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GPR37& GPR37L1: LIGAND IDENTIFICATION, CELL PROTECTIVE SIGNALING & DOPAMINERGIC CROSS-TALK

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By

Rebecca Christine Meyer

B.S. Creighton University 2007

Advisor: Randy A. Hall

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Abstract

GPR37 & GPR37L1: LIGAND IDENTIFICATION, CELL PROTECTIVE SIGNALING & DOPAMINERGIC CROSS-TALK

by

Rebecca Christine Meyer

G protein-coupled receptors (GPCRs) are essential to cellular communication in the central nervous system and constitute one of the largest classes of drug targets in the body. The ligands for many GPCRs are known, but there remains a subset of orphan GPCRs for which ligands have yet to be identified. These orphan receptors have the potential to be novel therapeutic targets; however, identification of endogenous ligands for orphan receptors is a crucial step toward targeting these receptors with therapeutics. Here, we present evidence that the orphan GPCRs GPR37 and GPR37L1 can be activated by prosaposin, a neuroprotective and glioprotective factor, as well as by the active fragment of prosaposin, prosaptide.

We found that prosaptide and prosaposin bind to GPR37 and GPR37L1 to promote receptor signaling and internalization. Prosaptide and prosaposin stimulation of cells transfected with GPR37 or GPR37L1 induced significant increases in ERK phosphorylation and reductions in cAMP levels in a pertussis toxin-sensitive manner, indicating that the receptors are Gαi-coupled. Work in primary cultured astrocytes in which GPR37 and/or GPR37L1 were depleted via siRNA knockdown indicated that prosaptide induces ERK phosphorylation through stimulation of endogenous GPR37. Additionally, we identified that GPR37-mediated ERK phosphorylation occurs through transactivation of the EGF receptor. Furthermore, we found that both GPR37 and GPR37L1 contribute to the ability of prosaptide and prosaposin to protect cortical astrocytes against oxidative cell death.

In additional studies examining receptor cross-talk with the dopaminergic system, we identified a physical interaction between GPR37L1 and the dopamine D1 receptor. Furthermore, co-expression of these receptors altered D1-mediated dopamine stimulation of cAMP and pERK, indicating the potential for GPR37L1 to regulate dopaminergic signaling. In conclusion, the work in this dissertation describes both ligand-dependent and potentially ligand-independent functions for GPR37 and GPR37L1 as protective receptors with the ability to influence dopaminergic function. Their ligand, prosaposin, has previously been shown to be neuroprotective in animal models of Parkinson's disease and focal cerebral ischemia, and has also been shown to promote nerve remyelination after injury. Thus, establishing GPR37 and GPR37L1 as receptors for prosaptide and prosaposin identifies novel therapeutic targets for the treatment of neurodegeneration and myelination disorders.

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LIST OF ABBREVIATIONS

6-OHDA..... 6-Hydroxy-Dopamine

AC ..... Adenylyl Cyclase

Akt ..... Protein Kinase B

AR ..... Adrenergic Receptor

AR-JP ..... Autosomal Recessive Juvenile Parkinson's disease

BIM ..... Bisindolylmaleimide

BSA..... Bovine Serum Albumin

CAMKII..... Calcium/ calmodulin-dependent protein kinase II

cAMP ..... Cyclic Adenosine Monophosphate

CART..... Cocaine- and Amphetamine - Regulatory Transcript

Co-IP ..... Co-Immunoprecipitation

DA..... Dopamine

DAT ..... Dopamine Transporter

DMEM ..... Dulbecco's Minimal Eagle Medium

ECL ..... Enhanced Chemiluminescence

EDTA .. Ethylenediamin-Etetraacetic Acid

EGF ..... Epidermal Growth Factor

EGFR..... Epidermal Growth Factor Receptor

ERK..... Extracellular Signal-Regulated Kinase

ETB .....Endothelin B Receptor

FBS..... Fetal Bovine Serum

GALT ..... Galactose-1-Phosphate Uridylyltransferase

GLD..... Globoid Cell Leukodystrophy

GPCR .....G Protein-Coupled Receptor

GRK ..... G Protein-Regulated Kinase

GPR37L1.....GPR37 Like-1

H_2O_2 ..... Hydrogen Peroxide

HA .....Head Activator

HB-EGF ..... Heparin Binding EGF-like Growth Factor

HEK ..... Human Embryonic Kidney

HEPES ..... (4 (2-hydroxyethyl)-1-piperazineethanesulfonic acid

Het..... Heterozygous

IBMX 3-isobutyl-1-methylxanthine

IP .....Immunoprecipitation

KD.....Knockdown

KO.....Knockout

LDH .....Lactate Dehydrogenase

LDL.....Low-Density Lipoprotein

L-DOPA....L-3,4-dihydroxyphenylalanine

LPA..... Lysophosphatidic acid

LRP-1.... low density lipoprotein receptor-related protein 1

MAOB..... Monoamine oxidase B

MDD ..... Major Depressive Disorder

MMP .....Matrix Metalloproteinase

mMSC -NPC..... marrow stromal cell-derived neuroprogenitor cells

MPTP .....

.....1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Mrg.....Mas-related Gene

MS..... Multiple Sclerosis

MT.....Melatonin

Pael-R... Parkin- Associated Endothelin-Like Receptor

PAGE ..... Polyacrylamide Gel Electrophoresis

PBS.....Phosphate Buffered Saline

PD.....Parkinson's disease

PDZ .....

... Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zona occludens-1 protein (zo-1)

PDL ..... Poly- D- Lysine

pERK..... Phospho-Extracellular Signal-regulated Kinase

PFA .....Para-Formaldehyde

PI3K ..... Phosphatidylinositide 3-Kinase

PKC..... Protein Kinase C

PLG..... L-prolyl-L-leucyl-glycinamide

PSAP.....Prosaposin

PTX.....Pertussis Toxin

RTK.....Receptor Tyrosine Kinase

WT ..... Wild Type

SDS .....Sodium Dodecyl Sulfate

SGP-1 .....Sulfated Glycoprotein-1

SOC.....Superior Olivary Complex

SP1-7 ..... Substance P Fragment 1-7

Tris .....Tris (hydroxymethyl) Aminomethane

β-arr.....β-arrestin

Chapter 1:

Introduction to G Protein-Coupled Receptor Function and Signaling

Section 1.1 G Protein-coupled Receptors

1.1.1 GPCR Signaling

All cells require a means to detect external stimuli and facilitate cell-cell communication to ensure the health and survival of the organism. There are a plethora of extracellular molecules that function as environmental indicators including biogenic amines, amino acids, peptides, chemokines, lipids, nucleotides, hormones, as well as olfactory and gustatory molecules (Civelli et al., 2013). To exert their effects, these molecules must bind to a cell surface receptor, which can then transduce the information into an intracellular signal. The largest and most diverse family of cell surface receptors are G protein-coupled receptors (GPCRs), which are able to facilitate cell-cell communication for a variety of molecules and, in doing so, represent a large component of cellular signaling.

G protein-coupled receptors are structurally characterized through their shared feature of a seven-transmembrane region of the receptor as well as an extracellular N terminus and an intracellular C terminus. There are 5 major families of human GPCRs: the rhodopsin family, the adhesion family, the glutamate family, the secretin family, and the frizzled/taste2 family (Fredriksson et al., 2003). Although the seven-transmembrane region is similar between families, the extracellular N terminus and extracellular loops vary widely both between and within families, leading to incredible diversity of receptors and the molecules to which they are able to bind (Katritch et al., 2013).

To facilitate intracellular signaling, GPCRs typically bind to an extracellular molecule, also known as a ligand, which induces a conformational change in the receptor eliciting a downstream signaling cascade. This intracellular signaling cascade is initiated by interaction of the receptor with cytoplasmic signaling intermediates such as heterotrimeric G proteins. The G protein complex consists of 3 subunits: α, β, and γ. Upon the binding of an extracellular ligand,

the GPCR increases its affinity for G proteins allowing the receptor to bind G proteins and facilitate the exchange of GTP for GDP on the Gα subunit. This guanine nucleotide exchange promotes the dissociation of Gα from Gβγ and the subunits are released to promote intracellular signaling (Pierce et al., 2002). Although there is also great diversity in the number of G protein subunits, most Gα subunits fall into four classes with unique downstream effectors, and therefore Gα subunits are often used to classify the receptors to which they bind. GPCRs that bind Gαs stimulate adenylyl cyclase (AC) to produce cylic adenosine monophosphate (cAMP), Gαi-coupled receptors inhibit adenylyl cyclase, Gαq-coupled receptors activate phospholipase C resulting in calcium stimulation, and Gα12/13-coupled receptors stimulate RhoGEF (Figure 1-1).

Section 1.1.2 GPCRs as Pharmaceutical Targets

Approximately forty percent of all drugs in current clinical use act on GPCRs (Lagerstrom and Schioth, 2008). GPCRs are outstanding drug targets for a variety of reasons, including their localization at the cell surface, their natural propensity for being activated by small molecules (neurotransmitters, hormones, sensory stimuli, etc.), and in many cases their discrete patterns of distribution in the body, which can allow for tissue-specific targeting of therapeutics. The expression of GPCRs on the surface of the cell allows small molecules to affect the cell without having to cross the plasma membrane. As most pharmaceuticals are developed to mimic endogenous hormones, peptides, and proteins, they are therefore are unable to cross the plasma membrane and must bind to a cell surface receptor to exert their effects.

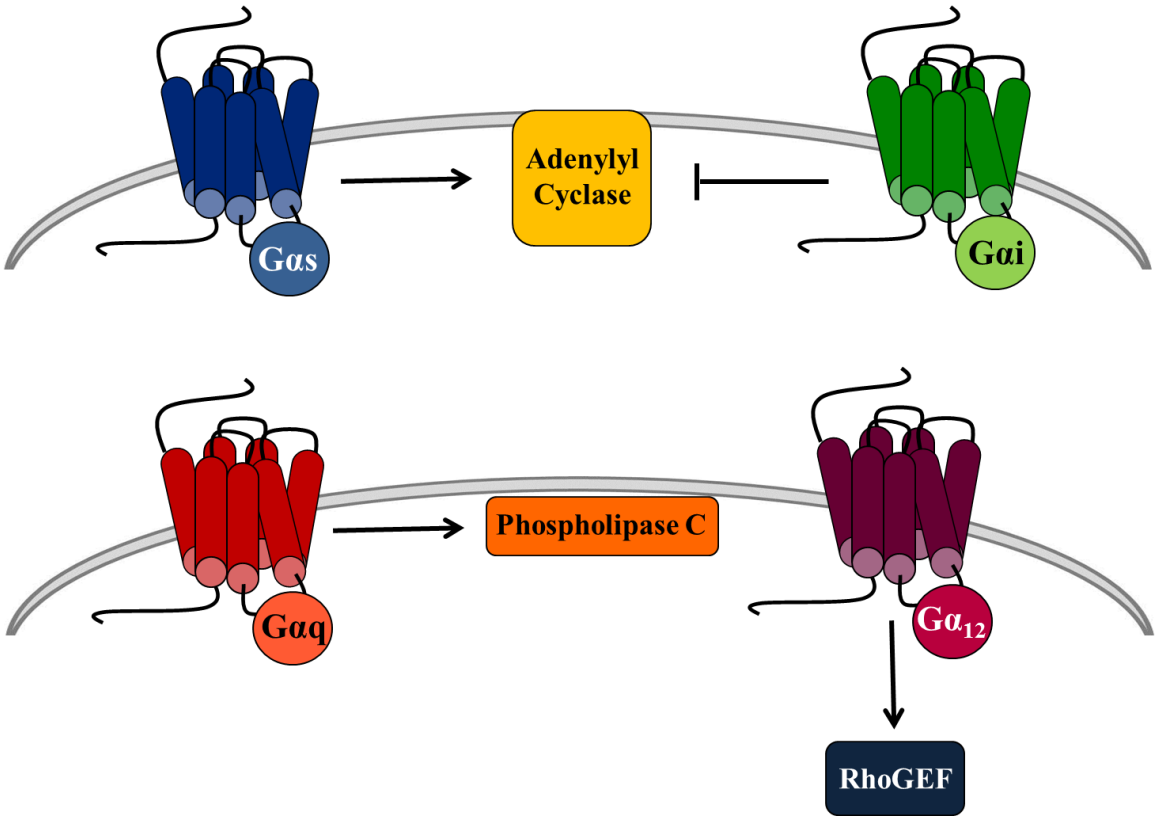


Figure 1-1 GPCRs couple to four main classes of G proteins

The G protein profile of the approximately 800 human GPCRs can be divided into four broad groups, with certain exceptions. These groups are 1) Gαs-coupled receptors which stimulate adenylyl cyclase, 2) Gαi-coupled receptors which inhibit adenylyl cyclase, 3) Gαq-coupled receptors which stimulate phospholipase C, and 4) Gα12/13-coupled receptors which stimulate Rho-GEF

Additionally, GPCRs signal through second messenger systems where in many cases the signal is amplified as it continues through the cascade. This signaling amplification allows a single receptor to have a profound effect on the cell in a relatively rapid manner. Furthermore, GPCRs and their signaling pathways are highly regulated by intracellular proteins to create a unique spatial and temporal signaling pattern with many points for regulation.

One example of the complexity of GPCR signaling with multiple regulation points is the ability of Gαi-coupled receptors to signal through extracellular signal-related kinase (ERK) (Figure 1-2). This signaling pathway is promoted in two ways. First, the Gαi subunit inhibits adenylyl cyclase which removes protein kinase A inhibition of c-Raf, allowing Ras to induce the signaling cascade to phosphorylate ERK (Radhika and Dhanasekaran, 2001). Additionally, the βγ subunit of the Gαi-coupled receptor is able to activate phosphoinositide-3-kinase (PI3K), which activates Ras as a downstream effector, again initiating pERK signaling. Furthermore, the βγ subunit is able to activate phospholipase C-β which in turn stimulates calcium release and initiates a Src and Shc signaling cascade capable of activating Ras to induce ERK phosphorylation (Goldsmith and Dhanasekaran, 2007) (Figure 1-2A).

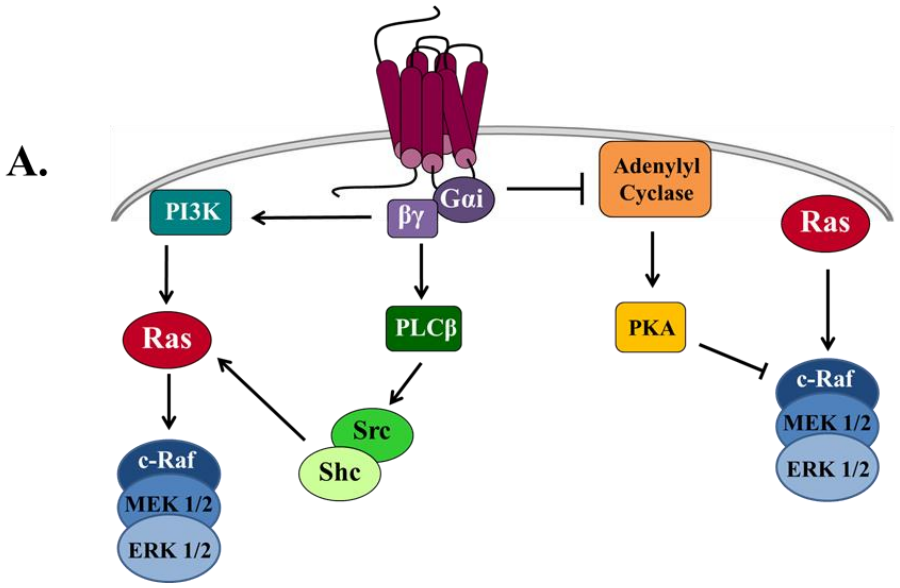
Furthermore, many GPCRs stimulate ERK phosphorylation through transactivation of receptor tyrosine kinases (RTKs) (Daub et al., 1997, Shah and Catt, 2004, George et al., 2013). In this way, ligand binding to a GPCR is able to induce intracellular signaling that causes the activation of an RTK which stimulates its downstream signaling cascade (Figure 1-2B). Although many RTKs can undergo transactivation, one especially prominent example is the epidermal growth factor receptor (EGFR). EGFR is expressed in many cell types in various brain areas including the cerebral cortex, hippocampus, and cerebellum, where it plays roles in multiple cellular functions such as cell proliferation, migration, and survival (Yamada et al., 1997, Jorissen et al., 2003). EGFR transactivation has been reported for GPCRs including the lysophosphatidic acid receptor (Cunnick et al., 1998), the angiotensin II receptor (Moriguchi et al., 1999,

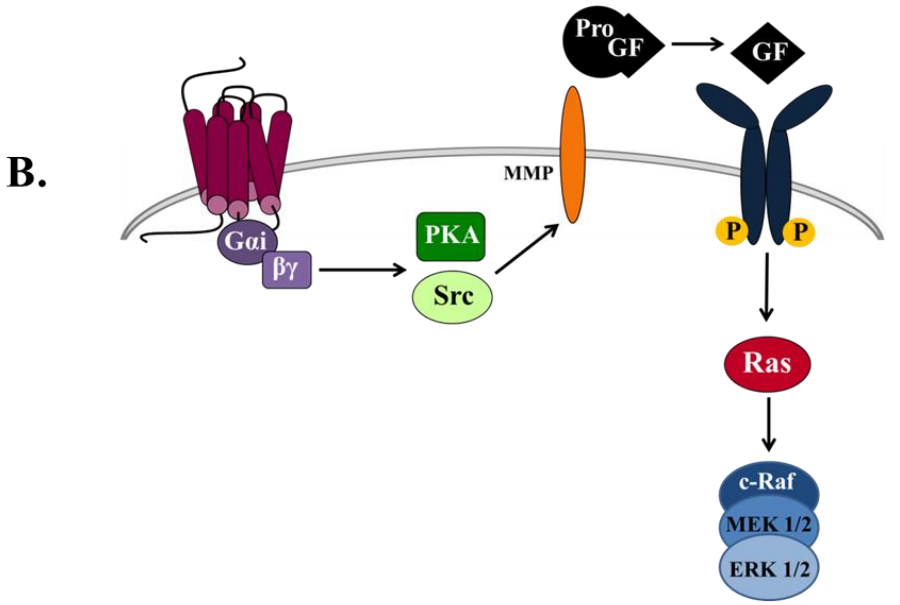
Bokemeyer et al., 2000, Eguchi et al., 2001), and the endothelin receptors (Vacca et al., 2000) indicating that this cross talk is essential for cellular communication. Furthermore, it has been reported that GPCR transactivation of EGFR often involves other intracellular mediators such as Src, protein kinase C (PKC), and calcium mobilization that stimulates metalloprotease proteolytic cleavage of pro-heparin-binding EGF precursor ((HB)-EGF) into EGF, which then binds to and activates EGFR (Daub et al., 1997, Prenzel et al., 1999).

Finally, GPCRs are able to bind a class of intracellular proteins known as β-arrestins to induce ERK phosphorylation by directly linking the GPCR to ERK machinery (Quan et al., 2008, Cervantes et al., 2010). Furthermore, it has been shown that some GPCRs, such as the μ-opioid receptor, are able to activate ERK phosphorylation through two distinct mechanisms. Short-term ligand stimulation of the mu-opioid receptor (2 minutes) causes ERK to be activated and translocated to the nucleus through a PKC-dependent mechanism while longer ligand stimulation (10 minutes) induces β-arrestin2-mediated ERK phosphorylation, with the kinase remaining cytosolic (Zheng et al., 2008). This use of multiple cascades to reach a common intracellular activator protein allows select GPCRs to exert control over a variety of signaling pathways, creating a complex signaling mosaic to exert a functional effect. The complexity and intricacy of the highly regulated GPCR signaling pathways indicates not only an important role for the receptors in normal human physiology, but also multiple points of intervention for pathological treatment.

GPCRs are also regulated in a number of ways, most rapidly through the phosphorylation of the GPCR by G protein-coupled receptor kinases (GRKs) and the subsequent binding of β-arrestins, which promotes the endocytosis of the receptor from the cell surface. While the binding of β-arrestins is able to induce ERK phosphorylation through bringing ERK machinery directly into contact with the receptor, it also effectively prevents G protein-mediated signaling by creating steric hindrance which prevents the GPCR from further interactions with G proteins

(Pierce et al., 2002). Furthermore, only a receptor that has previously bound a ligand is able to become desensitized in this manner, adding specificity to this mechanism of receptor regulation (Pierce et al., 2002). GPCRs can be downregulated through intracellular protein interactions, a type of regulation that occurs over a longer timeframe. Following prolonged receptor stimulation, GPCRs can become ubiquitinated and degraded through trafficking to the lysosome, however both ubiquitin and β-arrestin can also modulate signaling cascades without inducing receptor internalization and degradation (Pierce et al., 2002, Alonso and Friedman, 2013).


A.
PI3K
βγ
Gαi
Adenylyl Cyclase
Ras
Ras
PLCβ
PKA
c-Raf
MEK 1/2
ERK 1/2
Src
Shc
c-Raf
MEK 1/2
ERK 1/2


B.
Pro GF
GF
MMP
Gαi
βγ
PKA
Src
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c-Raf
MEK 1/2
ERK 1/2

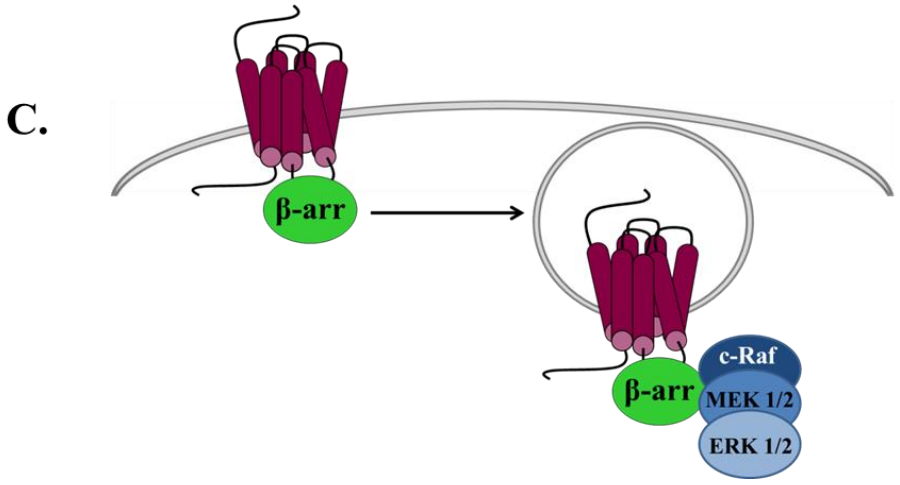

C.
β-arr
β-arr
c-Raf
MEK 1/2
ERK 1/2

Figure 1-2 The activation of pERK through Gαi-coupled receptors

A) GPCRs are able to activate intricate intracellular signaling pathways. These pathways overlap and are subject to regulation at many points. One example of the diversity of intracellular signaling is the activation of pERK through the Gαi-coupled receptor. This receptor is able to stimulate pERK through different mechanisms involving different G protein subunits. The Gαi subunit inhibits adenylyl cyclase thus removing the basal inhibition of Ras which is able to stimulate pERK. Additionally, the βγ subunits are able to activate PI3 Kinase and PLCβ which can signal through Ras as well to induce ERK phosphorylation (Figure adapted from Goldsmith & Dhanasekaran, 2007). B) GPCRs are able to induce ERK phosphorylation through transactivation of an RTK. This is mediated through an intracellular signaling cascade induced by the βγ subunits of the GPCR which leads to matrix metalloproteinase cleavage of pro-growth factors into growth factors which then bind to the RTK, causing phosphorylation and Ras activation of ERK. C) GPCRs are able to induce ERK phosphorylation through binding to β-arrestin which causes endocytosis of the GPCR and mediates the binding of the ERK machinery to the receptor, directly inducing ERK phosphorylation.

Section 1.1.3 GPCR Heterodimerization as a Mechanism for Signaling Regulation

Another way of regulating GPCR signaling and function is through dimerization of the receptors. It is thought that GPCRs can form functional homodimers with two of the same receptors physically interacting, or heterodimerization of two unrelated receptors to create a novel signaling entity. Dimerization can have multiple effects of GPCR signaling and function, ranging from being required for function, enhancing function, or altering signaling pathways. One example of dimerization requirement for function is the example of the $GABA_B$ receptors, $GABA_{B1}$ and $GABA_{B2}$. The dimerization between these two receptors is necessary for their function (Jones et al., 1998, White et al., 1998) and this observation was further refined to include the understanding that the $GABA_{B2}$ receptor interacts with the $GABA_{B1}$ receptor to mask an ER retention motif on $GABA_{B1}$, allowing the trafficking of the dimer to the cell surface where it can act as a functional signaling receptor (Margeta-Mitrovic et al., 2000).

Other examples of functional heterodimers are those that occur between two different receptors that function as individual receptors but are also able to take on new signaling and functional properties when they bind together. Heterodimers can form between receptors from the same family as well as unrelated receptors. The first example of related receptor heterodimerization was the δ- and κ-opioid receptors which take on different properties when they physically interact and synergistically bind ligands (Jordan and Devi, 1999). Additionally, the first reported example of heterodimerization between unrelated receptors was the dimerization of the somatostatin 5 receptor and dopamine D2 receptor, in which dimerization produced enhancement in ligand binding and signaling (Rocheville et al., 2000).

Furthermore, heterodimerization between two receptors can alter fundamental properties of the receptors. This can occur through altering the G protein binding of the receptors in the heterodimer. It has been suggested that when two receptors form a heterodimer, the receptors

collectively couple to a G protein that binds to the pair rather than each receptor binding its own G protein. One example of this is the dimerization of the dopamine D1 and D2 receptors. When D1 is expressed alone, it binds Gαs and stimulates cAMP production(Collier et al., 1983, Maeno et al., 1983). Conversely, D2 expressed alone binds to and signals through Gαi to inhibit cAMP production (Missale et al., 1998). However, when the two receptors are co-expressed and heterodimerize, they collectively bind Gαq and stimulate calcium/calmodulin-dependent kinase II (CAMKII) (Rashid et al., 2007). This strongly suggests that both receptors bind a single G protein when in complex, indicating that dimerization fundamentally alters their G protein coupling profile and downstream signaling pathways.

Section 1.1.4 Orphan G Protein-coupled Receptors

The advent of molecular cloning, including the Human Genome Project, has resulted in the identification of genes coding for proteins that have the characteristics of G protein-coupled receptors, but yet do not have a known ligand (Stadel et al., 1997). The first of these GPCRs to be cloned but not matched to a ligand was a protein known as G-21, originally identified through a genetic screen (Kobilka et al., 1987) and then "deorphanized" as the 5-HT_{1A} receptor through screening for potential ligand binding (Fargin et al., 1988). There are still more than 100 GPCRs with unknown ligands, and these so-called orphan receptors represent a large pool of potential targets for novel therapeutics (Civelli et al., 2006, Civelli et al., 2013). Traditionally, ligands and receptors were matched by purifying a ligand with a known function and then using that ligand to screen for the receptor mediating the effects (Stadel et al., 1997). However, with the advent of orphan receptors, the receptors themselves are often the starting point for discovery, and an alternative method, known as "reverse pharmacology" is employed to "deorphanize" orphan GPCRs. In this method, an orphan receptor with no known function is cloned from a DNA

library and expressed in a heterologous recombinant system to be used as bait to screen for ligands. A variety of assays may be employed to identify a potential ligand, screening for both signaling pathways and physiological effects (Stadel et al., 1997, Ozawa et al., 2010).

To identify potential ligands, orphan receptors can be screened against large libraries of potential GPCR ligands including peptides, lipids, and hormones. These high-throughput screens have been effective at identifying ligands for orphan receptors, although the rate of receptor deorphanization has slowed considerably in recent years (Civelli et al., 2006). There are a number of reasons for the decline, including difficulty in identifying signaling cascades utilized by orphan GPCRs, the potential for non-signaling roles of orphan GPCRs, and also issues with heterologous expression and trafficking of orphan GPCRs in a model system appropriate to screen ligands (Levoye et al., 2006b, Dunham and Hall, 2009)

Potential ligands can also be predicted through analysis of expression patterns and/or knowledge of physiological significance (Wise et al., 2004). An example of this method includes the identification of high and low affinity receptors for nicotinic acid (Wise et al., 2003, Tunaru et al., 2003). In addition to the necessity of choosing how potential ligands will be identified, the use of reverse pharmacology to identify ligand-receptor pairs hinges on the ability of the receptors to be properly expressed and trafficked in the heterologous expression systems that are typically utilized screen ligands (Mills and Duggan, 1994, Dunham and Hall, 2009). To allow analysis of total and cell surface expression, antibodies against the orphan receptor can be developed; alternatively, receptors can be generated with epitope tags expressed on the N-terminus, allowing for a more universal approach to examining receptor trafficking (Wise et al., 2004). Finally, the use of molecular chaperones or interacting proteins can be used to help facilitate receptor cell surface expression in heterologous recombinant systems (Dunham and Hall, 2009). Screening orphan receptors for potential ligands can be an arduous process, but one that is helped by identifying criteria to streamline the screens. A major approach to speed this process is the

identification of characteristics of various orphan receptors that might provide clues as to identities of their ligands.

Section 1.2 GPR37 and GPR37L1

Section 1.2.1 Expression and Receptor Homology

GPR37 and GPR37L1 are a pair of particularly intriguing orphan GPCRs that have been studied intensively with regard to Parkinson's disease. Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States, affecting approximately 1% of Americans over the age of 65 and 4% of the population over the age of 80 (Olanow and Tatton, 1999, Nussbaum and Ellis, 2003) and is identified through pathological indicators, the most pronounced of which is the loss of dopaminergic neurons from the substantia nigra, and in some cases, the development of aggregates known as Lewy bodies (Olanow and Tatton, 1999, Nussbaum and Ellis, 2003). While most PD is idiopathic, there are genetic mutations linked to the development of PD. One widely studied mutation is the mutation in *PARK2* which encodes parkin (Olanow and Tatton, 1999, Imai et al., 2000, Toda et al., 2003, Hasegawa et al., 2008, Dawson and Dawson, 2010). Parkin is an E3 ubiquitin ligase that is believed to function mainly to designate proteins for degradation via the ubiquitin-proteosome system (Imai et al., 2000, Xiong et al., 2009, Dawson and Dawson, 2010).

GPR37 and GPR3L1 are most closely related to the Endothelin B receptor with which GPR37 shares a 52% similarity and a 27% identity (Zeng et al., 1997). They are found mainly in the central nervous system (Zeng et al., 1997, Marazziti et al., 1997, Marazziti et al., 1998, Donohue et al., 1998, Valdenaire et al., 1998, Leng et al., 1999, Imai et al., 2001) and are expressed in multiple cell types including neurons and glia (Marazziti et al., 1997, Zeng et al.,

1997, Marazziti et al., 1998, Valdenaire et al., 1998, Leng et al., 1999, Imai et al., 2001, Cahoy et al., 2008). GPR37 is alternatively identified as the Parkin-Associated Endothelin-Like receptor (PAEL-R) (Imai et al., 2001), hET_BR-LP (Zeng et al., 1997), and GPCR/CNS1 (Leng et al., 1999). GP37L1 is named for its homology with GPR37 as the receptors share a 68% similarity and 48% identity (Valdenaire et al., 1998), and has been alternatively named ET_BR-LP-2 (Valdenaire et al., 1998), and GPCR/CNS2 (Leng et al., 1999) in keeping with it being named as a second member of the GPR37 family.

At the RNA level, GPR37 is expressed in multiple areas of the brain including the amygdala, caudate nucleus, corpus callosum, cortex, hippocampus, hypothalamus, putamen, substantia nigra, subthalamic nuclus, and thalamus (Zeng et al., 1997, Marazziti et al., 1997). Additionally, GPR37 appears to be fairly specific as a CNS-expressed protein, with the only peripheral RNA detection occurring in the testes, liver and placenta (Zeng et al., 1997, Marazziti et al., 1997, Marazziti et al., 1998). Further work using in situ hybridization in the cerebellum identified GPR37 as strongly expressed in the Purkinje cells (Zeng et al., 1997). Northern blot analysis found GPR37L1 RNA is concentrated in the cerebellum, cortex, brainstem, spinal cord, and putamen while expressed at low levels through all lobes of the brain, however no detectable signal was identified in various peripheral tissues (Valdenaire et al., 1998). In situ hybridization of human tissue further identified high levels of expression in the cerebellum, particularly Bergmann glia (Valdenaire et al., 1998) creating distinction from GPR37, which as mentioned above is expressed in Purkinje cells.

Since GPR37 and GPR37L1 exhibit their strongest sequence similarity to the endothelin receptors and other GPCRs activated by peptides, it has been viewed as likely that these receptors must be peptide-activated. Indeed, it has been reported that GPR37 can bind to a neuropeptide known as “head activator” (HA) (Rezgaoui et al., 2006, Gandia et al., 2013), which is derived from the invertebrate *Hydra*. However, following a handful of reports three decades ago about

the potential existence of an HA ortholog in mammalian brains (Bodenmuller et al., 1980, Bodenmuller and Schaller, 1981) no further evidence for an HA ortholog in vertebrates has come to light. Furthermore, some groups attempting to replicate the use of HA to activate GPR37 have been unsuccessful (Dunham et al., 2009, Southern et al., 2013). Additionally, groups have explored the possibility that GPR37 and/or GPR37L1 might be activated by endothelins or endothelin-related peptides, but all studies of this type have yielded negative results (Zeng et al., 1997, Valdenaire et al., 1998).

Section 1.2.2 Physiological Function and Role in Neuropathology

GPR37 first attracted interest due to its identification as a substrate of the E3 ubiquitin ligase parkin (Imai et al., 2001), which, as mentioned, is the product of a gene that plays a role in PD pathology. Due to the connection between parkin and GPR37, much of the early work on GPR37 focused on the propensity of this receptor to misfold in the absence of parkin or in overexpression models to ultimately induce cell stress and degeneration (Imai et al., 2001, Yang et al., 2003, Kitao et al., 2007, Wang et al., 2008a, Dusonchet et al., 2009, Marazziti et al., 2009). Also, upregulation of GPR37 occurs in a dopaminergic cell line exposed to MPP+, the active metabolite of MPTP (Wang et al., 2008b). In addition to its link to the autosomal recessive juvenile form of Parkinson's disease associated with parkin dysfunction, GPR37 is further linked to sporadic Parkinson's disease through its presence in Lewy bodies (Murakami et al., 2004). This may suggest that GPR37 has a role in promoting the progression of Parkinson's disease.

In addition to its potential role in PD pathology, GPR37 has also been found to be of importance for the normal function of the dopaminergic system, as GPR37-deficient mice exhibit a lower dopaminergic tone (Marazziti et al., 2004, Imai et al., 2007). Additionally, GPR37 has been shown to physically interact with proteins essential for proper dopamine signaling, the

dopamine transporter (DAT) (Marazziti et al., 2007) as well as the dopamine D2 receptor (Dunham et al., 2009). These interactions could help to explain why GPR37-deficient mice exhibit decreased conditioned place preference for psychostimulant drugs like cocaine and amphetamine (Marazziti et al., 2011). Furthermore, recent evidence has established the potential for GPR37 and GPR37L1 to act as protective receptors under conditions of cell stress with normal parkin expression (Lundius et al., 2013). In fact, GPR37 has been shown to protect against cell death induced by the dopaminergic toxins MPP+, rotenone, and 6-OHDA (Lundius et al., 2013) and it is possible that the ability of GPR37 to be either toxic or protective might be regulated through the cysteine-rich C terminus of the receptor which seems to have the potential to regulate receptor signaling (Gandia et al., 2013).

