Ligand-Gated Ion Channels in Sensory Neurons.

A thesis for the degree of PhD in Biochemistry,

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I, John Julian Willoughby, hereby certify that this thesis is entirely my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

The work described in chapters 3 & 6 is entirely my own work, although Dr N.N. Ninkina participated in some of the library screening in chapter 3. The studies described in chapters 4 & 5 are part of group collaborative projects to which I contributed with practical and intellectual input.

Analyses of clones isolated in chapter 4 performed jointly with Dr J.N. Wood and Dr N.N. Ninkina are described. Further analyses of clones performed by Dr J.N. Wood and Dr N.N. Ninkina are reported in brief in order to facilitate discussion of other results.

Signed: John Julian Willoughby

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Ligand-Gated Ion Channels of Sensory Neurons.

"O for a life of sensations rather than of thoughts!"

John Keats.

Macd: 'I know this is a joyful trouble to you;
But yet 'tis one'
Macb: 'The labour we delight in physics pain....'

Macbeth (ii.i) William Shakespeare.

"... life is a permanent possibility of sensation"

Robert Louis Stevenson.
This thesis is dedicated to the memory of

Ralph Thomas Willoughby (1928-1989)

- politician and philanthropist.
Abstract.

A subset of sensory neurons whose cells bodies lie within the dorsal root ganglia (DRG) are nociceptive - their activation in response to noxious stimuli can lead to the sensation of pain. The focus of this work was to try to clone ligand-gated ion channels (LGICs) that might be involved in the activation of these neurons, in particular, the capsaicin receptor which is expressed only in nociceptive neurons.

Firstly, a human cDNA library made from material containing sensory ganglia was screened by low stringency homology hybridisation using LGIC cDNA probes containing conserved transmembrane domains. This approach was devised to identify novel LGICs, including the capsaicin receptor. The strategy resulted in the cloning of the novel human homologue of the nicotinic acetylcholine receptor (nAChR) β3 subunit.

Secondly, a rat DRG cDNA expression library was screened for fusion proteins that would bind to resiniferatoxin (RTX), a toxin which binds to the capsaicin receptor. Several RTX-binding proteins were isolated, but none exhibited properties that suggested they were components of the capsaicin receptor.

NGF levels are raised following tissue injury and increases in NGF levels can cause hyperalgesia. The effect of NGF on mRNA levels of nAChR subunits in cultures of rat DRG neurons was investigated to see if NGF increased nAChRs, contributing to hyperalgesia. The complement of nAChR subunits in these cells was determined, and NGF was shown to have no effect on levels of mRNA of nAChR subunits.

The intracellular signalling pathways by which NGF induces hyperglesia are unknown. Transcription factors can be a downstream target of tyrosine receptor activation. The effects of NGF on the expression of the neuronal transcription factors Brn-3 and Oct-2 in DRG cultures was examined. NGF was shown to have no effect on Brn-3, however, Oct-2 levels increased four-fold, a change reflected in Oct-2 protein levels.
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Chapter 1. Sensory neurons.

1.1 Introduction - Dorsal root ganglion neurons.

Peripheral sensory neurons transmit information to the central nervous system. The primary neurons which react to sensory stimuli in the muscles, joints and skin of the limbs and trunk have their cell bodies within the vertebral column and immediately adjacent to the spinal cord in the dorsal root ganglia (DRG) and are known as dorsal root ganglion neurons (Martin and Jessell, 1991b). They have a single branching axon or fibre. One end of this axon enters the spinal cord at the tip of the dorsal horn and branches extensively, forming synapses with interconnecting neurons in different areas of the cord as well as with neurons which are part of ascending pathways to the brain (Perl, 1984, Dodd and Jessell, 1985, reviews: Maxwell and Rethelyi, 1987, Brown et al, 1992, Fyffe, 1992). The other branch of the axon is a peripheral ending which may terminate in a specialised sensory structure.

1.1a Physical and functional heterogeneity.

DRG sensory neurons are a heterogenous population, and include mechano-, thermo-, proprio-, and nociceptive receptive neurons. Mechanoreceptive neurons respond to mechanical perturbation of tissues, thermoreceptors to alterations in, or extremes of, temperature, proprioceptive neurons carry information about the position of limbs and nociceptive receptors carry information about potentially harmful stimuli. Different DRG neurons involved in reacting to the different modalities of stimuli, have particular properties adapted for reacting to the various modalities (Andres and During, 1990, Martin and Jessell, 1991a). Some DRG neurons have specialized receptor structures at their peripheral endings which are associated with these diverse sensory functions (Iggo and Andres, 1982, Halata, 1990, Andres and During, 1990, Halata and Strasman, 1990), while others appear to terminate in a 'naked' nerve ending (Iggo and Andres, 1982).

Such specialised peripheral structures, visualised using light and electron microscopy, are associated with various types of mechano-receptors (Iggo and Andres, 1982, Andres and During, 1990, Halata and Strasman, 1990, Levitan and Kaczmarek, 1991, Perl, 1992); many are in close association with muscles and joints
(Burke and Gandevia, 1990), these include Pacinian corpuscles, Ruffini corpuscles, hair follicles and merkel cells (see Table 1.1, page 28).

Although thermoreceptors and nociceptors have 'bare' endings, the membranes of these 'bare' endings nevertheless contain many distinct receptor proteins which can respond to different chemical signals, and define the function of the neuron.

Nociception is the transmission of sensory signals from nociceptive neurons in response to potentially harmful or damaging stimuli. The activation of nociceptive neurons may lead to the sensation of pain. The definition of nociceptive neurons is not clearly defined, as some primary sensory neurons seem to have a specifically nociceptive function, whereas others may be activated by harmless as well as harmful stimuli (Jessell and Kelly, 1991).

The sensory receptor population in different types of skin (e.g. hairy or glabrous) varies, and the proportion of sensory neurons in different dorsal root ganglia responding to different modalities may vary (Halata, 1990). In addition, there is a different ratio of the various sensory cell types innervating the muscle, skin or joints (O'Brien et al, 1989). DRG Neurons can be categorised by a number of criteria, for instance according to the properties of the type of fibre that they project. Fibres can differ in diameter, the extent of their myelination and their conduction velocities. Generally the larger the diameter of the fibre, the higher the conduction velocity (Harper and Lawson, 1985, Koester, 1991, Martin and Jessell, 1991a, Perl, 1992). Another criterion used is the size of sensory neuron cell bodies within the DRG, since there is a good correlation between fibre diameter and cell body diameter (Schoenen and Grant, 1990).

Although distinctions can be drawn using conduction velocities (Lawson et al, 1987, Lawson, 1992), they are not always a reliable indicator of function because there is considerable overlap in the conduction velocities between different classes of neurons (see Table 1.2, page 29). For instance low threshold mechanoreceptors are usually associated with the most rapidly conducting fibres, while the fibres associated with nociceptors tend to be slowly conducting (Perl, 1992). Nociceptive neurons are mainly associated with small diameter, thinly myelinated A-δ and unmyelinated C-fibres (Iruchijima et al, 1960, Bessou and Perl, 1969, Kumuzawa and Perl, 1977, Raja et al, 1988).
A class of nociceptive neurons with C-fibres, termed polymodal nociceptors, are most abundant. These are activated by moderately intense mechanical stimuli, noxious heat and irritant chemicals (Iriuchijima and Zotterman, 1960, Bessou and Perl, 1969, Kumazawa and Perl, 1977). The next most abundant group of nociceptive neurons are fine myelinated fibres (Aδ fibres), known as A-mechano-heat fibres which also respond to noxious mechanical and heat stimuli (Szolcsanyi et al, 1988). A proportion of the nociceptors with Aδ fibres also contain neuropeptides and are responsive to capsaicin and bradykinin as described below (Szolcsanyi et al, 1988, Rang et al, 1991 and see Chapter 4).

**Peptides in sensory neurons.**

DRG neurons can be divided into two groups, small dark (SD) and large light (L), using light microscopy analysis of cell bodies (Winter, 1987, Lawson et al, 1992), although the sizes of the two populations overlap. The two populations can be distinguished further by their biochemistry, L neurons contain significant amounts of neurofilaments, while the SD neurons contain only small amounts.

The SD neurons can be further subdivided on the basis of their sensitivity to capsaicin, a non-endogenous substance which acts on a subset of sensory neurons with specifically nociceptive function (Jancso et al, 1985, Marsh et al, 1987, Winter, 1987, Holzer, 1991 and see chapter 4). The capsaicin-sensitive cells are a subset of the SD neurons which contain several neuropeptides (Lawson et al, 1987, Pierau et al, 1987, Weihe, 1990). There are some contradictions in reports of cellular localisation of these substances which have resulted from cross-reactivity of antibodies raised to some of the neuropeptides (Lawson et al, 1987, Weihe, 1990).

Many neuropeptides have been demonstrated to be present in capsaicin sensitive cells (e.g., Hua et al, 1985, Jeftinija et al, 1992, see chapter 4) and most neurons recognised as having nociceptive function contain many of the peptides. SD neurons are often described as being a neuropeptide-rich population (Dodd et al, 1984, Holzer, 1988, 1992, Maggi and Meli, 1988), although substance P and particularly CGRP (calcitonin gene-related peptide) are probably also found in some L neurons (Gibbins et al, 1987, O’Brien et al, 1989, Hunt et al, 1992).
Local effector functions of primary sensory neurons.

Nerve injury, whether peripheral tissue damage (Dubner and Ruda, 1992) or axotomy (Hokfelt et al, 1994), can result in significant and long-lasting alterations in the vicinity of the injury and also in the nervous system. This may involve apparent changes in the sensitivity of afferent neurons, and development of sensitivity to new types of stimulation (Sato and Perl, 1991, Simone, 1992). These changes are mediated, at least in part, by release and changing levels of neuropeptides.

In addition to their role as primary afferent cells which respond to sensory input from their environment, DRG neurons have an efferent function in which they can alter the peripheral environment by the release of neurotransmitters and mediators from internal stores (Maggi and Meli, 1988, Holzer, 1988, 1992, 1993). These substances can induce vasodilation and are associated with increased vascular permeability and extravasation of plasma proteins (Holton and Perry, 1951, Jancso et al, 1967). This involves exerting effects on the local vascular system which may include increasing blood flow and altering the permeability of small vessels to allow plasma proteins to invade sites of injury (Reviews: Holzer, 1992, 1993).

Antidromic stimulation (electrical stimulation of fibres contrary to their normal direction of conduction) of afferent nerves or chemical excitation of their peripheral fibres stimulates release of neuropeptides such as substance P, neurokinin A and calcitonin gene-related peptide (Holzer, 1988). These three substances are considered to be the principal mediators of sensory afferent effector function (Bauman et al, 1991, Holzer, 1988, 1992, 1993). Unmyelinated nerves are mainly responsible for these responses (Kenins, 1984).

The release of these substances from the nociceptive neurons can be demonstrated by the capsaicin sensitivity of the release (Jancso et al, 1967, Juan et al, 1980). In addition, it has been shown that when the capsaicin-sensitive subset of DRG neurons has been destroyed by administration of large doses of capsaicin to neonatal animals, increases in gene expression in the spinal cord normally seen following peripheral inflammation are attenuated (Hylden et al, 1992).
Substance P can modulate the properties of some ligand-gated channels, such as nACh receptors (Simmons et al, 1990) and glutamate receptors (Randic et al, 1990) which may potentially contribute to the sensitizing of primary afferent neurons. There are complex and often additive interactions between some of these released substances and other mediators which may be released from cells of the immune (Lotz et al, 1988) and sympathetic nervous system (Hornyak et al, 1990). The interactions between some of these released substances and cells in the periphery may result in the release of other mediators, for instance cytokines, which are released from monocytes in response to substance P release (Lotz et al, 1988).

Levels of nerve growth factor (NGF) the first neurotrophin to be described (see Chapter 6) are increased in damaged tissue (Weskamp and Otten, 1987). NGF can regulate of the mRNA levels of some but not all of the above neuropeptides in primary sensory neurons (Lindsay and Hamar, 1989, Lindsay et al, 1989, Mulderry and Lindsay, 1990), this regulation may be the most significant biological consequence of this injury-induced increase in NGF.

In addition to the neurogenic effects of primary sensory neurons on the periphery, changes in the spinal cord during inflammation and hyperalgesia may also be mediated by a subset of these cells (Simone et al, 1991).

1.2 Channels and receptors in DRG neurons.

DRG neurons have a variety of channels and receptors on their surface (see Table 1.3, page 30). Some of these receptors may be involved in cell to cell communication and be clustered at pre-synaptic locations, while others involved in activation of the neuron may be distributed over the cell body or the axon. DRG neurons contain a variety of receptors and ion channels, including receptors that act through G-proteins (reviews: Brown and Birnbaumer, 1990, Piomelli and Greengard, 1990, Hille, 1992), channels that are activated by ionic movement (reviews: Catterall, 1988, 1991, Jan and Jan, 1989, Jan et al, 1992), and receptors for chemical messengers that are directly coupled to channels (reviews: Karlin, 1993, Sargent, 1993). These various channels play different roles in primary sensory neurons. These roles include activation of the neurons, propagation of signals within the neuron and transmission of
signals to other neurons in the spinal cord. In addition, some of the functions carried out by such channels are less direct and harder to define, such as inhibitory effects that may be exerted on the activation of neurons.

DRG neurons contain several types of ligand-gated channels: each type is made up of similar but distinct subunits. While second-messenger-independent rapid responses to neurotransmitter substances have been recorded in DRG neurons (see Table 1.3, page 30), which of these ligand-gated channel subunits are expressed in these cells and exactly what subunit combinations are assembled into functional receptors are largely unknown (see Table 6.1, chapter 6, page 196 for a list of known subunits expressed in DRG neurons).

**Ion channels in nociceptive neurons.**

DRG neurons contain various ligand-gated ion channels (see Table 1.3, page 30). The presence of both inhibitory and excitatory amino acid receptors has been studied in relation to their involvement in nociceptive pathways (review; Zieglgansberger and Tolle, 1993). While the presence of several of these in sensory neurons can be demonstrated, their function is often not clear. For instance, the presence of nicotinic receptors on primary sensory neurons has been demonstrated (Morita and Katayama, 1984, 1989), and the ability of nicotine (Brown and Gray, 1948) and acetylcholine to stimulate primary sensory neurons has been well documented (Douglas and Gray, 1953, Skouby, 1953), as well as the ability of high levels of acetylcholine to elicit painful sensations (Emmelin and Fielding, 1947). Despite this, a significant presence of acetylcholine or evidence for a physiological involvement in nociceptor activation has not been demonstrated, although it may play a part in modulation of nociceptive information in the spinal cord (Pert, 1987).

In addition, although nearly all known glutamate ligand-gated channel subunits are expressed in the spinal cord (Arancio and MacDermott, 1991, Tolle et al, 1993), only a few have been shown to be expressed in DRG neurons (Keinanen, et al 1990, Bettler et al, 1990). While glutamate agonists appear to be likely transmitters for signalling from the DRG neurons to the spinal cord (Salt and Hill, 1983, Dray and Perkins, 1986, Urban, 1991) the function of glutamate
receptors on DRG neurons is not known, although a population of glutamate channels responsive to kainate in DRG neurons has been demonstrated (Huettner, 1990).

GABA$_\lambda$ ligand-gated ion channels are also present on primary sensory neurons (Gallagher et al, 1978).

1.3 DRG neuron activation and nociception.

Nociceptive neurons are activated by noxious stimuli to the organism, and may result in pain as a conscious sensation only following complex sensory processing in the higher nervous system.

Chemical mediators, released from damaged cells, or as part of the inflammatory response, can activate or sensitize nociceptive neurons. The activation of nociceptive DRG neurons is ultimately the result of activation of membrane ion channels (reviews, Jessell and Kelly, 1991, Rang et al, 1991).

Chemical mediators.

A subset of nociceptive neurons are activated by mediators released from damaged tissue. Some of these mediators, such as 5-HT, protons and ATP can act through ligand-gated ion channels (Maricq et al, 1991, Krishtal and Pidoplichko, 1981, Bevan and Yeats, 1990, Bean, 1990, Bean et al, 1990). Other mediators released from damaged tissue, in contrast, exert their effects by activating second-messenger systems which can operate a number of cellular mechanisms (Dohlman et al, 1987, 1991, Rang and Ritchie, 1988, Piomelli and Greengard, 1990, Hille, 1992). It has also been proposed that chemoreceptors responding to non-endogenous mediators exist (LaMotte et al, 1988).

Some of the main activating and sensitizing mediators of sensory neurons are listed below.

5-hydroxytryptamine.

5-HT can be released from platelets and mast cells during tissue damage and can act on a variety of receptors to either sensitize or activate peripheral sensory neurons (Richardson et al, 1985, Berge et al, 1980, Rueff and Dray, 1992). The 5-HT$_3$ receptor is a directly gated ion channel which is expressed in DRG neurons (Tecott et al, 1993).

The actions of 5-HT on sensory neurons have been reported, (e.g., Higashi et al, 1982, Higashi, 1983), and some evidence
indicates that the 5-HT$_3$ receptor may play a role in nociceptor sensitization (Richardson et al, 1985). However, given the variety of receptor subtypes through which 5-HT can act (Frazer et al, 1990, Julius, 1993, Tecott and Julius, 1993), this conclusion should be viewed with caution until further studies of some of the newer subtypes have been performed (Rang et al, 1991).

5-HT also acts on a variety of receptor types which act via second messenger systems (Frazer et al, 1990, Rang et al, 1991, Julius, 1993, Tecott and Julius, 1993) to either sensitize or activate peripheral sensory neurons (Berge et al, 1980, Land et al, 1990, Rueff and Dray, 1992). There is evidence that subtypes of 5-HT$_1$ and 5-HT$_2$ receptors are involved in nociceptive processes (e.g., Wilcox and Alhaider, 1990).

**Adenosine triphosphate.**

The universal cellular role of ATP as a carrier of free energy means that a quantity of ATP is released in the event of cellular damage. Intradermal injection of ATP can elicit a painful sensation, and ATP has been demonstrated to depolarise the membranes of DRG neurons (Dodd et al, 1984, Krishtal et al, 1988).

ATP appears to activate two types of neuronal receptors, one which gates a cationic channel that has some properties of other ligand-gated channels (Bean, 1990, Bean et al, 1990) and one which acts via second messengers (review: Illes and Norenberg, 1993).

**Protons.**

Acidic substances can elicit painful sensations, and in addition inflammatory exudates are often acidic (Rang et al, 1991). Protons are able to produce a brief depolarization in some neurons (Krishtal and Pidoplichko, 1981), and in DRG neurons a depolarizing response is prolonged (Bevan and Yeats, 1990, Kovalchuk et al, 1990).

In addition to this, protons are able to alter the characteristics of a voltage gated calcium channel in DRG neurons, changing both the ion selectivity and the gating characteristics of the channel (Konnerth et al, 1987, Davies et al, 1988, Morad, 1988).

**Capsaicin.**

Capsaicin is a non-endogenous vanilloid substance which activates and then desensitizes a subset of DRG neurons which are specifically nociceptive in function (Szolcsanyi, 1985, Dray and Dickenson, 1993).
Capsaicin has been extensively used in the study of nociceptive neurons, but the physiological ligand which activates the receptor is not known. Capsaicin does not appear to activate second messenger systems (Wood et al, 1989) and may act on a specific receptor which appears to have many features of a ligand-gated ion channel (review: Bevan and Szolcsanyi, 1990, Bevan and Docherty, 1993, and see chapter 4).

**Bradykinin.**

Bradykinin is a peptide produced by cleavage of kininogens at the site of tissue injury which exerts several actions on a variety of cell types through two types of receptor, B₁ and B₂ (Regoli and Barabe, 1980), as well as activating sensory neurons via second messenger systems (Burgess et al, 1989).

Bradykinin is a major inflammatory agent and is a mediator of nociception (Guzman et al, 1962, Steranka et al, 1988, Dray and Perkins, 1988, 1993). In sensory neurons bradykinin acts by activating protein kinase C (Dray et al, 1988) and increasing hydrolysis of phosphoinositol (Gammon et al, 1989). It also activates enzymes such as phospholipase C and phospholipase A₂ through membrane receptors which appear to be of the B₂ type (Steranka et al, 1988).

**Histamine.**

Histamine may act through several receptor types in performing its various biological effects (Schwartz et al, 1991) and is released by mast cells in the vicinity of tissue injury. Histamine receptors have been described on the fibres of visceral (Higashi et al, 1982, Higashi, 1983) and cutaneous (Fjallbrant and Iggo, 1961) afferent neurons.

Histamine can induce antidromic vasodilation, neurogenic plasma extravasation as well as interacting with nerve endings (Rang et al, 1991). At higher concentrations it can produce varying subjective sensations of pain and itch, but greatly enhances the painful sensations evoked by other mediators (Emmelin and Fielding, 1947).

**Eicosanoids.**

This is a term used for some of the metabolites of arachidonic acid metabolism, most notably prostaglandins which can cause painful sensations at high doses and sensitize nociceptors at lower doses (Mizumura and Kumazawa, 1987, Lang et al, 1990, review:
Moncada et al, 1978). Prostaglandins are released by damaged tissue and the release may also be facilitated by nociceptor activation (Juan et al, 1980).

**Leukotrienes and hydroxy acids.**

Leukotrienes are products of arachidonic acid metabolism which appear to exert an indirect effect on sensory neurons (Rang et al, 1991). In particular, leukotriene D₄ stimulates release of substance P (Bloomquist and Kream, 1987), and 15-lipoxygenase products of arachidonic acid such as (8R,15S)-diHETE can elicit hyperalgesia (Levine et al, 1986).

**Cytokines.**

These are substances such as tumour necrosis factor, interleukins and interferons which are synthesized and released by cells of the immune system as mediators of inflammation. Their release is specifically stimulated by neuropeptides such as substance P and neurokinin A which originate in sensory neurons (Lotz et al, 1988).

**Potassium.**

Accumulation of potassium ions at areas of tissue damage may cause the sensitization of sensory neurons via the release of CGRP and substance P (Franco-Cerecda et al, 1992) and activation of primary neurons can occur with high concentrations (Jeftinja et al, 1992).

**Voltage-gated channels.**

In addition to receptors for the mediators mentioned above, DRG neurons contain a variety of channels activated by changes in membrane potential (Table 1.4, page 31). These are involved in generation of the action potential in these cells, including several types of sodium, potassium and calcium channels (review: Nowycky, 1992). Many of the above mediators may exert effects on these voltage-gated channels, in particular those which act through second messengers (Nairn and Shenolikar, 1992).

The heterogeneity of distribution of voltage gated channel in subsets of DRG neurons is the basis of the differences in the properties of their membrane currents (review: Koerber and Mendell, 1992).

**Nociceptive processing.**

These substances that act on these networks may include some of the mediators which also act on the primary sensory neurons, including 5-HT, GABA and opioids. Apart from its effects on primary afferent neurons, 5-HT is involved in nociceptive processes in the spinal cord (Lucas et al, 1993). Although the receptors for opiates are most abundant in the spinal cord (LaMotte et al, 1976) and the effects of these substances are exerted mainly in this region, primary neurons also contain some opioid receptors (Hiller et al, 1978, Fields et al, 1980), and some of these substances may exert a depressive effect directly on these cells (Suzuze and Jessell, 1980).

1.4 General Aims.

The general aim of this project was to try to find novel ligand-gated channel subunits in primary sensory neurons which might be relevant to activation of sensory neurons, particularly those involved in nociception. This involved in the first instance screening a cDNA library, which was likely to contain material from primary sensory neurons, by DNA homology with a pool of cDNAs encoding ligand-gated ion channel subunits. The clustered areas of homology between members of this receptor family suggested that similar but distinct subunits might be identified using this approach.

Another part of the project was an involvement in a group project which aimed to clone components of the capsaicin-gated channel by screening a DRG cDNA expression library for proteins that bound to resiniferatoxin, a toxin which also binds to the capsaicin receptor.

A further aim was to investigate possible effects of elevated nerve growth factor (NGF) levels in DRG neurons. Elevated NGF following tissue injury appears to play a role in the hyperalgesia or increased sensitivity of these neurons. NGF plays a role in both the development and in the maintenance of the phenotype of mature nociceptive DRG neurons, and regulates capsaicin sensitivity in
these cells. It seemed possible that increased sensitivity of DRG neurons following tissue injury might involve NGF increasing the numbers of ligand-gated ion channels in these cells. The presence in DRG neurons of ligand-gated ion channels which are regulated by NGF in other cell types supported this theory. An investigation of which nAChR subunits were expressed in DRG neurons was followed by a study of the effect of NGF on LGIC mRNAs in DRG neurons to see if raised NGF might induce an increase in the number of receptors on these cells.

Another part of the project was to investigate possible mechanisms by which NGF might alter the properties of DRG neurons. The presence of transcription factors which are expressed only in sensory neurons suggested a possible route by which NGF might exert its effects. Sequence motifs which resemble transcription factor binding sequences have been found upstream of the gene encoding a ligand-gated ion channel subunit. It seemed a possibility that increases in levels of NGF following tissue damage might increase the levels of specific transcription factors which in turn might regulate the expression of genes in DRG neurons which would contribute to the hyperalgesic effect, among them possibly ligand-gated ion channels and the capsaicin receptor.

The expression in DRG neurons of transcription factors and the effect of nerve growth factor on the levels of mRNA encoding these genes was examined.
<table>
<thead>
<tr>
<th>Tissue Type/name</th>
<th>Most effective stimulus</th>
<th>Fiber</th>
<th>Receptive terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glabrous RA</td>
<td>Moving displacement (&quot;rapidly adapting&quot;)</td>
<td>Aa</td>
<td>Meissner corpuscle</td>
</tr>
<tr>
<td>Glabrous SA, A2</td>
<td>Displacement—moderate to slow (&quot;slowly adapting&quot;)</td>
<td>Ad</td>
<td>Ruffini corpuscle</td>
</tr>
<tr>
<td>PC</td>
<td>Transient/vibration (acceleration)</td>
<td>Aa</td>
<td>Pacinian corpuscle</td>
</tr>
<tr>
<td>G hair</td>
<td>Transient (stiff hairs)</td>
<td>Aa</td>
<td>Hair follicle</td>
</tr>
<tr>
<td>G hair</td>
<td>Intermittent displacement velocities</td>
<td>Ad</td>
<td>Hair follicle</td>
</tr>
<tr>
<td>Field A2</td>
<td>Moving displacement (contact with hairy skin)</td>
<td>Ad</td>
<td>Hair follicle</td>
</tr>
</tbody>
</table>
| Type i           | Moving displacement (hairy skin) | Aa    | Merkel cells, hair 
| Type II          | Moderate velocity displacement (hairy skin) | Ad    | Ruffini corpuscle  |
| D—"hair"        | Moving displacement—slow skin distortion | Ad    | Hair follicle      |
| Mechanical nociceptor (HTMR) | Marked mechanical distortion (hair & glabrous skin) | Ad, B | Schwann cell, covered |
| Heat-mechanical nociceptor | High temperature marked distortion (papillary, suprapapillary) | Ab, C |            |
| Cooling thermoreceptor | Decrease in skin temperature | Aa—C | Cutaneous complex |
| C-mechanoreceptor | Slow distortion/very low threshold | C     |                  |
| Warming thermoreceptor | Increase in skin temperature | C     |                  |
| C-polymodal nociceptor | Noxious mechanical, heat, chemical | C     |                  |
| Cold nociceptor  | Very low skin temperature | C     |                  |
| C-mechanical nociceptor | Marked distortion | C     |                  |
| Muscle           |                        |       |                   |
| Spindle         | Intramuscular length (phasic dominant) | II (AA) | Nociceptor, Pacinian, C-polymodal |
| Spindle         | Intramuscular length (static dominant) | II (Ad) | Pacinian, C-polymodal |
| Tendon organ    | Muscle tension | II (AA) | Collagen organ |
| Pressure         | Pressure/distortion | III (AA) |               |
| Mechanical nociceptor | Severe distortion | III (Ad) |                |
| Noceceptor      | Severe distortion & ? | IV (C) |                |
| Chemical nociceptor | Inflammation (? normally inexhaustable) | IV (C) |                |
| Joint/Ligament   |                        |       |                   |
| End movement     | Marked flexion/extension (static) | Ad    | Pacinian corpuscle |
| Position         | One direction position (static) | AAd, D |                |
| Phase            | Joint movement (velocity detection) | Ad    | Pacinian corpuscle |
| Transient        | Changing velocity (acceleration) | AAd   | Pacinian corpuscle |
| Mechanical nociceptor | Beyond normal limit (sensitized by inflammation) | Ab    |                |
| Chemical nociceptor | Inflammation (? normally inexhaustable) | C     |                |
| Viscera          |                        |       |                   |
| PC-mesentery     | Transient (acceleration) | Aa    | Pacinian corpuscle |
| Small intestine movement | Moving deflection | Aa—C |                |
| Renal venous     | Increased venous pressure (also high urine pressure) | Aa    |                |
| Renal arterial baroreceptor | Increased perfusion pressure | Aa—C |                |
| Renal R1         | Renal ischemia | A?    |                |
| Renal R2         | Urine souces | Aa    |                |
| Urethral flow    | Flow turbulence | Aa—C | Pacinian corpuscle |
| Bladder distension | Bladder wall tension (contraction/relaxation) | Aa—C |                |
| Colon stretch    | Increase in lumen | Aa—C |                |
| Colon contraction | Colonic wall tension | Aa, C |                |
| Testicular polyvalent | Intense mechanical/thermal/chemical | Aa—C | Tunica planaris complex |
| Vaginal          | Moving distortion | Aa—C |                |
| Ureter           | Pressure/distortion | Aa—C |                |

Partial lists of mammalian primary afferent units according to the name or terminology given in the literature. Reference for sources are given in the text and bibliography. "—" after a category indicates that the listed values for the conduction velocity are less than the modal value for the indicated component. "?" indicates that the identity of the receptive terminal is not well established.
Table 1.2  Receptive Units and Afferent Fiber Category

<table>
<thead>
<tr>
<th>Fiber Category</th>
<th>Most Rapidly Conducting</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aα</td>
<td>Aβ</td>
<td>Aδ</td>
<td>C</td>
</tr>
<tr>
<td>Muscle spindle annulosp.</td>
<td></td>
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<tr>
<td>M. spindle flower spray</td>
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</tr>
<tr>
<td>G1 hair</td>
<td></td>
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<tr>
<td>G2 hair</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Golgi tendon organ</td>
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<tr>
<td>Skin type II</td>
<td></td>
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<td></td>
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<tr>
<td>Joint-end movement</td>
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<tr>
<td>Pacinian corpuscle</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Skin type I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Glabrous RA</td>
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<tr>
<td>Glabrous SA2</td>
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<td></td>
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<tr>
<td>Urethral flow</td>
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<tr>
<td>Joint-phasic</td>
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<tr>
<td>Muscle pressure</td>
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<tr>
<td>Muscle mechanical nociceptor</td>
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<tr>
<td>Cooling thermoreceptor</td>
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<tr>
<td>Joint nociceptor</td>
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<tr>
<td>Skin mechanical nociceptor–(HTRM)</td>
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<td></td>
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<tr>
<td>D-hair</td>
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<tr>
<td>Skin heat/mechanical nociceptor</td>
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<tr>
<td>Bladder distention</td>
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<tr>
<td>Intestinal movement</td>
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<tr>
<td>Skin C-mechanoreceptor</td>
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<tr>
<td>Muscle chemical nociceptor</td>
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<tr>
<td>Skin polymodal nociceptor</td>
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<tr>
<td>Chemical nociceptor</td>
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<td></td>
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<tr>
<td>Skin mechanical nociceptor</td>
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<td></td>
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<tr>
<td>Warming thermoreceptor</td>
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</tr>
<tr>
<td>Vaginal mechanoreceptor</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine press/distention</td>
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</tr>
</tbody>
</table>

This table is a representative listing of mammalian primary afferents in relation to the category of the peripheral afferent fiber. Position on the horizontal axis indicates the approximate range of conduction velocities for each kind of afferent unit in relation to the major components of the compound action potential. Not all categories of primary afferent units shown in Table 1-1 are indicated.
Table 1.3

**Channels/receptors detected in DRG cells.**
Some of the main receptors and receptor-channels expressed in DRG neurons.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Subtype</th>
<th>method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td>whole-cell recordings.</td>
<td>Higashi et al, 1982</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-HT₁</td>
<td>In vitro recording</td>
<td>Rueff and Dray 1992</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-HT₂</td>
<td>In vitro recording</td>
<td>Rueff and Dray 1992</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-HT₃</td>
<td>In vitro recording</td>
<td>Higashi et al, 1982</td>
</tr>
<tr>
<td>Nicotinic acetylcholine</td>
<td></td>
<td>Intracellular recordings</td>
<td>Morita and Katayama, 1989</td>
</tr>
<tr>
<td>Proton-gated channel</td>
<td></td>
<td>Whole cell voltage clamp</td>
<td>Krishnal and Pidoplichko, 1980, 1981</td>
</tr>
<tr>
<td>Capsaicin receptor</td>
<td></td>
<td>patch clamp</td>
<td>Bevan and Forbes, 1988</td>
</tr>
<tr>
<td>GABA</td>
<td></td>
<td>In vitro recording</td>
<td>Higashi et al, 1982</td>
</tr>
</tbody>
</table>
Table 1.4

Voltage-gated channels in DRG neurons.
(Information summarized from Nowycky, 1992)

<table>
<thead>
<tr>
<th>Ion selectivity, channel subtype</th>
<th>Channel characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺ L-type</td>
<td>High threshold, non activation, fast deactivation</td>
</tr>
<tr>
<td>Ca²⁺ T-type</td>
<td>low threshold, inactivation, slow deactivation.</td>
</tr>
<tr>
<td>Ca²⁺ N-type</td>
<td>high threshold, inactivation.</td>
</tr>
<tr>
<td>K⁺ - delayed rectifier</td>
<td>low inactivation, fast activation.</td>
</tr>
<tr>
<td>K⁺ - fast transient</td>
<td>fast activation and inactivation.</td>
</tr>
<tr>
<td>K⁺ (Ca²⁺ - activated)</td>
<td>Ca²⁺ dependent.</td>
</tr>
<tr>
<td>K⁺/Na⁺ (inward rectifying K⁺)</td>
<td>allows K⁺ flow at negative potentials only.</td>
</tr>
<tr>
<td>'fast' Na⁺ (TTX sensitive)</td>
<td>fast activation and inactivation.</td>
</tr>
<tr>
<td>'slow' Na⁺ (TTX insensitive)</td>
<td>slow activation and inactivation.</td>
</tr>
<tr>
<td>Cl⁻ (Ca²⁺ - activated)</td>
<td>slow inward, tail current, Ca²⁺ dependent.</td>
</tr>
</tbody>
</table>

2.1 Materials and Chemicals.

All chemicals were supplied by BDH unless otherwise stated. All media components and 'superscript' (modified reverse transcriptase) were supplied by (Gibco BRL) unless otherwise stated. GeneClean was purchased from Bio101. Restriction and modifying enzymes were purchased from Promega, library kits and lambda packaging extracts were purchased from Stratagene, Taq DNA polymerase, pGEM-T kits and labelling kits were purchased from Promega. All radioactively labelled molecules were purchased from Amersham.

Oligonucleotides.

These were synthesized on a model 391 PCR-Mate EP™ DNA synthesizer (Applied Biosystems Inc.), using 200 nM columns (or 40 nM columns in the case of sequencing primers or those for limited use) on the 63 step synthesis cycle. Columns were manually flushed through with ammonium hydroxide for an hour and then flushed out into screw-top eppendorf tubes which were incubated tightly sealed at 55°C overnight to remove the protecting groups. Prior to use the oligonucleotide mix was evaporated at 40°C in a rotary vacuum-dryer, and then resuspended to an appropriate concentration, usually 12.5 pMoles/µl for PCR primers and 1.25 pMoles/µl for sequencing primers.

2.2 Bacterial strains and growth conditions.

The Escherichia coli K12 strains used as cloning hosts were XL1, HB101, JM83, BB4 and DH4a. Bacterial strains HB101, JM83 and DH4a were used for growth and preparation of plasmid clones and were grown in Luria broth (LB ingredients, -Pharmacia, Difco) or on LB plates with the following additions. Transformed E.coli strains were cultured with appropriate concentrations of antibiotics (ampicillin (Amp) at 25 mg/ml, tetracycline (Tet) at 50 mg/ml) (Sigma) for isolation of recombinants possessing selectable characteristics.

Where appropriate, recombinants were identified by plating transformed E.coli on Amp LB plates that contained 5-bromo 4-
chloro 3-indolyl β-D-galactoside (BCIG) at 60 mg/ml and isopropyl 
β-D-thio galactopyranoside (IPTG) at 72 mg/ml (Sambrook et al, 
1989).

BB4 cells were used for plating of all libraries, and XL1 cells 
were used in the plasmid rescue procedures. Both of these cell 
types were grown in NZYCM media (Gibco BRL) or on agar plates 
made up in NZYCM media. Additions of IPTG and BCIG to plates 
for XL1 were as above. To induce expression of proteins in λZapII 
cDNA expression libraries, bacterial plates containing phage 
were overlayed with Hybond-C membranes (Amersham) 
impregnated with 10 mM IPTG (Ninkina et al, 1994).

2.3 Transfection of bacterial cells by electroporation.

Cells for electroporation were prepared from XL1 or DH4α 
strain E.coli, which had been grown shaking overnight at 37°C 
from a single colony in LB medium. 2.5 ml of this overnight 
culture was then added to 500 mls of fresh LB medium in a sterile 
flask and grown shaking at 37°C until the OD600 was between 0.5 
and 0.7 absorbence units (from three to five hours).

The cells were then chilled on ice for ten minutes in pre-
chilled sterile centrifuge bottles, before being centrifuged at 4,000 x 
g in a GSA rotor in a Sorval RC5B at 2°C for 20 minutes.

The supernatant solution was then poured off and replaced 
with 500 mls of ice-cold sterile distilled water, and the cells were 
resuspended on ice and then recentrifuged as above. Once again 
the supernatant solution was then poured off and replaced with a 
further 500 mls of ice-cold water, and the cells were resuspended 
on ice and then recentrifuged as above.

This time after removal of the supernatant solution, the 
cells were resuspended in a small volume of ice-cold sterile 
distilled water, around 1 ml for the cells from a 500 ml culture. If 
some cells were to be frozen at -70°C for use at a later date, then 
resuspension was in 1 ml of 10% glycerol in ice-cold sterile 
distilled water. 400μl aliquots of these cells were then put into pre-
chilled tubes on ice for either storage or use. Cells to be stored 
were snap-frozen with a dry-ice and ethanol bath and then stored 
at -70°C.

Plasmid DNA (typically ~10 pg) was mixed with 400 μl 
aliquoted of either freshly prepared cells, or with frozen aliquots of
cells that had been thawed slowly on ice (see above). After mixing, DNA and cells in chilled sterile tubes were left on ice for twenty to thirty minutes (n.b. only up to 1 µl of ligation reactions were used, as higher salt concentrations reduced efficiency). The cell mixes were then transferred to pre-chilled electroporation cuvettes (Gene pulser Cuvettes, 0.2 cm electrode gap, BioRad).

The electroporator (BioRad) was used at settings of 2.5 kV, 25 µF with the pulse controller set to 400Ω. After pulsing, the cell mix was immediately transferred from the cuvettes to a tube containing pre-warmed SOC medium (Sambrook et al, 1989), incubated at 37°C with gentle shaking for 30 minutes and then plated onto either LB or NZYCM agarose.

2.4 Mammalian cells and cell lines.

PC12.

These were grown from a frozen stock of clonal cells, described as PC12.5k, derived from a PC12 line. The media used was:- DMEM (Gibco BRL) containing 10% (v/v) fetal calf serum (Sigma, mycoplasma free), penicillin and streptomycin (100 IU/ml and 100 units/ml) and L-glutamine at a final concentration of 2 mM.

IMR32.

The media used for culture of IMR32 was:- L15 (Gibco BRL) containing 10% (v/v) 'myocline' (mycoplama free) fetal calf serum (Gibco BRL), 100 IU/ml (International units/ml) final concentration of penicilllin and 10 µg/ml streptomycin, L-glutamine at a final concentration of 2 mM, NaHCO₃ at a final concentration of 3 mM and L-glucose at a final concentration of 15mM.

Dissection of neonatal and adult rats to obtain DRG.

Neonatal Sprague-Dawley rats up to twenty four hours old were decapitated and then, in a laminar flow hood, opened along the dorsal edge of the spinal cord with a small pair of scissors. DRGs were removed from all spinal levels into sterile media with a pair of flamed fine forceps.

Adult rats were dissected as above, but dissecting scissors were required to cut the DRGs free from the cord, and trimming of connective tissue was required.
Culture of neonatal rat DRG.

Aseptically obtained DRGs were collected into Hams F14 (Imperial Labs), containing USG ('Ultroser G' serum substitute, Gibco Labs.) at 4%, the DRGs from about six or eight neonatal rats being collected into 2 mls of the medium in a sterile 10 ml tube.

After dissection, the media were poured off and 1.8 mls of F14/USG and 0.2 mls of collagenase (at 12.5mg/ml) was added, and the tube incubated at 37°C for thirty minutes. The collagenase was then poured off and the DRGs were washed in 10 mls of F14 (without USG) to remove the collagenase and serum. The tube was then briefly centrifuged at 1000 x g in a benchtop centrifuge, and the wash was carefully poured off the pellet, and replaced with 1.8 mls of F14 (without USG) and 0.2 mls of trypsin (at 25 mg/ml) and incubated at 37°C for thirty minutes.

The trypsin was then poured off and the DRGs were washed in 10 mls of F14 (without USG) to remove most of the trypsin solution. The tube was then briefly centrifuged as above, and the wash was carefully poured off the pellet, and replaced with 1.6 mls of F14/USG and 0.4 mls of DNase/trypsin inhibitor (20 mg of DNase I, 25 mg of soy bean trypsin inhibitor, 1.04 g of MgSO₄.7H₂O in 45 mls of distilled water).

A flame-narrowed sterile pasteur pipette was then used to triturate the cell suspension ten to fifteen times, or until an even suspension was obtained. This was then passed through sterile 90 μm gauze to remove the large debris, and the resulting cell suspension centrifuged as above. After excess media had been removed, the solution was made up to 2 mls with F14/USG and the cell density estimated by counting a small amount on a haemocytometer slide on an inverted microscope. About 60 - 100,000 cells were required for each Terasaki plate, in a volume of 600 μl, and so the cell density was adjusted accordingly. NGF prepared from mouse salivary glands (the kind gift of J.Winter) to a concentration of 50 ng/ml or commercial NGF (Promega) to a concentration of 5-50 ng/ml was added, and also AraC (cytosine arabinoside) at 10 μM. This mix was then pipetted onto poly-D-ornithine coated covered Terasaki plates which had also been treated with 5 μg/ml laminin, to a density of at least 1000 cells/well and grown overnight at 37°C with 3% CO₂.
After overnight culture, the plates were filled with 6 mls of medium consisting of 50% F14/USG and 50% conditioned medium (obtained form the C6 glioma cell line) plus NGF (50 ng/ml).

The media was changed after three to four days in culture, culture continuing for up to two weeks.

**Culture of adult rat DRG.**

Aseptically obtained DRGs were collected into 2 mls of F14/USG in a sterile 35 mm plastic petri dish, the product from a single rat per dish. After the DRGs had been collected, they were trimmed of connective tissue and moved to a fresh dish.

After dissection, the medium was poured off and 1.8 mls of F14/USG and 0.2 mls of collagenase (Boehringer Mannheim, at 12 mg/ml) was added, and the tube incubated at 37°C for three hours. The collagenase was then poured off and the DRGs were washed in 10 mls of F14/USG to remove the collagenase. The tube was then briefly centrifuged at 1000 x g in a benchtop centrifuge, and the wash was carefully poured off the pellet, and replaced with 1.8 mls of F14/USG.

A flame-narrowed sterile pasteur pipette was then used to triturate the cell suspension ten to fifteen times, or until an even suspension was obtained. This was then centrifuged through BSA (bovine serum albumin - 3 mls of 15% BSA was gently run down the side of the tube and then the whole was centrifuged at 1000 x g for a minute) to remove the large debris.

The DRGs were washed in 10 mls of F14/USG and centrifuged as above. After excess media had been removed, the solution was made up to 1 ml with F14/USG and the cell density estimated by counting a small amount on a cell counting slide. About 180-240,000 cells were obtained per adult rat. Cells were plated in the same way and at the same density as the neonatal DRG neurons.

**2.5 NGF+/- adult DRG culture.**

For this, the cells were prepared and plated on pairs of Terasaki plates exactly as above, for the initial overnight incubation at 37°C with 3% CO₂. After twelve to fourteen hours, the media was poured off, and replaced on one plate of each pair (for NGF+) with 50% F14/USG, 50% conditioned medium.
containing NGF at 10 ng/ml (Promega), and on the other plate of each pair with 50% F14/USG, 50% conditioned medium without NGF and containing anti NGF antibody MAb 23d, at a dilution of 1:40.

The (+) and (-) NGF cultures were paired, and were always produced from the same animals. Usually three or four adult rats were dissected at a time, and the resulting isolated DRG neurons were pooled and resuspended in the final culture medium. This cell suspension was then split into two and plated on 30mm sterile petri dishes, after which NGF was added to one culture and the anti-NGF antibody to the other. The RNA preparations were made from the cultures after three days, they were always performed in parallel. Although the number of cells appeared to be similar in the cultures, a larger yield of RNA was invariably isolated from the culture grown in the presence of NGF.

The concentration of RNA obtained was estimated by measuring the absorbence of a small portion of the preparation at 260 nm using a spectrophotometer. Using these estimates, 1 μg of total RNA was used as the template in first-strand cDNA synthesis reactions. A fraction of this reaction (one-twentieth) was then used to perform control PCRs using primers to the G6PDH gene (see Chapter 6 for discussion of comparative PCR).

2.6 45Ca Uptake assay.

The calcium uptake assay was performed on DRG neurons grown in culture in Terasaki dishes at a density of ~1,000 cells/well. Cells were grown in culture in the presence of NGF (isolated from mouse salivary glands) at 50 ng/ml for four days before assaying (Wood et al, 1988).

For experiments where antisense oligonucleotides were used to examine the effect of deleting specific genes on the capsaicin response, cells were first grown in culture without NGF and in the presence of anti-NGF antibody for four days. Following this the cells were washed and then fresh medium was added which contained NGF, and also synthetic oligonucleotides at a concentration of 5 μM. Cells were grown for four or five days with NGF and oligonucleotide with daily changes of medium and then assayed.
A buffer solution (assay buffer) was used to wash the cells at all stages of the assay. This consisted of:
1 x BSS (without magnesium and phenol red), containing 2 mM CaCl$_2$, 5 mM HEPES buffer at pH 7.4 -
hot buffer contained 10 $\mu$Ci/ml $^{45}$Ca (Amersham, 60 Ci/mMol) instead of CaCl$_2$.

Capsaicin in DMSO at a concentration of 2 $\mu$M was used to achieve a maximal response in activating the non-selective cation channels. In desensitization experiments, capsaicin at 2 $\mu$M, glutamate at 2mM and nicotine at 100 $\mu$M in dilute DMSO were used to pretreat cells prior to the uptake assay.

**Basic assay.**
1) The medium was poured off and then the cells were washed in assay buffer three times after which excess buffer was gently aspirated off.
2) If performing desensitization experiments, dilutions of agonists in assay buffer with DMSO were then added to the wells and the cells were incubated for 10 minutes at 37$^\circ$C with 3% CO$_2$.
3) Plates were then washed three times in assay buffer after which excess buffer was again gently aspirated off. Assay buffer containing $^{45}$Ca was then put onto the cells. This also contained capsaicin in DMSO at 0.5 $\mu$M except for the negative controls which nevertheless contained the same concentration of DMSO.
4) Plates were left for ten minutes at room temperature and then rinsed five times in assay buffer.
5) Plates were then oven dried at 70$^\circ$C for fifteen minutes. 10 $\mu$l of 1% SDS (sodium dodecyl sulphate) was then added and the plates were left at room temperature for twenty minutes.
6) The contents of the wells were transferred to individual scintillation vials containing 1 ml each of CP scintillation fluid (Beckman) and counted in a scintillation counter.

