Identification of Accessory Subunits that Interact with the Sensory Neurone-Specific Sodium Channel, SNS ($Na_V1.8$)

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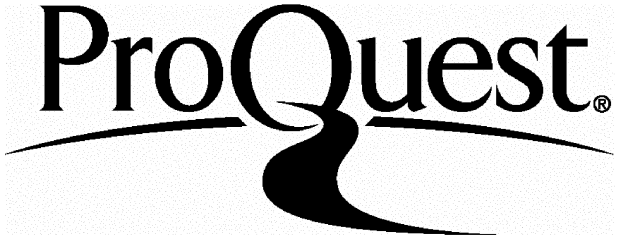

ABSTRACT

Voltage gated sodium channels initiate and propagate action potentials in excitable cells. $Na_V1.8$/SNS is a tetrodotoxin resistant (TTX-r) voltage-gated sodium channel expressed predominantly in the small diameter sensory neurones in the dorsal root ganglia. This channel is involved in transmission of nociceptive information from sensory neurones to the central nervous system. Recent evidence suggests that in addition to accessory β subunits, $Na_V1.8$ requires some distinct subunit to help produce functional expression on the plasma membrane in sensory neurones. Applying Yeast two-hybrid analysis, 27 clones have been identified as interactors for $Na_V1.8$ from a rat DRG cDNA library. Co-immunoprecipitation studies with $Na_V1.8$ have confirmed most of the clones bind strongly to $Na_V1.8$. All the clones were expressed in small diameter sensory neurones as indicated by *in situ* hybridisation. One clone was chosen for further analysis, I-1 or p11 protein. The 11 kDa protein p11 is a light chain in the annexin II (also known as calpactin I or lipocortin II) complex which is a member of the calcium and phospholipid-binding family of proteins known as annexins. Functional experiments were performed to investigate the tissue distribution and subcellular localization in sensory neurones of this clone by Northern blot analysis. Northern blot analysis revealed high level of expression of p11 mRNA in the DRG, a modest level in the heart and low level in the brain. This confirms the co-expression role of p11 with $Na_V1.8$ as these TTX-r channels are expressed in DRG where there is a high level of p11 expression. Introduction of antisense cDNA of the clones into primary culture of sensory neurones by microinjection methods revealed one clone in particular, p11 protein, caused a significant decrease in the sodium current hence showing that p11 is essential for the expression of a functional $Na_V1.8$ channel in DRG. When p11 is transfected into stably transfected $Na_V1.8$ expressing

CHO cells (which do not express a functional $Na_V1.8$ channel despite the existence of a considerable amount of $Na_V1.8$ mRNA and protein in the cytosol), $Na_V1.8$ is translocated to the plasma membrane. p11 elicits the functional expression via a direct interaction to the N-terminus of $Na_V1.8$. This expressed channel displayed a $Na_V1.8$ like current thus indicating that p11 is acting as regulatory protein that is necessary for the expression of a functional channel. Regulation of $Na_V1.8$ expression by distinct subunits is a good target for possible pain management.

CONTENTS

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ABBREVIATIONS

AD	Transcriptional activation domain
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CNS	Central nervous system
DB	DNA-binding domain
DIG-11-UTP	Digoxigenin-11-uridine-5'triphosphate
DMEM	Dulbecco's modified Eagles medium
DRG	Dorsal root ganglian
E	Embryonic
GDNF	Glial derived neurotrophic factor
GFP	Green flourescent protein
GST	Glutathione S-transferase
HA	Haemagglutinin
HAMS	Nutrient Mixture F-12 medium
HIS	Histidine
IASP	International Association for the Study of Pain
IB4	Isolectin
IPTG	Isopropyl-β-D-thiogalactoside
LB	Luria Broth
LEU	Leucine
LMT	Low threshold mechanoreceptive neurone
MS	Multiple sclerosis
NBT	Nitroblue tetrazolium salt
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PC	Phenol/Chloroform
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
TRP	Tryptophan
TTX-r	Tetrodotoxin resistant sodium channel
TTX-s	Tetrodotoxin sensitive sodium channel
UAS_G	Upstream activating sequence
URA	Uracil
X-Gal	5bromo-4chloro-3indolyl β D-galacto pyranoside
YNB	Yeast Nitrogen Base

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Primary Antibodies:

Rabbit Anti-p11 polyclonal antibody - Biodesign International.

L-periaxin polyclonal antibody - Gift from Dr. Peter Brophy, Edinburgh University.

Anti-HA monoclonal mouse antibody - AutogenBioclear, Wiltshire, UK

Anti-GFP monoclonal mouse antibody - Roche, UK

Anti-SNS11 polyclonal antibody - Dr. Kenji Okuse, UCL, London

Secondary Antibodies:

Anti-mouse IgG conjugated with Horseradish Peroxidase - Jackson Laboratories, UCL, London.

Anti-Rabbit IgG conjugated with FITC - Jackson laboratory, UCL, London

CHAPTER 1.
GENERAL INTRODUCTION

1.1. General:

The remarkable range of human behaviour depends on a sophisticated array of sensory receptors providing input to the brain. The continuous stream of information is organised by the brain into perception and appropriate behavioural responses. The brain accomplishes all this by using electrically excitable neurones and the connections between them to relay information.

1.2. Nervous system:

1.2.1. Central Nervous System:

The spinal cord receives information from the skin, viscera, joints and muscles of the trunk and limbs and contains motor neurones responsible for both voluntary and reflex movements. The spinal cord is divided into grey matter and surrounding white matter. The grey matter which contains nerve cell bodies is divided into dorsal and ventral horns. The dorsal horn contains sensory relay neurones, which receive information from the periphery while the ventral horn contains motor nuclei which innervate specific muscles.

1.2.2. Peripheral nervous system:

The brain processes a continuous stream of information about the environment both external and internal. This information is supplied by the peripheral nervous system (PNS) and the PNS also carries out motor commands generated in the brain and spinal cord. The peripheral nervous system is divided into sensory and autonomic nervous systems.

1.2.2.1. Autonomic Nervous System:

The autonomic nervous system mediates visceral sensation as well as motor control of the viscera, smooth muscles and exocrine glands. It consists of sympathetic and parasympathetic and enteric systems. Sympathetic neurones participate in the body's response to stress, while the parasympathetic system acts to consume body resources and restore homeostasis. The enteric system controls the function of smooth muscle of the gut.

1.2.2.2. Sensory Nervous System:

The sensory nervous system includes the sensory neurones that innervate the skin, muscle and joints. The cell bodies of these sensory neurones lie in the dorsal root ganglia (DRG) and the cranial ganglia. Receptors associated with DRG and cranial ganglia provide sensory information to the CNS about muscle and limb position and about touch and pressure at the body surface.

1.3. Nociceptors:

Nociceptors are damage sensing neurones. Information from the internal and external environment is conveyed to the central nervous system via primary afferent neurones. These include low intensity, low threshold neurones that gives rise to sensations other than pain and high threshold or nociceptive neurones that are attributed by high intensity potentially damaging stimuli and often result in the sensation of pain. Harmful stimuli to the skin or subcutaneous tissue such as joints or muscle activate several classes of nociceptor terminals, the peripheral endings of primary sensory neurones whose cell bodies are located in dorsal root ganglia and trigeminal ganglia. Free nerve terminals contain receptors capable of transducing chemical, mechanical

and thermal signals. There are two main groups of nociceptive somatic primary afferents defined according to their fibre size/conduction velocity. These groups are the A fibres which are myelinated and C fibres are unmyelinated. C fibre neurones are thought to be the most abundant nociceptive neurones. Nociceptive DRG have fibres that conduct in the C, Aδ and Aα/β fibre conduction velocity.

Properties of nociceptive compared with non-nociceptive DRG neurones are categorised into two groups. Group A properties, which are the common type of property of nociceptive neurones, include longer action potential duration and slower maximum rate of fibre firing. The properties of group A are graded according to conduction velocities group of the fibres. The most prominent form of group A properties is shown by C fibres and these become less prominent for Aδ fibres and the least prominent is the Aα/β fibres. Some of the A-type properties are also expressed in the same manner in groups of non-nociceptive low threshold mechanoreceptive neurone (LMT) (Lawson, 2002). Several studies have confirmed that a substantial proportion of A-fibre nociceptive neurones conduct in the Aα/β fibre range (Ritter & Mendel, 1992, Djouhri *et al,* 1998). Aδ and C fibres can be further divided according to the stimuli that they sense. They can respond to mechanical, chemical or thermal (heat and cold) stimuli, or to a combination of these (polymodal). Nociceptors membranes are thought to contain proteins that convert thermal and mechanical or chemical energy of noxious stimuli into a depolarising electrical potential. One such protein is the capsaicin receptor VR1 (Caterina *et al,* 1997). This is found exclusively in primary afferent nociceptors and mediates the pain producing actions of capsaicin, low pH and noxious heat. Thermal nociceptors are activated by extreme temperature. Mechanical nociceptors are activated by intensive pressure applied to the skin whilst

polymodal nociceptors are activated by high intensity mechanical, chemical or thermal stimuli.

1.4. Pain:

Pain is defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage" (IASP). It serves an important protective function. The pain system consists of nociceptors that are specialised sensory neurones in the peripheral nervous system that detect noxious stimuli. Primary nociceptive afferent fibres, normally Aδ and C fibres, transmit information regarding noxious stimuli to the dorsal horn of the spinal cord. Ascending nociceptive tracts convey nociceptive stimuli from the dorsal horn to the thalamus and cortex and descending pathways allow higher centres of the CNS to modify nociceptive information. Hyperexcitability and increased baseline sensitivity of primary sensory neurones following nerve injury can lead to abnormal activity associated with pain. Studies have demonstrated that following axonal injury, neurones display changes in excitability associated with altered sodium channel expression.

Multiple sodium channels are expressed within DRG. Changes in sodium channel gene expression can produce electrophysiological changes in DRG neurones which cause them to fire spontaneously or at high frequencies (Amir *et al,* 2002). The dynamic nature of sodium channel gene expression in DRG neurones and the changes occurring in sodium channels and current in these cells following axonal injury and in inflammatory pain models suggest that abnormal expression of sodium channels contributes to the molecular pathophysiology of pain. Altered patterns of sodium channel transcripts as well as post-translation modification of α-subunits have been

observed in different pain states. (Black *et al,* 1999; Waxman, 1999; Wood & Baker, 2001).

1.4.1. Acute pain:

The perception of acute pain is a complex interaction that involves sensory, emotional and behavioural factors. Many forms of pain arise from direct activation or sensitisation of primary afferent neurones, especially C-fibres. Surgical trauma or other noxious stimuli associated with acute pain are associated with an injury response or inflammatory response. Acute pain can arise from cutaneous, deep or visceral structures. Acute pain is initiated by the high threshold nociceptors innervating peripheral tissues.

1.4.1.1. Peripheral Sensitisation:

The sensitivity of the peripheral terminals of DRG neurones is not fixed and activation by repeated peripheral stimulation increases the excitability of the terminal and decreases the threshold for initiation of an action potential in the primary sensory neurone. This phenomenon is referred to as peripheral sensitisation.

1.4.2. Chronic pain:

Chronic pain is a major symptom of peripheral neuropathy whether induced by disease or by direct physical trauma to the peripheral nerves. Spontaneous and/or evoked hyperexcitability of the peripheral nerve after injury is considered to be a principal feature of the underlying pathophysiology associated with many chronic pain syndromes. Such neuropathic pain is often debilitating and resistant to therapeutic intervention. A prominent molecular basis for this abnormal, repetitive firing of

injured primary afferents is an increased membrane density of sodium channels at focal sites of injury (England *et al,* 1996). After axotomy the TTX-resistant current is reduced in small diameter neurones with the mRNA and protein expression reduced in parallel (Dib-Hajj *et al,* 1996; Novakovic *et al,* 1998). Porreca et al (1999) used antisense oligonucleotides to target $Na_V1.8$ expression and found there was a knock-down of $Na_V1.8$ protein in DRG neurones and this prevented hyperalgesia and allodynia caused by spinal nerve ligation injury.

1.4.3. Neuropathic pain:

Neuropathic pain results from direct injury to nerves in the peripheral or central nervous system and can often result in a burning sensation. Neuropathic pain also includes the syndromes of reflex sympathetic dystrophy and post-herpetic neuralgia, a severe pain that occurs in patients after a bout of shingles.

1.4.4. Inflammatory pain:

Inflammation is a critical protective reaction to irritation; injury characterised by swelling, redness and pain. The pain is due to activation and sensitisation of primary afferent nerve fibres. Inflammatory mediators like bradykinin and prostaglandins act on specific targets and cause the local release of other mediators from leucocytes and also further attract leucocytes to the site of inflammation. Inflammatory pain is experienced predominantly when the site of inflammation is mechanically stimulated by being moved or touched and this tenderness or lowered threshold for stimulation induced pain is known as hyperalgesia thought to be produced by inflammatory mediators. Allodynia is a pain response to light tactile stimuli such as touch or brushing of the skin.

1.5. Voltage-gated Sodium Channels:

Voltage-gated sodium channels are responsible for the rising phase of the action potential in membranes of neurones and electrically excitable cells. Voltage clamp studies were used by Hodgkin and Huxley (1952) to identify two major components in ionic current with separate kinetics (I_{Na} and I_K). Both have voltage dependent kinetics and account for the action potential. The Hodgkin and Huxley model of nerve action potential is based on electrical measurements of the flow of ions across the membrane of an axon, using the voltage clamp technique. Hodgkin and Huxley stated that sodium channels activate and then inactivate and these ions were responsible for the inward component of current in response to a change in voltage. Activation is the rapid process that opens sodium channels during depolarisation. A quick reversal of activation accounts for the rapid closing of the channels. Inactivation is a slower process than activation.

Models for sodium channel function were obtained from studies carried out on giant squid axons in the 60's and 70's. Biochemical methods led to the discovery of the sodium channel protein in 1980. Photoaffinity labelling with a photoreactive derivative of an α-scorpion toxin identified the principal α-subunit and the auxiliary β1 subunit of the brain Na^+ channel (Beneski & Catterall, 1980). Subsequent studies revealed that the brain sodium channel is made up of α-subunit, β1 and β2 subunits (Hartshorne & Catterall, 1981). Partial purification of tetrodotoxin ((TTX) a neurotoxin purified from puffer fish) binding proteins from electric eel electroplax revealed correlations between TTX binding activity and the presence of sodium channels (Agnew *et al,* 1980).

Noda et al (1986) isolated cDNAs encoding the entire polypeptide from expression libraries of electroplax eel mRNA. The deduced amino acid revealed a large protein with four internally homologous domains each containing six transmembrane segments. RNA encoding the α-subunit proved sufficient for functional expression of sodium currents in Xenopus oocytes (Goldin *et al,* 1986; Noda *et al,* 1986) but β-subunits are required for normal kinetics and voltage dependence of gating as shown by Isom et al (1992, 1995). β1 and β2 subunits have similar structures but are not closely related in amino acid sequence. Both β subunits have a large glycosylated extracellular domain, a single transmembrane segment and a small intracellular domain. Comparison of the primary structures of β1 and β2 subunits to those of other proteins revealed a structural relationship to the family of proteins that contain Ig-G like folds (Isom 1995). The extracellular domain of the subunits was predicted to fold like myelin protein P_O.

All sodium channels have similar permeation properties and are therefore expected to have similar selectivity filters. However cardiac sodium channels bind TTX with a lower affinity because of a change of a tyrosine or phenylalanine residue located in brain and skeletal muscle α-subunit to cysteine in cardiac sodium channel α-subunit (Backx *et al,* 1992). A serine in this position in some peripheral nervous system sodium channels causes even larger decreases in TTX binding (Sivilotti *et al,* 1997).

DRG are known to express multiple sodium currents with different voltage dependence and pharmacological properties. These include $Na_V1.1$ and $Na_V1.6$ which are tetrodotoxin sensitive (TTX-s) channels and expressed in high levels in DRG and also in other neuronal cell types within the CNS. $Na_V1.7$ (PN1) is expressed

preferentially in DRG and produces a fast transient and slowly inactivating TTX-s current (Toledo-Aral *et al,* 1997). $Na_V1.8$ (SNS) is expressed in the small diameter DRG neurones and encodes a tetrodotoxin-resistant (TTX-r) sodium current. $Na_V1.9$ (NaN) is expressed preferentially in small DRG and trigeminal neurones and encodes for a TTX-resistant sodium channel (Dib-Hajj *et al,* 1998). Another sodium channel cloned from an astrocyte library and at first considered to be glial specific is also expressed preferentially at high levels within the DRG neurones (NaG) (Black *et al,* 1996). In normal DRG, the levels of $Na_V1.2$ and $Na_V1.3$ is very low but after spinal nerve ligation the level of $Na_V1.3$ is increased while the levels for $Na_V1.2$ is decreased (Kim *et al,* 2001).

1.5.1. Voltage dependent activation:

The S4 segments of the α-subunit contain repeated motifs of positively charged amino acid residues followed by two hydrophobic residues potentially creating a cylindrical α-helix with a spiral ribbon of positive charge around it (see figure 1.1). The positively charged amino acids residues are stabilised by forming ion pairs with negatively charged residues in adjacent transmembrane segments. When the membrane is depolarised the S4 segment moves outwards initiating a conformational change that opens the pore (Yang *et al,* 1996). Stuhmer *et al,* 1989, provided the first experimental evidence that the S4 segment was the voltage sensor of the sodium channels and the positive charge in the S4 segments are the gating charges.

1.5.2. Inactivation:

Sodium channels inactivate within a few milliseconds of opening. The loop connecting domains III and IV forms an activation gate which folds into the channel structure

during inactivation (Vassilev *et al,* 1988). Cutting the loop between these domains greatly slows inactivation (Stuhmer *et al,* 1989). Mutagenesis studies of this region revealed a hydrophobic triad of isoleucine, phenylalanine and methionine residues that is critical for fast inactivation (West *et al,* 1992) and peptides containing this motif can serve as pore blockers and restore inactivation to sodium channel having a mutated inactivation gate.

1.6. Sodium channel protein family:

As illustrated in table 1.1 there are at least 10 sodium channel genes that have been identified and functionally expressed and are more than 50% identical in amino acid sequence in the transmembrane and extracellular domain. Sodium channels $Na_V1.1$ (type I), $Na_V1.2$ (type II), $Na_V1.3$ (type III) and $Na_V1.6$ (typeVI) are very similar to each other in amino acid sequence and are highly expressed in brain and spinal cord. Sodium channel $Na_V1.4$ (type μ1) is primarily expressed in skeletal muscle, and sodium channel $Na_V1.5$ (type h1) is primarily expressed in adult heart and developing skeletal muscle. $Na_V1.7$ (PN1), $Na_V1.8$ (SNS) and $Na_V1.9$ (NaN) are primarily expressed in DRG neurones. All the above 9 sodium channel isoforms are considered to be members of one family. $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.7$ are highly TTX sensitive (TTX-s) and broadly expressed in neurones. Their genes are all located on human chromosome 2 consistent with a common evolutionary origin. $Na_V1.5$, $Na_V1.8$, and $Na_V1.9$ are also closely related and are TTX resistant (TTX-r) to varying degrees, due to changes in amino acid sequences at a single position in domain 1, and they are expressed in heart and DRG. The genes for these channels are located on chromosome 3, consistent with a common evolutionary origin. In addition to these 9 sodium channels that have been functionally expressed, closely related sodium channel like

proteins have been cloned from mouse, rat and human and expressed in glial cells as well as in the DRG neurones (e.g NaG see table 1.1).

1.7. Accessory subunits:

The α-subunits of the voltage-gated sodium channels, form the central aqueous pore, and are associated with accessory β subunits that modulate channel properties and interact with cytoskeletal and extracellular matrix proteins. The biochemical purification of two proteins, β1 and β2, that associate with α-subunits allowed the properties of these accessory factors to be investigated in heterologous expression systems. Molecular cloning has recently identified a β1-like member of the family named β3 (Kazen-Gillespie *et al,* 2000), which shows a complementary pattern of expression to the β1 subunit and a splice variant of β1 named β1A (Morgan *et al,* 2000). Early studies focused on the role of these subunits in enhancing levels of channel expression and altering kinetic properties. However recent evidence suggests that β subunits may play an important role as cell adhesion molecules in determining the pattern of expression of functional α-subunits (Malhotra *et al,* 2000). β subunits are transmembrane proteins that contain an extracellular immunoglobulin domain that is homologous to the V-set of the immunoglobulin superfamily that includes cell adhesion molecules (Isom & Catterall, 1996). Figure 1.1 shows an overall view of the α-subunit of the sodium channel with accessory subunits.

Channel Name	Gene Symbol	Original Name	Tissue	Present in DRG	TTX Sensitivity
$Na_V1.1$	SCN1A	Type I	CNS	YES	+
$Na_V1.2$	SCN2A	Type II	CNS	YES	+
$Na_V1.3$	SCN3A	Type III	CNS	YES	+
$Na_V1.4$	SCN4A	SkM1, μ1	Skeletal Muscle	NO	+
$Na_V1.5$	SCN5A	SkM2, rH1	Heart	NO	--
$Na_V1.6$	SCN8A	NaCh6, PN4	CNS, PNS	YES	+
$Na_V1.7$	SCN9A	PN1	PNS	YES	+
$Na_V1.8$	SCN10A	SNS, PN3	PNS	YES	--
$Na_V1.9$	SCN11A	NaN, SNS2	PNS	YES	--
Na_X	SCN7A	NaG	PNS	YES	

Table 1.1. Mammalian sodium channel α-subunits showing the new nomenclature along with the gene and old name. Distribution in the nervous system is also displayed and the sensitivity to tetrodotoxin (TTX) toxin is also indicated.

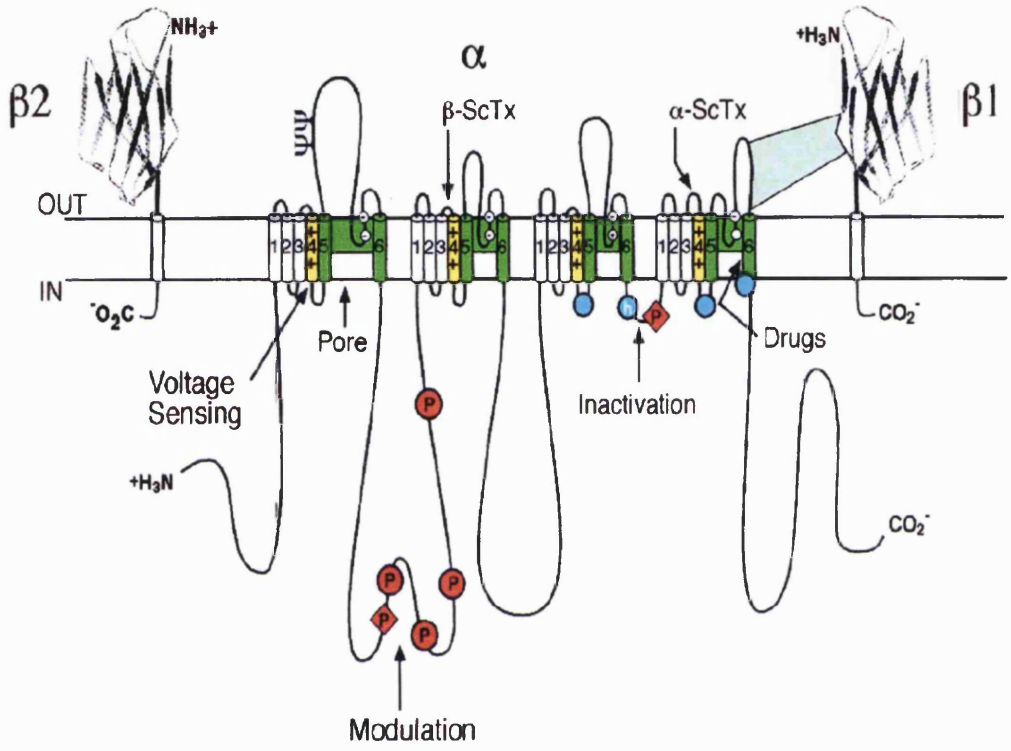


Figure 1.1. The primary structure of the voltage-gated sodium channels. The extracellular domains of β1 and β2 subunits are shown as Ig-like folds. ψ-sites of probable N-linked glycosylation. P-sites of demonstrated protein phosphorylation by PKA (circles) and PKC (diamonds). Sites of interaction between α and β1 subunit is also shown. α-ScTx and β-ScTx are sites of α and β-scorpion toxin respectively. [Reproduced from Catterall, 2000].

1.7.1. Role of β subunits

The β1 and β2 subunits from rat brain are not closely related in terms of amino acid sequence, but each contains a single membrane spanning segment that separates a large extracellular amino terminal from a smaller intracellular carboxy terminal (Isom, et al 1992, 1995).

1.7.1.1. β1:

The β1 accessory subunit is non-covalently associated with the α-subunit. Co-expression of β1 subunit with rat brain $Na_V1.2$ (type II) sodium channel or skeletal muscle ($Na_V1.4$) α-subunit in *Xenopus* oocytes increased the size of the peak sodium current, accelerated its activation, and shifted the voltage dependence of inactivation to more negative potentials. This indicates that β1 is crucial in the assembly, expression and functional modulation of the rat brain sodium channel heterotrimeric complex. (Isom *et al,* 1992; Smith & Goldin, 1996, 1998; Patton *et al,* 1994; Schreibmayer *et al,* 1994). Chen & Cannon (1995) showed through deletion analysis that the intracellular domain of human β1 is not required for modulation of the skeletal muscle α-subunit, whereas the extracellular domain is sensitive to deletion mutagenesis. In addition, the β1 extracellular domain together with proximal residues of transmembrane domain, was found to be sufficient for modulation of the skeletal muscle Na^+ channel in chimeric subunits formed with β2 subunits (Makita *et al,* 1996). McCormick *et al,* 1998 demonstrated through deletion mutagenesis and chimeric protein analysis that the extracellular domain of β1 forms an Ig fold that is essential for expression and function of this subunit. Co-expression in mammalian cells of $Na_V1.2$ (SCN2a) α-subunit and β1 increased the level of sodium channels at the plasma membrane two- to four fold (Isom *et al,* 1995) showing that β1 subunit

expression is a critical regulator of sodium channel density in the plasma membrane of transfected cells. β1 does not appear to modulate all sodium channel α-subunits. This is seen from the observation that $Na_V1.8$ (SNS) α-subunits are not affected by co-expression of β1 (Sangameswaran *et al,* 1996). These results suggest that other β1-like subunits may be present in sensory neurones. β1 is only expressed after birth in the developing brain (Patton *et al,* 1994). From developmental time course studies, β1 expression in rat forebrain showed multiple size bands at earlier time points; these bands were also present in adrenal glands, heart and skeletal muscle. Qu et al (1999) demonstrated through studies with chimeras that segment IVSS2-S6 of the α subunit played an important role in modulation of gating by β1. They proposed that this segment may be one site of interaction for the β1 molecule.

1.7.1.2. β1A:

In an attempt to identify other β1-subunit isoforms a rat adrenal cDNA library was screened from which a cDNA clone was isolated that had identity to the 5' region of β1 followed by a novel 3' end. This β1 isoform, β1A, was a splice variant of β1 (Kazen-Gillespie *et al,* 2000). The developmental time course of β1 vs. β1A mRNA expression in rat brain showed β1A is expressed early in embryonic development. Its expression declines to undetectable levels after birth, concomitant with the expression of β1. Immunohistochemical analysis of β1A expression revealed that it is expressed in adult DRG, spinal cord and heart. Functional co-expression of $Na_V1.2$ (SCN2a) with β1A in transfected Chinese hamster lung fibroblast resulted in a 2.5 fold increase in current density compared with cells expressing α-subunit alone. This increase in current density reflected two distinct effects of β1A, one is the increase in the

proportion of cells expressing detectable sodium currents and secondly an increase in the level of functional sodium channels expressing cells (Karen-Gillespie *et al,* 2000). These increases in $Na_V1.2$ sodium channel expression in mammalian cells with β1A are similar to the ones obtained for β1 (Isom *et al,* 1995) and in *Xenopus* oocytes (Isom *et al,* 1992).

1.7.1.3 β2:

Partial proteolytic maps showed that β1 and β2 subunits of brain Na^+ channel are distinct and unrelated in terms of amino acid sequences (Messner & Catterall, 1985).

β2 is associated to the α-subunit via a disulphide link (Hartshorne *et al,* 1982). Sequence analysis of β2 revealed that its extracellular domain contains an immunoglobulin like fold and an extended region with similarity to cell adhesion molecule contactin (Isom *et al,* 1995).

Co-expression of β2 with α-subunits in *Xenopus* oocytes caused an increase in functional expression of sodium channels, an increase in the fraction of α-subunits gating in a fast mode, and a small negative shift in the voltage dependence of channel inactivation similar to the effects observed with β1 (Isom *et al,* 1995). Expression of higher levels of β2 also caused a four fold increase in the capacitance of *Xenopus* oocytes, which resulted primarily from an increase in the number and surface area of the plasma membrane microvilli. This β2 mediated increase in membrane capacitance did not depend on co-expression with the α-subunit. β2 is not required for Na^+ channel function as it is only found in neuronal Na^+ channels and not in muscle. The sequence similarity of β2 subunits to contactin, their ability to expand the cell surface

membrane, and their appearance in developing neurones and axons suggested that they may modulate cell surface expression and function of sodium channels during neurogenesis and synaptogenesis (Isom *et al,* 1995).

1.7.1.4. β3:

Morgan *et al,* (2000) have identified a rat and human form of β3 which is closely related to β1 but exhibits a complementary distribution in the rat central nervous system. The coding region of rat β3 nucleotide sequence is 57% identical to rat β1 and 40% identical to rat β2 and is a separate gene product. As with β1 and β2, β3 has an extracellular domain which shows homology to proteins that adopt a V-type Ig fold (Harpaz & Chothia, 1994). Of these proteins, the extracellular domain of β3 has the highest sequence identity to myelin P_0 (Shapiro, 1996). The known structure of myelin P_0 was used as a template to model β3 and thus identify regions of functional importance and facilitate a comparison with the previously predicted β1 structure (M^{C}Cormick *et al,* 1998). When co-expressed in *Xenopus* oocytes with rat $Na_V1.2$ α-subunit, β3 causes slower inactivation of Na^+ channel opening than β1 (Morgan *et al,* 2000).

1.8. Sodium channel expression after axonal injury:

Waxman *et al,* (1994) studied the pattern of expression of sodium channel gene following axonal injury. They found that there was an up-regulation of previously silent $Na_V1.3$ (Type III) gene in DRG neurones following axotomy within the sciatic nerve. This was followed by down-regulation of $Na_V1.8$ gene expression which can persist for about 210 days after axotomy and there was also down-regulation of $Na_V1.9$ gene. Since the genes are down-regulated, it was expected that the TTX-r

sodium currents will also be reduced in these cells and this was found to be the case. There was an attenuation of the TTX-r sodium currents after axotomy, but in addition to this there was a switch in the properties of TTX-s currents. There was an emergence of a rapidly-repriming current (a current that recovers rapidly from inactivation) (Cummins & Waxman, 1997).

1.9. Developmental expression of TTX-r Sodium Channels in primary sensory neurones:

Primary sensory neurones show three specific patterns of action potentials in development. During a period of neurite outgrowth in early embryonic development DRG neurones are electrically unexcitable. This is followed by a period of low frequency spontaneous activity at embryonic (E) day 16-E20. Finally, after the formation of the central synapses and peripheral terminals, the spontaneous activity is replaced by higher frequency sensory evoked activity at which time the neurones adapt to a more differentiated pattern of excitability (Fitzgerald *et al,* 1987). The sodium currents of DRG are divided into 2 kinds depending on their basis to sensitivity to TTX. All primary sensory neurones express a low activation threshold, fast inactivating TTX-s current (Caffrey *et al,* 1992). Small diameter neurones also display a high activation threshold, slowly activating and inactivating TTX-r current (Elliot & Elliot, 1993). The TTX-s current is mediated by $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.6$, $Na_V1.7$ whereas TTX-r current is mediated by $Na_V1.8$ and $Na_V1.9$.

1.10. Regulation of sodium channels:

1.10.1. Phosphorylation:

The properties of voltage gated sodium channels that determine both the threshold and frequency of the generated action potential can be modulated by phosphorylation. In the intracellular regions of these channels there are several consensus phosphorylation sites for protein kinase A and C. In cultured neurones and intact synaptosomes substantial cAMP dependent phosphorylation of $Na_V1.2$ was seen to occur (Costa & Catterall, 1984). Smith & Goldin (1996) have also shown that brain $Na_V1.2$ sodium channel can be phosphorylated by protein kinase A on the four serine residues on the loop between domain I and domain II. Dascal & Lotan (1991) have also shown that phosphorylation by protein kinase C on the occurs on serine residue in domain III of $Na_V1.2$ sodium channel.

1.10.2. Inflammatory mediators:

England *et al,* 1996 and Gold *et al,* 1996 in their studies have independently shown that several inflammatory mediators released in response to injury are capable of directly sensitising a subpopulations of primary afferent neurones. PGE2, serotonin and adenosine were seen to decrease the activation threshold and increase the rate of activation and inactivation and increase the magnitude of TTX-r Na^+ currents.

Studies performed *in vivo* suggested that directly acting inflammatory mediators over a period of few minutes including PGE_2, serotonin and adenosine produce hyperalgesia via a cAMP/protein kinase A (PKA) second messenger cascade. The effect of these compounds were mimicked by compounds that increase intracellular

concentration of cAMP, prolonged by agents that blocked the breakdown of cAMP and blocked by agents that inhibit adenylate cyclase or PKA (Aley & Levine, 1999).

It is well established that inflammatory mediators can modulate TTX-r currents in DRG. Recently it has been shown that sodium channel gene expression is altered in inflammatory models of pain. Tanaka *et al*, (1998) have shown that there is an increase in $Na_V1.8$ mRNA expression following carageenan injection into the hind paw with an increase in the amplitude of the TTX-r sodium current consistent with the idea of abnormal sodium channel expression to the pathophysiology of inflammatory pain, Tate et al (1998) have reported an increase in $Na_V1.9$ mRNA following injection of complete Freunds adjuvant and Gould et al (1998) reported an increase in sodium channel immunoreactivity following injection of Freund's adjuvant.

Fitzgerald *et al*, 1999 showed with site-directed mutagenesis of the TTX-r sodium channel $Na_V1.8$ that this channel is phosphorylated after activation of PKA. PKA induced phosphorylation results in changes in gating properties similar to those induced by inflammatory mediators suggesting that inflammatory mediator induced modulation of TTX-r currents reflects a direct phosphorylation of the channel.

1.10.3. NGF:

Damage sensing small diameter neurones fall into two categories with distinct trophic factor requirements. About one-half of the neurones express the nerve growth factor (NGF) specific tyrosine kinase receptor trkA and p75 and are $IB4^-$, whereas a separate population which can be surface labelled with isolectin B4 ($IB4^+$) are trkA and p75 negative and have a trophic requirement for glia-derived neurotrophic factors (GDNF)

and express another tyrosine kinase receptor c-ret. $Na_V1.8$ is expressed in these two distinct types of sensory neurones, one bearing trkA and p75 receptors and another expressing GDNF receptor c-ret.

As well as inflammatory mediators, there is evidence that NGF is a key regulator of nociceptive thresholds. NGF application has been shown to cause dramatic decreases in thermal thresholds of pain perception in animal models and NGF levels rise in damaged tissues. NGF is known to act through the high affinity receptor trkA to increase the TTX-resistant current in PC12 cells (Omri & Meiri, 1990) in culture. Omri & Meiri have also demonstrated the induction of Na^+ currents by NGF in DRG neurones in serum-free medium. Recent evidence has shown that NGF up-regulates the expression of $Na_V1.8$ transcript *in vivo* in axotomised neurones in rat (Waxman *et al,* 1999). They found that *in vitro* NGF delivered to DRG cell bodies acts to down-regulate $Na_V1.3$ mRNA and maintains high levels of $Na_V1.8$ mRNA. Dib-Hajj *et al,* (1998a) extended the study *in vivo,* and showed that following axotomy, the administration of exogenous NGF to the proximal nerve stump results in partial rescue of $Na_V1.8$ mRNA levels and of TTX-resistant currents in small DRG neurones. These observations suggest that some of the changes observed following axotomy in the sciatic nerve are due to loss of access to peripheral pools of neurotrophic factors. Although $Na_V1.8$ does not appear to be completely dependent on NGF for expression, there is up-regulation of both the transcript and the protein together with the appearance of an unusual and non-functional splice variant in addition of NGF to sensory neurones in culture (Okuse *et al,* 1997).

In contrast to the up-regulation of TTX-r currents with inflammatory mediators, a variety of manipulations that leads to neuropathic conditions leads to down-regulation of $Na_V1.8$. This suggests that $Na_V1.8$ may play a more significant role in inflammatory than neuropathic pain states. Altered sodium channel activity in peripheral neurones is associated with the development of inflammatory and neuropathic pain where altered patterns of sodium channel transcripts, as well as changes in post-translational modifications, have been observed (Waxman *et al,* 1999; Black *et al,* 1999; Dib-Hajj *et al,* 1999). Neuropathic pain that results from direct damage to peripheral nerves seems to result from ectopic action potential propagation initiated at the site of injury, this activity is tetrodotoxin sensitive (Lyu *et al,* 2000). Trophic factors such as NGF are known to have important effects in regulating sodium channel expression. Overexpression of NGF results in large increases in $Na_V1.2$ and β2 transcripts in hyper-NGF mice and mRNA levels for $Na_V1.1$, Na_X, $Na_V1.6$, $Na_V1.8$, $Na_V1.9$ and β1 were also greater (Fjell *et al,* 1999). When peripheral nerve are damaged, there is loss of expression of $Na_V1.8$ and $Na_V1.9$ transcripts, but an increase in $Na_V1.3$ type sodium channel. Ectopic application of glial derived neurotrophic factor can reverse these changes, and is also able to reverse neuropathic pain behaviour in animal models (Boucher *et al,* 2000).

1.11. $Na_V1.8$ (SNS):

Sodium channel modulation is one mechanism that controls neuronal excitability. Evidence for selective distribution of a unique voltage-gated sodium channel among sensory neurones has been obtained both *in vivo* and *in vitro*. In *vivo* intracellular recordings from cell bodies of neurones indicate that action potentials of high threshold receptors (unmyelinated slow conducting axons) are resistant to TTX but

there is some TTX-r current seen in A fibres as well (Ritter & Mendell, 1992). Similar results were obtained *in vitro*. Jeftinija (1994) has suggested that TTX-r channel plays a role in transmission of nociceptive information. Electrophysiology studies on cultured sensory neurones have demonstrated that while TTX-s Na^+ currents are distributed throughout the population of spinal sensory neurones, TTX-r currents are primarily restricted to a subpopulation of small diameter sensory neurones likely to be involved in nociception (Gold, 1996).

Identification of a gene encoding a TTX-r sodium channel in small diameter neurones confirmed the electrophysiology data indicating the existence of unique Na^+ current in a subpopulation of sensory neurones. This cloned channel was known as SNS/PN3 ($Na_V1.8$) (Akopian *et al,* 1996; Sangameswaran *et al,* 1996). A second TTX-r channel to be cloned is also present in small diameter neurones and is called NaN/SNS2 ($Na_V1.9$) (Dib-Hajj *et al,* 1998b).

Increased voltage gated $Na_V1.8$ channel activity may contribute to the hyperexcitability of neurones in inflammatory and neuropathic pain states. There are several TTX-s sodium channel encoding genes expressed in DRG neurones (Black *et al,* 1996). There are only two TTX-r genes and these are $Na_V1.8$ and $Na_V1.9$. The molecular basis for the TTX-r property exhibited by $Na_V1.8$ has been addressed by site-directed mutagenesis experiments. A critical residue in the pore of the channel in TTX-s voltage gated sodium channels that is normally hydrophobic is transferred to a cysteine residue in the cardiac TTX-r channel. In $Na_V1.8$ at the same position a serine is present. When this serine is altered to phenylalanine, the sensitivity to TTX is

dramatically increased to a level shown by most other sodium channels (Sivilotti *et al,* 1997).

Okuse *et al,* (1997) tested levels of transcripts encoding $Na_V1.8$ in DRG in a range of inflammatory and neuropathic pain models in rat. They found that local Freund's adjuvant or NGF induced inflammation did not substantially alter the total levels of $Na_V1.8$ mRNA. TTX-r currents produced by $Na_V1.8$ were thought to be restricted to small diameter C fibers only but Renganathan *et al,* 2000 have studied the $Na_V1.8$ current in large diameter neurones. They used $Na_V1.8$ knockout mice and wild type mice to determine the identity of channel responsible for the slow TTX-r and found that these currents were produced by $Na_V1.8$. Okuse *et al,* (1997) used a number of pain models of neuropathic pain including the streptozotocin induced diabetic mice and the tight spinal nerve ligature to examine the relationship between changes in the level of expression of $Na_V1.8$ and the development of allodynia. Pain that results from stimuli that are normally innocuous, is known as allodynia, like a light stroking of sunburned skin. Patients with allodynia do not feel pain in the absence of appropriate stimuli. It was found with streptozotocin induced diabetic mice that the $Na_V1.8$ levels were reduced. Okuse *et al's* study revealed that all forms of nerve injury produced reductions in $Na_V1.8$ mRNA. However not all forms of nerve injury produced allodynia. Capsaicin pre-treatment had no effect on the development of allodynia. They concluded that that a reduction in $Na_V1.8$ transcript levels seen in nerve injury is unlikely to cause allodynia. Chaplan *et al,* (1995) have also reported the lack of effect of capsaicin depletion on the development of allodynia in the rat after L5/L6 tight nerve ligation. This evidence supports the hypothesis that allodynia involves

myelinated fibres and that C fibres are not necessary for its expression. Although it does not remove the possibility $Na_V1.8$ in A-fibres may be responsible for allodynia.