Genetic screens have identified dysregulation of GPR37 expression in neuropathologies other than Parkinson's disease. For example, a down-regulation of RNA expression for GPR37 was observed in patients with Major Depressive Disorder (MDD) (Aston et al., 2005). This potentially matches with the observation that aged GPR37 knockout mice demonstrate stronger anxiety and depression phenotypes than their wild-type counterparts (Mandillo et al., 2013). Additionally, a lowered startle response was observed in GPR37 knockout mice (Mandillo et al., 2013). The lower threshold for the startle response has a strong correlation to a diagnosis of schizophrenia (Braff et al., 1978), and the alteration to the startle response for GPR37 knockout mice potentially matches the observation that GPR37 is genetically downregulated in schizophrenic patients (Logotheti et al., 2013). Furthermore, higher expression of GPR37 has been observed in rat lines prone to high ethanol consumption (McBride et al., 2013), and a mutation in GPR37 has been linked to autism spectrum disorder (Fujita-Jimbo et al., 2012). Finally, GPR37 gene expression is upregulated in a model of Schwann cell tumors (Koelsch et al., 2013).

Both GPR37 and GPR37L1 appear to play a role in development, with GPR37L1

recently identified as a regulator of Bergman glia and Purkinje cell maturation in the cerebellum (Marazziti et al., 2013). Furthermore, GPR37 and GPR37L1 expression has been shown to be strongly upregulated in the superior olivary complex (SOC) in mice that have developed the ability to process auditory signals (Ehmann et al., 2013), identifying a potential physiological role in the auditory system. Recently GPR37 was shown to be highly expressed in ghrelin-positive cells, suggesting a possible role for the receptor in the regulation of metabolism (Engelstoft et al., 2013). With the numerous functions of GPR37 and GPR37L1 in the central nervous system, it is crucial to identify ligands that might stimulate receptor function. This dissertation proposes that the neuroprotective and glioprotective factors prosaposin and prosaptide are ligands for GPR37 and GPR37L1.

Section 1.3 Prosaposin and Prosaptide

Section 1.3.1 Prosaposin as a Lysosomal Protein

Prosaposin was initially identified as the precursor protein for four lysosomal activator proteins known as the saposins A-D (Kishimoto et al., 1992). Saposins were named due to their actions as sphingolipid activator proteins that facilitate the hydrolysis of sphingolipids by acting on several lysosomal hydrolases (Kishimoto et al., 1992). Interest in the saposins began in the 1960s with the discovery of saposin B (Mehl and Jatzkewitz, 1964) followed by the discovery of saposin C in 1971 (Ho and O'Brien, 1971). Saposin B, also known as SAP-1, was isolated and cloned in the mid-1980s (Isemura et al., 1984, Dewji et al., 1986). Not long after, the prosaposin precursor protein was identified and sequenced (O'Brien et al., 1988) and identified as a homologue of the rat sulfated glycoprotein 1 (SGP-1) (Collard et al., 1988) and the mouse testicular sulfated glycoprotein 1 (Morales et al., 1998).

Each of the four saposins has a distinct role in enhancing lysosomal enzyme function. Saposins A and C have been shown to enhance β-glucosylceramidase-mediated hydrolysis of glucocerebroside as well as hydrolysis of galactocerebroside through activation of galactosylceramidase (Wenger et al., 1982, Morimoto et al., 1989). Saposin A primarily acts by optimizing the activity of ß-galactosylceramidase (Harzer et al., 1997) whereas saposin C enhances ß-glucosidase activity and protects the enzyme from proteolytic degradation (Qi and Grabowski, 1998, Sun et al., 2003). Saposin B enhances hydrolysis of galactocerebroside sulfate (Fischer and Jatzkewitz, 1975), GM1 ganglioside (Inui and Wenger, 1984), and globotriaosylceramide (Gartner et al., 1983, Li et al., 1985), and also promotes glycerolipid hydrolysis (Li et al., 1988). Finally, saposin D has been shown to enhance the activity of sphingomyelin phosphodiesterase to enact the hydrolysis of sphingomyelin (Morimoto et al., 1988). The precise molecular mechanisms by which the saposins promote the lysosomal processing of lipids are still a point of significant research interest, but direct saposin interactions with lipids appear to be important (Kishimoto et al., 1992, Soeda et al., 1993). Similarly, saposin binding to lipids has been shown to be crucial for lipid loading of CD1, which is necessary for CD1-mediated antigen presentation and immune system recognition of lipid-based antigens on pathogens (Zhou et al., 2004, Kang and Cresswell, 2004, Leon et al., 2012).

Dysfunction or loss of saposins can result in an assortment of lysosomal storage diseases. Saposin A dysfunction has been linked to development of globoid cell leukodystrophy (GLD), also known as Krabbe disease (Matsuda et al., 2001, Spiegel et al., 2005), largely through reports that mice deficient in saposin A also demonstrate the phenotype of Krabbe disease (Matsuda et al., 2007) and exhibit nervous system deficits including neurological deficits, hindlimb weakness, and a demyelination phenotype (Matsuda et al., 2001). This was further confirmed by a clinical report of saposin A deficiency which detailed an infant exhibiting abnormal myelination as diagnosed with Krabbe disease (Fujita et al., 1996). Furthermore, leukocytes from this patient

demonstrated an abnormality of galactocerebrosidase activity, which was linked to a three base pair deletion in the saposin A coding region of prosaposin (Spiegel et al., 2005).

Mice deficient in saposin A as well as saposin B display motor deficits including tremor and foot slips as well as aberrant locomotor activity (Sun et al., 2013). Additionally, these mice show motor neuron deterioration and an accumulation of the autophagy marker LC3-II in the brainstem (Sun et al., 2013), underscoring the importance of saposin A function. Saposin B deficient mice have a distinct motor neuron deterioration phenotype, which is displayed as a head tremor that manifests itself by 15 months of age (Sun et al., 2008b). Histological analysis of saposin B-deficient brains also shows activated microglia and reactive astrocytes suggesting a proinflammatory response. Furthermore, cells in the brain and kidney accumulate fatty acid sulfatides (Sun et al., 2008b), which is suggestive of a lysosomal storage disorder. In humans, saposin B deficiency is autosomal recessive with afflicted patients accumulating cerebroside sulfate metachromatic leukodystrophy (Kretz et al., 1990).

Saposin C deficiency can lead to different forms of Gaucher's disease in humans, predominantly type 2 and 3, which are known for neuropathological symptoms (Schnabel et al., 1991, Vaccaro et al., 2010, Tamargo et al., 2012). Similarly, the mouse model of saposin C deficiency mimics type 3 Gaucher's disease (Sun et al., 2010) while the loss of the full-length prosaposin most closely resembles type 2 Gaucher's disease (Hulkova et al., 2001).

The phenotype for saposin D deficient mice is also degenerative in nature with mice developing ataxia at four months of age and progressive degeneration of Purkinje cells resulting in a complete disappearance of Purkinje cells by twelve months of age (Matsuda et al., 2004). A primary function of saposin D is to enhance acid ceramidase activity, a key regulator of ceramide concentration (Azuma et al., 1994), and thus saposin D deficiency leads to ceramide accumulation, most prominently in the cerebellum (Matsuda et al., 2004). Saposin D deficiency

has also been reported in a patient diagnosed with Gaucher's disease (Diaz-Font et al., 2005). Together, this suggests that deficiencies in each of the saposins as well as full length prosaposin produce symptoms of neurodegeneration.

1.3.2 Prosaposin as a Secreted Factor

In addition to serving as a precursor protein for the saposins, full-length prosaposin can also be released as a secreted factor into many secretory fluids including cerebrospinal fluid, semen, milk, pancreatic juice, and bile (Hineno et al., 1991). This extracellular presence of full-length prosaposin suggests a discrete function for full-length prosaposin beyond its role as the saposin precursor protein. Moreover, prosaposin was identified as a neurotrophic factor capable of promoting cell survival, neurite outgrowth and differentiation within a cholinergic cell line (O'Brien et al., 1994, O'Brien et al., 1995). The neurotrophic sequence of prosaposin was identified as a 12 amino acid peptide sequence (LIDNNKTEKEIL) located within the saposin C region of prosaposin (O'Brien et al., 1995). In addition to prosaposin, both saposin C and several "prosaptides", peptides containing the neurotrophic sequence of prosaposin, were found to promote neurite outgrowth, differentiation and cell survival in the same cholinergic cell line (O'Brien et al., 1995). Prosaptides were also shown to be capable of protecting primary cerebellar cells and hippocampal CA1 neurons *in vitro* (O'Brien et al., 1995, Kotani et al., 1996b).

Studies on human patients with prosaposin mutations suggest that prosaposin may exert neuroprotective and glioprotective actions *in vivo*, although these *in vivo* analyses are complicated by the fact that prosaposin also exerts effects on lysosomal function in addition to its potential actions as a secreted factor. Several reports describe the postmortem phenotypes of a 16-week old patient and his 20-week old fetal sibling, both of which had a novel mutation in the coding region of the prosaposin gene leading to a complete deficiency of the full-length

prosaposin and all four saposins (Harzer et al., 1989, Paton et al., 1992, Bradova et al., 1993). In addition to the lysosomal storage deficits, a striking lack of cortical neurons, myelin and mature oligodendrocytes as well as an increase in reactive astrocytes and microglia were found within the brains of these patients (Hulkova et al., 2001). Additional case studies report similar findings for other patients with prosaposin mutatons (Elleder et al., 2005, Sikora et al., 2007, Kuchar et al., 2009). Brain morphogenesis was also altered in most of these cases with the identification of gray matter heterotopias (Elleder et al., 2005, Kuchar et al., 2009). A later study focusing on the neuropathology of three additional cases described an exaggerated sensitivity of the cortical neurons to the loss of prosaposin (Sikora et al., 2007). Whereas non-neuronal cells showed a foamy appearance, characteristic of lysosomal lipid storage and sphingolipid hydrolase insufficiency, neuronal cells contained finely granular lysosomes with compacted inclusions and ubiquitinated proteins (Sikora et al., 2007). These results indicate a distinct phenotype for neuronal cells from prosaposin-deficient patients, above and beyond the pathology that might be expected purely from lysosomal dysfunction. Similar to human patients who do not survive past five months of age, prosaposin-deficient mice have a shortened lifespan and survive for only 30 days (Sun et al., 2008a). These animals have significant lysosomal dysfunction and begin to show neurological deficits around postnatal day 20, which rapidly progresses until death. Furthermore, mutations in the neurogenic region of saposin C also demonstrate a neurodegenerative phenotype (Yoneshige et al., 2010), adding to the evidence that prosaposin may exert effects *in vivo* as a secreted neurotrophic factor.

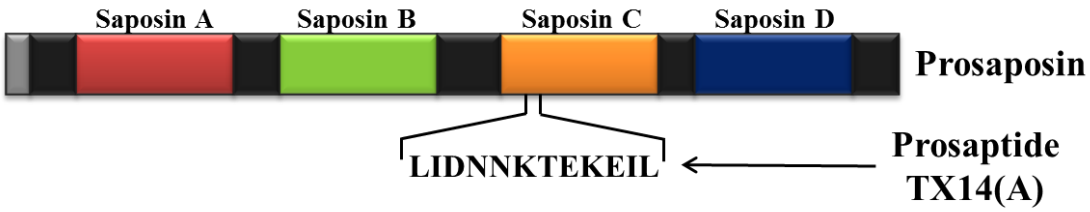


Figure 1-3. Prosaptide is derived from the saposin C region of prosaposin

Prosaposin is a 70kDa protein with four distinct saposin regions A-D. The saposin C region has neurotorphic and neuroprotective properties and a set of synthetic peptides known as prosaptides are derived from the neurotrophic sequence. The most commonly used prosaptide is TX14(A), shown in the figure as “prosaptide”.

1.3.3 Prosaposin Exocytosis Following Injury/Stress

Like other lysosomal proteins (Blott and Griffiths, 2002), prosaposin can be secreted into the extracellular space with this secretory process being enhanced under conditions of cellular stress. In models of peripheral nerve cut and crush, for example, prosaposin release is significantly elevated (Hiraiwa et al., 1999). Similarly, total levels of prosaposin mRNA and protein are increased following nerve injury in motoneurons, beginning on the third day following injury and continuing to change until post-injury day 21 (Unuma et al., 2005, Chen et al., 2008). Furthermore, prosaposin expression and release are greatly upregulated in animal models of focal cerebral ischemia (Yokota et al., 2001, Hiraiwa et al., 2003, Costain et al., 2010) , and prosaposin release from the retinal pigment epithelial cells in the eye can be elevated by cell stress induced by either light or hydrogen peroxide (Toyofuku et al., 2012).

1.3.4 Mechanisms Controlling Prosaposin Release

As a soluble lysosomal protein, prosaposin must associate with a sorting receptor that can interact with adaptor proteins necessary for the formation of cargo vesicles for trafficking to the lysosome (Lobel et al., 1989, Puertollano et al., 2001). Sortilin has been shown to be the key sorting receptor for prosaposin, as cells deficient for sortilin exhibit impaired trafficking of prosaposin to the lysosomal compartment and enhanced release of prosaposin into the extracellular medium (Lefrancois et al., 2003, Hassan et al., 2004, Zeng et al., 2009). The binding of prosaposin to sortilin has been localized to a 17-amino-acid region of the prosaposin carboxyl-terminus (Yuan and Morales, 2010). Additionally, prosaposin must be in its monomeric form to interact with sortilin and be targeted to lysosomes, as it has been shown that prosaposin oligomerization blocks interaction with sortilin and leads to prosaposin secretion (Yuan and Morales, 2011).

Work on retinal pigment epithelial cells has revealed that Sema4A is another prosaposin-interacting protein which blocks prosaposin interaction with sortilin and promotes prosaposin exocytosis (Toyofuku et al., 2012). The association of Sema4A with prosaposin was found to be increased when the cells were exposed to hydrogen peroxide to induce oxidative stress (Toyofuku et al., 2012). These findings reveal a specific molecular mechanism underlying enhanced release of prosaposin following cellular stress.

Section 1.4 Effects of Secreted Prosaposin in the Nervous System

1.4.1 Prosaposin Rescues Ischemic Damage

Given the aforementioned studies demonstrating that prosaposin expression and release are enhanced following ischemia (Yokota et al., 2001, Hiraiwa et al., 2003, Costain et al., 2010), a number of groups have explored the possibility that prosaposin might protect cells from ischemic damage. For example, the infusion of prosaposin into the lateral ventricles of gerbils prior to the induction of ischemia was found to prevent learning disabilities as a result of ischemic damage (Sano et al., 1994). Similar results were found when an 18-amino-acid prosaptide was infused into the lateral ventricles of gerbils (Kotani et al., 1996b, Morita et al., 2001). These behavioral effects were found to be accompanied by cellular changes consistent with protection of cells from apoptotic death (Sano et al., 1994, Morita et al., 2001). The infusion of the 18-amino-acid prosaptide into the lateral ventricle or the injection of the retro-inverso prosaptide D5 were also able to prevent secondary degeneration in the thalamus following occlusion of the left middle cerebral artery (Igase et al., 1999, Lu et al., 2000). However, the timing, concentration and/or method of delivery of prosaptide treatment may be important for its protective effects, as one study found that peripheral injections of prosaptide D5 exacerbated behavioral deficits in a spinal cord model of ischemia (Lapchak et al., 2000).

1.4.2 Prosaposin Rescues Dopaminergic Neurons

In addition to the protective actions exhibited by prosaposin and prosaptides in ischemic models, prosaptides have also shown protective effects on dopaminergic neurons *in vitro* and in models of Parkinson's disease *in vivo*. For example, prosaptide D5, the aforementioned retro-inverso peptide generated from the neurotrophic sequence of prosaposin that is resistant to protease cleavage and capable of crossing the blood-brain barrier, was found to protect dopaminergic mesencephalon neurons from the neurotoxins MPP+ *in vitro* and MPTP *in vivo* respectively (Liu et al., 2001). Doses as low as 1 ng/ml prosaptide D5 were found to be capable of rescuing primary dopaminergic neurons from 20μM MPP+, whereas inert versions of the peptide had no effect. Similarly, 200 μg/kg Prosaptide D5 rescued dopaminergic cells when given every other day for two weeks, 24 hours after 1 injection of 40 mg/kg MPTP (Liu et al., 2001). A subsequent study found similar results in cultured SH-SY5Y human neuroblastoma cells when treated with an 18-amino-acid version of prosaptide termed PS18 (Gao et al., 2013b). In addition, this group also found that 2.0 mg/kg PS18 was able to significantly improve behavioral deficits induced by MPTP, rescue dopaminergic neurons, and reduce the reactivity of astrocytes within the substantia nigra and striatum MPTP-treated mice. Prosaposin treatment was shown in these studies to upregulate the anti-apoptotic factor Bcl-2, down-regulate the pro-apoptotic factor BAX and inhibit MPTP-induced cleavage of caspase-3, suggesting an action on signaling pathways that inhibit apoptosis (Gao et al., 2013b). In terms of relevance to human Parkinson' disease, prosaposin was found to be upregulated in the substantia nigra of human Parkinson's patients compared to non-Parkinsonian control patients (Miller et al., 2006)

1.4.3 Prosaposin Facilitates Nerve Regeneration and Alleviates Sensory Neuropathy

Injections of prosaptide TX14(A) were found to shorten recovery time from sciatic nerve crush as well as alleviate thermal hypoalgesia and formalin-evoked hyperalgesia in diabetic rats (Jolivalt et al., 2008b). Prosaptide TX14(A) was also able to enhance nerve regeneration distance and axonal diameter of the regenerated axons following sciatic nerve injury (Jolivalt et al., 2008b). Prosaposin applied via a collagen-filled nerve guide after sciatic nerve transection in a guinea pig model increased the number of regenerating nerves, for both motor and sensory nerve types (Kotani et al., 1996a).

In addition to promoting nerve regeneration, prosaptide treatment has also been reported to reduce neuropathic pain. For example, prosaptide TX14(A) was found to reverse thermal hyperalgesia in a sciatic nerve ligation model (Otero et al., 1999). Prosaptide D5 was also able to reverse hyperalgesia in the same model in a manner that was blocked by pertussis toxin treatment, suggesting the activation of G protein pathways by prosaptide (Yan et al., 2000). Additionally, TX14(A) was shown to protect diabetic rats against progressive decline in sensory function and also relieve tactile allodynia and paw thermal hyperalgesia (Calcutt et al., 2000). In separate studies, prosaptide TX14(A) was found to protect against TNF-induced hyperalgesia (Wagner et al., 1998), paclitaxel-induced thermal hypoalgeisa (Campana et al., 1998a) and allodynia induced by the HIV envelope glycoprotein gp120 (Jolivalt et al., 2008a). The models of pain were further expanded in a report comparing the ability of prosaptide TX14(A) to mediate protection in a diverse array of pain models (Jolivalt et al., 2006).

1.4.4 Prosaposin Protects Myelinating Glial Cells

The aforementioned ability of prosaposin and prosaptides to enhance nerve repair may be

mediated via actions on the nerve fibers themselves and/or via actions on myelinating glial cells that surround the nerves. For example, treatment of Schwann cells in culture with prosaposin or prosaptides has been found to promote Schwann cell survival in the face of cellular insults (Hiraiwa et al., 1997b, Campana et al., 1999) and also increase synthesis of sulfatide, a marker for myelin production (Campana et al., 1998b, Hiraiwa et al., 1999) . Prosaptide TX14(A) was also shown to increase sulfatide concentration and reduce cell death, but not promote proliferation, in the CG4 oligodendrocyte cell line and the iSC Schwann cell line (Hiraiwa et al., 1997b). Moreover, prosaptide was shown to increase the expression and enzymatic activity of galactose-1-phosphate uridylyltransferase (GALT), an enzyme predominantly found in myelinating Schwann cells (Hiraiwa et al., 1999). These findings from work on cultures of myelinating glial cells are intriguing given that prosaposin-deficient mice and human patients are known to exhibit severe central and peripheral hypomyelination and paucity of mature oligodendrocytes (Harzer et al., 1989, Schnabel et al., 1992, Fujita et al., 1996).

1.4.5 Prosaposin Influence Cerebellar Development and Survival

Multiple studies have identified effects of prosaposin on cerebellar development and cerebellar cell survival. Prosaposin is expressed at high levels in cerebellar regions across diverse species including rat, mouse, and pigeon (Kondoh et al., 1993, Sun et al., 1994, Islam et al., 2013). Furthermore, mice that are hypomorphic for prosaposin exhibit Purkinje cell loss and cerebellar deficits prior to birth (Sun et al., 2008a). Prosaposin mRNA levels reach their peak during the developmental period that marks granule cell proliferation and maturation, suggesting potential effects of prosaposin on granule cell development (Tsuboi et al., 1998). Consistent with this idea, a 22-amino-acid prosaptide was found to promote neurite outgrowth in granule cells (O'Brien et al., 1995). Furthermore, both prosaposin and prosaptide TX14(A) have been shown to rescue cerebellar granule cells from programmed cell death, an effect mediated through the

activation of PI3K (Tsuboi et al., 1998). Prosaposin may also promote synaptic development and protection in regions of the brain beyond the cerebellum, as for example treatment with prosaposin has been shown to increase synaptic density in cultured hippocampal neurons (Cove et al., 2006).

1.4.6 Prosaposin Protects Diverse Cell Types from Cellular Insults

In addition to the aforementioned abilities of prosaposin/prosaptides to exert protective actions on myelinating glial cells and cerebellar neurons, prosaposin/prosaptides have also been shown to exert protective effects on a number of other cell types in culture. For example, prosaposin/prosaptides have been shown to protect pheochromocytoma PC12 cells from various cellular insults (Misasi et al., 2001, Sorice et al., 2008, Ochiai et al., 2008). Additionally, the aforementioned enhancement in prosaposin release induced by oxidative stress in retinal pigment epithelial cells has been shown to lead to prosaposin-mediated protection of photoreceptor cells from oxidative damage (Toyofuku et al., 2012). Prosaposin also protects U937 monocytic cells from tumor necrosis factor-α-induced cell death (Misasi et al., 2004) and cortical astrocytes from death induced by hydrogen peroxide-mediated oxidative stress (Meyer et al., 2013). Finally, in a comprehensive study of the ability of conditioned medium from mouse bone marrow stromal cell-derived neuroprogenitor cells (mMSC-NPCs) to protect neurons from 6-OHDA-induced cell death, prosaposin was identified as the essential component of the mMSC-NPC-conditioned medium that exerted the neuroprotective effects (Li et al., 2010).

Section 1.5 Receptors Controlling Prosaposin Uptake

1.5.1 Importance of Prosaposin Uptake

Following secretion into extracellular regions, prosaposin can be re-uptaken by either the same cell or neighboring cells (Hermo et al., 1992, Igdoura et al., 1993, Vielhaber et al., 1996, Hiesberger et al., 1998). The purpose of this endocytic process is unclear. One possibility is that it may serve to limit the time course of prosaposin action in extracellular spaces, thereby serving as a constraint on the physiological effects of secreted prosaposin described above. Another possibility is that prosaposin uptake may serve as a mechanism for shuttling gangliosides and/or other membrane lipids from one cell to another. Prosaposin robustly binds to gangliosides and can promote ganglioside transfer from donor liposomes to acceptor erythrocyte ghosts (Hiraiwa et al., 1992). Further work is needed, however, to assess whether prosaposin can actually mediate such ganglioside shuttling between live cells.

1.5.2 Prosaposin Uptake Mediated by LRP1

The low density lipoprotein receptor-related protein 1 (LRP1) has been shown to mediate most of the uptake of prosaposin into cells (Hiesberger et al., 1998) in a manner that is regulated by the LRP adaptor protein GULP (Kiss et al., 2006). LRP1 is one of seven members of the low-density lipoprotein (LDL) receptor family (May et al., 2007). All members of this superfamily share structural similarity but have varied functions. The six other members of this family, in addition to LRP1, are LRP1b, LRP2 (also known as megalin), LDL receptor, very low-density lipoprotein receptor (VLDL receptor), LRP4 (also known as MEGF7) and LRP8 (also known as apolipoprotein E receptor 2) (May et al., 2007). LRP1 was the second receptor in the family to be cloned and has a broad expression pattern throughout the body (Herz et al., 1988). This endocytic receptor appears to function mainly as a trafficking protein and regulator of cell surface receptors and proteases (Herz et al., 1988, Loukinova et al., 2002, Boucher et al., 2003, Deane et al., 2008, Marzolo and Bu, 2009). The other LRP receptors transport diverse cargo including lipoproteins, proteases, vitamins, bacterial proteins, viruses and signaling molecules, recognizing

many dozens of individual ligands (Herz and Bock, 2002, Lillis et al., 2005). A small fraction of prosaposin uptake in certain cells can be mediated by receptors other than LRP1, for example the mannose-6-phosphate receptor (Hiesberger et al., 1998), but nonetheless it is clear that LRP1 mediates the vast majority of prosaposin endocytosis (Hiesberger et al., 1998).

Section 1.6 Receptors Mediating Prosaposin Signaling via G proteins

1.6.1 Prosaposin Stimulates ERK and Akt Phosphorylation

Prosaposin and prosaptide treatment can stimulate extracellular signal-regulated kinase (ERK) phosphorylation in a variety of different cell types. ERK1 and ERK2 are widely-expressed protein kinases that mediate pleiotropic effects, with promotion of cell survival being one prominently-reported effect following transient ERK activation, although prolonged ERK activation can have deleterious consequences (Subramaniam and Unsicker, 2010). In primary Schwann cells, as well as an immortalized Schwann cell line, prosaptide TX14(A) was shown to promote ERK phosphorylation to promote the aforementioned increase in sulfatide synthesis (Campana et al., 1998b). Additionally, prosaptide D5 was found to induce ERK phosphorylation in the immortalized iSC cells (Hiraiwa et al., 2001). In an androgen-independent prostate cancer cell line, prosaptide TX14(A) was shown to induce ERK phosphorylation in a manner that resulted in enhancement in the proliferation, survival, migration, and invasion of the cancer cells (Koochekpour et al., 2004). In the immortalized PC12 cell line, prosaposin induced cells to enter the S phase of the cell cycle through the ERK signaling pathway (Misasi et al., 2001) and ERK phosphorylation was one of the signaling mechanisms by which prosaposin was found to protect PC12 cells against oxidative cell death (Ochiai et al., 2008). Finally, saposin C was shown to prevent cell death in the monocytic U937 cell line by preventing necrosis and apoptosis via promotion of both ERK phosphorylation and sphingosine kinase activity (Misasi et al., 2004).

Akt is another protein kinase that typically promotes cell survival (Brunet et al., 2001), prosaposin and prosaptides can induce Akt phosphorylation in a variety of cells types. In primary Schwann cells, for example, prosaptide TX14(A) was shown to stimulate Akt phosphorylation and promote Schwann cell survival in an manner that was dependent on activation of Akt and phosphatidylinositide 3-kinases, which are upstream activators of Akt (Campana et al., 1999). Furthermore, prosaposin was shown to use stimulate the Akt pathway to prevent oxidative cell death in PC12 cells in addition to stimulating signaling through the ERK pathway (Ochiai et al., 2008). Finally, saposin C was shown to activate Akt phosphorylation in a dose-dependent manner in prostate cancer cells, resulting in protection of the cells from caspase activation and apoptosis (Lee et al., 2004).

1.6.2 Prosaposin Can Stimulate G Protein-Mediated Signaling

Some of the signaling pathways stimulated by prosaposin and prosaptides have been shown to be dependent on G protein activation. In some cases, the evidence has been direct, such as prosaposin treatment promoting the activation of Gαi and Gαo G proteins (Hiraiwa et al., 1997a, Yan et al., 2000). In other cases, the evidence has been more indirect, as many of the signaling pathways stimulated by prosaposin and prosaptides can be blocked by pertussis toxin, which is an inhibitor of Gαi and Gαo G proteins. Notably, analgesis induced by prosaptide is pertussis toxin-sensitive (Yan et al., 2000), as is the ability of prosaposin to induce neurite sprouting (Misasi et al., 1998). Moreover, the aforementioned ability of prosaposin and prosaptides to induce ERK phosphorylation has been shown in multiple cell types to be pertussis toxin-sensitive (Campana et al., 1998b, Lee et al., 2004, Misasi et al., 2004). Therefore, prosaposin binds to two types of receptors in the nervous system, unknown GPCRs and the LRP-1 receptor (Figure 1-4).

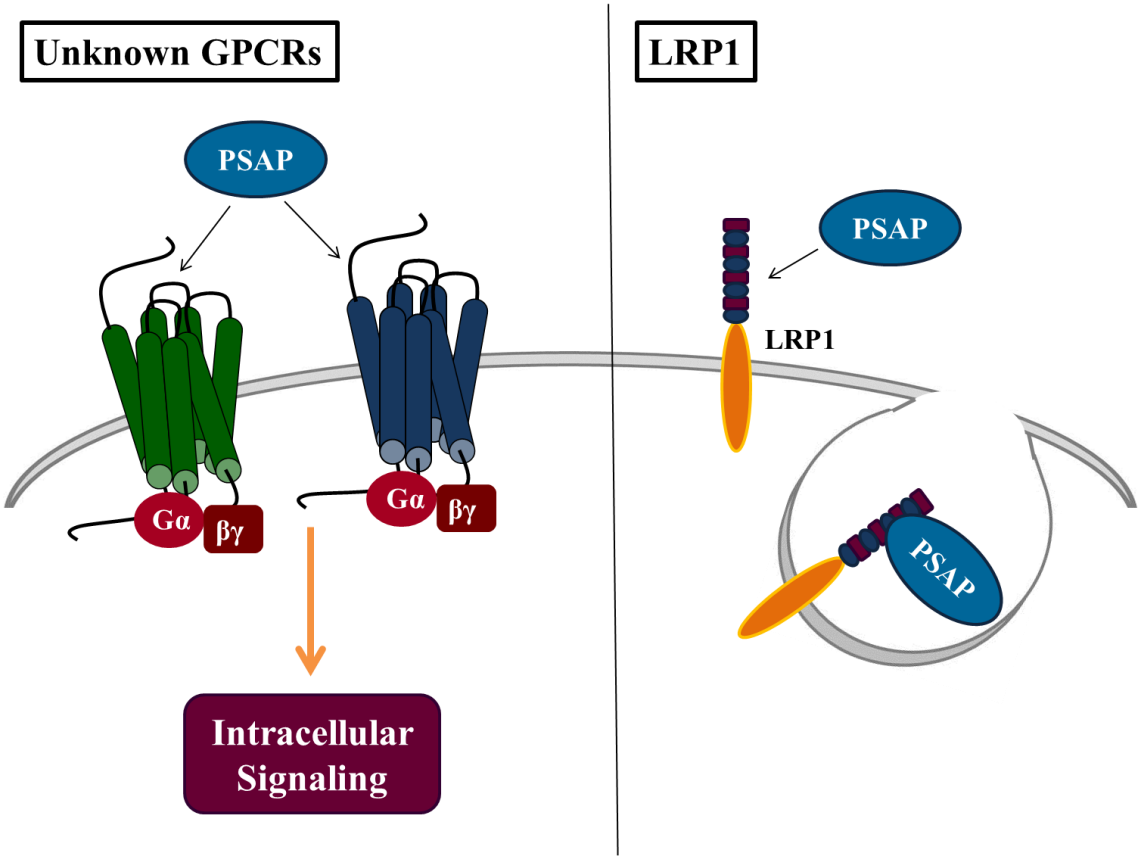


Figure 1-4. Prosaposin binds two distinct types of receptors

Prosaposin has been identified to signal through unknown GPCRs to stimulate a variety of intracellular signaling including ERK and Akt phosphorylation. Additionally, prosaposin has been identified to bind to the LRP-1 receptor, which mediates prosaposin endocytosis into the cell. However, this interaction has an unknown functional consequence.

Section 1.7 Dissertation Goals

The purpose of the studies described in this dissertation was to identify functional roles for GPR37 and GPR37L1. This includes both ligand-dependent and -independent roles as well as signaling mechanisms that might mediate the receptors' physiological actions.

Chapter 2 of the dissertation establishes that prosaptide and prosaposin are ligands for GPR37 and GPR37L1. Prosaposin and prosaptide are able to induce the endocytosis of GPR37 and GPR37L1, but not other related receptors. Additionally, the binding of prosaptide to GPR37 and GPR37L1 stimulates the receptors to signal through Gαi-coupled mechanisms. In this way, prosaptide is able to inhibit the production of cAMP and induce pERK signaling through GPR37 and GPR37L1. Furthermore, prosaposin is able to induce signaling through the receptors to initiate the phosphorylation of ERK.

Chapter 3 assesses prosaposin and prosaptide as ligands for GPR37 and GPR37L1 in a native cell type, cortical astrocytes. It was found that prosaposin and prosaptide induce ERK phosphorylation in cortical astrocytes, but siRNA knockdown of GPR37 prevents either ligand from signaling to pERK. Interestingly, knocking down either GPR37 or GPR37L1 prevented prosaptide and prosaposin from mediating protection of cortical astrocytes from cell death induced by oxidative stress. Furthermore, the signaling mechanism by which GPR37 signals to ERK in cortical astrocytes was dissected and found to involve transactivation of the EGF receptor through protein kinase C and Src.

Chapter 4 of this dissertation addresses a potential ligand-independent role of GPR37L1. We had previously demonstrated that a physical interaction between GPR37 and the dopamine D2 receptor functionally alters the response of D2 to agonists and antagonists (Dunham et al., 2009). Therefore, the last part of this dissertation was aimed at further exploring the idea that there might be a similar relationship between GPR37L1 and D1 as that between GPR37 and D2,

thereby identifying differential roles for the closely related receptors GPR37 and GPR37L1. Indeed, it was found that co-expresison with GPR37L1 can enhance D1 internalization upon ligand stimulation and also promote D1-mediated pERK signaling. However, co-expression with GPR37L1 inhibited the ability of D1 to induce cAMP production. This inhibition was pertussis-toxin sensitive, suggesting that the heterodimer of D1 and GPR37L1 couples to Gαi more robustly, while D1 alone couples to Gαs. As the interaction between GPR37L1 and D1 alters D1 function without the addition of a ligand for GPR37L1, it appears as though this represents a ligand-independent function of GPR37L1. The final chapter of this dissertation discusses the larger implications of the data presented in Chapters 2, 3 and 4.