Six wells for each treatment were used in each assay, and the results were averaged. Control wells which were not treated with capsaicin were prepared to measure the background uptake of $^{45}$Ca. Positive control wells in which calcium uptake in the cells was stimulated by capsaicin were prepared with each of the test conditions.
$^{45}$Ca is a moderately high-energy $\beta$-particle emitter (0.26 MeV) with a long half-life (165 days). Quenching of disintegrations and decay are not normally problems and efficiency of counting is above 90%. The counter used was already calibrated for $^{45}$Ca assays involving similar volumes of aqueous cell suspension and scintillant. A $^{45}$Ca standard curve previously calculated and stored on the machine was used in a program to convert the printout from the counter into disintegrations per minute (dpm) from counts per minute (cpm).

2.7 Extracting total RNA from mammalian brain and DRG.

When RNA was to be isolated directly from DRG or brain, the tissue was collected directly into a container filled with liquid nitrogen so that it was snap frozen immediately. When all the tissue was collected, it was transferred into a mortar in liquid CO$_2$ and ground up using a pestle until it resembled powder. Guanidinium denaturing mix was then added to the mortar, and the material was thoroughly resuspended, producing an opaque, viscous mixture. This material was often processed immediately, but immediate processing did not seem to be critical - the denaturing mix should rapidly destroy nucleases present. The denatured cell mix could be stored at -70°C for months before processing with no noticeable change in the result.

Making total RNA from cell cultures.

This was performed essentially as described (Curr. protocols in mol. biol., 1989), briefly: Cells to be harvested for RNA were grown in 30mm culture dishes (DRG neurons) or flasks (PC12, IMR32) (Nunc).

Cells were removed from petri-dishes by adding the denaturing mix straight to the dish and pipetting it up and down several times using a Gilson pipette until it had become viscous. Adhering cells in culture flasks were dislodged by tapping the sealed flasks sharply three times and then pouring off the medium into a centrifuge tube. This was then centrifuged at 1000 x g for ten minutes. Denaturing mix was then added to the cell pellet and mixed well.

The denatured cell mixture (volume ~1 ml) was then transferred to a 40 ml polypropylene tube, and 0.1 x vol. of 2M
sodium acetate (pH 4.0) was added, and the contents of the tube thoroughly mixed. 1 ml of water-saturated phenol was then added and the contents again mixed thoroughly, followed by 0.2 mls of 49:1 chloroform:isoamyl alcohol, followed again by mixing. The suspension was then incubated at 0°C to 4°C for fifteen minutes.

The tube was centrifuged at 9,000 rpm in an SS-34 rotor (10,000 x g) for twenty minutes at 4°C.

The upper aqueous phase (~1 ml) containing the RNA was then removed to a clean RNase-free tube and precipitated by the addition of an equal volume of isopropanol. The tube was stored at -20°C for thirty minutes and then centrifuged in an SS-34 rotor as above, for ten minutes after which the supernatant solution was discarded.

The RNA solution was then resuspended in 0.3 mls of denaturing solution in an RNase-free 1.5 ml eppendorf tube, vortexed and then precipitated with an equal volume of isopropanol at -20°C for thirty minutes, followed by centrifugation at 13,000 rpm in an MSE 'Microcentaur' for ten minutes at 4°C.

The resulting pellet was then resuspended in 75% ethanol, vortexed, and left to stand for ten minutes at room temperature to dissolve remaining guanidinium. After five minutes of centrifugation at room temperature in a 'Microcentaur' at 13,000 rpm and discarding the supernatant solution, the RNA pellet was dried in a rotary drier for ten minutes.

70% ethanol was added to the sample for storage at -70°C, at which point, a small amount of the suspension (typically 5 µl or 10 µl) was taken after vigorous vortexing, diluted in DEPC water* and used to make a spectrophotometer estimate of the RNA yield.

DEPC water* is double distilled water which has been treated to inactivate nucleases by autoclaving it after the addition of 200 µl/l diethyl pyrocarbonate (Sigma).

2.8 Transcribing RNA from a cloned gene fragment.

This was performed using an In Vitro transcription kit (Promega), according to instructions. Product from PCR amplification of the rat nAChR β4 gene performed using primers X5B4 and TMD4a cloned into pGEM-T was used as the plasmid template. The DNA fragment was in an orientation which would
allow transcription from the SP6 promoter to produce a 'sense' RNA transcript. A 'sense' transcript refers to a length of RNA similar to part of the mRNA from the gene sequence as opposed to a length of RNA sequence that would be the inverse complement of it. The template was linearised by digestion with the restriction enzyme Not 1.

In an RNase-free tube at room temperature was mixed:-

4 µl of 5 x transcription buffer.
2 µl of 100 mM DTT.
4 µl of 2.5 mM rNTP mix containing all four rNTPs.
1 µl of RNasin (Promega, 1 unit/µl).
1 µl of linearized template at 0.5 mg/ml
7 µl of DEPC water.
1 µl of SP6 polymerase at 20 u/µl.

These ingredients were vortexed and then incubated at 37°C for one to two hours.

2.9 Synthesising single stranded cDNA from total RNA.
This was done using components from the Stratagene λ-Zap II cDNA synthesis kit (unless other supplier is specified) according to the protocol, briefly:

In an RNase-free tube was mixed:-

3 µl of 10 x first strand buffer.
1.5 µl of 10 mM dNTP mix.
0.4 µl of Pharmacia random hexamers at 5 units/555 µl.
0.6 µl of RNase block II (Promega, 1 unit/µl).
[x] µl of DEPC water.
[y] µl of poly A+/total RNA (around 10-20 ng if using poly A+, or 1 µg if using total)
(volumes [x] = [y] = 23 µl).

These ingredients were vortexed and then annealed at room temperature for ten minutes. Then the following was added:
2.5 µl of ‘Superscript’ reverse transcriptase (BRL) at 4 units/µl.
Final reaction volume = 30 µl.

Incubation was at 37°C for one hour, followed by extraction with phenol and chloroform, and then precipitation with tRNA (to 50 µg/ml), sodium acetate and ethanol (Sambrook et al, 1989).

2.10 32P labelling of probes.
Oligonucleotides were labelled with $^{32}$P γATP using T4 polynucleotide kinase as described (Sambrook, et al 1989). All oligonucleotides were synthesized unphosphorylated.

PCR products were labelled with $^{32}$P αdCTP by using a random primer labelling kit (Promega) according to the manufacturer's instructions. These were briefly as follows; DNA to be labelled (10-50 ng) was diluted as required and to this was added an appropriate amount of 10 x buffer containing random hexanucleotides, dATP, dTTP, dGTP and the reaction salts. This was heated to 65°C for 2 minutes and then immediately incubated at 37°C for 20 minutes. $^{32}$P dCTP (10 μCi/10 ng of DNA) and T4 polynucleotide kinase were then added and the whole was incubated at 37°C for a further 60-90 minutes. Reactions were then stored at -20°C until separation.

**Separation of labelled probe.**

Unincorporated label was removed from all of the above labelling reactions by passing the reaction down a Sephadex G-50 fine (Pharmacia) separation column. This consisted of a glass pasteur pipette drawn out at the tip, partially plugged with glass wool and filled with a suspension of Sephadex G-50 fine (Pharmacia) in 5 x SSC, 0.1 % SDS. After applying the reaction to the column, follow-through washes of 5 x SSC, 0.1 % SDS were added and the column effluent was collected in 140 μl portions and counted to determine the peak of labelled DNA and the peak of unincorporated label.

One or two of the portions containing the peak of labelled probe were pooled and heated in a water bath at 90°C for three minutes prior to adding them to the hybridization mix in the case of double stranded DNA, or, added straight to the hybridization mix without heating, in the case of oligonucleotide probes.

2.11 **Southern Blotting.**

DNA was digested with 20 units of either EcoR1 or Not 1 restriction enzymes for 3 hours using recommended conditions (all restriction enzymes used were Promega), electrophoresed on 0.7% agarose gels containing ethidium bromide and then blotted onto Hybond-N (Amersham) using standard procedures (Sambrook et al, 1989). Hybridizations were carried out for 24
hours in a 5 x SSC and 0.2% SDS with 100μg/ml sonicated salmon sperm DNA at various temperatures, depending on the percentage of homology expected. Low stringency washing of blots was carried out in 5 x SSC, 0.2% SDS at 42°C and at various higher temperatures. Stringent washing was performed in 0.1 x SSC, 0.2% SDS at 60°C. Fuji X-ray film was exposed to the air-dried blots with single back intensifying screens for 3 days at -70°C.

2.12 Northern Blotting.

Northern blots were performed as described (Willoughby et al, 1992, Ninkina et al, 1994). Total RNA was fractionated on 1.2% agarose/formamide gels and blotted onto Hybond-N filters (Amersham). After blotting, the RNA was cross-linked to the membrane by exposure to 254 nM UV irradiation for 2-3 minutes followed by baking at 80°C for 1 hour. Baked filters were prehybridized for four hours in 50% formamide 5 x SSC, 5 x Denhardt’s solution, 100 mg/ml salmon sperm DNA, 50 µg/ml yeast RNA, 0.1% SDS at 42°C - hybridization with radioactive probe was for 40 hours in the same solution. Stringent washing was performed at 67°C in 0.2 x SSC, 0.2% SDS.

2.13 cDNA library information.

Three rat DRG libraries were used in the capsaicin receptor project, all of them directionally cloned into λZap II between the Xho I and Eco RI sites. The first of these was a generous gift from J.Boulter (Salk Institute), and was oligo dT primed and unamplified, with an average insert size of ~ 1Kb, containing 1 x 10⁷ clones. The other two were constructed by N.Ninkina (SIMR) and were primed with random hexamers and with oligo dT primers.

The human brainstem library (Stratagene, Cat. No. 935206) was oligo dT primed and inserted into the Eco RI site of Lambda Zap II. The library had an average insert size of 1 Kb.

The human substantia nigra library (Clontech, Cat. No. HL1093b) was produced was random-primer and oligo dT primed from material obtained from a normal adult, and inserted into the Eco RI site of λgt10. This library contained 1.8 x 10^6
independent clones with an average insert size of 1.4 Kb (from 0.6 to 4.0 Kb).

2.14 Screening of libraries with DNA probes.

' Lifts' were taken from the plates of transformed E.coli onto 82 mm diameter circular Hybond-N (Amersham) or BioTrace (Gellman) filters as described (Sambrook et al, 1989). Filters were baked at 80°C under vacuum for 2 hours and then washed extensively in 0.1 x SSC, 0.1 % (v/v) SDS at 65°C before prehybridization.

Prehybridization and hybridization was carried out at 55°C in 5 x SSC and 0.2% (v/v) SDS with 100mg/ml sonicated salmon sperm DNA. Prehybridization was for 2 hours and hybridization was for 24 hours. Low stringency washing of blots was carried out in 5 x SSC, 0.2% (v/v) SDS at 42°C and stringent washing in 0.1 x SSC, 0.2% (w/v) SDS at 60°C. Fuji X-ray film was exposed to the air-dried blots with intensifying screens for 3 days at -70°C.

Plasmid rescue.

This was performed on clones isolated from λZap II-based libraries according to the manufacturer's instructions (Stratagene).

Basically this involves superinfection of bacterial XL1 cells (infected with the purified phage) with a helper phage - VCSM13 (f1). A single stranded version of a part of the λZap II phage is then made circularized and secreted from the cells. These single stranded circles can be used to infect new cells and can replicate as a double stranded plasmid.

Rescue of plasmids from λZap II involves f1 (filamentous) bacteriophage-derived proteins sequences cloned into the λZap phage. The proteins from f1 phage recognise a DNA sequence present in λZap II which normally serves as the bacteriophage 'origin of replication' for positive strand synthesis. However, in λZap II this sequence has been engineered in in two separate parts, and so the sequence for initiation of replication and the site for termination of synthesis are separated by the central part of the lambda phage which includes the cloning region. By simultaneously infecting a bacterial cell with both the isolated λZap II clone and the f1 helper phage, these sequences are made
accessible to 'helper' proteins from the f1 phage. These cause a nick or break in one of the strands of phage DNA and allows the DNA downstream of it (i.e. 3' of it) to be duplicated by other f1 proteins as far as the termination sequence. Other f1 proteins recircularize the single stranded sequence.

This resulting single stranded circular DNA contains all of the plasmid Bluescript II sequences and the cloned insert. This molecule can now be 'packaged' as a phagemid and secreted by the E.coli cell. The cells in the mixture can be killed by briefly heating to 70°C. The 'packaged' Bluescript is then mixed with fresh XL1 cells and infects them. The resulting molecule contains an origin of replication and can replicate as a double-stranded plasmid. Selection with Ampicillin is used to isolate plasmid transfected cells.

The purified plaque of interest was removed as a core of agar from an agar plate and vortexed in a tube containing 500 µl of SM buffer and 20 µl of chloroform. The tube was then incubated at room temperature for 2 hours.

100 µl of this phage suspension was then added to:-
200 µl of XL1 cells (OD600 = 1.0)
1 µl of VCSM13 'helper phage' (>1 x 10⁶ pfu/ml), the mixture was then incubated at 37°C for 15 minutes.

To this, 3 ml of 2 x yt media was added, and then the tube was incubated shaking at 37°C for 2 hours.

The tube was then heated to 70°C for 20 minutes and then centrifuged at 400 x g for 15 minutes. The decanted supernatant fluid from the tube contained the single-stranded plasmid 'packaged' as a phage and was used as a stock solution.

To plate the rescued phagemid, two tubes, containing:-

200 µl of XL1 cells (OD600 = 1.0)
1 µl of phagemid stock solution

and
200 µl of XL1 cells (OD600 = 1.0)
50 µl of phagemid stock solution

were incubated at 37°C for 15 minutes.
200 µl from each tube was then plated on 15% agar LB plates containing ampicillin at 50 µg/ml and incubated at 37°C overnight.

2.15 Screening of libraries using photaffinity labelled RTX.

Briefly, an amplified rat DRG library constructed in λZap II was plated in BB4 cells and duplicate filter replicates from plates were made and incubated inverted on NZYCM plates. The filters were pre-soaked in IPTG to induce expression of insert cDNAs as β-galactosidase fusion proteins. Filters were then incubated with resiniferanol-9, 13, 14-orthophenylacetate-20-(-3-azido,4-methoxyphenyl) acetate (RTX-PAL), the photoaffinity label, (see Figures 4.2 and 4.4, pages 140 and 142, and James et al, 1993) and U.V. irradiated. This was followed by incubation with a rabbit polyclonal antiserum that had been raised to RTX photoaffinity proteins (4-aminoethyl resiniferatoxin conjugated to thyroglobulin with gluteraldehyde was used for immunization, see Harlow and Lane, 1990 for method) and then with an alkaline phosphatase-linked sheep anti-rabbit immunoglobulin antiserum (Miles) which was detected using nitroblue tetrazolium and bromochloroindolyl phosphate (Sigma - for detailed description of methods see Ninkina et al, 1994). Control filters were not irradiated, but were otherwise treated identically. For a further description and discussion of this method, see Ninkina et al, 1993 and also Chapter 4, section 4.3b, page 129, and section 4.4a, page 134.

Plasmid rescue was performed as above.

2.16 DNA Sequencing.

All sequencing was by the dideoxy chain termination method, performed using modified T7 DNA polymerase (Sequenase) with the sequencing kit from USB Corp., with either 32P dATP or 35S dATP.
PCR products intended for sequencing were either digested and then ligated into the cloning site of Bluescript II or cloned straight into the modified EcoR1 site of pGEM-T.

Plasmid purified by caesium chloride density centrifugation (Sambrook et al, 1989) or caesium chloride DNA minipreparation (Saunders et al, 1990) was produced for double stranded sequencing (Zang et al, 1988). Some additional oligonucleotides corresponding to internal sequences were synthesized to facilitate sequencing (see sequencing strategy).

Sequencing reactions were electrophoresed on denaturing 8% polyacrylamide gels in Tris borate buffer as per standard methods (Sambrook, et al 1989) for ~1650 volts, 90 mA for 2-7 hours. After running, one of the plates was prised off and 3MM chromatography paper was carefully placed against the gel. Gels were then peeled off the gel plate with the 3MM paper, fixed in 10% (v/v) methanol, 10% (v/v) acetic acid before being dried with a heated vacuum drier. Dried gels were used to expose X-ray film (Kodak, XAR-5) at -70°C without a screen for 14 (32P labelled dATP) or 36 hours (35S labelled dATP).

2.17 Sequence analysis.

Intelligenetics software was used throughout to manipulate new nucleotide sequences obtained. The 'Sequence', 'Site' and 'Match' functions were used for assembly of overlapping and contiguous stretches of sequence read from sequencing radioautographs. 'Fast database search' and 'Gene align' were used to compare new sequences with known sequences in the EMBL and Genbank nucleotide and protein sequence databases.

2.18 Standard polymerase chain reaction (PCR).

Optimal conditions for carrying out PCR reactions were explored in relation to the amount of template and primer as well as other parameters such as magnesium ion and dNTP concentrations. Standardised PCR reactions contained 50 pM of each primer, 1 mM each dNTP, up to 100 ng purified template DNA or single stranded cDNA from the equivalent of 50 ng of total RNA, 1 x PCR buffer (10 mM Tris.hydrochloride pH 8.3, 6 mM MgCl2, 50 mM NaCl) and 3 units of Taq DNA polymerase (Promega) in a volume of 50 μl. 100 μl reactions contained double
amounts of all components. 20 μl of liquid paraffin was added to each tube to prevent evaporation of the reaction mixture.

Temperature cycling was on a programmable heating block (Hybaid), using an external temperature probe built into a reaction tube identical to those used for the reactions. A typical programme would be - denaturation at 93°C for 20 seconds, annealing at 55°C for 30 seconds, and an extension period at 72°C for 40 seconds, repeated for 30 or 35 cycles. 4 units of Taq DNA polymerase were used for the 30 cycle PCR; for PCRs of 40-45 cycles, 3 units of enzyme were added at the start and an additional 3 were added after cycle 25. Typically 10 μl of the reaction was loaded onto a 1% agarose gel containing ethidium bromide and electrophoresed at 75 volts for 30 minutes before being photographed under UV light.

PCR amplification of larger fragments of DNA were essentially similar, but longer extension periods in the temperature cycle were programmed. This longer extension time was in proportion to the length of fragment being amplified assuming the speed of addition of dNTPs by Taq polymerase to be in the region of 24 bases per second at 55°C and above (Innis et al, 1988).

2.19 Screening of libraries using PCR.

The human substantia nigra library was screened using PCR to amplify sequences that would correspond to the 5' ends of clones isolated from the human brainstem library. 2 μl of the library (at 1 x 10⁶ clones/μl) was used per reaction, with one primer designed to match sequences close to the cloning site in λgt10, and one specific to the inverse complement of a sequence within the clone of interest. Two primers to λgt10 were used in different reactions to enable clones in either orientation to be detected, the orientation of inserts in the library being random. The two vector primers were called 5'gt0 and 3'gt10 and were 3' or 5' of the Eco RI site respectively (see Table 2.1, pages 56-58).

These screening PCRs were similar to the standard PCR mix, but contained 100 pM of the vector primer and 50 pM of the specific primer. Control PCRs between two primers internal to the clone contained these primers at 25pM each.
Material in SM buffer was quickly pelleted by centrifuging in a microcentrifuge tube for a minute, the pellet was resuspended in 50 μl distilled water, and the tube was incubated at 95°C for at least six minutes. The reaction mix was then added, and the reaction set up. Successful control PCRs with a single gene-specific primer and a vector primer indicated that the method was functional.

The temperature profile was as follows:
- 93°C for 30 seconds
- 60°C for 60 seconds
- 72°C for 60 seconds, for 32 cycles.

The only particular problem encountered in amplifying directly from phage was adequate denaturation of the template DNA. This was probably due to the presence of phage proteins. This problem was overcome by heating the template to 93°C for an extended period (7 minutes) prior to adding the template material to the reaction mix.

2.20 Comparative PCR.

See chapter 4 for discussion of methods and rationale.

Pairs of primers were designed from the known sequence of several rat neuronal nAChR subunits, the glutamate GluR5 subunit, glucose-6-phosphate dehydrogenase gene, ribosomal protein L27 and the preprotackykinin gene so that each product would amplify a unique sequence that crossing introns. Genes not mapped for introns had additional controls to show that no DNA was present.

The sequences of these specific primers were screened against all the other subunits and against all other sequences in the Genbank and EMBL databases (using Intelligenetics) to ensure that there were no similar sequences (including the other nAChR subunits) that they might prime from under the PCR reaction conditions.

PCRs were performed on single stranded cDNA made from randomly primed total RNA isolated from tissue or cultured cells, the equivalent of 50 ng of RNA was added to each PCR reaction. The first denaturation step was 93°C for an extended time of 6 minutes, to allow complete separation of the RNA-DNA duplexes. Annealing temperatures for the reaction cycles were 60°C lower
than the lowest theoretical annealing temperature of either of the primers of the pair used. Extension times were always 30 seconds at 72°C - the largest of the amplified fragments was less than 600 bases and these conditions are sufficient for synthesis of up to 1400 bases.

Reactions to be tested at different numbers of cycles were 100 µl volume, and 12 µl of the sample was removed from the tubes at the designated number of cycles while the reaction was running. 10 µl of reaction samples was loaded onto agarose gels of 1.8 - 30 % in 1 x TAE buffer (Trizma buffer, sodium acetate, EDTA, pH 8).

The initial PCR amplified fragment from the subunits was varied in size (see Table 2.2, page 59 and Figure 6.4, page 202) allowing the products to be initially identified when electrophoresed on a simple 1.2% agarose gel containing ethidium bromide.

2.21 Template preparation for RACE (Rapid amplification of cDNA ends).

The RACE template for this study was produced from RNA isolated from human adult pons and medulla tissue, and from human fetal whole brain and dorsal root ganglia. The adult material was dissected from a whole brain (stored at -70°C for several months), the fetal material was dissected on ice from fetuses which had been stored on ice for between two and eight hours. In both cases, dissected material was placed immediately into guanidinium denaturing solution and stored at -70°C until RNA extraction was carried out.

PCR was performed on single stranded cDNA preps as in the comparative PCR. However, following the first strand reaction, the cDNA was tailed with a single deoxy base. The first-strand reaction was phenol+chloroform extracted, and then precipitated with a standard ethanol precipitation with the addition of tRNA to 25 µg/µl as a carrier to facilitate the process. The resulting pellet was then redissolved in 50 µl of water or TAE buffer.

**Column purification of first-strand material.**
A Quiagen-5 column was equilibrated by adding 2 ml of Q0.4 buffer to it, and then 0.8 ml of Q0.8 containing 20 µg of tRNA followed by a further 2 mls of Q0.4.

The first-strand material in 50 µl of water or TAE was heated to 55°C for five minutes and then added immediately to the column.

50 µl of Q0.8 was then added to the column, followed by 400 µl of Q0.4. The effluent from these last three was retained and reapplied to the column three more times.

The column was then washed with 5 ml of Q0.8.

DNA was eluted with three separate washes of 400 µl each, of Q1.5. The second two fractions were retained and processed separately. Each was first precipitated with 0.85 volumes of isopropyl alcohol at -70°C for ten minutes and microcentrifuged in an MSE microcentaur at 4°C for ten minutes at 13000 rpm.

The pellets were washed by centrifugation in 70% ethanol, and then vacuum-dried before being resuspended in 20 µl of distilled water.

**Buffers used with Qiagen columns.**

As recommended for use with qiaegen columns, used in first strand cDNA synthesis.

**Q0.4**

0.5 M MOPS pH 7.0 1 ml
5M NaCl 0.8 ml
100% ethanol 1.56 ml
distilled water 1.64 ml

**Q0.8**

0.5 M MOPS pH 7.0 1ml
5M NaCl 1.6 ml
100% ethanol 1.56 ml
distilled water 5.84 ml

**Q0.4**

1 M MOPS pH 7.6 0.5 ml
5M NaCl 1.5 ml
100% ethanol 0.76 ml
distilled water 2.24 ml

**Tailing reactions.**

These contained the following:-
10 μl of column-purified first-strand cDNA.
4 μl of 5x terminal transferase buffer.
3 μl of cobalt chloride at 15mM.
1 μl of dGTP at 15mM.
2 μl of terminal transferase at 5 units/μl.

This was incubated at 37°C for fifteen minutes, then 40 μl of STE was added, and the suspension was extracted with phenol and chloroform, and then precipitated with sodium acetate and ethanol. The pellets were washed by centrifugation in 70% ethanol and vacuum-dried, before being resuspended in 100 μl of nuclease free water. This was then aliquotted, and 10 μl aliquots were stored at -70°C.

2.22 RACE PCR.

These reactions contained:

5 μl of tailed cDNA.
2 μl of dNTP at 25 mM each dNTP.
5 μl of 10 x reverse transcriptase buffer.
6.5 μl of pC primer.
6.5 μl of specific primer.
25 μl of sterile distilled water.

On top of this was layered about 30 μl of mineral oil.

This mix was then heated to 95°C for 5 minutes, cooled to the annealing temperature and held there while 2-3 units of Taq DNA polymerase was added (Promega) usually 0.5 μl.

A typical cycle would be: 93°C for 45 seconds (denaturation)
65°C for 1 minute (annealing)
72°C for 1.5 minutes (extension)

(The annealing temperature depended on the specific primer being used).

The number of cycles was 35 to 50.

2.23 Cloning of RACE products.

10 μl from the above reaction was loaded onto a 1.2% agarose gel along with φX174-Hae III fragment size-marker and the product sizes analysed after electrophoresis. The gel was then photographed under UV light, denatured with a sodium hydroxide and sodium chloride solution (see southern blotting
methods) and then blotted onto either Hybond-N or Hybond-N+ (Amersham) without neutralisation.

pC primer consists of a twelve base poly dC string on the 3' end of a Not I and an Eco RI site, plus a few other random bases at the 5' end to avoid having a restriction site at the extreme end of the strand (see Table 2.1, pages 56-58). Not I, being an 'infrequent cutter', was the enzyme of choice for one end of the RACE fragments, while the specific primers would either incorporate a restriction site in their 5' end, or would be designed to prime across a natural restriction site in the sequence at the other end. This latter offered any easy option for reconstructing complete coding regions from overlapping clones.

If there were any amplified fragments of interest, the remaining PCR reaction would be subjected to double restriction enzyme digest with an excess of enzyme for just one hour, or to two serial digests with a phenol and chloroform extraction and ethanol precipitation in between. The resulting solution was then electrophoresed on 1% LMP (low melting point) agarose (Gibco, BRL) and the band of interest was excised with a scalpel over a U.V. light source.

DNA was extracted from the agarose by adding three to four times the volume (of gel) of TE buffer and heating it to 55°C for five minutes and then extracting with phenol, phenol and chloroform, and then chloroform alone before ethanol precipitation with sodium acetate, often with the addition of tRNA.

Alternatively, 'Geneclean' (Bio 101) was used to purify the DNA from slices of LMP agarose. This was done strictly according to the manufacturer's instructions.

Restriction digestion of the PCR products left phosphorylated 3' ends, and so the fragments were then dephosphorylated before ligating them into vector DNA which had been digested with compatible restriction enzymes.

**Dephosphorylation of PCR product digests.**

Using CIP (calf intestinal phosphatase), a typical reaction would contain:-

- 20 mM Tris.Cl, pH 8.0.
- 1 mM MgCl₂
1 mM ZnCl₂
~20 pM of DNA termini.
0.1 unit of CIP.

After incubation for thirty minutes at 37°C, the reaction was stopped by heating to 75°C for ten minutes and then precipitating the DNA.

The CIP (Boehringer-Mannheim) was used with either the supplied buffer or with a custom made one as above.

2.24 Cloning of other PCR products.

PCR products from primers not designed to include restriction site sequences were cloned into pGEM-T (Promega). DNA from PCR reactions was cut out of LMP agarose gels after electrophoresis. 'Geneclean' (Bio 101) was used to purify the DNA from slices of LMP agarose, performed strictly according to the manufacturer's instructions.

The resulting fragments were then ready to be ligated into the vector pGEM-T (Promega), a vector specifically designed for the rapid cloning of PCR products which contains a single overhanging 3’ dT deoxyribonucleotide base which is compatible with the single overhanging 3’ dA base often produced by Taq DNA polymerase. The pGEM-T kit was used for this purpose, and manufacturer's instructions were followed. Ligation of unphosphorylated double stranded PCR product into the phosphorylated vector was performed for around six hours at 12°C.

This was followed by electroporation of bacterial cells, using 1 μl of the ligation mix, the equivalent of 0.5 ng of vector DNA.

2.25 Screening of RACE clones.

A quick method of screening colonies was devised for secondary screening of RACE clones which involved using a small amount of individual colonies as templates in a PCR. Standard PCR reaction mixes minus template DNA, Taq polymerase and paraffin oil were prepared using primers internal to the fragment being amplified and aliquoted into 0.75 μl eppendorf tubes. Sterile toothpicks were touched to individual colonies to be tested and then the tips were broken off into separate mixes as prepared above. The tubes were sealed and then placed
in a programmable heating block (Hybaid) and subjected to 95°C for 10 minutes. They were then centrifuged in a microcentaur at 1300 rpm for a minute, and 2 units of Taq polymerase was added, and paraffin oil was layered onto the reaction mixture which was then put in a programmable heating block for a standard 30 cycle PCR (see above). 10 µl portions of these reactions were then analysed by electrophoresis on a 1% agarose gel containing ethidium bromide).

Gels were Southern-blotted and probed with an appropriate probe to test for the presence of amplified sequences from the genes of interest.
Table 2.1  Oligonucleotides used.

Mixed bases are represented by IUPAC code letters. Underlined bases are restriction sites, bases in lower case have been engineered in for cloning purposes. Triplet grouping of bases is for ease of reading and does not relate to codons.

Primers used in cloning and sequencing of human genes.
(Primers made to the sequence of human nAChR β3 gene.)

<table>
<thead>
<tr>
<th>Oligo. size</th>
<th>Name</th>
<th>b.p.</th>
<th>Temperature (°C)</th>
<th>Sequence</th>
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</thead>
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<tr>
<td>NA2</td>
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<td>18</td>
<td>54</td>
<td>5'-TCT.TGT.ACT.ACC.TGG.CTG-3'</td>
</tr>
<tr>
<td>NA3</td>
<td></td>
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<td>5'-ACT.TTG.GAA.GAC.GAT.GGG-3'</td>
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<tr>
<td>NA4</td>
<td></td>
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<td>58</td>
<td>5'-GAA.GGC.TCC.CTG.ATG.ACC-3'</td>
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<tr>
<td>NA5</td>
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<td>62</td>
<td>5'-TAC.CCT.CTT.TCT.CAT.CAT.CCC-3'</td>
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<tr>
<td>NA6</td>
<td></td>
<td>19</td>
<td>58</td>
<td>5'-CCA.TCA.GAA.TCT.CTG.TGG.GC-3'</td>
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<tr>
<td>NA7</td>
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<td>5'-TGC.AGG.AGC.TTT.TGT.AGC.TGG-3'</td>
</tr>
<tr>
<td>NA8</td>
<td></td>
<td>29</td>
<td>68</td>
<td>5'-AA.Aga.re.CTA.TGT.CAG.GAA.GCCACA.GAG-3'</td>
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<tr>
<td>NA9</td>
<td></td>
<td>26</td>
<td>82</td>
<td>5'-TA.AAG.GCA.GGC.GTC.TCA.GGA.GGAAGG-3'</td>
</tr>
<tr>
<td>NA10</td>
<td></td>
<td>26</td>
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<td>5'-TG.ACC.CCT.ATC.AGC.AGG.GAG.CCT.TGC-3'</td>
</tr>
<tr>
<td>NA11</td>
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<tr>
<td>5pr1</td>
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(Primers made to the sequence of human nAChR β2 gene/β3 clone.)

<table>
<thead>
<tr>
<th>Name</th>
<th>b.p.</th>
<th>Temperature (°C)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB1</td>
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<td>5'-TGG.CTG.ACC.CAG.GAG.TGG.G-3'</td>
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<td>5'-TGA.AGA.AAG.TTC.GGC.TTC-3'</td>
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<tr>
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<td>72</td>
<td>5'-ATC.GTA.ACC.AGC.GTG.TGC.GTG.C-3'</td>
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<tr>
<td>NB4</td>
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<td></td>
</tr>
<tr>
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<td>5'-CAT.AAG.CTG.TAC.TGT.CAC.CAG.C-3'</td>
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<tr>
<td>NB23</td>
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<td>72</td>
<td>5'-TCT.TGC.ATG.CGC.TCT.TGT.AGA.TGG-3'</td>
</tr>
<tr>
<td>B2UN</td>
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<td>68</td>
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</table>

(Primers made to the sequence of human nAChR β4 gene.)

<table>
<thead>
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<th>Name</th>
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<th>Temperature (°C)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
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<td>76</td>
<td>5'-TCC. aag.cct.GCA.GCT.TGA.TGG.AGA.TGA.GCT.GTG-3'</td>
</tr>
<tr>
<td>P3</td>
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<td>5'-TCT.CGC.TCA.TTC.ACG.CTG.ATA.ACG.TGG-3'</td>
</tr>
<tr>
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<td>82</td>
<td>5'-CA.GGG.ATC.CTC.AGG.ATG.TTC.ACA.CCC-3'</td>
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<tr>
<td>P5</td>
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<td>76</td>
<td>5'-CAC.AGC.TCA.TCT.CCA.TCA.AGC.TGC3'</td>
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<tr>
<td>P6</td>
<td>23</td>
<td>76</td>
<td>5'-GCA.ACC.AGA.TGC.GCT.TTG.CAG.G-3'</td>
</tr>
</tbody>
</table>

Primers used for quantitative PCR.
(Primer to rat nAChR α3 and α5 subunit genes)
TMD3a 25 68 5'-CAC.YAT.GAT.YTT.YGT.SAC.CYT. VTC.C-3'
(Primer to rat nAChR a2 subunit genes)
TMD3b 23 60 5'-CTC.TTC.AYY.ATY.TTY.GTC. AC-3'
(Primer to rat nAChR a7 subunit genes)
TMD3d 23 68 5'-CY.AGC.ACC.ATG.gTY.ATY.GTT.GGC-3'
(Primer to rat nAChR β2,β3 and β4 subunit genes)
TMD4a 24 68 5'-RAA.SAB.CCA.CAG.GAA.SAK.GCG. GTC-3'
(Primer to rat nAChR a3 subunit genes)
TMD4b 26 64 5'-AR.Acc.ATG.GYR.AAG.ASS.GWA.AAB. GCC-3'
(Primer to rat nAChR a2 subunit genes)
X6A2 21 68 5'-ATG.GTC.CCC.GAA.AAC.GAG.ACG-3'
(Primer to rat nAChR a3 subunit genes)
X6A3 22 66 5'-CG.TTC.TAA.AAT.GCA.CAC.CAG.G-3'
(Primer to rat nAChR a4 subunit genes)
X6A4 21 68 5'-KMW.SMW.ASC.AGC.CAG.CCA.GGG-3'
(Primer to rat nAChR a5 subunit genes)
X6A5 25 72 5'-A.CAG.GAA.CAA.AAA.GCC.CTA.AAG. TCC-3'
(Primer to rat nAChR a2 subunit genes)
X6A7 21 68 5'-GCC.TCC.TAA.AAT.GCA.CAC.CAG.G-3'
(Primer to rat nAChR a4 subunit genes)
X5A4 24 74 5'-TCC.CAA.GAA.GCA.GCA.GAA.GAT. GGC-3'
(Primer to rat nAChR a5 subunit genes)
X5B2 21 70 5'-CC.TGT.GCT.CTT.CCT.GCA.GCA.GCC-3'
(Primer to rat nAChR a6 subunit genes)
X5B3 23 72 5'-TTG.CAT.GAA.GGA.CCC.CAT.GGACC-3'
(Primer to rat nAChR a5 subunit genes)
X5B4 25 70 5'-A.GCT.GCA.CTT.GGC.CAC.AGC.TGA. TAC-3'
(Primer to rat nAChR a2 subunit genes)
X5B5 25 70 5'-A.GCT.GCA.CTT.GGC.CAC.AGC.TGA. TAC-3'
(Primers to rat glutamate-gated ion channel GluR-5 subunit gene.)
G5-a 26 74 5'-TTT.CGG.TTG.CTG.AAT.ATT.GAC.AAC. CC-3'
G5-b 26 76 5'-GA.TAT.TGT.TGG.ACC.TGT.CTC.TGT. TGC3'
(Primers to gene encoding rat transcription factor Brn-3a.)
pBr3a 22 64 5'-C.GGA.TAA.GTG.CAG.ACC.TAT.TTG-3'
pBr3b 22 64 5'-C.AAA.ATA.GGT.CTG.CAC.TTA.TCC.G-3'
pBr3c 23 60 5'-TTG.GAT.TAT.TAG.TAT.GAG.ATA.CC-3'
(Primers to gene encoding RTX binding protein RTX13/42.)
p13a 22 60 5'-CAG.GTT.ACG.GAA.CCG.AAG.AAG.G-3'
p13b 22 60 5'-C.CTT.CTG.TCC.CTG.CTG.AAC.CTG-3'
p13c 21 60 5'-AAG.AAG.AAC.ACA.ACT.ATT.GGG-3'
p13f 21 60 5'-A.ACC.AAA.GCA.TAG.TTC.ACC.AC-3'
p13g 21 60 5'-AGA.TGT.GTC.ATC.ATC.TTC.CTG-3'
p13h 22 64 5'-C.AAA.ATA.GGT.CTG.CAC.TTA.TCC.G-3'
(Alternative primers to rat nAChR β4 subunit gene, spanning exons 4 an 5, used as a control in comparative PCR)
UB4X4 22 70 5'-GAA.TTG.ACT.GAC.TAC.CGC.CTG.G-3'
UB4X5 23 70 5'-GTC.AAT.CTC.CGT.GTG.GTC.ATA.GG-3'
(Primers to rat ribosomal L27 gene used as a control in comparative PCR)
Rib1  20  60  5'-ATC.GCT.CCT.CAA.ACT.TGA.CC-3'
Rib2  21  60  5'-AAA.GCC.GTC.ATC.GTA.AAG.AAC-3'

(Primers to rat glucose-6-phosphate dehydrogenase gene used as a control in comparative PCR)
G6PDH-a  20  60  5'-CAC.CTC.AAC.AGC.CAC.ATG.AA-3'
G6PDH-b  20  60  5'-GAG.GTG.GTT.CTG.CAT.GAC.AT-3'

(Primers to rat preprotachykinin gene used as a control in comparative PCR)
PPT1  24  72  5'-GGA.GAC.CCA.AGC.CTC.AGC.AGTC-3'
PPT2  23  70  5'-TGC.ATY.RCA.CTC.CTT.TCA.TAA.GCC-3'

Primers used for cloning and screening.
(Primers to Bluescript II)
M13  17  54  5'-GTT.TTC.CCA.GTC.AC-3'
RevM13  18  52  5'-G.AAA.CAG.CTA.TGA.CCA.TG-3'
T7L  22  64  5'-CG.TAA.TAC.GAC.TCA.CTA.TAG.GG-3'
BS3'  22  68  5'-CTA.GGA.ACT.AGT.GGA.TCC.CCC.G-3'
KS  17  56  5'-CGA.GGT.CGA.CGG.TAT.CG-3'
SK  19  56  5'-GCT.CTA.GAA.CTA.GTG.GAT-3'

(Primers to λgt10)
5'gt10  24  66  5'-A.GTA.TTT.CTT.CCA.GGG.TAA.AAA.GC-3'
3'gt10  24  66  5'-CAA.GTT.CAG.CCT.GGT.TAA.GTC.C-3'

(Primers for RACE and first-strand synthesis)
pC  27  -70  5'-T.AGC.GGC.CGC.GAA.TT(C)i 3' -3'
pT  30  -60  5'-ACT.AGT.CTC.GAG(T)i8 -3'

Thiol linked antisense primers used to ‘deplete’ transcripts in cells in culture.

Oligo. size  T_m  Sequence.
Name  b.p.  °C.
(Primer to complement of RTX42 sequence)
RTXT  18  56  5'-GAT.GGT.GCC.TTC.CAT.TCC-3'
(Primer to complement of neuronal Oct-2 sequence)
Oct-2  18  52  5'-CAT.GCT.GGA.ATG.AAC.CAT-3'
(Nonsense primer containing the same bases as the RTX42 primer, but in a scrambled sequence, used as a control)
102-Control  18  58  5'-AAG.TTC.TCC.GAC.GCG.CCT-3'
Table 2.2
Pairing of primers used in comparative PCR study, showing theoretical annealing temperatures (Tm), product size and restriction enzyme sites in product.

<table>
<thead>
<tr>
<th>gene</th>
<th>sense primer</th>
<th>Tm in °C</th>
<th>antisen. primer</th>
<th>Tm in °C</th>
<th>product size in b.p.</th>
<th>restrict. sites</th>
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</thead>
<tbody>
<tr>
<td>nAChR α2</td>
<td>TMD3b</td>
<td>60</td>
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<td>68</td>
<td>570</td>
<td>NcoI(4), Sac I</td>
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<tr>
<td></td>
<td>α3</td>
<td>68</td>
<td>X6A3</td>
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<td>NcoI, SacI</td>
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<td>α4</td>
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<tr>
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<td>pBr3c</td>
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<td>p13f</td>
<td>60</td>
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Figure 2.1
Sequencing strategy for human clones 12c, 12d and Jβ. Arrows indicate direction of sequencing runs, boxes indicate position of primers.
Figure 2.2
Sequencing strategy for clone 8a
(human nAChR β3 subunit). Arrows
indicate direction of sequencing runs.
Small arrows indicate position of
primers used for sequencing.
Boxes indicate position of
primers used for sequencing.

Transcribed DNA

5' to 3' poly d(A)

approx. scale in base-pairs
Figure 2.3
Sequencing strategy for clones RTX13 and Brn-3a(72) isolated from rat DRG cDNA libraries. Arrows indicate direction of sequencing runs, boxes indicate position of primers.
Figure 2.4

Schematic diagram showing the use of primers T7L, BS3', 5'gt10 and 3'gt10 paired with specific primers to amplify DNA sequences from cDNA libraries using PCR. Dotted lines indicate the amplified sequences. Screening with T7L and BS3' separately on 'Bluescript II' libraries and with 5'gt10 and 3'gt10 separately on λgt10 libraries ensures that clones present in only one orientation are not missed.
Figure 2.5.

Schematic diagram showing the principle of the RACE method.

(A) RNA corresponding to the partial gene sequence is obtained.

(B) Using random hexamer primers, a strand of DNA complementary to the RNA is synthesised using reverse transcriptase.

(C) A 'tail' of dGTP nucleotides is added to the 3' end of the cDNA using terminal transferase.

(D), (E) and (F) are all stages in the final PCR reaction.

(D) During the denaturation step the RNA and cDNA separate. The pC primer (consisting of a string of dCTP molecules with a restriction enzyme recognition sequence at the 3' end) anneals to the dGTP tail and is able to prime DNA synthesis from any cDNA in the reaction mix that possesses a dGTP 'tail'; thus non-specific single-stranded DNA rises linearly.

(E) The other primer in the PCR reaction is an oligonucleotide designed to match a sequence within the previously characterised region of the target gene. This primes synthesis only from single-stranded DNA sequences synthesised from primer pC that correspond to the target gene.

(F) Amplification between the specific primer and primer pC proceed giving near exponential amplification. n.b., as partial cDNAs synthesised from less than full-length RNA will be present in the process, RACE PCR normally produces products with a range of sizes.

From these, genuine products produced from the target gene must be identified (for instance, by probing a Southern blot of the RACE products using a probe corresponding to a part of the known sequence that will be amplified), and the largest products corresponding to the target gene can then be isolated.
Figure 2.5
Schematic diagram showing the basic principle of the RACE PCR method.
Chapter 3. Cloning human ligand-gated ion channel subunits.

Section 3.1 - Introduction - Ligand-Gated Ion Channels.

3.1 a Different classes of ligand-gated Ion channels.

The ligand-gated ion channel superfamily consists of a large group of proteins (subunits) with structural homology which can assemble into multimeric channel structures that are gated by a variety of ligands (reviews: Stroud, 1990, Nickel et al, 1990, Karlin, 1993, Unwin, 1993). The ligands define four main classes of ion channel gated by (1) Acetylcholine. (2) Glutamate. (3) Glycine. (4) GABA (gamma amino butyric acid). It should be noted that most of the ligands mentioned also interact with other receptor types which are not coupled directly to channels and which activate second messenger molecules inside the cell via G-coupled proteins. Ligand-gated ion channels (LGICs) are sometimes referred to as ionotropic receptors and the G-protein coupled receptors are referred to as metabotropic (Schoepp et al, 1990).

Glutamate and acetylcholine receptors which allow a flux of cations into the cell are referred to as ‘excitatory’ in reference to their ion selectivity. The GABA_A and Glycine channels which permit a flux of anionic chloride ions are conversely referred to as ‘inhibitory’ channels. However, the location of these various classes of channels on different cell types may give any of them specific inhibitory or excitatory roles in particular synaptic circuits.

The relationship between channel classes becomes more apparent when the protein sequences of individual subunits are compared. Several subunits from each of the above LGIC classes have been cloned so far, and it is likely that more remain to be discovered. Amino acid sequence comparison shows that there is usually more than 40% amino acid homology between many subunits of the same type of channel, for example between subunits of the neuronal nicotinic acetylcholine receptor (neuronal nAChR - Claudio, 1989), and around 20% or less residue homology between subunits of different channels (Unwin, 1993). Although showing a moderate level of homology within the group, the glutamate channel subunits are dissimilar in sequence to all the other channels. This suggests that the Glycine, ACh and GABA-gated channels, although permitting different fluxes, are all part of a related group, while the glutamate channels represent a separate group of LGIC.
While there is sequence diversity between the groups, the glutamate channels have structural features which are conserved throughout the LGIC superfamily, and so may resemble the other members in structure if not in sequence (see Fig. 3.1, page 98). The classes are further divided by distinctive pharmacology, thus making a number of related subclasses (Figs. 3.3a and 3.3b, page 102).

In addition to the four main agonist classes above, there are other channels that have similar functional characteristics to the LGIC superfamily, for instance, the capsaicin/proton activated channel (Bevan and Yeats, 1990, and Chapter 4), and the ATP-gated channel (Bean, 1990, Bean et al, 1990). In addition, a functional LGIC-like subunit has been cloned from a receptor activated by serotonin (5-hydroxytryptamine) known as the 5-HT$_3$ receptor (Maricq et al, 1991).

Whether the other above LGIC-like responses are produced by multimeric structures similar to the ligand-gated superfamily or arise from channels which are structurally dissimilar remains to be discovered.

### 3.1 b Structure of ligand-gated channels.

Ligand-gated channels are multimeric structures composed of proteins which span the cell membrane and define a pore which can be transiently opened in response to a chemical signal (reviews; Stroud et al, 1990, Unwin, 1993, Karlin, 1993). This pore then allows a direct flow of ions across the membrane along their electrochemical gradients, effecting immediate changes to the potential across the membrane. LGICs are integral receptor-channels, having both receptor and channel functions on the same structure. The opening of the channel therefore reflects an allosteric interaction in which the binding of a messenger molecule at a specific receptor site on the structure causes a conformational change which allows the opening of the channel pore at a discrete site (Galzi et al, 1991, reviews: Changeux, 1991, Lena and Changeux, 1993, Karlin, 1993).

LGICs are thus distinct from the large superfamily of G protein-coupled channels which open in response to altered levels of second messenger molecules or through direct interactions with G-proteins (reviews; Dohlm an et al, 1987,1991, Hille, 1992). They are also distinct from voltage-gated channels, which are mainly gated by changes in the membrane potential (review; Catterall, 1988).
Using *Torpedo* postsynaptic membranes which contain high densities of nAChRs crystalised in the form of flattened vesicles, electron microscopy has been used to obtain information about the subunit configuration of the nAChR at the neuromuscular junction, and three-dimensional image reconstruction has been possible (Toyoshima & Unwin, 1988). This reveals the channel to be a pseudo-symmetrical pentamer of subunits which cross the cell membrane and are arranged around a central pore. Use of hybrid density maps using X-ray diffraction data taken from different planes of latticed nAChR (Mitra et al 1989), has shown that the receptor has a relatively large tubular extracellular domain which extends approximately 60Å into the synapse and has an opening which is about 25Å wide. At about the level of the cell membrane, this opening is constricted to a small pore, and then there is a stretch of intracellular domain of about 20Å in diameter which is again about 20Å long.

