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Characterisation of calcium signalling and functional development in an immortalised human neural progenitor cell line

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Peter James Morgan

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Reviwers

1. Prof. Dr. Dieter Weiss

Institute for Cell Biology and Biotechnology, University of Rostock

2. Prof. Dr. Arndt Rolfs

Albrecht-Kossel-Institute for Neuroregeneration

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Rostock, 21.07.2011

Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
AP5	D(−)-2-Amino-5-phosphonopentanoic acid
ATP	adenosine tri-phosphate
βIII	βIII-tubulin
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
Cbx	cabenoxolone
CaMK	calmodulin-dependent protein kinase
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CREB	cAMP response element-binding protein
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DD	days differentiation
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DREAM	downstream responsive element antagonist modulator
ECM	extracellular matrix
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ESC	embryonic stem cell
FACS	Fluorescence Activated Cell Sorting
FGF-2	fibroblast growth factor-2
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HBS	HEPES buffered saline
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hNPC	human neural progenitor cell
HSA	human serum albumin
$InsP_3$	inositol 1,4,5-trisphosphate
iPSC	induced pluripotent stem cell
MAPK	mitogen-activated protein kinases
MEF-2	myocyte enhancer factor-2
MEM	minimum essential medium
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NGS	normal goat serum
NMDA	*N*-methyl-D-aspartate

NSC	neural stem cell
PBS	phosphate buffered saline
PFA	paraformaldahyde
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PPADS	pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate
PreP	preaggregation culture protocol
PSC	post-synaptic current
RuR	ruthenium red
RyR	ryanodine receptor
TH	tyrosine hydroxylase
Thap	thapsigargin
TRP	transient receptor potential
TTX	tetrodotoxin
StdP	standard culture protocol
Vtr	Veratridine

Contents

1. Introduction

Stem cells are the precursor cells from which all mature cell types derive. As it has become possible to isolate and culture these cells hopes have been raised that they could be used to treat neurodegenerative disorders, such as Parkinson's, Alzheimer's and Huntington's diseases, spinal cord injury and stroke. With this in mind, understanding the biology of stem cells *in vitro* and their behaviour once transplanted into the brain is of great interest. In this study I have characterised the functional properties of an immortalised human fetal neural progenitor cell line (hNPC), ReNcell VM, and investigated its integration into an *in vitro* model neural environment.

1.1 Stem cells

Two characteristics define stem cells: self renewal and the ability to differentiate into other cell types. Whether stem cells proliferate, renew or differentiate is reflected in their mode of division. Proliferating stem cells divide symmetrically to produce two identical copies of themselves, while self renewal is characterised by asymmetric division, where one copy and one more differentiated cell are produced. Finally, at later stages of development stem cells can undergo a second type of symmetric division, where two differentiated cells are produced (Fig. 1; Götz and Huttner 2005).

During development stem cells go through a precisely coordinated program of signals, both extracellular and intrinsic, through which each cell population emerges at specific times and locations (for reviews see Wurst and Bally-Cuif 2001, Lupo et al. 2006, Kreigstein and Alvarez-Buylla 2009). As a consequence of this, the age and location from which stem cells are derived informs their self renewal capacity, their differentiation potential and the instructive signals they require to adopt a specific phenotype.

The initial stem cell population is pluripotent, they can differentiate into all tissue types. Pluripotent embryonic stem cells (ESCs) are derived from the inner mass of the blastocyst, before germ layer formation and the initial steps towards tissue specification. When cultured *in vitro* these cells have robust self renewal capacity and can be maintained over long periods of time. However, their proliferative nature means they have a high risk of forming teratomas, which is a significant barrier to their clinical application. These cells also

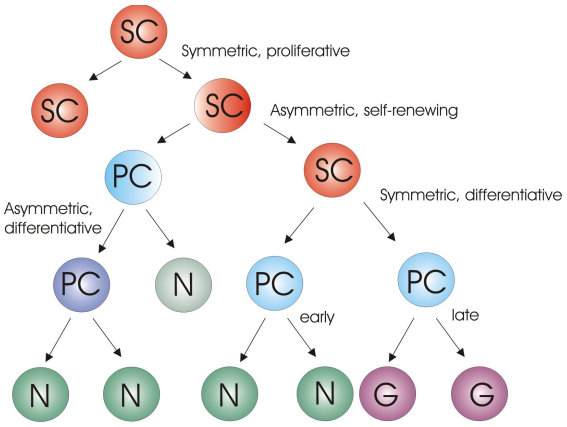

Figure 1. Model of stem cell division

A model demonstrating the different types of division stem cells undergo. The same stem cell population produces neurons (early) and glia (later), depending on the time at which they differentiate. Abbreviations: SC stem cell, PC progenitor cell, N neuron, G glia. Modelled on Götz and Huttner (2005).

require more guidance to differentiate into specific cell types, though protocols are continually improving and directed neuronal differentiation can be achieved *in vitro* (Bibel et al. 2004, Yan et al. 2005, Cho et al. 2008, Lee et al. 2010). Recently, induced pluripotent stem cells (iPSCs) have been created by reprogramming adult cells to an earlier developmental state (Takahashi and Yamanaka 2006). These cells are very similar to ESCs, but have the advantage of not requiring embryonic tissue. They could also be derived from individual patients, negating the need for immunosuppression to prevent rejection of transplanted cells. However, the low efficiency of induction and differentiation pose significant technical challenges to be overcome (Hu et al. 2010). As with ESCs, these cells are highly proliferative and have a high risk of tumor formation, which appears enhanced by the genetic modifications which induce pluripotency (Okita et al. 2007).

Following the formation of germ cell layers, cells become ever more restricted to specific lineages and regional identities. The first neurally committed stem cells (NSCs), neuroepithelial cells, emerge with the formation of the neural plate. The neural plate subsequently forms the neural tube, in which stem cell identity is regionally specified along the dorso-ventral and rostro-caudal axes by gradients of morphogens (Hynes and Rosenthal 1999, Wurst and Bally-Cuif 2001). Stem cells derived from these regions are less proliferative than ESCs, and therefore are less likely to form tumors, however they are also less able to maintain their 'stemness' over long periods *in vitro*.

After the formation of the neural tube stem cells increasingly undergo asymmetric divisions, producing progenitor cells which are more restricted in their differentiation potential. Like stem cells, progenitor cells can divide and differentiate into one or several cell types, but are distinct in that they lack the ability to self renew, and with each division

produce more differentiated daughter cells (Götz and Huttner 2005, Kreigstein and Alvarez-Buylla 2009). The distinction between stem and progenitor cells during natural development is not well defined, here the phrase 'stem cells' will refer generally to both stem and progenitor cells. When cultured *in vitro* progenitor cells can only propagate for a few passages before becoming permanently quiescent. Their advantages for stem cell therapies are their restricted potency and differentiation into defined cell types, and their limited ability to proliferate means they pose little risk of forming tumors. A significant disadvantage is their inability to be maintained in culture, meaning that new tissue donors would be required for each application, precluding their use as a routine therapy. This might be overcome by immortalisation with the oncogenic factor c-Myc or its viral homologue v-myc (Dang et al. 1999, Lee and Reddy 1999), and several immortalised human neural progenitor cell lines have been established using such methods (Flax et al. 1998, Villa et al. 2000, Lotharius et al. 2002, De Filippis et al. 2007, Donato et al. 2007, Villa et al. 2009). Furthermore, although v-myc immortalised cell lines cannot be transplanted into humans, a cell line conditionally immortalised with c-myc has been approved for clinical trials in stroke (ReNeuron Ltd 2010). Cell lines immortalised using v-myc have been shown to be stable over long periods in culture (Villa et al. 2004), however, v-myc and c-myc can induce genomic instability, increase apoptosis and impair differentiation (Coppola and Cole 1986, Dang et al. 1999, Lee and Reddy 1999). Despite this, these cell lines offer a useful model for neural progenitor development and have relevance for clinical stem cell therapies.

1.2 Therapeutic potential of stem cells

The use of stem cells as a therapy will depend on their ability to provide a safe, efficacious treatment which improves on the other available options. Where diseases are characterised by loss of specific cell populations, such as dopaminergic neurons in Parkinson's disease or motor neurons in amyotrophic lateral sclerosis, it might be possible to replace lost cells through transplantation of stem cells directed to a specific fate. More generally, stem cells could provide support to at-risk cell populations, reduce pathological inflammation, and promote endogenous mechanisms of brain repair (Goldman 2005, Lindvall and Kokia 2010).

Clinical trials have been performed in Parkinson's disease patients using grafts of human fetal tissue, but despite promising results from early trials double blind controlled studies

found only marginal improvements, and in some cases patients developed adverse side effects (Freed et al. 2001, Olanow et al. 2003, reviewed in Winkler 2005). In contrast, much greater success has been seen in animal models of Parkinson's disease, where functional benefits have been found using human and mouse embryonic stem cells (Kim et al 2002, Takagi et al. 2005, Roy et al. 2006, Yang et al. 2008), neural stem and progenitor cells (Studer et al. 1998, Yasuhara et al. 2006, Redmond et al. 2007), and iPSCs (Wernig et al 2008, Hargus et al. 2010).

There is evidence for several mechanisms which could underlie the therapeutic effects. Stem cells have been shown to secrete neuroprotective and trophic factors and to increase the survival of endogenous dopaminergic neurons (Lu et al. 2003, Yasuhara et al. 2006, Redmond et al. 2007). Functional improvements, and lack thereof, have also been correlated with the number of grafted tyrosine hydroxylase (TH) positive cells, suggesting action through dopamine release (Brederlau et al. 2006, Yang et al. 2008).

Despite their successes, several issues emerge from animal studies. Graft survival tends to be low, requiring a large volume of cells to achieve sufficient cell numbers, and the majority of surviving cells remain at the graft site, which limits their therapeutic reach. The differentiation of dopaminergic neurons from human cells is also generally low, and the survival of neurons has been seen to decrease over time (Roy et al. 2006, Yasuhara et al. 2006). Though engrafted neurons have been shown to develop functionally and to receive synaptic input from the host (Kim et al. 2002, Wernig et al. 2004, 2008), it remains unclear if these cells have any functional significance, and whether the environment will allow stem cells to functionally replace lost neurons. Finally, graft growth and tumor formation have been seen with ECSs and iPSCs, even where the cells had been pre-differentiated prior to transplantation (Brederlau et al. 2006, Roy et al. 2006, Yang et al. 2008), indicating that techniques would have to be improved before transplantation into humans. To overcome these issues a greater understanding of the processes underlying stem cell development and their integration into the host environment is required.

1.3 Calcium signalling in the development of stem cells

Of the many messengers cells use, Ca^{2+} is one of the most multi-faceted. It has well established roles in proliferation, apoptosis, survival, migration, differentiation and neurite

growth (Santella 1998, Berridge 2000, Orrenius et al. 2003, Gomez et al. 2006), and therefore understanding the Ca^{2+} signalling active in stem cells may offer insight into aspects of their development important for stem cell therapies.

1.3.1 Encoding calcium signals

The variety of Ca^{2+}'s roles results from its interaction with different Ca^{2+} sensing proteins, and their subsequent effect on intracellular signalling pathways. For example, calmodulin and calmodulin-dependent protein kinases (CaMKs), mitogen-activated protein kinases (MAPKs), several protein kinase C (PKC) isoforms, and calcineurin, to name but a few, are all regulated by Ca^{2+} (Berridge 2000), and gene transcription networks dependent on NFAT, NF-κB, DREAM, CREB and MEF-2, again to name but a few, are also regulated by Ca^{2+} and Ca^{2+}-activated signal cascades (Hogan et al. 2003, Mellström et al. 2008).

Having so many processes regulated by a single ion requires a coding system that allows signals to be discriminated. This is achieved through a combination of the frequency and amplitude properties of the Ca^{2+} signal, its spatial location, and the differing Ca^{2+} affinities of proteins (Berridge 2003, Rizzuto and Pozan 2006). This was demonstrated in B lymphocytes and Jurkat T cells, where the frequency and amplitude of Ca^{2+} signals differently activated NFAT and NF-κB dependent gene transcription (Dolmetsch et al. 1997, 1998). For this purpose cells have developed a broad range of ion channels, pumps, exchangers and buffers through which intracellular Ca^{2+} can be tightly controlled (Berridge 2003).

When a Ca^{2+}-permeable channel opens the increase in cytoplasmic Ca^{2+} will be greatest around the pore of the channel, and will decrease with distance as the Ca^{2+} diffuses through the cytoplasm. Unopposed, Ca^{2+} would then diffuse through the cytosol to reach a uniform concentration. However, due to the presence of Ca^{2+}-binding proteins much of this Ca^{2+} will be rapidly sequestered, producing mircodomains of differing Ca^{2+} concentrations within the cell (Naraghi and Neher 1997, Faas et al. 2011). Because of this the location of Ca^{2+}-binding proteins in relation to the source of Ca^{2+} will effect the Ca^{2+} dynamics they observe, and the subsequent activation of downstream signalling pathways. A stark example of this with relevance to cell replacement therapies is the activation of synaptic versus extrasynaptic *N*-methyl-D-aspartate (NMDA) receptors in neurons. Ca^{2+} influx through NMDA receptors has been shown to promote survival by activation of CREB and PI(3)K/Akt pathways, whereas

Ca^{2+} influx through extrasynaptic NMDA receptors opposed CREB activation and promoted apoptosis (Hardingham et al. 2002, Papadia et al. 2005). Therefore, the neuroprotective versus neurotoxic actions of glutamate are dependent on the type and location of the receptors activated, and a single ligand can have opposing effects in the same cell depending on those receptors activated.

It can be concluded that not all Ca^{2+} signals are equivalent, and that characterising each step, from entry through cascade activation to downstream effects, is important for understanding their biological role.

1.3.2 Routes of calcium entry

Ca^{2+} can be mobilised from two sources: the extracellular space and internal stores in the endoplasmic reticulum. Ion channels in the plasma and endoplasmic reticular membranes allow rapid Ca^{2+} influx into the cytosol in response to various stimuli, both extracellular and intracellular. On a much slower timescale, ion exchangers and pumps take up or extrude Ca^{2+} to maintain the resting concentration (Fig. 2; Berridge 2003, Clapham 2007).

Release from intracellular stores is mediated by inositol 1,4,5-trisphosphate receptors ($InsP_3$-Rs) and ryanodine receptors (RyRs). The activity of these channels is modulated through various mechanisms, but the principle agonists are $InsP_3$, for InsP3-Rs, and Ca^{2+} itself for both types of receptor. Many modifications (including $InsP_3$ binding) activate the channels by altering their sensitivity to Ca^{2+} (for in-depth reviews see Fill and Copello 2002, Foskett et al. 2007). With the appropriate conditions this enables Ca^{2+}-induced Ca^{2+} release and the propagation of Ca^{2+} signals across cells through activation of adjacent receptors (Cheng and Lederer 2008).

There are several ion channel families which allow influx of Ca^{2+} from the extracellular space. Of these, voltage-sensitive Ca^{2+} channels (Ca_v) are the best characterised They are divided into L-type ($Ca_v1.1$-1.4), P/Q-types ($Ca_v2.1$), N-type ($Ca_v2.1$-2.3) and T-type ($Ca_v3.1$-3.3) channels, all of which are activated by depolarisation and are highly Ca^{2+} selective (summarised in Catterall et al. 2005). In the brain they are principally found in neurons, where they shape neural activity patterns and underlie the Ca^{2+}-induced exocytosis of neurotransmitter at synapses (Reuter 1996, Bean 2007).

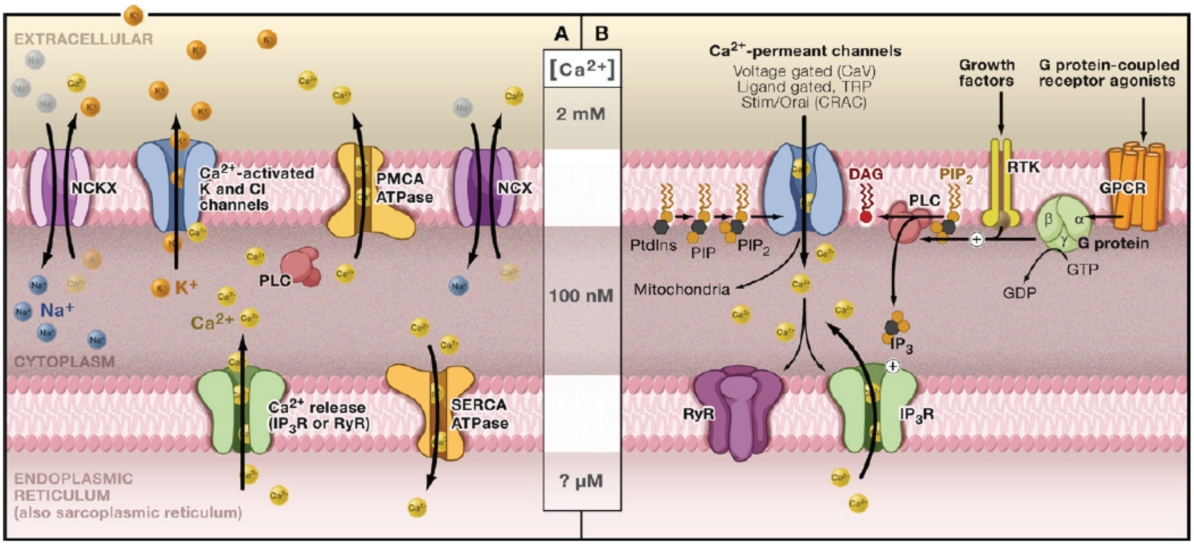

Figure 2. Mechanisms controlling cytosolic Ca^{2+}

The cytosolic Ca^{2+} concentration is set by ion exchangers and pumps (A). The Na^+/Ca^{2+}-K^+ (NCKX) and Na^+/Ca^{2+} (NCX) exchangers swap Na^+ ions for Ca^{2+} (and K^+), the direction of transport is dependent on the Na^+ gradient. Ca^{2+}-ATPases at the plasma membrane (PMCA) and endoplasmic reticulum (SERCA) remove Ca^{2+} from the cytoplasm. SERCA pumps are responsible for replenishing Ca^{2+} in the endoplasmic reticulum. (B) Ca^{2+} signal can originate from the opening of Ca^{2+}-permeable ion channels in the plasma membrane or via activation of RyR or $InsP_3$-Rs. Activation of metabotropic receptors, such as receptor tyrosine kinases (RTK) or G protein-coupled receptors can initiate release from internal stores via PLC-mediated $InsP_3$ production. From Clapham 2007.

Neurotransmitters and other ligands can activate Ca^{2+}-permeable ion channels in the plasma membrane or induce release from internal stores, depending on the type of receptor activated. Glutamate activates NMDA receptors, which are one of the principle glutamate receptors found at synapses and their Ca^{2+} conductance is vital for some forms of synaptic plasticity (Malenka and Bear 2004). Glutamate can also induce Ca^{2+} release from internal stores through activation of G protein-coupled metabotropic glutamate receptors, which activate phospholipase C (PLC) and production of $InsP_3$ (Masu et al. 1991, Abe et al. 1992).

Extracellular ATP can also induce Ca^{2+} increase through ionotropic (P2X) and metabotropic (P2Y) receptors (North 2002, Hussl and Boehm 2006). These receptors are widely expressed by many cell types and have wide ranging roles in development (Zimmerman et al. 2006).

The largest, and least understood, of Ca^{2+}-permeable ion channel families is the transient receptor potential (TRP) channel family. There are approximately 28 members of the

mammalian TRP family, all but two of which conduct Ca^{2+}. They are divided into three main subfamilies: canonical (TRPC), vanilloid (TRPV) and melastatin (TRPM), alongside the more distantly related ankyrin (TRPA), polycystin (TRPP) and mucolipin (TRPML) channels. These are distributed widely throughout many tissue types, and have been identified in the brain during development (Strübling et al. 2001, Zechel et al. 2007). TRP channels show complex gating properties, responding to a diverse range of stimuli variously including temperature, osmolarity, stretch, pH, voltage, and chemical compounds (Caterina et al. 1997, Voets et al. 2004, Maroto et al. 2005, Yeh et al. 2005, Spassova et al. 2006, Leffler et al. 2007 Dhaka et al 2009), though as yet few endogenous extracellular ligands have been identified. They can also be regulated by various intracellular mechanisms, such as G protein-coupled receptors, PLC activation, diacylglycerol, and phosphorylation through PKA, PKC and PKG (Zhu et al. 1998, Li et al. 1999, Kim et al. 2003, Hofmann et al. 1999; for general reviews see Pedersen et al. 2005, Ramsey et al. 2006).

Ca^{2+} can also enter cells via connexins. Pairs of connexins form gap junctions between cells which allow the passage of ions and small metabolites between cells, including Ca^{2+} and inositol 1,4,5-trisphosphate ($InsP_3$; Goldberg et al. 2004). Uncoupled connexins can also act as "hemi-channels" opening onto the extracellular space, allowing influx of Ca^{2+} and release of signalling molecules such as ATP (Cotrina et al. 1998, Goodenough and Paul 2003), and these have been implicated in the initiation of Ca^{2+} waves in the ventricular zone of the developing neocortex (Weissman et al. 2004).

1.3.3 Roles of calcium signalling during stem cell development

The actions of Ca^{2+} are fundamental to many processes during development, including proliferation, apoptosis, migration and neural development, each of which has important consequences for the development of stem cell therapies.

Proliferation has long been known to be regulated by Ca^{2+} and calmodulin (Rasmussen and Means 1987, 1989), though still relatively little is known about how it regulates the cell cycle machinery. The cell cycle consists of four sequential stages through which cells pass as they proliferate: G1, S, G2 and M. G1 and G2 are gap phases where the cells synthesise proteins in preparation for DNA synthesis in S phase and mitosis in M phase, respectively. During the cycle Ca^{2+} signals are required for entry into G1, and for transition at the G1/S and G2/M

boundaries (Santella 1998). Entry of quiescent cells into G1 is dependent on Ca^{2+} influx stimulated by growth factors, for fibroblast growth factor-2 (FGF-2) this has been associated with TRPC1 channels in rat NSCs (Pla et al. 2005), and in mouse ESCs epidermal growth factor (EGF) has been shown regulate proliferation via Ca^{2+}-dependent MAPK and PKC signalling (Heo et al. 2006).

The rate of progression through G1 was found to be proportional to the concentration of calmodulin (Rasmussen and Means 1987, 1989), and therefore could potentially be controlled by Ca^{2+} signalling. This might be of particular interest for stem cell development, as there is evidence that the speed of G1 has a direct influence on neurogenesis (Calegari and Huttner 2003, Lange et al. 2009). Ca^{2+} might be linked to this process through hyperpolarisation of the membrane by K^+ channels, which appears to be required for G1 progression (Wonderlin and Strobl 1996). If so, methods of manipulating this process could be useful for stem cell therapies.

The requirement for Ca^{2+} influx at the G1/S and G2/M boundaries may form part of the checkpoint controls that prevent damaged cells from progressing. At these checkpoints damaged cells will either attempt DNA repair or initiate apoptosis, depending on the extent of the damage (Zhou and Elledge 2001).

Initiation of apoptosis can occur through several Ca^{2+}-dependent mechanisms present in the cytosol, endoplasmic reticulum and mitochondria (Orrenius et al. 2003). In one situation, deregulation of Ca^{2+} homeostasis results in an overload of Ca^{2+} in the endoplasmic reticulum and mitochondria, leading to the release of apoptotic signals by the mitochondria (Pinton et al. 2008). Anti-apoptotic proteins Bcl-2 and Bcl-X_L act to reduces levels of Ca^{2+} in the endoplasmic reticulum, preventing stress and subsequent activation of apoptotic signalling pathways (Pinton et al. 2000), and over-expression of Bcl-X_L in human NSCs was shown to increase the survival of dopaminergic neurons (Liste et al. 2004), indicating this pathway is active in stem cell survival.

Migration and neurite growth are important aspects of stem cell integration into their host environment. In developing neurons Ca^{2+} signalling controls saltatory migration, neurite growth and turning, and neurite retraction (Gomez et al. 2006, Zheng and Poo 2007). Global increases in Ca^{2+} can promote neurite outgrowth and migration, and inhibition of Ca^{2+} signalling impairs migration (Komuro and Rakic 1996). In accordance, Ca^{2+} increases have been shown to promote neurite outgrowth in differentiating mouse primary NSCs (Ciccolini

et al. 2003), and knockout of TRPC1 or TRPC4 was found to reduce neurite length in human stem cell-derived neurons (Weick et al. 2009). However, TRPC5 channel activity has been shown to inhibit neurite growth (Greka et al. 2003, Hui et al. 2006), and in some cell types Ca^{2+} decrease has been shown to promote neurite outgrowth (Tang et al. 2003), suggesting that intracellular regulation of Ca^{2+} signals also regulates growth versus retraction. There is also evidence that different types of channel may differentially contribute to responses to guidance cues, possibly through differing Ca^{2+} conductances. Netrin is a chemoattractant which induces Ca^{2+} signals via L-type Ca_v channels, and blocking these channels switches the response from attraction to repulsion (Hong et al. 2000). However, this repulsive action also requires Ca^{2+} influx as lowering extracellular Ca^{2+} or applying Cd^{2+} (which blocks L-types Ca_v channels and some TRP channels) prevented both attractive and repulsive responses (Song et al. 1998, Hong et al. 2000), indicating that both are mediated by Ca^{2+}-dependent signalling pathways.

