IMIDAZOLINE RECEPTOR ANTISERA-SELECTED PROTEIN: A UNIQUE MODULATOR OF NEURONAL DIFFERENTIATION

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Abstract

The imidazoline I_1 receptor (I_1-R) is a novel receptor found primarily in the brain and nervous tissue where it modulates neurotransmission. It is named for its high affinity for compounds with an imidazoline structure such as the anti-hypertensive drugs, clonidine and moxonidine.

The imidazoline receptor antisera-selected protein (IRAS) is the putative clone of the I_1-R. IRAS has a unique structure, which does not resemble any other receptor protein. IRAS is present throughout the body with highest levels in the brain. There is a growing body of research examining the physiological roles of IRAS as an I_1-R, in cell survival, migration and protein trafficking. However, there is little research into its neuronal functions.

IRAS interacts with other membrane receptors: the mouse homologue of IRAS reorganises the actin cytoskeleton through interaction with the $\alpha5\beta1$ fibronectin receptor. IRAS also binds insulin receptor substrate 4 and enhances insulin-induced extracellular signal-regulated kinase1/2 (ERK1/2) activation. Actin reorganisation and ERK1/2 activation are important for the development of neurites during neuronal differentiation. Therefore, the work described in this thesis aimed to investigate the effects of IRAS on neuronal differentiation. Studies reported in this thesis also aimed to investigate whether IRAS affected ERK1/2 signalling of other receptors involved in neuronal differentiation such as the NGF receptor, TrkA, and lysophospholipid receptors.

The above aims were carried out in neuronal model PC12 cells transfected with either IRAS or a vector plasmid. Fluorescence microscopy and Western blotting techniques were used to examine the effect of IRAS on cell morphology and ERK1/2 signalling.

The work described in this thesis found that IRAS reorganises the actin cytoskeleton and enhances growth cone development in PC12 cells. This study also shows that IRAS differentially enhances or inhibits NGF-induced PC12 cell differentiation depending on the presence or absence of serum in the media. In full-serum conditions, IRAS enhanced neurite outgrowth and this was accompanied by an increase in ERK1/2 activation. In serum-starved cells, IRAS inhibited neurite outgrowth with similar levels of ERK1/2 activation observed in vector- and IRAS-transfected cells. Finally, studies presented in this thesis found that IRAS enhances lysophosphatidic acid-induced ERK1/2 activation and that IRAS interacting with lysophospholipid receptor agonists present in serum is a potential mechanism by which it enhances NGF-induced ERK1/2 activation in full-serum conditions.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Abbreviations

Alzheimer’s disease	AD
Central nervous system	CNS
Diacylglycerol	DAG
Epidermal growth factor	EGF
Extracellular signal-regulated kinase	ERK
G-protein coupled receptor	GPCR
I_1 Receptor	I_1-R
Imidazoline antisera-selected	IRAS
Lysophosphatidic acid	LPA
Lysophospholipid	LP
Mitogen activated protein	MAP
μ opioid receptor	MOR
Nerve growth factor	NGF
Phosphatidylcholine-specific phospholipase C	PC-PLC
Phosphatidylinositol-3-phosphate	PI3P
Phosphoinositide	PI
Protein kinase C	PKC
Protein tyrosine kinase	PTK
Rat embryonic fibroblast	REF
Rostral ventrolateral medulla	RVLM
Sorting Nexin	SNX
Sphingosine-1-phosphate	S1P
Sphingosine kinase	SphK

1 Introduction

Imidazoline receptors are a novel family of receptors that are widely distributed in the body. Imidazoline receptors are named for their high affinity for compounds with an imidazoline structure such as the anti-hypertensive drugs, clonidine and moxonidine. These receptors exist as multiple subtypes. I_1 receptors (I_1-Rs) are plasma membrane bound receptors found mainly in the brain and nervous tissue where they modulate neurotransmission (Ernsberger, 1999; Ernsberger *et al.*, 1995; Ernsberger *et al.*, 1997b; Reis *et al.*, 1997; Szabo, 2002). I_2 receptors (I_2-Rs) are present on the mitochondria of a wide variety of tissues. In the brain, they are mostly present in glial cells where they modulate the expression of glial fibrillary acidic protein (Regunathan *et al.*, 1996). An I_3 receptor has been found in the endocrine pancreas and this is believed to have a role in glucose metabolism (Efendic *et al.*, 2002).

The Imidazoline Receptor Antisera-selected protein (IRAS) is the putative clone of the I_1-R (Piletz *et al.*, 2000). IRAS has a unique structure which does not resemble any other receptor protein. IRAS is present throughout the body with highest levels in the brain (Alahari *et al.*, 2000; Piletz *et al.*, 1999). There is a growing body of research examining the physiological roles of IRAS as an I_1-R, in cell survival, migration and protein trafficking. However, there is little research into its neuronal function. This review will give a brief summary of the physiological roles and signal transduction of the I_1-R. The discovery of IRAS and evidence to suggest that it is an I_1-R will be examined. Finally, this review will highlight research into other physiological roles of IRAS and look at its potential role in neuronal development.

1.1 The imidazoline I_1 receptor

1.1.1 Discovery

Before the discovery of I_1-Rs, it was thought that only α_{2a}-adrenoceptors mediated central sympathoinhibition. The existence of imidazoline receptors was postulated when Bousquet *et al.* (1984) showed that drugs with an imidazoline structure (clonidine, cirazoline and St-587), when injected into the rostral ventrolateral medulla (RVLM) of cats, elicited a decrease in blood pressure, whereas microinjections of the non-imidazoline α_{2a}-adrenoceptor agonist, α-methylnoradrenaline (α-MNA), had little effect. This meant that the vasodepressor response could not be due to α_{2a}-adrenoceptor activation and so distinct receptors, the I_1-Rs, were postulated. This functional distinction was confirmed by using a wide range of drugs for radioligand binding studies as discussed below.

1.1.2 Radioligand binding studies

I_1-Rs are claimed to mediate sympathoinhibition by actions in the RVLM. The RVLM is involved in maintaining resting sympathetic tone and arterial pressure and is involved in the baroreflex pathway. I_1 binding sites were first detected in RVLM membranes using [^{3}H]-clonidine saturable binding (Bricca *et al.*, 1988; Ernsberger *et al.*, 1987). α-MNA was unable to displace [^{3}H]-clonidine, but drugs with an imidazoline structure potently inhibited clonidine binding (Bricca *et al.*, 1988). Importantly, the order of antihypotensive potency of imidazoline drugs upon microinjection into the RVLM paralleled their potency to bind to RVLM membranes, clearly establishing the I_1-R as a functional receptor (Ernsberger, 1999).

1.1.3 I_1-R compounds

It is important to consider the selectivity of the different agonists and antagonists used in I_1-R research (tables 1.1 and 1.2). Separating the functional effects of the I_1-R from α_{2a}-adrenoceptors in animal studies has been difficult, as the imidazoline drugs used do not have strong selectivity for I_1-Rs. Clonidine is a first generation imidazoline agonist. It has roughly equal affinity for I_1-R and α_{2a}-adrenoceptors (Ernsberger *et al.*, 1993). Second generation imidazoline agonists, such as rilmenidine and moxonidine, are more selective for I_1-Rs over α_{2a}-adrenoceptors. Of the antagonists used, idazoxan has slightly higher affinity for α_{2a}-adrenoceptors whereas efaroxan has similar affinities for I_1-Rs and α_{2a}-adrenoceptors. 2-methoxyidazoxan (2MI) is a highly selective α_{2a}-adrenoceptor antagonist. Importantly, until recently there were no selective I_1-R antagonists. The newly developed compound 2-endo-amino-3-exo-isopropylbicyclo[2.2.1.]heptane (AGN192403) was the first selective I_1-R antagonist (Munk *et al.*, 1996).

Clonidine, moxonidine and rilmenidine are clinically used antihypertensives. All three drugs lower blood pressure and heart rate. However, clonidine, which has equal affinity for I_1-Rs and α_{2a}-adrenoceptors, also has unwanted effects such as inhibition of saliva secretion and sedation. Moxonidine and rilmenidine, which are I_1-R-selective, have fewer adverse effects (Fillastre *et al.*, 1988; Planitz, 1987). Therefore, selectivity for I_1-Rs has been proposed as a major determinant in reducing adverse effects associated with centrally acting, antihypertensive imidazoline drugs. The following will discuss the mechanisms by which I_1-Rs mediate sympathoinhibition.

1.1.4 I_1 Receptors and blood pressure

There has been considerable debate as to whether I_1-Rs mediate the sympathoinhibition of imidazoline drugs (Ernsberger *et al.*, 1990; Ernsberger *et al.*, 1997b; Szabo, 2002). It is well established that activation of α_{2a}-adrenoceptors in the brainstem causes sympathoinhibition by reducing the release of sympathetic transmitters, noradrenaline (NA) and adrenaline, in peripheral tissue (Szabo, 2002). Consequently, it was hypothesised that I_1-Rs mediate sympathoinhibition via a similar mechanism. Recently, it was shown that moxonidine could still produce a dose dependent fall in plasma NA levels in rats under irreversible α_{2a}-adrenoceptor blockade (Raasch *et al.*, 2003). This suggests that moxonidine acts through central I_1-Rs, reducing NA release in a similar fashion to α_{2a}-adrenoceptors.

Administration of imidazoline compounds in animals has confirmed an I_1-R role in sympathoinhibition. Extensive studies in rabbits by Head *et al*. (2000; 1998) have demonstrated this. Firstly, it was shown that central administration of rilmenidine resulted in a hypotensive response, which was reduced by idazoxan. Secondly, rilmenidine was most effective when injected directly into the RVLM. When α-MNA was injected directly into the RVLM, hypotension was produced but only at very high doses. Finally, idazoxan reversed the effect of rilmenidine but not α-MNA, which suggests that idazoxan produced antagonism mainly at I_1-Rs in the RVLM despite its higher affinity for α_2-adrenoceptors.

The D79 mouse model, a transgenic mouse model with greatly reduced α_{2a}-adrenoceptor function, has been used to assess the importance of I_1-Rs in sympathoinhibition. It was originally found that intravenously administered moxonidine and rilmenidine did not produce a hypotensive response (Zhu *et al.*, 1999). However, Tolentio-silvia *et al.* (2000) showed that these mice had a large

hypotensive response to moxonidine microinjected into the RVLM. The hypotensive response was reversed by efaroxan. Overall, both radioligand binding studies and animal physiological studies demonstrate that I_1-Rs are present in the RVLM and centrally mediate sympathoinhibition.

1.1.5 Endogenous ligands

The role of I_1-R in sympathoinhibition has been determined by observing the effects of imidazoline drugs. However, an endogenous ligand for the I_1-R has not been determined. Several endogenous ligands of I_1-Rs have been proposed. The first of these to be proposed was agmatine (Li *et al.*, 1994). Agmatine is widely distributed in the brain and binds with high affinity to I_1-Rs. It is formed from arginine by arginine decarboxylase and deactivated by agmatinase (Reis *et al.*, 2000). Agmatine is stored within synaptic vesicles, which implies that it is a neurotransmitter (Reis *et al.*, 2000). However, agmatine does not lower blood pressure when injected into the RVLM (Reis *et al.*, 2000). It does lower blood pressure when injected intravenously, mostly via vasodilation (Sun *et al.*, 1995). It is unclear whether this is physiologically relevant.

Another putative endogenous ligand is the beta-carboline, harmane, which also has high affinity for I_1-Rs. Importantly, when injected into the RVLM, harmane produced a dose dependent fall in blood pressure similar to clonidine (Musgrave *et al.*, 2000). This effect was blocked by efaroxan, suggesting that harmane is potentially an endogenous neurotransmitter for I_1-Rs (Musgrave *et al.*, 2000).

Finally, Imidazoleacetic acid-ribotide (IAA-RP) was found to bind with high affinity to I_1-Rs (Prell *et al.*, 2004). Curiously, microinjection of IAA-RP into the RVLM increased hypertension, which was reversed by moxonidine. Further studies are required to confirm IAA-RPs exact role at I_1-Rs.

1.1.6 I_1-Rs and signal transduction

1.1.6.1 PC12 cells

Studies into I_1-R signal transduction have mainly been conducted in neuronal model rat pheochromocytoma PC12 cells. PC12 cells were developed from rat adrenal medullary tumours (Greene *et al.*, 1976). It was observed that these cells developed a phenotype similar to sympathetic neurons with nerve growth factor (NGF) treatment. This was characterised by the elaboration of neurites, which are axonal-like protrusions from the cell body. The authors concluded that PC12 cells contain properties of a progenitor that can differentiate to either chromaffin cells or sympathetic neurons (Greene *et al.*, 1976). PC12 cells have now become the main cell line for studying the mechanisms underlying neuronal differentiation by NGF. This is discussed in more detail in section 1.3.2.

PC12 cells are an ideal model for I_1-R research as they are neuronal and I_1-Rs are expressed highest in the brain. [^{125}I]p-iodoclonidine binds saturably to PC12 membranes and this binding is potently inhibited by other I_1-R ligands but not by specific α_{2a}-adrenoceptor compounds, which demonstrates that these cells express I_1 binding sites (*Separovic et al.*, 1996). Importantly, both NGF differentiated cells and naïve (undifferentiated) cells do not express α_{2a}-adrenoceptors (Molderings *et al.*, 2002; Separovic *et al.*, 1996). This implies that the effects of I_1-R agonists on PC12 cells are through I_1-Rs.

1.1.6.2 Early studies

Initial studies into I_1-R signal transduction mechanisms were based on the hypothesis that these were G-protein coupled receptors (GPCRs). However, these studies showed that stimulation of bovine adrenal medulla cells with clonidine did

not result in the accumulation of either cAMP or inositol phosphates (Regunathan *et al.*, 1991). Clonidine induced increases in cGMP at very high concentrations; however, this was orders of magnitude above its affinity for I_1-Rs (Regunathan *et al.*, 1991). This study suggested that I_1-Rs are unlikely to act via conventional GPCR pathways. However, it was later shown that nanomolar concentrations of moxonidine and another I_1-R agonist, benazoline, inhibited cAMP production in PC12 cells (Greney *et al.*, 2000), although this may be a downstream effect of a non-GPCR signalling mechanism. Furthermore, clonidine does increase $^{45}Ca^{2+}$ influx in bovine adrenal medulla cells although only at high concentrations (Regunathan *et al.*, 1991). It is still unclear from the results of these studies whether I_1-R agonists stimulate GPCR signalling in non-α_{2a}-adrenoceptor-expressing cell lines.

1.1.6.3 Diacylglycerol production/ phosphatidylcholine-specific phospholipase C activation

The first major breakthrough in understanding the signal transduction pathways of I_1-Rs came when it was found that I_1-R agonist stimulation of PC12 cells resulted in phosphatidylcholine-specific phospholipase C (PC-PLC) activation and accumulation of the second messenger, diacylglycerol (DAG) (Separovic *et al.*, 1996; Separovic *et al.*, 1997). Moxonidine treatment caused increased DAG production in a concentration dependent manner. The increase in DAG was prevented by the PC-PLC inhibitor, D609 (Separovic *et al.*, 1996). In a subsequent study, Separovic *et al.* (1997) directly demonstrated PC-PLCs activation as its by-product, phosphocholine, increased in response to moxonidine treatment. Both activation of PC-PLC and production of DAG were inhibited by efaroxan, which suggests that these were I_1-R mediated effects. I_1-R activation did not result in accumulation of PI-PLC and PLD by-products, inositol phosphates (Regunathan *et*

al., 1994) or phosphatidic acid (Separovic *et al.*, 1996), which implies that the I_1-R does not activate PI-PLC or PLD.

1.1.6.4 Arachidonic acid production/ protein kinase C activation/MAP kinase activation

DAG is known to produce arachidonic acid (Kennerly *et al.*, 1979) and to activate classical and novel isoforms of PKC. It was first shown that treatment with moxonidine increased [^{3}H]-arachidonic acid release from PC12 cells preloaded with [^{3}H]-arachidonic acid in a concentration dependent manner; an effect that was blocked by efaroxan (Ernsberger, 1998). Secondly, it was found that moxonidine treatment of PC12 cells increased membrane binding, and therefore activation, of both the classical isoform cPKCβII and the atypical isoform aPKC, but not of the novel isoform nPKCε (Edwards *et al.*, 2001). The activation of aPKCζ is surprising and may be due to the DAG-induced increase of arachidonic acid. Arachidonic acid has been shown to activate atypical PKC isoforms (Huang *et al.*, 1993).

PKC is known to activate mitogen activated protein (MAP) kinases, a family of intracellular mediators including extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). Two studies in the same year (Edwards *et al.*, 2001; Zhang *et al.*, 2001) investigated I_1-R-induced activation of ERK1 and ERK2. In both studies, PC12 cells were treated with I_1-R selective drugs (moxonidine or rilmenidine) and used Western blot analysis with antibodies to detect phosphorylated (and so active) MAP kinases. Both studies demonstrated a concentration and time dependent increase in ERK1/2 activation following imidazoline treatment. Edwards *et al.* (2001) also showed similar results for JNK activation. In each case, these increases were prevented by efaroxan. Furthermore, the PKC inhibitor, H7, also blocked ERK1/2 activation suggesting that PKCs are responsible in I_1-R induced

ERK1/2 activation (Edwards *et al.*, 2001). Activated MAP kinases may phosphorylate and activate transcription factors involved in cell growth and differentiation. Consequently, I_1-R activation led to a 20-50% increase in cell growth (Edwards *et al.*, 2001). The effects of I_1-R activation on neuronal differentiation have not been investigated. The I_1-R signal transduction pathway is summarised in figure 1.1.

1.2 IRAS

1.2.1 Discovery

Despite extensive research distinguishing the I_1-R from the α_{2a}-adrenoceptor, controversy still exists over whether the I_1-R regulates blood pressure and even if it exists at all. One of the major reasons this controversy still exists has been the difficulty in cloning the I_1-R. The first purified imidazoline binding protein was isolated from chromaffin cells in the bovine adrenal medulla (Wang *et al.*, 1992). Using affinity chromatography techniques (with clonidine and idazoxan), a 70 kDa protein was discovered with high affinity for imidazoline ligands but not catecholamines (Wang *et al.*, 1992). This protein was named imidazoline receptor binding protein (IRBP) and was used to generate polyclonal antibodies which could detect I_1 binding sites in neurons and glia (Wang *et al.*, 1993). Greney *et al.* (1994) developed antibodies raised against anti-idazoxan IgG. This antiserum was named anti-idiotypic imidazoline receptor (AIRP) antisera and was used to detect a 43 kDa imidazoline binding protein in the human brainstem. The IRBP and AIRP antisera were used to screen a λ phage human hippocampal cDNA library from which a truncated cDNA clone was found (Ivanov *et al.*, 1998). The truncated clone was used

to design PCR primers to obtain the full clone which the authors called IRAS (Piletz *et al.*, 2000).

In the same year, the murine homologue of IRAS, nischarin, was discovered. Nischarin was discovered independently of IRAS, as an integrin-binding protein rather than an imidazoline-binding protein. Nischarin was identified using a yeast two-hybrid screen to identify proteins binding the α5 subunit of integrin (Alahari *et al.*, 2000). Further yeast two-hybrid experiments confirmed that nischarin bound the α5 subunit with high affinity and also bound α2 and αv subunits with much lower affinity (Alahari *et al.*, 2000). The authors concluded that nischarin predominantly binds the α5β1 integrin which is the fibronectin receptor.

1.2.2 IRAS structure

IRAS is a 1504 amino acid protein with a unique structure (Piletz *et al.*, 2000). Western blots for the full-length form of human IRAS reveal a 167kDa protein. This distinguishes it from endogenous rat IRAS in PC12 cells, which is closer to 210 kDa (Piletz *et al.*, 2003). Similarly, nischarin is a 206kDa protein (Alahari *et al.*, 2000).

Analysis of the structure of IRAS provides insights into how IRAS may signal. The major signalling domains are shown in figure 1.2. This review will focus on the PX domain, the protein tyrosine kinase (PTK) binding domains and the integrin binding domain.

The first characterised domain of IRAS was the integrin binding domain. As mentioned above, the mouse homologue of IRAS, nischarin, was discovered as an integrin binding protein, binding the α5 subunit of the fibronectin receptor (Alahari *et al.*, 2000). Further studies revealed a 99 residue sequence at positions 464 to 562 (709 to 807 For IRAS) to be the minimal integrin binding domain. This domain on

nischarin binds a 14 amino acid sequence of the α5 subunit which resides partly in the cytoplasm and partly in the plasma membrane (Alahari *et al.*, 2004a).

The only other well characterised domains of IRAS are the PX domain and coiled-coil region. The PX domain of IRAS is located near its N-terminus. PX domains bind phosphoinositides (PIs), which are located on plasma and vesicular membranes such as endosomes and lysosomes. In fact, IRAS associates with endosomes through a combined action of its PX domain and coiled-coil region (Lim *et al.*, 2004). Therefore the PX domain is important for localising IRAS within the cell. PX domains are commonly found in a class of proteins called sorting nexins (SNXs). SNXs have roles in protein trafficking and localising proteins to specific vesicles in the cell (Sato *et al.*, 2001; Seet *et al.*, 2006). It has been postulated that IRAS may belong to this class of proteins (Lim *et al.*, 2004). One important distinction between nischarin and the human IRAS clone is that nischarin is a truncated clone and lacks the PX domain near the N-terminus (Piletz *et al.*, 2000). So while studies on nischarin reveal probable mechanisms of IRAS signalling, they should be treated with caution given the importance and potential role of the PX domain in signalling and membrane targetting. For example, nischarin may be acting as a dominant negative inhibitor of some of the functions of IRAS.

IRAS has other potentially important signalling domains as indicated by BLAST searches (Piletz *et al.*, 2000). IRAS contains a proline rich domain, a serine rich domain and an acidic residue. These regions have been found in the cytokine receptor interleukin2β (IL2β) where they are sites for interaction with non-receptor PTKs (Miyazaki *et al.*, 1996). PTKs such as c-Src are involved in a wide variety of signalling pathways and have been implicated in the activation of PC-PLC and PKC (Rao *et al.*, 1994). Furthermore, IL2β has also been implicated to activate PC-PLC

(Lu *et al.*, 1999). IRAS also contains a haem binding domain although it is not clear what role this may have. It has been suggested that the haem domain or acidic region may form part of an imidazoline binding site.

1.2.3 Location

Analysis of IRAS and nischarin mRNA expression shows that IRAS is expressed throughout the body, with highest levels being in the brain (Alahari *et al.*, 2000; Piletz *et al.*, 1999). IRAS is also highly expressed in the kidneys, heart and lungs (Piletz *et al.*, 1999). IRAS/nischarin is well expressed in PC12 cells (Alahari *et al.*, 2000; Dontenwill *et al.*, 2003a). IRAS is also endogenously expressed in 3T3, BC3(H)1, neuro2A and HeLa cells (Alahari *et al.*, 2000; Lim *et al.*, 2004). IRAS has little or no endogenous expression in SF9, COS-7 and CHO cells (Piletz *et al.*, 2000).

There is some debate as to where IRAS localises in the cell. As described above, IRAS has a PX domain which localises to membrane structures within the cell. This is crucial if IRAS is to be considered a receptor protein, particularly since it lacks the N-terminal sequence for insertion into the plasma membrane (Piletz *et al.*, 2000). Initial studies in COS-7 and transfected NIH 3T3 cells showed that nischarin resides almost exclusively in the cytoplasm (Alahari *et al.*, 2000). This is not surprising given that it lacks the PX domain. In contrast, IRAS was found to be equally distributed between the cytoplasm and membranes of transfected CHO cells (Piletz *et al.*, 2000). Lim and Hong (2004) found that IRAS strongly localised to small and recycling endosomes in Human embryonic kidney (HEK) 293 cells and HeLa cells through a combined action of its PX domain and coiled-coil region. A subsequent study on nischarin found that it localised to both vesicular structures and throughout the cytoplasm in NIH 3T3 cells (Reddig *et al.*, 2005). The authors suggested that the coiled-coil region may be sufficient for membrane localisation.

Taken together, these results suggest IRAS localises to membranes (both plasma and endosomal) in the cell. It is still unclear to what extent IRAS resides in the cytoplasm as well.

1.2.4 IRAS as an I_1-R

IRAS was discovered as an imidazoline binding protein and is the putative clone of the I_1-R. There is evidence to suggest that IRAS is an I_1-R. Initial studies using the truncated clone of IRAS showed that IRAS mRNA expression correlated with I_1-R density throughout the body with highest levels being in the brain (Piletz *et al.*, 1999). In the brain, IRAS mRNA is distributed with highest density in the hippocampus, which again correlates with I_1-R radioligand binding studies (Grigg, 2000). In IRAS-transfected CHO cells, [^{125}I]p-iodoclonidine bound saturably (Piletz *et al.*, 2000). This binding was inhibited by addition of the I_1-R agonists moxonidine and naphazoline, but not by specific α_2-adrenoceptor agonists adrenalin and rauwolscine, suggesting the accumulation of I_1-binding sites rather than α_2-adrenoceptors (Piletz *et al.*, 2000, figure 1.3). Furthermore, IRAS-transfected PC12 cells have a higher I_1-Bmax than untransfected PC12 cells with the level of IRAS mRNA corresponding to the number of I_1-binding sites (Piletz *et al.*, 2003). Moreover, the I_1-B_{max} is significantly reduced in PC12 cells transfected with antisense oligodeoxynucleotides (ODNs) to IRAS (Sun *et al.*, 2007). However, COS-7 and Sf9 cells transfected with IRAS did not bind [^{125}I]p-iodoclonidine. The authors concluded that the generation of I_1-Rs from IRAS is host-cell specific and may be due to differences in post-translational processing of IRAS (Piletz *et al.*, 2003). This highlights the need for further studies to establish the link between IRAS and the I_1-R.

Recent evidence shows that IRAS couples to the established I_1-R signalling pathway. In IRAS-transfected CHO cells, both rilmenidine and moxonidine caused increases in PC-PLC activation, accumulation of DAG and activation of ERK1/2 (Li *et al.*, 2006). Furthermore, the increased ERK1/2 activation was blocked by both efaroxan and D609 (Li *et al.*, 2006). In PC12 cells, rilmenidine- and moxonidine-induced increases in ERK1/2 activation were prevented with IRAS/nischarin antisense ODNs (Sun *et al.*, 2007; Zhang *et al.*, 2006). Taken together, these results suggest that IRAS is the clone of the I_1-R.

1.2.5 Cell migration and Rac signalling

As described above, nischarin was discovered independently of IRAS as an integrin binding protein. Studies on nischarin have focussed on its role in cell migration and interaction with Rho GTPases, rather than on its properties as an imidazoline binding protein.

Nischarin affects both the actin cytoskeleton and cell migration. When transfected into rat embryonic fibroblasts (REFs), nischarin inhibited stress fibre formation and reorganised actin in rings throughout the cytoskeleton (Alahari *et al.*, 2000, figure 1.4). Overexpression of nischarin also resulted in decreased cell migration (Alahari *et al.*, 2000). Critically, decreasing endogenous levels of nischarin in PC12 cells through RNA interference led to an increase in cell migration (Alahari *et al.*, 2004b).

Nischarin inhibits cell migration by interacting with Rho GTPases. Rho GTPases are members of the RAS superfamily of small GTPases. They are primarily involved in actin cytoskeletal regulation and control processes such as cell spreading and migration, stress fibre formation, and growth cone and neurite formation (Burridge *et al.*, 2004). The best studied Rho GTPases involved in regulating actin

dynamics are RhoA, Rac1 and Cdc42. Rac1 is responsible for lamellipodia formation and Cdc42 is responsible for filopodia formation. Lamellipodia and filopodia are composed of polymerised actin near the cell membrane and promote cell migration and cell spreading. In neuronal cells, this leads to the stimulation of growth cone development and the formation of neurites. RhoA, on the other hand, promotes the formation of stress fibres and so is important in cell attachment and contractility. In neurons (which do not form stress fibres) RhoA inhibits the formation of growth cones and neurite elongation. A summary of Rho GTPase signalling pathways is given in figure 1.5.

Nischarin inhibits cell migration by inhibiting Rac signalling pathways (Alahari, 2003; Alahari *et al.*, 2000; Alahari *et al.*, 2004b). Nischarin binds directly to Rac and inhibits activation of downstream effectors such as NF-κB and the cyclin D1 response element (Reddig *et al.*, 2005). Nischarin also blocks Rac-induced p21 activated kinase (PAK) activation. It was observed that nischarin only inhibited cell migration induced by Rac mutants that can activate PAK and JNK (a downstream effector of PI3-kinase) (Alahari, 2003). Moreover, nischarin binds directly to PAK. Interestingly, nischarin only bound PAK in its active state (Alahari *et al.*, 2004b). Furthermore, the formation of this PAK/nischarin complex was enhanced when cells were transfected with constitutively active Rac (Alahari *et al.*, 2004b). The authors concluded that nischarin prevents uncontrolled PAK signalling and is a key regulator of cell migration.

Nischarin may inhibit Rac by affecting its subcellular localisation. It was observed that nischarin colocalises with Rac in endocytic compartments away from the cell membrane (Reddig *et al.*, 2005). Rho GTPases require localisation at the cell membrane to be active (Del Pozo *et al.*, 2004; Del Pozo *et al.*, 2002; Del Pozo *et al.*,

2000). Del Pozo *et al*. (2000) demonstrated that Rac translocates to the cell membrane in cells adherent to fibronectin but not in suspended (non-adherent) cells and that this adhesion is necessary for downstream activation of PAK. Furthermore, when Rac was mutated to prevent it binding to the cell membrane, it could not activate PAK. Therefore, nischarin may prevent the activation of Rac by inhibiting its localisation at the cell membrane.

1.2.6 Cell survival

IRAS may be an anti-apoptotic protein (Dontenwill *et al.*, 2003a). When IRAS was transfected into PC12 or COS-7 cells, it prolonged cell survival against apoptotic stimuli such as serum-withdrawal, thapsigargin and staurosporine treatment (Dontenwill *et al.*, 2003a). Furthermore, a decrease in caspase-3 activity and phosphatidylserine translocation was observed meaning IRAS acts to downregulate downstream apoptotic stimuli (Dontenwill *et al.*, 2003b). The mechanisms by which IRAS does this are yet to be discovered.

1.2.7 Interaction with other receptors

As described above, nischarin was discovered as an integrin binding protein. Subsequent research has shown that IRAS interacts with or influences the signalling of a number of different receptors. It was next discovered that IRAS interacts with the insulin receptor. IRAS binds directly to insulin receptor substrates (IRSs) and forms a complex with Grb-2, PI3-kinase and the insulin receptor (Sano *et al.*, 2002). Interestingly, when IRAS and IRSs were transfected into the HEK293 kidney cell line, a four-fold increase in ERK1/2 activation was observed (Sano *et al.*, 2002). IRAS also affects ERK1/2 activation of another growth factor, NGF. In contrast to its

effect on insulin, IRAS decreased NGF-induced ERK1/2 activation when overexpressed in PC12 cells (Piletz *et al.*, 2003).

Recently IRAS was shown to influence the signalling of the μ opioid receptor (MOR) (Wu *et al.*, 2006; Wu *et al.*, 2005). The MOR is a GPCR, which when activated, causes decreased activation of cAMP and ERK1/2 as well as a decrease in Ca^{2+} entry into cells. Chronic administration of morphine induces both tolerance and dependence in CHO cells transfected with the MOR. This is characterised by a compensatory upregulation of adenylyl cyclase and intracellular calcium. This can be demonstrated by administration of the MOR antagonist naloxone, which causes the levels of cAMP and its downstream effectors to rise dramatically as the cells undergo withdrawal. Interestingly, the putative I_1-R endogenous ligand, agmatine, concentration dependently inhibited the naloxone induced increase in cAMP, Ca^{2+} and their downstream effectors cAMP responsive element, c-Fos and ERK1/2 (Wu *et al.*, 2006; Wu *et al.*, 2005). Moreover, the efficacy of agmatine was greatly increased in cells which had been co-transfected with IRAS (Wu *et al.*, 2006; Wu *et al.*, 2005). Importantly, the effects of agmatine and IRAS were blocked by efaroxan, which provides further evidence that IRAS is an I_1-R.

A study by Lim and Hong (2004) showed that IRAS may have a role in receptor trafficking. This study showed that IRAS bound small and recycling type endosomes via its PX domain and coiled-coil region. This occurred through an interaction with phosphatidylinositol-3-phosphate (PI3P) found in the lipid membranes of endosomes. Furthermore, when IRAS was overexpressed in HEK293 cells, it caused a redistribution of the α5 integrin subunit from the plasma membrane to intracellular compartments. The authors postulated that IRAS may belong to the sorting nexin (SNX) family of proteins. SNXs are involved in protein trafficking and

are characterised by their PX domain. Therefore, IRAS may affect the signalling of the above receptors by regulating their trafficking. This suggests that IRAS may not be a conventional (classical) receptor but rather an enzyme or scaffolding protein with an imidazoline binding site.

1.3 IRAS and neuronal development

Given that IRAS is highly expressed in the brain, there has been surprisingly little focus as to what its neuronal function may be. There is some evidence to suggest that IRAS may interact with receptors involved in neuronal development. Firstly, IRAS may associate with the NGF receptor, TrkA. As described above, overexpression of IRAS in PC12 cells affected NGF-induced ERK1/2 activation (Piletz *et al.*, 2003). Furthermore, the actions of I_1-R agonists in PC12 cells only occurred when the cells had been pretreated with NGF (Edwards *et al.*, 2001; Separovic *et al.*, 1996; Separovic *et al.*, 1997; Zhang *et al.*, 2001). Secondly, IRAS associates with integrins. Integrins mediate changes in cell morphology which are important for the development of neurons. Finally, it has been proposed that I_1-R ligands may signal through lysophospholipid (LP) receptors which have roles in neuronal development (Molderings *et al.*, 2007a). The following will focus on the roles of these three groups of receptors in neuronal development followed by a proposal of how IRAS may interact with these receptors to affect changes in neuronal development.

1.3.1 Growth cones and neuronal cell growth

The study of neuronal development is important not only in understanding how neurons form, but also because many of the same mechanisms underlying neuronal growth and differentiation are necessary for neuronal survival and repair.

