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**An investigation of the
mechanisms underpinning the
effect of anti-inflammatory drugs
on neural stem cells**

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Thesis submitted for Doctor of Philosophy in

Cell and Tissue Engineering

October 2017

Keele University

SUBMISSION OF THESIS FOR A RESEARCH DEGREE

Degree for which thesis being submitted: Doctor of Philosophy

Title of thesis: **An investigation of the mechanisms underpinning the effect of anti-inflammatory drugs on neural stem cells**

This thesis contains confidential information and is subject to the protocol set down for the submission and examination of such a thesis? NO

Date of submission	18/05/2017
Original registration date	06/01/2014
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Abstract

Anti-inflammatory drugs such as corticosteroids (CSs) and minocycline (MINO) are widely used in the treatment of a range of clinical conditions and to suppress graft rejection in stem cell transplantation therapy. However, such treatment is associated with adverse effects on brain development. The effects of anti-inflammatory drug on neural stem cells (NSCs) are largely unknown and the molecular mechanisms underlying these effects are poorly documented. The focus of this project is to systematically investigate the effects of different anti-inflammatory drug at different concentrations on the fate of NSCs using two different *in vitro* models.

In this thesis, it is shown that all three types of CSs (dexamethasone, prednisone and methylprednisolone) affect NSCs propagated in monolayers and neurospheres. Comparison of the monolayer and neurosphere growth formats for NSCs following CS treatment revealed that CS decreased NSCs proliferation and neuronal differentiation while accelerated the maturation of oligodendrocytes without concomitant effects on cell viability and apoptosis. The findings suggest that the difference in the physical format of NSCs does not impact on CS influences on these cells with similar results obtained for both culture systems.

Further, label-free quantitative proteomics was used to study methylprednisolone effects on NSCs at the cellular and molecular levels in monolayer cultures. Proteomics and bioinformatics analyses revealed that methylprednisolone induced downregulation of growth associated protein 43 and matrix metalloproteinase 16 with upregulation of the cytochrome P450 family 51 subfamily A member 1. These

findings support the hypothesis that neurological deficits associated with CS treatment mediated via effects on NSCs, and highlight putative target mechanisms underpinning such effects.

Finally, the organotypic spinal cord slice model was used to investigate the efficacy of MINO as a combinatorial therapy with transplanted NSCs. The data from neurosphere culture showed that MINO had no direct effect on key regenerative properties of NSCs such as proliferation and differentiation. While, the findings from organotypic spinal cord slice culture showed the astrogliosis and activated microglia were reduced and the outgrowth of the nerve fibres was increased following a combinatorial therapy. This study demonstrates the utility of the organotypic spinal cord slice model to test the efficacy of MINO as a combinatorial therapy with transplanted NSCs.

Abbreviations

11β-HSD2	11 beta–hydroxysteroid dehydrogenase types 2
Ambic	Ammonium Bicarbonate
ANOVA	Analysis of variance
ASCI	Acute spinal cord injury
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BETA	Betamethasone
BSA	Bovine serum albumin
Corticosterone	CORT
CNS	Central nervous system
CSs	Corticosteroids
Cy-3	Cyanine-3
CYP 51 A1	Cytochrome p450 51 A1
DAPI	4', 6-diamidino-2-phenylindole
DDA	Data Dependent Analysis
DEX	Dexamethasone
dH₂O	Deionised water
DIA	Data independent analysis
DiD	1,1'Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt
DIV	Days in vitro
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EBSS	Earle's balanced salt solution
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
ETOH	Ethanol
FBS	Foetal bovine serum

FGF-2	Basic fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAP-43	Growth associated protein 43
GCs	Glucocorticoids
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GRE	Glucocorticoid response elements
GRs	Glucocorticoid receptors
GSK-3β	Glycogen synthase kinase 3
HPA	Hypothalamic- pituitary- adrenal axis
HPLC	High performance liquid chromatography
Hsp90	Heat shock protein 90
IPA	Ingenuity Pathway Analysis
iPSC	Induced pluripotent stem cell
LC-MS^E DIA	Liquid chromatography mass spectrometry “everything”, data independent analysis
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
MBP	Myelin basic protein
MCs	Mineralocorticoids
MCT	Multiple comparison tests
MEM	Minimum essential medium
MINO	Minocycline
MMP-16	Matrix metalloproteinase 16
MMPs	Matrix metalloproteinases
MPRED	Methylprednisolone
MRM	Multiple reaction monitoring
MRs	Mineralocorticoids receptors
MS	Mass spectrometry
MSCs	Mesenchymal stem cells
NDS	Normal donkey serum
NF-κB	Nuclear factor- κ B
Non-ESCs	Non-embryonic stem cells
NPCs	Neural progenitor cells

NPSCs	Neural progenitor/stem cells
NSCs	Neural stem cells
OCT	Optimum cutting temperature
OECs	Olfactory ensheathing cells
OPCs	Oligodendrocyte progenitor cells
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3K/Akt	Phosphatidylinositol 3-kinase/Akt
PRED	Prednisone
RDS	Respiratory distress syndrome
RT	Room temperature
SCI	Spinal cord injury
SGZ	Subgranular zone
SVZ	Subventricular zone
S.E.M	Standard error of the mean
TBI	Traumatic brain injury
TUJ-1	β -tubulin
WHO	World Health Organization

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Acknowledgements

“Forget the good you do, but never forget the good that was done to you”

First and foremost, I would like to express my sincere gratitude to my supervisors Professor Divya Chari for her expertise, support and motivation. Her training and guidance helped me in all the time of research and writing of this thesis. What I learn from Divya are not only many techniques, but also the attitude to research work and I hope to take the skills she has taught me into my future laboratory. I would also like to thank my co-supervisor Dr. Sarah Hart for her helpful suggestions and guidance for the proteomics study and writing for this thesis. In this regard, I also want to give my great thanks to my advisor Dr. Mark Pickard for his fruitful discussion and critical reading of the manuscript.

I owe a huge thanks to my country, Iraq and particularly to the Iraqi Ministry of Higher Education and Scientific Research for giving me this opportunity to study my PhD and providing me with the support and help throughout my PhD duration.

I owe my gratitude to Dr. Stuart Jenkins for his excellent guidance on experimental technique and data analysis, to him I owe too much. I also thank Dr. Alinda Fernandes for teaching me cell culture techniques; her knowledge helped me throughout my time in the laboratory.

I also wish to thank Dr. Christopher Adams for the time he spent on helping me in all sorts of matters in the laboratory and providing valuable suggestions and advices to my experiments. I wish to extend my warmest thanks to Dr. Jacqueline

Tickle for her excellent assistance in the laboratory and useful guidance in terms of both personal and professional and she always answer my questions and made time for me. My sincerest thanks go to Dr. Alan Weightman for expert assistance, both technically and scientifically with regards to the organotypic spinal cord slice model.

In addition, I would like to thank Dr. Joanne Connolly and Dr. Richard Emes for their help with proteomics and bioinformatics analyses, respectively. Also, I am very thankful to Dr. Mirna Maarabouni for providing me with access to equipment, which without; my work would not have been possible.

Also, I express my gratitude to the following people for their friendship and scientific help during the project work: Zainab Al-Mnaseer, Arwa Al-Shakli, Farhana Chowdhury, Sama Al-Shaheeb, Wafaa Al-Jasim, Suad Ibrahim, Sile Griffin, Daniel Weinberg, Luke Sterio, and Aina Mogas.

I am grateful to all staff at Keele University School of Life Sciences and a special thanks to Jayne Bromley, Ian Wright, Nigel Bowers, Ron Knapper and Chris Bain. I am thankful to Stefanie Jones, Dave Bosworth, Zoe Bosworth and Debbie Adams at the animal facility; also, I extend thanks to Phil and Chris, who kept our lab clean.

Big thanks to my long-suffering husband, Wa'il, thank you for all his patience and help throughout this crucial time of my life. Thank you, Wa'il, for always being my biggest supporter. Thank you to my wonderful children, Mostafa, Ali and Hassan, who have kept me motivated and smiling through the highs and the lows of my PhD. Thank you all for bearing me even with my mood swings and being my greatest supporters.

There are no proper words to convey my deep gratitude to my parents, my constant source of inspiration. Particularly, my forever interested, encouraging and always enthusiastic my father he was always keen to know what I was doing and how I was proceeding. Massive thanks to my sisters and my brothers for their long distance support and unconditional love. Words cannot explain how grateful I am to my family. Finally, my especially warm gratitude and respect to Celia Jarrett, who has surrounded me with love and hugs. I am fortunate to have the support and friendship from you Celia, thank you for everything.

“Do not let your difficulties fill you with anxiety. After all, it is only in the darkest nights that the stars shine more brilliantly”

Chapter 1

General Introduction

1.1 Central nervous system: injury and disease

The nervous system is vulnerable to various injuries and diseases. It can be damaged by mechanical and chemical causes or affected by inherited genetic abnormalities. Neurotrauma [including traumatic brain injury (TBI) and spinal cord injury (SCI)] is one of the most main causes of central nervous system (CNS) damage (Han et al., 2014). Further, neuroinflammatory diseases such as multiple sclerosis, neurovascular diseases such as stroke and neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease are other important causes of CNS damage (Burda and Sofroniew, 2014). The World Health Organization (WHO) reported that CNS related injuries and disorders such as stroke, neurodegenerative disorders and depression are all in the top 20 causes of death globally (World Health Organization, 2013).

Neurological disorders affect millions of people worldwide, for instance, about 2.5 million people were suffering from SCI and around more than 130,000 new injuries every year globally (Thuret et al., 2006). Further, increasing life expectancies are predicted to increase the prevalence of neurological disorders in both developed and developing countries (World Health Organization, 2006). Neurological injury and disease have a negative impact on the lives of patients and their carers through stress and the requirement for provision of long-term care (Tsukamoto et al., 2013). In addition, there is a great impact on the economy for example, in the USA, the annual cost of SCI is estimated around \$7.7 billion and around £500 million in the UK (Adams and Cavanagh, 2004).

The CNS consists of different types of cells; **neurons**, the main functional and cellular component of the CNS, are specialised cells that transmit electrical signals between different parts of the body. The second type of cells is **glial cells**, which support the neurons. Glial cells are divided into three types: **astrocytes**, **oligodendrocytes** and **microglial** cells, each type has a special function and different structure (Verkhratsky and Butt, 2007). Astrocytes play a main role in providing structural and metabolic support to neurons, in addition to their role in synapse formation and maintenance (BrosiusLutz and Barres, 2014; Verkhratsky and Butt, 2007). Oligodendrocytes are responsible for myelin production in CNS (Fonseca et al., 2013; Salzer, 2013). Myelin is the fatty protective sheath around nerve fibres, which plays an important role in increasing the conduction velocity of impulses along the axon (White and Krämer-Albers, 2014). Microglial cells are the immune cells of the CNS, and have highly similar functions to macrophages (Weightman et al., 2016). They play an important role in neuroinflammatory and neurodegenerative diseases and neuroprotective and anti-neuroinflammatory processes (Banati, 2003; Burda and Sofroniew, 2014). Another important CNS subtype is **neural stem cells (NSCs)** which will be described in greater detail in **section 1.3**.

CNS injury and disease affects all CNS cell types and can result in progressive loss of neurons and glia, therefore the structure and function of the brain or the spinal cord is permanently affected. In general, the injury results in three phases (BrosiusLutz and Barres, 2014; Burda and Sofroniew, 2014):

(1) Cell death and inflammation: The response to CNS injury in this phase occurs immediately from about a number of seconds to hours. These events

include cell death (for neurons and oligodendrocytes) and blood brain barrier (BBB) damage.

(2) Cell proliferation and tissue replacement: This response to CNS injury occurs from about two to ten days after the injury. During this phase, the inflammatory cells such as microglia and different types of glia cells such as astrocytes respond by proliferation and migration into the sites of injury.

(3) Tissue remodelling: This phase is characterised by tissue remodelling which includes the formation of astrocyte scar and extracellular matrix (ECM). These events are in response to tissue damage at the end of the first week after the injury.

The capacity of the nervous system to repair or restore function after injury and disease is limited, and the regeneration of neuronal axon in the adults CNS is limited but can occur in both embryonic and early postnatal nervous system (Ming and Song, 2005).

Various treatment methods have been studied in neurological laboratories to improve the axonal regeneration process. Despite the extensive efforts focused on developing therapies which can restore function to the neurodegenerative diseases and CNS injuries, no treatment is currently effective and adequate to tackle this problem (Cao et al., 2002). The treatment of CNS disorders is one of the most challenging areas of modern medicine (Tsukamoto et al., 2013). Cell transplantation and drug therapy using anti-inflammatory and immunosuppressive drugs are major therapeutic approaches used in neurological diseases treatment. In this chapter, the role of cell therapy in neurological repair will be discussed in

sections 1.2 and 1.3, and then the role of anti-inflammatory and immunosuppressive drugs will be discussed in greater detail in **section 1.4**.

1.2 Cell therapy as potential therapeutic strategies in neurology

Cell transplantation is one of the most important approaches that are widely used to repair damaged tissue and restore neurological function after CNS injury and disease. Cell transplantation can enhance axon growth by two ways: **(1)** replacement of damaged neurons and **(2)** release of molecules such as neurotrophic factors to improve the regeneration process (Vishwakarma et al., 2014). There are many different types of cells that have been used in cell therapy due to their potential role in the neurological treatment, such as Schwann cells, olfactory ensheathing cells (OECs) and stem cells (Ke et al., 2006).

Schwann cells: They represent an attractive source for spinal cord repair in animal experimental studies. These cells have the ability to de-differentiate, migrate, proliferate, express growth promoting factors, and myelinate regenerating axons after transplantation into the injury site. Importantly, Schwann cells play important role in the endogenous repair of peripheral nerves, and can migrate into the injury site (Fortun et al., 2009; Kanno et al., 2015). Also, when Schwann cells are transplanted into the lesioned spinal cord they are able to myelinate both regenerating and intact central axons (Franklin et al., 1996).

OECs: A newer method is the transplantation of OECs into areas of SCI to enhance functional recovery. OECs are produced from autologous mucosal biopsies and can be propagated in culture. After transplantation into the damaged tissue, these cells are able to migrate over long distances in the sites of injury and

enhance functional recovery such as myelination and sprouting of axons (Ibrahim et al., 2006; Ramón-Cueto et al., 2000).

Uncommitted stem cells: The transplantation of stem cells is another alternative method that used to improve the regeneration process (Table 1.1). Stem cells are defined as undifferentiated and self-renewing cells which have the ability to proliferate and differentiate into specialised types of cells (Fonseca et al., 2013).

In the 1960s, McCulloch and Till discovered stem cells: they injected cells from bone marrow into irradiated mice and they found that the transplanted cells could migrate to spleen and form nodules. According to the linear relationship between the number of transplanted cells and the number of colonies, they concluded that each nodule generated from a single transplanted bone marrow cell (Bajada et al., 2008; Sharkis, 2005).

Several types of stem cells are commonly used in transplantation therapies to repair damaged tissue after neurological injury and disease. According to the potential differentiation of the stem cell, they can be classified into three types: totipotent, pluripotent and multipotent (Filippis & Binda 2012; Lodi et al. 2011). They can be further divided into two types: embryonic stem cells (ESCs) and non-embryonic stem cells (Non-ESCs) (Bajada et al., 2008).

ESCs: They are pluripotent and differentiated cells which can be obtained from aborted fetuses. These cells have the ability to generate any undifferentiated cell in the body. Although these cells have a great potential in neurological repair, their use is still limited due to their ability to form teratoma after transplantation (Ali et al., 2014; Bajada et al., 2008).

Induced pluripotent stem cell (iPSC): They are generated by reprogramming somatic stem cells. The pluripotency of iPSC enables them to differentiate into cells of all three germ layers, which makes them extremely valuable tool for the potential design of cell therapy protocols (Yamanaka, 2007). These cells are identical to ESCs in gene expression pattern and the ability to differentiate. Therefore, the using iPSC in regenerative medicine has prevented lots of problem such as immune rejection in autologous transplantations, ethical problems and tumour formation (Fu et al., 2015; Yamanaka, 2012).

Non-ESCs: They are multipotent and differentiated cells which can be derived from several sources including amniotic fluid, umbilical cord tissue and bone marrow (Bajada et al., 2008). For instance, mesenchymal stem cells (MSCs) are multipotent cells which can be derived from different sources such as blood and bone marrow. MSCs have the ability to differentiate into another type of cells, for example, fibroblasts, osteoblasts, chondrocytes, myoblasts, and adipocytes (Guilak et al., 2009; Halleux et al., 2001). Most importantly, the transplantation of MSCs in the brain show great promise in neurological disease such as stroke, because they have the ability to generate neural cells and secrete several growth factors (Liu et al., 2009).

Table 1.1 Clinical trials of stem cell in neurological treatment				
Trial Sponsor (Location)	Disease Target	Cell Therapy	No. Patients	Phase
City of Hope (CA, USA)	Recurrent high grade gliomas	E. Coli CD-expressing neural stem cells	24	phase I
	Recurrent high grade gliomas	Carboxylesterase-expressing neural stem cells	53	phase I
Neuralstem Inc. (MD, USA)	Amyotrophic lateral sclerosis	Foetal-derived neural stem cells	18	phase I
	Amyotrophic lateral sclerosis	Foetal-derived neural stem cells	18	phase II
	Chronic spinal cord injury	Foetal-derived neural stem cells	4	phase I
ReNeuron Ltd. (UK)	Stroke	Human neural stem cells	12	phase I
	Stroke	Human neural stem cells	41	phase II
	Lower limb ischemia	Human neural stem cells	9	phase I
Stem Cells Inc. (CA, USA)	Neuronal ceroid lipofuscinosis	Human CNS stem cells	6	phase I
	Cervical spinal cord injury	Human CNS stem cells	50	phase II
	Macular degeneration	Human CNS stem cells	15	phase I/II
	Thoracic spinal cord injury	Human CNS stem cells	12	phase I/II
	Pelizaeus-Merzbacher disease	Human CNS stem cells	4	phase I
TRANSEURO (UK)	Parkinson's disease	Foetal-derived dopaminergic cells	40	phase I
Wroclaw Medical University (Poland)	Spinal cord injury	Olfactory ensheathing cells, autologous	10	phase I

Table adapted from (Trounson and McDonald, 2015).

1.3 What are NSCs?

NSCs are one of the most important cells in the CNS because of their ability to self-renew and proliferate (De Filippis and Binda, 2012; Lee et al., 2015). After some years of debate, it is now accepted that the subventricular zone (SVZ) and subgranular zone (SGZ) regions are the main sites of genesis of NSCs in the adult mammalian brain (Hao et al., 2014; Qin, 2004). The SVZ lies adjacent to the lateral ventricles and provides the olfactory bulb with neurons, whereas the SGZ lies within the dentate gyrus of the hippocampus and produces new neurons in the granular layer of the dentate gyrus. These neurons are involved in networks that modulate mood and short term learning and memory (Santos et al., 2012). Also, the periventricular tissue region of the spinal cord is another source for NSCs (Mothe and Tator, 2013). NSCs play an important role in endogenous repair and transplantation to treat different CNS pathologies because their ability to generate region specific neurons, or differentiate primarily into oligodendrocytes when taken from the spinal cord (Mothe and Tator, 2013).

1.3.1 Biological properties of NSCs

The fundamental features of NSCs include: **(1) self-renewal**, because of their ability to proliferate and generate new cells and **(2) multipotency**, because of their ability to differentiate into a number of different neural cell types such as astrocytes, neurons and oligodendrocytes (Casarosa et al., 2014; Dai and Sottile, 2008; Fonseca et al., 2013) (Figure 1.1). NSCs properties have been studied *in vitro* by growing them as a monolayer in medium containing growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (FGF-2).

Interestingly, NSCs can also be grown *in vitro* as spherical aggregates, called neurospheres. Further, these types of cells can be differentiated into neurons, astrocytes and oligodendrocytes after the removal of growth factors (Louis et al., 2013). Both culture systems are described in detail in **section 1.3.3**.

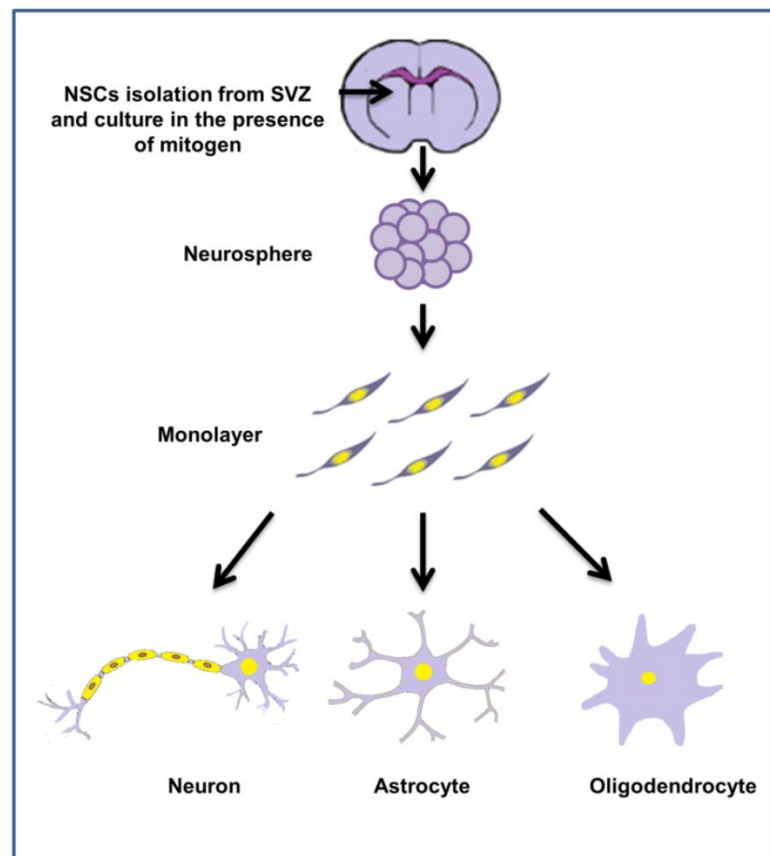


Figure 1.1 The fundamental properties of NSCs. *NSCs are isolated from SVZ and then cultured in neurosphere medium in presence of mitogens to form non-adherent spherical clusters of cells called neurospheres that can be passaged several times (self-renewal). These are then dissociated mechanically and enzymatically to a single cell to form secondary neurospheres. The NSCs differentiate in the absence of mitogens and in the presence of serum differentiate to give rise to three different types of neural cell (multipotency).*

1.3.2 Terminology used in the classification of uncommitted cells

There are various terms used by neurobiologists to describe undifferentiated cells of the CNS, such as stem cell, precursor cell and progenitor cell. This leads to confusion and misunderstanding in the field of NSCs research. To clarify these terms, this section refer to the most common terms used in the NSCs field (Lee et al., 2015).

1) Neural progenitor cells (NPCs): These have the ability to proliferate and differentiate into more than one type of cell. The most important difference between NPCs and the stem cell is that their ability to proliferate is limited and they do not exhibit self-renewal (unlike stem cells). NPCs can be unipotent, bipotent or multipotent.

2) NSCs: These cells are self-renewing, multipotent cells which have the ability to proliferate without limit, to give rise to multiple types of neural cell.

3) Neural precursor cells: This term refers to the mixed population of NPCs and neural stem cells.

1.3.3 Methods used to isolate and propagate NSCs under laboratory conditions

Neurobiological research is focusing on the problems of isolation, maintenance, propagation and differentiation of NSCs in both culture and after transplantation into the injury sites (Poltavtseva et al., 2002). There are different methods designed to isolate and propagate NSCs. NSCs are isolated from CNS of the embryonic, postnatal and adult in many species such as mice, rats and humans. There are two basic system commonly used to expand NSCs: **neurospheres** (floating cell aggregates, 3-dimensional cultures) and **monolayers** (adherent, 2-dimensional cultures) (Louis et al., 2013).

1) The Neurosphere culture system

Here, NSCs are cultured in the presence of growth factors (e.g. EGF and FGF-2) and hormonal supplement (e.g. N2 and B27) to generate “floating” large aggregations of cells called “neurospheres” which are produced from a single cell. Following 24 h, the single cells begins to proliferate and form clusters. The clusters grow in size and become bigger. Following seven days, the clusters called neurosphere which typically measures 100-200 μm in diameter and consists of around 10,000 cells (Louis et al., 2013). At this stage the neurosphere becomes ready to be passaged by dissociation in order to form new neurospheres, under the same conditions (Louis et al., 2013) (Figure 1.2).

2) The Monolayer culture system

NSCs may be cultured as an adherent monolayer by mechanical and enzymatic dissociation of the neurospheres to give single cells. These are then cultured as a monolayer in presence of growth factors (i.e EGF and FGF-2) and by using poly-L-ornithine and laminin for coating the coverslips. The NSCs from neurosphere and monolayer cultures can be differentiated to other types of neural cell by removing growth factors and adding serum to the medium (Louis et al., 2013).

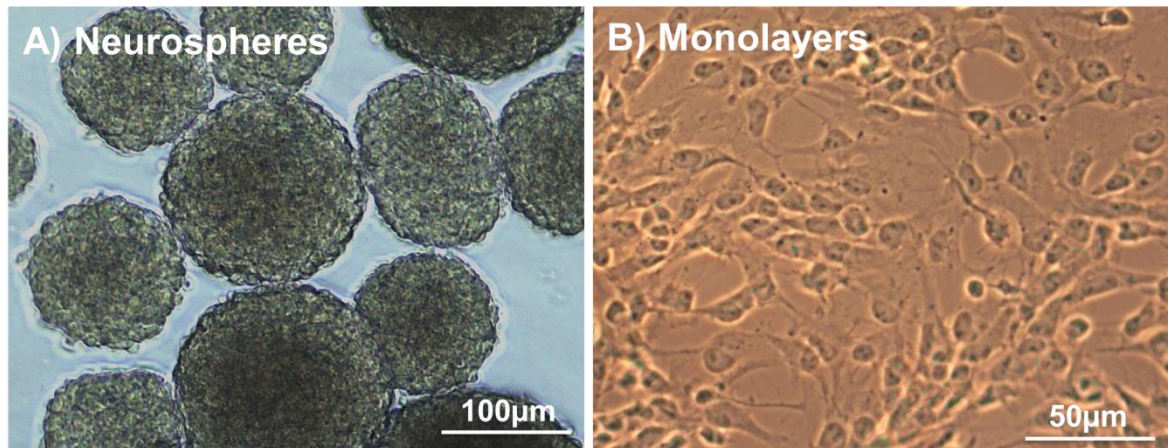


Figure 1.2 The culture systems of NSCs. (A) Phase contrast image showing the NSCs can be propagated as neurospheres. (B) Phase contrast image showing monolayer of NSCs which adopt a typical bipolar morphology.

1.3.4 NSCs as a promising therapeutic approach in neurology

NSCs have a very important role in CNS during brain development due to their ability to generate the main phenotypes of the nervous system, i.e. neurons, astrocytes and oligodendrocytes. Experimental studies demonstrate that both exogenous and endogenous NSCs can be used to repair neurological injuries/diseases. NSCs can reach the site of injury in the adults CNS and successfully differentiate to give rise to neurons and glia (Imitola et al., 2004; Martino and Pluchino, 2006).

The progress in studying stem cell biology has opened a new avenue to therapeutic strategies to replace lost and damaged neural cells in neurological disorders and traumatic nervous system lesion such as stroke, multiple sclerosis, SCI and Parkinson's disease (Bellenchi et al., 2013; Björklund et al., 2003; Lindvall and Kokaia, 2006). There are two ways to integrate the functional new neurons after neurological injury and disease: **(1)** by the induction of endogenous stem cells (Nakatomi et al., 2002) or **(2)** by the transplantation of stem cells to repair damaged cells (Björklund et al., 2003; Dai and Sottile, 2008; Dietrich and Kempermann, 2003; Saha et al., 2012) (Figure 1.3). The role of endogenous NSCs and exogenous NSCs transplantation in neurological repair will be discussed in more detail in **sections 1.3.4.1 and 1.3.4.2**, respectively.

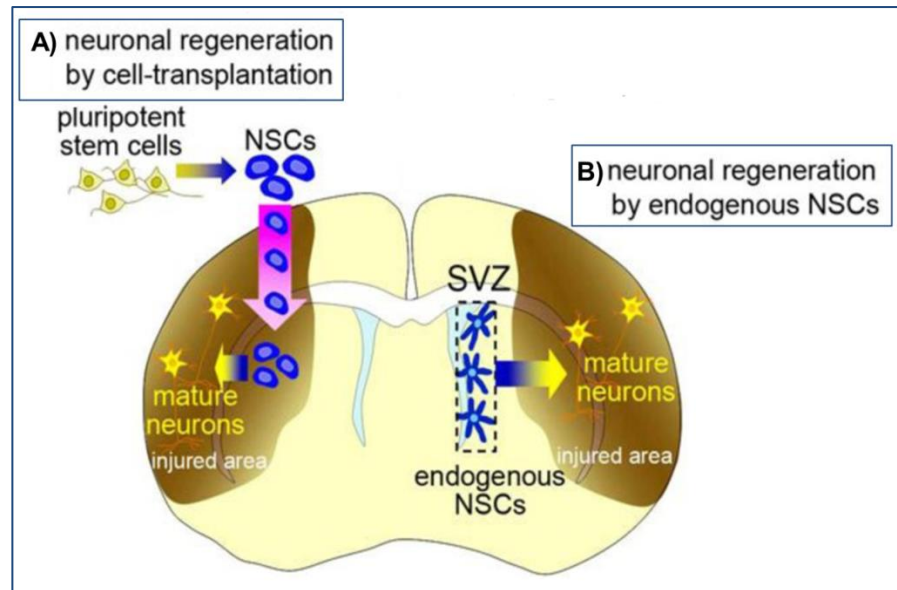


Figure 1.3 Exogenous and endogenous NSCs strategies in brain repair. (A) Schematic drawing representing the transplantation of exogenous NSCs into the damaged brain which can generate neurons to replace lost and damaged neural cells. (B) Representing the endogenous NSCs found in the SVZ which can migrate into the site of injury and differentiate into neurons. Figure adapted from (Kaneko et al., 2011).

1.3.4.1 The role of NSCs in endogenous repair

After the discovery of neurogenesis in the adult CNS, extensive research has demonstrated that NSCs and progenitor cells can be used to repair neurodegenerative disease because of their ability to produce neuronal and glial cells (Picard-Riera et al., 2004; Skardelly et al., 2013; Wu and Wang, 2012). Neurogenesis persists throughout life time in the adult mammalian brain especially, in the SVZ and SGZ and that is due to the persistence of the proliferation and differentiation of the NSCs (Bellenchi et al., 2013).

Many studies are focusing on the role of NSCs in endogenous repair after neurological injuries. Endogenous NSCs have a potential role in treatment of several neurological conditions because they can migrate into the site of injury and produce neurons and glial cells (Bellenchi et al., 2013; Lindvall and Kokaia, 2010). Efforts have been made to use endogenous NSCs in neurological disease treatment such as SCI, Parkinson's disease and stroke (Arvidsson et al., 2002; Dietrich and Kempermann, 2003; Ke et al., 2006; Kokaia et al., 2006). It was observed that the proliferation activity of endogenous NSCs increased and new neurons appeared in the site of injury to replace the damaged neural cells in several neurological conditions (Kaneko et al., 2011). For instance, Ke and colleagues demonstrated that the proliferation of endogenous NPCs in the mouse SCI model increased after SCI and these cells can differentiate into neurons but not astrocytes or oligodendrocytes (Ke et al., 2006).

Importantly, it has been demonstrated that the endogenous NSCs are involved in myelin repair, as they migrate toward the site of injury and differentiate into glial cells (Dietrich and Kempermann, 2003). Also, the experimental models of stroke in

the adult rat demonstrated that the proliferation of endogenous precursor cells in the SVZ increased in order to generate new neurons. The new neurons migrated from the SVZ into the damaged area and replaced the neurons destroyed by induced stroke (Arvidsson et al., 2002; Lagace, 2012).

The neuroregenerative process using endogenous NSCs avoids several problems such as ethical issues, immunogenicity and risk of tumour formation (Kaneko et al., 2011). However, a number of studies have suggested several limitations that are associated with the use of endogenous NSCs in neurological repair. For example, it was found that endogenous NSCs have limited ability to replace lost neural cells and that they become more gliogenic than neurogenic following insult. There is evidence that only 0.2% of the dead neurons are replaced due to death of most of the new migrating neurons before differentiating into functional neurons. Furthermore, several studies suggest that these cells cannot differentiate to specific types of neurons which is a potential problem in treatment of specific diseases such as Parkinson's disease, where dopaminergic neurons are lost (Kaneko et al., 2011).

1.3.4.2 The role of NSCs in transplantation

The transplantation of NSCs offers a promising strategy to treat several neurological conditions by **1)** replacing lost neural cells (i.e. neurons and oligodendrocytes) **2)** secreting a vast array of proteins such as cytokines, growth factors, or neurotrophins that could support the survival of remaining cells and promote neuronal repair (Drago et al., 2013).

Several cell transplantation studies have been attempted; some of them have been successful and others have not. Rodent experimental studies have shown the potential role of NSCs transplantation in treatment a range of neurological conditions. For example, multiple sclerosis is one of the most important examples of neurological disease characterised by loss of oligodendrocytes. It has been demonstrated that adult mouse NSCs derived from the periventricular region forebrain transplanted into a mouse model of multiple sclerosis are involved in myelin repair, as they differentiated into oligodendrocyte progenitors (Pluchino et al., 2003). Further, Yandava *et al.* transplanted NSCs into the *shiverer* mouse brain. The *shiverer* mouse is an autosomal recessive mutant characterised by the deletion of a large portion in the myelin basic protein (MBP) gene resulting in extensive CNS dysmyelination. They observed that the NSCs integrated into the SVZ after 24 h, and migrated after 1-2 weeks and then differentiated. Their results suggested that NSCs differentiated into oligodendrocytes and that is very useful in the treatment of several types of neurological diseases involving white matter degeneration (Yandava et al., 1999). In their study, Fricker and colleagues successfully transplanted NPCs into the neurogenic region in the adult rat brains. They obtained these cells from the embryonic human forebrain and then cultured them as a neurospheres (Fricker et al., 1999). It was shown that NSCs play a

potential role in Parkinson's disease treatment due to their ability to generate dopaminergic neurons which are affected by Parkinson's disease; this disease causes damage to the nerve cells that generate dopamine (Kitada and Dezawa, 2012). In addition, the transplantation of NSCs has been shown to promote locomotor recovery in SCI which characterised by loss of motor function. It was found that human NSCs derived from foetal brain tissue have the ability to generate into new neurons and oligodendrocytes, which promote locomotor recovery following transplantation into SCI mouse model (Cummings et al., 2005).

A large number of studies have used NSCs in preclinical studies. Several attempts are already being made to translate these studies into the clinical setting (Table 1.2). There are several clinical trials involving NSCs derived from different cell lines and other studies used iPSC, mesenchymal stem cell-derived NSCs, human spinal cord-derived NSCs (Barreau and Lépinoux-chambaud, 2016). Phase I clinical study employing foetal NSCs have been performed. Children with advanced stage Batten's disease tolerated high doses of foetal NSCs in several sites in the brain. They were observed the transplanted cells enhance replacement enzyme and provide renewal for cell replacement (Trounson et al., 2011).

The company ReNeuron in the UK also used foetal NSCs for treatment of stroke patients. NSCs were transplanted in patients for 6 to 24 months after stroke using a straight-forward neurosurgical implantation into the brain. The NSCs promote revascularization by expressing several trophic and pro-angiogenic factors which may improve tissue repair after stroke (Trounson et al., 2011). In 2011, clinical study in Switzerland used NSCs for chronic thoracic spinal cord injury. They observed the NSCs are injected into the spinal cord and migrate to the site of injury and generate neurons and oligodendrocytes (Trounson et al., 2011).

Furthermore, the company Neuralstem used foetal NSC for the treatment of amyotrophic lateral sclerosis. Patients received NSCs in multiple (five to ten) grey matter sites of the lumbar region of the spinal cord. The company NeuroGeneration Inc. used autologous NSCs which derived from patient brain biopsies to treat Parkinson's disease. These NSCs are cultured *in vitro* for several months and the expanded NSCs differentiated into neurons, astrocytes and oligodendrocytes. Patients showed some motor improvement and promoted dopamine uptake (Trounson et al., 2011).

However, several problems make cell therapy a distant goal. One of the unresolved issues is that the transplanted cells have a tendency to differentiate into astrocytes more than neurons and oligodendrocytes. For example, in a rat SCI model, most of the transplanted NSCs differentiated into astrocytes, not neurons and oligodendrocytes (Wu et al., 2001). Also, Ogawa *et al* found that about 5.9% of the transplanted cells differentiated into neurons, 4.4% into oligodendrocytes, and 32.6% into astrocytes at 5 weeks after transplantation (Ogawa et al., 2002). Increased cell death of NSCs after transplantation, and rejection of neural grafts are other problems of NSCs transplantation. Furthermore, cells source is another important factor that affects the transplantation. As mention in section 1.2, there are several types of cells derived from different sources, each type has advantages and disadvantages features that contribute to successful transplantation. This includes safety issue (e.g., absence of tumour formation) and immunogenicity (Pruszek, 2014). Therefore, the use of anti-inflammatory and immunosuppressive drugs is necessary to enhance the survival of transplanted NSCs and reduce the rejection of grafted cells, as discussed in next section.

Table 1.2 NSCs clinical trials in neurological treatment	
StemCells Inc., CA	HuCNS-SC® (foetal derived human NSCs)
Phase I – completed	Batten’s Disease (NCL), USA
Phase I	Discontinued for lack of enrolment
Phase I	Pelizaeus-Merzbacher Disease (PMD), USA
Phase I/II	Chronic Spinal Cord Injury, Switzerland
NeuroGeneration, CA	Autologous NSC-derived Neurons
Phase I–completed	Advanced Parkinson’s Disease, USA
Phase II – clinical hold	
Neuralstem Inc., MD	Foetal derived human spinal cord
Phase I	Amyotrophic lateral sclerosis, USA
ReNeuron, UK	ReN001 Immortalized human NSCs
Phase I	Stroke, UK
Targeted Delivery of Therapeutics	
City of Hope, CA	HB1.F3.CD Immortalized human NSCS
Phase I	Recurrent High Grade Glioma, USA

Table adapted from (Trounson et al., 2011).

1.4 Why do anti-inflammatory and immunosuppressive drugs have to be used in neurology?

Inflammation contributes to the pathogenesis of many neurodegenerative diseases and the presence of this process leads to the continued loss of neurons in CNS (Krause and Müller, 2010). Therefore, the use of anti-inflammatory drugs is necessary to reduce the cumulative effects of inflammation in the brain. For instance, Alzheimer's disease is characterised by the presence of many inflammatory proteins, which led to the hypothesis that brain inflammation is implicated in the pathology of Alzheimer's disease (Krause and Müller, 2010). Anti-inflammatory drugs such as corticosteroids (CSs) and minocycline (MINO) are widely used to reduce inflammation to avoid any further damage, in addition to their use following transplantation to limit cell rejection (Becker, 2013; Coutinho and Chapman, 2011).

The experimental strategies for CNS injury and disease consist primarily of allogeneic transplantations. Although the CNS is considered as an immunologically privileged site, the immune rejection of transplanted cells and the use of immunosuppressive drugs are a major constraint accompanied with allogeneic transplantations and may compromise the effectiveness of transplantation. Therefore, the autologous transplantation is considered as the paradigm of choice for cellular therapy (Barker and Widner, 2004; Schwab et al., 2006).

The anti-inflammatory drug, CS is clinically used for treatment of adult and paediatric conditions. Importantly, the synthetic CSs such as dexamethasone (DEX), prednisone (PRED) and methylprednisolone (MPRED) are widely used in

the treatment of neurological disease/injuries such as SCI and also to prevent inflammation and rejection following transplantation (Freed et al., 1992; Giusto et al., 2014; Mazzini et al., 2015). CSs will be discussed in further detail in **section 1.5**.

Also, MINO is another anti-inflammatory drug which has neuroprotective properties and also has the ability to enhance survival of grafted cells (Sakata et al., 2012). Animal and clinical studies overwhelmingly suggest that MINO is more effective in improving functional outcome when compared with CSs therefore its described for many patients with SCI (Wells et al., 2003). This type of anti-inflammatory drug will be discussed in detailed in **section 1.6**.

1.5 What are CSs?

CS is one of the most important groups of anti-inflammatory and immunosuppressive drugs (Rang et al., 2012; Romich, 2010; Vane and Botting, 1987). These groups are small lipid-soluble molecules which can pass through BBB and exert physiological effects on CNS (Riedemann et al., 2010). CSs can be divided into four major types which differ in the number of carbon atoms they contain, type of receptors they bind, and in their biological activities (Corsini et al., 2014).

The basic chemical structure of steroids consists of four rings fused together; three six-membered rings (cyclohexane A, B, C) and one five-membered ring (cyclopentane D). (Figure 1.4 and Figure 1.5) show chemical structures of several synthetic CSs (Corsini et al., 2014; Matura and Goossens, 2000). In general, CSs are classified into four groups based on their chemical structure:

Group A: Including cortisol, methylprednisolone and prednisone.

Group B: including triamcinolone, memetasone and budesonide.

Group C: including betamethasone, dexamethasone and fluocortolone.

Group D: including betamethasone dipropionate and its ester and clobetasone and its ester.

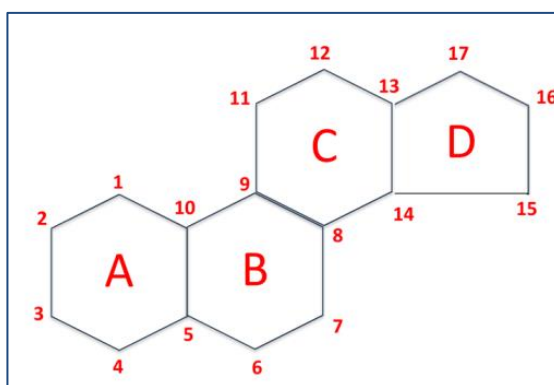
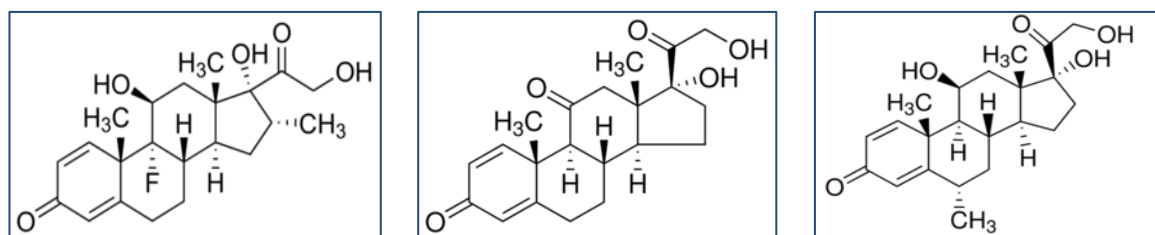


Figure 1.4 The basic chemical structures of steroid hormones. *The basic chemical structure of steroid consists of four fused rings, three of cyclohexane and one of cyclopentane. Figure adapted from (Shahidi, 2001).*



Dexamethasone

Prednisone

Methylprednisolone

Figure 1.5 The chemical structures of selected synthetic CSs to be employed in this studies. *Chemical structures of several synthetic CSs: dexamethasone, prednisone and methylprednisolone (Becker, 2013; Inaba and Pui, 2010).*

Further, CSs can be classified into two main groups dependent on the types of receptor: **glucocorticoids (GCs)** (primarily regulate carbohydrate metabolism) and **mineralocorticoids (MCs)** (primarily regulate electrolyte homeostasis) (Gulino et al., 2009; Riccardi, 2002; Shaikh et al., 2012). The CSs exert their effects via interaction with intracellular receptors. There are two types of receptors: **glucocorticoid receptors (GRs)**, which are highly expressed in various regions of the brain such as the hippocampus and **mineralocorticoids receptors (MRs)**, which are also found in high numbers in the hippocampus. Both types of receptors are members of the nuclear hormone receptor super family of ligand-activated transcription factors (Parker, 1993).

GR and MR show different affinities to CS (De Kloet, 2004; Falkenstein et al., 2000a; Hwang et al., 2006). It was found that the synthetic CSs tend to bind on one receptor or the other, for example, MR binds corticosterone (CORT) and cortisol with a 10 fold higher affinity than GR (Korz and Frey, 2003; Myers and Greenwood-Van Meerveld, 2007), while, GR binds preferentially to the synthetic CSs, DEX and betamethasone (BETA) (Heberden et al., 2013).

1.5.1 By what mechanism do CSs act?

The anti-inflammatory and immunosuppressive effects of GC are mediated by two different mechanisms, genomic (classical) and non-genomic (non-classical) mechanisms (Kleiman and Tuckermann, 2007).

1.5.1.1 The genomic (classical) mechanism of CSs

The GR is found in the cytoplasm in an inactive form as it binds with different proteins such as heat shock protein 90 (hsp90). These proteins dissociate and the GR become active after it binds with its ligand (Sandi, 1998). Briefly, the genomic action of GCs is elicited via binding of GC with GR to form a GR complex within the cytoplasm. This complex migrates to the nucleus and binds directly to specific DNA sequences called glucocorticoid response elements (GRE) in the promoter region of target genes, or interact with other DNA bound transcription factors such as nuclear factor- κ B (NF- κ B) (Ayroldi et al., 2012; Samarasinghe et al., 2012). This process can either induce the expression of anti-inflammatory-proteins (transactivation), or reduce the production of pro-inflammatory proteins (transrepression); this process can take hours or days (Heitzer et al., 2007; Kleiman and Tuckermann, 2007) (Figure 1.6).

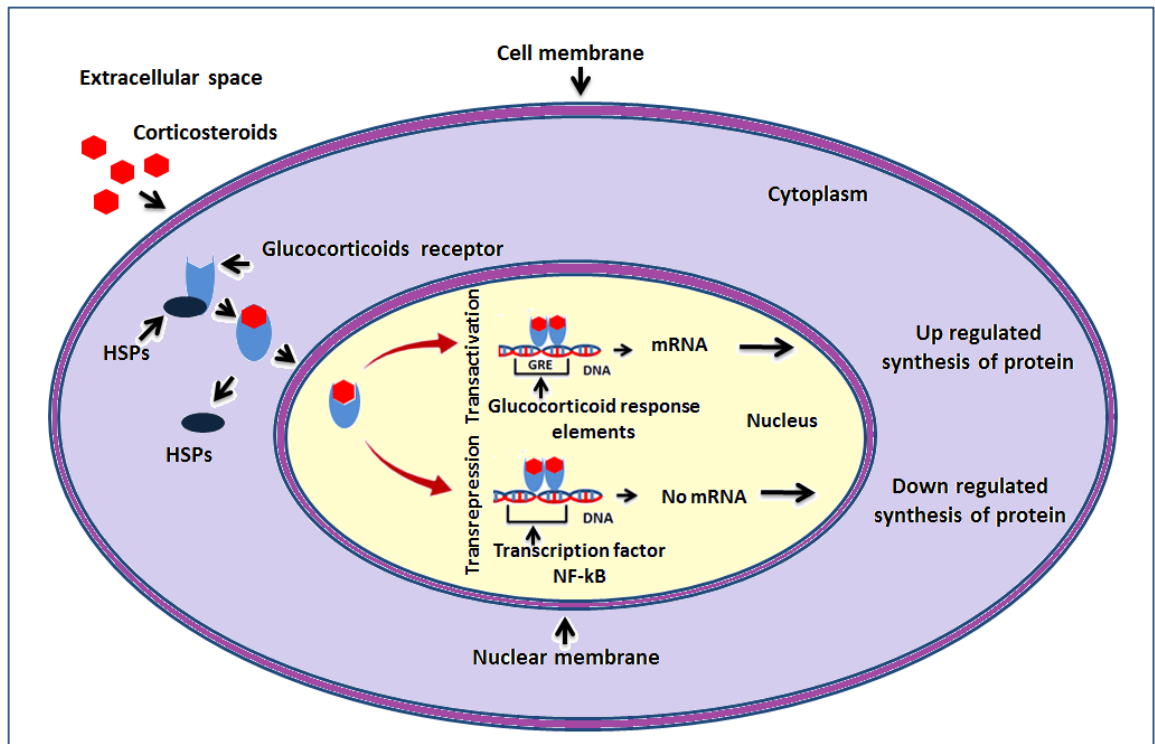


Figure 1.6 The genomic (classical) action of CSs. GCs are fat soluble molecules and can therefore cross plasma membrane of target cells. Once inside, they bind with the appropriate receptors in the cytoplasm of the cell to form complexes of steroid and receptor, these complexes move into the nucleus and bind either to specific DNA binding-sites (GRE) or bind to nuclear factor (NF-κB) lead to activate or repress the synthesis of proteins. Figure adapted from (van der Goes et al., 2014).

1.5.1.2 The non-genomic (non-classical) action of CSs

The mechanism of non-genomic actions of GC is characterised by their rapid immunosuppressive and anti-inflammatory effects (within a seconds or minutes) (Makara and Haller, 2001; Mikics et al., 2004). Many studies show that these rapid effects do not involve interaction with genes but are instead mediated by a different pathway: **1)** GCs can interact with the cytosolic GR; **2)** GCs interact with membrane-bound GRs; **3)** GCs interact with intracellular protein. These receptors then activate the second messengers (cellular signalling pathways) that can lead to these rapid effects (Makara and Haller, 2001; Mikics et al., 2004) (Figure 1.7). However, the physiological relevance of these non-genomic effects of GC remains uncertain (Tasker et al., 2006). Non-genomic actions of steroids are strongly suspected to exist, although the exact mechanisms are unclear (Falkenstein et al., 2000b).

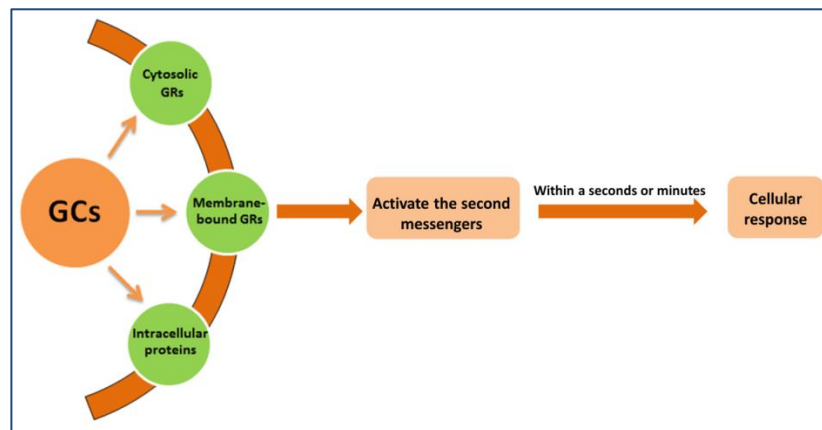


Figure 1.7 The non-genomic (non-classical) action of CSs. *The non-genomic action of GCs is characterised by rapid second messenger activation, which mediates the cellular responses within seconds.*

1.5.2 Anti-inflammatory and immunosuppressive effects of CS

The therapeutic effects of CS as anti-inflammatory and immunosuppressive drugs have been known and employed since the 1950's to treat diverse pathologies such as respiratory distress syndrome (RDS), asthma, allergies and rheumatoid arthritis (Chari, 2014; Rhen and Cidlowski, 2005; Schäcke et al., 2002; Shinwell and Eventov-Friedman, 2009).