Growing axons and dendrites must determine the appropriate synaptic partners from the many others they come into contact with. In the process of synaptogenesis highly localised Ca^{2+} signals occur in filopedia of dendrites within minutes of their contacting an axon. These signals appear to stabilise the structure of the filopedia, as filopedia with higher frequency transients were more likely to stabilise into a dendritic spine, whereas with lower frequencies filopedia were more likely to retract (Lohmann and Bonhoeffer 2008). The mechanism underlying these Ca^{2+} signals is unknown, but might be activated by adhesion molecules (Nishimune et al. 2004, Sanes and Yamagata 2009), and therefore could act as a mechanism of selecting appropriate synaptic partners, where inappropriate cell types fail to induce sufficient Ca^{2+} signals. In stem cells this process may be informative for their ability to integrate correctly into the host neural networks, and may lead to methods of engineering cells to integrate specifically into the host.

The expression of recognition molecules and responsiveness to different guidance molecules will depend on the identity adopted by differentiating neurons. What role Ca^{2+} has in determining neuronal identity is unclear; in Xenopus it is well established that the frequency of Ca^{2+} transients determines the expression of GABA, glutamate and acetylcholine in spinal neurons (Gu and Spitzer 1995, Borodinsky et al. 2004), however, limited data is available from mammalian cells. Ciccolini et al. (2003) reported that Ca^{2+} signals regulated the adoption of a GABAergic phenotype in mouse primary striatal NSCs, but how widespread

this mechanism may be is unclear. There have also been reports of Ca^{2+} regulating neurogenesis in mouse neural crest cells and primary cortical NSCs via InsP3-Rs and Ca_v channels, respectively (Carey and Matsumoto 1999, D'Ascenzo et al. 2006), but again, the general importance of such mechanisms has yet to be established.

It is clear that Ca^{2+} signalling plays important roles at different stages of cell development, and that understanding what these roles are and the mechanisms underlying them could lead to methods of guiding development. This requires characterisation of the Ca^{2+} signalling mechanisms present in different cell types, and of the influence promoting or inhibiting these signals has on stem cell development.

1.4 Functional development of stem cell-derived neurons

Any stem cell-derived neuron must be capable of developing the appropriate functional properties, particularly for therapies that aim to replace lost cells. The most fundamental of these are the voltage-gated Na (Na_v) and K (K_v) currents which underlie action potential generation (Hodgkin and Huxley 1952), and the expression of neurotransmitter receptors and release of neurotransmitter in response to stimulation, which underlie synaptic communication.

Neurons derived from ESCs and iPSCs have been shown to develop functionally *in vitro*, generating action potentials and forming synaptic connections (Johnson et al. 2007, Wernig et al. 2008, Kock et al. 2009, Kim et al. 2011). Detailed characterisation of non-immortalised hNPCs has shown they could differentiate into GABAergic and dopaminergic neurons *in vitro*, and that they develop key functional properties of neurons, such action potential generation and expression of neurotransmitter receptors (Milosevic et al. 2006, 2007, Wegner et al. 2008, 2009). However, functional development in v-myc immortalised cell lines appears limited, and has not been shown to be stable over long periods in culture. Spontaneous and evoked action potentials have been described (Lotharius et al. 2002, DeFilippis et al. 2007, Donato et al. 2007, Tonnesen et al. 2010), but where more detailed analyses were performed they showed limited maturation and expression of voltage-gated currents (Donato et al. 2007). Identifying the cause of this and methods to compensate would enhance the potential of immortalised hNPCs for cell replacement therapies.

1.5 Activity-dependent neuronal survival

The functional development of neurons is not just important for their mature role. In the developing brain an extensive period of cell death coincides with a period of synaptogenesis, during which immature neurons that fail to establish themselves in developing neural networks undergo apoptosis (Oppenheim 1991, Buss et al. 2006). This process of programmed cell death is crucial in establishing the connectivity of neural networks and removing unwanted neurons. As differentiating stem cells are not immune to this process it may have consequences for the success of cell replacement therapies.

Synaptic activity can mediate survival through activation of Ca^{2+}-dependent processes, via NMDA receptors or Ca_v channels (Koike et al. 1989, Hardingham et al. 2002, Léveillé et al. 2010), and through release of neurotrophic factors at synapses (Davies 2003). In the developing substantia nigra, dopaminergic neuron survival is mediated through the release of GDNF from their post-synaptic targets in the striatum (Oo et al. 2003, Burke et al. 2004), and excitation has been linked to the survival of developing substantia nigra dopaminergic neurons in primary cultures of mouse ESCs through a Ca^{2+}-dependent mechanism (Douhou et al. 2001, Salthun-Lassalle et al. 2004), and in midbrain slice cultures (Katsuki et al. 2001, 2003). These observations suggest that the survival of stem cell-derived neurons after transplantation could be influenced by their ability to integrate into the host neural networks, and therefore demonstration of activity dependencies in human stem cell-derived neurons and subsequent investigation of the underlying mechanisms may lead to methods of enhancing survival *in vivo*.

1.6 Organotypical slice cultures as a model neural environment

The environment into which stem cells would be transplanted differs considerably to both that of cell cultures and of the developing brain. The signals which would regulate differentiation and processes such as migration, axon growth and synapse formation are largely absent, and very little is known about the mechanisms underlying host-graft interactions.

Organotypical slice cultures provide a model neural environment in which interactions between stem cells and their host can be investigated, while maintaining the advantages of *in vitro* systems, namely a controllable environment, ease of manipulation, high throughput and

accessibility. The co-culture system does not replicate *in vivo* transplantation *per se*, as cultures must be made using embryonic or postnatal tissue, at which times the nervous system is still developing. Many of the basic structural features are present however, including neural networks, and in the case of hippocampal cultures there is a considerable literature on the cellular and chemical composition of the environment (Gähwiler et al. 1997, Holopainen 2005, Förster et al. 2006, Lossi et al. 2009). It should also be noted that the environment is that of injured rather than healthy tissue, as the process of slicing induces an inflammatory response from the tissue. This can be considered beneficial for modelling stem cell-host interactions since inflammation accompanies neurodegenerative disorders, including Parkinson's disease (Przedborski 2007).

It has previously been demonstrated that when transplanted onto organotypic hippocampal slice cultures mouse embryonic stem cells can differentiate into functional neurons which receive synaptic input (Benninger et al. 2003, Tonnesen et al. 2011), and into glia (Scheffler et al. 2003, Husseini et al. 2008). Expression of neuronal markers has also been induced in mouse bone marrow stromal cells (Abouelfetouh et al. 2009) and human cord blood-derived stem cells (Sarnowska et al. 2009), however, NPCs derived from adult rats predominantly differentiated into astrocytes when co-cultured with hippocampal slices (Rivera et al. 2009). It is clear that the responsiveness to environmental cues varies between cell types, and as yet it is not clear how such environmental conditions might influence human cells.

1.7 Aims

In this study I have used the ReNcell VM cell line (Millipore, US), a hNPC line derived from the ventral midbrain of a 10-week old fetus and immortalised with v-Myc (Donato et al. 2007). *In vitro*, these cells can be propagated by the addition of EGF and FGF-2 to the culture medium, and differentiation can subsequently be induced by their withdrawal.

The aims of this project were:

1. To examine the mechanisms of spontaneous Ca^{2+} signalling present in the ReNcell VMs and their biological roles, and to investigate the potential for manipulation of these mechanisms for the improvement of cell development *in vitro*.

2. To characterise the functional development of the ReNcell VM cells, with a focus on their ability to develop functional neuronal properties.

3. To examine the ability of ReNcell VM cells to differentiate and functionally integrate within an organotypical model neural environment.

2. Materials and Methods

2.1 Materials

Standard chemical reagents were acquired from Sigma (Germany), Merck (Germany) or Roth (Germany). All drugs were acquired from Sigma (Germany) or Tocris Biosciences (UK). Stock solutions were prepared by dissolving in water or DMSO and stored at -20°C.

2.1.1 Culture media and solutions

Culture media and supplements

	Company	Product ID
DMEM:F12 1:1 (without L-Glutamine, pyridoxine)*	Invitrogen	21331-020
Custom DMEM:F12*	PAA	T1080
GlutaMax I (100x)	Invitrogen	35050-038
Heparin sodium salt	Sigma	H3149
B27 Supplement*	Invitrogen	12587-010
Gentamicin	Invitrogen	15750-037
EGF	PeproTech	100-15-0,5mg
FGF-2	Roche	11120417001
mouse Laminin 1 Protein 1mg/ml	Trevigen	3400-010-01
MEM	Sigma	M0769
Penicillin / Streptomycin	Sigma	P0781
Vitamin C	Sigma	A4403
L-Glutamine	Gibco	11012-028
Trisma base	Merck	1083820100
Insulin	Roche	11074547001

Buffers and miscellaneous

	Company	Product ID
HBSS (with & without Ca/Mg)*	Invitrogen	24020 / 14170
PBS*	Biochrom	L1825
Trypsin/ EDTA 1x in HBSS w/o Ca and Mg	Gibco	25300-054
Benzonase	Merck	1016540001
Human Serum Albumin (HSA)	Grifols	720611
DMSO	Sigma	D2650
Normal goat serum (NGS)	Dako	X0907
Bovine Serum Albumin (BSA)	Roth	8076.3

*Formula can be found in appendix A.

Other solutions were prepared as follows:

FACS wash buffer 0.5% BSA, 0.02% NaN_3 in PBS

HBS	14mM HEPES, 0.9% NaCl, pH 7.4 with NaOH
Trypsin / Benzoase solution	Trypsin/EDTA, 25U/ml Benzoase
Trypsin inhibitor / Benzoase	1% HSA, 25/ml Benzonase, 0.55mg/ml trypsin-inhibitor in DMEM/F12
Saponin buffer	0.5% Saponin, 0.02% NaN_3, 0.5% BSA in PBS

The slice culture medium consisted of 50% MEM, 25% HBSS (+Ca^{2+}/Mg^{2+}), 25% horse serum plus 0.12% glucose, 4mM L-glutamine, 1% penicillin/streptomycin, 0.8ng/ml vitamin C, 10ng/ml insulin, 0.0058% $NaHCO_3$, 5mM trisma-base. When gassed the medium had a pH of 7.4. Media was stored at 4°C and used within 3 weeks. The slice preparation medium consisted of HBSS (with Ca^{2+} and Mg^{2+}) with 6mg/ml glucose.

For patch clamp experiments in cell cultures the standard intracellular solutions consisted of (in mM) 140 KCl, 10 HEPES, 11 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 2 Mg-ATP, pH 7.2 with KOH, ≈300mOsm. For patch clamp recordings in slice cultures the standard intracellular solution consisted of 130 KCl, 10 NaCl, 10 HEPES, 11 EGTA, 2 $CaCl_2$, 1 $MgCl_2$, 2 Mg-ATP, pH 7.2 with KOH, ≈300mOsm.

For Ca^{2+} imaging and patch clamp recordings from coverslips the standard extracellular solution consisted of 125 NaCl, 2.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 20 glucose, 20 saccharose, pH 7.4 with NaOH, ≈305mOsm. Changes in KCl concentration were balanced by an equimolar adjustment of the NaCl concentration. In experiments where KCl was removed completely it was replaced with either LiCl or Choline chloride. For Ca-free solution $CaCl_2$ was replaced by 2 EGTA. Recordings were made at room temperature.

For patch clamp recordings from slice cultures the extracellular solution consisted of 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 20 glucose, ≈305mOsm, pH 7.4, adjusted and maintained by bubbling with carbogen (95% O_2, 5% CO_2). Recordings were made at 37°C.

2.1.2 Antibodies

Primary Antibody	Concentration	Species	Company	Product ID
βIII-tubulin	1:500	mouse	Santa Cruz Biotechnology	SC-51670
GFAP	1:1000	rabbit	DAKO	Z0334
Nestin	1:50	mouse	R&D Systems	MAB1259
MAP2ab	1:1000	mouse	Chemicon	MAB378

NeuN	1:100	mouse	Millipore	MAB377
Ki67	1:200	rabbit	Santa Cruz Biotechnology	SC-15402
Olig2	1:200	goat	Santa Cruz Biotechnology	SC-19967
Tyrosine Hydroxylase	1:500	rabbit	Millipore	AB152
Secondary Antibody	**Concentration**	**Species**	**Company**	**Product ID**
Alexa Fluor 568	1:1000	goat anti-mouse	Invitrogen	A11031
Alexa Fluor 568	1:1000	goat anti-rabbit	Invitrogen	A11029
Alexa Fluor 568	1:1000	rabbit anti-goat	Molecular Probes	A11979
Alexa Fluor 488	1:1000	goat anti-mouse	Molecular Probes	A11029
Alexa Fluor 647	1:1000	goat anti-mouse	Molecular Probes	A21235

2.1.3 Hardware

Patch clamp and imaging system

Item	**Type/Model**	**Manufacturer**	**Location**
Patch clamp amplifier	EPC-10/2	HEKA	Germany
Patch clamp probes	EPC10	HEKA	Germany
Controller	SM-1	Luigs and Neumann	Germany
Controller	SM-5	Luigs and Neumann	Germany
Micromanipulators	mini 25	Luigs and Neumann	Germany
Microscope	Axioskop2	Carl Zeiss	Germany
CCD camera	SensiCam	PCO Imaging	Germany
Light source	SNT 12V 100W	Carl Zeiss	Germany
Fluorescent light source	Polychrome V	Till Photonics	Germany
Dodt gradient contrast system		Luigs and Neumann	Germany
40x objective	Achroplan	Carl Zeiss	Germany
10x objective	Achroplan	Carl Zeiss	Germany
GFP Filter	41017	Chroma	US
Fura-2 Filter	Fura-2 Filter set	Till Photonics	Germany
Bath heater	Temperaturcontroller III	Luigs and Neumann	Germany
Vibration table		Newport	USA
Pipette puller	DMZ Universal Puller	Zeitz Instruments	Germany

General

Item	**Type/Model**	**Manufacturer**	**Location**
Fluorescence microscope	Biozero	Keyence	Germany
Cell counter	CASY Model TT	Roche	Germany
Incubator	C Series	Binder	Germany
Sterile workbench	HERAsafe	Heraeus	Germany
Sterile workbench	Microflow Laminar workstation	Astec-Microflow	UK
FACS	FACSCalibur	Becton Dickinson	USA
Centrifuge	Z223 Mk2	Hermle	Germany
pH meter	HI 223	Hanna Instruments	Germany
Osmometer	Osmomat 030	Gonotec	Germany
Water bath	AL 5	Lauda	Germany
Balance	MCBA 100	Sartorius	Germany
Pipettes	Reference	Eppendorf	Germany
Vortexer	MS1	IKA	Germany

2.2 Methods

2.2.1 Cell Culture

Cells were cultured as either a monolayer cell culture (standard protocol), or as neurospheres (preaggregation protocol).

In the standard protocol cells were grown to 70-80% confluency in laminin coated culture vessels with proliferation medium. For patch clamp and immunocytochemistry experiments, cells were plated on glass coverslips coated with laminin (1mg/ml) and poly-D-lysine (25ng/ml) at a density of 20,000 cells per well and proliferated for a further 3 days. After washing with HBSS (without Ca^{2+}/Mg^{2+}), differentiation was induced by withdrawal of the growth factors and the addition of cAMP (1mM) and GDNF (2ng/ml).

For FACS, CASY and cell cycle analysis experiments, cells were cultured following the standard protocol. To harvest the cells they were first washed with warm HBSS (without Ca^{2+}/Mg^{2+}) before being incubated in trypsin/benzonase at 37°C for 2-3 minutes. The reaction was stopped by the addition of trypsin-inhibitor/benzoase.

The preaggregation protocol was described by Dontato et al. (2007). Cells were cultured for 7 days in uncoated 96 well plates with proliferation medium consisting of DMEM/F12, B27 media supplement (2%), Glutamax (2 mM), gentamycin (50µg/ml) and heparin (10 units/ml) and the growth factors EGF (20µg/ml) and FGF-2 (10µg/ml), which resulted in aggregation of the cells in to neurospheres. Single neurospheres were transferred to laminin (1mg/ml) and poly-D-lysine (25µg/ml) coated glass coverslips and cultured for a further 4 days in proliferation medium. Differentiation was induced by withdrawal of the growth factors and the addition of cAMP (1mM) and GDNF (2ng/ml). Where used, veratridine (Vtr, 1µM) and tetrodotoxin (TTX, 1µM) were present throughout differentiation.

For altering the KCl concentration in culture custom DMEM/F12 medium without KCl, NaCl, $NaHCO_3$, NaH_2PO_4, Na_2HPO_4 and sodium pyruvate was obtained from PAA Laboratories (Austria). These were subsequently added to the different media as follows, standard media (in mM): 4.16 KCl, 120.61 NaCl, 29.02 $NaHCO_3$, 0.453 NaH_2PO_4, 0.5 Na_2HPO_4 and 0.5 sodium pyruvate; low KCl: 1 KCl, 123.77 NaCl, 29.02 $NaHCO_3$, 0.453 NaH_2PO_4, 0.5 Na_2HPO_4 and 0.5 sodium pyruvate; high KCl: 15 KCl, 109.77 NaCl, 29.02

$NaHCO_3$, 0.453 NaH_2PO_4, 0.5 Na_2HPO_4 and 0.5 sodium pyruvate. Cells were prepared according to the standard protocol. For varying KCl during proliferation all cells were plated using standard media and allowed 24 hours to settle before changing to either standard, low or high KCl media. The cells were differentiated in standard media. For varying KCl during differentiation cells were proliferated in standard media, then differentiated for 24 hours in standard media to allow the cells to exit the cell cycle, so as not to conflate effects of KCl concentration during proliferation with those during differentiation. After 24 hours the media was changed to the experimental conditions.

2.2.2 Generation of GFP expressing ReNcell VM line

A GFP expressing ReNcell VM line was provided by Dr. Rayk Hubner (Albrecht-Kossel-Institute for Neuroregeneration, University of Rostock, Germany). ReNcell VM cells were transfected with pCAGIP-GFP (Alexopoulou et al., 2008) using Nucleofector technology (Amaxa) according to manufacturers recommendations. 18 hours after transfection, cells were passaged and selection was started using 2.5 µg/ml Puromycin in medium containing growth factors for 2 weeks. A GFP-positive sub-population was subsequently cultured as described above.

2.2.3 Organotypical Hippocampal Slice Cultures

Organotypical slice cultures enable the three dimensional organisation of the brain to be maintained in an *in vitro* culture system. In the interface culture method (Stoppini et al. 1991) slices of tissue are cultured on a semi-porous membrane, of which the underside is in contact with the culture medium and the surface exposed to the air. This allows uptake of nutrients from the medium while maintaining sufficient oxygenation of the slice, and with this method hippocampal slices from postnatal rats can be cultured for up to 1 month. Tissue from adult animals does not survive in culture due to insufficient oxygenation.

Slice cultures were prepared from P6 Wistar rats (Charles River, Germany) using the interface method described by Stoppini et al. (1991), with some modifications. All experiments were performed in accordance with the German Animal Welfare Act.

Before beginning the preparation the 6-well plates were prepared and the medium allowed to gas to the correct pH in the incubator. 1.1ml of slice culture medium was placed in each well. The tissue chopper cutting surface was covered with a sterile aclar sheet cut to fit. Each blade edge was used for no more than two rats. First, the rat pups were decapitated, the cranium was carefully cut along the midline and peeled back exposing the brain. This was removed into a petri dish containing cold preparation medium. The cerebellum and forebrain were removed, then the brain was hemisected along the midline and the subcortical regions were removed to reveal the hippocampus. The hippocampi were dissected out by cutting along the cortex above and to either end. A drop of preparation medium was applied to each hippocampus and the excess removed before slicing the tissue. The slices were then separated under the stereoscopic microscope and inspected for integrity. Only those slices showing a clear hippocampal structure without any sign of damage were used. Three or four slices were placed on each insert, and the excess preparation media was removed. The plates were then transferred to the incubator.

The culture medium was changed the first day after preparation and every 2-3 days thereafter. Before changing the medium the fresh medium was gassed to the correct pH in the incubator. To prevent slices drying out while the medium was being changed a drop of culture medium was placed over each slice. The excess solution was then removed before returning the slices to the incubator.

2.2.4 Transplantation of hNPCs onto Slice Cultures

GFP labelled ReNcell VM cells were prepared according to the standard protocol. To prepare for transplantation cells were trypsinised and centrifuged at 1300rpm and 4°C for 5 minutes, then resuspended in CASY-ton. The number of cells was measured by CASY, before re-centrifuging the cells and suspending them in slice culture medium at 100,000 cells per µl. 0.05-0.1µl of suspended cells were transplanted by hand onto each slice using a 0.5µl syringe. The hNPC suspension was placed centrally over the hippocampus, and typically covered approximately two thirds of the hippocampus (Fig. 17A). To avoid contact between the syringe and the slice, as the syringe approached the slice some solution was ejected to form a small blob at the tip, when this blob was seen to touch the slice the remaining solution was ejected. This minimised the risk of damaging the slice.

2.2.5 Immunocytochemistry

2.2.5.1 Cell Cultures

Coverslips were fixed in 4% paraformaldahyde (PFA) for 15 minutes at room temperature and then washed three times with PBS. They were stored in 0.02% NaN_3 in PBS at 4°C. Non-specific binding sites were blocked by incubating the coverslips in 5% normal goat serum (NGS) and 0.3% Triton X-100 in PBS for 30 minutes. They were then incubated with primary antibodies for a further 30 minutes at room temperature. Primary antibodies were diluted in PBS containing 1% NGS, except in the case of Olig2, where NGS was replaced with 1% bovine serum albumin (BSA). After washing 3x in PBS they were incubated with secondary antibodies at room temperature for 30 minutes in a dark, humidified environment. Secondary antibodies were diluted in PBS containing 1% NGS, or BSA in the case of Olig2. After washing 3x in PBS coverslips were mounted on glass slides using Vectashield mounting medium containing DAPI (Vector Labs, US). The slides were stored in the dark at room temperature.

2.2.5.2 Slice cultures

Slices were fixed in 4% PFA in PBS for 30 minutes at room temperature, washed 3x in PBS and stored at 4°C in PBS containing 0.02% NaN_3. For immunostaining, slices were cut out of the well inserts and processed on sections of the insert membrane. Blocking was performed by incubating slices in PBS containing 10% NGS (or BSA depending on the primary antibody) and 0.3% triton X-100 for 24 hours at 4°C. Slices were incubated with the primary antibody overnight at 4°C with 2% NGS (or BSA) and 0.3% triton X-100. Slices were then washed 6x in PBS for 30 minutes each step before incubation with the secondary antibody. Secondary antibodies were incubated overnight at 4°C in the dark with 2% NGS (or BSA) and 0.3% triton X-100. Slices were again washed in PBS 6x for 30 minutes each step, then incubated in DAPI (250ng/ml in PBS) for 20 minutes, before briefly washing 3x in PBS. The slices were gently removed from the membrane and mounted on slides using Vectashield mounting medium (Vector Labs, US). Fluorescence pictures were taken using a Biozero 8000 microscope system (Keyence, Germany).

2.2.6 Flow Cytometry

Flow cytometry was used to quantify the number of cells expressing βIII. The cells were cultured in 6-well plates up to 80% confluency and subsequently differentiated. For FACS analysis, cells were trypsinised and centrifuged at 1500rpm at 4°C for 10 minutes, washed with PBS without Ca^{2+} and Mg^{2+} and fixed with 1% PFA in PBS for 15 minutes. Cells were then resuspended in washing buffer and stored at 4°C in the dark. For the staining, cells were centrifuged and resuspended in saponin buffer containing βIII-tubulin (1:100) in which they were incubated for 2 hours at room temperature. Subsequently cells were washed and incubated with the secondary antibody (Alexa Fluor 647) for 1 hour in saponin buffer. Cells were washed twice with saponin buffer and resuspended in wash buffer for analysis. Experiments were performed using FACSCalibur Becton Dickinson, San Jose, USA) in combination with Cell Quest Pro software. These experiments were performed by a research assistant in the Albrecht-Kossel-Institute for neuroregeneration.

