed to 50 μM glutamate compared to control conditions. Only 10% of the cells differentiated into GFAP-positive

cells compared to control conditions, in which roughly 30-40% of the cells differentiated into GFAP-positive cells. Contrarily, the proportion of SVZ NPCs that differentiated into GFAP-positive cells was similar in both 50 μM glutamate and control conditions. Roughly, 40% of the SVZ NPCs differentiated into GFAP-positive cells in both conditions. These results raise the question about how the proportion of GFAP-positive cells would differ if they were exposed to both 50 μM glutamate and 0-500 μM of ketamine simultaneously.

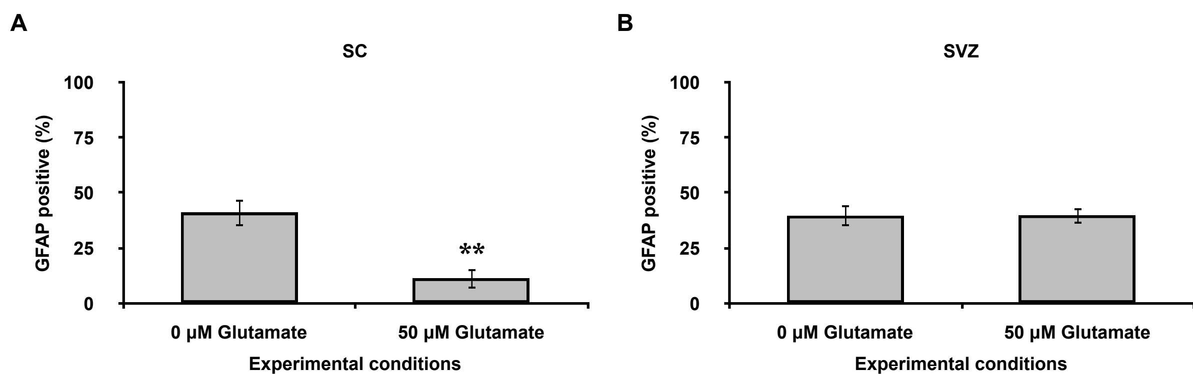

Figure 3.10 – Glutamate decreases SC NPCs derived GFAP-positive astrocyte differentiation. Glutamate did not significantly affect the percentage of GFAP-positive cells derived from the (B) SVZ NPCs. Cells were treated with culture medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for GFAP. Each cell type was compared with a one-way ANOVA single factor analysis, statistical significance shown with ** $p<0.01$.

Figure 3.11 shows the changes in GFAP-positive cell proportions when the NPCs are cultured with both ketamine and glutamate simultaneously. There was a significant increase in SC NPC derived GFAP-positive cell proportion when cells were incubated with both 50

μM glutamate and 500 μM of ketamine compared to when the neurospheres were exposed to 50 μM glutamate alone (Fig. 3.12) or 0 μM glutamate+0 μM ketamine. However, there was no significant difference in GFAP-positive cell proportion when NPCs were exposed to concentrations of ketamine less than 500 μM with 50 μM glutamate. Again, similar to when the SVZ NPCs were exposed to only ketamine, there was no visible difference in the proportion of GFAP-positive cells across the different ketamine concentrations.

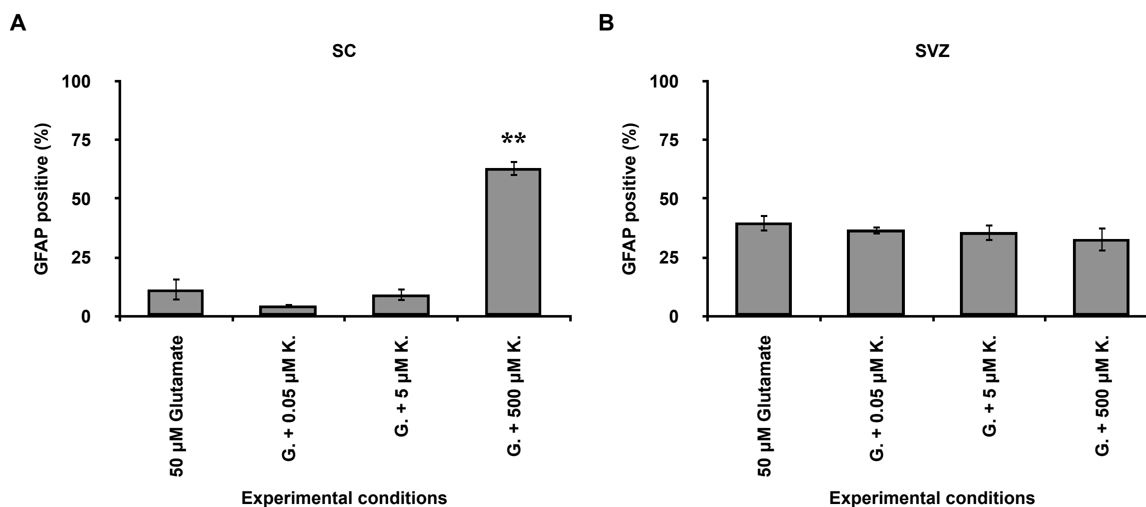

Figure 3.11 - 500 μM ketamine reverses the influence of 50 μM glutamate on SC NPCs derived GFAP-positive astrocyte differentiation. The influence of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's ability to differentiate into GFAP-positive astrocytes in the presence of glutamate. In the presence of glutamate, exposure to 500 μM ketamine increases differentiating SC derived NPCs' ability to differentiate into astrocytes. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells

positive for GFAP. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.0001$). Statistical significance shown with ** $p<0.01$. G. = Glutamate, K. = Ketamine

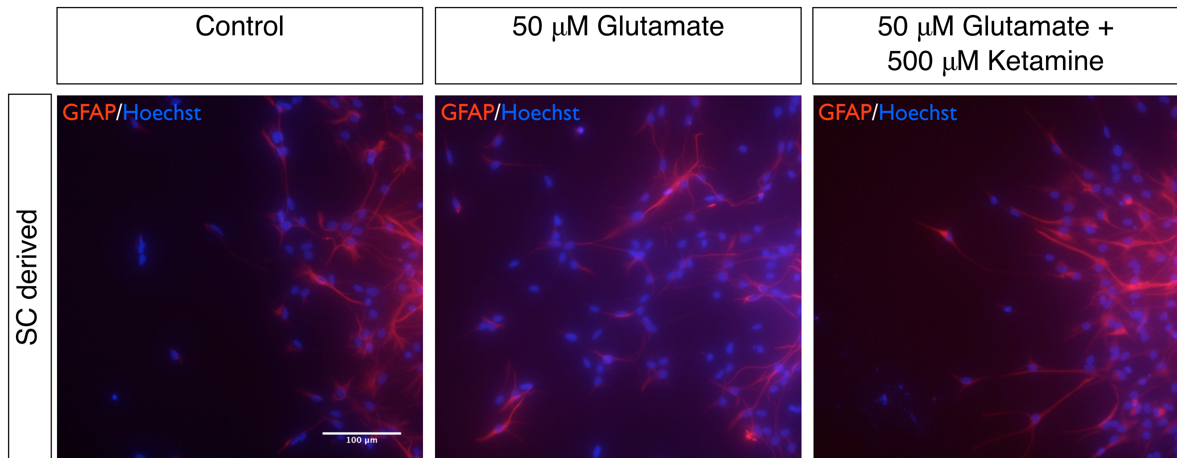

Figure 3.12 - Exposure to ketamine increases SC derived NPCs' ability to differentiate into GFAP-positive astrocytes. Sample SC derived NPC fluorescent images used to assess the proportion of GFAP-positive cells. Scale bar = 100 μm

Figure 3.13 shows the proportion of BLBP-positive when NPCs from the SC and SVZ are exposed to the selected concentrations of ketamine. Similar to GFAP-positive cells, there was no significant difference in radial glial proportions across the different ketamine concentrations compared to the control. When SC derived NPCs were exposed to ketamine, around 40% of the cells differentiated into BLBP-positive cells. However, the proportion of BLBP-positive cells derived from SVZ NPCs is roughly around 20-30%.

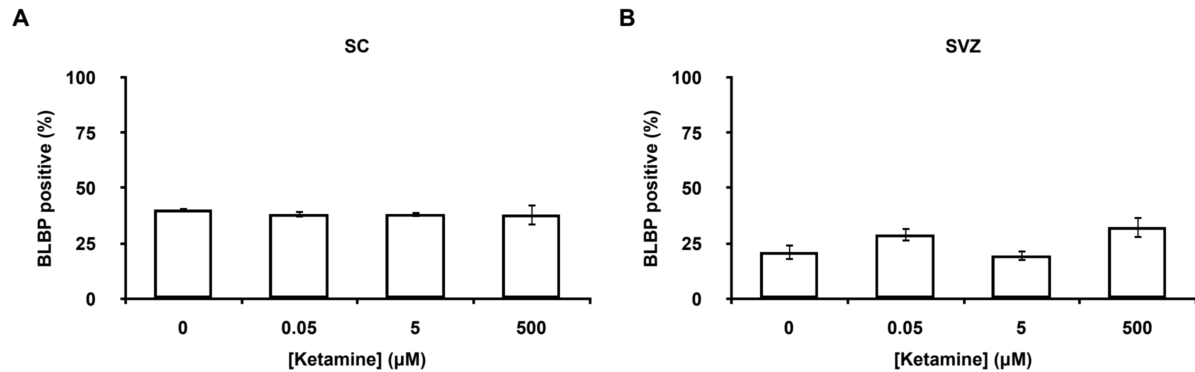

Figure 3.13 - The proportion of BLBP-positive radial glial cells is not affected by the presence of ketamine. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's ability to differentiate into BLBP-positive radial glia. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for BLBP. Each cell type was compared with a one-way ANOVA single factor analysis ($p>0.05$).

