MOLECULAR, CELLULAR AND DEVELOPMENTAL STUDIES OF SENSORY HYPERINNERVATION FOLLOWING NEONATAL SKIN WOUNDING

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ABSTRACT

A close interaction exists between the nervous system and the skin during development and injury. During development target derived factors are important for patterning the neuronal elements present in later life. After injury, at critical developmental time points this patterning is subject to change for long periods, and can subsequently affect pain processing. Sensory hyperinnervation is profound in the skin of postnatal animals wounded at birth (Reynolds & Fitzgerald 1995).

Here I have examined the development of this sensory hyperinnervation and the neurotrophins as possible causal factors. In vitro models for this phenomenon and histochemical characterisation of neurite outgrowth has also been assessed.

NGF, NT-3 and GDNF are all upregulated after skin wounding in the above circumstance. However, neither NGF nor NT-3 are solely responsible for the hyperinnervation (Reynolds et al. 1997; Alvares et al. 1999). In vitro studies have demonstrated that NT-3 may be partly responsible, although trkC IgG fusion proteins show no effect in vivo. In vitro models have also demonstrated the importance of the dermis/epidermis for determining extent of neurite outgrowth.

To add a new dimension to this study, I have constructed a neonatal wounded/naive skin cDNA library. By using a differential screening approach with mPCR probes, genes regulated in the above situation have been isolated. Clones generated have been sequenced and Northerns, and in situ hybridisation studies undertaken for genes of interest. Clones of particular interest include an endoplasmic reticulum stress protein-like gene (Erp29), an alpha globin-like gene, an ephrin (EPHRIN-A4) and a guanine nucleotide releasing protein-like gene. In addition several novel clones have been isolated which have yet to be characterised.

This research will provide further information and understanding to both wound healing in neonates, and the peripheral patterning of cutaneous innervation during development, and lead to the discovery of the essence of hyperinnervation a largely unexplored event.

CHAPTER 1

GENERAL INTRODUCTION

1.1 SKIN STRUCTURE

The skin is an organ in its own right. It forms the main protective barrier of the human body, as well as serving in thermoregulation, and the catabolism of steroids and synthesis of vitamin D. Skin may be hairy or non-hairy/glabrous. Hairy skin is less sensitive than glabrous skin, since hairs prevent contact of stimuli with the epidermis, although hairs themselves are sensitive to movements. The epidermis is much thicker in glabrous skin and the epidermal-dermal junctions are more complex.

The layered structure of the skin is shown in figure 1. The stratified epidermis, the outer layer, is tough and scaly and contains β keratin. Beneath it lies the wide stratified dermis containing collagen, elastin, nerves, blood vessels and lymphatics. Bundles of smooth muscle are scattered in the upper dermis and insert into the bulge of hair follicles At the dermo-epidermal junction there is a basal lamina which in mammals is poorly defined (see Montagna & Parakkal 1974). Below the dermis is an adipose/fatty cell layer.

1.1.1 The epidermis

The epidermis itself is divided into organised layers. The innermost layer, called the Malpigian layer, is further subdivided into a basal germinative layer which comprises dividing daughter cells which are displaced towards the periphery, a stratum spinosum or prickle cell layer, a stratum granulosum containing keratohyalin granules and a thick stratum lucidum. It is this set of layers which is responsible for the active transition of nucleated keratin cells into the enucleated keratin cells of the surface. The outermost layer of the epidermis, adjacent to the surface, is the stratum corneum or horny cell layer with its compact flat cells. In laboratory animals, unlike humans the epidermis is relatively thin with a thin stratum corneum and a poorly developed stratum granulosum (Montagna & Parakkal 1974).

The epidermal cells, known more correctly as keratinocytes, undergo a cycle of mitotic division, differentiation and exfoliation. The average turnover time for the stratum corneum is two days in man with a minimal transit time from basal to stratum corneum of 12-14 days (see Montagna & Parakkal 1974). With migration comes the loss of mitotic potential and the synthesis of keratohyalin, a fibrillar, amorphous protein. Terminal

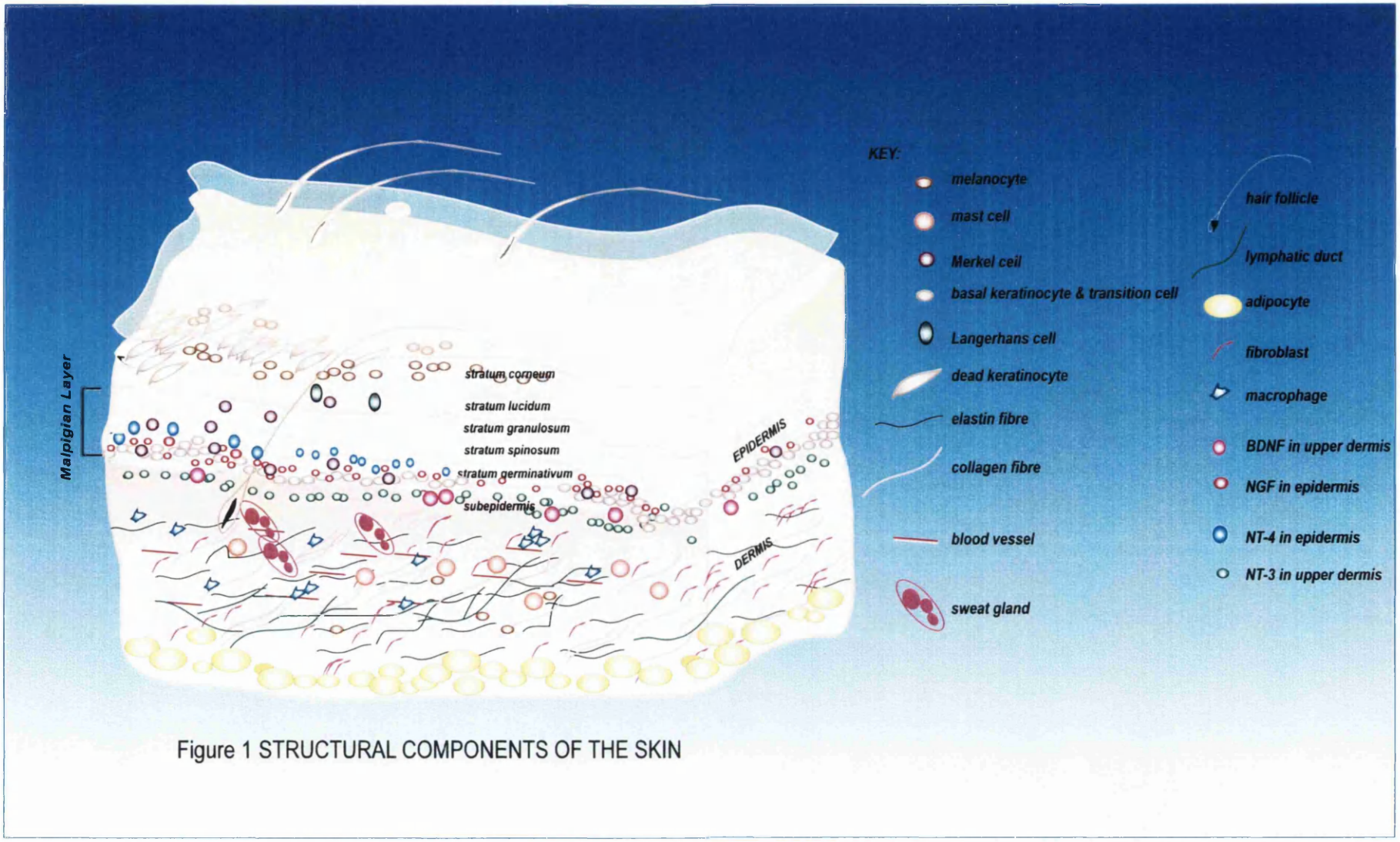


Figure 1 STRUCTURAL COMPONENTS OF THE SKIN

keratinisation also results in the thickening of the cell envelope of the cells in the stratum corneum, and the resorption of nuclei and organelles, with final exfoliation.

Melanocytes are found packed between keratinocytes. These cells are well known for their production of the pigment melanin which is thought to be transferred to the keratinocytes to be used as an ultraviolet screen. Melanocytes are derived from neural crest cells and invade the epidermis at 12 to 14 weeks gestation. At 4-6 months few remain in the dermis (see Montagna & Parakkal 1974).

Langerhans cells and Merkel cells are also scattered among the keratinocytes. These will be discussed later with respect to their neural relations.

1.1.2 The dermis

This consists of a matrix of loose connective tissue, collagen, elastin and reticulin in an amorphous ground substance. It is traversed by blood vessels, nerves and lymphatics, and it is penetrated by epidermal appendages, i.e. the eccrine sweat glands and the pilosebaceous units. The cellular matrix composition consists of fibroblastic cells, with abundant mast cells, macrophages, melanocytes and extravasated leucocytes. With the exception of the *arrectores pilorum* muscle, the dermis is free of muscle components.

Collagen fibres are laid down in an irregular mesh parallel to the epidermis. They are divided into an upper papillary layer, located immediately underneath the epidermis, with thin fibers and much ground substance, and a lower reticular layer with a network of thick collagen fibers.

Coarse fibers of elastin are entwined with the collagenous fibers of the dermal reticular layer, the papillary dermis and around the cutaneous adnexa and blood vessels. In sub human species there is a relation between the number of elastin fibers and the number of hair follicles, and as such they serve to attach the *arrectores pilorum* muscle to the bulge of the hair follicle, as well as anchoring blood vessels and sweat glands to the reticulum. In the reticular layer a palisade of elastic fibers run perpendicular to the epidermis, and fan out which may serve to anchor the dermis to the epidermis. In addition, fine branching fibers of reticulin are closely associated with collagen in the dermis.

The extracellular matrix (ECM) contains not only ions and proteins that pass into it via an osmotic gradient from the blood, but metabolic products of cells, and the fibrous proteins proteoglycans, glycoproteins, and glycosaminoglycans (GAGs) (see Montagna & Parakkal 1974). GAGs themselves incorporate the monosaccharides hyaluronic acid, chondroitin sulphate, dermatin sulphate, keratin sulphate and heparin sulphate. The

cellular component of the ECM consists of fibroblasts and macrophages. Fibroblasts are responsible for the synthesis of collagen, elastin and ground substance, and macrophages for tissue morphogenesis by phagocytosis throughout fetal and neonatal development. Mast cells are ubiquitous in the connective tissue and are often found around blood vessel walls.

1.1.3 Blood supply

The skin is metabolically overperfused i.e. supply is in excess of demand (see Montagna & Parakkal 1974). Branches of segmental arteries pass through muscles as perforator arteries and give off muscular vessels then continue peripherally to the skin. The segmental arteries closely relate to nerves. The skin is also supplied by direct cutaneous vessels which perforate the muscle to supply it, mainly at the extremities. Major trunks for these travel parallel to the surface. Blood is distributed from major vessels to surrounding dermis by superficial and deep plexuses, which are interconnecting vessels of different sizes. Long and straight capillary loops have been described, with metarterioles which emerge from arterioles and are surrounded by one layer of smooth muscle. These form a direct connection to the venous supply via precapillary sphincters and venules and are involved in regulation of temperature and blood pressure. These arteriovenous anastomoses are innervated by cholinergic nerves (see Montagna & Parakkal 1974).

1.1.4 Lymphatics and glands

These are as extensive as the blood vessels within the skin. Capillaries which end in the papillary dermis drain into a subpapillary lymphatic plexus which empties into a deeper plexus with valves.

In addition, glands are present in the stratum corneum and within the dermis such as the sebaceous glands which secrete sebum to lubricate the hair follicle and shaft, and the apocrine glands with their pale milky secretions (see Montagna & Parakkal 1974). The latter are located entirely within the dermis, larger ones extending into the subcutaneous fat. The duct of each gland rests in close proximity and in parallel to the hair follicle and opens inside the pilary canal above the sebaceous gland. These develop at 5-6 month gestation in man, but remains functionless in the neonate and child until the 7th year.

1.2 SKIN DEVELOPMENT

The epidermis and dermis arise from the embryonic epithelium and mesenchyme respectively. In the human, skin development begins in the first trimester at 5-7 weeks, when the dermis is predominantly cellular with mesenchymal cells separated by extracellular matrix. By 8-9 weeks collagen has increased in the dermis and a border is formed with the subcutaneous tissue. Between 10-20 weeks collagen bundles of fibrils are apparent and both papillary and dermal ridges are visible. The dermis then becomes invaded by downward invaginations of epidermis. Keratinisation takes place from six months gestation to birth (see Montagna & Parakkal 1974).

The collagen component of the fetal dermis is fine and fibrillar and gets coarser with age. The amount and thickness of elastin also during development. The GAG and hyaluronate content of fetal skin however, is much higher than that of the adult human although the dermatin sulphate content is lower (see Montagna & Parakkal 1974).

1.3 MORPHOLOGY AND STRUCTURAL ASPECTS OF SKIN INNERVATION

The skin is innervated by primary afferent sensory nerves, postganglionic cholinergic parasympathetic nerves and postganglionic adrenergic and cholinergic sympathetic nerves (Rossi & Johansson, 1998). Nerve fibres innervate the skin throughout all layers of the skin, but at different densities. Cutaneous cells are contacted by axon terminals containing neurosecretory vesicles (an intercellular distance of less than 300nm), which could be considered a synapse (Misery, 1997). As yet no electrical current has been detected in skin cells, except that between these axon terminals and Merkel cells (Misery, 1997). A schematic representation of some aspects of skin innervation is shown in figure 2.

1.3.1 The dermal nerve network

The major feature of cutaneous innervation is the dermal nerve network. It comprises parallel nerve trunks deep in the subcutaneous tissue where the papillary dermis joins the reticular dermis, accompanied by blood vessels which run in the connective tissue septa between fat lobules. The most distal of these nerve networks gives off collateral branches at irregular intervals which rise up to the dermal subcutaneous junction and often connect with each other. The networks occur in a random fashion and consist of both myelinated and unmyelinated fibres (see Winklemann 1960).

SCHEMATIC REPRESENTATION OF SKIN INNERVATION

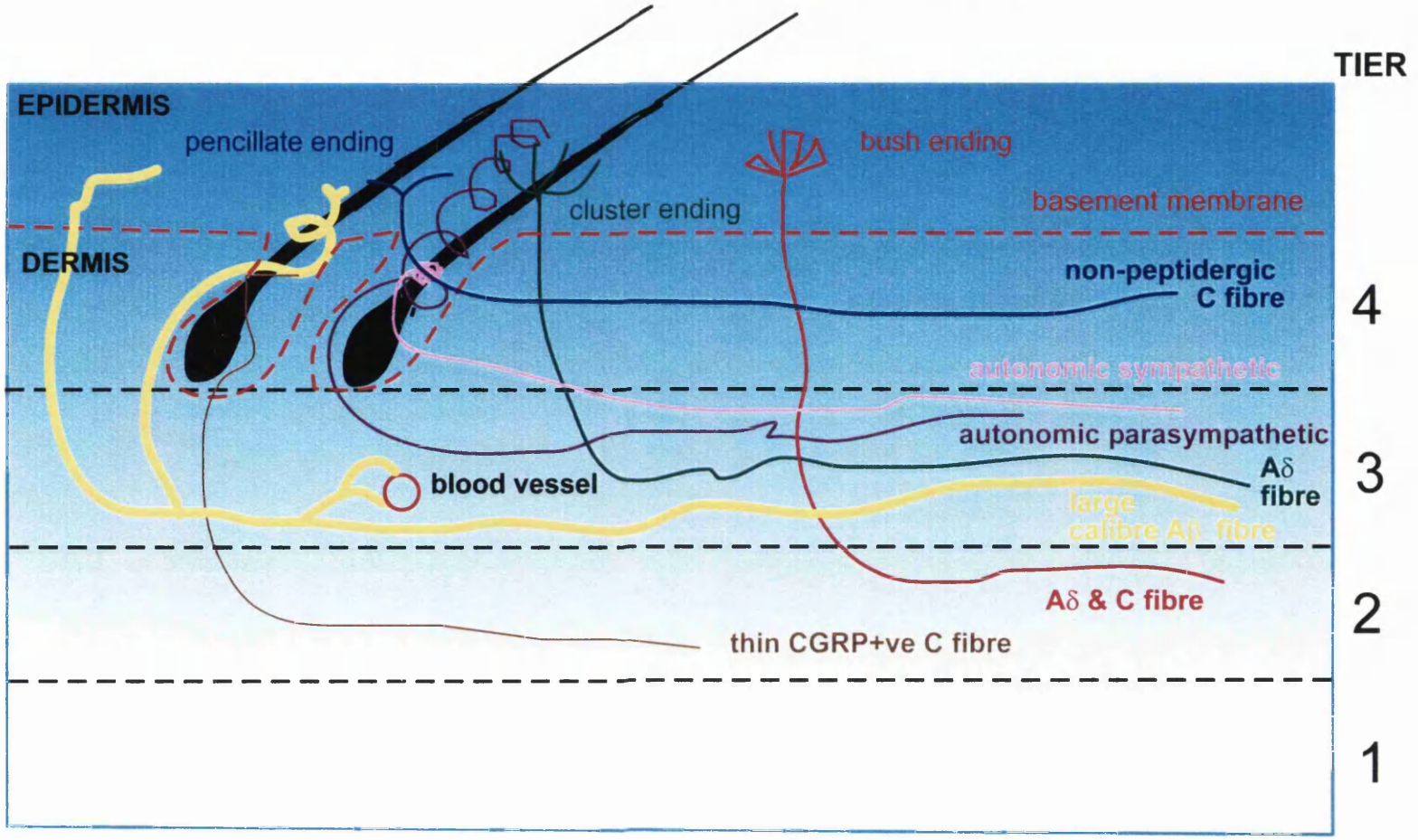


Figure 2 : Schematic representation of the unmyelinated and lightly myelinated groups of nerve fibres which innervate the skin. (Adapted from Rice et al,1999)

Deep within the dermis the network is heavily myelinated and with larger diameter fibres creating a larger network. In hairy skin this is less dense in the middle dermal area. The density of the nerve networks is also greater in developing animals compared to adults, although the myelination is less. Towards the surface unmyelinated fibres predominate, with light myelinated fibres with small chain networks.

The final termination of the dermal network is as fine unmyelinated subepidermal network with fine beaded terminal ramifications into the epidermis. The superficial nerves interact to a greater extent with other structures such as blood vessels and end organs (Winklemann 1960). Thin CGRP-IR axons are frequently scattered among clusters of mast cells and direct contact between nerve fibres and mast cells and axons in the dermis has been reported (Misery, 1997). Vascular related calcitonin gene related peptide (CGRP)-immunoreactive (IR) fibres ring around veins within the dermis and in glabrous skin there is also a non-vascular component with free endings near Meissners corpuscles. In the reticular layer of the dermis and subcutaneous adipose tissue elaborate CGRP-IR is found around acinar sweat glands (Kruger et al. 1989).

The course of nerve buds during development follows the path of least resistance i.e. within gaps in the connective tissue. The nerve networks are the first structures to be formed in the dermis in mouse embryos of 12mm, and there are more axons making contact with the basal layer of the epidermis in young animals compared to adults (Fitzgerald, 1966).

1.3.2 Papillary nerves

The majority of dermal papillae are shallow, but some project deeply into the epidermis. Their apices reach the epidermis in areas where the stratum basale is separated from the stratum granulosum by only one or two prickle cells (so called because they are attached to their neighbours by cytoplasmic processes, called prickles) (Fitzgerald, 1966).

Papillary nerves course in and out of the papillae from the subepidermis. Together with their associated capillaries they may pierce the epithelium in distal glabrous skin. As such they are common in hairless skin areas, and their development is chiefly in late life. Sometimes the papillary nerve network is so convoluted that it forms a kind of nerve ending. This resembles the Meissner corpuscles at the mucocutaneous junction of glabrous skin. The development of these endings is related to that of the dermal papillae and rete ridges.

Dermal papillae contain CGRP-IR axons surrounding each vascular loop: other papillary axons end freely or occasionally surround Meissners corpuscles. The dermal papillae are frequently innervated by axons surrounding blood vessels (Kruger et al. 1989).

The sub-epidermal plexus forms from the superficial cutaneous nerve bundles, wherein axons leave the plexus and innervate the epidermal pegs, many ending in the stratum spinosum. Subepidermal nerve fibres are numerous in rat and pig embryos (Fitzgerald, 1966) and decrease with age.

1.3.3 Intraepidermal nerves

Intra-epithelial fibrils with a varicose appearance are found in thick glabrous skin and enter the thick epithelium and zigzag between epithelial cells up to the granular layer, along with the tactile cells of Merkel-Ranvier, and the star-like Langerhans cells (see later). The most striking feature is the variability in thickness, with the finest axons penetrating to only the lower prickle cell level and without division, whereas medium to large diameter fibres reach the upper prickle cells of the stratum granulosum and form small branches (Fitzgerald, 1966). In the adult rat, thick axons with branches also terminate on the basal cells and innervate Merkel cells with a greater proportion in hairy skin (Kruger et al. 1985).

Intraepithelial axons enter glabrous epidermal pegs, branch and terminate throughout the stratum spinosum. This pattern is similar in hairy skin with additional innervation entering the base and surrounding lower third of each follicle (Kruger et al. 1989).

1.3.4 Epidermal Nerves

Some axons innervate the dermis and then form separate branches for innervating the epidermis (Kruger et al. 1985). Ten subpopulations of unmyelinated and small myelinated fibres innervate the epidermis, eight are unmyelinated or Aδ sensory axons and two are autonomic. Four out of the eight are varicose large unmyelinated terminals at the necks of hair follicles, blood vessels and within the upper dermis, which are supplied by layer 3 of the dermal plexus. One population, the CGRP and SP unmyelinated fibres is supplied by layer 2 of the dermal plexus. The remaining three sets are non-peptidergic unmyelinated and Aδ fibres supplied by layers 2, 3 and 4 (Rice et al. 1998 see figure 2).

Thick axons branch near apical ridges and innervate Meissner's corpuscles. Thin axons are more numerous here and with a variety of forms.

1.3.5 Hairy skin

Fundamentally there is not much difference between the patterns of innervation of the hairy and glabrous skin.

Nerve networks are well developed in the hairy skin, and rise close to the surface epithelium. Along the sides of hair follicles in the digits nerves may form fine narrow fibrillar networks in the region of the follicular neck. Terminal enlargements may also be present. More than one type of ending may innervate a hair follicle (Halata & Munger, 1983). Less innervation is seen in the epidermis of hairy animals, although intraepidermal axons are extensive with respect to hair bulbs, superficial dermis and overlying thin epidermis and distally the hairy paws of animals are heavily innervated by sensory mechanoreceptive fibres around the hair follicles (Kruger et al. 1989).

1.3.6 Hair follicle innervation

The basic structure of hair follicle innervation is present throughout the entire cycle of the hair. Hairs consist of compactly cemented keratinised cells produced by the follicles. In rats the cycling of hair varies in pattern and in location, but on average the total growth phase is between 21 and 26 days.

A double ring of myelinated and unmyelinated nerves is first seen surrounding the neck of the hair follicle (Winklemann, 1960). Deep dermal and subcutaneous fibres reach the hair follicles with 5-9 fibres per hair follicle in humans. They approach from many directions below the level of the sebaceous gland entrance with the hair follicle. At the neck, a heavy myelinated fibre network lies within the dermal connective tissue. Rising above these are the unmyelinated filamentous nerve fibres which encircle the hair follicle. The nerve supply varies directly to the size of the hair follicle (Winklemann, 1960).

Autonomic nerves supply sympathetic innervation to the hair follicle and via a terminal reticulum to the papillae of the hair follicle. In quiescent follicles the bulb degenerates, and the nerve plexus in the space vacated by the degenerated bulb collapses in a train below the dermal papillae, but remains intact and rich in cholinesterase (Montagna & Parakkal, 1974).

Hair follicles subserve the sensation of touch and tactile stimulation. The disk of Pinkus, an epidermal thickening close to the hair follicle into which nerves ramify and lose their myelin sheath is also present in some animals such as rats. Its function remains unknown. Lanceolate endings are characteristic of hair innervation with an axon and Schwann cell and a rapidly adapting mechanoreceptive response (Kruger et al. 1985) (see figure 2).

1.3.7 Glabrous skin

The skin here is thrown into verrucal folds, such that the papillae extend above the common level of the cutaneous surface.

The dermal nerve networks in these areas are pronounced. From the deep layer arise individual myelinated fibres arise supplying Meissners corpuscles and Merkel cells. They are most dense in the extremities.

In the middle or lower part of the dermis are the Vater-Pacini corpuscles (see later), with the convoluted types being deeper in the subcutaneous tissue.

1.3.8 Autonomic supply

The autonomic innervation of the skin subserves three main functions, sudomotor, pilomotor and vasomotor. Innervation is mainly in the form of a reticulum, with a sympathetic plexus in the middle dermis. Sympathetic fibres are mainly adrenergic NPY containing and cholinergic VIP/CGRP containing.

Adrenergic fibres supply the blood vessels, smooth muscle and the glands, with cholinergic fibres supplying the eccrine glands. Autonomic fibres accompany the blood vessels, with the sympathetics accompanying the cutaneous nerves into the dermis. The autonomic nerves consist of large fibres innervating the necks of hair follicles, originating from layer 3 of the dermal plexus and smaller fibres innervating the upper parts of hair follicles, originating from layer 2 (Rice et al. 1998). Autonomic endings consist of axoplasmic filaments that break up into a fine granular network comprising the ending itself. These axoplasmic fibrils are embedded in plasmatic ground substance and surrounded by fibrillar tissue of neural origin (see Montagna, 1960).

There is a species difference in the level of autonomic innervation and differences between glabrous and hairy skin components, such that in rat hairy skin there is very little autonomic supply (for species differences in autonomic fibres see Montagna, 1960). There are also species differences with respect to the responsiveness of rat pups to thermal gradients compared to other vertebrates (Bereiter-Hahn et al. 1986).

1.3.9 Nerves to blood vessels

A close proximity exists between blood vessels and the subepithelial plexus of nerves, thus nerves may to some extent follow the course of blood vessels. In addition the arteriovenous anastomoses, consisting of afferent arteriole connected by a muscular connecting channel of epithelial cells to a dilated venule, have a special innervation

network of fine unmyelinated fibres, which penetrate between muscle cells (Winklemann, 1960).

Fetal vasculature is present at birth as a disordered capillary network in the human, which gains similarity to that of the adult 60-80 days after birth (Johnson & Holbrook, 1989). Major vascular organisation of the dermis occurs in the first trimester of life, with mesenchymal tissue forming vascular cords which gain lumen and transform into the capillary network. Perivascular nerves are confined to the interface between adventitia and the smooth muscle layer of the tunical media (Misery, 1997). SP-IR is found in relation to blood vessels and SP is a known vasodilator (Kruger et al. 1989).

1.3.10 Other Autonomic Nerves

Nerve fibres lie in close relation to myoepithelial fibres of sweat glands, which have a cholinergic, sympathetic catecholaminergic and sensory innervation depending on species and developmental parameters (Schotzinger & Landis 1990a).

Adrenergic nerve fibres pass near and along the *arrectores pilorum* muscle (see Winklemann, 1960). The greater the muscle mass the more prominent the nerve.

Nerves are found in close proximity to sebaceous glands, heading for the neck of the hair follicles and the surrounding capillaries.

1.4 CUTANEOUS NERVE ENDINGS AND SPECIALISED CELLS

1.4.1 The Meissner Corpuscle

Deep in the papillae of the skin lies a heavily myelinated oval encapsulated nerve ending supplied by up to 9 nerve fibres, and approximately 0.08 by 0.03mm. This is the Meissner corpuscle. It exhibits pronounced lobulation and each lobe is surrounded by a connective tissue capsule. On reaching the capsule the nerves divide, lose their myelin sheaths, coil or loop, and penetrate the end organ in any direction. They end as terminal enlargements or within a nerve network (Winklemann, 1960). They are most common in distal glabrous skin, with the palm being preferred to the sole of the foot, and a 7 month fetus has as many as a mature adult.

They can first be seen in the 4 month old fetus as a cellular mass, but their development is not completed until birth. The number of neurofibrillar endings to the corpuscle diminishes with age and the corpuscle forms coarse fibres, and thus involutes gradually (see Winklemann, 1960). Its function is that of a touch receptor, a rapidly adapting mechanoreceptor (Airaksinen et al. 1996).

1.4.2 The Merkel-Ranvier or Hederiform Ending

At the base of the rete region ridge on distal glabrous skin or hairy skin, are one or more heavy myelinated fibres from the deep dermal network, that course along the rete ridge, whose collaterals lose their myelin and terminate as varicose leaf- like enlargements. Their terminations lie between and about basal cells of the epidermis and are surrounded by a perikaryon. They receive accessory innervation from subepidermal nerve networks. Their development is similar to that of the Meissner corpuscle, but they develop with the base of the rete ridge rather than along with the papillae. Similarly, they are also involved with touch perception, and produce a type I slowly adapting mechanoreceptor response (Airaksinen et al. 1996).

1.4.3 Merkel cells

The origin of these cells, whether epithelial or neuronal remains a subject of some controversy. They are located throughout the epidermis and adjacent to hair follicles, scattered between keratinocytes, typically at the base of the epidermis where they occur in groups (Airaksinen et al. 1996). The cells have lobulated, irregular nuclei with electron-dense cytoplasm compared to adjacent keratinocytes (Winklemann, 1960). Their cytoplasm contains scattered granules (densest juxta-axonally) and neurosecretory vesicles containing neuropeptides, which are located on the epidermal side. Merkel cells have a resting membrane potential of -54mV (Misery, 1997) and mechanical deformation activates membrane channels and allows the release of neurotransmitters. Other neuronal characteristics are the presence of S100 protein, neuron specific enolase, neurofilament protein, protein gene product 9.5, chromogranin and synaptophysin in Merkel cells. In addition, they synthesise NGF and its receptor (Misery, 1997). Finally, the Merkel cell has also been shown to be surrounded by a Schwann sheath which becomes continuous with the basal lamina of the epidermis (Montagna & Parakkal, 1974).

1.4.4 Ruffini Endings

These structures are responsible for the effects of stretch in hair shafts. They are located in the reticular dermis, and are a common feature of primate hairy skin, with a slowly adapting type II mechanoreceptive response (Munger et al. 1983; Halata & Munger, 1983).

1.4.5 The Vater-Pacini Corpuscle

This is the largest sensory receptor in the skin. Its chief location is in the connective tissue of the skin below the fingers, palm, toes and soles. It is also found along nerve

trunks. It is predominantly found in glabrous skin as an encapsulated oval or round ending which may be bent or coiled, with a variable size from 1-4mm, and 0.5-1mm in diameter. It is divided into two parts, a stalk and a body. The sheath of the stalk grows out of the neural sheath, and the nerve makes 4-16 right angled turns within the capsule and then enters the body of the corpuscle. The central nerve loses its myelin sheath on entering the stalk.

Each of the approximately 50 layers of the capsule is composed of a double membrane linked to lamellae with or without fibrillar connections. The inner bulb, the central zone surrounding the nerve, contains a granular substance. The fluid of the end organ is largely albuminous and it has a close association with blood vessels.

The Vater-Pacini corpuscle functions as a pressure receptor. Its deeper location suggests that it cannot be an acute sensation detector.

1.4.6 Langerhans cells

These characteristically dendritic cells of the upper layers of the epidermis are also obscure in origin. They have a markedly indented nucleus and clearer cytoplasm containing granules (Winklemann, 1960), S100 protein and neuron specific enolase, and they have the ability to produce the precursors of MSH, ACTH and β endorphin. They also possess receptors for SP, GRP and αMSH. If denervated they synthesise PGP (Misery, 1997). Direct connections between nerves and Langerhans cells occur via dendrites and cell bodies.

1.5 DEVELOPMENT OF SKIN INNERVATION

In the rat hindlimb peripheral nerves reach the base of the limb bud at E13 (Reynolds et al. 1991). In the DRG, A cells are born in advance of C cells (Lawson et al. 1974) and A fibres arrive in the skin first. In the late fetal and early neonatal period there are many changes in innervation of the epidermis and dermis, as fibres are retracted from the epidermis, and the complex pattern described below emerges (Fitzgerald, 1966; Jackman & Fitzgerald, 2000). Merkel cells for example are not innervated until birth in the rat (English et al. 1980) with Meissner's corpuscles appearing at P8 (Zelena et al. 1990). At P3 fibres are mainly restricted to the dermis with small bundles of 1-3 axons able to leave it and ascend into the epidermis. Sensory axons leave the dermal plexus and grow towards hair follicles at P3. Innervation is achieved at P7 and bundles of 20 fibres divide into 2 branches each and supply adjacent follicles in a circumferential arrangement (Payne et al. 1991). The adult pattern of a palisade formation is seen by P19. The number of axons innervating a particular follicle increases until P19, whereas the percentage of hair follicles innervated decreases from the second postnatal week onwards.

Cutaneous innervation of the rat hindlimb begins in the upper lateral leg at E14-15 and reaches the tip of the toes by birth (Reynolds et al. 1991). RT97 labelled fibres are clearly detectable at E14 with a single bundle entering the hindlimb and dividing into medial and lateral branches immediately. The medial branch innervates the epidermis locally whilst the lateral branch subdivides a third of the way down the hindlimb to innervate the epidermis here. No extensive arborisation is see at this stage (Jackman & Fitzgerald, 2000). C fibres are not in the epidermis at E13-14, and neither is there immunolabelling for CGRP or IB4, although trk A labelling is observed but not within the epidermis.

By E15-16 the medial bundle of RT97 positive fibres has reached and innervated the proximal leg with increasing epidermal innervation. Trk A+ve C fibres are seen as fine structures within the epidermis, with proximal and medial fibre bundles branching into the epidermis at right angles to the skin surface. No CGRP or IB4 labelling is seen.

By E17-18 RT97 fibres are extensively ramifying with parallel and perpendicular branches within the epidermis reaching the whole foot and branches reach the toes. Loosely fasciculated trk A fibres enter the dermis and branch finely near the epidermal edge. These continue to increase over the E18-19 period when CGRP expression begins (Jackman & Fitzgerald, 2000). IB4 is never found in peripheral axons. From thence on there is a decrease in innervation of the epidermis with a concomitant increase of trk A

fibres formed sub-dermally and extending into the epidermis at regular intervals. Towards birth and postnatally innervation becomes predominantly subepidermal. Terminal degeneration of epidermal fibres increases with age, with no degeneration in the embryo or neonate. The number of fibres entering the epidermis per unit area is maximum in young animals and peaks after birth, with a sharp postnatal increase, then decreases to the adult phenotype. The increase is thought to arise from bifurcations from the dermal plexus i.e. collaterals (Fitzgerald, 1966). Intraepidermal nerve fibre development takes place at 3 months in the human fetus and increases in density by the sixth, with a subsequent decrease towards adulthood (Fitzgerald, 1966). Myelination of these fibres occurs after the fifth month (see Winklemann, 1960).

The development and innervation of sensory end organs generally follows that of the arrival of cutaneous nerves. Merkel cells and their endings are already present at birth, but the number of cells and their complexes increases from birth to P14 (Airaksinen et al. 1996). At 7 months gestation, the Vater-Pacini corpuscle is complete and can be seen in a human fetus. In the adult the multilayered capsule of the end-organ expands with fluid and forms its typical structure, compressing the convolutions and irregularities formed early on.

Sympathetic neurons innervating sweat glands switch phenotype postnatally from noradrenaline to cholineacetyltransferase, acetylcholinesterase and VIP expression (Stevens & Landis, 1987). However, in the thoracic skin of the developing and adult rat no cholinergic innervation is evident (Schotzinger & Landis 1990b).

1.6 DEVELOPMENT AND HETEROGENEITY OF THE DORSAL ROOT GANGLION NEURONS: CELL BODIES OF CUTANEOUS NERVES

Segments of skin (dermatomes) are supplied by specific dorsal root ganglia (DRG). DRG neurons begin as a mass of cells arising from the neural crest in the developing embryo. As time proceeds these neural crest cells become separated and migrate (due to influence from blocks of mesenchymal tissue called somites) to various areas. In the rat, crest cells form distinct DRG over the period of E12-15, primary sensory neurons of the lumbar DRGs being produced between E11-14 (Lawson et al. 1974; Kitao et al. 1996; Altman & Bayer, 1984). Nearly half of the DRG neurons that are born and begin to differentiate, die at about the time when they begin to project to peripheral targets (Coggeshall et al. 1994). Programmed cell death (PCD) occurs shortly after nerves begin to connect functionally with their targets. Along with innervation comes the transformation of epithelium to epidermis.

Traditionally DRG neurons have been classified with respect to size, physiological properties, neuropeptide expression, target innervation and neurotrophin dependency. DRG neurons vary in size from 18-75μm reflecting their phenotype and axonal diameter.

Every DRG contains the so-called large L light neurons and the small dark SD neurons. The former are cell bodies of large diameter A fibre afferents, with the latter those of unmyelinated C fibre afferents. Characteristically, these A fibres have been thought of as the myelinated proprioceptive and mechanoreceptive fibres, which exhibit rapid conduction velocities, whilst C fibres are the detectors of pain and temperature. All sensory neurons with myelinated axons are primarily activated by mechanical stimuli of the receptive field (Lewin & McMahon, 1991). Aβ fibres conduct at greater than 10m/s and Aδ fibres at less than 10m/s. There are two subtypes of Aβ fibre, the rapidly adapting RA fibres which respond to mechanical movement of the skin hair follicles and the slowly adapting SA fibres which are subdivided again into types I and II, the former being the innervators of Merkel cells (Airaksinen et al. 1996). Aδ fibres are classified as those that have a low mechanoreceptor for mechanical stimuli and a mechanoheat sensitive group (Lawson, 1996). C fibres are in general the polymodal nociceptors (PMN's) which respond to noxious heat and mechanical stimuli, and those which respond to noxious mechanical stimuli only. All types of afferent fibres are present in adult mice and distinguishable at P14, but at younger ages RA fibres have SA responses at high stimulus intensities,

indicative of a undecided state of affairs which becomes committed in the following two weeks (Koltzenburg et al. 1997).

By E14 both A and C types of neurons are present in the rat. Markers for the large neurons and small SD neurons have been used to study the development and plasticity of DRG neurons. RT97 is a monoclonal antibody against a phosphorylated neurofilament heavy chain subunit which labels the majority of large A fibre cells, including those with Aα, Aβ, and Aδ fibres (Lawson et al. 1984). In the periphery RT97 has been localised to large myelinated endings for Meissner's corpuscles, Merkel cells, hair follicle receptors, Pacinian corpuscles and free nerve endings (Sann et al. 1995). 28% of RT97 immunopositive cells are CGRP positive, 38% of these being Aδ and 17% Aα/β (McCarthy & Lawson, 1989). Some labelling of SD cells is also apparent with RT97. RT97+ve cells are born at E12-15 peaking at E13 and IB4 +ve cells at E13-16 peaking at E14 (Kitao et al. 1996). In the adult 40% of the DRG neurons express RT97 (Lawson et al. 1993).

Calcitonin gene related peptide (CGRP) is a 37 amino-acid neuropeptide related to the gene encoding the hormone calcitonin. It is present in primary afferent C fibre neurons (small to medium sized) and has an α and β form. In human skin it is co-localised with substance P in dermal papillae and epidermal free endings of glabrous skin and with somatostatin in the epidermis and perivascular spaces (Rossi & Johansson, 1998). CGRP expression begins in the DRG at E16 and is maintained into adulthood (Marti et al. 1987). 46.5% of all DRG neurons are positive for this marker (McCarthy & Lawson, 1990) with mainly a small medium diameter distribution, and 50% of small, dark fibres in the skin are positive for CGRP (O'Brien et al. 1989). CGRP positive neurons exhibit both fast and slow conduction velocities (Lawson, 1995). It is also co-localised with SP in and around blood vessels in the dermis of hairy skin and in the plantar aspect of the foot with penetrating epidermal fibres (Gibbins et al. 1987; Kruger et al. 1989). CGRP-like-immunoreactivity is also present around eccrine sweat glands and around hair follicles with SP (Tainio et al. 1987). However, Dalsgaard demonstrated these fibres as free endings in the dermis and epidermis. Merkel cells in the cat are also positive, but in the rat this is not true (Alvarez et al. 1988). CGRP is also detected in the epidermis associated with Langerhans cells and haemopoeitic cells (see Rossi & Johansson, 1998).

A third population of cells unlabelled with either RT97 or CGRP is apparent from E14-15 (Kitao et al. 1996), the IB4 population. The percentage of IB4 positive neurons

increases in the early postnatal period (Molliver et al. 1997). Trk A positivity is seen at E13-14 in small C fibre cells (Carroll et al. 1992; Bennett et al. 1996).

In the adult, the SD population of neurons are divided roughly in half with a group expressing CGRP and a group expressing IB4. There is also a small population of neurons with overlapping expression of CGRP and IB4 (Averill et al. 1995). The majority of CGRP positive neurons express trkA, whereas the IB4 neurons do not express trk A (McMahon et al. 1994) but do express ret (the GDNF receptor), and the ATP receptor $P2X_3$ (Bradbury et al.1998). Hence, there is a small population of neurons expressing CGRP, IB4 and $P2X_3$.

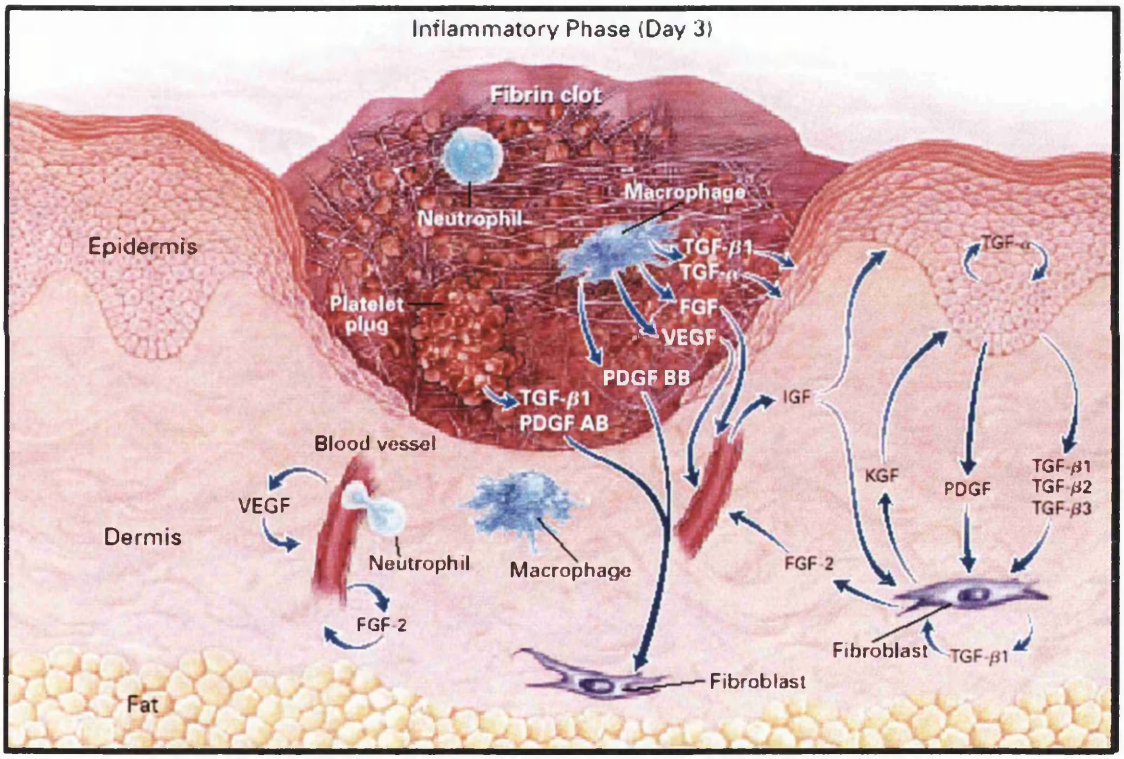


Figure 3 showing the development of the fibrin clot and the various interactions of growth factors and cells during inflammation. Taken from Epstein et al,1999.

1.7 SKIN INJURY AND INFLAMMATION

Tissue damage can result in one of three outcomes. Either the damaged area restores its structure and function completely, or it undergoes partial restoration with repair, or it continues to be inflamed. In the majority of cases partial restoration with repair occurs.

Acutely, the damaged area is filled with an inflammatory exudate consisting of proteins, antibodies, fibrin, macrophages, activated neutrophils and lymphocytes and fluid from leaky and dilated local blood vessels which respond to the substances released by the damaged site (see table 1 below). The effects of inflammation are seen in the redness, and swelling of the tissue as well as the perceived pain and heat.

1.7.1 Inflammation And Inflammatory Mediators

Table 1

CELLULAR MEDIATORS OF ACUTE INFLAMMATION	
Stored	Histamine
Active synthesis	Prostaglandins, leukotrienes, platelet activating factor (PAF), cytokines, nitric oxide (NO)
PLASMA-DERIVED MEDIATORS OF ACUTE INFLAMMATION	
kinin system	bradykinin
clotting pathway	activated Hageman factor
Thrombolytic pathway	plasmin
Complement pathway	C3a, C3b and C5a

1.7.2 Cellular Mediators

The activation of the endothelium of local blood vessels is of critical importance for plasma leakage and the transport of the above mediators to sites of damage (see Stevens & Lowe, 1995). The endothelium itself secretes factors such as nitric oxide (NO) and prostacyclin which induce vascular relaxation and inhibit platelet aggregation, as well as tromboxane A_2 (part of the arachidonic acid pathway),, and angiotensin II which cause vascular constriction. Mast cells are also important cell components of the wounding process and predominate where hyaluronate is present and this being upregulated after

wounding. They degranulate in wounds releasing heparin, a clotting agent, and histamine which has effects on capillary permeability, smooth muscle contraction and stimulates macrophages.

Under conditions of injury the endothelium synthesised platelet activating factor (PAF), increases vascular permeability and NO synthesis, and promotes vasodilatation. Nitric oxide is synthesised locally by the endothelium and macrophages through the activity of nitric oxide synthase (NOS). It causes vasodilatation, and increases vascular permeability, and can mediate bacterial and cell killing.

The properties of endothelium are also altered due to the presence of IL-1 and TNF (tumour necrosis factor), and the increased expression of adhesion molecules e.g. P-selectin (see Dressler et al. 1999). E-selectin, ICAM-1 and VCAM-1 on the endothelium can promote the adhesion of neutrophils, monocytes and lymphoid cells.

1.7.3 Cytokines

The role of the neutrophil is one of phagocytosis of pathogens, and proteolytic digestion by the release of lysosomal granules. Neutrophil survival is thus short. Later on in the inflammatory reaction macrophages take part in the phagocytosis of dead material, and remnants of neutrophils. Extracellular matrix proteins and monocyte chemoattractant proteins allow monocytes to infiltrate and turn into activated macrophages, releasing platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) for granulation tissue formation and expressing colony stimulating factor 1 (CSF1) for survival (Epstein, 1999). Activated macrophages and lymphocytes also secrete a group of polypeptides called cytokines. The main cytokines participating in acute inflammation are interleukin-1 (IL-1), interleukin 8 (IL-8) and TNFα (Hubner et al. 1996). They are responsible for the induction of cell adhesion molecules on endothelium (for a review see Meager 1999), induction of PGI_2 (prostacyclin) synthesis (Chen et al. 1994), the induction of PAF synthesis as well and being induced by PAF themselves (Pei et al. 1998; Lacasse et al. 1997) and stimulation of the acute-phase protein synthesis by the liver (Moshage, 1997), as well as fibroblast proliferation and secretory activity (see Heckman et al.1993), and the attraction of neutrophils to the damaged area (IL-8 dependent) (Bickel, 1993).

1.7.4 Clotting

Hageman factor (factor XII) in the clotting cascade activates the conversion of prekallikrein to kallikrein which allows the conversion of kininongen to bradykinin.

Bradykinin mediates increased vascular permeability, activates C fibres resulting in pain and activates the complement system (see below). Clotting factors are also responsible for the conversion of the soluble fibrinogen to insoluble fibrin in clots, and the production of fibrinopeptides which increase vascular permeability and are chemotactic for neutrophils (for a review see McGilvray & Rotstein, 1998).

1.7.5 Complement

Complement activation with the production of the C5a fragment induces increased expression of complementary cell adhesion molecules on neutrophils. The complement system comprises a set of plasma proteins with important roles in immunity and inflammation. Members are responsible for mediating vascular permeability by releasing histamine from mast cells/platelets (C3a and C5a), being chemotactic to neutrophils and adhesion molecules (C5a), and being chemoattractant to neutrophils (C345), and opsonizing bacteria, thus facilitating phagocytosis (see Kaplan et al. 1981).

1.7.6 Thromobolysis

During resolution of inflammation the thrombolytic pathway is activated such that plasminogen activator from the endothelium, causes the enzyme plasmin to activate complement, activate Hageman factor and lyse fibrin clots to form fibrin degradation products (FDPs), which also increase blood vessel permeability.

1.8 SKIN WOUND HEALING

With extensive damage to the skin as in full thickness skin wounds, the tissue heals by a process of organisation and repair, leading to the formation of a scar.

Initially, the formation of a blood clot re-establishes haemostasis and provides a provisional ECM for cell migration (Epstein, 1999). Platelets aid plug formation.

Re-epithelialisation begins within hours of injury with retraction of intracellular monofilaments and formation of peripheral cytoplasmic actin filaments (see Nodder & Martin, 1997). Migrating basal keratinocytes dissect the wound by secretion of collagenase, and separate the desiccated eschar (dead scar material) from viable tissue. The majority of interactions are mediated by integrins (see Nodder & Martin, 1997). During day 1 and 2 the leading edge of the wound begins to show keratinocyte proliferation brought on by loss of adjacent contacts or the release of growth factors such

as keratinocyte growth factor, TGFα, EGF (although EGF has been shown not to be mitogen for keratinocytes Hancock et al. 1988). This is accompanied by increasing gradients of glycogen from the marginal epidermis to the epithelial tip (see Montagna & Parakkal, 1974). There is also an increase in activity of glycolytic enzymes, as glucose utilisation and lactate production increases. Alkaline phosphatase is also present in damaged epidermis, decreasing once repair has been achieved (see Montagna & Parakkal, 1974). The principal role of it in the skin appears to be dephosphorylation for adsorption and transport of chemical substances necessary for growth and maintainence of the pilary system and glandular adnexa, but it may regulate scar formation (see Hui & Tenebaum 1995), being elevated in granulation tissue (Alpaslan et al. 1997).

1.8.1 Growth Factors

The synthesis of numerous growth factors is important for the wound healing response. PDGF is secreted by the endothelium as well as by macrophages and platelets (Ashcroft et al. 1997a). It acts as a potent chemoattractant and mitogen for fibroblasts and inflammatory cells and mesenchymal cells, stimulating fibronectin, glycosaminogylcans (GAG) and collagenase synthesis (see Ashcroft et al. 1997). Other growth factors such as epidermal growth factor (EGF) whose expression at the wound site correlates with the time of epithelialisation, allows the migration of keratinocytes (Hudson & McCawley, 1998), stimulates granulation tissue formation (see below), and cellular proliferation. Basic fibroblast growth factor (bFGF) a member of the heparin binding growth factors, produced by macrophages and endothelial cells is found in the basement membranes of all ages of animals (Whitby & Ferguson, 1991b; Whitby & Ferguson, 1991a). It is able to induce angiogenesis, ECM synthesis and stimulate wound healing in diabetic mice (see Ashcroft et al. 1997). TGFβ is released not only by monocytes, but by the degranulating platelets, and is thought to enhance tissue repair by attracting monocytes and fibroblasts as well as increasing ECM deposition (see Ashcroft et al. 1997). TGFβ decreases NGF mRNA in Schwann cells in newborn rat cultures (see Matsuoka et al. 1991).

Platelets are responsible for the release of IGF-I, as are fibroblasts and polymorphonuclear leucocytes. mRNA for IGF-I and II is increased in fibroblasts of wounded skin (Gartner et al. 1992), and fibroblasts both secrete and respond to IGF-I. Moreover, diabetic wounds have a delayed expression of IGF-II in epithelial cells and granulation tissue, although levels are much higher than in normal wounds (Brown et al.

1997). For a more detailed description of sites of production and biological activities of growth factors see (Blitstein-Willinger, 1991; Lindsay et al. 1985) and figure 3.

Pre-existing capillaries in the undamaged tissue bud into the damaged area forming new vessels and allow access to macrophages, fibroblasts and myofibroblasts. Macrophages binding to ECM components by their integrin receptors can then begin to phagocytose the inflammatory exudate and necrotic tissue. Macrophage depleted animals have defective wound healing (Epstein, 1999).

This mixture of capillaries, macrophages and support cells is known as vascular granulation tissue, and it is believed that the rate-limiting step in the formation of granulation tissue is the appearance of fibronectin and integrin receptors. Granulation tissue contracts to tug the wound edges together whilst epidermal cells migrate over the surface. Wound contracture is firmly in place by the second week.

As the fibroblasts, (synthesisers of matrix) and myofibroblasts proliferate, and the macrophages migrate away, collagen synthesis begins and capillaries regress, leaving only a few vascular channels. Some may acquire smooth muscle in their walls and remain as functioning venules and arterioles once healing is complete. Collagen is deposited by fibroblasts in an ordered fashion, and contraction of this granulation tissue often occurs with the aid of myofibroblasts. Dense collagen deposition finally forms a collagenous scar, and fibroblasts return to resting activity. The tensile strength of an incised wound continues to increase long after the initial increase in collagen content has levelled off (Montagna, 1960).

Surgical resection of cutaneous nerves, results in delayed wound healing in animals (Ansel et al. 1998).

1.8.2 FETAL WOUND HEALING

In the fetus, wound healing has been described as completely regenerative, without subsequent scarring, with a lack of neovascularisation and with increased efficiency (Whitby & Ferguson, 1991a). Some of the responsibility for this lies in the differential expression of factors related to wound healing in the fetus compared to the adult.

Fetal wounds appear to produce collagen type I by increasing the number of cells in an area of wounding, rather than by induction of the procollagen type I gene as in adults. The levels of expression are highest in the fetus on day 3 and 5, whereas that in the adult peaks at day 7 (Nath et al. 1994). The collagen in fetal wounds is indistinguishable from the

surrounding tissue and is laid down in the normal orientation, unlike that of the adult which is lies perpendicular to the surface (Whitby & Ferguson, 1991a). Ageing is thought to impair wound healing by the slow rate of re-epithelialisation, basement membrane formation and disordered collagen deposition.

βFGF responsible for angiogenesis, decreases in wounds with time (Whitby & Ferguson, 1991), and it's application to wounds produces an immature collagen free wound with continuing matrix deposition (see Ashcroft et al. 1997). It's presence in the fetus may be a factor in lack of scar formation.

Whilst in adult wound healing the first epidermal barrier between granulation tissue and the fibrin clot is formed by the crawling in of epidermal cells. In contrast, in the embryonic epidermis the area is fenced off by the contraction of actin filaments in the epidermal cells around the wound site, and has been likened to the action of a "purse-string". It is believed that this difference is caused by the lack of myofibrils in early wound healing in embryos, which are proliferative in the adult (Nodder & Martin, 1997). TGF is thought to be responsible for the differentiation of fibroblasts into myofibroblasts (see Nodder & Martin, 1997) and as such the lack of TGF in embryos would suggest a lack of these specialised cells, evidence for a different method of barrier establishment. Scar formation has also been attributed to this cellular type (Estes et al. 1994). However, recent studies by (Liu et al. 1999) indicate that in neonates where full thickness skin wounds heal more rapidly than in young adults, the proliferation of myofibroblasts is much greater in the former.

Ashcroft and co-workers (1997) have also shown that many of the growth factors which participate in wound healing are temporally altered in ageing wounds as compared to fetal wounds. For example TGFβ is low in embryonic mice (Whitby & Ferguson, 1991a), and highly expressed in young animals as are PDGF and EGF, but not continuously throughout wound healing as that of adult animals. IGF-II is also felt to exert its influence during fetal development with a role in increasing the scar resistance of fetal skin wounds (Sancho et al. 1997).

Very recently (Ashcroft et al. 1999) have shown that mice lacking SMAD 3 protein which transduces signals from TGFβ have impaired inflammatory responses and accelerated wound healing. Thus the increased healing seen in fetuses and the lack of inflammation in embryos, could be attributed to a lack in TGFβ components, and indeed TGFβ is not present in fetal wounds (see Whitby & Ferguson, 1991a), Early embryos also lack platelets, and exhibit little cell death within the wounded area. Neutrophils are absent

from fetal wounds until E19, prior to birth in the rat which may lead to a decrease in the overall extent of the inflammatory reaction, leading to faster healing (see Stelnicki et al. 1999). Platelets are necessary for clot formation, but their persistence may impair healing and lead to scarring. Hyaluronate which provides a permissive environment for various developmental cell types, persists in relatively large amounts and for longer in the fetus as apposed to the adult wounded environment, and as such may inhibit the function of platelets and limit scarring (see Nodder & Martin, 1997; Olutoye et al. 1997).

In addition the fetal environment is wholly different to that of the adult with differences in pH, oxygen saturation and the presence of amniotic fluid, all of which will aid or inhibit wound healing. Furthermore, there are differences in matrix metalloproteinases (important in matrix remodelling) during ageing in normal skin and wounded skin (Ashcroft et al. 1997b), the distribution of homeobox genes in epidermis and dermis of the fetal and adult skin (important in patterning and differentiation during development (Stelnicki et al. 1997)), the change of heat shock proteins (protein protectors activated by stress events) (Laplante et al. 1998) during skin wound healing, and the change in activin (members of the TGFβ superfamily) expression during inflammation (Munz et al. 1999). These all clearly demonstrate the variety of factors which may be altered between the immature and mature animal, as well as under conditions of cutaneous insult.

Thus embryonic skin is fundamentally different to that of the adult, and the altered response to wound healing is just one example of this difference. The neonatal period represents the transition between embryonic and adult life and thus may exhibit aspects of both, leading to complex aberrations if disturbed. The synthesis of cellular types and factors which were absent in the embryo, may be triggered by and overproduced in the neonate after skin wounding. Such an overproduction may be responsible for the sensory hyperinnervation and this hypothesis is discussed in the next section.

1.9 NEUROTROPHINS AND THE SKIN

The development of innervation of the skin is a prime example of support for the neurotrophic hypothesis. This states that the innervation of a target is dependent on a neurotrophic factor(s) produced by that target in limited amounts, so that a certain proportion of neurons which do not gain access to it will die. Recently, the neurotrophic hypothesis has been modified to suggest that neurotrophins are not the sole mediators of final patterning and neuronal death, and that other developmental cues for nerve growth are involved (see Chapter 3). In addition, the neurotrophins themselves have a wide variety of roles other than determining cell survival.

The skin is a continually renewable organ for development, maintenance and repair of the primary barrier of animals (Ibanez, 1998). Early removal of the skin results in the absence of cutaneous nerves that normally supply the denuded region (Martin et al. 1989), suggesting that the skin must release some nerve growth promoting agents.

The neurotrophins nerve growth factor (NGF) (Levi-Montalcini & Angeletti, 1968), brain-derived neurotrophic factor (BDNF) (Barde et al. 1982), neurotrophin-3 (NT3) (Maisonpierre et al. 1990) and neurotrophin-4 (NT4-5) (Hallbook et al. 1991) belong to a family of growth factors which are responsible for the survival, differentiation and maintenance of developing neurons of sensory, sympathetic and motor modalities. In fetal development, they are expressed in both epithelium and mesenchyme of developing skin. In addition, within the skin, non-neuronal phenotypes e.g. the keratinocytes (Paus et al. 1994), melanocytes (Yaar et al. 1994) and mast cells (Tal & Liberman 1997) are NGF responsive. Glial-derived neurotrophic factor (GDNF) has been recently discovered, and is a member of the transforming growth factor β (TGFβ) family and like the neurotrophins is important for survival and development of specific populations of neurons (Henderson et al. 1994; Yan et al. 1995).

The neurotrophins have all in recent years been ascribed specific receptors (Barbacid, 1994), Trk A for NGF, Trk B for BDNF and NT-4/5 and Trk C for NT-3 (Bothwell et al. 1991), as well as the non-specific p75 receptors (Johnson et al. 1986), although p75 itself is thought to distinguish among the neurotrophins with respect to its actions (Frade et al. 1996). Additional studies have shown that during development these receptors are less specific and co-localisation as well as co-action is well described (McMahon et al. 1994). The trk receptors are all tyrosine kinase receptors and dimerise when bound, leading to receptor phosphorylation and receptor complex internalisation by endosomes. They are then retrogradely transported by fast axonal transport from axonal terminations to somata

(Hendry et al. 1974; Stoeckel et al. 1975) with resultant signalling through phosphatidylinositol 3 kinase, phospholipase C and MAP kinase cascades (Bothwell, 1998), (for a review see Klesse et al. 1999). Neurotrophins may regulate mRNA levels and protein synthesis and or phosphorylation of cytoskeletal proteins via these pathways.

Neurotrophin receptors are selectively expressed by different subpopulations of neurons. Nociceptive neurons possessing the trk A receptor are responsive to the NGF secreted by keratinocytes as well as other cells. Proprioceptive neurons with their trk C receptors are responsive to the NT-3 released by muscle cells, and low threshold mechanoreceptors are likely to respond to both NT-3 and BDNF in the dermis since they possess both trkB and trkC receptors (Davies et al. 1987).

GDNF signalling is mediated through a two component system, a GPI-linked α or β component (see Widenfalk et al. 1997) along with the tyrosine kinase receptor ret (Trupp et al. 1996) which is expressed in small and medium sized DRG neurons with exception of some small neurons (Honda et al. 1999).

1.9.1 NEUROTROPHIC FACTORS AND SURVIVAL DEPENDENCE OF SENSORY NEURONS

In NGF, NT-3, BDNF, trkA, tRkB, trkC and p75 null mutants there is a loss of sensory neurons well before target innervation (for a review see Snider, 1994 or Liebl et al. 1997). 70-80% of small diameter sensory neurons in the DRG are lost in NGF/trkA knockouts. The cells that are lost subserve pain (Crowley et al. 1994). NT-3/TrkC knockouts show a loss of 50-60% of DRG neurons of proprioceptive Ia afferents, and those innervating D hairs and Merkel cells (Ernfors et al. 1994; Airaksinen et al. 1996). BDNF/trkB knockouts show a 35% loss of neurons and exhibit postnatal deficits (see Bull et al. 1998; Farinas, et al. 1998). In contrast, NT-4 knockouts show no loss in DRG neurons (see Stucky et al. 1998).

During the prenatal period, neurons from the trigeminal ganglion are responsive to NGF secreted by the keratinocytes in the epidermal skin and whisker follicles of the whisker pad of rodents (Davies, 1990). NGF administered at the period of naturally occurring cell death can prevent the loss of sensory neurons in vivo (Henderson et al. 1994), and NGF expression correlates with the time these axons reach the vibrissae (Davies et al. 1987). However, the arrival itself does not initiate NGF synthesis which takes place even if innervation is prevented (Rohrer et al. 1988). Active competition for neurotrophins by innervating axons is thus responsible for determining survival and hence patterning. In rats

sensory neuron survival dependency for NGF ends around postnatal day 2 (Lewin et al. 1992).

NT-3 is a prime factor in the survival of sensory neuron precursors. Overexpressors of NT-3 display increased in neuronal survival (Albers et al. 1996). NT-3 mRNA is present in the DRG at E11 and E17 but not detected at P15, suggesting a local survival role early on with downregulation postnatally (Elshamy & Ernfors, 1996). Consistent with this is a downregulation of the trkC receptor after the period of neurogenesis (see Elshamy & Ernfors, 1996). Cutaneous afferents in the chick show survival responsiveness to NT-3 in vitro (Hory-Lee et al. 1993). NT-3 may signal via trkA to prevent apoptosis when NGF is limiting, when trkC is also deficient (see White et al. 1996).

BDNF is a survival factor for various neuronal populations of sensory, cerebellar, and spinal motor neurons (Barde, 1994; Lindholm et al. 1997). Early in development, NT-4/5 is also a survival factor for mouse cranial sensory axons as well as for neurons supported by BDNF (Davies et al. 1993).