2.2.7 Cell Cycle Analysis

After 16, 28 and 40 hours of proliferation the distribution of cells across the cell cycle was analysed using flow cytometry. The cells were grown on six-well plates according to the standard protocol. After plating cells were allowed 24 hours to settle in standard medium before switching to the experimental conditions. The time of this medium change was taken as 0 hours for the experiments. To retrieve, the cells were trypsinised and centrifuged at 1300rpm at 4°C for 5 minutes, then suspended in 100µl HBS. To fix, the cell suspension was placed in 70% ethanol solution at -20°C for one hour, and stored in those conditions until use. RNase solution (1mg/ml in HBS) was activated by heating to 37°C for 1 hour. The cells were centrifuged for 10min at 1300rpm and 4°C, then suspended in RNase solution and incubated for 30 minutes at 37°C. Cells were stained with propidium iodide (50µg/ml) dissolved in HBS for 30 minutes at 37°C. Flow cytometry measurements were made as described above.

2.2.8 Cell counting and viability

Cell number and viability was measured using a CASY cell counter. The viable cells are distinguished from dead cells by their size, calculated from the change in resistance as they

pass through an electric field. Because the membrane of viable cells acts as an insulator, they increase the resistance relative to their volume. In dead cells, the cell membrane is no longer intact and offers little impedance, and therefore their apparent cell size is smaller – that of the nucleus. Cells were cultivated in 48 well plates following the standard protocol. For CASY measurements the cells were harvested with trypsin and suspended in the solution. For each measurement 50µl of the cell suspension was added to 10ml of CASY ton. 3 wells were measured per condition, with one measurement per well.

2.2.9 Patch Clamp

Patch clamp is used to record the activities of ion channels present in cell membranes (Hamill et al. 1981). A high resistance seal of several gigaohms, the gigaseal, is formed between the cell membrane and a glass pipette. This allows resolution of small currents of only a few picoamperes across the patch of membrane at the pipette tip. After formation of the gigaseal the patch of membrane at the tip of the pipette can be removed by suction, giving low resistance access to the cell interior. Under this condition the whole cell membrane can be voltage clamped, hence "whole cell" patch clamp. In voltage clamp current is passed through an electrode to maintain a set membrane potential, as the conductance of the cell membrane changes due to the opening or closing of ion channels the current required to clamp the membrane potential changes. Because the membrane potential is held constant and current through each open ion channel is the same, the current recorded is proportional to the number of open channels. By stepping the membrane potential to different values the voltage- and time-dependent properties of different currents can be characterised.

Whole cell patch clamp recordings were made using an EPC 10/2 amplifier controlled by PatchMaster software (v2.35, HEKA, Germany). Pipettes were pulled from borosilicate glass capillary tubes (GB150F-10 with filament, Science Products, Germany) using a DMZ Universal puller. They were backfilled with intracellular solution and had resistances between 3-8MΩ. Data was low-pass filtered at 2.9kHz and sampled at 10kHz, and series resistance compensation was set to 60%. Liquid junction potentials were not corrected. Cells were visualised using a 40x objective and a CCD camera controlled by TillVision software (v4.0, Till Photonics, Germany). For GFP targeted recordings cells were illuminated with 475nm light.

Upon accessing the membrane potential was recorded in current clamp mode (I = 0pA), averaged over a 12 second period. To elicit voltage-dependent currents IV steps were applied from -60mV to +50mV in 10mV increments from holding potentials of -60mV and – 80mV. Short duration and long duration IV protocols were used, with steps lasting for either 100ms or 500ms. Inward rectifying currents were tested for by applying 100ms voltage steps from -120mV to +50mV in 10mV increments from a holding potential of -80mV. Synaptic activity was recorded by continuous voltage-clamp at -80mV and was low-pass filtered at 3kHz. P/N leak subtraction was performed offline. Action potential generation was tested by current injection in current clamp mode, current was applied in steps of 30pA, up to 150pA.

2.2.10 Calcium Imaging

The level of intracellular Ca^{2+} can be measured using Ca^{2+} sensitive fluorescent dyes. Binding of calcium to these dyes induces a change in their fluorescent properties, either a change in intensity, emission wavelength or excitation wavelength, which can be measured and used to infer changes in intracellular Ca^{2+} concentration. The ratio of shifts in excitation or emission wavelength provide a measurement which is independent of dye concentration and illumination intensity, and which can be used to calculate the actual intracellular Ca^{2+} concentration (Grynkiewicz et al. 1985). Accurate determination of intracellular Ca^{2+} concentration is dependent on the K_d value of the dye, which is affected by pH, temperature, viscosity and the presence of other divalent ions, notably Mg^{2+}. As the ReNcell VMs cells are a heterogeneous population in which these properties are not necessarily constant between cells, and as this study was principally concerned with the mechanism of Ca^{2+} signalling, only the relative change in ratio was calculated.

Ratiometric imaging was performed using Fura-2AM dye (Invitrogen, Germany). The dye was dissolved in extracellular solution to a final concentration of 5µM. Coverslips were incubated for 30 minutes at room temperature, then washed three times in extracellular solution and left for a further 30 minutes to allow the dye to de-esterise. Coverslips were stored in the dark at room temperature when not in use.

The imaging was performed on the same setup as patch clamp experiments. The system was controlled by PC running TillVision (v4.0, Till Photonics, Germany). Illumination was provided by a Polychrome V (Till Photonics, Germany). The excitation wavelengths were

340nm and 380nm and the exposure time was typically 15ms, adjusted on occasion depending on the signal intensity. Images were acquired at 1Hz over a period of 1000 seconds. Ratiometric movies were produced off-line and background subtraction was performed per frame based on a specified region of the image containing no cells. Recordings were made from the somas of cells and regions of interest were marked by hand. The resultant traces were extracted as text files and then converted to axon binary file format using the ABF File Utility program (v2.1.76, Synaptosoft, US). Transients were marked by hand in Mini Analysis (v6.0.7, Synaptosoft, US). The same gain and display settings were used for analysis of all traces.

2.2.11 Statistical Analysis

Statistical analysis was carried out using Prism 5 (GraphPad Software Inc, US). Data is given as mean ± SEM. Unless otherwise stated Student's t-test was used to test for significance. Findings were considered statistically significant when $p < 0.05$, indicated by * (** $p < 0.01$, *** $p < 0.001$). N is given as the number of passages and n as the total number of trials or cells. For manual quantification of immunostainings 2 to 4 coverslips per passage were analysed, with at least 10 areas per coverslip.

Antagonists

Drug	Concentration	Described Targets	References
GdCl3	100µM	TRPC1, 3, 6, TRPM3, 4, TRPV2, 4, 5, 6, TRPA1, TRPP1, TRPML1, Ca_v, P2X	Nakazawa et al. 1997, Zhu et al. 1998, Beedle et al. 2002, Clapham et al. 2005, Leffler et al. 2007, Vriens et al. 2009
LaCl3	100µM	TRPC1, 3, 4** (mM), 6, 7, TRPM3, 4, 7, TRPV2, 3, 4, 5, 6, TRPP1, 2, Ca_v, P2X	Nakazawa et al. 1997, Zhu et al. 1998, Beedle et al. 2002, Clapham et al. 2005, Leffler et al. 2007, Vriens et al. 2009
NiCl2	2.5mM	Ca_v, TRPC3, TRPML1	Zhu et al. 1996, 1998
SKF 96365	5µM	TRPC1, 3, 6, 7, TRPV2, TRPP1? Ca_v, STIM1 and internal store release	Kiselyov et al. 1998, Zhu et al. 1998, Li et al. 1999, Bootman et al. 2002, Kim et al. 2003, Juvin et al. 2007, Malkia et al. 2007, Harteneck et al. 2011
2-APB	100µM	TRPC1, 2, 3, 4, 5, 6, TRPM2, 3, 8, Connexins, InsP3-R, K_v	Clapham et al. 2005, Bai et al. 2006, Togashi et al. 2008, Harteneck et al. 2011
Ruthenium Red	1, 10µM	TRPC3, TRPM6, TRPV1, 2, 3, 4, 5, 6, TRPA1, K_{2P}, Ryr-R, Ca_v	Cibulsky and Sather 1999, Clapham et al. 2005, Vriens et al. 2009
Carbenoxolone	100µM	Connexins, VRAC, P2X7, Cav, NMDA-Rs	Vessey et al. 2004, Suadicani et al. 2006, Chepkova et al. 2008, Benfenati et al. 2009, Ye et al. 2009
TTX	1µM	Na_v	
PPADS	5, 50µM	P2X1, 2, 3, 4*, 5, 7, 2/3, P2Y1, 13, InsP3-R	Vigne et al. 1996, Ralevic and Burnstock 1998, North and Suprenant 2000, Khakh et al. 2001, Garcia-Guzman et al 2007
Suramin	100µM	P2X1, 2, 3, 5, P2Y1, 2, (6), 11, 12, 13	Beindl et al. 1996, North and Suprenant 2000, Khakh et al. 2001, Klinger et al. 2001
CNQX	20µM	AMPA-R	
AP5	50µM	NMDA-R	
Thapsigargin	1µM	SERCA	
Apyrase	10U/ml	Degrades ATP to ADP and AMP	

* active on human but not rat P2X4

** agonist at 100µM

Agonists

2-APB	100µM	TRPV1, 2*, 3	Neeper et al. 2007
NMDA	50µM	NMDA-Rs	
2-methylthio-ATP	20µM	P2X, $P2Y_1$	

* not active on human TRPV2

Table 1. Description of drugs used in this study. Note that the pharmacology of many TRP channels is not well established and should be treated with caution; the antagonists may act at channels other than those listed, and in cases listed there may be unidentified species and expression system specificity.

3. Results

3.1 Immunocytochemical characterisation of ReNcell VM cells

To characterise the differentiation of the ReNcell VM cell line under cell culture conditions I made immunocytochemical stainings for the neuronal marker βIII-tubulin (βIII), the catecholaminergic marker TH (in midbrain cells a marker for dopaminergic neurons, Goridis and Rohrer 2002), the glial marker GFAP, the oligodendrocyte marker olig2 and the progenitor marker nestin (Fig. 3).

ReNcell VM cells were grown as neurospheres (preaggregation protocol, PreP) before being plated on glass coverslips coated with laminin and PDL. Immunocytochemical stainings were performed in proliferating cells and at several timepoints up to 7DD. Proliferating cells expressed nestin and GFAP (Fig. 3A-C), which are indicative of progenitor cells (Hartfuss et al. 2001). After differentiation cells could express βIII, indicating putative neurons, and TH, suggesting that some cells adopted a dopaminergic phenotype (Fig. 3D-F, see also Fig. 15 for a quantification). The majority of cells, however, expressed GFAP and nestin throughout differentiation. No Olig2 positive cells were found.

The morphology of the cells changed upon differentiation. During proliferation the cells had large cell bodies and short or no processes, and after differentiation the cells developed compact cell bodies and extended long, thin processes (Fig. 3D-F, Fig.4D-F).

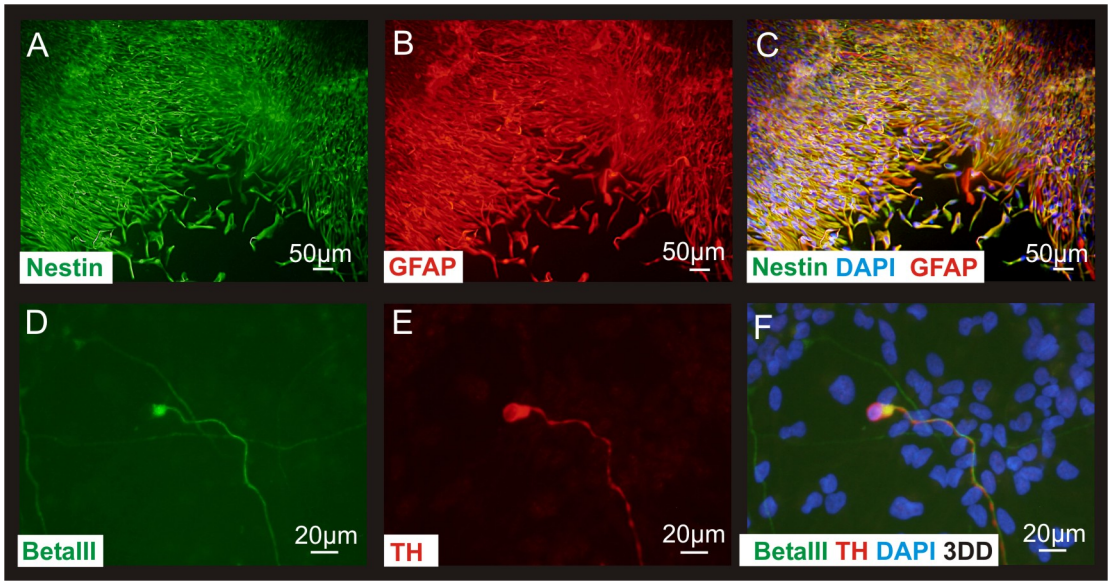

Figure 3. Immunocytochemical characterisation of ReNcell VM cells.

ReNcell VM cells were grown according to the PreP. A-C) During proliferation the cells co-express nestin and GFAP. D-F) After differentiation the cells can express βIII and TH, suggesting differentiation into dopaminergic neurons.

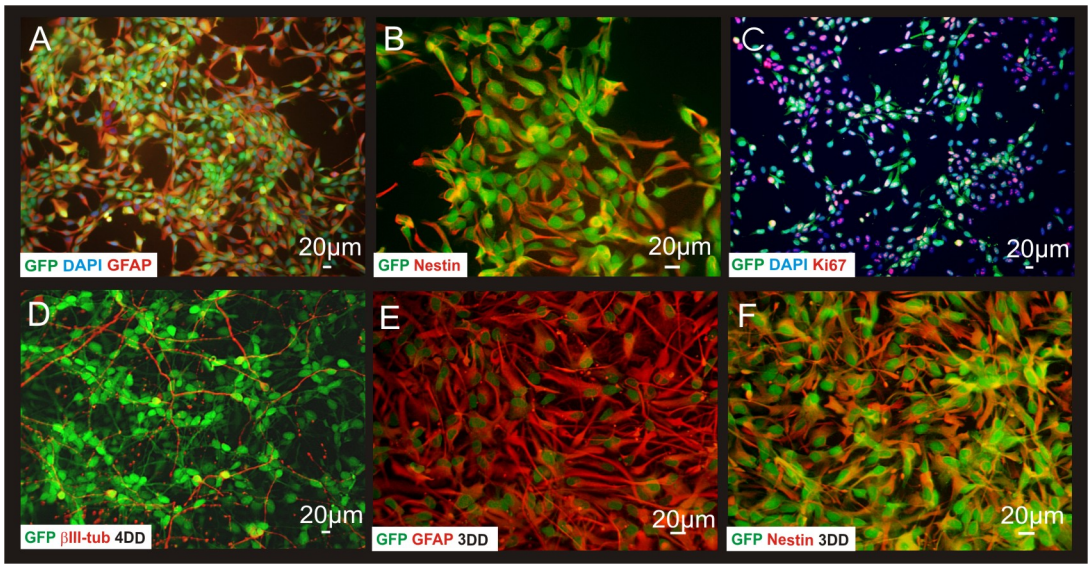

Figure 4. Immunocytochemical characterisation of GFP expressing ReNcell VMs

Cells were cultured according to the StdP. During proliferation they expressed GFAP (A), nestin (B) and Ki67 (C). After differentiation βIII^{+} cells could be detected (D). Most differentiating cells continued to express GFAP (E) and nestin (F).

3.2 Immunocytochemical characterisation of GFP expressing ReNcell VM cells

A GFP expressing ReNcell VM line was made through transfection with pCAGIP-GFP. Flow cytometry analyses showed that 94.4 ± 3.6% of proliferating cells and 90.5 ± 3.8% of 3DD cells were GFP-positive. To confirm this cell line had the same differentiation properties as the standard ReNcell VM cells I repeated the immunocytochemistry stainings for βIII, GFAP, nestin, Olig2 and Ki67. These cells were cultured as monolayers (standard protocol, StdP), as this is how they were to be prepared for co-culture experiments. As with non-GFP expressing cells, during proliferation the cells expressed nestin, GFAP and the proliferation marker Ki67 (Fig. 4A-C). βIII-positive cells were observed after the induction of differentiation (Fig. 4D). Flow cytometry analysis revealed no difference in the amount of βIII-positive cells between GFP-expressing cells and non-transfected cells (A Liedmann, unpublished data). During differentiation the cells developed more mature morphologies, characterised by compact cell bodies and extensive processes, however most continued to express GFAP and nestin (Fig. 4E,F). No cells positive for Olig2 were detected.

3.3 Spontaneous Calcium Signals in hNPCs

Calcium is a vital second messenger, interacting with multiple signalling pathways at different developmental stages. Understanding the role and potential regulation of calcium

signalling during hNPC development requires characterisation of the signalling activity present and the underlying mechanism(s). To this end, I made calcium imaging experiments from hNPCs loaded with the ratiometric calcium indicator Fura-2AM.

Spontaneous calcium transients were present in both proliferating and differentiating cells with a variety of waveforms, suggesting multiple mechanisms were present (Fig. 5A,B). Notably, high frequency oscillations were seen in some proliferating cells (Fig. 5A). In proliferating cells 54.5 ± 3.2% (N = 3, n = 19) were active per trial, which was significantly higher than in differentiating cells (3DD 27.8 ± 2.4%, N = 4, n = 13; 5DD 25.6 ± 2.2%, N = 2, n = 4; 7DD 23.4 ± 2.2%, N = 5, n = 16; $p < 0.001$, ANOVA with Tukey's multiple comparison test), with the exception of 4DD (44.6 ± 4.4% (N = 4, n = 8, $p > 0.05$). In turn, the level of activity at 4DD was significantly higher than at 3 and 7DD ($p < 0.01$ vs 3DD, $p < 0.001$ vs 7DD). Between 3, 5 and 7DD there were no statistically significant differences (Fig. 5C). The reason for the discrepancy at 4DD is unclear. As there was no difference between the other timepoints, for comparison of pharmacological agents they were pooled and 4DD excluded (except for some $GdCl_3$ and $LaCl_3$ experiments).

Comparison of the frequency of transients in active cells showed no difference between 3, 4 and 7DD (3DD n = 118 cells, 4DD n = 104, 7DD n = 121, $p > 0.05$, Kolmorogov-Smirnov 2 sample test, Fig. 5D), while in proliferating cells a there was a significant increase in the number of high frequency cells (proliferating (n = 408) vs differentiating (n = 343), $p < 0.001$, Kolmorogov-Smirnov 2 sample test, Fig. 5D).

3.4 Pharmacology of Spontaneous Calcium transients

The next step was to determine the mechanism(s) underlying these transients (Fig. 6). Pooling the data from differentiating cells gave a mean of 25.4 ± 1.5% cells active per trial (3, 5 and 7DD, N = 7, n = 33). Removal of extracellular calcium substantially reduced the number of active cells in both proliferating (7.9 ± 2.8%, N = 2, n = 10, $p < 0.001$) and differentiating cells (0.5 ± 1.3%, N = 3, n = 7, $p < 0.001$; Fig. 6A-D), indicating that the transients were almost entirely due to influx of Ca^{2+} from the extracellular space. Application of 100µM $GdCl_3$ or $LaCl_3$ similarly substantially reduced activity in both proliferating ($GdCl_3$ 3.9 ± 1.4%, N = 3, n = 6, $p < 0.001$, $LaCl_3$ 3.3 ± 1.8%, N = 3, n = 6, $p < 0.001$) and differentiating cells ($GdCl_3$ 2.4 ± 2.8%, N = 3, n = 8, $p < 0.001$, $LaCl_3$ 0.7 ± 1.2%, N = 3, n = 7, $p < 0.001$; Fig. 6A,B,E,F). Gd^{3+} and La^{3+} inhibit a broad spectrum of Ca^{2+} channels, notably many TRP channels, Ca_v channels and P2X receptors (see Table 1 for a description of

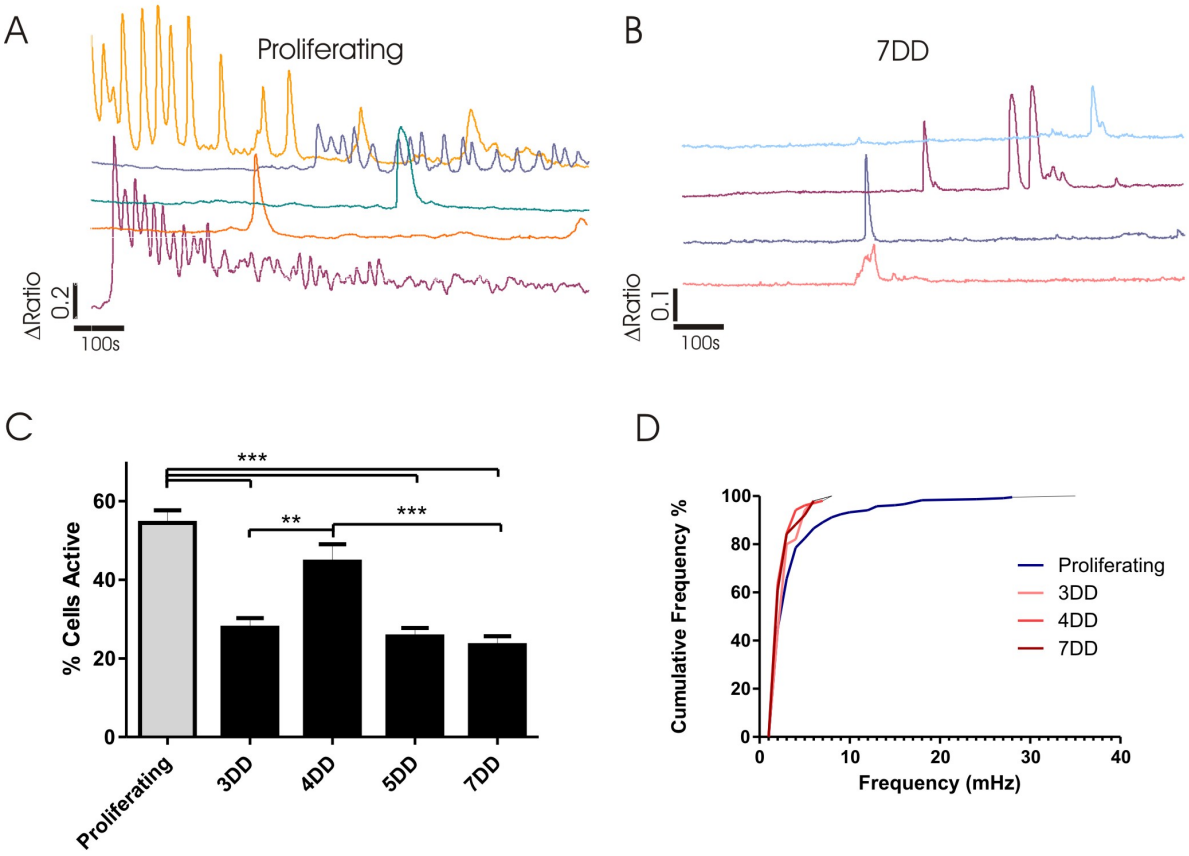

Figure 5. Spontaneous Ca^{2+} transients in ReNcell VM cells.