1.12. $Na_V1.8$ Knockout mouse:

Akopian *et al*, (1999) have generated a knockout mouse for $Na_V1.8$ channel. It was found that such mice are normal, healthy and fertile apart from partial deficits in perception of noxious thermal, mechanical and inflammatory stimuli. Using antibodies to define the neurones it was found that there was no loss of sensory neurones in the null mutants. TTX-s and TTX-r voltage-gated Na^+ currents in wild type and null mutants were also examined. TTX-s and TTX-r currents were found in the wild type but only TTX-s currents were found in the null mutants. The deletion of the TTX-r channel was not seen to effect the pattern of expression of the TTX-s channel in the DRG neurones although there was a significant up-regulation of the TTX-s currents. Cummins *et al*, (1999) found that DRG neurones from null mutant mice produced a TTX-r Na^+ current with novel properties which is also present in wild type neurones. This current was activated at relatively hyperpolarised voltages and was a persistant current as compared to $Na_V1.8$. It was concluded that this current was due to the TTX-r sodium channel NaN ($Na_V1.9$).

1.13. Abnormal $Na_V1.8$ expression in disease state:

The taiep rat mutant provides a model system in which myelination within the CNS initially progresses relatively normally followed by demyelination which appears to be due to a defect in oligodendrocytes. Black *et al,* 1999 have described the abnormal expression of $Na_V1.8$ protein in Purkinje cells of adult taiep rats which have lost myelin.

Multiple sclerosis (MS) has classically been considered to be a demyelinating disease in which clinical abnormalities are the result of damage to the insulating myelin sheath which causes axonal conduction block. Black *et al,* 2000 have shown that $Na_V1.8$ which is not normally present in the brain is expressed within the cerebellar Purkinje cells of patients with MS but not in normal control subjects. These studies indicate that mistuning of neurones due to abnormal ion channel expression may contribute to clinical abnormalities such as ataxia found in some demyelinating diseases.

1.14. $Na_V1.8$ as a Central Regulator of Inflammatory Pain:

The tetrodotoxin-resistant sodium channel ($Na_V1.8$) is expressed exclusively in sensory neurons and plays an important role in nociception. Several types of evidence suggest that $Na_V1.8$ requires a regulatory protein(s) to be expressed as a functional sodium channel on the plasma membrane. In order to develop greater understanding of the role of $Na_V1.8$, it is important to express these channels in heterologous systems in order to study their functions. $Na_V1.8$ mRNA is expressed predominantly in small-diameter neurons in DRG *in vivo*. Microinjection of $Na_V1.8$ cDNA into the nuclei of superior cervical ganglion (SCG) neurons results in the robust expression of a sodium current which shows exactly the same channel properties as observed in DRG neurons (England *et al,* 1998). In contrast, CHO, COS-7, HEK293 and other mammalian cell lines do not express the microinjected $Na_V1.8$ cDNA well (England *et al.*, 1998), and the expressed channel shows different properties from the endogenous DRG current in these cell types. It is also difficult to express functional $Na_V1.8$ in *Xenopus* oocytes and the expressed channel's properties are different from endogenous DRG current. In the case of sensory neuron specific $Na_V1.8$, $\beta1$ does not help the expression of functional $Na_V1.8$ channel expression in mammalian cell system although it helps in

Xenopus oocytes (Vijayaragavan *et al*, 2001). β3 seems to up-regulate the functional $Na_V1.8$ expression in mammalian cells, however, we found that all four known β–subunits do not help the functional $Na_V1.8$ channel expression in COS-7 and CHO cells (Baker *et al*, personal communication). This implies the need for new accessory subunits that bind to the channel and modulates the expression of the channel.

1.15. Two-hybrid proteins:

Fields and Song (1989) exploited the properties of transcriptional activators to develop a genetic screen to identify interacting proteins. Using yeast transcriptional factors, they realised that the DNA binding domain and the transcriptional activation domains can be separated, yet remain functional. Creation of a hybrid system where one protein is attached to the activating domain and another is fused to the DNA-binding domain brings the activating region to its site of action and allows transcription.

1.16. Two-hybrid system:

In the yeast-two-hybrid system a plasmid is generated that expresses a bait protein as a fusion to the DNA binding domain (DB) derived from a bacterial repressor protein, LexA. After construction, the bait is tested for its suitability for use in the system. In parallel to this a specific prey is also constructed as a fusion to an activation domain (AD) derived from bacterial sequence B42. Interaction between the bait and prey is assessed both by the transcriptional activation of a colorimetric reporter, LacZ and by a positive growth selection on media lacking amino acid leucine, LEU2. In this system the prey is expressed under the control of the Gal1 promoter making its expression galactose-dependent. This way the positive clones can be discerned based on the fact that growth and colorimetric phenotypes are dependent on the presence of galactose as

a carbon source in the growth medium. With a suitable bait chosen and an optimal reporter set selected, a library of prey is coexpressed along with bait and reporter plasmids in yeast to select for interacting proteins. Following identification of candidate interactors, the prey plasmid is recovered from the $LacZ^+$ $LEU2^+$ (i.e. gave blue colonies on X-gal plates and also were able to grow on media lacking leucine) colonies and retested for its interaction with the bait. Figure 1.2 shows a schematic diagram of the yeast two-hybrid systemS along with the different plasmids used in the interaction trap.

1.17. Different Hybrid systems:

Over the past few years the yeast-two hybrid system has been modified to study a broader spectrum of applications. One-hybrid system is used to study protein-DNA interaction. In this derivative of the two-hybrid, an AD-fused library is screened for proteins that bind and activate transcription through a regulatory motif of interest inserted directly up-stream of reporter genes in yeast (Wang *et al,* 1996).

Closely related to the one-hybrid is the one-and-a-half hybrid which makes possible the identification of proteins that conditionally bind to specific DNA motifs only in the presence of a co-expressed auxilliary proteins (Dalton & Treisman, 1992). The trihybrid system is used where there is a need to mimick the higher eukaryotic modification state of particular baits, like for example, when phosphorylation of the bait is relevant to its interaction with the prey, a protein kinase is coexpressed along with the bait and prey in yeast to generate a similar binding site in the trihybrid system (Osborne *et al,* 1995). Recent developments have also enabled the two-hybrid system to address protein interaction using dual-baits systems where two independent baits

are fused to different DNA-binding domains to activate separate but parallel reporter genes on interaction with a prey. Under such conditions it is possible to screen for proteins that can bind one bait but not another or for mutations that abolish or enhance the binding of a prey to one of closely related baits. Other techniques such as reverse and split two-hybrid systemS modify the basic forward two-hybrid system to instead allow screening for a protein that disrupts defined interactions between two proteins.

1.18. Background to the Interaction Trap:

1.18.1. Location and function of the upstream activating sequence:

The expression of Gal1 and Gal10 genes of *Saccharomyces cerevisiae* is tightly regulated by galactose. The two proteins are divergently transcribed and separated by 680bp of DNA which contains a sequence called the Gal upstream activating sequence (UAS_G) which is required for this induction. The UAS_G is the site of action of Gal4 product, a protein required for Gal1 and Gal10 transcription. The product of a second Gal regulatory gene Gal80 blocks Gal4 action. Glucose represses transcription of the Gal genes. West *et al,* 1984 studied various deletions in the UAS_G and found that they decreased Gal1 and Gal10 expression. They found that Gal4 activated transcription from the Gal1 and Gal10 only when the UAS_G was present. All internal deletions effect both genes equally indicating that a single functional unit works bidirectionally.

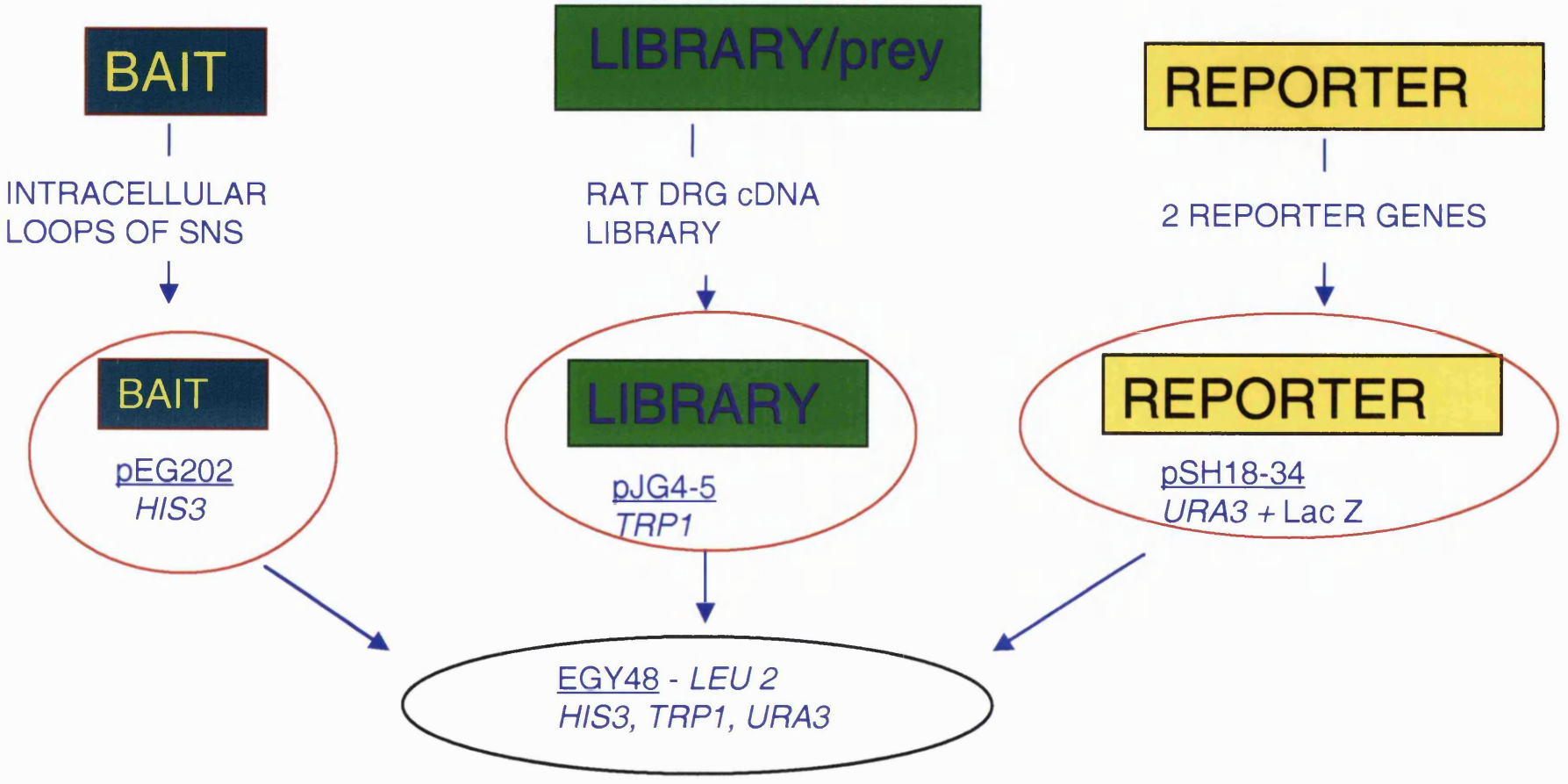


Figure 1.2. Schematic diagram displaying the steps involved in the yeast two hybrid system. The plasmids are underlined in the red circles and the yeast strain used is shown in the black circle. Each plasmid carries a specific marker gene allowing colonies to grow in the absence of that particular amino acid. *His3* allows selection on media lacking histidine; *Trp1* is tryptophan; *URA3* is uracil.

1.18.2. LexA:

In *E.coli* LexA represses many genes by binding to operators. The first 87 amino acids of LexA contain the information necessary for binding to the LexA operators. Brent & Ptashne, (1985) have reported the synthesis of LexA in yeast. If a lexA operator is inserted upstream of Gal1 between UAS_G and the transcription start, LexA represses Gal1 transcription. They have also generated a LexA-Gal4 gene that encodes for a LexA-Gal4 protein in *E.coli* that binds to lexA operators and activates transcription. In their experiments they found that in contrast to wild type Gal4 the LexA-Gal4 activity did not depend on the presence of galactose in the medium. This may be because LexA-Gal4 retains portions of the Gal4 that interacts with Gal80 and hence titrates out Gal80 and then the excess LexA-Gal4 is free to activate transcription.

1.18.3. Yeast Transcriptional Activators:

Endogenous yeast Gal4 binds DNA with its amino terminus and activates transcription using other parts of the protein. A fusion protein bearing the DNA-binding domain of the bacterial repressor LexA in place of Gal4 activates transcription from a lexA operator positioned upstream of a yeast gene. The carboxy terminal of Gal4 is involved in inhibition by Gal80. The activating regions of Gal4 are highly acidic. Ma & Ptashne, (1987) generated a new class of yeast activators which were encoded by genes bearing *E.coli* genomic DNA fragments fused to the coding sequence of the DNA-binding portion of Gal4. When one of these activators B42 is attached to the DNA-binding domain of LexA, a protein containing no yeast protein sequence, is capable of ctivating yeast transcription. All the activating sequences encoded by the *E.coli* DNA fragments are acidic.

1.18.4. Proteins isolated using the yeast two hybrid system:

Using this sytem we have isolated several positive clones of which p11 is studied in detail. This protein was chosen for further studies as in the initial screening with the antisense experiments and the translocation studies carried out in CHOSNS revealed an effect on the expression of $Na_V1.8$. p11 protein also known as annexin II light chain and belongs to a family of S100 calcium binding proteins and can exist as a monomer or as a tetramer in association with p36 (annexin II heavy chain). In this thesis I present evidence that p11 plays an important physiological role in regulating $Na_V1.8$ expression. An introduction to the properties of annexins is presented below.

1.19. Annexins:

The annexins are a family of cytoplasmic Ca^{2+}-dependent phospholipid binding proteins. While more than one has been assigned to each member of the protein the standard nomenclature suggested by Klee (1988) is commonly used. Annexin I also called calpactin II, lipocortin I or p35, annexin II known as calpactin I, lipocortin II or p36. Annexin IV is also known as lipocortin IV, protein II, p32.5 calelectrin or endonexin I. Annexin V also known as lipocortin V, 35K calelectrin or endonexin II and Annexins VI known as lipocortin VI, p68 or 67K clelectrin. Annexin VII is also known as synexin. Studies have shown that annexins are localised in discreet set of cells and structures and there are differences in annexin expression between species (Hamre *et al,* 1995).

These proteins are water-soluble and have the common property to bind to negatively charged phospholipids such as those in the plasma membrane, and this is calcium dependent. The primary structure of annexins is composed of highly homologous

repeats of 70 amino acid residues known as the annexin core (Geisow *et al,* 1986, Moss *et al,* 1992). The calcium binding affinity of the annexin is enhanced when they are bound to the phospholipid membranes and calcium binding induces conformational changes in the molecule. Annexins bind calcium via the annexin core (Moss, 1995). Each annexin contains the annexin core repeated four times at the C-terminal except for annexin VI in which the motif is repeated eight times. The N-terminals are unique and diverse for each annexin and can be modified by tyrosine and serine/threonine phosphorylation (Gerke, 1990). The conserved repeating segments of the annexin core contains the common calcium and phospholipid binding sites, whereas the unique N-terminal is involved in mediation of the biological functions specific to each annexin (Gerke & Moss, 1997).

1.20. Annexins Expression:

Using antibodies against the annexins, Hamre *et al,* 1995 have shown the distribution of the annexins in the CNS. The antibodies to annexin V and VI displayed little immunoreactivity in the CNS and hence only the other three annexins were studied.

Annexin I displays the most restricted distribution of the three proteins Annexin I, II and IV. The only structure labelled by the anti-annexin I antibody was the midline raphe of the brainstem. Annexin I was found in the spinal cord. Annexin I immunoreactivity is also present in some non-neuronal cells such as the lens of the eye and the olfactory neuroepithelium. In contrast annexin II immunoreactivity is found in midline structures throughout the spinal cord, brain stem and mesencephalon. Annexin II was also seen in the DRG neurones of all sizes. Annexin IV is the most widely

distributed of the three annexins found throughout the spinal cord and brainstem. Annexin IV is also present in the DRG neurones.

Naciff *et al* (1996) carried out a study where they studied the expression of annexins in the DRG and spinal cord by using indirect immunofluorescence.

1.20.1. Annexin I:

Annexin I is present in a high concentration in small sensory neurones throughout the DRG with a perinucleur distribution that follows a pattern similar to that shown by endoplasmic reticulum. This annexin is restricted to the soma and does not extend into the axon length either towards the central or towards the peripheral branch. Annexin I is expressed in the grey matter in the spinal cord and in neurones it is found through out the cytoplasm. It is also expressed in the oligodendrocytes ensheathing the axons.

Annexin I is found to have pharmacological activity in the CNS, as when exogenous annexin I was administered intravenously into rabbit it was found to prevent febrile reactions (Davidson *et al,* 1991). Intracerebral injection inhibits cytokine induced fever in rat (Carey *et al,* 1990). Using models of cerebral ischaemia, Relton *et al,* (1991) observed increased expression of annexin in the infarcted areas, and a cerebral injection of the N-terminal region of annexin I was able to reduce the infarct size. They suggested that annexin I could be used as a therapeutic agent in the treatment of excitotoxic cell death or damage.

Annexin I is a substrate for epidermal growth factor (EGF) receptor/kinase and is phosphorylated on a tyrosine residue (Fava & Cohen, 1984). An important feature of

the EGF receptor/kinase phosphorylation of annexin I is that the reaction is of high affinity and is calcium dependent.

1.20.2. Annexin II:

Most sensory neurones express this annexin but it is highly concentrated in the small diameter sensory neurones (Naciff *et al*, 1996). Endothelial cells lining the blood vessels are also rich in annexin II. It is also expressed by Schwann cells but is absent from the axon and from the cells of the perineurium surrounding the axon bundles. Annexin II is highly expressed throughout the spinal cord by glial cells although the strongest immunoreactivity is found in the white matter. It is also expressed by oligodendrocytes surrounding the axons and by ependymal cells lining the central canal but not by astrocytes (Naciff *et al*, 1996).

Annexin II in the amino terminal domain contains phosphorylation sites for tyrosine kinase and protein kinase C, kinases that are important in multiple signal transduction pathways. This annexin is also able to interact through its amino terminal with p11 to form tetramers and this complexs' calcium requirement for membrane aggregation and fusion activity is reduced. The annexin II-p11 tetramer translocates to the plasma membrane and the cytoskeleton and has been associated with the regulation of calcium dependent exocytosis (Sarafian *et al*, 1991).

1.20.3. Annexin III:

This annexin is localised in most of the sensory neuronal bodies regardless of size, although it is highly concentrated in the small neurones. It is evenly distributed throughout the cytoplasm of the small neurones and in a granular pattern in the large

neurones. This annexin is also highly concentrated in endothelial cells lining the small blood vessels that surrounds the axon bundles. In contrast with I and II, annexin III is present in axons and glial cells particularly the Schwann cells. This annexin has a striking distribution along the spinal cord, being highly concentrated in astrocytes located throughout the spinal cord. There is no expression in the ependymal cells lining the canal nor the oligodendrocytes.

1.20.4. Annexin IV:

The distribution pattern of annexin IV is very different. Peripheral glial cells are separated into two distinct groups: satellite cells and Schwann cells. Both types of cells are rich in annexin IV where it is mainly localised in the cytoplasm. The satellite cells surrounding the sensory neurones are richer in annexin IV than the neurones. In the sensory neurones this annexin is highly localised in the nucleus but absent from the nucleolus. In the cytoplasm of the neurones this annexin is found in lesser amounts than in the nucleus and is distributed evenly in a granular pattern. Annexin IV is primarily expressed by the oligodendrocytes ensheathing the axons and is also present in ependymal cells of the central canal and in glial cells throughout the white and grey matter and the astrocytic processes that surround the axon bundles.

1.20.5. Annexin V:

This annexin is highly expressed in most of the small sensory neurones of the DRG. In very few large neurones it is found heavily concentrated around the nucleus and follows a distribution pattern similar to the endoplasmic reticulum. It is also found at the plasma membrane. In some small sensory neurones annexin V is within the

nucleus. It is absent from the endothelial cells and glial cells. Annexin V is highly expressed by ependymal cells lining the central canal and by oligodendrocytes.

1.20.6. Annexin VI:

Most of the sensory neurones throughout the DRG express annexin VI in relative high concentration regardless of cellular size. Some of the large neurones have less immunoreactivity than neighbouring small neurones. In the cytoplasm this annexin is also found with a perinucleur distribution following the pattern of the endoplasmic reticulum. It is also concentrated at the plasma membrane and is also found along the axolemma of the central and peripheral branches of the sensory axons. Schwann cells ensheathing the axons contain annexins but the satellite glial cells and endothelial cells do not show annexin VI immunoreactivity. Annexin VI is concentrated at the plasma membrane of most of the large neurones in the ventral horn of the spinal cord. It is not found in the oligodendrocytes or astrocytes or in glial cells.

1.21. Role of annexins in the secretory pathway:

For multicellular organisms intercellular communication is important in order to coordinate various activities of the cells. One process which is important is exocytosis where intracellular components such as proteins are taken up in membrane vesicles shuttled to the plasma membrane and expelled from the cell by vesicle fusion. There are two forms of exocytosis, these are constitutive and regulated. In constitutive exocytosis vesicle formation is followed by immediate fusion while in regulated exocytosis vesicles wait in the cytoplasm for signals before initiating fusion. Constitutive exocytosis is common to all cell types whereas regulated exocytosis is restricted to endocrine and neuronal cells (Burgoyne & Morgan, 1993). Exocytosis can

be triggered by activation of membrane bound proteins or by depolarisation of the plasma membranes. In both instances vesicle fusion follows a rise in cytosolic free calcium.

Annexin I was the first member to be cloned and has been shown to be an inhibitor of phospholipase A_2. It has also been shown to co-localise with insulin containing granules where, when β-cells are stimulated to secrete insulin, the process requires translocation of annexin I to the vesicles. The process of insulin secretion is also seen to be dependent on the phosphorylation of annexin I by PKC along with other factors. In diabetic rats the level of annexin I in pancreatic islets is significantly decreased. Neutrophil degranulation is also a secretory response and is preceded by an increase in cytosolic calcium, annexin I may play a role in this process (Meers *et al,* 1992).

Most studies on the role of annexin II in the secretory process have focused on the adrenal medulla chromaffin cells. Following stimulation these cells secrete catecholamines which requires calcium. All annexins are responsive to calcium but annexin II has the lowest calcium requirement for binding to phospholipid vesicles. When cells are stimulated to undergo secretion, there is an increase in the phosphorylation of some proteins thought to be associated with chromaffin granules. Following phosphorylation on tyrosine-23 in the N-terminal region of annexin II by tyrosine kinase, the binding of annexin II to phospholipid vesicles is reduced. This effect is calcium independent and does not influence the annexin II binding to p11. As well as phosphorylation by tyrosine kinase, annexin II has been shown to be phosphorylated on serine residues by serine/threonine kinases and also by PKC. Phosphorylation by PKC on serine-25 does not effect the phospholipid binding ability

of annexin II but does decrease the level of annexin II-mediated vesicle aggregation (Johnstone *et al,* 1992). Annexin II was first identified as one of the major phosphorylation targets of $pp60^{v\text{-}src}$, the tyrosine kinase encoded by the *src* oncogene of the transforming Rous sarcoma virus (RSV) (Erikson & Erikson, 1980).

The cytoskeleton plays an important role in exocytosis, where actin forms a network beneath the plasma membrane. Disassembly of the actin layer is important for secretory granules to undergo fusion to the plasma membrane and this only occurs in areas where the actin layer is absent (Senda *et al,* 1994). Interactions have been reported between annexin II and actin and sequence analysis of annexin II revealed a region showing strong homology to the actin-binding domain of myosin. It may be that by interacting with the cytoskeletal proteins annexin II clears a path for secretory vesicles to move to the membrane allowing fusion to occur.

Annexin VI is the only annexin to have eight rather than four copies of the annexin repeats and has been implicated inhibiting rather than potentiating the secretory process. Zaks & Creutz (1990) showed annexin VI inhibits vesicle aggregation and fusion mediated by annexin I and annexin VII but the mechanism of this inhibition is unclear.

1.23. Aim:

We used the yeast two hybrid system to identify proteins that can bind to the $Na_V1.8$ channel and modulate the function of this channel. In this thesis I put forward the case that an accessory subunit p11 associated with the annexin family plays an important physiological role in the regulation of $Na_V1.8$ expression.

The existence of factors other than accessory β subunits that can alter inactivation kinetics has been suggested by studies in which Na^+ channel α subunits are expressed in cells lacking endogenous Na^+ channel α and β1 subunits. Expression of rat cardiac $Na_V1.5$ and rat brain $Na_V1.2$ in mammalian cell lines results in Na^+ channel with rapid activation and inactivation characteristic of native neuronal Na^+ channel (Scheuer *et al,* 1990; Qu *et al,* 1994). Functional expression of $Na_V1.8$ has been achieved in *Xenopus* oocytes by Akopian *et al,* 1996 and Sangameswaran *et al,* 1996 and Fitzgerald *et al,* 1999 have achieved the functional expression of $Na_V1.8$ in a mammalian system. They expressed the channel in COS-7 cells and produced a sodium current with similar properties to the native TTX-resistant channels in DRG but with a depolarised shift in the voltage dependence of activation. This shift was also seen to occur in *Xenopus* oocytes. The $Na_V1.8$ currents were also found to have a slower rate of inactivation. Akopian *et al,* 1996 suggested that these discrepancies may be due to the fact that $Na_V1.8$ were expressed without the β1 subunits which are normally present in the DRG. β1 has been shown to increase the rate of inactivation and cause a hyperpolarising shift of $Na_V1.2$, $Na_V1.4$ and $Na_V1.5$ α subunits (Patton *et al,* 1994; Isom *et al,* 1995; Qu *et al,* 1995). However co-expression of human sodium channel β1 failed to accelerate the inactivation kinetics of $Na_V1.8$ expressed in *Xenopus* oocytes (Rabert *et al,* 1998).

England *et al*, 1998 have also shown that if $Na_V1.8$ cDNA is microinjected into superior cervical ganglion neurones, a TTX-resistant current which has the same characteristics as the native currents in DRG is reproduced. We have also generated stably $Na_V1.8$ transfected CHO cell lines, but despite having the $Na_V1.8$ transcript and protein in the cytoplasm as confirmed by antibodies against $Na_V1.8$, there is no functional channel expressed on the plasma membrane. Co-expression with β1, β2 or β3 did not help translocate the $Na_V1.8$ to the plasma to help produce a functional channel (Baker *et al*, personnel communication). This suggests the possibility of another element needed to help the translocation of the $Na_V1.8$.

The aim of this project was therefore to identify necessary cofactors for high level expression of $Na_V1.8$ using a yeast two hybrid screening strategy.

CHAPTER 2.
MATERIALS AND METHODS

2.1 Molecular biology

2.1.1. PCR primers:

Primers were ordered from Genosys and then diluted with distilled water (dH_2O) to stock solutions of 500μM and then further diluted to either 20 or 40μM stocks. The solutions were kept at -20^0C until use. Polymerase chain reaction (PCR) primers were designed using the intracellular loops of $Na_V1.8$. Restriction sites for EcoRI were included in the 5' primer and for NotI in the 3' primer. Figure 2.1 shows the α-subunit of $Na_V1.8$ with the location of all the baits (green loops) used in our system.

BAIT I from position 1 to position 125 amino-acids using the following 5' forward and 3' reverse primers respectively.

SNSYH-1 Forward - GCGAATTCATGGAGCTCCCCTTTG

SNSYH-2 Reverse - TATAGCGGCCGCTTTGATGGCTGTTCTTC

BAIT II from position to 399 to position 659 amino acids using the following 5' forward and 3' reverse primers respectively.

SNSYH-3 Forward - GCGAATTCGAAGAGCAGAGCCAGG

SNSYH-4 Reverse - TATAGCGGCCGCGAACAGCGCCATCTTG

BAIT III from position 893 to position 1148 amino acids using the following 5' forward and 3' reverse primers respectively.

SNSYH-5 Forward - GCGAATTCAGCGCGGACAACCTCAC

SNSYH-6 Reverse - TATAGCGGCCGCGCGGTAGCAGGTCTTG

BAIT IV from position 1419 to position 1472 amino acids using the following 5' forward and 3' reverse primers respectively.

SNSYH-7 Forward - GCGAATTCGACAACTTCAACCAACAG

SNSYH-8 Reverse - TATAGCGGCCGCGTCAAACACGAAGCCTTG

BAIT V from position 1723 to position 1956 amino acids using the following 5' forward and 3' reverse primers respectively.

SNSYH-9 Forward - GCGAATTCGAGAACTTCAACGTAGCC

SNSYH-10 Reverse - TATAGCGGCCGCTCACTGAGGTCCAGG

2.1.2. PCR:

Using the full length $Na_V1.8$/SNS cDNA as a template PCR was carried out to amplify the baits with the 5 sets of forward and reverse primers. The following were added to give a final volume of 50µl; 5µl 10X Mg^{2+} Free Taq Buffer, 3µl 25mM $MgCl_2$, 2µl 5mM dNTP, 1µl 40µM 5' primer, 1µl 40µM 3' primer, 0.1µl of 1µg/µl DNA template ($Na_V1.8$/SNS), 37.4µl distilled water (dH_2O), 0.5µl Taq DNA Polymerase. The solutions were mixed and mineral oil added.

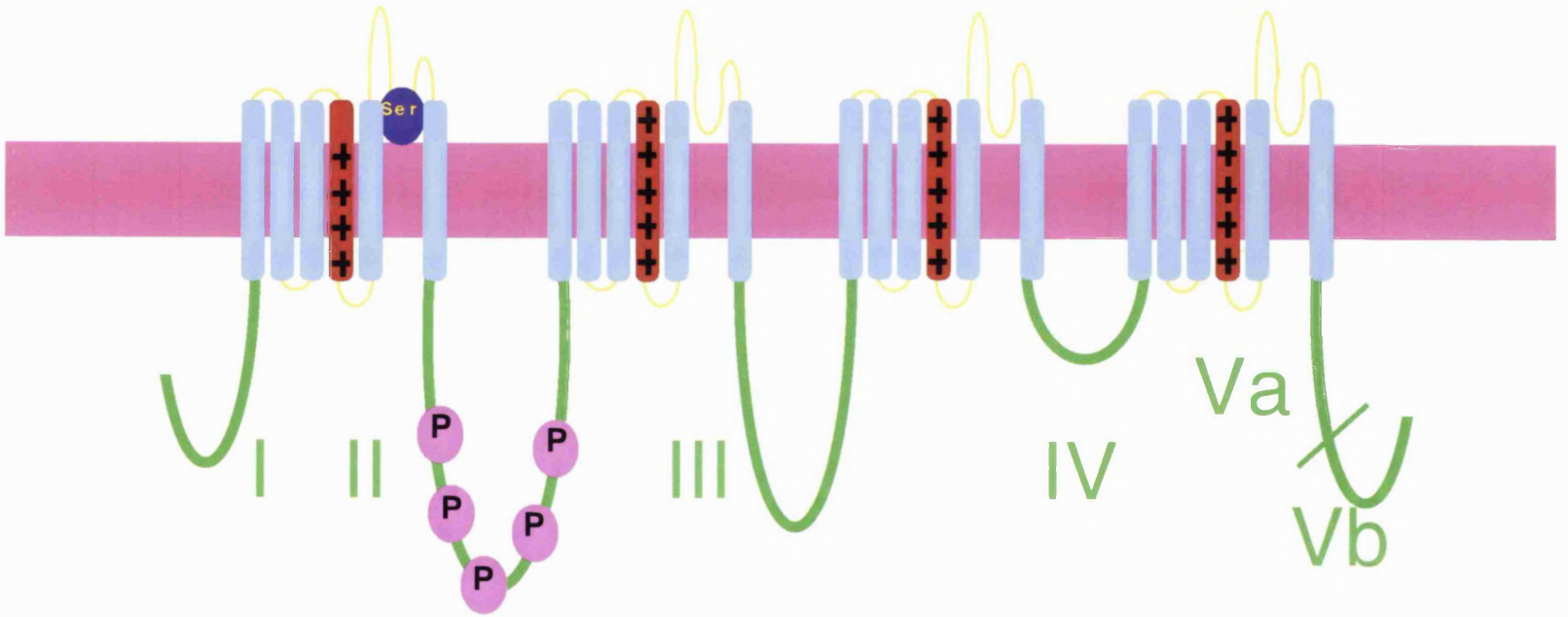


Figure 2.1. Schematic presentation of the baits used in the interaction trap. The intracellular loop of $Na_V1.8$ was used to generate baits I to V.

PCR was carried out in a Techne/Genius PCR machines using the following conditions.

5 CYCLES:	step 1	94°C	1 minute
	step 2	48°C	2 minutes
	step 3	72°C	2 minutes
25 CYCLES:	step 1	94°C	1 minute
	step 2	58°C	2 minutes
	step 3	72°C	2 minutes

At the end of the cycle the mineral oil was removed and a phenol/chloroform extraction was performed.

2.1.3. Phenol/Chloroform extraction:

Phenol/chloroform (PC) extraction was to ensure any Taq polymerase present after the PCR was removed. Tris-phenol (pH 8) (Tris and phenol are obtained from Sigma) was mixed with chloroform (obtained from BDH) to give 50%v/v.

The extraction was performed as follows, 100µl PC was added to the reaction tubes. The tubes were vortexed and then spun for 5 minutes at top speed. The top hydrophilic layer that contains the DNA was decanted for further use. 5µl of loading dye (10X loading buffer containing 500µl glycerol (Sigma), 100µl Bromo-phenol blue (Sigma), 400µl dH_2O) were added and the mixture was loaded onto to 1.5% agarose gel.

2.1.4. Agarose Gel:

1.5% agarose (BDH) was dissolved in TAE. The PCR products from the previous reaction are loaded onto the gel and run for 1 hour at 100V, 200mA using the power pack. The expected size of band for each of the 5 baits was as follows:

BAIT I - 381bp

BAIT II - 780bp

BAIT III - 765bp

BAIT IV - 159bp

BAIT V - 699bp

2.1.5. DNA Extraction:

Using the Gene Clean II kit (QBiogene), DNA extraction was performed as follows. The band size corresponding to the appropriate bait was excised from the gel in 3 X (volume/ weight of gel) of NaI (6M solution), and the solution incubated at 55^0 C for 5-10 minutes to dissolve the gel. 5-10µl glass beads (Aqueous suspension of silica matrix) were added, and the mixture was left on ice for 5 minutes. The glass bead/DNA was pelleted with a brief spin, and the pellet washed 3X with the new wash solution (concentrated solution of NaCl, Tris and EDTA and mixed with 100% ethanol). After the final wash, the pellet was resuspended in 20µl dH_2O and the DNA eluted by incubating for 5 minutes at 55^0C. The mixture was spun briefly to pellet the glass beads and the supernatant decanted and stored as it contains the DNA.

2.1.6. Restriction enzyme digestion:

The fragments for each of the baits I-V were cut in a final volume of 10µl containing 1µl Buffer H (Boehringer), 0.5µl EcoRI (10 Units) (Boehringer), 0.5 µl Not I (10 Units) (Boehringer), 5µl DNA fragment (Bait I-V), 3µl sterile distilled water. The solutions were mixed and tubes incubated for a minimum of 2 hours at 37^0C followed by 15 minutes incubation at 75^0C to heat inactivate the enzymes.

The pEG202 plasmid used (Gift Alan Halls' lab, LMCB, UCL) to ligate the baits was also cut in a final volume of 10µl containing 1µl Buffer H, 0.5µl EcoRI, 0.5µl NotI, 2µl vector (pEG202), 6µl sterile distilled water.

2.1.7. Ligation of Bait into plasmid:

In a final volume of 10µl the following were added in separate tubes for each of the baits; 1µl 10X ligation buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM dithiothretol, 1mM ATP, 25µg/ml Bovine serum albumin (BSA) (New England Biolabs)), 1µl T4 DNA ligase (New England Biolabs), 3µl restriction enzyme cut pEG202 from above and 5µl restriction enzyme cut DNA fragment corresponding to the 5 baits. The solutions were mixed and incubated at 16^0C overnight to ensure that the baits were ligated into the plasmid.

2.1.8. Bacterial Transformation:

XL1-Blue competent bacterial cells (100µl/tube) (See method later) were thawed on ice, 5µl bait DNA (ligated in pEG202 plasmid as mentioned above) was added into 5 separate tubes corresponding to the 5 different baits and containing the XL1-blue cells, the mixture incubated on ice for 30 minutes. Heat shock treatment was applied for 1

minute at 42^{0}C to encourage the bacteria to pick up the DNA. After the heat shock treatment, the tube was incubated for 2 minutes on ice before addition of 450µl SOC solution (In 950ml distilled H_2O (dH_2O), the following were added 20g bacto-tryptone, 5g bacto-yeast extract, 0.5g NaCl, the solutions stirred well and the rest added as follows, 10ml of 250mM KCl, adjust to pH 7 with 5N NaOH, then add 5ml of 2mM $MgCl_2$. The solution was sterilised by autoclaving for 15 minutes and cooled to 60°C prior to the addition of 20ml 1mM glucose). The mixture was shaken at 37^{0}C for 1 hour prior to plating on LB agar plates (5g Luria Broth powder, 1.5%(w/v) agar (3g) and 200ml distilled water. The solutions were autoclaved, cooled and SOC solution was added along with 50µg/ml ampicillin prior to pouring into petri dishes for setting and incubating overnight at 37^{0}C.

2.1.9. Bacterial Mini prep:

Colonies were picked from the XL1-blue bacterial transformation LB plates and shaken in 3ml LB broth (12.5g Luria Broth powder (Gibco BRL), 500ml dH_2O, autoclaved for 15 minutes, before addition of 50µg/ml Ampicillin(1/1000)) overnight at 37^{0}C. The next day stock solutions of each of the baits were frozen in 40% glycerol and kept at –70°C till further use (500µl bait solution/500µl 80% glycerol solution). The rest of the solution in the tubes was centrifuged at 4800rpm for 5 minutes to pellet the DNA. The pellet was resuspended in 150µl solution I (50mM glucose, 25mM Tris-HCl pH 8, 10 mM EDTA, 100µg/ml RNAse A). 150µl solution II (0.2M NaOH, 1% SDS) was added and the tubes incubated at room temperature for 5 minutes prior to addition of 150µl solution III (3M KoAc, pH 5.5 (60ml of 5M stock and 11.5ml glacial acetic-acid and 28.5ml dH_2O). The tubes were vortexed and left on ice for 5 minutes prior to centrifugation at 14000rpm for 10 minutes. The pellet discarded and

400µl PC added to the supernatant, before centrifuging for 10 minutes at 14000rpm. The supernatant was transferred to a new tube and 1ml 100% ethanol was added, and the tubes incubated at -20^{0}C for 30 minutes to precipitate the DNA. After the incubation, the tubes were centrifuged for 20 minutes at 14000rpm, and the pellet washed with 70% ethanol. The pellet was dried using speed vac and dissolved in 20µl dH_2O and stored at –20°C.

2.1.10. Midi prep:

Once it was confirmed by performing a restriction enzyme analysis with EcoRI and XhoI on the bacterial mini prep DNA that the insert was present in the plasmid, it was necessary to purify the DNA before the next step. A stock solution (1µg/ml) of each of the baits was made in 50% glycerol solution and stored at –70°C prior to performing a midi prep using the Qiagen (Qiagen -tip 100 (Midi)) kit and following the manufacturers protocol as follows:

From the glycerol stocks frozen in –70°C in previous section, the tubes containing the insert in the plasmid were used for the midi prep. Using a dip stick a small amount of stock was scraped out and inoculated in 5ml LB broth. The mixture was shaken at 37^{0}C until $O.D_{600}$ of 1 was achieved, the mixture was then diluted in 200ml of LB broth to achieve O.D 0.2 and shaken until O.D 0.6 was reached. This corresponds to cells in the log phase of growth. The Xl1-blue bacterial cells were harvested by centrifuging at 4800rpm in the Sigma 3K10 centrifuge at 4^{0}C for 10 minutes. The bacteria were resuspended in 4ml of Buffer P1, 4ml of Buffer P2 was added, the tubes mixed gently by inversion and incubated at room temperature for 5 minutes. 4ml of chilled Buffer P3 was added and the tubes mixed immediately by gently inverting 4-6

times and incubated on ice for 15 minutes. The tubes were centrifuged for 30 minutes at 4^0C and the supernatant containing the DNA was recentrifuged for another 30 minutes at 4°C, after which the pellet was discarded. Meanwhile applying Buffer QBT and allowing the column to empty by gravity flow equilibrated a Qiagen-tip 100. Apply the supernatant from the previous step and allow it to enter the resin by gravity flow. The Qiagen-tip 100 was washed two times with 10 ml of Buffer QC. The DNA was eluted in fresh tubes with 5ml of Buffer QF. Adding 3.5ml room temperature 100% isopropanol precipitated the eluted DNA. The DNA was mixed and incubated for 30 minutes at -20^{0}C before centrifuging at 4^0C for 30 minutes at 4800rpm. The supernatant decanted off and the DNA pellet washed twice with 2ml of room temperature 70% ethanol and centrifuged for 10 minutes at 4800rpm. The supernatant was decanted off carefully and the pellet air dried for 5-10 minutes and resuspended in a suitable volume of water and kept in -20^{0}C until use.

