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Role of CXCR4 dynamics and activity in neural crest migration

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I, Roger Singleton Escofet confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Abstract

During early embryonic development the neural crest migrates from its site of induction to various locations within the embryo; a peculiarity of this movement is that cells move collectively. Contact Inhibition of Locomotion (CIL) is required for directional migration of the neural crest cells. In addition, negative and positive signals present in neighbouring tissues help guide the group. Previous experiments have shown that group of cells are able to respond to these signals more efficiently than single cells, but how clustering neural crest cells contribute to a better response to chemoattractants is poorly understood.

The major neural crest chemoattractant is SDF1 that activates the receptor CXCR4 expressed in the neural crest cells and is required to guide neural crest migration. In this thesis we analysed the dynamic of CXCR4 during neural crest chemotaxis. We found that the CXCR4 is constantly internalised and trafficked within the cell, even in absence of SDF1. However, treatment of neural crest cells with SDF1 promotes a change in the endocytic pathways of the receptor. Importantly, we found that endocytosis of CXCR4 is polarized in neural crest cells, with higher rates of endocytosis at the free edge compared to the cells contact. The overall effect is that the free edge membrane is primed respond efficiently to SDF1. We show that this endocytic polarity is Rac1 and RhoA dependent, two GTPases whose activity is controlled by cell-cell contact.

In order to study the role of CXCR4 endocytosis in neural crest migration several mutant forms of the receptor were expressed and the response to SDF1 was analysed in vivo and in vitro. When a mutated CXCR4 that cannot be internalized is expressed, a dramatic effect on directional cell migration was observed. Cells are still able to respond to SDF1 but they exhibit a diminished capacity to adapt to changes in the position of the SDF1 source. When a constitutively active CXCR4 receptor is expressed in neural crest cells, their migration is also affected. Cell migration becomes independent on SDF1 and they disperse colonizing tissues that never contain neural crest cells.

In conclusion, our results show that cell-cell contact controls CXCR4 endocytosis, which is likely to explain why groups of cells respond better than single cell to the ligand SDF1. In addition, we show that the endocytosis and activity of CXCR4 need to be tightly regulated to allow a correct neural crest migration in vivo and in vitro.

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Abbreviations

CIL	Contact Inhibition of Locomotion
CISK	Cytokine Independent Survival Kinase
CME	Clathrin Mediated Endocytosis
CXCR4	CXC Receptor 4
ECM	Extracellular Matrix
EMT	Epithelial to Mesenchymal Transition
GFP	Green Fluorescence Protein
GPCR	G-Protein Coupled Receptor
GRK	G-Protein Coupled Receptor Kinase
IP_3	Inositol 1,4,5-tisphosphate
MO	Morpholino Oligomer
NAM	Normal Amphibian Medium
NC	Neural Crest
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-kinase
SDF1	Stromal Derived Factor 1
TM	Transmembrane
WHIM disease	Warts, Hypogammaglobulinemia, Infections and Myelokathexis disease

Part I

Introduction

Chapter 1 - Introduction

Early embryological development is a complex process through which all multicellular organisms must go through to grow from the initial zygote to a complex organism. The process is characterised by the extreme complexity of the many events that take place simultaneously and help specify a variety of cells that will become the various organs and tissues of the organism. These events take place at precise times of embryo development and may require intricate rearrangement and movement of cells. This is a common feature of the developing embryos in which similar cells will arise in the same area of the embryo and migrate through clearly signposted paths to their final destination where they will specify to become a particular cell type.

One such movement is the one undertaken by the neural crests which migrate following discreet pathways throughout the embryos form dorsal to ventral positions of the embryo. The study of this movement highlights the many signals involved in each individual event during embryological development and how tight coordination is necessary for it to occur correctly; failure to do so will impair future development

1.1 The Neural Crest

The neural crest is a pluripotent cell population which is specified at the border of the neural plate and later migrates to a range of areas of the embryo where

the cells differentiate. Although all the cells are specified in a similar manner they can give rise to multiple cell types, these range from parts of the peripheral nervous system to various facial structures and therefore their correct development is crucial for the normal growth and survival of all vertebrates (Knecht & Marianne Bronner-Fraser 2002; P. Kulesa et al. 2004).

The migration of these cells is regulated through mechanisms which ensure that they arrive to the correct destination in a timely manner (Crane & Trainor 2006; Minoux & Rijli 2010). In many instances this migration occurs collectively, in *Xenopus laevis* the cephalic neural crest migrate following discreet streams in which the cells remain in contact with one another until they arrive to their target in the ventral side of the embryo (Carmona-Fontaine et al. 2008). Chick Neural Crest show a similar migratory phenotype with the cells moving as a group remaining in close contact which is important to aid the migration (Teddy & P. M. Kulesa 2004). The neural crest therefore provides an excellent model to study how cells interact with each other and how these interactions help the cells move collectively (Theveneau & Roberto Mayor 2011c). This is of particular interest as cancer cells show similar migratory mechanisms during metastasis (Friedl et al. 2012; Friedl & Darren Gilmour 2009). There has been evidence linking processes such as EMT undergone by Neural crest that are hijacked in breast cancer (Haslehurst et al. 2012). The collective nature of the migration which neural crest exhibits has also been seen in cancer (Friedl et al. 2004)

1.1.1 Neural Crest induction

The induction of the neural crest occurs during the gastrula stages of the early development of the embryo. The site of induction of this cell type is at the neural plate border; here it receives various signals from the two adjacent tissues (neural plate and underlying mesoderm); these signals are required to induce tissue to become the neural crest (R Mayor et al. 1995). Various genetic cascades become active as a result of this signalling leading to the expression of particular transcription factors required for each step of the induction and alter specification of the neural crest (Sauka-Spengler & Marianne Bronner-Fraser 2008a).

The inductive events that take place can be split into various stages; an initial inductive process, then a specification event followed by a final maintenance phase. Each of these episodes have different signalling pathways involved which are switched on and off at particular moments; these fluctuations in signalling pathways mediate the transition between each step of the process. It is known that a combination of low and high levels of BMP and Wnt are crucial in determining these different phases as well as FGF signalling which also plays a role in inducing neural crest (Sauka-Spengler & Bronner-Fraser 2008a; Steventon et al. 2009).

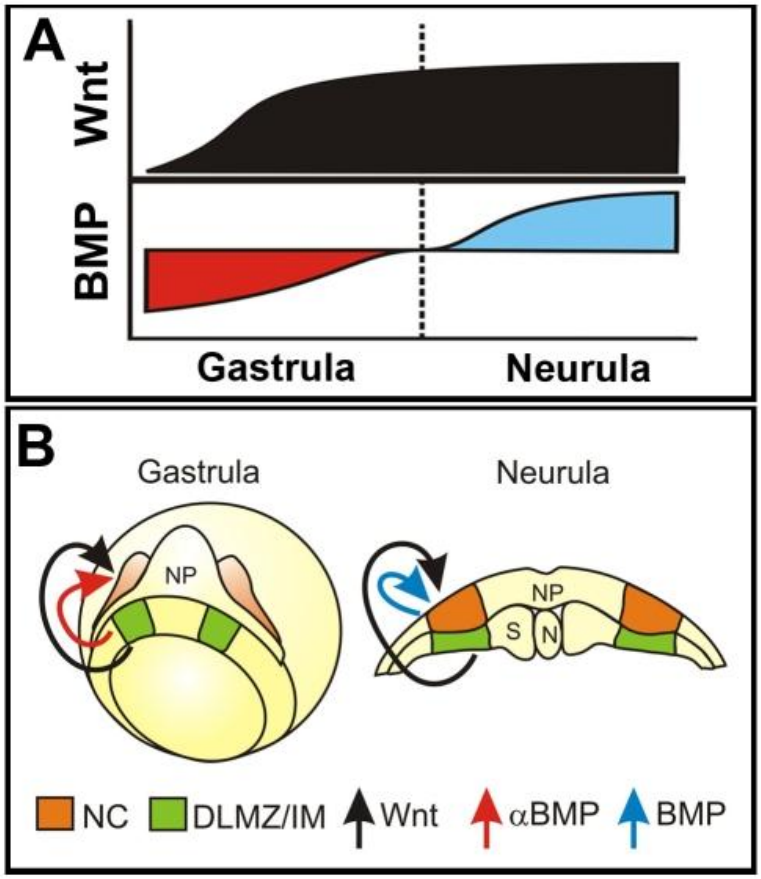


Figure 1.0-1 – Model of neural crest induction. The process takes gastrula and neurula stages. Wnt and anti-BMP signals from the dorso lateral marginal zone (DLMZ) and intermediate mesoderm (IM) are initially required to induce the tissue to become neural crest. A surge of BMP then follows which drives the specification of the cells at the neural plate border to become neural crest. The final maintenance phase requires high levels of both BMP and Wnt. Figure from Steventon et al. 2009.

Wnt signalling is low before the initial inductive event at the region of neural plate border; this is necessary to prime the area to become neural crest (Steventon & Mayor 2012); the actual induction can then take place. The ectoderm cells interpret the signals they receive from the adjacent tissues, in particular from the dorso-lateral marginal zone of the mesoderm which underlies the prospective NC and the adjacent epidermal tissue (R Mayor et al. 1995; Mancilla & R Mayor 1996). This inductive process combines a surge of Wnt signalling alongside an inhibition of BMP signalling which triggers the initial specification of the neural plate border to become neural crest. In the later maintenance phase the anti-BMP signal is replaced by an increase in BMP signalling, which allows for the maintenance of the NC region until later delamination and migration (García-Castro et al. 2002; Steventon et al. 2009); this step by step model of induction is summarised in Figure 1.0-1.

Other than the BMP and Wnt signals described; other pathways are also required to the process of specifying the neural crest. FGF signalling from the underlying mesoderm is necessary to induce the neural crest and is capable of inducing neural crest markers in particular *Slug* (R Mayor et al. 1997; Monsoro-Burq et al. 2003). Another important signal comes in the form of Retinoic Acid which is provided by the mesoderm and signals to complete neural crest specification (Villanueva et al. 2002).

Once the neural crest is specified it delaminates and migrates away from the now closing neural tube; this process starts at around stage 19 in *Xenopus*. The migration follows clear pathways in a stream like structure from the dorsal to ventral positions in the embryo (Theveneau & Roberto Mayor 2011b). Once

they arrive to their destination where they terminally differentiate into a wide variety of cell types. These include components of the peripheral nervous system, glial and sensory neurones, cartilage and connective tissue in the cranial regions and melanocytes throughout the embryo (Knecht & Marianne Bronner-Fraser 2002; Taylor & Carole LaBonne 2007). The terminal differentiation is governed by different Sox transcriptions factors which determine the terminal cell fate (Sauka-Spengler & Bronner-Fraser 2008b).

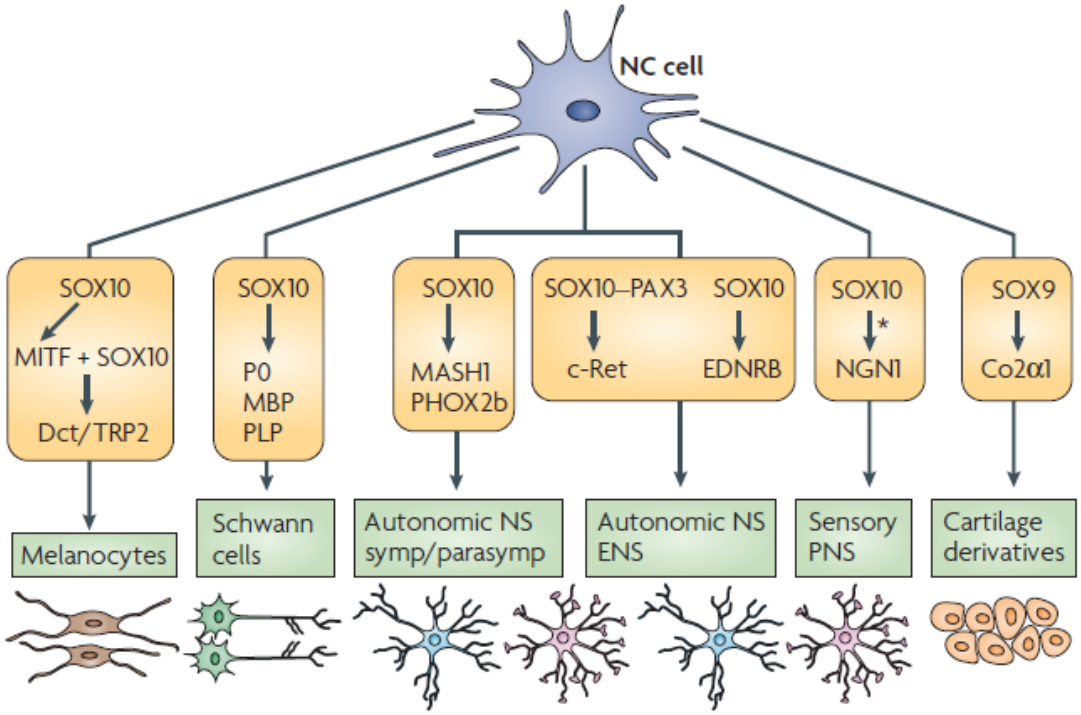


Figure 1.0-2 – Neural crest's different fates. Sox genes regulate the differentiation of NC cells into a variety of different other cell types at the various sites across the embryo. Figure extracted from Sauka-Spengler & Marianne Bronner-Fraser 2008.

1.2 Neural Crest Migration

The neural crest starts of as an epithelial tissue tha' delaminates away from the neural tube. Initially the cells can start migrating as an epithelial sheet which means migration is slow and the cells are closely packed together. A later process called EMT will allow cells to change from epithelial to mesenchymal characteristics; this will allow cells to change shape and become more independent. The now mesenchymal cells will migrate ventrally as a close group forming a series of streams form anterior to posterior positions. The cells stop migrating at predetermined locations where they will terminally differentiate.

1.2.1 NC delamination and EMT

Throughout the migration of neural crest many signals act to tightly regulate the process; the main reason is that the cells migrate through specific spaces and the movement has to be coordinated with other events taking place at the same time in development (Krull 2001); this includes many tissue rearrangements. As the cells start delaminating away from the neural they begin to lose their epithelial identity; this occurs through a process called epithelial to mesenchymal transition (EMT) and changes the characteristics of the cells allowing them to migrate as mesenchymal cells; this involves changes in cell shape and adhesion permitting the cells to leave the neuroepithelium and behave more like individual entities. (Thiery & Sleeman 2006).

One of the key features of EMT is the change of adhesion molecules which maintain cells together, in particular a switch between E to N-cadherin is

believed to be an important feature of EMT in Neural crest (J.-L. Duband 2010). This change of cadherin expression is characteristic of EMT not just in neural crest but other tissues that undergo this process (Maeda et al. 2005).This switch together with the expression of different integrin molecules which change the cells affinity to the substrate (Testaz et al. 1999) and MMPs allow the neural crest to change shape and slowly migrate out of the now folding neural plate and attach to the extracellular matrix in a stream like manner (Theveneau & Roberto Mayor 2012a). EMT is of interest since cancer cells are believed to be capable to undergo a similar process which allows them to change their migratory capabilities (Brabletz 2012; Micalizzi et al. 2010; Craene & Berx 2013; Friedl & Wolf 2003).

The changing adhesion properties of neural crest and the timing of these events are of great interest in order to understand the mechanism that governs this event and how tumours cells might replicate this. Transcription factors such as Twist have recently been involved in the suppression of E-cadherin expression (Barriga et al. 2013); the switching on of twist is a hallmark of the migratory neural crest. Further experiments; not yet published; show that the switch between E to N-cadherin is fairly complimentary and may occur in a wave along the neural crest and importantly there are distinct migratory capabilities of cells expressing either one or the other cadherin.

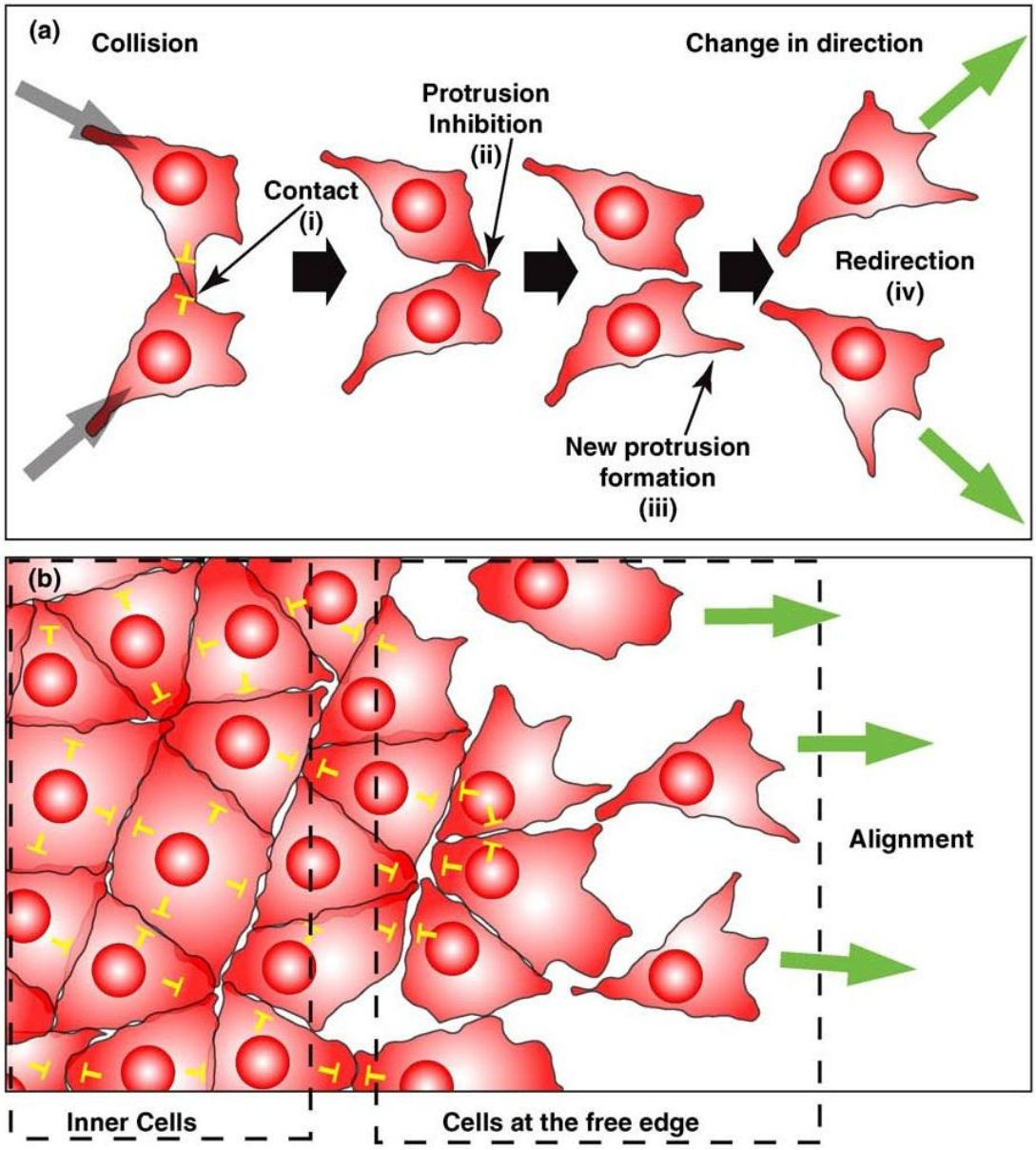
An important transcription factor involved in this transition and expressed in the neural crest is *Slug*; its role is to repress the expression of E-cadherin and triggers EMT (Bolos et al. 2002). When *Slug* is inhibited, the neural tube fails to close properly and the neural crest are unable to emigrate away from the neural

plate (Nieto et al. 1994). *Slug* is transcribed as a result of neural crest induction and represses gene expression through a zinc finger motif (C LaBonne & M Bronner-Fraser 2000). *Slug* and other neural crest markers necessary for EMT have been related to cancer progression (Pérez-Mancera et al. 2005; Haslehurst et al. 2012).

1.2.2 Collective Migration of the Neural Crest

After delamination and undergoing EMT the neural crest cells move away from the neural plate; these cells migrate as a closely packed group and still maintain many contacts between each other; this has been shown to be an important feature of the migration of neural crest (Theveneau & Roberto Mayor 2012b; Carmona-Fontaine et al. 2008). In *Xenopus* as in most organisms Cephalic neural crest form a series of streams in the mandibular, hyoid and branchial arches through which the cells migrate ventrally (Sadaghiani & Thiébaud 1987).

Figure 1. 3 – Summary of the Contact Inhibition of Locomotion model. (a) when a single cell exhibiting CIL comes in contact with it will collapse its protrusion once A new protrusion will be formed causing the cell to repolarise and migrate away in this new direction. (b) when cells in a group exhibit CIL; such as the neural crest; there are two possibilities depending on the location of each cell. Cells in the inside of the cluster cannot form protrusions and stay still. Cells at the edge can only form protrusions away from the centreand will migrate in that direction. In the tightly packed embryo this also drives to aligning the cells and therefore contributes to directional movement. Extracted from Mayor & Carmona-Fontaine, 2010


(a)
Collision
Change in direction
Protrusion
Inhibition
(ii)
Contact
(i)
Redirection
(iv)
New protrusion
formation
(iii)
(b)
Alignment
Inner Cells
Cells at the free edge

1.2.2.1 Contact Inhibition of Locomotion

The contacts formed by the group of migrating cells are themselves a characteristic that drive migration. The contacts formed allow the cells to polarise through a process known as Contact Inhibition of Locomotion (CIL). CIL was initially identified by Michael Abercrombie during the decades spanning from the 1950-70s (Abercrombie & J. Heaysman 1953; Abercrombie et al. 1970), during this period he described how migrating cells would collapse their protrusions when they came into contact with another cell causing them to stop migrating. The colliding cell would then repolarise its protrusion and migrate in a new direction. He identified that the cells collapse their protrusions as a direct result of the contact and hence suggested that cell contact inhibits migration (Figure 1.2a).

This same phenomenon has been found to take place in the migrating neural crest; here the cells migrate as groups so the result is slightly different. The cells in the middle will form contacts in all directions therefore these cells exhibit CIL throughout the membrane and are unable to produce protrusions and therefore do not move and remain in the tightly packed formation (Mayor & Carmona-Fontaine 2010). This contrasts with the cells at the edge of the group which solely make contacts with other cells at the back. The other side of the cell facing outwards is free to form protrusions and migrate in that direction; the result of this is that the leading line of cells can move forward and away from the group. CIL also contributes to give directionality to the group as the neural crest cells continuously delaminate from the dorsally positioned neural fold the leading cells will be facing ventrally and drive the migration in that direction

down the embryo (Carmona-Fontaine et al. 2008), this is explained in Figure 1.2b.

CIL explains how directionality can be conferred to the cells by being in a group but as soon as the first line of cells moves away these cells will lose this advantage of having contact inhibition to help them guide them; this effect would therefore be fairly short lived. To overcome this problem the NC have developed a mechanism which allows the cells to regroup by being attracted to one and other through a process called Co-Attraction (Carmona-Fontaine et al. 2011). This process involves cells secreting the complement protein C3a which acts as a chemoattractant; the NC also express the receptor to C3a (C3aR) and hence will be attracted to each other. The result of this is that the cells that may venture away from the group are attracted back to the group therefore allowing cells to remain in a group (Carmona-Fontaine et al. 2011).

1.2.2.2 Positive and Negative Signals help guide the Neural Crest

The migration of the neural crest as a group of cells can be explained by the mechanisms of CIL and Co-attraction but these fail to make clear how the neural crest can move through such predetermined routes on their way to ventral side of the embryo. Indeed that is the case and a combination of external positive and negative signals play a significant role in guiding the NC to allow them to migrate in streams and follow their characteristic pathway, as summarised in figure 1.3 (Theveneau & Roberto Mayor 2011c).

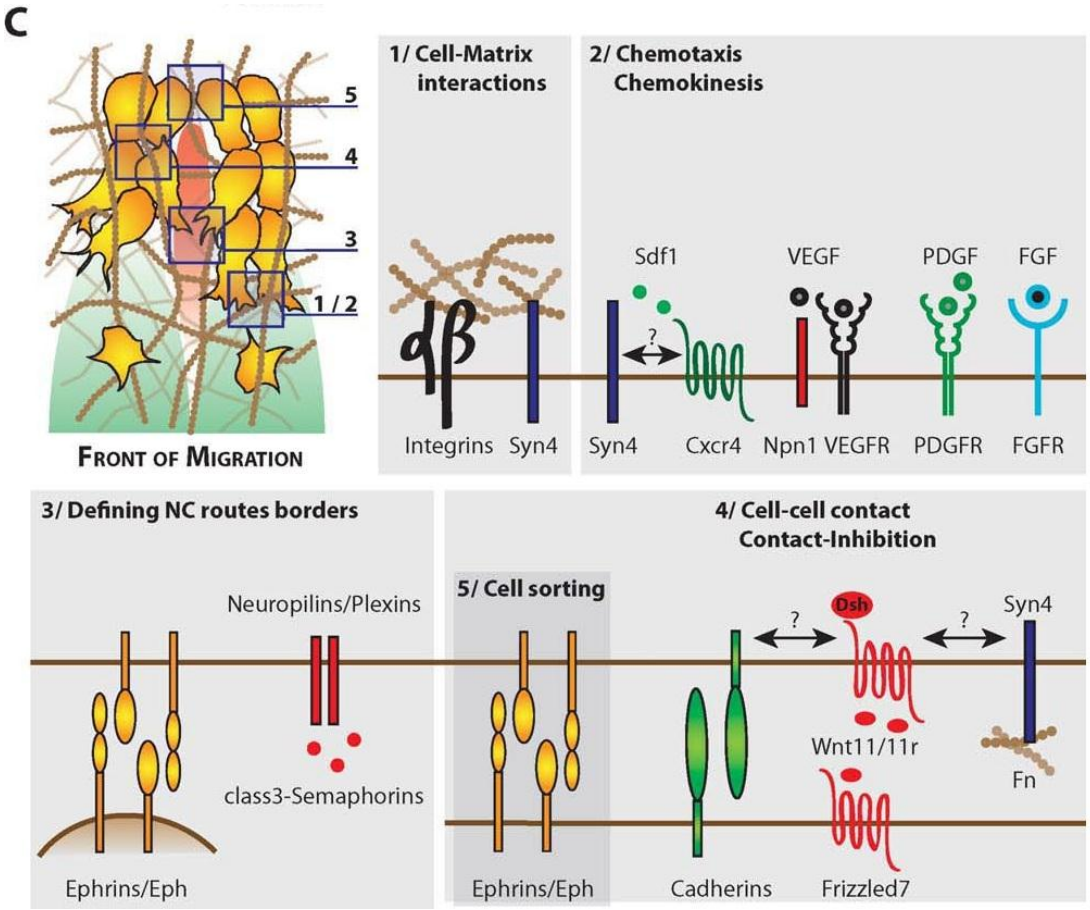


Figure 1. 4 – A summary of the signals involved in NC migration. The first panel shows the migrating NC in yellow migrating as streams through the ECM and the sites of positive (green) and negative (red) signals and where they act on and effect they have in the collective migration of NC These signals are then shown and classified in the panels alongside. Integrins play a role interacting with the substrate positive and negative signals help guide the cells and define the rout that they must take. CIL and its downstream effectors can repolarise the cells and drive migration. Extracted from Theveneau & Mayor, 2011

A negative signal described to be play a role in the migration of both cranial and trunk neural crest is that provided by semaphorins (Gammill et al. 2006; Yu & Moens 2005). Semaphorins can be secreted or transmembrane proteins which bind to their receptors (neuroplexin and neuropillin), they signal to stop the migration of cells by regulating Rho GTPases which control the migratory machinery (Kruger et al. 2005). During neural crest migration semaphorins are expressed in the regions between the migratory streams. The neural crest express the receptors for these semaphorins and hence avoid migrating in those regions helping form the stream structure (Yu & Moens 2005).

