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The role of olfactory ensheathing cells, MRI, and biomaterials in transplant-mediated CNS repair

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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Preface

This thesis is submitted to the Norwegian University of Science and Technology (NTNU) in partial fulfilment of the requirements for the academic title of PhD in Neuroscience. The research work presented in this thesis was carried out at the MR Centre, Department of Circulation and Medical Imaging, NTNU and also utilised the facilities of the Institute for Laboratory Medicine, Women's and Children's Health, NTNU, Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology, NTNU, as well as Department of Cancer Research and Molecular Medicine, NTNU. Financial support for this work was provided by the Medical Imaging Laboratory, Centre for Research-based Innovation, Norwegian Research Council.

Bruk av olfaktoriske ensheathing celler, MRI og biomaterialer i transplantasjonsmediert reparasjon av CNS skader

Den beskrevne studien har brukt en interdisiplinær tilnærming for å evaluere transplantasjons mediert CNS reparasjon i en skademodell av synsnerven. Ved å integrere forskjellige MRI metoder har vi analysert olfaktoriske ensheathing celler's (OEC) evne til å integrere og overleve in vivo i vår skademodell. Cellenes evne til å modulere regenerasjonen av den skadede synsnerven er også dokumentert ultrastrukturelt med elektronmikroskopi (EM). Studien har også omfattet *in vitro* analyser av interaksjonen mellom OEC og modifiserte biopolymerer i 2-og 3-dimensjonale matriser.

Den spesifikke målsetningen med denne studien har vært: (a) Utvikle protokoller for effektiv merkning av OEC med mikron store jern partikler; (b) Kombinere cellulær MRI og mangan-forsterket MRI (MEMRI) for spatiotemporal monitorering av intravitreal (*ivit*) og intra-optisk nerve (*iON*) transplantasjon av OEC; (c) Studere celleimplantatenes evne til å promotere regenerasjon av synsbane aksoner etter skade i synsnerven både (i) longitudinelt *in vivo* med MRI og (ii) ultrastrukturelt med transmisjons elektron mikroskopi (TEM); (d) Produsere og teste modifiserte alginat strukturer som plattformer for kontrollert frigjøring av mangan (Mn^{2+}) brukt som kontrastmiddel ved MEMRI; (e) Utvikle arginin-glycin-aspartat (RGD)-peptid alginater og karakterisere interaksjonen med OEC dyrket på disse modifiserte aliginat matrisene.

I avhandlingen presenteres gjennomføringen av disse målene og belyser potensialet av å integrere MRI, biomateraler og celleterapeutiske teknologier i studier av transplantasjons-mediert reparasjon av skader i CNS.

Navn kandidat: *Ioanna Sandvig*
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Finansieringskilde: *MI Lab, Senter for forskningsdrevet innovasjon, Norges Forskningsrådet*

Ovennevnte avhandling er funnet verdig til å forsvares offentlig for graden
Philosophiae Doctor i Nevrovitenskap
Disputas finner sted i auditoriet MTA, MTFS, NTNU
fredag 14. oktober 2011 kl 12:15

Η Ιθάκη σ' έδωσε το ωραίο ταξίδι.
Χωρίς αυτή δε θα 'βγαινες στο δρόμο.
Άλλα δεν έχει να σου δώσει πια.

Κι αν φτωχική τη βρεις, η Ιθάκη δε σε γέλασε.
Έτσι σοφός που έγινες, με τόση πείρα,
Ήδη θα το κατάλαβες οι Ιθάκες τι σημαίνουν.

Κωνσταντίνος Π. Καβάφης

Ithaka gave you the marvellous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have deceived you.
Wise as you will have become, so full of experience,
you will have understood by then what these Ithakas mean.

Constantine P Cavafy (1863-1933)

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(submitted to *Contrast Media and Molecular Imaging*)

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(this paper was submitted to *Contrast Media and Molecular Imaging on 27.01.11, has been through a first revision, and is being considered for publication*)

Paper IV

Sandvig I, Karstensen K, Rokstad AM, Aachmann F, Sandvig A, Skjåk-Bræk G, Strand BL

Effects of RGD peptide-modified alginates on olfactory ensheathing cells and myoblasts
(manuscript for submission to *Acta Biomaterialia*)

Contribution to other papers, not included in this thesis:

(i) Sandvig A*, **Sandvig I***, Berry M, Olsen Ø, Pedersen TB, Brekken C and Thuen M

Axonal tracing of the normal and regenerating visual pathway of mouse, rat, frog, and fish using manganese-enhanced magnetic resonance imaging (MEMRI)
Journal of Magnetic Resonance Imaging 2011; doi:10.1002/jmri.22361

* These authors have contributed equally.

(ii) **Sandvig I,** Berry M, Sandvig A.

The role of olfactory ensheathing cells in the repair of the rat visual pathway.
(Manuscript for submission to *Glia*)

Abbreviations

AIM-MRI	activation-induced MEMRI
ALS	amyotrophic lateral sclerosis
BDNF	brain derived neurotrophic factor
CEST	chemical exchange saturation transfer agents
CLSM	confocal laser scanning microscopy
CSF	cerebrospinal fluid
CNR	contrast-to-noise ratio
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CNTFRα	ciliary neurotrophic factor receptor α
CSPG	chondroitin sulphate proteoglycans
DCC	deleted in colorectal cancer
DIC	differential interference contrast
PDMAAm	poly(N,N-dimethylacrylamide) maghemite
DREZ	dorsal root entry zone
DRG	dorsal root ganglia
dpl	days post-lesion
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EM	electron microscopy
E-NCAM	embryonic neural cell adhesion molecule
ESCs	embryonic stem cells
FGF	fibroblast growth factor
FLEX	frequency-labelled exchange
GalC	galactocerebroside
GAP-43	growth associated protein
GBCs	globose basal cells
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GRID	gadolinium rhodamine dextran
HBCs	horizontal basal cells
hESCs	human embryonic stem cells
hNSCs	human neural stem cells

hOECs	human olfactory ensheathing cells
hpl	hours post-lesion
ICAM-1	intracellular adhesion moldecule-1
IL (-1, -2, etc)	interleukins
iON	intra-optic nerve
iPSCs	induced pluripotent stem cells
ivit	intravitreal
LV	lentivirus; lentiviral
MAG	myelin-associated glycoprotein
MEMRI	manganese-enhanced MRI
MGE	multi-gradient echo
MMPs	matrix metalloproteinases
MMP2	matrix metalloproteinase 2
MPIO	micron-sized particles of iron oxide
MRI	magnetic resonance imaging
MS	multiple sclerosis
NCAM	neural cell adhesion molecule
NG2	Neuron-Glia antigen
NGF	nerve growth factor
NMDA	N-methyl-D-aspartic acid
NPCs	neural progenitor cells
NSCs	neural stem cells
NT(-3, -4/5, 6)	neurotrophin
OB	olfactory bulb
OE	olfactory epithelium
OECs	olfactory ensheathing cells
OMpg	oligodendrocyte myelin glycoprotein
ON	optic nerve
ONC	optic nerve crush
ONF	olfactory nerve firbroblasts
ONL	olfactory nerve layer
ORN	olfactory receptor neurons
OPCs	oligodendrocyte precursors
PET	positron emission tomography
PLGA	poly(lactide-co-glucolide) acid
PN	peripheral nerve

PNG	peripheral nerve graft
PNS	peripheral nervous system
PSA-NCAM	polysialylated neural cell adhesion molecule
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RARE	rapid acquisition with relaxation enhancement
RGC	retinal ganglion cells
RGD	arginine-glycine-aspartic acid
SAPNS	self-assembling nanofibre peptide scaffold
SEM	scanning electron microscopy
Sema	semaphorine
SGZ	subgranular zone
SLA	sprout-like axons
SNR	signal-to-noise ratio
SPECT	single-photon emission computed tomography
SPIO	superparamagnetic iron oxides
SVG	subventricular zone
T	Tesla
TEM	transmission electron microscopy
TGF (-α, -β, etc)	transforming growth factor
TNF(-α, -β, etc)	tumour necrosis factor
trk	tyrosine kinase (neurotrophic) receptor
USPIO	ultra-small paramagnetic iron oxides
VB	vitreous body
Wpl	weeks post-lesion

Ethical aspects

All procedures involving laboratory animals were approved by the Norwegian Ethics Committee and were in accordance with local, regional, and site guidelines that apply.

1. Introduction

1.1. CNS damage and repair

Damage to the central nervous system (CNS) as a result of trauma or neurodegenerative disease, such as Parkinson's, Alzheimer's, and Huntington's disease, multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS), constitutes one of the major causes of morbidity and mortality in the human population today, with serious implications for the quality of life of millions of people and significant associated socio-economic costs. Yet despite intense research effort from translational neuroscientists and clinicians over several decades, CNS regeneration after injury still remains elusive.

Research progress over the last decades, has questioned the old dogma of an immutable CNS, which, unlike the peripheral nervous system (PNS), lacks the ability to regenerate once development has been completed [1]. This dogma is best expressed in the words of the pioneering Spanish neuroscientist Santiago Ramón y Cajal: '… the functional specialisation of the brain imposed on neurones two great lacunae; proliferative inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated'. However, as Ramón y Cajal himself observed, 'the incapability of regeneration is not a fatally irresistible law, but a secondary outcome of a physical or chemical environment unfavourable for the growth of the sprouts' [1]. This led to the hypothesis that CNS axon regeneration should be possible provided that there is a permissive environment supporting growth, demonstrated, for the first time, as the intrinsic regenerative capacity of axotomised CNS neurons to regrow their axons in the presence of peripheral nerve grafts (PNG) [1-4].

These early experiments in the 1980s signified a new era in neuroscience research, underpinned by the effort to elucidate the mechanisms of CNS damage and repair and to develop appropriate therapeutic interventions aimed at CNS regeneration, with the ultimate goal of achieving neuroanatomical restitution and re-establishment of lost synaptic connections. It is, nonetheless, the immense complexity of CNS degeneration and regeneration mechanisms that renders the development of effective regenerative approaches highly challenging.

1.1.1. CNS lesion pathology and axon growth inhibition

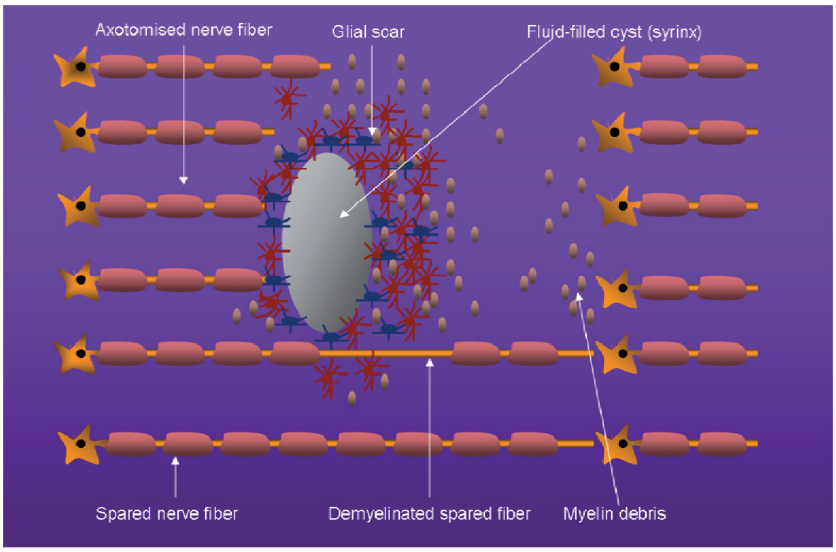
Insult to the CNS results in loss of neurons and/or damaged neuronal axons and the consequent disruption of neuronal circuits, leading to neurological deficits which are permanent and irreversible.

Neuronal cell death is due to either necrosis, or apoptosis [5-11]. Necrotic cell death occurs as a result of anoxia or mechanical damage to the cell membrane, causing severe disruption of metabolic mechanisms and cellular homeostasis [8]. On the other hand, neuronal cell death due to apoptosis is genetically programmed cell death precipitated, in the case of CNS lesions, by extracellular events, such as withdrawal of trophic support, free-radical damage and DNA damage [8, 12]. During apoptosis, the cell membrane initially remains intact, while cellular components start being digested inside the cell. As the apoptotic cycle progresses, the cell membrane becomes convoluted and cellular fragments separated and subsequently ingested by macrophages and other scavenger cells [8, 13]. Excitotoxicity, metabolic imbalance, oxidative stress, and calcium influx are events that, depending on the type of CNS lesion, may induce cell death *via* either apoptotic or necrotic mechanisms [14, 15]. Such events are determined by intricate molecular mechanisms and affect specific ligands and receptors and intracellular and extracellular signalling cascades, with major implications for synaptic connectivity and neurotransmission.

The vast majority of CNS lesions invariably affect neuronal axons, whose length of extension (i.e. up to 1m) from their somata, makes them highly vulnerable to insult [16]. Following axotomy, the distal segment of the injured axon gradually degenerates and becomes demyelinated, a process known as Wallerian degeneration [16]. Depending on the distance of the lesion from the cell body, axotomy may lead to retrograde neuronal death *via* either apoptotic or necrotic mechanisms, with rapid necrotic death occurring when the neuronal axon is damaged close to the soma. Additionally, anterograde cell death as a result of apoptosis may occur in target neurons, with which the axotomised neurons form synapses [16]. In all cases, trophic support from/to axotomised neurons is disrupted and eventually lost, leading to neuronal death [16]. On a molecular level, axotomy induces the upregulation and down-regulation of a host of different genes, including (i), transcription factors, such as c-jun, jun D, Krox-24; (ii), growth-associated proteins, i.e. GAP-43; (iii), cytoskeletal proteins, such as F actin; (iv), growth factor receptors and growth factors, including the tyrosine kinase (trk) family of receptors for brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), nerve growth factor (NGF), and its low affinity receptor, $p75^{NTR}$; (v), cytokines, including interleukins (i.e. IL-

6, IL1β), and tumour necrosis factor (i.e. TNFα); as well as (vi), the myelin-associated inhibitors NogoA, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) [16-25].

Apart from mechanical damage to axons at the lesion site, with subsequent disruption of the neuronal circuitry and gradual degeneration of the affected neurons, CNS lesion pathology is further complicated by a cascade of secondary cellular and molecular events which evolve up to several weeks after the injury. These include an inflammatory response with microglia activation, as well as macroglia responses, characterised by reactive astrocytosis, infiltration by oligodendrocyte precursors (OPCs), and the formation of the glial scar [26-34] (Figure 1.1).



(I Sandvig - Adapted from Barnett & Riddell, 2007)

Figure 1.1 – Pathological features of CNS injury. Illustration showing process of demyelination, reactive astrocytosis, and glial scar formation after CNS axotomy.

Microglia are immune-competent cells intrinsic to the adult CNS [35]. In response to CNS injury, resident microglia within the CNS parenchyma, become activated and are recruited to the lesion site within minutes, followed in the next few days by proliferation of macrophages and lymphocytes [16, 34-36]. Additionally, in injuries that damage blood vessels and result in extensive haemorrhage, there is massive macrophage infiltration from the blood, as well as some neutrophil recruitment, which, however, is limited given that the α-chemokines involved in the recruitment of leukocytes are absent in the CNS [16, 32, 35, 37]. Microglia assume

phagocytic and macrophage-type roles in response to the presence of cellular and myelin debris, however, as a result of the fact that microglia/macrophage populations in the injured CNS are much smaller than in the PNS, clearance of such debris in the CNS is significantly slower [16, 32]. Furthermore, activated microglia accumulate around the cell body of injured axons whose input synapses have withdrawn, and remain there for several weeks [34]. When the microglia subsequently retract, they are replaced by astrocytes, which become interposed with the neuronal terminals and cell body, effectively blocking re-innervation [16, 34].

Mechanical disruption of the lesion microenvironment inadvertently results in astrocytic death [38]. Moreover, in response to injury, surviving astrocytes in the lesion area become reactive, leading to a series of biochemical changes, including elevated levels of glial fibrillary acidic protein (GFAP), cytoskeletal changes and upregulation of nestin and vimentin production, as well as changes in the levels of cytokines, proteases and protease inhibitors, cell surface matrix and other molecules [29, 39-45]. In the event of injury that penetrates the meninges, interactions between reactive astrocytes and invading meningeal cells affect the process of gliosis and the re-formation of the glia limitans, in which meningeal cells form a layer around the inside of the injury, preventing molecular diffusion [29, 46-48]. The resulting tissue, described as the 'glial scar', is densely packed with hypertrophic astrocytes with interdigitating processes [16], extracellular matrix (ECM), and gap and tight junctions [16, 32]. In addition to astrocytes, macroglia responses to CNS injury include the recruitment of oligodendrocyte precursors to the lesion. OPCs express growth inhibitory molecule NG2 as well as other chondroitin sulphate proteoglycans (CSPGs). A host of CSPGs, including tenascin-R, tenascin-C, neurocan, versican, brevican, and phosphacan are also expressed in the ECM, as well as in reactive astrocytes and neurons at the lesion site [16, 32, 49-66]. As a result, intricate interactions between cellular and molecular constituents of the injury microenvironment pose a physical and chemical barrier to regenerating CNS axons and migrating myelinating cells.

In addition to the extrinsic factors described above, a major complicating factor in CNS axon growth after injury is the upregulation of a host of axon growth inhibitory molecules of the semaphorin (Sema), ephrin, and netrin families and their respective receptors [50, 67-74]. These ligands and receptors are axon guidance molecules that regulate pathfinding and chemotaxis and mediate synapse selection during development [50, 67]. Interestingly, in the adult CNS, the same ligands and receptors are implicated in mediating plasticity and stabilising synaptic connections [50]. Upregulation of expression of inhibitory ligands and receptors in reactive glia and fibroblasts in the lesion microenvironment triggers downstream signalling cascades in the Rho family of small GTPases, including RhoA, Cdc42, and Rac1, which regulate

the actin cytoskeleton, change the motility of outgrowing neurons, and thus arrest neurite outgrowth by inducing growth cone repulsion or collapse [50, 75, 76]. Finally, intrinsic factors such as reduced plasticity associated with neuronal maturity, as well as changes in evolutionarily conserved molecular mechanisms, for example, the PTEN-dependent inactivation of the mTOR pathway that promotes axon extension in CNS neurons, greatly impinge on the capacity of the adult CNS to regenerate after injury [77-81].

1.1.2. CNS recovery: mechanisms and strategies

Ability to promote CNS recovery after injury is contingent on a multitude of factors, including the nature, extent and location of the insult, as well as the time elapsed between the insult and the therapeutic intervention [80]. At the early stages after injury, damaged CNS neurons enter a programme of abortive regeneration [81-83]. The degree of spontaneous axonal sprouting in these cases is dependent on neuronal cell subtype and maturity, while neurite outgrowth is often aberrant, extends over short distances and, consequently, lacks the ability to promote axon regeneration towards correct synaptic targets distal to the lesion [81, 82]. Furthermore, collateral sprouting from the terminals of intact neurons, or the proximal segment of injured mature CNS axons is marginal and thus unlikely to effectuate repair of neuronal circuits *via* compensatory routes [81].

As discussed earlier, the regenerative failure of CNS axons can be attributed to interplay between intrinsic and extrinsic factors. Hence, therapeutic strategies may target either endogenous or exogenous constituents of CNS lesion sequelae or, alternatively, involve combinatorial approaches addressing both. Such strategies must be able to meet a number of challenges, mainly: (i), stimulate robust neurite outgrowth, (ii), circumvent the chemical and physical barriers posed by the non-permissive CNS neuropil to promote sustained growth cone advance through it, and (iii), achieve restitution of lost synaptic connections with functional outcome.

One approach to CNS recovery is limiting neuronal cell death by adopting neuroprotective strategies which aim to reduce excitotoxicity, metabolic imbalance, and oxidative stress after injury [14]. Such approaches may include pharmacological modulation of calcium influx using NMDA antagonists/calcium blockers, administration of free-radical scavenging agents, promotion of cellular metabolic activity, and use of anti-apoptotic and anti-inflammatory treatments [14, 84-93]. The latter aim to limit secondary damage by preventing extensive cavitation and gliosis. It is noteworthy, however, that in certain circumstances, inflammation

may actually exert a neuroprotective effect on lesioned CNS neurons, as demonstrated by increased neurite outgrowth induced by lens injury and zymosan injections after visual pathway lesion [94-98].

In addition to the above, infusion with neurotrophic factors, including BDNF, NT, glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF), transforming growth factor (i.e. TGFβ), and neurturin, may prevent axonal death and/or promote anatomical and biochemical plasticity in target CNS neurons [97-108]. Another approach is overcoming the inhibitory nature of the lesion microenvironment. This may be effectuated by modulation of the molecular and/or cellular components of axon growth inhibition. Examples include the use of neurotrophic factors, antibodies/recombinant protein treatments, and gene therapy to neutralise/modulate the expression of CSPGs [109-114], myelin-associated inhibitors [115-121], and axon guidance molecules [122-124], and to influence specific signalling cascades and signal transduction mechanisms [79, 122, 125-131].

Such interventions have varying degrees of success, but overall fail to achieve significant or sustainable functional outcomes. Furthermore, the complexity of CNS lesion pathology and body of experimental evidence strongly suggest that better results are to be derived by applying combinatorial approaches, rather than individual treatments [81, 132-135]. Furthermore, while some treatments yield promising results, they may not be clinically applicable. One such example is conditioning lesion of the peripheral branch of dorsal root ganglia (DRG) in the form of sciatic nerve injury, prior to spinal cord or dorsal column lesioning, to promote regeneration of centrally ascending afferent fibres [136-139].