Initially, GDNF was found to be a survival and developmental factor for motorneurons in culture (Henderson et al. 1995), but GDNF also promotes the survival of small and large 4a
cutaneous sensory neurons without any change in mRNA over the time of GDNF responsiveness which increases from E10-12 in chick cultures, ie it is not an increase in GDNF that determines survival but a change in neuronal responsiveness (Buj-Bello et al. 1995). It also supports cutaneous sensory neurons in culture (Buj-Bello et al. 1995), and two subpopulations of neonatal neurons are able to retrogradely transport GDNF (Matheson et al. 1997). GDNF deficient mice have deficits in DRG neurons (Moore et al. 1996). Neurturin, a relative of GDNF, supports survival of sympathetic neurons and DRG neurons in culture (Kotzbauer et al. 1996). Proteins not structurally related to GDNF e.g. CNTF, LIF, BFGF are also able to support neurons in cultures.

Neurons can change their preference for neurotrophins during development (Verdi & Anderson, 1994, for further examples see discussion). In the adult there is no overlap of cutaneous DRG neurons expressing trkA with those expressing trkC, but trkA and trkB expressing neurons do overlap. Earlier in development trkA and trkC are overlapping populations (Philips & Armanini, 1996).

Cutaneous afferents of the saphenous nerve are 50% trk A, 25% trkB and 16.6% trkC (McMahon et al. 1994). mRNA levels within DRG neurons are about 35-40%, 5%, and 15-20% for trkA, trkB, and trkC mRNAs, respectively (Kashiba et al. 1995). In addition, there exists a small population of small diameter sensory neurons which are unlabelled by trk

receptors (34% McMahon et al. 1994), the IB4 population described by Bennet et al, 1998. These cells do initially express trkA and are initially dependent on NGF, but then become GDNF responsive when there is a downregulation of trkA postnatally (Molliver et al. 1997).

In addition to the survival of neurons other cells and nerve endings in the skin are also dependent on neurotrophins for survival and development, for example the Merkel cells which depend on NT-3, but are also responsive to BDNF (Le Master et al. 1999) and D hair receptors dependent on NT-3 and which are also lost in NT-4 mutants (Stucky et al. 1998).

In addition to roles in cell survival which are most pronounced, neurotrophins can enhance the activation of the p75 receptor which promotes apoptosis, as well as a facilitating role in trk functioning (Rabizadeh et al. 1993; Carter & Lewin 1997). NGF causes expression of bcl-2 which prevents apoptotic cell death of melanocytes and keratinocytes (Misery, 1997).

Neurotrophins also have mitogenic actions on neuronal cells such as the action of NT-3 on proliferation of neural crest cells (Kalcheim et al. 1996), and sympathetic neuronal precursors (Verdi & Anderson, 1994), and on the non-neuronal phenotypes mentioned above. NGF is a mitogen for keratinocytes (Pincelli & Yaar, 1998) to an even greater degree than epidermal growth factor (Misery, 1997).

Although we have seen that neurotrophins can be produced by the neuronal target of innervation, for example the skin keratinocytes governing sensory innervation by production of NGF (Harper & Davies, 1990), NT-3 production by muscle cells for motorneuron innervation (Klein, 1994), it has also been established that neurones themselves can produce neurotrophins in an autocrine fashion (Acheson et al. 1995). The proposed reason for such an occurrence is to promote the survival of sensory neurons during embryonic development as they are making their long journey towards their target. Indeed anterograde transport of neurotrophins has been seen in sensory neurons (Zhou & Rush, 1996). It is also well known that cells associating with sensory neurons are able to produce neurotrophins, for example Schwann cells produce both NGF and BDNF (Watabe et al. 1995).

1.9.2 NEUROTROPHIC FACTORS AND NEURITE OUTGROWTH AND ARBORISATION IN SENSORY NEURONS

Neurotrophins are well-known for their ability to promote outgrowth from ganglia as well as for the extensive arborisation of axon terminations and dendrites especially within the target tissue (Diamond, 1987). This has lead to the suggestion that neurotrophins may be involved in, and may allow the regeneration of damaged axons. The growth of neurites in the presence of different neurotrophins is different at varying concentrations and for different populations of neurons. NGF via trkA signalling is important in the outgrowth and proliferation of sensory endings as is NT-3 (Rice et al. 1998). Lentz et al. 1999 have also shown that in BAX knockouts (such that DRG survival occurs without neurotrophin presence), the morphology of the growing neurons is determined by culture with particular neurotrophins. NGF for example results in a characteristic bipolar elongated morphology, whereas NT-3 produces end axonal branching, rather than elongation. In vitro cultures of adult mouse DRG with GDNF show increased numbers and lengths of axons (Leclere et al. 1998).

NT-4 can cause neurite outgrowth from ganglia (Ibanez et al. 1993). Very early on in the CNS, NT-3 can promote the differentiation of cultured neuroectodermal cells into neurons (Ghosh & Greenberg, 1995) suggesting a role in patterning, and even earlier than this NT-3 has been found to promote neurite outgrowth from chick neural plate, but with subsequent apoptosis (Li & Bernd, 1999). Thus, NT-3 could be selecting the neurons which grow.

1.9.3 NEUROTROPHIC FACTORS AND CONTROL OF SENSORY NEURON PHENOTYPE

The morphological maturation of chick DRG neurons is accelerated by exposure to NT-3 in vitro (Elshamy & Ernfors, 1996).

BDNF also plays a role in phenotypic alterations centrally and peripherally, both during development and after injury. Neural crest cells are stimulated to form sensory neuron specific markers by the action of BDNF (Sieber-Blum et al. 1993).

Antibody to NGF administered postnatally results in the conversion of Aδ-MR's to D hair afferents without the death of any neurons (Lewin et al. 1992). Thus NGF is required for the production of the normal population of Aδ mechanoreceptors during normal development.

NGF can influence nociceptor development. Nociceptors are converted into low threshold mechanoreceptors if NGF is supplied in excess when axons are innervating their target (Lewin & Mendell, 1994).

1.9.4 NEUROTROPHIN CONTROL OF NEUROTRANSMITTER SYNTHESIS

Neurotrophins can promote neurotransmitter synthesis in neurons. NGF has been shown to promote the expression of the choline acetyltransferase gene as well as the tyrosine hydroxylase gene and hence aid in the synthesis of acetylcholine and noradrenaline, resulting in effects presumably on cholinergic and sympathetic nerves (Bothwell, 1998). Peptides acting as neurotransmitters, such as substance P (SP) have also been shown to be stimulated by neurotrophins (Lindsay & Harmar, 1989). BDNF and NT-3 also both enhance transmitter release from neurons (Levine et al. 1995; see Besser & Wank, 1999). In neonatal neurons neurotransmitter release is of even greater importance considering the activity dependent plasticity of the environment. Neurotrophins increase levels of SP during the middle and late stages of avian DRG development (Yao et al. 1997) and promote or inhibit levels of other peptides in cultures of newborn sensory neurons (Mulderry, 1994).

BDNF itself can act as a neurotransmitter transported anterogradely to be released from nerve terminals (Zhou & Rush, 1996), and it's expression in DRG cells is controlled by access to NGF (Zhou, 1999).

1.10 PRODUCTION AND EXPRESSION OF NEUROTROPHINS AND THEIR RECEPTORS IN THE SKIN

1.10.1 Nerve growth factor (NGF), p75 and tyrosine kinase A (trkA)

In mice NGF mRNA has been shown to increase in cutaneous structures during embryonic stages 11 to 14, with an associated increase with respect to its receptors and level of innervation. Both mesenchyme (presumptive dermis), and epithelium (presumptive epidermis) express NGF mRNA, with a high level in the epithelium during development in both surface and follicular parts (see Davies et al. 1987). Normal levels of NGF increase within the skin from P0 to P20. At P3 approximately 30 pg/mg tissue of NGF is present (Constantinou et al. 1994).

The predominant production seems to occur in the epithelium (Davies et al. 1987), cutaneous periderm and epidermis having the highest levels of expression. Merkel cells and basal keratinocytes also produce NGF and possess the low affinity p75 receptor as do

all cutaneous nerves, as well as Meissner's and Pacinian corpuscles of human digital skin (see Hashino et al. 1999) and debate still governs whether the trkA receptor is also expressed by them (see Pincelli & Yaar, 1998). Trk A is confined to the basal keratinocytes in normal skin, with very little detectable in normal human nerves within the dermis, which raises the intriguing question of whether trk A does in fact govern the responses of cutaneous nociceptive sensory neurons (Bull et al. 1998). P75 is also expressed in skin mesenchyme (Chao, 1994). Mice with mutations to this receptor, with the production of an inactive protein are found to have skin ulcers and hair loss (Lee et al. 1994). In the dermis p75 positive neurons are numerous, but few are seen in the epidermis, with the majority of p75 being expressed by Schwann cells at their connective tissue facing membrane surface, suggesting their ability to respond to neurotrophins secreted by cellular components of the skin (Misery, 1997). Melanocytes however, express trk A mRNA and protein as well as the p75 mRNA and protein (Peacocke et al. 1988). Primary chick fibroblasts also have the capacity to secrete NGF in culture (Young et al. 1975) and express high levels of trk A (Klein et al. 1991).

1.10.2 Neurotrophin-3 (NT-3) and tyrosine kinase C (trk C)

Early on NT-3 has been found in the epidermis of branchial arches of avian embryos (Hallbook et al. 1993). The embryonic rodent dermis contains NT-3 (Schecterson & Bothwell, 1992) and NT-3 and BDNF mRNAs are equally distributed in the skin, detected in developing skin as early as E9.5. NT-3 transcripts in the skin are maximal at E13 and decline to original levels by E16, although NT-3 mRNA is three-fold higher originally to that of BDNF (Schecterson & Bothwell, 1992). At E14 mRNA for NT-3 is present in the presumptive dermis and surface epithelium (see Ibanez et al. 1993). Later it is localised to the keratinocytes of the suprabasal epidermis where many unmyelinated C fibres terminate (Kennedy et al. 1998). NT-3 is also present in developing skeletal muscle and later in muscle spindles (Escandon et al. 1994). It has ceased to be synthesised by sensory neurons themselves by birth (Schecterson & Bothwell, 1992), but immunoreactivity is present in the adult localised mainly, to large diameter neurons in the ganglia approximately 30% (Zhou & Rush, 1995) indicating its retrograde transport from the periphery. This study also demonstrated that 17% of total sensory innervation is by NT-3 projections to the subdermis and epidermis, and that only 8.2% of neurons projecting to the footpad are NT-3 IR. Zhou & Rush, 1993 showed that NT-3 mRNA levels are highest at one week after birth and

lowest in the adult. NT-3 seems to be more important for the SA I type afferents that subserve tactile sensation, than the D-hair afferents (Airaksinen et al. 1996).

Trk C receptors in the periphery are upregulated during development from the embryo to birth in the whisker pad of mice (Wyatt et al. 1999). Early expression of trkC is in the full-length form in the brain at least (Escandon et al. 1994). In adult rats 15% of sensory neurons retrogradely labelled from the skin express trkC (McMahon et al. 1994). Null mutants for NT-3, demonstrate that it regulates the expression of its receptor trkC in trigeminal sensory neurons at least, but not in the cutaneous peripheral tissue. Recently, trkC immunoreactivity has been localised to the suprabasal layer of the epidermis (see Terenghi et al. 1997), and trkC mRNA has been found at low levels in cultured melanocytes (Yaar et al. 1994). Truncated trk C receptors are expressed postnatally at higher levels than full-length receptor and may downregulate the full-length receptors by dimerising to them (Escandon et al. 1994). The presence of truncated receptors may on the other hand be important for the sequestration of neurotrophins (Biffo et al. 1995).

1.10.3 Brain derived neurotrophic factor (BDNF) and tyrosine kinase B (trk B)

BDNF expression begins at E11 in the developing skin and increases up to E12, decreasing by E16 (Buchman & Davies, 1993). It is normally expressed in the upper dermis (Rice et al. 1998), but not the epidermis (Ernfors et al. 1993). BDNF mRNA is found in the epidermis derived follicle proper with none in the mesenchyme surrounding the follicle (Botchkarev et al. 1999).

BDNF is synthesised by a subpopulation of DRG sensory neurons. 9.8% of sensory neurons projecting to the epidermis contain BDNF immunoreactivity, quite similar in fact to that of NT-3 (see above), with a small percentage to hair follicles (Zhou, 1999). Little information is known about the types of cutaneous sensory endings BDNF supports.

Full length trkB receptors are expressed in many nervous tissues before the expression of truncated receptors, and are downregulated at the time of programmed cell death (Escandon et al. 1994).

1.10.4 Neurotrophin-4 (NT-4)

NT-4 mRNA is expressed in the whisker pad skin the target of trigeminal ganglion neurons at E13 decreasing towards E20 along with the occurrence of programmed cell death. It is normally localised to the upper dermis and epidermis (Ernfors et al. 1992). NT-

4 mRNA in skin is expressed at E13 and declines during development (Timmusk et al. 1993). NT-4 neurons are trkA and B positive (Ibanez et al. 1993).

1.10.5 Glial cell-line derived neurotrophic factor (GDNF), the ret tyrosine kinase receptor, and GFRα and β receptors

GDNF is a member of the TGFβ family, and is neurotrophic to many neuronal populations. Early on in development, it is expressed by the condensing mesenchyme (Trupp et al. 1995). In the trigeminal ganglion at E11 when axons are just reaching the target and GDNF and neurturin (NTN), (a related member of the TGFβ family) have mRNA in the epidermis in discrete sectors. At E13 as most axons have reached the target, the levels of NTN are downregulated and restricted within the epidermal derived whisker follicles. By E16 downregulation has proceeded further, and at E18 GDNF is present in the outer root sheath of the hair follicle where NTN levels have diminished. Postnatally GDNF is found in the inner conical body of the hair follicle complex, mainly with the unmyelinated and Aβ myelinated mechanoreceptors (Fundin et al. 1999).

The interaction between GDNF, its receptors, arriving axons and terminal Schwann cells is important in the first postnatal week for the patterning of nerve endings. NGF which is important for the development of the myelinated mechanoreceptive nerve endings i.e. the reticular and transverse lanceolate endings, require GDNF during ending formation and maintenance postnatally (Fundin et al. 1999). Most primary sensory neurons express the ret tyrosine kinase receptor for GDNF during development, and terminal Schwann cells have apical fine finger like processes which express GFRα1. Schwann cells also have the ability to synthesise GDNF (Henderson et al. 1994) and are thought to play a role in sequestering neurotrophins. For a more detailed look at Schwann cell expression of growth factors and responsiveness (see Watabe et al. 1995).

Distinct populations of DRG neurons express GDNFα and β (Widenfalk et al. 1997). Peripherally, both mRNA forms are highly expressed in developing whisker pad skin, and in general GDNF mRNA has a higher level in peripheral organs than in neuronal tissues (Trupp et al. 1995).

1.11 NEUROTROPHINS, INFLAMMATION AND THE IMMUNE SYSTEM

The association between neurotrophins and the skin is also strengthened by their co-involvement in inflammation and interaction with the immune system. Increasing evidence is bringing the neuronal and immune systems together.

Table 2 (see Otten & Gadient, 1995) illustrates the variety of interactions between NGF and immune cells.

Mast cells	Proliferation, degranulation, expression of functional NGF receptors
Basophils	Regulation of inflammatory mediator release, expression of functional trk receptors
Monocytes/ macrophages	Promotion of monocyte differentiation, increase in oxidative burst, expression of functional trk receptors
T-lymphocytes	Proliferation, expression of functional trk receptors, T-cell dependent antibody synthesis
B-lymphocytes	Proliferation, differentiation into plasma cells, expression of functional NGF receptors
Other immune cells and functions	Differentiation of granulocytes, chemotaxis for neutrophils, shape change of platelets, increase vascular permeability

Macrophages are known to increase neuronal survival and neonatal DRG neurite extension (Hikawa et al. 1993), and brain macrophages are known to secrete NGF *in vitro* (Mallat et al. 1989). Macrophages also secrete interleukin-1 (IL-1) which acts on Schwann cells to promote NGF secretion and, IL-1 is a potent regulator of NGF mRNA expression in fibroblasts (see Matsuoka et al. 1991).

Macrophages express neurotrophins as well as other growth factors such as TGFβ, PDGF, EGF, insulin-like growth factors and βFGF (Zhou & Rush, 1994). They secrete over 100 factors promoting nerve regeneration (Hikawa et al. 1993). BDNF is expressed at low levels in macrophages, but is pro-proliferative to microglia (Zhou & Rush, 1994), and GDNF is present in increasing amounts in macrophages at CNS wounded sites (Batchelor et al. 1999). NT-3 immunolike reactivity is present in brain macrophages at P10 in 80% of those examined, but not in the adult, suggesting differing roles in during development

(Zhou & Rush, 1994). The morphology of the macrophage/microglia is also different within these two ages. This may be useful to assess in our phenomenon to see whether there is a change in morphology due to tissue state, since NT-3 treated microglia display a flattened large surface with extended processes (Zhou & Rush, 1994). NT-3 itself is a mitogen for macrophages, increasing microglial survival and inducing phagocytic activity. Hence, microglia both express and respond to NT-3.

Lymphocytes and monocytes express both receptors for NGF, p75 as well as trkA (see Carlson et al. 1998). In addition, NGF is produced by lymphocytes (Ehrhard et al. 1993). Trk A is also present on activated T lymphocytes and basophils which are recruited to inflammatory sites. Human immune cells produce mRNA for NT-3, secrete BDNF, and express trkB and C, with the latter being clonally restricted and only in activated populations (see Besser & Wank, 1999). BDNF is produced by these cells *in vitro* and during inflammatory brain lesions, and is thought to have a neuroprotective role (Kerschensteiner et al. 1999).

Cytokines IL-2 and IL-4 natural players in an immune reaction, also interact with neurotrophins and their receptors, such that IL-2 will activate trkB mRNA synthesis, and IL-4 will increase NT-3 mRNA specifically. Therefore, it is most likely that BDNF and NT-3 will have a role in repair after injury.

1.10.7 THE ROLE OF NGF IN CONTROLLING SKIN INNERVATION DENSITY

The extensive roles of neurotrophins and related molecules indicate that they are good candidates to investigate when looking for the possible initiators of hyperinnervation. Of this family, NGF is the first one to consider.

NGF levels in the skin continue to increase to P18 in normal tissue (Constantinou et al. 1994). It is of prime importance in neuronal survival during development as well as being a target derived factor and a chemotactic agent and has been well studied. In the trigeminal system for example the density of sympathetic innervation correlates with NGF mRNA levels (Shelton et al. 1984), suggesting this may be a limiting factor. NGF and trkA are important in the development of most sets of Aβ and Aαβ afferents (Albers et al. 1994).

NGF promotes outgrowth from both neonatal and adult DRG cultures, but NGF induces branching in cultures of neonatal sensory neurons, compared to the elongation seen in adult cultures (Lentz et al. 1999).

NGF levels in the DRG fluctuate after axonal injury and ligation. Initially a decrease in seen, followed by an increase in mRNA expression within the DRG cells (Shen et al.

1999). Deprivation of NGF during cut regeneration in the adult rat does not affect the time course of regeneration even though NGF mediates the sprouting (Diamond et al. 1992b). In the iris sympathetic and sensory fibres are increased with the accompanying increase in NGF levels after denervation (Ebendal et al. 1980). NGF can also prevent the behavioural and biochemical effects of toxic neuropathy in mice (Apfel et al. 1991).

Collateral sprouting in the absence of any denervation can be evoked by excess NGF (Bennett et al. 1996). That of mature intact cutaneous nociceptive Aδ and C afferents after denervation also occurs via an increase in NGF and its mRNA in this state (Mearow, 1998). Systemic antibody to NGF blocks the collateral sprouting but, the regeneration of injured afferents is not influenced by NGF (Diamond et al. 1992a).

It is also well-known to be involved in inflammatory pain, increasing in inflammatory tissue (Weskamp & Otten, 1987) and it's hyperalgesic effect and the sensitization of nociceptive afferents can be blocked by specific antibodies to it (Koltzenberg et al. 1999). Local administration of NGF to neonatal and mature animals results in hyperalgesia in the adult, and hyperalgesia with sensitization of Aδ fibres to mechanical stimuli in the neonate (Lewin et al. 1993). Systemic administration of anti-NGF does not alter sensory thresholds, suggesting that NGF levels do not play a role in maintaining normal sensory terminal sensitivity (see Lewin et al. 1993).

NGF ELISA studies on neonatal wounded skin have shown that there is an increase in NGF content in the skin peaking at 3 days after wounding, although NGF level are increasing naturally during this developmental period (Constantinou et al. 1994). Hyperinnervation after wounding is best documented between P7 and P14. Therefore, at later time points, either there is a saturating amount of NGF or it is unlikely NGF is to be of importance. Levels do not go much higher than 140pg/mg of tissue in both normal and wounded animals. Since levels are already increasing during this period, any prior increase in NGF after wounding may not lead to an overall change. Thus, NGF levels might be more important in the adult after wounding, since the developmental increase has ended.

High molecular weight NGF in saliva has also been shown to accelerate wound healing, as part of the licking response to wounds (Li et al. 1980). In our study, the animals were very young so this seems unlikely, but one cannot dismiss maternal care of the wounds. NGF is also a prominent feature of the fibroblasts within granulation tissue (Li et al. 1980), and both p75mRNA and receptor protein are increased in human keratinocytes during their

exponential growth phase suggesting an increase after the exuberant proliferation of these cells during wound healing.

NGF overexpressing mice have significantly increased unmyelinated and myelinated fibres in the skin as well as functional terminals of high threshold mechanoreceptors (Albers et al. 1994; Stucky et al. 1999). Large nerve fibres and bundles are seen in the reticular dermis and hyperinnervation is present in regions of the epidermis, dermis and around hair follicles (Albers et al. 1994; Rice et al. 1998).

NGF and TrkA knockouts show a loss of unmyelinated and Aδ endings in the epidermis and upper dermis and hair follicles (Rice et al. 1998), although developmentally sensory neurons lack receptors to NGF when growing to their targets (Davies et al. 1987), suggesting that NGF is not required for their growth. Another organ demonstrating sensory hyperinnervation during wound healing, the cornea, shows decreased nerve trunks and branches in trkA homozygous knockout mice (de Castro et al. 1998).

NGF overexpressed in the skin also results in sympathetic hyperinnervation of the spleen postnatally, described as robust by P14 and present into adulthood (Carlson et al. 1998). Sympathetic fibres like cutaneous afferents innervate the rat spleen in the first two postnatal weeks. Innervation of lymphoid tissues is important for immune function, and the changes governed by increasing NGF and hyperinnervation in the spleen may be a route for the increased wound healing in our phenomenon of hyperinnervation if NGF was the major contributor. NGF cDNA is also increased in the sympathetic hyperinnervation of islet cells of the pancreas (Edwards et al. 1989).

Administration of systemic antibody to NGF in the fetus causes a lack of epidermal innervation postnatally with a predominantly dermal innervation (see Jackman Phd thesis). This would suggest that the lack of NGF was responsible for the lack of ingrowth into the epidermis of these animals.

In splenic hyperinnervation, p75 is increased postnatally in normal development (Carlson et al. 1998). P75 can modulate trkA binding so that NGF is more effective, but only at low ligand levels or when trkA is down-regulated (Ryden et al. 1997). When knocked out this low affinity receptor for the neurotrophins p75 shows an increase in noxious threshold, but a decrease in epidermal innervation density and CGRP immunoreactivity in 3 month old animals (see Bergmann et al. 1997; Lee et al. 1992) with fibres entering the epidermis exhibiting little arborisation, especially unmyelinated sensory fibres, although p75 is not essential for sensory innervation to occur (Rice et al. 1998). There is also a loss of sympathetic innervation to hair follicle necks although innervation to the epidermal, and

dermal portions of hair follicles is increased from birth to four weeks with loss in the adolescent/adult animal, suggesting a maintenance role for p75 (Rice et al. 1998). Knockout animals also display a decreased skin thickness and few small dermal fibres with large sparse sweat gland innervation on the glabrous side. This would suggest that p75 may play a part in hyperinnervation, although all nerve terminals are affected as well as some anti-nociceptive end organs e.g. the Meissners corpuscles (see Bergmann et al. 1997).

In view of all the above evidence, it might be argued that NGF is the most likely neurotrophic factor to be responsible for wound-induced hyperinnervation. However, there is also evidence to suggest otherwise.

In vitro co-culture studies have shown NGF plays little part in mediating the increase in outgrowth from neonatal DRGs co-cultured with wounded skin (Reynolds et al. 1997). In addition, *in vivo* systemic antibody to NGF in these animals does not alter the hyperinnervation, even though serum values of antibody are able to block 5ng/ml of NGF (unpublished data Reynolds et al.).

The majority of NGF overexpressors show increases in CGRP fibres rather than heavily myelinated fibres, and it may be that NGF is more important in development rather than formation (Rice et al. 1998). In addition an increase in NGF is known to give rise to an increase in CGRP (Lindsay & Harmar, 1989), and nerves immunoreactive to CGRP are not greatly increased in hyperinnervation *in vivo* (see Reynolds & Fitzgerald, 1995), nor do we see a large change in CGRP fibres in co-cultured ganglia *in vitro* (see Chapter 3), although large diameter dermal fibres are increased in NGF overexpressors (Albers et al. 1994).

Indeed, NGF's chemoattractant action is only after sensory axons have reached their target, and early sensory neurons are therefore attracted by something other than NGF (Davies & Lumsden, 1984). Regional differences in innervation density are not governed by NGF synthesis but maintained by it (Harper & Davies, 1990). Along with the increase in NGF in the skin accompanying collateral sprouting are increases in mRNA expression of GAP-43, p75 and trkA (Mearow, 1998), such that it may be useful to look at these in our phenomenon to determine a role for NGF.

In view of this *in vitro* and *in vivo* evidence, NGF's role in hyperinnervation does not appear to be critical. It may interact with other neurotrophins, or maintain the response but other factor(s) are likely to be important. As such, in this study, the role of the other sensory neuron related neurotrophins, BDNF, GDNF and NT-3 were examined.

CHAPTER 2

MORPHOLOGY AND DEVELOPMENT OF HYPERINNERVATION

INTRODUCTION

2.1 SENSORY HYPERINNERVATION

That sensory innervation responds to skin injury has been known for some time. Julius in 1926 (see Fitzgerald, 1966) showed that painting coal tar on hairy skin of mice caused an increase in cellular activity of subcutaneous sensory proliferative endings especially around hair follicles with no penetration of the overlying epithelium.

Since these initial findings, major damage to the skin of mice causing marked hyperplasia of the epidermis has been shown to produce proliferative changes in innervation with penetration to the overlying epithelium (Fitzgerald et al. 1975). Superficial skin wounds in adult rats also cause a transient hyperinnervation (Aldskogius et al. 1987). Other hyperinnervation phenomena have also been reported e.g. that of sympathetic fibres after NGF overexpression (Davis et al. 1994; Albers et al. 1994) (this is discussed in more detail in chapter 4), and the abundance of sensory fibres after corneal injury, initially from axonal collaterals which subsequently degenerate and are replaced by regenerating neurites from transected stumps (Buerman & Rozsa, 1984). Indeed the cornea of animals who undergo laser ablation remains abnormally sensitive to touch for up to 10 weeks after the wound (Ishikawa et al. 1992).

In 1995 Reynolds & Fitzgerald documented a phenomenon of 'sensory hyperinnervation', which occurred in the skin of postnatal rat pups that had been subjected to full thickness skin wounds at birth. The hyperinnervation encompassed an area in and around the previously wounded site, and consisted of both large diameter myelinated RT97 labelled fibres as well as unmyelinated small diameter CGRP positive fibres. Very little sympathetic contribution was noted, and the effects of capsaicin only partially decreased the extent of innervation. The fibres seemed to arise from deep within the skin and extend into both the dermis and epidermis, and were still apparent 12 weeks after the initial wound had been made. The structural changes were most prominent in animals wounded at birth and diminished if older animals were wounded. Only weak, transient hyperinnervation that disappears after the wound is healed is observed in adult animals (Reynolds & Fitzgerald, 1995; Aldskogius, 1987).

Accompanying the structural change in skin innervation is a behavioural sensitivity and a functional decrease in thresholds for flexion withdrawal of the hindlimb to mechanical

sensory stimuli. This hypersensitivity could be observed even up to a period 3 weeks after wounding (Reynolds & Fitzgerald, 1995; de Lima et al. 1999).

Thus skin trauma within a neonatal 'plastic' developmental period results in a long-term sensory structural and functional change, which will influence pain processing and behaviour in the grown animal. Such a phenomenon will have implications for man particularly following infant surgery. The long-term hypersensitivity is of interest to anyone seeking to understand developmental changes and the maturation and formation of an adult sensory nervous system.

Before investigating mechanisms underlying the neonatal skin hyperinnervation response to skin wounding, it is important to fully describe the pattern and time course of the phenomenon.

- In this Chapter, we confirm the hyperinnervation response.
- We also describe its onset and timing in detail using immunohistochemistry.

2.2 METHODS

2.2.1 Dorsal foot wounds

Under halothane anaesthesia with oxygen newborn Sprague-Dawley rat pups were anaesthetised. Bilateral dorsal foot wounds of full thickness were made to hindpaws by pinching skin with forceps and cutting and removing a 2mm by 2mm flap of skin. Pups were allowed to recover and returned to their mother. Control pups were anaesthetised only.

24hrs later control pups and wounded pups were sacrificed and perfusion fixed with saline, 4% paraformaldehyde (in distilled water pH 7.4 with 40% sodium hydroxide), followed by 30% sucrose. The hindpaws were removed and stored in 4% paraformaldehyde overnight and then transferred to 30% sucrose with 0.3% azide until required.

2.2.2 Sectioning for immunostaining

Lateral foot sections of 100μm were cut with a freezing microtome. Hindpaws were trimmed to size, edges of wounds marked with pen, and mounted in tissue tek (SIGMA), prior to sectioning. Sections were collected in 1xPBS (7.45g sodium hydrogen orthophosphate anhydrous, 2.15g potassium hydrogen phosphate and 45g sodium chloride made up to 1 litre with distilled water and pH restored to 7 with 40% sodium hydroxide: working solution diluted 1:5 pH 7.4), and then transferred to 0.6% H_2O_2 for endogenous peroxidase quenching for 1 hour. Sections were then ready for immunostaining.

2.2.3 Immunostaining with PGP 9.5

Sections were washed with dilute PBS for 30 minutes, followed by blocking with 10% normal goat serum (VECTOR) in dilute PBS for 30 minutes, with overnight incubation in PGP 9.5 1:1000 (Ultraclone) with 0.1% NGS in PBS and 2% triton at room temperature.

The following day sections were washed three times in PBS with triton for 2 hours followed by incubation in anti-rabbit biotinylated IgG (VECTOR) 1:250 in PBS for 1-2 hours at room temperature. Three further washes with PBS and triton were then conducted over a 2 hour period, after which avidin biotin complex (1:1) was added (ABC kit VECTOR made at least 20 minutes before use) for 1 hour. A 30 minute wash in dilute PBS followed after which a 30 minute TRIS (30.3g Tris in 500mls distilled water pH to 7.6 with concentrated HCl) wash was conducted, and staining visualised using

diaminobenzidine (DAB, SIGMA), 10mg in 20 mls of TRIS, filtered with 7µl H_2O_2 added before use.

Once staining was apparent the reaction was stopped with distilled water, sections washed for 5-10 minutes in distilled water and then mounted onto gelatinised slides. Slides were left to air dry overnight, after which they were dehydrated in a series of alcohols (70%, 95%,100%, 100%), cleared with histoclear twice (BDH) and coverslipped in DPX (BDH).

2.2.4 Counterstaining

Where counterstaining was undertaken to visualise structural components of the skin in relation to nerve fibres, slides with mounted and dried sections were dipped in toluidine blue (170mg toluidine blue, 170mg borax in 100mls distilled H_20) for 10 seconds, followed by immersion in 70% through to 100% alcohols and thence cleared and coverslipped as above.

2.3 RESULTS

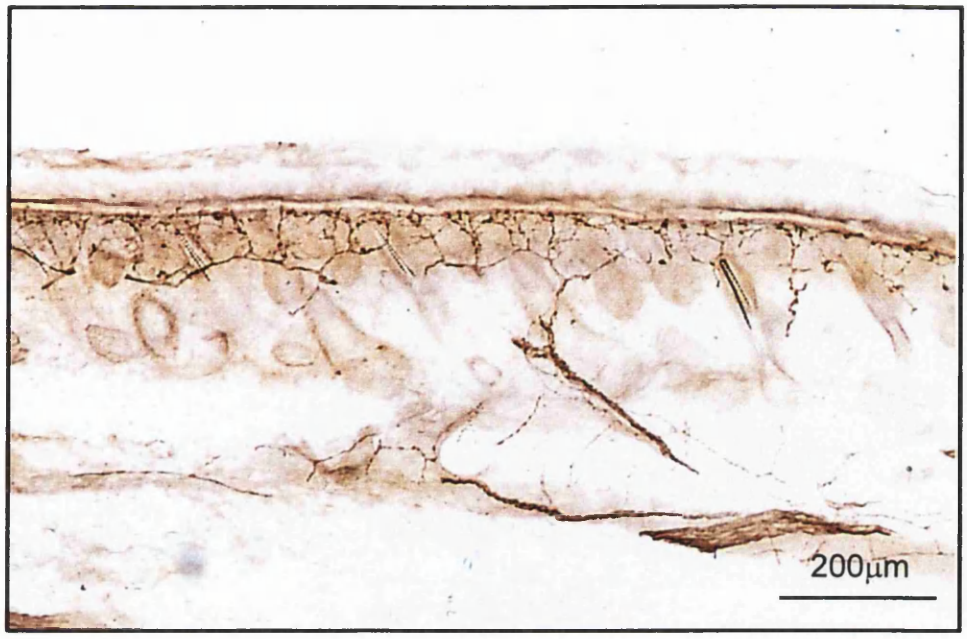
2.3.1 Development of hyperinnervation

In the naive one day old rat pup, the innervation of the dorsal hindlimb hairy skin can be clearly observed. Thick deep dermal bundles of nerves can be seen giving rise to finer fibres which in turn branch and innervate the developing hair follicles (see figure 4). At this stage no fibres have penetrated into the epidermis. By 3 days little change is observed, other than maturation of hair follicles. At 5 days, shafts of hair follicles seem to be encompassed by nerve fibres with regular branches from the dermal plexus. Normal 7 day old skin shows the development of fibres coiling around hair follicle bases and connecting to the dermal plexus, as well as branching into the epidermis.

After neonatal skin wounding, this normal development of innervation is disrupted. Figures 5,7,9 and 12 illustrate the developmental time course of hyperinnervation. The inflammatory response is evident 24 hours after wounding, with an enlarged wounded area distorting the uniform layer of the epidermis, such that in some animals it is hardly visible. The layered structure of the dermis is also distorted such that only a homogenous mass of inflammatory substances is visible. Scab formation and disrupted structure are still apparent at 3 days, but now the inflammatory and necrotic area is being pushed outwards with concomitant wound contracture (see figure 7).

The wound area itself is notable for its absence of hair follicles. At P3 only a few fibres are seen, these are mainly around remnants of hair follicles and deep within the dermis. Fine axons are also visible as seen in figure 7. Greater numbers of fibres appear at 5 days and dominate in and around the wound, with deeper fibres drawn up towards the wounded surface. The epidermis seems almost fully restored and smooth in appearance. Clear hyperinnervation is evident at P7 in and around the wounded area. The fibres are again drawn up towards the wound, with a lack of organisation resulting in a tangle of fibres within the dermis. Thick coarse fibres can be seen deep within the dermis, leading to finer branched fibres superficially and penetrating the epidermis in much greater numbers than that are seen in the naive animal (see figures 11-14). The keratinised scab has already been shed at P5 or is near shedding and an almost normal epidermal layer is apparent underneath it.

Counterstaining shows the relation of nerve fibres to cellular components of the epidermis and dermis, with numerous nerves in relation to both blood vessels and other cells. Some incoming fibres to the wounded area are from collaterals from adjacent hair



Figue 4: 1 day old uwounded skin showing numerous small nerve fibres encasing hair follicles and penetrating the epidermis. The deep dermal plexus and a branch to the superficial dermis is visible.

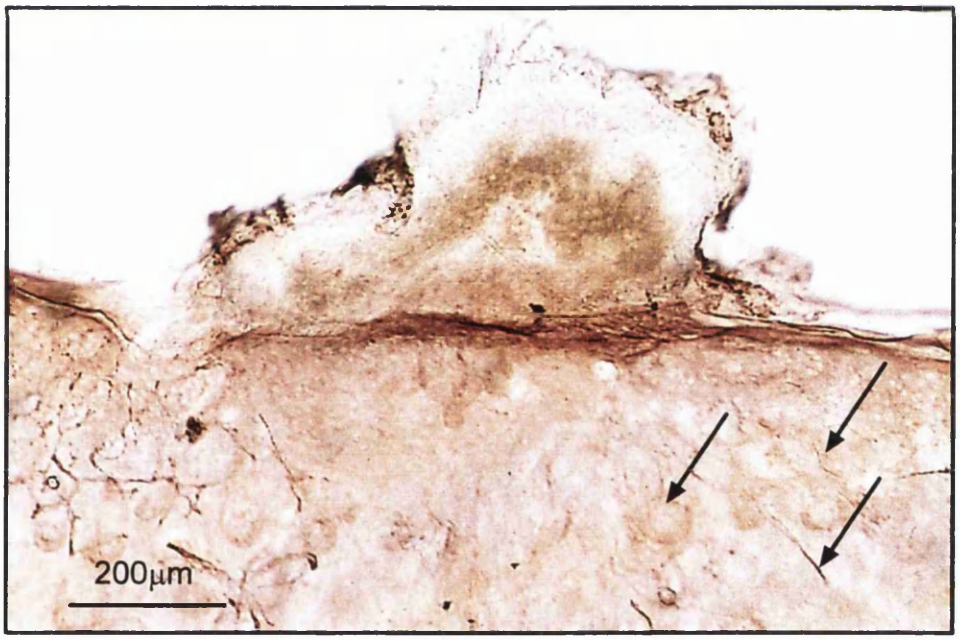


Figure 5: 1 day old skin wounded at birth showing homogenous demal area under wound, and keratin scab formation. Wound contracture is evident with a scattering of small nerve fibres and few hair follicle remnants (arrows).

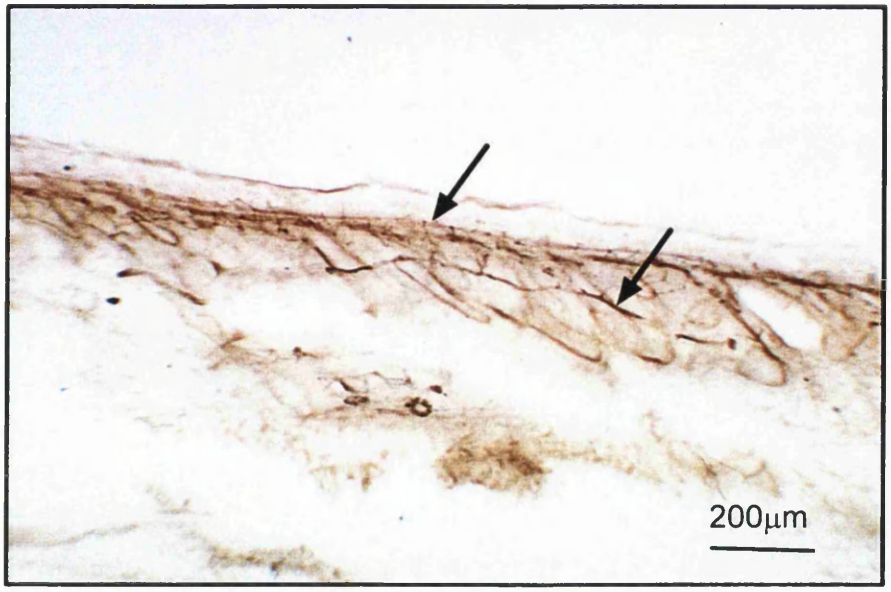


Figure 6: Unwounded skin 3 days old: A smooth epidermal surface, with intact hair follicles and nerve fibres encapsulating hair shafts and branching into the overlying epidermis.

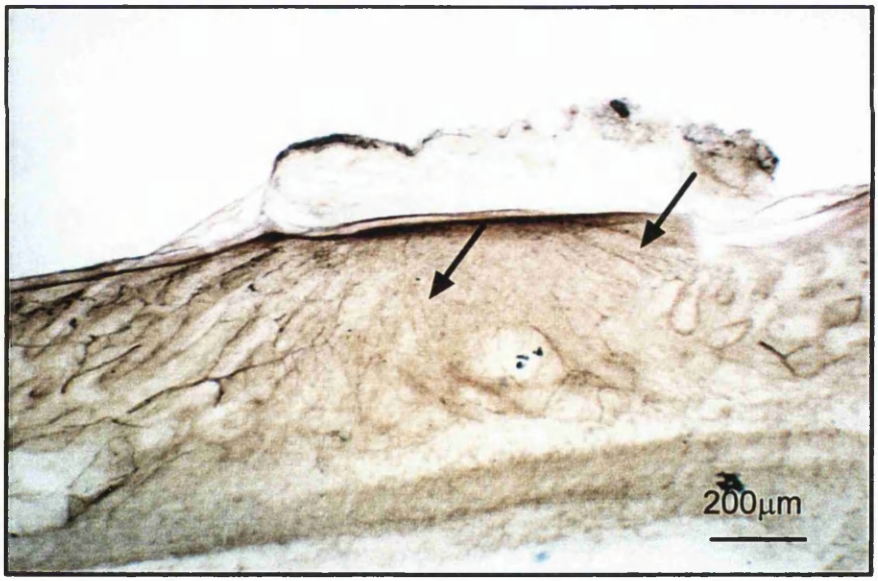


Figure 7: Wounded skin 3 days old showing wound contracture and keratinised scab overlying collagenous infiltrate. Very fine nerve fibres can be see diagonally stretching to reach the scab from axon collaterals and deeper fibers (arrows).

Figure 8: 5 day old unwounded skin stained with PGP9.5 and counterstained with toluidine blue labelled fibres, counterstained with toludine blue.
Fibres encompass hair follicles and their shafts.

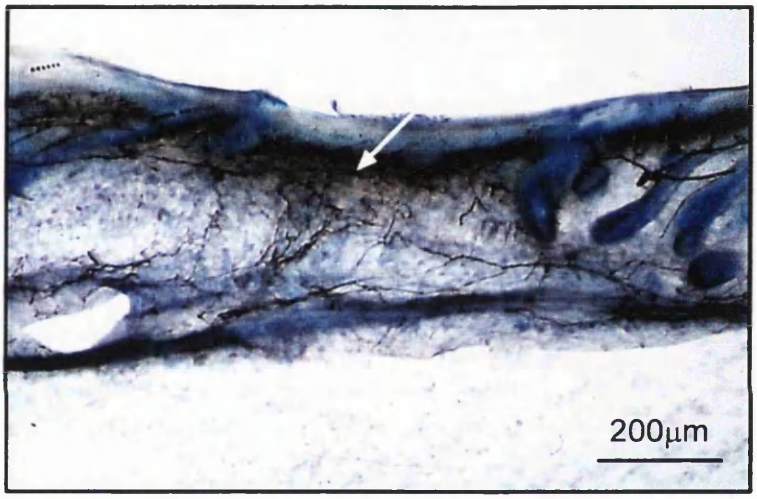


Figure 9: 5 day old wounded skin. PGP9.5 labelled fibres can be seen clearly innervating the wounded area which is devoid of hair follicles.

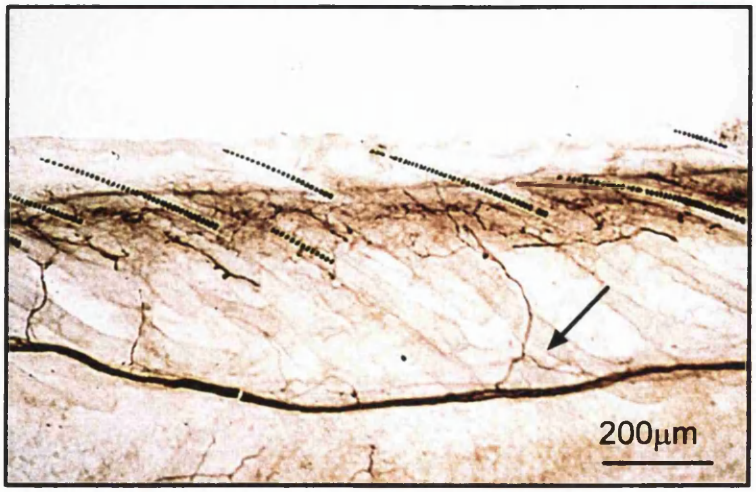


Figure 10: P5 naive skin stained with PGP9.5, no counterstain. A deep dermal nerve bundle is clearly visible projecting fine axons to hair follicles and the superficial dermis.

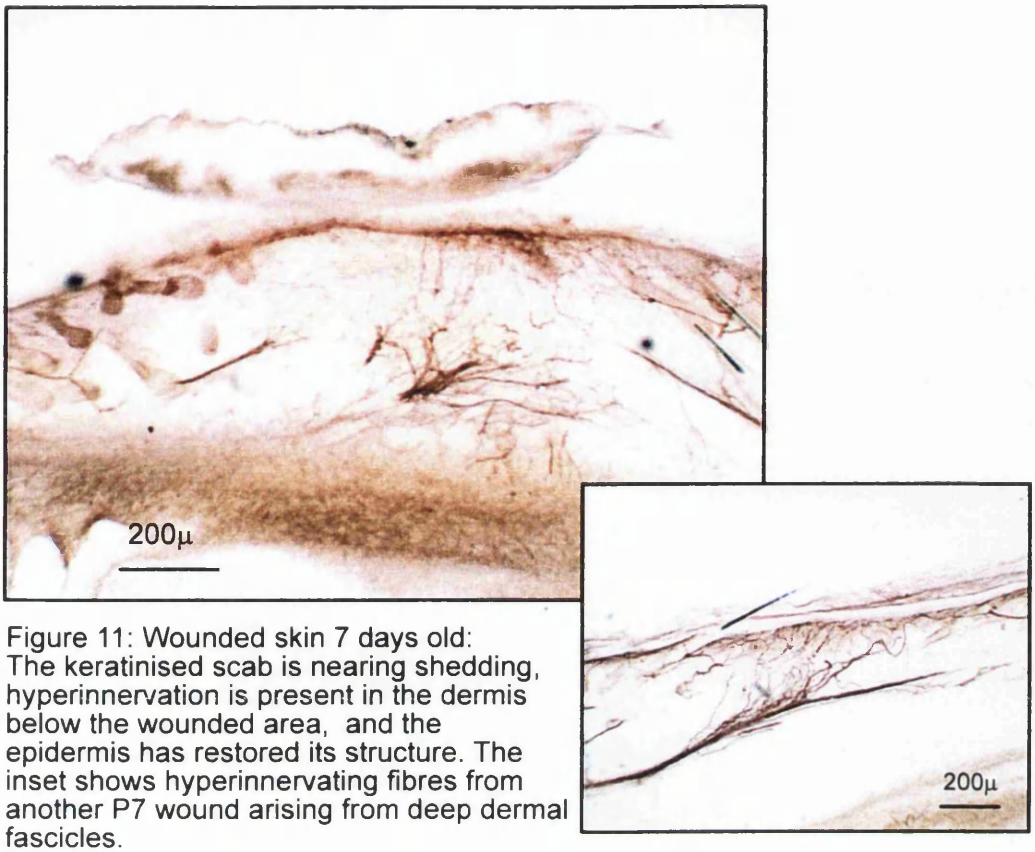


Figure 11: Wounded skin 7 days old: The keratinised scab is nearing shedding, hyperinnervation is present in the dermis below the wounded area, and the epidermis has restored its structure. The inset shows hyperinnervating fibres from another P7 wound arising from deep dermal fascicles.

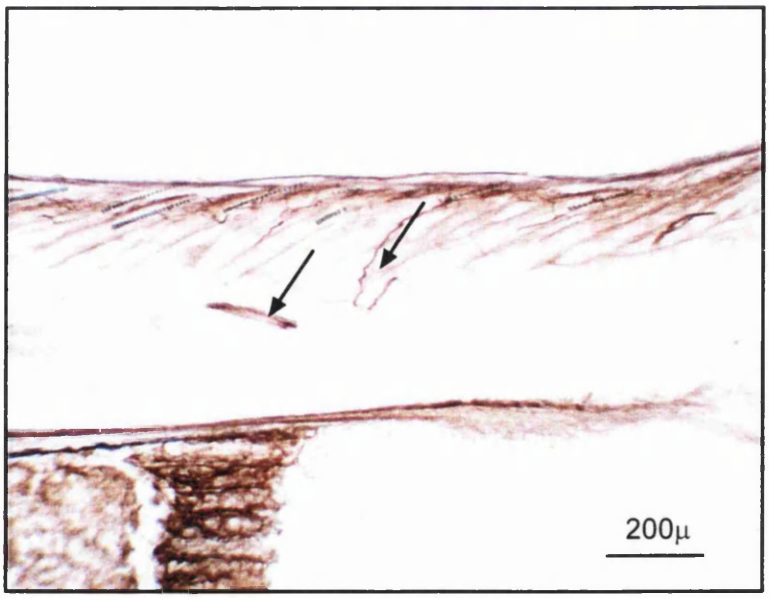


Figure 12: Unwounded skin 7 days old. Deep dermal nerve bundles give rise to thick fibres innervating hair follicles.

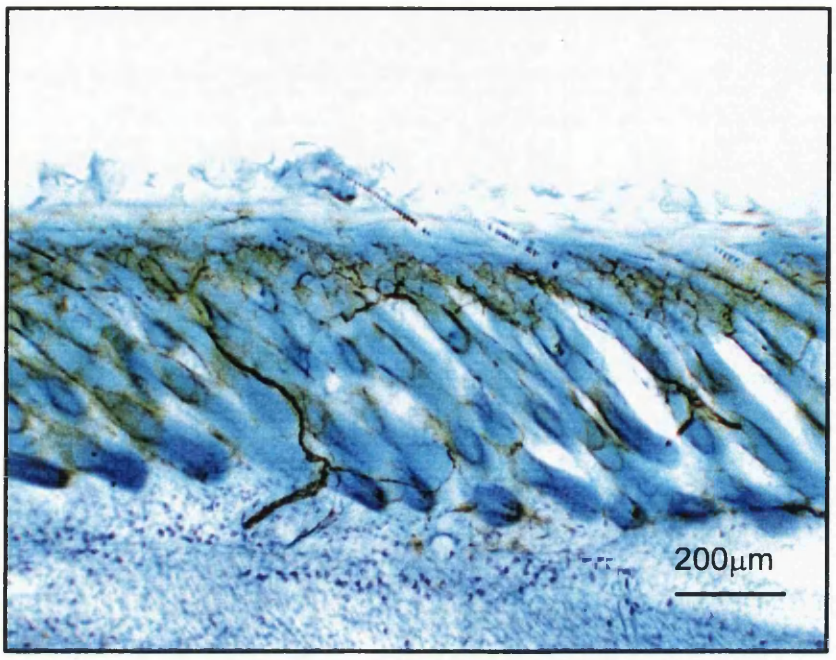


Figure 13: Naive skin 7 days old stained with PGP 9.5 and counterstained with toludine blue.

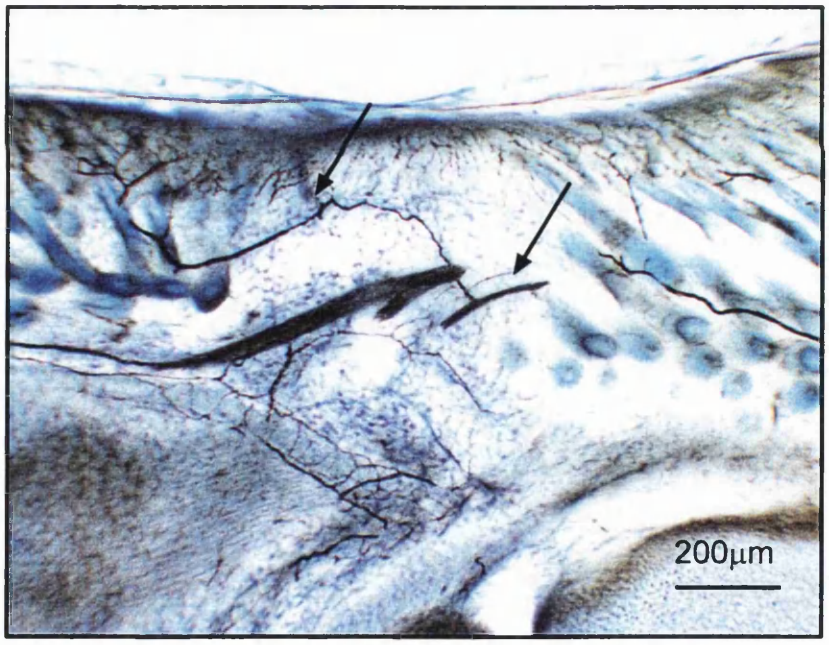


Figure 14: 7 day old wounded skin section stained with PGP 9.5 and counterstained with toludine blue, showing axon innervating wounded area and collateral innervating area adjacent to wound.

follicle fibres (see figure 14), but the majority are from deep nerve bundles. In addition in some sections nerves within the dermal plane, seem to follow the course of blood vessels and granular cells which are probably mast cells.

2.4 DISCUSSION

These results demonstrate that hyperinnervation is a robust phenomenon and consistently follows skin wounding at P0. It manifests over a time frame of 5-7 days with few fibres being evident at 3 days. This hyperinnervation has been shown to persist until at least 12 weeks after wounding (Reynolds & Fitzgerald, 1995).

Is neonatal wound healing faster than in adults and is there less scarring? In the fetus faster wound healing is ascribed, among other reasons mentioned in chapter 1, to the lack of immunoglobulins in circulation producing less inflammation (Whitby & Ferguson, 1991). We noted here that neonatal wound healing is also remarkably rapid and leads to the formation of a very small scar, discernible only by the loss of hair follicles. In the adult animal the area is largely undetectable. During this period in the naive animal there is postnatal cycling of hair follicle growth, but after wounding the loss of hair follicles is never recovered.

Wound healing is thought to be aided by the ingrowth of nerves, thus one would expect a hyperinnervated wound to heal much better. In conditions such as diabetic neuropathy, spinal cord injury, cut nerves, wound healing has been shown to be impaired (see Ansel et al. 1998). Poor wound healing in capsaicin treated rats (see Kruger et al. 1989) suggests that C fibres may have an important role. Intradermal capsaicin eliminates unmyelinated small sensory fibres of the DRG, with the result that there is a decrease in both labelled intraepidermal and dermal axons (Kruger et al. 1985). The contribution of these fibres to hyperinnervation may have a role in the little scarring that is seen in these animals.

A feature of the hyperinnervation is its lack of organisation or pattern. During development nerves may follow a path of least resistance. The keratin skeleton is preferentially arranged in the spinous layer with fibres weakly birefringent and intersecting and vertical or oblique, none being horizontal. Thus nerves are directed by structural barriers. In the stratum granulosum and outer 3 or 4 prickle cell rows of the stratum spinosum, the keratin has a horizontal axes with cells, therefore the passage to nerves is restricted and few epidermal fibres are seen here (Fitzgerald, 1998). The massive destruction and reorganisation that accompanies wound healing may result in the loss of such restrictions and result in hyperinnervating fibres forming a disordered arrangement. Indeed keratinocytes exert a directional influence on regenerating blood vessels and nerves which follow regenerating keratinocytes when re-epidermalisation is taking place (Terenghi, 1995), such that after wounding the keratinocyte population proliferates to address wound closure and may be a stimulus for the growth of fibres to that area. Nerve

growth precedes that of blood vessels during development and nerves are found in greater proximity to the epidermis than are blood vessels (Johnson & Holbrook, 1989). Therefore during development, nerves may promote angiogenesis or cause the epidermis to promote it. In injury this cannot be the case as angiogenesis precedes nerve growth and nerves here may indeed follow blood vessels (Gu et al. 1995; Kangesu et al. 1998). To assess this further markers of blood vessels need to be used and the time course and pattern of angiogenesis followed over the wound healing period.

Is this really hyperinnervation by local cutaneous axons or is it collateral sprouting from different nerve bundles or perhaps, a combination of the two? Hyperinnervation implies there are more nerves per given area than in comparison samples, whereas collateral sprouting suggests new fibres that normally innervate other areas branch and enter a territory that they would normally not innervate.

In 1941 Weddell et al reported axonal sprouting in response to partially denervated areas of skin of the rabbit's leg. Denervated areas were invaded by sprouts from intact, nearby nerve fibres. The collateral sprouting from adjacent nerves after nerve degeneration has been shown to be mediated by NGF (Doubleday & Robinson, 1992). Since NGF is upregulated in damaged skin (Constantinou et al. 1994) one might expect sensory neurons in damaged skin to be able to reinnervate denervated areas. This effect could be mediated by neurotrophins which are responsible for the initial patterning during development (for further illustrations see chapter 3). The removal of a full thickness piece of skin is a form of denervation although more than just nerves are removed. From inspection of numerous sections, it is evident that although there is some contribution from fibres from adjacently innervated hair follicles, the majority of hyperinnervating fibres arise from deeper nerve trunks (see figure 12).

Interestingly, in the adult rat there are few fibres branching deep within the epidermis. This is not so after wounding where many fibres are found in the epidermis (see figure 14). This is analogous to the 'transient' epidermal innervation seen earlier in development (Jackman & Fitzgerald, 2000).

Immunostaining has already revealed that the hyperinnervation is both CGRP and RT97+ve with no contribution from sympathetic nerves (see Reynolds & Fitzgerald 1995). However, the exact extent of the contribution of each of these two groups has not been documented, nor have their terminations within layers, nor specific relations to other structures, cells and end-organs within the area. This needs to be examined further.

In conclusion, factors responsible for sprouting are likely to be upregulated post wounding, over the period before clear evidence of hyperinnervation is seen, i.e. before P5. Therefore, the following studies of the mechanisms underlying hyperinnervation concentrated upon the period 3 days post wounding.

CHAPTER 3

3.*IN VITRO* MODELS OF HYPERINNERVATION

3.1 INTRODUCTION

The hyperinnervation that follows neonatal skin wounding must result from exuberant growth of sensory axons into the injured region. The phenomenon has been examined *in vivo* to some extent, but *in vitro* models would prove useful in investigating the actions of individual factors under controlled conditions.

For many years tissue culture models have been used successfully in the quest to discover whether factors promoted directed growth from DRG explants or not. The use of a collagen gel matrix for a substrate dates back as early as 1958 and nervous tissue use as early as 1907 (see Scott & Fisher, 1970). In 1983 Lumsden & Davies first described a culture system whereby explants of embryonic trigeminal ganglia were cultured with pieces of whisker pad skin. Since then many others have used skin as well as other target tissues to examine the effects on DRG neurons. Sharma et al. 1994 used the collagen gel system to examine the development time factors for sensory neurons within the DRG to correctly grow into spinal cord slices. Spinal cord cultures were also used by Fitzgerald et al. 1993 to show the repulsive effects of ventral spinal cord on specific subtypes of sensory neurons. Much more recently, Tonge et al. 1998 have reviewed the used of peripheral nerves attached to DRG explants to study axonal regeneration in culture. Skin explants in culture had been used for serum immunoantibody detection by many workers e.g. Mutasim et al. 1993.

Reynolds et al, 1997 adapted this method to look at the effects of wounded vs unwounded skin on lumbar newborn DRG neurons. The alternative to explanted ganglia was the use of dissociated neurons in culture, which had been used to characterise morphology and electrophysiology as early as 1969, and which in principle, methodologically has not changed over the last 30 years (Scott & Fisher, 1970). Modifications have been used for separating neurons with respect to size and maintaining adult neurons in culture over time, with supplements (Delree et al. 1989). Dissociated neurons provided the means to assess individual neuronal subtypes, quantify outgrowth and examine effects of growth factors and target tissue.

Hyperinnervation of the skin following neonatal injury was described in 1995 by Reynolds & Fitzgerald *in vivo*. Since then, an *in vitro* model was developed by Reynolds et al, in 1997. The model used newborn explant DRG co-cultures with skin from 3 day old

unwounded or 3 day old skin from animals wounded at birth, and was consistent in its effects both chick and rat species (see Reynolds et al. 1997). This model proved useful in the assessment of increased neurite outgrowth with wounded skin compared to control unwounded skin, and provided a comparison for *in vivo* and *in vitro* applications. However, disadvantages of this model remain the untrue situation of an *in vitro* application, the employment of 'unwounded' tissue as control, and the relative age and unpredictable nature of outgrowth of newborn DRG. In addition, the effect of skin on individual neurons and subtypes could not be investigated. Thus, a dissociated culture model was proposed.

Behaviour of a growth cone is influenced by a number of factors e.g. adherence to laminin (which in the intact chick embryo is abundant in the basement membrane that separates the epidermis and dermis, but barely detectable within the tissues themselves, see Cahoon & Scott, 1999) and other substrates, the expression of receptors for these molecules, adhesion to other neurons and to the environment, involving cell adhesion molecules and extracellular matrix molecules (N-CAM, fibronectin and collagen known substrates for axon growth, being largely restricted to the dermis), and collapsing factors and receptors for these substances, and levels of intracellular calcium and second messengers (see Honig & Burden, 1993). All of these are in turn influenced by the state of reactivity of the environment and the neurite i.e. a normal resting state or a reactive injured state.

During development various factors are thought to be responsible for axonal guidance to appropriate targets. Local cues such as other neurons (motorneurons developing before sensory neurons could serve as a guide, Honig & Burden, 1993), extracellular matrix molecules, cell adhesion molecules, blood vessels, and cues from the target e.g. collapsing factors (avian epidermis contains collapsin I, see Cahoon & Scott, 1999) which is expressed in the epidermis at the time when innervation is being established, and eph receptors (unpublished observations Cahoon & Scott, 1999) and various neurotrophins are all involved. The role of nerve growth factor (NGF) in mediating hyperinnervation, an anatomical and functional change had already been addressed *in vitro* (Reynolds et al. 1997), and this and the other neurotrophins are discussed in the introductory chapter, as well as chapter 4.

In injury where loss of peripheral innervation to these targets occurs, activation of these same mechanisms may be used to guide severed axons back to their targets. The over-exuberant nature of the axons in once again reaching their target, suggests that some of

these mechanisms have been altered to an abnormal state. Factors responsible for this could be directly or indirectly from the injured area. Some guidance molecules have positive and negative effects on the growth cone depending on the developmental time, and the type of neurons (Schwab, 1996). The optimal point at which to localise such substances will be before the onset of the innervation into the wounded area. In chapter 1, I showed that this had occurred at postnatal day 5. Therefore I have concentrated my studies of factors underlying hyperinnervation upon a time period of 3 days post wounding.

The aims of this chapter were:

- To assess the contributions of epidermis and dermis to the increased outgrowth produced by neonatal skin wounding and cultures of P0 DRG. This method could then be further refined for specific cellular types e.g. fibroblasts, macrophages, etc as has been done by many authors (Normand & Karasek, 1995).
- To assess neurochemical specificity within the co-culture model.
- To establish a dissociated culture model for the hyperinnervation phenomenon.
- To use this model to assess morphology and neurochemical specificity of neurons in wounded circumstances.

3.2 METHODS

3.2.1 Tissue culture of whole ganglia

Under sterile conditions lumbar dorsal root ganglia were rapidly dissected from newborn Sprague-Dawley rat pups killed by decapitation. The ganglia were transferred to minimum essential media (MEM) Eagles Hepes (SIGMA) a defined buffer medium, roots removed and ganglia cut into thirds. Each piece was placed in a bleb of Hepes in 3mm tissue culture dishes.