2.1.11. DNA sequencing (Cycle sequencing method):

DNA sequencing was carried out using the dideoxy-mediated chain termination method (Sanger et al, 1977). Sequencing was carried out to confirm the DNA insert was present in the correct orientation in the plasmid and it was also in the correct frame using the sequence kit (DNA sequencing kit from ABI Prisms). Samples were prepared as follows: 3µl sequencing buffer, 1µl reaction mixture, 0.5 µg DNA, 3.2 pmol of appropriate primer and made up to 10µl with distilled water. A drop of mineral oil was added and a sequencing reaction performed using the Techne/Genius machine with the following conditions:

25 CYCLES step 1 96°C 35 seconds

step 2 49°C 45 seconds

step 3 60°C 4 minutes

At the end of the cycle the mineral oil was removed and a chloroform extraction done to remove all traces of mineral oil. 50µl chloroform was added to the aqueous phase and the tubes vortexed prior to centrifuging the tubes at top speed for 5 minutes. 1µl of 3M sodium acetate (pH 5.5) was added and the aqueous phase containing the DNA was transferred to the tube. 25µl of 100% ethanol was added, the tubes vortexed and incubated on ice for 30 minutes to precipitate the DNA. After the incubation the tubes were centrifuged at 14000rpm for 20 minutes and the supernatant discarded. The pellet was washed with 70% ethanol and dried using speed vac. The pellet was resuspended in 4µl of loading buffer (80% formamide, 20% 50mM EDTA containing 30mg/ml dextran blue) in a 1:5 ratio of sequencing dye to formamide. The samples were loaded onto AccuGel (National Diagnostics) sequencing gel and electrophoresed for 7 hours in an ABI Prism 377 DNA sequencer. The data was collected and the sequences analysed by performing a BLAST search at NCBI.

2.2. Yeast-two-hybrid system

2.2.1. Solutions and media

2.2.1.1. LB medium:

12.5g of Luria broth (LB) powder obtained from BDH was added to 500 ml of distilled water and autoclaved for 15 mins at 120^0 C. The broth was cooled to 60^0 C prior to addition of 50ng/ml ampicillin.

2.2.1.2. Ampicillin:

A stock solution of 50mg/ml was obtained from Sigma. We used a final working concentration of 50ng/ml, so a 1000X dilution was performed.

2.2.1.3. L.B Agar:

5g L.B powder was mixed with 1.5%(w/v) agar and dissolved in 200ml distilled water. The solutions were autoclaved, and cooled prior to pouring into petri dishes for setting.

2.2.1.4. SOB solution:

A litre of solution contained the following 950ml distilled water, 20g bacto-tryptone, 5g bacto-yeast extract and 0.5g NaCl. The solution was shaken well and then the following added: 10ml of 250mM KCl and pH adjusted to 7 with 5N NaOH, before adding 5ml of 2mM $MgCl_2$. The solution was sterilised by autoclaving.

2.2.1.5. SOC solution:

Made up identically to SOB solution. After cooling to 60^0, 20ml of 1M glucose added.

2.2.1.6. X-Gal solution:

A stock solution of 20mg/ml was used at a concentration of 80μg/ml. 200mg 5bromo-4chloro-3indolyl β D-galacto pyranoside (X-Gal) was added to 10ml dimethylformamide.

2.2.1.7. X-Gal plates:

For 1 litre, the following were mixed in 800ml distilled water: 6.7g Yeast nitrogen base (YNB) without amino acids, 1.5g drop out supplement (DOS), 20g agar (Difco bacto agar), the solution autoclaved and the following added, 20% carbon source. The

solutions allowed to cool to 60^0 before adding 100ml 10X BU salts (see below) and 4ml of 20mg/ml X-Gal.

2.2.1.8. 10X BU salts:

For 1 litre, the following were added in distilled water: 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4, the pH adjusted to 7, solution autoclaved and stored at room temperature.

All plasmids used in the yeast-2-hybrid system were a kind gift from Alan Hall (LMCB, UCL).

2.3. Constructing the bait and assessing suitability:

2.3.1. pEG202:

The first step in a two-hybrid screen is to construct the plasmid expressing a LexA fusion to the bait protein. This is achieved by cloning the DNA encoding the protein of interest into the pEG202 plasmid. Figure 2.2 shows the map of the pEG202 plasmid. This plasmid is a yeast-*E.coli* shuttle vector and is a multi-copy plasmid containing the yeast 2μm origin of replication. The plasmid also contains the selectable marker gene *HIS3*, along with yeast promoter *ADH1* gene, followed by full length LexA coding region. This is followed by the *ADH1* terminator sequences. Bait proteins expressed from this plasmid contain the amino acids 1-220 of the bacterial repressor protein LexA, which includes the DNA binding domain. The plasmid also contains the *E.coli* origin of replication and the ampicillin resistant gene. Downstream of the LexA coding region are unique restriction enzyme cloning sites EcoRI, BamHI, SalI, NcoI, NotI and XhoI (Gyuris *et al*, 1993).

2.3.2. LacZ reporter systems:

The bacterial LacZ gene encoding the enzyme β-galactosidase (β-gal)is a ubiquitous reagent applied to the study of problems in genetics, cell and molecular biology by its induction. Due to the ability of β-gal being readily assessed, its induction has become a standard means of assaying gene expression.

Reporters for measuring activation were derived from the pLR1Δ1 plasmid, in which the Gal1 upstream activating sequences (UAS_G) have been deleted. LexA operators have replaced the UAS_G. The LacZ reporter plasmid resides on the yeast origin of replication 2μ plasmids containing *Ura3* gene and the Gal1 TATA transcription start. It also contained the *E.coli* origin of replication and the ampicillin resistant gene. Figure 2.3 shows in detail the various LacZ reporter plasmids. In the absence of interacting activation-tagged proteins, the yeast strain bearing these reporters do not make β-galactosidase and therefore appear white on X-Gal plates. Use of LacZ reporters provides 2 advantages as any false positive can be identified which may arise from activation of LEU2 reporter gene but which fail to activate the LacZ reporter. Secondly the LacZ reporters provides a relative measure of the amount of transcription caused by interaction of activation tagged cDNA protein with a bait as seen by a visual assay. The sensitivity of the LacZ reporters depends on the number of LexA operators positioned upstream of LacZ (West *et al*, 1984).

2.3.2.1. pSH18-34:

This LacZ plasmid was derived from the pLR1Δ1 plasmid where the UAS_G have been replaced by LexA operators and was used as a reporter gene to measure activation. The plasmid contained 8 of the high affinity overlapping type of *colE1* LexA operator

located upstream of the LacZ gene and was more sensitive than plasmids which contain only 1 operator. This plasmid also contained the URA3 selectable marker gene

2.3.2.2. pJK101:

This plasmid was used to measure repression by LexA fusions and was used as a positive control for the repression assay as it has the LacZ reporter insert. It is the same as pSH18-34 except it contains most of the UAS_G and two colE1 operator between UAS_G and the Gal1 TATA transcription start upstream of the LexA operator sites. The plasmid also contained the selectable marker URA3 gene (Brent & Ptashne, 1984).

2.3.3. LEU2 Reporter System:

The interaction trap uses a yeast strain, EGY48 that has an integrated LEU2 gene with its upstream regulatory region replaced by LexA operators. This strain cannot grow in the absence of leucine unless the LexAop-LEU2 gene is transcribed. The LEU2 reporter is very sensitive which is due to the presence of three high affinity LexA operators positioned near the LEU2 transcription start. The operators are from the colE1gene and each can potentially bind two LexA dimers (Ebina *et al,* 1983). The sensitivity of EGY48 can be of an advantage in isolating weak interactors, but it can also be too sensitive to use with baits that are themselves weak transcription activators. In addition to the mutation in the endogenous LEU2 gene, EGY48 carries mutation in three other marker genes, HIS3, TRP1, URA3, which are needed to allow selection.

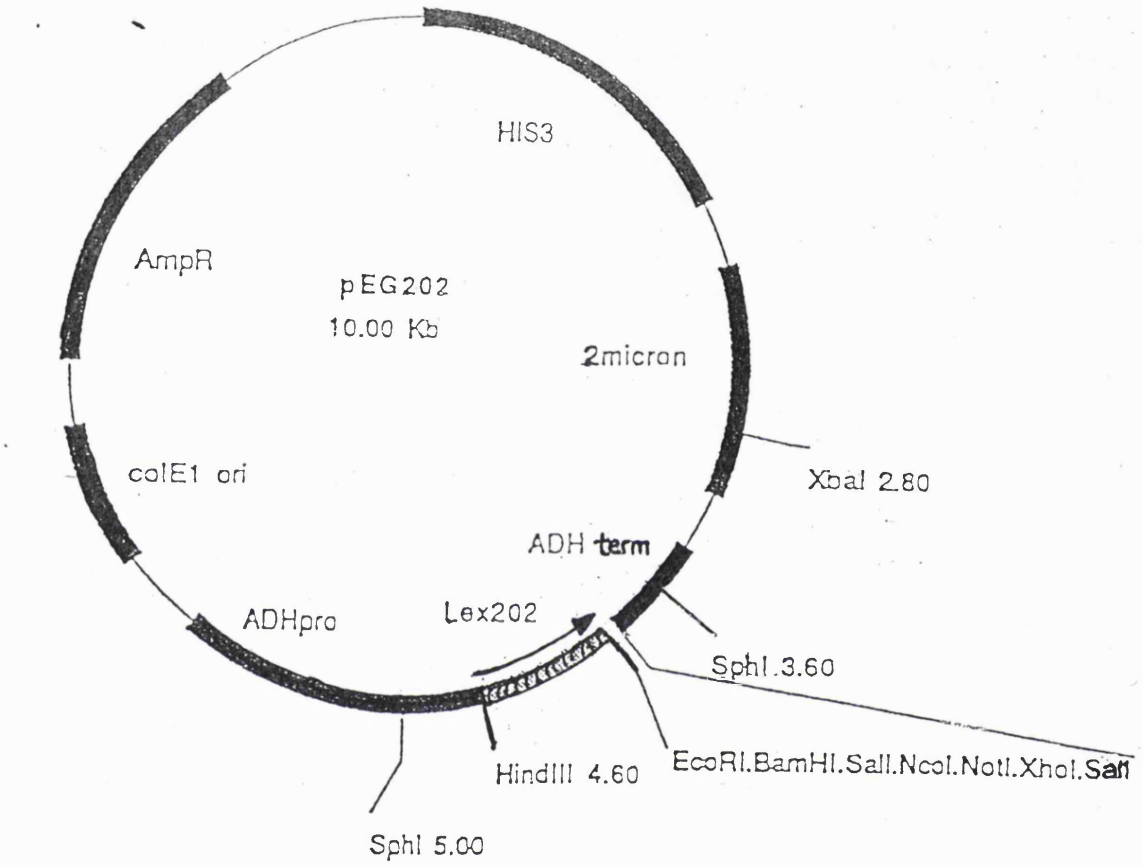


Figure 2.2. Map of plasmid pEG202, which is a yeast *E.coli* shuttle vector and is a multi-copy plasmid containing the yeast 2μm origin of replication. The plasmid also contains the selectable marker genes *HIS3*, along with yeast promoter *ADH1* gene which encodes for amino acids 1-202 of the bacterial repressor protein LexA. Bait proteins expressed from this plasmid contain amino acids 1-202 of LexA, which includes the DNA binding domain. The plasmid also contains the *E.coli* origin of replication and the ampicillin resistant gene. Our baits were cloned into EcoRI and NotI sites. The numbers indicate relative map positions.

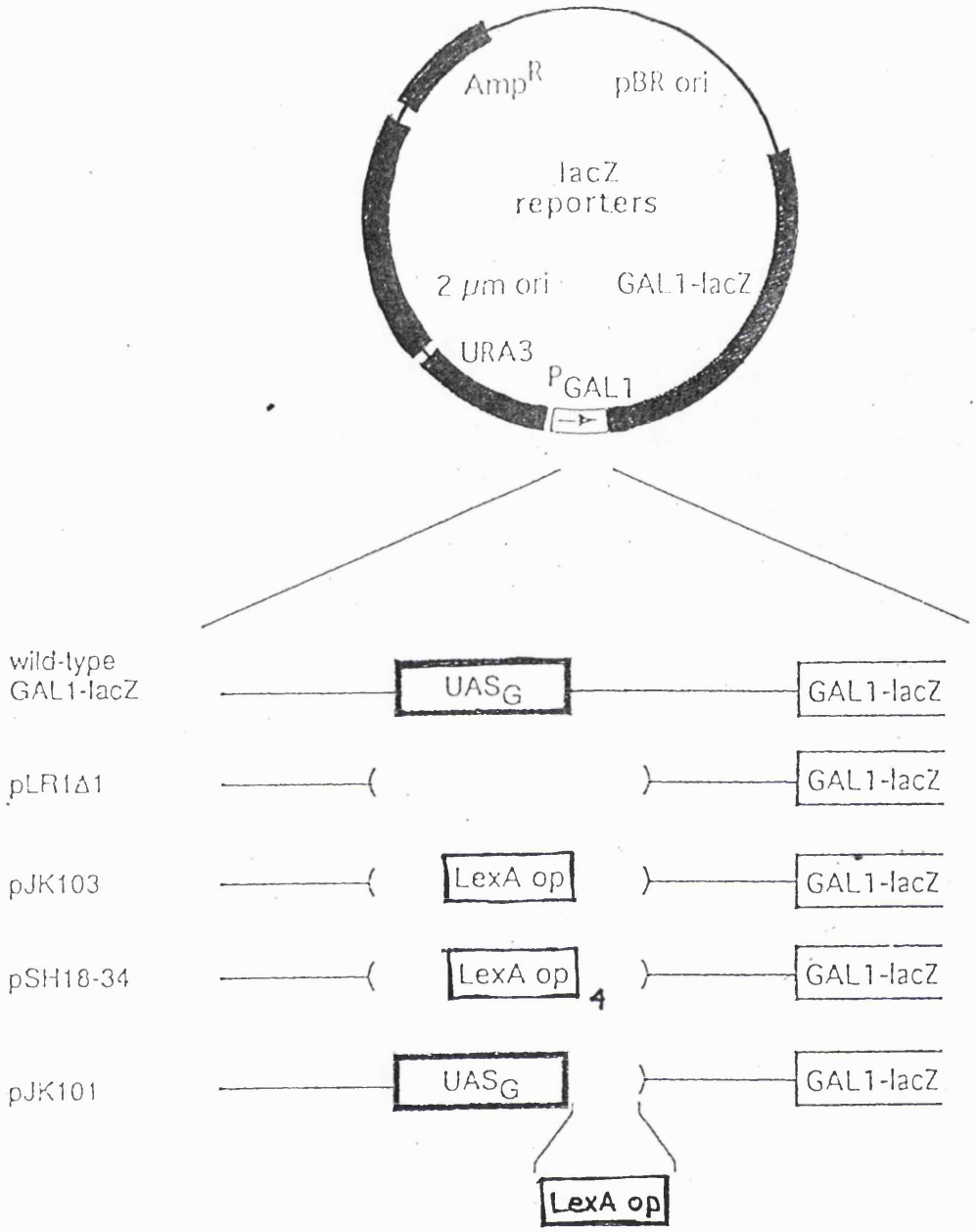


Figure 2.3. Map showing the different LacZ reporters which are derived from a plasmid that contains the wild-type Gal1 fused to LacZ. Reporters for measuring activation are derived from pLR1Δ1, in which the Gal1 upstream activation sequences have been inserted in place of UAS_G to create LacZ reporters with different sensitivities.

2.3.4. Control Plasmids:

2.3.4.1. pSH17-4:

This was a HIS3 2μm plasmid encoding LexA fused to the activation domain of the yeast activator protein Gal4. Yeast bearing this plasmid will produce colonies within 24 hours on medium lacking leucine and yeast that additionally contain pSH18-34 will turn deep blue on X-Gal plates. This fusion protein strongly activates transcription and was used as a positive control in the activation assay (Gyuris *et al,* 1993).

2.3.4.2. pRFHM-1:

This plasmid was a 2μm plasmid encoding LexA fused to the C-terminus of the drosophila protein bicoid.. This plasmid contained the selectable marker gene *HIS3.* So yeast containing this plasmid along with pSH18-34 do not grow on leucine minus media and remain white on X-Gal plates. This fusion protein has no ability to activate transcription and can be used as a negative control for the activation assay and a positive control for the repression assay (Gyuris *et al,* 1993).

2.3.4.3. pEG22:

pEG22 was derived from the plasmid pEG202, where a region was deleted from restriction enzyme SphI to SphI site that included the whole of LexA region. Once the LexA region was deleted the resulting plasmid can be used as a negative control for the repression assay since it is incapable of activating trasncription.

2.3.5. Library plasmid:

pJG4-5 is a yeast *E.coli* shuttle vector that contains a yeast expression cassette that includes the promoter from the yeast Gal1 gene followed by sequences that encode the 106 amino acid fusion moiety and the transcription terminator sequences from the

yeast ADH1. cDNA can be inserted into the EcoRI and XhoI sites so that encoded proteins are expressed with the fusion moiety at their amino terminus. The fusion moiety includes the nuclear localisation signal, the B42 transcription activation domain and the haemagglutinin (HA) epitope tag (surface antigen recognised by the anti-HA antibody). The plasmid also contains an *E.coli* origin of replication, the ampicillin resistant gene, and a yeast selectable marker gene for TRP1 and yeast origin of replication (Gyuris *et al*, 1993). The map for pJG4-5 is shown in figure 2.4.

2.3.6. Yeast Strain:

The interaction trap uses a yeast strain, EGY48 that has an integrated LEU2 gene with its upstream regulatory region replaces by LexA operators. This strain cannot grow in the absence of leucine unless the LEU2-LexA gene is transcribed (Gyuris *et al*, 1993). The LEU2 reporter in this strain is very sensitive and this is due to the presence of six high affinity LexA operators positioned near the LEU2 transcription start. The operators are from the *colE1* gene and bind to the LexA operatrors (Ebina *et al*, 1983). The sensitivity of EGY48 can be of advantage in isolating weak interactors, but it also be too sensitive to use with baits that are themselves weak trasncription activators. In addition to the mutation in the endogenous LEU2 gene, EGY48 carries mutations on three other marker genes HIS3, URA3, TRP1 that are needed for selection.

2.4. Yeast Transformation of reporter and bait plasmid:

The following protocol was used for the introduction of bait DNA (in pEG202) and reporter plasmid containing the *LacZ* reporter gene (in pSH18-34) into yeast EGY48 (Alan Halls lab, UCL) using the lithium acetate method.

The bait DNA was obtained from the bacterial transformation carried out with the 5 baits previously as described previously. Yeast EGY48 colonies were picked and inoculated into YPD medium (For 1 litre, mix in 900ml distilled water: 10g Yeast extract (Difco Bacto yeast extract), 20g peptone (Difco Bacto peptone), 100ml sterile 20% glucose) until OD 0.4-0.6 was reached. To culture in mid-log phase growth, enough of the overnight culture was transferred into fresh medium to produce an OD_{600} of 0.2-0.3. The solution was shaken at 37^0 for 3-5 hours until OD reached 0.4-0.6, then pSH18-34 transformation was carried out into the yeast. The cells were harvested by centrifugation at 4800 rpm for 5 minutes and the pellet resuspended in 10ml dH_2O. The solution was centrifuged again at 4800rpm and the yeast pellet was resuspended in 1.5ml buffered LiAc solution ([1 vol 10X T.E buffer (10mM), 1 vol 10X lithium acetate (100mM), 8 vol sterile distilled water]; [T.E Buffer 10X solution: 10mM Tris, 1mM EDTA, pH 7.5, filter sterilised. Lithium Acetate 10 X stock solution (1M): 5.1g lithium Acetate powder, 50ml sterile distilled water, filter sterilised]). 18µl carrier DNA (Salmon sperm DNA 5mg/ml stock solution (coresponding to 200µg) (Promega) was mixed with 5µg of transforming DNA (either bait DNA in pEG202 or reporter DNA in pSH18-34)) was mixed with 2µl reporter plasmid DNA (pSH18-34) and added to 200µl of buffered LiAc/EGY48 solution. 1.2 ml of PEG solution (40% Buffered PEG solution: 1 vol 10X T.E buffer, 1 vol 10X lithium acetate, 8 vol 50% PEG solution) was added, the tube shaken at 30^0C

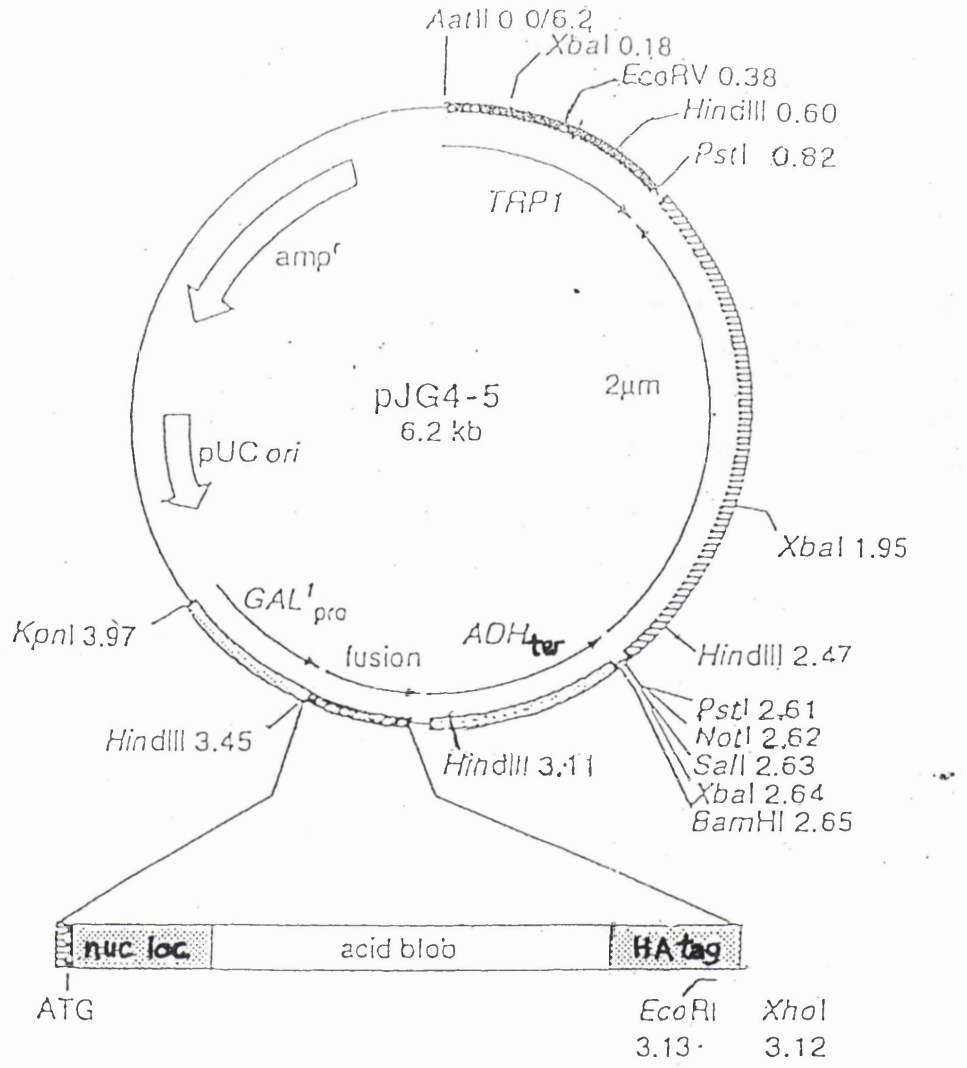


Figure 2.4. Map of pJG4-5 library plasmid expressing cDNAs inserted into EcoRI and XhoI sites consisting of the nucleur localisation sequence, the acid blob B42, and the hemagglutinin epitope tag (HA). Expression of the sequences is under the control of the Gal1 promoter. This plasmid contains the *TRP1* selectable marker and the 2µm origin of replication. The numbers indicate relative map positions.

for 30 minutes, which was followed by heat shock treatment at 42^0C for 15 minutes. The mixture was microcentrifuged for a few minutes and the pellet was resuspended in 1ml of 1X T.E buffer. 200µl was spread onto Glu Ura$^-$ (lacking uracil) drop out agar plates and colonies grown at 30^0C. After 2-5 days a colony was picked and inoculated into Glu Ura$^-$ minimal medium (Minimal medium: For 1 litre, mix in 850ml distilled water: 6.7g YNB without amino-acids (Difco), 2g Drop out supplement powder (DOS) lacking the appropriate nutrients i.e. uracil (Ura$^-$), appropriate carbon source from sterile 20% stocks. For Galactose/Raffinose add galactose to 2% and raffinose to 1% final concentration; for glucose add glucose to 2% final concentration.) and shaken at 30^0C overnight. The suspension was diluted to OD 0.2 and grown until OD 0.4-0.6 was reached, the transformation repeated as before and the EGY48/pSH18-34 transformed with the bait DNA and colonies grown on Glu Ura$^-$ His$^-$ (lacking uracil and histidine) agar plates at 30^0C.

2.4.1. Activation Assay:

The protocol below describes how to verify that the bait used in the yeast-2-hybrid system does not directly activate transcription of the *LEU2* reporter gene integrated in EGY48. To construct the selection strain, 4 colonies were picked from each of the EGY48/pSH18-34/Bait or EGY48/pSH18-34/positive (pSH17-4) or EGY48/ pSH18-34/negative (pRFHM1) controls from the Glu Ura$^-$His$^-$ plates constructed above and each colony used to inoculate 5ml Glu Ura$^-$His$^-$liquid cultures. At the same time the same 4 transformants were streaked to another Glu Ura$^-$His$^-$ master plate for storage. These plates were incubated at 30^0C until colonies formed and then stored at 4^0C.

The liquid cultures were grown at 30^0C shaking to OD 0.5 (corresponding to 10^7 cells/ml and was mid-log phase), 10^2 and 10^3-fold dilutions was made of each cultures

in water. 10µl of the cultures and 10µl of the dilutions were spotted onto two plates containing

-Gal/Raf Ura^-His^-

-Gal/Raf $Ura^-His^-Leu^-$

The yeast containing the bait plasmid being tested and yeast containing the control plasmids were spotted on the same plates for side-by-side comparison and incubated at 30^0C. The growth of the cells was monitored for several days. The above steps establish whether a bait can be used in an interactor hunt. For a bait to be used, the selection strain containing it must not grow on Gal/Raf Ura^- His^- Leu^- (lacking uracil, histidine and leucine) plates for 2-3 days. Only the positive control should grow.

A secondary assay employing the *LacZ* expression was also performed on the selection strain to establish an inert bait. Individual transformants from the Glu Ura^- His^- liquid cultures used in the step above were patched onto Glu Ura^- His^- X-Gal plates (Glu Ura^-His^--X-Gal) and incubated at 30^0C. Yeast with positive control should turn blue overnight while the negative control and the transcriptionally inert bait will remain white after many days.

2.4.2. Cleavage of Bait V:

Due to bait V plasmid growing on medium lacking leucine and therefore directly activating the LEU2 reporter gene prior to library transformation. Primers were designed to truncate bait V into two separate fragments V_a and V_b. The new baits were tested by repeating the activation assay. For V_a the primers used were the following forward primer SNSYH-9 (GCGAATTCGAGAACTTCAACGTAGCC) and the following reverse primer SNSYH-11 (TATAGCGCCGCGGCTATTGGTTCATAG) to give fragment V_a (360bp) from amino-acid position 1724 to position 1844. V_b was

generated using forward primer SNS-YH12 (GCGAATTCCCAATAGCCACCACCC) and the reverse primer SNSYH-10 (TATAGCGGCCGCTCACTGAGGTCCAGG) to give fragment V_b (315bp) corresponding to position 1842aa to 1947aa.

2.4.3. Repression assay:

To test the viability of the bait and to ensure that it is not toxic, a repression assay was performed. A colony from Yeast EGY48 plates was inoculated into 5ml YPD medium and grown at 30^0C until OD_{600} 0.5 was reached. The yeast strain was transformed with the reporter plasmid, pJK101 as described above and transformants selected on Glu Ura^- plates. 3 colonies were picked from the Glu Ura^- plates and inoculated into 5ml Glu Ura^- His^- medium at 30^0C overnight until OD_{600} reached 0.5. Bait plasmid (pEG202) or positive control (pRFHM1) or negative control (pEG22) was transformed using the lithium acetate method as described above into the liquid culture. The transformants were selected on Glu Ura^- His^- plates.

4 individual colonies from the Glu Ura^- His^- plates were picked and streaked onto Glu Ura^- His^- X-Gal and Gal/Raf Ura^- His^- X-Gal plates. These plates were examined after one, two and three days.

2.5. cDNA library transformation:

The yeast EGY48 which had been transformed in the previous step with the bait and the reporter gene was grown in 400ml Glu Ura^- His^- medium at 30^0C with shaking to an OD_{600} of 1, corresponding to about 3 X 10^7 cells/ml. The culture was centrifuged at 2000g for 5 minutes and the supernatant poured off. The cells were washed twice with 20ml distilled water. The yeast were resuspended in 5ml of sterile LiOAc/TE. The cells were pelleted again by centrifuging and resuspending in 1.2ml sterile LiOAc/TE

and dispensed into four 100µl aliquots. To 3 aliquots, 2µg of cDNA Rat DRG library was added (along with 60µg of carrier SS DNA in a total volume of 20µl). The postnatal day 1 rat cDNA library was a kind gift from Professor Moses Chao, NYU and was subcloned into EcoRI and XhoI sites in the plasmid pJG4-5 as described by Kong et al, 2000. To the last aliquot 10µg cDNA was added along with 30µg SS DNA, 10% (v/v) DMSO and 600µl of sterile 40% PEG 4000 in LiOAc/TE. The tubes were gently inverted to mix and incubated at 30^0C for 30 minutes. Heat shock treatment was applied at 42^0C for 15 minutes to encourage library transformation. The total number of transformants was determined by removing 10µl from each tube and making serial dilutions (10^2, 10^3) in sterile water. 100µl of each dilution was plated onto 10cm Glu Ura^- His^- Trp^- dishes (lacking uracil, histidine and tryptophan) and incubated at 30^0C.

A few days after cDNA library transformation, colonies were scraped from the yeast plates and transferred to 50ml falcon tubes. The cells were washed twice with 15ml of TE solutions and centrifuged at 2000g for 4 minutes. The cells were resuspended in 4.5ml of glycerol solution and some aliquots were frozen at -70^0C to use later. From the remaining resuspended cells the plating efficiency was determined, by making serial dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} and plating 100µl onto Gal/Raf Ura^- His^- Trp^- plates. Colonies were grown for 2/3 days and counted to work out the cfu/unit volume of frozen cells.

2.5.1. Selecting Interactors:

Library transformants containing cDNAs that encode proteins that interact with the bait will exhibit galactose-dependent growth on media lacking leucine (Leu^+) and also show galactose-dependent β-galactosidase activity ($LacZ^+$). The library transformants

were now selected by inducing synthesis of cDNA encoded proteins. From the serial dilutions frozen in the previous step, a 10^6 dilution was thawed and grown for 4 hours at 30^0C by diluting tenfold in Gal/Raf Ura$^-$ His$^-$ Trp$^-$ medium. The cells were pelleted by centrifugation at 2000g for 4 minutes and resuspended in sterile water and grown on Gal/Raf Ura$^-$ His$^-$ Trp$^-$ Leu$^-$ plates at 30^0C. The final amount plated corresponded to 10^6 transformants. Colonies will grow for 2-5 days and then picked with sterile toothpicks and streaked onto another Gal/Raf Ura$^-$ His$^-$ Trp$^-$ Leu$^-$ plate and left to grow for another 2/3 days. Also to ensure that the Leu$^+$ phenotype was galactose dependent, the Leu$^+$ yeast were patched onto Glu Ura$^-$ His$^-$ Trp$^-$ master plates to turn off the Gal1 promoter and stop expression of activation tagged cDNA protein and grown at 30^0C for 24 hours. A 4 dish selection was done by making replicas from the master plates onto the following 4 plates: Glu Ura$^-$ His$^-$ Trp$^-$-Xgal , Gal/raf Ura$^-$ His$^-$ Trp$^-$-Xgal, Glu Ura$^-$ His$^-$ Trp$^-$ Leu$^-$ and Gal/Raf Ura$^-$ His$^-$ Trp$^-$ Leu$^-$. The plates were grown at 30^0C and results examined after one, two and three days. Colonies were picked that are Leu$^+$ and LacZ$^+$ on galactose.

2.5.2. Rescue of library plasmid from yeast:

2.5.2.1. Yeast Mini-Prep:

The Leu$^+$/LacZ$^+$ yeast was scraped from the plates and resuspended in 1ml of TE buffer (10mM Tris-HCl pH 7.5, 1 mM EDTA) in a microcentrifuge tubes (the OD_{600} of this suspension should be between 2 and 5). The suspension was centrifuged briefly to pellet the cells and resuspended in 0.5 ml of S buffer (10mM K_2HPO_4, 10mM EDTA pH 7.2, 50 mM 2-mercaptoethanol, 0.5µg/ml Lyticase). The yeast was incubated at 37^0C for 30 minutes before addition of 0.1 ml Lysis (25 mM Tris-HCl pH 7.5, 25 mM EDTA, 2.5% SDS) solution. After vortexing the mixture was incubated at

65^0C for 30 minutes. 166 µl of potassium acetate (3M KoAc pH 5.5) was added and the yeast mixture left to chill on ice for 10 minutes. The mixture was centrifuged and the supernatant was poured into new tubes, adding 0.8ml of cold ethanol and the tubes incubated on ice for 10 minutes precipitated the DNA. The DNA was pelleted by spinning for 10 minutes and washed by centrifugation with 0.5 ml 70% ethanol. After drying the pellet was resuspended in 20 µl distilled water. Before transforming *E.coli* with the crude yeast mini-preps it was useful to determine which yeast minipreps contain the same library plasmid so that fewer need to be characterised. This was done by restriction analysis of the library plasmids.

2.5.2.2. Restriction enzyme digest:

It was useful to reduce the number of library plasmids to be rescued by determining which ones contain identical cDNAs. Comparing restriction enzyme digests of PCR products containing the cDNA achieved this. 2µl yeast miniprep from the above step was used as a template in PCR reactions (5U/µl *Taq* polymerase, 10X Mg^{2+} free *Taq* DNA polymerase buffer, $MgCl_2$ 1.5 mM, dNTP mix (all 4 dNTPs at 2.5 mM each), 5' primer (0.1µg/µl)- BC01, 3' primer (0.1µg/µl)- BC02-2)) with forward primer BC01 (CCAGCCTCTTGCTGAGTGGAGATG) and the following reverse primer BC02-2 (GACAAGCCGACAACCTTGATTGCAG). These primers were derived from sequences in the library plasmid flanking the cDNA insertion site in the pJG4-5 expression vector. The PCR was carried for 25 cycles at 92^0C for 30 seconds, 65^0 C for 2 minutes, 72^0 C for 2 minutes and 30 seconds. Two tubes were set up for each PCR reaction, one for AluI digestion and one for HaeIII digestion. 8µl of PCR reaction was mixed with 1µl of appropriate restriction enzyme and the corresponding buffer. The tubes incubated at 37 ^{0}C for 2 hours. The digest was analysed by

electrophoreses on 1.5% agarose gels. It should be apparent from this analysis which cDNAs were identical. The individual cDNAs were picked for sequencing.

2.5.2.3. Sequence reaction:

The sequence reaction was carried out as mentioned previously using the chain termination method (Sanger *et al*, 1977). The solution was mixed in 0.5ml microcentrifuge tubes to a final volume of 10µl (3µl sequence buffer, 1µl reaction buffer, 0.32pmol primer, 1µg/µl DNA), mineral oil was added and the sequence reaction performed. After the reaction, chloroform extraction was carried out by adding 50µl chloroform and the tubes vortexed prior to centrifugation for 5 minutes in a benchtop centrifuge. 1µl 3M NaOAc was added to fresh tubes and the bubble containing the DNA was transferred to them from the centrifuged tubes. 25µl of 100% ethanol was added and the tubes incubated on ice for half an hour. The cells were washed with 70% ethanol by centrifuging for 10 minutes at 140000rpm. The cells were pelleted by centrifuging at 14000rpm for 20 minutes and the pellet resuspended in 4µl of dye:formamide mixture and left at -20^0C until sequenced as mentioned above.

2.5.2.4. Transformation into KC8 competent cells:

To select the library plamid, the cDNAs rescued from the yeast mini-prep and the PCR reactions that show the library plasmid in the yeast were transformed into KC8 electrocompetent cells using the gene pulser II machine. The Gene Pulser apparatus is a pulse generator that uses capacitor discharge to produce controlled exponential pulses for cell electroporation. The resistance of the electroporation media is dependent on its ionic strength. As ionic strength increases, resistance of the medium decreases. A pulse delivered into a medium of higher ionic strength (low resistance)

will have a shorter time constant if all else remains constant. A buffered saline solution has a resistance about 10 x that of PBS containing no Ca^{2+} or Mg^{2+}.

40µl KC8 competent cells (see section for making electrocompetent cells) and electroporation cuvettes are placed on ice prior to transformation. 3µl DNA was added to the 100µl KC8 cells and placed in the cuvettes. The solution was electroporated at 1.8kV, 10µF and 600Ω. 1ml SOC solution was added and the mixture incubated at 37^0C for 1 hour. The solution was spun briefly to pellet the cells and resuspended in 50µl SOC solution and plated onto M9 plates (M9 plates: In 1 litre solution add the following 200ml 5X M9 salts (M9 salts: 5X Solution, in 1 litre distilled water add the following 64g Na_2HPO_4, 15g KH_2PO_4, 2.5g NaCl, 5g NH_4Cl, mix and autoclave), 700ml distilled water, 15g Bactoagar, 0.74g DOS medium (trp^-). The solution was autoclaved and cooled before adding 100ml 20% glucose and 1ml of thiamine. Pour into plates and allow to set. The plates were grown at 37^0C. Colonies were picked from these and a bacterial mini prep was performed on them prior to sequencing.

2.6. KC8 Electrocompetent cells for electroporation:

All glassware was autoclaved prior to use and pre-chilled. Cells are kept on ice at all times. 1 litre of L.B Broth was inoculated with 50ml overnight KC8 culture in a 2l bottle and grown to OD_{600} of 0.5-0.6. The broth was transferred to chilled bottle and kept on ice for 30 minutes. The broth was then spun at 4800rpm for 15 minutes at 0^0C, the broth was decanted and the tubes placed on ice. The pellet was resuspended in distilled water and respun at 4800rpm for 15 minutes. The water decanted and pellet washed with distilled water by resuspending and centrifuging. The water was decanted and the pellet resuspended in ice-cold 10% glycerol. This solution was spun at

4800rpm for 10 minutes at 0^0C and the pellet resuspended in a final volume of 4ml glycerol. 40µl of cells were then aliquoted into microcentrifuge tubes and kept on dry ice until stored in -70^0C.

2.7. XL1-Blue Competent Cells for transformation:

E.coli was streaked on LB plates without ampicillin and grown at 37^0C. Next day colonies were picked from the E.coli plates and inoculated into 250ml SOB (Bactotryptone Bacto Yeast Extract, NaCl, 2.5 mM KCl, 4N NaOH, 2 mM $MgCl_2$) medium in a 2l flask and shaken at 18^0C for 50-60 hours until OD_{600} of 0.4-0.8 was reached. The flask was then cooled on ice and the solution centrifuged at 4800rpm at 4^0C for 5 minutes. The pellet was resuspended in a third of the volume of ice-cold TB solution (Transformation Buffer: 10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250mM KCl, 55mM $MnCl_2.4H_2O$) and incubated on ice for 10 minutes prior to spinning at 4800rpm for 5 minutes. The pellet was resuspended in 10ml ice-cold TB and 750µl DMSO was added. The solution was incubated on ice for 10 minutes and 100µl aliquoted into sterile microcentrifuge tubes and frozen in liquid nitrogen before storing in -70^0C.

2.8 Cell Culture

2.8.1 COS-7 cell culture:

COS-7 cells were removed from the plates by addition of 1ml trypsin-EDTA (Gibco BRL), the cells were washed with Dulbecco's modified Eagles medium (DMEM) (Gibco BRL) by centrifuging for 5 minutes at 1500rpm and resuspending the cells in DMEM (with L-glutamine (Gibco BRL), 10% fetal calf serum (Sigma), 250 Units penicillin/streptomycin (Gibco BRL)). The cells were plated onto petri-dishes and incubated at 37°C.

2.8.2 CHO cell culture:

The $Na_V1.8$ stably transfected chinese hamster ovary cell line (CHO-SNS22) was kept in Nutrient Mixture F-12 (Ham) medium (Gibco BRL) with 2.5% fetal bovine serum, 250 Units streptomycin/penicillin and 1mg/ml Geneticin G418 sulphate (Gibco BRL). Cells were subcultured by removing from plates by addition of 1ml trypsin-EDTA and incubating for a few minutes prior to washing with Ham's medium by centrifuging for 5 minutes at 1500rpm and resuspending the cells in 10ml Ham's medium before plating.