Therefore, the study of neuronal development may have therapeutic potential in the treatment of neurodegenerative disorders.

Neuronal development is characterised by axonal growth, the process by which axons grow from cell bodies and project over long distances. Therefore, there is particular importance in understanding the factors which control axonal development. At the leading edge of the axon is the growth cone which controls their establishment and guidance. Growth cones are composed of polymerised actin near the cell membrane called lamellipodia from which long protrusions form called filopodia (figure 1.6). The regulation of actin polymerisation in the growth cone controls its rate of advancement (Huber *et al.*, 2003). The development and advancement of growth cones are regulated by extracellular guidance molecules such as neurotrophins, netrins, ephrins, slit proteins and cell adhesion proteins such as integrin (Huber *et al.*, 2003). This review will focus on the receptors mentioned above that have roles in neuronal development and which have been implicated in IRAS/I_1-R action: TrkA, integrins and LP receptors.

1.3.2 NGF/TrkA

NGF is one of the neurotrophin family of growth factors, which also includes brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5. NGF was the first neurotrophin to be discovered. It was originally isolated from mouse sarcomas and snake venom and was observed to promote neuronal growth of chick embryo sensory ganglia (Levi-Montalcini, 1987). It was later found that NGF was necessary for the survival of sensory and sympathetic nerves cultured in vitro (Levi-Montalcini *et al.*, 1963).

NGF is produced in sympathetic neurons and the target organs which they innervate (Campenot *et al.*, 2004). NGF is also produced within the central nervous

system (CNS) with highest levels being in the hippocampus (Conner *et al.*, 1992). NGF has roles in cell survival as well as neuronal development, plasticity and regeneration (Huang *et al.*, 2001; Lim *et al.*, 2003). In neurodegenerative disorders such as Alzheimer's disease (AD), there is disrupted NGF signalling (Fahnestock *et al.*, 2001; Hellweg *et al.*, 1998; Lad *et al.*, 2003; Salehi *et al.*, 2004). Reduced neurotrophic support has been proposed as one of the mechanisms leading to cell death in these disorders. Therefore, much research has focussed on the signalling mechanisms by which NGF mediates neuronal survival.

Target derived NGF binds to its receptor, TrkA, on presynaptic nerve terminals. TrkA then signals both locally at the growth cone and is also retrogradely transported down the axon to the cell bodies. NGF binding to TrkA results in the phosphorylation of ten specific tyrosine residues (Huang *et al.*, 2003). These provide docking sites for recruitment of specific second messengers and adaptor proteins. There are three major signalling pathways: PLC-γ-Ca^{2+}, Ras-Raf-ERK and PI3-kinase-Akt. TrkA signalling is summarised in figure 1.7.

1.3.2.1 PLC-γ1 signalling

PLC-γ1 binds directly to activated TrkA and is in turn phosphorylated and activated. PLC-γ1 hydrolyses PtdIns(4,5)P_2 to generate inositol tris-phosphate (IP3) and DAG. IP3 stimulates the release of internally stored Ca^{2+}. Both Ca^{2+} and DAG activate PKC. In PC12 cells, PKC activates ERK1/2 which is required for neurite outgrowth (Corbit *et al.*, 1999).

1.3.2.2 MAP kinase signalling

MAP kinases (including ERK1/2 and ERK5) are activated by several different TrkA mediated pathways. Activation of MAP kinases is essential for the

differentiation of PC12 cells (Cowley *et al.*, 1994). MAP kinases activate transcription factors such as CREB (Xing *et al.*, 1998), c-Fos (Pellegrino *et al.*, 2006) and ELK1 (Groot *et al.*, 2000; Vanhoutte *et al.*, 2001; Yao *et al.*, 1998), which are important in the differentiation process. Recent research has shown that MAP kinases also signal in growth cones and activate scaffolding proteins that regulate the cytoskeleton such as paxillin, synapse-associated protein 90 and vinexin (Cai *et al.*, 2006; Ito *et al.*, 2007; Sabio *et al.*, 2004). Importantly, sustained ERK1/2 activation is necessary for the differentiation of PC12 cells whereas transient ERK1/2 activation, such as that produced by epidermal growth factor (EGF), causes proliferation in PC12 cells (Kao *et al.*, 2001; Peraldi *et al.*, 1993; Qui *et al.*, 1992; Vaudry *et al.*, 2002). This is an example of how the activation of the same pathway can result in different cell fates depending on the strength and duration of the signal.

Transient activation of MAP kinases is mediated by the small GTPase, Ras (York *et al.*, 2000; York *et al.*, 1998). TrkA binds the adaptor protein Shc which recruits the adaptor Grb2. Grb2 is complexed with SOS which is an exchange factor for Ras. Ras activates Raf which phosphorylates and activates MEK1 which activates ERK1/2. Ras also activates MEKK3 which activates MEK5 which activates ERK5.

In addition, TrkA also activates MAP kinases through the small GTPase Rap1. Activation of Rap1 is necessary for sustained activation of ERK1/2 by TrkA (Kao *et al.*, 2001; York *et al.*, 2000; York *et al.*, 1998). In contrast, the EGF receptor only mediates transient ERK1/2 activation through Rap1 (Kao *et al.*, 2001). Activated TrkA recruits the adaptor fibroblast growth factor receptor substrate 2 (Frs-2) which binds the adaptor Crk. This activates the guanyl nucleotide exchange factor for Rap1. Activated Rap1 activates B-Raf which activates ERK1/2.

1.3.2.3 PI3-kinase signalling

TrkA activation of PI3-kinase is important for cell survival and actin cytoskeletal organisation. TrkA activates PI3-kinase by the adaptor Gab1 and may also be activated by Ras (Holgado-Madruga *et al.*, 1997; Korhonen *et al.*, 1999; Vaillant *et al.*, 1999). PI3-kinase generates PIs which activate Akt which promotes cell survival.

PI3-kinase generated PIP3 recruits Vav2 and Vav3 to growth cones (Aoki *et al.*, 2005). These are guanine nucleotide exchange factors for the small GTPase Rac and Cdc42. Rac and Cdc42 regulate changes in the actin cytoskeleton for growth cone development and neurite outgrowth to occur (Huber *et al.*, 2003).

Numerous studies have shown that NGF induces growth cone formation through a Rac1/Cdc42 dependent manner in PC12 cells (Aoki *et al.*, 2004; Daniels *et al.*, 1998; Posern *et al.*, 2000; Sebok *et al.*, 1999; Yamaguchi *et al.*, 2001). This is a rapid process with lamellipodia formation observed just 3 minutes after NGF addition (Posern et al, 2000). Furthermore, NGF-induced neurite outgrowth in PC12 cells is inhibited by expressing dominant negative Rac1 or Cdc42 (Daniels *et al.*, 1998; Nusser *et al.*, 2002).

As well as activating PI3-kinase, Rac1 and Cdc42, NGF also prevents RhoA activation in PC12 cells. NGF treatment of PC12 cells greatly decreased active membrane-bound RhoA and reduced RhoA association with Rho-associated kinases (Nusser *et al.*, 2002). Furthermore, this process was prevented by expressing dominant negative Rac1, which suggests that Rac1 is necessary for inhibition of RhoA during initiation of neurite outgrowth.

1.3.2.4 Retrograde TrkA signalling

In vivo, TrkA receptors are located on presynaptic axonal terminals. These can be metres away from the cell body, yet many of the effects of NGF, particularly those of cell survival, involve activation of transcription factors at the nucleus and changes in gene expression. Therefore, an important question in understanding NGF signalling is how does TrkA signalling at the distal axon retrogradely travel down axons to affect changes in the cell body?

Upon NGF binding, TrkA dimerises and undergoes clathrin-mediated endocytosis. This process involves the GTPase Dynamin 1 and the pinocytotic chaperone Pincher (Shao *et al.,* 2002). Studies have shown that NGF and TrkA colocalise with ERK1/2 in both clathrin-coated vesicles and endosomes and that these undergo retrograde transport (Howe *et al.*, 2001). In PC12 cells, immunofluorescence studies showed colocalisation of TrkA, Rap1 and the early endosome marker EEA1 throughout the cell (Wu *et al.,* 2001). Furthermore brefeldin A (BFA), which disintegrates golgi and endosomal membranes, prevented the redistribution of TrkA and Rap1 to the perinuclear region and sustained ERK1/2 activation (Wu *et al.*, 2001). This suggests that association with endosomes is critical for NGF signalling. Subsequently, it was shown that early endosomes retrogradely transport NGF-TrkA in dorsal root ganglion neurons (Delcroix *et al.*, 2003).

These studies support the idea of a “signalling endosome” in which NGF bound to TrkA is transported in endosomes from the distal axon to the cell body (Campenot *et al.*, 2004). Second messengers are recruited to the TrkA in the endosomes and signal from the endosome as it travels down the axon (figure 1.8). Thus, TrkA can initiate and propagate different signals depending on its location in the neuron. For example, TrkA can modulate actin organisation in the growth cone to

extend the axon and change gene expression in the cell body for cell survival (Zweifel *et al.*, 2005).

1.3.3 Integrins

1.3.3.1 Structure

The integrin family of transmembrane proteins are the major receptors for extracellular matrix proteins such as fibronectin, laminin, and collagen (Aplin *et al.*, 1998). They are heterodimers consisting of an α and β subunit, each with an extracellular domain, a single membrane spanning region and a short cytoplasmic domain (Juliano, 2002). There are 18 alpha chains and 8 beta chains which determine the binding specificity of integrin ligands (Belkin *et al.*, 2000). For example, fibronectin binds preferentially to the α5β1 integrin whereas laminin binds a wider range of integrins depending on cell type (Belkin *et al.*, 2000). In PC12 cells, α1β1 integrin is the predominant laminin receptor (Belkin *et al.*, 2000).

1.3.3.2 Mediation of cell morphology

Integrins regulate cell shape, cell motility and adhesion by modulating actin cytoskeletal organisation. Binding of integrin ligands causes integrin clustering and the formation of smaller contact points called focal complexes and larger, more adherent contact points called focal adhesions. Focal adhesions mediate stress fibre formation. Stress fibres consist of long actin filaments which transverse the cell and cause contraction. In contrast, focal complexes mediate cell spreading and migration through the formation of lamellipodia and filopodia. Whether focal complexes develop into focal adhesions depends on the time of adhesion (DeMali *et al.*, 2003) and the concentration of integrin ligand (Cox *et al.*, 2001). For example, after short time periods, cells on integrin ligands form focal complexes, which develop into

focal adhesions over time (DeMali *et al.,* 2003). Likewise, cells on high concentrations of fibronectin develop stress fibres whereas cells on low-intermediate concentrations of fibronectin develop lamellipodia and filopodia (Cox *et al.,* 2001).

1.3.3.3 Signal transduction

Integrins play important roles in transmembrane signalling and activate a variety of signal transduction pathways (Juliano, 2002). Given that the major role of integrins is to connect the actin cytoskeleton to the extracellular matrix, integrins also signal to organise actin in the cell. Focal complex mediated cell spreading, migration and neurite outgrowth are associated with upregulation of Rac and Cdc42 whereas focal adhesion mediated contraction and stress fibre formation are associated with RhoA activity.

Evidence suggests that integrin-induced lamellipodia and filopodia formation (and associated Rac and Cdc42 activation) occurs in association with PI3-kinase activation (Keely *et al.,* 1997; Sarner *et al.,* 2000). Furthermore, integrins mediate the localisation of Rac and Cdc42 to the cell membrane, which is necessary for their activation (Del Pozo *et al.*, 2000; Del Pozo *et al.,* 2002; Del Pozo *et al.*, 2004).

Integrin clustering also results in the recruitment of integrin-associated factors such as focal adhesion kinase (FAK) and integrin linked kinase (ILK). Binding and activation of FAK leads to downstream activation of MAP Kinases through the Ras/Raf pathway and Src activation (Cobb *et al.*, 1994; Juliano, 2002).

Integrins form complexes with other membrane receptors (figure 1.9). For example, focal adhesion complexes may couple to GPCRs which leads to Ras-dependent ERK1/2 activation (Della Rocca *et al.,* 1999) and lysophosphatidic acid (LPA) receptors interact with FAK leading to downstream activation of ERK1/2 (Luttrell *et al.,* 1997). Integrins also form signalling complexes with growth factor

receptors (Juliano, 2002). Integrins complex with the EGF receptor leading to activation of Src which then phosphorylates the EGF receptor (Moro *et al.*, 2002).

Integrin signalling is necessary for adhesion-dependent cell growth and survival. For example, neurons grown on laminin show increased resistance to glutamate induced cell death by signalling through ILK and the PI3-kinase/Akt pathway (Gary *et al.*, 2001; Gary *et al.*, 2003). Specific adhesion to fibronectin also prevents apoptosis through upregulation of the anti-apoptotic Bcl-2 protein (Zhang *et al.*, 1995) and ERK1/2 activation (Gu *et al.*, 2002).

1.3.4 Lysophospholipid receptors

LP receptors comprise a group of structurally related GPCRs which bind LPs such as LPA, sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPC) and sphingosylphosphorylcholine (SPC) (Ishii *et al.*, 2004; Meyer zu Heringdorf *et al.*, 2007). LP receptors are ubiquitously expressed and mediate a wide range of functions such as nervous system development, angiogenesis and lymphocyte trafficking (Ishii *et al.*, 2004; Meyer zu Heringdorf *et al.*, 2007). Table 1.2 summarises the main signalling pathways of LP receptors. This review will focus on LPA and S1P receptors, their roles in the nervous system and their possible interactions with imidazoline ligands.

1.3.4.1 LPA

LPA is produced in serum by several enzymes including monoglycerol kinase, phospholipase A1 and phospholipase A2, and lysophospholipase D (Fukushima *et al.*, 2001; Pages *et al.*, 2001). LPA is degraded by lysophospholipase, lipid phosphate phosphatase and LPA acyl transferase (Pages *et al.*, 2001). LPA is present in human serum at concentrations between 1-5μM (Baker *et al.*, 2001).

LPA receptors are present throughout the developing nervous system and are believed to play important roles in regulating neurogenesis (Fukushima, 2004). LPA induces neurite retraction and cell rounding in a number of cell lines including PC12 cells, N1E-115, NG108-15 and immortalised CNS neuroblast cells (Ishii *et al.*, 2000; Jalink *et al.*, 1993; Jalink *et al.*, 1994; Tigyi *et al.*, 1996; Tigyi *et al.*, 1992). In NGF-differentiated PC12 cells this process occurs through activation of RhoA and enhanced Ca^{2+} signalling (Tigyi *et al.*, 1996). In contrast, LPA appears to enhance NGF signalling in PC12 cells. Co-stimulation of PC12 cells with LPA and NGF leads to greater ERK1/2 activation than with either alone (Moughal *et al.*, 2004). This occurs through an interaction between TrkA and the LPA_1 receptor. This suggests that specific LPA receptors promote different effects on neuronal cell growth, however, this remains to be elucidated.

1.3.4.2 S1P

S1P is produced in cells by sphingosine kinases (SphKs), which phosphorylate sphingosine to form S1P. S1P is degraded by S1P phosphatases. S1P is stored in platelets at high concentrations and is present in human serum at concentrations of 0.5-0.8μM (Okajima, 2002).

Interestingly, S1P receptors can be transactivated by growth factor receptors. TrkA, platelet-derived growth factor-β and insulin-like growth factor receptors induce translocation of SphKs to the plasma membrane, leading to increased production of S1P and activation of the $S1P_1$ receptor (El-Shewy *et al.*, 2006; Pyne *et al.*, 2003). The interaction of S1P receptors with TrkA can influence neuronal development. Overexpression of $S1P_1$ in PC12 cells enhanced NGF-induced Rac activation and neurite outgrowth whereas neurite outgrowth was inhibited by siRNA to $S1P_1$ (Toman *et al.*, 2004). In contrast, overexpression of the $S1P_2$ receptor

inhibited neurite outgrowth and induced cell rounding of PC12 cells (Van Brocklyn *et al.*, 1999). These studies demonstrate that S1P has roles in neuronal development with $S1P_1$ promoting, and $S1P_2$ inhibiting, neuronal development. A recent study showed that activation of the $S1P_1$ receptor in neural stem cells promoted their migration to areas of spinal cord injury in a mouse model (Kimura *et al.*, 2007). Therefore, activation of $S1P_1$ may be important in the treatment of spinal cord injuries and neurodegeneration disorders.

1.3.4.3 Interaction with clonidine

Recently it has been proposed that the actions of imidazoline agonists in PC12 cells may be through activation of lysophospholipid receptors (Molderings *et al.*, 2007a). This was originally based on the finding that LPA inhibited NA release from PC12 cells in a similar manner to clonidine (Molderings *et al.*, 2002). A subsequent study showed that LPA and S1P inhibited [^{3}H]clonidine binding in a concentration dependent manner (Molderings *et al.*, 2007a). Moreover, the binding of clonidine was significantly reduced in PC12 cells transfected with siRNA to the $S1P_1$ and $S1P_3$ receptors but not to $S1P_2$ or LPA_1 (Molderings *et al.*, 2007a). Taken together these results suggest that clonidine may act through specific S1P or LPA receptor subtypes in PC12 cells. However, these results must be interpreted cautiously, as the affinity of imidazoline ligands in this study was much lower than previously reported (μM rather than nM). It remains to be seen if clonidine binding to LP receptors leads to activation of the I_1-R signal transduction pathway in PC12 cells and if clonidine acts through these receptors *in vivo*.

1.4 Aims and objectives

IRAS is expressed primarily in the brain, however, there has been little research into its neuronal function. As described above, studies have shown that transfection of the mouse homologue of IRAS, nischarin, into REF cells induces changes in actin formation and cell morphology (Alahari *et al.*, 2000, figure 1.4). While nischarin may be a dominant negative inhibitor of IRAS, nonetheless these results indicate that IRAS has some role in actin organisation. The experiments described in chapter 3 aim to see whether IRAS has a similar effect in neuronal model PC12 cells. Furthermore, in neuronal cells, actin organisation is important for the development of growth cones and neurite outgrowth. Therefore, it is hypothesised that IRAS affects this process and thus, has a role in neuronal development.

A previous study found that IRAS decreases NGF-induced ERK1/2 activation in PC12 cells (Piletz *et al.*, 2003). In contrast, I_1-R agonists enhance ERK1/2 activation in PC12 cells pretreated with NGF (Edwards *et al.*, 2001; Zhang *et al.*, 2001). However, the functional consequence of this is unknown. The studies reported in chapter 4 aim to better characterise the effect of IRAS on NGF-induced ERK1/2 activation and, importantly, to examine the effect of IRAS on NGF-induced PC12 cell differentiation.

Studies reported in this thesis will also investigate whether IRAS influences neuronal development by interacting with other receptors. IRAS has been hypothesised to be a SNX and so may have a role in receptor trafficking (Lim *et al.*, 2004). IRAS interacts with the insulin receptor and enhances insulin induced ERK1/2 activation (Sano *et al.*, 2002). Given the links between IRAS, NGF, I_1-R agonists and ERK1/2, this suggests that IRAS interacts with TrkA. Furthermore,

nischarin was originally described as an integrin binding protein. However at the time of this discovery, it was unclear what the function of this was. Integrins regulate actin organisation in growth cones. Therefore, the studies presented in chapters 4 and 5 will investigate the hypothesis that IRAS influences growth cone development and neurite outgrowth through interaction with receptors involved in neuronal differentiation: integrin and TrkA.

It has been proposed that the actions of clonidine in PC12 cells is through LPA and/or S1P receptors (Molderings *et al.*, 2007a; Molderings *et al.*, 2002). LP receptors are known to interact with TrkA and enhance NGF signalling (Moughal *et al.*, 2004; Toman *et al.*, 2004). Experiments reported in chapter 6 aim to investigate whether IRAS interacts with LP receptors as a mechanism by which it influences NGF signalling.

The above aims will be carried out using the PC12 cell model. As previously described, PC12 cells are the most common cell line for studying processes in neuronal differentiation, especially by NGF. PC12 cells have also been used to study the I_1-R signal transduction pathway. Therefore, it will be possible to determine whether IRAS influences this pathway. The PC12 cells used in this study were kindly donated by Prof. John Piletz (Loyola Medical School, Chicago, USA). These cells have been permanently transfected with either human IRAS or an empty plasmid vector. The IRAS-transfected cells have a 3-4 fold increase in the expression of IRAS compared to vector-transfected cells. The IRAS-transfected cells also have a higher I_1-B_{max} (Piletz *et al.*, 2003). These cells have previously been used to investigate the effects of IRAS on NGF-induced ERK1/2 activation (Piletz *et al.*, 2003) and apoptosis (Dontenwill *et al.*, 2003a; Dontenwill *et al.*, 2003b).

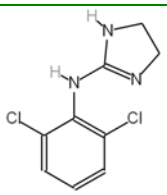
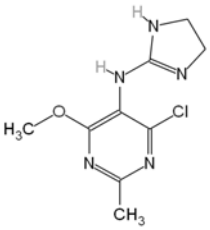
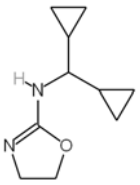
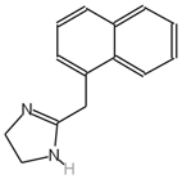
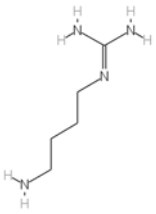
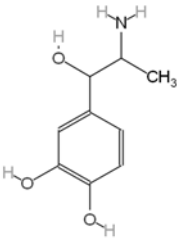
Compound	Structure	I_1-binding sites K_i (nM)	α_2-binding sites K_i (nM)	Tissue	Reference
Clonidine		1.0±0.3	3.8±1.0	Bovine RVLM	(Ernsberger *et al.*, 1993)
Moxonidine		2.3±0.5	75±8	Bovine RVLM	(Ernsberger *et al.*, 1993)
Rilmenidine		6.1±1.5	180±14	Bovine RVLM	(Ernsberger *et al.*, 1993)
Naphazoline		121.6±81	14.3±10.5	Human platelet	(Piletz *et al.*, 1996)
Agmatine		33.0±19.0	0	Human platelet	(Piletz *et al.*, 1996)
α-MNA		89000±34000	73±16	Bovine RVLM	(Ernsberger *et al.*, 1990)

Table 1.1. Affinities of various agonist drugs for I_1-Rs and α_{2a}-adrenoceptors.

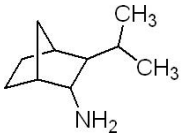
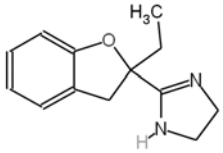
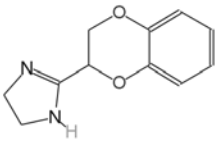
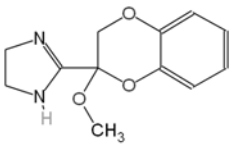
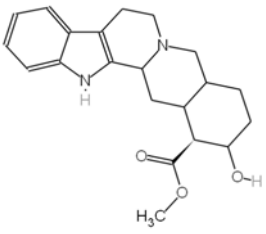
Compound	Structure	I_1-binding sites K_i (nM)	α_2-binding sites K_i (nM)	Tissue	Reference
AGN192403		42±17	>2000	Bovine RVLM	(Munk *et al.*, 1996)
Efaroxan		0.15±0.06	5.6±1.4	Bovine RVLM	(Haxhiu *et al.*, 1994)
Idazoxan		186±17	3.6±0.85	Bovine RVLM	(Ernsberger *et al.*, 1990)
2MI		400±85	2.1±0.8	Bovine RVLM	(Ernsberger *et al.*, 1997a)
Rauwolscine		>100000	5.6±3.6	Bovine RVLM	(Ernsberger *et al.*, 1997a)

Table 1.2. Affinities of various antagonist drugs for I_1-Rs and α_{2a}-adrenoceptors.

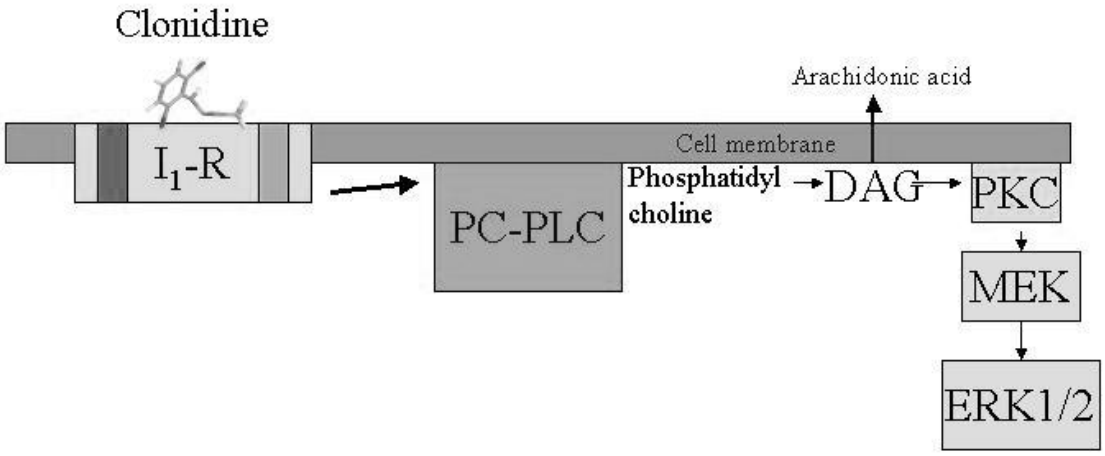


Figure 1.1. I_1-R signal transduction.

Activation of I_1-R leads to stimulation of PC-PLC, which leads to DAG production, activation of PKC and then ERK1/2.

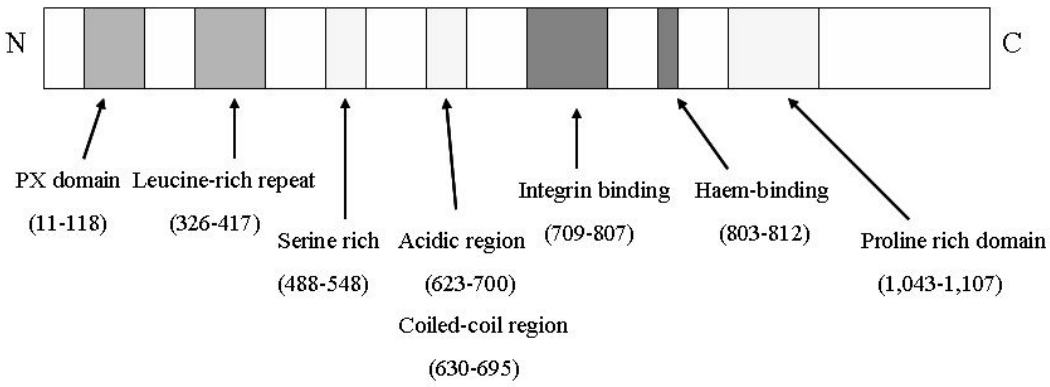


Figure 1.2. Schematic diagram of IRAS structure showing relative positions of important structural domains.

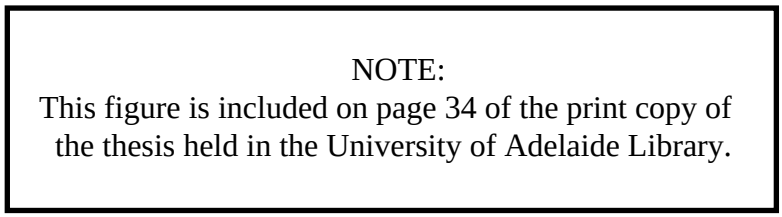
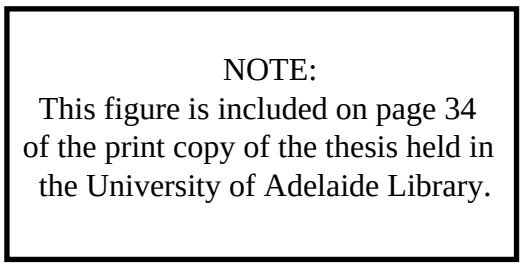


Figure 1.3. Inhibition of [^{125}I]p-iodoclonidine binding in IRAS-transfected CHO cells (graph from Piletz *et al.*, 2000).

A



B

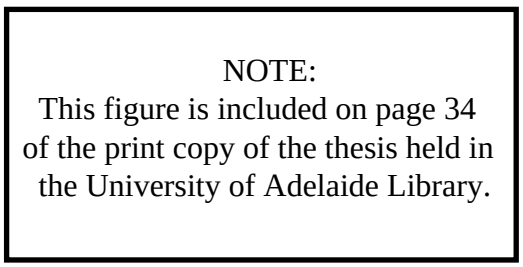


Figure 1.4. Effect of Nischarin on actin organisation in REF cells.

F-actin was stained in REFs transfected with nischarin (A) and untransfected cells (B). The nischarin-transfected cells inhibit stress fibre formation and reorganise actin into ring-like structures throughout the cytoplasm (figure from Alahari *et al.*, 2000).

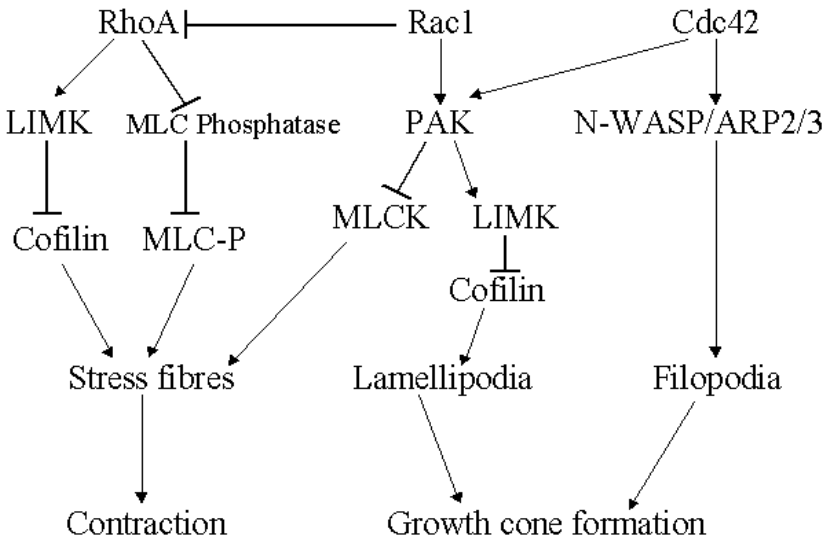


Figure 1.5. Downstream pathways from RhoA, Rac1 and Cdc42 that control stress fibre and growth cone formation.

Both Rac1 and Cdc42 activate PAKs. PAKs are a family of serine/threonine kinases with PAK1 and PAK3 being the most common in neurons (Bokoch, 2003). PAK activates LIM kinase (LIMK) which inhibits cofilin, an enzyme involved in actin severing (Edwards *et al.*, 1999). This has the effect of depolymerising actin at the minus (non-leading) end. PAK also inhibits myosin light chain kinase (MLCK), preventing myosin phosphorylation (Sanders *et al.*, 1999). This reduces cell contractility and prevents the formation of stress fibres. Cdc42 also binds directly to N-WASP, which is a member of the WASP family of proteins that control actin polymerisation. N-WASP activates the Arp2/3 complex (Ma *et al.*, 1998) which controls actin branching and when activated through Cdc42, leads to filopodia formation (Rohatgi *et al.*, 1999). RhoA activates Rho-associated kinases. These activate LIMK and inhibit MLC phosphatase leading to myosin phosphorylation and contraction, generation of tension and the formation of stress fibres and inhibition of growth cone formation (Chrzanowska-Wodnicka *et al.*, 1996; Kimura *et al.*, 1996; Maekawa *et al.*, 1999).

A

B

Figure 1.6. Growth cone dynamics and morphology.

A. Fluorescence microscope image of a single growth cone showing filopodia protruding from the growth cone (Picture from Grabham *et al.*, 1997).

B. Actin organisation within a growth cone. Extracellular guidance molecules control the formation of lamellipodia and filopodia at the leading edge of the growth cone by controlling actin polymerisation (figure from Huber *et al.*, 2003).

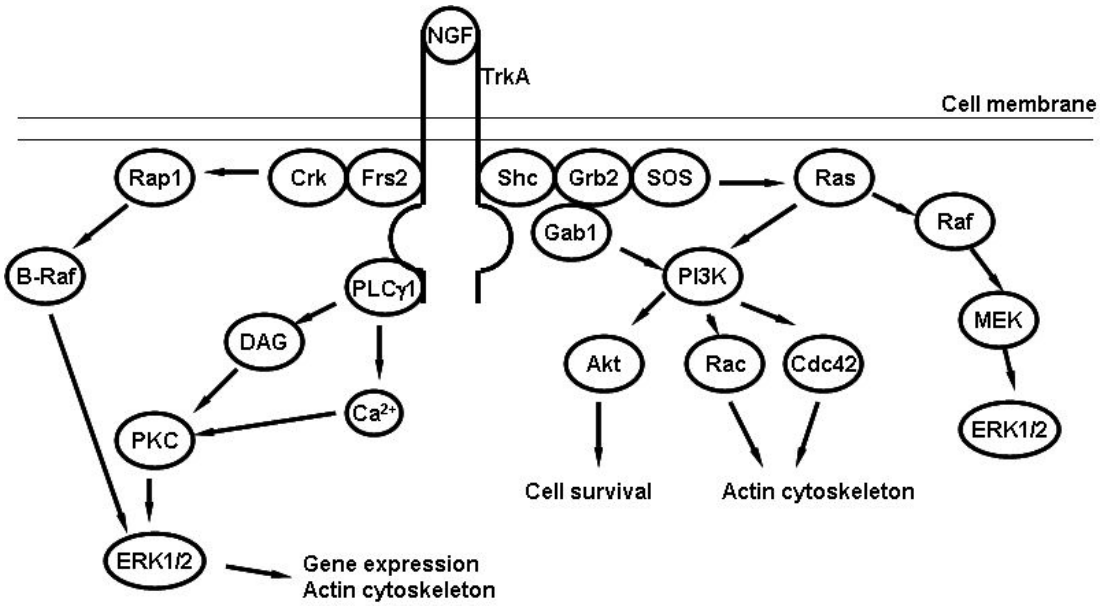


Figure 1.7 NGF signalling pathways

Figure 1.8 NGF signalling from endosomes.

