MULTIPLE ROLES FOR THE CHEMOKINE SDF-1 IN FOREBRAIN DEVELOPMENT

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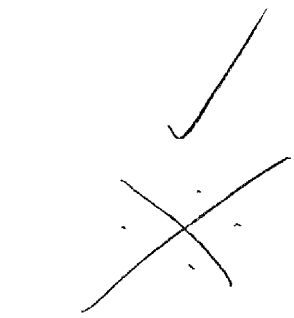

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Abbreviations

ABTS – 2.2-Azino-di(3-EthylBenzThiazoline Sulphonic Acid)

BAPTA – BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)

BF-1 – Brain Factor 1

bHLH – basic helix-loop-helix

BMP – Bone Morphogenetic Protein

BSA – Bovine Serum Albumin

BrdU – Bromodeoxyuridine

CNS – Central Nervous System

CP – Cortical Plate

CR – Cajal-Retzius Cell

Cx – connexin

DEPC - diethylpyrocarbonate

Dcx – Double Cortex

Dlx – Distaless

DMEM – Dubecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

E – embryonic day (day of vaginal plug = E1)

EDTA - ethylendiaminetetraacetic acid

EGL – external granule cell layer

ELISA – enzyme linked immunosorbant assay

Emx – Empty Spiracles

Erk – Extra cellular-signal regulated protein kinase

FGF – Fibroblast Growth Factor

GABA – γ-Amino-Butyric Acid

GAD – Glutamic Acid Decarboxylase

Gcm – Glial Cells Missing

GE – ganglionic eminence(s)

GFAP – Glial Fibrillary Acidic Protein

GFP – Green Fluorescent Protein

Gja – gap junction membrane channel protein

Gli3 - GLI-Kruppel family member

GPI – Glycosylphosphatidylinositol

Gsh – GS homeo box protein

Hes – mammalian hairy and enhancer of split homologues

HEK – Human Embryonic Kidney Cells

HIV – Human immuno-deficiency virus

IZ – Intermediate Zone

LC – lateral cortex

LGE – Lateral Ganglionic Eminence

Lhx – LIM Homeobox Protein

LIF – Leukaemia Inhibitory Factor

Lis – Lissencephaly

MAP – Microtubule Associated Protein

MAPK – Mitogen activated protein kinase

Mash – Mammalian Achaete Schute Homologue

Math – Mammalian Atonal Homologue

MC – medial cortex

MCD – Malformations of cortical development

MGE – Medial Ganglionic Eminence

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MZ – marginal zone

NCAM – Neural cell adhesion molecule

NeuroD – Neurogenic Differentiation

Ngn – Neurogenin

Nkx – NK Homeobox Protein Homologue

Nud – Nuclear Distribution Mutant

Nudel – NudE Like

Pax – Paired Box Transcription Factor

PBS – Phosphate Buffered Saline

PBSF – Pre-B-Cell Growth Stimulating Factor

PCR – Ploymerase chain reaction

PI3K – Phosphoinositide-3-kinase

PI-PLC – Phosphatidylinositol Specific Phospholipase C

PNS – Peripheral Nervous System

PP – Preplate

PSA-NCAM – polysialic acid-NCAM

qRT-PCR – quantitative real time PCR

SDF-1 – Stromal Derived Factor-1

SDS - Sodium dodecyl sulphate

Sfrp – secreted frizzled-related protein

Shh – Sonic Hedgehog

Smad1 – Mothers against decapentaplegic homolog 1

SP - Subplate

STAT3 – Signal Transducer and Activator of Transcription 3

TAG-1 – Transient Axonal Glycoprotein-1

Tbr – Brachyury Homolgue

Tuj1 – Neuronal Class III β-Tubulin

UNG – uracil-N-glycosylase

VZ – Ventricular Zone

Zfp – zinc finger protein

Abstract

SDF-1 (stromal cell derived factor-1), a proinflammatory chemokine has been shown to provide chemotactic signals in the central nervous system. Although SDF-1 and its receptor CXCR4 are constitutively expressed in the developing cerebral cortex, their function in this region is yet to be elucidated. Thus to investigate the regulatory role of SDF-1 signalling during early cortical development, primary cell cultures derived from embryonic rat cortex were treated with CXCR4 antagonist (AMD3100). Alternatively, cultures were exposed to non-endogenous levels of chemokine obtained from transfected HEK cells that over express SDF-1.

Immunocytochemistry data shows that SDF-1 exerts a role in the regulation of cell proliferation and differentiation. Primary cortical cultures exposed to SDF-1 conditioned medium showed a significant increase in the number of proliferating cells as revealed by Ki67 staining. Conversely, treatment with AMD3100, the receptor antagonist, yielded fewer cycling cells. Signalling via gap junctions has been suggested as a mechanism for regulating the proliferation of neuronal precursors. In support of this hypothesis treatment with carbenoxalone (10μM), a widely used gap junction blocker caused an apparent decrease in the number of Ki67 positive cells. This effect was reversed by SDF-1 and compounded by AMD3100, suggesting that SDF-1 signalling is likely to regulate cell proliferation via GAP junction signalling. The findings were further confirmed using dye transfer and calcium imaging studies.

We next investigated the regulatory role of chemokine signalling in the differentiation of cortical neurons. Analysis of cultures, using a panel of neuronal markers, revealed that exposure to SDF-1 significantly increased the GABAergic population, while total neuronal output remained largely unaffected. Likewise, treatment with antagonist reduced the number of GABAergic neurons. SDF-1 was also shown to regulate both axonal growth and cell migration *in vitro*. Using microarray technology in conjunction with qRT-PCR several genes of interest were identified with potential roles in these physiological events.

Taken together, the present study demonstrates that SDF-1/CXCR4 signalling exerts differential effects on distinct cell populations of the developing cortex.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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1 Introduction

The human brain is arguably the most complex structure known and the cerebrum alone contains over 10 billion neurons. The formation of such a complex organ requires the temporal and spatial orchestration of many developmental events, such as the specification of cell phenotypes, the patterning of specific regions, the correct migration of cells, and the formation of the appropriate neuronal connections. The cellular components of the brain fall into two major categories; neurons and glia. The neurons are responsible for nerve transmission, whereas the glia are thought primarily to be supporting structures. On the basis of morphology, physiological properties and neurochemical content, neurons can be further categorized into multiple phenotypes. Glial cells can be divided into 3 types, astrocytes, oligodendrocytes, and microglia. Astrocytes are involved in formation of the blood brain barrier. Oligodendrocytes provide the myelin sheath which covers axons. The microglia are thought to function as the phagocytes of the brain (McGeer *et al.*, 1987).

1.1 Structure of the mammalian forebrain

The forebrain or telencephalon consists of two paired hemispheres. Pallial (dorsal) regions give rise to the cerebral cortex and the hippocampus, whereas subpallial (ventral) regions are collectively known as the ganglionic eminences (GE) and eventually give rise to the basal ganglia. The GE is divided into 3 distinct territories, lateral (striatum), medial (pallidum) and caudal GE. During development the lateral and medial GE contains proliferative zones which give rise to neurons destined for various final destinations throughout the forebrain (see figure 1).

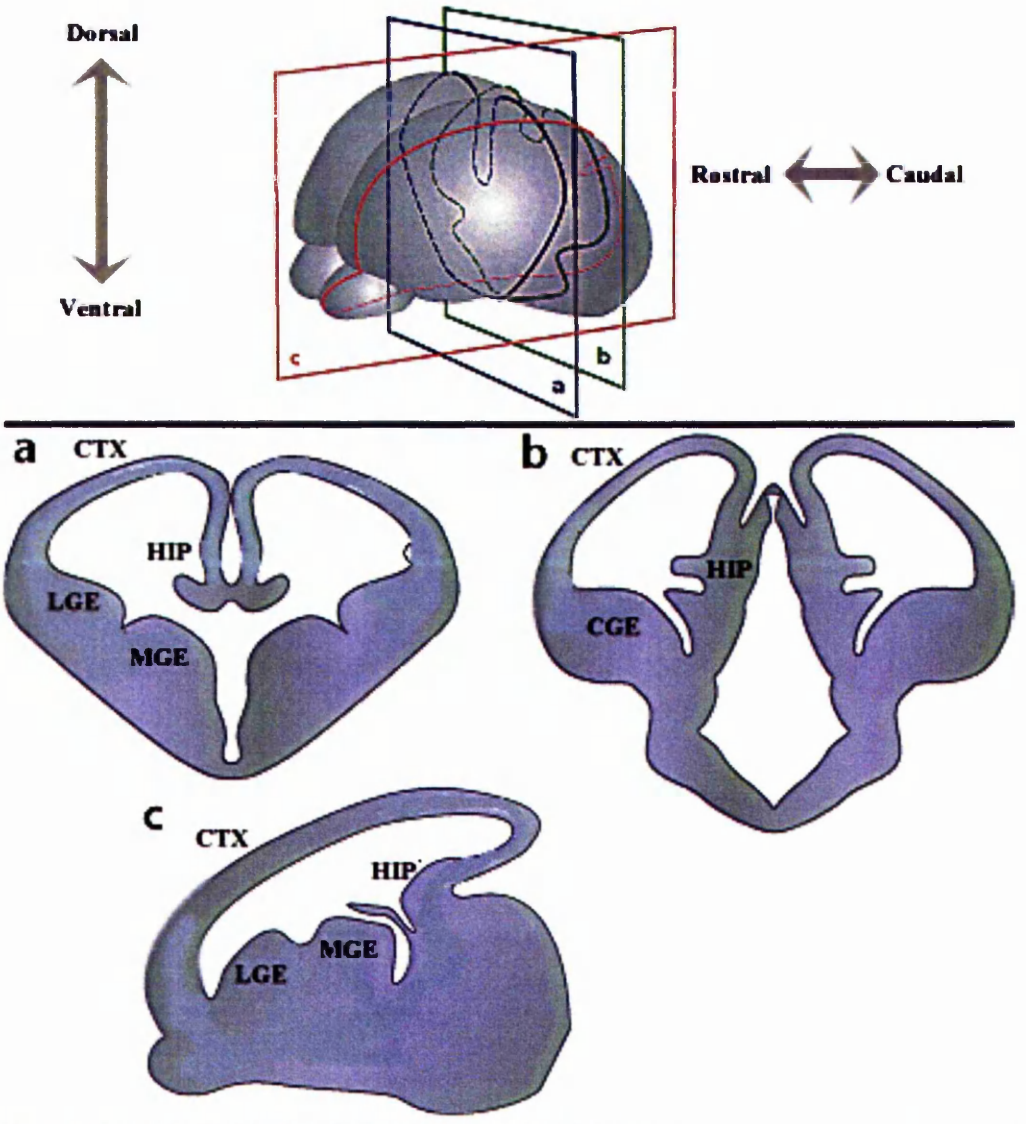

Figure 1: The embryonic telencephalon is shown in coronal (a, b) and sagittal (c) cutaways. CGE – caudal ganglionice eminence. CTX – cortex. HIP – hippocampus. LGE – Lateral ganglionic eminence. MGE – Medial ganglionic eminence. (Adapted from Corbin *et al.* 2001).

1.1.1 The cytoarchitecture of the cerebral cortex

The adult cerebral cortex is a laminated structure with distinct layers arranged parallel to the surface of the brain. Each layer consists of neurons that show similar size, shape and physiological properties. Layer I, immediately below the pial surface is sparsely populated with neurons. Layers II and III have no distinct boundary and contain small to medium sized cells with triangular or ovoid cell bodies. Layer IV is the granular layer and is noticeable because of the small, tightly packed neurons it contains. In contrast layer V is made up of loosely packed neurons with triangular or polygonally shaped cell bodies.

Finally layer VI contains small and closely packed neurons, of which a proportion are horizontally oriented.

Within the layers of the cerebral cortex there are two principal types of neurons. Projection neurons make up 75% of the neuronal population, have a pyramidal morphology, are excitatory and use glutamate as neurotransmitter. Local circuit interneurons make up the remaining 25%, are inhibitory and use GABA for their transmission. Subtypes of interneurons can be distinguished by expression of different neuropeptides (Cavanagh and Parnavelas, 1988;Cavanagh and Parnavelas, 1989;Cavanagh and Parnavelas, 1990), whose expression can be identified by immunohistochemistry. These include somatastatin and neuropeptide Y. Different populations of GABAergic interneurons have also been characterised by the expression of the calcium binding proteins calbindin, calretinin and parvalbumin. These are thought to function as "buffer" proteins thereby providing a passive system for decreasing the amplitude of calcium signals. Hof *et al* (Hof et al., 1999) reviewed the expression patterns of these molecules in several mammalian species. In the developing rat cortex the first calretinin and calbindin expressing neurons are found at E14. This includes Cajal-Retzius cells which are immunoreactive for both proteins. In the adult, calbindin expression is mostly restricted to layers II, III, V and VI. The highest calretinin expression is found in layers II and III.

1.2 Forebrain development

The development of the forebrain can be broadly divided into a series of ontogenetic steps that follow a hierarchy of differentiation, but not in a strict temporal sequence (Goetz, 1998). These stages include progenitor proliferation, cell specification, cell migration, and tissue regionalization. This could perhaps be best envisaged as a pyramidal hierarchy, in which cells show progressive restriction of their potential and become committed to a specific phenotype. This is made possible through the complex interplay of many intrinsic and extrinsic factors, throughout development.

1.2.1 Patterning of the developing forebrain

During development the telencephalon becomes divided into anatomically distinct regions. In the adult, the cerebral cortex is divided into many functionally distinct areas, in which neurons have different connections and functionality to those in neighbouring regions. However, the developing neocortex lacks any area specific distinctions, suggesting that mechanisms must exist to enable the regionalisation of the developing telencephalon into territories or areas. O'Leary (O'Leary, 1989; O'Leary *et al.*, 1992)

hypothesized that the adult cortex arises from a uniform "protocortex", which consists of comparable populations of cells derived from the neuroepithelium that can differentiate in comparable ways, and is patterned by external inputs such as those from the thalamus. The transformation from a protocortex to an adult cortex with distinct regions, involves regional differences in gene expression being translated into positional cues that allow the formation of different territories. Conversely, the protomap hypothesis put forward by Rakic postulates that region specific expression of intrinsic factors in the proliferative ventricular zones is maintained by radial migration of post-mitotic neurons (the radial unit hypothesis), and therefore gives rise to the regionalized structure of the adult cortex (Rakic, 1988). On a less localized level territories or zones of gene expression throughout the telencephalon give rise to the structures found in the adult forebrain; the cortex, the striatum, the pallidum, the septum, and the limbic system (Fishell, 1997). Unlike in the hindbrain, transplantation experiments seem to show that cells can move across the boundaries between these regions during development (Fishell, 1995), which was crucial in showing tangential migration could occur from the striatal ventricular zone to the developing neocortex. The prosomeric model (Rubenstein *et al.*, 1994; Puelles and Rubenstein, 2003) attempts to divide the telencephalon into the same distinct zones (prosomeres) in all vertebrates, based upon criteria such as gene expression patterns as well as obvious anatomical features, thus enabling comparisons to be made between species.

One result of the specification of these territories during development is the establishment of distinct proliferative zones that give rise to different classes of neurons; the cortex produces glutamatergic neurons; the lateral ganglionic eminence (LGE) produces GABAergic neurons; and the medial ganglionic (MGE) produces both GABAergic and cholinergic neurons (Wilson and Rubenstein, 2000). However, before this can occur, the telencephalon itself must be distinguished in some way from the other neural areas. *Brain Factor 1* (*BF-1*), as the earliest known telencephalic marker, presumably plays a role in this process (Fishell, 1997). Subsequent to this, the earliest obvious division of the telencephalon occurs longitudinally, splitting the telencephalon into the pallium (destined to be cortex) dorsally and the subpallium (destined to be striatum) ventrally (Lumsden and Krumlauf, 1996). The expression patterns of several genes are responsible for this division (figure 2), including *empty spiracles-1/2* (*Emx-1/2*), *Pax-6* (a paired box transcription factor) and *Brachyury homologue-1* (*Tbr-1*) (dorsally) (Fode et al., 2000) and *Distaless-1/2* (*Dlx-1/2*) (ventrally) (Lumsden and Krumlauf, 1996).

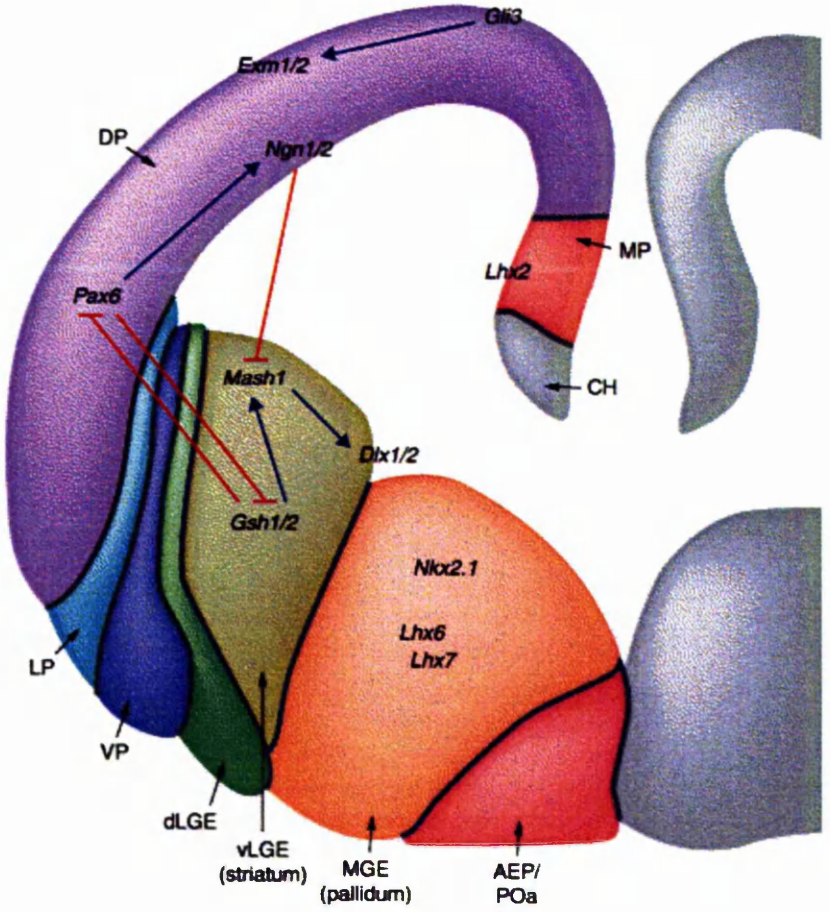

Figure 2 Genetic interactions underlying the regionalization of the mammalian telencephalon. Schematic coronal section through the telencephalic vesicles at E12.5 showing dorsal and ventral subdomains, as defined by their unique patterns of gene expression (*figure and caption taken from Schuurmans and Guillemot, 2002*). AEP – anterior entopeduncular, CH – cortical hem, dLGE – dorso-lateral GE, DP – dorsal pallium, LP – lateral pallium, MGE – medial GE, MP – medial pallium, vLGE – ventro-lateral GE, VP – ventral pallium.

1.2.1.1 Dorsal patterning

Interplay between several genetic factors is responsible for patterning the dorsal telencephalon. Genes that are responsible for defining dorsal regions include *Pax-6*, *Gli-3* (a zinc finger containing transcription factor), *Emx-2*, and *neurogenin-1/2* (*Ngn-1/2*), which are all expressed dorsally in the developing telencephalon (Wilson and Rubenstein, 2000).

Homeodomain proteins Emx 1 and 2 are expressed in the developing cerebral cortex and are involved in forebrain patterning (Cecchi and Boncinelli, 2000). One member of this family, Emx-2, is one of the earliest markers for the developing cerebral cortex and appears to be important for the regulation of neuroblast proliferation, migration, and differentiation (Cecchi and Boncinelli, 2000). Defects in its expression result in defects in the cerebral cortex (possibly partly due to a deficiency in neuronal migration (Mallamaci *et al.*, 2000)), olfactory bulbs (smaller), hippocampus (altered patterning), dentate gyrus (absent), and the marginal zone (lack of Cajal-Retzius cells) (Cecchi and Boncinelli, 2000).

Another homeodomain protein, Pax-6, is required for the formation of dorsal regions of the pallium (Wilson and Rubenstein, 2000). Pax-6 also antagonizes Nkx2.1 activity to establish the LGE/MGE border (Marin and Rubenstein, 2001). Pax-6 appears to function upstream of *Ngn-2*, which is a proneural gene. *Ngn2* suppresses ventral phenotypes and promotes neuronal differentiation by regulation of bHLH transcription factor activity (Schuurmans and Guillemot, 2002; Schuurmans *et al.*, 2004). Gli-3 may repress the expression of Sonic Hedgehog (Shh) target genes (Wilson and Rubenstein, 2000) and hence act as a dorsalizer. Gli-3 may also function upstream of the Emx family of homeodomain proteins (Schuurmans and Guillemot, 2002). In addition gradients of Pax-6 and Emx-2 expression have been proposed as a mechanism for defining positional identities within the dorsal telencephalon, and for the arealization of the neocortex (Wilson and Rubenstein, 2000; Bishop *et al.*, 2000; Bishop *et al.*, 2002). Furthermore, in mice lacking both Pax-6 and Emx2 expression, the entire pallium takes on striatal characteristics (Muzio *et al.*, 2002) demonstrating that these genes are crucial for the correct formation of dorsal regions.

1.2.1.2 Ventral patterning

Patterning of ventral regions of the telencephalon is regulated by FGF signalling and genes expressed ventrally include *Dlx1/2*, *Nkx2.1*, *GS homeo box protein 2* (*Gsh2*) and *mammalian achaete schute homologue 1* (*mash-1*) (Wilson and Rubenstein, 2000). Nkx2.1 is required for specification of the MGE (Marin and Rubenstein, 2001) and inhibits LGE formation, whereas Gsh2 maintains LGE identity (Wilson and Rubenstein, 2000). Nkx2.1 and Pax-6 are thought to antagonize each other in order to establish the MGE/LGE boundary (Marin and Rubenstein, 2001). Gsh2 and Pax-6 have complimentary patterns of expression and antagonize each other to set up distinct programs of gene expression in ventral and dorsal regions respectively (Toresson *et al.*, 2000; Yun *et al.*, 2001).

1.2.2 Formation of the Cerebral Cortex

Since the late 19th Century, using light microscopy and early histological techniques such as Golgi preparations, scientists have been studying the development of the brain with a view to elucidating the key ontogenetic events and the mechanisms underlying them.

One of the first theories was the spongioblast theory, which hypothesised that there were two types of progenitor cells in the proliferative ventricular zones of the developing cortex. Germinal cells were thought to give rise to neuroblasts, whilst glial cells were thought to be derived from spongioblasts. This theory was discounted after it was illustrated that the neural epithelium consists of a single type of epithelial cell. Hence, it became accepted that a homogenous population of cells gives rise to all the cell types of the adult cortex. The cells making up this homogenous population have asynchronous cell cycles and demonstrate a phenomenon called interkinetic nuclear migration.

From this early work it was determined that neurons migrate from their sites of origin in germinal centres to their final positions (Sidman and Rakic, 1973). This raises questions regarding the mechanisms used to achieve this, and about the time at which neurons commit to a certain phenotype; before, during, or after migration?

It was originally thought that neurons of the cerebral cortex arise from proliferative regions in the germinal ventricular zone where they exit the cell cycle and migrate radially. Radial migration occurs first by somal translocation (Nadarajah *et al.*, 2001; Nadarajah, 2003), then, as the cortex thickens and becomes more complex, by glial guided locomotion along a scaffold of radial glial fibres (Rakic, 1971; Rakic, 1972; Nadarajah *et al.*, 2003) to the margins of the cerebral wall. Here the earliest born neurons form the preplate layer. The preplate layer consists of two distinct cell types; the large stellate cells that have been

given the name Cajal-Retzius cells after the scientists who first described them, and the subplate cells. Later generated neurons accumulate within the preplate to form the cortical plate, splitting the preplate in two, to form the superficial marginal zone containing the Cajal-Retzius cells and the deeper subplate. The intermediate zone consists of nerve fibres. Later born neurons migrate past earlier-born neurons to reside in the more superficial layers (McConnell, 1988a). It is now accepted that the cortical layers are formed in an inside first, outside later spatio-temporal sequence such that later-born neurons reside in the more superficial layers (Rakic, 1972; Sidman and Rakic, 1973; McConnell, 1988a; Nadarajah *et al.*, 2001) (figure 3). This "inside out" generation of cortical layers was first demonstrated by cell birthdating studies using either [^{3}H]-thymidine or bromodeoxyuridine (BrdU) (McConnell, 1995b) and subsequently confirmed by analysis of mutant mice such as the reeler mutant (Rice and Curran, 1999). Indeed, laminar position has been shown to be determined by environmental cues in the surrounding tissue, just prior to the final mitotic cell division (McConnell, 1988b). The developing cortex can therefore be divided into 5 layers: the proliferative layer (consisting of the sub-ventricular and ventricular zones), intermediate zone, sub plate, cortical plate, and marginal zone.

However, several techniques have been used to demonstrate that radial migration is not the only route of migration employed during cortical development. These techniques include retroviral lineage analysis (Price and Thurlow, 1988; Parnavelas *et al.*, 1991), transplantation experiments (Fishell, 1995), cell labelling (de Carlos *et al.*, 1996), *in utero* fate mapping (Wichterle *et al.*, 2001), and live imaging of ex-vivo cortical slice cultures (Nadarajah and Parnavelas, 2002), all of which illustrate that a subpopulation of neurons arise in the proliferative zones of the ganglionic eminences and follow a tangential route of migration to their final positions in the cortex. Furthermore, neuronal phenotype is linked to the mode of migration. Tagentially migrating neurons have been shown to have a GABAergic phenotype using Dlx knockout mice (Anderson *et al.*, 1997). GABAergic interneurons and pyramidal neurons are therefore derived from distinct populations of progenitors in distinct forebrain regions that are intrinsically programmed to become either interneurons or pyramidal neurons, and consequently utilize different routes of migration to reach their target positions.

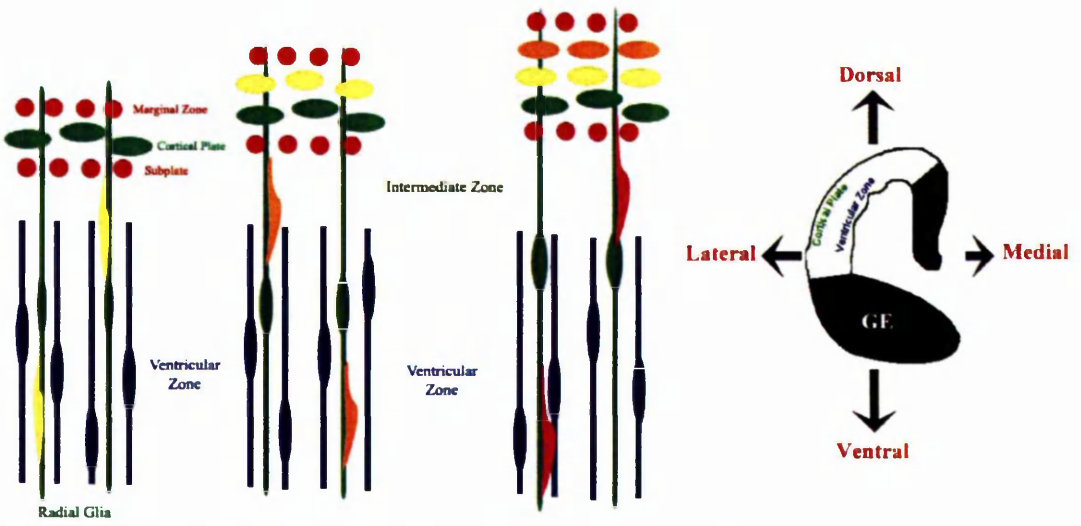

Figure 3: Neurons (green, yellow, orange and pink) migrate radially from the ventricular zone towards the pial surface. Later born neurons move beyond earlier formed layers. Thus, the cortical layers are generated in an inside out manner. As the cortex thickens, and migratory distances increase, neurons switch from somal translocation to glial guided locomotion along a scaffold of radial glial fibres. A coronal section through one hemisphere of the telencephalon is shown for orientation. The cortical plate and ventricular zones of the cortex are shown with text labels.

1.2.3 Neurogenesis and cell specification in the developing telencephalon

1.2.3.1 Neurogenesis

The mammalian brain comprises of a heterogeneous population of neurons. Regulatory mechanisms must therefore be in place to orchestrate the production of neural progenitors and control their differentiation, ensuring they give rise to specific populations of cells, in the correct quantities, in the right places, at the correct time in development. All the neuronal cell types, and glia, arise from the homogeneous population of progenitor cells that form the neural tube (McConnell, 1988a). At some point in development these cells commit to a certain phenotype. Indeed, the differentiation of neural precursors into neurons, astrocytes, and oligodendrocytes takes place in three overlapping but temporally distinct stages: the neurogenic phase, the astroglial phase, and the oligodendroglial phase (Ross *et al.*, 2003).

In 1988 McConnell (McConnell, 1988a) hypothesized that the early progenitors could give rise to glia and neurons by one of three methods:

a) A single type of pluripotent precursor gives rise to both neurons and glia.

b) Stem cells first give rise to neurons, switching to gliogenesis once neurogenesis is complete.

c) Two separate lineages of neuron and glial producing precursor cells coexist in the proliferative zone.

McConnell (McConnell, 1995a) also suggested that the potential of progenitor cells may become progressively reduced over time, and that early progenitors divide asymmetrically to give one neuron and one progenitor. The phenotype of each daughter cell would be determined by the differential inheritance of genetic markers (e.g.: numb protein in *drosophila*). So the early stages of cortical development involve rapid proliferation of cortical progenitors by symmetric cell division, during which time cell cycle times are short. During neurogenesis a subset of the progenitor population begins to divide asymmetrically resulting in one daughter cell from each division becoming restricted to a neuronal lineage (Ross *et al.*, 2003). It is believed that the progressive

restriction of progenitors is mediated by proneural and neurogenic genes through the process of lateral inhibition.

Birth dating studies have shown that during the initial expansion of the progenitor cell population cell cycle times are relatively short, and most cells divide asymmetrically to give rise to two mitotically active daughter cells. i.e.: two more progenitor cells. During corticogenesis the cell cycle times of progenitor cells gradually increase and more and more cells divide asymmetrically, so the number of progenitor cells gradually decreases (Caviness, Jr. and Takahashi, 1995).

It has been proposed that the regulation of cell cycle time is controlled by extrinsic factors which act on proliferating cells during the G1 phase of the cell cycle (Caviness, Jr. and Takahashi, 1995). The number of cells that divide asymmetrically and hence the number of cells that become committed to a neural fate appears to be under the control of notch signalling (Justice and Jan, 2002) via control of various transcriptional activators and repressors such as the Hes (mammalian hairy and Enhancer-of-split homologues) family of bHLH transcription factors (Ohtsuka *et al.*, 1999). Hes factors repress the activity of proneural target genes such as *mash 1* (Sasai *et al.*, 1992; Ross *et al.*, 2003). Indeed notch mutations have been shown to lead to an increase in neurogenesis in the mouse (de la Pompa *et al.*, 1997), whereas conversely activation of notch appears to promote a radial glial specification, in other words maintaining cells in a proliferative state (Gaiano *et al.*, 2000) as it has become accepted that radial glial cells are themselves progenitor cells (Malatesta *et al.*, 2000; Parnavelas and Nadarajah, 2001; Anthony *et al.*, 2004) as well as providing the scaffold for glial guided locomotion (see section 1.2.2). Timelapse images have been used to show that proliferative radial glia generate post-mitotic neurons which then migrate radially along clonally related radial glial cells (Noctor *et al.*, 2001). Hence a scheme of neurogenesis has been put forward in which progenitor cells gradually become more specified, firstly to a radial glial lineage, which through asymmetric division can give rise to both post-mitotic neurons and proliferative radial-glia, until symmetric divisions occur to give either neurons or at later stages astrocytes (Gotz and Huttner, 2005). Hence control over symmetric versus asymmetric cell division is crucial in governing both the number and types of cells generated during neurogenesis.

It has also become apparent that calcium transients between progenitor cells play a role in controlling precursor proliferation and hence the number of neurons generated (Weissman *et al.*, 2004). This has highlighted the potential importance of gap junction proteins expressed during forebrain development (Nadarajah *et al.*, 1997; Montoro and

Yuste, 2004). In particular connexin 43 which has been shown to have increased expression in primary cultures treated with FGF, which was linked to increased cell-cell coupling and a corresponding increase in proliferation (Nadarajah *et al.*, 1998).

1.2.3.2 Cell Specification

The subject of cell specification is extremely complex as cells can be specified, or classified, in many different ways; for example, by phenotype (e.g.: neurons versus glia or projection neurons versus interneurons), by their laminar position, or by their neurotransmitter content. A question that continues to pose a challenge is at what point does a cell become committed to a specific phenotype, or to express a certain neurotransmitter, and what are the factors responsible for these decisions? Equally can any progenitor give rise to any cell type within the cortex? Or are particular populations of cells responsible for generating a specific phenotype? If so, what are the factors responsible for restricting progenitors to a particular cell lineage?

Some of the factors responsible for controlling neuronal and glial differentiation have been identified. The bHLH family of transcription factors are especially important in defining the phenotypes of progenitor cells during corticogenesis (Nieto *et al.*, 2001; Sun *et al.*, 2001; Schuurmans and Guillemot, 2002; Ross *et al.*, 2003). Leukaemia inhibitory factor (LIF), bone morphogenetic protein 2 (BMP-2) and FGF appear to influence glial fates (Schuurmans and Guillemot, 2002). BMP-2 and its downstream effecter Smad1 promote astrocyte differentiation. This appears to work in conjunction with LIF and its downstream effecter STAT3, resulting in the formation of gliogenic Smad1/STAT3/p300 transcriptional complexes. Proneural genes appear to work by both promoting neuronal phenotypes and independently inhibiting glial differentiation (Nieto *et al.*, 2001; Sun *et al.*, 2001; Schuurmans and Guillemot, 2002). For example, the proneural gene *ngn1* promotes neurogenesis by transcriptional activation of genes required for neuronal differentiation, but also suppresses gliogenesis by binding Smad1/p300 in order to prevent formation of the gliogenic Smad1/STAT/p300 complexes (Sun *et al.*, 2001). In *ngn2* and *mash1* double mutants, progenitor cells failed to adopt a neuronal fate and instead remained pluripotent or entered the astrocytic differentiation pathway (Nieto *et al.*, 2001). Proneural genes are therefore involved in the lineage restriction of cortical progenitors by promoting neuronal fates whilst simultaneously inhibiting glial fates. Intriguingly, expression of the dorsal marker Pax6 was recently shown to cause the development of a neural phenotype in cultured HeLa cells (Cartier *et al.*, 2006). This is especially significant in light of the fact that Pax6 functions upstream of proneural genes such as *ngn2* (Scardigli *et al.*, 2003).

Dorsal neuronal phenotypes (pyramidal neurons) are distinguished by the expression of Tbr1 and bHLH proteins including NeuroD, NeuroD2, Mammalian atonal homologue (math) 2, math3 (Schuurmans and Guillemot, 2002), Pax-6 (Manuel and Price, 2005), ngn1/2 (Schuurmans *et al.*, 2004), and Emx-1/2, whereas ventral phenotypes (primarily GABAergic interneurons) seem to be derived from two different subpopulations in the LGE and the MGE (Marin *et al.*, 2000;Schuurmans and Guillemot, 2002). Ventral phenotypes hence appear to be defined a long distance from their ultimate destination in the cortex. It has been suggested that this is an evolutionary tactic to allow segregation of inductive cues, which increases the capacity for greater cell diversity (Wichterle *et al.*, 2001; Schuurmans and Guillemot, 2002). The LGE progenitor population is defined by expression of the bHLH proteins Dlx1 and Dlx2. Mice lacking Dlx1 and Dlx2 have a severe reduction in cortical neurons derived from the LGE (Wilson and Rubenstein, 2000). The MGE population is defined by expression of Nkx2.1, LIM homeobox gene-6 (Lhx6), and Lhx7 expression (Schuurmans and Guillemot, 2002). Lhx6 has since been shown to regulate the migration of cortical interneurons from the ventral telencephalon, but does not specify their GABA phenotype (Lavdas *et al.*, 1999; Alifragis *et al.*, 2004).

