Calcium-sensing Receptor and Integrin Protein Complexes in Cerebellar Development and Cancer Cell Migration

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Pharmaceutical Sciences
University of Toronto

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Doctor of Philosophy
Department of Pharmaceutical Sciences
University of Toronto
2014

Abstract

The calcium-sensing receptor (CaSR) is a widely expressed homodimeric G protein-coupled receptor that senses changes in extracellular divalent cations and maintains systemic calcium (Ca^{2+}) homeostasis. Recent studies suggest that CaSR activation can promote cellular migration. In the present study, we initially sought to identify novel CaSR interacting proteins that might be associated with cellular migration. Using a metastatic tumour model consisting of medullary thyroid carcinoma cells (MTCs), and a brain developmental neuronal migration model comprised of highly motile cerebellar granule-cell precursor neurons (GCPs), we established that the CaSR couples to the integrin family of extracellular matrix (ECM) binding proteins to promote cell adhesion and migration. Integrins are single transmembrane spanning heterodimeric adhesion receptors that mediate cell migration by binding to ECM proteins. Co-immunoprecipitation and co-localization studies established that the CaSR and β1-containing integrins were present in a macromolecular complex in MTC cells and GCP neurons. In the tumour cells, CaSR-mediated activation of phospholipase C and rise in intracellular Ca^{2+} was essential for integrin activation and potentiation of cell adhesion and migration. Conversely, CaSR mediated phosphorylation of ERK2 and AKT signaling, and the subsequent promotion of β1 integrin plasma membrane expression was crucial for cerebellar GCP migration. These in vitro results were corroborated
with *in vivo* application of a CaSR negative allosteric modulator which caused a reduction in GCP migration, while a CaSR positive allosteric modulator promoted GCP homing from its proliferative zone to its final destination. This work supports a model whereby CaSR-mediated activation of integrins operates as a mechanism underlying cellular migration in both cancer cells and in the developing brain. Our results indicate that CaSR/integrin complexes may function as a universal cell migration or homing complex, and that therapeutic manipulation of this complex may be of potential interest for treating metastatic cancers, and for developmental disorders pertaining to aberrant neuronal migration.
Acknowledgments

I would like to thank everyone who has helped and inspired me during my graduate education.

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I dedicate this thesis to my wife Pria, who has stood by me through my entire graduate studies and more. Her constant motivation and encouragement have been the pillars of my success. Pria has been my emotional support, and I am forever grateful for all her efforts and sacrifices. This thesis would not be possible without her.

My deepest gratitude goes toward my family for their constant love, support and patience. I am forever grateful to my mother who has sacrificed her entire life for my siblings and myself. She instilled in me the importance of discipline and dedication, and along with my father, have provided me with the best possible environment to foster learning. Words cannot express my gratitude to Periyappa, Periyamma, Maami and Maama for all their constant love and support. I am lucky to have my younger sister Meera whose innocence and encouragement have always brought happiness to my life. I thank Thambi, Surenthar and Arani for their support as well. I am truly grateful for all these people who have helped me through this endeavour.

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Through this academic pursuit, I have come to realize that the process of scientific endeavour requires a high degree of dedication and discipline. Patience, sustained effort and a willingness to fail are all essential life lessons I have learnt from my graduate school experience.
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<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACAP1</td>
<td>adenylate cyclase activating protein 1</td>
</tr>
<tr>
<td>ADD</td>
<td>CaSR monoclonal antibody targeting ADDDYGRPGEKFREEAEERDIC</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMSH</td>
<td>associated molecule with SH3 domain of STAM</td>
</tr>
<tr>
<td>ARF6</td>
<td>ADP-ribosylating factor 6</td>
</tr>
<tr>
<td>ARNO</td>
<td>Arf nucleotide binding site opener</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic growth factor</td>
</tr>
<tr>
<td>BRDU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ca-Glu-Glu</td>
<td>ECD of the rCaSR ligated to the TMD and tail region of mGluR1</td>
</tr>
<tr>
<td>CaSR</td>
<td>calcium-sensing receptor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>EC</td>
<td>effective concentration</td>
</tr>
<tr>
<td>ECD</td>
<td>extracellular domain</td>
</tr>
<tr>
<td>ECM</td>
<td>cell-extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptors</td>
</tr>
<tr>
<td>EGL</td>
<td>external granule-cell layer</td>
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<tr>
<td>ELMO</td>
<td>engulfment and cell motility protein</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GABABR</td>
<td>(\gamma)-amino butyric acid type B receptors</td>
</tr>
<tr>
<td>GCP</td>
<td>granule-cell precursor neurons</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone (GnRH) neuron</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRADSP</td>
<td>glycine-arginine-alanine-aspartate-serine-proline integrin control peptide</td>
</tr>
<tr>
<td>GRGDSP</td>
<td>glycine-arginine-glycine-aspartate-serine-proline integrin inhibitor peptide</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein receptor kinases</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HEK-293-MSR</td>
<td>human embryonic kidney 293 cells with macrophage scavenger receptor</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IBMX</td>
<td>3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione</td>
</tr>
<tr>
<td>IGL</td>
<td>internal granule-cell layer</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase/stress-activated protein kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>Kir</td>
<td>inward rectifying potassium channel</td>
</tr>
<tr>
<td>LC-MS-MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LDV</td>
<td>leucine-aspartate-valine</td>
</tr>
<tr>
<td>LIMBS</td>
<td>ligand-induced metal ion binding site</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MIDAS</td>
<td>metal-ion-dependent adhesion site</td>
</tr>
<tr>
<td>mgl</td>
<td>metabotropic glutamate receptor (<em>Caenorhabditis elegans</em>)</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor (mammalian)</td>
</tr>
<tr>
<td>ML</td>
<td>molecular layer</td>
</tr>
<tr>
<td>MTC</td>
<td>medullary thyroid cancer</td>
</tr>
<tr>
<td>NaF</td>
<td>sodium fluoride</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCL</td>
<td>Purkinje cell layer</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
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<tr>
<td>PNGase</td>
<td>peptide N-glycosidase</td>
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<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
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<tr>
<td>RAMP</td>
<td>receptor activity modifying proteins</td>
</tr>
<tr>
<td>R.F.U.</td>
<td>relative fluorescent unit</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartate</td>
</tr>
<tr>
<td>rMTC 44-2</td>
<td>carcinoma derived rat medullary thyroid C-cells</td>
</tr>
<tr>
<td>ROCK</td>
<td>rho-associated protein kinase</td>
</tr>
<tr>
<td>SDF</td>
<td>stromal cell-derived factor</td>
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<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
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<td>Term</td>
<td>Definition</td>
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<td>------------------------------------------------</td>
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<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>VFT</td>
<td>venus flytrap domain</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated Ca(^{2+}) channel</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>1,25-dihydroxyvitamin D3</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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</table>
Chapter 1. Introduction

1.1 Calcium ions: evolutionary aspects of calcium biology and physiology

The calcium ion (Ca\(^{2+}\)) is of fundamental importance for many biological processes. By mass, Ca\(^{2+}\) represents the most abundant mineral in the human body (Riccardi and Kemp, 2012), and is also the fifth most common element in the Earth's crust and oceans. The evolution and diversification of the animal kingdom has greatly benefitted from this vastly versatile, highly reactive, and readily available ion. The ability of prokaryotic unicellular organisms to bind, transport and store Ca\(^{2+}\) had allowed these primitive organisms to evolve complex Ca\(^{2+}\) based signaling mechanisms. Ca\(^{2+}\) also permitted single-cell organisms to develop cell cytoskeletal structures necessary for cell movement and migration, helped maintain plasma membrane stability, and activated enzymes involved in cellular communication and nutrient detection (Ehrstrom et al., 1973; Riccardi and Kemp, 2012). These adaptive characteristics provided by the Ca\(^{2+}\) ion played an essential role in the evolutionary development of complex multicellular eukaryotic organisms.

Ca\(^{2+}\) has become an indispensable second-messenger signal that is responsible for controlling a wide spectrum of cellular processes including fertilization, apoptosis, hormonal secretion, glycogen metabolism, cellular differentiation, proliferation, and motility (Chattopadhyay et al., 1996b; Chang and Shoback, 2004; Bouschet and Henley, 2005). Ca\(^{2+}\) is also an important enzymatic cofactor, and serves a multitude of physiological functions including bone formation, blood clotting, intercellular adhesion and regulation of neuromuscular excitability. Therefore, maintenance of appropriate concentrations of extracellular and intracellular Ca\(^{2+}\) is thus vital to the survival of all organisms. In humans, basal intracellular Ca\(^{2+}\) levels are approximately 100 nanomolar (nM) at resting state and can undergo large and rapid elevations to well over 1 µM upon cellular activation due to release of Ca\(^{2+}\) from intracellular stores and/or uptake of
extracellular Ca\(^{2+}\) ions (Breitwieser, 2008). On the contrary, extracellular Ca\(^{2+}\) concentrations measured in the blood remains nearly invariant under normal circumstances, and fluctuates from its mean value by only a few percent (Tfelt-Hansen and Brown, 2005). Proteins that buffer, sequester, store and exchange Ca\(^{2+}\) are therefore essential for maintaining proper intracellular Ca\(^{2+}\) levels.

The role of Ca\(^{2+}\) as an intracellular second messenger had been well recognized for years. However, the discovery that Ca\(^{2+}\) can also act as a hormone-like extracellular ligand was established with the molecular cloning of a Ca\(^{2+}\) receptive membrane spanning receptor, known as the calcium-sensing receptor (CaSR), from tissues involved in maintaining mineral ion balance (Brown et al., 1993). The initial studies determined that the CaSR plays an essential role in maintaining systemic Ca\(^{2+}\) homeostasis by sensing changes in blood Ca\(^{2+}\) levels and mediating the appropriate physiological feedback signals to preserve the blood extracellular Ca\(^{2+}\) concentration to near consistency (Brown, 1993; Armato, 2009).

1.2 Family C GPCRs and the CaSR

The CaSR is a seven transmembrane spanning cell surface protein that belongs to Family C of the G-protein coupled receptor (GPCR) superfamily. Members of this subfamily include the metabotropic glutamate receptors (mGluRs) of which eight isoforms are recognized in humans and rodents, \(\gamma\)-amino butyric acid type B receptors (GABA\(_B\)Rs) receptors, taste receptors, the GPRC6A cationic amino acid receptor and various orphan receptors including putative pheromone receptors (Conigrave and Hampson, 2010). The Family C GPCRs are characterized by a very large extracellular amino (N)-terminal domain (ECD) of around 450 to 600 amino acids (Conigrave and Ward, 2013). The structural feature of this domain is similar to the bilobed Venus Flytrap Domain Motif (VFT) of bacterial periplasmic binding proteins, and contains the
binding site for the endogenous orthosteric agonists. The large ECD is attached to a transmembrane domain (TMD) with seven spanning α-helices characteristic of all GPCRs, and an intracellular C-terminus domain important for intracellular signal transduction (Fig. 1.1). In addition, with the exception of GABA B Rs, members of the Family C GPCR subfamily contain a region of nine highly conserved cysteine residues located between the ECD and the TMD, known as the cysteine-rich region (Zhang et al., 2001).

Agonist binding to GPCRs elicits a wide range of intracellular enzymatic signaling cascades via activation of heterodimeric G-protein coupled signaling. G-proteins are composed of Gα, Gβ and Gγ trimers. They associate with GPCRs on the intracellular surface, and are inactive when reversibly bound to guanosine disphosphate and active when bound to guanosine triphosphate. Upon GPCR activation, the guanine nucleotide exchange factor domain of GPCRs facilitates the exchange of GDP for GTP at the Gα subunit of the G-protein, causing the trimer to dissociate into a Gα-GTP monomer and tightly interacting G-βγ dimer (Kobilka, 2007). The dissociated G-protein subunits activate a variety of secondary signaling proteins for further signal transduction and propagation. GPCRs can also function through G-protein independent pathways (Irannejad et al., 2013). For example, GPCRs can active intracellular signaling cascades via β-arrestin mediated signaling mechanisms without G-protein dissociation or activation (Reiter et al., 2012). Certain GPCRs can also transactivate growth factor receptors that couple to various tyrosine kinase signaling cascades without G-protein engagement (MacLeod et al., 2004).

1.3 Evolutionary comparison of Family C GPCRs

Homology based analysis at the level of the amino acid sequence demonstrated that members of the Family C GPCR share a high degree of conservation among mammals, birds, and
Figure 1.1. The human calcium-sensing receptor (hCaSR) amino acid sequence snake-diagram.

The positions of activating and inactivating mutations identified through human genomic screening and artificial mutation studies are illustrated. Transmembrane helical boundaries were determined using the crystal structure comparison of bovine rhodopsin transmembrane domain predicted by Petrel et al., 2004. Locations of signal peptide, beginning and end of Venous Fly Trap (VFT) Domain, four loops of VFT Lobe 1, Extracellular Loops 1 – 3, Intracellular Loops 1 – 3, and Cysteine Rich Domain (CRD) are shown.
amphibians (Cao et al., 2009). In fact, the CaSR gene that is highly homologous to the mammalian form have been identified in several aquatic species ranging from zebrafish to the dogfish shark (Nearing et al., 2002; Hentschel et al., 2003). Although the evolutionary analysis of the CaSR can be traced back to its early origin in the species of the chordate-vertebrate lineage, the existence of the mGluRs have been documented in even earlier evolutionarily primitive organisms of the nematode family (Dillon et al., 2006; Kuang et al., 2006). The CaSR shares approximately 30% sequence homology to the members of mGluR family, and analysis of their VFT structure suggests that the CaSR and mGluRs evolved in the broader context as nutrient sensors from a common ancestor of the bacterial periplasmic binding protein. Since mGluRs have been extensively studied and are largely conserved across phyla from nematodes to vertebrates (Perovic et al., 1999; Kuang et al., 2006), understanding mGluR evolution may shed insights into CaSR function and physiology.

In mammals, eight genes encoding mGluRs have been identified, and are further classified into three sub-groups based on their sequence similarity, signal transduction mechanism and pharmacology. Group 1 (mGluR1 and mGluR5) mGluRs activate $G_{q}\alpha$ and phospholipase C to induce intracellular Ca$^{2+}$ release similar to the CaSR, while Group 2 (mGluR2 and mGluR3) and Group 3 (mGluR4,6,7,8) mGluRs inhibit adenylyl cyclase (Mitri et al., 2004). The evolutionarily distant Caenorhabditis elegans genome of the nematode phyla contains three mGluR-like sequences referred to as mgl-1, mgl-2, and mgl-3. Sequence-function analysis established that each of the C.elegans mGluRs serves as a potential ortholog for the three mammalian mGluR subgroups (Groups 1, 2, and 3 mGluRs), indicating that the functional separation of the different mGluRs were present in the common primordial ancestor of nematodes and vertebrates (Mitri et al., 2004).
Phylogenetic dendrogram analysis of the full length and ECD amino acid sequences of the mammalian (Rattus norvegicus) CaSR and mGluRs, and the three *C.elegans* glutamate receptors (mgl-1 – 3) revealed that the rCaSR was most homologous to mgl-2 and Group 1 mGluRs (Fig. 1.2A, B). However, pharmacological comparison of the Ca$^{2+}$ sensitivity of the rat CaSR with mgl-2 and mGluR5 revealed that Ca$^{2+}$ did not potentiate glutamate-mediated activation of *C.elegans* mgl-2 or rat mGluR5, and conversely, L-glutamate did not affect Ca$^{2+}$ stimulated activation of the CaSR (Appendix A, Fig. 1A) (Tharmalingam et al., 2012). In addition, Ca$^{2+}$ did not directly activate mgl-2 or the rat mGluR5. These studies illustrated that in terms of orthosteric activity, mgl-2 is similar to the Group 1 mGluRs but not the CaSR, demonstrating that the CaSR ECD diverged from the mGluRs to incorporate binding sites for Ca$^{2+}$ ions and not L-glutamate. In addition, these studies provided evidence that the ECD of mGluRs have been conserved from nematodes to vertebrates, with selective pressures conserving the key residues important for L-glutamate binding (Perovic et al., 1999; Kuang et al., 2006).

In contrast, phylogenetic analysis of the TMD regions demonstrated that the CaSR was placed into a separate clad, distant from Group1 mGluRs and all three mgl-3s (Fig. 1.2C). Indeed, the TMD of Family C GPCRs have diverged considerably as evident by the selectivity of allosteric modulators which bind to various sites of the TMD. For example, pharmacological analysis of the mGluR subtype specific and CaSR allosteric modulators revealed that Group 1 mGluR antagonists that bind to the TMD were able to block L-glutamate activation of mgl-2, while the CaSR specific negative allosteric modulator was ineffective in inhibiting L-glutamate activation of mGluR5 and mgl-2 (Appendix A, Fig. 1B – D) (Tharmalingam et al., 2012). These studies demonstrated that the residues important for allosteric modulator antagonist activity are not conserved between the mammalian CaSR, Group 1 mGluRs and mgl-2, while the orthosteric ligand binding sites are conserved for mGluRs and mgl-2, but not the CaSR.
Figure 1.2. Phylogenetic relationships among the rat CaSR, and the rat and C. elegans mGluRs.

(A) Dendrogram of the aligned full length CaSR and mGluR sequences using ClustalW. Full length sequence alignments revealed that rCaSR clustered with Group 1 mGluRs and the mgl-2, mgl-1 grouped with Group 2 mGluRs, while mgl-3 had minimal resemblance to rat mGluRs or the CaSR. (B) LBD dendrogram of the rCaSR, Group 1 and C. elegans mGluRs. rCaSR LBD was most homologous to Group 1 mGluRs and mgl-2, and evolutionarily distant from mgl-1 and mgl-3. (C) TMD dendrogram. The CaSR TMD appears to have evolved separately and is distinct from TMDs of Group 1 and C. elegans mGluRs.
During the evolution of GPCRs, adaptive changes may have promoted a primitive, multi-nutrient-binding primordial Family C GPCR of the early metazoan lineage to diverge into several specific receptors which bind to individual nutrients and ions present in the surrounding environment (Perez, 2003). The ability to independently sense the complex nutrient milieu and direct appropriate cellular signals must have driven the evolution of Family C GPCRs to develop unique binding sites for specific agonists and TMD allosteric modulators (Kuang et al., 2005). The abundant availability of Ca^{2+} and the need to sense and control Ca^{2+} signaling may have prompted the divergence and formation of the CaSR from the other nutrient sensing Family C GPCR members.

1.4 CaSR gene and protein structure

The human CaSR is composed of 1078 residues with a predicted molecular weight of 120 kilo daltons (kDa) (Fig. 1.1). The first 612 amino acids comprise of a large, hydrophilic, amino terminal, ECD. Residues 36 – 513 are similar to the nutrient-binding VFT of bacterial periplasmic binding proteins; this region contains sites for Ca^{2+} binding and receptor dimerization (Ray et al., 2007). The end of the ECD contains a cysteine-rich domain consisting of 19 cysteines, nine of which are highly conserved among Family C GPCR members (Ray et al., 2007). The hydrophobic TMD stretches from amino acids 612 – 850, and is involved in signal transduction and allosteric modulation (Petrel et al., 2004). TMD also includes three intracellular and extracellular loops. The remainder of the CaSR comprises a carboxy terminal intracellular tail, which includes five protein kinase C (PKC) phosphorylation sites (Bai et al., 1998). Phosphorylation of these PKC sites inhibits coupling of the CaSR to the phospholipase C (PLC). In addition, the ECD contains 11 potential N-linked glycosylation sites that are important for cell surface expression (Bai et al., 1996). Furthermore, the CaSR is constitutively present in
a homodimeric configuration. Two identical protomers of the CaSR are linked by two disulfide covalent bonds between cysteine residues 129 and 131, as well as by additional non-covalent interactions (Garrett et al., 1995). The CaSR has also been shown to heterodimerize with mGluR5 and GABA<sub>B</sub>Rs in certain neuronal cell populations (Gama et al., 2001; Conigrave and Ward, 2013).

The CaSR human gene is located on the long arm of chromosome 3 at band 3q21-q24 as determined by in situ fluorescence hybridization (Brown and MacLeod, 2001). In mouse, rat and bovine the CaSR gene resides on chromosomes 11, 16 and 1 respectively (Janicic et al., 1995). The human CaSR gene contains seven exons, of which the first six encode the ECD and the upstream untranslated regions while a single exon codes for the TMD and intracellular C-terminus regions (Fig. 1.3). The CaSR gene has two alternately spliced transcripts containing either exon 1A or exon 1B (Chikatsu et al., 2000). The upstream promoters contain TATA and CAAT boxes, while the downstream promoter is GC content rich. Both transcripts yield identical proteins since both exon 1A or exon 1B are part of the 5’ untranslated region (UTR) that splices to exon 2, which contains the ATG start codon (Fig. 1.3) (Chikatsu et al., 2000). In addition, alternative splicing of noncoding exons within the 5’ upstream and 3’ downstream regions of the CaSR gene have been identified, yielding transcripts with varying sizes. Studies have identified several other forms of the CaSR transcript, including an in-frame splice deletion of exon-5 which lacks 77 amino acids that appears to be particularly important in keratinocyte differentiation (Oda et al., 1998). The exon-5 deleted transcript had altered glycosylation patterns compared to the full length CaSR and exhibited reduced responsiveness to extracellular Ca<sup>2+</sup> induced rise in intracellular Ca<sup>2+</sup> (Oda et al., 1998).
The CaSR gene has two promoters resulting in two alternately spliced transcripts containing either exon 1A or exon 1B. Both transcripts yield an identical protein since both exon 1A and exon 1B (grey rectangles) are part of the 5’ UTR that splices to exon 2, which contains the ATG start codon. The CaSR gene consists of upstream promoters such as TATA and CAAT boxes, and includes a downstream promoter that is GC content rich. The CaSR transcript comprises of 7 exons, and two 3’ UTR regions have been identified at 1300 or 180 base pairs. Double headed arrow marks intron sizes, shown in kilobase pairs. The number of amino acids produced from each exon is indicated. An in-frame CaSR splice variant which lacks exon-5 is also shown.
1.5 CaSR signaling

1.5.1 CaSR stimuli

The CaSR is activated by ionized Ca\(^{2+}\) and a variety of other positively charged organic and inorganic polycations. Divalent and trivalent cations activate the CaSR in an orthosteric fashion. The CaSR can also be activated orthosterically by a variety other compounds, including organic aminoglycoside polycations such as neomycin, and cationic peptides including polyarginine and polylysine (Nemeth et al., 1998; Conigrave et al., 2000b; Wang et al., 2006; Broadhead et al., 2010; Conigrave and Hampson, 2010). In addition, the CaSR is positively modulated by glutathione analogs and aromatic L-amino acids, and negatively influenced by high ionic strength and protons (Quinn et al., 1998; Conigrave and Hampson, 2006; Wang et al., 2006). Despite the wide pharmacological range, the most physiologically relevant stimulus of the CaSR is Ca\(^{2+}\), which binds to the CaSR with very low affinity (millimolar concentrations), but high cooperativity (Hill coefficients in the range of 4 – 6) (Brown and MacLeod, 2001). This phenomenon allows the CaSR to detect small changes in extracellular Ca\(^{2+}\) concentrations as found in most extracellular fluid compartments. Depending on the cell context and level of receptor expression, the CaSR is activated above Ca\(^{2+}\) threshold levels of 0.5 to 2.0 mM, and is inactive at Ca\(^{2+}\) levels below 0.2 mM (Conigrave and Ward, 2013).

Inorganic cations that activate the CaSR can be categorized depending on the rank order of potency. Trivalent cations are strong high-affinity CaSR ligands with affinities in the submicromolar to tens of micromolar range (Gd\(^{3+}\), La\(^{3+}\), Yt\(^{3+}\)) (Chang and Shoback, 2004). Divalent cations are medium-affinity agonists that have affinities in the micromolar and submicromolar range, including Zn\(^{2+}\), Ni\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), Co\(^{2+}\) and Fe\(^{2+}\) (Chang and Shoback, 2004). Low-affinity agonists are divalent cations with millimolar range affinities, including Ca\(^{2+}\), Mg\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\) and Mn\(^{2+}\). The cation rank order of potency reveals that binding of cations to the CaSR
orthosteric binding site depends on the ionic charge and the ionic radius of the cation, in that the potency of cations with larger radii increases with their charge number (Chang and Shoback, 2004). Despite the ability of numerous ions to activate the CaSR, Ca$^{2+}$ is the endogenous ligand since plasma levels of Ca$^{2+}$ hover around 1.2 to 1.5 mM, the range in which CaSR is partially activated, whereas the plasma levels of the others ions are not high enough to activate the receptor. Interestingly however, the second most physiologically relevant cation is Mg$^{2+}$ which differs significantly from the other cations in that it is only a partial agonist of the CaSR and its Hill coefficient is around 1. Mg$^{2+}$ positively modulates CaSR function and it can synergistically function with Ca$^{2+}$ to activate the CaSR. In this regard, hypermagnesemia can offset the systemic Ca$^{2+}$ homeostasis by activating the CaSR.

Organic polycations such as spermine and spermidine are effective CaSR agonists, and their potency is determined by the number of positive charges. Spermine (4 positive charges) is therefore more potent than spermidine (3 positive charges) while putrescine (2 positive charges) is ineffective at concentrations up to 10 mM (Quinn et al., 1997). High millimolar spermine concentrations are necessary to fully activate the CaSR; however at physiological Ca$^{2+}$ levels (~1.5 mM) 500 µM spermine can adequately activate the CaSR, demonstrating self-potententiating actions of the CaSR polycationic agonists. In vivo, spermine is present at mM concentrations, and in specific microenvironments such as gastrointestinal tracts which houses spermine producing bacteria, in the brain synaptic cleft where spermine is co-secreted with neurotransmitters, or in by-products secreted by some tumors, the spermine concentration can reach in excess of 1 mM (Quinn et al., 1997; Washburn et al., 1999). Polycations may serve as a physiologically relevant modulator of CaSR function during normal and pathophysiological conditions. Similar to spermine, polycationic aminoglycoside antibiotics potency for the CaSR correlates with the number of amino groups, with antibiotics with 6 amino groups like neomycin
showing affinity in the 100 µM range, while 5 and 4 amino group antibiotics displaying 500 µM and 1 to 2 mM affinity for the CaSR respectively (McLarnon et al., 2002). CaSR activation by these aminoglycoside antibiotics is physiologically relevant in antibiotic toxicity in the kidney where the CaSR is highly expressed. Here, the low luminal pH contributes to enhanced antibiotic ionization, which positively contributes to CaSR activation, causing kidney toxicity (Riccardi and Kemp, 2012). Finally, studies have shown that the changes in ionic strength produce parallel changes in CaSR affinity for orthosteric agonist binding (Quinn et al., 1998). For example, increasing ionic strength reduces CaSR sensitivity to agonists and vice versa. Similarly, studies using monovalent cations (e.g., sodium or choline) or anions (chloride or iodide) positively increased ionic strength, and therefore these ions negatively modulated orthosteric agonist binding to the CaSR.

In addition to orthosteric activation, the CaSR is allosterically modulated by free L-amino acids in the presence of CaSR polycationic agonists (Conigrave and Hampson, 2006). In particular, aromatic amino acids such as L-phenylalanine, L-tryptophan, L-tyrosine and L-histidine function as low affinity (millimolar range) allosteric potentiators of the CaSR in the presence of subthreshold extracellular Ca\(^{2+}\) levels. These amino acids potentiate the CaSR with low affinity, and Hill coefficient of 1, indicating lack of cooperative activation of the CaSR and distinct binding sites than that of orthosteric agonists (Conigrave et al., 2000a). Although the amino acids have low potencies for activating the CaSR, a mixture of amino acids in the stomach and intestine may collectively potentiate the CaSR expressed in the gastrointestinal tract to promote secretion of various gastric enzymes. Similarly, tripeptides such as glutathione (glutamate-cysteine-glycine) which share similar physical characteristics to the free amino acids also positively modulate CaSR activation in the presence of CaSR orthosteric agonists (Wang et al., 2006). However, compared to amino acids, glutathione analogs are much more potent
potentiators of the CaSR due to its large side chains. Therefore, these agonist studies demonstrate that the CaSR is more than a mineral ion receptor, but rather a multimodal nutrient sensor.

Importantly, synthetic phenylalkylamine derived allosteric modulators with very high affinity and specificity for the CaSR have been developed. These are referred to as type 2 calcimimetics to distinguish from type 1 orthosteric multivalent cationic CaSR agonists (Nemeth, 2004). Positive allosteric modulators such as NPS R-467 and NPS R-568 sensitize the CaSR to subthreshold concentrations of the CaSR orthosteric cationic agonists. Cinacalcet, the more readily absorbed analog of NPS R-568 is a positive CaSR allosteric modulator used for the treatment of secondary hyperparathyroidism during chronic kidney disease (Kawata et al., 2006). Similarly, phenyl-O-alkylamine derived negative modulators have been developed as calcilytics, including NPS 89636 and NPS 2143 (Conigrave and Ward, 2013). These allosteric antagonists inhibit CaSR activation with relatively high affinity in the nanomolar range. Together, the positive and negative CaSR allosteric modulators provide important pharmacological tools for studying CaSR function in normal and pathological conditions.

1.5.2 CaSR mediated intracellular signaling cascades

Signal transduction studies have shown that the CaSR is linked to a variety of diverse intracellular signaling pathways (Fig. 1.4). In many tissues expressing the CaSR, signal transduction occurs via \( G_{q}\alpha \) or \( G_{11}\alpha \) mediated mechanisms, where the hydrolysis of PtdIns(4,5)P\(_2\) by phospholipase (PLC) produces Ins(1,4,5)P\(_3\) and diacylglycerol (Kifor et al., 1997). Here, IP\(_3\) stimulates the IP\(_3\)-receptor to increase release of internal Ca\(^{2+}\) stores, while diacylglycerol activates PKC (Handlogten et al., 2001). In certain cells, CaSR mediated PKC activation stimulates the opening of non-selective cation channels which depolarizes the cell membrane.
The human CaSR is composed of 1078 residues, and is present constitutively in a dimeric configuration. The first 612 amino acids comprise of a large, hydrophilic, amino terminal, ECD. Residues 36 – 513 are similar to the VFT domain of bacterial periplasmic binding proteins. The end of ECD contains a cysteine-rich domain consisting of 19 cysteines, nine of which are highly conserved among Family C GPCR members. In addition, the ECD contains 11 potential N-linked glycosylation sites. The TMD consists of seven transmembrane spanning α helices, and also includes three intracellular and extracellular loops. The CaSR couples to G\(_{q}\alpha\)-mediated activation of phospholipase C (PLC) and intracellular Ca\(^{2+}\) release, G\(_{i}\alpha\)-mediated reduction in cyclic AMP (cAMP) production, G\(_{12/13}\alpha\) induced Rho kinase activation, as well as phosphorylation of MAPK/ERK, AKT and JNK pathways, and activation of a variety of pathways including nitric oxide (NO), arachidonic acid (AA), focal adhesion kinase, and β-arrestin mediated signaling. AC, adenylate cyclase; cPLA\(_2\), cytosolic phospholipase A\(_2\); DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; Ins(1,4,5)P\(_3\), inositol-1,4,5-trisphosphate; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ROCK, rho-associated protein kinase; PKC, protein kinase C. Figure adapted from Hofer et al., 2003.
thus opening voltage gated $\text{Ca}^{2+}$ channels which contribute to the $\text{Ca}^{2+}$ influx and subsequent vesicle release (Brown and MacLeod, 2001). Another outcome of the $\text{G}_{\text{q}}\alpha/\text{PLC}$ activation produces arachidonic acid via association with cytosolic phospholipase $\text{A}_2$ (PLA$_2$) (Handlogten et al., 2001). Furthermore, increases in CaSR-mediated PLC have been shown to activate phosphatidylinositol-4-kinase, creating phosphatidylinositol intermediates that have been implicated with apoptosis and ion-channel activity (Hofer and Brown, 2003).

The CaSR also couples to $\text{G}_{\text{i}}\alpha$, which results in adenylate cyclase (AC) inhibition and subsequent reduction in cyclic AMP levels (Kifor et al., 1997). Additional evidence links CaSR with $\text{G}_{12/13}\alpha$ proteins, which elicits phospholipase D activation and phosphatidic acid production (Huang et al., 2004a). $\text{G}_{12/13}\alpha$ proteins couple to RhoGTPase nucleotide exchange factors which activates small monomeric GTPases such as Rho. Rho stimulation activates rho-associated protein kinase (ROCK), which promotes actin polymerization and cell cytoskeletal reorganization.

CaSR activation also couples to mitogen-activated protein kinase (MAPK) signaling cascades via PKC activation, $\text{G}_{\text{i}}\alpha$ stimulation, or $\beta$-arrestin mediated mechanisms (Tfelt-Hansen et al., 2003; Holstein et al., 2004). MAPK cascade activation leads to phosphorylation of extracellular signal-regulated kinase (ERK1/2), and c-Jun NH2-terminal kinase/stress-activated protein kinases (JNK). These kinases control a variety of processes including transcriptional activities that promote cell growth, proliferation, and maturation. The CaSR can also activate the MAPK cascade by a unique triple-membrane spanning method involving epidermal growth factor receptors (EGFR) (MacLeod et al., 2004). Stimulation of the CaSR directly activates EGFRs, which then phosphorylates the MAPK proteins for further downstream signaling. Furthermore, CaSR activation has been attributed to a wide variety of other signaling cascades including nitric oxide production, AKT (also known as protein kinase B) phosphorylation, cytoplasmic tyrosine
kinase production such as Src kinase and focal adhesion kinase (FAK) activation, mTOR stimulation, etc (McNeil et al., 1998; Hofer and Brown, 2003). These intricate activation networks reveal that a single activator of the CaSR can have multiple intracellular functions.