Spontaneous Ca^{2+} signals were recorded over a 1000sec period. Example traces are shown from (A) 5 proliferating cells and (B) 4 differentiating cells. C) The number of cells active per experiment was higher in proliferating cells compared to differentiating cells. There was an increase in activity at 4DD. D) The frequency of transients per active cell was generally low, but there was a sub-population of proliferating cells that expressed high frequencies.

these and all other antagonists used here). Application of 2.5mM $NiCl_2$, at this concentration a general blocker of Ca_v channels, had no effect in proliferation (55.3 ± 8.3%, N = 3, n = 6, $p > 0.05$) or differentiation (20.5 ± 3.0%, N = 3, n = 13, $p > 0.05$). Combined application of P2X and P2Y receptor antagonists PPADS (50µM) and Suramin (100µM) also had no effect during proliferation (55.3 ± 4.0%, N = 3, n = 9, $p > 0.05$), while in differentiating cells a slight significant increase was seen (34.0 ± 5.9%, N = 3, n = 7, $p < 0.05$). Similarly, application of the ATP-degrading enzyme apyrase (10U/ml) to differentiating cells also resulted in an increase in the activity detected (33.4 ± 4.3%, N = 2, n = 8, $p < 0.05$). SKF 96365 (5µM), frequently used as an inhibitor of TRPC channels, was shown to inhibit spontaneous Ca^{2+} transients in human ESC-derived neurons (Weick et al. 2009), however, its application had no effect on proliferating (53.0 ± 6.6%, N = 3, n = 7, $p > 0.05$) or differentiating (24.1 ± 3.2%, N = 3, n = 11, $p > 0.05$) ReNcell VM cells. Application of 2-APB (100µM) had complex and

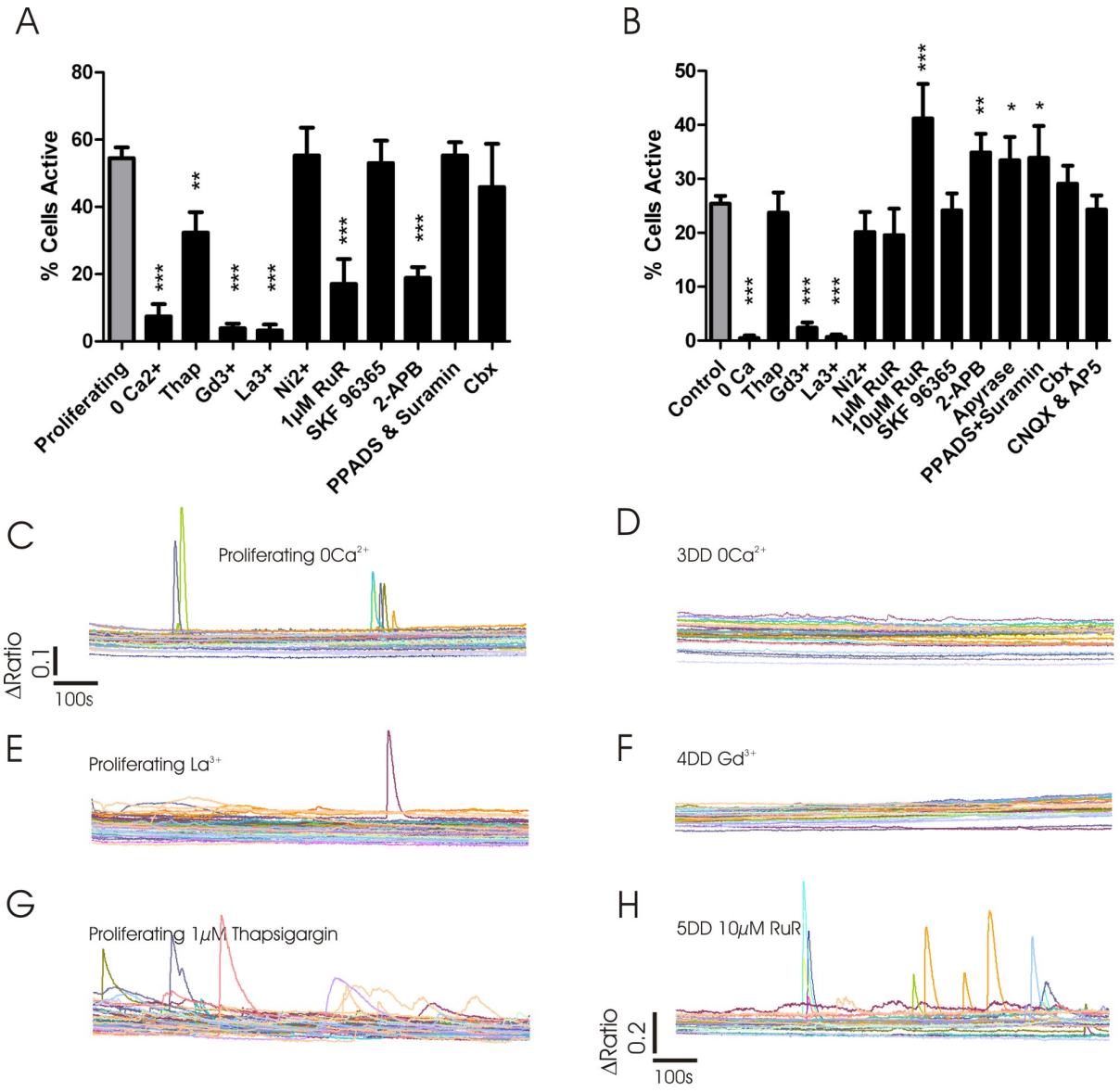

Figure 6. Pharmacological characterisation of spontaneous Ca^{+2} transients.

A) Effect of antagonists on the number of active cells during proliferation and B) differentiation. Example traces are shown for Ca^{2+}-free solution in (C) proliferating (27 cells) and (D) differentiating cells (25 cells), and for (E) La^{3+} in proliferating (34 cells) and (F) Gd^{3+} differentiating cells (28 cells). (G) 1µM thapsigargin reduced activity in proliferating cells (44 cells), while (H) 10µM RuR increased activity in differentiating cells (27 cells). Scales equivalent in C-G.

diverging effects on proliferating and differentiating cells. 2-APB is an agonist for TRPV1 and TRPV3 (note that human TRPV2 does not respond to 2-APB, unlike rat and mouse; Neeper et al. 2007), and upon wash in to proliferating cells a robust, transient influx of Ca^{2+} was seen (77.5 ± 7.8%, N = 3; Fig. 7), indicating that TRPV1 and/or TRPV3 channels are present. In contrast, upon wash in to differentiating cells few exhibited a transient influx (11.6 ± 11.6%, N = 3), suggesting that TRPV1 and/or TRPV3 channels are largely absent in

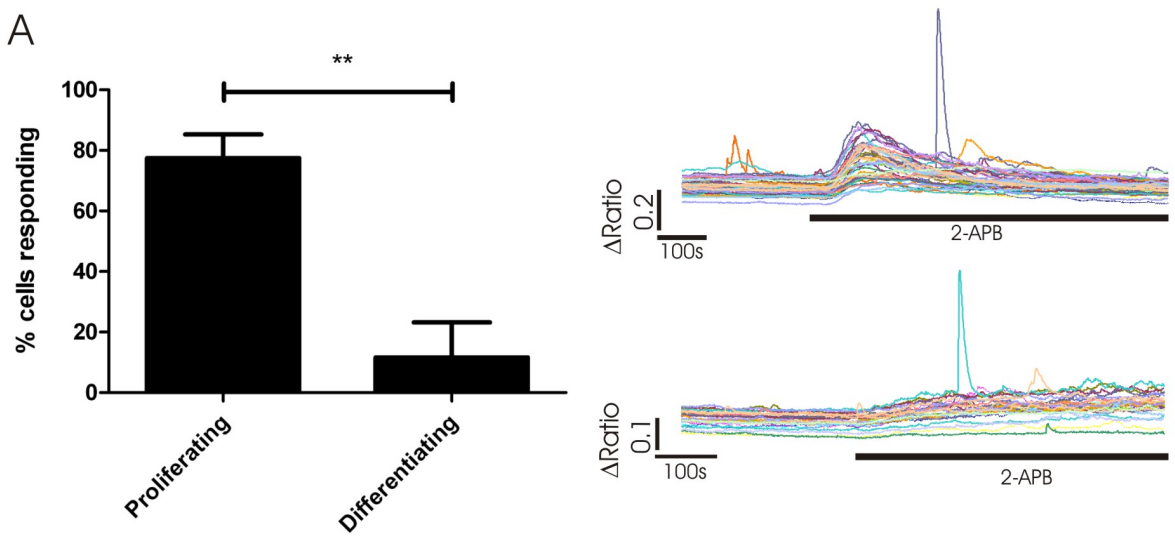

Figure 7. TRPV1/3 receptors are expressed in proliferating cells but down regulated during differentiation.

A) 2-APB strongly induced Ca^{2+} influx in proliferating but not differentiating cells. B) Example of 2-APB application onto proliferating cells (42 cells). C) Differentiating cells rarely produced a transient influx in response to 2-APB, but many showed a slight, continuous increase in baseline Ca^{2+} (27 cells).

differentiating cells, though many did show a small increase in their baseline Ca^{2+} level (Fig. 7). Measurement of activity levels with continued exposure to 2-APB showed a decrease in proliferating cells (18.9 ± 3.2%, N = 3, n = 8, $p < 0.001$), whereas in differentiating cells activity levels increased (34.9 ± 3.4%, N = 3, n = 8, $p < 0.01$). As shown in Table 1, 2-APB inhibits a variety of Ca^{2+} signalling mechanisms, including release from internal stores and gap junction coupling via connexins. Gap junction coupling allows the movement of Ca^{2+} and $InsP_3$ between cells, which may initiate Ca^{2+} transients in coupled cells, and unpaired connexins have been shown to initiate Ca^{2+} signals in the developing ventricular zone (Weissman et al. 2004). Gap junction coupling did not appear to influence the Ca^{2+} signalling in the ReNcell VM cells as application of the connexin antagonist carbenoxolone (100µM) had no effect on proliferating (45.9 ± 12.8%, N = 3, n = 6, $p > 0.05$) or differentiating (29.1 ± 3.3%, N = 3, n = 9, $p > 0.05$) cells. Inhibiting store release with thapsigargin (1µM) reduced activity in proliferating cells (32.4 ± 6.1%, N = 3, n = 6, $p < 0.01$, Fig. 6G) but not in differentiating cells (23.7 ± 3.8%, N = 3, n = 9, $p > 0.05$), suggesting that the reduction resulting from 2-APB in proliferating cells may involve inhibition of store release. Ruthenium red (RuR) can be used as a broad spectrum TRPV channel blocker. At 1µM it blocks TRPV1, 2, 3, 4 and 5, while TRPV6 is blocked by 10µM. Application of 1µM RuR to proliferating cells reduced the level of spontaneous activity (17.1 ± 7.4%, N = 3, n = 6, $p < 0.001$), whereas no effect was observed in differentiating cells (19.6 ± 4.9%, N = 3, n = 7, $p >$

0.05). Unexpectedly, 10µM RuR increased the level of activity in differentiating cells (41.2 ± 6.8%, N = 3, n = 8, p > 0.001, Fig. 6H). Addition of the glutamate receptor antagonists AP5 (50µM) and CNQX (20µM) to differentiating cells had no effect on the level of activity (24.3 ± 2.6%, N = 3, n = 9, p > 0.05).

3.5 Regulation of Calcium Signalling Activity by Extracellular K^+

During the course of investigating the transients I observed that alterations in the extracellular KCl concentration dramatically altered the level of spontaneous activity. Reducing the level of extracellular KCl from 2.4mM in controls to 1, 0.1 or 0mM (in the latter case replaced with either LiCl or choline chloride) increased the level of activity in both proliferating and differentiating cells, measured by the number of cells active and the frequency of transients in active cells (Fig. 8A,B,E). Conversely, increasing the KCl concentration to 15 or 35mM reduced the level of activity (Fig. 8A,B,E). This effect was highly non-linear in relation to Vm (Fig. 8D) approximated using linear regression from Vm measured in 6DD cells with 0, 2.4, 15 and 35mM KCl (R^2 = 0.91; Vm: 2.4K -79.9 ± 1.4mV n = 21, 0K -94.2 ± 4.3mV n = 12, 15K -42.5 ± 1.4mV n = 5, 35K -23 ± 1mV n = 4). The increase in activity resulting from removing extracellular K^+ could be blocked by 100µM $LaCl_3$, indicating that the source of the transients was the same as that in control cells (proliferating 0K: 99.4 ± 0.6%, N = 1, n = 3 vs 0K+La^{3+} 1.3 ± 0.7% N = 3, n = 4, p < 0.001; differentiating 0K: 93.1 ± 1.1%, N = 5, n = 18 vs 0K+La^{3+} 0.5 ± 0.5, N = 3, n = 6, p < 0.001; Fig. 8C). The increase in number of cells active corresponded to an increase in the frequency of transients per cell (proliferating 2.4KCl n = 408 vs 0.1KCl n = 275 p < 0.001, differentiating 2.4KCl n = 344 vs 0.1KCl n = 213 p < 0.001, Kolmorogov-Smirnov 2 sample test, Fig. 8A,B).

3.6 Influence of K^+ concentration on hNPC development

The ability to regulate the level or effect of endogenous calcium signalling by changing the extracellular K^+ concentration enables the manipulation of these signals in culture. This in turn allows determination of the influence of these signals without the use of antagonists which have non-specific actions, and maintains the spatial and temporal properties of the signals (including association with developmental state) which may be lost by applying agonists to the culture medium.

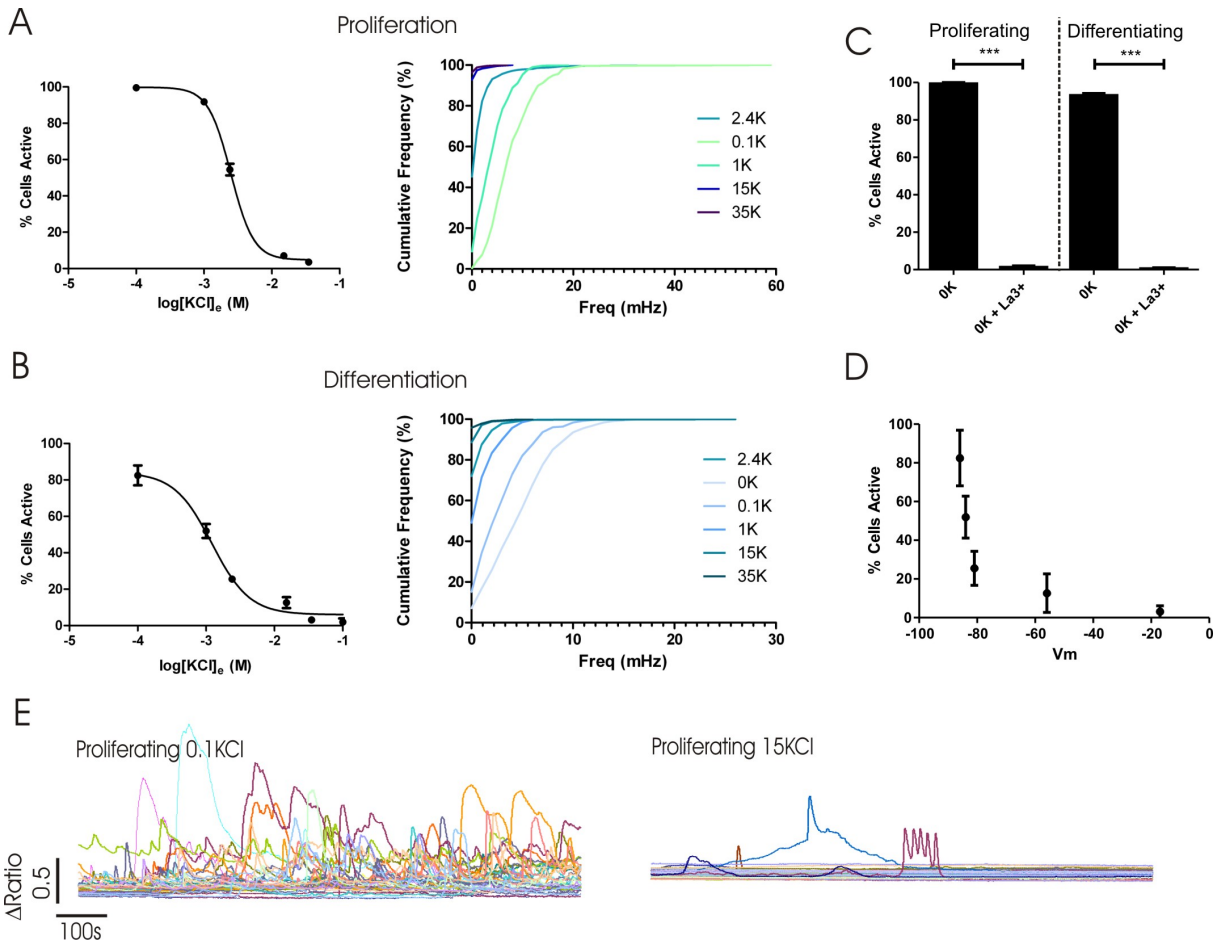

Figure 8. Effect of KCl concentration on Ca^{2+} signalling.

KCl had strong, non-linear effects on the number of cells active and the frequency of transients per cell in (A) proliferating and (B) differentiating cells. C) In both proliferating and differentiating cells the KCl induced increase could be blocked by La^{3+}, suggesting that the source of the transients remains the same. D) To examine the relationship between Ca^{2+} activity and membrane potential (Vm) the KCl concentration was plotted as Vm, calculated by linear regression from patch clamp recordings of Vm in differentiating cells at different KCl concentrations. E) Example traces of spontaneous activity in proliferating cells under 0.1mM (36 cells) and 15mM KCl (57 cells).

As both membrane potential and Ca^{2+} are involved in cell cycle regulation I examined the effect of lowering K^+ on proliferation rate and the cell cycle phase distribution. To examine the effect of K^+ on proliferation rate CASY measurements were taken from cells exposed to low (1mM), normal (4.16mM) and high (15mM) KCl in the culture medium after 24, 48 and 72 hours. Under low K^+ conditions the proliferation rate of the cells was slower than control or high K^+ treated cells (doubling time control: 17.4 hours, N = 5, low: 33.4 hours, N = 5, high: 17.2, N = 3; Fig. 9A), and resulted in significantly fewer cells at 72hrs ($p < 0.001$). There was no significant difference between control and high KCl conditions. There was a very small, but statistically significant reduction in the viability of cells under low KCl

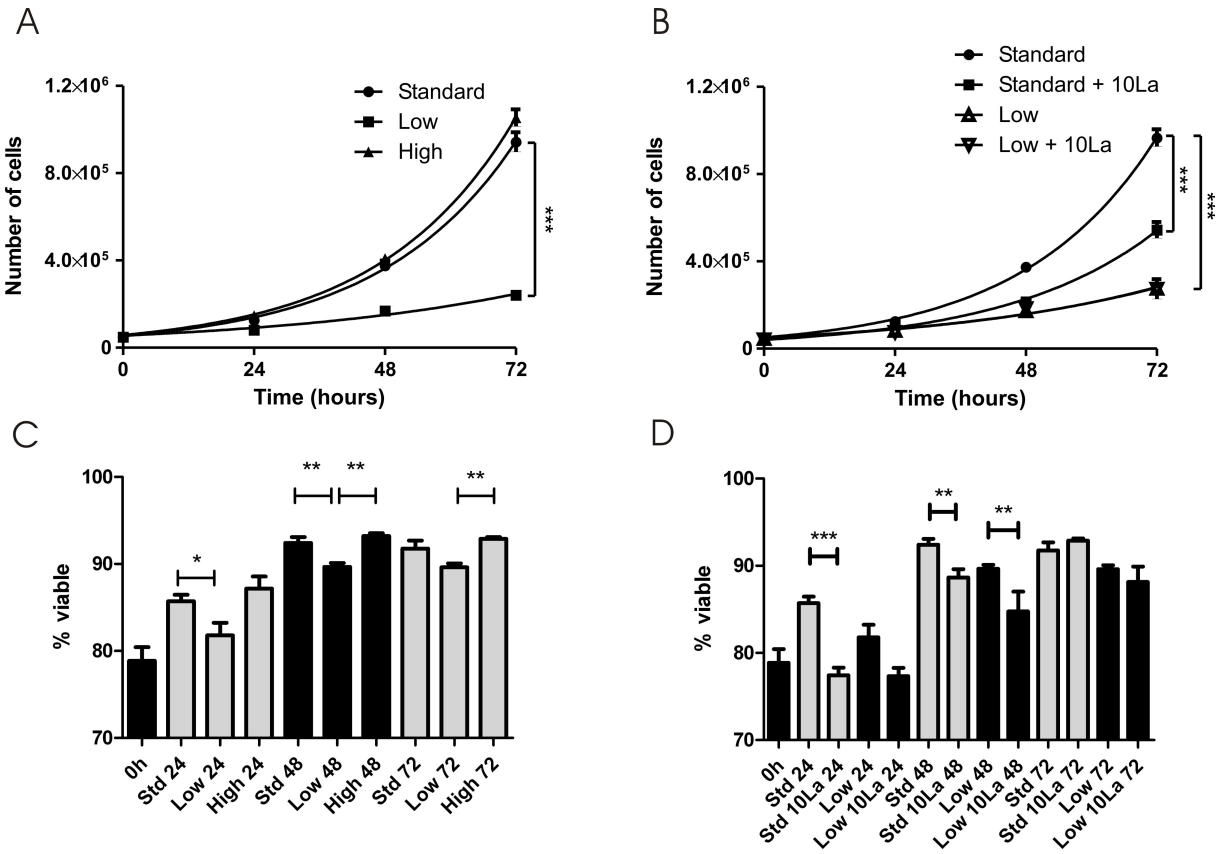

Figure 9. Effects of KCl concentration on proliferation rate and viability.

Cells were cultured according to the StdP in standard, low (1mM) and high (35mM) KCl medium for 72 upto hours. A) Low KCl dramatically reduced the rate of proliferation, while high KCl had little effect. B) Application of 10µM La^{3+} reduced the rate of proliferation in standard medium, but had no effect on cells cultured in low KCl. C) Low KCl slightly reduced the viability of hNPCs. D) 10µM La^{3+} reduced the viability of cells at 24 and 48 hours, but this effect was no longer present by 72 hours.

compared to standard and high KCl media ($p < 0.05$, ANOVA with Tukey's multiple comparison test), while there was no significant difference between high KCl and standard medium (Fig. 9C). This effect of low K^+ could be due to the increased average age of cells rather than a direct reduction in cell survival.

To examine the role of Ca^{2+} transients in the effect of low K^+ the control and low KCl experiments were repeated with the addition of 10µM La^{3+}, which reduced Ca^{2+} activity to a similar extent as 15mM KCl (data not shown). In standard medium the addition of La^{3+} reduced the proliferation rate (doubling time standard: 16.9 hours, N = 7, standard + La^{3+}: 19.2 hours, N = 3) and resulted in significantly fewer cells at 72hrs ($p < 0.001$; Fig 9B). In contrast, addition of La^{3+} to low KCl medium had no effect on the doubling time (low: 29.4 N = 7 hours low + La^{3+}: 28.8 hours, N = 3) or total number of cells ($p > 0.05$). There was a small reduction in viability under La^{3+} at 24 and 48 hours (Fig. 9D), but this is insufficient to

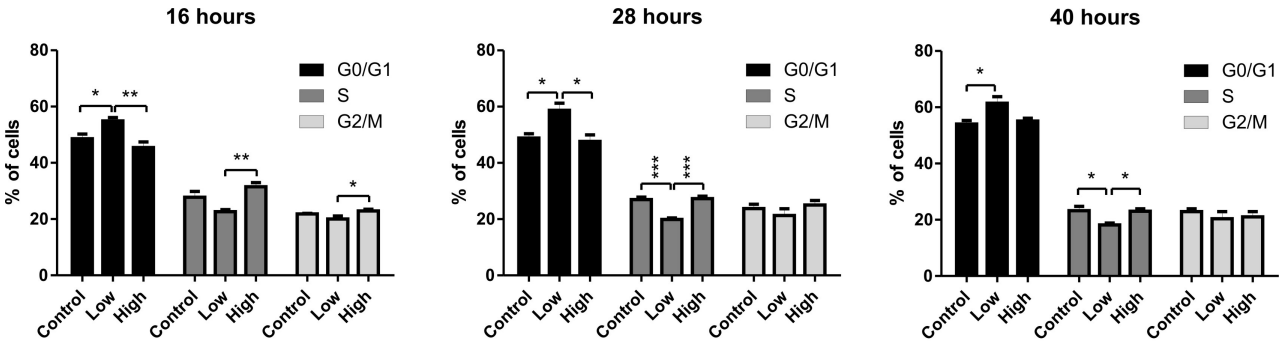

Figure 10. Effect of K^+ concentration on cell cycle progression

Standard protocol cells were proliferated in control, low or high KCl concentrations or 16, 28 and 40 hours. The cell cycle distribution remained even during this time, indicating that there was no arrest in any one phase. The increase in G1 with low KCl therefore can be attributed to a lengthening rather than arrest. N 16hrs = 6, 28hrs = 4, 40hrs = 4.

account for the reduction in proliferation rate under standard conditions. Ca^{2+} signals and membrane potential are associated with transition between cell cycle phases and progression through G1 (Wonderlin and Strobl 1996, Santella 1998). To determine if lowering KCl was acting at a specific point in the cell cycle, distribution within the cycle was analysed after 16, 28 and 40 hours. Low K^+ appeared to slow all phases of the cell cycle and did not induce arrest, however there was a slight increase of cells in G1/G0 phase compared to standard medium (16hrs N = 6, 28hrs N = 4 , and 40hrs N = 4; $p < 0.05$, ANOVA with Tukey's multiple comparison test). There was also a reduction in the length of S phase ($p < 0.05$, ANOVA with Tukey's multiple comparison test; Fig. 10).

Both lengthening of G1 phase and shortening of S phase have been associated with neurogenesis (Calergari and Huttner 2003, Arai et al. 2011), therefore to determine if low K^+ increased neurogenesis cells were proliferated under standard, low and high KCl medium for 16 hours prior to inducing differentiation in standard medium. After 3DD βIII expression was analysed using flow cytometry, and no statistically significant difference was found ($p > 0.05$, ANOVA with Tukey's multiple comparison test; Fig. 11A).