NGF bound TrkA is internalised into endosomes where it recruits and activates second messengers as it travels down the axon (figure from Huang *et al.*, 2003).

Figure 1.9. Integrin signalling.

On binding to components in the extracellular matrix such as fibronectin (Fn), integrin recruits associated proteins α-actin, talin and vinculin leading to recruitment and polymerisation of F-actin. Integrins can form complexes with growth factor receptors leading to ERK activation via the Ras-Raf pathway. Integrins also activate other small GTPases such as Rac and Cdc42 (figure from Juliano, 2002).

Receptor	G-protein coupling	Signal transduction
LPA_1	$G_{i/o}$, G_q, $G_{12/13}$,	ERK↑, PI3K/Akt↑, Rho↑, AC↓, PLC↑, Ca^{2+}↑
LPA_2	$G_{i/o}$, G_q, $G_{12/13}$	ERK↑, PI3K/Akt↑, Rho↑, AC↓, PLC↑, Ca^{2+}↑
LPA_3	$G_{i/o}$, G_q, G_s	ERK↑, AC↑↓, PLC↑, Ca^{2+}↑
LPA_4		AC↑, Ca^{2+}↑
$S1P_1$	$G_{i/o}$	ERK↑, PI3K/Akt↑, Rac↑, AC↓, PLC↑, Ca^{2+}↑
$S1P_2$	$G_{i/o}$, G_q, $G_{12/13}$, G_s	ERK↑, Rac↓, Rho↑, AC↑, PLC↑, Ca^{2+}↑
$S1P_3$	$G_{i/o}$, G_q, $G_{12/13}$, G_s	ERK↑, PI3K/Akt↑, Rac↑, AC↑↓, PLC↑, Ca^{2+}↑
$S1P_4$	$G_{i/o}$, $G_{12/13}$, G_s	ERK↑, Rho↑, AC↑, PLC↑, Ca^{2+}↑
$S1P_5$	$G_{i/o}$, G_q, $G_{12/13}$, G_s	ERK↓, AC↓, PLC↑, Ca^{2+}↑
GPR_3	$G_{i/o}$, G_s	AC↑, Ca^{2+}↑
GPR_6	$G_{i/o}$, G_s	AC↑, Ca^{2+}↑
GPR12	$G_{i/o}$, G_s	AC↑, Ca^{2+}↑
GPR4	$G_{i/o}$,	ERK↑, PLC↑, Ca^{2+}↑
OGR1	$G_{i/o}$, G_q,	ERK↑, PLC↑, Ca^{2+}↑
G2A	$G_{i/o}$, G_q, $G_{12/13}$, G_s	ERK↑, AC↑, PLC↑, Ca^{2+}↑
TDAG8	$G_{i/o}$, G_q, G_s	Rho↑, AC↓, PLC↑, Ca^{2+}↑

Table 1.2. Lysophospholipid receptor G-protein coupling and signalling. Abbreviations used: AC, adenylyl cyclase; PLC, phospholipase C (table adapted from Ishii *et al.*, 2004; Meyer zu Heringdorf *et al.*, 2007).

2 Methods and materials

2.1 PC12 cell culture

Experiments were performed on PC12 cells transfected with a plasmid containing either the IRAS protein or an empty plasmid vector as a control group. Both IRAS and vector plasmids contained geneticin resistance genes. Geneticin is toxic to cells and only the transfected cells, with the geneticin resistance gene, could survive. This ensured that only IRAS- and vector-transfected cells were studied. Cells were grown in RPMI 1640 medium supplemented with 10% horse serum, 5% foetal calf serum, 10 U/ml penicillin, 10 μg/ml streptomycin, 50 U/ml of geneticin and 2 g/L of sodium bicarbonate. Cells were cultured in uncoated 75cm^2 plastic flasks in an incubator with 95% air and 5% CO_2 at 37^{0}C. The medium was refreshed every 2-3 days.

To achieve the desired concentration of cells for each experiment, cell counting was performed by pipetting equal volumes of cell suspension and trypan blue (4%) into an Eppendorf tube. 20 μl of this mixture was placed on a Neubauer counting chamber and observed under a microscope. The number of cells/ml was determined and cells were plated at the desired concentrations as described in the experimental protocols below.

2.2 F-actin staining

2.2.1 Preparation of coverslips

Coverslips were washed overnight in hydrochloric acid (2 M). The following day, the coverslips were rinsed in running de-ionised water for 15 minutes and then

washed in ethanol (70%) for 60 minutes. The coverslips were air-dried under sterile conditions and placed in 6-well culture dishes. The coverslips were then coated with laminin (10 μg/ml), fibronectin (10 μg/ml) or poly-L-lysine (PLL, 10 μg/ml) and incubated overnight at 37^0C. The following day, the solutions were aspirated, the coverslips rinsed twice in sterile milliq water and air-dried.

2.2.2 Preparation of cells

IRAS- and vector-transfected PC12 cells were plated onto laminin (10 μg/ml), fibronectin (10 μg/ml) or PLL, (10 μg/ml) coated-coverslips in 6-well culture dishes at a density of $2x10^5$ cells per well in 1 ml of RPMI 1640 medium. In some experiments, cells were treated as described in the following chapters. The cells were then incubated at 37^0C for 24-96 hours depending on experimental design.

After the incubation period, the RPMI 1640 medium was removed by aspiration, and the cells washed several times in cold PBS (pH 7.4). The cells were fixed with 3.7% formaldehyde for 10 minutes at room temperature followed by two more washes with PBS. The cells were permeabilised in 0.1% triton-X 100 for 10 minutes at room temperature. The cells were washed three more times in PBS and non-specific binding sites were blocked with 2% BSA for 1 hour at room temperature. The cells were then incubated with 1.5 μM TRITC-Phalloidin F-actin stain in the dark for 15 minutes at room temperature. The cells were washed several times in PBS and coverslips were mounted on glass slides using 50% glycerol. Coverslips were observed on a fluorescence microscope (BX50, Olympus) using 20X, 40X and a 100X oil immersion objective through a Narrow Green (NG) filter. Images were recorded using a SPOT camera and a computer with SPOT image analysis software (Diagnostic Instruments, Inc. NSW, Australia). Images were

analysed using NIH imaging software (National Institutes of Health, USA) to measure growth cone development and neurite outgrowth.

2.3 Western blotting

2.3.1 Preparation of cell lysates

6-well plates were coated with PLL (10 μg/ml) and incubated for 5 minutes at 37^0C. The PLL solution was aspirated, wells rinsed twice in sterile milliq water and air dried.

Cells were plated onto the PLL-coated plates at a density of 1-2 million cells per well and grown for 24 hours to recover. The cells were then treated as described in the following chapters. Following incubations, the wells were cooled on ice, the medium removed and 100μl of ice cold lysis buffer (0.9% NaCl, 20 μM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium vanadate, 1% triton-X 100, 1% igepal, 1 mM PMSF, protease inhibitor cocktail (1 mM AEBSF, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, 14 μM E-64), pH 7.4) was added to each well. The wells were scraped and contents transferred to Eppendorf tubes and sonicated to shear DNA and decrease lysate viscosity. The lysate was stored at -20^0C until required for bicinchoninic acid (BCA) protein concentration assay, SDS PAGE and Western blotting.

2.3.2 Protein concentration assay

The BCA assay was used to determine protein concentration. Working reagent was prepared by mixing 50 parts of Reagent A (1.0% BCA-Na_2, 2.0%

Na_2CO_3, 0.16% NaK tartate, 0.4% NaOH, 0.95% $NaHCO_3$, pH 11.25) with 1 part reagent B ($CuSO_4$). A BSA standard (20 mg/ml) was diluted to obtain a standard concentration curve (0, 80, 160, 240, 320, and 400 μg/ml). 5 μl of each lysate sample and BSA solution were mixed with 1ml of working reagent. The samples were then incubated at 37^0C for 30 minutes. After the incubation, absorbance was measured at 570 nm on a Hitachi U-2000 spectrophotometer

2.3.3 Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the cell lysates. Aliquots (20 μl) of cell extracts were added to 20 μl of 2X electrophoresis sample buffer (62.5 mM Tris, 4% SDS, 10% glycerol, 0.05% bromophenol blue, 200 mM Dithiothreitol, pH 6.8) and then boiled for 5 minutes. After cooling, the samples were loaded into each well of a Laemmli discontinuous polyacrylamide gel system consisting of 4% stacking gel and 7.5% resolving gel. The amount of sample loaded to each well was determined using the BCA assay (described above) so that 15 μg were loaded into each well. Sizes of bands were determined using a broad range molecular weight protein ladder (Bio-Rad, Dual colour). The samples were run at 200V for approximately 1 hour in running buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3) in a Bio-Rad Mini PROTEAN II electrophoresis system (Bio-Rad, California, USA). Following electrophoresis, the gels were then electrophoretically transferred to nitrocellulose membrane at 100V for 70 minutes in transfer buffer (25 mM Tris 192 mM glycine, 20% methanol, pH 8.3) in a Bio-Rad Mini Trans-Blot Electrophoresis Transfer Cell system.

2.3.4 Immunodetection

Following transfer, membranes were washed in Tris buffer saline with Tween (TBS-T, 20 mM Tris, 137 mM NaCl, 0.1% Tween-20). Non-specific binding sites on the membranes were blocked for 1 hour at room temperature with blocking reagent (TBS-T with 5% skim milk powder). The membranes were then incubated overnight at 4^0C with blocking reagent containing primary antibody (Monoclonal antibodies were diluted 1:2000 in TBS-T with 5% skim milk powder; polyclonal antibodies were diluted 1:1000 in TBS-T with 5% BSA). The primary antibodies used were: phospho-p44/42 MAPK (Thr202/Tyr204) mouse monoclonal IgG which recognises dually phosphorylated p44 MAPK (ERK1) and p42 MAPK (ERK2); and phospho-Src (Tyr527) rabbit polyclonal antibody which detects Src only when phosphorylated at Tyr527. The membranes were then washed three times for 5 or 10 minutes with TBS-T according to antibody manufacturer's instructions to remove excess unbound primary antibody. The membranes were then incubated for 1 hour at room temperature with blocking agent containing secondary antibody conjugated to horseradish peroxidase (secondary antibodies diluted 1:2000 in TBS-T with 5% skim milk powder). The secondary antibodies used were Goat anti-rabbit IgG-HRP and Goat anti-mouse IgG-HRP. Membranes were once again washed repeatedly with TBS-T. The membranes were incubated for 3 minutes with Bio-Rad Immun Star HRP substrate kit or LumiGlo reagent and peroxide (Cell Signaling, Massachusetts, USA) to visualise protein bands. These bands were visualised by exposure to X-ray film. The X-ray film was scanned on to computer and the density over the area of individual bands was measured using NIH imaging software (National Institutes of Health, USA)

2.3.5 Membrane stripping and re-probing

Following immunodetection, the nitrocellulose membranes were washed three times for 5 minutes in TBS-T. The membranes were then incubated with stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM Dithiothreitol) for 30 minutes at 50^0C to remove bound primary and secondary antibodies. The membranes were then washed six times for 10 minutes with TBS-T followed by incubation with blocking reagent for 1 hour at room temperature. The membranes were washed three times for 5 minutes in TBS-T followed by incubation overnight at 4^0C with p44/42 rabbit polyclonal antibody (1:1000 in TBS-T with 5% BSA). This recognises all forms of ERK1/2 (phosphorylated and unphosphorylated). The total levels of ERK1/2 are constant in all cells. Therefore, the relative amount of protein loaded into each lane could be detected. The membranes were washed, incubated with secondary antibody and subject to chemiluminescence detection as described above.

Results of ERK1/2 activation were expressed as a ratio of density units between phospho-ERK1/2 and total ERK1/2.

2.4 Drugs and reagents

RPMI 1640 powder, FBS, HS and L-Glutamine were purchased from Thermo Electron Corporation (Victoria, Australia). Penicillin/Streptomycin solution was purchased from Sigma Chemical Company (Missouri, USA). Geneticin was purchased from A.G. Scientific Inc. (California, USA). Trypan Blue was purchased from Hopkins & Williams (Essex, England).

Rilmenidine and AGN192403 were purchased from TOCRIS (Missouri, USA). Efaroxan, NGF, LPA, D609, wortmannin PLL and laminin were purchased

from Sigma Chemicals. SEW2871 was purchased from Cayman Chemical (Michigan, USA). Fibronectin was purchased from Thermo Electron Corporation.

Acrylamide/Bis, Ammonium persulphate and TEMED were purchased from Bio-rad (California, USA). PMSF, protease inhibitor cocktail and BCA were purchased from Sigma Chemicals. BSA was purchased from Bovogen Biologicals (Victoria, Australia).

All other chemicals were obtained from Sigma Chemical or MERCK Pty, (Victoria, Australia).

3 Chapter 3: Effect of IRAS on growth cone formation

3.1 Introduction

The I_1-R is a novel receptor with wide distribution throughout the body. It is best characterised for its effects in the brain where it is believed to mediate sympathoinhibition (Ernsberger, 1999; Ernsberger *et al.*, 1995; Ernsberger *et al.*, 1997b; Reis *et al.*, 1997; Szabo, 2002).

IRAS is the putative clone of the I_1-R. IRAS is a 167kD (1504 amino acid) protein with a unique structure that does not resemble any other receptor protein. It is expressed throughout the body with highest distribution in the brain (Alahari *et al.*, 2000; Piletz *et al.*, 1999). There is evidence to suggest that IRAS is an I_1-R. In IRAS-transfected CHO cells, the I_1-R agonist [^{125}I]p-iodoclonidine bound saturably (Piletz *et al.*, 2000). This binding was inhibited by the addition of the I_1-R agonists moxonidine and naphazoline which suggests the accumulation of I_1-binding sites (Piletz *et al.*, 2000). Furthermore, IRAS-transfected PC12 cells have a higher I_1-B_{max} than untransfected PC12 cells with the level of IRAS mRNA corresponding to the number of I_1-binding sites (Piletz *et al.*, 2003). IRAS mRNA expression correlates with I_1-R density throughout the body (Piletz *et al.*, 1999). In the brain, IRAS mRNA is distributed with highest density in the hippocampus, which again correlates with I_1-R radioligand binding studies (Grigg, 2000). Recent evidence shows that IRAS activates the same signal transduction pathways as I_1-R agonists (Li *et al.*, 2006; Sun *et al.*, 2007; Zhang *et al.*, 2006).

The mouse homologue of IRAS, nischarin, was discovered independently of IRAS as an integrin binding protein- binding the α5 subunit of the α5β1 fibronectin receptor (Alahari *et al.*, 2000). Interestingly, when transfected into REF cells,

nischarin inhibited stress fibre formation and reorganised actin in rings throughout the cytoskeleton (Alahari *et al.*, 2000). Further studies showed that nischarin inhibited Rac-induced cell migration in fibroblasts and PC12 cells (Alahari, 2003; Alahari *et al.*, 2000; Alahari *et al.*, 2004b). These studies highlight that IRAS may have a role in cell development and morphology through interaction with the actin cytoskeleton and Rho GTPases.

As mentioned above, the highest expression of IRAS occurs in the brain. However, little is known about the effects of IRAS on cell morphology in a neuronal model. Actin organisation plays an important role in neuronal cell development. In particular, the control of actin organisation is necessary for growth cone development and neurite outgrowth. Growth cones are located at the leading edge of neurites and are important for their establishment and guidance. Growth cones are composed of polymerised actin near the cell membrane called lamellipodia from which long protrusions form called filopodia. The regulation of actin polymerisation in the growth cone controls its rate of advancement (Huber *et al.*, 2003). The development and advancement of growth cones are regulated by extracellular guidance molecules such as neurotrophins, netrins, ephrins, slit proteins and cell adhesion proteins such as integrin (Huber *et al.*, 2003).

Integrins are receptors for extracellular proteins such as fibronectin, laminin and collagen. Intergrins regulate cell shape by binding to and modulating actin organisation within the cells. Furthermore, integrins regulate Rho GTPase signalling and influence the signalling of other membrane receptors (Juliano, 2002). As IRAS/nischarin is an integrin binding protein that modifies the actin cytoskeleton, IRAS could influence growth cone development in neuronal cells.

PC12 cells are an ideal neuronal model cell line for studying the regulation of the actin cytoskeleton in growth cone development and neurite outgrowth. As such they are an ideal cell line for studying the effects of IRAS on this process. Furthermore, both NGF differentiated cells and naïve cells do not express α_2-adrenoceptors (Molderings *et al.*, 2002; Separovic *et al.*, 1996). This implies that the effects of I_1-R agonists on PC12 cells are through I_1-Rs/IRAS. Therefore, the aims of this study were to observe the effects of IRAS on actin organisation and growth cone development in PC12 cells. As IRAS is the putative clone of the I_1-R, this study also aimed to observe the effects of I_1-R ligands on this process.

3.2 Methods

3.2.1 F-actin staining

IRAS- and vector-transfected PC12 cells were plated onto laminin (10 μg/ml) or poly-L-lysine (PLL, 10 μg/ml) coated-coverslips in 6-well culture dishes at a density of $2x10^5$ cells per well in 1 ml of serum containing RPMI 1640 medium. In some experiments, the I_1-R agonist, rilmenidine (10 μM), or the I_1-R antagonist, AGN192403 (10 μM), were added. The cells were then incubated at 37^0C for 24 hours. After the incubation period, F-actin in the cells was stained as described in the general methods. For some experiments, cells were viewed and recorded using a Leica SP5 spectral scanning confocal microscope (Leica Microsystems, Germany).

3.2.2 Growth cone assay

Images were taken using a fluorescence microscope (BX50, Olympus) with a 40X objective. These images were analysed using NIH image (National Institutes of Health, USA) to assess growth cone development. A growth cone was defined as membrane projections having multiple, organised actin filaments with a width greater than 2 μm at the base of the growth cone. This ensured single filopodia, which typically have a base width of less than 1 μm, were not measured. To assess growth cone development, both the percentage of cells expressing growth cones and growth cone length were measured. A cell was counted as growth cone positive if containing one or more protrusions with the parameters described above. Typically, 50-100 cells were counted each experiment. To measure growth cone length, the growth cone was measured from its base to the tip of the longest filopodia on that

growth cone. Growth cones from 3-6 cells were measured for each experiment to assess average growth cone length.

3.2.3 Statistics

One way analysis of variance (ANOVA) was performed with Bonferroni and Dunnetts post-hoc tests depending on experimental design.

3.3 Results

3.3.1 IRAS modulates F-actin organization in PC12 cells.

The IRAS homologue, nischarin, was shown to reorganise the actin cytoskeleton in REF cells (Alahari *et al.*, 2000). Therefore, to determine if IRAS had the same effect in PC12 cells, IRAS- and vector-transfected PC12 cells were grown on PLL and Laminin coated coverslips for 24 hours and F-actin stained with TRITC-phalloidin.

Figure 3.1 shows confocal microscope images of vector- and IRAS-transfected cells grown on laminin. These images show the distribution of actin through the midpoint of the cells surrounding the nucleus (figure 3.1A,B) and at the base of the cells where growth cones and filopodia develop (figure 3.1C,D). This demonstrates that the distribution of F-actin in vector-transfected cells was predominantly around the cell membrane, whereas F-actin in IRAS-transfected cells was distributed throughout the cytoplasm (figure 3.1A,B). These images also show that vector-transfected cells tended to form small filopodia protruding from the cell membrane (figure 3.1C) whereas IRAS-transfected cells formed organised growth cones (figure 3.1D). Therefore, like nischarin, IRAS alters actin organisation in PC12 cells.

3.3.2 IRAS increases growth cone development

Altered F-actin formation is a key component for growth cone formation in PC12 cells. This study therefore examined whether IRAS could affect growth cone formation in PC12 cells by examining a series of fluorescence microscope images (figure 3.2A). Like the confocal images, the fluorescence microscope images show

that growth cone development in the IRAS-transfected cells are at a more advanced stage of development with both an increased percentage of cells forming growth cones and an increased growth cone length compared to the vector-transfected cells.

IRAS-transfected cells grown on laminin formed significantly more growth cones than both vector-transfected cells when grown on laminin (35.5 $\pm$ 2.3% vs 23.1 $\pm$ 2.2%, $p < 0.05$, figure 3.2B) and IRAS-transfected cells grown on PLL (35.5 $\pm$ 2.3% vs 20.5 $\pm$ 2.1%, $p < 0.05$, figure 3.2B). IRAS-transfected cells had 40% longer growth cones than vector-transfected cells when grown on PLL (8.2 $\pm$ 0.6 μm vs 5.9 $\pm$ 0.6 μm, $p < 0.05$, figure 3.2B) and 34% longer growth cones when grown on laminin (8.9 $\pm$ 0.5 μm vs 6.7 $\pm$ 0.3 μm, $p < 0.05$, figure 3.2B). Therefore IRAS promotes growth cone development in PC12 cells and this process is enhanced by laminin.

3.3.3 Effect of rilmenidine and AGN192403 on growth cone development in PC12 cells

To investigate further whether IRAS is an I_1-R, the IRAS- and vector-transfected cells were treated with I_1-R agonists and antagonists to observe their effect on IRAS-mediated growth cone development. Treatment of vector-transfected cells with the I_1-R agonist, rilmenidine, increased the percentage of cells expressing growth cones (26.5 $\pm$ 0.8% vs 35.7 $\pm$ 4.5%, $p < 0.05$, figures 3.3A and 3.4A). Rilmenidine also significantly increased growth cone length in vector-transfected cells (6.7 $\pm$ 0.3 μm vs 9.8 $\pm$ 0.5 μm, $p < 0.05$, figure 3.4B) whereas the I_1-R antagonist, AGN192403, had little effect on growth cone development in vector-transfected cells (figures 3.3C and 3.4). Importantly, the rilmenidine-induced

increase in growth cone development was blocked by AGN192403 in vector-transfected cells (figure 3.3E, G and 3.4).

Treatment of IRAS-transfected cells with AGN192403 reduced the percentage of cells expressing growth cones (38.6 $\pm$ 3.4% vs 27.4 $\pm$ 2.2%, $p < 0.05$, figure 3.3D and 3.4A) and reduced growth cone length (8.9 $\pm$ 0.5 μm vs 6.5 $\pm$ 0.5 μm, $p < 0.05$, figure 3.4B). Not surprisingly, rilmenidine had little effect on the IRAS-transfected cells as these cells already had increased growth cone expression (figures 3B and 3.4).

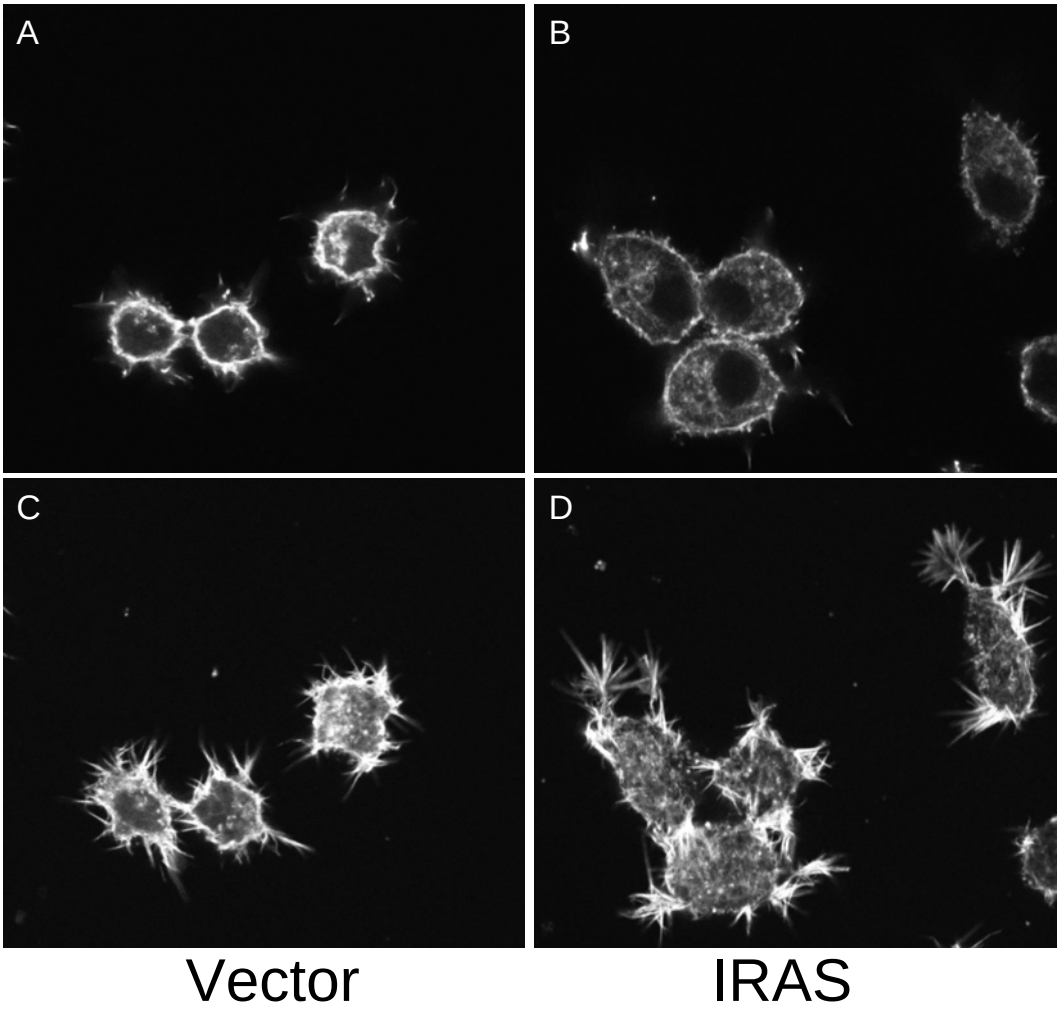


Figure 3.1. Effect of IRAS on F-actin distribution.

Confocal microscope images of vector-transfected cells (A, C) and IRAS-transfected cells (B, D) on laminin. Images were taken of the same cells through the mid-plane (A, B) and base of cells (C, D).

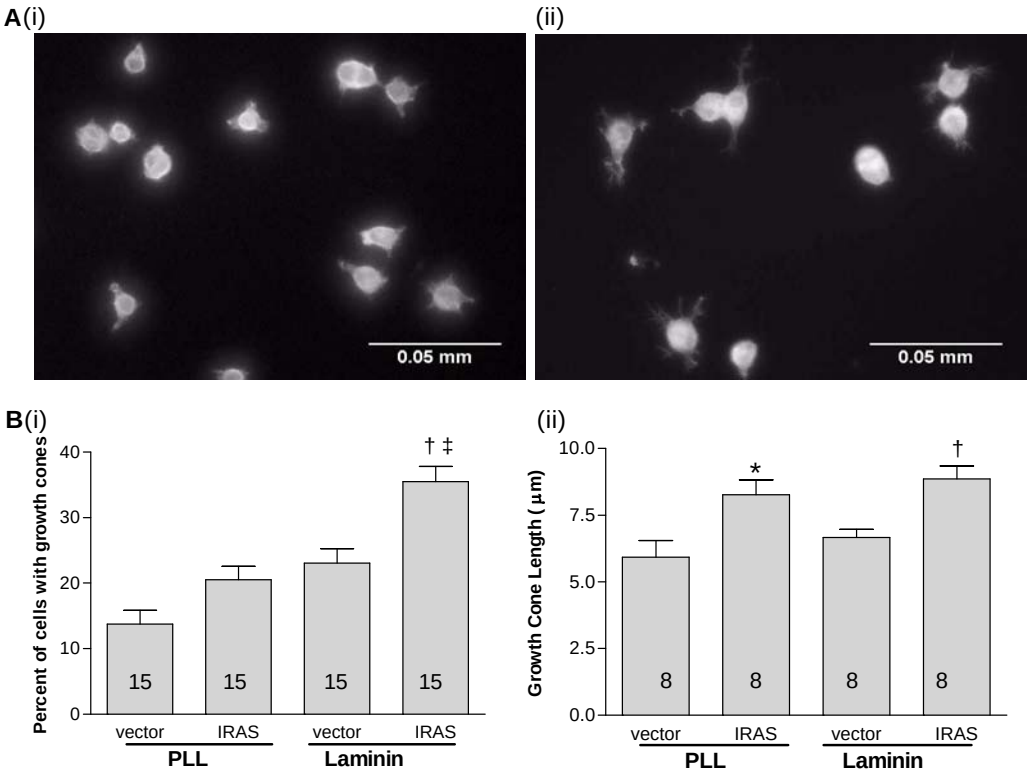


Figure 3.2. Effect of IRAS on growth cone development.

A. Representative fluorescence microscope images of vector-transfected cells (i) and IRAS-transfected cells (ii) grown on laminin. **B.** (i) Percentage of cells with growth cones (50-100 cells were measured per experiment). (ii) Average growth cone length (3-6 cells were measured per experiment). Values are expressed as mean $\pm$ SEM (number of experiments are in columns). *****, $p < 0.05$ compared with vector-transfected cells grown on PLL. †, $p < 0.05$ compared with vector-transfected cells grown on laminin. ‡, $p < 0.05$ compared with IRAS-transfected cells grown on PLL (one-way ANOVA, Bonferroni).

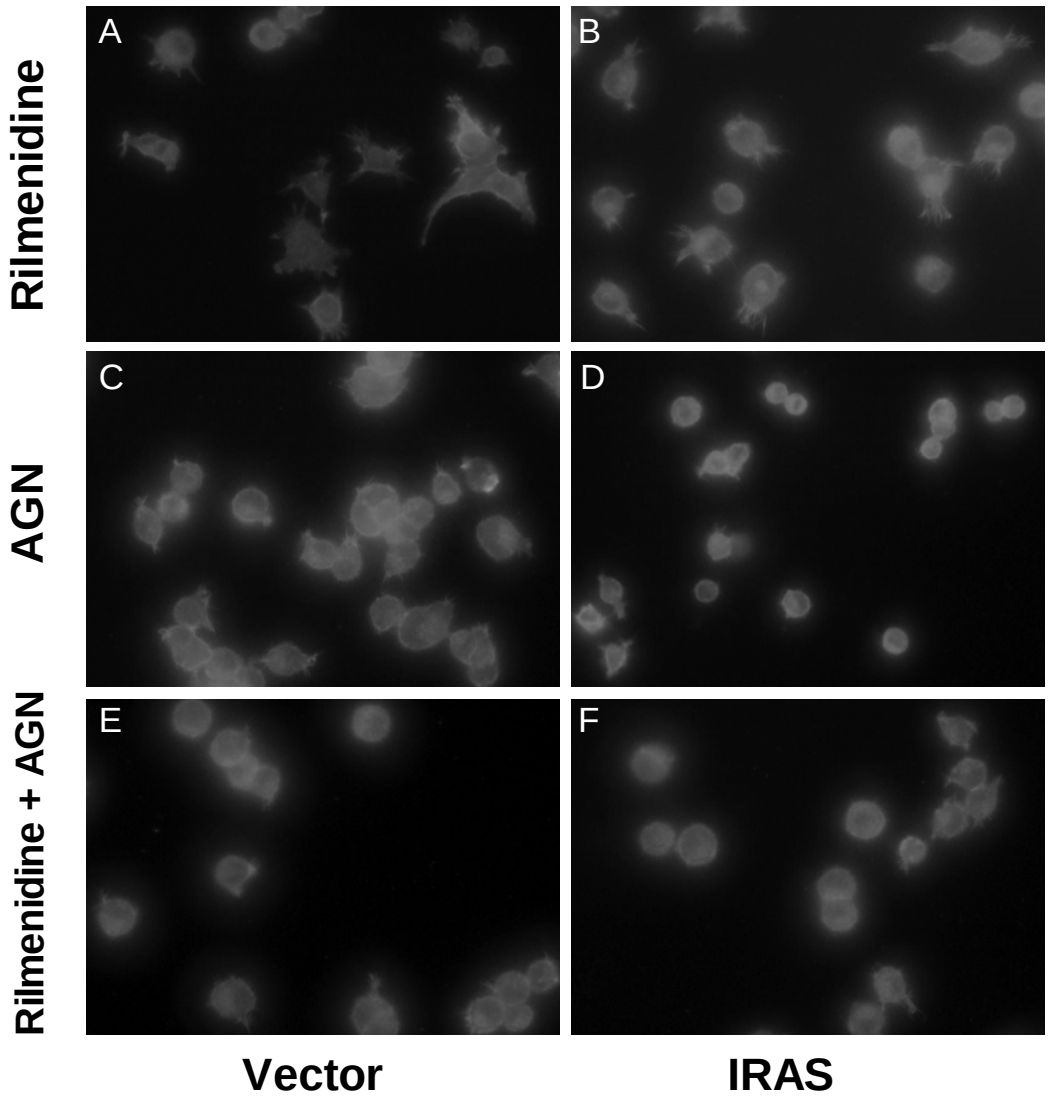


Figure 3.3. Effect of rilmenidine and AGN192403 on growth cone development. Representative fluorescence microscope images of vector-transfected cells PC12 cells (A,C,E) and IRAS-transfected cells (B,D,F) treated with rilmenidine (10μM, A,B), AGN192403 (10μM, C,D) or rilmenidine and AGN192403 (10μM, E,F).

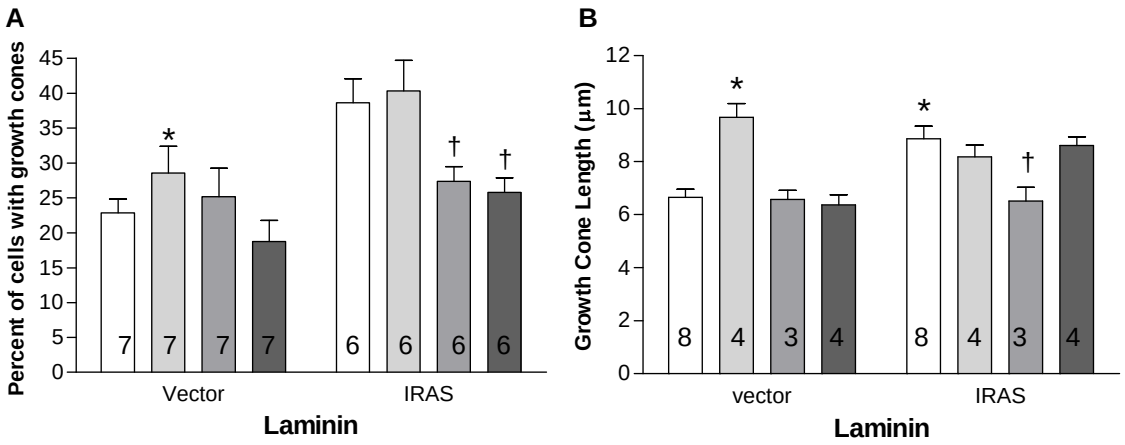


Figure 3.4. Effect of rilmenidine and AGN192403 on percentage of cells with growth cones and growth cone length.