Three days previously newborn rat pups were wounded as described in section 1. Pieces of skin from these 3 day old pups wounded at birth and naive P3 skin were added to dishes as close as possible to the DRG, and the Hepes replaced with clots of 0.8% collagen in 0.1M acetic acid (90µl collagen and 10µl 10x Dulbecco's modified eagle medium (DMEM) (GIBCO), with 5µl 7.5% HCO^{3-} (GIBCO) added immediately before use). (Skin pieces were removed from the dorsal hindpaw of each animal, such that full thickness skin of the wound and a 1mm area around the wound was removed, and each piece then cut into 4 equal pieces and placed near to a piece of DRG).

Once gels had set, 2mls of 1x Hams Nutrient Mixture F14 (GIBCO) with 0.5% L-glutamine (GIBCO) and 1% penicillin/streptomycin (GIBCO) medium with 1% Ultroser G, a serum substitute for the *in vitro* culture of anchorage dependent cells (USG reconstituted in sterile water) (GIBCO) was then added to each dish and the dishes placed in a 37 °C incubator with 5% CO_2 overnight.

3.2.2 Dermis and epidermis separation

Full thickness skin from the dorsal hindpaw of three day old pups half of which had been wounded at birth was removed under sterile conditions. The skin was then washed twice in calcium and magnesium free saline (Parkfields Pharmaceuticals Ltd), followed by incubation at 4°C on ice with 25% trypsin (10x SIGMA) for approximately an hour and a half. Following this pieces of skin were washed with F14 Hams medium containing 5% BSA (SIGMA), and placed in a dish containing this same medium, on a cold stage. Using forceps and under a dissecting microscope, the epidermis and dermis were teased apart, and then divided as before into 4 equal pieces for explant co-culture (see above).

3.2.3 Dissociated cell culture

All reagents used were filter sterilised before use.

Approximately 20 newborn rat lumbar DRG were dissected under sterile conditions and placed in 2mls of Hams F14 medium with 0.125% w/v collagenase type IV (Worthington), and placed in a 37°C incubator for 30 minutes. 10mls of F14 was then added the tube inverted twice gently and spun at 1000rpm for 1 minute. The supernatant was discarded and 2mls of F14 with 0.25% trypsin (Worthington) added to the pellet which was resuspended and then placed in an incubator at 37°C for 45 minutes. After this the tube was spun again at 1000rpm for 1 minute and the supernatant discarded. 2 mls of F14 with 4% USG and 20% DNase Trypsin Inhibitor (made up with 20mg DNase I (deoxyribonuclease I) (SIGMA), 25mg trypsin inhibitor (SIGMA) and 1.043g magnesium sulphate heptahydrate (BDH) in 45mls F14) was then added, the pellet resuspended and the solution pipetted into a column of 5% BSA in F14. The tube was then spun at 750 rpm for 4 minutes. The supernatant was again discarded and the pellet resuspended in an appropriate amount of F14 with USG ready for plating.

200µl of cells were plated onto 2% poly-DL-ornithine (hydrobromide) (SIGMA, 1:50 diluted in sterile distilled water) pre-treated, washed 2% laminin (SIGMA 1:50 diluted in sterile PBS) coated coverslips, with laminin removed just before plating. Cells were then placed in a 37°C incubator to adhere for 30 minutes to 1 hour.

After this time 2mls of F14 with 4% USG were added to neurons cultured alone, or membrane platforms containing 2mls of F14 with USG and 3 day old wounded or naive skin placed carefully on top of the plated neurons, and the neurons allowed to grow for 24 hours at 37°C and 5% CO_2. A second series of experiments was performed as above except that the co-cultured skin was removed at various time points (0.5, 1, 2, 5 and 24 hrs) after onset of incubation. This series was performed since preliminary data indicated no difference in neurite numbers from dissociated neurons co-cultured with naïve or wounded skin, but on visual inspection neurons from cultures with wounded skin did not look as robust and healthy as those of naïve co-cultures. Hence, it was hypothesised that a toxic or an excess of a factor in wounded skin may be the cause of such a difference, and by removing the skin at various intervals an optimum period in co-culture would be obtained for wounded skin, as well as any difference between naïve co-cultures.

After 24hrs in culture, neurons were fixed with 4% paraformaldehyde and immunostaining procedures conducted essentially as described in chapter 2. However, both stages of the protocol could be done in one day, hence primary antibody was only incubated for one hour, and all washes and incubation periods were halved. Coverslips

were finally mounted in glycerol jelly (10g gelatine (BDH), 60mls distilled water warmed until dissolved with 70mls of glycerol (BDH) added and 0.25g of phenol/preservative, warmed when required).

3.2.4 Immunohistochemistry with NF200 and CGRP

Where DRG or dissociated cells were to be immunostained for NF200 and CGRP, experiments were conducted as above and explants/dissociated cells fixed with paraformaldehyde overnight, washed the following day in PBS for 1hour. Incubation in 10% NGS was followed by incubation in both primary antibodies in PBS with triton, NF200 1:1000, CGRP 1:500 at room temperature or 4 C overnight.

The following day gels/neuron plates were washed in PBS 3x for one hour and incubated in anti-mouse FITC (SIGMA) 1:200 together with biotinylated anti-rabbit IgG (VECTOR) 1:200 in PBS with triton for one hour. Three PBS washes for 30 minutes were conducted, followed by incubation with streptavidin Texas red-HRP (VECTOR) 1:200 and an additional 1:200 anti-mouse FITC for one hour.

After 3 subsequent 10 minute washes in PBS, gels were mounted onto gelatinised slides, air dried overnight and coverslipped in Vectasheild mounting medium (VECTOR). Neuronal plates were coverslipped in fluromount (BDH).

3.3 ANALYSIS

(i) Microscope images of DRG explants co-cultured with epidermis or dermis were captured by a video camera and imported into a Leica Image Analysis System. The overall extent of neurite outgrowth was calculated by drawing around the total radial neurite outgrowth and the area of coverage was measured. In addition the longest neurite was also measured. Statistical analysis was performed using a two-way ANOVA with Bonferroni post tests (Prism version 3).

Further analysis for these cultures involved importing images into Freehand software making sure scales were not altered and then superimposing a circular grid of concentric circles having radii corresponding to uniform increasing distances away from the centre over each DRG image (see figure 15). The numbers of neurites within each radius of the circle was measured by drawing over the neurites with the mouse. Both co-cultures with epidermis and dermis, naive and wounded were assessed, and statistical analysis performed using Minitab version12.

(ii) For double-labelling experiments images were captured using a Leica laser scanning confocal microscope with elimination of bleed-through by using FITC and TRITC channels independently. Images were then imported into Freehand and overlayed so that those cells that were double-labelled could be counted.

(iii) Analysis for dissociated cells was extremely labour intensive. Initially, assessment of outgrowth involved measuring the total area covered by each neuron and its neurites and its longest neurite for many neurons on a coverslip under each treatment using See-Scan software. For the second series, total area, branch points, and total neurite outgrowth for each neuron were assessed using a Leica image analysis system with a Wacom tablet to draw neurons. 25 neurons on each coverslip were analysed under each condition. Neurons chosen were isolated from their neighbours and where neurite outgrowth was greater than three times the cell body diameter.

Statistical analysis was performed using a two-way ANOVA with Tukey post tests (Minitab version 12).

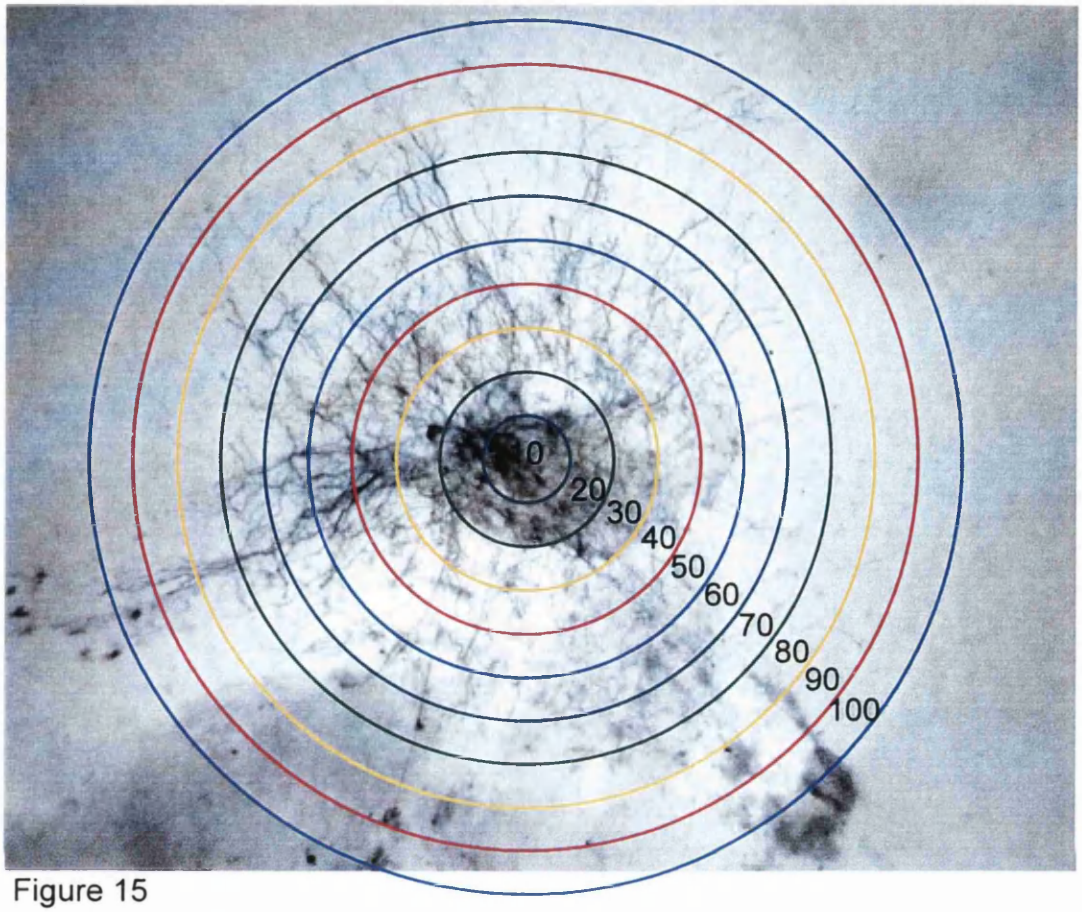


Figure 15

Figure Legends:

Figure 15: P0 DRG explant, showing the grid used for quantifying neurite outgrowth.

Figure 16: P0 DRG co-cultured with 3 day old unwounded dermis. Numerous fine, long neurites are seen extending away from the skin.

Figure 17: P0 DRG co-cultured with 3 day old dermis wounded at birth. Again numerous fine, long neurites are seen directed towards, penetrating, and growing well into the dermis.

Figure 18: P0 DRG co-cultured with 3 day old unwounded epidermis. Neurites appear straighter in this example and do not extend far away from their cell bodies.

Figure 19: P0 DRG co-cultured with 3 day old epidermis wounded at birth. The neurites show a typical mass, tortuous pattern and do not stray far from their cell bodies.

NB. Magnification was such that skin explants are outside the field of view.

3.4 RESULTS

3.4.1 Explant experiments

(i) The total area of neurite outgrowth of DRG explants was not significantly different in naive versus wounded epidermis or dermis co-cultures. The same was true of the length of the longest neurite in DRG co-cultured with naive or wounded epidermis or dermis. There was however a substantial difference between both longest neurite and total area of neurites when co-cultured with dermis compared to epidermis for both states of tissue. DRG explants co-cultured with dermis showed greater maximum neurite length ($p<0.0001$ two-way ANOVA with Bonferroni post test $p<0.001$ for naïve dermis and epidermis & $p<0.05$ for wounded dermis and epidermis) and total area of neurite outgrowth ($p<0.0001$ two-way ANOVA with Bonferroni post test $p<0.05$ for naïve dermis and epidermis & $p<0.001$ for wounded dermis and epidermis) $n=3$ animals each naive and wounded experiment (see figures 20 & 21 and figures 16 & 17 compared with 18 & 19).

(ii) The number of DRG neurites decreased with increasing distance from the skin for both epidermis and dermis co-cultures ($p<0.0001$, ANOVA Minitab version 12) (see figures 22 & 23).

There was a significant difference between naive and wounded epidermis neurite promoting activities when cultured with newborn DRG and analysed at concentric distances from the DRG body ($p=0.016$ using a two factor ANOVA analysis) $n=3$ animals for each naive and wounded experiment. Wounded epidermis was therefore better at promoting outgrowth compared to naive epidermis. Dermal co-cultures showed no statistical difference between naive and wounded samples ($p=0.366$, ANOVA Minitab version 12).

(iii) Double-labelling studies showed cells expressing NF200 and cells expressing CGRP. Very few if any were overlapping in any of the experiments studied and large cells were also labelled with CGRP. Neurites were NF200 positive. CGRP cells did not send out neurites at all. There was also no difference in the expression of NF200 or CGRP in naive/wounded epidermis/dermis co-cultures (see figures 24, 25, 26 & 27) $n=7$ ganglia for each $n=4$ animals for each experiment).

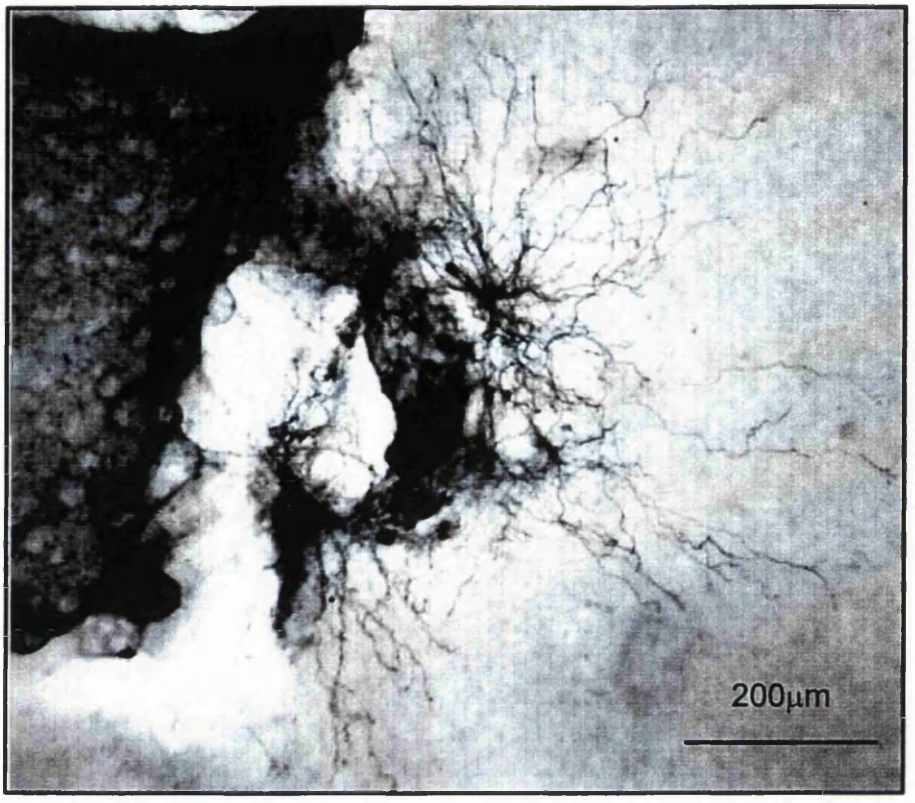


Figure 16

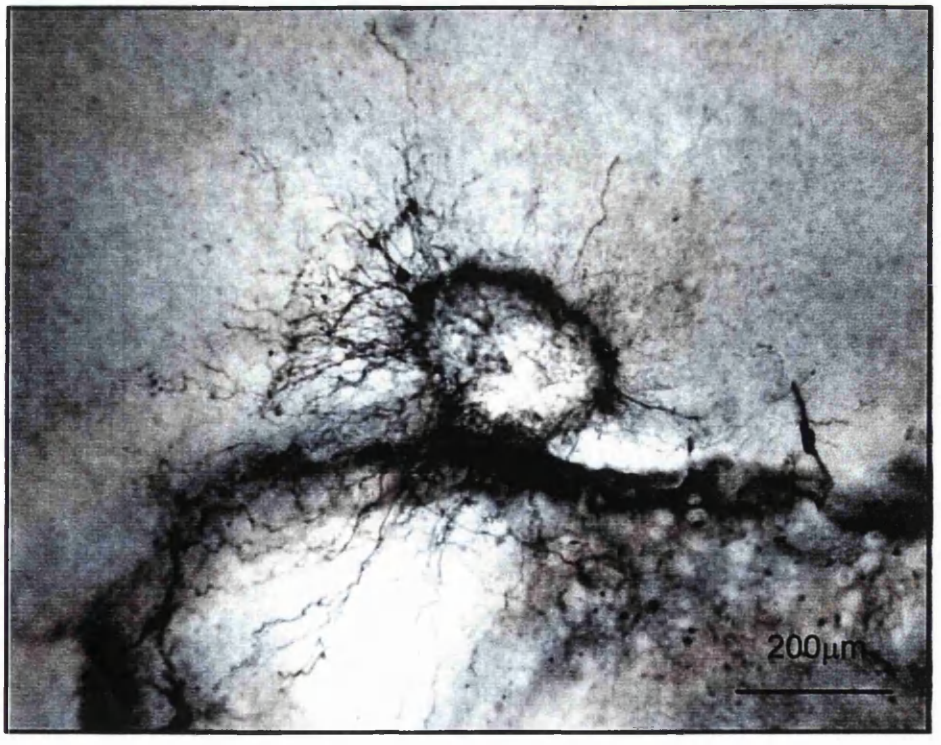


Figure 17

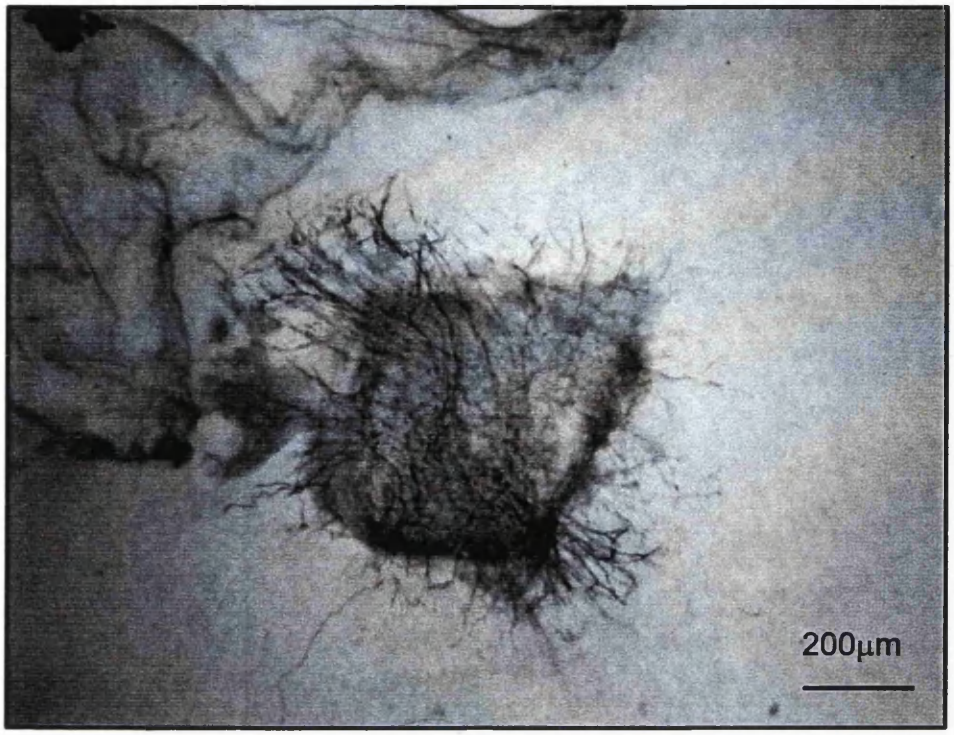


Figure 18

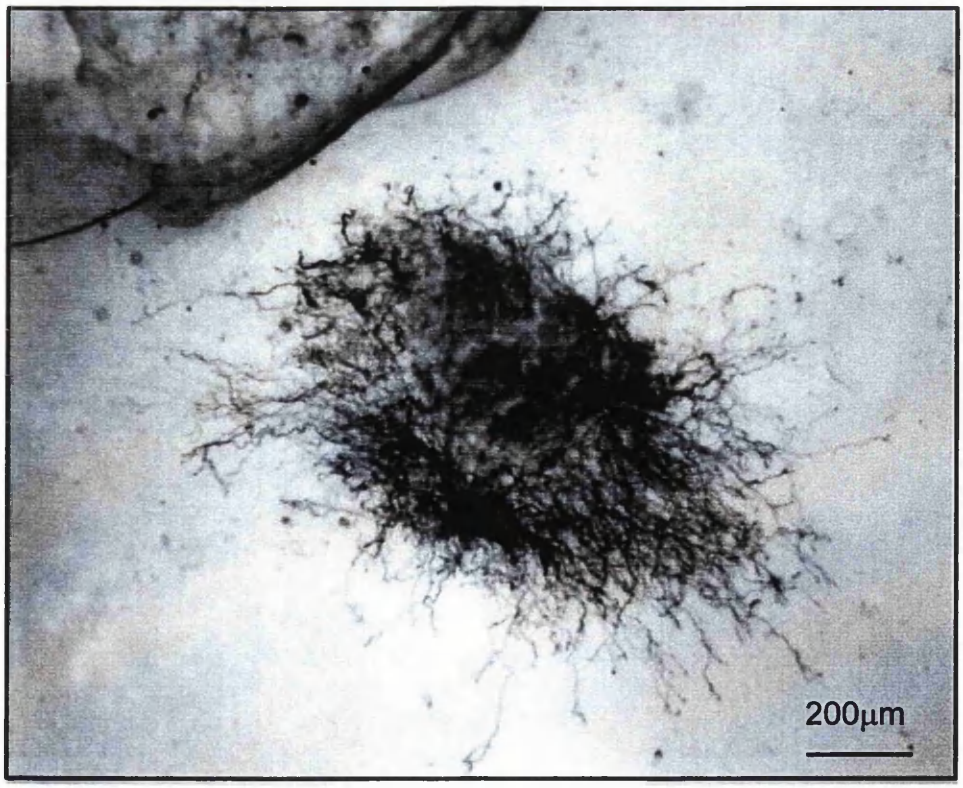


Figure 19

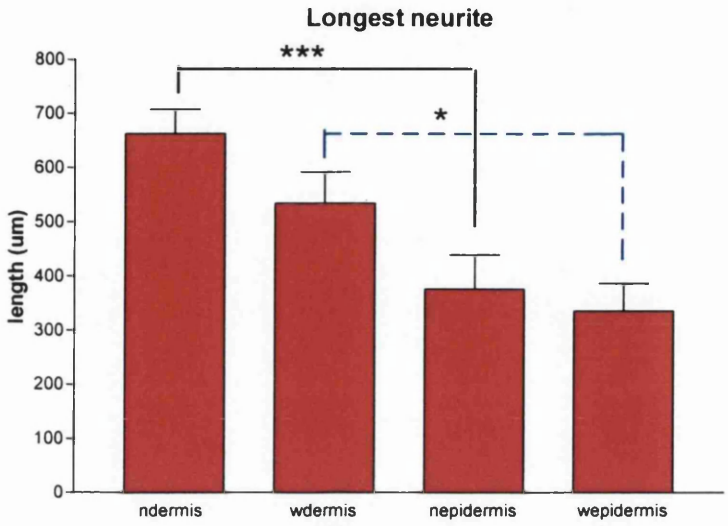


Figure 20 showing the mean length of neurites from P0 DRG co-cultured with the dermis of P3 rat pups unwounded or wounded at birth. There is a significant difference between dermal and epidermal elongation under both conditions, ($P<0.001$ for naive and $P<0.05$ for wounded, two-way Anova followed by Bonferroni post test).

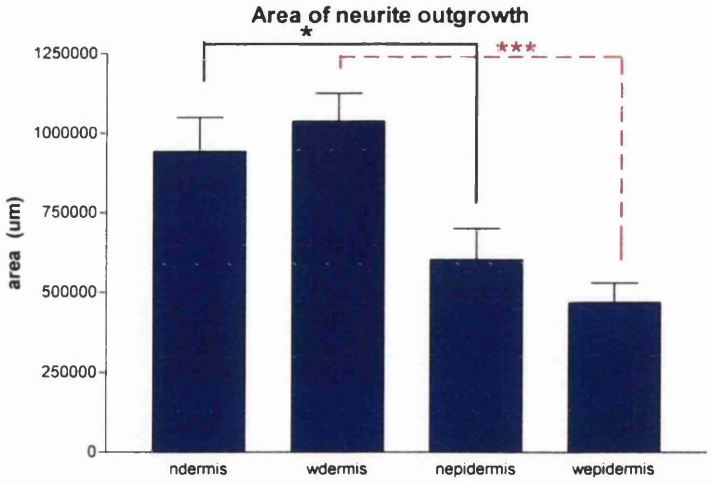


Figure 21 showing the effect of culturing P0 lumbar DRG with dermis or epidermis from naive and wounded P3 rat pups, on the overall area of neurite outgrowth. There is a significant difference between dermal and epidermal co-culture experiments,($P<0.05$ for naive, $P<0.001$ for wounded, two-way ANOVA with Bonferroni post test).

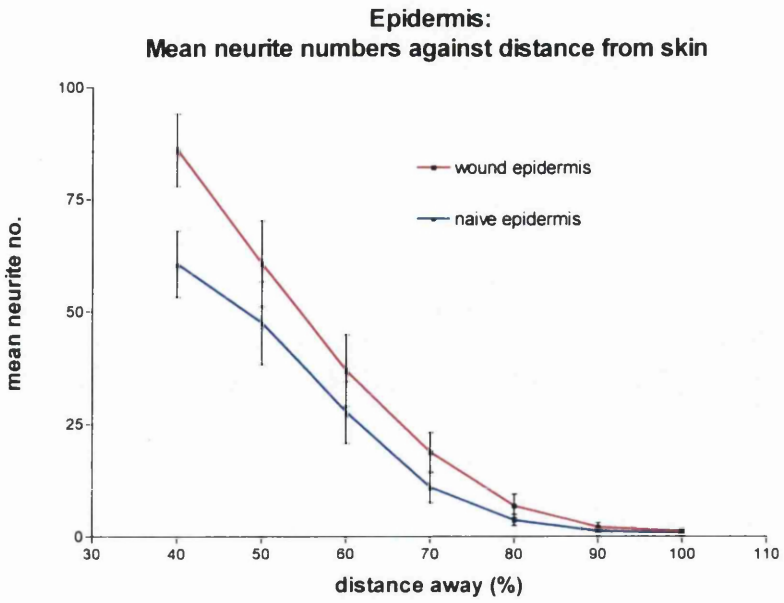


Figure 22 showing the change in mean neurite number at increasing distances away from the DRG for DRG co-cultured with naive P3 epidermis or P3 epidermis wounded at birth. ANOVA analysis shows a significant difference between naive and wounded outgrowth from the DRG p=0.016.

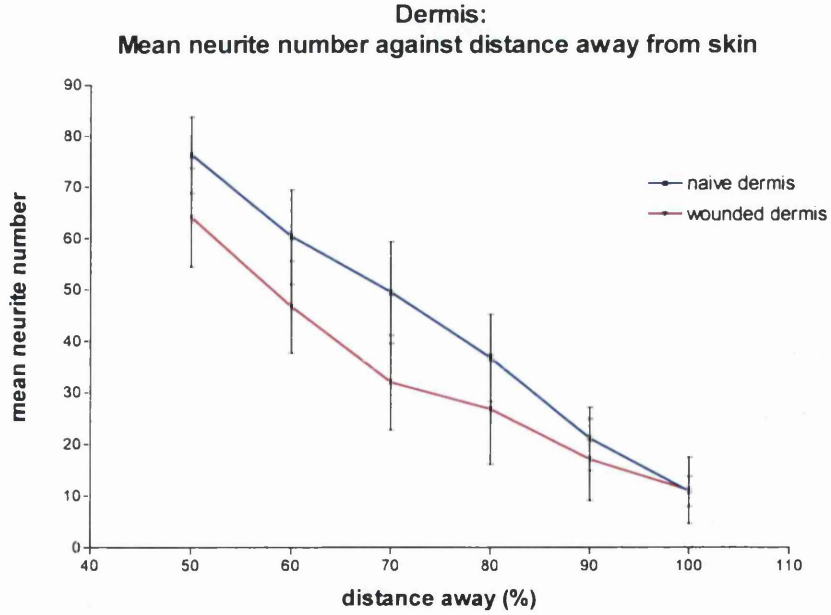


Figure 23 showing the effect of co-culturing P0 DRG with P3 naive dermis or dermis of P3 animals wounded at birth. At increasing distances from the DRG, fewer neurites are apparant, but ANOVA reveals a non-significant difference between naive and wounded dermis outgrowth promoting activities.

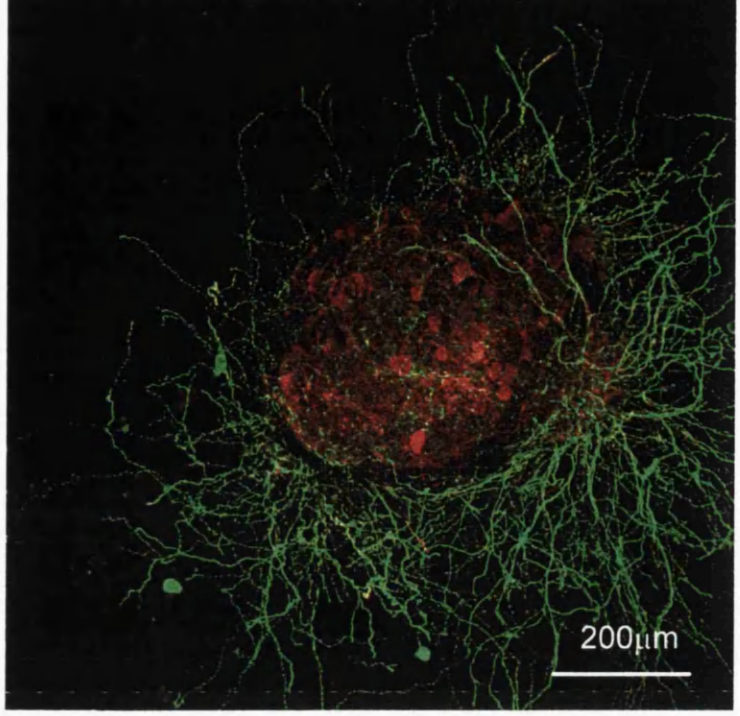


Figure 24:P0 DRG co-cultured with P3 naive epidermis immunostained for CGRP (red) and NF200 (green). Neurites cluster around the cell body, and there is little or no extension of CGRP positive neurites.

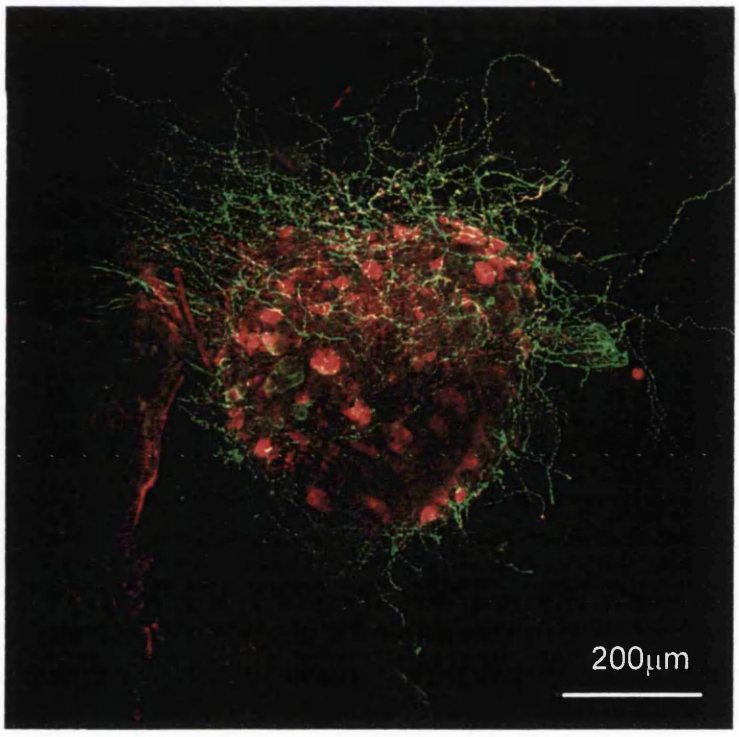


Figure 25:P0 DRG co-cultured with P3 wounded epidermis and immunostained for CGRP (red) and NF200 (green).
As with naive epidermis co-cultures there are clusters of neurites around the DRG cell body with little extension of CGRP positive neurites.

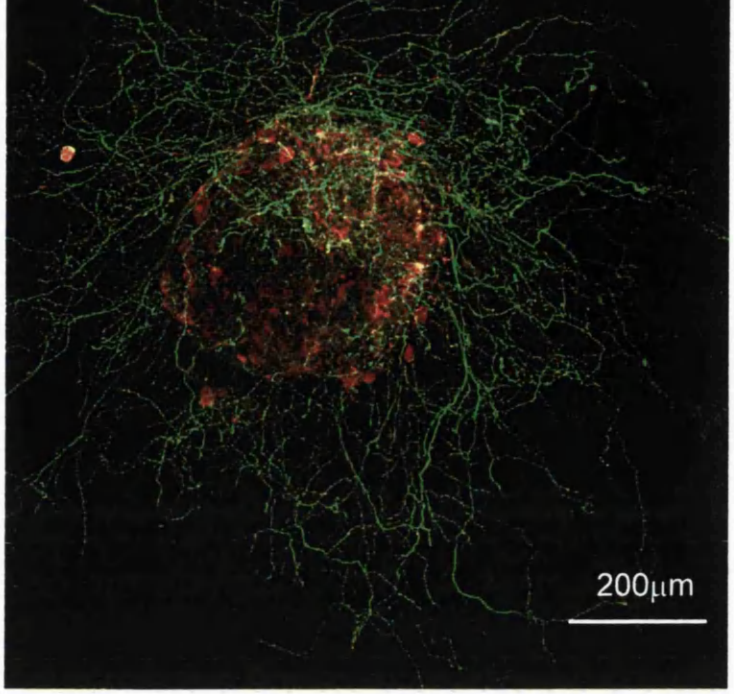


Figure 26: P0 DRG co-cultured with P3 naive dermis double-stained with CGRP(red) and NF200(green). In contrast to epidermis co-cultures, neurites are seen to extend far away from the cell body. Again little or no CGRP neurites are visible.

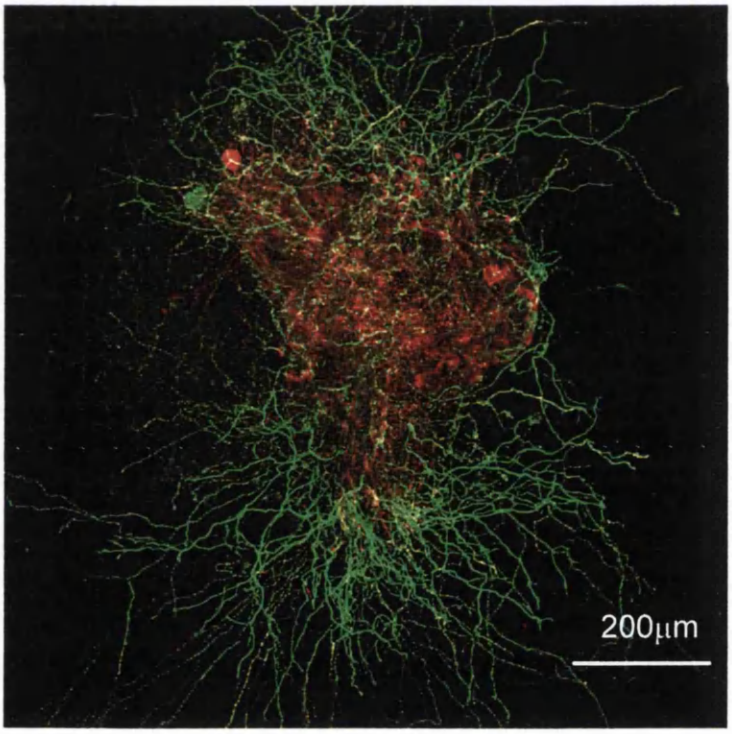


Figure 27:P0 DRG co-cultured with P3 wounded dermis double-stained with CGRP and NF200. As with naive dermal co-cultures, neurites extend into the periphery with no CGRP positive labelling of processes.

3.4.2 Dissociated cultures

(i) The first set of experiments using See-Scan software yielded no difference in the area of outgrowth ($p=0.8007$, $t=0.2527$, $DF=238$) and mean length of the longest neurite ($p=0.9991$, $t=0.001128$, $DF=238$ unpaired two tailed Student's t test, Instat), of dissociated neurons co-cultured with wounded and control skin for 24hrs. ($n=120$ neurons per treatment, $n=5$ coverslips per treatment, $n=2$ animals (see figures 28 & 29)).

(ii) For the second set of experiments using Leica Image software, a significant difference was noted between wounded, control and naive skin treatments in the total number of neurite branches, total length of neurites, longest neurite and total area of outgrowth examined. This was only seen if the skin was removed 1 hour after plating (for branches $p=0.006$, for total length $p=0.004$, for longest neurite $p=0.052$, for total area $p=0.013$, (see figures 30, 31 and 33), $n=50$ neurons per time point, $n=4$ animals, 2 experiments). Figures 34 and 35 show some of the neuronal morphologies obtained in this experiment.

(iii) In preliminary experiments, dissociated P3 DRG neurons co-cultured with skin unwounded or wounded at birth, were double-labelled with NF200 and CGRP. Large diameter neurons tended to predominate in outgrowth production, and exhibited various morphologies e.g. radial outgrowth, linear outgrowth, branching, elongative growth, pseudounipolar, bipolar etc (see figures 36 & 37). CGRP and NF200 co-localised to a number of cell bodies, and to a lesser extent in neurites.

Graph showing the mean length of neurites from newborn lumbar DRG co-cultured for 24 hours with P3 skin and P3 skin from rat pups wounded at birth.

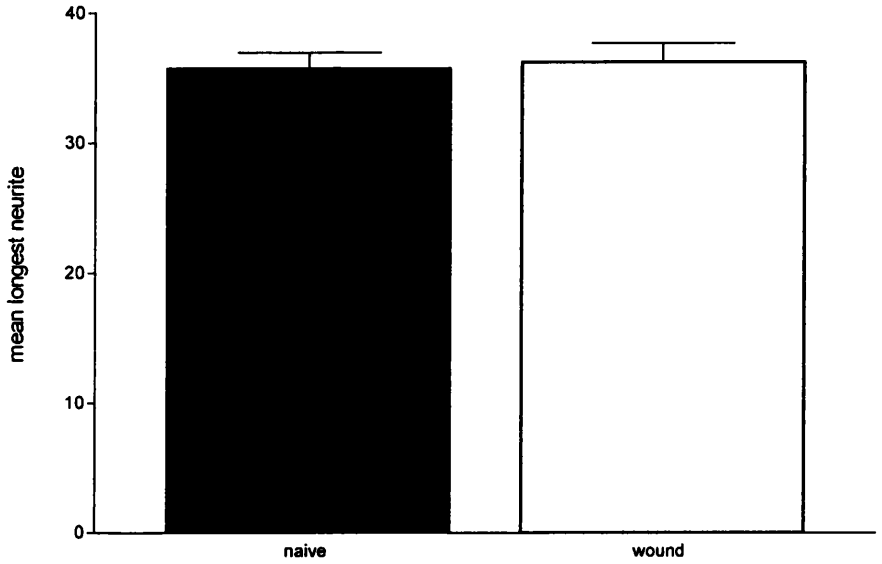


Figure 28 showing the effect of co-culturing newborn dissociated neurons with naive and wounded skin explants on mean length of neurite. No significant difference is seen under the two conditions after 24hrs incubation, p=0.8007, t=0.2527 and DF=238 (Unpaired Students t test Instat).

Graph showing the area of neurite outgrowth measured from dissociated newborn lumbar DRG in co-culture with 3 day old naive or 3 day old skin wounded at birth.

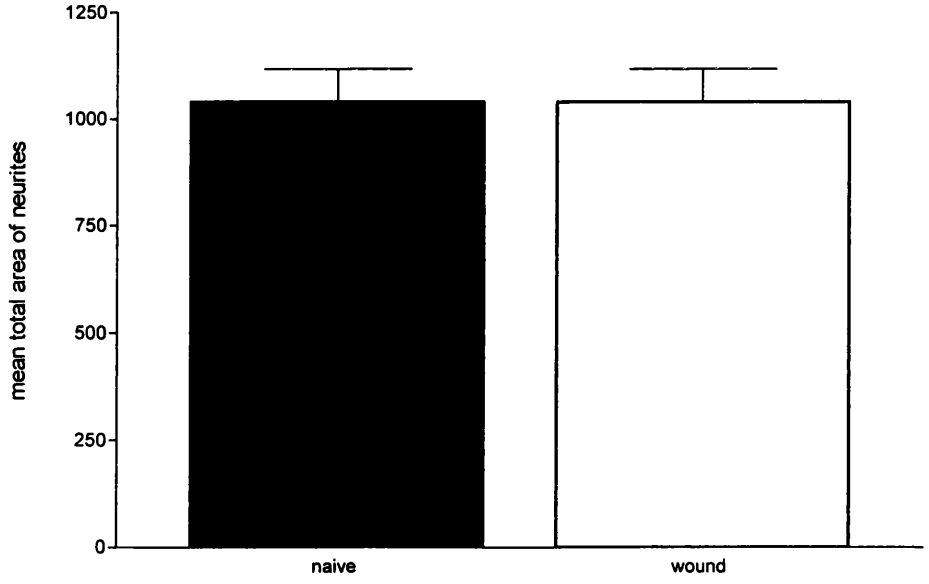


Figure 29 showing the effect on neurite area of co-culturing newborn dissociated neurons with naive or wounded skin. There is no significant difference between outgrowth produced under the two conditions when co-cultured for 24 hrs, two-tailed p value=0.9991, t=0.001128, and DF=238 (Unpaired Students t test Instat).

Graph showing the change in branches of neurites from P0 DRG co-cultured with 3 day old naive skin, or 3 day old skin wounded at birth at various lengths of time

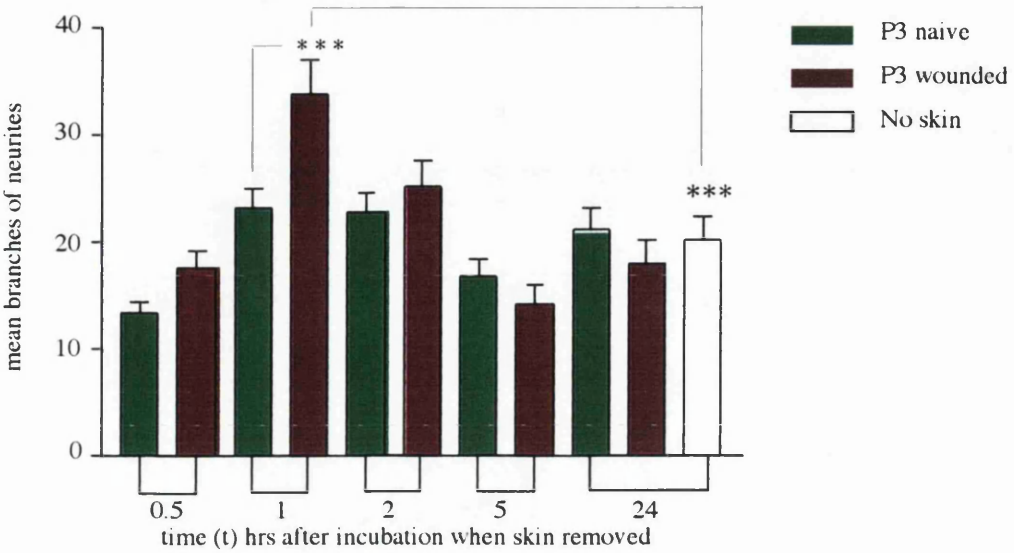


Figure 30: The graph shows significant differences between DRG branch numbers in co-cultures with wounded skin as compared to naive skin, when the skin is removed at t=1hr (P=0.006, n=50, two factor ANOVA).

Graph showing the total length of neurites of P0 DRG co-cultured with 3 day old skin, and 3 day old skin wounded incubated for various lengths of time.

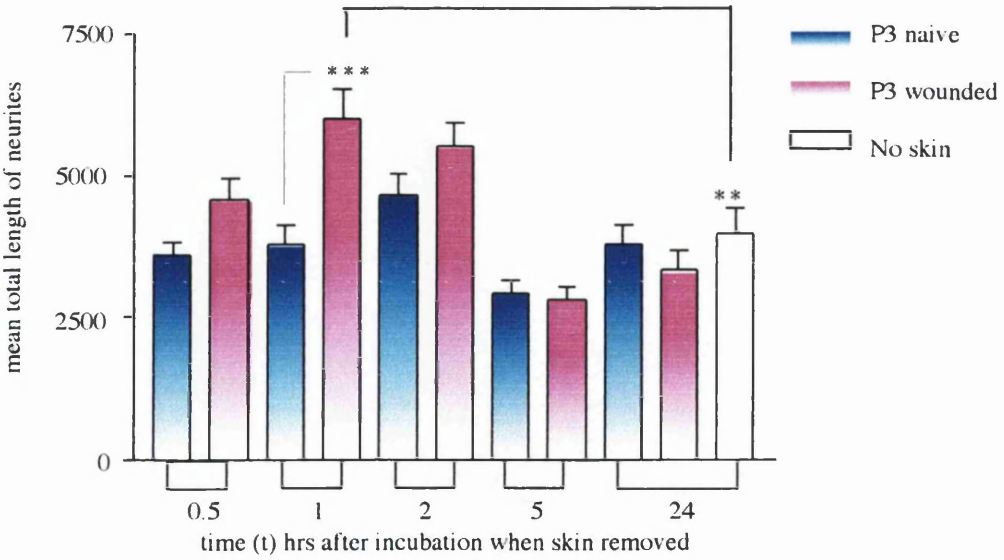


Figure 31: shows significantly different means for P0 DRG co-cultured with wounded skin compared to naive skin, when the skin is removed at t=1hr (P=0.004, n=50 two factor ANOVA).

Graph showing the longest neurite in co-cultures of P0 DRG and 3 day old naive skin, or 3 day old skin from animals that had been wounded at birth.

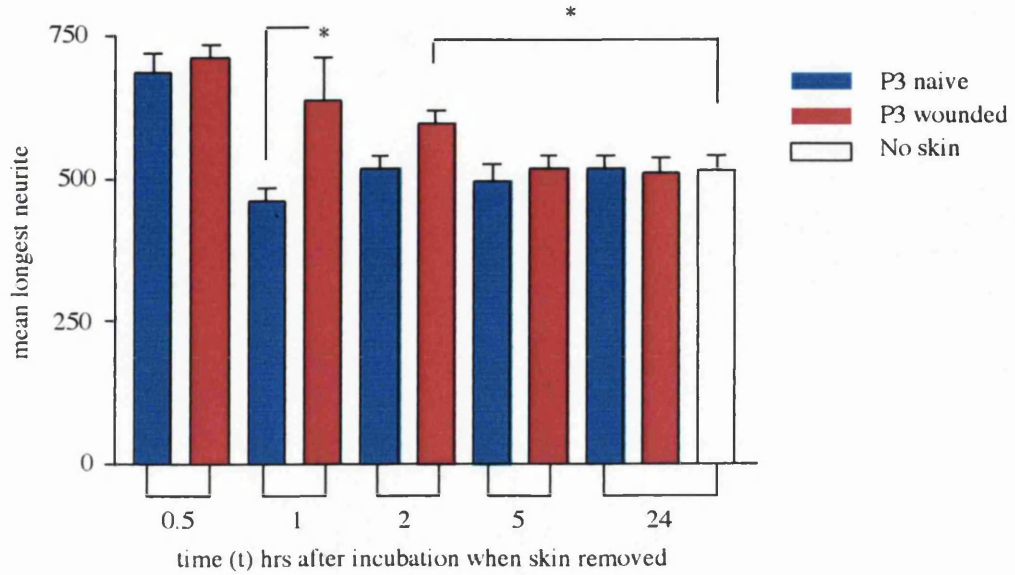


Figure 32 showing the increase in length of neurites with wounded skin removed at 1 hour compared to naive skin (P=0.052, n=50 at 1hr, two factor ANOVA), p<0.05 for comparison with no skin (Student T test, Instat).

Graph showing the effect of co-culturing P0 DRG with 3 day old naive skin or 3 day old skin wounded at birth, and removed at various times

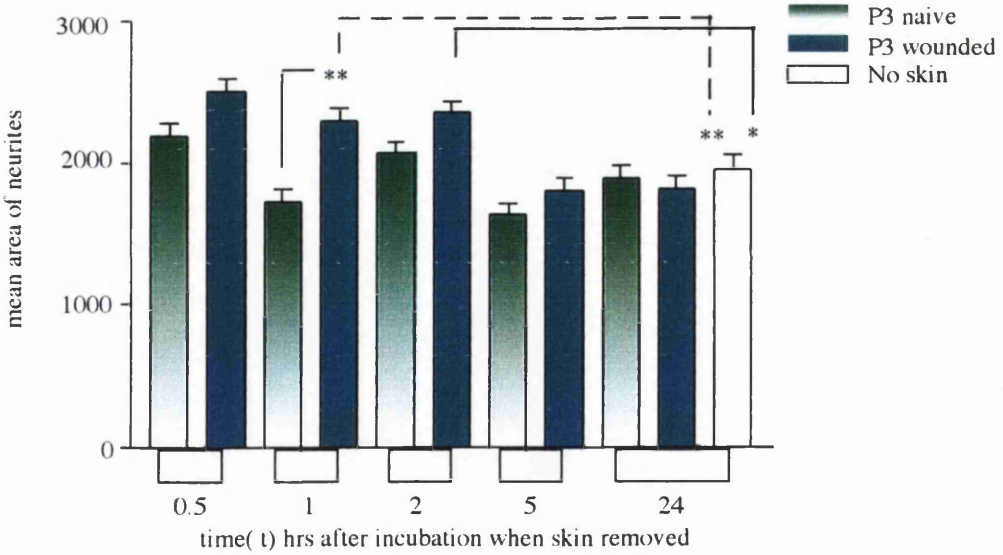


Figure 33 showing the significance of co-culturing wounded skin with P0 DRG on increasing the area of neurite outgrowth at t=1hr (P=0.013, two-factor ANOVA, n=50) compared to that of naive skin. There is also a significant difference between this time-point and no skin controls.

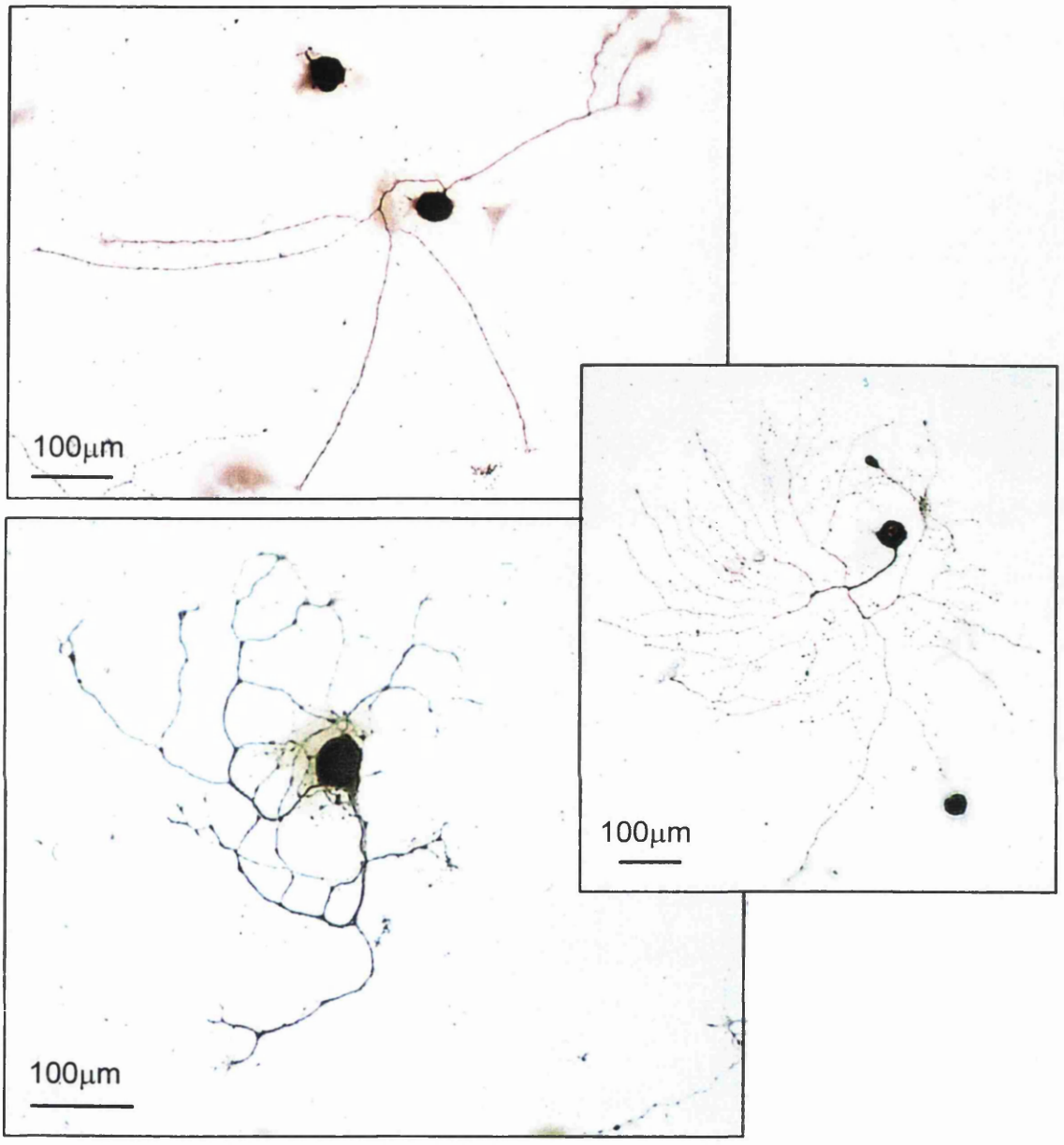


Figure 34 showing a sample of dissociated lumbar DRG neurons co-cultured with 3 day old naive skin and stained with PGP9.5. Plating density was such that single isolated neurons could be analysed.

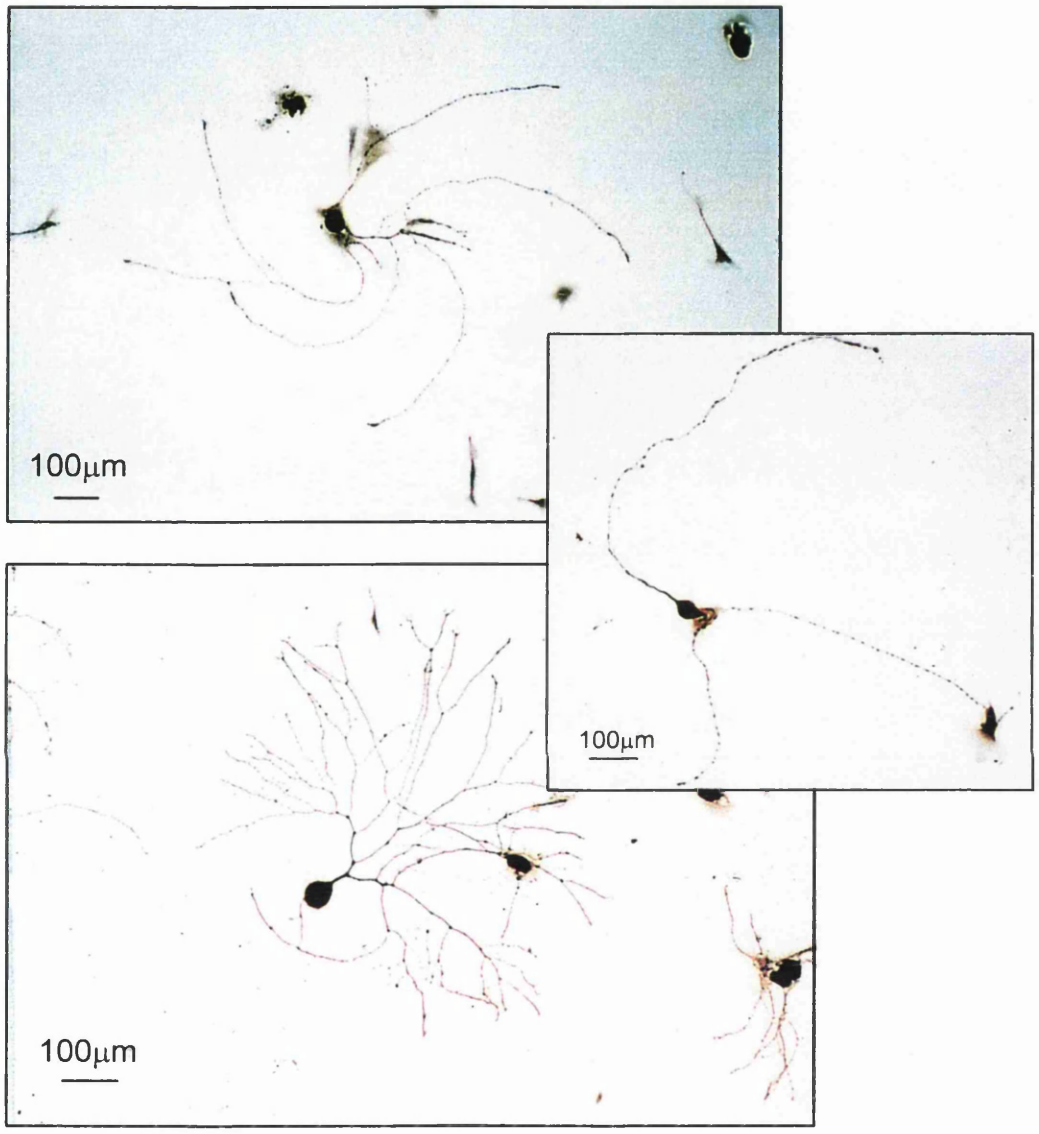


Figure 35 showing a sample of dissociated lumbar DRG neurons co-cultured with 3 day old wounded skin and stained with PGP 9.5. Plating density was such that single isolated neurons could be analysed.

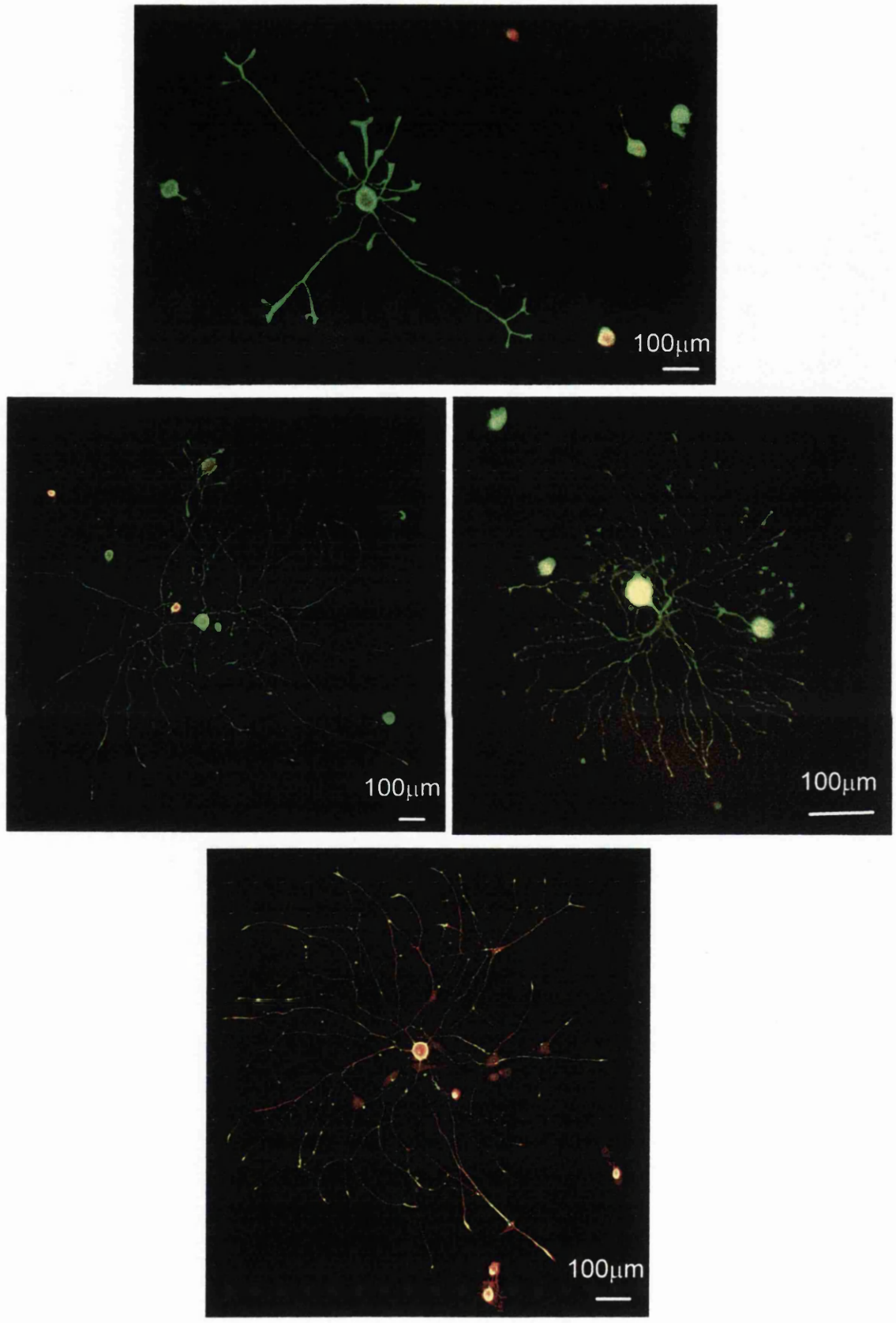


Figure 36 showing the various morphologies of dissociated lumbar DRG neurons from 3 day old rats co-cultured with unwounded skin, double-stained with CGRP (red) and NF200 (green).