To examine if K^+ had any effect on neural development after differentiation the cells were allowed 24 hours to exit the cell cycle before switching to standard, low or high KCl media. Again, after 3DD βIII expression was analysed using flow cytometry, and no statistically significant difference was found ($p > 0.05$, ANOVA with Tukey's multiple comparison test; Fig. 11B).

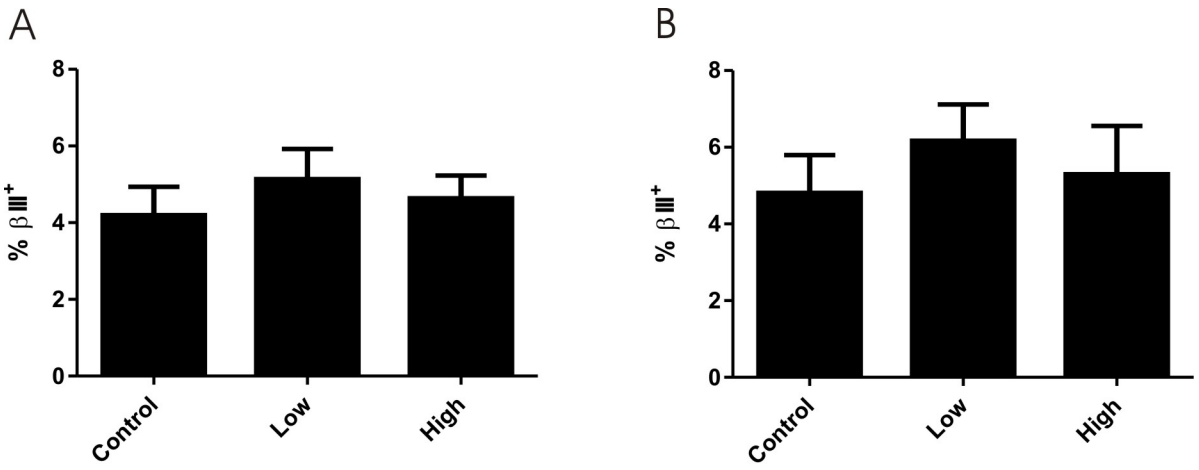

Figure 11. Effect of K^+ on neuronal differentiation

A) To examine the influence of KCl concentration during proliferation had on neurogenesis ReNcell VM cells were proliferated in standard, low and high KCl medium for 16 hours prior to differentiation in standard medium. After 3DD flow cytometry experiments showed no effect on βIII expression. B) Low, high and standard KCl medium was applied to the cells after 24hours differentiation. βIII expression was analysed after 3DD and showed no significant difference compared to control medium.

3.7 Functional Development of ReNcell VM cells

Functional ion channels are fundamental to the operation of neurons, therefore it is necessary to examine the ability of stem cell-derived neurons to appropriately express these properties, since this will determine their ability to function as replacement cells. For this purpose I made whole cell patch clamp recordings from ReNcell VM cells grown as monolayer cultures (StdP) and as neurospheres (PreP) between 2 and 10DD.

Putative voltage-gated Na (Na_v) and K (K_v) currents were observed in both StdP (Na_v: 1/26, K_v: 3/26) and PreP (Na_v: 6/71, K_v: 8/60; Fig. 12) cultures. There was no statistically significant difference between the two protocols. In neither StdP nor PreP cultures could Na_v expressing cells generate action potentials, spontaneously or upon current injection in current clamp mode. Nor did any cells display post synaptic currents.

Curiously, an inactivating leak current was frequently observed, which could be abolished by application of 100µM Cbx (Fig. 13). Cbx also increased membrane resistance (control: 95.3 ± 23.5MΩ, Cbx: 1205 ± 292MΩ n = 4, $p < 0.05$, paired t-test; Fig. 13E) and decreased non-inactivating leak current amplitudes (Fig. 13C,D). In some cells Cbx unmasked slow inward currents, possibly Ca_v currents, and K_v currents hidden by the inactivating leak current.

Unfortunately, Cbx can block Ca_v channels (see Table 1) and therefore cannot reliably be used to uncover masked Ca^{2+} currents, nor could the cells be readily patched under Cbx. Therefore, to determine whether the cells could express functional Ca_v channels I stimulated

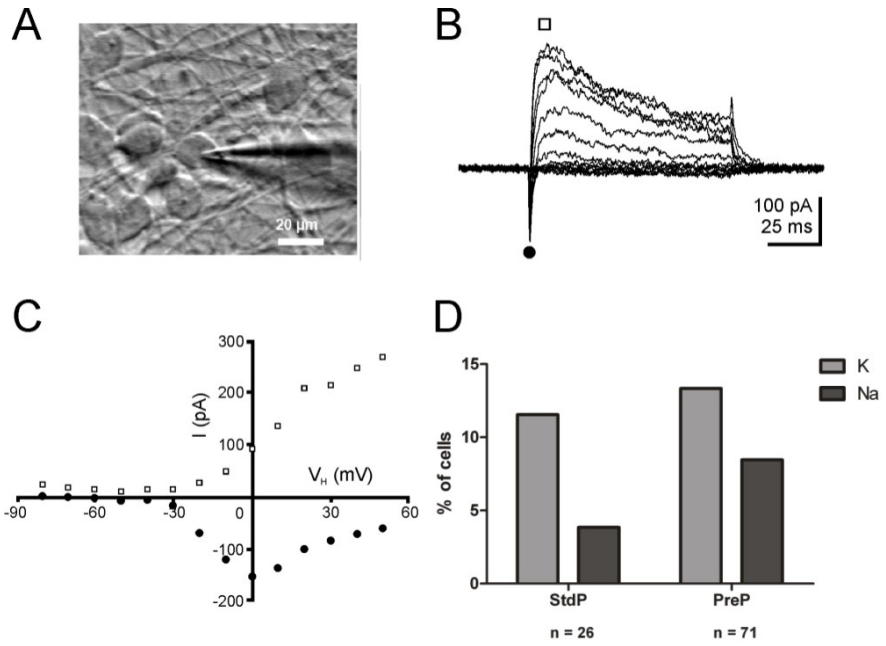

Figure 12. Functional development of ReNcell VM cells.

Whole cell patch clamp recordings were made from hNPCs between 2 and 10DD. A) A patched cell B) hNPCs could express Nav (black circle) and Kv (open square) currents. C) I-V curves for currents in B, showing typical relationships for Na_v (black circles) and K_v (open circles). D) Na_v and K_v currents could be detected in ReNcell VM cells cultured following either StdP or PreP. There was no statistically significant difference between the two conditions.

differentiating cells with 35mM KCl and measured the Ca^{2+} influx with Fura-2. Stimulation of StdP cultures produced only a slight influx of Ca^{2+} in a few cells (3.8 ± 0.9%, N = 2, n = 6; Fig. 14A), indicating little expression of functional Ca_v channels. In contrast, stimulation of PreP cultures produced a robust Ca^{2+} influx (66.2 ± 5.2%, N = 4, n = 10; Fig. 14A,B) which could be blocked by application of 2.5mM $NiCl_2$ (1.7 ± 0.8%, N = 2, n = 6, $p < 0.001$; Fig. 14A,B) or removal of extracellular Ca^{2+} (0/200 cells, N = 1), demonstrating the expression of Ca_v channels.

Another feature in the development of hNPCs is the expression of ligand-gated ion channels. As both NMDA glutamate receptors and purinergic P2X receptors lead to an increase in intracellular calcium, their expression can be readily tested through calcium imaging. Application of the P2X receptor agonist 2-methylthio-ATP (20µM) could induce a Ca^{2+} influx in 84.2 ± 4.9% (N = 4) of PreP cultured differentiating cells (Fig. 14C,D). This could be blocked by 5µM PPADS (2-meATP n = 142/168 cells, 2-meATP+PPADS n = 0/84, $p < 0.001$ Fisher χ^2 test). To test for the expression of NMDA receptors I applied 50µM NMDA in the presence of 15mM KCl, so as to depolarise the cells and prevent Mg^{2+} from blocking the

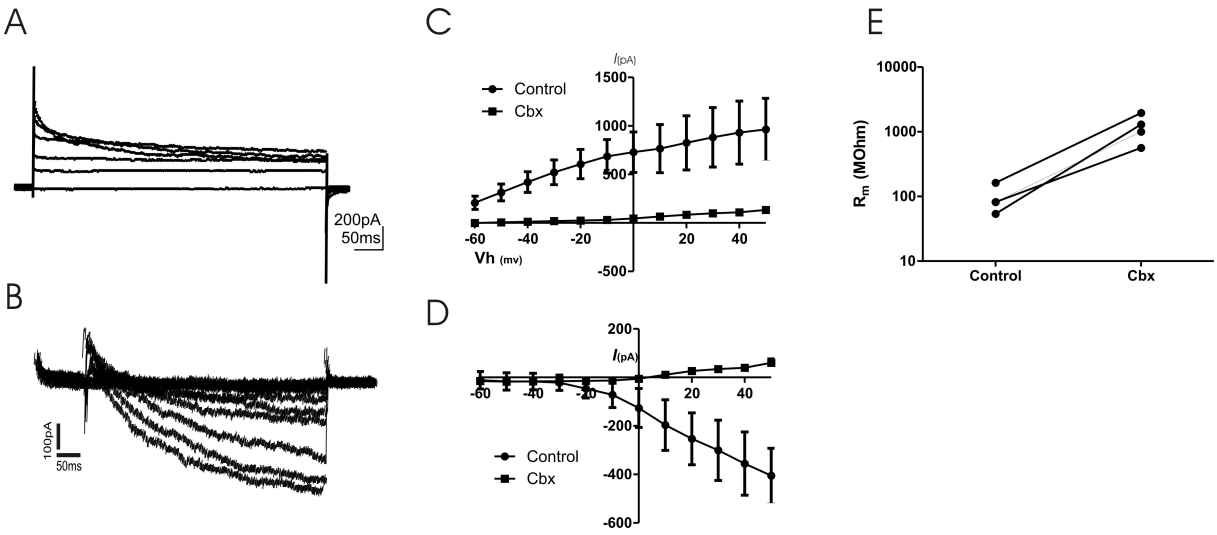

Figure 13. Inactivating leak current.

Many cells showed an inactivating leak current, shown without leak subtraction (A) and with p/n leak subtraction (B), which isolates the inactivating component. C) Steady-state IV curves for inactivating leak before and after Cbx application. D) Steady state IV curves with leak subtraction show abolition of inactivating component by Cbx. E) Rm recorded before and after Cbx application.

channel pore, which would prevent Ca^{2+} influx even when channels were activated. No differentiating cells responded to NMDA (N = 3).

3.8 Veratridine promotes neuronal survival in culture

Having established that the cells could express functional ion channels, and observing that the number of neuronal cells decreased after several days in culture, I next examined whether the survival of the neuronal hNPCs could be enhanced through stimulation by veratridine (Vtr). Vtr is a Na_v agonist which binds to the open channel to prevent its time-dependent inactivation (Ulbricht 2005). This provides a gentle depolarising stimulation which, as it does not directly activate the ion channels, has less risk of inducing excitotoxicity. To maximise the potential benefit of treating the cells with Vtr the pre-aggregation protocol was used and cells were differentiated in the presence of cAMP and GDNF. The cells were cultured with or without 1µM Vtr for the duration of their differentiation.

I quantified immunocytochemical stainings for βIII and TH at 1, 2, 3, 4 and 7DD. In control conditions βIII expression peaked at 3DD before declining (1DD 0.24 ± 0.04%, N = 4, 2DD 0.64 ± 0.05%, N = 4, 3DD 0.89 ± 0.07%, N = 10, 4DD 0.73 ± 0.05%, N = 7, 7DD 0.29 ± 0.05%, N = 4; Fig. 15A). Similarly, TH expression peaked at 2DD, after which it declined (1DD 0.05 ± 0.02%, N = 4, 2DD 0.11 ± 0.02%, N = 4, 3DD 0.09 ± 0.02%, N = 10, 4DD 0.08

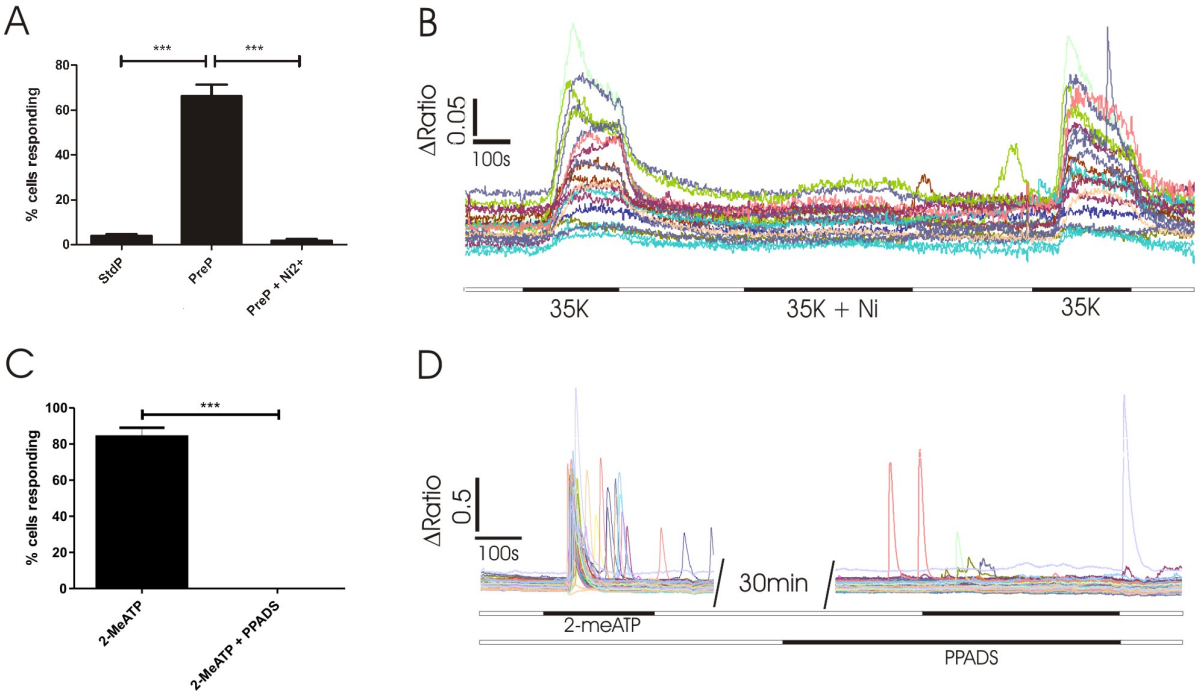

Figure 14. Ca_v channels and P2 receptors in differentiating hNPCs.

A)35mM KCl induced Ca^{2+} influx in a large proportion of PreP cultured cells but not StdP cultured cells. This influx could be blocked by 2.5mM Ni^{2+}, indicating involvement of Ca_v channels. B) Example traces (20 cells) showing response of PreP cells to 35KCl and block by Ni^{2+}. 35mM KCl experiments were performed using a 10x objective. C) 2-methylthio-ATP (2-Me-ATP) induced a Ca influx in the majority of PreP cultured hNPCs, this could be abolished by incubation in 5µM PPADS. D) Example traces showing 2-MeATP induced Ca^{2+} in PreP cultured hNPCs and abolition by PPADS (40 cells).

± 0.02%, N = 7 and 7DD 0.03 ± 0.01%, N = 4; Fig. 15C). In Vtr treated cells βIII expression was increased up to two fold at 4DD and 7DD compared to controls, prior to 4DD there was no statistically significant difference (1DD 0.34 ± 0.05%, N = 4, 2DD 0.86 ± 0.07, N = 6, 3DD 0.83 ± 0.07%, N = 14, 4DD 1.05 ± 0.07%, N = 6, 7DD 0.57 ± 0.06%, N = 8). Vtr treated cells also showed a significantly higher number of TH positive cells at 2 and 4DD (2DD 0.19 ± 0.03%, N = 4, $p<0.05$, 4DD 0.16 ± 0.03% N = 7, $p<0.01$; Fig. 15C) in comparison to untreated cells. Although at 1, 3 and 7DD the number of TH positive cells treated with Vtr was higher in comparison to control, these differences were not statistically significant (1DD 0.06 ± 0.02 N = 4, 3DD 0.13 ± 0.03% N = 10, 7DD 0.06 ± 0.03% N = 4, $p > 0.05$ vs control).

To confirm that the effects of Vtr in cell cultures were caused by its action on Na_v channels and not by other, non-specific effects the cell cultures were treated with the Na_v channel antagonist TTX (1µM) in the presence of Vtr. Stainings were made at 3DD, where no significant Vtr effects on βIII positive cells had been observed, and 7DD, where Vtr

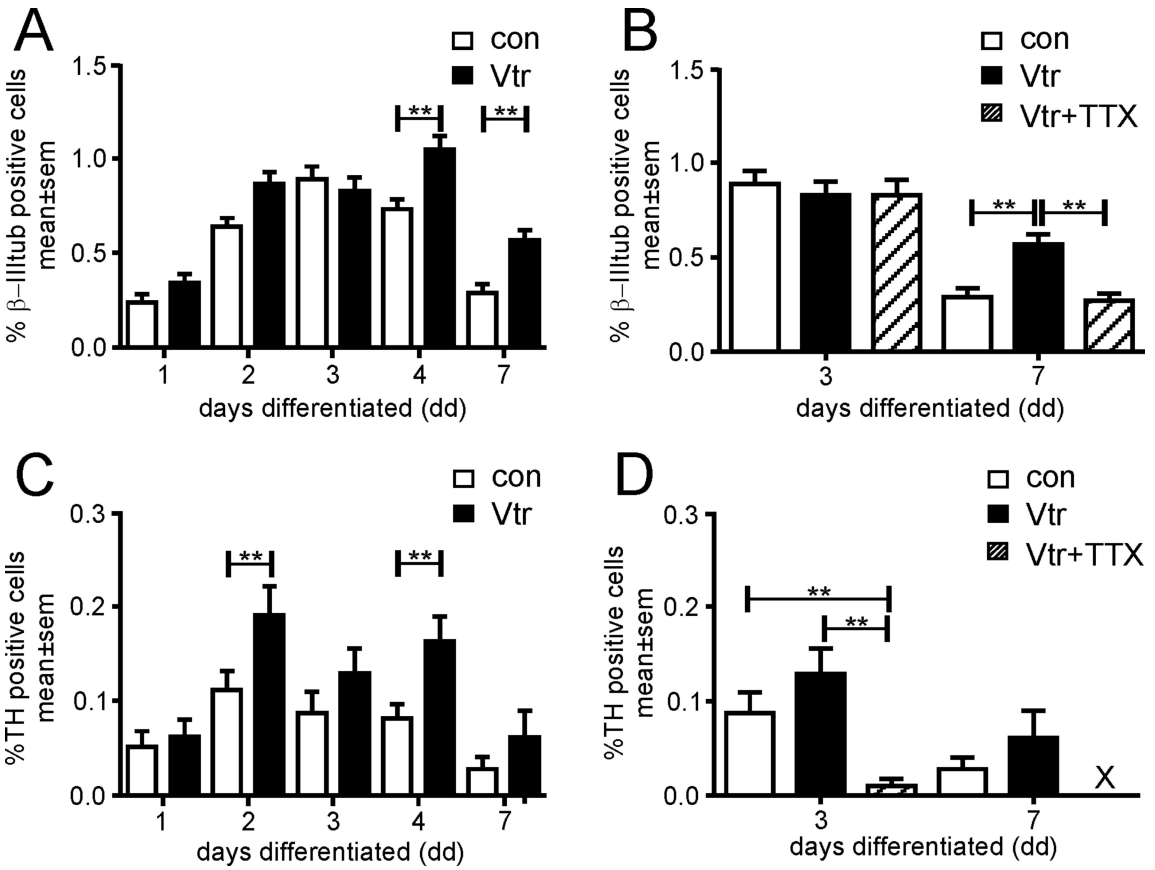

Figure 15. Vtr improves survival of hNPCs

(A) Percentage of β-III$^+$ cells over time. In the control experiments (white bars) there is an initial increase up to 3DD, after which the number of positive cells declines. Application of Vtr (black bars) significantly reduced the decline in cell numbers at 4 and 7DD. (B) In accordance with (A), treatment of cells with Vtr+TTX (striped bars) had no effect on cell numbers at 3DD, but prevented the Vtr induced increase at 7DD. (C) Percentage of TH$^+$ cells over time. The increase in cell numbers in the Vtr treated group (black bars) was statistically significant at 2 and 4DD compared to controls (white bars), the increase at 3dd did not reach significance ($p>0.05$). (D) Treatment with Vtr+TTX (striped bars) at 3DD reduced the number of TH$^+$ cells below control values, suggesting an activity dependence in survival is important at this stage. After 7DD with Vtr+TTX no TH$^+$ cells were found. Remarkably one could observe a shift in the peak of the expression of β-III and TH. ** = $p<0.01$.

significantly increased the number of βIII positive cells. Conforming with the effect of Vtr observed, at 3DD there was no significant difference in the number of βIII positive cells between Vtr treated and Vtr+TTX treated cultures (Vtr 0.83 ± 0.07%, Vtr+TTX 0.83 ± 0.08%, N = 4), whilst at 7DD there was a significant decrease in the number of βIII positive cells in the TTX treated culture ($p < 0.01$, Vtr 0.57 ± 0,06%, Vtr+TTX 0.27 ± 0.04%, N = 5; Fig. 15B). At 3DD the number of TH positive cells was significantly reduced in the TTX treated cultures ($p < 0.01$, Vtr 0.13 ± 0.03%, Vtr+TTX 0.01 ± 0.001%; Fig. 15D). At 7DD no TH$^+$ cells were found in Vtr+TTX treated cultures.

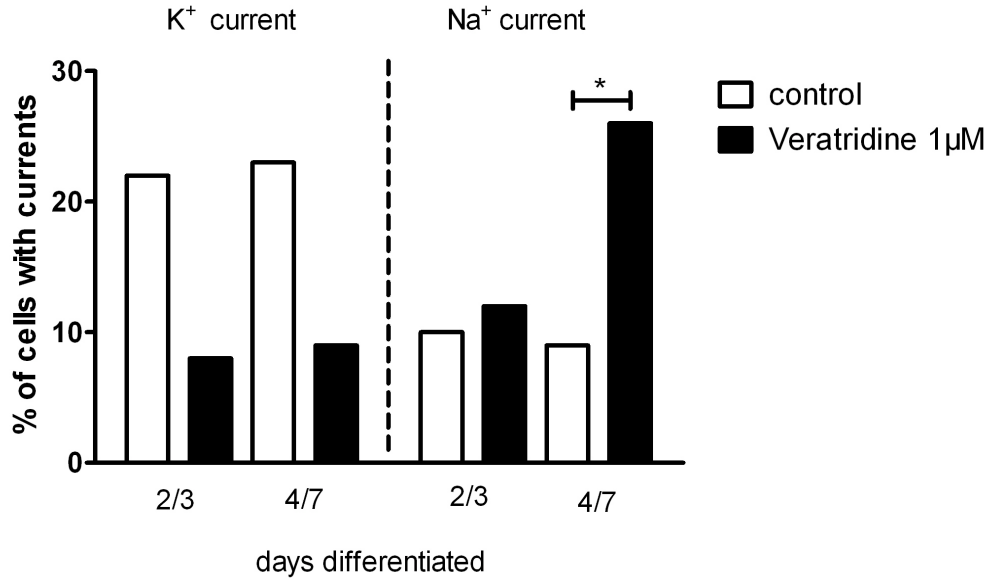

Figure 16. Vtr increases number of cells expressing Na_v currents

Patch clamp recordings were made from PreP cells cultured with and without 1µM Vtr. Vtr treated cells showed a significantly higher number of cells with Na^+ currents at 4/7DD ($p = 0.02$ Fisher χ^2 test), but not at 2/3DD. Vtr had no effect on K_v currents at either 2/3 or 4/7DD.

Patch clamp recordings from cells cultured with Vtr were made at 2, 3, 4 and 7DD. As no statistically significant difference was found between 2 and 3DD and 4 and 7DD the respective data sets were pooled. At 2/3DD 13% (2/15) of Vtr treated cells showed Na_v currents, which was not significantly different compared to control cells, where 10% (3/28) of cells expressed Na_v currents ($p = 0.8$, Fisher χ^2 test). After 4/7DD the percentage of cells expressing Na_v currents in the control group remained unchanged at 10% (3/29), whereas the number of Na_v currents in the Vtr group increased to 26% (10/28, $p < 0.05$, Fisher χ^2 test). Due to the low number of cells showing Na_v currents a more detailed analysis was not performed. Although the analysis of voltage-dependent outward currents, showing a typical IV-relationship of K_v currents (Fig. 16), revealed a higher number of cells with currents in the control group (2/3DD: 21%, 4/7DD: 14%) in comparison with the Vtr treated group (2/3DD: 7%, 4/7DD: 7%) the difference was not statistically significant ($p > 0.05$, Fisher χ^2 test; Fig. 16).