Another negative signal which works in a similar fashion to semaphorins is ephrin (Santiago & Erickson 2002). Ephrins and their receptor Eph work in a similar way to semaphorins but at a much closer range; both ligand and receptor are membrane proteins, therefore the binding of the ligand to its receptor leads to inhibition of cell migration in a cell contact dependent manner (Kuriyama & Roberto Mayor 2008). Ephrins are also important for sorting the NC into each of the different streams; this is important in insuring that the cells end up at the correct destination (Davy & Soriano 2007; Palmer & R. Klein 2003).

Apart from the negative signals described, there is also evidence for positive ones, one such signal has been identified to be Stromal Derived Factor 1 (SDF1); this chemoattractant plays a role in helping the neural crest migrate where it promotes migration of the NC ventrally (Theveneau et al. 2010a). SDF1 was originally described in the immune system and contributes to switching on the migratory machinery of cells through the receptor CXCR4 (Nagasawa et al.

1994; Bleul et al. 1996). Inhibition of either the receptor or ligand in *Xenopus* embryos leads to disrupted neural crest migration (Theveneau et al. 2010a); its role in in this process will be discussed in 1.3.4 CXCR4 in Neural Crest Migration.

1.3 The CXCR4/SDF1 axis

Most studies of CXCR4's structure and activity have been done in components of the immune system. The receptor is important in the homing of B Cells to the bone marrow until these mature to become fully functional (Q. Ma et al. 1999). It has since been found that CXCR4 plays a role in early development in various migratory events such as the migration of neural progenitors (Belmadani et al. 2005; Stumm & Höllt 2007). Another example is the part it plays in the migration of germ cells to the gonads where they will then mature and become part of the sex organs, this migration is a usually relatively long and is common in various vertebrates (Takeuchi et al. 2009; Molyneaux et al. 2003; Doitsidou et al. 2002).

1.3.1 The structure of CXCR4 and SDF1

CXCR4 is part of a larger family of CXC Receptors which in turn are a type of the large G-Protein Coupled Receptor (GPCR) family. GPCRs are 7 transmembrane receptors which help transduce signals across the membrane which ultimately switches on a G-protein; these then are responsible to activate a variety of signalling pathways (Katritch et al. 2013; Rosenbaum et al. 2009). CXC

receptors are mainly involved in the migration of cells belonging to the immune system (Plotkin et al. 2003).

The structure of CXCR4, as that of other GPCRs, structure contains a set of 7 α-helices which span the plasma membrane; the N terminus faces the extracellular space whilst the C terminus is located intracellularly (B. Wu et al. 2010a). The helices are linked together by small loops which help hold the protein together in the correct shape. These loops confer important properties to the receptor and in particular for the binding of other proteins to it; namely the ligand at the extracellular interface and the G-protein at the intercellular side. The C terminus tail is an integral part of the protein and required for its deactivation and posterior internalisation of the receptor itself and therefore is fundamental to regulate its activity (BK Kobilka 2007).

The ligand for CXCR4 is SDF1 which is a small chemoattractant also known as CXCL12; and is part of the larger family of ligands that bind members of the CXC receptors and act as chemoattractants (Zlotnik & Yoshie 2000). The CXCL family of ligands are small proteins no larger than 12kDa which are held in their tertiary structure by disulphide bonds (Veldkamp & Peterson 2005). Although there is no clear sequential homology between them they do follow similar folding patterns and invariably contain a short sequence of around 8 amino acids at their N terminus which acts as a signal sequence to be targeted for secretion (Fernandez & Lolis 2002). The rest of the structure is formed of α-helices and β-sheets; the key feature here is the C-terminus which is the site of identification and binding to the receptor; although individual structure of each

chemoattractant is yet to be determined and hence specific structural traits remain unknown (Allen et al. 2007).

1.3.2CXCR4 activation

Structural and mechanistic studies have been done to determine the actual process through which the binding of the ligand can ultimately lead to the activation of the downstream signalling not only by CXCR4 but by all GPCRs. A basic model for this involves the conformational change of the arrangement of the transmembrane domains when the ligand binds that leads to a change in the structure of the intracellular part of the receptor.

A look into the process in greater depth reveals the intrinsic mechanism of how this happens. Two transmembrane helices; 3 and 6; have been found to be the ones involved in the structural change (Peeters et al. 2011). The disulphide bridges formed between these TM domains and the extracellular loops are disrupted then the ligand binds and this leads to the change of their position (Weis & Kobilka 2008). The actual way in which specificity of each ligand for its respective receptor is achieved is not entirely known, although recent crystal structures of bound-state receptors will allow to start elucidating how this might occur (Granier et al. 2012).

The positional change of these two transmembrane helices has repercussions in the intracellular domain of the GPCR which now unlocks the G-protein binding and activation site (Oldham & Heidi E Hamm 2008). This results in a "pre-coupling" of the receptor with the G-protein forming a stable complex, this has been identified through FRET studies although the length of time they remain

together is debated (Hein et al. 2005; Galés et al. 2006). This transient complex is the one responsible to activate the heterotrimeric G-protein; the Gα subunit forms most of the contact with the receptor; this close interaction allows for its activation through the binding of GTP. This binding allows for the dissociation of the Gα an Gβγ subunits of the G-protein; these two subunits can then activate the downstream signalling pathways independently (Oldham & Heidi E Hamm 2008).

1.3.3 CXCR4 signalling

The bulk of the downstream signalling of the SDF1-CXCR4 axis occurs via the G-protein. Various signalling cascades are activated which lead to cell survival and the activation of the migratory machinery. Some signalling also occurs directly through CXCR4 and other accessory proteins; this takes place after the G-protein has dissociated it also triggers the receptor to be deactivate and internalised into the cell through endocytosis.

1.3.3.1 G-protein signalling

G proteins are made up of three subunits which join to form a heterotrimeric enzyme complex. Although made of three distinct components; α, β and γ units, they act as two signalling entities, this is due to the β and γ subunits remaining together upon activation (McCudden et al. 2005). The downstream signalling of these subunits is significantly different although they do work in a complementary manner.

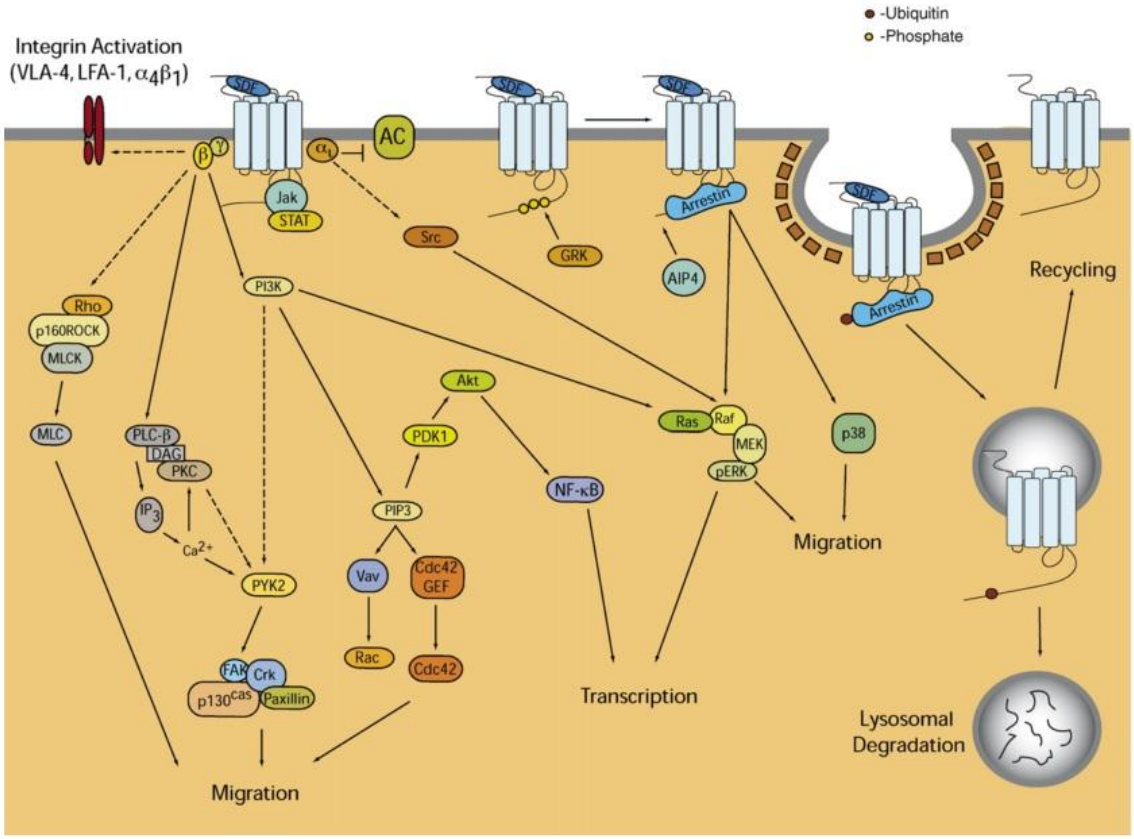


Figure 1.5 – Summary of CXCR4 signalling. Upon SDF1 binding to CXCR4 both the Gα and Gβγ subunits of the G protein become activate and can switch on downstream signalling leading to the activation of migratory machinery of the cell. The receptor is then phosphorylated allowing β-arrestin to bind and preventing any further G-protein activation and leading to the internalisation of the receptor which can then be sorted to degradation or recycling pathways. Figure extracted from Busillo & Benovic, 2007.

The Gα subunit is responsible for binding GTP which triggers the activation of the G-protein by releasing it from the GPCR and splitting both functional subunits. The α-subunit is a globular protein that can diffuse around the cytoplasm and reach its target (Cabrera-Vera et al. 2003). There are different types of α subunits; in the case of CXCR4 it activates the inhibitory type (α_i) subunit and it has an inhibitory effect on adenylyl cyclase (AC) which will therefore suppress the downstream signalling associated with cAMP which is produced by AC (Dwinell et al. 2004). The ultimate result is of the survival and growth of cells via the activation of a variety of signalling pathways summarised in figure 1.4 (Yang et al. 2007).

The β and γ subunits are responsible for the remainder of the G-protein signalling. One of the key characteristics of this complex is that it is held in the membrane via lipid anchors which are added during a posttranslational modification; this will therefore restrict the downstream signalling in the vicinity of the receptor (Wedegaertner et al. 1995). This is important in order to localise the response of the cell to the external cue; for a receptor such as CXCR4 this is important to localise the migratory machinery and hence give the cell directionality (Knaut et al. 2003).

The bulk of signalling by G-βγ is done in association with phospholipase C-β (PLCβ) which is recruited to the membrane. The main role of PLCβ is to catalyse the reaction that converts phosphatidylinositol 4,5-bisphosphateinto inositol to 1,4,5-trisphosphate (IP_3) (Kremer et al. 2011). IP_3 is small signalling molecule which is involved in calcium signalling and is able to trigger the release of $Ca^{2+}$from the various storage pools within the cell. Calcium signalling has wide

repercussions in the cell and a small fluctuation in the levels can activate various pathways that lead to migration (Wei et al. 2009). These such pathways include ERK signalling or PI3K signalling which is involved in small GTPase regulation; the signalling downstream of Gβγ is therefore known to be crucial for a chemotactic response of cells to SDF1 as it activates parts of the migratory machinery (Kremer et al. 2011). A summary of all the signalling downstream of CXCR4 appears in Figure 1.4.

1.3.3.2 Desensitisation and internalisation

Upon activation of CXCR4 and after the G protein leaves its docking site the receptor is deactivated and internalised (Signoret et al. 1997). The first step of this process is the phosphorylation of certain residues by GRK; this will help identify the protein to be internalised (S. Rajagopal et al. 2010). The site of this phosphorylation is at the C –terminus tail of the CXCR4 and it acts a signal for β-arrestin to bind the site.

β-arrestin is a small protein whose role is to desensitise the receptor and stop its signalling; it does so though blocking the G-protein binding site by attaching itself to the receptor, although it does not block the site directly it does prevent more G-protein accessing it; this results in diminished signalling of CXCR4 through G-protein signalling. The signalling however is not fully supressed; studies have shown that β-arrestin is able to signal and activate the MAPK pathway when bound to CXCR4 (Cronshaw et al. 2010; Sun et al. 2002), so in effect the first step of the deactivation leads to significant attenuation of the downstream signalling rather than full suppression (Lefkowitz & Shenoy 2005).

The binding of the arrestin to CXCR4 is a signal to alert the cell that the receptor is ready to be internalised. The main process through which activated CXCR4 is internalised through the clathrin dependent pathway (Y.-M. Kim & Jeffrey L Benovic 2002); this pathway is commonly used to internalise membrane proteins (Le Roy & Wrana 2005). The first step of this is the recruitment of AP2; this protein acts as an adaptor complex on which the scaffold of the clathrin endocytic machinery will sit on (Goodman et al. 1996).

The actual endocytic process relies on the curved shape of the clathrin protein which helps form a spherical shape. In addition this is aided by the also curved BAR proteins which bind to clathrin strengthening the structure (Doherty & McMahon 2009). As clathrin keep on binding around the protein it will slowly form what is known as a clathrin coated pit which is spherical shaped indentation of the membrane and will eventually bud off from the membrane with the aid of actin and fission proteins which can "snip off" the vesicle (S. Mayor & Pagano 2007). Once the vesicle is formed the coat will come off and the bare vesicle which is free to move with the cell.

1.3.3.3 Endocytic trafficking, degradation and recycling

B-arrestin remains bound to CXCR4even after internalisation and will still activate MAPK signalling until the vesicles has joined the sorting endosomes and multivesicular bodies for further processing (Katzmann et al. 2002). The intracellular sorting of vesicles is a process governed by Rab proteins which help identify each vesicle and can lead specific proteins to each of the compartments in which they should be in (Stenmark 2009).

Internalised material invariably ends up initially at sorting vesicle where it can then be directed to where the cell deems necessary. Degradation is the most frequent destination for internalised receptors such as CXCR4. The first step of this process consists in the ubiquitination of the CXCR4 (A Marchese & J L Benovic 2001); Ubiquitination is the process by which proteins are targeted to lysosomes or proteasomes to be degraded and is characterised by tagging the protein with ubiquitin (Hershko & Ciechanover 1998). Ubiquitination is done by the E3 ubiquitin ligase which adds ubiquitin to the C terminal domain of CXCR4 (Komander 2009). Once ubiqutinated the CXCR4 groups together on the sorting endosome and buds off to form a vesicle via the ESCRT pathway and this vesicle is then targeted to a lysosome (Welchman et al. 2005). The targeting of the vesicle to the lysosome is governed by Rab 7 which tags onto the vesicle. Rab proteins can use the microtubule array within the cell to transport its cargo to the lysosome; once there the vesicle fuses to and the protein is degraded by proteasomes (Neel et al. 2005).

Another fate that may await the receptor is to be processed and recycled back to the membrane therefore making it available again (C. a C. Moore et al. 2007). In certain circumstances receptors may be fast tracked via a fast recycling vesicle which will redeliver the protein back to the membrane as soon as it is internalised, this is a very rare processes and has been seen in seldom occasions for CXCR4 (Gage et al. 2001).

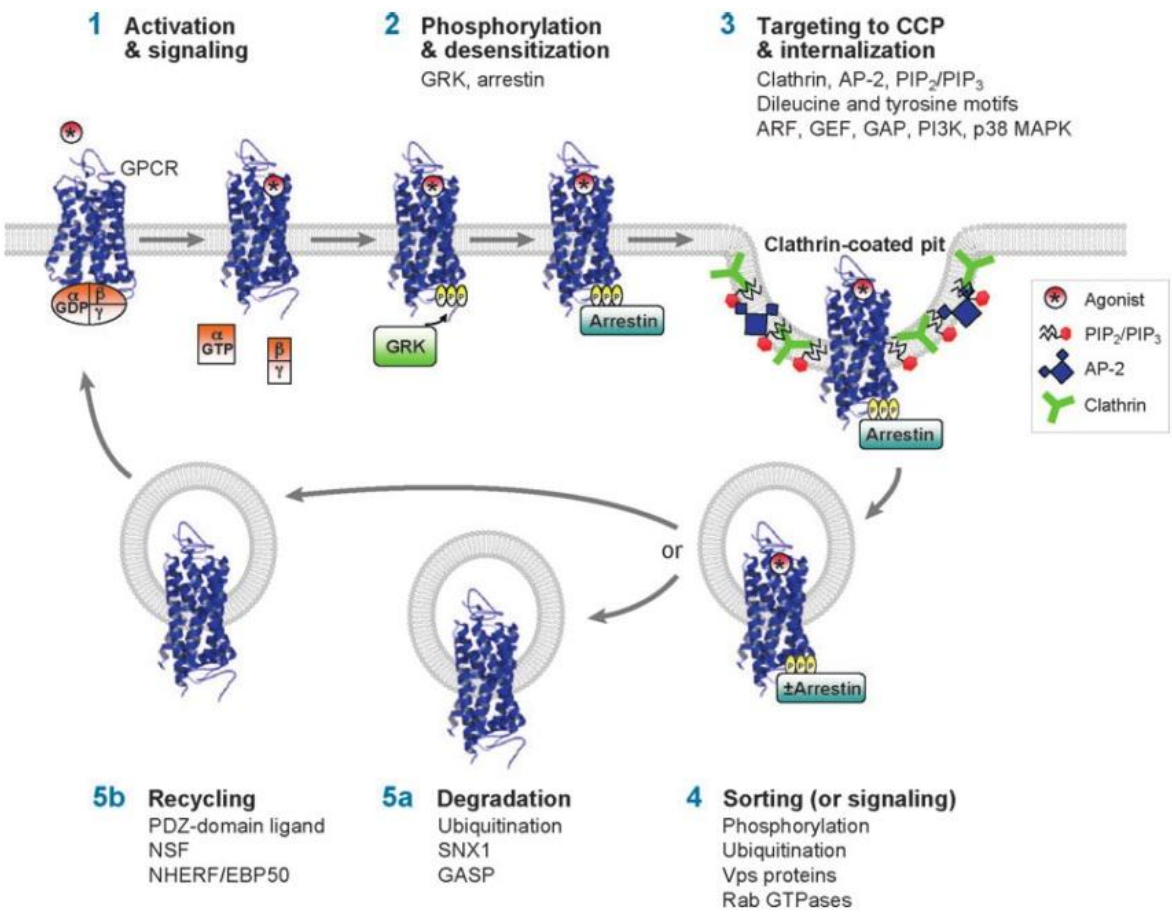


Figure 1.6 – Summary of CXCR4 desensitisation, internalisation and intracellular trafficking. After activation the receptor is phosphorylated by GRK allowing β-arrestin to bind; this targets the protein for internalisation. Clathrin mediated endocytosis follows and once finalised the receptor will be sent to a sorting endosome where it can be targeted for degradation or recycling. Figure extracted from Moore, Milano, & Benovic, 2007

This means that most recycling in which CXCR4 is involved requires a longer process and usually involves modifying the receptor in order to reactivate it (Förster & Kremmer 1998; Geminder et al. 2001). During this longer recycling route the receptor remains bound to β-arrestin until it has been removed by the cell; this allows for the continuous signalling downstream allowing endosomes to become signalling platforms (J. E. Murphy et al. 2009). Receptors destined for recycling are targeted to recycling endosomes which are identified by the Rab11 small GTPase (Stenmark 2009).

In order to target the receptor for recycling the degradation pathway is switched off; this is done by CISK which inhibits ubiquitin ligase and will prevent the CXCR4 form becoming ubiquitinated and targeted for degradation (C. Chen & Matesic 2007).CISK becomes active as a result of increased PI3K signalling and helps sustain cell survival signals by targeting the receptor to be recycled rather than degraded (Slagsvold et al. 2006). This process acts a positive feedback loop as high levels of CXCR4 signalling lead to greater PI3K which in turn activates CISK which increases the recycling of CXCR4 will be promoted (Adriano Marchese et al. 2008; Alkhatib 2009),CXCR4 not ubiqutinated and therefore targeted for recycling groups together in a particular region of the sorting endosome which is enriched with particular phospholipids. These phospholipids act as binding platforms for recycling Rabs such as 10 and 11 which help in the formation of a vesicle containing the proteins to be recycled (B. Chen et al. 2010).

The CXCR4 is trafficked to the recycling endosome; this is done again by using the structural components of the cytoskeleton of the cell; primarily actin and

microtubule networks (Rodriguez-Boulan et al. 2005). CXCR4 has been shown to co-localise with Rab11 in a site near the Golgi which is the usual location of the recycling endosome (Kumar et al. 2011). The phosphorylation can be reversed and is necessary to resensitise the receptor (W. Mueller et al. 2013). This recycling endosome also known as endocytic recycling compartment (ERC) is responsible to target proteins back to the plasma membrane; further processing of the receptor will take place here and the CXCR4 can be delivered back to the membrane (Grant & Donaldson 2009). A summary of the life cycle of CXCR4 can be seen in figure 1.5.

1.3.4 CXCR4 in Neural Crest Migration

Cells have various mechanisms to deal and regulate the activity of CXCR4; these all come together to help cells migrate and move to the correct place. Different migratory processes use the SDF1/CXCR4 axis for its effectiveness in guiding cells and the versatility in being able to modulate the signal which results in an efficient activation of the migration machinery (Kucia et al. 2004). The neural crest use this system to stimulate migration and give the migrating streams directionally (Theveneau et al. 2010b).

It has been shown that CXCR4 is expressed by the cranial neural crest of *Xenopus* embryos (Moepps et al. 2000). The study of this expression together with that of SDF1 shows how tightly coordinated and complementary they are during development (Braun et al. 2002). In addition it has been found to be of importance in migration in other organisms too (Jia & X.-H. Zhang 2009; Klymkowsky et al. 2010; Olesnicky Killian et al. 2009).

SDF1 is expressed by the placodal tissue which the neural crest follow whilst migrating ventrally (Braun et al. 2002; Theveneau et al. 2010b). As shown by Theveneau et al. 2010, neural crest are capable to respond to a localised source of SDF1 in vitro and it increases speed and directionality of the cells; suggesting a role for this signalling axis in the positive chemotaxis of the neural crest during *Xenopus* development.

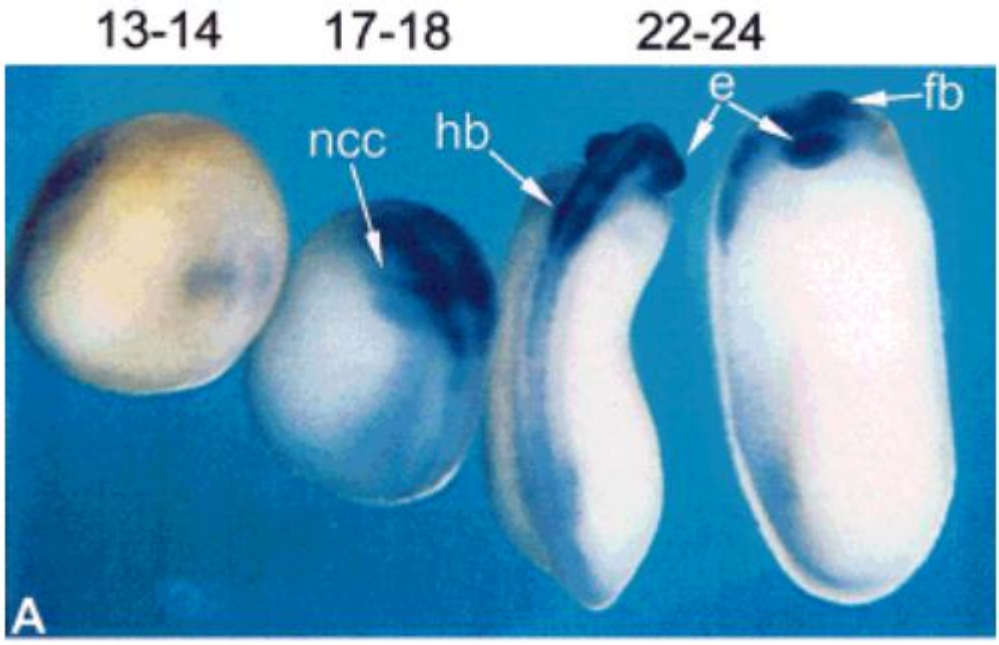


Figure 1.0-7 – CXCR4 expression in Xenopus embryos. In situ hybridisation against CXCR4; the protein is first expressed at the end of gastrulation; at stage 17-18 expression is confined to the neural crest cell region (ncc); later CXCR4 expands to the hindbrain (hb), eye (e) and forebrain (fb). Extracted from (Moepps et al. 2000)

Indeed disrupting the CXCR4/SDF1 interaction by using MO against either protein leads to the neural crest losing directionality both in vivo and in vitro; in the embryo the cells to remain in a dorsal position rather than migrating ventrally. It has also been seen that the cells respond better to a the chemoattractant when in groups rather than as individuals (Theveneau et al. 2010b), this might be due to the requirement of CIL during NC migration. Other collective migration events, such as the lateral line in zebrafish also involve a large number of cells responding to SDF1 signal via CXCR4; although here the migration is not dependent on CIL but rather differential distribution of CXCR4 along the group which can form a gradient that in turn guides the migration (Petra Haas & Darren Gilmour 2006).