1.1.3. Transplant-mediated CNS repair

One of the underlying assumptions in many CNS repair strategies is that regeneration may be achieved by recapitulation of normal neuronal development, i.e. that CNS neurons may be induced to switch to genetic programmes characterised by enhanced plasticity and regenerative capacity [140]. Thus the success of neuroprotection, neuronal replacement, stimulation of axonal sprouting and synaptogenesis, myelination, as well as *de novo* neurogenesis may be contingent on recapitulation of the relevant developmental mechanisms. Treatment of CNS lesions utilising tissue and cell transplants largely reflects this rationale.

As it was mentioned at the beginning of Section 1, the old dogma suggesting that the mature CNS is characterised by complete lack of regenerative capacity was challenged for the first time

when Aguayo and colleagues demonstrated that large numbers of lesioned dorsal root ganglia (DRG) and spinal cord neurons are able to extend axons into peripheral nerve (PN) segments engrafted into the injured rat spinal cord [1-4]. In later experiments, Aguayo and colleagues showed that intra-optic nerve (*iON*) PNG promote robust retinal ganglion cell (RGC) axon regeneration and re-innervation of the superior colliculus after visual pathway lesion in the adult rat [141, 142]. Similarly, Berry and colleagues demonstrated that intravitreal (*ivit*) PNG promotes RGC axon regeneration through the rat optic chiasm and into the optic tract [143, 144]. Additionally, a number of other studies demonstrated the neuroprotective and myelinating effects of Schwann cell-containing PNG alone, or in combination with other strategies, such as administration of neurotrophins and chondroitinase ABC [145-155]. The main limitation in such approaches, though, is that regenerating CNS axons growing through PNG generally fail to exit the Schwann-cell microenvironment of the graft and make the transition into the CNS [156]. In the same manner, regenerating sensory afferents from damaged DRG do not cross from the PNS into the CNS, but stop at the dorsal root entry zone (DREZ) [156]. Nonetheless, CNS axon regeneration through PNG into the distal spinal cord segment with partial restoration of functional recovery has been reported [155]. However, this promising outcome is largely attributed to additional manipulation of the non-permissive lesion microenvironment with FGF-1, rather than to the PNG alone [155-158].

Another transplantation strategy utilises foetal tissue for neuronal replacement and neuroprotection in experimental spinal cord and brain lesions. Foetal spinal cord transplants have been shown to promote functional recovery and to restore lost supraspinal and serotonergic input after spinal cord injury in the adult rat [157, 159, 160]. Again, improved effects were achieved in cases when foetal transplantation was combined with other strategies, including administration of BDNF, and NT-3 [161, 162].

Apart from transplantation of tissue segments, embryonic stem cells (ESCs), neural stem cells (NSCs)/neural progenitor cells (NPCs), and glia are attractive candidates for transplant-mediated CNS repair. Pluripotent ESCs are characterised by unlimited expandability and differentiation capacity [163, 164]. OPCs derived from human ESCs (hESCs) have been used to treat spinal cord injury patients in Phase I clinical trials with promising results [165-167]. However, the use of hESCs, is fraught with ethical concerns [168-170]. Furthermore, the pluripotent character of ESCs harbours the potential for aberrant growth after implantation into the host nervous system, resulting in overgrowth and formation of tumours, like teratomas [171]. Moreover, the use of ESC allografts carries the inherent risk of transferring viral, bacterial, fungal, and prion contaminants from the donor to the host [172, 173]. Finally, the risk

of rejection of tissue allografts necessitates the administration of immunosuppressive treatments, which may potentially precipitate side-effects and/or complications [173].

Mulipotent NSCs and neural stem cell progenitors (NSPs) are self-renewing mitotically-active cells harvested from special CNS locations in which neurogenesis is ongoing throughout adult life. These NSC niches include the subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the hippocampal dentate gyrus [174-179]. Alternatively, NSCs can be derived from induced pluripotent stem cells (iPSCs) [180-184]. NSCs/NPCs give rise to cells of neural lineage, i.e. neurons, astrocytes, and oligodendrocytes, *via* asymmetric division [179]. These stem cell populations have been utilised to treat neurodegenerative conditions such as experimental ALS, Parkinson's disease, and spinal cord lesions with promising results [185-195]. However, for safe translation of NSC-based therapies into the clinics a number of obstacles will need to be overcome, including the limited availability and expandability of human NSCs (hNSCs), genetic and epigenetic instability, tumorigenic potential, and other unwanted effects of NSC transplants, such as allodynias and dyskinesias [140, 157, 196-204].

1.2. Olfactory ensheathing cells in transplant-mediated CNS repair

1.2.1. Olfactory ensheathing cell properties and function

The observation, in the late 1970s, by Graziadei and Monti Graziadei that neurogenesis in the olfactory system continues throughout adult life and that mature olfactory receptor neurons (ORN) regenerate their axons and establish functional connections after injury [205-216] suggested that olfactory ensheathing cells (OECs), the glia that enwrap olfactory axons [217, 218], are possible candidate cells in transplant-mediated CNS repair.

OECs are a unique type of glia first identified by Golgi [219] and Blanes [220, 221]. OECs are found both in the olfactory epithelium and olfactory bulb (OB) of the primary olfactory system. The OECs that are located in the lamina propria of the nasal cavity associate closely with afferent receptor bundles of ORN, while intracranially, OECs enfold large numbers of contiguous, unmyelinated axons of the first cranial nerve [218, 222-235] (Figure 1.2).

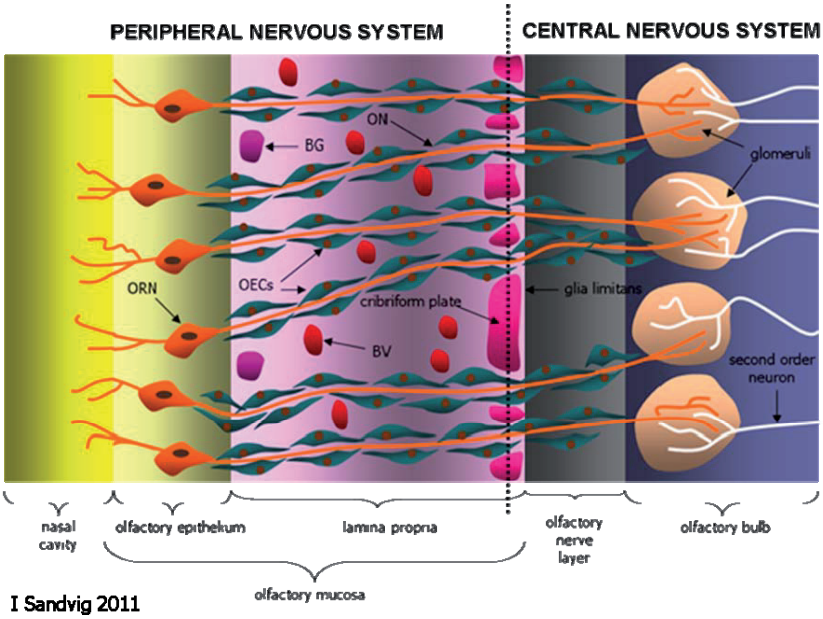


Figure 1.2 – OECs enwrap ORN along their trajectory from the PNS into the CNS; BV: blood vessel; BG: Bowman gland.

During mammalian development, ORN axon pathfinding and elongation in the lamina propria is partially attributed to chemotactic and mechanical support provided by OECs [236, 237]. OECs facilitate elongation and fasciculation of developing ORN axons by expression of cell adhesion and ECM molecules such as NCAM, E-NCAM, L1, collagen IV, galectin-1, and β2-laminin [236-241]. OECs migrate in tandem with developing ORN axons across the lamina propria and provide directional guidance during axon tract formation [236, 237]. Furthermore, $p75^{NTR}$-expressing OECs contribute to the formation of the glia limitans of the OB [241, 243], while at the ventral olfactory nerve layer (ONL), OECs guide ORN axons towards the olfactory bulb glomeruli by differential expression of galectin-1, β2-laminin, and Sema3A [236, 240].

ORN transmit odour signals from the olfactory mucosa to the OB, from where the signals are subsequently relayed to the olfactory cortex [244, 245]. It is established that detection and response to odours is crucial for the behaviour and survival of many species. This suggests that the continual turnover and regeneration of ORN in the primary olfactory system safeguards olfactory integrity against noxious stimuli [244]. Damage to the olfactory epithelium (OE) stimulates proliferation and differentiation of resident putative stem cells, i.e. horizontal basal cells (HBCs) and globose basal cells (GBCs) into ORN, while normal ORN turnover is attributed

solely to GBCs [244, 246, 247]. OECs provide distinct guidance cues and facilitate elongation of newly generated ORN axons along their trajectory, from the PNS into the CNS and OB, where the ORN establish synapses with second order neurons [217, 229, 248-250].

The localization, function and role of OECs in primary olfactory system development, adult neurogenesis and regeneration suggested that OECs are indeed stem cells [251]. Despite morphological and antigenic plasticity, however, OECs seem to lack pluripotency and ability for continuous self-renewal [244]. On the other hand, apparent similarities between OECs, Schwann cells, and also astrocytes have led to a degree of discrepancy in the relevant literature, with OECs often being identified as Schwann cells or astrocytes, especially in earlier publications [235, 251-254].

1.2.2. Comparison of OECs with Schwann cells and astrocytes

OECs display a malleable morphological and molecular phenotype and share many similarities with immature non-myelinating Schwann cells, as well as astrocytes both *in vitro* and *in vivo* [229, 245, 254-261]. Morphologically, OECs appear either spindle-shaped or stellate-shaped, closely resembling Schwann cells and astrocytes, respectively [256]. This heterogeneity is also reflected in the antigenic expression of OECs. In the early stages after isolation and culture, OECs express $p75^{NTR}$, a molecular marker also expressed by Schwann cells [221, 255]. However, *in vivo* $p75^{NTR}$ expression in both OECs and Schwann cells is low, but becomes upregulated in response to ORN and PN injury, respectively [221, 255, 260]. Furthermore, OECs expressing $p75^{NTR}$ are spindle-shaped and stain positive for the glial marker O4, but express no galactocerebroside (GalC) [255, 260], while stellate-shaped OECs are PSA-NCAM positive [221, 254, 255, 260]. Moreover, both OECs and Schwann cells express peripheral myelin protein P0 [261, 262]. This overlap in antigenic expression and morphology makes it difficult to distinguish OECs from other glia, especially Schwann cells.

A recent report by Boyd *et al* [263] claimed that calponin, an actin-binding protein, can be used as a marker to unequivocally distinguish OECs from Schwann cells both *in vitro* and *in vivo*. However, a different study by Ibanez *et al* [264] challenged this finding as the authors concluded that calponin is not expressed by OECs, but rather by fibroblasts and meningeal cells in the olfactory mucosa and olfactory bulb, respectively. Tomé *et al* [265] investigated whether the discrepancy in these findings could be attributed to differences in methodology or the developmental age of the OECs utilized in the studies [265] and showed that while calponin is expressed by subpopulations of connective tissue cells in the neonatal olfactory mucosa, OECs

do not express calponin either *in vitro* or *in vivo*, irrespective of methodology or developmental age [265]. It is, therefore, concluded that calponin is not a specific marker for OECs [265].

Despite the fact that OECs share common characteristics with other glia, they are a developmentally and functionally distinct cell type [248]. The general consensus is that OECs originate in the olfactory placode, as opposed to Schwann cells, which originate in the neural crest [235, 266]. It was recently claimed, however, that OECs are derived from the neural crest, which might potentially enable treatment of human patients by isolating autologous neural crest derived stem cells and directing their differentiation into OECs *in vitro* [267]. Although a shared origin between OECs and Schwann cells might explain the many similarities between the two types of glia [267], more research might be necessary to confirm and explore these findings.

Irrespective of ontogenic profile, a main distinction between OECs and Schwann cells is the fact that they display very different interaction profiles when co-cultured with astrocytes, as demonstrated by confrontation assays [268]. OECs intermingle with astrocytes *in vitro*, unlike Schwann cells, which induce boundary formation and a hypertrophic response [221, 268]. Similarly, transplanted OECs, not Schwann cells, intermingle with astrocytes *in vivo*, while increased deposition of CSPG by astrocytes is observed in response to Schwann cells only [269-277]. The exact mechanisms of the interactions of OECs and Schwann cells with astrocytes remain to be elucidated, however a number of different molecules, such as CSPG, ephrins, FGF2, and N-cadherin, are involved [221, 270-272]. Finally, *in vitro* assays demonstrated distinct interactions of OECs and Schwann cells with meningeal cells [278]. In a manner similar to that characterizing interactions with astrocytes, OECs intermingle with meningeal cells, as opposed to Schwann cells, which aggregate into clusters [278].

1.2.3. OEC transplants as mediators of CNS repair

The distinct interactions of OECs and Schwann cells with astrocytes and meningeal cells are highly relevant with regard to the capacity of either glia to promote CNS axon regeneration by negotiating the non-permissive chemical and physical boundary of the glial scar. Furthermore, the rich antigenic profile and ability of OECs to support continual ORN outgrowth throughout adult life render them superior candidates for transplant-mediated CNS repair.

OECs have been transplanted in different spinal cord injury models, including electrolytic and photochemical lesions, dorsal column lesions, complete spinal transection, contusion injury, and dorsal rhizotomy [277]. Each lesion paradigm is characterized by different cellular, molecular,

anatomical and functional sequelae, thus the inherent assumptions in the use of OECs, and also the mechanisms that determine transplant-mediated outcomes are quite different in each case [248, 277, 279]. OECs have been reported to repair lesion cavities and to promote axon regeneration of corticospinal, dorsal column, and monoaminergic neurons [248, 280-284]. However, a number of subsequent studies failed to confirm OEC-mediated axon regeneration through and beyond the lesion in some of these models [248, 277, 285-291]. Nonetheless, combinatorial approaches significantly enhance the regenerative effects of OECs in some of these lesions [248]. Examples include long-distance axon regeneration beyond the lesion site mediated by (i), transplanted OECs combined with stimulation of neuronal cell body with cAMP and injured axons with neurotrophins [292], and (ii), OEC engraftment in combination with fibroblasts and biomatrices [293]. Apart from the above, OEC transplantation after dorsal rhizotomy promoted entry of DRG axons into the DREZ and dorsal horn of the spinal cord [277, 294]. Furthermore, Li and colleagues [295] showed regeneration of afferent DRG axons across the DREZ. This led to the formulation of the 'pathway hypothesis' [295] , which proposes that transplanted OECs interact with astrocytic processes to form pathways that bridge CNS lesions, thus rendering the lesion microenvironment permissive to axonal growth [277, 295] (Figure 1.3). However, while modulation of the lesion microenvironment by transplanted OECs after dorsal root injury was confirmed by other groups, these investigators did not find evidence of axon regeneration [277, 296]. As a result, it has been suggested that OECs promote axon regeneration only in cases of homodirectional OEC and sprout-like axon (SLA) migration [277, 294, 297].

In addition to the above, a number of studies have reported functional recovery promoted by OEC transplants in different types of spinal cord lesions [298-304]. For example, OEC transplantation after electrolytic corticospinal tract lesion at the upper cervical level in rats promoted functional outcome by partially restoring skilled forepaw movement [298] while, after spinal cord contusion injury, OEC transplants promoted sparing of supraspinal axons and improvement of hindlimb function [304]. Overall, however, OEC-mediated functional outcomes, as assessed by behavioural tests, appear to be variable [285, 298, 299, 301, 304-306]. Toft *et al* [303] used electrophysiology for direct measurement of changes in the activity of spinal neurons in response to OEC transplantation after injury and found evidence of increased synaptic activity in the zone of partial preservation compared to that of corresponding sensory afferents in untreated animals [248, 303]. Given that rat motoneurons associated with walking retain considerable autonomic activity when disconnected from supraspinal centres [248], the findings by Toft *et al* [303] indicate that the additional functional outcomes observed after OEC transplantation involve responses in local neuronal networks at spinal levels below the site of

the injury [248]. In addition to promoting functional outcome after spinal cord injury (Figure 1.4), OECs have been reported to increase the dendritic length of sympathetic preganglionic neurons above the injury [307], reduce the duration of autonomic dysreflexia [307], and also mitigate neuropathic pain, including tactile allodynia, and thermal hyperalgaesia [308].

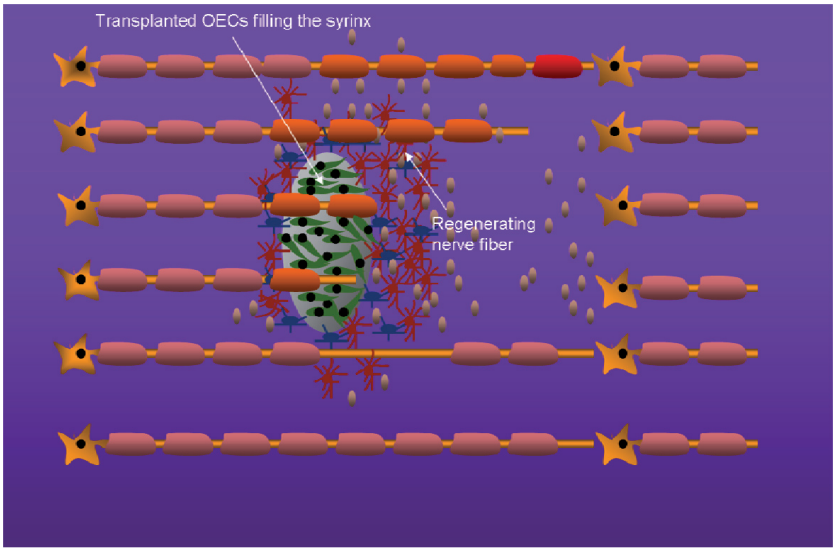
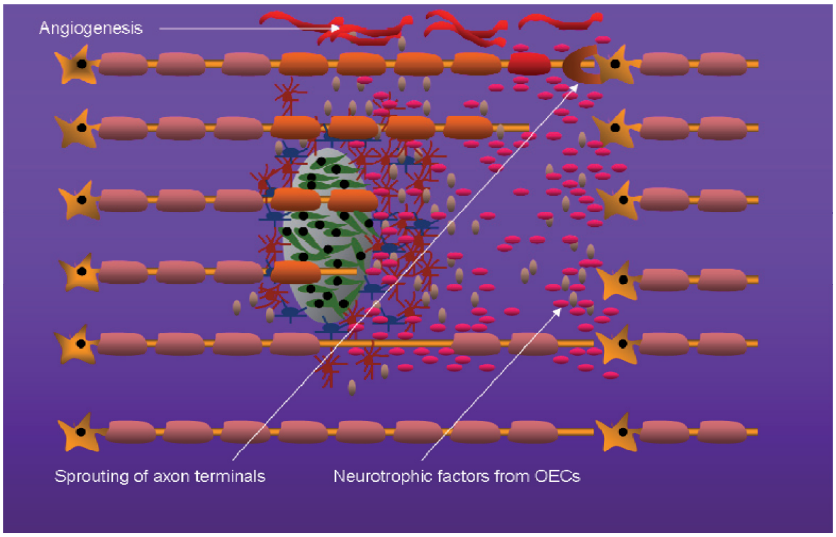


Figure 1.3 – Illustration showing reparative effects of OECs after CNS axotomy. Transplanted OECs fill the lesion cavity and modulate the lesion microenvironment through interactions with astrocytes, while they promote CNS axon regeneration and remyelination.



(I Sandvig - Adapted from Barnett & Riddell, 2007)

Figure 1.4 – Illustration showing axon regeneration and functional recovery promoted by transplanted OECs. OECs secrete soluble factors, stimulate angiogenesis, and provide trophic support to regenerating CNS neurons, leading to re-establishment of lost synaptic connections distal to the injury site.

The reparative potential of OECs has also been demonstrated in different models of brain lesions. OEC showed promising integration and promoted growth of axons in the septal-hippocampal pathway after fimbria-fornix pathway lesion [309], enhanced neuronal plasticity after stroke [310], and restored dopamine innervation and functional deficits in a rat Parkinson's disease model when co-transplanted with ventral mesencephalic cells [311, 312]. Moreover, several *in vitro* and *in vivo* studies showed that OECs play an important role in neuronal support, survival [313, 314], and differentiation [315-317]. Finally, the synergistic effects of OECs and NSCs in mediating CNS axon regeneration and functional recovery have been demonstrated in a number of co-transplantation studies [314, 316, 318]. The above effects are attributed to secretion of a host of different neurotrophic factors and cell adhesion/ECM molecules by OECs, including BDNF, GDNF, N-CAM, claudins, cadherins, and catenins [313-318].

Much of the interest in OECs as reparative candidates in CNS lesions stems from their similarity with non-myelinating Schwann cells and ability to assume a myelinating phenotype and produce peripheral type myelin upon association with large diameter axons [319-325]. As discussed

earlier in this chapter, CNS axotomy induces axon and myelin loss as part of the process of Wallerian degeneration [16]. Additionally, demyelination occurs as a result of oligodendrocyte damage and inflammatory disease, such as MS [320]. Demyelinated axons are characterized by impaired conductivity leading to neurological deficits [320]. While remyelination may occur as part of a spontaneous regenerative response, the myelin sheaths are not fully restored [326] and, in the case of MS and experimental autoimmune encephalomyelitis (EAE), remyelination is not robust enough to mediate significant and/or sustainable functional outcomes [320, 326]. Thus remyelination mediated by cell transplants aims at neuroprotection, and alleviation of functional deficits by restoring myelin sheaths and re-establishing rapid saltatory conduction [320, 326]. Although Schwann cell transplants can remyelinate CNS axons, their myelinating efficacy is often impaired in the presence of astrocytes [320, 327]. Therefore, the distinct ability of OECs to intermingle with astrocytes [268-270, 277] suggested that OECs, rather than Schwann cells, should be utilised in the treatment of demyelinating CNS lesions [248, 320]. A number of studies have demonstrated remyelination of demyelinated CNS axons promoted by OEC transplants in different CNS lesion models [283, 321-324, 328, 334]. However, myelination by OECs is indistinguishable from that mediated by Schwann cells [319-325]. Thus, in the absence of a specific marker for OECs [264-265], these results should be interpreted with caution, especially in cases when Schwann cell invasion into the lesion site cannot be excluded [327]. Furthermore, experimental evidence suggests that the myelinating capacity of OECs can be enhanced in the presence of non-myelinating cell types, such as meningeal cells [320].