1.5.3 CaSR interacting proteins

The CaSR receptor has been linked to a wide array of biological functions. To gain a better understanding of the cellular mechanisms mediating CaSR functionality, recent work has focused on determining the CaSR interacting proteins. Although limited in scope, a small number proteins have been identified to date that interact and influence CaSR signaling properties. The most established interaction of the CaSR is its association with filamin-A, identified through yeast two-hybrid library screens using the CaSR intracellular tail as bait (Awata et al., 2001). Filamin is a very large (280 kDa) actin binding protein and its primary function is to crosslink actin filaments to provide mechanical strength to the actin cytoskeleton. Filamin interacts with numerous cell surface receptors and anchors these transmembrane spanning receptors to the cytoskeleton (Calderwood et al., 2001). In addition, filamin also serves as a scaffolding protein for signal transduction proteins such as MAPKs, Rho GTPases, Rho guanine nucleotide exchange factors, Rho kinases, and phosphatases. Studies have shown that filamin-A interacts with the CaSR through two predicted β-strands from residues 962 to 981, and interactions between filamin-A and CaSR are greatly enhanced by exposure to 5 mM Ca$^{2+}$ (Zhang and Breitwieser, 2005). CaSR association with filamin-A has been shown to be important for CaSR mediated MAPK and JNK activation, and CaSR knockdown or disruption of the CaSR-filamin interaction using peptides abolishes CaSR mediated MAPK and JNK phosphorylation (Huang et al., 2006a). In addition, filamin-A protects CaSR from degradation since expression of CaSR in cells lacking filamin-A was almost doubled when filamin-A
expression was exogenously added (Zhang and Breitwieser, 2005). Finally, in keratinocytes, disruption of CaSR-filamin association prevents CaSR mediated Rho and E-cadherin activation, therefore inhibiting keratinocyte differentiation (Tu et al., 2011).

CaSR expression is also controlled by its intracellular association with Receptor Activity Modifying Proteins (RAMPs), a family of proteins that control cell surface trafficking, glycosylation and second messenger production. The CaSR interaction with RAMP is important for its cell surface expression and agonist sensitivity (Bouschet et al., 2008). Studies have shown that in cells lacking RAMP expression, the CaSR remains in the endoplasmic reticulum in an immature core glycosylated form. Exogenous RAMP expression facilitates CaSR exit from the endoplasmic reticulum to the Golgi where the receptor gets glycosylated and inserted into the plasma membrane (Bouschet et al., 2008). The CaSR-RAMP interaction is also important for understanding CaSR agonist affinity, since agonist binding depends on the level of CaSR surface expression. For example, the half-maximal effective concentration (EC$_{50}$) of Ca$^{2+}$ for the CaSR expressing parathyroid cells is approximately 1 mM, while the EC$_{50}$ for CaSR expressing thyroid cells is approximately 2.5 to 3 mM (Huang and Miller, 2007). The difference in Ca$^{2+}$ affinity for the CaSR in the two different cell-types has been attributed to the level of CaSR expression controlled by RAMPs. The CaSR interacts more efficiently with RAMP3 than RAMP1, and RAMP3 association increases cell surface expression of the CaSR compared to RAMP1. Since the CaSR associates with RAMP3 in parathyroid cells and RAMP1 in thyroid cells, the CaSR expressed in the parathyroid cells are more sensitive to Ca$^{2+}$ than the CaSR expressed in thyroid cells (Desai et al., 2014).

CaSR surface expression is also controlled by its association with β-arrestins, which are ubiquitously expressed cytosolic proteins that are known to mediate GPCR desensitization, internalization, and subsequent degradation. CaSR interaction with β-arrestins is induced when
the CaSR cytoplasmic tail is phosphorylated by PKC or G-protein receptor kinases (GRK), thus desensitizing CaSR coupling to the G proteins (Reiter and Lefkowitz, 2006). Binding of β-arrestins to the CaSR also results in CaSR internalization and degradation (Lorenz et al., 2007). In addition to the β-arrestins, two other CaSR interacting proteins identified through yeast two-hybrid analysis were dorfin, an E3 ubiquitin ligase, and AMSH (associated molecule with SH3 domain of STAM), a ubiquitin isopeptidase that has been shown to promote CaSR degradation (Herrera-Vigenor et al., 2006; Huang et al., 2006b). Therefore, tissue specific expression of the CaSR may be regulated by the complement of protein-protein interactions present in the cellular context.

Yeast two-hybrid analysis of CaSR expressed in kidney also identified inward rectifying potassium channels (Kir4.2) as a CaSR interacting protein (Huang et al., 2007). These studies established that the CaSR C-terminus interacts with and negatively modulates Kir4.2 channels in the thick ascending limb of Henle and the distal convoluted tubule of the kidney (Riccardi and Kemp, 2012). Kir channels contribute to the regulation of membrane potential and recycling of potassium ions for Na-K-ATPases. Although the physiological significance of the CaSR-Kir4.2 interaction is not well established, it is thought that CaSR interaction with Kir4.2 is mediated via filamin-A, a known scaffolding protein for Kir4.2 channels (Khan and Conigrave, 2010).

Finally, the CaSR colocalizes with caveolae, which are small flask-like membrane microdomains enriched in cholesterol and sphingolipids (Kifor et al., 2003). These microdomains are similar to lipid rafts with the addition of caveolin. The caveolae microdomains are enriched in components of G protein signaling (heterotrimeric G proteins, PKC isoforms, adenylyl cyclase, nitric oxide synthase, etc.) and therefore serve as signaling centers (Insel et al., 2005). In parathyroid cells, 80% of the CaSR is present within caveolae-like microdomains (Kifor et al., 1998). It is thought that surface stimulation of the CaSR promotes
further CaSR membrane expression by inducing the membrane insertion of the CaSR-caveolae pool (Grant et al., 2011). Additively, endocytosis of agonist bound CaSR into these signaling protein-rich microdomains can further enhance downstream signaling by providing the appropriate secondary signaling proteins in close proximity.

In addition to the CaSR associated proteins described above, other important CaSR interacting proteins identified through the yeast two-hybrid analysis includes 14-3-3 adaptor protein which negatively modulates Rho kinase activity, and calmodulin, a Ca²⁺ binding protein that serves as multifunctional intermediate messenger for a variety of signaling proteins that are unable to sense or detect intracellular Ca²⁺ levels (Huang et al., 2010; Arulpragasam et al., 2012). Nevertheless, the majority of CaSR interacting proteins identified to date are limited since these interactions were identified using the yeast two-hybrid approach with only the C-terminal tail of the CaSR. To obtain a better understanding of CaSR mediated functions, a large-scale proteomic analysis of the full length CaSR is required.

1.5.4 CaSR trafficking, recycling and degradation

The primary physiological role of the CaSR is to maintain systemic Ca²⁺ homeostasis, and is therefore under the control of unique protein trafficking mechanisms that allows the receptor to sustain chronic agonist stimulation. The CaSR demonstrates agonist-driven insertional signaling, whereby anterograde trafficking of the CaSR through the secretory pathway increases net plasma membrane CaSR expression (Grant et al., 2011; Breitwieser, 2013). The newly formed immature CaSR exits the endoplasmic reticulum immediately after the post-translational period by binding to p24A, a trafficking protein which cycles between endoplasmic reticulum and the golgi membranes (Stepanchick and Breitwieser, 2010). The CaSR is glycosylated to its mature form in the Golgi, and enters caveolae-rich microdomains with the aid of various cargo proteins
and RAMPS (as discussed above), where it awaits signals for insertion into the cell surface. The CaSR maintains minimal functional desensitization due to continual insertion of newly formed CaSR which balances the CaSR endocytosis process (Grant et al., 2011). This unique feature, which allows the CaSR to function under the presence of chronic agonist stimulation may be relevant for other receptors, but there are, as yet, no studies that have linked other GPCRs to this mechanism.

The CaSR endocytosis is a constitutive process and is initiated upon GRK or PKC mediated phosphorylation of the CaSR C-terminus, followed by β-arrestin mediated, dynamin independent endocytosis as described above (Brown and MacLeod, 2001). In addition, the CaSR displays rapid and constitutive endocytosis using ras superfamily of monomeric G proteins (Rab), namely Rab7 and Rab11a (Reyes-Ibarra et al., 2007; Breitwieser, 2013). Recycling of endocytotic CaSR to the plasma membrane has been noted in certain cells, but the mechanism is not well understood. Furthermore, the majority of CaSR that is internalized is degraded by proteosomes or lysosomes (Zhuang et al., 2012). In particular, the CaSR C-terminus contains a string of proline, glutamine, serine and threonine residues from 920 - 970 of the human CaSR that help target the CaSR to the lysosome for destruction (Zhuang et al., 2012). The CaSR can also be degraded in the caveolae via m-calpain (Kifor et al., 2003). Elevated extracellular Ca$^{2+}$ activates m-calpain which then targets the CaSR localized in the caveolae microdomains for cleavage and degradation. These studies demonstrates that in addition to CaSR mutations, mutations to proteins involved in CaSR anterograde transport, endocytosis and degradation can also contribute to systemic Ca$^{2+}$ homeostasis imbalances.
1.6 Biological roles of the CaSR

1.6.1 CaSR expression and maintenance of systemic Ca\(^{2+}\) homeostasis

The CaSR is an essential component of blood mineral ion homeostasis, and exerts its effect by sensing changes in circulating Ca\(^{2+}\) concentrations (Fig. 1.5). CaSR accomplishes this tight regulation through the actions of parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxyvitamin D3 (Vitamin D), and is thus expressed in parathyroid hormone producing Chief cells of parathyroid gland, calcitonin producing C-Cells of the thyroid gland, the cells lining the kidney tubule and intestine (Brown, 1993; Riccardi et al., 1995; Kameda et al., 1998; Brown, 2000; Brown and MacLeod, 2001).

In the Chief cells of the parathyroid gland, acute increases in extracellular Ca\(^{2+}\) activates the CaSR which suppresses the secretion of PTH within minutes (Brown, 1991). Upon detecting elevated extracellular Ca\(^{2+}\), the CaSR activates a number of secondary messengers which includes \(G_q\alpha\) mediated PLC activation and \(G_i\alpha\) mediated ras tyrosine kinase activation (Wettschureck et al., 2007). Both pathways synergistically activate the MAPK cascade, which then activates PLA\(_2\) to release arachidonic acid (Corbetta et al., 2002). The Chief cells convert the elevated arachidonic acid to leukotriene metabolites that inhibit PTH secretion (Bourdeau et al., 1992; Bourdeau et al., 1994).

Under resting conditions, PTH is constantly released in a pulsatile manner (Schmitt et al., 1996). The primary function of PTH is to increase serum Ca\(^{2+}\) levels. PTH acts on the kidney to increase renal Ca\(^{2+}\) resorption and phosphate excretion by blocking reabsorption of phosphate in the kidney proximal tubule while promoting Ca\(^{2+}\) reabsorption in the ascending loop of Henle, distal tubule, and collecting tubule (Riccardi and Kemp, 2012; Brown, 2013). PTH further increases systemic Ca\(^{2+}\) levels by acting on the bone to cause bone resorption. PTH induced bone resorption occurs in two phases. A rapid phase which includes PTH induced activation of
Figure 1.5. The role of the CaSR in the physiology of Ca\textsuperscript{2+} metabolism.

The CaSR maintains blood plasma Ca\textsuperscript{2+} homeostasis by regulating PTH secretion by the chief cells of the parathyroid gland upon sensing decreased plasma Ca\textsuperscript{2+} levels, and calcitonin secretion by the C-cells of the thyroid gland upon sensing increased plasma Ca\textsuperscript{2+} levels. PTH acts on the bone to stimulate Ca\textsuperscript{2+} release, and acts on the cells lining the kidney tubule to increase Ca\textsuperscript{2+} absorption and uptake. PTH also promotes the kidney to activate Vitamin D, which acts on the intestines to increase Ca\textsuperscript{2+} uptake. Calcitonin opposes the function of PTH. Calcitonin secretion stimulates bone deposition and reduces Ca\textsuperscript{2+} uptake in the kidneys. Figure adapted from Brown, 2002.
osteocytes that release Ca\(^{2+}\) ions from the interposed bone fluid, and a slow phase characterized by osteoclast proliferation and osteoclast mediated bone digestion (Lee and Partridge, 2009).

The final important function of PTH is activation of the enzyme 1-hydroxylase in the proximal tubules of the kidney, which promotes conversion of 25-hydroxyvitamin D to its most active metabolite, vitamin D (DeLuca, 2004). One important action of vitamin D is to promote absorption of Ca\(^{2+}\) in the intestine by stimulating the formation of calcium-binding protein within the intestinal epithelial cells. Vitamin D also promotes intestinal absorption of phosphate ion (Riccardi and Kemp, 2012), and participates synergistically with PTH to promote osteoclast proliferation and bone resorption (Brown, 2013).

Interestingly, activation of the CaSR suppresses PTH gene expression thus providing negative feedback on PTH secretion (DeLuca, 2004). The CaSR is so vital to systemic Ca\(^{2+}\) homeostasis that absence of CaSR results in lethality in CaSR knockout mouse model 2 - 3 days after birth due to hypercalcemia caused by insufficient inhibition of PTH secretion (Kos et al., 2003; Chang et al., 2008). In fact, numerous natural human mutations have been identified with clinical manifestations, including inactivating mutations that result in Familial Hypocalciuric Hypercalcemia or Neonatal Severe Hyperparathyroidism, and activating mutations that cause Autosomal Dominant Hypoparathyroidism (Fig. 1.1) (Hendy et al., 2000).

Unlike activation of the CaSR in Chief cells which inhibits PTH secretion, increased extracellular Ca\(^{2+}\) activates the CaSR on parafollicular C-cells of the thyroid gland to enhance calcitonin secretion (Kantham et al., 2009). CaSR activation in C-cells causes G\(_{q}\)α mediated stimulation of the PLC-DAG-PKC-Ca\(^{2+}\) intracellular signaling axis, and a rise in intracellular Ca\(^{2+}\) (Freichel et al., 1996). Ca\(^{2+}\) activation of PKC then induces activation of voltage-gated Ca\(^{2+}\) channels (VGCCs), causing influx of extracellular Ca\(^{2+}\). Ca\(^{2+}\) influx promotes classical vesicle release mechanisms that increase calcitonin secretion. The function of calcitonin is to
oppose that of PTH, and thus calcitonin inhibits Ca\(^{2+}\) absorption from the intestines, decreases osteoclast activity and promotes osteoblast function in bones, and inhibits renal tubular cell Ca\(^{2+}\) reabsorptions resulting in hypercalciuria (Sexton et al., 1999). Therefore, the primary role of calcitonin is to protect against Ca\(^{2+}\) loss by inhibiting osteoclastic bone resorption. The resulting rise in extracellular Ca\(^{2+}\) feedbacks on the CaSR to increase calcitonin secretion, and in conjunction with PTH provides tight plasma Ca\(^{2+}\) regulation of about 10mg/100ml in humans (Brown, 2002). Nevertheless, on the contrary to the CaSR expressed in Chief cells where plasma Ca\(^{2+}\) levels and PTH concentrations reveal an inverse sigmoidal relationship, activation of the CaSR in C-cells causes direct release of calcitonin. This demonstrates that the molecular and functional effects of CaSR are tissue dependent.

In addition to CaSR expression in the Chief and C-cells, the CaSR is also highly expressed throughout the kidney, where CaSR activation contributes to maintaining plasma Ca\(^{2+}\) levels by controlling Ca\(^{2+}\) secretion and reabsorption (Riccardi and Kemp, 2012). Immunohistochemical analysis revealed that the CaSR displays apical expression in the proximal tubule and the medullary collecting duct, basolateral expression in the thick ascending limb, and both apical and basolateral expression in the distal tubule and the cortical collecting ducts (Riccardi et al., 1998; Riccardi and Kemp, 2012). The unique CaSR cellular distribution in the kidney demonstrates that the CaSR is capable of monitoring Ca\(^{2+}\) levels in both the blood and the urine. The primary functions of the CaSR in the kidney involves divalent mineral ion homeostasis, water and salt reabsorption, modulation of the actions of PTH and vitamin D, and renin secretion, which regulates blood arterial blood pressure (Riccardi and Kemp, 2012). Activation of the CaSR in the apical side of proximal tubules reverses the inhibitory effects of PTH on phosphate reabsorption and 1α-hydroxylase activation (Ba et al., 2003). In the thick ascending limb of Henle's loop, ion transporters provide reabsorption of Na\(^{+}\), Ca\(^{2+}\) and Mg\(^{2+}\) ions under baseline
conditions. Here, activation of the CaSR under hypercalcemic conditions inhibits the function of these ion transporters, thus dampening the reabsorption of Na\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\) ions. Furthermore, in the distal tubule, CaSR activation on the apical membrane increases Ca\(^{2+}\) influx through coupling to transient receptor potential vanilloid membrane, and regulates Ca\(^{2+}\) exit into the urinary space by promoting plasma membrane Ca\(^{2+}\)-ATPase function (Topala et al., 2009). Additionally, in the kidney medullary collecting duct, basolateral binding of antidiuretic hormone vasopressin to the vasopressin GPCR promotes aquaporin 2 insertion into the apical membrane by increasing intracellular cAMP levels (Procino et al., 2008). Under hypercalciuric conditions, apical CaSR activation inhibits cAMP mediated apical insertion of aquaporin 2, hence reducing osmotic water permeability, and thus producing less concentrated urine that reduces the risk of Ca\(^{2+}\) stone formation (Riccardi and Kemp, 2012). Finally, CaSR activation in the juxtaglomerular apparatus participates in renin secretion and promotes glomerular cytoskeletal foot process formation which provides filter integrity (Oh et al., 2011).

Taken together, the coordinated effects of CaSR function in the kidney, parathyroid and thyroid glands provides tight control of plasma Ca\(^{2+}\) levels. Hence, it is not surprising that with the growing incidence of chronic kidney disease in the worldwide population, the CaSR positive allosteric modulator Cinacalcet has been approved by the FDA for treatment of secondary hyperparathyroidism in patients with chronic kidney disease on dialysis and hypercalcemia in patients with parathyroid carcinoma (Block et al., 2010).

1.6.2 CaSR knockout mice

CaSR\(^{-/-}\) knockout mice with targeted disruption of exon 5 of the CaSR gene were indistinguishable from wild-type littermates at birth. However, from second post-natal day, the CaSR\(^{-/-}\) mice growth was markedly reduced compared to wild-type or CaSR +/− animals. 72 hrs
after birth, CaSR<sup>−/−</sup> mice became hypercalcaemic, progressively lethargic and died by the age of 3 – 30 days. The CaSR<sup>−/−</sup> mice had markedly elevated serum Ca<sup>2+</sup> and parathyroid hormone levels, parathyroid hyperplasia, bone abnormalities, retarded growth and premature death (Ho et al., 1995). The CaSR<sup>−/−</sup> lethality was rescued when CaSR<sup>−/−</sup> mice were bred with mice lacking parathyroid glands or mice with an inability to synthesize PTH (Kos et al., 2003; Tu et al., 2003). This suggested that elevated PTH alone causes skeletal abnormalities in CaSR<sup>−/−</sup> knockout mice and that absence of the CaSR in bone and cartilage does not contribute to the skeletal pathology.

It has been shown that the CaSR<sup>−/−</sup> mice generated via disruption of exon 5 is an incomplete knockout of the receptor due to alternative splicing. An alternatively spliced CaSR which lacks exon 5 is naturally generated in certain tissues such as in the growth plate, skin and kidney (Oda et al., 2000; Rodriguez et al., 2005). This splice receptor compensates for the absence of full-length CaSR in certain tissues such as bone and cartilage and this renders the CaSR<sup>−/−</sup> knockout incomplete. Therefore, in order to establish the role of the CaSR in skeletal development, tissue-specific conditional knockouts of the CaSR in parathyroid, bone, and cartilage cells via loxP sites flanking exon 7 (which encodes for the seven transmembranes and the intracellular loops) were generated to address the issues raised by the CaSR exon 5 disruption. Tissue-specific deletion of CaSR in parathyroid gland and bone resulted in profound bone defects, whereas deletion of CaSR in cartilage-producing chondrocytes resulted in death before embryonic day 13 (Chang et al., 2008). Parathyroid gland-specific deletion of CaSR resulted in severe hyperparathyroidism which caused a drastic decrease in bone growth and mineralization. Mice with chondrocyte-specific deletion of the CaSR between E16 and E18 were viable, but demonstrated delayed growth plate development. This was attributed to the role of the CaSR in promoting chondrocyte differentiation. Analysis of the osteoblast-specific CaSR knockout mice revealed that the CaSR expression increased osteoblast proliferation, survival, and differentiation by stimulating the
release of osteoblast growth factors while inhibiting proapoptotic signals. Taken together, these studies revealed a critical role of the CaSR in early embryogenesis and skeletal development (Chang et al., 2008).

1.6.3 CaSR expression during development – anatomy and function

Evidence of CaSR expression in early embryonic mouse lung development during lung glandular formation throughout embryonic days (E) 11.5 to 16.5 provided initial evidence that CaSR expression is temporally regulated (Finney et al., 2008). Interestingly, the developing fetus is bathed in a hypercalcaemic environment of 1.7 mM Ca\(^{2+}\) sufficient to partially activate the CaSR, and within 48 after birth, the fetus regulates its Ca\(^{2+}\) concentration to the adult level of approximately 1.2 mM Ca\(^{2+}\) (Finney et al., 2008). CaSR stimulation in the developing lung was important for lung-branch morphogenesis. In addition, transient CaSR expression in the developing kidney around E13.5 emphasized the role of the CaSR in ureteric bud formation in the perinatal rat nephron (Chattopadhyay et al., 1996a). Furthermore, analysis of abundant but transient CaSR expression in the developing mouse superior cervical ganglion neuron between E18 to postnatal day (PND) 0 demonstrated that CaSR activation controls sympathetic axon innervation, neurite outgrowth and target field innervation (Vizard et al., 2008). *These studies emphasize the transient nature of CaSR expression during tissue embryonic development.*

Similarly, the CaSR is also temporally expressed throughout the developing postnatal nervous system. However the physiological importance and functional significance of the CaSR in most developing brain regions are unknown (Ferry et al., 2000). Intriguingly, during early postnatal CNS development the cerebrospinal fluid (CSF) Ca\(^{2+}\) concentration is around 1.6 mM, sufficient to partially activate the CaSR (Delivoria-Papadopoulos et al., 1967; Riccardi and Kemp, 2012).
Comprehensive characterization of a broad range of brain regions by Ferry et al., 2000 using *in situ* mRNA hybridization studies demonstrated a robust but transient increase in CaSR expression between PND 10 to 30, followed by a rapid and drastic decrease to adult levels (Fig. 1.6) (Chattopadhyay et al., 1997; Ferry et al., 2000). Similarly, rat hippocampal mRNA and protein expression analysis performed by Chattopadhyay et al., 1997 demonstrated peak CaSR expression from PND12 to PND24, followed by a 3-fold decrease to adult levels (Chattopadhyay et al., 1997). Interestingly, hippocampal CaSR expression parallels the ontogeny of hippocampal long-term potentiation, suggesting a role of the CaSR in learning and memory. Moreover, *in situ* hybridization studies in neurons, oligodendroglia, and microglia of the developing rodent brain identified moderate levels of CaSR expression, yet the roles of CaSR in these cells have not been characterized (Chattopadhyay et al., 1997). Conversely, postnatal CaSR expression is highest in the neurons of the subfornical organs, and unlike its transient expression profile in other brain regions, the CaSR expression in this region remain elevated into and throughout adulthood (Ferry et al., 2000). The subfornical organ is a thirst center and its neurons project onto supraoptic and paraventricular nuclei to control the activity of vasopressin-secreting neurons (Ruat and Traiffort, 2013). Therefore, subfornical organ expression suggests that CaSR is also involved in central fluid and electrolyte balance.

The CaSR expression profile in adult rodents differs drastically from immature brain since the regions that demonstrate transient CaSR expression during early development is absent in adult rodents. For example, substantial levels of adult CaSR expression are evident within the hippocampus, striatum, cingulate cortex, ependymal zones of the cerebral ventricles, and perivascular nerves around cerebral arteries, regions that do not express the CaSR during development (Bouschet and Henley, 2005; Ruat and Traiffort, 2013). The changes in temporal
In situ mRNA hybridization studies performed by Ferry et al., 2000 on a broad range of brain regions demonstrates a robust but transient increase in CaSR expression between postnatal days (PND) 7 to 25, followed by a rapid and drastic decrease to adult levels.
expression profile may be attributed to the functional role of the CaSR in immature versus adult brain.

The transient expression profile of the CaSR in the immature brain suggests the involvement in neuronal proliferation, differentiation, maturation or homing. In adults, CaSR expression reveals discrete punctate localizations that are associated with nerve terminals, and may regulate neurotransmitter disposition in response to elevated Ca\(^{2+}\) levels present in the synaptic space following neuron depolarization (Ruat et al., 1995). The adult cerebral arteries also display an intense network of CaSR immunoreactive fibers demonstrating possible functional role in vessel innervation. Studies on adult CaSR neuronal expression have determined that the CaSR controls neuronal excitability by regulating ion channel function (Phillips et al., 2008). For example, adult cortical neurons expressing the CaSR detects decreases in synaptic cleft Ca\(^{2+}\) upon high synaptic activity, and relays this to voltage-dependent, nonselective cation channels to increase neuronal activity. Here, excitatory postsynaptic currents were doubled in neurons from CaSR knockout (−/−) mice compared with those from wild-type mice. In addition, adult neuronal CaSR activation leads to tonic glutamate release (Vyleta and Smith, 2011), and this effect was absent in CaSR knockout (−/−) mice. Here, presynaptic extracellular Ca\(^{2+}\) in conjunction with Mg\(^{2+}\) activates the presynaptic CaSR to promote spontaneous glutamate release. Interestingly, the rise in intracellular Ca\(^{2+}\), and Ca\(^{2+}\) influx via VGCC was not necessary for tonic glutamate release, indicating that extracellular Ca\(^{2+}\) enhances spontaneous glutamate release via activation of the presynaptic CaSR (Vyleta and Smith, 2011). The above studies reinforce the idea that the CaSR modulates Ca\(^{2+}\)-dependent neuronal functions in the adult brain.
1.6.4 CaSR expression and function in cell types involved in chemotaxis and cell homing

The widespread expression of the CaSR in a broad range of cell-types unrelated to plasma Ca\(^{2+}\) regulation suggests that the CaSR regulates a variety of processes independent of systemic Ca\(^{2+}\) homeostasis. One such system where expression of the CaSR is unrelated to the regulation of blood mineral ion homeostasis is its involvement in directional cell migration. For example, during fetal development, stem cells from fetal liver migrate to the endosteal surfaces of the bone marrow cavity to undergo haematopoiesis (Nilsson et al., 2001). Several studies have demonstrated that the bone endosteal surface provides a niche for CaSR expressing haematopoietic stem cells to preferentially localize to the bone, and that haematopoietic stem cells prepared from mice lacking the CaSR were defective in localizing to the endosteal niche due to defective adhesion to the extracellular matrix protein, type I collagen (12, 13).

Similarly, CaSR expression has shown to be critical for recruitment of macrophages to the site of injury; an important step for the induction of the immunologic response following infection or tissue injury (Olszak et al., 2000). Extracellular Ca\(^{2+}\) concentrations are considerably elevated at the site of injury as compared to serum, and chronic inflammatory conditions have been associated with deposition of Ca\(^{2+}\) salts (Olszak et al., 2000). Olszak et al., 2000 demonstrated cell surface expression of the CaSR in adult mononuclear cells and established that extracellular Ca\(^{2+}\) modulates monocyte localization to the injury, therefore suggesting that Ca\(^{2+}\) is a primitive mediator of immune function.

Likewise, the CaSR has also been implicated in gonadotropin-releasing hormone (GnRH) neuron migration from its origin to its final resting position in the hypothalamus (Chattopadhyay et al., 2007). Chattopadhyay et al., 2007 have demonstrated CaSR expression in primary GnRH neuronal cultures from murine basal forebrain and two different GnRH neuronal cell line.
Activation of the CaSR with elevated extracellular Ca\(^{2+}\) promoted GnRH neuron directional chemotaxis. Here, CaSR stimulation resulted in Ca\(^{2+}\) influx through N-type VGCC and subsequent secretion of monocyte chemoattractant protein which synergistically promoted GnRH neuron migration towards extracellular Ca\(^{2+}\). In CaSR knockout mice, CaSR null GnRH resulted in approximately 27% fewer GnRH neurons in the final resting position in the preoptic area of the anterior hypothalamus compared to wild-type littermates.

Similarly, CaSR expression has been linked to bone cell migration (Yamaguchi, 2008). Studies have confirmed CaSR expression in both osteoclasts and osteoblasts, which are cells involved with resorption of the mineralized bone matrix and cells that replace the resorbed bone respectively (Sugimoto et al., 1993; Marie, 2009). The dynamic balance between osteoclasts and osteoblasts determine bone remodeling and long-term serum Ca\(^{2+}\) levels. The bone contains sites of enriched Ca\(^{2+}\) levels sufficient to activate the CaSR (Silver et al., 1988; Takahashi et al., 2002; Bushinsky, 2010), thus studies have shown that CaSR-expressing osteoblasts migrates to sites of osteoclast mediated bone resorption, which would allow replacement of the missing bone during the subsequent osteoblastic phase of bone turnover (Sugimoto et al., 1993; Theman and Collins, 2009). Activation of the CaSR in osteoblasts couples to PLC – ERK1/2 – JNK signaling cascade, whereas activation of the CaSR in osteoclasts couples to PLC-IP3 pathway, with both pathways contributing to cell migration, differentiation and survival (Marie, 2009).

Finally, the CaSR has been shown to promote plasma membrane ruffling, a precursor step to cell migration (Bouschet et al., 2007). Plasma membrane ruffling is characterized by cell morphological changes that result from the formation and retraction of cytoplasmic protrusions enriched in polymerised filamentous actin (F-actin) at the migrational cell leading edge. CaSR activation couples to a complex consisting of β1 arrestin, Arf nucleotide binding site opener (ARNO), ADP-ribosylating factor 6 (ARF6) and engulfment and cell motility protein (ELMO),
which collectively contribute to the actin polymerization, cytoskeletal reorganization and ultimately cell migration (Bouschet et al., 2007).

1.6.5 CaSR in cancer cell progression and metastasis

Advanced breast and prostate tumour cells primarily metastasize to bone (Coleman, 1997). Interestingly, the bone resorbing regions are enriched in extracellular Ca\(^{2+}\) at levels sufficient to significantly activate the CaSR (Silver et al., 1988; Takahashi et al., 2002; Bushinsky, 2010). Chemoattractant factors released during bone resorption mediate the preferential migration of cancer to the bone environment. Indeed, tumor metastasis preferentially attaches to regions of active bone turnover and remodeling, such as the femur, pelvis, ribcage, skull and humorous. One of the chemoattractant factors released during bone resorption is extracellular Ca\(^{2+}\), which has shown to provide a strong chemoattractant signal for CaSR expressing cancer cells to often colonize the bone tissue (Joeckel et al., 2014). Interestingly, CaSR expression has been shown to enhance the metastatic potential in various bone-preferring breast and prostate cancer cell lines (Liao et al., 2006; Mamillapalli et al., 2008; Saidak et al., 2009a). For example, \textit{in vitro} experiments have revealed that blocking CaSR function in PC3 prostate cancer cells and MDA-MB-231 breast cancer cells inhibits cell adhesion and migration (Liao et al., 2006; Saidak et al., 2009a). In addition, inhibition of CaSR mediated ERK1/2 or PLC activation abolished Ca\(^{2+}\) induced MDA-MB-231 cancer cell migration. Moreover, aggressive tumors with increased metastatic potential demonstrated increased CaSR protein expression compared to tumors with lower metastatic potential (Liao et al., 2006; Saidak et al., 2009a; Brennan et al., 2013). In addition to increasing the metastatic migrational properties of breast and prostate cancer cells, CaSR stimulation also promoted tumor proliferation by controlling parathyroid hormone-related protein secretion (Sanders et al., 2000; Sanders et al., 2001; Ardestipour et al., 2006). Taken
together, these findings indicate that the CaSR exhibits oncogenic properties in highly aggressive breast and prostate cancers. By comparison, CaSR expression in parathyroid and colon cancers is often reduced compared to normal tissue, and activation of the CaSR in these tumors decreases cell proliferation and tumor progression (Singh et al., 2013). These studies suggest that the CaSR expression decreases the tumourigenicity of parathyroid and colon cancers. Interestingly, increased bone resorption induced by dietary Ca\(^{2+}\) deficiency promotes breast cancer bone metastasis, while increased dietary Ca\(^{2+}\) intake has preventative effects in colon cancer and parathyroid tumour progression (Lipkin, 1999; Butler et al., 2010; MacLeod 2013).