The expression of ventral markers (Mash1 and Dlx1/2) appears to be controlled by the homeodomain protein Gsh2, whereas the expression of *Ngn1/2* is regulated by Pax6 expression in the early cortex (Scardigli *et al.*, 2003). *Gsh2* loss of function (LOF) mutations result in Mash1 and Dlx1/2 expression being replaced by ectopic Ngn expression. Conversely *Pax6* LOF mutations result in the loss of Ngn expression, which is replaced by ectopic expression of Gsh2, Mash1 and Dlx1/2 (Toresson *et al.*, 2000), indeed in Pax6 knockout mice ectopic GABA expressing cells in the dorsal forebrain have been shown to be derived from an *Emx1* lineage generated in the cortical ventricular zone (Kroll and O'Leary, 2005) further emphasising the role of Pax6 in not only promoting dorsal gene expression patterns, but also repressing the expression of ventral markers. Pax6 and Gsh2 control cortical and striatal development by regulating genetically opposed pathways that regulate the expression of each other as well as the regionally expressed developmental cues Mash1, Ngns and Dlx. There is some evidence that the ventral marker Dlx1/2 regulates expression of the enzyme glutamic acid decarboxylase (GAD) (Schuurmans and Guillemot, 2002), responsible for GABA production by interneurons. So Dlx1/2 could be responsible for specifying GABAergic interneuron phenotypes. It has been hypothesized (Anderson *et al.*, 1997; Schuurmans and Guillemot, 2002; Stuhmer *et al.*, 2002a; Stuhmer *et al.*, 2002b) that this would work in a pathway where Mash1 expression regulates Dlx1/2

expression, which in turn promotes GAD expression. Interestingly, there also seems to be a link between Mash1 and Dlx1/2 expression and the balance between the differentiation of early and late neuronal phenotypes (Marin *et al.*, 2000). Mash1 expression is linked to early phenotypes whereas Dlx1/2 expression appears to define later produced neurons. This is a possible link between cell fate specification and the final laminar position of a particular cell.

1.3 A role for SDF-1 and CXCR4 in forebrain development?

It was originally thought that the brain was an immune-privileged organ, cut off from circulating signalling molecules in the periphery by the blood brain barrier. However, it is now known that various molecules first characterised in the peripheral immune system are present in the brain, for example inflammatory molecules such as cytokines (e.g.: Interleukin-1 (Rothwell, 1991)). Furthermore, some of these molecules and their receptors are expressed during CNS development, in particular the chemokine stromal derived factor-1 (SDF-1) and its receptor CXCR4 (McGrath *et al.*, 1999). Chemokines are a subset of the cytokine family of secreted proteins which are so called as they can induce chemotaxis (Asensio and Campbell, 1999). They were originally noted for their ability to regulate the migration and activation of leukocytes via G-protein coupled receptors in the immune system (Luster, 1998). Chemokines are known to be important during development, for correct homeostasis, for correct immune system function, for angiogenesis and angiostatic processes, during tumour and metastasis progression, and for functions within the CNS (Bajetto *et al.*, 2002).

Chemokines are divided into four groups on the basis of the relative position of the first N-terminal cysteine residues (see figure 4):

- CC (β-chemokines) – cysteines are adjacent
- CXC (α-chemokines) – separated by one residue
- CX3C (δ-chemokines) – there is only one – fractalkine
- C (γ-chemokines) – only have two of the four conserved cysteines found in the other families

Chemokines and their receptors are now known to be important in neuroinflammatory disease, and recent work has established that all the major cell types in the brain, including microglia, glia, and neurons, express chemokine receptors. They are therefore potential targets for chemokines (Tran and Miller, 2003). Chemokines are therefore thought to be

involved in various processes during both health and disease in the adult CNS (Tran and Miller, 2003; Lazarini *et al.*, 2003).

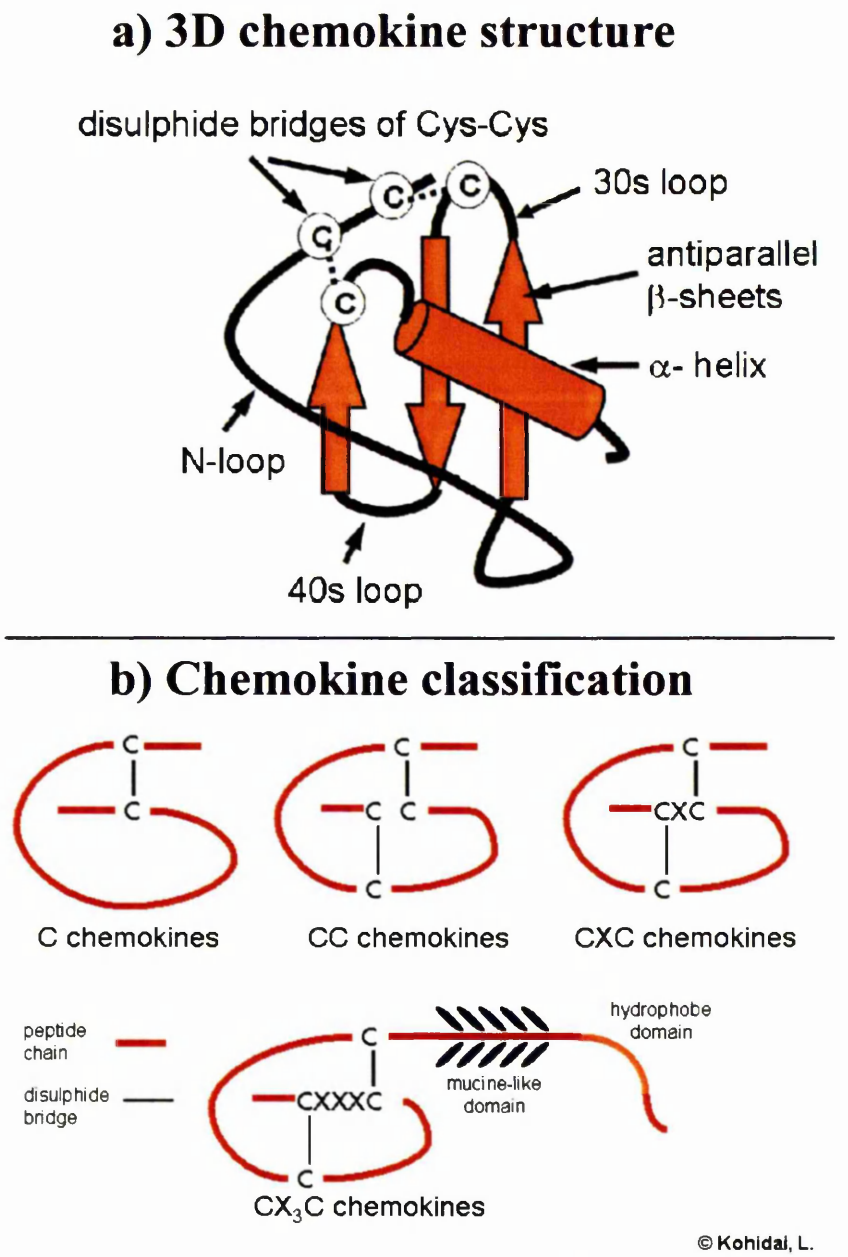

Figure 4: a) Diagram illustrating the 3D structural motif shared by all chemokines. b) The basis for the system of nomenclature used to classify chemokines. This is based on the positions of the first N-terminal cysteine residues. Original images taken from wikipedia: http://en.wikipedia.org/wiki/Chemokine (Copyright L. Kohidai).

1.3.1 Characterisation of SDF-1 and CXCR4

SDF-1 was first isolated and cloned from the bone marrow stromal cell line after which it is named (Tashiro *et al.*, 1993). There are three splice variants (see figure 5) of SDF-1 expressed in the CNS (Lazarini *et al.*, 2003). SDF-1α is a prototype of 89 residues including a 19 amino acid signal peptide. SDF-1β contains an additional four carboxy-terminal residues, whilst SDF-1γ contains a 30 residue c-terminal extension. SDF-1γ is expressed primarily in the adult, whilst the α and β splice variants are predominantly expressed during embryonic and postnatal development. SDF-1 is a member of the CXC group of chemokines and functions as a monomer. It has been shown to be extraordinarily well conserved throughout evolution (Shirozu *et al.*, 1995) suggesting it has a fundamental role in one or more processes, and this caused Ma *et al* (Ma *et al.*, 1998) to term it a "*primordial chemokine*". Its importance is further highlighted by the embryonic lethal phenotype of the murine knockout (Ma *et al.*, 1998).

The SDF-1 receptor (CXCR4) was first noted for its function as a co-receptor for HIV-1 fusion and cell entry (Feng *et al.*, 1996). It is a G-protein coupled 7 transmembrane (TM) domain protein previously termed fusin. Due to its role in HIV infection CXCR4 is a therapeutic target. As such there are several synthetic ligands for CXCR4 that block HIV binding to the receptor. One example is the bicyclam AMD3100 which was known to be an anti-HIV agent before it was shown to function by interacting exclusively with CXCR4 (Schols *et al.*, 1997). It was then demonstrated that SDF-1 signalled via CXCR4, causing an increase in intracellular free calcium and chemotaxis in CXCR4 transfected cells, as well as inhibiting HIV-1 infection of cells in culture (Bleul *et al.*, 1996).

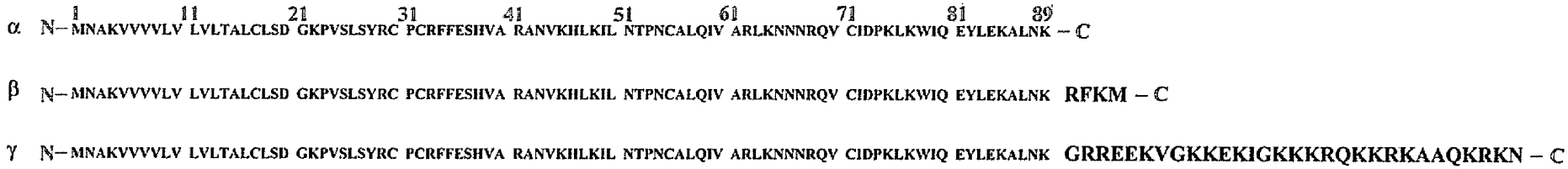

Figure 5: Protein sequences of the 3 isoforms of SDF-1

1.3.2 Known functions of SDF-1/CXCR4

SDF-1/CXCR4 signalling has been shown to have various physiological roles in both health and disease (figure 6). However, as described above, the first known biological function of SDF-1 was the stimulation of B-cell proliferation (Nagasawa *et al.*, 1994).

Since these experiments, SDF-1/CXCR4 signalling has been implicated in processes as diverse as angiogenesis (Salcedo and Oppenheim, 2003), inflammation, and organogenesis (Bajetto *et al.*, 2002), and also tumour metastasis in cancer (Fernandis *et al.*, 2004). Mice lacking either CXCR4 or SDF-1 die perinatally of defects in heart septum formation, as well as showing impaired B-cell lymphopoiesis, myelopoiesis and disrupted cerebellar development (Nagasawa *et al.*, 1994; Zou *et al.*, 1998; Ma *et al.*, 1998). Hence, SDF-1 was the first example of a chemokine to have an essential role in development, and perhaps even more intriguingly, the two knockouts exhibited identical phenotypes, which acts as genetic evidence supporting a monogamous relationship between CXCR4 and SDF-1.

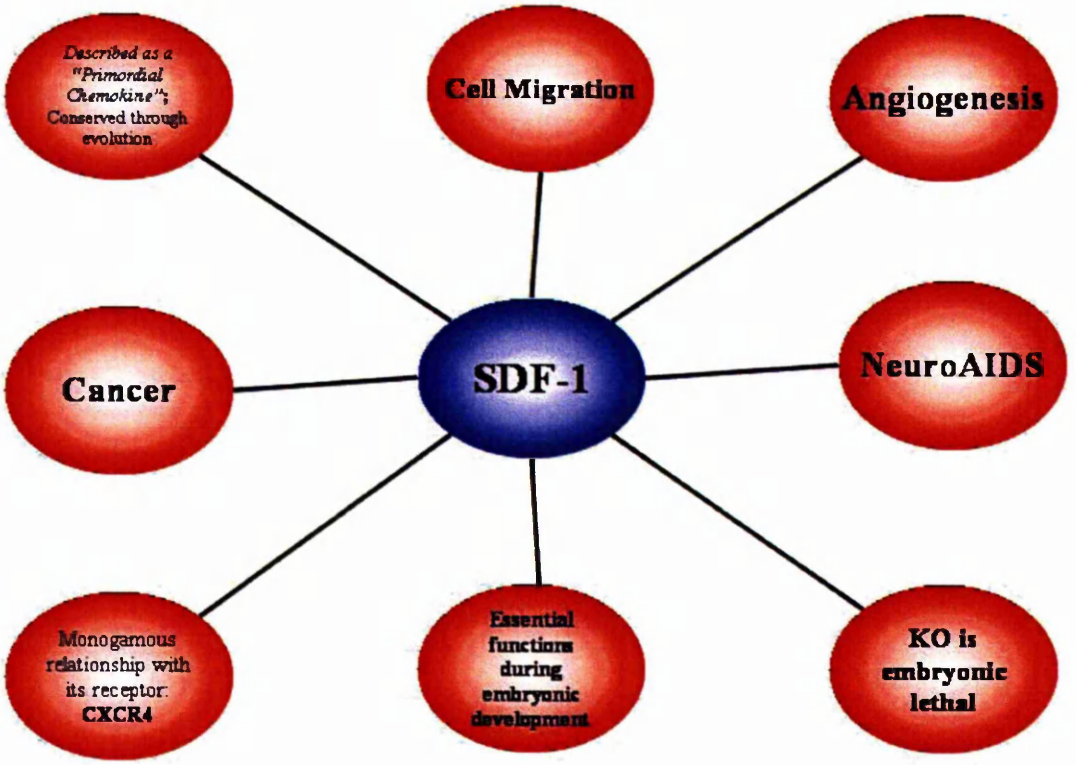

Figure 6 Diagram illustrating the diverse physiological roles of SDF-1 and highlighting some of its key characteristics.

1.3.3 SDF-1/CXCR4 Expression

The expression of SDF-1 and CXCR4 has been studied both in the adult CNS and during development, primarily by using immunohistochemistry and *in situ* hybridisation. The distribution of these two proteins and the mRNA messages encoding them gives clues to the likely functions played by SDF-1 and/or CXCR4 in the adult CNS and also during development.

In the adult CNS SDF-1 expression has been shown in astrocytes (Banisadr *et al.*, 2003), microglia and neurons but not in oligodendrocytes. This expression was found in several brain structures including the cerebral cortex, basal forebrain, hippocampus, thalamus, hypothalamus, substantia nigra, and pons. This expression appears to be largely co-distributed with CXCR4 expression. In the cortex there are differences in the laminar distribution of SDF-1, for example in the motor cortex it is primarily confined to a subpopulation of pyramidal cells in layers III-IV.

During development CXCR4 is the predominantly expressed chemokine receptor throughout the embryo (McGrath *et al.*, 1999). Using *in situ* hybridisation in mouse embryos, McGrath *et al* (McGrath *et al.*, 1999) showed both SDF-1 and CXCR4 genes have dynamic and complementary patterns of expression during organogenesis, not just in the developing CNS, but also in the cardiac, vascular, haematopoietic and craniofacial systems. Using this technique, CXCR4 transcripts were detected in mouse embryos as early as E7.5, the onset of haematopoiesis. These experiments also show that CXCR4 and SDF-1 may have fundamental roles during gastrulation, as their expression patterns delineate migrating versus non-migrating tissue. In the CNS, CXCR4 transcripts first accumulate in the neural epithelium in an anterior-posterior wave, between E8.5 and E9.5. Indeed, the CNS is the major site of CXCR4 expression during development. This expression can be divided into two types. In the spinal chord and hindbrain it correlates with cells that innervate muscle and viscera. In the midbrain and forebrain it is associated with a subset of expanding neuronal structures. CXCR4 expression also appears to define the midbrain/hindbrain border.

CXCR4 mRNA expression is associated with ventricular zones of neural cell proliferation throughout development in the mouse CNS (Tissir *et al.*, 2004), and this includes the ganglionic eminences of the telencephalon. Within the developing cortex CXCR4 mRNA appears to be produced by all the derivatives of the preplate, with expression found in the marginal zone, subplate and intermediate zone (Tissir *et al.*, 2004).

Both astrocytes and neurons have been shown to express SDF-1 and CXCR4 in primary cortical cultures (Bajetto *et al.*, 1999), and SDF-1 appears to stimulate astrocyte proliferation (Bajetto *et al.*, 2001). During forebrain development SDF-1 is expressed at the pallial-subpallial boundary of the telencephalon, extending dorsally and medially into the cortex, with no detectable expression in subpallial regions. The highest expression is in the intermediate zone, which correlates with polysialylated neural cell adhesion molecule expression (PSA-NCAM), a marker for tangentially migrating interneuron precursors (Daniel *et al.*, 2005). Furthermore, both human and rat neuronal precursor cells (derived from cortical tissue) have been shown to express CXCR4 and respond to SDF-1 (Peng *et al.*, 2004; Gong *et al.*, 2006).

These patterns of expression of SDF-1/CXCR4 and their timing during development of the forebrain, point to a possible role for SDF-1 in mediating some of the key events of forebrain development, including proliferation, migration and differentiation.

1.3.4 SDF-1/ CXCR4 signalling in CNS Development

SDF-1 was the first example of a chemokine with an essential function during development. Indeed, SDF-1 is synthesized constitutively in the developing brain (Asensio and Campbell, 1999), and expression of its receptor CXCR4 is highest during embryonic brain development (Lazarini *et al.*, 2003).

The first clues as to the role played by SDF-1/CXCR4 signalling in the developing CNS came from observations made in mouse knockouts for SDF-1 or CXCR4; both genotypes had defects in cerebellar development (Zou *et al.*, 1998). This was subsequently investigated further by Ma *et al.*, who noted the appearance of a disorganized cerebellum in both SDF-1 and CXCR4 deficient mice, characterised by an attenuated external granule layer (EGL), which they stated was due almost entirely to disrupted neuronal migration (Ma *et al.*, 1998). SDF-1 was then shown to elicit chemotactic responses in granule precursor cells, as well as enhancing the proliferation of granule cell precursors in response to Shh (Klein *et al.*, 2001). Zhu *et al* (Zhu *et al.*, 2002) then demonstrated that SDF-1 secreted from non-neuronal meningeal cells acts as a chemoattractant to control migration of embryonic EGL cells in the cerebellum. Thus SDF-1 appears to function as an attractant holding granule cells in the EGL until the appropriate time. Indeed, SDF-1 has been shown to become concentrated locally in the EGL by heparan sulphate proteoglycans, and thereby attracts granule cell precursors to the proliferative compartment (Reiss *et al.*, 2002). This is comparable to the role played by SDF-1 during haematopoiesis.

As well as having a role in the development of the cerebellum, SDF-1 has also been implicated in the formation of other areas of the CNS, including both the hippocampus and the cerebral cortex. In both these regions SDF-1 and CXCR4 seem to be crucial for the correct migration of neurons. Lu *et al* showed defects in migration in the dentate gyrus in mice lacking CXCR4 (Lu *et al.*, 2002). This is supported by Bagri *et al* who showed that SDF-1 and CXCR4 expression patterns are consistent with a role for SDF-1 in dentate granule cell migration in the developing hippocampus. Using ectopic SDF-1 expression in explants they were able to demonstrate the direct regulation of dentate granule cell migration by SDF-1 (Bagri *et al.*, 2002).

More recently, CXCR4 expression has been identified in interneurons migrating from the ventral forebrain towards the neocortex, and SDF-1 has been shown to act as a potent chemoattractant for isolated striatal precursors, selectively regulating the migration and layer specific integration of CXCR-4 expressing interneurons during neocortical development (Stumm *et al.*, 2003). This sentiment appears to be supported by the fact that SDF-1 produced in the intermediate zone of the cortex is ideally placed to exert an influence on tangentially migrating cells expressing CXCR4 (Tissir *et al.*, 2004). This influence could be to impart layer information, chemoattractive signals, or even to prompt cell specification. Indeed, work by Peng *et al* showed that neuronal precursors in the cerebral cortex exhibit chemotactic responses to SDF-1 (Peng *et al.*, 2004), and intracerebral injection of SDF-1 enabled intravenously injected neural stem cells expressing CXCR4 to migrate into the CNS where they were shown to differentiate into neurons (Corti *et al.*, 2005).

As well as roles in regulating migration, SDF-1/CXCR4 signalling has been shown to regulate axonal outgrowth in both cerebellar and hippocampal neuron cultures. Interestingly, it was shown that at differing concentrations SDF-1 had opposite effects on cultured cerebellar granule neurons. At low concentrations SDF-1 stimulated axonal elongation, but this was repressed at higher concentrations. These effects are mediated through 2 distinct Rho-GTPase dependent pathways (Arakawa *et al.*, 2003). Similar observations were made using cultured hippocampal neurons, where SDF-1 appeared to reduce growth cone number and outgrowth, but increase axonal branching (Pujol *et al.*, 2005).

1.4 Aims

As discussed above, SDF-1 is expressed in the cortex during key phases of development. This project aims to examine what function signalling by this chemokine may have during the formation of the cerebral cortex. As SDF-1 is expressed in the intermediate zone of the developing cerebral cortex (Daniel *et al.*, 2005), it could be involved in processes such as neuronal migration, differentiation, and also in the regulation of proliferation. Clearly, its role in other systems such as the immune system and the cerebellum, where it is involved in chemotaxis (Nagasawa *et al.*, 1996; Ma *et al.*, 1998), would suggest it may play a similar role in the migration of cortical neurons. Indeed, evidence for this has now been found (Stumm *et al.*, 2003). Hence the aim of this project is to address the following hypotheses:

- **SDF-1 regulates precursor proliferation during forebrain development**

SDF-1 may play a role in modulating proliferation in the developing cortex, as has been shown in the developing cerebellum. Indeed, it appears that signalling via SDF-1 can modulate the effects of sonic hedgehog (Shh) signalling on the proliferation of cerebellar granule cells (Klein *et al.*, 2001). As CXCR4 is also expressed by cells in the cortical ventricular zone, might SDF-1 expression have a similar function in controlling the proliferation of cortical progenitors?

- **SDF-1 regulates cell specification and differentiation in the developing cortex**

In other developmental systems, SDF-1 is involved in differentiation, hence, due to its temporal and spatial pattern of expression it may also function as a differentiating factor during cortical neurogenesis. Again, this could also be linked to signalling via other molecules such as Shh and bone morphogenetic proteins (BMPs).

- **SDF-1 functions as an axon guidance molecule in the developing cerebral cortex**

It has been shown that SDF-1 plays a role in axonal growth (Arakawa *et al.*, 2003; Pujol *et al.*, 2005) during the development of other brain regions. Thus it may have a parallel role during corticogenesis.

- **Genes downstream of CXCR4/SDF-1 will be identified using microarray technology**

In order to elucidate potential signalling pathways downstream of SDF-1, RNA from primary forebrain cultures exposed to the chemokine, or the CXCR4 antagonist AMD3100 will be screened using microarrays. This should enable the identification of genes whose expression is altered in response to SDF-1 signalling.

In order to address these hypotheses, primary cortical cultures from embryonic day 17 (E17) rat brains will be subjected to either SDF-1 over expression, or treatment with the CXCR4 antagonist AMD3100 (Schols *et al.*, 1997). These cultures will then be used for basic immunocytochemistry experiments using markers of both proliferation (BrdU indexing, Ki67) and differentiation (Neu-N, GABA) to assess what role, if any, SDF-1 has in these processes. Similarly, GFP transfections will be used in conjunction with the above treatments in order to visualise the role of signalling via CXCR4 in neurite outgrowth. It is then hoped to elucidate the downstream mechanisms responsible for any observed affects using techniques such as qRT-PCR and microarray technology to identify potential target genes for SDF-1 mediated events during cortical development.

2 Materials and Methodology

2.1 Experimental design

Primary neural cultures prepared from rat embryonic day 16 or 17 (day of vaginal plug is embryonic (E) day 1) brains were used. Cells from dorsal (cortical) and ventral (ganglionic eminence) telencephalic regions were cultured to determine the regulatory role of SDF-1 signalling during development. Medium collected from transfected human embryonic kidney (HEK) cells over expressing SDF-1α, was used to treat primary neural cultures to model SDF-1 over expression. To inhibit the receptor signalling, AMD3100, a specific antagonist of CXCR4 was used. In addition, cultures from mutant mice that lack the receptor ($CXCR4^{-/-}$) were used to further validate observations made with SDF-1 over expression or CXCR4 antagonist treatment studies.

2.2 Materials

All reagents not listed below were from Sigma-Aldrich (UK).

- NaCl and Tris were from VWR International Ltd. (Leicestershire, UK)

2.2.1 HEK Cell Culture and Transfection

- 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and ABTS buffer were from Roche
- Calcium phosphate mammalian transfection kit – Clontech (Mountain View, CA, USA)
- ELISA plates – Nunc-immuno maxisorp 96-well plates, Nalge Nunc International (Rochester, NY, USA)
- SDF-1 ELISA Kit, Antibodies and streptavidin conjugated horse radish peroxidase (Strep-HRP) – R&D Systems (Minneapolis, MN, USA)

2.2.2 Plasmids and Plasmid Purification

- Endofree plasmid maxikit – Qiagen Ltd. (West Sussex, UK)
- pCMS-EGFP – Dr Liapi, University College London (UK)
- pcxSTOP-GFP – Dr Rannala, University of Alberta (Edmonton, Canada)
- pRK5-SDF-1 – Dr Liapi, University College London (UK)
- Restriction endonuclease EcoRI – Roche

2.2.3 Primary Cell Culture

- AMD3100 – NIH Aids Reagent Program (Germantown, MD, USA)
- Artificial cerebro-spinal fluid (ACSF):
    - $CaCl_2$ and sucrose from Sigma-Aldrich (UK)
    - D-glucose, HEPES, KCl, KH_2PO_4, and $MgSO_4$ all from VWR International Ltd. (Leicestershire, UK)
- Chromic sulphuric acid – VWR International Ltd (Leicestershire, UK)
- Culture plates – Corning costar (Corning, NY, USA)
- Deoxyribonuclease (DNase) I – Roche
- Lipofectamine – Invitrogen Ltd. (Paisley, UK)
- Non-radioactive cytotox assay kit – Promega UK (Southampton, UK)

2.2.4 Genotyping: DNA Extraction, PCR and Electrophoresis

- dNTP mix, PCR buffer, and proteinase K – Fermentas International Inc. (Ontario, Canada)
- *Taq*-DNA polymerase - Roche

2.2.5 Immunocytochemistry

- Mounting media – Vectashield, Vector Labs (Burlingame, CA, USA)
- Secondary antibodies – Molecular Probes, Invitrogen Ltd. (Paisley, UK)
- Primary antibodies:
    - Brdu – Progen Biotechnik GmbH (Heidelberg, Germany)
    - Calbindin – Swant (Bellinzona, Switzerland)
    - Calretinin – Swant (Bellinzona, Switzerland)
    - Connexin 43 – Zymed Laboratories, Invitrogen Ltd. (Paisley, UK)
    - CXCR4 – Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
    - GABA – Sigma-Aldrich (UK)
    - Ki67 – Vector Labs (Burlingame, CA, USA)
    - MAP-2 – Sigma-Aldrich (UK)
    - Nestin – Susan Hockfield, Developmental Studies Hybridoma Bank (University of Iowa, USA)
    - Neu-N – Chemicon Millipore (Billerica, MA, USA)
    - Neurofilament – T Jessel and J Dodd, Developmental Studies Hybridoma Bank (University of Iowa, USA)
    - SDF-1 – R&D Systems (Minneapolis, MN, USA)

- Tag1 – M Yamamoto, Developmental Studies Hybridoma Bank (University of Iowa, USA)
- Tuj1 – Chemicon, Millipore (Billerica, MA, USA)

2.2.6 Real Time Imaging and Dye Coupling

- Lucifer yellow, Oregon green and pluronic acid were all from Molecular Probes, Invitrogen Ltd. (Paisley, UK)

2.2.7 Microarrays and Taqman

- DNase – Invitrogen Ltd. (Paisley, UK)
- dNTP's – Fermentas International Inc. (Ontario, Canada)
- qPCR Mastermix Plus – Eurogentec (Belgium)
- Genechip rat expression array 230v2 – Affymetrix (Santa Clara, CA, USA)
- qRT-PCR 96-well microplates – Axygen (Union City, CA, USA)
- RNAse inhibitor – Promega UK (Southampton, UK)
- Trizol – Invitrogen Ltd. (Paisley, UK)

2.3 Generation of SDF-1 media

To provide a source of secreted SDF-1, HEK cells were transfected with a plasmid containing the SDF-1 insert using a calcium phosphate transfection kit (Graham and van der Eb, 1973). Conditioned medium from these cultures was collected, assayed for SDF-1 content by ELISA and stored at -20°C until use.

2.4 Plasmid purification

E. Coli transformed with a pRK5 expression vector containing the SDF-1 gene cloned into the EcoR1 restriction site (figure 7) were used to inoculate ampicillin containing agar plates and incubated overnight at 37°C. A single colony was used to inoculate 5ml of lysogeny broth (LB Broth) (Bertani, 1951) and incubated for 4 hrs at 37°C. This was added to a further 250ml of LB Broth and incubated overnight at 37°C. Bacteria were lysed and plasmid DNA purified using a Qiagen Endofree Plasmid Maxikit. This is based on an alkaline lysis procedure followed by plasmid binding to an anion-exchange resin, under low-salt and pH conditions. RNA, proteins and low molecular weight impurities are then removed by a medium salt wash, followed by elution of plasmid DNA in a high salt buffer. Plasmid is then concentrated and desalted by precipitation in isopropanol. Purification products were then analysed using a restriction endonuclease

EcoR1 digest, followed by electrophoresis to verify extraction of plasmid DNA. DNA concentration was quantified using a NanoDrop ND1000 spectrophotometer. The same protocol was used to purify both pCMS-EGFP (Dr Liapi, UCL) and pcxSTOP-GFP (Dr Rannala, University of Alberta) plasmids.

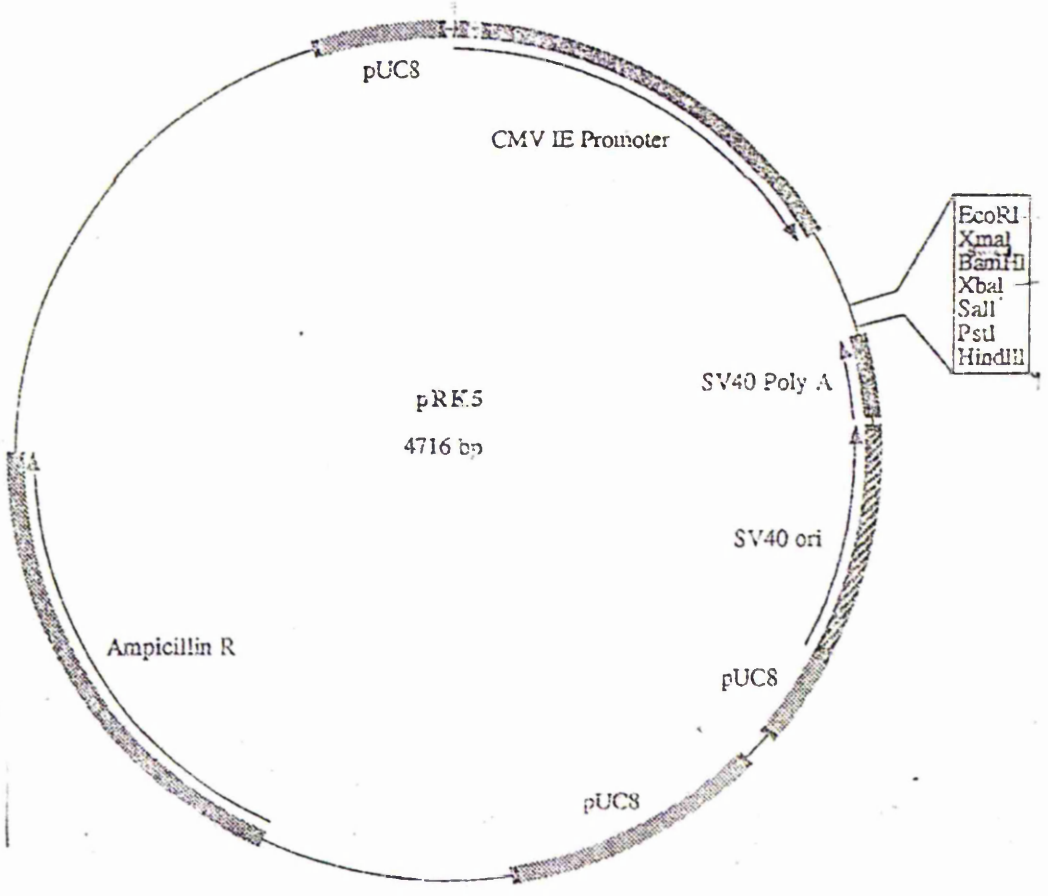

Figure 7: The pRK5 vector in to which the SDF-1 gene was cloned at the EcoR1 site (Dr Liapi, UCL).

2.4.1 Culture and transfection of HEK Cells

HEK-293 cells were cultured in DMEM with 2mM L-glutamine, 10% FBS and 1% antibiotic/antimycotic until confluent. They were transfected with prk5, containing the SDF-1 gene cloned into the EcoR1 site, using a calcium phosphate mammalian transfection kit. Control HEK cells were "dummy" transfected using the prk5 plasmid without the SDF-1 insert. Medium was collected from both SDF-1 transfected (SDF-1 medium) and dummy transfected (control medium) cultures 48 hrs after transfection, pooled and stored at –20°C.

2.4.2 Quantification of SDF-1 expression

Medium collected from both transfected HEK cells and primary cultures was assayed for SDF-1 using R&D Systems SDF-1 ELISA kit, with modifications by Dr Owen Jones (University of Manchester). 96 well plates were coated overnight with mouse anti-human SDF-1 antibody at 2μg/ml. Plates were washed in three changes of 0.05% tween in PBS (pH 7.4) and blocked (300μl per well) for 2 hours using 1% bovine serum albumin (BSA), 14mM sucrose in PBS, then washed as described. Samples were diluted appropriately using assay diluent (0.1% BSA, 0.05% tween, 20mM Tris, 150mM NaCl, pH7.3) and incubated in the plate for 2 hours at room temperature. Plates were washed and binding antibody (biotinylated rabbit ant-SDF-1 at 200ng/ml in assay diluent) added for 2 hours. After 3 further washes plates were incubated with Streptavidin conjugated horseradish peroxidase, diluted 1:200, for 1 hour. After washing ABTS peroxidase substrate was added and absorbance measured at 450nm after 30 minutes.