A. Percentage of cells with growth cones (50-100 cells were measured per experiment). **B.** Average growth cone length (3-6 cells were measured per experiment). Treatment groups are rilmenidine (10µM,), AGN192403 (10µM,), rilmenidine and AGN192403 (10µM,) or PBS (control,). Values are expressed as mean ± SEM (number of experiments are in columns). *****, $p < 0.05$ compared with control vector-transfected cells grown on laminin. † $p < 0.05$ compared with control IRAS-transfected cells grown on laminin (one-way ANOVA, Dunnetts).

3.4 Discussion

The mouse homologue of IRAS, nischarin, was previously shown to inhibit stress fibre formation and reorganise the actin cytoskeleton in REF cells (Alahari *et al.*, 2000). However, the functional significance of this is unknown. As IRAS is located primarily in the brain (Piletz *et al.*, 2000; Piletz *et al.*, 1999) it was hypothesised that IRAS would alter actin organisation in neuronal model PC12 cells. Furthermore, as actin organisation is important in growth cone formation and neurite outgrowth, this study sought to determine whether IRAS would affect this process.

The confocal microscopy images clearly show that in the vector-transfected cells, F-actin is primarily located at the cell membrane, whereas for the IRAS-transfected cells, there is a partial redistribution of actin from the membrane to the cytoplasm. Furthermore, this altered actin organisation is associated with enhanced expression of growth cones in the IRAS-transfected cells. The development of growth cones is an important morphological change in neuronal development. These results therefore suggest that IRAS has a role in PC12 cell differentiation. In fact, the change in actin distribution between vector- and IRAS-transfected cells is similar to that observed between naïve PC12 cells and PC12 cells undergoing differentiation by NGF. In naïve PC12 cells, F-actin is located diffusely around the cell membrane (Paves *et al.*, 1988). In contrast, NGF induces a rapid change in actin which becomes less uniformly distributed at the membrane with increased distribution throughout the cytoplasm and strong localisation in growth cones (Paves *et al.*, 1988). Therefore, the morphology of the IRAS-transfected cells is similar to that caused by rapid exposure to NGF. After prolonged NGF exposure, PC12 cells cease dividing and the growth cones guide the establishment of neurites. Neurite outgrowth and cessation of cell

proliferation was not observed in IRAS-transfected cells. Therefore, overexpressing IRAS itself does not cause differentiation.

3.4.1 Effect of integrin on growth cone formation

This study sought to determine whether the effect of IRAS on actin organisation and growth cone development was due to its integrin binding properties. Integrins connect actin to the cytoskeleton and activate signalling pathways to regulate its organisation (Juliano, 2002). Therefore, regulation of integrin and its signalling pathways is a likely mechanism by which IRAS could modulate actin and growth cone development. Nischarin was shown to alter the actin cytoskeleton when grown on fibronectin which suggests that this occurred through an interaction with integrin (Alahari *et al.*, 2000). However, the effect of fibronectin was not compared to a non-specific cell adherent, such as PLL. Therefore, it is unclear if IRAS requires integrin stimulation for it to affect actin organisation. To see if IRAS requires integrin stimulation for its activity, the PC12 cells were grown on the integrin agonist, laminin, and PLL. From the results of the present study, it is still unclear whether IRAS requires integrin stimulation to affect actin organisation. IRAS overexpression led to an increase in growth cone length compared with vector-transfected cells on both PLL and laminin. However, when IRAS-transfected cells were grown on laminin, there was an increase in the number of growth cones observed. This suggests that laminin enhances the ability of the IRAS-transfected cells to establish growth cones but not in the elongation of growth cones. Therefore, IRAS may enhance growth cone development through a weak association with laminin, however this association is not crucial for IRAS to affect the actin cytoskeleton. Alternatively, this may be due to a non-specific effect of laminin whereby growth cone development and neurite outgrowth is enhanced in neuronal

cells by integrin agonists compared with PLL. A limitation of this study is that it did not test the effects of fibronectin on growth cone development. Fibronectin may have had a greater effect on growth cone development in IRAS-transfected cells given that IRAS has a much higher affinity for the fibronectin receptor. This limitation is addressed in the following chapter.

IRAS may influence actin organisation by directly acting on the Rho family of GTPases. Nischarin inhibits stress fibre formation in fibroblasts, which suggests that it inhibits RhoA (Alahari *et al.*, 2000). Unlike fibroblasts, PC12 cells do not form stress fibres. In naïve PC12 cells, when RhoA is active, actin is located around the cell membrane (Yamaguchi *et al.*, 2001). This is the phenotype of the vector-transfected cells. The present study suggests that IRAS has a similar inhibitory effect on RhoA in PC12 cells. The enhanced growth cone development in the PC12 cells suggests that IRAS inhibits RhoA and activates Rac and cdc42. Surprisingly, nischarin was found to inhibit Rac and its downstream effector PAK (Alahari, 2003; Alahari *et al.*, 2000; Alahari *et al.*, 2004b; Reddig *et al.*, 2005). Nischarin binds both Rac and PAK in their activated states which leads to an inhibition of cell migration (Alahari *et al.*, 2004b; Reddig *et al.*, 2005). Interestingly, stimulating nischarin-transfected COS-7 cells with EGF led to an increased association of nischarin with Rac (Reddig *et al.*, 2005). Similarly, transfection of cells with constitutively active Rac led to enhanced association of PAK and nischarin (Alahari *et al.*, 2004b). This association largely occurred at lamellipodia near the cell membrane. The authors concluded that nischarin prevents uncontrolled PAK signalling and is a key regulator of cell migration.

A number of factors may explain these seemingly contradictory results. Nischarin lacks the PX domain of IRAS which may disrupt the localisation of

nischarin. The PX domain of IRAS is necessary in targeting IRAS to endosomes (Lim *et al.*, 2004) and the plasma membrane (Piletz *et al.*, 2000). Localisation at the cell membrane is necessary for activation of both Rac and PAK (Del Pozo *et al.*, 2004; Del Pozo *et al.*, 2002; Del Pozo *et al.*, 2000). Nischarin colocalised with Rac in vesicles away from the cell membrane (Reddig *et al.*, 2005). Therefore the effect of IRAS/nischarin on Rac activation may depend on where they colocalise. In this sense, the truncated nischarin may be acting as a dominant negative protein and inhibiting the function of Rac.

In addition, the growth conditions used may account for the different outcomes. In the present study, the cells remained in full serum media for the duration of the experiments whereas for studies on nischarin, the cells were typically grown in serum-free media or low serum media (0.5-1% FBS) for 12-24 hours before treatment (Alahari *et al.*, 2000; Alahari, 2003; Alahari *et al.*, 2004b). These different growth conditions may change the behaviour of IRAS. For example, IRAS may interact with components in serum which enhance growth cone development. The effect on serum withdrawal and IRAS on PC12 differentiation is examined further in the following chapters.

Interestingly, there is not a requirement for any additional stimulus (imidazoline or other) to see this effect of IRAS. This suggests that transfected IRAS has some basal activity. This type of constitutive activity has been observed in several different receptor types, especially in transfected receptor systems. A common theme in IRAS research is that IRAS interacts with a number of different receptors and often enhances their signalling. For example, IRAS associates with the insulin receptor by directly binding insulin receptor substrate proteins (Sano *et al.*, 2002). Furthermore, when IRAS and IRSs were transfected into the HEK293 kidney

cell line, a four-fold increase in ERK1/2 activation was observed (Sano *et al.*, 2002). Similarly, IRAS enhances signalling of the MOR. Therefore, IRAS may enhance the activity of receptors involved in differentiation. The most likely candidate is the NGF receptor, TrkA. TrkA is a growth factor receptor, like the insulin receptor, which induces neurite outgrowth in PC12 cells. Another possible candidate is the LP family of receptors. These are a closely related family of GPCRs which are activated by LPA and S1P. Furthermore, these receptors can be transactivated by NGF and enhance PC12 cell differentiation (Moughal *et al.*, 2004; Toman *et al.*, 2004). NGF, S1P and LPA are present in serum at low concentrations (Baker *et al.*, 2001; Martocchia *et al.*, 2002; Okajima, 2002). Therefore, IRAS may enhance these weak signals by enhancing receptor activity to induce an increase in growth cone development. This is examined further in the following chapters.

3.4.2 Effect of I_1-R ligands

This study shows that I_1-R ligands can affect growth cone development in PC12 cells. In vector-transfected cells, rilmenidine stimulated growth cone development. This meant that the vector-transfected cells displayed a phenotype similar to the IRAS-transfected cells. Importantly, AGN192403 blocked the effects of rilmenidine in the vector-transfected cells. These results suggest that I_1-Rs play a role in the mediation of growth cone development and provide evidence that IRAS is an I_1-R.

In IRAS-transfected cells, rilmenidine had no effect. Transfected IRAS is already active as clearly indicated by its dramatic effect on cell phenotype. Therefore, rilmenidine does not further activate IRAS to enhance growth cone development. AGN192403 blocked the IRAS mediated increase in growth cone length and reduced the percentage of IRAS-transfected cells expressing growth

cones. This seems unusual that an I_1-R antagonist could block the effects of IRAS given IRAS is constitutively active when overexpressed. One possible explanation for this is that AGN192403 was acting as an inverse agonist and so was able to reverse the effects of IRAS. The fact that an I_1-R antagonist (or inverse agonist) blocked an IRAS mediated effect suggests that IRAS is an imidazoline binding protein and possibly an I_1-R. A limitation of this study is that other I_1-R ligands were not tested. Therefore, this may be a non-specific effect of AGN192403. Further studies with other I_1-R agonists and antagonists are required to confirm if this is an I_1-R mediated effect.

These results add to a growing body of evidence connecting IRAS to the I_1-R signal transduction pathway. Research into I_1-R signal transduction has been primarily based on the effects of I_1-R agonists in NGF-differentiated PC12 cells. These studies have found that I_I-R agonists activate PC-PLC through an increase in DAG accumulation (Separovic *et al.*, 1996; Separovic *et al.*, 1997) and that this leads to an increase in PKC and ERK1/2 activation (Edwards *et al.*, 2001; Zhang *et al.*, 2001). Recent evidence has linked IRAS to this signalling pathway. IRAS/nischarin antisense oligodeoxynucleotides decrease rilmenidine and moxonidine-induced ERK1/2 activation in PC12 cells (Sun *et al.*, 2007; Zhang *et al.*, 2006). Furthermore, rilmenidine and moxonidine caused an increase in PC-PLC activation, ERK1/2 activation and DAG accumulation in IRAS-transfected CHO cells (Li *et al.*, 2006). These effects were blocked by efaroxan (Li *et al.*, 2006). Thus, these studies link IRAS to the established I_1-R signal transduction pathway and suggest that IRAS is under control of imidazoline ligands.

3.5 Conclusion

This study shows that IRAS reorganises the actin cytoskeleton and enhances growth cone development in PC12 cells. Furthermore, growth cone development can be influenced by I_1-R ligands, which suggests that IRAS is an imidazoline binding protein. This is the first study to show that IRAS may have a role in neuronal development.

4 Chapter 4: Effect of IRAS on NGF-induced neurite outgrowth and ERK1/2 activation

4.1 Introduction

The study described in the previous chapter showed that overexpressing IRAS in PC12 cells leads to actin reorganisation and enhanced growth cone development. The development of growth cones is necessary for the establishment and guidance of neurites and is an important part of PC12 cell differentiation. This suggests that IRAS may have a role in PC12 cell differentiation. It is unclear, however, if IRAS is acting itself to cause this increased growth cone development or through interaction with another receptor. IRAS may also be interacting with components in serum, such as growth factors, which enhance its activity.

PC12 cells typically undergo differentiation when exposed to NGF (Greene *et al.*, 1976). This process is characterised by neurite outgrowth. NGF binds to its receptor, TrkA, on the cell membrane. The activated TrkA recruits various adaptor proteins which in turn, activate several cell signalling pathways (Huang *et al.*, 2003). This includes activation of ERK1/2 which signal both locally to enhance growth cone development (Pullikuth *et al.*, 2007) and activate transcription factors involved in the differentiation process (Huang *et al.*, 2003). Interestingly, the intensity and duration of ERK1/2 signalling is crucial to cell fate. NGF causes prolonged ERK1/2 activation which leads to differentiation whereas EGF causes transient ERK1/2 activation which leads to the proliferation of PC12 cells (Kao *et al.*, 2001; Peraldi *et al.*, 1993; Qui *et al.*, 1992; Vaudry *et al.*, 2002).

Previous research in PC12 cells shows that there may be an interaction between NGF and I_1-R signalling pathways which enhance ERK1/2 activation. Treatment of PC12 cells with moxonidine, clonidine or rilmenidine enhanced ERK1/2 activation in PC12 cells (Edwards *et al.*, 2001; Zhang *et al.*, 2001). Crucially, this only occurred when the cells were pretreated with NGF; first with 50 ng/ml for 48 hours followed by a 5-10 ng/ml treatment before the I_1-R ligand treatment. This suggests a requirement of NGF for I_1-R activity in PC12 cells.

Transfection of IRAS into cell lines also affects ERK1/2 signalling. It was first shown that overexpression of IRAS in HEK cells caused a four-fold increase in insulin-induced ERK1/2 activation (Sano *et al.*, 2002). Subsequently, Piletz *et al*. (2003) investigated the effect of transfected IRAS on NGF-induced ERK1/2 activation in PC12 cells. Somewhat surprisingly, they found that IRAS deceased the magnitude of ERK1/2 activation after both 5 minutes and 90 minutes of NGF treatment. This appears in contrast to the findings presented in the previous chapter showing that IRAS enhances growth cone development; a process associated with increased ERK1/2 activation. These results may be related to the growth conditions of the cells. Prior to NGF treatment, the cells were grown in serum-free media for 12 hours. It is possible that IRAS exerts different effects on cell growth and signalling depending on the growth conditions.

Another possibility is that IRAS exerts its PC12 cell differentiation promoting properties through interaction with integrin. As described in the previous chapter, integrin connects the extracellular matrix proteins to the actin cytoskeleton, which is important for the development of growth cones in neuronal cells. However, it was unclear whether IRAS enhanced growth cone development by binding integrin. Growing IRAS-transfected cells on laminin increased the percentage of

cells expressing growth cones but did not affect the length of the individual growth cones. Moreover, the previous chapter did not test the effects of fibronectin, the specific ligand for the α5β1 receptor, to which IRAS binds with high affinity.

The primary aims of the experiments described in this chapter were to investigate the effects of IRAS on NGF-induced neurite outgrowth and ERK1/2 activation. This study also aimed to see how serum and different integrin substrates affected the role of IRAS in these processes.

4.2 Methods

4.2.1 Neurite outgrowth

To assess neurite outgrowth, the vector- and IRAS-transfected PC12 cells were grown in full-serum media or serum-free conditions for 4 hours prior to plating. The cells were then plated onto laminin (10 μg/ml), fibronectin (10 μg/ml) or PLL (10 μg/ml) coated-coverslips in full-serum media as described in the general methods. The cells were grown for 96 hours with NGF (100 ng/ml) added every 24 hours. The media was replaced after 48 hours. After incubation, the cells were stained for F-actin and viewed with a fluorescence microscope using a 20X objective as described in the general methods. For each treatment, 3-4 images were taken. Neurite outgrowth was assessed using NIH image. 50-1000 cells were counted. Cells with one or more neurites >1X cell body diameter were counted as neurite positive.

4.2.2 ERK1/2 activation

IRAS- and vector-transfected PC12 cells were plated onto PLL (10 μg/ml) coated 6-well plates and grown for 24 hours. The cells were then treated with NGF (100 ng/ml) or EGF (100 ng/ml) for between 1 and 120 minutes. In some experiments, the full-serum media was removed, the cells rinsed twice in serum-free media and then grown in serum-free media for 4 hours prior to treatment with NGF. Following treatment, the cells were lysed and protein concentration of the lysates determined. The cell lysates were subject to SDS-PAGE and immunodetection for phospho-ERK1/2 and total ERK1/2 as described in the general methods.

4.2.3 Statistics

Student t-tests were performed for comparisons of two groups. Student-t-tests or one way ANOVA with Bonferroni post-hoc tests were performed depending on experimental design.

4.3 Results

4.3.1 The effect of IRAS on NGF-induced neurite outgrowth

In full-serum conditions, IRAS-transfected cells formed significantly more neurites than vector-transfected cells (figures 4.1 and 4.2). This occurred when the cells were grown on PLL, laminin and fibronectin. Growing the cells on laminin or fibronectin did not significantly affect the percentage of vector- or IRAS-transfected cells forming neurites compared with growing the cells on PLL. When grown on PLL, 12.42 ± 0.34% of IRAS-transfected cells formed neurites compared to 2.52 ± 0.19% for the vector-transfected cells. On laminin, 10.73 ± 0.94% of IRAS-transfected cells formed neurites compared with 2.01 ± 0.184% of the vector-transfected cells and on fibronectin, neurite outgrowth was observed in 10.20 ± 0.96% of IRAS transfected cells compared to 3.26 ± 0.75% for the vector-transfected cells.

In contrast, when the cells were serum-starved before NGF addition, IRAS transfection caused a decrease in the number of cells forming neurites (figures 4.3 and 4.4). Similarly to the full-serum conditions, this occurred when the cells were grown on each substrate. Likewise, growing the cells on laminin or fibronectin did not significantly alter the number of vector- or IRAS-transfected cells forming neurites. On PLL, 5.87 ± 1.19% of IRAS-transfected cells formed neurites compared to 16.00 ± 2.93 % for the vector-transfected cells. 6.52 ± 1.31% of IRAS transfected cells formed neurites compared to 15.92 ± 2.33% for the vector-transfected cells when grown on laminin and 6.24 ± 0.91% of IRAS-transfected and 14.51 ± 2.07% of vector-transfected cells formed neurites on fibronectin.

4.3.2 The effect of IRAS on NGF-induced ERK1/2 activation

The effect of IRAS on NGF-induced ERK1/2 activation in full-serum conditions is shown in figure 4.5. For both vector- and IRAS-transfected cells, there were undetectable levels of phospho-ERK1/2 in untreated cells. For the vector-transfected cells, NGF caused the greatest ERK1/2 activation after 15 minutes. This was followed by a gradual decline in ERK1/2 activation over the 120 minute timecourse. In comparison, NGF caused significantly greater ERK1/2 activation in the IRAS-transfected cells after 5 minutes stimulation compared to the vector-transfected cells. There were similar levels of phospho-ERK1/2 in the IRAS- and vector-transfected cells between 15 and 120 minutes.

Figure 4.6 shows the effect of IRAS on NGF-induced ERK1/2 activation in serum-starved conditions. Similarly to the full-serum conditions, there were undetectable levels of phospho-ERK1/2 in untreated vector- and IRAS-transfected cells. For both vector- and IRAS-transfected cells, NGF caused greatest ERK1/2 activation after 15 minutes followed by a gradual decline over the timecourse. There were no significant differences between the vector- and IRAS-transfected cells at any of the timepoints under these conditions.

4.3.3 The effect of IRAS on EGF-induced ERK1/2 activation

The effect of EGF on ERK1/2 activation in IRAS- and vector-transfected cells under full-serum conditions was observed over a 60 minute timecourse (figure 4.7). This shorter timecourse (1 to 60 min) was chosen as EGF has been shown to activate ERK1/2 more quickly than NGF and over a shorter period. In both vector- and IRAS-transfected cells, EGF caused the greatest ERK1/2 activation after 5 minutes. ERK1/2 activation then declined sharply from 15 minutes onwards. There

were no significant differences between the vector- and IRAS-transfected cells at any of the timepoints.

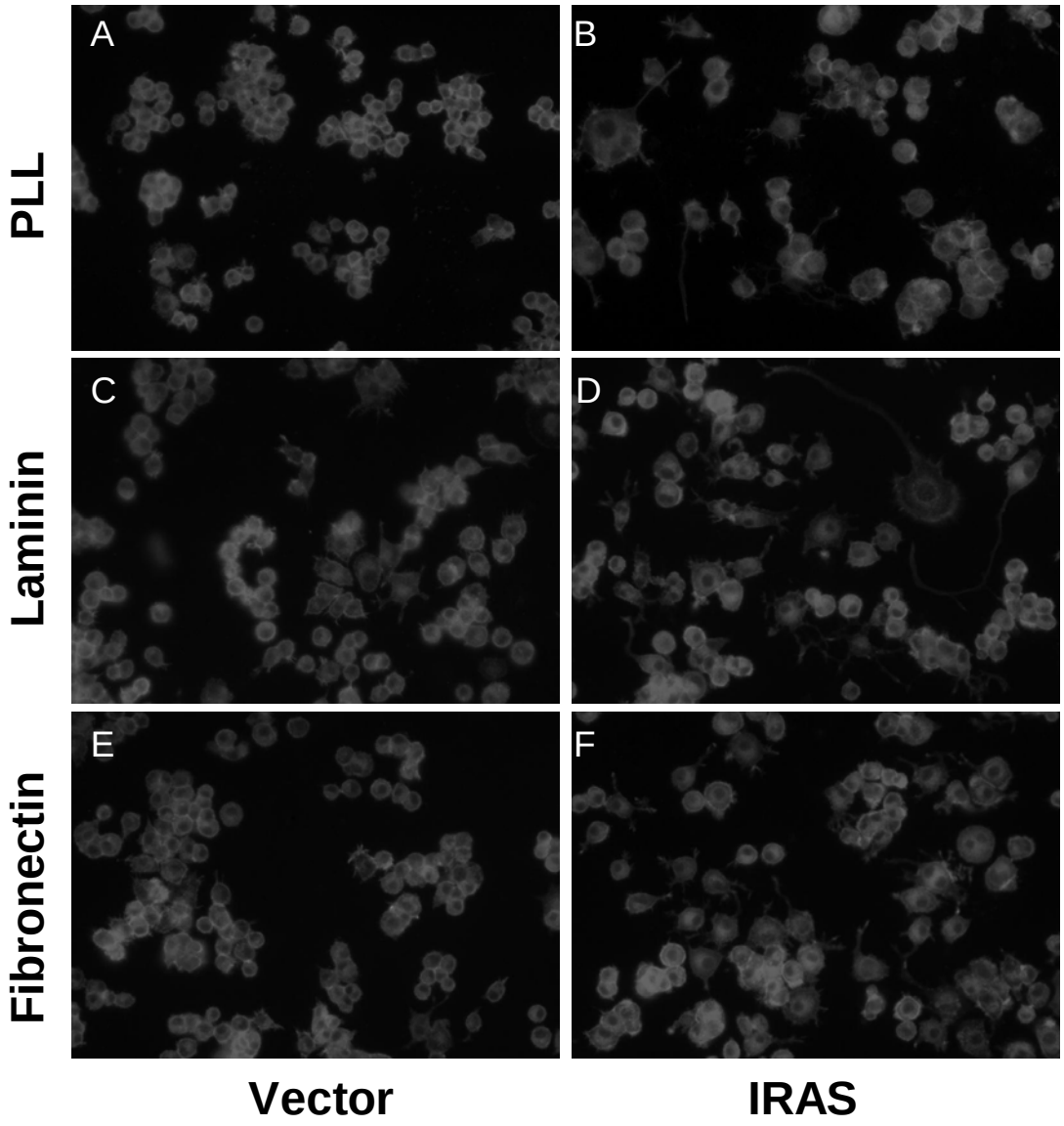


Figure 4.1. Effect of IRAS on NGF-induced neurite outgrowth in full-serum conditions.

Representative vector-transfected cells grown on PLL (A), laminin (C) and fibronectin (E). Representative IRAS-transfected cells grown on PLL (B), laminin (D) and fibronectin (F).

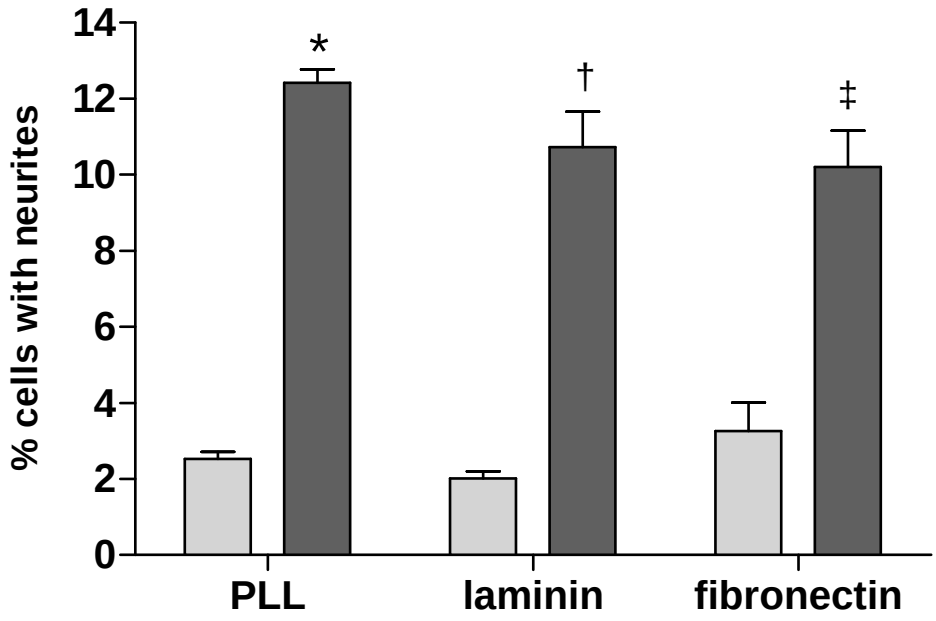


Figure 4.2. Percent of vector- and IRAS-transfected cells expressing neurites in full-serum conditions.

Vector- ( ) and IRAS- ( ) transfected cells were scored as neurite positive if containing one or more protrusions with length > the cell diameter. *****, $p < 0.05$ compared with vector-transfected cells grown on PLL (n=4, one way ANOVA, Bonferroni). †, $p < 0.05$ compared with vector-transfected cells grown on laminin (n=4, one way ANOVA, Bonferroni). ‡, $p < 0.05$ compared with vector-transfected cells grown on fibronectin (n=4, one way ANOVA, Bonferroni).

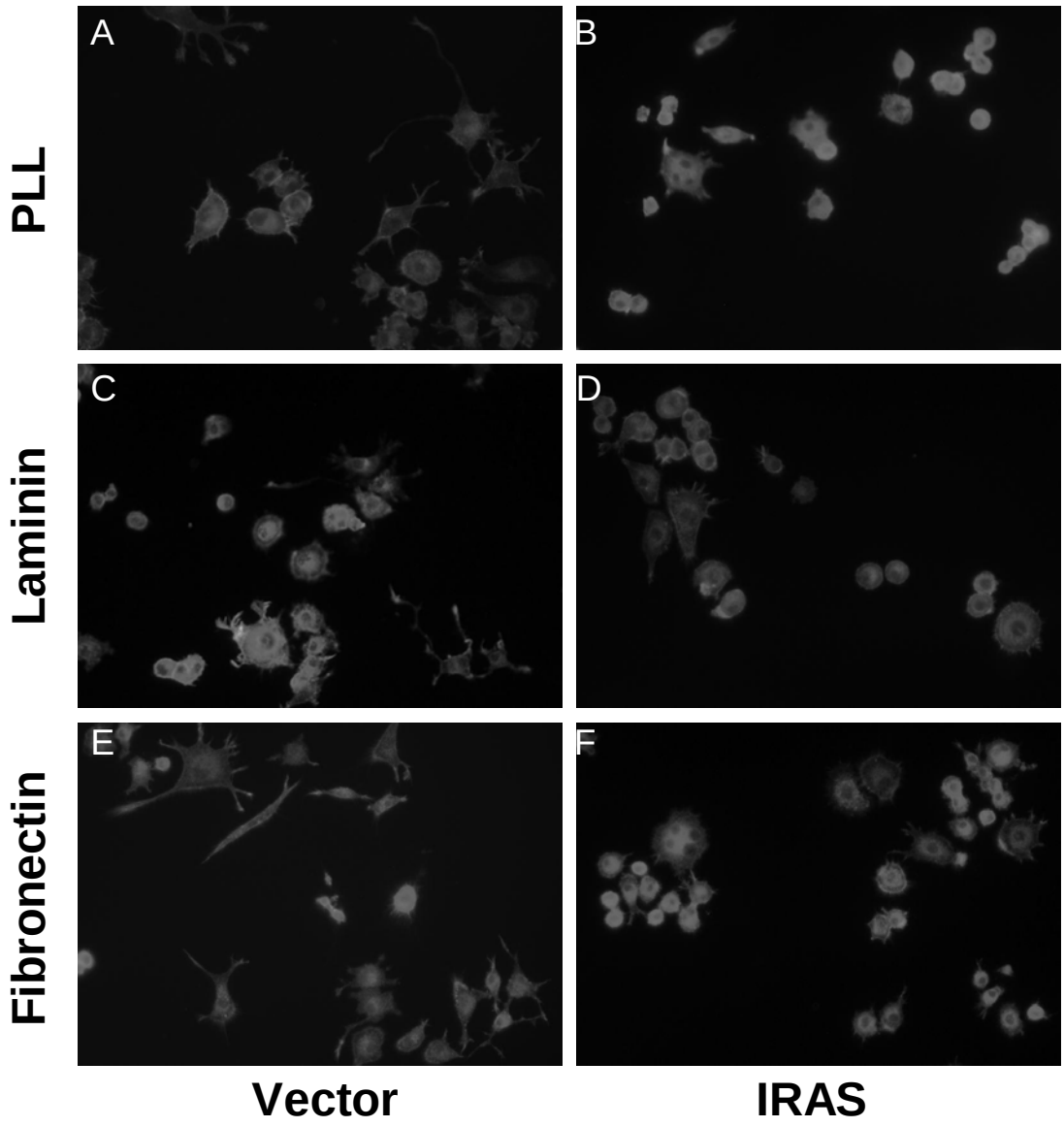


Figure 4.3. Effect of IRAS on NGF-induced neurite outgrowth in serum-starved conditions.

Representative vector-transfected cells grown on PLL (A), laminin (C) and fibronectin (E). Representative IRAS-transfected cells grown on PLL (B), laminin (D) and fibronectin (F).

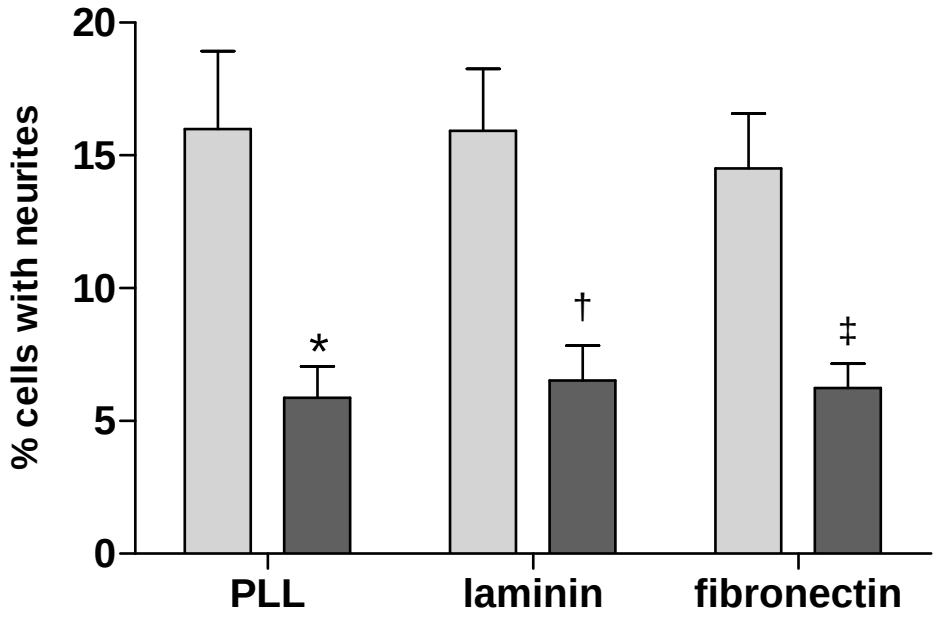


Figure 4.4. Percent of vector- and IRAS-transfected cells expressing neurites in serum-starved conditions.

Vector- (□) and IRAS- (■) transfected cells were scored as neurite positive if containing one or more protrusions with length > the cell diameter. *****, $p < 0.05$ compared with vector-transfected cells grown on PLL (n=4, one way ANOVA, Bonferroni). †, $p < 0.05$ compared with vector-transfected cells grown on laminin (n=4, one way ANOVA, Bonferroni). ‡, $p < 0.05$ compared with vector-transfected cells grown on fibronectin (n=4, one way ANOVA, Bonferroni).