Structure-function studies on LGICs have centred on the nicotinic acetylcholine receptor found at the neuromuscular junction as the prototype LGIC. This was first isolated from the electric organ of the marine ray *Torpedo californica* and related species. Because of the conservation of structural features, the results from work on the structure and mechanism of the neuromuscular nAChR have often been extrapolated and assumed to be true for other members of the family. Structural studies on the neuronal nAChR and glutamate channels are in progress, although the neuromuscular nAChR is still used for comparison (Claudio, 1989).

### 3.1 c Conserved structural features.

The neuromuscular nAChR channel is composed of four types of subunits, α, β, δ and either γ or ε with two non-adjacent α subunits separated by the γ subunit (Karlin et al, 1987). The conserved features of LGICs are often mapped against the corresponding numbered residues on the *T.californica* nAChR α and β subunits (Claudio, 1989, and see Fig. 3.1, page 100 and Fig. 3.2, page 101). The most striking of these is the four transmembrane domains (TMD1,2,3,4) which are hydrophobic stretches of residues which span the membrane. An amphipathic helix was at first thought to be a fifth TMD, but it is likely that this helix, not present in all channels,
lies along the inside side of the membrane and does not cross it (MA - Fig. 3.2, page 101).

This transmembrane topology contrasts with the common putative topology of G protein-coupled receptors which have a single receptor protein with seven TMDs (review; Dohlman et al, 1991, Hille, 1992), and with the putative topology and stoichiometry of voltage-gated channels which are either monomers with twenty four putative TMDs or tetramers with six putative TMDs to each subunit (review; Betz, 1990a, Catterall, 1991). Each receptor superfamily therefore has characteristic structural design (Strange, 1988, Betz, 1990a).

Experiments to try to map various regions of the subunits to the intra-, or extracellular side of the membrane have produced conflicting results, but the consensus locates both the amino- and carboxy-terminals of the protein extracellularly and seems to support the four TMD scheme. The presence of a signal peptide on all subunits supports the belief that the amino terminal is extracellular, and fusion protein studies have confirmed the location of the membrane-spanning regions (Chavez and Hall, 1992).

In work on the neuromuscular nAChR, which is assembled from four discrete types of subunit, domains involved in subunit interactions involved in assembly have been mapped to the extracellular amino-terminals of subunits (Yu and Hall, 1991, Verrall and Hall, 1992).

There is a high degree of conservation between the membrane spanning domains of the members of the LGIC superfamily, especially in the region of TMD1 and 3, which are thought to flank TMD2, with TMD4 on the outside of the subunit. Whether these transmembrane spanning regions are all α-helices remains to be demonstrated. In a recent alternative model, TMD2 is the only membrane-spanning α-helix, while TMD1,3 and 4 are membrane-spanning β-pleated sheets which surround it in the lipid bilayer (Sansom, 1993, Unwin, 1993, Gorne-Tscheinokow et al, 1994).

Mutation studies have indicated that TMD2 is the part of the subunit molecule that faces inwards towards the pore. Small uncharged residues which appear at regular four residue intervals along this region are thought to line the pore, and a conserved hydrophobic leucine residue is thought to mark the position of the ‘gate’ that regulates the pore. This leucine is present at the most constricted part of the pore throughout the AChR family.
It is also present in some glutamate receptor subunits, but others have a larger hydrophobic amino acid, phenylalanine. In the glutamate receptors, a residue position close to this phenylalanine seems to regulate the permeability to divalent cations (Burnashev et al, 1992).

Groups of charged residues at the ends of TMD2 are thought to play a part in ion selectivity, and again there are highly conserved residues at similar positions between subunits: for instance conserved asparagine, glutamate and lysine residues in the channels permeant to cations and arginine residues in the anion permeant channels (review; Changeux et al, 1992, Karlin, 1993).

Other features of the subunits include a conserved pair of cysteine residues that are thirteen residues apart known as the ligand-gated channel motif, and an N-linked glycosylation site within this which is present in all neuromuscular nAChR subunits and all GABA receptor β-subunits (review: Stroud et al, 1990, see Figs. 3.1 and 3.2, pages 100 & 101). Other glycosylation and phosphorylation sites vary between subunits.

A pair of adjacent cysteine residues found at positions 192 and 193 in the *Torpedo* α-subunit and in all neuromuscular nAChR α-subunits is involved in agonist binding, and is used to define α- and β-type neuronal nAChR subunits (see below).

There are several proline residues at conserved positions in LGIC subunits, including one residue present in all subunits cloned so far which lies between transmembrane domains two and three. These residues have been suggested to play a role in the folding of the subunits, and there is evidence that such folding may play an important part in the assembly of homo-oligomeric nAChR and 5HT₃ channels (Helekar et al, 1994).

**3.1 d Function of ligand-gated channels.**

The opening of LGICs is typically transient and lasts for only milliseconds, during which time millions of charged ions may pass. For instance, the open times for neuronal nAChR channels can range from values of around a tenth of a millisecond (0.1 ms) up to values nearer to 10 ms. The conductance of the channels when open also has a considerable range, from a single picosiemens (1 pS) up to values of 50 pS and above (review; Hille, 1984, Sargent, 1993).
Siemens (S) are a measure of conductance, and are defined by Ohm's law in simple conductors as:

\[ I = g E \]

Where current (I) equals the product of conductance (g) and (E) the voltage difference across the conductor (Hille, 1984).

The channels are usually only moderately selective, and are permeant to either monovalent anions, monovalent cations, or monovalent and divalent cations, as opposed to the voltage gated channels which tend to be selective for one type of ion. Several cationic channels are permeant to divalent ions including calcium (Ca\(^{2+}\)), among them are subtypes of neuronal nAChR, and subtypes of glutamate receptor, especially those sensitive to the agonist N-methyl-D-aspartate (NMDA).

3.1e Subunits and receptors.

Despite the fact that many component subunits of LGICs have been cloned, in most cases it is still unclear which of these subunits assemble to form channels in vivo, and how this process occurs.

The neuromuscular nAChR (found at nerve-muscle junctions) consists of four distinct but homologous subunits designated \( \alpha, \beta, \delta \) and either \( \gamma \) or \( \varepsilon \), arranged in a pentamer as described above, in the stoichiometry \( \alpha_2, \beta, \delta, \gamma \). Because ligand binding is associated with the \( \alpha \)-subunits, two ligand binding sites exist, at the side of the two \( \alpha \)-subunits which are not adjacent. The two binding sites differ in binding characteristics because binding is affected by the subunits adjacent to the \( \alpha \) subunits (Blount and Merlie, 1989).

Several subtypes of neuronal nAChRs have been described. These are found on neurons of the PNS and CNS, and consist of similar but distinct subunits, of which about ten have been discovered to date (review; Role, 1992). The subunits have been subdivided on the basis of the presence or absence of the adjacent cysteine residues described above (\( \alpha \)-type contain the paired cysteines, \( \beta \)-type do not), because they are not similar enough to the various neuromuscular subunits to be classified as homologous to \( \beta, \delta, \gamma \) or \( \varepsilon \) subunits. Indeed, it is not yet clear if there are neuronal homologues to all the different neuromuscular subunits, or how many different types of subunit assemble to produce neuronal nAChRs.
This problem of subunit function has been addressed by the use of expression systems, most notably the use of oocytes from the African clawed toad, *Xenopus laevis* (Sigel, 1990, Bertrand et al, 1991). Injection of mRNA, either extracted from neurons or transcribed from cloned channel subunit genes, can result in the encoded proteins being synthesised and inserted into the oocyte membrane as complexes which may function as channels in response to agonist (Boulter et al, 1987, Wada et al, 1988, Deneris et al, 1989, Couturier et al, 1990).

By examining the subunit combinations that result in functional channels, a start has been made in determining the role of the cloned subunits. For neuronal nAChRs for instance, a first working rule was that at least one type of α-subunit and one type of β-subunit was required to make a functional receptor, with the exception of the α7 subunit which is able to form a homo-oligomer (Couturier et al, 1990). An elegant study using mutated channel subunits with subtly altered properties has demonstrated that a channel constructed by combining the products of the neuronal α4 and β2 subunit genes is a pentamer consisting of two α4 and three β2 subunits (Cooper et al, 1991).

In further oocyte expression experiments, it has been shown that some of the neuronal α subunits can substitute for the neuromuscular α subunit and form functional channels if co-injected with the other subunits of the neuromuscular receptor. Similarly some of the neuronal β subunits can substitute for the neuromuscular β subunit.

Of the neuronal subunits functionally tested in oocytes, the α5, α6 and β3 subunits (Wada et al, 1990, Deneris et al, 1989) have yet to be demonstrated to function in a pairwise fashion with any of the other subunits, although the α5 does assemble together with two other subunits types (α3 and β4) in chick ciliary ganglion neurons (Conroy et al, 1992).

This association of three subunit types emphasises that it is still not clear exactly how many of the subunits are assembled into native receptors. It is common for neurons to express four or more neuronal nAChR subunits (for e.g., chick ciliary ganglion neurons, Corriveau and Berg, 1993; chick superior cervical ganglion neurons, Listerud et al, 1991, rat dorsal root ganglion neurons, see chapter 6).
It must still be considered a possibility that the function of pairs of the various LGIC subunits in oocytes could be fortuitous, or that lack of function in some instances may be due to the lack of modifications or the lack of accessory proteins required for assembly rather than a genuine lack of functional compatibility.

A similar problem exists with the other members of the LGIC superfamily. The subunits of other LGICs have been studied in a similar way by expression in oocyte systems, and functional homooligomers and other functional subunit combinations have been described, but once again, the relation of these combinations to in vivo channels is mostly not known (Sommer and Seeburg, 1992, Betz, 1990b, 1991, Seigel et al, 1990).

The glutamate receptor family of LGICs is the most abundantly expressed receptor family in the CNS and PNS (reviews: Betz, 1991, Gasic and Hollman, 1992, Sommer and Seeburg, 1992). The channels can be divided on the basis of whether they can be gated by \textit{n}-methyl-\textit{d}-aspartate (as NMDA or non-NDMA receptors).

Following the cloning of the first NMDA receptor subunit, NMDA-R1 or NR-1 (Moriyoshi et al, 1991), three further subunits have been described, NR-2A, NR-2B and NR-2C (Monyer et al, 1992). These share between 55 and 70\% protein similarity between each other but show only about 20\% homology to NR-1.

Although NR-1 is the only subunit which is able to form functional homomeric channels when expressed in Xenopus oocytes, the heteromeric channels formed by expressing NR-1 with any one of the three other subunits produces channels with amplitudes which are around 100 times larger. It seems likely that heteromeric channels exist in vivo (Monyer et al, 1992).

The non-NMDA receptors can be further divided, a distinct class of these has a high affinity for kainate and are known as kainate receptors, while the remainder are more sensitive to \textit{\alpha}-amino-3-hydroxy-5-methyl-isoxole-4-propionate and are known as AMPA receptors (Gasic and Hollman, 1992, Sommer and Seeburg, 1992). Functional expression screening was used to clone the first glutamate channel subunit, GluR1, which formed an AMPA receptor (Hollman et al, 1989). Homology screening has revealed several other related subunits, known as GluR1-GluR7 (Bettler et al, 1990) or GluR-A etc (Keinanen et al, 1990). These subunits can
function in different combinations to produce channels with differing properties (Boulter et al, 1990, Nakanishi et al, 1990).

The discovery that two alternatively spliced versions of an exon, termed 'flip' and 'flop', existed for several of these subunits increased the diversity of these channels (Sommer et al, 1990). In addition to this, it appears that cell-specific RNA editing may result in the conversion of a single amino acid in the crucial portion of the pore-lining TMD2 of some AMPA receptor subunits, thus affecting ion selection (Sommer et al, 1991).

Subunits corresponding to the high-affinity kainate-binding non-NMDA receptors have been identified as distinctly smaller proteins than the AMPA receptor subunits after peptide purification from receptors. Some of these have also been cloned (Egebjerg et al, 1991, Herb et al 1992), and while some can form functional homomeric channels in oocytes, others surprisingly produce receptors in oocytes only in combination with members of the low affinity kainate-binding class of subunits (Herb et al 1992).

Several component subunits of the ligand-gated chloride ion channels have so far been described, and biochemical analysis of the proteins purified from glycine receptors from different parts of the nervous system reveal only two or sometimes three species of polypeptide subunit (Betz, 1990b). Two homologous polypeptides were originally identified in purified glycine receptors from mammalian spinal cord, and photoaffinity labelling studies have mapped the ligand binding site to one of them, the 48 kDa α1 subunit (Graham et al, 1983). Since then, however, other variants of the α subunit have been cloned, and it has been demonstrated that alternative splicing of the mRNAs from these genes adds to the diversity of glycine receptors (Malosio et al, 1991, review; Betz, 1991).

The GABA_A channel subunits are similarly designated with greek letters on the basis of their relative homology to the prototypical (α1) subunit (Sigel et al, 1990, Burt and Kamatchi, 1991, Wisden and Seeberg, 1992). Several 'types' of subunit have been described, but the physiological significance of some of them is unclear. Expression in Xenopus oocytes reveals that homomeric channels can be produced with some subunits, although the efficiency of assembly is poor. Some pairs or more complex combinations of subunits can efficiently combine to form functional receptors in oocytes, and the presence of some subunits produces particular pharmacologies (Sigel et al, 1990).
The number of GABA\textsubscript{A} channel subtypes, based on pharmacological differences suggests that several different natural subunit combinations may exist (Sieghart, 1992).

Section 3.2
Cloning of a Novel Ligand-gated Channel Subunit.

3.2 a Initial Aims.

The initial aim of this project was to look for novel ligand-gated channel subunits expressed in sensory neurons. This was attempted by homology hybridisation screening of a cDNA library using LGIC cDNA clones. The high degree of nucleotide homology between the clustered transmembrane regions of subunits of the various classes of ligand-gated ion channel suggested that homology screening might allow isolation of novel subunits of the LGIC family. This was of particular interest, because there are receptor/channels expressed in primary sensory neurons which appear to be involved in their activation which have yet to be cloned and characterised, among them the capsaicin receptor and the proton-gated channel (see Chapter 1, section 1.3 d and 1.3 e, page 23).

When this work began, no DRG cDNA library was available. Of several commercial libraries available, a human brainstem cDNA library was chosen as the best source of primary sensory neuron material, as it would contain material from cranial sensory ganglia (see discussion).

The LGIC probes selected were rat cDNA clones, but because of the high degree of conservation between the human and rodent homologues of these genes (for e.g, see Fornasari et al,1990), heterologous probing (across species) was not likely to significantly reduce the chances of success.

The cDNA probes used were three neuronal nAChR genes, and a glutamate channel subunit. These clones represented the two LGIC families - nAChRs (including ACh-, GABA- and Glycine-gated receptors) and also Glutamate-gated LGICs. Of the nAChR genes used as probes, two were \(\beta\)-subunits, and one was the \(\alpha5\) subunit. The last was deliberately chosen because at the time it had yet to be demonstrated to contribute to functional nAChR channels, and thus it seemed possible that it might be a component of a separate class of
channel, possibly gated by a neurotransmitter other than acetylcholine.

The cDNA inserts from four cloned LGIC subunits were $^{32}$P-labelled by random-primed labelling (see methods, section 2.10, page 41), pooled, and used to screen a human brainstem library (Stratagene, Cat. No. 935206, average insert size 1.0 Kb, Oligo dT primed) at low stringency (hybridised at 57°C, washed in 2x SSC, 0.1% SDS at 57°C, Willoughby et al, 1993). The subunit probes were the rat nAChR α5, β2 and β4 subunits, (the generous gift of J. Boulter) and the rat glutamate receptor subunit, GluR-A (the generous gift of P. Seeburg). Standard molecular biology methods were used throughout (Sambrook, et al 1991, Willoughby et al, 1993, see also methods, section 2.14, page 44).

### 3.2 b Results of Library Screening.

From the first screening of approximately 1.3 x 10⁸ clones from the library, twelve corresponding positive signals were seen on the duplicate radioautographs. Agar discs from the position of the positive signals were removed from the plate with the blunt end of sterile pasteur pipettes and phage suspensions were made from this material which was then plated at a lower density of colonies and rescreened using the same probes.

Three out of the twelve positive signals were visible in this second screen on the duplicate radioautographs, these signals clearly corresponded to plaques when the radioautographs were matched with the bacterial plates. The plaques were picked with a toothpick and grown and purified by replating at lower density, reprobing and picking isolated single plaques. Plasmid rescue was performed so that the phage vector containing the three clones was converted into the plasmid Bluescript II for ease of manipulation (see methods, section 2.14, page 44).

Restriction digests using the enzyme Eco R1 were performed on each of the plasmid clones, and these were then electrophoresed on a 0.8% agarose gel adjacent to λ DNA digested with Hind III as size markers (see methods, section 2.11 page 42). The inserts from the plasmid clones were 0.5, 1.2 and 1.4 Kb, and were named 12c, 12d and 8a respectively.

The inserts to clones 8a and 12d were labelled and used to screen a fresh portion of the library using stringent conditions, to see
if longer versions of these clones were present. A single positive signal was identified from $1.3 \times 10^8$ clones screened. This was purified to an individual plaque, as above, isolated and rescued to plasmid form and found to contain a 1.7 Kb insert which was named j3.

3.2c Analysis of clones isolated.

There was a possibility that some of the clones isolated from the library screening could correspond to identical sequences, and that some of the clones could also prove to correspond to human nAChR subunit genes that had previously been identified. The analyses below were therefore performed in order to identify identical clones and to predict what cDNAs in the probe pool the clones most resembled at an early stage in order to minimise the amount of sequence analysis that would be necessary to identify already characterised or duplicated clones.

In order to determine which of the clones isolated during the library screening were similar, the labelled inserts from the newly isolated clones were used separately to probe southern blots containing the same three new clones using stringent conditions. The stringent conditions used meant that only clones representing identical or very closely related genes would hybridise to the clones present on the blot.

In order to investigate which of the pooled probe cDNAs the three newly isolated clones most resembled, labelled inserts from the new clones were also used separately to probe Southern blots containing the rat cDNA probes used to screen the library. This hybridisation was performed at lower stringency (see methods, section 2.11 page 42) as the hybridisation was performed between clones from a human library and rat cDNAs.

The cross-hybridisation of human clones revealed that clones j3, 12c and 12d were either identical or closely related to each other, and that clone 8a was distinct (see Fig. 3.4, page 103).

On the Southern blots containing rat cDNAs, human clone 12d hybridised strongly to the rat β2 and δ4 nAChR subunits and to a lesser degree to the α5 nAChR subunit (see Fig. 3.3b, page 102). Human clone 8a hybridised strongly to the α5 nAChR, and to a lesser degree to the β2 or δ4 nAChR subunits. Neither clone appeared to hybridise to the glutamate receptor subunit, GluR-A (see Fig. 3.3a
and b, page 102). This lead to the working assumption that jβ was a β-like nAChR subunit and 8a was an α-like nAChR subunit.

Sequence analysis started with oligonucleotides to the vector Bluescript II (standard sequencing primers, KS and SK, see Table 2.1) being used to obtain sequence information from the ends of the clones. Sequencing was performed on caesium chloride-purified double-stranded plasmid DNA (Zang et al, 1990) by the Sanger dideoxy chain termination method (Sanger et al, 1977) using USB sequencing kits and modified T7 polymerase 'Sequenase'™ (see methods, section 2.16 page 46). New primers were then synthesised to unique sequences within the clones as sequencing progressed (see Table 2.1, pages 56-58, and Figures 2.1, 2.2 and 2.3, pages 59-61, for primer sequences and for sequencing strategies). The whole of clone 8a and several hundred base-pairs from each end of clone 12d and clone jβ was sequenced (see Fig 3.5, pages 104-105, and Fig. 2.1 and 2.2, pages 59-60).

A comparison of these sequences with the sequences available in the Genbank/EMBL databases using Intelligenetics software (see methods, section 2.17 page 47) showed that human clone 8a had strong homology to the rat β3 nAChR subunit, and that it was probably the human homologue of that subunit (see Fig 3.5, pages 104-105, Willoughby et al, 1993 and discussion). Clone jβ showed 100% homology with the published sequence of the human nAChR β2 subunit (Anand and Lindstrom, 1990) at the 3' end of the coding region but a region of low homology to the human β2 subunit was found at the 5' end (Fig 3.4, page 103). This seemed to raise the possibility that it might be a splice variant of the β2 subunit.

Apart from the distinct 5' stretch, the regions of clone jβ that were sequenced had perfect homology to the published sequence of the human β2 subunit (Anand and Lindstrom, 1990), namely about four hundred base-pairs at the 3' end of the gene, including the untranslated region preceding the poly dA tail, and five hundred base-pairs near the 5' end of the gene.

Sequence comparison of both clones with database sequence also revealed that both were short of the full length protein coding region. The unusual end of jβ was in a position where it would be around the start of the signal peptide sequence on the β2 gene, probably around a hundred base pairs downstream of the coding region, and thus lacking about a hundred base pairs of the coding
region according to the previously published sequence (Anand and Lindstrom, 1990). The β3 gene terminated in a similar position, around ten bases short of the signal peptide (see Fig.3.5, pages 104-105), and was thus lacking about one hundred and twenty nucleotides of the protein encoding sequence by comparison to the rat homologue (Deneris et al, 1989).

3.2 d Expression of human nAChR subunits.

Portions of total RNA from various sources were electrophoresed on a formamide gel and (Northern-) blotted onto Hybond membranes using standard techniques for Northern blot analysis (Sambrook et al 1991, Willoughby et al, 1993, and methods, section 2.12 page 43).

The RNA was extracted using standard methods (see methods, section 2.7 page 39) from human adult pons and medulla, foetal brain and DRG, and, in addition from the human neuroblastoma cell line IMR32, shown to contain nAChRs (Lukas, 1993).

While the analysis of the clones isolated from the brainstem library was in its early stages, the complete inserts from both clones 8a (β3 - 1.4 Kb) and jβ (β2-like - 1.7 Kb) were labelled to high specific activity with 32P dCTP by random primer labelling (see methods, section 2.10 page 41, and Willoughby et al, 1993) and used to probe two Northern blots of total RNA from human foetal brain and foetal DRG as described above (containing about 40 μg per lane). These two probes were also used to probe adult pons and medulla and IMR32 total RNA (40 μg of each) on Northern blots. Similar blots of adult RNA were later probed with PCR amplified sequences. These were the 600 b.p. produced by primers NA10 and NA9 for β3, and the 250 b.p. fragment from NB22 and NB23, produced using jβ as a template for β2 (see Fig. 3.6, page 106). Probing (in formamide hybridisation mix) and washing was carried out under stringent conditions (Sambrook et al 1991, and methods, section 2.12 page 43).

Results of Northern analysis.

No hybridisation signal was observed on any of the foetal material with the 1.4 Kb 8a probe, but a signal from foetal human brain and a very weak signal from foetal DRG (both of about 3 Kb) was produced on a similar blot by probing with the 1.7 Kb jβ probe (Fig.
3.7, page 107). The brain signal was blurred, suggesting that it may have arisen from more than one transcript (see discussion).

The same jβ probe hybridised well to adult medulla, pons, and and IMR32 total RNA Northern blotted as above and probed with subunit-specific sequences from clone 8a and jβ which had been labelled with $^{32}$P dCTP, also as above, but again, an indistinct signal was seen, as though multiple transcripts were present (see Fig 3.7, page 107). Probing using the PCR amplified smaller probes produced single hybridisation bands. With the 300 b.p. 8a probe, a faint transcript of 1.7 Kb was visible with pons but not IMR32 RNA (see Fig. 3.7, page 107, and Willoughby et al, 1993). When reprobed with the β2 probe, a barely visible positive signal was seen with pons and with IMR32. With the 250 b.p. jβ probe, a transcript of 3.0 Kb was visible with pons and IMR32 RNA (see Fig. 3.7, page 107).

3.2 e PCR analysis of human brainstem and substantia nigra libraries.

A partial sequence to the human nAChR β4 subunit was obtained (Prof. F. Clementi, Dr. P. Tarroni, personal communication) enabling primers to the β4 sequence to be synthesised. The search for the 5' ends of the human genes was extended to include the β4 subunit (see Fig. 3.6, page 106).

In order to investigate quickly and easily the possible presence of clones covering the missing nucleotide sequences in the brainstem library, a polymerase chain reaction (PCR) screening was performed using primers at the 5' end of the clones (see Fig 3.6, page 106, and Fig. 2.4, page 63) paired individually with one or other of two PCR primers complementary to sequences within Bluescript II and flanking the Eco R1 cloning site. These primers, T7L and BS3', were situated about fifty b.p. away from the EcoR1 restriction enzyme site into which the cDNAs had been inserted (see Fig. 2.4, page 63).

An additional cDNA library was purchased from Stratagene, made from human substantia nigra (Cat. No. HL1093a containing 1.8 x 10⁶ individual clones, average insert size 1.4 Kb, size range from 0.6 to 4 Kb, primed using both oligo dT and random hexamer, inserted non-directionally into the Eco R1 site of λgt10 vector). Two primers for PCR-screening of the clones contained in this vector were also synthesised, named 5'gt10 and 3'gt10. These were complementary to sequences within λgt10, and 30 b.p. in from either
side of the EcoR1 restriction enzyme site into which the cDNAs had been inserted (see Methods, Figure 2.4 page 63).

The two sets of vector primers (T7L and BS3' for bluescript, and 5'gt10 and 3'gt10 for λgt10) enabled PCR to be used to isolate clones from the libraries that extended further in the 5' direction to those already isolated (see Methods, Figure 2.4 page 63). Amplification of sequences that existed between a gene-specific primer complementary to a sequence near the 5' end of each of the clones and a primer complementary to a sequence in the cloning vector that would be present in every clone in the library would be a more rapid and convenient screening method, and would only identify sequences corresponding to the end of the cDNA that was incomplete.

The use of the non-specific (vector) primers would mean that DNA synthesis would be primed from every clone in the library. This would result in a quantity of amplified unwanted sequences, but exponential amplification would only take place when sequences complementary to the gene-specific primers were present in an appropriate position, near to the common vector primer sequence. This unwanted amplification is insignificant because only one strand of each clone is synthesised, therefore the amount of this unwanted sequence grows linearly rather than exponentially.

Exponential amplification of the target sequences only occurs when two primers are in close proximity and are in the appropriate orientation (i.e., priming synthesis towards each other). This exponential amplification means that the amplified sequence becomes the dominant species in the reaction and other amplification becomes irrelevant. It is, however, not uncommon for gene-specific primers to cross-anneal to a small number of sequences other than those targeted, producing unwanted products.

The specificity of this reaction would therefore depend on the gene-specific primer having little or no cross-hybridisation with other sequences in the library. All primers for this study were designed to have high annealing temperatures (at least 65°C), and all PCRs were carried out with high annealing temperatures in an attempt to minimise the risk of such cross-annealing. Primers NA10 (β3 gene), P6 (β4), NB21 (β2) and NB20 (5' unique part of β2-like clone jβ) were designed for this library screening (see Fig. 3.6, page 106).

The primers specific for β3 and β4 subunits were complementary to the coding sequence of the two genes and designed
so that they would amplify the 5' ends of clones within the library, including 2-300 b.p. of known sequence and any sequence that lay 5' of this, bounded at the other end by about 30-50 b.p. of the vector sequence.

The position of these primers meant that a product of suitable size for PCR amplification (~400-500 b.p.) would be produced from a full length clone present in the library. This would also allow a few hundred base pairs of overlapping sequence, so that amplified products could be probed with identified DNA fragments containing the overlapping sequence to identify products corresponding to the genes as opposed to accidentally amplified sequences. In the case of β3, the small amount of unique sequence at the end of the clone was so small (60 b.p.) that only oligonucleotide size overlaps were possible. Primer NB22 was used for this function as a probe (see Fig 3.6, page 106).

Agarose gels containing the products of the brainstem and substantia nigra PCR screening were (Southern-) blotted and hybridised with β3, β3 and β4 probes matching overlapping sequences as described above to identify sequences genuinely corresponding to the genes targeted, and also to visualise larger and less abundant amplified DNA fragments present on the gel. The probes used were the oligonucleotide NB22 (β3) end-labelled with $^{32}$P γATP, and the PCR products of primers B2UN with NB23 (β2), NA11 with NA10 (β3) and P6 with P5 (β4) (see Fig 3.6, page 106), random-primer labelled as above.

The probe for β3 was amplified using the above primers in a PCR amplification of clone 8a, which produced a single product of the expected size. The probe for β2 was generated by using the primers B2UN and NB23 in PCR reactions performed on a clone of the 5' end of the human β2 subunit gene (clone HUMM1, the generous gift of J. Lindstrom). This reaction amplified a 560 b.p. sequence. The probe for β4 was generated by using the above gene-specific primers in PCR reactions performed on an aliquot of a human genomic library in EMBL3 (Clontech, Cat. No. HL1067J, 2.5 x 10⁶ individual clones, average insert size 15 Kb, size range from 8 to 21 Kb). The reactions with the above primers amplified products of the correct size, but the β2 and β4 products were also partially sequenced in order to positively identify them. To do this, the amplified fragments were electrophoresed in low melting temperature agarose, excised from
the gel and purified, before being ligated into the vector pGEM-T (Promega), and sequenced in the vector as purified double stranded plasmid (as above, and see methods, section 2.24 page 54). A hundred nucleotides of sequence was read from the two plasmid clones, and showed 100% homology to the published sequence of β2 (Anand and Lindstrom, 1990) and to the sequence of β4 (P.Tarroni, personal communication).

Control pairs of primers were included in the screening. These consisted of pairs of matched gene-specific primers (as opposed to a single gene-specific primer paired with a primer to the cloning vector), one of which was the gene-specific primer used for screening. These primer pairs amplified sequences within the clones already isolated from the brainstem library, namely NA10 with NA11 (amplifying a 330 b.p. sequence specific for β3), P6 with P5 (amplifying a 200 b.p. sequence specific for β4), NB22 with NB23 (amplifying a 250 b.p. sequence specific for jβ) and B2UN with NB23 (amplifying a 560 b.p. sequence specific for β2). These provided a positive control for hybridisations.

The control reactions were useful in assessing the value of the amplified products. As they all utilised the respective test primers, they were therefore identical at one end. Being defined by the test primers at one end provided a control fragment against which new products could be compared for size. Comparison of species newly amplified from the library with the corresponding control gave a good indication of whether any additional sequence would be present (i.e. species similar in size or smaller than the control PCR products would not contain any additional sequence to that already contained within the clones, while those that were larger would contain additional sequence).

**Results of PCR screening of brainstem and substantia nigra libraries.**

PCR amplified material from the brainstem and substantia nigra libraries was electrophoresed on an agarose gel stained with ethidium bromide and probed with the gene-specific sequences described above. The control reactions described above amplified fragments of the expected size from both libraries for β3 (see Fig 3.8a, lanes 9 and 20, page 109) and from the brainstem library only for β2. These were stronger in some reactions than in others, but were
visible on radioautographs of the probed filters (see Fig. 3.8e, page 110).

No products were amplified from the substantia nigra library with the β2-specific primers B2UN and NB23 or from either library by the use of the β4-specific primers P5 and P6 or jβ-specific primers NB22 and NB23. All of these primers were shown to function well in PCR. NB22 and NB23 were able to amplify a 250 b.p. fragment when clone jβ was itself used as template, P5 and P6 were used to amplify the 200 b.p. fragment used as the β4 subunit probe (see above, and Figs. 3.8a and 3.8d, lanes 6 and 17, pages 109-110) and B2UN and NB23 were used to amplify the 560 b.p. fragment used as the β2 subunit probe (see above, and Figs. 3.9a and 3.9c, lanes 3 and 14, pages 109-110).

The reaction temperature cycle was altered to see if larger amplified products could be produced, but even though changing such reaction conditions increased the efficiency of the amplifications (increasing the amounts of products already observed) it did not reveal new products. Increasing the extension time allowed for the polymerase to add nucleotide did not significantly affect the size of the products indicating that this was not a limiting factor. The longest extension time used was two minutes at 72°C, which according to previous estimates of the synthesis rate of Taq polymerase at this temperature (~1.4 Kb/minute - Innes et al, 1988) should allow for products of over two kilobases to be amplified. The parameter that most affected the PCR efficiency was the amount of library material included in the reactions, the optimum amount being about ~5 x 10^{12} clones.

The reactions were repeated five times each with separate aliquots of the libraries (each of 5 x 10^{12} clones) with each of the test primer pairs, and each of these was blotted and probed with appropriate probes. As two vector primers were used for separate reactions in this screening, this meant that in total, 5 x 10^{13} clones were screened in each library for the ends of each of the genes.

From what was visible on the gels before blotting, fragments were amplified from library material with most of the test primer pairs used, and in most cases several species were amplified (e.g. see Fig. 3.8a, page 109). These fragments amplified were of varied sizes, some larger than the control PCR products. The one gene-specific
primer that did not amplify any sequences from either library was the jβ-specific NB20.

When gels containing the products of the PCR reactions were probed with gene-specific probes as described above, these probes hybridised appropriately to the fragments amplified by control reactions, and also to a small number of the other fragments amplified. This indicated that some of the DNA visible on the gels was the result of amplification of sequences other than those targeted. Only products amplified by primers specific to β2 and β3 (see Fig. 3.8, pages 109-110) hybridised to their respective probes. Although the probes for β4 and jβ hybridised to the products from control reactions, no products corresponding to either were detected in any of the PCRs performed on the libraries.

Of fourteen amplified DNA fragments that hybridised to probes from either the β2 or β3 genes, eleven were identified by the β2 probe and only three by the β3 probe. All of the β2 products identified were smaller than the control. Of the amplified DNA fragments that hybridised to the β3 probe, two appeared to be close in size to the control PCR product, and one was considerably smaller (see Fig 3.8e, lanes 10, 11 and 22, page 110).

The two larger species were digested with the restriction enzymes Not I and Xho I, and then electrophoresed on a low-melting point agarose gel. The fragments were excised from the gel, purified, cloned into bluescript plasmid and sequenced. The cloned products were shown to correspond to the gene, but to be slightly smaller than the original clone by twenty two and fifty four base pairs respectively.

While it appeared that there were several clones in the library that corresponded to nAChR subunit genes, the β3 subunit clones were smaller in size than the clone originally isolated. From the number of clones screened, it was concluded that the library probably did not contain any β3 subunit clones longer than clone 8a.

3.2 f RACE analysis of human material for additional sequence.

As attempts to amplify the missing 5' nucleotide sequences from the human nAChR subunits from libraries was unsuccessful, I decided to attempt to obtain these sequences directly from the human total RNA described above using a RACE PCR protocol (name derived from rapid amplification of cDNA gnds, see Frohman, 1990 and methods, sections 2.21, 2.22 page 50, and Figure 2.5 page 36).
This technique involves modifying single stranded cDNA by adding an arbitrary sequence to either the 5' or 3' end of it so that PCR can be used to amplify sequences that may extend beyond the limits of the known sequence.

Following synthesis of single stranded cDNA from the RNA, a nucleotide 'tail' consisting of a single species of nucleotide is added to one end of it using the enzyme polynucleotide kinase (see methods, section 2.21 page 50, and Figure 2.5 pages 63-64 for schematic diagram of method). PCR amplification can then performed using a primer complementary to the string of nucleotides and a primer specific to the gene of interest.

Although the primer matching the nucleotide 'tail' will anneal to, and cause the synthesis from, a DNA strand from each of the tailed cDNA molecules, only cDNA molecules with a 'tail' and a specific sequence that matches the specific primer being used will be amplified at a near-exponential rate.

RACE was tried on various RNA preparations obtained from human neuronal tissue to try to get more sequence from the β3, β4 and jβ genes (see methods sections 2.21 and 2.22 for detailed description, page 50).

Briefly, 'first strand' cDNA was synthesised from the various human neuronal total RNA preparations using random primers. This produced material which could be used as a template for PCR amplification. The cDNA was then 'tailed' with a string of nucleotides (in this instance, dGTP) using the enzyme polynucleotide kinase. This 'tail' allowed the use of a primer which included an unbroken stretch of dCTP bases (primer pC, see Table. 2.1) to be used in conjunction with other gene-specific primers to amplify areas of the 5' end of the gene. The use of a non-specific primer would mean that DNA synthesis would be primed from every single stranded DNA fragment that possessed a dGTP sequence at its 3' end, but exponential amplification would only take place where two sequences complementary to primers were present in an appropriate, nearby position. As described above in the PCR screening of libraries, exponentially amplified sequences accumulate quickly and are able to dominate the reaction and make other amplifications irrelevant.

dATP is often used to 'tail' the cDNA in this method, but in this instance, dGTP was used as it offered greater specificity of synthesis in two ways. The stronger interaction between C+G
nucleotide bases would allow higher annealing temperatures to be used with a poly dC primer, decreasing the chances of mismatched primer annealing. Additionally, if dATP had been used with a poly dT primer, priming could occur from both ends of the cDNA sequence (i.e. from the 3' poly dA tail of mRNA present as well as the artificially added poly dA tail) - with poly dG, no unwanted priming from poly dA sequences would occur, and the amount of non-specific amplified sequences would be reduced (see methods, section 2.20 for description of RACE template preparation).

Once again, control amplifications were performed on the RACE template, using the same control primer pairs as described above for PCR library screening. After amplifications of the human material, the reaction products were electrophoresed on a gel, (Southern-) blotted and probed with gene-specific probes (as described in PCR library screening - the same probe sequences and primers were also used) to identify products which corresponded to the genes of interest.

The RACE template for this study was produced from RNA isolated from human adult pons and medulla tissue, and from human foetal whole brain and dorsal root ganglia (portions of the material used above for Northern analysis).

RACE Results.

The results of successful RACE amplification are not easy to predict from an ethidium bromide-stained agarose gel, as the range of sizes of RNA species inevitably present in a cDNA template produce a smear of single and double-stranded DNA on the gel. This must then be probed with an appropriate gene-specific probe, and material of a suitable size must then be isolated from the gel and inserted into a vector of some sort for analysis. The conditions of the first-strand reactions used to generate the single stranded cDNA template were varied by using different amounts of total RNA (from 1 to 10 µg) and of random-hexamer primers.

The only material that hybridised to the gene-specific probes was small in size, and far shorter (up to a hundred base pairs) than the length required to provide additional sequence to the genes under investigation (see Fig. 3.9, page 111). A similar amount and size of product was produced with the primers for β2, β3 and β4 (see Fig 3.9, page 111, result for β3 only shown).
Section 3.3. Discussion.

New clones isolated from the libraries.

The brainstem library was chosen because it would contain cell bodies of the fifth cranial nerve. The largest cranial nerve, the trigeminal has three sensory nuclei in the brainstem, the mesencephalic nucleus of the trigeminal nerve, the main sensory nucleus and the spinal trigeminal nucleus. Of these, the spinal trigeminal nucleus is involved with the sensations of pain and temperature. At the start of the project there was no dorsal root ganglion library in existence, and thus the brainstem library was the best available source of human sensory material.

Although there are areas of considerable diversity amongst the subunits of LGICs (nACh, GABA, glutamate and glycine receptors), the clustered transmembrane domains (see Fig 3.1, page 100, for schematic diagram) are a continuous region of high nucleotide homology between the subunits, and therefore also a region with potential homology to related novel subunits. The $^{32}$P labelled probes were hybridised and washed at low stringency, in conditions under which they would hybridise to sequences of $\sim 73\%$ homology and above. While it was unlikely that this percentage homology would be encountered over the whole probe length, this could be sufficient for hybridisation if only part of the probe was a continuous stretch of sequence with such a level of homology. The method of probe labelling (random priming, see methods, section 2.10 page 41) produces synthesis of sequences less than full length, increasing the likelihood of hybridisation between homologous stretches accounting for a fraction of the clone length.

At the time of the library screening, the $\alpha 5$ and $\beta 3$ subunits had yet to be demonstrated to participate in functional nAChRs and had failed to function in response to ACh or nicotine when expressed pairwise with other nAChR subunits in Xenopus oocyte systems (Deneris et al, 1989, Wada et al,1990). In terms of sequence, these two genes share more homology with each other than with other members of the neuronal nAChR family, and thus the possibility that they could be part of a channel gated by other ligands was a possibility that could not be discounted. Pursuing the possibility that these nAChR subunits were perhaps part of a separate class of
ligand gated channel, the α5 subunit was included in the pool in the hope that it might hybridise to new members of this putative class.

The GluR-A clone was also included in the initial screening in the hope of finding novel glutamate channel components. Previous studies have shown kainate activated channels in DRG neurons (Huettner, 1990) and that at least three glutamate receptor subunits are expressed in the dorsal root ganglia, namely GluR1, GluR2 and GluR5 (Bettler et al, 1990). GluR5, however, does not function effectively as a homo-oligomer when expressed in oocytes, but can form functional channels in such a system when co-expressed with KA-1 or KA-2, thought to be components of high-affinity kainate receptors in the CNS. The relationship between GluR-5 and other subunits that exhibit kainate binding properties but do not function effectively as homo-oligomers is unclear, and it seems possible that other subunits are yet to be discovered.

The only two ligand gated channel subunit genes to be isolated from the screening were the nAChR β3 and variants of the β2 subunit. The human version of the β2 had at that time been isolated but the human homologue of β3 had not (discussed below). However, one of the β2 clones isolated, clone jβ, had a novel sequence of 60 bases at its 5' end, and the possibility that this was a splice variant of the gene was considered.

3.3a Northern blots.

RNA was obtained from areas of an adult brain, which corresponded to those used in the construction of the brainstem library, in order to produce additional sequence to that found in the library clones. Foetal material was also available, but in these instances, whole brain including brainstem was removed, and dorsal root ganglion neurons were also isolated.

The foetal material was stored on ice for several hours before dissection, and although it was dissected straight into denaturation solution, it is likely that extensive degradation of the RNA had occurred. No hybridisation signals were seen with any of the LGIC probes in any of this material, and no hybridisation was observed from RACE amplified foetal cDNA template.

An adult brain that had been stored in a freezer (at -70°C) for an extended period was dissected to isolate areas rich in sensory neurons, for example the principal sensory trigeminal nuclei at the
level of the pons and the spinal trigeminal nuclei at the level of the medulla. Despite the period of storage, positive results were obtained with this adult material. Using a 600 b.p. probe generated by PCR from the 5' end of the β3 gene clone, an RNA species of about 1.7 Kb was faintly visible in pons material on a Northern blot while medulla and IMR32 cells did not show any signal. Material from the neuroblastoma cell line IMR32 was chosen for analysis by Northern blot because the human neuronal nicotinic α3, α5 and β4 subunits had previously been successfully cloned from this cell line (Fornasari et al 1990, Gotti et al, 1989, Tarroni, personal communication).

Double or 'blurred' hybridisation signals were observed on probing the human material with full-length clone inserts (e.g. see Fig 3.7, page 107). It is likely that this may be due to cross-hybridisation of the probe to mRNAs for other ligand-gated ion channel subunits. Despite the stringent probing conditions, cross-hybridisation is possible due to the high degree of homology between certain areas (especially the transmembrane regions) of β2 and β4.

Where the entire jβ clone insert was used as the probe, with the unique sequence comprising no more than 60 b.p. out of a probe length of 1700 b.p., the presence of the jβ-specific sequence was effectively irrelevant in this result, and it was effectively a β2 probe. It was impossible to compare the size of mRNA that hybridised to this probe with previous results, as no Northern analysis of the human β2 has been published. Analysis of Northern blots of rat central brain regions and of PC12 cells with a probe consisting of mainly untranslated 3' sequence from the β2 gene results in hybridisation to two distinct RNA species of 3.9 and 5.7 Kb (Deneris et al, 1988). Multiple transcripts are often obtained from Northern blots probed with nicotinic subunit genes (Boulter et al, 1990, Fornasari et al 1990, Chini et al 1992), even when unique untranslated sequences are used as the probe (Deneris et al, 1988). In some cases RNAse protection has revealed only a single protected species, suggesting that at least some of the multiple signals present on Northern blots may be nothing more than stable splicing intermediates (Boulter et al, 1990). However, when the two PCR products of 600 b.p. for β3 and 250 b.p. for β2 were labelled and used as probes for similar Northern blots, these probes were seen to hybridise to single but distinct species of RNA (see Fig 3.8, and Willoughby et al, 1993). These were of around 1.7 Kb
for β3 and 3.0 Kb for β2. This size of transcript for indicates that the β3 clone isolated from the brainstem library, predicted to lack around 120 b.p. of coding sequence including the signal peptide by comparison with the rat homologue (see Fig 3.10, page 112), also lacked about another 80 b.p. of 5' non-coding sequence.

Although the positive results with the Northern blot analysis are useful, the negative results are not informative without a positive control - the absence of these nAChR subunits from foetal brain and DRG should not be inferred from these results. Embryonic expression of nAChR subunits in chick has been widely reported (Moss et al, 1989, Couturier et al, 1990, Daubas et al, 1990, Matter et al, 1990, Jacob et al, 1991).

In most cases reprobing of blots of adult material produced no hybridisation, even in cases where it was expected (e.g., β2 showed a stronger hybridisation signal than β3 on identical blots, but when the β3-probed blot was stripped and reprobed with β2, no signal was observed). This suggests that degradation of RNA was probably occurring during manipulation of blots or that reprobing conditions were not ideal.

The expression of the β3 subunit, as described above has a very limited distribution in the rat (Deneris et al, 1989) and may be similarly limited in man. As discussed above, the relationship between neuronal nAChR subunits and assembled receptors is not known, although functional combinations have been produced in expression systems.

The α5 and β3 subunits have yet to be functionally expressed in this way, but recently the α5 subunit has been demonstrated to assemble with other nAChR subunits in chick ciliary ganglion neurons (Conroy et al, 1992), and is probably a component of one of the nACh receptors on chick ciliary ganglion neurons (Corriveau et al, 1993). Preliminary evidence points to the α5 subunit only assembling into functional receptors in the presence of other α- and β- subunit (Berg, D.K. and Conroy, W.G., personal communication). This raises the possibility that the α5 subunit may more analogous to the neuromuscular ε-δ- or γ-subunits.

There have been reports on the α5 subunits ability to bind to α-Bgt, based on the ability of a synthetic peptide corresponding to a portion of this subunit to bind $^{125}$I α-Bgt (McLane et al, 1990). Despite this, the α5 subunit-containing receptor detected in chick ciliary
ganglion neurons is not able to bind to α-Bgt and conversely the the α5 subunit has been shown not to contribute to the α-Bgt-binding receptor present on the same cells (Conroy et al, 1992, Vernallis et al, 1993). It is not clear if the complete α5 subunit is able to bind α-Bgt, but the evidence above, from ciliary ganglion neurons, suggests that if the α5 subunit is part of an α-Bgt-binding receptor, the ability of such receptors to bind the toxin is probably not conferred by the presence of the α5 subunit but is partially dependent on the presence of other subunits.

The β3 and α6 subunits are therefore the only known subunits not to have been shown to either function with other subunits in expression systems or to assemble with other subunits. The α6 subunit has only been partially characterised and its sequence has not been published (see within Deneris et al, 1991, Sargent, 1993).

Two likely possibilities present themselves, one being that the β3 is the minor subunit in an nAChR subtype, contributing to the receptor's properties but not able to substitute for the other subunits in oocyte systems. This would again raise the possibility that the neuronal nAChRs are like the neuromuscular nAChRs in stoichiometry, and that some of the neuronal subunits currently cloned and designated α- or β-type may turn out to be more correctly described as neuronal ε- or γ-subunits. The second possibility is that the β3 subunit is part of an nAChR whose other subunits have yet to be discovered.

Other more remote possibilities are that the subunit is non-functional, or is part of a channel that is gated by a ligand other than acetylcholine. However, the β3 and α5 subunits shows a higher level of homology to each other than to any other member of the nAChR family, though they show more homology to other nAChRs than to other members of the LGIC family. The α5 nAChR subunit shows 43-54% nucleotide homology to other nAChR subunits, and 69% homology to the β3 subunit. The α7 subunit shows least homology to the other members of the family, 38-41% similarity to the α subunits, 35-38% similarity to the β subunits (Seguela et al, 1993), while the homology between the rat α2, α3, and α4 nAChR subunits ranges from 68-80% (discussed in Nef et al, 1988).