Figure 3.14 shows the proportion of BLBP-positive when NPCs from the SC and SVZ are exposed to 50 μM glutamate. Interestingly, there was a significant decrease in the proportion of BLBP-positive cells when SC NPCs were exposed to 50 μM glutamate compared to control conditions. Only 18% of the cells differentiated into BLBP-positive cells compared to control conditions, in which roughly 35% of the cells differentiated into BLBP-positive cells. Contrarily, the proportion of SVZ NPCs that differentiated into BLBP-positive cells was similar in both 50 μM glutamate and control conditions. Roughly, 40% of the SVZ NPCs differentiated into BLBP-positive cells in both conditions. These results raise the question about how the proportion of BLBP-positive cells would differ if they were exposed to both 50 μM glutamate and 0-500 μM of ketamine simultaneously.

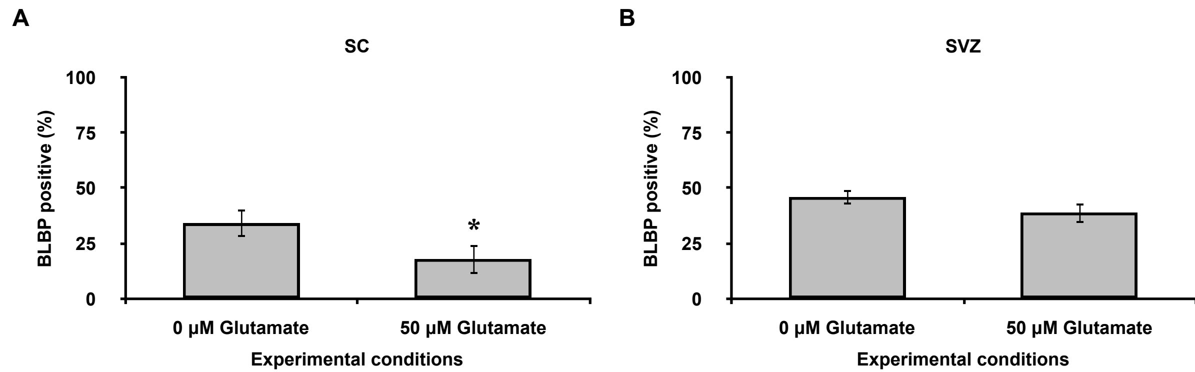

Figure 3.14 – Glutamate decreases SC NPCs derived BLBP-positive radial glial cell differentiation. Glutamate did not significantly affect the percentage of BLBP-positive cells derived from the (B) SVZ NPCs. Cells were treated with culture medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for BLBP. Each cell type was compared with a one-way ANOVA single factor analysis, statistical significance shown with * $p<0.05$.

Figure 3.15 shows the changes in BLBP-positive cell proportions when the NPCs are cultured with both ketamine and glutamate simultaneously. There was a significant increase in SC (Fig 3.15A) and SVZ (Fig 3.15B) derived BLBP-positive cell proportion when cells were incubated with both 50 μM glutamate and 500 μM of ketamine compared to when the neurospheres were exposed to 50 μM glutamate and control condition (0 μM glutamate + 0 μM ketamine). However, there was no significant difference in BLBP-positive cell proportion when NPCs were exposed to concentrations of ketamine less than 500 μM with 50 μM glutamate. Approximately, 70-75% of the SC and SVZ NPCs differentiated into BLBP-positive cells when exposed to 500 μM of ketamine in the presence of glutamate.

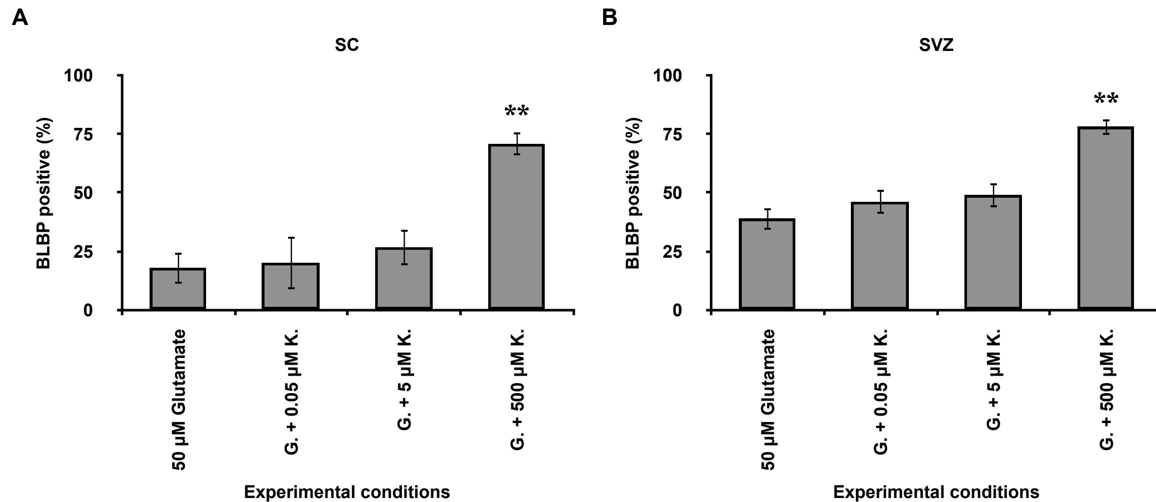

Figure 3.15 - 500 μM ketamine in the presence of glutamate increases the proportion of BLBP-positive radial glial cells. The influence of ketamine on SC (A) and SVZ (B) derived endogenous neural progenitor cell's ability to differentiate into BLBP-positive radial glial in the presence of glutamate. In the presence of glutamate, exposure to 500 μM ketamine increases differentiating SC and SVZ derived NPCs' ability to differentiate into radial glial. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for BLBP. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.0001$ for SC and $p<0.001$ for SVZ). Statistical significance shown with ** $p<0.01$. G. = Glutamate, K. = Ketamine

The changes in the proportions of GFAP-positive and BLBP-positive cells following exposure to glutamate and 500 μM ketamine, raises the question of the influence these factors would have on the proportion of cells that are positive for both GFAP and BLBP.

Therefore, SC and SVZ derived neurospheres were exposed to either 50 μM glutamate or 50 μM glutamate and 500 μM ketamine. There was a significant decrease in the proportion of GFAP-positive and BLBP-positive cells when SC neurospheres were exposed to 50 μM glutamate (Fig 3.16A). Contrarily, when these neurospheres were exposed to 50 μM glutamate and 500 μM of ketamine, there was a significant increase in the proportion of GFAP-positive and BLBP-positive cells. Interestingly, there were similar proportions of GFAP-positive and BLBP-positive cells in both control condition and 50 μM glutamate with 500 μM ketamine condition. This provides support for the notion that higher concentration of ketamine can overcome glutamate's influence on NPCs. The proportion of SVZ NPCs that differentiated into both GFAP-positive and BLBP-positive cells were similar under all experimental conditions. Figure 3.16C shows sample SC derived NPC fluorescent images used to assess the proportion of GFAP-positive and BLBP-positive cells.

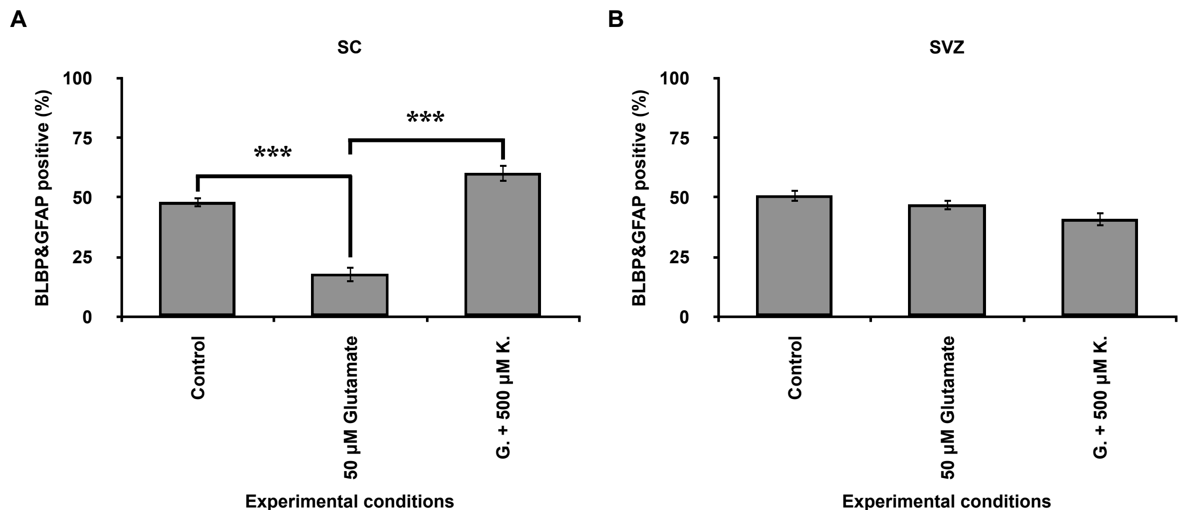

Figure 3.16 - Glutamate reduces the proportion of SC (A) NPCs derived BLBP-positive/GFAP-positive cells. The proportion of SVZ (B) NPCs derived BLBP-positive /GFAP-positive cells are not significantly affected by the experimental factors. Cells

were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with 500 μM of ketamine for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as % total number of cells positive for BLBP and GFAP. Each cell type was compared with a one-way ANOVA single factor analysis. Statistical significance shown with *** $p<0.0001$. G. = Glutamate, K. = Ketamine. Scale bar = 100 μm.