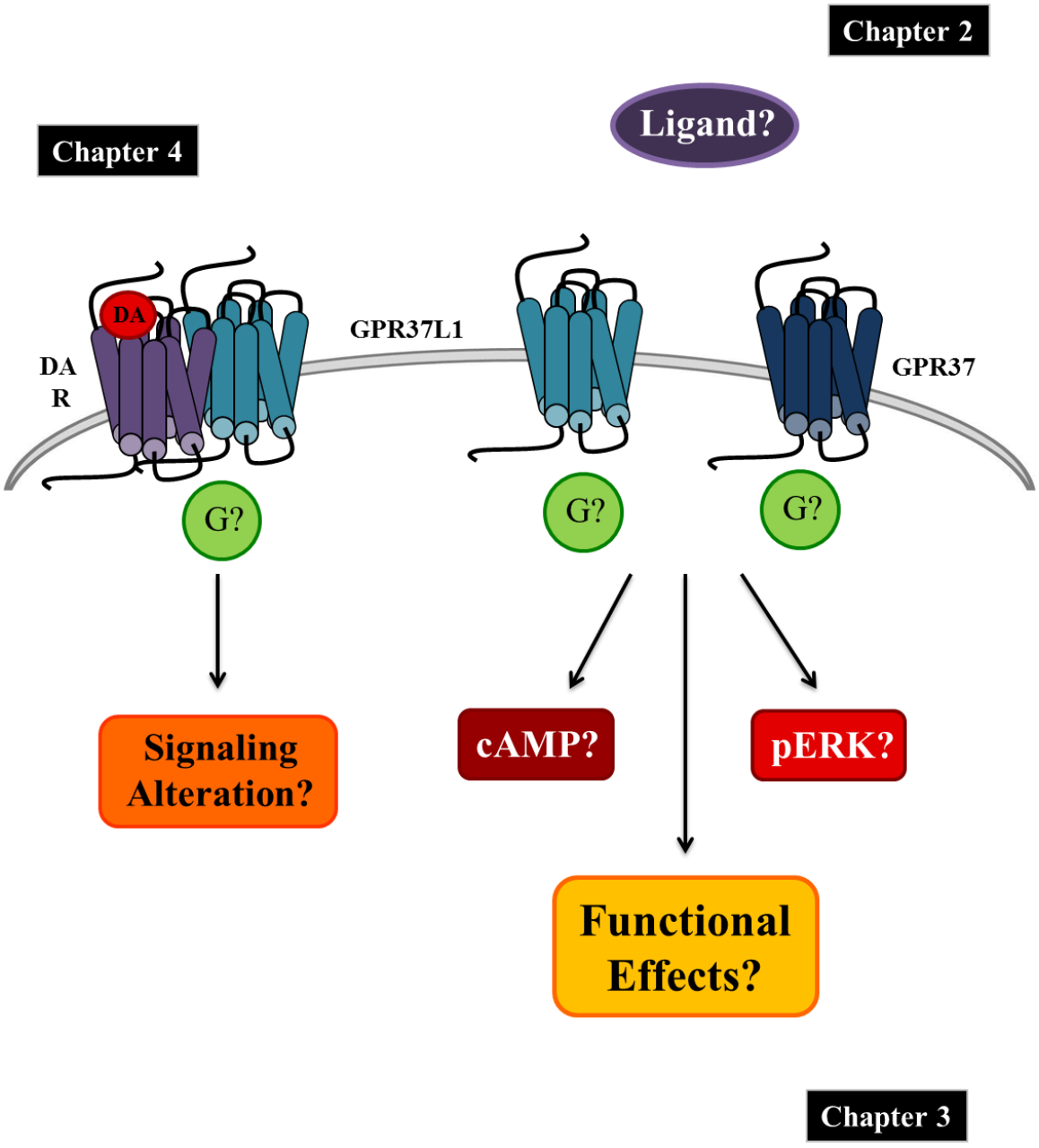

Chapter 2
Ligand?
Chapter 4
DA
DA R
GPR37L1
GPR37
G?
G?
G?
Signaling Alteration?
cAMP?
pERK?
Functional Effects?
Chapter 3

Figure 1-5. Schematic outline of the dissertation goals

The overall purpose of this dissertation was to identify functional roles for GPR37 and GPR37L1, including both ligand-dependent and -independent roles as well as downstream signaling mechanisms mediating receptor action. The studies shown in Chapter 2 identified a ligand for GPR37 and GPR37L1 and also elucidated key downstream signaling pathways, including the ERK pathway, in transfected cells. The studies shown in Chapter 3 demonstrated the importance of both GPR37 and GPR37L1 in mediating the protective effects of prosaptide and prosaposin in a native cell type, cortical astrocytes. Finally, the studies shown in Chapter 4 identified a ligand-independent function for GPR37L1 as an interacting partner and regulator of the dopamine D1 receptor.

Chapter 2:

Identification of Prosaptide and Prosaposin as Ligands for GPR37 and GPR37L1 in Transfected Cells

Section 2.1. Rationale

To screen for ligands for GPR37 and GPR37L1, we sought a suitable cell type and output to determine receptor activation. The initial experiments to identify a potential ligand for GPR37 and/or GPR37L1 were performed in transfected HEK293T cells, which we chose due to their rapid growth and high transfection efficiency. Furthermore, although GPR37 demonstrates trafficking deficits in heterologously-transfected HEK cells, previous studies demonstrated that co-expression of the scaffolding protein syntenin-1 can significantly enhance GPR37 cell surface expression (Dunham et al., 2009). Therefore, all experiments described below involving GPR37 used syntenin-1 co-expression unless otherwise noted. In order to screen for ligands in transfected HEK293T cells, receptor endocytosis was chosen as the initial output to identify ligand activation. Endocytosis, or internalization, of a receptor upon ligand stimulation is a common property of GPCRs and does not depend on knowing which G protein binds to the receptor (von Zastrow, 2001, Pierce et al., 2002, Civelli et al., 2006, Civelli et al., 2013). Thus, the monitoring of receptor internalization can be a useful output to assess the action of ligands on receptors in the absence of knowledge about the receptors' G protein coupling preferences.

In selecting ligands to screen for potential activation of the orphan GPCRs GPR37 and GPR37L1, several criteria were employed to focus initial screens. Due to the structural similarities of GPR37 and GPR37L1 to the endothelin B receptor and other peptide-activated receptors, an emphasis was placed upon screening orphan peptides. In particular, peptides that had been previously shown to exert G protein-mediated actions via unknown receptors were given priority in these screens. Moreover, as GPR37 had previously been identified as playing a role in the progression of Parkinson's disease and having interactions with the dopaminergic system, peptides known to exert effects on the dopaminergic system were preferentially selected for screening. Finally, we matched the nervous system expression pattern of potential ligands

with the expression patterns of GPR37 and GPR37L1 as a way to further focus the search for potential ligands.

Once ligands were selected for the screen, GPR37 and GPR37L1 were assessed for their endocytosis in response to ligand stimulation, with internalization being measured using cell surface luminometry to assess receptor expression on the cell surface in the presence and absence of peptide treatment. The single positive hit, prosaptide, was then run through a battery of signaling assays to assess its ability to activate GPR37 and GPR37L1. ^{35}S-GTPγS binding and cAMP were chosen to identify the G protein profile of the receptors and pERK was chosen due to its common downstream signaling pathway and its wide report as an output for prosaptide signaling. The ability of prosaptide TX14(A) to bind to GPCRs was tested using a biotin pulldown. Due to the unavailability of a radiolabeled version of prosaptide or the full length prosaposin, a version of prosaptide TX14(A) with a biotin attached to the N terminus was custom-made and attached to streptavidin beads. This technique has been used to great effect in the literature related to the study of the endothelin receptors (Akiyama et al., 1992, Saravanan et al., 2004). Together, these techniques were used to identify a potential ligand for GPR37 and GPR37L1, demonstrate the ability of that ligand to bind to and induce the endocytosis of the receptors, and stimulate G protein binding and signaling through GPR37 and GPR37L1.

Section 2.2. Experimental Methods

2.2.1 Materials

Materials used in the experiments were obtained from the following sources: GPR37 construct (originally obtained from Missouri University of Science and Technology cDNA Resource Center (Missouri S&T) and subcloned by Heide Oller); GPR37L1 construct

(Multispan); Syntenin-1 construct (gift from Paul Coffer); Gαi construct (Missouri S&T); Prosaposin construct (gift from Carlos Morales); β2 Adrenergic receptor construct (Missouri S&T); Human Embryonic Kidney (HEK) 293-T cells (ATCC); Dulbecco's Minimal Eagle Medium (DMEM) (Invitrogen); Fetal Bovine Serum (FBS) (Atlanta Biologicals); penicillin/streptomycin (Invitrogen); Lipofectamine 2000 (Invitrogen); M2 FLAG antibody (Sigma); Myc antibody (Santa Cruz); Prosaposin antibody (Santa Cruz); 3F10 HA antibody (Roche); HRP-conjugated secondary antibodies (GE Healthcare); Alexa-Fluor secondary antibodies (Li-Cor and Invitrogen); Prosaptide TX14(A) (Anaspec); Secretoneurin (Anaspec); SubstanceP(1-7) (Anaspec); L-prolyl L-leucylglycinamide (Sigma-Aldrich); Cocaine-and-Amphetamine-Regulated Transcript (CART) (American Peptide Company, Inc); 10,000 Da Ultrafiltration Filters (Millipore); Pro-Bond Nickel Resin (Thermo-Scientific); 35-mm uncoated cell culture plates (Corning); Uncoated 6-well cell culture plates (Corning); 4-20% Tris-glycine gels (Invitrogen); nitrocellulose (Bio-Rad); Odyssey blocking buffer (Li-Cor); Enhanced Chemiluminescence (ECL) and ELISA Pico Chemiluminescence reagents (Thermo Scientific); Autoradiography Film (Denville); Neutravidin (streptavidin) resin (Pierce); ^{35}S-GTPγS (Perkin Elmer); 30% ScintiSafe (Fisher); 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich); Colorimetric cAMP ELISA kit (Cell Biolabs); pertussis toxin (Sigma-Aldrich).

In all experiments, prosaptide TX14(A) was resuspended in an assay buffer containing 20mM HEPES, 100mM NaCl, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 5mM KCl, 0.5mM EDTA, and a protease inhibitor tablet (Roche).

2.2.2 Cell Culture

HEK293T cells were cultured and maintained in DMEM with 10% (vol/vol) FBS and 100µg/ml penicillin and streptomycin at 37 degrees Celsius and 5% CO and 5% CO_2. Cells were

used between passages 2 and 20 with signaling experiments performed between passages 2 and 12. HEK293T cells were transfected with Lipofectamine 2000 when approximately 70-90% confluent. Transfection was performed in serum and antibiotic-free DMEM for 4-5 hours and terminated by the replacement of transfection reagent with DMEM containing FBS and penicillin/streptomycin. All experiments were performed 48 hours following transfection. All experiments performed with GPR37 and GPR37L1 transfection used 2µg cDNA; GPR37 was co-transfected with 1µg syntenin-1 unless otherwise noted so that 3µg total of DNA was transfected per 10-cm plate. In experiments which included transfection of intracellular proteins (Gαi and prosaposin) in addition to receptor, transfections contained 2µg of receptor, 2µg of receptor, and 1µg of syntenin-1 or empty vector so that 5µg total was transfected per 10-cm plate.

2.2.3 Prosaposin Production

Prosaposin production and purification was performed by M. Giddens. HEK293T cells were transiently transfected with 5µg hexahistidine-tagged prosaposin using Lipofectamine 2000. Transfected cells were incubated in DMEM without serum for 48 hours. Media was removed from plates and spun down at 3000 × g for 10 minutes to remove dead cells and concentrated using 10 kDa ultrafiltration filters and centrifugation at 4000 × g for 15 minutes. The concentrated media was incubated with Pro-Bond nickel resin end-over-end at 4 degrees Celsius for 30 minutes to isolate the His-tagged prosaposin. The resin was washed 3 times with 10mM imidazole buffer (300mM NaCl, 50mM NaH_2PO_4, and PBS + Ca^{2+}, pH 7.4). Purified prosaposin was concentrated via ultrafiltration at 4000 × g for 15 minutes. The resulting prosaposin was assessed using the BSA protein assay to determine total protein concentration and run on an SDS-PAGE gel and stained with coomassie to estimate purity levels. Prosaposin presence was also confirmed using Western blot with antibodies against prosaposin and myc.

2.2.4 Luminometer Assay

Transfected HEK293T cells were plated on uncoated 35-mm plates 24 hours following transfection. The cells were grown overnight and were at approximately 50-60% confluency at the time of experimentation. When establishing cell surface expression, plates of cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed 3 times with PBS + Ca^{2+}, then blocked with a blocking buffer containing 2% nonfat milk in PBS + Ca^{2+} for 30 minutes, and incubated with HRP-conjugated FLAG or non-conjugated HA primary antibody for one hour at room temperature. Plates incubated with HRP-conjugated primary washed three times with blocking buffer. For experiments making use of HA-tagged receptors, following incubation with primary antibody and three washes, plates were incubated with HRP-conjugated secondary for 30 minutes at room temperature followed by three washes with blocking buffer. All plates were washed one time with PBS + Ca^{2+} and incubated with SuperSignal Pico ECL for 15 seconds. The luminescence of the plate was determined using a TD 20/20 luminometer and interpreted as a quantification of cell surface receptor expression. All experiments were done in triplicate. For studies examining endocytosis, unfixed plates were treated with peptides for 30 minutes at room temperature with a spike at 15 minutes to counteract peptide degredation. Following drug treatment, plates were fixed and the experimental procedure continued as described above.

2.2.5 Western Blotting

Samples for Western blotting were harvested in laemmli sample buffer containing 62.5mM Tris·HCl (pH 6.8), 25% glycerol, 2% SDS, and 0.01% bromophenol blue. Protein samples were resolved via SDS-PAGE on 4-20% Tris-glycine gels and transferred to nitrocellulose membranes. Gel transfer was done at 25V for 90 minutes. To blot for phospho-ERK (pERK), membranes were blocked with Odyssey blocking buffer for 30 minutes at room

temperature and overnight at 4 degrees Celsius with primary antibodies prepared in a buffer comprised of equal parts blocking buffer and PBS + 0.1% Tween-20. Membranes were washed 3 times in PBS + 0.1% Tween-20 and incubated with secondary antibodies for 30 minutes at room temperature. Blots were then washed 3 times in PBS + 0.1% Tween-20 and rinsed in PBS until visualization. Phospho-ERK blots were visualized on an Odyssey Imaging System (Li-Cor) and analyzed using Image-J. Because antibodies against phosphor-ERK and total ERK were raised in different species, blots could be incubated with both antibodies which were then visualized on the 700 channel for pERK and the 800 channel for total ERK rather than requiring two separate blots, allowing for more accurate normalization of phosphor-ERK to total ERK. For all other Western blots, membranes were incubated in a milk blocking buffer (2% nonfat dry milk, 50mM NaCl, 20mM HEPES (pH 7.4) and 0.1% Tween-20) for 30 minutes at room temperature followed by 1 hour incubation with primary antibody at room temperature. Blots were washed 3 times with blocking buffer and incubated with HRP-conjugated secondary antibody for 30 minutes at room temperature. Following secondary antibody incubation, blots were washed 3 times with blocking buffer and then incubated with enhanced chemiluminescence (ECL) reagent and exposed to autoradiography film.

2.2.6 Biotinylated Prosaptide Pulldown

Biotinylated prosaptide TX14(A) was incubated with a 1:1 streptavidin: PBS slurry for 30 minutes end-over-end at 4 degrees Celsius to allow peptide attachment to beads. For starting material, transfected HEK293T cells were harvested in a hypotonic high-salt harvest buffer (10mM HEPES, 150mM NaCl, 5mM EDTA, and a protease inhibitor tablet) and centrifuged at 15,000 × g for 10 minutes to isolate membranes. The resulting membranes were resuspended in high-salt harvest buffer with 1% Triton X-100 and incubated end-over-end at 4 degrees Celsius for 1 hour to solubilize membrane-bound protein. Samples were then centrifuged at 15,000 × g

for 15 minutes to isolate unsolubilized membrane which was then removed from the sample. Solubilized protein were divided equally between beads with prosaptide attached or streptavidin beads alone and incubated end-over-end for 2 hours at 4 degrees Celsius. Beads were then washed 2 times with high-salt harvest buffer with 1% Triton X-100 and boiled in sample buffer. Resulting samples were run on Tris-glycine gels for Western blotting.

2.2.7 ^{35}S-GTPγS Binding

Transfected HEK293T cells were harvested in a low-salt harvest buffer (10mM HEPES, 50mM NaCl, 5mM EDTA, and a protease inhibitor tablet) and membranes were isolated via centrifugation at 15,000 × g for 10 minutes. Membranes were resuspended in binding assay buffer (20mM HEPES, 100mM NaCl, 10mM $MgCl_2$, and a protease inhibitor tablet). Membranes were incubated with 0.1nM ^{35}S-GTPγS (1250 Ci/mmol), 10μM GDP, 0.001% saponin, and binding assay buffer with or without prosaptide TX14(A) for 30 minutes at room temperature. Membranes were then centrifuged at 15,000 × g for 10 minutes and supernatant decanted. The pellets were washed two times with binding assay buffer and the snap-cap tube was cut to isolate the pellet. The pellet-containing plastic was added to 30% ScintiSafe and vortexed. 24 hours later the samples were read in a scintillation counter for ^{35}S counts per minute. The assay was optimized prior to experimentation using transfected dopamine D2 receptors with 100μM dopamine in the presence of varying concentrations of GDP and ^{35}S-GTPγS.

2.2.8 cAMP Signaling

Transfected HEK293T cells were split into uncoated 6-well plates and were grown overnight. The following day, the cells were starved for 10 minutes in serum-free DMEM with 500µM IBMX to prevent cAMP degradation during the assay then treated with 100nM prosaptide TX14(A) for 10 minutes. Following prosaptide treatment, cells were stimulated with 100nM prosaptide and 1µM forskolin for 20 minutes. Cells were then lysed and cAMP was isolated through centrifugation at 15,000 × g for 15 minutes. Samples were analyzed using a colorimetric cAMP ELISA kit. To do this, samples were added to individual wells of an anti-rabbit antibody-coated 96-well plate along with a rabbit cAMP antibody and peroxidase cAMP tracer. All samples were assayed in replicates of five. The samples were incubated on the plate for 2 hours to allow antibody binding and tracer displacement to reach equilibrium. The wells were washed 5 times and incubated with substrate solution for 15 minutes. The substrate reaction was stopped and the absorbance was read using at a 450nm wavelength using a spectrophotomter.

2.2.9 pERK Signaling

Transfected HEK293T cells were plated on uncoated 6-well plates and allowed to grow overnight so that the cells reached a confluency of approximately 50-70%. The next day, cells were starved in serum-free DMEM for 2-3 hours prior to assay stimulation. To initiate signaling, DMEM was removed and replaced with fresh serum-free DMEM containing no drug, prosaptide, or prosaposin. Plates were then placed back into the incubator and incubated for the time indicated at 37 degrees Celsius with 5% CO_2. Cells were harvested in laemelli sample buffer, sonicated, and run on Tris-glycine gels. For studies using pertussis toxin, transfected cells were split into 6-well plates, allowed to attach for 6 hours, and incubated in the presence and absence

of 150ng/mL pertussis toxin for 14-18 hours. Plates were then starved in DMEM for 2 hours and the assay was completed as described above.

Section 2.3. Results

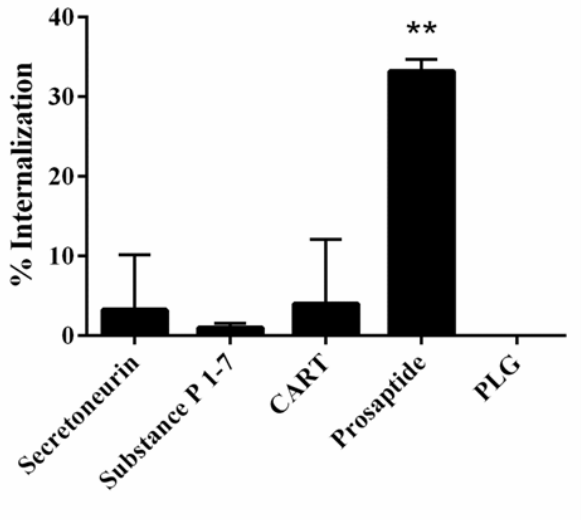
2.3.1 Prosaptide, but not other Peptides, Induces the Internalization of GPR37 and GPR37L1

In screens for potential ligand-induced endocytosis of GPR37 and GPR37L1, we examined five neuropeptides which are all known to exert physiological effects via G protein dependent pathways in cell types and/or areas of the brain that express GPR37 and GPR37L1—secretoneurin (Gasser et al., 2003), substance P 1–7 (Igwe et al., 1990), cocaine- and amphetamine-regulated transcript (CART) (Lakatos et al., 2005, Lin et al., 2011), prosaptide (Hiraiwa et al., 1997a, Campana et al., 1998b, Misasi et al., 1998, Taylor et al., 2000b, Yan et al., 2000, Lee et al., 2004) , and L-prolyl-L-leucyl-glycinamide (PLG) (Chiu et al., 1983). Cell surface luminometry identified a single positive hit for GPR37 and GPR37L1, a 14 amino acid peptide known as prosaptide TX14(A). This peptide was able to induce internalization of GPR37 and GPR37L1 (Figure 2-1A &B, respectively), indicating the potential for receptor activation by prosaptide. The ability of prosaptide to promote the endocytosis of GPR37L1 was confirmed via confocal microscopy using an antibody feeding assay (data not shown). Together the data demonstrate that prosaptide TX14(A) (hereafter referred to simply as “prosaptide”) is able to induce internalization of both GPR37 and GPR37L1.

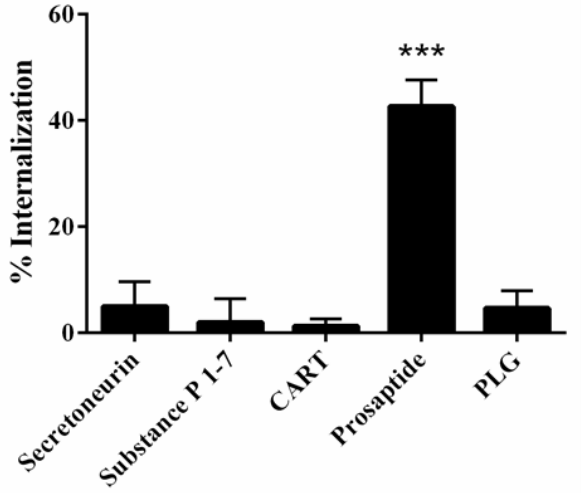
2.3.2 Prosaptide does not Induce Internalization of other Related Receptors

Given the ability of GPR37 and GPR37L1 to interact with dopaminergic receptors (Dunham et al., 2009) as well as the similarity of GPR37 and GPR37L1 to the Endothelin B receptor, we tested whether or not prosaptide is able to induce the internalization of any of these related receptors. We found that prosaptide did not induce internalization of either the dopamine D2 receptor or the endothelin B receptor (Figure 2-1C). However, in parallel experiments, prosaptide was again able to induce significant internalization of GPR37 and GPR37L1 (Figure 2-1C, GPR37 $p<0.001$ and GPR37L1 $p<0.01$, $n=3$).

A.


% Internalization
40
30
20
10
0
**
Secretoneurin
Substance P 1-7
CART
Prosaptide
PLG

B.


% Internalization
60
40
20
0
***
Secretoneurin
Substance P 1-7
CART
Prosaptide
PLG

C.

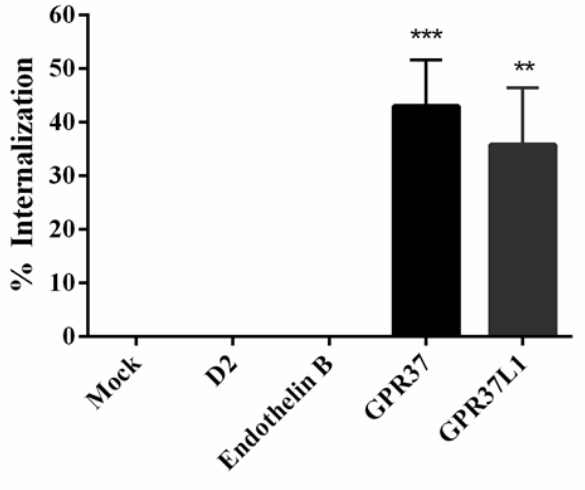

% Internalization
60
50
40
30
20
10
0
***
**
Mock
D2
Endothelin B
GPR37
GPR37L1

Figure 2-1. Prosaptide induces internalization of GPR37 and GPR37L1, but not related receptors

A-B) Five different peptides were examined for their abilities to induce internalization of FLAG-tagged GPR37 (A) and GPR37L1 (B) in HEK293T cells. Each peptide was added to the cells at 1 μM for 30 minutes. Only treatment with prosaptide induced significant receptor internalization (n=3-5 for each peptide; all points done in triplicate, ** $p<0.01$, *** $p<0.001$). C) 10μM prosaptide added for 30 minutes induces the internalization of FLAG-tagged GPR37 and GPR37L1 in transfected HEK293T cells, however 10μM prosaptide did not induce any internalization of HA-tagged dopamine D2 receptor or Endothelin-B receptor (n=3, *** $p<0.001$, ** $p<0.01$).

2.3.3 Prosaptide Binds to GPR37 and GPR37L1, but not other GPCRs

Following our observations of prosaptide-induced internalization of GPR37 and GPR37L1, we sought to determine whether prosaptide could bind to these receptors. Studies with the endothelin receptors, the most closely related receptors to GPR37/GPR37L1, have demonstrated that solubilized endothelin receptors can be efficiently pulled down by biotinylated endothelins coupled to streptavidin (Akiyama et al., 1992, Saravanan et al., 2004). Thus, we obtained a biotinylated version of prosaptide and pursued a similar approach for studying prosaptide binding to GPR37 and GPR37L1. As shown in Figure 2-2, solubilized GPR37 and GPR37L1 were robustly pulled down by biotinylated prosaptide but not by control streptavidin beads. In contrast, a number of other FLAG- or HA tagged transfected GPCRs (α1A-adrenergic, β1-adrenergic, D1 dopamine, and endothelin B receptors) were not detectably pulled down by biotinylated prosaptide.

2.3.4 Prosaptide Stimulates ^{35}S-GTPγS Binding to GPR37 and GPR37L1 Co-Transfected with Gαi

To examine the ability of prosaptide to induce G protein coupling to GPR37 and GPR37L1, we examined the binding of ^{35}S-GTPγS to the receptors upon prosaptide stimulation. In HEK293T cells in which the receptors were co-expressed with Gαi, stimulation with 10μM prosaptide induced a significant binding of ^{35}S-GTPγS to the lysed cell membranes (Figure 2-3, GPR37 $p>0.01$, GPR37L1 $p>0.05$, $n=3$).

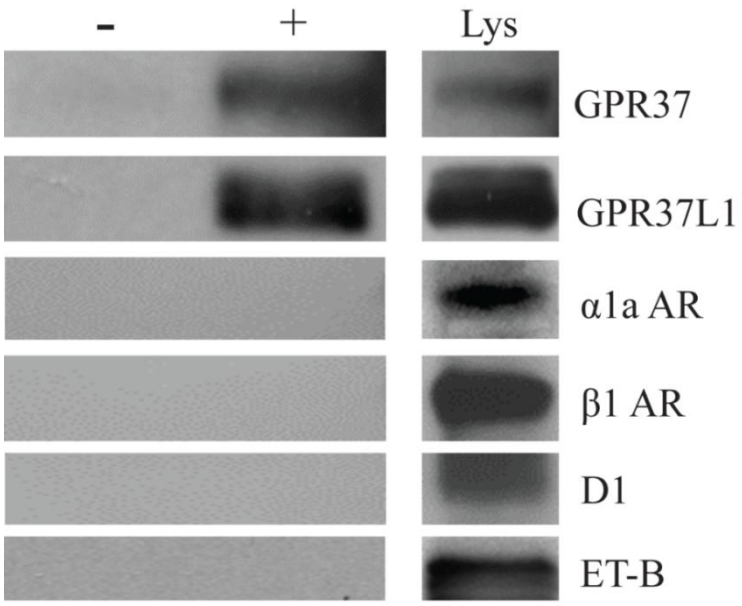


Figure 2-2. Prosaptide is able to pull down GPR37 and GPR37L1, but not other GPCRs

The ability of prosaptide to bind to different GPCRs was assessed using biotinylated prosaptide attached to streptavidin beads (+) or streptavidin beads alone (-). FLAG-tagged GPR37 and GPR37L1, but not other FLAG-tagged (α1A- and β1-adrenergic) or HA-tagged receptors (D1 dopamine and endothelin ET_B), were pulled down with the biotinylated prosaptide adsorbed to the beads, indicating specific binding of the peptide to GPR37 and GPR37L1. (n=3).

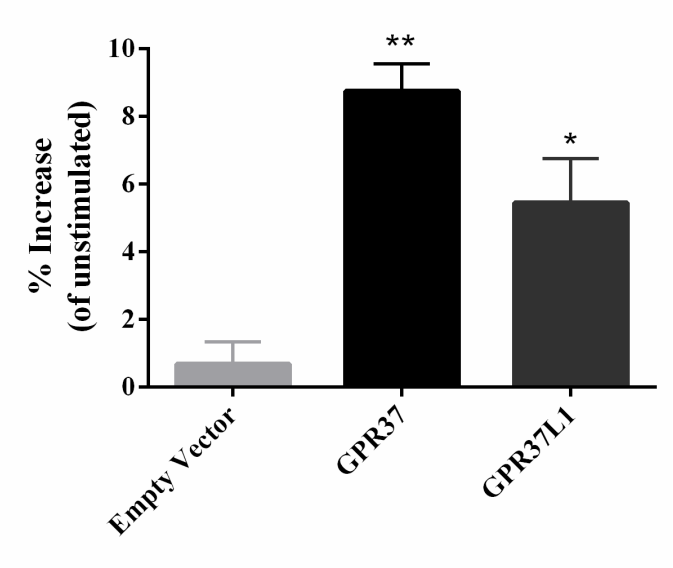


Figure 2-3. Prosaptide induces the binding of ^{35}S-GTPγS to GPR37 and GPR37L1

Effects of prosaptide treatment on ^{35}S-GTPγS binding to membranes derived from HEK293T cells transfected with $G\alpha_{i1}$ alone ("mock"), GPR37/syntenin-1/$G\alpha_{i1}$ or GPR37L1/$G\alpha_{i1}$. All experiments determined prosaptide-induced ^{35}S-GTPγS binding over vehicle treatment (n=3; all points done in duplicate, ** $p<0.01$, * $p<0.05$). Total counts for vehicle-treated samples averaged ~6000 CPM and did not vary significantly between transfection conditions.

2.3.5 GPR37 and GPR37L1 Mediate the Ability of Prosaptide to Inhibit cAMP Production

Given the results from the ^{35}S-GTPγS binding studies we next examined whether or not prosaptide stimulation of cells expressing GPR37 and GPR37L1 could affect the production of cAMP. GPR37- or GPR37L1-transfected cells were treated with forskolin to produce cAMP in the absence and presence of pretreatment with 100nM prosaptide. We found that for GPR37- and GPR37L1-transfected cells, but not mock-transfected HEK293T cells, prosaptide treatment significantly inhibited cAMP production (Figure 2-4, $p<0.01$, $n=5$).

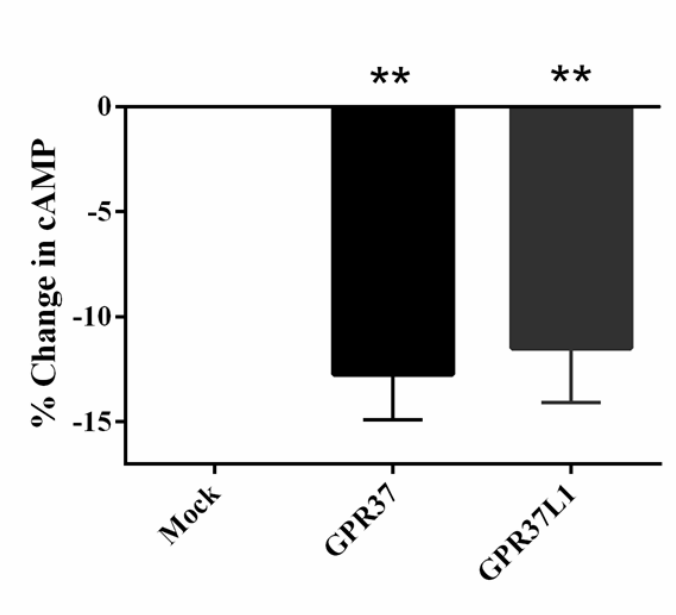


Figure 2-4. Prosaptide-induced inhibition of cAMP is mediated through GPR37 and GPR37L1

Effects of prosaptide treatment (30 min, 100 nM) on cAMP levels in HEK293T cells transfected with empty vector ("mock"), GPR37, or GPR37L1. All experiments determined prosaptide-induced cAMP inhibition compared to vehicle treatment (n=5; all points done in triplicate, ** $p<0.01$).

2.3.6 GPR37 and GPR37L1 Mediate the Ability of Prosaptide to Induce ERK Phosphorylation

We next examined the phosphorylation of ERK as a signaling output since phospho-ERK ("pERK") is the most frequently reported signaling output activated by prosaptide (26, 28, 53–55). Thus, we explored the ability of prosaptide to induce ERK phosphorylation in HEK293T cells transfected with either GPR37 or GPR37L1. Transfection of the receptors into HEK293T cells had no significant effect on basal levels of phospho-ERK or total ERK, but treatment with 100 nM prosaptide induced a significant increase in pERK for both GPR37- and GPR37L1-transfected cells, with no effect observed on mock-transfected cells (Figure 2-5A&B, *** $p<0.001$). Furthermore, dose–response studies revealed that prosaptide activates GPR37 with an apparent EC_{50} of 7 nM (Figure 2-6A) and GPR37L1 with an EC_{50} of 5 nM (Figure 2-6B).

We further identified that prosaptide is able to induce signaling through GPR37 and GPR37L1 in a time-sensitive manner with stimulation beginning at 2 minutes and completely desensitizing by 30 minutes of stimulation (Figure 2-7). These data indicate that GPR37 and GPR37L1 mediate only a short-term stimulation of pERK in response to prosaptide treatment.