1.3.5 Measuring activation of CXCR4

Measuring CXCR4 activation is of great interest in order to evaluate when the receptor is actually signalling and how this might be involved in the many processes it governs. It could be possible that there is differential regulation of CXCR4 within the migrating group and also when compared with individual cells. Therefore a suitable mechanism of doing this efficiently within the NC needs to be found.

A possible readout is to measure the stability of the protrusion since CXCR4 increases this as part of activating the migratory machinery and therefore see greater migration index of cells responding to SDF1 (Roland et al. 2003). Previous work has also shown that when exposed to SDF1 the protrusion stability increases, this is probably due to the activation of Rac1 downstream of CXCR4 which leads to increased actin polymerisation and hence the actin based

lamelleapodia and filopodia are more stable (Theveneau et al. 2010a). This in turn increases the persistence of migration of the cells responding to SDF1 and confers directionality (Minina et al. 2007).

As mentioned; another way of measuring activation is to look at the endocytosis of CXCR4. This internalisation can be measured and compared to the normal levels of CXCR4 endocytosis to determine if indeed the receptors internalisation increases when exposed to SDF1 and use this as readout of activation (Signoret et al. 1997). This would provide an immediate readout of signalling since the internalisation is fast upon ligand binding.

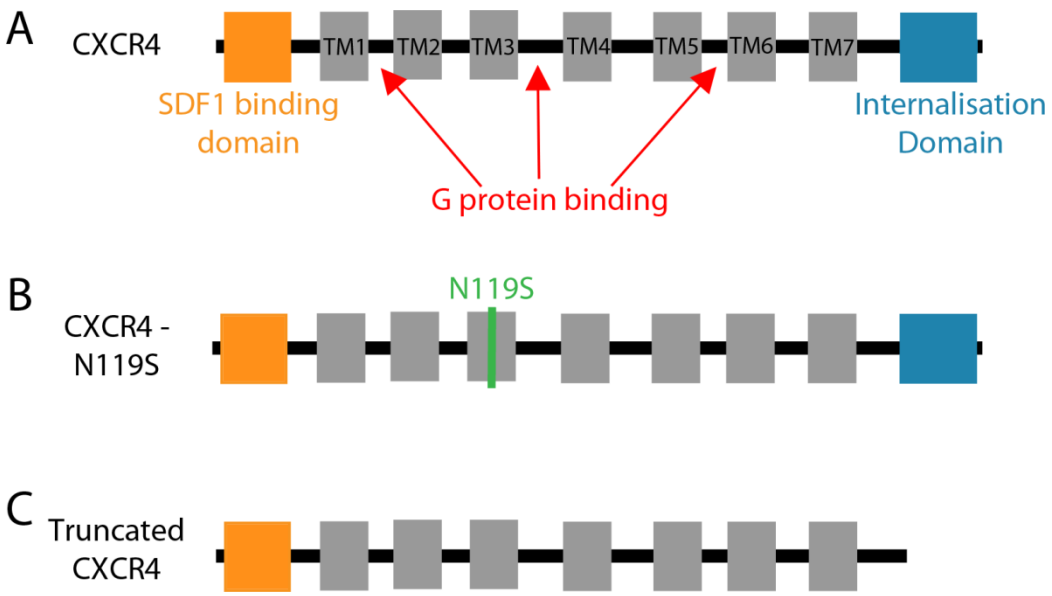


Figure 1.8– Summary of CXCR4 primary structure and mutations affecting activity. (A) Wild type CXCR4 contains the characteristic 7 transmembrane domains (TM); an SDF1 binding domain; an internalisation domain where β-arrestin binds and a G-protein binding domain formed from the three intracellular loops between TM domains. (B) A point mutation (N119S) in TM3 leads to a conformational change in the second intracellular loop allowing G-protein to be activated at all times rendering the protein constitutive active (W.-B. Zhang et al. 2002; Berchiche et al. 2007). (C) Truncated CXCR4 is caused due to a premature termination of the receptor which lacks the internalisation domain. This mutant receptor cannot be desensitised or internalised and has been linked to WHIM disease (P. a Hernandez et al. 2003; Balabanian et al. 2005).

1.3.6 CXCR4 mutations and disease

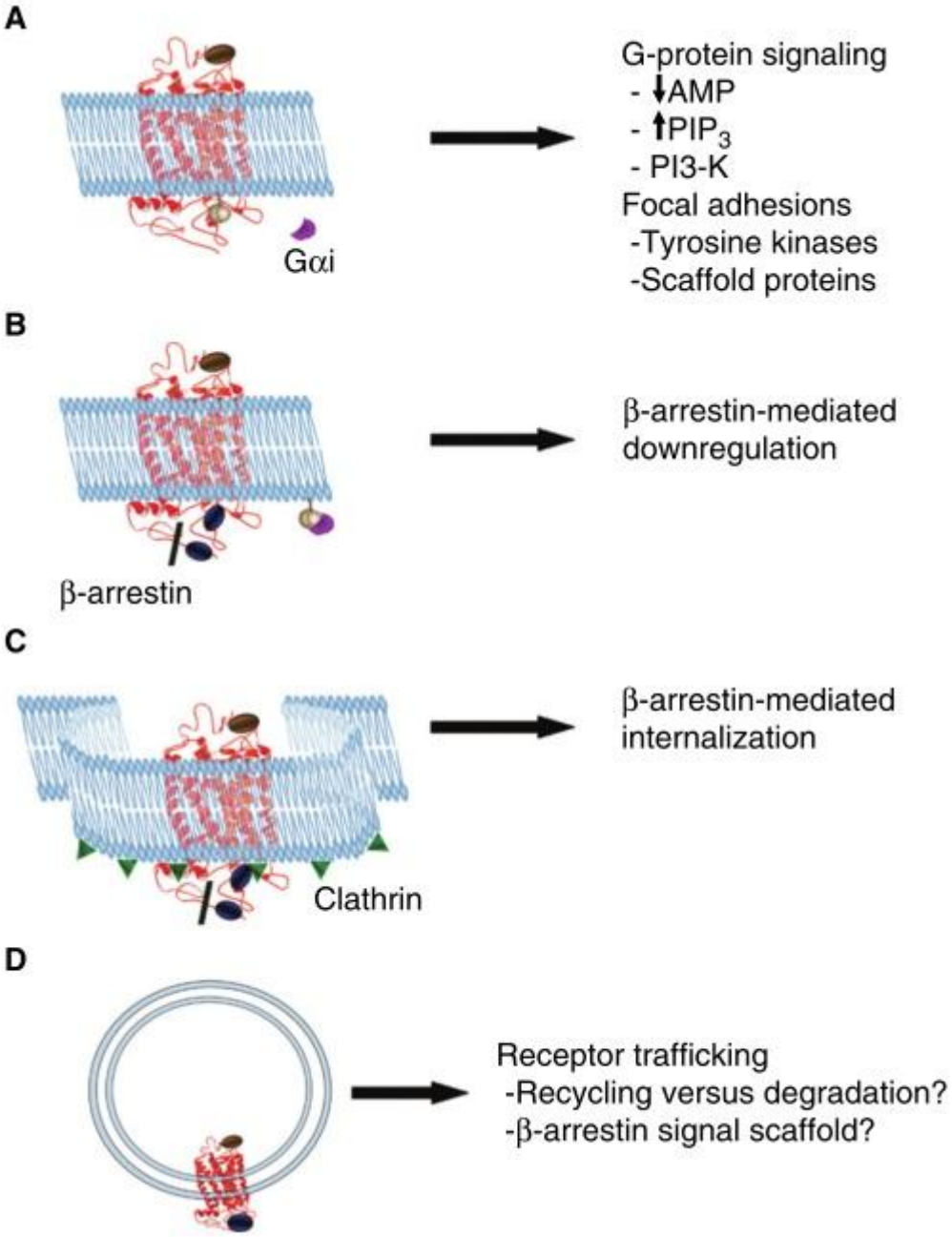
Due to its great importance in the immune system; such as correct development of B cells there are certain diseases which might arise in mutated forms. One such disease named warts, hypogammaglobulinemia, infections, and myelokathexis syndrome (WHIM) is due to a mutation at the C-terminus of CXCR4 which results in a truncated form of the protein (Diaz 2005). This truncation of between the last 10 to 19 amino acids results in an internalisation defect which leads to a decreased desensitisation in response to SDF1 (Balabanian et al. 2005). The chemical effect of these truncated mutants involves the normal activation of the receptor upon ligand binding. The G-protein site remains unaffected and its signalling is enhanced; as shown by increased calcium signalling; this is due to the receptors remaining at the membrane and not being desensitised (P. a Hernandez et al. 2003). The missing C-terminus tail is the docking site of β-arrestin which is involved in the clathrin dependent internalisation of the receptor; this does not happen leading to and the receptor not being internalised (Diaz 2005).

This autosomal dominant disease highlights the importance in the internalisation of CXCR4 in order to switch off its downstream signalling. Due to increased CXCR4 signalling WHIM patients show disrupted activity of lymphocytes and neutrophils; the cells are unable to leave the bone marrow where they mature as they cannot stop the signal that holds them there (Balabanian et al. 2005). Other chemokine receptors with similar desensitisation mutations also show disrupted signalling (Kraft et al. 2001; S. Mueller et al. 1997). Studies in development using this WHIM mutant have

shown impaired directionality of primordial germ cell in zebrafish with cells missing SDF1 targets due to prolonged signalling (Minina et al. 2007). A summary of the steps of the pathway that are affected by the pathway is found in figure 1.6.

A constitutive active CXCR4 form has also been found; this was done whilst determining the important residues involved in certain aspects of CXCR4 activity (Zhang et al. 2002). As mentioned; upon ligand binding the receptor undergoes a conformational change that can lead to the exposure of residues otherwise embedded in in the transmembrane domain this leads to the activation of G-protein and the phosphorylation of particular sites of CXCR4. Of particular importance is the DRY motif at the end of the 3rd helix which can bind to the G-protein (Ahr et al. 2005).

Figure 1.9 – Summary of how truncated CXCR4 can affect migration. (A) Increased G-protein signalling which can lead to sustained downstream signalling, (B) β-arrestin can't bind and block G-protein signalling desensitising the receptor, (C) Internalisation of the receptor via the clathrin dependent pathway is blocked, (D) Blocked intracellular trafficking including degradation and recycling are also blocked. Figure extracted from Diaz 2005.


A
Gαi
G-protein signaling
- ↓AMP
- ↑PIP3
- PI3-K
Focal adhesions
-Tyrosine kinases
-Scaffold proteins
B
β-arrestin
β-arrestin-mediated
downregulation
C
Clathrin
β-arrestin-mediated
internalization
D
Receptor trafficking
-Recycling versus degradation?
-β-arrestin signal scaffold?

The work done by Zhang et al. 2002 identified particular residues that affected the G-protein activation site; this was done by substituting candidate amino acids and measure the effect this had on CXCR4 activity. Through a screen of phenotypic mutations of CXCR4 the substitution of Asparagine 119 to a Serine was identified as a mutant conferring activated G-protein signalling. This substitution affects the shape of the third helix and the DRY motif at its end. This constitutive activity has been confirmed both in yeast and mammals (Rana & Baranski 2010; Berchiche et al. 2007). Although not much functional analysis has been done on it, this mutant form of CXCR4 can provide information upon the requirement of a tight control CXCR4's activity and its contribution to the migration of neural crest.

1.4 Project and aims

The collective nature and cell-cell interactions that occur during the migration of the neural crest provides a perfect platform on which to study how cells communicate with each other in order to aid the directional migration and the importance of the group in all of this. The improved migration of the group compared with the individual achieved through mechanisms such as contact inhibition and co-attraction does not answer fully why a single cell cannot respond to a chemoattractant as efficiently as when it is in a group so it might be possible that there is other intrinsic mechanisms which help the cells migrate.

This thesis will look at the dynamics and activity of CXCR4 and how this might be regulated to contribute to the improved migration of the neural crest in a cell cluster. The general aim of this project is to understand how the activity and dynamics of CXCR4 can help the group of neural crest respond more efficient to chemotactic cues as well as analysing how affecting CXCR4 may influence in the migration. This will be split in three specific aims:

- The study of the localisation and endocytosis of CXCR4 – including the intracellular trafficking of CXCR4, looking at internalisation upon activation and recycling of the receptor. This will define any underlying pattern in the CXCR4 distribution in the cell.
- Analyse the role of cell contact in governing CXCR4 dynamics of CXCR4; in particular its endocytosis and recycling differences between the cell contact and free edge membranes in addition to cell migrating singularly forming no contacts. The mechanism through which the endocytosis and subsequent trafficking might be regulated shall also be addressed; in particular looking at the the role small Rho GTPases might have in regulating this.
- Determine the role of the activity of CXCR4 by using the two mutant presented earlier. One is truncated at the C terminus and lacks the β-arrestin binding site; this will allow to understand the necessity of endocytosis of the receptor and why it can have an important role in regulating the downstream signalling which can affect migration. The other mutant corresponds to a constitutively active form of CXCR4. This mutated receptor will allow analysing how the cells would behave if

migration is promoted whilst ignoring the source of SDF1. It can also help us understand if the timing of when cells should start migrating is important and what happens when cells are capable of migrating whilst ignoring the chemotactic cue.

The ability of CXCR4 to promote the migration of single cells is proven in the immune system and it is difficult to explain why single neural; crest cells have difficulty in doing so. There is possibly a complex mechanism in the cluster that helps their migration which single cells take advantage of. This thesis will present a process by which cells in a group can respond more efficiently to SDF1 by regulating the turnover of CXCR4 differently to single cells allowing them to sense the SDF1 more efficiently at the leading edge of the cluster.

Part II

Experimental Procedures

Chapter 2 – Methods and Materials

2.1 Methods – Embryological procedures

2.1.1 *Xenopus* embryo isolation

Adult *Xenopus laevis* females were preprimed with 50 units of pregnant mare's serum gonadotropin (PMSG, Intervet) hormone 2-5 days before use. The night before use the females were induced to lay by injecting 500units Chorionic Gonadotrophin hormone (Chorulon, Intervet) and the females are kept at 17˚C overnight in MMR solution and then moved to 19˚C the following morning ; this causes an increase in egg laying.

Testes were removed from anesthetised males; the terminal anaesthesia was performed by placing the males in 0.5% Tricaine 1% bicarbonate solution. The testes were kept in Liebovitz L-15 Media; which contained 0.1% streptomycin. Fertilisation of eggs was done by mashing up a fragment of testes in water and spreading it on the isolated *xenopus* eggs, 30 minutes later 1/10 NAM is added to the egg containing plate. After cell division the embryo's jelly is removed by placing the embryos in a 2% cysteine solution for around 5 minutes, once the jelly is removed the embryos are washed in 1/10 NAM and kept in this solution ready for microinjection.

2.1.2 Microinjection of mRNA into *Xenopus* embryos

Borosilicate capillary tubes were pulled to make the microinjection needles using a Narishige PC-10 Dual-Stage Glass Micropipette Puller. Microinjections were done using Narishige IM300 Microinjectors; the needles were calibrated using these microinjectors under a Leica MZ6 microscope using an eye piece graticule in order to inject 5 or 10nl of solution.

Injections were performed on embryos at 2, 4, 8 or 16 cell stages. In all experiments only one half of the embryo was injected; this was done to keep one side as control and ensure a comparison for the experimental conditions was available. The stage of injection depended on the level of tissue targeting desired – the neural plate and neural crest can be targeted by injecting the dorsal cells on the animal pole.

Xenopus embryos were placed in a 3% Ficol solution in 3/8 NAM and then injected at the desired stage of development. The embryos are then kept in the ficol solution overnight in a 14.5°C incubator. The following morning embryos are moved to 1/10 NAM solution and kept at different temperatures to adjust the timing of the *Xenopus* development in order for the embryos to reach the desired stage.

2.1.3 Neural crest cell culture

Neural crest cell explants are placed on fibronectin coated plates. Plastic *Falcon 35-1006 petri dishes* were covered with 10µg/ml fibronectin (Fn, Sigma F2006) solution and placed on a heat block at 37°C for an hour; when plating glass surfaces the concentration of fibronectin was 50µg/ml. The fibronectin is then

removed and the plate washed in PBS. The area was further incubated using 0.1% BSA in PBS for 15-30minutes and finally filled with DFA.

Embryos were allowed to grow to stage 16-17 (neural crest premigratory stages) and moved to a NAM 3/8 solution. The Vitelline membrane is removed using forceps and embryos are allowed to heal for up to thirty minutes after this process. Embryos are embedded into plasticine in a position which makes the neural crest easily accessible; then using an eyebrow knife the neural crest is dissected out of the embryos and placed in DFA solution for 15-30 minutes. The neural crest explants are then placed onto the fibronectin coated plates and the plates are filled with DFA.

To disaggregate the cells and obtain single cells to study individual movement; the explants were placed in a calcium free DFA for 5-10 minutes; the explants were disturbed with an eyebrow knife to encourage the dissociation of the group of cells. The cells were then plated on the fibronectin coated dishes.

2.1.4 Visualisation of Neural crest migration

Neural crest migration was visualised using a LEICA DM5550B microscope and a Leica DFC300FX camera. Using a 10X objective the cells are viewed is using a widefield light and images were taken every 5 minutes for up to 12 hours. Greater magnification images were obtained using a 20X or 63X water immersion objective and pictures obtained every 30 secs taking 1µm slices to view the whole cell depth.

2.1.5 Chemotaxis assay of neural crest

When performing SDF1 chemotaxis assays heparin beads (Sigma H5263) are placed into a 5mg/ml SDF1 protein solution and allowed to be coated with the protein for over an hour. This concentration has been previously described in similar chemotaxis experiments published by Theveneau & Roberto Mayor 2011a A strip of vacuum free grease is laid on the fibronectin and the SDF1 coated beads are embedded in it. The neural crest explants are positioned a bead diameter away on the fibronectin and then visualised as explained previously.

2.1.6 Analysis of chemotaxis response to a movable chemoattractant

The protocol was adapted from Theveneau and Mayor, 2011. Briefly, the neural crest explants are placed on a fibronectin dish as described previously. SDF1 coated heparin beads are broken up into smaller pieces which contain straight edges and lightly attach to the fibronectin. The bead fragments are places in the vicinity of the neural crest. The movement of explants towards the bead was visualised using a Leica MZFLIII microscope 2.5X objective; which allowed viewing many positions simultaneously. Images were recorded every 5 minutes. After 90 minutes the bead fragment was gently moved to a new position using an eyebrow brush. The migration of different cells within the group was analysed as the chemoattractant source moves.

2.1.7 FRAP – fluoresce recovery after Photobleaching

FRAP was done using a Multi Photon UV Leica confocal microscope. Cells were visualised using a 63X water immersion objective and a region of interest was identified. Five prebleach frames were taken every 2 secs followed by five

bleach frames of the region of interest at full laser power, post bleach images were taken for at least 50 frames every 2 secs. Analysis and normalisation of FRAP recovery was done using ImageJ (NIH) and FRAP Analyser developed by the Cytoskeleton & cell plasticity laboratory (Evelyne Friederich , University of Luxemburg).

2.1.8 Dextran labelling of endocytic vesicles

The neural crest explants were plated on glass coverslips. They were then incubated in the absence and presence of 5mg/ml SDF1 solution and Rhodamine dextran beads 10000MW (Invitrogen D1824) for 1 hour – this concentration and Rhodamine size have been used in the past to label vesicles in a similar way Minina et al. 2007. The cells were carefully washed with DFA and fixed in 4% paraformaldehyde/PBS. In order to reinforce the red rhodamine immunohistochemistry was performed labelling dextran beads.

2.1.9 Immunohistochemistry of neural crest cells

Fixed cells were washed in 0.5% tween 20 in PBS followed by 0.1% tween 20/PBS. Blocking was performed by placing cells in a 20% serum/PBS-Tween solution for 1 hour. The primary antibody was diluted in the same blocking solution and incubated with the cells overnight. The antibody was washed out with PBS-T and then incubated for an hour in secondary antibody. This was followed by washes in PBS-T and a final wash in PBS. The cells were mounted onto slides with mowiol.

2.2 Methods – Molecular and Biochemical procedures

2.2.1 Transformation of DNA clones

DNA plasmids were obtained spotted on filter paper and resuspended in water. 5µl of DNA was incubated in to 100µl of competent Dh5α E. coli and placed in ice for between 20 to 30 minutes. The cells were heat shocked for 45 seconds at 42°C followed by a 1-3 minutes incubation in ice and 600µl of distilled water was added.

100µl of the cell mix was plated onto LB agar plates coated with ampicillin and left to grow overnight. Colonies were picked using a sterile pipette and transferred to 100ml of LB-ampicillin solution. The colonies are allowed to grow overnight at 37°C with agitation. The cells are pelleted by centrifugation and the supernatant disposed of. DNA was extracted and purified from the bacteria using a Plasmid Midi Kit (Qiagen, 12143). The amount of extracted DNA was measured using a Nanodrop Spectrophotometer (ND 1000).

2.2.2 Restriction Digest of DNA

A restriction digest was set up by placing 10µg of the DNA plasmid containing the gene of interest and 1µl of an appropriate restriction enzyme. The reaction mix also contained 10µl of 10X Buffer, 1µl BSA and was made up to 100µl by adding a suitable amount of H_2O; the mix was incubated for 2 hours at 37°C.

The digested DNA was purified using chloroform extraction. 100µl of Nuclease free water was added to the reaction mix followed by 200µl of Phenol-Chloroform-Isoamin and mixed through vortexing the tube. The solution was then centrifuged at 4°C for 10 minutes at a speed of 13000 rpm. The top layer

was transferred to a new tube and volume measured. Twice the volume of 100% ethanol was added and a 1/10 volume of 3M Sodium Acetate; this mix was incubated overnight at -20°C.

The DNA was spun down by centrifuging the tube at 13000 rpm at 4°C for 30 minutes. The supernatant was removed and the DNA pellet resuspended in 350µl 70% Ethanol and centrifuged for a further 15 minutes at 13000 rpm and 4°C. The supernatant was discarded and the DNA pellet was air dried for around 10 minutes and 20µl of TrisEDTA. The quality of the digestion was checked by agarose gel electrophoresis.

2.2.3 Transcription of sense mRNA

A reaction mix was prepared using an SP6 Message Machine Kit (Ambion); this involved mixing 1µg of linearised DNA; 10µl NTP Cap, 2µl 10X buffer, 2µl SP6 mix, 1µl GTP and made up to 20µl with water. This mix was incubated for 2 hours at 37°C; after which 1µl DNAase was added and further incubated for 30 minutes at 37°C.

The RNA was purified using Lithium Chloride precipitation. 100µl of TrisEDTA, 10µl of LiCl 4M and 200µl of 100% Ethanol was added to the mix and placed on ice for 30 minutes. The tube was then centrifuged at 13000 rpm at 4°C for 10 minutes. The supernatant was removed and the mRNA pellet resuspended in 300µl 70% Ethanol and centrifuged for a further 10 minutes at 13000 rpm and 4°C. Finally supernatant is discarded and the mRNA resuspended in nuclease free water.

2.2.4 Preparation of antisense probes for in situ hybridisation

Antisense RNA probes were made by mixing 1µg of linearised DNA, 4µl 5X buffer, 2µl 10X DTT, 2µl NTP-Dig mix, 0.5µl RNAsin, 1µl RNA polymerase and made up to 20µl with water. This mix was incubated for 2 hours at 37°C; after which 1µl DNAase was added and further incubated for 30 minutes at 37°C. The RNA probe was purified using Lithium Chloride precipitation and resuspended in 100µl of Hybridisation buffer and stored at 20°C.

2.2.5 Whole Mount In situ Hybridisation (WMISH)

The protocol is described in Harland, 1991. Briefly, the embryos were later washed in 100% methanol; at this point the embryos can be kept at -20°C for storage. To rehydrate the embryos they were washed in 75% methanol, 50% methanol, 25%methanol and PBT successively. The embryos were bleached; this was done by placing the embryos in bleaching solution for 10 minutes or until embryos look pale.

Following bleaching the embryos were quickly washed in PBT and fixed in formaldehyde 3.7% solution in PBS for 20-30 minutes. The embryos were again washed thoroughly in PBT and hybridisation buffer after which they were incubated in this buffer at 65°C for 3-6 hours. After this the digoxigenin labelled probe was added an embryos were kept at 65°C overnight.

The probe was removed (this solution can be recycled and reused up to 3 times in other in situ procedures). The embryos are washed in washing solution 1 to 5; these solutions contain successively lower amounts of Formamide/SSC

solutions. They were in each washing solution for 10 minutes with a final wash of 30 minutes and they are always kept at 65°C.

The embryos are further washed in PBT followed by a TBS wash and then incubated in blocking solution (10% serum in TBS) for 2 hours. The anti digoxigenin-Alkaline phosphatase (AP) antibody (Roche) was diluted 1:3000 in TBS-10% serum and the embryos were incubated overnight at 4°C. The antibody is washed away by washing the embryos in 5 thirty minutes successive washes in TBS-10% serum.