In summary, 500 μM ketamine was able to overcome glutamate's effect on SC NPC differentiation. Compared to control conditions, glutamate did not significantly influence both SC and SVZ NPC's ability to differentiate into O4-positive oligodendroctyes. However, in the presence of 50 μM glutamate, 500 μM ketamine was able to suppress SC and SVZ NPC's ability to differentiate into O4-positive oligodendroctyes. Moreover, glutamate and ketamine did not significantly influence SVZ derived NPC's ability to differentiate into GFAP-positive astrocytes. However, glutamate decreased the proportion of GFAP-positive astrocytes, while 50 μM glutamate with 500 μM ketamine increased the proportion of GFAP-positive astrocytes. Similarly, glutamate decreased the proportion of BLBP-positive radial glial cells, while 50 μM glutamate with 500 μM ketamine increased the proportion of BLBP-positive radial glial cells. Surprisingly, 50 μM glutamate with 500 μM ketamine increased the proportion of BLBP-positive radial glial cells derived from SVZ NPCs (Table 3.2).

Table 3.2 – Summary of the influence of glutamate and ketamine on SC and SVZ NPCs differentiation compared to baseline values[1]. G. = Glutamate; K. = Ketamine.

	SC			SVZ		
	500 μM K.	50 μM G.	50 μM G. + 500 μM K.	500 μM K.	50 μM G.	50 μM G. + 500 μM K.
Oligodendrocytes[a]	↓	-	↓	↓	-	↓
Astrocytes[b]	-	↓	↑	-	-	-
Radial glial[c]	-	↓	↑	-	-	↑

[1] control condition = 0 μM glutamate + 0 μM ketamine
[a] % total number of cells positive for O4
[b] % total number of cells positive for GFAP
[c] % total number of cells positive for BLBP

3.3 *In vitro*: differentiated NPCs' ability to migrate

Following SCI, precursor cells found in the SC are known to migrate away from the central canal region (Frisen, Johansson, Torok, Risling, & Lendahl, 1995; Frisen, Johansson, Lothian, & Lendahl, 1998; Johansson et al., 1999; Mothe & Tator, 2005; Namiki & Tator, 1999). Therefore, it is necessary to study the affect ketamine and glutamate would have on the NPCs' migratory potential. Neurospheres were randomly selected and plated on poly-l-ornithine coated 96-well plates. Following 48 hour exposure to 0-500 μM ketamine, 50 μM glutamate, or 50 μM glutamate + 0-500 μM ketamine, we noticed that the neurospheres remained largely intact, however many cells migrated radially out of the neurospheres. Cell migration phenotype *in vitro*, was quantified in two ways. First, the migration phenotype was quantified by counting the number of cells that migrated out from the neurosphere. There was no significant difference in the total number of cells that migrated from a neurosphere when derived from the SC and SVZ were exposed to 0-500 μM ketamine (Fig 3.17A and B),

50 μM glutamate (Fig 3.17C and D), or 50 μM glutamate + 0-500 μM ketamine (Fig 3.17E and F).

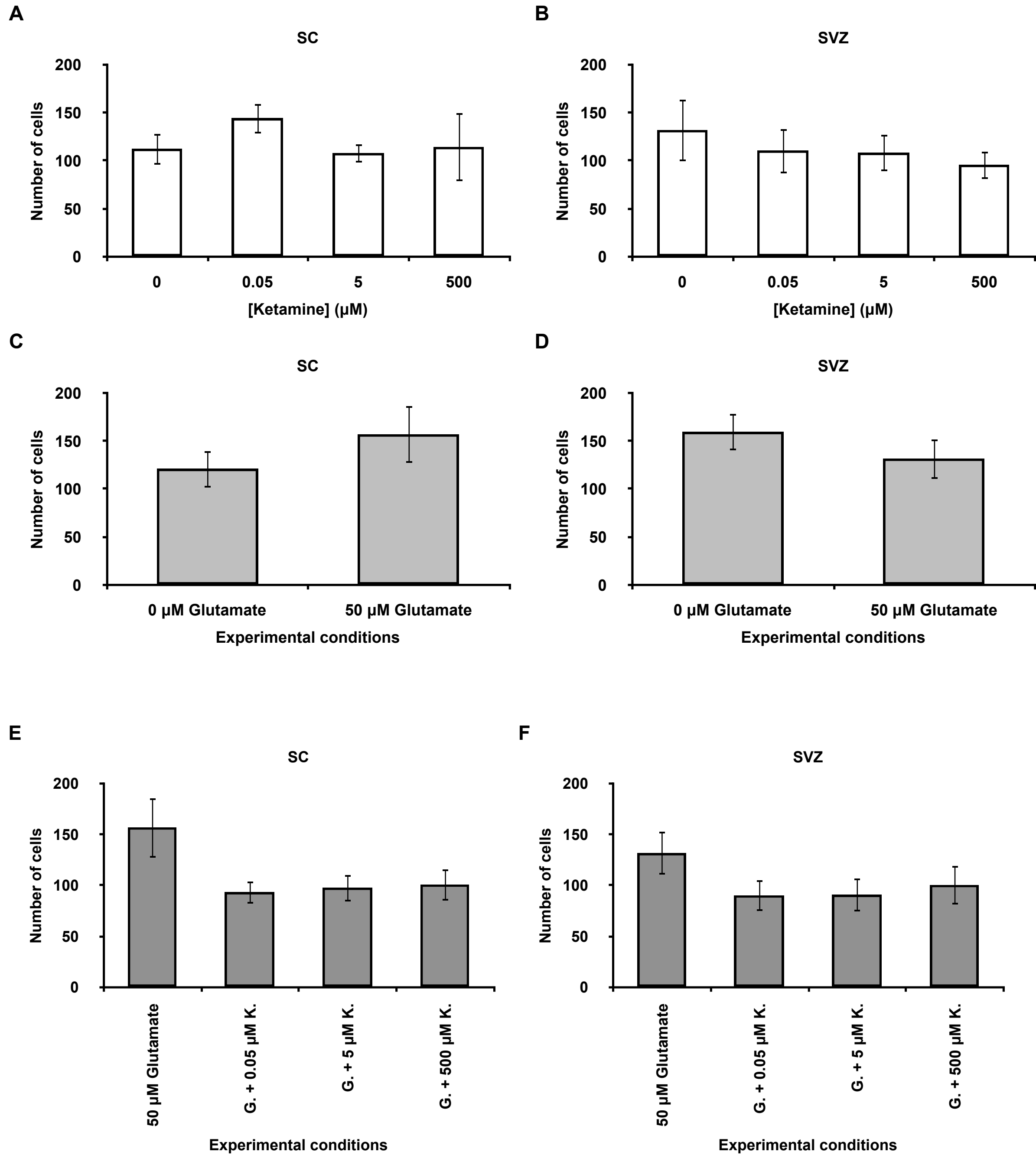

Figure 3.17 – Average number of migrating cells away from the mother neurosphere. Ketamine does not affect the total number of migrating cells. A) SC

and B) SVZ derived neurospheres were treated with culture medium containing ketamine (0-500 μM) for 48 hours. Glutamate does not affect the total number of migrating cells. C) SC and D) SVZ derived neurospheres were treated with or without medium containing 50 μM glutamate for 48 hours. In the presence of glutamate, ketamine does not affect the total number of migrating cells. E) SC and F) SVZ derived neurospheres were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean and are expressed as number of cells that migrated per neurosphere. Each cell type was compared with a one-way ANOVA single factor analysis ($p>0.05$). G. = Glutamate; K. = Ketamine.

When NPCs derived glial cells contribute to the formation of the glial scar, they migrate from the ependymal zone of the spinal cord to the injury site (Meletis et al., 2008; Mothe & Tator, 2005). Therefore, it is necessary to study the effect ketamine has on the migratory potential of the SC and SVZ derived differentiated cells. Therefore, we assessed migratory potential of oligodendrocytes and astrocytes by measuring the distance from the edge of the neurosphere to the nuclei of the differentiated cell in question.

Figure 3.18, shows the affect of ketamine on the migratory potential of differentiated NPCs, O4-positive oligodendrocytes derived from the SC and SVZ. Seven-day-old NPC derived neurospheres were exposed to the selected concentrations of ketamine (0-500 μM) to assess whether ketamine influenced cell migration and/or there was a dose response to the selected ketamine concentrations. It was found that there was a significant drop in the migratory

potential of O4-positive cells derived from the SC and SVZ when exposed to 500 μM of ketamine, Fig 3.18A and B, respectively. It should be noted that concentrations of ketamine less than 500 μM did not inhibit O4-positive cell migration.

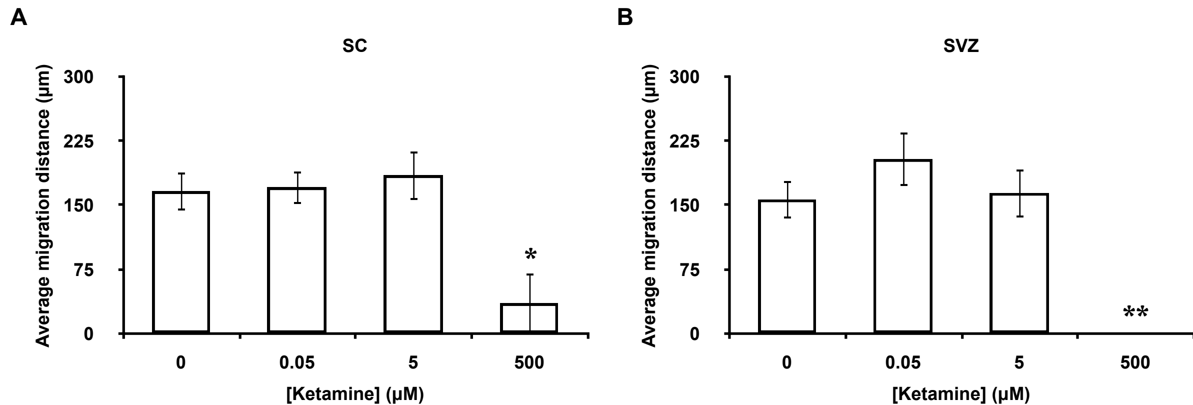

Figure 3.18 – 500 μM ketamine suppresses O4-positive oligodendrocyte migration. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived O4-positive cell's migration ability. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.05$ for SC and $p<0.01$ for SVZ). Statistical significance shown with * $p<0.05$ and ** $p<0.01$.