NB: Unless stated, for all reagents 100μl were used per well

2.5 Primary cell culture

2.5.1 Preparation of cover slips and cell culture plates

Glass cover slips were treated overnight with chromic sulphuric acid, to remove traces of silicon. Cover slips were then thoroughly rinsed in running tap water and boiled in 70% ethanol for sterilisation. Single cover slips were placed in a 24 well plate and allowed to dry for at least 1 hour. They were then coated with poly-l-lysine/laminin (1μg/ml) overnight. Coated cover slips were then rinsed with sterile H_2O and allowed to dry. 6-well plates were also coated with poly-l-lysine/laminin overnight before use.

2.5.2 Dissociation of forebrain tissue

Embryos were harvested in ice-cold artificial cerebro-spinal fluid (ACSF: 25mM KCl, 2mM KH_2PO_4, 25mM HEPES, 37mM D-glucose, 10mM $MgSO_4$, 175mM Sucrose, 0.5mM $CaCl_2$, 1% antibiotic/antimycotic, pH 7.4). Brains were freed from meninges and the cerebral cortex and ganglionic eminences separated. The resulting tissue was incubated for 90 minutes at 37°C in DMEM containing 21μM trypsin and 2.7μM DNase and mechanically dissociated by gentle trituration with a 1ml pipette. Cells were then plated out in 10% foetal calf serum in DMEM containing 2mM L-glutamine, and 1% combined antibiotic/antimycotic on poly-l-ysine/laminin coated glass cover slips in 24 well plates, or on coated 6-well plates. After 24 hours, cells were transferred to serum free medium

supplemented with N2 (1% N2, 2% antibiotic/antimycotic, 2mM L-glutamine in DMEM). To assay cell proliferation, after 48 hours *in vitro*, or when stated, cells were pulsed with 30μM bromodeoxyuridine (BrdU) for 1 hour. The following treatments were then used; control medium, SDF-1 medium, AMD3100 at 20μM or 40μM in N2 medium, 1μM carbenoxalone in N2 medium, or cyclopamine at 5μM or 10μM in N2 medium. Cells were treated once every 24 hours for 5 days, and medium collected prior to each treatment. For characterisation, cells were fixed using 4% PFA (pH 7) 24 hours after the final treatment, except cover slips used for connexin 43 (Cx43) immunocytochemistry. These were fixed for 10 minutes in ice cold methanol, and transferred to PBS.

2.5.3 Cultures from CXCR4$^{-/-}$ mice

CXCR4 heterozygous mice were obtained from Jackson Labs (USA), and their colonies were raised in house. Primary neural cultures from E15 mice were prepared as described above.

2.5.4 Genotyping of CXCR4$^{-/-}$ embryos/tail clips

Genotyping of either tail clips (to enable matings of heterozygotes with heterozygotes to generate homozygous foetuses) or of individual embryos was carried out as follows: DNA was extracted by incubation overnight at 55°C in lysis buffer (100mM Tris, 5μM EDTA, 0.2% SDS, 200mM NaCl, 0.5μg/μl proteinase K, pH 8). Following centrifugation at 1400rpm for 5 minutes, 1ml 100% ethanol was added to the supernatant. This was inverted 3 times to give a flocculus, and then centrifuged as before. The resulting pellet was resuspended in 70% ethanol and centrifuged for a further minute. The pellet was allowed to air dry for 15 minutes and resuspended in 100μl of dH_20. 5μl of DNA was then added to 20μl PCR reaction mixture (1x PCR buffer, 160μM dNTPs, 0.8% Taq DNA polymerase, 400nM primers). The PCR was carried out with the following parameters: 94°C for 3 minutes to activate the *taq*-polymerase, followed by 40 cycles of 90°C for 30 seconds (to denature the DNA), 62°C for 1 minute (for primer annealing), 72°C for 1 minute (for elongation).Primer sequences were as follows:

CXCR4 common – a g g c a g g t c a g t c t g a g a a t

CXCR4 knockout – a a g a a c g a g a t c a g c a g c c t

CXCR4 wildtype – c a a g g a a a c t g c t g g c t g a a

Electrophoresis was carried out on samples of extracted DNA and the PCR products using an agarose gel (1.5% agarose in TAE buffer: 200mM Tris, 1M acetate, 100mM EDTA) containing 0.125μM ethidium bromide.

2.5.5 Explant and slice culture/ex-vivo tissue culture

For slice cultures, E17 embryonic brains were embedded in 3% low gelling temperature agarose in PBS. Embedded brains were trimmed to square blocks and transferred to the sectioning chamber of a Vibroslice. 300μm coronal slices were cut in ice cold ACSF and placed on either millicell 0.4μm filter cell culture inserts (for immunocytochemistry) or millipore 0.45μm membrane filters (for real-time imaging). Slices were initially incubated in slice medium (5% FBS, 0.5% N2, 1mM L-glutamine, 13.3mM D-glucose, 1% antibiotic/antimycotic) overnight at 37°C, wherever applicable BrdU pulses were carried out as described earlier. Cultures were changed to either SDF medium or slice medium containing drug for 48 hours, with an additional medium change after 24 hours. Slices for immunocytochemistry were fixed overnight in 4% PFA in PBS.

For explant cultures, rat E17 cortices were dissected out and placed flat on 0.45μm nitrocellulose membrane filters with the ventricular zone surface uppermost. Treatment was commenced after 3 hrs incubation in warm ACSF.

2.5.6 Cryoprotection

Following fixation in 4% PFA, cultured slices or whole brains were transferred to 12% sucrose in PBS, and stored for at least 24 hours at 4°C. They were then placed in a 50:50 mix of OCT and 12% sucrose for no less than 24 hours, embedded in OCT, and sectioned at a thickness of 15μm using a cryostat. Sections were stored at -70°C until further use.

2.5.7 Quantification of neurite outgrowth

To visualise and quantify neuronal processes, primary cultures were transfected with a green fluorescent protein (GFP) plasmid construct (supplied by Dr Liapi, UCL, figure 8) after 3 days in vitro (DIV) using Lipofectamine 2000 according to the manufacturers instructions. Transfected cultures were fixed using a 50:50 mixture of 4% PFA and ACSF.

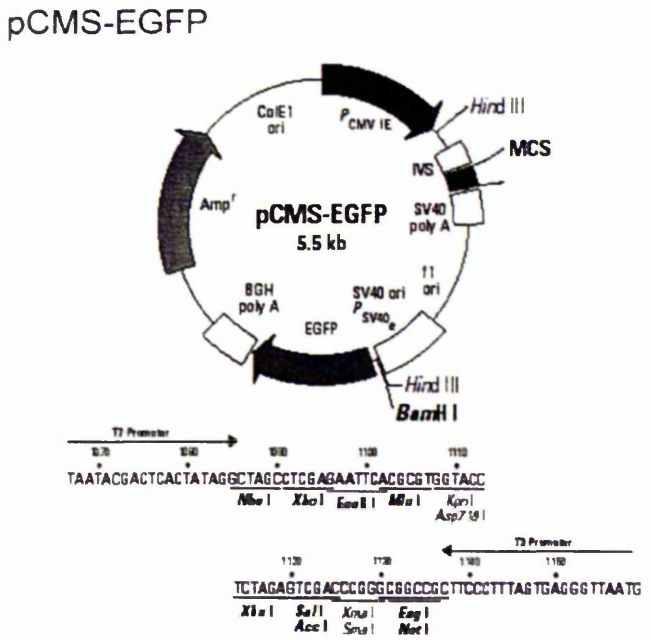

Figure 8: Restriction map and multiple cloning cite of pCMS-EGFP.

2.5.8 Cytotoxicity assay – using measurements of LDH activity

Medium collected from primary cultures was assayed for lactate dehydrogenase (LDH) activity to obtain an index of cytotoxicity using Promega's CytoTox 96 Non-Radioactive Cytotoxic Assay. This is based on the conversion of tetrazolium salt into a red formazan product by LDH released from lysed cells. Hence, the intensity of colour is proportional to the degree of cytotoxicity, which can be quantified by measuring absorbance at 492nm.

2.5.9 Cell survival assay using MTT

This assay is based on the conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan by active mitochondrial enzymes. This can be quantified by reading absorbance at 560nm, which is directly proportional to the number of viable cells. Cells were incubated with 1.2mM MTT for 2 hours at 37°C. Medium was removed and the purple formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was recorded at 560nm.

2.6 Immunocytochemistry

All images were captured and analysed using a Nikon Eclipse E600 microscope in conjunction with a Photometrics Coolsnap HQ black and white digital camera and

Metamorph imaging software (Universal Imaging Corporation). Images were subsequently edited using Adobe Photoshop.

Antibody dilutions were with PBS containing 5% normal goat serum (NGS), except where primary antibodies were raised in goat, in which case 5% normal donkey serum was used. For blocking solutions 0.1% triton was added. Secondary antibodies used were goat anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin conjugated to either FITC or rhodamine. For CXCR4 staining a donkey anti-goat immunoglobulin secondary conjugated to FITC was used.

For GFP transfected cultures single staining was carried out using only 2° antibodies conjugated to rhodamine.

The standard protocol followed was a 15 minute wash in PBS, followed by 1 hour's incubation in block. Primary antibody incubations were carried out overnight at 4°C, followed by three 10 min washes in PBS. Unless stated secondary antibodies were diluted 1:500 and incubated for 90 mins at room temperature. This was followed by a 10 min wash in 0.01% triton in PBS and 2 further 10 min washes in PBS. Counter staining of cells was with 3.75μM *bis*-benzamide in PBS. Cover slips were mounted using vectashield mounting medium. Exceptions to this protocol are noted in the sections below, in particular for BrdU staining.

2.6.1 BrdU immunocytochemistry: acid pre-treatment

BrdU, an analogue of thymidine, is incorporated into the DNA of proliferating cells during S-phase. It can therefore be used as a marker of proliferating cells. Prior to BrdU staining cells/sections were subjected to an acid pre-treatment to denature the DNA and allow antibody binding to BrdU incorporated during DNA synthesis.

Cover slips or slides were washed with PBS for 10 min, and then pre-treated with 2N HCl for 45minutes. This was followed by three 10 min washes with 0.1M boric acid, and a further 10 min wash with PBS. Cover slips were then incubated with blocking solution for 1 hour and the standard immunocytochemistry protocol outlined above was followed.

2.6.2 Immunocytochemistry on explant cultures

Due to the thickness of the tissue, explants were blocked for 4 hours, and 0.01% triton was added to the antibody diluent.

2.6.3 Primary antibody concentrations

Epitope	Raised In	Dilution Factor
CXCR4	Goat polyclonal	1:100
Cx43	Mouse monoclonal	1:100
BrdU	Mouse monoclonal	1:100
MAP2	Mouse monoclonal	1:100
Neuronal Nuclei	Mouse monoclonal	1:100
Neurofilament	Mouse monoclonal	1:100
SDF-1	Mouse monoclonal	1:250
Tag1	Mouse monoclonal	1:100
Calbindin	Rabbit polyclonal	1:500
Calretinin	Rabbit polyclonal	1:500
GABA	Rabbit polyclonal	1:3000
Ki67	Rabbit polyclonal	1:250

Table 1: Primary antibody concentrations

2.7 Cell cluster analysis: a modified method of lineage analysis

To further investigate the proliferation of cortical progenitors in treated cultures, a BrdU labelling assay was developed to follow the lineage of single cells. Several methods were initially tried, including the use of retrovirus with lacZ reporter (gift from Dr. Gotz, Germany), the cell tracer dye carboxyfluroscein diacetate, succinimidyl ester (CFDA SE) (Lyons, 1999); and transfection with a pcxSTOP-GFP plasmid (gift from Simon Ro and Dr Rannala, University of Alberta) (Ro and Rannala, 2004). However, the only method that gave reliable results was BrdU labelling of *ex-vivo* cortical tissue prior to dissociation, and subsequent mixing with a suspension of unlabelled cells. This allowed the culture of a subset of labelled cells within a larger unlabelled population, allowing for analysis of individual clusters of cells. Accordingly, individual pieces of cortical tissue were BrdU pulsed in serum free medium containing N2 for 3 hours prior to being dissociated as described previously. The resulting BrdU labelled cell suspension was then pooled with a suspension of unlabelled cells at a ratio of 12:1 (vol:vol, unlabelled:labelled); this gave a culture of cells containing individual BrdU positive cells, surrounded by unlabelled cells. Hence, the number of cells within a BrdU positive cluster is an index of the rate of proliferation within that culture. Cells were considered to be part of a cluster when they were within a distance of 15μm of another labelled cell from the same cluster.

2.8 Real time imaging of cells using Oregon green BAPTA labelling

Using Oregon green BAPTA, a calcium indicator, it is possible to label living cells and visualise changes in intracellular calcium transients in cells. Primary cortical cultures were labelled using 1μM Oregon green, 3.33μM pluronic acid in serum free medium for 20 minutes at 37°C. Cultures were then rinsed and incubated for at least 30 minutes at 37°C before commencing image acquisition. Explants and slice cultures were labelled for 2 hours in 8μM Oregon green, 6.66μM pluronic acid in serum free medium and rinsed as above.

2.9 Dye coupling: scrape load method

To visualise gap junction coupling in cortical explants, Lucifer yellow, a small molecular weight fluorescent dye, was used, as it has been shown to permeate gap junction channels (Lo Turco and Kriegstein, 1991). Briefly, explant cultures were scrape loaded (el Fouly et al., 1987) with 1μM Lucifer yellow in PBS, by making an incision in the ventricular zone surface with a scalpel blade. After 15 minutes incubation at 37°C they were rinsed with PBS and transferred back to the appropriate medium for image acquisition.

2.9.1 Confocal imaging of labelled explants

Following fixation, explants were imaged using a Leica DM IRE 2 confocal microscope and Leica Confocal Software v2.61.

2.10 Gene expression analysis using DNA microarrays

To analyse the regulatory effects of SDF-1 signalling on downstream gene expression, RNA was extracted from cortical cultures treated with SDF-1 medium or AMD3100 and probed with a "microarray" gene chip. This enabled a genome wide analysis of gene expression.

2.10.1 RNA extraction

RNA was extracted using a Trizol based protocol. Trizol is a mono-phasic solution of phenol and guanidine isothiocyanate, which maintains RNA integrity. This method is based on a previously published single-step protocol (Chomczynski and Sacchi, 1987).

Cultures were homogenized in 1ml trizol/50mg tissue and incubated for 5 minutes at room temperature. To this mix 0.2ml of chloroform was added per ml of trizol used.

Samples were shaken vigorously for 15 secs and incubated at room temperature for 3 mins. Following centrifugation at 12 000g at 4°C for 15 mins, RNA was precipitated from the aqueous phase by addition of 0.5ml isopropanol per ml of trizol used. Samples were incubated for 10 mins at room temperature, and then centrifuged as before for a further 10 mins. The RNA pellet was washed in 1ml 70% ethanol per ml of trizol used, vortexed and centrifuged for a further 5 mins at 7500g at 4°C. The pellet was then air dried and resuspended in 50µl DEPC H_2O per ml of trizol used. RNA concentrations were quantified using a NanoDrop ND1000 spectrophotometer.

2.10.2 Microarrays

To generate sufficient RNA for microarray experiments, many embryos were pooled together to obtain sufficient cells. The experiment was designed as outlined in figure 9, to generate 3 biological replicates.

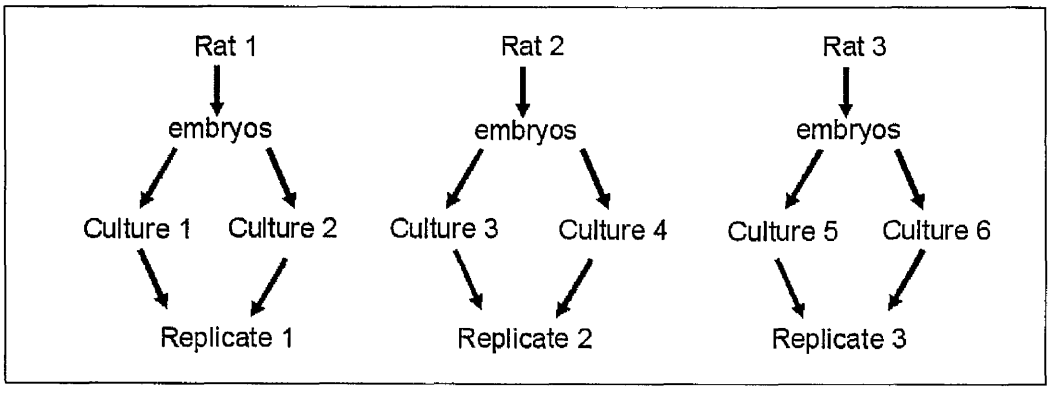

Figure 9: Experimental design for microarray experiments

Microarrays were used as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, 15µg of total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase with a poly(T) oligomer that incorporated the T7 promoter used as a primer. Subsequent second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of transcripts, and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nucleotides or less and incubated at 99°C for 5 min. Labelled fragmented cRNA was hybridized for 16 hours at 45°C to affymetrix rat genome RG-230v2 microarrays. The microarrays were washed at low and high stringency before staining with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional incubation with streptavidin-phycoerythrin stain.

2.10.3 Quantitative Real Time PCR

Using real-time quantitative PCR, the relative quantity of transcripts was measured in control and treated samples. All genes of interest were first normalised with GAPDH and β-actin, the house keeping genes, and subsequently the relative abundance of transcripts in treated samples was expressed as fold change compared to those measured in controls. To ensure there was no DNA contamination, RNA samples (1μg RNA used per sample) were first treated with DNase (using 1μl of DNase per μg of RNA). cDNA was then generated by reverse transcription: samples were incubated with 25ng/μl random primers and 0.77mM dNTPs for 5 minutes at 65°C. After 1 min on ice samples were centrifuged briefly and incubated with 50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$, 5mM DTT, 2 units RNase inhibitor, and 10 units reverse transcriptase for 5 mins at 25°C. For reverse transcription the mixture was left at 50°C for 45 mins, and the reaction inactivated by heating to 70°C for 15 mins. The prepared cDNA was stored at -20°C.

Taqman quantitative real time PCR (Heid *et al*, 1996) was performed in 96-well plates; 5μl per well of cDNA (this was substituted for H_20 in control wells) was added to 10μl 2x master mix (to give 5mM $MgCl_2$, and also containing uracil-N-glycosylase (UNG)), 0.8μM of each relevant primer, and 0.4μM of the relevant probe. PCR reactions and analysis were carried out using an ABI Prism 7700 Sequence Detector. Briefly, 50°C for 2 minutes (to allow UNG to eliminate any carry over contamination (Longo *et al*, 1990)), 95°C for 10 minutes (to heat inactivate UNG and activate Taq polymerase), followed by 35-40 cycles of 92°C (for template denaturation) for 15 seconds and 60°C for 60 seconds (for annealing and extension of primers). Primer and probes were designed and chosen using the Roche Applied Science Probe Finder software v2.10 (http://www.universalprobelibrary.com). Primer sequences and details of probes are shown in table 2.

Gene	Forward Primer	Reverse Primer	Roche Universal Probe Number
gap junction membrane channel protein alpha 1 (Gja1)	GCCTGAACTCTCATTTTTCCTTT	CCATGTCTGGGCACCTCT	75
hexokinase 2 (Hk2)	CCTGCCTTTGGGTTTCAC	ACTTGAGGAGGATGCTCTGG	12
zinc finger protein 238 (Zfp238)	ATCTTCATGTGCCCCTGT	GTGGATCTGCAGGATGTGG	60
β-actin (Actb)	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT	17
neural cell adhesion molecule 1 (NCAM1)	GGAGACCCCATTCCTTCC	TCTCTGGTCGAGTCCACGAT	80
secreted frizzled-related protein 1 (Sfrp1)	GGCTACAAGAAGATGGTGCTG	CTTCACCTCTGCCATGGTCT	67
DLX-2	CTCCAGCACGTACCACCA	TGCAGGTTGCTGTTGCTG	74
Gene Desert - To check for DNA contamination	GGAAAAGAGTTAGGAGGGAAGG	AAAGAAATTGCATTGTTCAGACAC	55
Gene Desert - To check for DNA contamination	TGGGGTACAGAGCTAAACAAAAAC	AGGTGTTTTTCAGGCATTCG	73

Table 2: Primer sequences and Roche Universal Probe Library probe numbers for qRT-PCR.

2.11 Statistical Analysis

Unless otherwise indicated, the symbols *, ** and *** used on figures indicate significant changes for the experimental condition indicated in comparison to the control, with p values of <0.05, <0.01 and <0.001 respectively.

2.11.1 Immunocytochemistry data

For BrdU and Ki67 counts were made of labelled cells, and expressed as a percentage of the total number of cells per field of view, given by counting the number of bis-benzamide labelled cells. For connexin 43 total cell counts were also performed, and the area of connexin 43 labelling was quantified using the thresholding function within Metamorph, and expressed as immunoreactivity per cell. For all other immunocytochemistry data thresholding was used for quantification of each label.

Statistical analyses were performed on all results where data had been pooled from at least 3 separate experiments. The values of n quoted are for the total number of fields of view analysed unless stated otherwise. Data was analysed using one-way ANOVA with Bonferroni's multiple comparison test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). However, for CXCR4 knockout studies, where wild type cultures were compared to knockout cultures, unpaired two-tailed t-tests were applied, again using GraphPad Prism version 4.00.

2.11.2 Axon elongation and branching data from GFP cultures

One-way ANOVA was used as described above to analyse both axon length, and also the number of branches per axon. However, to analyse counts of branched versus unbranched axons, the χ^2 test was used.

2.11.3 Live Imaging Data

A modified version of the method used by Pearson *et al* (Pearson et al., 2005) was applied to quantify changes in cell-cell coupling. Briefly, change in fluorescence of individual cells over time was normalized to initial fluorescence. Cell coupling was defined as 2 or more adjacent cells undergoing a spatially and temporally coordinated change in $[Ca^{2+}]_i$, of at least 10%. For statistical analysis one-way ANOVA was used as described above, *n* indicates the number of pairs of cells recorded, at least 5 explants were used for each condition. However, for acute treatments, an unpaired one tailed t-test was performed (also using GraphPad Prism version 4.00) based on the hypothesis that

following SDF-1 treatment there would be an increase in cell coupling. The same pairs of cells were analysed before and after acute exposure to increased SDF-1.

2.11.4 Scrape Loading Data

Where labelling had occurred, the area of Lucifer yellow dye labelling was expressed as unit area per unit length of scrape to give a coupling index. For each condition at least 3 explants were analysed. *n* refers to the total number of fields of view analysed. Again one-way ANOVA was used to test for statistical significance as described.

2.11.5 Microarray data processing and statistical analysis

Technical quality control was performed with dChip (V2005) using the default settings. Background correction, quantile normalization, and gene expression analysis were performed using RMA Express (Bolstad, 2003). Principal component analysis (PCA) and ANOVA were performed using Partek software (version 6.0, 2005, Partek Inc,) and maxdView software (http://bioinf.man.ac.uk/microarray/maxd/). Lists of significantly expressed genes were subjected to gene ontology analysis with GenMapp software (http://www.genmapp.org; (Doniger et al., 2003)). Parallel analysis was carried out using Genespring software. Genes identified as being of potential interest were subject to literature searches to uncover potential links with SDF-1 signalling using iHOP (http://www.ihop-net.org (Hoffmann and Valencia, 2004)).

3 Validation of experimental system

In order to provide a basis to study the potential role of SDF-1 signalling in the development of the cerebral cortex, several preliminary experiments were performed to both support our initial hypotheses, and also to validate the proposed experimental system.

3.1 Tissue specific concentrations of SDF-1 in the telencephalon

To estimate SDF-1 expression *in vivo,* tissue lysates were obtained from cerebral cortex, hippocampus and ganglionic eminences and assayed by ELISA. Quantitative measurements showed that cortical lysate SDF-1 concentration was 0.17nM, whereas in lysates of either hippocampus or the ganglionic eminences it was 0.19nM. While this reflects the SDF-1 concentration in gross anatomical areas of the embryonic telencephalon, there are likely to be localised cellular regions that display varying levels of expression within each of the areas (see chapter 4, figure 16).

3.2 Transfected HEK cells as a source of SDF-1

To provide a source of SDF-1 for treating primary telencephalic cultures, HEK cells were transfected with the pRK5 expression vector containing the SDF-1 gene. SDF-1 expression was demonstrated by both ELISA (figure 10a) and fluorescence immunocytochemistry (Figure 10b). Using ELISA, the average concentration of secreted SDF-1 in HEK cell medium was 4.83nM ($n=59$). In contrast the control medium contained less than 0.3nM ($n=28$). Hence, transfected HEK cell medium was considered a suitable source of SDF-1 for treatment of primary cell cultures.

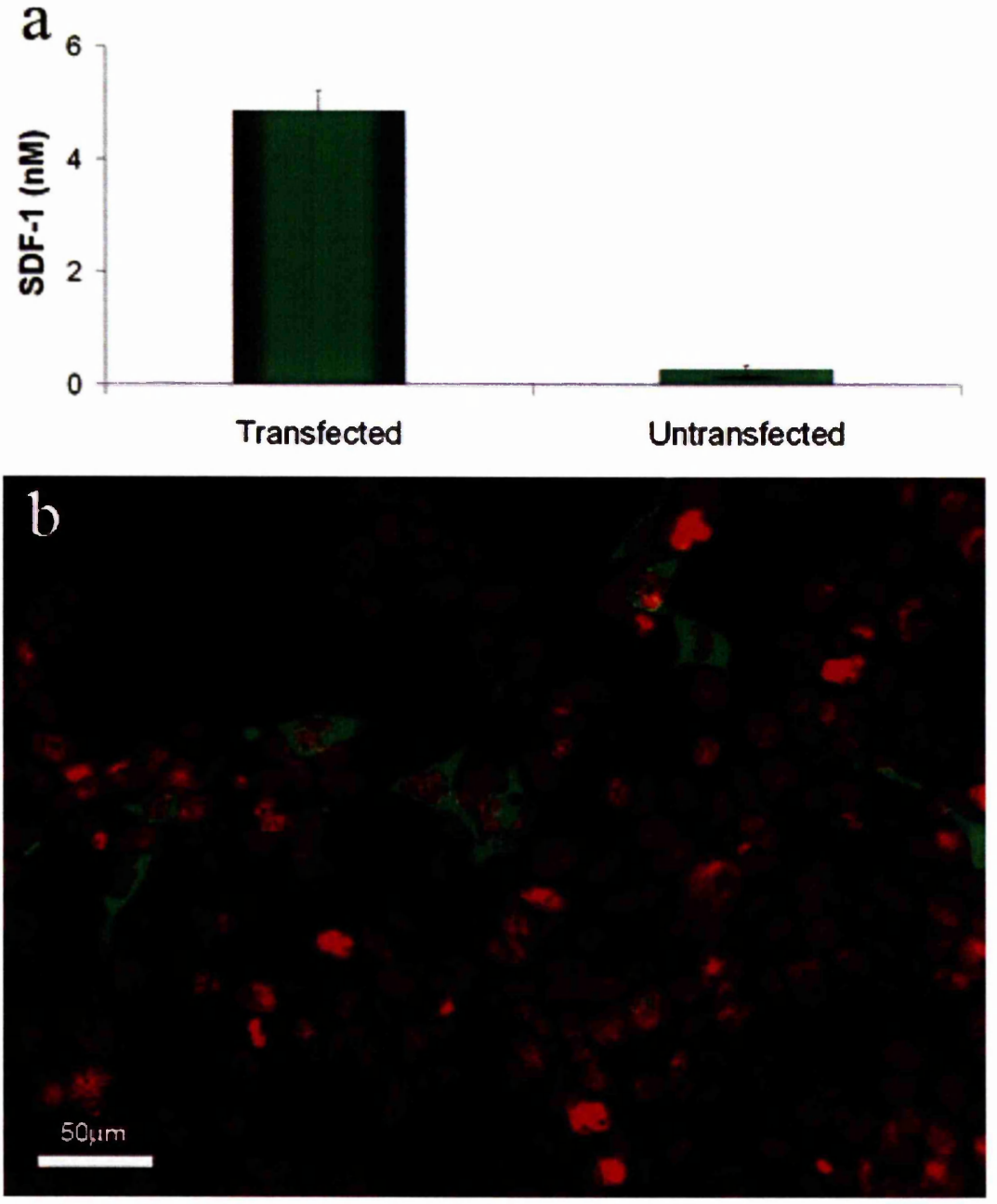

Figure 10: SDF-1 concentration in HEK cell conditioned media. **(a)** Illustrates that transfection of HEK cells with SDF-1 construct produces chemokine at a concentration more than 10-fold greater than control medium. **(b)** Immunostaining of transfected HEK cells shows cytoplasmic expression of SDF-1 (green); cells are counterstained with *bis*-benzamide, a nuclear stain (red). *Scale bar 50µm*

3.3 Characterisation of SDF-1 over expression model

To measure the level of chemokine in cortical cultures, medium was collected from E17 cultures 24 hr after each treatment (figure 11). In control cultures basal levels of SDF-

1 were approximately 0.15nM (*n=2* dissociated cultures), but after 3 days *in vitro* the levels increased to a maximum of approximately 0.4nM. However, treatment with CXCR4 antagonist (20 or 40μM AMD3100) appeared to prevent the basal SDF-1 levels from rising above 0.15nM. As expected, application of 2nM SDF-1 medium caused a cumulative increase in SDF-1 concentration over time to a maximum of approximately 0.8nM.

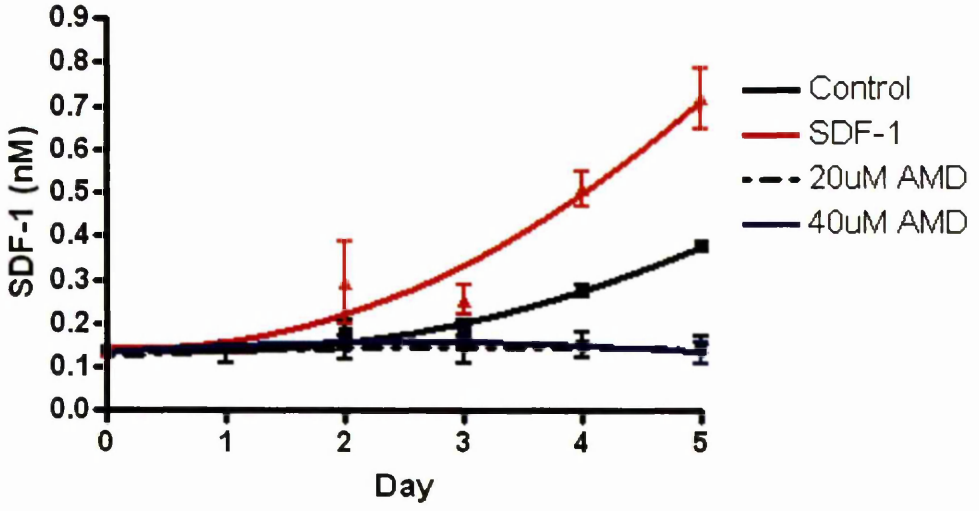

Figure 11: Illustration of SDF-1 levels in medium collected from E17 cortical cultures. Medium was collected daily prior to treatment. Hence day 0 measurements refer to chemokine levels prior to the application of any treatment.

To follow the kinetics of applied SDF-1 in cortical cultures, medium was collected at short intervals, up to 8 hours post treatment (*n=1* dissociated culture). Analysis showed that application of chemokine medium caused a sustained increase in the level of SDF-1, which remained approximately 10 times higher than basal levels up to 8 hours post treatment (figure 12). These results suggest that applied SDF-1 does remain in an active form at sufficient concentration to exert a regulatory effect on cortical cells. Indeed, after three or more treatments, the levels appeared to remain above those measured in control cultures (indicated by the measurements at time 0, made prior to treatment each day), mirroring the observations made in figure 11. Hence, our culture system appears to be an adequate model to study SDF-1 over expression.

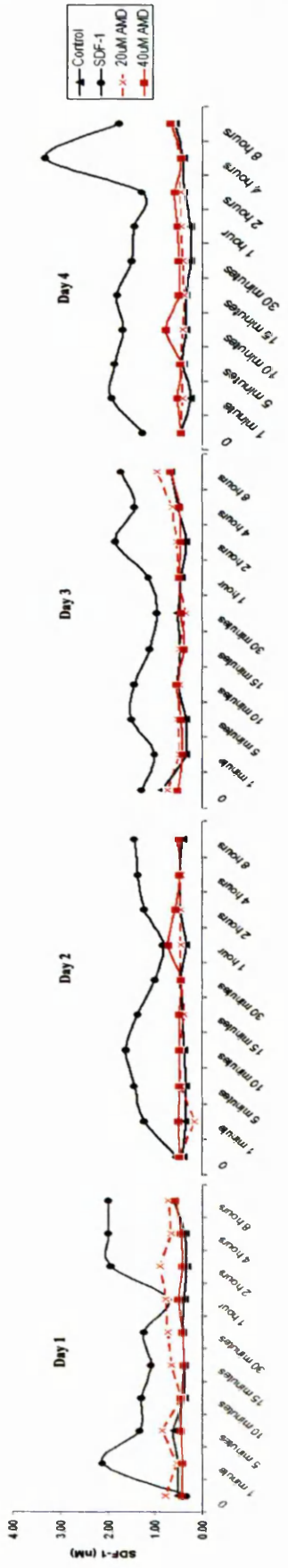

Figure 12: Graph showing SDF-1 concentration in medium collected from E17 cortical cultures at various timepoints after treatment. Readings made on 4 different days illustrate that SDF-1 levels are elevated after exposure to SDF-1 medium and persist between treatments. Treatment with SDF-1 medium elevated SDF-1 concentration by at least two-fold above control levels throughout treatment.

3.4 Cytotoxicity assay in control and treated cortical cultures

To measure the degree of cytotoxicity in chemokine and antagonist treated cultures, medium from exposed cells was assayed for LDH levels. Interestingly, analysis showed that the level of toxicity in treated cultures remained below that of control cultures (figure 13).

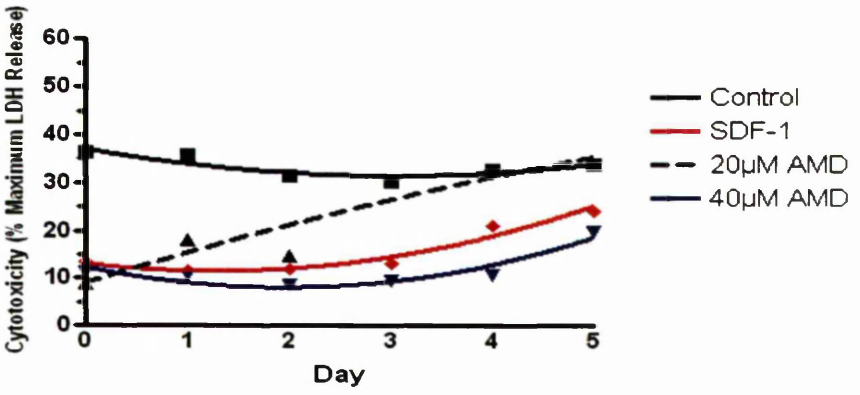

Figure 13: Graph illustrating the effects of treatment on cytotoxicity (as shown by LDH activity in collected media) in E17 cortical cultures ($n=3$).

3.5 Cell survival in control and treated cultures

To assess cell survival in control and treated cultures (figure 14), cells were processed for MTT colour assay. Absorbance measurements indicated that the viability of cells in control and treated cultures were comparable.