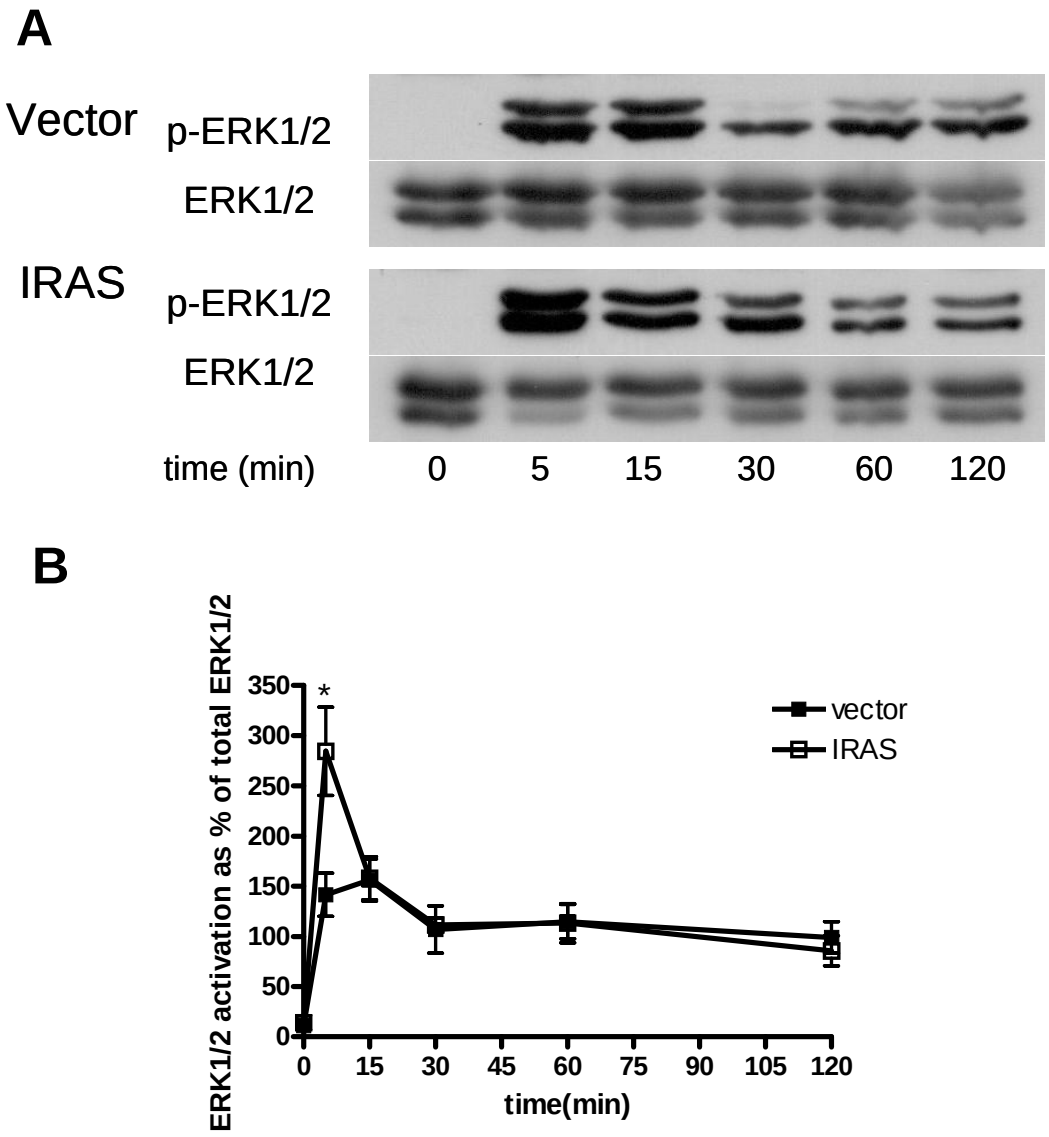


Figure 4.5. Effect of IRAS on NGF-induced ERK1/2 activation under full-serum conditions.

A. Representative Western blot of NGF-induced phospho-ERK1/2 and total-ERK1/2 in vector-and IRAS-transfected cells.

B. Densitometry of NGF-induced phospho-ERK1/2 for vector- and IRAS-transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM. *****, $p < 0.05$ compared with vector-transfected cells after 5 min NGF exposure (n=5, unpaired student-t-test).

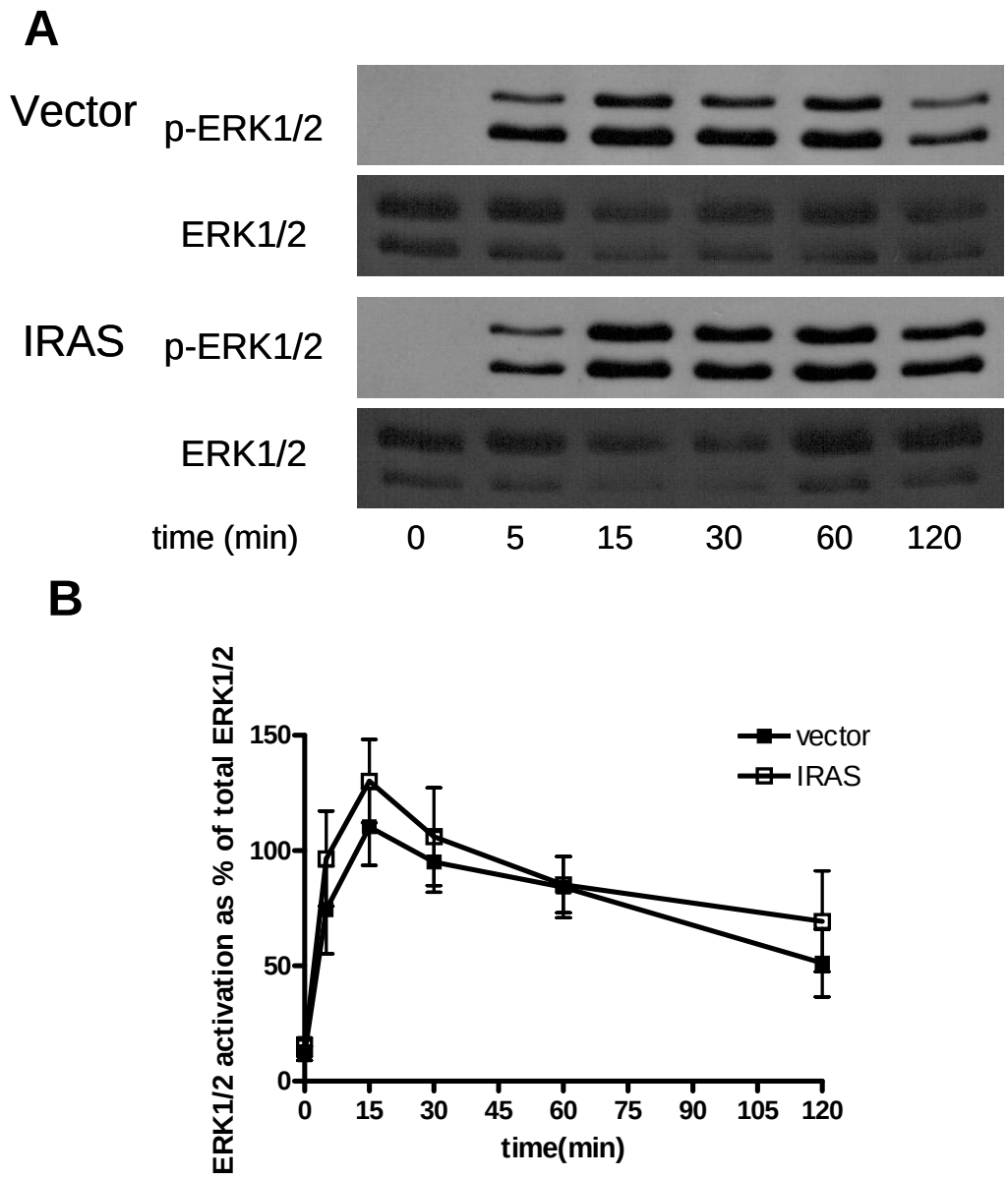


Figure 4.6. Effect of IRAS on NGF-induced ERK1/2 activation under serum-starved conditions.

A. Representative Western blot of NGF-induced phospho-ERK1/2 and total-ERK1/2 in vector-and IRAS-transfected cells.

B. Densitometry of NGF-induced phospho-ERK1/2 for vector- and IRAS-transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM (n=4).

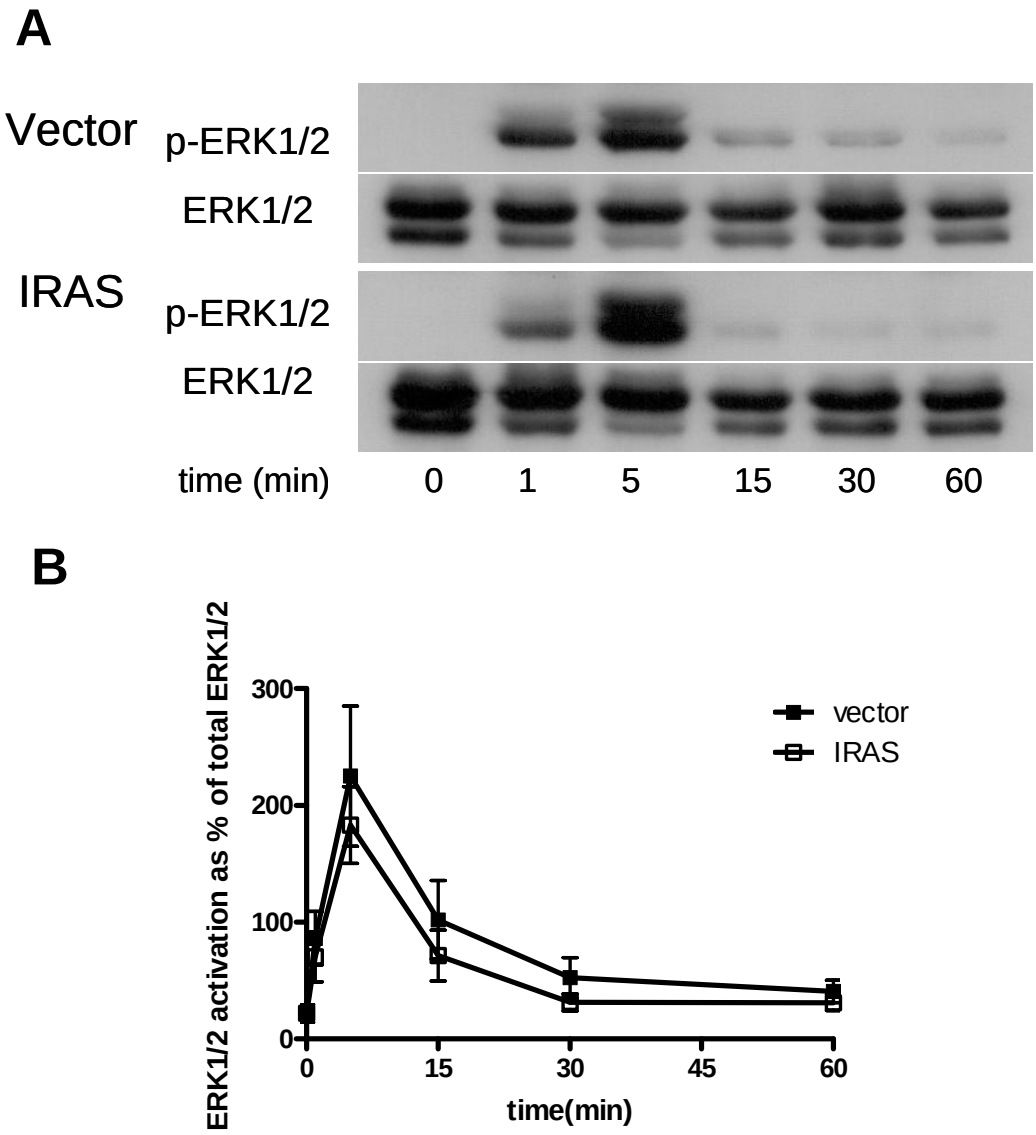


Figure 4.7. Effect of IRAS on EGF-induced ERK1/2 activation under full-serum conditions.

A. Representative Western blot of EGF-induced phospho-ERK1/2 and total-ERK1/2 in vector-and IRAS-transfected cells.

B. Densitometry of EGF-induced phospho-ERK1/2 for vector- and IRAS-transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 ± SEM (n=5).

4.4 Discussion

4.4.1 Neurite outgrowth

This study shows that IRAS differentially enhances or inhibits NGF-induced neurite outgrowth depending on the growth conditions. That is, IRAS enhances neurite outgrowth when cells are kept in full-serum media, whereas IRAS inhibits neurite outgrowth when the cells are serum-starved before NGF addition.

Under full-serum conditions, IRAS enhanced neurite outgrowth. This extends the finding in the previous chapter that IRAS enhances growth cone development in naïve PC12 cells. The experiments described in the previous chapter showed that overexpression of IRAS alone did not provide a great enough stimulus to induce differentiation. The results in the present chapter show that IRAS enhances the actions of NGF to develop these growth cones into neurites.

One possible mechanism by which IRAS could enhance neurite outgrowth is to facilitate the interaction of the NGF receptor, TrkA, with integrin receptors. Integrins have previously been shown to interact with growth factor receptors. Integrins can stimulate phosphorylation of the EGF receptor leading to activation of Src and ERK1/2 (Cobb *et al.*, 1994; Miyamoto *et al.*, 1996; Moro *et al.*, 1998). Furthermore, laminin and collagen were shown to induce neurite outgrowth in PC12 cells co-stimulated with EGF as well as enhance NGF-induced neurite outgrowth (Ivankovic-Dikic *et al.*, 2000). However, the results of the present study show almost no difference in the number of cells expressing neurites on laminin and fibronectin compared to PLL. This suggests that the effect of IRAS on neurite outgrowth is not due to its integrin binding properties but rather, is through an interaction with NGF.

Surprisingly, when the cells were serum-starved for 4 hours before NGF addition, IRAS inhibited neurite outgrowth. In studies observing the effects of NGF on neurite outgrowth in PC12 cells, the cells are often subject to a period of serum starvation before being exposed to NGF (for examples, see Piiper *et al.*, 2002; Sharma *et al.*, 2002; Watanabe *et al.*, 2004). This is done to induce growth arrest, which enhances the differentiating properties of NGF. This effect is seen in the vector-transfected cells. In full-serum conditions, NGF caused almost no differentiation of the vector-transfected cells over the 96 hour time period whereas 16% of cells formed neurites in the serum-starved conditions. This makes the effect of IRAS more surprising given that it inhibits neurite outgrowth in conditions that are more suitable for differentiation.

In other experiments on PC12 cell differentiation, cells may be serum-starved for between 12-24 hours. However, it has been observed in this laboratory and others that a significant number of these cells undergo apoptosis after 24 hours (Dontenwill *et al.*, 2003a). Also, given that IRAS protects PC12 cells against serum withdrawal-induced caspase activation and apoptosis (Dontenwill *et al.*, 2003a), this may affect its actions in regard to differentiation. This study aimed to see the effects of IRAS on differentiation independent of its anti-apoptotic properties. Therefore, 4 hours was chosen as long enough to slow proliferation without inducing apoptosis.

The levels of differentiation seen here are less than is typically reported where NGF often causes greater than 50% of cells to differentiate after 48-72 hours treatment (for examples, see Daniels *et al.*, 1998; Toman *et al.*, 2004; Aoki *et al.*, 2005; Saxena *et al.*, 2005). This is most likely due to clonal variation within PC12 cells. Originally, PC12 cells took 14 days of NGF treatment to achieve 80% differentiation, which was not practical in the present study (Greene *et al.*, 1976).

The cells used in the present research clearly do not differentiate as readily as other PC12 cells. Nevertheless, these results clearly show dramatic differences in the levels of neurite outgrowth between the vector- and IRAS-transfected cells.

4.4.2 Effect of IRAS on NGF signalling

This study demonstrates that in full-serum conditions, IRAS enhances NGF-induced ERK1/2 activation whereas in serum-starved conditions, this effect is abolished. These results suggest that the observed changes in neurite outgrowth by IRAS are, in part, due to its effect on ERK1/2 activation. In full-serum conditions, IRAS enhanced ERK1/2 activation. This was expected given that ERK1/2 activation is necessary for the differentiation of PC12 cells (Cowley *et al.*, 1994). Interestingly, IRAS only enhanced short-term ERK1/2 activation without affecting long-term ERK1/2 signalling (either increasing or decreasing). This is surprising as it is enhanced long-term ERK1/2 activation that is normally associated with NGF-induced differentiation. A previous study on PC12 cell migration found that EGF and NGF promoted migration when short-term ERK1/2 activation was above a certain threshold (Ho *et al.*, 2001). Similarly, there may be a short-term ERK1/2 activation threshold that is only reached in the IRAS-transfected cells, which, when combined with the long-term ERK1/2 activation, triggers the development of neurites.

When the cells were serum-starved, IRAS-transfected cells had similar levels of ERK1/2 activation compared to the vector-transfected cells. In comparison, Piletz *et al.* (2003) observed a decrease in NGF-induced ERK1/2 activation in IRAS-transfected cells after serum-starvation. In these experiments, cells were serum-starved for 12 hours before NGF addition. This suggests that the length of serum starvation is important in determining the effect of IRAS. Combined, these results suggest that while the effects of IRAS on ERK1/2 activation are important for

determining neurite outgrowth, there must be other factors involved. Firstly, in the vector-transfected cells, there was a similar pattern of ERK1/2 signalling in both full-serum and serum-starved conditions with peak ERK1/2 activation occurring after 15 minutes. Therefore, the relative levels of phospho-ERK1/2 in vector-transfected cells remain largely unchanged over this time period. However, serum starvation produced significantly more neurite outgrowth compared to the full-serum conditions in the vector-transfected cells. Secondly, while the enhanced ERK1/2 activation in IRAS-transfected cells mirrored the enhanced neurite outgrowth in full-serum conditions, the decreased neurite outgrowth under serum-starved conditions was not accompanied by a decrease in ERK1/2 activation compared to the vector-transfected cells. This implies that IRAS is affecting other cell signalling pathways necessary for neurite outgrowth.

One such pathway, which the IRAS homologue, nischarin, was shown to interact with, is the Rac/PAK signalling pathway. NGF activates this pathway to regulate the actin cytoskeleton and induce neurite outgrowth. Multiple studies have shown that nischarin inhibits Rac/PAK signalling, albeit in COS-7 and NIH3T3 cells rather than NGF treated PC12 cells (Alahari *et al.*, 2000; Alahari *et al.*, 2004b; Reddig *et al.*, 2005). Significantly, in each of these experiments, the cells were subject to a period of serum starvation of typically 12 to 24 hours before observations were made. Furthermore, under these conditions, nischarin inhibited migration (Alahari *et al.*, 2000; Alahari *et al.*, 2004b). The processes underlying migration are similar to those for neurite outgrowth. For example, both processes involve activation of Rac and formation of lamellipodia and filopodia. Therefore, these studies on nischarin support the finding in the present study that under serum starved conditions, IRAS inhibits neurite outgrowth. It would be interesting to see

whether the effects of IRAS on cell migration are reversed in full serum conditions. Likewise, the effects of IRAS on NGF-induced Rac/PAK signalling should be considered for future study.

There are a number of mechanisms by which the removal of serum could change the effect of IRAS. IRAS may interact with a component in serum which changes its behaviour. As discussed in the previous chapter, IRAS may interact with trace amounts of NGF in serum which causes growth cone development in naïve PC12 cells. This seems unlikely, however, as while this could explain the increased neurite outgrowth in the full-serum cells, it does not account for the decrease in neurite outgrowth in the serum-starved cells. Alternatively, IRAS may interact with the LPs, LPA and S1P, which are also found in serum. LP receptors have been implicated in clonidine action and with TrkA signalling (Molderings *et al.*, 2007a; Toman *et al.*, 2004). Therefore, the absence of these components in serum-starved cells may affect the nature of the interaction of IRAS with LP receptors and subsequently, TrkA.

Another possibility is that serum starvation changes the behaviour of IRAS. As described above, depriving cells of serum halts cell growth. This occurs partly through regulation of proteins that control the cell cycle. Nischarin inhibits Rac-induced activation of cyclin D1 and nuclear-factor κB (Reddig *et al.*, 2005). Cyclin D1 is a regulator of the cell cycle which arrests cells in the G1 phase of the cell cycle (Schwartz *et al.*, 2005). Cyclin D1 is upregulated by NGF in PC12 cells undergoing differentiation (Yan *et al.*, 1995). However, cyclin D1 is also activated during the apoptosis of neuronal N1E-115 cells (Kranenburg *et al.*, 1996). Moreover, overexpression of cyclin D1 enhances cells undergoing apoptosis by serum-withdrawal (Han *et al.*, 1996; Kranenburg *et al.*, 1996). Therefore, under serum-

starved conditions, IRAS may inhibit common signalling pathways of apoptosis and differentiation, such as cyclin D1. Serum withdrawal may also change the distribution of IRAS within cells. IRAS resides in the plasma membrane, endosomal membranes and the cytoplasm. In fact, nischarin was suggested to decrease Rac signalling by inhibiting its localisation to the cell membrane which is necessary for its activation (Reddig *et al.*, 2005). While nischarin lacks a PX domain, this result show that in principle IRAS could inhibit the membrane localisation of membrane receptors, such as TrkA, in serum-starved conditions if its cellular distribution alters.

These results show that IRAS does not affect EGF-induced ERK1/2 signalling in PC12 cells. This confirms a similar finding in HEK cells (Sano *et al.*, 2002). The EGF receptor is a growth factor receptor and so is structurally similar to both TrkA and the insulin receptor. Like the insulin receptor and TrkA, the EGF receptor activates ERK1/2 through a Ras-Raf pathway. This indicates that the effect of IRAS is not a general one for growth factor receptors or a general, direct effect on ERK1/2.

There are several possible mechanisms by which IRAS is enhancing ERK1/2 activation. IRAS may form a complex with TrkA and enhance its signalling in a similar manner to which it enhances insulin receptor signalling. Alternatively, IRAS may enhance ERK1/2 activation through a separate signalling pathway from TrkA but which is nevertheless dependent on TrkA for activation. For example, IRAS could also enhance the signalling of LP receptors which are transactivated by TrkA (Toman *et al.*, 2004). TrkA could also activate IRAS, which in turn activates the I_1-R signalling pathway to enhance ERK1/2 activation. These possibilities are examined in the following chapters.

4.5 Conclusion

This study showed that IRAS differentially enhances or inhibits NGF-induced PC12 cell differentiation depending on the presence or absence of serum in the media. This is, in part, due to an effect on ERK1/2 activation. In full-serum conditions, IRAS enhanced neurite outgrowth and this was accompanied by an increase in ERK1/2 activation. In serum-starved cells, IRAS inhibited neurite outgrowth, with similar levels of ERK1/2 activation observed in vector- and IRAS-transfected cells. Therefore, IRAS affects processes underlying neuronal differentiation and is dependent on components of serum for its activity.

5 Chapter 5: I_1-R and PI3-kinase signalling in IRAS modulation of NGF-induced ERK1/2 activation

5.1 Introduction

The results in the previous chapter showed that IRAS enhances NGF-induced ERK1/2 activation but only when the cells are grown in full-serum media. Experiments described in this chapter will focus on the potential mechanisms by which IRAS enhances ERK1/2 activation under these conditions.

As discussed in the previous chapter, one potential mechanism by which IRAS may enhance ERK1/2 activation is that it activates a separate signalling pathway from NGF-TrkA but is still dependent on NGF for its activation. This is similar to a model that has already been established between NGF and I_1-R ligands. In these studies, I_1-R agonists clonidine, moxonidine and rilmenidine enhanced ERK1/2 activation in PC12 cells but only when the cells had been pre-treated with NGF (Edwards *et al.*, 2001; Zhang *et al.*, 2001). It is unclear why this pre-treatment with NGF enhances the effects of I_1-R agonists. One possible explanation is that NGF enhances the expression and/or transactivates the I_1-R. This could explain why enhanced ERK1/2 activation is achieved through either treatment with I_1-R agonists as described above or through overexpression of IRAS as demonstrated in the previous chapter.

Interestingly, the I_1-R ligand-induced increase in ERK1/2 activation was dependent on activation of PC-PLC (Edwards *et al.*, 2001; Zhang *et al.*, 2001). PC-PLC is related to other phospholipases PI-PLC and PLD, neither of which are activated by I_1-R agonists (Regunathan *et al.*, 1994; Separovic *et al.*, 1996). PC-PLC specifically hydrolyses phosphatidylcholine to produce DAG. DAG activates PKC

which activates the MAP kinase pathway (Edwards *et al.*, 2001). Therefore, the present study aimed to see whether IRAS enhances ERK1/2 activation through a PC-PLC-dependent mechanism and thus, activates an established I_1-R signalling pathway.

Analysis of the structure of IRAS provides clues as to how IRAS may enhance ERK1/2 activation. IRAS contains a proline rich domain, a serine rich domain and an acidic residue. These regions have been found in the cytokine receptor interleukin2β (IL2β) where they are sites for interaction with non-receptor PTKs (Miyazaki *et al.*, 1996). PTKs such as c-Src are involved in a wide variety of signalling pathways and have been implicated in the activation of PC-PLC and downstream activation of PKC (Rao *et al.*, 1994). Therefore, in the model described above, NGF-activated IRAS may in turn activate ERK1/2 through a Src-PC-PLC-dependent mechanism.

Alternatively, rather than activating signalling pathways itself, IRAS may enhance ERK1/2 activation by enhancing the activity or regulating the trafficking of TrkA. IRAS contains a PX domain near its N-terminus. PX domains are predominantly found in the SNX family of proteins. SNXs associate with receptors and regulate their trafficking by localising to PIs found in the plasma membrane, endosomes and other vesicular compartments such as lysosomes and the Golgi apparatus (Carlton *et al.*, 2005; Ellson *et al.*, 2002; Sato *et al.*, 2001; Seet *et al.*, 2006). IRAS was found to associate with the small/recycling subtype of endosomes through an interaction with PI3P (Lim *et al.*, 2004). Furthermore, treatment of IRAS-transfected A4341 cells with the PI3-kinase inhibitor, wortmannin, disrupted the localisation of IRAS to endosomes (Lim *et al.*, 2004). This suggests that inhibition of PI3-kinase may disrupt IRAS signalling. A previous study found that IRAS bound

directly to insulin receptor substrates and formed a complex with the insulin receptor, PI3-kinase and Grb-2 (Sano *et al.*, 2002). Overexpression of IRAS in HEK293 cells also led to a 4-fold increase in insulin-induced ERK1/2 activation, however it was not investigated whether this was due to trafficking of the insulin receptor.

The aim of this chapter was to investigate the mechanisms by which IRAS enhances NGF-induced ERK1/2 activation. Specifically, it aimed to determine whether IRAS enhances ERK1/2 activation through a PC-PLC and Src dependent pathway or through a PI3-kinase dependent pathway and potential interaction with TrkA.

5.2 Methods

5.2.1 ERK1/2 activation

IRAS- and vector-transfected PC12 cells were plated onto PLL (10 μg/ml) coated 6-well plates and grown for 24 hours. The cells were then treated with NGF (100 ng/ml) for 5 or 60 minutes. Cells were pre-treated with AGN192403 (10 μM), D609 (10 μM) or wortmannin (100 ng/ml) for 10 minutes prior to NGF addition. Following treatment, the cells were lysed and protein concentration of the lysates determined. The cell lysates were subject to SDS-PAGE and immunodetection for phospho-ERK1/2 and total ERK1/2 as described in the general methods.

5.2.2 Src activation

IRAS- and vector-transfected PC12 cells were plated onto PLL (10 μg/ml) coated 6-well plates and grown for 24 hours. The cells were then treated with NGF (100 ng/ml) for between 1 and 60 minutes. Following treatment, the cells were lysed and protein concentration of the lysates determined. The cell lysates were subject to SDS-PAGE and immunodetection for phospho-Src(Tyr57) and total ERK1/2 as described in the general methods.

5.2.3 Statistics

Student t-tests were performed for comparisons of two groups.

5.3 Results

5.3.1 The effect of AGN192403, D609 and wortmannin on NGF-induced ERK1/2 activation

The effect of the I_1-R antagonist AGN192403, the PC-PLC inhibitor D609 and the PI-3 kinase inhibitor wortmannin on NGF-induced ERK1/2 activation in vector- and IRAS-transfected cells is shown in figures 5.1, 5.2 and 5.3. In these experiments, the cells were stimulated for either 5 or 60 minutes with NGF to observe the effects of these inhibitors on both short- and long-term ERK1/2 activation. For both vector- and IRAS-transfected cells, there were undetectable levels of phospho-ERK1/2 in untreated cells as observed in the previous chapter. Furthermore, there was consistently enhanced ERK1/2 activation in the IRAS-transfected cells compared to the vector-transfected cells after 5 minutes NGF stimulation but similar phospho-ERK1/2 levels after 60 minutes NGF stimulation. Neither AGN, nor D609 affected ERK1/2 activation in either the vector- or IRAS-transfected cells at either of the timepoints. Wortmannin significantly decreased ERK1/2 activation in both vector- and IRAS-transfected cells after 5 minutes NGF stimulation by 32% and 26% respectively. In vector-transfected cells, wortmannin also caused a significant 30% decrease in ERK1/2 activation after 60 minutes of NGF stimulation. This did not occur in the IRAS-transfected cells.

5.3.2 The effect of IRAS on NGF-induced Src activation

The effect of NGF on Src activation in IRAS- and vector-transfected cells under full-serum conditions was observed over a 120 minute timecourse (figure 5.4). Src is inactive when phosphorylated at Tyr57. Therefore, in these experiments, lower levels of phospho-Src indicates Src activity. In both unstimulated vector- and IRAS-

transfected cells, there were high levels of phospho-Src. NGF caused a small increase in Src activation after 5 minutes treatment in both the vector- and IRAS-transfected cells. This was followed by a steady decline in Src activation over the timecourse. There were no significant differences between the vector- and IRAS-transfected cells at any of the timepoints.

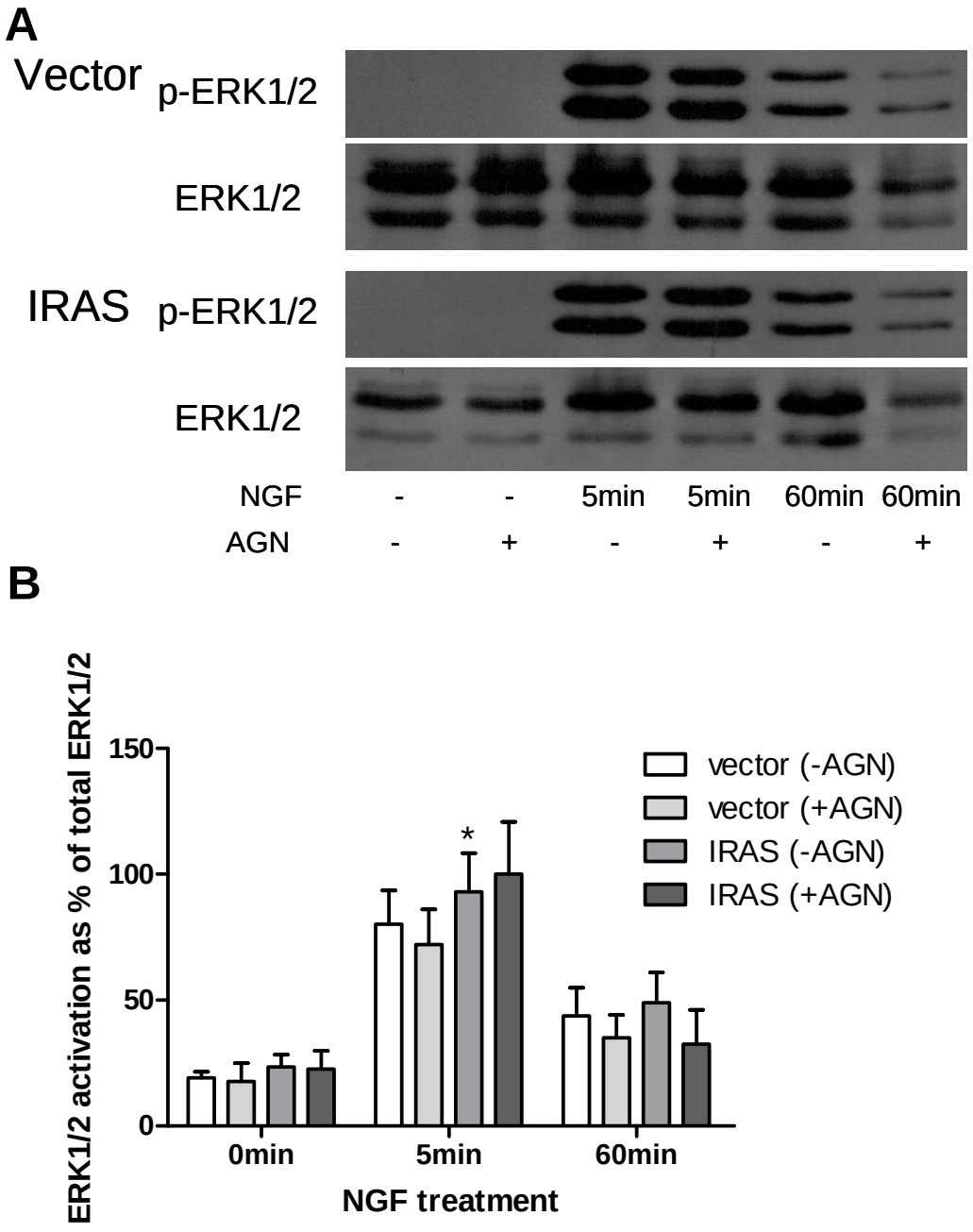


Figure 5.1. Effect of AGN192403 on NGF-induced ERK1/2 activation.

A. Representative Western blot of NGF-induced phospho-ERK1/2 and total-ERK1/2 in vector- and IRAS-transfected cells.

B. Densitometry of NGF-induced phospho-ERK1/2 for vector- and IRAS-transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM. *****, $p < 0.05$ compared with vector-transfected cells after 5 min NGF exposure (n=3, paired student-t-test).