The effect of glutamate on the migratory potential of differentiated NPCs, O4-positive oligodendrocytes derived from the SC and SVZ was also assessed. There was no difference in SC and SVZ O4-positive cell's migratory potential, when exposed to 50 μM glutamate compared to control condition (Fig 3.19). It is also interesting that the distance O4-positive cells derived from the SC and the SVZ migrated was not significantly different in both the control and 50 μM glutamate condition.

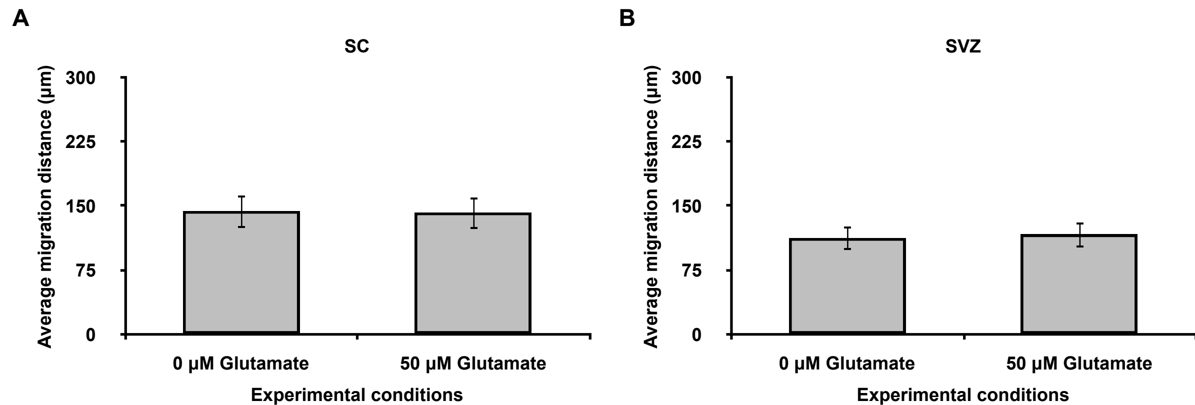

Figure 3.19 – Glutamate does not affect O4-positive oligodendrocyte migration. Cells were treated with culture medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis ($p>0.05$).

Figure 3.20 shows the changes in the migratory potential when the NPCs are cultured with both ketamine and glutamate. Interestingly, the migratory trend was similar to when the cells were exposed to ketamine alone. There was a significant decrease in SVZ and SC derived O4-positive cell migration when cells were incubated with both 50 μM glutamate and 500 μM of ketamine (60.2±31.8 μm and 36.3±17.1 μm for SC and SVZ, respectively) compared to when the neurospheres were exposed to 50 μM glutamate (142.2±18.9 μm and 116.6±15.1 μm for SC and SVZ, respectively). Again, it should be noted that concentrations of ketamine less than 500 μM did not inhibit O4-positive cell migration.

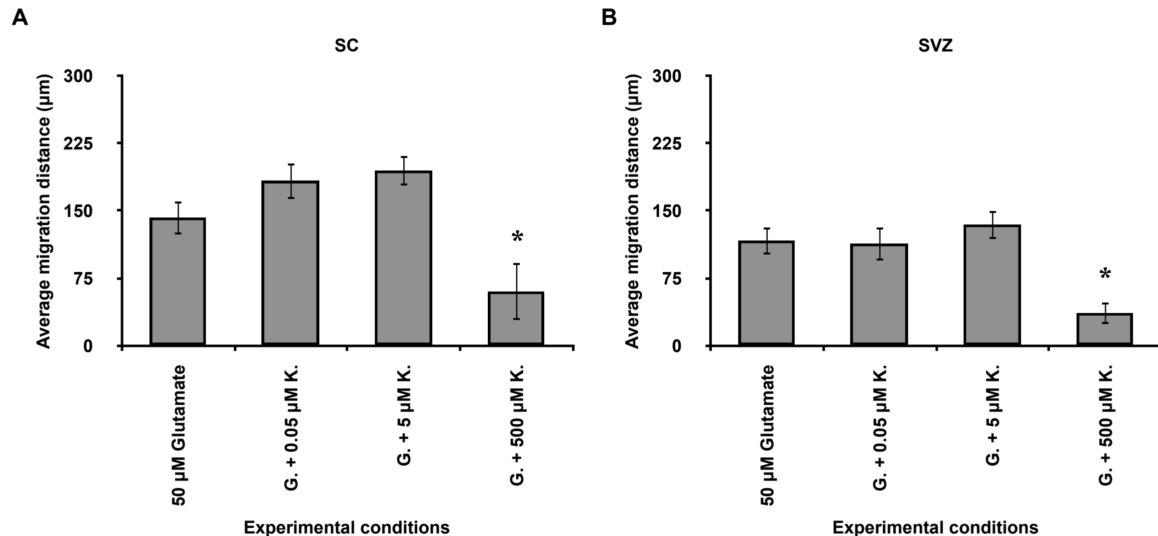

Figure 3.20 – 500 μM ketamine in the presence of glutamate suppresses O4-positive oligodendrocyte migration. The influence of ketamine on SC (A) and SVZ (B) derived O4-positive oligodendrocyte's ability to migrate in the presence of glutamate. In the presence of glutamate, exposure to 500 μM ketamine reduces O4-positive cell migration. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean of the mean. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.0001$ for SC and $p<0.01$ for SVZ). Statistical significance shown * $p<0.05$. G. = Glutamate, K. = Ketamine

Figure 3.21, shows the affect of ketamine on the migratory potential of differentiated NPCs, GFAP-positive astrocytes derived from the SC and SVZ. Two-day exposure to ketamine concentration of 5 μM or less did not significantly affect the migratory potential of GFAP-positive cells derived from the SC compared to control. However, exposure to 500 μM of

ketamine resulted in a significant decrease in GFAP-positive cell migration compared to control condition. Interestingly, it was found that the migratory potential of GFAP-positive cells derived from the SVZ was not affected by the presence of ketamine.

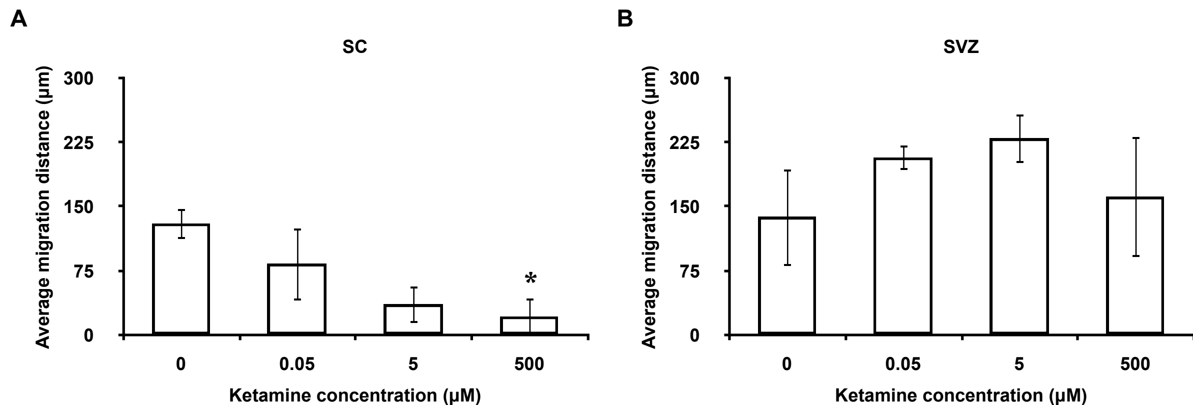

Figure 3.21 - 500 μM ketamine differentially affects the migratory potential of GFAP-positive astrocytes. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived GFAP-positive cell's migration ability. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.05$). Statistical significance shown with * $p<0.05$.

Two-day exposure to 50 μM glutamate induces a significant increase in SC derived GFAP-positive cell migration (Fig 3.22A). Under control conditions, SC derived cells migrated an average distance of 94.8±17.1 μm. However, when SC derived neurospheres were exposed to 50 μM glutamate, these cells tended to migrate an average distance of 154.1±15.9 μm. Contrarily, SVZ derived GFAP-positive cell's migrated roughly the same distance when they were exposed to control condition and 50 μM glutamate, 207.5±31.6 μm and 181.9±24.9 μm, respectively (Fig 3.22B). These results raise the question about how the migratory potential

of the cells would differ if they were exposed to both 50 μM glutamate and 0-500 μM of ketamine simultaneously.

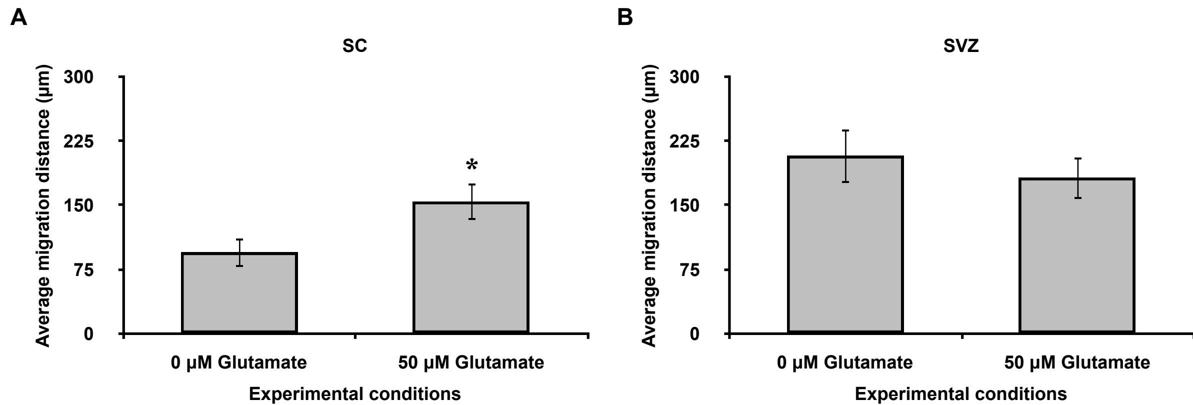

Figure 3.22 – Glutamate differentially affects the migratory potential of GFAP-positive astrocytes. The migratory ability of (A) SC NPCs derived astrocytes (GFAP-positive cells) is significantly increased in the presence of glutamate. Glutamate did not significantly affect the migratory ability of GFAP-positive cells derived from the (B) SVZ NPCs. Cells were treated with culture medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis. Statistical significance shown with * $p<0.05$.