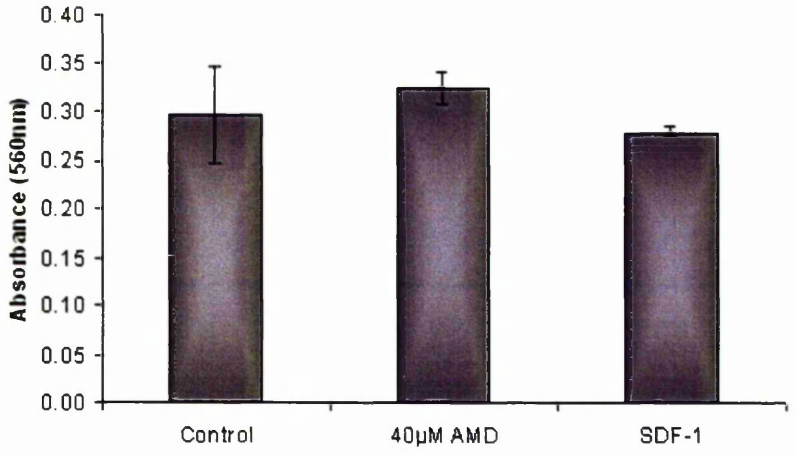

Figure 14: Graph illustrating the viability of E17 primary cortical cultures, as measured by MTT assay ($n=2$ dissociated cultures).

3.6 Comparison of control medium, with medium containing DMSO or neuronal supplement (N2)

To ensure that HEK conditioned medium did not induce any added effects on the physiology of neural cells, cultures grown in control HEK medium were compared to those grown in serum free N2 medium. Similarly, to ensure that the use of dimethyl sulfoxide (DMSO) to dissolve drugs had no adverse effects on neural cells, vehicle controls were carried out with 10µl/ml DMSO. As shown in figure 15, the neuronal output and the rate of proliferation of cortical cultures that were grown in control HEK medium or in medium containing DMSO was comparable to those maintained in N2 medium.

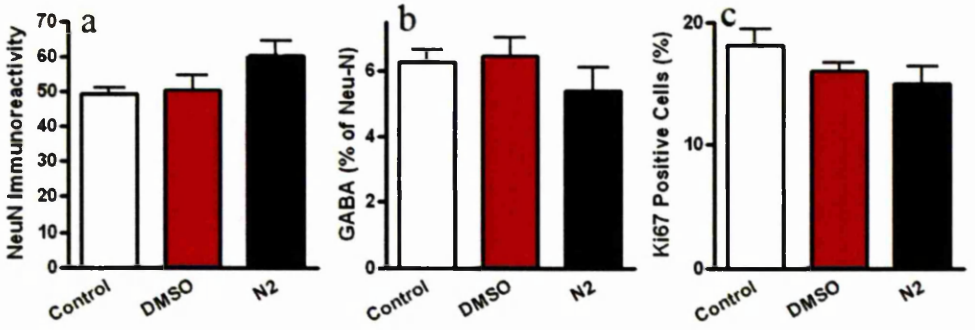

Figure 15: Graphs illustrating neuronal output, as indicated by Neu-N immunoreactivity (**a**), GABA immunoreactivity (**b**), and the percentage of proliferating cells, as indicated by Ki67 staining (**c**). All three graphs illustrate that the type of medium used had no effect on either proliferation or neuronal output (*n=3 dissociated cultures*).

3.7 Discussion

The data presented here supports the use of a primary cell culture system to investigate the regulatory role of SDF-1 signalling during forebrain development. To over express the chemokine *in vitro*, primary cultures were exposed to a chronic treatment of SDF-1 conditioned medium, collected from transfected HEK cells. Exposure of cortical cultures to chemokine medium did not result in elevated toxicity. Furthermore, treatment of primary cultures with SDF-1 medium led to a progressive increase in chemokine concentration that remained at higher levels between treatments. Although, the concentration of SDF-1 used in our experiments was 10 times higher than *in vivo* levels measured in the developing cortex, it compares favourably with other studies that have used the chemokine at an order of magnitude beyond the average concentration found across distinct anatomical regions such as the cortex or hippocampus (Lazarini *et al.*, 2000; Klein *et al.*, 2001; Bajetto *et al.*, 2001; Arakawa *et al.*, 2003; Stumm *et al.*, 2003; Peng *et*

al., 2004; Pujol *et al.*, 2005; Gong *et al.*, 2006), however, there are likely to be discreet well defined zones of higher expression within each region (see chapter 4, figure 16). Further, dose response tests for AMD3100 also illustrated that treatment with 40μM can elicit physiological responses in primary cultures without rendering significant cytotoxicity. Additional control experiments further confirmed that the vehicle used (DMSO) to dissolve many of the test compounds did not induce toxicity or alter the physiology of primary cells.

4 SDF-1 signalling regulates cell proliferation in the developing forebrain

4.1 Introduction

SDF-1 has been shown to regulate cell proliferation in the developing cerebellum (Klein *et al.*, 2001; Bajetto *et al.*, 2001; Bonavia *et al.*, 2003). Since it is expressed at a time when cell proliferation and migration are key events in the developing forebrain (Daniel *et al.*, 2005; Peng *et al.*, 2004), a regulatory role for chemokine signalling was hypothesised.

4.2 Cellular localisation of SDF-1 in the developing cerebral cortex

To establish the pattern of SDF-1 mRNA and protein expression in the developing cerebral cortex, sections from fixed E17 embryonic rat brains were examined. SDF-1 expression followed a lateral-to-medial gradient; strong expression of the protein was present in the lateral cortex (LC; figure 16a; arrows) while the medial cortex (MC) contained no or sparse staining (figure 16a). In the radial axis, the protein was expressed at high levels in the SP/upper intermediate zone (IZ) and extended to the lower IZ in a decreasing manner (figure 16c). Double-immuno labelling for SDF-1 with calbindin, a marker of SP and MZ cells, illustrated the presence of chemokine in SP zone (figure 16f). In contrast, co-staining with GABA, a marker of migrating interneurons, showed a non-overlapping spatial distribution for both SDF-1 and GABA+ cells, suggesting that the chemokine is not secreted by interneurons that course through the IZ (figure 16e). To further characterize the cellular localization of SDF-1, adjacent sections were stained for TAG1 to label the corticofugal axons in the IZ. Although, comparison of TAG1 staining with SDF-1 revealed a notable spatial similarity, the pattern of distribution varied as the chemokine was expressed in a decreasing intensity through the IZ in contrast to the homogeneous staining of axons for TAG1 (figure 16d). Interestingly, detailed examination of sections showed localized staining of SDF-1 in rounded cells that were located at the ventricular surface (figure 16g, h; arrows). To identify the cells that generate SDF-1 protein, *in situ* hybridization was carried out in rat E17 brain sections. These experiments showed the expression of SDF-1 mRNA in SP and CP cells (figure 16b; arrows and insert)

with a weak presence in the VZ (figure 16b). Further, it was noted that SDF-1 transcript preceded protein expression. Thus, while SDF-1 protein was barely detectable in the MC at E17 (figure 16a), the gene transcript levels were robust in the corresponding region (figure 16b). Collectively, these observations demonstrate that the localization of SDF-1 mRNA and protein closely follow the spatio-temporal maturation of the dorsal telencephalon.

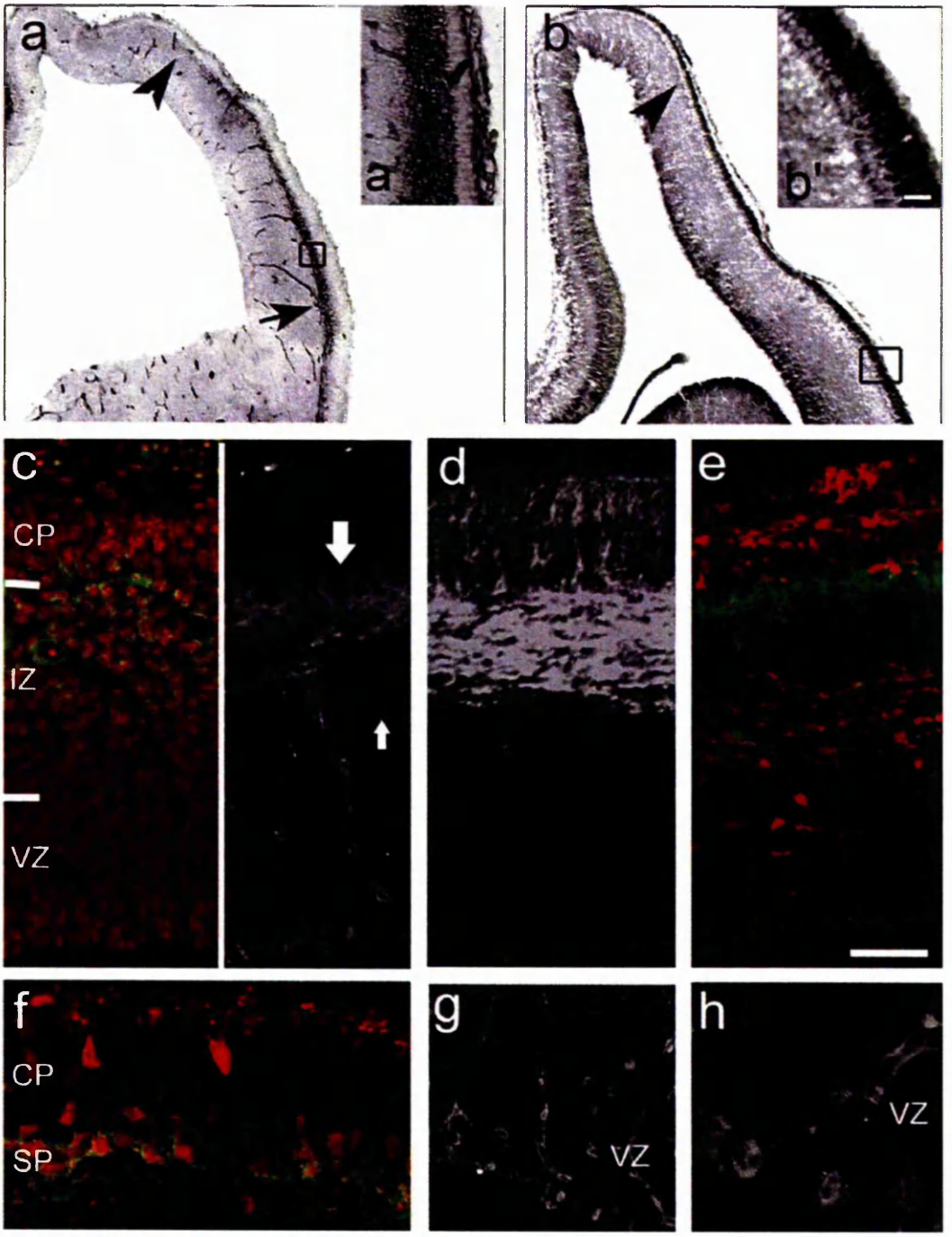

Figure 16: Cellular localization of SDF-1 in rat E17 cerebral cortex. (a) Pattern of expression of SDF-1 protein in the cortex extending from lateral to medial aspect of cortical anlage. The arrow points to high protein expression in the LC. The arrowhead in the MC indicates low levels of SDF-1 expression. (a') Higher magnification of the area denoted with a square in (a) showing the localization of SDF-1 protein. (b) in situ hybridization of a brain section illustrating the expression of SDF-1α mRNA; the arrowhead points to transcript localization in the MC. (b') Higher magnification of the square area in (b) illustrates SDF-1 mRNA in SP/CP region. (c) Spatial distribution of SDF-1 protein (green) through the radial axis of the developing cortex. The image is presented as a single confocal optical section (2μm) for better visualization of protein

staining. Red corresponds to propidium iodide counter stain. Adjacent grey scale image is a projection of a confocal stack collected through 20μm. Note the protein localized at high levels in the SP/upper IZ (large arrow) extends to the lower IZ in a decreasing gradient (small arrow). (d) Brain section illustrating TAG1 immunoreactivity in the developing cortex. (e) Brain section illustrating the stream of tangentially oriented GABA+ neurons (red) and their spatial relationship to SDF-1 (green) expression in the cortex. (f) Localization of SDF-1 protein (green) around the cell bodies of calbindin positive (red) SP neurons. The image is presented as a single confocal optical section (2μm) for better visualization of protein staining. (g-h) Localization of SDF-1 immunolabelled cells at the ventricular surface of developing cortex (arrows). Scale bars: a, b, 400 μm; (a'), (b') 75 μm, c-e, g 100 μm, f, g 25 μm.

4.3 *Altered SDF-1 signalling triggers ectopia in medial cortex of brain slices*

To determine whether SDF-1 signalling exerts a regulatory role in cell proliferation and radial migration during CP formation, we analyzed rat E17 brain slices that were pulsed with BrdU and maintained for 2 days in either SDF medium or the CXCR4 antagonist AMD3100. Sections (*n=10 slices*) stained for BrdU showed that, in control conditions, labelled cells were positioned well below the CP (figure 17a; arrow heads). By contrast, exposure to SDF-1 medium had a profound effect in the MC as BrdU positive cells were ectopically positioned across the entire thickness of the CP (n=16 slices) (figure 17b arrows; f). Quantitative analysis of the MC showed that 34% of BrdU immunoreactivity was distributed in the upper IZ and CP (bin 3 in figure 17f) compared to control sections that contained 16.5% immunoreactivity in the corresponding region (figure 17f; $p<0.001$). A similar trend but with lesser intensity was noted in the MC of slices (*n=20 slices*) that were exposed to AMD3100 (figure 17c arrows; f). However, exposure to SDF-1 medium or to the antagonists did not significantly alter radial migration in the LC (figure 17g). Subsequent staining of control and chemokine treated slices with Ki67, a marker of proliferative cells, illustrated that the ectopic cells in treated slices were non-mitotic in nature (figure 17d, e).

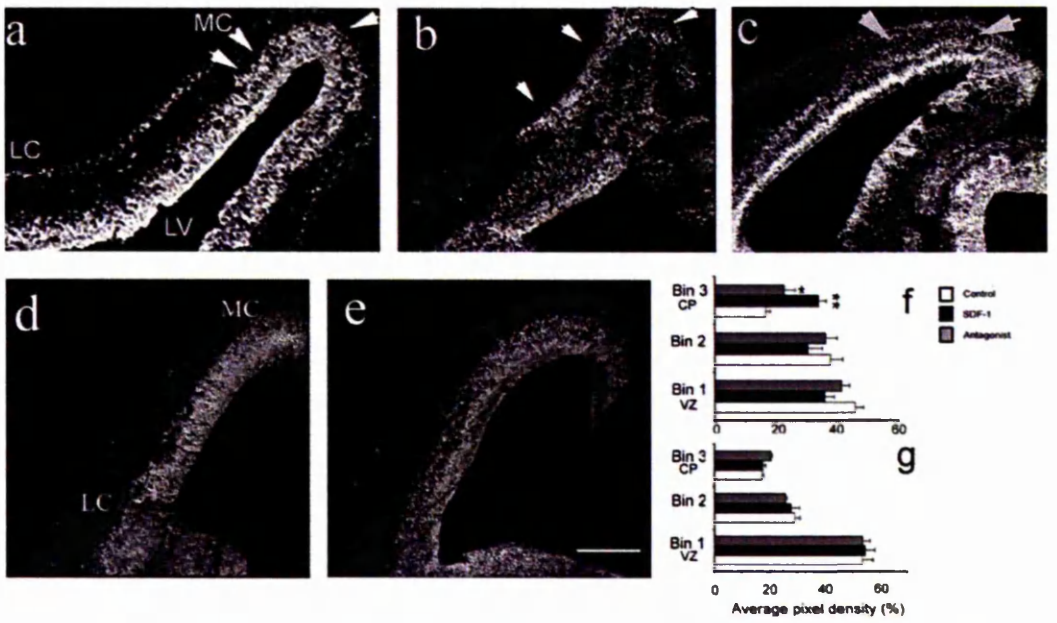

Figure 17: Altered SDF-1 signalling triggers ectopia in medial cortex of brain slices. (a-c) BrdU immunohistochemistry in slices that were exposed to control (a), SDF-1 (b) or AMD3100 (c) medium. Arrows in (b) and (c) point to ectopically placed BrdU+ cells in MC. (d-e) Ki67 immunohistochemistry in slices that received control (d) or SDF-1 medium (e). (f-g) Quantitative analysis of BrdU+ cell distribution in MC (f) and LC (g) of control and treated brain slices. Scale bar: 150 μm.

4.4 SDF-1 induces proliferation in primary forebrain cultures

To determine whether SDF-1 signalling exerts a regulatory role in cell proliferation and radial migration a primary cell culture system was used. Cultures derived from rat E17 or E16 cortex or ganglionic eminences were exposed to increased levels of chemokine, or treated with the CXCR4 antagonist AMD3100. Several different techniques were then used to quantify the effects of SDF-1 signalling on proliferation. Experiments using cultures derived from wild type and $CXCR4^{-/-}$ E15 mouse cortices were also carried out.

4.4.1 Chronic exposure to SDF-1 increases progenitor population in primary cultures: Ki67 immunohistochemistry

Ki67 is a nuclear marker expressed exclusively by proliferating cells. Using immunocytochemistry for Ki67 (figure 18), an increase in labelling was noted in primary forebrain cultures that were exposed to a chronic treatment with SDF-1 medium. In E17 cortical cultures, 11% (+/- S.E.M.) of cells were Ki67+, in control conditions, compared to a significant increase to 15% (+/- S.E.M.) in SDF-1 treated cultures. Notably, the proliferative activity was more significant in cultures that were exposed to higher

concentrations of SDF-1 (4-6nM: *p<0.01, n=28)* compared with lower levels of chemokine (0.5-3nM: *p<0.05, n*=20). Conversely, treatment with either 20μM or 40μM AMD significantly reduced the number of Ki67+ cells (*p<0.001, n=21* and *p<0.001, n=52* respectively). Interestingly, analysis of E16 cortical cultures that were exposed to chemokine medium showed a remarkable increase in the number of Ki67+ cells compared to controls (Controls: 7.1% +/-S.E.M., SDF-1: 15.7% +/- S.E.M., *n=47, p<0.001*), whereas AMD3100 had no significant effect. Similarly, primary cultures derived from the ganglionic eminences (both E16 and E17) showed a significant increase in Ki67 labelling after exposure to increased levels of chemokine (E16: Control; 13.7% +/- S.E.M., 1nM SDF-1; 25.5% +/- S.E.M., *n=35, p<0.001.* E17: Control; 13.4% +/- S.E.M., 1-3nM SDF-1: 20.19%, *n=21, p<0.001*), but no significant change following treatment with antagonist.

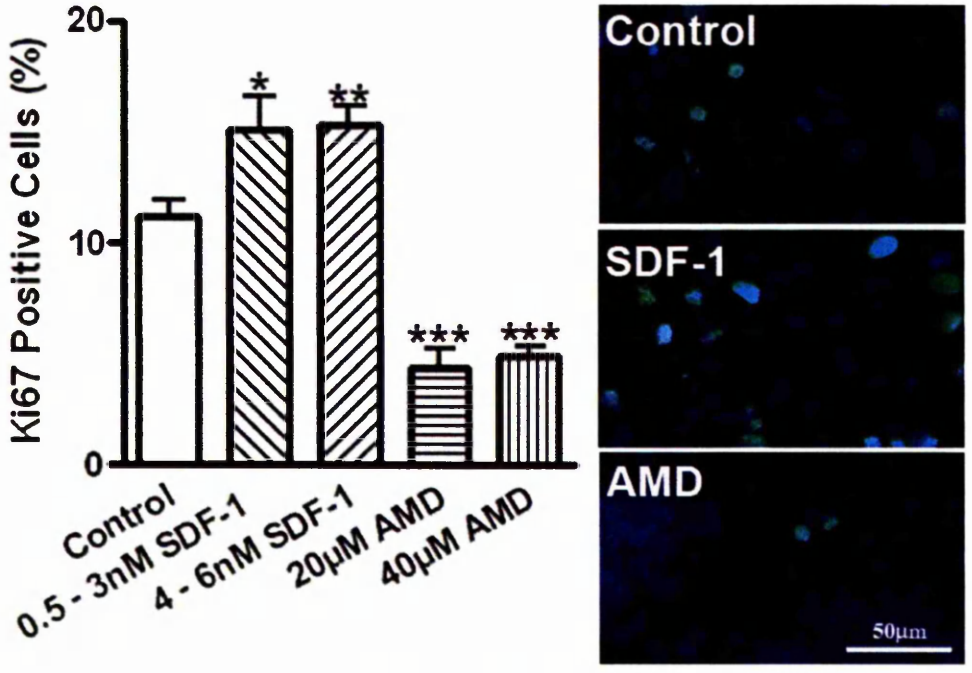

Figure 18: Proliferation in E17 primary cortical cultures. Images show Ki67+ cells appearing green, against blue *bis*-benzamide counter stain). Statistical significance between control and treatment is indicated as; * if p<0.05, ** if p<0.01, *** if p<0.001 (one-way ANOVA).

4.4.2 BrdU labelling index to follow cell proliferation in primary cultures

Since chronic exposure to chemokine medium lead to an increase in progenitor population as evidenced by Ki67 immunoreactivity, cortical cultures were BrdU pulsed to assess the rate of proliferation at different time points of the treatment. Thus, rat E17 cortical cultures were pulsed with BrdU for 1 hr on day 0 (prior to 1st treatment), day 2 (prior to 3rd treatment), day 4 (prior to 5th treatment) and day 5 (24 hrs after 5th treatment) and fixed immediately (see figure 19).

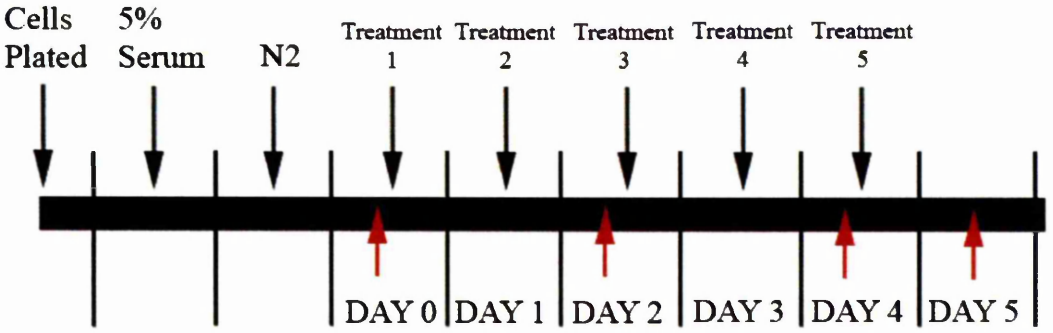

Figure 19: Diagram illustrating the experimental scheme for BrdU labelling in rat E17 primary cortical cultures. Black arrows indicate media changes. Red arrows show when cover slips were BrdU pulsed and fixed for immunocytochemistry.

Using the percentage of BrdU+ cells as an index of proliferation, this method clearly demonstrated that treatment with SDF-1 significantly induced proliferation at all the time points analysed (figure 20). Analysis showed that after 48 hours in SDF-1 medium, the rate of proliferation had risen by over 30% (7.37% +/- S.E.M. in control compared to 9.93% +/- S.E.M. in SDF-1 treated cultures), and this is maintained at both days 4 and 5 ($p<0.001$, $n=30$). Remarkably, by day 4 the rate of proliferation in the presence of chemokine had increased over 100% compared to control conditions. By contrast, after 48 hours in the presence of antagonist a decrease in the rate of proliferation was noted, with the effect more pronounced with 40μM AMD3100 (4.93% +/- S.E.M., $p<0.001$, $n=30$). However, by days 4 and 5, the number of BrdU+ cells in control cultures were significantly fewer than in antagonist treated cultures ($p<0.001$ and $p<0.05$, $n=30$ and $n=28$, for 20μM and 40μM AMD3100 respectively), suggesting that a negative feedback mechanism initiated by contact inhibition may have down regulated proliferation.

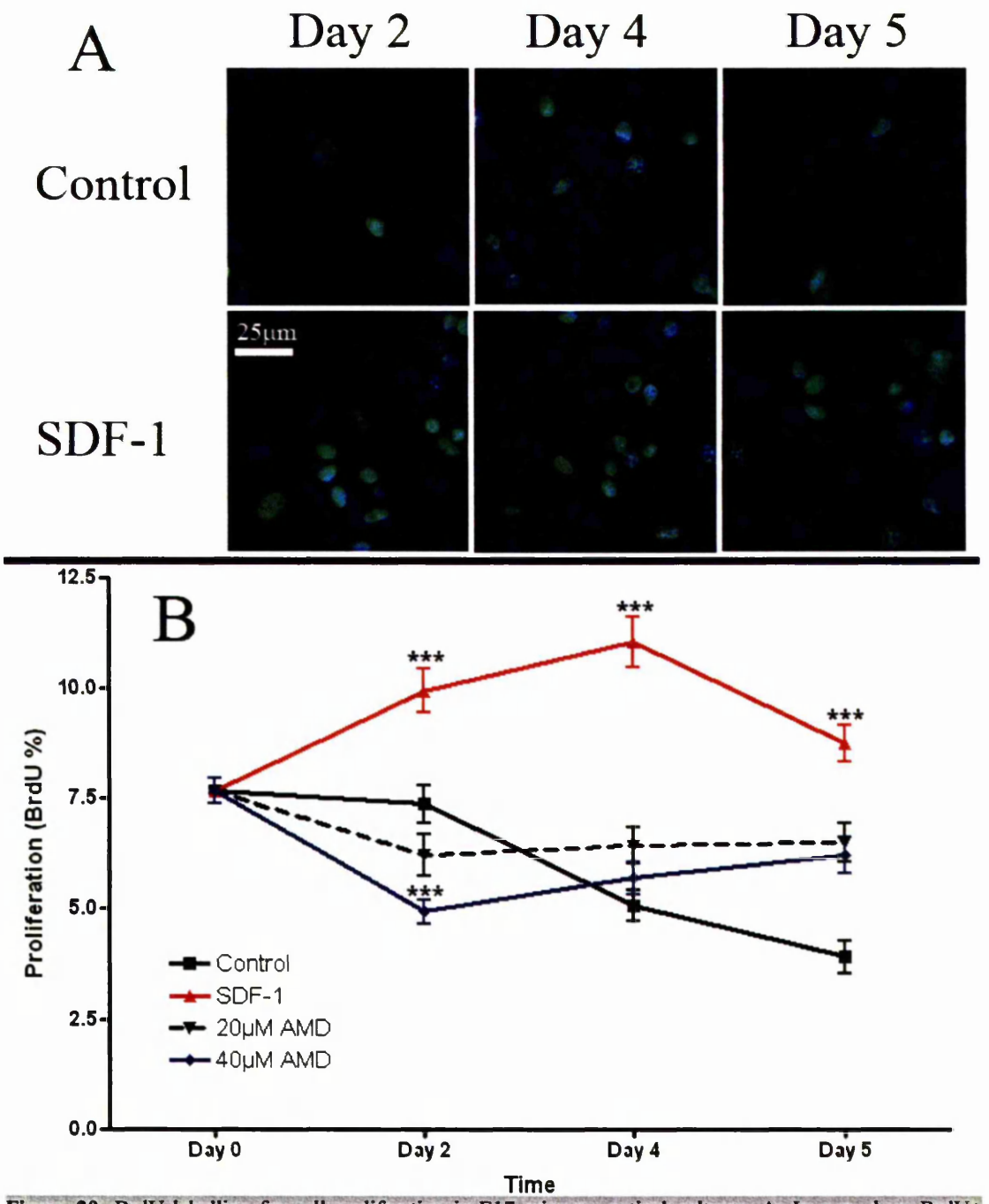

Figure 20: BrdU labelling for cell proliferation in E17 primary cortical cultures. **A:** Images show BrdU+ cells (green) against *bis*-benzamide counter stain (blue), from control or SDF-1 treated E17 cortical cultures after 2, 4 and 5 days' treatment. **B:** Graph illustrating changes in proliferation over time (mean percentage of BrdU labelled cells, +/- S.E.M.) in cultures that were treated with control media, 3nM SDF-1, or AMD3100. Statistical significance between control and treatment at each time point is indicated by *** if $p<0.001$ (one-way ANOVA).

4.4.3 Modified method of lineage analysis: SDF-1 increases cluster size

To further demonstrate the regulatory role of SDF-1 signalling in cortical proliferation, a cell cluster analysis was performed. In brief, rat E17 cortical tissue was BrdU pulsed for one hour prior to dissociation, and the resulting cell suspension was mixed with non-BrdU labelled cells. This resulted in a culture containing individual clusters of BrdU+ cells amidst a larger population of unlabelled cells. Figure 21 shows an example of one of these cultures after 1 day in vitro (DIV). Since BrdU is passed on to each daughter cell during mitosis, the number of progeny in each cluster provides a measure of the effect of treatment on proliferation.

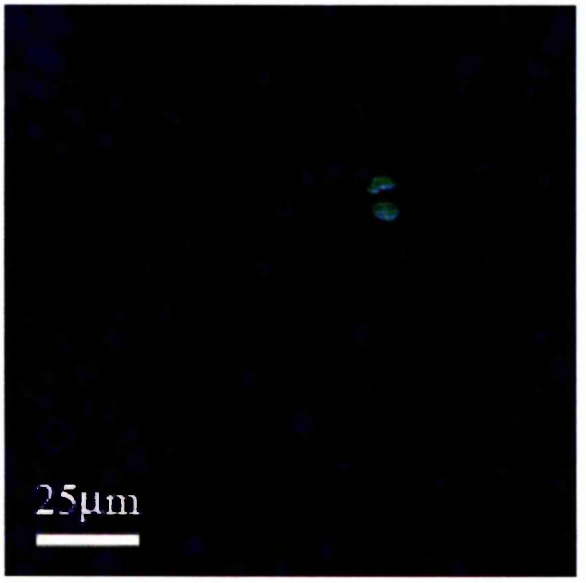

Figure 21: An example of a culture used for cluster analysis after 1 DIV. This shows a BrdU labelled cluster consisting of 2 cells (BrdU – green, *bis*-benzamide – blue).

Cultures treated with either SDF-1 or AMD3100 were analysed after 2 days and 5 days in treatment (figure 22). After 1 DIV each cluster of labelled cells contained on average 2 cells, this increased to 3 cells by 2 DIV (day 0, prior to treatment 1; see figure 22). Analysis of control cultures showed that the average number of cells per cluster remained no more than 4 cells per cluster throughout the course of the experiment (1 D.I.V.:2.3 cells; Day 0: 2.9 cells; Day 2: 4.0 cells; Day 5: 2.0, +/- S.E.M.). By contrast, exposure to SDF-1 medium caused a significant increase in cluster size by day 2 (6.5 cells, +/- S.E.M., $p<0.05$, $n=27$) and day 5 (6.2 cells, +/- S.E.M., $p<0.001$, $n=18$) compared to controls. Conversely, treatment with 20µM or 40µM AMD3100 caused a decrease in cluster size.

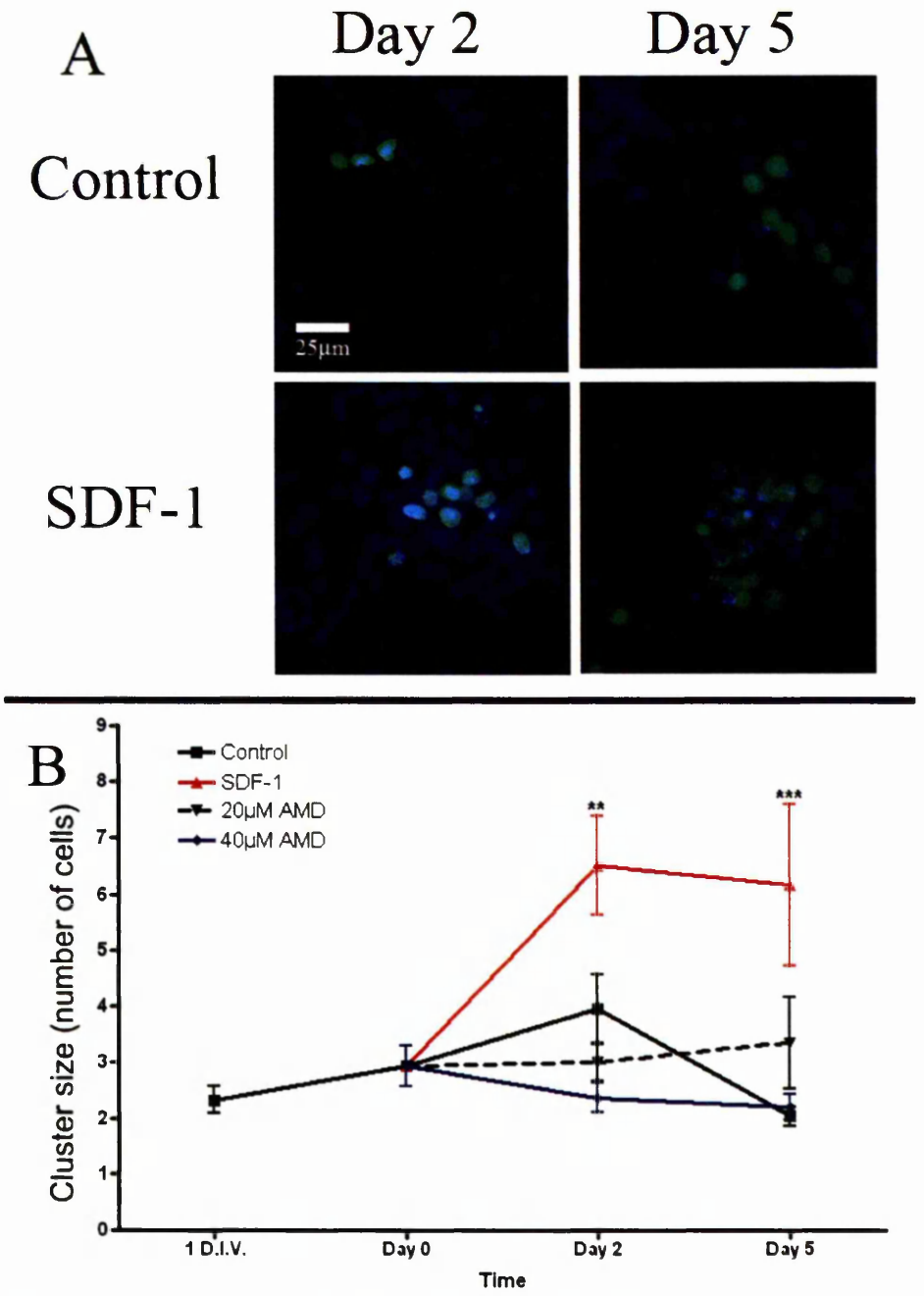

Figure 22: Lineage analysis shows increased cluster size due to increased SDF-1 **A:** Images illustrate larger BrdU+ clusters in SDF-1 treated cultures compared to controls at days 2 and 5 of treatment (BrdU – **green**, *bis*-benzamide – **blue**). **B:** Graph illustrating change in cluster size (number of BrdU labelled cells, +/- S.E.M) over time. Statistical significance between control and treatment at each time point is indicated as ** if $p<0.01$ and *** if $p<0.001$ (one-way ANOVA).

4.4.4 SDF-1 decreases cell cycle exit

To ascertain the proportion of cells that exited the cell cycle in the presence of chemokine, rat E17 primary cortical cultures pulsed with BrdU prior to treatment were exposed to SDF-1 medium for 5 days. Analysis of cultures for cells that contained BrdU but were negative for Ki67 (BrdU+/Ki67-) indicated the proportion of BrdU labelled cells that exited the cell cycle either to become post mitotic or quiescent. Accordingly, in control cultures, 91% of BrdU+ cells had exited the cell cycle, in comparison to 80% and 81% following treatment with 3nM and 6nM SDF-1 medium, respectively (3nM: $n=40$, $p<0.001$. 6nM: $n=26$, $p<0.001$, figure 23).