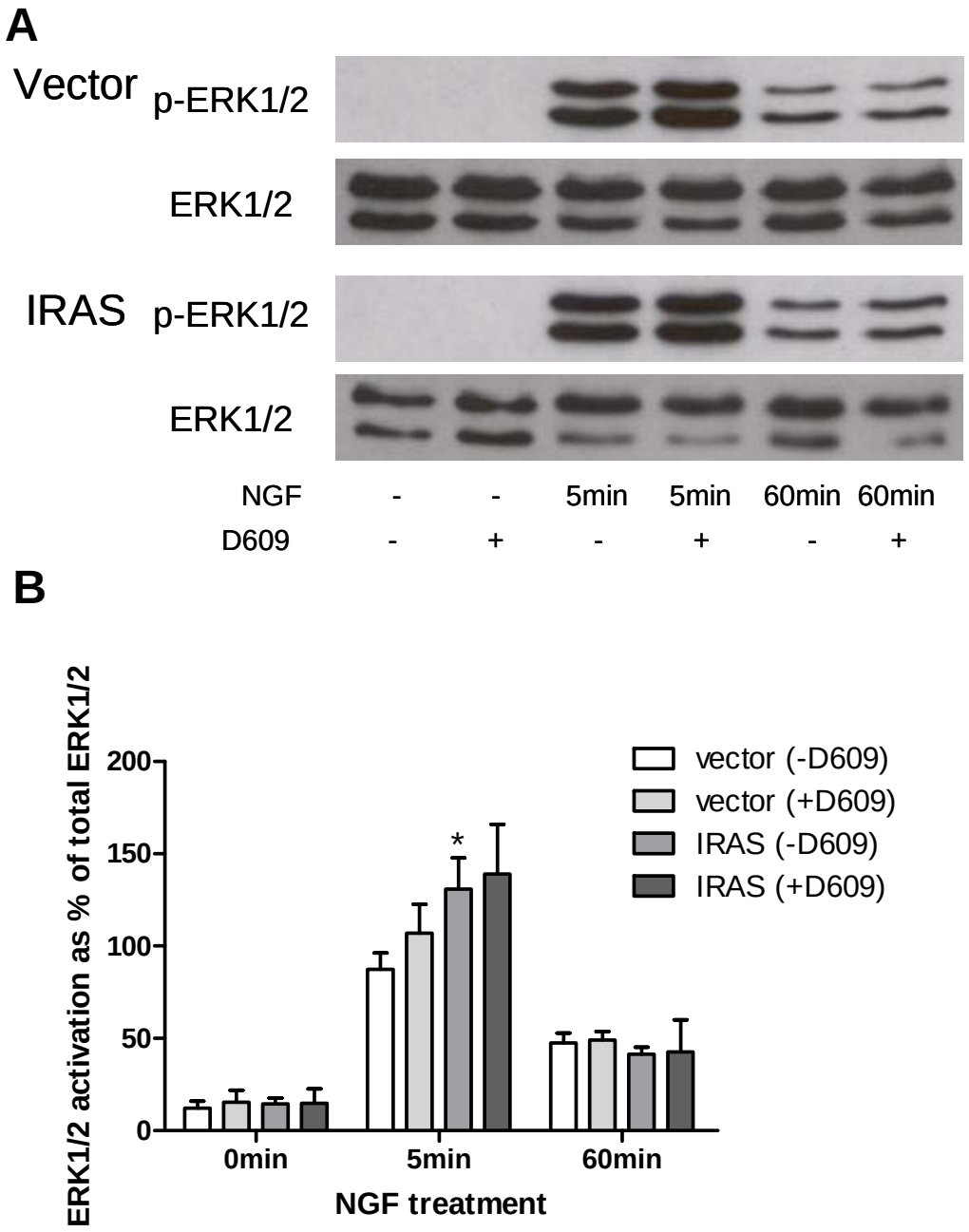


Figure 5.2. Effect of D609 on NGF-induced ERK1/2 activation.

A. Representative Western blot of NGF-induced phospho-ERK1/2 and total-ERK1/2 in vector- and IRAS-transfected cells.

B. Densitometry of NGF-induced phospho-ERK1/2 for vector- and IRAS-transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM. *****, $p < 0.05$ compared with vector-transfected cells after 5 min NGF exposure (n=3, paired student-t-test).

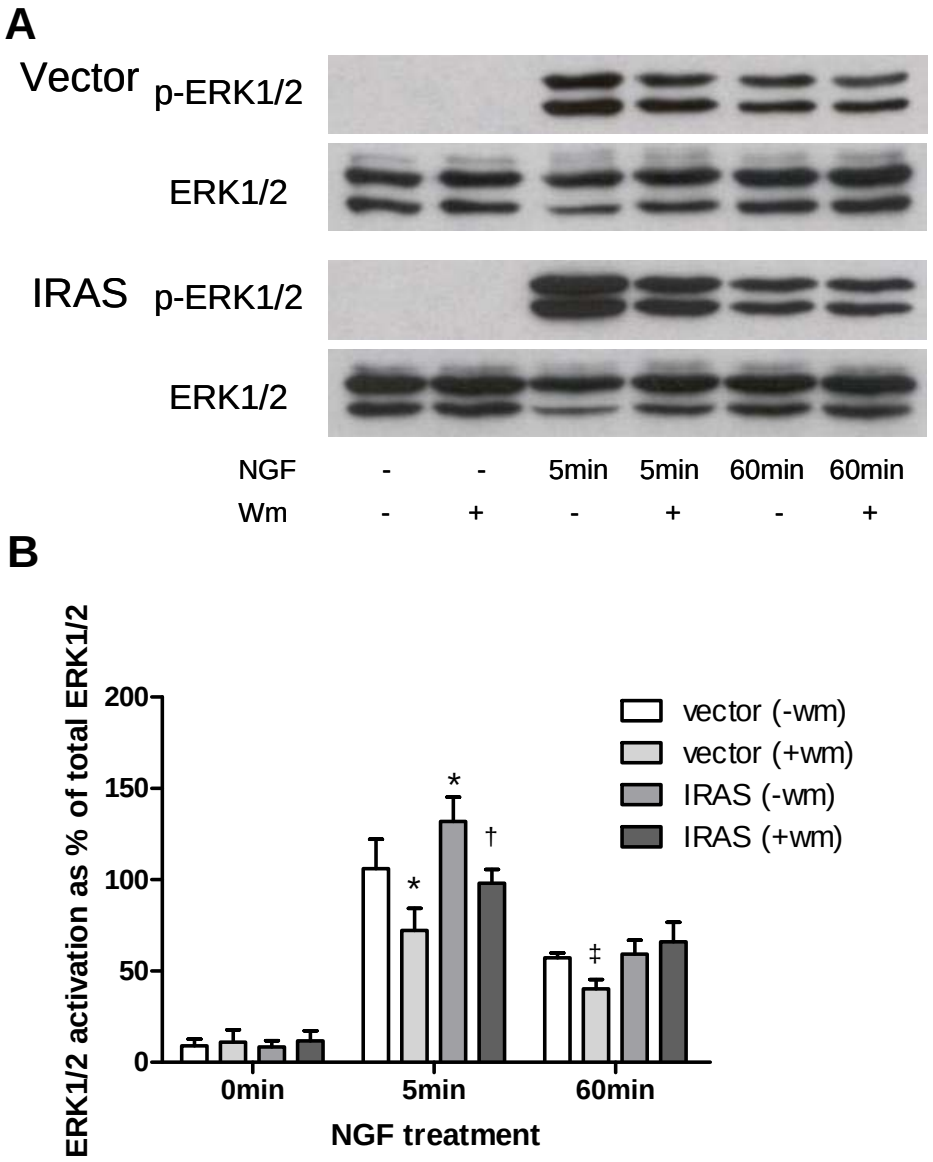


Figure 5.3. Effect of wortmannin on NGF-induced ERK1/2 activation.

A. Representative Western blot of NGF-induced phospho-ERK1/2 and total-ERK1/2 in vector- and IRAS-transfected cells.

B. Densitometry of NGF-induced phospho-ERK1/2 for vector- and IRAS-transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM. *****, $p < 0.05$ compared with vector-transfected cells after 5 min NGF exposure (n=5, paired student-t-test). †, $p < 0.05$ compared with IRAS-transfected cells after 5 min NGF exposure (n=5, paired student-t-test). ‡, $p < 0.05$ compared with vector-transfected cells after 60 min NGF exposure (n=5, paired student-t-test).

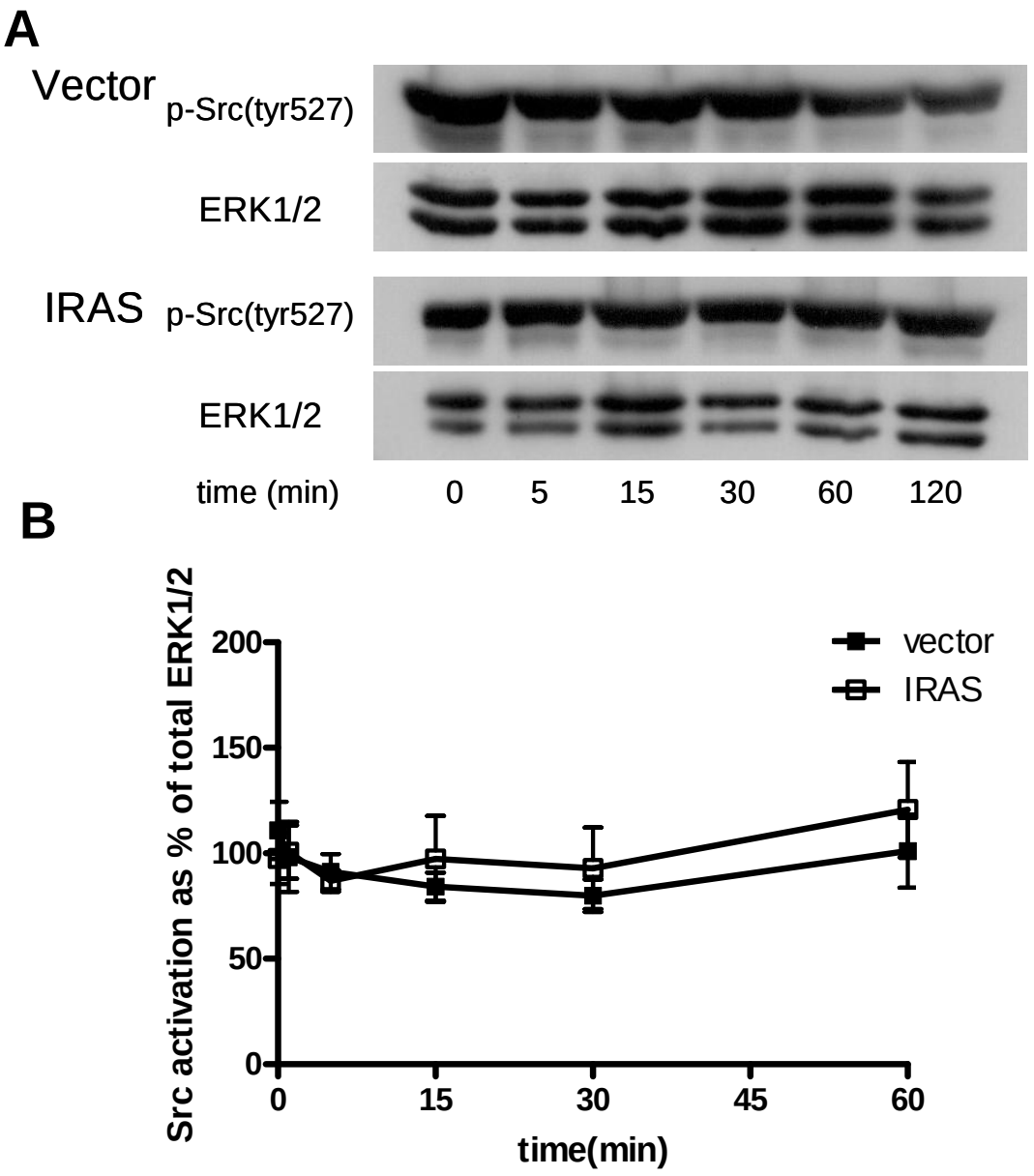


Figure 5.4. Effect of IRAS on Src activation.

A. Representative Western blot of phospho-Src(Tyr57) and total-ERK1/2 in NGF-treated vector- and IRAS-transfected cells.

B. Densitometry of phospho-Src(Tyr57) for NGF-treated vector- and IRAS-transfected cells. Values are expressed as mean of phospho-Src(Tyr57) relative to total-ERK1/2 $\pm$ SEM (n=3).

5.4 Discussion

This study aimed to investigate the mechanisms by which IRAS enhances NGF-induced ERK1/2 activation in full-serum conditions. The first hypothesis tested was that NGF transactivates IRAS which then activates the established I_1-R pathway (i.e. a PC-PLC dependent pathway) to enhance ERK1/2 activation. In previous research, treatment with the PC-PLC inhibitor, D609, was shown to inhibit I_1-R agonist-induced increases in DAG and ERK1/2, which implies that the I_1-R activates PC-PLC (Separovic *et al.*, 1996; Separovic *et al.*, 1997; Edwards *et al.*, 2001; Zhang *et al.*, 2001). Similarly, in the present study, the vector- and IRAS-transfected cells were treated with D609 to determine whether inhibiting PC-PLC would prevent the increased ERK1/2 activation in the IRAS-transfected cells. After 5 minutes NGF-stimulation, similar levels of phospho-ERK1/2 were observed in cells treated with D609 compared to untreated cells. Therefore, activation of PC-PLC is not responsible for the increased ERK1/2 activation observed after 5 minutes in IRAS-transfected cells. Similarly, D609 did not affect ERK1/2 levels after 60 minutes in either the IRAS- or vector-transfected cells.

To determine further if IRAS activates ERK1/2 via an I_1-R signalling pathway, the cells were treated with AGN to see if an I_1-R antagonist could prevent enhanced ERK1/2 activation in the IRAS-transfected cells. The results indicate that AGN, like D609, does not inhibit ERK1/2 activation in either IRAS- or vector-transfected cells. This is in contrast to AGN's effects on growth cone development where it reduced growth cone development in IRAS-transfected cells as discussed in chapter 3. Again, this finding implies that the IRAS-induced increase in ERK1/2 activation is not exclusively responsible for the increase in neurite outgrowth. For example, AGN may interfere with the association of IRAS with Rac/PAK signalling

pathways. A limitation of this study is that AGN was not compared to another I_1-R antagonist. This is addressed in the following chapter where it is found that efaroxan does not affect NGF-induced ERK1/2 activation in either vector- or IRAS-transfected cells.

In previous research on I_1-R signal transduction, Edwards *et al.* (2001) observed that moxonidine caused maximum ERK1/2 activation after 90 minutes. In this study, the PC12 cells were first treated with 50 ng/ml NGF over 48 hours to differentiate the cells, followed by treatment with 10 ng/ml NGF over 30 minutes before moxonidine treatment. Similarly, Zhang *et al.* (2001) treated PC12 cells with 50 ng/ml NGF over a 48 hour period, followed by 10 ng/ml NGF for 60 minutes. Under these conditions, rilmenidine caused maximum ERK1/2 activation after 15 minutes. In both these studies, the cells were treated with high concentrations of NGF over a long time period after which the ERK1/2 signal would not be maintained (Piletz *et al.*, 2003). This was followed by pre-treatment with low concentrations of NGF. It has been suggested that differentiated PC12 cells express more I_1-binding sites, and that pre-treating the cells with small doses of NGF upregulates I_1-R expression (Ernsberger, 1999). Treatment with low doses of NGF should, in itself, induce low levels of ERK1/2 phosphorylation. Thus, in the above studies, it would be easier to measure smaller changes in ERK1/2 activation induced by I_1-R agonists. In contrast, in the present study, the cells were administered with a single, high concentration of NGF, which produced high levels of ERK1/2 phosphorylation. If IRAS were to activate a signal transduction pathway to activate ERK1/2, this activation may be relatively weak compared to that produced by high concentrations of NGF and so undetectable in this system.

Also, in the present study, no I_1-R agonist was used. The use of an I_1-R agonist was deemed unnecessary as overexpression of IRAS itself is enough to induce changes in cell morphology, suggesting that IRAS is active in these cells. Furthermore, the I_1-R agonist, rilmenidine, did not induce further growth cone development in IRAS-transfected cells. Moreover, AGN decreased growth cone development in IRAS-transfected cells, suggesting that AGN alone can reduce the effects of IRAS. Surprisingly, in a study by Edwards *et al.* (2003), moxonidine (100 nM) caused a decease in ERK1/2 activation when co-administered with NGF (50 ng/ml) after 90 minutes in untransfected PC12 cell. This was due to increased expression of the ERK1/2 deactivating enzyme, MAP kinase phosphatase-2 (Edwards *et al.*, 2003). It would be interesting to see what effect co-administration of an I_1-R agonist would have on short-term ERK1/2 activation in IRAS-transfected cells.

In the present study, timepoints were taken at 5 and 60 minutes to observe ERK1/2 activation in both the short- and long-term. The 5-minute timepoint was taken specifically to see whether IRAS activation of PC-PLC was responsible for the increased ERK1/2 activation observed at this timepoint in IRAS-transfected cells. The 60-minute timepoint was taken to determine whether IRAS activated PC-PLC over a longer timeframe. This is especially important as production of DAG mediated by PC-PLC often occurs over a longer timeframe compared to PI-PLC (Exton, 1990). It was previously observed that moxonidine sustained DAG production beyond 10 minutes, which led to strongest ERK1/2 activation after 90 minutes (Edwards *et al.*, 2001; Separovic *et al.*, 1996). Therefore, IRAS activation of ERK1/2 through PC-PLC may occur over a longer timeframe. Also, IRAS may activate PC-PLC without any additional ERK1/2 activation via PC-PLC. One

limitation of the present study is that phosphocholine levels were not measured. Phosphocholine is produced by the breakdown of phosphatidylcholine by PC-PLC and so is a more direct measure and better indicator of PC-PLC activity. Regardless of whether IRAS activates PC-PLC in the present system, PC-PLC is not responsible for the short-term increase in ERK1/2 activation in IRAS-transfected cells as indicated by the lack of effect of D609.

The present study also sought to determine whether IRAS activates signal transduction pathways involving Src. The results show small levels of Src activation in both vector- and IRAS transfected cells, with no differences between the two groups. This Src activation is probably due to direct activation by NGF. NGF has been shown to activate Src directly, leading to activation of PKC (Wooten *et al.*, 2001). Similarly to PC-PLC, if IRAS were to activate Src directly, this contribution would be relatively small and unlikely to affect neurite outgrowth. One possibility, not tested here, is that IRAS activates other PTKs in the Src family. The proline rich domain, serine rich domain and acidic residue regions in IRAS are also found in the cytokine receptor, IL2β, where they interact with Lck, Syk and Jak3. Future studies could determine whether IRAS interacts with these or other PTKs.

This study began to investigate the hypothesis that rather than activating separate cell signalling pathways, IRAS enhances NGF-induced ERK1/2 activation by enhancing TrkA signalling. To this end, the cells were treated with the PI3-kinase inhibitor, wortmannin, to see whether disrupting the endosomal association of IRAS would inhibit ERK1/2 activation in the IRAS-transfected cells. Lim and Hong (2004) found that IRAS associates with small/recycling endosomes by binding to PI3P with its PX domain. PI3P production is regulated by PI3-kinase. Treatment with wortmannin inhibits PI3P production and consequently was found to inhibit the

association of IRAS with endosomes. The present study proposed that such a process could also prevent the association of IRAS with TrkA and thus, lead to a knockdown in ERK1/2 activation. However, the results showed a similar level of ERK1/2 inhibition in both the IRAS- and vector-transfected cells after 5 minutes of NGF stimulation. Surprisingly, after 60 minutes NGF stimulation, wortmannin inhibited ERK1/2 activation in the vector-transfected cells but not the IRAS-transfected cells.

While the PI3-kinase and ERK1/2 pathways are traditionally presented as separate TrkA signalling pathways, there is clearly crosstalk, given that PI3-kinase inhibition also leads to ERK1/2 inhibition in the vector-transfected cells. York *et al.* (2000) have previously studied the mechanisms by which this occurs in PC12 cells. They found that PI3-kinase inhibitors reduce ERK1/2 activation after 5 and 40 minutes stimulation by NGF, similar to the findings in the present study. Furthermore, they found that PI3-kinase inhibition inhibits TrkA internalisation from the plasma membrane to endosomes. Earlier research had found that ERK1/2 activation by NGF after 5 minutes is predominantly Ras-dependent whereas longer term ERK1/2 activation (after 30 minutes) is Rap1-dependent (York *et al.*, 1998). Interestingly, Ras is expressed predominantly at the plasma membrane, whereas Rap1 is predominantly vesicular and requires TrkA internalisation for its activation (York *et al.*, 2000). Thus, wortmannin inhibits ERK1/2 activation after 60 minutes of NGF stimulation in the vector-transfected cells as TrkA is unable to activate Rap1. After 5 minutes NGF stimulation, PI3-kinase inhibitors do not affect activation of the membrane-bound Ras. However, PI3-kinase activation is necessary for the coupling of Ras to the predominantly vesicular B-Raf. Therefore, PI3-kinase inhibition prevents short-term Ras-Raf activation of ERK1/2 by NGF, which was observed in both the vector- and IRAS-transfected cells.

The similar levels of ERK1/2 inhibition after 5 minutes in both vector- and IRAS-transfected cells suggests that binding to PI3P is not critical for IRAS action. Furthermore, while Lim and Hong (2004) showed strong endosomal localisation for IRAS, others have shown that IRAS resides in the cytoplasm as well as in membranes (Piletz *et al.*, 2000; Sano *et al.*, 2002).

The relative contributions of Ras and Rap1 to ERK1/2 activation by NGF suggest a possible mechanism for IRAS action. Transfection of IRAS into 293T cells causes a redistribution of α5β1 integrin from the plasma membrane to a more vesicular localisation (Lim *et al.*, 2004). If IRAS were to have a similar effect on TrkA, this could lead to faster activation of Rap1-ERK1/2 signalling pathways. After 60 minutes of NGF stimulation, overexpressed IRAS may induce trafficking of TrkA in the presence of the concentration of wortmannin tested here. This present study was unable to test whether the effects of wortmannin were concentration-dependent to determine if this is a possibility. Clearly, given the complex nature of PI3-kinase/ERK1/2 crosstalk, it is difficult to determine the function of IRAS in relation to TrkA from these results. A direct test of whether IRAS interacts with TrkA would require immunoprecipitation and immunofluorescence techniques. Appendix 1 outlines an attempt to do this. These techniques could be further employed to determine whether IRAS affects the localisation of TrkA and the relative contributions of Ras and Rap1 to enhance short-term ERK1/2 activation.

5.5 Conclusion

In summary, this study demonstrated that IRAS does not enhance ERK1/2 activation through the established I_1-R pathway in NGF treated PC12 cells. Disruption of the endosomal association of IRAS, through PI3-kinase inhibition, also

did not inhibit the enhanced ERK1/2 activation in the IRAS-transfected cells. However, PI3-kinase inhibition did lead to inhibition of long-term ERK1/2 signalling in the vector-transfected cells whereas this was not observed in the IRAS-transfected cells. Further study is required to determine if IRAS interacts with TrkA and if this affects activation of Ras-ERK1/2 or Rap1-ERK1/2 signalling pathways.

6 Chapter 6: Role of IRAS in NGF and LP receptor agonist interaction

6.1 Introduction

The experiments described in the previous chapter began to look at mechanisms by which IRAS enhances NGF-induced ERK1/2 signalling. These studies found that IRAS does not activate a Src-PC-PLC pathway. Whether IRAS interacts with TrkA remains unresolved. The current chapter investigates a third mechanism; that IRAS enhances ERK1/2 activation by enhancing the signalling of LP receptors.

LP receptors comprise a group of structurally related GPCRs which bind LPs. The two major classes of receptor within this group are the LPA receptors (LPA_{1-4}) and S1P receptors ($S1P_{1-5}$), which preferentially bind LPA and S1P respectively. LP receptors are expressed throughout the body and mediate a wide range of functions (Ishii *et al.*, 2004; Meyer zu Heringdorf *et al.*, 2007). LP receptors are especially important in regulating neurogenesis in the developing nervous system. LPA induces neurite retraction and cell rounding in a number of cell lines including PC12 cells, N1E-115, NG108-15 and immortalised CNS neuroblast cells (Ishii *et al.*, 2000; Jalink *et al.*, 1993; Jalink *et al.*, 1994; Tigyi *et al.*, 1996; Tigyi *et al.*, 1992). In NGF-differentiated PC12 cells, this process occurs through activation of RhoA and enhanced Ca^{2+} signalling (Tigyi *et al.*, 1996). S1P receptors, too, can influence neuronal development with different subtypes of receptors producing contrasting effects on neuronal cell differentiation. For example, overexpression of the $S1P_1$ receptor in PC12 cells promotes NGF-induced neurite outgrowth whereas

overexpression of the $S1P_2$ receptor has the opposite effect (Van Brocklyn *et al.*, 1999).

Intriguingly, both LPA and S1P receptors interact with TrkA. Toman *et al.* (2004) demonstrated that TrkA induces the translocation of SphKs to the plasma membrane leading to increased production of S1P as well as internalisation and activation of the $S1P_1$ receptor. Furthermore, overexpression of $S1P_1$ in PC12 cells enhanced NGF-induced Rac activation and neurite outgrowth whereas neurite outgrowth was inhibited by siRNA to $S1P_1$. Moughal *et al.* (2004) found that treatment of PC12 cells with either LPA or NGF stimulates the translocation of TrkA and LPA_1 to the nucleus and that co-stimulation of PC12 cells with LPA and NGF leads to greater ERK1/2 activation than does stimulation with either alone.

LP receptors have also been implicated in the actions of I_1-R agonists. In fact, it has been proposed that the actions of imidazoline agonists in PC12 cells may be through activation of LP receptors (Molderings *et al.*, 2007a). Both LPA and $S1P_1$ inhibited [^{3}H]clonidine binding in a concentration dependent manner (Molderings *et al.*, 2007a). Moreover, the binding of clonidine was significantly reduced in PC12 cells transfected with siRNA to the $S1P_1$ and $S1P_3$ receptors but not $S1P_2$ or LPA_1 (Molderings *et al.*, 2007a). Taken together, these results suggest that clonidine may act through specific S1P or LPA receptor subtypes in PC12 cells. However, these results must be interpreted cautiously, as the affinity of imidazoline ligands in this study was much lower than previously reported (μM rather than nM).

Interestingly, both LPA and S1P are present in serum. S1P is present in human serum at concentrations of 0.5-0.8 μM (Okajima, 2002), and LPA is present at concentrations between 1-5 μM (Baker *et al.*, 2001). Therefore, the contrasting

effects of serum on the IRAS- and vector-transfected cells may be due to the presence of these components of serum.

The aims of the present study were to investigate whether IRAS enhances ERK1/2 activation by LP receptor agonists and to investigate whether the presence of LPs in the serum affects the ability of IRAS to enhance NGF-induced ERK1/2 activation and neurite outgrowth.

6.2 Methods

6.2.1 ERK1/2 activation

IRAS- and vector-transfected PC12 cells were plated onto PLL (10 μg/ml) coated 6-well plates and grown for 24 hours. The cells were then rinsed twice in serum-free media and grown in serum-free media for 4 hours. The cells were then treated with NGF (100 ng/ml), LPA (1 μM) or the specific $S1P_1$ agonist, SEW2871 (100 nM), for 5 or 15 minutes. Cells were pre-treated with AGN192403 (10μM) and efaroxan (10μM) 10 minutes prior to NGF addition. In some experiments, the cells were grown in serum-free media containing LPA (30-1000 nM) or SEW2871 (3-100 nM) for 4 hours prior to NGF addition. Following treatment, the cells were lysed and protein concentration of the lysates determined. The cell lysates were subject to SDS-PAGE and immunodetection for phospho-ERK1/2 and total ERK1/2 as described in the general methods.

6.2.2 Neurite outgrowth

To assess neurite outgrowth, the vector- and IRAS-transfected PC12 cells were grown in serum-free media containing LPA (100 or 1000 nM) or SEW2871 (10 or 100 nM) for 4 hours prior to plating. The cells were then plated onto laminin (10 μg/ml) coated-coverslips in full-serum media as described in the general methods. The cells were grown for 96 hours with NGF (100 ng/ml) added every 24 hours. The media was replaced after 48 hours. After incubation the cells were stained for F-actin and viewed with a fluorescence microscope using a 20X objective as described in the general methods. For each treatment, 3-4 images were taken. Neurite outgrowth was

assessed using NIH image. 50-1000 cells were counted. Cells with one or more neurites >1X cell body diameter were counted as neurite positive.

6.2.3 Statistics

Student t-tests were performed for comparisons of two groups. One way ANOVA or two way ANOVA with Bonferroni post-hoc tests were performed depending on experimental design.

6.3 Results

6.3.1 The effect of LPA and SEW2871 on NGF-induced ERK1/2 activation

To determine the effect of LP receptor agonists and IRAS on NGF-induced ERK1/2 activation, the cells were co-treated with NGF and either the specific $S1P_1$ agonist, SEW2871, or the general LPA receptor agonist, LPA. These LP receptor agonists were chosen to see whether the effects of IRAS were specific to $S1P_1$ or were a general effect on LP receptors. In these experiments, the cells were grown in serum-free media for 4 hours prior to co-treatment with NGF and the LP receptor agonists. This was done in order to remove LPA and S1P present in serum. Also, as shown in chapter 4, vector- and IRAS-transfected cells have a similar level of ERK1/2 activation by NGF under these conditions, which makes it easier to compare the relative effects of the LP receptor agonists in the vector- and IRAS-transfected cells. Figure 6.1 shows that the IRAS- and vector-transfected cells did, indeed, have similar levels of ERK1/2 activation after 5 minutes when treated with NGF alone as expected. Treatment with neither SEW2871, nor LPA alone, induced ERK1/2 activation in the cells. After 5 minutes, co-treatment with NGF and LPA significantly enhanced ERK1/2 activation in the IRAS-transfected cells by 55% compared to treatment with NGF alone. LPA-NGF co-treatment also appeared to enhance ERK1/2 activation in the vector-transfected cells by 26% although the effect did not reach significance ($p = 0.08$). In contrast, SEW2871 did not significantly affect ERK1/2 activation in IRAS- or vector-transfected cells co-treated with NGF after 5 minutes. Neither SEW2871, nor LPA significantly affected NGF-induced ERK1/2 activation after 15 minutes.

To determine whether I_1-R antagonists could block the LPA-induced increase in ERK1/2 activation, the cells were pre-treated with AGN192403 or efaroxan before the addition of NGF and LPA. Neither AGN192403, nor efaroxan decreased ERK1/2 activation in cells treated with NGF only (figure 6.2). This confirms the findings concerning AGN192403 in the previous chapter. AGN192403 and efaroxan did not significantly affect ERK1/2 activation in the vector-transfected cells treated with LPA (figure 6.2B(i)). This was expected, given that LPA did not significantly enhance ERK1/2 activation in the vector-transfected cells. In the IRAS-transfected cells, LPA did not significantly increase NGF-induced ERK1/2 activation in cells treated with AGN192403 or efaroxan (figure 6.2B(ii)).

6.3.2 The effect of LPA and SEW2871 pre-treatment on NGF-induced ERK1/2 activation and neurite outgrowth

To test whether the presence of LP receptor agonists in serum are responsible for the IRAS enhancement of NGF-induced ERK1/2 activation and neurite outgrowth, the cells were grown in serum-free media containing various concentrations of LPA or SEW2871 for 4 hours prior to NGF treatment. Figure 6.3 shows the effects of this on ERK1/2 activation. LPA pre-treatment caused a concentration-dependent increase in ERK1/2 activation in the IRAS-transfected cells but had little effect in the vector-transfected cells. SEW2871 did not produce a concentration-dependent increase in ERK1/2 activation in either the vector- or IRAS-transfected cells, however, IRAS-transfected cells pre-treated with 3 nM SEW2871 had greater ERK1/2 activation than vector-transfected cells at this concentration.

Figures 6.4-6.6 show the effects of LPA and SEW2871 pre-treatment on NGF-induced neurite outgrowth. Neither SEW2871, nor LPA produced significant

changes in neurite outgrowth compared to NGF alone in either the vector- or IRAS-transfected cells.

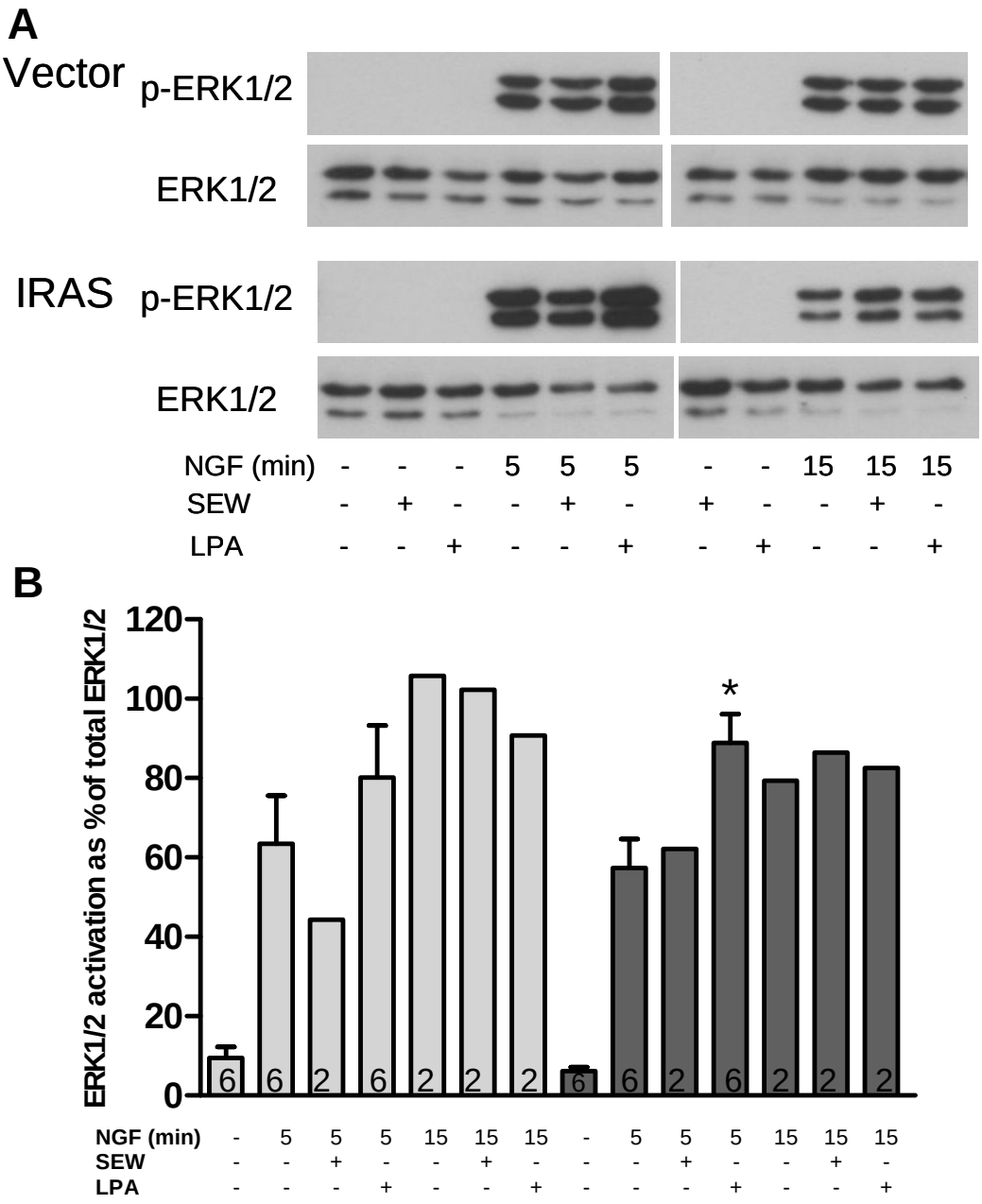


Figure 6.1. Effect of LPA and SEW2871 on NGF-induced ERK1/2 activation.