The 5-HT₃ receptor subunit (5HT₃R-A), first tentatively described as the nAChR β5 subunit (Isenberg, 1990) and then identified by its ability to form functional homo-oligomeric channels
gated by 5-hydroxytryptamine (Maricq et al, 1991), is only slightly more homologous to nAChR subunits (~32%) than to GABA<sub>A</sub> receptor (~22%) or Glycine receptor (~22%) subunits (Maricq et al, 1991).

However, the close homology between the α5 subunit which contributes to nAChRs in chick ciliary ganglion neurons and in brain (Conroy et al, 1992), and the β3 subunit suggests that β3 is most likely to be a part of a nAChR.

It was hoped that the results from in-situ hybridisation using nAChR subunit gene probes performed by several laboratories, in which the frequent co-localisation of some subunits is revealed, might have given clues as to which combinations are assembled in vivo. However, there is multiple overlap of expression, and many locations show expression of many different subunits (e.g. Wada et al, 1989). The most abundant and obvious overlaps are the α3 and β4 subunits which are commonly co-localized in the peripheral nervous system, and α4 and β2 similarly coinciding in the central nervous system. These subunit pairs have been postulated to play a major role in the receptors localised in these respective areas (Whiting et al, 1991). From the limited localization of β3 (Deneris et al, 1989), it is possible only to say that in the areas in which it has been shown to be expressed so far, there is generally a relatively high level of expression of α3 and β2 with lower levels of α4 (Wada et al, 1989, and see Chapter 6 results on expression of nAChR subunits in rat DRG neurons).

3.3b PCR library screening discussion.

In searching for additional sequence to complete the protein coding regions of the genes isolated, PCR was used to rescreen the brainstem library for a third time. This was a way of examining the library contents by size for the missing gene fragments quickly and efficiently. Hybridisation screening of the reactions would then identify the appropriate products from other amplified sequences.

The use of PCR in this way to screen such a library has not been widely described. The rationale of the PCR being able to specifically amplify the correct sequences out of such a large variety of sequences was not considered to be a problem, but the amplification of a DNA template encased in a bacteriophage capsid was a source of uncertainty. After some experimentation with pairs of control
primers, it was discovered that efficient amplification could be achieved once the library material was sufficiently denatured.

The difference of efficiency of various primer pairs at amplifying species from the libraries presumably reflected the different abundance of particular RNA species represented in the libraries. The number of positive products amplified out of $5 \times 10^{13}$ clones screened, may seem to be a low number, but with this method only clones containing the extreme 5' end would be detected as positive, and of the four positive clones identified out of $\sim 4 \times 10^9$ plaques screened using conventional methods, two of these would have been detected using the PCR method. Of these, three corresponded to the $\beta_2$ subunit or variants of it, and one to the $\beta_3$ subunit.

In addition to the screening of the brainstem library, a human substantia nigra library was screened. This library was chosen principally to look for the end of the $\beta_3$ gene, as the expression data from the original paper in which the rat $\beta_3$ had been cloned (Deneris et al 1989) revealed that the expression of the gene was confined to small discrete areas of the CNS, most notably the mesencephalic nucleus of the trigeminal, and two small strongly expressing areas corresponding to the substantia nigra pars compacta. Although the insert size of this library was small, the fact that it was random hexamer primed as well as oligo-dT primed allowed the possibility of cDNA synthesis from all areas of the mRNA rather than just from towards the 3' end. In a situation where some degradation of material has taken place, the priming of cDNA randomly has a greater chance of producing cDNA covering the whole message than has the priming of cDNA from one end of the molecule.

The several small amplified fragments corresponding to $\beta_2$ from library screening indicate that the PCR method was amplifying appropriate sequences, but that, as expected, the library contained many small partial cDNAs.

Apart from $\beta_2$ which was included in the study as a positive control, amplified products from the $\beta_3$ primer were the only other positive results from the screening. Although two of these appeared to be only similar in size to the control PCR product, they were found to be smaller, and it was concluded that it was unlikely that the library would contain any larger clones of the $\beta_3$ subunit.
**RACE**

Unfortunately most of the total RNA obtained from adult human pons and medulla was used up in making the Northern blots, and therefore only a few micrograms was available for the RACE attempt. Amplified products were produced from control PCR reactions using several primers for \( \beta_2, \beta_3 \) and \( \beta_4 \) on the cDNA RACE template material. That some of these corresponded to the nAChR genes was shown by probing with 5' probes produced by PCR, but no products big enough to extend the sequence beyond what was present in the original clones were obtained. These control RACE reactions were only successful in amplifying the smaller DNA fragments, (e.g., using either NA10 or NA 11, for RACE produced fragments of about 2-300 b.p.) which suggests that following the thawing and refreezing that occurred in making the Northern blots, the RNA had degraded until only small lengths were left and that by using RACE, it was thus only possible to amplify small sequences away from the ends of the strands.

Work was limited by the availability of the human material, and the cell line available did not contain the \( \beta_2 \) or \( \beta_3 \) subunits, although all but one of the previously cloned human nAChR subunits had been isolated from this cell line. As dorsal root ganglion (DRG) neurons were the specific area of focus of the group and the chances of obtaining human DRG material seemed to be poor, a study of the nicotinic subunit expression in DRG neurons was therefore undertaken in the rat because this was a more convenient source of material (See Chapter 6).

**3.3 c Sequence analysis.**

The fact that a poly dT primed library was used for the screening meant that the clones isolated all contained the 3' end of the cDNA up to the polyadenylation 'tail'. This meant that additional sequence from the 3' untranslated region that extended the published \( \beta_2 \) sequence was obtained. This sequence was identical for clones \( \beta \) and 12d, and was also in agreement with the partial sequencing of clone 12c. The untranslated sequence was surprisingly short, only ~90 b.p. (see Fig. 3.5, pages 104-105), as compared to more than 700 b.p. for the rat \( \beta_2 \) cDNA sequence (Deneris et al, 1988).
The more striking difference was a stretch of sixty bases at the extreme 5’ end of clone jβ which was GC-rich and which had no significant homology to the human gene (see Fig 3.4, page 103). This was a potentially interesting result, and it seemed possible that it might be a variant of the highly expressed β2 subunit. There have been two reported instances of splice differences in nAChRs, the neuromuscular α-subunit (Beeson et al, 1990) and the rat neuronal α4 (Goldman et al, 1987), one in glycine LGIC subunits (Betz, 1991) and several instances in the case of glutamate LGICs (review: Sommer and Seeburg, 1992).

Careful comparison of sequences within the database revealed that the intron-exon arrangement of nicotinic subunits of the neuronal type in several species had a conserved pattern of six protein-encoding exons (see Wada et al 1988, Nef et al, 1988, Claudio, 1989, Fig 6.1, page 199, and discussion). The α7 subunit, however, has recently been shown to have additional introns near the 3’ end of the coding region (Couturier et al, 1990, Fig 6.2, page 199). This pattern can be aligned where these genes have been isolated as genomic clones (Couturier et al, 1990, Nef et al 1988). It was also possible to align the chick non-α gene (the equivalent of the rat/human β2) with the sequence of jβ to see if the new sequence commenced at an intron boundary (making it likely to be intron sequence). However, the unique sequence commenced in the middle of the second exon. This removed the possibility that it was merely an immature mRNA which had not yet been fully edited.

To investigate jβ further, primers were made to sequences at the 5’ end of the clone, both in the unique part, in the β2-like part of jβ and at the boundary of these two areas, which would enable PCRs to be performed, one to recognise the β2 sequence and one to recognise jβ-specific sequence (see Fig. 3.6, page 106). On all material tested, including the original brainstem library, the PCRs on β2 were successful, but none of the reactions dependent on the jβ-specific sequence produced products of any kind.

There are several possible explanations for the novel stretch of sequence specific to clone jβ – that it is either a variant which is present at very low level, a variant with a very specific area of expression, or some accident, either of transcription or of cDNA synthesis. Although the quality of the human RNA was not consistent, it was consistently possible to PCR amplify small
stretches of cDNA using primers to β2, even if amplification of larger stretches was unsuccessful. Thus although this study was limited, it seems likely that the jβ clone was an artifact of cDNA synthesis or of some other step in the cloning procedure.

The human β3 nucleotide sequence (registered in the EMBL Data library under accession No. X67513) shows 77% homology to the rat sequence, the homology being greatest within the four hydrophobic regions and least in the presumed cytoplasmic loop between hydrophobic regions three and four.

A comparison of the deduced β3 protein sequence with the rat sequence (see Fig 3.10, page 112) shows an amino acid homology of 89%, close to but lower than the homologies between the rat and human homologues of other neuronal nAChR subunits. The deduced protein shows many of the features conserved in nAChR subunits, including the LGIC motif - two cysteines thirteen residues apart, corresponding to two similar residues at position 128 and 142 in the Torpedo neuromuscular α-subunit. The four conserved hydrophobic domains assumed to be membrane-spanning regions in nAChRs show particularly high homology between the two species, in particular the third hydrophobic domain which shows almost perfect homology.

Apart from a potential N-linked glycosylation site conserved at position 172 in the rat, two additional sites are present in the N-terminal domain of the human gene at positions 16 and 104, based on alignment with the rat gene. The two diagnostic adjacent cysteines associated with ACh binding used to distinguish between α- and β-type subunits are absent, indicating that this is probably not a ligand-binding subunit.
### Table 3.1

Selected pharmacology of the main ligand-gated channel families.

<table>
<thead>
<tr>
<th>Channel family</th>
<th>Flux</th>
<th>Agonists distinguishing LGIC from other receptors using same ligand.</th>
<th>Antagonists distinguishing LGIC from other receptors using same ligand.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>cations, including Ca$^{2+}$</td>
<td>AP4$^\wedge$ trans ACPD$^\wedge$</td>
<td>kyneuric acid$^\wedge$ L-AP4$^\wedge$</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>cations, including Ca$^{2+}$</td>
<td>nicotine * muscarine ^</td>
<td>D-tubocurarine * atropine ^</td>
</tr>
<tr>
<td>GABA_A</td>
<td>Cl$^-$</td>
<td>3-amino-propanesulphonic acid*</td>
<td>bicuculline* picrotoxin*</td>
</tr>
<tr>
<td>Glycine</td>
<td>Cl$^-$</td>
<td>glycine&quot;, β-alanine&quot;, taurine&quot;</td>
<td>strychnine &quot;</td>
</tr>
<tr>
<td>5-HT$_3$</td>
<td>cations, including Ca$^{2+}$</td>
<td>2-methyl-hydroxytryptamine*</td>
<td>MDL72222* ICS205-930*</td>
</tr>
</tbody>
</table>

(*) indicates where agonists/antagonists work only on ligand-gated channels, (') indicates where agonist/antagonist works only on G protein-coupled channel, (") indicates where agonists/antagonists distinguish between ligand-gated channel types in the absence of G protein-coupled channels.


Abbreviations: LGIC = ligand-gated ion channel, ACPD = trans-1-amino-cyclpentyl-1,3-dicarboxylate, AMPA = α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate, AP4 = 2-amino-4-phosphonobutyrate, CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione, CPP = 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, DNQX = 6,7-dinitro-quinoxaline-2,3-dione, GDEE= glutamate diethyl ester, 5-HT = 5-hydroxytryptamine, KA = Kaininate, Quis = quisqualate, NMDA = N-methyl-D-aspartate.
<table>
<thead>
<tr>
<th>Channel subtype/characteristics</th>
<th>Channel subtype/characteristics</th>
<th>Channel subtype/characteristics</th>
<th>Channel subtype/characteristics</th>
<th>Subunit assembles into channels, role uncertain.</th>
<th>Subunits not associated with known functional channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAChR channels.</td>
<td></td>
<td></td>
<td></td>
<td>α5</td>
<td>α6, β3</td>
</tr>
<tr>
<td>nAChR subunits.</td>
<td>α1, β1, δ1, ε1, γ1.</td>
<td>α7, α8</td>
<td>α2, α3, α4*, β2, β4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate channels.</td>
<td>NMDA, strong Mg2+ block</td>
<td>NMDA, weak Mg2+ block</td>
<td>Non-NMDA kainate/AMPA</td>
<td>Non-NMDA</td>
<td></td>
</tr>
<tr>
<td>Glutamate subunits</td>
<td>NR-1, NR-2A</td>
<td>NR-1, NR-2C</td>
<td>GluR-A/GluR-1*, GluR-B/GluR-2*, GluR-C/GluR-3*, GluR-D/GluR-4*</td>
<td>NR-2B, GluR-5, GluR-7, KA-1, KA-2</td>
<td></td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; channels.</td>
<td>Type I benzodiazepine pharmacology</td>
<td>α1</td>
<td>Type II benzodiazepine pharmacology</td>
<td>Insensitive to benzodiazepine</td>
<td></td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; subunits.</td>
<td></td>
<td>α5</td>
<td>γ1</td>
<td>α2, α3, α4, α6</td>
<td></td>
</tr>
<tr>
<td>Glycin&lt;sub&gt;e&lt;/sub&gt; channels.</td>
<td>Neonatal</td>
<td>Adult</td>
<td></td>
<td>β1, β2, β3, β4</td>
<td></td>
</tr>
<tr>
<td>Glycin&lt;sub&gt;e&lt;/sub&gt; subunits</td>
<td>α2*</td>
<td>α1*</td>
<td>γ2*, γ3, δ1, ρ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5HT&lt;sub&gt;3&lt;/sub&gt; channels/subunits</td>
<td>5HT&lt;sub&gt;3&lt;/sub&gt; / 5HT&lt;sub&gt;3&lt;/sub&gt;-1</td>
<td></td>
<td></td>
<td>β1</td>
<td>α3, α4</td>
</tr>
</tbody>
</table>

Table 3.2
Table listing some of the known subunits of the main classes of ligand-gated ion channels and the receptor subtypes or receptor properties that they are associated with. Asterisks (*) indicate where more than one splice variant of the subunit exists.

Figure 3.1
Schematic diagram of the conserved features of the ligand-gated ion channel superfamily. The putative signal peptides (SP) and the putative membrane spanning hydrophobic domains (M1-M4) are indicated by black areas. Cysteine residues are indicated by (s) and the cysteine motif by (ss). Branched structures represent potential glycosylation sites. Note that the extracellular domain (ED), cytoplasmic loop (CL) and N-terminal domain are often longer in the members of the glutamate family.
Figure 3.2
Schematic diagram of an nAChR subunit showing predicted areas of secondary structure, such as α-helices and β-pleated sheets. The cysteine motif and the adjacent cysteines at the toxin binding site are also shown (adapted from Stroud, 1990).
Southern blots of the individual rat ligand-gated channel subunit cDNAs which were pooled to screen the human brainstem library. Lane (1) GluR-A, (2) nAChR β2 subunit, (3) nAChR β4 subunit, (4) nAChR α5 subunit. The blots were probed with the human clones that were isolated in the screen, under conditions of low stringency (2x SSC, 0.1% SDS at 57°C). The clones used as probes are; (A) clone 8a and (B) clone 12d.
Figure 3.4  Schematic Diagram of Clones Isolated from Human Brainstem Library.

Rat β3 gene

- 86%
- 98%
- 81%
- 91%
- 27%

Clone 8a

Human β2 gene

1 21%
100%
ND
100%

Clone β

100%
ND
100%

Clone 12d

100%
ND
100%

Clone 12c

KEY TO SHADING.
- Untranslated Regions.
- Putative Transmembrane Domains
- Mature Protein Encoding Regions.
- Signal Peptide.
- Polyadenylation.
- Unique β Sequence.

Numbers with percentage symbol between dashed lines represents the approximate percentage nucleotide homology between clones in those areas. ND indicates areas not sequenced.
Figure 3.5 Nucleotide sequence alignment of human clone 8a with the published sequence of the rat nAChR β3 subunit (Deneris et al, 1989), showing the high level of homology. Horizontal lines denote putative transmembrane domains, arrows indicate position of the 'Ligand-gated channel motif'.

```
8a  CCGAGTG--GAATAAACAATCTCA---ACC--CTC--TGGCCG---AGTGTGCAGCTCTCTTTTACACAT
β3  CTGGTGCAGCTCTACGGCAACTGAGCTCAGGCTGCTGAAACCAAGAAGGC--ACTCC--TCAGCAGAT

70  80  90  100  110  120  130
TTGGTCCAGGTTATCAGAAAATGGGTCCGCCCTGTATTACATCTTAATGACACCCATAAAAATGATATTTTGAG
TTGGTCCAGGTTATCAGAAAATGGGTCCGCCCTGTATTACATCTTAATGACACCCATAAAAATGATATTTTGAG

140  150  160  170  180  190  200
TTGAAAATATCCCAGCTTGTAGATGTGGATGAAAAGAATCAGCTGATGACAACCAATGTGTGGCTCAAACAQ
TTGAAAATATCCCAGCTTGTAGATGTGGATGAAAAGAATCAGCTGATGACAACCAATGTGTGGCTCAAACAQ

210  220  230  240  250  260  270
GAATGGACAGACCAACATGTGTTAACAGCTGAGACTCAGATGACACCAATGTTGCTGGCCTACACAG
GAATGGACAGACCAACATGTGTTAACAGCTGAGACTCAGATGACACCAATGTTGCTGGCCTACACAG

280  290  300  310  320  330  340
GAATCTCTGTCGTTCCGCTCAGCATGTTCTCTTTTGGAAATGCTGACGAGGCCCCTTGAGCTGCTAGTACAGGC
GAATCTCTGTCGTTCCGCTCAGCATGTTCTCTTTTGGAAATGCTGACGAGGCCCCTTGAGCTGCTAGTACAGGC
```

350  360  370  380  390  400  410  420
AAAGTCATCTGAAAATCAGAGCACTTGCTGACCTTGAGCGCAGCTCTCGCTTACATATATGAGCTGAGCTGAG
AAAGTCATCTGAAAATCAGAGCACTTGCTGACCTTGAGCGCAGCTCTCGCTTACATATATGAGCTGAGCTGAG

430  440  450  460  470  480  490
GACGGTACAGTTTTTTCCCGTCTCGACAGCAGAATCTGGTACAGCTGAGACTCAGATGACACCAATGTTGCTGGCCTACAG
GACGGTACAGTTTTTTCCCGTCTCGACAGCAGAATCTGGTACAGCTGAGACTCAGATGACACCAATGTTGCTGGCCTACAG

500  510  520  530  540  550  560
ATGGGTACCTCATTTTCTGCTAATGAAATGGTACAGAAAGAAGCTCTCTTGAGAAGCAGAATGAGCAGAGATGAG
ATGGGTACCTCATTTTCTGCTAATGAAATGGTACAGAAAGAAGCTCTCTTGAGAAGCAGAATGAGCAGAGATGAG