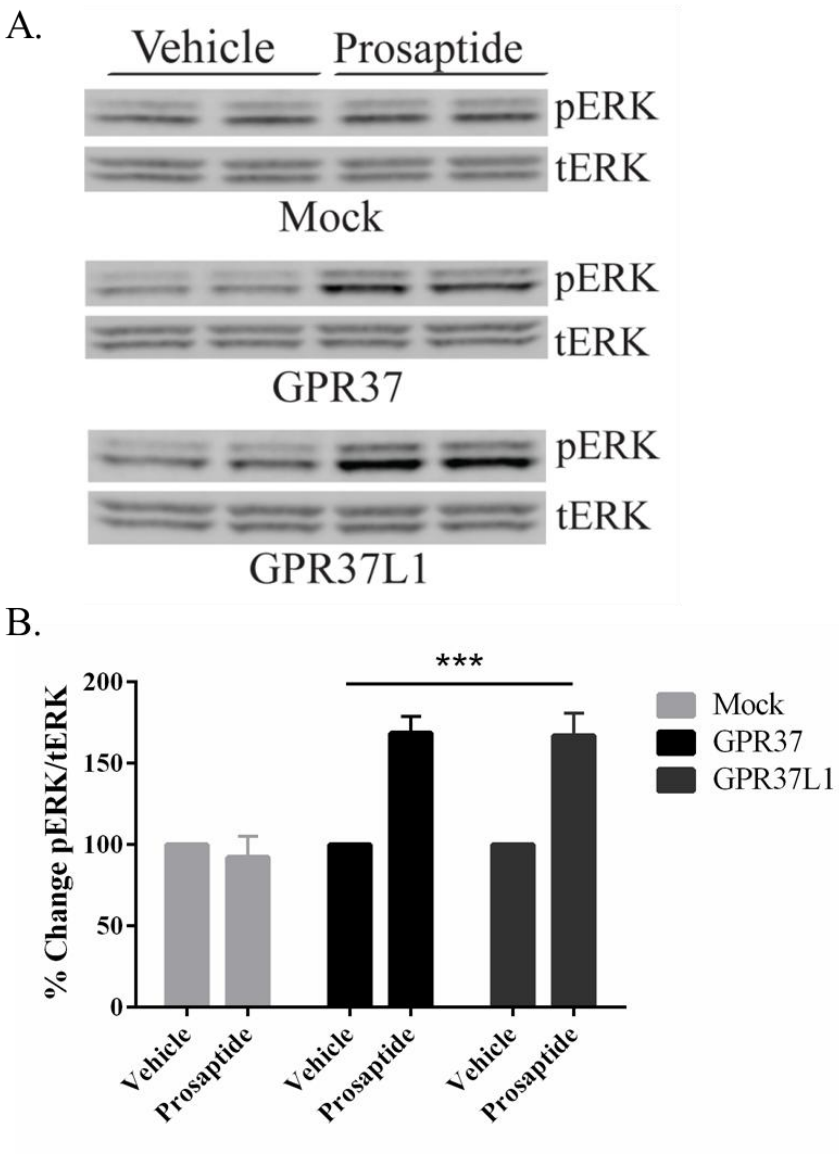


Figure 2-5. Prosaptide induces ERK phosphorylation in GPR37- and GPR37L1-transfected cells, but not mock transfected cells

A) HEK293T cells transiently transfected with either empty vector ("Mock"), GPR37 or GPR37L1 were treated for 10 min with 100 nM prosaptide and ERK phosphorylation was assessed (pERK= phosphorylated ERK, tERK= total ERK. B) Quantification of changes in pERK levels in response to prosaptide treatment. All experiments determined prosaptide-induced ERK phosphorylation over vehicle treatment (n=3; all points done in duplicate, *** $p<0.001$

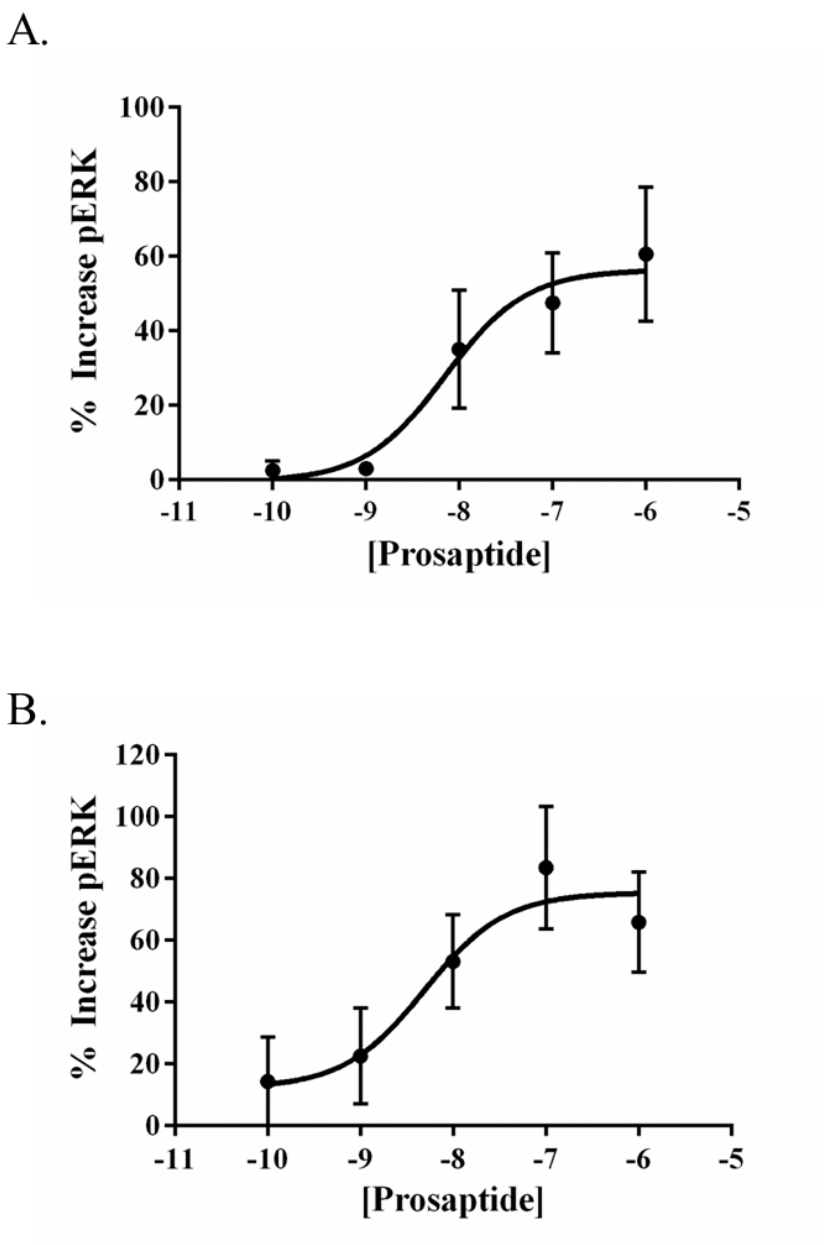


Figure 2-6. Prosaptide induces ERK phosphorylation in a dose-dependent manner for both GPR37 and GPR37L1

Dose-response curves for prosaptide stimulation of HEK293T cells transfected with either GPR37 (A) or GPR37L1 (B) were established. The EC_{50} for GPR37 was determined to be 5nM and the EC_{50} for GPR37L1 was determined to be 7nM. Curves were analyzed using an equation for log(agonist) vs. response (n=6).

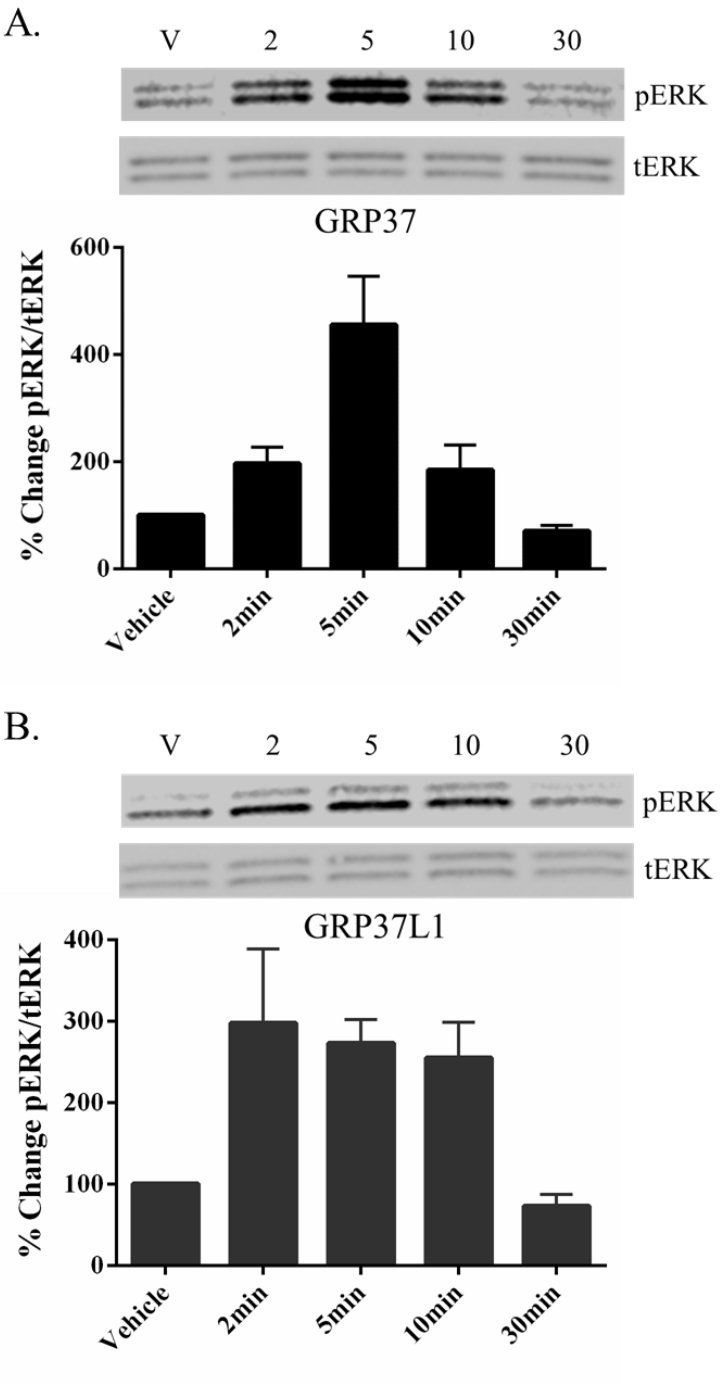

A.
V
2
5
10
30
pERK
tERK
GRP37
% Change pERK/tERK
600
400
200
0
Vehicle
2min
5min
10min
30min
B.
V
2
5
10
30
pERK
tERK
GRP37L1
% Change pERK/tERK
400
300
200
100
0
Vehicle
2min
5min
10min
30min

Figure 2-7. GPR37 and GPR37L1 mediate prosaptide-induced pERK signaling in a time-sensitive manner

Prosaptide treatment of transfected HEK293T cells at different time points induced differing levels of ERK phosphorylation. Cells were treated with 20nM prosaptide for 2, 5, 10, and 30 minutes and ERK phosphorylation was assessed. Prosaptide induced ERK phosphorylation beginning at 2 minutes for GPR37 (A) and GPR37L1 (B) , with ERK phosphorylation levels returning back to baseline by 30 minutes (n=3).

2.3.7 Prosaptide Induction of pERK through GPR37 and GPR37L1 is Pertussis Toxin-Sensitive

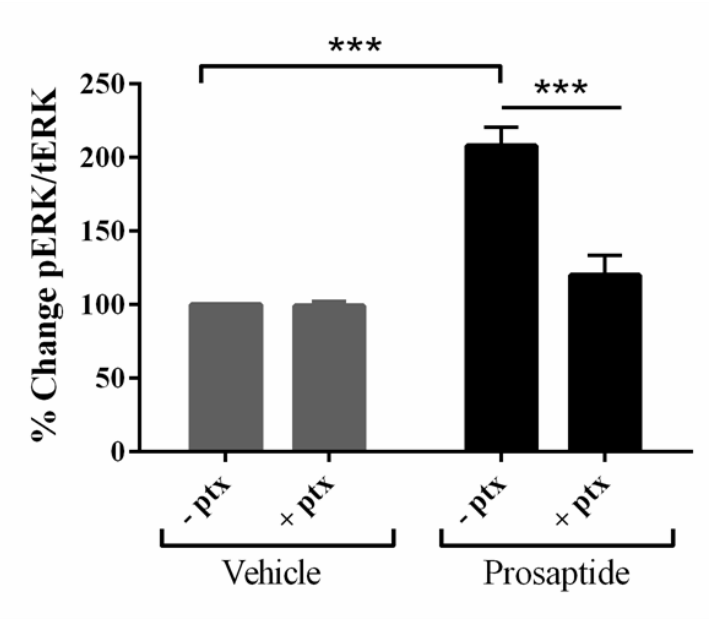
Because our ^{35}S-GTPγS binding studies and cAMP inhibition data suggested that GPR37 and GPR37L1 can couple to Gαi and because prosaptide has been reported to activate a pertussis toxin (PTX)-sensitive Gαi/o-coupled receptor in native cell types (Hiraiwa et al., 1997a, Misasi et al., 1998, Campana et al., 1998b, Misasi et al., 2001, Lee et al., 2004, Misasi et al., 2004, Koochekpour et al., 2007), we examined the effects of PTX pretreatment on prosaptide-induced pERK signaling. We found that PTX pre-treatment inhibited the ability of prosaptide to stimulate ERK phosphorylation in GPR37- or GPR37L1-transfected cells. Phosphorylation of ERK induced by prosaptide in the presence of PTX was significantly lower than prosaptide stimulated pERK without PTX for GPR37 and GPR37L1 (Figure 2-8A&B, respectively) . These data indicate a Gαi/o-dependent mechanism of ERK activation for these receptors.

2.3.8 Prosaposin is able to Stimulate Internalization and ERK Phosphorylation through GPR37 and GPR37L1

As mentioned earlier, prosaptide is a peptide fragment that mimics the trophic and protective actions of full-length prosaposin (O'Brien et al., 1995, Kotani et al., 1996b, Campana et al., 1996, Hiraiwa et al., 1997b, Campana et al., 1998b, Igase et al., 1999, Taylor et al., 2000a, Taylor et al., 2000b, Liu et al., 2001, Hiraiwa et al., 2001), but it is widely presumed that full-length prosaposin is the endogenous ligand for any receptor that can be activated by prosaptide. Thus we purified full-length prosaposin using a modified version of a previously described protocol (Xu et al., 2012) and assessed whether full-length prosaposin could induce the endocytosis of and/or activate ERK signaling via GPR37 and GPR37L1. As shown in Figure 2-9A, purified prosaposin was able to induce significant internalization of GPR37 and GPR37L1

($p<0.05$, n=3-4). This effect mirrored the internalization observed with prosaptide indicating that both the synthetic peptide and full length ligand have the similar effects on the receptors. To explore the effect of the receptors to mediate signaling, HEK293T cells transfected with empty vector, GPR37, or GPR37L1 were incubated with purified prosaposin (100 nM) for 10 minutes. Treatment with prosaposin induced ERK phosphorylation in the cells expressing GPR37 or GPR37L1, but not in mock transfected cells (Figure 2-9B), which was also similar to the results obtained earlier in the prosaptide stimulation experiments.

A.


***
***
250
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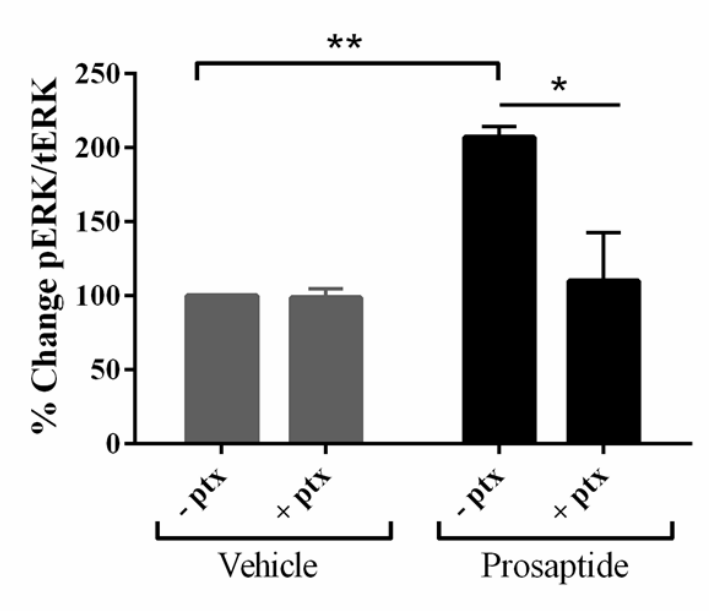
50
0
% Change pERK/tERK
- ptx
+ ptx
- ptx
+ ptx
Vehicle
Prosaptide

B.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% Change pERK/tERK
- ptx
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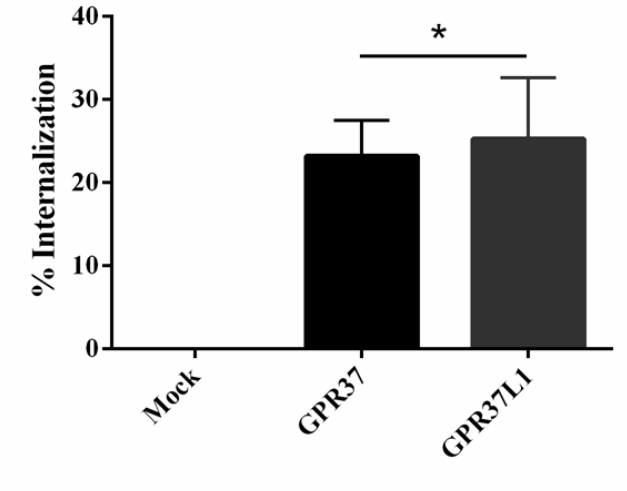
+ ptx
Vehicle
Prosaptide

Figure 2-8. The phosphorylation of ERK mediated through GPR37 and GPR37L1 pertussis toxin-sensitive

The ability of pertussis toxin (PTX) to inhibit prosaptide stimulation of pERK in cells transfected with GPR37 (A) and GPR37L1 (B) was assessed. Transfected HEK293T cells were pre-treated with PTX (14-18 hr, 150 ng/ml) and assessed for their ability to respond to 100nM prosaptide. In cells not exposed to PTX, prosaptide was able to induce a significant increase in pERK over vehicle-treated cells (*** $p<0.001$, ** $p<0.01$). In cells treated with pertussis toxin, however, prosaptide failed to induce a significant increase in pERK over vehicle, and thus cells treated with both PTX and prosaptide had significantly lower pERK levels than cells treated with prosaptide alone (*** $p< 0.001$, * $p<0.05$). All experiments were performed in duplicate (n=3).

A.


40
30
20
10
0
% Internalization
*
Mock
GPR37
GPR37L1

B.

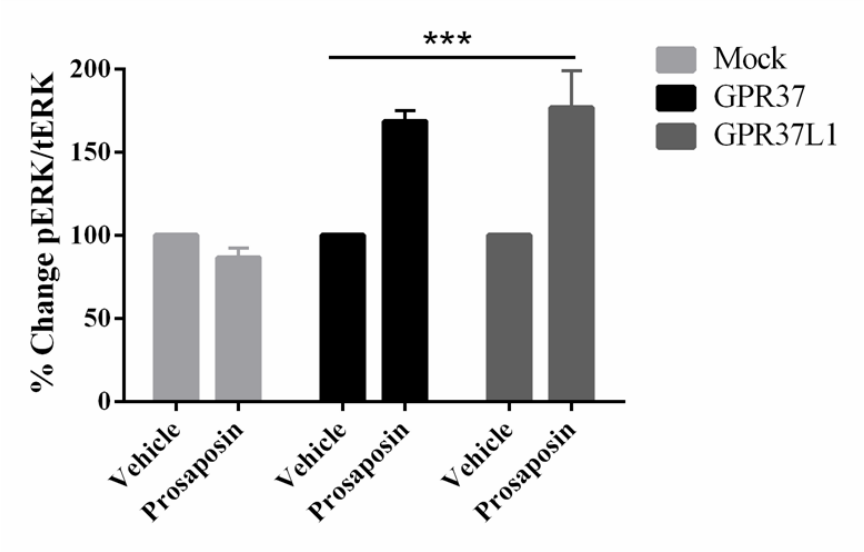

200
150
100
50
0
% Change pERK/tERK
***
Mock
GPR37
GPR37L1
Vehicle
Prosaposin
Vehicle
Prosaposin
Vehicle
Prosaposin

Figure 2-9. Prosaposin induces internalization of GPR37 and GPR37L1 and also stimulates pERK signaling through the receptors

A) Endocytosis of FLAG-GPR37 and FLAG-GPR37L1 expressed in HEK 293T cells was assessed following treatment with 1 μM prosaposin for 30 minutes. Prosaposin induced significant internalization of both GPR37 and GPR37L1 (n=3; all points done in triplicate, * $p<0.05$). B) Treatment of HEK293T cells transiently transfected with empty vector ("mock"), GPR37 or GPR37L1 with prosaposin (100 nM, 10 min) resulted in enhanced ERK phosphorylation in only GPR37- or GPR37L1-transfected cells (n=3; all points done in duplicate, *** $p<0.001$).

Section 2.4 Summary and Discussion

Through the experiments described in this chapter, we have identified that GPR37 and GPR37L1 are receptors for the protective factor prosaposin as well as its active fragment peptide prosaptide. Using receptor endocytosis as an output, we screened for potential ligands to activate GPR37 and GPR37L1. In this way, we identified the 14 amino acid peptide, prosaptide TX14(A), as capable of inducing GPR37 and GPR37L1 internalization. However, prosaptide was not able to induce internalization of other GPCRs, and other peptides were not able to induce internalization of GPR37 and GPR37L1 suggesting specificity between the ligand and receptors. Additionally, prosaptide specifically binds to GPR37 and GPR37L1, but not other GPCRs, further suggesting specificity in the interaction. Furthermore, we found that prosaposin, the endogenous protein from which prosaptide is derived, was also able to induce internalization of GPR37 and GPR37L1, and both prosaposin and prosaptide were able to induce ERK phosphorylation through GPR37 and GPR37L1. Prosaptide was able to induce the binding of Gαi proteins to bind resulting in the inhibition of cAMP. The Gαi-coupling of GPR37 and GPR37L1 was further confirmed through the inhibition of pERK through pertussis toxin pretreatment.

GPR37 and GPR37L1 have been linked to a myriad of pathologies in the central nervous system including Parkinson's disease (Yang et al., 2003, Marazziti et al., 2004, Murakami et al., 2004, Kitao et al., 2007, Wang et al., 2008b, Wang et al., 2008a, Marazziti et al., 2009, Dusonchet et al., 2009, Mandillo et al., 2013, Lundius et al., 2013), , substance abuse (Marazziti et al., 2011, McBride et al., 2013), CNS development (Marazziti et al., 2013), and psychiatric disorders (Aston et al., 2005, Fujita-Jimbo et al., 2012, Logotheti et al., 2013), indicating their potential as therapeutic targets. However, since GPR37 and GPR37L1 are classified as orphan receptors, their lack of a known ligand makes them difficult to study in a pharmacological context. The identification of prosaposin and prosaptide and ligands for the orphan GPCRs

GPR37 and GPR37L1 opens new doors for the study of these receptors with regard to their pharmacology and function.

Given the previous report of the ability of the invertebrate peptide head activator (HA) to activate GPR37 (Rezgaoui et al., 2006, Gandia et al., 2013), it is interesting to note that prosaptide and HA are similar in length and exhibit significant sequence similarity in their C-terminal regions (K-V-I-L for HA vs. K-E-I-L for prosaptide). HA does not appear to be a true ortholog of prosaptide, but nonetheless we propose that this invertebrate peptide may possess the ability to act as a GPR37 agonist due to its similarity to prosaptide and prosaposin. It is also interesting to note that a recent report regarding HA and GPR37 confirmed our finding that GPR37 is a Gαi-coupled protein as it was shown to inhibit cAMP in transfection-dependent manner (Gandia et al., 2013). However, it is also of interest to note that other groups have been unable to replicate the finding that HA can activate GPR37 (Dunham et al., 2009, Southern et al., 2013), casting doubt on its ability to activate the receptor. However, HA is a difficult peptide to study as it can dimerize in solution but requires monomerization for functional activity (Rezgaoui et al., 2006), and these difficulties inherent in working with HA may explain the discrepancies between studies assessing HA as a ligand for GPR37. Regardless, given the dearth of HA expression in mammalian tissue, HA is unlikely to be an endogenous ligand for mammalian GPR37.

The studies shown in this chapter demonstrate that GPR37 and GPR37L1 are individually sufficient to mediate the G protein-dependent signaling by prosaptide and prosaposin, using HEK293T cells that do not show any endogenous responses to prosaptide or prosaposin as a cellular model system. However, our experiments reveal that transfection of either GPR37 or GPR37L1 is sufficient to confer G protein-mediated signaling by prosaptide and prosaposin to HEK293T cells indicating that both are able to act as receptors for prosaposin and prosaptide.

Chapter 3:

Prosaptide and Prosaposin Induce Pro-Survival Signaling in Cortical Astrocytes via Stimulation of GPR37 and GPR37L1

Section 3.1. Rationale

The studies described in the preceding chapter made use of transiently-transfected HEK293T cells to screen for ligands for GPR37 and GPR37L1. These cells were chosen in part because they do not endogenously express GPR37 or GPR37L1, and thus the receptors could be introduced and compared to mock-transfected cells as a control. However, as valuable as studies in transfected cells can be, it is important to pair such studies with analyses of native cells that endogenously express the receptors of interest. Thus, we studied cortical astrocytes, which endogenously express both GPR37 and GPR37L1. In order to assess whether the receptors were necessary for the physiological effects of prosaptide and prosaposin on the astrocytes, we utilized an siRNA knockdown approach. Additionally, we chose to examine cell death through examining lactate dehydrogenase (LDH) release into the media, allowing for further examination of cells to determine mechanism of cell death and protection.

Once we identified that GPR37 was essential for prosaptide signaling in cortical astrocytes, we sought to expand our understanding of the mechanism through which GPR37 induces ERK phosphorylation in these cells. As crosstalk with receptor tyrosine kinases by GPCRs is a common mechanism to induce ERK phosphorylation, we chose to examine the ability of GPR37 to transactivate the EGF receptor as a means to stimulate pERK, and furthermore began to map out the intracellular mediators, including matrix metalloproteinases, βγ G protein subunits, protein kinase C and Src, that contribute to GPR37-mediated transactivation of the EGF receptor.

Section 3.2. Experimental Methods

3.2.1 Materials

0.25% Trypsin + EDTA (Invitrogen); DNase (Worthington); 100µm tissue strainer (BD Bioscience); Poly-D-lysine-coated T75 flask (BD Bioscience); Poly-D-lysine-coated 6-well cell culture plates (BD Bioscience); Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen); Fetal Bovine Serum (FBS) (Atlanta Biologicals); penicillin/streptomycin (Invitrogen); Prosaptide TX14(A) (Anaspec); 4-20% Tris-glycine gels (Invitrogen); nitrocellulose (Bio-Rad); Odyssey blocking buffer (Li-Cor); Enhanced Chemiluminescence (ECL) and ELISA Pico Chemiluminescence reagents (Thermo Scientific); Autoradiography Film (Denville); GPR37 siRNA (Qiagen); GPR37L1 siRNA (Qiagen); Mouse glia nucleofection kit (Lonza); Nucleofector (Amaxa); Caspase-3 antibody (Cell Signaling Technologies); CytoTox 96 nonradioactive cytotoxicity assay (Promega); AG1478 (CalBiochem); Bisindolylmaleimide IV (BIM IV) (Sigma-Aldrich); PP2 (Sigma-Aldrich); Gallein (Sigma-Aldrich)

In all experiments, prosaptide TX14(A) was resuspended in an assay buffer containing 20mM HEPES, 100mM NaCl, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 5mM KCl, 0.5mM EDTA, and a protease inhibitor tablet (Roche).

3.2.2 Cell Culture

To culture cortical astrocytes, cortices were dissected from C57BL/6 mice 1-4 days of age, meninges removed, and incubated in 0.25% trypsin + EDTA for 5-10 minutes at 37 degrees Celsius and 5% CO_2. Following initial trypsin dissociation, 600 U DNase was added to the trypsin and tissue and DMEM supplemented with 10% (vol/vol) FBS and 100µg/ml penicillin-streptomycin was added to the tissue to inhibit trypsin activity. Tissue was triturated with a glass pipette approximately ten times and dissociated tissue was transferred to a 50 mL conical tube and centrifuged for 10 minutes at 100 × g. The resulting tissue pellet was resuspended in 15 mL of DMEM with FBS and penicillin-streptomycin and triturated approximately 20 times to fully

homogenize the tissue. The homogenate was passed through a 100µm tissue strainer and plated in a poly-D-lysine-coated T75 flask. Cells were grown for 7-10 days at 37 degrees Celsius with 5% CO_2 to reach confuency. Once astrocytes formed a confluent monolayer they were either nucleofected or split into two 6-well plates for signaling experiments.

3.2.3 Prosaposin Production

Prosaposin production and purification was performed by M. Giddens. HEK293T cells were transiently transfected with 5µg hexahistidine-tagged prosaposin using Lipofectamine 2000. Transfected cells were incubated in DMEM without serum for 48 hours. Media was removed from plates and spun down at 3000 × g for 10 minutes to remove dead cells and concentrated using 10 kDa ultrafiltration filters and centrifugation at 4000 × g for 15 minutes. The concentrated media was incubated with Pro-Bond nickel resin end-over-end at 4 degrees Celsius for 30 minutes to isolate the His-tagged prosaposin. The resin was washed 3 times with 10mM imidazole buffer (300mM NaCl, 50mM NaH_2PO_4, and PBS + Ca^{2+}, pH 7.4). Purified prosaposin was concentrated via ultrafiltration at 4000 × g for 15 minutes. The resulting prosaposin was assessed using the BSA protein assay to determine total protein concentration and run on an SDS-PAGE gel and stained with coomassie to estimate purity levels. Prosaposin presence was also confirmed using Western blot with antibodies against prosaposin and myc.

3.2.4 siRNA Knockdown

Primary cortical astrocytes were trypsinized and resuspended in DMEM then pelleted through centrifugation for 15 minutes at 100 × g. The pellet was resuspended in supplemented

nucleofector solution with 200 nanomoles of siRNA against either receptor or a scrambled siRNA and nucleofected. Following nucleofection, cells were resuspended in supplemented DMEM and incubated at 37 degrees Celsius and 5% CO_2 to allow cell recovery before being plated on poly-D-lysine (PDL) coated 6-well plates. Cells were used for signaling or functional studies 72 hours following nucleofection.

3.2.5 pERK Assay

Cultured astrocytes were plated on poly-D-lysine-coated 6-well plates and starved in serum-free DMEM for 2-3 hours before assay stimulation. To initiate signaling, DMEM was removed and replaced with fresh serum-free DMEM or DMEM containing prosaptide or prosaposin. Plates were then placed back into the incubator and incubated for the time indicated at 37 degrees Celsius with 5% CO_2. Cells were harvested in laemelli sample buffer, sonicated, and run on Tris-glycine gels

For experiments involving pre-treatment with chemical inhibitors, cultured astrocytes were grown and split as described. The day of the assay, astrocytes in 6-well plates were starved in serum-free DMEM for 1 hour. The media was then exchanged for serum-free DMEM containing the inhibitor under study and plates were again incubated for 1 hour. Cells were then treated for 10 minutes with prosaptide or DMEM alone and harvested according to the protocol above. Inhibitor concentrations are as follows: AG1478 was used at 10µM, GM6001 was used at 10µM, BIM IV was used at 1µM, Gallein was used at 10oµM, and PP2 was used at 10µM.

3.2.6 Cytotoxicity

48 hours following nucleofection, astrocytes were treated with prosaptide or prosaposin in serum-free DMEM for 10 minutes at 37 degrees Celsius and 5% CO_2. Following the drug

pretreatment, the media was aspirated and replaced with serum-free DMEM containing 500µM H_2O_2 for 24 hours at 37 degrees Celsius and 5% CO_2. To examine LDH release, media was harvested and analyzed using the CytoTox 96 nonradioactive cytotoxicity assay. To do this, media samples were added to equal parts lactate dehydrogenase substrate in clear 96-well plates and incubated at 37 degrees Celsius in the dark for 30 minutes. Following incubation, the reaction was stopped and the plate was read at 490 nm to assess the colormetric change. Percent protection was identified by determining differences in total cell death in the presence and absence of drug. Cell death was calculated using the following formula:

$$\frac{(Drug\ Treatment\ LDH - Spontaneous\ Release\ LDH)}{(Total\ Kill\ LDH\ - Spontaneous\ Release\ LDH)}$$

3.2.7 Western Blotting

Samples for Western blotting were harvested in laemmli sample buffer containing 62.5mM Tris·HCl (pH 6.8), 25% glycerol, 2% SDS, and 0.01% bromophenol blue. Protein samples were resolved via SDS-PAGE on 4-20% Tris-glycine gels and transferred to nitrocellulose membranes. Gel transfer was done at 25V for 90 minutes. To blot for phospho-ERK (pERK), membranes were blocked with Odyssey blocking buffer for 30 minutes at room temperature and overnight at 4 degrees Celsius with primary antibodies prepared in a buffer comprised of equal parts blocking buffer and PBS + 0.1% Tween-20. Membranes were washed 3 times in PBS + 0.1% Tween-20 and incubated with secondary antibodies for 30 minutes at room temperature. Blots were then washed 3 times in PBS + 0.1% Tween-20 and rinsed in PBS until visualization. Phospho-ERK blots were visualized on an Odyssey Imaging System (Li-Cor) and analyzed using Image-J. Blots were then visualized on the 700 channel for pERK and the 800 channel for total ERK rather than requiring two separate blots. For all other Western blots,

membranes were incubated in a milk blocking buffer (2% nonfat dry milk, 50mM NaCl, 20mM HEPES (pH 7.4) and 0.1% Tween-20) for 30 minutes at room temperature followed by 1 hour incubation with primary antibody at room temperature. Blots were washed 3 times with blocking buffer and incubated with HRP-conjugated secondary antibody for 30 minutes at room temperature. Following secondary antibody incubation, blots were washed 3 times with blocking buffer and then incubated with enhanced chemiluminescence (ECL) reagent and exposed to autoradiography film.

Section 3.3. Results

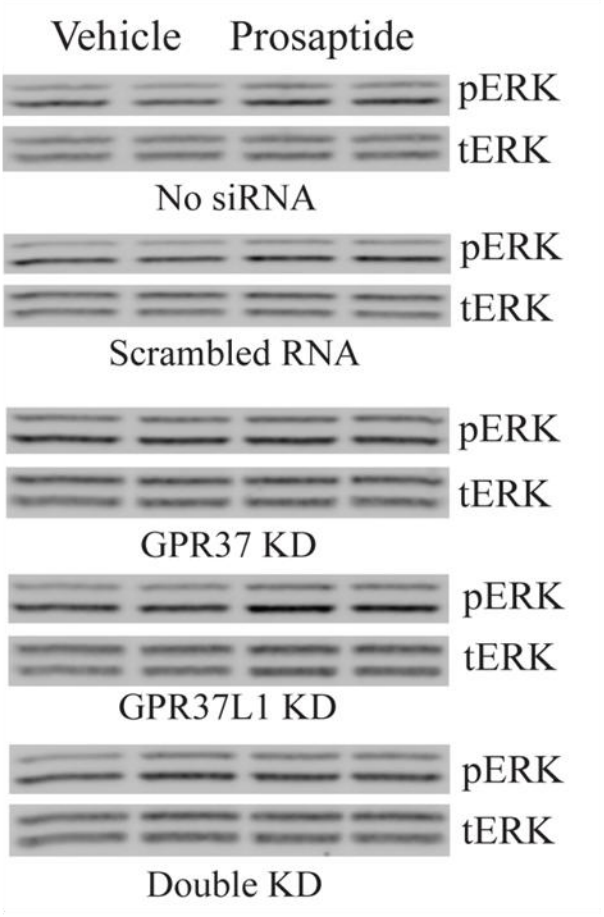
3.3.1 Prosaptide and Prosaposin Induce ERK Phosphorylation through GPR37, but not GPR37L1, in Cortical Astrocytes

To examine the role of GPR37 and GPR37L1 in the transduction of prosaptide signaling in cortical astrocytes, we used siRNA to specifically target GPR37 and GPR37L1, as well as both receptors together, and examined prosaptide signaling to identify any changes as a result of receptor knockdown. Treatment of cortical astrocytes with 100nM prosaptide for 10 minutes induced pERK signaling in astrocytes not treated with siRNA. The use of scrambled siRNA did not affect the ability of prosaptide to signal to pERK in cortical astrocytes as there was no change from the no siRNA condition (Figure 3-1A&B). Additionally, knocking down GPR37L1 from the astrocytes had no effect on the ability of prosaptide to induce a pERK response. However, knocking down GPR37 or a joint knockdown of both receptors together completely abolished the ability of prosaptide to induce ERK phosphorylation.

In addition to examining the ability of endogenous astrocytic GPR37 and GPR37L1 to mediate signaling induced by prosaptide, we also explored the importance of the receptors for

astrocytic ERK signaling induced by full-length prosaposin. As with the experiments using prosaptide, prosaposin was able to induce the phosphorylation of ERK in cortical astrocytes and this signaling was not disrupted using scrambled siRNA. Additionally, knockdown of GPR37L1 had no effect on prosaposin-induced pERK signaling. As with prosaptide, though, the knockdown of GPR37 or both receptors together completely prevented pERK signaling induced by prosaposin (Figure 3-2A&B).

A.