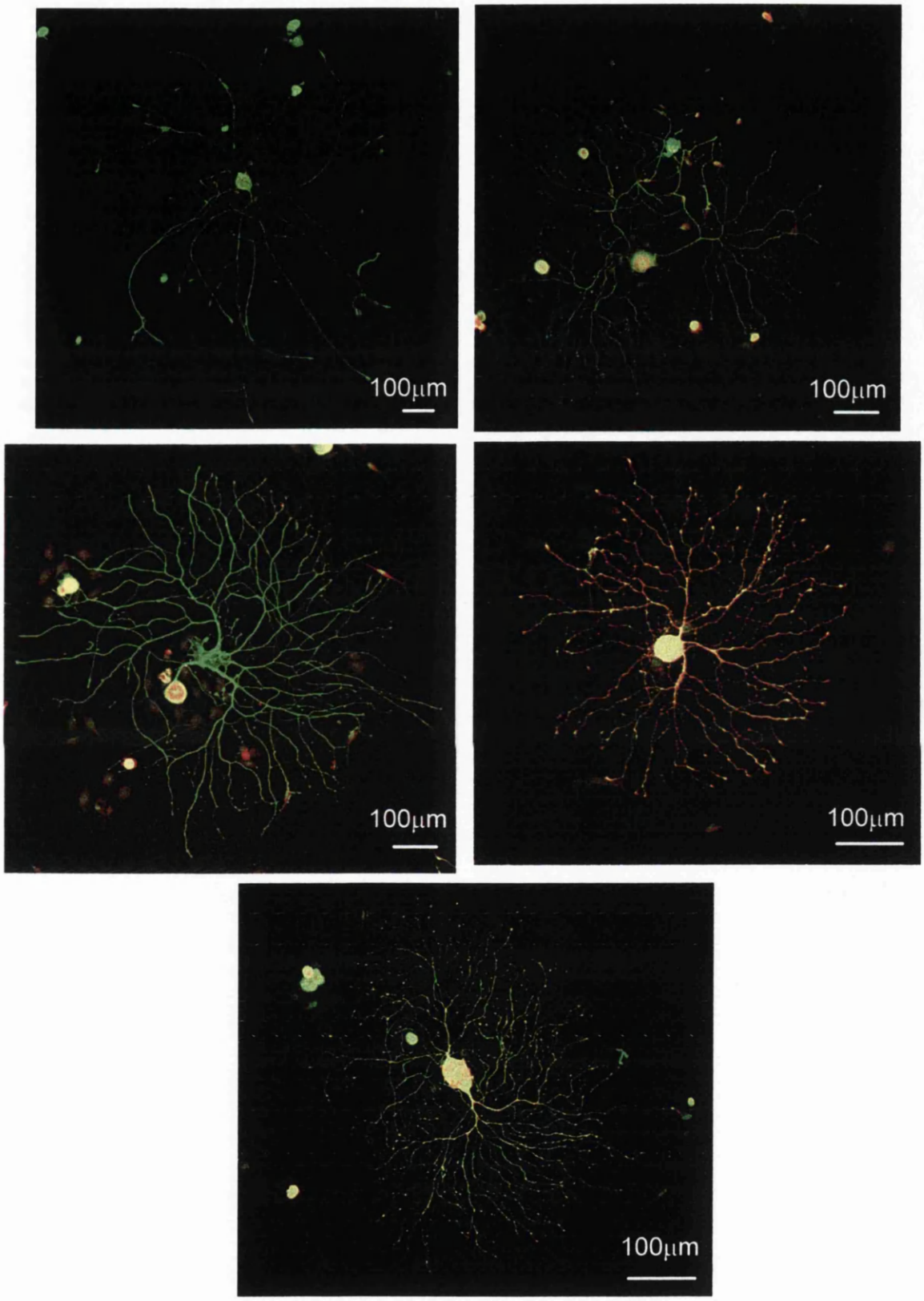


Figure 37 showing dissociated lumbar P3 DRG neurons from rats whose skin had been wounded at birth. Various morphologies and terminal arborizations are seen as well as differences in labelling. CGRP-IR (red) and NF200-IR (green).

3.5 DISCUSSION

The experiments above clearly showed differences between cultures of newborn DRG neurons and explants and naive of wounded skin. However, it is important to stress that culture conditions and the method of analysis play critical roles in assessing differences.

Here we have shown that the dermis promotes greater elongation of neurites from DRG explants co-cultured with both naive and wounded skin. We also show that wounded epidermis promotes greater branching from DRG explants than naive skin, and that wounded skin promotes greater effects on branching and length, early on in the culture period of dissociated neurons. The prominent outgrowth from both DRG cultured with naive and wounded skin explants seems to be NF200 positive, i.e. the large diameter axons, although CGRP is present within the ganglion cell bodies. The localised labelling is lost when neurons are dissociated and 3 days old. The predominant outgrowth observed *in vivo* was also shown to be of the large diameter axons, although contribution from small diameter fibres and few sympathetic fibres was also noted (see Reynolds et al. 1995).

Unlike Reynolds et al, 1997 we do not find a clear difference between DRG explant neurite outgrowth from wounded and unwounded co-cultures. This discrepancy can be explained by the numbers of neurites emanating from our co-cultures as compared with those of Reynolds et al,1997. In our study total neurite numbers regularly amassed greater than 100, whereas in the above study total neurite numbers approximated to 50 at their maximum. Reasons for this change in outgrowth could be attributed to methods of dissection and the rapidity of set-up. Since the first experiments were conducted in 1997, the method for obtaining lumbar DRGs has changed, such that the vertebral segment is no longer removed and dissected, instead the vertebral spines are cut, the spinal cord removed, exposing the DRG. This has lead to experiments being done much faster (change of hours) and more efficiently, thus increasing the survival of DRG neurons in explants. Hence, no difference is noted on the overall neurite number. However, when analysis is limited to the epidermis of wounded and naive skin, DRG clearly respond by increased branching to the former which clearly fits in with the increased branching noted in *in vivo* skin sections (see Reynolds et al. 1995, & chapter 2).

3.5.1 Methodological considerations

The tissue culture environment provides a good system for visualising and labelling cells or explants under controlled conditions. The collagen gel matrix slows diffusion, allows stable gradients to be established with explants representing *in vivo* conditions. Thus,

explants should show some degree of directed growth if gradients are allowed to be established, but in practice distances of 500μm or less are needed for good gradients (Honig & Zou, 1995), without contact of DRG with skin which is difficult to achieve consistently. The incubation period, the trypsinisation essential to separate the two layers of skin, and the fact that no skin can truly be naive in the culture system are all factors that need to be taken into consideration when formulating a working model. A 24 hour time point was chosen as a standard, since it already existed as a model (see Reynolds et al. 1997) and provided a comparison with earlier results. It was also easier to use for assessment, as later time points tended to yield DRG with high densities of neurites too difficult to count.

Explants are thought to select the fastest growing axons and therefore slower ones which may differ in characteristics and intracellular properties are not addressed. The other biases towards specific neurons are the retractive and repulsive forces occurring within the system which could also lead to differentiation in neurite outgrowth.

The dissociated cell-culture system is a diverse one of many varying neurons which develop numerous morphologies when allowed to grow in such a system (see figures 34-37). During development when sensory growth cones are in the process of extending to their targets and even after contacting them, it has been observed that behaviour towards other neurites can be one of four types, fasciculation, parallel growth, retraction or crossing (Honig & Burden, 1993). To avoid problems with contact of neurites in dissociated cultures, neurons were only scored if they were isolated from other neurons with growing processes (see figures 34 & 35).

For good comparisons between explants and dissociated neurons, models must try and keep conditions and other parameters constant. Growth cones respond differently when encountering other growth cones than when they are encountering neurites (see Honig & Burden, 1993) which implies that the whole DRG explants differ compared to dissociated cells in culture in their contact arrangement. Substrates within both systems also differ with DRG for explants being grown on plastic culture dishes within collagen gels and dissociated neurons being plated on a polyornithine laminin framework on coverslips. Furthermore, in a recent study (Chang & Popov, 1999), local actions and distant actions of neurotrophins have been examined and shown to act at the axonal shaft and soma to regulate distant processes, so that anterograde transport from the end terminal is not necessary. This has important implications for neurons dissociated in culture, which can respond therefore in any orientation, and alter their final patterns or arrangements.

Substrates have been shown to be of vast importance in the growth from dissociated cells, such that axons will adhere much better to polyornithine laminin substrates than laminin alone, which will cause fasciculation (Honig & Burden, 1993). Similar studies to ours in chick embryos have shown that coating the epidermis with laminin allows it to be permissive to ingrowth by axons (Cahoon & Scott, 1999) although laminin is not expressed by the dermis or epidermis in 24 hour culture systems.

The build up of toxins over time, (since the medium is not changed) may also lead to non-uniform results, or inconsistency between *in vivo* and *in vitro* situations. With dissociated neurons a difference lies at 24 hours after plating if the skin is removed at one hour incubation. This may suggest that substances released from both naive (N.B. not truly naive) and wounded skin are effective in promoting neurite outgrowth, but wounded skin is substantially better if it is removed at one hour. This may be because toxic levels of substances may build up if it is left in culture for longer, or that since naive skin is actually essentially wounded, it gains enough time to secrete the same substances at an optimal level, whilst the continual secretion by wounded skin becomes inhibitory or toxic.

A yet further consideration is the quantification of neurite outgrowth from explant ganglia and dissociated neurons. Many authors have used various methods for this and there is no consistent, uniform approach. Liu & Schmid 1988 for example have suggested a nerve growth index (NGI) for scoring outgrowth, which consists of the product of the average neurite length in mm and the total number of neurites. The complexity of the tissue culture environment and variability in neurite outgrowth from laboratory to laboratory depending on media and conditions of culture mean that unless numerous parameters are assessed initially similarities and differences may be overlooked. Here we chose to employ a grid system to detect any changes in branches and length of neurites simultaneously from explant DRG. For dissociated cells, we examined parameters of branching, total length and longest neurite, all in essence which produced the same result (see figures 30-33). Thus further work need only to examine one of these parameters.

3.5.2 Permissive and inhibitory molecules

Two general modes of neuritic guidance can be considered, one mode is by the establishment of a permissive environment of molecules which do not inhibit the in growth of axons. The other mode is by restriction or repression by inhibitory molecules. For example, semaphorin III (Sema III) is able to inhibit the collateral sprouting of nerves in

repithelialising corneal tissue if transfected into corneal epithelial cells (Tanelian et al. 1997), and is naturally expressed at the time of innervation (Shepherd & Raper 1999).

In our model of skin hyperinnervation using P3 skin, the time of innervation has passed and the majority of hyperinnervating fibres are not collaterals, so that sema III may not have a role here. In fact sema III directs its actions to the majority of NGF responsive Aδ and C fibre axons (Tanelian et al. 1997) (and at specific developmental time points), which are not the majority seen in hyperinnervation. Sema III is also downregulated in the skin after birth (Giger et al. 1996). Other authors (Rochlin & Farbman, 1998) however have shown that inhibitory target derived factors may be mediators of the directed outgrowth in embryonic trigeminal neurons, well before neurotrophic attractant activity is present.

Part of the inhibitory and permissive milieu is the expression of CAMS important as temporally and spatially regulated molecules on the surface of neurites e.g. TAG-1 (see Honig & Burden, 1993). Cutaneous and muscle afferents express different cell adhesion molecules in the trigeminal system (Scott et al. 1996).

The inhibitory, promotory balance or imbalance may be different in culture models when compared to *in vivo* levels. Events associated with culturing have been shown to affect mRNA levels of various factors including integrins (see Honig & Kueter, 1995). Neurotrophic factors *in vivo* are responsible for the complement of DRG neurons and development of subtypes, and peripheral proliferation across different developmental time points as evidenced by knockout studies (see Snider, 1994). *In vitro* they also support neuronal survival and outgrowth, but differences in physiologically active levels of neurotrophin in culture, extracellular matrix molecules, substrates and non-neuronal cells have important bearings on survival and chemotrophic guidance. Time period in culture and developmental age could also determine attractive or repulsive expression or response.

The expression of guidance molecules, repulsive molecules and molecules which can change function from attractive to repulsive e.g. MAG need to be addressed in further experiments.

3.5.3 Neurite outgrowth of neonatal DRG

Both the epidermis and dermis have important roles in development and wound healing. And the vascularity of skin wounds only returns to normal in the presence of epidermal cover (Terenghi, 1995; Kangesu et al. 1998). The epidermis is permissive to the ingrowth of fibres during early fetal life, which may be due to the expression of neurotrophins (see Jackman & Fitzgerald, 2000). We would expect in culture the epidermis from wounded

skin to be more neurite promoting compared with that of normal skin (since NGF for example is increased in inflammation, for detailed examples see general introduction and Davies et al. 1987), and indeed figure 22 shows this clearly. Although this result is significant as assessed by a two-way ANOVA, the labour intensity of such a model makes it unappealing to use in other situations. The overall trend is one that more branches or neurites emerge from newborn DRG co-cultured with wounded epidermis as compared to naive epidermis. Honig & Zou, 1995 also showed such an observation of increased branching at shorter distances (<400μm), and attributed this to the repulsive effect of the epidermis. Preliminary results also showed that size of explant tissue did not affect neurite outgrowth (data not shown).

The epidermis could be more important in patterning or producing the final arborisations. One possibility is that wounding triggers a re-expression or de-suppression of developmental factors that allows the epidermis to be permissive again and therefore hyperinnervation to be achieved.

7 day old chick embryo studies with back skin co-cultures, show a tortuous route of fibres when co-cultured with epidermis, with a straighter preference with dermis (Verna et al. 1986). In our experiments the dermis seems to promote a more extensive, elongative pattern, with neurites seeming to search for a source or target to grow towards (i.e. the normally wandering nature of growth cones, see Verna et al. 1986), with neurites in epidermal co-cultures preferring to branch, and not distance themselves from the cell body. Verna et al. 1986 suggest that there is a deflection of trajectories away from the epidermis with an additional slow growth rate of these neurites. The epidermal deflection may be due to a gradient of inhibitory diffusible molecule or to cell membrane incompatibility. In addition, the same authors suggest that at longer distances the epidermis may be growth promoting. This would be quite plausible, considering the above results, with numbers of neurites emanating from DRGs in the two situations not being very different. One could also hypothesise that the epidermis would be able to attract all neurons at large distances and then limit numbers and patterns close by. Lumsden & Davies in 1986 showed that directed growth of trigeminal neurons to the epithelium was apparent, but not to mesenchyme, suggesting an important role of the epidermis in attracting neurites. Indeed, results in chapter 2 show that there is a very directed growth of nerves within the dermis in the area under the wound directed towards the remaining scar tissue.

The epidermis may provide an inhibitory factor for the growth promoting activities of the dermis. During injury this equilibrium is disturbed and initially there is a complete loss of

the epithelium, and therefore inhibition, allowing dermal attractant factors to be left un-neutralised. A model could be proposed (see figure 38) of the dermis allowing the attraction and growth of all axons to it, with the epidermis finally determining which of these enter and form a final pattern of innervation. Thus during normal development, axons do not so much retract from the epidermis but rather a selection is made which determines the final pattern of arbours. The absence of attractive factors may be at fault. However, this model is rather speculative considering the greater innervation of the epidermis during rat development (see chapter 2).

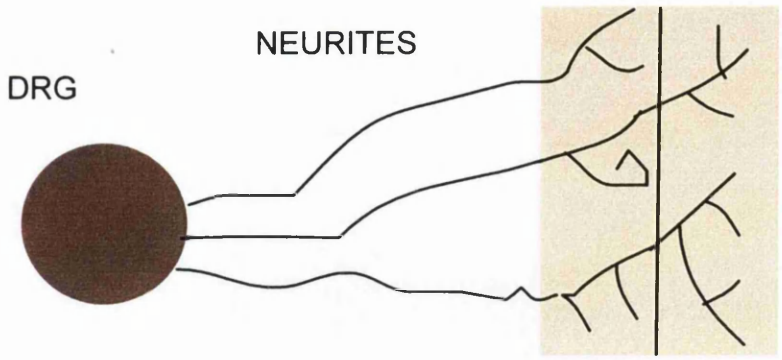
Studies in the chick have also shown that the epidermis is in some way inhibitory and the dermis promotes greater growth (Verna et al. 1986). Our study focuses on the rat and at a stage in development which has not previously been addressed. Previous studies also used target tissue at a time when axons were arriving to their targets, whereas in our experiments the axons have already reached their targets and innervated them. Target contact may specify previously unspecified neurons and these may then undergo differentiation. Hence, loss of contact may have unspecified neurons once again.

3.5.4 Neuropeptide expression of neurite outgrowth of neonatal DRG

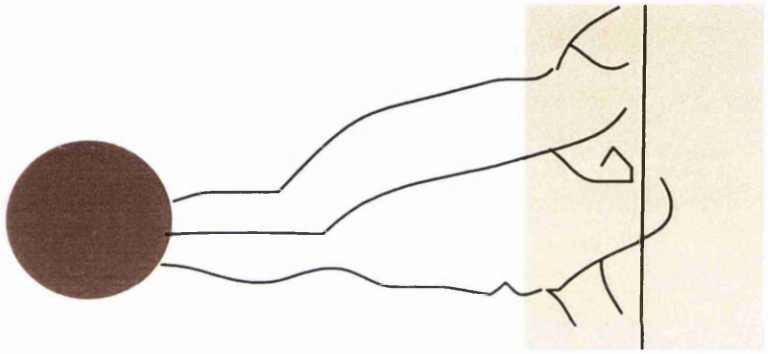
The hyperinnervation seen *in vivo* clearly shows a labelling of large diameter RT97/NF200 positive fibres as well as a contribution from the smaller diameter CGRP positive fibres (Reynolds & Fitzgerald, 1995).

Immunoreactivity to S100, PGP and CGRP in the nerves of skin is minimal in the absence of epidermal coverage. In addition studies such as that of (Honig & Zou, 1995) use neurons cultured in the presence of trophic/survival factors (where our studies have none), and growth is assessed at 12 hours rather than 24. In this model target tissues are attractant to sensory neurons but not specific to a particular type (Honig & Zou, 1995). This could be the result of culturing at earlier developmental stages in the presence of neurotrophic factors which are required for the survival of many types of neurons. This contrasts to the above data which clearly shows a preference for NF200 outgrowth compared to CGRP. Thus postnatally preferential growth is seen in the absence of external addition of neurotrophic factors.

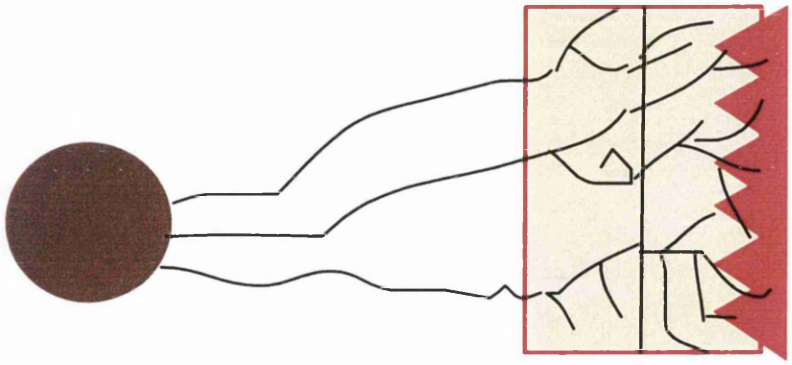
It has been reported that there is a late onset of CGRP staining along the whole length of the axon and into the skin during embryonic development (Jackman & Fitzgerald, 2000) increasing in postnatal week one (Reynolds & Fitzgerald, 1992). The implication is that



Normal early development: Ingrowth of axons into the periphery, making contact and branching into the epidermis.



Later in development axons retract from the epidermis.



After skin wounding postnatally, damage to the epidermis, and dermis allows axons to sprout and branch into the epidermis once more.

Figure 38 A diagrammatic representation of the proposal that skin injury causes axons to revert to their immature pattern of innervation.

there is a switch of axons to CGRP once they have reached their target. In culture the same scenario may exist. P0 DRG double labelling experiments with CGRP and NF200 showed that cell bodies are not double stained and few neurites are actually CGRP positive. This may be because they have lost contact with their target or they may be immature. By P3 dissociated cells co-cultured with skin seem to express CGRP within their neurites, and cell bodies are now double-stained (see figures 36-37). In the embryo depolarisation of cultured DRG leads to the expression of CGRP suggesting an activity dependent mechanism (see Jackman & Fitzgerald, 2000). Thus the change at P3 may be developmental or it may be activated by the removal and dissociation of DRG. The transport of CGRP within a DRG mass as with explant cultures may be wholly different from that of dissociated neurons, so that small diameter neurites may be present but unlabelled. Markers of small populations of neurons other than CGRP e.g trkA may be of use, as well as labelling of the non-CGRP positive unmyelinated population.

The time frame of analysis of outgrowth is also an important issue. Dissociated neurons that have not put out processes in culture may be at a later developmental stage. Indeed the majority of neurons which did not put out processes seem to be the small diameter peptidergic neurons which are known to begin peptide expression fairly late in development (Hall et al. 1997; Jackman & Fitzgerald, 2000). Although this seems plausible, it is important to note that all dissociated neurons in culture are essentially axotomized neurons and their neurites are regenerating rather than growing for the first time, and thus their neuropeptide expression may reflect this. Axotomy has been shown to diminish levels of CGRP and SP within the DRG in adults (Ju et al. 1987). As with explants the disconnection between the DRG and the periphery means that tissue culture can never mimic the *in vivo* situation (Hoyle et al. 1993).

We might expect that with the inflammation associated with skin wounding there may be a rise in neuropeptide expression in the P3 dissociated DRG examined. Further experiments and counts will help to establish whether this is indeed true.

CGRP and NF200/RT97 are not mutually exclusive and although they largely label separate populations of neurons, they cannot completely distinguish the small from the large neurons due to the degree of overlap. Other more selective markers and other markers for neuronal populations e.g. the isolectin IB4 need also to be examined. It is also possible that CGRP may not be a great marker for small neurons at the developmental stage examined.

It has been suggested that transported substances e.g. peptides may provide the CNS with a signal concerning the state of peripheral tissues, which can thereby control the connections of myelin afferents and exert a trophic effect on dorsal horn neurons (see Kruger et al. 1989). A noceffector role for CGRP has also been implied, such that peptides synthesised by the DRG could be transported to the periphery if damage has occurred and have a local effector response. Thus the function of CGRP in pain and its pathways may not be one of a transmission signal of the damage, but an effector important in vasodilatation, influencing the expression of receptors, adenylate cyclase activity (see Kruger et al. 1989) and many other systems. This is not to say its sensory activity is non-existent, but in the damaged state it may serve other functions.

There is also a large CGRP-IR network of thin axons within the periosteal capsule and the bone of the hindlimb (Kruger et al. 1989), and a rapid proliferation of CGRP-IR neurons during the healing of rat tibial fractures (Hukkanen et al. 1993). After epithelial wounding in the cornea topical application of CGRP increases the healing rate (Mikulec & Tanelian, 1996). Hence the postulation that it may be involved in tissue maintenance and renewal normally and after injury is highly likely. The destruction of C fibres by capsaicin with resultant delayed wound healing could also be due to the depletion of neuropeptides (Gallar et al. 1990), which could also mean that the rapid healing seen in hyperinnervation could be due to an increase in neuropeptide production, although our data in culture contradicts this model showing a decrease in neuropeptide expression. CGRP's intimate relation to blood vessels and lymphatic vessels may play a role in the modulation of the immune system to aid healing (Yamada & Hoshino, 1996). *In vitro* neuropeptides are lymphocytic and monocytic chemotactic agents, activate neutrophils and promote mast cell degranulation (see Rossi & Johansson, 1998).

Adult DRG neurons *in vitro* require NGF for the expression of SP and CGRP (Lindsay & Harmar, 1989; Mulderry, 1994), but there are low levels without NGF. Thus the presence, change or absence of these neuropeptides would give important insights into the production of NGF. Further experiments and counts will establish this.

Finally, the binding sites for CGRP are present in fetal life before its activity as a neurotransmitter, which again suggests its alternative or additional role in growth, differentiation and maturation (Nitsos & Rees, 1993).

3.5.5 Summary points

1. The 3 day old epidermis in wounded and naive states does not increase neurite length or number in newborn rat lumbar DRG explants in co-culture compared to dermal co-cultures.
2. However, wounded epidermis promotes the increased neurite branching of these neurons in DRG explants compared to naive epidermis.
3. The 3 day old dermis possesses a neurite outgrowth promoting/elongating or attractive effect of equal proportions in naive and wounded states to newborn lumbar rat DRG explants.
4. The expression of CGRP and NF200 is equal in both DRG co-cultured with wounded/naive dermis and epidermis.
5. The majority of neurites emanating from DRG co-cultured with dermis/epidermis, naive/wounded are NF200 immunopositive.
6. A dissociated culture model for increased neurite outgrowth in wounded skin co-cultures compared to naive cultures has been preliminarily established.
7. Dissociated postnatal day 3 DRG cells show expression of NF200, CGRP and co-localisation in the cell body, with expression in neurites as well.

CHAPTER 4

THE ROLE OF NEUROTROPHINS IN HYPERINNERVATION

Little is known about the neurotrophin response in the skin to the inflammation of skin wounding, which may be responsible for the aberrant growth of nerve fibres in neonates. The role of neurotrophins in innervation and development of innervation in the skin has been discussed in Chapter1.

- In this chapter we examine the role of NT-3, BDNF and GDNF in skin wounding and neonatal hyperinnervation.
- We characterise the expression of NT-3, BDNF and GDNF mRNAs in neonatal wounded rat skin and unwounded skin by Northern blotting, and *in situ* hybridisation.
- We also look at the NT-3 protein expression by ELISA and immunohistochemistry, and examine GDNF protein expression by ELISA.
- To address functional relevance we use the *in vitro* model developed by Reynolds et al,1997 to see if blocking NT-3 or GDNF has an effect on neurite outgrowth.
- Finally to assess causality *in vivo,* functional block of NT-3 using trkC IgG fusion bodies are used, and the development of hyperinnervation in BDNF knockout mice wounded at birth examined.

4.1 METHODS

Unless otherwise stated all reagents were of analytical grade quality from BDH.

4.2 MOLECULAR BIOLOGICAL ANALYSIS OF NEUROTROPHINS AND THEIR RECEPTORS

4.2.1a) DETECTION OF NEUROTROPHIN AND RECEPTOR CDNA

Reverse transcriptase polymerase chain reaction (PCR) amplification was used to determine whether neurotrophins and their receptors were present in the tissue of interest, and whether they occurred differentially in both naive and wounded states. This was not used as a quantitative measure, but for the detection of the presence or absence of neurotrophin and receptor cDNA, as well as any gross change.

4.2.1b) Reverse transcriptase PCR

This method allows one to take DNA, or RNA (having made a complementary first strand) and use this to reversely transcribe any genes of interest which may be present in the RNA by using known primers to these genes, and then amplify these genes to a

detectable and useful level. This technique is particularly useful for rare genes and small tissue samples (0.1-1μg of DNA can successfully be amplified).

The PCR reaction uses two oligonucleotide primers of approximately 17-30 base pairs in length which hybridise, one to each of the strands of DNA. The DNA is denatured by heating and the primers allowed to anneal at a specific temperature according to their sequence Tm (see below). Initially with the addition of an excess of deoxyribonucleotides and the DNA polymerase enzyme, the primers allow extension synthesis of complementary strands to the original DNA sample. Following this, subsequent cycles of denaturation and annealing, followed by extension synthesis allow the multiplication of the original strands as well as the newly synthesised strands. Final re-annealing means the procedure can synthesise DNA of interest in an exponential fashion.

4.2.1c) Primer selection

Primers were selected based on standard criteria, i.e oligonucleotide sequences from 13 to 22 bases long, with no running nucleotide sequences, with a GC content of about 50%, with little complementarity between the two primers (3' and 5'), and which would give a resultant fragment of about 200-300 base pairs and which had a Tm of about 58-60°C.

Tm=4x[G=C] + 2x[A+T]

NT-3 primers corresponding to base pairs 93 sense 5'-GTCCATCTTGTTTTATGT-3' and 303 anti-sense 5'-TGACCTGGTGGCCTCTCCCTGCTCTGGT-3', from the published sequence Maisonpierre et al, 1990, Genbank accession no. M33968, amplifying a band of 396 base pairs.

BDNF primers corresponding to base pair 194 sense 5'-GGACTCTGGAGAGCGTG-3' and 527 anti-sense 5'-GCTGTGACCCACTCGCTAAT-3', from the published sequence Maisonpierre et al, 1991, Genbank accession no. M61175, amplifying a 333 base pair band.

GDNF primers corresponding to base pair 271 sense 5'-TCACCAGATAAACAAGCGGC-3' and 686 anti-sense 5'-GTCAGATACATCCACACCGT-3', from the published sequence by Lin et al, 1993, Genbank accession no. L15305, yeilding a band of 415 basepairs .

TrkA primers corresponding to base pairs 157 sense 5'-GCCGCATCCTGTCGTGAGA-3' and 500 anti-sense 5'-CTGACAGGGTCAAGTCCTGTA-3', from the published sequence Meakin & Shooter, 1992, Genbank accession no. M85214, yielding a fragment of 343 basepairs.

TrkB primers corresponding to base pair 1779 sense 5'-GACAGATTTCTGCTCACTTCA-3' and base pair 2013 anti-sense 5'-AGCATCACCAGCAGGCAGAA-3', from the published sequence Middlemas et al. 1991, Genbank accession no. M55291, yielding a fragment of 234 basepairs.

TrkC primers corresponding to base pairs 201 sense 5'- GAACGCCAGCATCAACATCA-3' and 472 anti-sense 5'-CCAGTCTCAATTCCCGAAAGACT-3' of the published sequence Schaeren-Wiemers et al. 1995 Genbank accession no. L14447, yielding an amplified band of 271 base pairs.

4.2.1d) RNA extraction

RNA is notoriously difficult to keep from degradation. Contaminants can range from large pieces of dirt to skin and hair, which contain RNAses capable of digesting the RNA. In addition, any grease or oil tends to trap dirt and these enzymes, which are both stable and active.

All equipment used in the following experiments was cleaned thoroughly in 10% Decon detergent, and then washed with ultrapure de-ionised water. Dedicated RNA Gilsons were also essential, as well as sterile clean pipette tips and eppendorf tubes. Communal use of such equipment was forbidden. Solutions were prepared with autoclaved de-ionised water. To avoid contaminants from the skin, disposable gloves were worn at all times when dealing with RNA, and were changed frequently.

Under sterile conditions, full-thickness skin (~2mm x 2mm) was removed from the dorsal hindpaw of neonatal P3 Sprague-Dawley rats which had been wounded at birth, as well as skin from unwounded P3 litter mates. The skin was immediately snap frozen in liquid nitrogen and stored at -80°C until processing.

When required the skin was placed in a pre-cooled mortar on dry ice and ground to a fine powder with guanidinium thiocyanate (GTC) (4M guanidinium thiocyanate, 0.1M Tris.Cl, 1% β-mercaptoethanol), to immediately and directly prevent RNA degradation at a nuclear level.

Following this the powder was allowed to defrost with the addition of more GTC, and broken down further using a Polytron homogeniser at maximum speed for approximately 1 minute.

50µl of 2M sodium acetate was then added and the eppendorf tubes inverted three times, followed by the addition of 500µl pH 7.5 phenol and 200µl chloroform also with inversion

and shaking. The eppendorf tube was then placed on ice for 15 minutes, after which it was spun at 13000 rpm for 20 minutes at 4°C. The aqueous phase was removed and placed into a new eppendorf tube, and any protein left discarded. RNA was precipitated overnight at -20°C with 500µl isopropanol. The next morning the eppendorf tubes were spun at 13000 rpm at 4°C for 30 minutes, the isopropanol was aspirated off, and two 70% ethanol washes then performed, followed by spinning at 13000 rpm for 2 minutes for each wash. The ethanol was then aspirated off and pellets were vacuum dried and resuspended in de-ionised water to an appropriate volume. The resuspended RNA was then denatured at 65°C for 5 minutes followed by placement at -30°C until use.

A small sample was denatured as above and run on a 1% agarose gel with 1x TBE (10x :108g Tris, 55g borate and 9.5g EDTA/dm^3 of water) buffer to check RNA integrity.
Unless otherwise stated, all reagents were obtained from BDH and were of analytical grade.

4.2.1e) DNase treatment

To purify the RNA from residual DNA, it was treated with 1 unit of DNase enzyme (Stratagene), with 10x DNAse buffer (autoclaved 1M Tris pH 7.5 and 1M $MgCl_2$ with water to a final concentration 1x) at 37°C for 15 minutes. This was followed by 1 volume phenol:chloroform extraction as before, with spinning at 13000 rpm for 2 minutes, removal of the aqueous phase and addition of 1/10th of the volume 3M sodium acetate, and 2 1/2 volumes of 100% ethanol with overnight precipitation. After spinning at 13000 rpm for 30 minutes and two 70% ethanol washes the following day, the now DNA free RNA was vacuum dried and resuspend in its original volume of water, and a sample denatured and thence run on a 1% agarose gel to check integrity.

RNA was examined with a spectrometer and then 5µg DNase treated. However, due to experiencing problems with degradation, it was decided that Vanidyl complexes should be incorporated into the DNase treatment and following this to treat with an RNAid Kit (Bio101). 3 volumes of RNA binding salt were added to the complexed DNase treated RNA, followed by 1µl of RNA matrix per µg of RNA. Binding was allowed to take place for at least 5 minutes at room temperature with an occasional mix to prevent settling. The mix was spun for 1 minute at maximum speed and the supernatant removed. The pellet was spun again to remove residual supernatant. 500µl of RNA wash was then added, the pellet resuspended in this and spun for 1 minute at maximum speed. The supernatant was

again removed and an additional spin, wash, spin conducted. Finally the pellet was resuspended in 200µl of RNase free water, mixed and then eluted by incubating at 55°C for 5 minutes followed by spinning for 2 minutes for maximum speed. The RNA was eluted as a supernatant and transferred to a new eppendorf. It was then checked on a gel for integrity and amount, as well as read on a spectrometer.

4.2.1f) Reverse transcription

500ng of RNA together with 10µl of a 1:5 dilution of random hexamers (Pharmacia), and water made up to 25.6µl were heated at 65°C for 3 minutes, placed on ice for 1 minute and microfuged.

First strand reaction mixtures were set up for each reaction, or as a master mix. This involved the use of 1x lst strand buffer (GIBCO 0.05M Tris-HCl, pH8.3, 0.07M KCl, 3mM $MgCl_2$, 10mM DTT), 2.5mM of each dNTP (Promega), 40U RNAse block (Stratagene), 10mM DTT, and 40U MMLV-RT (GIBCO), a total of 14.4µl, with RNA to 40µl for each reaction. Together with the RNA, the mix was placed at 37°C for $1^1/_2$ hours. A 3 minute 95°C incubation was then conducted, followed by placement on ice for 1 hour. 160µl of water was then added to the above and mixed. 10µl of this mix could then be used for each PCR reaction.

4.2.1g) PCR reactions

Each PCR reaction incorporated 2U Taq polymerase enzyme (Promega), 1x magnesium free buffer (Promega), 1.5mM $MgCl_2$, 0.8mM of each dNTP, 0.5µg/µl of each primer per reaction.

Controls consisted of primer mix alone without RNA, and a 1:3 dilution of the original DNase treated RNA (not reversely transcribed) as a control for the DNase treatment.

PCR parameters used were a 94°C denaturation temperature for 5 minutes, followed by an annealing temperature of 58°C or 60°C (depending on which set of primers were used) for 30 seconds, synthesis at 72°C for 30 seconds, followed by 94°C for 20 seconds using a Perkin Elmer PCR machine. Final termination occurred at 58°C/60°C for 30 seconds followed by 72°C for 2-10 minutes. Between 30 and 40 cycles of PCR was conducted and 10µl samples removed for analysis at 30, 35 or 40 cycles.

4.2.2 REGULATION OF NEUROTROPHINS AT THE mRNA LEVEL

4.2.2a) PCR probe preparation

Probes for genes shown to be present by PCR were produced so that gene regulation after skin wounding could be investigated by Northern analysis.

PCR products from the above reactions were thus purified using the PCR purification kit (Quiagen). Briefly this involved adding 500µl of buffer PB to the PCR reaction, placement in a QIAquick spin column in a collection tube, centrifugation for 60seconds to bind DNA to the column. Flow-throughs were discarded and the column washed with 750µl of PE buffer by 60seconds of centrifugation. Flow-throughs were again discarded and an additional 60second centrifugation conducted to remove remaining wash buffer. Finally, the DNA was eluted with the addition of 50µl Tris-HCl (pH8.5) and centrifugation for 60seconds. The final products were then checked, and bands of the appropriate size cut out from a 1% agarose gel. As with the manufacture of other agarose probes (see Chapter 5) the gel band was weighed, diluted and dissolved in 3 volumes of water and stored at -30°C before use.

4.2.2b) Northern blotting

The technique of Northern blotting was essentially developed from the use of Southern blotting for DNA (as described later in chapter 5), for the transfer of RNA from gels to filter membranes. However, since RNA is single stranded the gel needs no pre-treatment.

When looking for rare RNA species it is best to load large amounts of RNA on gels. In this case we used 10µg of RNA per lane along with an RNA ladder of 5µg. This lead to the RNA having to be resuspended in very small volumes and we noted that the RNA ran differently according to varying salt concentrations. RNA as already discussed is a highly degradable material, and so for stable and undegraded gel running, it is important to run it in a MOPS formaldehyde gel, with 1xMOPS buffer (MOPS: 0.2M MOPS, 0.05M sodium acetate (anhydrous) and 0.01M EDTA made up to 500mls with ultrapure water and autoclaved and pHd to 7). Gels were run at 80V for 2-3hrs, after which they were photographed along with a ruler, for identification of band size following Northern hybridisation.

The gels were then washed in distilled, deionised water to remove formaldehyde and placed inverted on a wick of Whatman 3MM paper soaked in 10xSSC on a bed (see Sanbrook et al. 1998). The bed was placed in a tray of 10xSSC. A Hybond N+ nylon

membrane (Amersham), prewetted in deionised water, was then placed on the gel and air bubbles removed by careful rolling with a pipette. Cling film touching all four sides of the gel provided a barrier for preventing short circuiting. Three wet pieces of Whatman paper soaked in water, followed by seven dry pieces were placed on top of the nylon membrane. A stack of paper towels provided the final absorption material, after which a tray with a weight was added. The blot was left in this position overnight. The following day, once the gel looked flattened the weight, paper towels etc. were removed, and the filter membrane cross-linked and checked under UV for equal transfer. The gel was also checked for transfer.

4.2.2c) Northern hybridisation

The above nylon filter membranes were placed in canisters and prehybridised with 5mls (20x) SSC, 1ml (100x) Denhardts solution (2% Ficoll (Pharmacia), 2% polyvinylpyrrolidone, 2% BSA (SIGMA) in ultrapure water and autoclaved), 0.2mls (10ng/ml) herring sperm DNA, 10mls of formamide and 2mls (10%) SDS, with 1.8mls of water for 3hours.

Agarose probes were boiled for 10 minutes and placed at 37°C until required. Once the prehybridisation had been initiated, 29µl of the agarose probes were incubated with 1.5µl Klenow large fragment of DNA polymerase I, 10µl 5xRP buffer (450mM Hepes, 50mM $MgCl_2$ adjusted to pH6.6 with sodium hydroxide), 1µl each 100mM A, G and T deoxyribonucleotides, 3µl α^{32}PdCTP and 3.5µl 90unit random hexamers, (a total of 50µl) at 37°C for at least $2^1/_2$ hours.

Once prehybridisation was complete, the prehybridisation buffer was replaced and the radiolabelled probe added (after boiling for 10 minutes). Hybridisation was carried on overnight at 42°C and filters washed the following morning with 5xSSC for approximately 30 minutes, followed by 3xSSC down to 0.5xSSC as appropriate.

Filters were then blotted dry and placed in autoradiographic cassettes with film, and developed as appropriate.

4.2.2d) *In situ* hybridisation using antisense oligonucleotides

The increase in NT-3 and GDNF mRNA was investigated at a cellular and morphological level using *in situ* hybridisations using antisense oligonucleotides (Wisden & Morris, 1994).

4.2.2e) Oligonucleotide selection

Oligonucleotides were located for NT-3 and GDNF using NCBI Genbank, and forty to forty-five base nucleotides selected for synthesis on the basis of standard criteria i.e. 50% GC and AT residues, no repeat or runs of bases, etc. as for primer selection described before. Oligonucleotides were then ordered from Genosis, desalted. Once obtained they were diluted in diethylpyrocarbonate- (DEPC) treated ultra pure water to a final concentration of 5ng/μl using optical density (O.D) measurements and an oligonucleotide value of 20, where,

Dilution x absorbance at 260nm x 20 = concentration (μg/ml).

An O.D of 1 represents a concentration of 20μg/ml for a 1cm path length.

Rattus Norvegicus NT-3 45mer anti-sense oligonucleotide sequence 5'-TGTGTCTGTTGCAATCATCGGCTGGATTCTGACCTGGTGGCTCT-3' Genbank accession M34643, from the published sequence by Ernfors et al, 1990.

Rattus Norvegicus GDNF 45 mer oligonucleotide anti-sense sequence 5'-CACCAGGCAGACAGCCACGACATCCCATAACTTCATCTTAGAGTC-3'

corresponding to base pair 41, Genbank accession number X92495, from the published sequence by Suter-Crazzolara & Unsicker, 1998.

4.2.2f) Probe synthesis

10ng oligonucleotides (5ng/μl stock) were end-labelled with 10pmol ^{35}S dATP (1000mCi/mmol), in a 1x buffer (Promega: 100mM potassium cocodylate pH6.8, 1mM $CoCl_2$, 0.1mM DTT), with DEPC treated water using 45U terminal transferase enzyme. Addition was achieved at 37°C in a water-bath for 1hour. After this time, 40μl TE was added to each labelled probe and the probes spun down sephadex G25 (Pharmacia) columns (made at the time of use, 5g of G25 in 2-3 volumes of DEPC treated water, washed three times, supernatants removed, 50mls of TE (0.5M Tris pH 7.5 and 0.1M EDTA) added and autoclaved, with final removal of excess supernatant before use) to remove any unlabelled probe. Radiolabel was measured by placing 2μl of probe into 4.5μl of scintillation fluid in a vial and counts were recorded. Probes with counts less than 100,000 per μl were disposed of. Probes with adequate counts between 150,000 and 400,000 were stored at -20C with 2μl of DTT for up to one month.

4.2.2g) Tissue for *in situ* hybridisation

Hindpaws from P3 wounded and control animals (two for each experiment, see below) were sectioned fresh frozen at 15μm thickness using a cyrostat at -20°C, onto poly-L-lysine (poly-L-lysine hydrobromide SIGMA, made up to 0.1mg/ml with DEPC treated water) or silane coated slides (SIGMA). Once sectioned and mounted, slides were allowed to dry for 30 minutes to 1 hour and then placed in 4% paraformaldehyde fixative for 5 minutes followed by PBS made with DEPC treated water for 5 minutes and thence into 70% ethanol for 5 minutes. Slides were then stored at 4°C in 100% ethanol until required.

4.2.2h) Hybridisation

On the day of use slides were removed from ethanol and allowed to air dry for 1-2hours. The hybridisation buffer (50%v/v deionised formamide, 8xSSC, 50mM phosphate buffer Ph7, 2mM sodium pyrophosphate, 10x Denhardt's, 0.4mg/ml HSDNA, 200μg/ml polyadenylic acid, 0.2g/ml dextran sulphate vortexed and topped with DEPC treated water, with all buffers made with DEPC treated water and pH to 7) was warmed to 42°C and probes and unlabelled 5ng/ml oligonucleotides defrosted. The slides were placed in a hybridisation chamber with wetted tissue to prevent drying. For each slide 100ul of hybridisation buffer was mixed with 1-2μl probe and 0.02M DTT, vortexed and pipetted onto the slide. Using parafilm, the solution was carefully brushed over the sections and coverslipped with parafilm. For control slides to show extent of non-specific binding, the above solution was mixed with unlabelled probe and placed on a slide as before. The hybridisation chamber was then sealed with nescofilm and placed in a 42°C oven overnight.

4.2.2i) Washes

The next morning slides were washed in 1xSSC twice for 30 minutes each at 55°C, followed by a wash in 0.1xSSC for 1 minute at room temperature, followed by dehydration for 1 minute each in 70% and 100% ethanol. Slides were then allowed to air-dry for 1-2 hours before placing in a cassette with Biomax film (Kodak).

4.2.2j) Slide development

Autoradiographs were developed and examined one week later. Because of high background levels and poor resolution, slides were dipped in K5 emulsion in gel form

(Ilford) photographic emulsion (prepared with 0.5% glycerol v/v prewarmed to 30-42°C), and placed in the dark for approximately 2 months. When appropriate the slides were removed from 4°C storage and allowed to equilibrate to room temperature for 1-2 hours. Meanwhile photographic developer D19 (Kodak) was warmed to 22°C. Slides were dipped in developer for 2 minutes followed by immersion in distilled water for 30 seconds, acid fixative (1 part fix to 3 parts water AMFIX) for 4 minutes and 2 further washes in distilled water for 15 minutes or more. Slides were placed in thionin solution (acetate salt SIGMA: 1% stock solution of thionin in 0.1M acetic acid and 0.1M sodium acetate, buffered to pH 4.2, filtered before use), for approximately 2 minutes, followed by dehydration in a series of alcohols 70%, 95% and 2x 100%, after which they were cleared in histoclear x2 and coverslipped with DPX.

4.2.3 NEUROTROPHIN REGULATION AT THE PROTEIN LEVEL

4.2.3a) Enzyme-linked immunoasays (ELISAs) for neurotrophins NT-3 and GDNF

ELISAs were performed to determine whether the NT-3 and GDNF proteins changed following wounding. ELISAs provide fast, efficient and sensitive ways to assess protein levels in all types of tissue.

Immunoassays were performed in accordance with specifications and protocols of the NT-3 Emax™ Immnoassay system and the GDNF Emax™ Immunoassay system (PROMEGA). Essentially the procedure was as follows:-

4.2.3b) Sample preparation

DFW tissue and control skin was extracted from 3 day old, 5 day old and 7 day old animals weighed and placed in sterile cryotubes, before freezing on dry ice. Samples were then homogenised in homogenisation buffer (9.35g sodium chloride, 2g BSA, 0.02g benzethonium chloride, 1.49g EDTA, 0.004g aprotinin and 200ul Tween 20 in 400 mls of 1xPBS) using a polytron homogeniser at maximum speed for approximately 30 seconds. Samples were transferred to eppendorf tubes and centrifuged at 13000rpm at 4 C for one hour. Supernatants were collected and stored at -80 C.

4.2.3c) Plate preparation

For a 96 well plate primary coating antibody anti-human polyclonal NT-3 or anti-GDNF monoclonal antibody was diluted 1:500 or 1:1000 respectively, in carbonate coating buffer

(0.025M sodium bicarbonate and 0.025M sodium carbonate). After mixing, 100µl was added to each well and the plate sealed and left overnight at 4°C.

4.2.3d) Blocking the plate

The following day, the plate was tapped dry and washed with TBST wash buffer (20mM Tris-HCl (pH7.6), 150mM NaCl, 0.05%(v/v) Tween®-20) three times for NT-3 ELISA and no washes for GDNF ELISA. 200µl of block and sample buffer 1x was then added to each well. The plate was then incubated at room temperature for 1 hour.

4.2.3e) NT-3 and GDNF standard curves

For the reference values NT-3 and GDNF standards were prepared by diluting the supplied standards in serial dilutions from a concentration of 0 to 300 pg/ml for NT-3 and 0 to 1000 pg/ml for GDNF, and placing duplicate standards in wells of the ELISA plate.

4.2.3f) Sample, secondary antibody and horse radish peroxidase (HRP) conjugate addition

To the rest of the wells samples were added 100µl per well. Various samples, amounts and dilutions were used in duplicates such that values within the reference range with respect to the NT-3 standard could be obtained.

Plates were sealed and placed on a shaker at 500±100rpm for 6 hours at room temperature.

Wells were then washed five times with TBST buffer. Anti-NT-3 monoclonal antibody 1:4000 or 1:500 anti-human polyclonal GDNF antibody, in 1x block and sample buffer with 100µl added to each well, and the plate sealed and incubated overnight at 4°C. The following day five washes with TBST buffer were again performed. For NT-3 ELISAs, anti-mouse IgG, HRP was diluted 1:2000 in 1x block and sample buffer and 100 µl placed in each well, anti-chicken IgY, HRP conjugate was diluted 1:5000 for GDNF ELISAs, and the plates incubated at room temperature with agitation for $2^1/_2$ or 2 hours. Following incubation the plate were washed five times with TBST buffer.

4.2.3g) Colour development and analysis

30 minutes prior to the end of the incubation period, 3'3'5'5'-tetramethylbenzidine (TMB) substrate was prepared (TMB:stock 10mg/ml TMB in acetate buffer 100µl/ml

0.1M sodium acetate & 0.1M acetic acid, stock used at 10µl/ml) 1:1 TMB solution with peroxidase substrate.

Once incubation was complete 100 µl of TMB substrate was added to each well and incubated for 10-15 minutes. Once there was sufficient blue coloration the reaction was quenched with 1M phosphoric acid (67.6 mls of 85% phosphoric acid (14.8M) added to 1 litre of deionized water). A yellow colour developed and the plate was then placed in a pre-programmed Dynatech plate reader for standard curve and O.D measurements at 450nm.

4.2.3h) Immunohistochemistry for NT-3

Dorsal hindpaws from 3 day old rat pups and from 3 day old animals that had been injured at birth were taken, after sacrificing the animals, and fresh frozen with tissue tek, and sectioned at 30µm (using a cryostat at -22°C) onto silane coated slides (SIGMA). Slides were left to dry for 1-2 hours at room temperature and hair-dryered briefly.

Slides were then immersed into ice-cold acetone (BDH) for 10 minutes, followed by 3 washes in 1xPBS. Peroxidase treatment with 0.6% H_2O_2 (SIGMA) then ensued and 2 washes in 1x PBS.

Sections were then blocked with 10% NGS for one hour at room temperature and incubated with affinity purified rabbit polyclonal antibody to NT-3 (Santa Cruz Biochemicals) 1:400 (500ng/ml) overnight at room temperature.

The following day slides were washed 3 times in PBS with agitation, followed by incubation with anti-rabbit biotinylated IgG 1:250 for 2 hours. 2 subsequent washes with PBS were then followed by incubation with avidin-biotin complex (made 30 minutes before use) for 1 hour. Slides were further washed in PBS for 30 minutes followed by Tris for 30 minutes and staining visualised with DAB (as in chapter 2). Slides were allowed to air dry overnight. IgG controls of naive and wounded skin were always run alongside with antibody incubated sections.

4.2.3i) Haematoxylin and eosin counterstaining (H & E stain)

Slides for counterstaining were rehydrated in water for 15 minutes, and then immersed in haematoxylin (Ehrlich's acid solution BDH) for 15 minutes. Slides were then washed in running tap water for 15 minutes, after which they were dipped into 1% HCl followed by 70% ethanol for a couple of minutes. A wash in tap water for 15 minutes then ensued and

slides were dipped in 0.5% eosin (BDH) for 20 seconds, immediately dipped in water and then 70% ethanol. Dehydration, clearing and coverslipping was performed as usual.

H & E staining produced nuclei stained blue, red blood cells stained red, and connective tissue stained pink.

4.2.4 FUNCTIONAL STUDIES OF NEUROTROPHINS AND THEIR RECEPTORS USING NEUROTROPHIN NEUTRALISATION

4.2.4a) Neutralising antibodies *in vitro*

Newborn lumbar DRG explants were cultured in 3mm well dishes with naive or wounded skin preparations as described in chapter 3. After the collagen had set, 2.5 mls of medium (Hams F14 medium + 2% Ultroser G (USG) (GIBCO)) was added with antibody to NT-3 (2ng/ml or 200ng/ml Promega anti-human NT-3 polyclonal antibody, the latter detecting 200ng of human NT-3, assayed on newborn rat DRG cultured with 20ng/ml NT-3 (Promega)) or GDNF (500 ng/ml reconstituted in sterile PBS) (R&D assayed on E7-10 chick DRG explants cultured with 10ng/ml GDNF (gift from S.B.McMahon)) and dishes placed in an incubator at 37°C, 5% CO_2 for 24 hours.

Once this time had elapsed medium was poured off, and gels fixed with 4% paraformaldehyde overnight. Gels were then ready for immunostaining, as described in chapter 3.

4.2.4b) Receptor body neutralisation *in vivo*

4.2.4bi) Trk C receptor fusion body injections

TrkC-IgG (tyrosine kinase C immunoglobulin G) fusion bodies (a gift from S.P. McMahon) were used to bind NT-3 and hence prevent the action of NT-3. Previous characterisation of trk A-IgG fusion bodies by S.P. McMahon and colleagues 1995 has been successful in the block of NGF. In addition, work by Shelton et al, 1995 and Munson et al. 1997 have shown *in vitro* and *in vivo* block of neurotrophins by their respective fusion body. Xie et al, 1997 used trkC IgG molecules to partially block the frequency of spontaneous synaptic current due to NT-3 rise in conditoned medium from depolarised muscle cultures. Seebach et al, 1999 also used trkC-IgG fusion bodies to remove the effects of NT-3 using a dose of 10μg in 50μl injected subcutaneously in neonatal rats. It was suggested that a dose of 5mg/kg was adequate to block NT-3 activity *in vivo*.

4.2.4bii) Functional activity testing

The functional activity of the trk C -IgG was assessed on E7 chick lumbar DRG grown in 2, 200, and 1 μg/ml NT-3. 500ng/ml of receptor bodies were able to block the outgrowth produced by 2 and 200 ng/ml of NT3.

4.2.4biii) *In vivo* injections of trkC-IgG

The antibodies were diluted in sterile saline to a concentration of 0.5ng/μl such that 5mg/kg could be delivered with a single injection of 100μl.

Newborn rat pups were anaesthetised with halothane/oxygen mixture and injected intraperitoneally with 100μl of the saline diluted fusion bodies. Control animals were injected with saline. Animals were also ear or tail-clipped for identification purposes.

The animals were allowed to recover and placed with their mother for 3 hours. After this time animals were anaesthetised again and dorsal foot wounds performed as in chatper 2. After recovery animals were placed back with their mother.

Additional animals were given a single injection as before prior to wounding, followed by a second 5mg/kg dosage on day 3 post wounding.

At day seven animals were perfused, fixed, dorsal hindpaws sectioned and immunostained with the pan-neuronal marker PGP9.5 as in chapter 2.

4.2.4biv) BDNF knockout animals

Original BDNF knockout mice of the sv129 strain displayed altered neuropeptides, a decrease in myelination, a decrease in ganglion cell number and volume, with a loss of the nodose ganglion and died after 7 days (Ernfors, 1994). Because of this a cross-strain of an outbred strain was derived from a genetic background of C57BL/6, NMIR, and sv129 mice by P.Carroll.

Newborn mice were unilaterally wounded as with rat pups (animals kindly donated by Patrick Carroll). Dorsal hindpaws were sectioned and immunostained as before, except with 50μ sections rather than 100μ sections as the animals were smaller than the average P7 rat, and were only 6 days old when sacrificed and perfuse-fixed. Genotyping was performed in Marseille by P.Carroll.

4.3 DATA ANALYSIS

4.3.1 RT-PCR and northern blots

Autoradiographs were scanned into Adobe Photoshop using Fotolook and directly imported into Freehand 8. Where bands were apparent in only one lane e.g. GDNF and NT-3, no assessment of relative increase or decrease could be made, although all bands were compared by eye to cyclophilin controls (see figure 45).

4.3.2 *In situ* hybridisation

Slides with sections of 3 day old naive and 3 day old wounded skin and non-specific binding control sections were photographed under dark field, transparencies scanned into Adobe Photoshop and imported into Freehand 8. A relative comparison could then be made.

4.3.3 ELISAs

NT-3 and GDNF ELISAs were both performed twice. Standard curves were either plotted and individual samples assessed from this, or a plate reader was employed to generate a standard curve. The plate reader also provided the optical densities for all samples. Where sample values were out of range, samples were diluted. Statistical analysis was performed using Prism and Statview, for data over states and over developmental time.

4.3.4 Immunohistochemistry

Since staining is a variable process, no formal assessment was carried out. In all cases any differences were visually obvious (see figures 54-58). However, sections were incubated for the same time period.

4.3.5 DRG Explants

Analysis of any neurites is a complex and labour intensive process but if all aspects of neurite outgrowth are not assessed significant changes between treatments can easily be missed. Here we chose to analyse antibody co-culture experiments by counting neurites under light microscopy as described in chapter 3 and by Reynolds et al.1997.

4.3.6 Trk C receptor bodies

Four sections through the hyperinnervated region were examined under bright field and the sections scored overall for degree of hyperinnervation (where 0 represented no

hyperinnervation compared to adjacent innervation and 3 represented most pronounced outgrowth), and expansion into the epidermis (yes or no). The observer was blind to the treatment group.

4.3.7 BDNF knockout mice

Three sections through the hyperinnervated region were scored, and descriptive notes made of the extent of innervation and morphology. BDNF knockouts (n=3), heterozygotes (n=7), and wild type animals (n=2). The observer was blind to the treatment group.

4.4 RESULTS

4.4.1 REGULATION OF SKIN NEUROTROPHIN AND RECEPTOR cDNA, mRNA AND PROTEIN LEVELS FOLLOWING WOUNDING AT BIRTH

4.4.1a) RT-PCR shows the presence of NT-3, GDNF, BDNF, trkC, trkB, and trkA cDNAs in skin

Figures 39-44 show the presence of cDNAs for NT-3 at 40 cycles of PCR, GDNF at 30 cycles of PCR and BDNF at 30 cycles of PCR in all tissues i.e., 3 day naive skin, 24 hour wounded skin and 3 day old wounded skin. In addition, the receptor for NT-3, trkC was also present after 40 cycles of PCR, but just at 30 cycles. The trk B receptor was present from 30 cycles and showed some degree of increase in wounded samples (see figure 43). Trk A the receptor for NGF was also present at 40 cycles in 3 day naive and wounded skin, but was undetectable at 24 hours after wounding. Figures 39 & 40 also show some increase in BDNF and GDNF 3 days after wounding, and although these are duplicate samples, this effect is not quantifiable nor absolute. Thus, Northern blots were performed to show absolute regulation at the mRNA level.

4.4.1b) Northern blots show that GDNF and NT-3 mRNAs are upregulated following wounding

Northern blots for GDNF and NT-3 are shown in figure 45. It is clear that GDNF and NT-3 are up-regulated in wounded tissue, 3 days post wounding. Two transcripts for GDNF 555bp and 633bp could be detected, and a single transcript of 1.5kb for NT-3. BDNF was undetectable by Northern analysis in all samples examined.

Two BDNF transcripts of 4kb and 1.6kb were isolated by Maisonpierre et al, 1990. Alternative splicing of RNA generated at least three forms of trkB, all binding BDNF (Middlemas et al. 1991).

The NT-3 gene consists of 3 exons including two 5' short untranslated exons and a 3'long exons encoding the entire protein and giving rise to two transcripts of alternative splicing of the 5' exons to the 3'coding exon. Recently (Sekimoto et al. 1998) have demonstrated four new classes of transcript. Rat and human NT-3 transcripts are 1.5 kb in length (Sobue et al. 1998).

Human GDNF mRNA exhibits 2 or 3 splice variants with the receptor GFRα having three isoforms (see Suter-Crazzolara & Unsicker, 1998). In the newborn rat ubiquitous expression of the GDNF transcripts (700bp and 620bp) has been shown by (Suter-Crazzolara & Unsicker, 1998), with the smaller transcript having a deletion of 78bp.

4.4.1c) *In situ* hybridisation of GDNF and NT-3 mRNA localisation

GDNF in situ studies showed an overall increase in GDNF mRNA within the epidermis and scar tissue (see figure 48), but very little specific localised expression could be detected (n=2 animals).

In situ hybridisation studies show NT-3 mRNA in the suprabasal epidermis and within hair follicles (see figure 49). After wounding there seems to be a reduction in suprabasal epidermal labelling, but there appears to be a layer of labelling directly under the scar tissue and within the scar tissue itself (see figure 50) (n=2 animals).

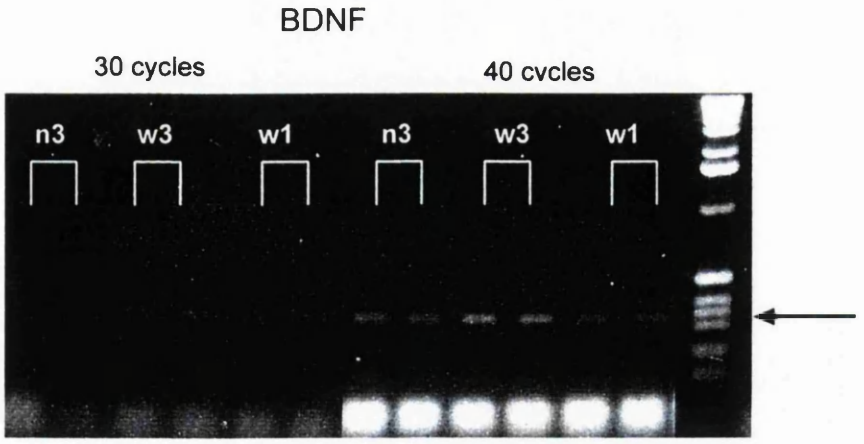


Figure 39: Photograph of a 1% ethidium bromide stained agarose gel of RT-PCR for BDNF. Both naive and wounded samples show BDNF at 30 and 40 cycles of PCR. BDNF seems to be greater in 3 day old wounded lanes at both cycles.

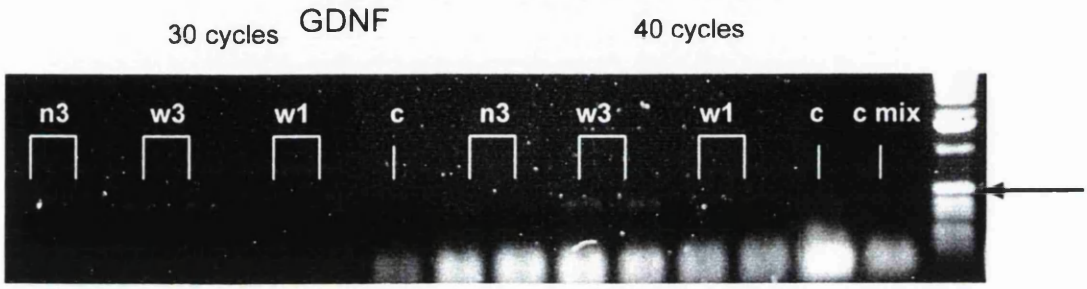


Figure 40: Photograph of a 1% ethidium bromide stained agarose gel of RT-PCR for GDNF. GDNF is seen in all samples naive and wounded skin at both 30 and 40 cycles. Wounded 3 day old samples seem to show greater expression at both cycles.

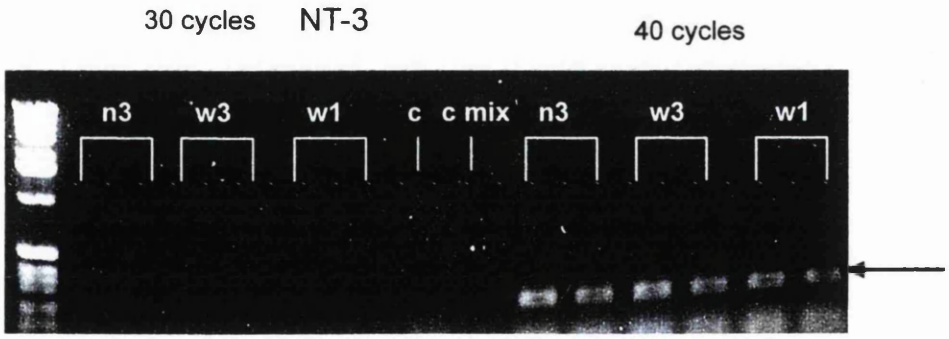


Figure 41: Photograph of a 1% ethidium bromide stained agarose gel of RT-PCR for NT-3. NT-3 is present in all samples of naive and wounded skin, but only at 40 cycles of PCR. No regulation is apparent.

For each gel c: control, DNAse treated RNA (naive +wounded)
c mix: RT mixture without RNA addition
n3: 3 day old naive skin
w3: 3 day old wounded skin
w1: 1 day old wounded skin

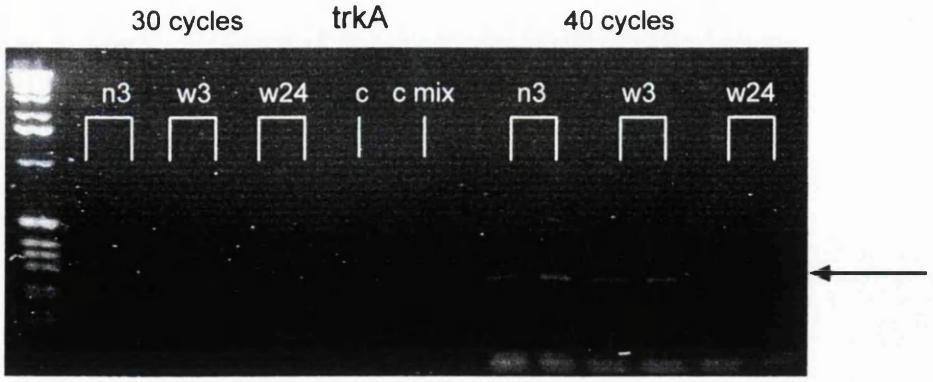


Figure 42: Photograph of a 1% ethidium bromide stained agarose gel of RT-PCR for trkA. Both naive and wounded 3 day old skin show expression at 40 cycles, but there is no expression for 1 day old wounded skin. No expression is seen at 30 cycles.