Figure 3.23 shows the changes in the migratory potential of GFAP-positive cells, when the NPCs are cultured with both 50 μM glutamate and 0-500 μM ketamine. Interestingly, similar to O4-positive cells, the migratory trend was similar to when the cells were exposed to just ketamine alone. There was a significant decrease in SC NPCs derived GFAP-positive cells migratory potential when exposed to both 50 μM glutamate and 500 μM ketamine (64.0±15.2 μm) compared to only 50 μM glutamate (154.1±15.9 μm). Again, it should be

noted that concentrations of ketamine less than 500 μM did not significantly inhibit GFAP-positive cell migration. Simultaneous exposure to 500 μM ketamine and 50 μM glutamate restores SC NPC derived GFAP-positive cells migratory potential back to similar levels as control conditions (absence of 50 μM glutamate and 500 μM ketamine). These results provide further support for the notion that high concentration of ketamine can overcome glutamate's influence on NPCs. It was found that the migratory potential of GFAP-positive cells derived from the SVZ neurosphere's were not significantly affected by the selected ketamine concentrations in the presence of glutamate.

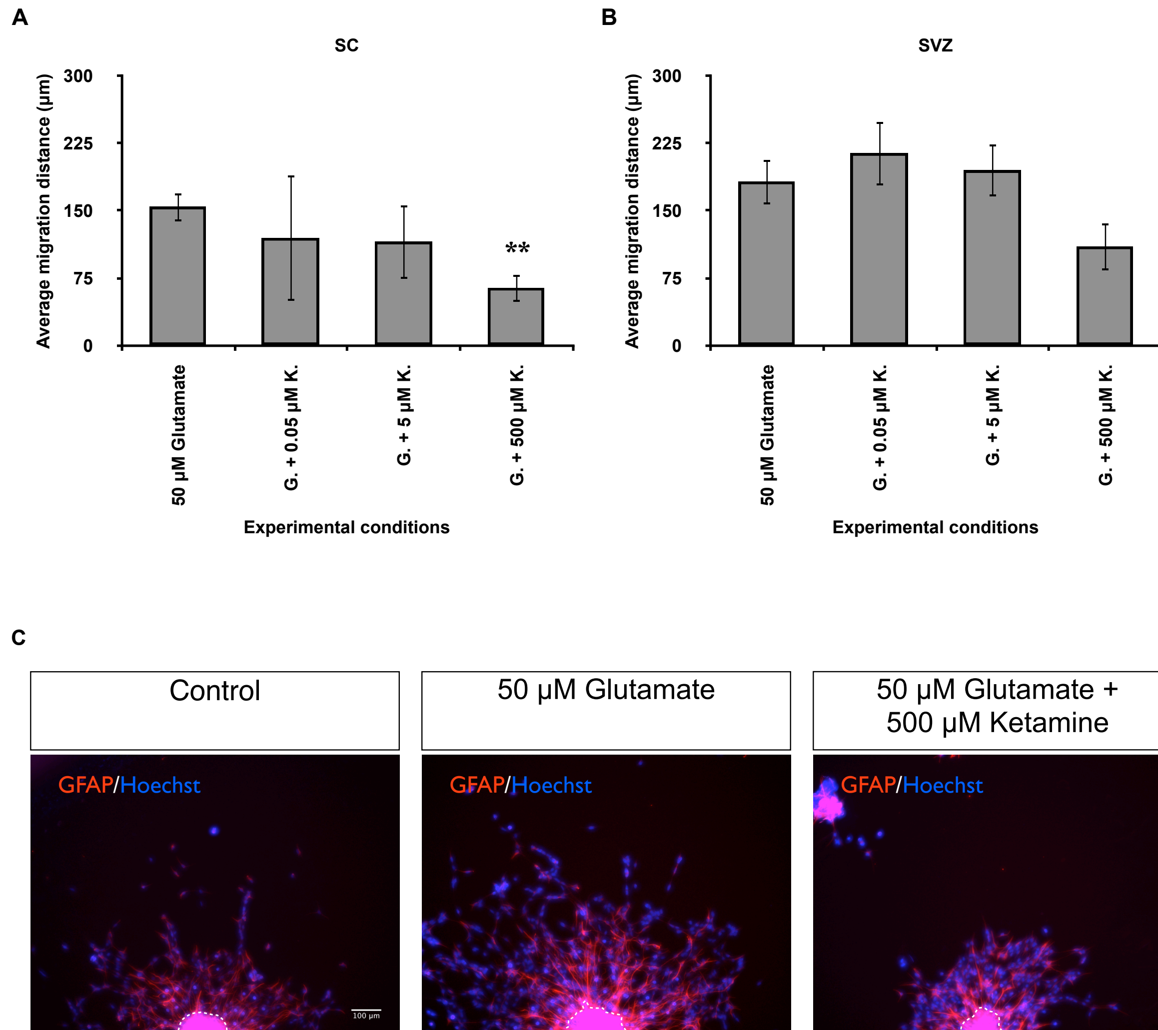

Figure 3.23 – 500 µM ketamine reverses the influence of 50 µM glutamate on SC NPCs derived GFAP-positive astrocyte migration. The influence of ketamine on SC (A) and SVZ (B) derived GFAP-postive astrocytes ability to migrate in the presence of glutamate. 500 µM of ketamine can overcome glutamate's induced SC NPCs derived GFAP-positive cell migration. (C) Sample SC derived NPC fluorescent images used to assess the migratory ability of GFAP-positive cells. Cells were treated with culture medium containing 50 µM glutamate or 50 µM glutamate with selected concentrations of ketamine (0-500 µM) for 48 hours (*n*=3). Results

presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis. Statistical significance shown with ** $p<0.01$. G. = Glutamate, K. = Ketamine. Scale bar = 50 μm

The effect of ketamine on the migratory potential of differentiated NPCs BLBP-positive radial glial cells derived from the SC and SVZ was also investigated (Fig. 3.24). Two-day exposure to ketamine concentration of 5 μM or less did not significantly affect the migratory potential of BLBP-positive cells derived from the SC compared to control. However, exposure to 500 μM of ketamine resulted in a significant increase in BLBP-positive cell migration compared to control condition. Furthermore, it was found that the migratory potential of BLBP-positive cells derived from the SVZ was not affected by the presence of ketamine.

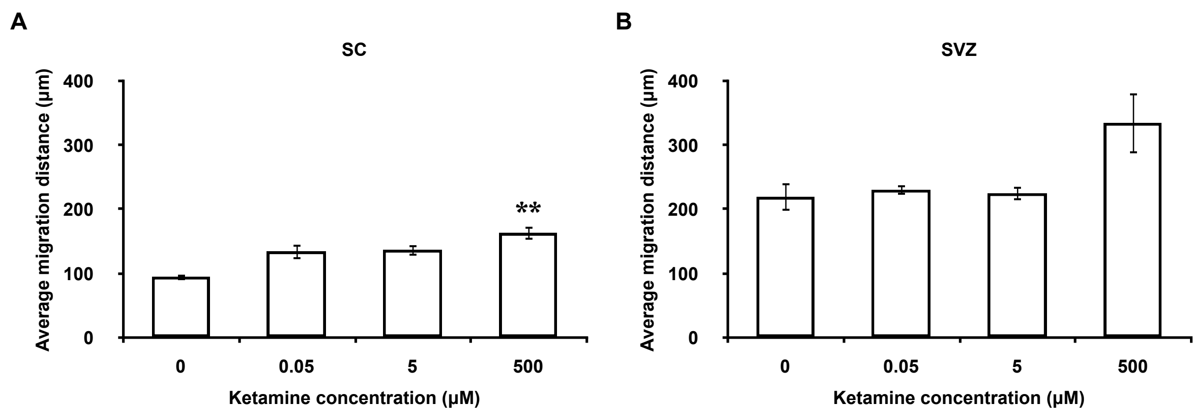

Figure 3.24 - 500 μM ketamine differentially affects the migratory potential of BLBP-positive radial glial cells. Concentration-response curves of ketamine on SC (A) and SVZ (B) derived BLBP-positive cell's migration ability. Cells were treated with culture medium containing ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way

ANOVA single factor analysis ($p<0.0001$). Statistical significance shown with ** $p<0.01$.

Two-day exposure to 50 μM glutamate induces a significant decrease in SC derived BLBP-positive cell migration (Fig 3.25A). Contrarily, SVZ derived BLBP-positive cells migrated roughly the same distance when they were exposed to control condition and 50 μM glutamate (Fig 3.25B). These results raise the question about how the migratory potential of the cells would differ if they were exposed to both 50 μM glutamate and 0-500 μM ketamine simultaneously.