570  580  590  600  610  620  630
CTGAAATGCCAAAAGGGGATAGAAGGGGAAACAGAAAAGGGGAGGCGCTGATCTCCTACATCCTCTGTACCTACGAC
CTGAAATGCCAAAAGGGGATAGAAGGGGAAACAGAAAAGGGGAGGCGCTGATCTCCTACATCCTCTGTACCTACGAC
```
Figure 3.5 (continued).
Figure 3.6
Schematic diagram of the primers used in RACE and PCR screening of libraries to find the 5' ends of the human nAChR subunit genes.
Figure 3.7

Northern blots of human total RNA probed with (A) and (B), the 250 b.p. probe for the human β2 nAChR subunit produced by PCR using primers NB22 and NB23 on clone jβ, and (C) a 330 b.p. probe for the human β3 nAChR subunit produced by PCR using primers NA10 and NA11 on clone 8a. Probing was under stringent conditions, (see methods).

Each of the following lanes contains 40 μg of total RNA as quantified by spectrophotometer and visualised on ethidium stained gels, (1) and (7) - adult pons, (2) - adult medulla, (3) - foetal brain, (4) - foetal DRG, (6) - IMR32 cell line. Lane (5) - size markers, (8) - rRNA markers. Two bands of RNA visible in each lane correspond to rRNA (compare with lane 8), the β2 and β3 mRNAs are arrowed.
Figure 3.8a

An agarose gel of PCRs performed on the human substantia nigra (Lanes 1-11) and brainstem (Lanes 12-22) cDNA libraries. Lanes marked with M contain DNA size markers.

<table>
<thead>
<tr>
<th>Lane No. / Gene</th>
<th>Specific primers</th>
<th>λgt10 primers</th>
<th>Lane No. / Gene</th>
<th>Specific primers</th>
<th>Bluescript primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (β)</td>
<td>NB20</td>
<td>5'gt10</td>
<td>12 (β)</td>
<td>NB20</td>
<td></td>
</tr>
<tr>
<td>2 (β)</td>
<td>NB20</td>
<td>3'gt10</td>
<td>13 (β)</td>
<td>NB20</td>
<td></td>
</tr>
<tr>
<td>3 (β2)</td>
<td>NB23 and (control)</td>
<td>5'gt10</td>
<td>14 (β2)</td>
<td>NB23 and (control)</td>
<td>p2UN</td>
</tr>
<tr>
<td></td>
<td>p2UN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (β2)</td>
<td>NB23</td>
<td>5'gt10</td>
<td>15 (β2)</td>
<td>NB23</td>
<td></td>
</tr>
<tr>
<td>5 (β2)</td>
<td>NB23</td>
<td>3'gt10</td>
<td>16 (β2)</td>
<td>NB23</td>
<td></td>
</tr>
<tr>
<td>6 (β4)</td>
<td>P6 and P5 (control)</td>
<td>5'gt10</td>
<td>17 (β4)</td>
<td>P6 and P5 (control)</td>
<td></td>
</tr>
<tr>
<td>7 (β4)</td>
<td>P6</td>
<td>5'gt10</td>
<td>18 (β4)</td>
<td>P6</td>
<td></td>
</tr>
<tr>
<td>8 (β4)</td>
<td>P6</td>
<td>3'gt10</td>
<td>19 (β4)</td>
<td>P6</td>
<td></td>
</tr>
<tr>
<td>9 (β3)</td>
<td>NA10 and (control)</td>
<td>5'gt10</td>
<td>20 (β3)</td>
<td>NA10 and (control)</td>
<td>NA11</td>
</tr>
<tr>
<td></td>
<td>NA11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (β3)</td>
<td>NA10</td>
<td>5'gt10</td>
<td>21 (β3)</td>
<td>NA10</td>
<td></td>
</tr>
<tr>
<td>11 (β3)</td>
<td>NA10</td>
<td>3'gt10</td>
<td>22 (β3)</td>
<td>NA10</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.8b

Southern blot of the above gel probed using stringent conditions with NB22, an oligonucleotide to the unique part of jβ.

Figure 3.8c

The same Southern blot stripped of probe and reprobed using stringent conditions with a 250 b.p. probe specific to the β2 nAChR subunit (product from primers NB22 and NB23).

Figure 3.8d

The same Southern blot stripped of probe and reprobed using stringent conditions with a 205 b.p. probe specific to the β4 nAChR subunit (product from primers P5 and P6).

Figure 3.8e

The same Southern blot stripped of probe and reprobed using stringent conditions with a 330 b.p. probe specific to the β3 nAChR subunit (product from primers NA10 and NA11).

See Figure 3.6 for schematic diagram of the primers and probe sequences used.
Figure 3.9

Southern blot of the products of a RACE amplification made using the primers NA10 and pC on two single stranded cDNA template preparations made from (A) human adult pons and (B) human foetal brain. Control PCRs performed on (C) fetal brain and (D) pons material using the β3-specific primers NA10 and NA11 are included. The blot is probed under stringent conditions with the 300 b.p. PCR product amplified from clone 8a using primers NA10 and NA11.

The strongest fragment seen in lane (D) is the control 300 b.p. product. The large smear of material in lane (A) is therefore around 50-100 b.p. in size.
Figure 3.10
Sequence comparison of the predicted human β3 sequence from clone 8a with the rat nAChR β3 sequence. Standard single letter amino acid code is used. Lines between residues indicate homology. The rat gene is numbered according to its open reading frame, the human sequence is numbered from the start of the clone. Lines flanking sequence indicate the position of putative membrane spanning domains.

Arrows mark the ligand-gated channel motif of cysteines.

Arrows mark potential N-linked glycosylation sites.
Chapter 4. The Capsaicin-Gated Channel.

4.1 Introduction - capsaicin.

A subset of peripheral sensory neurons, some involved in nociceptive signalling, are activated and then desensitised by application of capsaicin, an ingredient of hot chilli peppers which is not found in animal species. Capsaicin (8 methyl N-vanillyl 6-nonenamide) is the pungent principal of red peppers, described five hundred years ago by members of Columbus' crew in logbooks and letters (Colon, 1509) which had been utilised for their culinary and analgesic qualities by South American Indians for probably hundreds of years prior to that.

The desensitisation produced by the application of capsaicin can result in an analgesic state, while not affecting the ability to sense other stimuli such as gentle heat or touch (Simone and Ochea, 1991). This action appears to be due to the activation of a specific channel (see below). The characteristics of the capsaicin response therefore makes the cloning and further characterisation of the capsaicin receptor/channel of great interest in the study of nociceptive pathways, and from the point of view of designing capsaicin analogues which would desensitise sensory neurons without activating them, of practical and commercial interest as well.

4.1a Capsaicin-sensitive cells.


Primary nociceptive neurons, whose cell bodies lie within the dorsal root ganglia, are activated by noxious or damaging stimuli and release excitatory amino acids and neuropeptides in the dorsal horn of the spinal cord. Such activation can lead to the sensation of pain, a warning and, therefore, also a protective mechanism for the organism. In terms of function, capsaicin sensitivity in adult mammals is restricted to a subset of sensory neurons, activating only neurons with nociceptive function, including polymodal nociceptors and some warm receptors (Hayes and Tyers, 1980, Szolcsanyi, 1985).
However they have little or no effect on high threshold mechanoreceptors (Szolcsanyi et al., 1988); thus although the cells sensitive to capsaicin appear to be almost entirely nociceptive in function, not all nociceptive cells are sensitive to capsaicin (Holzer, 1991).

Because of its unusual functional specificity, capsaicin is frequently used to define a subset of primary afferent cells (e.g., Hua et al., 1985, Winter, 1987, Dray and Perkins, 1988, Holzer, 1993, Lundberg, 1993). The term 'capsaicin-sensitive neurons' is therefore commonly used in research to give an indication of whether the particular subset of cells being studied is likely to be involved in nociceptive processes. The neurons which are capsaicin-sensitive are difficult to define as they do not exactly overlap with any particular population of cells that has been otherwise defined by morphological, biochemical or functional means (Lawson and Nickel, 1980, Heyman and Rang, 1985, Hua et al., 1985 Winter, 1987, Holzer, 1991).

Most capsaicin-sensitive neurons have small or medium sized cell bodies and possess unmyelinated (C) or thinly myelinated (Aδ) nerve fibres (Heyman and Rang, 1985, Marsh et al., 1987, Urban and Dray, 1992) although in some studies, the responsiveness (and destruction) of Aδ fibres is either minimal or absent (Williams and Zieglgansberger, 1982).

In the DRG, neurofilament proteins are mainly restricted to the large light (L) neurons, and as capsaicin sensitivity is present in only a subset of the small, dark (SD) neurons, the capsaicin-sensitive population is therefore poor in neurofilament (Lawson and Nickel, 1980, Lawson et al., 1984, Winter, 1987, Wood et al., 1988, see also section 1.1 b, page 18).

Capsaicin-sensitive sensory neurons contain many neuropeptides, including substances such as neurokinin A, neuropeptide K, eledosin-like peptide, somatostatin, vasoactive intestinal peptide (VIP), Cholecystokinin-octapeptide (CCK), Calcitonin gene-related peptide (CGRP), Galanin (GAL), Corticotrophin-releasing factor (CRF), Arginin vasopressin, Bombesin-like peptides and substance P, but there is no consistent pattern to the peptide content of these cells (Hua et al., 1985, Gibbins et al., 1987, Winter, 1987, Maggi and Meli, 1988, Holzer, 1988, 1992 - and references within, O'Brien et al., 1989, Weihe, 1990). Indeed many of
the neuropeptides can also be found in some non-sensory neurons (Kirchgessner et al, 1988).

Responses to other activating substances may coincide largely with capsaicin sensitivity, but again, do not correlate exactly. For instance, many peripheral sensory cells sensitive to bradykinin are also capsaicin-sensitive, but a smaller portion of these are responsive to histamine (Lang et al, 1990).

Administration of capsaicin to neonatal animals results in an irreversible degeneration of about 50% of dorsal root ganglion cells, mainly small, dark neurons (Jancso et al, 1977) and their unmyelinated C-fibres (Jancso et al, 1985, Szolscanyi, 1985) with a corresponding loss of sensory function. In other studies either a small loss or no loss has been reported for small diameter, myelinated A-δ fibres (Jancso et al, 1977, 1985a, 1985b, Jancso and Kiraly, 1981, Hiura and Sakamoto, 1986). In the absence of outright destruction, a reduction in the diameter of some myelinated A-β and A-δ fibres has also been reported (Hiura and Sakamoto, 1986).

In adult mammals there is a similar but more selective degeneration mainly in small, dark neurons with a low neurofilament content (Jancso et al, 1977, Jancso and Kirally, 1981), this degeneration is thought to be more a destruction of nerve terminals and inhibition of axonal transport mechanisms than of total cellular destruction. Other work, however, indicates that a limited destruction of neurons does occur (Jancso et al, 1985a), but it is clear that, in adult animals at least, the cell bodies of sensitive cells are more resistant than are their axons (Jancso et al, 1985b).

4.1 b Neurons not sensitive to the neurotoxic action of capsaicin.

There is no evidence to show that capsaicin activates any peripheral neurons other than those mentioned, neither efferent neurons nor non-neuronal cells (Marsh et al, 1987, Wood et al, 1988). Sensitivity of cells in the enteric nervous system is uncertain, but is not likely to be extensive (Holzer, 1991). Most evidence points to capsaicin not exerting its specific effects directly on the membranes of spinal cord neurons (Urban and Dray, 1992). However, studies on capsaicin analogues administered to in vitro preparations of the neonatal rat spinal cord have indicated that attenuation of C-fibre input to the spinal cord may occur, but whether this is due to effects on primary afferents is not yet clear (Dickenson et al, 1990).
Few other excitatory effects of capsaicin have been recorded. Sensory neurons in the trigeminal nucleus caudalis and cerebellar neurons can exhibit a capsaicin-induced depression (Salt and Hill, 1980) upon application of mM amounts of capsaicin but this is a much larger amount than is required for the activation of primary afferent neurons. Long term effects can be seen in parts of the nervous system which are functionally related to primary afferent neurons. These are interpreted to be changes secondary to, and as a result of, capsaicin action on primary sensory neurons (Holzer, 1991).

4.1 c Central actions of capsaicin.

In other neurons in the central and enteric nervous systems, systemic administration of capsaicin can be shown to attenuate the function of neurons in a way which suggests a direct action (Holzer, 1991). Histological evidence of such permanent capsaicin-induced damage and degeneration in the adult rat has been shown in areas of the central nervous system including the ventral tegmentum and hypothalamus, areas not thought to contain extensions of primary sensory neurons of peripheral origin (Ritter and Dinh, 1988) and in rat retinal ganglion and bipolar cells (Ritter and Dinh, 1991). In preweaning rat pups the damage is more pronounced, (Ritter and Dinh, 1990) while some areas of the central and peripheral nervous systems appear to lose their sensitivity to capsaicin damage developmentally (Ritter and Dinh, 1992).

4.1 d Local effector functions.

The primary sensory neurons which are involved in the perception of noxious stimuli have efferent, as well as afferent, functions. That is, they not only react to stimuli by transmitting signals to other neurons in the central nervous system, but they can also release neuropeptides locally which may effect vascular tissue and cells of the immune system (reviews: Holzer, 1988, 1992, Maggi and Meli, 1988). An example of such a local effector response is the inflammation that can follow tissue irritation and injury.

4.1 e Actions of capsaicin on sensitive neurons.

Exposure to capsaicin can exert various effects on susceptible neurons, depending on the dose, delivery, and the developmental
stage of the animal (Fitzgerald, 1983, Szolcsanyi, 1993). At low doses, capsaicin excites and depolarizes a subset of sensory neurons including polymodal nociceptors (Peterson et al, 1987, Wood et al, 1988) causing an increase in cation permeability and the release of neuropeptides (Holzer, 1988) and of prostaglandins (Juan et al, 1980).

Capsaicin depolarises the membranes of mammalian sensory neurons transiently and then hyperpolarizes them, sometimes to a more negative value than the starting value. In mammals this is accompanied by a decrease in membrane resistance, suggesting that ion channels in the plasma membrane are transiently opened (Williams and Zieglgansberger, 1982, Heyman and Rang, 1985). The initial depolarisation and reduction in resistance of the membrane in response to capsaicin shows concentration dependence. As the hyperpolarization is dependent on extracellular calcium, it is thought to be due to subsequent activation of calcium activated potassium channels (Marsh et al, 1987).

Higher doses can result in long-term desensitization, degeneration and neuronal destruction. Primary neurons in neonatal animals are more susceptible to complete destruction (Jancso et al, 1977), while in adults the damage may be restricted to the axons of primary afferents with some studies indicating that the peripheral axon of these cells is the most vulnerable to damage (Chung et al, 1990).

4.1 f Mechanisms of toxicity.

Ion replacement experiments show that capsaicin neurotoxicity can be explained largely on the basis of ion fluxes evoked by capsaicin or resiniferatoxin, a potent capsaicin analogue (see below). The neurotoxic effects of capsaicin and RTX on cultured DRG neurons are reduced by replacement of sodium chloride in the medium with sucrose and are abolished by the removal of calcium (Jancso et al, 1977, Marsh et al, 1987, Santicioli et al, 1987, Winter et al, 1990). The neurotoxic effects of sodium influx are through the build-up of osmotic pressure in the cell; the uptake of sodium ions (Na+) also induces the passive entry of chloride ions (Cl-) into the cell, resulting in accumulation of NaCl which produces osmotic pressure, causing the cell to swell. Calcium-mediated damage involves a different mechanism, and it has been suggested that this may involve

4.1 g Capsaicin induced desensitization.

Capsaicin is able to confer two distinct states of desensitization on neurons, a desensitization of response to the repeated application of capsaicin, and a functional desensitization, which involves a loss of response to other stimuli (Holzer, 1991, Simone and Ochoa, 1991, Bevan and Docherty, 1993).

Activation may be followed by desensitization and, with systemic administration, the functional desensitization which can result in damage or inactivation of a variety of peripheral sensory systems (Dray et al, 1988, 1989). This more general desensitization may come about via different effects of capsaicin, including peptide depletion (Jeftinija et al, 1992), and a calcium-dependent inhibition of prostaglandin release (Juan et al, 1980). The localised calcium accumulation also causes inhibition of voltage activated calcium channels (Robertson et al, 1989, Bleakman et al, 1990, Docherty et al, 1991). Some of the desensitizing effects of capsaicin may be lessened or avoided by preventing calcium accumulation inside the cells (Santicioli et al, 1987).

Capsaicin challenge of primary afferent neurons results in a release of neuropeptides and other substances, leading to their depletion (Simone and Ochoa, 1991). This affects primary sensory neuron efferent function, preventing neurogenic inflammation (Jancso et al, 1967). This release seems to be mainly due to secondary activation of calcium channels and can be reduced by ruthenium red, a non-specific calcium channel blocker (Maggi et al, 1988). Other studies reveal that capsaicin may also affect the transport of some of the neurogenic mediators (Gamse et al, 1982).

Alterations in gene expression in the spinal cord following tissue injury and inflammation include upregulation of substance P, dynorphin and CGRP which are mediators of nociceptive signalling (review: Drubner and Ruda, 1992). The release of at least some of these mediators by spinal cord neurons may be induced by application of 0.1 mM capsaicin, an effect which can be inhibited by ruthenium red (Franco-Cerecda et al, 1992).

While serotonin1A receptor agonists activate the c-fos directed expression of dynorphin in the spinal cord (Lucas et al, 1993),
capsaicin treatment of neonatal DRG can prevent the spinal expression of both dynorphin and c-fos indicating that the loss of capsaicin sensitive cells deletes effector functions that operate at the spinal level as well as those that affect the periphery (Hylden et al, 1992).

4.1.1 The evidence for a specific receptor.

There are three major areas of study that show evidence for a specific receptor which is activated by capsaicin.

(1) The well-defined species and tissue-specificity of capsaicin action implies that a specific receptor is involved. Firstly, the action of capsaicin is restricted to mammals, having no effect in birds (Pierau et al, 1987, Wood et al, 1988). There are some differences in the receptor complement of mammalian and avian dorsal root ganglion neurons, for example, the mammalian neurons have voltage-gated sodium channels which are sensitive and insensitive to tetrodotoxin (TTX) while avian neurons lack TTX-sensitive group of channels (Yoshida and Matsuda, 1979, Lawson et al, 1987, Pieray et al, 1987, Aguayo et al, 1991, Nowycky, 1992).

(2) Structure function relationships indicate that there are strict structural requirements for agonist and antagonist activity (Szallasi et al, 1989, Walpole and Wrigglesworth, 1993).

(3) Most importantly, development of analogues and antagonists have been described (see below). In particular, the existence of a reversible antagonist, Capsazepine, provides very strong evidence for the existence of a specific capsaicin receptor.

4.1.1 The capsaicin receptor has ligand-gated channel-like properties.

Recently, electrophysiological analysis of the capsaicin sensitive subset of dorsal root ganglion neurons in vitro has allowed the investigation of the ionic basis of capsaicin-evoked currents to be made (Bevan and Forbes, 1988). A conductance increase associated with an inward current was observed upon capsaicin treatment of sensitive cells, suggestive of the opening of ion channels. Single channel properties of an ion channel gated by capsaicin have also been examined on isolated membrane patches from DRG cells using voltage-clamp (Forbes and Bevan, 1988).
Capsaicin was shown to elicit an inward depolarising current through a non-selective cation channel (review - Bevan and Szolscanyi, 1990). The capsaicin-induced ion flux is non-selectively cationic, calcium, potassium and sodium being the most significant contribution to the flux, with no detectable anion contribution. Both mono- and divalent cations are permeant, in the decreasing sequence of \( \text{Ca}^{2+} > \text{Mg}^{2+} > \text{K}^+ > \text{Na}^+ \). Under physiological conditions, there is a \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) flux into the cells and an outward \( \text{K}^+ \) flux, the net result being to depolarise the cell towards \(-0\) mV.

The size of the capsaicin channel complex has been estimated by elimination of binding of RTX (a potent capsaicin analogue, see section 4.11, page 124) in pig DRG membranes using high energy radiation inactivation. The value obtained from these studies was 270 kDa \([/- 25 \text{ kDa}] \) (Szallasi and Blumberg, 1991b). The size range for ligand-gated channel subunits is around 40 - 60 kDa, producing pentameric complexes of around 230 - 263 kDa (Strange, 1988), and so this is comparable.

All of the above characteristics are reminiscent of the general properties that one would associate with cation selective ligand-gated ion channels such as the nAChR and glutamate gated channels. Additionally, the channel behaves in a completely voltage-independent manner at negative potentials. Isolated DRG membrane patches have also revealed the single channel conductance to be \(-100\) pS at \(+60\) mV and \(20-30\) pS at \(-60\) mV even with identical sodium chloride solutions on either side of the membrane patch. The conductance of the capsaicin channel at \(-60\) mV is comparable with the conductance of other ligand-gated channels such as the nAChR which range from \(-5 - 50\) pS (Role, 1992, Sargent. 1993).

Although part of the calcium component of the flux is due to the secondary activation of voltage-gated calcium channels - an elevation of cGMP in response to capsaicin is a secondary effect due to calcium uptake through these voltage activated channels (Wood et al, 1989) - there is still a relatively large calcium component to the flux relative to most ligand-gated channels (Decker and Dani, 1990, Mulle et al, 1992, Vernino et al, 1992, Seguela et al, 1993), and the level of calcium accumulation in the mitochondria of sensory neurons in culture induced by capsaicin has been estimated to be as high as 12 mMol/litre (Wood et al, 1989). This is significant in terms
of activation of calcium-dependent enzymes (see Fig 4.1, page 139, and James et al, 1993). Much of the calcium taken up is probably stored in mitochondria, as blockade of mitochondrial uptake results in an inhibition of the flux (Wood et al, 1989, Dray et al, 1990).

Although some inhibition of the capsaicin-evoked calcium flux can be achieved by use of Cinnarizine and Trifluoperazine (calcium voltage-gated channel blockers), the effects are seen following preincubations rather than when administered with capsaicin.

Additional permeant ions for the channel include rubidium, guanidinium and cobalt (Wood et al, 1989), indicating that it has a relatively large pore size comparable with other ligand-gated channels. The uptake of cobalt is significant enough to be visualised using a histochemical stain where internalised cobalt in DRG neurons is precipitated with ammonium sulphide (Winter, 1987). The calcium uptake can also easily be measured in these cells using $^{45}$Ca accumulation in mitochondria (Wood et al, 1989) under conditions where the calcium and cobalt fluxes in the same cells evoked by exposure to nicotine, acetylcholine or glutamate is unmeasurable.

There is no evidence to suggest that the flux is effected through a second messenger system (Marsh et al, 1987, Wood et al 1988, 1989). Although it has been shown that capsaicin elevates cAMP in rat brain (Jancso and Wollemann, 1977), cAMP is not elevated in response to capsaicin in DRG cells (Wood et al, 1989). cGMP is elevated by capsaicin in rat DRG neurons, but it has been shown that dihydropyridine channel blockers can inhibit this elevation while at the same time not abolishing the calcium uptake (Wood et al, 1989) suggesting that cGMP stimulation is an indirect effect caused by activation of voltage gated calcium channels. The rise in intracellular cGMP cannot be blocked by staurosporin, a protein kinase C inhibitor which has been shown to have no effect on the capsaicin gated flux (Dray et al, 1988, Winter et al 1990), and RTX, although possessing some structural similarities to phorbol esters, does not appear to activate PKCs (Dray et al, 1988, Winter et al, 1990).

The activity of channels in isolated membrane patches where second messengers are not present strongly indicate that the receptor/channel operates independently of such molecules. Inhibitors of phospholipase A2 and lipoxygenase were also shown to
reduce the cGMP response without affecting the calcium accumulation, indicating that the cGMP response may be due to activation of eicosanoid metabolism pathways (Wood et al, 1989). Activation of second messenger systems such as protein kinases clearly does not mimic the effects of capsaicin (Wood et al, 1989).

4.1 j **Structural requirements for capsaicin analogues.**

There are strict structural requirements for analogues if they are to show capsaicin-like activity (Szolcsanyi and Jancso-Gabor, 1975). Some capsaicin analogues can exhibit noxious activity but are not as potent in terms of desensitising activity, and, in contrast, other analogues such as ovanil are able to desensitize against capsaicin in the absence of stimulant activity (Dickenson et al, 1990, Dray et al, 1990b). Furthermore, in some assays of capsaicin-like activity, the toxin RTX is orders of magnitude more potent in desensitising than in exciting sensory neuron fibres (Blumberg et al, 1993).

A possible explanation of these observations is that excitation, desensitization and neurotoxicity of capsaicin-like vanilloids are mediated by a common site of action and that mismatches in the activity of some congeners arise from differences in their pharmokinetic behaviour and/or metabolic stability or from differences in the ligand-receptor interactions with respect to binding forces and binding reversibility. Support for this comes from the finding that many capsaicin congeners grossly correlate in their ability to stimulate and desensitise sensory neurons (James et al, 1988) and in their potency at damaging afferent neurons in neonatal rats (Jancso and Kiraly, 1981).

One approach to studying the capsaicin structure-function relationship is to divide the molecule up into three regions (see Fig 4.2, page 140), varying only one of the regions at a time while keeping the others constant (review: Walpole and Wrigglesworth, 1993).

The 3-methoxy-4-hydroxybenzyl ring substitution in the ‘A’ region confers the highest potency, and changes to this configuration can have dramatic effects. Alkylation or removal of the hydroxyl group (4-OH) reduces or removes activity, and a reduced activity results from all other single or multiple substitutions at positions 2-, 5- and 6- on the aromatic ring. However complete agonist activity was retained if the methoxy group was replaced with a second hydroxy group to give

Alterations in the 'B' region may have a more variable effect, although there are some generalisations that can be made. The optimal distance for the group between the dipolar part of the 'B' region and the 'A' region appears to be one carbon atom, and N-methylation of either the amide or thiourea group leads to loss of activity (Szallasi et al, 1989, Chen et al, 1992, Walpole and Wrigglesworth, 1993).

Studies on the effect of chain length for the 'C' region have indicated that the optimal chain length is 8-10 carbon atoms for agonist activity, but that in some of the compounds with long chains there was enhanced desensitizing activity and a drop in pungency (Dickenson et al, 1990, Dray et al, 1990b), one such compound is NE 19550 ('Ovanil') which is being further investigated by Proctor and Gamble. The double bond and the branched side chain do not appear to be important for activity, but other alterations to the chain are significant, as analogues with short side chains, with long polar side chains, with polar functional groups attached to the end of hydrophobic chains, or with long hydrophobic chains, show a lack of activity. A 4-chlorophenyl version of the side chain however proved to be more potent than the octyl chain (Szallasi et al, 1989, Chen et al, 1992, Walpole and Wrigglesworth, 1993).

The precise structure-activity relationship described above provides strong evidence for the existence of a specific receptor.

4.1 k A specific capsaicin antagonist, capsazepine.

Because of capsaicin's selectivity of action, there has been an interest in finding analogues of the molecule which would exert the same desensitizing effects without activating nociceptive functions. This has been seen as a possible route to produce analgesic drugs (Campbell et al, 1998, Dray et al, 1990b, Maggi, 1991 Szallasi and Blumberg, 1993).

The appearance of agonists with differing activating and desensitizing properties has suggested the feasibility of producing analgesic drugs related to these compounds (Maggi, 1991). The development of capsaicin agonists with less marked abilities to activate peripheral fibres but with similar antinociceptive and anti-
inflammatory effects (Dickenson et al, 1990, Dray et al, 1990) illustrates the progress made so far with this approach.

A competitive antagonist, capsazepine, has been developed which is both specific and reversible (Bevan et al, 1991). The existence of this antagonist conclusively proves that the main effects of capsaicin described above are due to its actions on a specific receptor. Capsazepine is able to block the antinociceptive effects of capsaicin in vivo (Perkins and Campbell, 1992) and specifically the activation and desensitization of primary afferents (Dickenson and Dray, 1991).

Capsazepine contains the optimal 3,4-dihydroxy substitution on the benzyl ring but has a seven-member ring constraint behind the ‘A’ region which holds the relative coplanar orientations of the ‘A’ and ‘B’ regions. In addition a 4-chlorophenyl side chain replaced the octyl chain. The constraining ring was developed to investigate the theory that the lack of activity of simple N-methyl analogues could be due to their inability to adopt the coplanar orientation (Walpole and Wrigglesworth, 1993).

4.1.1 Resiniferatoxin is an potent analogue of capsaicin.

Resiniferatoxin (RTX) was first isolated from the latex of Euphorbia resinifera. It has structural similarity with capsaicin only in the 3-methoxy, 4-hydroxybenzyl ring region but elicits a similar response from the same subset of primary afferent neurons (Bettaney et al, 1989, Szallasi and Blumberg, 1990, Blumberg et al, 1993). All evidence points to RTX and capsaicin acting on the same site. The analgesic and anti-inflammatory actions of RTX and capsaicin in rodents are very similar (Campbell et al, 1989) and it evokes similar changes in membrane permeability in DRG neurons (Winter et al, 1990).

RTX-binding sites are found in areas where capsaicin-sensitive neurons are found (Bettaney et al, 1989, Szallasi and Blumberg, 1990) and RTX binding to DRG membranes is inhibited by capsaicin but not by phorbol esters to which RTX has some structural similarities (Szallasi and Blumberg, 1990, 1991). Additionally, desensitization of trigeminal and dorsal root ganglion neurons with RTX is associated with loss of capsaicin receptors (Szallasi and Blumberg, 1992). Cross-desensitisation experiments (Winter et al, 1990) and localisation of binding (Szallasi and Blumberg, 1990) indicate that RTX and capsaicin act on the same subset of sensory
neurons and it seems likely that their mechanism of action is similar (Winter et al, 1990). In addition, nerve growth factor (NGF) co-regulates RTX binding and sensitivity to capsaicin in DRG neurons in culture (Winter et al, 1993).

RTX is a potent analogue of capsaicin and is a hundred times more potent than capsaicin in most functional comparisons, exerting similar excitatory, analgesic and anti-inflammatory effects at very low doses (Bettaney et al, 1989, Campbell, et al, 1989). The specific capsaicin antagonist, capsazepine, is competitive in a number of in vitro assays against both capsaicin and RTX (Bevan et al, 1992).

Conventional purification of capsaicin-binding molecules has been hampered by a failure to develop binding assays owing to the low affinity and lipophilicity of capsaicin. The characterisation of RTX as a potent analogue of capsaicin (Szallasi and Blumberg, 1990, Szallasi et al 1991, Winter et al 1990) has allowed the development of binding assays and estimates of the size of the capsaicin/RTX receptor to be made (Szallasi and Blumberg, 1991). Such a binding assay, as well as allowing purification and characterisation of the capsaicin-binding site also provides a possible screen for the detection of recombinantly expressed resiniferatoxin-binding proteins.

4.1 Photoaffinity probes.

Prior to the discovery of capsazepine, indirect evidence for the existence of a receptor for capsaicin came from photoaffinity label analogues of capsaicin. One of these compounds causes long-lasting stimulation of calcium uptake in sensory neurons after irradiation in contact with cells, a stimulation that could be blocked by the presence of reversible capsaicin agonists during the irradiation step (James et al, 1988, 1993).

This suggested that the photoaffinity labels covalently bind to a specific capsaicin binding site. However, although proteins selective for DRG were labelled, capsaicin was unable to displace the label under assay conditions: thus, the proteins could not be identified as specific binding sites (Wood et al, 1990b).

The low affinity and lipophilicity of capsaicin has precluded the development of binding studies and the screening of expression libraries for proteins that bind capsaicin. A photoaffinity label derived from RTX was produced to enable such screening. The compound, RTX-PAL (see Fig 4.2, page 140), reversibly inhibits
binding of RTX to DRG membranes in the dark but irreversibly inhibits RTX binding after irradiation (James et al, 1993, Ninkina et al, 1994).

4.1 Physiological significance of the capsaicin receptor.

Despite the obvious specific excitatory effects of activation by capsaicin, it is unclear what the physiological significance of the receptor might be. This is partly because capsaicin and RTX are non-endogenous substances and the existence of a physiological ligand has not been proven. An anti-capsaicin antiserum characterised in a radioimmunoassay failed to detect material from either normal or damaged tissue that shared immunochemical determinants with capsaicin (Wood et al, 1990a).

Tissue damage can sometimes result in a local drop in pH (e.g. Corbe and Poole-Wilson, 1980). A membrane receptor which is activated by protons which has many similarities to the capsaicin channel exists in DRG neurons, and this has prompted the suggestion that that protons and capsaicin may act on the same channel (Bevan and Yeats, 1990, Bevan and Szolcsanyi, 1990). This does not exclude the possibility that other ligands act on the same channel.

The cloning of the capsaicin receptor would be of interest for a number of reasons. Firstly, knowing which family of receptors or channels that it is related to would help in deducing a physiological role in the activation of primary afferents. The expression pattern of the receptor could be determined which may contribute to the further definition of nociceptive pathways.

In addition, expression of the recombinant receptor in different systems could provide easily available material for use in binding studies for the development of partial capsaicin agonists which would desensitise sensory neurons without activating them. Finally, the expression of the capsaicin receptor in, for instance, *Xenopus* oocytes, could allow candidate ligands to be tested without having a significant number of other channels present. This might be approached by applying material from damaged tissue to oocytes expressing the receptor and testing for an electrophysiological response. In the presence of a detectable response, the tissue material could be fractionated as a first step towards purifying the receptor ligand.
4.2 An investigation into whether the capsaicin receptor can be desensitized by the application of some classical receptor ligands.

Investigation of capsaicin receptor desensitization using $^{45}$Ca Uptake assay.

This investigation of the capsaicin response involved preincubation of the cells with nicotine and glutamate in order to investigate possible desensitization of the capsaicin-evoked response. Desensitization of nAChR and glutamate channels has been well studied (Huettnner, 1990, Simmons et al, 1990, Cachelin and Jaggi, 1991, Gross et al, 1991, Revah et al, 1991) and it suggested a possible approach to getting evidence that the capsaicin receptor was related to either the glutamate or nAChR channel families prior to attempts at cloning the receptor.

4.2 a Use of a $^{45}$Ca Uptake assay.

As mentioned above, the calcium component of the flux induced by capsaicin is large enough to be measured with a calcium uptake assay using $^{45}$Ca (Wood et al, 1988). This functional assay was utilized in two separate types of experiment. In one, the assay was used to measure changes in capsaicin-evoked calcium uptake after preincubation of DRG neurons in culture in classical channel ligands to see if cross-desensitzation occurred.

For the desensitization experiments, the basic assay was performed as described in methods (section 2.6 page 37), but various substances were added to the cells in culture prior to assay in order to see if they could produce a desensitization to capsaicin. The substances used for preincubation were capsaicin at 2 $\mu$M, nicotine at 100 $\mu$M and glutamate at 2 mM. Dilutions of these agonists in assay buffer with DMSO were then added to the wells and the cells were incubated for 10 minutes at 37°C with 3% CO$_2$ before proceeding on to the standard assay (see methods, section 2.6 page 37).

Results of desensitization experiment.

The assay results were averaged from six wells for each treatment (see Fig 4.3, page 141). Control wells show that the preincubations did not affect calcium uptake. The results clearly show that preincubation with either of the two ligands had little effect
on the subsequent sensitivity of DRG neurons in culture to capsaicin as measured by uptake of radioactive calcium. In contrast, however, pretreatment with capsaicin effectively abolishes the subsequent response to a second application of capsaicin.

4.3 Cloning the Capsaicin-Gated Channel.

4.3a Cloning strategies.

Conventional purification of capsaicin-binding molecules has been hampered by a failure to develop binding assays owing to the low affinity and lipophilicity of capsaicin. As mentioned above, the characterisation of RTX has allowed the development of binding assays, allowing purification and characterisation of the capsaicin-binding site, and also providing a possible screen for the detection of recombinantly expressed resiniferatoxin-binding proteins.

As no clear response to capsaicin or RTX can be detected in Xenopus oocytes injected with total or mRNA from rat DRG using twin electrode whole cell voltage clamp recording methods (unpublished observations, Wood, J.N., and Boulter, J., respectively, personal communications), functional screening of fractionated RNA, the most direct of methods (for example, Hollman et al, 1989), is eliminated as a possible strategy for cloning of the capsaicin-gated channel.

In addition, no structural information to make nucleotide homology screening viable was available, so attention turned to the possibility of screening for the presence of binding sites on the channel as has been successfully employed in the cloning of some G-protein coupled channels (e.g., Sikela et al 1987).

Unfortunately, no high-affinity radio-labelled analogues of resiniferatoxin or capsaicin were commercially available, and so this made direct binding approaches less attractive. Attention turned to the possible use of a capsaicin-like photoaffinity label to identify RTX/Capsaicin-binding proteins (Wood et al, 1990b, James et al, 1993). By screening for proteins that can bind to RTX-PAL in a DRG expression library, it was hoped that the capsaicin/RTX-binding component of the channel could be identified.

An anti-capsaicin antiserum (Wood et al, 1990b) characterised by radioimmunoassay with a range of capsaicin congeners was utilised to detect proteins bound by the photoaffinity label.
4.3 b RTX screening protocol.

Briefly, an amplified rat DRG library constructed in λZap II was plated in *E.coli* BB4 cells and duplicate filter replicates from plates were made and incubated inverted on NZYCM plates. The filters were pre-soaked in IPTG to induce expression of insert cDNAs as β-galactosidase fusion proteins. Filters were then incubated with resiniferanol-9, 13, 14-orthophenylacetate-20-(-3-azido,4-methoxyphenyl) acetate (RTX-PAL), the photoaffinity label, (see Fig 4.2 and 4.4, pages 140 & 142 and James et al, 1993) and U.V. irradiated. This was followed by incubation with a rabbit polyclonal antiserum that had been raised to RTX photoaffinity proteins (4-aminoethyl resiniferatoxin conjugated to thyroglobulin with gluteraldehyde was used for immunization, see Harlow and Lane, 1990 for method) and then with an alkaline phosphatase-linked sheep anti-rabbit immunoglobulin antiserum (Miles) which was detected using nitroblue tetrazolium and bromochloroindolyl phosphate (Sigma - for detailed description of methods see Ninkina et al, 1994). Control filters were not irradiated, but were otherwise treated identically.

Results of photoaffinity screening of the expression library.

(Screening was performed collaboratively with N.N. Ninkina and J.N. Wood). Out of 1.3 x 10^6 clones screened, twenty plaques showed strong binding and were picked and purified to single clones by secondary screening. Of these twenty, five showed a significant difference between the U.V. irradiated and the non-irradiated halves of the lift filter and were plasmid rescued for further analysis. Cross-hybridising Southern blots showed that two of these clones RTX4 and 13 were similar in size (~1.2 Kb) and also probably identical, the other, RTX17 4.2 Kb was unique (N.N. Ninkina, personal communication).

4.3 c Analysis of RTX-binding clones - Sequence analysis.

Sequence data was obtained from the ends of each of the clones using standard sequencing methods (see methods, section 2.16 page 46). This was done so that the sequence could be checked against the genbank/EMBL databank to ascertain that it was a novel protein, and also so that PCR primers could be designed to match the
sequence, thus allowing comparative PCR analysis of rat DRG mRNA from cells cultured in the presence and absence of NGF.

A sequence search using one of these clones (RTX17), 4.7 Kb in size, against the nucleotide database identified it as a rat homologue of human topoisomerase II on the basis of homology, as it showed 87% homology at the nucleotide level and 78% homology at the amino acid level in a piece of sequence from the 5' end of the clone (see Fig 4.5, page 143).

Nucleotide sequence from another clone, RTX13 however showed no significant homology with anything in the database. Attention focussed on RTX13 because of its strong binding to the RTX-PAL and because two versions of it had been independently isolated from the same screen from separate plates.

A second 'RTX13' clone (RTX4) which had been isolated in a separate screening was able to hybridise to RTX13 on Southern blots when probed under stringent conditions and was slightly smaller in size (N.N. Ninkina, personal communication).

4.3d Obtaining a full length version of RTX13.

The insert to clone RTX13 was 32P-labelled and used to screen a second, non-amplified DRG library (oligo-dT and random hexamer primed) by nucleotide homology screening under stringent conditions (see methods, section 2.14 page 44) and further versions of the clone were isolated, the longest, RTX42, being 1.7 Kb in length.

Clone RTX42 was sequenced in its entirety using caesium purified plasmid as a template (see methods, section 2.16 page 46) and was shown to have a novel sequence in which the largest open reading frame was of 702 b.p., encoding a hypothetical protein of 26,548 daltons (see Fig 4.6, page 144). Three potential initiator methionine codons with preceding Kozak consensus sequences (Kozak, 1989) exist in frame with the original fusion protein RTX13.

4.3e Investigating the effect of NGF on regulation of RTX13.

A quantitative PCR to examine the possibility of NGF regulation of RTX42 was performed using primers to the 3' untranslated region of RTX42 (all experimental details of comparative PCRs are described in chapter 6).

It was found that RTX42 appeared to be unchanged in terms of mRNA levels in normalised rat DRG cultures grown in the
presence or absence of NGF (see chapter 6 for discussion of controls and results, Table 6.3, page 198 and Fig. 6.9, page 207 for results and graph. Discussion of result is below, section 4.4b, page 137).

4.3f Summary of further results.

Further analysis was performed on RTX42, the full length version of RTX13 (N. Ninkina, M.M. Beech and J.N. Wood, personal communication). They are detailed below.

RACE PCR.

RACE PCR (Frohman et al, 1988, Frohman, 1990, see also previous chapter) was used to determine whether additional upstream sequence existed in the corresponding message, although the apparent size of the mRNA on Northern blots indicated that RTX42 was probably almost full length. Several slightly larger clones were generated, the longest ones being 21 b.p. larger than RTX42 (see Fig 2., Ninkina et al, 1994). Several of these were sequenced and found to have identical sequence.

Primer extension.

To show that that the 5' end sequence of the RACE clones was the same as the end of the mRNA from DRG tissue, primer extension was performed with different preparations of DRG RNA (see Ninkina et al, 1994), using a 35-mer complementary to the 5' end of the clone.

In-situ hybridisation.

An investigation into whether specific cell types within the DRG express the gene was performed using an alkaline conjugated 31 b.p. oligonucleotide to 5' untranslated sequence on both cultured cells and on frozen sections. The marker dye could be seen clearly in neuronal cell bodies and less markedly in non-neuronal cell types (see Fig. 5, Ninkina et al, 1994).

In Vitro Translation.

Linearised RTX42 plasmid was used as a template for translation from the T3 promoter in a rabbit reticulocyte lysate system. [\(^{35}\)S] methionine was included in the reaction so that the protein products were labelled and could be visualised on 12.5% SDS-PAGE. Three products of approximate size 23, 25 and 26 kDa were seen when analysed by PAGE (see Fig. 6, Ninkina et al, 1994), corresponding reasonably well to the sizes of the three theoretical proteins of 24.4, 25.7 and 26.6 kDa predicted by the nucleotide sequence.
Expressed protein.
A synthetic peptide corresponding to a stretch of sequence from the open reading frame of RTX42 that showed no homology with any other known protein was made and coupled to thyroglobulin with gluteraldehyde and used to generate polyclonal antisera in rabbits. The antisera were assessed by solid phase immunoassay on peptide-coated 96-well PVC plates. The hyperimmune sera was affinity purified by chromatography on columns of peptide conjugated to Affigel-10. Acid and alkali washes were used for elution.

The purified antisera was used to probe Western blots of rat tissue homogenates that had been separated on 12.5% SDS-PAGE gels. Alkaline phosphatase conjugated anti-rabbit antisera was used to visualise the binding of the first antisera when developed with nitroblue tetrazolium/bromochloroindolyl phosphate.

The affinity purified antisera bound to a single protein of apparent molecular weight 36 kDa in both DRG and spleen homogenates (N. Ninkina personal communication, Ninkina et al, 1994).

Binding of [3H]-RTX to isolated proteins.
A number of bacterial clones were tested directly for [3H]-RTX binding in order to confirm that the screening method was isolating fusion proteins with RTX-binding ability. Several clones exhibited specific displaceable binding, most notably clone RTX-13 in which a 1.2 Kb insert encoding protein sequence corresponding to the C-terminal end of the predicted peptide of RTX42 in-frame with the fragment of β-galactosidase gene.

Subsequently, COS-7 cells were transfected with RTX42 subcloned from Bluescript II into the transfection vector pKS1 using calcium phosphate. Two days after transfection the washed harvested cells were sonicated in buffer and incubated in 2 nM [3H]-RTX with various competing concentrations of cold RTX (0.1-1µM) or capsaicin (10µM) at room temperature for forty minutes. The cell extract was chromatographed in gel exclusion columns and counts eluted in the void volume were used as a measure of binding. Capsaicin-displaceable binding was apparent in the cytoplasmic soluble fraction but not the membrane fractions. A gel filtration protocol was used to assess the binding of [3H]-RTX to COS cell expressed RBP26 (the protein product of RTX42), where bound and free ligand were separated on polyacrylamide gel columns. The half
maximal binding was shown to be 10 nM (see Fig. 7., Ninkina et al, 1994).

**Functional Analysis of RTX-binding protein.**
RNA transcripts from RTX42 alone and conjunction with purified DRG mRNA were injected into Xenopus oocytes and left for two days to be translated. A twin electrode voltage clamp system was then used to look for responsiveness to capsaicin or RTX. No clear current was elicited (J.N. Wood, unpublished data).

**Expression of RTX-binding clones.**
The expression distribution of the clones was investigated by probing Northern blots containing lanes of rat cortex, cerebellum, spinal cord, DRG, heart, lung adrenal gland and spleen RNA.

A Northern blot containing RNA from various tissues under stringent conditions was probed with the $^{32}$P-labelled insert of clone RTX13 under stringent conditions. Afterwards the blot was stripped of probe and reprobed with a PCR product for the ribosomal protein L27 (see chapter 6 for description of probe). Southern results for the different tissues with RTX13 were normalised against the signal received after probing with L27.

Results revealed that the gene was expressed as an RNA species of apparent size 1.6 Kb in several regions, most strongly in cerebellum and DRG, but clear expression was seen in cortex, spinal cord and adrenal gland (see Fig 4, Ninkina et al, 1994), tissues that are capsaicin insensitive.

The expression of other candidate clones was also investigated (N.N. Ninkina, personal communication, summarized in James et al, 1993).

**4.3 g Use of antisense oligonucleotides to RTX42 in $^{45}$Ca uptake assay.**

In this experiment, the assay described above was used to measure changes in capsaicin-evoked calcium uptake after the depletion of specific mRNAs was achieved by growing cells in the presence of antisense oligonucleotides. This was used in an attempt to get positive evidence that RTX42, the candidate clone isolated during the photoaffinity screening procedure, was involved in the capsaicin response. The method was also used to investigate the possible involvement of other genes in the capsaicin response (details
of the experiment are described in chapter 5 and methods, section 2.6 page 37).

The basic assay was performed as described in methods. DRG neurons were grown for 4 days in the absence of NGF, and then with NGF in the presence of a thiol-linked oligonucleotide complementary to the the N-terminal sequence of RTX42 (RTXT) which was added at a concentration of 5 μM. Fresh medium containing NGF and oligonucleotide was added every day for a further four days until the assay.

Results of antisense oligonucleotide assay.

The assay results show that the antisense oligonucleotide designed to block expression of the RTX42 gene did not affect the calcium uptake elicited by capsaicin (see Figure 5.4, page 160). This implies that clone RTX42 is not a component of the capsaicin receptor. A scrambled control nonsense oligonucleotide similarly did not affect the uptake of calcium.

Section 4.4 - Discussion of results and RTX-binding clones.

4.4 a RTX-binding clones.

On the basis of the evidence in the introduction to this section, it seems possible that the capsaicin-gated channel might be a directly ligand-gated channel, although other possibilities exist. For instance, although evidence has been presented to suggest that one of the channels gated by protons in DRG neurons may also be the capsaicin-activated channel (Bevan and Yates, 1991), another described response to protons is thought to be due to the proton conversion of a voltage-gated sodium channel (Bevan and Yates, 1991).

As capsaicin is a non-endogenous ligand, it is possible that it too may be causing a membrane-bound structure to be operated in an atypical manner, which may be non-physiological and irrelevant to the structure's normal function. Whether the capsaicin receptor is a ligand-gated channel, or whether, if it is a ligand gated channel, it resembles other ligand-gated channels in general design, remains to be determined.

The desensitization experiments were designed to see if the capsaicin channel might desensitize after exposure to glutamate or nicotine, ligands which can activate and desensitize members of
their respective families of ligand-gated channels. The concentrations of glutamate and nicotine used were higher than those that have been reported to cause receptor desensitization (Huettner, 1990, Zhang et al, 1994). The results show clearly that the two agonists have no effect on capsaicin response in cultured DRG neurons.

4.4b Cloning of the capsaicin-gated channel.

As mentioned, functional channels are not detectable after injection of rat DRG mRNA into Xenopus oocytes (unpublished observations, J.Boulter, J.Wood), and some of the possible reasons for this lack of function were important considerations when designing the cloning strategy.

If the capsaicin receptor is a member of the ligand-gated channel or even perhaps the voltage-gated channel superfamilies and consists of a multimer of discrete subunits like others already described (reviews, Betz, 1990a, Stroud, et al, 1990), it is possible that it may not be a homo-oligomer, and further, that individual subunits of the complex may not function as homo-oligomers. This possibility could in part explain the lack of capsaicin response in oocytes injected with DRG RNA (see below for further discussion).

The lack of capsaicin response in oocytes injected with DRG total RNA or mRNA may be due to logistical problems in getting enough RNA into the oocyte to allow the presence of several different subunits present in low abundance to be represented in sufficient quantity to allow channel formation. Alternatively, the oocyte may lack some species or tissue-specific processes which are necessary either to assemble components of the channel into a complete channel, insert the channel or its components into the oocyte membrane, or to modify an assembled precursor channel complex into a functional form.

It can be seen that the assay employed here as a screening method functioned effectively in detecting fusion proteins derived from a DRG cDNA library expressed in bacterial cells that had affinity for the photoaffinity probe RTX-PAL. Although some of the clones did not have reproducible RTX-binding properties when rescreened, several of these fusion proteins proved to be genuine RTX-binding proteins and were selected for further analysis.
However, the difficulties that applied to screening for the capsaicin receptor using functional approaches also applied to the investigation of the fusion proteins selected by the screening procedure.

Given the isolation of proteins possessing RTX- and capsaicin-binding properties that were not immediately identifiable as channel components either by sequence homology, or by structural similarity to other members of the various receptor superfamilies, there were a few direct functional tests that could be applied to these clones that could potentially provide positive functional evidence for their involvement in the capsaicin receptor.

One of these was expression of clones in the Xenopus oocyte system, but again, the explanations for a lack of capsaicin response to RNA from DRG cells would, if true, also apply to the testing of a single isolated clone (i.e. if more than one type of subunit was required, or if some other cellular function required to make a functional receptor was lacking in the oocyte.). The second possibility for obtaining positive evidence for involvement of the selected clones in the capsaicin receptor was via antisense oligonucleotide experiments. If by blocking the translation of the cloned proteins using antisense oligonucleotides in capsaicin-insensitive DRG cells (cultured in the absence of NGF) the return of the capsaicin response could be blocked after re-exposure of the cells to NGF, it would indicate that the proteins encoded by the clones were probably involved in the response to capsaicin.

The lack of a positive result in the antisense experiments should also not be taken as absolute proof that the clones isolated are not related to the capsaicin receptor, while positive results involving the abolition of function by two separate antisense oligonucleotides to one of the RTX-binding proteins would have been interesting and worthy of further study, negative results cannot be invested with the same importance. The success of antisense oligonucleotide-mediated blockage of mRNA translation can not be confidently predicted because the mechanism by which translation is blocked is not fully understood (Marcus-Sekura, 1988). Ideally an antibody against the targeted protein should be used to confirm that the levels of the protein have been altered.

It is also possible that the presence/absence of capsaicin responses and binding in DRG cells regulated by NGF could be due to
structural or allosteric modifications of the receptor rather than to the presence or absence of the receptor. In this instance a stable membrane bound protein in these experiments could be converted to a non-functional form in the absence of NGF and then converted back when NGF was reapplied, without being affected by the lack of synthesis of one of the components.

The methods of analysis left for the clones provided only indirect information about their possible relationship to the capsaicin receptor. RTX42 was the clone that had been independently selected twice from different library screens and was selected as a candidate clone to be analysed in antisense experiments and also in expression studies.

The clones isolated as Zapll were analysed by taking stretches of sequence from either end of the rescued Bluescript II clones using standard sequencing methods (methods, section 2.16 page 46). On this basis previously identified genes not related to channels were rejected, for instance, a rat topoisomerase II (see Fig. 4.5, page 143) with a wide distribution outside the nervous system (N.N. Ninkina, personal communication).

The remaining clones were analysed with a view to picking the best candidates as the screening continued, but RTX42 was selected early on because of its binding properties and because it had been isolated twice, and a full-length version of it was isolated.

Sequence analysis of RTX42 showed that it had only a single putative membrane spanning region unlike other members of the voltage- or ligand- gated channel families, although some membrane proteins with unusual structures have been reported, including some annexins, (Rojas et al, 1990, Cruetz, 1992) and ion antiporters (Sardet et al, 1989).

Expression studies with RTX42 demonstrated that these genes were not confined to the regions displaying capsaicin sensitivity, being found in several areas, including some non-neuronal tissues, although RTX42 is most highly expressed in DRG, and more specifically in DRG neurons as revealed by in situ hybridisation (Ninkina et al, 1994). NGF was not able to regulate the levels of RTX42 mRNA when examined by quantitative PCR, although as discussed above, the RNA level of channel components may not have much relevance to the amount of functional channel over a short period of time. The sequence of RTX42 possesses only a single hydrophobic
region large enough to span a cell membrane and thus does not resemble the conserved structure of either the known ligand-gated ion channel subunits or known voltage gated ion channels. Kinase-linked receptors such as the Trk receptors (see section 6.1 b, page 165) also possess a single large hydrophobic region, but RTX42 does not possess any homology to any known members of this family.

Recent work utilising antibodies raised against the putative coding region has confirmed that RTX42 is expressed in DRG cells, although it is not known which of the several possible forms is present. The transfection of cloned mammalian receptor genes into COS-7 cells and their successful expression and insertion into the cell membrane has been described (e.g. nAChRs, Gu et al, 1991b). The antisera also allowed a demonstration that the protein is not inserted into the membrane when transfected into COS-7 cells, suggesting that it is not a membrane-bound protein. It is again possible that some specific insertion mechanism could be lacking in these cells.

While the preceding studies could not conclusively demonstrate that the clones isolated are not part of the capsaicin receptor or channel, the accumulative circumstantial evidence suggests that the clones are probably not related to the capsaicin channel.

A possibility which would render this screening approach unlikely to work is that the binding of capsaicin and RTX to the receptor could be dependent on the presence of more than one subunit. This is the case with the neuromuscular nicotinic acetylcholine receptor, where ACh binds to the α subunit but binding is also determined by one of the adjacent subunits (Blount and Merlie, 1989). Additionally the agonist sensitivities of the neuronal nAChRs have been shown to be dependent on both β and α subunits (Luetje and Patrick, 1991) suggesting that more than one subunit may be involved in the agonist binding sites.

This cloning strategy has, however, demonstrated that there are a variety of proteins present in the mammalian nervous system which are able, to a greater or lesser extent, to bind to RTX. This must prompt increased caution in drawing conclusions about the distribution of the capsaicin channel from results of RTX or capsaicin-binding studies (Holzer, 1991).
CLOSED

Ligand?

Low pH?

Na, Ca