Vehicle
Prosaptide
pERK
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No siRNA
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GPR37 KD
pERK
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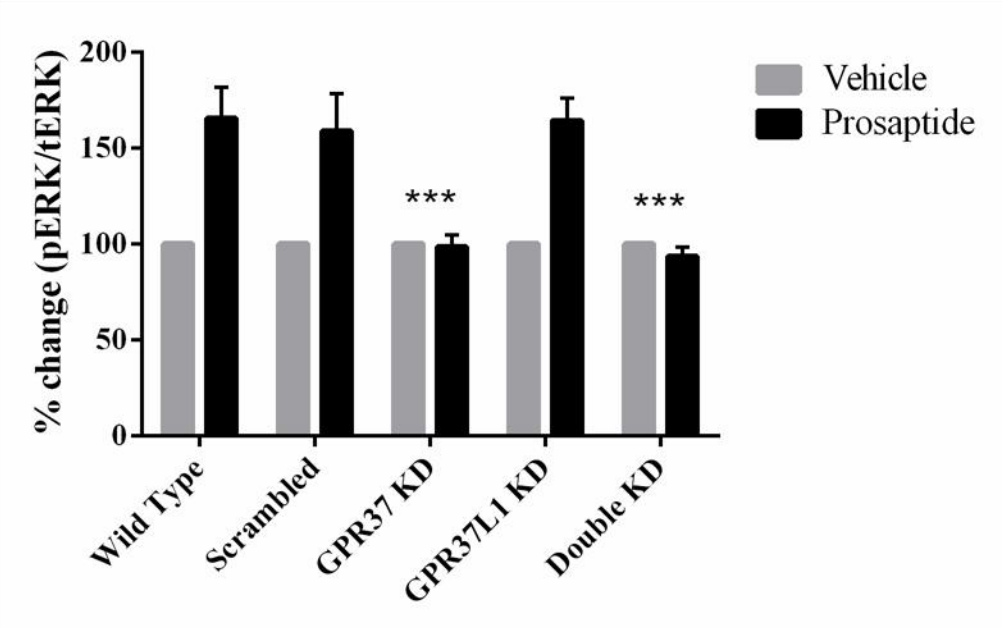
GPR37L1 KD
pERK
tERK
Double KD

B.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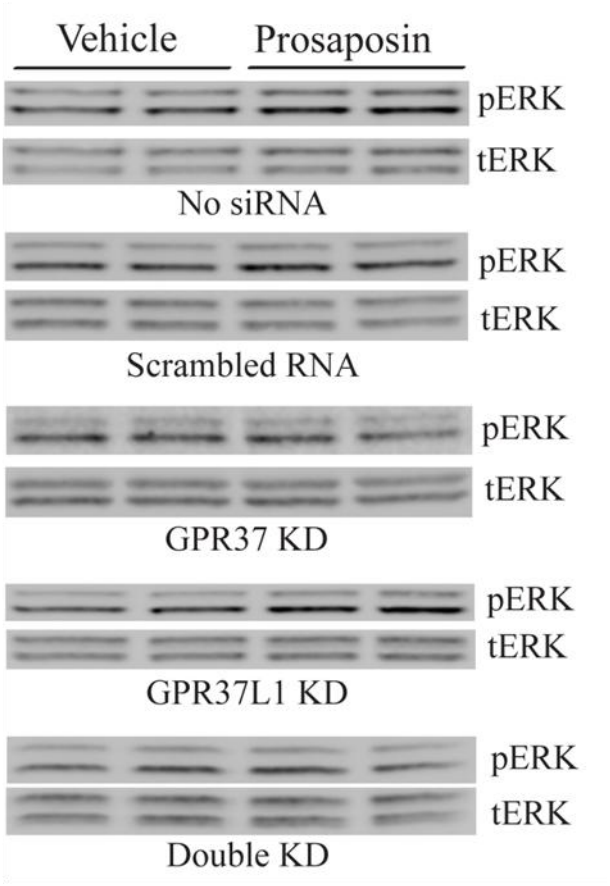
Prosaptide
Wild Type
Scrambled
GPR37 KD
GPR37L1 KD
Double KD

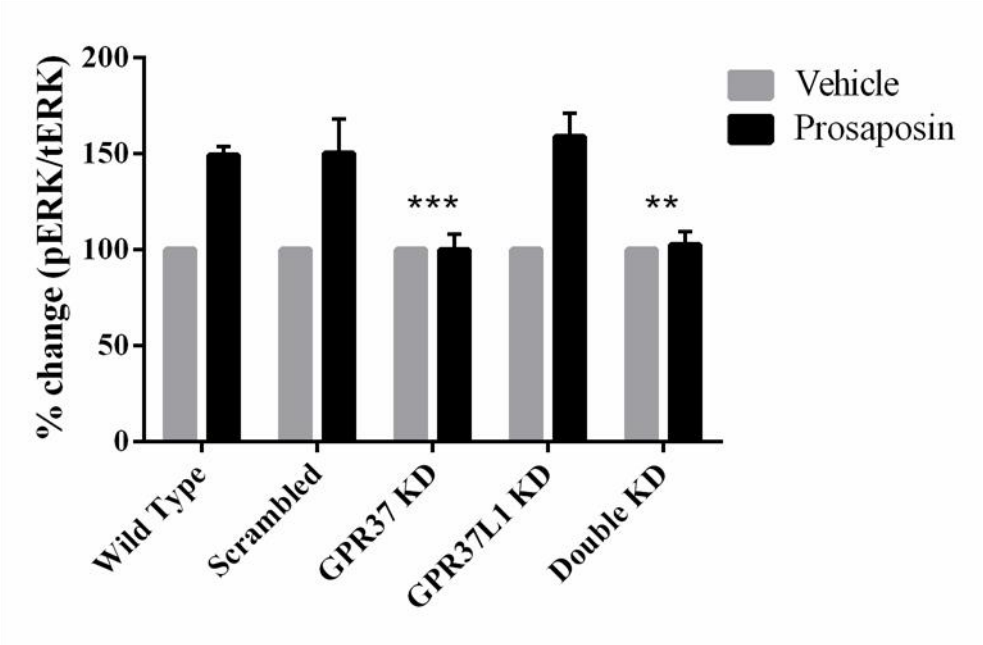
Figure 3-1. Knockdown of GPR37 but not GPR37L1 interferes with prosaptide-induced ERK phosphorylation in cortical astrocytes

A) Prosaptide-induced ERK phosphorylation in primary cortical astrocytes was assessed under several conditions: no siRNA treatment, treatment with a scrambled siRNA, or treatment with siRNAs directed against GPR37 and/or GPR37L1 ("KD" = "knockdown", "Double KD"= knockdown of both GPR37 and GPR37L1). B) Quantification of the studies illustrated in panel A (n=3; all points done in duplicate, *** $p<0.001$).

A.


Vehicle
Prosaposin
pERK
tERK
No siRNA
pERK
tERK
Scrambled RNA
pERK
tERK
GPR37 KD
pERK
tERK
GPR37L1 KD
pERK
tERK
Double KD

B.


% change (pERK/tERK)
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Vehicle
Prosaposin
Wild Type
Scrambled
GPR37 KD
GPR37L1 KD
Double KD

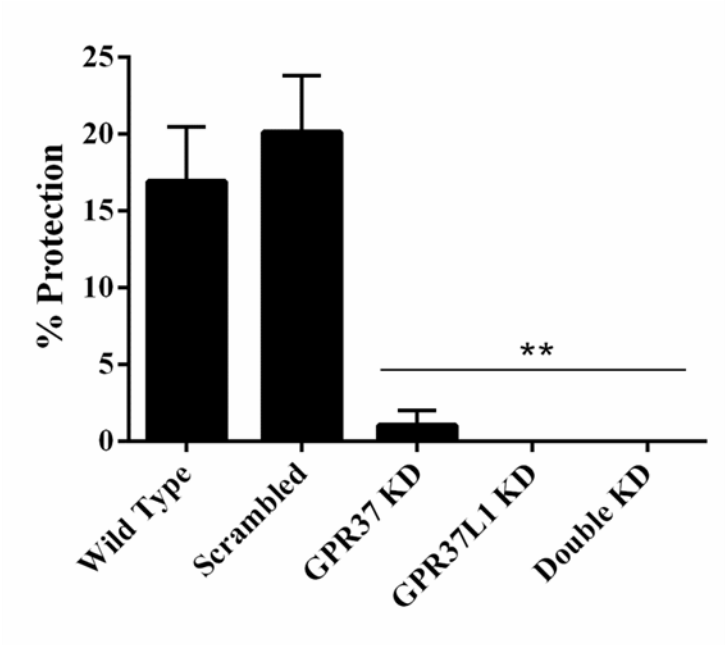
Figure 3-2. Knockdown of GPR37 but not GPR37L1 prevents prosaposin-induced ERK phosphorylation in cortical astrocytes

A) Primary cortical astrocytes were treated with either no siRNA, a scrambled siRNA, or siRNAs directed against GPR37 and/or GPR37L1. These astrocytes were then stimulated with prosaposin (100 nM, 10 min) and activation of ERK was assessed. D) Quantification of the experiments illustrated in panel A (n=3; all points done in duplicate, *** $p<0.001$).

3.3.2 GPR37 and GPR37L1 Mediate the Ability of Prosaptide and Prosaposin to Protect Against Cell Death Induced by Oxidative Stress in Cortical Astrocytes

Due to reports of prosaposin as a neuroprotective and glioprotective factor (O'Brien et al., 1994, O'Brien et al., 1995, Kotani et al., 1996b, Hiraiwa et al., 1997b, Campana et al., 1998b, Campana et al., 1998a, Misasi et al., 1998, Tsuboi et al., 1998, Campana et al., 1999, Igase et al., 1999, Calcutt et al., 1999, Campana et al., 2000, Lu et al., 2000, Yan et al., 2000, Calcutt et al., 2000, Liu et al., 2001, Morita et al., 2001, Jolivalt et al., 2008b, Li et al., 2010, Toyofuku et al., 2012), we examined the ability of prosaposin and prosaptide to protect cortical astrocytes from oxidative cell death induced by hydrogen peroxide (H_2O_2). Following H_2O_2 treatment, which induces oxidative stress, the release of lactate dehydrogenase (LDH) into the media was assessed and used to determine percent cell death as well as percent protection induced by prosaptide and prosaposin. Both prosaptide and prosaposin treatment significantly protected cortical astrocytes from H_2O_2-induced death in cultures treated with either no siRNA or scrambled siRNA. However, in astrocytes in which GPR37, GPR37L1, or both receptors had been knocked down, the ability of prosaptide or prosaposin to promote cell survival was significantly attenuated (Figure3-3A and Figure 3-3B, respectively).

A.


% Protection
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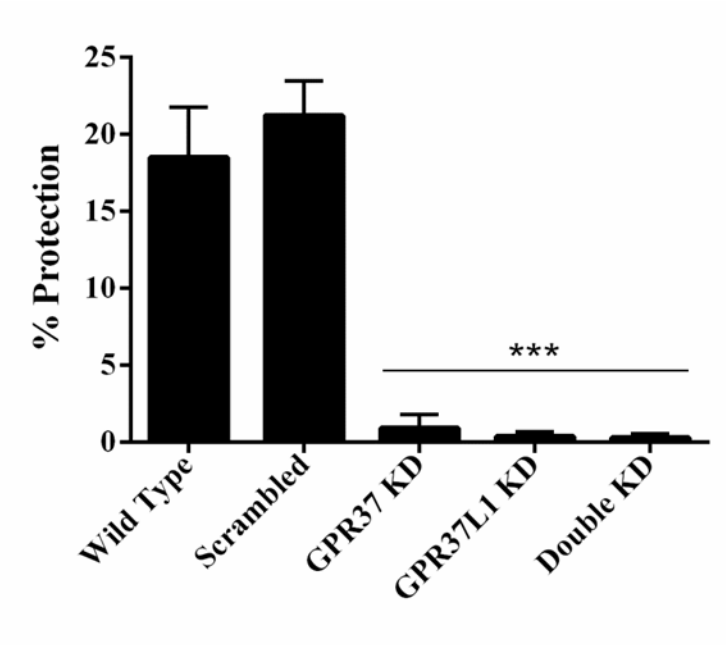
Wild Type
Scrambled
GPR37 KD
GPR37L1 KD
Double KD

B.


% Protection
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15
10
5
0
***
Wild Type
Scrambled
GPR37 KD
GPR37L1 KD
Double KD

Figure 3-3. Knockdown of either GPR37 or GPR37L1 prevents prosaptide- and prosaposin-induced protection of cortical astrocytes against oxidative cell death

A) Primary cortical astrocytes were exposed to H_2O_2, and cell death was assessed via measuring the release of lactate dehydrogenase into the media. Prosaptide (A) or prosaposin (B) pre-treatment (100 nM) protected the astrocytes from oxidative stress, but the effects of prosaptide and prosaposin were attenuated when GPR37 and/or GPR37L1 was knocked down via siRNA (n=3; all points done in triplicate, *** $p<0.001$, ** $p<0.01$).

3.3.3 GPR37 and GPR37L1 Mediate the Pro-Survival Effects of Prosaptide and Prosaposin Effects Partially through Preventing the Cleavage of Procaspase-3

Prosaposin has been previously reported to induce anti-apoptotic effects (Tsuboi et al., 1998, Campana et al., 1999, Misasi et al., 2001, Morita et al., 2001, Lee et al., 2004, Ochiai et al., 2008, Sorice et al., 2008, Li et al., 2010, Toyofuku et al., 2012, Gao et al., 2013b), and H_2O_2 treatment has been shown to induce apoptosis (Slater et al., 1995a, Slater et al., 1995b, Hampton et al., 1998). Thus, we examined the astrocytes that had been exposed to H_2O_2 in the aforementioned experiments for markers of apoptosis. In doing so, we found that in normal cortical astrocytes, both prosaptide and prosaposin were able to prevent the cleavage of pro-caspase 3 into caspase 3, which is a marker for apoptosis (Figure 3-4A). We furthermore found that knock down of both receptors was able to reverse the abilities of prosaptide and prosaposin to prevent pro-caspase 3 cleavage (Figure 3-4B, prosaptide $p<0.05$, prosaposin $p<0.01$, $n=3$).

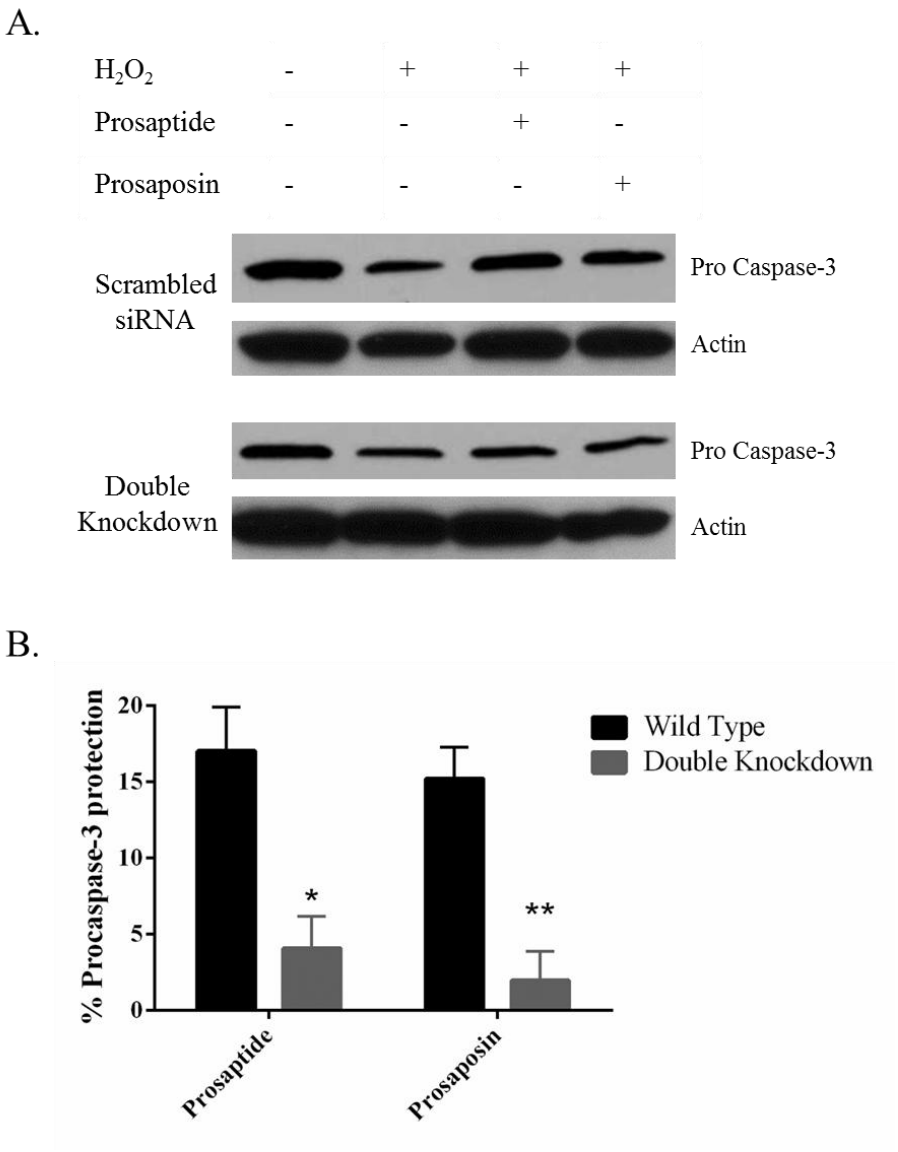


Figure 3-4. Knockdown of GPR37 and GPR37L1 inhibits prosaptide and prosaposin from preventing pro-caspase 3 cleavage

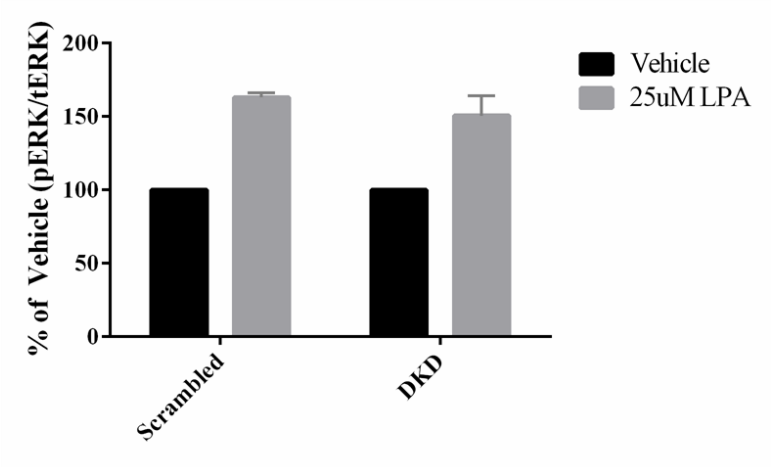
A) Prosaptide and prosaposin both prevented the cleavage of pro-caspase 3 into caspase 3 in astrocytes following hydrogen peroxide insult. However, knocking down both GPR37 and GPR37L1 negated the ability of prosaptide and prosaposin to exert protective effects. B) The effect of prosaptide and prosaposin on preventing H2O2-induced pro-caspase 3 cleavage was quantified (* $p > 0.05$, ** $p > 0.01$).

3.3.4 Knockdown of GPR37 and GPR37L1 does not alter Fundamental Cortical Astrocyte Signaling or Basal Cell Death from Hydrogen Peroxide

To establish the specificity of knocking down GPR37 and GPR37L1, we examined the astrocytes for any non-specific and off-target effects. With regard to signaling, we examined the effect of knocking down both receptors on the basal signaling by lysophosphatidic acid (LPA) receptors, which are found endogenously in cortical astrocytes and known to signal to pERK (Pebay et al., 1999). We found that knocking down GPR37 and GPR37L1 had no effect on LPA-induced signaling to pERK (Figure 3-5A, n=2).

Additionally, to examine whether or not knock down of GPR37 or GPR37L1 might exert effects on cell survival in the absence of prosaptide or prosaposin treatment, we examined the basal levels of cell death associated with exposure to hydrogen peroxide. We observed that knocking down GPR37, GPR37L1, or both receptors together had no effect on basal cell death induced by hydrogen peroxide (Figure 3-5B, n=3).

A.



B.

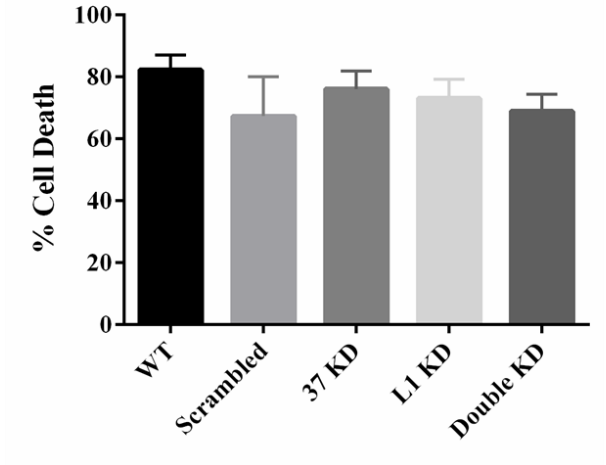


Figure 3-5. Knockdown of GPR37 and GPR37L1 does not interfere with global pERK signaling or basal cell death in cortical astrocytes

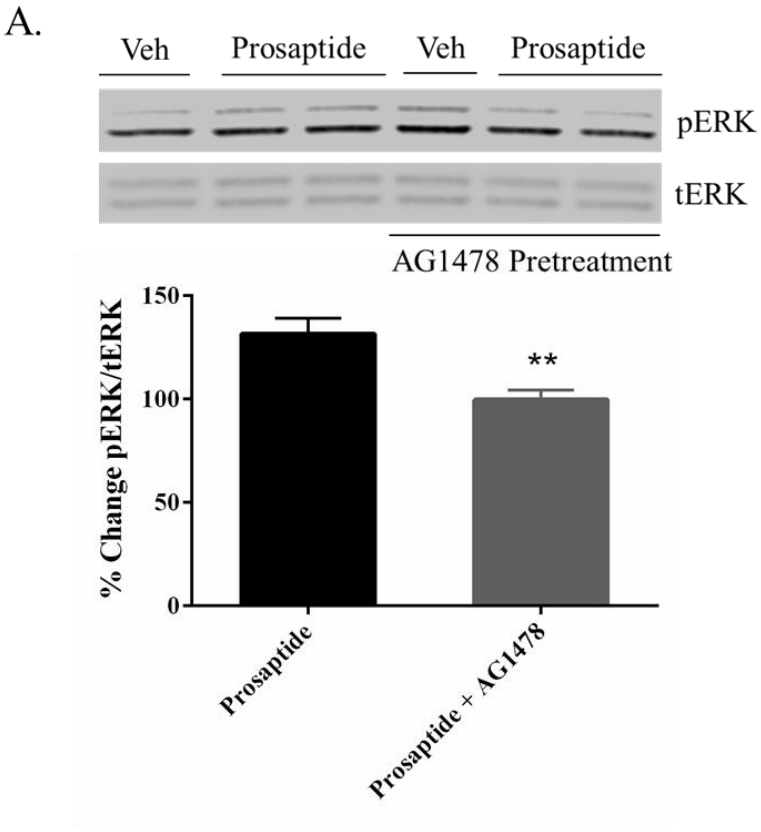
A) The ability of 25μM lysophosphatidic acid (LPA) to induce ERK phosphorylation was assessed in primary cortical astrocytes nucleofected with scrambled siRNA ("Scrambled") versus siRNA directed against GPR37 and GPR37L1 ("DKD" for "Double Knockdown"). There were no differences observed in the extent of pERK induction by LPA in the scrambled versus double knockdown conditions (n=2, all points performed in duplicate). B) The extent of the siRNA-mediated knockdown of GPR37 and GPR37L1 in primary astrocyte cultures was assessed via Western blot and quantified as percent knockdown. Error bars represent S.E.M. (n=3).

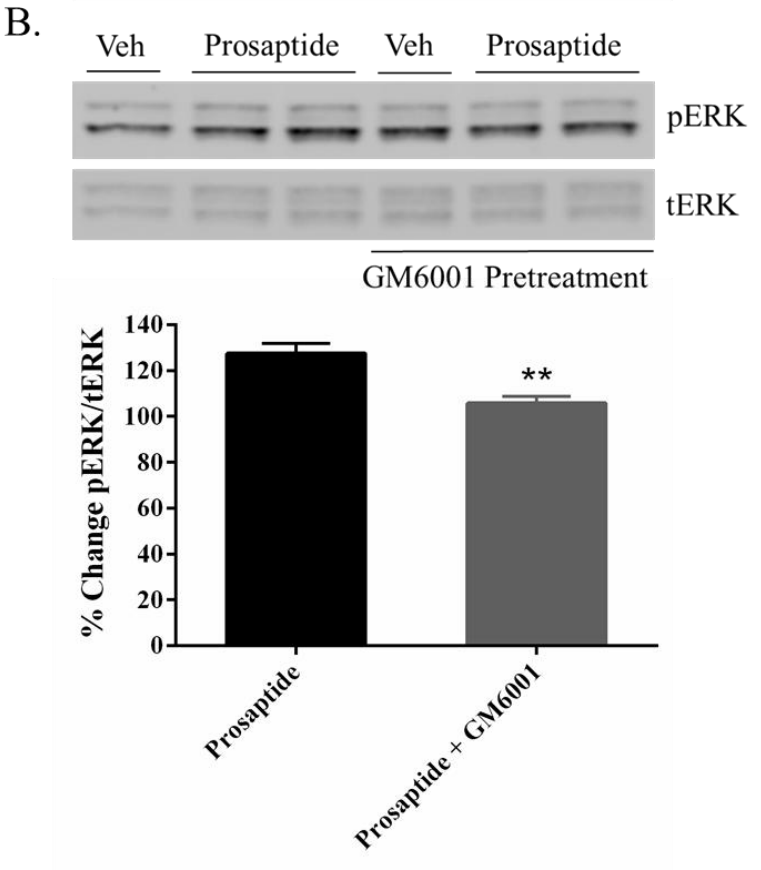
3.3.5 GPR37 Mediates Prosaptide Signaling to pERK through Transactivation of the EGF Receptor

Since we established that GPR37, but not GPR37L1, is responsible for prosaptide-induced pERK signaling in cortical astrocytes, we next sought to further elucidate the mechanism through which this signaling occurs. Many GPCRs activate ERK phosphorylation by transactivating receptor tyrosine kinases (Daub et al., 1997, Cunnick et al., 1998, Moriguchi et al., 1999, Bokemeyer et al., 2000, Vacca et al., 2000). To examine whether or not GPR37 might stimulate pERK signaling through receptor tyrosine kinase transactivation, we specifically examined the potential involvement of the epidermal growth factor receptor (EGFR), which is known to be expressed in astrocytes (Wagner et al., 2006, Liu and Neufeld, 2007). Interestingly, we found that pretreatment with the EGFR inhibitor AG1478 completely inhibited prosaptide-induced pERK signaling in astrocytes (Figure 3-6A, $p< 0.01$, $n=5$) suggesting that GPR37 mediates prosaptide signaling to pERK through transactivating EGFR.

We also assessed prosaptide-induced signaling to ERK in the presence of GM6001, a global inhibitor of matrix metalloproteinases (MMPs) (Nadal et al., 2002, Santiskulvong and Rozengurt, 2003). Inhibition of MMP activity should inhibit the cleavage of pro-EGF into EGF, which is a fundamental step in the EGFR transactivation pathway. Pre-treatment of the astrocytes with GM6001 completely prevented prosaptide signaling to ERK (Figure 3-6B, $p<0.05$, $n=3$), consistent with a requirement for prosaptide-induced EGF shedding leading to EGFR transactivation. Additionally, we examined the effect of pre-treatment with an inhibitor of the G protein $\beta\gamma$ subunits to alter prosaptide-induced ERK signaling in cortical astrocytes. Gallein is an inhibitor of $G\beta\gamma$ subunits and can be used to disrupt G protein signaling (Lehmann et al., 2008, Casey et al., 2010). We found that pre-treatment with galleon inhibited prosaptide-induced pERK signaling in cortical astrocytes (Figure 3-6C, $p<0.05$, $n=5$).

Finally, since EGFR transactivation by GPCRs often involves protein kinase C (PKC) (Tebar et al., 2002, Shah and Catt, 2004) and Src (Bokemeyer et al., 2000, Zhang et al., 2012, Ishibashi et al., 2013), we also assessed prosaptide-induced ERK signaling in the presence of the PKC inhibitor BIM IV and the Src inhibitor PP2. We found that pre-treatment of the astrocytes with either BIM IV (Figure 3-7A $p<0.05$, $n=4$) or PP2 (Figure 3-7B, $p<0.01$, $n=4$) was able to block ERK phosphorylation induced by prosaptide stimulation.


A.
Veh
Prosaptide
Veh
Prosaptide
pERK
tERK
AG1478 Pretreatment
% Change pERK/tERK
150
100
50
0
**
Prosaptide
Prosaptide + AG1478


B.
Veh
Prosaptide
Veh
Prosaptide
pERK
tERK
GM6001 Pretreatment
% Change pERK/tERK
140
120
100
80
60
40
20
0
**
Prosaptide
Prosaptide + GM6001

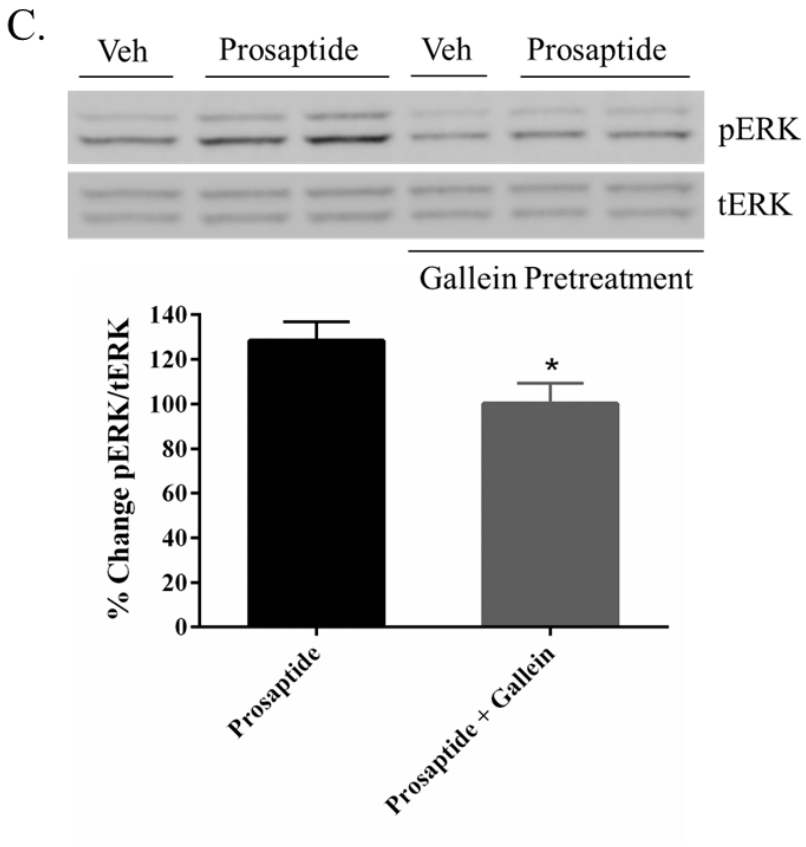
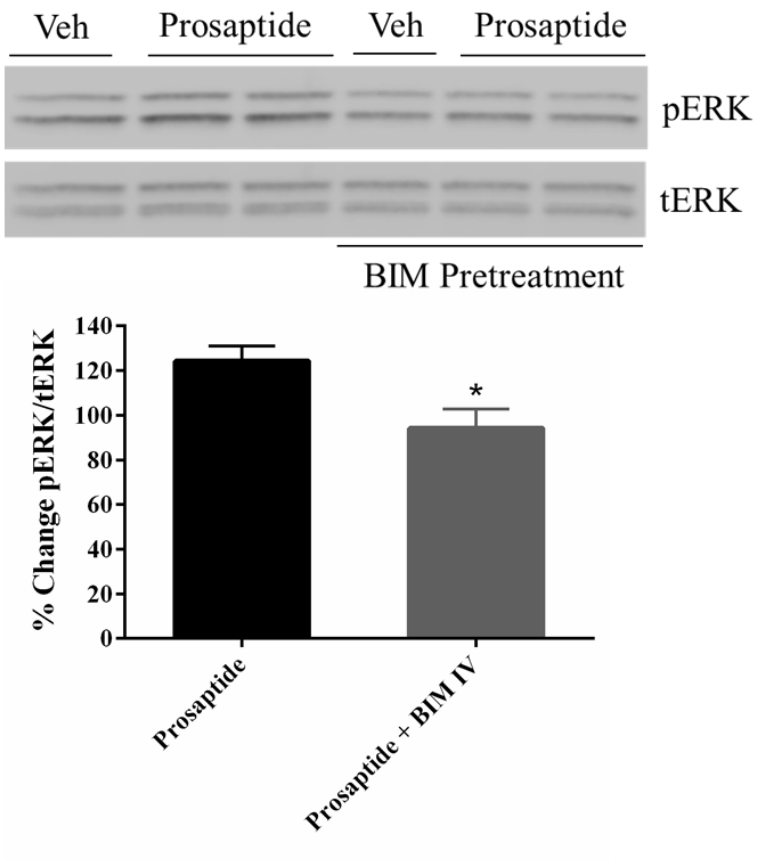


Figure 3-6. GPR37 mediates prosaptide signaling to pERK in cortical astrocytes through transactivation of the EGF receptor

A) Prosaptide-induced ERK phosphorylation in cortical astrocytes was inhibited by the pretreatment of AG1478, an EGFR inhibitor. (** $p<0.01$, n=5). B) Prosaptide-induced ERK phosphorylation in cortical astrocytes was inhibited by a global MMP inhibitor, GM6001 (** $p<0.01$, n=3). C) Prosaptide-induced ERK phosphorylation in cortical astrocytes was inhibited by a G protein βγ subunit inhibitor, gallein (* $p<0.05$, n=5).

A.


Veh
Prosaptide
Veh
Prosaptide
pERK
tERK
BIM Pretreatment
% Change pERK/tERK
140
120
100
80
60
40
20
0
*
Prosaptide
Prosaptide + BIM IV

B.

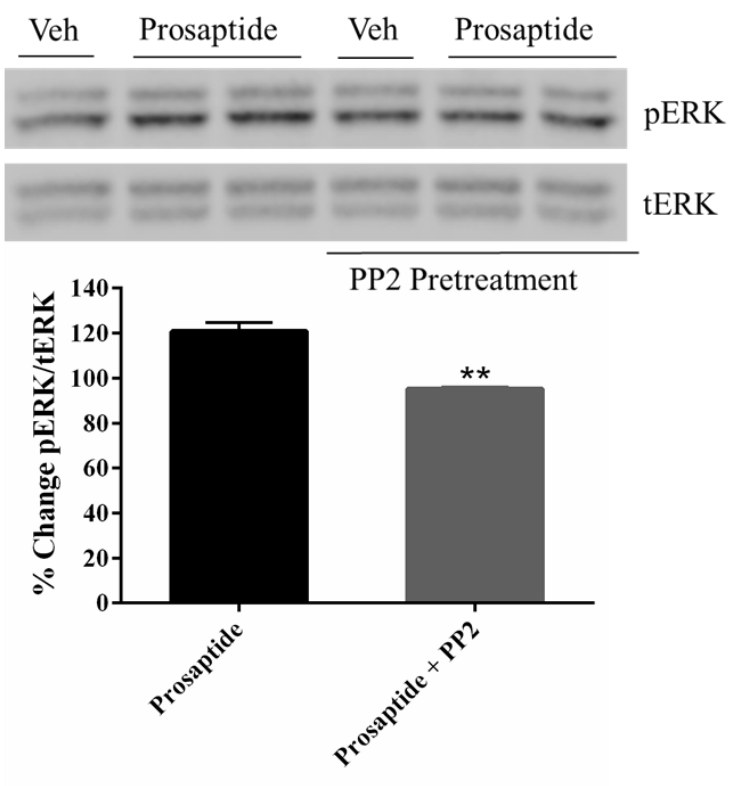

Veh
Prosaptide
Veh
Prosaptide
pERK
tERK
PP2 Pretreatment
% Change pERK/tERK
140
120
100
80
60
40
20
0
**
Prosaptide
Prosaptide + PP2

Figure 3-7. Prosaptide-induced pERK signaling is mediated through protein kinase C and Src

A) Pretreatment with the broad protein kinase C inhibitor, BIM IV, prevents prosaptide signaling to pERK in cortical astrocytes (* $p>0.05$, $n=4$). B) Pretreatment of cortical astrocytes with the Src inhibitor, PP2, prevents prosaptide-induced ERK phosphorylation (** $p>0.01$, $n=4$).