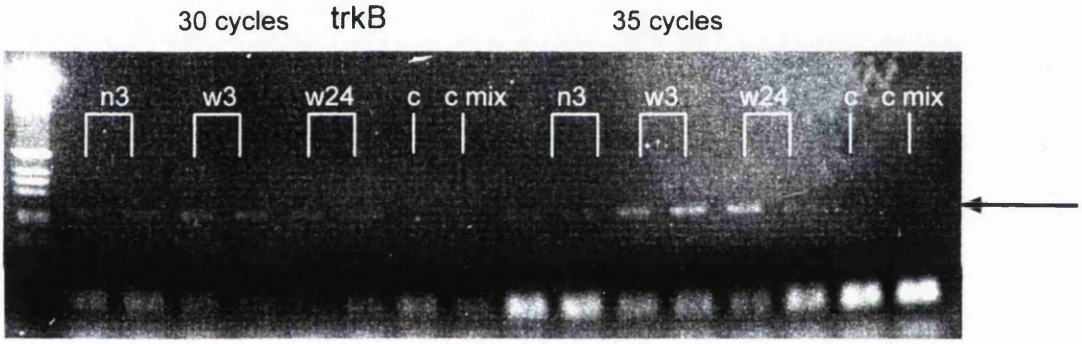


Figure 43: Photograph of a 1% ethidium bromide stained agarose gel of RT-PCR for trkB. All samples show the cDNA for trkB, at both 30 and 35 cycles of PCR. Some increased expression is noted in 3 day old wounded lanes at both cycle amplifications.

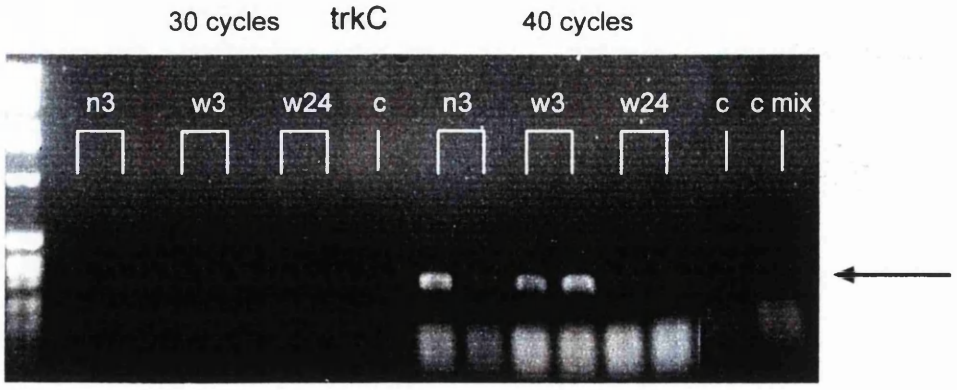
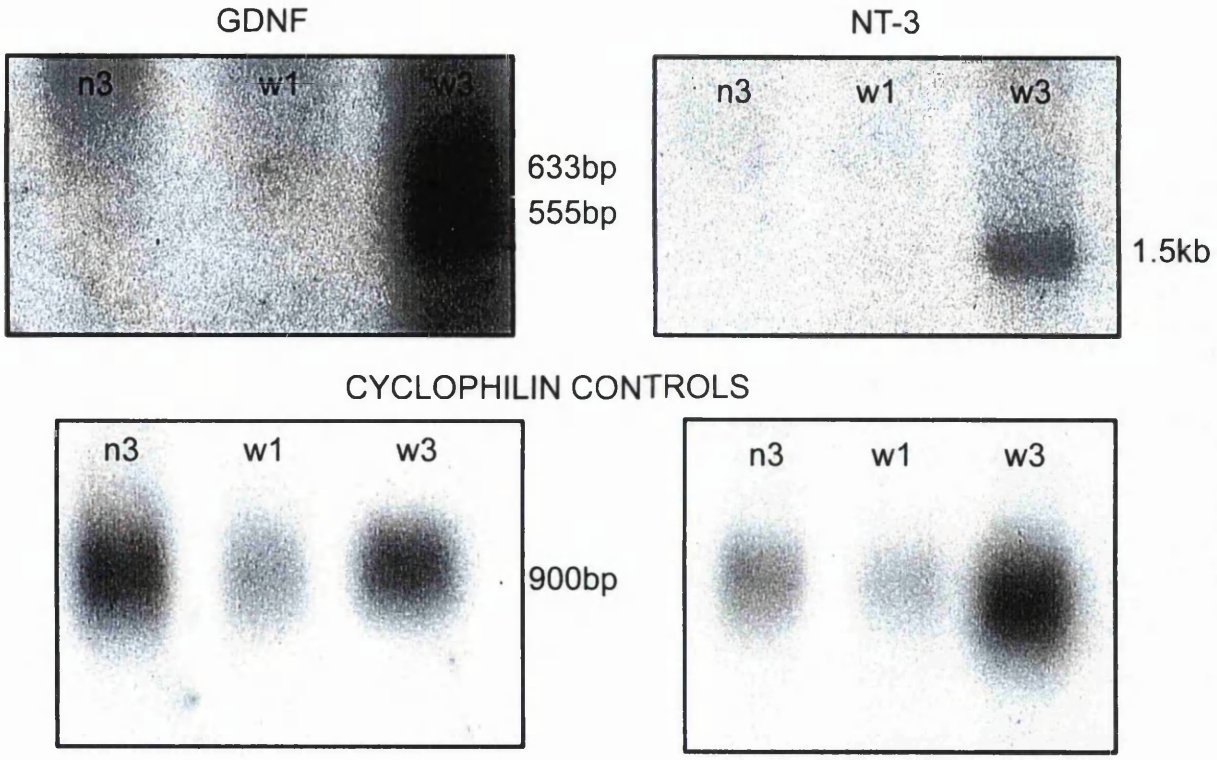


Figure 44: Photograph of a 1% ethidium bromide stained agarose gel of RT-PCR for trkC. Bands are only seen at 40 cycles of PCR in all samples.

(For key see previous page)

NORTHERN BLOTS FOR NT-3 AND GDNF



Where n3 represents RNA from 3 day old unwounded skin, w1 RNA from 1 day old wounded skin,and w3 RNA from 3 day old wounded skin.

Figure 45: Northern blots for GDNF and NT-3 show upregulation of mRNA for both species in 3 day old wounded skin. Both the 633bp and the 555bp transcript for GDNF are upregulated as is the 1.5 kb transcript for NT-3. Cyclophilin reprobing of the Northern blots demonstrates loading differences.

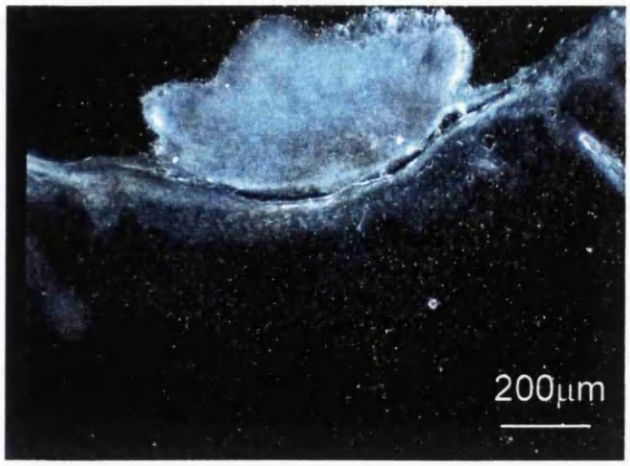


Figure 46: GDNF oligonucleotide non-specific probe binding to P3 wounded section.

Figure 47: GDNF probe binding to P3 naive skin. No marked difference from background levels in the epidermis or dermis.

Figure 48: GDNF probe binding to P3 wounded skin. There is some upregulation in the epidermis, and scar tissue.

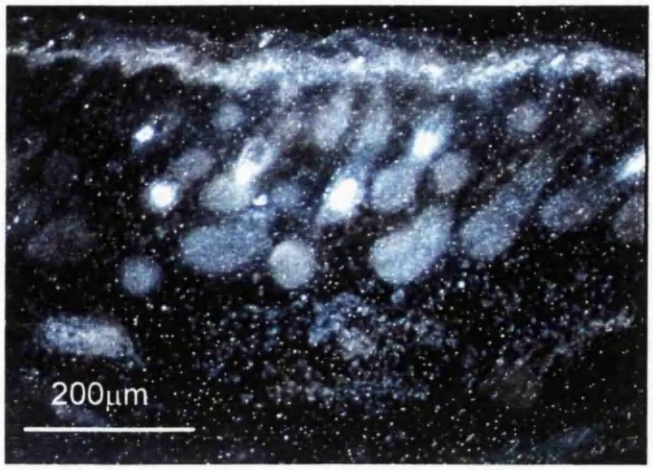


Figure 49: showing the distribution of NT-3 mRNA in naive 3 day old rat hindpaw skin. There is labelling within the epidermis and dermis, and around hair follicles.

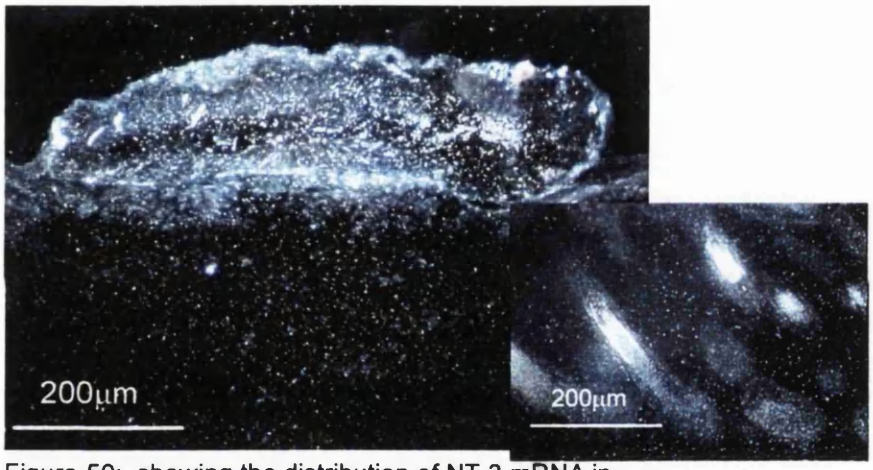


Figure 50: showing the distribution of NT-3 mRNA in wounded skin of 3 day old rat pups. Dense labelling is present within the scar tissue above the wound and some within the dermis below. Inset: Hair follicles also show high labelling.

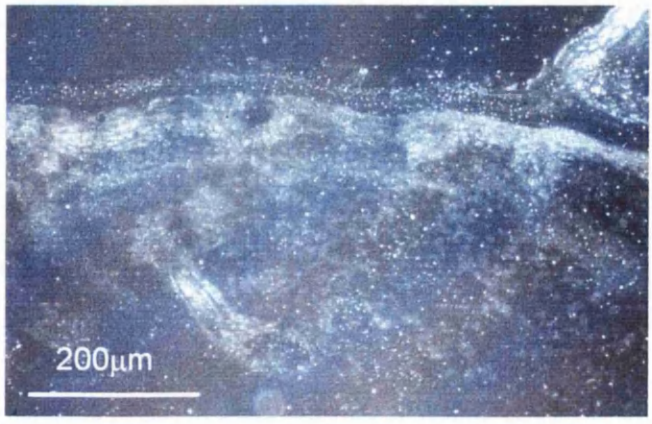


Figure 51: showing non-specific binding with NT-3 oligonucleotide around the wound edge of a 3 day old rat pupinjured at birth.

4.4.1d) ELISAs show that NT-3 but not GDNF protein is upregulated following wounding

Figures 52 & 53 demonstrate the results of ELISAs for NT-3 and GDNF (n=6-8 for each age and tissue state).

NT-3 protein was present in all tissue examined. It was greatly and significantly increased in the skin of wounded animals 3 days post wounding, to 3.5 times normal values ($p=0.0003$ T test Prism version 3). Levels dropped back to control values by P5. Naive tissue showed little change over this time period.

GDNF protein was present in all tissue examined. There was no difference between levels in naive and wounded skin samples, and no change over the P3 to P7 time period.

4.4.1e) Immunohistochemistry shows cellular distribution of NT-3 in skin

NT-3 like immunoreactivity was observed in both naive and 3 day post wound skin of rat pups. In naive animals, there was a clear band in the suprabasal epidermis, which stretched throughout this layer. On close examination this band consisted of densely labelled flattened cells, presumably keratinocytes. In addition, a lighter band of labelling was seen in the basal epidermis (see figures 54-58), which again stretched across the entire sectional layer. All sections exhibited such labelling to varying degrees (n=4 animals). Hairy and glabrous sides were labelled in the same manner, but with darker staining consistently within the glabrous side (see figures 55 and 58). Hair follicles were also labelled (see figure 54 inset).

In wounded tissue the labelling was also consistently found in the same two layers, but the suprabasal layer was less heavily stained and in fact very little if any staining could be seen closest to the wound margins. The newly formed suprabasal layer of the wounded epidermis was not labelled at all. The basal lighter band again was fainter and diminished towards areas near the wound. However, there was dense labelling of the remaining scar tissue above the area of the wound (see figure 56). In addition, in all animals studied (n=4), there was a patch of cells labelled in the superficial dermal layers directly under the wound (see figure 57). These cells only appeared in 2-3 sections from each animal, but were always present in an area directly under the wound and never in naive sections.

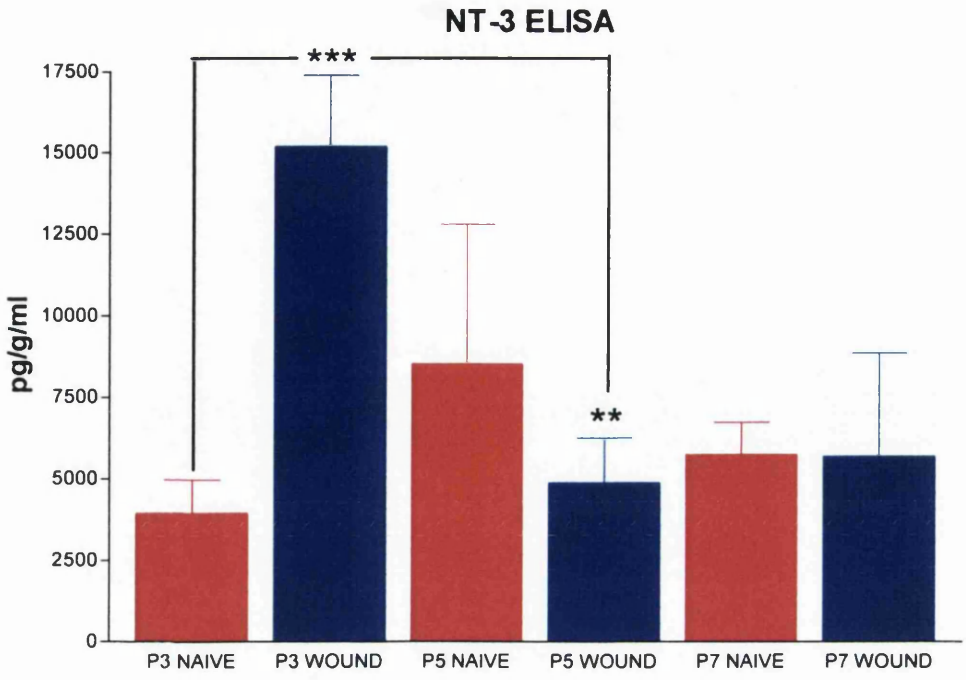


Figure 52 showing the levels of NT-3 protein in naive and skin wounded at P0 from thedorsal hindpaw of P3, P5 and P7 rats. Students T test and ANOVA reveals a significant increase between control P3 skin and wounded P3 skin (P=0.0003, t=5.222, DF=11) and a significant decrease back to control values, between 3 and 5 days after wounding (P=0.0074, t=3.724, DF=7). There is no difference between naive values at various developmental ages.

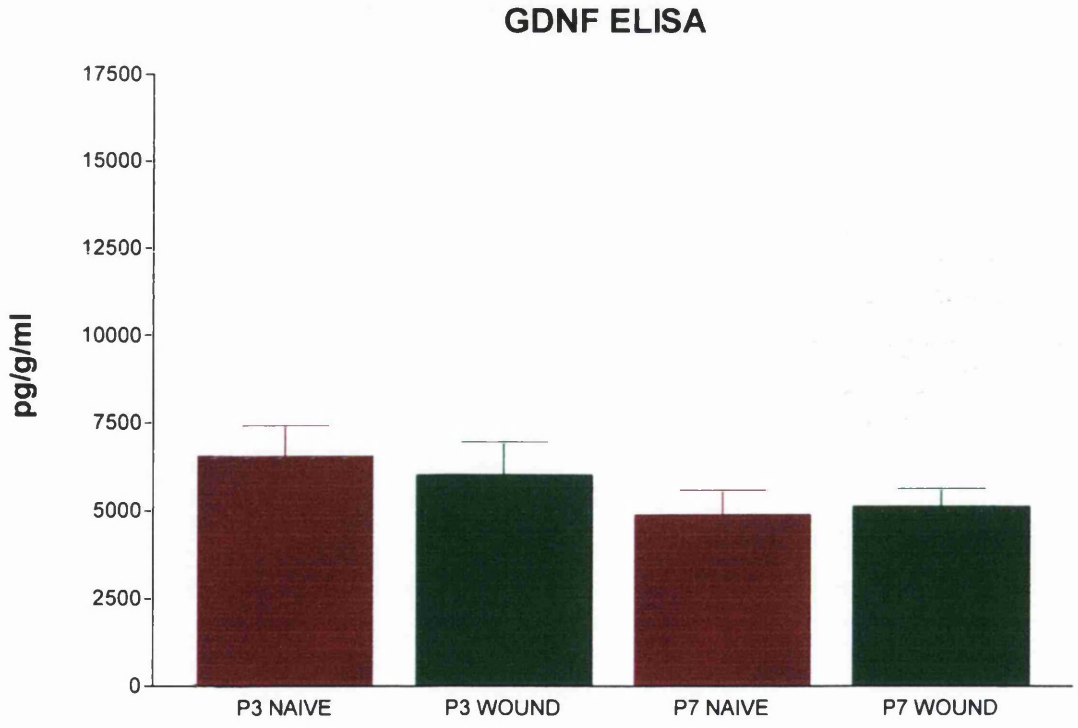


Figure 53 showing the GDNF protein content in P3 and P7 skin in both naive and wounded states. Students t test reveals there is no significant difference between any of the samples.

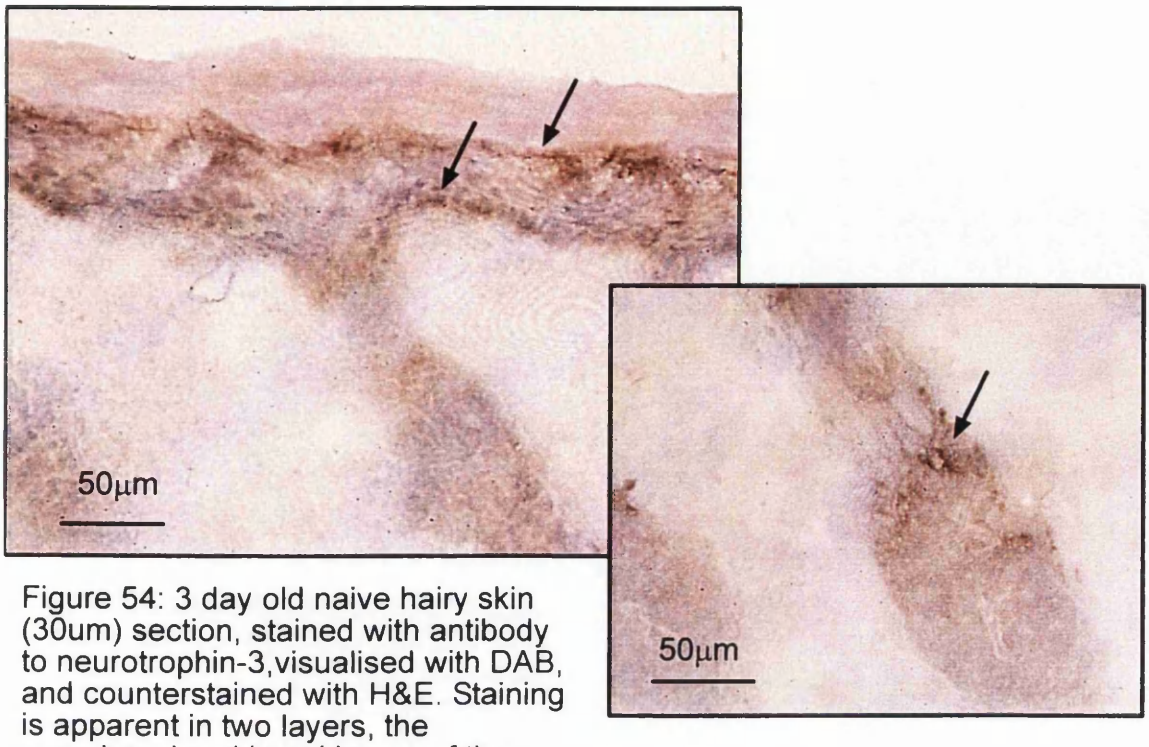


Figure 54: 3 day old naive hairy skin (30um) section, stained with antibody to neurotrophin-3,visualised with DAB, and counterstained with H&E. Staining is apparent in two layers, the suprabasal and basal layers of the epidermis. Inset shows a hair follicle end bulb showing a-NT3 labelling at higher magnification.

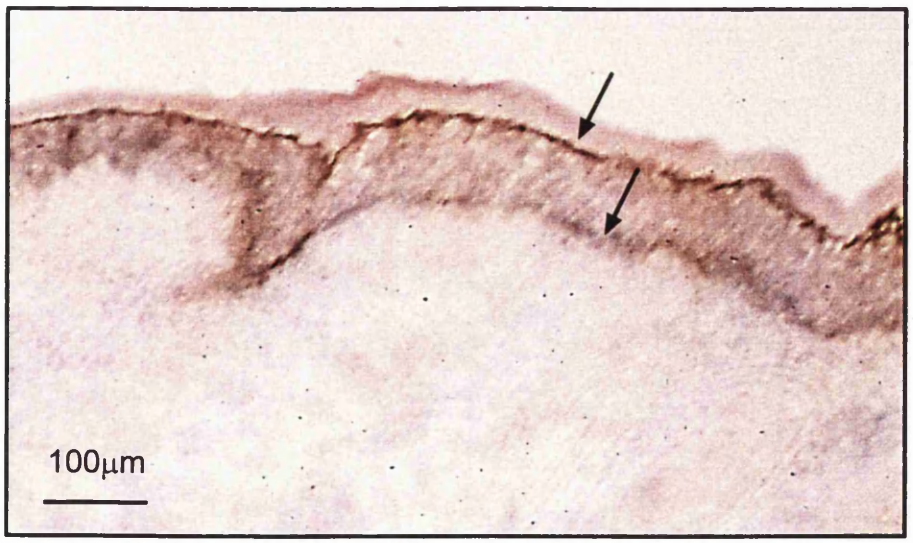


Figure 55: 3 day old naive skin, glabrous side of section above, showing a darker labelling of the two layers.

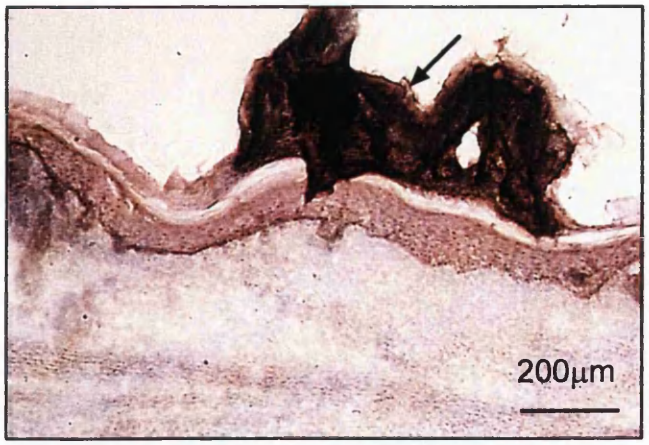


Figure 56: 3 day old hairy skin wounded at birth stained with a-NT3. Note the densely stained scab over the wounded area, the absence of hair follicles, and the double layer of NT-3 immunolabelling.

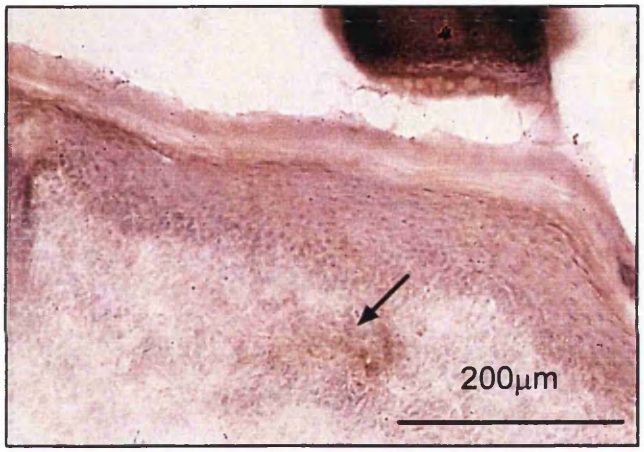


Figure 57: 3 day old hairy skin wounded at birth, showing a patch of immunopositive cells labelled for NT-3 in the dermis under the wound.The double layer labelling is fainter under the wound.

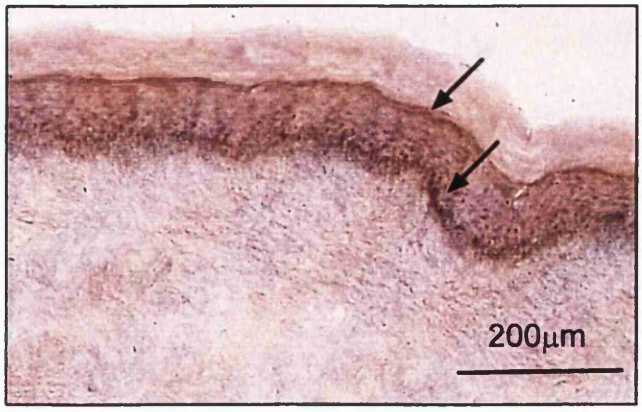


Figure 58: 3 day old glabrous skin from a rat hindpaw wounded on the hairy side at birth. Again as with naive glabrous tissue, NT-3 immunolabelling is darker.

4.4.2 FUNCTIONAL NEUTRALISATION OF NEUROTROPHINS

4.4.2a) GDNF and NT-3 antibodies reduce neurite outgrowth in DRG/ wounded skin co-cultures

Both naive and wounded 3 day old skin co-cultures resulted in a robust non-directional outgrowth from P0 lumbar DRG. Figures 59-69 show the effect of antibody to NT-3 or antibody to GDNF on neurite outgrowth from newborn DRG co-cultured with wounded or control skin.

Antibody to GDNF (500ng/ml) caused a significant decrease in neurite outgrowth in ganglia co-cultured with wounded skin ($P=0.0489$, $t=2.045$, $df=33$, $n=16$, unpaired two-tailed Student's t test Prism version 3). No effect of GDNF antibody was observed in naive skin co-cultures (see figures 59-63).

Antibody to NT-3 produced a significant reduction in outgrowth from wounded skin only, at both concentrations used ie. 2ng/ml and 200ng/ml, $P=0.0393$, $t=2.193$, $df=36$, $n=9$-26 for 2ng/ml of antibody and $P=0.0330$, $t=2193$, $df=50$, $n=10$-28 for 200ng/ml of antibody, (unpaired two-tailed Student's t test Prism version 3) (see figures 66-67 & 69). No significant effect of NT-3 antibody was seen in naive skin co-cultures (see figures 64-65 & 68).

4.4.2b) Trk C receptor body injections did not affect hyperinnervation following wounding

Injection of TrkC-IgG receptor bodies (5mg/kg) into newborn rat pups, caused no observable change in the degree or appearance of hyperinnervation (see figures 70 & 71). Saline controls and trkC-IgG treated wounds were hyperinnervated to an equal extent. Hyperinnervation extended into the epidermis, and exhibited similar morphology in control and treated tissue. Extent of innervation density in the dermis and epidermis were blind-scored as described in the methods. Figures 72-74 show the absence of any effect on hyperinnervation. A second injection of trkC-IgG was still ineffective at blocking hyperinnervation (see figures 72-74).

4.4.2c) Hyperinnervation after wounding is observed in BDNF knockout mice

Figures 75-76 clearly demonstrate hyperinnervation is still present in BDNF knockout mice. Morphologically, the hyperinnervation is similar to that of wild type and

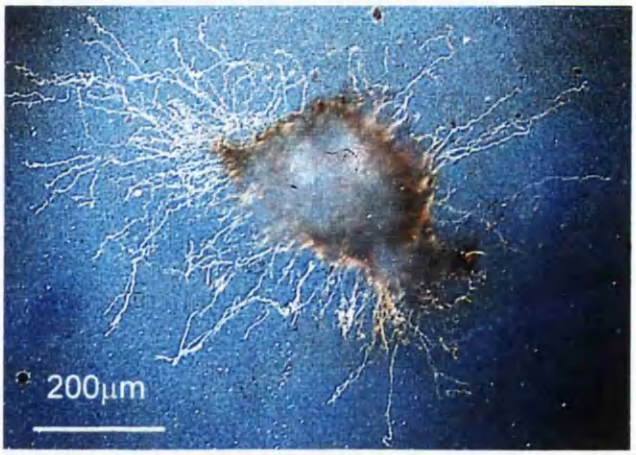


Figure 59:
P0 DRG co-cultured with unwounded 3day old dorsal hindpaw skin.

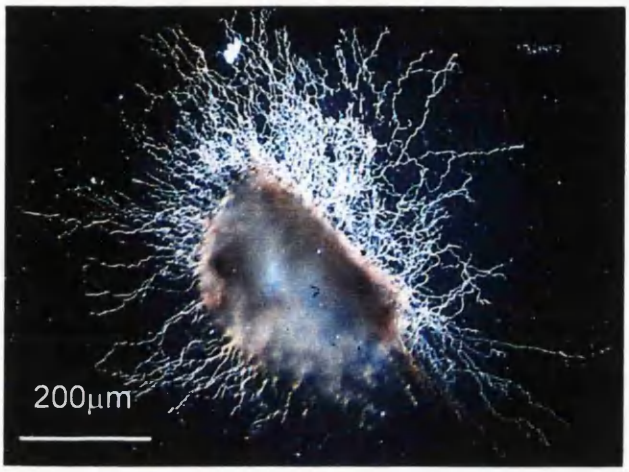


Figure 60:
P0 DRG co-cultured with unwounded 3 day old dorsal hindpaw skin, with the addition of 500ng/ml of antibody to GDNF. Neurite outgrowth is clearly visible and unperturbed.

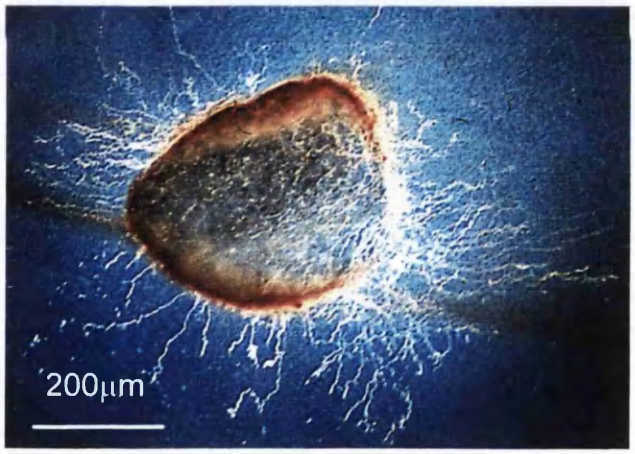


Figure 61:
P0 DRG co-cultured with 3 day old skin from the dorsal hindpaw of a rat pup wounded at birth.

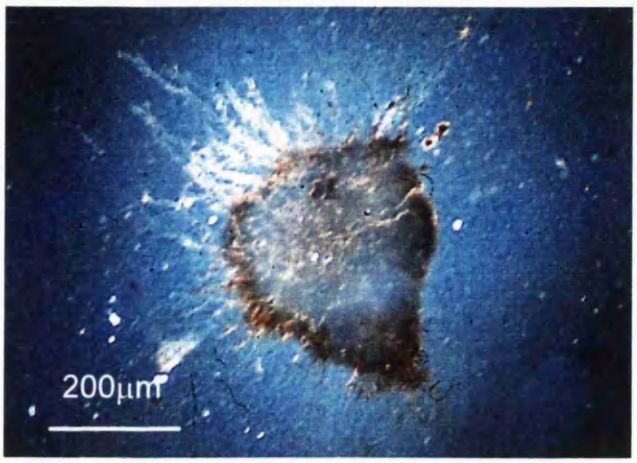


Figure 62:
P0 DRG co-cultured with 3 day old skin from the dorsal hindpaw of a rat pup wounded at birth, with the addition of 500ng/ml antibody to GDNF. There is marked loss in neurite outgrowth.

Due to magnification, skin pieces are out of the field of view.

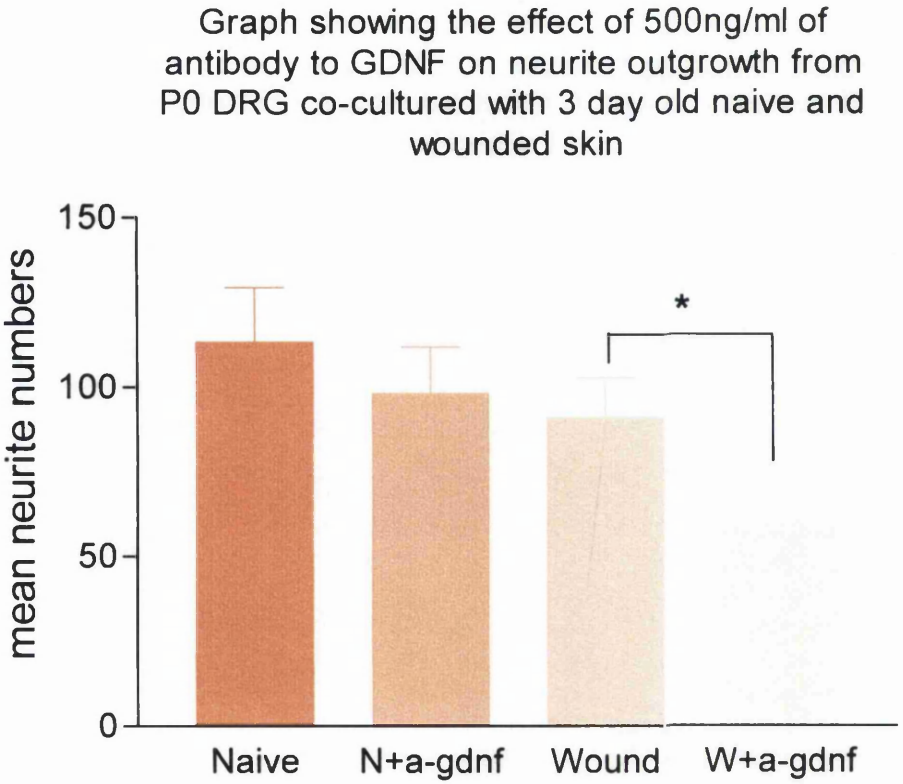


Figure 63: P0 DRG co-cultured with wounded skin produces neurite outgrowth which can be partially blocked by antibody to GDNF, (P=0.0489,t=2.045, df=33, n=17-18). Neurite outgrowth in naive skin co-cultures is not affected by antibody to GDNF.

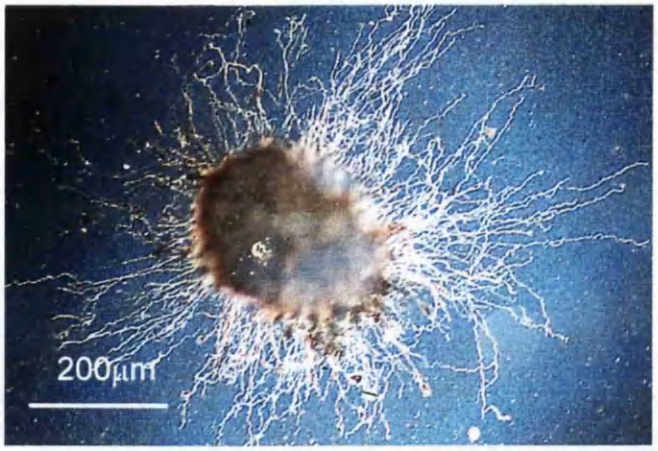


Figure 64:
P0 DRG co-culture with naive 3 day old skin stained with PGP9.5 and photographed under dark field. Numerous long neurites can be seen radiating from around the DRG.

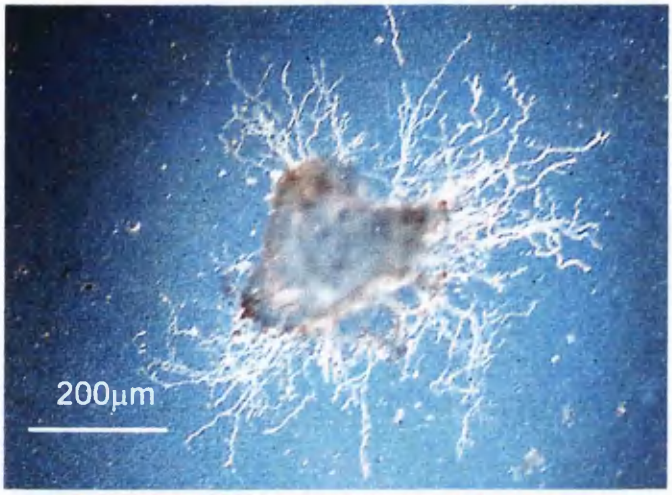


Figure 65:
P0 DRG co-cultured with 3 day old naive skin and 200ng/ml antibody to NT-3. No significant change in neurite outgrowth is observed.

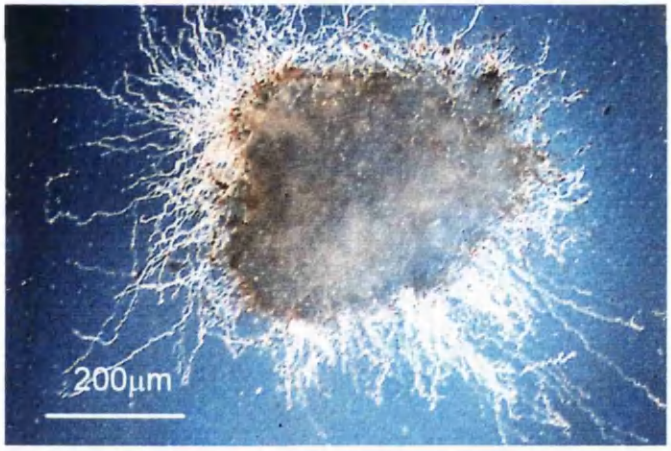


Figure 66:
P0 DRG co-cultured with 3 day old wounded skin.

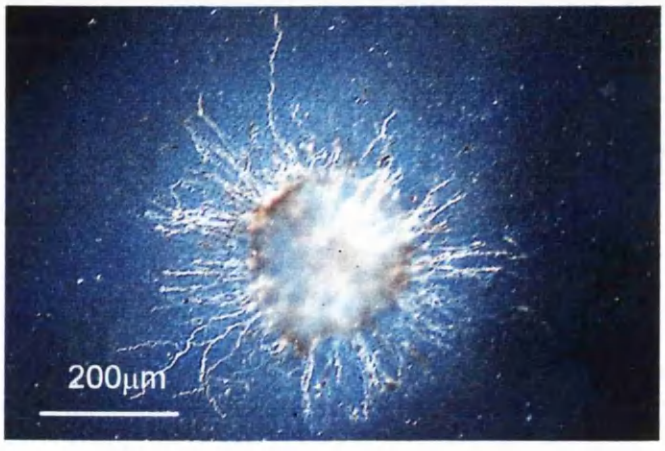


Figure 67:
P0 DRG co-cultured with 3 day old wounded skin and 200ng/ml antibody to NT-3. Fewer neurites are seen.

Due to magnification, skin pieces are out of the field of view.

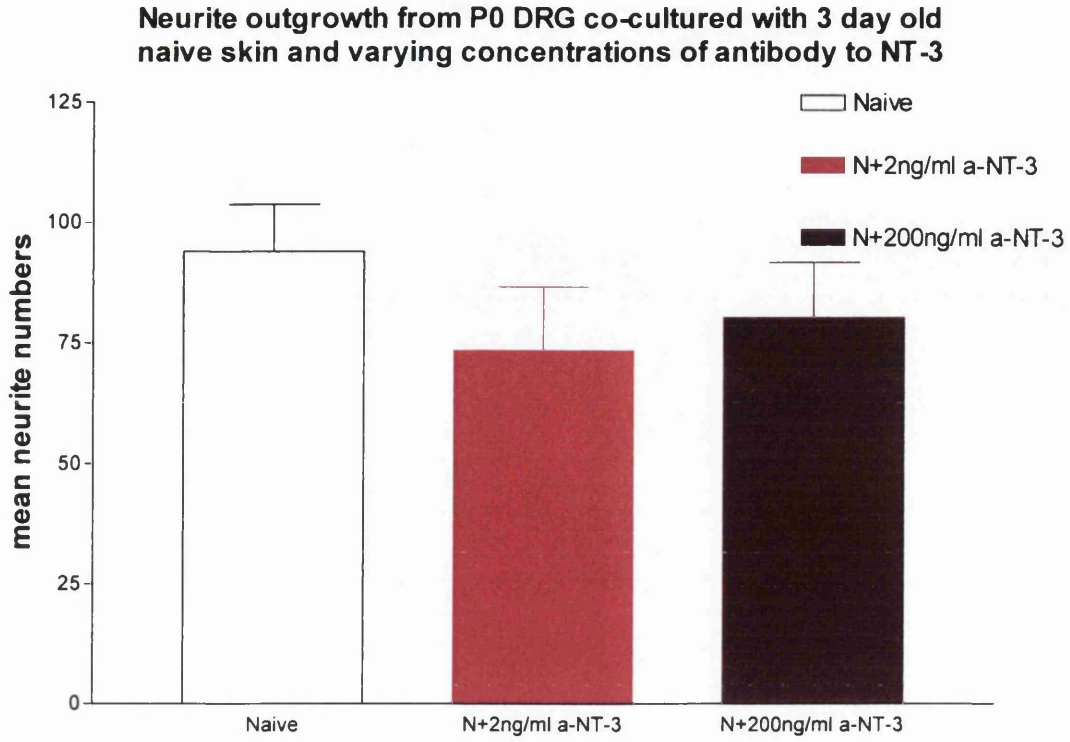


Figure 68 shows neither 2ng/ml or 200ng/ml of neutralising antibody to NT-3 affects the outgrowth from newborn DRG co-cultured with naive P3 skin.

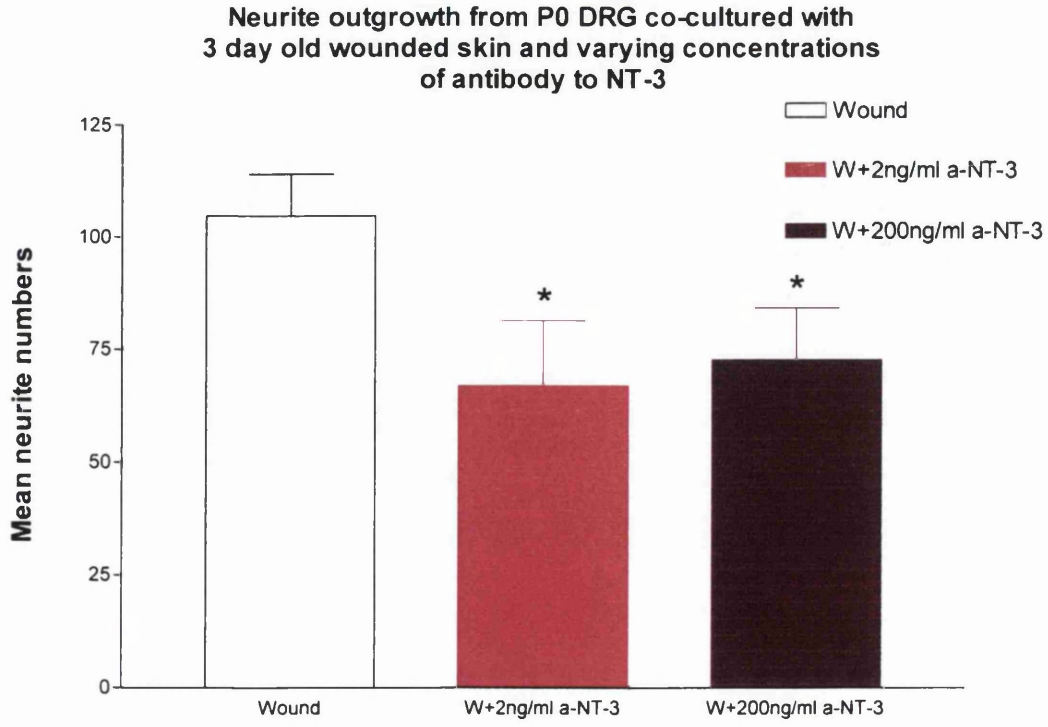


Figure 69 showing that neurite outgrowth from newborn DRG co-cultured with P3 wounded skin is significantly reduced by both 2ng/ml and 200ng/ml of neutralising antibody to NT-3 (P=0.0393, t=2.193, DF=36 for 2ng/ml, and P=0.0330, t=2.193, DF=50 for 200ng/ml, t test Prism).

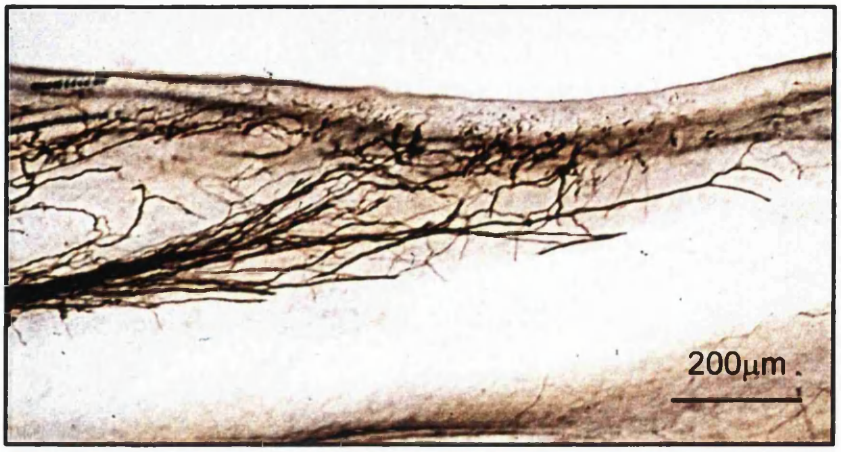


Figure 70:

P7 rat dorsal hind-paw, skin section from an animal injected i.p with saline prior to and again 3 days after wounding at P0.
100μm section stained with PGP9.5.
Dense hyperinnervation in the dermis and fibres extending into the epidermis from thick fascicles.

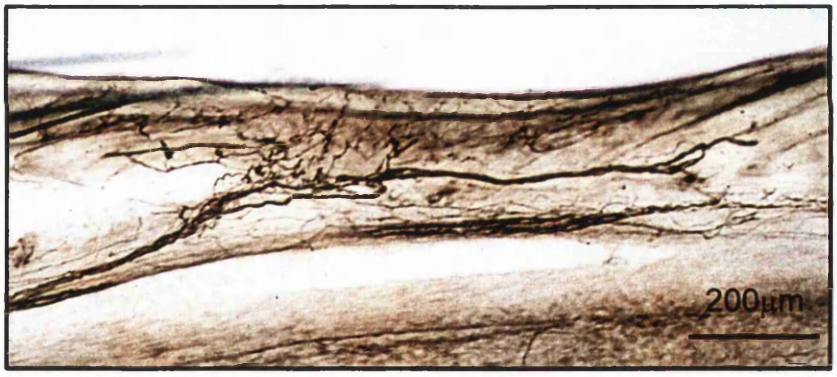


Figure 71:

P7 rat dorsal hind-paw skin section, from an animal which received trkCIgG fusion body injections i.p prior to and again 3 days after wounding at P0.
100μm section stained with PGP9.5.
Again hyperinnervation is evident with thick dermal fasicles giving riseto branches within thedermis and entering the epidemis are visible.

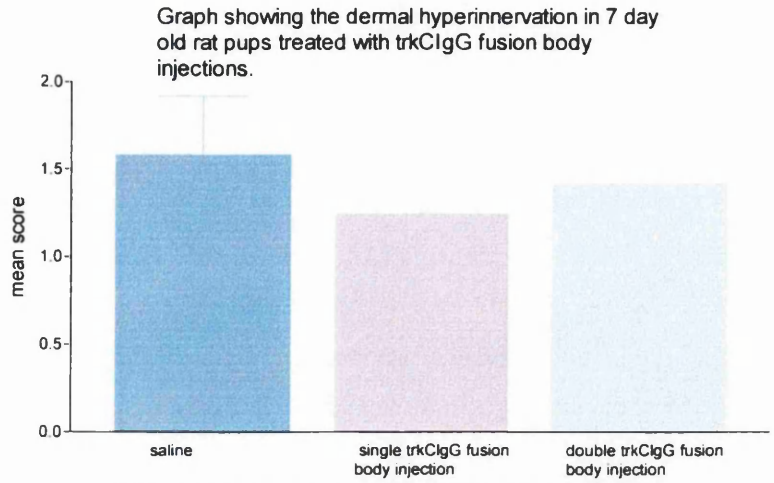


Figure 72 demonstrates that hyperinnervation within the dermis in animals which were given injections of trkCIgG fusion bodies prior to wounding is the same as those given saline injections .

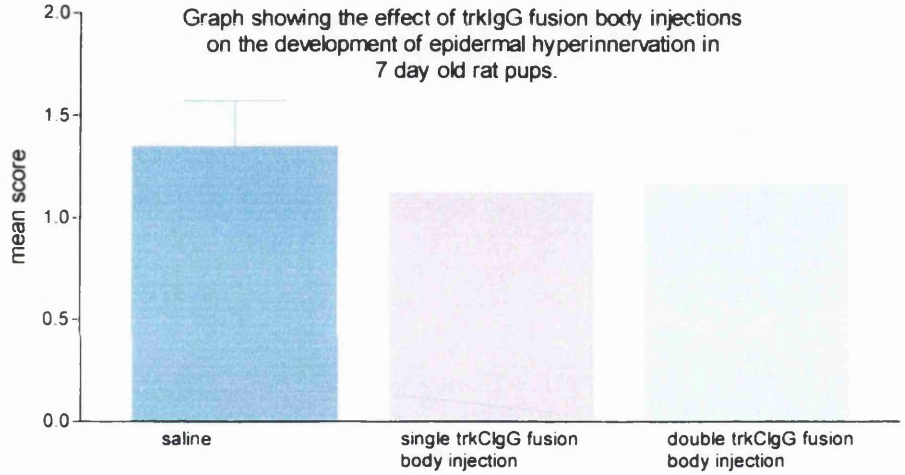


Figure 73 illustrates no difference between the epidermal hyperinnervation seen in saline injected wounded rat pups and trkCIgG injected rat pups.

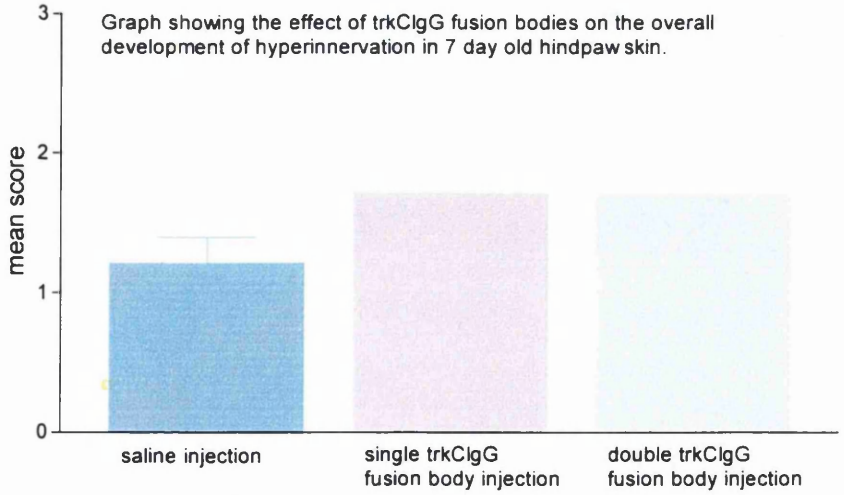


Figure 74 shows that there is no change in the overall levels of hyperinnervation in 7 day old rat pups which had been injected with trkCIgG fusion bodies.

heterozygous mice, although the number of sections obtained for knockout mice were fewer, and reflected the thin skin and smaller phenotype of the animal.

Generally, the hyperinnervation in mice differed in its morphological pattern from that in rats. In all mice (wild type, heterozygote and homozygote) fibres were not as fasciculated as in rats and curved into the wounded area with erratic trajectories within the dermis. Incoming fibres appeared from both proximal and distal deep bundles in all mice examined, which is never seen in wounded rat pups (see figure 76).

Figure 75: P6 contralateral hindpaw skin section stained with PGP9.5 showing normal innervation of the skin of a wild-type mouse, with numerous nerve fibers around the ends of hair follicles entering the epidermis.

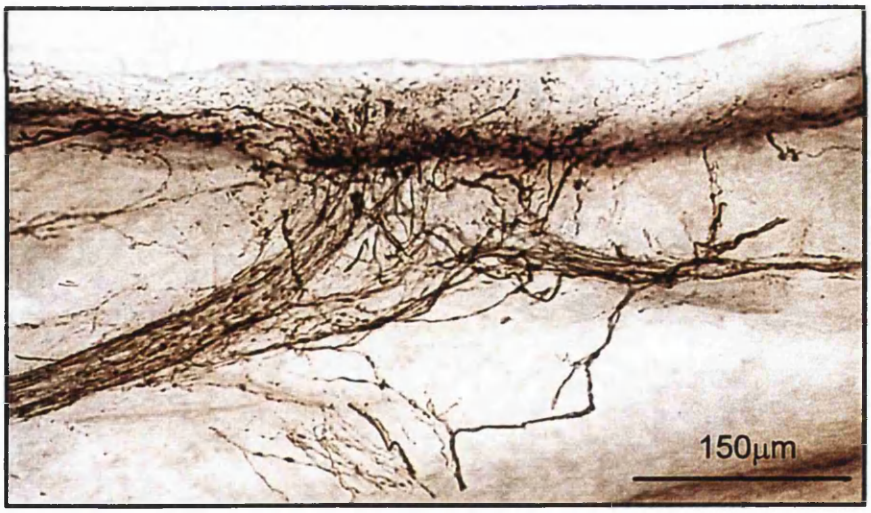


Figure 76: Wild type P6 mouse wounded at birth. The section (stained with PGP9.5) through the dorsal foot wound shows similar hyperinnervation to that of the rat, with deep coarse large bundles in the dermis, giving rise to finer proliferative sprouts into the epidermis.

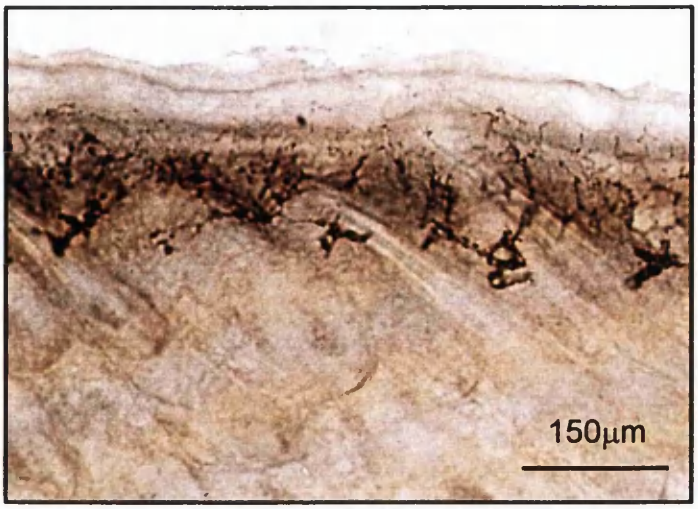


Figure 77: Contralateral unwounded hairy skin, from the dorsal hind-paw of a BDNFknock-out mouse, 100μmsection stained with PGP 9.5. Fine, branching fibres are seen encasing the tips of hair-follicles as shown by the arrow,and a deep thick nerve bundle is visible in the epidermis.

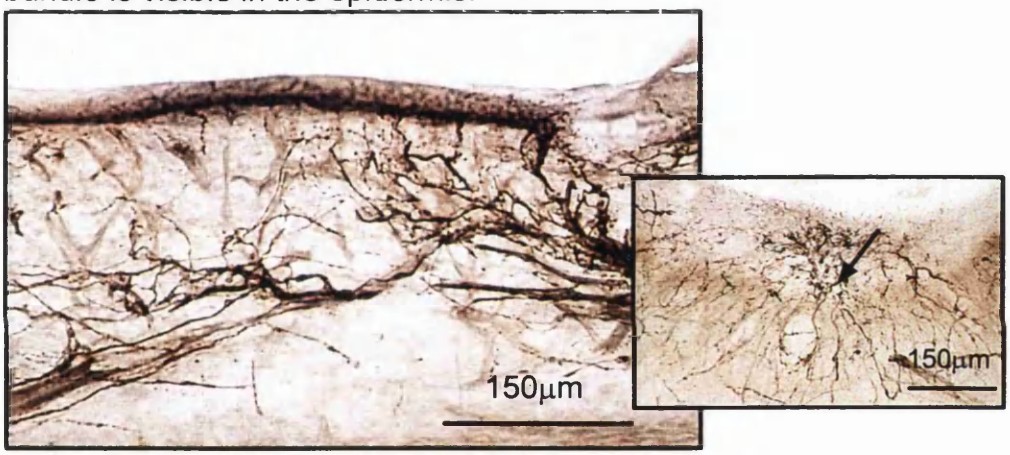


Figure 78: Ipsilateral wounded hairy skin, from the dorsal hind-paw of a BDNF knock-out mouse,100μm section stained with PGP9.5. Hyperinnervation comparable to the wild type animal is evident. Inset shows in some sections many fibres are seen within the dermis arising from deeper nerve bundles, and non-fasciculated within the dermis, penetrating and branching into the epidermis. The fibres are more chaotic than those seen in rat hyperinnervation (Chapter 2 figure 11).

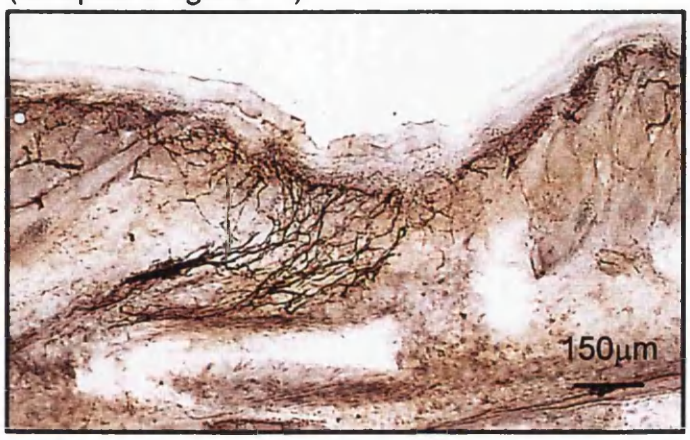


Figure 79: Heterozygous BDNF mutant mouse hairy skin section stained with PGP9.5. Again hyperinnervation is clearly present and resembles that of the wild-type. Note again the irregular nature of the outgrowth.

4.5 DISCUSSION

Skin wounding in neonates, in which neurons are in the process of finalising their peripheral patterns of innervation can lead to disruption of the normal levels of neurotrophins and other regulatory molecules, which may cause the permanent aberrant innervation depicted in this thesis. In the adult, these molecules are also disturbed leading to transient hyperinnervation after wounding, but since the initial state of tissue innervation is more static, reversal to the baseline state can occur.

Interesting links may be found in the association of hyperinnervation in the neonate to adult human neuropathy and psoriasis, all inflammatory lesions with associated immune responses. In the former clinical condition, nerves fail to re-innervate an inflamed area after denervation, although NT-3 increases in the skin (Kennedy et al. 1998). In the other two situations nerves are prolific (Jiang et al. 1998). NGF is also decreased in diabetic skin and increased in skin wounds (Anand, 1996).

In this chapter, the mRNA and protein expression of the neurotrophins BDNF, NT-3 and GDNF have been assessed by RT-PCR and *in situ* hybridisation, Northern blotting, ELISA and immunohistochemistry.

It has also been shown that neurotrophin levels are altered after wounding to the skin. BDNF expression is very low and undetectable at the mRNA level by Northern blots, but GDNF is upregulated at the mRNA level, but not at the protein level in wounded skin. NT-3 is both upregulated at the mRNA and protein level.

The role of GDNF and NT-3 in neurite outgrowth from newborn DRG co-cultured with wounded skin has also been examined. Both antibody to NT-3 and antibody to GDNF is able to partially block the neurite outgrowth exhibited by newborn DRG in wounded skin co-cultures.

In addition, *in vivo* studies reveal no change in occurrence or timing of hyperinnervation in BDNF knockout mice or rats treated with trkC IgG fusion bodies. This suggests firstly that BDNF is not necessary for the phenomenon to occur, and secondly that normal levels of NT-3 are also not critical. This is assuming that the fusion bodies are an effective means of blocking the action of NT-3, and are able to work *in vivo*.

The role of each neurotrophin in hyperinnervation will be discussed in turn with reference to the experimental evidence presented in this chapter. Some clinically relevant situations will also be examined.

4.5.1 BDNF AND SKIN SENSORY INNERVATION

BDNF levels within the skin increase embryonically up to E12, and then decline (Buchman & Davies, 1993). BDNF neurite growth morphology in culture has been suggested to be of a similar response compared to NT-3 i.e. with some degree of branching. But BDNF was also shown to promote a marked lamellipodial response along distal axon shafts (de Castro et al. 1998). Such neurons were seen in dissociated cultures in section 3 and a closer examination may reveal the degree of participation of BDNF in neurite outgrowth in these cultures.

Transgenic animals lacking BDNF have no loss of the myelinated axons innervating the hairy skin of the hindpaw compared with wild-type animals (see Lewin et al. 1996), but there is conflicting reports of the effects of BDNF overexpression. Overexpression has been reported to cause hypoinnervation of the epidermis (Rice et al. 1998) as do p75 knockouts (Bergmann et al. 1997) with concomitant decrease in skin thickness. Others have reported a decrease in innervation in BDNF, trk B and NT-4 overexpressors, although bush endings increase in size and density (Rice et al. 1998). Palisades of lanceolate endings that terminate below the sebaceous glands of hair follicles are decreased in both BDNF and trkB knockouts (Fundin et al. 1997). BDNF and NT-4 are thought to be inhibitory to innervation within the dermis and epidermis respectfully (Rice et al. 1998).

In contrast, Le Master et al. 1999 recently showed that BDNF overexpressing mice have an increased peripheral innervation density, especially with reference to hair follicles, with enlarged Meissners corpuscles overlying sweat glands in glabrous skin, and increased Merkel cell innervation at P7. An increase of the truncated trkB receptor was also noted. But, it was also interesting to find that there was a reported retraction of epidermal small unmyelinated fibres during development (Le Master et al. 1999).

BDNF may be important for regulation of sympathetic fibres, for example BDNF heterozygotes and knockouts show hyperinnervation of the sympathetic fibres within the pineal gland (Kohn et al. 1999).

BDNF is required for the mechanical sensitivity of SA-MR's, but not for postnatal survival of myelinated cutaneous afferents (Carroll et al. 1998).