CSs may inhibit inflammation by regulating many aspects of the inflammation process. They increase the synthesis of several anti-inflammatory proteins and decrease the synthesis of inflammatory proteins. CSs significantly reduce the survival of inflammatory cells such as eosinophils and T-lymphocytes and increase the phagocytosis of apoptotic leukocytes (Liu et al., 1999).

The survival of eosinophils is dependent on the presence of cytokines such as IL-5. It was found that CS treatment blocks the effects of these cytokines and leads to apoptosis of eosinophils (Liu et al., 1999). Additionally, CSs promote apoptosis of T-lymphocytes (Barnes, 1998; Coutinho and Chapman, 2011). A single dose of CSs can inhibit the proliferation of the lymphocytes within 4 h. It has found that a single dose of PRED induces lymphopenia and this involves both B and T lymphocytes (Fauci and Dale, 1974; Greaves, 1976). However, the molecular mechanism of action of CSs in promoting apoptosis in eosinophils and T-lymphocytes is still unknown (Barnes, 1998; Coutinho and Chapman, 2011).

Moreover, CSs cause depletion of monocytes and reduce the inflammation process by blocking responses to macrophage activation factors (Giles et al., 2001). It was observed that GR are expressed in the inflammatory cells, especially macrophages, which have a key role in inflammatory processes (Liu et al., 1999).

For instance, CS treatment in patients with asthma increased the synthesis of IL-10, an anti-inflammatory cytokine produced by macrophages lead to reduce the synthesis of many pro-inflammatory cytokines, chemokines and inflammatory enzymes (Barnes, 1998; Coutinho and Chapman, 2011; Wang et al., 1995). In addition, CSs attenuate the induction of neutrophils and granulocytes to the site of inflammation, and reduce their activation and proliferation (Barnes, 1998).

1.5.3 The role of signalling pathways in the anti-proliferative effects of CSs

There are several signalling pathways regulate the proliferation and differentiation of NSCs such as β -catenin/TCF, Wnt, and glycogen synthase kinase 3 (GSK-3 β). Wnt is the most important signalling pathways in neural development and plays a major role in the enhancement of the proliferation and differentiation of the NCSs (Ille and Sommer, 2005). In 2012, Moors and colleagues studied the effect of DEX on the proliferation and differentiation of hNPC. They found that the exposure to 1 μ M DEX leads to a reduction in the proliferation and neural differentiation of hNPC. The mechanisms underlying the DEX-induced inhibition of the proliferation and neural differentiation of hNPC is mediated by the inhibition of the canonical Wnt signalling pathway.

Further, Boku *et al.* treated NPCs which were obtained from the dentate gyrus of adult rats with DEX and lithium to study their effects on the proliferation of these cells. They found that DEX decreases the proliferation of these cells without any effect on their differentiation and apoptosis. They then treated the cells with different concentrations of lithium (the most common drug which is used in the

treatment of stress-related disorders). They observed that there are no effects of lithium on the proliferation of NPCs in the absence of DEX whereas in the presence of DEX, lithium recovered the proliferation of cells that were decreased by DEX. Their findings suggested that the reciprocal effects between DEX and lithium on the proliferation of precursor cells are regulated by β -catenin/TCF and GSK-3 β pathways (Boku et al., 2009). In 2012, Garza and colleagues exposed NCSs and NPCs which were isolated from the hippocampus of adult rats to DEX and different concentrations of leptin for 48 h in order to study the effects of leptin in reversing the action of DEX on neurogenesis and to explore the potential mechanisms underlying this process. Their results demonstrated the reciprocal effects between leptin and DEX on the neurogenesis process, DEX reduced the neurogenesis whereas leptin increased this process via activating the GSK3 β / β -catenin signalling pathways (Garza et al., 2012).

Several experimental studies were found that sonic hedgehog signalling pathways is involved in the effects of CSs on proliferation and neural differentiation of NSCs. Hedgehog signalling pathways play an essential role in the regulation of brain development by inducing the proliferation of NPCs of mice. For example, Anacker and colleagues studied the effects of cortisol on the proliferation and neural differentiation *in vitro*, in human hippocampal progenitor cells, and *in vivo*, in prenatal stressed rats. They observed that the low concentration of cortisol (1 nM-100 nM) increased the proliferation and reduced the neurogenesis whereas the high concentration of cortisol (1 μ M-100 μ M) reduced the proliferation and neural differentiation *in vitro* and *in vivo*. Their data identify the molecular signalling pathways which are involved in the effects of cortisol on proliferation and neural differentiation. They found that the low and high concentrations of the cortisol

inhibit the hedgehog signalling which is responsible for the enhancement of neural differentiation (Anacker et al., 2013). Several GCs such as halcinonide, fluticasone propionate, clobetasol propionate, and fluocinonide can activate the hedgehog pathway and stimulate stem cell growth by binding with the Smoothed receptor. In contrast to the other GCs that binds with the GRs such as DEX which inhibit stem cell growth (Wang et al., 2011).

The gap junction protein connexin-43 signalling molecules were found influence proliferation. Samarasinghe and colleagues observed that the exposure of embryonic day 14.5 mouse neurosphere cultures to DEX inhibits proliferation of NPCs by reducing S- phase progression and promoting cell-cycle exit. Also they were observed that a short time of DEX treatment (i.e 1 h) reduced gap junction intercellular communication. DEX effects on gap junction intercellular communication in NPCs are transcription-independent and mediated through plasma membrane GRs. They suggested that non-genomic pathway of DEX mediates via the lipid raft protein caveolin-1 associated GRs (Samarasinghe et al., 2011).

1.5.4 Clinical uses of CS

CSs are used clinically to treat several adult and paediatric pathologies such as asthma, allergy, and dermatological disorders (Ciriaco et al., 2013; Heine and Rowitch, 2009; Lipworth, 1999; Tauber et al., 2006). CSs are also widely used in treatment of acute and chronic inflammation such as rheumatoid arthritis, inflammatory bowel disease and eczema, as a result of their ability to reduce inflammation (Coutinho & Chapman 2011). In addition to their use in the treatment of neurologic trauma which consists of acute SCI (ASCI) and TBI because they

play an important role in the reduction of brain edema (Han et al., 2014). Of particular interest their clinical use during cell transplantation therapy to prevent graft rejection (Freed et al., 1992; Giusto et al., 2014; Mazzini et al., 2015).

DEX is clinically used to treat or prevent respiratory disease such as bronchopulmonary dysplasia in premature infants; it is more potent than the natural steroid cortisol and can cross the placenta (Seckl 2001; Mutsaers & Tofighi 2012). The preterm birth rates range from 6 to 15% of all pregnant women and is responsible for about 75% of all neonatal deaths in North America and Europe (Huang et al., 2001; Matthews et al., 2004, 2002). Premature babies commonly suffer serious complications such as RDS, intraventricular haemorrhage and necrotizing enterocolitis. Chronic lung disease or bronchopulmonary dysplasia is one of the most important causes of mortality and morbidity in preterm babies (Huang, 2011). Babies born before 32 weeks have an increased risk of developing RDS and the mortality rates are approximately (40-60%) (Peter et al. 2008; Huang et al., 2001). Between the end of 1960s and the beginning of 1970s, a series of experimental studies conducted by Liggins suggested adoption of CSs for the treatment of RDS in preterm born babies. They observed that DEX prevents RDS in preterm lambs by enhanced maturation of lung tissue, it was found that the foetal lung contains high concentration of GR compared to other organ such as foetal skin, kidney, heart, liver and gut (Ballard and Ballard, 1972). Following these studies, synthetic CSs have been widely used during antenatal and postnatal periods (Moss & Sloboda 2006).

In 1972, Liggins and Howie performed a landmark randomised controlled trial of antenatal CS treatment for pregnant women at high risk of preterm delivery. They demonstrated that antenatal CS had been successfully used to reduce

intraventricular haemorrhage, RDS, neonatal mortality and necrotizing enterocolitis (Crowley et al., 1990; Newnham and Jobe, 2009; Whitelaw and Thoresen, 2000), and as such, CSs are now standard treatment in women at risk of preterm delivery. A single course of antenatal CS was shown to accelerate lung maturation and thus improve infant survival rates. Antenatal CS therapy improves the clearance of fetal lung fluid and increase the secretion of lung surfactant (Ballard and Ballard, 1972; Mutsaers and Tofighi, 2012). This aids gas exchange, allows alveoli to expand on inspiration and prevents alveolar collapse on expiration (Grier & Halliday 2003; Bonanno & Wapner 2009; Murphy et al. 2001). There has been a trend to increase the number of treatments given to pregnant women at risk of preterm birth (Noguchi et al., 2008; Yeh et al., 2004). However, the CS treatment causes a detrimental influence in both adult and children as discussed in further detail in following **sections 1.5.5 and 1.5.6**.

1.5.5 Adverse effects of CS use - general effects

Although CSs have great potential in clinical therapies and treatment of a large number of conditions, they carry a risk of a number of general adverse effects (Whitelaw and Thoresen, 2000). These effects range from minor to severe. The nature and severity of unwanted effects can depend on many factors such as: the route of delivery, dosage administration type of CSs, and the length of time treatment (Dietrich et al., 2011; Doyle et al., 2005). Neonatal exposure to DEX leads to a reduction in foetal growth which is accompanied with several risks during the lifespan, such as a production of permanent hypertension and hyperglycaemia in rodents and other species of mammals (O'Regan et al., 2001;

Seckl, 2001). In several clinical studies, it was found that prenatal and postnatal of CSs treatment reduced birth weight and caused several adverse effect in adult life such as hypertension, insulin resistance, type 2 diabetes and cardiovascular disease deaths (Seckl, 2004). Based on these data, CS have a number of detrimental effects including, notably, neurological effects which will be discussed in detailed next **section 1.5.6**.

1.5.6 The neurological effects of CS

A wide range of experimental studies strongly suggest that CS treatment can adversely affect the CNS, particularly the hippocampus. These effects include reduced head circumference, neural progenitor cell death and cerebral palsy. Therefore, this section will discuss the neurological effects of CS treatment in both children and adults based on experimental and clinical research evidence.

1.5.6.1 The neurological effects of CS in paediatric life

The maternal CSs have little effect on the foetus because the mammalian placenta contains an enzyme called 11 beta-hydroxysteroid dehydrogenase type 2 (11 β -HSD2) which protects the foetus from the maternal CS; although at high levels of maternal CS and in some pathological conditions the foetus might be exposed to excess CSs. The synthetic CSs like DEX and BETA have a low affinity for 11 β -HSD2, meaning they can cross the placenta and exert greater effects on the foetus than maternal CSs (Bose et al. 2010). 11 β -HSD2 plays an essential role in

a protection of the foetus from CORT, hydrocortisone and prednisolone but not from synthetic CSs (Heine & Rowitch 2009) (Figure 1.8).

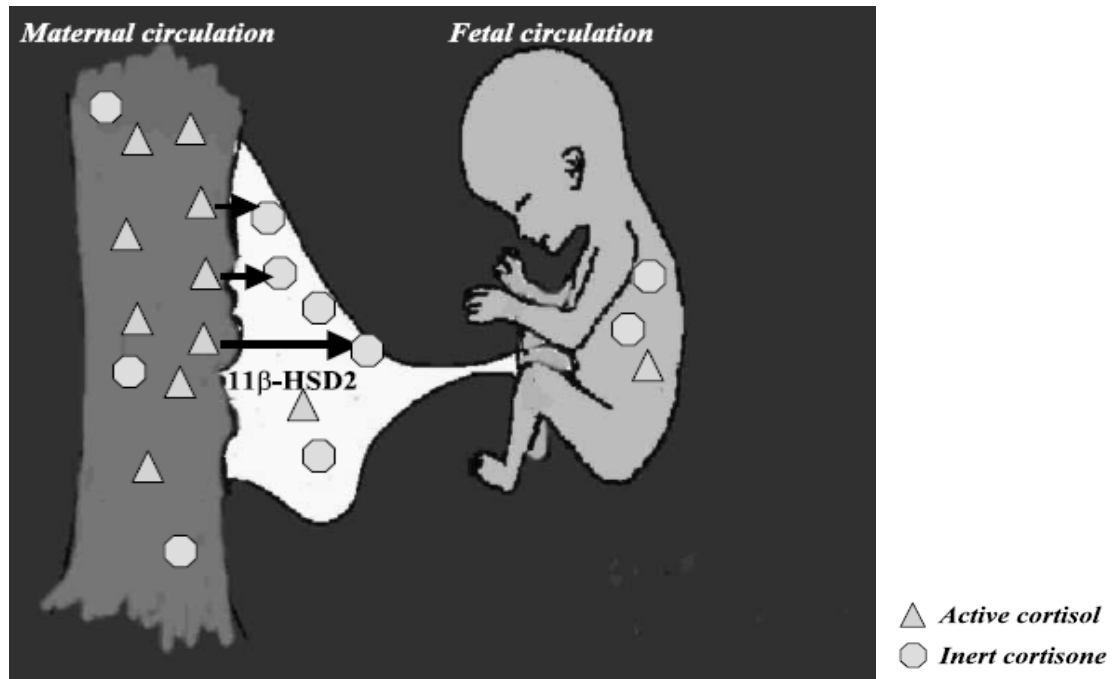


Figure 1.8 Some active maternal CSs pass through the placenta to the foetus. *11β-HSD* is an enzyme found in the mammalian placenta and plays an important role to protect the foetus from the high level of maternal CS during stress and in certain pathological conditions. In rodents, corticosterone is inactivated by conversion to 11-dehydrocorticosterone. In most other mammals, including humans, cortisol is inactivated by conversion to cortisone. Synthetic CS such as DEX and BETA have low affinity for *11β-HSD* therefore they can exert greater effect on the foetus compared to maternal CSs. Figure adapted from (Seckl, 2001).

It was demonstrated that the foetal exposure to CSs during the prenatal period caused several adverse effects including delayed brain development and also caused cognitive impairment, behavioural and emotional disturbances (Yossuck et al., 2006). The experimental research suggests that high levels of CS reduced the brain weight in neonatal rats, and caused alteration in social behaviour and learning processes (Sousa et al., 2000). Importantly, there is strong evidence that the administration of CS during the antenatal and postnatal period exerts effects on the hippocampus. The hippocampus is an important structure of the brain which contains high density of GR. It is responsible for regulation of cognitive function such as learning, memory storage and emotion, therefore any changes in the hippocampus composition could lead to mental illness (Karten et al. 2005; Cameron & McKay 1999; Boku et al. 2009). At high levels of CS, the brain development of the foetus is altered causing persistent structural and functional changes and reduced neurogenesis (Whitelaw & Thoresen 2000). The exposure of NPCs - obtained from the hippocampus of rats - to DEX during the neonatal life, increases the apoptosis of NPCs and reduces neurogenesis (Yu et al., 2010). Further, it was observed that high doses of CS cause impairment of neurogenesis and enhancement of apoptosis of oligodendrocytes in the brain of newborn animals (Chang, 2014). Additionally, the administration of CORT decreased brain derived neurotrophic factor (BDNF) in cultured rat hippocampal cells and DEX administration downregulating BDNF in all hippocampal regions *in vivo*, BDNF is important for neurogenesis and synaptogenesis, therefore the lower level of this protein led to a reduction in cell survival in the hippocampus (Franklin and Perrot-Sinal, 2006).

Moreover, a number of experimental animal studies show the effects of CS on the foetal brain development. For instance, Uno *et al.* gave 0.5, 5 and 10 mg/kg DEX to pregnant monkeys; cell counts showed that single and multiple doses of DEX led to a reduction in the number of neurons in dentate gyrus area of hippocampus. They observed that the severity of effect of DEX was increased with the injection of multiple doses (Uno *et al.*, 1990). Gould *et al.* treated rat pups with CORT and aldosterone during the first postnatal week and used [³H] thymidine to label the cells in the granule cell layers. They observed in this experiment that the density of labelled cells was decreased with both CORT and aldosterone treatment and they suggest, based on their results, that the adrenal steroid regulates dentate gyrus cell birth and cell death (Gould *et al.*, 1991). Huang *et al.* (1999) demonstrated that a single and repeated dose of BETA (0.5 mg/kg) to pregnant sheep led to a reduction of the weight of brain components, including cerebrum, cerebellum, and brain stem (Huang *et al.*, 1999). The neurotoxic effects of CS were studied by Heine and Rowitch (2009), who observed that the proliferation of the cerebellar granule neuron precursors was reduced when mouse pups were treated with multiple doses of DEX during the first week after birth (Heine and Rowitch, 2009). Table 1.3 summarises several experimental animal studies that investigating the effects of CS on the foetal brain development.

Table 1.3 Summary of the neurological effects of CS on foetal brain development in animal models

CS	Type of dose	Neurological effects	Region of effects	Animal species	Reference
DEX	Single and multiple	Decreases the number of neurons	Dentate gyrus of hippocampus	Pregnant monkeys	(Uno et al., 1990)
DEX	Multiple	Reduces the proliferation of granule neuron precursors	Cerebellar	Mouse pups	(Heine and Rowitch, 2009)
BETA	Single and multiple	Reduces the weight of brain components	Cerebrum, cerebellum, and brain stem	Pregnant sheep	(Huang et al., 1999)
CORT and aldosterone	Multiple	Reduce the proliferation of cells	Dentate gyrus	Rat pups	(Gould et al., 1991)

Most notably, several human studies have shown that CS therapy increased risk of cerebral palsy, along with differences in morphology and structure of the offspring's brain (Choi et al., 2004; Heine and Rowitch, 2009; Murphy et al., 2001; Noguchi et al., 2011; Shinwell and Eventov-Friedman, 2009). For example, a randomised controlled trial have been performed to study the effects of CS in human pregnancy and their outcome in the infant, finding that human infants treated with prenatal DEX administration had lower birth weight, lower IQ scores, and poor motor and visual coordination skills at school age compared with their peers (Stark et al., 2001; Yeh et al., 2004). Yeh *et al.* reported the two year outcome of a randomised controlled trial to study the effect of DEX on preterm infants. They administered 0.5 mg/kg/ day DEX to infants which had birth weight of 500 to 1999 g and had severe RDS. They found that a total of 25 of 63 children treated with DEX had neuromotor abnormalities, whereas only 12 of 70 control children had neuromotor abnormalities (Yeh et al., 1998). In 1999, O'Shea and colleagues reported one year neurological outcome on a randomised controlled trial of postnatal CSs for 42 days for preterm infants had low birth weight and had chronic lung disease. They observed that 25% of infants had cerebral palsy after DEX treatment compared with 7% of control (O'Shea et al., 1999). In 2001, Murphy and colleagues found that the cortical brain volume was reduced by 35% when compared to full term controls in human premature infants that were postnatally treated with DEX (Murphy et al., 2001).

Collectively, clinical and animal results overwhelmingly suggest that both single and multiple courses of CS cause neurological effect in children and increase the risk of brain damage.

1.5.6.2 The neurological effects of CS in adult life

A numbers of animals and clinical studies observed a clear link between prenatal exposure to CSs and adverse consequences in later life. For example, in animal models of antenatal, prenatal and maternal administration of DEX produce offspring that appear more anxious as adults. Also, it was found that the prenatal DEX exposure is associated with behavioural changes in adults (Welberg and Seckl, 2001). It was observed that the antenatal and postnatal treatments of CSs are associated with behavioural changes in adults which are frequently accompanied by alterations in the hypothalamic- pituitary- adrenal (HPA) axis. Importantly, these alterations in the offspring's HPA axis function have also been associated in humans (Wyrwoll and Holmes, 2012). It was found in several clinical studies that the prenatal exposure to CSs exerts long-term effects on brain structure and function, alters affective behaviours and leads to mental health in adults. Moreover, the postnatal treatment of CSs leads to maternal stress and behaviorual disorder such as hyperactivity in young people (Khalife et al., 2013).

Furthermore, a number of animals experimental studies showed that CSs treatment in adults are also associated with neurological effects. For example, CORT exposure has been associated with increased anxiety in adult rats (Myers and Greenwood-Van Meerveld, 2007) and impaired memory (Brabham et al., 2000). The CS administration has been shown to markedly reduce neurogenesis in the SVZ of the adult rat hippocampus. Much Experimental research has studied the adverse effects of CS on neurogenesis (Table 1.4). It was observed that the high exposure to CSs leads to structural changes in neurons and can reduce neurogenesis, especially in the hippocampus (Fuchs and Flügge, 1998; Heberden et al., 2013; Reagan and McEwen, 1997). Also, the high level of CORT has an

effect on the fate of the NSCs and causes a reduction in neurogenesis and an increase in oligodendrogenesis in the hippocampus of adult rats (Chetty et al., 2014). While, Anacker *et al.* found that low doses of cortisol causes a reduction of neurogenesis in stressed adult rats (Anacker et al., 2013).

Most importantly, several experiments have studied the neurological effects of CS after transplantation. For example, Skardelly and colleagues investigated the effects of immunosuppressive drug on the fate of the human neural progenitor/stem cells (NPSCs) *in vitro*. They found that the higher concentrations of prednisolone (100 mg/ml and 200 mg/ml) significantly compromised cell viability, increased the apoptosis rate, reduced cell proliferation and mainly increased differentiation of astrocytes without any effect on neurogenesis (Skardelly et al., 2013). Other studies have shown the effects of MPRED following neurological injury (Bracken, 2012). For example, Schröter and colleagues studied the effects of MPRED after SCI on the NPCs of adult mice. They reported that MPRED reduces the proliferation of NPCs in spinal cord and hippocampus, and this reduction leads to the inhibition the proliferation of oligodendrocyte progenitor cells (OPCs) (Schröter et al., 2009). Although CSs have great potential in clinical therapies and treatments of a large numbers of conditions, they carry a risk of a number of neurological deficits. Such deficits appear to be due to CS effects on NSCs.

Table 1.4 Summary of the CS effects on neurogenesis		
CS	Neurological effects	Reference
DEX	Increases the apoptosis of NPCs and reduces neurogenesis	(Yu et al., 2010)
DEX	Impairs neurogenesis and increases apoptosis of oligodendrocytes	(Chang, 2014)
CORT	Reduces neurogenesis and increases oligodendrogenesis	(Chetty et al., 2014)
Cortisol	Reduces neurogenesis	(Anacker et al., 2013)

1.6 MINO usage and the possible mechanisms of action

The therapeutic use of tetracycline has been described in the 1940's; various molecular modifications on tetracycline structure have been employed to promote and improve their antibacterial activity, their tissue absorption, and prolong their half-life (Garrido-Mesa et al., 2013). MINO is a second-generation semi-synthetic tetracycline that is used as an antibiotic and anti-inflammatory drug (Figure 1.9). Importantly, MINO shows better absorption with a longer half-life compared to 1st generation tetracycline. Recently, several studies have shown the neuroprotective potential of MINO (Karimi-Abdolrezaee et al., 2010; Sakata et al., 2012; Wells et al., 2003). It has been approved by the medicines and healthcare products regulatory agency and by the food and drug administration due to its low toxicity and low side effects. MINO is a small, highly lipophilic molecule which can readily pass through the BBB, and as such, is used clinically for the treatment of several neurological disorders such as cerebral ischemia, TBI and SCI (Festoff et al., 2006; Pinzon et al., 2008).

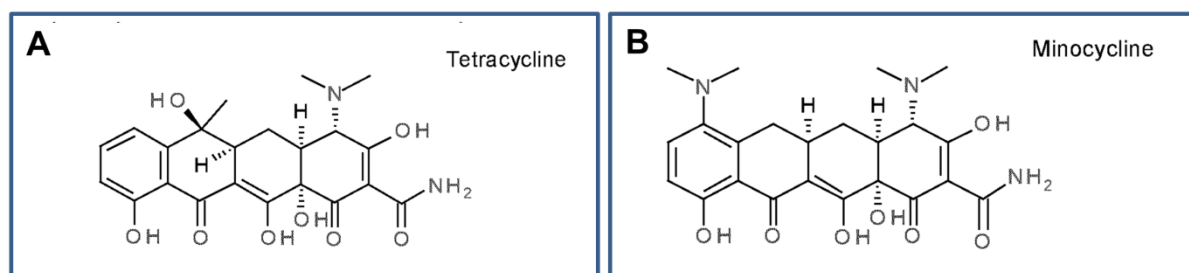


Figure 1.9 Chemical structures of tetracycline and MINO. (A) *chemical structure of first generation tetracycline.* (B) *chemical structure of second generation tetracycline, MINO.* Figure adapted from (Garrido-Mesa et al., 2013).

MINO has been demonstrated to exert its anti-inflammatory effects by reducing the activity of microglia, and subsequently their secretion of factors such as cytokines, chemokines, lipid mediators of inflammation and nitric oxide release (Lee et al., 2003; Yrjänheikki et al., 1998). MINO plays a neuroprotective role and has been used to inhibit microglia activation, and modify their toxic effects on NSCs. The neuroprotective properties of MINO were mediated by regulating chemokine activity causing a decrease in resident and immune cell migration to sites of inflammation (Kremlev et al., 2004). Also, several reports have demonstrated that the neuroprotective effects of MINO were mediated by regulating nitric oxide production. In general, the mechanisms of action of MINO appear to result from anti-inflammatory, anti-apoptotic, and antioxidant functions (Figure 1.10). The main biological effects of MINO, which are involved in the pathogenesis of several neurological conditions, include inhibition of microglial activation. Microglial inhibition results in, among others, such as the downregulation of MHC II expression, a decrease in cell motility, the inhibition of the p38 MAPK pathway, a reduction in prostaglandin E synthase expression or a reduction in the level of matrix metalloproteinases (MMPs). The anti-apoptotic effects of MINO include a decrease in the mitochondrial calcium overloading, MINO inhibits release several apoptotic factors into the cytoplasm such as cytochrome C, resulting in decreased caspase activation and nuclear damage. In addition to the antibiotic, anti-inflammatory and anti-apoptotic effects of MINO, this drug possesses antioxidant properties. It directly scavenges free radicals and inhibits molecules such as cyclooxygenase 2, induced nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate oxidase (Pinkernelle et al., 2013; Plane et al., 2010).

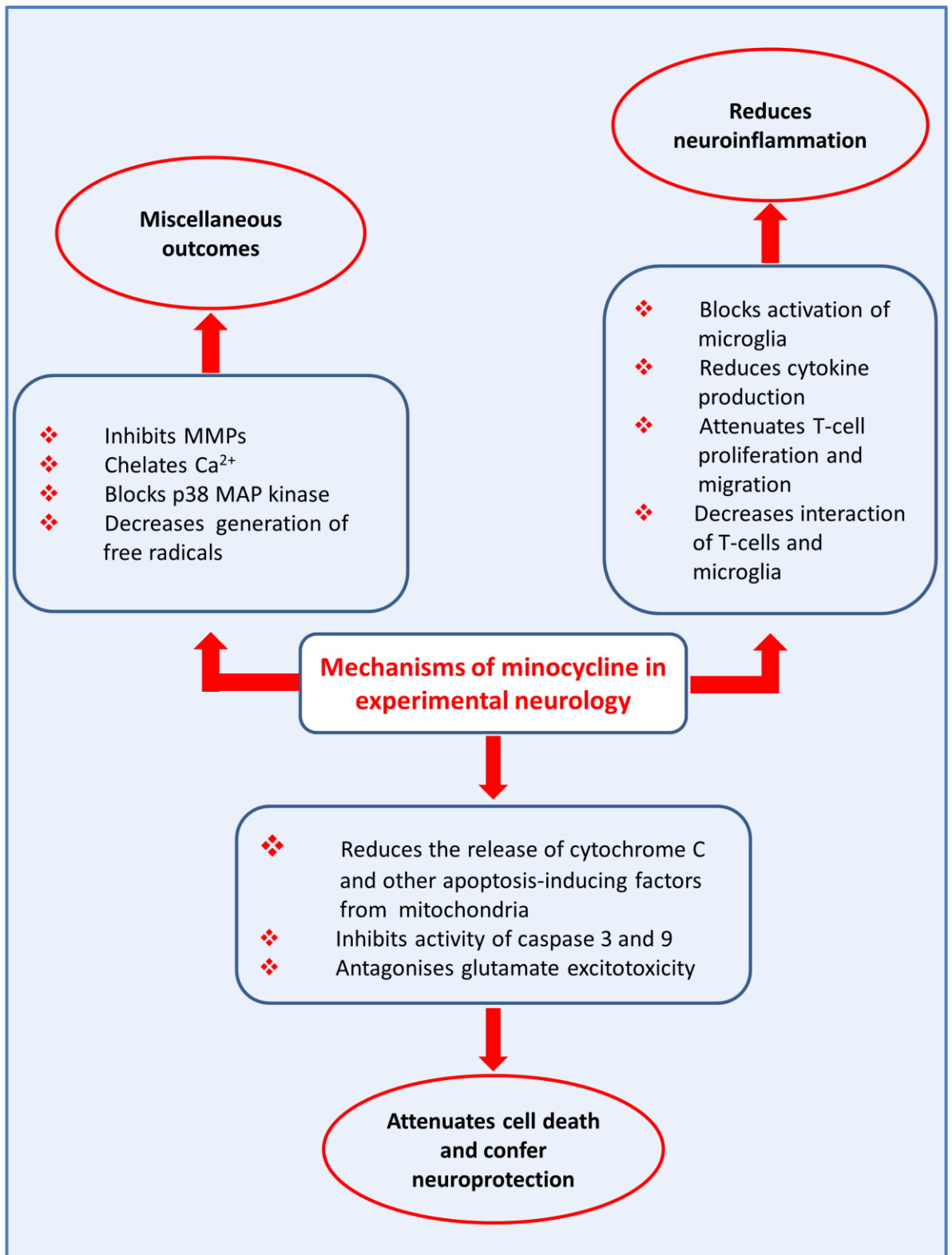


Figure 1.10 Multiple mechanisms of MINO account for its useful effects in experimental neurology. *Figure adapted from (Yong et al., 2004).*

Importantly, MINO has been successfully used to treat several neurological disorders in animal models such as ischemia, stroke, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and SCI (Yong et al., 2004). For example, the neuroprotective effects of MINO were investigated in an ischemia animal model, it was found that MINO markedly reduced the production of nitric oxide, TNF- α and IL-1 β (Suk, 2004). In a gerbil model of brain ischemia, Yrjänheikki *et al.* demonstrated that MINO significantly reduced nitric oxide induced by microglia activation and reduced loss of pyramidal neurons (Yrjänheikki et al., 1998). Another study in rat model of SCI showed that MINO reduced the level of TNF- α at 24 h after SCI (Festoff et al., 2006). Also, in a Parkinson's disease model, MINO was found to reduce microglia activation with minimal effects on the astrocyte. Further, in the brain of the R6/2 experimental mouse model of Huntington's disease, MINO showed effective neuroprotective features (Wu et al., 2002). Additionally, MINO has also been shown to inhibit the astrocytic response after SCI and subsequently the astrocyte-mediated release of pro-inflammatory mediators, and reduce scar formation (Teng et al., 2004).

1.7 Aims of experimental chapters

The aims of the series of experiments contained in this thesis are to study the effect of anti-inflammatory and immunosuppressive drugs on the fate of NSCs. Recently, NSCs represent an attractive source for regenerative and cell replacement therapies in several neurodegenerative diseases/injuries. These cells play an important role firstly, during brain development because of their ability to give rise to three main types of neural cells and secondly, as these cells have important regenerative properties, they play a major role in endogenous repair. Thirdly, they have a potential role in transplant-mediated repair.

In the context of neural cell therapy, the use of anti-inflammatory and immunosuppressive drugs following neural transplantation, is necessary to improve graft survival and to avoid cell rejection in host tissue (Sakata et al., 2012; Skardelly et al., 2013). Therefore it is of major importance to study the effects of these drugs on a variety of physiological processes of NSCs, such as proliferation, survival, apoptosis, and differentiation.

On the other hand, the administration of anti-inflammatory drugs such as CS during the antenatal and postnatal period leads to neurodevelopmental impairment and affects growth and development of the immature brain (Shinwell and Eventov-Friedman, 2009). A number of randomised controlled trials from the 1990s, observed that CS treatment resulted in elevated risks of neurodevelopmental impairment and cerebral palsy in humans (O'Shea et al., 1999). Further, it was found that the repeated and prolonged treatments of CS increase this risk (French et al., 2004; Wapner et al., 2007).

Animal experimental studies have shown that the administration of CS induces apoptosis and reduces the neurogenesis and the proliferation of NSCs (Sze et al., 2013). Also, there is evidence suggesting that CS treatment can also accelerate the apoptosis of neurons and oligodendrocytes, induce degenerative changes in oligodendrocytes, and delay the formation of myelin (Chari et al., 2006; Huang et al., 2001). However, the mechanisms underlying such neurological defects are unclear and largely unknown.

Based on the important role of both NSCs and the anti-inflammatory drugs, this thesis focused on the potential effect of anti-inflammatory drugs on NSCs. The effect of anti-inflammatory drugs on NSCs fate has not been sufficiently explored and the specific mechanisms behind these effects remain unclear and not fully understood.

This thesis studied the effects of different clinically relevant drugs at different concentrations and different time of treatments so, this study allowed for a systematic comparison of anti-inflammatory drugs on the key regenerative properties of NSCs (i.e proliferation, survival, apoptosis, and differentiation) and gained insight into the stages at which these drugs may exert their effects.

Chapter 3: Histological study to evaluate the effects of CS treatment on NSCs propagated in monolayer cultures

The aim of this chapter is to investigate the effects of CS on NSCs grown as a monolayer cultures. NSCs are routinely cultured using two distinct culture systems: as 2-D adherent cells called monolayers and as 3-D suspension cells called neurospheres. Both culture systems are commonly used to propagate and expand NSCs for cell transplantation. In this chapter monolayer cultures system used to study the effects of CS on NSCs using the histological analysis to address the question of whether CS effects are mediated by a direct influence on parameters such as the NSCs survival, self-renewal, and differentiation into astrocytes, neurons and oligodendrocytes.

Chapter 4: Histological study to evaluate the effects of CS treatment on NSCs propagated in neurosphere cultures

This chapter aims to investigate the effects of CS on NSCs in neurosphere cultures. The nature of NSCs population is an important factor that can affect the potential mechanisms of repair after transplantation and the type of culture system that is used in isolation and propagation of NSCs could impact the heterogeneity and differentiation of NSCs. Furthermore, the differences in the features 2-D and 3-D cultures impact the choice of culture system when using NSCs in transplantation after neurological disease/injury. Despite this, it is not known if the physical format by which these cells are propagated can impact on drug influences on these cells. Therefore it is of major importance to study the effects of CS on NSCs parameters such as proliferation, cell cycle, viability, apoptosis and differentiation in 3-D culture.

Chapter 5: Investigating the mechanisms underpinning the effects of CS on NSCs

CSs mediated their anti-inflammatory effects either by genomic or non-genomic mechanisms. Many researchers are convinced that most effects of GC are mediated by a nuclear genomic mechanism, in this mechanism the expression of key genes is regulated by GRs. The GR can modulate the expression of genes either by binding to GREs in the promoters of target genes or by binding with other transcription factors (Ayroldi et al., 2012; Samarasinghe et al., 2012). The non-genomic mechanisms are also mediated by the GR to mediate rapid cellular effects to GC in the absence of measurable alterations in gene expression (Makara and Haller, 2001; Mikics et al., 2004). Therefore it is of vital importance to investigate the expression of GRs in NSCs and their daughter cells used in this study.

Although many experiments have been performed to study the effects of CS using analysis of gene expression and microarray analyses, no in-depth study of the influence of CS treatment upon protein expression has yet been performed in NSCs. Proteomic analysis enables identification of potential proteins that show altered expression in drug treated cells. As a large number of proteins were obtained, sophisticated bioinformatics analysis used for these experiments to understand the mechanism behind these effects. Further, proteomic analysis enables us to derive novel hypothesis as to the mechanisms via which CSs exert their effect on cellular proliferation and differentiation capacity.

In this chapter, the first experiment was undertaken to investigate the GR expression in all types of cells used in this study. Then the molecular mechanisms

of CS were highlighted in NSCs monolayer cultures using proteomics and bioinformatics analyses.

Chapter 6: Testing NSCs in a combinatorial therapy using organotypic spinal cord slice model

The aim of this chapter is to investigate the utility of organotypic spinal cord slice model to study the effects of combinatorial therapy to promote spinal regeneration through the direct replacement of neuronal and glial cells using transplanted NSCs, with or without anti-inflammatory drug. The organotypic spinal cord slice models are widely used as high throughput approach in neurology research to study the therapeutic strategies in neurological injury/disease. This type of model has several advantages such as: it is easy amenable to experimental manipulation and allow control and experimental slices to be derived from the same animal, reducing experimental variability (Weightman et al., 2014). Interestingly, the organotypic spinal cord slice cultures are more mimetic of *in vivo* environment due to their ability to preserve the tissue architecture and maintain cell-to-cell contact (Cho et al., 2007; Sypecka et al., 2015).

Importantly, many drugs were found to improve behavioural and histological outcomes in the experimental animals SCI but have yet to be implemented after human SCI. Thus, it is important to evaluate the safety of these drugs in clinically relevant models to move from animals to humans. It should be noted that, the use of CS such as MPRED has now dramatically declined due to the detrimental side effects, also some clinical trials have failed to report its therapeutic benefits (Hurlbert, 2000; Sayer et al., 2006). Based on this, MINO was chosen in this

chapter as a combinatorial therapy with transplantation of NSCs in organotypic spinal cord slice model.

In the first part of this chapter, the direct effects of MINO have been tested on NSCs in neurosphere cultures. The fundamental properties of NSCs have been investigated: survival, proliferation and apoptosis, viability, and differentiation after MINO treatment. In the second part of this chapter, organotypic spinal cord slice culture has been used to investigate the regeneration of SCI following MINO treatment and NSCs transplantation.

Chapter 2

Materials and Methods

2.1 Materials

Cell and organotypic spinal cord slice culture: Cell culture reagents were from Life Technologies (Paisley, Scotland, UK) and Sigma-Aldrich (Poole, Dorset, UK). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine,4-chlorobenzenesulfonate salt (DiD), Foetal bovine serum (FBS), penicillin and streptomycin, Nunc T25 cell culture flasks, Nunc 24 well plates, 24 well suspension culture plates and other tissue culture-grade plastics were purchased from Fisher Scientific (Loughborough, UK). Omnipore membranes (JHWP04700) and Millicell culture inserts (PICM0RG50) were from Millipore (Watford, UK). Disposable scalpels and size 15 surgical blades were obtained from Swann-Morton (Sheffield, UK). DNase I was from Roche (Welwyn, UK). Accutase was from Sigma-Aldrich, human recombinant basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF) were purchased from Sigma-Aldrich and R&D Systems Europe Ltd (Abingdon, UK), respectively.

Drugs: Dexamethasone (DEX), 21-phosphate disodium (D4902; $\geq 97\%$ pure), prednisone (PRED), 17 α , 21-Dihydroxy-1,4-pregnadiene-3,11,20-trione (P6254; $\geq 98\%$ pure), methylprednisolone (MPRED), 6 α -methylprednisolone 21-hemisuccinate sodium salt (M3781; $\geq 98\%$ pure) and minocycline (MINO), minocycline hydrochloride (286710; $\geq 97\%$ pure) were from Sigma-Aldrich (Poole, UK).

Immunocytochemistry: The following primary antibodies were used: mouse anti-nestin was from BD Biosciences (Oxford, UK) and rabbit anti-SOX-2 was from Millipore (Watford, UK), mouse anti- β -tubulin (TUJ-1) and rabbit anti- β -tubulin were from Covance (Princeton, NJ), rabbit anti-gial fibrillary acidic protein (GFAP)

was from DakoCytomation (Ely, UK) and rat anti-myelin basic protein (MBP) was from Serotec (Kidlington, UK). A neuronal marker rabbit anti-growth associated protein 43 (GAP-43) was from (Abcam, UK). Biotin-conjugated lectin (microglial marker; from *Lycopersicon esculentum*, tomato) and monoclonal, anti-biotin FITC-conjugated antibodies were from Sigma-Aldrich (Poole, UK). Two glucocorticoid receptor antibodies H-300 and BuGR2 were from (Santa Cruz Biotech, USA) and (Abcam, UK), respectively. Secondary antibodies (Cy3- and FITC-conjugated) were from Jackson ImmunoResearch Laboratories Ltd (Westgrove, PA, USA). Normal donkey serum (NDS) was from Stratech Scientific (Suffolk, UK) and DAPI (4', 6-diamidino-2-phenylindole) mounting medium was from Vector Laboratories (Peterborough, UK).

Proliferation analysis: Click-iT® Plus EdU Alexa Fluor® 488 Flow Cytometry Assay Kit was from Life Technologies (Paisley, Scotland, UK). The muse cell cycle kit was from Millipore (Watford, UK).

Viability analysis: The LIVE/DEAD Viability/Cytotoxicity Assay Kit was from Invitrogen (Paisley, UK).

Apoptosis analysis: Kits for Annexin V and Dead cell was from Millipore (Watford, UK).

Proteomic analysis: Trifluoroacetic acid was from Fisher Scientific (Loughborough, UK). Acetonitrile was from VWR Chemicals (Lutterworth, UK), Iodoacetamide was from Acros Organics (Geel, Belgium) and Rapigest was from Waters Corporation (Milford, MA). Ammonium Bicarbonate (Ambic), protease inhibitor cocktail, dithiothreitol, Proteomics-Grade dimethylated trypsin and Bradford Reagent were from Sigma-Aldrich (Poole, UK).

Animals: Primary cultures were used for all experimental studies performed. These were derived from CD1 mice. The care and use of all animals used in the production of cell cultures were in accordance with the Animals Scientific Procedures Act of 1986 (UK) with approval by the local ethics committee.

2.2 Preparation of media used in primary NSCs culture

2.2.1 Preparation of neurosphere medium

Neurosphere medium was prepared with: 3:1 mix Dulbecco's Modified Eagle Medium (DMEM): F12, DMEM and F12 nutrient medium, 2% B27 supplement, 20 ng/ml FGF-2 and EGF, 4 ng/ml heparin, 50 U/ml penicillin, 50 µg/ml streptomycin. Neurosphere medium was stored at 4°C and used within 1 week (Pickard et al., 2017).

2.2.2 Preparation of monolayer medium

Monolayer medium was prepared with: 1:1 mix DMEM:F12, 1% N2 supplement, 20 ng/ml FGF-2 and EGF, 4 ng/ml heparin, 50 U/ml penicillin, 50 µg/ml streptomycin. Monolayer medium was stored at 4°C and used for up to 1 week after preparation (Pickard et al., 2017).

2.2.3 Preparation of differentiation medium

Differentiation medium was prepared with: 3:1 mix DMEM:F12, 2% B27 supplement, 4 ng/ml heparin, 50 U/ml penicillin, 50 µg/ml streptomycin, 1% FBS. Differentiation medium was stored at 4°C and used for up to 4 weeks after preparation.

2.3 Coverslip preparation and coating

It has been observed in our laboratory that NSCs more reliably adhere to nitric acid washed coverslips than non-washed glass. Therefore coverslips for all adherent NSCs culture were washed in 1% nitric acid and shaken overnight. These were then rinsed in deionised water (dH₂O) for 3 h (dH₂O changes every 30 min), and sonicated in dH₂O for 1.5 h, then in ethanol 70% for 1.5 h and finally in ethanol 95% for 1 h with solution changes every 30 min. Prepared coverslips were stored in 70% ethanol in large petri dishes at room temperature (RT). To coat coverslips for adherent NSCs culture, they were incubated with Poly-L-ornithine (MW 30-70 000 Da, 0.002%) at 37°C and 5% CO₂ for 1 h. Afterward, poly-L-ornithine was removed and coverslips were washed once with dH₂O. Then 5 µg/mL laminin was added to each coverslip, plates were incubated at 37°C and 5% CO₂ for 1 h. The laminin was removed and coverslips were washed 3 times with dH₂O.

2.4 Primary NSCs derivation and maintenance

NSCs were derived from the SVZ of CD1 mice pups, postnatal day 1-3 (the day of birth was designated as postnatal day 0) (Adams et al., 2013). Briefly, the SVZ was dissected out and the tissue was mechanically dissociated into a single cell suspension in the presence of DNase I. Dissociated cells were then counted and plated at 1×10^5 cells/ml in neurosphere medium, for routine maintenance, neurospheres were fed every 2-3 days and passaged every 5-7 days using a mix of accutase and DNase I. Cells from passages one to three were used for experiments.

For adherent cultures, neurospheres were dissociated and plated on poly-L-ornithine and laminin coated coverslips and maintained in monolayer medium.

For differentiation, the NSCs from both monolayer and neurosphere cultures can differentiate into three types of CNS cells, astrocytes, neurons and oligodendrocytes by removing the growth factors and adding serum to the medium. All stages of NSCs culture (neurospheres, monolayers and differentiated cells) have been used in the experiments described in this thesis and their generation is illustrated in (Figure 2.1).

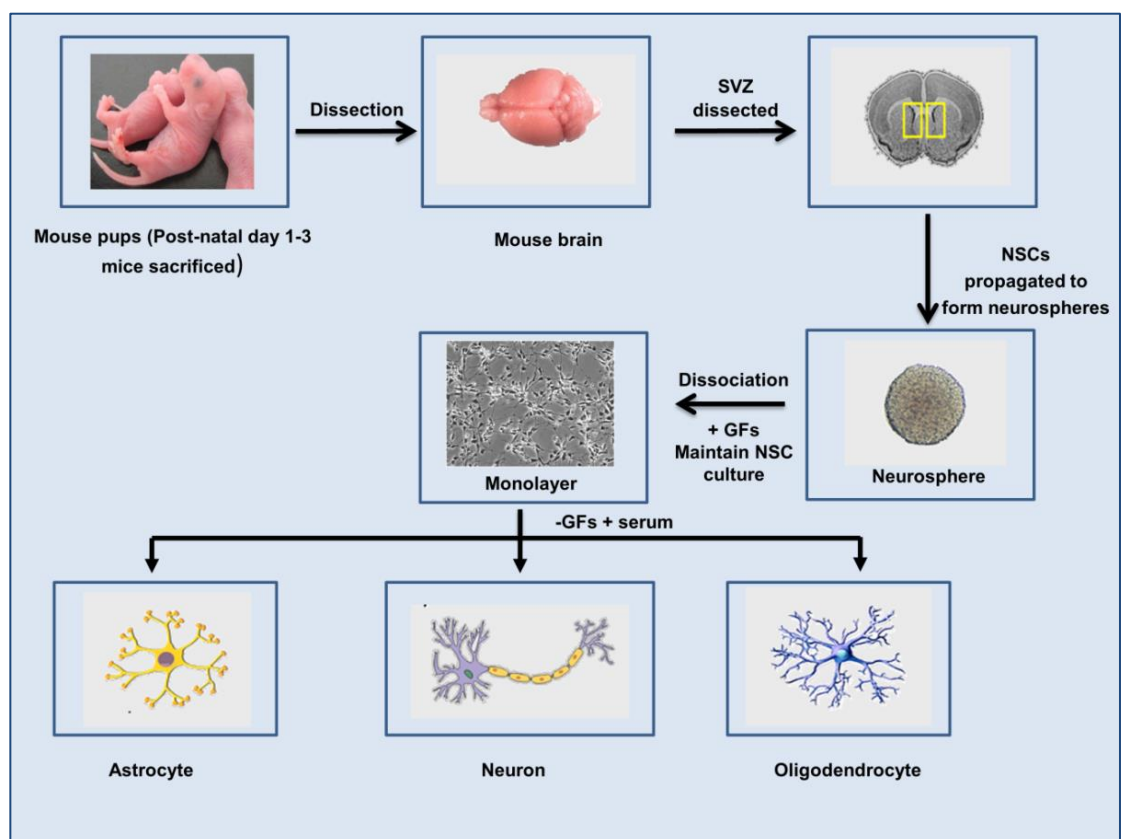


Figure 2.1 Schematic diagram illustrating the procedures for NSCs culture and differentiation. *This procedure was performed to setup cultures from different litters.*

2.5 Preparation and dose testing of drugs

2.5.1 Preparation of CSs

Three clinical synthetic CSs (DEX, PRED and MPRED) were used in NSCs primary culture. These drugs were selected for use in the experimental studies because they are widely used in treatment of a large number of clinical conditions in both adults and children, such as RDS, chronic lung disease, and in the treatment of neurological trauma (ASCI and TBI) (Crowley et al., 1990; Hall and Springer, 2004; Han et al., 2014; Moss and Sloboda, 2006; Sayer et al., 2006; Short et al., 2000). Stock solution of DEX was prepared at 31.5 mM in 100% ethanol (ETOH), while, the stock solutions of PRED and MPRED were prepared at 117 mM and at 118 mM respectively in Dimethyl sulfoxide (DMSO). Then the stock solutions of DEX, PRED and MPRED were diluted in appropriate culture medium to the indicated concentration. Cell cultures were treated with three different concentrations of CS (0.1 μ M, 1 μ M and 10 μ M). The final concentration of ETOH was 0.95% (v/v) at all doses of DEX and for ETOH vehicle controls and the final concentration of DMSO was 0.27% (v/v) at all doses of PRED and MPRED and for DMS vehicle controls.

2.5.2 Preparation of MINO

The clinical anti-inflammatory drug, MINO, has cytoprotective properties and promotes survival of grafted cells (Sakata et al., 2012). Further, MINO treatment has been described for many patients with SCI (Wells et al., 2003). MINO has the ability to improve the functional recovery following SCI by reducing lesion size, cell death, and altering cytokine expression (Lee et al., 2003; Wells et al., 2003).

Previously, it was demonstrated that MINO is more effective in improving functional outcome when compared with MPRED (Festoff et al., 2006; Wells et al., 2003). Therefore, this drug was selected for use in the experimental studies. Prior to examining the effect of MINO on NSCs transplantation in the organotypic SCI model, the direct effect of MINO was assessed on NSCs in neurosphere cultures. Stock solution of MINO was prepared at 33.7 mM in DMSO to obtain a stock solution, this solution was then diluted with the respective medium to the final concentration of 10 μ M. Primary NSCs and organotypic spinal cord slices were treated with 10 μ M MINO. The final concentration of DMSO was 0.88% (v/v) at MINO dose and for vehicle controls.

2.5.3 Verification of drugs concentration

Spectrophotometry (Genesys 10S UV-vis spectrophotometer, ThermoScientific, USA) was used to verify the concentration of drug solutions. The molar absorptivity (molar extinction coefficient) at a particular wavelength of each type of drugs was known; therefore the absorbance of the drug solutions was measured at the particular wavelength to calculate their concentrations using Beer-Lambert law: **$A = e L c$**

A = absorbance, **e** = molar extinction coefficient, **c** = concentration and **L** = light pathlength. Given this equation below, the concentration can be calculated by: **$c=A/eL$** .