The embryos are then washed in AP buffer in a series of 15 minutes washes. The embryos are then developed in AP buffer containing a mix of 75µg/mL BCIP (5-bromo-4-chloro-3-indoyl-phosphate; Roche) and 150µg/mL NBT (4-nitro blue tetrazolium chloride; Roche). The embryos were allowed to develop in the dark checking at regular intervals to analyse the levels of staining of the expression. The reaction was stopped by quickly washing in PBT; background staining was removed by washing the embryos in 100% Methanol. Finally embryos are fixed and stored in 3.7% formaldehyde in PBS. *For solutions refer to 2.3.2 Solutions for in situ Hybridisation.*

2.3 Materials

2.3.1 Solutions

10X Modified Ringer Solution (MMR) pH 7.6

1M	NaCl
20mM	KCl
10mM	$MgSO_4$
10mM	$CaCl_2$
50mM	HEPES
1mM	EDTA

Normal Amphibian Medium (NAM) A

1.1M	NaCl
20mM	KCL
10mM	$Ca(NO_3)_2$
10mM	$MgSO_4$
1mM	EDTA

NAM B pH 7.5

20mM	NaH_2PO_4

NAM C

100mM	$NAHCO_3$

NAM dilutions

	NAM A	NAM B	NAM C	Streptomycin (50mg/ml)
NAM 1/10	10ml	10ml	1ml	1ml
NAM 3/8	37ml	37ml	1ml	1ml

Danilchick's Solution (DFA) pH 8.3

53mM	NaCl
5mM	Na2CO3
4.5mM	K-Gluconate
32mM	Na-Gluconate
1mM	MgSO4
1mM	CaCl2
0.1%	BSA

50mg/ml	Streptomyocin

2.3.2 Solutions for in situ Hybridisation

MEMFA

4%	Formaldehyde
0.1M	MOPS pH7.4
1mM	$MgSO_4$
2mM	EGTA

Diluted in DEPC water

PBT

0.1% Tween 20 in PBS

Bleaching solution (for 1 ml)

600µl	Water
330µl	Hydrogen Peroxide
50µl	Formamide
25µl	SSC 20X

Hybridisation buffer (for 100ml)

50%	Formamide
5x	SSC
1x	Denhardt's Solution
1mg/ml	Ribonucleic acid
100_g/ml	Heparin
0.1%	CHAPS
10mM	EDTA
0.1%	Tween-20

Diluted in DEPC water (heated to dissolve).
The pH is adjusted to 5.5.

Washing Solutions

Washing solution	Formamide	SSC
1	50%	2X
2	25%	2X
3	12.5%	2X
4	-	2X
5	-	0.2X

All made up in 0.1% Tween 20 and made in DEPC water.

AP (Alkaline Phosphatase) buffer (for 10ml)

1ml	NaCl 1M
1ml	Tris HCl pH 9.5 1M
1ml	$MgCl_2$ 0.5M
10µl	Tween 20

Made up to 10ml in DEPC water

2.3.3 mRNA constructs & morpholinos

Constructs for CXCR4 and CXCR4 mutants were obtained from Barbara Moepps; University of Ulm.

Antisense morpholino was used for CXCR4:

5'-CAATGCCACCAGAAAACCCGTCCAT-3'.

This morpholino targets a splice site of the CXCR4 xenopus mRNA; it was injected at either 2 or 8 cell stage. The fact that this morpholino targeted a splice site allowed its effect to be rescued via both the CXCR4-GFP construct and mutant forms of the receptor. The morpholino works by binding the mRNA at the splice site and hence preventing the correct translation and protein synthesis(Theveneau et al. 2010b).

2.3.4 Antibodies & Inhibitors

Primary antibody for Rhodamine (Vector labs SP0602) was used together with a secondary Anti goat IgG-Alexa 555 (Invitrogen A21432).

Inhibitors against Rac 1 – NSC23766 (Calbiochem 553502); this inhibitor works by inhibiting the activation of Rac 1 by the Guanine exchange factor (GEF); it will therefore not target other mechanisms of activation via another

pathway(Gao et al. 2004). The RhoA pathway was inhibited by using a ROCK inhibitor – Y27032 (Calbiochem 688001); it inhibit the activity of the kinase and downstream signalling via this side of the pathway by RhoA (Davies et al. 2000); as with the Rac1 inhibitor the effect may be limited ad we do not know the full extent of both Rho GTPases activity.

2.4 Analytical Procedures

2.4.1 Analysis of migratory profiles of cells *in vitro*

The movement of cells *in vitro* was measured using ImageJ software (NIH). The cells were manually tracked using a manual tracking plugin (MTrackJ) which allows following cells through consecutive frames in the movie and recording their coordinates. Before tracking was started the movies were aligned so that the explant and bead were laying on the y-axis; this allows measuring the movement in this particular axis independently and measure forward migration index (chemotaxis index).

The velocities, directionality and chemotaxis index were then determined by using Chemotaxis and Migration tool software (ibdi, Germany). The software can process the migratory behaviour of each cell from the coordinates; the velocity was recorded in μm/min. The directionality measures the displacement over the actual distance moved of each cell as illustrated in figure 2.1. The chemotaxis index was also obtained and measures the relative migration of the cell in a particular direction in this case the y- axis towards the bead.

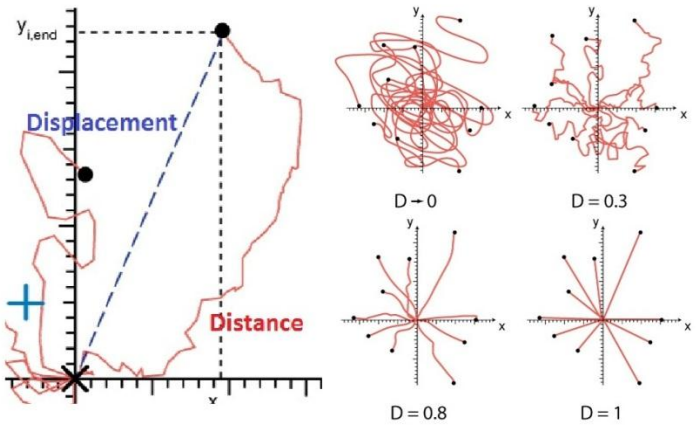


Figure 2.1 – Definition of Directionality. Directionality is a measure of the displacement; which is the value of the straight line distance between start and end point; divided by the distance; which is the value of distance actually migrated by the cell. A value close to 0 represents non directional movement and a value of 1 indicates a straight line.

2.4.2 Vesicle counting and tracking

The number of vesicles formed over a period of 30 minutes was counted using o the object counter plugin in ImageJ. The duration and destination of vesicles was also tracked using the manual tracking plugin and quantified in MS Excel.

2.4.3 FRAP Analysis

Analysis of the levels of FRAP Recovery was done using the FRAP Analyser tool (Evelyne Friederich , University of Luxemburg). The levels of fluorescence against time were measured in ImageJ in three areas; (1) FRAP region membrane; (2) control membrane and (3) background. The 3 sets of measurements were processed with the FRAP analyser which normalises the fluorescence recovery relative to the other two other regions and recovery curves were produced.

2.4.4 Statistical manipulation of data

Developmental biology experiments need to be reproducible and consistent in order to allow us to understand the full extent of the pathways and processes studied; therefore statistical analysis is required to ensure the results are reliable. In this thesis the statistical analysis was done using MS Office – Excel and the standard statistical formulas. The degree of significance of difference seen in experiments was analysed by performing the student t-test; this involves comparing the two sets of data and obtaining a p-value; values beneath 0.05 were significant and below 0.01 very significant.

Part III

Experimental Results

Chapter 3 – CXCR4 dynamics

As mentioned in the introduction; CXCR4 is required for the correct migration of the neural crest from the neural plate border to more ventral positions; the cells follow an SDF1 signal coming from the underlying placodal tissue (Theveneau et al. 2010b). The exact role that CXCR4 may have in guiding this movement is yet unknown although there might be differences in the way cells deal with the receptor within groups compared to single cells which allows a group of cells to migrate more effectively. Is it therefore possible for cells in groups to have differential CXCR4 localisation or dynamics to respond to SDF1 more efficiently compared to single cells?

3.1 CXCR4 localisation

Using a CXCR4-GFP construct; which has been shown to be functional and able to rescue the CXCR4-MO in *Xenopus* (Theveneau et al. 2010b); the movement of the receptor within the cell can be visualised; this will provide a mechanism to analyse the localisation and intracellular dynamics giving us an insight on how neural crest cells deal with the receptor before and after they respond to an SDF1 signal and if any mechanism exists to aid migration.

The CXCR4-GFP mRNA was injected into 8 cell stage *Xenopus* embryos targeting the neural crest; this is achieved by injecting specific blastomeres. The neural crest were then dissected from a stage 18 embryo and allowed to attach to a

fibronectin covered coverslips, these cells were incubated in the presence and absence of the SDF1 protein to analyse any possible differences in CXCR4 distribution. The cells were visualised after being fixed and results show that CXCR4 is located mainly at the membrane and vesicles containing CXCR4 are present within the cell in both conditions (Figure 3.1). This localisation follows what is known about the receptor; as atypical membrane protein it is mainly located there and when internalised it will be present in vesicles as seen in the figure (Kumar et al. 2011). These static images do not allow discerning major differences between cells incubated with SDF1 or not.

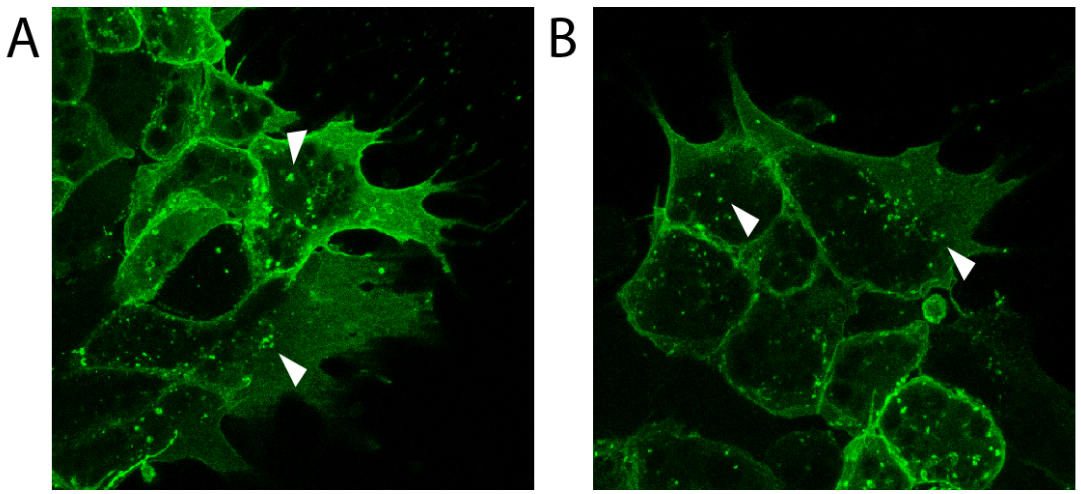


Figure 3.1 – CXCR4-GFP construct is expressed by neural crest cells. The location of CXCR4-GFP in neural crest incubated in the (A) absence and (B) presence of SDF1. Both situations show CXCR4 present at the membrane and within vesicles in the cell with no major differences. The arrows illustrate examples of vesicles analysed.

This lack of major differences may be due to the cells already having responded to SDF1 from the embryo before being dissected which causes CXCR4 internalisation and hence vesicles are visible in both conditions. Alternatively these vesicles correspond to a CXCR4 internalised even in the absence of SDF1, this would allow the cell to redistribute the receptor around membrane (Baudouin et al. 2006). From figure 3.1 it can also be seen that cells in the inner part of the explant have fewer vesicles which might be indicative of a reduced internalisation in cells with contacts all around. In order to study the dynamics of CXCR4 live tracking within the cell using microscopy techniques is required.

3.2 CXCR4 endocytosis

CXCR4 is internalised as a result of ligand binding; it is also known that the receptor is internalised in a ligand independent manner in order to regulate the levels at the membrane and help cells modulate the response (Yanyan Zhang et al. 2004; Futahashi et al. 2007). Using the CXCR4-GFP construct the dynamics of CXCR4 internalisation in neural crest can be analysed in vitro.

3.2.1 Internalised CXCR4 undergoes longer trafficking when exposed to SDF1

Once this receptor is internalised it remains in the cell in small vesicles; the GFP tag allows tracking the movement of these within the cell and discovers two distinct trafficking fates, a short one and a longer lived one. The short lived vesicles form at the membrane and disappear in the close vicinity lasting no longer than a few minutes; this probably indicates recycling back to the membrane or quick degradation. The destination of the longer vesicles leads

them to join together in larger vesicles, probably multivesicular bodies or sorting endosomes. From here they can be distributed to their final destination such as lysosomes, where they are degraded; or recycling endosomes which will take the receptor back to the membrane; it is hard to track these as once they cluster together and join sorting or recycling endosomes each specific vesicles' tracking is lost.

By using these two endocytic fates; short and long; differences can be seen in the endocytosis and trafficking of CXCR4 after responding to the chemotactic signal. Vesicle movement was analysed in single and group of cells in vitro when exposed or not to a localised source of SDF1. In order to define the two characteristics movies were compared and short movements were defined as lasting less than 3 minutes and long would last longer and usually travelled further in the cell. The length of time cells were analysed for was 30 minutes; this was done in order to reduce potential loss of signal due to bleaching and also loss of focus as cells moved.

Single cells in absence of SDF1 exhibit a higher proportion of short lived vesicles; these are formed at the edge of the membrane that last for just a few minutes (figure 3.2); longer movements are less. There are vesicles already present within the cytoplasm that do not form at the membrane during the movie; these are most likely just receptor stores or vesicles containing CXCR4 awaiting degradation.

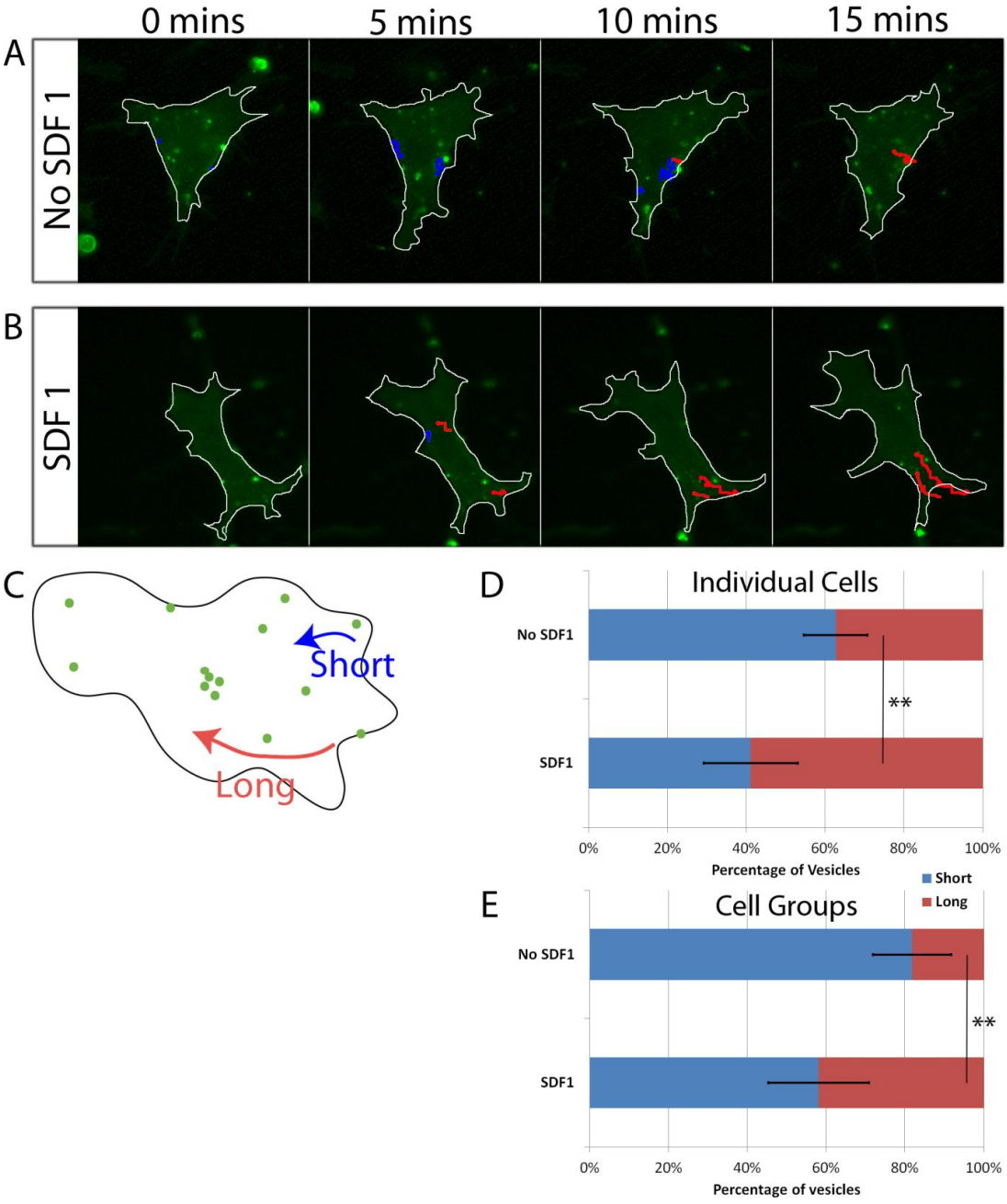


Figure 3.2 – CXCR4 vesicles are trafficked for longer when the cells are migrating towards an SDF1 source. Image stills from movies of single cells migrating in the (A) absence (n=86) and (B) presence of SDF1 (n=75). (C) The length of internalisation is quantified in short and long events in both (D) single and (E) cells with groups. The movement of vesicles within cells was measured in periods of 30 minutes with images acquired every 30 seconds.

When cells are migrating towards an SDF source the situation reverses and now the longer movements are more common. Many of these vesicles end up near the centre of the cell were they group together in the site in which the sorting endosome is found, suggesting that SDF1 promotes CXCR4 processing (figure 3.2B).

This pattern is also seen in cells migrating as part of a group; although in this case the proportion is always in favour of shorter movements (Figure 3.2E). When the cells are migrating towards SDF1, the longer trafficking events increase corresponding to the need to process the receptor once activated; where the receptor is phosphorylated and endocytosed via the AP2-β-arrestin pathway which will lead the proteins sorting endosomes (Busillo & Jeffrey L Benovic 2007; Borroni et al. 2010).

During the movies some vesicles cluster together in what might be a sorting endosome; from here they can be processed to be returned to the membrane or alternatively be ubiqutinated and targeted for degradation. The clustering of vesicles together in the centre of the cell body has also been seen in other cell types in which CXCR4 is similarly endocytosed and clusters in a similar way usually near the Golgi of the cell (Kumar et al. 2011).

When comparing single cells and clusters (Figure 3.2 D & E); we see that in cell groups the vesicle pathway is predominately shorter than individual cells. The shorter trafficking might signify an increase in the rapid recycling of CXCR4 in cell clusters this is particularly true in the absence of SDF1 where in cell clusters over 80% of vesicles are short lived. Alternatively in these cells the CXCR4 is

rapidly degraded or sent to a sorting endosome and the tracks are lost more rapidly; this would also signify higher receptor trafficking. An impact of this might be that the group has the ability to replenish membrane CXCR4 levels and using this rapid receptor trafficking.

It could be possible that the different localisation and arrangement of cytoskeleton proteins in single cells and groups has a role in this as it is known that cytoskeleton is important for vesicle trafficking and recycling (Millman et al. 2008; Scita & Di Fiore 2010). Cells in groups are more highly polarised (Matthews, Broders-Bondon, et al. 2008) and the NC could use this to their advantage when trafficking of proteins is required.

3.2.2 CXCR4 internalisation provides an underlying polarity of CXCR4 endocytosis

CXCR4 is constantly endocytosed both in the presence and absence of SDF1 (Orsini et al. 1999; Signoret et al. 1998) and described here. It is important for the receptor to be internalised upon activation in order to switch off its signalling (Signoret et al. 1997), but little is known about why it is internalised when inactive. It might be that the cell is simply sensing its immediate environment by pinocytosis forming small vesicles which contain CXCR4 present in the membrane. Alternatively endocytosis can be a mechanism through which the signalling and availability of the receptor at the membrane can be regulated, this has been described for other receptors (Sorkin & Mark von Zastrow 2009; Sigismund et al. 2012) and might play a role in regulating response to SDF1.

A cell at the edge of a migrating group of neural crest has two main classes of membrane; one at sites of cell contact and the other at the free edge. We know that cell contact is important to polarise cells and is necessary for the correct migration via CIL(Matthews, Broders-Bondon, et al. 2008; Carmona-Fontaine et al. 2008). The free edge on the other hand is able to sense the environment and help guide the cell towards the source of the chemoattractant, in this case SDF1. It is therefore possible that the cell would prefer to deal with CXCR4 differently at these two sites; favouring the free edge somehow.

Using the CXCR4-GFP construct the endocytosis from both membrane types, at the free edge and cell contact; can be analysed. The result shows the existence of an endocytic polarity with more vesicles being formed at the free edge membrane rather than at sites of contact (Figure 3.3). The overall effect is that CXCR4 is more readily endocytosed at the site where the external signal is most likely to be present priming the cells for this hypothetical signal.

In absence of SDF1 cells internalise CXCR4 predominately at the free edge; with just over 75% of the vesicles formed at the free edge of the cell (Figure 3.3D). Single cells obviously lack a cell contact and therefore comparison between these two situations is difficult. In the absence of SDF1 single cells are constantly repolarising and form protrusions in all directions; they also internalise CXCR4 form anywhere in the membrane and there is no clear pattern.

In cells migrating in the presence of SDF1 the endocytosis polarity is still existent; in this case 63% of vesicles are formed at the free edge. The decrease

in the difference in endocytosis between free edge and contact membrane can be explained due to the processing internalised CXCR4 must undergo before it can be recycled back or degraded. It could also be that the cell internalises CXCR4 from the contact membrane to redistribute to the free edge; this would result in increased internalisation at the contact side and reduce the endocytic polarity described. Also in other migrating cells movement of membrane from the back to the front is commonly seen (reviewed in Keren 2011) and this might be the case here too.

Cell groups can therefore use this to their advantage compared to single cells in regulating the dynamics and endocytosis of the receptor. This results in cell groups being better prepared to respond to the eventual positive cue by having the receptor at the correct place and the endocytic machinery already in place ready for when the ligand binds. The endocytic polarity can therefore add to other mechanisms that exist within the group such as CIL or Co-Attraction to make the neural crest collective an efficient migratory entity.

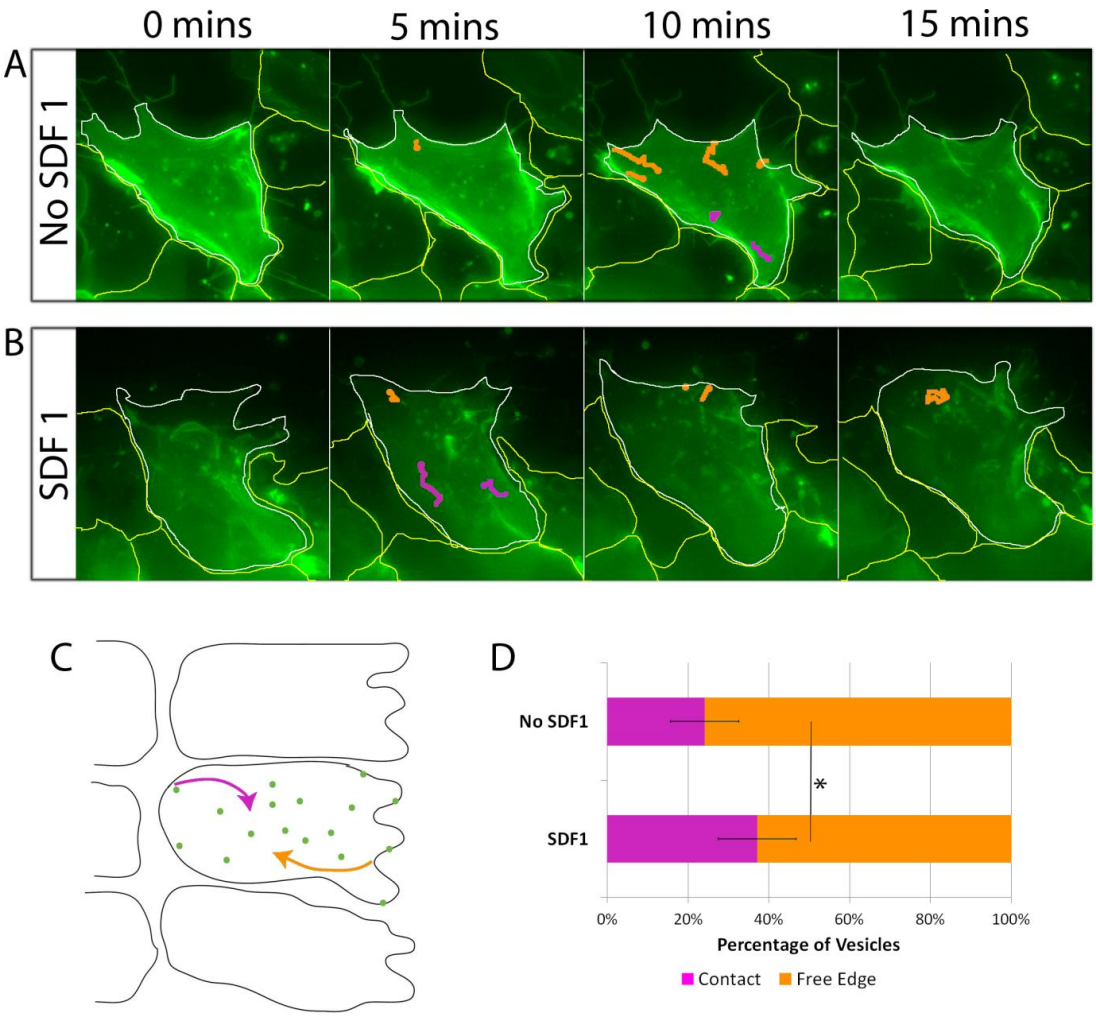


Figure 3.3 – CXCR4 is more likely to be internalised at free edge membrane than contact creating an internalisation polarity. Image stills from movies of cells at the edge of a neural crest explant migrating in the (A) absence (n=115) and (B) presence of SDF1 (n=98). (C & D) The membrane location at which internalisation occurs is quantified showing a preference to the endocytosis at the free edge both the absence and presence of SDF1.

3.2.3 Interfering with small GTPases loses the polarised endocytosis of CXCR4

The existence of an endocytic polarity requires the cell to be able to control endocytosis at different sites of the cell. Migrating neural crest are highly polarised cells; this polarity is set up in large part by CIL which forces the cells to form protrusions away from contact sites (Carmona-Fontaine et al. 2008; Theveneau et al. 2010b).

One of the characteristics of this polarity is the establishment of two distinct small Rho GTPase domains; namely Rac1 at the front and RhoA at the back (Matthews, Marchant, et al. 2008). These two small Rho GTPases are required for differential regulation of actin filaments Rac1 promotes the formation lamellipodia whilst RhoA favours stress fibres (Hall 1998). These two GTPases are complimentary in function and can inhibit each other; they are important in setting up a polarity in epithelial cells; in addition they have a role in cell division and regulate cell morphology (Etienne-Manneville & Hall 2002; Heasman & Ridley 2008). In migratory cells they are also important in polarity and mediate the directional response to chemoattractants by regulating protrusion formation and regulating the actin cytoskeleton (Ridley et al. 2003; Machacek et al. 2009).