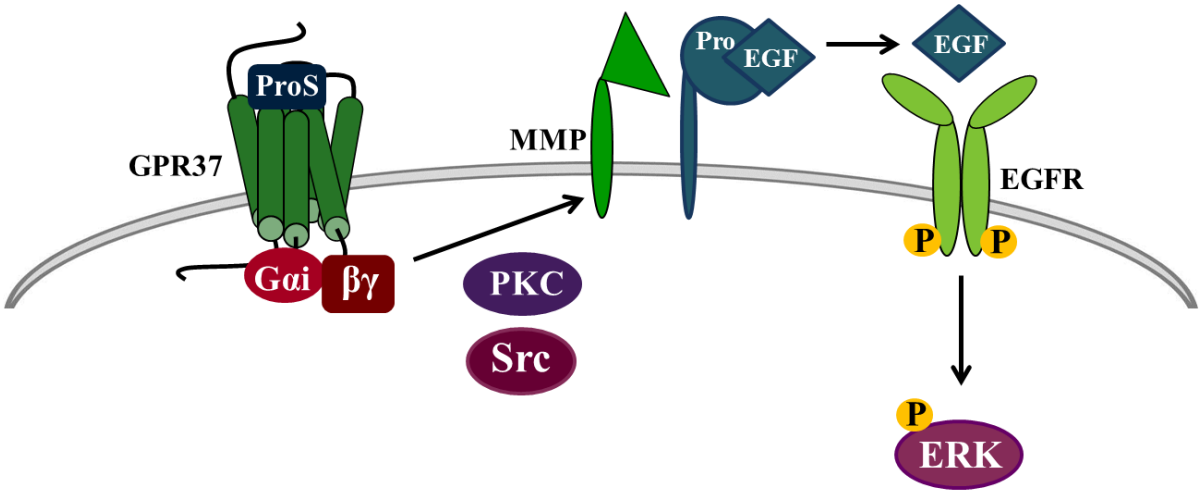


Figure 3-8. Summary of GPR37 signaling mechanism in cortical astrocytes

Upon stimulation with prosaptide, GPR37 induces ERK phosphorylation through transactivation of EGFR. This transactivation pathway is mediated though signaling from G proteins and involves the intracellular mediators PKC and Src as well as MMP cleavage of pro-EGF into an active form of EGF to stimulate EGFR signaling to pERK.

Section 3.4 Summary and Discussion

Following the identification of prosaptide and prosaposin as ligands for GPR37 and GPR37L1, we sought to determine if endogenous GPR37 and GPR37L1 mediate the physiological effects of prosaptide and prosaposin in primary astrocytes. In the experiments presented in this chapter, we demonstrated that GPR37 mediates prosaptide and prosaposin signaling to ERK in cortical astrocytes. This GPR37-mediated signaling depends on transactivation of the EGF receptor using the cellular activity of PKC and Src. Finally, we also demonstrated that GPR37 and GPR37L1 both mediate the cell protective effects of prosaptide and prosaposin in astrocytes, preventing apoptotic cell death induced by oxidative stress.

The ability of GPR37 and GPR37L1 to mediate the protective actions of prosaposin and prosaptide in cortical astrocytes provides new insights into the action of GPR37 and GPR37L1 as prosaposin receptors. While the work in Chapter 2 demonstrated that either GPR37 or GPR37L1 was sufficient to mediate prosaptide and prosaposin signaling, the studies shown in this chapter demonstrated that GPR37 and GPR37L1 are necessary to mediate the protective actions of prosaptide and prosaposin in cortical astrocytes. Furthermore, the ability of the cortical astrocytes to respond to prosaptide and prosaposin through the initiation of pERK signaling suggests that ERK phosphorylation at least partially mediates the protective response. The finding that only GPR37 mediates the ability of prosaptide and prosaposin to stimulate pERK in cortical astrocytes, whereas both receptors were necessary to mediate the protective response of prosaptide and prosaposin, suggests that GPR37 and GPR37L1 do not couple to exactly the same set of intracellular signaling pathways and that the protective actions of prosaptide and prosaposin via GPR37 and GPR37L1 are dependent on more than just downstream activation of ERK.

The demonstration that GPR37 can transactivate the EGF receptor to mediate pERK signaling provides added insight into the mechanism of prosaptide signaling in cortical astrocytes.

It has previously been reported that EGFR knockout mice display a unique type of neurodegeneration that primarily affects the cortex. This occurs due to an increased apoptosis of cortical astrocytes in a caspase-dependent manner resulting in neuronal loss (Wagner et al., 2006). This increased apoptosis in EGFR knockout astrocytes is not seen in the midbrain, suggesting cortical specificity of the mechanism of neuronal survival (Wagner et al., 2006). This observation raises a number of interesting questions with regard to the role of prosaptide signaling through GPR37 in cortical astrocytes, and the role this has in neuronal survival. Our observation that GPR37 is necessary for the protection of cortical astrocytes and that it mediates signaling to ERK through EGFR transactivation provides an intriguing parallel to finding of Wagner et al. It would be interesting for future studies to explore if the transactivation of EGFR results in enhanced cell survival of cortical astrocytes. Additionally, as our studies have dealt with protection upon a stressful insult, future studies might focus on identifying whether or not GPR37 is able to play a role in basal cell survival of astrocytes, or only act as a protective receptor. It would also be interesting to identify the effects of prosaptide and GPR37 for neuronal survival that is subsequent to astrocyte survival. Moreover, given the observations that GPR37 is also highly expressed in the midbrain and that prosaposin and prosaptide have been shown to exert cell survival effects in midbrain dopaminergic neurons, it would be interesting to see if EGFR signaling *in vivo* might be involved in the protective actions of prosaptide and prosaposin in this region.

Finally, we found that prosaptide and prosaposin can each prevent cleavage of pro-caspase 3 in H_2O_2-treated astrocytes, but knockdown of GPR37 and GPR37L1 attenuated this protective effect. The cleavage of pro-caspase 3 into caspase-3 is an indicator of apoptosis. There are two main groups of caspases involved in cell death: initiator caspases which include caspase-2, -8, -9, -10 that cleave the executioner caspases-3, -6, and -7 which go on to degrade proteins and other intracellular molecules (Hengartner, 2000, Kumar, 2007, Snigdha et al., 2012).

The activation of caspase-3 is considered one of the terminal events that occur before cell death (Snigdha et al., 2012). Therefore, GRP37 and GPR37L1 appear to function as receptors that can rescue cells from the apoptotic cascade or prevent the initiation of the apoptotic cascade. Double knockdown of the receptors was performed to observe the maximal effect, and future studies will determine if the signaling of one receptor or another predominates in the rescue of cortical astrocytes mediated by prosaptide and prosaposin. Future studies will also be necessary to elucidate the roles of GPR37 and GPR37L1 in the prevention of apoptotic cell death in other cell types such as neurons and oligodendrocytes. The prevention of caspase-3 activation is of great interest in the study of PD as it has been shown that MPTP is able to promote caspase-3 activation as part of the apoptotic cascade that promotes the death of dopaminergic neurons (Viswanath et al., 2001, Mochizuki et al., 2001)

Chapter 4:

Functional Cross-Talk of GPR37L1 and the Dopamine D1 Receptor

Section 4.1. Rationale

Previous studies demonstrated that GPR37 co-expression with the dopamine D2 receptor enhances D2 binding affinity for the agonists dopamine and quinpirole as well as the antagonists haloperidol, spiperone, and YM-09151; GPR37 also co-immunoprecipitates with the D2 receptor, suggesting that the ability of GPR37 to influence D2 binding is the result of a physical association between the two receptors (Dunham et al., 2009). This ability of GPR37 to interact with a specific subtype of dopamine receptor suggests that there may additional ligand-independent roles for GPR37 and GPR37L1. Thus, since the role of GPR37 in regulating D2 receptor ligand binding had already been established, we sought to determine whether GPR37L1 might also exhibit a capacity for heterodimerization with other GPCRs.

Part of the rationale for examining the ability of GPR37 to affect D2 function was based on the overlapping expression pattern of GPR37 and D2, with both receptors being expressed presynaptically on dopaminergic neurons (Filloux et al., 1988, Marazziti et al., 2007), thereby providing spatial rationale for their functional interaction. Our preliminary data suggest that GPR37L1 is expressed both presynaptically and postsynaptically in neurons (Appendix II), a pattern mimicking both D1 and D2 receptor expression (Filloux et al., 1987, Filloux et al., 1988). Therefore, we sought to identify whether there might be interactions between GPR37L1 and the dopamine D1 or D2 receptors.

Upon identifying that D1 appeared to preferentially interact with GPR37L1 over GPR37, we sought to elucidate potential roles of GPR37L1 on D1 physiological function. Using assays to measure cAMP and pERK signaling, we found that co-expression with GPR37L1 altered the signaling output of the D1 receptor in response to dopamine, suggesting a role for GPR37L1 in regulating dopaminergic signaling. Additionally, we undertook experiments to elaborate on the specificity of the interaction between GPR37L1 and D1. We found that co-expression with

GPR37 did not have any effect on D1 signaling to pERK or cAMP. Conversely, GPR37L1 altered D1 signaling but did not affect pERK or cAMP signaling of another Gαs-coupled receptor, the β2 adrenergic receptor. Therefore, the studies shown in this chapter suggest that GPR37L1 may selectively regulate dopamine D1 receptor signaling and function.

Section 4.2. Experimental Methods

4.2.1 Materials

Materials used in the experiments were obtained from the following sources: GPR37 construct (originally obtained from Missouri University of Science and Technology cDNA Resource Center (Missouri S&T) and subcloned by Heide Oller); GPR37L1 construct (Multispan); β2 Adrenergic receptor construct (Missouri S&T); D1 Dopamine receptor construct (Missouri S&T); D2 Dopamine receptor construct (Missouri S&T); Human Embryonic Kidney (HEK) 293-T cells (ATCC); Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen); Fetal Bovine Serum (FBS) (Atlanta Biologicals); penicillin/streptomycin (Invitrogen); Lipofectamine 2000 (Invitrogen);M2 FLAG resin (Sigma-Aldrich); Protein A/G resin (Pierce); M2 FLAG antibody (Sigma-Aldrich); Myc antibody (Santa Cruz); 3F10 HA antibody (Roche); Dopamine D1 antibody (Sigma-Aldrich); GPR37L1 antibody (Mab Technologies); HRP-conjugated secondary antibodies (GE Healthcare); Alexa-Fluor secondary antibodies (Li-Cor and Invitrogen); Prosaptide TX14(A) (Anaspec); Uncoated 6-well cell culture plates (Corning); 4-20% Tris-glycine gels (Invitrogen); nitrocellulose (Bio-Rad); Odyssey blocking buffer (Li-Cor); Enhanced Chemiluminescence (ECL) and ELISA Pico Chemiluminescence reagents (Thermo Scientific); Autoradiography Film (Denville); 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich); Isoproterenol (Sigma-Aldrich); Colorimetric cAMP ELISA kit (Cell Biolabs); Pertussis toxin (Sigma-Aldrich); (+)-Butaclamol (Sigma-Aldrich);

In all experiments, prosaptide TX14(A) was resuspended in an assay buffer containing 20mM HEPES, 100mM NaCl, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 5mM KCl, 0.5mM EDTA, and a protease inhibitor tablet (Roche). Dopamine was resuspended in PBS + Ca^{2+} with the addition of 1% ascorbic acid to prevent degradation. All dopamine was made fresh the day of the experiment and stored in the dark until use. Dopamine used for internalization had ascorbic acid added to prevent breakdown.

4.2.2 Cell Culture

HEK293T cells were cultured and maintained in DMEM with 10% (vol/vol) FBS and 100µg/ml penicillin and streptomycin at 37 degrees Celsius and 5% CO_2. Cells were used between passages 2 and 20 with signaling experiments performed between passages 2 and 12. HEK cells were transfected with Lipofectamine 2000 when approximately 70-90% confluent. Transfection was performed in serum and antibiotic-free DMEM for 4-5 hours and terminated by the replacement of transfection reagent with DMEM containing FBS and penicillin/streptomycin. All experiments were performed 48 hours following transfection. All experiments performed utilized a total of 4µg of cDNA total, 2µg of each receptor or empty vector.

4.2.3 Luminometer Assay

Transiently transfected HEK293T cells were split into 35mm plates 24 hours following transfection and grown to 50-60% confluency. All conditions were performed in triplicate. For the assay, cells were washed with PBS + Ca^{2+} and fixed with 2% paraformaldehyde. Cells were washed with PBS + Ca^{2+} three times and were incubated for 30 minutes in a blocking buffer containing 2% nonfat milk in PBS + Ca^{2+}. Plates were then incubated with HRP-conjugated M2

anti-FLAG or 3F10 anti-HA antibody for 1 hour at room temperature. Plates incubated with anti-HA antibody were then washed twice with blocking buffer and incubated with HRP conjugated secondary for 30 minutes at room temperature. Plates were washed two times with blocking buffer and one time with PBS + Ca^{2+}. Plates were incubated with SuperSignal ELISA Pico ECL reagent for 15 seconds, and the resulting luminescence of the plates was read by a TD20/20 luminometer. For internalization assay, cells were incubated with dopamine or PBS + Ca^{2+} for 2 hours at 37 degrees prior to fixing.

4.2.4 Co-Immunoprecipitation

Transiently transfected HEK293T cells were harvested 48 hours following transfection into a low-salt harvest buffer (5mM NaCl, 1mM HEPES, protease inhibitor), and membranes were isolated via centrifugation at 15,000 × g for 15 minutes. Membranes were solubilized in low salt harvest buffer (10mM HEPES, 50mM NaCl, 5mM EDTA, and a protease inhibitor tablet) with 1% Triton-X 100 for 30 minutes, the soluble lysate was added to FLAG-conjugated beads and incubated end-over-end for 1 hour at 4 degrees. Beads were spun down and washed 3 times with Triton-X 100 buffer. Beads were then boiled in laemelli buffer to release proteins, and samples were run on 4-20% SDS-PAGE gels. Western blotting was performed and ECL was used to visualize proteins on autoradiography film.

For the endogenous Co-IP, adult male mouse brains were isolated and homogenized. The homogenates were centrifuged at 15,000 × g for 20 minutes to isolate membranes. Membranes were then solublized in low-salt harvest buffer with 1% Triton-X 100 for 1 hour at 4 degrees Celsius. A/G beads were incubated with an anti-D1 antibody or PBS and Ca^{2+} for 30 minutes, then solubilized proteins were added to the bead slurry and incubated end-over-end at 4 degrees Celsius for 2 hours. Beads were washed 2 times with low-salt buffer and Triton X-100 and boiled

in laemelli sample buffer to release proteins. Western blotting was performed and proteins were visualized with ECL reagents on autoradiography film.

4.2.5 Western Blotting

Samples for Western blotting were harvested in laemmli sample buffer containing 62.5mM Tris·HCl (pH 6.8), 25% glycerol, 2% SDS, and 0.01% bromophenol blue. Protein samples were resolved via SDS-PAGE on 4-20% Tris-glycine gels and transferred to nitrocellulose membranes. Gel transfer was done at 25V for 90 minutes. To blot for phospho-ERK (pERK), membranes were blocked with Odyssey blocking buffer for 30 minutes at room temperature and overnight at 4 degrees Celsius with primary antibodies prepared in a buffer comprised of equal parts blocking buffer and PBS + 0.1% Tween-20. Membranes were washed 3 times in PBS + 0.1% Tween-20 and incubated with secondary antibodies for 30 minutes at room temperature. Blots were then washed 3 times in PBS + 0.1% Tween-20 and rinsed in PBS until visualization. Phospho-ERK blots were visualized on an Odyssey Imaging System (Li-Cor) and analyzed using Image-J. Because antibodies against phospho-ERK and total ERK were raised in different species, blots could be incubated with both antibodies which were then visualized on the 700 channel for pERK and the 800 channel for total ERK rather than requiring two separate blots. For all other Western blots, membranes were incubated in a milk blocking buffer (2% nonfat dry milk, 50mM NaCl, 20mM HEPES (pH 7.4) and 0.1% Tween-20) for 30 minutes at room temperature followed by 1 hour incubation with primary antibody at room temperature. Blots were washed 3 times with blocking buffer and incubated with HRP-conjugated secondary antibody for 30 minutes at room temperature. Following secondary antibody incubation, blots were washed 3 times with blocking buffer and then incubated with enhanced chemiluminescence (ECL) reagent and exposed to autoradiography film.

4.2.6 cAMP Assay

HEK293T cells were transfected, split into 6-well plates, and incubated overnight to allow adhesion. The next day, cells were serum-starved for 10 minutes in DMEM and incubated with 500μM IBMX for 10 minutes to prevent cAMP degradation during the assay. Following IBMX pretreatment, cells were incubated with 100μM dopamine for 20 minutes. Cells were lysed and cAMP was isolated through high speed centrifugation. Samples were analyzed using a colorimetric cAMP ELISA kit produced by Cell Biolabs, INC. cAMP samples were added to individual wells of an anti-rabbit antibody coated 96 well plate along with a rabbit anti-cAMP antibody and a peroxidase-bound cAMP tracer. The samples were incubated on the plate for 2 hours to allow antibody binding and tracer displacement to reach equilibrium. The wells were washed 5 times and incubated with substrate solution for 15 minutes. The absorbance was read using a spectrophotometer at 450nm wavelength. In experiments with pertussis toxin treatment, the cells were incubated in the 6-well plates with 150ng/ml pertussis toxin for 16-18 hours prior to the assay.

4.2.7 pERK Assay

HEK293T cells were transfected, split into 6-well plates, and allowed to adhere to the plates for overnight. The following day, plates were incubated in DMEM for 2 hours. Following starvation, the media was aspirated and wells were incubated with either DMEM alone as a vehicle control or 100μM dopamine in DMEM. Cells were incubated for 10 minutes at room temperature and then harvested in sample buffer. Samples were sonicated and run on Tris-glycine gels. Following transfer, nitrocellulose membranes were incubated in Odyssey blocking buffer for 30 minutes and in primary antibody overnight at 4 degrees. Mouse pERK antibody and Rabbit tERK antibody were used simultaneously. Following washing and incubation with Alexa-

Flour secondary antibodies, blots were imaged using a LI-COR imaging system and analyzed using ImageJ.

Section 4.3. Results

4.3.1 The dopamine D1 Receptor Preferentially Co-Immunoprecipitates with GPR37L1 over GPR37

Co-immunoprecipitation experiments revealed that the dopamine D1 receptor preferentially interacts with GPR37L1 but exhibits little or no associate with GPR37 (Figure 4-1), indicating that GPR37L1 and D1 possess the ability to form complexes, which might affect the pharmacology and function of the D1 receptor. The ability of GPR37L1 and D1 to heterodimerize was also observed in native brain tissue as well as transfected cells. Using solubilized mouse brain tissue as starting material, the dopamine D1 receptor antibody attached to A/G beads was able to robustly pull down D1 and co-immunoprecipitate GPR37L1. There was a marked enrichment of GPR37L1 in the co-immunoprecipitated samples relative to the starting lysate indicating a relatively robust interaction between the two receptors (Figure 4-2).

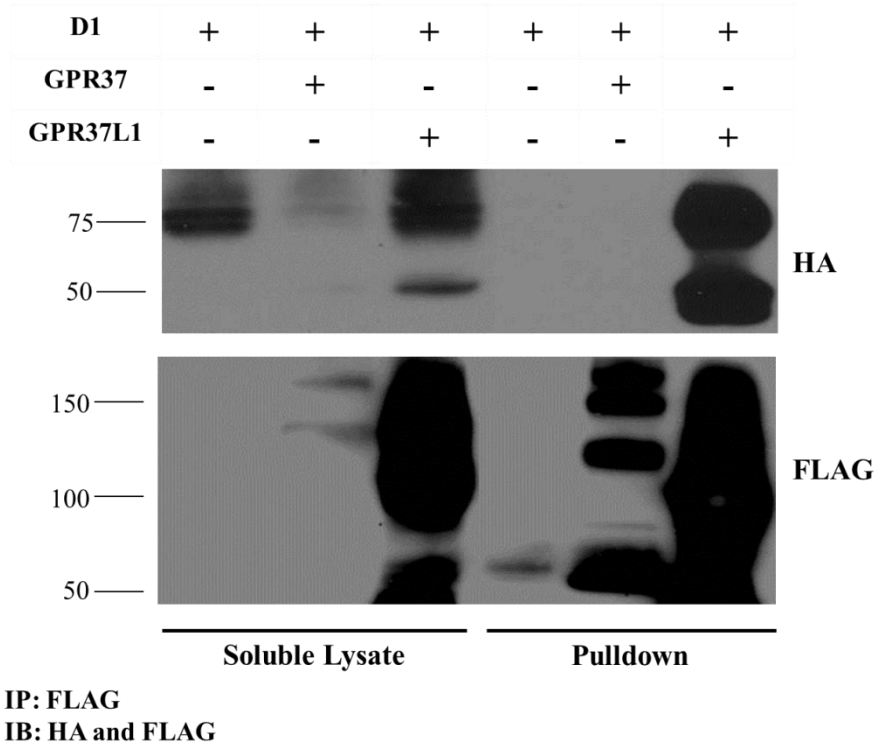


Figure 4-1. The dopamine D1 receptor preferentially interacts with GPR37L1 rather than GPR37

HEK293T cells were transfected with the dopamine D1 receptor and the orphan receptors GPR37 and GPR37L1. FLAG-conjugated beads were used to immunoprecipitate FLAG-tagged GPR37 and GPR37L1. HA-tagged D1 was only able to co-immunoprecipitate with GPR37L1. D1 was detected with an HA antibody; GPR37 and GPR37L1 were detected with a FLAG antibody. (n=3)

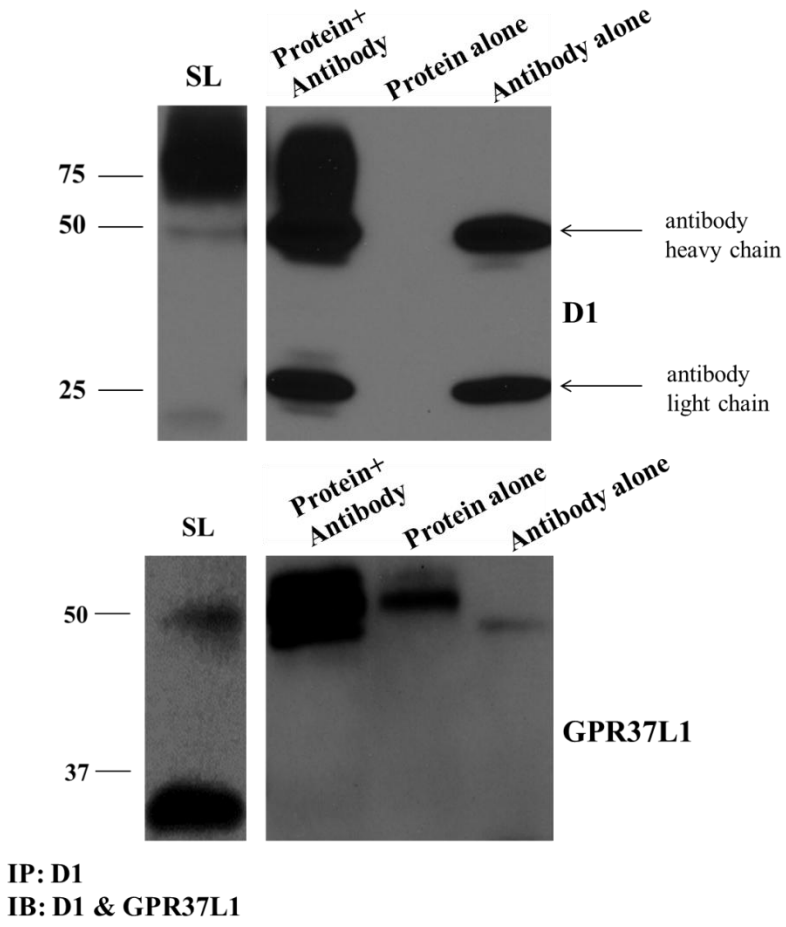


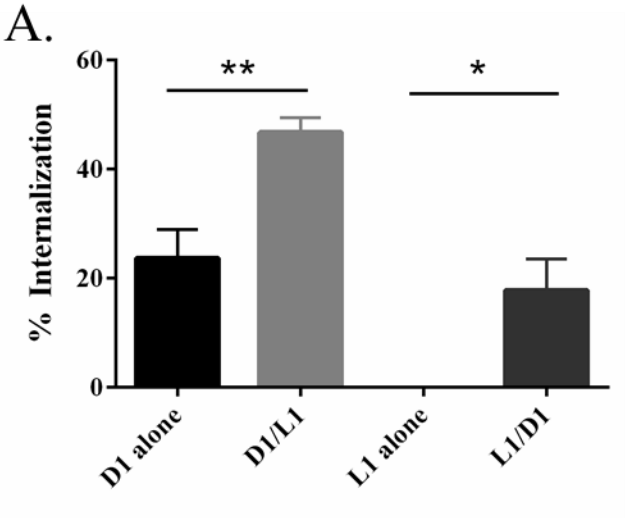
Figure 4-2. The physical interaction between GPR37L1 and D1 is present in native tissue

Mouse brain tissue was homogenized and solubilized for starting material. Using a D1 antibody, we immunoprecipitated the D1 receptor and blotted for the presence of GPR37L1. GPR37L1 demonstrated some background binding to A/G beads with D1 antibody attached, however the amount of GPR37L1 co-immunoprecipitated with D1 above the background signal was robust. The co-immunoprecipitation was able to greatly enrich the GPR37L1 in the sample. (n=2)

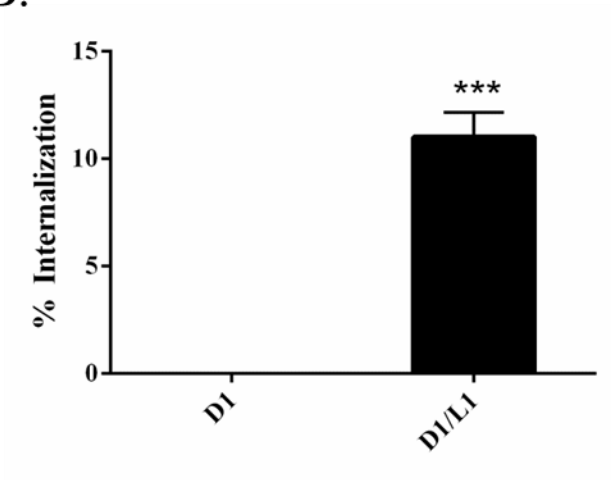
4.3.2 Ligand-Stimulated Cross-Internalization of GPR37L1 and D1

When receptors physically interact, their ligands often have the ability to induce cross-internalization of both receptors (Jordan and Devi, 1999, Prinster et al., 2005). Thus, potential cross-internalization of GPR37L1 and the D1 receptor was assessed using cell surface luminometry. This assay makes use of the differential tags on GPR37L1 and D1 to allow specific receptor populations to be monitored. GPR37L1 was tagged with FLAG and D1 is tagged with HA. These studies revealed that 100 μM dopamine did not induce any internalization of GPR37L1 expressed alone, but did induce significant internalization of GPR37L1 when GPR37L1 was co-expressed with the D1 receptor (Figure 4-3A, $p<0.05$, $n=3$). Furthermore, we found that there was a significant enhancement in the ability of 100μM dopamine to induce the internalization of the D1 receptor when GPR37L1 was co-expressed, further indicating an ability of the two receptors to alter each other's activity (Figure 4-3A, $p<0.01$, $n=3$). When examining the ability of prosaptide treatment to induce internalization of D1, we found that treatment with prosaptide was able to induce a modest but significant cross-internalization of D1 (Figure 4-3B, $p<0.001$, $n=3$). Additionally, we examined the ability of isoproterenol, an agonist for the β adrenergic receptors, to induce cross-internalization of GPR37L1. When GPR37L1 and β2AR were co-expressed and stimulated with 100μM isoproterenol, β2AR internalized but there no cross-internalization of GPR37L1 was observed (Figure 4-3C, $p<0.01$, $n=3$). The enhanced endocytosis of the D1 receptor seen with co-expression of GPR37L1 with D1 might plausibly correlate with increased signaling activity of the D1 receptor, given previous work demonstrating that the endocytosis of the D1 receptor can enhance D1 signaling (Kotowski et al., 2011). Therefore, we sought to examine the effect of GPR37L1 on D1 signaling activity.

A.


60
40
20
0
% Internalization
**
*
D1 alone
D1/L1
L1 alone
L1/D1

B.


15
10
5
0
% Internalization
***
D1
D1/L1

C.

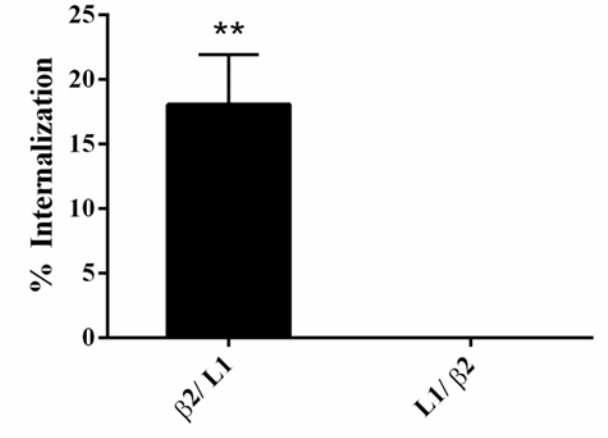

25
20
15
10
5
0
% Internalization
**
β2/ L1
L1/ β2

Figure 4-3. Co-expression of GPR37L1 and D1 is able to induce the cross-internalization of GPR37L1 with dopamine stimulation and enhances the internalization of D1 in response to dopamine

A) Transfected HEK293T cells were incubated with 100μM dopamine for 2 hours at 37 degrees Celsius. Cells were fixed and incubated with specific antibodies to detect either FLAG (GPR37L1) or HA (D1) presence on the cell surface. When both receptors were present, the first receptor listed in the figure is the one that was monitored. Dopamine stimulation induced cross-internalization of GPR37L1("L1"), providing further evidence for a physical interaction between the two receptors. Moreover, enhancement of D1 internalization was observed when GPR37L1 was co-expressed. (n=3, ** $p<0.01$, * $p<0.05$). B) Transfected HEK293T cells were incubated with 10μM prosaptide and monitored for internalization of D1. D1 did not internalize in response to prosaptide when expressed alone, but when co-expressed with GPR37L1, prosaptide treatment was able to induce internalization of D1 (n=3, ***$p<0.001$). C) Treatment with 100μM isoproterenol for cells co-transfected with GPR37L1 with the β2 adrenergic receptor induced internalization of β2 AR, but not GPR37L1 (n=3, **$p<0..01$).

4.3.3 Co-Expression with GPR37L1, but not GPR37, Alters the Ability of Dopamine to Stimulate cAMP Production through the D1 Receptor

We sought to examine the ability of GPR37L1 to enhance the most commonly-studied signaling output of D1, cAMP production. D1 is a Gs-coupled receptor and as such is able to stimulate the production of cAMP (Missale et al., 1998). Therefore, we sought to determine if co-expression of GPR37L1 might alter the ability of D1 to produce cAMP. As a control, we compared D1-GPR37L1 co-expression to both D1 alone and D1 co-expressed with GPR37, which as shown previously, does not detectably interact with D1. We found that co-expression with GPR37L1 inhibited the ability of D1 to signal to cAMP, whereas in contrast co-expression with GPR37 did not have any effect on D1 signaling (Figure 4-4A, D1 $p<0.001$, D1/GPR37 $p<0.01$, D1/GPR37L1 non-significant, $n=3$).

These data suggest that GPR37L1 might *i)* inhibit D1 surface expression, *ii)* globally suppress cAMP signaling, or *iii)* specifically alter D1 signaling in some manner. To discern between these options, we examined the ability of GPR37L1 to alter the signaling ability of another Gαs-coupled receptor, the β2 adrenergic receptor, and found that co-expression with GPR37L1 had no effect on β2 AR mediated-signaling (data not shown). This finding suggests that the effect of GPR37L1 is specific to the D1 receptor rather than representing a global inhibition of cAMP. Furthermore, using cell surface luminometry we found that co-expession with GPR37L1 does not alter D1 cell surface expression ($n=3$, data not shown), which suggests a specific effect of GPR37L1 on D1 signaling rather than an effect of D1 surface trafficking.

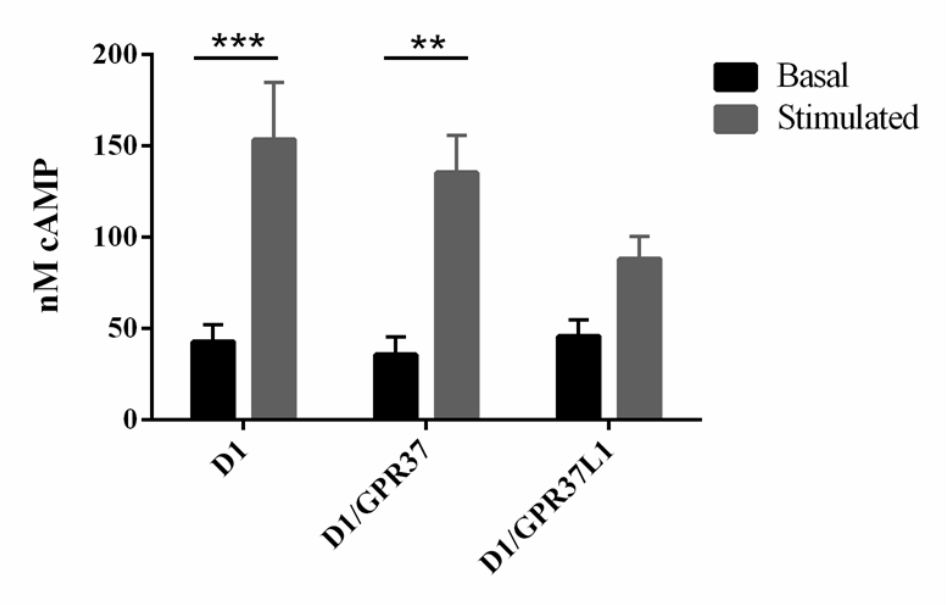
To test the hypothesis that the inhibition of D1 signaling to cAMP might be due to an alteration in G protein coupling preferences of the D1/GPR37L1 complex relative to D1 alone, we pretreated cells with pertussis toxin (PTX) prior to initiating cAMP signaling. The PTX treatment reversed the alteration in dopamine-mediated signaling induced by co-expression of

GPR37L1 (Figure 4-4B, D1 alone $p>0.05$, D1/GPR37 $p>0.01$, D1/GPR37L1 $p>0.05$, $n=3$). These data suggest that co-expression of GPR37L1 and D1 creates a functional heterodimer that exhibits enhanced coupling to Gαi, thereby leading to a reduction in cAMP signaling.

4.3.4 Co-Expression with GPR37L1 Enhances ERK Phosphorylation Mediated by Dopamine

To more comprehensively explore the ability of GPR37L1 to alter the signaling of D1, we examined the effect of co-expression of GPR37L1 and D1 on the ability of D1 to signal to pERK in transfected HEK cells. In order to compare the ability of 100μM dopamine to stimulate pERK signaling between D1-alone and D1-GPR37L1 co-expression, we calculated the percent increase in pERK over vehicle for each receptor combination. As shown in Figure 4-5, co-expression of GPR37L1 significantly enhanced the ability of dopamine to stimulate pERK signaling ($p< 0.05$, $n=4$). Dopamine did not induce any ERK phosphorylation in cells transfected with GPR37L1 expression alone, indicating that the increase in pERK signaling with D1/GPR37L1 co-expression was the result of increased D1 activity toward this pathway. Furthermore, cells transfected with GPR37 and D1 did not affect the ability of dopamine to induce ERK phosphorylation, suggesting that the receptors must physically interact to effect a change in pERK signaling. Additionally, cells co-transfected with the dopamine D5 receptor and GPR37L1 did not show a significant increase in pERK activation when compared to D5 transfected alone (data not shown). Interestingly, co-expression of GPR37L1 and D1 did not affect the ability of prosaptide to stimulate pERK through GPR3L1 suggesting that the ability of GPR37L1 to influence D1 signaling is not reciprocal in nature.