2.5.4 Assessment of the biological potency of selected drugs

Anti-inflammatory drugs such as CS and MINO are known to affect the proliferation and morphology of immune cells, including microglia, the major immune component of the CNS (Chao et al., 1992; Ganter et al., 1992; Jenkins et al., 2014; Sriram et al., 2006; Zhang et al., 2007). Therefore, primary microglial cultures were used as a positive control to assess the biological efficacy of all three types of CSs, as well as MINO. Primary microglia were derived from mixed glial cultures, which prepared from dissociated cerebral cortices of Sprague–Dawley rats at postnatal day 1-3 according to a standard protocol (Jenkins et al., 2014), (courtesy of Dr. Stuart Jenkins, Keele University). Microglia were plated in 24-well plates coated with poly-D-lysine at a density of 6×10^5 cells/ml (300 μ L/well) using D 10 medium (DMEM with 10% FBS, 2 mM glutaMAX-I, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μ g/mL streptomycin). After 24 h, 10 μ M of DEX, PRED, MPRED and MINO were added. Primary microglia cells were treated with CSs and MINO for 1-3 days. Cell numbers and morphology were monitored using phase-contrast microscopy.

2.6 Drug treatment of NSCs parent cells in 2-D monolayer cultures

NSCs were cultured as 2-D adherent monolayers in 24-well plates at a density of 0.65×10^5 cells/ml (600 μ L /well). NSCs were treated with CSs at one day post-plating by replacing medium with medium containing three different concentrations (0.1 μ M, 1 μ M and 10 μ M) of CSs followed by incubation for 48 h. Untreated NSCs just had medium replaced with fresh medium (without CS) and vehicle controls for DEX contained ETOH whereas vehicle controls for PRED and MPRED contained DMSO.

2.7 Drug treatment of NSCs parent cells in 3-D neurosphere cultures

NSCs were plated in 24-well suspension cell plates at a density of 1×10^5 cells/ml (0.5 mL suspension/well) using neurosphere medium. After 24 h, 0.1 μ M, 1 μ M and 10 μ M of CSs and 10 μ M of MINO were added for 48 h, the number and size of spheres were assessed from phase-contrast micrographs using four biological replicates with two technical repetitions in each experiment. Numbers of spheres per field were counted and the diameter of sphere was measured across four microscopic fields at 100 x magnification.

2.8 Cryostat sectioning

For cryostat sectioning, neurospheres from the culture plates were taken and placed in 1.5 ml Eppendorf tube and fixed by adding 500 μ L of 4% paraformaldehyde (PFA) for 15 minutes at RT. They were then centrifuged at 1000 rpm for 5 minutes and supernatant was removed and washed once with phosphate buffered saline (PBS) then centrifuged at 1000 rpm for 5 minutes and supernatant was removed. Neurospheres then were incubated in 15% sucrose in PBS and then left overnight in this solution at 4°C. The following day, sucrose was removed carefully and neurospheres were then embedded within optimum cutting temperature (OCT) compound and placed at -80°C. Serial cryostat sections (5 μ m) were cut with a cryostat microtome (Bright, OTF5000) and affixed to poly-lysine slides. Sections were processed for immunocytochemistry as described in section **2.20.2**. Cell counting was carried out in four biological replicates, with two technical replicates in each experiment. Two cryostat sections randomly were selected for each condition.

2.9 NSCs differentiation

The differentiation assay was performed to assess the effect of anti-inflammatory drugs on the proportion of each daughter cell type generated from NSCs parent cells. **For monolayer cultures**, cells were treated with CSs for 48 h then, the monolayer medium was replaced with differentiation medium containing the same concentrations of CSs. **For neurosphere cultures**, the spheres were dissociated using a mix of accutase and DNase I at 48 h of drug treatment and replated using differentiation medium containing 0.1 μM , 1 μM and 10 μM of CS and 10 μM of MINO. Differentiated cells from both cultures were cultured for a further 7 days (37 °C in 95% air: 5% CO₂) to produce a mixed cell population typically containing ca. 85% astrocytes, 10% neurons and 5% oligodendrocytes. Cultures were fed every 2-3 days then they were fixed with 4% PFA following 9 days of drug treatment. The experiments design of differentiation assay is illustrated in (Figure 2.2).

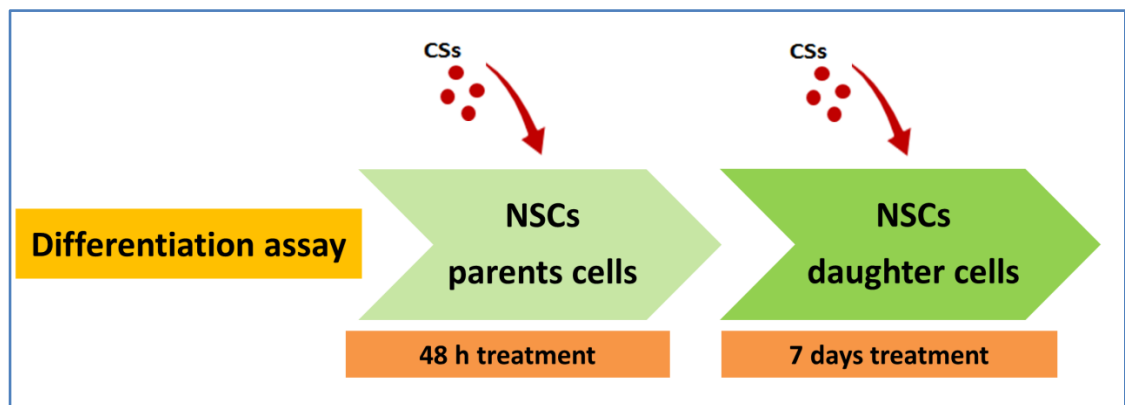


Figure 2.2 Schematic diagram illustrating the procedures for differentiation assay.

2.10 NSCs proliferation assay

The 5-ethynyl-2'-deoxyuridine (EdU) assay was used to measure the effect of anti-inflammatory drugs on cellular proliferation rates. Detection of EdU incorporation into the DNA was performed with Click-iT1 EdU Alexa Fluor proliferation assay kit, following the manufacturer's instructions. Briefly, 10 μ M EdU was added into each well followed by incubation at 37° C for 16 h. This incubation time was determined by the cell doubling rate for NSCs which is acknowledged to be around 20 h (Bose et al., 2010; Sun et al., 2009). The cells were fixed at 48 h of drug treatment in 4% PFA for 15 minutes at RT, and then they were washed twice with 3% bovine serum albumin (BSA). Then samples were incubated for 20 minutes in 0.5% Triton-X 100 in PBS (0.5 mL). Cells were washed twice with 3% BSA and the reagent cocktail for EdU detection was distributed over the cells (0.3 mL final volume/well). The cells were washed twice with 3% BSA after incubation at RT for 30 minutes in the dark. In preparation for imaging and analysis, the coverslips were mounted on slides using mounting medium containing DAPI and fluorescence images were captured from four random fields of the coverslip. Counts of nuclei co-expressing the EdU marker and nuclear counterstain (DAPI) were classified as proliferating cells (proliferation expressed as a percentage of the total cells counted).

2.11 Cell cycle analysis

Cell cycle analysis was performed using the Muse cell cycle kit according to the manufacturer's instructions. **For monolayer cultures**, cells were enzymatically detached using TrypLE, following a few minutes incubation, cells began to detach. Wells were washed with PBS to collect any remaining cells and then cells were pelleted by centrifugation and cells washed once in PBS and centrifuged again to remove and discard the supernatant.

For neurosphere cultures, spheres were collected following 48 h of drugs treatment. Cells were pelleted by centrifugation and then washed once in PBS. The supernatant removed and discard following centrifugation. The pellets were dissociated using a mix of accutase and DNase I and incubation at 37°C and 5% CO₂ for a few minutes. Following dissociation, cells were centrifuged and then washed with PBS and centrifuged again to remove and discard the supernatant. After centrifugation, NSCs (1×10^6) were fixed from monolayer or neurosphere cultures with 1 ml of 70% cold ethanol and incubated at -20°C for at least 3 h prior to staining, as per the Muse Cell Cycle Kit instructions. Subsequently, 200 µL of ethanol-fixed cells were washed in PBS and stained for 30 minutes at RT in the dark with 200 µL of Muse Cell Cycle Reagent. Cell suspension samples were transferred into 1.5 ml microcentrifuge tubes and were analysed using a Muse Cell Analyser (EMD Millipore, Darmstadt, Germany). In total, 5000 events were recorded for cell cycle analysis and the results are displayed the percentage of cells in G0/G1, S and G2/M phases.

2.12 Apoptosis assay

Muse Annexin V and Dead cell kit was used to quantify apoptotic cells. After 48 h treatment of NSCs, they were stained with Annexin V, following the manufacturer's instructions. TrypLE was used to detach monolayers, cells were collected and wells were washed with PBS to collect any remaining cells. Cells were pelleted by centrifugation and washed once in PBS. Then 100 μ L of cells in suspension were added to 100 μ L of the Muse Annexin V and Dead cell reagent and stained for 20 minutes at RT in the dark. Stained cells were detected using the Muse Cell analyser. In total, 2000 events were recorded for apoptosis analysis. The results are presented the percentage of apoptotic cells.

2.13 Viability assay

Cells and slice viability were measured of drug treatment using live/dead viability/cytotoxicity kit which contains calcein AM and ethidium homodimer-1. Cells and slices were washed with PBS, incubated for 15 minutes with a PBS solution consisting of 4 μ M calcein AM (produces green fluorescence in live cells) and 6 μ M ethidium homodimer-1 (produces red fluorescence in dead cells). Cells were washed again with PBS, and then mounted for fluorescence microscopy. Cellular viability was measured by counting green (LIVE) and red (DEAD) cells and expressing the number of LIVE cells as a percentage of total cells (green + red) from a total of four images taken at 400 x magnification.

2.14 Proteomic and bioinformatics analyses

In parallel with the histological safety assessments, a proteomics based analysis was performed to examine protein expression in NSCs monolayer cultures following MPRED treatment. The process of proteomic analysis is briefly illustrated in detailed below (Figure 2.3). Briefly, following the isolation of NSCs as detailed in **section 2.4**, they were then cultured as monolayers in 6 wells plates at 1.2×10^5 cells/ml in 1.5 ml. After one day, they were treated with 10 μ M MPRED for 48 h.

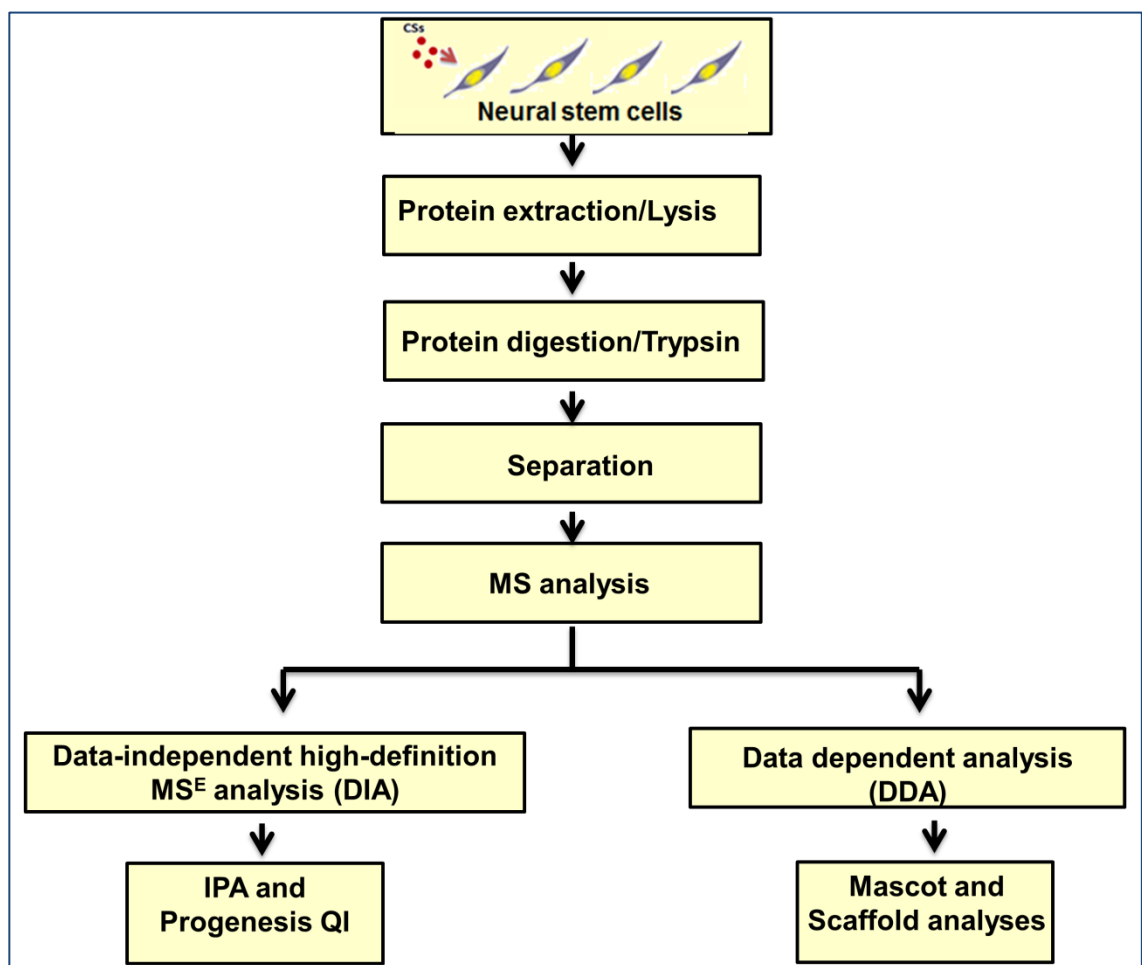


Figure 2.3 Schematic diagrams illustrating the procedure for the proteomic analysis.

2.14.1 Protein extraction

In order to obtain protein from the NSCs, the cells were enzymatically detached using TrypLE (<5 min, RT). Cells were pelleted by centrifugation and washed three times in 50 mM Ambic with centrifugation in between each wash. Cell pellets were lysed in 100 µL lysis buffer (0.1% Rapigest, 1% DNase I in 50mM Ambic). Probe sonication (Bandelin, Sonopuls) was performed on ice (3 cycles of 15 seconds on, 5 seconds off, amplitude 50%, pulse 0.5 on 1.0 off) to break apart cell membranes. Cell debris was removed by centrifugation and the protein concentration of the supernatant fraction of each lysate determined by Bradford assay according to manufacturer's protocol.

2.14.2 Tryptic digestion of cell lysate proteins

Protein concentrations were normalised to 100 µg for each sample. Each solution was incubated with 10 mM dithiothreitol with shaking (80°C, 15 min) before reduction using iodoacetamide (20 mM, 30 min, RT). Trypsin (2 µg) was then added to each sample, with incubation at 37°C for 16 h. Remaining tryptic activity was terminated, and Rapigest precipitated, by addition of trifluoroacetic acid (1%) and acetonitrile (2%) with shaking (60°C, 2 h). Rapigest was pelleted and removed by centrifugation and the supernatant taken for mass spectrometry analysis.

2.14.3 Mass spectrometry (MS)

2.14.3.1 Data Dependent Analysis (DDA)

Tandem mass spectrometry was performed to identify tryptic peptides. Peptide mass spectra were obtained using nanoflow electrospray ionization MS with tandem MS. A quadrupole time-of-flight mass spectrometer (QToF Premier,

Waters), coupled to nanoflow high performance liquid chromatography (HPLC) (Dionex Ultimate 3000, Thermo Fisher Corporation, Hemel Hempstead) was used to analyse peptides. Automated switching between MS (survey mode) and MS/MS (product ion mode) was used to generate sequence tag-specific product ion spectra. The data were analysed using Mascot and Scaffold software (Proteome Sciences, Toronto, ON). Mascot is a software tool widely used in mass spectrometry data analyses to identify peptides from DDA data and Scaffold was used to compare protein expression patterns between samples.

2.14.3.2 Data Independent Analysis (DIA)

We performed data-independent high-definition MS^E analysis (Rodriguez-Suarez et al., 2013), with ion mobility separation of precursor and mixed pseudo-product ion data using a Synapt G2Si instrument with associated NanoAcquity UPLC (Waters Corporation, Wilmslow, Cheshire) (Ansari et al., 2015; Burniston et al., 2014). Data were analysed using Progenesis QI for proteomics (Non-Linear Dynamics, Newcastle upon Tyne), with a High-N (n = 3) quantification being used to generate quantification data (Silva et al., 2005). An analysis of variance (ANOVA) p value of 0.05 was used as a cut-off for significance of differential protein identifications. Pathway analysis to identify differentially-regulated proteins was performed using Ingenuity Pathway Analysis (IPA; QIAGEN, Redwood City CA, www.qiagen.com/ingenuity). Statistical enrichment is calculated by a right-tailed Fisher's exact test.

2.15 Preparation of media used in organotypic spinal cord slices

2.15.1 Preparation of slicing medium

Slicing medium was prepared with Earle's balanced salt solution (EBSS), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B, and buffered with 25 mM HEPES. Slicing medium was stored at 4°C and used for up to 4 weeks after preparation.

2.15.2 Preparation of culture medium

Culture medium was prepared with 50% minimum essential medium (MEM), 25% heat-inactivated horse serum and 25% EBSS supplemented with 36 mM D-glucose, 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B. Culture medium was stored at 4°C and used for up to 4 weeks after preparation.

2.16 Production of organotypic spinal cord slices

Spinal cord slices were prepared from the spinal cord of CD1 mice pups, postnatal day 0-5 (Weightman et al., 2014). Briefly, under sterile conditions, mice were anaesthetised and rapidly decapitated. The limbs, tail and the abdominal organs were removed. The surface of the skin was sprayed with ETOH (95%), a scalpel was used to make a dorsal midline incision, and the skin flaps retracted to expose the spinal column along with retraction of the thick dorsal neck muscles to increase the ease of tissue extraction. Using fine microdissecting Vannas spring scissors (Stoelting UK), a midline incision was made along the length of the spine. The spinal cords were rapidly dissected out from the thoracolumbar region (using the point of attachment of the last true rib to define the upper margin for dissection) and placed in ice-cold slicing medium. To obtain the slices, the spine

was placed on the chopping plate of McIlwain tissue chopper and sliced lengthways in the parasagittal plane (350 μm thickness).

Five to seven slices could be generated from each spinal cord; slices from the extreme lateral margins of the cord showed a tendency to fragment therefore they were discarded. The dissection microscope was used to check intact slices to be used in experiments then intact slices were transferred to pre-cut Omnipore membrane 'confetti', resting on the Millicell culture insert membrane (2-3 slices on each). The culture inserts were placed within 35 mm Petri dishes to improve their isolation and reduce contamination risk (Figure 2.4). A single square Petri dish was used to hold 3-4 small Petri dishes to provide further protection against the spread of any infection present in the culture. Slices were cultured at the air- medium interface with an 80% medium change every two days. Spinal cord slices were lesioned after 1 day *in vitro* (DIV) and fixed at 7 days post-transplantation for a total of 9 DIV.

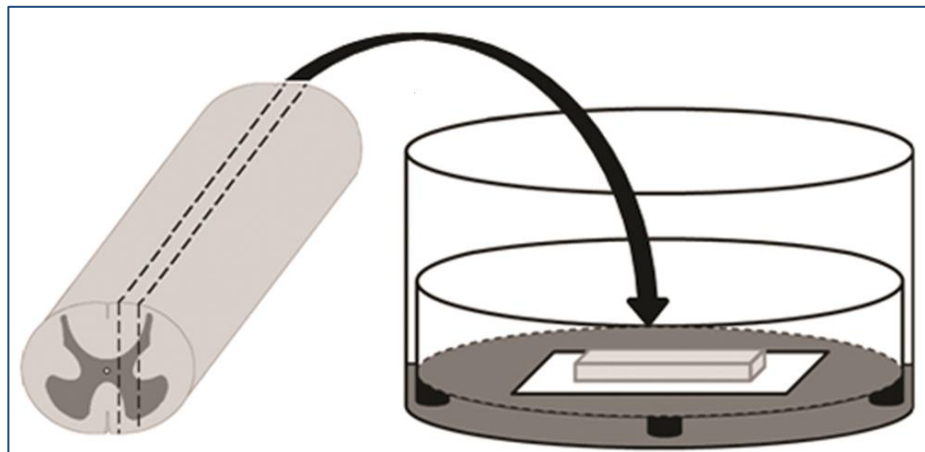


Figure 2.4 The production of organotypic spinal cord slices. *Schematic diagram illustrating the longitudinal section of spinal cords cut and transferred to culture inserts. Figure adapted from (Weightman et al., 2014).*

2.17 Lesioning spinal cord slices

The medium was refreshed prior to lesioning which was performed inside a laminar flow hood using a dissection microscope at 12.5 x magnification. To stabilize the slice during lesioning, the confetti shape was designed with elongated tabs on the sides to facilitate the use of forceps to hold the confetti and the wall of culture insert together as shown in [Figure 2.5 (A)]. Slices were lesioned after 1 DIV using a tool developed in the laboratory, constructed from two parallel surgical blades (size 15) secured into an empty scalpel holder [Figure 2.5 (B)], with small lateral movements made to ensure the complete severance of nerve fibre tracts (Weightman et al., 2014). Any remaining tissue between the two lesion margins was subsequently removed using an aspirator, fitted with a 200 μ L pipette tip.

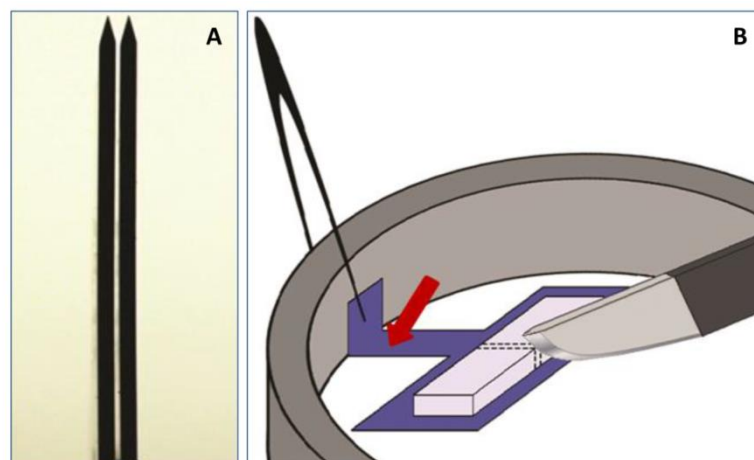


Figure 2.5 Inducing defined lesions in spinal cord slice cultures. (A) Photograph of the assembled scalpel blades, demonstrating equal spacing along the length of the blades which around 439 μ m. (B) Slices were lesioned by gripping an elongated section (red arrow) of confetti (purple) and the insert wall together (grey) using forceps to fix the slice. Figure adapted from (Weightman et al., 2014).

2.18 Transplantation procedure

2.18.1 Preparation of NSCs for transplantation

NSCs were isolated from the SVZ of CD1 mice pups as described in **section 2.4** and cultured in neurosphere medium. Briefly, on the day of transplantation, the neurospheres were dissociated into single cells using a mix of accutase and DNase I. Cells were resuspended at 1.2×10^4 cells in neurosphere medium and 0.25 μ L was pipetted onto the lesion site of spinal cord slices using a Hamilton syringe. After one day of lesion, 10 μ M MINO treatment and NSCs transplantation were started then the slices were incubated at 37°C in humidified 95% air/5% CO₂ for up to one week with the medium changed every two days. Lesioned slice cultures without any treatment were used as control and the treated cultures were compared to untreated controls from the same experiment.

2.18.2 Labelled NSCs transplantation into organotypic spinal cord slices

For transplantation of labelled NSCs into lesions of the organotypic spinal cord slice model, NSCs were labelled using two different ways:

2.18.2.1 DAPI labelling

Cell labelling is an essential component of various experimental protocols and DNA binding dyes such as DAPI are available for live cell labelling because they are membrane permeable, cost-effective, easy methodology for live staining and has no effects on NSCs viability (Leiker et al., 2008). Therefore, nuclear dye DAPI was used for monitoring transplanted NSCs in this study. DAPI can interact with both DNA and RNA, and thus can label the cytosolic as well as the nuclear

compartments. NSCs were labelled with DAPI at 20 µg/mL for 20 minutes and washed twice in PBS. NSCs were grafted into the sites of lesions as above in section 2.18.1. Slices were subsequently fixed 2 h and 1 week post-transplantation.

2.18.2.2 DiD labelling

1) Labelling NSCs with DiD in monolayer cultures: A lipophilic carbocyanine fluorescent tracking dyes such as DiD have the ability to label the cell membranes and stain the entire cell. Interestingly, they are widely used for cell transplantation in animal experiments due to their ability to trace neuronal connections in both live and fixed tissues. Most importantly, these types of dyes have lower cytotoxicity compared to cytoplasmic and nuclear dyes and they are easy and rapid to apply (Mohtasebi et al., 2014; Progatzyk et al., 2013), for these reasons DiD was chosen in this experiment.

NSCs in monolayer culture were labelled with DiD according to the manufacturer's description. Briefly, stock solution of the dye was prepared in DMSO at 2.5 mg/mL. Then 5 µL of stock solution was added to each 1 mL of cell suspension and incubated for 20 minutes at 37°C. The labelled suspension was washed twice by centrifugation, the supernatant was removed and then cells were resuspended in warm medium. Following the second wash, DiD-labelled NSCs were plated in 24-well plates with coated coverslip at a density of 0.65×10^5 cells/ml (600 µL/well). Images were taken immediately and at four days of labelling.

2) Labelling NSCs with DiD before transplantation: NSCs were stained with the DiD as above then implanted into the lesion site of spinal cord slices, the images were taken at the same day of transplantation and at four days post transplantation using dynamic time-lapse microscopy as described in **section 2.21.2**.

2.19 Fixation

Cells and slices were washed with PBS and fixed in 4% PFA for 15-20 minutes at RT. Following fixation, cells and slices were washed three times with PBS for 5 minutes to remove fixative, and stored at 4°C prior to immunolabelling.

2.20 Immunocytochemistry

2.20.1 Immunostaining of cells seeded as monolayers

Following fixation, non-specific staining was blocked (blocking solution: 5% NDS, 0.3% Triton-X-100 in PBS) for 30 minutes at RT. Primary antibodies were diluted as follows in blocking solution: nestin 1:200, SOX-2 1:1000, H300 1:100, BuGR2 1:100, β -tubulin III/TUJ-1 1:1000, GFAP 1:500, MBP 1:200, added to the cells and incubated overnight at 4°C. Stained cells were washed three times in PBS, blocked for 30 minutes at RT and incubated with the appropriate FITC- or Cy3-conjugated secondary antibody in blocking solution (1:200 dilution, RT; 2 h). Cells were then washed three times with PBS at RT, and mounted with DAPI. Table 2.1 summarises the antibodies used to detect specific cell types and the immunostaining protocols.

2.20.2 Immunostaining of cryostat sections

For staining cryostat sections stored in a freezer, the slides were thawed at RT for a few minutes. The samples then were surrounded with a hydrophobic barrier using a Pap pen to prevent the spreading of reagent over the entire surface of the slide and allow using small quantities of reagent with minimal risk of drying. Then samples were rinsed twice with PBS. Immunostaining was performed as above in **section 2.20.1**.

2.20.3 Immunostaining of organotypic spinal cord slices

Immunocytochemical staining of slices was performed following the protocol as described in **section 2.20.1**. However, the blocking solution for lectin consisted of 10% NDS in PBS to reduce non-specific background staining during the longer incubation periods. The incubation length of lectin was increased to 36 h (at 4°C) to facilitate antibody penetration through the tissue. Whereas the blocking solution for TUJ-1 and GFAP staining consisted of 5% NDS in PBS with 0.3% Triton X-100 and the incubation times were for 24 h at 4°C to further facilitate antibody penetration. Samples were incubated with lectin (1:200), (TUJ-1; 1:1000) and (GFAP; 1:500) in blocking solution. Following PBS washes, samples were incubated with FITC-conjugated secondary antibodies (1:160 anti-biotin, otherwise 1:200), the incubation times were increased from 2 h at 4°C, to 3 h at 4°C for anti-biotin and 4 h at RT or 4°C for TUJ-1 and GFAP. Slices were subsequently washed three times with PBS and mounted with Vectashield mounting medium containing DAPI.

Table 2.1 Antibodies and Immunostaining Protocols

Antibody	Supplier	Blocking solution in phosphate buffered saline (PBS)	Antibody concentration	Description
Mouse anti-nestin neural stem cell marker	BD Biosciences, Oxford, UK	5% serum, 0.3% Triton	1:200	NSCs cytoskeletal protein
Rabbit anti-SOX-2 neural stem cell marker	Millipore, Watford, UK,	5% serum, 0.3% Triton	1:1000	Transcription factor express in NSCs
Rabbit anti-GFAP astrocyte marker	DakoCytomation Ely, UK	5% serum, 0.3% Triton	1:500	Cytoskeletal protein in astrocytes
Rabbit anti-TUJ-1 neuron marker and mouse anti-TUJ-1 neuron marker	Covance, Princeton, TJ, USA	5% serum, 0.3% Triton	1:1000	Major constituent of microtubules in neuronal cell bodies and axons
Rat anti-MBP, oligodendrocyte marker	Serotec Kinlington, UK	5% serum, 0.3% Triton	1:200	Main component of myelin produced by oligodendrocytes

Rabbit anti-glucocorticoid receptor, H300	Santa Cruz Biotech, USA	5% serum, 0.3% Triton	1:100	Glucocorticoids receptor
Mouse anti- glucocorticoid receptor, BuGR2	Abcam, U.K.	5% serum, 0.3% Triton	1:100	Glucocorticoids receptor
Lectin (<i>Lycopersicon esculentum</i> , biotin-conjugated), microglial marker	Sigma-Aldrich, U.K.	5% serum	1:160	A protein lectin with specific affinity for poly-N-acetyl lactosamine sugar residues which are located on the plasma membrane and in the cytoplasm of microglia
Rabbit anti- growth associated protein GAP-43	Abcam, U.K.	5% serum, 0.3% Triton	1:500	Major component of the motile "growth cones" that form the tips of elongating axons

2.21 Microscopy and image analyses

2.21.1 Fluorescence microscopy

A Leica DM IL LED inverted microscope fitted with a DFC 420 C digital camera and a pE-300 W CoolLED fluorescence unit, was used to capture images from well-plates using the Leica Application Suite imaging software, version 3.3.1. Fluorescence images were taken using an Axio Scope A1 microscope equipped with an Axio Cam ICc1 digital camera and AxioVision software (release 4.7.1, Carl Zeiss MicroImaging GmbH, Goettingen, Germany). Photoshop CS3 (Adobe, USA) was used to merge counterpart images; three channels were merged to form 'triple merge' images. At least four microscopic fields were captured per condition for subsequent analysis using ImageJ software. To validate proteomic findings regarding GAP-43 expression, fluorescence micrographs of control and CS-treated NSCs and their daughter cells (neurons) were taken. The relative expression of GAP-43 protein was quantified using optical density measurements of individual cells (minimum of 30 nestin⁺ NSCs and TUJ-1⁺ neurons four images per condition per culture). Briefly, using ImageJ software, fluorescence images were converted to grayscale, inverted and calibrated using an optical density step tablet before fluorescent intensity readings were taken from each cell expressing GAP-43. Readings were taken to correct for background fluorescence, these values then subtracted from the mean fluorescence intensity value obtained from the cells, with the resulting value representing a measure of the extent of GAP-43 expression in NSCs and neurons.

2.21.2 Dynamic time-lapse microscopy

Lesioned spinal cord slices (within 35 mm Petri dishes) at two DIV were placed in an incubation chamber (preheated to 37 °C; 5% CO₂ /95% humidified air) on a time-lapse microscope (Axiozoom V16, AxioCam ICm1 camera Carl Zeiss, Germany). Time-lapse imaging commenced on the same day as transplantation of NSCs. Lesioned slices 4 days post-transplantation were similarly recorded to observe the NSCs labelled with DiD in the injury sites.

2.22 Histological analyses of NSCs culture properties

Assessment of cell health in monolayer and neurosphere cultures within each experimental conditions was quantified via culture characterisation such as average cell count (the number of nuclei/image), culture purity (percentage of nuclei co-localised with nestin⁺ or SOX-2⁺ expression) and incidence of pyknosis (as a percentage of healthy plus pyknotic nuclei) which was identified by nuclear shrinkage, fragmentation or DNA condensation. Cells were stained with DAPI and the percentage of DAPI-stained nuclei exhibiting pyknotic features was expressed as a proportion of total DAPI-stained nuclei.

For daughter cell types (neurons, astrocytes, and oligodendrocytes), the number of TUJ-1 (neuron), GFAP (astrocyte) or MBP (oligodendrocyte) expressing cells were expressed as a proportion of at least 100 viable DAPI-stained nuclei over a minimum of four microscopic fields per condition.

The culture characteristics, experimental outcomes and cellular assessments in NSCs parent cells (monolayer and neurosphere experiments) and their daughter cells were quantified using fluorescence images, which were taken from four random fields with at least 100 nuclei assessed from each experimental condition.

2.23 Histological analyses of organotypic spinal cord slice culture

2.23.1 Slice viability

An assay was used to investigate the proportions of live and dead cells within slices before and after lesioning, using live/dead staining (Weightman et al., 2016, 2014). Spinal cords were extracted from P0-P5 mice, lesioned after 1 DIV and fixed 8 days post-lesioning (n = 5). Fluorescence micrographs (live; green and dead; red) of each slice were captured at 50 x magnification with same exposure settings. The micrographs were converted into greyscale, ImageJ software was used to calculate the corrected integrated density from both live and dead stained micrographs (Weightman et al., 2014).

2.23.2 Astrocyte reactivity analysis

An assay was devised to quantify the relative expression of GFAP (as a function of immunofluorescence intensity) at slice lesion margins versus areas in the body of the slice. Spinal cords were extracted from P0-P5 mice, lesioned after 1 DIV and fixed 8 days post-lesioning (n = 5). Each half of a lesioned slice was stained with GFAP to quantify the relative expression of GFAP at slice lesion margins across experimental conditions. Separate images were taken for each half of a lesioned slice at 100 x magnifications with the same exposure setting across all images. Fluorescence images were converted to grayscale using ImageJ software and 400 μm lines were drawn into the slice body of each image. Then, three points were marked along the length of the lesion margins to show the part of the lesion edge. Optical density profiles were measured using ImageJ software (Weightman et al., 2014).

2.23.3 Quantification of nerve fibre outgrowth

The average outgrowth density and variation of outgrowth density of nerve fibres were quantified across lesion sites. Spinal cords were extracted from P0-P5 mice, lesioned after 1 DIV and fixed 8 days post-lesioning (n = 5). Fluorescence micrographs of slices immunostained with TUJ-1 antibodies were used to generate optical density profiles in ImageJ software of the number of TUJ-1⁺ nerve fibres parallel to the lesion margins, across the width of the slice regions demonstrating nerve fibre outgrowth. Fluorescence micrographs were generated at 200 x magnification. A rectangular grid was subsequently overlaid onto each image, with marks at 20 µm intervals along the length of the lesion site. Optical density profiles were then generated at the level of each interval, across the width of the slice regions with nerve fibre outgrowth (Weightman et al., 2014).

2.23.4 Microglial infiltration analysis

The number of microglia was quantified in the lesion sites to assess the microglia infiltration. Spinal cords were extracted from P0-P5 mice, lesioned after 1 DIV and fixed 8 days post-lesioning (n = 5). The numbers of lectin-positive (lectin⁺) microglia were counted within the lesion site of each slice. The total number of microglia per unit area per slice was averaged at each experimental condition (Weightman et al., 2014).

2.24 Statistical analysis

All experimental data were analysed using Prism statistical analysis software (GraphPad, USA; version 7.0) and all data are presented as mean \pm standard error of the mean (S.E.M). Data were analysed by one-way ANOVA with post-hoc analysis carried out using Bonferroni's multiple comparison test (MCT). Optical density measurements of GAP-43 expression were averaged for each culture/condition and then analysed by unpaired t test. The numbers of experiments (n) relate to the number of cultures, each generated from a different litter.

Chapter 3

Histological study to evaluate the effects of CS treatment on NSCs propagated in monolayer cultures

3.1 Introduction

CSs are small lipid-soluble molecules which can readily pass through the BBB and bind to intracellular receptor to exert their effects on the CNS (Joëls, 1997). CSs are widely used in the treatment of a range of paediatric/adult clinical conditions, in addition to their use following stem cell transplantation therapy to reduce inflammation and graft rejection (Garrison et al., 2000; Mazzini et al., 2015; Rhen and Cidlowski, 2005). However, a number of neurological studies demonstrate the detrimental effects of CS including cerebral palsy and neurodevelopment impairment (Chari, 2014; Shinwell and Eventov-Friedman, 2009). There is increasing evidence that CSs can adversely influence key biological properties of NSCs but the molecular mechanisms underpinning such effects are largely unknown (Melanie et al. 2014). This is an important issue to address given the key roles NSCs play during brain development and as transplant cells for regenerative neurology.

A number of *in vivo* and *in vitro* experimental studies have suggested that at least some of the adverse consequences of CS mediated via effect on NSCs. In this respect, several studies demonstrated that CSs alter the phenotype of NSCs such as **proliferation and differentiation**. For instance, it was found that the exposure of NPCs to DEX leads to a reduction of the differentiation of astrocytes without any effects on the neuronal differentiation and oligodendrocytes (Sabolek et al. 2006; Wagner et al. 2009). In 2010, Bose *et al.* found that DEX treatment reduces the proliferation of rat NSCs without any effects on the differentiation *in vitro* experimental model (Bose et al., 2010). Similar *in vitro* and *in vivo* results have shown that DEX reduces the proliferation of adult rat NPCs without any effects on differentiation (Kim et al., 2004). Another *in vitro* study observed that DEX reduces

the growth of NPCs without promoting cell death while, *in vivo* study found that DEX significantly reduces the somatic and brain weights (Ichinohashi et al. 2013). Also, it was found that CORT markedly decreases the survival and differentiation of embryonic NPSCs without any effects on the proliferation *in vitro* (Odaka et al., 2016). Additionally, *in vitro* SCI mouse model have observed the anti-proliferative effects of MPRED on the endogenous NPCs (Li et al., 2012). Similar *in vitro* studies have shown the anti-proliferative effects of CS on NPCs derived from the adult hippocampus (Obermair et al., 2008; Schröter et al., 2009; Wang et al., 2014).

Based on the above studies, CS-mediated reduction in the proliferation of NSCs and alteration of their differentiation may account for some of the observed behavioural and neurodevelopmental consequences after CS treatment.

In regard to CS effects on the **apoptosis** of NSCs and NPCs, in 2008, Noguchi *et al.* treated P7 (postnatal seven days) mice with DEX to examine its effect on NPCs. They found that DEX induces apoptosis specifically in the NPCs of the cerebellar external granule layer. Also, both DEX and BETA were found to induce the apoptosis of NPCs in the cerebellar external granule layer of mice (Noguchi et al., 2008, 2011). In 2012, Mutsaers and Tofighi studied the effects of CS on the phenotype of mouse NSCs and the mechanisms underlying their effect. They demonstrated that the exposure of mouse NSCs to CSs increased their susceptibility to 2,3-methoxy-1,4-naphthoquinone-induced apoptosis and this effect is mediated by the stimulation of GRs (Mutsaers and Tofighi, 2012). Using *in vivo* experimental rat model, Sze and colleagues gave injections of DEX to the animals on P1 and then sacrificed these on P2, P3, P5 and P7. They divided the

animals into two groups: the first one was treated with DEX and the second group injected with the same volume of normal saline as control group. Their findings demonstrated that the administration of DEX caused apoptosis of NPCs in the hippocampus of rat pups (Sze et al., 2013). Whereas, Bhatt and colleagues found that CSs induced the apoptosis of NPCs derived from the SVZ and dentate gyrus of the developing rat brain by increasing caspase 3 activity and DNA fragmentation. Further, they observed a reduction in the body and brain weights following CS treatment compared with the control (Bhatt et al., 2013). The discrepancy between these various experiments may be due to the variations in experimental design, differences in methodology and the different origin of cells. Table 3.1 summarises a number of *in vitro* and *in vivo* experiments investigating CS effects on neural cells.

Taken together, the findings from above studies suggest that CS treatment can detrimentally affect NSCs proliferation, differentiation and survival, potentially resulting in neurological deficits. However, the literature pertaining to CS effects on NSCs contains contradictory information regarding the adverse influence of CS on key biological properties of NSCs illustrating the need for a systematic study of CS effects on the fate of NSCs.

As described in the general introduction **section 1.3.3**, NSCs are routinely propagated in neuroscience laboratories using two distinct culture systems: as 2-D adherent cells termed **monolayers** and 3-D cell aggregates in suspension, termed **neurospheres**. Importantly, these two culture systems are used when expanding NSCs (including human NSCs) for pre-clinical and clinical cell transplantation. In

general, both culture systems of NSCs behave in different manner and that may cause an effect on the regenerative outcomes after CS treatment. It is therefore important to investigate the effects of CS on the fate of the NSCs propagated in 2-D and 3-D culture systems. This chapter aims to investigate the effects of three clinically relevant drugs of CS on NSCs when grown in monolayer culture.

Table 3.1 Summary of <i>in vitro</i> and <i>in vivo</i> experiments investigating effects of CS on neural cells					
Type of study	CS	Species	Type of cells	Neurological effects	Reference
<i>In vitro</i>	DEX	Rat	NPCs	Reduces the proliferation of NPCs and the differentiation of astrocyte	(Wagner et al., 2009)
	DEX	Rat	NPCs	Reduces the proliferation of NPCs	(Kim et al., 2004)
	DEX	Rat	NSCs	Reduces the proliferation of the parent NSCs and changes the expression of genes of the cell cycle, cellular senescence and mitochondrial function	(Bose et al., 2010)
	DEX	Rat	NPCs	Reduces the growth of NPCs without promote cells death	(Ichinohashi et al., 2013)
	MPRED	Mouse	NPCs	Reduces the proliferation of NPCs by changing gene expression	(Li et al., 2012)

<i>In vivo</i>	DEX	Mouse	NPCs	Induces apoptosis specifically in the NPCs of the cerebellar external granule layer	(Noguchi et al., 2008)
	DEX	Mouse	NSCs	Alters the phenotype of NSCs and increase their susceptibility to 2,3-methoxy-1,4-naphthoquinone-induced apoptosis	(Mutsaers and Tofighi, 2012)
	DEX and BETA	Mouse	NPCs	Induce apoptosis of NPCs	(Noguchi et al., 2011)
	DEX	Rat	NPCs	Reduces the body and brain weight and induces apoptosis of NPCs	(Bhatt et al., 2013)
	DEX	Rat	NPCs	Induces apoptosis of NPCs	(Sze et al., 2013)
	DEX	Rat	NPCs	Reduces the body and brain weight and induces apoptosis in the hippocampus	(Ichinohashi et al., 2013)

3.2 Chapter objectives

This chapter was undertaken to investigate the effects of three different types of CS at three different concentrations on NSCs in monolayer cultures.

The objectives of this chapter are:

- 1. To investigate the effects of CS on NSCs survival/proliferation.**
- 2. To investigate the effects of CS on the generation of daughter cells from NSCs.**

3.3 Results

3.3.1 Physiological potency of CS preparations

CSs are known to affect the proliferation and morphology of immune cells such as microglia (Jenkins et al., 2014). Therefore, primary microglial cells were used for positive-control experiments. Primary microglial cells treated with 10 μ M of all three types of CS (DEX, PRED and MPRED) to assess the biological efficacy of these drug preparations. Phase-contrast microscopy showed there is a reduction in cell number and alteration in the morphologies of microglial cells in CS treated microglia versus untreated cells. The treated cells were appeared as floating and rounded compared to branching ramified untreated cells, confirming the biological potency of the CS preparations (Figure 3.1). Also, the spectrophotometrically confirmed that drug doses were appropriate for eliciting cellular responses.

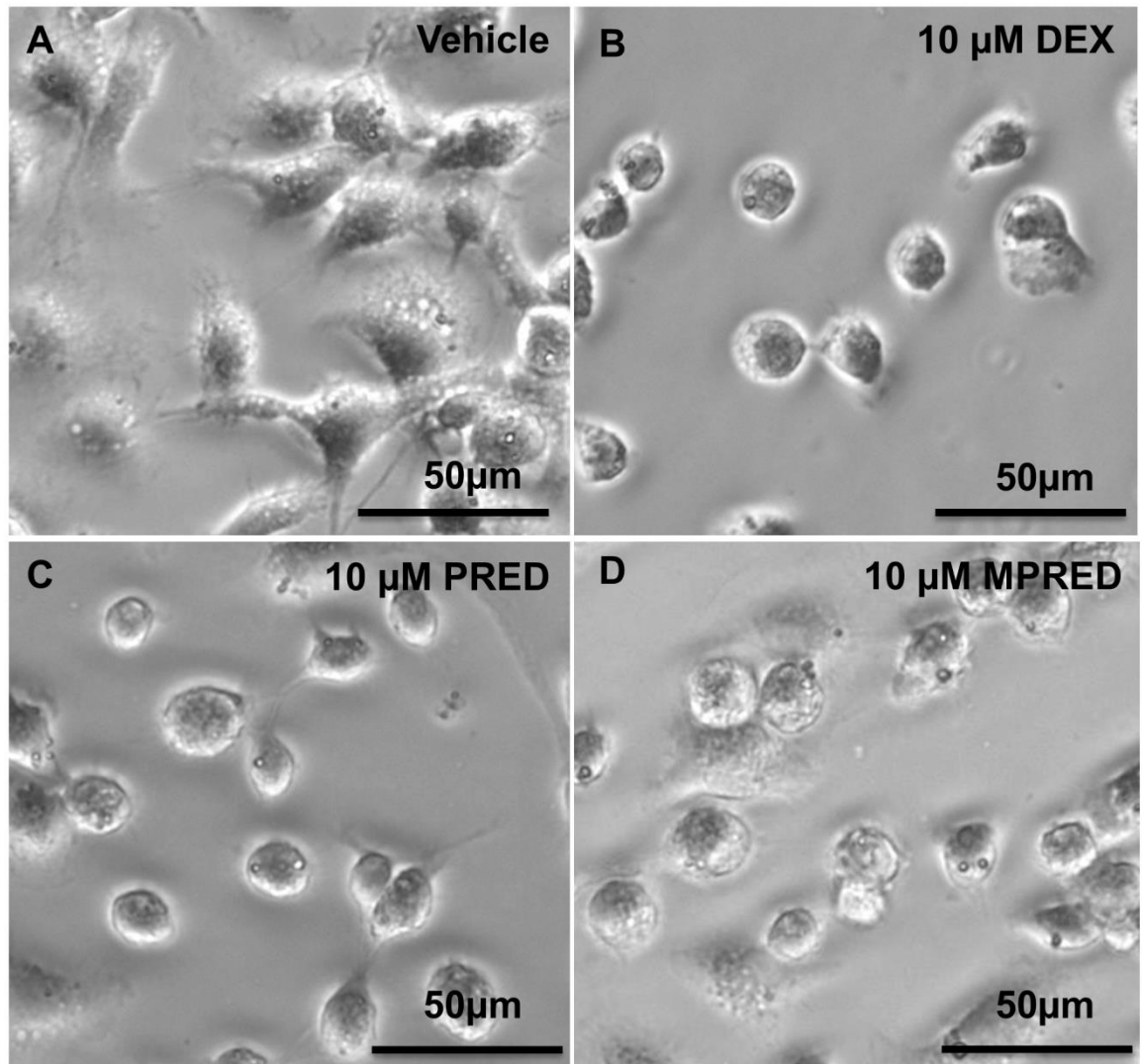


Figure 3.1 Assessment of the biological efficacy of CS preparations. (A) Phase contrast micrograph depicting primary culture of microglia in vehicle control. (B, C and D) are showing a reduction in cell number and altered morphologies of microglia following CSs treatment (48 h CSs, 10 μM).

3.3.2 Effects of CS on the number and proportion of NSCs

To determine the effects of CS on NSCs, the monolayer cultures were treated with three different types of CS (DEX, PRED and MPRED) in various concentrations (0.1 μ M, 1 μ M and 10 μ M). The numbers of NSCs were counted after 48 h of incubation. Immunostaining for nestin revealed that 10 μ M of all three types of CS significantly reduced the total counts of NSCs compared with control (Figure 3.2A-G). Monolayer cultures showed elongated and bipolar morphologies typical of NSCs and were of high purity as judged by immunostaining. The number of nestin positive cells was quantified in all conditions to assess NSCs 'stemness'. Here, >99% of cells were nestin positive across all conditions. Accordingly, no differences in proportions of nestin positive cells were observed between treatment conditions and controls (Figure 3.2H-J).