Small Rho GTPases have also been associated with regulating endocytosis and vesicle transport (reviewed in Ellis & Mellor 2000; Qualmann & Mellor 2003); therefore it is possible that this differential Rho GTPase localisation could also be regulating the endocytic polarity of the CXCR4. The exact way in which Rac1 and RhoA contribute to modulating endocytosis is sometimes contradictory;

initially it was shown that both GTPases inhibit CME of transferrin (Lamaze et al. 1996); although Rac1 has also been suggested to have a positive role (Kunita et al. 2007); and more recently Rac1 has been involved in holding CXCR4 in a specific conformation which allows internalisation (Zoughlami et al. 2012); this could be used by the NC to increase internalisation at the front where Rac1 is active.

To understand the effect these two GTPases might have on establishing the endocytic polarity of CXCR4 neural crest explants were analysed in the presence of Rac1 (NSC-23766) or ROCK (Y-27632) inhibitors. ROCK is a kinase responsible for the phosphorylation of RhoA and is required for its downstream signalling to become active; hence blocking it acts as a RhoA signalling inhibitor (Riento & Ridley 2003); more information about the inhibitors can be found in 2.3.4 Antibodies & . The cells were incubated for an hour in the inhibitor and then visualised under the microscope for 30 minutes to look at the vesicle movement in both the presence and absence of SDF1.

Cells incubated in either of these inhibitors result in a loss of the endocytic polarity. Now the CXCR4 is being internalised fairly evenly at the free edge and contact membranes as shown in figure 3.4. These results suggest that the polarized endocytic pathway described here for the neural crest, is dependent on Rac1 and RhoA activity. Although we cannot rule out a more general abrogation on cell polarity induced by these inhibitors.

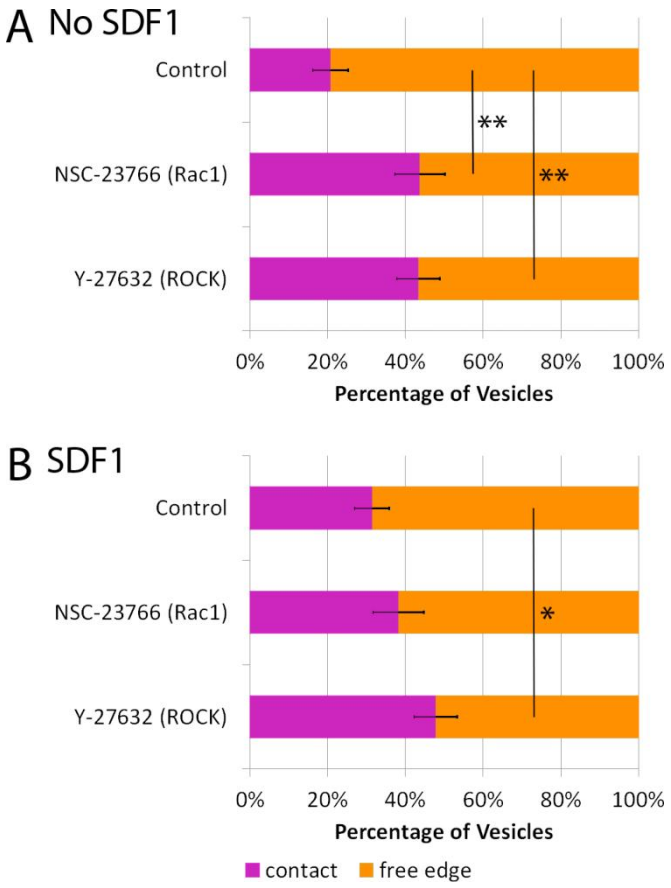


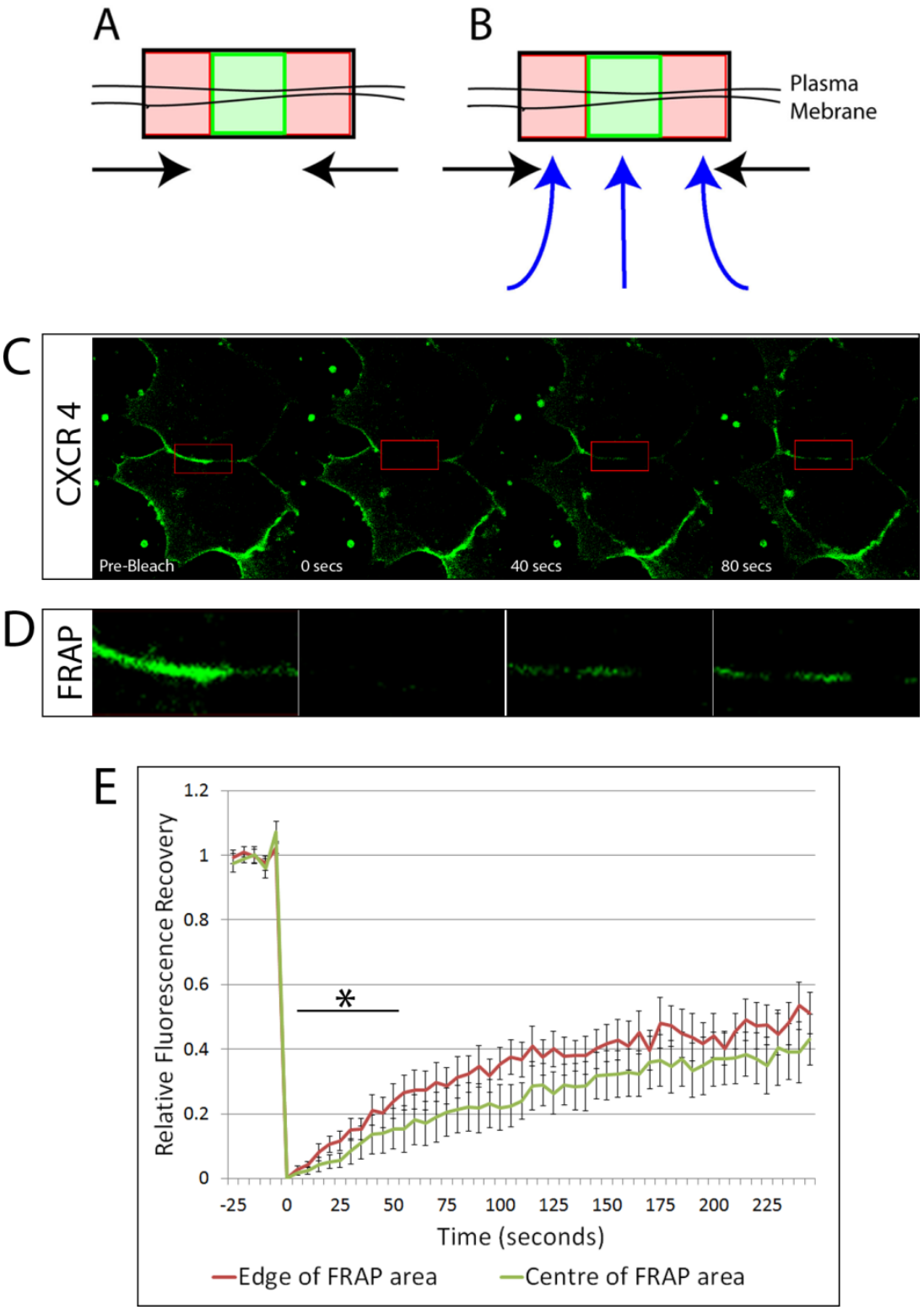
Figure 3.4 – Rho GTPases inhibition affects the endocytic polarity of CXCR4. Internalisation of CXCR4 is more evenly spread in either free edge or contact membranes both in the (A) absence and (B) presence of SDF1. (For control no SDF1n=115 and SDF1 n=98; inhibitor NSC-23766 no SDF1 n=46 and SDF1 n=40; for inhibitor Y-27632 no SDF1 n= 34 and SDF1 n=42)

3.3 CXCR4 membrane dynamics

A method that has been widely used to measure membrane dynamics of proteins is Fluorescence Recovery After Photobleaching (FRAP). FRAP involves bleaching a particular area and then measuring fluorescence recovery; it is used to look at dynamics and speed of movement of proteins. For example in the cytoplasm if a protein can freely diffuse the recovery is fast whereas if it is attached to a vesicle or the reticulum the recovery takes longer (Lippincott-Schwartz et al. 2003).

For membrane proteins FRAP can give information about the movement of the protein within the membrane as well as exocytosis of the protein (Dushek et al. 2008). When the recovery of the bleached are measured they can be split into two main regions, one at the edge of the bleached membrane and the other at the centre; by measuring the recovery in these two distinct areas it can be deduced if recovery occurs mainly through lateral diffusion; in which case the edge of the FRAP area will recover before the centre (Figure 3.5A). Alternatively it can also be used to determine if the protein is exocytosed back to the membrane in which case the central region of the FRAP area will recover as soon as the FRAP area is bleached as seen in figure 3.5B (Goehring et al. 2010).

Figure 3. 5 – FRAP recovery of CXCR4 at the membrane occurs both by lateral movement and exocytosis. FRAP at the membrane can be used to measure (A) membrane only recovery or (B) membrane and exocytosis recovery in which the green area will recover at the same time as the red area at the edge of the FRAP area. (C) Movie stills of pre and post bleach times and (D) a zoom of the FRAP area. (E) Graph showing the relative recovery of the membrane at both edge and centre of FRAP area; the recovery is faster at the edge although the centre region begins recovering immediately after bleaching. (n=54)


A
B
Plasma
Mebrane
C
CXCR 4
Pre-Bleach
0 secs
40 secs
80 secs
D
FRAP
E
Relative Fluorescence Recovery
1.2
1
0.8
0.6
0.4
0.2
0
*
-25
0
25
50
75
100
125
150
175
200
225
Time (seconds)
Edge of FRAP area
Centre of FRAP area

FRAP was performed on neural crest cells expressing CXCR4-GFP and recovery measured in the regions shown in figure 3.5 A & B. Quantification of recovery shows that it occurs faster in the edge of the bleached area than at the centre, suggesting that lateral diffusion of CXCR4 within the membrane is a major component of the CXCR4 redistribution. The central area also recovers, albeit at a slower rate. This recovery begins as soon as the area is bleached, this suggests that CXCR4 also returns to the membrane via exocytosis. It is worth noting that the recovery is not full this is due to the existence of a immobile fraction; a certain percentage of the receptor does not move around the membrane and hence when bleached it will not be replaced by a fluorescent form of the protein and therefore fluorescence recovery is not full (Reits & Neefjes 2001).

This FRAP experiment was done both in the presence and absence of SDF1 and no significant difference was seen in the recovery at both areas of membranes analysed and therefore data was pooled together. The membranes at the free edge and contact were also compared and again no significant difference was seen here either; this might mean that trafficking back to the membrane has no major spatial cue and the main goal of the cell is to make as much CXCR4 as possible .available at the membrane to alter distribute it via polarised endocytosis.

3.4 Summary

Our results investigating CXCR4 dynamics within cells reveal that the neural crest has developed a mechanism in order to prime the cells to respond to the SDF1 signal when in a group. By having increased internalisation of the

receptor at the free edge it ensures that the levels of CXCR4 are constantly being renewed at the site where the signal is most likely to act on. It also suggests an increased environment sensing which can help the group of neural crest to follow the correct pathway.

The results suggest that small Rho GTPases play a role in this as inhibition of these components leads the cells to lose the aforementioned polarity of endocytosis. It is difficult to analyse to what extent this will impairs the response to SDF1 and later migration because affecting the actin cytoskeleton already has an effect *per se*.

The endocytosis polarity which exists within the cells can work in conjunction with the other mechanisms the neural crest has in order to migrate such as CIL and Co-attraction. Single cells are unable to take advantage of this since they do not form contacts and no clear endocytic polarity is formed; this can contribute to the explanation of why single cannot migrate as efficiently to SDF1.

Other aspects of the dynamics follow previously published results such as the longer processing route CXCR4 undergoes when exposed to SDF1. This is mainly due to the alternative fates CXCR4 may have which need sorting of the receptor within the cell. FRAP analysis also shows the existence of both lateral diffusion of the receptor in the membrane as well as exocytosis which shows that there is constant renewal of the receptor at the membrane. FRAP is an excellent technique to determine membrane dynamics of proteins; but in this instance the best results might not have been obtained this may be due to the fast recovery of this particular membrane protein. Therefore to investigate this

further other protein dynamics techniques could be used such as photoactivatable version of the protein which would allow tracking the fate of CXCR4 in a more accurate manner. Also a more varied use of markers; particularly Rab markers; could give more information about colocalisation of CXCR4 to other cellular compartments such as Golgi and the various different endosomes within the cell.

Chapter 4 – CXCR4 Activity

The CXCR4-SDF1 axis has been shown to be very important to guide a large variety of cell types and its importance both in development and in the immune system has become very apparent through many studies (Y. R. Zou et al. 1998; R. S. Klein & Rubin 2004; Petit et al. 2007). Previous work from our group has shown that the receptor has a role in neural crest migration and inhibition of either CXCR4 or SDF1 leads to impaired migration (Theveneau et al. 2010b). The way in which the activity and regulation of the receptor contribute to the migration is yet to be elucidated, by using the CXCR4-GFP construct and mutants which affect the activity we can determine how important this is for the correct movement of the neural crest.

4.1 Overexpression of CXCR4 does not affect Neural Crest Migration

The CXCR4-GFP construct was injected into *Xenopus* embryos in order to understand the effect overexpressing the receptor might have on the migrating neural crest. The migration was analysed both by whole mount in situ hybridisation and in vitro chemotaxis assays (R. M. Harland 1991; Theveneau & Roberto Mayor 2011a).

In situ hybridisation was done on injected embryos to look at the effect the construct might have in the migration of neural crest in vivo. Analysing the

expression of *twist*, a migrating neural crest maker, shows that the migration takes place in a similar manner in both control and injected sides (Figure 4.1). At an early migratory stage (21) we see a slight delay in migration within some embryos although the stream domains are clearly visible. This delay is very mild and quantification by measuring the proportional length of control compared to disrupted sides (Figure 4.1D & E) indicates this delay is around 20% of the normal length. Later at mid-migratory stages (23) the NC still show a similar mild delay. This delay is not seen in late migration stage suggesting the cells overexpressing CXCR4 catch up later on.

An explanation for this delay lays on the increased amounts of the CXCR4 expressed by the cells which could make them more responsive to the SDF1 signal; this creates more migratory competition between the cells as now all of them will have increased response to SDF1. This cell competition will make the cells wanting to migrate ventrally at the same time creating a "bottleneck" at the top of the stream; this will slow down the cells and hence causes the delay. Once the cells are within the stream the cells can catch up.

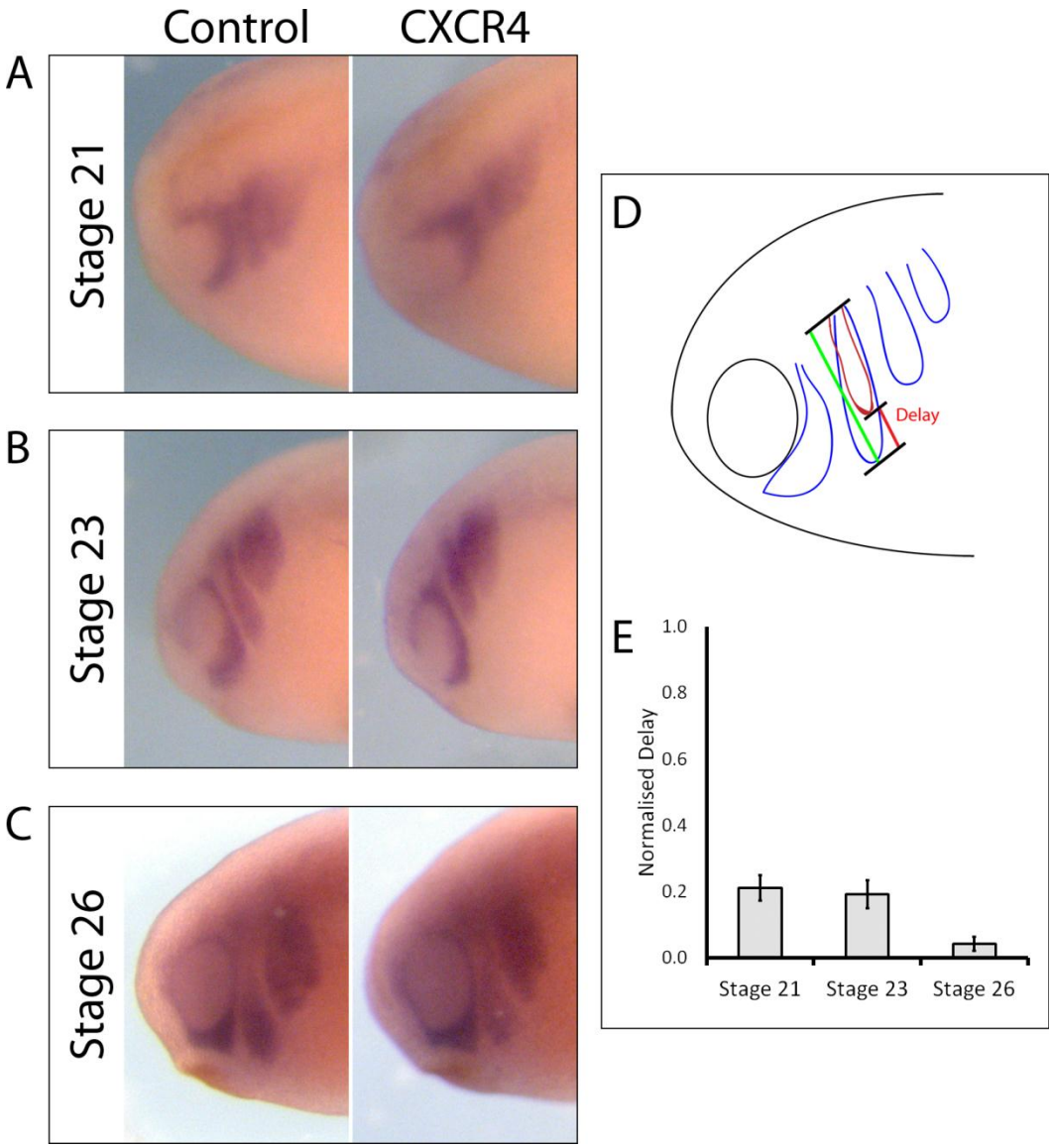


Figure 4. 1- Overexpressing CXCR4 causes a mild neural crest migration delay. In situ hybridisation showing expression of neural crest marker *twist* reveals the half of the embryo overexpressing CXCR4 at (A) early and (B) mid migratory stages have a mild migratory delay, at (C) later stages the neural crest catch up with the control side. The delay is quantified by measuring the normalised delay as shown schematically in (D). (E) shows a delay of around 20% at both stages 21 and 23. (Stage 21 n=103; Stage 23 n=129; stage 26 n=74)

To further test the possible implications of overexpressing the receptor in the migration of neural crest cells this was also analysed *in vitro*. Figure 4.2 shows how neural crest explants overexpressing the receptor follow similar migratory profiles to control cells and have a similar directional and chemotactic response to a localised source of SDF1. We do see a small difference in velocity of the receptor in response to SDF1; this might be due to increased migratory machinery being activated as a result of larger downstream signalling by CXCR4. This also supports the increased responsiveness of cells which in vivo leads to the delayed migration; although here there is no space restriction to form the possible bottleneck we see in the embryo and the migration can proceed normally.

These results show that the injection of the CXCR4-GFP construct and resulting overexpression of the receptor does not lead to any major migratory phenotype of the neural crest in vitro. In vivo there is a slightly delay in the migration of neural crest as a result of this increased amount of the receptor; the difference between these two can be explained due to the reduced space the cells have in the embryo which makes moving much more difficult when more cells are responding to the signal.

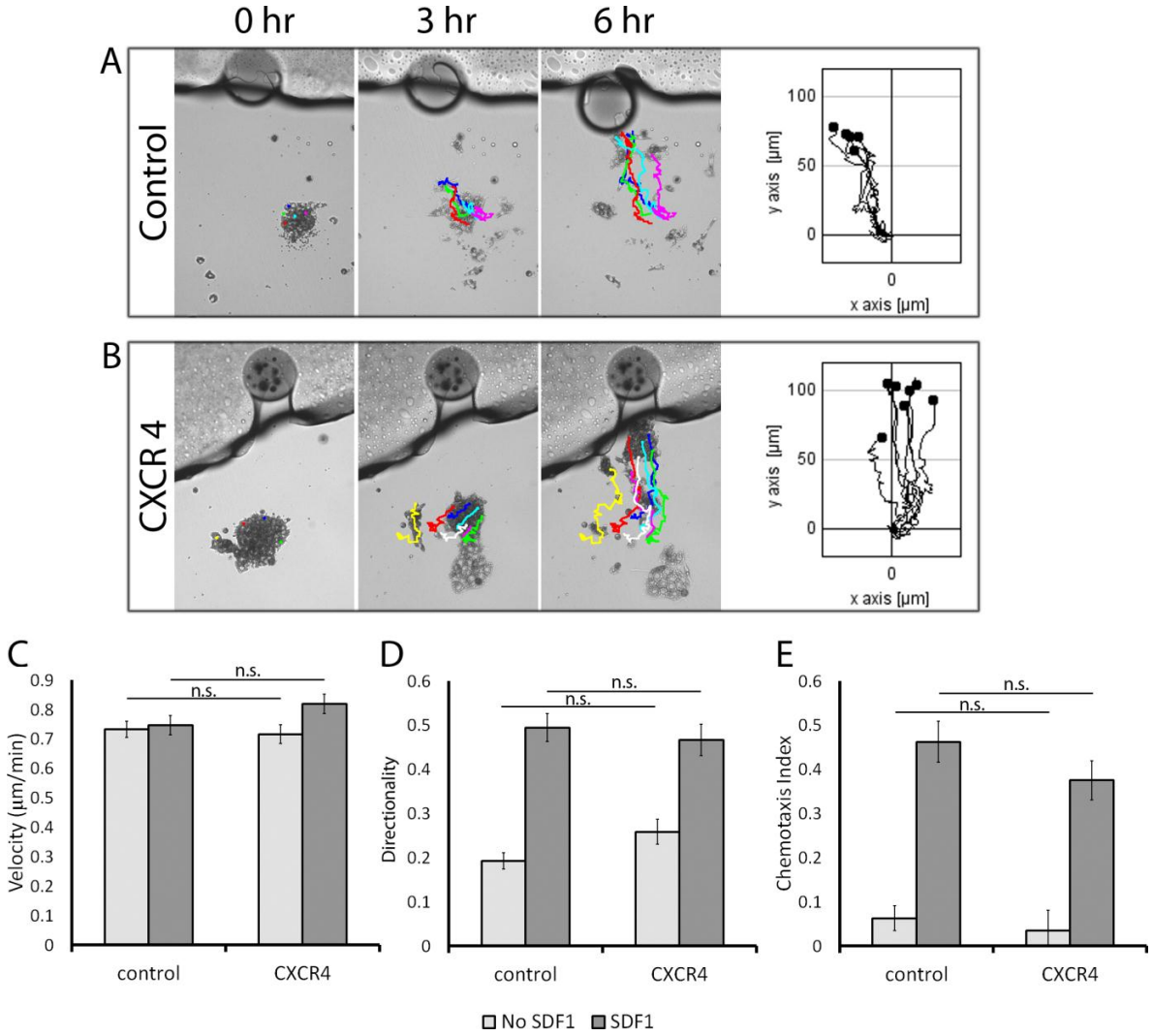


Figure 4. 2 – In vitro migration of explants overexpressing CXCR4 show no significant difference. Explants migration towards an SDF1 soaked bead is tracked for both (A) control and (B) CXCR4 injected explants. Quantification reveals similar (C) migratory velocity; (D) directionality and (E) chemotaxis index. (Control no SDF1 n=259; SDF1 n=301 –CXCR4 no SDF1 n=324; SDF1 n= 315)

4.2 Using CXCR4 mutants to affect activity

Work in other systems has been used to investigate the structure of CXCR4 and how this contributes to its downstream signalling (Doranz et al. 1999; B. Wu et al. 2010b). From all this information certain important components have been involved both in G-protein binding and posterior activation(Crump et al. 1997; Roland et al. 2003). The domains of the receptor important in its internalisation have also been identified (Cronshaw et al. 2010). Mutants affecting either of these two components have been involved in disease and can be used as tools to affect the function of CXCR4; allowing us to understand what role these might have in neural crest migration.

4.2.1 A point mutation gives constitutive activity to CXCR4

The binding of SDF1 to the receptor leads to a conformational change exposing an activation site which will activate the G protein (Berchiche et al. 2007). Previous work has shown that helix-3 is important for this activation, in particular a change in position of certain hydrophobic residues leads to a change in the second intracellular loop which is crucial to activate the G-protein (Percherancier et al. 2005).

A hydrophobic residue in this helix has been shown to play an important role in this process; via point mutations to the asparagine in position 119 which has been substituted into other amino acids which leads to varying effects on the receptor signalling. Of particular interest is the substitution of asparagine to serine which leads to a constitutive active mutant form of CXCR4 (Zhang et al. 2002); the position of this residue can be seen in Figure 1.8 (Introduction). This

mutant provides us with a tool to further understand the role of CXCR4 activity in neural crest migration and how important the regulation and timing of the activation is for the process to happen correctly.

4.2.2 A Truncated mutant impedes receptor desensitisation and internalisation

Another mutant which results in the premature termination of the protein and hence a truncation of its C terminal has been identified in WHIM disease (Balabanian et al. 2005). The mutation affects the internalisation of CXCR4 upon activation; this is a result of the β-arrestin docking site being absent (McCormick et al. 2009). The binding of the arrestin is an important step to desensitise the GPCR and the first step towards its internalisation; this mutant therefore leads to the continuous CXCR4 signalling and results in misguided signalling and disrupted migration (Diaz 2005).The domain affected by this truncation can be seen in Figure 1.8C.

This mutant will allow analysing the effect of not desensitising of CXCR4 through arrestin binding and the role this might have in the migration of neural crest. As the cells move along ventrally they follow the guidance cue from the underlying tissue; the source of the chemoattractant is also changing position and therefore it is important for the neural crest to be able to detect these changes. The renewal of CXCR4 at the membrane is therefore important to make sure there is enough fresh receptor to sense the changing signal. In addition the signal needs to be switched off as the migrating cells have to turn and fine tune the direction of migration and this mutant will allow us to analyse the important of this in helping the migration of the neural crest.