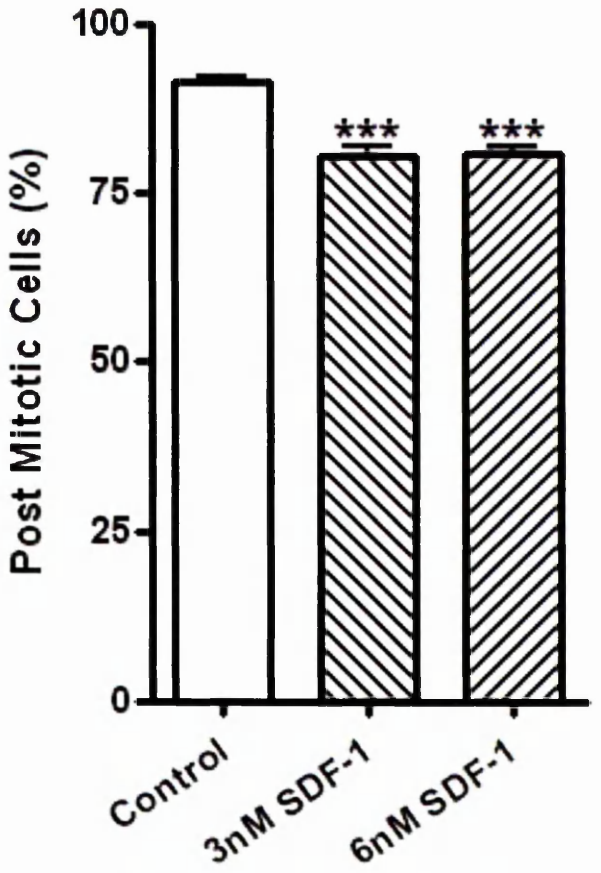

Figure 23: Graph to show cell cycle exit in rat E17 cortical cultures (+/- S.E.M.). Statistical significance between control and treatment is indicated by *** if p<0.001 (one-way ANOVA).

4.4.5 Proliferation is reduced in CXCR4-/- mutant cortical cultures

In agreement with the data obtained from rat E17 cortical cultures, examination of mouse E15 primary cortical cultures derived from wild type and $CXCR4^{-/-}$ embryos showed a significant decrease in proliferation as evidenced by Ki67 immunocytochemistry

(*p<0.001, n=47 fields of view from 7 mutants*; figure 24). Further, estimation of mitotic profiles also revealed a significant decrease in mitotic activity by 50% in $CXCR4^{-/-}$ cultures compared to wild type controls (*p<0.001, n=57 fields of view from 8 mutants*; figure 25). However, analysis of mouse E15 primary GE cultures from wild type and $CXCR4^{-/-}$ embryos showed no differences in Ki67 labelling (wild type: 8.56% +/- S.E.M. of cells were Ki67 positive, $CXCR4^{-/-}$: 8.64% +/- S.E.M. of cells were Ki67 positive, *n=50 fields of view from 5 mutants*).

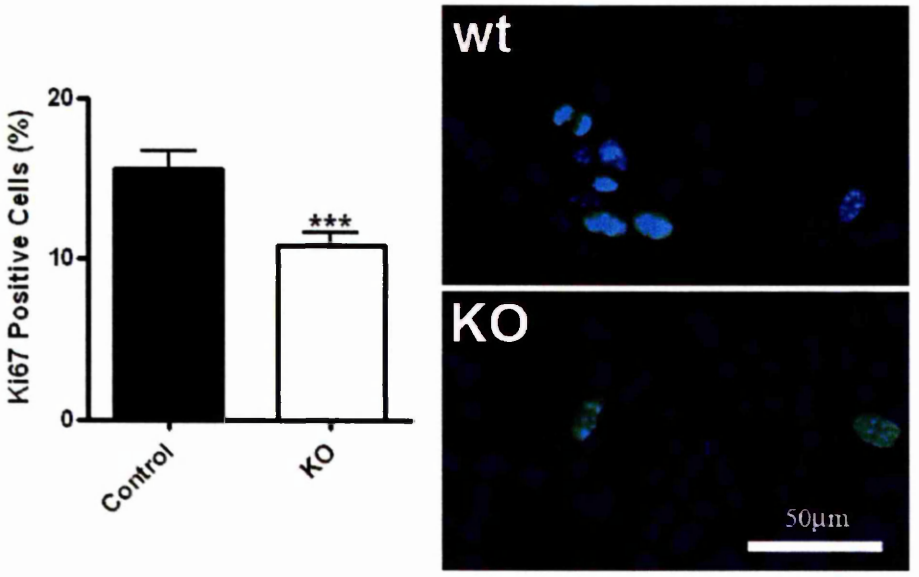

Figure 24: Proliferation in mouse E15 primary cortical cultures. Images show Ki67 staining (green, against blue *bis*-benzamide counter stain) in wild type (control) and $CXCR4^{-/-}$ (KO) cultures. Graph shows the mean percentage of Ki67 positive cells +/- S.E.M. Statistical significance between control and KO is indicated by *** if p<0.001 (Students t-test).

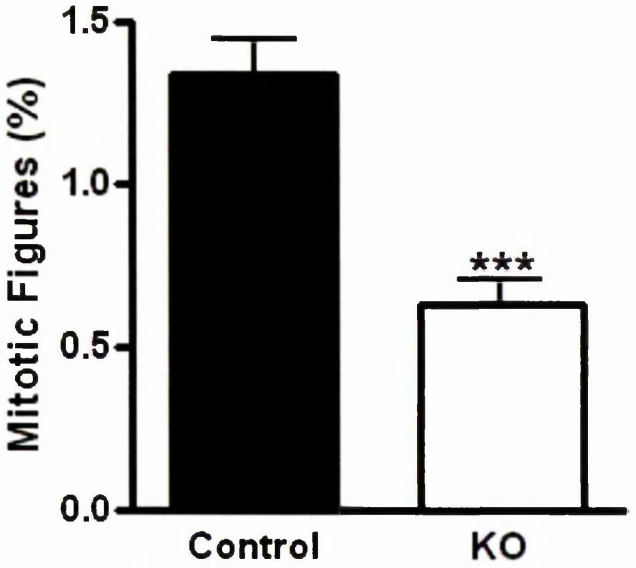

Figure 25: Frequency of mitotic figures in mouse E15 primary cortical cultures. Graph shows the mean percentage of mitotic profiles +/- S.E.M. Statistical significance between control and KO is indicated by *** if $p<0.001$ (Students t-test).

4.5 SDF-1 regulates intercellular coupling: a possible regulatory mechanism for cortical cell proliferation

It is evident from the above data that SDF-1, through its downstream signalling functions as a positive regulator for cell proliferation in primary neuronal cultures. Earlier reports have shown that developing cortical cells express gap junction proteins (Nadarajah *et al.*, 1997; Nadarajah *et al.*, 1998) that mediate the passage of calcium transients, known to regulate cell proliferation (Weissman *et al.*, 2004; Montoro and Yuste, 2004). In this context, SDF-1 has been shown to elevate Ca2+ transients in isolated cortical and cerebellar progenitors (Klein *et al.*, 2001; Peng *et al.*, 2004).

To elucidate the mechanisms that underlie SDF-1 signalling, Affymetrix microarrays were employed to investigate changes that may have occurred in gene expression following SDF-1 treatment in rat E17 cortical cultures (see chapter 6). Interestingly, array analysis revealed that the gene encoding the gap junction protein connexin 43 (*gja1*) was up-regulated; this was subsequently validated using quantitative real time PCR to analyse changes in gene expression following treatment with SDF-1 (see chapter 6).

Based on the premise of the gene array data, it was postulated that SDF-1 mediates cell proliferation, at least in part, through its downstream regulation of connexin 43 (Cx43), which in turn would provide a conduit for Ca2+ to effect cell proliferation. Given

that Cx43 is expressed in radial glial cells (Nadarajah *et al.*, 1997), the neuronal progenitors in the developing cortex (Noctor *et al.*, 2001; Anthony *et al.*, 2004), it is likely that SDF-1 signalling exerts a direct influence on cortical cell proliferation.

To test this hypothesis, a multi-experimental approach was adopted. First changes in Cx43 protein expression were measured in primary cortical cultures exposed to SDF-1 treatment. Second, cell proliferation was assayed using Ki67 immunocytochemistry in cortical cultures that were exposed to SDF-1 medium together with carbenoxalone, a widely used gap junction blocker. Third, scrape-load techniques were used to assess the intercellular dye-transfer in cortical explants that were maintained in SDF-1 medium. Finally, calcium imaging techniques were utilised to measure Ca2+ transients in cortical explants that were maintained in SDF-1 medium.

4.5.1 SDF-1 increases the expression of Connexin 43 protein in cortical cultures

To measure Cx 43 protein in rat E17 primary cortical cultures, quantitative immunocytochemistry was used. Figure 26 illustrates the typical punctate staining of Cx 43 on cell surface; the image shows strong expression of the protein (green) appearing as clusters around the cell membrane. Analysis showed an increase in expression following treatment with SDF-1. However, cortical cultures that were exposed to AMD3100 showed a significant decrease in immunoreactivity when compared to the levels measured following SDF-1 treatment (Figure 26; $p<0.05$, $n=40$).

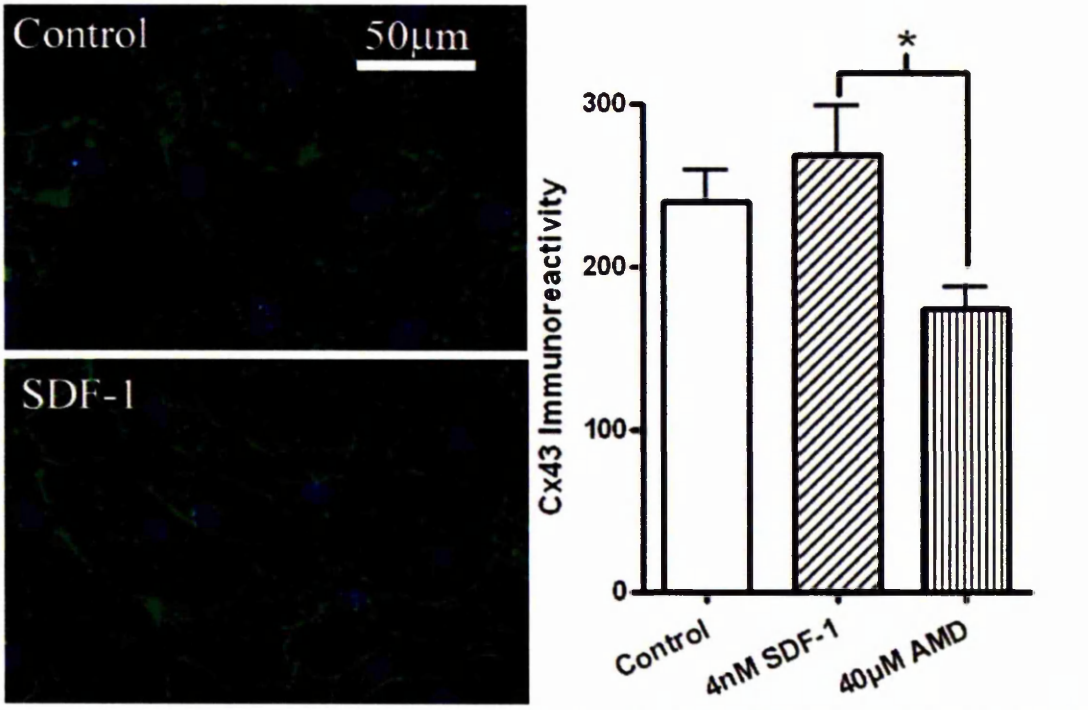

Figure 26: Cx43 immunocytochemistry (Cx43 (green) and *bis*-benzamide counter stain (blue)) in rat E17 primary cortical cultures. Graph illustrating Cx43 immunoreactivity (Area of Cx43 staining as a fraction of area of DAPI staining) in control and treated cultures (mean +/- *S.E.M.*). * indicates $p<0.05$. Note the more punctate membrane staining present in SDF-1 treated cultures, possibly indicative of the presence of more functional gap junction channels.

4.5.2 Treatment with carbenoxalone reduces SDF-1 induced proliferation in cortical cultures

To determine whether SDF-1 induced proliferation in cultures is mediated through gap junction channels, rat E17 cortical cultures were treated with 10µM carbenoxalone (figure 27). Subsequent analysis of control cultures revealed a significant reduction in the number of Ki67+ cells when treated with carbenoxalone ($p<0.001$, $n=35$). This corroborates the earlier data that gap junction coupling is required for regulating cell proliferation in the developing cerebral cortex (Weissman *et al.*, 2004). Interestingly, treatment of cortical cultures with SDF-1 showed an increase in Ki67 immunoreactivity, but cultures that were exposed to chemokine together with carbenoxalone showed a significant decrease in proliferation ($p<0.001$, $n=22$). This clearly illustrates that SDF-1 induced proliferation is regulated through gap junction channel communication. In agreement with this notion, further reduction in proliferation was noted in cultures that were grown together with AMD3100 and carbenoxalone.

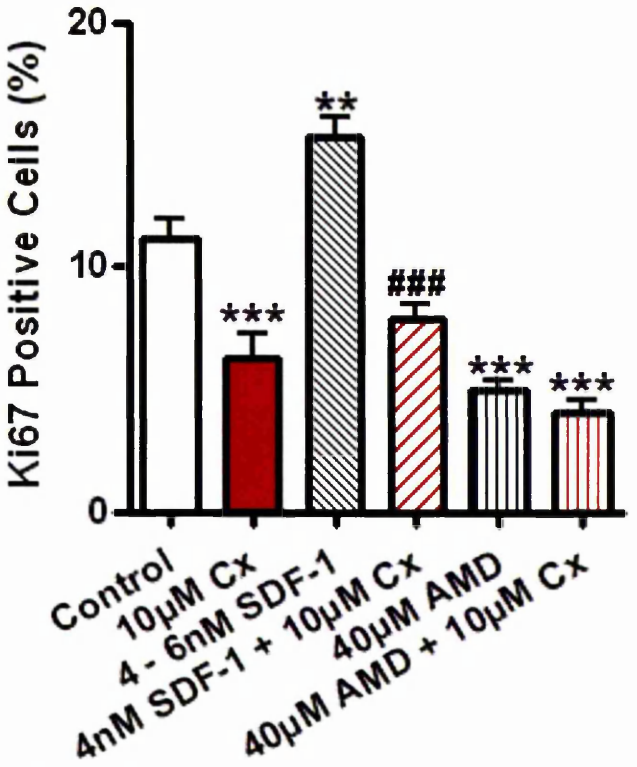

Figure 27: Graph illustrating the effect of carbenoxalone (Cx) on SDF-1 induced proliferation. Statistical significance is indicated between control and treatments (10μM Cx, SDF-1, AMD, AMD+Cx as; ** if $p<.001$, *** if $p<0.001$ (one-way ANOVA with Bonferroni's test). ### indicates statistical significance between SDF-1 treatment and SDF-1+carbenoxalone when $p<0.001$.

4.5.3 SDF-1 increases intercellular coupling as evidenced by Lucifer yellow dye-transfer

To determine whether exposure to SDF-1 treatment would alter intercellular coupling, a modified method of scrape-loading technique was adopted. Briefly, cortical explants that were whole mounted with ventricular zone facing up were maintained in control, SDF-1 medium or AMD3100 for 1-2 days. Explants were then scrape-loaded with Lucifer yellow and the extent of dye transfer was quantified to provide a coupling index (figure 28).

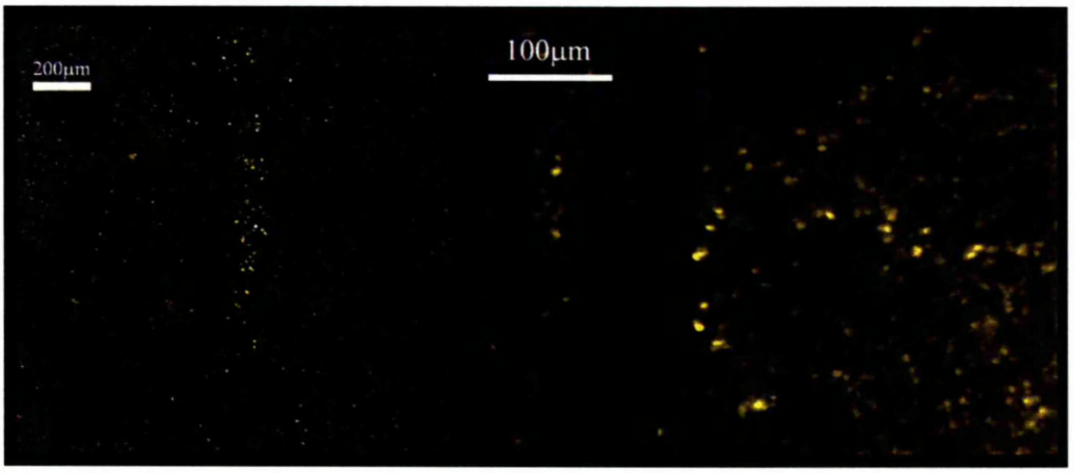

Figure 28: Representative images to demonstrate Ki67 immunocytochemistry (blue) and Lucifer yellow (yellow) dye transfer in cortical explants. Ki67 staining was performed to confirm that the explants were in the correct orientation (ventricular zone upper-most); the cortical plate should be devoid of Ki67 staining as it does not contain proliferating cells, hence the presence of Ki67 positive cells confirms that explants are in the correct orientation.

Analysis showed that cortical explants that were maintained in SDF-1 medium showed a 2-fold increase in dye-transfer compared to controls as measured by Lucifer yellow coupling index (figure 29; $p<0.00$, $n=15$). Although treatment with either AMD or carbenoxalone reduced dye-transfer, the effects were not measurably significant.

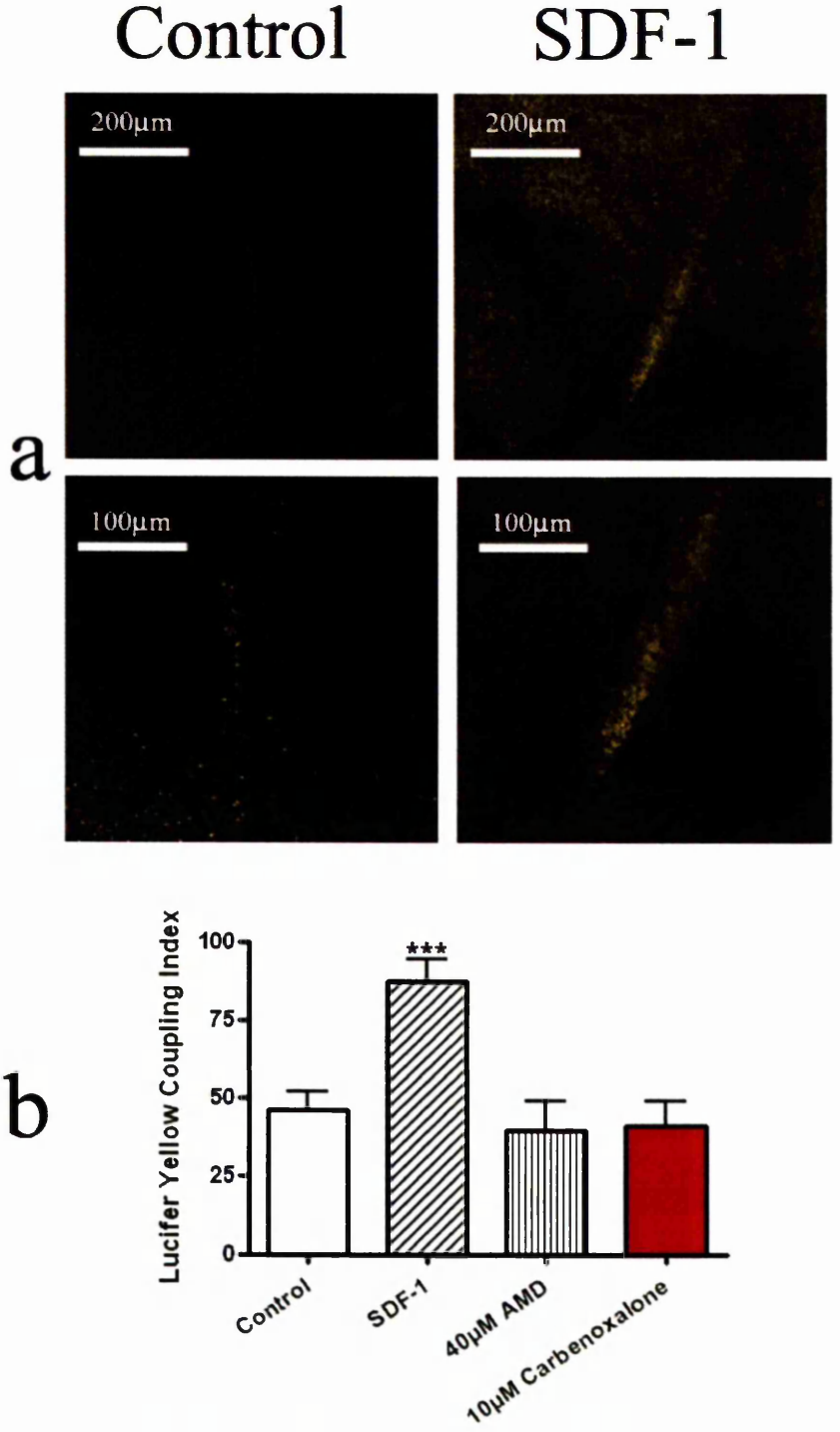

Figure 29: Lucifer yellow coupling index. **a**: Confocal images of Lucifer yellow dye transfer in rat E17 cortical explants following exposure to either control or SDF-1 medium. Images from one Control and one SDF-1 explant are shown at 2 magnifications. **b**: Graph illustrating the coupling index (+/- S.E.M.). *** indicated statistical significance between control and SDF-1 treatment ($p<0.001$, one-way ANOVA).

4.5.4 SDF-1 facilitates synchronised calcium activity between VZ cells in cortical explants

To determine whether exposure to SDF-1 would alter calcium activity, cortical explants that were exposed to either acute (15 min; see supplemental movies: Control.avi and Acute SDF-1.avi) or chronic (24 hrs; see supplemental movies: Control.avi, Chronic SDF-1 1.avi and Chronic SDF-1.avi) treatment of chemokine were loaded with Oregon Green BAPTA and analysed using real time imaging. Images were collected every second and changes in the average fluorescent intensity of selected cells were recorded for 3-5 min. Data was then converted to a 'cell coupling score', given by the number of coupling events per pair of cells analysed. A coupling event was defined as a 10% or greater fluctuation in fluorescent intensity between pairs of neighbouring cells. Analysis showed that exposure of cortical explants either to acute or chronic SDF-1 significantly increased the frequency of synchronised Ca^{2+} transients between adjacent VZ cells, as measured by cell coupling score (figure 30; acute treatment: *$p<0.05$, $n=19$ pairs of cells*, one-way ANOVA; chronic treatment: *$p<0.05$, $n=33$ pairs of cells*, unpaired *t-test).* However, chronic exposure to carbenoxalone appeared to have no effect on cell coupling, possibly due to the low coupling score measured in control cultures, so that to detect a further decrease would require a more sensitive method of analysis.

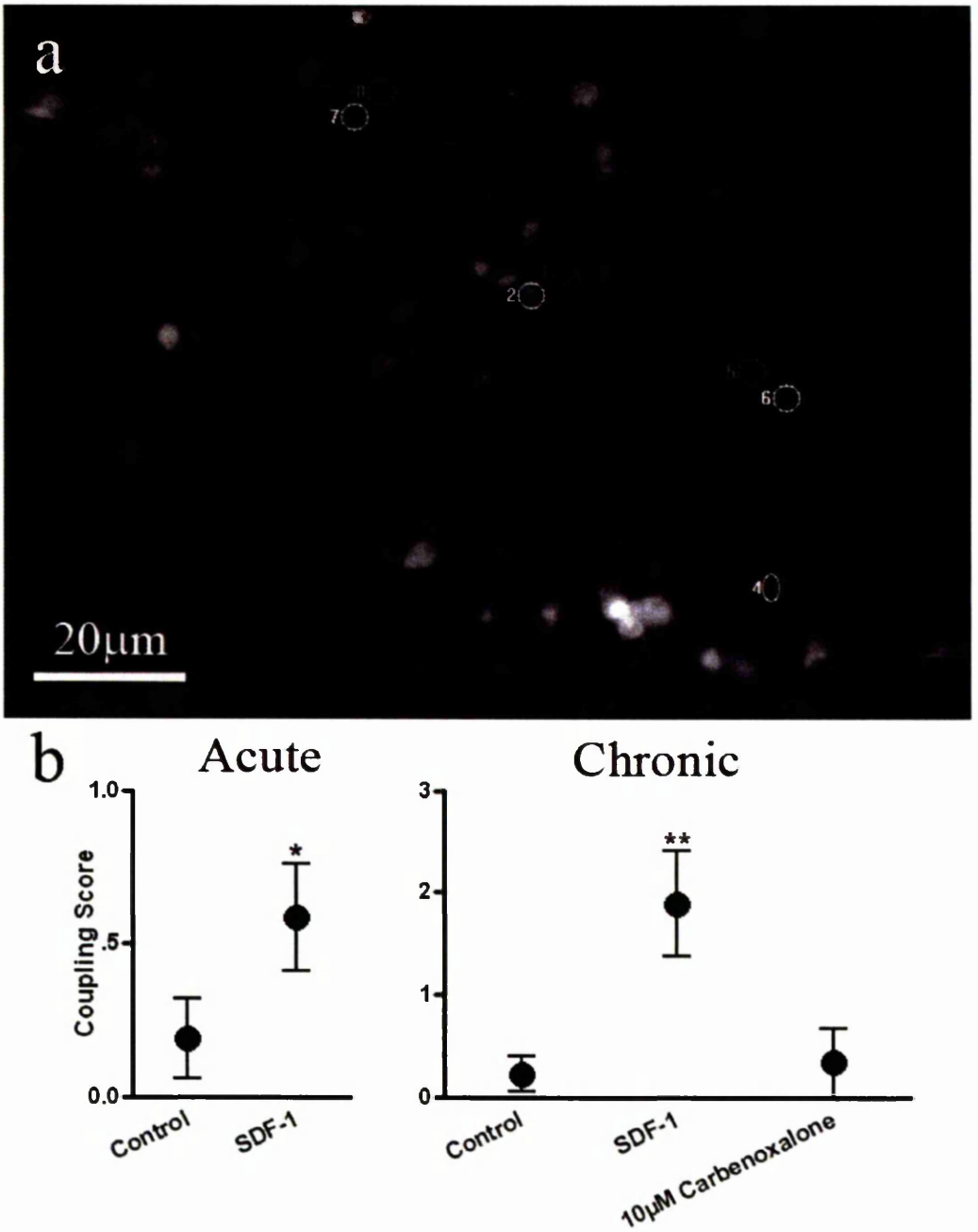

Figure 30: Measurement of calcium transients in rat E17 cortical explants. **a)** Image taken from a stack of time-lapse images, illustrating pairs of cells used to quantify cell coupling. **b)** Graphs illustrate that both acute (15 minutes) and chronic (24 hours) exposure to SDF-1 significantly increase the number of synchronised calcium transients between neighbouring cells (+/- *S.E.M.*). * if $p<0.05$, ** if $p<0.01$.

4.6 Discussion

4.6.1 SDF-1 signalling positively regulates proliferation

The data presented here demonstrates that SDF-1 signalling positively regulates cortical cell proliferation. This hypothesis was based upon the cellular distribution of SDF-1 within the developing cortex, at a time when cell proliferation and migration are at their peak. In support of evidence from *ex-vivo* slice cultures, we have shown, using Ki67 immunocytochemistry, that exposure of primary cultures from both the cortex and ganglionic eminences to SDF-1 leads to increased proliferation, at both E16 and E17. Indeed, at each time point studied, both BrdU indexing and also lineage analysis showed that SDF-1 over expression increased proliferation. Furthermore, observations made in cortical cultures from $CXCR4^{-/-}$ embryos also demonstrated that a lack of functional receptor caused a decrease in proliferation. However, cultures derived from the GE did not exhibit a measurable change in proliferation. Since SDF-1 expression does not extend below the pallial - sub-pallial border in wild type brain (Daniel *et al.*, 2005), it is less likely for the cells in the ventral telencephalic germinal zone to respond to SDF-1 signalling. Thus, deletion of the receptor gene is unlikely to perturb the proliferation in this region. These findings are supported by the established roles for SDF-1 in other systems, including in the development of the immune system (Nagasawa *et al.*, 1994; Nagasawa *et al.*, 1996; Ma *et al.*, 1998) and also of the cerebellum (Klein *et al.*, 2001). In both these systems SDF-1 is important for the correct regulation of proliferation. Furthermore, SDF-1 has since been shown to regulate the proliferation of both isolated neuronal precursor cells (Peng *et al.*, 2004; Gong *et al.*, 2006) and also of cortical astrocytes (Bajetto *et al.*, 2001) *in vitro*. It has been shown that these increases in proliferation are linked to G-protein coupled signalling cascades through changes in $[Ca^{2+}]_i$ (Bonavia *et al.*, 2003), as well as the ERK1/2 signalling pathway (Lazarini *et al.*, 2000; Bajetto *et al.*, 2001; Peng *et al.*, 2004; Gong *et al.*, 2006) and also the phosphatidylinositol 3-kinase (PI 3-Kinase) signalling cascade (Bajetto *et al.*, 2001). Perhaps most relevant were changes in the expression of Akt (also known as protein kinase B) after stimulation of astrocytes with SDF-1 (Bajetto *et al.*, 2001), as Akt is known to promote cell cycle progression from G2 to M phase (Liang and Slingerland, 2003). However, the data presented here is the first to show increases in proliferation in cultures containing the entire cell population of the developing cortex, one step closer to the *in vivo* situation. Thus, the present study demonstrates that SDF-1

signalling functions as a positive regulator for cortical cell proliferation during development.

4.6.2 A mechanism for SDF-1 mediated regulation of proliferation; modulation of gap junction coupling

It has been shown that in response to bFGF, cortical progenitors show an up-regulation in the expression of the gap junction protein Cx43, which is linked to cell coupling and to proliferation (Nadarajah *et al.*, 1998; Cheng *et al.*, 2004). Further it is widely known that calcium signalling can regulate cell proliferation (Berridge, 1995; Berridge *et al.*, 2000), primarily through activation of transcription factors such as NF-κB. Indeed, disruption of calcium transients between Cx43 coupled radial glial cells causes down regulation of proliferation in *ex-vivo* cortical preparations (Weissman *et al.*, 2004). Hence, the results of the microarray study detailed in chapter 6, which showed a significant increase in Cx43 expression following SDF-1 treatment were of interest as they gave rise to the hypothesis that SDF-1 regulates proliferation by directly or indirectly modulating Cx43 expression. Thus, using quantitative immunocytochemistry it was shown that SDF-1 increased the expression of Cx43 protein, thereby confirming the microarray and qRT-PCR data presented in chapter 6. Further experiments have illustrated that the gap junction channel blocker carbenoxalone that inhibits intercellular coupling decreased proliferation in cultures that were treated with SDF-1 medium. Importantly, functional assays employing calcium imaging and dye transfer techniques have demonstrated enhanced cellular coupling in cortical explants that were exposed to both acute and chronic chemokine treatment.

SDF-1 could potentially influence cell coupling via the interaction of several distinct mechanisms. SDF-1 is known to regulate several intracellular signalling pathways such as the ERK1/2 and PI-3 kinase pathways, either of which could modulate transcription of Cx43 as a direct response to SDF-1 signals. SDF-1 could also alter the functionality of gap junction channels by regulating the phosphorylation state of Cx43 subunits within functional channels. Perhaps most intriguingly, SDF-1 may provide the initial signal to trigger release of intracellular calcium stores to begin a calcium wave within coupled progenitor cells, thereby directly regulating proliferation. Indeed, it has been suggested that connexins alone are not sufficient for generation of calcium transients between radial glia within the ventricular zone, and that a diffusible signal is also necessary (Weissman *et al.*, 2004), might this signal be provided by SDF-1?

In summary, the data discussed here provides the first functional evidence for a mechanism by which SDF-1 signalling regulates the proliferation of cortical progenitor cells during development.

5 SDF-1 signalling regulates neuronal differentiation and migration in the developing cortex

5.1 Introduction

Differentiation in the nervous system is best described as a hierarchical ontogenetic process that extends from embryonic stages to postnatal life, during which precursors that are generated in the germinal zone mature to become specific cell types. In the developing telencephalon, post mitotic cells that are committed to a specific lineage migrate to their target destinations, undergo morphological changes to generate dendrites and axons and express specific neurochemical markers. Since SDF-1 is expressed in the developing telencephalon at a time when cell proliferation, migration and differentiation are at a peak, it is likely that signalling via this chemokine plays a role in regulating these events.

Thus, to examine whether SDF-1 signalling exerts a regulatory role in the neurochemical and morphological differentiation of dorsal and ventral telencephalic neurons, primary cortical and ganglionic eminence cultures were used. Further, to test the effect of chemokine signalling in regulating cell motility, time-lapse imaging was used to follow labelled cells in embryonic brain slices.

5.2 SDF-1 signalling differentially regulates neuronal output in distinct regions of telencephalon

To determine whether treatment with SDF-1 alters the output of postmitotic neurons, rat E16-17 primary cortical cultures were quantified using immunocytochemistry for Neu-N, a neuron specific marker (figure 31). Analysis of E17 cortical cultures showed no significant change in neuronal output compared to controls (figure 31). In contrast, exposure of E16 cortical cultures significantly diminished the number of neurons from 52.6% to 32.52% ($p<0.001$, $n=27$). However, treatment with AMD did not yield a significant change in either of the ages analysed.

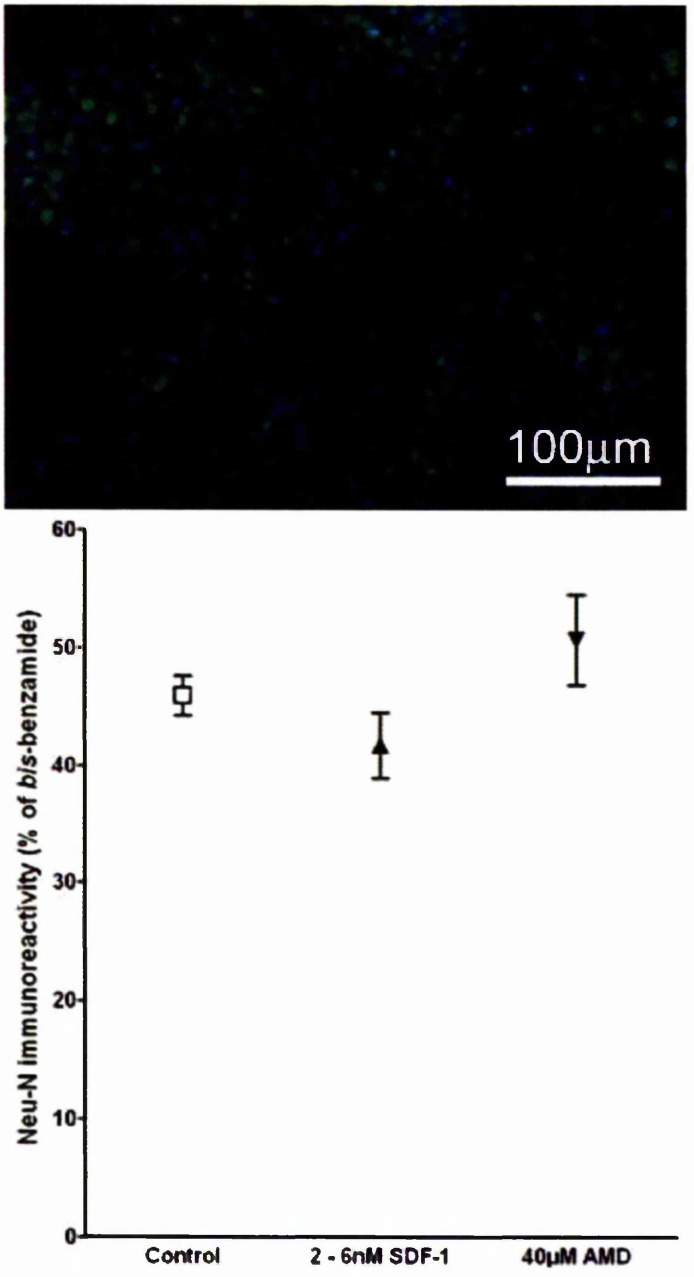

Figure 31: Image of Neu-N immunocytochemistry (green) with *bis*-benzamide counter stain (blue) in rat E17 primary cortical culture. Graph illustrates the percentage of Neu-N positive cells in E17 control and treated cortical cultures (*mean, +/- S.E.M.*).