1.7 Integrins

Integrins are cell adhesion molecules that mediate cell-extracellular matrix (ECM) interactions by providing a dynamic link between the extracellular adhesion molecules and the intracellular actin cytoskeleton. Integrins are unique proteins in that they are able to bind to ECM proteins and regulate appropriate intracellular signaling cascades, and are also capable of conveying intracellular signaling messages to the ECM by modulating their binding affinity for these ECM proteins. By mediating cell-ECM interactions, integrins play an essential role in cell migration, and have been directly attributed to a variety of cellular functions recently implicated with the CaSR, including cell differentiation, proliferation, chemotaxis, embryogenesis, CNS development, inflammation and cancer (Aplin et al., 1999; Tarone et al., 2000; Nikonenko et al., 2003).

1.7.1 Integrin α and β subunits

Integrins are heterodimers of type 1 membrane-spanning glycoproteins that are composed of an α and β subunits (Luo et al., 2007). In mammals, there are 18 α and 8 β subunits (Luo et al., 2007). The α and β subunits share no sequence homology or structural resemblance to each
other, although the different α and β subunits have conserved regions among themselves. Both integrin subunits are characterized by a very large extracellular domain of 700 - 1100 residues, an intermediate transmembrane domain, and a very short cytoplasmic domain of only 30 - 50 residues, with both subunits held together via non-covalent interactions (Humphries et al., 2003a).

The outermost N-terminal region of the α integrin subunit that faces the extracellular side of the cell is composed of a head structure with seven-bladed β-propellers, which connects to the leg structure composed of a thigh, calf-1 and calf-2 domains (Barczyk et al., 2010). A region between the thigh and calf-1 domain known as the α subunit “genu” plays a key role in providing the pivot point for integrin domain extension. The top face of the head structure contains the ligand binding sites, while the lower part of the head contains EF-hand like domains that bind to Ca^{2+} allosterically. Of the 18 integrin α subunits expressed in mammals, 9 contain an extra I-domain of approximately 200 amino acids that is located between blades 2 and 3 of the β-propeller head. Members of the integrin α subunit family are highly homologous within the αI domains, but are highly divergent in their cytoplasmic domains. On the contrary, the β integrins are highly homologous in their C-terminus cytoplasmic tails (Luo et al., 2007). The structure of these β integrin subunits consists of a plexin-sempahorin-integrin domain at the outermost N-terminal region, followed by tail structure consisting a hybrid domain, a βI domain and four cysteine-rich epidermal growth factor-like repeats (Springer and Wang, 2004).

Integrin heterodimerization has been associated with the area between the β subunit hybrid domain and the β-propeller of the α subunit (Humphries, 2000). The sequences adjacent to this interaction region contain binding motifs that characterize the different heterodimerization specificities. In addition, there are no free α or β subunits present at the cell surface since heterodimerization occurs intracellularly prior to trafficking to the membrane (Barczyk et al.,
In addition, due to excess β subunit expression, the level of α subunit expression controls the amount of functional integrin complex. Finally, there are no known interacting proteins of the integrin α subunit.

### 1.7.2 Prototypic integrin ligands and recognition sequences

Integrins are primarily activated by ECM proteins. In vertebrates, the integrin Family contains 24 functional α-β heterodimer combinations with distinct distribution, ligand preference, and signal transduction (Fig. 1.7A) (Humphries et al., 2006). Although some integrin subunits appear in a single heterodimer, 12 integrins contain the β subunit, while five heterodimers contain the αV subunit. Despite the complexity, integrin-ligand combinations are characterized into four main classes, (1) RGD-binding integrins, (2) LDV-binding integrins, (3) A-domain β1 integrins, and (4) non-αA-domain-containing laminin-binding integrins (Humphries et al., 2006). RGD-binding integrins are activated by the arginine-glycine-aspartate tripeptide containing ECM proteins such as fibronectin, vitronectin, fibrillin, fibrinogen, etc (Humphries et al., 2006). The LDV-binding integrins, also known as leukocyte receptors, are stimulated by the leucine-aspartate-valine (LDV) motifs present on proteins primarily from the immunoglobulin-like family of adhesion molecules. The A-domain β1 integrins bind to the collagen family of proteins, and certain laminin members. Finally, the non-αA-domain-containing laminin-binding integrins are highly selective laminin receptors. In addition to these four classes of ligands, the integrins are also activated by a variety of other ligands including matrix metalloproteinases, vascular endothelial growth factors, cartilage oligomeric matrix protein, etc (Barczyk et al., 2010). Figure 1.7B further details the integrin-ligand combinations, and their respective ligand specificity.
Figure 1.7. Integrin heterodimers and ligands.

(A) In mammals, there are 18 α and 8 β subunits that can form 24 functional heterodimers with distinct distribution, ligand preference, and signal transduction. Integrins are categorized into four groups depending on their ligand preference. Examples of ligands that contain the RGD (Arginine-Glycine-Aspartate) binding sequence motif include fibronectin and vitronectin. (B) Table shows integrin heterodimer combinations expressed in humans and the respective ligand specificity. Figure and table adapted from Barczyk et al., 2010.
1.7.3 Cation-binding sites in integrins

There are several cation binding sites within the integrin subunits that control its affinity for ECM proteins. In addition the Ca\(^{2+}\) binding sites of the α integrin EF-hand like domains, the αI domain contains a metal-ion-dependent adhesion site (MIDAS) motif that binds Mg\(^{2+}\), which promotes α integrin binding to the ECM proteins (Valdramidou et al., 2008). The integrins have an additional Mg\(^{2+}\) binding site on the adjacent βI domain of the β integrin subunit.

Another binding site adjacent to the MIDAS known as ADMIDAS, and a ligand-induced metal ion binding site (LIMBS) of the β integrin subunit binds Ca\(^{2+}\) ions, which negatively modulates integrin binding to the ECM proteins. The release of Ca\(^{2+}\) binding in the MIDAS, or binding of Mn\(^{2+}\) leads to integrin conformational changes resulting in an active form of the integrins (Humphries et al., 2003b). Binding of Ca\(^{2+}\) to the LIMPS has shown to positively affect integrin activation. The difference in Ca\(^{2+}\) binding to the MIDAS and LIMPS sites can account for the regulatory role of Ca\(^{2+}\) in integrin activation; at high Ca\(^{2+}\) concentrations, the integrins adopt an inactive conformation due to preferential binding of Ca\(^{2+}\) to the MIDAS which competes with Mn\(^{2+}\), whereas low concentrations of Ca\(^{2+}\) synergize with suboptimal Mg\(^{2+}\) at the LIMPS to facilitate integrin active conformation (Luo et al., 2007; Barczyk et al., 2010).

1.7.4 Integrin activation (inactive and active states) and bidirectional signaling

At resting state, the integrins adopt a bent conformation where the integrin leg structures are severely bent, generating a closed topology where the head domain are juxtaposed to the leg regions close to the cell membrane (Fig. 1.8) (Springer and Wang, 2004). Here, the α and β integrin subunit transmembrane domain and c-termini are only a few angstroms apart (Xiong et al., 2001). In this bent conformation, the integrin ligand binding sites are not optimal for binding ECM proteins, therefore the bent conformation represents a low-affinity state. A switch-blade
Integrins are type 1 membrane-spanning glycoproteins that mediate cell adhesion and migration. Each subunit has a large (>700 residue) NH₂-terminal extracellular domain. A single membrane-spanning domain links this extracellular domain to a short (13–70 residues) cytoplasmic domain tail. The large extracellular domain contains sites for ligand binding, while the small intracellular regions sense changes in intracellular signaling pathways and mediates the information to ECM proteins via conformational modifications. When inactive, integrins are present in a bent conformation (low affinity). Changes to intracellular activation signals induce integrins to take an upright position with two extended head-piece conformations. The upright conformation is characterized by an intermediate affinity state and high affinity, ligand bound state where the integrin α and β subunit tails are separated. Integrin inside-out signaling is accomplished when activation of intracellular signaling proteins push the β integrin leg away from the α integrin tail, allowing for formation of integrin high affinity activation state. Stabilization of integrin binding to ECM initiates outside-in signaling, where the integrins mediate downstream signaling that includes focal adhesion and actin stress fiber formations, activation of Rho GTPases, cell structural changes, gene transcription modifications, etc., which ultimately leads to promotion of cell adhesion and migration.
like extension of the head region away from the legs shifts the integrin into an intermediate-affinity conformation (Barczyk et al., 2010). The presence of ECM ligands induce integrin intermediate-affinity conformation (Luo and Springer, 2006). Destabilization of the $\alpha/\beta$ integrin association fully activates the integrins into a high-affinity conformation (O'Toole et al., 1991; Lu et al., 2001), and transmembrane domain separation is necessary for integrin signaling (Fig. 1.8) (Wang and Luo, 2010). Binding of the head domain of the cytoskeletal protein talin to the cytoplasmic intracellular tail region of $\beta$ integrin causes conformational changes and integrin activation, a process known as inside-out signaling (Fig. 1.8) (Kinashi, 2005). Following separation, the $\alpha$ subunit helix maintains its original structure, whereas the $\beta$ subunit helix has tilted by inserting 5 – 6 additional residues into the hydrophobic lipid membrane core (Humphries et al., 2003a).

Integrin inside-out signaling mediated by talin binding to the $\beta$ integrin C-terminal tail is controlled by small GTPase Rap1, and its effectors such as RapL and RIAM (Lee et al., 2009; Mehrbod et al., 2013). For example, in leukocytes and platelets, association of Rap1 and RIAM recruits talin to the integrins (Tadokoro et al., 2003). Interestingly, Rap1 is activated by various GPCRs that are activated by chemoattractant stimulation. Therefore, stimulation of membrane spanning receptors can mediate cell adhesion and migration by activating intracellular signaling cascades that lead to integrin inside-out signaling (Mehrbod et al., 2013). In addition to talin, the kindlin family of proteins also contribute to integrin inside-out signaling by serving as a coactivator for talin mediated activation of the integrins (Larjava et al., 2008; Kahner et al., 2012). Therefore, kindlin alone is incapable of activating inside-out integrin signaling, but rather has synergistic effects on talin mediated integrin activation. Similar to kindlins, other intracellular proteins activated by GPCRs can also activate integrin inside-out signaling.
Upon separation of the integrin α and β subunits, the integrins adopt a high affinity active conformation that allows binding of ECM proteins. Ligand occupancy and integrin clustering in-turn activates intracellular signaling cascades that mediate cell cytoskeletal changes, a process known as integrin outside-in signaling (Fig. 1.8) (Kinashi, 2005). Once integrins are tightly bound to the ECM proteins, they then cluster together laterally on the membrane via cell surface insertion of integrins localized in caveolae-rich microdomains (Maheshwari et al., 2000). Integrin clustering assembles highly organized intracellular signaling complexes that mediate actin filament assembly. The reorganization of actin filaments into larger stress fibers in turn causes further integrin clustering, providing a positive feedback loop that maintains enhanced matrix binding (Webb et al., 2002). The aggregation of ECM proteins, integrins, cytoskeletal proteins, and signaling kinases forms unique structures known as focal adhesion complexes (Parsons et al., 2010). In particular, integrin ligation and clustering activates focal adhesion kinase (FAK), which in-turn activates Src-family kinases (Mitra and Schlaepfer, 2006; Hu and Luo, 2013). Activated Src phosphorylates cytoskeletal proteins such as paxillin, actin bundling protein α-actinin and vinculin to form nascent adhesion structures. Binding of these initial cytoskeletal proteins recruits further actin nucleating proteins that result in focal adhesion complex and actin stress-fiber formation (Mitra et al., 2005; Luo et al., 2007). These cytoskeletal changes mediated by integrin outside-in signaling ultimately allow cells to adhere and spread over the ECM proteins.

1.7.5 Stimulation dependent integrin adhesion, disassembly and cell migration

Cell migration is a multistep process that requires continuous coordinated formation and disassembly of adhesions (Fig. 1.9). The basic migratory cycle includes protrusion extension towards a chemoattractant gradient, formation of stable adhesions at the leading edge of the
Figure 1.9. The molecular aspects of cell adhesion and migration.

This schematic depicts stepwise cytoskeletal changes that occur during directional cell migration towards a chemoattractant gradient. Upon plating of cells on ECM, integrins bind to the matrix substrate and allows the cell to spread. Cell spreading establishes cell polarity with the leading edge forming adhesion contacts and actin polymerization, while the tailing edge disassembles actin formation. Stimulation dependent redistribution of integrins and actin stress formation from the trailing edges to the migrating front is important for chemoattractant-guided cell migration. Receptors which sense the chemoattractant gradient causes activation of intracellular signaling pathway which leads to trafficking of β1 integrins and actin fibers to the leading edge of the cell, while limiting the level of integrins and microtubules available in the retracting edge. The accumulation of integrins at the leading edge facilitates the formation of a stable association between the cells and the ECM, thereby allowing directed cell migration. The dynamic assembly and disassembly, together with actomyosin contractility moves the cell towards the chemoattractant gradient. Figure adapted from Mukhtar Ahmed PhD thesis (University of Toronto, Copyright 2012).
protrusions via integrin engagement of ECM proteins, release of adhesions and retraction at the cell rear, and the translocation of cell body forward (Webb et al., 2002). The initial step in directional migration requires the binding of the chemoattractant molecules to its specific cell surface receptors, and the activation of intracellular signaling cascades that initiate integrin inside-out signaling. Inside-out integrin activation results in outside-in integrin signaling, whereby ligand bound integrins mediate actin cytoskeletal polymerization (Vicente-Manzanares et al., 2009). These cytoskeletal changes drive the initial protrusion extension of the plasma membrane at the cell front allowing the cells to spread (Webb et al., 2002). Integrins play a crucial role during this stage of cell migration by stabilizing the nascent adhesion sites by recruiting signaling and cytoskeletal proteins for focal adhesion complex formation (Mitra et al., 2005). Cell spreading further establishes cell polarity with the leading edge by forming adhesion contacts and actin polymerization, while the trailing edge disassembles actin formation. The stable adhesion points created by integrin activation serves as traction points for the propulsive forces that move the cell body forward. Release of integrin mediated adhesions at the rear allows net translocation of the cell in the direction of the chemoattractant gradient (Fig. 1.9) (Webb et al., 2002).

Stimulation dependent redistribution of integrins and actin stress formation from the trailing edges to the migrating front is important for chemoattractant-guided cell migration. Receptors which sense the chemoattractant gradient activates intracellular signaling pathways which leads to trafficking of β1 integrins and actin fibers to the leading edge of the cell, while limiting the level of integrins and microtubules available in the retracting edge (Fig. 1.9). The accumulation of integrins at the leading edge facilitates the formation of a stable association between the cells and the ECM, thereby allowing directed cell migration (Li et al., 2005). The dynamic assembly
and disassembly, together with actomyosin contractility moves the cell towards the chemoattractant gradient.

1.7.6 Integrin expression

Integrins are ubiquitously expressed, and every nucleated cell in the body expresses a specific set of integrin heterodimers (Barczyk et al., 2010). Due to their dynamic nature, integrin expression can fluctuate depending on the environmental cues, and are developmentally and spatially regulated. Therefore, integrin expression literature is highly variable and the majority of expression analyses to date have been identified as a result of integrin knockout studies.

Spatial and temporal changes in integrin expression in the developing organism parallel changes in neurogenesis, differentiation, and migration. Integrin heterodimers containing the β1 integrin are most important for these biological processes since β1 integrin knockout results in null mutant mice that die soon after implantation as a result of inner cell mass defects in blastocyst formation (Stephens et al., 1995). Similarly, αV and α5 integrin knockout mice demonstrate embryonic lethality due to abnormalities in the developmental processes that control cleft palate formation, epithelialization, posterior trunk formation and vasculature development (van der Flier et al., 2010). Integrin heterodimers containing β2, αL, αX, αD, αE and αIIb are expressed in tissues important for immune function, leukocyte adhesion and blood clotting (Bouvard et al., 2013). Integrins α1, α2, α10 and α11 are expressed in tissues involved in bone and cartilage formation. These integrins also mediate angiogenesis and wound healing. α3 integrins are expressed in tissues involving branch formation, as seen in kidney, lungs, and cerebral cortex. α7 integrins are important for embryonic vasculature formation, while α6 laminin integrins are important for neuroblastic outgrowth, brain development, skin epithelia formation, etc (Fassler et al., 1996). α6 integrin knockout mice also die at birth. These
expression studies reveal that integrins are important for the development and maturation of all stages of the organism lifespan, and alterations in their expression can have pathological effects.

1.7.7 Integrins in cancer progression and metastasis

Integrin mediated adhesion contributes to tumour progression and metastasis by promoting tumor cell migration, invasion, proliferation and survival (Desgroisellier and Cheresh, 2010). Although integrins alone are not oncogenic, their expression levels have been reported to be elevated in several classes of tumours including that of breast, prostate, pancreatic, lungs and melanomas (Feldman et al., 1991; Friedrichs et al., 1995; Maschler et al., 2005; McCabe et al., 2007). In particular, tumour vasculatures consisting of the vascular endothelium, perivascular cells, fibroblasts, bone marrow-derived cells and platelets have robust up-regulation of integrin expression. In fact, over-expression of α5β1, α6β4, αVβ3 and αVβ5 correlates with increased tumour progression and decreased patient survival (Nip et al., 1992; Mierke et al., 2011). Recent studies have found that many oncogenes require integrin signaling to be able to initiate their effects on tumour growth and invasion. This may in part be attributed to the role of integrins in activating pro-survival and inhibition of apoptotic signals even in the absence of integrin ligation (Desgroisellier et al., 2009). Importantly, the upregulation of these ligand independent integrin expression promotes activation of pro-survival signals including FAK, ras, raf, and MAPK pathways which contribute to anchorage independent cell growth, which in normal cells would result in programmed cell death (Cho and Klemke, 2000; Guo and Giancotti, 2004). These observations demonstrate that although integrins are not oncogenic, in concert with tyrosine kinases and other oncogenes may contribute to tumorigenesis.

Integrins also play a crucial role in maintaining the tumour environment by promoting angiogenesis and lymphangiogenesis. Tumour-associated blood vessels are structurally different
from the normal vessels, and are characterized by the expression of αVβ3 and αVβ5 integrins (Hood et al., 2003). These RGD receptors are beneficial for tumour angiogenesis since the tumour environment contains many RGD containing ECM proteins (Avraamides et al., 2008). In addition, integrin crosstalk with specific growth factor receptors is important for angiogenesis since integrin ligation to ECM proteins increases the responsiveness of these growth factor receptors to the angiogenic growth hormones present in the tumour environment (Miyamoto et al., 1996; Trusolino et al., 2001). Integrin crosstalk with growth receptors can also promote tumour cell migration and survival by potentiating the activation of downstream signals involved in metastasis.

Integrins contribute to tumour metastasis by enhancing the activation of matrix metalloproteinases (Hosotani et al., 2002; Desgrosellier and Cheresh, 2010). These endopeptidases contribute to ECM degradation and remodelling, allowing the tumour cells to invade the surrounding environment by making a path for tumour cells to move forward. Using the available ECM proteins, integrin binding provides the tumour cell with the necessary traction required for cell motility and invasion.

The importance of integrins in tumour progression has made them an appealing pharmaceutical target for cancer therapy. Currently, integrin antagonists for αVβ3 and αVβ5 are currently under Phase III trial for patients with gliablastoma (Reardon et al., 2008). Furthermore, monoclonal antibodies with integrin function blocking activity, RGD mimetics and small molecule inhibitors of integrin function are being developed for the treatment for a wide variety of cancers (Shannon et al., 2004; Bhaskar et al., 2008). In addition to treatment, these integrin antagonists are being employed for use as imaging markers for visualizing cancer progression by linking the antagonists to detectable small molecule probes (Winter et al., 2003). Lastly, integrin antibodies are being linked to nanoparticles containing chemotherapeutic agents targeting
various other oncogenes thereby providing a tumour selective delivery system (Murphy et al., 2008).

1.8 Cell migration model systems

1.8.1 Carcinoma derived rat medullary thyroid C-cells (rMTC 44-2 cells)

Carcinoma derived rat medullary thyroid C-cells (rMTC 44-2) endogenously express the CaSR and displays high level of cell migration. These neuro-endocrine cells are distinguished by the expression of calcitonin and neurotensin (Clark et al., 1995). Normal C-cells originate from the parafollicular cells of the developing vagal neural crest, along with sympathoadrenal progenitors and enteric neurons, and consist of 1% of the total thyroid cells (Barasch et al., 1987). During early development, C-cell progenitors co-migrate with the enteric neuroblasts into branchial arch mesenchyme, while sympatho-adrenal progenitors differentiate into sympathetic neurons and adrenal chromaffin cells (Barasch et al., 1987). As the C-cells mature, neuronal CGRP peptide levels decrease while calcitonin levels increase, thus changing the initial neuronal characteristics of these cells into an endocrine phenotype (Barasch et al., 1987). However, rMTC 44-2 cells retain certain neuronal properties such as serotonin biosynthesis, and ability to extend processes in response to NGF or laminin (Clark et al., 1995).

rMTC 44-2 cells are tumour derived, thus they mimic forms of medullary thyroid carcinoma (MTC), a rare but highly metastatic neuroendocrine neoplasm (Pinchot et al., 2009). In humans, MTCs account for approximately 5 – 10% of all thyroid malignancies, and are characterized by early metastasis to regional lymph nodes, and distant metastasis to bone, lung, liver, brain and soft tissue (Bergholm et al., 1997). Distant metastasis is observed in 7 – 23 % patients with MTC, and is the main cause of MTC related deaths with survival rates of 25% at 5 years after their detection or 10% at 10 years (Modigliani et al., 1998). At present, the best treatment
available for MTC management relies on surgical resection consisting of total thyroidectomy and lymph node dissection. However, recurrent disease develops in 50% of the patients due to early metastasis to regional lymph nodes, with high risks of developing distant metastasis (Sippel et al., 2008). Novel ways to prevent MTC metastasis and progression are of need.

Therefore, rMTC 44-2 cells are highly metastatic neuroendocrine cells that possess both neuronal, cancerous, and migratory characteristics. These cells have the potential to serve as a suitable model system to study cell migration and cancer progression.

1.8.2 Cerebellar granule cell precursor neurons (GCPs)

The early postnatal cerebellum provides an excellent model system to delineate the molecular properties of neuronal migration due to the well-established migrational patterns of immature cerebellar granule-cell precursor neurons (GCPs), which mature into the most numerous neurons of the vertebrate brain (Fig. 1.10). Our work established that these migratory cells express the CaSR. One striking feature of GCPs is that they originate in the rhombic lip during late embryonic stages of rodent development, and migrate rostrally to a secondary germinal layer known as the external granule-cell layer (EGL) of the cerebellum where they continue to proliferate even after birth (Chedotal, 2010). During early rodent development (PND 5 – 20), GCPs proliferate in the periphery of the EGL-cerebellar basement membrane interface. Various factors such as laminin (expressed on the basement membrane), sonic hedge-hog (released from Purkinje cells), BDNF (released from Bergmann glia) and insulin-like growth factors contribute to GCP proliferation (Klein et al., 2001; Borghesani et al., 2002; Taylor et al., 2002; Blaess et al., 2004; Behesti and Marino, 2009). GCPs that have proliferated and exited the cell-cycle undergo tangential migration within the EGL. Tangentially migrating GCPs attach to the Bergmann glial processes and undergo radial migration whereby the GCPs migrate radially along the Bergmann
Over PND 5 – 20, GCPs (blue spheres, top) proliferate in response to growth signals including sonic hedgehog (shh) released by Purkinje cells (PC, purple). Mitotic GCPs exit the cell cycle to become migratory post-mitotic bipolar GCPs (orange) that undergo tangential migration in the external granule-cell layer (EGL). The bipolar migratory GCPs sprout a third axon and attach to Bergmann glia (BG) fibers (red) to undergo radial migration through the molecular layer (ML) and the Purkinje layer (PL), and into the internal granule-cell layer (IGL; mature granule neurons, dark orange). GCPs undergo radial migration by sensing extracellular matrix cues (laminin, vitronectin, etc.) and radially expanding transglial Ca\(^{2+}\) waves (pink circles) released by Bergmann glial processes.
glia fibers through the cerebellar molecular layer (ML) and the Purkinje cell layer (PCL), and into their final resting position in the internal granule-cell layer (IGL) (Chedotal, 2010). After the first three weeks of birth, GCP proliferation ceases, and the remaining GCPs migrate into the IGL and the EGL disappears.

As with cancer cells, the growth, maturation, and homing of GCPs are controlled by various chemoattractants that bind to specific cell surface receptors. For example, stimulation for radial GCP migration may be provided by radially expanding transglial Ca^{2+} waves generated by Bergmann glia fibers which initiate at a centre point and expand radially to encompass multiple Bergmann glia processes (Hoogland et al., 2009). In addition, factors such as BDNF and stromal cell-derived factor (SDF-1) are also strong chemoattractants for GCP radial migration. These extracellular signals activate intracellular proteins that result in increased intracellular Ca^{2+} and phosphorylation of ERK and AKT kinase, cascades which ultimately lead to integrin activation and GCP migration (Klein et al., 2001; Dave and Bordey, 2009).

The role of β1 integrins in cerebellar GCP proliferation and migration has been well documented (Selak et al., 1985; Dahmane and Altaba, 1999; Graus-Porta et al., 2001; Blaess et al., 2004). For example, Blaess et al., 2004 have shown that in CNS-specific β1 integrin knockdown mice, absence of β1 integrin resulted in GCPs to lose adhesive contact with laminin expressed in the meningeal basement membrane, leading to decreased GCP proliferation, migration and premature differentiation (Blaess et al., 2004). Furthermore, potent stimulants of GCP proliferation such as sonic hedgehog (Shh) and SDF-1 have been shown to induce GCP proliferation only in the presence of β1 integrin mediated adhesion to laminin (Klein et al., 2001; Blaess et al., 2004). Similarly, others have shown that β1 integrins control GCP migration by binding to ECM proteins such as laminin, vitronectin, reelin, etc., which aid in the homing of GCPs from the EGL to the IGL (Rivas and Hatten, 1995; Pons et al., 2001; Borghesani et al.,
These studies demonstrate that neuronal migration relies heavily on integrin activation. Therefore, analysis of GCP migration in both in vitro purified GCP primary cultures using an ECM chemoattractant gradient, or in vivo tracking of live GCP migration in an intact developing cerebellum are good model systems to delineate the roles of proteins involved in neuronal migration.

Interestingly, defects in GCP migration result in medulloblastoma, the most common cause of malignant brain tumor in children aged under 4 years of age (Hatten and Roussel, 2011; Remke et al., 2013; Macdonald et al., 2014). Medulloblastoma is a highly heterogeneous group of tumors characterized by aberrant GCP proliferation and migration. Unlike most neuronal populations, GCPs remain mitotically active even after birth and constitute potential targets for transforming insults (Roussel and Hatten, 2011). Impairment of GCP migration allows GCPs to remain longer in the EGL proliferative-niche; thus GCPs are more prone to transformational insults due to increased exposure to proliferation molecules. To date, there are no effective ways to treat medulloblastoma. Therefore, identifying the important proteins regulating GCP migration and progression may provide novel therapeutic avenues for treating medulloblastomas.

1.9 Hypotheses, objectives and rationale

1.9.1 Overall objective and hypothesis

Recent findings suggest that the CaSR may function as a chemotaxis sensor in regulating cell movement and migration, with possible functional associations with other membrane proteins and complexes associated with cell adhesion and motility. For example, during fetal development, haematopoietic stem cells lacking the CaSR were found to be defective in localizing to the Ca$^{2+}$-rich endosteal bone niche (Adams et al., 2006). Similarly, elevated extracellular Ca$^{2+}$ concentrations detected by CaSR-expressing prostate and breast cancer cells
have been shown to act as a strong chemoattractant for cancer cell metastasis to bone cells (Saidak et al., 2009a). In addition, CaSR-expressing monocytes localize to sites of tissue injury by detecting elevated local Ca\textsuperscript{2+} gradients (Olszak et al., 2000). These studies demonstrate a role for the CaSR in chemotaxis and support the idea that the CaSR may participate in cellular homing. Despite the publication of several studies associating CaSR function to migration, the signaling mechanism and the protein-protein interactions of the CaSR involved in cell migration have not been characterized. To address this gap, we investigated the signaling proteins and underlying mechanisms involved in CaSR-mediated cell adhesion and migration.

The integrin family of ECM binding proteins mediate cell adhesion and migration. Stimulation dependent activation of the integrins control directional cell migration by allowing the cells to adhere, spread and migrate over ECM proteins. Therefore, the general goal of this study was to determine whether the CaSR couples to integrins to form functional protein complexes required for cell adhesion and migration. Thus, our general hypothesis was that stimulation of the CaSR would result in activation of integrins, whereby the CaSR acts as the chemoattractant nutrient sensor and uses the adhesion and migration properties of integrins to direct cell migration.

1.9.2 Cancer cell migration: rat medullary thyroid carcinoma cells (rMTC 44-2)

The CaSR is highly expressed in rat medullary thyroid carcinoma cells. We hypothesized that in these cancer cells, the CaSR physically couples to integrins to form functional macromolecular protein complexes. We predicted that stimulation of the CaSR would result in increased cell adhesion and migration via activation of the integrins, whereby the CaSR acts as the chemoattractant nutrient sensor and uses the adhesion and migration properties of integrins to direct rMTC 44-2 cell migration.
The first objective was to perform proteomic analysis on the endogenously expressed CaSR in the rMTC 44-2 cell model using CaSR immunoprecipitation coupled to mass spectrometry based protein identification method. By employing this proteomic method, candidate CaSR interacting integrin receptors and other novel CaSR signaling interacting partners involved in rMTC 44-2 cell migration were indentified. To determine the role of the CaSR and integrins in cell migration, ECM-based cell adhesion, cell spreading, and cell migration assays were conducted in the presence and absence of CaSR specific allosteric modulators and integrin inhibitors. Potential biochemical pathways involved in CaSR/integrin complex mediated cell adhesion and migration were analyzed using specific inhibitors of intracellular signaling pathways.

The CaSR regulates the migration, chemotaxis and homing of several cell-types, however the mechanisms linked to CaSR mediated migration is unknown. Despite the various studies associating CaSR function to migration, the signaling mechanism and the protein-protein interactions of the CaSR involved in cell migration have not been well characterized. Therefore, the primary goal of this study was to determine whether cell adhesion and migration proteins such as integrins are involved in CaSR-mediated cell adhesion and migration in rMTC 44-2 cells.

1.9.3 Neuronal migration: cerebellar granule cell precursor neurons (GCPs)

In the developing CNS, the CaSR is transiently expressed in several brain regions and includes the cerebellum. We hypothesized that the CaSR physically couples to the integrins in cerebellar GCPs of the developing cerebellum and promotes GCP migration and cell homing from the EGL into the IGL. As noted above, although several studies have demonstrated CaSR expression in the developing CNS, the role of the CaSR in CNS development has remained uncharacterized. Recent studies have shown that CaSR stimulation results in migration of a
variety of cell types ranging from cancer cells to hematopoietic stem cells (Drueke, 2006; Saidak et al., 2009b; Lam et al., 2011; Tharmalingam et al., 2011; Lee et al., 2012; Joeckel et al., 2014). Similarly, integrin activation is also important for the migration of a variety of cell-types, including GCPs ((Blaess et al., 2004). Therefore, the goal of this study was to identify and characterize the expression pattern of the CaSR and integrin complexes in the developing cerebellum, and to determine whether this complex is important for GCP migration during cerebellar development.

We hypothesized that stimulation for the GCP CaSR-β1integrin complex would be provided by the integrin ligand laminin present in the basement membrane surrounding the EGL, and spontaneous Ca$^{2+}$ release provided by the Bergmann glia processes. We predicted that the Ca$^{2+}$ waves produced by the Bergmann glia would locally activate the CaSR expressed on GCPs, causing intracellular signaling cascades that would result in integrin inside-out signaling and subsequent cell migration. We predicted that the subsequent CaSR-mediated rise in intracellular Ca$^{2+}$, or CaSR mediated promotion of ERK and AKT phosphorylation would result in integrin inside-out activation.

The primary objectives were to determine whether the CaSR and β1 containing integrins were present in a macromolecular complex in the cerebellar GCPs, and whether stimulation of the CaSR increased GCP migration towards an ECM gradient in vitro and cell homing in vivo. To establish the molecular mechanisms involved in CaSR mediated GCP migration, integrin cell surface expression analysis, and potential biochemical pathways involved in CaSR-integrin mediated cell migration were studied using specific inhibitors of intracellular signaling pathways. To determine whether CaSR modulation controlled GCP homing in vivo, allosteric modulators of the CaSR were injected into the CSF of developing rats and changes in GCP migration were analyzed.
CHAPTER 2. Materials and Methods

2.1 Reagents

The following compounds were purchased from Tocris Bioscience: L-glutamic acid (#0218), L-quisqualic acid (#0188), DHPG (#0342), ACPD (#0284), CHPG (#1049), Fenobam (#2386), LY 341495 (#1209), (S)-4-Carboxyphenylglycine (#0232), JNJ 16259685 (#2333), YM 298198 hydrochloride (#2448), CDPPB (#3235), NPS 2143 hydrochloride (#3626), and NPS R-568 hydrochloride (#3815). The CaSR negative modulator NPS 89636, CaSR positive modulator NPS R-467 and its control less potent isomer NPS S-467 were kindly provided by Dr. Edward F. Nemeth (NPS). MPEP was donated by Michael Trafaglia and FRAXA Research Foundation (synthesized by Technically Inc., Woburn MA). The rat and human CaSR cDNAs were kindly provided by Dr. A.D. Conigrave. The Flag-mgl2 cDNA was provided by Drs. Andrew Burns and Peter J. Roy. All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA, USA), and all other chemicals were procured from Sigma unless otherwise stated.