4.5.1a) BDNF is unlikely to be a candidate in initiating or maintaining wound-induced hyperinnervation

RT-PCR clearly demonstrated it's presence within naive and wounded skin as cDNA, and consistently shows a qualitative difference between the two (see figure 39). However, Northern analysis failed to detect either of the two transcripts for BDNF mRNA in all

samples, even though both transcripts are increased in nerves in human neuropathies, and correlate with the degree of macrophage and T cell invasion (Sobue et al. 1998).

Levels of BDNF mRNA and protein are thus very low and unlikely to be involved in hyperinnervation. The trkB receptor which mediates the actions of BDNF is expressed in all tissues, both naive and wounded, as shown by RT-PCR and some regulation is seen (see figure 43). Further studies on trkB mRNA expression and regulation following wounding may shed more light on the role of BDNF.

In addition, in our study BDNF knockout mice wounded at birth and examined at 6 days exhibit hyperinnervation, as do heterozygotes (see figures 78 & 79) in a normal time frame of analysis.

On observation the hyperinnervation in mice is somewhat different to that of rats, such that fibres tend to be more tortuous, curvaceous and less directed than those seen in rat skin wounding (see figures 76 & 78). The augmented branching and defasciculation (see figure 78) in the dermis of BDNF knockout animals may be due to the loss of BDNF inhibition.

In addition, new growth of fibres into the wounded area is bi-directional, giving the appearance of sprouting at both ends of a cut nerve.

It may indeed be, that a down-regulation in the expression of BDNF may be a causal factor since BDNF is inhibitory to dermal innervation (Rice et al. 1998). Ishida et al. 1984 noted that BDNF in the epidermis was repulsive to matured nerve endings. However, we found no evidence of this. Thus in conclusion, this factor is also unlikely to be responsible for the hyperinnervation.

4.5.2 GLIAL CELL-LINE DERIVED NEUROTROPHIC FACTOR AND SKIN SENSORY INNERVATION

GDNF is confined to the mesenchyme in early development (Hellmich et al. 1996), and since chapter 3 indicated the source of activity responsible for increased neurite outgrowth arising from the epidermis, GDNF may have other roles here. GDNFβ is expressed in peripheral nerves of the neonatal rat (Widenfalk et al. 1997), and its receptor expression as well as that of the other GDNF receptors, may yield more data on the regulation and location of any responsive neurons and the effect of injury on the GDNF responsive population in neonates.

During development, GDNF seems to take over the role of NGF postnatally, by its requirement in the final patterning of nerve endings within the epidermis (Fundin et al.

1999). Thus GDNF may be involved after the hyperinnervating nerves have grown into the dermis and are making contact with the epidermis (P5-6) i.e. affecting axon terminals rather than axon growth, chemotaxis and survival. Adult heterozygotes for GDNF display a decrease of Aβ sensory endings within the inner conical body of the hair follicle complex, with correlated decrease in RT97-IR. Aβ-mechanoreceptors (transverse lanceolate and reticular endings) are also affected (CGRP fibres are unaffected). Reticular endings show a decreased branching also. However GDNF knockouts, which die rapidly display a normal appearance of immature endings, suggesting that the GDNF switch of dependency is most likely postnatally (Fundin et al. 1999). More developmental switching can be observed in vestibular ganglion axons which switch from BDNF to GDNF for neurite outgrowth, after target innervation (Hashino et al. 1999). GDNF may also be involved in the fasciculation of fibres in the area of hyperinnervation since it is responsible for fasciculated growth in chick sympathetic neurons (Trupp et al. 1995).

GDNF can enhance axonal sprouting and hyperinnervation of neuromuscular junctions (Nguyen et al. 1998). In the CNS at least there seems to be a redundancy for neurotrophins such that more than one neurotrophin can support various neuronal populations. NT-3 can act on its own, but GDNF requires the co-operation of BDNF for the survival of axotomised corticospinal neurons (Giehl et al. 1998). Therefore GDNF may serve as an adjuvant or cofactor in many situations.

In cultures of adult DRG explants GDNF has been shown to promote the growth of IB4 positive axons as well as RT97 positive axons (Bennett et al. 1998; Leclere et al, 1998), both of which contribute to hyperinnervation.

GDNF is thought not to play a role in the development of cutaneous sensory neurons in rodents since mRNA is not detected in uwounded neonatal rat skin (Henderson et al. 1994). This is confirmed by our findings here. The fact that embryonic chick neurons become responsive to GDNF with age (Buj-Bello et al. 1995), but without increases in mRNA also implies that neurons themselves may intrinsically govern their state, or that receptor alterations on neurons may allow greater responsiveness without neurotrophin change. Tissue state may alter expression for example, GDNF mRNA for the 633bp transcript is also known to be increased in denervated muscle (Lie & Weis, 1998), and in our wounded skin.

A most exciting recent study by Batchelor et al, 1999 has shown that GDNF may be involved in the sprouting of neurons observed in wounded striatum, mRNA being

expressed by activated macrophages and microglia which accumulate at the site of injury. Macrophages are also seen to invade CNS wounds between 1-3 days post injury, which correlates well with the appearance of sprouting at later time points. Hence, skin wounding may also cause an upregulation of GDNF mRNA in macrophages.

4.5.2.a) GDNF is unlikely to be a candidate in initiating wound-induced hyperinnervation

In our study, RT-PCR demonstrated the presence of GDNF in neonatal unwounded and wounded tissue. Some qualitative degree of regulation was again observed but unquantifiable and indefinite. Northern analysis for GDNF mRNA confirmed this regulation, with an increase in the two GDNF transcripts, the 633bp and 555bp only at three days after wounding. In situ hybridisation studies showed an increase of GDNF in general, with increased localisation to areas of the epidermis and scar tissue of the skin (see figure 48). Further analysis by grain counts over certain areas e.g. epidermis, dermis, scar tissue etc. may yield greater insight.

GDNF ELISAs did not reveal an overall change in GDNF protein in wounded as apposed to naive tissue at 3 or 7 days post wounding. The lack of increase of GDNF even with an increase in mRNA may reflect a general increase in the production of neurotrophin mRNA by cells, an immediate response to injury or that GDNF protein is upregulated at a stage not examined with ELISA i.e. between day 3 and day 7, since levels of transcription and translation into protein can vary from hours to days. NGF mRNA is detected in the target just 12 hours before the protein (Davies et al. 1987; Rohrer et al. 1988).

Our tissue culture studies have shown that antibody to GDNF inhibit some neurite outgrowth in co-cultures of newborn DRG and wounded skin seen. Naive skin co-cultures with antibody to GDNF show no such inhibition (see figure 63), suggesting ELISA studies may have missed the appropriate time point. The intrinsic activity of GDNF protein in wounded skin may be activated by wounding ie. there could be no need for protein upregulation to elicit outgrowth. Culture situations must also bear in mind that different neuronal species respond differently to neurotrophins from within or across species. For example rat GDNF is 3 orders of magnitude less effective on chicken motorneurons than it is on rat (see Buj-Bello et al. 1995).

4.5.3 NEUROTROPHIN-3, A CANDIDATE FACTOR FOR HYPERINNERVATION

Our data supports NT-3 as the most likely neurotrophin involve in hyperinnervation. NT-3 is synthesised in mesenchyme along the pathways of developing sensory and sympathetic axon projections from E10, and could influence the route of such axons (Farinas et al. 1996). NT-3 is required for most sensory DRG neuron development, giving rise to cutaneous afferents, the D hair and SA-mechanoreceptors (which are lost in knockouts) (Airaksinen et al. 1996) whose loss and differentiation occurs in the first postnatal weeks of life. In knockouts there is also a loss of Merkel cells, but only after the 2nd postnatal day. Other innervation is also lacking, with the exception of CGRP positive fibres on the necks of hair follicles in tier 2 of the dermal plexus (Rice et al. 1998). Trk C knockouts show less of a decrease in skin innervation compared to NT-3 knockouts, with a loss of the CGRP positive fibres also, and an increase in non-peptidergic endings in the epidermis in the first two postnatal weeks (Rice et al. 1998).

Unlike NGF expression, NT-3 expression precedes the arrival of sympathetic nerve fibres to the heart suggesting a causal guidance role (see Maisonpierre et al. 1990). However, it is inhibitory to the sympathetic innervation of hair follicles, although being essential for sympathetic innervation (Rice et al. 1998).

NT-3 has been shown to be important for terminal branching postnatally, and heterozygotes show innervation of the skin but no terminal branching (Elshamy et al. 1996). The hyperinnervation observed will have some degree of a branching phenomenon and ~~if we look~~ at the results of chapter 2 and the *in vitro* model of wounded epidermis co-cultured with newborn DRG, the epidermis does promote increased branching.

The dissociated culture experiments in chapter 3 may help to determine contribution from NT-3, if for example only neurons with pseudounipolar morphology and branching end terminals are scored. Unfortunately contact with Schwann cells may be necessary for such growth (Mudge, 1984).

Overexpressors of NT-3 show an increase of mechanoreceptive afferents associated with touch dome sensory units of Merkel cells (Albers et al. 1996), and all unmyelinated and Aδ endings within the epidermis and dermis (Rice et al. 1998). The skin of such mice overexpressing NT-3 in the epidermis have touch domes that are hypertrophied and increased numbers of Merkel cells (Albers et al. 1996).

NT-3 can also promote collateral branching of trigeminal sensory axons into the brainstem (Ulupinar & Erzurumulu, 1988).

RT-PCR demonstrated the presence of NT-3 cDNA at 40 cycles of PCR in all tissues examined (see figure 41). Northern analysis showed NT-3 was upregulated at the mRNA level, and could therefore be important in mediating or contributing to hyperinnervation. The mRNA increase was so great that the 1.5kb transcript was undetectable in the naive lane and only seen in the 3 day post wound lane (see figure 45).

In situ studies substantiated the mRNA increase showing grains in the 2 layers of keratinocytes basally and superficially, as well as being present in the scab tissue elements and around hair follicles (see figure 50). NT-3 distribution in around hair follicles has also been reported by (Rice et al. 1998).

ELISA studies for NT-3 protein clearly showed the conversion of mRNA upregulation into protein upregulation 3 day post wounding. These levels returned to normal by 5 days. The data also showed no developmental increase in NT-3 over the period of birth to 7 days, thus reflecting a true increase after neonatal skin wounding, although Zhou & Rush, 1993 have reported that levels of mRNA are highest in the skin one week after birth with lowest levels in the adult. This protein profile seems similar to that of NGF (see Constantinou et al. 1994).

Immunolabelling for NT-3 in naive 3 day old hindpaw skin, showed a dense band throughout the suprabasal layer of the epidermis (also reported by Kennedy et al. 1998) continuous from the rete ridge collar of epidermal thickening with a fainter band in the basal layer, as well as immunoreactivity around hair follicles (see figures 54 & 55). This double-banding we observed could be a result of independent syntheses by these two layers, since there is no gradient of staining between them (see figure 54), or that the cells become flattened towards the surface and thus show increased expression. However, there was comparable or less staining in wounded animals in these same areas, which may reflect staining differences (although slides were incubated for an equivalent time in DAB), or a redistribution of the NT-3.

In fact there were two other locations for NT-3 like immunoreactivity in wounded sections. Firstly the dense labelling of the scar tissue above the wound itself. This looks most promising as it is directly above the site of the wound, and nerve fibres (as seen in Chapter 2) are always directed towards this area. (The scar tissue is not labelled in control IgG incubated sections). This area is used in co-culture studies and is separated as part of the epidermis in the studies conducted in Chapter 3. The cells producing NT-3 may indeed be macrophages located in the scar tissue at three days post injury, which are thought to participate in neuronal sprouting in the CNS (see Batchelor et al. 1999). The use of grafts

on wounded animals or suturing as well as grafts from knockout animals may give insight as to whether this is indeed true in our situation.

In addition, since the scar is lost by P5, it would also fit in with the ELISA studies where the levels of NT-3 return to normal at this age. One could also imagine the heaping up of adjacent epidermis after wounding and wound contracture such that above the damaged area will be a region of increased NT-3. NT-3 being a mitogen for macrophages and increasing phagocytic activity may explain the increase in NT-3 in wounded skin to remove debris and the rapidity of healing.

Secondly, there was labelling of a few cells in a few sections in the dermis under the wound (see figure 57). These could not be artifact since they were not seen in control animal sections, and were consistently under the wound of all wounded animals immunostained. It could be that the NT-3 increase seen in the ELISA studies could arise from such cells and only very small levels of neurotrophins are needed in the appropriate region. The fact that these cells are only seen in a few sections argues against their importance, but this may reflect the thickness (30μ) of the sections. Further studies would establish the cellular type displaying this immunoreactivity with various markers for macrophages etc. macrophages being known to express neurotrophins *in vitro* and *in vivo* and with regional specificity (Zhou & Rush, 1994 and introductory chapter). The increase in the 1.5kb NT-3 transcript in human neuropathies is also correlated with the degree of macrophage and T cell invasion (Sobue et al. 1998).

Tissue culture studies implicated a functional role, such that antibody to NT-3 had some effect on the blockade of neurite outgrowth from co-cultures of wounded skin and P0 DRG, but not of co-cultures of naive skin and P0 DRG. Thus, NT-3 has some role in promoting the outgrowth seen in wounded skin co-cultures, and this will be important in the *in vivo* hyperinnervation phenomenon. It would therefore also be interesting to find out whether the block observed was a result of reduction in branching, rather than absolute neurite numbers.

The fact that there is not total blockade, may indicate another component to hyperinnervation, or suggest the complexity of the *in vitro* culture system (problems and experimental considerations of this have been discussed in Chapter 3). It is also interesting that naive skin can lead to an independent, non-NT-3 mediated neurite outgrowth.

The injection of trkC IgG fusion bodies did not however, attenuate the proliferation of fibres in wounded skin. Various hypotheses can be brought forward to explain this. The NT-3 levels may be too great to block with fusion bodies, or the time period of the

injection may be at fault i.e. it's activity may peak and then decline. Other reasons could be the lack of absorption of receptor body and hence accessibility to nerves, with a low final concentration in the skin. It was assumed that since this antibody had been characterised for the adult rat in terms of systemic absorption and delivery concentration, an extrapolation to the neonatal animal would be appropriate, but this may not be so. The fusion bodies may have lost activity form extended storage but this seems unlikely since they effectively inhibited NT-3 mediated outgrowth in chick DRG assays. This is also unlikely considering ELISA data demonstrates that the skin produces only 150pg/mg/ml of NT-3 at maximal levels, and the receptor bodies are able to block at least 2ng/ml of NT-3 in culture at peak activity. Again, species differences may be responsible for discrepancies.

The blockade of NT-3 could lead to some other molecule, e.g. GDNF taking over the role of hyperinnervation, or that NT-3 is just one of the many contributors to the phenomenon.

One possibility is that NT-3 does not mediate the hyperinnervation at all, but that NT-3 upregulation is just an effect of the skin damage and loss of nerves after wounding. Some local NT-3 injected antibody may yield some light on this.

NT-3 is increased in the skin of patients with human diabetic neuropathy, a disease characterised by consistent reduction in conduction velocity, with minor morphological evidence of sciatic nerve axonal degeneration (see Conti et al. 1997). Patients exhibit altered levels of thermal pain (Navarro & Kennedy, 1991), and abnormalities of mechanical stimulation (Mackel, 1989). It is thought that the increase in NT-3 reflects the degree of denervation (Kennedy et al. 1998). Theoretically the increase in NT-3 may be due to the decreased uptake by absent nerves, or denervation could lead to the upregulation of NT-3 as suggested by Kennedy et al. 1998. Application of NT-3 has also been shown to attenuate the large fibre neuropathy in rats (Helgren et al. 1997) thus limiting degeneration rather than encouraging regrowth. Diabetes also increases the level of NT-3 mRNA in sensory nerves in the dorsal root and sural nerve (Cai et al. 1999), indicating that the nerves themselves may be responsible for the synthesis of NT-3. The patients with such a condition may have nerves that due to state or developmental age do not respond to the increase in NT-3.

Thus, neonatal hyperinnervation seen here could underly the basic principle of growth of nerves, which is disrupted or prevented in diabetic neuropathy.

4.5.4 Trk C RECEPTORS AND SWITCHING

If hyperinnervation is mediated by NT-3, the major receptor implicated would be trkC, although NT-3 can also activate trk A and B (Farinas et al. 1998).

In early trigeminal cutaneous neurons there is a switch from BDNF/NT-3 dependency from E10 to NGF dependency, and thus a change in receptor signalling from trkB to trkA. Interestingly trkA innervation is inhibited by trkB (Rice et al. 1998). In addition, NT-3 can act using the trkB receptor in these early neurons (Davies, 1997), such that one could suggest NT-3 could act via different receptors at any time or in different tissues states, although trkB transcripts are downregualted in human neuropathies (Sobue et al. 1998). What regulates and determines this switching is still unknown. Of much interest is the suggestion that neurotrophins need not be in a gradient for action, since they are not required for guidance, but for the invasion and differentiation of neurons (Ringstedt et al. 1997). In some early neurons trkA is replaced by trkC (see Elshamy et al. 1996), so NT-3 could be involved in the neurotrophin receptor switching. A subpopulation of NT-3 requiring neurons that do not use trk C as their receptor are present between E13.5 and E.15.5, and are thought to utilise trkA at this time (see Liebl et al. 1997), so that there seems no reason why at this developmental stage and after such a tissue insult this could occur again.

In vitro and *in vivo* studies have shown that exogenous neurotrophins can increase the expression of their receptors, and NT-3 can induce trk C postnatally (Elshamy et al. 1996). In diabetic neuropathy the levels of the trkA and trkC receptors are increased in keratinocytes in the skin (Terenghi et al. 1997), where some of the source of NT-3 is in the nerves themselves.

In NT-3 knockout studies however, there is no change in the mRNA expression of trk C in developing cutaneous tissues (Wyatt & Davies, 1993), hence it may be the effect of injury on nerves that promotes the expression of trkC, or the overabundance of NT-3 itself in the wounds of neonatal rat pups. In human neuropathies where trkC is in fact downregulated (Sobue et al. 1998), it may be that this downregulation is responsible for the inability for fibres to regenerate.

Several forms of the full-length trk C receptors containing tyrosine kinase insertions in the tyrosine kinase domain have been reported (Tsoulfas et al. 1993; Valenzuela et al. 1993), which give different responses with respect to proliferation, neurite outgrowth and survival.

Further work could thus involve the co-culture of wounded skin with DRG and the measurement of trkC levels after culture. NT-3 overexpressed in spinal cord, results in a loss of Ia projections, but a normal SP, CGRP and trk C expression on DRG neurons is found. This would indicate that if NT-3 were the factor responsible it may not be acting via trkC receptors as these are unchanged with central overexpression, although they may be changed peripherally. Furthermore, it is well established that NT-3 mutants show a loss of neurons greater than those expressing the trkC receptor (Ernfors et al. 1994). The constancy of the CGRP population also fits in with the lack of change seen in this population of neurons by tissue culture models see chapter 3.

Another interesting parallel to our studies, are the numerous nerve fibres seen in patients with psoriasis, an inflammatory skin condition which results in proliferation of keratinocytes with resultant raised plaques covered by thick white scales which appear on the knees, elbows, trunk and scalp of patients (Stevens & Lowe, 1995) (a comparison of neurotrophin and receptor distribution for psoriasis, neonatal skin wounding and diabetic neuropathy is shown in figure 81). There is much controversy over the distribution of nerve fibres, with some reports of decreased epidermal fibre density (Johansson et al. 1991), and other reports of increased SP and CGRP fibres in the epidermis (Jiang et al. 1998).

The levels of trkA-IR are decreased in tissue of such patients, and p75 receptor immunoreactivity normally found in nerve fibres throughout the dermis and some within the epidermis, with those of a thick variety in the middle and deep dermal layers is also decreased (Bull et al. 1998). Thus NT-3 could be acting via p75 in conjunction with trkA, rather than trkC. P75 becomes increasingly important in neurons undergoing a down-regulation in trk A (Ryden et al. 1997). High levels of NT-3 increase trkA mRNA in sympathetic neuroblasts in culture (Mendell, 1996). Recently, Brennan et al. 1999 have documented the influence of the p75 receptor on NT-3 responsiveness in sympathetic neurons. NT-3 can activate trkA in these neurons and if there is a loss of the p75 receptor there is increased affinity of trkA receptors for NT-3. NT-3 is noted to be increased in the skin of p75 knockout mice (see Brennan et al. 1999) and is thought to accumulate as a result of reduced sensory innervation which is a notion similar to the loss of innervation after skin wounding.

The vital next experiment though is of course skin wounding in newborn NT-3 mutant mice, although examining receptor distribution and regulation is also important. The problem with such studies is that homozygous mutants do not live very long at all, and at

least five days is necessary to show visible hyperinnervation. Heterozygotes however, may reveal dosage dependent protein effects.

Trk C mutants may yield further answers since these animals have a less severe phenotype than NT-3 knockouts (Tessarollo et al. 1994), and survive for several days.

4.5.5 CO-OPERATION, COMPENSATION AND SYNERGISM BETWEEN NEUROTROPHINS

It is well known that during development sequential support by neurotrophins determines the emergence, guidance and pattern of neurons within their target areas. For example in culture NT-3 stimulates survival of sympathetic neuroblasts (Birren 1993), these neurons becoming NGF dependent only after terminal mitosis (Birren 1993) i.e. the ganglia has NT-3 and the target has NGF. And in the skin, Merkel innervation is sequentially dependent on NGF and NT-3 (Fundin et al. 1997). NGF/trk A is thought to be important for major outgrowth and proliferation of sensory axons, but NT-3/trkC is thought to manage the formation of sensory endings (Rice et al. 1998).

In experimental models of bladder inflammation NGF, BDNF and NT-3 are elevated and the immunological studies show protein elevation of a similar time course as the mRNA with respect to NGF (Oddiah et al. 1998).

Could this be the same in wounding and hyperinnervation, i.e. that in some way NGF, NT-3, BDNF and GDNF interact sequentially to produce the phenomenon? Or are they just the effects rather than the initiators? The fact that NT-3 and GDNF the mRNA levels are upregulated well before the hyperinnervation is visually detectable suggests a causal role.

One could also hypothesise a situation where elongation is promoted by an upregulation of NGF with final terminal arbors in the epidermis governed by NT-3 expression. NGF could also aid fasciculation within the dermis as in BAX knockouts (Lentz et al. 1999), and potentially the bundles of fibres entering the dermal wounded area. With prolonged administration of NT-3, there is a decrease in NGF levels within the upper dermis, with consequent switch from axon growth to ending formation (Ernfors et al. 1992).

There could be co-operation between neurotrophins, for example NGF overexpressed in the skin is known to increase trkA and C in neurons of the trigeminal ganglion and DRG (Goodness et al. 1997) so that in hyperinnervation, NGF could increase in wounding and upregulate these receptors on the damaged neurons, thence they could respond to the increase in NT-3. Trk A and C receptors could feasibly be expressed on the same neurons e.g. 16% of DRG neurons co-express trkA and C (Wright et al. 1992). And in the

trigeminal ganglion trkC neurons are not restricted to large diameter cells, but are present on many small diameter cells also. The competitive nature between axons of the same neuron for one neurotrophin during the final patterning of the skin may be abolished with the rise in NT-3 associated with wounding. Synergism between p75 and NT-3 has already been reported for limb proprioceptors (Fan et al. 1999).

NT-3 and BDNF display reciprocal expression in early development, and suggested roles of innervation and maintenance respectively (Maisonpierre et al. 1990), and this indeed is true here where NT-3 is high and BDNF low, at least at the mRNA level. Again this suggests the neurons are back to their initial developmental parameters. Longitudinal lanceolate endings depend on NGF/trkA signalling prior to target innervation followed by BDNF/trkB dependency (Fundin et al. 1997). Thus trophic support maybe provided by more than one factor. Recently, Kohn et al. 1999 have shown that BDNF via p75 can be antagonistic to NGF trkA mediated target innervation. Furthermore, a recent study in the trigeminal sympathetic system by Walsh and colleagues 1999 indicates that p75 is not required to initiate or sustain the collateral NGF dependent growth of sympathetic axons into the ganglia, but plays a role in the directional regulation of axonal growth.

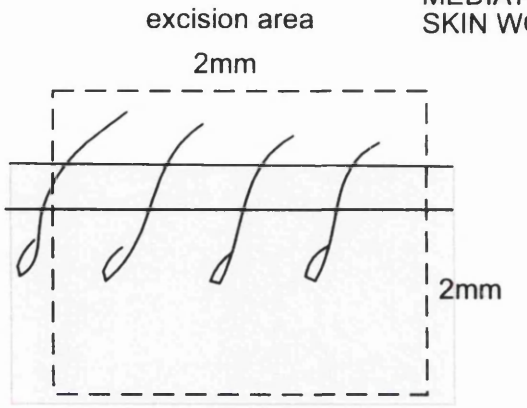
Neurotrophins are only one of a number of families that can mediate neurite outgrowth what of the others? Whilst it is only natural to assume the production of a neuritic promotion factor, one must also look at the expression of inhibitory or repulsive molecules, many of which could be down-regulated to allow hyperinnervation to proceed.

4.6 CONCLUSION

NT-3 seems to be the most likely candidate for the induction of neonatal hyperinnervation after skin wounding. NT-3 mRNA and protein levels are increased well before any visible appearance of nerve fibres, and it is localised by immunohistochemistry and *in situ* hybridisation to the scar tissue at 3 days post wounding, directly above the region of hyperinnervation. *In vitro* tissue culture studies also demonstrate its ability to partially block neurite outgrowth in a model of hyperinnervation. Neither BDNF, NGF nor GDNF are able to show consistency in all of the above parameters examined, and therefore we hypothesise that these may be important in regulating the hyperinnervation in morphology, distribution and maintenance once it has been induced by NT-3. Our proposed model encompassing the competition and balance between promoting and suppressing effects of the neurotrophins and other molecules is represented in figure 80. It is important to note that all the neurotrophins can contribute in someway to

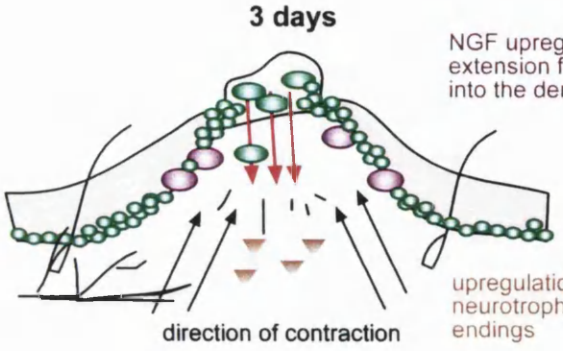
hyperinnervation with different neurotrophins regulating different fibres and endings, and various aspects of nerve growth.

FIGURE 80: POTENTIAL MODEL FOR NEUROTROPHIN MEDIATED HYPERINNERVATION AFTER NEONATAL SKIN WOUNDING



Newborn rat hindpaw skin showing extent of full thickness wound.Both epidermis and dermis are removed.

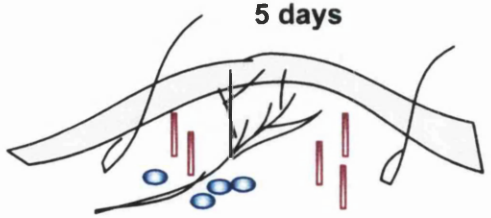
NT-3 (in macorphages) upregulation elicits hyperinnervation directs the fibres and promotes branching within the dermis



NGF upregulation allows fibre extension from severed axons into the dermis.

upregulation of receptors for neurotrophins on damaged nerve endings

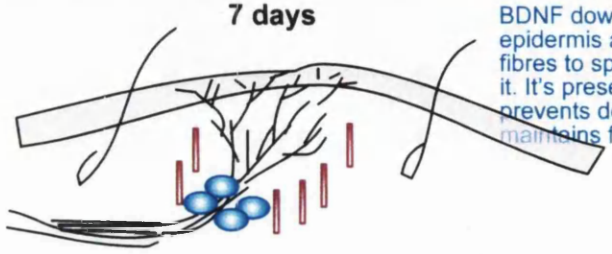
3 days later NT-3 is released from sites within the wounded area. Fine fibres appear under the wound, which is easily characterised by the presence of a scab and lack of hair follicles. The wound has contracted and pulled to a peak.



At 5 days the epidermis has re-established itself, and scabs have sloughed off the injured area. Nerve fibres are already numerous in the area under the wound, with branching and entry into the epidermis.

GDNF may participate in the fasciculationof fibres growing towards the wounded area, or be upregulated as a result of the developmental time point for the switch in dependency of IB4 neurons.

extracellular matrix and axon guidance molecules prevent sprouting into uwounded areas



BDNF downregulation in the epidermis allows fibres to sprout vigourously into it. It's presence in the dermis prevents defascicualation, and maintains function.

By 7 days the fibres have branched much more and well into the epidermis. Thick fascicular bundles are visible, but hair follicles are still not present.There is a continuous level of epidermal covering.

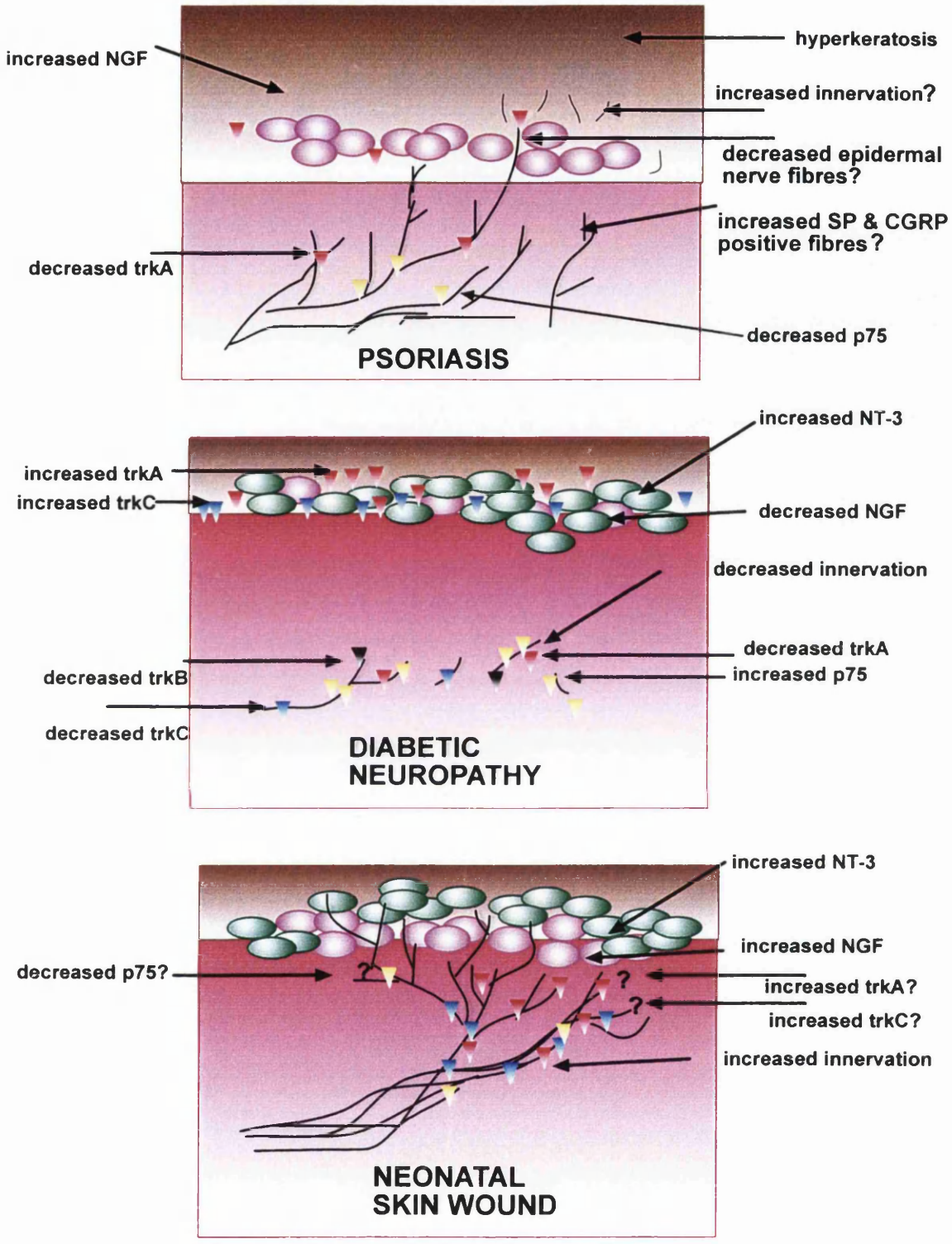


Figure 81 showing the neurotrophin and receptor links between Psoriasis, Diabetic Neuropathy and Neonatal Hyperinnervation

CHAPTER 5

ISOLATING DIFFERENTIALLY EXPRESSED GENES

5.1 INTRODUCTION

Identifying the gene that regulates a particular function of the nervous system is an important step in understanding that function during development and in normal and aberrant CNS adult behaviour. Such genes are also important tools for the manipulation and understanding of disease processes. The question of what causes a certain phenomenon, will inevitably lead to an investigation of the genes that regulate it.

In the development of the rat peripheral nervous system, the genes that regulate sensory nerve growth, morphology, branching, target recognition and guidance may be altered or disturbed by injury. An example of this is the overexpression of neurotrophins described in Chapter 4.

The dramatic hyperinnervation that follows skin wounding at birth, suggests many changes in the expression of genes involved in the regulation of peripheral nerve target innervation, as well as in those responsible for wound healing and the accompanying inflammatory reaction.

Several methods have been employed in the identification of regulated genes. They can be classified into two main categories, open and closed systems. The former being non-library based options such as differential display and RNA fingerprinting, with the latter necessitating the use of cDNA or oligonucleotide libraries.

5.2 DIFFERENTIAL DISPLAY

This method originally developed by Liang & Pardee, 1992; Liang et al. 1993 has been of great use in isolating novel genes between different cellular types and treatments. It is based on the principle of amplifying mRNAs from each tissue, after first strand reverse transcription (RT) synthesis by PCR, and then distributing them via their 3' radiolabelled termini on a denaturing polyacrylamide gel, and examining the lanes for band differences.

Advantages of this procedure include the ability to display mRNA species from extremely small sample amounts of RNA, which may or may not be polyA+ purified, (although contaminant DNA must be removed to avoid smearing), and the method is very tissue specific and reproducible (Liang et al. 1993).

Initially the method used a degenerate oligo-dT primer for RT, with a set of arbitrary 10mers (a string of 10 nucleotides). This caused several problems:- redundancy, under-representation of certain mRNA species and high false positives which could not be confirmed by Northern blot hybridisation. In 1994, Liang et al. introduced three one base anchored oligo-dT primers, and restriction sites on 5' ends on anchored and arbitrary primers to decrease these effects and to ease manipulation of discovered clones into vectors. However, many disadvantages still remain:- many clones have to be re-amplified after band extraction, and although this is good for probe synthesis, it is also time consuming. The generation of small products approximately 500bp in length meant that in some cases the coding region was absent. The number of false positives means that duplicate samples must be run, and in most places in the gel more than one DNA species occupies a band leading to problems with re-amplification and cloning into vectors - all making the process labour intensive. Scanning bands with the eye is error prone and although dyes for each sample using an automated sequencer have been used successfully to reduce this, it is unknown whether this causes some difference in priming abilities. Variation in expression is also difficult to detect, so the method is not quantitative. Denaturing gels produce crowded band patterns although Bauer et al. 1993 have used non-denaturing gels, but estimate greater than 300 PCR reactions are needed for 95% mRNA representation. (Mou et al. 1994) have used a slot blot manifold instead of Northerns to verify their bands increasing the efficiency. The process is labour intensive and bands often fail to be cloned or fail to bind to Northern blots. Laboratories have employed various strategies for overcoming this e.g. Mou et al. 1994 bound radiolabelled PCR amplified, differentially expressed bands to Northerns, purified the hybrid from the blot directly, cloned the fragment into a vector, and then reprobed another Northern. Again, this becomes very time consuming. Finally, low copy number genes are harder to isolate with this technique.

Many variations on this method exist. For example Ralph et al. 1993 adapted this to form an RNA fingerprinting technique whereby no oligo dT primer was used, only arbitrary primers, 'RNA fingerprinting using arbitrarily primed PCR' (RAP). Very small amounts of total RNA can be used, and it is unaffected by low levels of genomic DNA contamination. First strand reverse transcription is undertaken with an arbitrary 20 base primer for each tissue pool, followed by second strand cDNA synthesis PCR with the same arbitrary primer, at low stringency. The products are then run on an acrylamide gel and bands compared, cut, purified, re-amplified, run again, cut and cloned into vectors (see figure

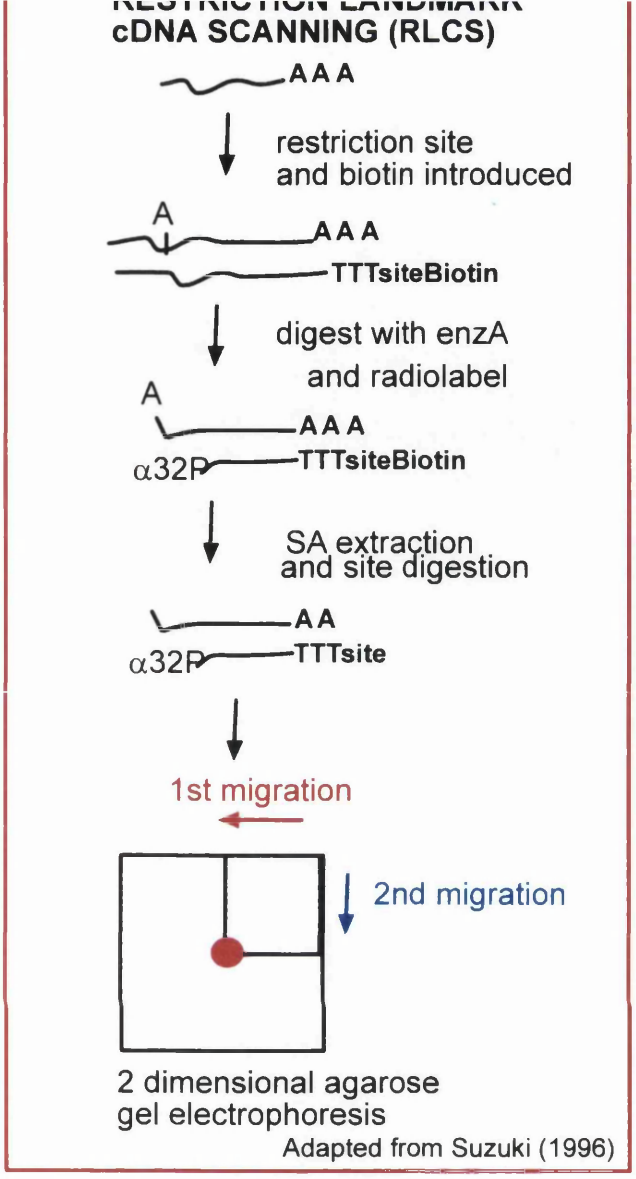
82). The technique therefore relies on the primer design and synthesis of both strands with the same primer. Welsh et al. 1992 stated that because each primer samples only a small subset of the RNA population, the method would be inappropriate in situations where one was looking for differences in a single message or only a very small number of messages. Ralph et al. 1993 in addition, modified the technique to use nested primers in second strand synthesis and PCR, so that there is a greater chance of obtaining rarer mRNA species.

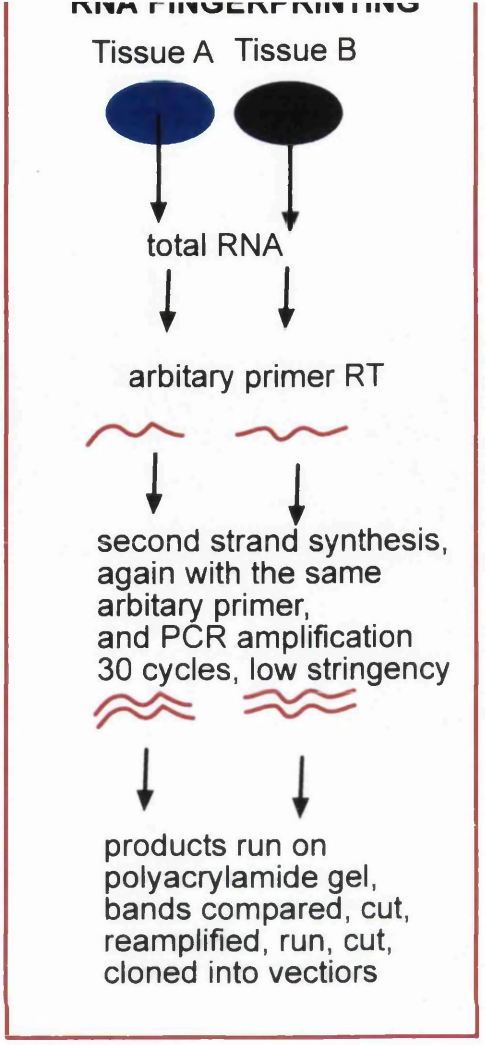
Even with the many hazards involved in differential display the technique has been successfully exploited to produce many regulated genes in a wide range of research e.g. Livesy & Hunt 1998 identified genes induced in regenerating neurons.

A short while ago Suzuki et al. 1996 reported on an alternative to differential display, called restriction landmark cDNA scanning (RLCS), using two dimensional agarose gels. Fundamentally, this concept was based on running cDNAs synthesised after RT from the tissue of interest on a two dimensional agarose gel. The fragments would run according to size and were then cut in the gel using a restriction endonuclease targeted to a specific site in all cDNAs. The fragments were then run in another orientation, thus migrating two dimensionally according to size (see figure 82). The fragments being radiolabelled provided an autoradiograph of distribution of cDNAs. If this was executed for treated and untreated samples two gels with identical spots of different regulation dependent on transcript amounts should be obtained. The method provides a more definite regulation since no PCR is involved, and allows easier detection of regulation, since products have to comply with both vertical and horizontal migration.

5.3 SUBTRACTIVE HYBRIDISATION

Subtractive hybridisation as the name suggests involves the subtraction of genes from one tissue/cell type from another, be it treated and untreated samples of the same tissue or entirely different tissues. The technique employs the use of 5-10μf of poly A^+ RNA. Modifications by PCR amplification (Brady et al. 1995), have lead to very minute amounts of RNA being necessary, although with any PCR based technique there is a degree of misrepresentation, bias towards more abundant species (a five fold change has been shown to be required by many workers, see Nishio et al. 1994) and mispriming.





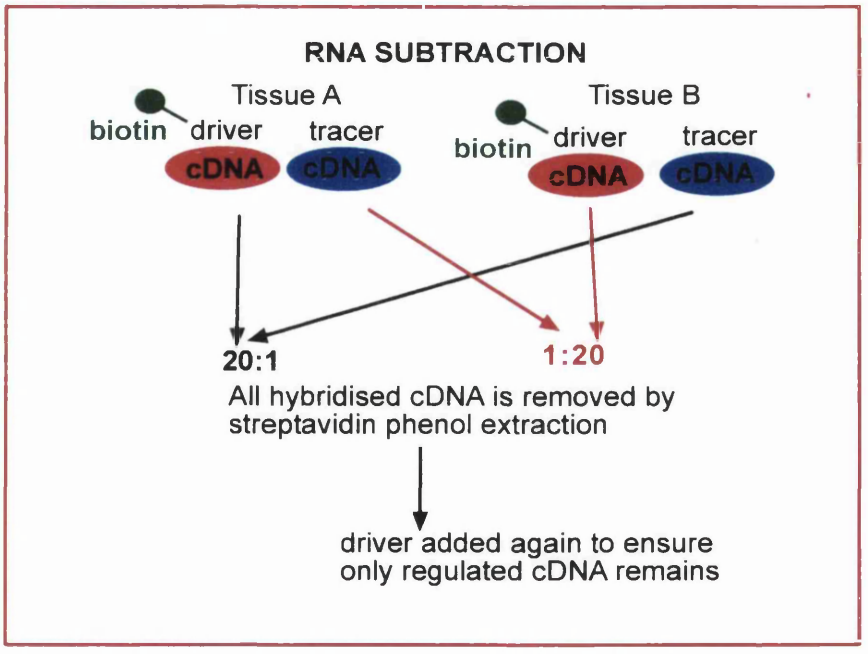


Figure 82 summarising three of the techniques used in isolating genes

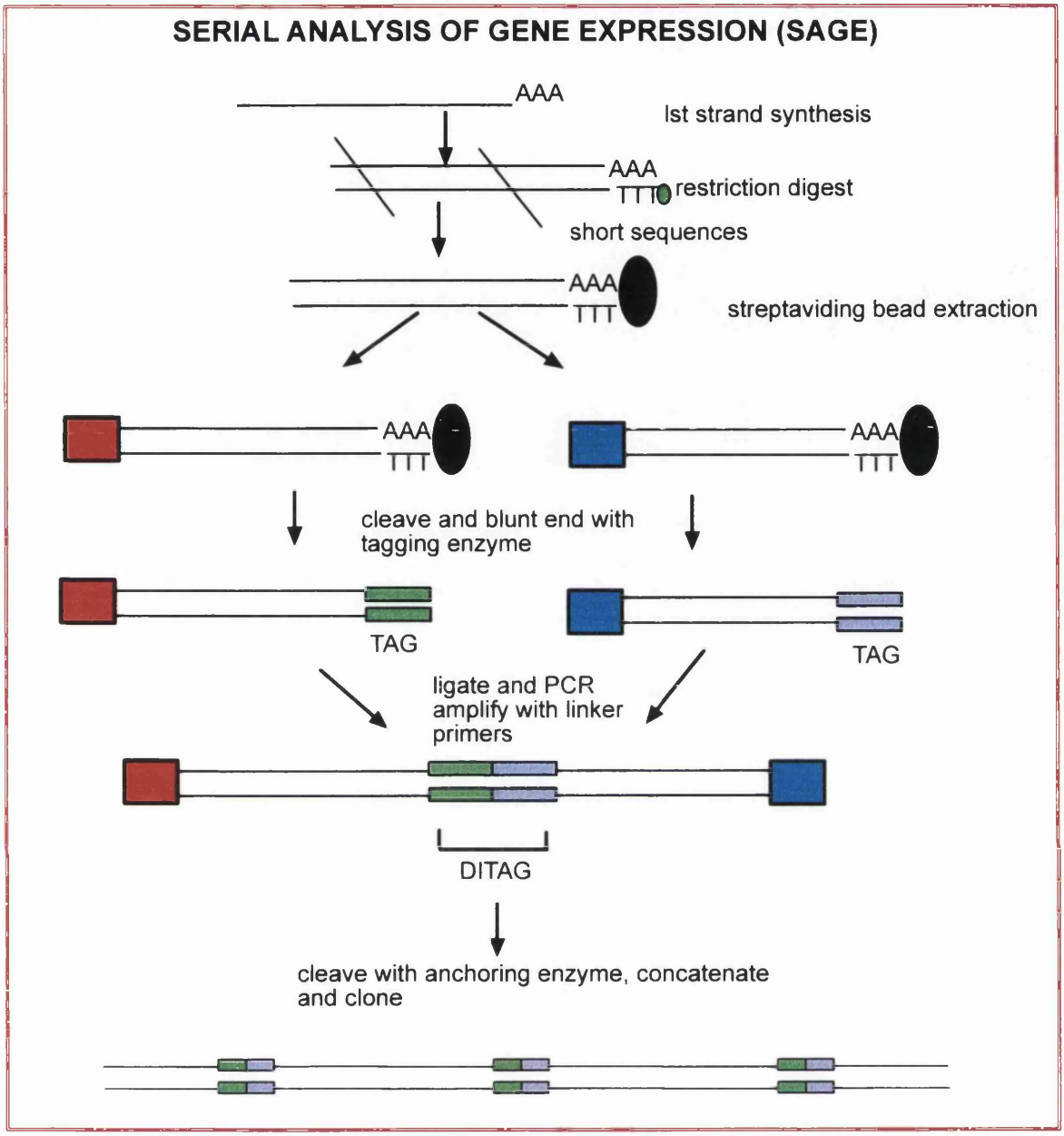


Figure 83 showing a summary of the SAGE technique, adapted from Velculescu et al,1995.

Essentially, the aim is to create two populations of mRNA and reversely transcribe them, and synthesise cDNA for each tissue under investigation. One population called the 'tracer' and the other the 'driver' are PCR amplified to generate the double stranded DNA, with drivers photobiotinylated and hybridised with tracer at 20:1.

Streptavidin is then used to remove the hybridised cDNA and phenol extracted. The remaining population now represents upregulated and downregulated clones, depending on which tracer and driver from the two tissues was used (see figure 82). The biotinylated driver can then be added again to minimise error, and increasingly select regulated genes.

The main disadvantage of this method is that it tends to leave non-regulated clones in the pool of cDNAs i.e. unhybridised tracer elements. In addition, the technique is inconsistent with limited reproducibility, and abundant cDNAs are favoured. Nevertheless, this method has recently been successful in the isolation of genes specific to DRG neurons (Akopian & Wood, 1995).

The subtractive principle has been applied to create many other methods, for example serial analysis of gene expression (SAGE) (Velculescu et al. 1995) (see figure 83). SAGE is based on the assumption that 9-10 bases are adequate to identify transcripts if the bases are at an appropriate site. Concatenation (serial linkage, see figure 83) of these 'tags' as they are called allows serial analysis of the isolated transcripts. First strand synthesis with an oligo dT primer is followed by restriction endonuclease digestion and 3' fragment isolation by streptavidin beads. The pool is divided in two and ligated to different linkers containing a type IIS restriction site, and then cleaved with a tagging enzyme, yielding linker, a short cDNA fragment and a tagged blunt end. The two blunt end pools are then ligated to each other and PCR amplified by primers to the linkers. The products can then be cleaved with an anchoring enzyme, and concatenated and cloned into vectors. Thus many genes can be determined in short spaces of time, with little effort and compared with concatenated clones from other tissues for abundance.

The enriched single-stranded cDNA probe, generated by phenol-emulsion-enriched DNA driven subtractive cDNA cloning (PERT) has modified subtraction to allow low abundance messages to be isolated and using only modest quantities of mRNA (Travis & Sutcliffe, 1988). Here, the cDNA probe (which would normally be used on a library) is hybridised to an excess of a phenol-emulsion mixture of denatured plasmid driver DNA made from amplification of a cDNA library not of the tissue of interest.

5.4 DIFFERENTIAL SCREENING

The generation of cDNA libraries has been a handy implement for the discovery of genes responsible for certain functions. The cDNA reflects the messenger RNA of a given tissue and encodes the genetic material responsible for the translation of genes into proteins. The cDNA is cloned into viral vectors and expressed in *E.coli* bacterial cells. Each colony plated out represents one cDNA insert. The plaques can then be imprinted onto filters in duplicates and probed with candidate genes, differential probes etc to isolate genes of interest.

One major drawback of the library based method apart from its time-consuming nature, is that if the gene is not inserted into the vector, there is no representation of it within the library, and it will not be isolated even if it is differentially regulated. Thus, any library based system is a 'closed' system. Besides this, large amounts of RNA are needed for library manufacture, and abundant species, as seen in the majority of techniques, are likely to be isolated first.

5.5 PROBES FOR DIFFERENTIAL SCREENING

5.5.1 cDNA targeted gene: If a gene of interest has been isolated in part sequence or in one species and not in another, this fragment can be used in the form of a cDNA probe to isolate the whole gene from a library, presumed to contain such a gene.

In the same way, cDNA probes with homologous sequences to a family of molecules can be used to locate other and novel members of the family e.g. the discovery of perserphin, a member of the GDNF and neurturin family isolated by Milbrandt et al. 1998.

5.5.2 cDNA differential probes: Classical differential screening uses cDNA made from all the mRNA in the two tissues of interest, and each tissue probe is used on the library, and the level of binding of each probe gives an indication of the change in expression of each gene. Proteins of interest, receptors and ion channels have all been isolated in this way (see Kato, 1992).

5.5.3 Subtractive probe: This is essentially used for the same types of experiments as 5.5.2 It saves time and does not rely on observer quantification, since the probe is a single probe made from subtracting the two tissues of interest. Only regulated genes are thus probed for by the subtractive probe. Probes enriched by subtractive hybridisation have been used by Baba et al. 1994 in differential screening to obtain novel mRNAs from oligodendrocytes.

5.5.4 mPCR probe:- (Kendall et al. 1996) This probe is essentially the same as the conventional cDNA probe. However, when there is very limited supply of mRNA and hence very few cDNA syntheses, mPCR allows the amplification of these, so that even with small amounts of tissue, differential regulation may be found.

The differential screening approach has been the traditional tool employed for many years. This broad based screening approach has been focused more constructively in recent years, by subtractive hybridisation methods for both library construction and probe generation. Companies such as Clontech now provide a kit form of the subtractive method, with the incorporation of nested PCR (see CLONTECH catalogue PCR select kit 1997) which increases priming efficiency), so that subtracted material can be isolated and used for, either the construction of libraries (although a vast amount of tissue is necessary for this), or the production of subtracted probes. The differential screening approach has been successfully used on a spinal cord library using a subtractive hybridisation technique involving naive and oligodendrocyte free spinal cord, and genes responsible for myelination by oligodendrocytes isolated (Schaeren-Wiemers et al. 1995). A classic differential screen has been used to identify a GTPase-activating protein (Baba et al. 1995).

A variation on this theme has been the large scale industry based silicon chip oligonucleotide library screening (Schena, 1996), whereby multiple genes are probed with fluorescently labelled probes within a silicon grid. After hybridisation and washes, the chips are automatically scanned by confocal, and signals quantified. This reduces many of the conventional disadvantages to library screening. Firstly, many genes can be scanned simultaneously, with multiple gene oligonucleotides minimising error, and there is no cloning into vectors, nor time consuming manual screening.

Libraries have also recently been utilised to determine regulated genes by functional expression differences, 'expression cloning', for example the transfection of a DRG library to isolate clones upregulated after calcium treatment, lead to the cloning of the capsaicin VR-1 receptor (Caterina et al. 1997).

cDNA libraries have now been used in mass screening projects, and novel sequences of cDNA in libraries i.e. expressed sequence tags (ESTs) have been used for EST subtractive analysis between two libraries e.g. PC12 cells with or without NGF treatment (Lee et al. 1995). ESTs have also been employed for functional homology searching. Computer

software packages have replaced the manual screening and therefore allow the generation of millions of regulated clones by electronic subtraction.

An adult rat skin wound healing library has already been made by Okada et al. 1995, and used by directly probing for tissue remodelling genes, leading to the isolation of matrix metalloproteinases (Okada et al. 1997).

Any genes isolated from the techniques dealt with above need to be independently verified by means of in situ hybridisation, Northern blotting or Reverse northerns. The two former methods are both used and discussed in Chapter 4 and here in Chapter 5.

The reverse Northern protocol is a technique which is not commonly used, but seems to have many merits over the others. It requires that the cloned gene be amplified and run on duplicate gels, blotted and subsequently probed with treated and untreated cDNA probes from the original tissues of interest. In such a manner regulation can definitively be observed and large quantities of RNA are not needed. Moreover, many clones can be assessed together using just two RNA probes.

- In this study we chose to examine the effects of skin wounding on P0 rat pups at the gene level.
- To achieve this a cDNA library of neonatal wounded and unwounded skin was constructed. This chapter provides a step by step approach needed to construct such a library and the problems encountered along the way.
- This library was then used as the basis for a classic differential screen, and the isolation of clones seemingly regulated at the DNA level in hyperinnervated wounded skin.
- Southern hybridisation was then used to confirm clones which were up- or downregulated, and the clones sequenced.
- A P3 time point was chosen because:

I) of the associated increased in neurite outgrowth seen at this time point in culture (see Reynolds et al. 1997).

II) P3 wounded skin showed increased levels of NT-3 seen in Chapter 4.

III) A time point before innervation is seen ie before P5 was thought to be necessary to find genes up-regulated before the phenomenon occurred.

5.6 METHODS

5.6.1 GENERATING A cDNA LIBRARY

(Protocol based on ZAP express ™ cDNA synthesis kit and ZAP express™ cDNA gigapack III gold cloning kit), see flow diagram figure 84.

5.6.1a) RNA extraction

RNA for naive and wounded dorsal foot wound skin was extracted as described in chapter 4 for both 1 day and 3 day old animals.

5.6.1b) Agarose gel electrophoresis

To visualise DNA or RNA, fragments were run on an agarose gel. Migration occurs according to the size of each fragment of DNA/RNA.

The agarose gel of required agarose percentage was prepared with the appropriate buffer, and the agarose was melted with its buffer, poured into the appropriate sized gel tank, with combs to mould the wells, and allowed to harden. The rest of the buffer was then poured over the gel. Samples were loaded into the wells after mixing with a gel loading buffer. The loading buffer used for DNA gels was 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll (Type 400 Pharmacia) in water.

With an electric current applied the negatively charged DNA fragments migrate towards the positive anode. The larger the size of the DNA, the slower the migration. The agarose concentration also affects migration, as does the voltage applied, and the concentration of the gel running buffer. The visualisation agent added, in the majority of cases, was 0.5μg/ml ethidium bromide (stock solution 10mg/ml in water) (Sharp et al. 1973), but more sensitive agents such as Sybr green can also be used. NB. These agents are highly mutagenic.

A ladder was usually run along with the samples as a size marker for the DNA/RNA bands. For DNA gels a 1kb DNA ladder was used (GIBCO). After the gel had run completely the bands were visualised under ultra-violet illumination and photographed.

In the above preparation the RNA was run with an RNAse free loading buffer (1ml glycerol, 0.08 ml, 10% (w/v) bromophenol blue, 0.15 ml 10x MOPS, 0.75ml deionised formamide, 0.1 ml deionised RNAse-free water and 0.24 ml formaldehyde, aliquotted and stored at -20°C), and ethidium bromide (10mg/ml stock diluted 1:10 in RNAse free water and stored at -20°C), added to each RNA sample rather than to the gel and buffer. RNA

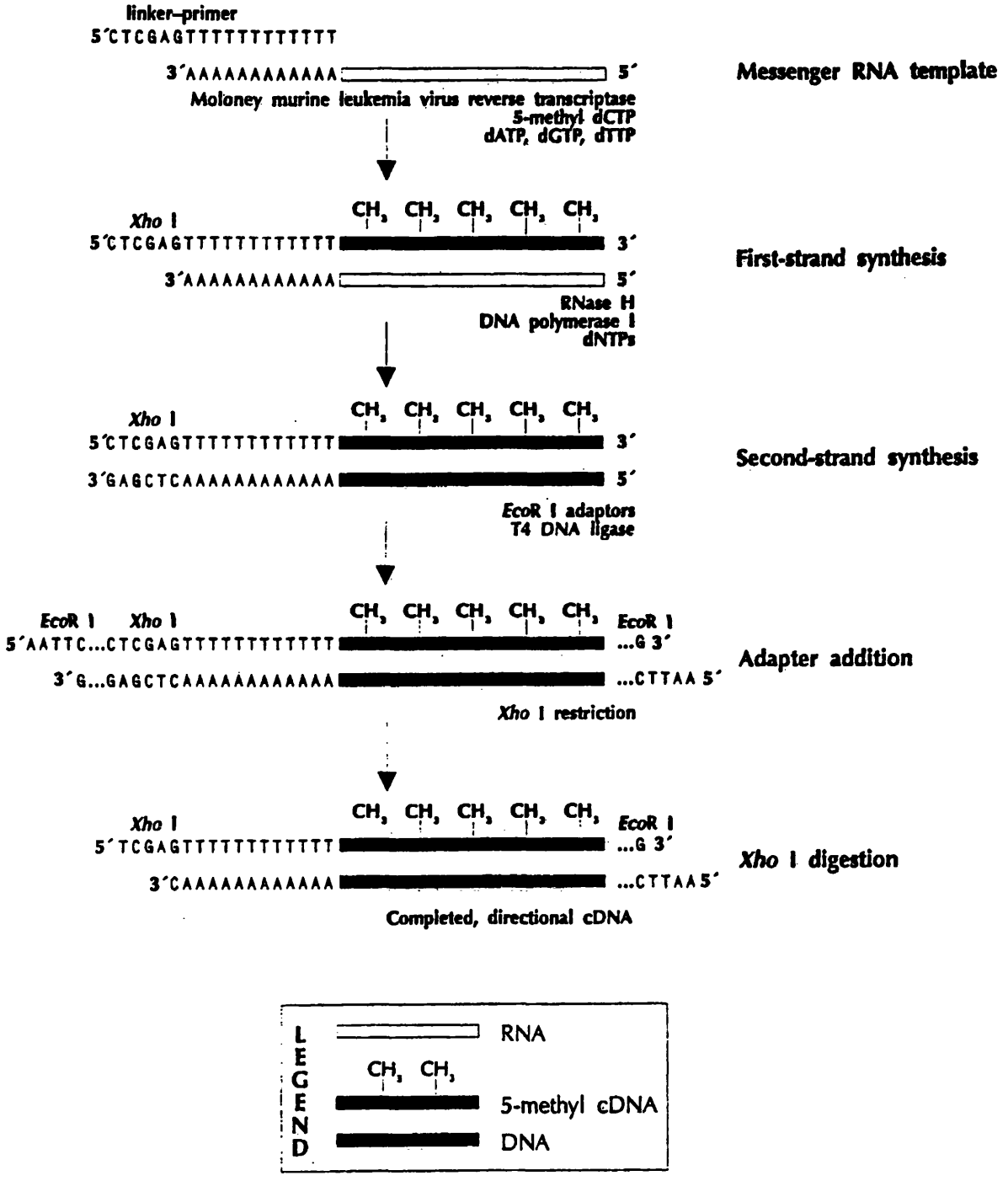


Figure 84 cDNA synthesis flow chart from Stratagene protocol 1997

gels should be run on a MOPS formaldehyde gel, however to check the integrity of the RNA this was not necessary, a 1xTBE gel sufficed. Minigels were run at 70V, and larger gels at 100-250V. Usually a voltage of 5V per cm of gel is maximal.

5.6.1c) Quantitation of RNA extracted

A spectrometer was used to measure optical density (O.D) at 260nm. 1 O.D represented 40μg/ml of RNA. An O.D was optimal with a 0.1-1 spectrometer reading at a path length of 1.

To calculate the amount of RNA present in a sample, the following formula was used:

$$\text{Amount of RNA in } \mu g/ml = O.D_{260} \times \text{Dilution factor } \left(\frac{\text{total volume}}{\text{sample volume}}\right) \times 40\mu g/ml$$

5.6.1d) DNase treatment

As described in Chapter 4.

5.6.1e) Purification of poly(A)$^+$ RNA

Only about 1-5% of the total cellular RNA is mRNA. This is responsible for the translation process, and the synthesis of polypeptide chains. Thus, enrichment of mRNA is important for the generation of cDNA libraries.

To yield an appropriate level of poly (A)$^+$ RNA for use as a template for construction of the library, an Oligotex™ mRNA kit (Quiagen) was used. The DNase treated RNA was mixed with DEPC (diethyl pyrocarbonate) treated water, binding buffer and oligotex suspension and incubated for 3 minutes at 65°C to disrupt the secondary structure within the RNA. Following incubation at room temperature for 10 minutes, the RNA was centrifuged for 2 minutes at 13000rpm and the supernatant aspirated off. The mRNA bound to the oligotex resin by it's poly-A tail was then resupended in wash buffer and placed in a spin column. After another wash, the flow-through was discarded, and the polyA$^+$ RNA resuspended and centrifuged at 13000rpm for 30 seconds, then, eluted twice with elution buffer preheated to 70°C. mRNA was stored at -20°C in elution buffer until use.

5.6.1f) First strand cDNA synthesis

Using murine reverse transcriptase isolated from a strain of *Escherichia coli* (*E.coli*), that express a cloned copy of the gene of the Moloney murine leukaemia virus (MMLV-RT 50U/μl) 1.5μl, with the addition of a 50 base oligo-dT linker-primer (1.4μg/μl) 2μl, with a Xho 1 site, 5μl 10x first strand buffer, and 3μl of a deoxyribonucleotide mix (including 40mM dTTP, dGTP, dATP and methylated dCTP), mRNA (up to 5μg) was incubated at 37°C for one hour, for the first strand of complementary DNA to be synthesised.