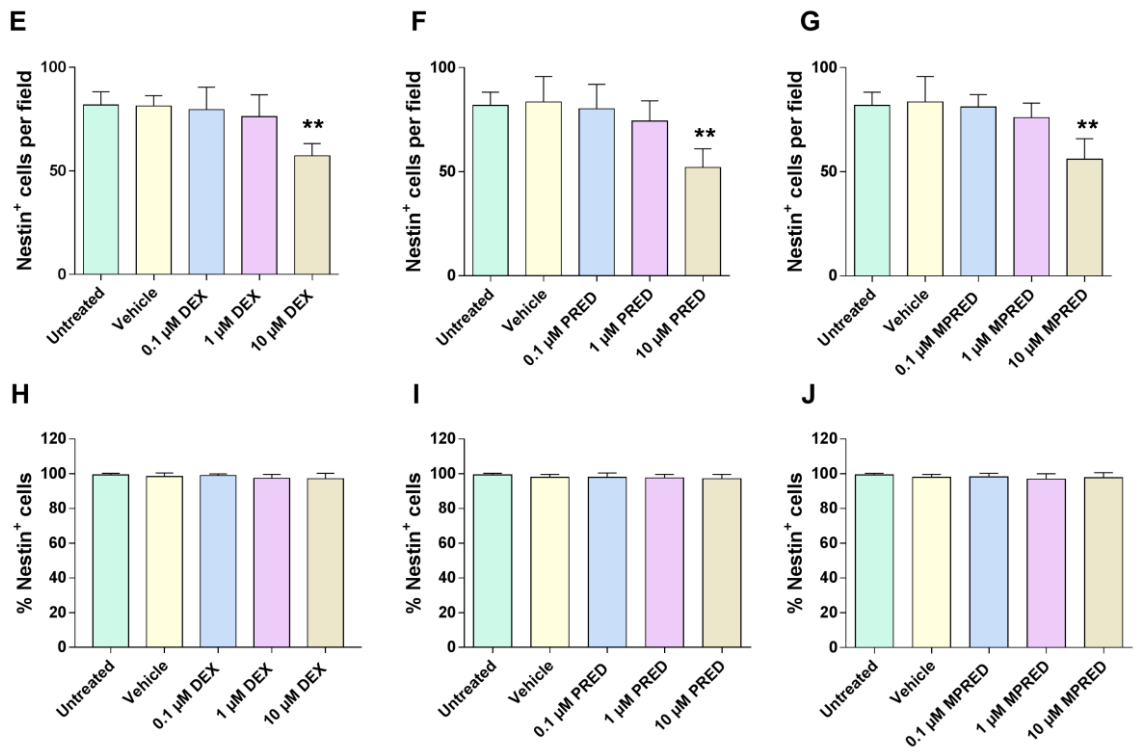
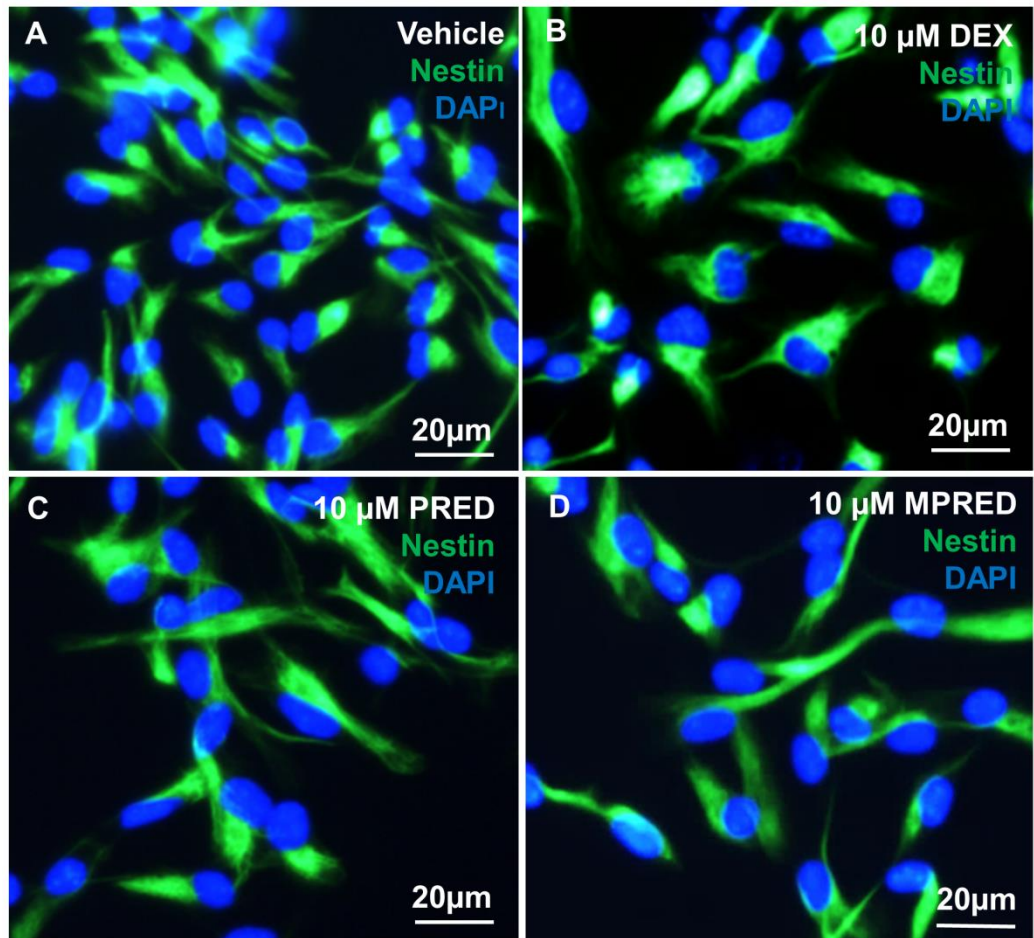


Figure 3.2 Effects of CS on the number and proportion of NSCs. *Fluorescence images showing nestin positive NSCs in vehicle control (A) and 10 μ M CSs treated NSCs (B, C and D). (E, F and G) Bar charts displaying the total number of NSCs per field across treatment conditions following CSs treatment. (H, I and J) Bar charts showing the proportions of nestin positive NSCs across all conditions following CSs treatment. (48 h CSs; 0.1 μ M, 1 μ M and 10 μ M); ** p <0.01 versus vehicle control NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in six biological replicates, with two technical replicates in each experiment.*

3.3.3 Effects of CS on the proliferation and cell cycle of NSCs

EdU proliferation assay was used to assess the proliferative capacity of the NSCs in monolayer cultures following CS treatment, as detailed previously in Chapter 2 **section 2.10**. The highest concentration (10 μM) of CSs significantly decreased the percentage of EdU⁺ cells i.e. decreased the proliferation of NSCs (Figure 3.3). Additionally, cell cycle analysis was employed to assess whether the reduction of NSCs proliferation is associated with changes in the proportions of cells in individual phases of the cell cycle after CSs treatment. The results showed that in parallel with reduced proliferation, the exposure of NSCs to the highest concentration (10 μM) of CSs led to a significant increase of cells in the G0/G1 phase and to a parallel decrease of cells in both S and G2/M phases, when compared with controls (Figure 3.4).

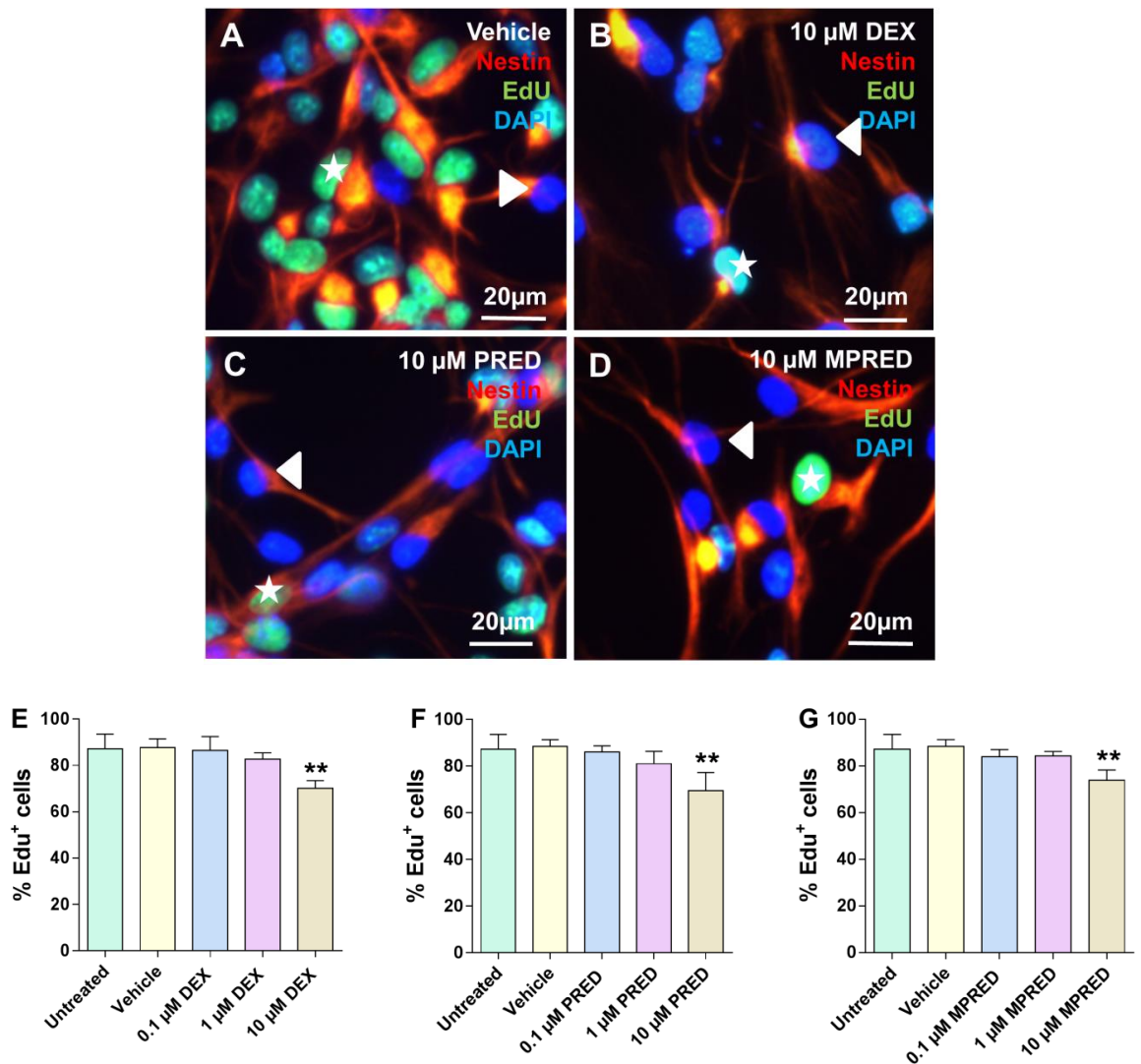


Figure 3.3 Effects of CS on the proliferation of NSCs. Triple merged fluorescence images depicting EdU positive NSCs in vehicle control (A) and 10 μM CSs treated NSCs (B, C and D), an asterisk denotes double-labelled cells; arrowhead indicates NSCs that did not express EdU marker. (E, F and G) Bar charts showing proportions of EdU incorporating cells across treatment conditions. (48 h CSs; 0.1 μM, 1 μM and 10 μM); ** $p < 0.01$ versus vehicle control NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Proliferation assay was carried out in three biological replicates, with two technical replicates in each experiment.

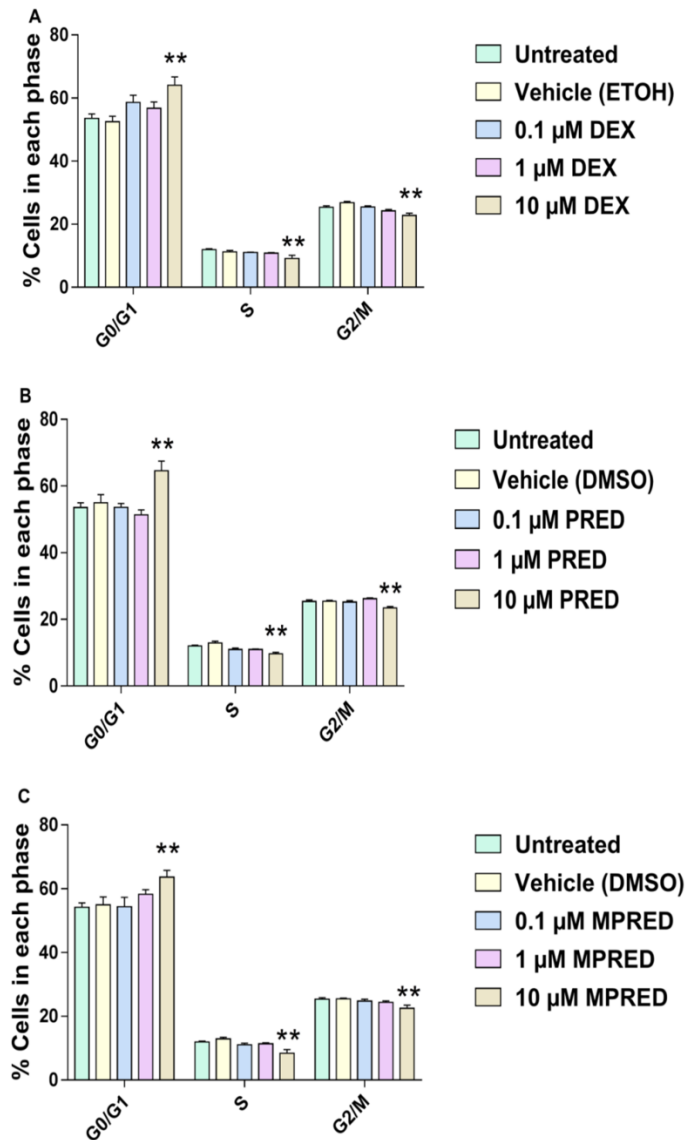


Figure 3.4 Effects of CS on the cell cycle of NSCs. (A) Bar chart showing the percentage of the cells in G0/G1, S and G2/M phases of cell cycle following DEX treatment. (B) Bar chart displaying the percentage of cells in G0/G1, S and G2/M phases of the cell cycle following PRED treatment. (C) Bar chart showing the percentage of cells in G0/G1, S and G2/M phases of the cell cycle following MPRED treatment. (48 h CSs; 0.1 μ M, 1 μ M and 10 μ M); ** p <0.01 versus untreated and vehicle control NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell cycle analysis was carried out in three biological replicates, with three technical replicates in each experiment.

3.3.4 Effects of CS on the viability of NSCs

Live/dead staining was used to assess the effects of CS on the viability of NSCs. NSCs were treated with DEX, PRED and MPRED at three different concentrations (0.1 μ M, 1 μ M and 10 μ M). The viability assay was performed following 48 h of CSs treatment on NSCs in monolayer cultures. After live/dead staining, the majority of cells had normal bipolar NSCs morphologies appearing green (LIVE) with small numbers of rounded cells appearing red (DEAD) in all conditions (Figure 3.5A-D). Live/dead staining reveals high cellular viability after CS treatment at all doses, similar to the viability of control cultures (Figure 3.5E-G).

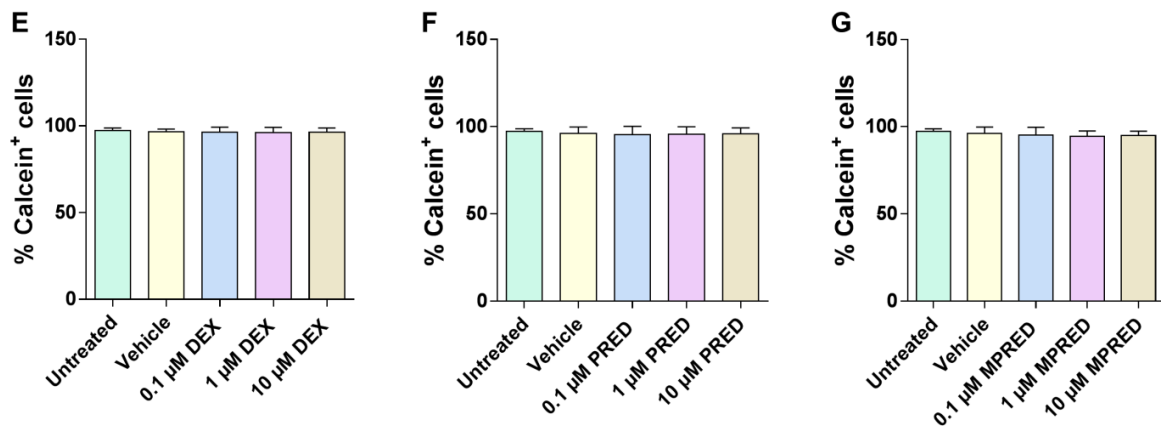
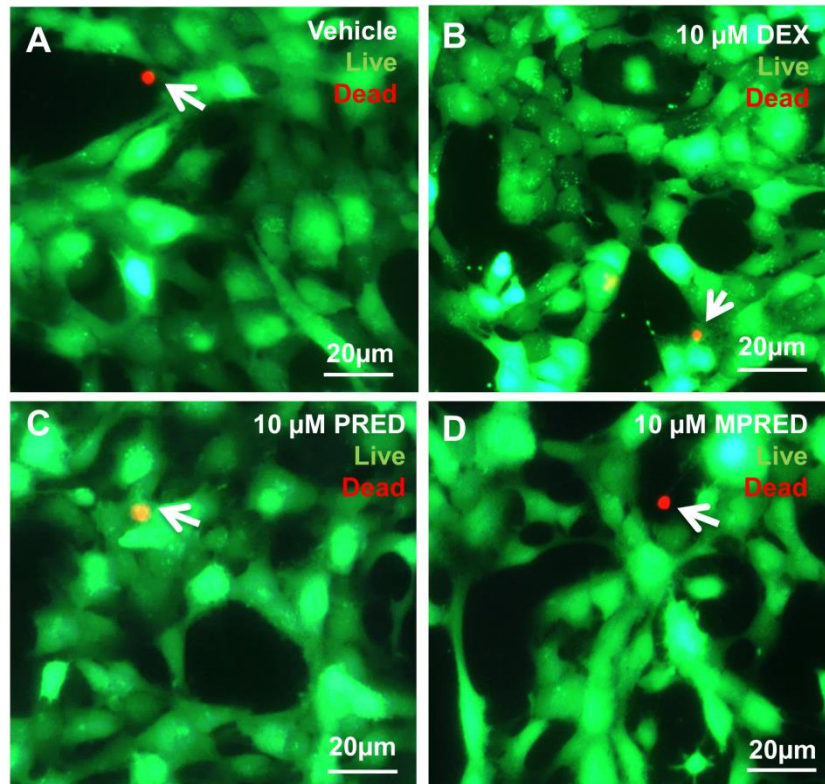


Figure 3.5 Effects of CS on the viability of NSCs. *Representative live/dead fluorescence images of vehicle control (A) and 10 μM CSs treated (B,C and D) NSCs 48 h after CSs treatment. LIVE cells appear green and DEAD cells appear red (arrows). (E, F and G) Bar charts showing the proportion of viable cells, (48 h CSs; 0.1 μM, 1 μM and 10 μM); no significance; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Viability assay was carried out in three biological replicates, with two technical replicates in each experiment.*

3.3.5 Effects of CS on the apoptosis and pyknosis of NSCs

Annexin V staining was used to assess the apoptosis of NSCs following 48 h of CSs treatment. NSCs were treated with DEX, PRED and MPRED at three different concentrations (0.1 μ M, 1 μ M and 10 μ M). The results showed that the apoptosis of NSCs remained low (*ca.* 4%) across all conditions following CS treatments (Figure 3.6A-C). The numbers of pyknotic nuclei (i.e. shrunken or fragmenting morphologies) in NSCs monolayer cultures were assessed by DAPI staining following CSs treatment. Across all treatment conditions a small proportion (<2%) of nuclei were observed as pyknotic (Figure 3.6D-H). These findings suggest that all three type of CS did not affect the apoptosis and pyknosis of NSCs in monolayer cultures.

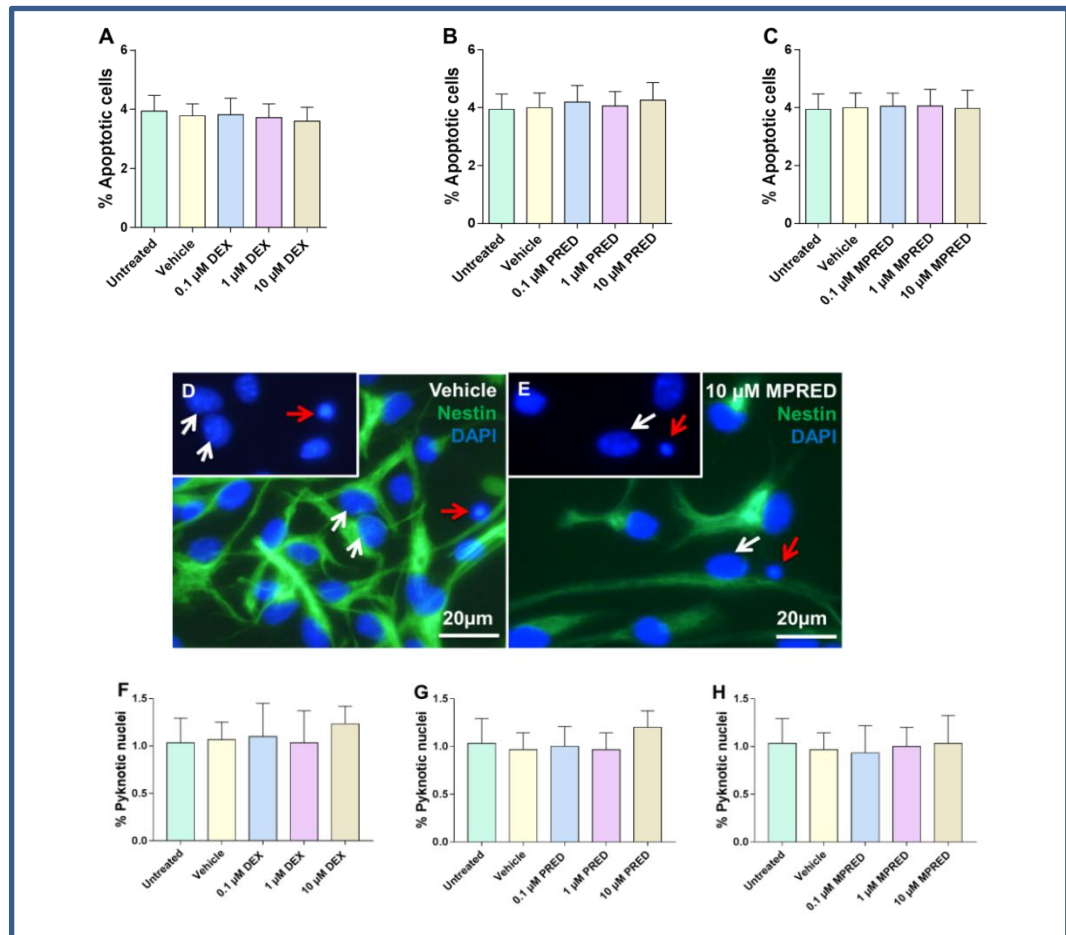


Figure 3.6 Effects of CS on the apoptosis and pyknosis of NSCs. (A, B and C) Bar charts displaying the percentage of apoptotic cells following CSs treatment. The pyknosis of NSCs in monolayer cultures were assessed by identifying cells with fragmenting and condensing nuclei. (D and E) fluorescent images showing the pyknotic nuclei (red arrows indicate same pyknotic nuclei in main image and inset). Healthy nuclei were associated with adherent cells and normal nestin staining (white arrows indicate same cells in main image and inset). (F, G and H) Bar graphs displaying the percentage of pyknotic nuclei following CSs treatment, (48 h CSs; 0.1 μM, 1 μM and 10 μM); no significance; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Apoptosis assay was carried out in three biological replicates, with three technical replicates in each experiment. Pyknotic nuclei were carried out in six biological replicates, with two technical replicates in each experiment.

3.3.6 Effects of CS treatment on the differentiation of NSCs

Part of the therapeutic potential of NSCs depends on their ability to produce their daughter cells for replacing neural cells that are damaged following neurological injury and disease. Therefore, the differentiation assay was conducted to assess the effects of CS on the proportion of each daughter cell type. Daughter cells produced from CS-treated NSCs populations were treated with three different types of CS (DEX, PRED and MPRED) in various concentrations (0.1 μ M, 1 μ M and 10 μ M).

Fluorescence micrographs of astrocytes showed similar staining profiles and morphologies in vehicle control and treated cultures, suggesting no significant difference on astrocyte morphology/maturation following 9 days of CS treatments (Figure 3.7A-D). There was also no significant difference in numbers, and proportion of astrocytes per field that were derived from NSCs across all treatment groups (Figure 3.7E-J).

In contrast to astrocytes, the highest concentration (10 μ M) of CSs reduced significantly the number and proportion of neurons compared to control (Figure 3.8A-J). 10 μ M CSs treatment also resulted in a significant reduction in the length of axons compared to the untreated cells (Figure 3.8K-M).

Additionally, the microscopic observations suggested that oligodendrocytes in 10 μ M of CSs-treated cultures had a greater membrane surface area with extensive and complex branching of processes (Figure 3.9A-D). The results also showed no difference in the number and proportion of oligodendrocytes generated per field after CSs treatment (Figure 3.9E-J). The maturation of oligodendrocytes was

confirmed by the total cell area measurements wherein control cells and vehicle showed less maturation of oligodendrocytes, while 10 μ M CSs-treated cells accelerated the maturation profile of oligodendrocytes (Figure 3.9K-M). These findings suggested that 10 μ M of DEX, PRED and MPRED reduced the number and the axonal length of neurons and increased the maturation of oligodendrocytes without any effects on the number and proportion of astrocytes and oligodendrocytes.

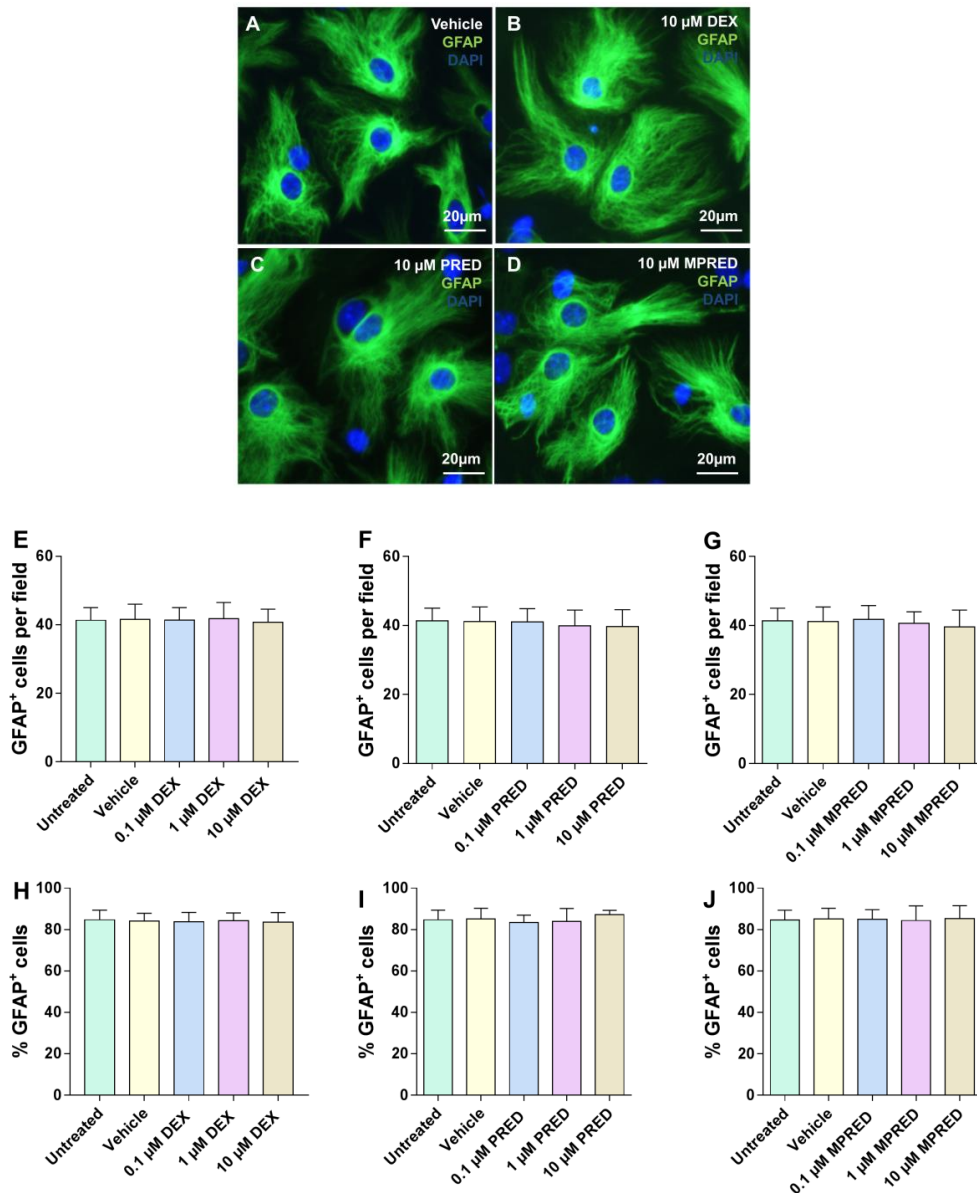


Figure 3.7 CSs treatment showed no effect on astrocytes generated from NSCs. Representative fluorescence micrographs depicting astrocytes generated from vehicle control (A) and 10 μ M CSs-treated (B, C and D) differentiated NSCs. (E, F and G) Bar charts displaying the total number of GFAP + cells per field. (H, I and J) Bar charts showing the proportion of astrocytes. (9 days CSs; 0.1 μ M, 1 μ M and 10 μ M); no significance; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in six biological replicates, with two technical replicates in each experiment.

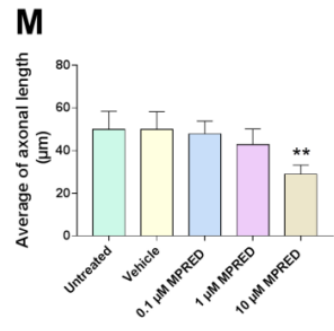
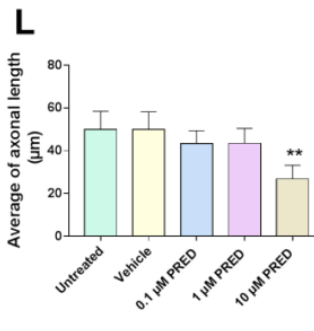
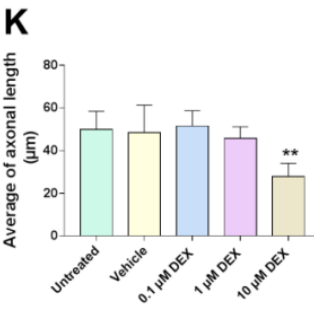
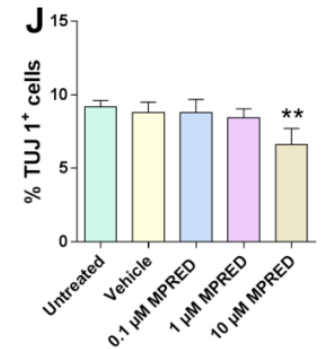
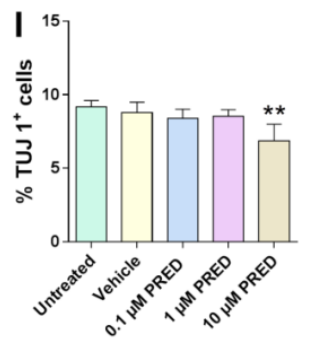
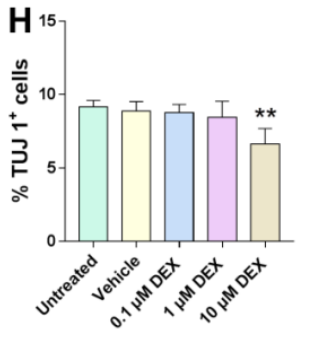
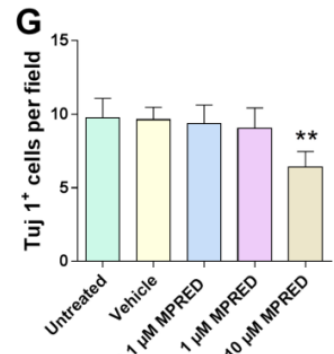
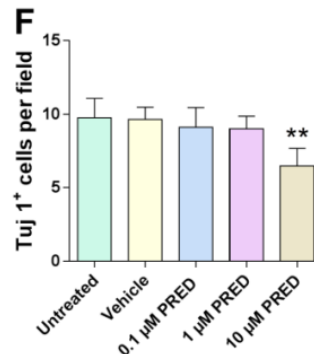
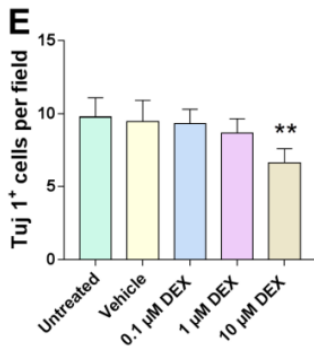
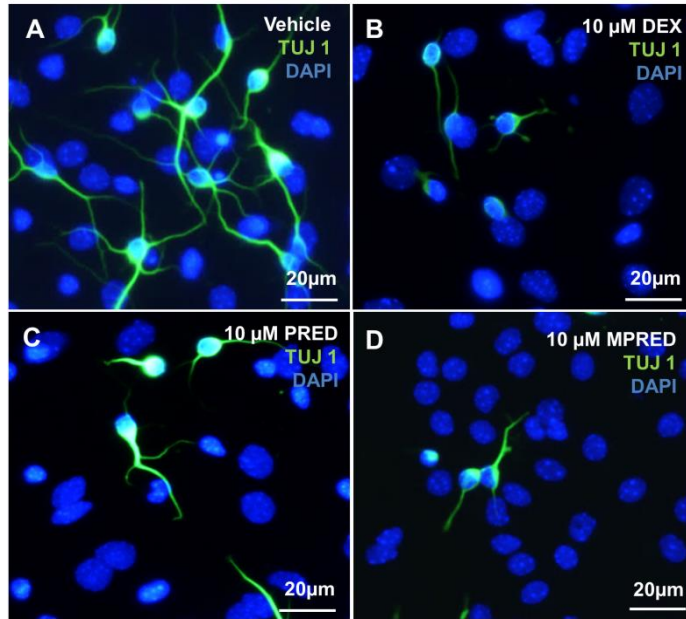


Figure 3.8 CSs treatment altered neuron cell number and morphology.

*Fluorescence micrographs of neurons derived from vehicle control (A) and 10 μ M CSs-treated (B, C and D) differentiated NSCs. (E, F and G) Bar charts quantifying the number of TUJ 1+ neurons per field across treatment conditions. (H, I and G) Bar charts showing the proportion of neurons across all treatment conditions. (K, L and M) Bar charts showing the axonal length of neurons across all conditions. (9 days CSs; 0.1 μ M, 1 μ M and 10 μ M); ** $p < 0.01$ versus vehicle control; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in six biological replicates, with two technical replicates in each experiment.*

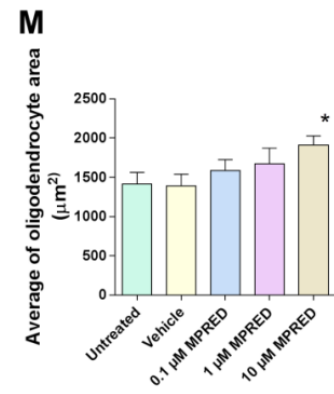
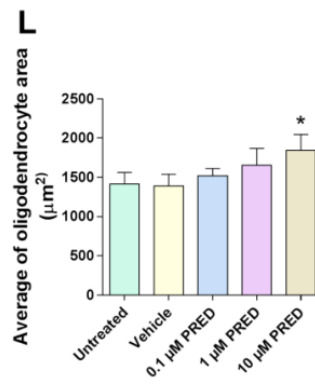
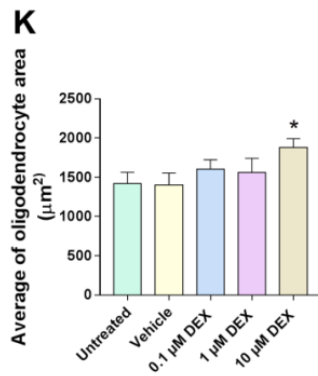
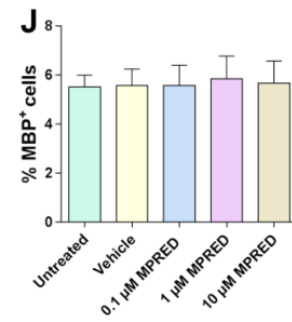
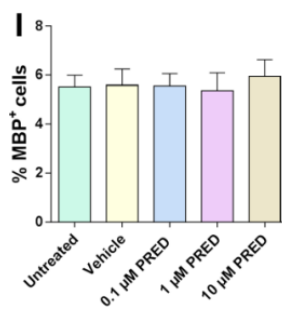
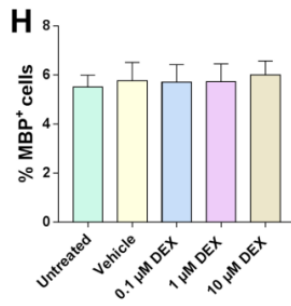
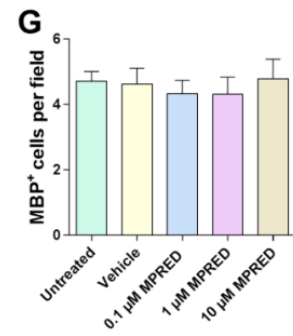
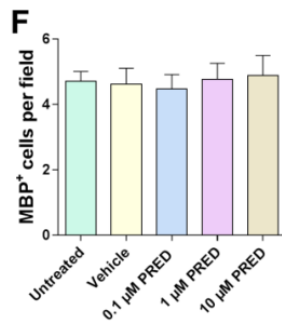
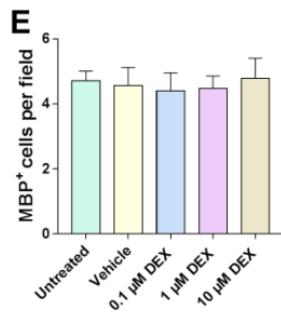
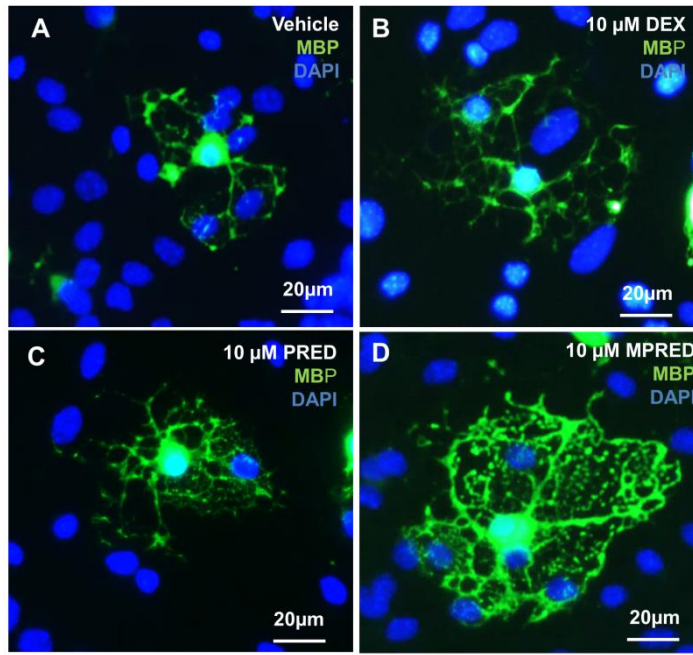


Figure 3.9 CSs treatment increased the maturation of oligodendrocytes. *Fluorescence micrographs of oligodendrocytes generated from vehicle control (A) and 10 μ M CSs treated (B, C and D) differentiated NSCs. Note the different morphologies and membrane surface areas of MBP+ oligodendrocytes in treated cultures. (E, F and G) Bar charts showing the number of oligodendrocytes per field across treatment conditions. (H, I and G) Bar charts showing the proportion of oligodendrocytes. (K, L and M) Bar charts showing the measurement of oligodendrocyte area across treatment conditions. (9 days CSs; 0.1 μ M, 1 μ M and 10 μ M); * $p < 0.05$ versus vehicle control; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in six biological replicates, with two technical replicates in each experiment.*

3.4. Discussion

This study was performed to investigate the impact of synthetic CSs on NSCs using isolated mouse NSCs as *in vitro* experimental model. All three types of CS that were used in this study have clearly affected the primary microglia culture proving that the drug concentrations used are physiologically active.

The data presented in this chapter suggests that the exposure to the highest CSs concentration induced a reduction in cell number and proliferation of NSCs without any effects on viability and apoptosis. The percentage of EdU⁺ NSCs was significantly reduced at this concentration. In parallel, cell cycle assay results suggest that CSs treatment on NSCs affect their cell cycle phases.

The findings from this study are in agreement with several previous studies, it was demonstrated that CS had inhibitory effect on the proliferation of NSCs. For example, the high concentrations of cortisol (100 μ M) decreased on the human foetal hippocampal progenitor reduce the proliferation and neuronal differentiation (Anacker et al., 2013). In contrast, Bose and colleagues found that 48 h treatment with 1 μ M of DEX led to reduce the proliferation of NSCs with no changes seen in the cell survival or differentiation. They observed that DEX changed the expression of genes of the cell cycle, cellular senescence and mitochondrial function (Bose et al., 2010). Also, Sippel and colleagues reported that DEX treatment resulted in reduced the proliferation of NPCs and reduced the expression of BRUCE/Apollon, an apoptosis inhibitor protein family member family. They demonstrated the essential role of BRUCE in controlling cell division in NPCs (Sippel et al., 2009). While, Li and colleagues showed that MPRED had

inhibitory effects on the proliferation of NPCs by decreasing the levels of endothelin receptor type B protein, which involved in regulating the proliferation and apoptosis of endogenous neural precursors cells (Li et al., 2012).

In terms of cellular processes, the present study found that all three types of CS at 10 μ M reduced neurogenesis and axonal length. The differentiation findings from this study are broadly in line with several previous studies, for example, Chetty et al. found that CORT caused a reduction in the percentage of neurons in the hippocampus of adult rats (Chetty et al., 2014). Most importantly, the neurological experimental studies have shown that CS at high levels induced changes in neurogenesis. Several preclinical studies have demonstrated that GCs, especially DEX impaired neurogenesis and induced apoptosis of mature neurons. For example, YU and colleagues were used phenotype-specific genetic and antigenic markers to examine the fate NPCs which were obtained from the hippocampus of rats after neonatal DEX treatment during neonatal development. They were found that neonatal DEX treatment induced apoptosis among the proliferating population of cells in the dentate gyrus (Yu et al., 2010). *In vivo* and *in vitro* experiments conducted by Kim and colleagues have studied the effects of DEX on two month-old-adults rats which were received daily injections of DEX for 9 days their results demonstrated that DEX reduced the proliferation and the neurogenesis of NPCs (Kim et al., 2004). The effects of CSs on neurogenesis process are implicated in the regulation of cognition, mood, depression and emotional dysfunction (Balu and Lucki, 2009; Drew et al., 2010; Saxe et al., 2006; Snyder et al., 2012).

Also, the present results showed that CSs treatment did not affect the proportion of NSCs differentiating into astrocytes or oligodendrocytes. In contrast, Sabolek *et al.* observed that CS exposure led to a reduction in astrocyte differentiation (Sabolek *et al.*, 2006). This discrepancy may be due to the variations in culture protocols, as they examined the effects of CS on NSCs derived from rat midbrain which were differentiated for 14 days in the absence of N2 supplement whereas in this present study CSs effect was examined on NSCs derived from SVZ region from mouse brain in the absence of growth factor and in the presence of N2 supplement. Therefore, the discrepancy in these results could be due to the differences in methodology and the different origin of NSCs.

Additionally, CSs were found to accelerate the maturation of newly generated oligodendrocytes, a finding with potential implications for the myelination process. Oligodendrocytes, the myelinating cells of the CNS pass through many stages of development before their final maturation to generate myelin sheath (Baumann and Pham-dinh, 2001). Therefore, any morphological changes of these cells could affect the process of myelination. Jenkins *et al.* studied direct actions of DEX on OPCs in culture. No effects were found on OPC proliferation and survival, or oligodendrocyte maturation following DEX treatment (Jenkins *et al.*, 2014). This discrepancy could be due to different cultures (mixed culture in this study versus purified culture in the study by Jenkins *et al.*) and detection times after CSs treatment (9 days in this study versus 72 h in the study by Jenkins *et al.*). The interesting suggestion of CS-based effects on the maturity of oligodendrocytes in this study may be due to direct effects of CS on the oligodendrocytes or indirect effect mediated via other neural cell types (NSCs or neurons). Further, the known

CS effects on myelin genesis (Chari et al., 2006; Clarner et al., 2011) may alternatively be mediated via changes in the interactions of newly generated oligodendrocytes with axons in the developing nervous system; this is normally a highly spatially and temporally controlled process. Premature maturation of oligodendrocytes can be predicted to result in aberrant myelination.

Taken together, the findings from the histological analysis of this study suggest that the low concentrations of CS did not show any effects on NSCs in comparison with control group. In contrast, all three types of CS at highest concentration reduced the proliferation and neural differentiation of NSCs without any effects on their viability and apoptosis.

Chapter 4

Histological study to evaluate the effects of CS treatment on NSCs propagated in neurosphere cultures

4.1 Introduction

The neurosphere is one of the most useful and commonly used techniques used in expanding and propagating NSCs. In 1992, Reynolds and Weiss successfully isolated neurospheres from adult mouse brains and showed their ability to differentiate into neurons and astrocytes (Reynolds and Weiss, 1992). Commonly, mouse and rat neurospheres are isolated from embryonic or adult SVZ (Conti and Cattaneo, 2010). A single-cell suspension is plated in low-attachment tissue culture plastic dishes in the presence of N2 or B27 supplemented and growth factors to form floating balls of cells (Conti and Cattaneo, 2010; Jensen and Parmar, 2006; Rahman et al., 2015).

In addition to the important role of neurosphere cultures in isolating and expanding NSCs, they have been shown to be very useful as an *in vitro* model system for neurogenesis and neural development studies (Jensen and Parmar, 2006). Interestingly, both monolayer and neurosphere culture systems are commonly used to propagate and expand NSCs for cell transplantation (Bose et al., 2015; Conti et al., 2006; Jensen and Parmar, 2006; Kornblum, 2007) and both systems have associated advantages and disadvantages for this purpose (Table 4.1).

Table 4.1 Summary of advantages and disadvantages between monolayer and neurosphere culture formats for NSCs	
Neurosphere format (3-D suspension NSCs)	Monolayer format (2-D adherent NSCs)
Technically easy to culture	Technically more challenging
High cell density in small surface area	Cells occupy greater surface area
Non-uniformity of cell exposure to environmental factors	Uniform exposure of cells to environmental factors
High survival post-transplantation	Lower viability post-transplantation
More difficult to monitor single cells	Single cells easily monitored

Table adapted from (Weinberg et al., 2015).

The differences in the features of each culture impact the choice of culture system when using NSCs in transplantation after neurological disease/injury. There are several factors which may influence the potential mechanisms of recovery after transplantation. For example, the nature of the NSCs population is one of these factors that could affect the capacity of the transplanted cells. It was observed that the original source of engrafted cells, isolation and preparation methods could influence the fate of transplanted cells (Hooshmand et al., 2009). Further, the type of culture system that is used in isolation and propagation of NSCs could impact the heterogeneity and differentiation of NSCs. For example, NSCs maintained as monolayer cultures are more homogenous when compared to neurosphere cultures, which are a heterogeneous population of cells (Conti and Cattaneo, 2010; Jensen and Parmar, 2006). It was found that each neurosphere contains in addition to NSCs, other types of cells at various stages of differentiation such as neurons and glia (Suslov et al., 2002). The heterogeneity of neurospheres increases with the sphere size and within longer time in culture because neurospheres produce more differentiated cell types (Jensen and Parmar, 2006).

Interestingly, the transplantation of NSCs grown as neurospheres displays several advantages. For example, cells derived from neurosphere cultures are thought to show greater survival after transplantation in the site of injury because of their ability to maintain cell-cell communication (Mothe et al., 2008). It was found that cell transplantation as a neurosphere shows more than a threefold greater survival rate after two weeks of transplantation in the intact spinal cord when compared to transplantation of dissociated cells. Therefore, transplantation of neurospheres is thought to be more useful for enhanced cell survival after transplantation versus cells derived from monolayers (Mothe et al., 2008).

Furthermore, neurosphere cultures generate more cells within a smaller surface area when compared to monolayer cultures (Conti and Cattaneo, 2010; Mothe et al., 2008). Importantly, the 3-D neurosphere culture system has been successfully applied in Alzheimer's and Parkinson's disease (Brito et al., 2012; Choi et al., 2013; Vishwakarma et al., 2014; Zhou et al., 2016). In 2005, Meissner and colleagues generated cells from the SVZ in the brains of 1-year-old enhanced green fluorescent protein (GFP) mice and produced neurospheres in culture. Then they transplanted the cryopreserved neurospheres into 6-hydroxydopamine model of Parkinson's disease. Three weeks after creating the 6-OHDA lesions, the cryopreserved neurospheres were thawed and expanded to form new spheres and transplanted into mice. They observed the ability of transplanted cells to differentiate to neurons and astrocytes (Meissner et al., 2005). Also, the neurospheres were successfully transplanted in a mouse model of Alzheimer's disease and restore the working memory decline. They were observed that the neurospheres grafts survived in the transplantation sites and differentiated into the cholinergic neurons following 12 weeks of transplantation (Wang et al., 2006).

With regard to NSCs transplantation for multiple patients, the production of large numbers of cells is one of most important issues. For instance, it has been found that 8-12 fetuses are required to treat one patient with Parkinson's disease (Ali et al., 2014). Neurospheres offer another advantage in addition to their high cell density; they are technically easy to culture (Weinberg et al., 2015).

In general, the two culture systems of NSCs behave in different manners which may cause an effect on the mechanisms of repair following transplantation. Immunosuppressant drugs play a pivotal role in the survival of transplanted NSCs by modulating the immune response following transplantation. It is therefore

important to investigate the effects of CS on the fate of NSCs in 3-D culture system, focusing on parameters underpinning regeneration such as proliferation, differentiation, apoptosis, viability and cell cycle.

4.2 Chapter objectives

The aim of this study was to systematically investigate the effects of three different immunotherapeutic drugs of CS using neurosphere growth formats, a second major format of NSCs. It is not known if the physical format by which NSCs are propagated can impact the drug influences on these cells. To the best of my knowledge, it has never been established whether the effects of CS differ when NSCs are grown as a 2-D or 3-D culture system– a question of significant importance for the neurobiology, neurotransplantation and regenerative medicine fields.

Q: Does CS treatment have different effects on NSCs propagated via different growth formats (i.e monolayers versus neurospheres)?

4.3 Results

4.3.1 Effect of CSs on the number and size of neurospheres

Three different types of CS (DEX, PRED and MPRED) were used at three different concentrations (0.1 μ M, 1 μ M and 10 μ M) to assess their effects on NSCs in neurosphere cultures. Following 48 h of CS treatment in NSCs cultured as 3-D suspension neurospheres, healthy spheres containing phase bright cells were observed in controls and other treatment conditions. Phase images and quantification analyses revealed no differences in sphere number per field but showed differences in sphere diameter when compared between control and the highest dose of CSs (10 μ M) (Figure 4.1).