OPEN

Calcium

PI

MITOCHONDRIA

Calcineurin

Calmodulin

Second messengers

cGMP, Arachidonic acid

PLA2 PLC PK

Figure 4.1

Diagram showing the capsaicin receptor as a putative ligand-gated ion channel and the effect of calcium influx into capsaicin sensitive cells. Cai = free calcium, ER = endoplasmic reticulum, PLA2 = phospholipase A2, PK = calcium activated kinases, PLC = phospholipase C, VSC = voltage sensitive channels. Reproduced from James et al, 1993.
Figure 4.2

Diagrams showing the structures of capsaicin, capsazepine, RTX (Resiniferatoxin) and RTX photoaffinity probe, 249-555 (RTX-PAL). The dotted lines indicate the regions into which capsaicin is sometimes notionally divided for purposes of structure-function studies.
Graph showing results of the $^{45}$Ca uptake experiment in which DRG neurons grown in culture in the presence of NGF were pretreated with the ligands nicotine and glutamate before the standard capsaicin-evoked calcium assay (see methods). Graph shows uptake of $^{45}$Ca (d.p.m.) in control (CON) and capsaicin-treated DRG cultures (caps-CON); capsaicin stimulated $^{45}$Ca uptake in capsaicin pretreated positive control (caps-CAPS), 'NIC' and 'GLU' indicate pretreatments with nicotine and glutamate, 'caps' (lower case) indicates capsaicin administered during assay.

No difference is seen between no pretreatment and pretreatment with nicotine or glutamate (no significant difference at the 5% level). Pretreatment with capsaicin abolishes subsequent capsaicin response, n=6, ± SD shown.
Figure 4.4

Diagram showing the screening strategy used to detect and isolate RTX-binding proteins from a rat DRG cDNA expression library. (A) IPTG-impregnated nitrocellulose filters are used to make replicas from a DRG library in λ ZapII. Presence of IPTG induces expression of β-gal fusion proteins (B) Filters are incubated with RTX-PAL and washed (C) Filters are UV irradiated (D) Filters are blocked by incubation with haemoglobin and then incubated in rabbit anti-RTX serum at 1:2500 dilution (E) Filters washed and incubated in alkaline phosphatase-conjugated second-stage anti-rabbit antiserum (F) positive clones visualised by use of BCIP/NBT reagents.
<table>
<thead>
<tr>
<th>RTX 17</th>
<th>GAC AGA -A GAA GCT CGC CCA AGC GGA AG-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hum Topo II</td>
<td>GAC TGA AGA GAA ACT AGC ACA AGC AGA AGC</td>
</tr>
</tbody>
</table>

| TGC CGG CCT GCA TAA AGT TTT TAA ACT CCA AAC CAC -CT |
|---------|------------------|
| TGC TGG ACT GCA TAA AGT TTT TAA ACT TCA AAC TAC TCT |

| TAC TIG CAA TIC CAT GGT ACC TTT GAA TCA TAT GGG ATG |
|---------|------------------|
| TAC TIG TAA TIC CAT GGT ACT TTT TGA TCA TAT GGG ATG |

| TCT GAA GAA GIA TGA AAC TGT GCA AGA CAT TTT GAA AGA |
|---------|------------------|
| TCT GAA GAA ATA TGA AAC TGT GCA AGA CAT TCT GAA AGA |

** Figure 4.5a **
Alignment of partial nucleotide sequence taken from RTX17 with human topoisomerase II showing 87% nucleotide identity (performed with Intelligenetics software). Asterisks indicate base changes.

<table>
<thead>
<tr>
<th>R-17</th>
<th>TPN HLT CNS MVP LNH MGC LKK YET VQD ILK EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topo</td>
<td>LQT TLT CNS MVL FDH MGC LKK YET VQD ILK EF</td>
</tr>
</tbody>
</table>

** Figure 4.5b **
Alignment of deduced partial amino acid sequence from partial nucleotide sequence of RTX17 with amino acid sequence of human topoisomerase II, showing that the proteins have 78% homology (performed with Intelligenetics software). Asterisks indicate residue changes.
**Figure 4.6** Complete nucleotide sequence of clone RTX-42, showing the sequence of the predicted protein RBP-26 (Ninkina et al, 1994). Possible initiator methionines preceded by upstream Kozak consensus sequences which are in frame with the initially identified fusion protein are underlined. The hydrophobicity plot of RBP-26 uses a window of six amino acids with Kyte and Doolittle analysis.
Chapter 5. Transcription factors in DRG neurons.

5.1 Introduction - transcription factors.

5.1a Transcription factors as potential mediators of hyperalgesia?

The transcription of cellular genes is regulated by special proteins which recognise and bind to specific DNA sequences, either enhancing or repressing expression of the gene. These proteins are called transcription factors, and are grouped into families on the basis of conserved domains which are involved in the interactions with DNA. These factors act by regulating the transcription of defined genes, for instance, the appearance of Oct-1 transcription factor protein in many cell types correlates with the transcription of many generally expressed genes that contain the octamer motif - a conserved DNA sequence targeted by Oct proteins as a binding site (Schaffner, 1989), while some versions of the Oct-2 transcription factor seem to be expressed specifically in B-cells of the immune system where they are important in the regulation of immunoglobulin genes (Scheidereit et al, 1987).

Some members of the POU family of transcription factors have a limited distribution, in the CNS and PNS (see Table 5.1, page 156), and have come under scrutiny as candidates for mediators of neuronal-specific transcriptional control. Members of the POU family are related by the possession of a conserved domain (reviews, Herr et al, 1988, Ruvkin and Finney, 1991), first described in (and named from) the mammalian transcription factors Pit-1, Oct-1, Oct-2, and the developmental control gene Unc-86 from the nematode C.elegans (Finney et al 1988, Finney and Ruvkin, 1990). Since the discovery of these prototypical POU domain genes, an increasing number of related but distinct genes has been described.

The conserved region of POU proteins consists of two domains, a carboxy-terminal POU-homeodomain and an amino-terminal POU-specific region separated by a short, less conserved linker region (Sturm and Herr, 1988, He et al, 1989, Latchman, 1990, and see Fig. 5.1, page 157). Deletion and mutation studies have indicated that both homeodomain and POU-specific domains are probably required for specific DNA binding - mutations in either the POU specific domain or the POU homeodomain strongly affect binding of Oct-1 and Pit-1 to their target sequences (Gerster et al, 1990, Latchman, 1990, Muller-Immergluck et al, 1990, Tanaka and
Members of the POU family are able to bind with high affinity to specific DNA sequences (review; Ruvkin and Finney, 1991). The DNA sequences which form these distinct, high affinity binding sites are found upstream of a variety of genes. POU proteins are capable of binding to single sites. Two molecules of both Oct-2 and Pit-1 bind cooperatively to natural sequences containing two binding sites (Ingraham et al, 1990).

Alteration of the Unc-86 gene from the nematode *C. elegans* has been shown to result in a behavioural mutant (Finney et al 1988, Finney and Ruvkin, 1990), and mutations in the gene result in animals which have a reduced ability to detect mechanical sensations. While Unc-86 alone may not define particular neuronal cell types, it is clear that it contributes to the phenotype of several related cell types (Finney and Ruvkin, 1990), and interacts with at least one other transcription factor, Mec-3 (Xue et al, 1993) which in turn is required for expression of Mec-4 and Mec-7 genes which are required for touch receptor function (Way and Chalfie, 1988, Chalfie and Au, 1989).

Other members of the POU family are expressed during development and in adult animals, and some of these are similarly thought to play a role in the development and maintenance of some cell phenotypes (reviews; Ruvkin and Finney, 1991, He and Rosenfeld, 1991). Further mammalian members of the POU family analogous to Unc-86, Brn-1, Brn-2, Brn-3 and Tst-1, were isolated by use of PCR with degenerate primers to conserved POU domain regions (He et al, 1989). New members of the family are still being discovered, including an inhibitory protein, I-POU, which has been identified in Drosophila (Treacy et al, 1991). While the DNA-binding and transcriptional activation domains in newly identified POU proteins can be predicted on the basis of sequence homology, the target sequences for many of them are not yet known.

The neuronal distribution of some members of the POU family in mammals suggests that these proteins may have a part to play in neural-specific gene regulation (see Table 5.1, page 156). Oct-2, which is associated mainly with transcriptional control of immunoglobulin genes in B-cells of the immune system, has several splice variants which appear to arise from a single gene (Wirth et al, 1990, Stoykova
et al, 1992). One of these forms - 'neuronal Oct-2', differs from the others in DNA-binding specificity and functional activity (Dent et al, 1991), and is expressed in dorsal root ganglion neurons and neuronal cell lines including ND7 cells (Lillycrop et al, 1991). In addition, Brn-3 (a homologue of Unc-86 - now called Brn-3a), has a limited distribution of expression and is mainly found in sensory neurons (He et al, 1989, and see table 5.1, page 156).

More recently, negative regulatory elements with an Octamer-like repeat have been identified in a novel exon upstream of the chick α2 neuronal nAChR subunit, (Bessis et al, 1993). This repeat is altered from the Oct motif, but has similarities with it. It did not bind to any of the members of the Oct family. However, expression studies with the Oct-like repeats revealed that they had a silencer activity as naturally arranged, but could result in enhancer activity if present in fewer copies (Bessis et al, 1993).

5.2 New clones of a transcription factor expressed in DRG neurons.

The presence of Oct-like motifs upstream of a neuronal ion channel subunit raises the possibility that octamer motifs might regulate other ion channel subunit genes. As NGF has been shown to play a part in the regulation of such ion channels and also to regulate sensitivity to capsaicin and the mRNA levels of the preprotackynin gene in DRG neurons (see Figure 6.9, page 207 and Chapter 6 section 6.3 f, page 192), it was planned to investigate the transcription of three POU proteins expressed in DRG neurons to see if these were similarly upregulated by NGF. A parallel increase in the level of any of these proteins would then be circumstantial evidence suggesting the possibility that NGF might exert its effects by upregulating a transcription factor(s) which in turn increased the transcription of specific genes.

A study of the levels of these transcription factors in adult rat DRG neurons grown in culture with either the addition of NGF or anti-NGF antibodies using comparative PCR was planned (details described in Chapter 2, section 2.20, page 49, and Chapter 6, section 6.2 f, page 177). Neuronal Oct-2 and Brn-3 were selected for investigation, and Oct-1, a ubiquitous transcription factor was added to the study for purposes of comparison.

However, although primers capable of amplifying the neuronal Oct-2 from DRG cells were available (Lillycrop et al, 1991),
the only sequences from rodent Brn-3 available at the time were partial sequences of the most conserved areas of the gene (He et al., 1989, Goldsborough et al., 1990, and see discussion below). As these sequences covered only the POU regions, it was not possible to design primers specific to Brn-3 that would not risk amplifying sequences from similar related POU proteins.

It was therefore decided to homology screen a neonatal rat cDNA DRG library with a probe to the POU region in order to isolate new Brn-3 clones containing sequences specific to the gene from outside the conserved POU regions that could be used to distinguish Brn-3 from other POU proteins (see discussion). The high degree of nucleotide homology between the DNA-binding regions of members of the POU protein family required high stringency hybridisation and washing conditions in order to ensure that the clones isolated corresponded mainly to the desired gene.

5.2 a Screening a DRG library for genes encoding transcription factors.

A neonatal rat cDNA DRG library (the kind gift of J. Boulter, Salk Institute) constructed in λ Zap II was plated at a density of 1-2 x 10^5 plaques per 13 cm-square plate on NZY agar and screened using conventional homology screening with the random-primer 32P-labelled insert of a partial clone of rat Brn-3, comprising the POU-specific domain, linker region and POU homeobox domain under stringent (0.2 x SSC, 0.2% SDS at 66°C) hybridisation conditions (see methods, section 2.14 page 44).

Results of library screening.

Of ~ 1.5 x 10^6 clones screened, between twenty and thirty positive signals were seen on the duplicate radioautographs. Agar discs from the position of ten of the strongest positive signals were removed from the plate with the blunt end of sterile pasteur pipettes and phage suspensions were made from this material which was then plated at a lower density of colonies and rescreened using the same probe in order to ensure than single plaques were isolated.

5.2 b Analysis of clones isolated.

Six of the strongest positive clones were rescued into Bluescript II plasmid (see methods, section 2.14 page 44), and the
size of their inserts compared. This was done by digesting purified plasmid versions of the clones with Xho I and Eco RI, and electrophoresing the digests on a 0.8% agarose gel. The largest of these positives, clone 72, had an insert of 650 b.p. (N.N. Ninkina, personal communication) and was selected for sequencing. Sequencing was performed using a sequenase 2.0 kit (USB) on caesium chloride-purified double-stranded plasmid DNA using first primers SK and KS to sequences in the vector cloning site and then primers made to sequences within the clone (see Fig. 2.3, page 62 for sequencing strategy).

A comparison of this sequence with the other sequences available using Intelligenetics software showed that clone 72 showed 100% homology with the partial rat Brn-3 sequence, but extended 3' beyond the published rat sequence (see fig 5.2, page 158 for relative positions of published rat and mouse Brn-3 sequences to rat clone 72 sequence).

The open reading frame of the sequence could be predicted because of the known amino acid sequence of the POU domains of other members of the family (see Fig 5.3, page 159). The sequence of clone 72 overlapped with the POU homeodomain of the partial rat sequence by 150 b.p., and extended beyond it in the 3' direction by 500 b.p.. The novel sequence continued the open reading frame for 57 b.p., encoding 19 additional amino acid residues before terminating in a double stop codon. Seven more stop codons are present in the same frame after the initial double stop. The 425 b.p. untranslated region containing the stop codons ends in an 18 b.p. polyadenylated sequence.

5.2c NGF Regulation.

Comparative PCR was performed between primers pBr3b and pBr3c, producing an amplified fragment of 170 b.p. on single stranded cDNA synthesised from total RNA isolated from primary cultures of adult rat DRG neurons. The levels of mRNA encoding clone 72 were compared for DRG neurons grown in two different conditions - either with the addition of mouse NGF to the cultures, or with the addition of an anti-NGF antibody (experimental details discussed in Chapter 6, section 6.2 f, page 177, and methods, sections 2.5 page 36 and 2.20 page 49).
NGF Regulation Results.

Reactions were normalised against ribosomal protein L27 and also against G6PDH, and controls against genomic DNA contamination were performed (see discussion in Chapter 6, section 6.2f, page 177 and section 6.3f, page 192).

Samples were taken at identical numbers of rounds of amplification, performed on equivalent amounts of template cDNA, electrophoresed on agarose gels, Southern blotted and probed with the isolated 170 b.p. random-primer labelled product of pBr3b and pBr3c amplified from clone 72. When the density of bands on radioautographs were normalised using the two 'control' genes L27 and G6PDH, no significant difference could be detected in the relative amounts of Brn-3/72 from cDNA produced from DRG cultures grown in the presence of added NGF or in the presence of an anti-NGF antibody.

Concurrent experiments performed on Oct-1 and Oct-2 indicated that the addition of NGF to cultures upregulated Oct-2 levels by approximately fourfold while Oct-1 levels remained unchanged (Wood et al, 1992, results discussed below).

5.2d Use of antisense oligonucleotides in $^{45}$Ca Uptake assay.

The basic assay was performed as described (methods, section 2.6 page 37). DRG neurons were grown on Terasaki dishes for 4 days in the absence of NGF, and then with NGF in the presence of a thiol-linked oligonucleotide complementary to the sequence of RTX42 which was added at a concentration of 5 μM. Fresh medium containing NGF and oligonucleotide was added every day for a further four days until assay.

The $^{45}$Ca uptake assay was performed as described on neonatal DRG neurons grown in the presence of antisense oligonucleotides to the sequence of Oct-2 intended to 'deplete' the amount of Oct-2 protein.

The level of capsaicin evoked $^{45}$Ca uptake was compared to the $^{45}$Ca uptake in identical cells grown in the absence of oligonucleotides and the presence of nonsense oligonucleotides included in the study to show as a control to show that the presence of non-specific thiol-linked nucleotides did not affect the cells.
Results of $^{45}$Ca uptake assay.

As can be seen from the graph of results, neither the nonsense control oligonucleotide nor the oligonucleotide complementary to the sequence of Oct-2 had any significant effect on the levels of capsaicin-evoked $^{45}$Ca uptake in DRG neurons (Fig. 5.4, page 160).

5.3 Discussion.

5.3a New clones isolated.

The neurotrophin NGF acts to maintain the phenotype of a subset of sensory neurons (discussed in Chapter 6, section 6.1f, page 168) and increases in the levels of NGF can lead to a hyperalgesic state. To investigate whether the effect of NGF is mediated by transcription factors, comparative and also quantitative PCR was performed on primary cultures of rat DRG neurons (Wood et al., 1992, also see Table 6.3, page 198 and Figure 6.9, page 207).

Oligonucleotide PCR primers to the sequence of Oct-1 and neuronal Oct-2 were readily available (Lillycrop et al., 1991), but the only sequences of Brn-3 at the time published were partial sequences of the POU homeodomain and POU specific domain from rat and mouse (He et al., 1989, Goldsborough et al., 1990). These sequences are highly homologous to other POU family proteins in this area (He et al., 1989) and there was some difficulty in designing either probes or PCR primers which would detect Brn-3 specifically.

The neonatal rat cDNA DRG library was homology screened in order to isolate new Brn-3 clones containing sequence from outside the conserved DNA binding regions that could be used to distinguish Brn-3 from other POU proteins. Most of the clones isolated from the library screening were similar in size (~ 0.5 Kb, N.N. Ninkina, personal communication), and the largest of these, clone 72 was only 650 b.p.. However, after sequencing this clone, it was found that it overlapped with the known sequences from Brn-3 clones by only 150 b.p. - it therefore extended from about 0.5 Kb to the 3' side of the DNA binding domains (see Fig 5.2, page 158). By extrapolating the deduced reading frame of the amino acid sequence of the Brn-3 POU homeodomain through this clone, it was possible to predict that the protein continued for only 19 residues beyond the homeodomain before a double stop codon occurred.
Not knowing if any other Brn-3-like POU proteins existed, the
primer sequences chosen for the PCR were designed to sequences at
the far 3' end of the untranslated region. This was done as a safety
measure, as untranslated regions are far less conserved between
genes than are the protein encoding regions (e.g., between species,
the nucleotide sequence of the human and rat nAChR β3 subunit
share ~77% homology in their coding regions, but no detectable
homology in the 3' untranslated regions, the rat 3' region is several
hundred b.p. in size, while the equivalent human 3' untranslated
sequence is only ~90 b.p.).

5.3 b NGF+/- comparative PCR.
The PCR results obtained for Brn-3, which demonstrated that
NGF had no effect on the mRNA levels of the gene, agreed with PCR
results obtained in parallel by co-workers (see Wood et al, 1992). Other
comparative PCRs on Oct-1, Oct-2 and Brn-3 were performed in
parallel by others. Comparative PCR demonstrated that the level of
Oct-2 mRNA is reduced by four-fold if NGF is removed from the
culture media while Oct-1 and Brn-3 are unchanged by removal of
NGF or elevation of NGF levels in the DRG neuron cultures (Wood et
al, 1992). These experiments were performed in a similar way to the
comparative PCR described in Chapter 6 (section 6.2f, page 177).
However, the measurement of Oct-2 was further investigated by
performing PCR reactions with an internal standard (e.g., Celi et al,
1993). However in this instance, the internal standard was provided
by titrated human Oct-2 RNA (transcribed from a plasmid clone of
the gene, Clerc et al, 1988) added to the test RNA before first strand
cDNA synthesis. The human and rat Oct-2 were co-amplified in the
same reaction by the same pair of primers to produce the same size
PCR product. However, after amplification, the reaction products
were digested using the restriction enzyme \( Bgl \) 1. This cleaves the
human product into two, but does not affect the rat Oct-2 PCR
product, and so the amounts of the two products can be readily
identified. When the amounts of product from the test template and
the titrated 'control' human template were compared a quantitation
of the relative amounts of rat Oct-2 was possible. Oct-2 was found to be
elevated by 2.7 - 3.5 fold in three NGF-treated cultures tested (Wood et
Confirmation that the change in level of this mRNA was reflected in terms of levels of Oct-2 protein available for binding was obtained from a DNA mobility shift assay. This was performed on protein extracts from NGF and anti-NGF treated DRG cultures. A radiolabelled oligonucleotide of a high affinity binding site for both Oct-1 and Oct-2 (Dent and Latchman, 1991) was used to detect the DNA binding ability of proteins expressed in DRG grown with NGF and with anti-NGF. The identity of Oct-2 protein was determined on the basis of its specificity of binding for different octamer oligonucleotides that distinguish between B-cell and neuronal Oct-2 (Dent et al, 1991). The lack of a consensus DNA binding sequence for Brn-3 meant that similar band-shift experiments could not be performed with this protein.

This result shows that addition of NGF to DRG neurons in culture produces an increase in the mRNA encoding the transcription factor neuronal Oct-2 and does not affect the mRNA levels of either Brn-3 or the ubiquitous transcription factor Oct-1. Under similar conditions, addition of comparable amounts of NGF induces capsaicin sensitivity (Winter et al, 1988) and increases the levels of preprotackkykinin mRNA (Lindsay and Hamar, 1989). The co-elevation of Oct-2 and preprotackkykinin with the appearance of the capsaicin response are interesting coincidences, but there is no positive evidence that any of these events are connected.

5.3 c Functional assay.

Use of the $^{45}$Ca uptake assay was an attempt to provide positive evidence that the NGF regulation of capsaicin sensitivity in DRG neurons is due to the elevation of Oct-2 levels by NGF. The results show no effect on the capsaicin-evoked $^{45}$Ca uptake (Fig 5.4, page 160). However, the experiment should ideally be repeated using either antisera raised against the Oct-2 protein or a band-shift assay to confirm that the levels of Oct-2 protein are reduced by the inclusion of antisense oligonucleotide in the culture medium.

5.3 d New Brn-3 proteins.

The decision to perform the PCR on the far 3' untranslated region of Brn-3a proved to be important, as subsequently two new Brn-3 related proteins have since been described. The three genes are now referred to as Brn-3a (originally Brn-3), Brn-3b and Brn-3c (Wood

The complete sequence of the murine versions of the three genes has recently been made available (Theil et al, 1994), and the absence of the sequences used for the Brn-3a PCR in the Brn-3b and Brn-3c sequences was noted, although inter species variation means that the sequences differ by a few nucleotides.

In addition, the subsequent use of probes which are derived from regions of the Brn-3a gene which overlap with the Brn-3/72 clone indicate that the PCR is likely to be specific for Brn-3a. The $^{32}$P-labelled Brn-3a probes were used to probe Southern blots of restriction endonuclease digested genomic DNA. Probing of identical blots with a sequence specific to Brn-3c and a probe that recognised the POU domains of all three Brn-3 proteins resulted in a distinct pattern of hybridisation (Ninkina et al, 1993).

A comparison of the blots probed with these three genes is informative. The DNA restriction fragments which hybridise to the Brn-3a-specific probe can also be easily identified on the blot probed with the non-specific POU probe. Similarly, a different but distinct pattern of hybridisation visible after probing with the Brn-3c-specific probe can be detected on the non-specific POU probed blot. In addition to the DNA fragments corresponding to the two specific probes, a third set of DNA fragments is visible. While these have not been positively identified, it could be tentatively predicted that they might correspond to the genomic sequence of Brn-3b. Whether or not this is so, the clear difference in the restriction pattern of DNA probed with Brn-3a and Brn-3c suggests that it is likely that these two genes are encoded by separate genes, unlike the different Oct-2 variants, at least several of which are produced by differential splicing of RNA from a single gene (Wirth et al, 1990).

The above work indicates that it is likely that the PCR primers used to study NGF regulation of Brn-3(a) would recognise only Brn-3a. Probes derived from an overlapping area recognised different restriction patterns in genomic DNA.

Brn-3a, Brn-3b and Brn-3c have a limited distribution in the central and peripheral nervous system. Brn-3b has been shown to be most strongly expressed in a subset of retinal ganglion cells (Xiang et al, 1993) while Brn-3c is expressed most strongly in a subset of
neurons in the spinal cord and dorsal root ganglia (Ninkina et al., 1993) suggesting that it may be specific for a subset of sensory neurons.

The possible involvement of transcription factors in the mediation of the effects of NGF on dorsal root ganglion neurons has been investigated above. Brn-3a, a transcription factor localised to spinal cord, cerebellum, brainstem and DRG neurons was not NGF regulated. Oct-1, a ubiquitous transcription factor found in many dividing cells was also unsurprisingly not affected by the presence of NGF. Interestingly, the neuronal form of Oct-2 was found to be upregulated fourfold in the presence of NGF. The use of a functional assay based on the sensitivity of DRG neurons to capsaicin indicates that the NGF-dependence of capsaicin sensitivity is not mediated through changes in the levels of neuronal Oct-2. However, the change in Oct-2 levels indicates that this transcription factor may mediate at least some of the effects of NGF. There are several neuronally-expressed genes which contain Oct-like control sequences upstream and so there are obvious immediate candidates for such a control pathway (e.g., Dawson et al., 1994).

Equally importantly, the isolation of a novel Brn-3 clone resulted in the discovery of nucleotide sequence outside the Brn-3 POU region. This allowed the construction of PCR primers and probes which were able to distinguish between Brn-3a and other Brn-3 factors and contributed to the later identification of three distinct Brn-3-like proteins.
### Table 5.1

**Distribution of POU transcription factors.**


<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Tissue-specificity</th>
<th>Brain distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-1</td>
<td>ubiquitous</td>
<td>hypothalamus; cerebellum</td>
</tr>
<tr>
<td>Oct-2</td>
<td>B-cells;brain</td>
<td>hypothalamus; cerebellum</td>
</tr>
<tr>
<td>Pit-1</td>
<td>neural tube;pituitary</td>
<td>-</td>
</tr>
<tr>
<td>Brn-1</td>
<td>brain</td>
<td>cortex; cerebellum</td>
</tr>
<tr>
<td>Brn-2</td>
<td>brain</td>
<td>cortex; cerebellum</td>
</tr>
<tr>
<td>Brn-3a</td>
<td>brain</td>
<td>brainstem, trigeminal ganglia, spinal cord, DRG</td>
</tr>
<tr>
<td>Brn-3b</td>
<td>brain</td>
<td>retinal ganglion cells, subset of DRG neurons, very limited expression in CNS.</td>
</tr>
<tr>
<td>Brn-3c</td>
<td>brain</td>
<td>brainstem, DRG</td>
</tr>
<tr>
<td>Tst-1/SCIP</td>
<td>brain;glia;testis</td>
<td>cortex; cerebellum</td>
</tr>
</tbody>
</table>
Figure 5.1  General structure of POU domains.

<table>
<thead>
<tr>
<th>Required for:</th>
<th>DNA Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>High affinity DNA binding</td>
<td>Low affinity</td>
</tr>
<tr>
<td>Site specificity</td>
<td>Relaxed specificity</td>
</tr>
<tr>
<td>Protein-protein interactions</td>
<td>Protein-protein interactions</td>
</tr>
</tbody>
</table>

POU-Domain:

- POU-Specific Domain
  - +++ helix A helix B
  - KQR8IKLG V K T
  - SQ7TCTR S S

- POU-Homeo Domain
  - +++ helix 1 helix 2 helix 3 +++
  - RKRKERTTI DKKK S
  - VRVIVFCNBOKR I
  - QRQ

DNA Binding Consensus Sites:

- Pit-1  AAAAAATATN CAT
  TTTT T
- Oct-1,2 AAAAATTTGC AT
  TTTT T
Figure 5.2
Schematic diagram of clone 72/Brn-3 (isolated from an adult rat DRG cDNA library by homology screening) aligned with the two partial Brn-3 clones available at the time.
Figure 5.3

Sequence of clone 72 (Brn-3a/72, isolated from neonatal rat DRG cDNA library by homology screening) - lower nucleotide sequence, aligned with the partial nucleotide sequence of the rat Brn-3a POU domains showing 100% homology in the area of overlap. The predicted amino acid sequence is shown above the nucleotide sequence in single letter code, (*) indicates a stop codon. Above the amino acid sequence for rat fim-3a/cIone 72 are the predicted POU domain amino acid sequences for the other Brn-3 POU proteins Brn-3b and Brn-3c (- dash indicates that residue is the same as Brn-3a) showing the high level of homology between the POU regions of the three proteins (from Lillycrop et al, 1992, Ninkina et al, 1993). The positions of primers pBr3b and pBr3c in the 3' untranslated region of clone 72 are marked.
Figure 5.4
Graph showing results of the $^{45}$Ca uptake experiment in which DRG neurons had been grown in culture in the presence of antisense oligonucleotides to specific genes (see methods). Graph shows uptake of $^{45}$Ca (d.p.m.) in control and capsaicin-treated DRG cultures; capsaicin stimulated $^{45}$Ca uptake in control, control nonsense (oligonucleotide 102), RTX antisense (RTXT) and Oct-2 antisense (Oct) -treated cultures was identical (no significant difference at the 5% level), n=6, ± SD shown.
Chapter 6. Neurotrophin regulation of genes in DRG neurons.
6.1 Introduction - Nociception and hyperalgesia.

Inflammation and tissue injury can result in a state called hyperalgesia, in which there is an increase in the response of sensory neurons to noxious stimuli and a decrease in pain threshold. Two distinct types of hyperalgesia exist, primary and secondary. These can be distinguished by their location relative to the site of injury, primary hyperalgesia referring to changes that occur within the site of injury, and secondary hyperalgesia refers to changes occurring in the undamaged area surrounding the injury (Lewis, 1935, 1942).

It is clear that a variety of different mechanisms or at least pathways produce hyperalgesia (Trede et al, 1992), as is indicated by the difference in delay of onset of heat hyperalgesia compared to mechanical hyperalgesia, and the ability of non-competitive NMDA antagonists to block heat but not mechanical hyperalgesia (Rueff et al, 1993). Recently attention has turned to the sensitisation of spinal cord pathways in response to a repetitive afferent input from injured tissue (Treede et al, 1992). NMDA receptors have been implicated in prolonged nociceptive activity (Dickenson and Sullivan, 1987, Haley et al, 1990, Zieglgansberger and Tolle, 1993) and spinal dorsal horn NMDA-induced currents are potentiated by substance P (Randic et al, 1990). Thus the neuropeptide substance P may be involved in a modification of central nociceptive processing as well as being involved in neurogenic peripheral processes.

There are, however, sensitised states in cutaneous receptors that contribute to hyperalgesia (LaMotte et al, 1984). The release of several substances from surrounding tissue may contribute to the activation or sensitisation of primary sensory neurons. Several of these, such as protons, ATP and 5-HT may act via ligand-gated channels.

Protons can produce a brief depolarization, which rapidly inactivates, in many neurons, but can produce a maintained depolarization involving an inward current in some capsaicin-sensitive sensory neurons which are probably nociceptive (Krishtal and Pidoplichenko, 1981, Bevan and Yeats, 1991). As inflammatory exudates are often acidic, this excitatory effect may follow inflammation.
ATP has been shown to activate a cation-selective ion channel in the membrane of sensory neurons (Krishtal, 1983, Bean, 1990, Bean et al, 1990). The universal cellular utilisation of ATP as an energy carrier means that the damage or destruction of any cells is likely to result in the release of this molecule in the vicinity.

5-hydroxytryptamine (5-HT) which is released from platelets and mast cells during tissue damage can act on a number of receptors in sensory neurons, and its effects are complex (Higashi et al, 1982). Some receptors are coupled to second-messenger systems, but 5-HT also activates a ligand-gated ion channel - the 5-HT_3 receptor (Derkach et al, 1989, Peters and Lambert, 1989, Sugita et al, 1992). It appears that activation of the 5-HT_3 receptor may be able to activate (Robertson and Bevan, 1991) and sensitise (Richardson et al, 1985) nociceptors.

There is good evidence that there are nAChRs on sensory neurons in a number of species, (Brown and Gray, 1948, Polz-Tejera et al, 1980, Higashi et al, 1982, Higashi, 1983, Morita and Katayama, 1989), and that there are also muscarinic, i.e., second-messenger coupled ACh receptors (Morita and Katayama, 1984).

Nicotine, acting through the nAChRs, can stimulate the release of neuropeptides such as substance P and CGRP from mammalian DRG neurons in culture (Franco-Cerecda et al, 1992). Nicotinic receptors can also cause a rapid transient depolarisation of DRG neurons in amphibians (Koketsu et al, 1969, Morita and Katayama, 1984): this occurs only in a subset of neurons (Morita and Katayama, 1989). The excitatory effects of ACh on mammalian sensory neurons has been demonstrated (Brown and Gray, 1948, Douglas and Gray, 1953, Fjallbrant and Iggo, 1961, Higashi et al, 1982, Steen and Reeh, 1993).

In addition, there is evidence that ACh acting through nAChRs can affect nociceptive thresholds (Holton and Gray, 1951, Armstrong et al, 1953, Skouby, 1953, Guzman et al, 1962,), and also that ACh can elicit painful sensations, although the mechanisms involved are not clear (e.g., Emmelin and Feldberg, 1947).

6.1a Neurogenic inflammation.

Small diameter neurons which are capsaicin sensitive may be involved in hyperalgesia by the release of neuropeptides like calcitonin gene-related peptide (CGRP), substance P (SP), and
tachykinins, following antidromic stimulation of peripheral primary 
afferent fibres after injury (Hamon et al, 1988). This may contribute to 
sensitisation of primary neurons, although indirectly as the peptides 
do not themselves activate primary neurons (Mizumura et al, 1987). 
The peptides may, however, stimulate release of other sensitizing 
substances such as prostaglandins, bradykinin, histamine and 
serotonin (reviews: Holzer, 1988, Rang et al, 1991, see also Chapter 1, 
sections 1.3 b, page 22 and 1.3 f, 1.3 g and 1.3 h, page 24).

6.1 b  Nerve growth factor (NGF) and the neurotrophins.

Work on neurogenesis by Viktor Hamburger and Rita Levi-
Montalcini in the 1930's and 1940's, lead towards the important 
observation that in the developing chick dorsal root ganglia there are 
more dying neurons in the ganglia that innervate a small peripheral 
field than there are in the ganglia innervating a large field 
(Hamburger and Levi-Montalcini, 1949). They showed that the 
amount of neuronal cell death can be greatly increased by removing 
target tissue early in development without affecting the number of 
neurons initially generated (Hamburger and Levi-Montalcini, 1949).

The theory that neuronal cell death during normal 
development results from the competition for limiting amounts of 
trophic molecules produced from target tissue was made testable by 
the purification of the first such survival factor, NGF (reviews; Levi-
Montalcini, 1987, Barde, 1989). NGF was first isolated as a protein of 
molecular weight 44 kDa from adult male mouse submandibular 
gland (Cohen, 1960). Antibodies raised against the new factor were 
shown to specifically destroy the peripheral nervous system when 
jected into newborn rodents, providing firm evidence that the 
molecule is a survival factor during development (Cohen, 1960).

Twelve years after the protein was sequenced (Angeletti and 
Bradshaw, 1971), the gene encoding it was cloned (Scott et al, 1983). 
Only after it became possible to measure the low amounts of NGF and 
its mRNA present in target tissues, and to correlate these levels with 
the density of innervation, was it firmly established that NGF was in 
fact target derived (Korsching and Thoenen, 1983, Heumann et al, 
1984). That NGF is retrogradely transported along neuronal axons 
from target tissue was earlier indicated by the demonstration that 
inhibition of retrograde transport in the axons of developing neurons 
had a similar effect on neuronal survival to that of application of anti-
NGF antibodies (Johnson, 1978). Aside from neuronal survival, NGF was also shown to influence the degree of axonal branching of sympathetic neurons in culture (Campenot, 1977).

NGF plays a major role as a survival factor for sensory as well as sympathetic neurons (review; Johnson, 1986). Administration of NGF to chick embryo DRGs resulted in an enhanced survival of sensory neurons (Hamberger et al, 1981). In addition, anti-NGF antibody treatment of fetal chicks can reduce the number of DRG and trigeminal neurons by up to 80% (Johnson et al, 1980, and see below for further discussion).

Though the gene is composed of several exons, is spread over 45 Kb in the mouse genome, and the mRNA exists as at least four different splice variants, the nucleotide sequence encoding almost the entire mature protein is present on a single exon (Selby et al, 1987). The relatively inactive glycosylated precursor protein is edited to an active form by trypsin-like enzymes (Edwards et al, 1988).

The NGF dimer consists of two identical proteins of 118 amino acids containing cysteine residues that form three disulfide bonds central in maintaining the dimer. Crystallographic analysis of NGF at 2.3Å resolution indicates that each protomer folds to produce three antiparallel β-pleated sheets which apparently form a flat surface (McDonald et al, 1991).

Other neurotrophins.

Other members of the neurotrophin family have now been identified, including BDNF - brain-derived neurotrophic factor (Barde et al, 1982, Hofer and Barde, 1988) neurotrophin-3 (NT-3) (Ernfors et al, 1990, Maisonpierre et al, 1990a), and neurotrophin-4/5 (NT-4 or NT-5) (Berkemeier, et al, 1991, Hallbrook, et al, 1991, Ip et al, 1992). The various neurotrophins are expressed at different times and locations during development of the nervous system and it is likely that interactions between them are involved in the normal development of the nervous system (e.g., Ernfors et al, 1990, Maisonpierre et al, 1990b).

Neurotrophin receptors.

The first neurotrophin receptor cloned was a low affinity receptor for NGF (known as LNPFRp75) (Radeke et al, 1987); its role in mediating the effects of NGF is unclear, however, targeted disruption of the gene results in defects in the PNS (Lee et al, 1992). More recently, other members of the neurotrophin family have been shown
to bind to and activate a series of receptors with tyrosine kinase activity - \textit{trkA}, \textit{trkB} and \textit{trkC} (Klein et al, 1989, Squinto et al, 1991, Carroll et al, 1992 Mu et al, 1993, Rosenthal et al, 1993, review; Barker and Murphy, 1992). The tyrosine kinase receptor \textit{trkA} is thought to be the main receptor through which NGF exerts its actions (Ernfors et al, 1990), \textit{trkB} is a receptor for both BDNF and NT-3 but not for NGF (Squinto et al, 1991), while \textit{trkC} is activated by NT-3 (Rosenthal et al, 1993, Valenzuela et al, 1993) and also NT-4/5 (Ip et al, 1992). The \textit{trk} receptors are expressed at different levels in overlapping locations during embryonic and postnatal development, and of some of them are also expressed as truncated forms which lack the cytoplasmic tyrosine kinase domains (Escandon et al, 1994).

6.1 c Neurotrophins as survival factors for DRG neurons.

Depletion of NGF in the DRGs of developing animals (e.g. by injection of anti-NGF antibodies) can result in neuronal loss (Ritter et al, 1991, Lewin et al, 1992) and functional impairment surviving neurons (Lewin et al, 1993). Axotomy in newborn rats results in the loss of nonmyelinated neurons, an effect which can be reversed by injection of NGF ((Myata et al,1986). NGF depletion of developing DRG neurons, whether in vivo by use of anti-NGF antibodies or by culturing embryonic or neonatal DRG cells in the absence of NGF, results in the death of a subset of neurons, which are characterised by their expression of substance P and sensitivity to capsaicin (Ruit et al, 1992). This selective sensitivity of some DRG neurons to NGF depletion has been correlated with their expression of \textit{trkA} receptors (Carroll et al, 1992).

In both developing and adult animals the \textit{trkA}, \textit{B} and \textit{C} receptors are expressed in DRG neurons (Verge et al, 1992, Mu et al, 1993). Although NGF is no longer a survival factor for most cells in the adult animal, it can facilitate the regeneration of axons (Lindsay, 1988, 1992).

6.1 d NGF regulation of neuronal gene expression.

In both developing and adult animals neurotrophin receptors are expressed in DRG neurons (Verge et al, 1992, Mu et al, 1993). The tyrosine kinase receptor \textit{trkA}, present in DRG, is thought to be the main receptor through which NGF exerts its actions (Ernfors et al, 1990). NGF depletion of developing DRG neurons, whether in vivo by
use of anti-NGF antibodies or by culturing embryonic or neonatal DRG cells in the absence of NGF, results in the death of a subset of neurons, which are characterised by their expression of substance P and sensitivity to capsaicin (Ruit et al, 1992). This selective sensitivity of DRG neurons to NGF depletion has been correlated with the expression of \textit{trk} receptors (Carroll et al, 1992). In the adult animal, although NGF is no longer a survival factor for most cells, it can facilitate regeneration of axons (Lindsay, 1988, 1992).

\textbf{The role of NGF in adult sensory neurons.}

In the adult animal, NGF acts to maintain the phenotype of a subset of sensory neurons. NGF regulates the mRNA levels of the neuronal-specific protein peripherin (Thompson and Ziff, 1989). NGF also regulates the mRNA levels of substance P (the preprotachykinin mRNA), CGRP and NGF receptor in DRG neurons (Lindsay et al, 1989, Miller et al, 1991, Lindsay, 1992). In addition, the capsaicin sensitivity which is exhibited by a subset of DRG neurons (see sections 1.3 e, page 23, and 4.1 a, page 113) is also entirely dependent on the presence of NGF (Winter et al, 1988). Interestingly, by contrast, the level of substance P transcript is independent of NGF in sympathetic neurons (refs within Jonakait, 1993).

Levels of NGF are rapidly elevated in damaged or inflamed tissue (Weskamp and Otten, 1987), an effect due amongst other things to increased synthesis of NGF by astrocytes and fibroblasts - probably stimulated by release of cytokines and growth factors (Yoshida and Gage, 1992, Lewin and Mendel, 1993).

Anti-NGF treatment in neonatal rats can result in reduced nociceptor function, and the factor appears to play a role in the development of some nociceptive neurons (Ritter et al, 1991, Lewin et al, 1992a, 1992b). Direct evidence for NGF-induced hyperalgesia has resulted from experiments in which levels of NGF were artificially raised in neonatal and adult rats causing a hyperalgesic affect (Lewin and Mendel, 1993).

The non-endogenous vanilloid, capsaicin, can induce hyperalgesia (e.g. Bauman et al, 1991, Simone et al, 1991a, LaMotte et al, 1992) and the capsaicin responsiveness of cultured DRG cells is entirely dependent on the presence of NGF. Cells grown in the absence of NGF lose capsaicin sensitivity after 2-3 days. This effect is reversible, as addition of NGF to the media after this time results in
the regaining of capsaicin sensitivity after a similar period of time (Winter et al, 1988, 1993).

The NGF-dependency of expression of neuropeptides and capsaicin sensitivity suggest that a continuous supply of target-derived NGF is required to maintain the levels of expression of several genes in mature DRG neurons. Studies on the effects of axotomy, which may affect the transport of NGF, support this view (review: Hokfeldt et al, 1994). In addition, the elevation of NGF levels in damaged and inflammed tissue coincides with a number of phenotypic changes in DRG neurons (Weskamp and Otten, 1987). It seems likely that the increase in NGF that occurs locally to tissue damage contributes to the changes, as NGF receptors are increased following tissue injury or as a result of infusion of NGF (Verge et al, 1992). Thus NGF is also likely to play a role in altering cell phenotype in response to certain stimuli.

6.1 General NGF regulation.

NGF has been shown to regulate a number of genes in different cell types. Increase in AChE (acetylcholine esterase) but not choline acetyl transferase activity in PC12 cells results from addition of exogenous NGF (Lucas et al, 1980). NGF also regulates the RNA level the peripherin gene in PC12 cells (Thompson and Ziff, 1989).

A number of regulatory NGF effects on ion channels has been described. Increased sodium currents through nAChRs (Amy and Bennet, 1983) may be due to NGF-induction of Na channels. An increase in the number of functional sodium channels in response to NGF has been demonstrated in PC12 cells (Mandel et al, 1988). Another study in PC12 cells found that while nAChR subunit mRNA was altered by addition of NGF, it did not appear to affect the amount of subunit protein present, although the number of cells responding to ACh increased (Rogers et al, 1992). NGF stimulated increases in nAChR current density have been shown to be independent of protein kinase A activity, as changes in current density are similar in protein kinase A-deficient and wild-type PC12 cells (Henderson et al, 1994). In sensory neurons nAChR currents are also increased by the administration of NGF (Cooper and Lau, 1986). In nodose ganglion neurons in culture, NGF increases the number of cells sensitive to ACh (Mandelzys et al, 1990), although the effect is suppressed in the presence of ganglionic satellite cells (Mandelzys
and Cooper, 1992). Conversely, both NGF and fibroblast growth factor (FGF) have been shown not to have any effect on the binding of α-bungarotoxin or neuronal bungarotoxin to ciliary ganglion neurons. Ciliary neurotrophic factor (CNTF) down regulates the number of surface α-Bgt-binding sites in these cells while not affecting the other nAChR receptor population (Halvorsen and Berg, 1989).

6.1 NGF effects on DRG.

Nociceptor responses are modified by NGF (Ritter et al, 1992, Lewin et al, 1992, Lewin et al, 1993, Ritter and Mendell, 1992), and in addition, NGF regulates the mRNA levels of neuropeptides such as substance P, substance A, and CGRP (Lindsay et al, 1989, Lindsay and Harmar, 1989), but not of vasoactive intestinal peptide (VIP - Mulderry and Lindsay, 1990).

In DRG neurons in culture, addition of NGF can increase the duration of action potentials (Chalazonitis et al, 1987) and TTX-sensitive action potentials are increased with increase of NGF levels, suggesting some effect on TTX-sensitive voltage-gated ion channels (Aguayo et al, 1991, Luis et al, 1991).

6.2 - PCR study of gene expression in DRG Neurons.

6.2 a Rationale for PCR.

There is limited evidence about which nAChR subunits are expressed in DRG neurons (Table 6.1, page 196), although there is evidence that primary afferent neurons are sensitive to ACh (Douglas and Gray, 1953, Fjallbrant and Iggo, 1961, Higashi et al, 1982, Higashi, 1983). It was decided to investigate the basis of this sensitivity to acetylcholine by determining which nAChR subunits are expressed in rat DRG neurons.

The effect of NGF on the mRNA levels of the nAChR subunits in DRG was then investigated as a possible contributing factor to the hyperalgesia induced following tissue injury. An NGF-induced increase in the mRNA levels of nAChR subunits could conceivably result in an increase in the number of receptors and an increase in the sensitivity of the neurons.

PCR was used to examine the expression of several genes in DRG neurons, firstly to confirm which of several genes of interest were present, and secondly to examine the possible regulatory effects
of NGF on the genes expressed. In both of these studies, specific oligonucleotide primers were used as primers in these reactions.

As culture of adult rat DRG neurons is possible in the absence of NGF, the study of regulation of expression was done on DRG neurons grown in culture either in the presence of NGF or a monoclonal antibody against NGF. Previous work on the NGF regulation of neuropeptides and action potentials was performed on DRG neurons cultured in a similar manner. The neuropeptide substance P was chosen to be a positive control for the study, as it is upregulated fifteen- to sixty-fold by addition of NGF to DRG neuron cultures (Lindsay and Harmar, 1989).

PCR was chosen because it would require the smallest amount of RNA as template material - the culture of DRG neurons produces a low yield of RNA and is labour intensive. The comparative PCR technique was devised to reveal consistent differences in levels of mRNA transcripts under the experimental conditions rather than to make quantitative measurements of any differences. Following this study, it was planned to analyse consistent differences in levels using other techniques.

6.2b PCR primer design.

PCR was first used to determine which LGIC subunits were present in rat DRG neurons, as there is limited evidence about which subunits are expressed in these cells (Table 6.1, page 196). Pairs of PCR primers were designed to be specific for single subunits.

In the presence of genomic DNA, any PCRs which amplify sequences within a single exon performed on cDNA will amplify DNA sequences identical in size from the cDNA and from any contaminating genomic DNA. Unless strict controls are utilised, the results will be meaningless in terms of expression of the gene. To avoid having to perform these controls, however, PCR primers can be designed to amplify a sequence which spans more than one exon; in this instance the PCR product produced from cDNA and that from genomic DNA will be distinguishable by size (differing by the length of the intron[s] which it spans). This allows the easy identification of sequences amplified from cDNA.

As the intron-exon structure of the nAChR is highly conserved (see Fig. 6.1, page 199), it was possible to design PCR primers so that PCR products would span an intron, thus reducing
the problems encountered with genomic DNA contamination of template cDNA. In addition, nucleotide sequence searches indicated that the most divergent regions of most of the subunits was the cytoplasmic loop between TMDs 3 and 4. Accordingly, for the nAChR subunits, primers were designed so that at least one primer of a pair corresponded to a unique sequence in the cytoplasmic loop while the other primer would be matched to either TMD3, TMD4 or another part of the cytoplasmic loop (see Fig. 6.2, page 200).

The α7 subunit possesses a unique intron-exon arrangement, which is distinct from all other nAChR subunits (Couturier et al., 1990, Seguela et al., 1993). The neuromuscular nAChR subunits are encoded by nine exons in the case of the α subunit and twelve exons in the case of the other subunits (Fig 6.1, page 199, Sawruk et al., 1988, Claudio Buonanno et al., 1989) while all known neuronal nAChR subunits (with the exception of α7) are encoded by six exons.

Although the first four protein-encoding exons of the nAChR α7 subunit coincide with the conserved arrangement of exons in all the other nAChR subunits, the intron-exon arrangement of the 3' end of the α7 subunit is distinct from the other genes; the coding region being divided into ten exons (see Fig 6.1, page 199). The primers to the nAChR α7 subunit were again located in the cytoplasmic loop region, amplifying a small sequence that nevertheless crossed an intron-exon boundary.

The nAChR α4 subunit has two alternative splice versions at the 3' end of the protein coding regions (Goldman et al., 1987), and one of the primers to this sequence crosses the junction of the alternatively spliced region (due to similarities between subunit sequences constraining the placing of primers). Although both splice variants of the α4 subunit gene have been reported to be present in the spinal cord (Wada et al., 1989) and α4 can be detected in DRG (Boyd et al., 1990), it was not known which of the versions was expressed in DRG. This primer was therefore made with mixed bases at some positions so that either of the two splice variants would be equally amplified. This design meant that no distinction would be made between the two variants, which would be amplified as DNA sequences of identical size.

The primers to the glutamate receptor subunit, GluR5, were the only ligand-gated channel subunit primers designed to amplify regions away from the cytoplasmic loops. In this instance the region
amplified is a distinct sequence at the 5’ extracellular domain of the subunit, spanning an exon boundary at which an optional exon may or may not be inserted, making two versions of the subunit, GluR5-1 and GluR5-2 (Bettler et al, 1990). As the primers spanned this area, two differently sized PCR products corresponding to mRNAs GluR5-1 and GluR5-2 were produced.

**Specificity of PCR and of probes.**

The following precautions were taken to demonstrate that any products amplified were produced from the targeted gene and not as the result of accidental cross-annealing to other sequences.

1. A control amplification using the cloned gene as template was first performed in which the size of the amplified fragment could be predicted from the published sequence. This product was then isolated and used as the probe for southern blots of the experimental PCRs.

   Where no control template was available (for the nAChR β3, GluR5, PPT, G6PDH and L27 genes, see overpage), a partial gene sequence was amplified out of appropriate material (first strand template from either DRG neurons, whole rat brain or PC12 cells), cloned into pGEM-T and sequenced to ascertain that it was the correct sequence. The cloned fragments from these materials were the expected sizes as predicted from the published sequences.

2. All Southern blots of experimental PCRs contained material from a positive control PCR utilising either cloned gene or cloned, identified product as a template. Thus products were identified by size and sequence homology.

**Specificity.**

The primers used were designed to match published sequences of the corresponding genes, namely nAChR α2 (Boulter et al, 1986), nAChR α3 and α4 (Boulter et al, 1990), nAChR α5 (Wada et al, 1990), nAChR β2 (Deneris et al, 1988), nAChR β3 (Deneris et al, 1989), nAChR β4 (Duvoisin et al, 1989), GluR5 (Bettler et al, 1990), RTX13/42 (Ninkina et al, 1994), Brn-3a (Wood et al, 1992, Ninkina et al, 1993) and ribosomal protein L27 (LeBeau et al, 1991).

In some instances, no published gene sequences from the rat were available. In this case, primers were based on sequences most conserved between species, and from the the nearest related species
available. In this instance, all products were identified by sequencing, as described below.

The chick nAChR α7 sequence (Couturier et al, 1990) was used to derive primers, and then the primers designed were later confirmed as appropriate upon the subsequent publication of the rat nAChR α7 sequence (Seguela et al, 1993). Primers for preprotackkykinin/substance P (PPT) were derived from areas conserved between the bovine (Nawa et al, 1983) and human (Hamar et al, 1986) sequences. Primers for glucose-6-phosphate dehydrogenase (G6PDH) were complementary to partial rat sequences (Rank et al, 1992) (Fig. 6.3, page 201).

Where cloned versions of these genes were available, the PCR primers were tested out on plasmid clones to ensure that they amplified DNA sequences of the correct size. This was done for nAChR subunits α2, α3, α4, α5, α7, β2 and β4 (clones were the generous gifts of J. Boulter, J. Connolly and J. Patrick), and on Brn-3a and RTX13/42.

Plasmid clones for nAChR β3, GluR5, PPT, G6PDH and L27 were not available, and so PCRs were performed on single stranded cDNA template material synthesised from rat whole brain (nAChR β3, G6PDH and L27), rat DRG (GluR5) or PC12 cell (PPT) total RNA (see methods, sections 2.7 page 39, and 2.20 page 49).

In these instances, the resulting products were cloned into the vector pGEM-T (Promega, see methods, section 2.24 page 54) and the ends of the cloned product were sequenced in order to positively identify that the correct RNA sequence was being amplified. Sequencing was performed as previously on purified double-stranded DNA template using sequenase 2.0 kits and sequenase enzyme. In addition to these genes, the PCR products of α7 and β4 were isolated and cloned in a similar fashion, and identified by sequencing (see discussion). In the later comparative PCR, the results of amplification were Southern-blotted and probed with specific probes (Fig 6.8, page 206) under stringent conditions, to positively identify products.

6.2c PCR amplification from cDNA.

This was carried out on single stranded cDNA made by reverse transcription of RNA isolated either from newly dissected dorsal root ganglia or from dorsal root ganglion neurons grown in
culture (see methods, sections 2.4, 2.5, 2.7 pages 34-40 and section 2.20 page 49). The starting material was the equivalent of the first-strand template from 50 ng of total RNA. The amount of RNA in paired samples was matched using a spectrophotometer and sometimes also adjusted after running control PCRs for G6PDH and L27, if the levels of product produced in paired samples were visibly different.

6.2 d nAChR subunits expressed in DRG neurons.

While there have been reports of nAChR receptor function in DRG neurons (Morita and Katayama, 1989), chemoreceptors for ACh in primary afferent neurons (Higashi et al, 1982), and expression of some nAChR subunits in DRG neurons (Wada et al, 1989, Boyd et al, 1991, and see Table 6.1, page 196), the nAChR subunit complement of DRG neurons has not been extensively catalogued.

An investigation into the possible regulation of nAChR and glutamate receptor subunits in DRG neurons was planned using established methods for the culture of adult rat dorsal root ganglion neurons with and without NGF (Lindsay and Harmar, 1989).

DRG neurons were dissected from adult and neonatal rats and placed immediately into guanadinium denaturing solution to prevent degradation of RNA. RNA extraction was by the single step method (Chomczynski and Sacchi, 1987, and see methods, section 2.7 page 39). In each reaction the amount of cDNA template used was one tenth of a first strand reaction, i.e the equivalent of first strand synthesis from around 50 ng of total RNA, and 25 - 40 cycles of amplification were performed.

In the first instance, samples were taken from the PCR tubes at five cycle intervals to check at what level of amplification the products became visible on an ethidium bromide stained agarose gel. Following this, similar reaction tubes were prepared and the relative amounts of products were compared after the same number of cycles of amplification.

Results of nAChR subunit expression in DRG neurons.

The first results were obtained from RNA isolated straight from adult or neonatal DRG neurons. Visible PCR products had been amplified from many of the subunits in reactions containing single stranded cDNA corresponding to ~50ng of RNA, after 30
amplification cycles, but by 35 cycles, all the subunits detectable had appeared, and performing 40 or 45 cycles, even in two reactions stages in which the reaction mix is replenished, did not result in the appearance of any more products, but increased the amounts of those products that were already visible at 35 cycles.

Because of the presence in the laboratory of cloned genes and PCR products, negative as well as positive controls were always included in all experiments for all reactions. Positive controls were performed on plasmids containing either full length or PCR products (see Fig 6.4, page 202, for control reactions).

Several products were amplified in some of the reactions, most notably in the neonate with primers for the nAChR α4 subunit. In all cases (except α7 and GluR5), probing of Southern blots of the gels using labelled PCR product as a probe under stringent or non-stringent conditions resulted in a single amplified sequence of the predicted size hybridising to the probe (sample results seen in Figs 6.8, page 206, and with comparative PCR). With the α7 and GluR5 genes, two DNA bands on blots hybridised to the probes. In the case of GluR5, this was simply the two spliced versions of the gene which were both present. In the case of α7, an unexpected band at 410 b.p. was found to hybridise to the α7 probe under stringent conditions (see discussion). None of the other additional DNA species seen after amplification hybridised, even faintly, to any of the probes when probed on Southern blots.

The results of the PCRs on the nAChR subunits and GluR5 on cDNA synthesised from neonatal rat DRG are shown in figure 6.5a (30 cycles) and 6.5b (35 cycles), page 203. The subunit that appears to be most abundant is the α7, while there is no discernible signal for α2 and β2. In addition, no signal for α2 or β2 was visible after 50 cycles of PCR. The subunits can be ranked according to their apparent abundance, as α7, β4, α4, α3, GluR5, α5, and β3. However several variables affect the efficiency of PCR reactions, and this should not be taken as more than a rough indication of the subunits that are abundant in DRG (see discussion).