2.2 Animals

All animal procedures were performed on Sprague-Dawley rats (Charles River Laboratories; males and females combined) in accordance with the national regulatory standards set forth by the Canadian Council on Animal Care and the University of Toronto Animal Care Committee.

2.3 Sequence alignment and phylogenetic analyses

The protein sequences of rat CaSR, rat mGluRs, and C. elegans mgl receptors were obtained from the National Centre for Biotechnology Information database. Full length multiple sequence alignments and the corresponding phylogenetic tree-diagrams were generated using ClustalW. To construct the ligand binding domain alignments, highly variable short segments of the N-
termini sequences (including the signal peptide) and the segments encompassing the cysteine-rich, transmembrane and cytoplasmic domains were excluded. Gly43 to Gly498 of mGluR1 was used as the reference sequence to obtain the ligand binding residues of the corresponding proteins. The following residues were used to obtain ligand binding domain alignments: mGluR1 (43 – 498), mGluR5 (33 – 484), rCaSR (30 – 509), mgl-1 (84 – 585), mgl-2 (35 – 478), and mgl-3 (43 – 515). To produce transmembrane domain alignments, mGluR1 transmembrane domain residues (Ser593 to Ala840) were used as the reference sequence. The following residues were used to construct the transmembrane domain alignments: mGluR1 (593 – 840), mGluR5 (579 - 826), rCaSR (613 - 862), mgl-1 (682 - 929), mgl-2 (578 - 826), and mgl-3 (614 - 861).

2.4  Cell culture

HEK-293-MSR and rMTC 44-2 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% glutamax, and 15% heat inactivated horse serum and 1.4 % glutamax respectively. Cells were maintained at 5% CO₂ at 37°C.

2.5  Primary cultures of GCPs

Primary GCPs were purified from postnatal day (PND) 8 – 10 rat cerebella using a two-step Percoll gradient (35%, 60%) separation method as previously described (Hatten, 1985). Briefly, 4 – 12 rat cerebella were isolated and the meninges were removed in ice-cold dissection solution (Hanks Balanced Salt Solution – HBSS, 2.5 mM HEPES, 35 mM glucose, 4 mM sodium bicarbonate, pH 7.4). The cerebella were washed three times in dissection solution and dissociated into single cell suspension by incubating the tissue in 1% trypsin with 0.1% DNase I (Roche) for 15 min. After centrifugation, the cell pellet was tritutated with HBSS, and the cell suspension was gently layered on top of a two-step Percoll gradient (35%, 60%). After
centrifugation at 1800 X g for 10 min, the astroglia were found between the HBSS and 35% Percoll interface, while the GCPs were located between the 35% and 60% Percoll interface. The GCP fraction was recovered, washed, and resuspended in complete media (Basal Medium Eagle supplemented with 10% horse serum, 5% fetal bovine serum, 2mM L-glutamine, 250 μM KCl, and 0.9% glucose). To remove astroglia contamination, the GCP fraction was pre-plated on 100 mm tissue culture dishes coated with poly-D-lysine (25 μg/ml) three times for 20 min each. After each preplating step, loosely adhered GCPs were lifted off by gentle agitation, while the heavier glia adhered to the plates. Following three rounds of preplating, the purified GCPs were collected, centrifuged, and plated on 500 μg/ml poly-D-lysine coated plates. In some assays, the GCPs were plated on 30 μg/ml laminin coated plates. GCPs were grown in 5% CO₂ at 37°C.

2.6 Transient transfections and intracellular Ca²⁺ measurements

80 to 90% confluent HEK-293-MSR cells grown on 6 well-plates were transiently transfected with cDNA plasmids at 2 μg/well using Lipofectamine2000 as described by the manufacturers. 20 h post-transfection, the cells were trypsinized and plated on poly-L-ornithine treated 96-well microtiter plates (Costar #3603) at 100,000 cells/well in normal culture media. In addition, untransfected rMTC 44-2 cells or PND 8 – 10 purified GCPs were plated on 96-well microtiter plates at 150,000 cells/well. After 24 h in-culture, the cells were equilibrated with Ca²⁺ assay buffer (20 mM HEPES, 146 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1mg/ml bovine serum albumin, 0.5 mM Ca²⁺ and 1 mg/ml glucose; pH 7.4), loaded with 6 μM Ca²⁺ sensitive fluorescent dye Fluo-4 AM (Invitrogen, #F14201) and incubated in the dark for 1 h at room temperature. The cells were gently washed three times and incubated with 150 μl assay buffer for 30 min at room temperature in the dark. Fluorescence measurements (R.F.U.) were recorded
at room temperature on a FlexStation scanning fluorometer (Molecular Devices Inc.) at 485 nm excitation and 525 nm emission (515 nm cut-off).

For agonist stimulation studies, baseline recordings were measured for 20 seconds followed by addition of buffer (control) or drugs for 2 to 5 minutes. For antagonist studies, the cells were incubated with the inhibitors for 5 min, followed by stimulation of the receptors for 2 minutes. For allosteric potentiation studies, the cells were incubated with the modulators for 5 min, and the cells were activated with an agonist concentration corresponding to the EC$_{20}$ or EC$_{50}$ of the receptor analyzed. GraphPad Prism was used to plot the R.F.U. measurements and calculate the EC$_{50}$ and Hill slope values. All data represented correspond to means ± standard error of means (S.E.M.) from representative experiments performed in triplicate.

Concentration-response curves were generated by analyzing the data via a non-linear regression method (GraphPad Prism). Individual experiments were standardized by normalizing the data to the maximum and minimum values identified by the fitted curves. The normalized data from several experiments were used to generate the final concentration-response curves.

2.7 cAMP assay

cAMP measurements were determined using Perkin-Elmer’s Alphascreen cAMP functional assay kit, based on the competition between endogenous cAMP and exogenously added biotinylated cAMP. Briefly, hCaSR transiently transfected HEK-293-MSR cells were plated on poly-L-ornithine treated 96-well microtiter plates at 80,000 cells/well in normal culture media. After 24 h in-culture, the cells were equilibrated with cAMP assay buffer (20 mM HEPES, 146 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1mg/ml bovine serum albumin, and 1 mg/ml glucose; pH 7.4) containing 0.2 mM Ca$^{2+}$, and incubated with 1 mM IBMX phosphodiesterase inhibitor for 15 min at room temperature. The cells were washed twice, and stimulated with various
concentrations of Ca$^{2+}$ dissolved in cAMP assay buffer and incubated at 37$^0$C, 5% CO$_2$. After 5 min, 5 µM forskolin was added to each well (to induce adenylyl cyclase mediated cAMP accumulation) and incubated for 20 min at 37$^0$C, 5% CO$_2$. The media was subsequently removed, and 150 µl mixture of cAMP assay buffer and lysis buffer (3% Tween-20, 5 mM HEPES, and 0.1% bovine serum albumin, pH 7.4) were added to each well (1:1 ratio). 20 µL from each well was transferred to a 384-well microtiter plate (Costar). 5 µL of Alphascreen acceptor beads were added to each well for 30 min at room temperature in the dark, followed by addition of 5 µl mixture of Alphascreen donor beads combined with biotinylated cAMP at room temperature in dark overnight. The 384-well plates were analyzed on an Alphascreen microplate reader (Perkin Elmer), with excitation set to 480 – 485 nm, and emission set to 520 – 535 nm. Concentration-response curve was generated using GraphPad by representing 5 µM forskolin stimulation with 0.2 mM Ca$^{2+}$ inhibition as maximal response. Each data point denotes mean ± standard error of four independent experiments, determined by normalizing each point to the Alphascreen signal obtained with 5 µM forskolin + 0.2 mM Ca$^{2+}$ stimulation.

2.8 Western blotting

The cerebella isolated from rats of various ages from PND 3 to adult (8-12 weeks PND) were rapidly homogenized using a glass/teflon homogenizer at 4$^0$C in 50 mM Tris-HCl supplemented with 1% sodium dodecylsulfate (SDS, w/v) and protease inhibitor cocktail (Roche). For analysis on cultured cells, cell pellets were resuspended in 0.1 M PBS containing protease inhibitor cocktail. The solubilized tissue samples and resuspended cell pellets were analyzed using the QuantiPro BCA Assay Kit (Sigma) to determine protein concentrations, and were diluted in electrophoresis buffer containing 2% SDS, 62.5 mM Tris, 10% glycerol, and 100 mM dithiothreitol to give a final protein concentration of 1 µg/ml. Equal amounts of protein (5 – 20
μg) were separated on 6% to 12% polyacrylamide-SDS gels and electrotransferred onto nitrocellulose (Pall) using a semi-dry transfer system (Bio-Rad). The membranes were blocked in 5% milk for 1 h and incubated overnight at 4°C with the following primary antibodies: extracellular epitope binding ADD mouse anti-CaSR (1:500; Thermo Fisher Scientific, #MA1-934), rabbit anti-β1 integrin (1:500; Millipore, #AB1952); rabbit anti-α6 integrin (1:500; Cell Signaling, #3750S), mouse anti-α3 ATPase (1:1000; Thermo Fisher Scientific, #MA3-915), mouse anti-IP3 receptor (1:1000; Neuromab, #75-035) and mouse anti-GAPDH (1:40,000 – 100,000; Sigma, #G3895). The blots were washed and incubated with horseradish-peroxidise conjugated secondary goat anti-mouse or goat anti-rabbit antibodies (Jackson Immuno-Research Labs). Immunoreactive bands were detected with Pierce West Pico ECL chemiluminescent detection kit and visualized using a FluorChem multi-image light cabinet (Alpha Innotech). Densitometry analysis of band intensities was performed using the AlphaEase FC software (Alpha Innotech). To account for loading errors, the band intensity of the target proteins were normalized to the corresponding band intensity for GAPDH. Cerebellar protein expression of different time points was presented as a percentage of adult expression. For integrin analysis, the cerebella were homogenized in 50 mM Tris-HCl in the absence of SDS, and subjected to membrane preparation followed by solubilization in 1% TX-100. Solubilized samples were prepared for electrophoresis under non-reducing conditions (absence of dithiothreitol in the electrophoresis buffer). For protein deglycosylation, PNGase F was obtained from New England Biolabs and used according to the manufactures instructions. Briefly, 10 μg of cell/tissue homogenate was denatured in glycoprotein denaturing buffer at 100 °C for 5 min, followed by incubation with 2 μl PNGaseF in G7 reaction buffer and 1% NP-40 for 2 h at 37 °C.
2.9 Cellular membrane preparation and protein solubilisation

All membrane preparation and immunopurification procedures were performed at 4°C in the presence of a protease inhibitor cocktail (Roche). rMTC 44-2 cells grown to 80-90% confluency on ten 150 x 25 mm dishes were isolated and lysed in 10 ml 50 mM Tris-HCl (pH 7.4) using a Polytron (setting 6, 5 seconds). For cerebellar membrane preparation, four to eight PND 10 rat cerebella were homogenized in 50 mM Tris-HCl (pH 7.4) using a glass-teflon homogenizer followed by a Polytron (setting 6, 5 seconds). The homogenized samples were centrifuged for 20 min at 48,000 X g. The supernatant was removed and the pellet was resuspended in 10 ml 50 mM Tris-HCl (pH 7.4), using a Polytron. The protein concentration of the suspension was determined using the Quanti-Pro BCA kit while the remaining suspension was centrifuged for 20 min at 48,000 X g. The supernatant was discarded and the pellet was resuspended in solubilization buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM NaH2PO4-7H2O, 10 mM CaCl2 and 1% Triton X-100 (pH 7.5) at a protein concentration of 2 mg/ml using a Polytron. The suspension was incubated at 4°C rocking for 1 h for cells and 16 h for tissue solubilisation, followed by centrifugation for 1 h at 48,000 X g. The supernatant containing the solubilized proteins were collected and immediately used for immunoprecipitation analysis or stored at -80°C for western blot analysis.

2.10 Immunoprecipitations

Solubilized tissue/cell samples were pre-cleared with 50 μL of Protein A and Protein G slurry (1:1 bead slurry; BioShop) per 1 ml aliquot and incubated for 1 h at 4°C rocking. In parallel, Protein A + G beads were blocked with 1 mg/ml bovine serum mixture for 1 h, washed and resuspended in equal volumes of wash buffer (20 mM Tris-HCl, 0.1% TX-100, 1% glycerol and 100 mM NaCl, pH 7.5). The precleared samples were centrifuged at 2,000 x g for 2 min, and the
supernatant was transferred to a new microcentrifuge tube with 1 μg of anti-CaSR ADD mouse monoclonal antibody (Thermo Fisher Scientific, # MA1-934), 2 μg of anti-CaSR 4640 rabbit polyclonal antibody, or 2 μg of anti-CaSR 4641 rabbit polyclonal antibody (kindly provided by Dr. Edward F. Nemeth; generated in two different rabbits in house at NPS Pharmaceuticals to the ADD sequence: ADDDYGRPGIEKFREEAEERDIC), 2 μg of N-term β1 integrin rat monoclonal antibody (Hybridoma Bank, #AIIB2), 2 μg of β1 integrin C-terminal rabbit polyclonal antibody (Millipore, #AB1952), or 2 μg of rabbit polyclonal GluR2/3 antibody (PharMingen, #60656N) for 1 h at 4°C rocking. 40 μL of the blocked Protein A/G slurry were added to the antibody containing solubilized samples and incubated for 16 h at 4°C rocking. The Protein A/G beads were collected by centrifugation, washed three times in wash buffer, resuspended in 50 μl of 2X sample buffer (4% SDS, 62.5 mM Tris, and 10% glycerol, pH 6.8) and 100 mM dithiothreitol, and incubated at 37°C for 15 min. The resulting eluates were subjected to SDS-PAGE and western blotting as described above. For detection of the CaSR, the western blots were probed with the mouse anti-CaSR ADD antibody (Thermo Fisher Scientific, #MA1-934), and for detection of the β1 integrin, the blots were probed with β1 integrin antibodies purchased from Cell Signaling (#4706) or Millipore (#AB1952).

2.11 Mass spectrometry – sample preparation and protein identification

The CaSR was immunopurified from rMTC 44-2 cells as described above with minor modifications. Briefly, 10 ml of the TX-100-solubilized membrane preparation was precleared with 500 μl of 50% Protein A/G slurry and incubated with 10 μg of the CaSR ADD antibody with 150 μl of Protein A/G overnight at 4°C rocking. The Protein A/G beads were collected, washed three times in wash buffer, then washed three times in 50 mM ammonium bicarbonate (pH 7.8) and eluted three times in 200 μl of ammonium hydroxide (pH 11.0) at 37°C for 15 min.
each. The combined eluates were dried in a SpeedVac (Thermo Fisher Scientific) and the proteins were resuspended in 50 mM ammonium bicarbonate, reduced in 25 mM dithiothreitol, alkylated with 100 mM iodoacetamide, and supplemented with 1 mM CaCl$_2$ prior to digestion with 1 μg of sequenced-grade trypsin (Promega, V511) at 37°C overnight. The resulting peptide mixture was loaded onto a C-12 reverse-phase nano-column packed in-house in a pressure bomb, eluted from an HPLC using a 2 h gradient of buffer A (95% acetonitrile, 0.1% trifluoroacetic acid) and buffer B (95% acetonitrile, 5% water, 0.1% trifluoroacetic acid) and subjected to electrospray ionization followed by analysis on a LTQ-XL Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific). The acquired tandem mass spectra were searched against the rat sequences obtained from National Center for Biotechnology Information using a normalized implementation of the SEQUEST algorithm running on the Sorcerer platform. The resulting peptide sequences identified by SEQUEST were filtered and assembled into protein identifications using peptide and protein prophets (ISB, Seattle).

2.12 Immunocytochemistry

Rats of various ages (PND 3 to adult) were anaesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5mg/kg), and intracardially perfused with 0.1M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA). Brains were dissected from the skull, post-fixed overnight in 4% PFA at 4°C, and cryoprotected by infiltration with 30% sucrose at 4°C overnight. A single sagittal cut was made along the midline of each brain, and the right side of the brain was embedded in optimal cutting temperature compound (Sakura Finetek) for sectioning on a cryostat (Leica). Free-floating whole-brain sagittal sections (30 μm) were rinsed in PBS and blocked for 1 h at 25°C in PBS solution containing 5% goat serum and 0.2% TX-100. Following washing, the sections were incubated with primary antibodies diluted in blocking
solution and incubated overnight at 4°C. The primary antibodies utilized include mouse anti-CaSR ADD (1:500; Thermo Fisher Scientific, #MA1-934), rabbit anti-β1 integrin (1:500; Millipore, #AB1952), rabbit anti-calbindin (1:10,000; Swant, # CB-38a) and mouse anti-BRDU (1:100; Abcam, # ab8152) antibodies. After five 10 min washes in PBS, the brain sections were incubated with DyLight 488-conjugated goat anti-mouse (1:1000; Jackson ImmunoResearch, #115-485-146) and DyLight 549-conjugated goat anti-rabbit secondary antibodies (1:1000; Jackson ImmunoResearch, #711-505-152) for 2 h at 25°C. Following extensive washes in PBS, the free-floating brain sections were mounted on glass slides with Prolong Gold Antifade (Invitrogen). For sections treated with the anti-BRDU antibody, antigen retrieval was performed. Free-floating sections were washed three times in 0.1M PBS solution containing 1%TX-100, incubated with 1 N HCl for 10 min on ice, followed by 2 N HCl treatment for 10 min at 25°C, and 20 min at 37°C. Immediately after the acid washes, the sections were treated with 0.1 M borate buffer for 15 min at 25°C. After three 10 min washes in PBS-1%TX-100 solution, the sections were blocked for 1 h at 25°C in PBS containing 5% goat serum, 1% TX-100 and 1 M glycine. The blocking buffer was removed and the sections were washed for an additional three times in PBS-1%TX-100 solution. The free-floating sections were then incubated with mouse anti-BRDU and rabbit anti-calbindin antibodies overnight at 4°C. The sections were washed, treated with secondary antibodies and mounted onto glass slides as described above.

Immunocytochemical analysis was also performed on purified GCPs and rMTC 44-2 cell cultures. Briefly, cells were plated on poly-D-lysine treated glass coverslips and grown for 2 days in normal culture media. On the day of experiment, the cells were fixed with 4% PFA for 10 min, blocked for 30 min using 5% goat serum and incubated with anti-CaSR ADD antibody (1:500; Thermo Fisher Scientific, #MA1-934) and rabbit anti-β1-integrin antibody purchased
from either Abcam (1:500; #AB59271) or Millipore (1:500; #AB1952) for 1.5 h. After four 10 min washes with PBS, the cells were incubated with DyLight 488-conjugated goat anti-mouse (1:1000; Jackson ImmunoResearch, #115-485-146) and DyLight 549-conjugated goat anti-rabbit secondary antibodies (1:1000, Jackson ImmunoResearch, #711-505-152) for 1 h. The cells were subjected to a final round of four 10 min washes and the glass cover slips were mounted onto slides using ProLong Gold Antifade (Invitrogen, #P36930) and viewed using a Zeiss LSM 510 confocal microscope equipped with a 100X oil immersion lens. Some slides were also imaged using a Nikon AR1 Confocal Laser Microscope System equipped with a 40X water and 100X oil immersion lens, driven by the Nikon Elements software package. Controls were performed in parallel by excluding primary antibodies.

2.13 ERK1/2 and AKT phosphorylation assay

Purified GCPs obtained from PND 9 rat cerebella were grown on 30 μg/ml laminin coated 24-well plates in complete media for 20 h at 37°C, 5% CO₂. On the day of the assay, confluent GCPs were serum starved for 6 h with assay buffer (20 mM HEPES, 146 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mg/ml bovine serum albumin, and 1 mg/ml glucose; pH 7.4 ) containing 1.8 mM Ca²⁺. The cells were then exposed to DMSO, 1 μM NPS R-467, 1 μM NPS S-467 or 5 mM Ca²⁺ for 2 – 5 min (ERK activation) or 10 min (AKT activation) at 37°C. For inhibitor treatment, cells were treated with 1 μM NPS 2143 for 1 h prior to stimulation. Reactions were terminated with two washes of ice-cold HBSS and the cells were lysed using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM NaF, 50 mM β-glycerophosphate, 0.25 mM Na-orthovanadate, 500nM okadaic acid, 1% TX-100, 0.5% Na-deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche). The contents of the wells were collected, solubilized for 15 min at 4°C rocking, and spun at 21,000 X g for 10 min. The supernatant was isolated and the protein
concentration was quantified using the QuantiPro BCA kit. Five to 8 μg equivalents of each protein sample were subjected to SDS-PAGE and western blotting. Anti-ERK1/2 antibody (1:2000; Cell Signaling, #4370) and anti-AKT antibody (1:1000; Cell Signaling, #9272) were used for the detection of phosphorylated ERK1/2 and AKT respectively, while anti-GAPDH antibody (1:80000; Sigma, #G3895) was used as an internal loading control.

For analysis of rMTC 44-2 cell ERK phosphorylation, confluent cells grown on 12 well plates were serum starved overnight in DMEM:F12 (1:1), and preincubated for 3 h with ERK assay buffer. The cells were then exposed to DMSO or 1 µM R-586 for 5 min at 37°C in the presence and absence of 50 μM PD 98059. Reactions were terminated with ice-cold PBS, lysed using ERK lysis buffer and analyzed for ERK stimulation as stated above.

2.14 Cell surface protein isolation

Plasma membrane biotinylation was performed using Pierce EZ-Link Sulfo-NHS-SS-Biotin cell surface protein isolation kit according to manufacturer’s instructions with slight modifications (SulfoLink; Cat. #89881, Pierce Biotechnology, Rockford, IL). Briefly, confluent rMTC 44-2 cells grown in T-75 flasks (BD Falcon) were incubated in the presence and absence of Sulfo-NHS-SS-Biotin in DMEM-F12 (1:1) at room temperature with gentle agitation. After 30 min, the reaction was quenched, and the cells were harvested, lysed and subjected to a streptavidin-agarose column. Cytoplasmic proteins unexposed to the biotinylation reaction were isolated into the “flow-through” fraction, and cell surface biotinylated proteins were eluted into the “eluate” fraction. The cytoplasmic and cell surface fractions were separated by SDS-PAGE and immunoblotted using various antibodies.

For isolation of membrane proteins from primary GCP cultures, GCPs purified from PND 9 rat cerebella were plated on 30 μg/ml laminin coated T-75 flasks (BD Falcon) at 20 x 10⁶ cells
per flask in complete media, and grown for 20 h at 37°C, 5% CO₂. Confluent GCPs were washed with fresh complete media and incubated for 8 h with DMSO control, 1 μM R-467 or 1 μM S-467 in complete media. For inhibitor studies, confluent GCPs were pretreated with various antagonists for 1 h prior to the 8 h agonist incubation. The GCPs were carefully washed with ice-cold HBSS, incubated in the presence of Sulfo-NHS-SS-Biotin in HBSS at 4°C with gentle agitation, and prepared as described above.

2.15 Cell adhesion assay

96-well flat bottom plates coated with various concentrations (0.0, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 μg/ml) of fibronectin (Sigma, #F1141) were washed and blocked with 2% filtered bovine serum albumin solution for 1 h at room temperature. rMTC 44-2 cells were dissociated, washed, resuspended in assay buffer (serum free DMEM supplemented with 20 mM HEPES and 0.1% bovine serum albumin, pH 7.4), and incubated at 37°C in a humidified 5% CO₂ incubator for 20 min to allow the cells to recover from the process of detachment. In parallel, the wells were aspirated, washed, and incubated with 50 μl of 2X DMSO, NPS R-568, or NPS 89636. For inhibition experiments, 50 μl of 2X NPS R-568 was incubated with 2X GRGDSP or 2X GRADSP peptides (EMD Biochemicals Inc.). 50 μl of cells diluted to 2.5 x 10⁵ cells/ml were plated in triplicates and incubated at 37°C. After 1 h, the cells were washed three times with warm PBS supplemented with 0.2% bovine serum albumin. For each condition, input-control wells were maintained without washes. The cells were fixed with glutaraldehyde, stained with 0.1% crystal violet, and quantified by measuring the absorbance at 590 nM in a plate reader (Perkin Elmer). Absorbance readings from blank wells were used to subtract the background binding of crystal violet to plastic. Cell adhesion was quantified by representing the background subtracted absorbance readings as percentage of input. GraphPad Prism was used to plot the %
input values and calculate the EC$_{50}$ and Hill slope values. All data represented correspond to means ± S.E.M. from representative experiments performed in triplicate.

2.16 Haptotaxis cell migration assay

The lower surface of two-chambered Boyden assay plates separated by a polyethylene-terephthalate transwell filter (8 μm pore size; BD Falcon, #353097) were coated with 30 μg/ml laminin (for GCP cultures) or 10 μg/ml fibronectin (for rMTC 44-2 cells) overnight at 4°C. The lower compartment of the transwell filters were washed with PBS, blocked with 10% bovine serum albumin, and incubated with complete culture media in the presence of 1 μM NPS R-568, 1 μM NPS R-467, 1 μM NPS S-467 or DMSO control. Purified GCPs from PND 8 – 10 rat cerebella resuspended at 200,000 cells in 200 μl of complete media, or rMTC 44-2 cells resuspended at 75,000 cells in 200 μl of culture media were added to the upper chamber. The cells were incubated at 37°C for 20 h to allow migration from upper chamber to the lower chamber. For inhibition studies, both the upper and lower chambers were incubated with 1 μM NPS 2143, 1μM NPS 89636, 500 μM GRGDSP, 500 μM GRADSP, or various other cell signaling inhibitors. The transwell filters were washed with PBS, and the cells were fixed with glutaraldehyde and stained with 0.1% crystal violet for 1 h. Non-migratory cells on the upper-side of the filters were removed using cotton swabs. GCPs that had migrated through the pores and adhered to the bottom surface of the membrane were quantified by taking 8 random images per well using an inverted phase-contrast microscope (Nikon T100) attached with a Canon digital camera. The cells were counted and expressed as % control.

2.17 Preparation of shRNA lentiviral system and virus transduction

Short hairpin (sh) interfering RNA constructs were delivered into rMTC cells by lentiviral transductions. shRNAs directed at the β1 integrin were generated by cloning the following
oligos into a second generation lentiviral packaging system (pLKO.1; Addgene) containing an internal ribosome entry site GFP sequence (generated in-house): \( \beta_1 \) integrin shRNA #1 forward – 5’CCGGGCCATTACTATGATTATCCTTCTCGAGAAGGATAATCATAGTAATGGCTTT TTG-3’; \( \beta_1 \) integrin shRNA #2 forward – 5’CCGGGCACGATGTGATGATTTAGAACTCGAGTTCTAAATCATCACATCTGTGCTTTTTTG-3’; control scrambled shRNA sequence. Lentivirus was produced by transfecting 30-40% confluent monolayers of HEK-293T cells in 10 cm plates with VSV-G (4 µg), psPAX2 (6 µg) and shRNAs cloned into pLKO.1-GFP vector (6 µg) by calcium phosphate transfection method (Ahmed et al., 2010). Media was changed 16 h post-transfection and virus subsequently collected after 24 and 48 h. Target cells were infected at 1:2 dilution of the virus in DMEM + 10% fetal bovine serum media followed by media replacement after 24 h. Efficiency of viral infection was assessed by monitoring GFP expression (typically >90%) and knockdown of target protein was verified by western blot analysis.

2.18 Rat intra-cerebroventricular drug injections and intraperitoneal BRDU labeling

PND 9 rats were anesthetized with isoflurane and immobilized on a heat pad set to 37°C. Each rat pup was placed over a fiber-optic light to help visualize the midline and transverse sutures. A 30-gauge needle connected through polyethylene-tubing to a 10 ml Hamilton syringe was inserted 3.0 mm deep perpendicular to the cranial surface, approximately 0.5 mm lateral to the sagittal suture and 2.0 mm rostral to the coronal suture (Gholizadeh et al., 2013). 5 µL unilateral injections of either DMSO control (0.2%), NPS S-467 (10 mg/ml), NPS R-467 (10 mg/ml) or NPS 2143 (10 mg/ml) dissolved in 0.9% saline solution was injected into the CSF through the right ventricle using a 10 ml Hamilton syringe controlled by an electronic syringe pump set to dispense 1 µL/min (circulation of the CSF would allow the injected drugs to
penetrate the cerebellum). Immediately after the injections, the needle was kept in place for an additional 1 min to prevent liquid backflow. The rat pups were allowed to recover in a warmed cage, and once the isoflurane had worn off, the pups were placed back with its dam. During the recovery phase from the anesthesia, the rats were administered intraperitoneal injections of 50 mg/kg 5-bromo-2’-deoxyuridine (BRDU). After 3 days, the rats were perfused and prepared for immunocytochemical analysis as described above.

Anti-calbindin was used to label Purkinje neurons and to identify the cerebellar molecular layer, and to distinguish the EGL and IGL regions of the cerebellum. The number of BDRU labelled GCPs/100μm² in each cortical layer was counted in 9 non-adjacent mid-sagittal sections on folia 6 of the primary fissure and folia 9 in the secondary fissure of each animal. 8-10 animals were used per treatment condition.

2.19 Data analysis and statistical significance

Data are reported as means ± S.E.M. and analyzed by t-test or one-way ANOVA followed by Tukey’s multiple comparison test using SPSS statistical software version 18. Statistical significance was accepted at p < 0.05 for all analyses.
CHAPTER 3. The CaSR and integrin protein complexes in medullary thyroid carcinoma cell adhesion and migration

3.1 Specific hypotheses, objectives, and rationale

We hypothesized that in medullary thyroid carcinoma cells the CaSR couples to integrins to form functional protein complexes required for tumour cell adhesion and migration. We predicted that stimulation of the CaSR would result in activation of integrins, whereby the CaSR acts as the chemoattractant nutrient sensor and uses the adhesion and migration properties of integrins to direct rMTC 44-2 cell migration.

Although the CaSR regulates the migration and homing of several cell-types, the cellular and molecular mechanisms linked to CaSR mediated migration are unknown. Despite several studies associating CaSR function to cell homing, the signaling complexes and the protein-protein interactions of the CaSR involved in cell migration have not been well characterized. Since integrins are known to mediate cell adhesion and migration, we sought to investigate whether the CaSR is in a macromolecular complex with integrins, and whether this complex is involved in CaSR-mediated cell adhesion and migration in rMTC 44-2 cells.

Interaction of integrins with the CaSR may be important for mediating cell migration because binding of chemoattractants to their specific cell surface receptors induces inside-out activation of the integrins. Activated integrins control directional cell migration by allowing the cells to adhere, spread and migrate over ECM proteins

Specific objectives:

1) The first objective was to establish and validate whether the CaSR endogenously expressed in rMTC 44-2 carcinoma cells is functionally coupled to its downstream signaling pathways. This
objective was met by using the intracellular Ca$^{2+}$ assay with stimulation of the CaSR with its endogenous ligand Ca$^{2+}$ and inhibition of this response by CaSR specific inhibitor NPS 89636.

2) The second objective was to identify the integrin subunits, and other novel CaSR signaling interacting partners associated with the endogenous CaSR expressed in rMTC 44-2 cells. This objective was met by performing immunoprecipitation of the CaSR followed by mass spectrometry (LC-MS/MS) based proteomic analysis.

3) The third objective was to determine the role of the CaSR and integrins in cell adhesion and migration. This was accomplished by conducting fibronectin-matrix based cell adhesion, cell spreading, and cell migration assays in the presence and absence of CaSR specific allosteric modulators. An additional objective was to determine whether the effects of the CaSR on cell adhesion and migration were mediated via activation of the integrins. This was addressed by performing fibronectin-matrix based cell adhesion and migration assays with CaSR stimulation in presence and absence of integrin specific peptide inhibitors and shRNA mediated knockdown of the integrins.

4) The final objective was to investigate the signaling pathways involved in CaSR/integrin complex mediated cell adhesion and migration.

3.2 Results

3.2.1 Functional activity of the CaSR in rMTC 44-2 cells

In parathyroid cells, stimulation of the CaSR activates $G_q$ and PLC and the subsequent release of intracellular $[Ca^{2+}]_i$ (Brown and MacLeod, 2001). To establish whether the CaSR endogenously expressed in rMTC 44-2 thyroid carcinoma cells is functionally coupled to this downstream signaling pathway, we stimulated the CaSR with its endogenous ligand Ca$^{2+}$, and measured $[Ca^{2+}]_i$. Upon receptor activation, a very large and robust concentration-dependent rise
in \([\text{Ca}^{2+}]_i\) was detected (Fig. 3.1A). The high level of expression of the CaSR in these cells was confirmed by western blot analysis (see below). Concentration-response analysis of the data revealed an \(EC_{50}\) value for \(\text{Ca}^{2+}\) of \(2.5 \pm 0.1\) mM and Hill slope of \(5.5 \pm 0.5\), similar to those in other cell types (Gama and Breitwieser, 1998; Hauache et al., 2000). To confirm that the extracellular \(\text{Ca}^{2+}\)-induced rise in \([\text{Ca}^{2+}]_i\) was mediated by the CaSR, the selective CaSR antagonist NPS 89636, was used to block the rise in \([\text{Ca}^{2+}]_i\) upon stimulation with extracellular \(\text{Ca}^{2+}\) (Nemeth et al., 2001). NPS 89636 exhibited a concentration-dependent inhibition of the rise in \([\text{Ca}^{2+}]_i\) induced by exposure to 5 mM \(\text{Ca}^{2+}\) with an \(IC_{50}\) value of \(271 \pm 37\) nM and Hill Slope value of \(-1.7 \pm 0.2\) (Fig. 3.1B). These results demonstrated that the CaSR is functionally coupled to the \(\text{Ca}^{2+}\) release pathway in rMTC 44-2 cells. The observation that the Hill coefficients for \(\text{Ca}^{2+}\) and NPS 89636 are greater than 1 is indicative of intrinsic receptor based cooperativity.