4.3 Measuring receptor activity

To ensure the mutants work in an effective way the activity of CXCR4 can be measured as readout of functionality and compare how the mutants affect this. A way of analysing this is to look at endocytosis of the receptor; this will increase upon ligand binding (Dar et al. 2005). CXCR4's effect in activating migratory machinery as a result of its activation and downstream signalling can also be looked at in order to analyse the activity of the receptor. One such way in which this can be done is by measuring protrusion formation which should increase as a result of CXCR4's downstream activation of the migratory machinery (Peled et al. 1999).

4.3.1 CXCR4 is internalised as a result of SDF1 response

A method of measuring internalisation of fluorescent protein is by incubating the cells in a media containing rhodamine dextran beads (Minina et al. 2007). When the receptor is internalised it will incorporate the beads from the environemnt into the vesicles; these can then be visualised since the beads fluoresce in the red region of the spectrum and will colocalise with the CXCR4-GFP. The process is summarised in Figure 4.4A.

This also allows us to compare the internalisation of the non-fluorescent mutant forms of CXCR4 when co-injected with the CXCR4-GFP mRNA; this is due to the fact that CXCR4 forms dimers when internalised (Terrillon & Bouvier 2004; Milligan 2010). In this case we would expect both mutants to affect CXCR4 internalisation. The activated mutant (N119S) would be expected to increase endocytosis since it has been reported that receptor activation promotes

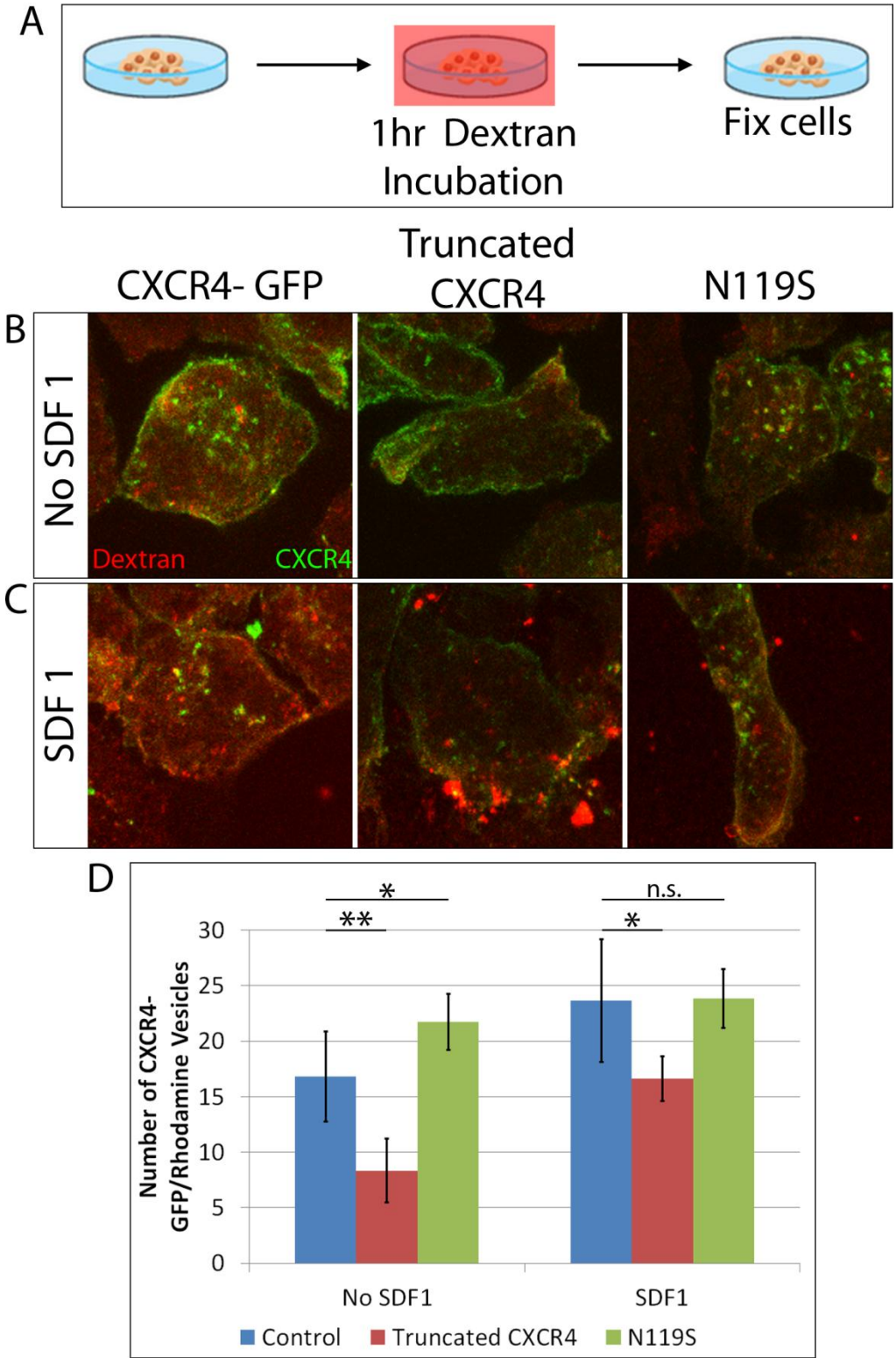
internalisation (Busillo & Benovic 2007). It can also be expected that the non-internalisable mutant will lead to decreased internalisation since dimers formed between the CXCR4-GFP and Truncated-CXCR4 will not be internalised; this will not give us absolute values of endocytosis but can provide evidence for the correct function of these particular receptors.

The total number of CXCR4-GFP/Rhodamine dextran is measured both in the absence and presence of SDF1; images of cells and the quantification can be seen in Figure 4.4. Results show internalisation of CXCR4-GFP occurs both in the absence and presence of SDF1; this is consistent with the analysis shown earlier concerning the endocytic polarity of CXCR4 which is set up even in the absence of SDF1. As expected, incubation with SDF1 increases the number of vesicles containing dextran beads and CXCR4-GFP.

When the cells are injected with both the fluorescent receptor and the mutant form there is a difference in the number of endocytosed vesicles. The activated from of the receptor (N119S) shows high levels of internalisation in both conditions. This indicates that dimers are formed between the N119S mutant and the CXCR4-GFP which will be internalised via the clathrin pathway; this activated mutant shows increased CXCR4 internalistion even in the absence of SDF1. It can also be explained by the downstream signalling of CXCR4 which increases the endocytosis of non-ligand bound receptor in order to modulate the response by regulating the amount of the receptor at the membrane (Busillo & Benovic 2007).

The effect of the truncated mutant is to decrease the levels of endocytosis in both conditions, as previously described (McCormick, Segarra & Gasperini 2009), and confirmed here. The dimers formed between the truncated mutant and CXCR4 will not always be internalised leasing to this phenotype in which internalisation is lower and confirms the expected functionality of the mutated receptor.

Figure 4.3 - Measurement of dextran beads internalisation together with CXCR4 vesicles. (A) Cells are plated on fibronectin, followed by 1hr incubation with dextran in the absence or presence of SDF1; they are finally washed and fixed. B & C show images of cells expressing CXCR4-GFPand mutants and dextran beads in red in the absence and presence of SDF1. Quantification is shown in D, internalisation of CXCR4 increases when exposed to SDF1 as indicating by the increase in the number of vesicles positive for both dextran and CXCR4-GFP. Truncated CXCR4 lowers the amount of internalisation whilst cells expressing N119S-CXCR4 have high levels of CXCR4 internalisation in both the absence and presence of SDF1. (Control no SDF1 n=34; SDF1 n=32– Truncated CXCR4 no SDF1 n=26; SDF1 n= 28 – N119S no SDF1 n=30; SDF1 n= 30)


A
1hr Dextran Incubation
Fix cells
CXCR4- GFP
Truncated CXCR4
N119S
B
No SDF 1
Dextran
CXCR4
C
SDF 1
D
Number of CXCR4-GFP/Rhodamine Vesicles
30
25
20
15
10
5
0
*
**
n.s.
*
No SDF1
SDF1
Control
Truncated CXCR4
N119S

4.3.2 Duration of protrusions increases upon ligand exposure

Another mechanism which can be used to measure the activation of the receptor is to look at the effect on the activation of the migratory machinery components. An easily measurable feature is the stability of protrusions which increase as a result of the downstream signalling cascade. In particular the activation of the PI3K pathway which in turn can activates both cdc42 and Rac1; these two GTPases are responsible for forming protrusion at the leading edge by regulating the actin filaments (Yoo et al. 2010).

The length of time a protrusion lasts for is measured following the scheme shown in figure 4.5A. The results quantified in figure 4.5B show an increase of the stability when exposed to SDF1 when comparing with cells migrating without an external chemoattractant; this is consistent with previously published data in neural crest (Theveneau et al. 2010a).

The effect of the activated mutant, N119S, shows no significant increase in protrusion stability after Sdf1 exposure; indicating that the activity of this receptor is SDF1 independent. The duration of the protrusion is longer even in the absence of SDF1 compared to the control indicating a possible increase in CXCR4 signalling which is consistent with the activity of this particular constitutive active mutant.

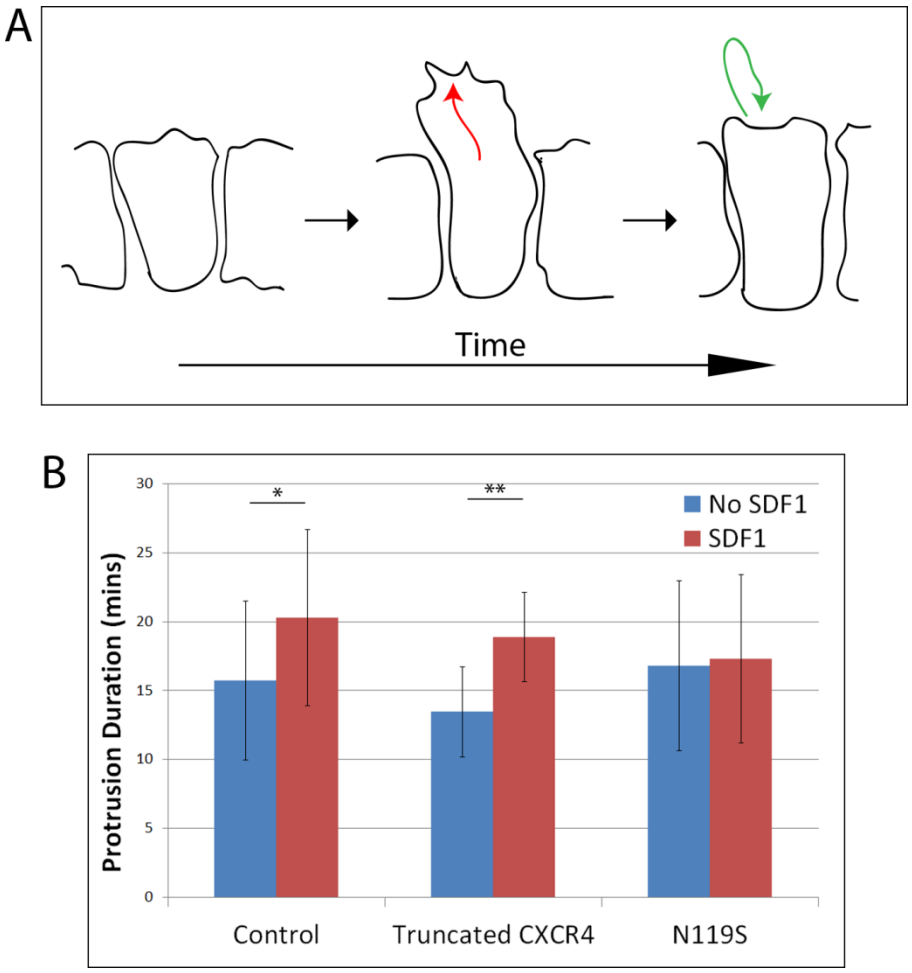


Figure 4.4 - Neural crest protrusion duration increases when exposed to SDF1. (A) the duration of the protrusion is measured from its original formation until its collapse. (B) The duration is longer when cells migrate towards SDF1 in both control and Truncated CXCR4 injected cells. Cells expressing N119S-CXCR4 do not show any variation when exposed to SDF1. (Control no SDF1 n=98; SDF1 n=95– Truncated CXCR4 no SDF1 n=100; SDF1 n= 102 – N119S no SDF1 n=95; SDF1 n= 102)

Cells injected with the truncated mutant; which cannot be internalised show an increase in stability when responding to SDF1, indicating that cells expressing the truncated receptor can still sense SDF1 in a similar way to the wild type receptor; this experiment simply confirms that the truncated CXCR4 is capable of activating its downstream signalling in a similar way to the control situation.

4.4 Summary

The neural crest provides a versatile system on which the activity of CXCR4 can be analysed. When the availability of CXCR4 is increased by overexpressing the receptor we see a slightly delayed migration indicating the importance in regulating the activity to respond to the chemoattractant. To further analyse the implications of CXCR4 in the correct migration of the NC, two mutants will be used to determine how particular aspects are important.

The activity of mutant forms of CXCR4 and the CXCR4-GFP construct has also been confirmed as shown by the effect they have on endocytosis as a result of SDF1 incubation. The mutants also have the power to alter the stability of protrusions in the way that would be expected of them. These two mutants provide the possibility of interfering with CXCR4-SDF1 signalling axis and determine the fine tuning required; let it be by activating the receptor or by internalising it; and the effect these two important processes have on migration.

Chapter 5 – CXCR4 Mutants and Migration

The downstream signalling of CXCR4 leads to an increased survival and migratory phenotypes (Busillo & Benovic 2007). Neural crest require this receptor to migrate ventrally (Theveneau et al. 2010a); although how the various aspects of the receptors regulation an activity affect the migration are unknown; the two mutants previously described can be used to further understand this. The constitutive active CXCR4 will let us determine if CXCR4 is required for directionality or if simply it is used as an initiator of migration and the full movement is dependent on the other signals such as CIL, Co-Attraction and negative signals. The truncated CXCR4 will give us an insight on the importance the receptor desensitisation and internalisation has on guiding the neural crest along the migratory streams.

5.1 Effect of constitutive activity of CXCR4 on migration

The neural crest starts migrating after undergoing EMT during neural tube closure after stage 18 in the *Xenopus* development. Their migration relies on mechanisms described previously such as CIL, which polarises cells at the front of the migrating stream allowing them to follow the SDF1 chemoattractant secreted by adjacent tissues. The constitutively active N119S-CXCR4 mutant can

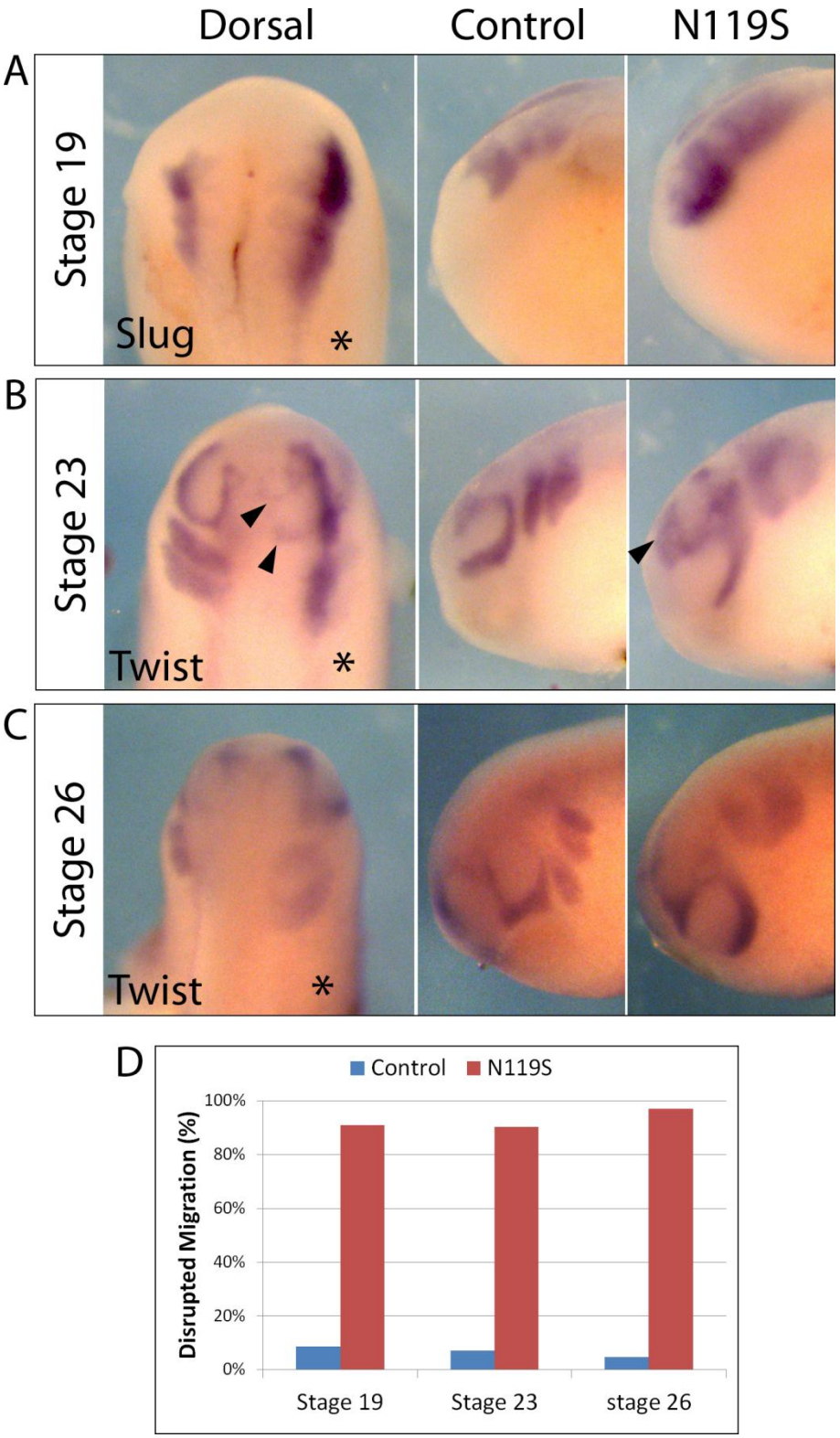
affect the response of the cells to the SDF1 signal. If the CXCR4-SDF1 axis role is to initiate migration and guidance is mainly regulated through negative signals this mutant might cause migration to start sooner although the neural crest will still migrate ventrally, if however SDF1 is not only required to initiate migration but also as a positive cue this cells expressing the mutant might not migrate correctly as they will ignore the chemoattractant location as the receptor will already be active. By analysing the effect this mutant has on migration both within the embryo and in vitro we will be able to clarify the extent of the positive cue provided by SDF1

5.1.1 Cells do not migrate ventrally when expressing constitutive active CXCR4

By using in situ hybridisation the effect this mutant has on the migration in vivo can be analysed; this process relies on using a specific neural crest gene marker in this case slug and twist and labelling their expression. In all cases only half the embryo was injected with a given mutant and their effect was compared with the untreated side of the embryo to ensure there was nothing intrinsically wrong with that particular embryos; as seen in the results there is always a small proportion of embryos that have their development disrupted.

The role SDF1 has in guiding the cells down the stream will become apparent as the N119S-CXCR4 will confer the NC with the ability to start migrating earlier. It could be that this together with the other mechanism used for migration is sufficient to form the migratory streams to ventral positions. The results show that injection of the N119S mutant leads to an altered migration of neural crest and these fail to move ventrally (Fig 5.1).

Figure 5.1 - N119S disrupts migration of Neural Crest. In situ hybridisation of neural crest markers expression in embryos at stages (A) 18, (B) 23 and (C) 26 viewed both dorsally, where (*) indicates the injected side and lateral views. At stage 23 cells migrate ectopically around areas of the neural tube and above the eye as indicated above by the arrows. (D) Quantification shows over 90% of embryos show disrupted migratory phenotypes, the neural crest fail to migrate ventrally and invade areas of the embryo in which they should not be at. (Stage 19 n=42; Stage 23 n=108; stage 26 n=50)


Dorsal
Control
N119S
A
Stage 19
Slug
*
B
Stage 23
Twist
*
C
Stage 26
Twist
*
D
Control
N119S
Disrupted Migration (%)
100%
80%
60%
40%
20%
0%
Stage 19
Stage 23
stage 26

This disrupted migratory phenotype is already evident at early stages even prior to the start of the migration (Figure 5.1A). The control side of the *Xenopus* embryo shows the neural crest positioned at dorsal positions next to the closing neural plate as would be expected. The injected side however shows a much different neural crest domain occupying a much larger area which finishes at a more posterior region. The dorsal view shows that the neural crest is also closer to the dorsal midline in an area that should be populated exclusively by the neural tube. Both these situations can be explained by an earlier activation of the migratory machinery which allows the cells to start moving earlier than what would be usual and invade areas which they should not have. The N119S-CXCR4 can activate this machinery and cells migrate throughout all permissive areas; these do not include ventral areas in which negative signals such as semaphorins and ephrins are present at (Santiago & Erickson 2002; Yu & Moens 2005).

At later migratory stages we see a stark contrast between both control and injected sides of the embryo (Figure 5.1B & C). Within the uninjected half of the embryo the neural crest migrate around the eye and ventrally through the characteristic streams. When expressing N119S-CXCR4 the migration is greatly disrupted; the neural crest cells are located in their majority at dorsal positions and do not migrate following the streams seen on the control side. Again they also occupy parts of the neural tube reaching all the way to the dorsal midline where they should not be at (figure 5.1B). At a late migratory stage they seem to be excluded from this area and limited to three dorsal domains and around the eye.

Lateral views of the embryos reveal an increased migration of neural crest in the area above the eye pushing it to a more ventral position. The neural crests also remain dorsal, restricted to domains which are probably separated by the negative signals expressed in between the streams (reviewed in Theveneau & Mayor 2012).,

5.1.2 N119S-CXCR4 injected cells ignore SDF1 signals in vitro

To further understand the effect this particular mutant has on the migration of neural crest their migratory characteristics were measured in vitro. Explants were removed from *Xenopus* embryos at stage 18 and allowed to attach and migrate on a fibronectin coated dish. Explants were then confronted to a localised source of SDF1 or allowed to migrate freely. In all these experiments explants are allowed to migrate freely around the dish unrestricted by any physical barriers, their migration is tracked using a manual computer software and the speed directionality and chemotaxis index is measured as explained in the methods. The experiments were repeated on various occasions to confirm the results were fully reproducible.

Control cells migrate towards the chemoattractant soaked bead (Figure 5.2A). The cells expressing the N119S mutant ignore the bead and move in no particular direction (Figure 5.2B). Measuring the migratory properties of these cells shows that when allowed to migrate in the absence of an external signal both control and injected cells migrate with low directionality and chemotaxis index. We do see an increase in the average velocity of injected cells this is most likely due to an increase in the activation of CXCR4's downstream signalling in a

similar fashion to what we saw previously when CXCR4 is overexpressed (Figure 5.2C).

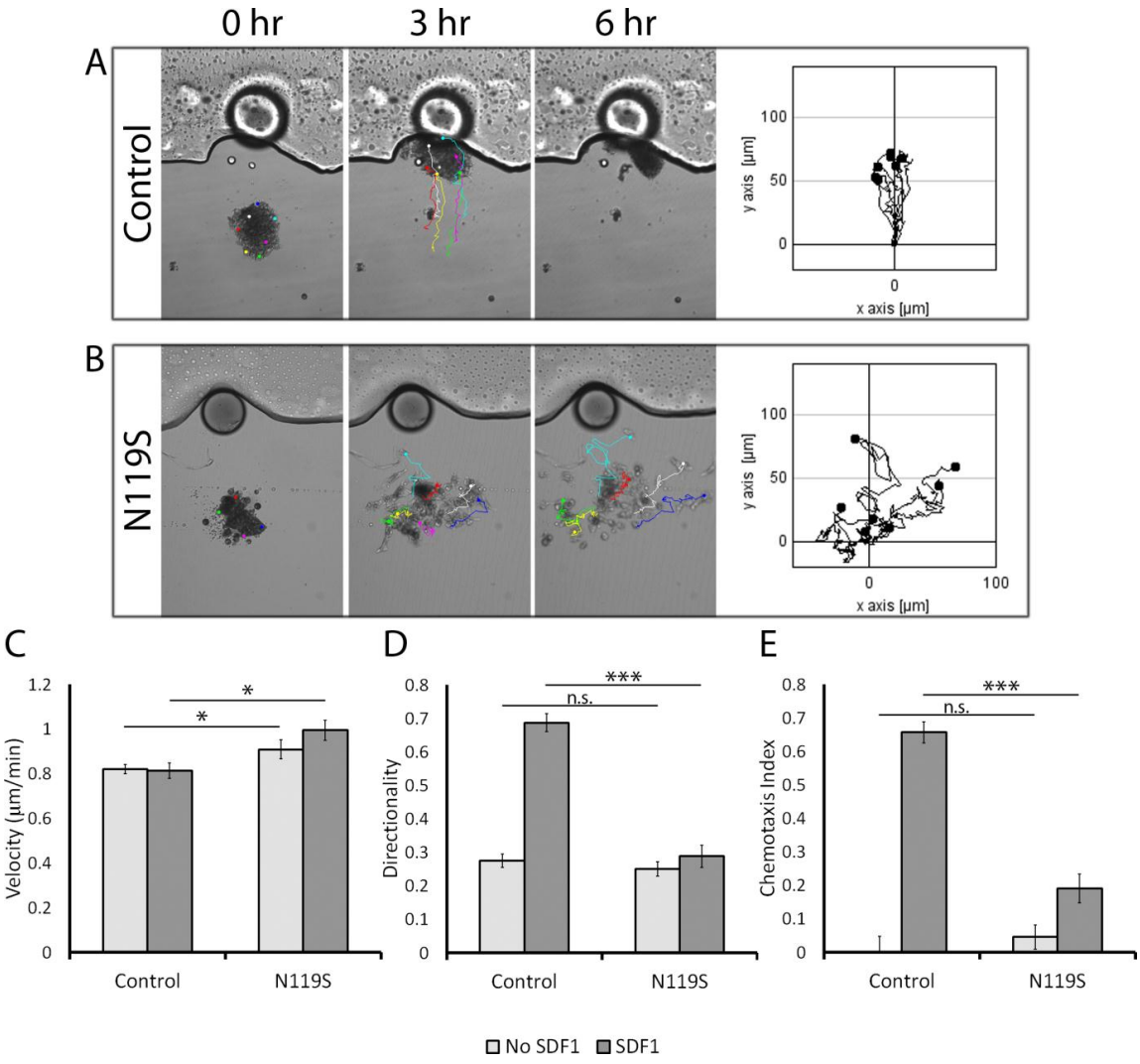


Figure 5.2 - CXCR4-N119S injected explants ignore a localised source of SDF1. Movie stills and tracks of (A) control and (B) injected neural crest cells migrating whilst exposed to a bead soaked in SDF1. Measurements of (C) velocity, (D) directionality and (E) chemotaxis index shows N119S increases the velocity of cells whilst cells ignore the SDF1 having low directionality and chemotaxis index. (Control no SDF1 n=215; SDF1 n=221 – N119S no SDF1 n=256; SDF1 n= 267)

When explants are confronted to a source of SDF1 we can see control cells migrating towards the bead with a high level of directionality and chemotaxis index; both these measurements remain low in injected cells. In fact the N119S injected cells show similar migratory properties in both conditions indicating that indeed the cells ignore the external positive cue as the results in vivo suggested.