2.8.3 DRG cell culture:

2 weeks old rat pups were killed by cervical dislocation and the whole spinal cord dissected out. The dorsal root ganglia (DRG) were extracted and placed in Phosphate buffered saline solution (PBS) (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1l dH_2O and pH adjusted to 7.4 prior to autoclaving). The surrounding axon and tissue were excised and the ganglia incubated in 2ml enzyme solution (50ml PBS, 30mg Collagnase Type XI (Sigma), 150mg Protease (Dispase) (Sigma), and 10 mM Glucose (BDH)) for 45 minutes at 37^0C. The ganglia were gently trituated a few times and passed through a 70μM Nylon strainer (Falcon) and rinsed with 8ml DMEM, 10% FCS, 250 Units Penicillin Streptomycin medium prior to 5 minutes centrifugation in Centaur-2 MSE centrifuge at 1000rpm. The ganglia were washed once with DMEM and resuspended in fresh medium and plated onto poly-lysine (0.1- 0.2 mg/ml) (Sigma) coated coverslips placed in 35mm petri-dishes. The neurones were grown in DMEM medium at 37°C.

2.9 DNA Microinjections

2.9.1 Cell preparation:

DRG neurones were cultured one day prior to microinjections as mentioned above, this was to ensure they are stuck down. Immediately before injection, the DMEM medium was removed and replaced by 2ml of microinjection buffer (PBS without Ca^{2+} and Mg^{2+} with 25mM glucose, and 2.5mM $MgCl_2$). It was found that the cells lasted longer and were in an healthier condition in calcium free enviroment. After completion the solution was replaced with normal DMEM medium and the cells incubated for 3 days prior to electrophysiology recordings.

2.9.2 Antisense DNA Preparation:

Various antisense expression vectors were used for microinjections. These were cloned in different sites of different expression vectors as explained below. The sense direction cDNA in the original pJG4-5 vector obtained from the yeast-2-hybrid interaction trap (For method see later) was used in the subsequent steps to generate the antisense expression vector. The antisense cDNA was incorporated into an expression vector in the 3' to 5' direction and expressed by a promoter in the DRG when it was microinjected and this expressed cDNA binds to the endogenous mRNA and inhibits protein synthesis. All the cDNA antisense generated were confirmed with DNA sequencing to ensure the antisense was ligated in the right direction (3' to 5') for the corresponding clone and also the correct cDNA was obtained. The antisense stocks were diluted to 1μg/μl and stored in –20°C. 20-40μg antisense DNA was added to 25μg GFP DNA (Green flourescent protein). The water was evaporated in speed vac and the DNA was resuspended in 5μl Texas red and 45μl KCl loading buffer (KCl 118 mM, HEPES 5 mM, $NaHCO_3$ 22.2 mM, $MgCl_2$ 1.2 mM). The solution was passed

through a Whatmans' vectaspin micro 0.2μm filter (Whatman labs) by centrifuging for 5 minutes on a bench top centrifuge to remove all impurities. The samples were kept on ice at all times during injection and stored at 4°C. Control was generated with 25μl GFP in pRK7 vector (1μg/μl) containing 20μl KCl injection buffer and 5μl texas red.

2.9.2.1. I-1:

The antisense cDNA for I-1 was ligated in 3' to 5' direction using 309bp NcoI fragment in pBS500 vector. 20μl of I-1 antisense (1μg/μl stock) was resuspended with 25μl GFP (1μg/μl stock). After drying the pellet was resuspended in 5% texas red and KCl buffer.

2.9.2.2. II5/II6/III27/IV7-40:

The antisense for II5 (244bp) was ligated in the 3' to 5' direction in pRK7 vector using restriction enzyme sites of HindIII/SalI. The antisense for II6 (1.4kb) was ligated in the 3' to 5' direction in the expression vector pcDNA3 using HindIII/XhoI sites. The antisense for III27 (407bp) was ligated in 3' to 5' direction in pRK7 using HindIII/SalI restriction enzyme sites. The antisense for IV7-40 (690bp) was ligated into pcDNA3 in 3' to 5' direction using HindIII/EcoRI restriction enzyme sites. Prior to the injection 60μl each of the above antisense cDNA was added to 50μl GFP in the same tube. The solution was evaporated in speed vac and the DNA resuspended in a final volume of 10μl Texas red and 90μl of KCl injection buffer. The solution was filtered through a Whatman filter to remove any impurities and the resultant solution stored on ice.

2.9.2.3. III42/IV40/A-145:

III-42 (600bp) was ligated in 3' to 5' direction in pcDNA3 using EcoRI/HindIII sites. IV-40 (1.5kb) was ligated in 3' to 5' direction in pcDNA3 using EcoRI/HindIII sites.

A-145 (1.1kb) was ligated from 3' to 5' direction into pcDNA3 using restriction enzyme sites for BamHI/HindIII.

2.9.2.4. A-10/A-32/A-165:

A-10 (2.3kb) cDNA antisense was ligated in the 3' to 5' direction in pBS500 using restriction enzyme site for NcoI. A-32 (845bp) was ligated in the 3' to 5' direction using restriction enzyme sites for HindIII/EcoRI in pcDNA3. A-165 (1kb) was ligated in the 3' to 5' direction using restriction enzyme site for NcoI in pBS500.

2.9.2.5. A-92/A-103/B-18:

A-92 (138bp) was ligated in the 3' to 5' direction using restriction enzyme sites for HindIII/EcoRI in pcDNA3. A-103 (543bp) was ligated in the 3' to 5' direction using restriction enzyme sites for XhoI/PstI in pRK7. B-18 (1.6kb) was ligated in the 3' to 5' direction using restriction enzyme sites for HindIII/EcoRI in pcDNA3.

2.9.2.6. A-140/A-148/B-25:

A-140 (1.8kb) was ligated in the 3' to 5' direction using restriction enzyme site for NcoI in pBS500. A-148 (1.4kb) was ligated in the 3' to 5' direction using restriction enzyme sites for BamHI/EcoRI in pcDNA3. B-25 (1.4kb) was ligated in the 3' to 5' direction using restriction enzyme sites for BamHI/KpnI in pRK7.

2.9.2.7. A123:

A-123 (1kb) was ligated in the 3' to 5' direction using restriction enzyme site for EcoRI in pcDNA3.

2.9.2.8. A-133:

A-133 (1.1kb) was ligated in the 3' to 5' direction using restriction enzyme site for HindIII in pcDNA3.

2.9.2.9. B-4:

B-4 (619bp) was ligated in the 3' to 5' direction using restriction enzyme sites for HindIII/XhoI in pRK7.

2.9.2.10. B-13/B-15/B-20:

B-13 (1kb) was ligated in the 3' to 5' direction using restriction enzyme site for HindIII in pRK7. B-15 (278bp) was ligated in the 3' to 5' direction using restriction enzyme sites for KpnI/XbaI in pRK7. B-20 (1.8kb) was ligated in the 3' to 5' direction using restriction enzyme sites for HindIII/EcoRI in pcDNA3.

2.9.3 Microinjecting:

Approximately 80-100 DRG neurones were injected with the antisense construct. Injection needles were pulled using thin wall glass capillaries (GC150TF-10, Harvard apparatus) with the Sutter P97 microelectrode puller. The DMEM medium was replaced by a calcium free buffer. The antisense solution (approximately 3μl) was loaded via an eppendorf micoloader into the injection needle and attached to the eppendorf microinjector. The solution was pressure injected into nucleus of the small diameter neurones (for the purpose of this thesis a small diameter neurone was taken to be of 22μm in size or below). After completion of the injections, the calcium free buffer was replaced by DMEM containing 3.3ng/ml aphidicolin, to discourage growth of non-neuronal cells. The neurones were incubated for 3 days at 37^0 C prior to electrophysiology recordings of the $Na_V1.8$/SNS sodium current.

2.9.4. Electrophysiology recording:

Tetrodotoxin resistant sodium (TTX-r Na) currents were recorded from DRG neurones following microinjection of antisense in the appropriate expression vector. The pRK7 expression vector incorporated with GFP acted as a control for microinjection with no

antisense present. Recordings were also made from non-injected neurones for another control. The extracellular recording solution contained the following: NaCl 43.3mM, TEA-Cl 96.7 mM, HEPES 10 mM, $CaCl_2$ 2.1 mM, $MgCl_2$ 2.21 mM, CsCl 10 mM, KCl 7.5 mM, 4-aminopyridine (4-AP) 0.5 mM, $CdCl_2$ 0.02 mM, tetrodotoxin (TTX) 250nM and was buffered to pH 7.2-3 with the addition of CsOH. The TEA, Cs and 4-AP block potassium currents and the TTX blocks the TTX sensitive currents endogenously present in the DRG in order for the TTX resistant current to be recorded. The intracellular solution contained the following: CsCl 130 mM, CsF 14.5 mM, EGTA 3 mM, NaCl 6 mM, TEA-Cl 10 mM, Hepes 10 mM, $CaCl_2$ 1.21 mM, $MgCl_2$ 1.21 mM, ATP-Mg 3 mM, GTP-Li 0.5 mM, and was buffered to pH 7.2-3 with the addition of CsOH. The combination of internal and external cadmium (Cd) ions was used to eliminate Ca^{2+} currents. Chemicals were supplied by BDH or supplied by Sigma. TTX was obtained from Alomone labs (TCS Biologicals, UK). Electrodes were fabricated from thin-wall glass capillaries (GC150TF-10; Harvard apparatus, UK), and had an access resistance of 2-4MΩ when filled with intracellular recording solution. Recordings were made using an Axopatch 200B patch-clamp amplifier (Axon Instruments, USA). Capacity transients generated in response to clamp-steps were eliminated using the resistant/capacitance (RC) circuit provided by the amplifier and an estimate of membrane capacity was read from the front panel potentiometer. Neurones were held at 90 mV the potential at which inactivation was removed from TTXr sodium currents. Voltage-clamp protocols incorporated a negative pre-pulse to 110 mV, and the cell was subsequently stepped to more depolarized potentials for 50ms (up to a final value of +80 mV), in 10 mV increments. Pulse protocols were generated and data stored to disk using pClamp6 software (Axon Instruments),

running on a PC. A p/n pulse protocol was employed in pClamp6, with n= 5, giving on-line leak subtraction. The recordings were performed by Dr. Mark Baker, UCL.

2.10. Antibody Staining:

Commercially bought antibody for p11 protein was utilised for antibody staining to confirm that p11 protein was down regulated after microinjections with the respective antisense. The antibody for I-1 (Rabbit anti-p11 IgG antibody) was obtained from Biodesign international. DRG neurones were cultured and plated onto polylysine coated coverslips, 24 hours later the I-1 antisense was microinjected into the nucleus of the DRG neurones as mentioned previously. The plates were incubated for 2-3 days after which the p11 antibody (1.3mg/ml in buffer solution (20mM Tris pH 7.5 and 150mM NaCl)) was applied as follows. Old medium from the plates was discarded and the plates washed with PBS solution. Cold 4% paraformaldehyde (PFA) (40g PFA added to 900ml dH_2O, and the solution heated to 65°C to dissolve the PFA. To this 100ml 10X PBS was added (76g NaCl, 9.93g Na_2HPO_4, 4.68g $Na_2HPO_4.2H_2O$ was added to 1l dH_2O and the pH adjusted to 7.4)) and the dishes incubated on ice for 10-15 minutes to fix the cells. The dishes were washed three times with PBS solution and incubated in 750µl of antibody solution. To optimise the conditions initially three different dilutions of the antibody were used 1/500 dilution, 1/1000 dilution and 1/1500 dilution (1/500 dilution: 1.5µl of antibody solution in 750µl of 10% sheep serum in PBS; 1/1000 dilution: 0.75µl of antibody solution in 750µl of 10% sheep serum in PBS; 1/1500 dilution: 0.5µl of antibody solution in 750µl of 10% sheep serum in PBS). The plates were incubated with primary antibody for 2 hours at room temperature. The dishes were washed three times with PBS and 750µl anti-rabbit IgG (conjugated with rhodamine) secondary antibody was applied at 1/150 dilution and the dishes incubated overnight at 4^0C. The dishes were washed three times in PBS

solution and citifluor solution was added and a glass coverslip mounted on the dish. The dishes were viewed under a green filter in the fluorescence microscope

2.11. Northern Blot:

2.11.1. Extraction of tissues:

RNA was isolated from various rat tissues by acid guanidinium thiocyanate-phenol-chloroform extraction method as described by Chomczynski & Sacchi (1987). Briefly DRG, heart, liver, kidney and brain were dissected from two weeks old rat pups, minced and frozen on dry ice immediately. Cells were homogenised at room temperature in a glass homogeniser using 5ml of denaturing solution (solution D containing: 4 M Guanidinium thiocyanate (Sigma); 25 mM Sodium citrate, pH 7 (Sigma); 0.5 % Sarcosyl (sigma); 0.1 M 2-Mercaptoethanol (BDH)) and transferred to 15ml tubes before addition of 500µl of 2M sodium acetate (pH 4). Cells were kept on ice and 5ml water-saturated phenol was added and the mixture was vortexed before addition of 1.2ml chloroform. The final homogenate was mixed thoroughly by vortexing and cooled on ice for 15 minutes. Samples were centrifuged at 4800rpm for 20 minutes at 4^0 C. The aqueous phase containing the RNA was transferred to fresh tubes and mixed with equal amounts of 100% isopropanol and placed in -20^0 C overnight to precipitate RNA. DNA and proteins present in the interphase and phenol phase were discarded. The homogenate was centrifuged at 4800rpm for 30 minutes and the RNA pellet washed with 75% ethanol by centrifuging for 10 minutes at 4800rpm prior to resuspending in autoclaved distilled water at a concentration of 1µl/µg. The dissolved pellets were kept in -20^0 C until use. The RNA concentration was determined using the UV spectrophotometer and measuring the absorbance at OD $_{260}$. The ratio for OD_{260}:OD_{280} was calculated to determine how pure the samples were.

A value of 2 represents a pure RNA sample, a value below 2 indicates protein contamination and a value above 2 indicates salt contamination.

2.11.2. Glyoxylation:

This step was carried out to denature the total RNA and linearise it so that it can be loaded onto the agarose gel and the band visualised, and also to separate the mRNA from the other RNA species. The glyoxylation step was performed with 10µg RNA and to this 12µl of glyoxal solution (6M glyoxal (deionised); 7.8µl DMSO (Sigma); 0.1M NaH_2PO_4, pH 6.5 (Sigma) was added. The solution was incubated for 30 minutes at 50^0 C before addition of 4µl loading dye (50% glycerol (Sigma); 0.01M NaH_2PO_4, pH 7; 0.4% bromophenol-blue (Sigma)). The RNA was fractionated by electrophoresis on 1.5% agarose gel (Agarose 1.8g; 120ml Distilled water; 1.2ml 1M NaH_2PO_4 pH 6.5) at 10V overnight in 10mM electrophoresis buffer (10mM NaH_2PO_4 pH 6.5). Next day RNA was blotted onto hybond N^+ nylon membrane in 20X SSC solution (175.3g sodium chloride; 88.2g Sodium citrate in 1 litre distilled water and pH adjusted to 7 and autoclaved) for 6 hours. Prior to washing, the RNA was fixed by placing the membrane under UV light and applying 1200 kJoules of energy for a few seconds. The membrane was placed in a glass tube and washed for a few minutes with 5% acetic acid. The membrane was stained with methylene blue staining solution (0.5M NaOAc; 0.04% methylene blue (Sigma)) by applying the solution for a few minutes and then destained by rinsing with distilled water to wash off the methylene blue.

2.11.3. Synthesis of Radiolabelled Probe for Northern blots:

2.11.3.1. Template fragment:

Annexin II light chain, p11 (I-1) was used as a template and a 1284bp probe generated by PCR (Genius) using the 5' primer, GFP-5: (ACCACATGGTCCTTCTTGAG) and

3' primer, CSF-R: (TGCTGTTTAAATATTAAACAGGG) in the 5' to 3' direction with a T_a of 58^{0}C and 28 cycles. The PCR product was loaded onto a 1.5% agarose gel and electrophoresed for 1 hour at 100V. The band corresponding to the I-1 band (1289bp) and cyclophilin (300bp) was excised and the DNA purified using the Gene clean II kit. The templates were stored in –20°C until use.

2.11.3.2. DNA probe:

The probe used was labelled by random priming with [α-^{32}P]dATP (0.925MBq) using the Stratagene prime-it II random primer labelling kit and following the manufactures protocol. Briefly in a final volume of 25µl: 50ng p11 template (I-1) and 5µl 5X dATP Random 9mer primers were mixed and denatured for a few minutes by incubating at 90^{0}C and then rest of the solutions were added from the Stratagene kit as follows 5µl dNTP mixture; 2.5µl [α-^{32}P]dATP; 0.5 µl Klenow enzyme and made upto 25µl with dH_2O. The labelled solution was incubated at 37^{0}C for 15 minutes before removal of unincorporated nucleotides using the QIAquick nucleotide removal kit (Qiagen) and following the manufacturer guide as follows. Briefly to the ATP[P^{32}] reaction mixture 250µl of PN buffer was added and the solution was placed into a QIAquick spin column in a 2ml tube. The tube was centrifuged for 1 minute at 6000rpm and the spin column was placed in a new 2ml tube. 500µl of PE buffer was added and the reaction centrifuged for 1 minute at 6000rpm, the flow through was discarded and the column washed again in a new tube with 500µl of PE buffer for 2 minutes by centrifuging at 6000rpm. The QIAquick spin column was placed in a 1.5ml eppendorf and the RNA eluted by addition of 100µl EB buffer and centrifuging for 2 minutes at 6000rpm. The QIAquick spin column was discarded and the eppendorf kept in a lead box until use.

2.11.4. Pre-hybridisation:

Non-specific binding sites were blocked for 1 hour at 43^0 C with 10ml of hybridisation buffer composed of 5ml 100% Formamide (deionised) (Sigma), 3ml 20X SSC solution, 1ml 0.5M NaH_2PO_4, pH 6.5, 20µl 0.5M EDTA, 1ml 10% SDS, 500µl 50X Denhardt's solution (1% BSA, 1% Ficoll 400, 1% polyvinylpyrollidone and filtered through 0.45µM filter), 250µl Herring sperm DNA (10mg/ml). The membrane was placed in a glass tube and pre-hybridised with 10ml of hybridisation buffer at 43^0 C for 1 hour.

2.11.5. Hybridisation:

To 10ml of hybridisation solution 100µl of purified [α-^{32}P]dATP probe was added and this solution replaced the pre-hybridisation solution and the membrane was hybridised overnight at 43^0 C. The next day the membrane was washed twice with 2X SSC/0.1% SDS for 5 minutes by shaking gently at room temperature. A second wash was done twice with 0.2 X SSC/0.1% SDS for 15 minutes at 53^0C. An autoradiograph was established by exposing the membrane for 72 hours to X-ray film (Kodak Biomax MS-1 film) at -70^0 C. The film was developed by washing firstly in developer (GBX Developer/replenisher, Sigma) and then in fixer (GBX fixer/replenisher, Kodak).

2.12. Direct interaction of p11 with $Na_V1.8$:

2.12.1. SNS-GST Fusion Proteins:

pGEX (Pharmacia) vectors can be used in bacterial systems to express foreign polypeptides as fusions with glutathione S-transferase (GST). These proteins can be purified from lysed cells by absorption with glutathione-agarose beads followed by elution in presence of free glutathione. Each pGEX vector contains an open reading

frame encoding GST, followed by unique restriction endonucleases sites for BamHI, SmaI, EcoRI followed in turn by termination codons.

SNS-GST fusion proteins were made with the 3 separate fragments of the SNS N-terminal region (Bait 1 corresponding to position 1 to 125 of the intracellular loop 1). The 3 separate fragments were generated by PCR (Genius) with 30 cycles at an annealing temperature of 58^0C using primers to generate N1 (25 amino acids) using forward primer -TCTACTGTCCACGAAATCCTG (N1-F) and reverse primer -CTCGATCTCTGCCAGTGACTC (N1-R). N2 (26 amino acids) was generated by using the following forward primer -AAGCAGATTGCTCACCGC (N2-F) and the following reverse primer -CTTCTCGCCTTGTCCTCCTG (N2-R) and N3 (77 amino acids) was generated with the following primer sequence for the forward primer -CCCAGGCCTCAGCTGGACTTG (N3-F) and the following reverse primer -TCAGTCGTCCCCACCACAC (N3-R). After a phenol chloroform extraction, the PCR products were electrophoresed on a 1.5% agarose gel, the corresponding bands were cut and purified using the Gene Clean II kit and restriction endonuclease cut performed at EcoRI and NotI sites. The 3 fragments were subcloned into EcoRI and NotI sites in pGEX 5X1 vector and the resultant plasmid transformed into competent *E.coli* XL1-Blue cells. The SNS-pGEX5X1 plasmid was selected on LB/ampicillin plates. The plates were incubated for 12-15 hours at 37^0C. Transformants were picked and a miniprep performed to confirm the presence of the right fragment. SNS (N1, N2, and N3)-GST fusion proteins were expressed by transforming the plasmids into *E.coli* BL21 cells and incubating the LB/ampicillin plates for 12-15 hours at 30^0C.

2.12.2. Production of Fusion Proteins:

A colony of pGEX5X1 transformants (N1, N2, N3 and GST only) was inoculated into 15ml LB/ampicillin medium and grown for 12-15 hours at 37^0C in a shaking incubator. Expression of fusion proteins was induced by addition of 1M isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1mM and continuing incubation at 37^0C for a further 4 hours. A 500µl aliquot was taken in 500µl 80% glycerol and frozen at -70^0C for further use and the rest was centrifuged for 10 minutes at 4800rpm at 4^0C. The pellet was resuspended in 2ml ice-cold PBS and the tubes immersed in ice and sonicated for 1 minute using a probe sonicator. 10% Triton X-100 was added to 1% and the tubes mixed and centrifuged for 10 minutes at 4800rpm at 4^0C. The supernatant was collected and 1ml of 50% slurry of glutathione agarose-beads (Pharmacia Biotech) was added and the tubes gently mixed at room temperature for 2 minutes. The solution was washed 3 times with ice-cold PBS solution and centrifuging for 1 minute at 1600rpm. The beads were resuspended in 1ml of ice-cold PBS and stored at -20^0C until use.

2.12.3. GST Pull Down Assay:

Full length rat cDNA for p11 in yeast expression vector pJG4-5 was subcloned as the NcoI-XbaI fragment into the pBS500 expression vector which results in *GFP*-p11 fusion protein expression driven by elongation factor 2α promoter. The resultant plasmid was designated as pBS-GFP/p11. COS cells were transfected with pBS-GFP/p11 by lipofection as described below. pBS-GFP-p11 fusion protein was extracted from transfected COS cells by lysis buffer (4% SDS, 10 mM sodium phosphate, pH 7.4) 3 days after transfection. The lysate was centrifuged at 10,000rpm at 4°C for 10mins. The supernatant was retained and centrifuged at 14,000rpm at 4°C

for 30 min. The supernatant was then transferred to a macrosep 30K omega centrifugal concentrator (Pall Filtron) and 10ml ice-cold PBS, pH 7.4, was added. It was centrifuged at 5,000rpm for 2 hours. The filtrate was discarded and the residue, containing the solubilized protein, was used in the pull-down assay experiment. GFP expression plasmid, pBS-GFP, was used for production of GFP protein for a negative control.

To examine the binding of p11 to the N-terminus of $Na_V1.8$ *in vitro*, glutathione-sepharose beads preincubated with purified GST, serving as control, or N1-GST, N2-GST and N3-GST obtained above were incubated at 4°C overnight with GFP-p11 fusion protein extracted from transfected COS cells in PBS. After intensive washing with ice-cold PBS, the bound proteins were denatured in sample buffer (100mM Tris-Cl pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol, 200mM DDT and incubation at 85°C for 10 minutes), spun briefly and 20µl loaded and separated on 12% SDS-PAGE gel for 4 hours at 160V in the electrophoreses buffer (10x Laemmli Buffer - 800 ml dH_2O, 30.3g Tris Base, glycine 144.2g, 10g SDS, and dH_2O to final volume of 1 litre and pH adjusted to 8.3. This solution was diluted 1:10 to use). The proteins were transferred to nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) by incubation in transfer buffer overnight (1X Laemmeli buffer with 2% methanol) at 10V, 7mA. The membrane was blocked for 1h in 5% nonfat dry milk in PBS-T at room temperature (0.1% Tween-20 in PBS, pH7.4). Primary anti-GFP antibody (Santa Cruz Biotechnology) in 1:800 dilution was applied for 1 hr at room temperature. Secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse IgG, Amersham Pharmacia Biotech) in 1:2000 dilution was applied for 1 hr at room temperature. ECL Western Blotting Detection Reagents (Amersham Pharmacia

Biotech) was applied according to the manufacturer's instructions and the blot was exposed to BioMax film (Kodak).

2.12.4. Preparation of Glutathione Sepharose 4B (50% slurry):

Glutathione sepharose 4B in tube was shaken gently to resuspend the matrix. 1.33ml of the original slurry per ml of bed volume required was dispensed into 50ml tubes. The matrix was sedimented by centrifugation at 4800rpm for 5 minutes. The supernatant was decanted and the glutathione sepharose 4B was washed with 10ml ice-cold PBS. The matrix was sedimented by centrifugation at 4800rpm for 5 minutes and the supernatant decanted. 1ml of ice-cold PBS was added which resulted in 50% slurry and this mixture was kept at 4^0 C.

2.13. In Situ hybridisation:

2.13.1. Preparation of linearised DNA:

The clones to be used for *in situ* hybridisation was excised from pJG4-5 vector and recloned into pBluescript using EcoRI and XhoI sites. The pBluescript has T3 and T7 primer sequences, which are T3/T7 RNA polymerase promoter sequences, and the DIG-11-UTP (digoxigenin-11-uridine-5'triphosphate) was used as a substrate for T3 or T7 RNA polymerases and for RNA labelling to replace UTP in *vitro* transcription. The T7 polymerase will transcribe from the 3' direction and make the antisense. To stop the antisense transcribing endlessly, the circular plasmid was linearised at the 5' prime end by endonuclease cut with EcoRI. The plasmid was incubated with EcoRI overnight at 37° C after which a phenol/chloroform extraction was performed. The solution was centrifuged for 10 minutes at 14000rpm and the supernatant decanted and 5µl 3M NaOAc added. 120µl 100% ethanol was added and the resultant solution

vortexed prior to incubation at –20° C for 30 minutes. After the incubation the tubes were centrifuged for 30 minutes at 14000rpm and the supernatant discarded. The pellet was washed with 70% ethanol and centrifuging at 14000rpm for 10 minutes. After evaporating off the ethanol the pellet was resuspended in 10µl dH_2O. This linearised DNA was used to generate the DIG-labelled probes.

2.13.2. Making DIG-labelled probes:

The following were added to give a final volume of 20µl. In an eppendorf tube on ice; 2µl of 1µg linearised DNA (clone III-42 or clone IV-40), 2µl of 10X dNTP mix- with DIG (10mM each of ATP, GTP, CTP; 6.5mM UTP, 3.5 mM DIG-11-UTP in TRIS neutralised solution, pH 7.5), 2µl of 10X transcription buffer (0.4M TRIS, pH 8; 60mM $MgCl_2$, 100mM DTT, 20mM spermidine, 100mM NaCl, 1 Unit/µl RNase inhibitor), 200 units T3, T7 RNA polymerase, 0.5µl RNase inhibitor, and 13.3µl dH_2O. The solution was incubated at 37°C for 2 hours and the transcription reaction stopped by addition of 0.2M EDTA, pH 8. Salt precipitation was carried out with 10µl NH_4OAc and was followed by 90µl of ice-cold 100% ethanol and incubating the mixture for 2h at –20°C. The mixture was centrifuged for 10 minutes at 14000rpm and the pellet washed with 70% ice-cold ethanol. The pellet was dried and resuspended in 50µl dH_2O. 1µl RNase inhibitor was added and the probe stored at –20°C.

2.13.3. Preparation of section:

Fresh frozen sections (15µm) from 2 week old rat pup DRG mounted in OCT compound were cut, and immediately mounted on superfrost plus charged slides (BDH). The sections were air dried for 1 hour followed by addition of ice-cold 4% Paraformaldehyde (40g PFA added to 900ml dH_2O, and the solution heated to 65°C to

dissolve the PFA). 100ml 10X PBS (76g NaCl, 9.93g Na_2HPO_4, 4.68g $Na_2HPO_4.2H_2O$ was added to 1l dH_2O and the pH adjusted to 7.4) to fix the sections for 10 minutes on ice. Sections were washed 3 times with 1X PBS and acetylated with triethanolamine (3.5ml triethanolamine and 750µl acetic acid in 300ml dH_2O) for 10 minutes. Sections were washed 3 times with 1X PBS.

2.13.4. Hybridisation of sections:

500µl hybridisation solution (50% formamide (1.5ml of 100% formamide), 4X SSC; 600µl of 20X SSC (175.3g NaCl and 88.2g Na citrate made upto 1l in dH_2O and pH adjusted to 7.4 before autoclaving), 2X Denhardt's solution; 120µl of 50X Denhardt's solution (1% BSA; 1% Ficoll 400; 1% polyvinylpyrollidone and filtered through 0.45µM filter), 15µl of 10µg/µl tRNA to give 50µg/µl, 90µl of 5mg/ml salmon sperm DNA to give 150µg/ml) was added and the sections incubated at room temperature for 1 hour. Then 500µl hybridisation solution containing 0.5µl DIG-labelled probe was added to the slides and incubated overnight at 66°C. Next day the slides were washed 3 times with 2X SSC for 10 minutes at 72°C. The slides were incubated at 72°C for 30 minutes with 2X SSC followed by 1 hour incubation at 72°C with 0.1X SSC solution. The sections were given a final wash for 10 minutes at room temperature with 0.2X SSC solution.

2.13.5. Detection of DIG-labelled probes:

A highly sensitive non-radioactive DNA labelling and detection system based on the ELISA principle was used here. The DNA was modified with digoxigenin (DIG) by enzymatic incorporation of digoxigenin labelled deoxyuridine-triphosphate (dUTP) with Klenow enzyme. Following hybridisation of membrane with a digoxigenin labelled probe (DIG-labelled probe), the hybrids were detected by an ELISA reaction

using DIG specific antibodies covalently coupled to the marker enzyme alkaline phosphatase. This binding of antibody: conjugated alkaline phosphatase was followed by an enzyme catalysed coupled redox reaction with the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) which gives rise to a dark blue coloured water-insoluble precipitate directly adhering to the sections.

The slides were incubated for 10 minutes at room temperature in buffer B1 (0.1M TRIS pH 7.5, 0.15M NaCl). The solution was replaced by buffer B2 (0.1M TRIS pH 7.5, 0.15M NaCl, 2% sheep serum) for 1 hour. Anti-DIG antibody (alkaline phosphatase conjugated) (Roche) solution was made up in B2 buffer and added to the slides for 90 minutes. At the end of the incubation buffer B3 was added for 5 minutes (0.1M TRIS pH 9.5, 0.1M NaCl, 50mM $MgCl_2$). Buffer B4 was added to each slide and the slides incubated overnight (4.5µl nitroblue tetrazolium salt (0.34mg/ml) (NBT), 3.5µl 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (0.18mg/ml) (BCIP), 25µl of 5mg/ml levamisole in 1ml solution B3). The next day the reaction was stopped by washing with 1X PBS solution.

2.13.6. I-1 In Situ hybridisation:

An 284bp p11 PCR fragment was subcloned into pGEM-T Easy (Promega), and DIG-UTP labelled sense or antisense cRNA probe were generated using T7 RNA polymerase as described above. Frozen DRG sections (10 µm thick) were fixed for 15 min in 4% paraformaldehyde on ice and were acetylated in 0.1M triethanolamine, 0.25% acetic anhydride for 10 minutes. Prehybridisation was carried out in 50% formamide, 4 x SSC, 100 µg/ml herring sperm DNA, 50 µg/ml tRNA, 2 x Denhardt's

solution at room temperature for 1 hr. Hybridisation was carried out in the same buffer containing 50 ng/ml cRNA probe at 65°C for 16 hrs. Sections were washed in 0.1 x SSC at 72°C and incubated with alkaline phosphatase conjugated anti-digoxygenin antibody. The same sections were then stained with anti-$Na_V1.8$ polyclonal antibody (SNS11) followed by rhodamine-conjugated anti-rabbit IgG antibody.

2.14. Immunohistochemistry:

Sections were cut (15μm) from 2 week old rat DRG and placed on superfrost plus charged slides. The sections were air-dried for 2 hours. The sections were washed 2 times for 10 minutes with PBS-triton (500ml of PBS, 500ml H_2O, 0.5ml DepC, 0.5ml Triton X-100 (PBS-T)). Non-specific binding sites were blocked with 20% sheep serum in PBS-T for 1 hour prior to the addition of the primary antibody (L-Periaxin-1/1500 dilution (Gift from Dr Peter Brophy, Edinburgh University). The primary antibody was incubated overnight at 4°C in an humidified chamber. The sections were washed 4 times for 5 minutes with PBS-T prior to the application for 2 hour at room temperature of the secondary antibody (Anti-Rabbit IgG – conjugated with FITC for Periaxin; 1/200 dilution in PBS-T). The sections were washed 4 times with PBS-T for 5 minutes and a coverslip mounted on the sections with a few drops of CITIfluor. The sections were examined under a fluorescent microscope.

2.15. Co-immunoprecipitation analysis using Western Blots

2.15.1 Transfection using Lipofectamine:

2.15.1.1. COS Cell Transfection:

Cells were sub-cultured 1 day prior to transfection in 100mm dishes as described above. They need to be 50-80% confluent before performing transfection. COS cells were transfected with pBS500 expression vector encoding GFP-HA tagged-full length p11 as follows. For each transfection of a 35mm dish, 1mg of DNA was diluted into 100µl serum-free DMEM. In a separate eppendorf, 5µl of lipofectamine (Gibco BRL) was diluted into 100µl serum-free medium. The two solutions were combined, mixed gently, and incubated at room temperature for 30 minutes. The cells were rinsed once with 2ml of serum-free medium. 800µl of serum-free medium was added to the DNA/lipofectamine mixture and the mixture applied to the rinsed cells. The cells were incubated at 37° C for 2 hours after which the medium was replaced with normal DMEM medium containing 10%FCS. For each 100mm dish, 8µg of DNA, 20µl of lipofectamine and 600µl serum-free medium was used. For the DNA/lipofectamine mixture 4.8ml of serum-free medium was added. p11 protein was extracted from transfected COS cells 3 days after transfection.

2.15.2 Lysis:

Dishes were washed with 10ml PBS and drained well. 1ml of lysis solution was added with 1µl of protease inhibitor cocktail containing PMSF (1000 X PMSF Stock solution for serine protease inhibition in isopropanol). Dishes were left on ice for half an hour, with occasional rocking to prevent cells drying out. After the incubation the cells were washed into 1.5µl eppendorfs and spun at 14000rpm for 10 minutes at 4°C. The lysate

was removed to fresh tubes and the tubes centrifuged at 14000rpm for a further 10 minutes at 4°C. The lysate was kept for antibody addition in subsequent steps.

2.15.2.1. Lysis Buffers:

2.15.2.1.1. 2% SDS Buffer:

2% SDS, 10% glycerol, 100mM DTT, 60mM Tris, pH 6.8 were mixed together.

2.15.2.1.2. 4%SDS/2% Triton/phosphate buffer:

10mM phosphate buffer (1/100 dilution of a solution of 1M phosphate buffer pH 6.5; 13.7ml of 1M NaH_2PO_4 and 6.3ml of 1M Na_2HPO_4), 0.5 mM EDTA, 20µl Triton, 400µl of 10% SDS, 1µ PMSF and 1µ protease inhibitor was mixed together.

2.15.2.1.3 NP-40:

1.5ml 5M NaCl, 500µl 1% NP-40, 2.5ml 1M Tris, pH 8 was mixed together and the solutions brought upto 50ml with dH_2O.

2.15.2.1.4. RIPA:

1.5ml 5M NaCl, 500µl 1% NP-40, 500µl 10% SDS and 2.5ml 1M Tris pH 8 were mixed together and the solution brought to 50ml with dH_2O.

2.15.3 Preclearing prior to antibody application:

This step was taken to ensure that there was no non-specific binding. Supernatant from the lysis step was taken and 100µl sheep serum was added. The tubes were incubated on ice for an hour prior to addition of 150µl protein A-sepharose beads. The mixture was then incubated on ice again for a further half an hour. The mixture was pelleted by centrifuging at 4800rpm for 10 minutes and the supernatant decanted and kept in the fridge for subsequent steps.

2.15.4 Antibody Addition and collection on Protein A beads:

1ml supernatant was taken and 2.5µl HA antibody added (1/400 dilution), this mixture was incubated on ice for 1 hour. 100µl protein A-Sepharose C1-4B mixture was added and the mixture left on ice for 1 hour. The mixture was spun for 30 sec at 4°C at 10500rpm, 70µl protein A bead mixture was added and this solution was washed 3 times by centrifuging for 30 seconds at 10500rpm at 4°C. The final wash was aspirated and 40µl SDS sample buffer was added for the COS cells and the tubes heated at 100°C for 5 minutes to denature all the proteins and to break up the antibody-antigen complex. For the DRG 4% SDS/2% Triton buffer was added along with phosphate buffer in order to denature the proteins. The solution was spun and the supernatant loaded onto the gel and run at 150V for 3 hours.

2.15.5 Gels:

10% Separation gel: In a final volume of 30ml the following were added:

40% acrylamide	7.5ml (Mixed with 20g/l Bis acrylamide)
1M Tris pH 8.8	11.25ml
10% SDS	0.3ml
H_2O	10.85ml
10% APS	0.1ml (amonium persulphate)
TEMED	20µl (Just prior to pouring gel)

12% Separation gel:

40% acrylamide	9ml (Mixed with 20g/l Bis acrylamide)
1M Tris pH 8.8	11.25ml
10% SDS	0.3ml
H_2O	9.35ml

10% APS	0.1ml (amonium persulphate)
TEMED	20µl (Just prior to pouring gel)

The gels were poured immediately and left to set for half an hour. To ensure a flat surface approximately 1ml of water was added to the top to make a layer and this can be poured off when gel has set and before stacking gel was added.

Stacking gel: In a final volume of 10ml add

40% acrylamide	0.94 ml
1M Tris-HCl pH 6.8	1.25 ml
10% SDS	0.1ml
H_2O	7.66ml
10% APS	0.05ml
TEMED	10 µl (Just prior to pouring)

The water was poured out and the stacking gel added and left to set.

2.15.6. Loading and running the gel:

The protein samples were separated via SDS-Polyacrylamide Gel Electrophoresis. 4 µl of Rainbow Marker (Amersham RPN 756) was used and the gel run at 10 mA, 200 mV, over night in the electrophoresis running buffer (10x Laemmli Buffer - 800 ml dH_2O, 30.3g Tris Base, glycine 144.2g, 10g SDS, and dH_2O to final volume of 1 litre and pH adjusted to 8.3. This solution was diluted 1:10 to use).

2.15.7 Coomassie blue staining:

One half of the protein gel was cut and placed in a fixer solution for 10 minutes (50% methanol, 10% acetic acid and 40% dH_2O) at room temperature. The fixer solution was replaced by coomassie blue stain (0.2g coomassie brilliant blue R-250, 50ml acetic acid, 125ml isopropanol, and made with dH_2O to 500ml) and the gel stained for

10-30 minutes at 55°C. After staining the gel till all the rainbow marker had turned blue, destaining solution (100ml methanol, 75ml acetic acid and made up with dH_2O to 1l) was added to remove excess coomassie blue in order to see the protein bands present for IV-40 and IV7-40. The destaining solution was added to the gel and a piece of tissue placed in the solution to blot up the stain for 30-120 minutes at 55°C. This solution was replaced several times until the protein bands become visible.

2.15.8. Gel to membrane transfer:

The electrophoresed proteins from the other half of the gel were transferred to Hybond-C extra membrane via electrophoretic elution. A sandwich was made consisting of a piece of transfer membrane and the gel between adsorbant filter paper. This was submerged into a transfer tank filled with transfer buffer (In a final volume of 1 litre the following were added: 39 mM Glycine, 2.9 g; 48mM Tris base, 5.8 g; 0.037% SDS (electrophoresis grade), 0.37 g; 20% Methanol, 200 ml; dH_2O to 1 litre, pH should be 8.3) with the membrane closest to the positive electrode (Anode) (red). Transfer occurred from the cathode to the anode between 5 hours to over night at around 10V, 7mA.

2.15.9. Detection using ECL:

The primary antibody was detected using highly sensitive ECL Western blotting solution. A secondary horseradish peroxidase antibody was capable of catalysing the oxidation of luminol in alkaline conditions, potentiated with phenol. Immediately after oxidation the luminol is in an excited state and will decay back to ground state via a light emitting pathway (emission at 428 nm). The protocol laid out in the ECL manual

was followed. This method will detect 1pg of antigen or less on Hybond-ECL, with high resolution.