To further establish whether the CaSR mediated rise in \([\text{Ca}^{2+}]_i\) is mediated by release of \(\text{Ca}^{2+}\) stores or influx of \(\text{Ca}^{2+}\) from the extracellular media, rMTC 44-2 cells were exposed to various CaSR orthosteric agonists and the \(\text{Ca}^{2+}\) assay was performed in the presence (0.5 mM) and absence (0.0 mM) of extracellular \(\text{Ca}^{2+}\). In normal thyroid C-cells, activation of the CaSR stimulated \(\text{Ca}^{2+}\) entry via VGCCs, therefore verapamil was used to block VGCC dependent \(\text{Ca}^{2+}\) entry. Analysis of CaSR activation in 0.0 mM \(\text{Ca}^{2+}\) assay buffer using 5 mM \(\text{Ca}^{2+}\), 100 \(\mu\)M gadolinium (Gd\(^{3+}\)), 100 \(\mu\)M lanthium (La\(^{3+}\)), 10 mM magnesium (Mg\(^{2+}\)), or 250 \(\mu\)M neomycin resulted in robust rise in intracellular \(\text{Ca}^{2+}\) levels similar to CaSR activation in the presence of extracellular \(\text{Ca}^{2+}\) (0.5 mM \(\text{Ca}^{2+}\) assay buffer) (Fig. 3.1C). Furthermore, 100 \(\mu\)M verapamil had no effect on CaSR activation, while 3 \(\mu\)M NPS 89636 blocked the effects of \(\text{Ca}^{2+}\), Gd\(^{3+}\), La\(^{3+}\), Mg\(^{2+}\) and neomycin on CaSR mediated increases in intracellular \(\text{Ca}^{2+}\) levels (Fig. 3.1C). Therefore activation of the CaSR in rMTC 44-2 cells promotes the release of intracellular \([\text{Ca}^{2+}]_i\).
Figure 3.1. Functional expression of the CaSR in rMTC 44-2 cells.

(A) Extracellular Ca\textsuperscript{2+} induced intracellular Ca\textsuperscript{2+} mobilization in rMTC 44-2 cells. Each data point represents the mean ± S.E.M. of four independent experiments performed in triplicate. EC\textsubscript{50} = 2.5 ± 0.1 mM and Hill Slope = 5.5 ± 0.5. (B) CaSR antagonist (NPS 89636) mediated inhibition of intracellular Ca\textsuperscript{2+} mobilization upon activation of CaSR with 5 mM Ca\textsuperscript{2+}. IC\textsubscript{50} = 271 ± 37 nM and Hill Slope = -1.7 ± 0.23 (N = 2). (C) Activation of the CaSR in rMTC 44-2 cells results in release of Ca\textsuperscript{2+} from ER stores. rMTC 44-2 cells incubated in assay buffer containing 0.0 mM or 0.5 mM Ca\textsuperscript{2+} were preincubated with control, 100 μM verapamil or 3 μM NPS 89636 for 5 min, and stimulated with various CaSR orthosteric agonists. Error bars represent mean ± S.E.M. of 3 – 5 independent experiments performed in triplicate.
stores, demonstrating that the CaSR is functionally coupled to the downstream signaling proteins involved in intracellular Ca\(^{2+}\) release.

3.2.2 Identification of integrins as CaSR interacting proteins in rMTC 44-2 cells

To identify novel CaSR signaling partners, the endogenous CaSR expressed in rMTC 44-2 cell was immunoprecipitated and subjected to LC-MS/MS analysis. Activation of the CaSR was required for efficient receptor solubilisation from rMTC 44-2 cell membranes because solubilisation in buffer containing low Ca\(^{2+}\) (1.5 mM which would induce little activation of the receptor), only low amounts of receptor were present in the solubilized fraction, whereas in solubilisation buffer containing high Ca\(^{2+}\) (10 mM, sufficient for full activation), the CaSR was highly concentrated in the solubilized fraction. The most prominent group of proteins identified in the LC-MS/MS analysis from the initial CaSR immuno-affinity pull-down experiments was the integrin family of ECM binding proteins (Table 1). Members of the integrin family were among the most abundant proteins detected in terms of the total number of peptides identified, the percentage of sequence covered, and probabilities of identified peptides corresponding to the predicted proteins. Several isoforms of α and β integrins were identified in the LC-MS/MS analysis (Table 1). β1 integrin was selected for further evaluation because it forms obligate heterodimers with several α integrin subunits.

Co-immunoprecipitation experiments were carried out using the solubilized rMTC 44-2 cell membrane fraction and three anti-CaSR antibodies (ADD, 4640, and 4641), targeting the CaSR ECD, and two different β1-integrin antibodies targeting the C-terminus and N-terminus of β1 integrin. The ADD monoclonal antibody labelled a doublet on western blots; deglycosylation of the CaSR caused a shift in the relative molecular weight of the upper 130 kDa band to the lower 120 kDa band (Fig. 3.2A). The β1 integrin N-terminal antibody immunoprecipitated the fully
Table 1. List of integrins identified from CaSR immunoprecipitation and LC-MS/MS experiments.

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<tr>
<th>Protein</th>
<th>Accession #</th>
<th>Total Peptides</th>
<th>Unique Peptides</th>
<th>Probability</th>
<th>Coverage (%)</th>
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<td>1</td>
<td>0.2569</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Integrins identified by mass spectrometry analysis from immunopurified CaSR endogenously expressed in rMTC 44-2 cells. The table lists integrins identified from 3 separate experiments.
Figure 3.2. Co-immunoprecipitation (IP) of the CaSR and β1 integrin from rMTC 44-2 cells.

Samples of 1% TX-100 solubilized (Sol) rMTC 44-2 cells were immunoprecipitated using three anti-CaSR specific antibodies (ADD, 4640, or 4641) targeting the extracellular domain of the
CaSR, and two different antibodies targeting the C-terminus or N-terminus of β1 integrin. The samples were subjected to immunoblotting (IB) using the ADD anti-CaSR monoclonal antibody and anti-β1 integrin antibodies. (A) deglycosylation of the CaSR with PNGase F showing that the upper band in the doublet is the glycosylated form of the receptor while the lower band is the core glycosylated form; (B) and (C), co-immunoprecipitation of CaSR and β1 integrin using the CaSR ADD antibody, and β1 integrin specific N-terminal antibody. (D and E), co-immunoprecipitation of CaSR and β1 integrin using CaSR specific 4640 and 4641 antibodies, and β1 integrin specific C-terminal antibody. The β1 integrin N-terminal antibody recognized primarily the immature 88 kDa form of the protein (panel B) while the C-terminal β1 antibody recognized primarily the fully glycosylated 130 kDa form (panel E). (F) the mouse anti-NMDA NR1 antibody and rabbit anti-GluR2/3 antibody immunoprecipitated their respective proteins but failed to co-immunoprecipitate the CaSR. The anti-CaSR ADD antibody immunoprecipitated the CaSR but failed to co-immunoprecipitate NMDA NR1 or GluR2/3. (G) rMTC cells were solubilized using more stringent conditions (compared with TX-100 only) with either 0.5% CHAPS or 0.5% deoxycholate + 0.1% SDS. Immunoprecipitation (using the 4641 anti-CaSR antibody) resulted in immunoprecipitation of the CaSR together with the β1 integrins. The blots are representative of 2 – 3 experiments with similar results.
glycosylated form of the CaSR ($M_r = 130$ kDa, Fig. 3.2B). Conversely, both the CaSR ADD monoclonal antibody and the β1 integrin N-terminal antibody immunoprecipitated the immature 88 kDa form, and to a lesser extent, the mature 130 kDa glycosylated form of β1 integrin (Fig. 3.2C). To further substantiate this co-immunoprecipitation result, two additional CaSR antibodies (rabbit polyclonals 4640 and 4641) and a C-terminal β1 integrin polyclonal antibody (Millipore) were used. The two rabbit anti-CaSR polyclonal antibodies (4640 and 4641) immunoprecipitated the fully glycosylated and core glycosylated forms of the CaSR in roughly similar amounts (Fig. 3.2D), while immunoprecipitation with the CaSR ADD monoclonal antibody preferentially immunoprecipitated the fully glycosylated form (Fig. 3.2B). The C-terminal β1 integrin antibody also immunoprecipitated the CaSR (Fig. 3.2D) and immunoprecipitated the mature 130 kDa β1 integrin (Fig. 3.2E), and in samples immunoprecipitated with the 4640 and the 4641 CaSR polyclonal antibodies, the mature 130 kDa form of β1 integrin was also detected when the blots were probed with the anti-β1 integrin antibody (Fig. 3.2E). To exclude the possibility of non-specific binding of the CaSR and integrins to the antibody complex, mouse anti-NMDA NR1 and rabbit anti-GluR2/3 glutamate receptor antibodies were used. Both antibodies failed to co-purify the CaSR or β1 integrins (Fig. 3.2F).

To further validate the co-immunoprecipitation results, the rMTC 44-2 cells were solubilized using more stringent conditions (compared with TX-100 only) with either 0.5% CHAPS or 0.5% deoxycholate + 0.1% SDS. Both conditions resulted in solubilisation of the CaSR and β1 integrins; more importantly, immunoprecipitation using the 4641 anti-CaSR antibody resulted in immunopurification of the CaSR together with the β1 integrins (Fig. 3.2G). Taken together, the co-immunoprecipitation results obtained from multiple co-immunoprecipitation experiments
demonstrated that the CaSR and β1 integrins are present together in a macromolecular complex in rMTC 44-2 cells.

3.2.3 Co-localization and cell surface expression of CaSR and β1-integrins

The cellular distributions of the CaSR and β1 integrins in rMTC 44-2 cells were assessed in immunolocalization experiments. Cells grown on poly-D-lysine treated glass coverslips in subthreshold Ca$^{2+}$ levels (1.8 mM) were fixed and incubated with a mouse anti-CaSR monoclonal antibody (ADD) targeting an extracellular epitope, and a rabbit anti-β1-integrin antibody (Abcam), and basolateral images were obtained using a confocal microscope. Although no permeabilization reagent was used, we found that fixation of the cells alone caused permeabilization (data not shown). The anti-CaSR antibody revealed an expression pattern of discrete punctate formations throughout the cells (Fig. 3.3A, green). Visible puncta of β1 integrin complexes were also abundantly expressed throughout the cell with a distribution similar to that seen with the CaSR (Fig. 3.3A, red). In the merged images, the distribution of the CaSR overlapped extensively (but not completely) with that of β1 integrin (Fig. 3.3A, yellow). The co-localization was observed at all focal planes (basolateral and apical).

Although the co-localization images suggested that the CaSR and β1 integrins were co-distributed, the membrane locations of the staining were unclear. Therefore, I next evaluated cell surface expressions of the CaSR and β1 integrins using a biotin-based cell surface protein isolation method in which biotin-labelled cell surface proteins were immobilised on agarose columns conjugated with streptavidin. To validate this method in rMTC 44-2 cells, the cell surface expressed α3 subunit of Na$^+$/K$^+$-ATPase was used as a positive control for surface expression, while IP$_3$ receptor expression was used as a negative control to ensure intracellular specific expression, and an additional control was performed whereby the presence or absence of
Figure 3.3. Cellular localization and cell-surface expression of the CaSR and β1 integrin in rMTC 44-2 cells.

(A) cells grown in subthreshold (1.8 mM) Ca\textsuperscript{2+} were immunolabelled with extracellular epitope mouse ADD anti-CaSR antibody (1:500) and a rabbit anti-β1-integrin antibody (1:400), and viewed using a confocal microscope (100X). CaSR expression was present in discrete puncta distributed throughout the cell (green). β1-integrin was also expressed throughout the cell (red) and showed extensive overlap with the CaSR expression (yellow). Photomicrographs are representative of similar results obtained in three experiments. (B) Cell surface proteins were covalently biotinylated and subjected to streptavidin-agarose chromatography. Cytoplasmic proteins appeared in the “Flow Through” fraction whereas cell surface proteins were eluted in the “Eluate”. Immunoblot analysis showed that the 150 kDa mature form of the CaSR was recovered in the cell surface fraction as expected, whereas the 130 kDa high mannose immature form was recovered in the ‘flow-through’ (N = 3). (C) Summary of biotinylation analysis. β1-integrin and α4 integrin as well as the α3 subunit of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (positive control for plasma membrane marker) were found on cell surface (✓), whereas β3 integrin and IP\textsubscript{3} receptor (intracellular membrane marker) were found in the intracellular fractions (x).
biotin was used to monitor non-specific protein binding. Using this method, the CaSR, β1 integrins and α4 integrins were all recovered in the cell surface fraction as identified by the presence of the α3 subunit of Na\(^+/K^+\)-ATPase, whereas β3 integrins were expressed exclusively in the intracellular fraction as identified by the presence of the IP\(_3\) receptor (Fig. 3.3B, C). Some cell surface proteins (CaSR, α3 subunit of Na\(^+/K^+\)-ATPase) were also detected at low levels in the intracellular fraction. However, the mature 150 kDa band of the CaSR appeared in the cell surface fraction, whereas the immature high mannose 130 kDa band appeared in the intracellular fraction (Fig. 3.3B). Therefore, the biotin-streptavidin affinity chromatography analysis supported the \([Ca^{2+}]_i\) release and immunolocalization studies described above by establishing that both the CaSR and β1 integrins are expressed on the cell surface.

### 3.2.4 Potentiation of fibronectin-mediated cell adhesion by CaSR positive allosteric modulator (NPS R-568)

Evidence from LC-MS/MS analysis, co-immunoprecipitation, and co-localization studies revealed that the CaSR and β1-containing integrins appeared to be components of a macromolecular protein complex. β1-containing integrin heterodimers bind to the ECM protein fibronectin. Therefore, fibronectin-matrix based adhesion assays were conducted to determine the effects of CaSR stimulation on integrin-mediated cell adhesion. Since integrins are affected by changes in divalent cation concentration including Ca\(^{2+}\) and Mg\(^{2+}\) (Mould et al., 1995; Vorup-Jensen et al., 2007; Puklin-Faucher and Vogel, 2009), we obviated the use of cations and instead used a CaSR-specific positive allosteric modulator (NPS R-568) in the presence of a submaximal concentration of extracellular Ca\(^{2+}\) (1.8 mM). Fibronectin was coated onto 96 well plates at various concentrations (0 – 15 µg/ml) and equal numbers of cells were then plated in the presence of 0.02% DMSO (vehicle control), 6 µM NPS R-568 and/or 6 µM NPS R-89636. Maximum cell adhesion levels were observed at fibronectin concentrations above 5 µg/ml. NPS
R-568 potentiated the effects of fibronectin-mediated cell adhesion (Fig. 3.4A); compared to control, 6 µM NPS R-568 significantly increased cell adhesion at fibronectin concentrations above 5 µg/ml, with the greatest impact observed at 7.5 µg/ml (166.1 ± 10.2% compared to control). Unlike NPS R-568, NPS 89636 alone had no significant effect on cell adhesion (Fig. 3.4A).

The concentration dependence of NPS R-568 stimulated cell adhesion was investigated at 1 and 2.5 µg/ml fibronectin, corresponding to the EC_{20} and EC_{40} values from fibronectin concentration-response experiments (Fig. 3.4B, C). Under these conditions, NPS R-568 stimulated cell adhesion in a concentration-dependent manner, with an EC_{50} value of 111 ± 29 nM and Hill Slope of 1.9 ± 0.8 at 1 µg/ml fibronectin (Fig. 3.4B) and an EC_{50} value of 38 ± 12 nM and Hill Slope of 1.6 ± 0.5 at 2.5 µg/ml fibronectin (Fig. 3.4C). Simultaneous addition of NPS R-568 and NPS 89636 abolished the effects of NPS R-568 alone (Fig. 3.4B, C). The DMSO vehicle control did not promote cell adhesion at either 1 or 2.5 µg/ml fibronectin. Thus, the CaSR positive allosteric modulator NPS R-568 potently stimulated rMTC 44-2 cell adhesion in the presence of fibronectin.

3.2.5 Inhibition of NPS R-568-mediated potentiation of cell adhesion by GRGDSP peptide

The GRGDSP peptide competes with fibronectin for integrin binding and serves as an inhibitor of integrin-mediated cell adhesion on RGD-containing ECM proteins such as fibronectin (Humphries, 2000). To investigate whether the effects of NPS R-568 on cell adhesion were dependent on integrin binding to the fibronectin-matrix, cell-adhesion was measured in the absence or presence of various concentrations of GRGDSP peptide and a single concentration of NPS R-568 (300 nM); as above cells were plated on either 1 or 2.5 µg/ml of the fibronectin-matrix. A related peptide GRADSP, which exhibits markedly reduced peptide
Figure 3.4. The CaSR positive allosteric modulator NPS R-568 potentiated fibronectin mediated cell adhesion.

(A) rMTC 44-2 cells were incubated on fibronectin-coated wells (0 – 15 μg/ml) in the absence or presence of 0.02% DMSO (control), 6 μM NPS R-568 or 6 μM NPS 89636. Treatment of rMTC 44-2 cells with 6 μM NPS R-568 significantly increased cell adhesion over control levels, whereas 6 μM of the CaSR antagonist NPS 89636 did not significantly affect cell adhesion. Data points were normalized to maximal cell adhesion level obtained under the control condition (i.e. in the absence of NPS R-568 or NPS 89636) (N = 5). *, p<0.05. (B) NPS R-568 increased cell adhesion in a concentration-dependent manner. rMTC 44-2 cells were incubated on 1 μg/ml fibronectin in the presence of various concentrations of NPS R-568 (1 nM to 10 μM) or DMSO control (3x10⁻⁶ – 3x10⁻³%). The data points (mean ± S.E.M.) were normalized by standardizing basal cell adhesion levels to 0% and maximal adhesion levels to 100% by analyzing the NPS R-568 treated data using GraphPad software. At 1 μg/ml fibronectin, NPS R-568 stimulated cell adhesion in a dose-dependent manner with an EC⁵₀ = 111 ± 29 nM and Hill slope = 1.9 ± 0.8 (N = 7). (C) At 2.5 μg/ml fibronectin, NPS R-568 displayed a more potent dose-dependent stimulation on cell adhesion, with EC⁵₀ at 38 ± 12 nM and Hill slope at 1.6 ± 0.5 (N = 7).
binding affinity (Pierschbacher and Ruoslahti, 1984; Lin et al., 2003) was used as a negative control.

At 1 µg/ml fibronectin, GRGDSP at concentrations at and above 50 µM reduced the stimulatory effect of 300 nM NPS R-568 on cell adhesion in a concentration-dependent manner (Fig. 3.5A). The peptide induced a statistically significant decrease in cell adhesion at concentrations at and above 100 µM compared to the GRADSP control peptide. Thus, 100, 200, and 500 µM of GRGDSP suppressed cell adhesion by 70.1 ± 2.1%, 73.8 ± 4.8%, and 79.8 ± 1.3% compared to control no-peptide treatments (p < 0.05). At 2.5 µg/ml fibronectin, the GRGDSP peptide inhibited adhesion at concentrations above 100 µM with 500 µM GRGDSP inhibiting 300 nM NPS R-568 mediated cell adhesion by 49.8 ± 6.3% (n = 4; p < 0.05, Fig. 3.5B). Therefore, GRGDSP inhibited NPS R-568-mediated potentiation of cell adhesion in a concentration-dependent manner. The results demonstrate that CaSR activation potentiates cell adhesion by promoting integrin binding to a fibronectin-rich matrix, at least in part by the RGD peptide sequence present in fibronectin.

3.2.6 CaSR stimulation increases cell spreading in rMTC 44-2 cells

Integrin mediated cell adhesion on ECM proteins results in cell spreading. Cell spreading is an important step in integrin mediated cell migration as it allows for the establishment of focal adhesion contacts, actin polymerization and microtubule elongation (Fig. 1.9). To determine whether stimulation of the CaSR results in increased cell spreading, rMTC 44-2 cells were incubated on 1 µg/ml fibronectin-coated glass coverslips for 1 h in the absence or presence of NPS R-568. Treatment of rMTC 44-2 cells with 1 µM NPS R-568 significantly increased cell spreading over fibronectin by 245 ± 27% compared to control (Fig. 3.6A, B). Immunolocalization analysis revealed that control and NPS R-568 treated cells demonstrated β1-
Figure 3.5. GRGDSP peptide inhibited NPS R-568-mediated cell adhesion.

rMTC 44-2 cells were incubated on 1 μg/ml fibronectin or 2.5 μg/ml fibronectin in the presence of 300 nM R-568 with varying concentrations of the integrin inhibiting peptide, GRGDSP, or a control peptide, GRADSP, that does not inhibit integrins. (A) At 1 μg/ml fibronectin, GRGDSP peptide significantly inhibited NPS R-568 mediated cell adhesion at peptide concentrations greater than 100 μM compared to the control GRADSP peptide (N = 2 – 4). (B) At 2.5 μg/ml fibronectin, 500 μM GRGDSP peptide was required to significantly reduce the potentiation effects of 300 nM NPS R-568 on cell adhesion compared to GRADSP peptide (N = 4). The control GRADSP peptide showed a small non-significant degree of inhibition on cell adhesion at all concentrations. Data points represent the mean ± S.E.M. normalized to cell adhesion levels obtained by stimulation with 300 nM NPS R-568 in the absence of peptides. *, p<0.05.
rMTC 44-2 cells were incubated on 1μg/ml fibronectin-coated glass coverslips for 1 h in the absence or presence of DMSO control or 1 μM NPS R-568. Cells were fixed, immunolabelled with ADD anti-CaSR antibody (1:500) and a rabbit anti-β1-integrin antibody (1:400), and viewed using a confocal microscope. (A and B) Treatment of rMTC 44-2 cells with 1 μM NPS R-568 significantly increased cell spreading over fibronectin by 2.5 fold compared to control. Both control and NPS R-568 treated cells demonstrated β1-integrin expression throughout the cell (red) and showed extensive overlap with the CaSR expression (yellow). Photomicrographs are representative of results obtained in three similar experiments. Each data point represents the mean ± S.E.M of 3 independent experiments, with 50 cells analyzed per experiment. *, p<0.05.
integrin expression throughout the cell (red) and showed extensive overlap with the CaSR expression (yellow) (Fig. 3.6A). Therefore, the CaSR positive allosteric modulator increased rMTC 44-2 cell spreading on fibronectin, and the cellular distribution analysis illustrated that the CaSR and β1 integrin maintained colocalization upon cell spreading.

3.2.7 NPS R-568 potentiates fibronectin-mediated cell migration via integrin activation

Human forms of medullary thyroid carcinoma are highly metastatic (Modigliani et al., 1998; Moley and DeBenedetti, 1999). The role of the CaSR-integrin protein complex in cell migration was further investigated in Boyden chamber experiments. rMTC cells did not exhibit chemotaxis in response to various stimuli, including fetal bovine serum, horse serum, or 1 µM NPS R-568. However, rMTC cells displayed directional chemotaxis in response to the ECM protein fibronectin. rMTC cells migrated towards fibronectin-matrix treated filters whereas no migration was observed in bovine serum-treated filters and 1 µM NPS R-568 significantly potentiated fibronectin-dependent cell migration by 218 ± 15% compared to a vehicle control (Fig. 3.7). Furthermore, the potentiating effect of 1 µM NPS R-568 on cell migration was eliminated by 500 µM GRGDSP peptide or 1 µM of the CaSR antagonist NPS 89636, with 98 ± 17% and 77 ± 15% cell migration respectively (P < 0.005). NPS 89636 (1 µM) had no effect on cell migration in the absence of NPS R-568 and the negative control peptide GRADSP did not reverse the positive effect of 1 µM NPS R-568 (Fig. 3.7).

To further validate the role of β1 integrins in NPS R-568 mediated potentiation of cell adhesion and migration, protein expression levels of β1 integrin in rMTC 44-2 cells were knocked-down by a shRNA-lentiviral based system. Robust protein knock down was observed
Figure 3.7. NPS R-568 increased fibronectin mediated cell migration via integrin activation.

rMTC 44-2 cells added to the upper surface of transwell filters migrated to the lower chamber coated with 10 μg/ml fibronectin. 1 μM NPS R-568 induced a 2-fold increase in cell migration toward the fibronectin matrix as compared to control conditions. The effects of NPS R-568 on cell migration were inhibited in the presence of 500 μM GRGDSP or 1 μM NPS 89636, but not by 500 μM of the GRADSP control peptide. 1 μM NPS 89636 treatment alone did not inhibit cell migration. Each data point represents the mean ± S.E.M of 3 – 6 independent experiments. *, p<0.005.
using β1 integrin shRNA sequence #2, whereas shRNA sequence #1 showed β1 integrin expression similar to that of the control shRNA (scrambled) condition (Fig. 3.8A). β1 integrin shRNA #2 inhibited NPS R-568 stimulated cell adhesion and migration on fibronectin, while scrambled and shRNA #1 treatment did not inhibit CaSR mediated cell adhesion and migration (Fig. 3.8B, C). These data further demonstrate that CaSR-stimulated cell migration towards a fibronectin matrix is mediated by β1 integrins.

3.2.8 The effects of intracellular signaling inhibitors on CaSR-mediated cell adhesion and migration in rMTC 44-2 cells

CaSR enhancement of cell adhesion and migration could occur through CaSR mediated intracellular second messenger signaling mechanisms. The CaSR couples to several G-protein-dependent signaling systems such as $G_q\alpha$-mediated activation of PLC and $Ca^{2+}_{i}$ release, $G_i\alpha$-mediated reduction in cyclic AMP production, $G_{12/13}\alpha$ induced Rho kinase activation, as well as phosphorylation of MAPK/ERK (Brown and MacLeod, 2001; Huang et al., 2004a; Davies et al., 2006; Conigrave and Hampson, 2010; Magno et al., 2010). These intracellular signaling mechanisms could induce inside-out signaling via an integrin-associated protein to facilitate the binding of integrins to ECM proteins.

To investigate potential biochemical pathways involved in CaSR-mediated cell adhesion and migration, inhibitors of intracellular signaling pathways were tested. It was anticipated that inhibition of the signaling pathway(s) involved in the CaSR-mediated effects would reduce or block CaSR promotion of cell adhesion and migration. Inhibition of $G_i\alpha$, PKC, and voltage-gated $Ca^{2+}$ channels using pertussis toxin (100 and 200 ng/ml), chelerythrine (5 µM), and verapamil (100 µM) respectively did not significantly affect NPS R-568 potentiation of cell adhesion or migration (Fig. 3.9). In contrast, inhibition of PLC, ERK, and Rho-associated
Figure 3.8. shRNA-mediated knock down of β1 integrins reduces CaSR-stimulated cell migration.

Cells were treated with one of three shRNA constructs: a scrambled control and two β1 integrins shRNAs (#1 and #2). (A) western blot probed with anti-β1 integrin antibody and anti-GAPDH antibody (loading control). (B) the effects of shRNA on cell adhesion (1 μg/ml fibronectin), (C) the effects of shRNA on cell migration (10 μg/ml fibronectin). In B and C, each column is the average ± S.E.M of 2 – 3 experiments. *P < 0.05.
Figure 3.9. Intracellular signaling inhibitors that did not block CaSR-mediated cell adhesion and migration.

(A) and (B), the effects of Giα inhibitor pertussis toxin (100 ng/ml and 200 ng/ml) on NPS R-568 mediated potentiation of cell adhesion and cell migration. (C) and (D), the effects of protein kinase C inhibitor chelerythrine (5 μM) on NPS R-568 enhancement of cell adhesion and migration. (E) the effect of voltage gated Ca^{2+} channel blocker verapamil (100 μM) on NPS R-568 mediated increase in cell adhesion. Each bar represents the mean ± S.E.M. of 3 – 4 independent experiments.
kinase (ROCK) affected CaSR-mediated adhesion and/or migration. The PLC inhibitor U73122 had no significant effect at 1 µM but significantly reduced NPS R-568 stimulation of [Ca^{2+}]_i release, and blocked both NPS R-568 potentiated cell adhesion and migration at 3 µM (Fig. 3.10A – C). Similarly, chelation of [Ca^{2+}]_i with BAPTA-AM significantly reduced the NPS R-568-induced rise in [Ca^{2+}]_i and blocked NPS R-568 potentiation of cell adhesion and migration (Fig. 3.10D-F).

Inhibition of ERK using PD 98059 (30 µM) reduced ERK activation (Fig. 3.10G) but had no effect on basal or NPS R-568 stimulated cell adhesion (Fig. 3.10H). The ROCK inhibitor Y27632 (30 µM) also had no effect on NPS R-568-mediated cell adhesion (Fig. 3.10J). Surprisingly, PD 98059 and Y27632 strongly potentiated the positive effect of NPS R-568 potentiation on cell migration (Fig. 3.10I, K).

In summary, the results of the signal transduction analyses indicated that in rMTC 44-2 cells, CaSR stimulation of PLC and the subsequent rise in [Ca^{2+}]_i is required for the positive CaSR effects on cell adhesion and migration.

3.3 Summary, discussion, and conclusions

3.3.1 Summary

Cell adhesion and migration are crucial events during organogenesis and abnormalities in cell movement and adhesion play an essential role in tumour growth and cancer cell metastasis. The CaSR has been previously implicated in the regulation of cell migration in the developing nervous system and in cancer metastasis (Vizard et al., 2008; Saidak et al., 2009a). Here, we identified the integrins as key protein interaction partners with the functionally active CaSR endogenously expressed in rat medullary thyroid carcinoma cells.
Figure 3.10. The effects of intracellular signaling inhibitors on Ca\(^{2+}\) release, ERK activation, and CaSR-mediated cell adhesion and migration.

(A) the effects of the PLC inhibitor U73122 and the inactive isomer U73343 on CaSR-induced increase in [Ca\(^{2+}\)], (B) the effects of U73122 on NPS R-568 potentiation of cell adhesion, (C) the effects of U73122 on cell migration, (D) BAPTA inhibition of [Ca\(^{2+}\)] release, (E) the effects of BAPTA on NPS R-568 enhancement of cell adhesion, (F) BAPTA inhibition of the NPS R-568-mediated potentiation of cell migration, (G) the effects of the ERK inhibitor PD 98059 on ERK activation, (H) the effects of PD 98059 on cell adhesion, (I) PD 98059 enhancement of NPS R-568 potentiation of cell migration, (J) the ROCK inhibitor Y-27632 had no effect on cell adhesion, (K) the effects of Y-27632 on cell migration. Each bar represents the mean ± S.E.M of 3 - 5 independent experiments. *P < 0.05.
The identification of the integrins as CaSR interacting proteins was initially determined from CaSR immuno-affinity pull-down experiments in rMTC 44-2 cells. Further confirmation of the CaSR-integrin interaction was provided by extensive co-immunoprecipitation experiments and cellular co-localization analysis. Pharmacological stimulation of the CaSR using the positive allosteric modulator NPS R-568 potently enhanced cell adhesion and migration. Evidence that these effects occurred via the integrins was provided by the demonstration that the enhancement of cell-ECM adhesion by NPS R-568 could be largely blocked by the addition of GRGDSP integrin blocking peptide, but not by a control peptide (GRADSP) that does not compete with fibronectin for β1-integrin binding. Further confirmation of the role of integrins was obtained in knock down experiments where shRNA-mediated knock down of β1-containing integrins reduced CaSR-mediated cell adhesion and migration. We conclude that the CaSR is associated with β1-containing integrins in a macromolecular protein complex and that stimulation of the CaSR promotes cell adhesion and migration via integrin activation.

3.3.2 Potential mechanisms

At the molecular level, a potential mechanism for functional cooperation between the CaSR and integrins would include a direct protein-protein interaction between the CaSR homodimer and the integrin heterodimers as part of a protein-protein tetramer. For this scenario we propose a model whereby an agonist-induced conformational change in the CaSR induces a shift of the integrin heterodimer into an active conformation (Fig. 3.11). Both classes of proteins possess large extracellular domains and although no direct studies examining conformation changes in the CaSR have been reported, other Family C GPCRs such as the GABA\textsubscript{B} and the mGluRs are known to undergo closure of their extracellular VFT domains upon agonist binding (Tsuchiya et al., 2002; Hampson et al., 2008; Pin et al., 2009). A Ca\textsuperscript{2+}-induced stimulation of the CaSR that
Figure 3.11. Proposed mechanistic scheme for CaSR/integrin interactions.