Interestingly, primary cultures derived from rat E17 ganglionic eminence (figure 32), showed comparable results to E16 cortical cultures. Accordingly, exposure of cultures to low (1-3nM) and high (4-9nM) levels of SDF-1 significantly reduced (47.7%) the percentage of postmitotic neurons (1-3nM: $p<0.001$, $n=34$; 4-9nM: $p<0.001$, $n=24$).

Conversely, treatment with AMD displayed a significant rise in the number of neurons (20μM AMD: $p<0.001$, $n=27$; 40μM AMD: $p<0.001$, $n=24$).

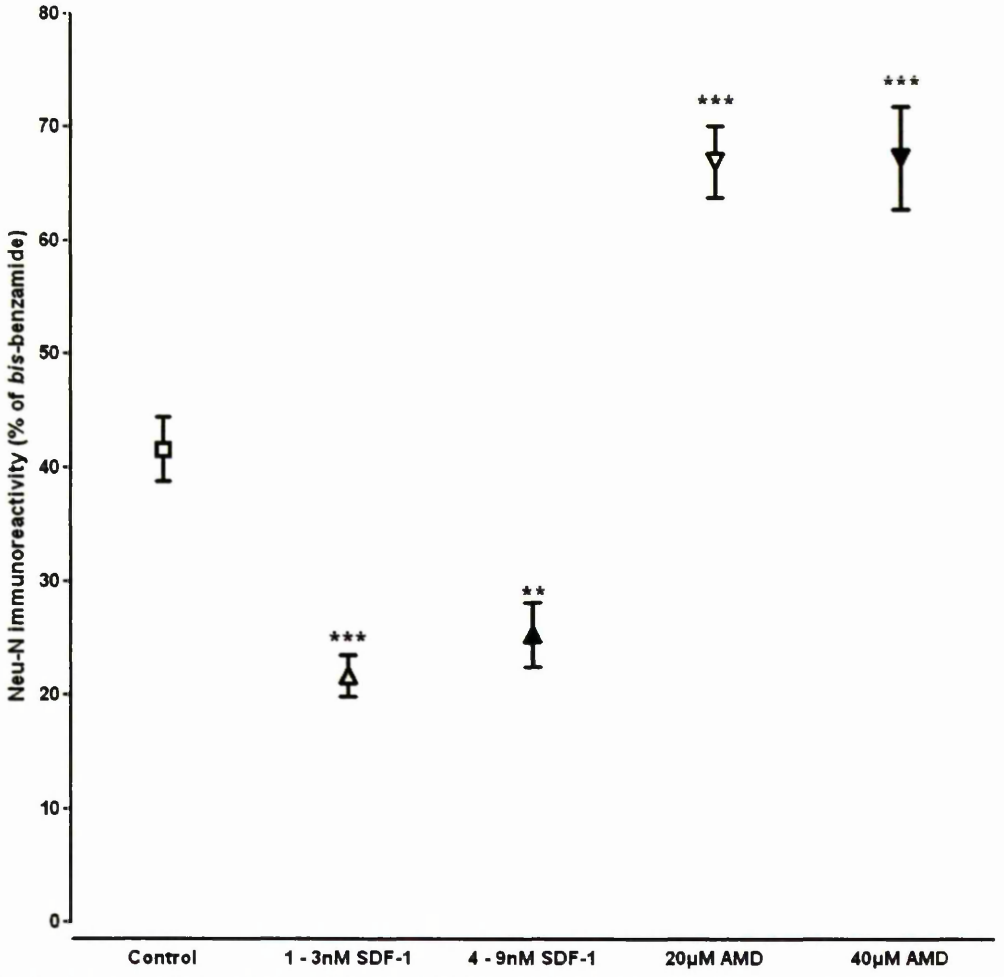

Figure 32: Graph illustrating the effects of SDF-1 and AMD on neuronal output in rat E17 ganglionic eminence cultures (*mean, +/- S.E.M.*). Significance between control and treatments is indicated by ** if $p<0.01$ and *** if $p<0.001$.

5.3 SDF-1 induces the differentiation of GABAergic neurons

5.3.1 Expression of GABAergic neurons

Since the total output of neurons in E17 cortical cultures remained unchanged, it became evident that the chemokine signalling did not affect the generation of glutamatergic (pyramidal) population, which constitutes 80% of all cortical neurons. Thus, to determine whether SDF-1 signalling regulates the differentiation of the cortical GABAergic population, rat E17 control and treated cultures were processed for GABA

immunocytochemistry. Interestingly, a two-fold increase was noted in GABA+ neurons compared to controls (figure 33, $p<0.001$, $n=47$), whereas treatment with 40μM AMD caused a reduction.

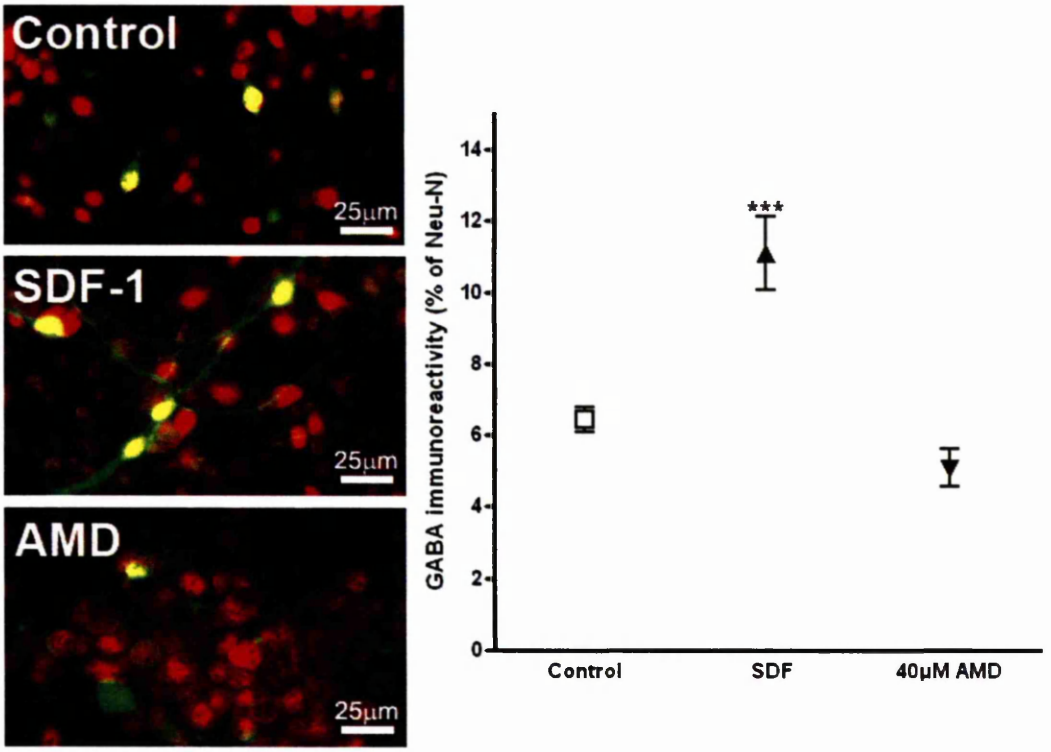

Figure 33: Images of rat E17 cortical cultures stained for *GABA – green and Neu-N – red*. Graph illustrates the effect of treatment (*mean, +/- S.E.M.*) on the differentiation of cortical GABAergic population. *** indicates significance of p<0.001between control and SDF-1 treatment.

Since cortical GABAergic neurons are generated in the ganglionic eminences (Anderson *et al.*, 1997; Nadarajah *et al.*, 2003), and likely to use SDF-1 as a migratory cue (Stumm *et al.*, 2003), the effects of SDF-1 on the differentiation of GABAergic neurons derived from E17 ganglionic eminence were also investigated (figure 34). These experiments illustrated that both exposure to SDF-1 and antagonist caused an increase in GABA + neurons (SDF-1: $p<0.05$, $n=21$; AMD: $p<0.05$, $n=21$).

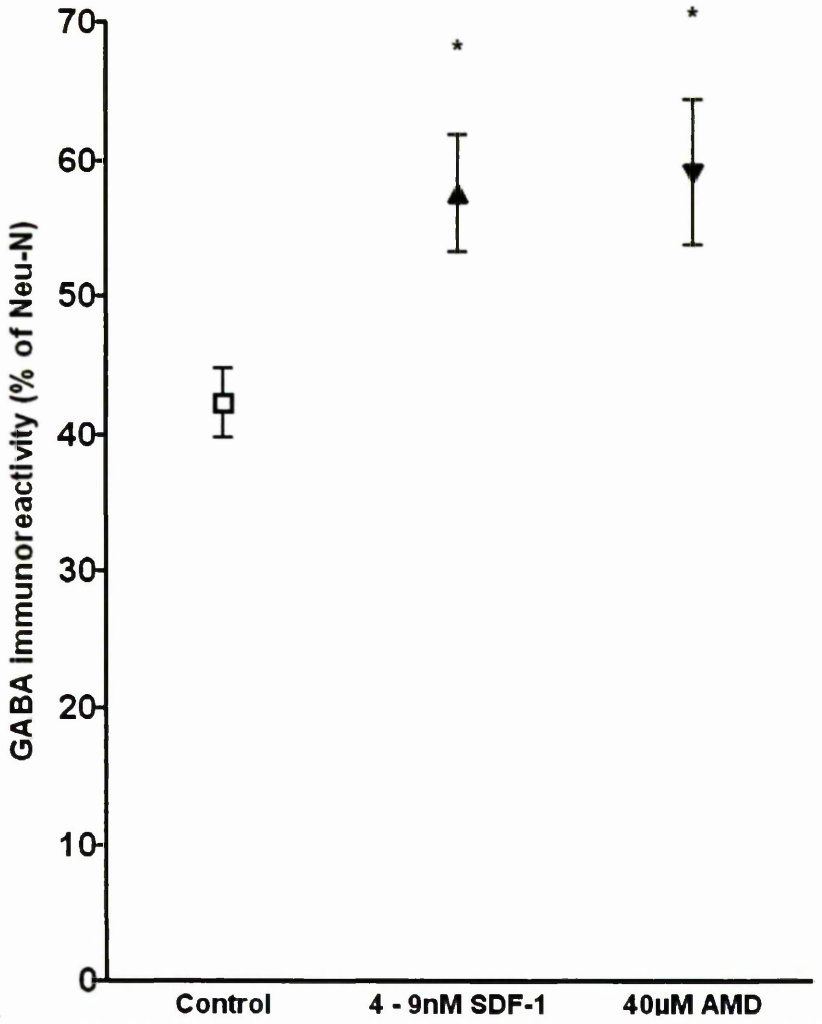

Figure 34: Graph illustrating the effect of treatment on the expression of GABAergic neurons in rat E17 ganglionic eminence cultures (*mean, +/- S.E.M.*). * indicates significance of $p<0.05$ between control and treatments.

5.3.2 SDF-1 differentially regulates the expression of calretinin and calbindin populations

On the basis of neurochemical content, cortical interneurons can be further categorised into calretinin+, calbindin+, parvalbumin+ and somatostatin+ populations. Among these, calretinin and calbindin containing GABAergic neurons are the major populations of interneurons that are present during embryonic development. Thus, to ascertain whether the noted increase in the GABAergic population is reflected in the respective interneuronal subtypes, E17 primary cortical cultures were examined following treatment with chemokine or antagonist. Remarkably, treatment with chemokine induced a

significant rise in calretinin+ neurons (*p<0.05, n=20,* figure 35), whereas the receptor antagonist further reduced the expression by 75% (*n=13, p<0.01,* figure 35).

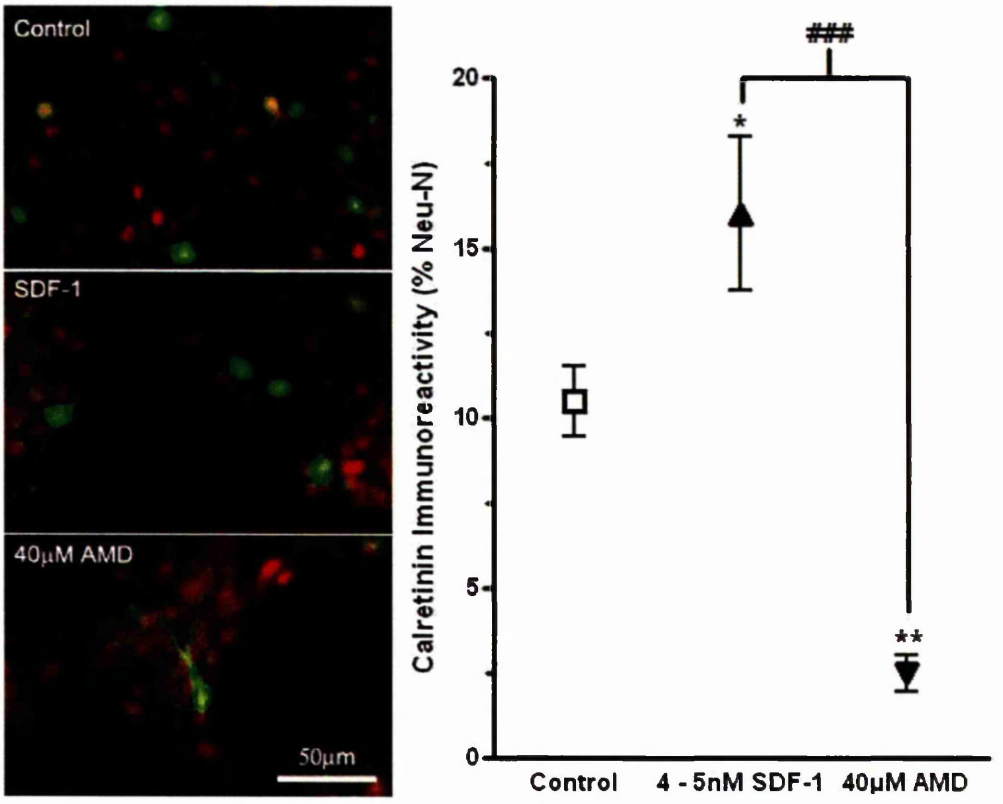

Figure 35: Images of calretinin staining – *green*, *Neu-N* – *red*, in rat E17 cortical cultures that were exposed to control, SDF-1 or AMD treatment. Graph illustrates the effect of treatment on the expression of calretinin immunoreactivity (*mean, +/- S.E.M.*). Statistical significance between control and treatment is indicated as; * if p<0.05, ** if p<0.01, *** if p<0.001. ### indicates statistical significance (p<0.001) between SDF-1 and AMD3100 treated cultures (one-way ANOVA).

Similarly, chemokine treatment also increased the expression of calbindin+ neurons compared to controls and AMD, with the latter showing a near 2-fold decrease (figure 36, *p<0.01, n=24*).

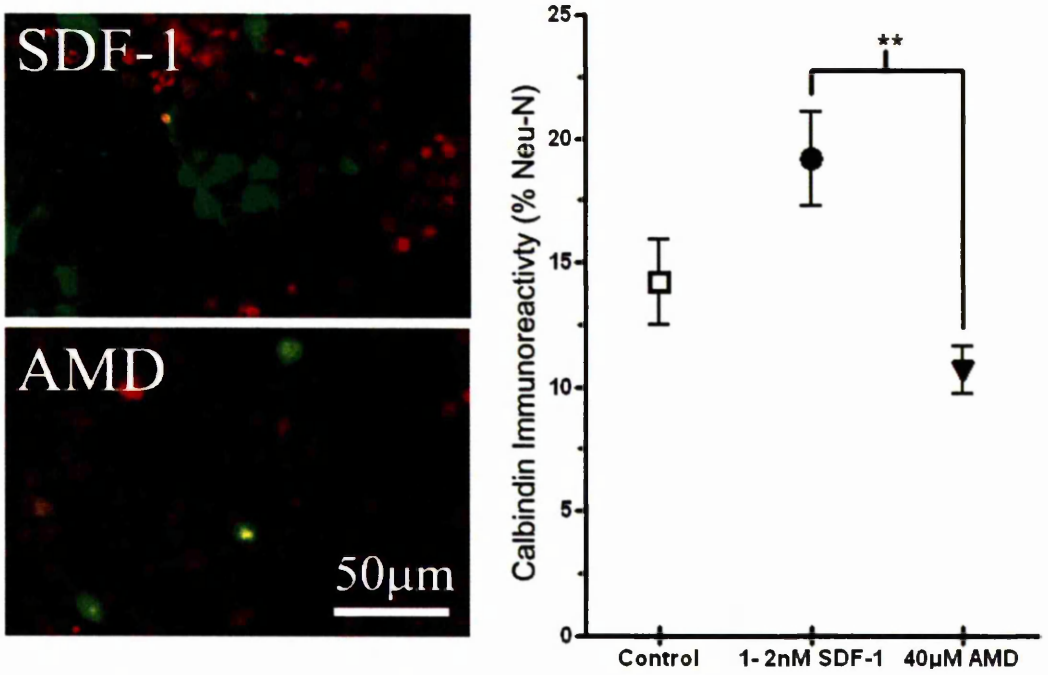

Figure 36: Immunocytochemistry for calbindin (green) and Neu-N (red) in SDF-1 and AMD treated E17 cortex primary cultures. Graph shows calbindin immunoreactivity (*mean +/- S.E.M.*). Statistical significance is indicated as; * if $p<0.05$, ** if $p<0.01$, *** if $p<0.001$ (one-way ANOVA).

5.4 Neuronal differentiation is altered in CXCR4$^{-/-}$ mutant cortical cultures

5.4.1 CXCR4$^{-/-}$ cortical cultures contain fewer post-mitotic neurons

Examination of E15 mutant cortical cultures showed a significant decrease in the number of postmitotic neurons, as indicated by Neu-N immunocytochemistry (figure 37, *$p<0.001$, $n=68$ fields of view from 9 mutants*).

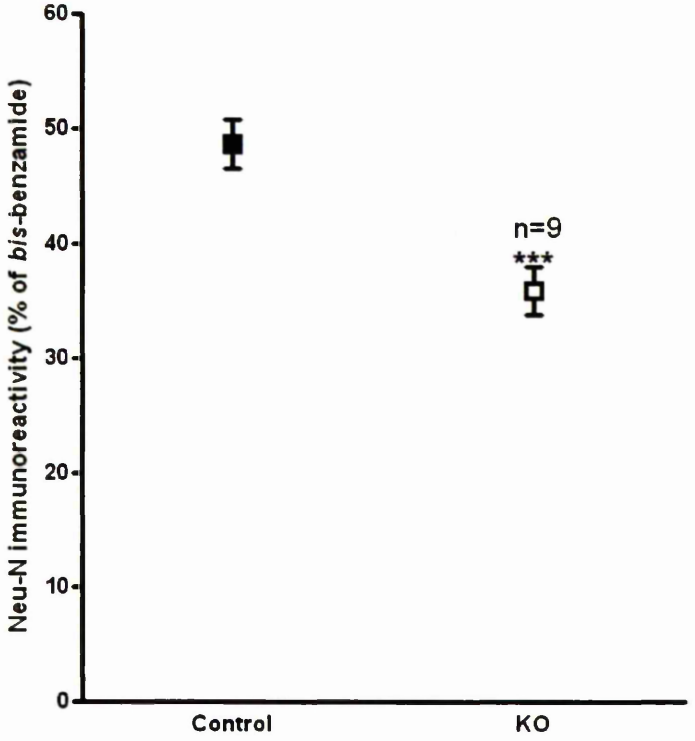

Figure 37: Graph illustrates the neuronal output in E15 wild type and CXCR4$^{-/-}$ primary cortical cultures (*mean, +/- S.E.M.*). *** indicates significance of $p<0.001$.

5.4.2 CXCR4$^{-/-}$ cortical cultures contain fewer GABA positive neurons

Further characterisation of E15 wild type and CXCR4$^{-/-}$ cortical cultures revealed a significant reduction in the number of GABA positive neurons, as indicated by GABA immunocytochemistry (figure 38, *$p<0.01$, $n=45$ fields of view from 9 mutants*).

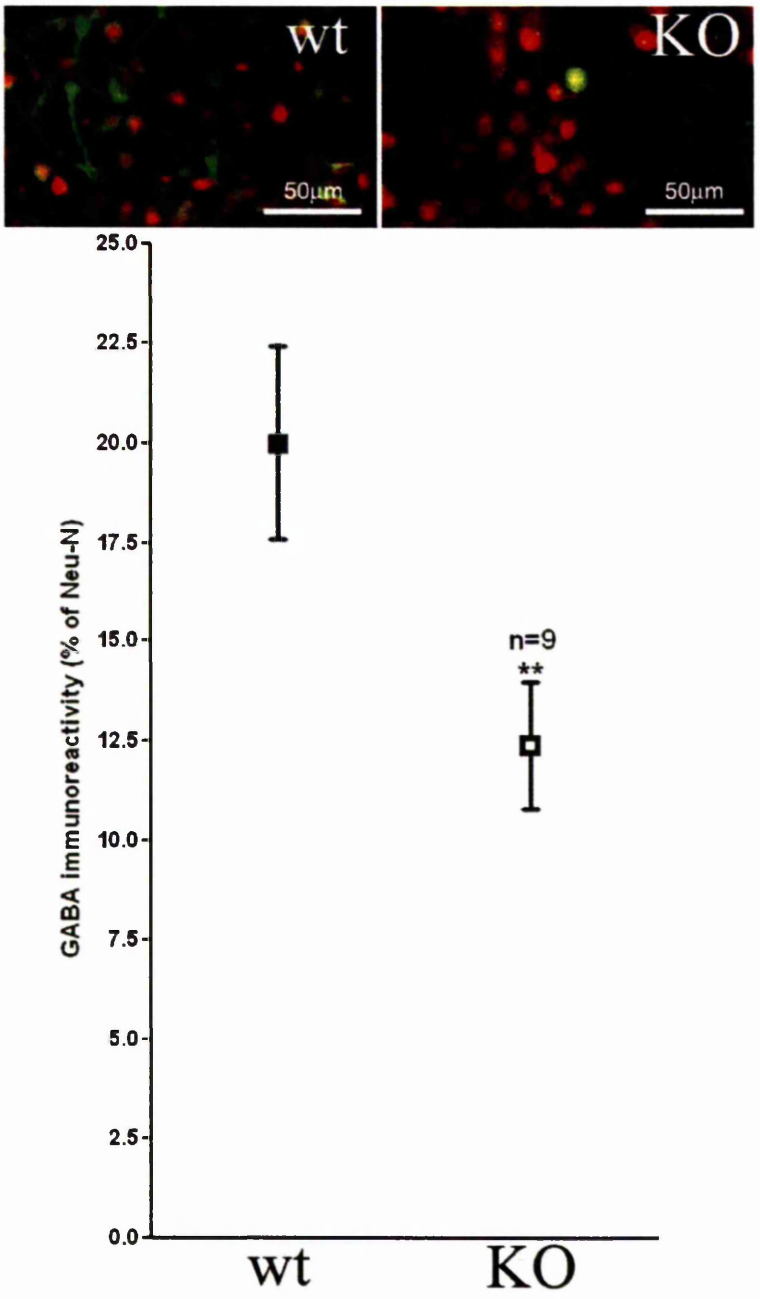

Figure 38: Images of GABA immunocytochemistry (green) and Neu-N (red) in E15 wild type and CXCR4$^{-/-}$ cortical cultures. Graph illustrates the proportion of GABA positive neurons in wild type versus CXCR4$^{-/-}$ cortical cultures; ** indicates significance of $p<0.01$).

5.5 SDF-1 increases Dlx-2 gene expression

Transcription factor *Dlx-2* has been implicated in the regulation of GAD 65 expression and consequently the synthesis of GABA (Stuhmer *et al.*, 2002a ; Stuhmer *et al.*, 2002b). Since, exposure to SDF-1 up regulated the expression of GABA+ neurons in cortical cultures, it was hypothesised that the chemokine signalling might exert its regulatory role through the activation of *Dlx-2* gene expression. Thus, using quantitative real time PCR, our experiments have demonstrated that application of SDF-1 medium to cortical cultures increases the expression of *Dlx-2* by over 3-fold (figure 39).

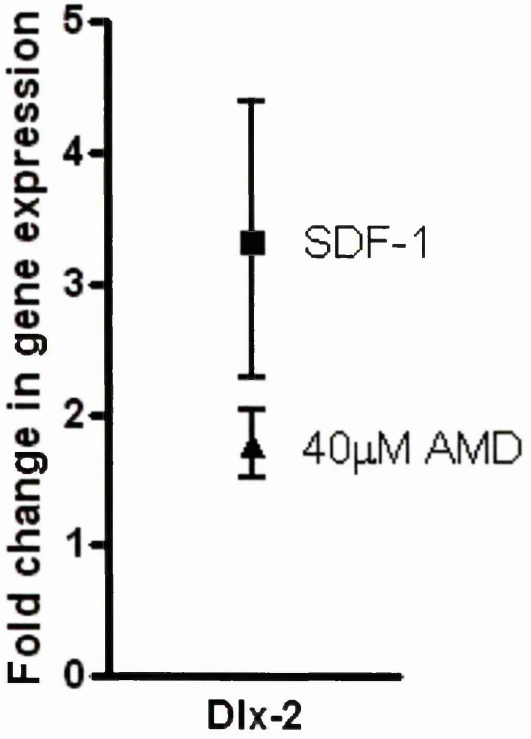

Figure 39: Graph illustrating fold change in the mRNA expression of *Dlx-2* (*mean +/- S.E.M.*).

5.6 Does SDF-1 modulate sonic hedgehog signalling?

The sonic hedgehog (Shh) signalling pathway is important for the patterning and differentiation of the developing CNS. Interestingly, several studies have now demonstrated a role for Shh in the differentiation of GABAergic neurons in the dorsal telencephalon (Gulacsi and Lillien, 2003; Viti *et al.*, 2003; Xu *et al.*, 2005; Gulacsi and Anderson, 2006). Since SDF-1 has been shown to modulate Shh signalling in the developing cerebellum (Klein *et al.*, 2001), and *Shh* gene expression has been shown in the developing cortex (Dahmane *et al.*, 2001), it was postulated that a similar mechanism

might be employed to regulate the differentiation of cortical GABAergic neurons. To test this hypothesis, rat E17 primary cortical cultures were exposed to the Shh antagonist cyclopamine (Incardona *et al.*, 1998; Cooper *et al.*, 1998) in the presence or absence of SDF-1 medium. Subsequent analysis of control cultures showed that treatment with 5μM cyclopamine had no effect on the total output of neurons, as indicated by Neu-N immunocytochemistry (data not shown). Nonetheless, a significant increase was noted in the GABAergic population (figure 40, $p<0.01$, $n=23$). Interestingly, cultures that were grown together with SDF-1 and cyclopamine showed a significant rise in the GABA+ neurons in a manner similar to that noted with either of the treatments alone ($p<0.001$, $n=33$).

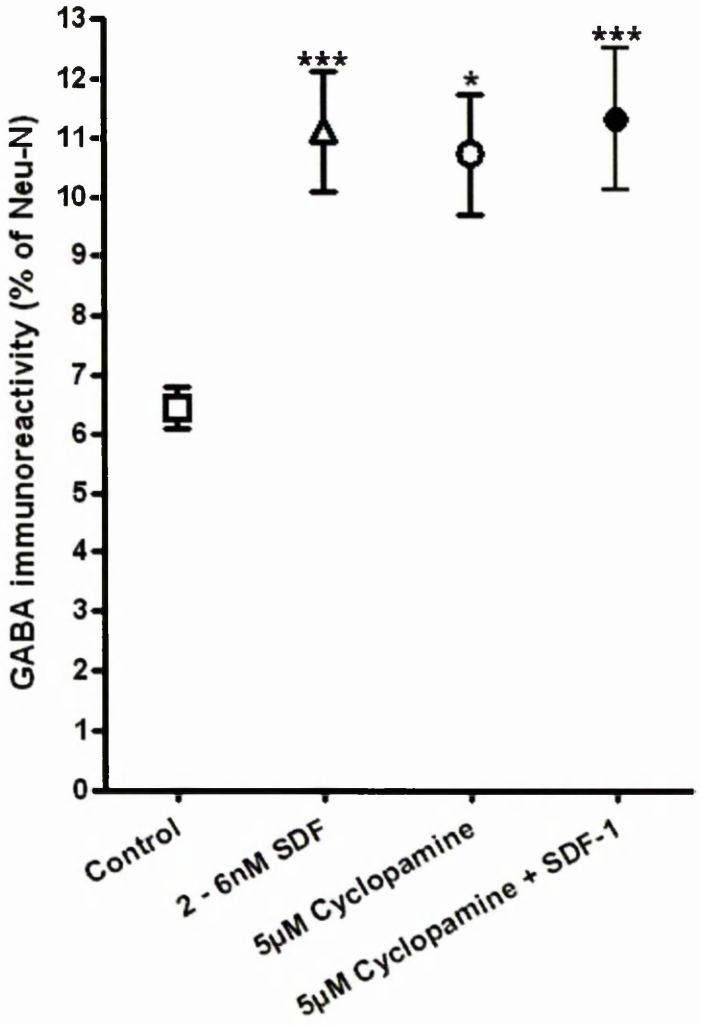

Figure 40: The effect of cyclopamine on GABA expression in rat E17 primary cortical cultures. Significance between control and various treatments is indicated by *, *** for $p<0.05$ and $p<0.001$, respectively.

5.7 SDF-1 regulates axonal growth in cortical neurons

5.7.1 SDF-1 increases axonal elongation

SDF-1 signalling has been shown to regulate axonal outgrowth in cerebellum and hippocampus during development (Klein *et al.*, 2001; Arakawa *et al.*, 2003; Pujol *et al.*, 2005). Thus, to examine whether the chemokine exerts a similar regulatory mechanism in the developing cortex, rat E17 cultures were transfected with GFP to randomly label cells. Following transfection, cortical cultures were maintained in control, SDF-1 or AMD as described previously. Subsequent analysis revealed a transfection efficiency of 20-25 GFP+ cells/cover slip, which was well suited for the morphological analysis of neurite outgrowth. Although, the longest neurite was defined as an axon (Pujol *et al.*, 2005), immunocytochemistry was performed to label mid weight neurofilaments that are typically prevalent in axons (figure 41, a – c). Quantitative analysis demonstrated that SDF-1 treatment induced axonal elongation by 60% compared to controls (figure 41, d – f, $p<0.05$, $n=45$).

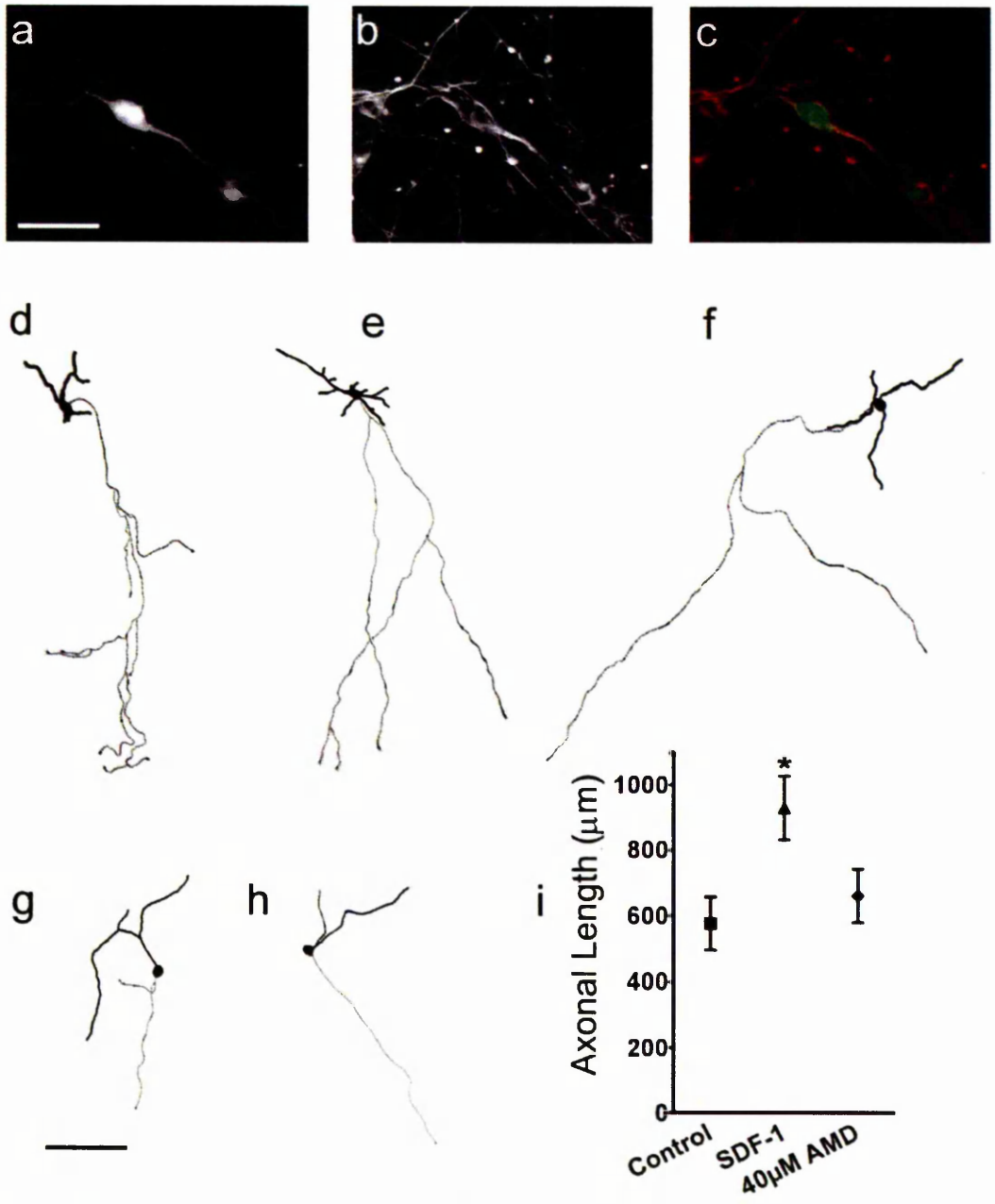

Figure 41: SDF-1 induces axonal outgrowth. Images a – c show a typical GPF transfected neuron (a) with processes positive for neurofilament (b). (c) is a merged image of the two channels (GFP – green, neurofilament – red). Scale bar for a –e is 50µm. d - h are traces taken from images of GFP + neurons reconstructed from multiple fields of view. d – f are images of neurons that were exposed to SDF-1 treatment whereas g, h were from control cultures. i. graph illustrates the effect of chemokine treatment on axonal growth (*mean, +/- S.E.M.*).* indicates significance of $p < 0.05$ between control and SDF-1.

5.7.2 SDF-1 induces axonal branching but does not affect the number of collaterals in cortical neurons

To determine whether chemokine treatment induces axonal branching in E17 cortical neurons, morphological analyses were performed on GFP transfected cells. Interestingly, exposure to chronic SDF-1 significantly increased the frequency of axonal branching compared to control conditions (figure 42; $p<0.001$, $n=45$, χ^2 test). Nonetheless, the treatment did not increase the degree of branching to a measurable significance, at least at the concentration of chemokine used (figure 43).