5'GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTT3'

"GAGA" sequence linker Xho1 Poly dT

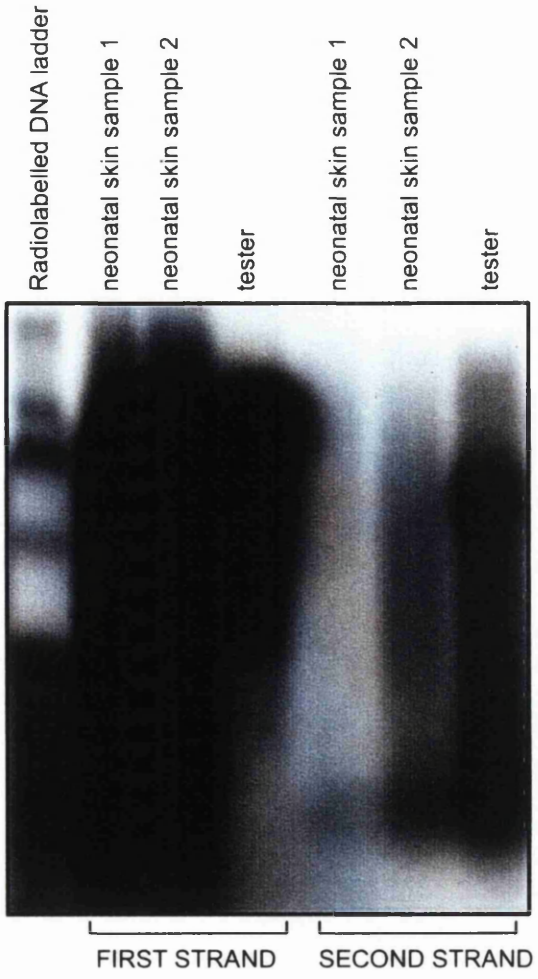
1μl of an RNase inhibitor (40U/μl) was used to bind and block any RNases present (See Stratagene kit).

To verify first strand synthesis, 0.5μl of a radioactive deoxynucleotide ([^{32}P]dATP 800Ci/mmol Amersham) was also added to a 5μl aliquot, and checked by electrophoresis on an alkaline 1% agarose gel with alkalysis buffer (1.2g NaOH, 0.74g EDTA per 100mls for 10x, used at 1x), and alkaline loading buffer (300mM NaOH, 6mM EDTA, 18%Ficoll (Type 400 Pharmacia), 0.15% bromocresol green, 0.25% xylene cyanol). A radioactive DNA ladder was used to mark the size of the first strand species. The first strand cDNA should run from about 700 bases to up to 8000 bases. Tester poly (A^+) RNA served as a control for the synthesis reaction (see figure 85).

5.6.1g) Second strand synthesis

Second strand synthesis was performed with the addition of 2μl of RNAse H (1.5U/μl), (a nick forming enzyme), 6μl of dNTP's (40mM with 2μl [α-^{32}P] dCTP 800Ci/mmol), 20μl of 10x second strand buffer, and 11μl *E. coli* DNA Polymerase I (9U/μl), made up to a total of 200μl with sterile water, and incubated at 16°C for 2.5 hrs to prevent hairpinning.

The cDNA was then blunt ended with 2μl Pfu DNA Polymerase I and 23μl of blunting dNTPs, vortexed, spun and incubated at 72°C for 30 minutes (see flow diagram). Another, standard pH 8 phenol:chloroform overnight precipitation with 3M sodium acetate followed, with 70% ethanol washes, and vacuum drying the next day.



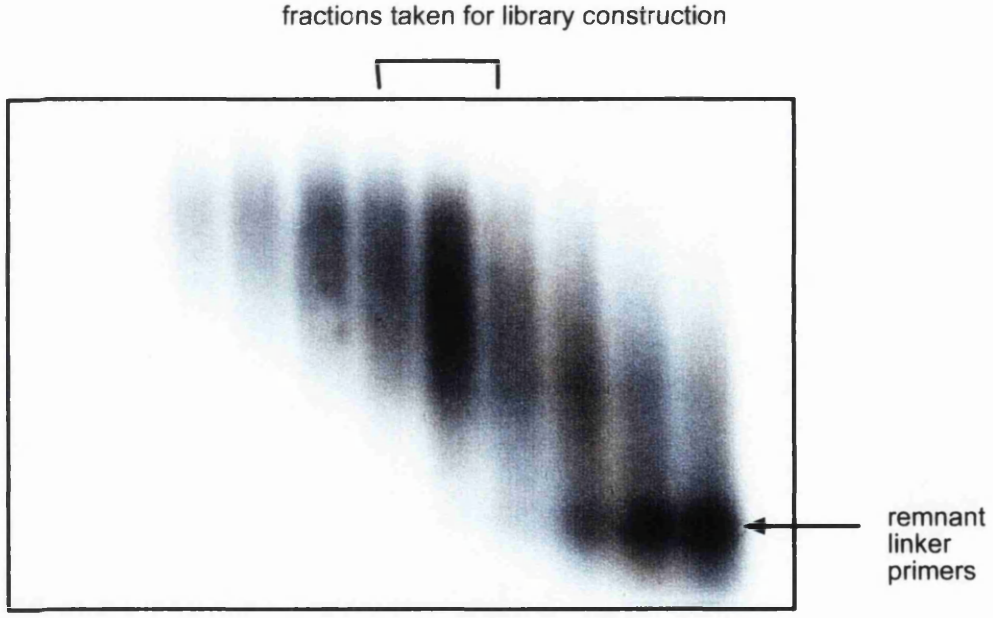


Figure 85 showing first and second strand cDNA, radioactively labelled and run on a formaldehyde gel. Size fractionation of a sample of radiolabelled 2nd strand cDNA, demonstrating the extent of DNA taken for ligation.

5.6.1h) Adapter ligation

The cDNA was then resuspended in EcoR1 adapters of 9- and 13-mer oligonucleotides for terminal ligation, and incubated at 4°C for 30 minutes.

A radiolabelled 1µl aliquot of the second strand synthesis reaction was then run along with the first strand on an alkaline agarose gel blotted, U-V cross-linked and developed by autoradiography the following day (see figure 85). If second-strand synthesis was self primed, synthesis yields cDNA from about 900 bases to 10,000 bases in size (see figure 85 also).

1µl T4 DNA ligase (4U/µl) with 1µl of 10x ligase buffer (500mM Tris-HCl (pH 7.5), 70mM $MgCl_2$, 10mM DTT Stratagene) and 1µl of 10mM rATP (ribonucleotide ATP) was added to the rest of the sample and the reaction incubated overnight at 8°C, to fill in the gaps formed by nicking and blunt-ending. The T4 ligase was then heat inactivated at 70°C for 30 minutes, the sample spun and cooled for 5 minutes to allow the ends to re-anneal.

Finally adapters (9-mer only), were phosphorylated with 1µl T4 polynucleotide kinase (10U/µl), 2µl 10mM rATP, 1µl of 10x ligase buffer and 6µl of sterile water, at 37°C for 30 minutes, after which the kinase was inactivated at 70°C for 30 minutes.

5.6.1i) Synthetic DNA linkers and adapters

To allow restriction sites to be placed at the end of the synthesised cDNA, so that insertion into an appropriate vector or plasmid can be brought about, synthetic linkers or adapters can be used. Single stranded 3′ overhangs are filled in with DNA polymerase and then incubated with the desired linkers and T4 DNA ligase which catalyses the linkage of these now blunt-ended cDNA molecules with the linkers. The molecules can then be cleaved at a restriction site in the linker, purified and ligated into the vector of choice.

Synthetic adapters differ to linkers in that they are shorter molecules with a cohesive end for blunt ending and a cohesive terminus for ligating into the vector. They do not require digestion with restriction enzymes prior to ligation. However, since they are phosphorylated they can form circular molecules or linear chimeras with themselves or the dephosphorylated vector DNA. To prevent this only the shorter of the two oligonucleotides that make up the adapter is phosphorylated before ligation to cDNA.

5.6.1j) XHO1 digest

Samples were digested with 3µl Xho1 (40U/µl) with 28µl buffer at 37°C for 1.5 hours to yield final second stranded cDNA, as well as residual adapter-primers.

5µl of TEN buffer (10mM Tris-HCl (pH7.5), 0.1mM EDTA, 25nM NaCl) was then added, and cDNA allowed to ethanol precipitate overnight at -20°C. The next day, samples were spun and dried and resuspended in TEN buffer.

5.6.1k) Size fractionation

The second strand cDNA was then run down sephacryl size fractionation columns (GIBCO) to yield appropriate sized cDNA, i.e. without unincorporated adapters, residual linker-primers, and lower molecular weight products.

Each column was washed with 0.8ml of TEN buffer three times, and the entire ^{32}P-labelled sample in 100µl of TEN buffer pipetted onto the column. After complete drainage a further 100µl of buffer was added and the column completely drained again. Following addition of another 100µl of buffer, a drop representing each fraction was collected, up to a total of 18 fractions. A small sample of each fraction was then checked on a 1% alkaline agarose gel by electrophoresis, blotted and visualised by autoradiography of labelled ^{32}P (see figure 85). The GIBCO columns contained 1ml of Sephacyrl® S-500 HR in a 2ml column.

Appropriately sized fractions corresponding to a certain range of molecular weights, without residual primer-linkers were pooled and phenol:chloroform, ethanol precipitated to remove any contaminating proteins or residual kinase carry over, and resuspended in TE buffer (10mM Tris pH 7.5 and 500mM EDTA pH7). Pellets were ethanol washed, dried and resuspended in sterile water.

An ethidium bromide plate assay was conducted in order to quantitate the cDNA (amounts in nanogrammes). Plates of 0.8% agarose in TBE were poured containing 10µl ethidium bromide (10mg/ml) under a fume hood, approximately 10ml/plate, and 0.5µl samples of diluted cDNA as well as a DNA diluted size marker ladder spotted on the plate, allowed to dry in for 10-15 minutes at room temperature, and then visualised under U-V.

5.6.1l) The Lambda Vector

In earlier times cDNA libraries were kept in bacterial colonies independently transformed at greater than 10^5. The advent of λ vectors allowed libraries to be packaged into these virus particles, amplified and kept for very long periods of time.

The λ ZAP vector used here, was developed by Short et al.1988, and carries a polycloning site downstream from the *E.coli* lac Z promoter (the lac Z gene when expressed allows the synthesis of the enzyme β galactosidase and conversion of X-gal into product, yielding a blue colour in bacterial colonies expressing it, in the presence of IPTG). cDNA's up to 12 kb may successfully be inserted into this vector which has been modified by Stratagene, to contain 12 unique cloning sites at the amino terminal region of the lac Z gene, which can be used to directionally clone cDNA molecules (see figure 86). The presence of the T3 and T7 promoters, and the 17 restriction sites within the multiple cloning region of the pBK-CMV, allows synthesis of specific RNA's complementary to the cDNA's, as well as being of use in PCR amplification and sequencing.

Recovery of the inserted cDNA is also facilitated by the presence of certain DNA sequences that Stratagene have inserted into the lambda phage genome and M13 phage proteins for *in vivo* excision and conversion of the cloned fragments into pBK-CMV phagemid vectors. Using the f1 bacteriophage "origin of replication", by placing the initiation and termination sties of this region at different points in the lambda phage vector, only exposure to the M13 helper phage proteins within the E.coli can allow nicking of the DNA within the vector, and subsequent synthesis of the single stranded DNA duplicate to the insert. A gene II product from the helper phage then allows circularisation of the DNA.

The unidirectional nature of insertion, makes sequencing and database analysis of clones much easier.

In addition the ZAP express vector system, by virtue of it's CMV promoter, allows the cDNA to be transfected into appropriate cell lines as expression vectors to synthesise the cDNA incorporated into them in situ.

5.6.1m) Ligation into the ZAP express vector

Up to 100ng of cDNA was ligated into 1μl of the ZAP express vector arms (1μg/μl) with addition of 0.5μl of 10x ligase buffer, 0.5μl of 10mM rATP (pH 7.5), sterile water and

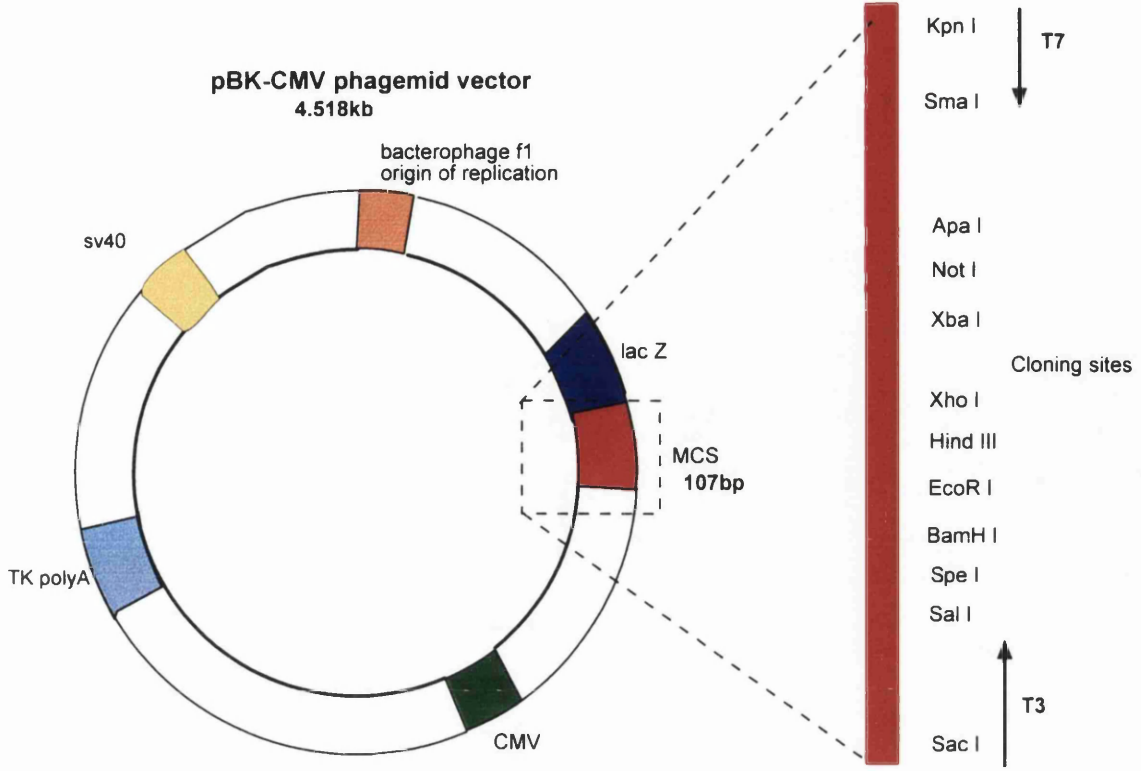


Figure 86 showing the phagemid vector after excision from the ZAP express vector.

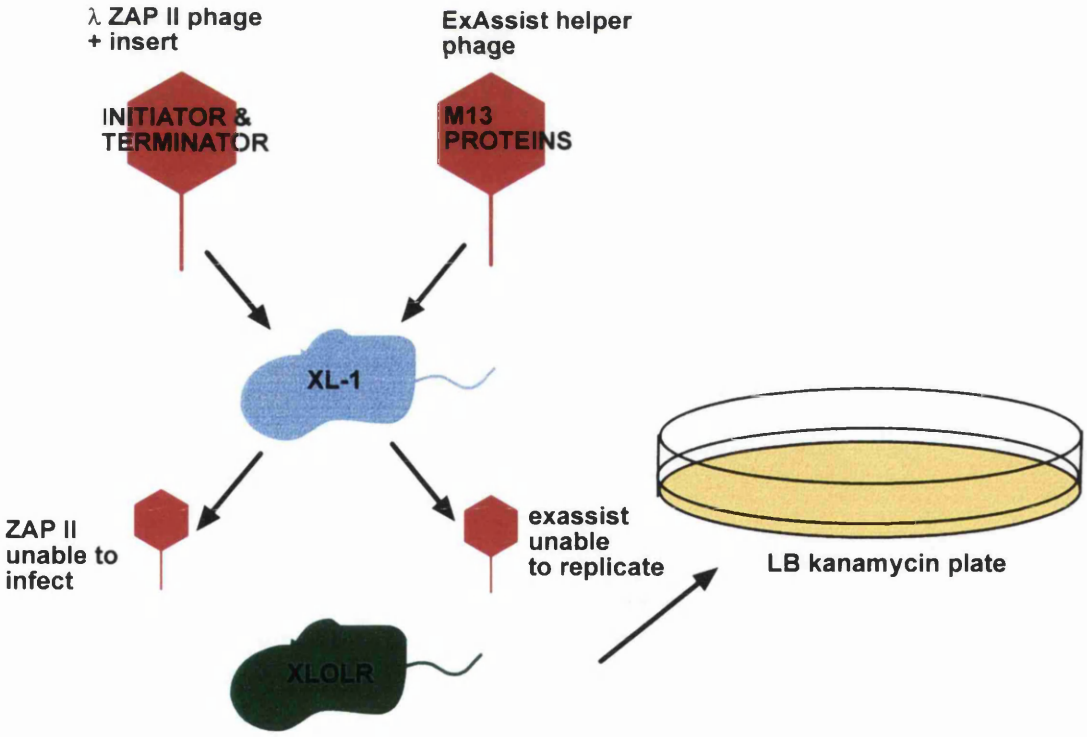


Figure 87 of the excision process required to obtain plasmid colonies with cloned inserts for DNA preparation.

0.5µl T4 DNA ligase (4U/µl), and incubated at 12°C overnight. Test insert served as a positive control for effective ligation.

5.6.1n) Packaging the vector and introduction into host bacteria

Packaging extracts (Gigapack III Gold Stratagene) containing the lambda phage heads and bodies, stored at -80°C were thawed and ligated to 1µl of cDNA at room temperature for 2 hours. One packaging extract typically provided three reactions. 500µl of SM buffer (5M NaCl, 1M $MgSO_4$, 1M Tris pH7.5, 2% gelatine, topped up with deionised water to 100mls, then filtered sterile through a 0.2µ filter) was then added and 2 drops of chloroform. After spinning, the supernatant was stored at 4°C and ready to be titred.

VCS257 host strain bacteria served as a control for the packaging extracts, provided the test λcI857 Sam7 wild type test DNA was used for packaging and plating.

5.6.1o) The host strain

XL1-Blue MRF' supplied by Stratagene was used as the host strain for the ZAP express vectors. This strain of bacteria carries a tetracycline resistance and thus initial bacteria were plated out on a 1.5% agar plate containing 12.5µg/ml of tetracycline.

The phage were then introduced to the *E.coli* XL1-Blue MRF' bacterial culture, isolated from a colony on a tetracycline agar plate, made up in 1M $MgSO_4$ and 0.2% maltose medium the previous day, and resuspended in 10mM $MgSO_4$ to yield an $O.D_{600}$ of 0.5.

The F' episome of the strain allows the lac Z gene within the vector to be fully complemented, so that vectors without inserts will appear blue. It also contains the lac Z repressor gene required to suppress lac Z activity in the absence of IPTG. Finally, it also houses the genes for the expression of filamentous pili, required for the phage infection.

5.6.1p) Plating and titering

Where titres of phage had to be established, dilutions ranging from 10^{-3} to 10^{-7} were made in SM buffer, and 5µl of each phage dilution with 100µl of XL1 cells (resuspended in 10mM $MgSO_4$ to an O.D of 1), incubated for 15 minutes at 37°C. 25ml, 1.5% agar plates were poured, dried and cooled, and then the cells plus phage added to 3mls of 0.7% top agarose (0.7g agarose in 100mls of LB) with $MgSO_4$ and maltose added in LB (10g bactotryptan, 10g NaCl, 5g yeast extract in 1L of deionised water, autoclaved) at 50°C,

poured and allowed to set onto each plate. The plates were then dried on the bench for 15 minutes and place in the 37°C incubator overnight.

Alternatively, if many titres were needed top agarose and cells were poured onto each plate as before and allowed to set. 3µl of each phage dilution was then spotted onto the plate, allowed to dry in, and then incubated at 37°C.

The following morning individual plaque forming units (pfu's) were counted. Each pfu represented the lysis of bacterial cells by one phage.

5.6.1q) Amplification of the cDNA library

Four plates 20x20 cm of 250mls of agar at 1.5% each were poured and allowed to cool and set. Plates were thoroughly washed in hot soapy water, followed by rinsing in distilled water, rinsing in 70% ethanol, and dried prior to use.

50mls of 0.7% top agarose per plate was made and kept at 55°C until required.

XL1 cells in 10mM $MgSO_4$ with 0.2% maltose were grown up prior to the day of use, and diluted 1 in 100 the next day and grown to an optical density of approximately 1 in 10mM $MgSO_4$ with 0.2% maltose. (Approximately 50mls of LB with 100µl of an overnight culture with the addition of magnesium and maltose grown for approx. 4hrs, will yield an O.D of 1). The cells were then pelleted at 3000rpm for 10 minutes and resuspended in 10mM $MgSO_4$ at an optical density of 2.

Late afternoon, 2mls of cells with up to 1ml of lambda packaged cDNA was placed at 37°C for 15 minutes for the virus to adhere to the cells.

Each 50mls of top agarose prepared earlier was aliquoted into a falcon tube and placed at 50°C. Once the temperature had been reached, the 2mls of the lambda packaged cells, 0.5ml of $MgSO_4$ (1M), and 0.5ml of 20% maltose was added, and the whole concoction poured over the pre-dried agar 20x20 plates. The plates were levelled and allowed to set, and then placed in an incubator at 37°C overnight.

The following day, 20mls of SM buffer were poured onto each plate, and the plates placed in the refrigerator overnight. The next day depending on the amount of SM absorbed, a further amount of SM was added, and the plates placed on a rocking table for 3-4 hours. The liquid from each plate was aspirated and pooled into sterile falcon tubes. The plates were then coated with an additional 5mls of SM buffer and this aspirated off and added to the rest of the liquid. The 50ml falcon tubes were then treated with 5% chloroform and placed at room temperature for 15 minutes. Following a 10 minute

centrifuge at 3000 rpm, if the supernatant was clear, it was poured into a clean 50ml falcon tube, 0.3% chloroform added and another 10 minute spin conducted. The final supernatant was then placed in 15 ml falcon tubes, containing 5ml each, with 0.3% DMSO, snap frozen in liquid nitrogen, and stored at -80°C. A small aliquot was kept refrigerated for blue and white selection, and plating for screening.

5.6.1r) Blue and white selection

To verify that cDNA incorporated into the viral vector library is indeed that from the tissue of choice, and not from non-recombinant DNA, a blue and white selection was conducted.

This involved plating out the library (1µl) with 200µl of XL1 cells at an O.D (600nm) of 0.5 (incubated for 15 minutes prior to the addition of, 0.0025M IPTG, (isopropyl-1-thio-β-D-galactopyranoside in water) (Calbiochem) and 4mg/ml X-gal bv (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in dimethylformamide (DMF)) (Calbiochem) added to 3mls of top agarose separately, which are required for the lac Z gene to be switched on, resulting in a bluish coloration of the plaques. This gene is disrupted by the insertion of cDNA from the tissue of choice. After allowing to set, overnight incubation at 37°C produces background blue plaques and white recombinant plaques.

5.6.2 SCREENING THE cDNA LIBRARY

To isolate clones of interest from the generated skin cDNA library, we decided to differentially screen the library with mPCR probes (Kendall et al. 1996).

5.6.2a) Making mPCR probes

5.6.2ai) First strand synthesis

RNA was extracted from naive and wounded skin as before. This was then diluted and the O.D measured for each sample. The O.D's were used to equilibrate the RNA from each sample for 100/500ng. Each sample was incubated at 65°C for 3 minutes with the addition of sterile water and a T7GpdT_{14} primer (Genosis) up to a volume of 25.6µl, and placed directly on ice for 1 minute.

T7GpdT_{14} 5'-GGTACCTAATACGACTCACTATAGGGAGATTTTTTTTTTTTTT-3'

1µl of each 10mM dNTP (Promega), 8µl of 5x MMLV buffer (0.25M Tris-HCl, Ph 8.3, 0.375M KCl, 15mM $MgCl_2$, 50mM DTT GIBCO), 0.4µl of 1M DTT ((dithiothreitol) GIBCO), 1µl RNase block (40U Stratagene), and 1µl of the Superscript II enzyme (200U), were then added and the samples kept at room temperature for 5 minutes, followed by incubation at 37°C for 30 minutes. More Superscript II (0.5µl), and 1µl of each 100mM dNTP were then added, and the samples placed back at 37°C for 1 hour. A 3 minute incubation at 95°C followed, and the samples were then placed on ice. 1µl of RNase A was then added and a 15 minute incubation at 37°C started. Finally, 1µl of 5M NaCl and 80µl of 100% ethanol was added for overnight precipitation at -20°C.

5.6.2aii) Second strand synthesis

First strand samples were spun at 12000 rpm for 30 minutes, and then washed in 70% ethanol, vacuum dried and resuspended in 38µl of deionised water.

To this the T3GN_6T primer (Genosis) was added in an equal amount to the original RNA, and incubated at 65°C for 3 minutes, and then placed on ice.

T3GN_6T 5'-CTCGAGATTAACCCTCACTAAAGGGAACNNNNNNT-3'

5µl of 10x Klenow buffer (100mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 7.5mM DTT) 1µl of each 100mM dNTPs, 0.4µl of 1M DTT and 2µl of Klenow large fragment of DNA

polymerase I (10U GIBCO) were the added and the samples incubated at room temperature for 30 minutes followed by 37°C for 1-3 hours.

The reaction was terminated by an incubation of 95°C for 5 minutes, and the samples placed on ice.

Each microlitre of now double stranded cDNA could then potentially be PCR-amplified using the primers T7GpdT$_{14}$ and T3G.

5.6.2aiii) PCR amplification of cDNA

For a 100µl reaction, 2µl of each 10mM dNTP, 10µl of 10x Taq buffer without Mg^{2+}, 6µl of 25mM $MgCl_2$, 0.5µl i.e. 2 units of Promega Taq, and 1µl of each primer i.e. T7GpdT$_{14}$, and T3G, were mixed and used for PCR.

T3G 5'-CTCGAGATTAACCCTCACTAAAGGGAAC-3'

The PCR reaction consisted of an initial cycle of 94°C for 4 minutes, 29 cycles of a hybridisation and annealing step at 50°C for 1 minute, followed by a synthesis reaction at 72°C for 3 minutes, then 94°C for 30 seconds, and a final cycle of 50°C for 1 minute, followed by 72°C for 10 minutes (Kendall et al. 1996), conducted in a Perkin Elmer thermal cycler.

Each PCR reaction was then purified of residual primers and small cDNA fragments less than 200 base pairs, using the Quiagen PCR spin purification kit. This involved, the addition of a buffer PB to the PCR reaction, a filter centrifugation to bind the DNA to the membrane, a wash in ethanol buffer and an elution with a pH 8 buffer. DNA was checked for integrity and primer removal.

5.6.2b) Primary screening

After titration of the amplified library, approximately one million pfu's from the library were plated out in XL1 cells, onto four agar gel plates with top agarose. The plates were then incubated at 37°C overnight and chilled. Filter lifts using hybond N^+ nylon membranes (Amersham), were taken from each plate, two lifts per plate. The filters were marked according to first or second lift and then placed in denaturing solution (219g NaCl, 50g NaOH /2.5dm^3 of water), followed by neutralising solution (302.75g Tris, 219.15g NaCl pH to 7.2 with conc. HCl /dm^3), and thence into 2x SSC (for 20x: 438g NaCl, 220g

NaCitrate /2.5 dm^3). The filters (DNA) were then auto-crosslinked with 1200kJ and allowed to dry.

Filters were then placed in a prehybridisation solution of 10% dextran sulphate, 6x SSC, 0.5% SDS, 100µg/ml herring sperm DNA (hsDNA) (boiled for 5 minutes, and cooled for 2 minutes before addition), 5x Denhardt's solution, all diluted in water to a volume of 50mls per canister, and placed in rotating canisters at 65°C for 3-4 hours. Meshes were placed in between filters to prevent adherence to one another.

Meanwhile the mPCR probes outlined before, were denatured and second stranded with 3.5µl of random hexamers (90U), and sterile water up to 16µl, heated to 100°C for 3 minutes, and then placed on ice for 2 minutes. Deoxyribonucleotides, 0.5µl of each GTP, ATP and TTP of 100mM, with 3µl of radioactive ^{32}PdCTP, 2µl Klenow large fragment of DNA polymerase I and 2.5µl of 10x Klenow buffer were added and the reaction incubated at 37°C for two hours.

Following this, probes were diluted to a volume of 225µl with water and passed down sephadex spin columns. Sephadex G50 (Pharmacia) (0.25g sephadex in 40mls of deionised water, washed and spun, the supernatant decanted off, and TE buffer added at a pH of 7.5 until the slurry was of the correct consistency) columns were made using 1ml syringes plugged at their ends, placed in 15ml falcons into which sephadex was poured. These columns were then spun up to 3000 rpm and then back down, topped up with more sephadex and respun to 3000 rpm. 200µl of water was then added and the columns spun again for 5 minutes. The flow-through was discarded and the columns placed in new 15ml falcons.

Alternatively, mPCR probes were purified using the Geneclean kit for the removal of primers, linkers and unincorporated labelled nucleotides. This procedure involved addition of the DNA solution to glassmilk in a filter tube, spinning to bind the DNA to the filter bed, a wash in wash buffer, and elution with a pH solution.

1µl of the flow-through from each 225µl probe was spotted on 3MM paper and examined with a scintillation counter. The probes were then aliquoted to produce equal radioactive emission.

After prehybridisation, the solution on the filters was replaced with new solution plus each probe, naive skin and wounded skin boiled for 10 minutes before addition to their respective filters. First lift filters were labelled with naive probe, and second lift filters

with wounded probe in order to ensure that any upregulation seen would be more of a definite result.

Thus two sets of filters each with a naive and wounded skin probe were hybridised overnight at 65°C.

The next day, the probes were removed and the filter washes conducted with 6x SSC, down to 1x SSC for 3-4 hours, or until the radioactivity was down to a reasonable level, and failed to be washed off.

Filters were then blotted dry and placed in cassettes with autoradiographic film. Cassettes were placed at -80°C and films developed after two days.

5.6.2c) Clone selection

Autoradiographs were then aligned with each other and any spots showing up or down-regulation in radioactivity identified by visual inspection and circled. The films were then aligned with the original plaque plates and primary plugs picked according to the up or downregulated spots. The plugs were placed at 4°C in 1ml of SM buffer with one drop of chloroform.

The plugs were then titred on (0.7% top agarose) gel plates and plated out at 100pfu's per plate.

5.6.2d) Secondary screening

Two filter lifts were taken from each of the above plates, labelled first or second, denatured, neutralised and soaked in SSC, U-V cross-linked as before, and a secondary screening undertaken as with the primary, under the same prehybridisation and hybridisation conditions with fresh solutions and fresh equalised mPCR probes. Autoradiographs of each naive plate were then aligned with their corresponding wounded plate, and secondary plugs now consisting of an individual plaque, chosen and removed (see figure 88). All secondary plaques were then placed in 500µl SM buffer and stored at 4°C.

Filter probed with naive skin mPCR probe

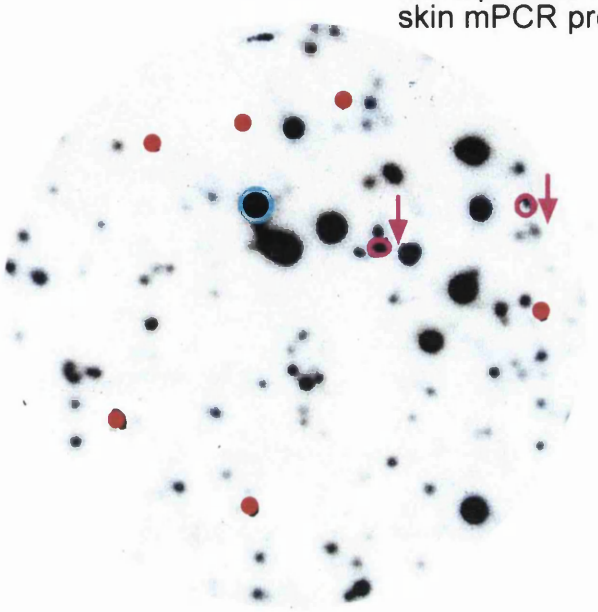
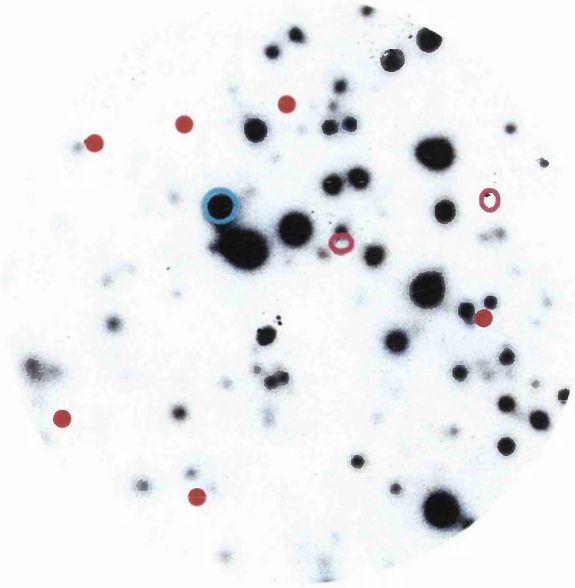


Figure 88 illustrating the secondary screening of primary plugs with naive and wounded probes. Red dots show plate allignment. The blue circle shows an unregulated clone, the pink circles clones downregulated in the wounded probed sample.

Filter probed with wounded skin mPCR probe



5.6.3 EXCISION OF cDNA

Unless the plaques were amplified, sufficient numbers of colonies with excised DNA in phagemid form could not be obtained. Therefore, secondary plugs were titrated and plated at a density of 50-100 pfu's per plate. Ten of these plaques were then used for each excision reaction.

250µl of phage stock, 200µl XL1 cells (grown in the usual magnesium sulphate and maltose medium to an O.D of 1) and 2µl of ex-assist helper phage were incubated for 15 minutes at 37°C. 3mls of LB was added, and the mixture incubated for a further 2.5-3 hours with agitation, to allow the expression of the kanamycin-resistance gene.

Afterwards the tubes were heated to 70°C for 20 minutes to lyse the bacteria, and spun at 3000rpm for 10 minutes. The supernatant was then decanted into a sterile tube and either stored or used immediately to plate out the excised phagemid.

To plate the phagemids, 100µl of the supernatant was added to 200µl of freshly grown XLOLR cells. Tubes were incubated at 37°C for 15 minutes again, and 300µl of LB then added, followed by a further incubation at 37°C for 45 minutes. 200µl of this mixture was then plated onto LB-kanamycin (50µg/ml) agar plates, and placed in a 37°C oven overnight (see figure 87).

5.6.3a) Helper phage

The helper phage ExAssist™ interference-resistant, provides the means of excising the cDNA in vector form and ready for infection into the XLOLR strain of bacteria, without co-infection and replication of itself. Co-infection within E.coli allows the trans-acting proteins from the helper phage to recognise the initiator and terminator domains within the ZAP express vector arms and new DNA strands are synthesised, displacing the existing strands which are then circularised and packaged by helper phage proteins, and secreted from the cell.

The phage contains approximately $1x10^{10}$ pfu/ml of supercoiled single stranded DNA, but was titred as indicated by the Stratagene protocol before use, since it had been stored at -80°C for some months.

5.6.3b) XLOLR strain of bacteria

The XLOLR strain of bacteria carry a kanamycin resistance gene and allow infection of the phagemid without subsequent lysis. Thus colonies of these bacteria can be grown

containing the appropriate phagemid and used for bacterial cell culture and DNA isolation. In addition, they are resistant to lambda infection, preventing lambda DNA contamination after excision. Helper phage is unable to replicate in Su^- (non-suppressing) cells such as the XLOLR's.

XLOLR's were obtained as a bacterial glycerol stock and streaked on a tetracycline LB agar plate with a wire loop, with subsequent overnight culturing with LB supplemented with MgSO4 and maltose.

5.6.4 Plasmid minipreps

Overnight cultures of a single colony in 10mls of LB with kanamycin were then prepared.

The next morning 5ml of each culture was spun at 3000 rpm for 10 minutes. The bacteria were then resuspended in 250µl of buffer P1, alkaline lysis buffer with RNAse added. Plasmid minipreps were prepared according to the Quiagen Miniprep Spin Kit. Briefly this involved, addition of lysis buffer P2, inversion and addition of a neutralising buffer N3, inversion and centrifugation. Supernatants were then transferred to spin columns and washed in an ethanol buffer PE, centrifuged, washed again, and the DNA eluted with water.

5.6.5 Restriction Digests

20µl restriction digests of the above plasmid preparations were performed, with the enzymes Kpn I (0.5µl NEB) and Sac I (0.25µl NEB), (with 2µl of NEB1 buffer, and sterile water), which flank the ends of the multiple cloning region of the vector (see figure 86) and run on 1% TBE agarose gels. This was done initially to check that the inserts could be retrieved. On confirmation, larger digests (100µl) were performed and checked, as well as a Xho I (1U/20µl digest), Xba I (1U/20µl digest) digest for a line 1 plasmid preparation (courtesy of M.Robinson, isolated from Quinn's ZAP library, yielding a 900bp insert) which is known to be a housekeeping gene i.e. stay unregulated in conditions of adult injury.

N.B. In subsequent digests and Southerns a clone from the library constructed above, known to be unregulated after neonatal skin wounding was used as a control.

In addition, a 24 hour wounded skin probe was constructed in further Southerns to allow for any developmental regulation of genes which may be up- or downregulated to be obtained.

5.6.6 SOUTHERN BLOTTING

Southern blotting is a form of blotting which allows DNA to be bound to filters, and was developed by Professor Southern in 1975 (For a detailed description see Old & Primrose, 1994). The gel on which the DNA is run must be pre-treated to denature the DNA, which can then be transferred onto a nylon membrane by capillary action.

Duplicate samples of each digest reaction (25µl) were run on an ethidium bromide gel along with duplicates of the line 1 fragment (unregulated clone) (see figures 89 & 91). The gels were then denatured, neutralised and placed in 2x SSC solution before blotting overnight (for a detailed protocol refer to Sambrook et al. 1998). The filters were then UV cross-linked, and pre-hybridised and hybridised in a mixture of 5x SSC, 5x Denhardt's solution, 1% SDS, 10ng/ml HSDNA and 50% formamide, with de-ionised water up to 20mls, at 42°C for 4 hours, and probed with equalised wounded and unwounded skin mPCR probes as before.

The filters were then washed the following day in 6x SSC with decreasing concentrations, until counts failed to be washed off. The membranes were then blotted dry, and placed in a cassette with autoradiographic film. After refrigeration at -80°C films were developed after 4-5 hours (see figures 89 & 91). Regulated clones were then partially sequenced (~200bp), or sent off to be partially sequenced (~800bp).

T3 primer 5'-AATTAACCCTCACTAAAGGG-3'

T7 primer 5'-GTAATACGACTCACTATAGGGC-3' (as indicated by Stratagene for their pBK-CMV vector).

Figure 89 showing part of the Southern blot of clones screened with dermal probes. Arrows indicate regulated clones, 1 and 3. Clone 12 is not shown, since the band was too faint.

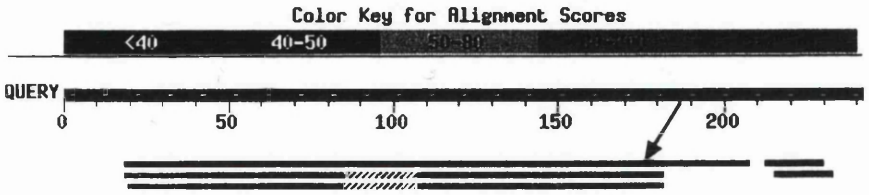


Figure 90 showing the homology between many of the initial clones isolated by screening and mouse epidermal keratin type I intermediate filament.

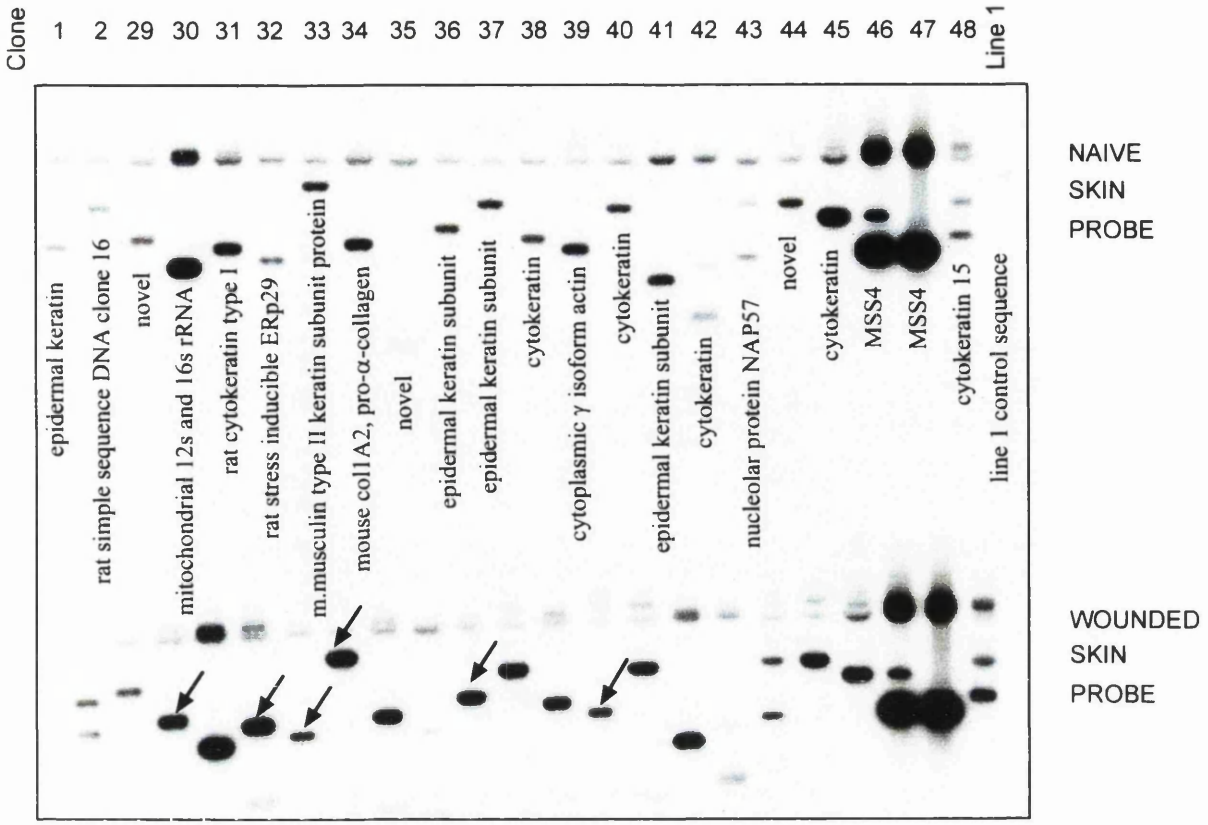


Figure 91: Southern Blot of plasmid inserts from secondary screening probed with naive and wounded skin. Duplicate samples probed. Arrows show some regulated clones. Labels show possible homologous sequences.

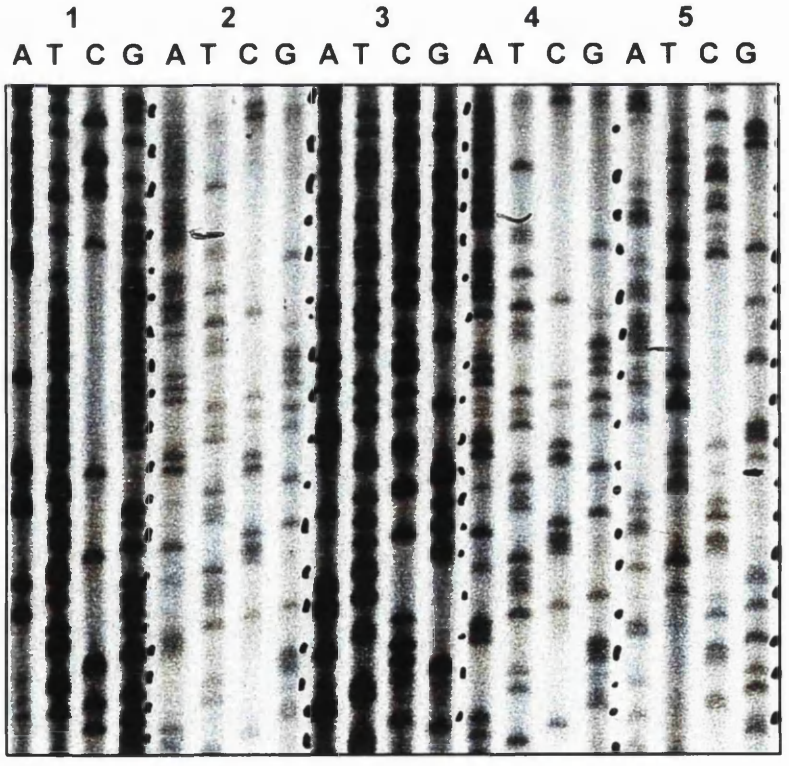


Figure 92: Sequencing gel autoradiograph, showing sequences for five clones isolated from dermal differential screening.

5.6.7 SEQUENCING

The genetic material encoded in DNA is characterised by its sequence of deoxyribonucleotide bases, namely, Adenosine, Cytosine, Guanine and Thymidine. The most common method employed to determine the genetic sequence has been by enzymatic digestion and chain termination (see Sambrook et al. 1998).

Thus, denatured plasmid cDNA can be used as a starting point for the enzymatic synthesis of second strands of variable length with the use of dideoxyribonucleotides for chain termination. Four radiolabelled synthesis reactions each with a different base dideoxyribonucleotide allow the manufacture of variable sized cDNA's with populations of oligonucleotides for each A, T, C, G in the desired template. These fragments can then be run on a polyacrylamide electrophoresis gel and the resultant genetic sequence determined in order of appearance on autoradiographic film (see figure 92).

1µl of each plasmid miniprep was diluted with water to 20µl, corresponding to 2µg of DNA template, and sent off to be sequenced to a Microchemical facility (The Barbraham Institute Cambridge or Oswel DNA Southampton for automated sequencing, or for short sequences manual sequencing by Mary Rahman, Central Lab UCL), with the use of T3 and T7 primers, for sense and antisense sequencing respectively.

A small sample of clones were sequenced using ^{35}SdATP (NEN), and T7 Sequenase Kit (Stratagene). NB. Sequenase T7 DNA polymerase (see Sambrook et al. 1998) is a modified without 3' to 5' exonuclease activity.

18µl of miniprep DNA diluted in water (corresponding to 5-6µg of template) was denatured using 1.8µl of 2M NaOH/2mM EDTA and incubated at room temperature for 5 minutes. A 2M ammonium acetate precipitation with 3 volumes of ethanol was then performed for 30 minutes at -20°C. Following centrifugation for 10 minutes at 13000rpm, pellets were washed with 70% ethanol and allowed to dry.

Meanwhile a 2µl of 1 pmole primer (T3 or T7 Genosis) per 10µl was prepared with 2µl sequencing buffer, and the pellets resuspended in this, and allowed to incubate at 37°C for 10 minutes.

Stock labelling mix was prepared with 10µl 0.1M DTT, 4µl labelling mix, 16.25µl sequenase dilution buffer, 5µl ^{35}SdATP, 16µl dH_2O, 1.25µl pyrophosphatase and 2.5µl undiluted sequenase enzyme added last. 5µl of this was then added to each annealing reaction and incubated at room temperature for 5 minutes.

2.5µl of each termination deoxyneucleotides were aliquoted into a microtitre plate, and incubated at 37°C. 3.5µl of labelling reaction mix was then added to each termination well and incubated at 37°C for a further 5 minutes. Finally, 4µl of stop solution was added to each well, the dish taped and placed in a -20°C fridge overnight.

5.6.7a) Sequencing gel preparation

Glass plates were cleaned with Decon, rinsed with water and wiped with ethanol. The smaller of the two glass plates was then silanised with dimethyldichlorositane (Promega), the larger plate being wiped with acyrlease (Stratagene). Sponge heads and spacers were then applied, the plates laid on top of each other, and clipped firm. The bottom of the plates was sealed with 6% acrylamide gel (stock solution; 75mls Acryl Promega, 50mls 10x TBE, 230g Urea ultrapure, dissolved at 37°C to a volume of 500mls with water and filtered with a 0.45µm filter, with a pH below 7, stored at 4°C used as 5mls acrylamide with 20µl of 20% ammonium persulphate and 5µl of Temed). The rest of the gel was then poured at an angle, and left to set, with combs in position until the following day.

Plates were then placed in the gel tank with 1x TBE buffer, and the sharks tooth combs inverted and gently pressed into the gel top. Residual urea was flushed out of the wells with a pipette. Leads were applied and the gel pre-run for 15 minutes at 65watts. Samples were heated at 80°C for 5 minutes, and 2.5µl of each sample was then loaded, and the gel run until the xylene cyanol (SIGMA) was 2 inches from the bottom of the gel.

The plates were then teased apart and a layer of 3MM placed on the gel to peel it off the plate. A layer of Saran wrap was laid on top of the gel, and the gel placed in a dryer at 80°C for 2 hours. Once the gel was dry the Saran wrap was removed and the gel placed in a cassette with Biomax film (Kodak) overnight. The film was developed the next morning (see figure).

A Genbank, Blast search of the sequence of A,G,C and T neucleotides corresponding to the clones in question was conducted (see figures 90 & 93).

5.6.8 Construction of a keratin probe

From the primary Southern, it was noted that some form of keratin or keratin subunit appeared frequently in clones upregulated after wounding. Since the abundance of this substance was deemed a hindrance to more significant clone isolation, it was decided that

this clone should be screened out on subsequent Southern blots. A keratin probe had to be made.

Initial database searches showed that the fragments incorporated into the plasmids were not of the same sequence, nor exactly the same gene. However, it was decided that the sequence of greatest homology be used to construct a probe. This clone was then bacterial transfected and restriction digested with Kpn 1 and Sac 1 in NE buffer 1, since digests had shown it to yield approximately a 1Kb band. The resultant digest was then ethanol precipitated, vacuum dried, resuspended in a smaller volume and run on a 1% low melting point agarose TBE gel, and the appropriate band excised. Three volumes of water were then added to the agarose fragment, with subsequent boiling for 10 minutes and vortexing. The resultant solution was stored at -20°C until required.

5.6.9 Bacterial transfection

XL1 cells were grown from a fresh tetracycline agar plate in LB, overnight at 37°C with agitation. The following morning, 50mls of this culture was placed in a 500mls flask and grown for 3^1/2 hours again with shaking at 37°C. Once the $O.D_{600}$ had reached approximately 0.5 the cells were pelleted at 3000rpm for 10 minutes and the supernatant poured away. The cells were allowed to dry and resuspended in ice cold $CaCl_2$ (100mM) from 1ml to 20mls total volume. They were then placed on ice for 20 minutes. The cells were then spun again for 10 minutes at 3000rpm and drained. Finally they were resuspended in 2.5mls of ice cold $CaCl_2$ (100mM). They could then be left for 1 hour to 7 days before use.

200µl of cells were then added to 50-1µg of DNA to be transformed. The components were mixed well and placed on ice for 30 minutes. The eppendorf tube was then heated to 42°C for 60-90 seconds and placed on ice for 2 minutes. The solution could then be spread directly onto a kanamycin (or appropriately selectable marker) plate or directly minipreped by taking half of the transformation reaction and growing it in 10mls of LB with the appropriate marker. (Plates were allowed to dry before incubation overnight at 37°C).

5.6.9a) Probing with keratin

When the agarose keratin probe was needed to hybridise to filters, the filters were pre-hybed as before and meanwhile, a Klenow reaction was performed with 10µl 5x RP buffer, 3µl α^{32} PdCTP, 1µl of each 100mM nucleotide A,T and G, 3.5µl of random hexamers

(90U), 1.5µl of the large fragment of Klenow DNA polymerase I, the probe DNA (29µl boiled for 3-5 minutes before use) and made up to a total volume of 50µl with water. This mixture was then incubated at 37°C for 3-12 hours.

Once prehybridisation was completed the Klenow reaction was made up to 200µl with water and placed with the hybridisation mixture to hybridise overnight. The next day filters were dealt with as before and assessed by autoradiography.

5.6.10 Epidermal and dermal cell layer separation

Due to the large number of keratin clones, it was decided that the library should be screened again, with pure dermal cell probes. Thus epidermis and dermis was separated as described in Chapter 3, again bearing in mind RNase free conditions where possible. The resultant dermal layer was placed in an eppendorf tube and snap frozen in liquid nitrogen.

mPCR probes for dermal wounded and naive skin were made as before, and primary and secondary screens were then repeated, with primary screens conducted at a lower phage density 250,000 plaque forming units per plate.

5.6.11 Independent verification of regulated clones

Northern blotting for clone 18 of the full screen, and clones 1, 2, and 12 dermal screen was conducted as described in Chapter 4. Probes for northern analysis were made directly from restriction digested bands excised from agarose gels.

In situ hybridisation was conducted for clones of known homology, 32 and 18 of the full screen, clones 1 and 2 of the dermal screen, and 2 novel clones 29 and 35 of the full screen (which had been sequenced), as described in Chapter 4. Antisense oligonucleotide sequences were selected with the same criteria as before, and were as follows:

CLONE FS 46, 40 mer anti-sense oligonucleotide corresponding to base pair 338 of the published sequence for MSS4 by Burton et al. 1993. Genbank accession no. X70496
5'-CAGTGAAATTGACCTTCCAGTGAAGAGGCTGGAATCTCCC-3'

CLONE DS1, 45 mer anti-sense oligonucleotide corresponding to base pair 4245 of the published sequence for rat alternative brain Ca^{2+}ATPase by Gunteski-Hamblin et al. 1992. Genbank accession no. J04024
5'-TCTTCTCAGGAACAGGAAACTAAGGCTCCGAGCTGAGAAGCTCAG-3'

CLONE DS 3, 45 mer anti-sense oligonucleotide corresponding to base pair 138 of the published sequence for rat major alpha globin mRNA by Satoh et al. 1987. Genbank accession no. M17083.
5'-GCTTACATCAATGTGAGAGAAGTAGGTCTTGGTGGTGGGAAGGCA-3'

CLONE FS 32, 40 mer anti-sense oligonucleotide corresponding to base pair 607 of the published sequence for *Rattus norvegicus* mRNA for ERP 29 protein, by Demmer et al. 1997. Genbank accession no. Y10264
5'-ACTTCTTGTCTGTCTCCTTCACACCTGAGAGGCCATCCTG-3'

CLONE FS 35, 45 mer anti-sense oligonucleotide from a novel sequenced clone
5'-AAGGCATCTAGAGGAAGCTCTCCATCCAGAAACTTCTCTGCCATG-3'

CLONE FS 29, 40 mer anti-sense oligonucleotide from a novel sequenced clone
5'-ACTTCTTGTCTGTCTCCTTCACACCTGAGAGGCCATCCTG-3'

5.7 RESULTS

5.7.1 Isolation of Regulated Clones

The initial library titre fell just short of the recommended million plaque number suggested for a representative library. It was decided to carry on and amplify this library anyway, even though it was possible that it did not represent all the genes within the tissue of interest. A great deal of tissue would be needed for a better yield and the process would be time-consuming and not necessarily problem free. The final amplified library titre was $2.2x10^9/\mu l$.

Initially, 80 regulated clones were isolated from differentially screening the library. In the first 20 clones sequenced 9 were keratin or keratin had some homology in their sequence to keratin and keratin related genes. Apart from these, 8 showed significant homology to known genes, and 3 were novel. From the dermal skin differential screening of the first 15 clones isolated, none were keratin or keratin related. Eight showed considerable homology to known genes and 7 were sequences of unknown identity.

Sequences obtained from the Microchemical facility were approximately 300-900 bp in length, those from Central Lab (UCL) were 150-300 bp in length. A basic blast search was conducted using Genbank for all sequences, the results of some are represented in figure 93.

The vast number of genes isolated and the wide scope of regulated genes (assessed by Southern blotting), meant that verification of all clones and further analysis was well out of the range of this project. Hence, only a few clones (those with greater homology to known genes, and those with evidence of regulation on Southern blotting) were selected to examine and verify primarily by *in situ* hybridisation and or Northern blotting.

5.7.2 Six clones chosen for further examination

Southern blotting showed that clones FS 29, 35 and 46 were increased in tissue hybridised to probes of wounded skin. Clone FS 46 was also picked up in dermal screening where it was unregulated (see figure 91). From dermal screening, clone DS 3 labelling was increased with wounded probe. Clones DS 1 and DS 12 of the dermal screen however showed a decrease in labelling with wounded probe (see figure 89).

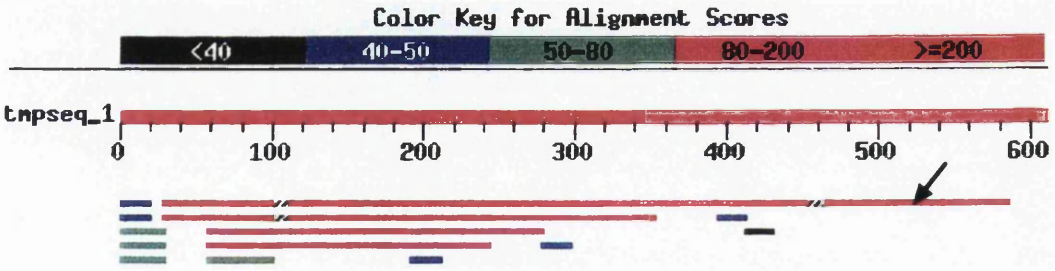
Northern blotting for clones FS 46, DS 1 and DS 3 showed that all three were regulated at 1 and 3 days after wounding (see figure 94). Northern blotting for clone DS 12 showed undetectable levels in all lanes.

In situ hybridisation for clones FS 32, FS 46, FS 29, FS 35 and DS 1 and DS 3, showed substantial changes in the levels and patterns of expression of mRNAs. Clone FS 32

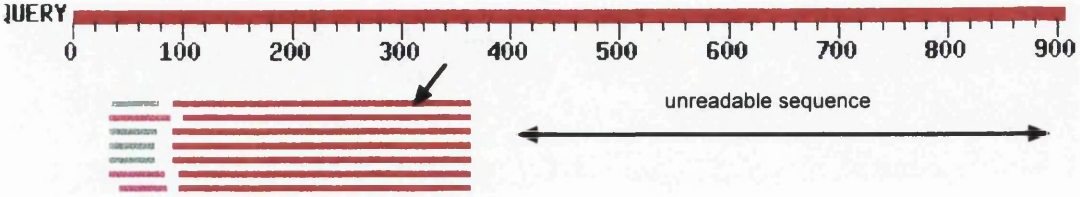
showed very little labelling of epidermis, dermis or hair follicles of naive or wounded sections (see figure 96d-f). Clone FS 46 showed very little labelling in naive sections, with increased labelling within the scar tissue, and epidermis in wounded sections (see figure 96a-c). Clone FS 29 also showed very faint labelling in control and naive sections, but had a marked increased in mRNA within the scar tissue in some sections (see figure 95a-c). Clone FS 35 showed a marked increase in labelling of the epidermis in wounded sections, with very little staining elsewhere in scar tissue or dermis (see figure 95d-f). Clone DS 1 showed expression in hair follicles in naive tissue sections, with increased mRNA expression within the hair follicles and epidermis adjacent to the wound itself, as well as some degree of increase within the scar tissue of wounded sections with less binding under the wound (see figure 97a-c). Clone DS 3 showed visible mRNA signal in the hair follicles and within the epidermis of naive tissue sections, with abundant signal in the scar tissue and under the scar tissue i.e. the dermis of wounded tissue sections (see figure 97d-f).

A summary table (3) of the clones and their regulation is shown below:

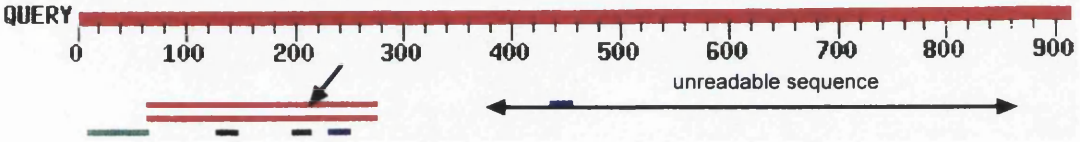
CLONE	Regulation in Southern	Regulation in Northern	*In situ* labelling in epidermis or scar tissue
FS29	↑	_	↑
FS32	↑	_	low in all tissue
FS35	↑	_	↑
FS46	↑	↑	↑
DS1	↓	↑	↑ in hair follicles
DS3	↑	↑	↑
DS12	↓	undetectable	_



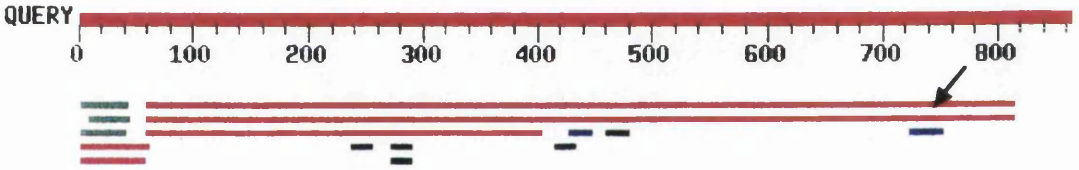
CLONE 12 dermal screen, corresponding to bases 2372 to 2715 of the Mus musculus LERK-4 (Epl4) gene, exons 2,3, 4, and complete coding sequence 3.226Kb. Cerretti et al,1998.



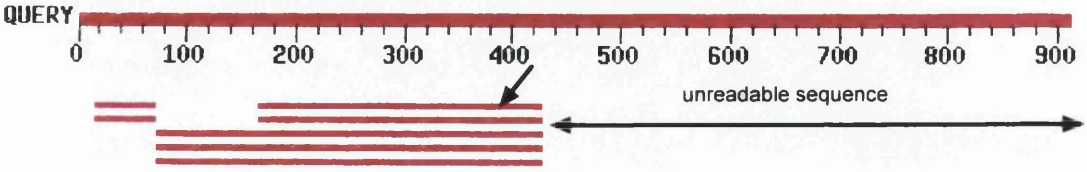
CLONE 1 dermal screen, corresponding to bases 28 to 299 of the rat major alpha-globin mRNA, complete coding sequence. 556bp mRNA. Satoh et al,1993.



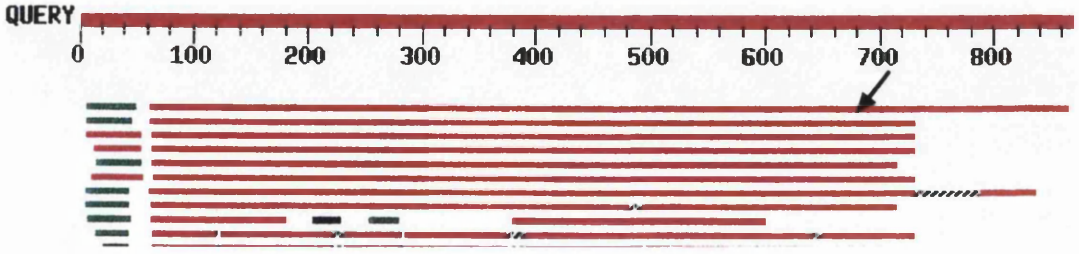
CLONE 3 dermal screen, corresponding to bases 4194 to 4403 of the rat alternative brain Ca2+-ATPase mRNA, complete coding sequence. 5576bp mRNA. Gunteski-Hamblin et al, 1993.