Finally, a pertinent issue in OEC-mediated CNS repair is the extent to which the therapeutic interventions described above are clinically translatable. Barnett *et al* [332] identified human OECs (hOECs) with ability to remyelinate demyelinated axons in a rat spinal cord injury model. Other studies have also confirmed the reparative potential of hOEC transplants in experimental CNS lesions in terms of promoting remyelination [334] and reducing the volume of the lesion cavity [335]. A promising role for hOECs has also emerged from the first clinical applications, including a PhaseI/IIa clinical trial [336-338], which demonstrated the feasibility and safety of autologous OEC transplantation in paraplegic patients [337, 338]. However, given the small numbers of patients involved, these results are considered preliminary and are treated with cautious optimism [336-338]. A large scale clinical trial in China has also reported positive outcomes of OEC-based therapies in the treatment of human spinal cord injury [339], however, an independent observational study challenged these findings as it revealed serious inadequacies in terms of application of inclusion-exclusion criteria, correlation of injection sites to the level of injury, reporting of complications, correct identification of cell type for transplantation, and conformity to international standards pertaining to the safety and efficacy

of clinical trials [340]. Moreover, experimental treatment of ALS patients using hOEC transplants in Beijing has come under severe criticism given that follow-up of these patients failed to find evidence of recovery and also identified serious debilitating side-effects of the treatment [341-342]. Despite these highly controversial cases, it is widely recognised that OECs hold great promise as reparative candidates in transplant-mediated CNS repair. Nevertheless, it is generally agreed that further elucidation of the reparative role of OECs in experimental CNS injury models is required before safe translation of experimental therapies into the clinics.

1.3. OECs in transplant-mediated repair of visual pathway lesion

1.3.1. The mammalian central visual system

The central visual system is part of the brain and includes the eye, retina, and retinofugal projection [343, 344]. RGC neuron somata are located in the retina, while RGC axons exit the retina at the optic nerve (ON) head, become myelinated at the lamina cribrosa, and form bundles inside the optic nerve (ON) [343, 344] (Figure 1.5). The ON, which is surrounded by meninges and cerebrospinal fluid (CSF), passes through the orbit, and optic foramen, into the cranial cavity [343, 344]. Immediately rostral to the pituitary gland, the left and right ON decussate at the optic chiasm, and form the optic tracts, which subsequently project to the lateral geniculate nucleus (LGN), superior colliculus, and midline pretectal area [343, 344] (Figure 1.6). Each of these areas mediates visual perception, visually guided movement, and pupillary reflexes by relaying information to the neocortex, motor nuclei in the brainstem and cord, and brainstem ocular autonomic and motor nuclei, respectively [344].

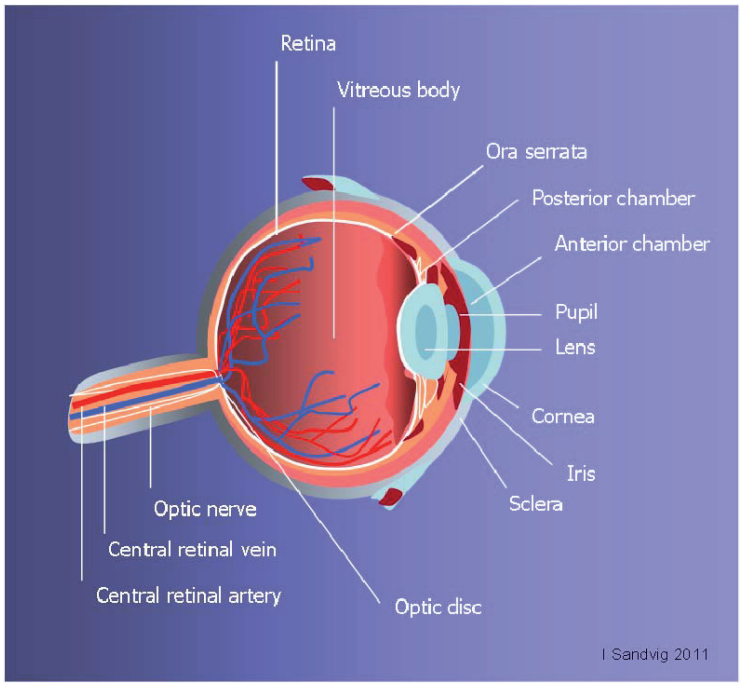


Figure 1.5 – Cross-section of the eye showing key anatomical features..

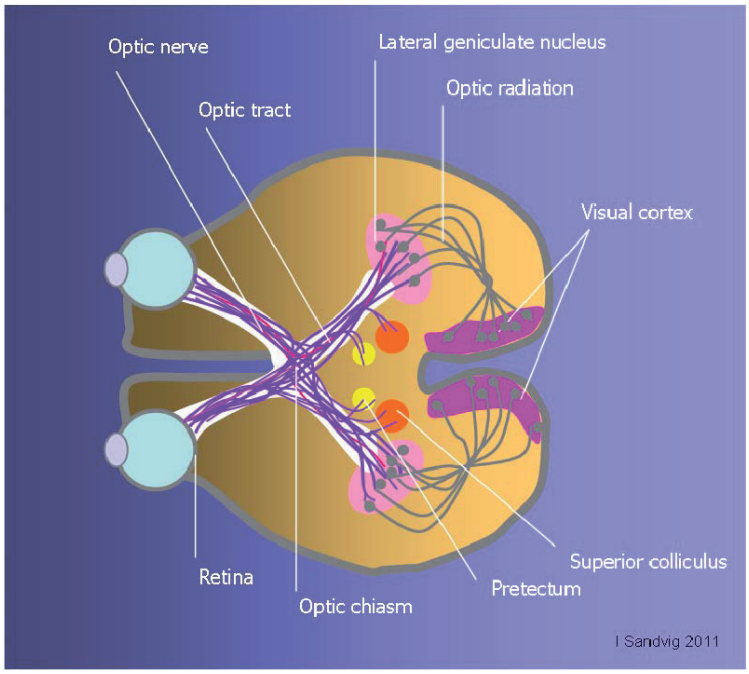


Figure 1.6 – Illustration showing the mammalian visual pathway. In rodents, 90% of RGC axons decussate at the optic chiasm and form the contralateral optic tract.

1.3.2. Visual pathway damage and repair

The rodent visual system is an excellent model for the study of CNS damage and repair, both in terms of clinical relevance, and anatomical and biological properties. Loss or impaired function of RGC neurons results from direct trauma or ischemia, as well as different types of chronic ophthalmic conditions related to diseases such as glaucoma and diabetes [345, 346]. Furthermore, various inflammatory and neurodegenerative diseases often present with demyelinating ON pathologies, for example, optic neuritis is a common symptom in MS [345]. In addition to the above, the special anatomy of the visual pathway, i.e. the exclusively centripetal direction of the RGC axon projection, absence of interneurons, and relative accessibility of the ON, vitreous body (VB), and retina allow targeted therapeutic interventions uncomplicated by inadvertent damage to surrounding neuroanatomical structures [344].

RGC can be axotomised at different distances from the cell body by means of intraorbital or intracranial ON crush (ONC) or transection [148, 347-349]. The cellular and molecular responses to ON injury are characteristic of those observed in other types of CNS lesions [344]. Following intraorbital axotomy, rapid apoptotic RGC death commences at 5-7 days post-injury (dpl) and results in 85-90% RGC loss by 15-20dpl [344, 350-352]. Concomitantly, a host of different factors and signaling cascades undergo dynamic changes, including upregulation of c-jun [353], CNTF receptor α (CNTFRα) [354], trk receptor [355], BDNF [355], GDNF [356], N-CAM [357], and GAP-43 [358], as well as transient upregulation of Sema3A [359], and downregulation of the netrin family receptors DCC, Unc5H1, and Unc5H2 [345, 360]. The glial scar is formed by 8dpl, and subsequently matures and contracts at 8dpl-18dpl [344]. In addition to the above, RGC axons at the proximal ON segment respond to the injury by entering a programme of abortive regeneration, in which sprouting RGC axons do not cross the lesion site and their growth is arrested by 20dpl [344].

Despite the fact that RGC axon regeneration is inhibited in a manner typical of other CNS lesions, the rodent ON injury model is one of the few models where robust axon regeneration has been achieved after therapeutic intervention [81, 135, 143, 144, 344, 361, 362], including activation of inflammatory cytokines [81, 94, 95, 97, 147, 362-364], *ivit* PNG [143, 144, 365], *ivit* administration of neurotrophic factors [135], as well as activation of the mTOR pathway [77-79, 127, 366]. Such strategies aim to promote RGC survival, stimulate axonal sprouting, and disinhibit RGC axon growth by neutralizing inhibitory ligands and signaling cascades, including Nogo, NgR1, epidermal growth factor receptor (EGFR), RhoA, and ROCK [344].

As discussed earlier, the glial scar around the injury site presents a physical and chemical barrier to regenerating CNS axons. Interestingly, though, in regenerating ON injury models in which RGC axons grow beyond the lesion site, anti-scarring treatments may not always be necessary [143, 144, 344]. Recruitment of fibrinogen to the lesion in the acute haemorrhagic phase of CNS scarring, activates RhoA-mediated inhibition of neurite outgrowth and causes growth cone collapse *via* formation of a fibrinogen/integrin/EGFR complex [344, 367]. TGFβ neutralisation reduces scar deposition and inhibits infiltration by meningeal fibroblasts to the lesion site [344], nevertheless, it does not prevent axon growth arrest given that myelin-associated inhibitors and GSPGs are still secreted by reactive glia [50, 344]. However, as demonstrated by Berry *et al* [143, 144], in regenerating ON lesion models fibrotic scarring is significantly reduced, even when fibrinogen influx is not inhibited. This suggested that regenerating RGC axon growth cones modulate their microenvironment by secretion of matrix

metalloproteinases (MMPs) which, in turn, induces MMP secretion by ON glia [132, 344, 368], thus disinhibiting axon growth and blocking migration of meningeal fibroblasts [344, 368]. It follows that, as long as a therapeutic strategy can promote robust RGC axon regeneration, which can effectively regulate growth inhibition, additional anti-scarring treatments may be redundant [344].

1.3.3. OECs in visual pathway repair

As discussed earlier, central visual pathway damage and repair are determined by the same mechanisms involved in degeneration and regeneration of other types of CNS lesions. As a consequence, successful strategies for visual pathway repair should not only address the issue of RGC neuron survival, but should also aim to induce RGC axons to enter a rigorous growth state, promote their growth through the inhibitory lesion microenvironment, direct them towards the right synaptic targets, remyelinate them and, ultimately, restore synaptic function. Key attributes of OECs render them particularly interesting in this context. These include: (i), secretion (natural and engineered) of a multitude of neurotrophic factors, such as BDNF, GDNF, CNTF, and NT4/5 [233, 285, 286, 317, 346]; (ii), superior ability to intermingle with astrocytes and meningeal cells and to modulate CSPG expression [268-278]; (iii), production of MMP2 [370] and expression of cell adhesion and ECM molecules [236-241]; (iv), remyelinating capacity [261, 262, 283, 321-324, 328, 334]; and (v), ability not to interfere with retinal target recognition [346, 371, 372].

Repair of visual pathway lesions using OEC transplants is an emerging field in neuroscience. As a result, there are currently only few studies that have explored the reparative potential of OECs after RGC axotomy. Li *et al* [373] transplanted a mixture of OECs and olfactory nerve fibroblasts (ONF) embedded in a matrix into the lesion site of the transected rat ON and showed RGC axon growth 10mm into the distal stump. In a later study, the same group reported myelination of the damaged ON by Schwann cells, but not by OECs [374]. Wu *et al* [375] showed improved survival of axotomised RGC at 7dpl after intraoptic OEC engraftment, however, they did not find evidence of improved RGC survival at 14dpl. Furthermore, Liu *et al* [376] reported long-distance RGC axon regeneration and functional recovery at 8 weeks post-lesion (wpl) after combined treatment with OECs and human recombinant GDNF. Finally, Plant *et al* [346] transplanted purified OECs transduced with LV-CNTF at 5dpl after ONC injury and showed RGC axon growth beyond the lesion site at 7wpl, but found no surviving OECs in the ON at the specific time point.

While these findings are very important in highlighting the potential and, perhaps, the limitations of OECs as mediators of visual pathway repair, it is clear that the relative novelty of the specific line of research provides amble opportunity for further investigation for the purpose of eliciting key mechanisms of OEC-mediated recovery, optimizing/controlling the viability and function of transplanted OECs *in situ*, and developing therapeutic strategies that can be translated into clinical applications for the treatment of different types of CNS lesions in the future.

1.4. Monitoring transplant-mediated CNS repair by MRI

A pertinent issue in translational neuroscience is the ability to monitor anatomical, biochemical, molecular, and functional changes mediated by therapeutic interventions in a serial, non-invasive manner. Magnetic resonance imaging (MRI) is highly-relevant in this context.

1.4.1. Manganese-enhanced MRI (MEMRI)

MEMRI utilises the paramagnetic properties of manganese ions (Mn^{2+}), which cause a strong attenuation in the T_1 spin-lattice relaxation time constant of water protons and thus produce positive contrast in areas where Mn^{2+} are accumulated when T_1-weighted imaging is applied [377-379]. Furthermore, MEMRI utilises the fact that Mn^{2+} enter excitable cells through L-type voltage-gated Ca^{2+} channels [380, 381], as well as other Ca^{2+} transport routes, including the Na^{2+}/ Ca^{2+} exchanger, the Na^{+}/ Mg^{2+} antiporter, and the active Ca^{2+} uniporter in mitochondria [382, 383]. Upon entry into the cell, Mn^{2+} are sequestered in the endoplasmic reticulum and subsequently transported along axonal microtubules to the synaptic cleft, where they are released and taken up by the next neuron, traversing the post-synaptic membrane through voltage-gated Ca^{2+} channels [378, 384, 385].

As a result of the above properties, Mn is an excellent contrast agent in translational neuroimaging rendering MEMRI a powerful, versatile tool for non-invasive *in vivo* imaging of brain cytoarchitecture and activity and for tracing axonal projections in studies of neuronal connectivity [386, 387]. Hence, MEMRI has been used for mapping the mature and developing brain in rodents and non-human primates [388-395], while activation-induced MEMRI (AIM-MRI) has been applied in a number of studies as a technique for imaging activity in the brain [396-405]. In addition to the above, utilisation of Mn^{2+} as an *in vivo* neuronal tract tracer enables application of the MEMRI technique for imaging the visual, olfactory, auditory, and somatosensory pathways in a variety of species [384, 385, 406-415].

For the purpose of such studies, aqueous solutions of $MnCl_2$ are administered systemically or directly into the vicinity where the target neuronal population resides. After administration to neuronal somata, Mn^{2+} are transynapticaly relayed to the specific neuronal circuit *via* kinesin-mediated anterograde transport mechanisms [416]. However, MEMRI may also exploit dynein-mediated retrograde axonal transport of Mn^{2+} [416, 417]. Although the exact Mn^{2+} transport

mechanisms require further elucidation, it is established that Mn^{2+} movement within neurons is *via* active transport, rather than passive diffusion [407].

1.4.2. MEMRI of the visual pathway

Given its neuroanatomical features, the central visual system constitutes an excellent model for MEMRI application. Mn^{2+} uptake by RGC is effected by *ivit* $MnCl_2$ injection. Upon entry into RGC somata, Mn^{2+} are anterogradely transported within RGC axon microtubules, transynapticaly relayed to tertiary neurons at the LGN, and subsequently to the primary visual cortex, thus enabling visualisation of the entire normal visual projection [407, 408, 413, 418-420]. Importantly, MEMRI also enables longitudinal *in vivo* monitoring of damaged and regenerating RGC axons [406, 408, 421-423], as well as studies of comparative physiology between regenerating and non-regenerating species [424], and is thus a powerful tool for assessing the efficacy of therapeutic intervention strategies, including transplant-mediated repair, in experimental models.

One of the main caveats in such investigations, however, is the risk of Mn^{2+}-induced toxicity as a result of repeated/high doses of $MnCl_2$ [425]. Thuen *et al* [407] demonstrated that *ivit* doses of 150-300nmol $MnCl_2$ are safe, yield optimal RGC axon enhancement, while they may also have a neuroprotective effect on RGC neurons. Furthermore, Olsen *et al* [419] showed that RGC axon contrast enhancement is contingent on prolonged availability of *ivit* Mn^{2+} for uptake by RGC neurons, rather than high dosage of $MnCl_2$. This suggested that manipulations, for example by use of biomaterials, which enable controlled release of *ivit* Mn^{2+} should achieve the dual purpose of optimising contrast enhancement and minimising the risk of Mn^{2+}-induced cytotoxicity. Alternatively, fractionated doses of $MnCl_2$ [426, 427] or dendritic manganese chelates [428] may be viable options, however, their utility in the visual system model remains to be tested. Considering that MEMRI is unlikely to be used for monitoring axon regeneration in human patients receiving regenerative treatments, it is envisaged that the knowledge acquired from modifications of the MEMRI technique in the rat visual system, will enable the development of clinically applicable derivatives, which circumvent the issue of Mn^{2+}-induced cytotoxicity.

1.4.3. Non-invasive *in vivo* imaging of cell transplants

Of central importance in studies of transplant-mediated repair is the ability to verify the location of transplanted cells, resolve their integration with host tissues, and monitor their temporospatial migration non-invasively *in vivo*. An array of technologies is currently available for such investigations, including optical, acoustical, and nuclear imaging, as well as MRI [429, 430]. Each method has advantages and disadvantages. For example, bioluminescence imaging enables *in vivo* tracking of transplanted cells and direct correlation between bioluminescent signal strength and number of live cells in the tissue, thus providing information about cell survival post-transplantation [429, 431, 432]. However, signal loss as a result of increasing tissue depth is a major limitation of the technique [429]. Cell graft rejection can also be imaged by ultrasound using, for example, phospholipid-based, perfluorobutane-filled microbubbles targeted to intracellular adhesion molecule-1 (ICAM-1), a marker for endothelial inflammatory cells, as demonstrated in a cardiac transplantation model [429, 433]. Nonetheless, this method does not allow quantification of transplanted cell death and is further limited by the fact that the ultrasound technique can only be applied to relatively small regions of interest, rather than for whole body imaging [429]. Nuclear imaging, including positron emission tomography (PET) and single-photon emission computed tomography (SPECT), allows imaging functional effects [434] and biodistribution of transplanted cells [429, 434-436]. While simultaneous detection of two probes for parallel assessment of cell graft function and host-tissue integration is possible using dual pinhole SPECT, the scope of this technique is confounded by the short temporal resolution of exogenous labels [429, 437, 438]. Moreover, potential radiotoxic, dilution, and leakage effects of radioligands on live tissues need to be thoroughly investigated [429].

1.4.4. Imaging cell transplants by MRI

Due to its superior spatial resolution, MRI is one the most widely applied technologies for non-invasive *in vivo* monitoring of transplanted cells. Visualisation of transplanted cells by MRI requires labelling of the cells with intracellular, or extracellular contrast agents. A variety of such agents are available including (i), bifunctional agents, such as gadolinium rhodamine dextran (GRID), which enable cell detection by MRI and identification in histological sections [439-441]; (ii), convertible contrast agents (based on MnO, MnO_2, Mn_3O_4, and $MnCO_3$), which switch from T2*-weighted contrast to T1-weighted contrast upon molecular dissolution inside lysosomes and endosomes [442]; and (iii), different types of metallic particles, including gold nanoparticles (Au_3Cu_1), aptamer-modified nanoparticles, and various types of functionalized derivatives, which enable delivery of antibodies, ligands and drugs to target tissues [443-448].

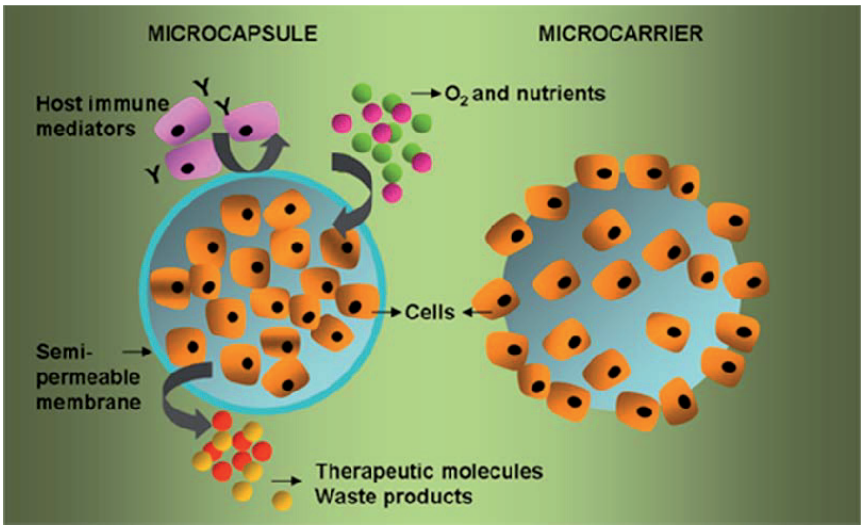
One of the most widely used classes of intracellular contrast agents comprises different types of ferumoxide-based microparticles/nanoparticles and their modified derivatives. These include poly(lactide-co-glycolide) (PLGA)-coated [449], poly(N,N-dimethylacrylamide) maghemite (PDMAAm)-coated [450], and $_{D}$-Mannose-modified nanoparticles [451] with ferumoxide cores, superparamagnetic iron oxides (SPIO), ultra-small paramagnetic iron oxides (USPIO) [450-463], and micron-sized particles of iron oxide (MPIO) [464-466]. Although some of these contrast agents have been approved for clinical use, for example, Endorem™, Feridex® (SPIO), and Sinerem®, Combidex® (USPIO), intracellular uptake of these particles by means of endocytosis often requires long incubation times, while it also necessitates surface modification or conjugation with transfection agents [455]. Another consideration is that for single-cell detection by MRI, which may be desirable in transplantation studies that involve monitoring of the temporospatial migration of transplanted cells, very large numbers of SPIO/USPIO are required to achieve sufficient intracellular iron uptake for visualisation by MRI. This suggests that the application of these agents carries the inherent risk of intracellular iron overload, with associated cytotoxic effects and potential interference with the cells' function and migratory capacity post-transplantation [467]. Some of these practical limitations can be overcome by using MPIO, which have a higher magnetite content than SPIO/USPIO and can thus achieve better iron loads using significantly less contrast agent [466, 467]. However, a major caveat in the use of intracellular magnetic labels is that imaging artefacts deriving from secondary uptake of the label by *in situ* macrophages cannot be excluded. As a consequence, correlation of MRI findings with those of histological/histochemical methods is imperative, as it adds interpretive acumen to the relevant investigations by evaluating the rigour of MRI data within an appropriate biological context.