A.


***
**
200
150
100
50
0
nM cAMP
Basal
Stimulated
D1
D1/GPR37
D1/GPR37L1

B.

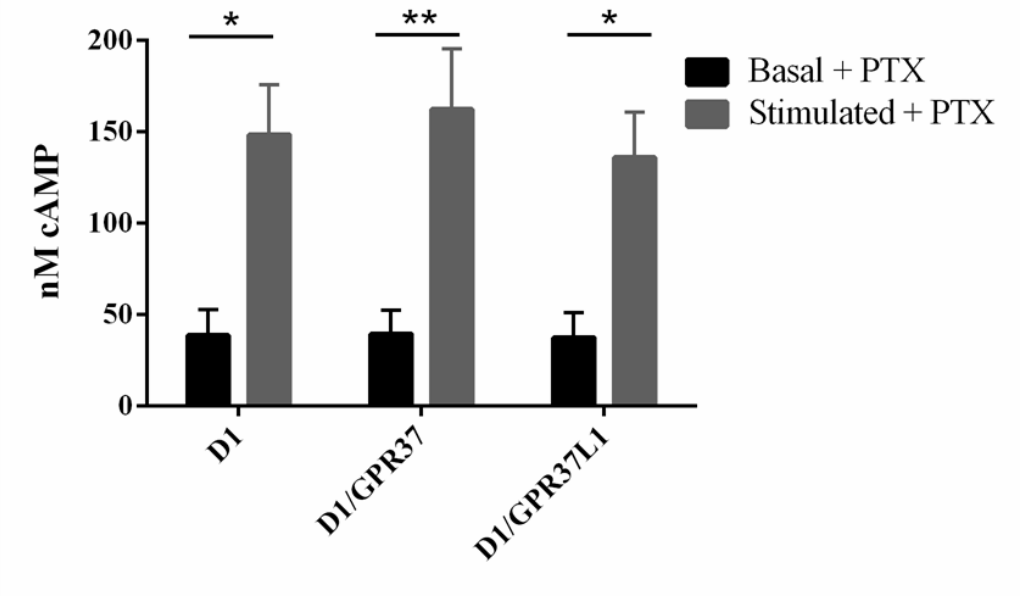

*
**
*
200
150
100
50
0
nM cAMP
Basal + PTX
Stimulated + PTX
D1
D1/GPR37
D1/GPR37L1

Figure 4-4. Interaction with GPR37L1, but not GPR37, alters D1 signaling to cAMP

A) HEK293T cells transfected with either D1 alone or D1/GPR37L1 were treated with 100μM dopamine for 20 minutes at 37 degrees Celsius. Co-expression with GPR37L1 inhibited the ability of D1 to stimulate cAMP production (n=4, *** $p<0.001$, ** $p<0.01$). B) Pertussis toxin treatment to inhibit Gαi-mediated signaling reversed the loss of D1 mediated-cAMP production (n=3, ** $p<0.01$, * $p<0.05$).

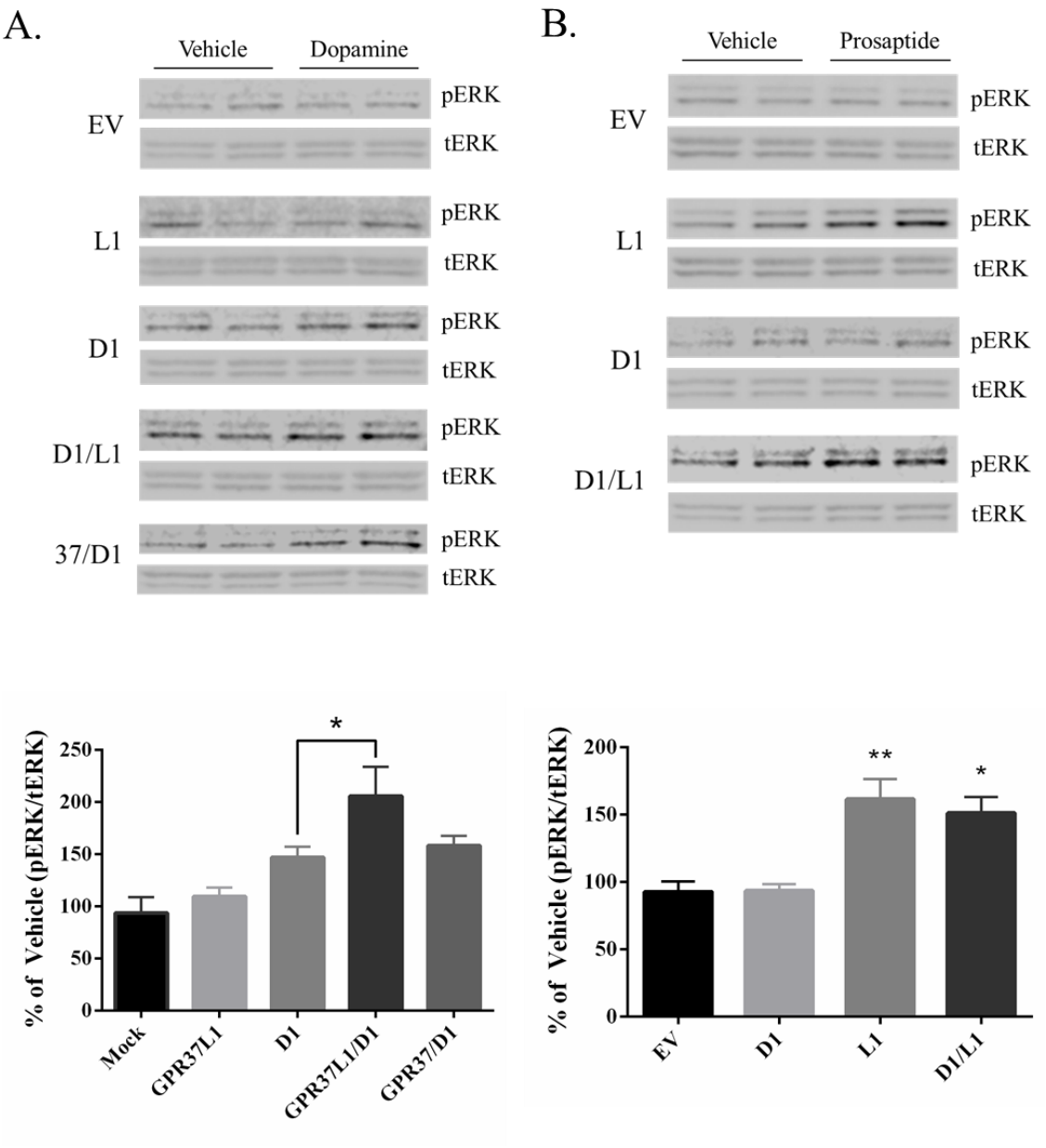

A.
Vehicle
Dopamine
EV
L1
D1
D1/L1
37/D1
pERK
tERK
B.
Vehicle
Prosaptide
EV
L1
D1
D1/L1
pERK
tERK
% of Vehicle (pERK/tERK)
250
200
150
100
50
0
*
Mock
GPR37L1
D1
GPR37L1/D1
GPR37/D1
% of Vehicle (pERK/tERK)
200
150
100
50
0
**
*
EV
D1
L1
D1/L1

Figure 4-5. Co-expression with GPR37L1 enhances the ability of D1 to induce ERK phosphorylation

A) HEK293T cells transfected with either D1 alone, GPR37L1 alone, D1/GPR37, or D1/GPR37L1 were treated with 100μM dopamine for 10 minutes at 37 degrees Celsius and levels of phospho-ERK and total ERK were assessed via Western blot. Mock- and GPR37L1-transfected cells did not demonstrate any response to dopamine. Cells transfected with D1 alone or D1 co-transfected with GPR37 exhibited a modest increase in pERK, an effect that was markedly enhanced in cells co-transfected with GPR37L1 and D1 (n=3, * $p<0.05$). B) Transfected HEK293T cells were treated with 100nM prosaptide for 10 minutes at 37 degrees Celsius and blotted for phospho-ERK and total ERK. Stimulation with prosaptide induced a significant increase in pERK for both GPR37L1 and GPR37L1/D1 co-transfected cells (n=3-4, ** $p<0.01$, * $p<0.05$), but not mock- or D1-transfected cells. Furthermore, there was no significant difference between the ability of prosaptide to induce ERK phosphorylation in GPR37L1- versus GPR37L1/D1-expressing cells, indicating that co-expression with D1 does not affect prosaptide signaling by GPR37L1.

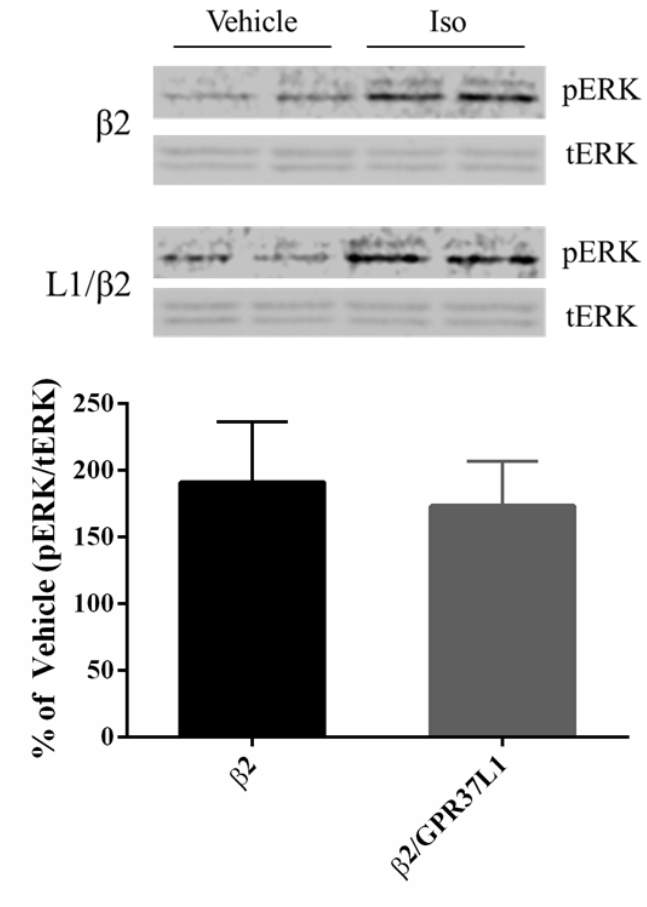


Figure 4-6. Co-expression with GPR37L1 does not alter the ability of β2 adrenergic receptor to induce ERK phosphorylation

A) ERK phosphorylation mediated through β2 AR alone or co-expressed with GPR37L1 following stimulation with 100μM isoproterenol was assessed. There was no significant difference between pERK signaling through β2 AR alone or β2 AR co-transfected with GPR37L1 (n=4).

Section 4.4 Summary and Discussion

The studies shown in Chapter 4 of this dissertation demonstrate that GPR37L1 can physically interact with the dopamine D1 receptor to alter dopaminergic signaling. The D1 receptor co-immunoprecipitated with GPR37L1 but not GPR37, suggesting a preferential interaction between GPR37L1 and D1. The preference for interaction between GPR37L1 and D1 provides an interesting parallel to a previous report indicating that GPR37 and the dopamine D2 receptor physically interact and that this interaction enhances GPR37 trafficking as well as D2 binding of agonists and antagonists to the D2 receptor (Dunham et al., 2009). This physical interaction between GPR37L1 and D1 manifested itself in both transfected cells and brain tissue, further indicating its potential for endogenous physiological relevance. Furthermore, the association between GPR37L1 and D1 also was further confirmed through the demonstration of cross-internalization of GPR37L1 induced by dopamine stimulation.

In addition to the physical interaction between GPR37L1 and D1, the internalization studies demonstrated a dopamine-induced increase in D1 receptor internalization upon GPR37L1 co-expression, however, the corresponding cross-internalization of D1 when stimulate with prosaptide was weak, suggesting that the effect of GPR37L1 on D1 internalization is largely ligand independent. The increase in ligand-induced internalization of D1 with GPR37L1 co-expression suggests that D1 may experience greater ligand stimulation when interacting with GPR37L1. When D1 signaling was assessed, it was found that co-expression with GPR37L1, but not GPR37, reduced D1-mediated cAMP signaling. Superficially, this would seem to suggest that interaction with GPR37L1 attenuated D1 signaling. However, this inhibition of D1-mediated signaling to cAMP was reversed with pertussis toxin pretreatment, which suggested that the inhibition of cAMP was due to altered G protein-coupling of the GPR37L1-D1 heterodimer. Although D1 is normally a Gs-coupled receptor, interactions with the Gi-coupled GPR37L1 may create a heterodimer with an enhanced ability to be Gi-coupled, relative to D1

alone. Furthermore, co-expression of GPR37L1 and D1 enhanced dopamine-induced phosphorylation of ERK, thereby suggesting further alteration to the signaling pathways downstream of D1 when the receptor is co-expressed with GPR37L1.

There have been previous reports of the effects of receptor cross-talk on cellular physiology, including reports of potentially "ligand-independent" effects of orphan receptors (Levoye et al., 2006b, Levoye and Jockers, 2008). One of the most commonly reported of such effects is the enhancement of forward trafficking (White et al., 1998, Jones et al., 1998, Kaupmann et al., 1998, Margeta-Mitrovic et al., 2000, Hague et al., 2004, Dunham et al., 2009). There are also reports of receptor cross-talk altering signaling properties of individual receptors, such as the association between GPR37 and D2 altering the binding affinity of D2. Another example of this phenomenon comes from the Mas-related gene (Mrg) family. In this family the MrgD receptor binds β-alanine while the MrgE receptor is considered an orphan (Shinohara et al., 2004). Despite the lack of ligand, MrgE is able to influence MrgD function. Co-expression of MrgD and MrgE enhances the ability of MrgD to induce ERK phosphorylation upon stimulation with β-alanine and also impairs ligand-stimulated MrgD internalization (Milasta et al., 2006). Another example comes from work on the melatonin (MT) receptors, where it has been shown that the orphan receptor GPR50, also known as the melatonin-related receptor, can associate with the MT1 receptor to alter the signaling properties of MT1 (Levoye et al., 2006a). The reports join the previous report of ligand-independent effects of GPR37 on D2, with regard to the ability of GPR37 to enhance the binding affinity of D2 (Dunham et al., 2009).

The preference for interaction between GPR37L1 and D1 provides an interesting parallel to the association between GPR37 and the dopamine D2 receptor. Thus, it is possible that GPR37 might selectively alter D2-like receptor function while GPR37L1 might selectively affect D1-like receptor function. In this scenario, GPR37 and GPR37L1 could have significant ligand-independent actions by which they alter signaling in the dopaminergic system. Moreover, the

associations between GPR37/D2 and GPR37L1/D1 receptors may provide a point of cross-talk between prosaposin signaling and dopaminergic signaling in the CNS.

An important caveat to the studies presented in this chapter is that it is unlikely that all co-transfected GPR37L1 and D1 receptors form heterodimers. Thus, the fact that these experiments are likely studying mixed populations of receptors means that care must be taken with the interpretation of the results. These studies revealed that when D1 forms a heterodimer with GPR37L1, that heterodimer gains the ability to couple more efficiently to Gαi, thereby altering D1 signaling. Further studies will be needed to clarify the G protein coupling preferences and pharmacological properties of the GPR37L1/D1 heterodimer. Further work will also be needed to determine the functional role of the GPR37L1-D1 heterodimer in cultured neurons as well as *in vivo*.

The ability of GPR37 to regulate dopamine signaling through the D2 receptor and the ability of GPR37L1 to regulate dopamine signaling through the D1 receptor suggest an ability for GPR37 and GPR37L1 to differentially regulate dopamine signaling through physical interactions with the dopamine receptors even in the absence of exogenous application of a ligand for GPR37 and GPR37L1. The selective interactions between GPR37/GPR37L1 and dopamine receptors may contribute to the dopaminergic phenotypes that have been reported for GPR37 and GPR37L1 knockout mice (Marazziti et al., 2004, Marazziti et al., 2007, Imai et al., 2007, Marazziti et al., 2011, Mandillo et al., 2013). Moreover, this cross-talk between receptors has a number of implications for neuropathic disorders that involve aberrant dopaminergic signaling such as Parkinson's disease, schizophrenia, and substance abuse.

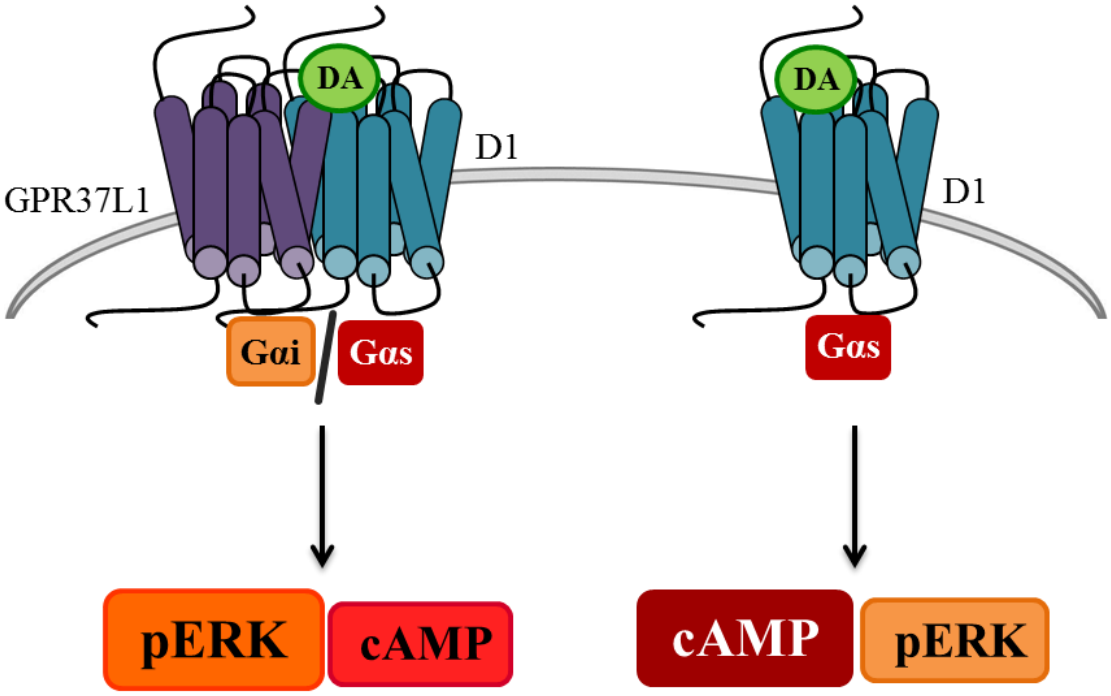


Figure 4-7. Schematic summary of D1 and GPR37L1 heterodimerization

The physical interaction between GPR37L1 and the dopamine D1 receptor alters the ability of D1 to stimulate cAMP, an effect that is reversed by pertussis toxin, suggesting an alteration in G protein binding upon receptor dimerization. Additionally, the co-expression of GPR37L1 and D1 enhances dopamine-induced ERK phosphorylation, suggesting another alteration to downstream signaling pathways emanating from the D1 receptor.

Chapter 5:

Further Discussion and Future Directions

Section 5.1 Summation of Dissertation Work

This dissertation has identified GPR37 and GPR37L1 as receptors for the neuroprotective and glioprotective factor prosaposin as well as its active peptide fragment, prosaptide. We demonstrated that prosaposin and prosaptide are able to bind to GPR37 and GPR37L1 to incite G protein binding, receptor internalization, and intracellular signaling to ERK. We confirmed this receptor-mediated signaling in cortical astrocytes where we found that GPR37, but not GPR37L1, was able to induce ERK phosphorylation in response to prosaptide and prosaposin treatment. Furthermore, we demonstrated that GPR37 induces pERK signaling through the transactivation of the EGF receptor using PKC and Src as intracellular mediators. Finally we demonstrated that both GPR37 and GPR37L1 mediate prosaptide- and prosaposin- induced protection against astrocyte death induced by oxidative stress. Furthermore, we identified that GPR37L1 plays a role in regulating dopaminergic signaling through association with the D1 receptor, subtly altering the signaling output for D1 by reducing cAMP production to potentiating D1-mediated stimulation of pERK. This work is broadly summarized in Figure 5-1.

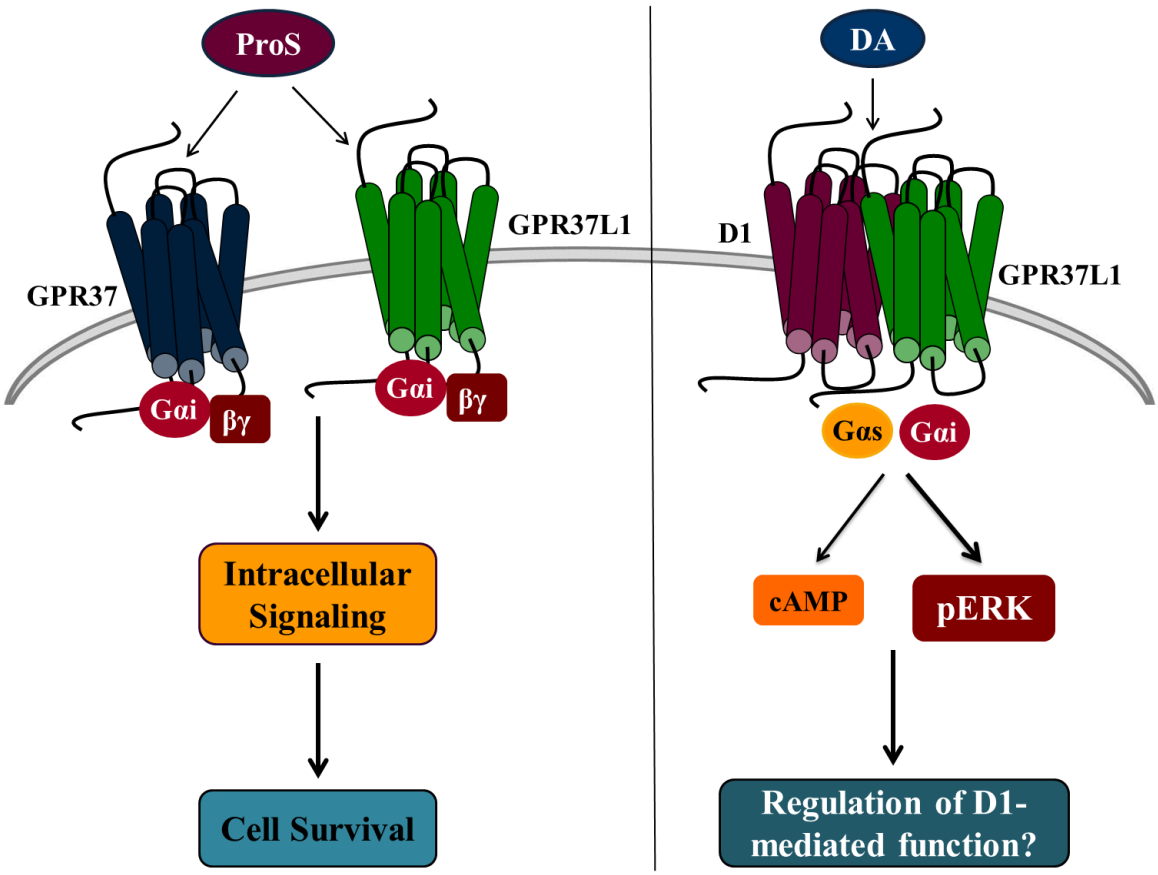


Figure 5-1 GPR37 and GPR37L1 are prosaposin receptors and GPR37L1 can modulate D1 signaling

In the studies described in this dissertation, GPR37 and GPR37L1 were identified as G protein-coupled receptors that mediate prosaptide and prosaposin signaling in a cell culture model system. These receptors were found to use a Gαi-coupled mechanism to mediate their intracellular effects. Furthermore, GPR37L1 was found to heterodimerize with the dopamine D1 receptor and alter D1 signaling upon dopamine stimulation.

Section 5.2 Theoretical Model of Prosaposin Function

Prosaposin has many diverse actions; thus it is challenging to synthesize the various physiological effects of this multifunctional protein into a coherent model of prosaposin activity. We propose the following: prosaposin evolved as a key lysosomal enzyme mediating the breakdown of lipids. Like many lysosomal enzymes, such as cathepsins, prosaposin can be secreted following cellular stress, with these secreted lysosomal enzymes having important roles in cleaning up cellular debris following injury (Blott and Griffiths, 2002). We furthermore suggest that secreted prosaposin came to gain an additional role as a signal of cellular injury/inflammation that cues nearby neurons and glia to initiate survival pathways. In this view, GPR37 and GPR37L1 evolved as sensors of secreted prosaposin, which thereby makes these receptors sensors of cellular injury/inflammation (Figure 5-2). If this model is accurate, it suggests that GPR37 and GPR37L1 may be outstanding targets for novel therapeutics aimed at promoting protection from injuries such as nerve damage or ischemia, since these receptors are part of the normal response of the nervous system to protect itself following injury.

Signaling through ERK phosphorylation has been shown to have a bi-modal effect on cell survival with transient ERK phosphorylation being associated with cell survival while long-term ERK phosphorylation is indicative of cell stress and death (Subramaniam and Unsicker, 2010). The ability of GPR37 and GPR37L1 to induce a transient increase in ERK phosphorylation (as shown in Chapter 2) is indicative of the ability of these receptors to induce protective cellular signaling, borne out in the ability of GPR37 and GPR37L1 to function as protective receptors in the hydrogen peroxide paradigm of cell death (as shown in Chapter 3).

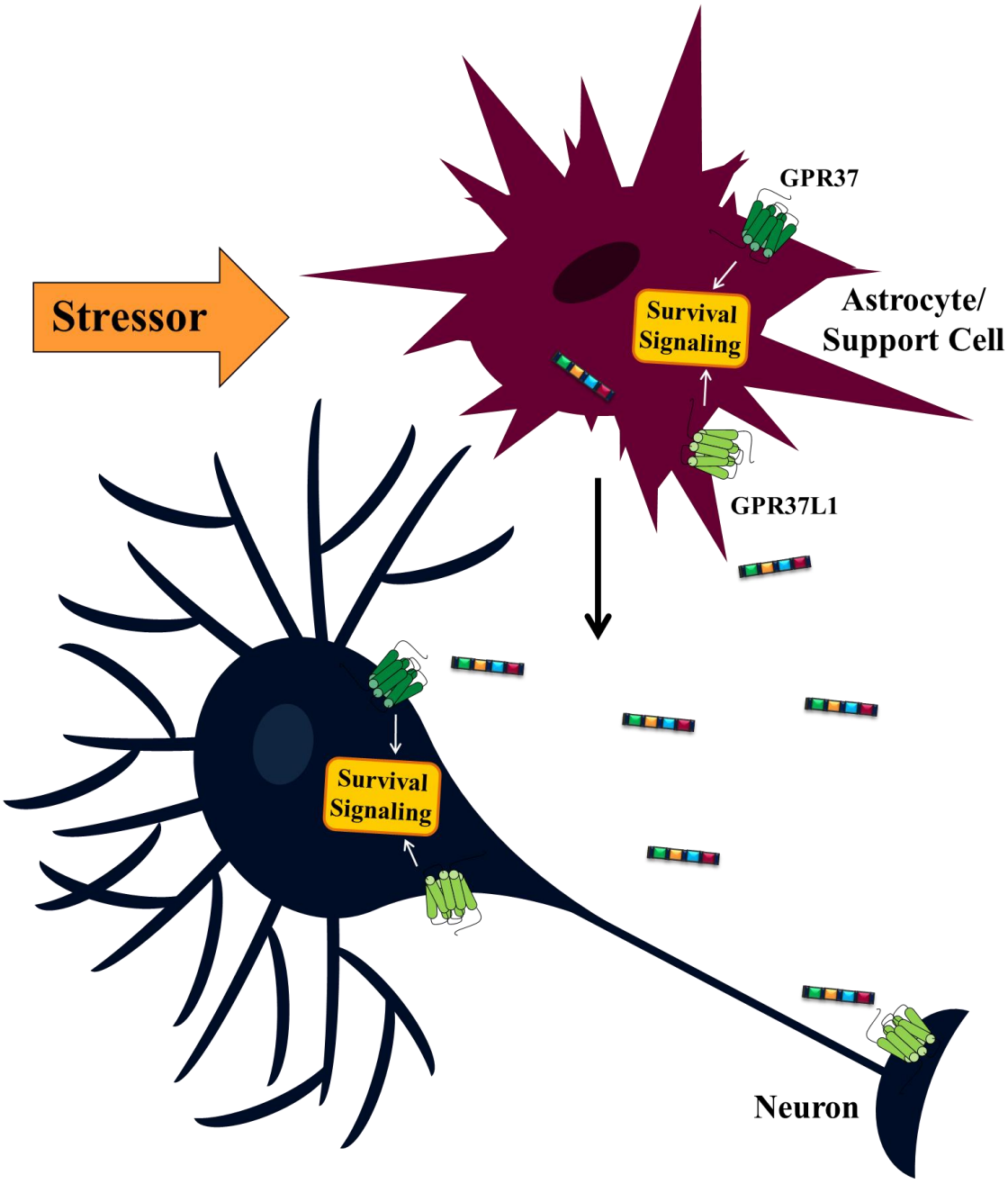


Figure 5-2. Theoretical model of prosaposin receptor function

A cell stressor such as an environmental toxin, oxidative damage, or other insult causes the release of prosaposin from support cells such as astrocytes or microglia. The released prosaposin is able to bind to GPR37 and GPR37L1 to induce pro-survival signaling, which protects cells in the nervous system from further damage.

Section 5.3 Relevance of Cross-Talk between GPR37, GPR37L1, and Dopamine Receptors

The ability of GPCRs to form heterodimers allows for profound functional diversity in receptor signaling and function of receptor combinations which fall into three broad groups: *i)* trans-inhibition of one receptor for the other, *ii)* asymmetric effects of one receptor on the other, *iii)* combinatorial effects where the two receptors together form a novel signaling entity. It is interesting to note that there seems to be asymmetry in several receptor heterodimers, with one receptor exerting a non-reciprocal inhibition of other receptors in the dimer. Examples of this include, but are not limited to, apelin and angiotensin AT1 (Siddiquee et al., 2013), the adenosine A2A and D2 receptor (Tanganelli et al., 2004), and GPR50 and the melatonin MT1 receptor (Levoye et al., 2006a), which demonstrate trans-inhibition where one receptor antagonizes the other. Furthermore, other receptor heterodimers demonstrate an effect on only one member of the dimer pair, rather than an inhibition. For example, physical interaction between the dopamine D3 and D1 receptors enables D3 to enhance D1 agonist activity with no corresponding effect of D1 on D3 signaling (Marcellino et al., 2008). Finally, receptors can form heterodimers that take on novel roles for each receptor. The adrenergic receptors α_{1B} and α_{1D} are able to dimerize resulting in enhanced trafficking of the α_{1D} receptor and the ability of BMY 7378, an α_{1D} receptor antagonist to bind to the α_{1B} receptor (Hague et al., 2004, Hague et al., 2006). Finally, the dopamine D1 and D2 receptors are able to form a heterodimer which creates a novel heterodimer entity; rather than binding Gαs as D1 does or Gαi as D2 does, the heterodimer of the two receptors binds Gαq (Rashid et al., 2007).

GPCR heterodimers have the potential to serve as novel targets for the treatment of neuropathologies. For example, there has been extensive work during the past decade on the functional consequences and therapeutic potential of complexes between the adenosine A2a and

dopamine D2 receptors with regard to Parkinson's disease. One consequence of dopaminergic neuron loss in PD is the loss of the reciprocal regulation of the direct and indirect pathways of the basal ganglia, effectively losing control of motor function. Dopamine exerts its control of the motor system of the basal ganglia by activating D2 receptors on corticostriatal glutamatergic neurons which inhibit glutamate release (Bamford et al., 2004), and also by signaling through D1 and D2 receptors expressed on medium spiny neurons which send projections through the direct and indirect pathways, respectively, allowing dopamine to stimulate the direct pathway while simultaneously inhibiting the indirect pathway to control movement (DeLong, 1990, Albin et al., 1995). The loss of dopaminergic innervation in PD causes an imbalance between the direct and indirect pathways through a hyperactivity of GABAergic striatopallidal neurons (DeLong, 1990), which can be alleviated through stimulation of D2 receptors but also, interestingly, through antagonism of adenosine A2a receptors (Fuxe et al., 2007).

The ability of A2a receptors to affect D2 signaling is at least partly attributable to dimerization between the two receptors (Franco et al., 2000, Canals et al., 2003), which results in a reduced binding affinity of D2 for agonists (Ferre et al., 1991). Furthermore, the receptors antagonize each other with regard to cAMP signaling (Premont et al., 1977, Kull et al., 1999). The alterations in signaling for the heterodimer have behavioral consequences as seen in the ability of A2a receptor antagonists to potentiate motor effects induced by D2 receptor agonists (Jiang et al., 1993, Popoli et al., 2000). By analogy with this work on cross-talk between adenosine and dopamine receptors, the interactions and cross-talk that we have observed between GPR37/GPR37L1 and dopamine receptors may provide novel therapeutic possibilities for the treatment of PD and other disorders that are associated with dysregulation of the dopamine system.

Section 5.4 GPR37 and GPR37L1 as Pharmaceutical Targets

The identification of GPR37 and GPR37L1 as prosaposin receptors suggests that small molecule agonists for these receptor may be able to mimic the protective role of prosaposin in disorders including stroke (Sano et al., 1994, Kotani et al., 1996b, Igase et al., 1999, Lu et al., 2000, Morita et al., 2001, Hiraiwa et al., 2003, Costain et al., 2010), Parkinson's disease (Liu et al., 2001, Gao et al., 2013b), myelination disorders (Bradova et al., 1993, Hiraiwa et al., 1997b, Calcutt et al., 1999, Hiraiwa et al., 1999, Hiraiwa et al., 2001, Elleder et al., 2005), and peripheral nerve repair (Gillen et al., 1995, Hiraiwa et al., 1999, Otero et al., 1999, Calcutt et al., 2000, Campana et al., 2000, Chen et al., 2008, Jolivalt et al., 2008b). Additionally, knowledge of the ligand for GPR37 and GPR37L1 will enhance drug discovery efforts aimed at GPR37 and GPR37L1 for the trteatment of disorders with which they have independently been associated, such as major depressive disorder (Aston et al., 2005), schizophrenia (Logotheti et al., 2013), autism (Fujita-Jimbo et al., 2012), and Parkinson's disease (Murakami et al., 2004, Miller et al., 2006, Wang et al., 2008b, Lundius et al., 2013, Mandillo et al., 2013). The identification of GPR37 and GPR37L1 as prosaposin receptors opens up new avenues for the novel treatment of a variety of different pathologies of the nervous system. One example of this is the potential for GPR37 and GPR37L1 to mediate treatment in Parkinson's disease.

Section 5.4.1 Treatment of Parkinson's disease

PD is traditionally treated through the administration of levo-dihydroxyphenylalanine (L-DOPA), a precursor for dopamine (Fehling, 1966, Cotzias, 1968) that helps to replenish lowered dopamine levels resulting from the death of dopaminergic neurons. The peripheral administration of L-DOPA, co-administered with carbidopa to reduce peripheral L-DOPA metabolism, has been the standard treatment for Parkinson's disease for decades, and remains the most effective treatment

against the motor symptoms of PD (Gazewood et al., 2013). Despite the efficacy of this approach, it is plagued by side effects. L-DOPA treatment eventually leads to the development of dyskinesia, or facial tics (Olanow et al., 2004, Goetz et al., 2005, Calabresi et al., 2008). Normally, dopamine neurons fire sporadically but continuously to maintain striatal dopamine levels. When dopamine is depleted through the loss of dopaminergic neurons, the introduction of dopamine through L-DOPA causes a burst of dopamine release followed by a dopamine deficit (Chase, 1998, Clarke and Guttman, 2002, Grace, 2008). The discontinuous dopamine release causes physiological changes in basal ganglia function which lead to the motor problems, like dyskinesias, that are seen during the late stages of PD treatment (Olanow et al., 2004, Olanow et al., 2006, Calabresi et al., 2008). This treatment problem has led to the exploration of other pharmacological therapies.