3.9 Co-culture of hNPCs on rat hippocampal brain slices

The cell culture environment provides controlled conditions in which to examine the differentiation of hNPCs, however, if hNPCs are to be successfully used in cell replacement

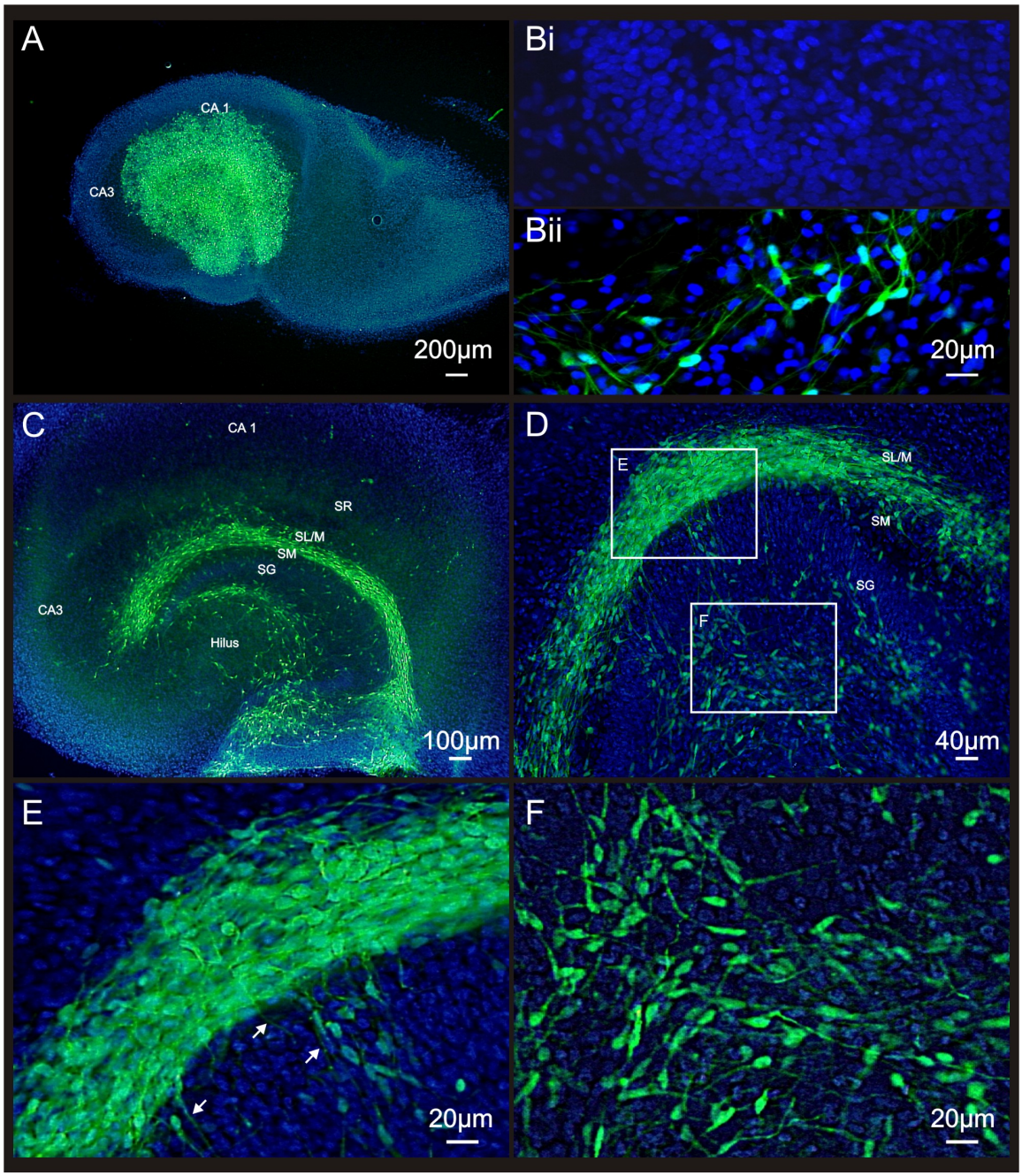

Figure 17. Distribution of hNPCs transplanted onto hippocampal slice cultures

GFP expressing cells were seeded on to the rat hippocampal slices and cultured for up to 24 days. (A) Distribution of the hNPCs 9hrs after seeding. The cells are located over a wide area of the slice with no clear regional preference. (Bi) Example of a confocal micrograph (provided by M Hovakimyan) of the surface of the slice showing no GFP-positive cells and (Bii) cells in the depth (approx. 40µm) of the same host tissue, demonstrating the ability of the hNPCs to penetrate the hippocampal tissue. (C) and (D) Examples of distribution of hNPCs along the stratum moleculare (SM) and lacumosum-moleculare (SM/L) regions. DAPI nuclear staining reveals the hippocampal structures. (E) Magnification of the region indicated by the inserted box in (D). The processes of most cells in the fissural region project in parallel to the hippocampal layers, but some processes can be seen to project perpendicularly into the dentate gyrus (white arrows). (F) Magnification of the dentate hilus area indicated by the box in (D). The orientation and distribution of hNPCs shows much less coherence compared to (E). SG: stratum granulosum, SR: stratum radiatum, SM: stratum moleculare.

therapies a detailed understanding of how they interact with and are influenced by their host environment is needed. To examine what influence a neural environment might have on hNPCs I co-cultured GFP expressing ReNcell VM cells on organotypic hippocampal brain slices from P6 rats and analysed their development between 3 and 24 days *in vitro* (DIV). Because neurospheres provide their own local microenvironment which can influence the development of the cells, and because they limit the potential for interaction between the slice and the hNPCs, standard protocol cells were used for the co-culture.

After transplantation the hNPCs formed a distinctive band along the regions around the hippocampal fissure, namely the stratum moleculare and stratum lacunosum-moleculare (Fig. 17C-E). This behaviour was highly reliable, occurring in almost all slices. Cells were also frequently found in the dentate gyrus, though with lower density. Occasionally cell bodies were present in the stratum radiatum, but cells were rarely found in the pyramidal cell layer or the stratum oriens. The hNPCs developed differentiated morphologies, typically uni- or bi-polar and extended processes throughout the slice. A clear pattern of projections emerged in the fissal region, with many running parallel to the laminar structure (Fig. 17E). Other cells in the fissal region extended processes outward into the stratum radiatum or dentate hilus. The cells located in the dentate gyrus showed no consistent pattern in their projections (Fig. 17F). The few cells seen in the stratum radiatum almost always sent their projections perpendicular to the laminar structure of the hippocampus. These observations suggest that the hNPCs are differentially responding to guidance cues present in the slice, and that characteristics of the hippocampal architecture, known to regulate the growth of axons during development, can also regulate the integration of hNPCs.

3.9.1 Immunocytochemical characterisation of hNPC differentiation in slice cultures

The fate of transplanted hNPCs was examined by immunostaining for βIII-tubulin, GFAP and Olig2. As with differentiation in cell cultures, a small proportion expressed βIII-tubulin (Fig. 18A,B). Similarly the detection of βIII-tub$^+$ cells was rarer and more variable at later time points, with only one βIII-tub$^+$ cell detected between 14 and 24DIV, consistent with the observation that in this cell line the number of neurons declines after several days in cell culture. In stark contrast to differentiation in cell culture, very few of the hNPCs expressed GFAP at any of the timepoints tested (Fig. 18C). As in cell cultures no cells positive for Olig2 were found (data not shown). Throughout the culture period most of the cells continued to express the progenitor marker nestin (Fig. 18E,F), indicating that they had not fully

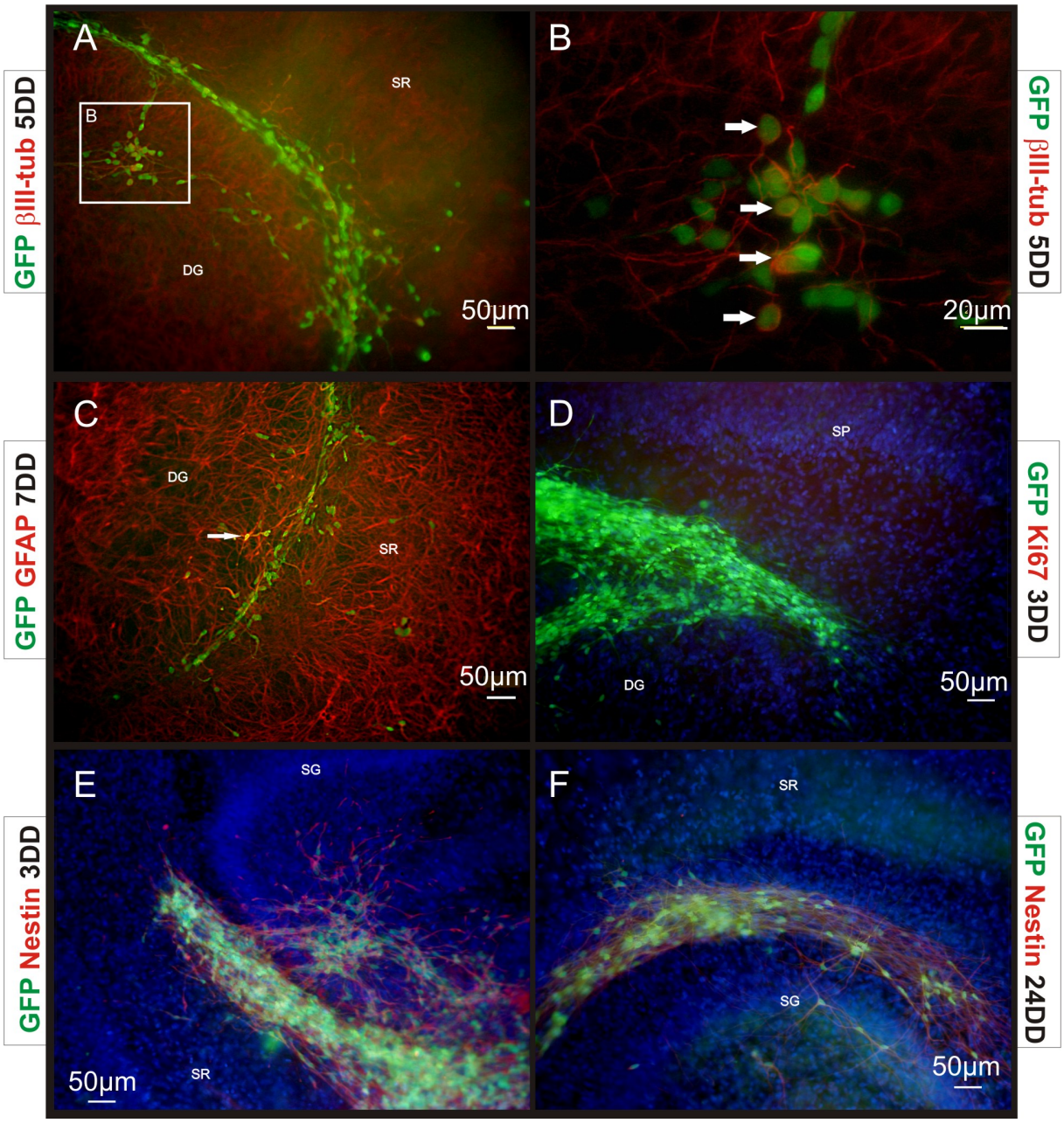

Figure 18. Differentiation of hNPCs transplanted onto hippocampal slice cultures

A) βIII^{+} hNPCs could be detected on the slices (B) magnification of region indicated in (A). (C) GFAP was largely absent, but occasional positive cells were detected, indicated by the arrow. (D) Ki67 was not expressed by the hNPCs, showing they were no longer proliferating. Nestin was expressed by most cells throughout the culture period, 3DIV shown in (E) and 24DIV in (F). SR:stratum radiatum, DG: dentate gyrus, SP: stratum pyramidale.

differentiated, though no cells continued to proliferate, shown by the absence of any Ki67 positive cells (Fig. 18D). Therefore most of the cells remained in an intermediate state and did not differentiate into any of the three major neural cell types.

In contrast to the hNPCs transplanted onto the slices those growing on the culture insert membranes, away from the slices, had immature morphologies and some continued to

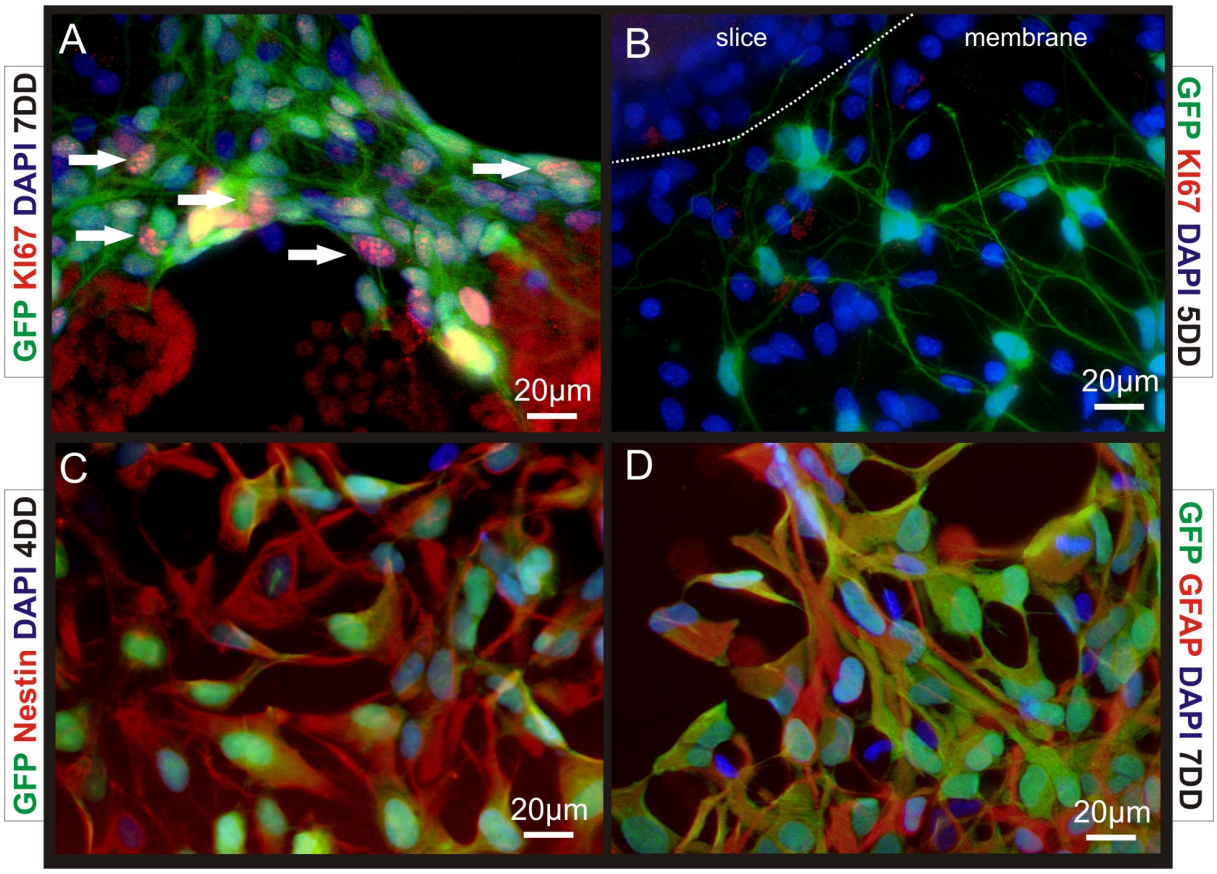

Figure 19. Development of hNPCs on culture insert membranes

hNPCs growing on the culture insert membrane away from the slice remained in an immature state. They continued to express the proliferation marker Ki67 (A), GFAP (C) and nestin (D) and had immature morphologies. Cells growing adjacent to the slice did not express Ki67, and had mature morphologies (B).

proliferate as demonstrated by expression of Ki67 (Fig. 19A). Those cells adjacent to the slice did not express Ki67 and had the fine processes and compact cell bodies of differentiated cells (Fig. 19B). The majority of hNPCs on the membrane also expressed nestin (Fig. 19C) and, unlike those on the slice, GFAP (Fig. 19D). These observations suggest that the cells on the insert membrane remained in either a quiescent or proliferative state, whereas the cells on the slice began to differentiate. As both hNPCs on the slice and the membrane are exposed to the medium, and hNPCs close to the slice show differentiated morphologies, these results suggest that it is the slice environment that is promoting the switch to differentiation.

3.9.2 Electrophysiological properties of transplanted NPCs

To examine the functional development of the transplanted hNPCs whole cell patch clamp recordings were made from GFP labelled cells. Voltage-dependent currents were elicited by a series of voltage steps from -60 mV to +50 mV. K_v and Na_v currents expected of neuronal

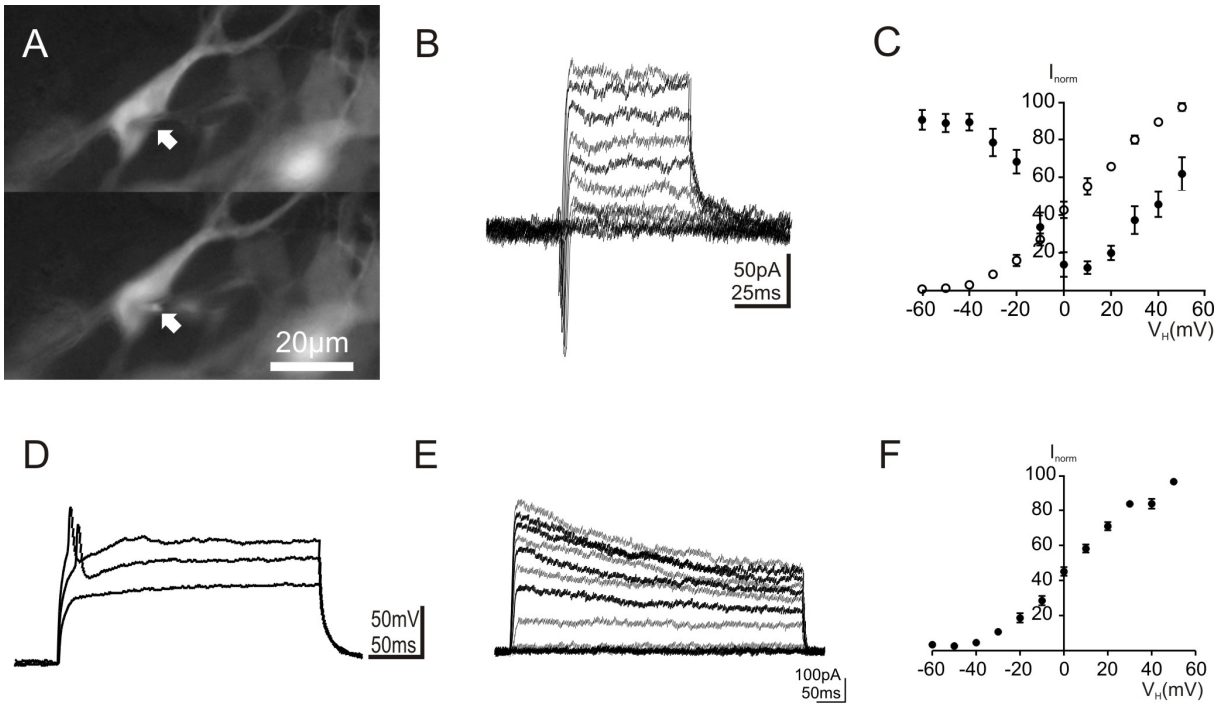

Figure 20. Electrophysiological properties of hNPCs on hippocampal slices

(A) Cell with patch pipette (upper panel) and GFP in tip (lower panel). (B) Current traces recorded from the cell in (A) in response to voltage steps from -60 to +50mV, showing Na^+ and K^+ currents. (C) Current-Voltage (*I*V) relationships of Na^+ and K^+ currents in cells expressing both currents. (D) Current clamp recording from the same cells showing an action potential evoked by current injection (30pA/step). (E) Outward current traces with *I*V relationships characteristic of K^+ channels shown in (F).

cells could be found (Fig. 20B,C), and upon current injection 6/10 of these cells produced a single action potential (Fig. 20D). Another population of cells expressed K_v currents alone (Fig. 20E). In many cases the current traces were dominated by the inactivating leak current, as was also observed in StdP and PreP cell cultures. The cells were categorised based on these currents. Na_v and K_v currents were found in 9% of cells (12/132, Fig. 21A). K_v currents alone were found in 39% (51/132) and 36% (48/132) expressed the inactivating leak current. In 14% (19/132) of hNPCs no voltage-gated currents were expressed. In comparison, the hNPCs with immature morphologies growing on the insert membrane did not express Na_v or K_v currents, consistent with their undifferentiated state. Several did, however, exhibit the inactivating leak current (8/14).

Comparison of the membrane potentials between groups (Fig. 21B) showed that neuronal cells tended to be depolarised relative to cells expressing K_v or inactivating leak currents (K_v -65.3 ± 2.8 mV, n = 46 vs Na_v -53.5 ± 3.7 mV, n = 11 and leak -74.0 ± 1.1 mV, n = 48, $p < 0.05$, vs none -54.3 ± 5.4 mV, n = 15, $p > 0.05$), consistent with reduced dependence on the potassium equilibrium potential. The mean membrane potential of K_v cells was significantly

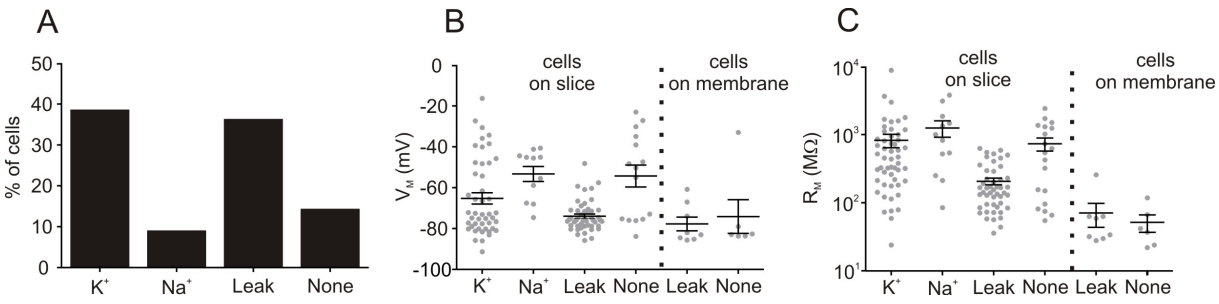

Figure 21. Current and membrane properties of transplanted hNPCs

(A) Percentage of transplanted cells showing Na^+, K^+, inactivating leak or no currents. (B) Distribution of membrane potentials and (C) membrane resistance of cells growing on the hippocampal slice or on the culture insert membrane, organised by current expression.

higher than the inactivating leak group, however, there was greater variability within the K_v group and many K_v cells also showed evidence of inactivating leak currents, therefore there is probably extensive overlap between these groups. The cells on the membrane also showed hyperpolarised membrane potentials (membrane leak: -77.8 ± 3.5 mV, n = 8, membrane none: -74.1 ± 8.3 mV, n = 6). No trend was seen over time within any groups (1-way ANOVA, $p > 0.05$).

The membrane resistance of cells expressing inactivating leak currents, both on the slice (207 ± 23 MΩ, n = 48) and membrane (52 ± 15 MΩ, n = 8), was lower compared to other cell groups (K_v 826 ± 184 MΩ, n = 52, Na_v 1252 ± 338 MΩ, n = 12, None 734 ± 159 MΩ, n = 19; Fig. 21C). The cells on the membrane without currents similarly had a low membrane resistance (71 ± 27 MΩ, n = 6).