In addition to the above, the emergence of new classes of nonmetallic chemical exchange saturation transfer (CEST) agents has been met with great interest. The specific contrast agents applied alone, or in combination with ferumoxide agents, enable simultaneous imaging of multiple cellular and molecular targets [468-471] and are thus extremely important for non-invasive monitoring of the effects and function of cell transplants *in vivo*. Finally, the evolution of other novel MRI-based methodologies, such as frequency-labelled exchange (FLEX) transfer [472], can be expected to be a driver for the development of sophisticated, versatile contrast agents which will elucidate dynamic changes in specific genes and proteins in response to cell transplants.

1.5. Biotechnology and nanotechnology in transplant-mediated repair

1.5.1. Biomaterials

A pertinent issue in transplant-mediated CNS repair is optimisation of cell graft function and survival by mitigating the deleterious effects of host immune responses post-transplantation [473]. Equally important is the ability to direct the fate/differentiation of transplanted cells *in situ*, enhance their therapeutic potential, for example, by graft functionalisation and controlled expression of genes and proteins for targeted delivery *in vivo*, and to provide regenerating CNS axons with molecular and structural support to promote directional growth towards appropriate synaptic targets. Such interventions are made feasible through advances in biomaterials science and nanotechnology, which allow manipulation of a multitude of natural and synthetic biomaterials for tailor-made applications in experimental models, as well as in regenerative medicine and tissue engineering. Examples include microcapsules/microcarriers for immunoisolation and delivery of cells and genes to specific tissues [473-480], as well as biomimetic matrices with ECM properties, which act as biodegradable scaffolds for bridging CNS lesion cavities [473, 474] (Figure 1.7).



I Sandvig – Adapted from Su et al. 2009

Figure 1.7 – Microcapsules and microcarriers can be utilised for cell transplantation. The semi-permeable membrane of microcapsules protects encapsulated cells from immune responses in the host microenvironment. Microcapsules allow entry of O_2 and nutrients into the capsule while they enable diffusion of therapeutic molecules and waste products out of the capsule. Microcarriers, on the other hand, support cell attachment to the surface of the capsule and can be utilised for scaffolding of CNS lesions.

Although the choice of type and form of biomaterials is dictated by the specific aims of the therapeutic approach in each case, desirable properties include biocompatibility, mechanical stability, permeability, and ease of handling [476]. Representative examples of applications of different types of biomaterials in regenerative neuroscience models include (i), attachment of NSCs to modified PLGA particles and transplantation in stroke-induced cavities [481, 482]; (ii), transplantation of hydrogel matrices seeded with engineered fibroblasts to promote ON axon growth after intracranial ON lesion [483]; (iii), use of a self-assembling nanofibre peptide scaffold (SAPNS) to promote ON regeneration in chronic optic tract lesion [423]; (iv), use of poly(D,L)-lactide matrices for stem cell tissue engineering [484] and for co-transplantation of OECs and ONF to stimulate axon regeneration after spinal cord hemisection [293]; and (v), transplantation of mesenchymal stem cells seeded in modified hydrogel matrices to treat chronic spinal cord lesions [485].

1.5.2. Tailor-made alginate matrices

Alginates are naturally occurring polysaccharides, comprised of unbranched copolymers of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) [486, 487], which are found in algae [488] and certain types of bacteria [489, 490]. Alginates are promising candidate biopolymers for cell encapsulation as a result of their ionotropic properties [491] and the fact that they lend themselves to structural and compositional modification for tailored applications through the conversion of M-residues to G-residues with the use of C-5 epimerases [492-496]. The specific arrangement of G- and M- monomers in the polymer chains, and the type of divalent cation used for ionotropic gelation, such as Ca^{2+}, Ba^{2+}, and Sr^{2+} in the gelling solution, determine the strength, stability, syneresis, and permeability of the alginate microbeads and microcapsules [497-499]. In addition to the above, further modification of the alginates can be effected by engraftment of functional peptides, such as arginine-glycine-aspartic acid (RGD) [500]. Such manipulations aim to ascribe specific functional properties to the alginates by mimicking ECM conditions, which are expected to promote cell adhesion, survival, and differentiation post-transplantation [500].

The biocompatibility of different types of alginate hydrogels for immunoisolation of cell transplants has been studied with promising results [499-504]. Equally promising are specific applications of modified alginate matrices in neurotransplantation and CNS repair, including the use of alginate hydrogels for differentiation of neural stem cells [505-510], promotion of neurite outgrowth *in vitro* [511], as well as directed outgrowth of regenerating CNS axons *in vivo* [512]. Furthermore, the ability to modulate alginate hydrogel properties such as mechanical strength and stability suggests that alginates may have additional roles in CNS repair studies, such as utilisation for controlled release of MRI contrast agents for tracing of regenerating CNS axons *in vivo*. Clearly, the functional attributes of the alginates in each case should be determined by the type of CNS lesion, the specific reparative or imaging approach and, importantly, the type of cell(s) used to mediate repair. It is therefore imperative to thoroughly test and develop alginate matrices tailored to the specific requirements of individual intervention strategies.

2. Overview of methods

This section describes the key methodology involved in the papers that comprise this thesis. A detailed description of the rest of the methods used is provided in the Materials and Methods section of each paper.

Table 1. Overview of key methodology presented in this thesis

	Method	**Paper**
1.	OEC purification and culture	I, II, IV
2.	Intracellular labelling of OECs with MPIO	I, II
3.	Optic nerve crush injury	II
4.	*Ivit* and *iON* OEC engraftment	II
5.	*Ivit* $MnCl_2$ injections	II
6.	*In vivo* and *in vitro* MRI	
6.1.	T_2*/T_2-weighted imaging	I, II
6.2.	3D T_1-weighted imaging	II, II, III
6.3.	2D T1-weighted imaging	I, III
7.	Formation of Mn-alginate beads	III
8.	RGD peptide coupling to alginates	IV
9.	Epimerisation of alginates	III, IV
10.	Cell encapsulation in alginates	IV

2.1. OEC purification and culture

The OECs utilised in this thesis were purified from neonatal Fischer rats as described by Barnett and Roskams [516]. Briefly, the olfactory bulbs of 4-5 P7/P8 rats were finely chopped, enzymatically digested in L-15 (Leibovitz) medium (Sigma), and triturated through a 26 gauge needle. Dissociated cells were incubated in a cocktail of the O4 (IgM at 1:4) and anti-galactocerebroside (IgG3 at 1:2.) primary antibodies, followed by their fluorochrome conjugated class-specific secondary antibodies. After rinsing, dissociated cells were incubated in goat anti-mouse IgM phycoerythrin and goat anti-mouse IgG3 fluorescein secondary antibodies (1:100, Southern Biotech). Fluorescence-activated cell sorting (FACS, Vantage Becton Dickenson) was used for OEC purification by selecting for galactocerebroside-negative and O4-positive cells. OECs were subsequently cultured in Dulbecco's Modified Eagle's Medium (DMEM GlutaMAX; Sigma) with 1.25% gentamicin (Sigma) and 5% FBS (Autogen Bioclear) on 13µg/ml poly-L-lysine- (PLL) (Sigma) coated cell culture flasks (Corning). The cultures were supplemented with 500ng/ml fibroblast growth factor 2 (FGF2) (Peprotech, London, UK), 50ng/ml heregulin (hrgβ1) (R&D Systems Europe Ltd, Abingdon, UK), and 10^{-6} M forskolin (Sigma). OECs were passaged at confluence. Purity of the OECs populations was assessed by $p75^{NTR}$ specific labelling and was always 98-100%.

2.2. Intracellular labelling of OECs with MPIO

MPIO with a diameter of 0.96µm (Bangs Laboratories, US) were used for intracellular labelling of OECs. The specific MPIO are comprised of a COOH-modified styrene-divinyl benzene inert polymer with a Fe_3O_4 magnetic core (27.8% w/w magnetite content) and a fluorescent label (Dragon Green; ex480, em520) (MC05F/8112; density 2.06g/cm^3, 1% solids, $1.278x10^{10}$ beads/ml; Bangs Laboratories, Inc, Fishers IN, US). After washing with PBS, the MPIO were resuspended in PBS or OEC media and added to the OEC cultures. Assessment of labelling efficiency was based on adjustment of the following parameters: (i), MPIO label concentration, expressed as number of MPIO particles per cell; minimum MPIO:OEC=10:1, maximum MPIO:OEC=100:1; (ii), degree of confluence of the OEC cultures, starting with 100% and reducing to 50%, (iii) duration of incubation, starting with 18h and reducing to 6h. After labelling, the media were removed by aspiration and the cultures extensively washed with PBS, before addition of new media.

2.3. Optic nerve crush injury

For ONC operations, inbred female Fischer rats (110-115g) were anaesthetised with subcutaneous injection of 0.4ml/100g of 2:3:3:4 mixture of Haldol/Midazolam/Fentanyl/sterile water respectively, under sedation with 4% isoflurane in 3% O_2. ONC was performed according to Berry *et al* [143]. Briefly, after intraorbital exposure through a superior palperbal incision, the dural sheath of the ON was incised longitudinally and the ON crushed for 10s, 2mm caudal to the lamina cribrosa using microforceps (AgnTho's AB, Lidingö, Sweden). Care was taken not to disturb the central retinal artery running inside the dural sheath along the ON. Post-surgical analgesia was maintained with subcutaneous injections of 1.667µl/g of 1:10 Buprenorphin (Tegmesic®, Schering-Plough, Brussels, Belgium) in sterile water. Animals were kept in a 12:12 artificial light-dark cycle and fed *ad libitum*.

2.4. *Ivit* and *iON* OEC engraftment

For *ivit* OEC engraftment, a 3µl suspension of $2.5x10^5$ OECs was injected acutely post-ONC into the left eye of anaesthetised animals, immediately posterior to the ora serrata. The same suspension volume and OEC concentration were used for *iON* injections, 1mm proximal to the injury, i.e. between the lamina cribrosa and the ONC lesion site. A pulled-glass capillary micropipette with a tip diameter of ~0.1-0.2mm was used for the injections. To minimise reflux, the micropipette tip remained inside both the vitreous body (*ivit* groups), and the ON (*iON* groups), for a few seconds, before being slowly withdrawn.

2.5. *Ivit* $MnCl_2$ injections

An aqueous solution of 3µl of 150nmol $MnCl_2$ was injected into the vitreous body of the left eye of anaesthetised animals, immediately posterior to the ora serrata, in the same manner as for *ivit* OEC engraftment above.

2.6. *In vivo* and *in vitro* MRI

MRI was performed on a 7T Bruker Biospec Avance 70/20 (Bruker Biospin MRI, Ettlingen, Germany) small animal scanner. A 72mm volume resonator was used for RF transmission and an actively decoupled (i), mouse head surface coil (MPIO OEC phantom study), and (ii), rat head surface coil (*in vivo* experiments) for RF reception. No surface coil was used for the *in vitro* experiments involving Mn-alginate beads.

2.6.1. T_2*/T_2-weighted imaging

For T_2*-weighted imaging, a multi-gradient echo (MGE) sequence was used with the following parameters: TR=1000ms, TE=5, 10, 15, 20ms, flip angle 30%, FOV=2.8x2.5cm^2, matrix=224x200, pixel resolution=125x125μm^2/pixel, number of slices=10, slice thickness=0.70mm (no gap), and an acquisition time of 16m40s with 5 averages.

For T_2-weighted imaging, a rapid acquisition with relaxation enhancement (RARE) spin echo sequence was applied with the following parameters: TR= 2000ms, TE=12, 36, 60ms, FOV=2.80x2.50cm^2, matrix=224x200, pixel resolution 125x125μm^2/pixel, number of slices=20, slice thickness=0.70mm (no gap), and an acquisition time of 16m40s with 5 averages.

2.6.2. 3D T_1-weighted imaging

For *in vivo* T_1-weighted imaging, a 3D FLASH sequence was used with the following parameters: TR=12ms, TE=3.5ms, FOV=3.5x2.2cm^3, acquisition matrix=224x192x141, voxel resolution 156x156x156 μm/voxel; 12 averages were obtained and the acquisition time was 33min 38s.

To correct for the gradually reduced RF signal detected by the surface coil, two additional T1-weighted 3D FLASH sequence scans were performed for *in vivo* MRI using coupled and single coil operations, respectively, with the same sequence parameters as in 2.5.3. above, but with reduced resolution and with an acquisition time of 2min 3s/scan.

2.6.3. 2D T_1-weighted imaging

For imaging release of Mn^{2+} from alginate beads in vitro, the following sequence was applied:
2D MSME evolution scanning: TE.=8.1ms, TR=500ms, FOV=30x40mm, matrix=128x64, slice thickness=1mm, NEX=1, 6 frames per h over 24h.

2.7. Formation of Mn-alginate beads

Five different alginate samples were used in the study of Mn^{2+}/alginate beads: two commercially available alginates and three alginates of extreme composition: (i), High-G alginate from *Laminaria hyperborea* (67% G, intrinsic viscosity 620 ml/g) and (ii), high-M

alginate from *Macrocystis pyrifera* (40% G, intrinsic viscosity 820 ml/g) were obtained from FMC biopolymer and Sigma Chemicals, respectively. (iii), A polymannuronan alginate (polyM, 0% G, intrinsic viscosity 800 ml/g) was produced by an epimerase-negative mutant ($AlgG^-$) of *Pseudomonas fluorescens* (28). (iv), A strictly alternating (polyMG) alginate (46% G, 0% GG, intrinsic viscosity 700 ml/g), and (v), a polyguluronan alginate (polyG, 88% G, intrinsic viscosity 1150 ml/g) were produced by epimerising the bacterial polyM alginate with the C-5 epimerases AlgE4 and AlgE6, respectively. An electrostatic bead generator was used to form alginate beads by dripping a 1.8% (w/v) solution of Na-alginate (filtrated through 0.8 µm filters, dissolved in ion free water) into gelling solutions containing divalent cations, i.e. (a), 0.1 M $MnCl_2$ + 1 mM $BaCl_2$, and (b), 0.1 M $MnCl_2$ + 10 mM $CaCl_2$.

2.8. RGD peptide coupling to alginates

RGD peptide was coupled to alginates using carbodiimide chemistry. 3.5g-5g of purified mannuronan were dissolved in 1% phosphate buffer saline (PBS) solution overnight. EDC was added to the solution at 1:20 molar ratio to the uronic acid monomers of the alginate. Sulfo-NHS was added as a co-reactant at a 1:20 molar ratio to the EDC and incubated for 2 hours (h). The peptide was then added at a concentration of 100µmol/ml, as described by Rowley *et al* [500] and allowed to react for 20h before the alginate solution was purified through extensive dialysis against 3 shifts of 50mM NaCl, and against distilled water, until the water measured <2µs. The alginates were subsequently freeze dried and stored at 4°C.

2.9. Epimerisation of alginates

Alginate consisting of G- and MG-blocks was made by epimerization using the C-5 epimerases AlgE4 (produced in *Hansenula polymorpha*) and AlgE6 (produced in *Eschericia coli*). To produce MG alternating alginate, AlgE4 enzyme was added to alginate at a 1:200 ratio in a mixture of 0.25% (w/v) alginate, 50mM MOPS, 2.5mM $CaCl_2$ and 10mM NaCl and incubated for 24h-48h at 37ºC. To produce alginates with a G-content of 50% and 70%, AlgE6 enzyme was added to MG alternating alginate at a ratio of 1:20 in a mixture of 0.25% alginate (w/v) and 50mM MOPS for 2.5h and 24h, respectively. To eliminate potential toxins, the epimerized alginates were purified through a carbon filter (PALL Corporation).

2.10. Cell encapsulation in alginates

2% (w/v) alginate solutions were prepared from peptide alginates and control alginates by mixing with 0.3M D-mannitol (VWR) in sterile water. 2% alginate solution and OEC suspension were mixed in a 10ml syringe at a final concentration of $1.5x10^6$ cells/ml in 1.8% alginate. Beads with encapsulated cells were formed by dripping the alginate solutions into a gelling bath containing 50mM $CaCl_2$, 0.15M Mannitol and 10mM MOPS buffer in 1L sterile endotoxin-free water using an electrostatic bead generator with electrostatic potential difference 7kV, needle size of 0.35mm outer diameter, and flow at 10ml/h. Beads containing encapsulated OECs/myoblasts were transferred to $75cm^2$ cell culture flasks containing appropriate culture media and incubated at 37ºC with 7% (for OECs) and 5% CO_2 (for myoblasts), respectively.

3. Aims

The aim of this project was to integrate MRI and biomaterials in the study of visual pathway repair mediated by olfactory ensheathing cell (OEC) transplants.

Key objectives include:

(i) Development of customised labelling protocols for efficient labelling of OECs with micron-sized particles of iron oxide (MPIO).

(ii) Combining cellular MRI and manganese-enhanced MRI (MEMRI) for monitoring intravitreal (*ivit*) and intra-optic nerve (*iON*) OEC grafts and damaged/regenerating retinal ganglion cell (RGC) axons after visual pathway lesion.

(iii) Testing the potential of *ivit* and *iON* OECs as mediators of visual pathway repair.

(iv) Producing and testing modified alginates as systems for controlled release of Mn^{2+} for MEMRI

(v) Producing and characterising arginine-glycine-aspartic acid (RGD)-peptide alginates and testing their interactions with OECs *in vitro* with a view to developing suitable matrices for future *in vivo* application.

4. Overview of papers

4.1. Paper I - Labelling of olfactory ensheathing cells (OECs) with micron-sized particles of iron oxide (MPIO) and detection by MRI

The aim of this study was to test the suitability of MPIO, a widely applied class of T_2* contrast agents, for intracellular labelling of OECs for detection by MRI *in vitro* and *in vivo*. We defined labelling efficiency in terms of (i), percentage of labelled OECs, and (ii), intracellular iron uptake, and proceeded to develop a tailored labelling protocol that can achieve both objectives without compromising OEC viability, proliferation, and migration capacity for application in future investigations of OEC-mediated repair after visual pathway lesion in rats monitored by MRI.

We found that OECs avidly endocytose MPIO, achieving a labelling efficiency of >90% with incubation times as short as 6h, while the intracellular MPIO uptake was contingent on MPIO label concentration, yielding intracellular iron loads between 1.90pg and 13.80pg. No adverse effects of the MPIO label in terms of cytotoxicity, altered cell morphology, proliferation and migration pattern in culture, were observed up to 96h post-labelling. Furthermore, MPIO-labelled OECs were resolvable by MRI at 7T *in vitro*, and also *in vivo*, after engraftment in the vitreous body of adult rats.

This study provides the first detailed protocol for safe, efficient labelling of OECs with MPIO for non-invasive imaging by MRI in conjunction with studies of CNS repair mediated by OEC transplants.

4.2. Paper II - *In vivo* MRI of olfactory ensheathing cell grafts and regenerating axons in transplant-mediated repair of the adult rat optic nerve

A key aim of this study was to combine cellular MRI and MEMRI to monitor OEC transplants and regenerating RGC axons *in vivo*, after visual pathway lesion in the adult rat, and to test whether MEMRI is sensitive enough to detect potential axon regenerative effects of OEC grafts.

We found that the T_1-weighted 3D FLASH sequence applied for MEMRI facilitates simultaneous visualisation of Mn^{2+}-enhanced regenerating retinal ganglion cell (RGC) axons and MPIO-labelled OEC grafts. Furthermore, the MEMRI technique was sensitive enough to detect axon regenerative responses to the *iON* grafts at 20dpl. However, the CNR profiles of animals with *iON* MPIO-labelled OEC transplants were partially distorted due to susceptibility effects of the MPIO proximal to the lesion. Ultrastructural analysis of tissue obtained at 40dpl revealed robust axonal sprouting and surviving *iON* OECs proximal to the lesion site. Importantly, formation of myelin of peripheral appearance proximal to the lesion site suggested a remyelinating role for *iON* OEC transplants. Finally, the ultrastructural study revealed secondary uptake of MPIO by macrophages and degradation of the MPIO label at >40dpl.