A. Representative Western blot of phospho-ERK1/2 and total-ERK1/2 in vector-and IRAS-transfected cells treated with NGF, LPA and SEW2871.

B. Densitometry of ERK1/2 activation in vector- (□) and IRAS- (■) transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 ± SEM (number of experiments are in columns). *****, $p < 0.05$ compared with IRAS-transfected cells after 5 min NGF exposure (paired student-t-test).

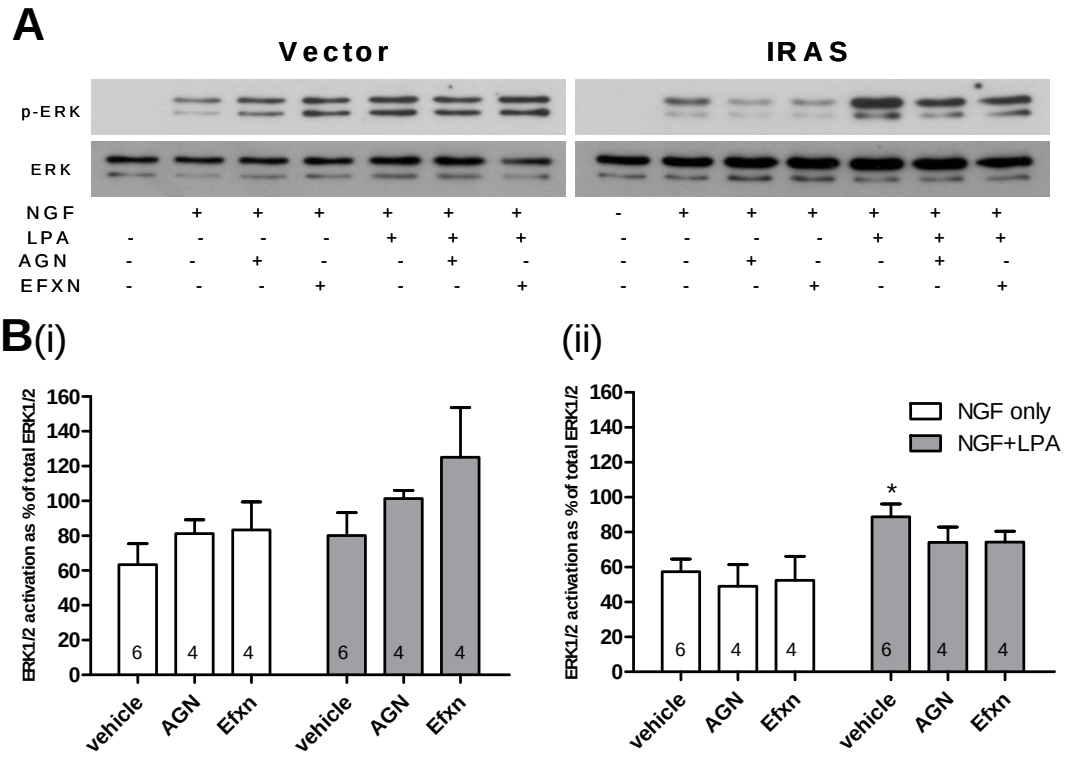


Figure 6.2. Effect of LPA, AGN192403 and efaroxan on NGF-induced ERK1/2 activation.

A. Representative Western blot of phospho-ERK1/2 and total-ERK1/2 in vector-and IRAS-transfected cells treated with NGF, LPA, AGN192403 and efaroxan.

B. Densitometry of ERK1/2 activation in vector- (i) and IRAS- (ii) transfected cells. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM (number of experiments are in columns). *****, $p < 0.05$ compared with IRAS-transfected cells treated with NGF only (two way ANOVA, Bonferroni).

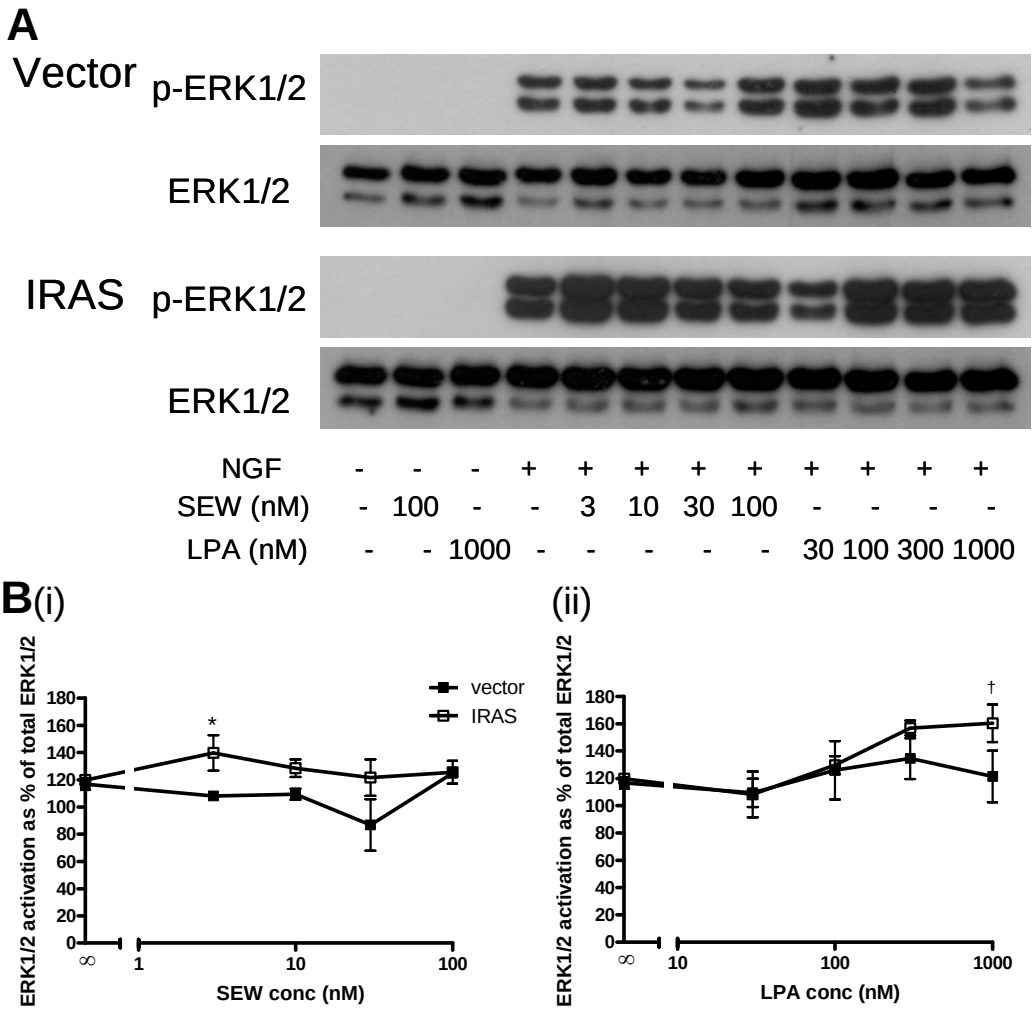


Figure 6.3. Effect of LPA and SEW2871 pre-treatment on NGF-induced ERK1/2 activation.

A. Representative Western blot of phospho-ERK1/2 and total-ERK1/2 in vector-and IRAS-transfected cells treated with LPA, SEW2871 and NGF.

B. Densitometry of ERK1/2 activation in vector- and IRAS-transfected cells. (i). Effect of SEW2871 pre-treatment on NGF-induced ERK1/2 activation. (ii). Effect of LPA pre-treatment on NGF-induced ERK1/2 activation. Values are expressed as mean of phospho-ERK1/2 relative to total-ERK1/2 $\pm$ SEM. *****, $p < 0.05$ compared with vector-transfected cells after 5 min NGF exposure and pre-treatment with SEW2871 (3 nM) (n=4, paired student-t-test). †, $p < 0.05$ compared with IRAS-transfected cells after 5 min NGF exposure (n=4, one-way ANOVA, Bonferroni).

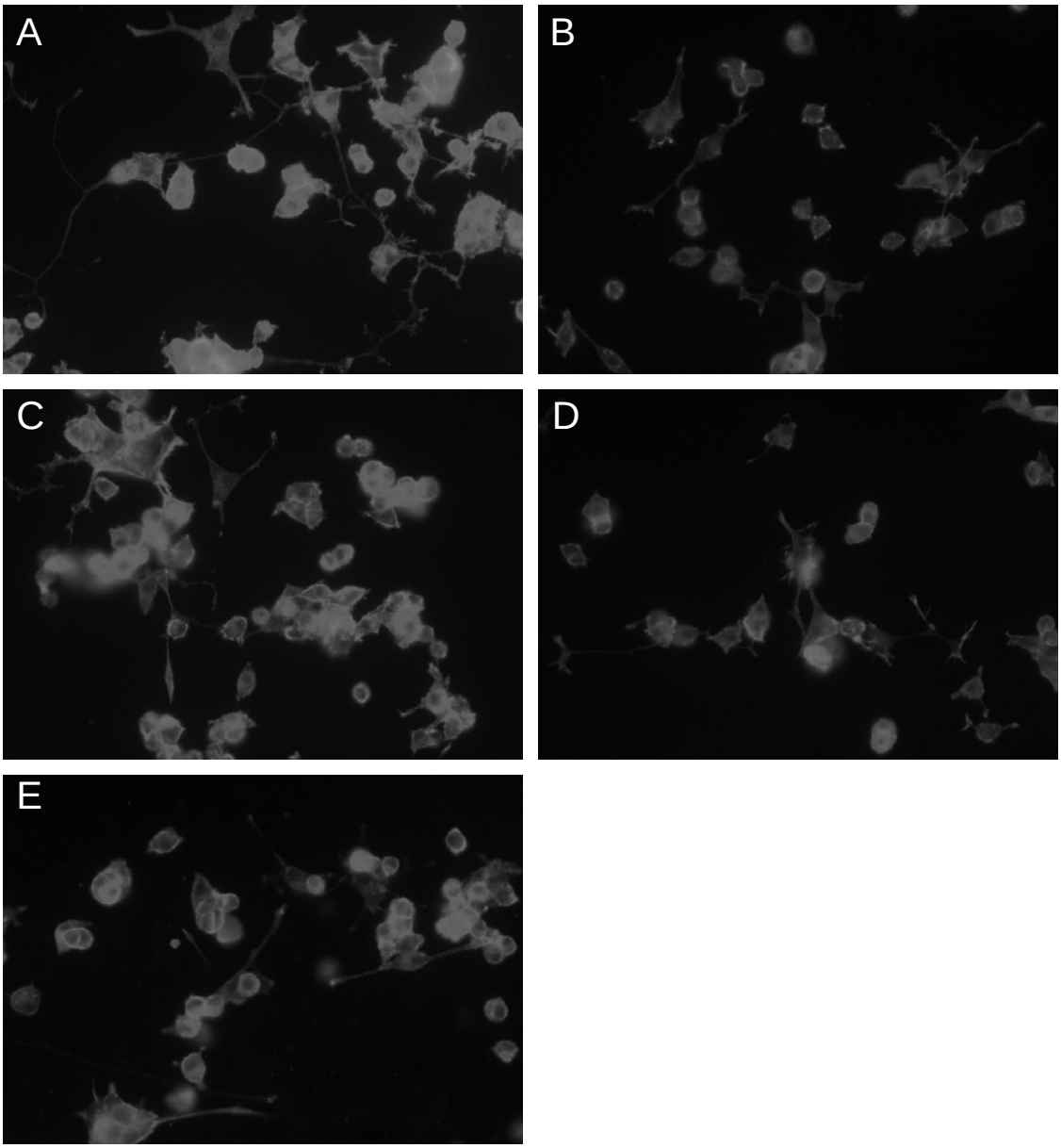


Figure 6.4. Effect of LPA and SEW2871 pre-treatment on NGF-induced neurite outgrowth in vector-transfected cells grown in serum-starved conditions. Representative vector-transfected cells treated with NGF (A), NGF and LPA (100 nM) (B), NGF and LPA (1000 nM) (C), NGF and SEW2871 (10 nM) (D), NGF and SEW2871 (100 nM) (E).

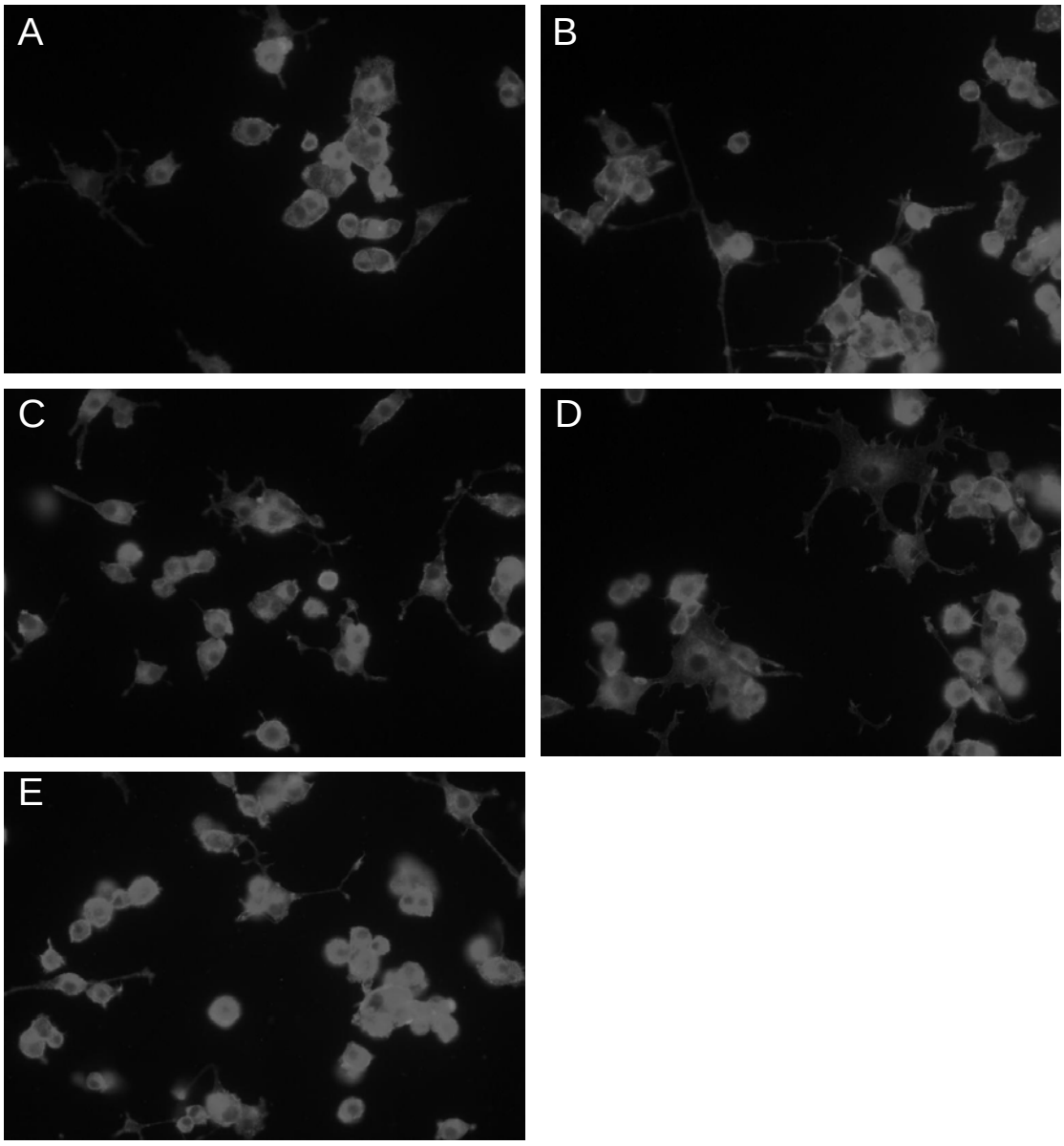


Figure 6.5. Effect of LPA and SEW2871 pre-treatment on NGF-induced neurite outgrowth in IRAS-transfected cells grown in serum-starved conditions. Representative IRAS-transfected cells treated with NGF (A), NGF and LPA (100 nM) (B), NGF and LPA (1000 nM) (C), NGF and SEW2871 (10 nM) (D), NGF and SEW2871 (100 nM) (E).

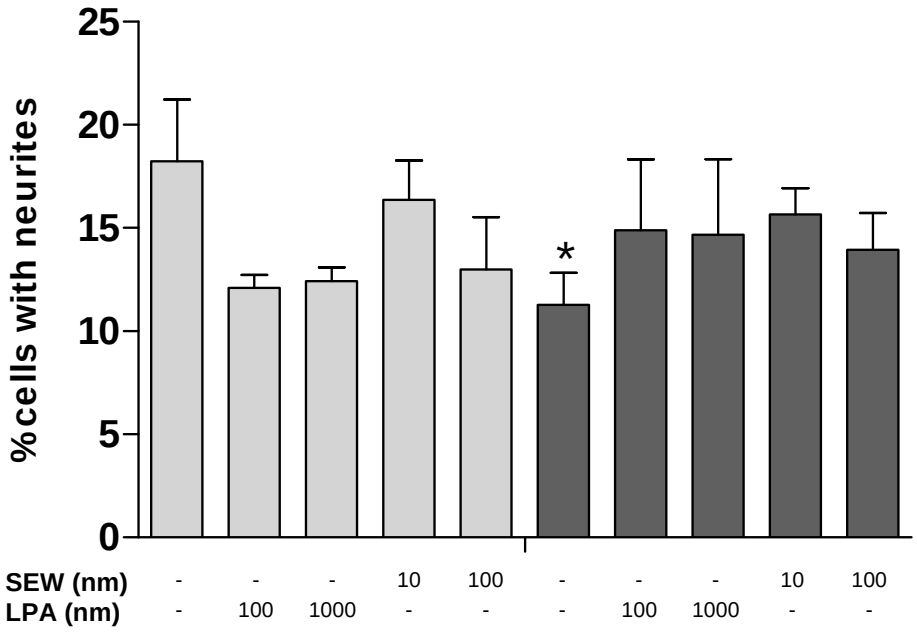


Figure 6.6. Effect of LPA and SEW2871 on percent of NGF-treated vector- and IRAS-transfected cells expressing neurites in serum-starved conditions. Vector- (□) and IRAS- (■) transfected cells were scored as neurite positive if containing one or more protrusions with length > the cell diameter. *****, $p < 0.05$ compared with vector-transfected cells treated with NGF (n=3, one way ANOVA, Bonferroni).

6.4 Discussion

6.4.1 Effect of IRAS on LP receptor agonist-induced ERK1/2 activation

This study investigated whether IRAS interacts with LP receptors and whether the enhanced NGF-induced ERK1/2 activation and neurite outgrowth in IRAS-transfected cells was due to IRAS interacting with LP receptors. The aims of the present study were based on previous research showing that the action of I_1-R ligands in PC12 cells may be through LP receptors (Molderings *et al.*, 2007a; Molderings *et al.*, 2002) and that TrkA enhances LP receptor action in PC12 cells (Moughal *et al.*, 2004; Toman *et al.*, 2004).

To test the hypothesis that IRAS interacts with LP receptors, the cells were co-treated with 100 ng/ml NGF and 1 μm LPA or 100 nM SEW2871. LPA itself typically induces strong ERK1/2 activation at concentrations of 10 μM (Della Rocca *et al.*, 1999). LPA, at concentrations of 0.5 μM, has been shown to induce larger increases in ERK1/2 activation in the presence of NGF (Moughal *et al.*, 2004). As the present study sought to establish a relationship between IRAS, LPA and NGF, a concentration of 1 μM was chosen. There has been little published research in cell models using SEW2871. Previous studies in CHO cells transfected with mouse $S1P_1$ have shown that SEW2871 has an EC_{50} of 21 nM in inducing GTPγS binding (Sanna *et al.*, 2004). Therefore, SEW2871 was used at a concentration of 100 nM.

Under the conditions in the present study, LPA did not significantly enhance ERK1/2 activation in the vector-transfected cells. In contrast Moughal *et al.* (2004) found that co-treatment with LPA and NGF led to a significant increase in ERK1/2 activation compared to treatment with either alone. There are several reasons for these different findings. Firstly, the concentration of NGF used in the present study

was higher than that used by Moughal *et al.* (2004) who used 1 ng/ml of NGF, which alone produced little ERK1/2 activation. Thus, it would have been easier to detect an enhancing effect of LPA in their study. Secondly, in the present study, LPA alone did not stimulate ERK1/2, whereas an effect for LPA alone was detected by Moughal *et al.* (2004). This suggests that LPA was not as effective in the present study. Thirdly, the effect of LPA in the vector-transfected cells did approach significance ($p = 0.08$). It is possible that LPA enhances ERK1/2 activation in the vector-transfected cells, but to a smaller extent than in IRAS-transfected cells.

In the IRAS-transfected cells, LPA significantly enhanced ERK1/2 activation when the cells were co-treated with NGF. It is important to note that in these experiments, the cells were serum-starved for 4 hours prior to treatment and this produced similar ERK1/2 activation in the IRAS- and vector-transfected cells treated with NGF as observed in chapter 4. This indicates that IRAS is enhancing ERK1/2 signalling through, or in partnership with, LPA. Furthermore, this effect was only observed after 5 minutes but did not extend to 15 minutes. Therefore, the effect of LPA is transient and similar to the effect of NGF alone in IRAS-transfected cells under full-serum conditions. This study did not investigate whether LPA or SEW2871 affects ERK1/2 activation over a longer timecourse.

Unlike LPA, the specific $S1P_1$ agonist, SEW2871, did not affect ERK1/2 activation in these conditions. Previous research demonstrated that siRNA to SIP_1 was the most effective at preventing clonidine binding in PC12 cells (Molderings *et al.*, 2007a). This study also demonstrated that siRNA to $S1P_3$ was also effective but siRNA to $S1P_2$ was not (Molderings *et al.*, 2007a). The results from the present study suggest that IRAS does not interact exclusively through the $S1P_1$ receptor, however, there are a number of other possible explanations for why SEW2871 did not induce

ERK1/2 activation. For example, it has not been shown that the $S1P_1$ receptor enhances TrkA-induced ERK1/2 activation. This seems unlikely, however, given that $S1P_1$ receptor activation enhances NGF-induced neurite outgrowth and Rac activation (Toman *et al.*, 2004). Moreover, the $S1P_1$ receptor does enhance PDGFβ receptor-induced ERK1/2 signalling (Waters *et al.*, 2003). Another possibility is that the concentration of SEW2871 used was too low. As mentioned above, SEW2871 has an EC_{50} of 21 nm for GTPγS activation in $S1P_1$-transfected CHO cells. SEW2871 may not be as sensitive in activating ERK1/2 in PC12 cells.

While these results show that IRAS enhances LPA signalling, it cannot be determined which LP receptor subtypes IRAS may interact with to achieve this effect. That IRAS enhances the effects of LPA suggests that IRAS interacts with LPA receptor subtypes. However, LPA also has micromolar affinity for the $S1P_1$ receptor (Lee *et al.*, 1998), so interaction with S1P receptors cannot be ruled out. Further immunoprecipitation and immunofluorescence studies are required to determine which LP receptors IRAS interacts with.

Interestingly, the effects of LPA in IRAS-transfected cells were partially blocked by I_1-R antagonists. Therefore, an important question is, where does this inhibition occur? For example, AGN192403 and efaroxan may bind directly to IRAS and prevent it from interacting with LP receptors. This is possible given that these antagonists had no effect in the vector-transfected cells. Alternatively, AGN192403 and efaroxan directly antagonise LP receptors. A recent study by Molderings *et al.* (2007b) suggests this might be the case. In this study, it was observed that both I_1-R agonists and antagonists concentration-dependently inhibited PC12 cell growth in a similar manner to S1P and that this inhibited growth was prevented with siRNAs to $S1P_1$, $S1P_2$ and $S1P_3$. Furthermore, the siRNAs to different S1P receptor subtypes

affected cell growth inhibition by the I_1-R ligands to varying degrees, suggesting that these ligands preferentially bind different S1P receptor subtypes. It is tempting to speculate that IRAS enhances imidazoline binding by enhancing the endosomal trafficking and recycling of LP receptors. In previous binding studies involving IRAS, clonidine bound saturably in IRAS-transfected CHO cells but not in COS-7 or Sf9 cells. S1P receptors and the LPA_1 receptor are well expressed in CHO cells (Holdsworth *et al.*, 2005). In contrast, Sf9 cells are unresponsive to LPA and S1P when testing for adenylate cyclase inhibition and GTPγS binding (Windh *et al.*, 1999; Zondag *et al.*, 1998) indicating their lack of expression in this cell line. In COS-7 cells, LPA but not S1P enhanced activation of ERK1/2 and nitric oxide synthase activity which suggests that LPA but not S1P receptor subtypes are present in this cell line (Kou *et al.*, 2002; Zondag *et al.*, 1998). Thus, it is possible that the increased clonidine binding in IRAS-transfected CHO cells was due to increased trafficking of endogenously expressed S1P receptors. Clearly, more binding studies need to be conducted to determine whether IRAS transfection enhances S1P and/or LPA binding and to determine the affinity of I_1-R ligands to S1P and LPA receptors.

6.4.2 Effect of LP receptor agonist pre-treatment on ERK1/2 activation and neurite outgrowth

As mentioned above, the LPA enhancement of ERK1/2 activation in the IRAS-transfected cells occurs after 5 minutes but is not present after 15 minutes. This observation is similar to the increased ERK1/2 activation observed in the IRAS-transfected cells in full-serum conditions. The results indicate that pre-treating IRAS-transfected cells with low concentrations of LPA in serum-free media moderately enhanced NGF-induced ERK1/2 activation after 5 minutes. This supports the hypothesis that the increased ERK1/2 activation in full-serum conditions is due to an

interaction between IRAS and LP receptor agonists present in serum. Interestingly, the lowest concentration of SEW2871 also produced greater ERK1/2 activation in IRAS-transfected cells compared to vector-transfected cells. However, it is unclear whether this was due to an enhancement of ERK1/2 activation in the IRAS-transfected cells or an inhibiting effect in the vector-transfected cells.

One limitation of this study is that the effect of S1P was not tested. S1P, unlike SEW2871, is present in serum and would give a better indication whether IRAS interacts generally with S1P receptors. Furthermore, it would be interesting to see the effects of LPA and S1P together and whether this would further enhance ERK1/2 signalling in IRAS-transfected cells.

Another limitation of this study is that the concentrations of LPA and S1P in HS and FBS are unknown. In human serum, LPA and S1P are present at between 1-5 μM and 0.5-0.8 μM respectively (Baker *et al.*, 2001; Okajima, 2002). Assuming that LPA has a similar concentration in HS and FBS, the LPA concentration in full-serum media would be between 150-750 nM, which is within the range of concentrations used in the present study. Another reason to test S1P in future experiments is that it is similarly potent at its probable concentration in full-serum media. For example, S1P caused maximum inhibition of PC12 cell growth at 1 nM (Molderings *et al.*, 2007b). Treatment of PC12 cells with 100 nM S1P was also found to enhance NGF-induced neurite outgrowth (Toman *et al.*, 2004).

In contrast to their effects on ERK1/2 activation, neither LPA nor SEW2871 significantly affected NGF-induced neurite outgrowth in the IRAS- or vector-transfected cells. There are several possible explanations for this finding. The effect of IRAS on ERK1/2 may be largely independent of neurite outgrowth. As discussed previously, the interaction of IRAS with the Rac/PAK pathway may be more

important for this. Furthermore, IRAS may interact with multiple factors other than LPA to enhance neurite outgrowth, whereas interaction with LPA alone is sufficient to enhance ERK1/2 activation. In fact, despite enhancing ERK1/2 activation, LPA inhibits neurite outgrowth and causes neurite retraction in PC12 cells (Tigyi *et al.*, 1996; Tigyi *et al.*, 1992). These results again highlight the need to examine S1P, which has a positive influence on neurite outgrowth.

6.5 Conclusion

This study demonstrated that IRAS enhances LPA-induced ERK1/2 activation, which strongly suggests a link between IRAS, TrkA and LP receptor signalling. Furthermore, this study indicated that IRAS interacting with LP receptor agonists present in serum is a potential mechanism by which IRAS enhances NGF-induced ERK1/2 activation in full serum conditions. These findings should form the basis for future study examining the relationship between IRAS, LP receptors, imidazolines and NGF.

7 Chapter 7: General discussion

7.1 Summary

The overarching aim of the work reported in this thesis was to investigate the role of IRAS in neuronal differentiation. Previous studies using fibroblast, CHO and HEK293 kidney cell lines have found that IRAS modulates actin organisation and enhances ERK1/2 signalling (Alahari *et al.*, 2000; Li *et al.*, 2006; Sano *et al.*, 2002). However, despite the high level of IRAS expression in the brain (Alahari *et al.*, 2000; Piletz *et al.*, 1999), there has been surprisingly little research into the neuronal functions of IRAS. Actin reorganisation and ERK1/2 signalling are important for the development of neurites during neuronal differentiation. Therefore, this project sought to investigate the role of IRAS on these main aspects of neuronal differentiation in neuronal model PC12 cells.

Experiments in chapter 3 showed that overexpression of IRAS in PC12 cells led to reorganisation of actin and enhanced growth cone development. Furthermore, this growth cone development was influenced by I_1-R ligands. The I_1-R agonist, rilmenidine, enhanced growth cone development whereas the I_1-R antagonist, AGN192403, decreased it. This chapter demonstrated that IRAS has a role in neuronal development and provided further evidence that IRAS is an imidazoline binding protein.

IRAS interacts with and influences the signalling of other receptors. Chapter 4 investigated whether IRAS affected the neurite outgrowth and ERK1/2 signalling induced by NGF. NGF, acting through its receptor, TrkA, induces differentiation in PC12 cells (Greene *et al.*, 1976) and has been implicated in I_1-R signalling (Edwards *et al.*, 2001; Zhang *et al.*, 2001). Results in this chapter found that IRAS enhanced

NGF-induced neurite outgrowth and ERK1/2 signalling, however, only when the cells were grown in full-serum conditions. Surprisingly, when the cells were serum-starved before the addition of NGF, IRAS inhibited neurite outgrowth and did not enhance ERK1/2 activation. Therefore, IRAS differentially enhances or inhibits neurite outgrowth and ERK1/2 activation depending on the growth conditions.

Chapter 5 began to investigate mechanisms by which IRAS-enhanced NGF-induced ERK1/2 activation. Studies in this chapter found that IRAS does not enhance ERK1/2 activation through an established I_1-R pathway (i.e., PC-PLC) or Src. Therefore, it is unlikely that IRAS activates ERK1/2 signalling pathways itself in the absence of ligand. As discussed in Appendix I, efforts to clarify the role of IRAS in TrkA trafficking were unsuccessful.

Experiments in chapter 6 investigated whether IRAS enhanced ERK1/2 activation through interaction with LP receptors. The rationale for this investigation was based on the findings that both LPA and S1P receptors crosstalk with TrkA (Moughal *et al.*, 2004; Toman *et al.*, 2004), I_1-R ligands appear to have affinity for LP receptors in PC12 cells (Molderings *et al.*, 2007a) and LP receptor agonists are present in serum (Baker *et al.*, 2001; Okajima, 2002). Experiments in this chapter found that the general LPA receptor agonist, LPA, enhanced ERK1/2 activation in IRAS-transfected cells co-stimulated with NGF whereas the specific $S1P_1$ agonist, SEW2871, did not. Furthermore, the I_1-R antagonists, AGN192403 and efaroxan, partially blocked this effect of LPA. Pre-treating the cells with LPA and SEW2871 in serum-starved conditions caused enhanced ERK1/2 activation but not neurite outgrowth in IRAS-transfected cells. The results in this chapter suggest that IRAS enhances NGF-induced ERK1/2 activation by enhancing the activity of LP receptors.

Several wider questions arise from the results reported in this thesis and other recent studies. These concern the concept of IRAS as a receptor or trafficking protein, imidazoline binding protein, as well as its potential role in the regulation of neuronal growth and survival. These topics will be discussed in the following sections.

7.2 Is IRAS a receptor or trafficking protein?

IRAS was originally cloned as the I_1-R. However, this thesis and other recent studies have demonstrated that IRAS does not resemble nor function as a classical receptor but rather as a protein which influences the signalling of other receptors, potentially through their trafficking.

Analysis of the structure of IRAS shows that it does not resemble a classical transmembrane receptor (Piletz *et al.*, 2000). IRAS contains hypothetical transmembrane domains but does not contain the N-terminal sequence for insertion into the plasma membrane (Piletz *et al.*, 2000). Furthermore, while IRAS does localise to plasma and endosomal membranes, it appears also to have some degree of cytoplasmic localisation (Alahari *et al.*, 2000; Piletz *et al.*, 2000). IRAS does contain a PX domain and this has been shown to localise IRAS to PIs on endosomal membranes (Lim *et al.*, 2004). In fact, the structure of IRAS has more in common with SNXs, a class of proteins involved in receptor trafficking (Lim *et al.*, 2004; Seet *et al.*, 2006).