3.9.3 hNPCs receive synaptic input

Synaptic connections are a fundamental part of neuronal functioning and therefore it is important for any NPC derived neuron to be capable of forming them. Spontaneous post-synaptic currents (PSCs) were observed at very low frequencies in several cells. These PSCs could be detected as early as 4DIV, similar to the timecourse of neuronal differentiation. To examine the extent to which hNPCs received synaptic input the slices were stimulated with 5µM kainate or 35 mM KCl. Both kainate (Fig 22) and KCl (data not shown) could induce synaptic activity. Stimulation of the cell shown in Fig. 22 resulted in an increase of the PSCs frequency from 0.14 Hz to 0.73 Hz (Fig 22B). A slightly higher amplitude was observed for the PSCs measured in the presence of kainate (control: 23.0 ± 0.9 pA, 42 events, kainate: 34.1

± 1.2 pA, 98 events). Analysis of the decay kinetics revealed a fast rise time in both groups (control 1.3 ms; kainate 0.6 ms) indicating that the currents originate from synaptic input rather than from an activation of postsynaptic glutamate receptors. The decay kinetics of the PSCs recorded in the control were best fitted by a mono-exponential function, whereas the PSCs in the presence of kainate were best fitted by a bi-exponential function (control τ: 2.6 ms, kainate:τ_1:11 ms, τ_2:37 ms, (Fig. 22C). The differences between the time constants may indicate different types of synaptic input, with the fast decay of the mono-exponential events suggesting excitatory input and the slow, bi-exponential decay suggesting inhibitory input. The difference in PSCs under control and kainate probably represents a change in network dynamics, where under broad stimulation inhibitory neurons dominate the network activity. The low number of cells exhibiting PSCs and the low frequency of this activity prevented further characterisation of their kinetic and pharmacological properties.

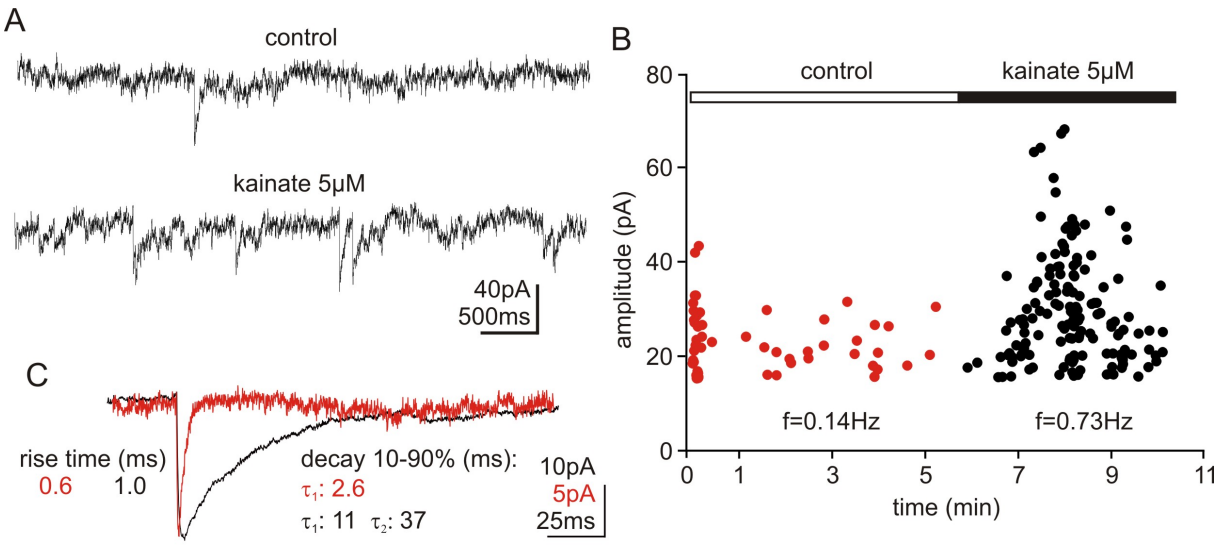

Figure 22. Synaptic connections between host tissue and hNPCs

Patch clamp recordings of PSCs demonstrating synaptic input on to the hNPCs. (A) Example of PSCs in a hNPC under control conditions (upper trace) and after to the application of kainate (lower trace). (B) The application of kainate induced a higher frequency of PSCs. (C) Superimposed average of PSCs recorded under control conditions (red trace) and in the presence of kainite (black trace). The analysis revealed a higher amplitude of the PSCs recorded under kainate and different decay kinetics, whereat the rise times were comparable. Although the number of analysed PSCs was to low for a detailed analysis, the different decay kinetics may hint to a different populations of PSCs originating from different sites of synaptic input.

4. Discussion

This study aimed to characterise the functional properties of an immortalised hNPC line, ReNcell VM, and to examine how these properties influence hNPC development. Spontaneous and evoked Ca^{2+} signalling mechanisms were investigated using Ca^{2+} imaging, and the level of spontaneous Ca^{2+} signalling was found to be controllable through altering the extracellular KCl concentration. The functional development of ReNcell VM cells was characterised in cell culture and organotypical hippocampal slice culture environments using whole-cell patch clamp recordings.

4.1 Calcium signalling in hNPCs

Ca^{2+} regulates many vital aspects of cellular functioning, and as such is tightly regulated by a range of mechanisms. Identifying the sources of Ca^{2+} signals in stem cells will contribute to understanding the role and regulation of Ca^{2+} signals during hNPC development, and will provide targets through which development might be guided.

4.1.1 Characterisation of spontaneous calcium signals

In the ReNcell VM cells spontaneous Ca^{2+} transients were observed during proliferation and differentiation. In both states, but more so during differentiation, most transients were dependent on extracellular Ca^{2+} and could be blocked by the lanthanide metals La^{3+} and Gd^{3+}. Combined with the inactivity of P2X and Ca_v channel antagonists, this suggests that TRP channels underlie the majority of spontaneous transients. It was also apparent with more detailed pharmacological characterisation that the channel types varied between proliferation and differentiation. During proliferation the cells were more active and there appeared to be a greater variety of sources, as RuR, 2-APB and thapsigargin treatment all decreased the level of activity. The inhibition of activity by RuR and the activation of Ca^{2+} influx by 2-APB indicate that TRPV1 and/or TRPV3 channels are present in the proliferating hNPCs and likely contribute to the spontaneous transients observed, while the inhibition by thapsigargin indicates that store release underlies some transients. Whether TRPC channels also contribute is unclear, as although continuous application of 2-APB inhibited activity, this was to a similar degree as RuR, and might be explained by inactivation of TRPV channels with continuous stimulation. Furthermore, 2-APB can also inhibit store release, and 5µM

SKF96365, another TRPC channel antagonist, had no effect on the level of spontaneous activity.

Some proliferating cells expressed high frequency Ca^{2+} oscillations, and although they were too infrequent to analyse their pharmacology, the regular oscillations are characteristic of release from internal stores. It may be inferred that these oscillations serve a different function to the low frequency, extracellular Ca^{2+}-dependent transients, and are highly likely to activate gene transcription networks (Mellström et al. 2008). Furthermore their absence in differentiating cells suggests they have a role specific to proliferation.

These results can be contrasted with the human NSCs described by Weick et al. (2009), as both are proliferative neural cell types. In both cell types the broad mechanism of Ca^{2+} transients appears to be TRP channels, which suggests some degree of functional equivalence, but the pharmacological characteristics indicate different channel types. In particular, the hNSCs were sensitive to block by Ni^{2+}, and insensitive to thapsigargin, 2-APB and RuR (Weick et al. 2009), in direct contrast to the proliferating hNPCs. This could represent redundancy in function between TRP channels, or alternatively there may be functional specialisations across cell types and developmental stages, either through differing properties of the channels or their associations with intracellular signalling pathways. It is also possible that immortalisation with v-myc induced changes in ion channel expression in the ReNcell VM cells, as occurs in cancer cells (Lehen'kyi et al. 2007), and which may contribute the effects of immortalisation on proliferation and differentiation.

These findings in hNPCs and hNSCs contrast to observations in human mesenchymal stem cells, where spontaneous Ca^{2+} transients were dependent on store release (Kawano et al. 2002, Sun et al. 2007, Resende et al. 2009). Ca^{2+} transients in mouse ESCs were also found to be dependent on store release via $InsP_3$-Rs (Kapur et al. 2007), while in mouse endothelial stem cells Ca^{2+} transients depended on a combination of store release and influx from the extracellular space (Sauer et al. 1998). Therefore it appears that the Ca^{2+} signalling apparatus changes as stem cells develop lineage specific identities, and that in hNSCs and hNPCs TRP channels are involved. It remains unclear whether these Ca^{2+} signals serve similar or different cellular functions.

In differentiating cells no TRP channel antagonists other than La^{3+} and Gd^{3+} inhibited the transients, leaving the specific mechanism unknown. Unlike in proliferating cells there was no effect of thapsigargin, indicating that internal store release is not involved in initiating the transients. Neither did the differentiating cells exhibit TRPV channel activation upon 2-APB application, although there was a slight, continuous increase in baseline Ca^{2+} levels. This

increase in baseline can be explained by a non-specific leak of Ca^{2+} from intracellular stores and inhibition of mitochondrial Ca^{2+} uptake (Missiaen et al. 2001). This increase might also explain the higher spontaneous activity, through hyperpolarisation by activation of Ca^{2+}-activated K^+ channels (Vergara et al. 1998) or Ca^{2+} sensitivity of the underlying channel. Despite the ineffectiveness of subtype specific antagonists it is probable that TRP channels underlie the transients, given that antagonists for Ca_v and P2X-Rs had no effect at concentrations which blocked their agonist-induced Ca^{2+} influx.

Curiously, in differentiating cells the application of ATP receptor antagonists slightly increased the level of spontaneous activity. As this was present with both receptor antagonists and the ATP degrading enzyme apyrase it appears that this is a biological effect, and I might tentatively speculate about a functional link between the putative TRP channel(s) and ATP receptors, as has been described for TRPV1 and $P2Y_2$ receptors (Moriyama et al. 2003). A more prosaic explanation may be that ATP receptor activation underlies small fluctuations in the baseline Ca^{2+} level, and abolishing these enhanced visibility of transients during analysis, though this was not apparent during visual inspection of the traces. Another expected antagonist, 10µM RuR, also increased the level of spontaneous activity, which might be consistent with a functional link between TRPV6 channels and P2 receptors. However, RuR is membrane permeable and acts at a very wide range of Ca^{2+} binding sites, including calmodulin, a major Ca^{2+} buffer (Sasaki et al. 1992). Therefore it is more probable that RuR reduced the intracellular Ca^{2+} buffering capacity enough to significantly increase the binding of Ca^{2+} to Fura-2. Although much stronger evidence would be required to draw any conclusions about interactions between different Ca^{2+} signalling pathways, should such interactions occur their identification would offer significant new insight into the biological regulation of Ca^{2+} signals, and may therefore warrant further investigation.

Pharmacological characterisation of TRP channel subtypes must be treated with caution, however, as none of the antagonists available are specific to TRP channels and the properties of most channels remain poorly understood. Most descriptions of TRP channel pharmacology come from artificial expression systems using HEK293 or CHO cells (see Ramsey et al. 2006), in which interacting proteins and other regulatory mechanisms could be absent, and species differences, heteromeric channels and splice variants have not been fully characterised, all of which could affect the gating properties of the channels. Molecular characterisation of the TRP channels expressed and the effect of their knockout are therefore needed to demonstrate the involvement of specific TRP channel subtypes.

4.1.2 Differentiating hNPCs express Ca_v channels and P2 receptors

In characterising the functional development of differentiating hNPCs I examined the ability of channel type specific stimuli to evoke Ca^{2+} influx. Depolarisation of the cells with 35mM KCl showed that the majority of hNPCs could express Ca_v channels when cultured as neurospheres but not as a monolayer, indicating that the neurosphere environment promotes some aspects functional development. The apparent absence of these currents in patch clamp recordings is most likely due to masking by the inactivating leak current, as discussed below. The presence of Ca_v channels in most cells when only a small percentage expressed βIII indicates that they are not limited to neuronal cells, and may potentially be a target for promoting neurogenesis (D'Ascenzo et al. 2006). Similarly, almost all cells responded to 2-methylthio-ATP, indicating the expression of P2 receptors. As this influx was blocked completely by 5μM PPADS it suggests any of $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ are involved. The metabotropic $P2Y_1$ receptor is also activated by 2-methylthio-ATP, but would not be expected to be completely inhibited by 5μM PPADS (Charlton et al. 1996). However, $P2Y_1$ receptors were found to mediate ATP induced Ca^{2+} influx in non-immortalised midbrain hNPCs (Rubini et al. 2009), and ATP was shown to inhibit proliferation and dopaminergic differentiation in hNPCs (Milosevic et al. 2006). In contrast, UTP, which acts at $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors, was found to increase proliferation and dopaminergic differentiation (Milosevic et al. 2006), whilst also inducing ATP release and activation of $P2Y_1$ receptors (Rubini et al. 2009), therefore it appears that purinergic receptors may have differing yet interconnected roles in hNPCs development.

It is intriguing that despite both Ca_v and P2 receptors being expressed they made little or no contribution to the level of spontaneous Ca^{2+} activity, whether the same it true under culture conditions and whether it represents a deficiency of the culture system requires further research. However, the presence of both mechanisms offer potential targets for regulating development, and a starting point to investigate the segregation and interaction of different Ca^{2+} signalling mechanisms, as despite being fundamental to the operation of Ca^{2+} signalling systems little is known about how this might influence stem cell development.

4.2 Modulation of calcium signalling via K^+

The level of Ca^{2+} signalling was strongly influenced by the extracellular KCl concentration. As Ca^{2+}-dependent gene transcription networks are tuned to the amplitude and frequency of

Ca^{2+} transients this may have important consequences for hNPC development (Dolmetsch et al. 1998, Li et al. 1998).

The most prominent effect of altering the extracellular K^+ concentration is the change in membrane potential. As cell membranes are generally more permeable to K^+ than other ions the equilibrium potential for K^+ (E_K; the potential at which the electrical and chemical gradients driving K^+ across the membrane are in equilibrium) dominates the membrane potential, and shifts in E_K will similarly shift the membrane potential. Depolarisation of the membrane by raising KCl will reduce the electrical gradient for other ions, such as Ca^{2+}, and therefore will reduce the amount of Ca^{2+} that enters the cell for a given ion channel opening. Conversely, hyperpolarising the membrane potential by lowering KCl will increase the driving force for Ca^{2+} entry and therefore increase Ca^{2+} entry for a given channel opening. Assuming the permeability of the channels is voltage independent this effect will scale linearly with Vm. However, the effect of KCl concentration on Ca^{2+} signalling appeared to be highly non-linear, and so cannot be explained purely by an increase of driving force.

Voltage dependence of the underlying channels could account for the increased frequency. Many TRP channels are sensitive to voltage, and several can show non-linear increases in conductance with hyperpolarisation, for example TRPC1 and TRPV6 (Yue et al. 2001, Maroto et al. 2005). Alternatively, there could be a gain modulation through Ca^{2+}-induced Ca^{2+} release from internal stores, whereby the increase in Ca^{2+} entry is amplified by (greater) activation of internal store release. In both cases the increase in frequency would result from an increase in detectable signals, either through amplitude or, for signals originating in processes, propagation to the soma.

Change in membrane potential is not the only consequence of changing extracellular K^+ however. The activity of ion transporters and exchangers will also be altered, which may then affect intracellular pH and cell volume (Hoffmann et al. 2009, Casey et al. 2010). Therefore another possibility is that the hyperpolarisation of the membrane affects intracellular processes that regulate the activity of TRP channels, for example stretch activated TRPC1 and TRPC6 channels (Maroto et al. 2005, Spassova et al. 2006), or pH sensitive TRPV1 and TRPV5 channels (Yeh et al. 2005, Dhaka et al. 2009). Acidification has also been shown to increase the conductance of TRPM7 channels (Jiang et al. 2005).

It is apparent that Ca^{2+} signals are present throughout the cell cycle, as with low K^+ concentrations virtually all proliferating cell showed activity. Whether this represents a role at every stage of the cell cycle or independence from the cycle will require further investigation. The ability of low K^+ to increase Ca^{2+} signalling in hNPCs provides a largely

unexplored mechanism to control this signalling while maintaining its spatial and temporal properties, which may be lost with agonist application. It has recently been shown that culturing human cardiomyoctes in low (2.4mM) KCl increased activation of Ca^{2+}-dependent signalling pathways and induced spontaneous differentiation (Vliet et al. 2010).

4.3 Cell cycle regulation by extracellular K^+

When cultured in low KCl medium (membrane hyperpolarisation and increased Ca^{2+} activity), the proliferation rate of the cells was dramatically slowed, whereas when proliferated in high KCl medium (membrane depolarisation and decreased Ca^{2+} activity), there was no, or possibly marginal, effect on proliferation rate. The effect of low KCl on proliferation corresponded to a general slowing of the cell cycle without inducing arrest, while also lengthening G1 and shortening S phase relative to standard medium.

This could be interpreted as being consistent with the effect of K^+ on Ca^{2+} signalling, where under standard conditions Ca^{2+} signalling is low, and lowering K^+ produces a dramatic increase. However, this interpretation is complicated by the slowing of proliferation by La^{3+}, which inhibits the Ca^{2+} transients and would therefore be expected to counteract the effect of KCl concentration on Ca^{2+} signalling.

Both K^+ channel activity and Ca^{2+} signalling are involved in proliferation (Wonderlin and Strobl 1996, Santella 1998, Ghiani et al. 1999, Yasuda et al. 2008), and lowering K^+ will affect multiple cellular processes. In most, but not all, cell types K^+ channel block inhibits proliferation (Wonderlin and Strobl 1996, Wang et al. 1998, Yasuda et al. 2008) and a transient hyperpolarisation is required for progression through early G1 (Wang et al. 1998). Somewhat contradictorily, most highly proliferative cells have depolarised membrane potentials, and it has long been known that depolarisation increases proliferation rate (Cone 1971, Cone and Tongier 1973, Binggeli and Weinstein 1986). Therefore it appears that many of the actions of K^+ channels on proliferation are independent of membrane potential.

Other cellular processes that could account for the slowing of the cell cycle include volume regulation, pH and slowing of metabolic and protein synthesis rates. In proliferating cells cell volume increases during G1 phase, and inhibition of this process slows proliferation (Rouzaire-Dubois et al. 2004, 2005). Reduced intracellular K^+ will reduce cell volume through an osmotic reduction in water content, and therefore lowering K^+ might slow the growth of cells during the G1 phase. It is also probable that low extracellular K^+ will cause acidification of the cytosol through reduced Na/K-ATPase activity. This would occur through

increased intracellular Na^+, which in turn would oppose the export of protons via the Na/H exchanger, lowering intracellular pH (Aronson 1985). Many cellular processes have an alkaline optimal pH, including protein and DNA synthesis (Houle and Wasserman 1983, Madshus 1988, Fuller et al. 1989), slowing which would slow the accumulation of cell cycle proteins and therefore proliferation. Consistent with this hypothesis Na/H exchanger activity is positively linked to proliferation (Kapus et al. 1994).

Ion homeostasis is not independent of membrane potential or Ca^{2+}, however. Ion channels in the cell membrane can be regulated by membrane potential and Ca^{2+}, and any changes in ion concentrations will also have osmotic effects on cell volume. The Na/H exchanger is also activated by Ca^{2+} (Manganel and Turner 1990, Wakabayashi et al. 1994) and regulates cell volume as well as pH (Hoffmann et al. 2009). Furthermore, almost all TRP channels are non-selective or weakly Ca^{2+} selective cation channels, therefore their activation will allow K^+ and Na^+ movement in addition to Ca^{2+}. This interlinking of K^+, cell volume, pH, membrane potential and Ca^{2+} makes isolating the consequences of specific mechanisms difficult. This is further complicated by the methods available to probe these channels, as all the pharmacological antagonists available have other, non-specific actions, and channel knockout with siRNA can cause compensatory changes in channel expression and may miss effects related to channel regulation. For these reasons, although TRP channel-mediated Ca^{2+} signals have been associated with increased proliferation in many cell types, including human and rat NSCs (Pla et al. 2005, Weick et al. 2009) and cancer cells (Lehen'kyi et al. 2007, Yang et al. 2009, Bomben and Sontheimer 2010), their precise roles remain unclear.

Although the specific mechanisms remain to be established, culturing cells in low KCl medium may provide a method for controlling hNPC development. Identifying the K^+ and Ca^{2+} channels involved and their functions could provide specific targets for regulating proliferation, and help further our understanding of these processes.

4.4 Influence of extracellular K^+ on neuronal differentiation

Neurogenesis in the developing brain is accompanied by lengthening of G1 and shortening of S phase (Calegari and Huttner 2003, Arai et al. 2011). According to the G1 length hypothesis, the prolongation of G1 should allow greater time for the accumulation of cell fate determinants and the chemical reactions that induce changes in fate (Calegari and Huttner 2003). However, flow cytometry analysis of neuronal differentiation showed no effect of low KCl during proliferation. There are several possible explanations for this: If lowering KCl

slows protein synthesis the additional time for accumulation of cell fate determinants would be counteracted by their reduced production rate. It is also possible that the extension of G1 was insufficient to produce detectable effects, or that the effects are not visible at 3DD, and therefore later timepoints should be tested. Alternatively, cell fate determination during G1 might not be a limiting factor in neurogenesis in the ReNcell VM cells. It should also be noted that the role of G1 length has not been directly tested, and therefore it remains possible that other factors, other than time, are responsible. If the mechanisms underlying the general slowing of proliferation and the specific effects on G1 and S phase length are separable the role of G1 length might be investigated further.

No effect of KCl concentration during differentiation on neurogenesis or survival was observed. The absence of increased neuronal survival by high KCl is most likely due to the time point tested, as stimulation with Vtr also had no effect at 3DD (discussed below). It is probable that post-differentiation Ca^{2+} signalling and membrane potential are associated with development of cellular properties rather than establishment of cell fate. Differentiating cells in a combination of hyperpolarising followed by depolarising medium has been shown to promote the functional development of hNPCs (Schaarschmidt et al. 2009), therefore varying K^+ concentration might enable promotion of functional development. The Ca^{2+} transients might be involved in process growth and migration (Gomez et al. 2006, Zheng and Poo 2007, Weick et al. 2009). It is also possible that the change in Ca^{2+} frequency affects neuronal identity, as has been described in developing xenopus neurons (Gu and Spitzer 1995, Borodinsky et al. 2004) and in mouse ESCs (Ciccolini et al. 2003). Further investigation into the effects of KCl concentration on differentiation of ReNcell VMs is required to examine these possibilities.

4.5. Neuronal development in the ReNcell VM cell line

In cell cultures the ReNcell VM cells showed rapid neuronal differentiation, expressing βIII and functional Na_v channels within three days of differentiation. However, as observed in other immortalised cell lines the extent of functional development in cell culture was limited. Although they could express Na_v and K_v currents, these did not reach sufficient density to produce regenerative action potential currents. Additionally, no cells responded to NMDA application, indicating they did not express NMDA-Rs, however neuronal cells account for only a small percentage of the total cell population and may not be represented in these experiments. These results contrast slightly with the original description of the cell line by

Donato et al. (2007), where a much greater number of neuronal cells was reported, and which generated single action potentials in response to current injection.

The functional development of the ReNcell VMs was enhanced when co-cultured on hippocampal slices, with over half of the Na_v-expressing cells able to generate action potentials upon current injection. This suggests that limitations of the cell culture environment impair functional development of the ReNcell VMs. Previous work in these cells has indicated that they require cAMP and GDNF in the differentiation medium to express Na_v currents (M Frech, unpublished data), and the neurotrophic factors BDNF and NT-3 have been shown to rescue the expression of voltage- and ligand-gated currents in primary hippocampal progenitors lost after several passages (Sah et al. 1997). It is therefore likely that the enhanced development seen in the slice environment is due to BDNF and other neurotrophic factors produced by neurons and glia. Vtr also enhanced functional development, increasing the number of cells expressing Na_v currents, which is consistent with observations that periods of depolarisation promote functional development (Schaarschmidt et al. 2009). The extracellular environment therefore is important for the functional development of hNPC-derived neurons, and additional modifications to the culture medium may be required to support their functional development *in vitro*.

In cell cultures the βIII-positive cell population declined rapidly after 4DD, most likely through cell death, as the total number of cells was also seen to decline during differentiation. This is probably a consequence of the immortalisation, as v-myc is known to render cells more vulnerable to insult (Lee and Reddy 1999), however phenotypic instability of differentiating cells cannot be ruled out from these results (for example see Paul et al. 2007). The loss of neuronal cells after 4DD may also contribute to the limited development observed, although no correlation between current expression and length of differentiation was seen during this period or in the co-culture experiments. It should be commented that the enhancement of survival by Vtr could only partly compensate for the decline in neurons, which, as discussed above, is probably caused by v-myc. If the negative effects of v-myc on the survival of differentiated cells are to be prevented a more specific intervention in the signalling processes affected might be required.

Promoting the differentiation and survival of transplanted stem cells will be an important factor in the success of stem cell therapies. Studies in animal models and human patients has shown that the adult brain environment is permissive for neurogenesis (Studer et al. 1998, Kim et al 2002, Takagi et al. 2005, Roy et al. 2006, Yasuhara et al. 2006, Redmond et al. 2007, Yang et al. 2008), and to the extent that cell fate might be pre-patterned *in vitro*,

optimising the cell population may not prove to be a major obstacle for clinical translation, although it should be noted that transplantation studies using human ESCs have had lesser success in replicating neuronal differentiation seen *in vitro* (Brederlau et al. 2006, Roy et al. 2006, Yang et al. 2008), and therefore the environment may still affect differentiation. Despite this, for therapeutic purposes promoting the survival of grafted cells and ensuring they integrate into the host appropriately may prove to be a greater obstacle.