The membrane was removed from the transfer tank and the marker bands marked onto the membrane. Non-specific binding sites on the membrane were blocked for 45 minutes using 50ml of blocking solution (PBS with 0.1%Nonidet-40 (PBS-Tween) detergent, 5% powdered milk) with gentle agitation. The membrane was washed 3 times with PBS-Tween before addition of Primary antibody (1/400 anti HA antibody or 1/1000 anti- $Na_V1.8$ antibody (SNS11) or 1/800 dilution of anti-GFP antibody) with membrane placed face down for 1 hour at room temperature). The membrane was then washed 3 times in 150 ml of Wash buffer (PBS-Tween) for 10 minutes. Anti mouse IgG (1/2000) conjugated with horseradish peroxidase secondary antibody was added for anti-GFP, anti-HA primary antibody or for anti-$Na_V1.8$ antibody and membrane placed face for 1 hour at room temperature. The membrane was washed 3 times with PBS-Tween. 1 ml each of ECL Solution 1 and 2 were mixed, the excess Wash buffer was drained from the membrane and it was placed face down on top of the 2 solutions and incubated for 2 minute at room temperature. After draining off excess solution the membrane was wrapped in Saran wrap, smoothing out any bubbles. The membrane was then exposed to X-ray film (Kodak Biomax MS-1 film) (Sigma) for 30s to a few minutes and the film developed by washing firstly in developer (GBX Developer/replenisher, Sigma) and then in fixer (GBX fixer/replenisher, Kodak).

2.16. Translocation of $Na_V1.8$ to the plasma membrane:

2.16.1. Transfection of p11 into CHO-SNS22:

A stably transformed CHO cell line (CHO-SNS22 cells) that expresses rat $Na_V1.8$ protein in the cytosol was transfected with the expression plasmid pBS-GFP/p11 by

lipofection. CHO-SNS22 cells were plated in 35mm dish containing F-12 medium with 0.5% fetal bovine serum, 250units/ml streptomycin/penicillin and 1mg/ml G418. Prior to transfection, cells in 35mm dish were rinsed twice with serum-free F-12 medium. 1.1μg of DNA was diluted in 100μl serum-free F-12 medium. 5μl of Lipofectamine was diluted into 100μl serum-free F-12 medium. The two solutions were mixed together and were incubated at room temperature for 30 minutes. The mixture was added to the pre-rinsed cells and incubated at 37°C for 2 hours after which the DNA/lipofectamin mixture was replaced with F-12 medium with 0.5% fetal bovine serum, 250 Units streptomycin/penicillin and 1mg/ml G418. Three days after transfection, the cells were fixed with 4% paraformaldehyde for 15 minutes on ice and subsequently incubated with anti-$Na_V1.8$ polyclonal antibody (SNS11) for 1 hour at room temperature. The cells were washed with three times with PBS and incubated with rhodamine-labelled anti-rabbit IgG. The cells were mounted in CITIfluor and analysed with a fluorescent and confocal microscope.

2.16.2. Electrophysiology:

Membrane currents were recorded from CHO-SNS22 cells using the whole-cell patch-clamp technique 3 days after trnasfection. The extracellular recording solution contained the following: 140mM NaCl, 10mM TEA-Cl, 10mM Hepes-NaOH pH7.2-3, 2.1mM $CaCl_2$, 2.12mM $MgCl_2$, 0.5mM 4-aminopyridine, 7.5mM KCl, 250nM tetrodotoxin (Alomone labs). The intracellular solution contained the following: 145mM CsCl, 3mM EGTA, 10mM Hepes-CsOH pH 7.2-3, 1.21mM $CaCl_2$, 1.21mM $MgCl_2$, 10mM TEA-Cl. Chemicals were either 'AnalaR' (BDH, Merk Ltd.) or supplied by Sigma. A minority of CHO-SNS22 cells generated an endogenous tetrodotoxin-sensitive (TTX-S) Na^+ current which was eliminated from all recordings

by including 250nM TTX in the extracellular media. No inward currents were recorded in non-transfected cells under these circumstances.

Electrodes were fabricated from thin-wall glass capillaries (GC150TF-10; Harvard apparatus, UK), and had an access resistance of 2-4 MΩ when filled with recording solution. Recordings were made using an Axopatch 200B patch-clamp amplifier (Axon Instruments, USA). Pulse protocols were generated using pClamp6 software (Axon Instruments). CHO-SNS22 cells were held at –90mV. Voltage-clamp protocols incorporated a negative pre-pulse to –110mV, and the cell was subsequently stepped to more depolarized potentials for 50ms (up to a final value of +80mV), in 10mV increments. Dr Mark Baker performed all experiments at room temperature.

CHAPTER 3.
RESULTS FOR POSITIVE INTERACTING CLONES

3.1. Results for positive clones achieved in the interaction trap:

Using the yeast-2-hybrid system, proteins were identified that interact with the $Na_V1.8$/SNS channel. The interaction trap was performed as described in chapter 2. Briefly the intracellular loops of $Na_V1.8$ were used as the baits (figure 3.1) and fused to the DNA binding domain of LexA. For the baits the plasmids were generated with PCR using $Na_V1.8$ as a template with different 5' forward and 3' reverse primers as detailed in chapter 2.

The amplified fragments were ligated into pEG202 plasmid at EcoRI–NotI sites as an in-frame fusion with the LexA–DNA binding domain. This plasmid contains the selectable marker gene HIS3, and the plasmid containing this gene can be maintained in the yeast strain and selected on media lacking histidine. Yeast strain, EGY48, was transformed with the pEG202 containing the bait fragment/LexA. The binding sites for the bait/LexA were located upstream of 2 reporter genes. Firstly the upstream activating sequences of the chromosomal LEU2 gene, required in the biosynthetic pathway for leucine, were replaced in EGY48 with LexA operators, permitting selection for viability when cells were plated on media lacking leucine. This yeast strain also harbours a plasmid pSH18-34 that contains a LacZ fusion gene, permitting discrimination based on colour. In addition to the mutation in the LEU2 gene, EGY48 carries a mutation in three other marker genes (HIS, TRP1, URA3) that are needed for selection of the plasmids used in the interaction trap.

The rat DRG cDNA library was cloned in the plasmid pJG4-5 at EcoRI-XhoI sites and fused to the transcription activation domain as described previously. This library containing plasmid also contained the selectable marker gene TRP1 allowing selection

of library plasmids on media lacking tryptophan. The interaction trap was performed where the EGY48/pSH18-34 containing the bait plasmid pEG202 was transformed with the conditionally expressed rat DRG cDNA library in pJG4-5. Expression of library encoded proteins was induced by plating transformants on galactose/raffinose(Gal/Raf) plates lacking uracil (Ura^-), histidine (His^-), tryptophan (Trp^-), and leucine (Leu^-). Galactose induces the expression of the library proteins and yeast containing library proteins that interact with the bait will form colonies within 2 to 5 days on media lacking leucine, histidine, uracil and tryptophan and the colonies will turn blue as these colonies produce β-galactosidase on plates containing X-gal. Using this selection system yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow in the absence of leucine. The plasmids were isolated and characterised by a series of tests to confirm specificity of the interaction with the initial bait protein. Those found to be specific were then sequenced.

3.2. Characterisation of the bait protein:

The major requirements for the bait protein were that it should not actively be excluded from the yeast nucleus and was capable of entering the yeast nucleus and binding LexA operator sites. Secondly it should not activate transcription of the lexA operator-based reporter genes on its own prior to the transformation of the library i.e it must not grow on media lacking leucine and the colonies should appear white on medium containing X-gal. The protocol is described in chapter 2.

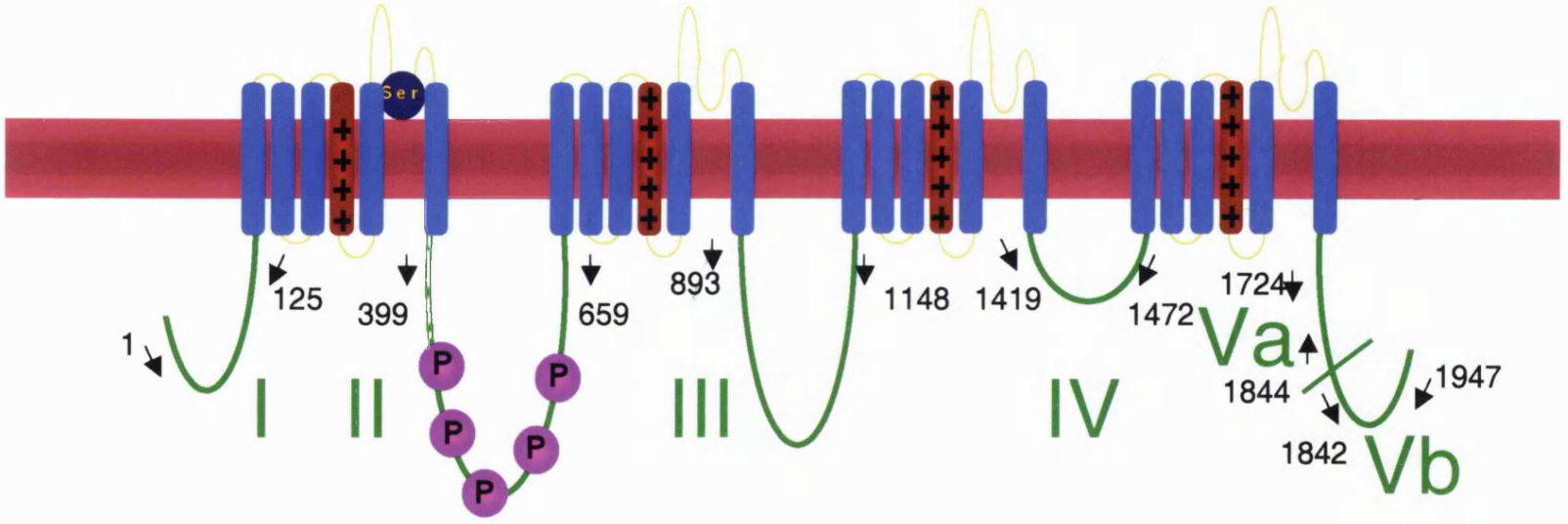


Figure 3.1. Structure of NaV1.8 α-subunit showing the four homologous domains I-IV each of which is composed of six membrane spanning segments. The location of the five baits is indicated by arrows and the numbers correspond to the amino-acid location. Bait II has the five sites for PKA phosphorylation at the serine residues.

3.2.1. Activation assay:

The activation assay confirmed that the bait proteins were not activating transcription on their own. The method is described in full in chapter 2. Yeast strain was transformed with the reporter plasmid (pSH18-34) and grown on glucose minus uracil (Glu Ura$^-$) plates. Colonies were picked and grown in Glu Ura$^-$ medium and the bait plasmid (pEG202), positive control (pSH17-4) and negative control (pRFHM1) were transformed and the transforants grown on Glu Ura$^-$ His$^-$ plates. Colonies were picked and grown onto Glu Ura$^-$ His$^-$-Xgal plates to look for LacZ expression. Colonies were also grown in Glu Ura$^-$ His$^-$ medium to select for the bait plasmid and plated on Gal/Raf Ura$^-$ His$^-$ and Gal/Raf Ura$^-$ His$^-$ Leu$^-$ plates to see if the bait was activating the reporter plasmid on its own. There was no library plasmid present to turn on activation and therefore the bait plasmid did not interact with the library and hence was unable to grow on media lacking leucine and transcribe the reporter gene.

In the Gal/Raf Ura$^-$ His$^-$ plates the positive and negative control as well as the bait plasmid gave colonies that grew at the same rate as was expected. As described previously baits I, II, III, IV and V were fused to the *LexA* operators in the plasmid pEG202. All 5 baits were PCR generated using rat $Na_V1.8$ cDNA as a template with bait I corresponding to position 1-127 amino acids in the intracellular loop (the N-terminal region). Bait II corresponded to position 399-659, bait III corresponded to position 893-1148, bait IV corresponded to position 1419-1472. The C-terminal region, bait V was from position 1724 to position 1947. There was no library transformation present hence the colonies were grown on plates that contain tryptophan and leucine in the media. This showed that the bait protein was not toxic and can enter the yeast and survive. In the Gal/Raf Ura$^-$ His$^-$ Leu$^-$ only the positive

control grew as there was no library plasmid present to turn on activation and allow colonies to be grown in the absence of leucine hence the negative control and the bait plasmids were not able to grow. In this assay only the positive control produced blue colonies on the Glu Ura^- His^--Xgal plates. This was what was expected. The bait plasmids did not produce blue colonies as the baits are not activating the reporter gene on their own and therefore there was no β-galactosidase activity and hence the colonies remain white on X-gal plates. The results for the activation assay are shown in table 3.1. It was concluded that baits I, II, III and IV did not activate transcription prior to library transformation and therefore could be used in the interaction trap. However bait V was seen to produce colonies in the absence of leucine, this showed that bait V was causing activation of the reporter gene on its own prior to library transformation. The next step in this stage was to cleave bait V into two separate fragments and produce new fusion proteins in pEG202 and repeat the activation assay. The protocol for the cleavage is described in chapter 2. Using this procedure it was observed that the two separate fragments of bait V, V_a and V_b, did not activate transcription on their own and therefore were able to be used in the interaction trap.

3.2.2. Repression Assay:

For bait-*LexA* proteins that do not activate transcription, it was important to confirm that the fusion protein was actually being synthesised in the yeast and was binding to the *LexA* operators by doing a repression assay. The repression assay was based on the observation that *LexA* and non-activating *LexA* fusions can repress transcription of a yeast reporter gene that has 1 LexA operator in between UAS_G and the TATA box. As mentioned previously that LacZ expression was induced by galactose and was detectable in the presence of glucose because the negative regulatory elements that

normally keep the Gal1 repressed in glucose were absent. The method is described in chapter 2. Yeast strain was transformed with the reporter plasmid pJK101 and selected on Glu Ura$^-$ plates, colonies were picked and grown in Glu Ura$^-$ medium and theplasmids containing the bait (pEG202), positive (pRFHM1) control and the negative (pEG22) control were transformed into yeast colonies. The transformants were plated onto Glu Ura$^-$ His$^-$ plates and grown for a few days. Colonies were picked and streaked onto Glu Ura$^-$ His-Xgal and Gal/Raf Ura$^-$ His-Xgal and grown at 30^0C. Yeast lacking LexA will begin to turn blue on the Gal/Raf Ura$^-$ His-Xgal after one day and will appear light blue on Glu Ura$^-$ His-Xgal after 2-3 days.

The repression assay is summarised and shown in table 3.2. An inert bait that makes LexA fused proteins, enters the nucleus and binds the lexA operators will block activation from the UAS_G repressessing the LacZ expression 2 to 20-folds in the presence of galactose. Yeast containing a bait that enters the nucleus and binds operators turn blue more slowly than yeast lacking LexA i.e. the negative control. Bait proteins that do not activate in the activation assay, and do repress in the repression assay, were good candidates for use in an interaction trap. All of our baits could be used as they were seen to repress the β-galactosidase activity in X-gal medium and the colonies appeared at a slower rate than the negative control. A schematic diagram of the repression assay is also shown in figure 3.2.

	Positive control	Negative control	Bait I	Bait II	Bait III	Bait IV	Bait V	Bait V_A	Bait V_B
Glu Ura^- His^- Xgal	Blue	White	White	White	White	White	White	White	White
Gal/Raf Ura^- His^-	+	+	+	+	+	+	+	+	+
Gal/Raf Ura^- His^- Leu^-	+	--	--	--	--	--	+	--	--

Table 3.1 Activation assay. The above table shows whether a bait can be used in the interactor hunt. For a bait to be used, the selection strain containing it must not grow on Gal/Raf Ura^- His^- Leu^-. For LacZ expression, the bait produces white colonies on Glu Ura^-His^--Xgal hence indicating the presence of an inert bait.

3.2.3. Interactor hunt:

An interactor trap involved large platings of yeast containing LexA-fused baits, the reporter gene and the library in pJG4-5 with a cDNA expression cassette under the control of the *GAL*1 promoter. In the first plating, yeast was plated on complete minimal medium Glu Ura^- His^- Trp^- dropout plates to select for the library plasmid. In the second plating, which selects for yeast that contains the interacting proteins, approximately $10^6 - 10^7$ colonies were plated onto Gal/Raf Ura^- His^- Trp^- Leu^- dropout plates. Library plasmids from colonies identified in the second plating were purified by bacterial transformation and used to transform yeast cells for the final screen. A miniprep was carried out to purify library containing plasmids and characterise them by sequencing.

Bait I

4 dish selection was carried out and colonies were plated onto Leu^+ dishes at 10^6cfu (colony forming units)/10cm dish on Gal/Raf Ura^- His^- Trp^--Xgal and Glu Ura^- His^- Trp^--Xgal plates. This is to elimate any colonies that can cause activation on their own or are toxic to the yeast. Only colonies which do activate the bait and therefore can be used in the activation trap were picked for further analysis. Colonies were picked from these plates and plated onto Leu^- dishes (Gal/Raf Ura^- His^- Trp^- Leu^- and Glu Ura^- His^- Trp^- Leu^-) and if interaction occurred between the library and bait colonies will be grown on Leu^- dishes. For bait I 1000 colonies were obtained on Leu^- plates out of which only 212 were blue on Xgal plates. These colonies were picked for further analysis.

Bait II

From the 4 dish selection only 1 colony was blue on Gal/Raf Ura^- His^- Trp^--Xgal. The number of cells plated onto Gal/Raf Ura^- His^- Leu^- was increased from 10^6 cfu to 10^7cfu/10cm dish. From these dishes there were 9 colonies which were blue on Gal/Raf Ura^- His^- Trp^-Xgal plates.

	Positive control	Negative control	Bait I-IV
Gal/Raf Ura^-His^--Xgal	Lex A binds to operators positioned between UAS_G and LacZ reporter hence block activation and therefore repressing β-gal activity.	No Lex A operators to bind hence promoter for LacZ transcribed and colonies turn blue.	Lex A binds to operators and inhibits activation from UAS_G and represses β-gal activity and colonies turn blue more slowly than negative control.

Table 3.2. Repression Assay. The above table shows whether a bait can be used in the interactor hunt. For a bait to be used it should repress β-galactosidase activity more slowly than the positive control as when LexA fused proteins are made they enter the nucleus and bind lexA operators. The reporter plasmid used for represion assay is pJK101. Binding of LexA will block activation from the Gal1 upstream activation sequences (UAS_G) repressing β-galactosidase activity by 3 to 20-fold.

Bait III

Dishes were plated at 10^6cfu/10cm. Colonies were picked and plated for the 4 dish selection out of which 51 were blue on Gal/Raf Ura$^-$ His$^-$ Trp$^-$-Xgal.

Bait IV

Dishes were plated at 10^6cfu/10cm dish and from the 4 dish selection 107 had blue colonies on Gal/Raf Ura$^-$ His$^-$ Trp$^-$-Xgal.

Bait V

This bait activated the LEU2 reporter gene on its own prior to library transformation and therefore the bait was truncated into 2 separate fragments by designing primers and repeating the PCR to generate 2 separate fragments. The first fragment, V_A was generated using forward and reverse primers and corresponded to amino acids position 1723 to 1844. V_B was generated using forward and reverse primers and corresponded to amino acids position 1842 to 1947. Bait V_A was plated at 10^6 cfu/10cm dish and 10 dishes were plated corresponding to 10^7 cfu in total. V_B was plated at 10^6 corresponding to 10^7 cfu. 1000 colonies were streaked for each fragment and from the 4 dish selection, V_A gave 27 blue colonies and V_B gave 99 blue colonies.

DNA sequencing was carried out on the positive colonies picked to confirm what clone it was and also to eliminate duplicate sequences. In the final selection 39 clones were obtained with the interaction trap out of which 12 of the clones obtained were non-specific and for the final selection 27 positive clones were picked, of which 1 clone was unknown, showing no homology to any known protein. The rest of the 26 clones isolated showed homology to known proteins. The results were tabulated and shown in table 3.3.

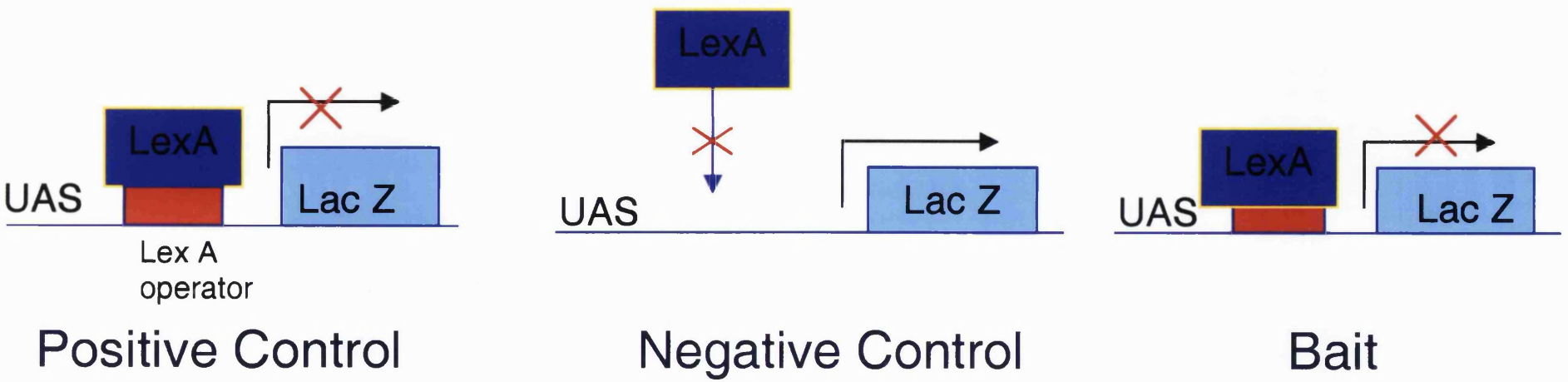


Figure 3.2. Schematic diagram representing the repression assay. In the positive control the plasmid harbours Lex A operators positioned between the Gal upstream activating sequence (UAS) and the LacZ reporter gene. When LexA binds to the operators, it represses the LacZ gene from transcribing and there are no blue colonies on Xgal plates. There is no LexA operator in the negative control and hence the LacZ is transcribed and the colonies turn blue on Xgal plates. The bait also has LexA operators and hences represses trancription of LacZ but more slowly than the positive control.

Bait		Positive Clone
Bait I	I-1	p11 Annexin II light chain (J03627)
Bait II	II-4	Huntingtin-interacting protein (AF05228)
	II-5	Zyxin related protein (AF097511)
	II-6	Follistatin related protein (Q62632)
Bait III	III-27	SAST (1905906)
	III-42	Papin (KIAA0300) (AB002298)
Bait IV	IV-40	Periaxin (myelinating protein) (2498801)
	IV-73	Cdc37 (D26564)
	IV7-40	Tctex-1(light chain of dynein) (AB010119)
V_A	A-10	PIPP (Inositol polyphosphate 5-phosphatase) (AB032551)
	A-32	$VDAC3_V$ (Voltage dependent anion channel) (AF048830)
	A-91	TM4SF (tetraspanins) (NM019656)
	A-103	Necdin (Nuclear protein) (NM010882)
	A-123	IC2 (Intermediate chain of dynein) (U39044)
	A-133	Cx43 (Connexin) (L10388)
	A-136	CGI-17 (comparative gene identification) (AF132951)
	A-140	Moesin (Cytoskeletal-membrane linker) (AF004811)
	A-145	Alpha-tubulin (Cytoskeletal protein) (V01227)
	A-148	HSPC025 (NM016091)
	A-150	Prnpb (long incubation prion protein) (U29187)
	A-165	TAO2 (thousand and one protein kinase) (AF140556)
	A-189	Unknown
V_B	B-4	Calmodulin III (NM012518)
	B-13	Calmodulin II (NM017326)
	B-15	Calmodulin I (AF178845)
	B-18	Zip (PKC Zeta-interacting protein) (Y08355)
	B-20	Beta-actin (X03672)
	B-25	KIAA0066 (D31886)

Table 3.3 Positive clones as identified by DNA sequencing for yeast two hybrid screening against $Na_V1.8$. The accession number for each clone is shown in the brackets.

3.3. Positive Interacting Clones: Identity and possible function:

3.3.1. p11 Protein (I-1):

The first positive clone showed 100% homology to the rat clone of S-100 related protein, calpactin I light chain (p11) protein. Our clone had the full length protein (96aa) and included a 51bp 5' UTR (untranslated region) and a 450bp 3'UTR. In the database there was only 240bp 3' UTR reported. The differences in size as from our gel picture for the insert for p11 arose from differences in the length of the 3' UTR. p11 is present in a variety of cells as a homodimer or as a heterotetramer. The heterotetramer is composed of two copies of the calpactin I heavy chain, also known as annexin II, lipocortin II, or p36, and two copies of p11. The association of p11 with p36 is Ca^{2+}-independent and of high affinity. It is suggested that calpactin-I complex may play a role in membrane trafficking events such as exocytosis, endocytosis and cell-cell adhesion (more details follow in next chapter).

3.3.2. Huntingtin interacting protein (HIP1) (II-4):

A second clone showed 93% homology to the human clone for huntingtin interacting protein 1 (HIP1). We cloned the full length transcript including 219bp 5' UTR, 2745bp coding region and 1.5kb 3'UTR. Huntington disease is associated with the expansion of a polyglutamine tract in the gene product, Huntingtin. It was found that even though the mRNA for HIP1 was ubiuqitously expressed, HIP1 protein was predominantly detected in the central nervous system. The function of huntingtin is unknown although it has been speculated that it has a role in endocytosis and organelle transport (Kalchman *et al,* 1997).

3.3.3. Zyxin related protein (II-5):

A further clone shows an homology of 92% to mouse clone zyxin related protein (ZRP) and has 301 amino acids out of 542 and has 180bp 3'UTR. Zyxin is a member of the family of proteins known as LIM domain containing proteins. Zyxin, the founding member of the family is a phosphoprotein that is localised at focal adhesions and along actin filaments (Macalma *et al,* 1996). This family is thought to mediate signals by shuttling between the nucleus and the cytoplasm (Beckerle, 1997). The protein displays an N-terminal proline rich region and three copies of the LIM motif at its C-terminal. This LIM domain is highly similar in all 3 members of the family zyxin, Trip6 and lipoma-preferred partner (LPP). LIM domains are zinc binding sequences involved in protein to protein interactions. LIM domains have been identified in proteins that play an important role in transcriptional regulation and cellular differentiation.

3.3.4. Follistatin related protein (II-6):

This clone had a 100% homology to a rat clone follistatin related protein (FRP). We isolated 150aa out of 306 and included a 1.4kb 3'UTR of which 389bp have been reported. I cloned full length FRP by performing PCR of the rat cDNA DRG library using forward primers and the reverse primer with an annealing temperature of 56°C and 30 cycles. Characterisation of FRP reveals that it is related to the follistatin gene and it also binds activin (Nakamura *et al,* 1990). Activin is a member of the transforming growth factor beta protein (TGF-β) family and stimulates release of pituitary follicle-stimulating hormone (FSH). Follistatin inhibits FSH release. FRP and follistatin binding to activin prevents activin's contact of its cell surface receptor and thus inhibits activins biological activity (DePaolo *et al,* 1991). FRP is expressed widely in most tissues but has the highest expression in placenta and testis. When it is

over-expressed in transgenic mice, FRP leads to interruption of follicular development and fertility in females but has only a modest effect on males. FRP is not as effective as follistatin in neutralising activins actions suggesting that additional features of follistatin contribute to its activities. This result suggests that FRP is a structural but not necessarily a functional homologue of follistatin (Schneyer *et al,* 2001).

3.3.5. SAST (III-27):

This clone had 87% homology to mouse clone mRNA for Syntrophin-associated serine/threonine kinase (SAST). We isolated 781 out of 1567aa, and our 2.5kb insert included 200bp 3'UTR although only 97bp has been reported. SAST binds via a PDZ domain to β2-syntrophin and co-localises in the cerebral vasculature, spermatic acrosomes and neuronal processes (Lumeng *et al,* 1999). Another homologue of SAST, which is microtubule associated S/T kinase (MAST205) also co-localised with syntrophin and utrophin at neuromuscular junctions. Among the cytoskeletal elements found at neuromuscular junctions are dystrophin, utrophin and the dystrophin/utrophin associated protein complexes (D/UAPC) (Lidov,*et al,* 1990, Kamakura *et al,* 1994) of which syntrophin is one.

Dystrophin/utrophins networks link actin filaments to extracellular matrix components throughout the muscle sacrolemma (Sunada & Campbell, 1995) and have been implicated in agrin-mediated signalling (Gee *et al,* 1994). SAST and syntrophin are highly associated with purified microtubules and microtubule-associated proteins, whereas utrophin and dystrophin were only partially associated with microtubules thus suggesting that SAST and MAST205 links the dystrophin/utrophin network with microtubule filaments via the syntrophins. The syntrophins are a family of cytoplasmic

proteins that bind to the C-terminus of dystrophins (α1 and β1-syntrophin) and utrophin (β2-syntrophin) and include a PDZ domain, two pleckstrin homology domains (PH) and a unique C terminal domain (SU) (Adams *et al,* 1995).

3.3.6. PAPIN (III-42):

This clone showed 100% homology to rat PAPIN. We had 210aa out of 2767aa and our 3kb insert included a 2.4kb 3'UTR. PAPIN is a protein which has six PDZ domains (4 in the N-terminal domain and 2 in the C-terminal domain) and is a p0071 binding protein. Since we isolated the C-terminal region of PAPIN, it is possible that $Na_V1.8$ binds to PAPIN via a PDZ domain located in the C-terminal. p0071 is an isoform of neural plakophilin-related armadillo repeat protein (NPRAP/δ-catenin) and hence this protein has been named plakophilin-related armadillo repeat protein-interacting PSD-95/Dlg-A/ZO-1 (PDZ) protein (PAPIN) (Deguchi *et al,* 2000). This is a member of a family of proteins known as $p120^{ctn}$ which are major substrates of tyrosine kinase phosphorylation enriched at adherens junctions and contains 10 armidillo repeats (Reynolds *et al,* 1992). $p120^{ctn}$ directly interacts with E-cadherins. The armidillo repeat is a repeated motif of about 40amino acids originally identified in Drosophola segment polarity gene (Hatzfeld, 1999). PAPIN was first identified by Deguchi *et al,* 2000 through the yeast two hybrid system screening using NPRAP/δ-catenin as a bait. They found that PAPIN interacted with NPRAP/δ-catenin and its isoform p0071 *in vitro* and in transfected cells and this interaction is mediated via the 2^{nd} PDZ domain of PAPIN and the PDZ binding motif of NPRAP/δ-catenin or p0071. The function of NPRAP/δ-catenin and p0071 is not known but since both proteins are localised at cell-cell junctions this suggests that they play roles as components of cell-cell junctions like $p120^{ctn}$ (Reynolds *et al,* 1992, Yap *et al,* 1998).

3.3.7. Periaxin (IV-40):

This clone had 100% match to rat clone mRNA for periaxin. We isolated 482 out of 1389aa but we obtained full length of the clone from Peter Brophy (Edinburgh University, UK). Periaxin was first described as a protein with a possible role in the later stages of myelinations (Gillespie *et al,* 1994). As myelin sheath matures, periaxin becomes more concentrated suggesting the possibility of its role in the stablisation of the myelin sheath. Scherer *et al,* 1995 have found that periaxin immunoreactivity was only detected in the Schwann cells and not the oligodendrocytes therefore it was only expressed in the peripheral nervous system and not the central nervous system. They also found that periaxin had similar mobility on SDS-PAGE to two proteins isolated from peripheral nerve myelin, p170 and SAG (Shuman *et al,* 1986; Dieperink *et al,* 1992). They also displayed similar staining of myelinating Schwann cells to antisera against P170 as that found for staining with periaxin antiserum, it was concluded that they were the same proteins. Scherer et al have found that periaxin was expressed by myelinating Schwann cells, and that its localisation changes during ensheathment and myelination and therefore it had a specific function in myelinating Schwann cells.

Dytrych *et al,* 1998 have shown that there are two isoforms of periaxin, L-periaxin and S-periaxin. Both proteins have an N-terminal PDZ protein binding domains. L-periaxin also possesses a tripartite (three basic sequences) nuclear localisation sequence (NLS) (Shermann *et al,* 2000). NLS are short sequnces that have the capacity to transport heterologous proteins into the nucleus (Nigg, 1997). Shermann et al have shown that NLS also localise L-periaxin to the Schwann cell nucleus when it is first expressed in the embryonic PNS and is then subsequently localised to the plasma membrane. They have targeted the NLS to the basic region found between amino acids

118 to 196 in the N-terminal domain by doing deletion of this region and transfecting cultured Schwann cells to see if the fusion protein is targeted to the nucleus.

3.3.8. Cdc37 (IV-73):

This clone had 100% homology to rat clone mRNA for cdc37 a homologue of a yeast cell cycle protein. We cloned the full length of the clone and included 37bp 5'UTR, 1140bp coding region and 377bp 3'UTR. A number of transcription factors and protein kinases involved in signal transduction exist in heterocomplexes with the chaperone protein hsp90. These signalling protein:hsp90 complexes are assembled by a multiple chaperone system comprising hsp90, hsp70, hsp40 and p23. For transcription factors, these heterocomplexes contain a high molecular weight immunophilin with tetratricopeptide repeat (TPR) motifs. In the case of protein kinases the heterocomplexes contain $p50^{cdc37}$ (Pratt *et al,* 1999). TPR are sequences of 34aa that are required for the binding of the receptor heterocomplexes of steroid hormones to hsp90 (Sikorski *et al,* 1990). The protein serine/threonine phosphatase PP5 is a TPR domain protein that accounts for a significant percentage of receptor:hsp90 heterocomplexes (Chen *et al,* 1996). Hsp90 is an abundant stress protein that is a highly conserved molecular chaperone essential for eukaryotic cell survival (Buchner, 1999). Hsp90 has been shown to be necessary for the function of the Src family of tyrosine kinases in yeast (Nathan & Lindquist, 1995) and for signalling in the sevenless receptor (Cutforth & Rubin, 1994), a protein tyrosine kinase required for the differentiation of the compound eye of drosophila. This protein kinase: hsp90 complex contains a 50kD phosphoprotein which has been identified as an homologue of the yeast cell cycle control protein Cdc37 and is called $p50^{cdc37}$.

3.3.9. Tctex-1 (IV7-40) and IC2 (A-123):

This clone (IV7-40) showed 100% homology to mRNA for Tctex-1. Our isolated full length 800bp clone included 15bp 5'UTR, 342bp coding region and 400bp 3'UTR of which only 6bp has been reported. The second clone A123 showed 98% homology to rat mRNA for intermediate chain of dynein (IC2) and we isolated 271aa out of 613-639aa depending on which type of IC2 (A,B or C) we isolated as there are 3 types. We do not know at this stage which binds to $Na_V1.8$. Dynein proteins are composed of heavy, intermediate and light chains. Dyneins are microtubule associated motor protein complexes that move molecules along microtubules. They are divided into 2 subclasses, axonemal (movement of flagella and cilia) and cytoplasmic (involved in trafficking of vesicles, retrograde axonal transport in nerve cells and nucleur migration in fungi) (Xiang *et al,* 1994). Cytoplasmic dyneins interact with a variety of structures such as lysosomes, the golgi complex, synaptic vesicles and the endoplasmic reticulum and is implicated in transport of these organelles and vesicle transport between these organeles (Holzbaur *et al,* 1994; Vallee & Sheetz 1996; Hirokawa 1998).

King *et al* (1996) first described that the mouse *t*-complex encoded protein tctex-1 was a component of brain cytoplasmic dynein. They found from sequencing a 16-residue peptide associated with bovine cytoplasmic dynein that there was a match both with mouse and human Tctex-1 and was a microtubule associated protein. They also found that Tctex-1 was specifically found in cytoplasmic tissue immunoprecipitated with the monoclonal antibody specific for the light chain of dynein, which led them to believe that Tctex-1 was a dynein light chain. Nagano *et al* (1998) describe Tctex-1 as a Doc-2 interacting protein which is ubiquitously expressed. Doc-2α isoform is specifically expressed in neuronal cells and localised on synaptic vesicles in nerve terminals and

has been implicated in Ca^{2+} dependent neurotransmitter release (Orita *et al,* 1999). Whereas Doc-2β is ubiquitously expressed but its function is unknown (Orita *et al,* 1995, Naito *et al,* 1997) though it may associate with some specific vesicles in nonneuronal cells (Nagano *et al,* 1998).

3.3.10. PIPP (A-10):

This clone showed 99% homology to rat clone mRNA for proline rich inositol polyphosphatase (PIPP). We isolated the full length clone which included 24bp 5'UTR, 2985bp coding region and 274bp 3'UTR though our clone lacked 7aa at position 35. Inositol phosphatidylinositol polyphosphates play an important role in a variety of signal transduction systems. Their intracellular levels are tightly controlled and changed by the enzymes in response to extracellular stimuli. There is evidence that phosphatidylinositol polyphosphates play important roles in the regulation of actin cytoskeleton (Sakisaka *et al,* 1997; Vollenweider *et al,* 1999). Mochizuki & Takenawa (1999) have cloned and characterised a novel 5-phosphatase, PIPP, that is an enzyme that specifically hydrolyses phosphates at the D-5 position of inositol and phosphatidylinositol phosphates at ruffling membranes and may be involved in the modulation of the functions of proteins present at the membrane ruffles (Mochizuki & Takenawa, 1999).

5-phosphatases can be categorised into three groups based on their substrate specificity. Type I phosphatases hydrolyses only water-soluble substrates such as inositol 1,4,5 triphosphate (Ins 1,4,5 P_3) and inositol 1,3,4,5 tetrakisphosphate (Ins 1,3,4,5 P_4) (Verjans *et al,* 1994; Zhang *et al,* 1995). SH2 containing inositol polyphosphate 5-phosphatase dephosphorylate Ins(1,3,4,5)P_4 phosphatidylinositol triphosphate (PtdIns3,4,5 P_3) (Damen *et al,* 1996). Type II 5-phosphatases, hydrolyse

both water-soluble substrates, Ins 1,4,5 P_3 and lipid substrates, PtdIns3,4,5 P_3 (McPherson *et al,* 1996). It has been reported that type I 5-phosphatase was activated when bound to platelet protein pleckstrin or 14-3-3ζ binding motif (Auethavekiat *et al,* 1997; Campbell *et al,* 1997).

3.3.11. VDAC3v (A-32):

This clone showed 98% homology to rat clone mRNA for a voltage dependent anion channel (RVDAC3v). Our full length clone included 774bp coding region and 26bp 3'UTR but had extra 9aa at the N-terminal region. VDACs are pore-forming proteins found in the outer mitochondrial membrane of eukaryotes and play a role in the regulated flux of metabolites across the mitochondrial membrane (Sorgato & Morgan, 1993). VDACs have been shown to bind cytosolic kinases (Adams *et al,* 1991) and this interaction allows the bound kinases preferential access to mitochondrial ATP derived from oxidative phosphorylation. Direct demonstration of voltage gated ATP-flux through VDAC was reported by Rostovtseva & Colombini (1996). VDAC have also been identified as a component of the peripheral benzodiazepine receptor complex which is linked to steroid synthesis (Papadopoulos *et al,* 1990). VDACs have also been shown to co-purify with brain $GABA_A$ receptor complex (Bureau *et al,* 1992).

3.3.12. TM4SF (A-91):

This clone corresponded to human genomic sequence from the genomic library and so had introns as well as exons. Tetraspanins named for the presence of four transmembrane domains are present in nearly all mammalian cells and many species including *C.Elegans* and *Drosophila* (Todd *et al,* 1998). Tetraspanins are expressed at the cell surface in association with each other and also with other molecules such as

integrins (Rubinstein *et al,* 1996). There are two recognised features and structures of tetraspanins which may be critical to their structure and function. The first feature is the conservation of charged residues in or near the transmembrane domains, similar to known ion channels, and the second feature is the conservation of cysteine residues in the second extracellular domain (EC2). Tachibana *et al,* 1997 have identified a new protein NAG-2 (novel antigen-2) that associates with the TM4SF protein CD-81 as well as with integrins too. NAG-2 is itself a new TM4SF member. TM4SF comprises a group of 19 cell-surface proteins including CD81 (Wright & Tomlinson, 1994). Although the precise function of TM4SF is still unknown, experiments have implicated the proteins in cell proliferation, activation, adhesion and motility (Hemler *et al,* 1996). Several TM4SF members may regulate signalling events involving tyrosine phosphorylation (Shaw *et al,* 1995) and intracellular calcium (Gil *et al,* 1992).

3.3.13. Necdin (A-103):

This clone showed 93% homology to mouse clone for necdin gene. We have isolated the full length of this clone which has 60bp 5'UTR, 977bp coding region and 544bp 3'UTR. In the vertebrate CNS neurones withdraw from the cell cycle after differentiation and remain in the post-mitotic state all their lives. Necdin is a nuclear protein expressed in post-mitotic neurones in the central and peripheral nervous systems of mice (Aizawa *et al,* 1992). Uetsuki *et al* (1996) have shown that necdin mRNA is expressed in all post-mitotic neurones in the central nervous system as well in SCG and DRG in the peripheral nervous system. They were unable to find any negative regulatory elements and concluded that necdin expression in neurones is not controlled by neurone-restrictive silencer elements such as those found in type II sodium channels (Kraner *et al,* 1992) and SCG 10 gene (Mori *et al,* 1992). These types

of silencer genes are expressed in cell-lines derived from neuroblastoma and pheochromocytoma which lack silencer-binding factor (Chong *et al,* 1995).