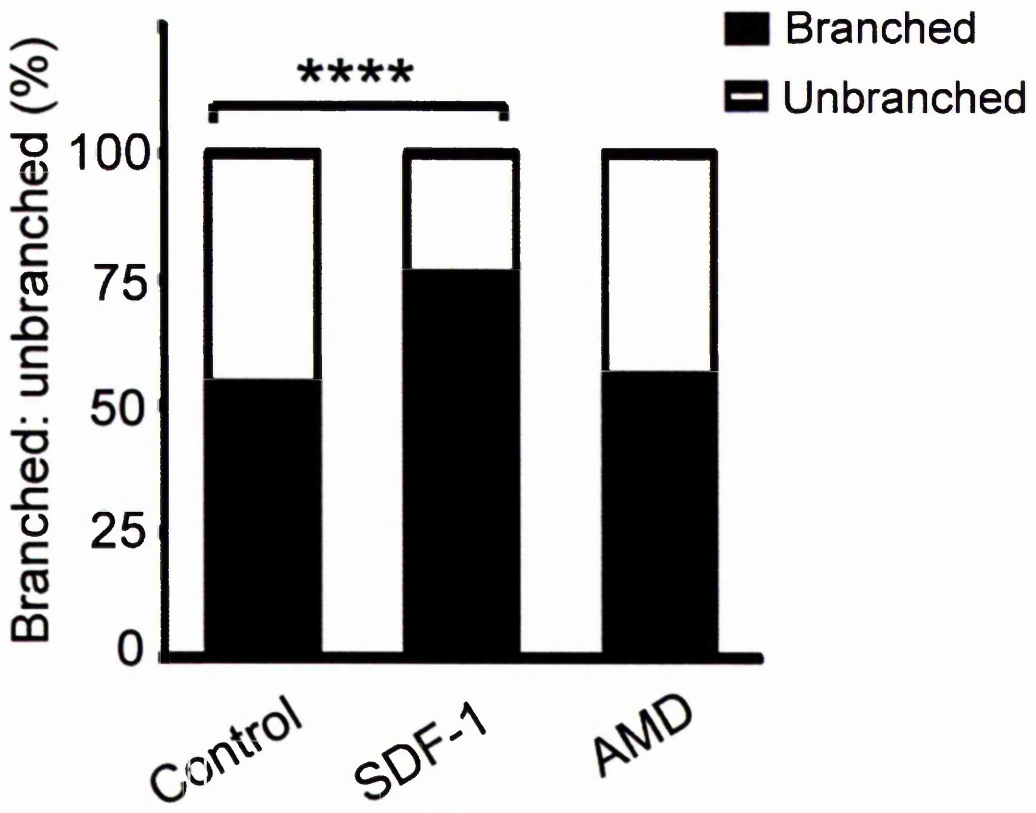

Figure 42: Axon branching frequency. SDF-1 increases the proportion of cells with branched axons, *** indicates statistical significance ($p<0.001$) between control and SDF-1 treated cultures (χ^2 test).

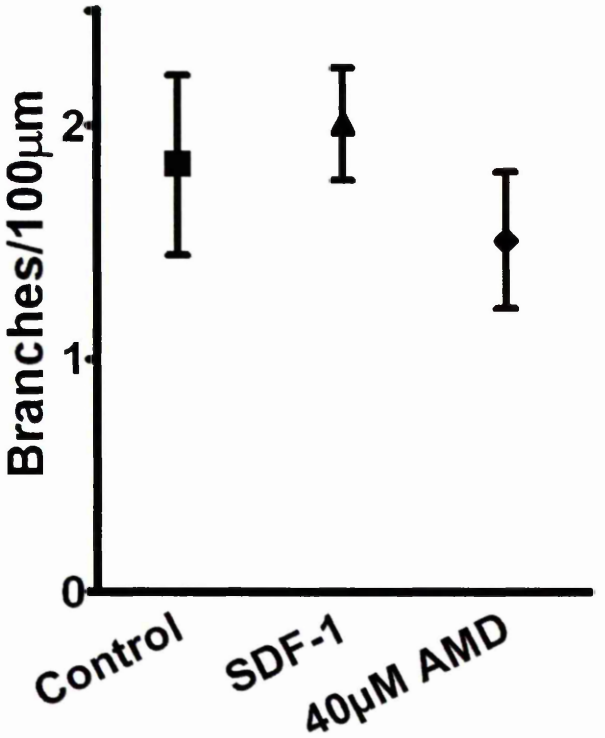

Figure 43: Effect of chemokine on axonal branching.

5.8 SDF-1 increases the motility of migrating cortical cells

It is widely known that SDF-1 functions as a chemoattractant in the immune system (Aiuti *et al.*, 1997). Recent reports have highlighted that SDF-1 exerts chemotactic responses in different regions of the developing CNS. For example, in the cerebellum, SDF-1 regulates the migration of precursor cells from the external to the internal granule layer (Ma *et al.*, 1998; Klein *et al.*, 2001; Reiss *et al.*, 2002; Zhu *et al.*, 2002), and also in the migration of dentate granule cells (Bagri *et al.*, 2002). More recently, at a concentration of 200nM, SDF-1 has been shown to function as a chemoattractant for isolated rat E14 striatal precursors (Stumm *et al.*, 2003). Taken together with our data (chapter 4), it is likely that SDF-1 plays a role in the migration of cortical neurons.

To investigate the role of SDF-1 in regulating neuronal migration, rat E17 *ex-vivo* brain slices were exposed to control, SDF-1 medium or to AMD. After 1-2 days, slices were labelled with Oregon green BAPTA and cell motility was assayed using time-lapse imaging (figure 44, see also time-lapse movies: control motility and SDF induced motility). Analysis showed that 16.2% of the labelled cells were motile in control slices, whereas, twice the number (30%) were actively motile in SDF-1 medium (figure 44, a – c). In contrast, treatment with antagonist significantly reduced the motility of cortical neurons

(figure 44c; *p<0.001*). Interestingly, chemokine treatment had the propensity to increase the average speed of movement of cortical cells (figure 44d). Taken together, these results show that SDF-1, a chemoattractant, has the potency to elicit motility in populations of cortical neurons even when applied in a non-gradient manner.

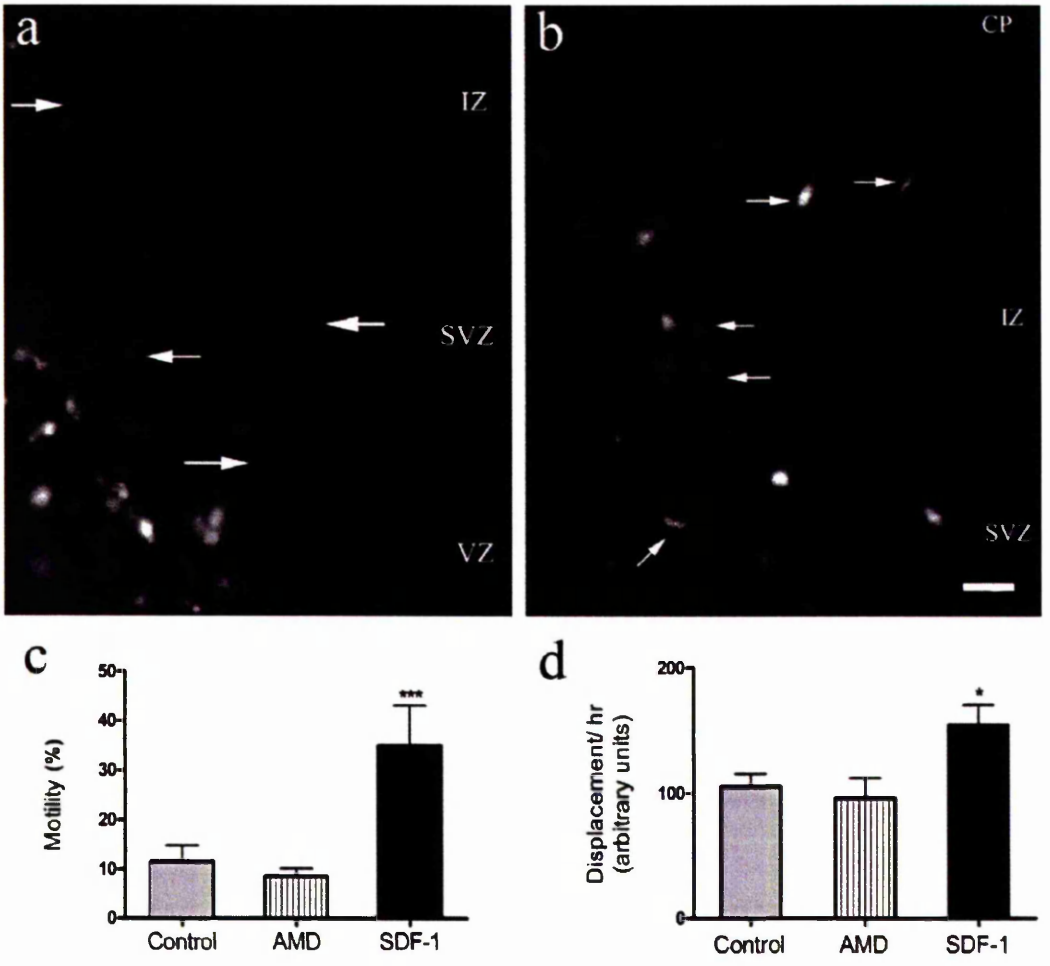

Figure 44: SDF-1 induces motility in cortical neurons. (a, b) E17 brain slices that were maintained in control (a) or SDF-1 medium (b) were labelled with Oregon Green BAPTA (arrows) for imaging. Time-lapse imaging showed a significant level of motility (c; $p<0.001$) and increased displacement (d; $p<0.01$) in SDF-1 treated slices compared to controls. Scale bar: 25 μm.

5.9 Discussion

5.9.1 SDF-1 and neuronal differentiation

The data presented here demonstrates that SDF-1 signalling influences the differentiation of E17 telencephalic neurons. Since earlier experiments (chapter 4) illustrated that exposure to SDF-1 increases cortical cell proliferation, it was perceived this might consequently lead to an increase in the output of neurons. Contrary to this notion, chronic exposure to SDF-1 did not alter the output of cortical neurons in E17 cultures, whereas a significant reduction was apparent in cultures derived from both E16 cortex and E17 ganglionic eminence. Since symmetric cell division is more prevalent in E16 cortex, it is plausible that applied SDF-1 might have exerted a mitogenic effect in these cultures. This consequently may have enabled the generation of a progenitor population with fewer postmitotic neurons. In E17 GE cultures, treatment with chemokine diminished the output of neurons, whereas the antagonist opposed the effect. Since the GE is a composite of lateral, medial and caudal ganglionic eminences, it is likely that SDF-1 in concert with other local signalling molecules such as Shh and Fgf2 functions as a mitogen. Thus, exposure to chemokine treatment may have reduced the neuronal output, whereas inhibition with the antagonist is likely to have promoted the differentiation pathway. Intriguingly, mouse E15 $CXCR4^{-/-}$ cortical cultures also showed a decrease in the number of post-mitotic neurons, apparently a consequence of impaired proliferation (chapter 4). It is also possible that lack of chemokine signalling may have resulted in a population of undifferentiated quiescent cells, as SDF-1 has been shown to promote cell cycle progression (Lataillade *et al.*, 2002).

Further studies revealed that SDF-1 signalling up regulates the expression of cortical GABAergic population. In this context, evidence shows that GABAergic neurons express CXCR4 (Bajetto *et al.*, 1999; Stumm *et al.*, 2003) and respond to SDF-1. Our experiments also demonstrated an up regulated expression of *Dlx-2* in cortical cultures that were exposed to chemokine. Taken together, it is likely that SDF-1 functions at a transcriptional level where the activation of *Dlx2* leads to a downstream regulation of GAD65 (Stuhmer *et al.*, 2002a; Stuhmer *et al.*, 2002b), and consequently to GABA expression. Treatment with AMD also appeared to increase the expression of *Dlx2*, although to a lesser degree. Antagonist mimicking the effect of ligand has been previously observed, particularly in *ex-vivo* brain slices (see chapter 4). While AMD3100 is a commonly used receptor antagonist of CXCR4, a recent study has reported that it can exert

a weak partial agonistic effect depending on the concentration applied (Zhang *et al.*, 2002). Moreover, it is pertinent to note that CXCR4 is not ubiquitously expressed in all cortical cells. Thus, treatment with AMD may be of inconsequence to some cell populations that continue to express *Dlx2*. Interestingly, characterisation of SDF-1 treated cultures illustrated a significant increase in the calretinin+ population, suggesting that the signalling regulates the differentiation of a subtype of cortical neurons. Since, calretinin is also expressed by preplate neurons it is unclear whether the chemokine signalling has altered the differentiation of early preplate neurons, or a subset of GABA+ neurons that are generated in the cortical VZ.

5.9.2 Unravelling SDF-1 and Sonic hedgehog (Shh) interactions

It is now widely accepted that cortical interneurons are generated in the ventral telencephalon (Anderson et al., 1997). Nonetheless, it is also evident that cortical progenitors are competent to generate as many as 20-30% of GABAergic interneurons (Gotz and Bolz, 1994). This raises the question why the neocortex fails to generate large numbers of interneurons *in vivo*. A plausible explanation would be that the chemical environment of the cortex does not support the generation of interneurons due to the absence of inductive signals and/or the presence of inhibitory signals. In this context, Shh, expressed at high levels in the ventral telencephalon (Kohtz *et al.*, 1998; Nery *et al.*, 2001), is also expressed in the dorsal telencephalon during mid corticogenesis (Dahmane *et al.*, 2001). While a number of roles have been attributed to Shh signalling, recent reports have demonstrated that it induces the generation of cortical GABA+ neurons in concert with BMPs or through non-autonomous mechanisms (Hattori *et al.*, 1999; Yung *et al.*, 2002; Gulacsi and Lillien, 2003; Xu *et al.*, 2005; Gulacsi and Anderson, 2006).

Given that SDF-1 has been shown to interact with Shh in the cerebellum (Klein *et al.*, 2001), we reasoned a similar mechanism might enable the up regulation of GABAergic population following treatment with chemokine medium. Notably, Shh signalling has been implicated to function through the activation of *Dlx* genes (Kohtz *et al.*, 1998, Kohtz *et al.*, 2001) that are essential for the specification of interneurons. Thus, E17 cortical cultures were exposed to the Shh antagonist cyclopamine in the presence or absence of SDF-1. Interestingly, treatment with either of the conditions alone or together increased the proportion of GABA+ neurons. Since, Shh promotes the GABAergic lineage (Yung *et al.*, 2002; Xu *et al.*, 2005) it was thought that blocking the signalling with cyclopamine would diminish the GABAergic population as has been reported (Gulacsi and Lillien, 2003).

Based on our results, it is evident that the SDF-1 induced GABA expression is independent of Shh signalling pathway. However, this does not preclude the fact that SDF-1 and Shh might interact to regulate other developmental events in the cortex.

5.9.3 Branching and elongation – SDF-1 and axonal growth

The data presented here demonstrates that SDF-1 signalling regulates the elongation of cortical axons in E17 primary cultures, as has been previously documented in cerebellum (Arakawa *et al.*, 2003) and hippocampus (Pujol *et al.*, 2005). Since, glutamatergic cells constitute 80% of cortical neurons, the probability of labelling this population with a low efficiency GFP transfection was significantly high compared to the chances of GABAergic neurons being randomly labelled. However, to confirm the cell type of transfected neurons, GFP transfected cells were immunolabelled for GABA. As predicted, 99% of the GFP labelled neurons were negative for GABA immunoreactivity, thus confirming their glutamatergic phenotype. Interestingly, in cerebellar cultures, a lower concentration (10nM) of SDF-1 induced axonal elongation, whereas treatment with higher concentration (50nM) opposed the effect (Arakawa *et al.*, 2003). This is in agreement with our experiments that used relatively low concentrations of SDF-1 (3nM) to effect axonal elongation. Interestingly, in hippocampal cultures 50nM SDF-1 not only caused a decrease in axonal length but also induced branching of collaterals (Pujol *et al.*, 2005). In our experiments treatment with SDF-1 significantly increased the frequency of branching in cortical axons; however the number of collaterals was not significantly increased compared to controls. Recent reports have implicated a role for the secreted frizzled related protein 1 (sfrp1) in the regulation of retinal axons (Rodriguez *et al.*, 2005; Drescher, 2005). In this context, treatment of cortical cultures with SDF-1 medium lead to an up regulation of this gene (see chapter 6), thus suggesting a potential mechanism for SDF-1 in regulating axonal growth. As signalling via the small GTPase Rho has been implicated in SDF-1 mediated regulation of axon growth, it was proposed (Arakawa et al., 2003) to make use of pull down assays to ascertain whether this was also the case in our culture system. Small GTPases function as biological switches. The GTP-bound, or active state, rapidly converts back to the inactive GDP-bound state due to their intrinsic GTPase activity. Pull down assays have the advantage over normal western blots of allowing the quantification of changes in the amount of active GTP bound Rho following treatment. This uses a GST-rhotekin fusion protein containing a Rho binding domain from mouse rhotekin, in conjunction with immobilized glutathione discs to bind to ("pull-down") active Rho in

protein lysates, and then detection using Rho antibodies. However, using lysates from primary cortical cultures this was unsuccessful, possibly due to the small amounts of protein involved. Optimisation of this technique would allow further elucidation of the pathways in SDF-1 mediated control of elongation, and possible interactions between SDF-1, sfrp1 and Rho-GTPase.

5.9.4 SDF-1 regulates neuronal migration in the developing forebrain

Our experiments provide the first evidence that SDF-1 can elicit cell motility in *ex-vivo* cortical tissue. Using chemotactic chambers, previous studies (Stumm *et al.*, 2003) have demonstrated the ability of SDF-1 to elicit a response in isolated E14 striatal precursors. However, the concentration of chemokine used in that study was 200nM, in contrast to the physiologically relevant levels used in our experiments (<10nM). Using time-lapse imaging the present study illustrates that SDF-1 induces motility in the migrating population of cortical neurons. Earlier work has shown that focal placement of SDF-1 beads in the ventral telencephalon elicits strong chemotactic response in local neurons, while placement of beads in the cortico-striatal border attracts the migrating GABA+ neurons (Liapi *et al.*, 2006).

Further, the present study demonstrates that SDF-1 exerts a regulatory role in the radial migration of cortical neurons. This is further supported by the expression of SDF-1 protein in the developing cortex (Chapter 4, figure 16), where it is strategically localised to provide a chemotactic gradient between the ventricular zone and cortical plate. Consistent with this view, exposure of E17 brain slices to chemokine medium lead to a localized neuronal ectopia in the medial cortex, suggestive of increased proliferation with altered radial migration (Chapter 4, figure 17; Liapi *et al.*, 2006).

6 SDF-1 mediated changes in gene expression – a microarray analysis

6.1 Introduction

It is known that SDF-1 signalling activates the downstream intracellular signalling cascades, such as the ERK1/2 pathway that can regulate a number of genes (Gong *et al.*, 2006). Thus, to investigate the effects of SDF-1 induced gene expression a genome wide approach was used. Accordingly, total RNA was extracted from rat E17 cortical cultures after 48 hours of treatment to quantify changes in gene expression that might have enabled the regulatory function of SDF-1 in cell proliferation and differentiation as described in chapters 4 and 5.

6.2 Analysis of microarray data

Normalised intensities from Affymetrix rat expression arrays (230v2) were processed for fold change in the expression of a given transcript in control and treated (SDF-1 or AMD) cultures. A two-step criterion was then used to select genes that were regulated by SDF-1 signalling. Transcripts that showed a consistent expression of at least 50 Affymetrix units in all three biological replicates of each condition were first selected. From the selected gene list, transcripts that displayed an expression intensity of at least 1.5 folds above or below control condition with significance less than $p<0.1$ (ANOVA) were further short-listed. This list was then analysed as described below.

6.2.1 SDF-1 signalling alters downstream gene expression

To assess the overall changes in gene expression mediated either by chemokine treatment or receptor antagonist, median raw intensity values were averaged for each condition (figure 45). Analysis showed that SDF-1 signalled a global increase in the gene expression, whereas treatment with AMD opposed the effect.

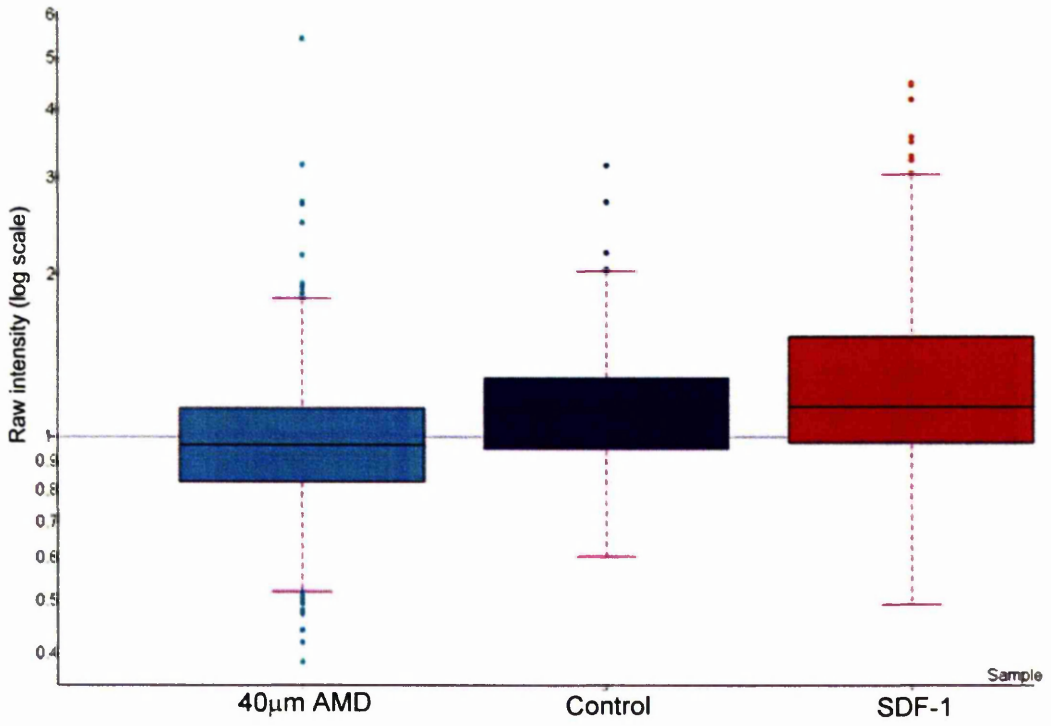

Figure 45: Box plots of median (median is marked by solid line, box outline indicates 95% confidence intervals, and error bars show S.E.M.) gene expression in control, AMD or SDF-1 treated cultures. Gene expression given as raw array labelling intensity for each condition (average values, experiments performed in triplicate).

6.2.2 SDF-1 signalling up regulates gene expression

The list of 335 transcripts that met the quality control criteria was further shortened based on their responses to SDF-1 signalling. Table 3 illustrates a list of 100 genes that were significantly up regulated in response to SDF-1 treatment. Genes of relevance to the regulatory function of SDF-1 signalling discussed in earlier chapters are highlighted on the volcano plot in figure 46. To assess the overall effects of SDF-1 treatment on downstream gene expression, fold changes ($\log_{10}$ [mean SDF-1 gene expression] – $\log_{10}$ [mean control gene expression]) were plotted against significance (-$\log_{10}$ [p value]) to generate a volcano plot (figure 46).

Number	Gene Title	Gene Symbol	p-value	Fold change
1	protein kinase, cAMP dependent, catalytic, beta (predicted)	Prkacb	0.0465439	4.098338
2	protein phospatase 3, regulatory subunit B, alpha isoform,type 1	Ppp3r1	0.0207437	3.024627
3	zinc finger protein 238	Zfp238	0.0329205	2.988048
4	glutaminase	Gls	0.0151413	2.866382
5	similar to transmembrane protein TM9SF3	LOC309475	0.0404629	2.847079
6	caspase 2	Casp2	0.0482366	2.774742
7	lin-7 homolog C (C. elegans)	Lin7c	0.0260049	2.753361
8	potassium channel tetramensation domain containing 12 (predicted)	Kctd12_predicted	0.0223921	2.676994
9	protein kinase inhibitor, alpha	Pkia	0.0164314	2.668696
10	similar to hypothetical protein DKFZp586A1524 (predicted)	RGD1305961_predicted	0.0265606	2.638761
11	similar to RIKEN cDNA 2700038I16 (predicted)	RGD1309158_predicted	0.0184892	2.570391
12	guanine nucleotide binding protein, alpha inhibiting 3	Gnai3	0.0211209	2.550937
13	CDC23 (cell division cycle 23, yeast, homolog) (predicted)	Cdc23_predicted	0.0370374	2.543075
14	apoptosis inhibitor 5 (predicted)	Api5_predicted	0.00814035	2.536787
15	similar to Ac2-190 (predicted)	RGD1306456_predicted	0.0379693	2.529796
16	zinc finger protein 68 (predicted)	Zfp68_predicted	0.0174021	2.526037
17	meningioma expressed antigen 5 (hyaluronidase)	Mgea5	0.0436885	2.514499
18	GPI-anchored membrane protein 1 (predicted)	Gpiap1_predicted	0.0409538	2.500592
19	Secreted frizzled related protein 1	Sfrp1	0.0196011	2.473321
20	stearoyl-Coenzyme A desaturase 2	Scd2	0.0620805	2.464894
21	ADP-ribosylation factor-like 6 interacting protein 2 (predicted)	Arl6ip2_predicted	0.0186805	2.454219
22	Potassium channel tetramensation domain containing 12 (predicted)	---	0.0337601	2.417893
23	asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase) (predicted)	Alg2_predicted	0.0833984	2.408374
24	amyloid beta (A4) precursor protein	App	0.0827745	2.384874
25	Similar to hypothetical protein MGC52110	---	0.0108844	2.362878
26	Eukaryotic translation elongation factor 1 alpha 1	Eef1a1	0.0457711	2.357639
27	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (predicted)	Ddx17_predicted	0.0647225	2.35301
28	germ cell-less protein	Gcl	0.00852363	2.348803
29	microtubule-associated protein 1b	Map1b	0.0681903	2.343789
30	glutamyl-prolyl-tRNA synthetase (predicted)	Eprs_predicted	0.0592263	2.340401
31	activating transcription factor 2	Atf2	0.0264141	2.326877
32	ATP/GTP binding protein 1 (predicted)	Agtpbp1_predicted	0.0571222	2.315474
33	Toll interacting protein (predicted)	---	0.0846873	2.298866
34	pleckstrin homology domain containing, family B (evectins) member 2 (predicted)	Plekhb2_predicted	0.080313	2.277113
35	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	Ddx3x	0.0310649	2.260117
36	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	Eif2s3x	0.0205031	2.250085
37	RAB5A, member RAS oncogene family	Rab5a	0.0181145	2.242339
38	LOC499526	LOC499526	0.0162544	2.238875
39	similar to Vps41 protein	LOC306991	0.00859611	2.229051
40	similar to ADP-ribosylation factor-like 10C	LOC500282	0.0217277	2.221707
41	Neural cell adhesion molecule 1	Ncam1	0.0190936	2.216564
42	septin 2	Sept2	0.0330108	2.216322
43	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	Ogt	0.0389822	2.214842
44	solute carrier family 1 (glial high affinity glutamate transporter), member 3	Slc1a3	0.0515366	2.196056
45	RAB6A, member RAS oncogene family	Rab6a	0.0266474	2.192746
46	ARP3 actin-related protein 3 homolog (yeast)	Actr3	0.0395366	2.190225
47	transcription factor 12	Tcf12	0.0663954	2.178724
48	N-ethylmaleimide sensitive fusion protein	Nsf	0.0610114	2.175178
49	sperm associated antigen 9 (predicted)	Spag9_predicted	0.0637102	2.174366
50	glycosylphosphatidylinositol specific phospholipase D1	Gpld1	0.045857	2.168987
51	RN protein	Opa1	0.0422389	2.15772
52	gamma-aminobutyric acid receptor, subunit beta 3	Gabrb3	0.0702749	2.141065
53	opioid-binding protein/cell adhesion molecule-like	Opcml	0.0497681	2.136093
54	origin recognition complex, subunit 3-like (S. cerevisiae) (predicted)	Orc3l_predicted	0.0430596	2.13468
55	Dihydropyrimidinase-like 3	Dpysl3	0.0621746	2.126934
56	similar to polybromo-1	LOC306254	0.0617496	2.121856
57	Similar to 4921517L17Rik protein (predicted)	---	0.0586624	2.109262
58	protein kinase, cAMP dependent, catalytic, beta (predicted)	Prkacb	0.0192333	2.108533
59	glia maturation factor, beta	Gmfb	0.0193211	2.096897
60	similar to dJ1033H22.1 (KIAA0554 protein) (predicted)	RGD1305386_predicted	0.0263579	2.095696
61	calnexin	Canx	0.0357595	2.074859
62	similar to RIKEN cDNA 2700038I16 (predicted)	RGD1309158_predicted	0.0730563	2.073811
63	solute carrier family 31 (copper transporters), member 1	RGD:620059	0.043329	2.073776
64	similar to Sperm 1 POU-domain transcription factor (SPRM-1) (predicted)	RGD1305526_predicted	0.0612639	2.064606
65	Imprinted and ancient	---	0.0118874	2.060976
66	caspase 3	Casp3	0.0265001	2.056867
67	similar to B230212L03Rik protein	MGC95152	0.0378747	2.052298
68	similar to RIKEN cDNA 1110067D22 (predicted)	RGD1307414_predicted	0.0185318	2.052033
69	ring finger protein 4	Rnf4	0.095306	2.050778
70	mitogen-activated protein kinase 9	Mapk9	0.055191	2.049729
71	CDNA clone MGC:93828 IMAGE:7110880	---	0.0122573	2.045555
72	Mitogen activated protein kinase 1	Mapk1	0.0056879	2.044553
73	CUG triplet repeat,RNA-binding protein 2	Cugbp2	0.0317953	2.041223
74	iron responsive element binding protein 2	Ireb2	0.0292926	2.040693
75	similar to mKIAA0267 protein	LOC302863	0.0540716	2.040614
76	similar to SOX2 protein	LOC499593	0.0926326	2.039808
77	methionine adenosyltransferase II, alpha	Mat2a	0.0496027	2.039595
78	solute carrier family 3, member 1	Slc3a1	0.0236058	2.029352
79	protein kinase C, iambda	RGD:620961	0.0209527	2.029118
80	S-adenosylhomocysteine hydrolase-like 1 (predicted)	Ahcyl1_predicted	0.0362666	2.014186
81	similar to Selenoprotein T precursor	LOC499625	0.0112761	2.013433
82	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa (predicted)	---	0.00155897	2.008439
83	Similar to hypothetical protein FLJ22965	---	0.0129648	1.996743
84	adaptor-related protein complex 2, beta 1 subunit	Ap2b1	0.0876272	1.993523
85	kinase D-interacting substance 220	Kidins220	0.0572641	1.991169
86	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3	Pik3r3	0.0990782	1.988935
87	neural precursor cell expressed, developmentally down-regulated gene 4A	Nedd4a	0.0283529	1.984545
88	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1	Galnt1	0.0163439	1.980875
89	similar to RIKEN cDNA 5730410E15 gene	LOC500865	0.0774842	1.979063
90	guanine nucleotide binding protein, alpha inhibiting 1	Gnai1	0.0680978	1.972639
91	Protein kinase, cAMP-dependent, regulatory, type 2, alpha	Prkar2a	0.0561347	1.97228
92	casein kinase 1, gamma 3	Csnk1g3	0.0638749	1.965769
93	CDNA clone MGC:105701 IMAGE:7309421	---	0.0275576	1.956493
94	dynamin 1-like	Dnm1l	0.0159999	1.952672
95	N-myristoyltransferase 2	LOC291318	0.00587368	1.951552
96	similar to RIKEN cDNA 5330414D10 (predicted)	RGD1307986_predicted	0.0461407	1.950522
97	Sprouty protein with EVH-1 domain 1, related sequence (predicted)	---	0.00457499	1.947516
98	gap junction membrane channel protein alpha 1	Gja1	0.0805725	1.945132
99	coated vesicle membrane protein	Rnp24	0.0172137	1.940236
100	tropomodulin 2	Tmod2	0.091905	1.935593

Table 3: A list of 100 genes that were significantly up regulated following treatment with SDF-1. Genes highlighted in red were selected for validation by qRT-PCR due to their biological relevance to chemokine signalling. The results of the cluster analysis are described below. Other genes of interest are marked in green.

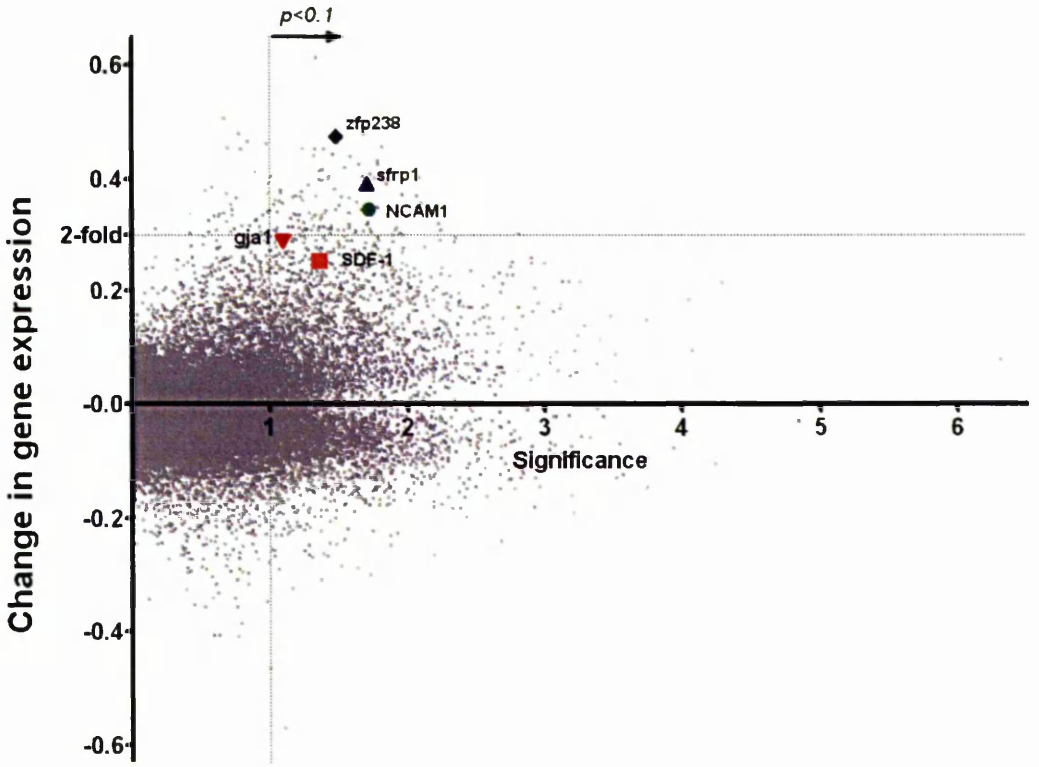

Figure 46: SDF-1 mediated changes in gene expression. Volcano plot illustrates the significance of fold change in expression for 31, 043 probes that were hybridised. X axis: - log_{10}*[p value]*, y axis; log_{10} *[mean SDF-1 gene expression]* – log_{10} *[mean control gene expression]*). 2-fold: Points above this line indicates the transcripts that were up regulated by more than 2-fold. Dotted line at x=1 indicates the significance of p=0.1. For points right of this line $p<0.1$ (ANOVA).