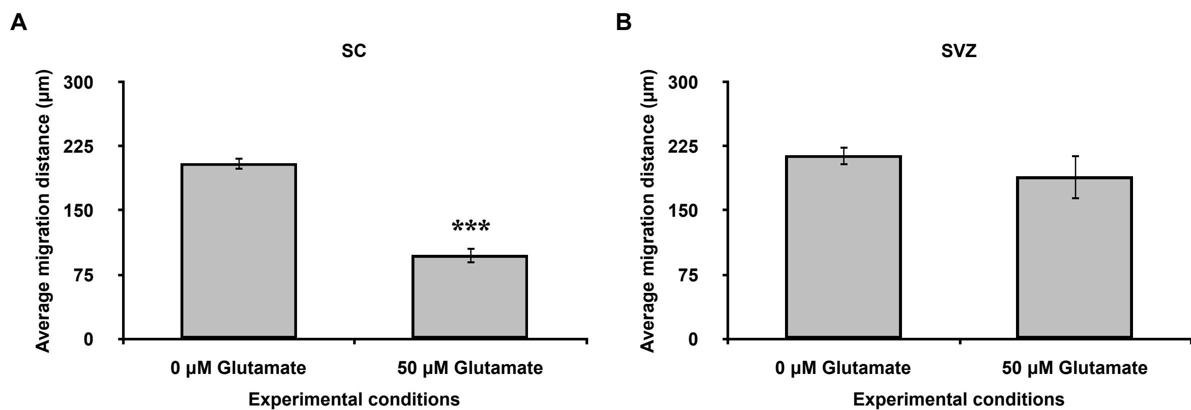

Figure 3.25 – Glutamate differentially affects the migratory potential of BLBP-positive radial glial cells. The migratory ability of (A) SC NPCs derived radial glial cells (BLBP-positive cells) is significantly decreased in the presence of glutamate. Glutamate did not significantly affect the migratory ability of BLBP-positive cells derived from the (B) SVZ NPCs. Cells were treated with culture medium containing 50 μM glutamate for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis. Statistical significance shown with *** $p<0.0001$.

Figure 3.26 shows the changes in the migratory potential of BLBP-positive cells, when the NPCs are cultured with both 50 μM glutamate and 0-500 μM ketamine. There was a significant increase in SC NPCs derived BLBP-positive cells migratory potential when exposed to both 50 μM glutamate and ketamine compared to only 50 μM glutamate. It is interesting that concentration of ketamine (0.05, 5 and 500 μM) all induced an increase in radial glial cell migration. It is important to note that simultaneous exposure to ketamine and 50 μM glutamate restores SC NPC derived BLBP-positive cells migratory potential back to similar levels as control conditions (absence of both 50 μM glutamate and 500 μM ketamine). These results provide further support for the notion that high concentration (500 μM) of ketamine can overcome glutamate's influence on NPCs. It was found that the migratory potential of BLBP-positive cells derived from the SVZ neurospheres was not significantly affected by the selected ketamine concentrations in the presence of glutamate.

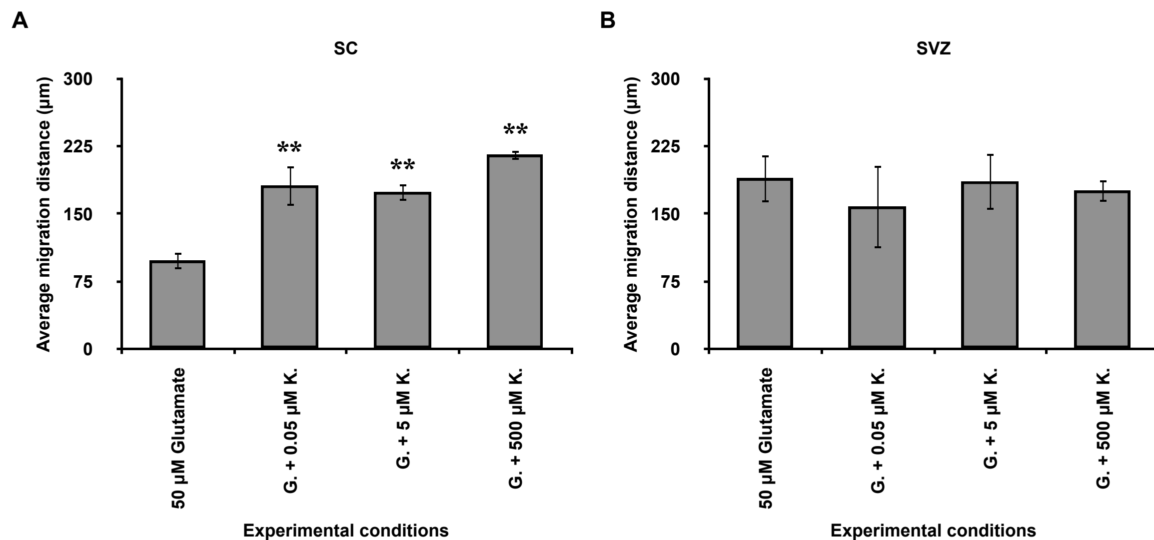

Figure 3.26 – In the presence of glutamate, ketamine increases SC NPCs derived BLBP-positive radial glial cell migration. The influence of ketamine on SC (A) and

SVZ (B) derived BLBP-positive radial glial cells ability to migrate in the presence of glutamate. Ketamine can overcome glutamate's induced SC NPCs derived BLBP-positive cell migration. Cells were treated with culture medium containing 50 μM glutamate or 50 μM glutamate with selected concentrations of ketamine (0-500 μM) for 48 hours (*n*=3). Results presented as mean±standard error of the mean. Each cell type was compared with a one-way ANOVA single factor analysis ($p<0.0001$). Statistical significance shown with ** $p<0.01$. G. = Glutamate, K. = Ketamine.

In summary, 500 μM ketamine alters the migratory potential of differentiated SC and SVZ NPCs. In the absence and presence of 50 μM glutamate, 500 μM ketamine suppresses the migratory potential of SC and SVZ NPC derived oligodendrocytes. Compared to control conditions, SC NPC derived GFAP-positive astrocytic migratory potential is suppressed and enhanced by 500 μM ketamine and 50 μM glutamate, respectively. However, simultaneous exposure to 500 μM ketamine and 50 μM glutamate restores SC NPC derived GFAP-positive astrocytic migratory potential to baseline value. Alternatively, SVZ NPC derived GFAP-positive astrocytic migratory potential is not affected by exposure to 0-500 μM ketamine, 50 μM glutamate, or 50 μM glutamate + 0-500 μM ketamine. On the other hand, compared to control conditions, SC NPC derived BLBP-positive radial glial cell migratory potential is enhanced and suppressed by 500 μM ketamine and 50 μM glutamate, respectively. Moreover, simultaneous exposure to ketamine and 50 μM glutamate restores SC NPC derived BLBP-positive radial glial cell migratory potential to baseline value. Furthermore, SVZ NPC derived BLBP-positive radial glial cell migratory potential is not

affected by exposure to 0-500 μM ketamine, 50 μM glutamate, or 50 μM glutamate + 0-500 μM ketamine.

Table 3.3 – Summary of the influence of glutamate and ketamine on SC and SVZ NPCs migration compared to baseline values[1]. Ketamine can overcome glutamate's influence on the migration of astrocytes derived from SC NPCs. G. = Glutamate; K. = Ketamine.

	SC			SVZ		
	500 μM K.	50 μM G.	50 μM G. + 500 μM K.	500 μM K.	50 μM G.	50 μM G. + 500 μM K.
Oligodendrocytes	↓	-	↓	↓	-	↓
Astrocytes	↓	↑	-	-	-	-
Radial glial	↑	↓	-	-	-	-

[1] control condition = 0 μM glutamate + 0 μM ketamine

CHAPTER 4.0 - DISCUSSION

4.1 *In vitro*: differentiating NPCs' proliferation and survival

It has been shown that ependymal cells proliferate in the normal and injured spinal cord (Mothe & Tator, 2005; Namiki & Tator, 1999). In this study, we examined the influence ketamine and glutamate had on differentiating NPCs proliferation and survival. Cell proliferation appears to be influenced by NMDA receptor agonists and antagonists, although there are discrepancies in the results published to date partly because of the different models used. Although the detailed mechanism is still unclear, previous studies have shown that the activation of the NMDA receptor influences the proliferation rate of neural progenitor cells (Dong, Rovnaghi, & Anand, 2012; Joo et al., 2007; Kitayama, Yoneyama, & Yoneda, 2003; Kitayama, Yoneyama, Tamaki, & Yoneda, 2004; Luk, Kennedy, & Sadikot, 2003; Luk & Sadikot, 2004; Mochizuki et al., 2007; Sah, Ray, & Gage, 1997; M. Suzuki et al., 2006; Yoneyama et al., 2008).

It has been shown that blocking the NMDA receptor increases the number of proliferating cells (Hirasawa, Wada, Kohsaka, & Uchino, 2003; Kitayama, Yoneyama, & Yoneda, 2003; Maekawa et al., 2009; Nacher, Rosell, Alonso-Llosa, & McEwen, 2001) in certain areas of the rodent central nervous system. The inhibition of NMDA receptors by a competitive antagonist, CGP43487, has been shown to increase cell proliferation in the dentate gyrus of the hippocampus and the SVZ of young adult rats (Nacher, Rosell, Alonso-Llosa, &

McEwen, 2001). Similarly, in another study, when adult mouse dentate gyrus cells were exposed to the NMDA receptor agonist, NMDA, there was a significant reduction in the incorporation of BrdU into cells, while exposure to MK-801, a NMDA receptor antagonist, resulted in an increase in the incorporation of BrdU into cells. However, under the same experimental settings, it was found that there was no effect on SVZ derived cells (Kitayama, Yoneyama, & Yoneda, 2003). The reason for the discrepancy regarding cell proliferation in the SVZ might be the difference in the NMDA antagonist used in both studies, Nacher *et al.* (Nacher, Rosell, Alonso-Llosa, & McEwen, 2001) used a competitive antagonist whereas Kitayama *et al.* (Kitayama, Yoneyama, & Yoneda, 2003) used a non-competitive antagonist. It is interesting that NMDA receptor activation and inhibition show drastic different results depending on the source of cell in question. In this study we found that there was no effect on differentiating SC and SVZ derived neural progenitor cell's proliferative ability in the presence of 0.05-500 μM ketamine, a non-competitive NMDA receptor antagonist. This coincides with the above-mentioned study, which found that there was no effect on SVZ derived cells when exposed to a non-competitive NMDA receptor antagonist (Kitayama, Yoneyama, & Yoneda, 2003).