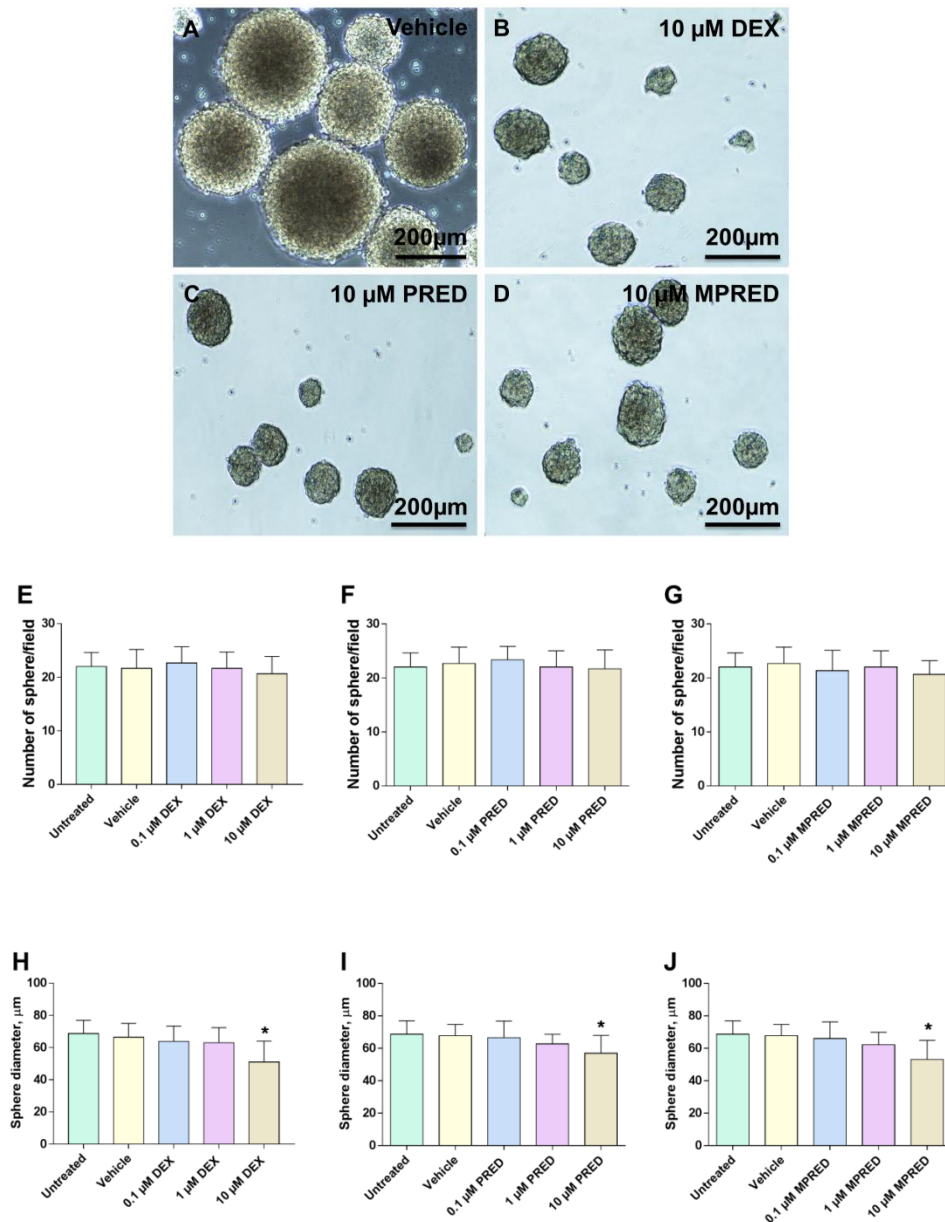


Figure 4.1 Effect of CSs on the number and size of neurospheres. (A) Phase-contrast micrograph of neurospheres in vehicle control, (B, C and D) treated neurospheres with CSs. (E, F and G) Bar charts showing the average sphere number per microscopic field and (H, I and J) bar charts showing neurosphere size across all treatment conditions. (48 h CSs; 0.1 μM, 1 μM and 10 μM); * $p < 0.05$ versus vehicle control of NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Number and size of spheres were assessed in four biological replicates, with two technical replicates in each experiment.

4.3.2 Effect of CSs on the number and proportion of NSCs in neurosphere cultures

To assess the effects of all three types of CS studied in this thesis, the numbers of NSCs were counted after 48 h of CS-treatment in four biological replicates, with two technical replicates in each experiment. Cell counts for the 3 D spheres were carried out in two cryostat sections taken through the sphere and randomly selected for each condition. Immunostaining for nestin and SOX-2 revealed that the number of NSCs per field was reduced only at the highest concentration of CSs used (Figure 4.2 and Figure 4.3).

'Stemness' was assessed by analysing the proportion of SOX-2 positive cells. SOX-2 is a transcription factor that is expressed by self-renewing and multipotent stem cells. Therefore, SOX-2 was used to assess the purity of NSCs instead of nestin which is intermediate filament protein. Immunostaining for SOX-2 positive cells revealed that spheres yielded high purity populations of NSCs across all conditions. Importantly, cells following CS treatment also displayed normal patterns of NSCs marker staining and regular circular nuclei as judged by DAPI staining, indicating NSCs purity was not altered following CS treatment (Figure 4.3).

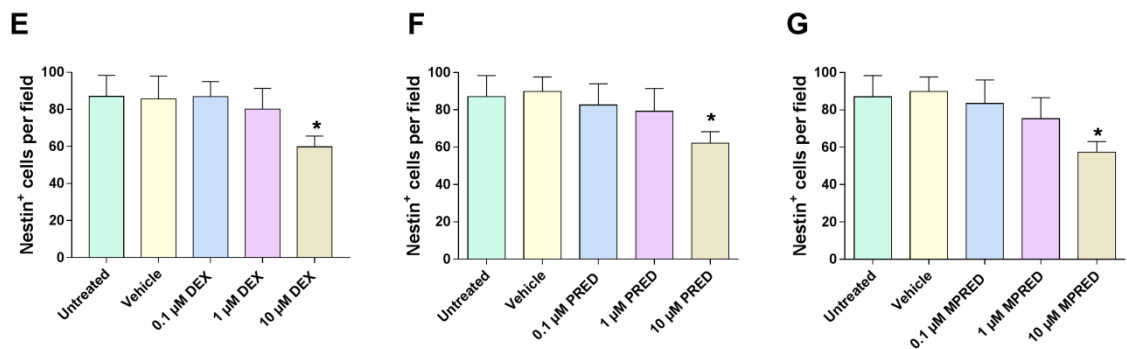
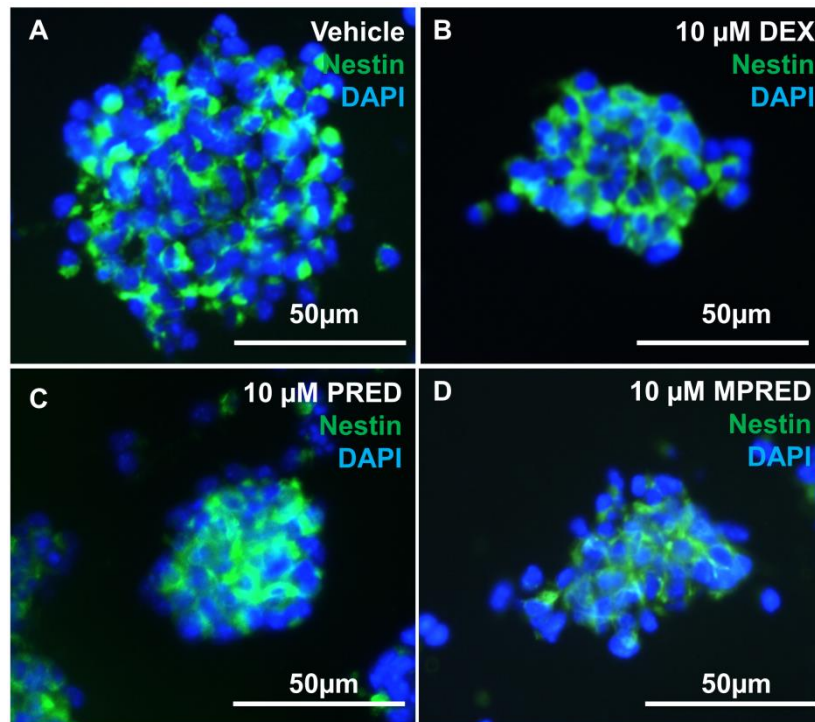


Figure 4.2 Effect of CSs on the number of NSCs using nestin marker in 3-D neurosphere cultures. (A) Fluorescence micrograph displaying nestin positive NSCs neurospheres in vehicle control, (B, C and D) treated neurospheres with 10 μM CSs. (E, F and G) Bar charts showing the total number of NSCs per field across all conditions. (48 h CSs; 0.1 μM , 1 μM and 10 μM); * $p < 0.05$ versus vehicle control of NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was assessed in four biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition.

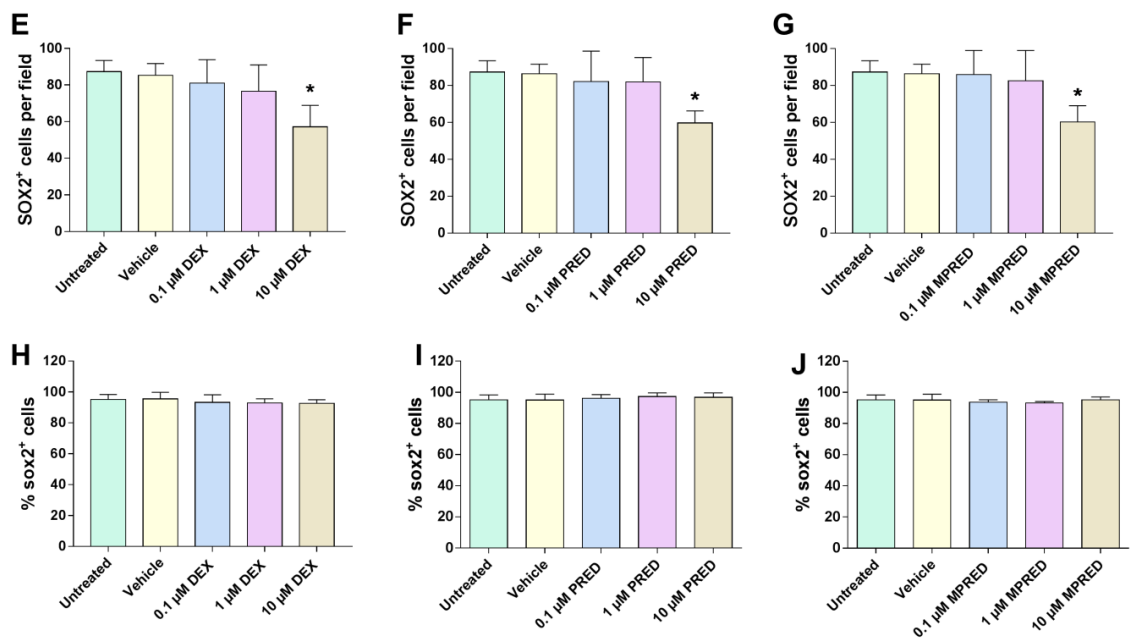
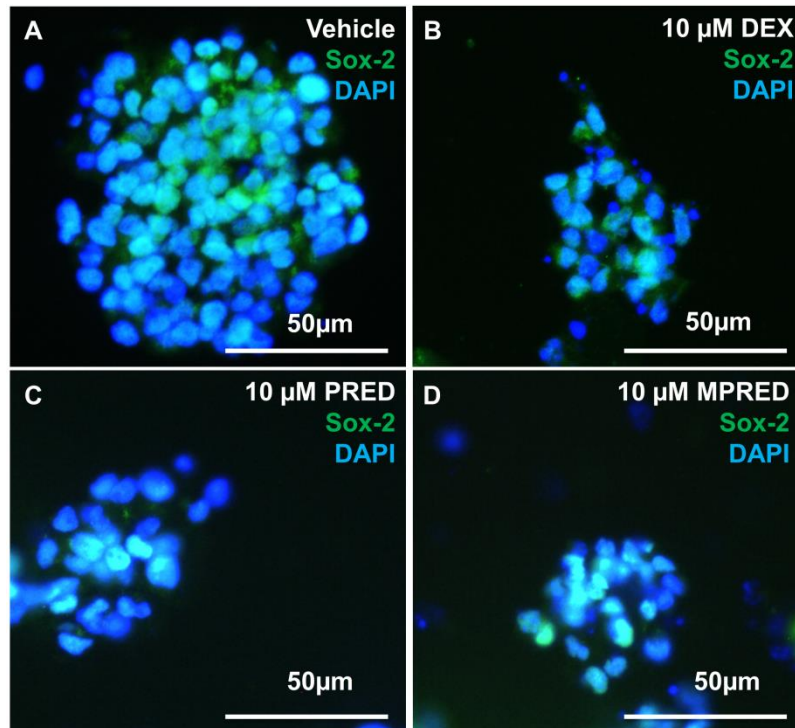


Figure 4.3 Effect of CSs on the number and proportion of NSCs using SOX-2 marker in 3-D neurosphere cultures. (A) Fluorescence micrograph displaying SOX-2 positive NSCs neurospheres in vehicle control, (B, C and D) treated neurospheres with 10 μ M CSs. (E, F and G) Bar charts showing the total number of NSCs per field across all conditions. (H, I and J) Bar charts displaying

*quantification of the proportions of cells positive for NSCs marker SOX-2 following CS treatment across all conditions. (48 h CSs; 0.1 μ M, 1 μ M and 10 μ M); * $p < 0.05$ versus vehicle control of NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in four biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition.*

4.3.3 Effect of CSs on the proliferation and cell cycle of NSCs

To assess the effects of CS on the proliferation of NSCs in neurosphere cultures, an EdU proliferation assay was performed. The percentage of EdU⁺ cells was significantly decreased post CS treatment at the highest concentration (10 μ M) when compared with controls (Figure 4.4). The cell cycle analysis was performed to confirm the EdU assay results. The data showed that the percentage of NSCs increased in G0/G1 and reduced in S and G2/M phases at 10 μ M of CSs (Figure 4.5). These findings suggest that all three types of CS at the highest concentration had anti-proliferative effects on NSCs in neurosphere cultures.

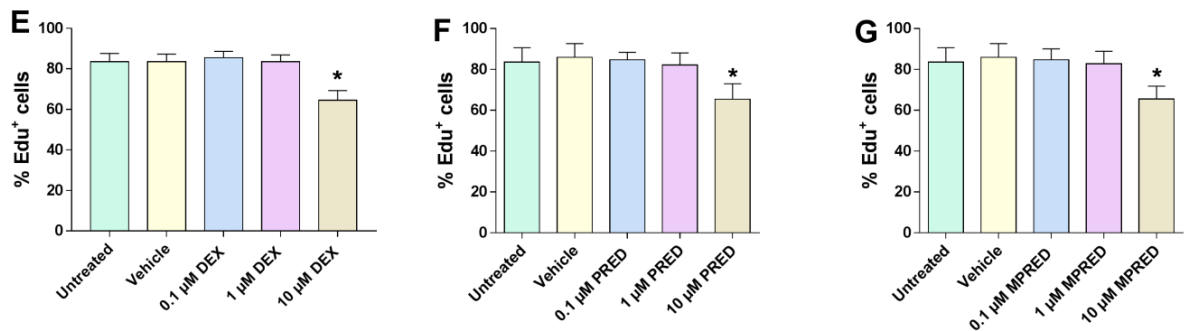
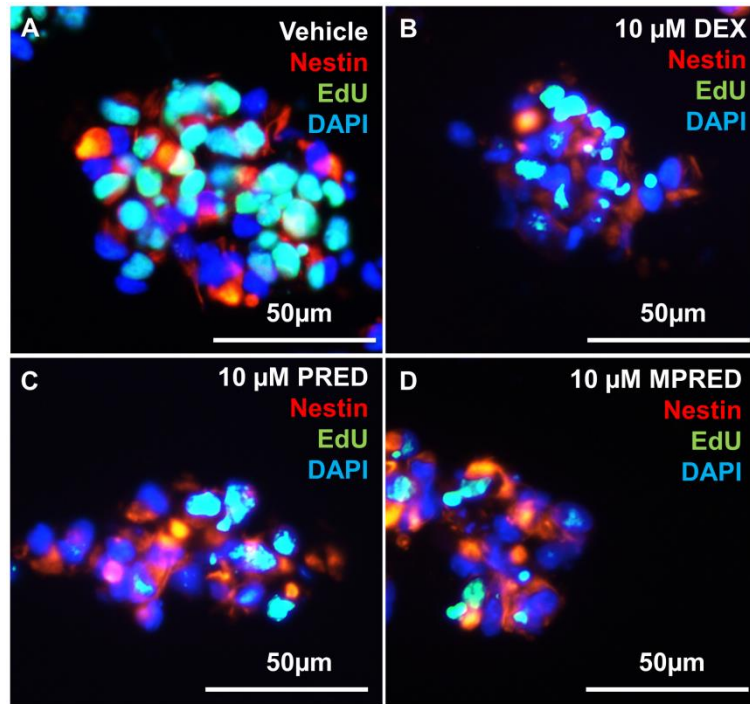


Figure 4.4 Effect of CSs on the proliferation of NSCs in 3-D neurosphere cultures. (A) Triple merged fluorescence images depicting EdU^+ NSCs in vehicle control, (B, C and D) treated neurospheres with 10 μM CSs. (E, F and G) Bar charts showing proportions of EdU incorporating cells across all treatment conditions. (48 h CSs; 0.1 μM , 1 μM and 10 μM); * $p < 0.05$ versus vehicle control of NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Proliferation assay was carried out in three biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition.

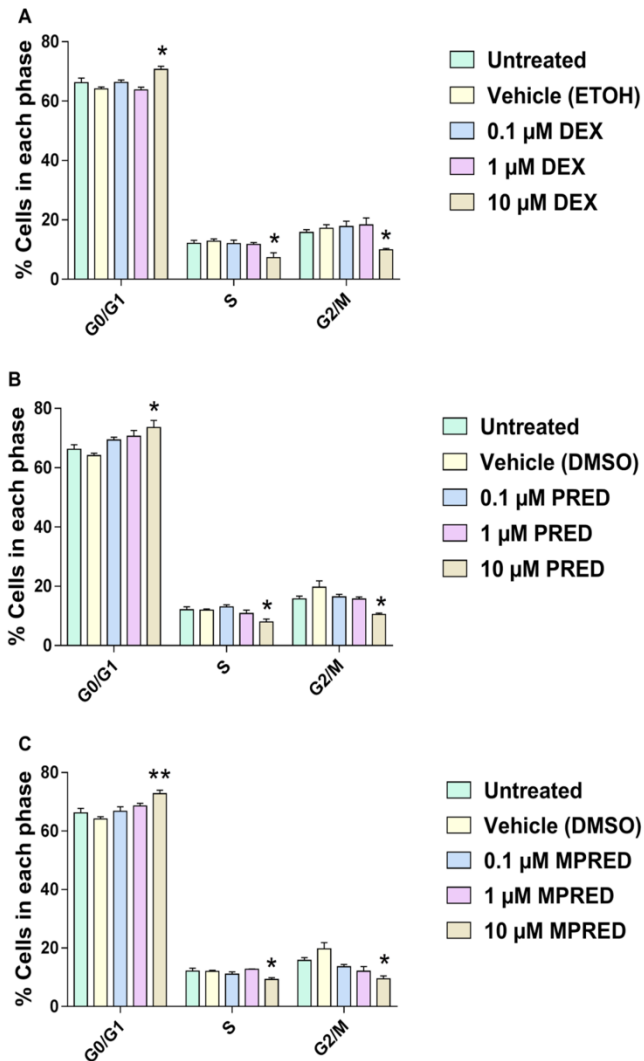


Figure 4.5 Effect of CSs on the cell cycle of NSCs in 3-D neurosphere cultures. (A) Bar chart showing the percentage of cells in G0/G1, S and G2/M phase following DEX treatment. (B) Bar chart showing the percentage of cells in G0/G1, S and G2/M phase following PRED treatment. (C) Bar chart displaying the percentage of cells in G0/G1, S and G2/M phase following MPRED treatment in neurosphere cultures. (48 h CSs; 0.1 μM, 1 μM and 10 μM); * $p < 0.05$ and ** $p < 0.01$ compared to untreated and vehicle control of NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell cycle analysis was carried out in three biological replicates, with three technical replicates in each experiment.

4.3.4 Effect of CSs on the viability, apoptosis and pyknosis of NSCs

To study the effects of CS on NSCs viability, NSCs were cultured as neurospheres in the presence of DEX, PRED and MPRED at different concentrations (0.1 μ M, 1 μ M and 10 μ M). The viability of NSCs was conducted at 48 h of drug treatment. The findings showed that NSCs displayed high viability following CS treatment as judged by live/dead staining across all conditions, suggesting there is no effect of CSs on viability of NSCs in neurosphere cultures (Figure 4.6). Further, Muse Annexin V and Dead cell kit was used to assess the percentage of apoptotic cells in neurosphere cultures at 48 h of CS treatment. No significant differences were observed in the apoptosis of cells following CS-treatment at all investigated concentrations (Figure 4.7). Additionally, the incidence of pyknosis as judged by counting the percentage of cells exhibiting pyknotic nuclei (i.e. shrunken or fragmenting morphologies) was also low (<3%) across all conditions, indicating no significant differences between CS-treated and untreated (control) NSCs cultures (Figure 4.8). These findings suggest that all three types of CS used in this study had no effects on the viability, apoptosis and pyknosis of NSCs in neurosphere cultures.

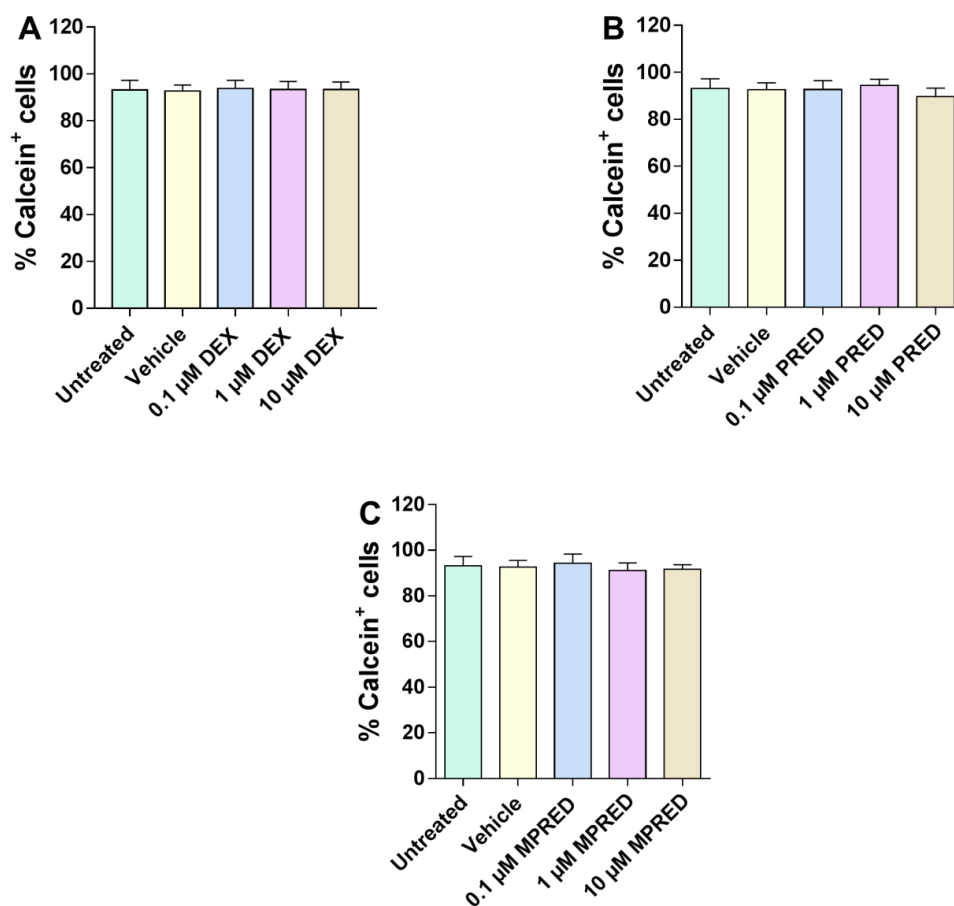


Figure 4.6 Effect of CSs on the viability of NSCs in 3-D neurosphere cultures.

(A) Bar chart showing quantification of LIVE cells as a percentage of total cells in neurosphere cultures following DEX treatment. (B) Bar chart showing quantification of LIVE cells as a percentage of total cells in neurosphere cultures following PRED treatment. (C) Bar chart displaying quantification of LIVE cells as a percentage of total cells in neurosphere cultures following MPRED treatment. No significant differences in the viability were noted between all conditions. (48 h CSs; 0.1 μ M, 1 μ M and 10 μ M); one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Viability assay was carried out in three biological replicates, with two technical replicates in each experiment.

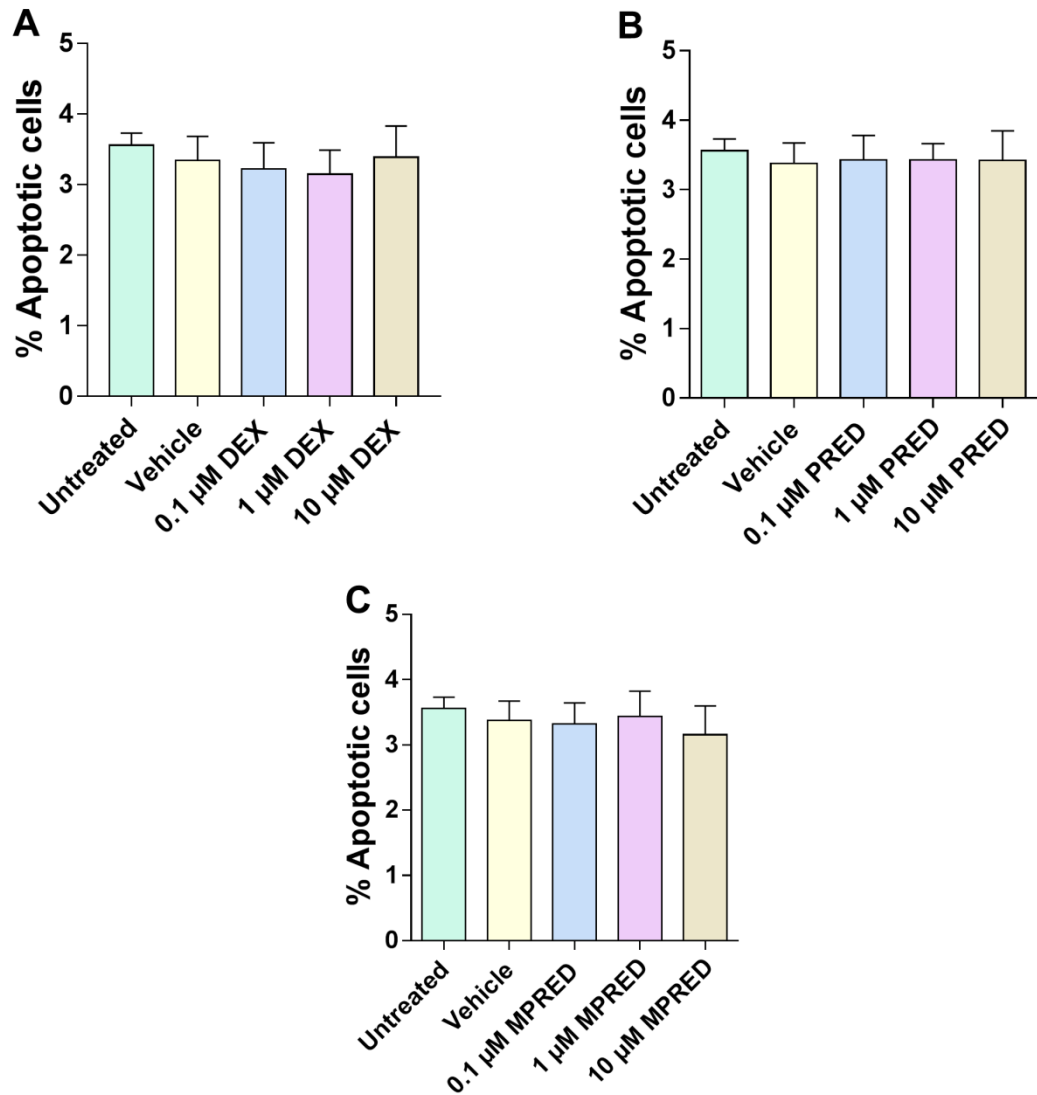


Figure 4.7 Effect of CSs on the apoptosis of NSCs in 3-D neurosphere cultures. (A) Bar chart displaying the percentage of apoptotic cells post DEX treatment. (B) Bar chart showing the percentage of apoptotic cells post PRED treatment. (C) Bar chart showing the percentage of apoptotic cells post MPRED treatment. No significant differences in the apoptosis were noted between all conditions. (48 h CSs; 0.1 μ M, 1 μ M and 10 μ M); one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Apoptosis assay was carried out in three biological replicates, with three technical replicates in each experiment.

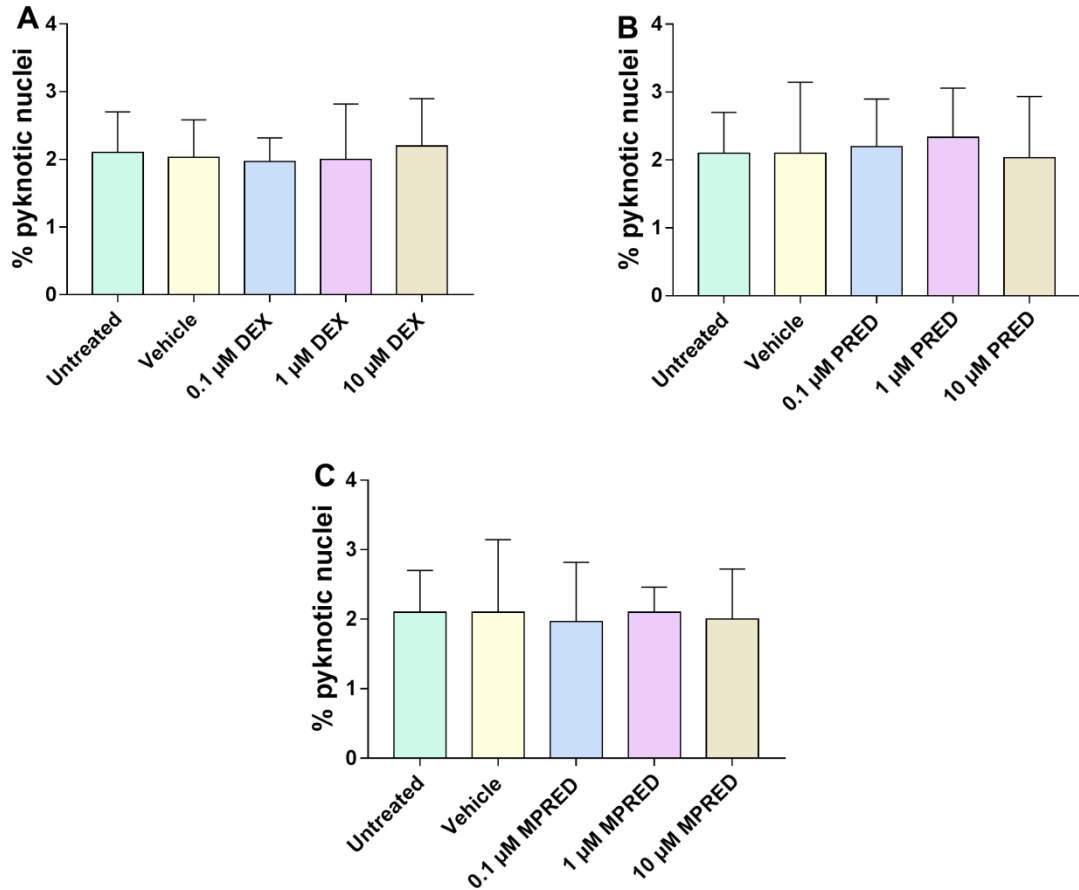


Figure 4.8 Effects of CS on the pyknosis of NSC in 3-D neurosphere cultures.

(A) Bar chart displaying the percentage of pyknotic nuclei counts for all conditions following DEX treatment. (B) Bar chart showing the percentage of pyknotic nuclei counts for all conditions following PRED treatment. (C) Bar chart displaying the percentage of pyknotic nuclei counts for all conditions following MPRED treatment. No significant differences between CSs-treated and untreated. (48 h CSs; 0.1 μM, 1 μM and 10 μM); one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Pyknotic nuclei was carried out in four biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition.

4.3.5 Effect of CSs treatment on the differentiation of NSCs

The influence of CS treatment on the differentiation profile of NSCs in neurosphere cultures was a final goal in this study. To study the role of CSs in more detail, daughter cells generated from CS-treated neurospheres were treated for further 7 days with DEX, PRED and MPRED at different concentrations (0.1 μM , 1 μM and 10 μM). A quantitative histological analysis of daughter cell populations revealed that astrocytes were produced in similar numbers and proportions and displayed normal morphologies across all conditions following CS treatment (Figure 4.9). In contrast to astrocytes, the number and proportion of neurons was significantly reduced per field. The length of axons was also significantly reduced compared to the control cells after 10 μM CS treatments (Figure 4.10). In addition, the results also showed no difference in the number and proportion of oligodendrocytes generated per field following CSs treatment. However, the microscopic observations demonstrated that oligodendrocytes in 10 μM of CS-treated cultures had a greater membrane surface area with extensive and complex branching of processes. The total cell area measurement of oligodendrocytes treated with 10 μM of CSs showed less maturation compared to control cells (Figure 4.11). These findings suggested that all three types of CS at 10 μM reduced the neuronal differentiation and affected the morphology of neurons and oligodendrocytes without any effects on the number and proportion of astrocytes and oligodendrocytes.

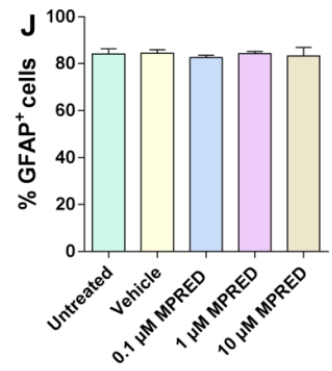
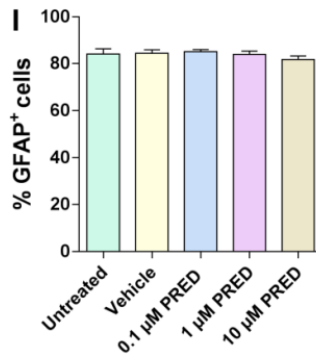
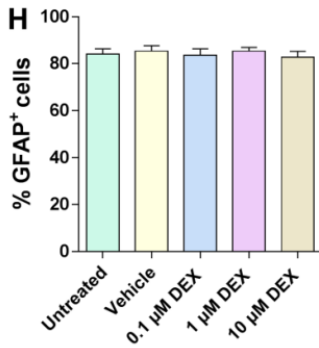
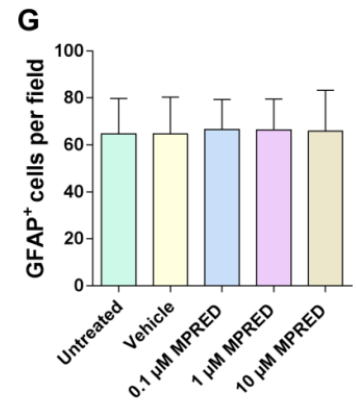
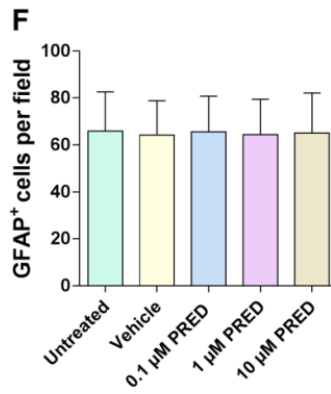
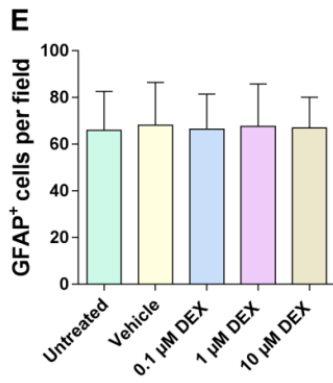
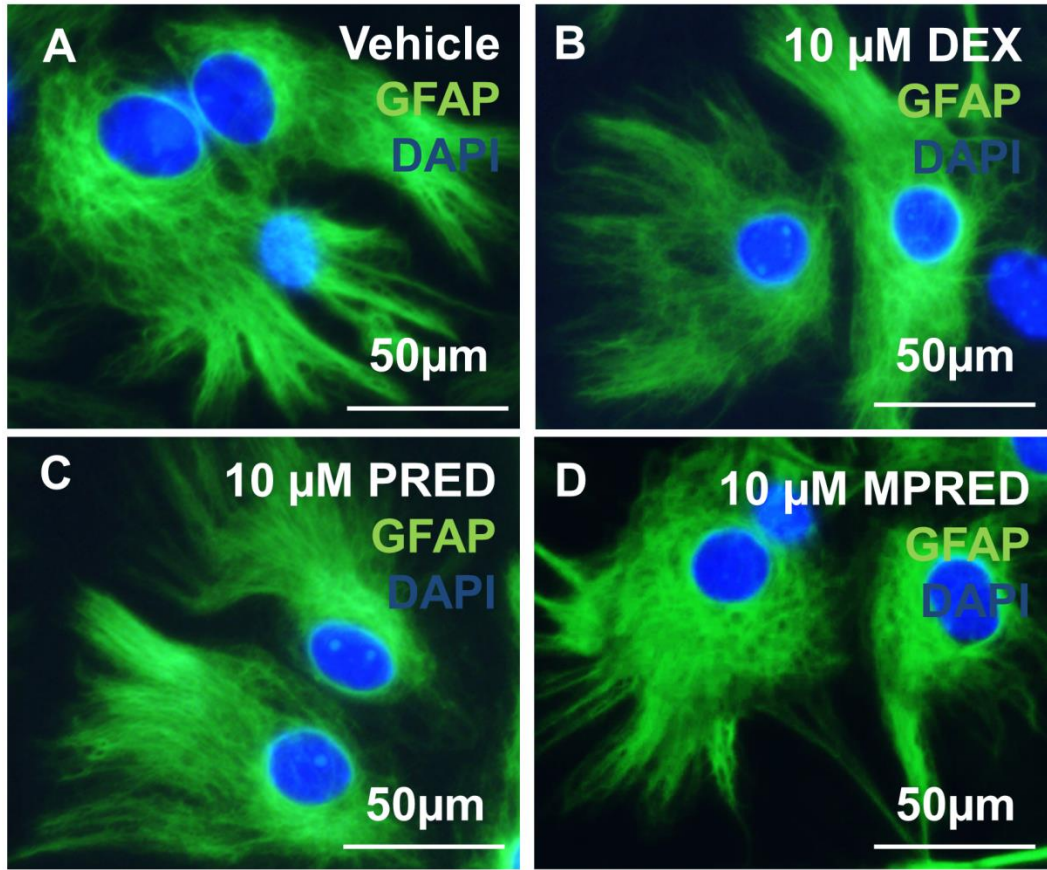


Figure 4.9 CSs treatment showed no effect on the proportion and morphology of astrocytes. (A) Representative images of cells positive for the neural cell markers GFAP for astrocytes derived from vehicle control neurosphere cultures, (B, C and D) treated astrocytes with 10 μ M CSs. (E, F and G) Bar charts depicting the total number of GFAP⁺ cells per field. (H, I and J) Bar charts quantifying the proportion of cells positive for astrocytes marker GFAP⁺ after CS treatment across all conditions. No significant differences in the morphology and proportion of astrocytes were noted between all conditions (9 days CSs; 0.1 μ M, 1 μ M and 10 μ M); one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cells counting were carried out in four biological replicates, with two technical replicates in each experiment.

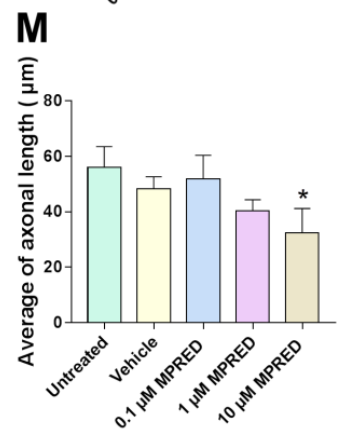
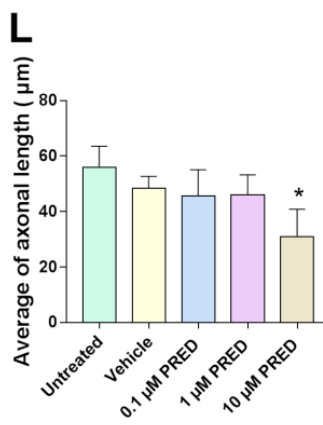
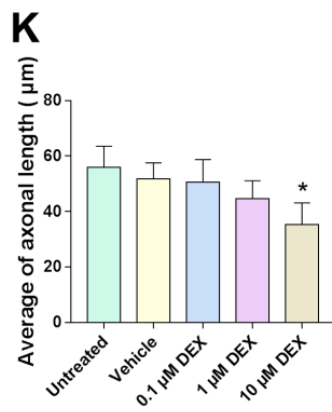
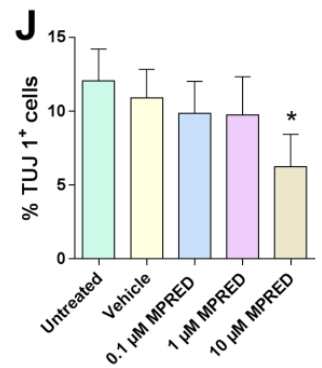
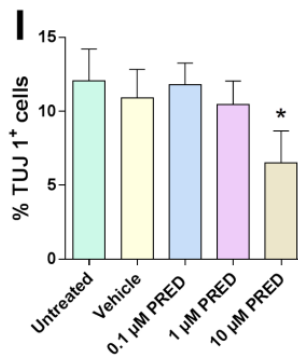
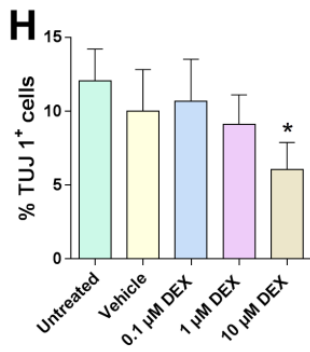
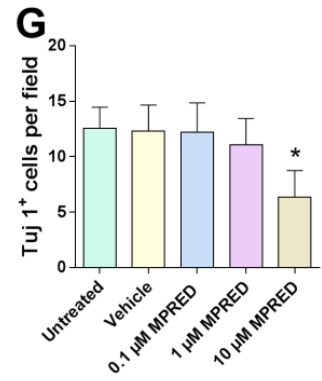
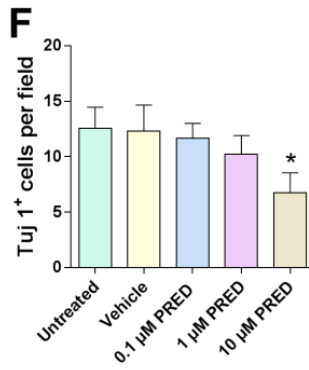
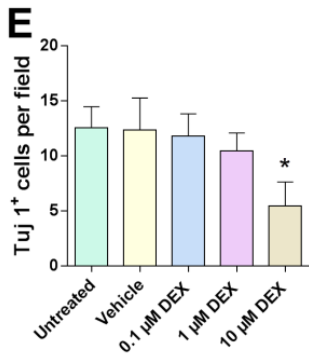
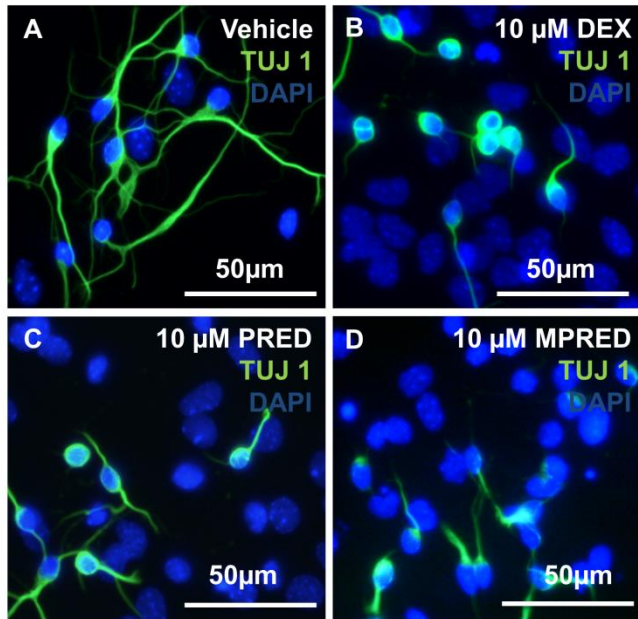


Figure 4.10 CSs treatment altered neuron cell number, proportion and morphology. (A) Fluorescence micrographs of neurons derived from vehicle control neurosphere cultures, (B, C and D) treated neurons with 10 μM CSs. (E, F and G) Bar charts quantifying the total number of TUJ 1⁺ neurons per field across treatment conditions. (H, I and J) Bar charts showing the proportion of TUJ 1⁺ neurons per field following CS treatment. (K, L and M) Bar charts showing mean axon length of neurons across all conditions. (9 days CSs; 0.1 μM , 1 μM and 10 μM); * $p < 0.05$ compared to vehicle control; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in four biological replicates, with two technical replicates in each experiment.

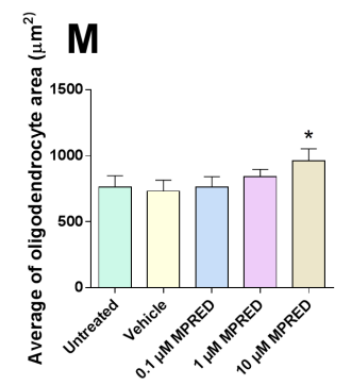
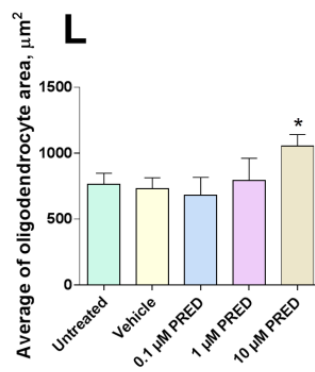
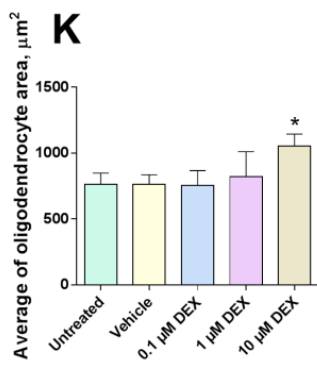
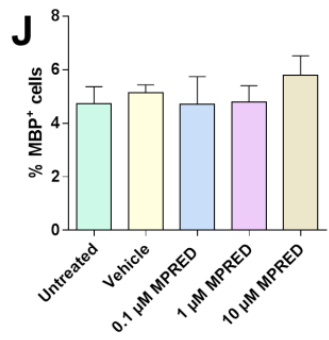
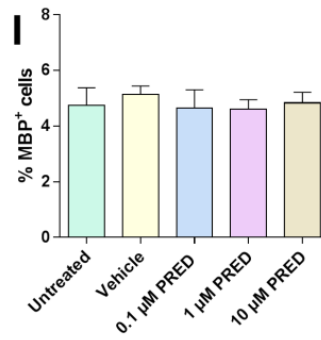
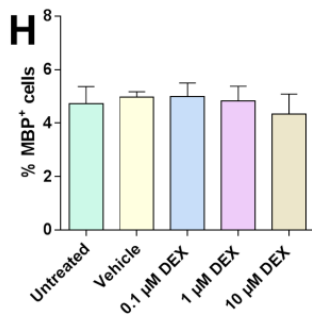
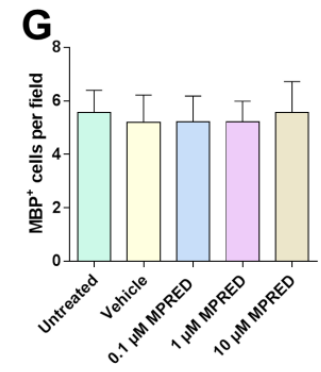
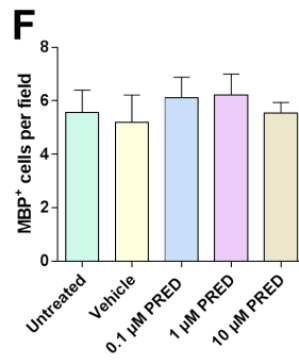
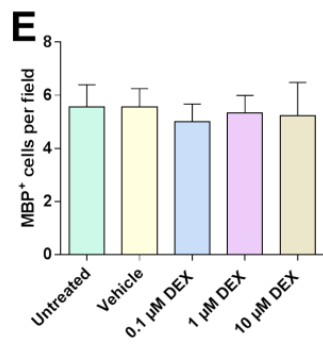
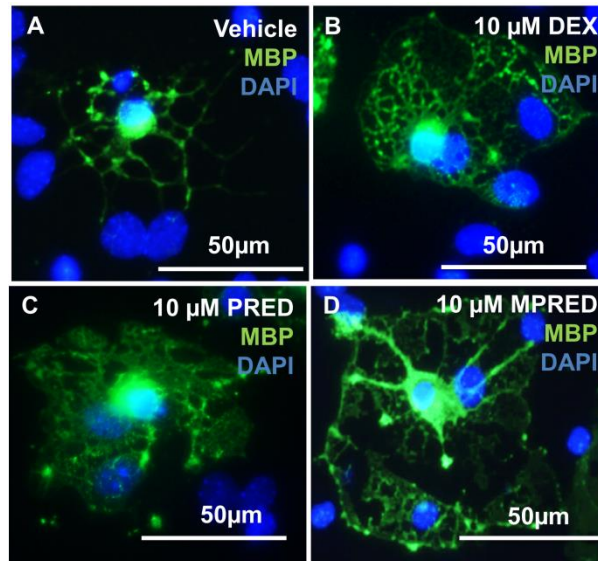


Figure 4.11 CSs treatment increased the maturation of oligodendrocytes. (A) Fluorescence micrographs of oligodendrocytes generated from vehicle control neurosphere cultures, (B, C and D) treated oligodendrocytes with 10 μM CSs. (E, F and G) Bar charts showing the total number of MBP⁺ oligodendrocytes per field across treatment conditions, (H, I and J) bar charts showing the proportion of oligodendrocytes across all conditions. (K, L and M) Bar charts showing the measurement of oligodendrocytes area across all conditions. (9 days CSs; 0.1 μM , 1 μM and 10 μM); * $p < 0.05$ compared to vehicle control of NSCs; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in four biological replicates, with two technical replicates in each experiment.

4.4 Discussion

The use of immunosuppressive drugs such as CS after transplantation of NSCs is necessary to ameliorate engrafting outcomes by increasing cells survival and limiting cell rejection in host tissue (Mazzini et al., 2015). The control of stem cell activity depends on a diverse array of environmental factors. Mainly, the genetic and molecular mediators such as growth and transcription factors which play pivotal role in the control of stem cells fate either *in vivo* or *in vitro*. For example, cell shape is one of the main physical factors that could significantly influence the growth and physiology of NSCs (Guilak et al., 2009). Therefore, this study was undertaken to evaluate the effects of CS on NSCs properties when they are cultured as 3-D suspension neurospheres. It has never been established if the effects of CS differ when NSCs are grown as 3-D or 2-D culture system, a very important question for the fields of neurobiology, neurotransplantation and regenerative medicine.

Emerging knowledge of CS effects on NSCs and the molecular mechanisms behind these effects in both 3-D and 2-D growth formats will refine the judgment of when and how to use the anti-inflammatory and immunosuppressive drugs combined with NSCs. Furthermore, within this knowledge the evaluation of the clinical efficacy of NSC transplants will be improved by learning how to reduce the cell death rate of these cells and provide a more permissive environment and niche in neurological injuries/diseases.