One commonly used alternative in early stages of PD is the use of dopamine D2 receptor agonists, which are able to avoid the dopamine bursting phenomenon and by proxy avoid the neuroplastic changes in the basal ganglia and unwanted motor effects (Clarke and Guttman, 2002, Olanow et al., 2004, Calabresi et al., 2008). Although these medications show a lower incidence of dyskinesia, they increase incidences of non-motor side effects including hallucinations and nausea, and are ultimately less effective in treating motor symptoms in most cases (Stowe et al., 2008). Alternatively, monoamine oxidase-B (MAOB) inhibitors, which slow dopamine metabolism, can be administered. This class of drug demonstrates the lowest efficacy in treating motor symptoms when compared to L-DOPA and dopamine agonists, however MAOB inhibitors benefit from fewer adverse side effects than other treatments (Miyasaki et al., 2002, Caslake et al., 2009).

As pharmacological replacement of dopamine ultimately becomes less successful as dopaminergic neurons continue to die due to disease progression, surgical implantation of electrodes into either the globus pallidus or subthalamic nucleus for deep brain stimulation can be

utilized as a treatment (Bronstein et al., 2011). However, despite the temporary improvements in motor function and quality of life, deep brain stimulation ultimately does not prevent further loss of dopaminergic neurons or slow disease progression (Bronstein et al., 2011). Therefore, surgical treatment joins dopamine-replacement therapies as symptomatic treatments for PD, further indicating a need for novel therapeutics with the potential to slow disease progression.

5.4.2 GPR37 and GPR37L1 as Pharmaceutical Targets for Parkinson's disease

As described in the introductory chapter, Parkinson's disease treatment currently focuses on dopaminergic replacement and symptomatic treatment, rather than preventing further loss of dopaminergic neurons (Clarke and Guttman, 2002, Olanow et al., 2004, Goetz et al., 2005, Caslake et al., 2009, Obeso et al., 2010). Therefore, therapeutics that focus on neuronal protection would be of great benefit as an addition to dopaminergic replacement therapy. The data shown in this dissertation suggest that GPR37 and GPR37L1 may be excellent targets for protecting dopaminergic neurons in PD, as we found that both GPR37 and GPR37L1 can mediate protective actions of prosaptide and prosaposin against oxidative damage, a major contributing factor to PD progression. Additionally, prosaptide has previously been shown to rescue dopaminergic neurons from insults *in vitro* and *in vivo* (Liu et al., 2001, Gao et al., 2013b) while GPR37 has been shown to be protective in a cellular model of PD damage (Lundius et al., 2013).

Our proposal that GPR37 and GPR37L1 mediate protective actions of prosaposin and are therefore excellent targets for treating neurodegenerative disorders like PD might seem upon first consideration to be at odds with much of the literature on GPR37, which has focused on the propensity of overexpressed GPR37 to induce neurodegeneration (Imai et al., 2001, Yang et al., 2003, Kitao et al., 2007, Wang et al., 2008a, Dusonchet et al., 2009, Marazziti et al., 2009). However, it is important to appreciate that published studies linking GPR37 to neurodegeneration

have been performed in cells where parkin was knocked out (Kitao et al., 2007, Wang et al., 2008a) or where GPR37 was highly overexpressed to artificially overwhelm cellular trafficking machinery (Imai et al., 2001, Yang et al., 2003, Dusonchet et al., 2009, Marazziti et al., 2009). As mentioned in the first chapter, parkin is an E3 ubiquitin ligase necessary for targeting misfolded substrates for degradation (Walden and Martinez-Torres, 2012). If parkin is inactivated and its misfolded substrates are allowed to build up and aggregate, this can induce endoplasmic reticulum stress and ultimately cell death (Walden and Martinez-Torres, 2012). Thus, although overexpression of GPR37 can be deleterious in certain cell types of parkin function is lost owing to mutation or knockdown, our data suggest that GPR37 and GPR37L1 play predominantly protective roles in neurons and glia under normal conditions, and this has been confirmed by another study (Lundius et al., 2013).

Given the propensity of GPR37 to misfold and cause ER stress, targeting GPR37 for the treatment of PD has appeal even beyond the potential for GPR37-mediated neuroprotective signaling. In addition to stimulating protective signaling, GPR37 agonists could potentially act as pharmacological chaperones to assist in proper GPR37 folding to enhance receptor trafficking and reduce ER stress in dopaminergic neurons in which parkin is deficient or down-regulated. Pharmacological chaperones are small molecules able to cross the plasma membrane to interact with misfolded GPCRs, promoting proper folding of the otherwise functional receptor and promoting its trafficking to the cell surface (Ulloa-Aguirre et al., 2004, Bernier et al., 2004, Conn et al., 2007). A small molecule that acted as both a pharmacological chaperone to promote the correct folding of GPR37 and an agonist to stimulate surface-expressed GPR37 would be doubly beneficial to dying dopaminergic neurons, as it would reduce ER stress while simultaneously activating neuroprotective signaling pathways.

5.4.3 GPR37 and GPR37L1 as Pharmaceutical Targets for the Treatment of Ischemia

Ischemic stroke is caused by a disruption of blood flow resulting in cell death in the affected area. Neurons are especially susceptible to excitotoxicity brought on by the loss of glucose and resultant cell stress (Barreto et al., 2011). While work in this area has traditionally focused on protecting neurons from damage following ischemia, these lines of inquiry have largely been unsuccessful in terms of developing new therapies, and thus there is new interest in examining the role of glia in protecting neurons following ischemia (Barreto et al., 2011). With regard to the role of glia in ischemia, astrocytes play an important role in protecting neurons following ischemic damage, taking up excess glutamate and releasing neurotrophic factors to support neuronal health (Swanson et al., 2004, Nedergaard and Dirnagl, 2005).

Prosaposin and prosaptide have been shown to be effective in promoting recovery from stroke in animal models (Sano et al., 1994, Igase et al., 1999, Lu et al., 2000, Morita et al., 2001). The evidence presented in this document suggests that the protective actions of prosaposin and prosaptide are likely to be mediated through GPR37 and GPR37L1, and therefore the targeting of these receptors may be beneficial in the treatment of human stroke. A necessary step in this direction would be the development of small molecules with longer half-lives *in vivo* than prosaptide. Such small molecule ligands for GPR37/GPR37L1 might effectively protect astrocytes from ischemic damage, allowing the astrocytes to exert protective actions on surrounding neuronal populations. GPR37 and GPR37L1 are also expressed in neurons in some brain regions (Cahoy et al., 2008), indicating that these receptors might mediate both neuroprotection and glioprotection in response to ischemic insults.

5.4.4 GPR37 and GPR37L1 as Pharmaceutical Targets to Repair Peripheral Nerve Injury

Peripheral nerve injury is major clinical problem with many causes ranging from traumatic injury to damage resulting from surgical intervention (Scholz et al., 2009). While peripheral nerves are able to regenerate and innervate targets, several factors contribute to poor clinical outcomes following injury. These include the long distance required for regenerating neurons in human limbs, poor targeting which leads to misdirection of the regenerating axon, and loss of trophic support at critical stages of regeneration which leads to incomplete repair (Gordon et al., 2011).

Following injury, one of the most common obstacles to successful nerve repair is that axon lose their regenerative ability as a function of time and are subsequently unable to reach their target (Gordon et al., 2003). In order to overcome this obstacle, methods of intervention have been developed including electrical stimulation to promote axon growth and targeting, stem cell implantation, and surgical intervention with graft nerve segments (Khuong and Midha, 2013). These current methods fall short, however, in that they are invasive (in the case of surgical intervention) or difficult to pursue on a large scale due to regulatory issues (stem cell implantation), leaving a gap in treatment availability (Khuong and Midha, 2013). Another consequence of the length of time required to reinnervate the neuron target is the loss of trophic support which occurs when Schwann cells atrophy following axon denervation and are subsequently unable to support axon regrowth (Fu and Gordon, 1995).

As time is a limiting factor in neuronal repair, successful therapies focus on shortening the time required for axonal regrowth. Schwann cell support can guide and speed the repair and regeneration of damaged axons, and thus the targeting of receptors capable of maintaining Schwann cell health and function may be advantageous in promoting peripheral nerve repair (Gordon et al., 2003). Prosaposin and prosaptide have previously been shown to enhance

peripheral nerve repair, at least partially through targeting Schwann cells (Kotani et al., 1996a, Campana et al., 1998b, Campana et al., 1999, Campana et al., 2000, Jolivalt et al., 2008b). Thus, studying GPR37 and GPR37L1 may allow for the development of small molecule ligands capable of promoting peripheral nerve repair.

5.4.5 GPR37 and GPR373L1 as Pharmaceutical Targets to Treat Multiple Sclerosis

Multiple sclerosis (MS) is characterized by lesions that form on the myelin sheath surrounding axons, leading to destruction of the myelin and damage to the axons (Putnam, 1934). The disease onset occurs in young adulthood and continues to progress, often with periods of remittance, as patients progressively lose motor function (Lisak, 2001, Rubin, 2013). As the lesions that cause myelin and axon damage are the result of an autoimmune inflammatory response, the current therapeutics most commonly used to treat this disorder are immunomodulatory drugs that suppress inflammation and the overactive immune response in MS patients. However, these therapies do not prevent the continuing axonal damage and degeneration as a result of lesion formation (Haghikia et al., 2013, Rubin, 2013).

As GPR37 and GPR37L1 were shown in the studies presented in this dissertation to mediate the protective actions of prosaposin in glial cells, these receptors may be excellent targets for protective therapeutics to be used as part of a combinatorial therapy with current immunomodulatory drugs for the long-term treatment of MS. GPR37 and GPR37L1 are both highly expressed in oligodendrocytes (Cahoy et al., 2008), and prosaptide and prosaposin have been shown to promote myelin production in oligodendrocytes and Schwann cells (Hiraiwa et al., 1999, Hiraiwa et al., 2001). Furthermore, prosaposin deficient mice and humans demonstrate hypomyelination (Fujita et al., 1996, Oya et al., 1998, Elleder et al., 2005), suggesting that prosaposin and its receptors are important in proper myelin maintenance *in vivo*. Small

molecules designed to mimic prosaposin action on GPR37 and GPR37L1 might therefore be valuable therapeutic tools in promoting oligodendroglial survival in MS.

Section 5.5 Future Directions

5.5.1 Future Directions in the Study of GPR37L1 and D1 heterodimerization

The studies shown in Chapter 4 utilized transfected HEK293T cells to begin the characterization of the interaction between GPR37L1 and D1. However, these experiments in transfected cells are limited in scope as they did not assess endogenous receptors in native tissues for any functional studies. Therefore, future studies will be necessary to identify the functional role(s) of the GPR37L1 and D1 heterodimer in native cell types and *in vivo*. Additionally, as we observed that GPR37L1 can preferentially interact with D1, further studies will be required to identify if that preference is extended to D5, the other D1-like receptor.

The dopamine D1 receptor plays an important role in learning and plasticity, notably in cue-induced learning (Valjent et al., 2000, Runyan and Dash, 2004, Girault et al., 2007, Hamilton et al., 2010, Xing et al., 2010, Fricks-Gleason and Marshall, 2011, Gangarossa et al., 2011, Hansen and Manahan-Vaughan, 2012, Herold et al., 2012, Xing et al., 2012, De Bundel et al., 2013, Roggenhofer et al., 2013, Wei et al., 2013). The ability of D1 to mediate plasticity has been linked to the ability of D1 to signal through pERK (Valjent et al., 2000, Runyan and Dash, 2004, Girault et al., 2007, Fricks-Gleason and Marshall, 2011, Gangarossa et al., 2011). D1-expressing neurons in the nucleus accumbens have been demonstrated to induce an increase in pERK in response to cocaine-paired cues which was inhibited by pretreatment of the D1 antagonist SCH 23390 (Fricks-Gleason and Marshall, 2011). Additionally, SCH 23390 has been shown to prevent cocaine-induced reward through inhibiting ERK phosphorylation (Valjent et al.,

2000) and also impaired association of fear condition through the reduction of pERK in the prefrontal cortex (Runyan and Dash, 2004). Furthermore, the D1 receptor agonist SKF 81297 induced ERK phosphorylation in the dentate gyrus which lead to increases in levels of Arc/Arg3.1 and Zif268, an early immediate gene and transcription factor associated with activity-dependent plasticity (Gangarossa et al., 2011). Therefore, pERK signaling mediated through the D1 receptors appears to be important for cue-related learning in a number of brain areas.

The ability of GPR37L1 to interact with the D1 receptor to enhance D1 signaling to pERK suggests that GPR37L1 might influence D1-mediated plasticity. Future studies to determine whether GPR37L1 influences D1-mediated cue-induced learning may include comparing GPR37L1 knockout mice with their wild type counterparts with regard to their ability to undergo cue-based tasks like fear conditioning and conditioned place preference. Additionally due to the reports of D1-mediated changes in the hippocampus (Hamilton et al., 2010, Xing et al., 2010, Gangarossa et al., 2011, Hansen and Manahan-Vaughan, 2012, Xing et al., 2012, De Bundel et al., 2013), spatial tasks such as the Morris water maze may also be utilized to examine behavioral consequences of the influence of GPR37L1 on D1-mediated function. Furthermore, it might be of interest to explore the importance of GPR37L1/D1 cross-talk for neuronal changes associated with learning and plasticity, such as dendritic spine formation and regulation.

5.5.2 Future Directions in the Study of GPR37 and GPR37L1 as Prosaposin Receptors

There are clear directions for future studies regarding GPR37 and GPR37L1 as prosaposin receptors, with these directions falling into two main categories: *i)* further elucidation of the ability of GPR37 and GPR37L1 to mediate prosaposin-induced signaling through intracellular pathways beyond ERK and *ii)* examination of the roles of GPR37 and GPR37L1 in mediating responses to prosaposin and prosaptide *in vivo*.

Although most of the signaling studies shown in this dissertation focused on ERK phosphorylation, prosaposin has been shown to stimulate protective signaling through other intracellular pathways such as phosphorylation of Akt (Campana et al., 1999, Lee et al., 2004, Misasi et al., 2004, Ochiai et al., 2008), and sphingosine-1 kinase (Misasi et al., 2001, Misasi et al., 2004, Sorice et al., 2008). The ability of prosaposin to exert cell-protective effects through multiple signaling pathways is especially salient given our data that GPR37L1 is responsible for protective signaling in cortical astrocytes despite not mediating ERK phosphorylation, thus suggesting an alternative signaling pathway. The observation that GPR37 and GPR37L1 do not compensate for each other, despite their homology, suggests that prosaposin mediates cell survival through multiple signaling cascades in addition to pERK. In order to address this issue, cell culture model systems provide the simplest avenue to elucidate cellular signaling cascades. Comparing the ability of prosaposin to induce receptor-mediated signaling in astrocytes, neurons, oligodendrocytes, and microglia will be an important next step in eludicating the role of GPR37 and GPR37L1 in mediating the protective effects of prosaposin and prosaptide. In addition to examining cell-specific signaling, it will be important to examine region-specific signaling to comprehensively examine the ability of prosaposin to signal through GPR37 and GPR37L1. These spatial differences in receptor expression and signaling mechanisms prosaposin will provide valuable insight into the complexity of cell protection mediated by GPR37 and GPR37L1.

To conclusively demonstrate that GPR37 and GPR37L1 are physiologically-relevant receptors for prosaposin, further studies *in vivo* will be necessary. In keeping with our observation that GPR37 and GPR37L1 mediate protective effects of prosaposin and prosaptide *in vitro*, models of neuronal damage and degeneration are a priority for examining the roles of GPR37 and GPR37L1 *in vivo*. Additionally, the use of these animal models will provide further insights into the pharmaceutical potential of GPR37 and GPR37L1. Indeed, a study published

following our initial identification that GPR37 and GPR37L1 are prosaposin receptors demonstrated that both prosaposin, GPR37, and GPR37L1 are reduced in brain areas of a mouse model of Duchene muscular dystrophy (DMD) (Gao et al., 2013a). Additionally, this group found that prolonged treatment with prosaposin neurotrophic fragment PS18 was able to enhance expression of GPR37 and GPR37L1 in SHSY5Y cells, again indicating a positive relationship between ligand and receptors (Gao et al., 2013a). Future studies should focus on the ability of GPR37 and GPR37L1 to mediate the protective actions of prosaposin in models of cerebral ischemia, as this is the most commonly reported model of prosaposin protection (Sano et al., 1994, Kotani et al., 1996b, Igase et al., 1999, Lu et al., 2000, Morita et al., 2001, Yokota et al., 2001, Hiraiwa et al., 2003, Costain et al., 2010). The use of GPR37, GPR37L1, and double receptor knockout mice in models of ischemia will provide insight into the ability of GPR37 and GPR37L1 to mediate the effects of prosaposin as well as providing a model system to test potential small molecule agonists of the two receptors. As the most commonly used of the prosaptides, TX14(A) has a relatively short half-life *in vivo* (Taylor et al., 2000a, Taylor et al., 2000b), it is important to develop agonists that mimic the actions of prosaptide and prosaposin but can be administered peripherally for ease of use.

The studies shown in this dissertation concerning the importance of GPR37 and GPR37L1 in native cell types focused on astrocytes, and thus important next steps will also include examination of the ability of these receptors to mediate the effects of prosaposin in other types of glia, most notably myelinating glia. As it has been previously shown that prosaposin can promote myelination (Hiraiwa et al., 1997b, Hiraiwa et al., 1999, Hiraiwa et al., 2001), and that GPR37 and GPR37L1 are highly expressed in oligodendrocytes at the mRNA level (Cahoy et al., 2008), it will be interesting to explore whether the receptors are able to mediate prosaposin-induced stimulation of myelin production and survival in oligodendrocytes. Furthermore, as the report of GPR37 and GPR37L1 mRNA expression in oligodendrocytes suggests that GPR37L1 is

most highly expressed in oligodendrocyte precursor cells and immature oligodendrocytes while GPR37 is most highly expressed in myelinating oligodendrocytes (Cahoy et al., 2008), it will be important to confirm this at the protein level and then examine the potentially distinct roles played by each receptor in the oligodendrocyte maturation process. GPR37L1 has recently been shown to play a role in cerebellular development through modification of sonic hedgehog signaling (Marazziti et al., 2013), suggesting the potential for GPR37L1 to regulate the maturation of multiple cell types, including perhaps oligodendrocytes.

Finally, future work will examine the role of GPR37 and GPR37L1 in mediating prosaposin- and prosaptide-mediated protection of dopaminergic neurons in models of Parkinson's disease. Both GPR37 and prosaposin have been implicated as protective mediators in models of Parkinson's disease (Liu et al., 2001, Gao et al., 2013b, Lundius et al., 2013), however it remains to be determined whether or not GPR37 mediates the protective effects of prosaposin and prosaptide in dopaminergic neurons. Both cell culture models and animal experiments will be important components in the identification of the role that GPR37 and GPR37L1 might play in PD. Additionally, given the propensity of GPR37 to misfold in models of cell stress, it will be interesting to assess whether prosaposin might be able to regulate GPR37 function by promoting the proper folding and signaling of the receptor.

Section 5.6 Concluding Thoughts

The work presented in this dissertation is the first report of prosaposin and its active fragment prosaptide as ligands for the orphan GPCRs GPR37 and GPR37L1. In these studies we identified several novel properties of GPR37 and GPR37L1 to extend our understanding of the pharmacology and function of these orphan receptors. We demonstrated that prosaposin and prosaptide bind to GPR37 and GPR37L1 to activate Gαi-coupled signaling, most notably to

pERK via transactivation of EGFR. Additionally, we demonstrated that both GPR37 and GPR37L1 mediate pro-survival actions of prosaptide and prosaposin in cortical astrocytes by preventing apoptosis. Finally, we identified a potentially ligand-independent function of GPR37L1 as a regulator of D1 receptor signaling through heterodimerization of the receptors. Given the protective effects that have been described over the past two decades for prosaposin and prosaptide *in vivo*, the work shown in this dissertation has broad implications for the treatment of disorders such as Parkinson's disease, ischemic stroke, multiple sclerosis and peripheral nerve damage.

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Appendix I:

Generation of GPR37 and GPR37L1 Double Het Mice

Section I.1 Rationale

Although phenotypes for GPR37 and GPR37L1 knockout mice have been described (Marazziti et al., 2004, Marazziti et al., 2007, Imai et al., 2007, Marazziti et al., 2011, Marazziti et al., 2013, Mandillo et al., 2013), there has been no description of mice which lack both receptors. Given our new understanding of the ability of GPR37 and GPR37L1 to bind the same ligand, we sought to generate GPR37 and GPR37L1 double heterozygous mice to ultimately create GPR37 and GPR37L1 double knockout mice to study their phenotype. Prosaposin-deficient mice have been described to have a unique neurodegeneration phenotype (Paton et al., 1992, Bradova et al., 1993, Hulkova et al., 2001, Sikora et al., 2007, Sun et al., 2008a, Kuchar et al., 2009, Yoneshige et al., 2010), however it is unclear whether or not the prosaposin deficiencies cause neurodegeneration as a result of a deficiency in the lysosomal prosaposin or the secreted prosaposin. Generating mice deficient in receptors known to mediate actions of the secreted prosaposin will be a major step in identifying the role of secreted prosaposin in neurodegeneration *in vivo*.

Section I.2 Materials and Methods

GPR37 -/+ and GPR37L1 -/+ mice were bred together to develop mice heterozygous for both GPR37 and GPR37L1 (Double -/+) on a mixed background of C57/Bl6 and 129S5. The progeny were genotyped using the following primers:

GPR37:

mutant forward: GGGTGGGATTAGATAAATGCCTGCTCT
wildtype forward: AACGGGTCTGCAGATGACTGGGTTC
common reverse: GGCCAAGAGAGAATTGGAGATGCTC

GPR37L1:

wildtype forward: CACAGCTACTACTTGAAGAG

common reverse: ACACCTGCCTGTTCATCTGG

mutant forward: GCAGCGCATCGCCTTCTATC

To genotype mice, tails were digested overnight at 55 degrees Celsius in a protease buffer (100mM NaCl, 100mM EDTA, 50mM Tris-base, pH 8.0, and 1% SDS). DNA was extracted using isopropanol and resuspended in water. PCR reaction was performed using the Qiagen Taq PCR Core Kit.

GPR37 PCR cycle:

1	95 C	7 min	
2	94 C	30 sec	
3	60 C	30 sec	
4	68 C	1.5 min	Repeat 2-4 for 35 cycles
5	68 C	7 min	
6	4 C	Hold	

GPR37L1 PCR cycle:

1	94 C	15 sec	
2	65 C	30 sec	Decrease 1C/cycle
3	72 C	40 sec	Repeat 1-3 for 10 cycles
4	94 C	15 sec	
5	55 C	30 sec	
6	72 C	40 sec	Repeat 4-6 for 30 cycles
7	4 C	Hold	

Section I.3 Results

Mendellian genetics would indicate a ratio frequency of 0.25 for each genotype, with 0.125 for each genotype with accounting for animal gender. However, there were significant discrepancies in expected and observed frequencies. Wild type male mice demonstrated a ratio frequency of 0.203 (162% expected), female GPR37L1 -/+ mice demonstrated an observed frequency of 0.083 (66% expected), and finally double -/+ females demonstrated a genotype frequency of 0.042 (33% expected) (Table I-1, n=214, Chi-square 15.86, df=7, $p>0.0265$). The lack of female double -/+ mice are compensated for through the increased frequencies of male wild type mice (shown graphically in Figure I-1).

	WT Male	WT Female	GPR37Het Male	GPR37Het Female	GPR37L1Het Male	GPR37L1Het Female	Double Het Male	Double Het Female
Expected	27	27	27	27	27	27	27	27
Observed	45	27	30	31	32	18	26	9

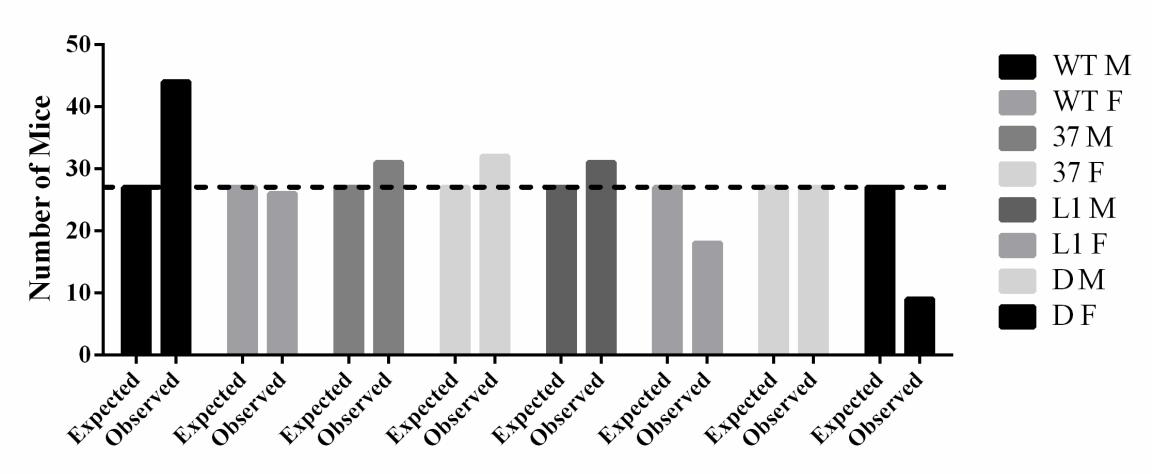

Figure I-1: Genotype Frequencies of GPR37 and GPR37L1 Het Mice

Genotype frequencies for mice generated from GPR37 and GPR37L1 -/+ breeder pairs are mapped. Table and chart abbreviations are as follows: WT M= wild type male, WT F= wild type female, 37 M= GPR37 Het male, 37 F= GPR37 Het female, L1 M= GPR37L1 Het male, L1 F= GPR37L1 female, D M= double Het male, D F= double Het female. It is expected that breeder pairs which conform to mendellian genetics will produce progeny in equal proportion with wild type, GPR37 -/+, GPR37L1 -/+, and double -/+ with a frequency of 0.125 for each genotype with regard to sex. However, we observed a frequency as follows: WT M= .208, WT F= .125, 37 M= .139, 37 F= .144. L1 M= .148, L1 F= 0.083, D M= .120, D F= 0.042 (n=214, Chi-square 15.86, df=7, p>0.0265).

Section I.4 Conclusions and Discussion

For the effect observed, we note a significant genetic disparity in the production of female double -/+ mice and male +/+ mice. It is unclear if this alteration of genotype frequency is a survival effect as there is anecdotal evidence of increased pup death in cages breeding for double -/+ mice, but no generated survival curves. Further studies will be undertaken to identify the cause of the genetic shift as well as the consequence it has. Given that prosaposin deficient mice and humans suffer from survival issues (Paton et al., 1992, Bradova et al., 1993, Sikora et al., 2007, Sun et al., 2008a, Kuchar et al., 2009), it is intriguing speculate that double -/- mice will display a similar phenotype to prosaposin deficient mice, indicating that the phenotype of prosaposin deficient mice is the result of a loss of GPR37 and GPR37L1 signaling. Further studies will be required to confirm that the disruption in genetic frequencies is a reliable effect and whether or not there is a discrepancy in generation of double knockout mice.

Appendix II:

Brain Expression of GPR37L1

Section II.1 Rationale

Very little is known about GPR37L1, and no study has comprehensively compared the protein expression of GPR37L1 within regions of the brain, or confirmed reports based on analyses of mRNA that GPR37L1 is only expressed in the CNS (Leng et al., 1999). Therefore, using samples from various brain regions and peripheral tissue, we examined the protein expression of GPR37L1 to provide clues to its function in the body. Additionally, although GPR37 has been reported to be a presynaptic receptor (Marazziti et al., 2007), nothing is known about the subcellular localization of GPR37L1.

Section II.2 Materials and Methods

Brain region samples (courtesy of S. Ritter) were dissected from male mice and homogenized in a hypotonic harvest buffer. Brain samples as well as peripheral tissue samples (courtesy of the lab of C. Yun) were assessed for protein concentration using the BCA assay (Pierce) and normalized to a concentration of 1µg protein/ µl and diluted in lammaeli sample buffer. Twenty µl of protein were loaded in Tris-Glycine gels and run through SDS-PAGE electrophoresis as described in pervious chapters. Using an antibody raised against the C-terminus of GPR37L1 (MAb Technologies) we probed samples of brain tissues as well as peripheral tissue to identify expression of GPR37L1.

Brain fractionation was performed by J. Stephenson as described (Stephenson et al., 2013). Briefly, brain tissue was homogenized and centrifuged at low speed to remove extraneous cell material. The supernatant was extracted and placed on a Percoll gradient and centrifuged at 31,000 x g. The fraction between 15% and 23% Percoll was extracted and centrifuged at 20,000

x g for 30 minutes resulting the isolation of the synaptosome. The post-synaptic density was extracted from this fraction using 1% Triton and centrifugation.

Section II.3 Results

We observed that GPR37L1 exists in two main forms in brain tissue, a species that is 50 kDa in size and another fragment that is approximately 35 kDa in size (Figure II-1, n=3). Despite equal protein loading confirmed through Ponceau staining, the actin levels in different organs vary. Nonetheless, GPR37L1 was only seen in brain tissue samples, not in any of the peripheral tissue samples tested. Analyses of synaptosome fractions revealed that GPR37L1 is present in both the synaptosome fraction as well as the post-synaptic fraction, suggesting that GPR37L1 is present both pre-synaptically and post-synaptically (Figure II-2, n=1). However, we also noted that GPR37L1 is not enriched in either fraction, indicating that GPR37L1 may be a peri-synaptic protein or enriched in areas of the neuron besides the synapse. Alternatively, GPR37L1 could be enriched in cell types that do not form synaptic connections.

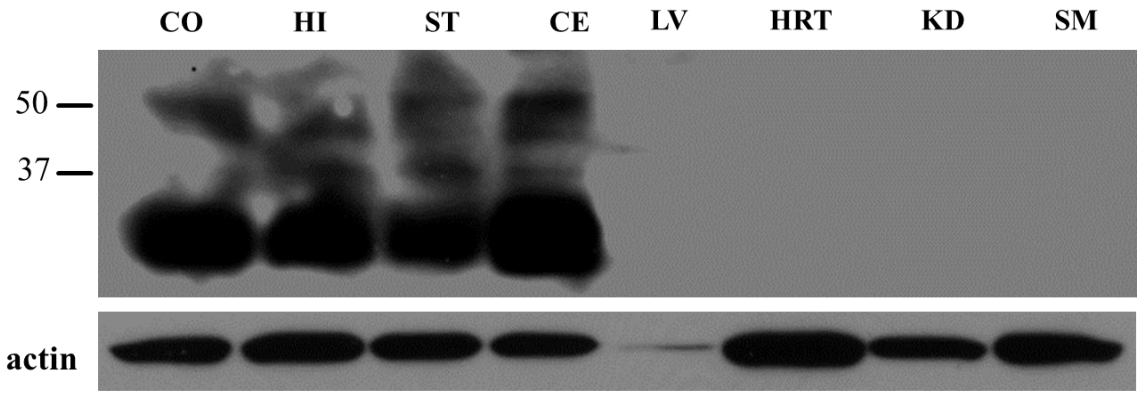

Figure II-1: GPR37L1 is a brain-specific protein

Expression of GPR37L1 is seen only in brain tissue, not in peripheral tissue. Furthermore, GPR37L1 appears as two distinct bands on the Western blot, one corresponding with the size of 50 kDa and the other at approximately 35 kDa, which may be a proteolytic fragment. The tissue regions are as indicated: CO= cortex, HI= hippocampus, ST= striatum, CE= cerebellum, LV= liver, HRT= heart, KD= kidney, SM= smooth muscle. (n=3, representative image shown)

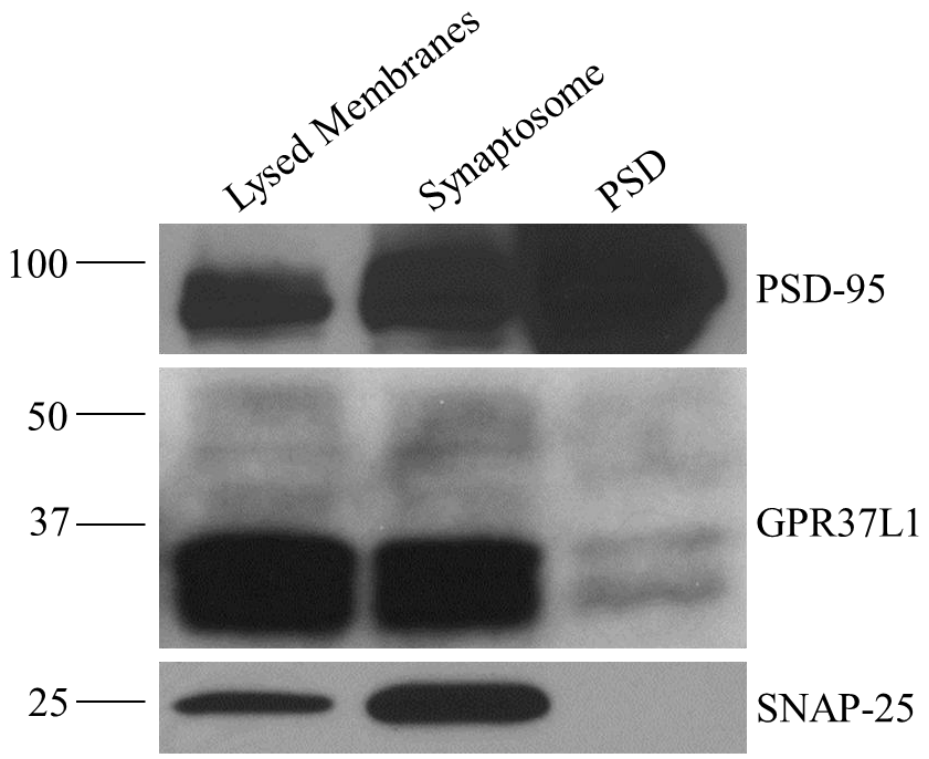

Figure II-2: GPR37L1 is present both pre-synaptically and post-synaptically in the brain

Adult male mouse brains were homogenized and fractionated using a Percoll gradient. Lysed membranes were compared to the synaptosome fraction and the post-synaptic density (PSD) fraction. SNAP-25 is a pre-synaptic marker that was enriched in the synaptosome but not present in the PSD. PSD-95 is a post-synaptic marker enriched in the synaptosome and even more in the PSD. GPR37L1 is present in both the synaptosome and the PSD, but not enriched in either (n=1).

Section II.4 Conclusions and Discussion

As the antibody used in these studies was raised against the C terminus of GPR37L1, the smaller fragment seen in samples is likely the result of the loss of amino acids from the N terminus. No receptor expression was seen in peripheral tissue, indicating that GPR37L1 is a brain-specific receptor, however the studies here did not exhaustively examine all peripheral tissue. Additionally, preliminary studies identified that GPR37L1 is present in the synaptosome and post-synaptic density, but not enriched in either fraction. However, GPR37L1 might be a perisynaptic receptor, present within near proximity to the synapse, but not in the fraction isolated in these studies. Additionally, GPR37L1 might be enriched in glia present near synapses, but not part of the synaptosome, or in other nearby support cells. Finally, GPR37L1 might primarily be a receptor present in the cell soma. Further studies will be required to shed more light on the subcellular localization of GPR37L1, as well as its expression pattern in mammalian tissues.