The specific study demonstrated the applicability of MEMRI as a tool for monitoring repair of the rat visual pathway mediated by OEC transplants in terms of visualising MPIO-labelled OEC grafts and regenerating RGC axons, as well as detecting axon regenerative responses to OEC transplants. Being the first study to combine the application of two different contrast agents in the rat visual pathway lesion model, the study also revealed some of the methodological challenges involved in the specific MR imaging approach. Furthermore, the evidence of secondary uptake of the MPIO label by macrophages constitutes a reminder of the fact that relevant artefacts in such investigations cannot be excluded. Finally, the suggestion of a remyelinating role for *iON* OECs after ONC injury is a very promising finding, but in the absence of supplementary data to unequivocally confirm this observation, the specific result is regarded with cautious optimism.

4.3. Paper III - Mn-alginate gels as a system for controlled release of Mn^{2+} in manganese-enhanced MRI

The aim of the study was to evaluate alginate beads of different composition in combination with different cross-linking divalent ions as systems for controlled release of Mn^{2+} *in vitro* and to provide proof of principle for potential application of such systems *in vivo*, by testing the utility of controlled release of Mn^{2+} from *ivit* Mn-alginate beads for MEMRI of the normal rat visual projection.

We utilised different types of commercially available and modified alginates to form Mn-alginate beads and imaged the release of Mn^{2+} from the Mn-alginate beads *in vitro*, as well as *in vivo*, after unilateral *ivit* injection in rat, using a 7T MR scanner. Compartment model simulation and analysis found that the time constant (τ_1) that represents the rate of release of Mn^{2+} from the alginate beads greatly varied between the different types of alginates and had values between 600min and 100min, corresponding to the order high-G ≥ high-M > polyMG, as well as Ba^{2+}>Ca^{2+}. This means that reductions of *ivit* Mn^{2+} concentration up to 85% can be effected by selective adjustment of alginate composition and choice of cross-linking divalent ions.

These findings are highly significant as they demonstrate the feasibility of designing and utilising alginate-based systems for controlled release of Mn^{2+}, which enable optimal contrast enhancement and potentially circumvent the issue of Mn^{2+}-induced toxicity. Furthermore, the pliability and versatility of alginates indicate that such systems can be used to adjust Mn^{2+} uptake, transport and clearance to the requirements of specific neuroanatomical and biophysical properties of the system under investigation, thus enabling the refinement of the MEMRI technique through the development of sophisticated derivative approaches.

4.4. Paper IV - Effects of RGD-peptide modified alginates on olfactory ensheathing cells and myoblasts

The aims of the study were to produce RGD-peptide alginate capsules of different flexibility, characterise the modified alginates, test their effects on OECs in terms of cell survival, cell-cell, and cell-matrix interactions using both 3D and 2D cultures, and compare these interactions with those of myoblasts using the same culture substrates.

Chemoenzymatic modification enabled RGD peptide coupling to the alginate by first introducing RGD peptide to mannuronan and subsequently introducing MG- and G-blocks using C-5 epimerases, thus preventing interference with the G-blocks as a result of peptide coupling. Analysis using NMR spectroscopy determined the degree of RGD-peptide coupling, alginate composition, as well as downstream processes on the RGD-coupled alginates. 2D cultures of OECs and myoblasts revealed dynamic responses to the RGD-coupled alginates in terms of altered morphology and adhesion of the cells to the alginate substrate by extension of processes. Encapsulation in RGD-coupled alginates did not promote survival and/or differentiation of the cells.

The present study demonstrates the utility of our protocol for alginate modification and characterisation, however it does not confirm improved OEC or myoblast survival in response to RGD-peptide modified alginates. While RGD-coupled alginates induced dynamic responses to OECs and myoblasts in terms of cell-cell, cell-matrix interactions, encapsulation in RGD-coupled alginates did not enhance survival/differentiation of either cell type *in vitro*. This suggests that further modification of the alginates is necessary, for example, by coupling more RGD peptide to the alginates, or by further modulating their composition. Clearly the ability to modify and thoroughly characterise the alginates is a major advantage in developing tailor-made matrices for transplantation of OECs and other cell types in different experimental models.

5. Discussion

In the present study, we adopted an interdisciplinary approach in the investigation of transplant-mediated CNS repair, by (i), defining the biological context of the investigation in terms of experimental model and cell of interest, (ii), predicting potential outcomes of cell transplantation in the specific model, (iii), integrating MRI methodologies for monitoring cell transplants and regenerating CNS axons, and (iv), modifying biopolymers to address specific methodological and biological aspects of the study.

5.1. Regeneration of RGC axons mediated by OEC transplants

A key hypothesis in our study was that RGC axon regenerative effects can be mediated by transplantation of OECs after ONC injury. Initial assessment of the reparative effects of OEC transplants after ON lesion was based on analysis of MEMRI data at 20dpl. Intraorbital ON lesioning results in loss of 90% of RGC [344], while the spontaneous regenerative response of <10% of the remaining RGC is abortive and, thus, not sustained beyond 20dpl [344]. Based on previous studies by our group, in which the regenerative effects of LI and PNG after ONC in mouse and rat, respectively, were assessed by MEMRI [407, 418, 424], we predicted that RGC axon regeneration in response to *ivit* and *iON* OEC transplants, would be manifested as elevated CNR profiles along the ON proximal and, possibly, distal to the lesion site. The designations proximal and distal correspond to the ON segment from the lamina cribrosa up to the lesion site, and from the latter towards the chiasm, respectively.

Based on the above assumptions, the observation at 20dpl that the CNR profiles of animals with *ivit* OECs were not significantly different ($P>0.05$; two-tailed t test) from those of untreated controls indicated that the *ivit* OEC grafts had no neuroprotective/regenerative effects. It can be argued that the lack of RGC axon response to the *ivit* OEC grafts may be attributed to poor graft function and/or survival, or insufficiency of the *ivit* transplants in terms of number of transplanted cells. However, a thorough investigation of the regenerative effects of *ivit* OECs after ONC injury was not implemented, as it did not constitute a primary goal within the framework of this study. In light of the above, it can only be concluded that potential neuroprotective/regenerative effects of *ivit* OECs on RGC may be marginal and, thus, not detectable by MEMRI [407].

Contrary to the above, analysis of the MEMRI data obtained from the *iON* groups, revealed differences ($P \leq 0.05$; two-tailed t test) between the CNR along the ON proximal, as well as distal to the ONC lesion site, compared to those of untreated controls, indicating a regenerative response to the *iON* transplants.

These observations suggested that it would be interesting to investigate whether the axon regenerative effects detected by MEMRI in the *iON* transplanted animals would be sustained beyond 20dpl. However, given methodological considerations relating to potential toxic effects of repeated doses of $MnCl_2$ on RGC neurons and OEC grafts (discussed in section 5.6), we decided that MEMRI should not be used beyond the 20dpl time point. Evaluation of the reparative potential of OECs at >40dpl, was thus based on ultrastructural analysis using TEM.

Ultrastructural analysis at >40dpl revealed SLA proximal to the injury site in the *iON* transplanted animals, consistent with an axon regenerative response, thus confirming the MEMRI observations from 20dpl. Qualitative comparison with untreated controls showed that the degree of axonal sprouting observed in the *iON* transplanted group was far more extensive and, thus, consistent with axon regenerative effects of the *iON* OECs, rather than an abortive regenerative response triggered by the ONC injury. Small numbers of SLA were also observed in the *iON* transplanted animals distal to the lesion site. Furthermore, we identified OECs extending long processes in close proximity with SLA and blood vessels proximal to the injury, providing indirect evidence of long-term survival and integration of OEC transplants with the host tissue. Thus, based on the observations at 20dpl and >40dpl, we conclude that there is an axon regenerative response to *iON* OECs in terms of increased axonal sprouting proximal, and to a far lesser extent, distal to the lesion site.

Our findings partially confirm findings by Plant *et al* [346] and Liu *et al* [347] of axon regenerative responses to OEC transplants after rat visual pathway lesion. Liu *et al* [347], however, reported long-distance RGC axon regeneration, and also functional recovery at 8wpl. This difference may be attributed to the fact that Liu *et al* [347] utilised human recombinant GDNF, in addition to OEC transplants. Although we recognize that combinatorial approaches may be better aligned towards promoting visual pathway repair, as they simultaneously address different factors involved in CNS recovery, our reparative strategy, on this occasion, was based on OEC transplants only. This is because one of the main aims of our study was to test the suitability of MEMRI as a method for monitoring OEC-mediated repair in the visual pathway. This implied that transplantation of OEC, unassisted by other factors, would allow investigation of the regenerative capacity of OECs *per se* and, by the same token, would provide a clearer

measure of MEMRI sensitivity in detecting the relevant responses. In addition to the above, our observation of surviving *iON* OECs at >40dpl contrasts with the findings by Plant *et al* [346] as these authors did not identify any surviving OECs in the ON at 7 weeks post-transplantation. However, differences in our corresponding experimental protocols may account for this discrepancy, including number of transplanted cells and time point for transplantation. In our study, $2.5x10^5$ OECs were transplanted acutely after ONC lesion, compared to $5x10^4$ cells transplanted at 5dpl in the study by Plant et al [346].

Finally, additional methodological considerations require clarification. The OECs utilised in our study did not express GFP and would not have been readily identifiable in histological sections. This precluded correlation of MRI data with findings from *ex vivo* examination of tissue collected from OEC transplanted animals. Alternatively, immunohistochemistry could have been used, however, in the absence of a specific marker for OECs [264, 265], unequivocal identification is not possible. Furthermore, identification of OECs by immunoreactivity with $p75^{NTR}$ gives variable results and $p75^{NTR}$ expression is lost once the cells have differentiated into a myelinating phenotype, while Gap-43 immunoreactivity for detection of regenerating RGC axons was considered redundant in the presence of ultrastructural data.

5.2. Can OECs play a remyelinating role in visual pathway repair?

The ultrastructural study provided evidence of remyelination of ON axons by myelin of peripheral appearance, as indicated by its hue and lamellar periodicity [374]. This finding is promising, however, it should be interpreted with caution, given that remyelination by OECs is indistinguishable from that by Schwann cells [319-325]. In a previous study, Li *et al* [374] reported that remyelination of ON axons is mediated by *iON* Schwann cell transplants only, not by *iON* OECs. In our study, we utilised 98-100% pure OECs obtained exclusively from the OB, which precludes the presence of contaminating Schwann cells in the OEC transplant. Furthermore, we did not observe any new myelin formation in ON collected from untreated controls, while the observed myelin in the *iON* tissue was always apposed to OECs. Based on these observations, we may attribute the presence of new myelin to the transplanted OECs. However, the possibility that the observed myelin may be due to Schwann cells migrating from meningeal fibres, as a result of the ONC injury, cannot be excluded [327]. Immunostaining for P0 might have confirmed the type of myelinating cell. Such investigation, however, would be confounded by the absence of an unequivocal marker for OECs [264, 265] and the fact that the OECs used in this study did not express GFP. Finally, a pertinent issue in this discussion is myelination by oligodendrocytes, given that the presence of *iON* OECs may either stimulate

myelin formation by endogenous oligodendrocytes, or impair it through a mechanism of competitive inhibition. Clearly, additional investigations are necessary to further explore and confirm a remyelinating role for OECs in visual pathway repair.

5.3. To what extent can MPIO uptake by OECs be controlled?

Labelling efficiency and cellular responses to intracellular labels can be expected to be contingent on the particular type of label and cell under investigation. A main consideration with regard to ferumoxide-based intracellular labels is the risk of cytotoxicity associated with intracellular iron overload [467]. It is therefore imperative to develop safe intracellular labelling protocols tailored to specific cell types and imaging requirements.

Despite the fact that OECs are promising candidates in transplant-mediated repair, very few studies have utilised cellular MRI of OEC transplants [513, 514], thus little is known about the suitability of specific contrast agents for intracellular labelling of OECs for MRI. In these earlier studies, the OECs were labelled using SPIO [513] and magnetodendrimers [514], while incubation time was 48h and 24h, respectively. Cell viability was assessed at one time point only, i.e. immediately after labelling, and no adverse effects of the label were reported, except for concentrations of SPIO of 6mg/ml, which were cytotoxic and resulted in complete loss of the OECs from the *in vitro* cultures [513]. Using the protocol developed in Paper 1, however, high labelling efficiency (>90%) was achieved with incubation times as short as 6h. Furthermore, cell proliferation, migration and survival were monitored over 96h post-transplantation, thus excluding potential delayed adverse effects of the intracellular label *in vitro*. No adverse effects of the intracellular MPIO label were observed at any time during the observation period.

Labelling efficiency was defined as 90% MPIO-labelled OECs and a minimum intracellular iron load of 0.90pg, which allows single cell detection by MRI [442]. The definition of a minimum threshold for intracellular MPIO uptake for single cell detection by MRI was based on an earlier study by Shapiro and Koretsky [442]. For the purpose of future MRI studies, we wanted to ensure that our labelling protocol enables sufficient intracellular iron load to detect potential migration of individual or small numbers of OECs post-transplantation *in vivo* by MRI.

We found that optimal labelling efficiency of >90% is achieved by incubation of 50% confluent OEC cultures with MPIO over a period of 6h. Individual OECs displayed differential intracellular MPIO uptake, while MPIO uptake was also contingent on the MPIO concentration used for labelling and was in the range of 12-115 endocytosed MPIO. This corresponds to 1.90-13.80pg

of intracellular iron load, which is considered sufficient for single-cell detection by MRI at a resolution of 100µm, based on previous findings [442]. Monitoring of the MPIO-labelled OECs over a period of 96h post-labelling and comparison with unlabelled control OECs did not reveal any differences in cell survival, proliferation, and migration capacity *in vitro*, showing no adverse effects of the MPIO label.

The observation of differential uptake of the MPIO label by individual OECs, irrespective of MPIO label concentration and length of incubation is hardly surprising, given that intracellular MPIO uptake by individual cells cannot be fully controlled under experimental conditions. At the same time, the specific finding confirms the assumption that partial control of intracellular iron load can be effected by using smaller-diameter MPIO with lower magnetite content, and shorter incubation times, thus limiting the risk of cytotoxicity associated with intracellular iron overload.

Another important consideration is MPIO label retention after endocytosis. In our study, the MPIO were retained by the OECs throughout the incubation period. Interestingly, however, the specific MPIO type and labelling protocol developed in our study were utilised by a different research group for intracellular labelling of myeloma cells. After initial uptake of the MPIO label, these investigators found that the MPIO were rejected by the myeloma cells *n vitro* , possibly *via* an efflux mechanism (Dr Therese Standal; personal communication). Although this response may be explained by specific biochemical properties pertaining to cancer cells, the particular observation further emphasises the importance of thoroughly testing the suitability of different intracellular labels and protocols for specific cell types. Intracellular MPIO retention by specific cell types may be another factor that cannot be fully controlled under experimental conditions. In such cases, potential rejection of the MPIO label by transplanted cells post-transplantation *in situ* would seriously impact the usefulness of relevant MRI data.

5.4. Viability and function of MPIO-labelled OECs *in vivo*

The protocol developed in Paper 1 ensured that labelling of OECs with MPIO does not compromise cell viability, proliferation, and migration capacity *in vitro*, as demonstrated in a series of assays performed up to 4d post-labelling. While these results confirm the safety and efficiency of MPIO-labelling for the purposes of the MRI protocols used in Paper 2, they do not constitute undisputable predictors of MPIO-labelled OEC behaviour post-transplantation *in vivo*. Considering the framework of our study, a discussion on *in vivo* function and survival of transplanted MPIO-labelled OECs, can only be based on indirect measures.

As discussed earlier, MEMRI did not detect any RGC axon responses to *ivit* OEC transplants. If we were to assume that this lack of regenerative response can be attributed to poor OEC survival and/or function post-transplantation, MPIO-labelling *per se* does not appear to be a contributing factor. This is because equivalent observations were made in the animal groups with *ivit* unlabelled OEC, i.e. both MPIO-labelled and unlabelled *ivit* OEC groups had CNR profiles that were not significantly different from those of untreated controls (i.e. $P>0.05$; paired t test).

On the other hand, MEMRI detected axon regenerative responses to both MPIO-labelled and unlabelled *iON* OECs, providing indirect evidence of MPIO-labelled OEC function post-transplantation. On this occasion, the CNR profiles of animals with unlabelled *iON* OECs were significantly higher than those of untreated controls ($P=0.02$; paired t test). The differences in the CNR of MPIO-labelled *iON* OEC and untreated control animals were marginally significant ($P=0.05$; paired t test). It can be argued that this may be an indicator of reduced survival and/or efficacy of the *iON* MPIO-labelled OEC graft as a result of intracellular MPIO. However, the lower CNR profiles observed in the MPIO-labelled OEC group can be explained by distortion of the CNR of the Mn^{2+}-enhanced ON as a result of T2* effects from MPIO proximal to the lesion site (please see section 5.5), rather than impaired *iON* graft function and/or survival as a result of MPIO labelling.

Finally, ultrastructural analysis identified MPIO-labelled OECs extending long processes in close apposition with SLA and blood vessels, thus providing evidence of long-term survival, function and integration of *iON* MPIO-labelled OEC grafts with host tissue. Although the above observations suggest that intracellular MPIO may not compromise the viability and function of MPIO-labelled OEC grafts *in vivo*, potential adverse effects of intracellular MPIO post-transplantation should be thoroughly investigated in a different study, designed specifically for this purpose.

5.5. Monitoring MPIO-labelled OEC transplants by MRI. Fact or artefact?

Cellular MRI provides information about cell graft localisation immediately post-transplantation, while it also enables longitudinal monitoring of transplanted cell movements and survival *in situ*, albeit with certain caveats.

MPIO-labelled OECs can be imaged both with T_2*- and T_2-weighted MRI. T_2*-weighted gradient echo sequences are highly sensitive to the MPIO label. However, consistent with our

expectations, better image quality was achieved with T_2 imaging. In the pilot experiment described in Paper 1, the T_2*-weighted sequence revealed a large hypointense region in the ipsilateral eye of *ivit* transplanted animals consistent with *ivit* MPIO-labelled OECs. No hypointense region was present in the contralateral eye, in which unlabelled OECs had been transplanted. Similarly, in Paper 2, the *ivit* MPIO-labelled OEC graft was unequivocally detected with T_2*-weighted imaging, while no hypointense region was revealed in the contralateral eye of the animals after saline injections.

Unequivocal detection of the *iON* MPIO-labelled OEC transplants based on T_2*-weighted images alone was confounded by the fact that potential blood/air artefacts in the region of the injury could not be excluded. Nonetheless, analysis of SNR comparing the ipsilateral and contralateral ON of *iON* engrafted animals and untreated controls revealed signal attenuation proximal to the injury side consistent with the MPIO-labelled OEC graft.

An important finding in our study is the evidence of secondary MPIO uptake by macrophages revealed by the ultrastructural study. This observation was not entirely unexpected. It reiterated, however, one of the main limitations associated with the use of intracellular contrast agents such as MPIO, i.e. imaging artefacts derived from secondary uptake of the label by *in situ* macrophages and other scavenger cells. Furthermore, the evidence of MPIO degradation revealed by the ultrastructural study questions the suitability of the MPIO label for longitudinal studies beyond 40dpl. Apart from false positives derived from secondary MPIO uptake, potential degradation of the label within transplanted cells might significantly compromise the efficacy of cellular MRI, resulting in signal loss/ dilution, while it may also have deleterious effects on long-term graft function and survival.

Additionally, although tracking of cell movements by MRI may be desirable, *in vivo* monitoring of potential migration of the *ivit* and *iON* transplanted MPIO-labelled OECs by MRI was not possible in our study. Although individual cells had enough intracellular MPIO for single cell detection by MRI, the large susceptibility effects induced by the intracellular MPIO label limited resolution to the macroscopic graft.

5.6. MEMRI in combination with T_2* contrast agents – imaging, interpretation, and toxicity

In the present study, MEMRI was applied for *in vivo* monitoring of RGC axons after visual pathway lesion and transplantation with OECs. Consistent with previous findings, MEMRI enabled visualisation of damaged and regenerating RGC axons 24h after *ivit* injection of 150nmol of $MnCl_2$, demonstrating the efficacy of the technique for CNS axon tracing *in vivo* [406-408, 418-419, 424]. Analysis of the MEMRI data using a semi-automatic segmentation technique [418] provided the CNR profiles of the Mn^{2+}-enhanced ON in the different experimental groups.

As discussed earlier, based on previous studies [408, 421-424], we predicted that potential axon regenerative effects of OEC transplants would be reflected in increased CNR proximal and, possibly, distal to the lesion site. Furthermore, previous work by our group [407] determined that the lowest axon density resolvable by MEMRI is 125 000 axons/mm^2 and that anterograde transport of Mn^{2+} in RGC axons is *via* kinesin-mediated anterograde transport mechanisms, thus requiring viable axons [419]. As discussed in section 5.1, the fact that no significant differences were found in the CNR of the *ivit* engrafted groups compared to untreated controls at 20dpl, suggested that the *ivit* OEC transplants did not have a neuroprotective/regenerative effect on RGC neurons. Contrary to this finding, analysis of the CNR profiles along the ON of animals with *iON* MPIO-labelled OEC transplants compared to untreated controls, revealed marginally significant differences at 1-3mm from the lamina cribrosa, indicative of an axon regenerative response to the graft. However, the CNR profile of the Mn^{2+}-enhanced ON of *iON* MPIO-labelled OEC animals was distorted by susceptibility effects induced by the presence of MPIO proximal to the lesion site. Comparison of the CNR profiles of the *iON* unlabelled OEC group with untreated controls, revealed statistically significant differences at 0.6mm-2mm (P=0.02; paired t test) from the lamina cribrosa, consistent with an axon regenerative response to the *iON* OEC transplant.