3.3.14. Cx43 (A-133):

This clone has 88% homology to mouse connexin gene, Cx43. Our clone had 349aa out of 1217aa. It is the principal gap junctional protein expressed in a variety of mammalian tissues including in mammalian heart. Intracellular communication via gap junctions composed of connexin 43 (Cx43) is known to be regulated by phosphorylation of this protein. The relationship between connexin phosphorylation and gap junctional intracellular communication has yet to be fully clarified but it has been suggested that dephosphorylation of Cx43 in astrocytes *in vitro* resulted in reduced gap junction communication (Hossain *et al,* 1994). Gap junctions in hearts co-ordinate cellular functions by providing a pathway for passage of electrical and or second messenger signals form one cell to another (Hotz-Wagenblatt & Shalloway, 1993) and changes in Cx43 expression are associated with hypertension (Bastide *et al,* 1993) and the development of atherosclerosis (Polacek *et al,* 1997). Rash *et al* (2001) have shown that gap junctions in neurones, astrocytes and oligodendrocytes have distinct nonshared complements of the 4 known connexins. They established that oligodendrocytes share intracellular gap junctions only with astrocytes and not detectably with other oligodendrocytes. They also showed that neurones only shared gap junctions with neurones and not with oligodendrocytes or astrocytes.

3.3.15. CGI-17 (A-136):

Our isolated rat clone showed 89% homology to human mRNA for CGI-17 gene. Our full length clone had 9bp 5' UTR, 1158bp coding region and 181bp 3' UTR. Comparative gene identification (CGI) is a term used to describe the identification of

new human genes by searching expressed sequence tags (EST) databases with query sequences of xenolog origins. ESTs are nucleotide sequences generated from single pass cDNA sequences of the ends of randomly selected clones from many different cDNA libraries (Adams *et al,* 1991). Lai *et al* (2000) describe the use of CGI approach to assist human gene identifications. They use the *C.elegans* sequence as a template to identifying novel human genes. The main focus of the study was to generate full-length continuos contigs by linking fragmented ESTs. They performed gap closure experiments to identify gap sequences by designing specific primers. They selected 12 genes containing 5' end initiation ATG sites with only one gap for assembling a full length transcript. 11 out of the 12 genes were amplified successfully and CGI-17 gene was one of them. When CGI-17 was used as a hybridising probe against human tissue blots it was seen to be expressed in most tissues but is more abundant in adrenal gland, placenta, lung, kidney and spleen.

3.3.16. Moesin (A-140):

This clone showed 97% homology to rat gene for moesin. Our clone had 564aa out of 578aa and also a 267bp 3' UTR. The rat and human clone have 15aa extra at the N-terminal as compared to the mouse clone. The rat and human clone are highly conserved and show an homology of 98%. Moesin along with ezrin, radixin of proteins and merlin (moesin-ezrin-radixin-like proteins) form a family of proteins called ERM. It is now widely accepted that ERM proteins do function as general cross-linkers between the plasma membrane and the actin filaments (Arpin *et al,* 1994). Members of this family have been shown to interact with cell adhesion molecules such as CD44 via the highly conserved NH_2 terminal (Yonemura *et al,* 1998) and also directly to F-actin and also involved in microvillar formation (Takeuchi *et al,* 1994). Phosphorylation appears to regulate the binding of this sub-family

(Vaheri *et al,* 1997) and there is evidence that cross-linking activity of ERM is regulated through Rho dependent signalling pathway through binding to Rho-GDP dissociation inhibitor and/or Rho-dependent phosphorylation (Hirao *et al,* 1996; Matsui *et al,* 1998).

The signal transduction events that mediate platelet cytoskeletal rearrangements have proved to be largely mediated through phosphorylation and dephosphorylation of cytoskeleton-associated and membrane proteins (Haimovich *et al,* 1993). Nakamura *et al,* 1995 have shown that only moesin and not ezrin or radixin is expressed in human platelets. They found that moesin is phosphorylated only on a single threonine residue and this is in response to activation by thrombin and in presense of inhibitors of S/T kinase and tyrosine phosphokinases and phosphatases, and moesin phosphorylation is rapidly modulated. They found that moesin was distributed throughout the platelet and in after activation the platelets were seen to alter their shape by forming filopodia and lamellopodia. Doi *et al* (1999) have generated a knockout mouse with null mutation of the moesin gene located on the X chromosome. Using this mouse they have shown that all the mice developed normally and were fertile. Moesin is known to be involved in platelet aggregation and Doi et al found that in moesin-deficit mice, immunoblotting showed the disappearance of the moesin band with no compensatory up-regulation of either ezrin or radixin and no difference in the aggregation ability between wild-type and moesin-deficit mice. this indicates that moesin is not required for normal mouse development or for survival. Selective suppresion of one or two of the ERM proteins by oligonucleotide antisense did not effect cell-cell or cell-matrix adhesion or microvillar formation. These were only affected when expression of all family members was suppressed.

3.3.17. α-Tubulin (A-145):

Our isolated rat clone showed 100% homology to rat clone for α-tubulin. Our isolated clone had 290aa out of 452aa and has 195bp 3'UTR. This is a protein that has a role in the regulation of microtubule assembly and function in the ciliate *tetrahymena thermophila* which can be used as a model to study microtubule functions *in vivo* (Hai & Gorovsky, 1997). *Tetrahymena* cells maintain diverse microtubule systems and express one type of α and β-tubulin protein (Gaertig *et al,* 1993). These tubulins possess most of the conserved post-translational tubulin modifications which play a role in the regulation of microtubule assembly and functions (Bre *et al,* 1994; Redeker *et al,* 1994). Microtubules are fibrous elements in the cytoplasm of eukaryote cells where they have diverse functions. Microtubules are involved in cell motility, division, and in organelle transport. Microtubule dynamics in vivo are based on intrinsic dynamic properties of the polymers themselves determined by the properties of the microtubule building blocks the αβ tubulins.

Tubulins are able to interact with a variety of proteins and small molecules such as nucleotides and drugs. Tubulin is a GTPase and GTP hydrolysis during tubulin assembly is of central importance for microtubule physiology (Valiron *et al,* 2001). α and β monomers are proteins of about 450aa. Microtubule dynamics are regulated by many microtubule-associated proteins (MAP) (Hirokawa, 1994) which either stabilise or destabilise the microtubules. Members of the stabilising proteins are Tau and MAP-2 which localise to the axon and dendrites respectively and MAP-4 which is present in all non-neuronal cells. Doublecortin and Lis1 are also MAP and mutations in either of these proteins cause type I lissencephaly, a human brain malformation characterised by smooth cerebral surface and a disordered organisation of the cortical layers resulting in a defect in neuronal migration (Gleeson, 2000). Several types of proteins

have been identified which destablise the microtubule. SCG10 is a neurone specific protein highly enriched in growth cones and it has been shown to bind to microtubules to inhibit their assembly and induce their disassembly (Riederer *et al,* 1997).

3.3.18. HSPC025 (A-148):

This clone showed 88% homology to human HSPC025 gene. Our full length clone had 21bp 5' UTR, 564aa coding region, and 1.7kb 3' UTR. This is a protein whose function is unknown but when a homology search was performed our clone appeared to match G-protein coupled receptor for the protein rhodopsin found in the eye. The rhodopsin phosphatase, RDGC, participates in Drosophila phototransduction (Steele & O'Tousa, 1990) which culminates with $Ca2^{+}$ and Na^{+} influx via the TRP channels (Montell & Rubin, 1989; Xu *et al,* 2000) and the Ca^{2+} rise is crucial for termination of the photoresponse (Hardie, 1995). Lee & Montell (2001) have recently identified RDGC as a calmodulin-regulated protein phosphatase. They showed that RDGC bound directly to calmodulin and this interaction disrupted an association between the N-terminal domain of RDGC and the catalytic domain. They found that association of RDGC to calmodulin is required for RDGC phosphatase activity in vivo.

3.3.19. Prnpb (A-150):

Our isolated rat clone showed 88% homology to mouse genomic sequence of chromosome 10, clone RP21-39C4. So when the 1^{st} 30 bases were compared we found an 86% homology to mouse long incubation prion protein . The prion protein was first identified in scrapie-infected rodents. The cellular prion protein PrP^{C} is a copper binding glycosylphosphatidylinositol (GPI)-anchored glycoprotein abundant in neurones as well as in other cells (Castagna *et al,* 2002). In normal human brain PrP^{C} undergoes a proteolytic cleavage within the N-terminal region with generation of two

major truncated fragments. A misfolded pathological isoform PrP^{SC} (sc=scrapie) is characterised by partial resistance to protease digestion and insolubility in non-denaturing detergents, this seems to represent the essential infectious component of transmissible spongiform encephalopathies or prion disease (Prusiner, 1998). These fatal neurodegenerative disorders, which include Creutzfeldt-Jakob disease (CJD) in human as well as scrapie in sheep and bovine spongiform encephalopathy, are neuropathologically characterised by the cerebral deposition of PrP^{SC}, neuronal loss and spongiform degeneration.

3.3.20. TAO2 (A-165):

This clone showed 100% homology to rat clone for thousand and one amino-acid protein kinase 2 (TAO2). Our isolated clone had 531aa out of 1236aa. TAO2 is rat protein that activates mitogen-activated protein/extracellular signal regulated kinase kinases (MEK/ERKs) of the stress responsive mitogen-activated protein (MAP) kinase pathways. TAO2 like TAO1, a rat homologue of the *saccharomyces cerevisiae* protein kinase sterile 20 protein (Ste20p), phosphorylates and activates MEKs from the MAP kinase cascade (Chen *et al,* 1999). Chen et al found that when TAO2 was expressed in Sf9 cells, the full length enzyme was significantly less active than the truncated enzyme. It was concluded from their studies that *in vitro* TAO2 activates MEK3,4 and 6 towards their substrates p38 MAP kinase c-Jun N-terminal kinase/stress activated protein kinase. Another kinase named A-kinase which showed 90% homology to TAO2 was identified in a screen for RNAs overexpressed in human prostate carcinoma (Moore *et al,* 2000). A third kinase named JIK for JNK inhibitory kinase has a similar organisation and is over 60% identical to TAOs (Tassi *et al,* 1999). Since phosphorylation by TAO increases their activities toward the downstream MAP

kinases, p38 kinases and the JNK/SAPKs suggests that TAO1and TAO2 function in stress responsive pathways within the cell.

3.3.21. Calmodulin (B-4, B-13, B-15):

The B4 rat clone showed 99% homology to the rat calmodulin III gene. Our full length clone had the 32bp 5' UTR and 450bp coding region and 33bp 3' UTR. Calmodulin III is mainly expressed in skeletal muscle and brain. Calmodulin is the most widely distributed and versatile member of a family of calcium-binding proteins and plays a role in many physiological processes. It acts as an intracellular calcium sensor that translates calcium signals into cellular responses by interacting with target molecules. Our isolated B13 rat clone had the full length clone that showed 95% homology to calmodulin II and had 21bp 3' UTR, 450bp coding region and 592bp 3' UTR. Calmodulin II is expressed in the brain. Our isolated B15 rat clone showed 86% homology to calmodulin I and we had 92aa out of 150aa of the coding region and 3kb 3'UTR. This type of calmodulin is found in liver, skeletal tissues and brain in similar amounts. Calmodulin I, II and III have the exact protein sequence although their genes are different.

Calcium plays a critical role in the normal function of the central nervous system. The processing of a Ca^{2+} signal requires its interactions with specific intracellular proteins. Calmodulin is the major Ca^{2+} binding protein and it modulates Ca^{2+} dependent enzymes and participates in relevant cellular functions (Cheung *et al,* 1980). Calmodulin has four similar domains each containing one Ca^{2+} binding site. The binding of Ca^{2+} alters the conformation of calmodulin and the resulting complex can interact with target proteins and modulate their activity. Calmodulin is highly abundant in the mammalian nervous system especially in sites involved in

neurotransmission such as postsynaptic membranes and synaptic vesicles (DeLorenzo, 1980). It also controls the function of the cytoskeleton, where it regulates the assembly and disassembly of microtubules (Marcum *et al,* 1978). Mori *et al,* 2000 have found a novel interaction between the C-terminal domain of type II brain α-subunit and calmodulin, they have shown that this binding occurs in the presence and absence of calcium.

Calmodulin is known to bind to at least 2 consensus sequences like the Baa motif (O'Neil *et al,* 1990) and the IQ motif (Cheney & Mooseker, 1992). Mori et al found that in type II C-terminal region there were 2 regions, region 1, that contained a Baa region and region 2, that contained a Baa and an IQ motif and they concluded that region 2 was the calmodulin binding region. Calmodulin bound to the Baa motif in an Ca^{2+} dependent manner, whereas the calmodulin binding to the IQ motif was in presence of Ca^{2+} as well as in the absence of calcium. Carlier *et al,* 2000 have shown that sodium channels of cerebellar granule cells can be regulated by calmodulin dependent protein kinase II (CAM Kinase II). Tan *et al* (2002) have shown the binding of calmodulin to the C-terminal of cardiac Na^{+} channels in an calcium dependent manner. This binding interaction significantly enhances slow inactivation. Altered slow inactivation has been proposed as a mechanism for arrhythmia risk in $Na_V1.5$ mutations (Veldkamp *et al,* 2000) and a rare form of idiopathic ventricular fibrillation known as Brugada syndrome (Brugada & Brugada, 1992).

3.3.22 ZIP (B-18):

Our isolated rat clone showed 90% homology to rat mRNA for PKC-zeta-interacting protein (ZIP). Our full length clone had 5bp 5' UTR, 1320bp coding region and 279bp 3'UTR. ZIP is a cytoplasmic and membrane associated protein that interacts with the

regulatory domain of atypical protein kinase C (PKC-ξ) but not classic PKC. It mediates complexes between PKC-ξ and other proteins and is phosphorylated by atypical PKC (Puls *et al,* 1997). The atypical PKC has been implicated in several signal transduction pathways regulating differentiation, proliferation or apoptosis of mammalian cells. Most cells express several PKC enzymes and while the classic and novel members of this family participate in signal transduction from cell surface receptors that trigger the generation of diacylglycerol by activating phospholipase C, the mode of activation and function of the atypical members is much less clear (Nakamura & Nishizuka, 1994). Atypical PKC has been connected with two distinct signalling pathways. The first one is where phosphatidylinositol-3,4,5-triphosphate can activate atypical PKC in vitro pointed to the possibility that atypical PKC participates in phosphorylation events downstream of phosphatidylinositol-3-kinase activation by receptor tyrosine kinases (Nakanishi *et al,* 1993). Atypical PKC has also been proposed as a mediator of the growth inhibitory and apoptotic action of ceramide, an intracellular messenger generated by hydrolysis of sphingolipids (Hannun & Obeid, 1995). Puls et al also investigated the interaction *in vivo* between Zip and atypical PKC and found that amino acids 41-105 of Zip contained the binding site for atypical PKC. Amino acids 79-145 of atypical PKC mediated binding to Zip. They also found that Zip displayed strong binding to itself and this interaction was mapped to the same binding site as for the atypical PKC and competes for the site.

3.3.23. Beta-actin (B-20):

Our isolated rat clone showed 97% homology to mRNA for the mouse cytoskeletal beta-actin. Our isolated full length clone had 53bp 5'UTR 1127bp coding region and 684bp 3' UTR. At least six actin isoforms are known in mammals, each encoded by a

separate gene and they differ by less than 10% of their amino acid sequence (Vandekerckhove & Weber, 1978; Erba *et al,* 1988). During diferentiation of myoblasts to form myotubes the expression pattern of the different actin isoforms change. The cytoplasmic β and γ are down regulated and the muscle specific isoforms are upregulated (Shani *et al,* 1981). Gu *et al* (2002) have shown that localisation of β-actin mRNA to the leading lamellae of chicken fibroblasts, where actin polymerisation drives cell motility, and neurite growth cones of developing neurones requires a localisation signal (the Zipcode) within the 3' UTR. Fibrobalsts with localised β-actin mRNA migrate significantly further than those with non-localised β-actin mRNA (Kislauskis *et al,* 1997). In cultured rat and chicken developing neurones, the sorting of β-actin mRNA to neurite growth cones has also been observed (Bassell *et al,* 1998; Zhang *et al,* 1999) and mRNA localisation is necessary for enrichment of β-actin protein and forward movement of growth cones. Bassell et al have shown the first evidence for the presence of β-actin mRNAs within developing dendritic and axonal growth cones and also have shown that β-actins interact with microtubules within the growth cones. Zhang *et al* (1999) showed that localisation of β-actin mRNA and protein to growth cones of forebrain neurones is stimulated by neurotrophin-3 (NT-3). They also found that a similar response occurred when neurones were exposed to forskolin or dibutyryl cAMP suggesting the involvement of cAMP dependent signalling pathway. The NT-3 treatment resulted in rapid and transient stimulation of PKA activity that preceded the localisation of β-actin.

3.3.24. KIAA0066 (B25):

Our isolated rat clone showed 85% homology to human mRNA for KIAA0066. Our clone had 233aa out of 983aa of coding region and 687bp 3' UTR. This gene is related to transcriptional factors.

3.3.25. A-189:

This a completely unknown clone which shows no homology to any known protein registered in the database.

3.4. Non-specific Clones:

From the interaction trap we employed, we also isolated some non-specific clones which were eliminated from further analysis. Non-specific clones can be isolated very easily as they stick to any prey. These clones were determined to be non-specific by checking the published list of well known non-specific clones in the database. From the DNA sequncing results it was confirmed that a lot of the non-specific clones were in the wrong frame and hence were eliminated. Wrong frame is where the bases have shifted during sequences or there is mutation in one of the bases and is due to experimental error. Clone A102 had 91% homology to human eukaryotic translation initiation factor 3 (eIF3). We have isolated 550aa out of 874aa. Clone A107 had homology to rat DAD-1 (defender against death) gene which suppresses apoptosis. This clone was in the wrong frame and was eliminated. Clone A113 had 98% homology to mouse translational regulatory factor alpha Nac (Nascent polypeptide-associated complex). Clone A119 was only 6aa long and was in the wrong frame. Clone A142 had 94% homology to mouse Sin3 (transcriptional corepressor) associated polypeptide 18 (SAP-18). Clone A167 had 91% homology to rat Sec16 a key component of protein translocation associated with ribosomes and nascent polypeptides during translocation. Clone B16 was a rat phosphatidylethanolamine

binding protein (Pbp) associated with opioid binding protein. We also isolated β-globin and cytochrome C which are are known non-specific clones.

3.5. Functional experiments:

3.5.1. Expression of clones in DRG Neurones:

To determine whether the clones were expressed in $Na_V1.8$-positive small diameter neurons in DRG, *in situ* hybridization was performed on 2 weeks old rat DRG sections.The clone was excised out of the yeast expression vector pJG4-5 and sub-cloned into EcoRI and XhoI sites in pBluescript vector. Linearised cloned DNA (EcoRI digested at 5' end) was used to generate antisense from the 3' to 5' direction using T7 RNA polymerase and sense 5' to 3' probe using T3 polymerase. Digoxigenin–11-uridine-5' triphosphate was used as a substrate for T7, or T3 RNA polymerase to label RNA in *in vitro* transcription in place of UTP. Digoxigenin is linked to UTP via the C-5 position on the nucleotide. This Dig-labelled nucleotide can now be incorporated into nucleic acid probes RNA. A highly sensitive non-radioactive labelling and detection system based on the ELISA principle was used here. The DNA was modified with cardenolide-hapten digoxigenin (DIG) by enzymatic incorporation of digoxigenin labelled deoxyuridine-triphosphate (dUTP) with Klenow enzyme. Following hybridisation of membrane with a digoxigenin labelled probe (DIG-labelled probe), the hybrids were detected by an ELISA reaction using DIG specific antibodies covalently coupled to the marker enzyme alkaline phosphatase. This binding of antibody: conjugated alkaline phosphatase was followed by an enzyme catalysed coupled redox reaction with the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) which gives rise to a dark blue coloured water-insoluble precipitate directly adhering to the tissue. The sections were

hybridised with the DIG-labelled probes overnight at 66°C. After washing the sections were visualised with alkaline phosphatase conjugated anti-digoxygenin antibody (Roche) and the sections viewed using the fluorescent microscope. The principle was that the DIG-labeled antisense mRNA probe will bind to the endogenous sense direction mRNA for the appropriate clone as they have complementary sequences. An anti-Dig antibody conjugated to alkaline phosphatase will bind to the probe and this can be viewed in the microscope following a colorimetric reaction with the salts BCIP and NBT. These results are shown in figure 3.3A where antisense III-42 probe demonstrated strong staining in both small and large diameter neurones showing that III-42 is expressed in neurones that have endogenous $Na_V1.8$. We also tested several other clones (II-4, II-5, II-6, IV-40, IV7-40) and IV-40 is also shown in figure 3.3B and the rest of the clones also all showed expression in small and large diameter neurones. These particular clones were the first to be sequenced after the interaction trap and hence the reason they were chosen for In situ hybridisation.

3.5.2 Expression of Protein in DRG Neurones:

Immunohistochemistry studies were carried out to see if the protein of the clones isolated actually were expressed in the small diameter neurones. Cryosectioned tissues are fixed in paraformaldehyde and primary antibody applied followed by a secondary antibody and the sections viewed. Figure 3.4 shows one such clones' protein expression in the DRG. Periaxin (IV-40) staining was seen both in the small diameter and the large diameter neurones. The periaxin antibody was a gift from Professor Peter Brophy (University of Edinburgh, UK). 1/1500 dilution of anti-L-Periaxin polyclonal antibody along with 1/10 dilution of anti-peripherin monoclonal antibody was applied to 2 weeks old sections of rat DRG. 1/200 dilution of secondary antibody, anti-rabbit

IgG conjugated with FITC was used for periaxin and 1/50 dilution of anti-mouse IgG conjugated with texas red was used for peripherin. Fluorescent microscopy was used with a blue filter to view the periaxin sections and a green filter to view the peripherin antibody. From the results it was seen that peripherin which acts as a positive control in this study was expressed in the small diameter neurones as expected. Periaxin has been shown to express in Schwann cells during myelination. We confirmed that periaxin was expressed in nerve fibres as well as in small and large diameter neurones. These results indicate that periaxin protein isolated in the yeast-hybrid system was actually expressed in neurones where $Na_V1.8$ is expressed i.e. small diameter neurones.

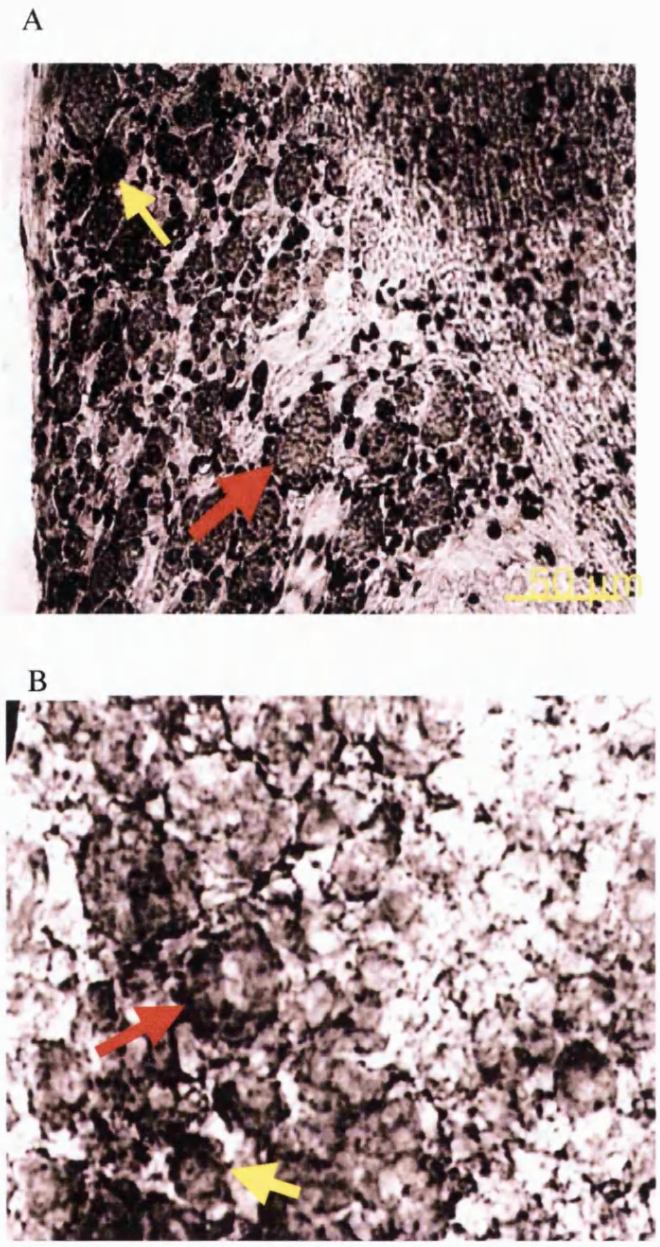


Figure 3.3. *In situ* hybridisation with A: PAPIN (III-42) antisense using DIG-labelled probes showing expression in small diameter neurones (yellow arrow) and large diameter neurones (red arrow) and B: Periaxin (IV-40) antisense showing expression in small diameter and large diameter neurones.

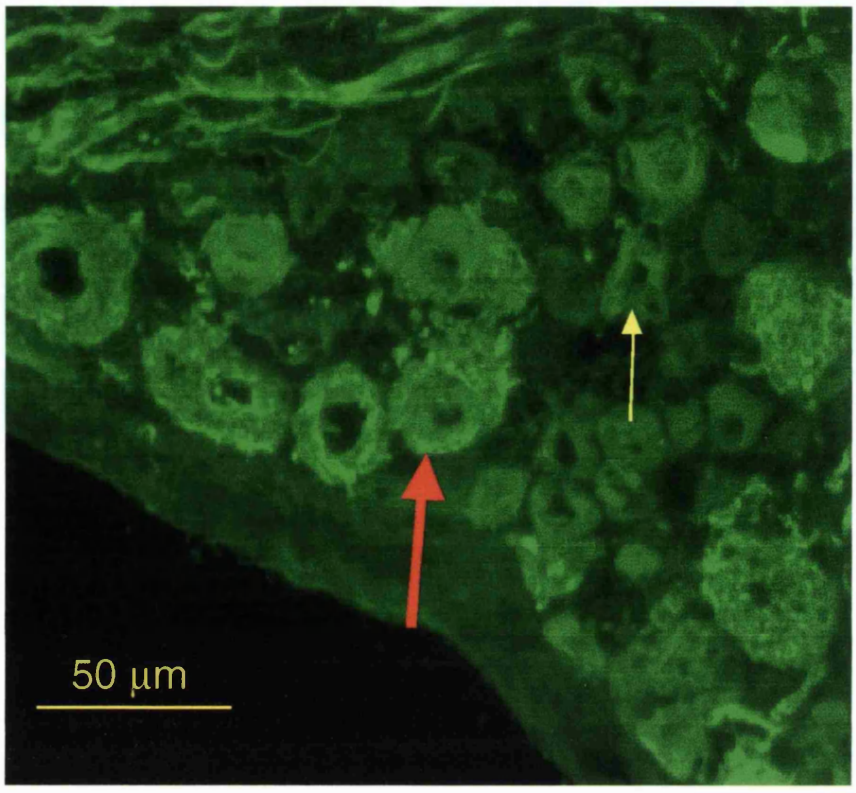


Figure 3.4. Immunohistochemistry data showing staining for periaxin protein in small diameter neurones (yellow arrow) and in large diameter neurones (red arrow) using 1/500L-periaxin polyclonal antibody (gift from Peter Brophy).

3.5.3 Functional Expression of $Na_V1.8$ after Antisense Injections:

To test the function of the clones on $Na_V1.8$, a fragment of each clone was cloned in 3' to 5' direction into restriction sites in appropriate vectors which resulted in a expression system for an antisense mRNA when microinjected into nuclei of DRG neurons as described in chapter 2. DNA sequencing was done to confirm the direction of the mRNA expression as well as to see the whether the correct expression vector was generated. Some of the clones were microinjected individually, but some clones were pooled together and microinjected. This was a screening experiment to eliminate the antisense which did not have an effect. The principle of this method was that the generated 3' to 5' direction mRNA will bind to the endogenous sense direction mRNA for the corresponding clone and inhibit appropriate protein production.

The list in table 3.4 shows the total number of cells recorded for each pooled/individual antisense and the number of cells that did not exhibit $Na_V1.8$/SNS current. The mean peak sodium current is also shown and the last column measures the mean current density as compared to GFP mean current density which are used as the controls and consist of GFP injected cells with no antisense expression vector. The mean peak sodium current density was significantly reduced for the triplicate III-42/IV-40/A-145 (Papin/Periaxin/α-tubulin). The current density was reduced (46.7 ± 14.3 pA/pF (n=7)) when compared to the mean peak sodium current density for GFP (179.2 ± 40.3 pA/pF (n=9)) controls showing significance at $P< 0.05$. Another set of triplicate which was significantly reducing the peak sodium current was the A-140/A-148/B-25 (Moesin/HSPC025/KIAA0066) set and was significant at $P<0.02$. Figure 3.5 shows the log current density distribution recorded in cultured DRG neurones for both sets of triplicates. Another antisense which was seen to down regulate the current was

A-123, this is the intermediate chain of dynein and it was significant at $P<0.05$ (students unpaired two-tailed t-tests). The rest of the clones tested were not significantly altering the mean peak sodium current (apart from I-1-see chapter 5). The significant triplicate have to be microinjected individually to confirm which clone was necessary for expression of a functional $Na_V1.8$ channel. The electrophysiology experiments were performed by Dr. Mark Baker, UCL.

3.5.4 Direct Interaction of Clones with $Na_V1.8$:

Direct interaction of the clones with $Na_V1.8$ was studied (IV-40 & IV7-40) with transfections done in COS cell lines. The clones were sub-cloned into pBS500/GFP vector and Westerns performed to visualise the interaction bands with anti-HA antibody conjugated with horseradish peroxidase as described in detail in chapter 2. Protein A beads were used to collect the antibody-antigen complex and SDS buffer added to denature the protein and break up the antibody-antigen complex. Anti-HA antibody was used as the library expressed in pJG4-5 has an HA epitope tag upstream to insertion site of the library. IV-40 and IV7-40 displayed a strong interaction with $Na_V1.8$ when precipitated with either anti-$Na_V1.8$ or anti-HA and visualised with anti-HA antibody. However when visualised with anti-$Na_V1.8$ (1/500 dilution) antibody there were no bands present. Due to solubility problems it was decided to make shorter versions of the intracellular $Na_V1.8$ region. The bait fragments I to IV were ligated to Bait V to make fragments I-V, II-V, III-V and IV-V. They were ligated to Bait V as anti-$Na_V1.8$ antibody recognises this region. Figure 3.6 shows the band obtained with IV-40 and IV7-40 when the shorter bait IV-V fragment of $Na_V1.8$ was used for the co-immunoprecipitation and precipitated with either anti-HA or anti-$Na_V1.8$ and visualised with anti-$Na_V1.8$. Ms. Poon, UCL, is carrying on with the rest of the bait

fragments I-V, II-V, II-V and examining interaction for the rest of the clones with $Na_V1.8$.

3.5.5. cDNA Expression in CHO-SNS22 Cells:

CHO-SNS22 cells are a stably transfected cell line using the lipofectamine method which express full length rat $Na_V1.8$ cDNA. They do not have $Na_V1.8$ current despite the fact they express high amount of full length $Na_V1.8$ mRNA. Immunocytochemical studies using anti-$Na_V1.8$ polyclonal antibody SNS11 showed $Na_V1.8$-like immunoreactivity in the cytosol of CHO-SNS22 cells but not on the plasma membrane. To study whether any of our positive clones helped produce a $Na_V1.8$-like current, we transfected CHO-SNS22 cells with GFP/cDNA fusion protein. Table 3.5 shows the results obtained with the positive clones transfected. The clones were chosen in no particular order, they were transfected as soon as the full lenght clone was produced. So far two clones have produced a $Na_V1.8$ current after transfection (p11 is discussed in detail in next chapter). HSPC025 (A-148) was seen to generate a TTX-r current. The expression of the clones were detected as green fluorescence signal due to fused GFP. The rest of clones did not help produce a $Na_V1.8$ current. It is interesting to see that HSPC025 was also one of the triplicate antisense that had an effect on the $Na_V1.8$ current. The next step is to microinject this antisense on its own and see whether it down regulates the Na^+ current in DRG neurones also.

ANTISENSE	Number of cell recorded	Number without $Na_V1.8$ Current	Mean current density /GFP current density
I-1* (p11)	8	0	0.35
II5/II6/III27/IV7-40 (ZRP/FRP/SAST/Tctex-1)	5	0	0.70
III42/IV40/A-145* (Papin/Periaxin/α-tubulin)	7	1	0.26
A-10/A-32/A-165 (PIPP/VDAC/TAO2)	10	1	0.67
A-91/A-103/B-18 (TM4SF/Necdin/Zip)	9	3	0.60
A-140/A-148/B-25* (Moesin/HSPC025/KIAA0066)	8	1	0.28
A-123 (IC2)	8	2	0.40
A-133 (Connexin 43)	5	4	--
B-4 (Calmodulin III)	9	3	0.54
B-13/B15/B-20 (Calmodulin II & III/ β-actin)	7	1	0.49
Control:			
GFP	10	1	1.00
Non-injected	55	5	1.01

Table 3.4 Results of the different antisense microinjections into the nucleus of cultured DRG neurones. Antisense cDNA were subcloned into an expression vector in the 3' to 5' direction and left for 3 days prior to electrophysiology recording to investigate the $Na_V1.8$ current. * denotes the antisense results displayed as histograms in the Results obtained form Dr Mark Baker, UCL.

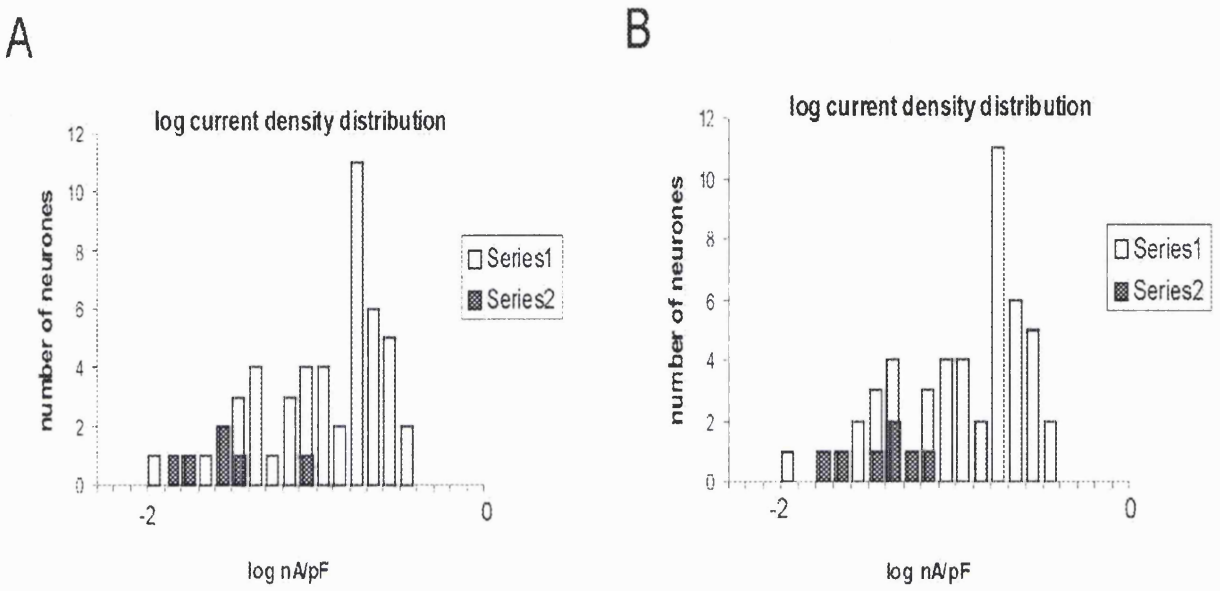


Figure 3.5. Log current density distribution for $Na_V1.8$ recorded in rat cultured small sensory neurones (injected cells were >25μm). A, Series1: control GFP-injected; series 2: III42 / IV40 / A145 antisense. Comparison of triplicate antisense injected and GFP injected reveals a significant difference in log current density ($P < 0.01$, Student's t-test, unpaired, two-tailed). B, Series 1: control GFP-injected; series 2: A140 / A148 / B25 antisense. Comparison of triplicate antisense injected and GFP injeccted shows a significant difference in log current density ($P < 0.02$, Stuident's t-test, unpaired, two-tailed). Results obtained from Dr Mark Baker, UCL.

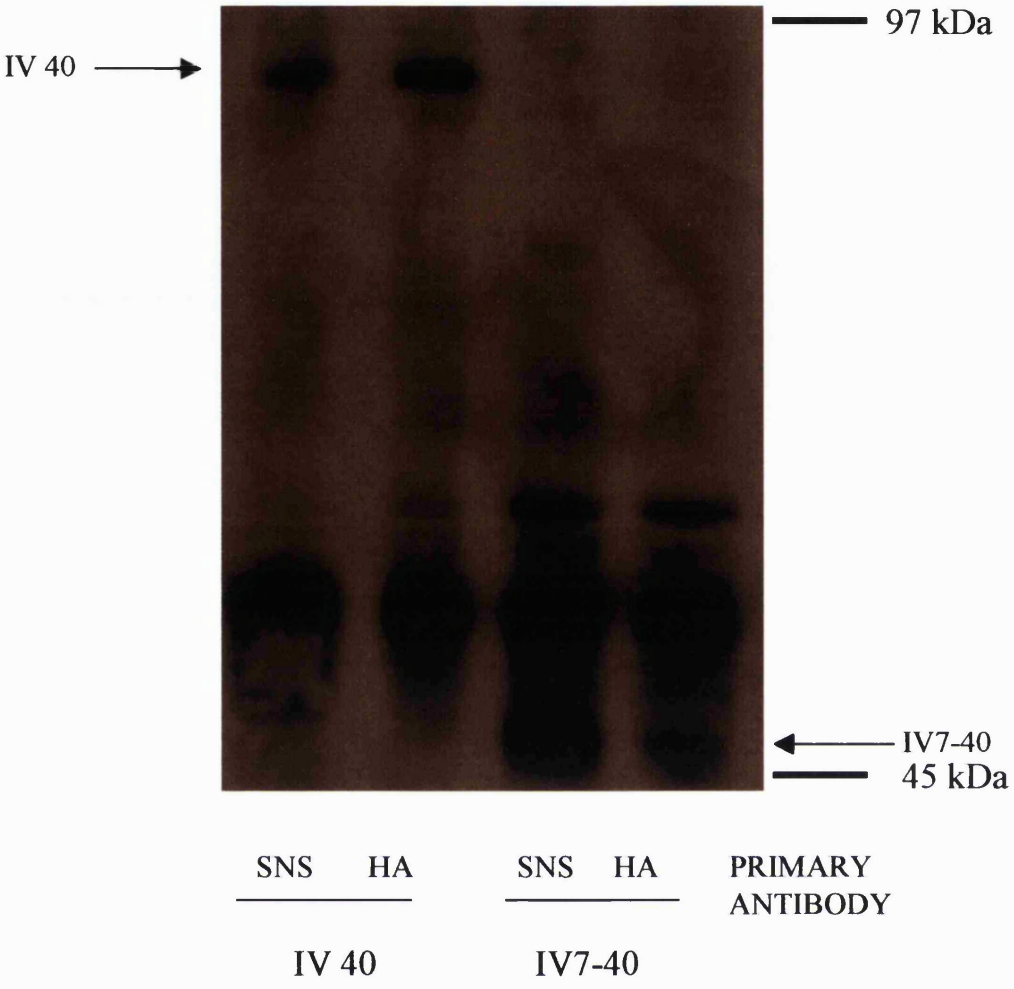


Figure 3.6. Results for the co-immunoprecipitation done with IV-40 and IV7-40. Primary antibodies used were anti-SNS and Anti-HA and viewed with Anti-HA secondary antibody. The arrows indicate the position of the bands seen.

Positive clone	Number of cells recorded	Cells with currents > 50pA
A-10	5	0
A-32	6	0
A-103	1	0
A-123	6	0
A-140	11	0
A-148	3	1*
B-4	8	0
B13	5	0
B-18	7	0
B-20	6	0
IV-40	5	0
IV-73	6	0
IV7-40	7	0

Table 3.5 Full length cDNA transfected into CHO-SNS22 cell line and $Na_V1.8$ current recorded from 1 clone (*), HSPC025 (A-148) Electrophysiology recordings were done by Dr Mark Baker, UCL.

CHAPTER 4.
RESULTS FOR P11

4.1. Annexin II light chain p11 interacting with $Na_V1.8$:

Using the yeast-2-hybrid system and performing an interaction trap with bait I (N-terminal region of the intracellular loop of $Na_V1.8$ from position 1 to 127 amino-acids) and the rat cDNA DRG library, five identical full length positive clones were obtained encoding annexin II light chain, p11, in yeast expression vector pJG4-5. The clones included 51 bp 5'-UTR, 288 bp coding region, and 450 bp 3'-UTR of rat p11 light chain of annexin II gene.

The bait I plasmid was generated by PCR with rat $Na_V1.8$ cDNA as a template with a forward primer and a reverse primer. The amplified bait was ligated as an in frame fusion into pEG202 EcoRI and NotI sites and used to transform a yeast strain, EGY48 and perform the interaction trap as detailed in chapter 2. The isolation of p11 clones was confirmed by DNA sequencing. To verify that p11 interacts specifically with the N-terminal intracellular domain of $Na_V1.8$, the rescued p11 encoding plasmid DNA was re-introduced into the other strains of yeast containing different intracellular domains of $Na_V1.8$ as baits. This showed the interaction between p11 and $Na_V1.8$ is specific to the N-terminal intracellular domain of $Na_V1.8$. Figure 4.1 reveals the binding of p11 to $Na_V1.8$. Control experiments done showed no band for p11 with GST pull down assay with GST fusion proteins only but a band was present when GST- SNS fusion protein was used in the pull down assay.