The results of the PCRs on cDNA synthesised from adult rat DRG are shown in figure 6.6a (30 cycles) and 6.6b (35 cycles), page 204. The results are distinct from those in the neonatal DRG. Firstly, the α7 and α5 subunits which were clearly present in the neonatal animal are present at much lower levels in the adult, and only traces
of each can be seen after 35 cycles. The α4, in addition to the α2 and β2 subunits is not visible at 35 cycles. The apparent rank order of subunits, starting with the most abundant is β4, GluR5, α3 and β3, with trace amounts of α7 and α5.

The initial results were obtained from RNA isolated directly from adult and also neonatal rat DRG neurons which were dissected out and placed straight into guanadinium denaturing mix, as this resulted in much greater yield than growing the neurons in culture and then harvesting the RNA by immersing the cultures in denaturing solution.

The results from PCRs performed on cDNA synthesised from the DRGs of adult and neonatal animals were repeated using cDNA synthesised from DRG cells isolated from adult and from neonatal animals grown in culture for three days in the absence of NGF before being harvested. The results obtained using cDNA from neonatal DRGs and from cultures of neonatal DRGs showed that DRG cells grown in culture expressed the same compliment of LGIC subunit genes as the DRG cells that were isolated directly from neonatal animals. Likewise, the adult DRG cells grown in culture expressed the same compliment of LGIC subunit genes as DRG cells that were isolated directly from the adult animals.

The results also show that the two splice variants of the GluR5 subunit are present in neonatal and adult DRG, with GluR5-1 (containing the additional optional exon) more abundant than the GluR5-2 mRNA.

6.2 e Estimate of amounts of subunits present.

A rough estimate was made of the amounts of β4, the subunit which appeared to be most abundant in adult DRG neurons, as an indication of the levels of expression of subunits in these cells. This was done by comparing the relative efficiency of amplification of single stranded DRG template with titrated amounts of the β4 PCR sequence RNA transcribed from the cloned β4 PCR product. RNA corresponding to a portion of the rat nAChR β4 subunit was synthesised for use as a standard against which to measure levels of β4 subunit in DRG total RNA. Transcription of this RNA from a plasmid containing a cloned partial sequence of was initiated from the SP6 promotor using a standard kit (Stratagene) after the plasmid template had been digested with Xho 1 to limit the size of transcript
produced to 500 b.p.. First strand reactions to synthesize cDNA from the transcribed RNA were performed on three concentrations of sense RNA. In each case, tRNA solution was used to dilute the β4 subunit sense RNA so that the overall concentration of RNA was similar (see methods, section 2.8 page 40).

Identical PCR reaction mixes lacking template were prepared, and aliquots of the first strand reactions produced from titrated β4 sense RNA were added to three tubes. One of the tubes was a negative control, and the last contained an identical aliquot of the test cDNA template to those used in the other PCRs above, the equivalent of 50ng of total RNA.

**Results of estimated β4 subunit quantitation.**

The amount of PCR product produced from the titrated reaction mixes could be seen to increase with the amount of RNA used in the first strand reactions in a non-linear manner. When a portion of the reactions was electrophoresed on an agarose gel, Southern-blotted and probed with the β4 probe using stringent conditions, it was apparent that the amount of product in the test PCR sample was closest to the amount of product in the PCR mix that had contained first strand cDNA synthesised from ~5 fg of β4 RNA (see Fig 6.7, page 205). The number of copies of the target sequence in this amount of β4 transcript RNA (and therefore in cDNA synthesised from 50 ng of DRG total RNA) was calculated to be in the order of 18,500.

The average yield of total RNA from the DRGs isolated from a single adult rat was in the order of 30 µg, therefore if 50 ng of total DRG RNA contains 18 500 target sequences, then there would have been 1.1 million target sequences isolated from each animal.

The average number of DRGs removed from each animal to obtain the yield of RNA quoted above was 25, so each dorsal root ganglion contained ~444,000 target sequences. As there are around 10,000 cells in each ganglion, there would be ~44 target sequences/cell, assuming that all cells express nAChR subunits. However, the percentage of individual cells in chick DRG in which nAChR α3 and α4 subunit transcripts can be detected has been reported to be 8% and 6% while around a quarter of the cells produce responses to ACh (Boyd et al, 1991). If the percentage of cells expressing β4 is guessed to be ~10%, the final estimated number of
transcripts per cell would be ~ 400. This calculation makes several assumptions and estimations, and is merely a guide to the possible level of expression (see discussion).

6.2 f Investigation of NGF regulation of DRG gene expression by comparative PCR.

Competitive PCR methods, in which titrated control sequence added to the reaction mix is co-amplified with the test sequence, have been employed to allow a quantitative approach to PCR (e.g., Dallman and Porter, 1991, Siebert and Larrick, 1992, Bouaboula et al, 1992, Celi et al, 1993). This can yield results which give a measurement of the amount of target sequence. However, rigorous controls must be carried out with each reaction performed.

In most instances methods which do not involve an amplification process which results in a near-exponential increase of the substance to be measured, such as quantitative Northern blotting (e.g., Miller et al, 1992, Ma et al, 1992) or RNase protection (e.g., Corriveau and Berg, 1993), would be preferred for obtaining quantitative information. The difficulty in obtaining enough material from the dissection and culturing of DRG neurons from rats made the use of quantitative Northern blotting or RNase protection a possibility, but would have involved a considerable number of animals. It was therefore decided to conduct preliminary experiments using comparative PCR to see if NGF appeared to exert any consistent change in the levels of the subunit mRNAs, and then to confirm this with RNase protection or Northern blot analysis if NGF appeared to be exerting a significant effect on expression.

Preliminary experiments were carried out using a comparative PCR method in which specific sequences were amplified from first-strand cDNA samples that had been synthesised from an equivalent amount of starting RNA, and the amount of product compared at different points in the reaction. In order to make any apparent differences in the amount of transcript for a specific sequence meaningful in terms of differences in the amount of amplified product from that sequence, several criteria had to be met in the experimental procedure.
Contaminating genomic DNA.

Amplified sequences had to be shown to be produced from cDNA and not from genomic DNA. Most of the primers used amplified sequences that included one or more introns (see figure 6.2, page 200 and 6.3, page 201), thus amplification of contaminating genomic DNA would produce an easily recognised product of significantly larger size or no product. However, great care was taken to remove contaminating genomic DNA from the samples, as this might have altered the resulting levels of reaction products.

Screening for genomic DNA was done by performing PCR reactions with two primers to exons 4 and 5 of the nAChR β4 subunit (UB4a and UB4b) in excess on an aliquot of RNA. Although the complete β4 subunit gene, tightly linked with the α3 and α5 subunit genes in the rat genome, is known to span 19 Kb (Boulter et al, 1990) the intron separating protein encoding exons 4 and 5 is around 500 b.p..

The two above primers define and amplify a sequence of 300 b.p. in β4 cDNA and a sequence of ~800 b.p. from genomic DNA which includes the intron of around 500 b.p.. By performing a PCR of 45 amplification cycles with these primers under conditions that would amplify a sequence as large as 800 b.p. from genomic DNA (the extension time for DNA synthesis was ~1 minute, allowing amplification of fragments of up to 1.4 Kb - Saiki et al, 1985, 1988), a sensitive test for the presence of genomic DNA was produced.

In cases where such a PCR revealed amplification of the genomic sequence, the RNA preparation was DNAsed and then retested with the PCR. Only when the ‘genomic PCR’ showed a negative result was the RNA used for first strand cDNA synthesis. Some RNA preparations tested contained so much DNA relative to RNA that they could not be cleaned of DNA without the total loss of the RNA content, and so they were discarded.

Where the amplified sequence did not span an intron, extra controls which lacked reverse transcriptase were included. Absence of product from the control reactions indicated that amplification was occurring only from cDNA reverse-transcribed from the RNA in the sample. None of these reverse-transcriptase control reactions resulted in amplification from RNA preparations previously tested for genomic DNA.
Negative controls.

A designated 'sample room' was set up in which to deal with reaction preparation. This area contained an isolation cupboard which allowed PCR mixes to be segregated from the rest of the room when template was added.

Other areas were designated 'dirty' areas for manipulation of completed reactions, running of gels and preparation of positive controls. All solutions for PCR were made up in sterile disposable tubes in very small aliquots in the 'clean room', including paraffin oil and distilled water.

Consumable materials were only allowed to travel downwards through the system (i.e. from clean to sample to dirty). Samples were stored in a separate freezer from the reaction components. PCR reaction mixes were always made and aliquoted in the 'clean' room and then transported to the 'sample room' where the template was added to the mix.

In addition to this, a strict scheme of negative controls was devised. Several negative control tubes would be prepared in the 'clean room' with the other tubes. One of these tubes would remain unopened in the 'sample room' as a check that contamination did not occur at the level of the 'clean room'. One negative control for every ten sample PCR reactions was included. These tubes having sterile distilled water added to them in place of sample, and the negative controls were sampled during the reaction run as a check that no major sample spill or aerosol had occurred while taking portions from reactions in progress.

NGF effect on cell morphology.

The effects of NGF on the cultures of DRG neurons could just be discerned visually upon observation through an inverted microscope - cultures lacking NGF contained a small population of cells that were rounded and lacked processes, while cultures containing NGF contained very few cells that did not possess axonal processes. Similar levels in cultures of a clonal line from the rat pheochromocytoma cell line PC12 however, produced a clearly visible change. PC12 cells cultured in the absence of NGF were rounded, while cells grown in the presence of NGF put out long branching processes.
Obtaining values from PCR results.

After a test PCR had been performed, with 10 µl portions taken from the reactions at either 3, 4 or 5 cycle intervals, the portions were electrophoresed on an 2 - 3.5 % (w/v) agarose gel containing ethidium bromide and photographed under U.V. light (% agarose differed depending on the size of product amplified).

If it was seen that negative and positive controls had produced the appropriate results, the gel was Southern-blotted onto Hybond-N using standard techniques (see methods, section 2.11 page 42), fixed using U.V. light and baking, and sealed in clean plastic until probing. Batches of ten or more blots containing the same PCR product were probed together.

After test PCRs had been performed, a further test G6PDH PCR was performed. The results from this were electrophoresed, Southern-blotted and probed with labelled, purified G6PDH probe. In nearly all cases where the levels of reaction products from the G6PDH PCR performed before the test PCRs were similar, the levels of reaction products from the G6PDH PCR performed after the test reactions was similar.

The radioautographs for all of the probed PCR blots were scanned using a laser densitometer, and the density of the area of X-ray film in the areas of radioactive probe hybridisation recorded (a non-linear relationship exists between band density and the signal). The results for all test PCRs were adjusted by dividing them by the values obtained from the same first-strand template preparation with the G6PDH PCR. In instances where the G6PDH reaction performed at the end of the test reactions produced different levels of product from the initial G6PDH PCR, all the test results were discarded.

In order to guard against any errors caused by the preparation or storage of RNA, all test PCRs were carried out on material from three different batches of +/- NGF RNA. In addition, test PCRs were often carried out on several different first-strand cDNA preparations from each batch of RNA. When the results from these cross-analyses were examined, neither the first-strand template synthesis nor the RNA preparation caused a consistent alteration of the transcript levels by more than 5 %, and the standard errors from averaged results were insignificant (see Fig. 6.9, page 207). Following adjustment by the levels of G6PDH from the same
cDNA preparation, the value of the (+)NGF sample was expressed as a percentage of the (-)NGF sample for each test PCR.

**Results of comparative PCR.**

The results of the comparative PCR were all normalised against G6PDH. To double check this, test PCR amplifications of L27, a ribosomal protein expressed at a constant level (Le Beau et al, 1991) were performed with the other test reactions, and afterwards normalised against the G6PDH values. When L27 values from NGF + and - cultures were expressed as a percentage difference, the values always differed by +/- 5% or less. In addition, the averaged L27 values for NGF- as a percentage of the value for NGF+ reactions when eleven pairs of test reactions were examined was 100.11%, i.e., a change in levels of a little over 0.1% (see Table 6.3, page 198, and Fig. 6.9, page 207).

With the positive PCR control, the results were consistent in showing higher levels of PPT transcript in cultures grown with NGF. However, the values obtained varied, the lowest increase being a fourfold increase, the largest being unmeasureable because when the product from the NGF+ reaction was at a very high level, the sample from the NGF- reaction at the same number of cycles was so low as to be unmeasurable. The measurable results only are shown (Fig. 6.9, page 207).

The results for all the ligand-gated channel subunits and the other DRG neuron genes tested indicate that the presence or absence of NGF in DRG neuron culture does not affect the levels of RNA transcript of these genes. Individual samples show slight variations in levels, the difference in values of NGF+ versus NGF- cultures is mostly less than +/-10%. The averaged variations for each of the genes indicates that no consistent regulation occurs (see Fig. 6.9, page 207)

A comparison of the results of all test genes for each individual RNA preparation or for individual first-strand reactions (see Table 6.3, page 198) indicates that no significant biasing errors were contributed by either process.

6.3 Discussion of expression and regulation of ligand-gated ion channel subunits in DRG neurons.

6.3 a Comparison of mRNA levels.
The control genes used in this study were chosen carefully for the equalisation of amounts of RNA. Many genes often considered to be expressed at constant levels can vary developmentally or under certain circumstances (e.g., β-actin, LeBeau et al, 1991) and many 'housekeeping genes' that might be considered candidates for this function are present in multiple copies (Chan and Wool, 1991) with related sequences occurring as pseudogenes present in hundreds of copies in the genome.

As with all the PCRs performed, aliquots were taken from the reaction tubes after set numbers of cycles, and then run in adjacent lanes on an agarose gel for analysis. Comparison was made between reactions containing template from (+) and (-) NGF cultures. This comparison was made between samples from points in the reaction where the amounts of products were increasing in near linear proportion to the number of amplification cycles performed. This was a precaution against taking results from reactions which had reached 'saturation'. This refers to the stage at which the PCR has reached a point where the reaction ingredients are so depleted, that the amplification rate is reduced.

Given inexhaustible Taq polymerase and a large number of amplification cycles, two PCR reactions which contain different amounts of starting template but are identical in every other respect could finish with an approximately similar amount of product, as the limitations of the amount of product are governed largely by the limitations of the reaction components. Thus to ensure that the levels of product measured are an expression of the relative amounts of starting material, samples must be measured or compared at a point when amplification in all reactions is near-linear.

If there was a visible difference in the amounts of amplified G6PDH product obtained with template from (+) and (-) NGF cultures, the first-strand reaction was discarded and repeated with an adjusted amount of RNA added to the reaction. Only when the amounts of product from the G6PDH PCR appeared to be similar as judged by eye from an ethidium bromide stained agarose gel, were test PCRs performed.

After test PCRs had been performed, a further test G6PDH PCR was performed. The results from this were electrophoresed, Southern-blotted and probed with labelled, purified G6PDH probe. In nearly all cases where the levels of reaction products from the
G6PDH PCR performed before the test PCRs were similar, the levels of reaction products from the G6PDH PCR performed after the test reactions was similar.

On several occasions the G6PDH reaction performed at the end of the test reactions produced different results to the one carried out before the test PCRs. Usually this took the form of one of the reactions failing to produce any product, or showing a much smaller amount of product than the test PCR. This was probably due to degradation of the single stranded cDNA, which is vulnerable to nuclease digestion. The matching of results from G6PDH reactions performed before and after the test PCRs confirmed that the status of the template material had not changed during the study. Following adjustment by the levels of G6PDH from the same cDNA preparation, the value of the (-)NGF sample was expressed as a percentage of the (+) NGF sample for each test PCR.

The PCR was designed to allow only a direct comparison of the levels of product amplified from a test gene in paired samples that had been equalised for their mRNA content. The results indicate no significant change in test genes, with a small margin of experimental error. The reactions performed with the PPT gene indicate that increases in the levels of gene transcripts could be detected by the method, although changes of more than 20-fold were unmeasureable. With the small margin of error, a change in levels of only 2- or 3-fold would have been detected by the method. A study conducted in parallel in which a similar comparative PCR was used to investigate the effect of NGF on levels of mRNA encoding the transcription factor neuronal Oct-2 in rat DRG neurons in culture, showed a consistent increase in levels of Oct-2 mRNA in the presence of NGF (Wood et al, 1992). This increase was between three and four-fold, and was measured using an internal standard in the PCR (Wood et al, 1992). That this increase is significant in terms of the levels of protein was confirmed by the use of quantitative band shift assays which showed a similar increase in Oct-2 protein in the presence of NGF (Wood et al, 1992).

If any of the results on test genes had indicated a small consistent change in levels, then this result would have been investigated further by the use of RNAse protection or quantitative Southern blotting (e.g. Ninkina et al, 1994). However, the results
indicated that no significant change in the levels of any of the test transcripts occurred.

RNA degradation was not checked following RNA preparation because only small RNA fragments (between 200 and 500 b.p.) were required for the PCR to work. It was assumed that any RNA degradation that would significantly affect the results of the PCR study would be apparent using the control PCRs to equalise the RNA levels.

6.3b Specificity of probes and primers.

The PCR amplification of single stranded cDNA made from cDNA isolated from neonatal and adult rat DRG neurons and from primary culture of adult rat DRG neurons described above was used to investigate the expression of several nAChR subunits and a glutamate ligand-gated ion channel subunit in these cells.

The probes used were produced in one of two ways from oligonucleotide PCR primers that were designed to match published cDNA sequences. Where cDNAs of the genes were available, PCR reactions were performed on plasmids containing full-length cDNAs of the respective genes, and the products were identified on the basis of their size. In this instance, where the nucleotide sequence of the cloned genes and the vectors into which they had been cloned were known, it could be predicted that the only one product of the correct size would be amplified, corresponding to the cytoplasmic or other sequences targeted. In all cases with these reactions, other products of high molecular weight were also produced, corresponding to 'run-on' of the DNA synthesis through the plasmid. This could be minimised by adjusting the amount of template plasmid downward. No products of less than 2 kb in size were seen except for the intended product in all cases except the α7 gene (see below). In all cases the DNA fragments to be used as probes were carefully excised from low-melting-point (LMP) agarose gels so that no contamination with vector sequence was possible.

Where cloned genes were unavailable, PCR primers designed to published sequences were used to perform PCR on adult rat whole brain cDNA preparations. Reaction products of the correct size were excised from LMP agarose and purified as above. The fragments were cloned into pGEM-T and sequenced. The resulting sequences were compared to sequences available in the Genbank and EMBL
databases using 'Intelligenetics' software (see methods, section 2.17 page 47) and all of them proved to be a 100% match to the published nucleotide sequences.

The level of nucleotide homology between the PCR amplified products was calculated to be less than 50% between any two products or between any of the products and any other sequence from other known ligand-gated channel subunits (see Table 6.2, page 197).

As all the probes used were identified as described above, and all the probing of blots was carried out in stringent conditions, the amplified products described must reasonably be assumed to correspond to the genes intended, or to sequences very closely related to those sequences. Additional amplified products were seen in only one reaction, that for the α7 subunit, where an amplified product of 410 b.p. was seen in addition to the expected product of 270 b.p.. Upon probing with the 270 b.p. α7 probe, this product was shown also to hybridise to the probe.

Upon a computer sequence search of the newly published rat α7 subunit cDNA sequence (Seguela et al, 1993), it was discovered that one of the primers (X6A7) could anneal to two discrete positions in the cytoplasmic loop of the α7 gene, and therefore two separate PCR products of different size, one 410 b.p. and one of 270 b.p. would result. The near duplication of this sequence does not occur in the chick α7 gene.

The second sequence to which the primer could bind had one mismatch out of 21 b.p. while the original sequence targeted had two mismatches when compared against the published rat sequence. The two products corresponded in size with the two products resulting from the α7 PCR. As probing of these products with the α7 probe under stringent conditions results in hybridisation to both fragments, it seemed likely that the second product seen was a result of this. However, both products were also isolated and cloned into pGEM-T and sequenced (as above) using primer X6A7 as a sequencing primer. The resulting sequence from the ends of these cloned fragments confirmed that the unexpected smaller product was indeed from the α7 gene and overlapped with the 410 b.p. product originally designed. The two overlapping products can reasonably be assumed to result from the hybridisation of the primer to two similar sequences in the cytoplasmic loop, separated by only ~140 b.p.. The amplification was more efficient for the smaller product, and by
increasing the annealing temperature in the amplification cycle, the reaction was altered so that amplification of the larger product became negligible.

6.3c Subunits expressed in DRG neurons.

The GluR5 subunit primers were made as a control for the cDNA template preparations, as the clear expression of this subunit has already been demonstrated in rat DRG neurons (Bettler et al, 1990). The above study confirmed this, and showed the presence of both splicing variants of this mRNA. As described above, the PCR products from each reaction produced an amplified product of distinctive size. However, to positively identify the products amplified, each test PCR was electrophoresed on an agarose gel, Southern-blotted and probed using labelled, purified PCR product under stringent conditions (for example results, see Fig 6.8, page 206).

The results showed several nAChR subunits being expressed at different levels in neonatal DRG neurons, fewer subunits expressed in the adult, and some subunits which did not seem to be expressed at all. The results showing that α3 is expressed, α4 is weakly expressed and that α2 and β2 are absent agree with previously reported results using Northern blot analysis and RNAse protection (Boyd et al, 1991).

Although the above analysis was non-quantitative, the number of cycles of PCR required to amplify an equivalent amount of DNA product from a single preparation of first-stranded cDNA using the various pairs of primers was noticeably different (see Fig. 6.5, page 203 & Fig. 6.6, page 204). This gives an apparent rank order of expression of these genes in DRG. This rank order is an indication of the likely rank order of those subunits expressed at the highest level in DRG to those expressed at the lowest level. Again, this cannot be taken as an absolute result because of several parameters of the amplification reaction which cannot be assessed without a separate internal control for each reaction. It is possible that these factors might affect the rank order, although probably not by a large amount, as all of the PCR primer pairs appeared reasonably efficient at amplifying their respective control DNA templates.

There are several differences in the nAChRs subunits present in neonatal and adult DRG, most notably the much higher levels of α7 subunit in the neonatal animal. Work on expression of nAChRs during development has focussed mainly on sympathetic ganglia.
Developmental regulation of nAChR subunits in ciliary ganglia and also the brain, including regulation of the a7 subunit has been described previously (Daubas, 1990, Couturier et al, 1990, Corriveau and Berg, 1993).

Developmental changes in sympathetic ganglia of ACh-activated currents (Moss et al, 1989, Engisch and Fishbach, 1990, 1992, Gardette, et al, 1991, Mandelzys et al, 1994) and mAb35 binding (Wang and Schmidt, 1976, Jacob and Berg, 1988, Margiotta and Gurantz, 1989, Jacob, 1991) have been described. Experiments in which removal of target tissue has been shown to reduce the ACh-evoked response from extending axons have suggested that nAChRs may play some role in synapse formation (Gardette et al, 1991, Englisch and Fischbach, 1990), however there is conflicting data (see Mandelzys et al, 1993).

Survival of some neurons during development has been linked with elevated levels of free cytoplasmic Ca^{2+} ions (review; Franklin and Johnson, 1992). Bearing in mind that the α-Bgt-binding receptor containing the a7 subunit in sympathetic ganglia is often non-synaptic in location (Listerud et al, 1991, Vernallis et al, 1993) and that this receptor influences the levels of intracellular Ca^{2+} (Vijayaraghaven et al, 1992), one possibility is that the a7 subunit plays a role in a nAChR that is opened in response to developmental signals to regulate the level of Ca^{2+} ions in the cell.

6.3 d Estimated β4 subunit quantitation.

The levels of expression of subunits can be only very roughly estimated due to the non-linear nature of PCR amplification. For a comparative guide to the amounts of mRNA for the various subunits, the product from β4, the most abundant nAChR subunit, was titrated and used as template for a PCR reaction (see Fig. 6.7, page 205).

With a similar fraction of a comparable PCR performed on a simple DNA template, products are visible on an ethidium bromide-stained agarose gel with thirty rounds of amplification from ~ 4 x 10^3 copies of the target sequence while ~40 copies of the target sequence are visible at forty cycles (Willoughby et al, 1991). The relative number of target sequences in the RNA required to produce a visible product here is 18 x 10^3 rather than 4 x 10^3 - a figure which probably reflects the efficiency of a reverse transcriptase reaction performed on total RNA with random hexamers.
The efficiency of two different sets of PCR primers cannot be assumed to be similar - they may have different annealing characteristics, and the size of fragment amplified and its sequence affects efficiency. But as the β4 levels in this study were calibrated against control reactions performed using the test primers on RNA transcribed from cloned β4 PCR product, these inaccuracies were not encountered.

However, there are several areas where differences between the sample and the calibrated template might arise. One of these areas is likely to be the reverse transcriptase reactions, where the reactions performed on total cellular RNA may have differed in their efficiency relative to the reactions performed on β4 transcript diluted in tRNA. The tRNA was added in sufficient quantity to make the percentage of β4 transcript to other RNA similar to that in the total RNA, a dilution of 1/10 000, assuming that mRNA is ~1% of total RNA and that β4 transcripts might comprise ~1% of the mRNA population. Despite this, the presence of tRNA sequences alone competing for the random hexamer primers (as opposed to tRNA, rRNA and the other cellular mRNA) may have introduced a factor of error.

While the use of control β4 RNA gives an indication of the efficiency of the first-strand reaction on RNA, there is no measure of the efficiency of the RNA extraction procedure from the animals. This is likely to be the largest area of error, as the yield of RNA differed from animal to animal.

The number of copies of individual nAChR subunit transcripts per neuron of the chick ciliary ganglion neurons has been estimated at around 1000 (Corriveau et al, 1993). After making allowances for several variables in this calculation which reduce the value obtained to a rough estimate, the results indicate that the level of expression may be similar to levels of nAChR in chick ciliary ganglion neurons.

The discrepancy between the amount of RNA isolated from freshly dissociated DRGs and the amount of RNA isolated from a similar number of DRGs isolated and then grown in culture is considerable. Although some of the difference may be due to general unavoidable cell loss during manipulations and to the clear difference in efficiency of RNA isolation from whole ganglia as opposed to cells from a culture dish, it is possible that some
differences might exist between the two populations of cells from which RNA was extracted for this work.

It was inevitable that non-neuronal cells were isolated in the dissection of the ganglia, and only rough trimming of the ganglia was possible, because rapid processing is necessary in order to obtain a good yield of RNA. Cytosine arabinoside (AraC) was added to the cells isolated from DRG in culture. This substance is a mitotic inhibitor and so maintains the proportion of neuronal cells in the cultures. The use of AraC to minimise the number of non-neuronal cells in the cultures was to prevent non-neuronal cells from dividing and therefore becoming abundant in the cell population; thus 'diluting' the signal from neurons expressing LGICs. Although LGICs can be expressed in astrocytes and other non-neuronal cells (e.g., Jensen and Chiu, 1993), in situ hybridisation on chick DRG cells in culture has demonstrated that the α3 and α4 nAChR subunits are expressed exclusively in a subset of neurons and are not in non-neuronal cells (Boyd et al, 1990).

In addition to this, a number of cells are lost during culture. It is not clear whether this loss of cells is entirely due to general loss of cells due to damage received during the isolation process, or whether a particular population of cells fails to survive. As NGF acts as a survival factor for a specific subset of DRG neurons during development, it is also possible that it remains a survival factor for a smaller subset of DRG neurons in the adult animal. However, if this is the case, the subset of cells is not yet identifiable by specific features and is likely to be fairly small.

6.3 e Subunits and receptors.

Despite rapid progress in the functional analysis of neuronal nAChRs, and the molecular characterisation of ten putative neuronal nAChR subunits, major questions about which subunits are expressed and assembled into receptors and how the selection of these subunits is achieved are still unanswered.

Work on nAChRs in neuronal culture has permitted studies on interactions of subunits and channels in the presence of possible neuronal accessory proteins which may be involved in assembling receptors or converting them into an active state. While two types of subunit expressed together is sufficient to make a functional channel in oocytes (Papke et al, 1989), it is still not clear whether neuronal
nAChRs are normally composed of only two types of subunit or more, as in the case of the neuromuscular nAChR. In addition, in some cells showing nicotinic responses which express several subunits, not all of the subunits appear to contribute to functional channels, for instance the β2 subunit in ciliary ganglion neurons (Vernallis et al, 1993) and by inference, the α4 subunit in superior cervical ganglion neurons (Listerud et al, 1991).

Thus the simplest possible model where any of the subunits expressed in a particular cell can assemble with equal affinity does not hold. The discovery that the subunit complement of a cell is not necessarily related to the receptor complement of a cell, reduces predictions about the receptor types that may be present on nAChR subunit containing cells to speculation unless other analyses are performed.

If neuron-specific mechanisms are responsible for directing the assembly of receptors from their constituent subunits, the subunit complement of a cell may be less important than the cell-specific mechanisms themselves in terms of the receptors produced by the cells.

However, it can be said from the above work, that DRG neurons contain a sufficient nAChR subunit complement, at least in terms of the different types of subunit transcripts that are present in the cells, to make several types of receptor, including the abundant α3- and β4- containing receptor found in the peripheral nervous system (Whiting et al, 1991), and the α-Bgt-resistant nAChR found on ciliary ganglion neurons (Vernallis et al, 1993).

It is not clear, however, that all of these subunits are present in the same cell types, as in situ work with probes for nAChR subunits has indicated that only a small percentage of cells (6 - 8%) express nAChR subunits, and that α3 and α4 subunit mRNAs are expressed in a subset of both large and small neurons (Boyd et al, 1991).

The further investigation of nAChRs on DRG neurons requires other experimental work to be done, either functional testing of the receptors, or using toxins or monoclonal antibodies (mAbs) to determine which subunits assemble together into surface complexes. mAb35, a monoclonal antibody that was originally described as recognizing assembled nACh receptors containing the α3 subunit (Halvorsen and Berg, 1990) has been used to examine chick DRG
neurons for the presence of nAChR protein. Around 16% of the DRG neurons from 18-20-day old embryos contained detectable levels of specific labelling, contrasting with labelling of ~85% of chick ciliary ganglion neurons from animals of the same age (Boyd et al, 1991). The labelling seen in ciliary ganglion neurons is however, partly due to a large intracellular pool of receptors (Jacob et al, 1986, Stollberg and Berg, 1987). This level of mAb35 immunoreactivity was seen in DRG neurons from several levels in the spinal cord.

Subsequent work with mAb35 suggests that it more generally recognises nAChR subunit complexes which contain one or more of either the α3, α5 and β4 subunits (Conroy et al, 1992, Vernallis et al, 1993), all of which are expressed in DRG to some degree.

Neurotoxin-binding studies performed on DRG neurons support the suggestion that binding sites are not abundant. Low levels of neuronal bungarotoxin (bungarotoxin 3.1, Toxin F) are found in cultures of spinal cord and DRG neurons (Halvorsen and Berg, 1986). This toxin is able to bind to nAChRs containing α3 and β2, less strongly to α4 and β2 (Luetje et al, 1990). It has also been demonstrated to bind to the chick ciliary ganglion neuron nAChRs (Ravdin and Berg, 1979, Halversen and Berg, 1986, 1987) which have been shown to contain principally the α3, α5 and β4 subunits (Conroy et al, 1992, Veranallis et al, 1993).

Testing nAChR function on DRG neurons is unlikely to be possible using simple assays because of the low density of nicotinic receptors on rat DRG neurons. Only around a quarter of the neurons produce an easily measured conductance in response to application of 100-200 μM ACh (Boyd et al, 1991), but the mean response was 5.5 (± 1.4) nS, about one fifth of the whole cell ACh response for ciliary ganglion neurons with patch-clamp recording (Margiotta and Gurantz, 1989). d-tubocurarine was able to reversibly block the response, thus confirming that this ACh response was nicotinic in nature.

A useful investigation of the types of receptors present on the surface of DRG neurons and the possible regulation of their numbers under different conditions might be made by measuring the surface binding of 125I-labelled α-bungarotoxin and neuronal bungarotoxin on neurons grown in culture with different concentrations of NGF and with anti-NGF mAbs.
The α-bungarotoxin would give an indication of the amount of α7-containing receptors and complexes on the cell surface - it should be remembered that the possible presence of the other α-bungarotoxin-binding subunit, α8 (e.g. Schoepfer et al, 1990), in the cells has not yet been investigated. Neuronal bungarotoxin (also known as toxin F, κ-bungarotoxin, bungarotoxin 3.1) binds to other nAChR receptor types, including those that contain the α3 subunit (e.g., Luetje et al, 1990, McLane et al, 1990). Previous work has indicated that there are a small number of neuronal bungarotoxin binding sites in DRG neurons (Halvorsen and Berg, 1987).

6.3 Regulation of ligand-gated channel subunit expression in DRG neurons.

Various culture conditions have been shown to regulate the number and type of nAChR subunits. In some of these instances the regulation of subunits happens synchronously with receptor numbers suggesting that the subunit and functional receptor content of a cell may be linked, in other instances one is affected without a change in the other, suggesting that separate regulatory mechanisms also exist. The concurrent changes in numbers of subunits and receptors that occurs in some cell types suggests the possibility that regulation of key subunits, present in these cells in limiting amounts may be one mechanism of receptor regulation.

In chick ciliary ganglion neurons, CNTF specifically down regulates the α-Bgt-binding receptors while not affecting the other nACh receptor population (Halvorsen and Berg, 1989). The effect of CNTF on individual subunits was not investigated. The failure of NGF and FGF to differentially regulate the receptors indicates that the down regulation in these neurons is not a general property of growth factors.

However, the nACh receptors on the cell surface of ciliary ganglion neurons represent only a proportion of those present in the cell (Stollberg and Berg, 1987) suggesting that the assembly of receptors may occur in several stages. Furthermore, treatment of ciliary ganglion neurons with cAMP causes the number of functional surface receptors to increase without detectable protein synthesis or any apparent change in the number of receptor complexes on the cell surface (Margiotta et al, 1987). This suggests
that a pool of receptors in the membrane may exist in inactive and active states.

Pre-ganglionic denervation and post ganglionic axotomy of ciliary ganglion neurons were found to have different effects on receptors and subunits, implying different regulatory pathways. Where the former reduced the number of intracellular nAChR proteins without detectably changing the number of receptors on the cell surface, post-ganglionic axotomy produced a marked reduction in both the cytoplasmic and synaptically located nACh receptors (Jacob and Berg, 1988).

It seems likely from these results that there are several levels of receptor regulation, some of which may link the regulation of subunits with receptors, and some in which the regulation of receptors is independent. The possibility that the availability of key subunits is part of the regulatory mechanism exists is supported by the varied levels of expression of the subunits expressed in many cell types (e.g., in ciliary ganglion neurons transcripts of the α5 subunit, a participant in one receptor type, are present at much lower levels than those encoding the other two subunits with which it principally assembles - Corriveau and Berg, 1993).

The possibility that regulation of a single subunit might change the properties of assembled nAChRs on neurons by altering the subunit composition of receptors has been raised by work on chick sympathetic ganglion neurons in culture in which antisense oligonucleotides were used to specifically deplete the neurons of single subunits. In these cells an array of subunits is expressed (α3, α4, α5, α7, β2, β4) and several classes of nAChR channels can be detected by patch-clamp studies (Listerud et al, 1991).

When the antisense oligonucleotides were used to 'delete' specific subunits, several classes of channel were lost, but new channel classes appeared. Thus the subunit composition of nAChRs may be to some extent flexible, and if significant changes in the levels of single subunits may occur, it is possible that this may alter the properties of the surface receptors. No example of this sort of regulatory mechanism has yet been described, but it could potentially be a functionally important mechanism, as substitution of a single subunit could affect properties such as the agonist sensitivity of the receptors (e.g., Luetje and Patrick, 1991).
The results from the quantitative PCR analysis do not indicate any consistent differences in the mRNA levels of any of the ligand-gated channel genes in cultures grown with either NGF or with the anti-NGF mAb (see Fig. 6.9, page 207). In contrast to the LGIC subunits, the positive control showed clear induction by NGF of the PPT gene (see Fig. 6.9, page 207). The PPT mRNA is a precursor mRNA molecule that encodes a peptide which is eventually edited into the neuropeptide substance P (Harmer et al, 1986). Two splice variants of the molecule exist, α-PPT and β-PPT (Nakanishi, 1986, Nawa et al, 1983), both of which encode peptides which contains the substance P peptide. However, one version of the peptide, β-PPT, contains an additional exon which encodes NkA (neurokinin A).

The PCR for PPT was designed so that both versions of the gene could be amplified to give a different size of product. Only β-PPT was found in DRG neurons, either in material isolated directly from neurons or from material cultured in the presence or absence of NGF. The levels of this product were found to increase dramatically when cells were cultured in the presence of NGF. This agrees with previous reports of Southern blot analysis of the PPT mRNA in DRG neurons which indicated that the presence of NGF significantly upregulates this mRNA (Lindsay and Harmer, 1989).

Higher PPT mRNA levels in the presence of NGF were seen consistently in this study, but the amounts by which the PPT mRNA levels were raised in the presence of NGF were not consistent, varying from between 4-fold to levels that were unmeasureable (>25-fold). The highest measurable result was a 20-fold increase in mRNA. The published Southern blot analysis measured the increase as being between 15- and 60-fold (Lindsay and Harmer, 1989). This study was performed in cultures of DRG neurons grown in similar conditions, with the major exception that a concentration of 25 ng/ml of NGF prepared from mouse submaxillary gland was used in the media in the published study, whereas in this study, commercial, purified NGF (Promega) was added to a concentration of 5 ng/ml.

Short and long-term regulation of RNA levels in response to NGF has been described, ranging from effects seen over a period of hours (e.g., Kendall et al, 1994), to effects which achieve their maximum levels only after several days (Lindsay and Harmer, 1989).
In this study a moderately high NGF concentration was used for a time period of three days before cell cultures were harvested. This was designed to examine the effects of a prolonged elevation of NGF.

The PPT gene included in the study as a positive control of NGF activity was clearly elevated by this treatment. It is not clear why the changes in level of PPT mRNA are so variable in the study. However, near exponential amplification of sequences are involved and so small errors in the reaction mixes are likely to result in some variation. It should also be pointed out that in the published Southern blot analysis, the change in RNA levels varied for unknown reasons. This may be partly due to drifting NGF levels in some of the cultures used, as neurons are grown either with or without NGF in the previous study. This might allow NGF synthesis by some cells present in the culture, resulting in trace amounts of NGF in the (NGF-) culture. In this work, however, neurons are grown either with NGF or with an anti-NGF antibody immediately upon isolation, so that any NGF in the NGF- cultures would be neutralised: thus the difference in NGF levels between the (NGF+/−) cultures may be more marked. The relative increase in PPT mRNA level is often unmeasurable in the study as they are too high for the range of the technique, and it is unclear whether they do reach levels as high as those reported previously. Concentration-dependent regulation of expression by NGF has been reported previously, (e.g., Miller et al, 1991, Ma et al, 1992), and so it is possible that the lower levels of NGF used in this study would result in a smaller increase in PPT transcripts. However, the PPT PCR did confirm the biological activity of the NGF, and as such provided confidence in the very consistent negative results obtained with all other genes tested. Results for RTX13/42 and Brn-3a are discussed separately in chapters 4 and 5 respectively.

The PCR results in this study show that several nAChR subunits are expressed in DRG neurons, although to what extent these assemble into receptors, and on which neurons in the ganglia is not known. The results from the quantitative PCR analysis show that the levels of nAChR subunit mRNA in DRG neurons are probably not regulated by NGF. As no functional studies of nAChRs were performed, no comment can be made on the effect of NGF on the nicotine and ACh sensitivity of the cells.
Table 6.1

Ligand-gated channel subunits localised to DRG neurons.

<table>
<thead>
<tr>
<th>Channel/receptor</th>
<th>Subunit</th>
<th>method of detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAChR</td>
<td>α2 - not present</td>
<td>in-situ, RNAse protection</td>
<td>Boyd et al 1990</td>
</tr>
<tr>
<td></td>
<td>α3</td>
<td>in-situ, RNAse protection</td>
<td>Boyd et al 1990</td>
</tr>
<tr>
<td></td>
<td>α4</td>
<td>in-situ, RNAse protection</td>
<td>Boyd et al 1990</td>
</tr>
<tr>
<td></td>
<td>β2 - not present</td>
<td>in-situ, RNAse protection</td>
<td>Boyd et al 1990</td>
</tr>
<tr>
<td>Glutamate</td>
<td>GluR1</td>
<td>Northern blot</td>
<td>Keinanen et al, 1990</td>
</tr>
<tr>
<td></td>
<td>GluR2</td>
<td>Northern blot</td>
<td>Keinanen et al, 1990</td>
</tr>
<tr>
<td></td>
<td>GluR5</td>
<td>In situ</td>
<td>Bettler et al, 1990</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5HT-3</td>
<td>In situ</td>
<td>Tecott et al, 1993</td>
</tr>
</tbody>
</table>
Table 6.2.

Table showing the approximate percentage homology between the products amplified from the rat neuronal nAChR subunits by PCR. The products of test PCRs were electrophoresed, Southern blotted and probed with purified, $^{32}$P-labelled PCR product from the appropriate gene. The highest level of homology between any two subunits is 57%, and the probing of blots using stringent conditions did not result in any cross-hybridisation to nAChRs. As the size of the products from each gene was also distinctive, there is little doubt that the gene sequences amplified are those intended.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>α2</th>
<th>α3</th>
<th>α4</th>
<th>α5</th>
<th>α7</th>
<th>β2</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α3</td>
<td>52%</td>
<td>100%</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>57%</td>
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<td>39%</td>
<td>42%</td>
<td>49%</td>
<td>51%</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2</td>
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<td>48%</td>
<td>56%</td>
<td>52%</td>
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<td>48%</td>
<td>56%</td>
<td>52%</td>
<td>48%</td>
<td>57%</td>
<td>52%</td>
<td>100%</td>
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</table>
Table 6.3

Table of results showing the relative levels of various test genes in cells grown in NGF+ or NGF- conditions. The data shown is the density of the area of hybridization seen on radioautographs from Southern-blotted gels which have been probed with a specific probe (see methods). The values were measured in arbitrary units on a scale of 500 points (5.00 = 500) using a laserdensitometer (LKB).

The results are from three different RNA preparations (RNA preps 1, 2, 3) and from each of these several different cDNA template preparations were made. Five different cDNA preparations numbered 1.1, 1.2 etc. as separate rows are represented for RNA prep 1, from top to bottom, three cDNA preps (2.1, 2.2, 2.3) for RNA preparation 2 and three cDNA preps for RNA preparation 3. Values shown were later adjusted using the corresponding value for G6PDH shown in the left hand column of figures*.

<table>
<thead>
<tr>
<th>Preps</th>
<th>cDNA</th>
<th>L27</th>
<th>PPT</th>
<th>α3</th>
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<th>α7</th>
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<th>β4</th>
<th>G5-1</th>
<th>G5-2</th>
<th>RTX</th>
<th>Brn-13</th>
<th>3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA prep 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1.1 NGF+</td>
<td>1.34</td>
<td>0.57</td>
<td>4.87</td>
<td>0.21</td>
<td>1.31</td>
<td>1.90</td>
<td>0.17</td>
<td>0.97</td>
<td>1.06</td>
<td>1.69</td>
<td>-</td>
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| RNA prep 2 |
| 2.1 NGF+ | 3.63 | 0.09 | 0.74 | 4.50 | 0.86 | 0.59 | 1.41 | 0.78 | 1.56 | 3.79 | -     | -     |     |
| NGF-   | 3.20 | 0.09 | 0.05 | 4.43 | 0.97 | 0.55 | 1.44 | 0.93 | 1.55 | 3.52 | -     | -     |     |
| 2.2 NGF+ | 2.62 | 0.13 | 2.25 | 0.93 | 1.80 | 4.41 | 1.68 | 0.87 | 1.26 | 4.82 | -     | -     | -     |
| NGF-   | 2.82 | 0.14 | 0.41 | 1.02 | 1.79 | 4.51 | 1.71 | 0.92 | 1.35 | 4.27 | -     | -     | -     |
| 2.3 NGF+ | 1.11 | 0.80 | 3.23 | 0.18 | -     | 2.66 | 1.15 | -     | 4.43 | 3.28 | -     | -     | 3.33 |
| NGF-   | 1.21 | 0.74 | 0.43 | 0.18 | -     | 2.57 | 1.03 | -     | 4.12 | 3.33 | -     | -     | -     |

| RNA prep 3 |
| 3.1 NGF+ | 1.09 | 0.82 | 4.34 | 1.56 | -     | 0.92 | -     | 2.54 | -     | -     | -     | -     |     |
| NGF-   | 1.17 | 0.79 | 0.14 | 1.43 | -     | 0.85 | -     | 2.91 | -     | -     | -     | -     |     |
| 3.2 NGF+ | 1.99 | 0.25 | 4.65 | 0.69 | 2.77 | -     | 1.08 | 0.49 | -     | -     | -     | -     |     |
| NGF-   | 1.02 | 0.26 | 0.43 | 0.74 | 2.69 | -     | 1.01 | 0.47 | -     | -     | -     | -     |     |
| 3.3 NGF+ | 1.00 | 0.93 | 4.91 | 1.20 | -     | 0.75 | 0.64 | 0.73 | 0.93 | 3.49 | 3.38 | 3.46 | -     |
| NGF-   | 1.07 | 0.91 | 1.96 | 1.41 | -     | 0.65 | 0.65 | 0.69 | 0.94 | 3.28 | 3.63 | 3.18 | -     |
Figure 6.1
Schematic diagram (not to scale) of the intron-exon arrangement of mammalian neuronal and neuromuscular nAChR subunit genes.
Black triangles indicate the position of introns, roman numerals denote numbering of protein-encoding exons. White boxes indicate transmembrane regions, stippled boxes indicate signal peptides, s-s indicates the position of the ligand-gated channel motif. Only the protein-encoding region of the genes is represented.

Neuromuscular α-subunits.

Neuromuscular β-, γ-, ε-, σ- subunits.

Neuronal subunits.

Neuronal α7 subunit.
Figure 6.2
Schematic diagram of the PCR primers used in the comparative PCR study of ligand-gated ion channel subunits in DRG neurons. Hatched boxes indicate signal peptide, clear boxes indicate transmembrane regions, long triangles indicate intron positions, arrows indicate location of primers.
Preprotachykinin.

* Introns

1 2 3 SP 4 5 SK 6 7

PPT1 (product 210 b.p. with these exons) PPT2

PPT1 (shorter 156 b.p. product)

G6PDH

* Introns

5 6 7 8

G6PDH-A (433 b.p. product) G6PDH-B

Figure 6.3

Schematic diagram showing the position of primers used in PCR amplification of preprotachykinin (PPT) and glucose-6-phosphate dehydrogenase (G6PDH) gene sequences in relation to the exon structure of the two genes. Boxes represent exons, dotted lines represent introns. Arrows denote primers. The dark stippled areas are the peptides substance P (SP) and substance K (SK).
Figure 6.4

Ethidium bromide stained agarose gels containing portions of control PCR reactions. (A) is a 3% agarose gel, (B) is a 1.6% agarose gel. Product size is indicated in b.p. - reactions are as follows:

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<th>template</th>
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<td>pBr3b + pBr3c</td>
<td>plasmid clone 72</td>
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<tr>
<td>2</td>
<td>PPT</td>
<td>PPT1 + PPT2</td>
<td>cloned PCR product</td>
</tr>
<tr>
<td>3</td>
<td>nAChR β3</td>
<td>X5B3 + TMD4a</td>
<td>cloned PCR product from rat DRG</td>
</tr>
<tr>
<td>4</td>
<td>GluR5-2</td>
<td>G-5a + G-5b</td>
<td>purified PCR product from rat DRG</td>
</tr>
<tr>
<td>5</td>
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<td>G-5a + G-5b</td>
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</tr>
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</tr>
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Figure 6.5
Ligand-gated ion channel subunit expression in neonatal rat DRG neurons after (A) 30 and (B) 35 cycles of PCR amplification. PCR's were performed on a single stranded cDNA Template synthesised from 50 ng of neonatal rat DRG total RNA, in a reaction volume of 100 µl. 10 µl of the reactions are shown electrophoresed on a 1.8% agarose gel containing ethidium bromide. Lanes are as follows:

(1) = nAChR α2, (2) = α3, (3) = α4, (4) = α5, (5) = α7, (6) = β2, (7) = β3, (8) = β4, (9) = glutamate subunit, GluR5, (M) = DNA markers.
Figure 6.6
Ligand-gated ion channel subunit expression in adult rat DRG neurons after (A) 30 and (B) 35 cycles of PCR amplification. PCR's were performed on a single stranded cDNA Template synthesised from 50 ng of adult rat DRG total RNA, in a reaction volume of 100 µl. 10 µl of the reactions are shown electrophoresed on a 1.8% agarose gel containing ethidium bromide. Lanes are as follows:

(A) 1 = nAChR α2, (2) = α3, (3) = α4, (4) = α5, (5) = α7, (6) = β2, (7) = β3, (8) = β4, (9) = glutamate subunit, GluR5, (M) = DNA markers.
Figure 6.7

Titrated PCR on the nAChR β4 subunit. Southern blot of PCRs performed with primers X5B4 and TMD4a for the rat nAChR β4 subunit. 35 cycles of amplification have been performed for all PCRs. Positive and negative controls are lanes (1) and (5) respectively. The standard PCR reaction (lane 6) has been performed on single stranded cDNA synthesised from 50 ng of adult rat DRG total RNA (the same amount of material used in comparative PCRs). Lanes 2, 3 and 4 contain single stranded cDNA made from 100, 5 and 0.25 fg of nAChR β4 subunit mRNA transcribed from a cloned fragment of the gene diluted in tRNA solution (see methods).
Figure 6.8

Example radioautographs of probed Southern blots containing PCR products from NGF+/- experiments. In each case, identified, purified PCR product was used as a probe to identify specific products. Blots contain samples taken from PCR reactions at either three of four-cycle intervals. The blots contain negative controls (lacking template) at either end, and a positive control which is a PCR performed on cloned template sequences. Gels shown are PCRs to amplify fragments of (A) nAChR 53 subunit and (B) Preprotachykinin as a 210 b.p. product (β-PPT).
Figures 6.9a and 6.9b. Results of NGF +/- experiment.

The values are the % change for all test genes from each RNA preparation was averaged, with standard deviation. Averaged values for negative control (L27) and positive control (PPT) are likewise shown for comparison. The two graphs show the same data, but 6.12a does not include PPT. Three of the data points for PPT are (>2500%) rather than a real value, and so the PPT value and SD are an underestimation.

Data is the level of mRNAs in NGF(+) cultures expressed as a % of the level of mRNAs in NGF(-) cultures. The numbers of values are as follows:

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Figures 6.9a and 6.9b. Results of NGF +/- experiment.
Conclusions and possible future directions for research.

7.1 nAChRs in DRG neurons and the nAChR β3 subunit.

The capsaicin receptor is of great interest because of its specific expression in nociceptive neurons. The first screening procedure based on nucleotide homology hybridisation was likely to succeed in isolating components of the capsaicin receptor only if the receptor resembled other LGICs. However, any novel LGIC expressed in DRG would be of interest, and the approach of screening with a pool of cDNA clones allowed the possibility of isolating new members of the LGIC family. The novel nAChR β3 subunit clone isolated is a homologue of a gene which has been isolated from both rat (Deneris et al, 1989) and chick (referred to in Sargent, 1993), and sequenced. The gene encodes a protein which is assumed to be a member of the nAChR subunit family, but whose function has yet to be determined. Experiments in which the nAChR β3 subunit gene is co-injected as RNA into Xenopus Oocytes along with other LGIC subunit genes or co-transfected along with other LGIC subunit genes as plasmids into cell lines is still being performed in several laboratories as attempts are made to find a combination of subunits that will assemble into functional receptors incorporating the β3 subunit (Bertrand, D., personal communication). This approach may prove unfruitful because the subunits that assemble with the β3 subunit have yet to be isolated.

Perhaps the best method of pursuing the possible function of the gene would be to try to isolate the subunits that may assemble with it before attempting to reassemble the receptor in expression systems. This could be approached by choosing a cell type in which the subunit is normally expressed and then purifying receptors which contain the nAChR β3 subunit.

To do this, primary cultures of the neurons would be infected with replication-deficient adenovirus (Le Gal La Salle et al, 1993) expressing a mutated form of the nAChR β3 subunit carrying an epitope tag. Such a tag, typically consisting of a string of around ten amino acids (e.g., Davis et al, 1991), allows the resulting fusion proteins to be isolated by monoclonal antibodies (mAbs) specific to the epitope tag peptide. With such a tag attached to the C-terminus of the
β3 subunit protein, it may prove possible to isolate membrane-bound receptors that contain the β3 subunit. Once such receptors are isolated as complexes, the proteins comprising the complexes could be separated by electrophoresis on denaturing polyacrylamide gels and their molecular weights determined as has been done previously with nAChR subunit (Whiting et al, 1987). These gels could be than Western-blotted and probed with subunit-specific mAbs which recognise various denatured subunit proteins to determine whether any of the isolated proteins are novel or whether they correspond to characterised LGIC subunits (Conroy et al, 1992).

Although the epitope tagging method may appear to be elaborate, it may prove to be a more straightforward route than attempting to raise antibodies to nAChRs in solubilized membranes. In the past attempts to generate such antibodies by immunizing animals with ‘nAChR complexes’ purified from membrane have not produced mAbs that recognise nAChRs, or have produced mAbs that recognise only the denatured subunits on Western blots which are therefore not useful for isolating native receptors from cell fractions (Whiting and Lindstrom, 1986a, 1986b).

In the event of the functional significance of the being identified, the existence of the human cDNA sequence of nAChR β3 subunit will facilitate attempts to find specific antagonists to the receptor should it prove to be of pharmacological interest.