From both in vivo and in vitro experiments, we can see that the correct activation of the receptor is important to guide cells. If the cells activate their migratory machinery too early they can migrate into regions in which they should not be at, therefore the time at which the cells are allowed to begin their migration needs to be tightly regulated in order to make sure it occurs correctly.

The results also indicate the importance of having a localised positive cue; this was already known from the morpholino analysis of both CXCR4 and SDF1 (Theveneau et al. 2010a); but it was not clear if the role of this was simply to activate the migratory machinery and the cells could be guided through other mechanisms described in the introduction. Using this mutant we can conclude that activating CXCR4 signalling is not sufficient and the external guidance cue is necessary to guide cells ventrally. The results also confirm the importance of negative signals in shaping the different streams as the three domains are clearly visible at the dorsal part of the embryo.

5.2 Truncated-CXCR4 effect on Neural Crest Migration

The internalisation of the receptor is important to regulate its activity once the ligand binds. Results discussed previously in Chapter 3 also point to a role in

internalisation of the receptor in priming the cells to respond to SDF1 more efficiently by having an endocytic polarity which renews the protein more favourably at the free edge membrane. A truncated CXCR4 mutant was used in order to understand how interfering with this internalisation might hinder neural crest migration. This mutant lacks the C terminal domain which is used as the docking site for the internalisation machinery and remains in the membrane even upon activation; it has been linked with WHIM disease where cells are misguided (Balabanian et al. 2005).

5.2.1 Migration in vivo is delayed by the truncated-CXCR4 mutant

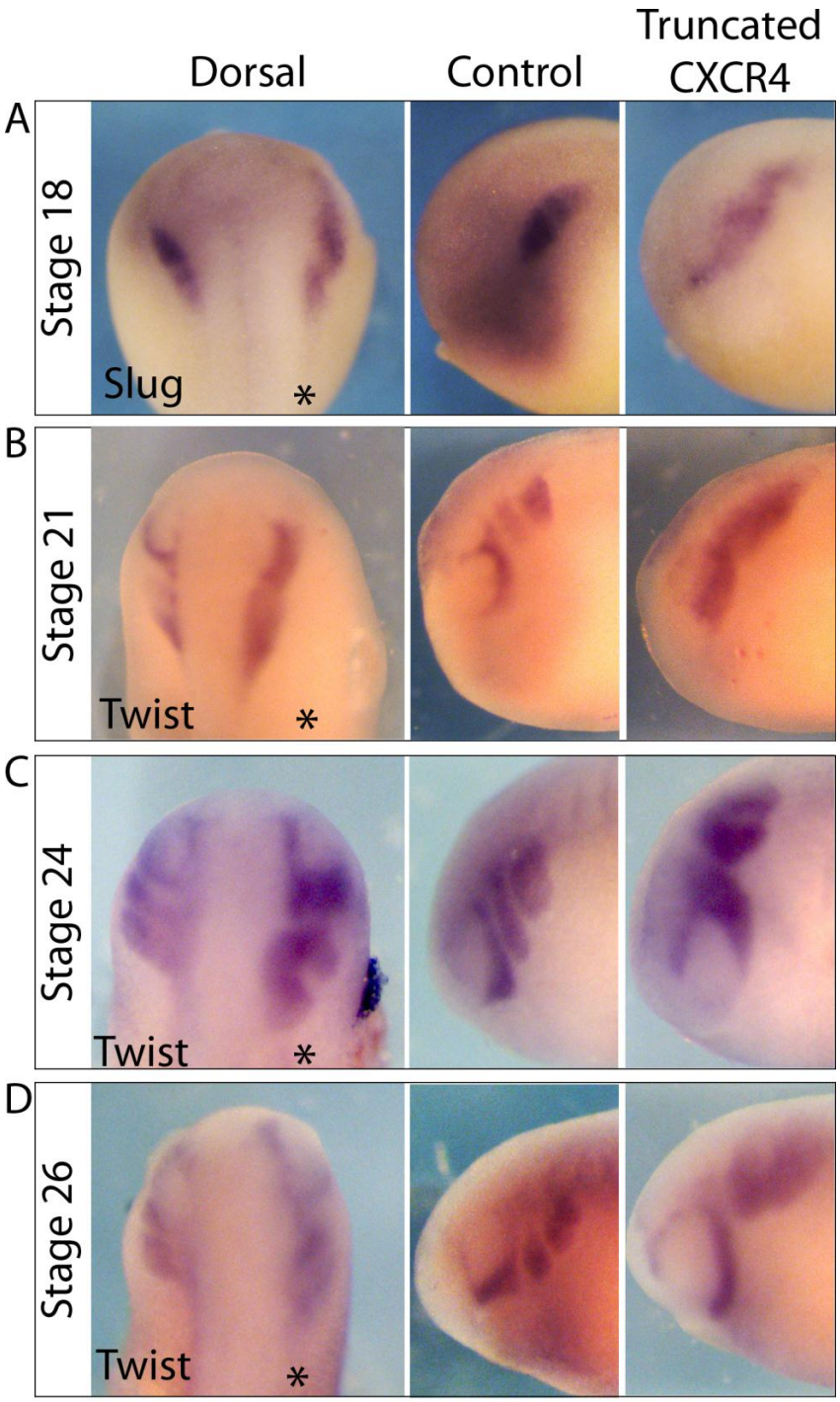
The truncated-CXCR4 was injected into embryos and the migration was analysed by whole mount in situ hybridisation labelling expression of different neural crest markers at different stages. Figure 5.3 shows that the migration is indeed impaired at all stages of the migration.

At pre-migratory stages 18 (Figure 5.3A) we see the neural crest on the control side is mainly located at the edge of the neural plate with the characteristic shape, they are grouped together at this stage and will be delaminating from the closing neural plate. The cells on the injected side occupy a slightly larger area; this may be due to some SDF1 being secreted from the underlying tissue which has started to activate migration. Since the migration is not switched off the cells can stats migrating and this appears to be magnified allowing the cells to spread more. Unlike the activated form of CXCR4 the migration does not occupy dorsal areas where the neural tube is closing since no SDF1 comes from this area.

At early migration stages (Figure 5.3B) we see a clear phenotype in which migration of the neural crest is delayed. In the wild type situation we see the streams are emerging from the dorsal positions; around the eye the neural crest have migrated a significant distance. This is different to the injected side; here the streams are also discernible but their migratory ability is reduced. This is particularly evident in the cells around the eye where the cells have migrated a much smaller distance when compared to the control.

A similar phenotype is seen again at a mid-migration stage with up to 90% of embryos showing disrupted migration. As shown in Figure 5.3C the embryos display short streams with very little migration in a ventral direction in stark contrast with the control side of the embryo which shows streams migrating as expected. The longer streams do appear to be slightly misshaped and sometimes appear to be joining together; this phenotype will be discussed in further detail later.

Figure 5.3 - Truncated CXCR4 delays migration of Neural Crest. In situ hybridisation of *slug* or *twist* expression in embryos at stages (A) 18, (B) 21, (C) 24 and (D) 26 showing a dorsal view of the embryo, (*)indicates the injected side; and lateral views of both control and injected sides of the embryo. (E) At the pre migratory stage (18) over half the embryos show a disrupted distribution of neural crest cells. At migratory stages (21-26) the neural crest streams are delayed and remain at dorsal positions. (Stage 18 n=58; Stage 21 n=109; Stage 24 n=123; stage 26 n=62)


Dorsal
Control
Truncated CXCR4
A
Stage 18
Slug
B
Stage 21
Twist
C
Stage 24
Twist
D
Stage 26
Twist
E
Control
Truncated CXCR4
Disrupted Migration (%)
100%
80%
60%
40%
20%
0%
Stage 18
Stage 21
stage 24
Stage 26

During the final stages of migration where the streams should have migrated ventrally, the injected side is severely delayed and misshaped. The streams are located in a dorsal position and although the different domains are separated there is certain level of overlap at the leading edge of the stream as seen at the earlier stage too, which is likely due to the misguided signalling.

Overall the in situ results show the importance that desensitising the receptor and switching off CXCR4 downstream signalling has in directing the correct migration of neural crest. If the cells are unable to modulate their response to the positive cue they are delayed, this can be caused by the inability of the cells to sense the changing location of SDF1 as the cells move ventrally.

5.2.2 Migration of Neural Crest expressing Truncated-CXCR4 is delayed in vitro

To further assess the impact the truncated mutant of CXCR4 has on migration the behaviour of cells in vitro was measured in both the absence and presence of SDF1. Our results show that neural crest explants migrating on fibronectin do so normally in the absence of SDF1. Both control explants and mutant injected cells show similar speed and directionality which we would expect since in both situations the CXCR4 would remain inactive and therefore would not affect the migration; this we already saw by analysing protrusion formation. The cells also migrate in a similar fashion to controls with some cells remaining as a group and others dissociating from the main group; this is a common feature of neural crest explants.

When the same experiment is performed with explants exposed to SDF1 we see different migratory properties. Both control and injected cells sense and migrate towards the bead as seen in figure 5.4 A & B, but whilst control explants show a high level of directionality and a chemotaxis index of above 0.6; the neural crest expressing the truncated-CXCR4 has a significantly smaller directionality and a chemotaxis index below 0.5.

The explanation for this lower directionality is that the explants take a much longer time and path to get to the bead. The main reason for this is that as the group approaches the chemoattractant source it misses it and migrates straight past it just to turn at a later time to eventually migrate towards the bead. Missing the target is likely the result of not being able to switch off the signal and hence the cells continue migrating in that particular direction. The cells are not as fast at changing direction when they deviate from the direct pathway to the bead and the explant migrates too far; eventually signalling in the correct direction helps the cells reach the target which will overrule the previous signal; this will be looked in more detail later by using a movable source of SDF1

In the wild type situation the group of cells is able to fine tune direction of migration to make sure the source of the chemoattractant is reached by being able to regulate the activity and internalisation of the receptor. This constant directional adjustment has been seen in other systems where the periods of migration shorten near the target so that the cells can repolarise in order to update its migration information and make sure it is sensing the correct direction of the chemoattractant (Minina et al. 2007).

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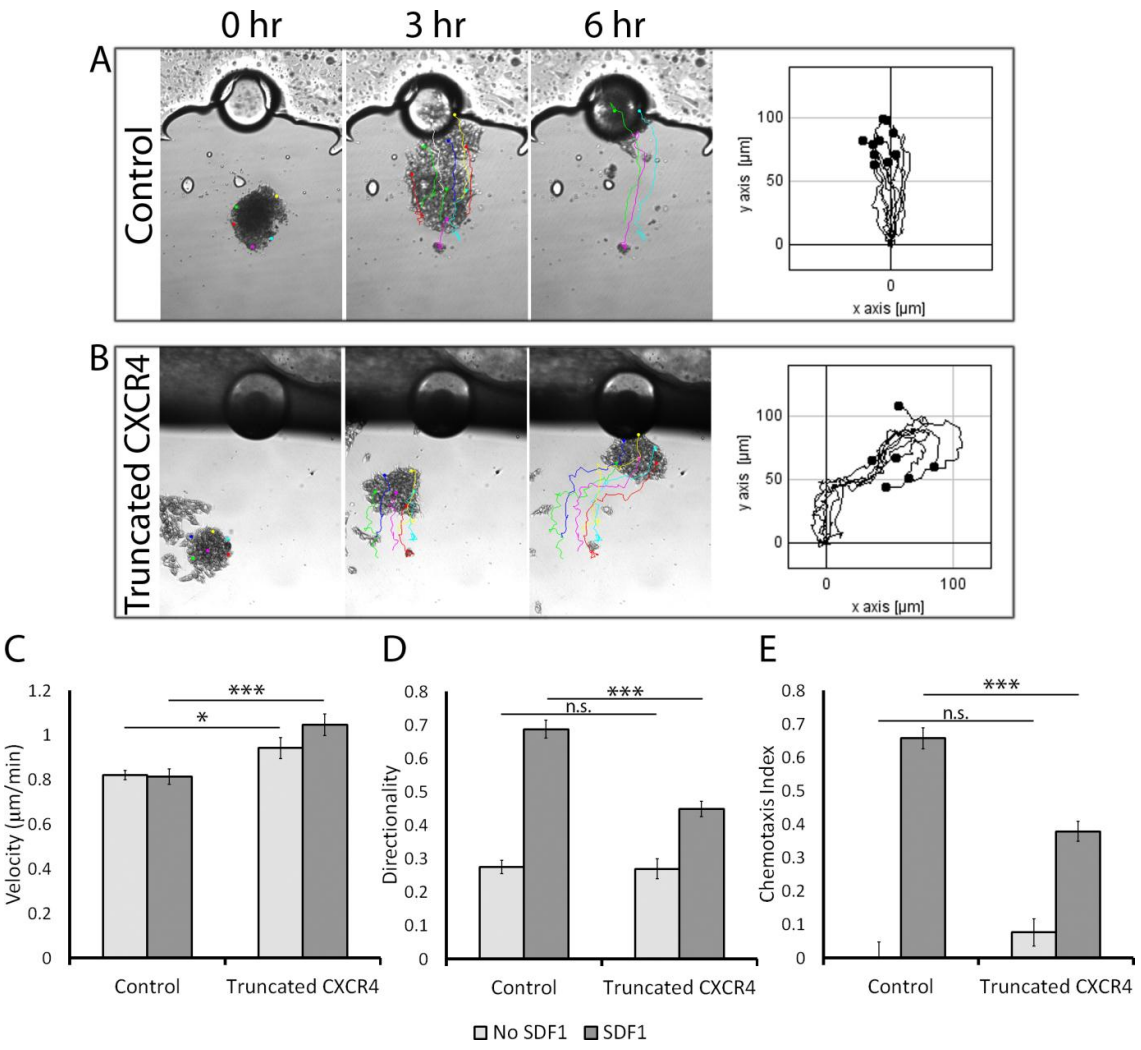


Figure 5.4 7 - Truncated CXCR4 injected explants respond to SDF1 with reduced directionality in vitro. Movie stills and tracks of (A) control and (B) injected neural crest cells migrating towards and SDF1 soaked bead. Measurements of (C) velocity, (D) directionality and (E) chemotaxis index shows that cells expressing truncated CXCR4 migrate with greater velocity but significantly reduced directionality and Chemotaxis index towards the bead. (Control no SDF1 n=215; SDF1 n=221 – Truncated CXCR4 no SDF1 n=282; SDF1 n= 276)

5.2.3 Truncated CXCR4 leads to disrupted streams *in vivo*

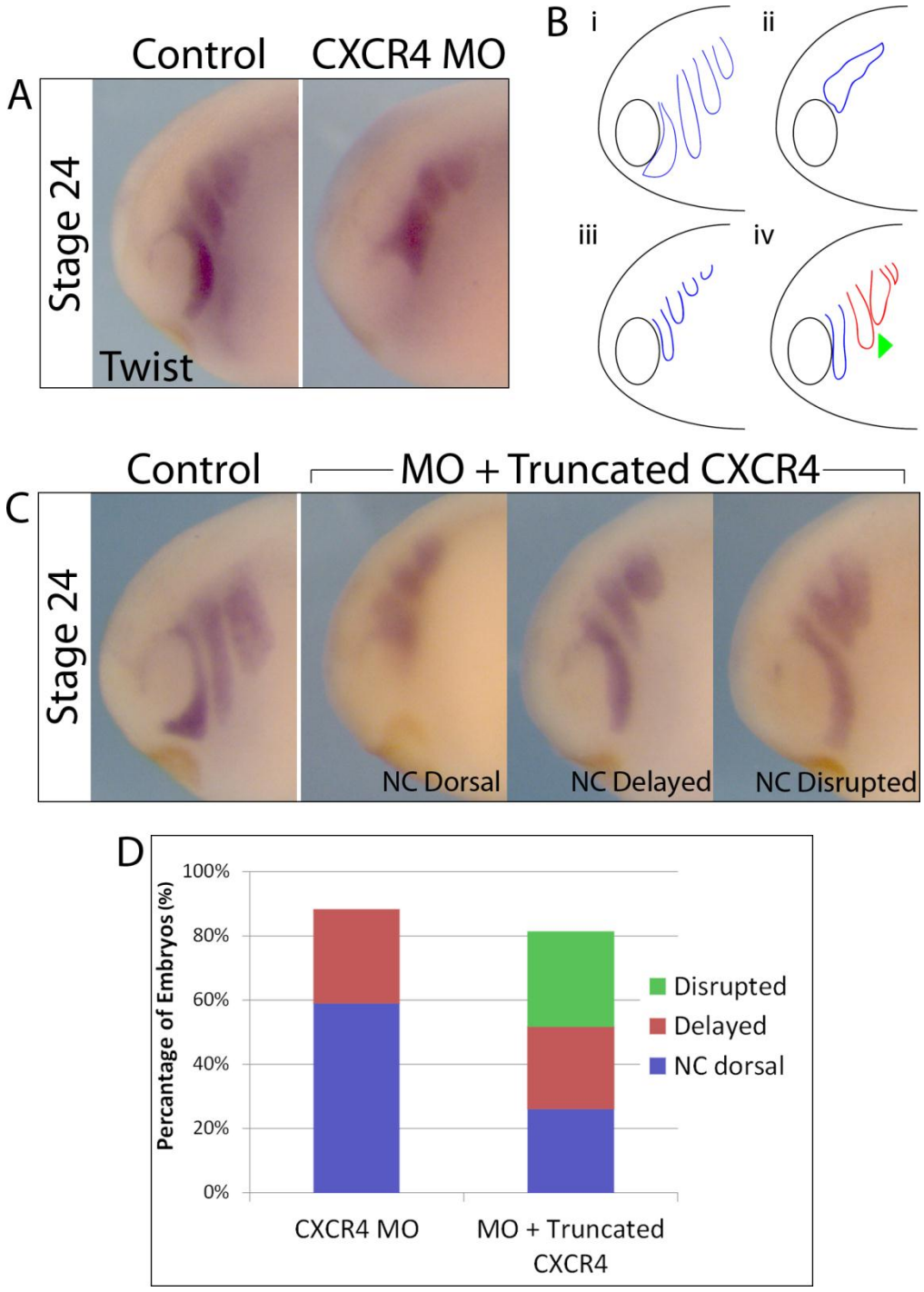
From both in vitro and in vivo results it is clear that attenuating signalling via β-arrestin binding and posterior internalisation is important in directing cells. Cells unable to switch the signal off show altered migration which is misguided and delays the ventral migration of neural crest. A phenotype we see during the migration in vivo is streams getting closer than usual and their shape becoming slightly disrupted.

To investigate this particular characteristic further the truncated CXCR4 construct was injected together with CXCR4-MO and the neural crest stream formation was analysed at stage 24 when the streams should have already formed and migrated a substantial distance ventrally. CXCR4-MO leads to a stop or severe delay in the migration NC as seen in figure 5.5 A; this is consistent with previously published results (Theveneau et al. 2010a).

When the rescue of this phenotype was attempted using the truncated CXCR4 we see an improvement in the migration of neural crest although in the majority of embryos this migration is disrupted. In order to quantify these various disruptions the number embryos were classified using the system shown in figure 5.5B. Over 30% of the embryos have a kink in one of the streams which in many cases causes the streams to invade each other's path. Also in most embryos the stream around the eye migrate straight ventrally without turning this suggests that the neural crest fails to adapt to a changing source of SDF1 when this one has changed.

This result might be due to the fact the signal is not switched off and the migratory machinery persists in its migration in a particular direction. In early development cells need to be constantly sensing the environment and adapt as it rapidly changes. If we inhibit the ability of cells to be able to modulate the response to external factors, as this truncated mutant does, the embryo fails to develop correctly. This highlights the requirement of signals to be switched on and off at the appropriate time to orchestrate all the morphogenetic movements in the embryo.

Figure 5.5- Truncated CXCR4 causes misshaped neural crest streams to form. (A) In situ hybridisation of embryos injected with CXCR4 MO show supressed migration. (B) Phenotypes shown by embryos injected with CXCR4-MO and truncated CXCR4 show a variety of migratory paths: (i) normal, (ii) NC dorsal, (iii) NC delayed and (iv) NC stream disruption; in particular at the third stream where the stream is kinked and misguided as shown by the green arrow. (C) Shows examples of embryos with the 3 defective migratory phenotypes. Quantification shown in D indicates close to 90% of embryos injected with CXCR4MO have abolished or delayed NC migration, this phenotype is partly rescued by co-injecting mutant1 with under 30% showing NC at exclusively dorsal positions; however over 50% of embryos have a delay or disruption of neural crest. (MO n=28; MO+Truncated CXCR4 n=51)


A
Control
CXCR4 MO
Stage 24
Twist
B
i
ii
iii
iv
C
Control
MO + Truncated CXCR4
Stage 24
NC Dorsal
NC Delayed
NC Disrupted
D
Percantage of Embryos (%)
100%
80%
60%
40%
20%
0%
CXCR4 MO
MO + Truncated CXCR4
Disrupted
Delayed
NC dorsal

5.2.4 Cells expressing truncated CXCR4 do not respond to a moving source of SDF1 in vitro

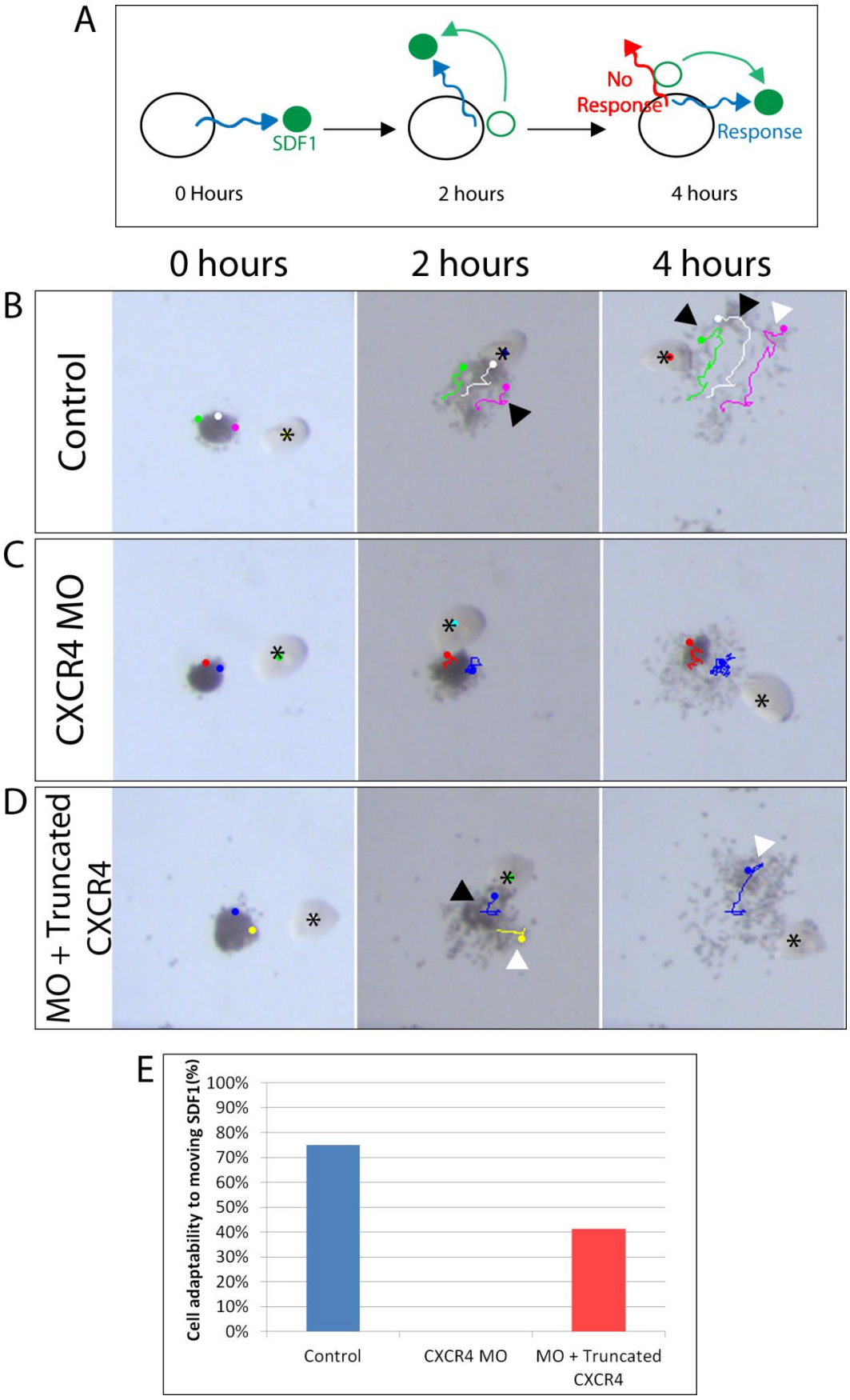
In order to further understand this misguided migration the cells were analysed in vitro. A problem of using beads embedded in grease is that it lacks the flexibility to replicate a dynamically moving chemoattractant source as they are fixed to a particular location. Therefore a method is needed which would allow to change the location of the SDF1 source as the cells migrate; this would replicate a moving source of chemoattractant within the embryo.