Notwithstanding the distortion of the CNR profiles of the Mn^{2+}-enhanced ON as a result of the *iON* MPIO, the MEMRI technique was sensitive enough to detect axon regenerative responses to MPIO-labelled OEC transplants. On the other hand, the challenges in the interpretation of MEMRI data indicate that combination of different contrast agents in future studies may require the development of sophisticated post-processing tools that can compensate for the loss of T_1 signal as a result of susceptibility effects derived from T_2* contrast agents. Furthermore, spin echo sequences, such as RARE, are less sensitive to T_2* effects than gradient echo sequences,

such as FLASH. However, gradient echo sequences are faster and yield higher SNR, thus enabling better resolution and significantly reducing scanning times. These factors were taken into account in applying gradient echo sequences for the purposes of the specific study.

An important result in our study was that the T_1-weighted 3D FLASH sequence applied for MEMRI of RGC axons, simultaneously detected the MPIO-labelled *ivit* and *iON* OEC transplants. This finding can be explained by the fact that T_2* susceptibility effects derived from the MPIO destroy the Mn^{2+}-derived T_1 signal. Although simultaneous detection of MPIO-labelled grafts by MEMRI was not a primary goal of our investigation, the specific effect may be useful in terms of reducing scanning times for each animal in similar experimental protocols. On the other hand, the interplay between the two contrast agents poses certain methodological challenges.

A main consideration in the above study was that serial *in vivo* imaging of RGC axons using MEMRI would necessitate co-localisation of $MnCl_2$ with *ivit* and *iON* labelled OEC grafts at specific time points. Although the *ivit* dose of 150nmol of $MnCl_2$ used in the study does not induce toxicity, while it has also been reported to have a neuroprotective effect on RGC neurons [407], potential toxic effects of repeated *ivit* doses of $MnCl_2$ on OEC transplants and RGC neurons could not be excluded. Another consideration was that Mn^{2+}-induced toxicity might be exacerbated in the presence of intracellular iron from the MPIO label, and *vice versa*. We therefore decided to postpone MEMRI until 20dpl so as not to interfere with OEC graft function and survival by introducing $MnCl_2$ to the tissue acutely post-transplantation. The same considerations indicated that MEMRI beyond the 20dpl time point should not be used in the specific experimental protocol. As a result, ultrastructural analysis rather than MEMRI was used for follow up of the animals at >40dpl.

Taken together, the above findings illustrated some of the opportunities and challenges involved in integrating MEMRI as a tool for monitoring transplant-mediated repair after visual pathway lesion. While the technique indeed enabled detection of axon regenerative responses to the *iON* OEC transplants, correlation of the MEMRI data with ultrastructural findings was essential for correct interpretation and/or verification of the MEMRI observations. Furthermore, the risk of cytotoxic effects as a result of co-localisation of Mn^{2+} and OEC transplants, as well as interplay between T_1 and T_2/ T_2* contrast agents, are factors that must be taken into consideration in future studies involving serial *in vivo* MEMRI of RGC axons after transplantation of MPIO-labelled OECs.

5.7. Controlled release of Mn^{2+} from alginate beads - what can it achieve?

A novel approach in our study was the development of tailor-made alginates for controlled release of Mn^{2+} for future MEMRI applications in studies of CNS damage and repair. The rationale behind this approach was that such systems should enable optimal contrast enhancement in MEMRI and also minimise the inherent risk of Mn^{2+}-induced toxicity associated with repeated and/or high doses of Mn^{2+}. This rationale was based on earlier observations regarding Mn^{2+} uptake into neurons [419], as well as differential structural, mechanical, and functional properties of modified alginate gels in physiological conditions [492-494, 497, 498, 515].

Earlier findings suggested that there is a plateau of maximum Mn^{2+} entry into neurons [419], a corollary of which being that increased Mn^{2+}-induced signal enhancement is contingent on prolonged Mn^{2+} availability, rather than on Mn^{2+} dose. This indicated that regulation and adjustment of Mn^{2+} release to the neuronal uptake threshold can be expected to dramatically reduce exposure of neurons to Mn^{2+}.

Furthermore, previous studies demonstrated that the compositional and structural properties of alginate can be modulated by conversion of M-residues to G-residues using mannuronan C-5 epimerases [492, 493, 494, 497]. Additionally, it is known that alginates display different affinities to divalent ions such as Ba^{2+} and Ca^{2+} [498] and that the binding between guluronic acid residues and Ca^{2+} or Ba^{2+} is characterised by strong and specific auto-cooperativity [515]. In physiological conditions, alginate hydrogels have a tendency to swell and dissolve, as divalent crosslinking ions are released in competition with non-gelling ions, with each crosslinking ion being replaced by two Na^{+}. However, more stable gels can be produced using high G-content alginates, or divalent ions, such as Ba^{2+}, which have a high affinity to alginate. At the same time, given that the affinities of Ba^{2+}, Ca^{2+}, and Mn^{2+} to alginate are in the order $Ba^{2+}>Ca^{2+}>>Mn^{2+}$, we predicted that formation of alginate gels using gelling solutions containing (i), $BaCl_2$ and $MnCl_2$, and (ii), $CaCl_2$ and $MnCl_2$, will result in differential rates of release of Mn^{2+} under physiological conditions.

Consistent with our predictions, elemental analysis and stability studies revealed distinct differences in the ion binding capacity of the different Mn-alginate gels. As expected, Mn/Ba-alginate gels were most stable. Analysis of the MRI data and simulation of the *ivit* Mn^{2+} release and concentration in the beads and in the vitreous using a compartment model confirmed the above findings. Two time constants for *ivit* Mn^{2+} release were extracted from the model, i.e. a

long and a short time constant (τ_1 and τ_2, respectively), corresponding to slow and fast rates of release. While the short time constant hardly varied between the different alginates, there were significant differences in the long time constant (i.e. 600min-100min) in the order high-G ≥ high-M > polyMG, as well as Ba^{2+}>Ca^{2+}, i.e. contingent on the crosslinking ions.

Unilateral *ivit* injection of Mn-alginate beads resulted in clear enhancement of the rat visual projection consistent with uptake of Mn^{2+} released from the beads by RGC neurons and active anterograde transport within RGC axons [416-417]. Simulation of the *ivit* Mn^{2+}concentration showed that controlled release of Mn^{2+} from alginate with a time constant of 600min, representing the rate of Mn^{2+} release, effectively reduced maximum *ivit* Mn^{2+} concentration to 57%, 21%, and 15%. This relates to an initial amount of bound Mn^{2+} stipulated at 20%, 80%, and 100%, respectively. On the other hand, a time constant of 100min, led to a corresponding reduction of *ivit* Mn^{2+} to 63%, 46%, and 44%. Furthermore, we predict that the use of Mn-alginates for controlled release of Mn^{2+} can achieve reduction of maximum *ivit* Mn^{2+} concentration by ~85%. Considering that a bolus injection results in *ivit* Mn^{2+} concentration of 100%, the large reductions in the *ivit* Mn^{2+} concentration facilitated by controlled release of Mn^{2+} from tailor-made alginates are highly significant in terms of reducing the risk of Mn^{2+} toxicity to RGC axons.

In light of the above, we provide proof of principle for the design of alginate-based systems for controlled release of Mn^{2+} for MEMRI. We propose that such systems can be customised for use with specific experimental models, taking into account the particular bioanatomical/biophysical properties of the neuronal populations under investigation in terms of Mn^{2+} uptake, transport and clearance. Such applications can be expected to enhance the versatility and scope of the MEMRI technique for safe application in longitudinal studies of CNS damage and repair.

5.8. Modification of alginates with RGD-peptides – developing an ideal matrix?

One of the main considerations in studies of transplant-mediated repair is cell graft rejection as a result of immune responses. Although this type of host response may be modulated by the use of immunosuppressive treatments, or circumvented by utilising autologously derived or syngeneic transplants, it is often the case that the function, survival and integration of the transplanted cells with the host tissue is poor. With regard to OECs, survival post-transplantation can be expected to be as low as 50% [248]. Furthermore, our observations in Paper 2 indicated that a possible reason for the lack of neuroprotective/regenerative effects of *ivit* OEC transplants may have been poor function and/or survival of the OEC graft post-

transplantation in the vitreous. This suggested that enhanced benefits of transplanted OEC might be derived through utilisation of biopolymer matrices for cell encapsulation.

Based on the knowledge that the structural and mechanical propertied of alginates lend themselves to substantial modification [492, 493, 494, 497] and that RGD-peptide coupling to alginates promotes differentiation, adhesion, and survival of encapsulated cells by mimicking ECM conditions [500], we proposed to test the effects of RGD-peptide modified alginates as substrates for the culture of OECs *in vitro* with a view to future applications in connection with *in vivo* transplantation studies. Given that the interactions between RGD-alginates and myoblasts are well-characterised [500, 517], we used myoblasts as a reference cell line in the study.

The strategy for alginate modification adopted in our study involved introduction of bioactive ligands to the M-residues of the alginate using a chemoenzymatic approach. G-residues were subsequently introduced to the alginate by epimerisation with mannuronan C-5 epimerases (as in Paper 3). The particular approach ensured that there is no interference with G-blocks as a result of the peptide coupling, thus allowing the G-blocks to be the main contributor to the formation of the gels.

Another advantage in our methodological approach is that the proportions of M- and G-sequences and grafted functional peptide in our modified alginates, as well as downstream effects, were determined by NMR spectroscopy, while molecular weight characterisation was achieved by intrinsic viscosity measurements. This type of characterisation is very important given that the structural and compositional properties of the modified alginates may thus be correlated with observations from *in vitro* assay studies to elicit specific interactions with the cells under investigation, also with a view to *in vivo* applications.

Characterisation of the alginates showed that modification of the alginates' composition by epimerisation was consistent with previous findings [492, 493, 494, 497]. Utilisation of the RGD-coupled alginates and their non-coupled equivalents as substrates for OEC and myoblast culture in the form of flat gels (2D cultures) revealed dynamic responses to the RGD-coupled alginates in terms of altered OEC and myoblast morphology, formation of large clusters scattered across the surface of the gels, and adhesion of the cells to the alginate substrate by extension of bipolar processes. The specific morphological phenotype is largely unseen for OECs [256], while it denotes an intracellular response in terms of gene/protein expression affecting the cytoskeletal structure. On the other hand, encapsulation in RGD-coupled alginates did not

promote survival and/or differentiation of OECs and myoblasts *in vitro*. It is postulated, however, that further modification of the alginates, for example by coupling more functional peptide, or decreasing the G-content of the alginates may be necessary.

The above findings are interesting with regard to future applications of RGD-peptide alginates in experimental studies of CNS repair mediated by OEC transplants. They indicate that introduction of RGD-peptide to the alginates may have an epigenetic effect on OECs, elucidation of which may lead to the development of tailor-made, functionalised alginate matrices for encapsulation of OECs with a view to promoting OEC survival and specific cell functions, such as increased secretion of neurotrophic factors. Furthermore, it can be envisaged that modification of alginate matrices with functional peptides can by used to produce scaffolds which promote enhanced function and survival of seeded OECs, alone or in combination with other cell types. Such scaffolds may also provide molecular guidance and structural support to regenerating axons, facilitating directional growth towards correct synaptic targets distant to the lesion site.

6. Conclusions

The aim of this thesis was to integrate MRI and biomaterials in the study of visual pathway repair mediated by OEC transplants. Based on the findings of the relevant investigations, we can draw the following main conclusions:

(i) MRI enables simultaneous *in vivo* monitoring of OEC transplants and regenerating RGC axons, however, signal distortions as a result of interplay between T_1 and T_2* contrast agents, as well as false positives in terms of imaging artefacts derived from secondary uptake of intracellular T_2* contrast agents *in situ*, necessitate careful correlation of the MRI data with ultrastructural findings for correct interpretation and/or verification of MRI-based observations.

(ii) OEC transplants have an axon regenerative effect and, possibly, a remyelinating role after *iON* transplantation following ONC injury.

(iii) Tailor-made Mn-alginate beads can be utilised for controlled release of Mn^{2+} as a means of optimising contrast enhancement and minimising the risk of Mn^{2+}-induced cytotoxicity, thus providing a significant refinement of the MEMRI technique.

(iv) RGD-modified alginates induce dynamic responses on OECs in terms of morphology, cell-cell, and cell-matrix interactions, suggesting that further modification of the alginates may produce tailor-made, functionalised alginate matrices with ECM properties to be used for OEC encapsulation/seeding in models of CNS repair mediated by OEC transplants.

7. Future directions

Integration of imaging, biomaterials, and nanotechnologies is important in investigations of CNS damage and repair. However, although advances in these technologies create opportunities for innovation and thus enable investigations in neuroscience which might have been inconceivable only some decades ago, their application should be hypothesis-driven rather than technology-driven. In other words, technology alone, no matter how powerful, may not be expected to elucidate key mechanisms of CNS damage and repair unless it is supported by rigorous biological principles and ability to address specific biological questions.

The complexity of the mechanisms of CNS damage and repair suggests that multi-factorial approaches may be better-aligned towards promoting repair of the damaged CNS after injury. Future research may thus benefit from the utilisation of biomaterials and nanotechnologies to optimise the effects of cell transplants *in situ*, deliver genes and proteins to appropriate targets, modulate the inhibitory nature of the lesion microenvironment, and promote axonal regeneration and re-establishment of lost synaptic contacts. Furthermore, emerging technologies such as bioprinting may be important for producing customised functional matrices for scaffolding of different types of CNS lesions.

Ability to monitor and elucidate the efficacy of transplant-mediated CNS repair *in vivo*, can be expected to rely on multimodal imaging and to integrate different MRI technologies, such as cell tracking, MEMRI, fMRI, and DTI/tractography, with MR spectroscopy and PET, to evaluate different aspects of the reparative process. Smart contrast agents, which circumvent the vagaries of imaging artefacts and which enable visualisation of genes and proteins post-transplantation are also of great interest. However, appropriate correlation of *in vivo* imaging data with the findings of more traditional methods, such as EM, immunohistochemistry and *in situ* hybridisation, may still be necessary for rigorous evaluation of the efficacy of therapeutic interventions in experimental CNS lesion models.

Finally, successful integration of interdisciplinary approaches in regenerative medicine may, in the future, contribute towards resolving the lack of regenerative capacity in the CNS.

In the words of Ramón y Cajal: 'It is for the science of the future to change, if possible, this harsh decree. Inspired with high ideals, it must work to impede or moderate the gradual decay of neurons, to overcome the almost invincible rigidity of their connections, and to re-establish normal nerve paths, when disease has severed centres that were intimately associated'.

8. References

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9. Contributions

Papers 1-4 are included in the following pages.

Paper I

Is not included due to copyright

Paper II

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Paper III

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Paper IV

Effects of RGD-peptide modified alginate matrices on olfactory ensheathing cells and myoblasts *in vitro*

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Abstract

One of the main challenges in tissue engineering and regenerative medicine is the ability to maintain optimal cell function and survival post-transplantation and to protect the transplanted cells from immune responses and rejection in the host-tissue microenvironment. Biomaterials such as alginates can be utilised for immunoisolation, while they may also provide structural support to the cell transplants by mimicking the extracellular matrix. In the present study, we produced RGD-peptide coupled, stable alginates of variable flexibility by adopting a unique strategy for controlling the peptide coupling and G-content of the alginates. We characterised the alginates and proceeded to test their interactions with olfactory ensheathing cells (OECs) and myoblasts in 2D and 3D cultures. We found that RGD-peptide modified alginates induced distinct cell-substrate interactions, demonstrated as marked morphological changes in 2D cultures of OECs and myoblasts, and partially enhanced survival, compared to unmodified and control alginates. 3D cultures, however, did not demonstrate substantial benefits of RGD-modification in terms of improved encapsulated cell viability. Taken together, our findings suggest that further modification, for example, by coupling more RGD peptide to the alginate and/or reducing the rigidity of the 3D network, might be necessary in order to significantly enhance the survival of encapsulated OECs and myoblasts, with a view to future transplantation studies.

1. Introduction

In the last decades, cell therapy has emerged as a promising approach in translational medicine, with potential applications in the treatment of a multitude of conditions, ranging from diabetes (1-5), and bone defects (6), to cancer (7), and central nervous system (CNS) lesions as a result of trauma [8-10] or neurodegenerative disease (11-13). Contingent on cell type and disease/injury under treatment, cell transplantation may aim to replace and/or regenerate damaged tissues, promote *de novo* tissue formation, or deliver secreted factors and therapeutic molecules *in situ* (14, 15). The success of cell therapy, however, is often confounded by poor cell function, survival, and integration with host tissue post-transplantation, as well as by the need for immunosuppression in experimental and clinical protocols which involve the use of allografts or xenografts (16). To circumvent some of these issues, biomaterials are increasingly used in tissue engineering and regenerative medicine as they may provide the dual benefits of immunoisolation and structural support to encapsulated cells, mimicking extracellular matrix (ECM) conditions for enhanced cell-cell and cell-tissue interactions (17,18). Such matrices may also enable controlled cell proliferation, fate and differentiation *in situ* (17, 18).

Alginates, a generic term for naturally occurring polysaccharides found in algae (19) and some bacteria (20, 21), are widely used for microencapsulation of a variety of cell types (22-27). Alginates are linear unbranched copolymers of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) and their superior ionotropic properties (28) and biocompatibility render them particularly suitable for formation of hydrogel beads for cell encapsulation. Furthermore, alginates can be structurally and compositionally modified by converting M-residues to G-residues using mannuronan C-5 epimerases, while they can also be grafted with functional peptides such as arginine-glycine-aspartic acid (RGD), to enhance cell adhesion (29). Such manipulations enable tailoring and optimisation of the alginates to meet specific requirements for mechanical stability and functionality (30-35).

In the present study, we aimed to (i), produce peptide-coupled, stable alginate microbeads of variable flexibility by adopting a unique strategy for controlling the degree of peptide grafting as well as the G-content of the alginates, and (ii), characterise the modified alginates and monitor and compare their interactions with two different cell types, both in 3D and 2D cultures. We used a chemoenzymatic strategy (32, 36) to introduce bioactive ligands exclusively to the M-residues of alginate in a two-step process. First, a pure mannuronan was grafted with RGD peptides using carbodiimide chemistry, and then, it was treated with mannuronan C-5 epimerases, which convert non-substituted M-residues into G-residues in the alginate chain (32-34). We determined the proportions of M- and G- sequences and the degree of peptide substitution in the modified alginates using NMR spectroscopy, and further characterised the molecular size of the grafted alginates by intrinsic viscosity measurements. The alginates were purified by active coal filtration. We proceeded to test whether the modified alginates, in the form of flat gels (2D culture) and microbeads (3D culture), enhance cell survival and/or differentiation and whether they thereby render themselves as suitable substrates for immobilisation of olfactory ensheathing cells (OECs), a promising candidate cell type in transplant-mediated CNS repair (37), with a view to future *in vivo* transplantation studies in experimental CNS lesion models. C2C12 myoblasts, a well-characterised cell line in immobilisation studies utilising RGD-coupled alginates (29), were used as a reference cell line in the study.

2. Materials and Methods

2.1. Alginate

Mannuronan isolated from an epimerase-negative mutant (ALG^-) of *Pseudomonas fluorescens* (batch 512-215-01 TP, FM=1, [η]=1614ml/g) was modified by coupling a hexapeptide with the sequence GRGDSP to the C-5 carboxyl group of mannuronic acid monomers. UPLVG (batch FP603-04, NovaMatrix™, Norway) was used as control alginate.

2.2. Peptide coupling

Two batches of alginates, i.e. batch 1, and 2, were produced by peptide coupling of sequences GRGDSP (AlBioTech, US) to mannuronan using carbodiimide chemistry. 3.5g (batch 1), and 5g (batch 2) of purified mannuronan were dissolved at 1% in phosphate buffer saline (PBS) solution overnight. EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride) was added to the solution at 1:20 molar ratio to the uronic acid monomers of the alginate. Sulfo-NHS (*N*-hydroxysulfosuccinimide) was added as a co-reactant to the EDC at a 1:20 molar ratio and incubated for 2 hours (h). The peptide was then added at a concentration of 100μmol/ml, as described in Rowley and Mooney (29) and allowed to react for 20h before the alginate solution was purified through extensive dialysis against 3 shifts of distilled water, 3 shifts of 50mM NaCl, and against distilled water, until the water conductivity measured <2μs. The alginates were subsequently freeze dried and stored.

2.3. Epimerisation

Alginate consisting of G- and MG-blocks was made by epimerizing the peptide-coupled mannuronans with the mannuronan C-5 epimerase AlgE4 from the alginate producing bacterium *Hansenula vivelandii* (produced recombinantly in *Hansenula polymorpha*) and AlgE6 (produced in *Eschericia coli*) (33). To produce MG alternating alginate, AlgE4 was added to the mannuronan at a 1:200 (w/w) ratio in a mixture of 0.25% (w/v) alginate, 50mM MOPS, 2.5mM $CaCl_2$ and 10mM NaCl and incubated for 24h-48h at 37ºC. To produce alginates with a G-content of 50% and 70%, AlgE6 was added to MG alternating alginate at 1:20 ratio in a mixture of 0.25% alginate (w/v) and 50mM MOPS for 2.5h and 24h, respectively. To eliminate potential toxins, the epimerized alginates were purified through a carbon filter (PALL Corporation). Control samples were prepared by epimerizing an uncoupled alginate (i.e. pure mannuronan).

2.4. NMR spectroscopy

In order to reduce the viscosity of the alginate samples prior to the NMR measurements, the samples were depolymerised by mild acid hydrolysis to a final average Dp_n~30 residues (38).

3-(trimethylsilyl)-proprionic-2,2,3,3,-d4 acid sodium salt (Aldrich, Milwaukee, WI, USA) was used as internal standard for the chemical shift and triethylenetetra-amine hexa-acetate (Sigma Aldrich) was added to chelate residual calcium ions in end-point epimerased samples. Preliminary tests of depolymerisation by mild acid hydrolysis on the peptide grafted alginate showed no detachment of the RGD-peptide.