Apart from the nAChR α8 subunit and the α6 subunit (for which no sequence has been released), this was a comprehensive study on the nAChR subunits expressed in DRG neurons, and showed that several subunits are expressed in these cells. If the subunits are expressed in the same cell types, there is the potential for some cells to make several different types of nAChR. A useful piece of work would be to extend this study to perform in situ hybridisation on DRG cells in culture so that it could be determined if all the subunits were expressed in a single subset of cells, or whether the subunits are expressed in non-overlapping populations of cells. Another interesting study would be to compare the cell types that express nAChRs with the subset of DRG neurons that exhibit capsaicin sensitivity. This perhaps could be performed by doing in situ hybridisation for nAChR subunits on neonatal DRG cells in culture and on neonatal DRG cells in culture which had been pretreated with a high level of capsaicin. The increased sensitivity of
neonatal cells to capsaicin exposure means that most of the sensitive cells are killed (Wood et al, 1988). If the in situ signal for nAChRs was reduced or abolished after the capsaicin treatment, this would indicate to what degree nAChRs were present on capsaicin sensitive cells.

7.2 The capsaicin receptor.

The screening for proteins that bound to RTX was targeted specifically to isolate the capsaicin receptor or RTX-binding components of it. This screening method was a valid approach, although because the structure of the receptor is uncertain in terms of whether the RTX binding domain is contained within a single protein, there is a possibility that it is ultimately unlikely to succeed. The successful isolation of RTX-binding proteins indicates that the method as used was functional.

However, because of the uncertain structure of the RTX-binding site on the capsaicin receptor and the problem of how to further investigate fusion proteins that are isolated as RTX-binding proteins, other approaches may be more likely to succeed.

One strategy that would aid in reducing the number of clones to be screened by different methods, would be to construct a DRG subtraction library, that is, a DRG cDNA library in which all the mRNAs which are expressed in another cell type are removed during the construction. Such a subtraction could be, for example, the subtraction of mRNA from sympathetic neurons (for instance PC12 cells) from DRG neurons. Such a subtraction would produce a library with much fewer clones in it, but would include sequences specific to sensory neurons and therefore present a much smaller population of clones to be screened.

Such a library would be useful for screening with the two methods used in this study, that is, homology screening using LGIC clones and screening for proteins that bind RTX. Once again, the possibility that the receptor may be composed of several different subunits must be considered, as not all of these may necessarily be specific to sensory or even capsaicin-sensitive neurons.

Although the construction of a subtraction library may reduce the number of clones that are to be screened, the problem of how to identify clones that are isolated as contributing to the capsaicin receptor remains.
The most direct route to cloning the receptor, functional screening (e.g., Maricq et al, 1991) should perhaps be investigated further. Although no functional response has been detected in *Xenopus* Oocytes, other expression systems, for instance COS cells, could be investigated. This would necessitate construction of an expression library which could be transfected into the cells. In the first instance a method other than looking for an electrophysiological response, such as histological methods for detecting uptake of divalent cations, for instance cobalt (Davis, 1982, Pruss et al, 1991) might be utilised.

### 7.3 Transcription factors in DRG neurons.

The study of *Unc 86*-like transcription factors in DRG neurons was at an early stage during the project, and the isolation of new Brn-3 clones from the rat DRG library was a useful contribution to the identification of several related genes, now referred to as Brn-3a, Brn-3b and Brn-3c. All of these are expressed to varying degrees in DRG neurons (Ninkina et al, 1993).

As the originally described *C.elegans* mutant with an *Unc 86*-deletion was defective in terms of its touch receptor function, it is tempting to speculate that the three related mammalian POU proteins, Brn-3a, -b and -c, may be similarly specific in determining the fate of primary sensory neurons which detect other modalities, for instance, perhaps temperature, or nociception. However, as there is often a 'cascade' of transcription factors which are switched on during the determination of cell fate, it is also possible that these transcription factors may function at an earlier stage in development or contribute to a part of several neuronal phenotypes which would be more difficult to define.

It would be interesting to look at the effect on the neurons of deleting one or more of these genes, for example, to see if there was any loss of a subset of neurons, and also to determine whether there was still a capsaicin-sensitive population of cells. The presence of three closely related genes raises the possibility that there may be some overlap of function, and that experiments that involve deletion of one of these genes may not produce clear cut results.
7.4 NGF regulation.

As discussed briefly at the end of chapter 6, the possible effects of NGF on nAChR levels in DRG might be pursued by determining if NGF changes the amount of surface receptor protein rather than mRNA levels. It is possible that NGF could influence the turnover of nAChRs on the surface, for example, by either increasing receptor stability or decreasing endocytosis. Surface nAChR could be measured using nAChR antibodies, such as mAb35 (Jacob et al, 1986), or a-BGT binding in DRG neurons (e.g. Wang and Schmidt, 1976, Loring and Zigmond, 1987).

The analysis of levels of surface proteins in this manner may be more relevant in terms of the effect that NGF has on the cell than a study of mRNA levels; regulation of expression of nAChR subunit genes does not appear to necessarily affect the number of functional receptors present on the cell surface (review: Berg et al, 1989).

In addition, the effects of BDNF, NT-3 and NT-4/5 on cultures of DRG neurons could be examined. The expression of receptors for these members of the neurotrophin family on developing DRG neurons suggests that they play a part in the development of at least a subset of these cells (Mu et al, 1993). Some work has indicated that BDNF, like NGF, may play a part in the response of mature neurons to injury (Lindsay, 1988). It is possible that other neurotrophins are responsible for the regulation of transcription factors and LGIC subunits in these cells.
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(alphabetically by first author, and then chronologically).


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Glossary of abbreviations.

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<td>5-bromo 4-chloro 3-indolyl-(\beta)-D-galactoside</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BK</td>
<td>bradykinin</td>
</tr>
<tr>
<td>b.p.</td>
<td>nucleotide base pairs</td>
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<tr>
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<td>cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA, i.e. made by reverse transcription from RNA</td>
</tr>
<tr>
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<td>ciliary ganglion</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitro-quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CPP</td>
<td>3-((\pm))-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotrophin-releasing factor</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dNTPs</td>
<td>a mixture of all four deoxyribonucleotide triphosphates, dATP, dCTP, dGTP, dTTP.</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
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<td>dCTP</td>
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<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl-sulphoxide</td>
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<tr>
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<td>DNQX</td>
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<td>double stranded DNA</td>
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<td>dorsal root ganglion</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
</tr>
</tbody>
</table>
G6PDH: glucose-6-phosphate dehydrogenase
GABA: gamma amino butyric acid
GAL: galanin
GDEE: glutamate diethyl ester
G-protein: guanosine triphosphate-binding protein
5HT: 5-hydroxytryptamine
IPTG: isopropyl-β-D-thiogalactopyranoside
KA: kainate
Kb: kilobases
kDa: kilodaltons
LGIC: ligand-gated ion channel
LMP agarose: low melting point agarose
MOPS: 3-(N-morpholino)-propanesulfonic acid
nAChR: nicotinic acetylcholine receptor
NGF: nerve growth factor
NKA: neurokinin A
NMDA: N-methyl-D-aspartate
NT: neurotrophin
ODx: optical density (number, x, following shows wavelength of light at which measurement is made)
ORF: open reading frame
PCR: polymerase chain reaction
pfu: plaque-forming units
PKA/PKC: protein kinase A/C
PNS: peripheral nervous system
PPT: preprotachykinin
Quis: quisqualate
RACE: rapid amplification of cDNA ends
RNA: ribonucleic acid
RNase: ribonuclease
mRNA: messenger (polyadenylated) RNA
rRNA: ribosomal RNA
RTX: resiniferatoxin
RTX42,13,17: RTX-binding proteins (identified by number)
RTX-PAL: RTX photoaffinity label
SDS: sodium dodecylsulphate
SSC: saline sodium citrate
ssDNA: single stranded DNA
PAGE: polyacrylamide gel electrophoresis
TAE: Tris-acetate-EDTA buffer (Sambrook, 1982)
TBE: Tris-boric acid-EDTA buffer (Sambrook, 1982)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>Tris/Trizma</td>
<td>tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VGC</td>
<td>voltage-gated channel</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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</table>
Molecular cloning of a resiniferatoxin-binding protein

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Key words: Capsaicin; Resiniferatoxin; Dorsal root ganglion; Nociception

INTRODUCTION

The sensory neurotoxin capsaicin (8 methyl N-vanillyl 6-nonenamide) is the pungent principle of red peppers. It activates a sub-population of peripheral sensory neurons, some of which respond to a variety of noxious stimuli. Because of this selectivity of action, capsaicin has been a useful tool in elucidating the function of these neurons. At low doses, capsaicin excites and desensitises a variety of peripheral sensory systems, whilst high doses of capsaicin, particularly in neonatal animals, can lead to the loss of sensitive cells through calcium-mediated damage. Evidence for a specific capsaicin receptor comes from structure-function studies of capsaicin congeners, the well-defined species and tissue specificity of capsaicin action, and the development of a functionally-defined competitive antagonist of capsaicin, capsazepine. Capsaicin has been shown to induce cation fluxes in sensitive cells through a direct mechanism not involving second messengers. Using isolated membrane patches from sensory neurons, capsaicin has been found to activate a non-selective cation channel. Evidence from whole cell studies that the capsaicin-gated channel may also be activated by protons has been obtained. pH values lower than 6.5 are found in inflamed tissue suggesting that the capsaicin-activated channel may play a physiological role in the response to tissue-damaging stimuli. This idea is strengthened by the analgesic actions of a number of capsaicin analogues that are likely to act at the same site as capsaicin. The identification of the capsaicin receptor and its associated channel is therefore of practical and theoretical interest.

Conventional purification of capsaicin binding molecules has been hampered by a failure to develop binding assays owing to the low affinity and lipophilicity of capsaicin. The recent discovery that resiniferatoxin (RTX) is an ultrapotent analogue of capsaicin has allowed the development of binding assays and an estimate of the size of the capsaicin/resiniferatoxin receptor to be made. Such a binding assay, as well as allowing purification and characterisation of the capsaicin binding site also provides a possible screen for the detection of recombinant expressed resiniferatoxin binding proteins. A number of approaches to the molecular cloning of cell surface recep-
tors and associated ion channels have been developed which could be applied to the identification of the capsaicin receptor.\(^{3,4,35}\) Of the various strategies available, expression cloning using RNA injected into *Xenopus* oocytes and electrophysiological detection of functional channels is the most direct\(^{19}\). Using twin electrode whole cell voltage clamp recording, no reproducible capsaicin-gated ion fluxes have been detected in *Xenopus* oocytes injected with total or poly(A)\(^+\) RNA from dorsal root ganglia (DRG) sensory neurons, however (unpublished observations and J. Boulter, personal communication), precluding this approach to identifying the receptor. In the absence of a simple in vitro system to detect functional resiniferatoxin-gated channel activity, a screen for resiniferatoxin binding became an attractive approach to identification of the capsaicin receptor.

Recent studies on the expression of seven transmembrane G-protein-coupled receptors in bacteria has suggested that binding sites normally expressed in eukaryotic cells are also functionally expressed in prokaryotic systems.\(^{6,36}\) We therefore devised a binding screen to detect high affinity RTX binding proteins in a bacterial expression system. Because RTX has only been radiolabelled to low specific activities (approximately 30 Ci/mmol) with tritium, making detection of direct binding problematic, we used an indirect, sensitive screen based on the immunochemical detection of RTX-derived photoaffinity labels to screen expressed proteins encoded by rat dorsal root ganglia libraries. We describe the identification and characterisation of a soluble sensory neuron protein, RBP-26, which shows specific displaceable resiniferatoxin binding, but exhibits a broader distribution of expression and distinct pharmacology from that found for capsaicin sensitivity.

**MATERIALS AND METHODS**

**DRG cDNA library screening**

Poly(A)\(^+\) RNA was isolated from newborn Sprague–Dawley rat DRG from all spinal levels\(^3\) and two oligo(dT)-primed cDNA libraries (one the generous gift of Dr. J. Boulter) were constructed in \(\lambda\) Zap-II (Stratagene).

The photoaffinity label resiniferanol-9,13,14-orthophenylacetate-20-(3-azido-4-methoxyphenyl)acetate (RTX-PAL) is a capsaicin-like agonist with an EC\(_{50}\) of 7 nM (James, I., Walpole, C. and Wood, J.N., in preparation). A rabbit polyclonal antiserum that recognises DRG cDNA library screening became an attractive approach to identification of the capsaicin receptor.

In vitro translation

RTX-42 plasmid DNA was linearised with KpnI, and purified on a LMP agarose gel. Transcripts of cRNA were produced using T3 polymerase and an in vitro transcription kit (Stratagene). Nucleoside-treated rabbit reticulocyte lysates (Amersham) were used to translate the cRNA into \(^{35}S\)methionine-labelled proteins which were analysed on 12.5% SDS–PAGE.\(^{22,29}\)

**In situ Hybridisation**

In situ hybridisation was carried out with directly conjugated alkaline phosphatase-linked probes to a specific sequence in the 5'
untranslated sequence of RTX-42. The 31-mer 5' CAAAGCATAGTCCACCAAAAGATGAAAG 3' was coupled to alkaline phosphatase by AFFINITI Research products Ltd. and in situ hybridisation carried out as described[15].

Northern blots
Neonatal rat tissue total (20 μg) or poly(A) + RNA (2 μg) was fractionated on 1.2% agarose/ formaldehyde gels and blotted on to Hybond N filters (Amersham). After blotting, the RNA was cross-linked to the membrane by exposing the moist blot to 254 nm UV irradiation for 3 min, followed by baking at 80°C for 1 h. Baked filters were prehybridised for 4 h in 50% formamide 5 × SSC. 5 × Denhardt's solution, 100 μg/ml salmon sperm DNA, 50 mg/ml yeast RNA 0.1% SDS and hybridised for 40 h in the same solution containing 3 × 10^6 cpm/ml of a denatured random primer-labelled probe at 42°C. Filters were washed at room temperature for 20 min in 2 × SSC and for 3 × 20 min in 2% × SSC/0.2% SDS and finally in 0.2% SSC. 0.2% SDS at 65°C for 20 min. Washed filters were exposed to Kodak XAR-5 film at −70°C overnight.

After hybridization, radiolabelled probe was stripped from the filters by washing in 0.1 × SSC 0.2% SDS at 98°C for 20 min. Control hybridizations were carried out with a oligo-labelled probe specific for the ribosomal protein L-27 mRNA[16]. A 300 bp fragment of cDNA encoding this gene was amplified in the PCR reactions with primers

rib1 5' ATGGCTCTCTAAACTTGACC 3'
rib2 5' AAAGCCGTATCGTAAGAAAC 3'

purified by 1% agarose gel and labelled by random priming. Northern blot radioautographs were scanned and the amount of RNA in each track normalised using signal detected with the L-27 probe as a measure of the quantity of RNA in each sample.

RTX binding assays
COS-7 cells were transfected with RTX-42 sub-cloned from Bluescript II SK into the expression vector pKSI using EcoRI and Xhol sites. pKSI is a derivative of CDMS with a pUC backbone (K. Smith, P. McIntyre, M. Rolfe and G. Starke, unpublished). DNA (9 μg/60 mm dish) was introduced using calcium phosphate[17,18]. Two days after transfection, the cells were washed in PBS, harvested with a rubber policeman and sonicated in 25 mM Tris pH 7.4. Cells were briefly microfuged and 50 μl of cell extract was then incubated with 2 mM [3H]RTX and various concentrations of cold resiniferatoxin (0–1 μM) or capsaicin (10 μM) at room temperature for 40 min. The cell extract was chromatographed on 1 ml BioRad P-6 gel exclusion columns pre-equilibrated with 50 mM NaCl, 25 mM Tris pH 7.6, and counts eluting in the void volume (1.1 ml) used as a measure of binding.

RESULTS
Expression cloning of RTX binding proteins
Resiniferatoxin binding proteins expressed by a directionally-cloned rat DRG λ Zap-II were detected as shown in Fig. 1. Expressed fusion proteins were photoaffinity labelled with the resiniferatoxin-derived agonist and photoaffinity label (RTX-PAL). Proteins that were covalently labelled by RTX-PAL were detected with a polyclonal antiserum raised against 4- aminoethoxy-resiniferatoxin.

Of 20 positive clones, 4 showed UV-dependent immunoreactivity after photoaffinity labelling and were further characterised after plaque purification. Two clones — RTX-4 and RTX-13 — showed very strong cross-hybridization and sequence analysis showed that they were identical apart from their lengths. Both RTX-4 and RTX-13 had 1.2 kb inserts, but RTX-4 has an approximately 50 bp shorter poly(A) tail and a corresponding longer 5' coding region.

The cDNA insert from RTX-13 was used to screen an additional non-amplified DRG cDNA library by hybridisation. The biggest positive clone — RTX-42 — contained a 1.7 kb insert and was used for further investigation and analysis. The nucleotide sequence of RTX 42 cDNA was determined and found to contain an open reading frame encoding a hypothetical protein of molecular weight 26,548. There are three potential initiator methionine codons preceded Kozak consensus sequences[21], and these potential initiation sites were in frame with the original fusion protein detected in RTX-13. However, there was no stop codon upstream of the first potential initiator methionine. To be sure that there was no additional upstream start codon we identified the most 5' sequence of the corresponding mRNA using a RACE protocol[13]. Several clones were
found to be of nearly the same length and were 18–21 bp longer than RTX-42. The complete sequences of these clones revealed no differences in amino acid structure and no additional initiator methionine codons were found.

Primer extension analysis using different preparations of RNA showed that all the transcripts of RTX-42 present in total RNA from the DRG of newborn rats have the same start site that corresponds exactly to the first nucleotide of the cDNA for the RACE clone c7 (Fig. 2). Thus, the full-length cDNA sequence encoding a possible RTX binding protein was established and is shown together with the predicted protein sequence in Fig. 3. The longest open reading frame predicts a protein with a molecular weight of 26,600 and a pl of 5.9, whilst the two possible shorter proteins have molecular weights of 25,700 and 24,400 with pl's of 6.53 and 9.32, respectively, reflecting the high density of aspartate and glutamate residues within the N-terminal region of the protein. Hydrophobicity analysis (Fig. 3) demonstrated that only one region of the protein could be membrane-spanning, and the overall structure of the protein did not correspond to that known for any class of ion channel.

**Distribution of expression of RTX-42**

Northern blot analysis revealed a single RNA transcript with a size of approximately 1.6 kb in RNA extracted from rat DRG. This corresponds to the size predicted from the sequencing of the cloned cDNA (Fig. 4). A single similarly-sized transcript was identified in different tissues of newborn rats by Northern hybridization, although the level of expression varied in different tissues. The highest intensity of signal was detected in cerebellum and DRG, and strong hybridization was observed in cortex, spinal cord and adrenal glands. In other tissues, the gene was expressed at lower levels (Fig. 4).

In order to determine which cell types within dorsal root ganglia contained RTX-42 mRNA transcripts, we used in situ hybridisation to probe both cells in culture, as well as frozen sections of neonatal rat dorsal root ganglia. Using non-radioactive directly-conjugated alkaline phosphatase-specific 35-mers, we found positive signals of variable intensity in a large proportion of neuronal cell bodies in both frozen sections and cells in culture (Fig. 5), whilst non-neuronal cells did not show equivalent levels of hybridisation.

**RTX-42 protein expression**

Rabbit reticulocyte lysates were used to translate cRNA transcripts of RTX-42 mRNA, in order to determine the size of possible encoded translation products. Polyacrylamide gel electrophoresis revealed three [35S]methionine labelled translation products of sizes 23, 25 and 26 kDa. These sizes correspond to those predicted from the DNA sequence, which contains two additional possible initiator methionines preceded by 'strong' upstream Kozak consensus sequences (GCCGA/G−3 GCC AUG G+) downstream of the first possible initiator codon. The predicted molecular weights of the three putative proteins (24.4, 25.7 and 26.6 kDa) correspond reasonably well with the observed sizes of the translation products.

The structure of the protein is unique. Scanning of databases revealed little homology with known proteins. The protein contains a single hydrophobic domain of 18 amino acids (Fig. 3), and a variety of consensus post-translational modification sites, including phosphorylation, myristoylation and glycosylation sites, as well as nuclear localisation signals and possible nucleotide binding sites identified using the Intelligenetics Keytool and Keysite data bases (Intelligenetics Inc., Mountain View, CA). These are tabulated in Table I.

**Binding and functional studies**

In order to confirm that the photofinity-based screening method was indeed identifying RTX binding proteins, we analysed the binding characteristics of a number of clones in a direct binding assay using [3H]RTX. Specific displaceable binding in the nM range was apparent with clone RTX-13, in which a 1.2 kb
Fig. 3. Nucleotide sequence and predicted protein sequence of RBP-26. The full-length sequence of RTX-42 together with the encoded amino acid sequence for RBP-26 is shown. Possible initiator methionines preceded by upstream Kozak consensus sequences, which are in frame with the initially identified fusion protein sequence are underlined. Lower right panel: the hydrophilicity analysis using Kyte and Doolittle parameters and a window of 6 amino acids is shown.
Fig. 4. Distribution of expression of RBP-26 mRNA by Northern analysis. Northern blots of rat tissue RNA were probed with RTX-42 (lower blot), and blots subsequently stripped and re-probed for the expression of a constitutively expressed ribosomal protein L-27 (upper blot). The relative expression of mRNA was then normalised with respect to the content of L-27 RNA, and expression presented in arbitrary units on the bargraph. The size of the transcript was estimated at 1.6 kb with respect to RNA markers (not shown).

Fig. 5. In situ hybridisation with an RTX-42 specific probe. Transverse frozen sections of adult rat DRG (A), or fixed neonatal rat DRG cultures (C) were hybridised with a specific RTX-42 antisense oligomer conjugated to alkaline phosphatase. Arrows indicate typical positive neuronal cell bodies in sections (A) and in primary cultures of adult rat sensory neurons (C). Ribonuclease treatment of DRG sections (B) abolishes the specific staining.

Fig. 4. Distribution of expression of RBP-26 mRNA by Northern analysis. Northern blots of rat tissue RNA were probed with RTX-42 (lower blot), and blots subsequently stripped and re-probed for the expression of a constitutively expressed ribosomal protein L-27 (upper blot). The relative expression of mRNA was then normalised with respect to the content of L-27 RNA, and expression presented in arbitrary units on the bargraph. The size of the transcript was estimated at 1.6 kb with respect to RNA markers (not shown).

Insert encoding protein sequence corresponding to RBP26 was in frame with the β-galactosidase gene (Fig. 7B). This suggests that the binding site for RTX resides towards the C-terminal region of RBP-26. To further elucidate this point, we expressed the full-length sequence of RTX-42 in a eukaryotic expression vector pSk1, containing an SV40 origin of replication where transcription is driven by a highly-efficient CMV pro-
TABLE I

<table>
<thead>
<tr>
<th>Consensus signal sites in RBP-26</th>
<th>First residue number</th>
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<tr>
<td>Phosphorylation sites</td>
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<td>Casein kinase II site</td>
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<td>Tyrosine kinase site</td>
<td>132</td>
</tr>
<tr>
<td>Glycosylation sites</td>
<td>40, 92, 98, 109, 136, 176, 193, 198</td>
</tr>
<tr>
<td>Myristoylation sites</td>
<td>29, 86, 121</td>
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<tr>
<td>Nuclear translocation signals</td>
<td>62, 141, 148</td>
</tr>
<tr>
<td>Nucleotide binding sites</td>
<td>29, 118, 189</td>
</tr>
</tbody>
</table>

pressed in COS cells (Fig. 7C), where bound [3H]RTX was separated from free ligand on small polyacrylamide gel columns. The maximal level of binding does not reflect the Bmax because the concentration of radioligand used is lower than the half-maximal binding concentration of approximately 10 nM (Fig. 7C); no displaceable binding was detected in mock-transfected cells.

DISCUSSION

The non-selective cation channel activated by capsaicin in a subset of DRG sensory neurons has a

Fig. 6. In vitro translation of RBP mRNA transcripts. A radioautograph of a 12.5% polyacrylamide gel is shown with molecular weights of markers in kilodaltons shown on the gel. Rabbit reticulocyte lysates were used to translate RTX-42 cRNA into [35S]methionine labelled proteins (lanes a and b with different amounts of cRNA in the translation mix). Lane c is a control lane where no cRNA has been added. Three bands of the apparent molecular weights 23, 25 and 26 kDa (arrowed) were identified corresponding to the use of all three possible initiation methionines (Fig. 3).

Fig. 7. Binding characteristics of expressed RBP-26 protein. A: sub-cellular fractions of COS-7 cells transfected with the expression vector CMV containing a full-length RTX-42 clone. Specific capsaicin-displaceable [3H]RTX binding was apparent in cytosolic supernatant fractions (P = 0.0074 compared with capsaicin treated supernatant or P = 0.0373 compared with membrane fractions) but not membrane fractions prepared according to ref. 37. B: displaceable [3H]RTX binding to β-galactosidase fusion protein RTX-13 containing the C-terminal region of RBP26 assessed in a filter-based binding assay13 using IPTG-induced bacterial culture lysates. C: soluble proteins from RTX-42-transfected (□) and mock-transfected (■) COS cells were assessed for [3H]RTX binding using a gel filtration assay with increasing concentrations of cold resiniferatoxin.
unique pharmacology\textsuperscript{1,46}. The same channel is activated by resiniferatoxin with an IC\textsubscript{50} of 2 nM\textsuperscript{44}. Binding studies using tritiated RTX have shown a highly restricted distribution of membrane associated binding sites that are co-expressed with capsaicin sensitivity\textsuperscript{38}. It thus seems likely that the binding site and channel are intimately related. Certain characteristics of the channel are reminiscent of ligand-gated ion channels. For example, the cation selectivity, voltage dependence and conductance of the capsaicin channel are quite similar to some glutamate and nicotinic-gated ion channels\textsuperscript{31}. In addition, the molecular weight of the RTX binding site as determined by radiation inactivation (\textasciitilde 280 kDa) is similar in size to that of nicotinic and kainate-gated receptors\textsuperscript{39} and both nicotinic and capsaicin-gated channels are blocked by ruthenium red\textsuperscript{12}. Finally, NGF which is known to up-regulate the capsaicin binding site also up-regulates nicotinic receptor expression\textsuperscript{26,44}. Taken together these diverse pieces of information are consistent with an action of capsaicin on a ligand-gated channel-like site. However, unlike known classes of ligand-gated ion channels, we have not been able to express a capsaicin-activated channel in \textit{Xenopus} oocytes injected with DRG RNA.

One explanation for this phenomenon is provided by the observation that charged membrane impermeant analogues of capsaicin are much more effective on inside/out than outside/out patches of DRG membrane, suggesting that the capsaicin binding site is intracellular (R. Docherty, personal communication). Bath application of capsaicin to intact oocytes could thus lead to a slow ingress of the drug which could desensitise the channel or cause undetectably small levels of channel activity. Another possible explanation is that the three-dimensional organisation of channel and receptor are not appropriately expressed in oocytes, possibly because the quaternary structure of the channel receptor complex may involve a number of protein subunits that do not assemble appropriately in the oocyte.

The initial binding screen identified four different positive clones, two of which were identical, giving support to the view that the assay was detecting a specific class of binding molecules. Of the other positive clones as yet incompletely characterised, partial sequence analysis suggests that one is related to topoisomerase 1, whilst the other is a novel transcript. Northern analysis of their distribution showed expression in neuronal and non-neuronal tissue, with no correlation of mRNA expression with capsaicin-sensitive tissues (data not shown).

Both the mRNA transcript and the RTX binding protein RBP-26 are found in dorsal root ganglion neurons, but are also expressed in a range of neuronal and non-neuronal tissues where high affinity RTX binding has not been found. In addition, when expressed in COS cells, RBP-26 appears to be a cytoplasmic protein. This suggests that it is unlikely to participate in a functional capsaicin receptor/channel complex. The possibility exists that the protein is specifically-associated with the membrane in DRG neurons, perhaps by post-translational modification (e.g. myristoylation), and that this explains its cell type specificity as a channel-associated receptor.

Comparison of the primary sequence of RBP-26 with other proteins provides few clues to its function. The single hydrophobic region from residues 23 to 40 is not homologous with known receptor transmembrane regions, and binding studies suggest that the protein is expressed in a soluble form. Sequence comparison at the protein level reveals no protein with more than overall 20\% sequence homology and at the nucleic acid level no database sequence was more than 52\% homologous with RBP-26. The expression of sequence motifs, such as potential glycosylation, myristoylation and phosphorylation sites was unexceptional, and no unusual features of the predicted protein structure were apparent. Putative nuclear localisation signals for example (Table I) are present in a variety of cytoplasmic or membrane associated proteins (e.g. nicotinic acetylcholine receptors). Secondary structure prediction using the Chou Fassman algorithm identified a number of potential alpha helical regions and \(\beta\) sheet structures, and hydrophobicity analysis showed a hydrophilic, extensively charged molecule with an overall pI of 5.9. One interesting facet of the three possible start sites for translation is that the three products differ markedly in their pI because of the preponderance of acidic residues at the N-terminal.

Given the lack of channel-like characteristics associated with RBP-26, one might postulate that it could play an accessory role in gating a cation-selective channel. However, we could not obtain functional evidence that RBP-26 played a role in capsaicin receptor activity. Down-regulating the capsaicin response in NGF-depleted adult sensory neurons followed by adding back antisense thiol-linked oligonucleotides that should complex with RBP-26 mRNA did not lead to inhibition of capsaicin sensitivity, as measured by calcium incorporation into sensitive neurons. We made further attempts to establish a functional role for RBP-26 in capsaicin sensitivity, for example by microinjection of oocytes with RBP-26 transcripts alone or in combination with DRG mRNA. The rationale behind these experiments was that although DRG RNA has not been found to encode proteins that respond to cap-
sacain functionally in oocytes, such RNA when injected with large amounts of putative receptor transcript might be induced to express a capsacain response if a receptor/channel interaction is necessary for activity. However, RBP-26 transcripts either alone or in conjunction with DRG poly(A)+ RNA did not lead to functional expression in oocytes (data not shown). The relationship between the cytoplasmic resifierator binding protein RBP-26 and the sensory neuron-specific membrane associated capsacain receptor is thus still uncertain. However, the broad distribution of expression of RBP-26 and the failure to obtain functional evidence for a role in capsacain sensitivity would suggest that this binding protein is unlikely to be the functional capsacain receptor.

The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ nucleotide sequence databases under the accession number X67877.

Acknowledgements. We thank our friends and colleagues at the Sandoz Institute, and Armen Akopian, Jim Boulter, and David Latchman for helpful discussions. We are particularly grateful to Vladimir Buchman for helpful suggestions throughout the course of this work. We are very grateful to Jane Yeats for help with DRG isolation, and to Chris Walpole for photoaffinity probe synthesis.

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Molecular cloning of a human neuronal nicotinic acetylcholine receptor \( \beta 3 \)-like subunit

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Key words: Nicotine; Acetylcholine; Human; Neuron; Ion channel

Three cDNA clones homologous to rat neuronal nicotinic receptors were identified in a human brain stem library by screening at low stringency with a mixture of rat \( \alpha 5 \), \( \alpha 2 \) and \( \beta 4 \) subunit probes. Positive clones were further analysed by hybridisation at higher stringency with individual rat nicotinic receptor probes. One positive clone was found to encode a protein that exhibited characteristic features of a member of the ligand-gated ion channel class of protein subunits, and showed 89% homology with a rat \( \beta 3 \) neuronal nicotinic receptor subunit. Northern blots demonstrated the presence of a 1.7 kb transcript in RNA extracted from adult human Pons. This clone has therefore been designated the human \( \beta 3 \) neuronal nicotinic receptor subunit.

Fast excitatory neurotransmitters, such as acetylcholine, gate multimeric cation-selective ion channels [2]. The structure of the Torpedo nicotinic acetylcholine-gated receptor has been extensively investigated. It has been found to comprise five related subunits arranged pseudosymmetrically around an axial channel, by means of biochemical cross-linking and electron microscopic studies [19]. Each individual subunit probably has 4 transmembrane domains, and the second transmembrane region of each subunit (TM2) faces the central pore of the channel. This model has been applied to other ligand-gated ion channel receptors, the subunits of which show a similar primary sequence organisation. A number of related chick, rodent and human nicotinic receptor subunits have been identified by homology screening. The muscle and neuronal forms of vertebrate nicotinic receptor subunits are the products of separate genes and differ in primary sequence [18]. The \( \alpha \) subunits of neuronal nicotinic receptors (nAChRs), which have a characteristic sequence motif of two adjacent cysteines in their N-terminal extracellular domain, are necessary for ligand binding to functional receptors. Related neuronal nAChR subunits that do not express this motif are defined as \( \beta \) subunits. The recent discovery [15] that the 5-HT3 serotonin-gated ion channel is the previously designated neuronal nAChR \( \beta 5 \) subunit [11] demonstrates the close homology between receptor subtypes throughout the class of ligand-gated ion channels.

Little is known about the functional role of nicotinic receptors within the CNS, mainly because of the rapid desensitisation associated with channel activation [6]. Actions of acetylcholine on Renshaw cells and motor neuron activity, and presynaptic nicotinic activation in the interpeduncular nucleus have been described [4]. Some studies suggest that a consistent and severe loss of nAChRs also occurs in Alzheimer's disease [21]. The CNS distribution of expression and role of nAChRs in normal and pathological states is thus of potential clinical interest. In order to identify novel subtypes of human neuronal nAChRs, and study their distribution and function, we have used cloned rat nicotinic receptors to identify human ligand-gated ion channels by homology screening. We report here the sequence of a novel human \( \beta 3 \)-like nAChR subunit.

A commercial human brainstem cDNA library in Lambda Zap II (Stratagene) plated at a density of \( \sim 10^5 \) plaques/12 cm\(^2\) dish on E. coli BB4 cells was screened at low stringency (2 \( \times \) SSC, 0.1% SDS, 57°C), with a pool of random-primed \( \alpha 5 \), \( \beta 2 \) and \( \beta 4 \) rat neuronal nAChR
subunit probes (kindly provided by Dr. J. Boulter, Salk Institute). From 1.3 million plaques screened, three positive signals were obtained. The three positive clones were plaque-purified and isolated as plasmids by excision into the Bluescript 11. Random-primed inserts from the positive clones were used in turn as probes at moderate stringency (2 × SSC, 0.1% SDS, 60°C) on a Southern blot of the three rat clones (data not shown). Two of the positive inserts were found to be strongly homologous to the rat β2 nAChR gene, whilst the largest clone (1.4 kb) encoded an α5-like transcript. However, when this clone was sequenced (Sequenase USB) using the dideoxy method [17], a stronger homology at the DNA level with the rat α3 subunit (77%) than with the α5 subunit (65%) was apparent. The apparent anomaly that a subunit hybridises more strongly to an α than other β subunits is explained by the fact that rat α5 and β3 nAChRs are more closely related to each other than to other α and β subunits. The nucleotide sequence of α3, α5 and β2 subunits show 83%, 82% and 86% homology respectively between human and rat. The greatest divergence is exhibited in the cytoplasmic loop between TM3 and TM4. As the human β3 clone was isolated from an oligo dT-primed library, the 3' poly-A tail is present. However, the clone is not full length and by analogy with the rat β3 subunit probably lacks about 120 bp of coding sequence at the 5' end, most of which is likely to encode a signal peptide. The 3' untranslated region of the clone (90 bp from the single stop codon to the poly-A sequence) is considerably shorter than that found in the rat gene, where it is more than 700 bp long.

The deduced protein sequence (Fig. 1) shows features found in other nAChR subunits [18], including four conserved hydrophobic regions presumed to be transmembrane (TM) domains (shown by Kyte and Doolittle hydrophathy plot), of which the first three are perfectly conserved between rat and human clones. The ligand gated channel motif of two cysteine residues thirteen residues apart, corresponding to two cysteines at position 128 and 142 in the torpedo α subunit is also present. The two adjacent cysteine residues which are diagnostic of nAChR α subunits are absent from the predicted peptide however, confirming the β subunit classification. The potential N-glycosylation site at residue 172 in the rat gene is conserved, and in addition, two new potential sites at residues 16 and 104 are present within the N-terminus. As the human β3 clone was isolated from an oligo dT-primed library, the 3' poly-A tail is present. However, the clone is not full length and by analogy with the rat β3 subunit probably lacks about 120 bp of coding sequence at the 5' end, most of which is likely to encode a signal peptide. The 3' untranslated region of the clone (90 bp from the single stop codon to the poly-A sequence) is considerably shorter than that found in the rat gene, where it is more than 700 bp long.

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Fig. 2. Northern blot of (a) IMR32 and (b) adult human pons total RNA probed with a fragment of the human β3 clone. Lane (c) contains ribosomal RNA. The 1.7 kb fragment is arrowed. The lane on the left contains RNA marker fragments of 7.4, 5.3, 2.8, 1.9 and 1.6 kb, respectively (Boehringer Mannheim).

terminal extracellular domain. The overall sequence homology at the amino acid level with the rat β3 subunit is 89%.

A 600 bp PCR product from the 5' end of the β3 clone, (defined by internal primers) was used to probe a Northern blot containing 40 µg of total RNA from the human neuronal cell line IMR32 [5] and 40 µg of human adult pons total RNA, under stringent conditions (42°C, 50% formamide, washed in 0.2x SSC, 0.2% SDS at 67°C). A faint transcript of 1.7 kb was found in pons, but no expression of the gene was detected in IMR32 RNA (Fig. 2), although this cell line was the source of two of the three human nAChR subunits so far cloned [1, 5, 10].

Comparison of signal intensity on Northern blots of adult and foetal human brain and dorsal root ganglia using a probe derived from a human β2-like clone showed that, as in the rat, the levels of β3 mRNA are considerably lower than those encoding β2-like subunits [1, 3]. It is known that some rat nAChR subunits are developmentally regulated (e.g. refs. 12, 16) and it is possible that β3 mRNA is more abundantly expressed at developmental stages other than those studied here.

No clues to the function of the human β3 nicotinic receptor are provided by studies of the rat homologue, as this subunit does not assemble into functional neuronal nAChRs when coexpressed with nAChR α subunits alone [8], raising the possibility that β3 type subunits may function in ion channels gated by ligands other than acetylcholine and nicotine. The two apparently 'non-functional' nAChR subunits, α5 and β3 are more limited in their distribution in the rat than other neuronal subunits [8, 20], and show little overlap in distribution of expression. The β3 subunit in particular is restricted in its expression to well-defined small areas, such as the trigeminal mesencephalic nucleus in the pons, and pars compacta of the substantia nigra. The close sequence homology between α5 and β3 subunits, combined with their inability to participate in functional nicotinic channels, suggests that they may comprise a distinct sub-group of receptor subunits whose physiological role has yet to be determined.

Recent evidence [7] indicates that chick α5 subunits are able to co-assemble into receptors based on α3 or α4 subunits, raising the possibility that α5 and β3 subunits may play an accessory role in modulating the gating characteristics of neuronal nAChRs. Thus, although functional nAChRs cannot contain only the β3 subunit in conjunction with a subunit, the inclusion of β3 with other β-type subunits may result in receptors with distinct gating or ligand binding properties. It is known that both α and β subunits contribute to agonist sensitivity [13], but the electrophysiological properties of neuronal nicotinic receptors that comprise more than two types of subunit are only now being investigated [12].

The diversity of neuronal nicotinic subunits, and their restricted pattern of expression suggests that distinct functions are subserved by different combinations of nicotinic receptor subunits including the human β3-like subunit described here. The possibility remains that this apparently non-functional β3-like subunit may also participate in ion channels gated by ligands other than acetylcholine, and oocyte expression studies with a full-length clone will help to resolve this issue.

The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ nucleotide sequence databases under the accession number X67877.

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Regulation of Expression of the Neuronal POU Protein Oct-2 by Nerve Growth Factor*

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POU proteins are a class of homeobox-containing transcription factors that regulate tissue-specific gene expression and influence cell differentiation and function. We have investigated the possible role of such factors in mediating the actions of nerve growth factor (NGF) on sensory neurons. NGF has been found to have differential effects on the levels of three POU protein transcription factors that are expressed in adult rat sensory neurons. A sensory neuron octamer-binding protein with the properties of the transcription factor Oct-2 is up-regulated 3-4-fold by NGF, as measured by mobility shift assays using nuclear extracts from adult rat dorsal root ganglion neurons grown in the presence or absence of NGF. Quantitation of Oct-2 mRNA by polymerase chain reaction amplification of RNA from such cells shows a parallel increase in Oct-2 mRNA levels. In contrast, the levels of mRNA encoding the ubiquitous POU protein Oct-1 or the neuron-specific POU protein Brn-3, also present in sensory neurons, are unaffected by NGF. These observations suggest a role for Oct-2 in mediating transcriptional effects induced by NGF. In particular, as Oct-2 is known to inhibit herpes simplex virus immediate-early gene expression in neuronal cells, these findings provide a mechanism for the known action of NGF in the maintenance of latent herpes virus infections in sensory neurons.

Nerve growth factor (NGF) is one of a family of neurotrophic factors that, among other actions, promote the survival of developing peripheral sensory and sympathetic neurons. Neuronal survival in the developing peripheral nervous system has been demonstrated to depend in part upon the presence of limiting amounts of NGF (Barde, 1989). A family of related factors (BDNF, NT3, NT4/5) with remarkably conserved protein sequences between species that show subtly differential binding properties from NGF (Oberheim, 1991). A high affinity receptor for NGF has also been identified and been demonstrated to be a transmembrane protein with intrinsic tyrosine kinase activity (TrkA) (Kaplan et al., 1991; Klein et al., 1991). Structurally related transmembrane tyrosine kinases (TrkB, TrkC) are good candidates as cognate receptors for the other neurotrophins so far identified (Squinto et al., 1991; Lamballe et al., 1991). Despite these advances, and the cataloguing of NGF-induced second messenger changes and effects on gene expression in susceptible cells (Chao, 1992), the physiological role of neurotrophic factors in the mature nervous system and the mechanism of their action are poorly understood. Artificial elevation of NGF levels has been shown to alter the phenotype of nociceptive sensory neurons that play a role in the neuronal component of inflammatory responses, and effects on the expression of various neuronpeptides and ion channels at the mRNA level in these cells have been described (Lindsay and Harmer, 1988). It therefore seems likely that many actions of NGF are mediated through effects on transcriptional regulation. Consistent with this, a number of NGF-regulated transcription factors have been identified in the pheochromocytoma-derived PC12 cell line by differential screening methods (Milbrandt, 1987; Oppenheim, 1991).

We have used primary cultures of peripheral neurons as an in vitro system to investigate the action of NGF on the POU protein class of transcription factors that appears to have particular importance in tissue-specific gene regulation (Herr et al., 1988; He et al., 1989). Adult rat sensory neurons are known to express a number of such proteins, including Oct-1, Oct-2, Brn-3, and a number of Brn-3-related factors, the partial sequences of which have been identified by PCR (Latchman et al., 1992). Two such factors, Brn-3 and Oct-2, are of particular interest because of their restricted cellular distribution. The octamer-binding protein Oct-2, a factor first identified as a B cell immunoglobulin-specific transcription factor has subsequently been found in a number of types of neurons including sensory neurons (He et al., 1989; Lillycrop et al., 1991). The brain-derived putative transcription factor Brn-3 is additionally interesting because of its close structural similarity to a protein that determines the developmental fate of some sensory neurons in Caenorhabditis elegans, first identified in the unc-86 mutant (Herr et al., 1988). We therefore analyzed the effects of NGF on the levels of mRNA encoding the neuron-specific class IV POU protein Brn-3 and the tissue-specific octamer binding protein Oct-2 as well as Oct-1, a ubiquitously expressed POU protein, that is known to play a critical role in regulating the expression of a number of cellular and viral genes by binding to the octamer sequence ATGCAAAT in their promoters (Falkner et al., 1986). Adult rat sensory neurons, unlike their neonatal counterparts,
can survive in culture without NGF (Lindsay et al., 1989; Winter et al., 1988). We were therefore able to examine the effect of depleting NGF on the expression of transcription factors that have been identified in adult rat sensory neurons without compromising the viability of the cultures. We report here that studies using quantitative polymerase chain reaction amplification of sensory neuron RNA demonstrate a specific up-regulation of Oct-2 mRNA levels by NGF in adult rat sensory neurons, together with an increase in Oct-2 protein levels measured in mobility shift assays. This effect is specific for Oct-2, as the POU proteins Brn-3 and Oct-1 as well as other non-POU transcription factors such as TFIIIC are unaffected by alterations in the levels of NGF.

MATERIALS AND METHODS

Cell Culture—Dorsal root ganglia from all spinal levels of adult male Sprague-Dawley rats were dissected aseptically and collected in Ham's F14 medium supplemented with 1,176 μg/liter sodium bicarbonate, 1 mM glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin. Ganglia were digested with 0.125% collagenase (Boehringer) and mechanically dissociated through a fire polished Pasteur pipette. Cells were then plated overnight on polyornithine-coated Petri dishes. After 24 h, lightly adherent neurons were removed from the dishes in a stream of medium and replated on 13-mm glass coverslips previously coated with polyornithine and laminin (Bethesda Research Laboratories) at a density of 20,000 neurons per coverslip. Cultures were then supplemented with 0.1 μg/ml 2.5 S NGF prepared from mouse saliva glands (Suda et al., 1978) or grown in the absence of NGF and presence of neutralizing sheep anti-NGF antiserum at concentrations capable of neutralizing 0.2 μg/ml NGF to block any endogenously synthesized NGF produced by nonneuronal cells in the cultures. An equivalent concentration of normal sheep serum was added to cultures grown in the presence of NGF. Cytochrome arabinoside (10 μM) was included in the medium for the first 2 days to kill dividing cells, then removed, and 5 days after plating either total RNA was extracted from the cells by a guanidine chloride/phenol extraction procedure (Chomczynski and Sacchi, 1987) or nuclear extracts were prepared for mobility shift assays.

cDNA Probe Synthesis and Northern Blot Analysis—A neonatal rat dorsal root ganglion (DRG) cDNA library constructed in λ zap 11 (the kind gift of Dr. J. Boulter) was plated at 1–2 × 10⁶ plaque-forming units per 132-mm-square plate and plaques grown for 7 h at 42 °C before binding to Hybond N membranes (Amerham). The filters were denatured and neutralized and UV-irradiated. The filters were then hybridized to a 4-kb mRNA (Fig. 1). We therefore used additional primers across the 3'-untranslated region of Brn-3 (30 s at 95 °C, 45 s at 57 °C, 1 min at 72 °C used 50 μl of primers 5'CAATAGTCTGCATCTATCCG3' and 5'TTGGATTTAGTATGAGTACCC3' in a 50-μl volume containing 0.4 units of Taq polymerase (Promega), 1 mM dNTPs, 50 mM Tris-HCl, pH 8.5, 50 μM NaCl, 5 mM MgCl₂, 2 mM DTT, and cDNA synthesized from 0.1 μg of DRG RNA reverse-transcribed with superscript (BRL) and primed with random hexanucleotides (Promega). Rat ribosomal protein L27 primers were 5'AATCTACAACCTCTGATACCC3' and 5'ATCGCTCTCCACTAAGTGC3'. The PCR reactions were analyzed by Southern blotting or spliced with 1 μl of [α-⁴²P]dATP and reaction products separated on a 6% polyacrylamide gel, dried, and autoradiographed.

RESULTS AND DISCUSSION

Actions of NGF on PO U Protein mRNA Levels—RNA was extracted from adult rat sensory neurons grown in the presence or absence of NGF, with anti-NGF antiserum added to NGF-free cultures to neutralize any NGF released from non-neuronal cells. Because of the limited amounts of material available from the DRG cultures and the low abundance of transcription factor mRNAs, the mRNAs were quantitated using PCR with oligonucleotide primers specific to each of the POU domain sequences of the Oct-1, Oct-2, or Brn-3 mRNAs. Because a variety of Brn-3-like clones with similar but not identical POU domain sequences have been identified in neuronal cells by PCR amplification (Latchman et al., 1992), we screened a rat DRG library using the originally described Brn-3 mRNA as a probe (He et al., 1989) and isolated and sequenced overlapping clones, in order to identify PCR primers uniquely directed at Brn-3 itself. Those clones that contained partial sequence with 100% homology to the original clone were used to probe Northern blots of DRG RNA. Both the original clone and a 3′ overlapping clone hybridized to a 4-kb mRNA (Fig. 1). We therefore used additional primers across the 3′-untranslated region of Brn-3 sequence derived from the new clone, as well as primers for the POU domain of Brn-3 to be certain that we were measuring Brn-3 mRNA levels. As an external control, primers specific for a constitutively expressed control mRNA encoding the ribosomal protein L27 were also used (LeBeau et al., 1991). In each experiment, the identity of the PCR product was...
confirmed both by digestion with appropriate restriction enzymes and by hybridization with Oct-1, Oct-2, or Brn-3-specific cDNA clones. To ensure that the PCR was quantitative, preliminary experiments were carried out by varying the amounts of mRNA and cycle numbers to identify conditions in which the signal obtained was linearly related to the amount of input RNA. In addition, in some experiments we included equal amounts of a control human Oct-2 RNA template prepared by the transcription of a human Oct-2 plasmid whose PCR product could be distinguished from that of rat Oct-2 through sequence differences encoding distinct restriction enzyme recognition sites.

We found that NGF did not alter the levels of Oct-1 or Brn-3 mRNA, or that of the control ribosomal protein mRNA. In contrast, Oct-2 mRNA levels were elevated 2.7-3.5-fold in NGF-treated cells than in control cells cultured without NGF (Fig. 3A). The protein producing this complex was identical to that produced by the Oct-2 protein also present in both B cells and a neuronal cell line were 3-4-fold greater in both clones of Brn-3 has yet to be determined, precluding identification of the indicated sizes. The expected sizes of the Oct-1 and Oct-2 PCR products is 250 base pairs, b, PCR amplification of Oct-2 mRNA using a control internal template prepared by the transcription of a human Oct-2 cDNA, separated on a 1.8% agarose gel. The product from the control human Oct-2 RNA and from the rat Oct-2 mRNA in the sample was distinguished by restriction enzyme digestion with BglI which cuts only the human Oct-2 PCR product to produce fragments of 142 and 108 base pairs, while not affecting the rat Oct-2 product. The 250-base pair product of the rat Oct-2 mRNA is indicated by arrows, c, PCR amplification of both Brn-3 and 170-bp cDNA fragment, and a ribosomal protein L27 130-bp cDNA used as a measure of the amount of input RNA (LeBeau et al., 1991). Amplifications were carried out using cDNA derived from 0.1 µg of RNA from DRG cultures grown in the presence (+) or absence (−) of NGF for 22 (C), 26 (B), and 30 (A) cycles using PCR primers across the 3'-untranslated region of Brn-3 (arrow B) or from rat L27 primers (arrow L). PCR reactions were spiked with 1 µCi of [α-32P]dATP, and reaction products were separated on a 6% polyacrylamide gel, dried, and autoradiographed.

**NGF Effects on POU Protein Levels**—In order to test whether the alteration in mRNA levels was reflected in changes in the levels of Oct-2 DNA binding, nuclear extracts were isolated from adult sensory neurons grown in the presence or absence of NGF, and DNA mobility shift assays using the radiolabeled octamer ATGCTAATGATAT, which is a high-affinity binding site for both Oct-1 and Oct-2 (Dent and Latchman, 1991), were used to quantitate the levels of octamer binding proteins. The DNA binding specificity of full length clones of Brn-3 has yet to be determined, precluding the measurement of this protein by DNA mobility shift assays. In these experiments the levels of a complex with a mobility identical to that produced by the Oct-2 protein also present in both B cells and a neuronal cell line were 3-4-fold greater in NGF-treated cells than in control cells cultured without NGF (Fig. 3A). The protein producing this complex was identified as the neuronal form of Oct-2 on the basis of its sequence specificity for different octamer oligonucleotides which we have previously shown distinguish neuronal Oct-2 from the B cell form of Oct-2 and other octamer binding proteins (Dent et al., 1991). Thus in competition experiments the protein bound strongly to the octamer oligonucleotide ATGCTAATGATAT but less strongly to an oligonucleotide...
The role played by the Oct-2 protein in B cells in positively regulating the expression of immunoglobulin genes (Scheider, 1987) suggests that neuronal Oct-2 may play a similarly significant role in gene regulation in sensory neurons and their response to NGF. Although the cellular genes regulated by Oct-2 in sensory neurons remain to be identified, one obvious candidate is the gene encoding the nucleotide CGRP which contains two octamer motifs in its promoter (Broad et al., 1989) and whose mRNA increases in abundance in adult sensory neurons treated with NGF (Lindsay and Harmer, 1989). It is probable however, that Oct-2 may act primarily to inhibit rather than activate gene expression in neuronal cells. A number of differently spliced variants of Oct-2 have been identified (Wirth et al., 1991), and there is evidence that the forms expressed in neuronal cells may differ in their activity from those expressed in B cells (Dent et al., 1991). Thus unlike B cell Oct-2, neuronal Oct-2 has been shown to be ineffective at activating reporter constructs containing an octamer motif and can interfere with the activation of such constructs by Oct-1 (Dent et al., 1991), suggesting that Oct-2 acts as an inhibitor of octamer-mediated gene regulation. This potential inhibitory role of neuronal Oct-2 is of particular interest with respect to the mechanism of infection of these cells by herpes simplex virus (HSV). Sensory neurons support a latent form of asymptomatic HSV infection in an NGF-dependent manner (Wilcox and Johnson, 1988). Depleting the supply of nerve growth factor to the cells results in the re-expression of virus which lyrically infects cells innervated by the infected neuron. Evidence for the existence of an NGF-regulated repressor of viral reactivation has been obtained in rat, monkey, and human sensory neurons (Wilcox et al., 1990). The failure of the HSV lytic cycle in neuronal cells with the consequent production of a latent infection has been shown to be dependent on the weak activity of HSV immediate-early promoters in sensory neurons, due to the presence of an inhibitory factor that binds to the viral regulatory octamer-related motif TAATGARAT. This inhibitory factor has been identified as neuronal Oct-2 by mobility shift assays, and elevation of the levels of Oct-2 in neuronal cell lines has been shown to exert an inhibitory action on immediate-early gene expression using reporter gene constructs (Kemp et al., 1990; Lillycrop et al., 1991). This inhibitory action reflects the competition for the viral octamer binding site by Oct-1 which forms a productive trans-activating transcriptional complex with the HSV virion protein Vmw65 and Oct-2 which does not (Gerster and Roeder, 1988). The demonstration that NGF up-regulates Oct-2 without altering the levels of Oct-1 thus provides a mechanism for the repression of HSV immediate-early gene expression and maintenance of latent infection by NGF and is consistent with the view that Oct-2 is the inhibitor of herpes virus expression. Failure of retrograde transport of NGF caused by tissue damage would lead to a fall in Oct-2 levels with a consequent activation of HSV immediate-early gene transcription and production of virus. It will therefore be important to determine the precise molecular structure of sensory neuron Oct-2 and to test the actions on Oct-2 mRNA levels of other neurotrophic factors such as NT3 and BDNF as well as mediators such as glucocorticoids that play important homeostatic roles and may also influence HSV expression.

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NGF Regulation of POU Proteins