The figure depicts one possible scenario where the CaSR may be associated with integrin heterodimers in a signaling complex. However, the actual protein-protein interaction may be indirect and involve additional proteins. Left, the complex prior to CaSR stimulation; right, elevated extracellular cation concentrations activate the CaSR causing its Venus flytrap domain to undergo a conformational change. This protein domain movement together with increased $[\text{Ca}^{2+}]_i$, from $G_\alpha$ activation of PLC and the IP$_3$ receptor, could induce the integrin to flip it into an activate state promoting binding to the ECM resulting in enhanced cell adhesion and migration. The “X” in the right hand panel denotes a hypothetical protein that may participate in facilitating the CaSR-mediated activation of integrins subsequent to increased $[\text{Ca}^{2+}]_i$. 
drives integrins into the active conformation could promote binding to ECM proteins, thereby transmitting conformational changes to other downstream proteins and adjacent cells. Phosphorylation of focal adhesion kinase and subsequent activation of Src kinase is one such pathway by which integrins mediate outside-in signaling (Mitra et al., 2005; Mitra and Schlaepfer, 2006). These kinases form the backbone of the adhesion and migration complex, and the observation that CaSR can activate Src (McNeil et al., 1998) suggests a possible link between this tyrosine kinase, the CaSR, and integrins in the context of cell adherence.

Alternatively, the CaSR homodimer and integrin heterodimer could functionally associate indirectly as part of a macromolecular complex containing additional signaling proteins. A calcium-induced conformational change may be followed by a second stage that involves the activation of second messenger-mediated pathways that ultimately mediate inside-out activation of the integrins. The results of experiments using inhibitors of intracellular signaling systems demonstrated that activation of PLC participates in CaSR-mediated cell adhesion and migration. The PLC inhibitor U73122 and Ca\(^{2+}\) chelator BAPTA were the only signaling pathway inhibitors tested that reduced or blocked the CaSR promotion of cell adhesion and migration. Previous studies have shown that elevated [Ca\(^{2+}\)], including Ca\(^{2+}\) contributed by the PLC/IP\(_3\)/IP\(_3\) receptor pathway, increases cell adhesion and migration (Sjaastad et al., 1996; Valeyev et al., 2006). Ca\(^{2+}\) imaging experiments have shown that increased [Ca\(^{2+}\)]\(_i\) can specify the location and timing of cell process retraction. Moreover, a gradient of [Ca\(^{2+}\)]\(_i\) that increases towards the rear in migrating cells has been reported in migratory blood cells and fibroblasts (Brundage et al., 1991; Brust-Mascher and Webb, 1998), and this appears to be linked to Ca\(^{2+}\)-dependent cell detachment mechanisms such as increased contractility, enzymatic adhesion disassembly or increased actin severing to the rear (Doyle and Lee, 2002; Valeyev et al., 2006; Wong et al., 2007). The role of Ca\(^{2+}\) in triggering retraction is particularly evident in cells that display...
transient increases in \([\text{Ca}^{2+}]_i\) where \(\text{Ca}^{2+}\) transients are often observed immediately before a retraction; this process requires the \(\text{Ca}^{2+}\)-dependent protease calpain (Robles et al., 2003).

Despite strong evidence for the role of the \(G_q/\alpha/\text{PLC/Ca}^{2+}\) pathway described above, we cannot rule out the possibility that the effects may also involve \(G_{12/13}\alpha\) (and \(p115\) or other Rho-GEF-mediated signaling). Due to the inability to transfect the rMTC 44-2 cells (despite multiple attempts with different transfection protocols), we were not able to test the effects of inhibitory dominant negative constructs targeting \(G_{12/13}\alpha\) in this system.

In addition to the \(G_q/\alpha/\text{PLC/IP}_3\) pathway, I also investigated the ERK and ROCK signaling pathways. In contrast to inhibitors of intracellular \(\text{Ca}^{2+}\) release which reduced or blocked NPS R-568-mediated adhesion and migration, the ERK and ROCK inhibitors had no effect on rMTC-44-2 cell adhesion but potentiated the R-568 enhancement of migration. Previous studies examining the effects of ERK and ROCK inhibitors on cell migration showed variable effects on cell migration. For example, \textit{in vitro} and \textit{in vivo} experiments on breast cancer tissues or cell lines showed that Y27632 inhibited cell migration and metastasis (Liu et al., 2009), while other studies such as those conducted on trophoblast cells (Fafet et al., 2008) have shown a potentiating effect, as shown here. There is evidence that the effects of ROCK depend on the mode of cell migration (e.g. “rounded” vs. “elongated protrusion” modes of cell motility) in different cell types (Sahai and Marshall, 2003).

Our results with the ERK and ROCK inhibitors were unexpected and differ from some previous reports of the effects of ERK or ROCK on cell migration (Huang et al., 2004b; Lester et al., 2005; Zohrabian et al., 2009). The effect of ROCK inhibition might be due to the fact that ROCK phosphorylates and inactivates myosin phosphatase preventing myosin light chain phosphorylation and subsequent cell outgrowth. Inhibition of Rho kinase (ROCK) increases neurite outgrowth by preventing the activity of myosin light chain, therefore obstructing F-actin
stabilization and actin-myosin cross-linking (Hirose et al., 1998; Lingor et al., 2007). Thus destabilization of F-actin promotes stress fiber formation and may have caused increased cell migration in our experiments. In the case of ERK, it has been shown that filamin A binding to the C-terminus of the CaSR stabilizes the receptor and attenuates its degradation rate, thereby facilitating MAPK/ERK signaling (Zhang and Breitwieser, 2005). Our data appear to indicate that CaSR activation of ERK works in opposition to the \(G_q\alpha/PLC/IP_3\) pathway in the context of promoting cell migration. We speculate that in rMTC 44-2 cells, the net effect of CaSR stimulation in the absence of signaling inhibitors is the dominance of intracellular Ca\(^{2+}\) release in promoting migration over the inhibitory effect of ERK activation. Further studies are required to fully elucidate the molecular mechanisms mediating the effects of CaSR and ERK on cell migration.

Taken together, our findings reveal that CaSR-expressing cells can participate in cellular mobility by converting an increase in extracellular Ca\(^{2+}\) levels into a compartmentalized intracellular Ca\(^{2+}\) signal (release from the endoplasmic reticulum via IP\(_3\) receptor activation) that culminates in enhanced cellular movement. Our data suggest that cation-induced protein conformational changes in the CaSR-integrin complex likely operate in conjunction with PLC-mediated [Ca\(^{2+}\)], release to stimulate the movement of tumour cells.

3.3.3 Implications of the CaSR-integrin complex in cancer metastasis

The rMTC 44-2 cell line was derived from a rat medullary thyroid carcinoma originating from the calcitonin expressing thyroid C-cells (Gagel et al., 1980; Fajtova et al., 1991). Our results demonstrate that rMTC 44-2 cells express high levels of the CaSR. Human C-cell carcinomas are metastatic and often colonize bone tissue, an environment enriched in extracellular Ca\(^{2+}\) at levels sufficient to significantly or fully activate the CaSR. The mechanism
of medullary thyroid carcinoma metastasis to bone has never been identified despite clinical data indicating that distant metastasis is observed in 7 – 23% of patients with MTC, and is a major cause of MTC-related deaths with survival rates of 25% at 5 years after detection, and 10% at 10 years (Bergholm et al., 1997; Modigliani et al., 1998). Our findings suggest that the CaSR may participate in medullary thyroid carcinoma bone metastasis by activation of a CaSR-integrin signaling complex that becomes preferentially activated in the high Ca\(^{2+}\) environment of bone and thereby promoting cellular adhesion of the tumour cells within bone. Breast prostate, and renal cell tumour cells also metastasize to bone; 65 - 75% of advanced breast and prostate cancer patients and 30% of renal cell carcinoma patients have secondary tumours in bone, with a 5 year survival rate of only 20 - 25% (Coleman, 1997; Joeckel et al., 2014). Interestingly, the CaSR has been shown to enhance the metastatic potential of various bone-preferring breast, prostate and renal cancer cell lines (Liao et al., 2006; Mamillapalli et al., 2008; Saidak et al., 2009a). For example, \textit{in vitro} experiments have revealed that blocking CaSR function in PC3 prostate cancer cells and MDA-MB-231 breast cancer cells inhibits cell adhesion and migration (Liao et al., 2006; Saidak et al., 2009a). Moreover, analysis of bone metastasized renal carcinoma cells demonstrated that stimulation with extracellular Ca\(^{2+}\) increased cell migration and proliferation via activation of highly expressed CaSR and its downstream signaling pathways including AKT and JNK kinases (Joeckel et al., 2014). Therefore, the key features of the CaSR-integrin complex identified in the current study in medullary thyroid carcinoma cells might be applicable to other cancer cell types that robustly express the CaSR and that metastasize to bone.
CHAPTER 4. CaSR and integrin protein complexes in the developing cerebellum

4.1 Hypotheses, objectives, and rationale

Our study on medullary thyroid carcinoma cells revealed that the CaSR couples to integrins to form functional signaling protein complexes that promote cell adhesion and migration. Mechanisms that underlie cancer cell migration and progression are also important for neuronal precursor cell migration during brain development. Precursor neuronal cells originate at specific proliferative zones in the central nervous system and often migrate long distances to reach their final destination.

Although several studies have demonstrated CaSR expression in the developing CNS, the physiological role of the CaSR in CNS development remains relatively uncharacterized. To investigate whether the CaSR and β1 integrin protein complex identified in the medullary thyroid carcinoma cells plays a role in neuronal migration during CNS development, we screened for the expression of CaSR protein in several brain regions of the rat CNS; we identified a robust but transient increase in CaSR expression in the developing cerebellum (Fig. 4.1A and B). Moreover, the early postnatal cerebellum provides an excellent model system to delineate the molecular properties of neuronal migration due to the well established migrational patterns of cerebellar GCPs (Fig. 1.10).

We hypothesized that the CaSR couples to the integrins in cerebellar GCPs of the developing cerebellum and promotes GCP migration and homing from the EGL into their final resting position in the IGL. We expected that stimulation for the GCP CaSR-β1 integrin complex is provided by the integrin ligand laminin present in the basement membrane surrounding the EGL, and spontaneous Ca^{2+} release provided by the Bergmann glia processes. Ca^{2+} waves produced by Bergmann glia could locally activate the CaSR expressed on GCPs and provide stimulation
for CaSR mediated rise in intracellular Ca\textsuperscript{2+}, or CaSR-mediated promotion of ERK and AKT phosphorylation (Klein et al., 2001). Thus, we predicted that GCPs might migrate by binding to the Bergmann glia processes, and use the spontaneous Ca\textsuperscript{2+} release provided by the Bergmann glia (Hoogland et al., 2009) as stimulation for the CaSR-integrin complex to guide GCP homing from the EGL into the IGL.

Previous studies have shown that β1 integrin is important for GCP migration since binding of GCPs to ECM proteins such as laminin, vitronectin, reelin, etc., aid in the homing of GCPs from the EGL to the IGL (Rivas and Hatten, 1995; Pons et al., 2001; Borghesani et al., 2002; Porcionatto, 2006). Interestingly, the increase in CaSR expression during early cerebellar development parallels the period of rapid proliferation and migration of GCPs. Our work and others have shown that CaSR stimulation results in migration of a variety of cell types (Drueke, 2006; Saidak et al., 2009b; Lam et al., 2011; Tharmalingam et al., 2011; Lee et al., 2012). Therefore, the goal of this study was to extend this to identify and characterize the expression patterns of the CaSR and integrins in the developing cerebellum, and to determine whether this complex is important for GCP migration.

**Specific objectives:**

1) The first objective was to determine the temporal protein expression profile of the CaSR and β1 integrin in the developing rat cerebellum using western blotting.

2) The next objective was to determine whether the CaSR and integrins were present in a macromolecular protein complex in the developing rat cerebellum. This objective was met by performing reciprocal co-immunoprecipitations.

3) The third objective was to establish the cellular distributions of the CaSR and β1 integrins in the developing cerebellum. Immunocytochemical analyses were performed on purified GCPs, and on fixed cerebellar sections at various developmental ages.
4) The next objective was to identify the effects of CaSR stimulation on integrin-mediated cell migration. Laminin-matrix based cell migration experiments were conducted on purified GCPs in the absence and presence of CaSR allosteric modulators.

5) The fifth objective was to evaluate the cell surface and intracellular distribution of the CaSR and \(\beta_1\) integrins in the presence and absence of CaSR stimulation. This objective was met by using purified GCPs and a biotin-based cell surface protein isolation assay.

6) The sixth objective was to establish whether the CaSR that is endogenously expressed in purified GCPs is functionally coupled to rise in intracellular Ca\(^{2+}\) levels, and/or phosphorylation of ERK and AKT pathways. Intracellular Ca\(^{2+}\) and phosphorylation assays were performed to address this objective.

7) The seventh objective was to investigate potential biochemical pathways involved in CaSR-integrin mediated cell migration. This objective was tested using a panel of inhibitors of intracellular signaling pathways.

8) The final objective was to determine whether CaSR modulation affected GCP migration \textit{in vivo}. This objective was demonstrated by injecting CaSR allosteric modulators into the CSF of developing rats and measuring changes in GCP migration.

4.2 Results

4.2.1 Developmental expression of the CaSR and \(\beta_1\) integrins in the rat cerebellum

I first examined the temporal protein expression profile of the CaSR and \(\beta_1\) integrin in the developing rat cerebellum by western blotting. Relatively low levels of the CaSR were detected at postnatal day (PND) 3 followed by a robust but transient increase from PND 7 to 18, with peak expression between PND 10 to PND 13 (Fig. 4.1A, B). The increase in CaSR expression
Figure 4.1. Developmental expression and immunoprecipitation of the CaSR and integrins in the rat cerebellum.

(A) Representative western blot analysis of the rat cerebellum during development, and the (B) corresponding quantitation. Each data point represents the mean ± S.E.M of two independent
experiments. *, CaSR, †, β1 integrin; ‡, α6 integrin indicate statistically significant differences (p < 0.05) compared with adult expression levels. (C) Co-immunoprecipitation of the CaSR and the β1 integrin in PND13 rat cerebellum. Samples of PND13 rat cerebella were immunoprecipitated using an anti-CaSR antibody targeting the extracellular domain of the CaSR, and an antibody targeting the C-terminus of β1 integrin. Polyclonal anti-GluR2/3 glutamate antibody was used as a negative control. The blots are representative of three experiments with similar results.
was followed by a rapid decrease to adult levels, with PND 21 through adult stages expressing relatively low levels. CaSR expression at PND 10, 13, and 18 was 284 ± 5%, 345 ± 13%, and 268 ± 75% (mean ± SEM) of adult levels respectively (p < 0.05), whereas the expression levels at PND 3 and PND 24 were 102 ± 12% and 126 ± 14% of adult. Thus, CaSR expression is strongly up-regulated during the period of peak GCP migration from PNDs 7 to 18.

To determine whether the transient rise in CaSR expression coincides with elevated integrin expression, rat cerebella of various ages were subjected to membrane purification followed by solubilization and western blot analysis. Both β1 integrin and α6 integrin expression, the heterodimer subunits that bind cerebellar laminin, were highly expressed throughout all ages, with slightly elevated levels of expression in the first two postnatal weeks of birth compared to adult levels (Fig 4.1A, B). β1 integrin expression at PND 3, 7 and 10 were 133 ± 10%, 170 ± 2% and 149 ± 2% of adult expression respectively, with expression tapering off to adult levels from PND 13 onwards (p < 0.05). α6 integrin expression was 148 ± 6%, 162 ± 11% and 136 ± 6% at PND 3, 7 and 10 relative to adult expression respectively, with expression leveling off to adult levels after PND 13. Although β1 and α6 integrin expression did not mimic the transient increase in CaSR expression, both integrin proteins are abundantly expressed throughout all ages, with significantly elevated levels of expression at PND 3 to PND 13 compared to adult.

4.2.2 The CaSR and β1 integrins form macromolecular protein complexes in the developing rat cerebellum

To determine whether the CaSR and integrins were present in a macromolecular protein complex in the developing rat cerebellum, reciprocal co-immunoprecipitations were performed. Given that β1 integrin forms obligate heterodimers with several α integrin subunits including the α6 integrins, β1 integrin was used for co-immunoprecipitation studies. The CaSR and β1 integrins were immunopurified using the solubilized cell membrane fractions of PND 13 rat
cerebella, and a polyclonal antibody targeting the extracellular domain of the CaSR, or a polyclonal β1-integrin antibody targeting the C-terminus of the β1 integrin. To exclude the possibility of non-specific binding, a no-antibody control, and an anti-GluR2/3 AMPA glutamate receptor polyclonal antibody were used as negative controls.

The ADD CaSR monoclonal antibody labelled a presumably mature glycosylated 130 kDa band and the immature unglycosylated 120 kDa band of the CaSR (Fig. 4.1C). The CaSR polyclonal antibody and the β1 integrin C-terminal antibody immunoprecipitated the mature 130 kDa glycosylated form, and to a lesser extent, the immature 120 kDa form of the CaSR (Fig. 4.1C). Similarly, the C-terminal β1-integrin antibody labelled the 110 and 130 KDa glycosylated forms of β1 integrin. The β1 integrin antibody and the CaSR 4641 antibody immunoprecipitated 110 and 130 kDa forms of the β1 integrin in roughly similar amounts (Fig. 4.1C). The no-antibody control and a polyclonal anti-GluR2/3 antibody failed to co-immunoprecipitate the CaSR or β1 integrins. These results demonstrated that the CaSR and β1 integrins are present together in a macromolecular complex in the PND 13 rat cerebellum.

4.2.3 Co-localization of the CaSR and β1 integrins in rat cerebellar GCPs

To determine the cellular distributions of the CaSR and β1 integrins in the developing cerebellum, cerebella sections at various ages were immunostained with an antibody to an extracellular epitope of the CaSR, and a C-terminus targeting anti-β1-integrin antibody. In agreement with the protein expression analysis (Fig. 4.1), CaSR and β1 integrin immunolabeling was low at PND 3 (Fig. 4.2A). However, abundant expression and co-localization of the CaSR and β1 integrin in the EGL and IGL was seen at PND 10, PND 13, and PND 18 (Fig. 4.2A). Higher magnification images of PND 13 cerebellar sections revealed discrete punctate expression of both the CaSR and β1 integrins throughout the cerebellar GCPs of the EGL; the
Figure 4.2. Immunocytochemical analysis of CaSR and β1 integrin in the developing cerebellum.

(A) Rat cerebellar sagittal sections at PND 3, 10, 13 18, and adult immunostained with anti-CaSR and β1-integrin antibodies. Both proteins displayed similar developmental expression
patterns in the cerebellar cortex. Sections from PND 10, 13 and 18 rat cerebella illustrate abundant expression and co-localization of the CaSR and β1 integrin in the EGL and IGL, whereas sections from PND 03 and adult showed relatively low levels of the CaSR and β1 integrin expression. The CaSR and β1 integrins were expressed in the Purkinje cells at all time points after PND 03. (B) High magnification (100X) images of PND 13 rat sagittal cerebellar sections illustrate abundant co-localization of the CaSR and β1 integrins in the GCPs of the EGL.
merged images illustrate extensive co-localization of the CaSR and β1 integrin expression (Fig. 4.2B). Analysis of PND 21 (data not shown) and adult (Fig. 4.2A) cerebellar cortex revealed relatively low levels of CaSR and β1 integrin expression. Interestingly, both CaSR and β1 integrin expression were observed in the Purkinje neurons in all ages from PND 3 to adulthood (Fig. 4.2A).

Western blot analysis of purified GCPs isolated from PND 8, 9, and 10 rat cerebella confirmed robust expression of the CaSR, β1 integrin and α6 integrin (Fig. 4.3A). Cellular localization analysis on PND 8 purified GCPs illustrated discrete CaSR punctate formations throughout the cells (Fig. 4.3B, green). β1 integrin complexes were also visible as punctate formations and were abundantly expressed throughout the cell soma with a distribution similar to that seen with the CaSR (Fig. 4.3B, red). In the merged images, the distribution of the CaSR overlapped extensively, but not completely, with that of β1 integrin (Fig. 4.3D, yellow). The CaSR-integrin puncta expression pattern of the GCPs was very similar to that present in rMTC 44-2 cells (Fig. 3.3). Thus, immunocytochemical analysis revealed that the CaSR and β1 integrins are co-localized in GCPs of the developing cerebellum, and in conjunction with the co-immunoprecipitation results, demonstrates that the CaSR and β1-containing integrins are components of a macromolecular protein complex.

4.2.4 Potentiation of laminin-mediated GCP migration by CaSR positive allosteric modulator

GCPs are highly migratory cells and use environmental cues such as laminin to guide their migration. Therefore, to determine the effects of CaSR stimulation on integrin-mediated cell migration, cell migration experiments were conducted. These experiments used Boyden chambers coated with a laminin matrix. Since integrins are affected by changes in divalent cation
Figure 4.3. Expression of the CaSR and β1 integrins in purified GCPs.

(A) Western blot analysis demonstrating expression of the CaSR, β1 integrin and α6 integrin in purified GCPs isolated from PND 8, 9 and 10 cerebella. (B) PND 8 GCPs cultured for 48 h and immunostained with CaSR and β1-integrin antibodies. CaSR expression was present in discrete puncta distributed throughout the cell (green). β1-integrin was also expressed throughout the cell (red) and showed extensive overlap with the CaSR expression (yellow). Photomicrographs are representative of similar results obtained in three experiments. Scale bar represents 10 µm.
concentration including Ca\(^{2+}\) and Mg\(^{2+}\), we avoided the use of cations and instead used two CaSR-specific positive allosteric modulators (NPS R-568 and NPS R-467) in the presence of a submaximal concentration of extracellular Ca\(^{2+}\) (1.8 mM) (Mould et al., 1995; Vorup-Jensen et al., 2007; Puklin-Faucher and Vogel, 2009; Tharmalingam et al., 2011).

GCP cells added to the upper surface of transwell filters displayed directional chemotaxis toward the lower chamber coated with 30 µg/ml laminin. 1 µM R-568 and 1 µM R-467 significantly potentiated laminin-dependent cell migration by 165 ± 15% and 153 ± 9% respectively, compared to vehicle control (p < 0.05; Fig. 4.4). In addition, the potentiating effect of 1 µM R-467 on cell migration was eliminated by 1 µM of NPS 2143 (a CaSR antagonist), with 97 ± 3% cell migration compared to control (p < 0.05). Furthermore, NPS 2143 (1 µM) had no effect on cell migration in the absence of CaSR stimulation. Lastly, 1 µM NPS S-467, the less-potent control isomer of NPS R-467, did not potentiate GCP migration towards laminin, showing 101 ± 3% cell migration of control (Fig. 4.4). Therefore, the cell migration experiments using the CaSR pharmacological modulators demonstrated that CaSR stimulation promotes GCP migration towards laminin.

4.2.5 CaSR stimulation increases cell-surface expression of β1 integrins in GCPs

One mechanism by which CaSR modulation may increase GCP migration towards laminin is by promoting the plasma membrane expression of β1-containing integrins (Qin et al., 2011; Lin et al., 2013). To evaluate the cell surface and intracellular distribution of the CaSR and β1 integrins in the presence and absence of CaSR stimulation, PND 9 GCPs grown on 30 µg/ml laminin were subjected to a biotin-based cell surface protein isolation assay. CaSR immunoblot analysis showed that the mature 130 kDa band of the CaSR appeared in the cell surface (eluate) fraction, whereas both the mature and immature 120 kDa bands appeared in the intracellular
Figure 4.4. CaSR stimulation increased GCP migration towards laminin, an extracellular matrix protein present in the cerebellum.

GCP cells added to the upper surface of transwell filters (20 hrs at 37°C) migrated to the lower chamber coated with 30 μg/ml laminin. CaSR positive modulators 1 μM NPS R-568 and 1 μM NPS R-467 demonstrated 1.5–fold increase in GCP migration toward the laminin matrix as compared to control conditions and 1 μM NPS S-467 (the less potent isomer of NPS R-467). The effects of NPS R-467 on cell migration were inhibited in the presence of 1 μM NPS 2143. 1 μM NPS 2143 treatment alone did not inhibit GCP migration. Each data point represents the mean ± S.E.M of 3 – 5 independent experiments. *, p<0.05. Asterisks indicate statistical significance compared with DMSO control treatment.
fraction (Fig. 4.5A). Quantitation of the cell surface band revealed that the cell surface expression of the CaSR was not significantly affected by 1 μM NPS R-467 CaSR stimulation, showing 87 ± 17% of control surface expression (p < 0.05; Fig. 4.5B). Others have also shown that unlike most GPCRs which are internalized upon agonist activation, the CaSR maintains cell surface expression due to unique cellular trafficking mechanisms that promote agonist driven membrane insertion of the recycled receptor (Grant et al., 2011). In addition, the α6 integrin immunoblot showed cell surface expression of the 110 and 130 kDa forms, and quantitation of these bands demonstrated that CaSR stimulation induced a relatively small increase in α6 integrin surface expression, showing an increase of 133 ± 24% of control (Fig. 4.5A, D). Surprisingly, 1 μM NPS S-467 (the less active isomer) did not significantly increase the cell surface expression of β1 integrins, whereas CaSR stimulation with 1 μM NPS R-467 resulted in a 230 ± 33% increase in β1 integrin cell surface expression compared to the DMSO control treatment (Fig. 4.5A, C). Thus, biotin-streptavidin affinity chromatography analysis established that the CaSR, β1 integrins and α6 integrins are expressed on the cell surface of GCPs, and more importantly, that specific pharmacological activation of the CaSR resulted in increased cell surface expression of the β1 integrins - but not of the CaSR itself.

4.2.6 CaSR activation in purified GCPs induces ERK2 and AKT phosphorylation, but not intracellular Ca^{2+} release

In normal thyroid and parathyroid cells, and in thyroid carcinoma cells, stimulation of the CaSR activates G_{qα} and PLC and induces release of intracellular [Ca^{2+}], (Nemeth and Scarpa, 1986; Nemeth et al., 1986; Conigrave et al., 2000b; Brown and MacLeod, 2001; Tharmalingam et al., 2011). To establish whether the CaSR that is endogenously expressed in purified rat GCPs is functionally coupled to this downstream signaling pathway, the CaSR was stimulated with
Figure 4.5. CaSR stimulation increases cell-surface expression of β1 integrins in GCPs.

(A) PND 9 GCPs grown on 30 μg/ml laminin were treated with DMSO control, 1 μM NPS S-467 or 1 μM NPS R-467 for 8 hrs. Cell-surface proteins were covalently biotinylated and isolated using a streptavidin-agarose column. Cytoplasmic proteins appeared in the “Flow Through (F.T.)” fraction whereas cell surface proteins were eluted in the “Eluate”. CaSR stimulation resulted in 2-fold increase in β1 integrin cell surface expression, whereas the CaSR and α6 integrin cell surface expression did not change. (B) Band intensities present in the eluted fractions of the CaSR, β1 integrin and α6 integrin blots were analyzed and are presented as a percent of control (n = 3). Band intensity of the target proteins were normalized to the corresponding band intensity for GAPDH. Asterisks indicate statistical significance (p < 0.05) compared with DMSO control treatment.
NPS R-467 in conjunction with its endogenous ligand Ca\(^{2+}\) at subthreshold concentration of 1.8 mM, and changes in intracellular [Ca\(^{2+}\)]\(_i\) were measured. L-glutamate was used as a positive control since GCPs express metabotropic glutamate receptors which are also coupled to G\(_{q}\) and intracellular [Ca\(^{2+}\)]\(_i\) release (Pickering et al., 1993; Copani et al., 1998; Di Giorgi Gerevini et al., 2004). Treatment of PND 8-10 GCPs with 100 μM L-glutamate elicited a robust rise in intracellular [Ca\(^{2+}\)]\(_i\), whereas 5 mM Ca\(^{2+}\) or 1 μM NPS R-467 did not induce changes in intracellular [Ca\(^{2+}\)]\(_i\) (Fig. 4.6A). Thus surprisingly, in purified GCPs, the CaSR does not couple to intracellular [Ca\(^{2+}\)]\(_i\) release, as it does in other non-neuronal cell types (Tharmalingam et al., 2011).

CaSR activation has also been shown to increase phosphorylation of ERK and AKT in other cell types (Kifor et al., 2001; Tfelt-Hansen et al., 2003; Leach et al., 2013). To determine whether stimulation of the CaSR activates ERK and AKT signaling in GCPs, PND 9 purified GCPs grown on 30 μg/ml laminin were stimulated with 1 μM NPS R-467 or 5 mM Ca\(^{2+}\). ERK1/2 immunoblot revealed two bands at 42 kDa and 44 kDa, indicating forms of phosphorylated ERK1 and ERK2 respectively. Stimulation of the CaSR with 1 μM NPS R-467 or 5 mM Ca\(^{2+}\) increased GCP ERK2 phosphorylation by 211 ± 34% and 185 ± 12% of DMSO control respectively (p < 0.05; Fig. 4.6B, D), whereas ERK1 phosphorylation was unaffected by CaSR stimulation (Fig. 4.6B, C). Similarly, phospho-AKT immunoblots revealed that stimulation of the CaSR with 1 μM NPS R-467 increased AKT phosphorylation by 145 ± 9% of control. Furthermore, the potentiating effect of R-467 mediated AKT phosphorylation was eliminated by 1 μM NPS 2143 (Fig. 4.6E, F). In addition, specificity of CaSR selectivity towards the CaSR was further demonstrated by the observation that NPS S-467, the relatively inactive isomer of NPS-467, did not elicit significant elevation of ERK2 or AKT phosphorylation (Fig. 4.6B – F).
Figure 4.6. CaSR stimulation induces ERK2 and AKT phosphorylation, but not intracellular Ca$^{2+}$ release.

(A) The CaSR does not couple to intracellular Ca$^{2+}$ release in purified GPCs. Stimulation of PND 8–10 GCPs with 5 mM Ca$^{2+}$ or 1 μM NPS R-467 did not induce changes in intracellular Ca$^{2+}$ levels, whereas 100 μM L-glutamate elicited a robust rise in intracellular Ca$^{2+}$. (B) Representative western blot analysis of ERK1/2 phosphorylation in GCPs. Stimulation of CaSR with 1 μM NPS R-467 or 5 mM Ca$^{2+}$ increased GCP ERK2 phosphorylation compared to control and 1 μM NPS S-467 treatment. (C) Quantitation for the phosphorylated ERK1; ERK1 phosphorylation was unaffected by CaSR stimulation. (D) Quantitation of ERK2; immunoblots were normalized to GAPDH (n = 3). (E) Representative western blot analysis of AKT phosphorylation. 1 μM NPS R-467 increased AKT phosphorylation in PND 9 purified GCPs compared to control and 1 μM NPS S-467 treatment. Pretreatment of GCPs with 1 μM NPS 2143 blocked 1 μM NPS R-467 induced AKT phosphorylation. (F) Bar graphs represent quantitation of phosphorylated AKT band intensities (n = 3). Asterisks indicate statistical significance (p < 0.05) compared with DMSO control treatment.
To summarize, the phosphorylation assay results demonstrated that activation of the CaSR induces phosphorylation of AKT and ERK2 in purified GCPs.

4.2.7 Positive allosteric modulation of the CaSR potentiates β1 integrin cell surface expression and GCP migration via ERK and AKT activation

CaSR-mediated potentiation of GCP migration could occur through intracellular second messenger signaling mechanisms coupled to the CaSR that affect integrin expression and trafficking. These intracellular signaling proteins activated by the CaSR could induce an integrin-associated protein to facilitate the binding of integrins to ECM proteins by shifting the integrin conformation to the active state (Hu and Luo, 2013). To investigate potential biochemical pathways involved in CaSR-mediated enhancement of β1 integrin cell surface expression and promotion of cell migration, a panel of inhibitors of intracellular signaling pathways were tested. It was anticipated that inhibition of the signaling pathways involved in the CaSR-mediated effects would reduce or block CaSR mediated promotion of cell migration and β1 integrin cell surface expression. Inhibition of PI3 Kinase, G\(\alpha\)\(_i\), protein kinase C, rho-associated protein kinase, phospholipase C, nitric oxide synthase, protein tyrosine phosphatase and focal adhesion kinase using LY 294002 (10 μM), pertussis toxin (200 ng/ml), chelerythrine (5 μM), Y-27632 (30 μM), U73343 (3 μM), L-NAME (1 mM), NSC 87877 (10 μM) and FAK Inhibitor 14 (0.1 μM) respectively did not significantly affect NPS R-467 mediated potentiation of GCP migration towards laminin (Fig. 4.7A). In contrast, inhibition of ERK and AKT blocked CaSR-mediated cell migration and promotion of β1 integrin cell surface expression (Fig. 4.7B–D). The ERK inhibitor PD 98059 (10 μM) and AKT inhibitor GSK 690693 (3 μM) significantly reduced NPS R-467 potentiated GCP migration by 32 ± 2% and 37 ± 4% respectively (p < 0.05; Fig. 4.7B), and blocked the promotion of β1 integrin cell surface translocation by 48 ± 4% and 42 ± 12% respectively (p<0.05; Fig. 4.7C, D). These findings indicated that CaSR-mediated
Figure 4.7. CaSR stimulation increases GCP migration towards laminin via ERK and AKT signaling.