The results demonstrated that the low concentrations of CS do not show any effects on NSCs in comparison with the control group in neurosphere culture systems. At a high concentration (10 μ M), all types of CS prominently reduced NSCs proliferative capacity (EdU incorporation) in 3-D cultures, followed by an alteration of the percentage of the cell cycle phases. Cell viability and cell apoptosis remained mostly unaffected under these conditions for all CSs. A number of studies have observed that CSs reduced the proliferation of NSCs in neurosphere cultures (Abdanipour et al., 2015; Moors et al., 2012; Sundberg et al., 2006). These findings are in accordance with the present results that show a significant reduction in the size of neurospheres which reflects the CSs effect on the number and proliferation of NSCs in neurosphere cultures. Further, the data showed that the percentage of EdU was markedly reduced within spheres that were exposed to (10 μ M) CSs at 48 h. A second method used in this study to detect NSCs proliferation was cell cycle analysis; there was a prominent increase in the percentage of cycling cells entering G0/G1 and reduction in S and G2/M phases. Previously, a number of studies have shed light on the inhibitory effects of CSs on NSCs cell cycle in neurosphere cultures; they proposed that CSs reduce the percentage of cells in S phases via their effects on cell-cycle regulating genes (Samarasinghe et al., 2011; Sundberg et al., 2006). Further, the results from the current study are in line with several previous studies. For example, Sundberg *et al.* showed the effects of CS on the embryonic rat NSCs. They observed in their study, the ability of CSs to reduce the proliferation of embryonic NSCs accompanied by a decrease in cyclin D1 (Sundberg et al., 2006). Also, it was found that DEX had anti-proliferative effects on the NPCs derived from embryonic day 14.5 mouse neurosphere cultures and this effect was confirmed by both a

reduction in S- phase progression and enhanced cell-cycle exit (Samarasinghe et al., 2011). Another group found that the exposure of NPSCs derived from individual male and female mouse embryos (embryonic day 14.5) to 100 nM of DEX reduces the proliferation of NPSCs in neurosphere culture and also *in vivo* (Frahm et al., 2016). In 2012, Moors and colleagues studied the effect of CSs on the proliferation and differentiation of hNPC. They found that the exposure to 1 μ M DEX leads to a reduction in the proliferation and neural differentiation of human NPCs. The mechanism underlying the DEX-induced inhibition of the proliferation and neural differentiation of human NPCs is mediated by the inhibition of the canonical Wnt signalling pathway (Moors et al., 2012). Furthermore, Ekthuwapranee and colleagues found that three different concentrations of DEX (0.1 μ M, 1 μ M and 10 μ M) lead a reduction in the number of neurospheres for NPCs derived from adult rats (Ekthuwapranee et al., 2014). Peffer and colleagues demonstrated that lipid raft protein caveolin-1 is required to induce the anti-proliferative effects of DEX in NPCs (Peffer et al., 2014).

The results presented here also show that the process of neurogenesis is negatively affected by CS treatment resulting in effects the number and morphology of neurons. Moreover, the findings from the differentiation assay shows that CSs treatment increased the maturity of oligodendrocyte which points to complex effects of the CS. This finding is of particular interest because any alteration in neuron and oligodendrocytes morphology could affect the reciprocal interaction between neurons and oligodendrocytes, and the communication between neurons and oligodendrocytes which is important in myelin synthesis and repair. The detrimental effect of CS on NSCs progeny has been reported. For

example, the exposure of human NPCs to DEX led to a reduction of the differentiation of neurons without any effects on the differentiation of astrocytes and oligodendrocytes (Moors et al., 2012). In contrast, Sundberg and colleagues found that 1 μ M of DEX and CORT did not affect cell differentiation of embryonic NSCs (Sundberg et al., 2006).

At all three different concentrations, none of the three examined CSs induced apoptosis or affected viability of NSCs in neurosphere culture systems. In contrast to these results, several previous studies demonstrated that the CS does induce the apoptosis of NSCs and reduce the viability of these cells. For example, Abdanipour and colleagues have found that the high concentration of cortisol decrease the viability and proliferation of adult NSCs in neurosphere culture and increases the apoptosis and necrosis of these cells (Abdanipour et al., 2015). The conflicting results attained by this body of experiments may be related to different cell types or CS concentrations being utilised.

The findings presented in this chapter reveal no distinct differences between monolayer and neurosphere cultures post CSs treatment. The present study showed *ca.* 40% reduction in the number of NSCs with the highest CSs in monolayers culture while only *ca.* 20% reduction in the number of NSCs with the highest CSs in neurospheres culture. Therefore, we suggest the ability of CS to induce their effect on NSCs in neurosphere cultures was not as high as that observed in the monolayer cultures (Chapter 3). The 3-D nature of neurospheres means that cells within the neurosphere may be 'hidden' from CS effects, in contrast, to monolayer cells which are all exposed to CSs in the media. In general,

the cell shape in a 3-D culture system is more rounded in comparison to 2-D culture system which generally induces flattened shapes. The surface area that is exposed to CSs in monolayer cultures is greater in comparison to neurosphere cultures and this may lead to higher influences of CS in 2-D culture. Furthermore, the difference in the physical characteristics of neurospheres and monolayers may impact the gene expression and cellular function of stem cells after CS treatment, resulting in differences in mechanisms that are involved in CS effects.

Chapter 5

Investigating the mechanisms underpinning the effects of CS on NSCs

5.1 Introduction

The findings from Chapter 3 and 4 found an effect of CS on the proliferation and differentiation of NSCs in both culture systems. However, the molecular signalling mechanisms by which CS effect proliferation and neurogenesis of NSCs are unknown. Therefore, this chapter was undertaken firstly, to assess GR expression in NSCs and their daughter cells and if they could be a target of drugs action and secondly, to evaluate the molecular mechanisms underpinning the effects of CS using proteomics and bioinformatics analyses.

As discussed previously within the main introduction, CSs exert their effect by binding to specific type of receptor. The action of CS is mediated by its binding to GR which is activation or repression transcription of target genes. In general, the regulation of gene expression by the GR occurs by (1) either the direct binding of a GR-ligand complex to specific DNA sequences called GREs that are present in target genes, (2) or indirect interaction of GR with other DNA-bound proteins or DNA-bound transcription factors, this mechanism called genomic (classical) (Samarasinghe et al., 2012; van der Goes et al., 2014). However, the observation of rapid effects of CS which cannot be mediated by activation of RNA and protein synthesis has prompted the investigators to search for alternative mechanisms, this mechanism called non-genomic (non-classical) mechanisms (Peffer et al., 2014; Samarasinghe et al., 2011; Simoncini and Genazzani, 2003). In non-genomic mechanism, CSs can interact with the cytosolic and membrane-bound GRs to induce modulation of cytoplasmic or cell membrane-bound regulatory proteins (Simoncini and Genazzani, 2003). Previously, a number of experimental studies have suggested the role of GRs as the main regulator of CS effects on cell proliferation and differentiation (Garcia et al., 2004). GRs are found in different

brain areas such as the dentate gyrus of the hippocampus. Also, it was found that the expression of GR in neuronal precursor cells is higher than that of the MR both *in vivo* and in primary neuronal precursor cell cultures (Boku et al., 2009; Garcia et al., 2004).

Importantly, a number of experimental studies observed that most actions of CS are largely considered to be mediated via the GRs involving changes in gene expression. For example, they found the pre-treatment with mifepristone (GR antagonist) significantly reduced the apoptosis of murine NSCs, suggesting the involvement of GRs (Mutsaers and Tofighi, 2012; Sze et al., 2013).

Most importantly, a number of researches have studied the molecular mechanisms underpinning the effects of CS on neural cells by using genomic study. In this regards, there are several signalling pathways were involved in the regulation of cell proliferation and differentiation of NSCs such as β -catenin/TCF, Wnt, and glycogen synthase kinase beta 3 (GSK-3 β). Wnt is the most important signalling pathways in neural development and plays a major role in the enhancement of the proliferation and differentiation of the NSCs (Ille and Sommer, 2005). Moors *et al.* (2012) studied the effects of CS on the proliferation and differentiation of human NPC. They found that the exposure to 1 μ M DEX leads a reduction in the proliferation and neural differentiation of human NPCs (Moors et al., 2012). The mechanism underlying the DEX-induced inhibition of the proliferation and neural differentiation of human NPCs is mediated by the inhibition of the canonical Wnt signalling pathway.

In 2012, Garza *et al.* exposed NSCs and NPCs which were isolated from the hippocampus of adult rats to CSs and different concentrations of leptin for 48 h in

order to study the effects of leptin in reversing the action of CSs on neurogenesis and to explore the potential mechanisms underlying this process. Their results demonstrated the reciprocal effects between leptin and CSs on the neurogenesis process, CSs reduced the neurogenesis whereas leptin increased this process via activating the GSK3 β / β -catenin signalling pathways (Garza et al., 2012).

Sonic hedgehog is a soluble signalling protein that plays an important role in regulating processes during nervous system development and adult hippocampal neurogenesis. Further, it was found that Hedgehog signalling pathways plays an essential role in the regulation of brain development by inducing the proliferation of NPCs of mice. In 2013, Anacker and colleagues studied the effects of cortisol on the proliferation and neural differentiation *in vitro*, in human hippocampal progenitor cells, and *in vivo*, in prenatal stressed rats. They observed that the low concentration of cortisol (100 nM) increased the proliferation, reduced the neurogenesis and increased the differentiation of astrocytes, whereas the high concentration of cortisol (100 μ M) reduced the proliferation and neural differentiation *in vitro* and *in vivo*. Their data identify the molecular signalling pathways which are involved in the effects of cortisol on proliferation and neural differentiation. They found that the low and high concentrations of cortisol inhibit the hedgehog signalling (Anacker et al., 2013).

Additionally, the lipid raft protein caveolin-1 was found to involve on the inhibitory effects of DEX on NPCs (Peffer et al., 2014). While, the analysis of signalling pathway in another *in vitro* study revealed that CORT induces downregulation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signalling pathways, indicating their important roles in the reduction of NPSCs viability and their differentiation into neurons and astrocytes

following CORT treatment (Odaka et al., 2016). Table 5.1 summarises a number of signalling pathways which regulate the proliferation and differentiation of NSCs following CS treatment.

Table 5.1 The role of signalling pathways in regulating of the proliferation and differentiation of NSCs following CS treatment				
Compound	Type of cells	Neurological effects	Type of signalling pathways	Reference
DEX	Human NPCs	Reduces the proliferation and neural differentiation	Canonical Wnt	(Moors et al., 2012)
DEX and leptin	NSCs and NPCs	Reduces the neurogenesis whereas leptin increase this process	GSK3 β / β catenin	(Garza et al. 2012)
Cortisol	NPCs	Reduces the proliferation and neural differentiation	Hedgehog	(Anacker et al., 2013)
DEX	NPCs	Reduces the proliferation of NPCs	Caveolin-1	(Peffer et al., 2014)
CORT	NPSCs	Reduces the viability and differentiation of NPSCs	ERK and PI3K/Akt	(Odaka et al., 2016)

All the above studies give several different mechanisms of action of CS in the developing brain using gene expression and microarray analyses. This contradiction is the driving force for the intensive search for more accurate analysis to determine the mechanism of CSs. Given widespread clinical CS use, and the key roles of NSCs in developmental and regenerative processes, there is a significant need to investigate the mechanisms mediating CS effects on NSCs. It should be noted that the overwhelming majority of studies investigating CS effects on NSCs rely exclusively on histological analyses alone. While useful, such assays cannot provide an unbiased detailed insight into the molecular mechanisms underpinning the observed neurological effects of NSCs. In this chapter, proteomic analysis was carried out to provide an insight into the molecular changes induced by CS in NSCs. This allows generating independently validated and corroborative analyses of CS effects at both the morphological and molecular levels, in order to investigate the mechanisms by which this major class of anti-inflammatory drugs impact NSCs development and function. Moreover, this approach was used to sample cellular proteins in parallel with histological observation of cells in culture and provide an unbiased survey, unhindered by prior expectation.

Many types of information cannot be obtained from analysis of transcript levels or genetic sequencing. For example, it is impossible to understand the mechanisms of disease, aging, and effects of the environment by studying the genome because proteins are (largely) responsible for the phenotypes of cells, rather than the encoding genes. There is a recognised disparity between alterations in transcription and resultant changes to protein levels (Lawless et al., 2016). Proteome analysis can, if designed appropriately, characterise protein expression

alterations, post-translational modifications and identify the targets of drugs (Aebersold and Mann, 2016). The proteomics approach gives a fuller picture of the actual state of NSCs following CS treatment and provides a global integrated view of cellular processes by studying protein properties on a large scale. Most importantly, the proteomics analysis provides more information about the proteins and their subcellular localizations compared to genomic analysis (Lawless et al., 2016; Nagaraj et al., 2011; Shoemaker and Kornblum, 2016). Protein localization is one of the most important regulatory mechanisms. In more complex analyses, proteomics can aid in the further identification of the subcellular location of each protein and create a complete three-dimensional map of the cell indicating where proteins are located. Proteomics can also help to develop a complete 3-D map of all protein interactions in the cell (Graves and Haystead, 2002). The understanding of protein-protein interactions is important information in biology. For example, the process of cell growth, programmed cell death and the decision to proceed via the cell cycle are all regulated by signal transduction through protein complexes (Graves and Haystead, 2002). Also, protein pathways and networks could be investigated by using this analysis.

Mass spectrometry represents one of the most comprehensive and versatile approach in proteomics (Yates et al., 2009). Therefore, proteomics analysis was chosen in this study to investigate the molecular mechanism of CSs on NSCs in monolayer culture by using mass spectrometry and bioinformatics analysis.

5.2 Chapter objectives

This chapter was undertaken to explore the potential mechanisms underlying the effects of MPRED on NSCs in monolayers culture using proteomic and bioinformatics analyses.

The objectives of this chapter are:

- 1. To assess GR expression - the target of drug action, in NSCs and their differentiated cells (astrocytes, neurons, and oligodendrocytes).**
- 2. To assess molecular changes in NSCs after CSs treatment using proteomics and bioinformatics analyses.**
- 3. Validate the expression of a key protein found to be altered in proteomic analysis**

5.3 Results

5.3.1 GR expression in NSCs and their daughter cells

In order to show the presence of GR in NSCs parent cells, NSCs were treated with 10 μ M of three different types of CS (DEX, PRED and MPRED). Immunostaining showed positive staining for GR in NSCs after stimulation with the CS (Figure 5.1A). The results showed that 10 μ M CS treatments induced nuclear translocation of GR, with greater intensity of nuclear staining observed following drug application (Figure 5.1 B-D). Double staining of cells showed that GR was present in cells positive for the NSCs marker nestin. Similar findings were observed in NSCs differentiated cells following CS treatment. Double staining of the differentiated cells showed that GR was present in cells positive for the astrocyte marker GFAP, neuron marker TUJ 1 and oligodendrocyte marker MBP (Figure 5.2). These results show that GRs are present in NSCs and all their daughter cell types, suggesting that these cell types are capable of responding to CS treatment.

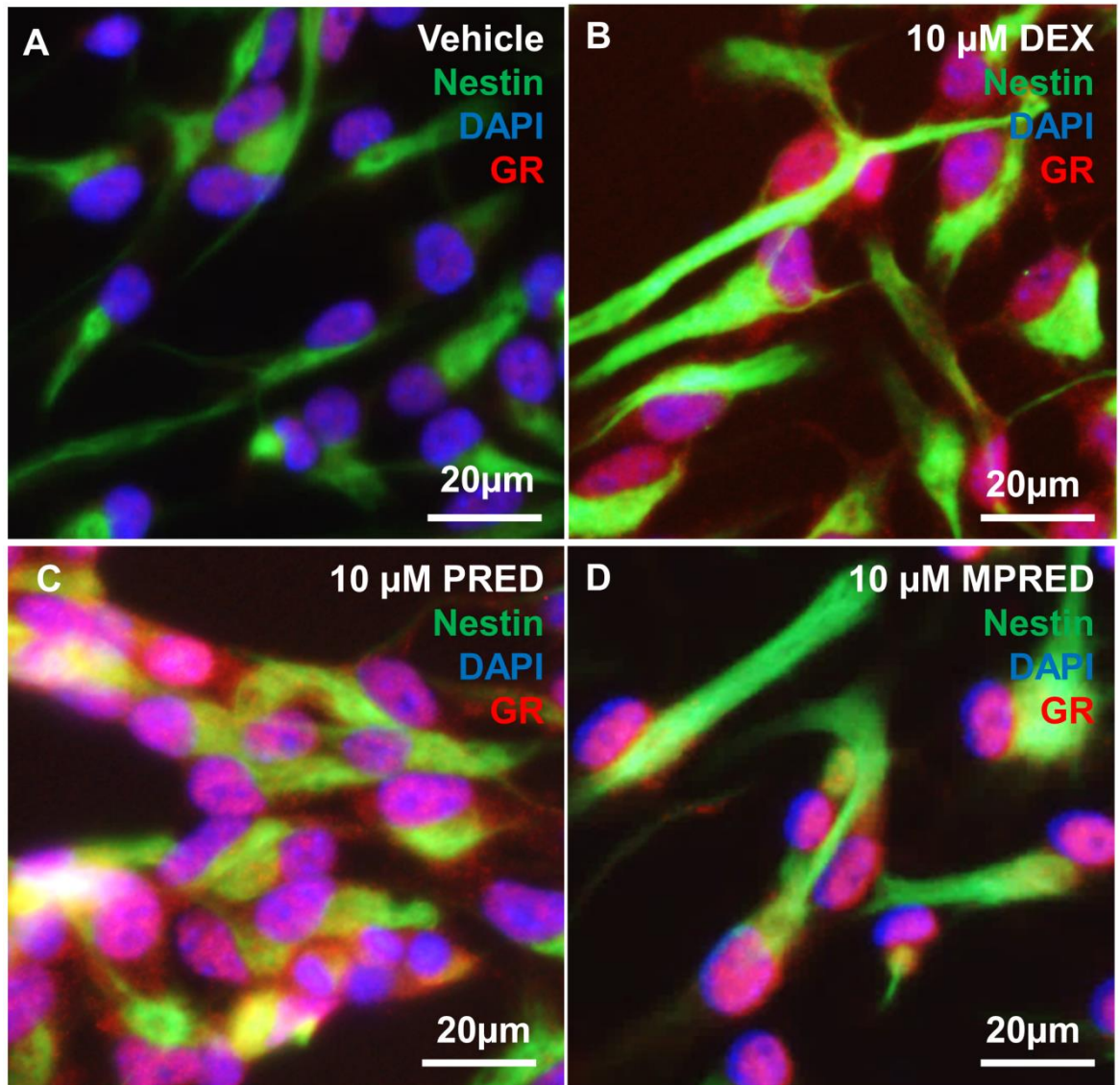


Figure 5.1 NSCs express GR. (A) Representative fluorescence micrographs showing *nestin*⁺ NSCs and GR expression in vehicle control cells. (B-D) Representative fluorescence micrographs showing *nestin*⁺ NSCs and GR expression in treated cells. (48 h CSs, 10 μM); *n* = 3.

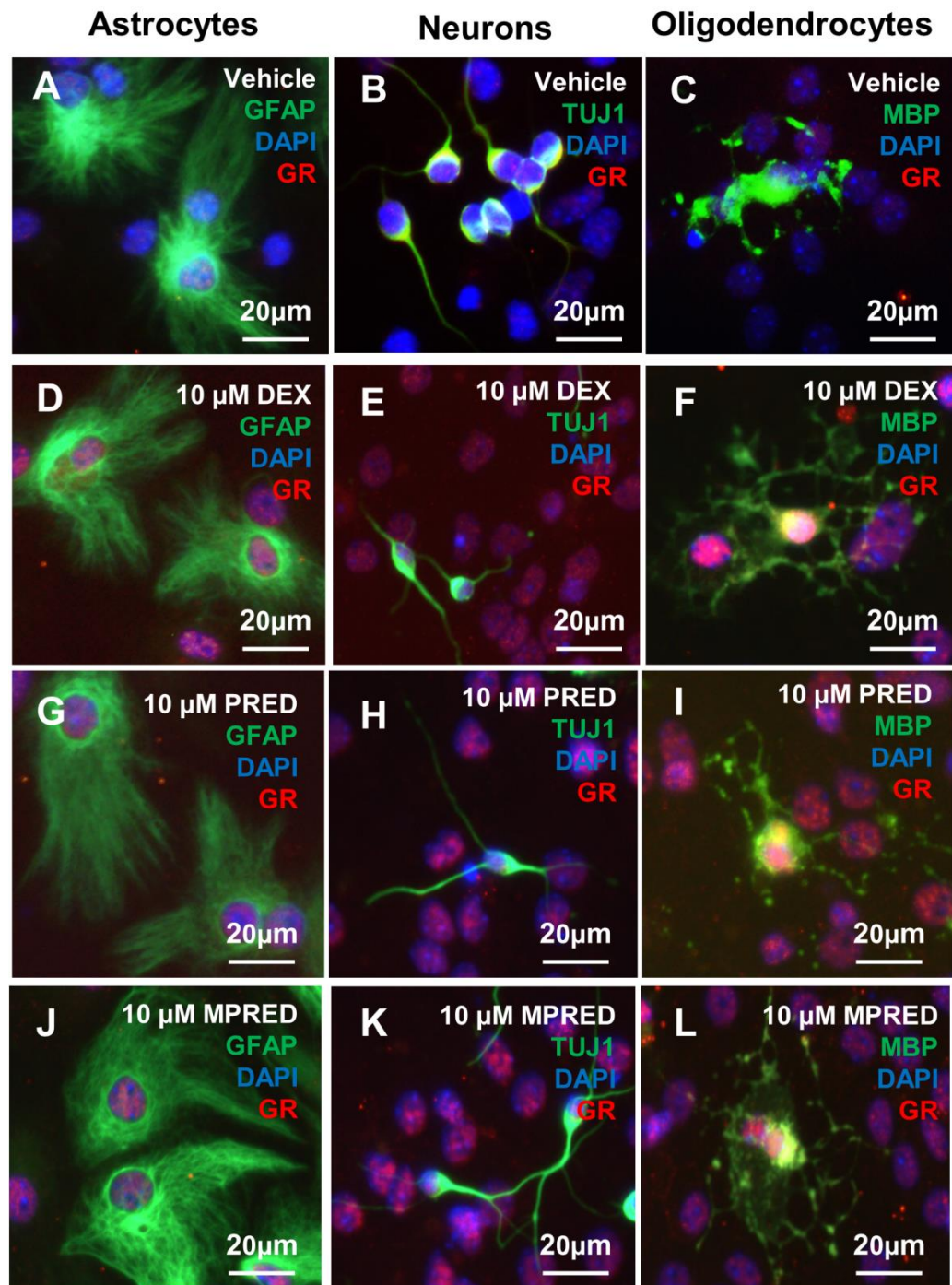


Figure 5.2 Differentiated cells express GR. Representative fluorescence micrographs showing GR expression in vehicle control (A-C): GFAP⁺ astrocytes (A), TUJ 1⁺ neurons (B), and MBP⁺ oligodendrocytes (C), compared with CS-treated cells GFAP⁺ astrocytes (D, G and J), TUJ 1⁺ neurons (E, H and K) and MBP⁺ oligodendrocytes (F, I and L), (48 h CSs, 10 µM); n = 3.

5.3.2 Molecular analysis of the effect of MPRED on NSCs in monolayer culture

A proteomics analysis was performed to assess the effects of 10 μ M MPRED on protein expression in NSCs monolayer culture. MPRED is one of the most clinical relevant drugs which are widely used in ASCI and TBI treatments due to their ability to reduce swelling and inflammation. Also, MPRED used following transplantation to improve grafts survival and to avoid cells rejection in the host tissue. However, there is much controversy over the use of MPRED, a number of previous studies have shown the neurological effects following MPRED treatment and these effects are mediated by NSCs. For example, the inhibition effects of MPRED on the proliferation of endogenous NPCs following SCI was demonstrated in several preclinical studies (Li et al., 2012; Obermair et al., 2008; Schröter et al., 2009; Wang et al., 2014). Also, through the findings obtained from this present study, it has been proved that MPRED has inhibition effect on the proliferation of NSCs and neuronal differentiation. The literature pertaining to MPRED effects on NSCs contains contradictory information regarding the mechanisms underlying the detrimental effects of MPRED. Therefore, the purpose of this section is to investigate the potential mechanism of MPRED in primary NSCs monolayer culture by studying protein expression profiling. LC-MS/MS DDA and LC-MS^E DIA were both performed in this study.

5.3.3.1 DDA analysis

The performance of DDA analysis was evaluated to identify key proteins involved in the changes observed in NSCs cellular proliferation capability following CS treatment. In DDA analysis, peptide ions are selected for fragmentation using the initial quadrupole MS analyser; these are then activated by collision with inert argon gas molecules at high energy; the unimolecular dissociation products are analysed in the time-of-flight mass analyser to observe the product ions. DDA analysis was used in this study to verify our protein extraction and digestion procedures. DDA analysis generated a large number of MS/MS spectra and the Scaffold analysis of DDA data confirmed a core proteome. These 66 'core' proteins were found in all conditions (see Appendix 1). A small amount of variation was observed in protein profile between conditions (Figure 5.3). This variation is likely to result from undersampling resulting from the switching nature of the DDA experiment, undersampling being a common observation in QToF experiments on instruments of a similar generation (Wang et al., 2010).

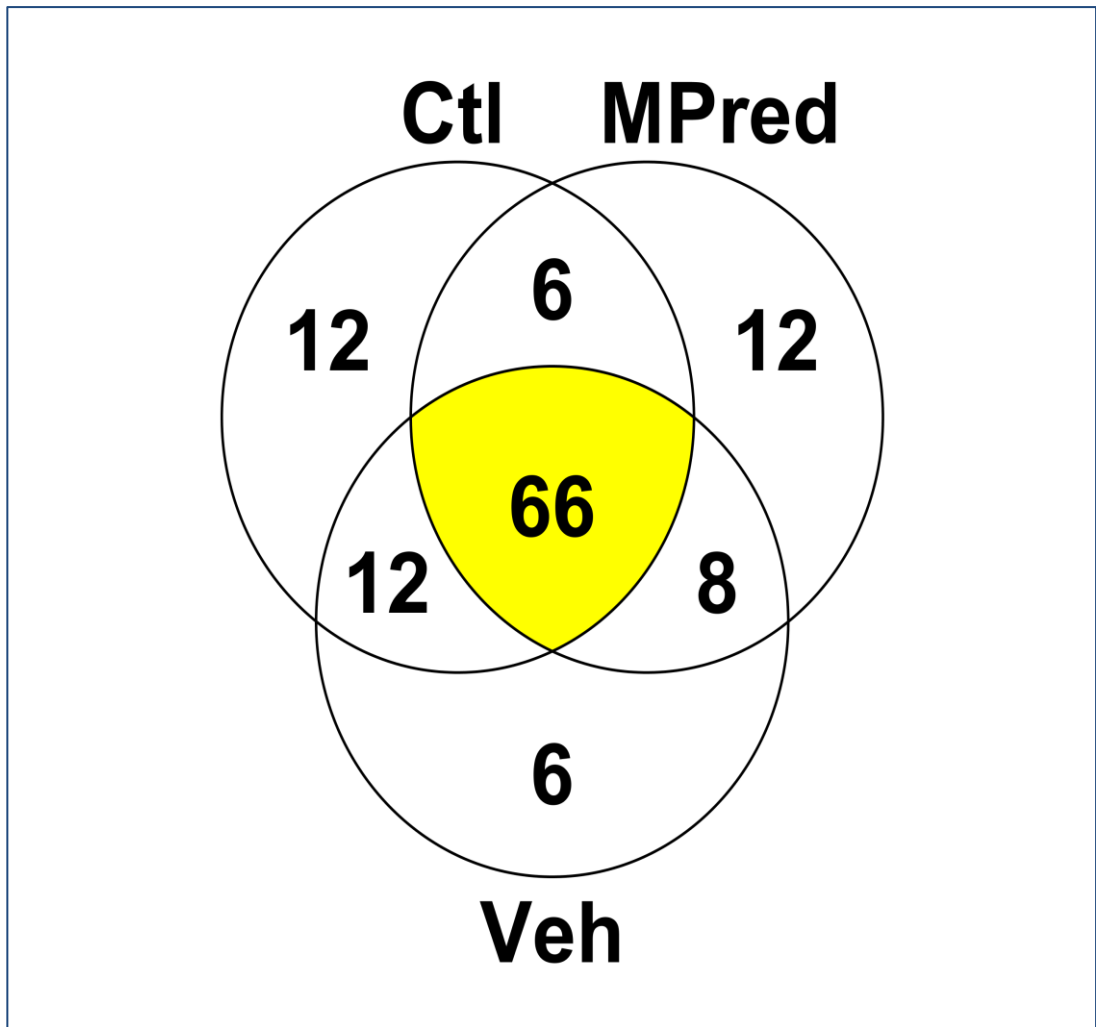
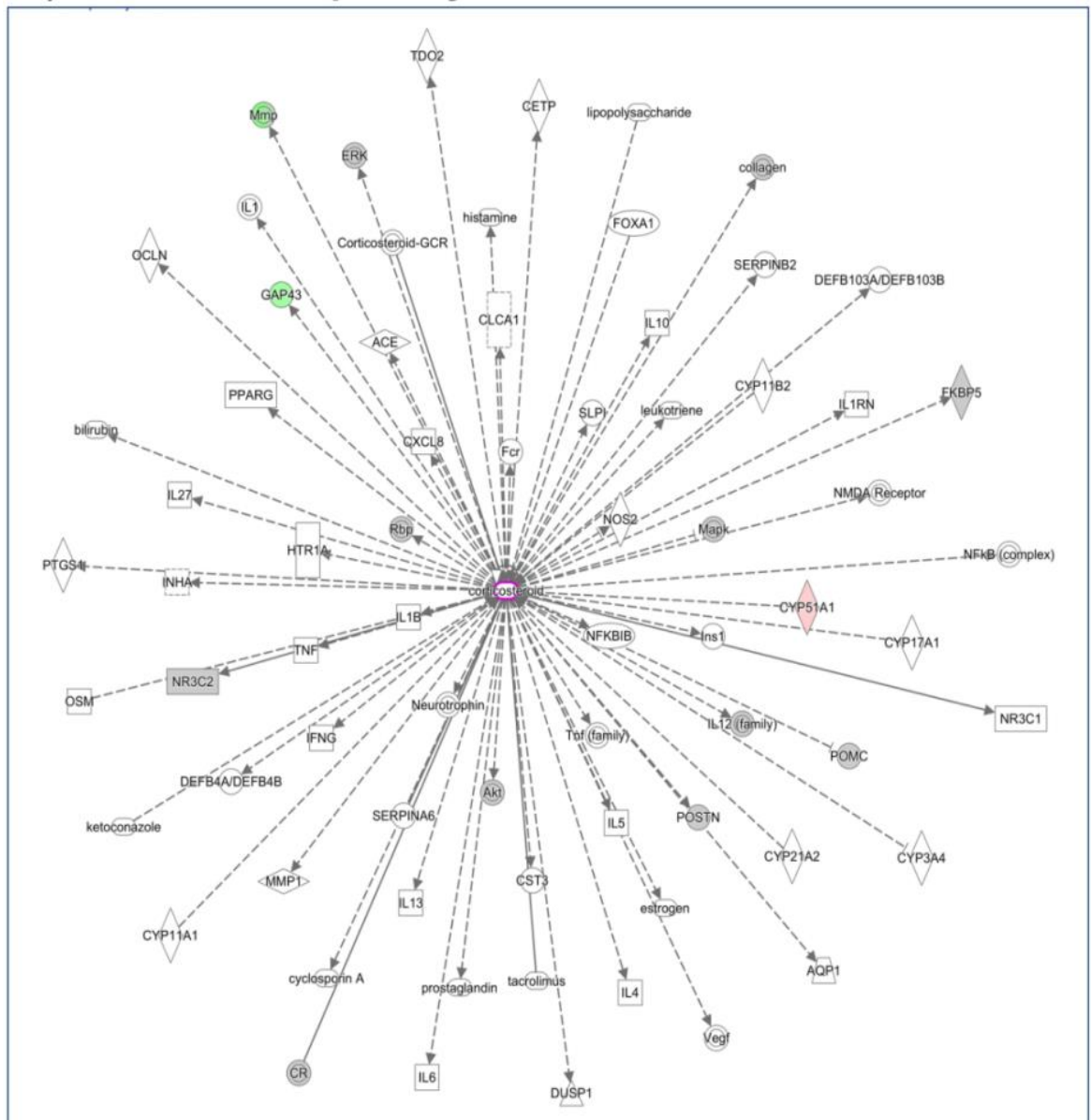


Figure 5.3 Scaffold analysis of DDA. Venn diagram showing comparative data on proteins identified using Scaffold analysis across all conditions; (48 h MPRED; 10 μ M); $n = 3$.

5.3.3.2 DIA analysis

DIA was used as alternative approach to DDA analysis for identifying and quantifying proteins in the samples. DIA analysis isolates and fragments all of the molecular species within a given mass-to-charge window, without a precursor ion selection step. Therefore, the main differences between the DDA and DIA analyses are that the window selection of the quadrupole mass analyser is dynamic during DDA, while it is used to scan the complete spectrum during DIA. Proteomic analysis was used in this study to provide an unbiased readout of molecular phenotype following MPRED treatment. A total of 3,220 quantifiable proteins from >20,000 peptide features were identified across the biological and technical replicate analyses. IPA was used to examine clustering of differentially-expressed proteins as nodes within molecular networks (Kalayou et al., 2016). A number of proteins showed significantly altered expression levels, however these were isolated entities within otherwise unaffected pathways; no entire pathways showed strong evidence of being significantly dysregulated. Whilst not linked in a canonical pathway, we next sought to identify dysregulated proteins that are known to have direct interaction with CSs. There are 72 CS interacting molecules identified by IPA, 14 of which were proteins identified by the proteomics approach used here. Three of these demonstrated significant differential expression: down-regulation of matrix metalloproteinase 16 (MMP-16) and GAP-43, and up regulation of cytochrome p450 51 A1 (CYP 51 A1) (Figure 5.4A). Relative quantification of the peptide features as performed by Progenesis Q1 showed consistent patterns of regulation for the aforementioned CS primary interactors (Figure 5.4B).

A) corticosteroid.primary.interactors



Legend

- | | | | |
|---|-----------------------------------|-------|-------------------------------|
| ◇ | Enzyme | △ | Phosphatase |
| ⊙ | Complex | ◇ | Peptidase |
| □ | Chemical | ○ | Other |
| □ | Cytokine | — | Direct interaction |
| △ | Transporter | - - - | Indirect interaction |
| ○ | Transcription regulator | ● | Downregulated |
| □ | G-protein couple receptor | ● | Upregulated |
| □ | Ligand-dependent nuclear receptor | ● | Neither up nor down-regulated |

B) Progenesis QI for proteomics normalised expression profiles

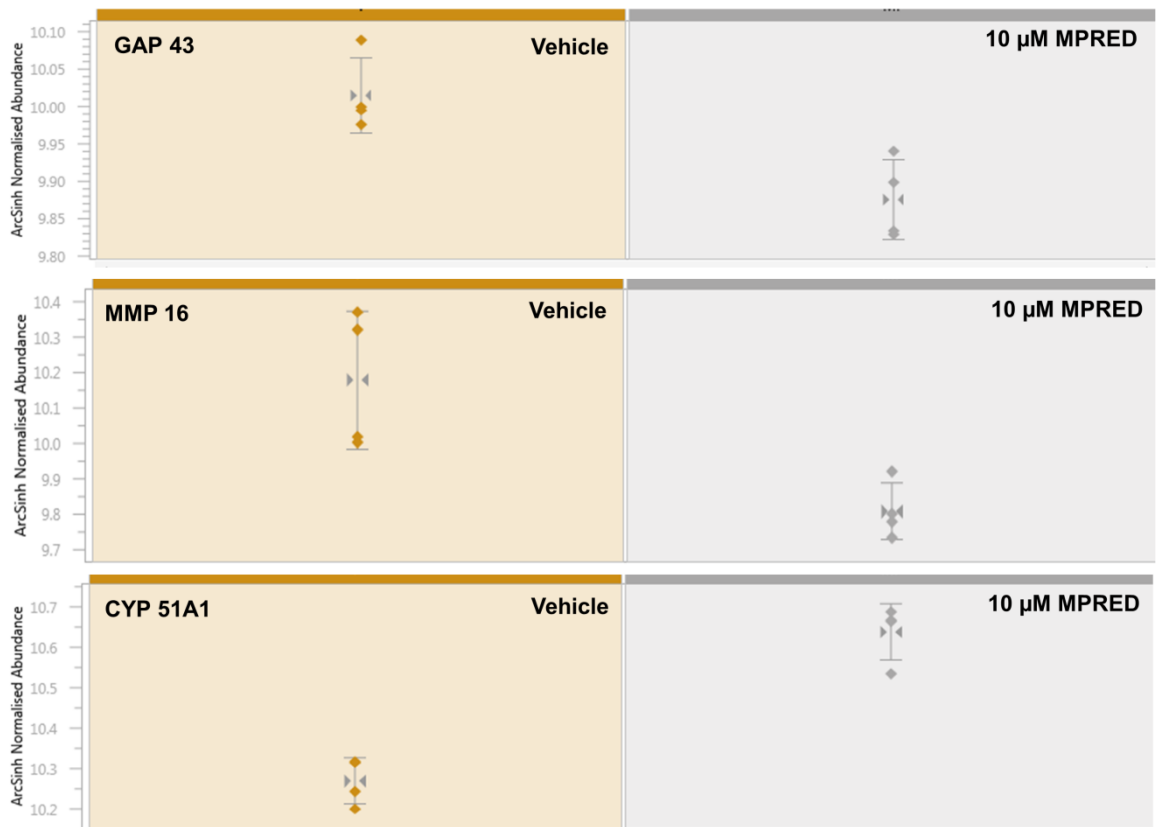


Figure 5.4 Differential protein expressions by NSCs following MPRED treatment. (A) *Ingenuity™ Pathway Analysis* was used to cluster identified proteins according to biochemical pathways differentiating controls from MPRED-treated cells. Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins (ANOVA $p < 0.05$, min. 2 fold). Grey nodes indicated protein detection without differential expression. Progenesis QI for proteomics normalised expression profiles of (B) MMP-16, (C) GAP-43 and (D) CYP51A1 illustrating protein abundance in MPRED-treated cells compared with controls, (48 h MPRED; 10 µM). Progenesis QI was carried out in three biological replicates, with three technical replicates in each experiment.

5.3.3.3 MPRED reduce GAP-43 expression in NSCs and their differentiated cells

GAP-43 is a pre-synaptic protein located on the growth cones of axons, plays important roles in cytoskeletal dynamics such as axonal growth and guidance and in modulating synapse formation (Latchney et al., 2014). The synthesis of GAP-43 is correlated with the development and regeneration of neurons (Hoffman, 1989). Therefore, GAP-43 down regulation could explain the reduced neuronal number and axonal growth in our histological analyses. GAP-43 considers as an important finding impacting neural development, and hence this protein was chosen for further detailed immunohistochemically analysis.

The results from proteomic analysis were confirmed using immunostaining to detect GAP-43. Both NSCs parent cells and their differentiated cells expressed GAP-43 in treated and untreated cells. However, a significant reduction in GAP-43 expression was evident in MPRED treated NSCs and in the neurons derived from treated NSCs compared to controls. Quantification of the fluorescence intensity of GAP-43 immunostaining revealed that MPRED treated NSCs and neurons consistently demonstrated significantly lower optical density values than controls (Figure 5.5).

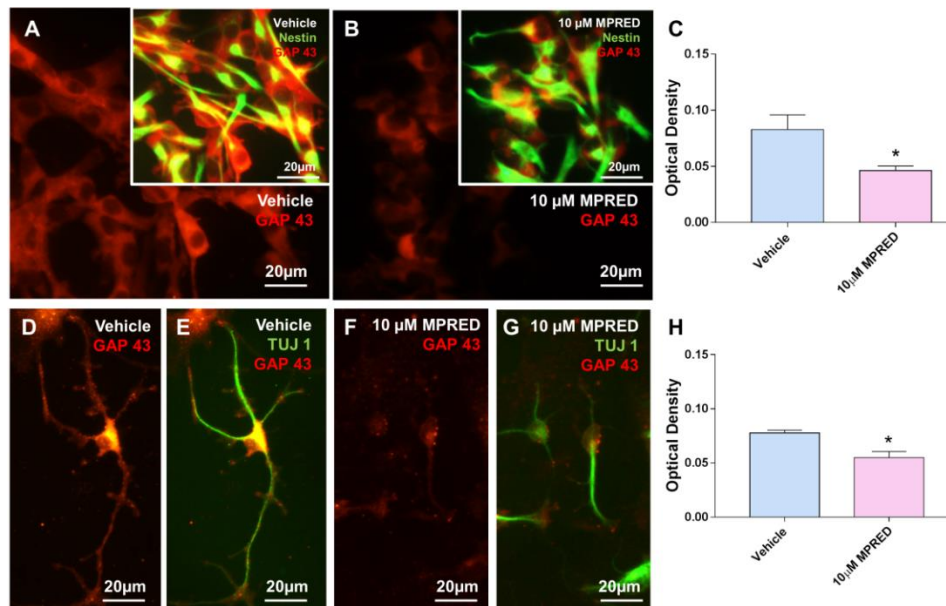


Figure 5.5 NSCs and their differentiated cells show a reduction in GAP-43 expression following MPRED treatment. *Fluorescence micrographs showing GAP-43 expression in vehicle control (A) and 10 μM MPRED treated (B) NSCs. Note the marked reduction in GAP-43 expression following MPRED addition. (A and B insets are the same fields with addition of nestin). (C) Bar graph showing the optical density measurements of GAP-43 expression in MPRED treated NSCs over vehicle controls. (D) Representative fluorescence micrograph of neurons derived from untreated NSCs showing extensive GAP-43 expression. (E) Fluorescent counterpart to D showing co-localisation of GAP-43 staining with TUJ-1. (F) Representative fluorescence micrograph of neurons derived from 10 μM MPRED treated NSCs showing marked reduction in GAP-43 expression. (G) Fluorescent counterpart to F with the addition of TUJ 1. (H) Bar graph showing the optical density measurements of GAP-43 expression in MPRED of neurons derived from treated NSCs culture over vehicle controls. (48 h MPRD; 10 μM; unpaired t test with error bars representing SEM; *p < 0.05. Experiment was carried out in three biological replicates, with three technical replicates in each experiment.*

5.4. Discussion

This study was performed to elucidate the key proteins involved in the changes observed in NSCs monolayer culture following CS treatment. The molecular mechanisms of the effects of CS remain unclear and not fully understood. A complementary histological and proteomic analyses were carried out to take the first step towards a global identification of the molecular changes induced by CS, as a basis for understanding the underlying mechanisms of their adverse neurodevelopmental effects.

CSs exert their effect via binding with GRs by genomic and non-genomic mechanisms (De Kloet, 2004; Falkenstein et al., 2000; Hwang et al., 2006). Many experimental studies have shown that the adverse effects of CS are mediated by the activation of GRs. Therefore, it is very important to investigate the presence of GRs in all cell types studied in this research. The findings of this current study demonstrate that both NSCs and all three of their daughter cell types were found to express the GR receptor and therefore, all cell types studied here, can be considered to be CS-responsive. Also, this result suggests that NSCs and their daughter cells can be directly influenced by CSs, since we found GR to be expressed *in vitro*.

Mutsaers and Tofighi (2012) demonstrated that the pre-treatment with mifepristone could abolish the apoptotic effect of CS and Bose *et al.* (2010) observed that the blocking GR with mifepristone could prevent the effects of CS (Bose et al., 2010). Also, Ekthuwapranee and colleagues found that the pre-treatment with mifepristone prevent the inhibitory effects of DEX on the number of neurospheres (Ekthuwapranee et al., 2014). Moreover, it was demonstrated that the high level of cortisol reduced the proliferation of human hippocampal progenitor cell line via

GRs (Anacker et al., 2013). All these studies and several similar studies revealed that CSs mediate their effect by activation of GRs (Boku et al., 2009; Kim et al., 2004).

These previous studies were performed using gene expression and microarray analyses, but as far as I am aware, no in-depth study of the influence of CS treatment upon protein expression has yet been performed in NSCs. This is important as the correlation between transcript and protein expression changes is relatively poor, for example only 40% of altered protein expression can be directly predicted by changes in transcript levels (Vogel and Marcotte, 2013). Therefore, the proteomic analyses are beneficial in this regard, as it enables unbiased detection of molecular mechanisms potentially mediating the observed CS effects on NSCs proliferation and differentiation. Follow up hypothesis-driven investigations can then be performed on specific identified dysregulated proteins and their phenotypic influence; these will be the goal of future research.

Mass spectrometry based proteomics was used in the present study to identify the molecular mechanisms underpinning the effects of MPRED on NSCs. Two types of analysis, DDA and DIA were performed. The major advantage of DDA analysis is the generation of primary structural information from the peptide precursor ion selected for fragmentation. However, the DDA process is limited by the reproducibility, sensitivity and speed by which the mass spectrometer can sequentially acquire product ion spectra. Therefore, DIA analysis was used as an alternate method with the capacity to overcome this limitation. DIA systematically parallelizes the fragmentation of all precursor ions within a wide mass-to-charge ratio (m/z) range regardless of intensity, thereby providing broader dynamic range of detected signals, improved reproducibility for identification, better sensitivity,

and accuracy for quantification, and, potentially, enhanced proteome coverage (Geromanos et al., 2009). Therefore, the proteomic analysis opens up the possibility that understanding the mechanisms underlying CSs may allow its pharmacological reversal and improve outcome.

The data presented in this chapter suggest that a potential mechanism underpinning the effects of MPRED could be via reduced GAP-43 levels – a nervous tissue-specific protein highly expressed in neurons and glial cells. The failure of NSCs to express GAP-43 can reduce neurogenesis, increase apoptosis of neurons and affect their maturation (Shen et al., 2004). The downregulation of GAP-43 can be considered as an important finding that impacted neural development, and hence I chose GAP-43 expression for further detailed immunohistochemical analysis. The findings showed a reduction in the expression of GAP-43 in both NSCs and newly generated neurons following MPRED treatment. The synthesis of GAP-43 is correlated with the development and regeneration of neurons (Hoffman, 1989), therefore GAP-43 down regulation could explain the reduced neuronal number and axonal growth in the histological analysis.

Also, MPRED reduced levels of MMP-16, a member of a family of proteinases which regulate biological functions such as neurogenesis, axonal extension, differentiation and cell migration in the developing and adult nervous systems. MMPs play an essential role by ECM remodelling (Stamenkovic, 2003); the ECM and its remodelling regulate many aspects of cellular behaviour such as proliferation, migration and differentiation of neural cells and NSCs (Faissner et al., 2010; Fujioka et al., 2012). The involvement of MMPs in ECM remodelling enabling axonal extension and repair after brain injury suggests a contribution to

the effects on neurogenesis and growth observed in this study; specifically, overexpression of MMPs has been shown previously to increase neurite extension and migration of neurons during neuronal development (Larsen et al., 2003; Reeves et al., 2003; Reichardt, 1991).

Additionally, the findings from proteomics analysis showed that MPRED increased levels of CYP51A1 (aka lanosterol 14 α -demethylase). These finding suggests a possible link between the upregulation of CYP51A1, key protein involved in cholesterol biosynthesis and the maturation of oligodendrocytes that observed following MPRED treatment on NSCs in monolayer culture (Chapter 3). CYP51A1 is involved in important steps in the biosynthesis of cholesterol (Björkhem et al., 2004; Debeljak et al., 2003) which is found in high levels in myelin, (Orth and Bellosta, 2012). Therefore, any alterations in cholesterol biosynthesis could be associated with impairments to oligodendrocyte development and the myelination process.

To the best of our knowledge, the effects of MPRED on these three identified proteins in NSCs have not been reported to date. Several previous studies demonstrated that MPRED exerted inhibitory effects on the proliferation of endogenous NPCs *in vitro* and *in vivo* after SCI (Schröter et al., 2009). In their study, Wang *et al.* indicated that MPRED reduced the proliferation and effects on differentiation of spinal cord-derived NPCs under both normoxic and hypoxic conditions. Also, they used polymerase chain reaction and western blot analysis to study the molecular mechanisms responsible for the effects of MPRED on NPCs and suggest that the down- regulation of HIF-1 α and Hes1 play a vital role in this

effect (Wang et al., 2014). While gene expression profiles in another study showed different molecular mechanism underpinning the anti-proliferative effects of MPRED on NPCs. They suggested EdnrB is involved in this effect (Li et al., 2012). Microarray analyses comparing gene expression profiles of CSs-treated NSCs have yielded important, yet limited, information of the pathways responsible for CSs effects. Although, many different genes were identified as CSs-regulated, only few genes were reproduced in more than one publication. This suggests that RNA-based methods may be limiting and there is growing evidence that levels of mRNA transcripts do not necessarily reflect protein amounts (Unwin et al., 2006).

Taken together, this is the first study to use discovery mass spectrometry based proteomic approach to investigate the effects of MPRED on NSCs. The proteomics and bioinformatics analyses following MPRED treatment suggest that the down regulation of GAP-43 and MMP-16, along with upregulation of CYP51A1 proteins, provide an explanation for the observed histological effects of CS on NSCs. However, further systems biology approaches will be required to elucidate the differences in the modes of action of all three types of CS on NSCs fate in more detail.

Chapter 6

Testing NSCs in a combinatorial therapy using organotypic spinal cord slice model

6.1 Introduction

As mentioned previously in the main introduction, several types of stem cell transplantation strategies such as OECs, Schwann cells and foetal /adult NSCs have been widely tested to promote functional recovery following neurological injury (Hooshmand et al., 2009). NSCs transplantation offers great potential for repair and regeneration in SCI. These cells have the ability to replace lost or damaged neural cells and provide neurotrophic factors. Further, NSCs can be induced to differentiate into enriched populations of neurons or glial cells (Cao et al., 2002). For instance, the transplantation of NSCs populations has been shown to promote functional recovery in SCI through integration of myelinating oligodendrocytes, neuronal differentiation or by inducing endogenous repair (Hooshmand et al., 2009). In 2002, Ogawa and colleagues transplanted *in vitro*-expanded neurosphere cells that were derived from rat embryonic spinal cord (embryonic day 14.5) into the site of injury in rat spinal cord model. They found that transplanted NSCs had the ability to generate neurons and improved motor function *in vivo* following transplantation. Furthermore, they observed the formation of synaptic structures of neurons that were differentiated from the grafted cells (Ogawa et al., 2002). Another study has shown that human foetal NSCs survived and differentiated into neurons and oligodendrocytes following transplantation into the contused rat spinal cord at 9 days after injury (Tarasenko et al., 2007). All these previous experimental studies support the idea of NSCs serving as a promising cell replacement therapy for CNS injury.

Previously, several studies demonstrated the ability of NSCs to secrete neurotrophic factors. For example, Kamei and colleagues clarified the mechanism that underlies promotion of axonal regeneration following transplantation of NPCs in the injured rat spinal cord. They found that NPCs secreted BDNF, NT3 and NGF before and after differentiation. This result suggested that NPCs may promote axonal regeneration via these three types of neurotrophic factors (Kamei et al., 2007). In 2008, Bottai and colleagues successfully transplanted GFP-labelled NSCs into the injured mouse spinal cord and their results showed that NSCs significantly improved functional recovery. They observed that the level of BDNF, NGF, LIF and NT3 were significantly increased at 48 h after NSCs transplantation (Bottai et al., 2008).