6.2.3 Cluster analysis of gene expression patterns

To further analyse gene expression, cluster analysis was performed to group genes into sets or clusters based upon their responses to treatment with either SDF-1 or AMD3100. Interestingly, several of the genes that were singled out based on their biological relevance to the regulatory role of SDF-1 were grouped with a cluster of genes that were down regulated in response to AMD but up regulated following exposure to chemokine (figure 47).

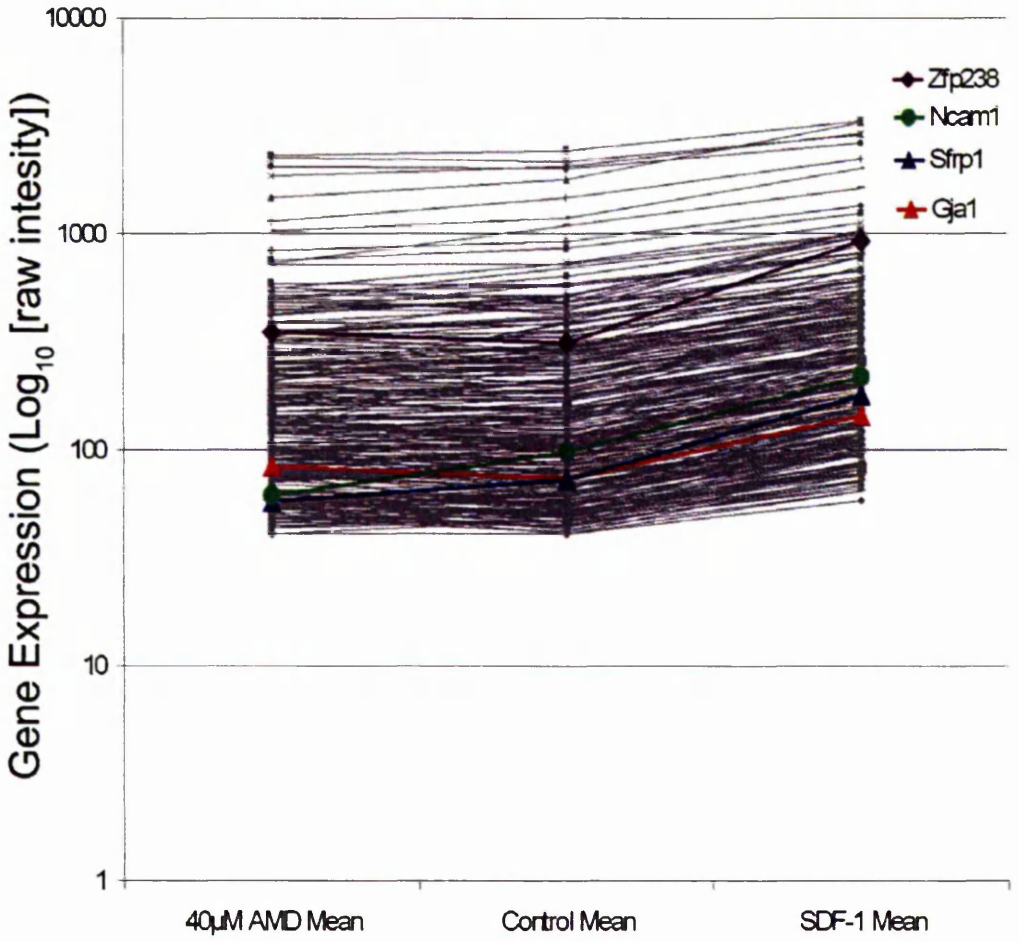

Figure 47: A cluster analysis illustrating a group of genes that were up regulated in SDF-1 treatment and conversely down regulated by AMD3100. Genes of interest are highlighted.

6.2.4 Validation of microarray data using quantitative real time PCR

To validate the data obtained with microarrays, qRT-PCR was employed. Genes were selected based on their putative relevance to SDF-1 signalling discussed in earlier chapters and also on the basis of their response to chemokine and antagonist treatments. Hence, several transcripts that displayed a significant up regulation in response to SDF-1 treatment were chosen from the cluster of genes shown in figure 47. The four genes that were selected for validation were: zinc finger protein 238 (*zfp238*), secreted frizzled related protein 1 (*sfrp1*), neural cell adhesion molecule 1 (*NCAM1*), and gap junction membrane channel protein α 1 (*gja1* – which encodes the protein connexin 43). All these genes met the stated quality control criteria, are present in a cluster of genes showing up regulation in response to SDF-1, and down regulated in response to AMD, are amongst the top 100

genes in terms of SDF-1 induced up-regulation, and may provide functional mechanisms linked to the observed effects of SDF-1 on primary forebrain cultures.

qRT-PCR demonstrated that all 4 genes were up regulated by SDF-1 treatment, although the magnitude of fold change was less than that observed in microarrays (figure 48). Notably, SDF-1 treatment up regulated the expression of all four genes by at least 10%; *gja1 (Cx 43)*: 1.2 fold, *NCAM1*: 1.8 fold, *sfrp1*: 1.2 fold, *zfp238*: 1.1 fold, *n=3 dissociated cultures*. By contrast, treatment with AMD down regulated the expression of Cx 43 and *sfrp1; Cx 43*: -1.2 fold, *sfrp1*: -1.5 fold, *n=3 dissociated cultures*. Changes in expression between SDF-1 and AMD treated cultures are illustrated in figure 49.

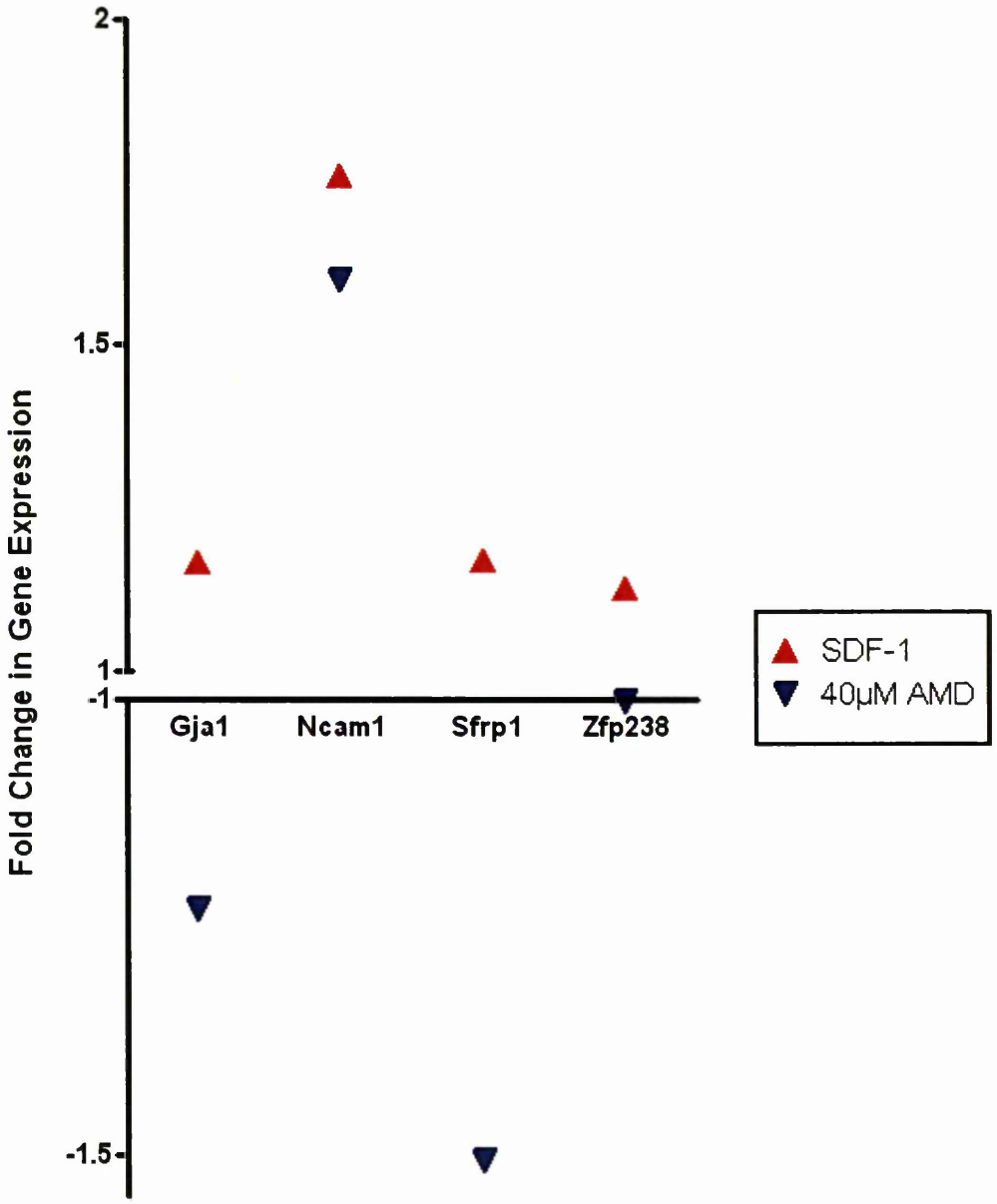

Figure 48: Fold change in gene expression between treated and control cultures. Calculated from normalised Taqman gene expression values in SDF or AMD treated cultures versus controls (average values, *n=3*).

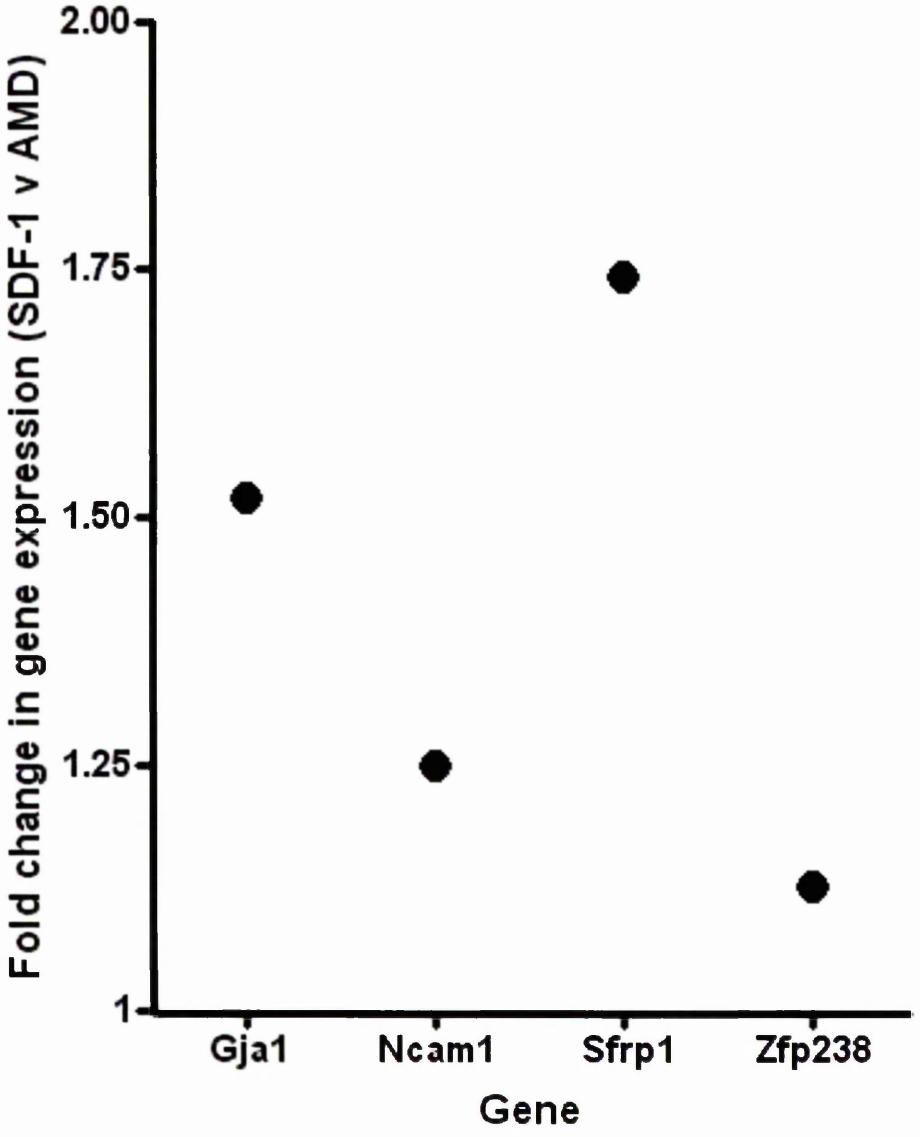

Figure 49: Fold change in gene expression in SDF-1 versus AMD treated cultures. Calculated from normalised Taqman gene expression values for SDF versus AMD treated cultures (average values, *n=3*).

6.2.5 Validation with In situ hybridisation for Sfrp1 and zfp238 in E15 mouse brain

The images presented here are taken from the St Jude Brain Gene Expression Map (BGEM, http://www.stjudebgem.org, (Magdaleno *et al.*, 2006)). Figure 50 illustrates the mRNA expression of both *zfp238* (a) and *sfrp1* (b) in mouse E15 cortex, equivalent to rat E17 cortex. Hence, both these genes have spatio-temporal patterns of expression that would enable them to play roles in cell proliferation and differentiation during cortical development.

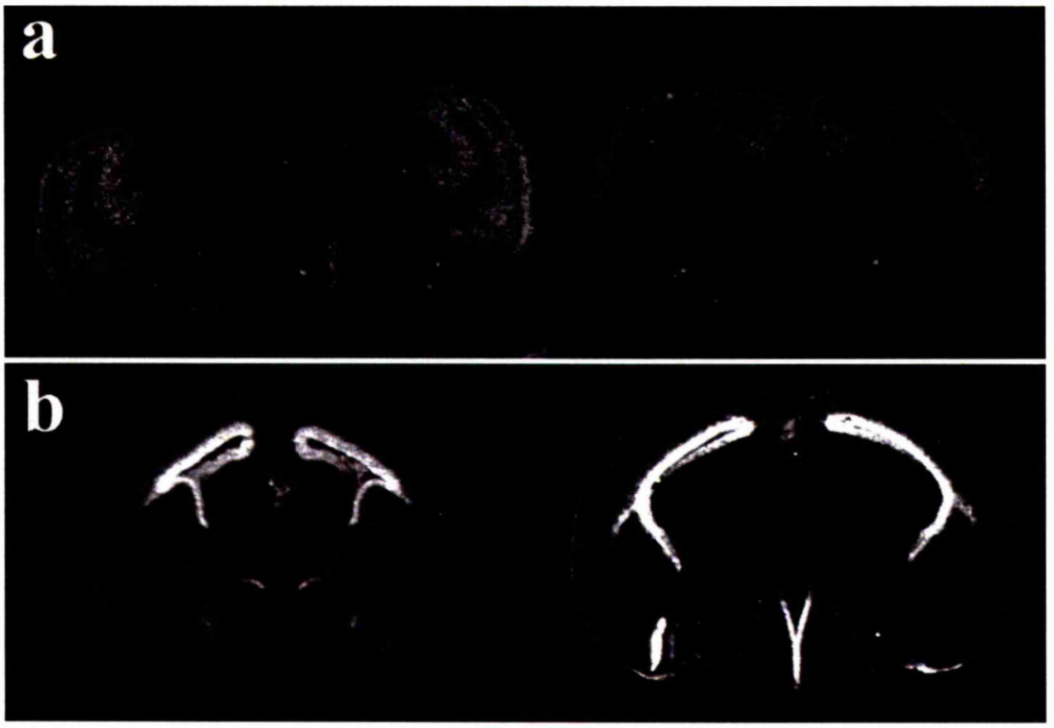

Figure 50: In situ hybridisation for *zfp238* (a) and *sfrp1* (b) in mouse E15 forebrain. Images obtained from the St Jude Brain Gene Expression Map (BGEM, http://www.stjudebgem.org, (Magdaleno *et al.*, 2006)).

6.3 Discussion

This section will discuss the relevance of the genes highlighted in table 3 with regard to the regulatory function of SDF-1 signalling during forebrain development. However, for *gja1* refer to chapter 4 for data relating to the potential role of this gene in SDF-1 mediated regulation of cortical proliferation. Each of the genes described here, could potentially play a role downstream of SDF-1 and enable the regulatory function of this chemokine seen in chapters 4 and 5.

6.3.1 Zfp238 – a potential regulator of SDF-1 target gene transcription

NB: This gene has the following aliases: 58 kDa repressor protein (*RP58*), and *ZNF238* (http://www.ihop-net.org/UniPub/iHOP/gs/95569.html).

The data presented here illustrates that the expression of *zfp238* was up regulated in cultures treated with SDF-1 and conversely down regulated in response to AMD. *Zfp238* encodes a 58kDa protein that functions as a transcriptional repressor (Aoki *et al.*, 1998). It is highly expressed in neural tissue (Zhang *et al.*, 2004) particularly in the cerebral cortex (Redmond, Jr. *et al.*, 2003; Magdaleno *et al.*, 2006). Intriguingly, its pattern of expression in the developing cortex was found to be similar to that of both *Emx1* and *NeuroD*

(Redmond, Jr. *et al.*, 2003). Given that *Zfp238* transcripts are abundantly expressed in the developing cortex, it is likely to provide a potential mechanism for SDF-1 to regulate its target genes that are involved in cortical proliferation and differentiation.

6.3.2 Sfrp1 – a role in SDF-1 mediated axonal growth?

Sfrp1 is known to antagonise Wnt signalling (Finch *et al.*, 1997; Kawano and Kypta, 2003), and could therefore potentially play a role in regulating processes such as proliferation, differentiation and axonal growth that are known to be influenced by Wnt signalling (Ille and Sommer, 2005). As illustrated in figure 47, *Sfrp1* gene is strongly expressed in the germinal zones of the developing cortex and ganglionic eminence *in vivo* (Augustine *et al.*, 2001; Magdaleno *et al.*, 2006). Intriguingly, Sfrp1 protein has been shown to promote neurite outgrowth in retinal neurons *in vitro*, independent of Wnt inhibition (Rodriguez *et al.*, 2005). Since *Sfrp1* expression was up regulated following treatment with SDF-1, it is likely that it may have promoted axonal extension in cortical neurons in a manner similar to that shown in retinal explants (see chapter 5).

6.3.3 Microtubule associated protein 1b – a role in SDF-1 mediated neuronal differentiation and migration?

Microtubule associated protein 1b (MAP1b) initially prevalent in immature neurons diminishes with progressing ontogeny. Interestingly, mutant mice that lack this protein have a delayed neuronal development (Takei *et al.*, 1997). Given that MAP1b is associated with axonal elongation and neuronal migration (Gonzalez-Billault *et al.*, 2001, 2005), it is plausible that SDF-1 exerts its regulatory function, at least in part, through the downstream activation of MAP1b.

6.3.4 NCAM1 – a possible component downstream of SDF-1 signalling that regulates axonal growth and neuronal migration

NCAM1, in its polysialic acid (PSA) bound moiety, is thought to regulate neuronal migration during development (Ono *et al.*, 1994; Ulfig and Chan, 2004). In this context, recent studies have demonstrated a role for NCAM1 in neuronal differentiation and plasticity (Pillai-Nair *et al.*, 2005; Seidenfaden *et al.*, 2006). Although, treatment with SDF-1 up regulated NCAM1 gene expression in cortical cultures, the function of this cell adhesion molecule is dependent on it's binding to PSA. Intriguingly, microarray data showed that a gene encoding one of the enzymes (*SIAT8B* encoding sialyl transferase 8B)

responsible for the transfer of PSA to NCAM was also up regulated following treatment with SDF-1. However, its expression was too low for it to be considered significant for further analysis.

6.3.5 Glial maturation factor-β and SDF-1 mediated differentiation

Glial maturation factor-β (GMFβ), originally purified from bovine brain, was shown to promote the differentiation of both neurons and glia in neuroblastoma and glioma cell lines (Lim *et al.*, 1989; Lim *et al.*, 1990). In the adult brain it is expressed in the cortex (Inagaki *et al.*, 2004). Phosphorylation of GMFβ by protein kinase A has been shown to strongly inhibit signalling via the ERK1/2 (p44/p42) subfamily of MAP kinases (Zaheer and Lim, 1996). In addition, GMFβ is known to promote the activity of p38 MAP kinase (Lim and Zaheer, 1996) *in vitro*. However, less is known about the expression and function of GMFβ during brain development. Interestingly, a recent study has reported on the expression of this protein following injury and its potential as a putative tumour suppressor because of its anti-proliferative effects (Hotta *et al.*, 2005). Taken together, it is possible that SDF-1 induced up-regulation of GMFβ expression might have enabled a cross talk between the components of the MAP kinase cascade that are known to regulate cell proliferation and differentiation.

6.3.6 Mitogen activated protein kinases 1 and 9: regulation of cell proliferation

SDF-1 is known to function upstream of MAP kinase signalling cascades, and as discussed in chapter 4, signalling via these pathways is one mechanism by which the chemokine might influence cell proliferation (Bajetto *et al.*, 2001; Bonavia *et al.*, 2003; Peng *et al.*, 2004; Gong *et al.*, 2006). Thus, SDF-1 mediated up regulation of MAP kinases is likely to provide a downstream mechanism for the chemokine signalling to exert its regulatory effects during cortical development.

6.3.7 Phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (PIK3R3) - regulation of intracellular signalling

As discussed in chapter 4, PI-3 kinase is a key player in the regulation of intracellular signalling that has been implicated in cell proliferation (Liang and Slingerland, 2003). Since PIK3R3 or $p55^{PIK}$ is expressed by neurons in the developing brain (Pons *et al.*, 1995; Shin *et al.*, 1998), up regulation of this gene could provide a

potential mechanism by which SDF-1 could exert its regulatory role in cortical cell proliferation.

6.3.8 Neural precursor cell expressed, developmentally down-regulated gene 4A (Nedd4a)

Nedd4a was one of the 10 cDNA clones originally isolated from neuronal precursors whose mRNA expression decreased with progressing brain development (Kumar *et al.*, 1992). Subsequently it was shown to be expressed in other adult tissues and to contain an ubiquitin ligase domain (Kumar *et al.*, 1997), implicating it in the regulation of turnover of membrane channels, receptors and transporters (Harvey and Kumar, 1999). Interestingly, it has been shown to bind to and down regulate the TrkA neurotrophin receptor (Arevalo *et al.*, 2006). Hence, up regulation of this gene by SDF-1 could potentially be a feedback mechanism to control the activity of CXCR4 by ubiquitination.

7 Discussion: A wider view of SDF-1 signalling during forebrain development

7.1 Future directions and technical limitations

Although the work presented here illustrates that SDF-1 plays key roles in regulating proliferation and differentiation during cortical development, many questions still remain unanswered. As mentioned previously, it seems reasonable to suggest that SDF-1 mediated effects on axonal growth are likely to function via Rho-GTPase linked pathways. However, further experiments using pull down assays to quantify changes in Rho activation downstream of SDF-1 signalling are required. In order to look for interactions between SDF-1 and other signalling pathways, immunoprecipitation or yeast two-hybrid screens could be used. However, these approaches could only reveal direct physical interactions between SDF-1 and other proteins, and potential candidates must first be identified. One such candidate is PSA-NCAM, which is expressed in a spatially related pattern to SDF-1 in the intermediate zone (Daniel *et al*, 2005), and associated with tangentially migrating interneurons (Ulfig and Chan, 2004), and its expression appears to be up-regulated by SDF-1 (see section 6.2). If PSA-NCAM were to bind directly to SDF-1, it could perhaps sequester SDF-1 protein to localised regions that might serve as guidance cues for tangentially migrating interneurons.

The microarray data also provides an obvious start-point for further research, and there remains much to be done to elucidate which signalling pathways are modulated by SDF-1 during forebrain development. Perhaps most intriguing is the data relating to *sfrp1* and *MAP1b*, both of which seem to be linked to the role played by SDF-1 during axonal growth. In addition, the function of axonal growth may also be linked to *NCAM1* expression, which could be further tested by Western blotting.

Since AMD3100 has been shown to function as a weak partial agonist (Zhang *et al*, 2002), despite blocking the binding of SDF-1 to CXCR4 (Schols *et al*, 1997), other approaches may cause a stronger inhibition of SDF-1 signalling. For example, neutralizing antibodies for SDF-1 could be used to inhibit receptor activation. However, the availability of antibodies limits this approach. The use of RNAi technology (reviewed by Genc *et al*, 2004) to silence the expression of SDF-1, CXCR4, or both, may give better results. Indeed, RNAi techniques have been successfully applied in cultured primary neurones (Krichevsky

et al, 2002) and also *in vivo* in mouse brain (Xia *et al*, 2002). Nevertheless, the success of these methods largely depends on achieving adequate transfection efficiency without inducing cytotoxicity.

The generation of conditional knockout mice for tissue specific gene deletion of SDF-1 or CXCR4 would provide the most powerful tool to investigate the chemokine signalling in the developing forebrain. Using the Cre/loxP technology, it would be possible to ablate the expression of the chemokine or receptor, by generating a target mouse line with a modified allele of the gene flanked by two loxP sites. Subsequent crossing of this target mouse line with a transgenic line carrying the Cre recombinase, under the control of a promoter that is specific to dorsal forebrain, will result in a tissue specific ablation of SDF-1 or its receptor in the dorsal telencephalon. In this context, *EMX1*-cre or *BF1* (brain factor 1)-cre mouse lines are currently available. Using such models it would be feasible to investigate the role of SDF-1 signalling in the postnatal cortex, which at present is impossible due to the embryonic lethality of $CXCR4^{-/-}$ and $SDF\text{-}1^{-/-}$ mutants. Furthermore, *in vivo* injection of BrdU into CXCR4 null embryos, would allow further analysis of the effect of disrupted SDF-1 signalling on proliferation and neuronal migration *in vivo*.

Further, to dissect the intracellular signalling pathways downstream of SDF-1, specific kinase cascade inhibitors, such as wortmannin (PI3K inhibitor) and pertussis toxin (G-protein coupled receptor inhibitor) could be used to rule in or out the involvement of various kinase cascades. This method has previously been used to illustrate the role of PI3K downstream of SDF-1 in the regulation of proliferation of neuronal progenitors *in vitro* (Bajetto and Adriana, 2001; Gong *et al.*, 2006).

7.2 Multiple roles for SDF-1 signalling in forebrain development

The aim of this project was to investigate the role of SDF-1 in the developing forebrain. We proposed that SDF-1 regulates proliferation, differentiation, cell migration and axonal growth in the developing cerebral cortex. This was based on observations made in other systems; notably within the developing cerebellum (Arakawa *et al.*, 2003; Klein *et al.*, 2001; Ma *et al.*, 1998; Reiss *et al.*, 2002; Zhu *et al.*, 2002; Zou *et al.*, 1998) hippocampus (Bagri *et al.*, 2002; Lu *et al.*, 2002), and ganglionic eminences (Stumm *et al.*, 2003). The observations discussed here, highlight parallel roles for SDF-1 signalling in the developing cortex in all these processes.

Perhaps the most compelling evidence relates to cortical progenitor proliferation in primary cortical cultures, in which SDF-1 promotes proliferation, whereas inhibition of

signalling via CXCR4 (either in antagonist treated cultures, or cultures lacking the receptor) showed a converse effect. This has been supported by other studies, although these used isolated cortical neuronal precursors (Gong *et al.*, 2006), whereas the primary cultures used here contain all the cell types of the developing cortex and are hence one step closer to the *in vivo* situation. Furthermore, the data presented highlights a putative mechanism by which SDF-1 might regulate proliferation. It is plausible that SDF-1 could provide the initial signal required to start the calcium waves which propagate between radial glia across the ventricular zone (Weissman *et al*, 2004), and possibly also increase the number of functional gap junctions through regulation of *Cx43* gene expression. This provides a mechanism by which SDF-1 present in the intermediate zone *in vivo* could activate CXCR4 receptors on radial glial fibres, causing release of Ca^{2+} from intracellular stores, thereby starting a wave of calcium transients via gap junction coupling, and hence regulating proliferation *in vivo*. The observations of calcium transients in the ventricular zone (Weissman *et al.*, 2004), and the reduced cortical size in $CXCR4^{-/-}$ mice (Lu *et al.*, 2002) both support our findings. Furthermore, the importance of gap junction signalling during cortical development has been highlighted using RNAi knockdown of Cx43 expression within rat embryos *in vivo*, demonstrating a disruption in radial migration. Indeed, neurons in which *Cx43* expression was knocked down were unable to migrate across wild type cortex following transplantation experiments (Elias *et al.*, 2006).

The expression pattern of SDF-1 in rat E17 cortex shows that the chemokine is ideally placed to exert a response in radially migrating neurons, via a mechanism again possibly involving Cx43 expression, and coupling between radial glia and radially migrating neurons. Given that SDF-1 is localized to the subplate/cortical plate, with weak expression extending into the intermediate zone, it could serve as a chemoattractant for migrating neurons, impart signals for layer integration and regulate phenotype specification. Although it has been suggested that SDF-1 could serve as a chemoattractant for tangential migration, this theory was based on its effects on isolated E14 striatal precursors *in vitro* (Stumm *et al.*, 2003). Based upon the data available now, it seems equally likely that SDF-1 acts as a chemoattractant for radially migrating neurons. It may also impart layer information to tangentially migrating neurons derived from the GE once they reach the dorsal forebrain. This could be dependent upon other environmental cues, such as those provided by the reeler signalling pathway, which has been shown to influence tangential (Morante-Oria *et al.*, 2003), as well as radial migration.

As has been shown, SDF-1 regulates axonal growth *in vitro*. Since it is present in the cortical plate and the intermediate zone it is plausible that the chemokine functions as a guidance cue for cortical afferents. Given that neurons express CXCR4 (Bajetto *et al.*, 2001), concentrated at the leading edge of neuronal processes (Pujol *et al.*, 2005), it is likely that SDF-1 provides a chemotactic gradient for growing cortical axons.

7.3 SDF-1 is a primordial signalling molecule

As SDF-1 and its receptor CXCR4 are well conserved, it has been suggested that the chemokine is a primordial signalling molecule (Ma *et al.*, 1998), a statement which is supported by the parallel roles for SDF-1 signalling observed in other systems both during development, and in mature tissues. As described previously, SDF-1 regulates proliferation, differentiation, axonal growth and migration in the developing cortex, with similar functions having been observed in both the hippocampus and the cerebellum. However, similar regulatory functions for SDF-1 have been alluded to in non-neural systems and disease states, and in the developing and adult immune-system (reviewed by Klein and Rubin, 2004), during cancer, where SDF-1 may regulate cell proliferation and migration in the development of metastases (Muller *et al.*, 2001; Rubin *et al.*, 2003; Woerner *et al.*, 2005), and during angiogenesis (reviewed by Salcedo and Oppenheim, 2003) where SDF-1 is known to act as a chemoattractant for endothelial cells *in vivo* (Salcedo *et al.*, 1999). Indeed, patterns of angiogenesis and innervation (reviewed by Carmeliet and Tessier-Lavigne, 2005) are known to be extremely similar, possibly providing further potential for parallel functions of SDF-1 signalling in PNS axon guidance and angiogenesis.

7.4 The importance of understanding SDF-1 signalling during forebrain development

Given that the cortex of $CXCR4^{-/-}$ mice is reduced in size (Lu *et al*, 2002), and diseases such as schizophrenia have been associated with disorders of cortical development (Lewis, 2002), it raises the possibility that aberrant SDF-1 signalling might contribute to the underlying pathology of many developmental brain disorders. Malformations of cortical development (MCD, reviewed by Barkovich *et al*, 2005), vary greatly in severity and symptoms, ranging from minor learning difficulties to schizophrenia and epilepsy and through to severe retardation. MCD is classified based on the developmental process where the defect is first noted, in cell proliferation, migration or differentiation. Many arise from

defects in neuronal migration, including periventricular heterotopia (neurons fail to migrate radially from the VZ), subcortical band heterotopia and lissencephaly. Molecular genetics studies focussing on MCD have identified several genes in which mutations lead to specific clinical disorders. A notable example is *Lis1*, mutations in which cause lissencephaly (an absence (agyria) or partial absence (pachygyria) of convolutions in the cortical surface to give a smooth cortex, reviewed by Guerrini and Marini, 2006). Hence, the changes in neuronal migration noted following exposure to elevated levels of SDF-1 indicate that abnormal signalling via CXCR4 could potentially result in similar defects in cortical development *in vivo*.

Many forms of MCD can also give rise to epilepsy (reviewed by Sisodiya, 2004), and intriguingly, gap junctions have also been implicated in this disease state (Szente *et al.*, 2002). Since SDF-1 regulates the expression of gap junction proteins and intercellular coupling, it is conceivable that the chemokine signalling might directly or indirectly contribute to the pathophysiology of epilepsy.

Schizophrenia is associated with changes in GABAergic interneurons (Lewis, 2000; Lewis *et al.*, 2005), and the expression of *GAD65/67* mRNA is known to decrease in patients with schizophrenia (Akbarian *et al.*, 1995; Akbarian and Huang, 2006). Interestingly, these changes have been linked to NCAM, and transgenic mice over-expressing this protein (from a neuron specific promoter) were shown to have decreased synaptic connectivity between GABAergic interneurons (Pillai-Nair *et al.*, 2005). Based on the premise that SDF-1 regulates the expression of *ncam1* and *Dlx*, as well as the differentiation of GABAergic interneurons, it is tempting to speculate that altered chemokine signalling might indirectly contribute to pathologies relating to schizophrenia.

It is now evident that SDF-1 promotes the proliferation of neural progenitors. In this context, *SDF-1* and *CXCR4* mRNA are up regulated in paediatric brain tumours, while systemic administration of the CXCR4 antagonist AMD3100 inhibited the growth of intracranial xenografts derived from glioblastomas or medulloblastomas (Rubin *et al.*, 2003). These results suggest that aberrant signalling or expression of SDF-1 and CXCR4 during development may therefore trigger the development of paediatric brain tumours.

CXCR4 is known to be one of the receptor proteins by which HIV gains entry into host T-cells (Bleul *et al*, 1996; Feng *et al*, 1996; Schols *et al* 1997), and was therefore identified as a therapeutic target for the treatment of AIDS, which lead to the development of the CXCR4 antagonist AMD3100 (Donzella *et al*, 1998, De Clercq, 2003). Given that the HIV coat protein gp120 binds to CXCR4, which results in neurotoxicity (Bezzi *et al*,

2001), it would be interesting to know what effects, if any, HIV infection may have on CNS development. Since HIV infection appears to increase activation of CXCR4, one could hypothesize that the effects would be similar to those seen *in vitro* in response to increased levels of SDF-1. However, to date, no such study has been undertaken.

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Appendix – Publications Arising

Paper: Liapi A, Pritchett JP, Jones O, Fujii N, Parnavelas JG, Nadarajah B (2006): SDF-1 signaling regulates radial and tangential migration in the developing cerebral cortex (manuscript accepted, October 2006). Developmental Neuroscience

Paper: Pritchett JP, Wright C, Zeef L, Nadarajah B (2006), Stromal derived factor-1 exerts differential regulation on distinct cell populations of the cerebral cortex (manuscript submitted July 2006). BMC Developmental Biology.

Poster: Pritchett JP *et al* (2006), SDF-1/CXCR4 signalling regulates proliferation during cortical development International Society for Developmental Neuroscience Annual Meeting (Banff, Canada).

Meeting Report: Pritchett JP (2006), British Neuroscience Association Bulletin 53, 28-29.

Poster: Pritchett JP *et al* (2005), SDF-1/CXCR4 signalling regulates proliferation and differentiation during cortical development, Society for Neuroscience Annual Meeting (Washington DC).

Poster: Pritchett JP *et al* (2004), SDF-1alpha/CXCR4 signalling in cell proliferation and differentiation of forebrain neurons, International Society for Developmental Neuroscience Annual Meeting (Edinburgh).