1D and 2D homonuclear experiments were carried out on a BRUKER Avance DPX 300 or 400 spectrometer equipped with a 5mm QNP and 5mm z-gradient DUL (C/H) probe, respectively. 2D homonuclear and heteronuclear experiments were performed on a BRUKER Avance 600 spectrometer equipped with a 5mm z-gradient CP-TCI (H/C/N) probe. The NMR data were recorded with BRUKER XwinNMR Ver. 2.6 or 3.5 software. The NMR data were processed and analysed with BRUKER Topspin Ver. 3.0 software.

For quantification of the peptide grafted alginate and the M/G ratio in alginate, 1D spectrum was recorded using a pulse with a 30 degree flip angle at 25°C, 40ºC, or 90°C. For assignment of the peptide and peptide grafted alginate 2D homonuclear in-phase correlation spectroscopy (IP-COSY) (39), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY) with mixing time 100ms, and heteronuclear ^{13}C heteronuclear single quantum coherence were recorded at 25°C.

2.5. Intrinsic viscosity measurements

Intrinsic viscosity was measured at the flow time through a capillary, using an Ubbelohde (type 0a), a titrator (ABU91 Autoburette, Radiometer, Copenhagen), and a viscometer (AVS310, Schott Gerte). 10-15mg of alginate were dried in a chemical dehydrator overnight. Alginate was dissolved in 10ml water and 10ml 0.1M NaCl. 15ml filtrated 0.1M NaCl were added to a clean ubbelohde connected to a viscometer and the efflux time measured at a constant temperature of 20°C. After filtration through a 5µm filter, 15ml of the alginate solution was added to a clean ubbelohde connected to a viscometer, the titrator turned on, and the efflux time measured at a constant temperature of 20°C. The sample was diluted 5 times and 4 parallel measurements performed at each dilution.

2.6. Cell seeding on flat alginate gels

To form flat alginate gels, 16.2mM GDL were added to autoclaved solutions of 0.7% alginate, 2mM HEPES and 8.1mM $CaCO_3$. The solutions were subsequently transferred to 24-well cell culture plates (Corning) in volumes of 400µl/well and allowed to gel for 1h at room temperature. OECs and myoblasts were seeded on the gels at concentrations of $1x10^4$cells/cm^2.

To compare cell cultures on alginate flat gels with cell cultures on their standard substrates, the same concentrations of OECs and myoblats were seeded on empty wells coated with poly-L-lysine and untreated plastic surface (Corning), respectively. OEC and myoblast cultures were fed with appropriate media and incubated at 37ºC with 7% and 5% CO_2, respectively.

2.7. Cell encapsulation in alginate beads

2% (w/v) alginate and 0.3M D-mannitol (VWR) in sterile water were prepared from peptide alginates and control alginates. 1.8ml of 2% alginate solution and 0.2ml of cell suspension were mixed in a 10ml syringe at a final concentration of $1.5x10^6$ cells/ml of alginate. Beads with encapsulated cells were formed by dripping the alginate solutions into a gelling bath containing 50mM $CaCl_2$, 0.15M Mannitol and 10mM MOPS buffer using an electrostatic bead generator with electrostatic potential difference 7kV, flow 10ml/h, and distance between needle and gelling solution surface 1.7cm. Beads containing encapsulated myoblasts and OECs were transferred to 75cm^2 cell culture flasks containing their respective culture media and incubated at 37ºC with 5% and 7% CO_2, respectively.

2.8. OEC purification and culture

Neonatal rat OECs were purified as described by Barnett and Roskams (40). Briefly, the olfactory bulbs of 4-5 P7 Fischer rats were finely chopped, enzymatically digested in L-15 (Leibovitz) medium (Sigma) and triturated through a 26 gauge needle. Dissociated cells were incubated in a cocktail of the O4 (IgM at 1:4) and anti-galactocerebroside (IgG3 at 1:2.) primary antibodies, followed by their fluorochrome conjugated class specific secondary antibodies. After rinsing, dissociated cells were incubated in goat anti-mouse IgM phycoerythrin and goat anti-mouse IgG3 fluorescein secondary antibodies (1:100, Southern Biotech). OECs were purified by fluorescence-activated cell sorting (FACS, Vantage Becton Dickenson) by selecting for galactocerebroside-negative and O4-positive cells. Purity of the OEC populations was assessed by $p75^{NTR}$ specific labelling and was always 98-100%. OECs were subsequently cultured in Dulbecco's Modified Eagle's Medium (DMEM GlutaMAX; Sigma) with 1.25% gentamicin (Sigma) and 5% FBS (Autogen Bioclear) on 13µg/ml poly-L-lysine- (PLL) (Sigma) coated 25cm^2 flasks. The cultures were supplemented with 500ng/ml fibroblast growth factor 2 (FGF2) (Peprotech, London, UK), 50ng/ml heregulin (hrgβ1) (R&D Systems Europe Ltd, Abingdon, UK), and 10^{-6} M forskolin (Sigma). The cells were maintained in sub-confluent cultures prior to the experiments.

2.9. Myoblasts

C2C12 mouse skeletal myoblasts (ATCC) were cultured in DMEM (Sigma) supplemented with 10% FCS, 4mM glutamine (Sigma) and 20µg/ml gentamicin (Sanofi Aventis). The cells were maintained in sub-confluent cultures prior to the experiments.

2.10. Live/Dead assay

Cell viability was qualitatively assessed post-seeding/encapsulation by visualisation of live and dead cells, stained by calcein and ethidium homodimer-1 (EthD-1) using LIVE/DEAD® assay (Molecular Probes). A working solution consisting of 3ml PBS, 1.8µl calcein and 8µl EthD-1 was freshly made before each experiment and added to cell culture media in a 1:1 ratio. The 2D cultures and 3D cultures were studied using confocal laser scanning microscopy (CLSM) (LSM510, Carl Zeiss). Three-dimensional images were constructed by a 3D projection function after digital sectioning through the entire microbeads (Z-stacks). A quantitative assessment of the relative proportion of live and dead cells was performed by counting live and dead cells through the Z-stacks obtained from the microbeads (n=10).

2.11. MTT assay

Mitochondrial activity was assessed by MTT assay. Cell cultures on flat alginate gels ore encapsulated cells were incubated with culture media conditioned with 0.5mg/ml MTT for 4h at 37ºC. For the flat gels, the media was harvested and cells sampled by centrifugation. Subsequently, 400µl DMSO and 50µl 0.1M glycine/0.1M NaCl were added to the cell pellets. For the microbeads, the media were removed and further processes as described (41). For each condition, 200µl of the colour solution were analysed at 570nm on a Victor^3 Multilabel Plate Reader (Perkin Elmer).

3. Results

3.1. Characterisation of modified alginates

Coupling of RGD-peptide (GRGDSP) to mannuronan was characterised by NMR spectroscopy using homo- and heteronuclear spectra (Figure 1). The anomeric signals for mannuronan and both Hβ, Hγ from proline and arginine were used to determine the degree of substituted C-5 mannuronan units with RGD peptide. Due to relatively large differences in concentration between mannuronan and RGD-peptide, which resulted in a high signal-to-noise ratio in combination with complex coupling for the peptide proton, this will only give a rough estimate for the specific coupling reaction. The NMR spectra also revealed some by-products from the coupling reaction which, based on NMR spectra, could be assigned to urea derivatives and N-

acylurea bound to mannuronan. The remaining urea derivatives were removed during the activated carbon filtration, while the N-acylurea was still bound to the alginate. A negative control experiment with N-acylurea-bound alginate did not show any effects on viability and morphology of OECs in 2D culture. Prior to the coupling of RGD-peptide to mannuronan, both compounds were characterised using standard 1D and 2D homonuclear spectra in order to check their purity (data not shown).

NMR spectroscopy was also used to determine the composition of the alginates as previously described (34). Figure 2 shows the anomeric regions of alginate before and after epimerisation. From the spectra, we calculated the molar fractions of monomers, the four possible diads (F_{GG}, F_{MM}, F_{MG}, F_{GM}), and the G-centered triads (F_{GGG}, F_{MGG}, and F_{GGM} and F_{MGG}), which allow calculation of the average length of the G-blocks ($N_G>1$), a value that correlates well with the gel forming capacity of the polymers. The sequence data, the peptide content, and intrinsic viscosities are provided in Table 1. The epimerisation reactions were tailored to produce alginates containing alternating structures of M and G interspacing G-blocks. Indeed, high-G and low-G alginates with and without peptides were produced in both batches of alginates for 2D and 3D experiments. As can be seen for the alginates produced for 2D experiments, the F_{MM} was lower than for the naturally occurring alginate used as controls. Hence, about every second M was converted to G by the AlgE4 epimerase. For the alginates produced for the 3D experiments, the conversion of M-blocks to MG-blocks is not that obvious, indicating inefficient treatment of the mannuronan by AlgE4. However, the efficiency of AlgE6 is shown given that long G-blocks ($N_{G>1}\geq2$) were produced for all high-G samples. As described above, the peptide content was estimated by NMR spectroscopy to be about 0.2% and 0.1% of the uronic acid units grafted with peptide for the 2D and 3D experiments, respectively. As expected, the content of peptide was the same within each batch, as the same background (i.e. mannuronan with peptide) was used for the production of epimerised alginates. The grafted peptide did not seem to affect the epimerisation reaction as a higher G-content (F_G) was found for the peptide grafted alginates compared to the non-grafted alginates. Table 1 also shows that a slight reduction in intrinsic viscosity was seen for all samples compared to the mannuronan starting material, probably as a result of handling and hydrolysis.

3.2. Interactions between alginates and OECs

3.2.1. Flat gels

Microscopic examination of the OEC 2D cultures after Live/Dead staining revealed distinct morphological differences in OECs contingent on the type of substrate used (Figure 3). On the

RGD substituted alginates, i.e. RGD LG and RGD HG, the OECs hade formed small clusters and they were rounded, with short bipolar processes attached to the gel surface. By contrast, OECs seeded on the non-substituted alginates, i.e. Alg LG, Alg HG, and UPLVG, were spherical and had formed aggregates floating in the culture media, These cells demonstrated poor survival, with all cells being lost, especially those cultured on UPLVG gels, by 72h post-seeding. Control OEC cultures on PLL-treated surfaces displayed normal morphology, i.e. the majority of cells were spindle-shaped, interspersed with some stellate-shaped cells.

The mitochondrial activity of OECs cultured on different substrates was assessed between 24h and 72h post-seeding. Irrespective of type of alginate substrate, the mitochondrial activity of OECs decreased by 48h, possibly reflecting a relatively low attachment ratio. An increase in mitochondrial activity was observed, however, for OECs cultured on PLL-coated surfaces, consistent with better attachment and survival.

3.2.2. Microbeads

Live/Dead assay on encapsulated OECs (3D cultures) showed that the cells were able to survive within the microbeads, irrespective of alginate type or modification used. Interestingly, OECs encapsulated in the coupled as well as non-coupled alginates had arranged themselves within channels formed in the alginate in a characteristic fireworks-like pattern. The relative proportions of live cells/microbead were estimated from digital sectioning through the microbeads (Z-stacks) using CLSM. Overall OEC survival, however, inside the microbeads deteriorated between 9d and 16d post-encapsulation (Figure 3).

3.3. Interactions between alginates and C2C12 myoblasts

3.3.1. Flat gels

Microscopic examination of the C2C12 myoblasts cultured on RGD-coupled alginate , i.e. RGD LG, RGD HG, small clusters of cells (Figure 4). Similar to OECs, the myoblasts were rounded and extended protrusions attached to the gel surface. However, no myoblast attachment was observed on the non-coupled alginates, i.e. Alg LG, Alg HG, and UPLVG). Instead, large cell aggregates were observed, floating in the media (Figure 4). On cell culture plastic, however, the myoblasts were attached and spread across the gel surface (Figure 4).

The mitochondrial activity of myoblasts was higher when cultured on the RGD-coupled alginates, compared to non-coupled ones. For myoblasts cultured on RGD HG, the

mitochondrial activity was consistent during the 72h period, compared to decreasing mitochondrial activity observed on the RGD LG gels. The highest levels of mitochondrial activity though were observed on myoblasts on cell culture plastic (Figure 4).

3.3.2. Microbeads

Live/Dead assay demonstrated long-term myoblast survival throughout the observation period (41d), irrespective of type of alginate used for the 3D cultures, although dead cells were also observed in the microbeads (Figure 4). Similar to OECs, myoblasts cultured in RGD LG and Alg LG had arranged themselves within channels, showing a distinct fireworks-like pattern. While mostly dead cells were observed in these channels at 1d-10d, the channels were subsequently filled with live myoblasts.

The mitochondrial activity of myoblasts encapsulated in RGD-coupled (RGD HG, RGD LG, batch1) and non-coupled (Alg HG, Alg LG, and UPLVG) alginates was monitored over a period of 38d. At 1d-14d post-encapsulation, The mitochondrial activity of myoblasts in RGD HG and Alg HG alginates was lower than in RGD LG, Alg LG, and UPLVG. After 20d, the mitochondrial activity of myoblasts ad increased in all types of microbeads. Compared to unmodified alginates, the RGD-modified alginates did not seem to impact myoblast viability in the 3D cultures, rather the differences observed seem to relate more to the M and G content of the alginates.

DISCUSSION

Although there is intense interest in peptide coupled alginates in the field of tissue engineering and an increasing number of studies are using RGD-coupled alginates (REFs), little has been described regarding the composition of the polymers utilised in these studies. Hence, the aim of this study was to monitor cell attachment and survival using structurally-designed and well-characterised RGD-coupled alginates. The peptide alginates were produced by chemoenzymatic modification, ensuring peptide coupling to the M-units in the alginate by first introducing peptide to mannuronan and subsequently introducing MG- and G-blocks by epimerisation. In this way, the peptide coupling on the carboxyl group of the uronic acids does not interfere with G-blocks being the main contributor to alginate gel formation. This chemoenzymatic strategy has previously been shown for galactose (36) and methacrylate substituted alginates (32).

The peptide coupling was characterised by NMR showing that 0.1-0.2% of the monomers were substituted by peptide. As 5% of the carboxyl groups were activated by EDC and Sulfo-NHS,

and excess of peptide was added, the yield is 2-4%. The basis of this coupling reaction was the work of Rawley and Mooney (29), reporting 55-60% incorporation efficiency using carbodiimide chemistry. Even though the results from the NMR characterisation are a rough estimate, this cannot explain the difference in the coupling efficiency (29). Furthermore, NMR spectroscopy allows identification of other products from the carbodiimide coupling reaction. This information was used to adapt downstream protocols for processing of the RGD-coupled alginate. Especially, some of the by-products and non-reacted peptides were not removed during dialysis. This can lead to an apparently higher incorporation efficiency. The NMR data show that the active coal filtration was efficient to remove most of these products from RGD-coupled alginates, except for N-acylurea bound to alginate. Nonetheless, it can be concluded that NMR spectroscopy can be used to determine the coupling efficiency, as well as to monitor the effects of the downstream process on the RGD-coupled alginates.

In addition to the above, NMR was used to characterise alginate composition. The relevant findings were consistent with what is previously shown for enzymatic modifications of mannuronan with the mannuronan C-5 epimerases AlgE4 and AlgE6 (33,34). The alginate was tailored to contain a majority of G- and MG-blocks forming stable gels with divalent ions (33). Furthermore, we aimed to produce alginates with different contents of G-blocks, as this has been demonstrated to give gels of different stiffness (34), which has been shown to impact differentiation of C2C12 myoblasts (29). As expected, compared to UPLVG alginate, the epimerized alginates had a reduced fraction of M dimers and trimers and a higher number of MG-blocks. Furthermore, F_{MM} values in peptide coupled and non-coupled equivalents were similar, indicating that the peptide did not significantly interrupt the epimerization reaction at a degree of substitution of 0.1%-0.2%.

Compared to batch 2, epimerised alginates in batch 1 had similar F_G values, and showed better homology between the RGD-coupled alginates and their non-coupled equivalents. However, while batch 2 alginates had similar F_G values, the G-content in the RGD LG and Alg LG alginates was much lower than that of the LG alginates in batch 1 (i.e. $F_G \approx 0.3$, compared to $F_G \approx 0.5$). This difference in the G-content can be explained by the fact that the reaction time for batch 2 alginates was 24h, as opposed to 48h used for batch 1. Thus the stability of batch 1 alginate gels should be expected to be higher than that of batch 2 alginates given that substituting M-blocks with MG-blocks has been shown to produce more stable alginates (33, 34, 42).

The intrinsic viscosity of the alginates was found to decrease slightly during peptide coupling and epimerization. Vold et al (43) showed that there are no differences in chain stiffness

between poly M, poly G, and poly MG alginates. The coupling and epimerization reactions *per se* do not result in chain breakage, however a degree of hydrolysis upon handling of the gels may explain the observed reduction in viscosity. Nonetheless, intrinsic viscosity values, irrespective of sample type, are comparable.

Subsequent to characterization, we proceeded to test the interactions of the modified alginates with OECs and myoblasts, two different cell types with therapeutic potential in regenerative medicine and tissue engineering.

OECs are unique glia that have emerged as promising candidates in the repair of CNS lesions (37, 44, 45), however, OEC survival post-transplantation may be expected to be as low as 50% (44). Despite the fact that encapsulation of OECs in biopolymer matrices may improve cell survival, few studies have explored this line of research (46, 47), while, to our knowledge, there is only one study of OEC culture on alginate (48). The specific study utilised unmodified alginate alone, and in combination with fibronectin, for culture of OEC monolayers and showed that OECs were transformed to atypical cells and displayed inhibited metabolic activity (48).

In our study, OECs cultured on RGD alginate flat gels formed large clusters of spherical cells with bipolar protrusions attached to the gels. The particular morphotype was similar to that of myoblasts cultured on RGD alginates, and also consistent with a previous report, in which OEC morphologic plasticity and motility were associated with rapid changes in mitotic activity (49). There were no obvious differences in OEC morphology and pattern between RGD LG and RGD HG alginates, suggesting that while the RGD peptide may facilitate OEC adhesion, gel rigidity does not inhibit or promote OEC attachment.

The above observations indicate that the presence of RGD in the alginate, induced an effect to the OEC 2D cultures in terms of cell-cell, cell-substrate interactions. It can be argued that the specific morphology and pattern observed in OEC cultured on RGD-coupled alginate gels are indicative of a dynamic intracellular response in terms of altered gene/protein expression, transcription and translation, causing a modulation of cytoskeletal structure. It would be interesting to elucidate which factors in OECs are upregulated and downregulated in response to RGD-coupled alginates, however, such investigations would necessitate the use of proteomic and/or genomic methods and were, therefore, beyond the scope of the present study.

Encapsulation of OECs in RGD-coupled alginates (3D cultures) did not induce an effect on OECs in terms of improved survival. This suggests that further modifications may be required to

develop a tailor-made alginate substrate for optimisation of OEC function and survival with regard to future transplantation studies. Such modifications may include coupling more RGD-peptide to the alginate, further modulating the mechanics of the alginate microbeads, as well as testing whether higher concentrations of encapsulated OECs may display improved survival.

Myoblasts are proposed for repair of skeletal muscle and myocardial tissue (50, 51) and have been widely used in conjunction with biopolymer matrices (32, 50, 51, 52). Rowley and Mooney (29) have established that RGD alginates mediate myoblast differentiation into multi-nucleated myofibrils. In our study, however, we were not able to replicate these findings as the myoblasts remained undifferentiated, displaying unaltered morphology throughout the observation period. The lack of differentiation may be attributed to the fact that the RGD densities in our alginates were significantly lower than those used by Rowley and Mooney (29). However, control myoblast cultures, using plastic as a substrate, supplemented with differentiation media, also remained undifferentiated in this experiment. Therefore, we cannot exclude the possibility that the myoblasts utilized in this study had lost their differentiation capacity.

There was a discrepancy between the low mitochondrial activity of myoblasts encapsulated in RGD HG and Alg HG alginates and the relatively large proportion of live cells observed in these microbeads during the first week after encapsulation. The comparatively low metabolic activity may have denoted a differentiation process, however, CLSM observations did not confirm any signs of cell fusion.

The overall survival/growth of the myoblasts during the 42d observation period showed only small differences in mitochondrial activity as well as the proportion of live and dead cells within the Alg HG and UPLVG microbeads. We interpret these findings as indicative of 1-2 cell divisions during the 41d encapsulation period, as shown previously (32).

It is noteworthy that relatively improved myoblast survival was observed in the RGD LG and Alg LG microbeads, where the cells had arranged themselves in a characteristic fireworks-like pattern inside channels formed inside the microbead. The channels formed in the microbeads could be attributed to viscous drag that may have occurred during gelling. Myoblasts, have been previously shown to have inhibited growth after encapsulation (32, 52). Our observations thus indicate that myoblasts may have been able to expand within these channels, which are assumed to contain lower gel concentrations, or may even represent gel-free pockets. Interestingly, there is a better correlation between MTT data and Live/Dead CLSM data from the LG alginate microbeads throughout the observation period, compared to the other alginate

types. Furthermore, the overall metabolic activity of myoblasts encapsulated in the LG alginates was consistently higher than that of myoblasts in HG and UPLVG alginates. It is known that increased solid stress on cells may affect proliferation and survival (41). On the other hand, a higher level of capsule stability in terms of increased G-content may be desirable with a view to *in vivo* transplantation (53). Our findings suggest, however, that a lower degree of network rigidity, rather than grafting the alginates with RGD peptide alone, may be a key component for improved myoblast survival post-encapsulation *in vitro*.