4.2. Tissue distribution of p11:

Because $Na_V1.8$ is specifically expressed in sensory neurons, we examined the tissue distribution of the p11 transcript. Total RNA was isolated from various rat tissues by acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) and fractioned by electrophoeresis on 1.5% agarose gel in 10mM

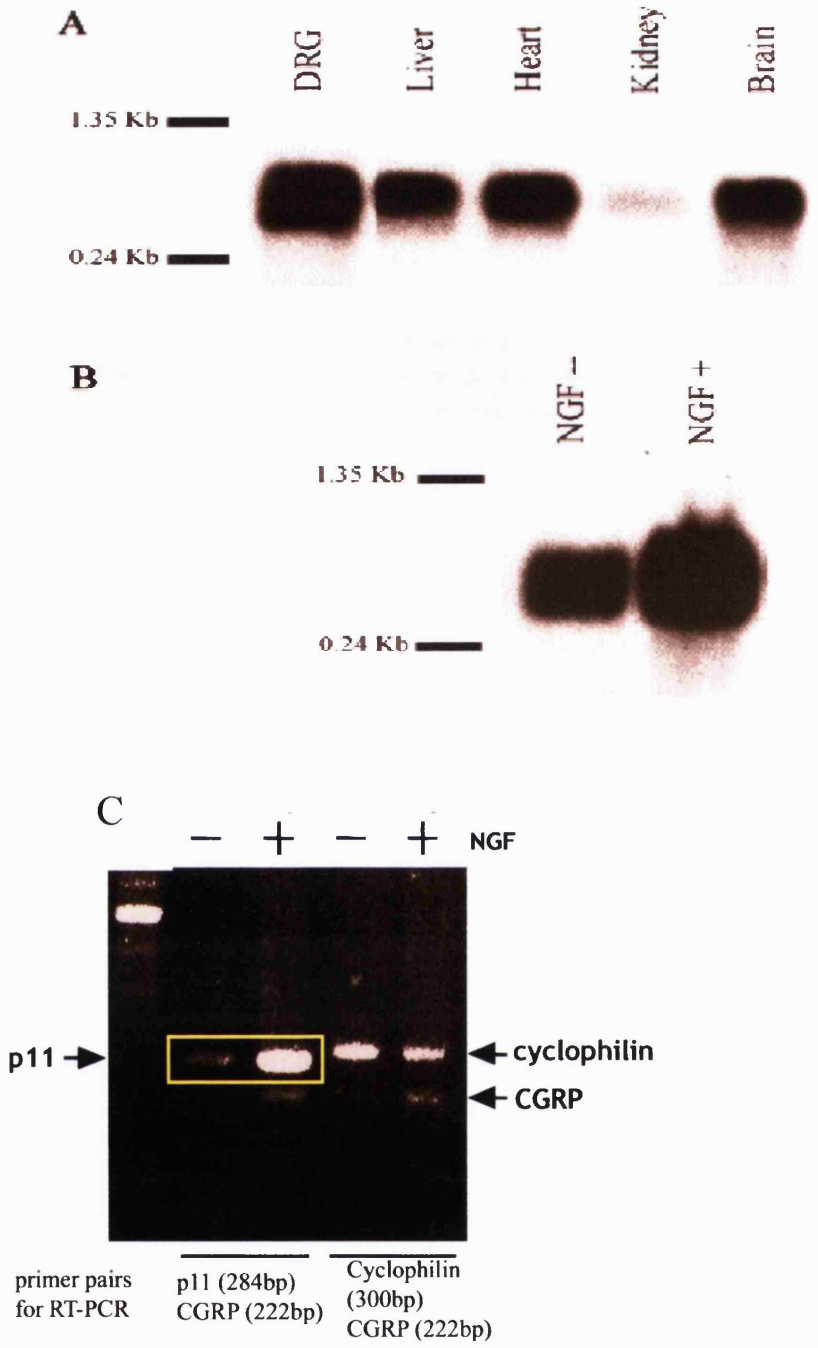


Figure 4.1. Tissue distribution of p11 using ^{32}P-labelled DNA probe generated with 284bp p11 PCR fragment and hybridised at 43^{0}C for 18 hours. A: Northern blot analysis revelas high expression of p11 mRNA in DRG, modest amount in heart, liver and brain and low expression in kidney. B: Expression of p11 mRNA after NGF treatment for 3 days to cultured DRG neurones. Anti-NGF antibody was added for 3 days at 1/200 dilution to sequester the endogenous NGF. NGF is seen to up-regulate p11 expression. C: RT-PCR data confirms the upregulation of p11 after NGF application. Primers pairs used are indicated and negative control with cyclophilin and positive control with CGRP are also shown (RT-PCR data obtained from Dr. Kenji Okuse). For a detail explanation see section 4.3.

phosphate buffer (pH 6.5). RNA was blotted onto hybond N^+ nylon membrane in 20 x SSC solution for 6 hours and fixed by UV irradiation. The membrane was stained with methylene blue to confirm recoveries of mRNA. Thereafter, the membrane was prehybridized and hybridized with ^{32}P-labeled p11 DNA probe (50 ng, specific activity 2 x 10^9 c.p.m. per μg DNA) at 43°C in hybridization buffer (50% formamide, 6 x SSC, 50mM NaH_2PO_4, 1mM EDTA, 1% SDS, 2.5 x Denhardt's solution, 250μg herring sperm DNA, pH 6.5) for 18 hrs. The ^{32}P-labeled p11 DNA probe was generated from a 284bp (p11) PCR fragment using Klenow enzyme and random primer in the presence of [α-^{32}P] ATP at 37°C for 15 min. Unincorporated nucleotides were removed using the QIAquick nucleotide removal kit. Hybridized membrane was washed with 0.2 x SSC/0.1% SDS at 53^0C and exposed to Biomax film at -70^0C.

Figure 4.1A reveals that there was a high expression of p11 in DRG, a modest level in heart and liver and low levels in brain. There was very low expression in the kidneys. In the brain there is high level of expression of TTX-sensitive sodium channels. Our results show a low level of expression of p11 in the brain suggesting that p11 is not required for the expression of TTX-sensitive channels. There is modest expression of p11 in the heart, which have a TTX-resistant channel, $Na_V1.5$. p11 may be necessary for the expression of the TTX-resistant channels in the heart. There is high expression of p11 in the DRG where $Na_V1.8$, and $Na_V1.9$ are expressed exclusively hence suggesting that p11 is necessary for the expression of TTX-r channels in the DRG.

4.3. NGF Regulation of p11:

The effect of NGF was tested on p11 expression. DRG cells were cultured in the presence of 50ng/ml NGF or in the absence of NGF (anti-NGF antibody added at 1/200 dilution) for 3 days prior to extracting the RNA as described previously. After

loading 10μg mRNA and observing even recoveries of mRNA by methylene blue staining, the membrane was hybridised with a 284bp p11 probe labelled with ^{32}P. From the results obtained it can be seen in figure 4.1B that NGF was up-regulating the expression of p11 mRNA in DRG cells. This set of experiment was reppeated twice, with similar results obtained. The results were also confirmed with RT-PCR and there was an up-regulation of p11 mRNA by NGF as shown in figure 4.1C. Cyclophilin acts as a negative control and is not up-regulated by NGF whereas CGRP acts as a positive control where upregulation by NGF is seen.

4.4. Expression of p11 in DRG neurones:

To determine whether p11 was expressed in small diameter neurones in DRG where $Na_V1.8$ is expressed, *in situ* hybridization was performed on sections of 2 weeks old rat DRG. A 284bp p11 PCR fragment was subcloned into pGEM-T Easy vector, and DIG-labelled sense or antisense cRNA probe were generated using T7 RNA polymerase as detailed in chapter 2. The sections were hybridised with the DIG-labelled probes overnight at 66°C. After washing the sections were visualised with alkaline phosphatase conjugated anti-digoxygenin antibody. The same sections were then stained with anti-$Na_V1.8$ polyclonal antibody (SNS11) followed by rhodamine-conjugated anti-rabbit IgG antibody. The reaction was stopped by washing with PBS and the sections viewed using the fluorescent microscope.

Figure 4.2A showed antisense p11 probe demonstrating strong staining in small and large diameter neurones. Combined immunohistochemistry with anti-$Na_V1.8$ polyclonal antibody SNS11, showed that most of the $Na_V1.8$ positive cells expressed p11 mRNA, supporting an association between $Na_V1.8$ and p11 in nociceptive small diameter DRG neurons. The results are shown in figure 4.2B.

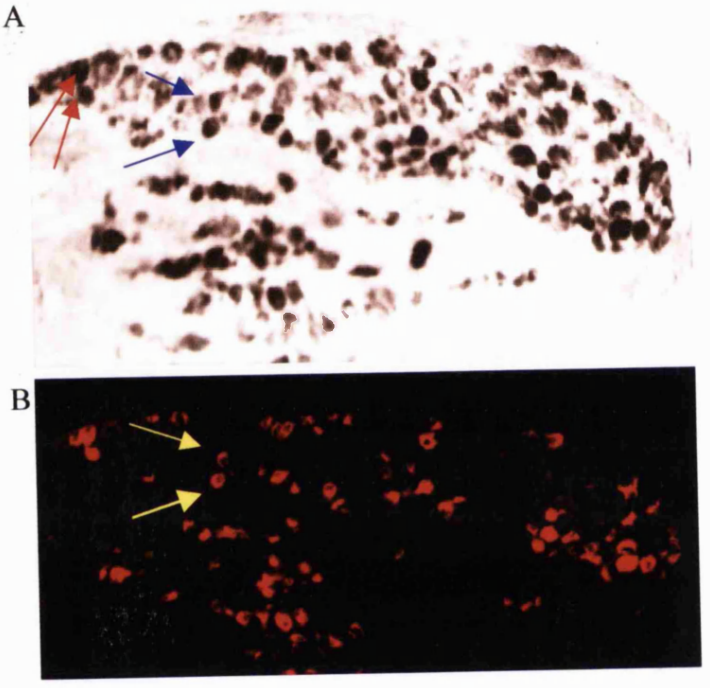


Figure 4.2. A: Insitu hybridisation on 10mm section of 2 weeks old rat DRG showing positive staining with p11 antisense in the small diameter neurones (blue arrows) and large diameter neurones (red arrows) using DIG-labelled probes and signal visualised with alkaline phosphatase conjugated anti-digoxygenin antibody. B: Immunohistochemistry section counterstained with SNS11 polyclonal antibody showing $Na_V1.8$ protein (yellow arrows) in the same DRG neurones as panel A.

4.5. Direct Interaction of p11 with $Na_V1.8$:

Previously work showed a direct interaction between N-terminal region of $Na_V1.8$ and p11 with GST-fusion pull down assay (Personal communication WYLP). The results are shown in figure 4.3A. To verify that p11 interacts specifically with the N-terminal intracellular domain of $Na_V1.8$, the rescued p11 encoding plasmid DNA was re-introduced into other strains of yeast containing different intracellular domains of $Na_V1.8$ as baits. It was seen that the interaction between p11 and $Na_V1.8$ was specific to the N-terminal intracellular domain of $Na_V1.8$ and p11 did not bind to the other baits.

The next stage was to determine which region of the N-terminal domain of $Na_V1.8$ in vitro the p11 protein binds to. This was done by generating three separate fragments of the N-terminal region using forward and reverse primers with $Na_V1.8$ as a template and performing a PCR reaction with the following conditions; 30 cycles at an annealing temperature of 58^0C using primers to generate N1 (position 1-25 amino acids corresponding to 25 amino-acids which is 75bp long). N2 (Position 26 to 52 amino acids corresponding to 26 amino-acids and 78bp long) was generated by using the forward primer and reverse primers. N3 (Position 53 to 127 amino-acids corresponding to 76 amino acids and 222bp long) was generated with the forward and reverse primers. The 3 fragments were subcloned into EcoRI and NotI sites in pGEX5X-1 vector and the resultant plasmid transformed into competent *E.coli* XL1-Blue cells. GST-N1, GST-N2 and GST-N3 fusion proteins were expressed by transforming the plasmids into *E.coli* BL21 cells. GFP-p11 fusion protein was generated in COS-7 cells and the affinity-purified GST and GST-N1, GST-N2 and GST-N3 immobilized on glutathione-sepharose beads were incubated with cell lysates

overexpressing GFP-p11proteins, the fusion proteins denatured with SDS sample buffer prior to separation on a 12% SDS-PAGE gel.

The GST fusion protein on its own has a size of 30.5kDa, so N1, N2 and N3 GST fusion proteins sizes were:

N1: has 25 amino acids corresponding to 33.5kDa.

N2: has 26 amino acids corresponding to 33.6kDa.

N3: has 76 amino acids corresponding to 39.7kDa.

GFP-p11 should give a band of approximately 45kDa when visualised with anti-GFP monoclonal antibody.

Protein extraction was performed after generating the fragments with PCR and cloning them into pGEX5X-1 and expressing the fusion proteins. Once it was determined the protein was present, a pull down assay was performed by incubating the fusion proteins with GFP-p11 at 4^0C overnight. After intensive washing the proteins were loaded onto the SDS gel. After transfer overnight to a nitrocellulose membrane, 1/800 anti-GFP antibody was applied and viewed with 1/2000 anti-mouse HRP conjugated IgG and applying ECL before exposing to a Biomax Kodak film for 5 minutes.

The results in figure 4.3 reveal that p11 binds to the N3-GST fusion protein. This region in the N terminal of $Na_V1.8$ is 76 amino acids long from amino acid positions 53 to 127. As seen from the Western blots there is a 45kDa band obtained with GFP-p11 cell lysate and GST-N3 fusion protein when incubated with GFP-p11 and visualised with anti-GFP. This band corresponded to the GFP-p11 protein which has been pulled down by GST-N3 fusion protein.

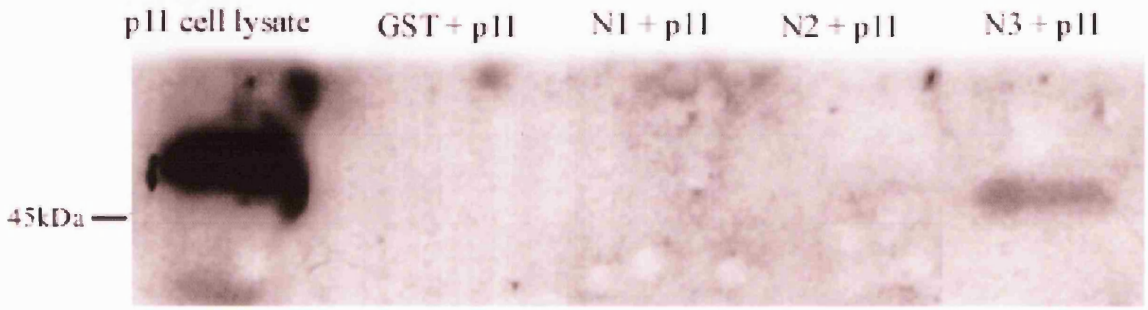


Figure 4.3. Direct interaction of p11 to the N-terminal of $Na_V1.8$ using GST fusion proteins to perform the pull-down assay. The $Na_V1.8$ N-terminal region was truncated into three separate fragments. p11 was seen to bind to N3 (amino-acids 53-127) region as indicated by the 45kDa band. The primary antibody used was anti-GFP and the band visualised with anti-mouse IgG conjugated with horseradish peroxidase.

4.5.1. Specificity of p11 binding to $Na_V1.8$:

To test whether the binding of p11 is specific to $Na_V1.8$, the N-terminal regions of other sodium channels were used to test whether p11 binds to them too. Fusion proteins of the N-terminal region of $Na_V1.2$, $Na_V1.5$, $Na_V1.7$ and $Na_V1.9$ sodium channel α-subunit were made in pGEX5X-1 vector as described in chapter 2. The fusion proteins were used to perform the pull down assay with p11 to determine whether it binds to any of these channels. Preliminary results indicate that it binds weakly to $Na_V1.2$ and strongly to $Na_V1.9$. To test the action of p11 on $Na_V1.2$, ND7 cells were transfected with p11 antisense expression vector. ND7 is an immortalised cell line derived from DRG and N18 neuroblastoma therefore this cell line has some characteristics of DRG neurones but are also able to grow in culture. Electrophysiology studies revealed that p11 antisense did not effect the TTX sensitive currents in these cells. These results were obtained from Dr Baker.

4.6. Translocation of $Na_V1.8$ to plasma membrane by p11:

CHO-SNS22 cells are stably transfected cell line with full length rat $Na_V1.8$ cDNA. They do not have $Na_V1.8$ current despite the fact that they express high amount of full length $Na_V1.8$ mRNA. Immunocytochemical study using anti-$Na_V1.8$ polyclonal antibody SNS11 showed $Na_V1.8$-like immunoreactivity in cytosol of CHO-SNS22 cells but not in the plasma membrane. Figure 4.4A (arrows) shows the immunoreactivity of $Na_V1.8$ in the cytosol. To study whether p11 changes the cellular localization of $Na_V1.8$ protein, we transfected CHO-SNS22 cells with GFP-p11 fusion cDNA. The p11 expression was detected as green fluorescence signal due to fused GFP. The green fluorescence specifically localized in the plasma membrane (Figure 4.4B, arrow head). In the same cell, $Na_V1.8$ immunoreactivity, red fluorescence, also shows signal in the plasma membrane as well as cytosol (Figure 4.5A, arrow head).

The merged picture shows co-expression of p11 and $Na_V1.8$ in the plasma membrane as indicated in yellow colour (Figure 4.4C, arrow head).

Densitometric analysis (Figure 4.5) of $Na_V1.8$-like immunoreactivity of the GFP-p11 fusion or GFP protein expressing CHO-SNS22 cells showed that 16% (n=30) of $Na_V1.8$-like immunoreactivity moved to the plasma membrane fraction after the expression of GFP-p11 fusion protein (Figure 4.5A), while only 4% (n=30) of $Na_V1.8$-like immunoreactivity localized on the plasma membrane in the GFP expressed CHO-SNS22 cells (Figure 4.5B).

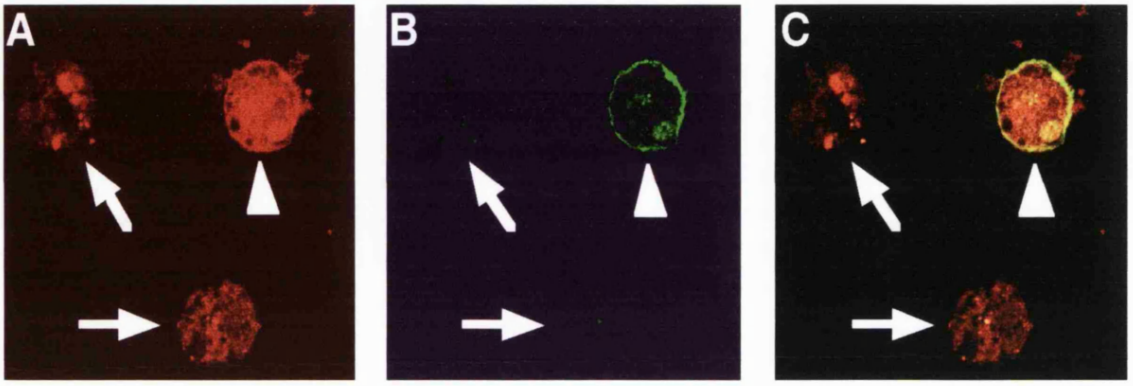


Figure 4.4. CHO-SNS cell line expressing full-length rat $Na_V1.8$ cDNA. Immunocytochemistry studies using anti-$Na_V1.8$ SNS 11 polyclonal antibody showed $Na_V1.8$ like immunoreactivity in the cytosol but not on the plasma membrane of the CHO-SNS cell line (A arrows). After transfection withGFP/p11 fusion cDNA the expression was detected as a green fluorescence signal due to the fused GFP which specifically localised to the plasma membrane (B arrow head). In the same cell, $Na_V1.8$-like immunoreactivity showed signal (red fluorescence) in the plasma membrane as well as the cytosol (A arrow head). The merged picture shows co-expression of GFP/p11 and $Na_V1.8$ in the plasma membrane as indicated by the yellow ring (C arrow head).

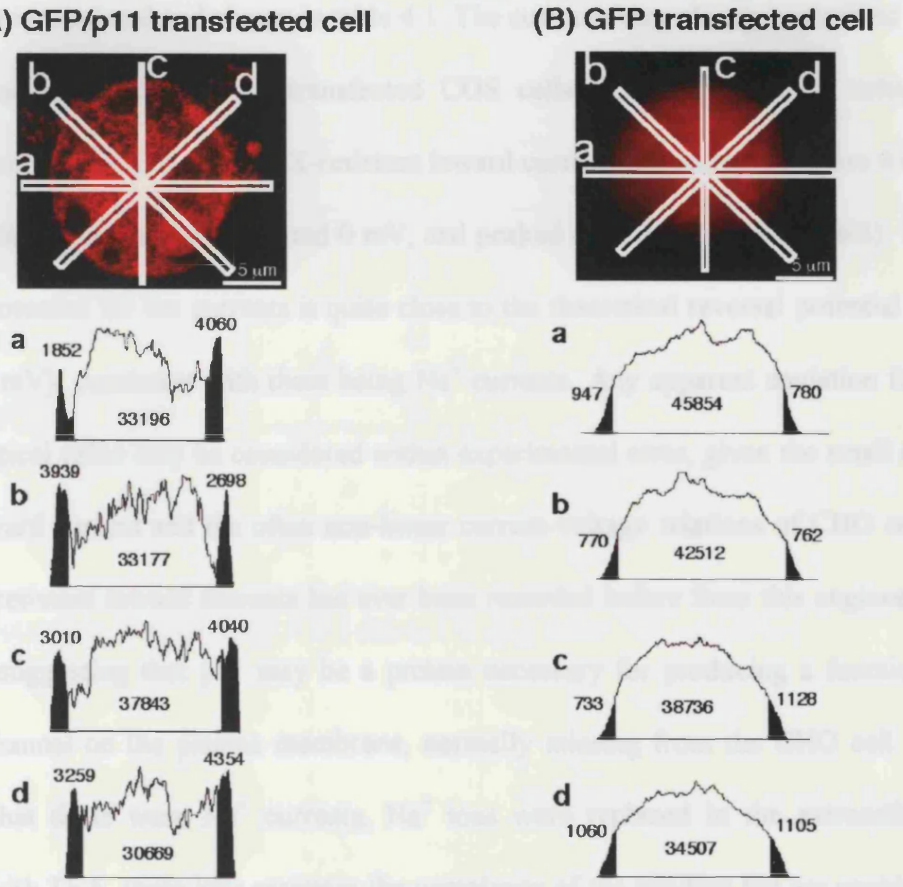


Figure 4.5. Densitometric analysis of NaV1.8-like immunoreactivity in CHO-SNS22 cells Microscopic images were quantitated using public domain NIH Image program. Typical pictures of $Na_V1.8$-like immunoreactivity obtained from GFP-p11 fusion protein (A) or GFP control protein (B) expressed CHO-SNS22 cells.

4.7. p11 Induces $Na_V1.8$ Current in CHO-SNS22 Cells:

In 9 from a total of 46 CHO-SNS22 cells transfected with GFP-p11 cDNA expression vector, TTX-resistant currents were found that resembled neuronal $Na_V1.8$ current. This result is tabulated and shown in table 4.1. The currents very closely resembled the $Na_V1.8$ current recorded from transfected COS cells, both in terms of voltage-dependence and kinetics. The TTX-resistant inward currents are shown in figure 4.6A. The current began to activate around 0 mV, and peaked at +40 mV (Figure 4.6B). The reversal potential for the currents is quite close to the theoretical reversal potential for Na^+ (+81 mV), consistent with them being Na^+ currents. Any apparent deviation from the theoretical value may be considered within experimental error, given the small size of the inward current and the often non-linear current-voltage relations of CHO cells. No TTX-resistant inward currents has ever been recorded before from this engineered cell line, suggesting that p11 may be a protein necessary for producing a functional $Na_V1.8$ channel on the plasma membrane, normally missing from the CHO cell. To confirm that these were Na^+ currents, Na^+ ions were replaced in the extracellular solution with TEA, these ions maintain the osmolarity of the solution but are unable to cross the plasma membrane and therefore unable to depolarise the membrane. This resulted in the abolishment of the inward sodium current hence confirming that the currents obtained with transfected CHO-SNS22 cells were sodium currents (Figure 4.6B). These results were obtained from Dr. Mark Baker, UCL.

4.8. Down-Regulation of $Na_V1.8$ expression after antisense microinjections:

The 309bp fragment of p11 was cloned in 3' to 5' direction into NcoI restriction site in pBS500 vector which resulted in an expression system for an antisense-p11 fusion RNA, pBS-GFP/AS(p11). 40 μg/ml of pBS-GFP/AS(p11) together with 10% Texas red in injection buffer was injected into nuclei of 2-week-old rat DRG small diameter

	Number of cells recorded	Cells with currents >50 pA
p11-GFP-pBS500 vector	46	9*†
Control non-transfected	41	0
GFP-pBS500 vector	40	0

*$P < 0.002$ – vs. control non transfected. Fisher exact test, one-tailed.
†$P < 0.002$ – vs pBS500 GFP, CRE deleted. Fisher exact test, one-tailed.

Table 4.1. The results for full length transfection of p11 into CHO-SNS22 cell line showing a total number of cells recorded and how many of these cells had $Na_V1.8$, also displaying control non-transfected cells and cells transfected with GFP only. The recordings were carried out by Dr Mark Baker, UCL.

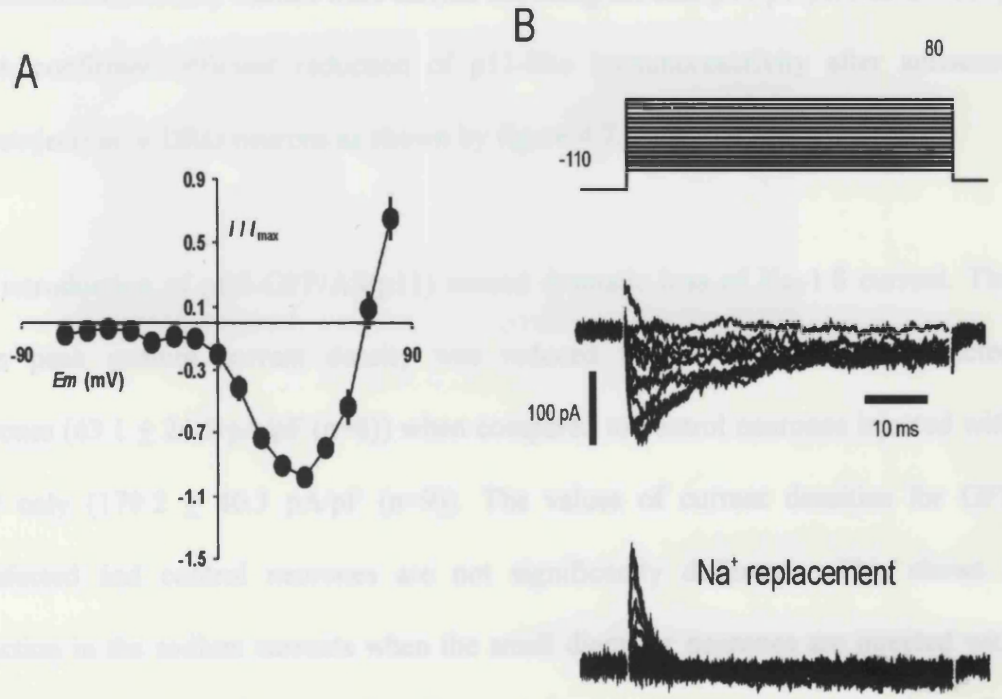


Figure 4.6. Stably transfected CHO-SNS cell line displaying $Na_V1.8$ current after transfection with GFP-p11 that translocated $Na_V1.8$ to the plasma membrane and expressed a functional channel. A: IV plot displaying the TTX-r inward current I/I_{max} versus voltage E_M. ● represents Mean ± SEM (n=5). B: The voltage was stepped up from -110mV to +80mV and displayed a TTX-r current of 100pA which disappeared when sodium was replaced with another cation. Results obtained from Dr. Mark Baker, UCL.

neurones. After completion of the injections the neurones were incubated for 3 days at 37^0C prior to electrophysiology recordings.

To validate the antisense experiments by antibody staining, and to confirm the reduction of the p11 immunoreactivity by the introduction of the antisense, immunohistochemistry studies were carried out using the anti-p11 polyclonal antibody which confirmed efficient reduction of p11-like immunoreactivity after antisense microinjection in DRG neurons as shown by figure 4.7.

The introduction of pBS-GFP/AS(p11) caused dramatic loss of $Na_V1.8$ current. The mean peak sodium current density was reduced in pBS-GFP/AS(p11) injected neurones (63.1 ± 24.5 pA/pF (n=8)) when compared to control neurones injected with GFP only (179.2 ± 40.3 pA/pF (n=9)). The values of current densities for GFP transfected and control neurones are not significantly different. This shows a reduction in the sodium currents when the small diameter neurones are injected with the p11 antisense therefore indicating that p11 plays a role as a permissive factor for $Na_V1.8$ in DRG neurons and that p11 is necessary for a functional $Na_V1.8$ channel. Figure 4.8 shows the graph for log current density distribution for $Na_V1.8$ in cultured DRG neurones after p11 antisence microinjections.

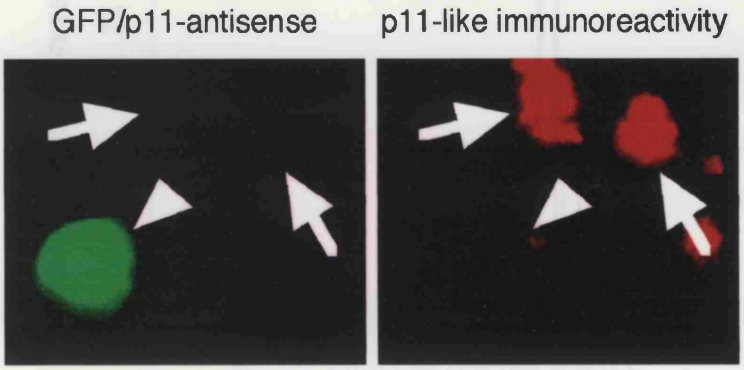


Figure 4.7. Showing p11 antibody staining after GFP-p11AS microinjection in cultured DRG neurones. The green cell represents the GFP staining (white arrowhead) indicting the antisense microinjected cell and the red cells show the staining with the anti-bovine p11 antibody at 1/1000 dilution (white arrows) and visualised with rhodamine conjugated anti mouse IgG at 1/150 dilution. There is no red staining in the microinjected cell indicating the down regulation of the p11 protein.

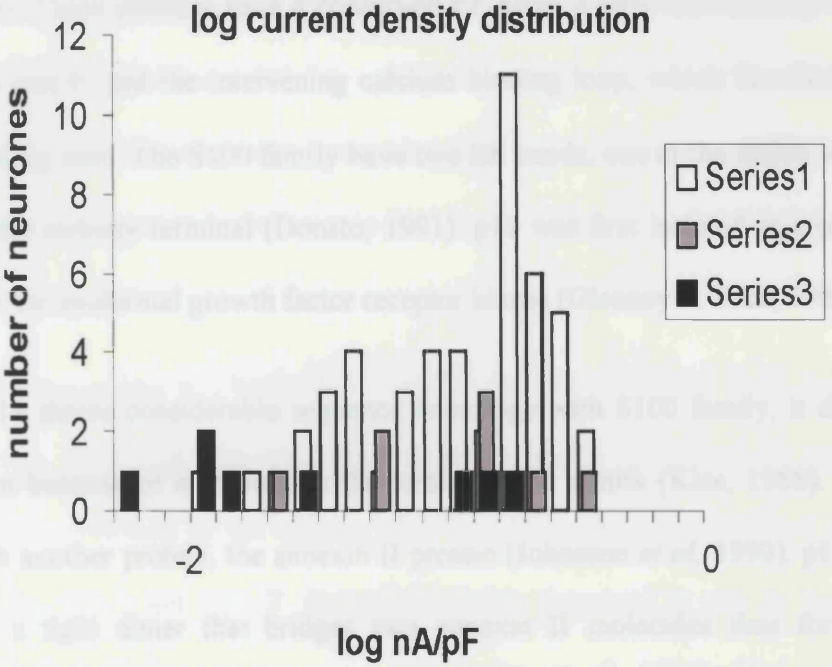


Figure 4.8 Log current density distribution for $Na_V1.8$ recorded in rat cultured DRG sensory neurones. Series1: control non-injected; series 2: GFP injected; series 3: GFP and p11 antisense injected. The values of current densities for GFP injected and control neurones are not significantly different ($P = 0.9$, Student's t-test, unpaired, two-tailed), whereas comparison of p11 antisense injected with GFP injected reveals a significant difference ($P < 0.02$, Student's t-test, unpaired, two-tailed). These results were obtained from Dr Mark Baker, UCL.

4.9. p11:

p11 is a unique member of the S100 family of calcium binding proteins. The S100 proteins are a group of low molecular mass acidic calcium binding proteins. (Kligman & Hilt 1988). These proteins have a conserved EF-hand, a term representing the two α-helices, E and F, and the intervening calcium binding loop, which function as the calcium binding sites. The S100 family have two EF hands, one at the amino terminal and one at the carboxy terminal (Donato, 1991). p11 was first isolated as a potential substrate for the epidermal growth factor receptor kinase (Glenney & Tack, 1985).

Although p11 shares considerable sequence homology with S100 family, it does not bind calcium because of mutations in the two EF-hand motifs (Klee, 1988). It does interact with another protein, the annexin II protein (Johnsson *et al,* 1990). p11 on its own forms a tight dimer that bridges two annexin II molecules thus forming a heterotetrameric annexin II_2-$p11_2$ complex that is present in many eukaryotic tissues and cells (Waisman, 1995). While annexin II exists as a monomer or binds to p11 forming the tetramer complex, p11 has often been found as part of a complex with annexin II in many p11 positive tissues where annexin II is present in similar or excess amounts (Waisman, 1995). The expression of p11 and annexin II is not always co-ordinated and the ratio of p11 to annexin II varies with different cell types (Saris *et al,* 1987). In the F9 teratocarcinoma cells, there exists a significant amount of p11 but almost no annexin II both at the RNA and protein levels, with the unbound p11 distributed in the cytoplasm (Harder & Gerke 1993). Under standard cell culture conditions F9 cells remain undifferentiated but can form cellular layers resembling early embryonic tissue upon induction of differentiation by retinoic acid and cyclic AMP (Harder & Gerke 1993). They found that in contrast to annexin II mRNA, the level of p11 mRNA is unaffected by the differentiation process.

Another protein that p11 has an effect on is phospholipase A_2 (PLA_2), an enzyme that catalyses the hydrolysis of phospholipids, resulting in the production of free fatty-acids and phospholipids (Wu *et al,* 1997). These lipids products can serve as intracellular second messengers or can be further metabolised to potent inflammatory mediators. The release of arachidonic acid from membranes by PLA_2 (Mayer & Marshall, 1993) and its subsequent conversion into leukotrienes, prostaglandins, and other eicosanoids play an important role in the process leading to inflammation.

We have also shown that p11 binds to the $Na_V1.8$ channel which has been implicated in nociception. The fact that p11 may have a role in inflammation and pain transmission is confirmed by the binding of p11 to PLA_2 and $Na_V1.8$. There are two types of PLA_2, type I and type II. Type II has been suggested to play a role in the pathogenesis of inflammatory reactions (Mukherjee *et al,* 1994). PLA_2 has two functionally distinct regions, the NH_2 terminal containing the Ca^{2+} dependent lipid binding motif and COOH terminal containing the catalytic domain (Nalefski *et al,* 1994). Wu et al have shown that p11 binds to the C-terminal region of PLA_2, and inhibits its activity *in vitro.* They also found that the annexin II did not alter the inhibitory effect of p11 on PLA_2 and p11 itself was sufficient to bind and inhibit PLA_2 and the p11-annexin II complex was not required for the inhibition. We have also shown that p11 helps translocate the $Na_V1.8$ channel to the plasma membrane and that annexin II is not needed for this expression. The current produced in the CHO-SNS cell lines is similar to TTX-resistant current recorded from DRG neurones though not exactly the same, so it may be that other factors are required to achieve an exact replica of the TTX-resistant current.

Another protein that p11 binds to is PCTAIRE-1 a cyclin dependent kinase like (Cdk) protein found in mammalian brain (Sladeczek *et al,* 1997). They suggested that p11 links PCTAIRE-1 to annexin II and plays a role with the protein networks responsible for the transduction of multiple signals in eukaryotic cells. Hsu et al. 1997 have also identified p11 as a binding protein for apoptotic Bcl-2 family member BAD and shown that it preferentially binds to the unphosphorylated form. They found in CHO cells that BAD induces apoptosis and this is dampened down with overexpression of p11.

p11 has also been shown to link annexin II to the cytoskeleton. The cortical region of the eukaryotic cells is made up of the plasma membrane and the underlying protein meshwork of the cytoskeleton. The cortical cytoskeleton plays a role in signal transduction events that regulate membrane trafficking, cell migration and growth regulation (Yin & Stull, 1999). A major portion of the cortical cytoskeleton is made up of actin filaments whose physiological state is determined by the actin-binding proteins (Pollard *et al,* 1994). Annexin II like other members of the annexin family bind to actin in a calcium dependent manner (Waismann, 1995). Annexin II monomer is localised throughout the cell whereas the annexinII-p11 tetramer is localised to the plasma membrane-actin cytoskeleton interface (Sagot *et al,* 1997). Filipenko & Waisman, 2001 have localised the actin-binding site to the C-terminal of annexin II by using site-directed mutagenesis to create annexin II mutants with truncations in the C-terminal. Due to its ability to bind simultaneously to actin and phospholipids annexin II is thought to act as a membrane-cytoskeleton linking protein. Filipenko & Waisman found that truncation of the C-terminal had no effect on the phospholipid binding of annexin II.

4.10. Conclusion:

To summarise we have isolated p11 protein using the yeast -two hybrid system and a rat DRG library. We have shown that p11 binds directly to the N-terminal region of $Na_V1.8$. p11 antisense was seen to down regulate the $Na_V1.8$ current and this was validated by antibody staining which was seen to be reduced with anti-bovine p11 antibody in antisense injected cells. In stably transfected CHO-SNS which does not express a functional $Na_V1.8$ despite the presence of $Na_V1.8$ mRNA was seen to produce an $Na_V1.8$-like current when p11 was transfected into these cell lines. This shows that p11 is acting as a regulatory protein that is needed for a functional $Na_V1.8$ channel. Although in our hands p11 does not specifically bind to $Na_V1.8$, this seems to be the only channel in which it helps produce a functional channel on the plasma membrane.

We have shown that p11 binds strongly to both $Na_V1.8$ and $Na_V1.9$ TTX resistant channels, both these channels are very difficult to express in heterologous systems. Maybe the system is lacking an essential subunit and this subunit may be p11. Therefore in conclusion from our studies p11 can act as a regulatory subunit and may be necessary for the functional expression of both $Na_V1.8$ and $Na_V1.9$. Since p11 does not bind with any other sodium channel apart from $Na_V1.2$, it means that this protein can be used as a basis for developing an analgesic drug since it regulates the TTX resistant current only that have been implicated in nociception transmission.

CHAPTER 5.
DISCUSSION AND CONCLUSION

5.1. Discussion

The yeast two-hybrid system takes advantage of eukaryotic transcriptional activators which have two discrete molecular domains, a DNA binding domain and a transcriptional activation domain that can be exchanged from one transcription factor to another and still retain function. The DNA binding domain binds to a specific promoter sequence and the transcriptional activation domain directs the RNA polymerase II complex to transcribe the downstream gene. There are several variations of yeast two-hybrid systems which can be distinguished by their utilisation of each domains. Fields and Song (1989) first demonstrated the use of transcription factors when they reported protein-protein interactions by showing the interaction of two proteins if one was fused to the DNA binding domain and the other to an activation domain. They used yeast transcription factor Gal4 for both the DNA binding domain and transcriptional activation domain. Because of its strong transcriptional activity and endogenous expression of Gal4 in yeast, this method gives high sensitivity with high background. Gyuris et al. (1993) modified this method altering the Gal4 DNA binding domain to the bacterial repressor LexA and Gal4 transcriptional activation domain to yeast activation domain B42. This was based on the system developed by Ma and Ptashne (1987) where they generated a new class of yeast transcriptional activators (B42) encoding *E.coli* genomic DNA fragments fused to the coding sequence of the DNA-binding domain of Gal4. They also generated a LexA fusion protein containing the new class of activating sequences fused to the DNA-binding domain of LexA. The acid blob B42 has relatively weaker transcriptional activity compare to Gal4 activation domain. Due to its bacterial origin, no endogenous yeast proteins bind to the LexA operators hence giving a system with low sensitivity and low background. In addition to Gal4 and B42, the Herpes simplex virus protein VP16 is also used as a

transcriptional activation domain in combination with Gal4 (Fearon et al., 1992) or LexA (Vojtek et al., 1993) DNA binding domain, which does not have a nuclear localisation signal. The VP16 activation domain is fused to a nuclear localisation signal. Due to its higher transcriptional activity than Gal4 and B42, the systems which utilize VP16 are likely to have the highest sensitivity among the different yeast two-hybrid systems. In order to minimise the chance to clone non-specific interactor, we used the least sensitive system, LexA DNA binding domain and B42 transcriptional activation domain.