(A) Inhibitors to the following cell signaling pathways did not affect NPS R-467 mediated GCP migration towards laminin: PI3 Kinase (10 μM LY 294002), G\(_i\)α (200 ng/ml pertussis toxin), protein kinase C (5 μM chelerythrine), rho-associated protein kinase (30 μM Y-27632), phospholipase C (3 μM U73343), nitric oxide synthase (1 mM L-NAME), protein tyrosine phosphotase (10 μM NSC 87877) and focal adhesion kinase (0.1 μM FAK Inhibitor 14). Each data point represents the mean ± S.E.M of 3 – 5 independent experiments. (B) Inhibition of ERK and AKT signaling using 10 μM PD 98059 and 3 μM GSK 690693 respectively blocked the effects of CaSR stimulation (1 μM NPS R-467) on PND 8 – 10 purified GCP migration towards laminin (n = 5). (C) Representative western blot analysis and quantitation (D) of β1 integrin cell surface expression of PND 9 GCPs grown on 30 μg/ml laminin. CaSR-stimulation (1 μM NPS R-467) mediated increase in cell surface expression of β1 integrin was inhibited by pretreatment with inhibitors of ERK (10 μM PD 98059) and AKT (3 μM GSK 690693) inhibitors (n = 3). Asterisks indicate statistical significance compared with NPS R-467 treatment in the absence of inhibitors.
activation of AKT and ERK phosphorylation, and the subsequent promotion of β1 integrins to
the cell surface are required for the positive CaSR effects on GCP migration.

4.2.8 In vivo pharmacological modulation of cerebellar GCP migration

To determine whether CaSR modulation affects GCP migration in vivo, allosteric modulators
of the CaSR were injected into the CSF of PND 9 rats and changes in GCP migration were
analyzed 72 h later. GCP migration was visualized by administering BRDU i.p. and then tracing
its uptake into GCPs. Figure 4.8A depicts representative confocal images of the cerebellar
cortex from DMSO control, NPS S-467, NPS R-467, and NPS 2143-treated animals.
Immunocytochemical analysis revealed that BRDU-labelled GCPs (green) migrated from the
EGL into the IGL over the span of 72 h (Fig 4.8A). Cell count analysis of the EGL
demonstrated that blocking CaSR function with NPS 2143 reduced movement of GCPs out of
the EGL. The NPS 2143 treated animals had 140 ± 12% more BRDU-positive GCPs in the EGL
compared to the DMSO control (p < 0.05; Fig. 4.8B). NPS S-467 (96 ± 11% of control) and
NPS R-467 (77 ± 5% of control) injected rats had similar or a slightly lower number of BRDU-
positive GCPs in the EGL compared to control respectively. Cell count analysis of the molecular
layer (ML) showed that there was no difference in BRDU-positive GCP numbers in all treatment
groups, probably due to the transient nature of GCP migration through this layer. Cell count
analysis of the IGL revealed that the CaSR positive allosteric modulator (NPS R-467) promoted
BRDU-positive GCP migration from the EGL into the IGL. Here, NPS R-467 treated animals
had 159 ± 8% more BRDU-positive GCPs in the IGL compared to control. NPS S-467 (94 ± 7%
of control) and NPS 2143 (88 ± 9% of control) injected rats both showed no significant effects
with similar numbers of BRDU-positive GCPs in the IGL compared to control (Fig. 4.8B).
Taken together, the in vivo studies with CaSR allosteric modulators indicated that blocking CaSR
Figure 4.8. *In vivo* modulation of cerebellar GCP migration.

(A) CaSR allosteric modulators were injected into the right ventricle of PND 9 rats and GCP migration was tracked by simultaneous intra-peritoneal injections of BRDU. 72 h post-injection, the brains were analyzed by immunocytochemistry using an anti-BRDU antibody (green) and anti-calbindin antibody (red) was used as a marker to distinguish the EGL, IGL, and molecular layer (ML) regions in the immature cerebellum. Photomicrographs illustrate representative cerebellar images from DMSO, NPS S-467, NPS R-467 or NPS 2143 treated animals (40X). (B) The number of BRDU labelled GCPs/100μm² in each cortical layer is shown. Inhibiting CaSR function (NPS 2143) prevented migration of GCPs into the IGL, whereas the CaSR positive allosteric modulator (NPS R-467) promoted GCP migration compared to DMSO and NPS S-467 treated animals. Asterisks indicate statistical significance compared with DMSO control treatment (p < 0.05).
activation prevented the exit of GCPs from the EGL, whereas facilitation of CaSR activation promoted GCP migration from the EGL into the IGL.

4.3 Summary, discussion, and conclusions

4.3.1 Summary

Our findings demonstrate that the CaSR, together with integrins, functions as a cell-surface chemoattractant receptor expressed on the surface of cerebellar GCPs to guide their movement during cerebellar maturation. We observed a robust but transient increase in CaSR expression in GPCs that peaked during the second postnatal week, the period of maximal GPC migration. Our results support a model whereby the CaSR acts as a chemoattractant sensor to detect a gradient of extracellular Ca$^{2+}$, while integrin heterodimers in conjunction with ECM proteins function as the driving force to mediate cell movement. Our data also indicate that CaSR-mediated stimulation of ERK2 and AKT phosphorylation provides a biochemical link between calcium-sensing and the integrin-mediated movement of GCPs (Fig. 4.9).

4.3.2 Stimulation–dependent β1 integrin cell surface expression controls GCP migration

The vertebrate nervous system cytoarchitecture is formed by a complex array of dividing neuronal progenitors that migrate to specific target locations. In many cases the final resting place of the migrating cells is located a considerable distance from the initial pool of dividing cells. The migration and homing process is guided by gradients of various chemoattractants that bind to specific cell surface receptors that are expressed in a coordinated spatial-temporal fashion. During early cerebellar development, newly formed immature GCPs migrate towards
Summary of the role of the CaSR/integrin complex in cerebellar development depicting cross-talk between CaSR (blue), integrin heterodimers (red), and intracellular protein kinases. Left, the CaSR/integrin complex prior to stimulation; right, elevated extracellular Ca\(^{2+}\) levels (and/or polyamines) activate the CaSR which then increases phosphorylation of ERK2 and AKT promoting \(\beta_1\) integrin cell surface expression. The accumulation of integrins to the leading edge of GCPs allows for stable attachments to be formed between the GCPs and the ECM, thereby enhancing cell migration.
the IGL by extending growth cones to guide axon establishment, and by sensing the extracellular environment (Marin et al., 2010).

Stimulation-dependent redistribution of β1 integrins from the retracting edges to the migrating front is important for chemoattractant-guided cell homing. In the case of GCPs, a CaSR-stimulated increase in β1 integrin surface expression promoted an integrin-laminin interaction as demonstrated by increased GCP migration towards laminin. Biochemical analysis using signaling inhibitors revealed that CaSR promotes β1 integrin cell surface expression by activation of ERK2 and AKT signaling. Previous work has shown that AKT phosphorylation activates adenylate cyclase activating protein 1 (ACAP1), an endosomal GTPase activating protein that plays an essential role in cargo sorting by recycling surface receptors (Li et al., 2005). Moreover, activated ACAP1 is known to associate with β1 integrins in the recycling endosome to promote β1 integrin cell surface expression. In GCPs, the stimulation of the CaSR and subsequent induction of AKT phosphorylation in our experiments may have facilitated ACAP1 interaction with endosomal β1 integrins to promote β1 integrin cell-surface expression.

Our results show that CaSR activation in GCPs induced increased phosphorylation of ERK2, but not ERK1. ERK phosphorylation is known to control β1 integrin cell surface expression. It has been reported that ERK1 function is dispensable for this process while ERK2 is required for cell migration (Lai et al., 2001). Phosphorylated ERK2 supports cell polarization by controlling the proper orientation of centrosomes, which is an important step for the accumulation of proteins involved in cell movement at the leading edge of the migrating cell (Bisel et al., 2008; Imamura et al., 2010). In GCPs, activation of the ERK and AKT signaling pathway by the CaSR may cause trafficking of β1 integrins to the leading edge of the cell, while limiting the level of integrins available in the retracting edge. The accumulation of β1 integrins at the leading edge
could facilitate the formation of a stable association between the GCPs and the ECM, thereby allowing the CaSR to control GCP migration.

4.3.3 The CaSR couples to ERK and AKT signaling via G-protein-independent mechanisms

Our analysis of the intracellular signaling pathways suggests that the CaSR couples to ERK and AKT activation via G-protein-independent mechanisms. For example, our data in GCPs showing (1) the lack of \( G_{q} \alpha \) coupled intracellular \( Ca^{2+} \) release upon CaSR activation, (2) the ineffectiveness of the \( G_{i} \alpha \) inhibitor pertussis toxin in preventing the CaSR mediated promotion of \( \beta 1 \) integrin cell surface expression and migration, and (3) the absence of an effect of \( G_{12/13} \alpha \) pathway inhibition by ROCK kinase inhibitor all suggest that in GCPs, the CaSR does not couple to \( G_{q} \alpha \), \( G_{i} \alpha \) or \( G_{12/13} \alpha \). Other studies have thoroughly documented that GPCRs also signal via G-protein independent mechanisms through \( \beta 1 \) arrestin (Reiter et al., 2012; Zimmerman et al., 2012; Walther and Ferguson, 2013). Traditionally \( \beta \)-arrestin is known to desensitize GPCR signaling by preventing G-protein interaction with its receptor. In addition to desensitization, \( \beta \)-arrestin also acts as a scaffolding protein and can phosphorylate both ERK and AKT upon GPCR activation (Luttrell et al., 2001; Beaulieu et al., 2008). \( \beta \)-arrestin signaling has been shown to induce chemotaxis in the absence of G-protein engagement in several cell types (Reiter and Lefkowitz, 2006). Thus, in GCPs the CaSR could couple to ERK and AKT phosphorylation via G-protein independent, \( \beta \)-arrestin mediated signaling.

4.3.4 CaSR stimuli in the developing cerebellum

A key question to be addressed is, what is the source of the CaSR stimulus in the immature cerebellum? One possibility is that activation of the CaSR may be mediated by local extracellular \( Ca^{2+} \) released by the Bergmann glia. Tangentially migrating unipolar GCPs in the
EGL sprout a third axon that allows the GCPs to attach to Bergmann glial fiber tracts to undergo radial migration into the IGL. Bergmann glial fibers have been shown to generate radially expanding transglial Ca$^{2+}$ waves that initiate at a central point and expand radially to encompass multiple Bergmann glial processes (Hoogland et al., 2009). The Ca$^{2+}$ waves produced by Bergmann glia could locally activate the CaSR expressed on GCPs to promote radial migration (see Fig. 1.10).

Another potential source of CaSR activation may arise from elevated polyamine levels during early development. The CaSR is a multimodal nutrient sensor; it is activated not only by its primary ligand Ca$^{2+}$, but is also activated or potentiated by a variety of compounds including other di- and trivalent cations, polyamines, L-amino acids, glutathione, and \(\gamma\)-glutamyl peptides (Nemeth et al., 1998; Conigrave et al., 2000b; Wang et al., 2006; Conigrave and Hampson, 2010). Polyamines such as spermine and spermidine are potent activators of the CaSR, and polyamine levels are elevated during postnatal cerebellar development, and then subsequently decline to adult levels (Jasper et al., 1982). Interestingly, the period of increased polyamines corresponds to the peak period of CaSR expression and GCP migration, suggesting that polyamines may act to guide GCP migration.

4.3.5 Implications of the CaSR-integrin complex in medulloblastoma

Our \textit{in vivo} results demonstrated that stimulating the CaSR with positive allosteric modulation increases GCP homing to the IGL, while negative modulation of the CaSR prevents GCP exit from the EGL. Unlike most neuronal populations, GCPs remain mitotically active even after birth and constitute potential targets for transforming insults. Impairment of GCP migration allows GCPs to remain longer in the EGL proliferative-niche; thus GCPs are more prone to transformational insults due to increased exposure to proliferation molecules. GCPs are exposed
to a variety of proliferation cues in the EGL, including sonic hedgehog, Wnt, BDNF, Notch, stromal-derived factor 1, and over-expression of these factors have been associated with medulloblastoma etiology (Klein et al., 2001; Borghesani et al., 2002; Taylor et al., 2002; Blaess et al., 2004; Behesti and Marino, 2009). Medulloblastoma is the most common cause of malignant brain tumor in children aged less than 4 years of age (Macdonald et al., 2014). Medulloblastoma is a highly heterogeneous group of tumors characterized by aberrant GCP proliferation and migration. Although a role for the CaSR in medulloblastoma has not been established, highly metastatic breast, prostate, renal and thyroid cancer cells express the CaSR and preferentially metastasize near resorbing bone regions by sensing the Ca\textsuperscript{2+} enriched bone environment (Liao et al., 2006; Mamillapalli et al., 2008; Saidak et al., 2009a; Tharmalingam et al., 2011). In these cancers, it may be predicted a priori, that inhibition of the CaSR may reduce metastasis to bone. Therefore, work presented in this thesis and others provide evidence that modulation of the CaSR-β1 integrin complex using CaSR allosteric drugs may provide novel ways to control cancer cell migration and metastasis.

4.3.6 The CaSR-integrin complex may be a universal cell migration system

It has been well documented that common mechanisms underlie both tissue development and tumor formation. The results obtained in the present study extend our earlier findings demonstrating that CaSR-β1/integrin complexes mediate thyroid carcinoma cell migration (Tharmalingam et al., 2011), by documenting its importance for GCP cell homing in the developing cerebellum. The role of CaSR-β1 integrin complex in GCP chemotaxis may also be pertinent to our understanding of the molecular mechanisms underlying the translocation of other cells during development. For example, during fetal maturation, haematopoietic stem cells from fetal liver migrate to the endosteal surfaces of the bone marrow cavity to undergo haematopoiesis
(Drueke, 2006; Lam and Adams, 2010). Haematopoietic stem cells prepared from CaSR knockout mice were defective in localizing to the endosteal niche due to defective adhesion to the extracellular matrix protein, type I collagen, an integrin ligand. In addition, the temporal elevation of the CaSR in other brain regions during development such as embryonic expression in sympathetic neurons, or postnatal expression in the developing hippocampus, coincides with increased neuronal migration, synaptogenesis, and axon formation, all of which require β1 integrin mediated cell adhesion and migration (Chattopadhyay et al., 1997; Vizard et al., 2008). Taken together, these studies suggest that CaSR/integrin complexes may function as a universal cell migration/homing complex for a variety of cell types during CNS development and in tumor metastasis.
CHAPTER 5. General discussion

5.1 CaSR-integrin signaling cross-talk

Earlier proteomic studies to identify CaSR interacting proteins employed the yeast two-hybrid approach with only the C-terminal tail of the CaSR and were thus somewhat limited in scope (Awata et al., 2001; Hjalm et al., 2001; Huang and Miller, 2007; Magno et al., 2011; Arulpragasam et al., 2012). To obtain a better understanding of the role of the CaSR in cell migration, in the current study we immunoprecipitated the full length CaSR endogenously expressed in rat MTC cells and subjected to mass-spectrometry based proteomic analysis. With this experimental strategy, we demonstrated a novel macromolecular interaction between the CaSR and integrins and the importance of this complex in cell adhesion, directed cell migration and homing.

Our work has established that the CaSR couples to integrins to mediate cell adhesion and migration via activation of cell-type dependent intracellular signaling cascades. For example, in tumour cells, CaSR-mediated activation of PLC and rise in intracellular Ca$^{2+}$ was essential for integrin activation and potentiation of cell adhesion and migration. In contrast, CaSR mediated phosphorylation of ERK2 and AKT signaling, and the subsequent promotion of β1 integrin plasma membrane expression was crucial for cerebellar GCP migration. In fact, the CaSR also couples to numerous other intracellular signaling cascades upon activation, and the type of intracellular signaling is dictated by the complement of protein-protein interactions present in the cellular context. Interestingly, many of the signaling cascades initiated by CaSR activation are also downstream signaling effectors in integrin mediated outside-in signaling. A potential conceptual framework could be stated as follows: stimulation of the CaSR activates integrins, which bind to the ECM, and binding of ECM causes integrin outside-in signaling which activates
another set of intracellular signaling sequences. In this situation, secondary signaling cascades attributed to the CaSR may actually result from transactivation of the integrins. In effect, certain unique CaSR signaling pathways may be attributed to the CaSR-integrin complex present in the cell. Thus, the diverse intracellular functions and molecular effects mediated by the CaSR could be in part mediated by the integrins.

A possible role of integrins in mediating CaSR signaling can be attributed to the finding that integrins can directly bind and activate $G_{13}\alpha$ proteins. The C-terminal tails of $\beta_1$ and $\beta_3$ integrin subunits bind to $G_{13}\alpha$ upon ligation to ECM proteins, and inhibition of this interaction prevents integrin inside-out signaling of Src and RhoA mediated cell spreading (Gong et al., 2010). This demonstrates that integrins are non-canonical $G_{13}\alpha$ coupled receptors. Since the CaSR has been shown to couple to $G_{12/13}\alpha$ proteins (Huang et al., 2004a), stimulation of the CaSR could couple to $G_{13}\alpha$ via integrin activation. Furthermore, integrin-ligation mediated activation of FAK, Src and RhoA proteins which are important for cell spreading and migration have also been linked to CaSR stimulation (Pi et al., 2002). These cell structural changes attributed to the CaSR may result from CaSR activation of the integrins. In addition, CaSR coupling to MAPK signaling cascades can also be credited to integrin stimulation (Hood et al., 2003; Kudirka et al., 2007). For example, binding of integrins to ECM proteins increases phosphorylation of ERK, which is important for integrin mediated cell proliferation and survival (Lai et al., 2001). Here, activation of the CaSR may promote ERK phosphorylation via activation of integrins.

Another possible function of the CaSR-integrin complex is that CaSR transactivation of EGFR could occur through integrins, since integrins are known to cross-talk with numerous growth factor receptors (Bouvard et al., 2013). In prostate cells, integrins mediate ERK and Src phosphorylation via activation of EGFRs (Edick et al., 2007). The CaSR has been shown to be expressed in prostate cells, therefore activation of the CaSR in these cells can contribute to ERK
signaling via transactivation of EGFR mediated by the CaSR-integrin interaction. Therefore, the complex signaling cascades mediated by the CaSR can be due to its interaction with integrins. Thus, structural changes in cells that have been attributed to CaSR stimulation may be dependent on the complement of integrin expression present in the cell. Hence, cells that express the CaSR-integrin complex can control cell adhesion and migration, whereas cells that do not express the correct complement of integrins for CaSR-integrin complex formation may mediate signaling cascades independent of cell structural changes.

5.2 Integrin metal ion binding sites and importance in CaSR-integrin function

The CaSR is activated by Ca$^{2+}$; however, excessive extracellular Ca$^{2+}$ has inhibitory effects on integrin activation. At high Ca$^{2+}$ concentrations, the integrins adopt an inactive conformation due to preferential binding of Ca$^{2+}$ to the integrin MIDAS binding motif which competes with Mn$^{2+}$, whereas low concentrations of Ca$^{2+}$ synergize with suboptimal Mg$^{2+}$ at the integrin LIMPS binding site to facilitate integrin active conformation (Luo et al., 2007; Barczyk et al., 2010). Therefore a key question for the CaSR-integrin complex arises as to how elevations in extracellular Ca$^{2+}$ activate the CaSR, but do not inhibit integrin activation. Several plausible explanations may resolve this dilemma.

Due to its high cooperative binding characteristic, the CaSR is known to bind to 4 to 6 Ca$^{2+}$ ions (Appendix B). Therefore, the high cooperative binding of Ca$^{2+}$ to the CaSR binding sites of the CaSR-integrin complex could compete with and displace the Ca$^{2+}$ bound at the MIDAS site of integrins, thereby enabling Mn$^{2+}$ association with MIDAS and subsequent integrin activation. In addition, activation of the CaSR mediates influx of extracellular Ca$^{2+}$ via various Ca$^{2+}$ channels (Yamaguchi et al., 2000; Chow et al., 2011), further decreasing the amount of Ca$^{2+}$ ions on the extracellular side available for inhibiting integrin activation.
Another possible role of Ca\(^{2+}\) binding to the MIDAS site of integrins may help to prevent unwanted intracellular integrin activation. The α and β integrin subunits assemble in the endoplasmic reticulum, and both subunits are required for proper integrin folding. Integrins cannot be transported from the endoplasmic reticulum to the plasma membrane unless integrins have reached their native structure. In the endoplasmic reticulum, integrins are found in an inactive, bent conformation due to engagement of MIDAS site by the Ca\(^{2+}\). Ca\(^{2+}\) binding to integrins in the endoplasmic reticulum is essential for correct folding and assembly, and is also important for maintaining the integrins in an inactive form before they reach the golgi apparatus and until they reach the cell surface (Tiwari et al., 2011). Inhibiting integrin activation during transport to the cell membrane is important for preventing unwanted signaling cascades that could cause unnecessary cell structural changes. Constitutively active integrins can be found at the cell surface, indicating that quality control measures do not discriminate between active and inactive conformations. It should be noted that Ca\(^{2+}\) ions are present in millimolar concentrations in the endoplasmic reticulum which can sufficiently inactive the integrins. It is possible that Ca\(^{2+}\) binding to integrins keeps it in an inactive conformation in the intracellular side, but once on the extracellular side, CaSR helps to displace the Ca\(^{2+}\) ions from the integrins allowing integrin activation.

In contrast to Ca\(^{2+}\) ions which activates the CaSR but not integrins, both proteins are activated by Mg\(^{2+}\) ions. Mg\(^{2+}\) binding to the CaSR differs significantly from the other cations in that it is only a partial agonist of the CaSR and its Hill coefficient is around 1 (Chang and Shoback, 2004). Perhaps the binding of Mg\(^{2+}\) to the CaSR is impeded by its interaction with integrins. Mg\(^{2+}\) may be bound to the integrins, and therefore higher Mg\(^{2+}\) concentration are needed to activate the CaSR. Another possibility is that the effect of Mg\(^{2+}\) on CaSR activation is the result
of Mg\(^{2+}\) binding to integrins rather than the CaSR itself. Here, activation of integrins by Mg\(^{2+}\) could transactivate the CaSR to allow further intracellular signaling cascades.

5.3 Agonist-driven insertion of CaSR may contribute to integrin surface expression and function.

The CaSR and integrins are both abundantly localized in caveolae-rich microdomains (Breitwieser, 2013). My immunocytochemical analysis of the CaSR and integrins in GCPs illustrated this microdomain-type expression pattern by revealing discrete punctate present throughout the cell cytosol. The integrins and the CaSR are co-localized in these microdomain punctate structures. We demonstrated that in GCPs, activation of the CaSR resulted in increased cell migration by promoting cell surface expression of β1 integrins. Surprisingly however, stimulation of the CaSR did not increase the cell surface expression of the CaSR itself. This discrepancy might be attributed to the trafficking mechanisms which control receptor cell surface expression. For example, CaSR and integrin cell surface expression is driven by agonist stimulation, in that activation of the surface receptor activates signaling cascades which allows further insertion of caveolae-rich microdomain containing receptors to the surface, thus allowing persistent signaling in the presence of chronic agonist stimulation as previously reported (Grant et al., 2011). We propose that stimulation of the CaSR activates phosphorylation of ERK2 and AKT, which then signals the insertion of the microdomains that contain the co-localized CaSR-integrin complex. Upon entering the cell surface, the CaSR agonists activates the newly inserted complex, which then transactivates the integrins, allowing the integrins to engage in ECM ligation. Here the ligated integrins remain on the cell surface to mediate cell adhesion and migration, whereas the endocytotic mechanisms remove the CaSR from the cell surface and degrade the receptor using lysosomal enzymes. In this proposed mechanism, accumulation and
clustering of integrins would take place at the cell surface, whereas the CaSR would serve as an integrin trafficking protein whose net cell surface expression would not change.

5.4 Transactivation of the CaSR gene during development and cancer metastasis

The CaSR expression profile in the developing cerebellum presented above, and studies performed by others emphasize the transient nature of CaSR expression during tissue development, and illustrate that CaSR expression and function is completely ineffective outside their respective developmental window. In addition, CaSR expression is up-regulated in certain cancers and down-regulated in others. Therefore, it is important to understand the mechanisms which control temporal and spatial CaSR expression during development and tumour formation.

The CaSR gene is under the transcription control of two promoters, P1 and P2, which gives rise to transcripts 1A or 1B respectively. P1 (transcript 1A) produces an mRNA of approximately 5.4 Kb and 10 Kb, whereas P2 (transcript 1B) provides a transcript of 4.2 Kb (Garrett et al., 1995; Hendy et al., 2013). Interestingly, the developing hippocampus contains both the 10 Kb (P1; transcript 1A) and 4.2 Kb transcripts (P2; transcript 1B), where the large 10 Kb transcript (1A) is upregulated and parallels the increases in CaSR expression during early hippocampal development, whereas the 4.2 Kb transcript (1B) levels remain constant during all time points. Similarly the levels of CaSR transcript 1A were up-regulated in prostate cancers (Sanders et al., 2001) but reduced in parathyroid adenoma and colon cancer (Chikatsu et al., 2000). In these prostate, parathyroid and colon cancer cells, the transcript 1B levels remained unchanged (Hendy et al., 2013). More importantly, cells which regulate systemic Ca$^{2+}$ homeostasis (Chief cells, C-cells, cells lining the kidney) express exon 1B and its expression controlled by Promoter 2 is 2.5 fold more active than P1 in most cells (Hendy et al., 2013). Therefore, it is evident that temporal CaSR changes seen during development and cancer are attributed to promoter 1 and transcript 1A, whereas the CaSR expression present in tissues
involved in maintaining systemic Ca\(^{2+}\) homeostasis are under the control of promoter 2 and transcript 1B. Thus evolutionary pressures must have resulted in the creation of two promoters with one controlling the transient CaSR expression levels during developmental processes, while the other controls serum Ca\(^{2+}\) levels throughout all temporal stages. Indeed, the human gene promoter and exon organization was evident evolutionarily as early as in aquatic vertebrates and more distantly related invertebrates (Naito et al., 1998; Hendy et al., 2013).

5.5 The CaSR-integrin complex in cell migration and the effects of CaSR mutations

Our analysis of the CaSR-integrin complex in MTC chemotaxis and GCP homing demonstrated that stimulation of the CaSR resulted in directed cell migration via activation of the integrins. Our results are compatible with the idea that this novel complex may be involved in the migration of a variety of cell types and offers the first mechanistic description of CaSR in cell migration. A salient question that needs to be addressed is the relative importance of this interaction in the various cell types that express the CaSR-integrin complex. For example, several chemoattractant factors have been implicated in GCP homing (e.g. SDF-1, BDNF, etc). In addition to the Ca\(^{2+}\) and ECM present in the bone environment, metastasis of MTC to bone tissue is also stimulated by numerous bone-derived growth factors including insulin-like growth factors and transforming growth factors. Therefore, evolution has provided various mechanisms for controlling cell migration. In fact, the homing of cells from the site of origin to its final destination is a multifaceted process that is influenced by a variety of intricate array of spatially and temporally controlled regulators and chemoattractant cues. Therefore, the CaSR-integrin complex is likely one of several contributors to cell homing during development and cancer metastasis.
Understanding the engraftment of hematopoietic stem cells from their origin in the fetal liver to the bone marrow endosteal niche is of therapeutic interest to enhance stem cell engraftment in disorders pertaining to blood disorders. The CaSR has been implicated in the engraftment of hematopoietic stem cells, and although the interaction of the CaSR with integrins was not specifically investigated in these studies, the activation of the CaSR resulted in binding of hematopoietic stem cells to integrin ligands such as collagen and fibronectin present in the bone environment (Lam et al., 2011). Therefore, the CaSR-integrin complex identified in MTC and GCP cells is likely to also contribute to hematopoietic stem cell homing. However, the contribution of CaSR was synergistic with CXCR4 chemokine receptor and its agonist SDF-1 present in the bone. Here, hematopoietic stem cells derived from CaSR knockout mice did not completely abrogate cell homing, but rather reduced the overall number of cell engraftment (Adams et al., 2006; Lam et al., 2011). Hence, the engraftment of hematopoietic stem cells is most probably the result of several chemoattractant receptors that collectively contribute to proper cell homing, with each system fine tuning and contributing to the overall cell migration. This phenomenon is most likely true for GCPs, MTC, and other cells expressing the CaSR-integrin complex.

Numerous CaSR activating and inactivating mutations have been identified in the human population. Although the role of these mutations in systemic Ca\(^{2+}\) homeostasis have been well established, the contributions of these mutations to CaSR mediated cell migration and development have not been studied. In CaSR mutations that alter the affinity of the CaSR for its agonists, integrin activation would likely be altered too therefore modulating the level of cell migration. In terms of GCP homing in relation to our results, positive or negative allosteric modulation of the CaSR, which would mimic CaSR activating and inactivating mutations respectively, resulted in changes to the overall cell migration by approximately 30% compared to
control (Fig. 4.8). In terms cerebellar development, such CaSR mutations would result in changes to the total number of mature cerebellar granule neurons. Although individuals which possess these CaSR mutations may not have lethal cerebellar effects, the CaSR mutation induced changes to the overall mature cerebellar granule neuron numbers may have more complex implications in terms of altered motor coordination or higher order cognitive changes usually associated with altered cerebellar function. Similarly, other cell types such as hematopoietic stem cells which may express the CaSR-integrin complex could also be affected by the CaSR mutations, with the overall number of stem cell engraftment altered depending on the type and intensity of the CaSR mutation. Therefore mutations which affect CaSR function may not necessarily cause drastic morphological alterations due to impaired cell migration, but rather significant changes to the overall number of cells that have migrated and matured.

5.6 The CaSR-integrin complex and cancer

Analysis of the CaSR-integrin complex in MTC cells which abundantly expresses both the CaSR and integrins, demonstrated that positive allosteric modulators of the CaSR promote cancer cell migration. However, the role of the CaSR-integrin complex in other tumour cells is likely cell-type dependent. For example, the CaSR expression suppresses parathyroid and colon cancers, whereas CaSR expression positively correlates with breast and prostate cancer bone metastasis. The discrepancy in these tumour outcomes could result from differences in the expression and the formation of the CaSR-integrin macromolecular protein complex. In fact, CaSR expression in parathyroid adenomas and colon cancers is often reduced compared to normal tissue, and activation of the CaSR in these tumors decreases cell proliferation and tumor progression (Singh et al., 2013). Therefore parathyroid adenomas and colon cancers may not express a CaSR-integrin complex. On the contrary, metastatic breast and prostate cancer cells are highly aggressive and motile cells which have increased CaSR and β1 integrin expression

(Sanders et al., 2001; Yano et al., 2004; Liao et al., 2006; Saidak et al., 2009a; Pontes-Junior et al., 2010). The increased CaSR-integrin protein complex expression and formation may contribute to the overall metastatic potential of the breast and prostate cancer cells. However, as mentioned above the CaSR-integrin interaction is perhaps just one of many signaling complexes that control the metastatic migration of these cancer cells.

A report of a family with CaSR activating mutation resulted in several cases of neoplasms compared to individuals of the family that did not have mutations to the CaSR (Hoff et al., 1999). These neoplasms included prostate cancer, medulloblastoma, leukemia and pituitary adenoma. Furthermore, in breast and prostate cancer cells which have increased CaSR and β1 integrin expression, individuals that express CaSR activating mutations might be at a higher risk of developing increased bone metastasis. Under normal circumstances, these individuals would only have clinical symptoms of systemic hypoparathyroidism, however in the presence of other proto-oncogenic transforming mutations which bring about tumour formation, the CaSR activating mutations could further contribute to the tumorigenicity by increasing the metastatic potential of these cancer cells. Likewise, CaSR inactivating mutations in breast and prostate cancer would provide preventative actions on metastasis. In terms of parathyroid and colon cancers, CaSR activating mutations would be theoretically preventative, whereas CaSR inactivating mutations could exacerbate these cancers. CaSR null cells obtained from colon cancers are non-adherent and have increased propensity for anchorage-independent growth (Singh et al., 2013). Interestingly, increased bone resorption induced by dietary \( \text{Ca}^{2+} \) deficiency promotes breast cancer bone metastasis, while increased dietary \( \text{Ca}^{2+} \) intake has preventative effects on colon cancer and parathyroid tumor progression (Lipkin, 1999; Butler et al., 2010; MacLeod, 2013). This further emphasizes the role of \( \text{Ca}^{2+} \) in breast and prostate cancer
metastasis since the CaSR-integrin interaction will be fully stimulated by the elevated bone Ca\(^{2+}\) levels resulting in increased migration towards the bone environment.

5.7 CaSR-integrin complex in cell differentiation

The role of the CaSR-integrin complex identified in cell migration may also be applicable in cell differentiation. The CaSR has been shown to mediate differentiation of a wide variety of cell types including keratinocytes, osteoclasts, colonic crypt cells, etc (Hendy et al., 2013). For differentiation to occur, cells need to have proper anchorage to the ECM proteins provided by integrin ligation (Hu and Luo, 2013). In fact, in CNS-specific β1 integrin knockdown mice, absence of β1 integrin resulted in GCPs to lose adhesive contact with laminin resulting in abnormal differentiation (Blaess et al., 2004). In GCPs, CaSR-integrin complex-mediated differentiation may contribute to GCP migration by promoting the transition from proliferative phase to the migratory form. Moreover, further differentiation of the GCPs may alter the phenotype of the precursors into mature granule neurons, thereby effectively turning off CaSR expression. Thus, the CaSR-integrin complex may not only mediate cell migration, but might also contribute to cell differentiation, ultimately causing transcriptional changes which would reduce the CaSR expression.
CHAPTER 6. Concluding remarks and future directions

6.1 CaSR therapeutics

It is now clear that the biological and physiological roles of the CaSR extend beyond its function in regulating systemic Ca\textsuperscript{2+} homeostasis. We have demonstrated a novel role of the CaSR in mediating tumour cell and cerebellar neuron migration and homing via activation of the integrins. Similarly, others have established that CaSR is involved in a plethora of cellular functions ranging from stem cell engraftment and differentiation, to neurite outgrowth and tumour cell metastasis. Therefore, in order to effectively treat specific disorders that may benefit from CaSR manipulation, CaSR pharmacological agents need to be administered without affecting systemic Ca\textsuperscript{2+} homeostasis given that the CaSR is promiscuously expressed in a wide variety of tissues.