Taken together, the findings from the myoblast and OEC 2D and 3D studies did not reveal enhanced cell adhesion and survival *in vitro* as a result of RGD grafting, despite observed attachment of cells, morphological changes and partially improved viability in 2D cultures for some of the surfaces . Nonetheless, we predict that improved attachment and survival of OECs and myoblasts should be achieved with higher percentage of coupled peptides in the alginates. We also recognise that further modifications and tailoring of the alginates may be required with a view to transplantation of encapsulated OECs and myoblasts *in vivo,* especially with regard to the former as little is known about OEC interactions with modified alginates. Furthermore, given that different types of CNS lesions require different reparative strategies, it is important to consider that the alginate substrates used in conjunction with OEC transplantation need to be tailored accordingly. For example, if OEC transplantation aims at neuroprotection, as in the case of intravitreal grafts to promote optic nerve regeneration after injury, optimisation of OEC survival and function post-transplantation, in terms of sustained secretion of neurotrophic factors, may be effected by encapsulation and associated changes in the 3D microenvironment of the cells. If, however, OEC transplants aim to stimulate directed axonal growth towards synaptic targets distal to the lesion site, as for example in a spinal cord injury scenario, an appropriate RGD-coupled alginate matrix should be in the form of a scaffold which, should serve the dual purpose of mediating improved function and survival of seeded OECs, as well as providing an appropriate substrate for regenerating RGC axons in terms of structural and molecular support, by mimicking ECM functions. Finally, other types of CNS lesions, such as stroke-induced cavities, might require transplantation of microbeads with cells seeded on the surface, to act as scaffolds and induce *de novo* neurogenesis within the lesion cavity (54). The importance of unique strategies for alginate modification as well as appropriate characterisation of the modified alginates are of great relevance in this context.

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TABLE 1

		F_G	F_M	F_{GG}	$F_{MG/GM}$	F_{MM}	$F_{GGM/MGG}$	F_{MGM}	F_{GGG}	$N_{G>1}$	[η] (ml/g)	Peptide substitution
Mannuronan		0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1614	
Batch	**Alginate type**											
1 (3D cultures)	Alg LG	0.52	0.48	0.16	0.36	0.13	0.04	0.34	0.12	4.00	1117	
	RGD LG	0.55	0.45	0.20	0.35	0.10	0.04	0.34	0.16	5.00	909	<0.20%
	Alg HG	0.66	0.34	0.40	0.26	0.08	0.04	0.25	0.36	12.0	1087	
	RGD HG	0.71	0.29	0.49	0.22	0.07	0.03	0.20	0.46	15.0	1290	<0.20%
2 (2D cultures)	Alg LG	0.36	0.64	0.19	0.16	0.48	0.03	0.15	0.17	7.00	1290	
	RGD LG	0.31	0.69	0.14	0.16	0.53	0.03	0.14	0.12	5.00	1210	~0.10%
	Alg HG	0.76	0.24	0.65	0.12	0.12	0.03	0.09	0.61	21.0	1040	
	RGD HG	0.69	0.31	0.54	0.14	0.17	0.04	0.11	0.56	16.0	1060	~0.10%
Control	UPLVG	0.67	0.33	0.55	0.12	0.21	0.05	0.09	0.50	12.0	1072	

Table 1 - Sequence data, peptide content, and intrinsic viscosities of modified alginates calculated from the NMR spectra.

Figure 1

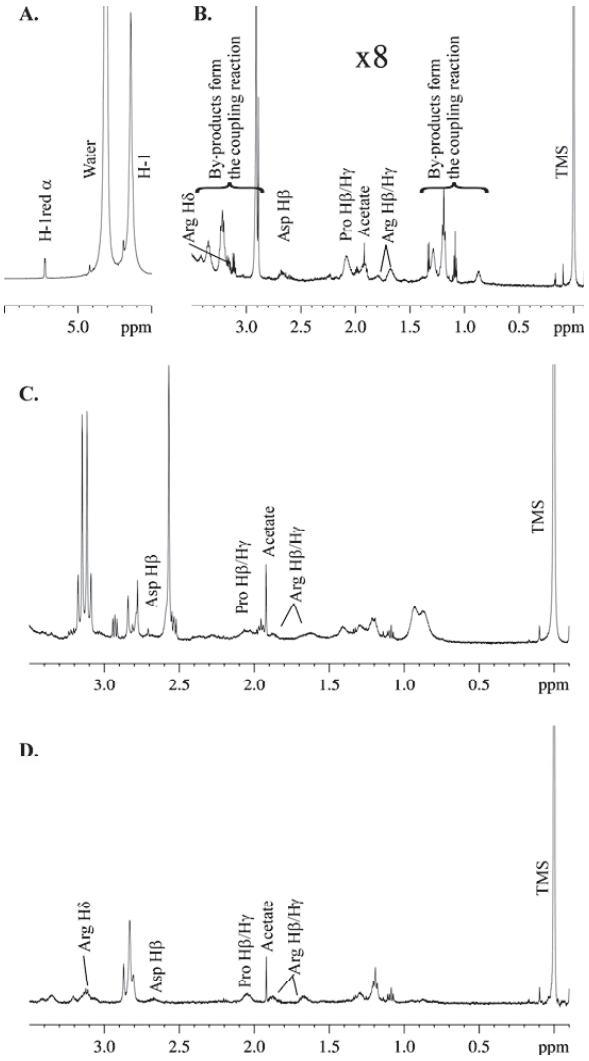


Figure 1 - 1D proton spectrum RGD-peptide grafted mannuronan at 40 °C. **A.** The anomeric region. **B.** The aliphatic region. The anomeric proton of mannuronan and both Hβ, Hγ from proline and arginine were used to determine the degree of substituted C-5 mannuronic acid units with RGD-peptide. By-products were assigned to consist mainly of urea derivatives and N-acylurea bound to mannuronan. **C.** The aliphatic region before activated coal treatment **D.** The

aliphatic region after the activated coal treatment. The chemical shifts vary slightly given the equal pH of the samples. Acetate was an impurity from the freeze dryer.

Figure 2

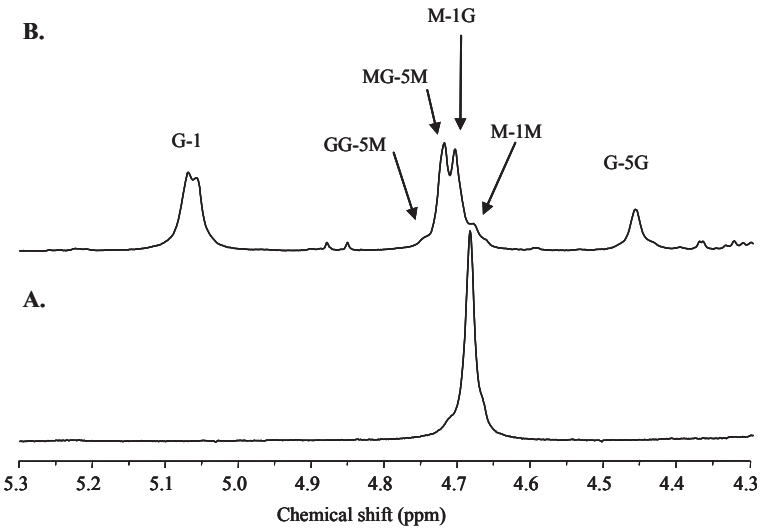


Figure 2 - 1D proton spectrum of epimerased RGD-peptide grafted alginate at 90 °C. **A.** The anomeric region for the RGD-peptide grafted alginate before epimerisation **B.** after epimerasation.

Figure 3

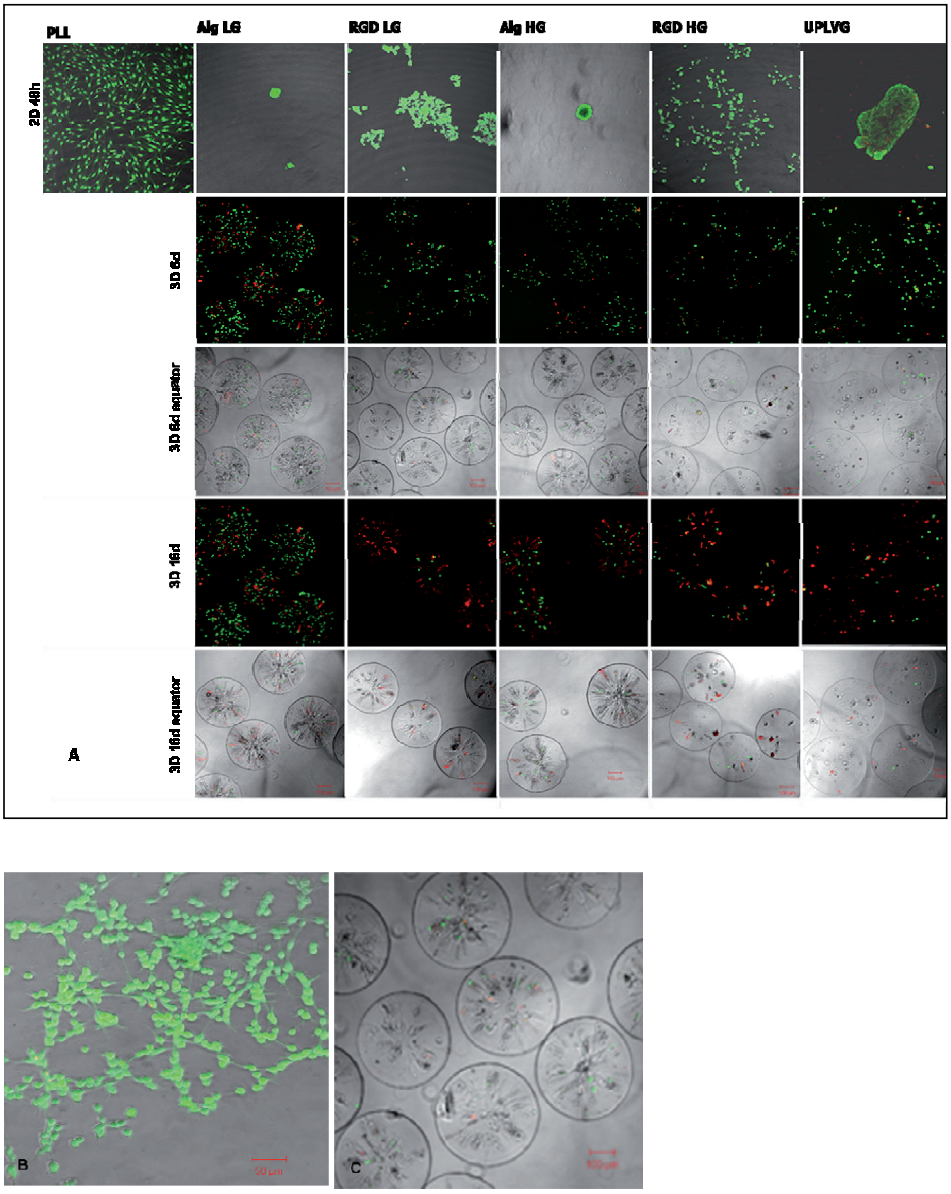
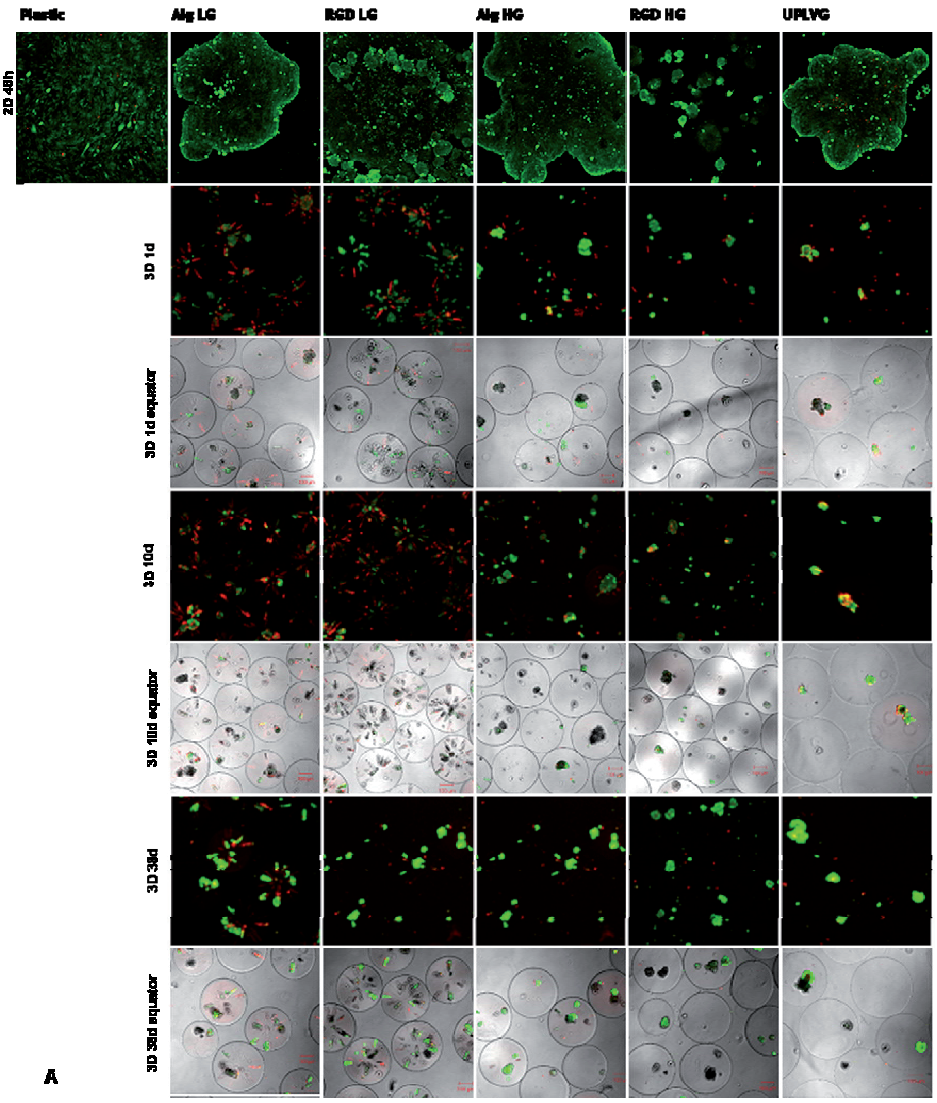


Figure 3 - Live/dead assay showing overall OEC survival and interaction profiles with different alginate substrates. **A.** Representative images of 2D ad 3D cultures. Upper row shows 2D OEC cultures at 6d post-seeding. OECs seeded on RGD-modified alginates display altered

morphology compared to controls (PLL substrate). Instead of spindle shaped, the cells are rounded and extend bipolar protrusions attached to the gel surface (shown in higher magnification in **B**). Second and fourth rows show representative images from Live/Dead assays on 3D OEC cultures at 6d and 16d. Overall OEC survival declines between 6d and 16d, irrespective of substrate. Equator images, representing cross sections through the different types of alginate microbeads at the same time points, show that the size of the microbeads is stable over time. **C** Higher magnification of RGD LG microbeads at 6d post-encapsulation showing characteristic arrangement of OECs in a fireworks-like pattern.

Figure 4


Plastic
Alg LG
RGD LG
Alg HG
RGD HG
UPLVG
2D 48h
3D 1d
3D 1d equator
3D 10d
3D 10d equator
3D 30d
3D 30d equator
A

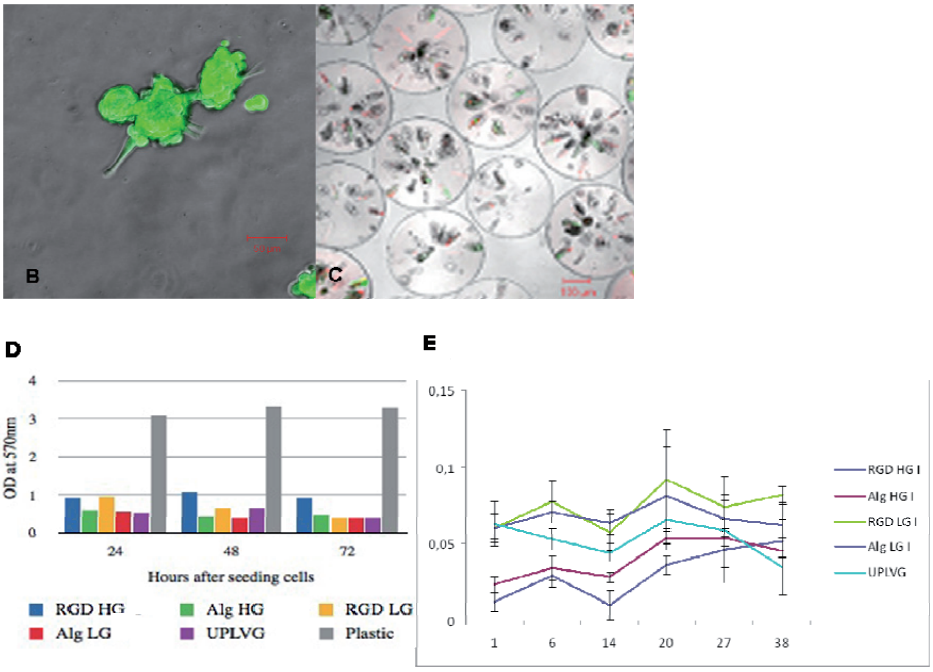


Figure 4 - Interaction profiles of myoblasts with different alginate substrates. **A.** Representative images of 2D ad 3D cultures. Upper row shows 2D myoblast cultures at 48h post-seeding. Myoblasts seeded on RGD-modified alginates display altered morphology compared to controls (plastic substrate). The myoblasts are rounded and extend bipolar protrusions attached to the gel surface (shown in higher magnification in **B**). Second to sixth row showing Live/Dead assay from 3D myoblast cultures at 1d, 10d, and 38d post-encapsulation. Relatively improved myoblast survival was observed in the RGD LG (shown in higher magnification in **C**) and Alg LG microbeads, where the cells had arranged themselves in a characteristic fireworks-like pattern inside channels formed inside the microbead. Equator images from the three different time points demonstrate that the different types of microbeads were stable throughout the observation period. **D, E** show mitochondrial activity of myoblasts from the 2D and 3D cultures respectively. The overall survival/growth of the myoblasts during the observation period showed only small differences in mitochondrial activity as well as in the proportion of live and dead cells within the Alg HG and UPLVG microbeads.

Dissertations at the Faculty of Medicine, NTNU

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1978

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151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.

162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
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168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
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184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
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187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
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189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
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192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
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199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
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204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
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206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
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208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
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223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
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227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
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234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
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238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY

243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
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246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
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252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE

271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
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274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
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276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
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285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OF PATHOLOGICAL GAMBLING IN NORWAY
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289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
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292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
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296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN

297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
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308. Mathias Toft: GENETIC STUDIES OF LRRK2 AND PINK1 IN PARKINSON'S DISEASE
309. Ingrid Løvold Mostad: IMPACT OF DIETARY FAT QUANTITY AND QUALITY IN TYPE 2 DIABETES WITH EMPHASIS ON MARINE N-3 FATTY ACIDS
310. Torill Eidhammer Sjøbakk: MR DETERMINED BRAIN METABOLIC PATTERN IN PATIENTS WITH BRAIN METASTASES AND ADOLESCENTS WITH LOW BIRTH WEIGHT
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312. Olav Magnus Søndenå Fredheim: HEALTH RELATED QUALITY OF LIFE ASSESSMENT AND ASPECTS OF THE CLINICAL PHARMACOLOGY OF METHADONE IN PATIENTS WITH CHRONIC NON-MALIGNANT PAIN
313. Anne Brantberg: FETAL AND PERINATAL IMPLICATIONS OF ANOMALIES IN THE GASTROINTESTINAL TRACT AND THE ABDOMINAL WALL
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315. Elin Tollefsen: RESPIRATORY SYMPTOMS IN A COMPREHENSIVE POPULATION BASED STUDY AMONG ADOLESCENTS 13-19 YEARS. YOUNG-HUNT 1995-97 AND 2000-01; THE NORD-TRØNDELAG HEALTH STUDIES (HUNT)
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318. Torbjørn Dahl: CAROTID ARTERY STENOSIS. DIAGNOSTIC AND THERAPEUTIC ASPECTS
319. Inge-Andre Rasmussen jr.: FUNCTIONAL AND DIFFUSION TENSOR MAGNETIC RESONANCE IMAGING IN NEUROSURGICAL PATIENTS
320. Grete Helen Bratberg: PUBERTAL TIMING – ANTECEDENT TO RISK OR RESILIENCE ? EPIDEMIOLOGICAL STUDIES ON GROWTH, MATURATION AND HEALTH RISK BEHAVIOURS; THE YOUNG HUNT STUDY, NORD-TRØNDELAG, NORWAY
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322. Olav Sande Eftedal: ULTRASONIC DETECTION OF DECOMPRESSION INDUCED VASCULAR MICROBUBBLES
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324. Svein Brekke: TECHNIQUES FOR ENHANCEMENT OF TEMPORAL RESOLUTION IN THREE-DIMENSIONAL ECHOCARDIOGRAPHY
325. Kristian Bernhard Nilsen: AUTONOMIC ACTIVATION AND MUSCLE ACTIVITY IN RELATION TO MUSCULOSKELETAL PAIN
326. Anne Irene Hagen: HEREDITARY BREAST CANCER IN NORWAY. DETECTION AND PROGNOSIS OF BREAST CANCER IN FAMILIES WITH *BRCA1*GENE MUTATION
327. Ingebjørg S. Juel : INTESTINAL INJURY AND RECOVERY AFTER ISCHEMIA. AN EXPERIMENTAL STUDY ON RESTITUTION OF THE SURFACE EPITHELIUM, INTESTINAL PERMEABILITY, AND RELEASE OF BIOMARKERS FROM THE MUCOSA
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