Unfortunately, NSCs application in neurological treatment remains a distant goal, as there are many unsolved problems. One of these problems is that the majority of implanted NSCs have a tendency to differentiate into astrocytes, but not into neurons and oligodendrocytes (Ogawa et al., 2002; Wu et al., 2001).

Cell death following transplantation is another problem faced in NSCs application. Several studies have suggested that the high concentration of extracellular glutamate, and increased levels of inflammatory cytokines immediately after injury may contribute to the poor survival of NSC transplants (Bottai et al., 2008; Tarasenko et al., 2007).

Also, cell source is one of the main limitations of stem cell transplantation. Currently, a wide range of stem cells from different sources are being investigated for their potential role in treatment several neurological disorders. For example, there are a number of obstacles are associated with the clinical applications of

transplantation of both foetal and adult NSCs *in vivo* including ethical concerns, host immune rejection in the absence of anti-inflammatory and immunosuppressive drugs and the formation of teratocarcinomas (Steinbeck and Studer, 2015; Yu and Silva, 2008).

Finally, the activation of microglia can also affect NSCs transplantation. Microglia, the brain's resident immune cells, become activated following pathogens, injury, or damage, resulting in rapid proliferation and migration towards the lesion site (Tikka and Koistinaho, 2001). Activated microglia are able to phagocytose cellular debris and toxic substances that have an inhibiting effect on axonal regeneration and produce pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α , chemokines and nitric oxide into the lesion site (Chao et al., 1992; Merrill et al., 1993; Smith et al., 2012; Tikka and Koistinaho, 2001). It was found that the neurotoxic molecules that are produced by microglia can contribute to further neuronal damage leading to permanent neurological deficit (Festoff et al., 2006). For example, a number of neurobiological studies show that activated microglia can affect NSCs transplantation. It was observed that both microglia and macrophages surround the transplanted NSCs, with the macrophages phagocytosing most of the grafted cells (Bottai et al., 2008; Su et al., 2007). An *in vitro* study was conducted by Cacci and colleagues to study the direct effects of microglia on NSCs. They found an increase in the apoptosis of NPC and a reduction in neuronal differentiation following activation of microglia, due to the high level of pro-inflammatory cytokines (Cacci et al., 2008). Also, it was demonstrated that activated microglia could be one factor responsible for the low survival rate of grafted NSCs, in that they are found to work with T cells to reject transplanted cells (Tambuyzer et al., 2009). In 2011, Darsalia and colleagues found that transplantation at 48 h resulted

in higher cell survival than transplantation at six weeks following stroke in rat brain, because the number of activated microglia was increased at this later time point, reaching maximum levels at 1 to 6 weeks following stroke (Darsalia et al., 2011). Furthermore, it was found that activated microglia may affect oligodendrocytes and myelination (Bosco et al., 2008). Previous study showed that microglial activation could impair neurogenesis due to their deleterious effect on the newly formed neurons which is mediated via the action of cytokines (Ekdahl et al., 2003).

For these reasons, the use of anti-inflammatory and immunosuppressive drugs following transplantation is necessary to reduce the inflammation and limit cell rejection. CSs such as MPRED are used in treatment of neurotrauma including SCI, due to their ability to reduce swelling and inflammation and also used to suppress graft rejection in stem cell transplantation therapy (Jablonska et al., 2013; Wells et al., 2003). However, the adverse effects of CS on NSCs proliferation and neuronal differentiation that were found in the current study are in agreement with previous reports. It is therefore of vital importance to use another anti-inflammatory drug, MINO which render improved SCI outcome, without the risks associated with high dose CS therapy using clinically relevant animal models. MINO would play a pivotal role in several neurological injuries and disease, due to their ability to increase the survival of transplanted NSCs, limit cell rejection and reduce neuroinflammation such as activated microglia. MINO has demonstrated neuroprotective effects in several animal and clinical studies of CNS. For example, MINO reduced lesion size and apoptotic pattern following mild contusion injury of rat spinal cord models (Lee et al., 2003). Clinical trials have shown the beneficial effects of MINO treatment in several neurological disease such as SCI, multiple sclerosis, and Huntington disease. A number of clinical trials are under way for the

use of MINO to treat the neurological conditions including stroke, multiple sclerosis, spinal cord injury, Huntington disease, and Parkinson disease (Plane et al., 2010). **Based on these evidences MINO was chosen in this chapter as a combinatorial therapy with transplantation of NSCs using organotypic spinal cord mouse model.**

Organotypic slices have been successfully generated from different regions of the brain such as hippocampus, striatum, cortex, cerebellum and spinal cord (Cho et al., 2007). They consist of thin, 3-D sections of animal brain and/or spinal cord tissue. These types of slices can be cultured for long periods (weeks or even months) in a dish. Additionally, organotypic slice cultures can preserve their cytoarchitecture and cellular inter-relationships during this period of time (De Simoni et al., 2006).

Organotypic spinal cord slices offer unique opportunities to study axonal regeneration after traumatic injury. Recently, there have been some organotypic spinal cord models developed. One example of this type of model is the one developed by Weightman and colleagues (Weightman et al., 2016, 2014), which has been used to generate traumatic SCI models. They can provide numerous slices in the parasagittal plane from each spinal cord, and have been successfully lesioned to generate a SCI model. Also, these contain all neural cell subclasses which can be easily detected using standard histological procedures. Furthermore, this model allows for the study of different pathological responses such as reactive astrocytosis, axonal regeneration and microglial infiltration (Weightman et al., 2014). The organotypic spinal cord slice cultures offer other advantages, for

instance, they allow live microscopic observation of transplanted cells introduced into slices, In addition, this model allows easy visualization of the outgrowth of nerve fibres using immunofluorescence, scanning and transmission electronic microscopy (Sypecka et al., 2015). Also, the slices cultures are amenable to molecular biology methods such as polymerase chain reaction, Western-blot, the enzyme-linked immunosorbent assay, chromatography, and spectroscopy to gain a more detailed understanding of the underlying mechanisms controlling the process of nerve fibre regeneration and lesion repair (Marsh et al., 2000; Sypecka et al., 2015). Electrophysiological recordings (De Simoni et al., 2006) and time lapse video microscopy can also be conducted in such slices (Seidl and Rubel, 2010; Weightman et al., 2016).

It has to be mentioned, however, that the organotypic spinal cord slices cannot represent all the *in vivo* features including an incomplete immune system and the degeneration of the majority of long white matter tracts surrounding gray matter indicating that contribution of myelin cannot be mimicked in this type of culture. In addition, the use of this model is limited because of lack of uniformity across culture substrates. For example, the differences in anatomy between lateral and central slices. However, the advantage of organotypic spinal cord slices for being easily amenable to experimental manipulation may offset a large part of the differences between the *in vivo* tissue and the organotypic spinal cord slice (Kim et al., 2010).

The SCI is a multifactorial process and complex combinations of therapy are most likely to be of benefit for the repair of such injuries, for example use of anti-inflammatory therapy plus cell transplantation. However, a key point to be noted is that the experimental studies into this area of research are very dependent on live

animal models of neurological injury. These have a number of disadvantages such as ethical issues, expense, technical complexity, large animal numbers and lack of reproducibility. In contrast, *in vitro* experiments have several advantages compared to *in vivo* study. For example, they reduce animals pain and suffering, are technically easy to culture, do not require government permission, reduce cost, finally, fewer numbers of animals are sufficient to give the data statistical significance (Weightman et al., 2016, 2014). Therefore, it would be useful to have a 'dish' model of spinal cord in which to test such complex combinatorial therapy.

6.2 Chapter objectives

This chapter was undertaken to examine whether an *in vitro* model of SCI based on the use of organotypic slices, can be used to evaluate the effects of the combination therapy of MINO and NSCs. However, it is important to investigate the direct effect of MINO on NSCs proliferation, differentiation, viability and apoptosis, to test whether MINO has any negative effect on NSCs before using it as a combinatorial therapy with transplanted NSCs.

The objectives of this chapter are:

- 1. To investigate the direct effects of MINO on NSCs survival/proliferation in 3-D neurosphere cultures**
- 2. To investigate the effects of MINO on the differentiation of NSCs in 3-D neurosphere cultures.**
- 3. To investigate the utility of longitudinal organotypic spinal cord slice model to study the effects of a combinatorial therapy (MINO and NSCs transplantation) for neurological repair.**

6.3 Results

6.3.1 Effect of MINO on NSCs in neurosphere cultures

6.3.1.1 Physiological potency of MINO preparations

Primary microglia cultures were used as positive control to assess the biological efficacy of MINO concentration as detailed in Chapter 2 **section 2.5.4**. Phase-contrast microscopy showed there was a reduction in cell number of microglia following MINO treatment (10 μ M) versus untreated cells. Also, the morphologies of microglial cells were changed in MINO treated microglia versus untreated cells. MINO-treated cells appeared as round cells compared to untreated cells which exhibit ramified morphology, proving that the MINO concentration used is physiologically active (Figure 6.1). Additionally, the spectrophotometrically confirmed that MINO concentration was suitable for eliciting cellular responses.

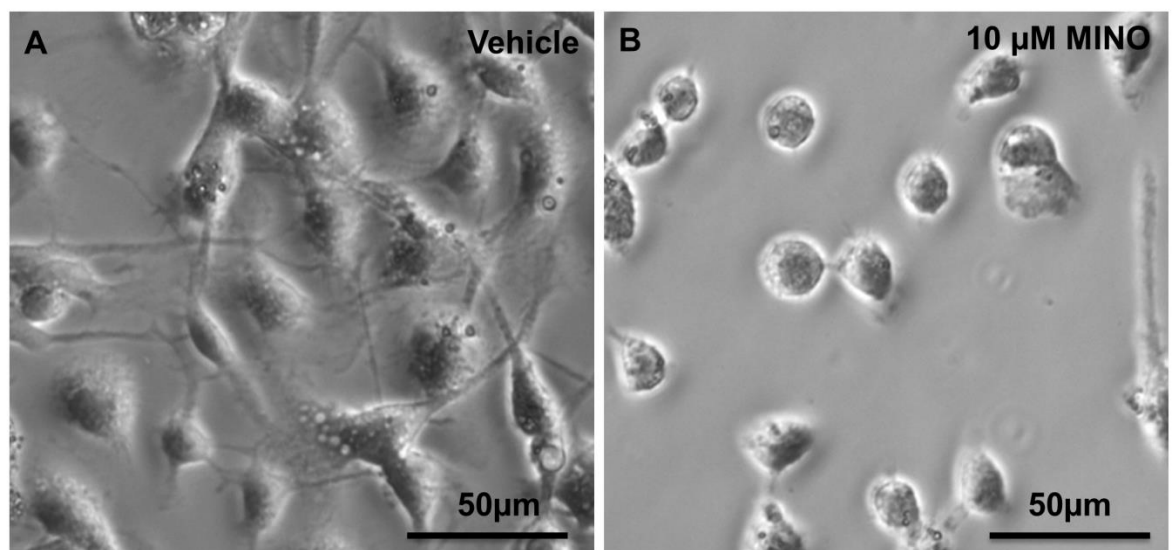


Figure 6.1 Assessment of the biological efficacy of MINO preparations. (A) Phase contrast micrograph showing primary culture of microglia in vehicle control. (B) Phase contrast micrograph depicting a reduction in cell number and changed morphologies of microglia post MINO treatment (48 h MINO; 10 μ M).

6.3.1.2 Effect of MINO on the number and size of neurospheres

To assess the direct effects of MINO on NSCs in neurosphere cultures, 10 μM of MINO was added to NSCs in neurosphere cultures. Following 48 h of MINO treatment, phase images and quantification showed that neurospheres number and size were similar between control and treated samples, indicating that MINO treatment had no adverse effects on NSCs self-renewal (Figure 6.2).

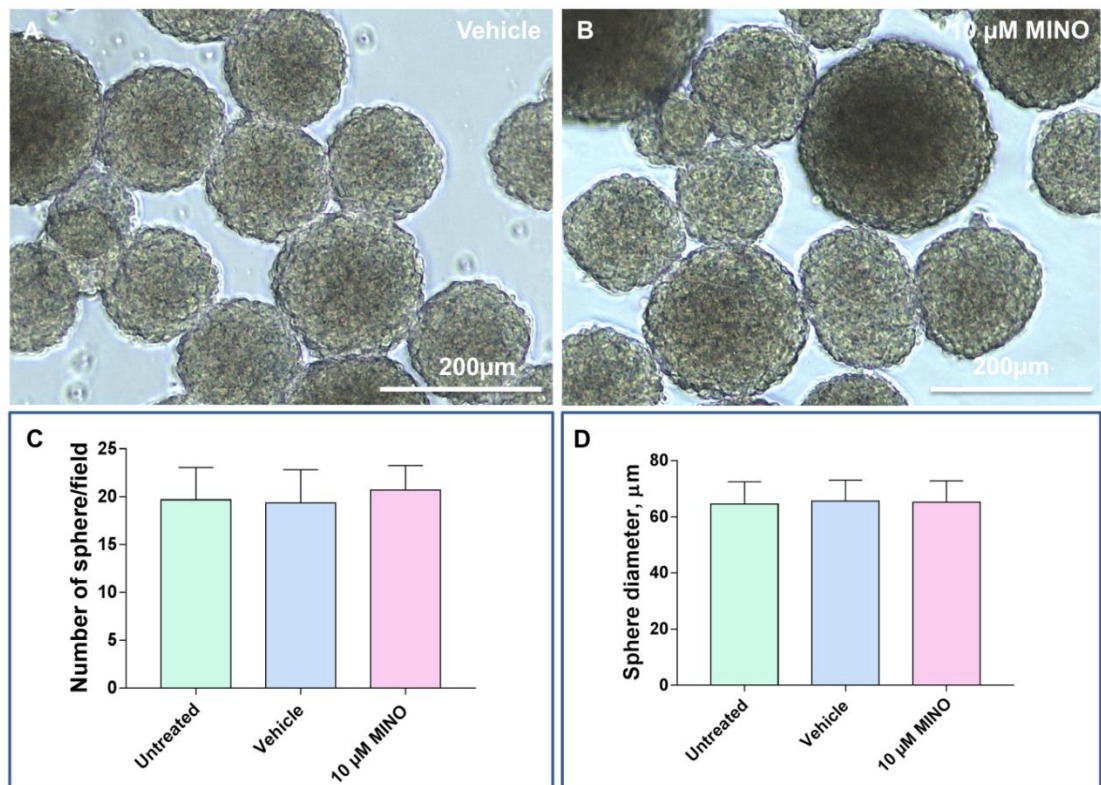


Figure 6.2 Effect of MINO on the number and size of neurospheres. (A) Representative phase-contrast micrograph of neurospheres in vehicle control. (B) Treated neurospheres with MINO. (C) Bar chart representing the average sphere number per microscopic field and (D) bar chart showing the sphere size. No significance; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Number and size of spheres were carried out in three biological replicates, with two technical replicates in each experiment, (48 h MINO; 10 μM).

6.3.1.3 Effect of MINO on the number and proportion of NSCs

Immunostaining for nestin and SOX-2 revealed that 10 μ M MINO had no significant effects on the total number of NSCs (Figure 6.3 and 6.4). To assess neurospheres purity, the proportion of SOX-2 positive cells were analysed and the immunostaining for this marker revealed that the neurospheres displayed high purity populations of NSCs across all conditions (Figure 6.4). Importantly, cells after MINO treatment also displayed normal patterns of NSCs marker staining with regular rounded nuclei as judged by DAPI staining, indicating 'stemness' was not changed following MINO treatment (Figure 6.4).

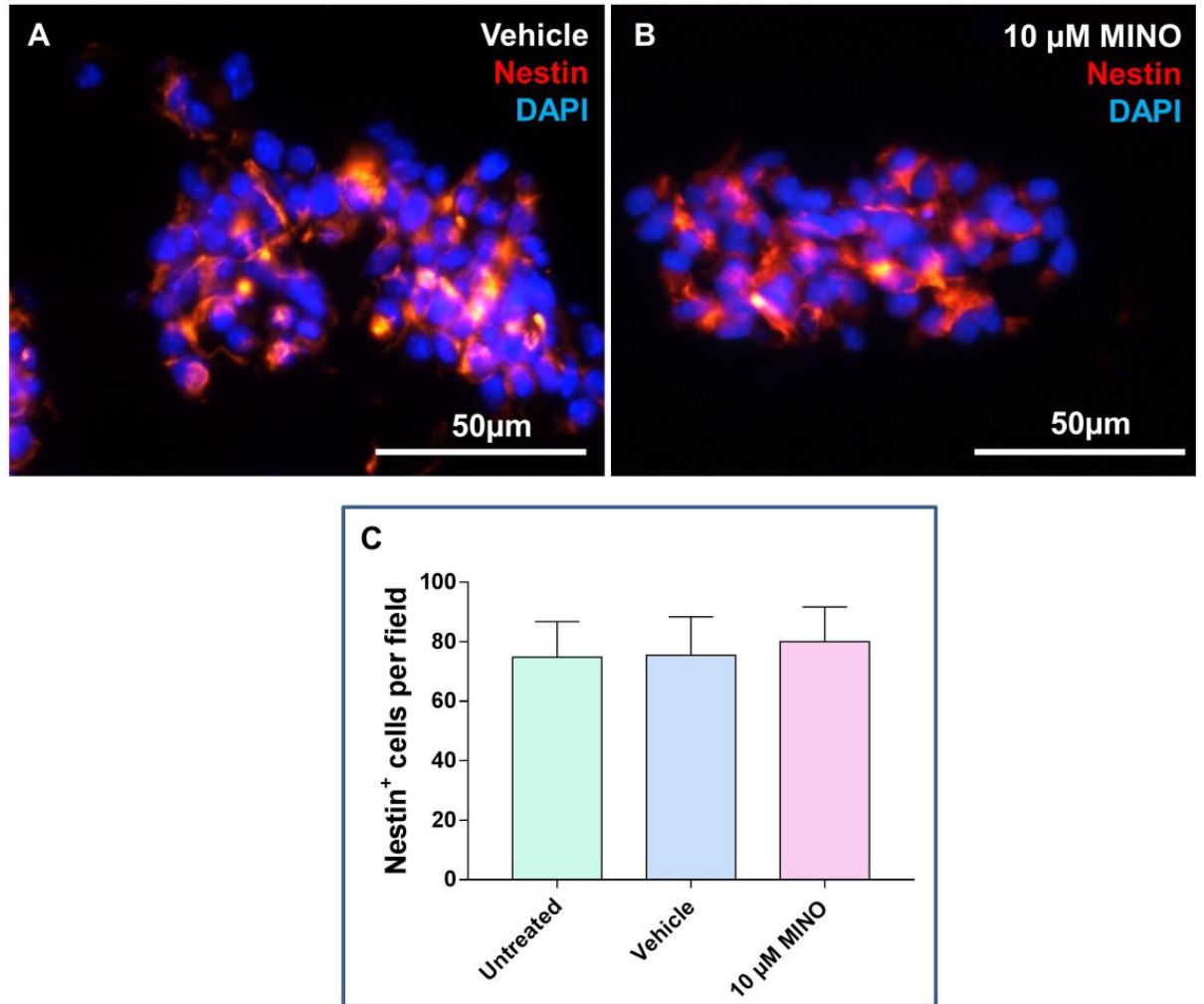


Figure 6.3 Effect of MINO on the number of NSCs using nestin marker. (A) Fluorescence micrograph displaying nestin positive NSCs neurosphere in vehicle control, (B) treated neurospheres with 10 μ M MINO. (C) Bar chart showing the total number of NSCs per field across all conditions. No significance; one-way ANOVA, Bonferroni's post-test with error bars indicating SEM. Cell counting was carried out in four biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition, (48 h MINO; 10 μ M).

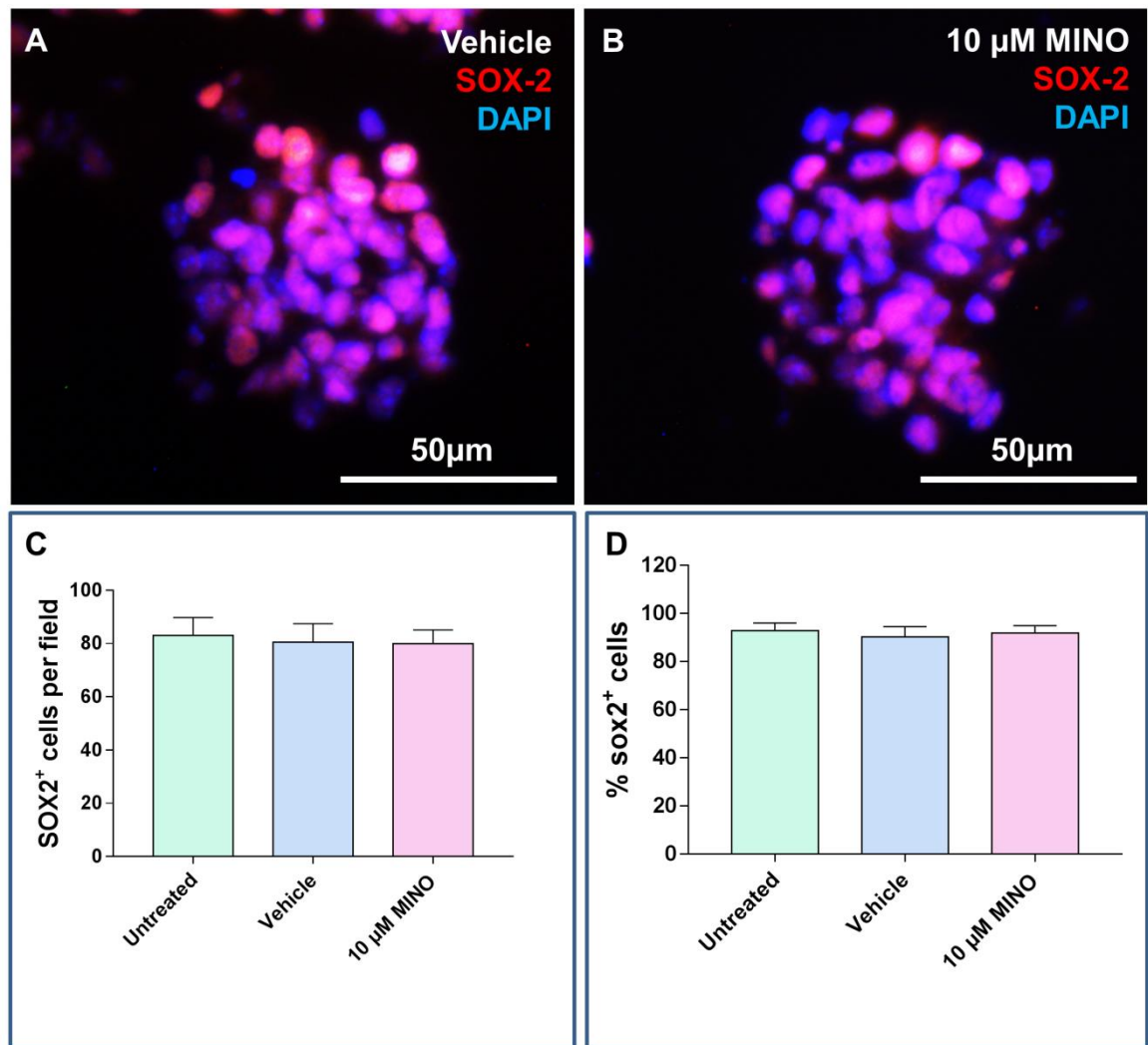


Figure 6.4 Effect of MINO on the number and proportion of NSCs using SOX-2 marker. (A) Fluorescence micrograph displaying SOX-2 positive NSCs neurosphere in vehicle control, (B) treated neurospheres with 10 μM MINO. (C) Bar chart showing the total number of NSCs per field across all conditions. (D) Bar chart showing quantification of the proportions of cells positive for NSCs marker SOX-2 following MINO treatment across all conditions. No significant differences; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in four biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition, (48 h MINO; 10 μM).

6.3.1.4 Effect of MINO on the proliferation and cell cycle of NSCs

In order to study the effects of MINO on NSCs in more details, EdU proliferation assay was used to assess the proliferation capacity of NSCs in neurosphere culture following MINO treatment. The results showed that 10 μ M of MINO had no effect on NSCs proliferation. The percentage of EdU⁺ cells showed there was no significant differences between MINO-treated and untreated cells (Figure 6.5). Additionally, cell cycle analysis was conducted to assess the effects of MINO on the proportions of cells in each individual phase of the cell cycle. The results showed that 10 μ M of MINO had no effect on the proliferation and cell cycle phases of NSCs (Figure 6.6).

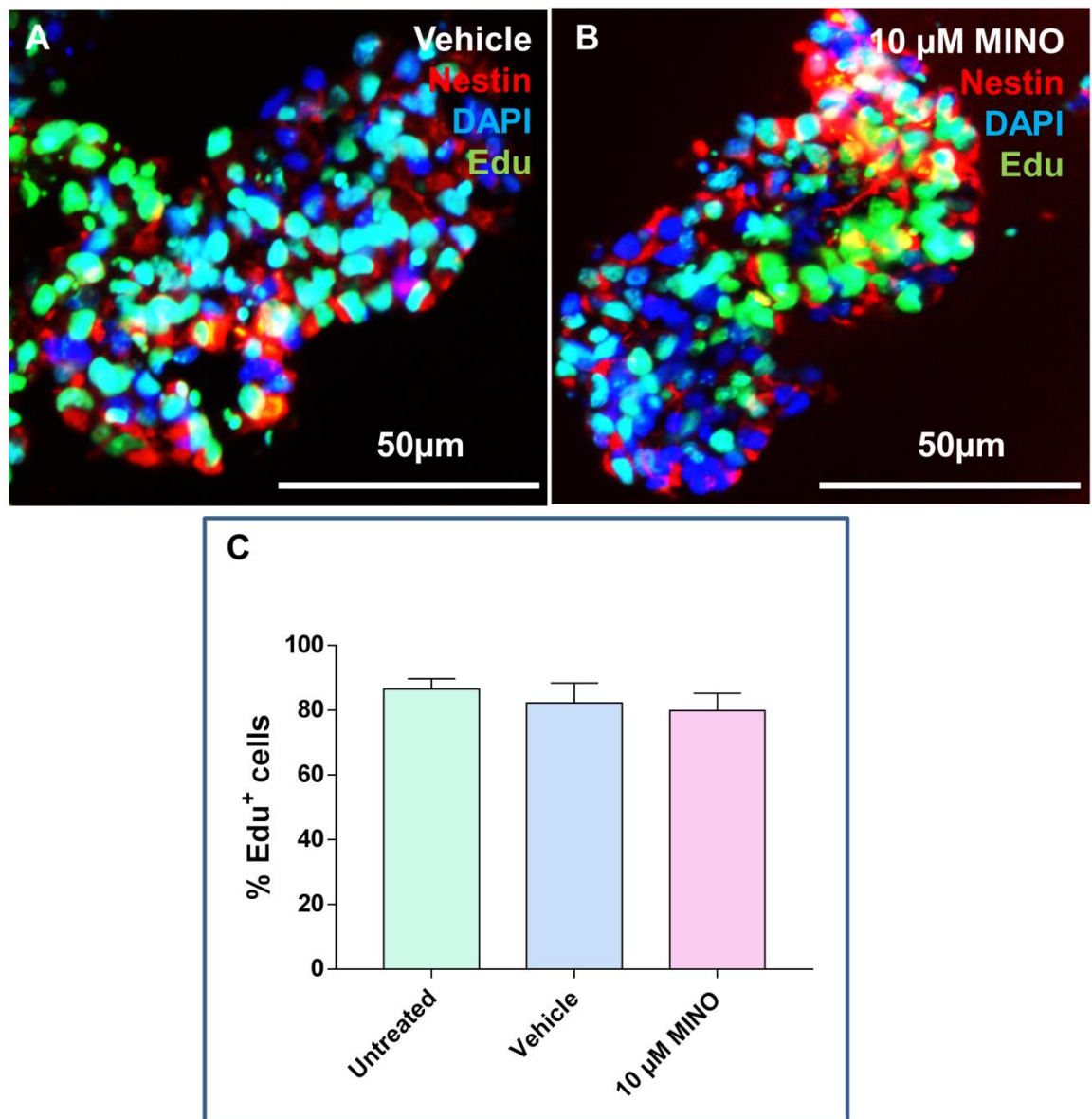


Figure 6.5 Effect of MINO on the proliferation of NSCs. (A) Triple merged fluorescence images depicting Edu positive NSCs in vehicle control and (B) 10 μM MINO treated NSCs, (C) Bar chart showing proportions of Edu incorporating cells across treatment conditions. No significant differences; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Proliferation assay was carried out in three biological replicates, with two technical replicates in each experiment, two cryostat sections were used for each condition, (48 h MINO; 10 μM).

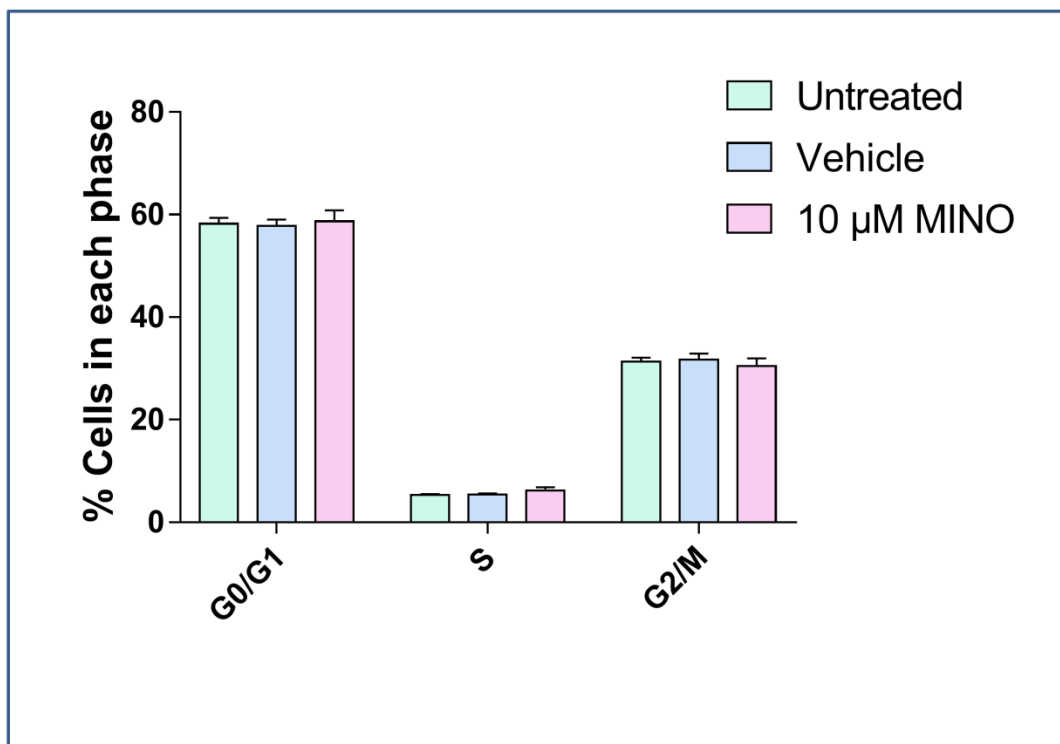


Figure 6.6 Effect of MINO on the cell cycle of NSCs. Bar chart representing the percentage of the cells in the G0/G1, S and G2/M phase of the cell cycle following MINO treatment. No significant differences; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cell counting was carried out in three biological replicates, with three technical replicates in each experiment, (48 h MINO; 10 μM).

6.3.1.5 Effects of MINO on the viability, apoptosis and pyknosis of NSCs

Live/dead staining was used to assess NSCs viability at 48 h following MINO treatment. The findings showed that NSCs from neurosphere cultures displayed high viability post-MINO treatment (Figure 6.7). The percentage of apoptotic cells of NSCs were also assessed using muse Annexin V and Dead cell kit. No significant differences were observed in the apoptosis of cells post MINO-treatment across all conditions (Figure 6.8A). Further, the incidence of pyknosis after MINO treatment was determined by assessing the percentage of pyknotic nuclei, as judged by DAPI-staining (shrunken, fragmenting nuclei were classed as pyknotic). The results showed that the number of pyknotic nuclei was low (<2%) across all conditions, indicating no significant differences between MINO treated and untreated cells (Figure 6.8B). These results suggest that MINO had no effects on the viability and apoptosis of NSCs in neurosphere cultures.

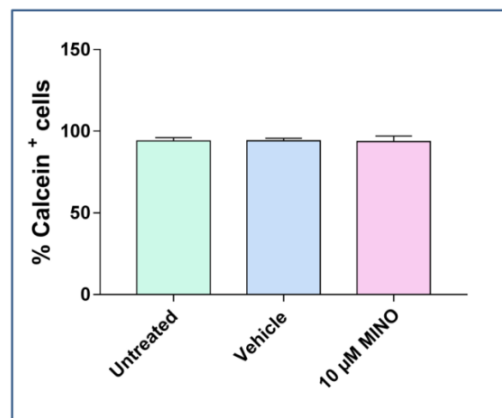


Figure 6.7 Effect of MINO on the viability of NSCs. Bar chart showing the proportion of viable cells. No significance; one-way ANOVA, Bonferroni's post-test; with error bars representing SEM. Viability assay was carried out in three biological replicates, with two technical replicates in each experiment, (48 h MINO; 10 µM).

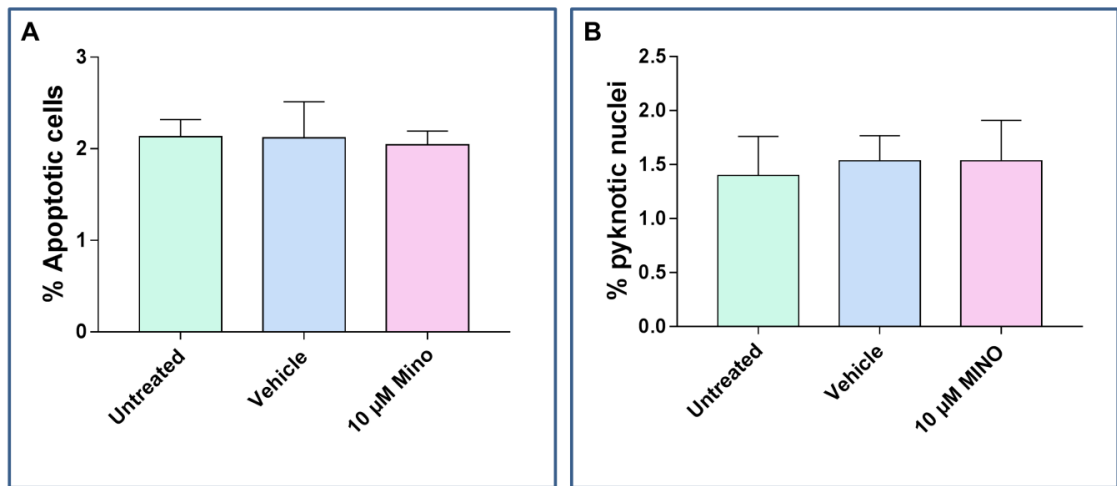


Figure 6.8 Effect of MINO on the apoptosis and pyknosis of NSCs. (A) Bar chart displaying the percentage of apoptotic cells in neurosphere cultures. (B) Bar charts showing the percentage of pyknotic nuclei for all conditions. No significant differences were noted between all conditions; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Apoptosis assay was carried out in three biological replicates, with three technical replicates in each experiment. Pyknotic nuclei were carried out in three biological replicates, with two technical replicates in each experiment, (48 h MINO; 10 µM).

6.3.1.6 Effect of MINO treatment on the differentiation of NSCs

In order to test the effects of MINO on the differentiation profile of NSCs, the neurospheres were dissociated at 48 h of MINO treatment and resuspended in differentiation medium and treated with MINO for further 7 days. Astrocytes, neurons and oligodendrocytes were all produced from MINO-treated NSCs culture in similar numbers and proportions across all conditions. Further, all cell types showed normal morphologies across all conditions following MINO treatment (Figure 6.9).

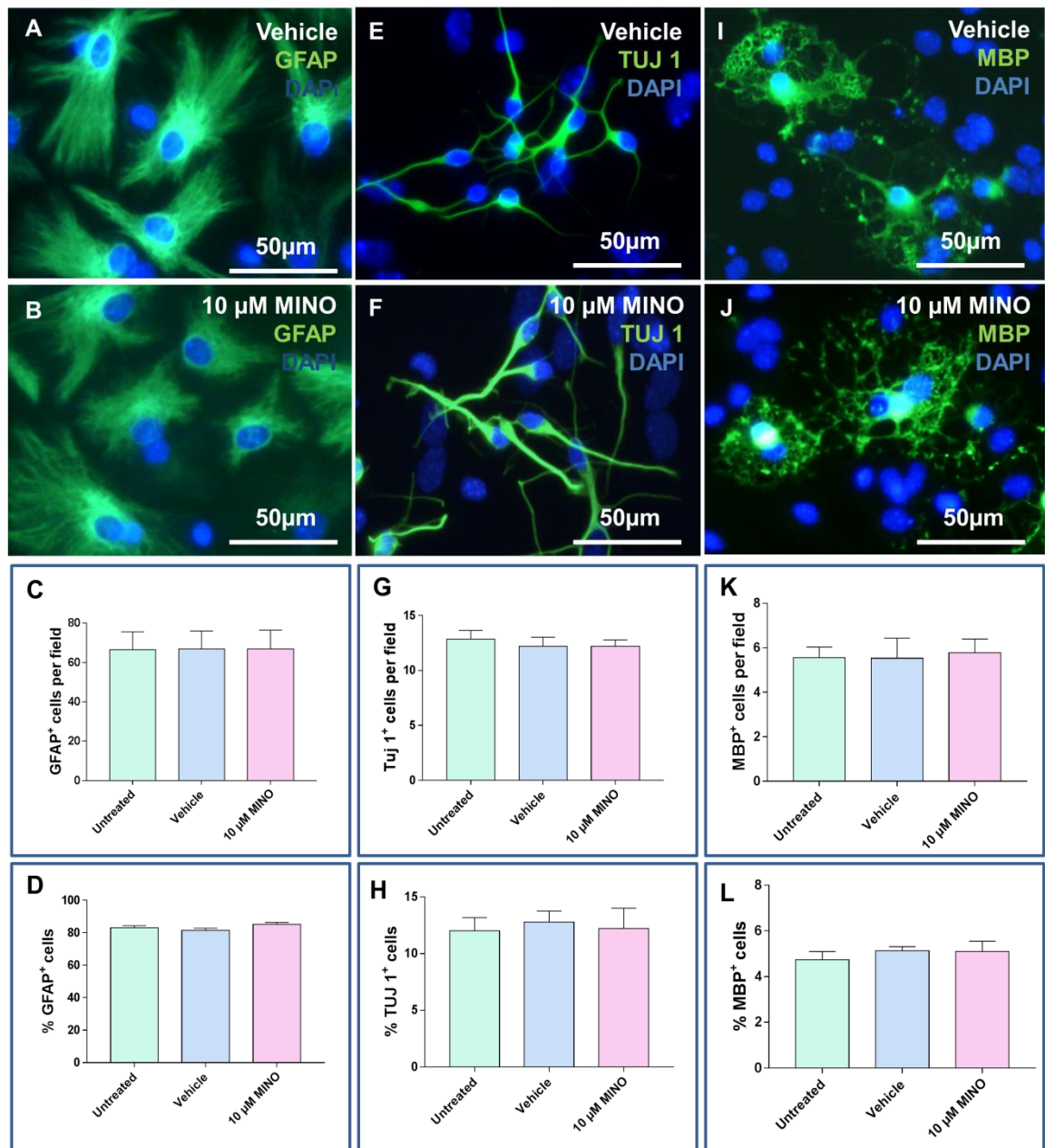


Figure 6.9 Effects of MINO treatment on the differentiation of NSCs. (A) Representative fluorescence micrographs depicting astrocytes generated from vehicle control and (B) 10 μ M MINO treated differentiated NSCs. (C and D) Bar charts displaying the total number and the proportion of GFAP⁺ cells per field, respectively. (E) Fluorescence micrographs of neurons derived from vehicle control and (F) 10 μ M MINO treated differentiated NSCs. (G and H) Bar charts quantifying the number and the proportion of TUJ 1⁺ neurons per field across

treatment conditions, respectively. (I) Fluorescence micrographs of oligodendrocytes generated from vehicle control and (J) 10 μ M MINO treated differentiated NSCs. (K and L) Bar charts showing the number and the proportion of MBP⁺ oligodendrocytes per field across treatment conditions, respectively. No significance; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. Cells counting were carried out in three biological replicates, with two technical replicates in each experiment, (9 days MINO; 10 μ M).

6.3.2 NSCs transplantation and MINO as a combinatorial therapy in longitudinal organotypic spinal cord model

Following examination of the direct effects of MINO on NSCs in neurosphere cultures, longitudinal organotypic spinal cord slice model was used to assess the neuroprotective effects of the combination treatment of MINO and NSCs post-slice lesioning. A series of neuropathological assessments were conducted to evaluate the features of SCI pathology in lesioned slices. At 1 day post-lesioning, the slices were randomly divided into four groups: group **SCI** (control), group **SCI + MINO** (in which 10 μ M of MINO was added with culture medium into spinal cord slices at 2 DIV), group **SCI + NSCs** (in which NSCs were transplanted in the sites of injury of the slices at 2 DIV) and group **SCI + NSCs + MINO** (in which NSCs were transplanted and 10 μ M of MINO was added with culture medium into spinal cord slices at 2 DIV) (Figure 6.10).

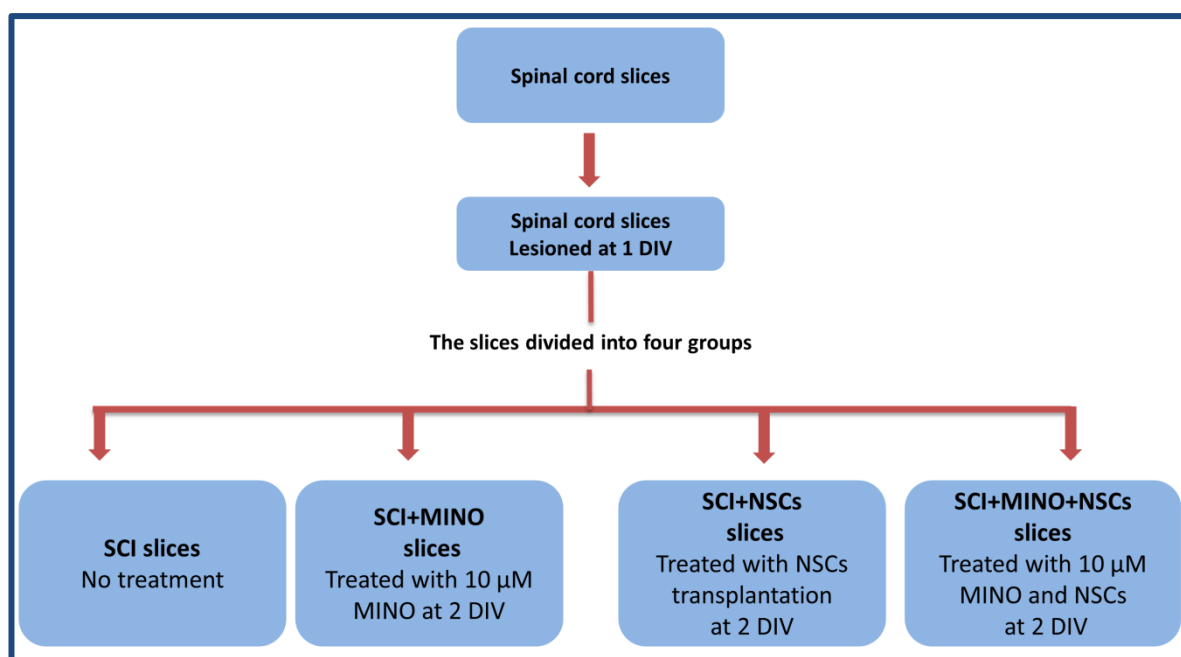


Figure 6.10 schematic showing the experimental design of MINO treatment and NSCs transplanted in organotypic spinal cord slice culture.

6.3.2.1 Slice viability before and after lesion induction

In order to assess the viability of slices, live/dead staining protocol was used to assess the slice viability before and after lesioning. The fluorescence microscopy provided high viability of intact slices. As expected, dead cells generated by the slicing procedure were accumulated around the edges of the slices. The results showed that the viability of the slices before lesion was $96.8 \pm 1.4\%$ (Figure 6.11A, B and C). Also, the viability of lesioned slices was investigated at 8 days post lesioning. The fluorescence microscopy showed some dead cells within the lesion with high viability in the main body of the slice (Figure 6.11D). The results showed the high viability of lesioned slices which found to be approximately $93.3 \pm 0.4\%$. These findings demonstrated that the procedures used to induce lesioning did not affect slices viability.

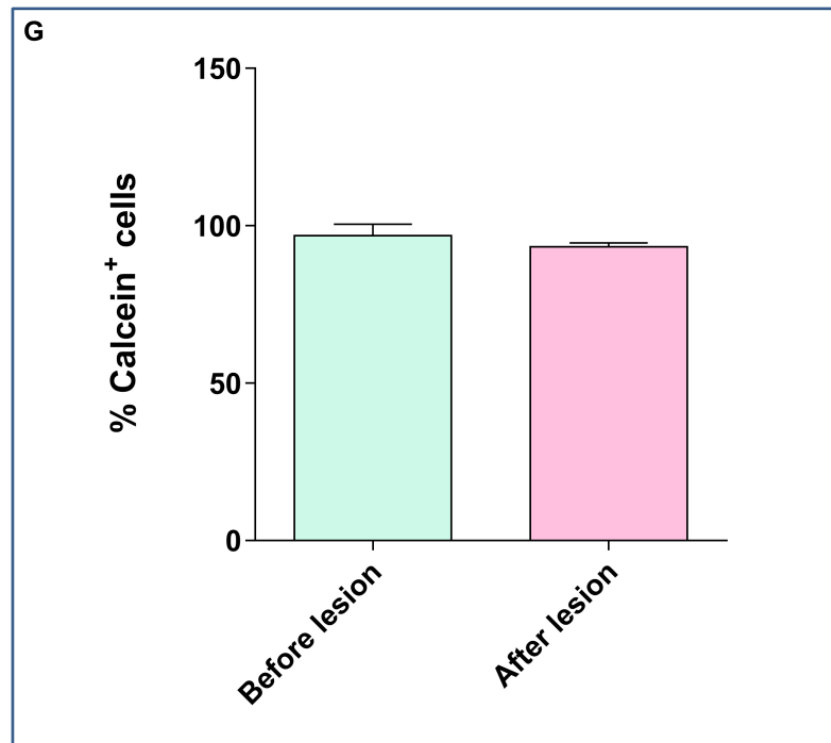
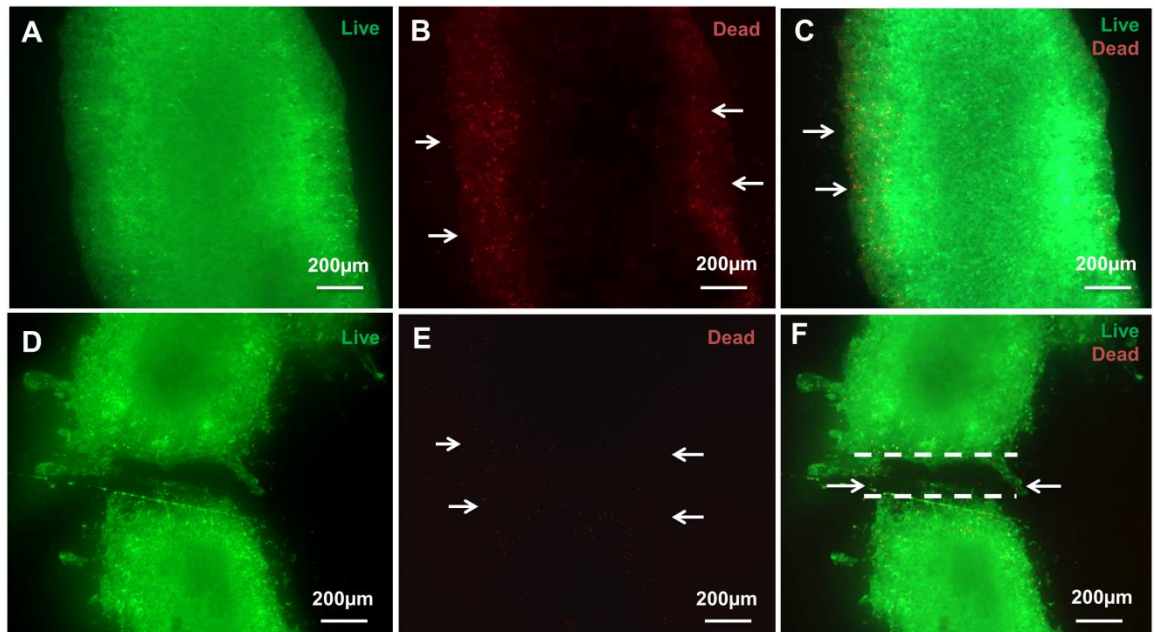


Figure 6.11 Slice viability before and after lesion induction. (A) Representative fluorescence micrographs of a slice stained at 1 DIV with calcein for live cells. (B) The same field as (A) stained with ethidium homodimer-1 for dead cells. (C) Corresponding merged micrograph of (A) and (B). Dead cells were found around the slice edges (white arrows), live cells were found in the main body of the slice.

(D) Fluorescence micrographs of a lesioned slice stained at 8 days after lesioning with calcein for live cells. (E) The same field as (D) stained with ethidium homodimer-1 for dead cells. (F) Corresponding merged micrograph of (D) and (E). Few dead cells interspersed around the site of injury (white arrows), with high viability in the main body of the slice (white broken lines demarcate lesion margins). (G) Bar chart showing the proportion of viable cells. (P5; unpaired t-test no significance, with error bars representing SEM). Viability assay was carried out in five biological replicates, with three technical replicates in each experiment.

6.3.2.2 Labelling the transplanted NSCs

6.3.2.2a DAPI labelling

NSCs were labelled with DAPI to distinguish transplanted cells from host cells. At 1 day after injury, NSCs labelled with DAPI were transplanted into lesion sites of organotypic spinal cord slices. Phase and fluorescent images show dissociated single NSCs displaying DAPI label at the same day of transplantation (Figure 6.12A). At 1 week after transplantation, DAPI staining had leaked out from the transplanted NSCs. As mentioned previously in Chapter 2, DAPI is a fluorescent dye that binds to DNA and stains the nucleus. Therefore, this observation suggested that the nuclear staining was lost during cell division (Figure 6.12B).