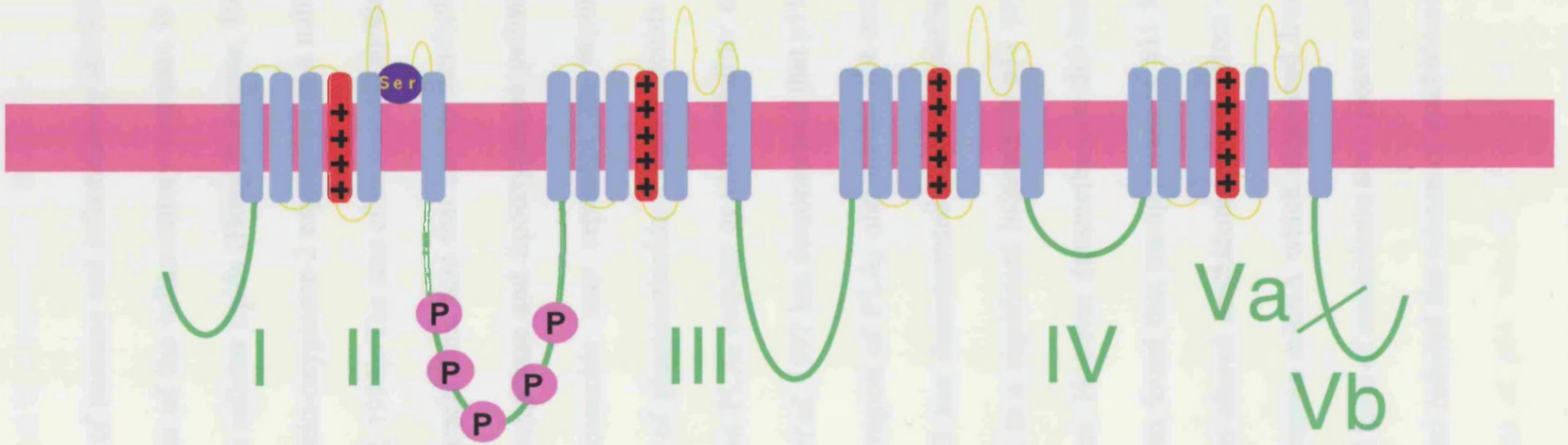
The sensitivity of the yeast two-hybrid systems also depends on reporters. Most systems use two reporter genes, one for enzymes required for the biosynthesis of an amino acid such as HIS3, LEU2 or URA3 genes and the other for enzymes which produce colour such as LacZ or CAT (chloramphenicol acetyl transferase). Using selectable markers for growth on a particular media has marked advantages of providing a selection for cDNA that encode interacting proteins rather than a visual assay which produce coloured colonies. The intensity of the expression of each reporter gene depends on the number of operators on the promoter region. The yeast strain we used, EGY48, has an integrated LEU2 gene with its upstream regulatory region replaced by six LexA operators. This was a very sensitive assay and can be activated by weak transcription activators fused to LexA. In our case we found this to be happening with bait V, so we truncated bait V into two separate fragments. For a second reporter, we chose the plasmid pSH18-34 as this has eight LexA operators positioned upstream of LacZ as compared to other plasmids such as pJK103 and pRB1840 which only have two and one LexA operator respectively. The advantage of using two reporter genes was to rule out possible false positives which can arise by

activation of Leu2 gene by binding of weak activators to Leu2 promoters. This means our system utilized the most sensitive reporter system driven by least sensitive DNA binding domain/transcriptional activation domain complex.

Using this system we found several clones some of which were non-specific, and from the final 27 clones, 3 showed no homology to any known protein and 24 showed homology to known proteins. Figure 5.1 shows a diagram of the intracellular loops of $Na_V1.8$ along with the clones isolated from each of the baits I - V.

The first clone we isolated was p11 (I-1) and to date several proteins have been shown to bind to p11. The first one is annexin II which forms a heterotetramer complex with p11 (Johnsson *et al,* 1990). Annexins are a large family of proteins which are expressed in the majority of cells mammalian cells. Each member of the family has a conserved core domain consisting of either four or eight 70-amino acid repeats and a unique N-terminus. The other proteins shown to bind to p11 are phospholipase A_2 (Wu *et al,* 997) PCTAIRE-1 (Sladeczek *et al,* 1997) and also to BAD (Hsu et al , 1997).

We have now found another protein namely the p11 protein that binds to $Na_V1.8$. We have shown that p11 directly binds to the N-terminal of $Na_V1.8$ and selective inhibition of p11 expression by antisense RNA caused a reduction in the $Na_V1.8$ current. p11 was also seen to help translocate $Na_V1.8$ to the plasma membrane in CHO-SNS cell line where it was expressed and produced a sodium current. We carried out Northern blot analysis and found that NGF up-regulated p11 expression in cultured DRG neurones. Masiakowski & Shooter (1988) have reported that p11 is up-regulated



I-1

II-4
II-5
II-6

III-27
III-42

IV-40
IV-73
IV-7-4

A-10
A-32
A-91
A-103
A-123
A-133
A-136
A-140

A-145
A-148
A-150
A-165

B-4
B-13
B-15
B-18
B-20

Figure 5.1. Schematic diagram of the baits used in the interaction trap with the location of all positive clones identified

by NGF in PC12 cells and the p11 mRNA reached maximal level 24hours after NGF exposure.

Glucocorticoids are effective in the treatment of immune and inflammatory disorders. One mechanism of glucocorticoid modulation of the inflammatory response is the inhibition of release of arachidonic acid from cellular lipids (Hong & Levine, 1976) and inhibition of prostaglandin synthase or cyclo-oxygenase-2 expression in a number of tissues (O'Banion *et al,* 1992, Newton *et al,* 1998). The rate of eicosanoid synthesis may be regulated by the availability of free arachidonic acid that can be metabolised into prostanoids and leukotrienes via cyclooxygenase and lipooxygenase pathways. The decreased synthesis of bioactive eicosanoids may represent an important mechanism of the anti-inflammatory action of glucocorticoids. Glucocorticoids can induce annexins which may be able to inhibit PLA_2 activity *in vitro* (Wallner *et al,* 1986; Wu *et al,* 1995). Studies done by Wu *et al,* 1997 has demonstrated that p11 can directly interact with the carboxy terminal region of PLA_2 and inhibit the enzyme activity. Another study by Yao *et al,* 1999 has demonstrated that dexamethasone treatment of human epithelial cells resulted in a significant increase in p11 protein expression with no effect on PLA_2 expression. However dexamethasone did increase the amount of p11 bound to PLA_2. They also found that manipulation of p11 levels independent of corticosteroid treatment also altered arachidonic release from cells. This was shown by expression of p11 antisense mRNA which expressed less p11 protein in transfected cells and had enhanced level of arachidonic acid release and over expression of p11 inhibited PLA_2 activity and reduced the release of arachidonic acid from the cells.

Another function of the p11-annexin II tetramer is in the lung. Annexin II is abundant in the lung and can exist as a monomer or a tetramer with p11. Lung surfactant is a complex of phospholipids and proteins secreted by alveolar type II cells in the lung. The major function of the surfactant is to lower the collapsing pressure imposed on the alveoli caused by surface-tension at the air-liquid interface. The secretion of lung surfactant is via exocytosis of lamellar bodies (Rooney *et al,* 1994). Annexin II-p11 complex has been shown to mediate lamellar body fusion in lung type II cells. It promotes in vitro fusion of lamellar bodies with liposomes at micromolar concentrations of calcium (Liu *et al,* 1995). The lung surfactant secretagogue, arachidonic acid stimulates annexin II-p11 mediated fusion. Annexin-p11 complex also partially restores surfactant secretion from permeabilised type II cells and is also able to translocate from cytoplasm to the plasma membrane of type II cells in response to stimulation (Liu, 1999). Nitric oxide (NO) is an important signalling molecule and alters surfactant metabolism in isolated alveolar type II cells by inhibiting disaturated phosphatidylcholine synthesis and decreasing cellular ATP levels. Studies have also shown that low levels of NO stimulate surfactant secretion in type II cells but high levels inhibit it (Beckman *et al,* 1998). In vivo high levels of NO can potentially form peroxynitrite ($ONOO^-$) a strong nitrating agent by reacting with superoxide and $ONOO^-$ can inhibit surfactant secretion by inactivating annexin II-p11 complex via nitration of the protein (Rowan *et al,* 2002). Rowan et al's study showed that annexin II core domain was nitrated by $ONOO^-$ and p11 was also nitrated at the two tyrosine residues it contains. Since p11 modulates the function of the annexinII-p11 complex by binding to the N-terminal of p36, they concluded that the nitration of p11 is likely to alter its binding characteristics to those of p36 subunit. The in vitro results confirmed that $ONOO^-$mediated nitration of tyrosine residues on annexin II-p11

inhibits the proteins activity directly. NO can affect nociception as peripheral administration of nitric oxide synthase (NOS) inhibitor attenuates PGE2-induced hyperalgesia (Aley *et al,* 1998). NO can also activate cyclooxygenase enzymes to stimulate the production of prostaglandins (Salvemini *et al,* 1993).

Annexin II possesses the unique property among the other annexins to aggregate chromaffin granules at micromolar calcium concentration (Drust & Creutz, 1988). Annexin II is closely associated with the inner face of the plasma membrane in chromaffin cells. In cultured chromaffin cells thin strands were found cross-linking the chromaffin cells to the plasma membrane after stimulation with acetylcholine (Nakata *et al,* 1990). Similar thin strands were also observed between aggregated chromafin vesicles when they were mixed with annexin in presence of calcium. These data strongly suggests that after stimulation conformational changes occurred in annexin II to cross-link the vesicles and the plasma membrane. When chromaffin cells were stimulated by nicotine, annexin II was phosphorylated by protein kinase C. It has been shown in resting chromaffin cells the annexin II p36 heavy chain was localised in the cytosol while p11 light chain was concentrated in the cortical subplasmalemma and upon nicotine stimulation annexin II translocated to the cell periphery where p11 was localised (Chasserot-Golaz *et al,* 1996). All these results suggests that annexin II may be involved in exocytosis.

Of the other positive clones isolated there are several cytoskeletal binding proteins involved in microtubule assembly like ZRP (II-5) which is a recruiting protein that contributes to the regulation of actin polymerisation at the plasma membrane. The precise mechanism by which zyxin enhances cell motility has not been clarified. However one suggestion for the function is that zyxin acts as a molecular scaffold

which functions to facilitate the formation of a molecular complex that promotes site-specific actin assembly. This view is consistent with zyxin's ability to interact with several proteins involved in cytoskeletal regulation (Beckerle, 1997) as well as its ability to promote the assembly of actin-rich structures when targeted to the plasma membrane (Golsteyn *et al,* 1997). Zyxin is a phosphoprotein composed of 3 C-terminal LIM domains, a proline rich N-terminal and at least one nulear export signal (NES) (Aplin *et al,* 1998). The N-terminal region of zyxin displays docking sequences for several proteins implicated in actin assembly and organisation including α-actinin and members of the Ena (enabled)/ VASP (vasodilator-stimulated phosphoprotein) family (Ahern-Djamali *et al,* 1998). Ena/VASP family members are profilin-binding proteins that have been proposed to regulate actin assembly and dynamics (Gertler *et al,* 1996). To date only 2 proteins have been shown to dock on the LIM region of zyxin, these are CRP (cysteine-rich proteins) which bind to the 1st LIM domain (Louis *et al,* 1997) and H-warts/LATS1 tumour supressor protein which is an important regulator of mitotic progression (Hirota *et al,* 2000), though they may not be the proteins that zyxin requires to associate with the focal adhesions. The N-terminal of zyxin also binds to α-actinin and members of the Ena/VASP family. The binding of proteins to zyxin LIM domain may contribute to conformational regulation that can effect focal adhesion targeting and protein docking functions (Hirota *et al,* 2000). The $Na_V1.8$ binding site on zyxin is not known, since we isolated a zyxin binding clone which did not have the N-terminal region. It is a possibility that $Na_V1.8$ binds to one of the LIM domains of zyxin found at the C-termianl.

Dynein is also a microtubule associated protein that is involved in axonal transport in nerve cells. Tctex-1 (IV7-40) which is the cytoplasmic light chain of dynein has been

shown to associate with the C-terminal of rhodopsin, and is involved in transport of visual pigment to the base of the connecting cilium within the photoreceptor (Tai *et al,* 1998). Tctex-1 also binds to the Fyn tyrosine kinase in B lymphocytes (Campbell *et al,* 1998). It also associates with and can be phosphorylated by Fyn in *Torpedo* electric organ (Mou *et al,* 1998) and also interacts with Doc2 protein where it potentially mediates dynein attachment to specific vesicular structures (Nagano *et al,* 1998). There are several additional members of the tctex-1 family including the rp3 protein that is a component of cytoplasmic dynein (King *et al,* 1998) thought to be involved in X-linked retinitis pigmentosa. The tctex-1 and rp3 light chains are differentially regulated in both a developmental and tissue-specific manner. For example tctex-1 message is abundant in foetal brain but is down-regulated in the adult (Kai *et al,* 1997) whereas rp3 light chain reveal the opposite pattern (Roux *et al,* 1994). It has been shown that tctex-1 is also expressed in primary cultures of SCG and DRG, which are neurotrophin responsive cells (Yano *et al,* 2001). Primary ciliary dyskinesia (PCD) is a congenital respiratory disease usually associated with male infertility (Afzelius, 1976). The disease phenotype results from axonemal structural and functional abnormalities of cilia and flagella due to abnormal dynein arms (Afzelius *et al,* 1985). Cilia and flagella share a common complex structure the axoneme. In the axoneme nine peripheral microtubule doublets are arranged around a central pair of microtubules and outer and inner dynein arms are attached on the peripheral microtubule doublet. By means of an ATP-dependent reactions the dyneins arms enable the sliding of adjacent peripheral doublets to each other and this gives rise to ciliary and flagellar beating (Porter 1996, Shingyoji *et al,* 1998). Yano *et al,* 2001 have shown that trkA receptors that bind NGF associate with the light chain of dynein suggesting a critical role for dynein in neurotrophin transport. Neurotrophins like NGF require signals that are

conveyed over long distances from the nerve terminal to the cell body. They bind to transport membrane receptors and undergo internalisation and transport from axon terminal to neuronal cell bodies. Trafficking of neurotrophins is believed to be required not only for survival but also modulatory effects on neuronal; activity and synaptic function. Binding of neurotrophins to Trk receptors results in autophosphorylation and association with adapter proteins (Kouhara *et al,* 1997). These interactions give rise to downstream phosphorylation cascades involving phosphoinositide lipid phosphorylation and activation of GTPases such as Ras and Rap1 (York *et al,* 1998). Experiments conducted in PC12 cells have indicated that NGF-trkA complex could be found in clathrin-coated vesicles and endosomes associated with the tyrosine kinase substrates, such as phospholipase Cγ (Grimes *et al,* 1997). Several tyrosine-phosphorylated proteins are associated with the trkA receptor during transport suggesting that signalling by neurotrophins persists after internalisation of their receptors (Ehlers *et al,* 1995) (Neurotrophins bind to trans-membrane receptors and undergo internalisation and transport from axon terminals to neuronal cell bodies (Hendry *et al,* 1974)). For example activation of the nuclear transcription factor cAMP response element-binding protein in sympathethic neurones depends on transport of the neurotrophin-trk complex (Riccio *et al,* 1997). SNS/$Na_V1.8$ is an NGF regulated channel (Dib-Hajj *et al,* 1998) and dynein binding to NGF receptors implies that this protein may indirectly regulate SNS/$Na_V1.8$ through its association to the NGF binding receptor TrkA. Cytoplasmic dynein consists of two heavy chains, three intermediate chains and three light chains. Of these chains the heavy chain has the ATPase activity and interacts with microtubules. Dynactin is a multi-subunit complex that binds both to the microtubules and to cytoplasmic dynein (Vaughan & Vallee, 1995). Disruption of the dynein-dynactin interaction blocks

dynein mediated transport both *in vitro* and *in vivo* (Waterman-Storer *et al,* 1997). One role for the interaction between dynein and dynactin is that dynactin links dynein to its cellular cargoes (Holleran *et al,* 1996).

α-tubulin is another protein involved in the regulation of microtubule assembly and function. Microtubule associated proteins' function on the stability of the microtubule structure depends on phosphorylation and all structural microtubule associated proteins are in vitro substrates for several protein kinases. Lis1, a protein whose mutation causes a human brain disorder, type I lissencephaly interacts with tubulin and is distributed along microtubules. This protein has been shown to interact with dyneins and increases retrograde movement of cytoplasmic dynein and leads to peripheral accumulation of microtubules. This suggests that the amount of Lis1 protein in neurones may stimulate specific dynein functions in neuronal migration and axon growth (Smith *et al,* 2000). Proteins have recently been identified that have the capacity to link actin, intermediate filaments and microtubule networks. The best characterised are plakins, a family of cross-linker proteins, which include plectins, the bullous pemphigoid antigen-1 proteins (BPAG1s) (Ruhrberg, 1997). In mice deletions within the BPAG1 gene results in severe neuronal generation throughout the peripheral nervous system. The DRG axons exhibit dramatic disorganisation of neuronal intermediate filaments and microtubules accompanied by abnormal axonal myelination and axonal swelling (Fuchs & Karakesisoglou, 2001). In neurones immunohistochemical localisation of β-actin in tissue sections of developing rat brain has shown that β-actin protein is specific to growing axons and is deplete in mature neurones (Weinberger *et al,* 1996). Actin and tubulin have been found in a cDNA library from squid axoplasm (Kaplan *et al,* 1992) and more recently in biochemical

fractions of rat sympathethic axons (Olink-Coux & Hollenbeck, 1996). Bassell *et al,* 1998 have also shown an association of β-actin with microtubules within the growth cones and have suggested that microtubules are involved in some component of β-actin mRNA localisation in neurones. In our system we have isolated β-actin and microtubules component α-tubulin and they both were seen to interact with the C-terminal of $Na_V1.8$.

Many studies have addressed the question of how the neurone targets cytoskeletal precursors over considerable distances to reach the growth cone for their use in filopodial information and control of process outgrowth. One established mechanism to provide axon and growth cones with specific cytoskelatal proteins is to actively transport them by slow transport mechanism into processes and growth cones after their synthesis within the cell body (Okabe & Hirokawa, 1990; Mills *et al,* 1996). In addition evidence has accumulated that mRNA are localised into dendrites and axons, suggesting that local protein synthesis may provide an important mechanism to influence the distribution of cytoskeletal proteins (Garner *et al,* 1988; Olink-Coux & Hallenbeck, 1996).

Moesin (A-140) is a member of a sub-family of proteins acting as linkers between the plasma membrane and the cytoskeleton and the regulation of this protein is through phosphorylation. There are several other clones that are also regulated through phosphorylation like the connexin-43 (A-133) protein which is a principal gap junctional protein involved in intracellular communication (Liao *et al,* 2001). Changes in CX-43 expression have been associated with hypertension (Bastide *et al,* 1993). It has proven difficult to get a CX-43 knock-out mice as these mice die shortly after birth

(Reaume *et al,* 1995). All connexin genes have similar structures consisting of 2 exons separated by a long intron. All of the coding information lies in the second exon (Finbow & Pitts, 1993) and so based on this information Liao et al (2001) created a mouse cell line in which exon 2 was flanked by loxP sites (VEC Cx43 KO mice). These mice were hypotensive and bradycardic. The hypotension may be due to nitric oxide elevation in the blood and the synthesis of NO may depend on gap junctional exchange between vascular smooth muscle and endothelium.

HIP1 (II-4) is a cytoplasmic protein of unknown function but which localises to the cytoplasm of neurones and associates with vesicle membranes and microtubules in brain where it is present in abundance (Difiglia *et al,* 1995). Duyao *et al,* 1995 found that in models of Huntingtons disease (HD), where the mouse HD homologue Hdh was inactivated by gene targeting, mice heterozygous for Hdh inactivation were phenotypically normal, whereas homozygous mice resulted in embryonic death. Thus showing that huntingtin is critical early in embryonic development. Kim *et al,* 1999 have shown in striatal cell lines that huntingtin is distributed to endosomal membranes after forskolin stimulation, and this treatment increased huntintin localization on membranes where clathrin coated vesicles were also located. Forskolin also increased the presence of HIP1, clathrin and huntingtin in neurites and membrane fractions. Clathrin coated vesicles function at the cell surface in the early stages of endocytosis to internalise ligands and their receptors and to mediate intracellular signalling (Pley *et al,* 1993). Forskolin which directly stimulates adenylyl cyclase and therefore increases cyclic AMP action is known to influence neurite outgrowth and membrane trafficking in neurones. Also brief stimulation with dopamine D1 agonists which are known to induce rapid internalisation of D1 receptors into endosomes and increase cyclic AMP

activity also increased huntingtin intracellular localisation. Kim et al suggested that since huntingtin co-distributed with clathrin in the neurites of striatal X57 cells it has a role in membrane transport during development. They also concluded since forskolin affected huntingtin localisation in X58 cell lines (devoid of dopamine D1 receptors) suggests that other ligand-receptor systems in striatal neurones that activate adenylyl cyclase could affect huntingtin's localisation. Huntingtin appears to regulate receptor membrane trafficking in developing and mature neurones at cell surface and one way of finding a function for huntingtin is through its association to other proteins. HIP1 association to $Na_V1.8$ may be that it contributes to the trafficking of this channel to the plasma membrane.

Mitogen-activated protein (MAP) kinases have been shown to be activated by various stresses and growth stimuli and to be involved in transmitting signals from outside of cells into cell-nuclei (Sturgill & Wu, 1991). In mammalian systems, three major types of Map kinases have been identified, namely extracellular signal-regulated kinase (ERK), p38, and c-Jun N terminal kinase/stress activated protein kinase (JNK/SAPK). A number of protein kinases are known to associate with chaperones like hsp90 and cdc37 (IV-73). Hsp90 associates with various cellular proteins including transcription factors and protein kinases thus assisting correct folding and functions of target proteins (Yahara *et al,* 1998) but cdc37 is a unique chaperone that is involved in the folding and regulation of protein kinases (Grammatikakis *et al,* 1999). Protein kinases are known to regulate the function of $Na_V1.8$ and it may be that cdc37 acts as a chaperone for kinases which regulates this channel since we have shown that it interacts $Na_V1.8$.

Voltage dependent anion channels are known to express in nearly all tissues and are known to bind various kinases, but the functional roles of the VDAC in metabolic homeostasis are not well understood. The importance of VDAC proteins for normal metabolic homeostasis was emphasised by the report of a child with a mitochondrial myopathy who was shown by western analysis to have a partial deficiency of VDAC1 protein (Huizung *et al,* 1996). This disorder is tissue-specific with a greater deficiency in skeletal muscle than fibroblasts. Several of the other clones isolated are kinases, SAST (III-27) is a syntrophin associated serine/threonine kinase which associates via a PDZ domain to β-syntrophin and has been shown to co-localise in the neuronal process (Lumeng *et al,* 1999). This group has shown that SAST links the dystrophin/utrophin network (DAPC/UAPC) with microtubule associated protein kinases via the syntrophins. DAPC/UAPC also bind to sodium channel proteins (Gee *et al,* 1998) and links them to the actin cytoskeleton. Gee et al have shown that syntrophins bind to adult skeletal (SKM1) and cardiac muscle (SKM2) sodium channels by direct binding of the PDZ domain of syntrophins to (S/T)XV C terminal of these channels. Their studies demonstrated that the C terminal SXV sequences were important for binding syntrophins PDZ domains because brain sodium channels which do not bind to syntrophin PDZ domains but have a C terminal domain that is highly similar to muscle channels bind to the C terminal region (SU) thus suggesting that other domains of syntrophins can contribute to binding of brain channels. If this binding of SU to brain channel is direct Gee et al suggested that it may occur via binding to other intracellular domains of sodium channels. We found that $Na_V1.8$ binds via the intracellular loop between domain II and III, i.e. bait III to SAST which in turn is known to bind to syntrophins therefore confirming the fact that syntrophins might bind to other sodium channel intracellular domains too. The functional

significance of the association of sodium channels to syntrophins and dystrophins can be likened to the association of nicotinic acetylcholine receptors (ACR) to the protein rapsyn. The synaptic accumulation of ACR in skeletal muscle involves the association of ACR with the postsynaptic cytoskeleton in response to signals initiated by neurally derived agrin (McMahan, 1990; Bowe & Fallon, 1995). Rapsyn is thought to play a central role in this process and has been shown to cluster ACR in heterologous cells (Froehner *et al,* 1990). It has been seen that in rapsyn deficit mice ACR are distributed unevenly and do not cluster in response to agrin (Gautam *et al,* 1995). Sodium channels have been shown to aggregate between points of contact between skeletal muscle cells and CHO cells expressing a neural form of agrin suggesting similar mechanisms mediate sodium channels anchoring (Sharp & Caldwell, 1996). A protein with a function similar to rapsyn has yet not been identified for sodium channels though syntrophins might play an analogous role. As dystrophins binds actin (Hemmings *et al,* 1992) and α-dystroglans (extracellular protein of DAPC) bind both laminin and agrin (Bowe *et al,* 1994), the interaction with syntrophin may be sufficient to link sodium channels actin network and the extracellular matrix. (Gee *et al,* 1998). It has been suggested that syntrophins may modulate sodium channel functions since a demonstration by Jing *et al,* 1997 showed that the interaction of SXV-containing C terminal of the potassium channel α-subunit, $K_V1.1$ with cytoskeleton regulates the extent of inactivation conferred by β1-subunit. Such functional changes may be important in certain pathological conditions or myopathies and as seen by the important role for dystrophin in Duchenne muscular dystrophy (DMD) where patients suffer from conduction disturbances and heart block and the absence of dystrophins leads to a marked reduction in sacrolemmal syntrophin which in turn leads to disturbances in sodium channel distribution and function of cardiac sodium channels.

Sodium cardiac channels display the highest homology to $Na_V1.8$ and since they are so similar, syntrophins reductions may also have a deleterious effect on the function of $Na_V1.8$. Further tests have to be performed to see whether syntrophins can link to $Na_V1.8$ and have an effect. There is a possibility that syntrophins will also bind to $Na_V1.8$ though so far this has not been demonstrated.

PAPIN (III-42) is a member of a $p120^{ctn}$ family of proteins which have been identified as major substrates of tyrosine kinase phosphorylation enriched at adherens junctions (Reynolds *et al,* 1992). NPRAP/δ-catenin also interacts with E-cadherin and β-catenin (Lu *et al,* 1999). PAPIN has 6 PDZ domains and may act as a scaffolding protein connecting components of epithelial junctions with p0071. The exact function of NPRAP/δ-catenin and p0071 is not known but since they are localised at cell-cell junctions suggests they may play a role as components of cell-cell junctions like $p120^{ctn}$. So far there has been three reports for the interactions of PDZ domain-containing proteins and armadillo repeat-containing proteins. Adenomatous popyosis coli gene product interacts with PSD-95/SAP90 and SAP97/human discs-large tumour repressor gene. NPRAP/δ-catenin interacts with synaptic scaffolding molecule (Ide *et al,* 1999) and NPRAP/δ-catenin and p0071 bind to PAPIN. As both the PDZ containing proteins and the armadillo repeat containing protein are localised at cell-cell junctions, their interaction may be important for the maintenance of the cell-cell junctions. Our isolated clone for PAPIN only had the last 210aa which contained the 2 PDZ domain in the C terminal of PAPIN and it is likely that $Na_V1.8$ binds to this region.

Differentiated neurones are incapable of dividing even in the presence of chemical and physical stimuli that promote cell-cycle progression of proliferative cells. Little is known about the mechanism underlying the quiescence displayed by all neurones. A few studies have suggested that the retinoblastoma protein (RB) a growth suppressor protein is involved in neuronal differentiation-associated growth arrest. In the brain of RB-deficit mice embryos, abnormal mitotic figures accompanied by massive neuronal death are observed particularly in the spinal cord, hindbrain and sensory ganglion (Lee *et al,* 1992). Maruyama *et al,* 1991, isolated a novel protein termed necdin (A-103) and have shown that it is a growth suppressor protein found in postmitotic neurones that is functionally similar to RB. They found that necdin like RB interacts with the transcription factor E2F1, which directly regulates the transcription of a diverse set of genes involved in DNA replication and cell growth control (Adams & Kaelin, 1995). Muscatelli *et al,* 2000 have generated a necdin deficit mice and found that the mice had abnormal phenotype. The lack of the necdin gene is correlated to an early postnatal lethality. They also presented a characteristic behaviour profile with higher skin scrapping. Muscatelli et al concluded that lack of necdin gene expression resulted in respiratory distress and neonatal lethality that resembles the respiratory problems often associated with Prader-Willi syndrome. This syndrome is a complex neurogenetic disorder with considerable clinical variability that is thought to be the result of hypothalamic defect. Gerard *et al,* 1999 have also shown that Prader-Willi syndrome, a neurobehavioural disorder is characterised by neonatal respiratory depression which resulted in early neonatal death.

Inflammatory pain that is characterised by a decrease in mechanical (and thermal) nociception threshold (hyperalgesia) arises through actions of inflammatory mediators.

Hyperalgesia can occur through two pathways involving protein kinases. England *et al,* 1996 and Gold *et al,* 1996 both independently showed that the inflammatory mediators prostaglandin E_2 (PGE2), serotonin and adenosine produce hyperalgesia through cAMP-dependent protein kinase A (PKA) phosphorylation of the TTXr channels. Okuse *et al,* 1997 investigated the expression of $Na_V1.8$ in inflammatory and neuropathic pain models. They investigated the level of mRNA $Na_V1.8$ in DRG after treatment with inflammatory stimuli such as Freund's adjuvant which involves a range of inflammatory mediators or NGF which acts directly on sensory neurones to exert hyperalgesic effect (Lewin *et al,* 1994). They found 72 hours after Freund's adjuvant was injected into the footpad there was no change in the expression of $Na_V1.8$ mRNA in L4 and L5 DRG although there was profound hyperalgesia. In the presence of NGF there was a small increase in membrane associated $Na_V1.8$ protein in DRG although the mRNA expression did not alter. They concluded that NGF was not necessary for the expression of $Na_V1.8$ mRNA in experiments and $Na_V1.8$ mRNA was not up-regulated in peripheral inflammatory states. They also found that in neuropathic states such as spinal nerve ligature and streptozotocin diabetic rat that leads to allodynia there was a down regulation of $Na_V1.8$ mRNA levels. They concluded that $Na_V1.8$ was not necessary for development of allodynia produced by neuropathic pain. Cesare *et al,* 1999 has shown that bradykinin induced sensitisation of nociceptive heat receptors is through protein kinase C (PKC). PKA and PKC mediate nociceptive sensitisation by modulating the activity of TTXr sodium currents (Gold *et al,* 1996). One of our clones isolated TAO2 (A-165) phosphorylates and activates MEKs from the MAP kinase cascade. Another clone isolated ZIP (B-18) is a cytoplasmic and membrane associated protein that interacts with the regulatory domain of atypical protein kinase C (PKC-ξ) but not classic PKC. The atypical PKC has been implicated in signal transduction

pathways regulating differentiation and proliferation of mammalian cells (Puls *et al,* 1997) though an exact mode of activation and function is not clear. Nakanishi *et al,* 1993 have shown that phosphatidylinositol-3,4,5-triphosphate can activate atypical PKC *in vitro* which could imply that atypical PKC participates in phosphorylation events downstream of phosphatidylinositol 3-kinase activation by receptor tyrosine kinases. ZIP acts as a scaffolding molecule between atypical protein kinase C and tyrosine kinase and shows no homology to any other PKC-binding proteins. ZIP protein has a ZZ motif which has been defined as a novel zinc finger found in several proteins including dystrophins (Ponting *et al,* 1996). Binding to atypical PKC does not involve the ZZ finger but was mapped N-terminal to the ZZ domain. This region contains another amino-acid motif with the consensus sequence $YXDEDX_5SDEE/D$ (where X can be any amino-acid) shared by four other proteins so far. The two homologous proteins scd1 and cdc24 contains this motif in a region that binds to Ras-type and Rho-type GTPase (Chang *et al,* 1994). Also binding of $p40_{Phox}$ and $p67_{Phox}$ subunit of NADPH oxidase has been mapped to a region on $p40_{Phox}$ containing this motif. The same sequences are in MEKs and a novel gene of unknown function called Trk-fused gene (TFG) that was isolated as part of a rearranged oncogenic form (named TRK-T3) of the nerve growth factor receptor (TrkA) that forms oncoprotein complexes (Greco *et al,* 1995). ZIP has also been cloned as a p62 protein interacting with the SH-2 domain of the tyrosine kinase $p56^{lck}$ in a phoshotyrosine-independent manner (Joung *et al,* 1996). Puls et al concluded that due to the presence of 2 protein binding motifs and its association with the atypical PKC and p62 this particular protein was like the yeast protein STE-5 that acts as a scaffold for at least four protein kinases of the mitogen-activating protein kinase cascade in yeast. We have also shown

that ZIP binds to the C-terminal region of $Na_V1.8$ and it would be interesting to see if it binds via the ZZ zinc finger or via the YXDED motif.

Schwann cells primary function is to myelinate nerve fibres and to promote rapid nerve impulse transmission, but they also have a role in providing trophic support for spinal motorneurones and DRG neurones. Periaxin was first identified as a protein of myelinating Schwann cells in a screen for novel cytoskeleton-associated proteins with a role in peripheral nerve myelination (Gillespie *et al,* 1994). Like P_O, the major integral membrane protein of peripheral nervous system myelin, periaxin is detectable at early stages of peripheral nervous system development (Scherer *et al,* 1995). The developmentally regulated nucleocytoplasmic redistribution of L-periaxin in embryonic Schwann cells is the first such example for a PDZ domain protein. Data has suggested that the nucleocytoplasmic distribution of several proteins that undergo active nuclear uptake is affected by cell-cell contact (Pedraza *et al,* 1997). The appearance of appropriate binding partners at the cell surface of Schwann cells may be the stimulus for the translocation of L-periaxin from the nucleus to myelinating processes as they ensheath the axon. Shermann *et al,* 2000 suggest that nuclear targeting of L-periaxin in embryonic Schwann cells may sequester the PDZ domain from inappropriate interactions in the cytoplasm until the correct ligand becomes available at the cell cortex of the maturing myelin-forming Schwann cells. It has been shown that the stimulus that influences nucleocytoplasmic distribution is cell-cell contact (Gottardi *et al,* 1996), though zyxin a LIM domain protein which also shuttles between the nucleus and the focal contacts does so in response to cell-substrate interaction (Nix *et al,* 2001). PDZ domains are known to be involved in protein-protein interaction but in our case we found that $Na_V1.8$ does not bind to the PDZ

domain of periaxin as our isolated clone did not contain this region. Further experiments have to be done to see which region of periaxin the $Na_V1.8$ binds to. Studies carried out with periaxin gene knockout mice (Gillespie *et al,* 2000) have shown that mice assemble compact PNS myelin but it is unstable, leading to demyelination and reflex behaviours that are associated with the painful conditions caused by peripheral nerve damage. Older animals were seen to display extensive peripheral demyelination and a severe clinical phenotype with mechanical allodynia and thermal hyperalgesia which can be reversed by intrathecal administration of a selective NMDA receptor antagonist. Gillespie et al found that when they examined the peripheral nerves of periaxin deficit mice to check whether the myelin sheath was affected, the demyelination was not apparent at 6 weeks. However at 6 months sensory, motor and autonomic nerves were extensively demyelinated. They found that saphenous nerves (sensory) were hypermyelinated but that C-fibres bundles that are unmyelinated were normal. The damage is confined to the myelin sheath and there was no difference seen in the number of L5 dorsal root ganglian between wild-type and periaxin deficient mice. Periaxin was one of a triplicate for the antisense expression vector microinjections that was seen to reduce the peak of the sodium current. The next stage would be to separate out the triplicates and inject the antisense alone to discover which clone was necessary for the functional expression of $Na_V1.8$.

Many of the clones isolated seem to be scaffolding proteins or cell adhesion molecules and its possible that they may act as anchoring proteins between the $Na_V1.8$ channel and the cytoskeleton. The function of these proteins is regulated by phosphorylation. $Na_V1.8$ channel is also regulated by phosphorylation, this was shown by Fitzgerald et al where they mutated the 5 PKA consensus sequences responsible for PKA

modulation of the $Na_V1.8$ channel by converting serines to alanines. This produced an SNS mutant which did not produce phosphorylation when compared to the wild-type. In rat brain there are also 5 consensus PKA phosphorylation sites, 4 of which are involved in PKA phosphorylation (Murphy *et al,* 1993, Chen *et al,* 1995). Cardiac smooth muscle α-subunit has also been shown to be involved in phosphorylation as well (Murphy *et al,* 1996). The brain sodium channel is known to be phosphorylated by cAMP dependent protein kinases (Costa *et al,* 1982) and also can be dephosphorylated by calcineurin, a calcium and calmodulin dependent protein phosphatase, at c-AMP dependent phosphorylation sites. Recently Tan et al (2002) have shown that calmodulin also binds to C-terminal IQ domain of human cardiac sodium channels in a Ca^{2+} dependent manner. It was found that the binding significantly enhanced slow inactivation, a process linked to life threatning idiopathic ventricular arrhythmias. The binding had no effect on the voltage dependence of channel activation We have also shown that the C-terminal region of $Na_V1.8$ can bind to type I, type II and type III calmodulins.

5.2. Conclusion:

$Na_V1.8$ mRNA is expressed predominantly in small-diameter neurones in DRG *in vivo*, microinjection of $Na_V1.8$ cDNA into the nuclei of superior cervical ganglion (SCG) neurones resulted in the robust expression of a sodium current which showed exactly the same channel properties as observed in DRG neurones (England et al., 1998). In contrast, CHO, COS-7, HEK293 and other mammalian cell lines did not express the microinjected $Na_V1.8$ cDNA well (England *et al.*, 1998), and the expressed channel showed different properties from the endogenous DRG current in these cell types. It is also difficult to express functional $Na_V1.8$ in *Xenopus* oocytes and the

expressed channels' properties are very different from endogenous DRG currents (Akopian *et al.*, 1996). Recently, we obtained several stable transformants of CHO cells which express $Na_V1.8$ mRNA and immunoreactive protein. None of these transformants showed TTX-resistant $Na_V1.8$ currents, despite the existence of a considerable amount of $Na_V1.8$ mRNA and strong immunoreactivity for $Na_V1.8$ detected by anti-$Na_V1.8$ monoclonal and polyclonal antibodies (Okuse et al., 1997). The $Na_V1.8$-immunoreactivity was mainly localized in the cytosol and not on the plasma membrane. This suggests that correct trafficking of SNS from endoplasmic reticulum in the cytosolic fraction into plasma membrane did not occur in CHO cells. One reason for this failure of transport of $Na_V1.8$ protein is the lack of an essential regulatory protein for $Na_V1.8$ in CHO cells which exists in DRG and SCG neurons. β1, β1A, β2, or β3 did not alter the cellular localization of $Na_V1.8$-immunoreactivity and did not help to produce sodium current in the stable $Na_V1.8$ transformants. These observations suggested that $Na_V1.8$ required a distinct specific subunit or permissive factor different from known beta subunits for folding, transport, or as a stabilizer of $Na_V1.8$ to help its functional expression on plasma membrane which normally occur in DRG neurons. We have isolated at least two clones from the yeast two-hybrid system which interact with $Na_V1.8$ and helps its translocation to the plasma membrane in the CHO-SNS22 cell line where a $Na_V1.8$ current is seen. This is an unknown protein HSPC025 (A-148) but which has been shown to have homology to G-protein coupled receptor for the protein rhodopsin (Recently Lembo *et al,* 2002 have cloned a GPCR which is uniquely localised in the human and rat small diameter sensory neurones in DRG and named them SNSR and found that these receptors preferentially associated with IB4 class of nociceptors. The SNSR constitute a previously unknown family of GPCR with the seven predicted α-helical transmembrane domains containing highly

conserved motifs as reported for other members of the rhodopsin class I family (Bockaert & Pin, 1999). To date no other GPCR have demonstrated such a localised distribution exclusive to the sensory ganglia. Lembo et al concluded that the receptors were involved in the function of nociceptive neurones like other proteins such as the TTXr Na^+ channels and $P2X_3$ and vanilloid receptors which have selective distribution in small diameter neurones). The other was the p11 annexin II light chain which is a member of the S100 protein family. In this thesis evidence has been presented that p11 is essential as a regulatory protein for the normal level of expression of $Na_V1.8$ in sensory neurones.

5.3. Future Work:

In the immediate future experiments will be carried out to test the function of the other 26 positive clones on the expression of $Na_V1.8$.

The binding site on $Na_V1.8$ has been localised to 28 amino acids in the N-terminal region and this can be resolved further by point mutation studies to determine which region is essential for the binding. It is also essential to determine the binding region on p11where $Na_V1.8$ binds.

The regulatory function of p11 can also be determined by generating p11 knock-out mice which will allow further insight into the functional role of p11 in nociception.

Possible studies to determine suitable drug targets for future analgesic drugs will also benefit from further studies on p11/$Na_V1.8$ interaction.

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