Some studies have shown that NMDA receptor activation increases the proliferation of NPCs (Haydar, Wang, Schwartz, & Rakic, 2000; Joo et al., 2007; Luk, Kennedy, & Sadikot, 2003; Luk & Sadikot, 2004; Mochizuki et al., 2007; Sah, Ray, & Gage, 1997; M. Suzuki et al., 2006) depending on the cell's origin. One study found that glutamate activation of NMDA receptors, promotes proliferation of precursor cells in the ventral telencephalon (Luk, Kennedy, & Sadikot, 2003). More specifically, exogenously administered glutamate has been shown to increase proliferation in the embryonic rat ventricular zone, while inhibiting

proliferation in the SVZ (Haydar, Wang, Schwartz, & Rakic, 2000). Contrarily, another study found that glutamate enhances the survival and proliferation of neural progenitors derived from the SVZ (Brazel, Nunez, Yang, & Levison, 2005). Similarly, we found that exposure to glutamate resulted in a significant increase in differentiating SVZ cell's proliferative ability compared to control conditions. However, in the presence of 50 μM glutamate and 500 μM ketamine, there was a significant decrease in differentiating SVZ cell's proliferation rate. This indicates that in terms of cell proliferation, the higher ketamine concentration can overcome glutamate's influence on the SVZ progenitor cells. It is also important to mention that when the cells were exposed to 50 μM glutamate it did not stimulate apoptosis. This is consistent with previous studies that found that high levels of glutamate, up to 1 mM are not toxic to neural precursor cultures (Brazel, Nunez, Yang, & Levison, 2005). Under the same experimental settings, we found that there was no effect on SC derived NPC's proliferative and survival ability following exposure to glutamate and ketamine. Therefore, the influence of glutamate and ketamine on cell proliferation is not the same for all cell types.

Once again focusing on the change in SVZ derived NPC's proliferative ability following glutamate and ketamine exposure; it was predicted that there would also be a change in the proportion of nestin-positive progenitor cells. A previous study found that when the number of proliferating cells increased, there was also an increase in the number of nestin-positive cells (Nacher, Rosell, Alonso-Llosa, & McEwen, 2001). Likewise, another study found that when the number of proliferating cells decreased, there was also a decrease in the number of nestin-positive cells (Kitayama, Yoneyama, & Yoneda, 2003). Taking these studies into account, since, there was an increase in the SVZ's proliferative ability following glutamate

exposure, it was expected that likewise there would be an increase in the proportion of nestin-positive progenitor cells. Furthermore, it was expected that there would be a decrease in the proportion of nestin-positive progenitor cells following exposure to 50 μM glutamate with 500 μM of ketamine. Similarly, we found that exposure to glutamate resulted in a significant increase in the proportion of nestin-positive progenitors compared to control conditions. Furthermore, in the presence of 50 μM glutamate+ 500 μM ketamine, there was a significant decrease in the proportion of nestin-positive progenitors compared to 50 μM glutamate condition. Interestingly, simultaneous exposure to 50 μM glutamate and 500 μM ketamine restored the proportion of nestin-positive progenitor cells to values comparable to control conditions. This further indicates that the higher ketamine concentration can overcome glutamate's influence on the SVZ NPCs. However, the mechanism by which this occurs is not known. Since, there was no affect on SC derived NPC's proliferative ability; it was predicted that there would be no significant change in the proportion of nestin-positive progenitor cells. As expected, there was no difference in the proportion of SC derived nestin-positive progenitor cells across all experimental conditions. These results are indicative that post SCI functional recovery via ketamine administration is not due to an increase or decrease in spinal cord cell population.

4.2 *In vitro*: differentiating NPCs' phenotype

SC and SVZ NPC derived neurospheres were differentiated in the absence of mitogens, as whole neurospheres to study the effects ketamine and glutamate have on their multipotency. It has been previously demonstrated that neural progenitor cells often differentiate into glial

cells (Brazelton, Rossi, Keshet, & Blau, 2000; Cao et al., 2001; Gritti et al., 1996; Palmer, Markakis, Willhoite, Safar, & Gage, 1999). In our study we found that following a two-day differentiation period there were no visible βIIIT –positive cells derived from both the SC and SVZ neurospheres following exposure to 0-500 μM ketamine, 50 μM glutamate, and 50 μM glutamate + 0-500 μM ketamine. This is not surprising since neuronal lineage cells expressing βIIIT appear approximately five days into the differentiation period (Cavallaro et al., 2008). Therefore, a longer differentiation period is needed to detect neuronal markers in culture. Preliminary data indicate the presence of βIIIT –positive cells in both control and experimental conditions following a seven-day differentiation period (Fig. 4.1).

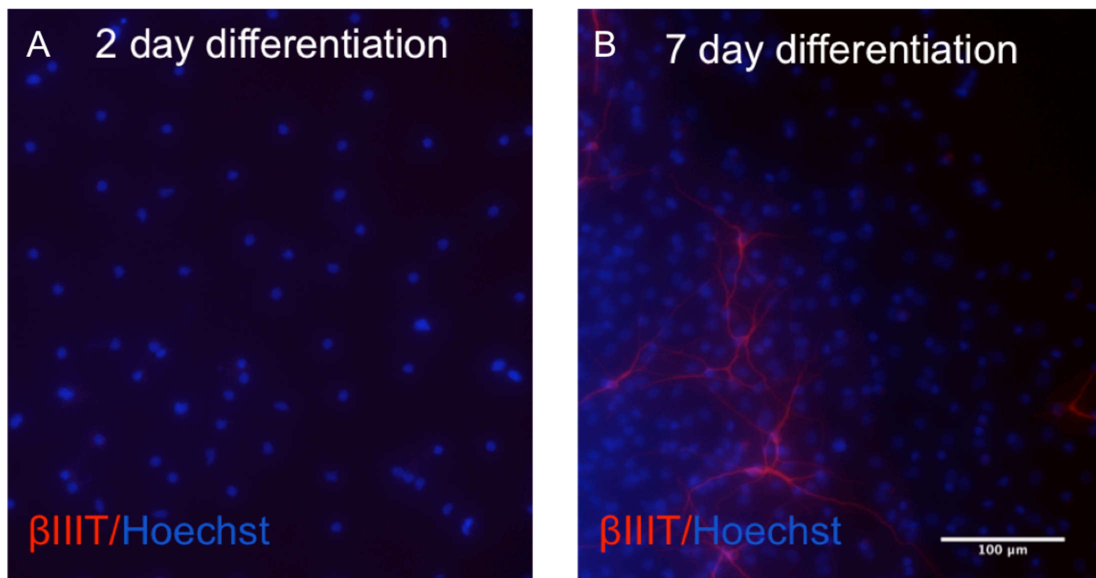

Figure 4.1 – Immunofluorescence for neuronal marker at differentiation (A) day two and (B) day seven. Scale bar = 100 μm. βIIIT, Neuronal Class III β-tubulin,

It has been previously shown that SC derived NPCs preferentially differentiate into glial cells, with the highest percentage of the total cells becoming oligodendrocytes and radial glial cells (Kulbatski et al., 2007). Moreover, it has been found that the proportion of

differentiated oligodendrocytes, astrocytes, and neurons from the cervical SC derived neurospheres do not differ significantly from the SVZ brain-derived neurospheres. However, it has been shown that lumbar SC derived neurospheres produce significantly higher proportion of neurons than cervical SC derived neurospheres (Kulbatski & Tator, 2009). We found that there was no significant difference in the proportion of GFAP-positive astrocytes between the two cell types. However, under basal conditions there is significant difference in the proportion of O4-positive oligodendrocytes between the two cell types. A potential reason for this discrepancy is the difference between the assessment period used by Kulbatski and Tator (2009) and the assessment period we used in this study. They differentiated the neurospheres in the absence of mitogens for seven days, while we differentiated the neurospheres for two days.

There was a significant decrease in the proportion of O4-positive oligodendrocytes, when SC and SVZ derived neurospheres are exposed to 500 μM ketamine. This decrease that we observed may not be beneficial since remyelination of axons is needed post SCI. These results are surprising considering previous research has found that non-competitive NMDA antagonist, MK-801, did not influence gliogenesis in the SGZ of the hippocampus (Petrus *et al*., 2009). Petrus *et al* (2009) assessed the effects of MK-801 on gliogenesis after a 4-week time point, which is a significantly longer time point. These results indicate that NMDA receptor antagonists might have a time-dependent influence on gliogenesis. Moreover, MK-801 is a more specific NMDAR antagonist than ketamine.

A recent study demonstrated that extracellular glutamate activates NMDA receptors of cells derived from the SVZ of adult rats, to enhance oligodendrocyte differentiation. When 1 mM

glutamate was added to the culture medium there was a significant increase in the proportion of O4-positive oligodendrocytes. However, exposure to concentrations of glutamate less than 1 mM did not affect oligodendrocyte differentiation (Cavaliere, Urra, Alberdi, & Matute, 2012). Consistently, when cells from the SC and SVZ were exposed to 50 μM glutamate there was no significant difference in the proportion of O4-positive oligodendrocytes. Moreover, in the same study they found that the pro-oligodendrocyte differentiation effect of glutamate was abolished by MK-801 (Cavaliere, Urra, Alberdi, & Matute, 2012). These results further support our findings, where we saw a significant decrease in the SVZ and SC derived O4-positive cell proportions when cells were incubated with both 50 μM glutamate and 500 μM of ketamine. However, there was no visible difference in the proportion of O4-positive cells following exposure to 50 μM glutamate and concentrations of ketamine less than 500 μM. Taken together, these data indicate that a higher concentration of ketamine is needed to over come glutamate's influence on NPC differentiation. Furthermore, these results indicate that post SCI functional recovery is less likely to have been due to remyelination through and increase in oligodendrocyte differentiation.