4.6 Veratridine promotes survival of hNPC-derived neurons

Activity-dependent survival during neural development helps sculpt the immature neural networks (Oppenheim 1991). The survival of dopaminergic neurons is particularly sensitive to activity levels (Michel et al. 2007), and therefore their survival upon differentiation may be significantly affected by their functional development and synaptic activity. In ReNcell VM cell cultures the neuronal cell population declined over time, I therefore asked whether stimulating the cells with Vtr might prevent this decline, as has been shown for mouse embryonic stem cells (Salthun-Lassalle et al. 2004).

The survival of both βIII- and TH-positive cell populations could be prolonged by culturing with Vtr, and this effect was blocked by TTX indicating its dependence on Na_v channels. Furthermore, the effect of TTX on survival was correlated with the emergence of Vtr's protective effects; at 3DD neither Vtr nor TTX had an effect on βIII-positive cell survival, whereas by 7DD neuronal survival was significantly increased by Vtr and decreased by TTX. In the TH-positive cell population TTX severely reduced survival at both 3 and 7DD, indicating that the putative dopaminergic neurons require excitation early in their differentiation for survival. Unlike in previous studies (Douhou et al. 2001, Salthun-Lassalle et al. 2004) the effect in ReNcell VM cells was not limited to TH-positive cells. This could be due to activity-dependent survival in non-dopaminergic hNPC-derived neurons, or alternatively TH might not be expressed in all dopaminergic progenitors at this early developmental stage. It also cannot be excluded from this study that Vtr is increasing neuronal differentiation rather than survival, though given the dependence on Na_v channel activation it seems improbable that Vtr could act on cells prior to neuronal differentiation. Supporting this, Vtr had no effect on differentiation in mouse ESCs (Douhou et al. 2001, Salthun-Lassalle et al. 2004).

These observations demonstrate that stimulation of cultured hNPCs during early differentiation may help promote neuronal survival, with particular emphasis on

dopaminergic neurons, as the *in vitro* environment may provide insufficient stimulation to maintain these cells. More broadly, it has been seen in transplantation studies using hNPCs and hNSCs that the number of TH cells in the grafts is often low (Yashuhara et al. 2006, Takagi et al. 2008, Yang et al. 2008), and can decrease over time (Roy et al. 2006, Yasuhara et al. 2006). One cause of this might be programmed cell death regulated by activity-dependent mechanisms, initiated as a result of the cells failing to integrate into their local environment – either within the host neural networks or synaptic networks between grafted neurons. This would suggest that a major goal of cell replacement therapies for Parkinson's disease should be to promote synaptic integration into the host.

4.7 Synaptic integration of hNPCs into organotypical slice cultures

In addition to the enhanced functional development of hNPCs seen when co-cultured on hippocampal slices, it was also apparent that the hNPCs were competent to receive synaptic input rapidly, within four days of transplantation. This input may be assumed to originate from the hippocampal neurons given the low number of hNPC-derived neurons and their immature state.

It is notable, therefore, that the hippocampal neurons recognised the human midbrain-derived hNPCs as appropriate synaptic partners, since neural connectivity is highly specific and tightly regulated during development (Shen and Scheiffele 2010, Williams et al. 2010). Positive selection of synaptic partners can be achieved through expression of adhesion molecules such as n-cadherin or Dscam (Fannon and Coleman 1996, Inoue and Sanes 1997, Yamagata and Sanes 2008), while chemotactic signals control target matching by attracting or repulsing axons to guide them to the correct target (Kennedy et al 1994, Lyukusyutova et al. 2003). Synapse formation can also be promoted or inhibited by morphogens, such as wnts (Hall et al. 2000, Ahmed-Annuar et al. 2006. Klassen and Shen 2007), and by local signals provided by glial cells (Christopherson et al. 2005, Johnson et al. 2007, Ango et al. 2008).

Relatively little is known about how the expression of these cues is regulated during cell development. The formation of synapses onto hNPCs suggests they express appropriate receptors, but whether the type of receptors is intrinsic to midbrain cells or is influenced by their environment would be important to determine, as progenitor cells cultured outside of their native region can partially adopt phenotypic feature of their host environment (Rolando et al. 2010). The responsiveness of stem cell-derived neurons to guidance and adhesive cues will determine how, and to what extent, they integrate into the host environment. Therefore,

understanding how their expression develops and is regulated may lead to mechanisms by which stem cell integration can be guided.

Early synaptic integration of transplanted stem cells may be important as during development many neurons undergo programmed cell death, regulated in part by the level of synaptic input (Oppenheim 1991, Mennerick and Zorumski 2001), and as shown in this study, cultured hNPCs are sensitive to activity levels early in their differentiation. Furthermore, in an environment with active, mature neurons failure to form synapses might also leave neurons vulnerable to cell death induced by activation of extrasynaptic receptors through volume transmission of glutamate (Bading et al. 2002, Papadia et al. 2005). As yet there have been no studies of the role of activity in the survival of transplanted stem cells, an issue which should be addressed considering the limited survival and decline of stem cell-derived dopaminergic neurons reported in some transplantation studies.

One reason for a lack of such studies may be the technical difficulty of investigating the influence of activity on survival *in vivo*. The demonstration of synapse formation in this and earlier studies (Benninger et al. 2003) shows that organotypical slice cultures are a promising system to investigate synaptic integration and the influence on hNPCs. Compared with *in vivo* models they are highly accessible and easily manipulated, whilst compared to *in vitro* cell cultures they maintain the tissue structure, and subsequently the organisation of extracellular signalling molecules.

4.8 hNPCs co-cultured on hippocampal slices show strong regional preference

When transplanted onto the hippocampal slices the hNPCs formed a very specific distribution along the fissural regions, which could be caused by the host environment restricting migration and process growth, or by preferential survival of cells within specific regions. Selective adhesion may also contribute, though that the cells were found to adhere across the slice shortly after seeding indicates this is not the principle cause.

This distribution has not been seen in other co-culture systems using different cell types (Benninger et al. 2003, Husseini et al. 2008, Sarnowska et al. 2009, Rivera et al. 2009, Abouelfetouh et al. 2009), which may indicate cell type specific interactions with the host environment. A similar behaviour was described for mouse ESC-derived astrocytes transplanted onto the entorhinal cortex of hippocampal slice cultures, which preferentially migrated into the hippocampus along the perforant path and schaffer collaterals in the stratum radiatum (Scheffler et al. 2003). The stratum moleculare and lacunosum-moleculare contain

several features which may influence the migration of hNPCs. In the postnatal hippocampus neural connections are still being established, including entorhinal afferents projecting from the entorhinal cortex along the stratum moleculare and lacunosum-moleculare, and the commisural/associational afferents which originate from the polymorphic layer of both the contra- and ipsi-lateral dentate gyri (Witter and Amaral 2004). These connections are severed (excepting the ipsilateral associational afferents) when the entorhinal cortex is removed, and therefore will degenerate rapidly after slice culture preparation. The cues that guide them will still be present however, and the extension of processes by hNPCs in parallel to the hippocampal fissure is suggestive of their influence. Cajal-Retzius cells act as guideposts for entorhinal afferents, and their ablation prevents entorhinal fibres entering the hippocampus (Del Rio et al. 1997). Independent of this role, these cells are also the main source of the ECM molecule reelin during development, the loss of which causes catastrophic defects in neuronal migration throughout the hippocampus and cortex (D'Arcangelo et al. 1995). Reelin appears to act as a stop signal for migration through stabilisation of the cytoskeleton (Dulabon et al. 2000, Chai et al. 2009), however any interaction would necessarily be more complex than simply preventing changes in cell morphology, as the hNPCs manage to extend processes throughout this region. Not all hNPCs in the fissural region followed this pattern, some cells sent their projections up through the layers, oriented parallel to the projection neurons, which suggests that within the hNPCs there are populations that differentially respond to guidance cues present in the hippocampus.

Another ECM molecule, hyaluronan, acts as an anchor for many signalling molecules and has also been associated with adhesion and lamination in the stratum moleculare and stratum lacunosum-moleculare (Förster et al. 1998, 2001). It could, therefore, result in greater hNPC adhesion in these regions, and should the cells migrate extensively across the slice after transplantation they may aggregate in these regions.

Preferential survival might also contribute to the distribution. One possibility is that the availability of trophic factors is greater in the fissural and dentate gyrus regions, thereby increasing survival. The availability of nutrients and protective factors might also be increased by selective penetration into the slice. To definitively determine the roles of migration, adhesion and survival time-lapse microscopy experiments will be required.

The regulation of hNPC migration by the neural architecture will affect their ability to integrate into the host and therefore their therapeutic potential. Ideally the cells would migrate throughout the target region to reach all afflicted cells, but too extensive migration increases the risk of aberrant integration and adverse side effects. In a recent study using both

striatal organotypic slice cultures and *in vivo* mouse models, gap junction connections between nestin positive mouse NSCs and host cells were identified as a mechanism by which transplanted cells provide protection (Jäderstad et al. 2010). Whether a similar mechanism is present with hNPCs requires further study. Because gap junction coupling requires contact between transplanted and host cells the extent of any therapeutic benefit will depend on the distribution of cells throughout the afflicted region, which, as I have shown here, may be strongly influenced by the structure of the environment.

4.9 Inactivating leak current

Many of the ReNcell VMs, in both cell and slice cultures, expressed a voltage-dependent inactivating leak current which has not been previously described in the literature. This current would inactivate with depolarisation above approximately -20mV, and correlated with a hyperpolarised membrane potential and low membrane resistance. It could be abolished by carbenoxolone, along with most of the non-inactivating leak current, which suggests that the current is only weakly inactivating with voltage. Carbenoxolone also massively increased membrane resistance, consistent with the closure of open channels, though curiously this would occur several minutes after the current had been blocked, indicating distinct actions. One possible cause of the current is heterotypic gap junction coupling (White et al., 1995), which may also explain the low membrane resistance. A volume-regulated chloride current that also appears superficially similar has been described in glial cells (Parkerson and Sontheimer, 2004), and volume regulation processes are involved in differentiation. Both of these channel types are blocked by carbenoxolone (Benfenati et al. 2009). But, as equi-osmolar solutions were used for the patch clamp recordings it is unlikely that this current is activated in response to changes volume, though it is possible that the current is activated by hNPCs to induce changes in volume. Further studies are required to determine the identity of the channel and its biological significance.

A technical issue raised by the presence of this current is its potential to mask slow inward and outward currents, such as Ca_v and delayed rectifier K_v currents. Indeed, in some experiments application of Cbx unmasked such currents. This may account for the discrepancy in the number of cells expressing Ca_v currents between patch clamp recordings and Ca^{2+} imaging experiments.

4.10 Non-neuronal differentiation in cell and slice cultures and the potential of hNPCs

Upon differentiation in cell and slice cultures most of the ReNcell VMs did not differentiate into neurons or oligodendrocytes. In cell cultures they continued to express GFAP and nestin, similar to proliferating cells. This might either be representative of astroglial differentiation, or alternatively most of the cells might be arrested in an intermediate or quiescent state. After transplantation onto slice cultures the majority of hNPCs similarly did not differentiate into neurons or oligodendrocytes and expressed nestin throughout the culture period, however unlike in cell cultures there was a downregulation of GFAP in most cells, possibly indicating a change in the state of these cells. This downregulation of GFAP in slice co-cultures does not appear to result from differences in the medium composition, as hNPCs growing on the insert membrane continued to express both markers. Interestingly, a similar suppression of GFAP expression was reported for human cord blood stem cells (Sarnowska et al. 2009), whereas studies using mouse stem cells observed astrocytic differentiation (Scheffler et al. 2003, Husseini et al. 2008, in hippocampal cultures and Jäderstad et al. 2010 in striatal cultures), hinting at species specific responses to the environment. Further evidence of interaction between hNPCs and the slice during differentiation was provided by comparison of cells differentiating on the slice with those on the insert membranes. Cells growing on the membranes retained an immature morphology and continued to proliferate, whilst cells on or adjacent to the slice ceased proliferation and developed more mature morphologies, indicating that the slice environment is suppressing proliferation and promoting differentiation. This could be caused by short-range diffusible factors or contact-mediated signals present on the debris that surrounds the slices, for example the ECM component hyaluronan has been shown to inhibit proliferation of progenitor cells through the activation of toll-like receptors (Okun et al. 2010, Sloane et al. 2010). How the hNPCs are altered by the neural environment requires further investigation, as this may have implications for their differentiation potential once transplanted *in vivo*.

The continued expression of nestin by the hNPCs in both systems suggests that they have retained their progenitor status, and the downregulation of GFAP supports the hypothesis that after the removal of growth the majority of ReNcell VMs remain in an intermediate, quiescent state and do not adopt a mature cell phenotype. The failure to differentiate into astrocytes or oligodendrocytes whilst showing some degree of neurogenesis would hint that those undifferentiated cells might remain neurogenic, as neurogenesis and gliogenesis are temporally segregated in the developing brain (Morrow et al. 2001). This limited differentiation may be attributable to v-myc immortalisation (Coppola and Cole 1986, Lee

and Reddy 1999), and therefore methods of directly compensating for the undesirable effects of v-myc (and c-myc) upon differentiation might significantly improve neuronal differentiation and development in immortalised cell lines, and would greatly enhance the potential of hNPC lines for stem cell therapies.

5. Summary

5.1 Summary of results

Immortalised hNPCs are a potential source of cells for stem cell therapies. In this study I have investigated the functional properties of the ReNcell VM cell line, a human fetal midbrain-derived NPC line immortalised with v-myc.

Proliferating and differentiating ReNcell VM cells express spontaneous Ca^{2+} transients which appear to be mediated by TRP channels, but via differing sub-types. TRPV1 and/or TRPV3 channels are present in proliferating cells, but are downregulated after differentiation. Differentiating cells did not respond to any TRP sub-type selective antagonist, leaving the specific mechanism unknown. Despite little or no contribution to spontaneous activity, functional Ca_v and P2 receptors were present in differentiating cells, indicating there are at least three distinct routes of Ca^{2+} entry.

Ca^{2+} signalling activity was highly dependent on the extracellular K^+ concentration. Culturing the ReNcell VM cells in low K^+ slowed their proliferation rate without inducing cell cycle arrest, whilst also lengthening G1 and shortening S phases. These effects are associated with increased neurogenesis during development, however initial characterisation found no subsequent increase in neuronal differentiation.

Characterisation of ReNcell VM differentiation showed that they underwent rapid neuronal differentiation and could express functional Na_v and K_v currents, although functional maturation and survival was limited. Their survival could be enhanced by stimulation with the Na_v agonist Vtr, demonstrating activity-dependent survival of hNPC-derived neurons and an environmental manipulation which may support their development *in vitro*.

Co-culture of the ReNcell VM cells on rat hippocampal slices indicate that the neural environment promotes functional development of the ReNcell VM cells, and that the cells were competent to receive synaptic input from host neurons. The distribution and process growth of the ReNcell VM cells was highly dependent on the hippocampal architecture, suggesting that the neural architecture could regulate the potential of hNPCs to integrate into the host environment.

5.2 Conclusions

The change in TRPV1 and/or TRPV3 channel expression between proliferating and differentiating states suggests a role specific to proliferation, and may provide a target for regulation of hNPC development.

The presence of several distinct sources of Ca^{2+} signals during proliferation and differentiation suggests that Ca^{2+} signalling has multiple roles in hNPCs at each stage of development. The characterisation performed in this study provides a platform for investigation of the roles specific Ca^{2+} channels have in hNPC development and how these different Ca^{2+} signalling pathways interact.

Regulation of Ca^{2+} signalling and proliferation by extracellular K^+ offers a novel method of controlling hNPC development *in vitro* which could be used prior to transplanting stem cells. Further investigation is required into the underlying mechanisms and effect on neurogenesis in relation to the G1 length hypothesis.

As a model system the ReNcell VM cells show rapid neuronal differentiation and express key functional properties of neurons, indicating they are a viable system for investigating early aspects of hNPC differentiation. Their limited functional development and survival are partly attributable to immortalisation with v-myc, and as immortalised cell lines offer a potential source of stem cells for clinical transplantation, understanding the consequences of immortalisation and identifying methods of compensating for negative effects may have clinical relevance.

The effects of Vtr on neuronal survival indicate activity-dependent survival emerges early in hNPC differentiation, and is particularly important for the survival of TH^+ cells. Despite programmed cell death being fundamental to neural development no studies have directly addressed the consequences of this for stem cell transplantation. The rapid formation of synapses onto hNPCs in slice cultures demonstrates that this system can be used to investigate the functional integration of hNPCs and the consequences for their survival.

5.3 Future Directions

Several aspects of this work raise questions for further research, the most significant of which I will address here.

The role of Ca^{2+} signals in stem cell development is complex and not well understood. To continue the work of this study requires molecular characterisation to identify and confirm the specific TRP channel types present in the ReNcell VM cells, this will further enable siRNA

knockout studies to investigate the involvement of each channel type in development. With identification of channel types the work could be expanded to their associations with intracellular signalling pathways, which would provide greater insight into how Ca^{2+} signalling regulates development. There are hints in this study of an interaction between the putative TRP channels and P2 receptors, further functional and molecular characterisation will enable investigation of this possibility and the influence it may have on intracellular signalling pathways.

The findings of this study suggest that lowering KCl in the culture medium might allow control of hNPC proliferation, and consequently development and cell fate. The actions of low K^+ and the consequences for differentiation require further investigation. In particular, the extension of G1 and shortening of S phase is consistent with observations of the cell cycle during neurogenesis. The G1 length hypothesis postulates that this effect is directly linked to neurogenesis, however no such effect was detected in the ReNcell VM cells. Whether this is due to additional effects of low K^+ or is evidence against the G1 length hypothesis will be important to determine. Extension of the method into other cell types and investigation of the underlying mechanisms will help determine the utility of this method for guiding stem cell development.

The demonstration of activity dependent survival early in hNPC differentiation raises the question of the involvement of this mechanism of hNPC survival after *in vivo* transplantation. This topic has yet to be addressed in *in vivo* models, which may partly be due to the technical difficulty of studying this process *in vivo*. The formation of synapses onto hNPCs transplanted onto rat organotypical slice cultures demonstrates that this system can be used to investigate the process of hNPC integration *in vitro*, and to investigate the consequences for their survival.

6. References

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Appendix

A. Commercial solution formulae

DMEM/F12	Invitrogen		
COMPONENTS	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	18.75	0.25
L-Alanine	89	4.45	0.05
L-Arginine hydrochloride	211	147.5	0.699
L-Asparagine-H2O	150	7.5	0.05
L-Aspartic acid	133	6.65	0.05
L-Cysteine hydrochloride	176	17.56	0.0998
L-Cystine 2HCl	313	31.29	0.1
L-Glutamic Acid	147	7.35	0.05
L-Histidine hydrochloride	210	31.48	0.15
L-Isoleucine	131	54.47	0.416
L-Leucine	131	59.05	0.451
L-Lysine hydrochloride	183	91.25	0.499
L-Methionine	149	17.24	0.116
L-Phenylalanine	165	35.48	0.215
L-Proline	115	17.25	0.15
L-Serine	105	26.25	0.25
L-Threonine	119	53.45	0.449
L-Tryptophan	204	9.02	0.0442
L-Tyrosine disodium salt	261	55.79	0.214
L-Valine	117	25.85	0.221
Vitamins			
Biotin	244	0.0035	0.0000143
Choline chloride	140	8.98	0.0641
D-Calcium pantothenate	477	2.24	0.0047
Folic Acid	441	2.65	0.00601
Niacinamide	122	2.02	0.0166
Pyridoxine hydrochloride	206	2	0.00971
Riboflavin	376	0.219	0.000582
Thiamine hydrochloride	337	2.17	0.00644
Vitamin B12	1355	0.68	0.000502
i-Inositol	180	12.6	0.07
Inorganic Salts			
Calcium Chloride	111	116.6	1.05
Cupric sulfate	250	0.0013	0.0000052
Ferric Nitrate	404	0.05	0.000124
Ferric sulfate	278	0.417	0.0015
Magnesium Chloride	203	61	0.3
Magnesium Sulfate	246	100	0.407
Potassium Chloride	75	311.8	4.16
Sodium Bicarbonate	84	2438	29.02
Sodium Chloride	58	6995.5	120.61

Sodium Phosphate dibasic	268	134	0.5
Sodium Phosphate	138	62.5	0.453
Zinc sulfate	288	0.432	0.0015
Other Components			
D-Glucose (Dextrose)	180	3151	17.51
Hypoxanthine Na	159	2.39	0.015
Linoleic Acid	280	0.042	0.00015
Lipoic Acid	206	0.105	0.00051
Phenol Red	376.4	8.1	0.0215
Putrescine 2HCl	161	0.081	0.000503
Sodium Pyruvate	110	55	0.5
Thymidine	242	0.365	0.00151

Customised DMEM/F12 PAA

Inorganic Salts	**Concentration (mg/L)**
Calcium Chloride 2 H_2O	154.45
Copper(II)-Sulphate 5 H_2O	0.0013
Iron(III) Nitrate 9 H_2O	0.05
Iron(II) Sulphate 7 H_2O	0.417
Magnesium Chloride 6 H_2O	61.20
Magnesium Sulphate	69.76
Zinc Sulphate 7 H_2O	0.432
Amino Acids	
L-Alanine	4.45
L-Alanyl-L-Glutamine (E15-889)	539.00
L-Arginine HCl	147.50
L-Asparagine H_2O	7.50
L-Aspartic Acid	6.65
L-Cystine	25.51
L-Cysteine 2 HCl H_2O	17.56
L-Glutamic Acid	7.35
Glycine	18.75
L-Histidine HCl H_2O	31.48
L-Isoleucine	54.47
L-Leucine	59.05
L-Lysine HCl	91.25
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	17.25
L-Serine	26.25
L-Threonine	53.45
L-Tryptophan	9.02
L-Tyrosine 2 Na	48.10
L-Valine	52.85
Vitamins	
D(+)-Biotin	0.0037
D-Calcium Pantothenate (Vitamin B5)	2.24
Choline Chloride	8.98

Folic Acid	2.66
myo-Inositol	12.61
Nicotinamide	2.02
Pyridoxal HCl	2.031
Riboflavin	0.219
Thiamine HCl	2.17
Vitamin B12	0.68
Other Components	
2´-Deoxythymidine	0.365
D-Glucose anhydrous	3151.00
Hypoxanthine	2.04
Linoleic Acid	0.042
DL-α-Lipoic Acid	0.105
Phenol Red	8.13
Putrescine 2 HCl	0.0805

B27 Invitrogen

Vitamins: Biotin, DL Alpha Tocopherol Acetate, DL Alpha-Tocopherol
Proteins: BSA, fatty acid free Fraction V, Catalase, Human Recombinant Insulin, Human Transferrin, Superoxide Dismutase
Other Components: Corticosterone, D-Galactose, Ethanolamine HCl, Glutathione (reduced), L-Carnitine HCl, Linoleic Acid, Linolenic Acid, Progesterone, Putrescine 2HCl, Sodium Selenite, T3 (triodo-I-thyronine)

HBSS Invitrogen
(with / without Ca^{2+} and Mg^{2+})

COMPONENTS	Molecular Weight	Concentration (mg/L)	mM
Inorganic Salts			
Calcium Chloride	111	140 / 0	1.26 / 0
Magnesium Chloride	203	100 / 0	0.493 / 0
Magnesium Sulfate	246	100 / 0	0.407 / 0
Potassium Chloride	75	400	5.33
Potassium Phosphate	136	60	0.441
Sodium Bicarbonate	84	350	4.17
Sodium Chloride	58	8000	137.93
Sodium Phosphate	142	48	0.338
Other Components			
D-Glucose (Dextrose)	180	1000	5.56
Phenol Red	376.4	10	0.0266

PBS Biochrom

137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4

B. Publications

Morgan, P. J., Ortinau, S., Frahm, J., Kruger, N., Rolfs, A., & Frech, M. J. 2009, "Protection of neurons derived from human neural progenitor cells by veratridine", *Neuroreport*, vol. 20, no. 13, pp. 1225-1229.

Morgan, P. J., Liedmann, A., Hübner, R., Hovakimyan, M., Rolfs, A., & Frech, M. J. 2011, "Human neural progenitor cells show functional neuronal differentiation and regional preference after engraftment onto hippocampal slice cultures", *Stem Cells Dev*, In Press

Morgan, P. J., Rolfs, A., & Frech, M. J. 2011, “Spontaneous calcium signalling in immortalised human neural progenitor cells”, *In preparation*.

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