The concept of biased ligand and agonist functional selectivity provides an opportunity to identify therapeutic agents that demonstrate intracellular signaling pathway selectivity. Each CaSR agonist/modulator may adopt a unique receptor conformation that preferentially activates a certain signaling pathway over another (please also see Appendix B). The goal here would be to identify CaSR modulators that mediate intracellular signaling bias for integrin-directed cell adhesion and migration without affecting signaling cascades which control systemic Ca\textsuperscript{2+} homeostasis. For example, CaSR modulators which positively affect CaSR stimulated ERK2 or AKT phosphorylation might be useful in treating medulloblastoma. A priori; these phosphorylation-specific CaSR modulators would promote the translocation of GCPs from its proliferative zone to its final destination. The most beneficial aspect of these modulators is that the control of PTH and calcitonin hormone secretion would be unaffected since the secretion of
these hormones is not controlled by ERK2 or AKT phosphorylation. Therefore, biased signaling in CaSR pharmacology needs to be further characterized.

Biased signaling studies conducted on Cinacalcet, the FDA approved CaSR positive modulator, demonstrated greater allosteric modulation for intracellular Ca\(^{2+}\) mobilization relative to ERK1/2 phosphorylation (Davey et al., 2012). The ligand bias of Cinacalcet for intracellular Ca\(^{2+}\) mobilization is not surprising considering that most small molecule screens for CaSR allosteric modulation used the intracellular Ca\(^{2+}\) release assay. Therefore, ligand bias signaling must be incorporated into the characterization of future CaSR modulators, and more broadly to all GPCRs. Understanding such complex CaSR pharmacology directed biased signaling provides intriguing possibility that cellular signaling can be controlled with unprecedented precision and specificity.

Another approach to specifically control CaSR function is to identify peptide inhibitors that can disrupt the CaSR-integrin interaction. Identification of the key residues important for CaSR-integrin association can be used to create peptide inhibitors that can treat CaSR-integrin expressing tumors. Metastasis of MTC, breast, prostate and renal cancers to the Ca\(^{2+}\) rich bone environment can be potentially reduced with the CaSR-integrin peptide interaction inhibitor. Furthermore, these inhibitors will have minimal side effects since the biological importance of the CaSR-integrin involvement in cell migration is largely an early developmental process whereas the use of these inhibitors can be restricted to the adult population where developmental migration process of the CaSR-integrin complex is no longer necessary. Finally, the inhibitors will also not affect systemic Ca\(^{2+}\) homeostasis, therefore serving as a safe and elegant method of treating CaSR-integrin mediated cancer metastasis to the bone.

In a more speculative model, CaSR modulators might also be targeted specifically to the tumours by utilizing the specific complement of integrins upregulated in metastatic cancers. By
coupling the CaSR modulators to the integrin specific antibodies, a tumour selective delivery system can be achieved. Currently, nanoparticles containing chemotherapeutic agents targeting various oncogenes have been selectively delivered to the tumours using integrin antibodies targeting αVβ3 and αVβ5 integrins (Murphy et al., 2008). These integrins are also upregulated in breast, prostate and thyroid cancers. Thus the CaSR negative modulators can be specifically targeted to the metastatic tumors by coupling to the αVβ3 and αVβ5 targeting integrin antibodies without affecting CaSR function in other systems.

6.2 Future studies

6.2.1 Tissue-specific CaSR knockout

The role of the CaSR-integrin complex in cell migration was extensively established using the various CaSR pharmacological modulators and integrin peptide inhibitors. However, tissue-specific CaSR knockout animal studies need to be performed to obtain a better understanding of the importance of the CaSR-integrin complex in cell migration and homing, and perhaps higher order cognitive functions. Complete tissue-specific CaSR knockdown as opposed to pharmacological inhibition will demonstrate the contribution of the CaSR in mediating integrin activation and subsequent cell migration. Cell migration is a complex process which requires coordinated and synergistic activation of a variety of chemoattractant factors, therefore tissue-specific CaSR knockout animal models will provide the best system to study the contribution of the CaSR-integrin complex in cell migration. Selective CaSR knockout in GCPs using the cerebellar Math-1 promoter would be an excellent model system to solidify the role of the CaSR-integrin complex in cell migration and homing.
6.2.2 In vivo analysis of the CaSR-integrin complex in cancer metastasis

In vivo analysis of the CaSR-integrin interaction in cancer progression is crucial to delineate the contribution of this complex in metastasis. It would be especially important to identify the magnitude of influence the CaSR-integrin complex has on aggressive breast and prostate cancers, given that these cancers account for the highest metastatic tumour related deaths in women and men respectively. In addition, it would be interesting to determine whether systemic administration of the CaSR modulators will affect tumour metastasis in vivo. Although this would cause changes to the systemic Ca\(^{2+}\) homeostasis, such a study can easily provide proof-of-principle that modulation of the CaSR-integrin complex may control cancer metastasis. In addition, luciferase based in vivo bioluminescence analysis can also be used to determine whether disruption of the CaSR-integrin interaction in cancer cells would affect the in vivo metastases to the bone or lymph nodes. For example, silencing CaSR or β1 expression, vs. CaSR-wildtype luciferase labeled rMTC cells injected intracardiacally can be analyzed 21 days later using bioluminescence and X-ray (superimposed) to measure the sites of tumor and the tumor burden.

Furthermore, the CaSR mutations present in the human population need to be correlated with cancer incidence and likelihood of cancer metastasis. Activating and inactivating CaSR mutations largely go unnoticed due to the benign effects of these mutations on systemic Ca\(^{2+}\) homeostasis. However, individuals with CaSR activating mutation may be at higher risk of cancer metastasis, thus such correlation studies need to be performed to understand the contribution of the CaSR-integrin complex in metastasis.
6.2.3 Mapping the CaSR-β1 integrin interaction

The CaSR-integrin complex is contained within an oligomeric macromolecular interaction consisting of a homodimeric CaSR and the heterodimeric integrin α and β subunits. There are several options for the possible sites of a direct interaction between the CaSR and the integrins. A hypothesized site of interaction between the two proteins could involve the extracellular VFT domain of the CaSR and the large globular domain of the β1 integrin (Hu and Spiegel, 2007; Canaff et al., 2008; Wang and Luo, 2010). The CaSR undergoes closure of the extracellular VFT domains upon agonist binding, whereas the integrins demonstrate considerable bending-like motion when the integrins are triggered to undertake the active conformation (Liddington and Ginsberg, 2002). Here, conformational changes to the CaSR upon agonist binding could provide direct changes to the integrin extracellular domain if the two proteins had interacting sites in this region. An alternative hypothesis would include interacting domains within the TMD or the C-termini of the two proteins. An interaction in the C-termini could be mediated by a similar mechanism to the heterodimerization of GABA<sub>B</sub> receptor subunits which utilize the C-terminus coiled-coil alpha-helices (Kammerer et al., 1999). It is also possible that the interaction can occur at multiple domains throughout both proteins.

In addition to a possible direct physical association, the CaSR and integrins can be part of a signaling complex that are in close proximity in caveolae-rich microdomain structures and both receptors are abundantly expressed in these structures. Indeed the role of the CaSR-integrin complex in MTC and GCP migration established above do not necessarily require a direct physical interaction. The association of the CaSR-integrin complex in these microdomain structures would allow access to various intracellular signaling molecules and can mediate integrin membrane insertion and inside-out signaling. Nevertheless, to conclusively determine
whether the CaSR and integrins are in a physical protein-protein interaction, fluorescent/bioluminescence resonance energy transfer methods need to be employed.

6.2.4 Regulation of the CaSR gene transcription

Transcriptional factors which are critical for altered CaSR P1 and P2 gene activity in neoplasia and development are unfortunately not well known. Studies which aim to correlate altered usage of CaSR gene promoters P1 and P2 with changes in CaSR protein levels need to be addressed. In addition, the functional and tissue-specific use of alternate splicing of the CaSR gene transcripts remains to be fully elucidated. A comprehensive knowledge of CaSR transcriptional control may be beneficial for treating disorders which have altered CaSR expression.

6.3 Concluding statement

Our work is the first to demonstrate that an ion-sensing G-protein coupled receptor functionally couples to the integrins. Our findings also provide the first mechanistic understanding of how CaSR mediates migration of various cells types. Taken together, we established that the CaSR is associated with β1-containing integrins in a macromolecular protein complex and that stimulation of the CaSR promotes cell adhesion, migration and homing via integrin activation. The work presented here emphasizes the role of the CaSR-integrin complex as a cell-surface chemoattractant macromolecule, and provides a novel mechanism for understanding the process of neuronal and cancer cell migration.

Our study brings to light the importance of understanding the effects of CaSR mutations on cancer progression and development. Under normal circumstances, individuals with CaSR mutations are largely asymptomatic and rarely diagnosed. However in the presence of other proto-oncogenic transforming mutations which bring about tumour formation, the CaSR
activating mutations could further contribute to the tumorigenicity by increasing the metastatic potential of these cancer cells. With numerous activating and inactivating CaSR mutations present in the human population and the increasing prevalence of metastatic cancers, it is essential to elucidate the role of the CaSR-integrin complex in tumour metastasis.

The role of the CaSR/integrin complex in cell migration and homing may be applicable to other cancer and neuronal cells. Perhaps the CaSR/integrin complexes may function as a universal cell migration/homing system for a variety of cell types during CNS development and in tumor metastasis. Our in vivo experiments demonstrated that pharmacological modulation of the CaSR-integrin complex can fine-tune cell migration and homing. Therefore, therapeutic manipulation of this complex may be of potential interest for treating various disorders with aberrant cell migration such as metastatic cancers and developmental disorders pertaining to abnormal neuronal migration.
APPENDIX A. The evolution and pharmacology of the CaSR and Family C GPCRs

The evolutionary role of the CaSR in cell migration may be attributed to its wide nutrient sensing ability. In addition to the CaSR, members of Family C GPCRs contain nutrient-sensing VFT that have evolutionary ties to the multimodal nutrient-sensing bacterial periplasmic proteins. Therefore, I hypothesized that selective evolutionary pressures on Family C GPCR protein structures have resulted in conservation of the orthosteric binding sites which are structurally similar to the nutrient-sensing bacterial proteins. We predicted that the CaSR evolved from a common ancestor with the mGluRs, and diverged early and lost its ability to bind L-glutamate, but obtained the ability to bind aromatic amino acids and various cations. On the contrary, although these receptors share structural similarities in their seven α-helices spanning TMD, selectivity of transmembrane binding allosteric modulators suggest that this domain has largely diverged during evolution. Therefore, we hypothesized that the allosteric modulator sites within the TMD have been subjected to greater divergent evolutionary changes than the orthosteric ligand binding sites situated within the ECD.

The primary objective was to determine the evolutionary divergence of the ECD and TMD domain of the CaSR and the mGluRs. This objective was established by comparing the pharmacological profile of orthosteric agonists and allosteric modulators of the CaSR and the mGluRs.

Results:

The phylogenetic analysis of the ECDs placed the CaSR equidistant to Group1 mGluRs and mgl-2 (Fig. 1.2B). To compare the Ca$^{2+}$ sensitivity of the rat CaSR with mgl-2 and mGluR5, the receptors were exposed to glutamate and/or Ca$^{2+}$. Transiently transfected HEK-293-MSR cells were stimulated and increases in intracellular Ca$^{2+}$ were analyzed on a FlexStation scanning
fluorometer. 10 mM Ca\(^{2+}\) robustly activated the CaSR but was ineffective at mGluR5 and mgl-2 (Appendix A, Fig. 1A). 100 μM L-glutamate strongly activated mGluR5 and mgl-2 but did not activate the rCaSR. To determine whether Ca\(^{2+}\) or L-glutamate could potentiate mgl-2 or CaSR respectively, both receptors were stimulated with a combination of 100 μM L-glutamate and 10 mM Ca\(^{2+}\). Ca\(^{2+}\) did not potentiate glutamate-mediated activation of mgl-2 or mGluR5, and conversely, L-glutamate did not affect Ca\(^{2+}\) stimulated activation of the CaSR (Appendix A, Fig. 1A). Hence, the pharmacological profile of mgl-2 is similar to the Group 1 mGluRs but not the CaSR, demonstrating that the L-glutamate ligand binding sites are conserved among the mammalian Group 1 mGluRs and the \textit{C.elegans} mgl-2, while the CaSR ECD diverged to incorporate binding sites for Ca\(^{2+}\) ions and not L-glutamate.

Phylogenetic dendrogram analysis suggested that the TMDs of the CaSR, mgl-2 and the Group 1 mGluRs are more divergent than the ECD. GPCR transmembrane regions are known to contain the sites of action of allosteric modulators. Here, several negative modulators of the CaSR and Group 1 mGluRs were analyzed in the intracellular Ca\(^{2+}\) assay. mGluR5 specific allosteric antagonists MPEP (20 μM) and fenobam (10 μM) were effective in inhibiting 100 μM L-glutamate mediated activation of mGluR5, but were ineffective in blocking 100 μM mediated stimulation of mgl-2 and mGluR1 (Appendix A, Fig. 1B). The potent mGluR1 specific allosteric antagonists JNJ 16259685 (10 μM) and YM 298198 (10 μM) were effective in inhibiting 100 μM L-glutamate activation of mGluR1 but had no effect on the activation of mgl-2 at 100 μM L-glutamate stimulation (Appendix A, Fig. 1C). In addition, the CaSR specific allosteric negative modulator NPS 8963 at 3 μM and 10 μM, blocked 1.5 mM Ca\(^{2+}\) induced activation of the CaSR, but did not inhibit 100 μM L-glutamate mediated stimulation of mGluR5 and mgl-2 (Appendix A, Fig. 1D). Thus, none of the Group 1 mGluR antagonists that bind to the TMD
were able to block L-glutamate activation of mgl-2, and the CaSR specific negative allosteric modulator was ineffective in inhibiting L-glutamate activation of mGluR5 and mgl-2.

These results demonstrate that the residues important for allosteric modulator antagonist activity are not conserved between the CaSR, Group 1 mGluRs and mgl-2, while the orthosteric ligand binding sites are conserved for mGluRs and mgl-2, but not the CaSR.
Appendix A, Figure 1. Evolutionary divergence of the ECD and TMD of the CaSR with members of the mGluRs.

Transiently transfected HEK-293-MSR cells were stimulated and increases in intracellular Ca\textsuperscript{2+} were analyzed on a FlexStation scanning fluorometer. (A) Summary of the effects of Ca\textsuperscript{2+} and L-glutamate on CaSR, mGluR5 and mgl2. (B – D) Transiently transfected HEK-293-MSR cells were preincubated with modulators for 5 minutes and stimulated with 100 μM L-glutamate or 1.5 mM Ca\textsuperscript{2+}, and increases in intracellular Ca\textsuperscript{2+} were measured. (B) mGluR5 negative modulators MPEP and fenobam inhibited glutamate mediated mGluR5 activation, but did not block glutamate mediated mgl-2 activation. (C) the mGluR1 specific antagonists JNJ 16259685 and YM 298198 inhibited glutamate mediated mGluR1 stimulation, but did not block glutamate mediated mgl-2 activation. (D) The CaSR antagonist NPS 89636 inhibited extracellular Ca\textsuperscript{2+} induced CaSR activation, but did not inhibit glutamate mediated stimulation of mGluR5 or mgl-2. Each bar represents the mean ± S.E.M. of 2 – 3 independent experiments performed in triplicate.
APPENDIX B. CaSR pharmacology

1.1 Results: orthosteric agonist activation and allosteric modulation of the mammalian CaSR

Identification of important pharmacological allosteric modulators of the CaSR led by Dr. Edward F. Nemeth and others have resulted in high affinity and very specific phenylalkylamine calcimimetics such as cinacalcet, NPS R-568, NPS R-467 (positive CaSR allosteric modulators), NPS 2143 and NPS 89636 (negative CaSR allosteric modulators) (Nemeth et al., 1998; Nemeth et al., 2004). These drugs are important tools for understanding and identifying CaSR biology and physiological function.

To study the pharmacological profile of the CaSR, concentration-response curves were generated for the CaSR orthosteric agonist Ca\(^{2+}\) and several allosteric modulators using the intracellular Ca\(^{2+}\) assay. Transiently transfected HEK-293-MSR cells were stimulated with Ca\(^{2+}\) in the presence and absence of CaSR specific allosteric modulators, and increases in intracellular Ca\(^{2+}\) were analyzed on a FlexStation scanning fluorometer.

Human embryonic kidney 293 cells (HEK-293) provide an excellent heterologous system to study CaSR pharmacology due to the ease of growing and transfecting these cells. In particular, HEK-293-MSR cells, which are HEK-293 cells genetically engineered to expresses the human macrophage scavenger receptor (MSR) allows the cells to strongly adhere to standard cell culture plates. The addition of the MSR protein allows the HEK-293-MSR cells to undergo experimental procedures that require extensive washes, similar to the type of intracellular signaling analysis that would be needed to study CaSR signaling and pharmacology. Furthermore, HEK-293-MSR cells do not endogenously express the CaSR, therefore comparison of untransfected and heterologously transfected CaSR-containing plasmids allows for an excellent system to analyze CaSR pharmacology.
Results:

Ca\textsuperscript{2+} concentration-response curves for hCaSR demonstrated an EC\textsubscript{50} value of 1.23 ± 0.22 mM and Hill Slope of 6.41 ± 1.92, similar to the previously reported values (Appendix B, Fig. 1A) (Nemeth et al., 2001). Positive CaSR allosteric modulator NPS R-568 (1μM) potentiated the Ca\textsuperscript{2+} concentration-response curve for hCaSR, with an EC\textsubscript{50} value of 0.72 ± 0.03 mM (Hill Slope = 5.28 ± 2.38), and negative modulation of the CaSR (NPS 89636) decreased affinity for extracellular Ca\textsuperscript{2+} by shifting the Ca\textsuperscript{2+} concentration-response curve EC\textsubscript{50} to 1.69 ± 0.04 mM (Hill Slope = 4.24 ± 1.41). To generate NPS R-568 concentration-response curve for hCaSR, the positive modulator was incubated with an EC\textsubscript{50} Ca\textsuperscript{2+} stimulating concentration of 1.2 mM Ca\textsuperscript{2+} (Appendix B, Fig. 1B). NPS R-568 was a potent positive modulator of the hCaSR, with EC\textsubscript{50} of 238.8 ± 14.2 nM (Hill Slope = 1.01 ± 0.30). CaSR negative allosteric modulator NPS 89636 concentration-response curve was generated by using an EC\textsubscript{80} Ca\textsuperscript{2+} stimulating concentration value of 1.5 mM Ca\textsuperscript{2+} (Appendix B, Fig. 1C). NPS 89636 inhibited 1.5 mM Ca\textsuperscript{2+} mediated rise in intracellular Ca\textsuperscript{2+} with an IC\textsubscript{50} of 121.6 ± 9.1 nM (Hill Slope = -1.49 ± 0.23).

Analysis of the rCaSR Ca\textsuperscript{2+} concentration-response curve demonstrated an EC\textsubscript{50} value of 1.19 ± 0.08 mM (Hill Slope = 8.07 ± 1.09) similar to that of the hCaSR (Appendix B, Fig. 1D). To provide additional controls for pharmacological modulation of the CaSR, positive allosteric modulator NPS R-467 and its less-potent control isomer NPS S-467 were analysed. In the presence of 0.75 mM Ca\textsuperscript{2+}, rCaSR had a 1.5 log order lower affinity for NPS S-467 (EC\textsubscript{50} = 460.5 ± 16.3 μM and Hill Slope = 1.32 ± 0.49) compared to NPS R-467 (EC\textsubscript{50} = 682.3 ± 10.8 nM and Hill Slope = 1.16 ± 0.19) (Appendix B, Fig. 1E). NPS R-467/S-467 concentration-response curves were also generated at 1.8 mM Ca\textsuperscript{2+} to mimic the behaviour of the CaSR in normal cell culture media, which contains 1.8 mM Ca\textsuperscript{2+}. In the presence of 1.8 mM Ca\textsuperscript{2+}, rCaSR had two log order lower affinity for NPS S-467 (EC\textsubscript{50} = 392.4 ± 23.3 μM and Hill Slope = 1.33 ±
Appendix B, Figure 1. Orthosteric agonist activation and allosteric modulation of hCaSR, rCaSR, mGluR1 and CaSR-mGluR1 chimera.

Transiently transfected HEK-293-MSR cells were preincubated with modulators for 5 minutes and stimulated with agonist, and increases in intracellular Ca^{2+} were analyzed on a FlexStation scanning fluorometer. (A) Ca^{2+} concentration-response curves for hCaSR in the presence of control (EC_{50} = 1.30 ± 0.22 mM and Hill Slope = 6.41 ± 1.92), 1 μM NPS R-568 (EC_{50} = 0.72 ± 0.03 mM and Hill Slope = 5.28 ± 2.38) or 120 nM NPS 89636 (EC_{50} = 1.69 ± 0.04 mM and Hill Slope = 4.24 ± 1.41). (B) NPS R-568 concentration-response curve for hCaSR in the presence of 1.2 mM Ca^{2+} (EC_{50} = 238.8 ± 14.2 nM and Hill Slope = 1.01 ± 0.30). (C) Inhibition of 1.5 mM Ca^{2+} mediated rise in intracellular Ca^{2+} by NPS 89636 (IC_{50} = 121.6 ± 9.1 mM and Hill Slope = -1.49 ± 0.23). (D) Ca^{2+} concentration-response curve for rCaSR (EC_{50} = 1.19 ± 0.08 mM and Hill Slope = 8.07 ± 1.09). (E) rCaSR had 1.5 log order lower affinity for NPS S-467 (EC_{50} = 460.5 ± 16.3 μM and Hill Slope = 1.32 ± 0.49) compared to NPS R-467 (EC_{50} = 682.3 ± 10.8 nM and Hill Slope = 1.16 ± 0.19) in the presence of 0.75 mM Ca^{2+}. (F) In the presence of 1.8 mM Ca^{2+}, rCaSR had two log order lower affinity for NPS S-467 (EC_{50} = 392.4 ± 23.3 μM and Hill Slope = 1.33 ± 0.23) compared to NPS R-467 (EC_{50} = 385.1 ± 14.7 nM and Hill Slope = 0.86 ± 0.19). (G) Ca^{2+} activation of chimeric construct consisting of ECD of rCaSR, ligated to mGluR TMD and ICD (EC_{50} = 1.31 ± 0.13 mM and Hill Slope = 3.36 ± 0.56). (H) L-glutamate mediated Ca^{2+} release activation of mGluR1 (EC_{50} = 0.98 ± 0.33 uM and Hill Slope = 2.40 ± 0.72). Each data point represents the mean ± S.E.M. of 3 – 5 independent experiments performed in triplicate.
0.23) compared to NPS R-467 (EC$_{50}$ = 385.1 ± 14.7 nM and Hill Slope = 0.86 ± 0.19) (Appendix B, Fig. 1F).

To delineate the role of the ECM and TMD in CaSR activation, Ca$^{2+}$ activation was performed on a chimeric construct (Ca-Glu-Glu) consisting of the ECD of the rCaSR ligated to the TMD and intracellular tail region of mGluR1 (EC$_{50}$ = 1.31 ± 0.13 mM and Hill Slope = 3.36 ± 0.56) (Appendix B, Fig. 1G). As a control, L-glutamate mediated Ca$^{2+}$ release activation of mGluR1 was also performed (EC$_{50}$ = 0.98 ± 0.33 uM and Hill Slope = 2.40 ± 0.72) (Appendix B, Fig. 1H). Here, activation of the Ca-Glu-Glu receptor with extracellular Ca$^{2+}$ behaved similar to the r/hCaSR in the intracellular Ca$^{2+}$ assay, illustrating that the Ca$^{2+}$ binding sites are located in the ECD of the CaSR.

1.2 Results: CaSR biased signaling

Amino acids such as L-phenylalanine allosterically potentiate the rise in intracellular Ca$^{2+}$ mediated by CaSR activation (Conigrave et al., 2007). Recent studies have demonstrated that GPCRs undergo ligand bias, whereby receptors demonstrate ligand-dependent selectivity for certain signal transduction pathways. Interestingly, in addition to coupling to $G_q$-mediated activation of PLC and intracellular Ca$^{2+}$ release, the CaSR is also known it couple to $G_i$-mediated inhibition of adenylyl cyclase and the subsequent reduction in cyclic AMP (cAMP) production. To determine whether the CaSR demonstrated agonist functional selectivity and biased agonism, Ca$^{2+}$ and L-phenylalanine were analyzed via the cAMP assay. cAMP measurements were determined using Perkin-Elmer’s Alphascreen cAMP functional assay kit, based on the competition between endogenous cAMP and exogenously added biotinylated cAMP. Here, HEK-293-MSR cells transiently transfected with hCaSR or fish 5.24/mouse GPRC6A chimeric receptor (positive control; VFT domain of fish 5.24 fused to the TMD of
mouse GPCR6A) were stimulated with 5 μM forskolin to induce cAMP formation. The HEK-293-MSR cells were preincubated with Ca\textsuperscript{2+}, L-phenylalanine or L-arginine to determine the level of cAMP inhibition. Figure 2A (Appendix B) demonstrates that Ca\textsuperscript{2+} inhibits 5 μM forskolin stimulation with an IC\textsubscript{50} of 2.53 ± 0.31 mM, and Hill slope value of -5.11 ± 0.82. Interestingly, L-phenylalanine inhibited 5 μM forskolin stimulation without the presence of extracellular Ca\textsuperscript{2+}, with an IC\textsubscript{50} of 1.01 ± 0.18 mM (Hill slope value of -2.29 ± 0.62) (Appendix B, Fig. 2B). As a positive control, fish 5.24 orthosteric agonist L-arginine inhibited 5 μM forskolin stimulation with an IC\textsubscript{50} of 0.83 ± 0.19 mM, and Hill slope value of -2.20 ± 0.87 (Appendix, Fig. 2C). Thus, these results illustrate that Ca\textsuperscript{2+} serves as an orthosteric ligand for the CaSR to promote rise in intracellular Ca\textsuperscript{2+} and inhibition of cAMP production. However, L-phenylalanine is an allosteric potentiator of the CaSR in mediating increases in intracellular Ca\textsuperscript{2+}, whereas it is an orthosteric agonist for the CaSR in coupling to the adenylyl cyclase and cAMP pathway. Therefore, the CaSR agonists demonstrate functional selectivity for the CaSR depending on the signal transduction pathway.

1.3 Summary, discussion, and conclusions of CaSR pharmacological studies

The Ca\textsuperscript{2+} concentration-response curves for the rCaSR and hCaSR validated previous findings that the CaSR binds to Ca\textsuperscript{2+} with low affinity (mM EC\textsubscript{50} values) and high positive cooperativity (please refer to Appendix B, Table 1 for summary) (Miedlich et al., 2002; Brown, 2007). Hill coefficient analysis of the rCaSR and hCaSR illustrated that the CaSR has 6 to 8 binding sites for Ca\textsuperscript{2+}. Furthermore, analysis of the Ca-Glu-Glu CaSR-mGluR1 chimeric receptor (consisting of the ECD of the rCaSR ligated to the TMD and ICD of mGluR1) demonstrated that ECD contains Ca\textsuperscript{2+} binding sites, since activation of Ca-Glu-Glu with Ca\textsuperscript{2+} resulted in EC\textsubscript{50} values similar to rCaSR. Furthermore the Ca-Glu-Glu Ca\textsuperscript{2+} concentration-response Hill slope of 4 suggests that
Appendix B, Figure 2. Inhibition of 5 μM forskolin induced cAMP formation.

HEK-293-MSR cells transiently transfected with hCaSR or fish 5.24/mouse GPRC6A (F/M) chimeric receptor were incubated with 0.2 mM Ca²⁺ and activated with various concentrations of Ca²⁺ (A), L-phenylalanine (B) or L-arginine (C) for 5 minutes, then stimulated by 5 μM forskolin, and cAMP measurements were determined. Concentration-response curve was generated using GraphPad. ★ represents 5 μM forskolin stimulation with 0.2 mM Ca²⁺ inhibition (A and B), or no L-arginine inhibition (C), while each data point (●) denotes mean ± S.E.M. of 4 – 5 independent experiments, determined by normalizing each point to the alphascreen signal obtained with 5 μM forskolin. (A) Ca²⁺ inhibits 5 μM forskolin stimulation with an IC₅₀ of 2.53 ± 0.31 mM, and Hill slope value of -5.11 ± 0.82. (B) L-phenylalanine inhibits 5 μM forskolin stimulation with an IC₅₀ of 1.01 ± 0.18 mM, and Hill slope value of -2.29 ± 0.62. (C) L-Arg inhibits 5 μM forskolin stimulation with an IC₅₀ of 0.83 ± 0.19 mM, and Hill slope value of -2.20 ± 0.87.
two of the Ca$^{2+}$ binding sites of the CaSR may be located in the TMD, since comparison of the Ca$^{2+}$ concentration-response curves for r/hCaSR revealed Hill slope values of 6 to 8. Of particular note is the observation that for inhibition of cAMP the Hill slope is approximately 6, similar to the Ca$^{2+}$ assay. In contrast the Hill slope is about 2 for both L-phenylalanine at CaSR and for L-arginine at the 5.24/mouse GPRC6A chimeric receptor. Here, the concept of 6 to 8 cation binding sites and 2 amino acid sites per functional receptor complex could fit well into several models that consider the quaternary oligomeric structure and G-protein coupling arrangements (Ferre et al., 2014). Thus, a dimeric (or oligomeric) CaSR may function in a complex to promote cooperative binding of multiple Ca$^{2+}$ ions and two amino acid binding sites per complex.

The allosteric modulator concentration-response curves generated for the CaSR revealed that NPS R-568 is more potent than NPS R-467 in potentiating CaSR activation, while NPS S-467 is a suitable control for NPS R-467 since NPS S-467 has a 1000-fold lower affinity for the CaSR compared to NPS R-467. In addition, analysis of CaSR negative modulator revealed that NPS 89636 is a potent inhibitor of the CaSR. Importantly, the Hill coefficient analysis of the above four CaSR allosteric modulators demonstrated values of approximately 1, indicating a single binding site distinct from Ca$^{2+}$ binding.

CaSR analysis of L-phenylalanine using the cAMP assay demonstrated that L-phenylalanine demonstrated functional selectivity; L-phenylalanine is an allosteric potentiator of the CaSR for the Ca$^{2+}$ release pathway, while it is an orthosteric agonist for the cAMP pathway. The identification of L-phenylalanine in biased signaling provides an example of the complexity in understanding CaSR pharmacology and signaling since the CaSR is activated/modulated by a variety of agonists and is known to couple to a multitude of signaling pathways. Each CaSR agonist/modulator may adopt a unique receptor conformation that preferentially activates certain
signaling mechanisms over another pathway, making receptor efficacy characterization a very difficult and multidimensional process to understand. However, identifying such complex pharmacology directed signaling pathways for GPCRs like the CaSR provides intriguing possibility that cellular signaling can be controlled with unprecedented precision and specificity. Development of this theme could provide new classes of therapeutic agents with fewer side effects (Reiter et al., 2012).
Appendix B, Table 1. CaSR pharmacology summary in transiently transfected HEK-293-MSR cells.

<table>
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<th>Transient Transfected HEK-293-MSR Cells</th>
<th>Assay</th>
<th>Receptor</th>
<th>Agonist</th>
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<th>EC$<em>{50}$/IC$</em>{50}$</th>
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<td>5.28 ± 2.38</td>
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<td>3</td>
<td>460.5 ± 16.3 μM</td>
<td>1.32 ± 0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rCaSR</td>
<td>[NPS R-467] + 1.8 mM Ca$^{2+}$</td>
<td>3</td>
<td>385.1 ± 14.7 nM</td>
<td>0.86 ± 0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rCaSR</td>
<td>[NPS S-467] + 1.8 mM Ca$^{2+}$</td>
<td>3</td>
<td>392.4 ± 23.3 μM</td>
<td>1.33 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Ca-Glu-</td>
<td></td>
<td>[Ca2+]</td>
<td>4</td>
<td>1.31 ± 0.13 mM</td>
<td>3.36 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>mGluR</td>
<td></td>
<td>[L-Glu]</td>
<td>4</td>
<td>0.98 ± 0.34 uM</td>
<td>2.40 ± 0.72</td>
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</tr>
<tr>
<td>cAMP Inhibition Assay (PerkinElmer Alphascreen)</td>
<td>hCaSR</td>
<td>[Ca2+]</td>
<td>4</td>
<td>2.53 ± 0.31 mM</td>
<td>-5.51 ± 0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hCaSR</td>
<td>[L-Phe]</td>
<td>4</td>
<td>1.01 ± 0.18 mM</td>
<td>-2.29 ± 0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fish 5.24/ mgprc6</td>
<td>[L-Arg]</td>
<td>3</td>
<td>0.83 ± 0.19 mM</td>
<td>-2.20 ± 0.87</td>
<td></td>
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Ref Type: In Press


Ref Type: Edited Book


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2. The study described in Appendix B and Figure 1.2 was published in September 2012 issue of Neuropharmacology. Copyright 2012, Elsevier.