We saw that while exposure to 50 μM glutamate reduced the proportion of GFAP-positive cells, simultaneous exposure to both 50 μM glutamate and 500 μM of ketamine resulted in a significant increase in SC derived GFAP-positive cell proportion, which was comparable to control conditions. Again, this supports the view that *in vitro* a high concentration of a NMDA receptor antagonist can prevent the influence of glutamate. The aforementioned observations indicate that glutamate plays a role in regulating GFAP-positive cell differentiation. However, the precise mechanism used by glutamate to alter cell differentiation is not well known.

Interestingly, we found that glutamate suppressed radial glial differentiation of SC derived NPCs, whereas ketamine in the presence of glutamate facilitated radial glial differentiation. It is important to point out that majority of the GFAP-positive cells derived from SC neurospheres were also BLBP-positive. Moreover, we show that following exposure to 50 μM glutamate and 500 μM of ketamine, SC neurospheres differentiate abundantly into GFAP-positive/BLBP-positive cells compared to other conditions. Following SCI, it has been shown that there is an upregulation of radial glial cells, which may play an important role in neural regeneration (Shibuya, Miyamoto, Itano, Mori, & Norimatsu, 2003). Moreover, acutely transplanted radial glial cells *in vivo* form bridges and promote functional recovery following SCI (Hasegawa et al., 2005). Therefore, radial glial cells play an important role in recovery post SCI. As such, the increase in radial glial differentiation we see following ketamine exposure could be beneficial following SCI. Moreover, BLBP is usually expressed during cell migration (L. Feng, Hatten, & Heintz, 1994), which suggests that maybe the BLBP-positive cells in our study is functioning to aid migration *in vitro*.

4.3 *In vitro*: differentiated NPCs' ability to migrate

Following SCI, precursor cells found in the SC are known to proliferate and migrate away from the central canal region (Frisen, Johansson, Torok, Risling, & Lendahl, 1995; Frisen, Johansson, Lothian, & Lendahl, 1998; Johansson et al., 1999; Mothe & Tator, 2005; Namiki & Tator, 1999). A standard *in vitro* cell migration assay (Courtes et al., 2011; Ishido & Suzuki, 2010; Kong et al., 2008) was used to assess NPCs migratory behavior *in vitro;* the migratory ability of these cells types has not yet been extensively described. The cells

migrated from the neurosphere in chains or as individual cells. Through this study, it was demonstrated that following a 48 hour differentiation period, neurospheres remain largely intact, however many cells migrated radially out of the neurospheres. Quantitative analysis of the migratory potential demonstrated that ketamine played an important role in limiting astrocyte migration away from neurosphere.

In our study, the migration of O4-positive cells was not altered by glutamate, but in the presence of 50 μM glutamate and 500 μM ketamine there was a significant suppression of O4-positive cell migration. In the presence of 50 μM glutamate and 500 μM ketamine, the migration range of O4-positive oligodendrocytes were suppressed to levels below the base line value. These cells were restricted to an area close to the edge of the neurosphere. This decrease in O4-positive oligodendrocyte migration that we observed may not be beneficial since remyelination of axons is needed post SCI. These results support the differentiation data, where we saw a decrease in O4-positive oligodendrocyte cell proportion following exposure to 500 μM ketamine and 50 μM glutamate with 500 μM ketamine. Since, less O4(+) oligodendrocyte cells are migrating out of the neurosphere, there is a decrease in the proportion of oligodendrocytes. Or it is possible that the cells are differentiating into oligodendrocytes but are not migrating out of the neurosphere. Another possibility is that the O4-positive cells are neither differentiating nor migrating.

Glutamate differentially affects the migratory potential of GFAP-positive cells derived from the SC and SVZ neurosphere. While it enhanced the migratory potential of the GFAP-positive cells derived from the SC, it did not influence the migratory potential of the cells derived from the SVZ. Thus, *in vitro* glutamate seems to only promote SC-derived GFAP-

positve cell migration. Furthermore, as seen with O4-positive cells, in the presence of glutamate and 500 μM ketamine there was a significant suppression of SC derived GFAP-positive cell migration. Again, this exemplifies that a concentration of 500 μM of ketamine can overcome glutamate's influence on NPCs. These results also suggests that blocking or activating NDMA receptors promote or inhibit GFAP-positive cell migration in only certain areas of the central nervous system.

Similar to astrocytic migration, glutamate differentially affects the migratory potential of BLBP-positive cells derived from the SC and SVZ neurosphere. Glutamate reduced the migratory potential of the BLBP-positive cells derived from the SC, but it did not influence the migratory potential of the cells derived from the SVZ. Interestingly, ketamine can overcome glutamate's suppressive effects on radial glial cell migration. These results are indicative that post SCI functional recovery is due to delaying astrocyte migration while inducing radial glial cell migration. The suppression of astrocyte migratory ability may be beneficial in delaying the contribution NPC derived GFAP-positive cells have at the glial scar. Furthermore, ketamine's ability to overcome glutamate's suppressiveness of radial glial cell migration is beneficial since radial glial cells can act as cellular scaffolds for axonal guidance. (Nomura et al., 2010).

This is the first report that demonstrates greatly enhanced SC derived GFAP-positive astrocyte cell migration while suppressing BLBP-positive radial glial cell migration following glutamate exposure. It is essential that more research be done to discover the mechanism that coordinates GFAP and BLBP-positive cell migration, as it will be useful in improving our knowledge about NMDA receptor mediated cell migration.

Taken together, these results however do not fully explain why we saw an increase in functional recovery in spinalized rats following ketamine administration. Our results suggest that ketamine may play a role in keeping the NPCs in an astroglial state (GFAP-positive and BLBP-positive) instead of fully differentiated astrocytes (GFAP-positive and BLBP-negative). Furthermore, ketamine may also play a role in delaying the contribution NPC derived GFAP-positive cells have at the glial scar. However, the glial scar is usually fully formed by four weeks post SCI (R. Hu et al., 2010; Leal-Filho, 2011). We saw an improvement in hind-limb locomotor function when ketamine was administered 4 weeks post SCI. The reasons for this locomotor function improvement are unknown. Given the variability in the type of receptors ketamine can activate, its' influence on functional recovery is probably an interplay involving many different mechanisms and cell types found in the environment. However, the results obtained through this study are still promising, since ketamine administration at an early time point following SCI might have a more profound effect at both the cellular and functional level.

4.4 Limitations

It has been noted that cells expressing βIIIT appear approximately five days into the differentiation period (Cavallaro et al., 2008); therefore, one limitation of this study is that we were not able to investigate the influence of ketamine and glutamate on neuronal lineage cells. Therefore, a longer differentiation period is needed to examine the effect ketamine and glutamate has on neuronal cell differentiation. In this study, a two-day assessment period was chosen to ensure that there was no direct interaction between the cells migrating away from

two distinct mother neurospheres, since the migration distance was assessed in respect to the mother neurosphere.

In terms of the pure affect of ketamine and glutamate on cell proliferation, in this study mitogens were removed and then the cells were labelled with BrdU. Due to the removal of mitogens from the system, the cells would have been induced to differentiate during the cell proliferation assessment. To fully study the influence on cell proliferation, it would have been beneficial to expose the cells to mitogens during BrdU labeling. Moreover, although, the neurosphere culture system allows us to study the full potential of a cell *in vitro* due to the lack of cell-cell interactions, it is likely that the cell may respond differently i*n vivo*. As such, it is critical that an *in vivo* study using a SCI model is used to verify whether ketamine has the same or similar influence on NPCs *in vivo*.

4.5 Future Directions

In addition, to future experiments investigating the influence of ketamine and glutamate on NPCs proliferation, differentiation, and migration *in vivo*, this study has opened the door for more possible studies. It will be interesting to elucidate the mechanism through which ketamine influences gliogenesis and glial cell migration. Furthermore, it is critical to look into possible focal delivery methods that can be used to deliver ketamine *in vivo*.

CHAPTER 5.0 - CONCLUSIONS

Majority of the previous studies have only focused on the influence NMDA receptor agonists and antagonists have on cell proliferation and neuronal differentiation. These results indicate that blocking or activating NMDA receptors seem to promote or inhibit cell proliferation in only certain areas of the central nervous system. Our study illustrates that NMDA receptor agonists and antagonists play a role in gliogenesis. Furthermore, our results support the notion that a higher concentration of ketamine (500 μM) can overcome glutamate's influence on NPCs. These results are promising since ketamine administration may help alleviate some of the adverse affects glutamate has on the NPCs found in the spinal cord following SCI. Also, ours results are indicative that SC and SVZ derived neurospheres can be unique in terms of their response to certain ketamine concentrations. Therefore, the influence of ketamine on cell proliferation, gliogenesis and cell migration would not be the same for all cells types. Ketamine's regulatory role will differ depending on the location of the cell population. These results have great implication on the plausible delivery method used to administer ketamine post SCI. Since SC and SVZ NPC respond differently to ketamine, an optimized therapeutic administration requires targeted focal delivery to the injured spinal cord. Since so little is know about the influence of ketamine on cell migration, the present study uncovers a potentially significant function of ketamine in the central nervous system. We found that both glutamate and ketamine play a role in O4-positive, GFAP-positive, BLBP-positive cell migration. The enhancement or suppression of cell migration when the NMDA receptor is blocked or activated depends on the cell type.

Furthermore, these results indicate that post SCI functional recovery through ketamine administration is neither due an increase or decrease in cell population or remyelination. Instead, it is likely that delaying astrocyte maturation and migration, while promoting radial glial cell migration, may have lead to improved functional recovery. Our findings suggest ketamine as a potential candidate for a therapeutic cocktail following SCI.

Chapter 6.0 - References

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