A system by which we can do this is by adapting a technique previously used in which the beads are placed freely in the medium allowing cells to migrate towards it; as the neural crest migrate towards it they push the bead to a new location and follow it eventually reaching the bead and "kicking it" or pushing it to a new location (Theveneau & Roberto Mayor 2011a). This modified method involves manually moving the bead at particular times and measure the ability of the cells leading the migration to change to the new location of the bead; this is shown schematically in figure 5.6A.

The results show that control explants follow the SDF1 bead, when this bead is moved the cells quickly follow and migrate to the new location (Figure 5.6 B). Cells injected with CXCR4-MO do not respond to SDF1 and migrate without any particular direction in the area where they were initially plated (Figure 5.6 C). When cells injected with truncated-CXCR4 are placed in a similar situation they do respond to the SDF1 source and migrate towards it. Once the bead is moved to a new position the explants generally do not respond to the new signal

location; the most common fate is for the cells to continue migrating for a short period and then stop (Figure 5.6 D).

Figure 5.6 – Truncated-CXCR4 affects response to moving source of SDF1. (A) Neural crest explants are allowed to migrate towards an SDF1 soaked bead, after 90mins the bead is moved to a new location and the ability of explants to turn to the new location measured. Movie stills of (B) control, (C) CXCR4-MO and (D) MO+Truncated CXCR4 injected cells migrating in the presence of a movable source of SDF1 (*), explant turning is shown with a black arrow and inability to turn in white. (E) Measuring the percentage of times the explant does turn as a result of the new location of SDF1 reveals cells expressing truncated CXCR4 are much less able to turn and usually stop migrating. (Control n=52; MO n=28; MO+Truncated CXCR4 n=51)


A
SDF1
No Response
Response
0 Hours
2 hours
4 hours
0 hours
2 hours
4 hours
B
Control
C
CXCR4 MO
D
MO + Truncated CXCR4
E
Cell adaptability to moving SDF1(%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Control
CXCR4 MO
MO + Truncated CXCR4

The explants unable to desensitise CXCR4 are disorientated once the chemoattractant moves; the cells cannot respond with the versatility and speed that control cells show. Obviously in the growing embryo the change of position would not be as sudden, but the results do provide evidence for the inability of cells expressing non-internalisable CXCR4 to dynamically sense a changing chemotactic cue and contribute to explain the in situ data.

5.3 Summary

Using both constitutive active and non internalisable mutants the role CXCR4 plays in aiding Neural Crest migration is clearer. The data from the activated receptor show that the neural crest require a localised chemoattractant in order to proceed with the ventral movement. The role of the attractant is not exclusively to activate the migratory machinery but to make sure it is activated in the correct orientation. It could have been that SDF1 simply switched on migration and the cells could then move directionally using mechanisms discussed in the introduction; but by using N119S-CXCR4 it is evident that neural crest are capable of migrating in many other directions and require this ventral positive cue do so directionally.

What does seem apparent is that stream formation is more reliant on negative signals as the domains are formed in the presence of both mutants. It might be that over-activating the positive signal can disrupt the streams as shown with the non-internalisable mutant which causes misguided movement and may lead to overlap between adjacent streams.

Switching signalling off is also vital in order to guide the cells ventrally. The truncated CXCR4 shows that cells are unable to migrate properly in vivo and are massively delayed when responding to a chemoattractant in vitro. In addition internalisation allows cells to constantly sense the environment and fine tune the direction which they are migrating, when the receptor cannot be internalised this fine tuning does not occur and the target is missed.

All the small components of downstream signalling of CXCR4 come together in order to modulate the response to the positive cue and allow for directional migration in an orderly fashion. Here there is clear evidence that activation, desensitisation and internalisation are all important in aiding this collective movement of cells.

Part IV

Conclusions

Chapter 6 – Discussion

The coordinated movement of tissues during embryo development is crucial to ensure structures and organs form in an orderly fashion. It makes sense to induce similar tissues in one location and then allow the cells to move to their destination; rather than have many separate induction events around the embryo. The neural crest is a clear example of this; they are all formed at the border of the neural plate and later migrate together to a variety of locations around the embryo where they will terminally differentiate into components of the peripheral nervous system, cartilage and pigment cells amongst others (Knecht & Marianne Bronner-Fraser 2002). The migratory path the cells take around the embryo needs to be tightly controlled to make sure cells end up in the correct place.

Cephalic neural crest in *Xenopus laevis* migrate as a collective and their migration involve many mechanisms which guide the cells and maintain the group structure. The cells use cell contacts formed with each other to help polarise via CIL; in order to remain as a group they co-attract each other by secreting C3a (Carmona-Fontaine et al. 2008; Theveneau et al. 2010a; Carmona-Fontaine et al. 2011). Positive and negative signals also play a role in guiding the cells and form the migratory streams (reviewed in Theveneau & Mayor 2012). This thesis analyses the role the CXCR4-SDF1 axis has in this process and how regulating the activation and signalling of it is necessary for the migration

to take place correctly. There is also evidence of a novel mechanism by which the group of cells are primed to respond to an external signal giving them an advantage over single cells.

6.1 Cells in groups are primed to have increased receptor renewal at the free edge

Neural crest cell groups migrate towards SDF1 more efficiently than single groups. One of the reasons for this is explained by the contribution of CIL which helps polarise the first row of cells allowing them to move forward; this however does not fully explain why a single cell cannot respond to the chemoattractant and migrate quickly to the source.

This thesis presents a mechanism by which the cell group not only contributes to polarise cells but also to create a polarized endocytosis of CXCR4. This results in increased turnover of the receptor at the free edge which can prime the cells to respond to SDF1 more efficiently at the free edge. The differential endocytosis is lost when either Rac1 or RhoA signalling are inhibited; both have been involved in regulating internalisation of proteins. These two GTPases also have a polarised presence in the cell as a result of CIL meaning that all processes work together to ensure that neural crest are ready to migrate.

Single cells lack contacts and therefore do not set up such strong cell polarity via CIL and the results do not show any clear preference in the internalisation of CXCR4 in any regions of the cells; hence cells do not set up the endocytic polarity and are not primed to respond to SDF1. In addition cells repolarise quite often and the front of the cell is constantly changing; this explains why

these cells have such difficulty in migrating effectively. Cells in a group are able to amplify the response to SDF1 by having an increased turnover of the receptor at the free edge; this will act as a strong guidance cue. The fact that single cells show such randomised endocytosis of CXCR4 means that such a mechanism is not available to them and a positive signal will not be maintained once the receptor is internalised making it more transient and requiring the cell to sense the signal again.

Rho GTPases have also been involved in the trafficking of components required for clathrin mediated endocytosis during T cells migration (Samaniego et al. 2007). CXCR4 is internalised via clathrin dependent mechanism so it could be possible that the polarised distribution of RhoA can transport the proteins required for internalisation to the front and help from the endocytic polarity.

The concept of polarised trafficking during chemotaxis is not entirely new; it has been suggested that cells responding to chemoattractant will recycle the receptor predominately at the front where the signal is coming from (S. J. Fletcher & Rappoport 2010). This thesis does not present any evidence that single neural crest cells can do this in the presence of chemoattractant; but presents a mechanism through which cells within a group can polarise the dynamics of a receptor in a similar way. This results in group of cells to be primed to have increased receptor endocytosis and renewal at the free edge which will be responsible to sense the SDF1 signal. Therefore the group has a an advantage over single cells which do not have such a mechanism (Figure 6.1)

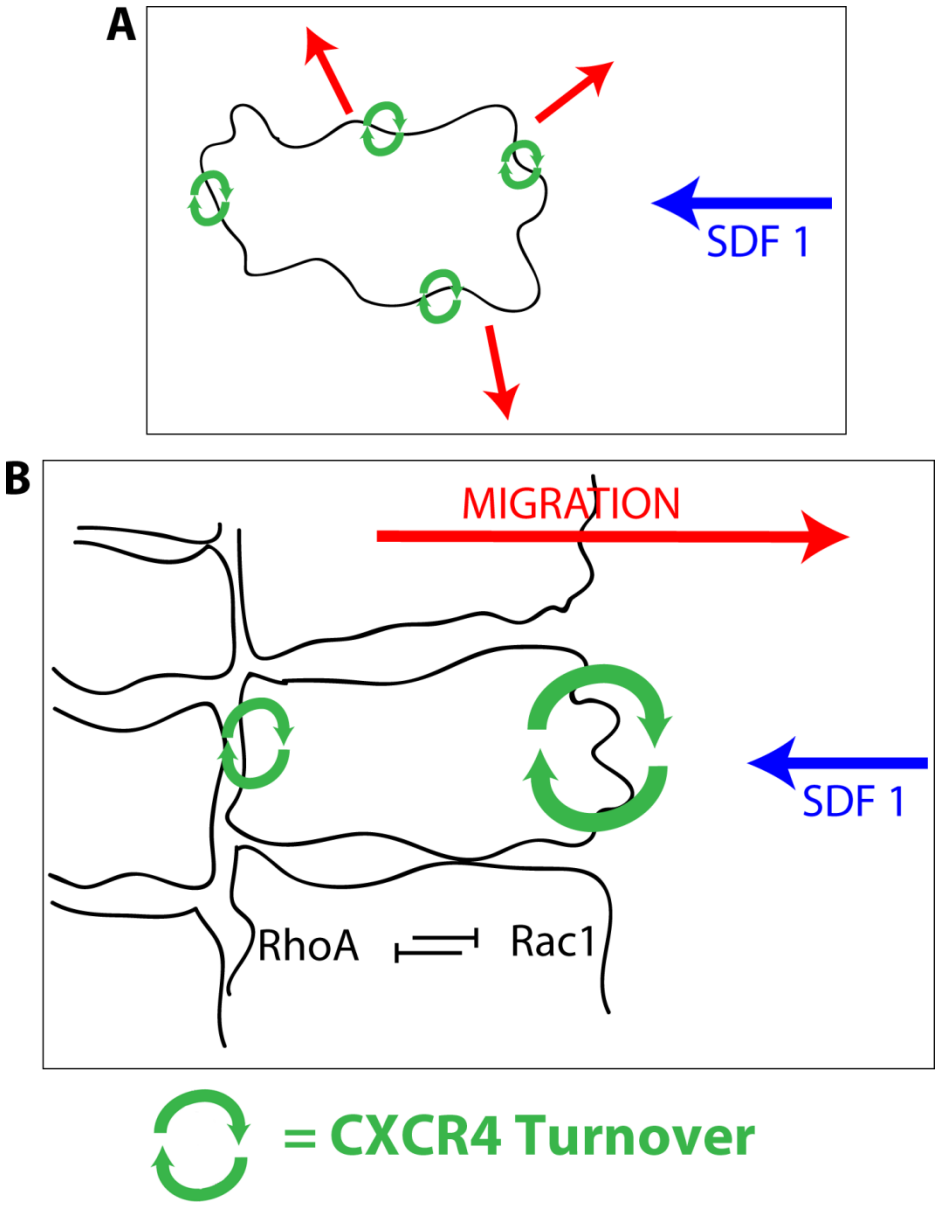


Figure 6.1 – Increased recycling at the free edge can prime cells for improved SDF1 response. (A) Single cells show no preference in CXCR4 recycling and show frequent changes in direction when migrating towards SDF1. (B) Neural crest cells at the edge of the groups have a mechanism by which the internalisation and renewal of CXCR4 at the free edge is greater when compared to cell contact; this prepares the membrane to respond to SDF1 from the underlying tissue. This differential internalisation of the receptor is lost when the balance of RhoA-Rac1 is disrupted.

6.2 SDF1 activates and guides the migration of the Neural Crest

Results using a constitutively active mutant form of CXCR4 allow understanding the importance SDF1 has in guiding the ventral motion of the neural crest. CXCR4-SDF1 has already been shown to be important in guiding a variety of other tissues during development such as the posterior lateral line in zebrafish (David et al. 2002; P Haas & D Gilmour 2006); or germ cells in *Xenopus*, mouse and zebrafish (Knaut et al. 2003; Molyneaux et al. 2003; Takeuchi et al. 2009). In *Xenopus* neural crest migration; this signalling axis is required as inhibition of either CXCR4 or SDF1 by using morpholino leads to the cells remaining dorsal (Theveneau et al. 2010a). The full role of the receptor-ligand pair in this process is not known; it is possible that the SDF1 signal can activate migration and the cells use CIL, co-attraction and negative signals to guide the group. Otherwise the signalling is required not only to trigger the migration, but also to guide the cells ventrally.

This thesis uses an activated CXCR4 mutant to show that SDF1 is necessary not only to activate migration but also to drive the migration ventrally. Cells expressing N119S-CXCR4 are completely misguided and invade areas where the neural crest should not be migrating into and the neural crest does not migrate ventrally as summarised schematically in figure 6.2. They do however form distinct domains which correspond to the migratory streams indicating the importance of negative signals.

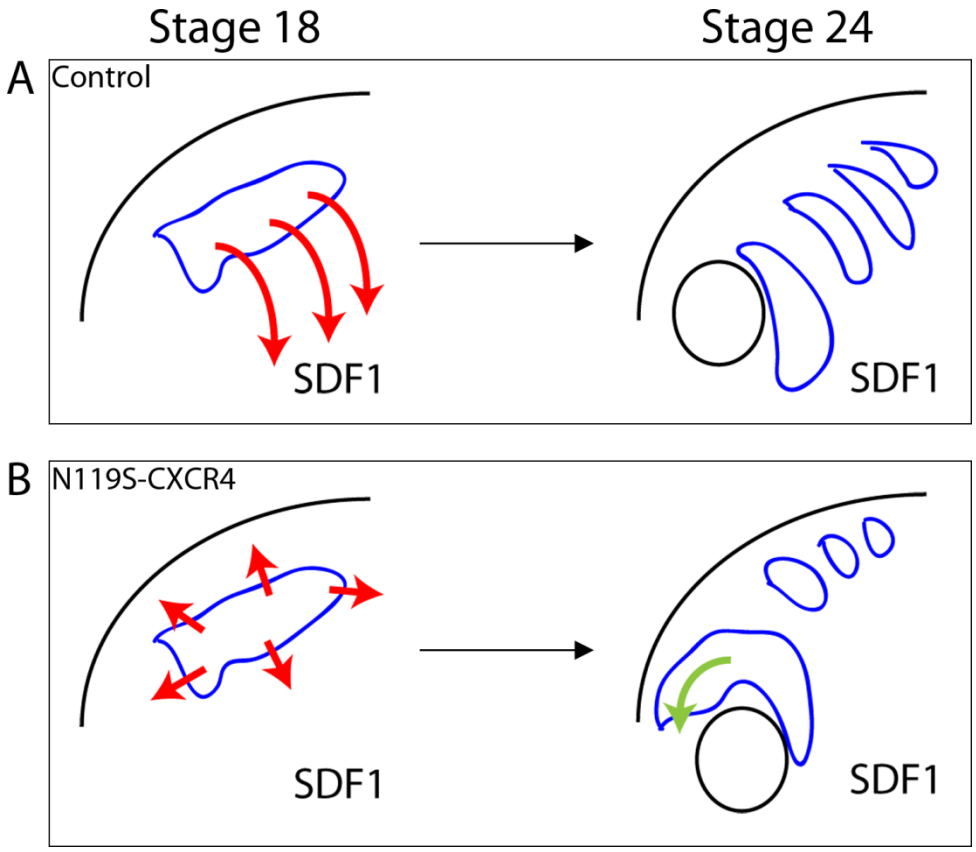


Figure 6.2 – Constitutive activity of CXCR4 misguides migration. (A) In normal conditions Neural crest delaminating from the neural plate will migrate ventrally towards the SDF1 coming from the ventrally located placodal tissue. Certain areas contain negative signals and hence streams are formed. (B) Neural crest cells expressing constitutively active CXCR4 are capable of migrating in all directions regardless of the location of the positive cue. The cells migrate in all permissive directions, this includes dorsally and anteriorly above the eye; but most streams rarely migrate ventrally due to the existence of the negative migratory signals which make it harder to migrate and ignoring the positive signal.

A result of this misguided migration is the disruption to other tissues such as the forming eye which is located more ventrally; this might be due to the neural crest restricting the site at which the eye is induced at. This highlights the importance of coordinating the different rearrangements that take place in early development and how disrupting one tissue has an impact on other tissues. The collective nature of the migrating neural crest is what gives it sufficient force to be able to push other tissues and hence their migration ventrally might also rely on this to open up their way into the streams in the wild type situation.

It is also interesting that neural crest injected with this mutant are capable to invade the closing neural tube and do not migrate ventrally. This suggests there are no negative signals limiting their migration in that region which means the embryo relies simply on the positive signals from the ventral areas of the embryo. Therefore the formation of streams has to be governed by a fine balance between the negative and positive signals what come from the ventral tissues modulating the movement and patterning of the neural crest.

6.3 CXCR4 desensitisation and internalisation is required to guide the Neural Crest.

All migratory events in the embryos are dynamic and the cells need to be constantly sensing the environment in order to ensure that the direction taken is correct. Previous work (Diaz 2005) has shown this is the case in other cells using CXCR4 for guidance; a truncated mutant preventing the desensitisation and internalisation of the receptor and hence disrupting its signalling has been

linked as the cause of WHIM disease. The mutant CXCR4 misguides mature neutrophils which are now unable to leave the bone marrow causing problems with the immune system of affected patients which fail to combat infection in affected patients (P. a Hernandez et al. 2003; Kawai & Malech 2009). The misguided cells fail to switch off CXCR4 signalling keeping them at the bone marrow and are unable to sense the correct signal provided by CXCR2 (Martin et al. 2003).

In development this same mutant has also been shown to misguide migrating germ cells during zebrafish development (Minina et al. 2007); suggesting the importance the correct sensing of the environment has in reaching the destination. This thesis investigates the effect this mutant on migrating neural crest and shows how it has the power to delay migration and misguide the morning streams.

The results show how the migratory group of cells are unable to form the streams correctly and they are misdirected; even invading the adjacent path. The streams never seem to merge and stripes of cells probably expressing negative signals remain between them. This is a result of the cells being unable to adapt to the dynamically changing signal which directs the cells as shown in figure 6.3; this is particularly clear from the in vitro data.

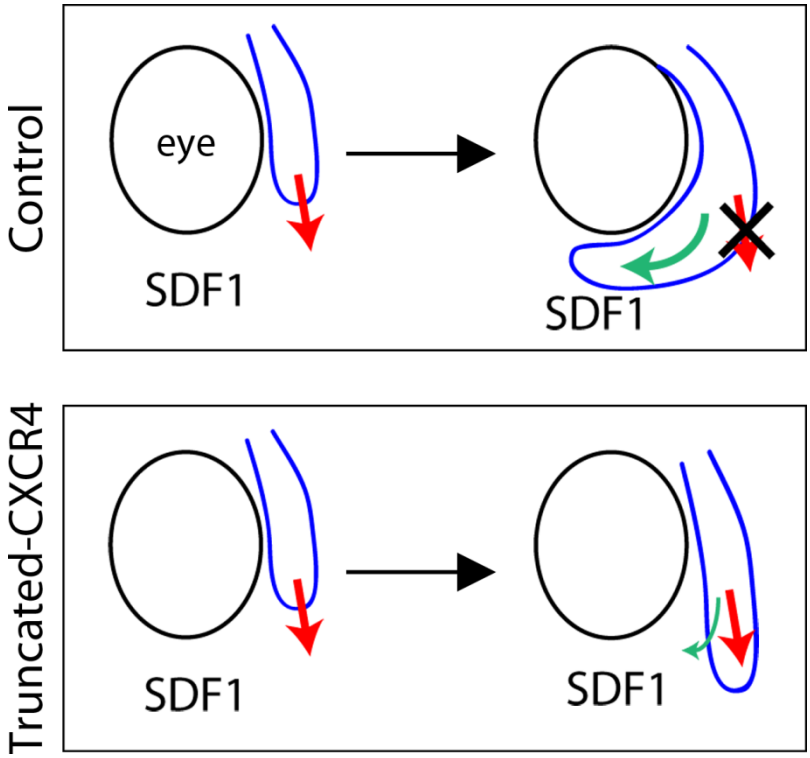


Figure 6.3 – Truncated CXCR4 causes misguided streams unable to turn. (A) In control embryos the stream adjacent to the eye turns around the forming optic vesicle following SDF1 signalling. (B) Cells injected with the Truncated CXCR4 do not turn around the eye; the cells do not switch off the original signal (red arrow) and continue migrating in that direction rather than adapting to the new environment and SDF1 signal. In the other streams this results in misshaped paths.

The ability of the cells to sense the changing environment is crucial when the movement is occurring in such a dynamic setting. From the previous results the internalisation of the receptor at the leading edge is important to set up an endocytic polarity that can contribute to improved migration of cells in the group; this mutant could also lead to lessened endocytic polarity impairing the migration.

6.4 Future Directions

This thesis has tried to understand how a single receptor can be used by cells to help their migration in particular looking at the trafficking within the cells can influence it. When working with live embryos there are always many limitations that need to be taken into account and readjustments need to be made to satisfy these.

One of the principal goals of this thesis was to determine the life cycle of CXCR4 within neural crest cells this was not achieved and only part of the dynamics were determined; to further understand the complete life cycle further experiments would be required. To give a few examples on how these could be determined I believe a better method to track the protein should be used; as mentioned previously a photoactivatable CXCR4 could be used; this would allow to track the protein from any part of the membrane and confirm if the pathways taken indeed correlate with the results presented here. This mechanism would also provide a more flexible tool to measure recycling back to the membrane which was one of the limitations to FRAP that was presented earlier.

A more extensive use of immunochemistry techniques could also give more information about the exact localisation of CXCR4 within cells. This could be achieved by using specific lysosomal, endosomal and Golgi markers; to name a few; and map the full localisation taking snapshots at the different stages of the migration. This would not be dissimilar to experiments done following the localisation of other internalised proteins such as transferrin (Wacker et al. 1997).

Another goal of this thesis was to understand when the receptor is active and achieve a reliable method of understanding the exact timing and extent of activity. In this thesis; indirect methods of measuring such as internalisation of the receptor or protrusion formation were presented; but in addition to these as part of this project we attempted more direct methods of activity detection; in particular looking at calcium fluctuations which may occur downstream of CXCR4 signalling.

Calcium signalling is a key second messenger which triggers numerous downstream signalling events in many pathways. Various optical methods exist that can detect changing levels of calcium within cells reliably (Pinton et al. 2007). Initially we chose to use Fura 2; this chemical is absorbed by cells and can be excited at two different wavelengths; at both these lengths the degree of excitation depending on levels of Calcium change and a ratio of the change at both wavelengths can be used to confirm the change in calcium levels(Grynkiewicz et al. 1985).

By measuring calcium release as a result of CXCR4 activation we wanted to compare wild type cells with those injected with the mutant and use this as an alternative confirmation of mutant activity. The use of Fura 2 however proved unsuccessful the levels of Calcium change were not detectable when the cells were exposed to SDF1; a possible reason for this was that Xenopus cells autofluoresce meaning that the slight changes of absorption at the two measured wavelengths were not accurate. We also see the fura 2 dye building up in internal compartments within the cell; we were not sue what those were; possible candidates are internal vacuoles or glycogen storage granules; however we did not pursue this.

Since Fura 2 had proved unsuccessful we attempted to use a construct which has been used in Chick embryos with better results (McKinney & P. M. Kulesa 2011). This construct; GCaMP3; is a modified GFP which contains a calmodulin subunit which separates the fluorophores; when calcium bind the calmodulin it changes shape allowing the GFP to fluoresce; hence calcium release can be measured by looking at the changes in the green channel (Akerboom et al. 2009).

The use of GCaMP3 did allow us to see; to some degree; calcium within cells. Although no clear patterns emerged; we found it was very hard to get cells to express the construct and only a few cells appeared to show fluctuation in the green levels. We also encountered technical problems since calcium release is a very fast process and the rate of image acquisition we had available made it impossible to use this type of construct to make the measurements we required.

I believe that a future challenge to confirm receptor activity is being able to measure its activity; as just exposed we attempted to use calcium in its role of second messenger to try to discern this for CXCR4 but were unsuccessful. A refinement of Calcium visualisation techniques might allow us to do this in the future; but maybe other downstream targets should be measured. I also think measuring the site at which the activation occurs would be interesting it may be possible that there is a pattern among the group of cells which aid the collective movement; for this we would require a live imaging technique that would allow us to follow activation accurately and a sufficiently long time period to allow drawing conclusions.

Another direction the project could go from here is to look at CXCR7; this receptor also binds SDF1 and has been shown to be complimentary to CXCR4 in some migratory events. Its mechanism is still not clear although recent literature suggests it may have a scavenger role in regulating SDF1 in the surrounding area of the migrating cells (Naumann et al. 2010). A system in which CXCR7 is thought to be important is in the migration of the lateral line in zebrafish; this is also a collective migration with CXCR4 expressed by leading cells and CXCR7 by trialling cells; the scavenger activity of CXCR7 at the back creates a localised gradient that allows the group of cells to move forward (Valentin et al. 2007).

Looking at expression patterns in Xenopus might give a clue as to where CXCR7 is expressed in the embryo development and if it is possible for the receptor to have a similar role in the migration of Neural crest. It could also be possible that CXCR7 has a different role and signalling to CXCR4 that has yet to be

determined; some recent papers are starting to doubt that its only role is to act as a scavenger receptor and does indeed have some internal signalling (Y. Wang et al. 2011; Décaillot et al. 2011). A more detailed analysis of the complementarity of both receptors could open a new level of complexity in how neural crest migration is regulated.

6.5 Final Remarks

The work in this thesis shows the level of complexity within early development and in particular embryonic cell movements. This process requires many signals which can guide the cells and help them reach their destination. An interesting aspect of this is the interaction amongst cells that is required for the migration to take place and how cells can integrate information from the environment and neighbouring cells to aid these complex processes.

The collective nature of this migration shows how important cell communication is and how widespread it actually is. The coordination of the many development processes taking place at the same time make it necessary for cells to know where they are and where they are going. Disrupting this fine balance that govern this can unsettle the normal development and affects multiple tissues.

The study of the mechanisms underlying these important embryonic processes is necessary to understand the nature of disease such as Cancer, which hijacks many of these. In particular the communication of cancer cells with its neighbouring tissues is disrupted allowing cells to invade and metastasise

throughout the body and a thorough understating of the process from a developmental perspective can be useful in treating such diseases.

The CXCR4-SDF1 axis illustrates the complexity of the dynamics that are required for development to occur normally. As is the case with many other developmental processes the organism can use the same axis to govern different process and therefore its important they are rightly regulated and that the embryos uses all its resources to contribute to maximal efficiency during embryogenesis.

Part V

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Chapter 7 – Bibliography

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