Numerous studies have shown that IRAS affects the signalling of various classes of receptors including integrin receptors, growth factor receptors and GPCRs (Alahari *et al.*, 2000; Sano *et al.*, 2002; Wu *et al.*, 2006; Wu *et al.*, 2005). Furthermore, these studies demonstrate that agonist stimulation (imidazoline or

other) of IRAS is not required for it to influence the signalling of these receptors. The present study shows that IRAS influences the signalling of receptors involved in neuronal differentiation: TrkA and LP receptors. Thus, the findings in the present study are consistent with IRAS having a role in receptor trafficking or as a signalling-scaffold protein.

Combined, these studies demonstrate that the structure and function of IRAS are unlikely to be those of a classical transmembrane receptor. One possibility, which has been postulated, is that IRAS complexes with other proteins to form a functional imidazoline receptor (Piletz *et al.*, 2000; Sano *et al.*, 2002). Further immunoprecipitation, 2-D gel and mass spectrometry techniques could be employed to find which other proteins associate with IRAS, as a first step to determine whether this is so.

7.3 Is IRAS an Imidazoline binding protein?

As described above, IRAS is unlikely to be a transmembrane receptor. Rather, IRAS may be an intracellular target for imidazolines which affect the action of IRAS upon binding. In this sense, IRAS may be similar to the I_2-R which is found colocalised with monoamine oxidases on mitochondrial membranes (Regunathan *et al.*, 1996). Alternatively, IRAS may not even bind imidazolines but associate with and traffic receptors that do.

Results presented in chapter 3, showing that growth cone development can be influenced by I_1-R ligands rilmenidine and AGN192403, support the notion that IRAS is an imidazoline binding protein. Other studies show that IRAS enhances rilmenidine- and moxonidine-induced PC-PLC activation and ERK1/2 activation (Li *et al.*, 2006). Furthermore, IRAS/nischarin antisense ODNs decrease rilmenidine and

moxonidine-induced ERK1/2 activation and [^{125}I]p-clonidine binding in PC12 cells (Sun *et al.*, 2007; Zhang *et al.*, 2006). In a recent study by Zhang and Abdel-Rahmen (2008), IRAS antisense ODNs were administered intracisternally 2 days before treatment with rilmenidine. This caused a significant reduction in the rilmenidine-induced hypotension. Therefore, this study links IRAS to centrally-mediated sympathoinhibition, which is perhaps the most important criterion for determining whether IRAS is an I_1-R.

While the studies mentioned above strongly support the idea that IRAS is an imidazoline binding protein (or forms part of the I_1-R), these results could still be interpreted that overexpression or knockdown of IRAS causes the increase or decrease in trafficking of imidazoline binding receptors endogenously expressed in PC12 cells, CHO cells and the RVLM. One study which supports this found that transient transfection of IRAS into α_{2a}-adrenoceptor-expressing CHO cells led to an increase in both the number of imidazoline and specific α_{2a}-adrenoceptor binding sites (Chen *et al.*, 2003). Furthermore, IRAS was found to colocalise with α_{2a}-adrenoceptors in membranes. Results from this study suggest that IRAS associates with the α_{2a}-adrenoceptor, a known imidazoline binding receptor.

It must be considered that neither CHO cells nor PC12 cells endogenously express the α_{2a}-adrenoceptor, however, transfection of IRAS into these cell lines enhances imidazoline binding. Therefore, if IRAS is not an imidazoline binding protein itself, it must traffic other imidazoline binding receptors. As described in chapter 6, LP receptors have been proposed to have affinity for I_1-R ligands (Molderings *et al.*, 2007a). While results in this chapter support the proposal that IRAS interacts with these receptors, it is still unclear whether I_1-R ligands have affinity for and activate these receptors. For example, the present study found that I_1-

R antagonists partially inhibited the LPA-induced increase in ERK1/2 activation in the IRAS-transfected cells, however, it is unclear if this was due to antagonism at LP receptors or through an interaction with IRAS. Similarly, efaroxan was found to block the effects of agmatine on opioid dependence in IRAS-transfected CHO cells (Wu *et al.*, 2006; Wu *et al.*, 2005). Efaroxan does not have affinity for opioid receptors, which suggests that this antagonism occurred through IRAS.

If imidazolines were to act at LP receptors, it would be expected that these compounds cause increases in GTPγs binding and intracellular Ca^{2+} similar to LPA and S1P and consistent with GPCR activation. It is still unclear whether this is the case. Studies with rilmenidine and moxonidine have found that these compounds do not stimulate GTPγS binding in α_{2a}-adrenoceptor-lacking cell lines, even when transfected with IRAS (Li *et al.*, 2006). However, it has been reported that moxonidine and clonidine do stimulate increases in intracellular Ca^{2+} levels in $S1P_3$-transfected HEK293 cells (Molderings *et al.*, 2007a). Furthermore, moxonidine and benazoline have been shown to inhibit cAMP production in PC12 cells, although clonidine does not have this effect in either PC12 cells or bovine adrenal chromaffin cells (Greney *et al.*, 2000; Regunathan *et al.*, 1991).

In summary, results in this thesis support the hypothesis that IRAS traffics LP receptors. However, further research is needed to determine whether I_1-R ligands have affinity for IRAS and LP receptors. To test the hypothesis that IRAS transfection enhances imidazoline binding through LP receptors, both overexpression of IRAS and siRNAs to IRAS could be employed to determine the effect this has on LPA and S1P binding. Further IRAS siRNA studies could determine whether I_1-R agonists bind in the absence of IRAS and whether this binding is reduced in the

presence of efaroxan and AGN192403. This would also help determine whether efaroxan is inhibiting IRAS or LP receptors.

7.4 IRAS in neuronal cell signalling

7.4.1 TrkA signalling

Results in chapter 3 showed that transfecting PC12 cells with IRAS enhances growth cone development but does not cause neurite outgrowth. Results in chapter 4 showed that in full-serum conditions, IRAS enhances NGF-induced neurite outgrowth and ERK1/2 activation. Combined, the results from these two chapters indicate that IRAS enhances the differentiating properties of NGF but does not cause PC12 cell differentiation without this additional stimulus. Studies in chapter 5 show that IRAS, in the absence of I_1-R agonists, does not enhance ERK1/2 activation through an I_1-R pathway. As discussed above, IRAS itself is unlikely to be a transmembrane receptor. However, IRAS does interact with and enhances the ERK1/2 signalling of the insulin receptor and MOR (Sano *et al.*, 2002; Wu *et al.*, 2006). Similarly, the results in this thesis suggest that IRAS interacts with and enhances the signalling of TrkA. This thesis did attempt to determine whether IRAS formed a complex with TrkA but was unsuccessful. This should form the basis of future study to determine whether IRAS enhances TrkA signalling through increased internalisation and recycling of this receptor.

Internalisation and trafficking of TrkA is especially important for its signalling. NGF binds to TrkA located at presynaptic nerve terminals. The NGF-bound TrkA is then transported down the axons to the cell body where it can affect changes in gene expression. This retrograde TrkA signalling is necessary for cell

survival (Zweifel *et al.*, 2005). Interestingly, only a small percentage of TrkA is transported down the axon (Ye *et al.*, 2003). The majority of TrkA signalling occurs within the growth cone, which promotes changes in actin cytoskeletal organisation for axonal growth.

A high degree of TrkA trafficking occurs in small and recycling endosomes. Retrograde transport of TrkA has been shown to occur in small endosomes (Delcroix *et al.*, 2003). Furthermore, internalised TrkA can be recycled back to the plasma membrane via endosomes (Chen *et al.*, 2005). TrkA recycling has been proposed as one of the reasons why TrkA promotes long-term ERK1/2 activation compared to the EGFR, the majority of which is transported to lysosomes for degradation (Qiu *et al.*, 2004). It is interesting to note that IRAS does not enhance the signalling of EGF. Moreover, receptors that are influenced by IRAS, the MOR and α5β1 integrin, show some degree of recycling (Caswell *et al.*, 2006; Tanowitz *et al.*, 2003). Thus, IRAS appears to enhance the signalling of receptors that are recycled and not degraded.

How might IRAS enhancing TrkA trafficking lead to enhanced ERK1/2 signalling? Two potential mechanisms are shown in figure 7.1. Firstly, IRAS may enhance internalisation of the TrkA receptor similar to its effect on α5β1 integrin. This enhanced internalisation could lead to an increase in short-term Rap1 signalling. This additional Rap1 signalling, combined with Ras signalling usually associated with short-term growth factor signalling, may account for the enhanced ERK1/2 signalling observed in the IRAS-transfected cells. Secondly, IRAS may enhance the recycling of TrkA. Enhanced recycling of TrkA is often accompanied by greater long-term ERK1/2 activation (Saxena *et al.*, 2005). However, in the present study, this was not observed in the IRAS-transfected cells. Therefore, this second mechanism is less likely. It should be noted that IRAS overexpression did not

diminish long-term ERK1/2 signalling either, so if IRAS were to enhance TrkA internalisation, such a process does lead to enhanced degradation of TrkA.

7.4.2 LP receptor signalling

The above model offers an explanation as to how IRAS enhances ERK1/2 activation under full-serum conditions. However, the model does not take into account the finding that IRAS does not enhance ERK1/2 activation under serum-starved conditions. The results in chapter 6 suggest that IRAS, rather than enhancing ERK1/2 signalling exclusively through TrkA, enhances ERK1/2 activation by facilitating crosstalk between TrkA and LP receptors. This is based on the findings that IRAS enhances LPA-induced ERK1/2 activation in the presence of NGF. Moreover, supplementing serum-free media with LPA before NGF addition also causes increased ERK1/2 activation in the IRAS-transfected cells.

Both LPA_1 and $S1P_1$ receptors interact with TrkA, although the nature of this interaction differs slightly. For the TrkA/LPA_1 interaction, either LPA or NGF can cause internalisation of both TrkA and LPA_1 (Moughal *et al.*, 2004). The TrkA/$S1P_1$ interaction differs in that TrkA stimulates SphK and S1P production which in turn activates the $S1P_1$ receptor and increases its endocytosis (Toman *et al.*, 2004). In both cases, this leads to an increase in downstream signalling from TrkA. The TrkA/LPA_1 interaction was shown to lead to increased ERK1/2 activation (Moughal *et al.*, 2004) whereas the TrkA/$S1P_1$ interaction led to an increase in Rac activation and neurite outgrowth (Toman *et al.*, 2004).

IRAS may fit into these models of TrkA/LP receptors signalling as a trafficker of LP receptors. In this model (figure 7.2), LP receptors would have some level of activation due to LP agonists that are present in full-serum media. Following addition of NGF, crosstalk would occur between NGF-activated TrkA and LP

receptors, leading to enhanced LP receptor activity. LP receptor interaction with IRAS would lead to increased internalisation and ERK1/2 signalling. In the absence of serum, the lack of LP agonists would reduce LP receptor activity and therefore the effect of the LP receptor/TrkA interaction. Thus, any trafficking of the LP receptor by IRAS would be unlikely to affect ERK1/2 signalling.

While the above model provides a plausible explanation for the effect of IRAS on ERK1/2 activation, how IRAS affects neurite outgrowth is less clear. Whereas levels of ERK1/2 activation in IRAS- and vector-transfected cells were similar in serum-starved conditions, IRAS, unexpectedly, caused a dramatic decrease in neurite outgrowth. Supplementing the serum-free media with LPA or SEW caused only subtle changes in neurite outgrowth which did not reflect the dramatic changes observed in chapter 4. Therefore, the presence and absence of LP agonists alone is unlikely to account for the differences in neurite outgrowth observed. Perhaps, the combination of reduced ERK1/2 signalling due to absence of LP agonists combined with the inhibitory effects of IRAS on Rac/PAK signalling leads to the observed decrease in neurite outgrowth.

7.4.3 Potential clinical implications

Previous imidazoline-related research has focussed primarily on the role of the I_1-R in blood pressure regulation. The present study introduces a new role in IRAS/imidazoline research: the modulation of neuronal cell signalling and growth. Clinically, this is important, as stimulating neuronal cell growth is a potential treatment for neurodegenerative disorders such as AD. Although research into the role of IRAS in neuromodulation is at an early stage, IRAS may be a potential drug target due to its possible imidazoline binding site. Furthermore, currently used I_1-R antihypertensives penetrate the blood-brain barrier very effectively. The following

paragraphs will briefly discuss NGF-TrkA signalling in AD and how IRAS may be a potential therapeutic target.

AD is characterised by a loss in cholinergic neurons in the basal forebrain and, consequently, a loss in cognitive function and memory. NGF is important for the maintenance and survival of these neurons. For example, mice expressing anti-NGF show an age-dependent loss of basal forebrain cholinergic neurons (BFCNs) (Capsoni *et al.*, 2000). Imbalances in NGF signalling and loss of TrkA signalling has been proposed as one of the main mechanisms for progression of AD. Studies in aged rats and Down syndrome mice have shown that this occurs early on in the disease progression and is related to the accumulation of amyloid β (Salehi *et al.*, 2006; Williams *et al.*, 2006b). Interestingly, studies of AD brain tissue show that NGF production in the hippocampus is not reduced, however there is an increased ratio of pro-NGF to NGF (Fahnestock *et al.*, 2001; Hellweg *et al.*, 1998). Pro-NGF is the precursor to NGF and has higher affinity for the pro-apoptotic p75NTR receptor. Furthermore, while NGF levels in the hippocampus remain constant, there are reduced levels in BFCNs, suggesting the impairment of retrograde transport of NGF to this area (Hellweg *et al.*, 1998; Mufson *et al.*, 1989). In fact, studies in a Down syndrome mouse model have shown that retrograde transport of TrkA is reduced (Salehi *et al.*, 2006). In aged rats, there is a decrease in TrkA-induced Erk1/2 activation and this correlates with an increase in cognitive decline (Williams *et al.*, 2007; Williams *et al.*, 2006a). Therefore, these studies suggest that decreased trafficking and retrograde transport of TrkA is one of the major reasons behind cholinergic loss in AD.

NGF treatment presents an attractive therapy for the treatment of AD symptoms. Recent results from a phase 1 clinical trial show that patients implanted

with NGF producing fibroblasts demonstrate improved cognitive function (Tuszynski *et al.*, 2005). However, there are problems with NGF therapy such as the need for surgery. Also, NGF has affinity for the pro-apoptotic p75NTR receptor, which may limit its effectiveness. An alternative therefore, may be specifically to enhance the signalling of the pro-survival TrkA. One way do this is to increase its trafficking and retrograde transport. In this respect, IRAS may be a good target to enhance TrkA signalling and thus, neuronal growth and survival. Regardless of the mechanism by which IRAS enhances ERK1/2 signalling - via TrkA or LP receptor - this may be important for the stimulation of TrkA signalling. For example, if the primary action of IRAS is at LP receptors, IRAS could potentially enhance TrkA/LP receptor crosstalk, which has the effect of enhancing TrkA internalisation and ERK1/2 activation.

It must be considered that IRAS only enhances ERK1/2 and neurite outgrowth in full-serum conditions. While results in this thesis provides good evidence that this is due to the presence of LP receptor agonists in the serum, this is still not conclusive. The contrasting finding that, in serum-starved conditions IRAS inhibits neurite outgrowth, highlights the need to further the current findings in PC12 cells to primary cell culture and *in vivo* models. To date, there has only been one *in vivo* study of IRAS function which, as described above, showed that IRAS is linked to rilmenidine-induced hypotension (Zhang *et al.*, 2008). Interestingly, infusion of rilmenidine to the RVLM also enhances ERK1/2 activation (Zhang *et al.*, 2005). Although this study did not link rilmenidine-induced ERK1/2 activation to IRAS, it nonetheless suggests that IRAS has a positive influence on ERK1/2 activation *in vivo*.

7.4.4 Future directions

The models described in figures 7.1 and 7.2 provide a starting point for future investigation into the role of IRAS in neurite outgrowth. To determine whether IRAS interacts with TrkA, it is necessary to repeat the immunoprecipitation and immunofluorescence experiments detailed in the appendix. These studies should be extended to determine whether IRAS also interacts with LP receptor subtypes. The LPA_1 and the $S1P_1$ receptors are the best documented to interact with TrkA (Moughal *et al.*, 2004; Toman *et al.*, 2004). Furthermore, the $S1P_1$ receptor appears the most likely to interact with imidazolines (Molderings *et al.*, 2007a). Therefore, these two subtypes are the best candidates to interact with IRAS. Studies of this type could not only determine whether IRAS colocalises with TrkA and/or LP receptors but also whether IRAS affects the localisation of these receptors in a similar manner to the $\alpha5\beta1$ receptor.

To confirm the role of IRAS in neurite outgrowth, siRNAs to IRAS could be employed to determine whether reducing expression of IRAS inhibits or enhances neurite outgrowth and under what conditions. Furthermore, results in this thesis suggest that the effect of IRAS on ERK1/2 activation is not solely responsible for the neurite outgrowth observed. Therefore, it is necessary to observe whether IRAS affects NGF-induced Rac/PAK activation in a similar manner to nischarin.

Finally, as suggested above, it is important to extend these findings in PC12 cells to other models such as primary neuronal cell culture and *in vivo* studies. This could include the establishment of an IRAS knockout mouse to determine how important IRAS is in neuronal development. Also, further use could be made of IRAS ODN infusion into specific brain regions to observe whether this affects ERK1/2 levels, TrkA phosphorylation and localisation.

7.5 Conclusion

This thesis investigated the role of IRAS in neuronal cell growth and signalling. It aimed to show that IRAS, through modulation of the actin cytoskeleton, could affect growth cone development and NGF-induced neurite outgrowth in PC12 cells. Furthermore, this research aimed to show that IRAS could affect the signalling of receptors involved in neuronal differentiation: TrkA and LP receptors. It was found that IRAS differentially enhances or inhibits NGF-induced neurite outgrowth and that this is, in part, due to an effect on ERK1/2 signalling. While it was demonstrated that IRAS affects the signalling of both NGF and LP receptor agonists, the exact mechanism by which this occurs has still to be identified. A frequent assertion of this thesis, and one supported by the results herein, is that IRAS enhances ERK1/2 signalling through the trafficking of other receptors rather than acting as a transmembrane receptor itself. If this is the case, one of the neuronal functions of IRAS may be to enhance the internalisation and signalling of TrkA and/or LP receptors and therefore, influence both neuronal development and survival.

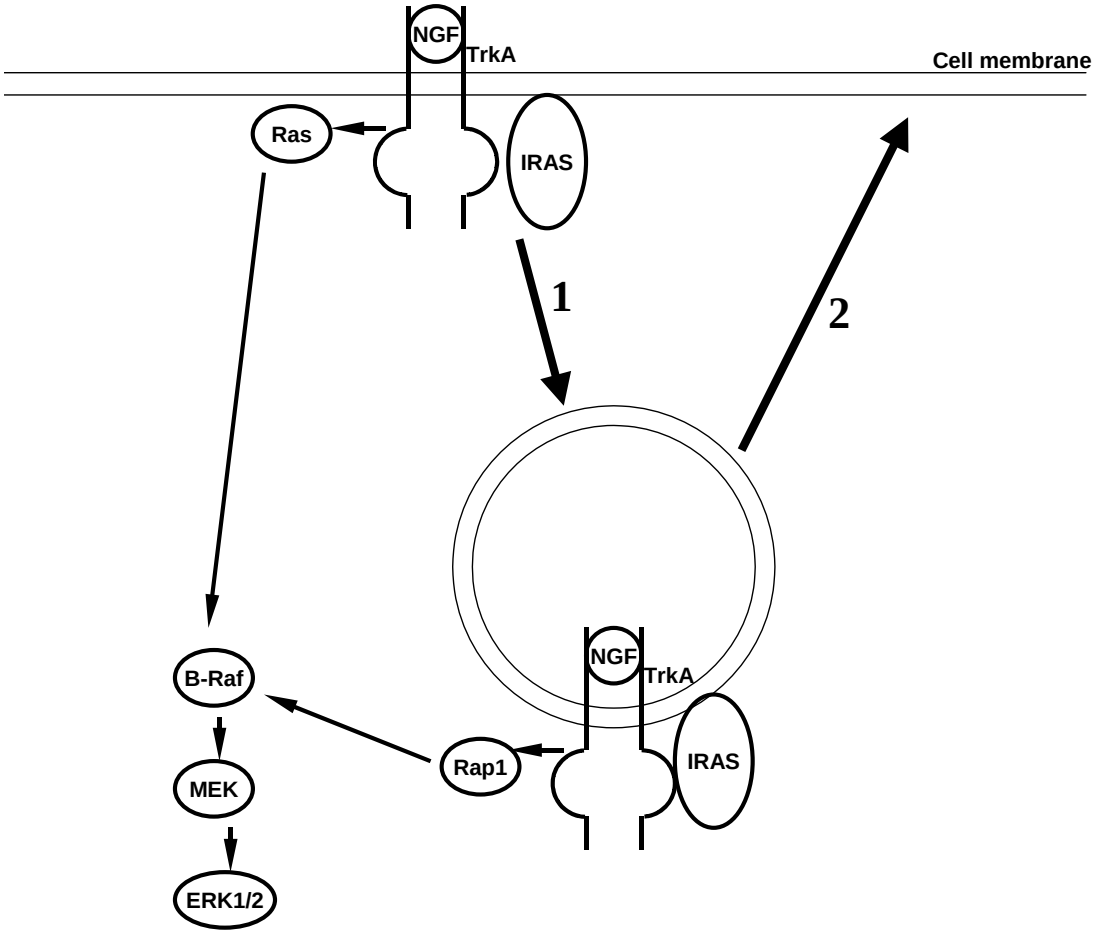


Figure 7.1. Model of IRAS signalling through TrkA trafficking.

IRAS might enhance internalisation of TrkA (1) leading to enhanced short-term Rap1 activation. This Rap1 activation, together with Ras activation would combine to enhance short-term ERK1/2 activation as observed in the IRAS-transfected cells. Furthermore, IRAS may enhance the recycling of TrkA to the cell membrane to enhance ERK1/2 activation (2).

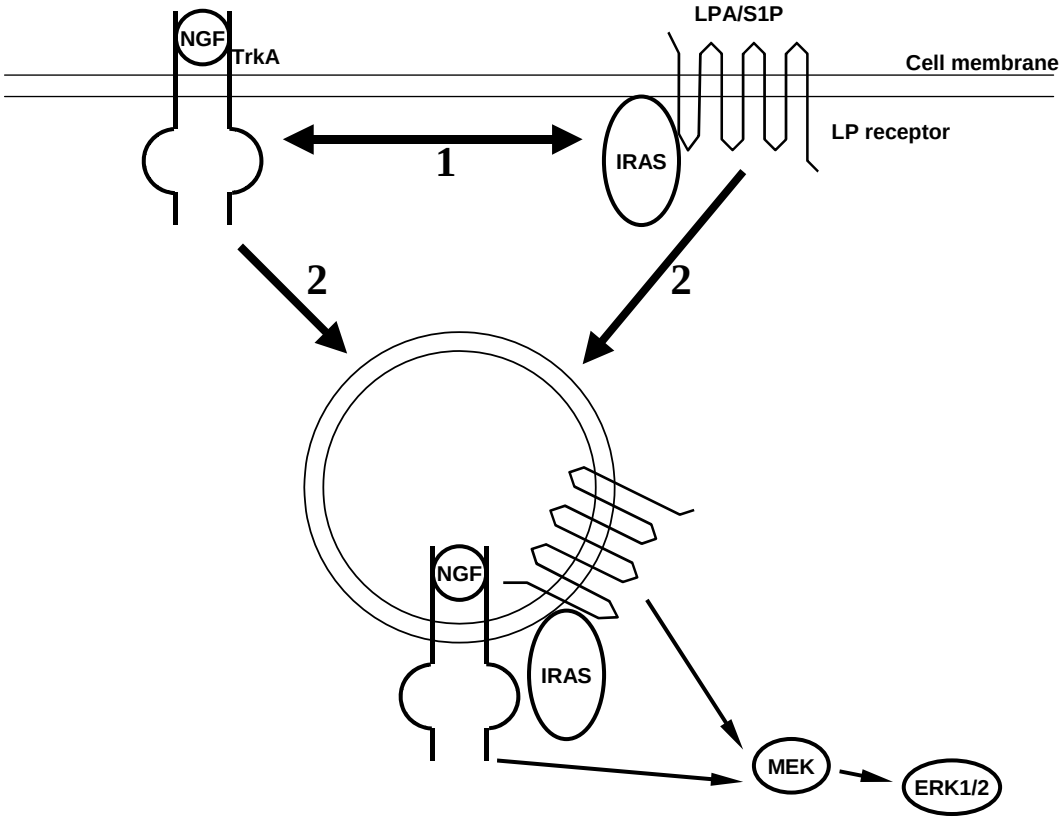


Figure 7.2. Model of IRAS signalling through LP receptor trafficking.

NGF enhances crosstalk between TrkA and LP receptors (1). LP receptors have low levels of activation due to the presence of LPA and S1P in full-serum media. The internalisation and signalling of LP receptors by TrkA and LP receptor agonists is enhanced by IRAS (2). This leads to enhanced short-term ERK1/2 activation.

Appendix I

As discussed in chapter 5, IRAS may enhance NGF-induced ERK1/2 activation by associating with TrkA and regulating its trafficking. To determine whether IRAS forms a complex with TrkA, immunoprecipitations and immunocytochemistry were performed with antibodies to IRAS and Trk. The (rat polyclonal) IRAS antibody was kindly donated by Prof. John Piletz (Loyola Medical School, Chicago, USA). This antibody recognises both human and rat IRAS (Piletz *et al.*, 2000). The mouse-monoclonal Trk antibody (Invitrogen, USA) recognises TrkA, TrkB and TrkC, however, only TrkA is present in PC12 cells.

Immunoprecipitations were carried out in untransfected PC12 cells. The PC12 cells were plated onto PLL (10 μg/ml) coated 6-well plates and grown for 24 hours. The cells were then treated with NGF for between 1 and 60 minutes. Following treatment, the cells were lysed and the lysates incubated with either TrkA antibody or mouse IgG overnight at 4^{o}C. The following day, protein A sepharose CL-4B beads were added to the lysate and incubated for 3 hours at 4^{o}C. The beads were then microcentrifuged for 30 seconds and washed with cold lysis buffer. Following 4 washes with lysis buffer, the lysis buffer was removed and 2X loading buffer added to the beads. The beads were boiled for 5 minutes at 100^{0}C, cooled on ice and microcentrifuged for 1 minute. The loading buffer supernatant was subject to SDS-PAGE and Western blotting using Trk and IRAS antibodies as described in the general methods.

Immunocytochemistry was performed on untransfected PC12 cells plated on laminin (10 μg/ml) coated coverslips in full-serum media as described in the general methods. After 6 hours growth, media was removed and the cells were fixed with 4%

paraformaldehyde, permeabilised with 0.1% triton-X 100 and non-specific binding sites blocked with 2% BSA. The coverslips were incubated with IRAS and Trk antibodies (antibody dilutions ranging from 1:100 to 1:1000 in PBS with 2-10% BSA) in a humidified environment overnight at 4^{o}C. The following day, the cells were washed 3 times for 10 minutes in PBS and then incubated with goat anti-rabbit antibody conjugated to FITC and goat anti-mouse antibody conjugated to Texas red fluorophore (antibody dilutions ranging from 1:200 to 1:2000 with 2-10% BSA) for 1 hour at room temperature in the dark. The coverslips were then washed in PBS 3 times for 10 minutes and mounted on glass slides using 90% glycerol. Cells were viewed and recorded using a Leica SP5 spectral scanning confocal microscope (Leica Microsystems, Germany).

Figure A-1 shows the results of Western blots and immunoprecipitations using the IRAS and Trk antibodies. The IRAS antibody was reported to detect human IRAS at 167 kDa and rat IRAS at 206 kDa (Piletz *et al.*, 2000). However, in the present study bands were detected only at 110 and 65 kDa (figure A-1A). Furthermore, there appeared little difference in the amount detected between vector- and IRAS-transfected cells, suggesting that these may be non-specific bands. It should be noted that the IRAS-transfected cells have only a moderate increase in the expression of IRAS, which may be represented by the increase in the 110kDa band. The Trk antibody was reported to detect TrkA at 140 kDa. However figure A-1B shows that this antibody detected a band at 60 kDa which is unlikely to be TrkA. Unsurprisingly, the immunoprecipitations did not show any relationship between IRAS and TrkA (figure A-1C(i)), with bands at 50 kDa and 25 kDa detected using both the Trk antibody and mouse IgG. These bands are most likely the heavy and

light chains of IgG. Moreover, Western blotting using the Trk antibody failed to detect immunoprecipitated TrkA (figure A-1C(ii)).

Representative images of PC12 cells stained with IRAS and Trk antibodies are shown in figure A-2. Both these antibodies show cytoplasmic staining, however, as demonstrated with the Western blots for these antibodies, this staining is unlikely to be specific for IRAS and TrkA. This is especially true for TrkA, which numerous studies have shown is strongly localised to the plasma membrane and vesicles (Chen *et al.*, 2005; Delcroix *et al.*, 2003; Saxena *et al.*, 2005; Valdez *et al.*, 2005; Valdez *et al.*, 2007; Wu *et al.*, 2001). Similar results were observed using different antibody dilutions (data not shown). Almost no bleed-through was observed.

In summary, the results are inconclusive due to the non-specific binding of the antibodies used. These experiments should be repeated with new antibodies to TrkA and the very recently developed commercial antibodies to IRAS/nischarin.

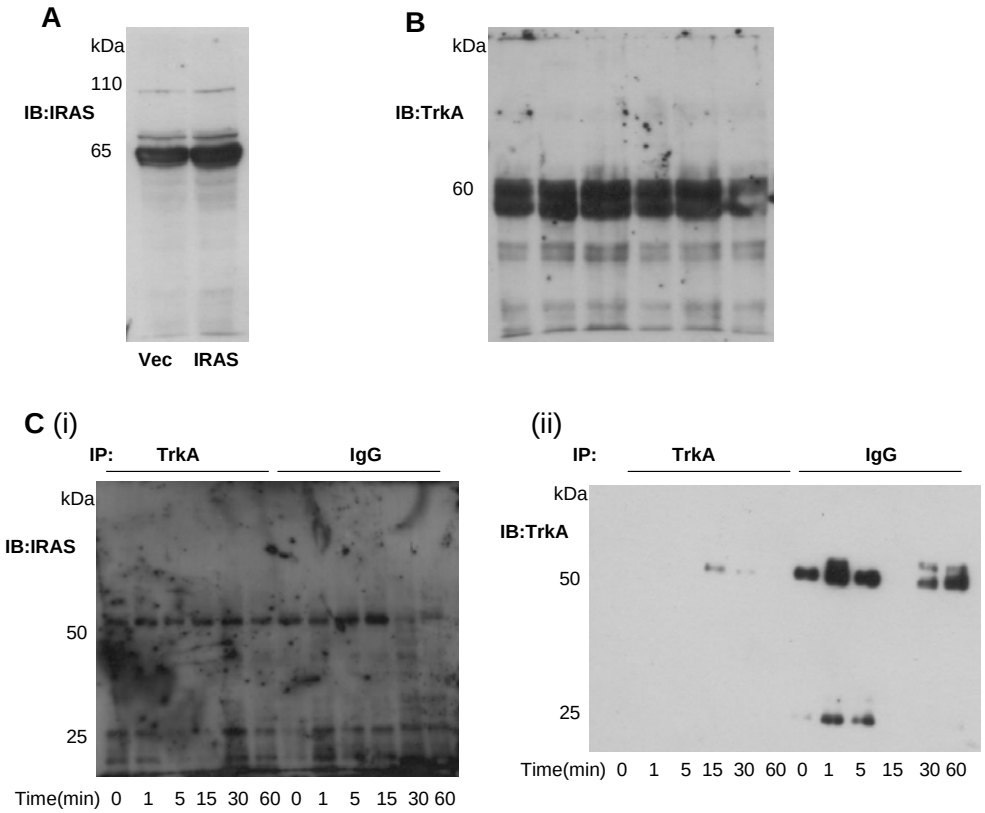


Figure A-1. Western blots and Immunoprecipitations using IRAS and Trk antibodies. **A**. Western blot using IRAS antibody in vector- and IRAS-transfected cells. **B**. Western blot using Trk antibody in untransfected PC12 cells. **C**. Immunoprecipitations of PC12 cell lysates with Trk antibody or mouse IgG followed by Western blotting for IRAS (i) or Trk (ii).

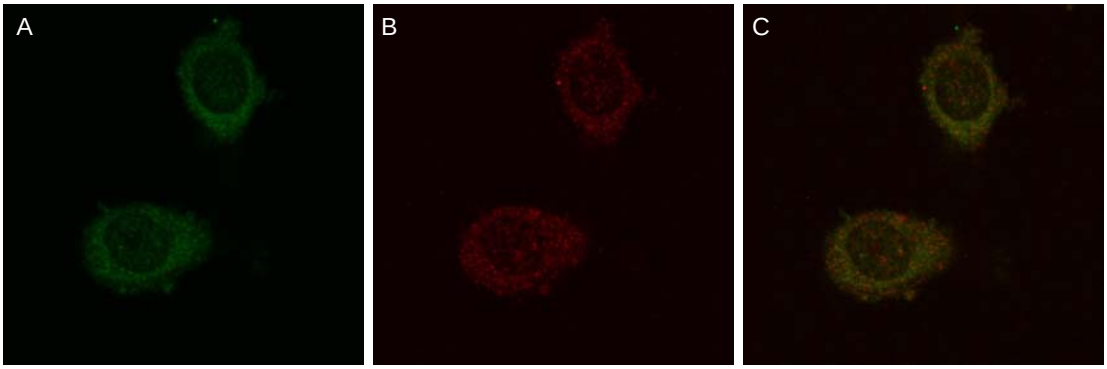


Figure A-2. Immunocytochemistry using IRAS and Trk antibodies

Representative PC12 cells co-stained for IRAS (**A**) and Trk (**B**). Merged image (**C**).

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