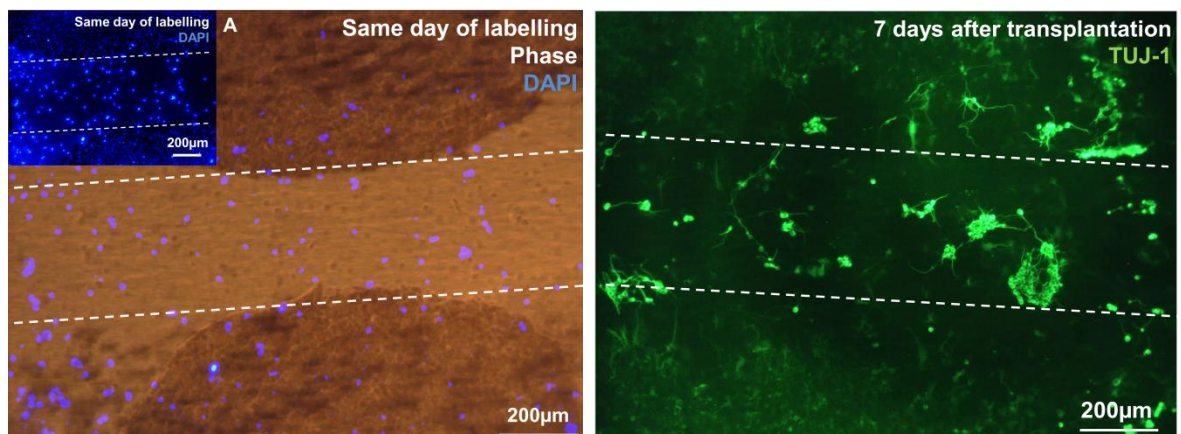


Figure 6.12 DAPI labelling of NSCs transplantation in organotypic spinal cord slices. (A) Phase contrast micrograph depicting DAPI staining of transplanted NSCs in lesioned slice at the same day of labelling. (B) Fluorescent micrograph showing TUJ-1⁺ neurons in the lesion sites (DAPI staining had leaked out from cells) in lesioned slice differentiated into neurons post 7 days after transplantation.

6.3.2.2b DiD labelling

DiD was used as an alternative dye to DAPI staining to label NSCs, as mentioned in detailed in Chapter 2 **section 2.18.2.2**. NSCs were labelled with DiD in monolayer culture before transplanting into spinal cord slices in order to assess the ability of DiD to label NSCs. Phase and fluorescent images showed that DiD labelled NSCs were attached to the surface of coated coverslip and showed normal cell morphology at the same day of labelling, suggesting the ability of DiD to label NSCs in monolayer culture (Figure 6.13A). At 4 days post labelling, the dye had leaked out from the cells (Figure 6.13B). Then single NSCs were labelled with DiD and then transplanted into the site of injury of organotypic slices. Dynamic time lapse microscopy was used to trace the transplanted NSCs in the lesioned slices. Dynamic time-lapse images showed DiD labelled cells in the lesion site of the organotypic SCI model at same day and four days following transplantation (Figure 6.13C and D). The observations from these images confirmed that cells have been delivered into the lesion.

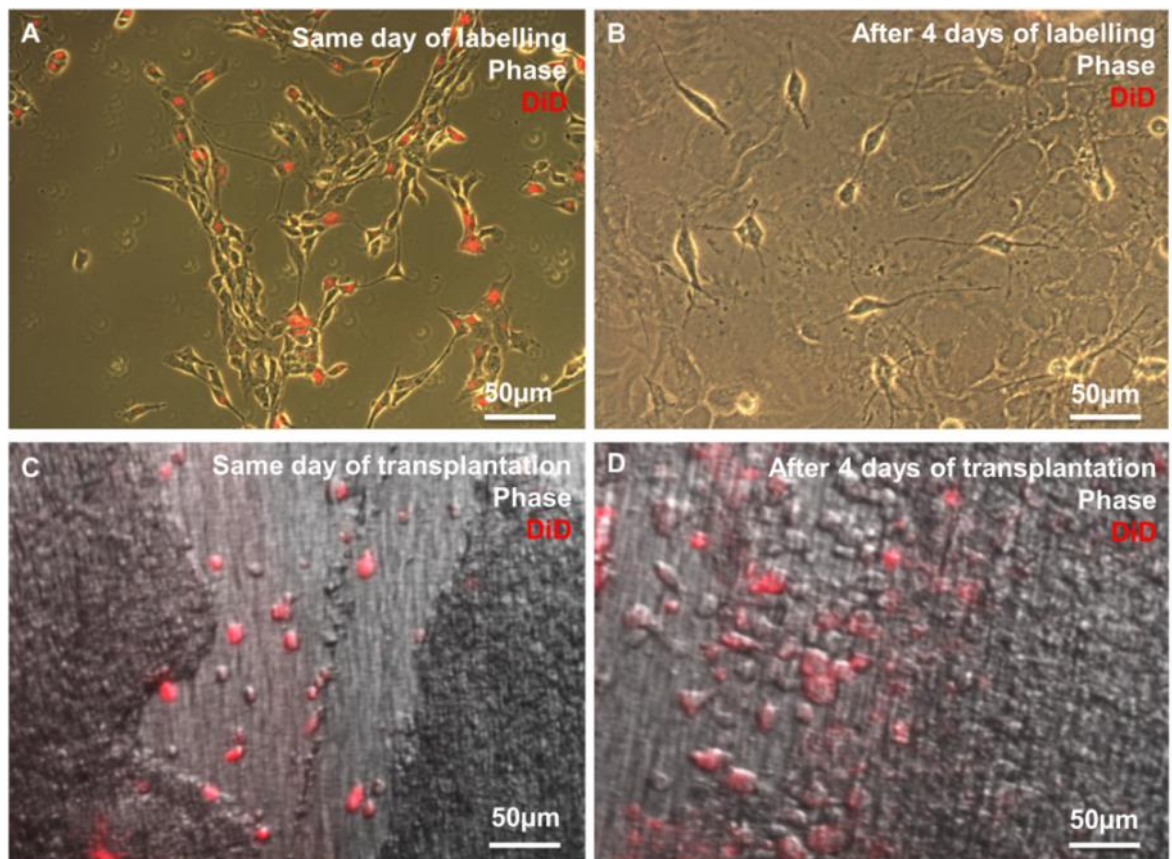


Figure 6.13 DiD labelling of NSCs transplantation in monolayer and organotypic spinal cord slices. (A) Phase contrast micrograph depicting DiD staining of NSCs cultured in monolayer at the same day of labelling. (B) Phase contrast micrograph depicting DiD staining of NSCs cultured in monolayer following four days of labelling. (C) Still image taken from dynamic time-lapse microscopy representing NSCs labelled with DiD at same day of transplantation in organotypic spinal cord slice culture. (D) Still image taken from dynamic time-lapse microscopy representing NSCs labelled with DiD following four days of transplantation in organotypic spinal cord slice culture.

6.3.2.3 The effect of the combinatorial therapy on astrogliosis at lesion margins

Immunostaining of GFAP positive cells was performed to investigate whether the combination therapy had any effects on astrogliosis. GFAP staining was observed clearly throughout slices and the fluorescent micrograph showed the intense reactive of GFAP⁺ cells at lesion margins (hallmark features of the glial scar) (Figure 6.14A-D). Mean fluorescence intensity profiles were used to quantify GFAP expression at the first 100 μ m adjacent to lesion margins across all treatment conditions. The results showed an evident reduction in astrogliosis in (SCI + MINO) and (SCI + MINO + NSCs) groups compared to the (SCI) and (SCI + NSCs) groups, (Figure 6.14E). These findings suggest the beneficial effects of MINO in astrogliosis reduction compared to transplant NSCs.

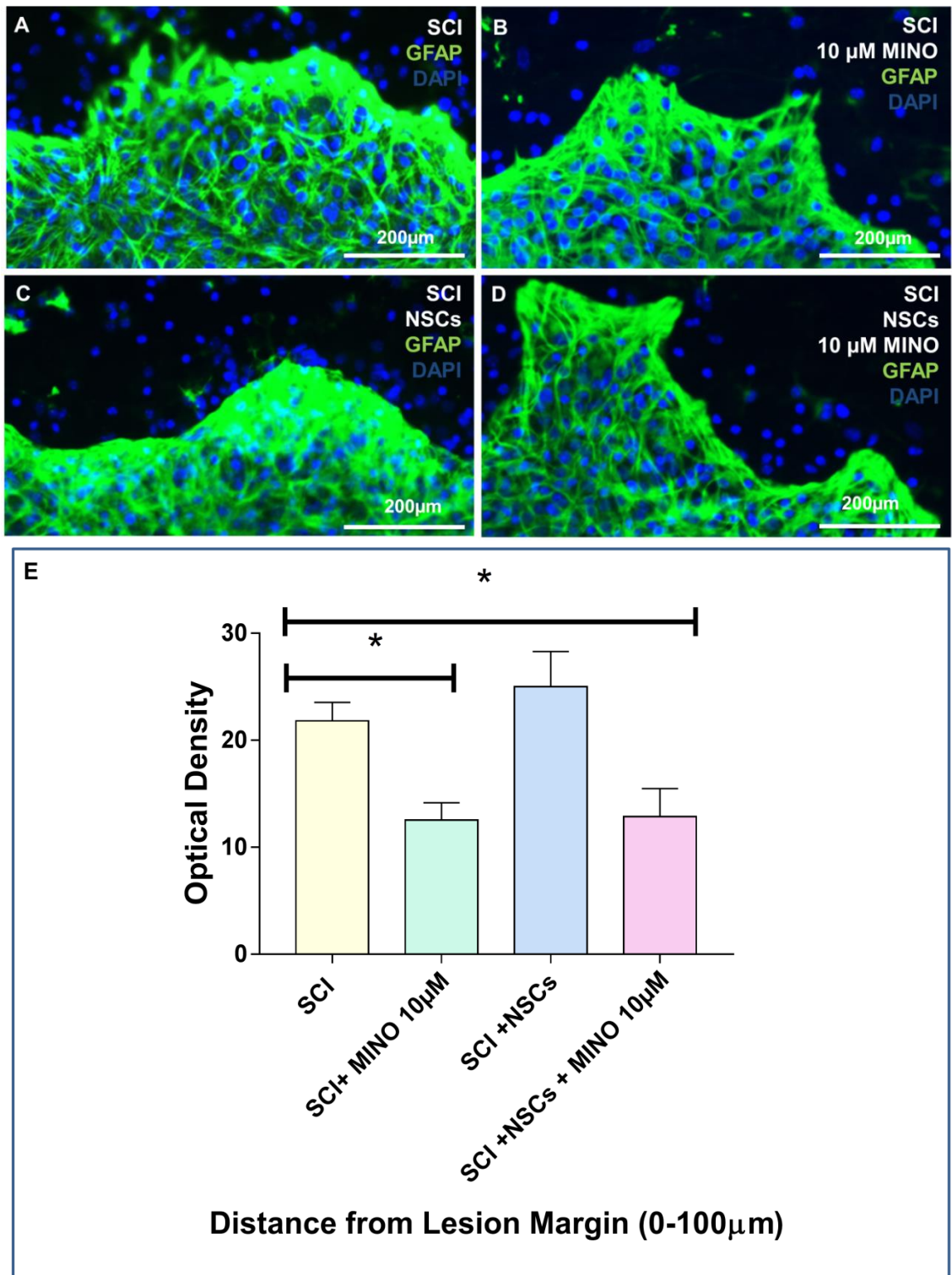


Figure 6.14 Astrogliosis assessment in lesioned slices. (A) Representative fluorescence micrograph showing intensely reactive astrocytes and increased GFAP expression at the lesion margin in untreated group. (B) Representative fluorescence micrograph showing GFAP expression at the lesion margin following

*MINO treatment. (C) Fluorescence micrograph displaying intensively reactive of GFAP positive cells at the lesion margin following NSCs transplantation. (D) Fluorescence micrograph displaying GFAP expression at the lesion margin following NSCs transplantation and MINO treatment. (E) Bar graph showing a significant reduction in the average optical densities for GFAP⁺ slices in (SCI + MINO) and (SCI + MINO + NSCs) groups compared to the (SCI) and (SCI + NSCs) groups. (P5; stained 7 days post treatment); * $p < 0.05$; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. The experiment was carried out in five biological replicates, with three technical replicates in each experiment.*

6.3.2.4 The effect of the combinatorial therapy on microglial infiltration into lesion sites

Immunostaining of lectin positive cells was performed to assess the effects of combinatorial therapy on microglial activation and infiltration within the site of injury. Microglia displayed rounded activated morphologies within the lesion site. The quantification from immunocytochemistry showed a significant reduction in the number of microglia within the lesion site in (SCI + MINO) and (SCI + MINO + NSCs) groups compared to the (SCI) and (SCI + NSCs) groups (Figure 6.15). These results suggest the beneficial effects of MINO compared to NSCs.

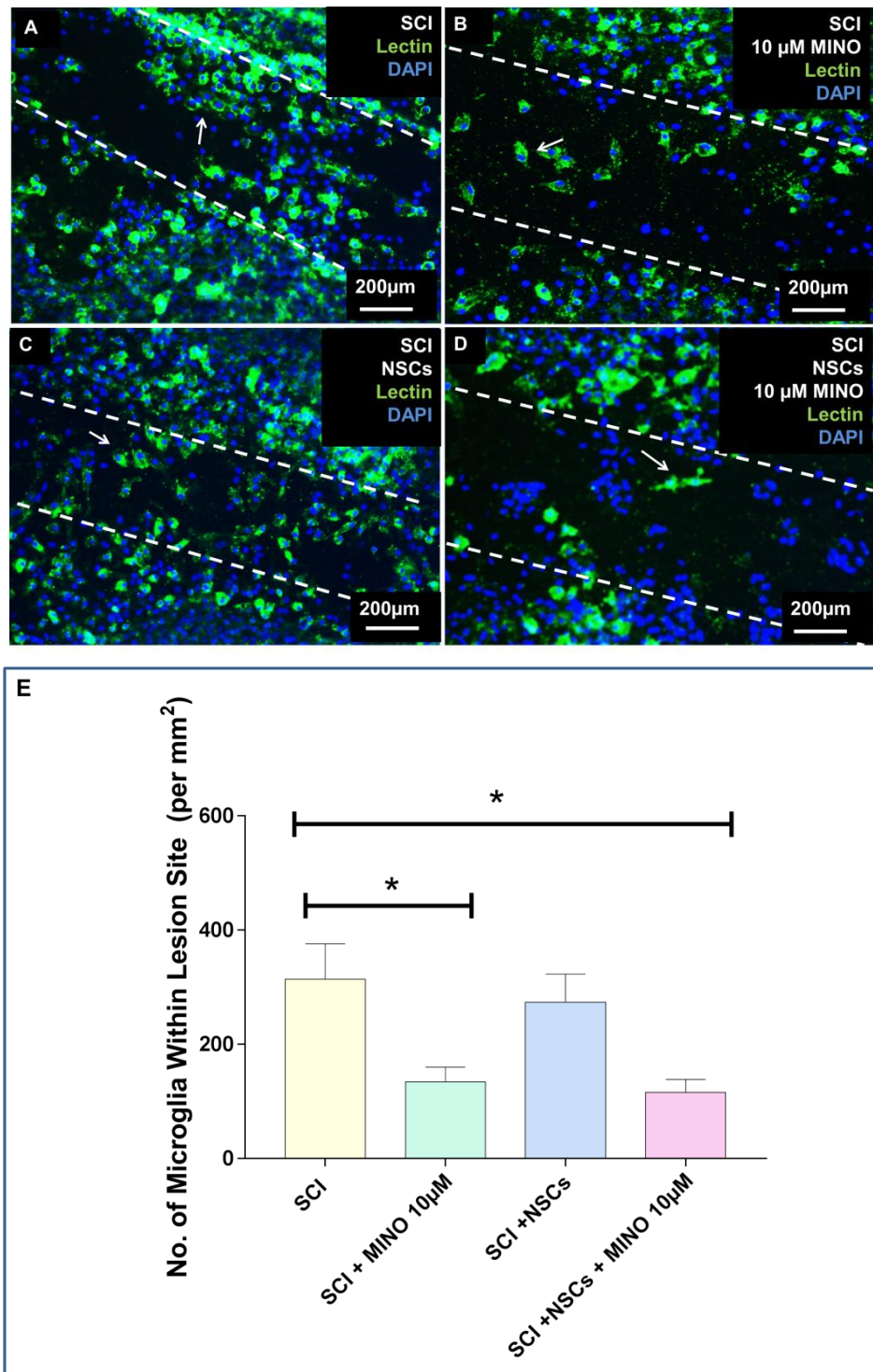


Figure 6.15 The effect of the combinatorial therapy on infiltration of microglia into lesion sites. (A) Representative fluorescence micrograph of a lesion margin 8 days post-lesioning shows the infiltration of lectin⁺ microglia into the lesion site in untreated slices (SCI), (white dotted line: lesion margin). (B) Representative fluorescence micrograph showing lectin⁺ microglia in the lesion margin following

*MINO treatment (SCI+MINO). (C) Fluorescence micrograph showing lectin⁺ microglia in the lesion margin post NSCs transplantation (SCI+NSCs). (D) Fluorescence micrograph displaying lectin⁺ microglia in the lesion margin following MINO treatment and NSCs transplantation (SCI+MINO+NSCs). (E) Bar graph quantifying numbers of lectin⁺ microglia in lesion sites across all treatment conditions, (P5; stained 7 days post treatment); * $p < 0.05$; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. The experiment was carried out in five biological replicates, with three technical replicates in each experiment.*

6.3.2.5 The effect of the combinatorial therapy on the nerve fibre outgrowth from lesion margins

After 9 days of culture, nerve fibre outgrowth of TUJ-1⁺ in untreated slices was relatively limited from both margins of the lesion suggesting limited regeneration. By contrast, the combined treatment with transplanted NSCs and MINO, slices showed slight but significant increase of the process of TUJ-1 positive cells compared with (SCI), (SCI + MINO) and (SCI + NSCs) groups. These data indicate that the transplanted NSCs, in the presence of MINO made a significant improvement in neuronal outgrowth in the organotypic SCI model (Figure 6.16A-D), as confirmed by quantification of the outgrowth of nerve fibres (Figure 6.16E).

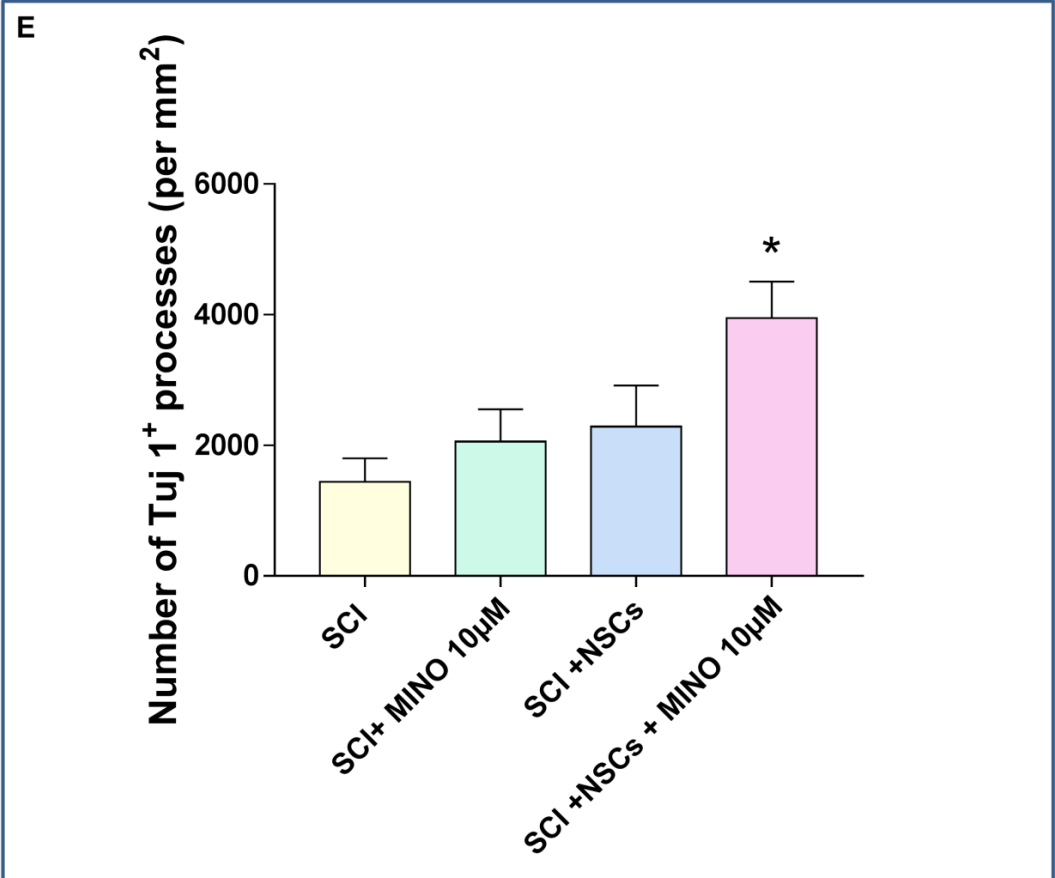
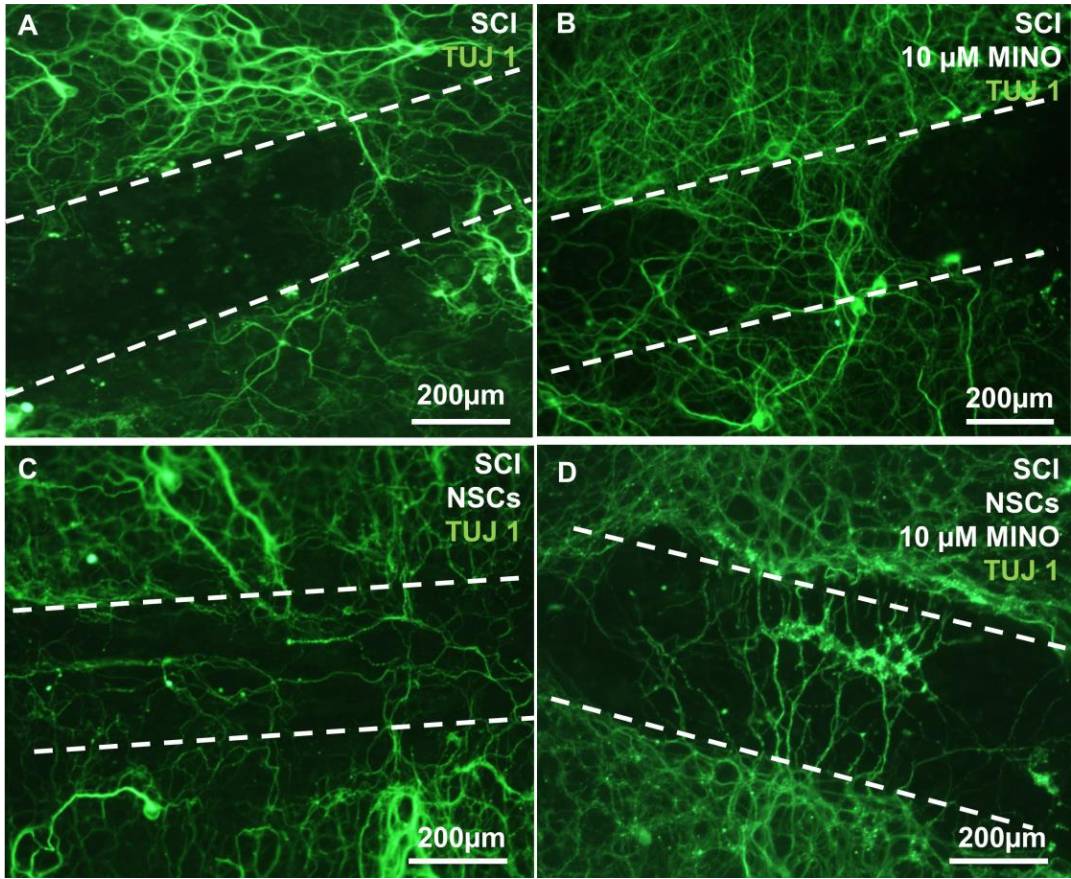


Figure 6.16 The effect of the combinatorial therapy outgrowth of nerve fibres from lesion margins. (A) Representative fluorescence micrograph of TUJ-1⁺ nerve fibres in lesions in untreated slices (SCI), (white dotted line: lesion margin). (B) Representative fluorescence micrograph of limited outgrowth of TUJ-1⁺ nerve fibres in lesions following MINO treatment (SCI+MINO). (C) Representative fluorescence micrograph of TUJ-1⁺ nerve fibres in lesions following NSCs transplantation (SCI+NSCs). (D) Representative fluorescence micrograph of outgrowths of TUJ-1⁺ nerve fibres in lesions following MINO treatment and NSC transplantation (SCI+MINO+NSCs). (E) Bar chart quantifying TUJ-1⁺ nerve fibre outgrowth density across all treatment conditions, (P5; stained 7 days post treatment); * $p < 0.05$; one-way ANOVA, Bonferroni's post-test with error bars representing SEM. The experiment was carried out in five biological replicates, with three technical replicates in each experiment.

6.4 Discussion

Here, the utility of a SCI mouse model was investigated to study the effects of a combinatorial therapy consisting of NSCs transplantation and MINO in neurological repair.

In the first part of this chapter, the direct effects of MINO on NSCs in neurosphere culture were studied to assess whether MINO has any adverse effect on the fate of NSCs. The results showed that 10 μ M MINO does not affect NSCs neurosphere cultures proliferation, survival, apoptosis and differentiation. Of particular interest were the results from the second part- the combinatorial therapy (NSCs and MINO) with testing in organotypic spinal cord mouse model.

MINO, the anti-inflammatory drug, was used as a combinatorial therapy with NSCs in organotypic spinal cord mouse model. Much criticism has been generated towards the use of CS, a widely used treatment available to patients suffering from an ASCI (Bracken, 2012; Hall and Springer, 2004; Wells et al., 2003). The results from this current study (Chapter 3 and 4) showed the detrimental effects of CS including MPRED on NSCs and these findings are in agreement with many previous studies. Interestingly, the clinical trials from the National Acute Spinal Cord Injury Study II and III failed to demonstrate the beneficial effects of MPRED treatment (Hurlbert, 2000). The harmful effects of MPRED, particularly after high doses, have prompted the search for more efficacious neuroprotective agents. Importantly, several human trials have shown the clinical potential of MINO in treatment of neurodegenerative disorders such as Huntington's and Parkinson's disease following transplantation of foetal neurons (Blum et al., 2004). It was observed that MINO is more effective in improving functional outcomes following

SCI when compared with MPRED (Festoff et al., 2006; Wells et al., 2003). MINO has the ability to reduce lesion size, cell death, and alter cytokine expression following SCI (Lee et al., 2003; Wells et al., 2003).

MINO is an antibiotic drug that has been widely used in various models of neurodegenerative diseases, because it has neuroprotective, anti-inflammatory and anti-apoptotic effects (Pinkernelle et al., 2013). Most importantly, MINO was shown to have the ability to enhance the survival of grafted cells. In 2006, Karimi-Abdolrezaee and colleagues demonstrated the ability of transplanted adult brain-derived NSCs to differentiate into either OPCs or mature oligodendrocytes and repair myelin in the presence of growth factors, MINO and cyclosporine A (Karimi-Abdolrezaee et al., 2006). In 2012, Sakata and colleagues found that the transplantation of the MINO-preconditioned NSCs markedly increased the number of survived cells and improved neuroprotection in ischemic stroke in rats, compared with non-preconditioned NSCs (Sakata et al., 2012). *In vitro* and *in vivo* experiments conducted by Rueger and colleagues, have shown that MINO significantly increased the number of transplanted NSCs in a rat stroke model (Rueger et al., 2012).

Transplantation of NSCs holds promising therapeutic strategy for the treatment of SCI, therefore NSCs were used as a combined treatment with MINO to improve regeneration outcome following SCI. Cells labelling can be used to distinguish transplanted cells from host cells, monitor distribution and migration of target cells following transplantation, and understand their biologic mechanisms and the therapeutic effect (Kang et al., 2014). However, the labelling of these cells is one of the major problems faced in clinical applications. In this study, DAPI and DiD dyes were used to label NSCs *in vitro*. The results from labelling methods found

that both methods were of limited use for these experiments given their leakage out from the cells within a short time period. Numerous techniques have been used to label and track transplanted stem cells *in vivo* and *in vitro*. In general, there are two types of methods used to label transplanted cells: **directly or indirectly**. Several agents such as fluorescent probes, super-paramagnetic iron oxide, and radio- tracers, have been used for direct labeling and tracking method (Kang et al., 2014). For example, membrane dyes such as DiD are incorporated into cell membrane lipids through hydrophobic interaction. DNA binding dyes such as DAPI and Hoechst stains, are used for live cell labeling because they are membrane permeable. However, these methods have limitations as a result of the phagocytosis of dead cells by host macrophages. Therefore, it is not clear whether the imaging signal comes from targeted cells or from macrophages. In order to overcome the limitations of direct labeling, indirect labeling method have been used such as genetic modifications using nonviral and viral systems (Leiker et al., 2008). However, genetic modifications have several limitations such as difficulties in controlling gene expression and the possibility of tumor formation (Kang et al., 2014; Leiker et al., 2008).

The presented data here revealed that 10 μ M MINO reduces astrogliosis, a functional barrier to nerve fibre regrowth, in the spinal cord slices. The presence of astrocytes in the site of injury limits the spinal cord's ability to repair. Following SCI, the astrocytes become hypertrophic, proliferate, and organise into a dense astrocytic rich border at the site of lesion to form a glial scar (Fawcett and Asher, 1999; Yiu and He, 2006). This glial scar forms a barrier to axonal regeneration. Its function is to seal the lesion sites and the BBB to prevent further damage occurring around the site of the injury (Yiu and He, 2006). The high level of

chondroitin sulfate proteoglycans which are produced by reactive astrocytes and other neural cell types within the injury site, also act as inhibitors for neurite outgrowth and regenerative activity (Siebert et al., 2015). In 2012, Sakata and colleagues found that of transplanted NSCs, the majority differentiated into astrocytes in rat models of ischemic stroke at 28 days after transplantation. Further, they observed some of these cells have the ability to produce glial scar, which could delay the recovery process (Sakata et al., 2012). More specifically, astrogliosis could produce a physical barrier and block access of oligodendrocyte progenitors to demyelinated axons resulting in failure of the remyelination process (Coutts and Keirstead, 2008). Therefore, the reduction in astrogliosis may improve regeneration following SCI.

The findings also showed a reduction in activated microglia following MINO treatment. This result is in line with other previous results. For example, it was found that MINO could effectively reduce neuroinflammation including activated microglia and prevent the production of inflammatory cytokines resulting in improved neuronal survival (Lee et al., 2003; Tikka and Koistinaho, 2001).

Finally, the results show that a treatment for SCI with MINO combination with NSCs can significantly enhance nerve fibre outgrowth. This finding may be due to the inhibitory effects of MINO on astrogliosis and microglia activation post-SCI. The findings presented here are in accordance with previous *in vitro* and *in vivo* studies. For example, in 2013, Pinkernelle and colleagues conducted an *in vitro* model of organotypic spinal cord culture (prepared from neonatal rats, postnatal day 4) to investigate effects of MINO on motor neuron survival, astrogliosis and microglia. They used two different concentrations of MINO (10 and 100 μM) at two different time points (DIV 1 and DIV 4). They found that early treatment 10 μM

MINO (DIV 1) resulted in a significantly reduced number of surviving motor neurons and microglia activity whereby astroglia activity was unchanged. Whereas, MINO treatment from DIV 4 onwards did not significantly alter the number of surviving motor neurons. Earlier treatment of 100 μ M MINO significantly increased the percentage of anti-pan-NF stained area (neurofilaments) of organotypic cultures compared to control slices. Anti-pan-NF is a general neuronal marker and is commonly used to identify neurons in tissue sections and cultures. Also, they were found that a high concentration of 100 μ M reduced activated microglia and impaired the formation of astrogliosis (Pinkernelle et al., 2013). *In vivo* studies have shown that the survival rates of grafted cells were increased and the activated microglia reduced after pre-treatment of these animals with MINO (Festoff et al., 2006; Zhang et al., 2003). Further, MINO was found to enhance the survival of transplanted adult NPCs in SCI model, in the presence of growth factors and immunosuppressant cyclosporine A (Karimi-Abdolrezaee et al., 2006).

Taken together, the findings presented in this chapter demonstrate the utility of an *in vitro* SCI mouse organotypic model to investigate the efficacy of the combinatorial drug and cell therapy in neuropathology. This type of model has a number of advantages, for example, slices generated from the same animals can be subjected to different treatment conditions and different analyses. The differences between slices which are produced from the same litter or even the same animal could more confidently be supposed to be due to treatment. Also, this type of model is easier to learn and perform compared with *in vivo* models, and moreover, allows some refinement of the treatment before *in vivo* handling. Also, this model demonstrates that three cardinal pathological properties of SCI *in vivo* (astrogliosis, microglial infiltration and nerve fibres outgrowth) can be

mimicked in the lesions of organotypic spinal cord slice (Jin et al., 2012; Sofroniew, 2009; Zhou and Snider, 2006). This model showed hypertrophic and reactive astrocytes in the lesion model which represents hallmark features of the glial scar *in vivo* (Fawcett and Asher, 1999). Microglial infiltration dynamics as seen within the lesion model here are broadly comparable with their acute infiltration characteristics *in vivo* (Jin et al., 2012; Loane and Byrnes, 2010). Therefore, the acute inflammatory responses can be mimicked within the lesion sites *in vitro*. Furthermore, *in vivo* studies show that a second, more sustained wave of microglial infiltration can occur later within ca. 60 days after lesioning. It will be of interest to assess if microglial infiltration is mimicked in this model (Beck et al., 2010). Therefore, this study can provide clinically relevant insights into the role of combinatorial therapy in neuropathology and can be predicted to be of high use in pharmaceutical research.

Chapter 7

Conclusions and Future Directions

7.1 Summary of key thesis findings

The work presented in this thesis has studied the effects of the anti-inflammatory and immunosuppressive drugs on the fate of NSCs. The findings obtained in this thesis can be summarised as follows:

Chapter 3: Histological study to evaluate the effects of CS treatment on NSCs propagated in monolayer cultures

This chapter was undertaken to investigate the effects of three different clinically relevant drugs of CS at three different concentrations and different time of treatments, therefore this study allowed for a systematic comparison of CSs on the fate of NSCs in monolayer culture.

The highest concentration of CSs used (10 μ M) was found to reduce the number and proliferation of NSCs in monolayer culture. Cell cycle analysis showed that 10 μ M of CSs led to a significant increase of cells in the G0/G1 phase with a parallel decrease of cells in S and G2/M phases, compared with controls. In contrast, viability and apoptosis analyses of NSCs did not show any significant effects following CS treatment. CS treatment of differentiated cells showed that the highest concentration of CSs reduced the genesis of neurons and increased the maturation of oligodendrocytes.

Chapter 4: Histological study to evaluate the effects of CS treatment on NSCs propagated in neurosphere cultures

I have investigated the effects of three different clinically relevant CSs on the fate of the NSCs neurosphere culture focusing on parameters underpinning regeneration such as proliferation, differentiation, apoptosis, viability and cell cycle. The results from this chapter showed that the highest concentration of CSs used reduced the number and proliferation of NSCs without any effects on viability and apoptosis. The number and proportion of neurons that were generated from treated-NSCs was also reduced. In contrast, the maturation of oligodendrocyte was increased following 10 μ M of CSs. These findings suggest that the differences in the physical format of NSCs did not impact on the CS influences on these cells with similar results obtained for adherent (monolayer) and suspension (neurosphere) cultures.

Chapter 5: Investigating the mechanisms underpinning the effects of CS on NSCs

The results from this chapter showed that both NSCs and their daughter cells express the GRs and therefore can be considered to be CS-responsive. Further, the potential mechanisms underlying the detrimental effects of MPRED on NSCs in monolayer culture were explored by using proteomics analysis. The proteomics and bioinformatics analyses suggest a hypothesis that the down-regulation of major neural development proteins MMP-16 and GAP-43, along with MPRED induced upregulation of CYP51A1 proteins could explain the observed histological effects of this drug on NSCs. Without significant further work we cannot however

conclusively demonstrate a causal relationship between these dysregulation events and downstream impact on cellular behaviour. The clustering of these proteins within a single network suggests MPRED treatment acts by altering a common upstream regulator of these proteins, rather than individual disparate nodes being affected in unrelated molecular pathways. Immunocytochemistry of GAP-43 confirmed the findings from proteomic results, with both NSCs parent cells and the neurons derived from treated NSCs showing a significant reduction in GAP-43 compared to controls.

Chapter 6: Testing NSCs in a combinatorial therapy using organotypic spinal cord slice model

In this chapter, the utility of a new longitudinal organotypic spinal cord slice injury model was investigated to study the effects of another type of anti-inflammatory drug, MINO, delivered as a combinatorial therapy with NSCs.

In the first part of this chapter, several key regenerative properties of NSCs such as survival, proliferation and differentiation were evaluated with respect to the direct effects of MINO on NSCs in neurosphere culture. The findings from this chapter showed that the concentration of 10 μM , MINO did not affect NSCs proliferation, viability, apoptosis and differentiation. In the second part of this chapter, the 3-D neural tissue arrays (organotypic spinal cord slice cultures) were used to examine whether the combination therapy of MINO and NSCs could be evaluated in this *in vitro* injury model. Histopathological analysis demonstrated that MINO inhibited astrogliosis and microglia infiltration, and increased nerve fibre outgrowth.

Collectively, these findings demonstrate the utility of organotypic spinal cord slice culture as a useful screening model for testing the effects of the combinatorial therapy before commencing pre-clinical animal experiments. This model can mimic the *in vivo* tissue environment and it would be feasible to examine the influence of the environmental factors on the transplanted NSCs. Therefore, such 3-D *in vitro* models may bridge gaps between isolated cell cultures and *in vivo* investigations.

7.2 Future directions

There are a number of avenues by which the work presented in this thesis could be expanded and built upon to further our understanding of the detrimental effect of anti-inflammatory and immunosuppressive drugs on the fate of NSCs and the molecular mechanisms underpinning these effects.

The major challenge lies in the understanding of the mechanisms of action of CS, and it is unclear how CS treatments might exert their effects on NSCs. In the work (Chapter 5), the findings showed three different proteins *clustered within a single biological pathway* are differentially expressed, and hence could be responsible for the mechanisms of action of MPRED on NSCs in monolayer culture. A further step in this regard could be conducted; we can expand this study by knocking out the three candidate proteins that participate in mediating the histological observations such as the reduction in NSCs proliferation and neuronal differentiation following CS treatment. The knockout model is a powerful tool to provide information that can be used to validate the function of suggested proteins. A variety of methods can be employed to knock down or knockout gene expression. For example, the

short interfering RNAs (siRNAs) offer a useful tool for studying gene function and validating drug targets by knocking down the expression of specific genes (Cejka et al., 2006). Also, this technique can be used to silence gene expression with no need for genomic manipulation.

Further, it would be interesting to investigate the mechanisms that underlie the anti-proliferative effects of DEX and PRED not only in the monolayer but also in neurosphere cultures. This kind of work will provide a comparative study of CSs effect and their molecular mechanisms. Proteomics studies of NSCs following CSs treatment have the potential to delineate pathways and the means by which CSs can induce their effects on NSCs. Also, it will be beneficial to use multiple reactions monitoring (MRM) analysis, which is a form of tandem mass spectrometry with highly throughput targeted strategies. This analysis allows investigating the dysregulated nodes identified in this study. Importantly, this approach allows quantification of various proteins in a shorter time (around 10 proteins in 20 minutes) (Prasad, 2014). Further, the researchers used MRM proteomics to analyse the targeted proteins of interest in multiple samples with great high-throughput, unlike the traditional mass spectrometry which attempts to detect all proteins.

Also, the aim of this thesis was not only to understand the molecular mechanisms behind the effects of CS on NSCs, but also to expand our knowledge and understanding of how CSs can influence human health. The findings from this study have shown that CS treatment cause a reduction in NSCs proliferation, alteration in the cell cycle phases and effects on the differentiation process. These *in vitro* results are certainly far-away from human clinical application; however, they could provide a program of experiments that can be useful in the clinical

trials. Therefore, it will be interesting to expand this study using human cells. Although, the use of primary human cells has several difficulties such as ethical considerations and supply, they provide a several advantages compared to the use of animal cells. This type of cells has more relevant morphological, physiological and biochemical features such as receptors. Further, the human NSCs have same advantages as animal cells in culture. For example, human NSCs have been propagated as monolayers and neurospheres and provide different cell types from different tissues (Lévesque et al., 2009; Liras, 2010; Xu et al., 2009).

In the context of combination therapy, the results from Chapter 6 demonstrated the utility of organotypic spinal cord culture to assess the efficacy of NSCs transplantation combination with MINO in SCI treatment. For future work, it would be beneficial to study the key parameters of transplanted NSCs such as survival, proliferation and apoptosis post combinatorial therapy using this slice culture model. Additionally, electrophysiological recordings can be made before and after lesioning, and used to evaluate functional improvement following experimental treatments, including transplantation of NSCs. Also, dynamic time-lapse microscopy could also be utilised to study the migration of stem cells and provide more accurate data compared to fixed tissue.

In Chapter 6, a slice model of the spinal cord was used as host tissue to test the transplantation of NSCs. To further enhance the clinical relevance of this model, human cells (e.g. NSCs and ESCs) can be used in slice culture to examine if there are any differences in cellular responses following combinatorial therapy

compared to the mouse NSCs used in this research. Furthermore, the clinical relevance of this model can be improved by using spinal cord slices derived from human tissue to test combinatorial therapies for SCI treatment. Our lab is now developing human slice models and this could be useful to study the effects of combined drug and cell therapy. This type of slice models may provide very useful information on human tissue responses to such therapies before their use in humans. Significant collaboration with clinical colleagues is necessary to develop this pre-clinical model. Finally, it will be of interest to perform proteomics analyses using mass spectrometry. Mass spectrometry based proteomics is a powerful and versatile tool to profile proteomes, investigate biological regulation through post translational modifications, discover biomarkers, study protein interaction and protein degradation. For all of these goals proteome coverage, reproducibility and quantitative precision are the key to gain a comprehensive and full picture of the biology. Therefore, using this approach can examine cellular changes at the genetic and molecular level following MINO treatment and NSCs transplantation in organotypic slice model. This type of analysis could detect alterations in regeneration-associated gene and protein expression from the cells in slice culture.

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Appendices

Appendix 1

Core proteome identified in all conditions using DDA experiment	
Accession	Protein Name
1433E_MOUSE	14-3-3 protein epsilon OS=Mus musculus GN=Ywhae PE=1 SV=1
1433F_MOUSE	14-3-3 protein eta OS=Mus musculus GN=Ywhah PE=1 SV=2
1433Z_MOUSE	14-3-3 protein zeta/delta OS=Mus musculus GN=Ywhaz PE=1 SV=1
A2A6F8_MOUSE	60S ribosomal protein L23 (Fragment) OS=Mus musculus GN=Rpl23 PE=3 SV=1
A2ACG7_MOUSE	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 OS=Mus musculus GN=Rpn2 PE=4 SV=1
A2BH06_MOUSE	60S ribosomal protein L11 (Fragment) OS=Mus musculus GN=Rpl11 PE=3 SV=1
ACTB_MOUSE	Actin, cytoplasmic 1 OS=Mus musculus GN=Actb PE=1 SV=1
ACTC_MOUSE	Actin, alpha cardiac muscle 1 OS=Mus musculus GN=Actc1 PE=1 SV=1
ADT2_MOUSE	ADP/ATP translocase 2 OS=Mus musculus GN=Slc25a5 PE=1 SV=3
ALDOC_MOUSE	Fructose-bisphosphate aldolase C OS=Mus musculus GN=Aldoc PE=1 SV=4
ANXA5_MOUSE	Annexin A5 OS=Mus musculus GN=Anxa5 PE=1 SV=1
AT5F1_MOUSE	ATP synthase subunit b, mitochondrial OS=Mus musculus GN=Atp5f1 PE=1 SV=1
ATPA_MOUSE	ATP synthase subunit alpha, mitochondrial OS=Mus musculus GN=Atp5a1 PE=1 SV=1
ATPB_MOUSE	ATP synthase subunit beta, mitochondrial OS=Mus

	musculus GN=Atp5b PE=1 SV=2
B2M1R6_MOUSE	Heterogeneous nuclear ribonucleoprotein K OS=Mus musculus GN=Hnrnpk PE=2 SV=1
CALR_MOUSE	Calreticulin OS=Mus musculus GN=Calr PE=1 SV=1
COF1_MOUSE	Cofilin-1 OS=Mus musculus GN=Cfl1 PE=1 SV=3
D3YTT7_MOUSE	MCG2650 OS=Mus musculus GN=Rpsa-ps10 PE=3 SV=1
D3YUQ9_MOUSE	Elongation factor 1-delta (Fragment) OS=Mus musculus GN=Eef1d PE=4 SV=1
D3YYM6_MOUSE	40S ribosomal protein S5 (Fragment) OS=Mus musculus GN=Rps5 PE=3 SV=1
D3YZQ9_MOUSE	L-lactate dehydrogenase (Fragment) OS=Mus musculus GN=Ldha PE=3 SV=1
DHSA_MOUSE	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial OS=Mus musculus GN=Sdha PE=1 SV=1
E0CZ27_MOUSE	Histone H3 (Fragment) OS=Mus musculus GN=H3f3a PE=3 SV=1
E9PZ00_MOUSE	Sulfated glycoprotein 1 OS=Mus musculus GN=Psap PE=4 SV=1
E9Q4P0_MOUSE	Protein 2810422J05Rik (Fragment) OS=Mus musculus GN=2810422J05Rik PE=4 SV=1
EF1A1_MOUSE	Elongation factor 1-alpha 1 OS=Mus musculus GN=Eef1a1 PE=1 SV=3
EF1B_MOUSE	Elongation factor 1-beta OS=Mus musculus GN=Eef1b PE=1 SV=5
EF2_MOUSE	Elongation factor 2 OS=Mus musculus GN=Eef2 PE=1 SV=2
ENOA_MOUSE	Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3
F6VW30_MOUSE	14-3-3 protein theta (Fragment) OS=Mus musculus GN=Ywhaq PE=3 SV=1

F8WIX8_MOUSE	Histone H2A OS=Mus musculus GN=Hist1h2a1 PE=3 SV=1
FAS_MOUSE	Fatty acid synthase OS=Mus musculus GN=Fasn PE=1 SV=2
FPPS_MOUSE	Farnesyl pyrophosphate synthase OS=Mus musculus GN=Fdps PE=2 SV=1
G3P_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase OS=Mus musculus GN=Gapdh PE=1 SV=2
G3UXJ6_MOUSE	Heterogeneous nuclear ribonucleoprotein Q OS=Mus musculus GN=Syncrip PE=4 SV=1
G3UYV7_MOUSE	40S ribosomal protein S28 (Fragment) OS=Mus musculus GN=Rps28 PE=4 SV=1
G3UZK4_MOUSE	60S ribosomal protein L18 OS=Mus musculus GN=Rpl18 PE=4 SV=1
GRP78_MOUSE	78 kDa glucose-regulated protein OS=Mus musculus GN=Hspa5 PE=1 SV=3
H2AX_MOUSE	Histone H2A.x OS=Mus musculus GN=H2afx PE=1 SV=2
H2B1B_MOUSE	Histone H2B type 1-B OS=Mus musculus GN=Hist1h2bb PE=1 SV=3
H32_MOUSE	Histone H3.2 OS=Mus musculus GN=Hist1h3b PE=1 SV=2
H4_MOUSE	Histone H4 OS=Mus musculus GN=Hist1h4a PE=1 SV=2
HS90A_MOUSE	Heat shock protein HSP 90-alpha OS=Mus musculus GN=Hsp90aa1 PE=1 SV=4
HS90B_MOUSE	Heat shock protein HSP 90-beta OS=Mus musculus GN=Hsp90ab1 PE=1 SV=3

HSP7C_MOUSE	Heat shock cognate 71 kDa protein OS=Mus musculus GN=Hspa8 PE=1 SV=1
J3QNR5_MOUSE	Uncharacterized protein OS=Mus musculus PE=3 SV=1
KCRB_MOUSE	Creatine kinase B-type OS=Mus musculus GN=Ckb PE=1 SV=1
MDHM_MOUSE	Malate dehydrogenase, mitochondrial OS=Mus musculus GN=Mdh2 PE=1 SV=3
PDIA6_MOUSE	Protein disulfide-isomerase A6 OS=Mus musculus GN=Pdia6 PE=1 SV=3
PGAM1_MOUSE	Phosphoglycerate mutase 1 OS=Mus musculus GN=Pgam1 PE=1 SV=3
PP1A_MOUSE	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit OS=Mus musculus GN=Ppp1ca PE=1 SV=1
PPIA_MOUSE	Peptidyl-prolyl cis-trans isomerase A OS=Mus musculus GN=Ppia PE=1 SV=2
PROF1_MOUSE	Profilin-1 OS=Mus musculus GN=Pfn1 PE=1 SV=2
Q14AA6_MOUSE	MCG49183 OS=Mus musculus GN=1700009N14Rik PE=2 SV=1
RL12_MOUSE	60S ribosomal protein L12 OS=Mus musculus GN=Rpl12 PE=1 SV=2
RL30_MOUSE	60S ribosomal protein L30 OS=Mus musculus GN=Rpl30 PE=2 SV=2
RLA1_MOUSE	60S acidic ribosomal protein P1 OS=Mus musculus GN=Rplp1 PE=1 SV=1
RLA2_MOUSE	60S acidic ribosomal protein P2 OS=Mus musculus GN=Rplp2 PE=1 SV=3
SERPH_MOUSE	Serpin H1 OS=Mus musculus GN=Serpinh1 PE=1 SV=3
SMD1_MOUSE	Small nuclear ribonucleoprotein Sm D1 OS=Mus musculus GN=Snrpd1 PE=2 SV=1

TBA1A_MOUSE	Tubulin alpha-1A chain OS=Mus musculus GN=Tuba1a PE=1 SV=1
TBA1C_MOUSE	Tubulin alpha-1C chain OS=Mus musculus GN=Tuba1c PE=1 SV=1
TERA_MOUSE	Transitional endoplasmic reticulum ATPase OS=Mus musculus GN=Vcp PE=1 SV=4
VIME_MOUSE	Vimentin OS=Mus musculus GN=Vim PE=1 SV=3
sp P52480 KPYM_MOU USE	Pyruvate kinase isozymes M1/M2 OS=Mus musculus GN=Pkm PE=1 SV=4
sp P63038 CH60_MOU SE	60 kDa heat shock protein, mitochondrial OS=Mus musculus GN=Hspd1 PE=1 SV=1

Appendix 2

Rawaa S. Al-Mayyahi, Luke D. Sterio, Joanne B Connolly, Christopher F. Adams, Wa'il A. Al -Tumah, Jon Sen, Richard D. Emes, Sarah R. Hart, Divya M. Chari. A proteomic investigation into mechanisms underpinning corticosteroid effects on neural stem cells. Manuscript submitted.