CLONE 32 full screen, corresponding to bases 319 to 1101of the rattus norvegicus endoplasmic reticulum protein ERp29 precursor mRNA, complete coding sequence. 1139 bp mRNA.. Mkrtchian et al, 1998.



CLONE 46 full screen and dermal screen, corresponding to bases 462 to 719 of the rattus rattus guanine nucleotide-releasing protein (mss4) mRNA completed coding sequence. 2.490 Kb mRNA.Burton et al,1993.



CLONE 34 full screen corresponding to bases 3038 to 3841 of the mouse COL1A2 mRNA for pro-alpha-2 (I) collagen. 4.270Kb mRNA. Phillips 1992.

Figure 93 showing alignments for some of the clones isolated from whole skin and dermal skin differential screening.

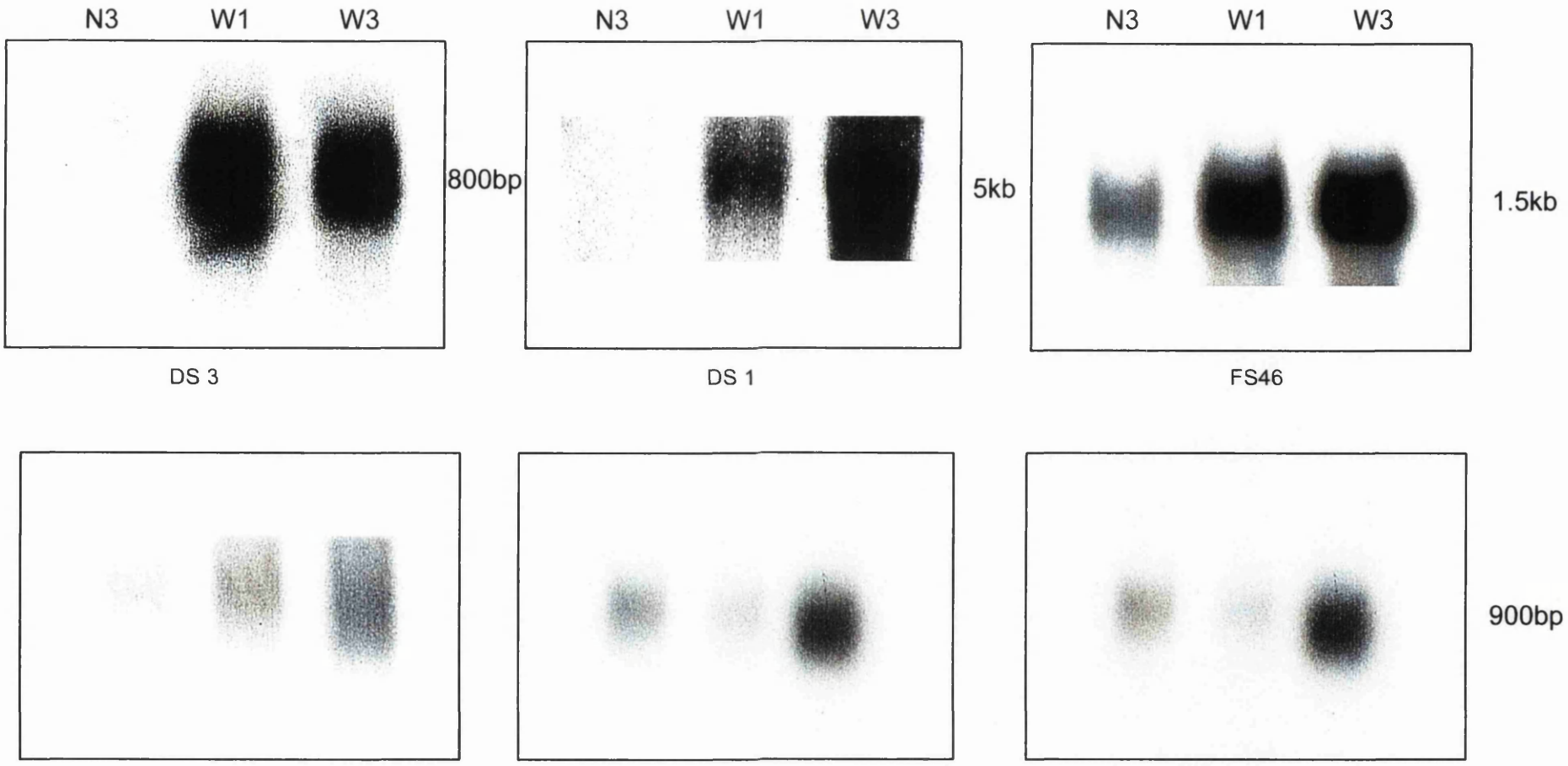
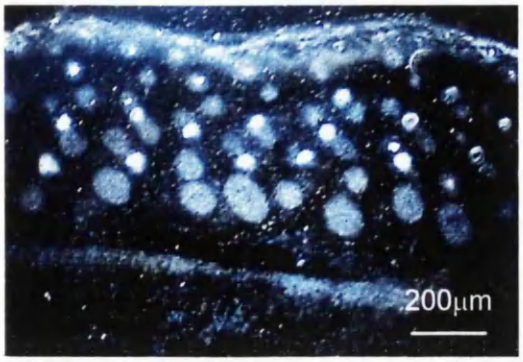
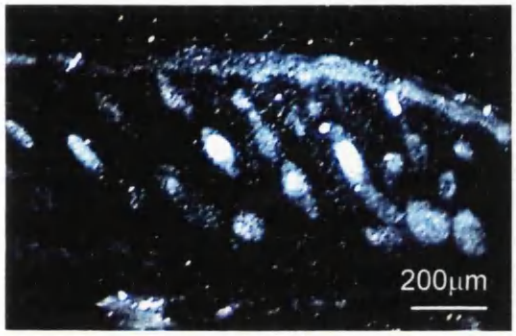


Figure 94 showing Northern blots of 3 clones isolated from library screening shown to be regulated by Southern blotting. Both clones DS 1 and DS 3 were clones isolated from dermal screening and show upregulation at 1 and 3 days after wounding, compared to cyclophilin controls below. Clone FS 46 was isolated from the full screen, and is also upregulated at both 1 and 3 days after wounding.

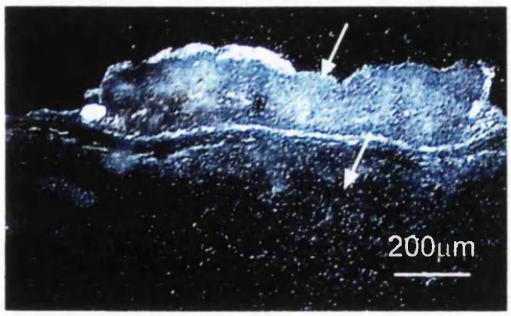
CLONE FS 29



a) P3 skin section showing non-specific binding for clone FS 29.

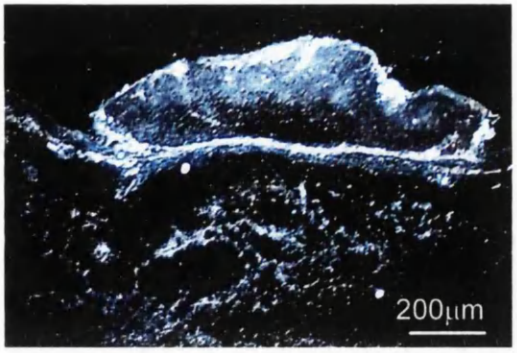


b) P3 naive skin section showing labelling for clone FS 29 mRNA. Very little labelling is apparent.



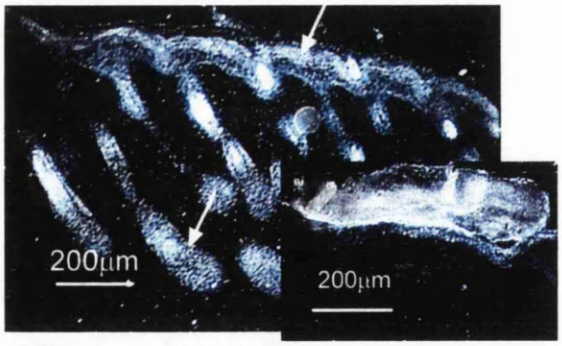
c) P3 wounded skin section showing the increasein labelling for clone FS 29 mRNA within the scar tissue and under the region of the wound itself.

CLONE FS 35



d) P3 skin section showing non-specific labelling for clone FS 35.

e) P3 naive skin section showing distribution of mRNA for clone FS 35 within the dermis and within hair follicles.

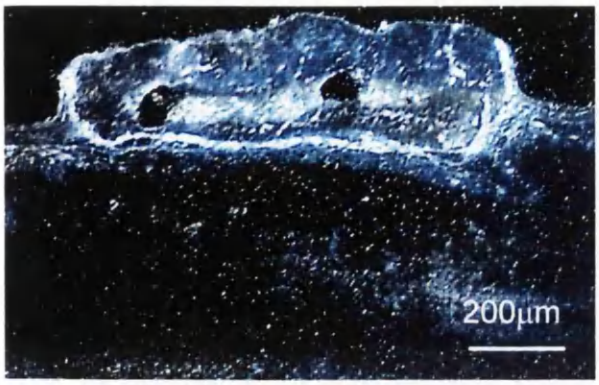


f) P3 wounded skin section demonstrating a marked increase in labelling of epidermis and hair follicles with clone FS 35. Very little mRNA is seen in the scar tissue or directly under the wound (see inset).

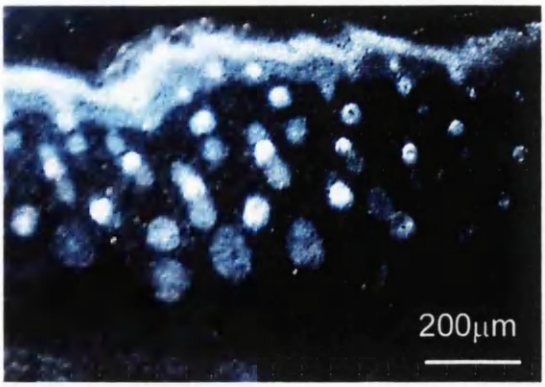
Figure 95: In situ hybridisation for clones FS 29 and FS 35 from whole skin differential screening.

CLONE FS 46

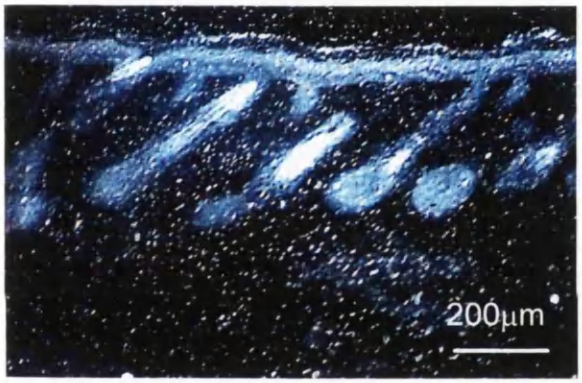
CLONE FS 32



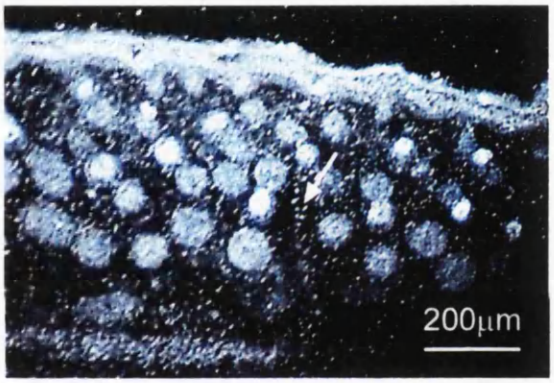
a) P3 section of skin from a wounded animal, showing non-specific labelling for clone FS 46.



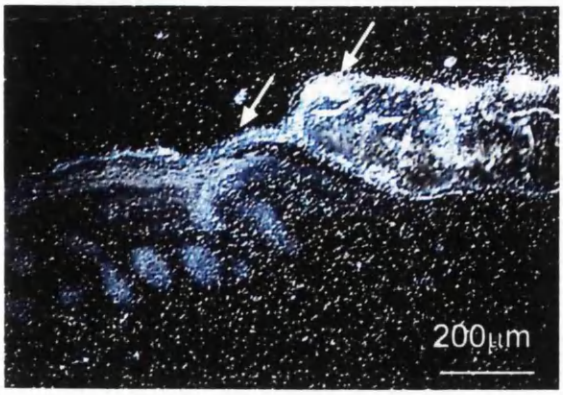
d) P3 section of skin from a wounded animal, showing non-specific labelling for clone FS 32.



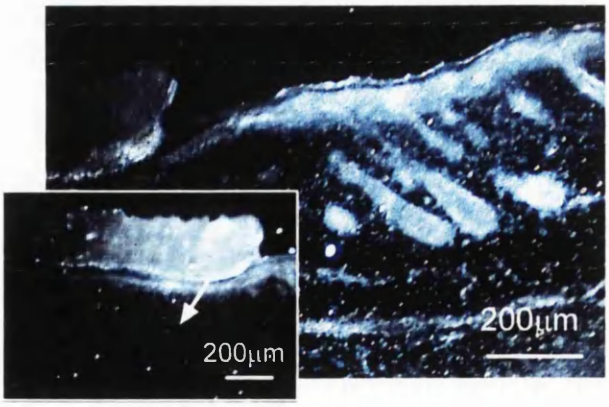
b) P3 section of naive skin showing labelling with clone FS 46 in the dermis and epidermis.



e) P3 section of naive skin showing mRNA labelling for clone FS 32 within the dermis, and hair follicles.



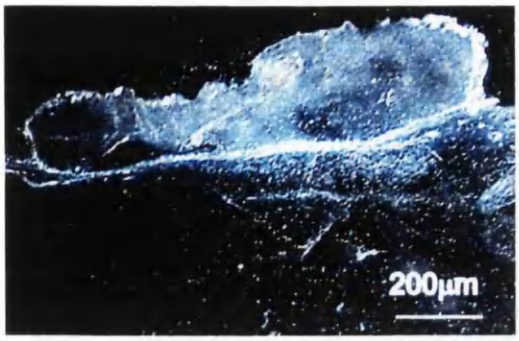
c) P3 section of skin from a wounded animal showing increased mRNA labelling for clone FS 46 (arrows), scar tissue and epidermis.



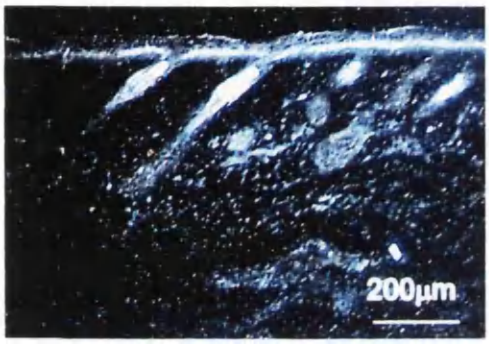
f) P3 section of skin from an animal wounded at birth. Very little labelling with clone FS 32 is present, with none in the region of scar tissue or below the wounded area (inset).

Figure 96: In situ hybridisation for clones FS 46 and FS 32 isolated from whole skin differential screening.

CLONE DS 1



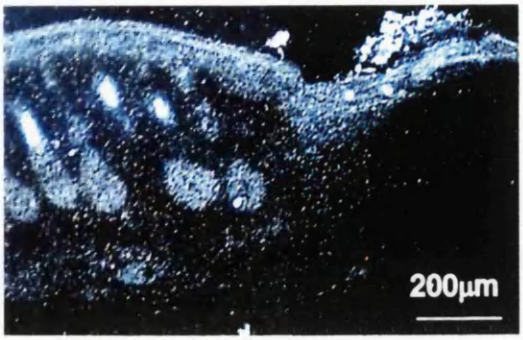
a) P3 wounded sectiion of dorsal hindpaw skin showing non-specific labelling with clone DS 1 (calcium ATPase) oligonucleotide probe.



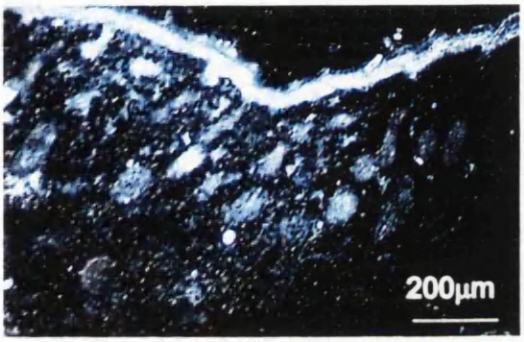
b) P3 naive section of dorsal hindpaw skin showing labelling for clone DS 1 within the dermis.

c) P3 skin from a neonate wounded at birth, demonstrating increased mRNA for clone DS 1 within the scar tissue, hair follicles and within the epidermis adjacent to the wound, with very little labelling of the underlying dermis.

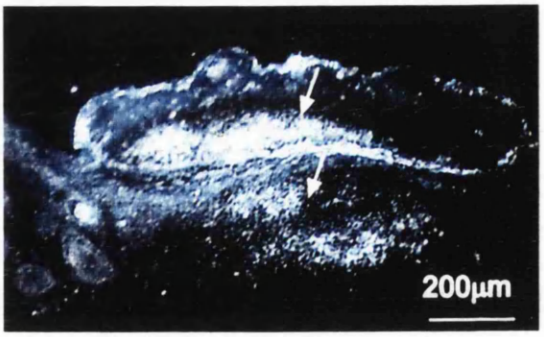
CLONE DS 3



d) P3 wounded sectiion of dorsal hindpaw skin showing non-specific labelling with clone DS 3 (alpha-globin) oligonucleotide probe.



e) P3 naive dorsal hindpaw skin section showing mRNA labelling for clone DS 3. Little difference is apparent between this and the non-specific binding.



f) P3 skin section from an animal that was wounded at birth demonstrating increased mRNA labelling for clone DS 3 within the wound scar tissue itself and beneath this.

Figure 97: In situ hybridisation for clones DS1 and DS3 isolated from differential screening of dermal skin.

5.8 DISCUSSION

5.8.1 METHODOLOGICAL ISSUES

The introduction to this chapter illustrates just some of the techniques now employed by research workers and industry for the isolation of differentially expressed genes. The vast numbers of tools available, and the ever increasing advances and modifications to methods, make the task of choosing which one to use very difficult. Most research workers utilise techniques based on available materials, costs, established methods within a laboratory, and advice from experienced colleagues. Such choices are thus not always made on the basis of whether a technique is suitable for the project in question and too often scientists speak of an element of 'luck'.

Every technique has advantages and drawbacks and the published accounts of their use in the search for differentially regulated genes within different treatments and tissues, should now provide us with a classification system, as well as some broad criteria for the use of the above techniques in different scenarios. Every technique can theorhetically be used in any scenario, but successful and efficient application is more difficult to achieve.

It is not enough to understand the molecular biology behind a technique to apply it. One must also have full comprehension of the tissue, model and hypothesis in question, and plan carefully whether one technique would be more beneficial in all aspects compared to another. Here it was clear that, as with the majority of tissues, certain clones are more abundant than others. In our case it was the keratins. The fact that 9 out of 20 first analysed clones bore some relation to this family, meant that it needed to be screened out. This was done with 100% effectiveness by the generation of dermal probes, although genes regulated within epidermal compartments were therefore selected out.

Classical differential screening has been employed successfully for many many years and is a method utilised by many researchers e.g De Leon et al. 1991. Here we have successfully constructed a neonatal rat wounded and unwounded cDNA library and used the differential screening approach with mPCR probes to isolate regulated genes in neonatal skin wounding. The increase in keratins has served to verify the technique, as keratin is known to be upregulated after wounding (see below), as have other clones, such as pro-alpha-2-collagen (see figure 93). We have established that some of these genes as truly regulated by *in situ* hybridisation and Northern blotting and shown that they bear sequence homology to known genes. We have also isolated some novel genes which are also regulated, but bear no homology to known genes. This technique has been most suitable for this study, considering the tissue of interest and the developmental and tissue

state parameters. Differential display would not have deselected the large keratin upregulation, and has indeed been used to isolate keratin genes (see Takaishi et al. 1998), and neither would have subtractive hybridisation, since keratin is upregulated and remains so even if some is removed. Other workers have commented on this with respect to genes of abundant upregulation within their own tissue types e.g. tubulin in oligodendrocytes (Baba et al. 1994). Wound healing is an essential part of the hyperinnervation process and clones involved in one could be involved in the other. Subtraction techniques may therefore remove vital genes.

5.8.2 REGULATED GENES

The number of genes isolated in the two differential screens conducted here i.e. the first full screen with naive and wounded whole skin cDNA probes and the second with dermal naive and wounded cDNA probes are far too many to give detailed descriptions.

This discussion will concentrate on the known or like genes isolated, some of which have been verified to be regulated and some of which have not, but are of interest in the area of neonatal hyperinnervation and skin wound healing.

Table 4 summarises the results of clones found, both regulated, and unknown but of interest, some with a possible role for them in neonatal skin wounding and hyperinnervation.

It should be noted that sequenced clones did not necessarily have complete, readable sequences, ie the sequence data was unreadable for some clones after ~200bp of sequence, such that homology searching could not match each clone fully.

CLONE	PRODUCT & LENGTH	INSERT SIZE ESTIMATE	FUNCTIONAL RELATION	HOMOLOGY	REFERENCE
FS 1	Homo sapiens keratin 6 isoform K6e 1.989Kb	3.8Kb	Increased under conditions of enhanced cell proliferation and abnormal differentiation	211 of 241bp match from 868bp sequenced	(Watanabe et al. 1995)
FS 2	Rat simple sequence cDNA clone 16 542bp	1.9Kb	?	188 of 189 bp match from 875bp sequenced	(Ivanova et al. 1984)

FS 29	Novel	2.3Kb		No match 859bp sequenced	
FS 30	Rat mitochondrial 12s and 16s rRNA 796bp	0.8Kb	Shown to be increased in the contralateral nerve after sciatic nerve crush	674 of 695bp match from 847bp sequenced	(Kobayashi et al,1981) (De Leon et al. 1991)
FS 31	M.musculus type II keratin subunit protein 2.341Kb	1.44Kb		492 of 542bp match from 859bp sequenced	(Steinert et al. 1983)
FS 32	Rat stress-inducible endoplasmic reticulum protein ERp 29 1.139Kb	950bp	Induced in conditions of stress to aid synthesis within the E.R	712 of 761bp match from 822bp sequenced	(Mkrtchian et al. 1998); (Demmer et al,1997)
FS 33	Mouse epidermal keratin type I intermediate filament 867bp	3.3Kb		487 of 556bp match from 861bp sequenced	(Steinert et al. 1983)
FS 34	Mouse col A2 for pro-α2-collagen (I) 4.474Kb	1.5Kb	Induction in adult skin wounds for collagen synthesis	783 of 827bp match from 867bp sequenced	(Phillips et al. 1991);(Nath et al. 1994)
FS 35	Novel	2.72Kb		No match 828bp sequenced	
FS 36	Mouse epidermal keratin subunit 847bp	1.5Kb		542 of 603bp match from 871bp sequenced	(Steinert et al. 1983)
FS 37	Mouse epidermal keratin subunit 847bp	2.1Kb		616 of 711bp match from 874bp sequenced	(Steinert et al. 1983)

FS 38	Mouse epidermal keratin subunit 847bp	1.3Kb		511 of 604bp match from 860bp sequenced	(Steinert et al. 1983)
FS 39	Rat cytoplasmic γ isoform actin 1.880Kb	1Kb		628 of 640bp match from 843bp sequenced	(Brown et al. 1990)
FS 40	Mouse epidermal keratin type I intermediate filament 847bp	2.05Kb		672 of 778bp match from 880bp sequenced	(Steinert et al. 1983)
FS 42	Rat mRNA for nucleolar protein NAP57 1.803Kb	1.2Kb	Chaperone to ribosomal proteins	687 of 711bp match from 845bp sequenced	(Meier, Blobel, 1994)
FS 43	Novel	3.4Kb		No match 864bp sequence	
FS 45	Skin keratin type I 1.539Kb	1.8Kb		224 of 238bp match from 846bp sequenced	(Sato et al. 1999)
FS 46	Rattus rattus guanine nucleotide releasing factor 2.5Kb	3Kb		462 of 483bp match from 829bp sequenced	(Burton et al. 1993)
FS 47	Rattus norvegicus MSS4 protein 2.5Kb	1.2Kb	secretory aid for Rab proteins	469 of 489bp match from 843bp sequenced	(Burton et al. 1993)
FS 48	Novel	3.3Kb		No match 854bp sequenced	
DS 1	Brain Ca^{2+}ATPase 5.576Kb	1.5Kb	increased efficiency of intracellular pathway	208 of 210bp match of 300bp sequenced	(Gunteski-Hamblin et al. 1992)

DS 2	Novel	450bp		No match 350bp sequenced	
DS 3	Rat major α-globin 556bp	700bp	provides increased oxygen needs	262 of 272bp match from 373bp sequenced	(Satoh et al,1987)
DS 4	Novel	450bp		No match 347bp sequenced	
DS 5	Rattus rattus MSS4 2.5Kb	1.1Kb		242 of 262bp match from 512bp sequenced	(Burton et al. 1993)
DS 6	Mouse proteasome Z subunit 969bp	750bp		178 of 182bp match from 190bp sequenced	(Kasahara et al. 1996)
DS 8	Rat ribosomal phosphoprotein P1 467bp	600bp		274 of 279bp match from 354bp sequenced	(Wool et al. 1991)
DS 9	Novel	3.7Kb		No match 180bp sequenced	
DS 10	Novel	800bp		No match 160bp seqeuenced	
DS 12	Mouse EPHRIN-A4 3.226Kb	1Kb	Ephrin involved in axon guidance	494 of 540bp match from 600bp sequenced	(Flenniken et al. 1996)
DS 16	Novel	1.8Kb		No match 130bp sequenced	
DS 17	Novel	600bp		No match 309bp sequenced	

DS 20	Novel	500bp		No match 150bp sequenced	

5.8.2a) Keratin

From this study many keratin and cytokeratin genes have been isolated. These were not checked for regulation, since it was clearly obvious they were upregulated after wounding (see Southern blot figure 91), a factor first documented by Pinkus in 1960's cited in M.J.T Fitzgerald 1975, and confirmed by many workers. One form, keratin 6 isolated here has also been induced in regenerating epidermis in recent studies by Paldini et al. 1996.

These molecules are the major differentiation products of mouse epidermis, and are typically from 40-70 kiloDaltons (kD) in size. They constitute a family of at least 10 related α-helix rich structural proteins, which make up intermediate filaments, and are the major components of the cytoskeleton of most epithelial cells. Different sized keratins are expressed in newborn epidermis as opposed to culture systems (Roop et al. 1983). The sheer enormity of the populations of keratins and cytokeratins, and the fact that individual keratin genes are present in multiple genes in mammalian cells, wonderfully illustrates the complexity of wound healing and re-creating skeletons for many cellular types to re-establish the skin barrier (see Paladini et al. 1996; Watanabe et al. 1995). The increased wound healing and re-epithelialisastion seen in neonates, may be partly due to the differing expressions of keratins. One must not overlook this important role, nor the many other roles that cytokeratins and keratins play e.g. cytokeratin 20 is increased in the proliferation associated with Merkel cells (Scott & Helm, 1999) and expression of some of the keratins is altered during experimental skin carcinogenesis (Winter et al. 1980), providing a diagnostic tool for screening, as well as being altered during wound healing (Betz et al. 1993). In conjunction with this are the many growth factors that keratinocytes secrete, which are important in wound healing e.g Soler et al. 1999, (see Chapter 1).

5.8.2b) CLONE DS 1 with homology to Calcium ATPase

This clone was isolated from the dermal screening of our library as clone DS 1, and was shown to be homologous to calcium ATPase after partial sequencing, with approximately a 200bp match of the 300bp sequenced. On Southern blotting the insert was approximately 1.5kb and showed downregulation after probing with the wounded skin mPCR probe. There was an upregulation on Northern hybridisation, which showed an increase in 1 and 3 day old wounded skin, with a transcript of approximately 5kb (see figure 94). This discrepancy is most likely due to the Southern only assessing dermal regulation, whereas the Norhern gel loaded both epidermis and dermis, although the probes were purely dermis. This transcript was also reported by Gunteski-Hamblin in 1992, along with a 3.9kb

transcript, which we did not detect. *In situ* hybridisation studies further confirmed the upregulation of mRNA in wounded skin, and localised it to an area immediately adjacent to the wound and within hair follicles. There was also some degree of expression within the scar area, but this was not apparent in all sections and further studies are needed to confirm this.

Cytoplasmic levels of free calcium are regulated by activity of Ca^{2+}ATPases, within the endoplasmic reticulum (ER). Three types exist, first that of skeletal muscle, which is not found in brain, a variety found in brain and heart, and a ubiquitous form with low expression in the brain (see Miller et al. 1991).

The gene isolated above encodes a calcium pump that was isolated from rat stomach and testis by Gunteski-Hamblin in 1988, and is homologous to the yeast calcium pump, implicating it in maintaining calcium concentrations within one or more components of the secretory pathway within a cell. Since all cells require this pathway it is impossible to delegate the function to a particular cell type. However, areas adjacent to the wound and the scar tissue itself and hair follicles are the likely sources demonstrating the increased turnover of proteins and activity of cells required, to allow healing and re-epithelialisation. Calcium concentration is a known regulator of certain aspects of epidermal differentiation (Hennings et al. 1980), and epithelial growth after wounding begins at the areas adjacent to the wounded site, suggesting a possible function for this upregulation.

5.8.2c) CLONE FS 46 with homology to MSS4

Clone FS46 was partially sequenced and correlated with 462bp of the 849bp sequenced, to the MSS4 protein first described by Burton et al. 1993, and isolated from a rat brain cDNA, the brain being the tissue of most abundance. This mammalian protein was identified as a suppresser of a yeast secretory mutant harbouring a mutation in the GTPase Sec 4. MSS4 allows the dissociation of GDP from this protein (Burton et al. 1994).

Rab GTPases are members of the ras GTPase superfamily. MSS4 binds GTPases specifically the Rab 10 proteins allowing the release of GDP. Recombinant MSS4 and Rab 3a form a stable complex in solution, and Rab 3a is known to be specifically targetted to neuronal and neuroendocrine synaptic vesicles (Fischer von Mollard et al. 1990).

MSS4 injected into squid giant axon terminals enhances the release of neurotransmitter by synaptic vesicle mediated exocytosis. Studies suggest that MSS4 may serve as a guanine nucleotide exchange factor (GEF) and be important for vesicular transport and secretion. The release of GDP then, is required for the docking and fusion of synaptic

vesicles (see Burton et al. 1994). MSS4 may be important as a modulator for this, since hydrolysis occurs at low levels under normal conditions.

The amino acid sequence for MSS4 shows it is a hydrophilic protein with no membrane spanning domain, indicative of natural solubility.

Southern blotting illustrated upregulation in a full screen, giving a total insert size of approximately 2.8kb. It was also isolated by dermal screening, and appeared as an uregulated band. By northern analysis it was upregulated 1 day and 3 days after wounding yielding a 1.5kb transcript. The other transcripts (780bp, 1.2kb) shown by Muller-Pillasch et al. 1997 and colleagues in the pancreas were not seen.

Since it's mRNA expression cannot be localised to neurons which have their cell bodies in the DRG, cells within the skin must upregulate this protein, and from the epidermal and dermal screens, it should be upregulated within the epidermis, aiding it's likely role in the secretion of substances. *In situ* hybridisation detected increased mRNA in some sections of wounded skin, within the scar tissue surface itself (see figure 96c), and a general increase within the epidermis, as compared to naive and control samples. However, the mRNA increase detectable by Northern analysis was substantial compared to that obtained by in situ. This may reflect the techniques themselves, or the distribution of mRNA over area of section, and number of sections. More detailed analysis and further experiments will aid definition.

Chapter 1 described the numerous cell types within the skin, and the cells which have potential secretory capacities for neurotransmitters, such as the Merkel cells and Langerhans cells. The increase in collagen production, keratin turnover etc will also necessitate the synthesis and secretion of proteins to re-establish epithelial and mesenchymal frameworks. In 1997 Muller-Pillasch and colleagues reported on the increase in expression of MSS4 in pancreatic cancer cells, especially the 12kb transcript, suggesting additional roles in the regulation of intracellular transport, important in growth. This could be likened to the growth and division of cells necessary for wound healing. Indeed, in the yeast, from which it was originally isolated, it is involved in organisation of the actin cytoskeleton (Desrivieres et al. 1998). Hence, MSS4 may have many potential roles in wound healing.

5.8.2d) CLONE DS 3 with homology to α-Globin

Clone 3 from the dermal screen showed upregulation at the level of a Southern blot (see figure 89). The fragment isolated was approximately 700bp. This clone was partially sequenced and shown to be homologous with 262 of the 373bp to rat major α-globin mRNA, from the published sequence by Satoh et al. 1987. It was confirmed to be upregulated at 1 and 3 days after wounding by northern analysis, producing an approximately 800bp band, whose signal was apparent at 5hrs room temperature incubation. Ohyagi et al. 1994 isolated a ~800bp clone by Northern analysis from neurons. Our increase was also confirmed by *in situ* hybridisation, see figure 97f, which showed a dramatic upregulation of the mRNA within the scar tissue and under the wounded area itself.

Alpha-globin was first isolated from an adult rat reticulocyte library (Satoh et al. 1987). Interestingly, it was also isolated by differential screening, as a novel protein developmentally regulated in neurons, by Ohyagi et al. 1994, who showed it is more highly expressed in embryonic than adult rat brain.

It was also found in a differential screen of sciatic nerve injury in the contralateral nerve i.e. a repressed clone by De Leon et al, 1991. Here, it was also suggested that the source was haemopoietic cells. Haemoglobin is well known for its oxygen transport within erythrocytes, but less well known for it's free radical generating properties which are thought to be implicated in neurodegeneration (see Ohyagi et al. 1994). Its increase may reflect the increased oxygen consumption of a regenerating tissue, or the increased requirement of such a tissue.

The increase depicted here cannot be within neurons but the distribution of mRNA strongly suggests its involvement in stimulating or sustaining growth into the injured area.

5.8.3 UNVERIFIED CLONES

5.8.3a)CLONE FS 32 with homology to Endoplasmic reticulum protein, ERp29

712bp of 822bp of CLONE FS 32's sequence was homologous to the endoplasmic reticulum protein Erp29. This protein was isolated from the livers of rats stressed under metabolic conditions (Mkrtchian et al. 1998). It was found to be associated with an abundant molecular chaperone/stress protein within the cytosol of the hepatoma cells. ER stress-inducible proteins belong to structurally diverse families including glucose-regulated proteins, similar to the cytosolic heat shock proteins.

In our study the increase of such a protein is only natural in the stressful action of wounding. The protein may provide part of a cascade involved in the processing of proteins, regulating the synthesis of chaperone proteins and to increase the folding of enzymes important in wound healing and the development of hyperinnervation. Transcripts are present in many tissues with highest expression within the brain (Mkrtchian et al. 1998).

However, *in situ* hybridisation showed no difference in labelling in naive and wounded sections, and the overall level of binding was low (see figure 96d-f). This could mean that there is no regulation of this molecule after wounding, even though Southern blotting suggested there was, showing an approximately 950bp insert (see figure 91), although this is subject to equalisation of probes and cDNA. The oligonucleotide probe may not have been appropriate for the *in situ* process, it's labelling reaction showing scintillation counts of 125-175 cpm. Finally, it may be that the regulation is slight and further analysis of specific areas of sections, i.e. grain analysis may be necessary, rather than a gross examination of sections. A northern blot may also be useful in verifying this clone.

5.8.3b) CLONE DS 12 with homology to Ephrin-A4 (LERK-4)

Southern analysis of Clone 12 from the dermal screen revealed a single 1Kb insert which was downregulated when probed with wounded skin, but barely visible at 1 weeks autoradiographic exposure at -70°C. On partial sequencing, the clone was highly homologous (494bp of 600bp sequenced) to the mouse ephrin-A4 gene, obtained by Flenniken et al. 1996.

Although Northern analysis did not reveal expression of ephrin-A4, this was probably due to low levels of expression, and quantitative PCR or reverse northerns may be better strategies for determining regulation.

Unfortunately, this clone was isolated and sequenced when time constraints were high, and therefore future work would involve the above techniques as well as *in situ* hybridisation, and other experiments to look at a funtional role. In addition, the rat ephrin-A4 sequence has not yet been cloned.

LERKS belong to a family of molecules known as the ephrins. Ephrins are ligands of the Eph family of receptor tyrosine kinases. They mainly act as repellents throughout development, in axonal pathfinding, fasciculation and provide barriers between cell and tissue types. Their receptors are divided into two groups, glycosyl-phosphatidylinositol (GPI)-linked e.g., ELF-1, RAGS, and our clone 12, ephrin-A4, and the transmembrane

group e.g. ELK-1. Ephrin-A4 has the ability to bind both classes of Eph-A and B ligands. It is widely expressed in the embryo and becomes restricted as development proceeds and has therefore been suggested to play a part in tissue patterning (Flenniken et al. 1996). In the developing brain it is localised in the E18 forebrain, P1 hippocampus and P1 cerebral cortex, with no expression at P8 (Carpenter et al. 1995). Thus, ephrin-A4 seems a very exciting prospect with respect to a role in hyperinnervation. Other family members, such as RAGS have been wonderfully studied to demonstrate a role in the guidance of retinal axons to the optic tectum (Drescher et al. 1995). Ephrin-A4 may play a similar role in the guidance of cutaneous nerves to the skin, and disturbances in early development e.g. wounding may cause it's downregulation and permit or restrict hyperinnervation to only wounded areas only. Moreover, ephrin-A4 expression has been shown to sort cells into the boundaries between rhombomeres in zebrafish embryos (Xu et al. 1999), and it may thus establish the boundaries for nerves during development and injury.

5.8.4 NOVEL CLONES

Time constraints prevented thorough analysis of novel clones, however two which were shown to be upregulated by Southern analysis, clones FS 29 and FS 35 (see figure 91), were characterised to some extent by *in situ* hybridisation. Thus, clone FS 29 demonstrated marked increase within the scar tissue of wounded sections compared to naive skin sections, although further experiments are needed to confirm this. Clone FS 35, showed a definite increase in the epidermis of wounded skin sections compared to naive skin sections, with little or no staining of the scar tissue or the area under the wound itself (see figure 95d-f). This clone may therefore be one of general epidermal increase after trauma to the skin, and may be part of the initiation and reforming of the epidermis after skin wounding. Further, studies are needed to establish the function, structure and location of these unknown clones as well as the other novel clones isolated in this study.

5.8.5 FUTURE DIRECTIONS

The isolation of so many regulated known and unknown clones is both exciting and overwhelming. Each clone will hold the key to part of an important process in neonatal skin wounding and/or hyperinnervation. Additional clones as yet undiscovered with respect to regulation and function remain as phage inserts at 4°C!

The next step will be to take each clone and conduct many experiments to look at its role in wound healing and hyperinnervation. To aid this the plasmid vector can be directly

expressed into cells and the insert expressed, which will allow functional studies to be conducted quite quickly. Anti-sense transfection and knock-outs, both conditional and conventional, will also aid the understanding of function of these genes.

The combination of molecular and non-molecular techniques e.g. the use of DIG-labelled *in situ* and immunohistochemistry will localise genes to specific areas and or cells. RNase protection and quantitative PCR as well as reverse Northern strategies will allow genes which cannot be shown to be regulated by conventional Northern analysis to be detected and assessed. The scope is endless, but the door to discovery is now firmly open.

6. GENERAL CONCLUSIONS AND SUMMARY

In introducing the skin and its structure, we have demonstrated the complexity of the largest organ in the body. The development of its structure and its innervation is highly organised and disturbances lead to permanent changes both in neonate and adult, ie. hyperinnervation and scarring. A sound knowledge of the structure and development can explain numerous changes in pathology fore example, intraepidermal nerves are correlated in number with the thickness of the stratum corneum, and are thought to increase sensitivity as thickness of the epidermis is increased. Thus, the observable increase seen in psoriatic skin.

The complex interactions of the immune system, the nervous system, skin components, and growth factors is clearly demonstrated, as well as the temporal and spatial regulations of these elements in the accomplishment of wound healing. To aid further understanding of the rapid healing in neonates as well as the possible implications of hyperinnervation, the role of growth factors, extracellular matrix components and immune elements need to be explored.

In addition the role and expression of the neurotrophins in establishing innervation of different neuronal phenotypes and formation of specific endings was addressed.

In Chapter two, the phenomenon of sensory hyperinnervation was examined.

- The phenomenon of hyperinnervation after neonatal skin wounding was confirmed.
- Hyperinnervation was shown to be well established by 5 days after wounding, and most prominent at 7 days post wounding.
- It arose from deep nerve bundles running beneath the skin.
- It was not due to collateral sprouting from existing undamaged adjacent fibres.
- Re-epithelialisation and the re-establishment of a barrier between granulation tissue and the dermis was present by 1-3 days post wounding.

The hyperinnervation seen following neonatal wound healing (and the impaired healing seen with the loss of nerves in diabetes), suggests that innervation, inflammation and healing are closely linked. Whether this is due to a change in levels, distribution or temporal regulation of growth factors or a change in the responsiveness to existing levels of such factors remains to be ascertained. The results reported here and the related discussion will lead to a better understanding of this process.

In Chapter 3, tissue culture models for this process were examined.

- A model was established implicating epidermal production of a factor which promoted increased neurite outgrowth. This was seen in co-cultures of neonatal wounded skin and newborn DRG.
- Only NF200 positive neurites were observed emanating from DRG co-cultured with naïve or wounded skin, although CGRP positive cell bodies were present.
- Dissociated cultures were also established to aid further classification.
- Dissociated neurons showed a variety of morphologies in culture.
- Dissociated neurons were NF200 and CGRP positive and had neurites with one or both of these markers.
- Removal of co-cultured wounded skin after one hour in culture led to increased neurite outgrowth from neurons in all parameters of neurite outgrowth examined.

In Chapter 4 the role of neurotrophic factors in mediating neurite outgrowth was examined.

- RT-PCR showed the presence of the cDNA for the neurotrophic factors NT-3, GDNF and BDNF as well as cDNA for the receptors of the neurotrophins, trkA, trkB and trkC, within naïve and wounded skin.
- *In vitro* antibody blocking studies indicated a role for NT-3 and GDNF in eliciting neurite outgrowth from wounded co-cultures, such that both antibodies were able to block some of the neurite outgrowth seen. Thus, culture models when combined with *in vivo* studies have substantiated hypotheses of growth factor production.
- Both NT-3 and GDNF mRNAs were also shown to be increased in wounded skin at 3 days post wounding by the use of Northern blotting and *in situ* hybridisation techniques.
- BDNF mRNA was undetectable by Northern analysis.
- The protein for GDNF was shown not to increase at 3 days post wounding by ELISA.
- The protein for NT-3 was shown to be increased in wounded skin by ELISA at 3 days post wounding, decreasing by 5 days post wounding.
- NT-3 protein was localised to epidermal layers and scar tissue using immunohistochemistry.
- BDNF homozygous knock-out mice showed hyperinnervation after neonatal skin wounding. Hyperinnervation is therefore not BDNF dependent.

- *In vivo* studies with trkC-IgG fusion bodies showed no effect in reducing or inhibiting the hyperinnervation. This may be a reflection of the access of antibody to the skin, rather than conclusive data to suggest NT-3 does not mediate the event.

Thus, NT-3 was identified as a prime candidate for mediating neonatal sensory hyperinnervation.

In Chapter 5 we explored other factors in wound healing and neonatal hyperinnervation.

- We successfully constructed a cDNA library.
- We successfully differentially screened this library for regulated genes in neonatal skin wounding and/or hyperinnervation.
- We examined a few clones by *in situ* hybridisation and Northern blotting to show regulation at the mRNA level.
- We found that Clones FS 46, DS 1 and DS 3 were all upregulated at the mRNA level by Northern analysis and *in situ* hybridisation.
- These clones have some similarity to known genes, a guanine nucleotide releasing protein alpha globin and calcium ATPase respectively.
- We also isolated a gene Clone DS 12 closely resembling the mouse ephrin-A4, which is likely to be the rat homologue. Since this is an inhibitory axon guidance molecule, it and other inhibitors of neurite outgrowth present a new domain for further investigation.

It has been shown that neonatal hyperinnervation after skin wounding is a complex phenomenon encompassing interactions between anatomy, developmental biology, neuroscience, clinical pathology and immunology. We have provided insight into the development of skin innervation, wound healing, nerve regeneration, clinical conditions such as psoriasis and diabetes, and explored the roles of neurotrophins and other molecules involved in neuronal development, patterning and wound healing.

Using anatomical, functional and molecular techniques, we have gradually narrowed our search by elimination, to implicate neurotrophin-3 in sensory hyperinnervation after neonatal skin wounding.

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LIST OF ABBREVIATIONS

ACTH –adrenocorticotrophic hormone
Aδ-MR- A delta mechanoreceptor
ATPase – adenosine triphosphatase
BCL-2 –B cell lymphoma related gene
BDNF – brain derived neurotrophic factor
βFGF- beta fibroblast growth factor
BSA- bovine serum albumin
CAMS- cell adhesion molecules
cDNA- complementary deoxyribonucleic acid
CGRP- calcitonin gene related protein
CGRP-IR- calcitonin gene related protein-immunolike reactivity
CLONE DS – clone dermal screen
CLONE FS – clone full screen
CNS – central nervous system
CNTF – ciliary neurotrophic factor
CSF-1 – colony stimulating factor -1
DAB – diaminobenzidine
dATP – deoxyadenosine triphosphate
dCTP- deoxycytosine triphosphate
DEPC - diethylpyrocarbonate
DMEM – Dulbeccos minimum essential medium
DMF - dimethylformamide
DMSO - dimethylsulphoxide
DNA –deoxyribonucleic acid
DNTP – deoxyribonucleotide triphosphate
DRG – dorsal root ganglion
DTT - dithiothrietol
E – embryonic day
ECM – extracellular matrix
E.coli – *Escherichia coli*
EDTA - ethylenediaminetetraacetate
EGF – epidermal growth factor
ELF-1 – eph ligand family-1
ELISA –enzyme-linked immunosorbent assay
ELK-1 – eph like kinase
Eph A/B –ephrin A/B
ER – endoplasmic reticulum
ERP-29 – endoplasmic reticulum binding protein-29
EST – estimated sequence tag
FDP – fibrin degradation product
FITC – fluorescein isothiocyanate
GAG -glycosaminoglycan
GAP-43 – growth associated protein-43
GDNF – glial cell derived neurotrophic factor
GDP – guanine diphosphate
GEF –guanine nucleotide exchange factor
GPI – glycosyl-phosphatidylinositol
GRP- gastrin releasing peptide

GTC –guanine thiocyanate
GTPase – guanine triphosphatase
H&E – haematoxylin and eosin
HRP – horse-radish peroxidase
HSDNA –herring sperm deoxyribonucleic acid
IB4 – isolectin B4 from *Griffonia simplicifolia I*
ICAM-1 – intercellular cell adhesion molecule-1
IGF –insulin-like growth factor
IL-1/2/4/8 –interleukin-1/2/4/8
IPTG – isopropyl-1-thio-β-D-galactopyranoside
ISH – *in situ* hybridisation
kD - kiloDaltons
L –large light
LB – Luria-Bertani medium
LERK-4 – ligand for eph related kinase-4
LIF – leukaemia inhibitory factor
MAG – myelin associated glycoprotein
MAP kinase – mitogen activated protein kinase
MMLV – murine moloney leukaemia virus
MOPS – 3-[N-morpholino] propanesulphonic acid
mPCR – messenger RNA polymerase chain reaction
mRNA – messenger ribonucleic acid
MSH – melanocyte stimulating hormone
MSS4 – mammalian suppresser of sec4
N-CAM – neural cell adhesion molecule
NCBI Genbank – National centre for biotechnology information
NF200 – neurofilament 200
NGF – nerve growth factor
NGI – nerve growth index
NGS – normal goat serum
NO – nitric oxide
NOS – nitric oxide synthase
NPY – neuropeptide Y
NT-3 – neurotrophin-3
NT4/5 – neurotrophin 4/5
NTN - neurturin
O.D – optical density
P – postnatal day
PAF – platelet activating factor
PB – phosphate buffer
PBK-CMV - cytomegalovirus
PBS – phosphate buffered saline
PC12 – phaeochromocytoma cells-12
PCD – programmed cell death
PDGF – platelet derived growth factor
PERT - phenol-emulsion-enriched DNA driven subtractive cDNA cloning
PGI_2 - prostacyclin
PGP – protein gene product
PMN – polymodal nociceptors
RA – rapidly adapting

RAGS – repellant axon guidance signal
RAP - RNA fingerprinting using arbitrarily primed PCR
rATP – ribonucleotide adenosine triphosphate
RLCS – restriction landmark cDNA scanning
RNA – ribonucleic acid
RP – random priming
RT-PCR – reverse transcriptase- polymerase chain reaction
SA – slowly adapting
SAGE – serial analysis of gene expression
SD – small dark
sema III – semaphorin III
SMAD3 – vertebrate member of *Drosophila* mothers against decapentaplegic gene product-3
SP – substance P
SP-IR - substance P immunolike-reactivity
TAG-1 – transient axonal glycoprotein
Taq –*Thermus aquaticus*
TGF – transforming growth factor
Tm - melting temperature
TMB - 3'3'5'5'-tetramethylbenzidine
TNF-α - tumor necrosis factor- alpha
TRITC –tetrarhodamine isothiocyanate
TrkA – tyrosine kinase A
TrkB – tyrosine kinase B
TrkC – tyrosine kinase C
TrkC-IgG– tyrosine kinase C- immunoglobulin G
USG –Ultroser G
UV –ultra-violet
VCAM-1 –vascular cell adhesion molecule-1
VEGF – vascular endothelial growth factor
VIP – vasoactive intestinal polypeptide
VR-1 – vanilloid receptor-1

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Research report

Neonatally wounded skin induces NGF-independent sensory neurite outgrowth in vitro

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DEVELOPMENTAL BRAIN RESEARCH

Research report

Neonatally wounded skin induces NGF-independent sensory neurite outgrowth in vitro

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Abstract

An in vitro model was established to investigate factors underlying the sensory hyperinnervation of neonatal rat skin wounds that has been observed in vivo (Reynolds and Fitzgerald, J. Comp. Neurol. 358 (1995) 487–489). Explants of normal and wounded rat dorsal foot skin were co-cultured with explants of embryonic chick or newborn rat dorsal root ganglia for 24 h and the number of sensory neurites counted. Explants of skin surrounding a wound made at birth were taken 3 (P3) or 10 (P10) days later and compared with normal skin of the same age. In addition, explants were taken from adult skin wounded 3 and 10 days earlier. At P3, normal skin induced weak neurite outgrowth (mean 13.1 ± 2.1 neurites per ganglion explant) but skin that had been wounded 3 days earlier, at birth, induced three times more neurite outgrowth (37.8 ± 3.3). Ten days after wounding at birth, neurite outgrowth was still substantial (40.9 ± 3.3) although at that age (P10), even normal skin stimulates substantial growth (37.4 ± 2.9). Normal adult skin also stimulated neurite outgrowth (28.7 ± 0.45) but this was not increased by wounding 3 or 10 days earlier, and this was enhanced 3 days but not 10 days after wounding. Anti-NGF (nerve growth factor) added to the culture medium blocked the constitutive neurite stimulating activity from normal P10 and adult skin but was ineffective in blocking the neurite stimulating activity produced by neonatal wounding. It is concluded that skin wounding at birth results in release of one or more sensory neurotrophic factors that stimulate rat and chick dorsal root ganglia neurite outgrowth for at least 10 days, but which do not include NGF. © 1997 Elsevier Science B.V.

Keywords: Sensory neuron; Sprouting; Skin; Wound; Nerve growth factor; Neonate

1. Introduction

Skin wounds made during the neonatal period result in long lasting sensory hyperinnervation of the wound area [34], this in contrast to the adult, where similar injury results in a slight and transient increase in innervation [2]. After wounding at birth (postnatal day 0, P0), sensory nerve terminals show a dramatic sprouting response over the following 14 days; fibres appear to be drawn towards the surface of the skin leaving the affected area hyperinnervated and hypersensitive for a prolonged period after the wound has healed. Sympathetic axons do not contribute to this sprouting but both capsaicin sensitive sensory C fibres and RT97 positive sensory A fibres are involved [34].

Sprouting is likely to result from the release of neurotrophic factors and cytokines from the damaged skin [10,13,33,37] and the profile of these factors and the sensitivity of sensory neurons to them may differ between neonatal and adult neurons. During the first 3 days after wounding at P0 the levels of nerve growth factor (NGF) in the skin around the wound have been shown to increase sharply compared to smaller and shorter-lived increases seen in adult skin after wounding [7].

In this study we have aimed to establish the part played by diffusible neurotrophic factors released by wounded skin in initiating and maintaining the observed sensory hyperinnervation and to determine the role of NGF in this response. To do this we have used the neurite outgrowth of chick and rat sensory ganglion explants cocultured with neonatal and adult wounded skin or normal age matched skin. The contribution of NGF secreted by the tissues to neurite outgrowth was assessed by using NGF specific blocking antibodies.

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2. Materials and methods

Embryonic day 7 (E7) chick and newborn, postnatal day 0 (P0) rat dorsal root ganglia (DRG) were cocultured with explants of wounded or normal neonatal or adult rat skin and the consequent neurite outgrowth analysed using the following procedures:

2.1. Preparation of skin wounds

Sprague–Dawley rats, either newborn (P0), P10, or adult, were anaesthetised with halothane. Full thickness skin wounds were made in the mid-dorsal region of the hind paw by carefully separating from underlying tissues and removing a piece of skin about 2 mm in diameter at P0 and P10, or about twice this size in the adult. Wounds were aligned between the first and fifth digit for comparability. The animals were allowed to recover and wounds healed with no sepsis. After 3 or 10 days the animals were sacrificed by terminal anaesthesia with sodium pentobarbitone. The skin was swabbed with 70% alcohol, and where appropriate shaved with a sterile blade, before sampling. A 1 mm wide strip was taken from immediately around the wound, or from the equivalent area in controls, and cut into equal sized pieces, approximately 1 mm, long under sterile conditions

2.2. Preparation of cocultures

Lumbar DRG were dissected from E7 chicks and P0 rat pups. Using sterile techniques E7 chick embryos were placed in minimum essential medium containing HEPES buffer (MEM/HEPES, Gibco) on ice. P0 rat pups were given an overdose of barbiturate and the vertebral column removed also into MEM/HEPES on ice. In both cases lumbar DRG were carefully dissected out and subdivided into 3–4 explants. Each piece was placed in a drop of MEM/HEPES, with two DRG explants in each 35 mm culture dish. One piece of skin was positioned 1.5–2 mm from each DRG. The droplet of MEM/HEPES was aspirated and replaced by 30 μl of rat tail collagen containing 5 μl 7.5% solution of NaHCO (Gibco) and 10 μl Dulbecco's minimum essential medium × 10 (Gibco) per 90 μl collagen. Once the collagen had set at room temperature, 2.5 ml of Ham's nutrient mixture F14 plus 0.5% L-glutamine (Gibco), 1% penicillin–streptomycin (Gibco) and 2% Ultroser G (Gibco) were added to each dish. The cocultures were incubated in humidified air containing 5% CO_2 at 36°C for 24 h. In some cases rabbit anti-NGF (generously provided by C.J. Woolf) or normal rabbit serum at final dilution of 1/1000 or 1/500 was added to the medium. This was a polyclonal anti-NGF raised in rabbits against HPLC purified mouse NGF with established specificity and shown not to cross react with BDNF or NT3 [37]. Antibody was added to the medium and well mixed, before aliquoting into the culture dishes containing the cocultures.

The following gels were set up (P = postnatal day):

- P3 normal skin; in control medium ($n = 23$ gels with chick DRG; $n = 17$ gels with rat DRG)
- P3 skin, wounded at P0; in control medium ($n = 44$ with chick DRG; $n = 17$ with rat DRG) or in medium + anti-NGF ($n = 24$ with chick DRG; $n = 20$ with rat DRG)
- P10 normal skin; in control medium ($n = 50$ with chick DRG; $n = 11$ with rat DRG) or in medium + anti-NGF ($n = 29$, chick DRG)
- P10 skin wounded at P0; in control medium ($n = 64$ with chick DRG; $n = 10$ with rat DRG) or in medium + anti-NGF ($n = 27$, chick DRG)
- Adult normal skin; in control medium ($n = 39$, chick DRG) or in medium + anti-NGF ($n = 26$, chick DRG)
- Adult skin wounded 3 days earlier; in control medium ($n = 36$, chick DRG) or in medium + anti-NGF ($n = 32$, chick DRG)
- Adult skin wounded 10 days earlier; in control medium ($n = 38$, chick DRG) or in medium + anti-NGF ($n = 23$, chick DRG)

To ensure constant culture conditions for comparison of neurite outgrowth, cocultures were always arranged so that tissue with or without wounds in control medium or medium with or without anti-NGF and the same tissue type, were incubated in the same run. In all experiments control gels containing only E7 chick or P0 rat DRG were set up as above and cultured in medium containing 10 ng/ml NGF or 10 ng/ml NGF with the addition of α-NGF, in the dilution used in the experiment.

2.3. Analysis of neurite outgrowth

After 24 h incubation the cocultures were washed for 3–4 h in cold medium, fixed in 4% paraformaldehyde for 24 h then washed in phosphate buffered saline pH 7.4. Gels were removed from the culture dishes into multiwell staining dishes and neurites stained free floating using polyclonal rabbit anti-PGP 9.5 (Ultraclone) and the Vectastain ABC kit, as previously described [34]. Using a Nikon Optiphot microscope with camera lucida, the neurite growth was divided into quadrants from the centre of the DRG, so that neurites growing in the quadrants facing towards, away from or parallel to the skin could be separately counted. PGP staining allows neurites to be clearly distinguished from the pale staining underlying flat cells. The relatively sparse neurite outgrowth meant that individual neurites could be easily counted.

The effectiveness of the α-NGF antiserum was measured in each experiment, by estimating the reduction in total neurite outgrowth area from ganglia grown in medium + 10 ng/ml NGF plus anti-NGF, compared with control ganglia grown with 10 ng/ml NGF alone, using a Seescan image analysis program for area measurement. Areas were established by joining points marking the four longest neurites in each quadrant.

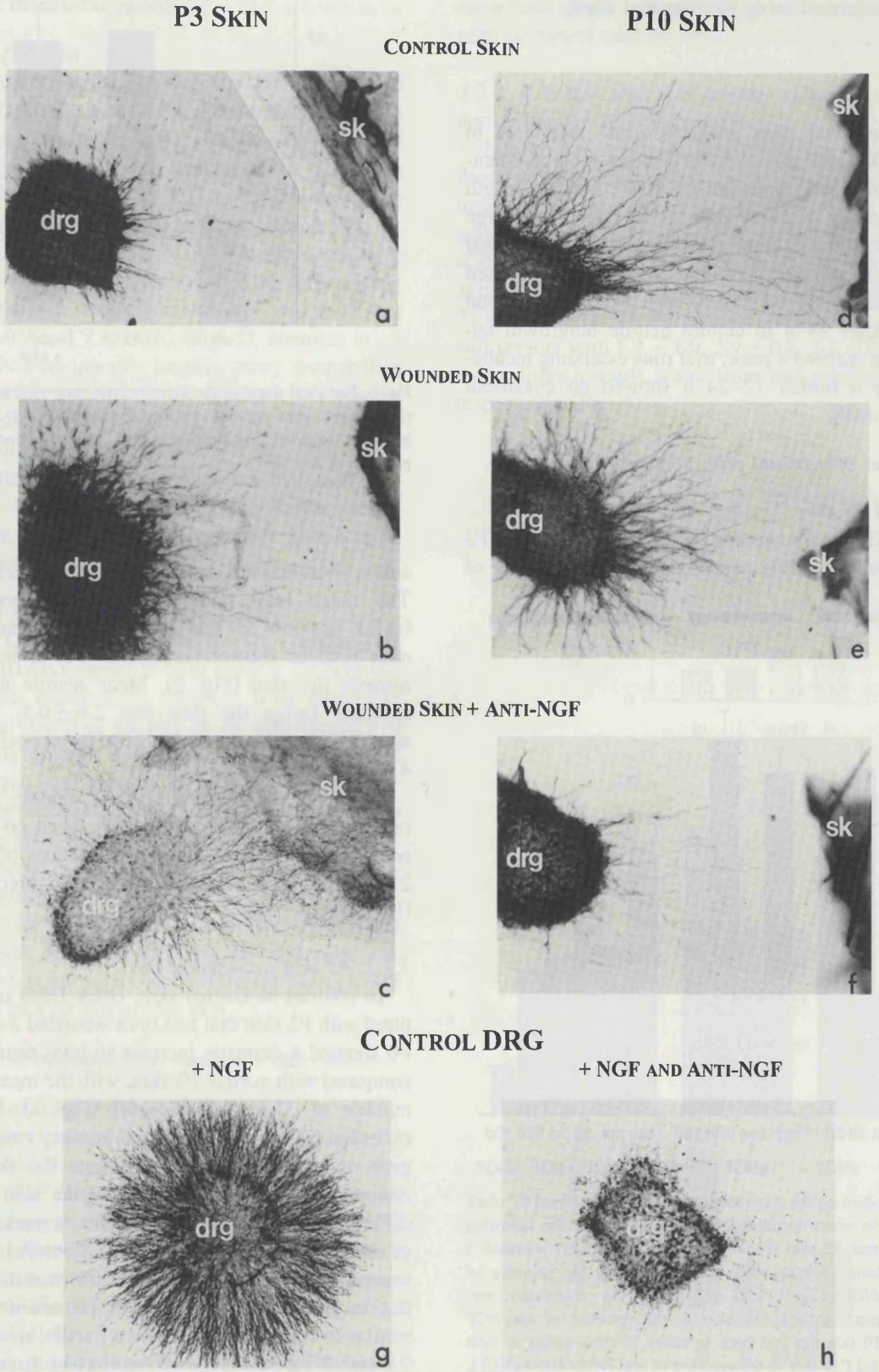

Fig. 1. Top three panels: photomicrographs of cocultures of E7 chick dorsal root ganglia (drg) and rat skin (sk) explants immunostained with the pan-neuronal marker, PGP 9.5. On the left: the skin explants are from 3 day old rat pups (P3) (a) normal P3 skin, (b) P3 skin that was wounded 3 days earlier at birth, (c) P3 wounded skin as in (b) but in the presence of 1 : 1000 anti-NGF. On the right: the skin explants are from 10 day old rat pups (P10) (d) normal P10 skin, (e) P10 skin that was wounded 10 days earlier at birth, (f) P10 wounded skin as in (e) but in the presence of 1 : 1000 anti-NGF. Bottom panel: control dorsal root ganglia explants grown alone in (g) 10 ng/ml NGF and (h) in 10 ng/ml NGF plus 1 : 1000 anti-NGF. Magnification ×125.

Results are expressed as the mean number of neurites ± S.E.M. from the number of gels stated. Tests of significance were performed using the unpaired *t*-test.

3. Results

Neurite outgrowth was analysed from cocultures in which the DRGs appeared phase bright and some non-neuronal cell growth was apparent (> 90%). Neurite length was not measured in every case as in many gels large numbers of neurites, often growing over non-neuronal cells, made this estimate impossible. Neurites were counted when processes could be clearly seen extending beyond these cells. After 24 h in culture neurite outgrowth appeared to have reached a peak; trial runs extending incubation times by a further 12–24 h showed no consistent increase in growth.

3.1. Cocultures of neonatal skin: control medium

3.1.1. Normal P3 skin

Chick DRG sensory neurons cocultured with normal P3 rat skin showed very little neurite outgrowth consisting of a few, short neurites, between 150–350 μm long (Fig. 1a). The mean total number of neurites was 13.2 ± 2.1 (± S.E.M., $n = 23$) and comparison of neurite counts in each quadrant showed no indication of preferential growth towards the skin (Fig. 2). Mean neurite number in the quadrant facing the skin was 2.6 ± 0.5, which is not significantly different from that in the opposite quadrant of 4.4 ± 1.0 (Fig. 4).

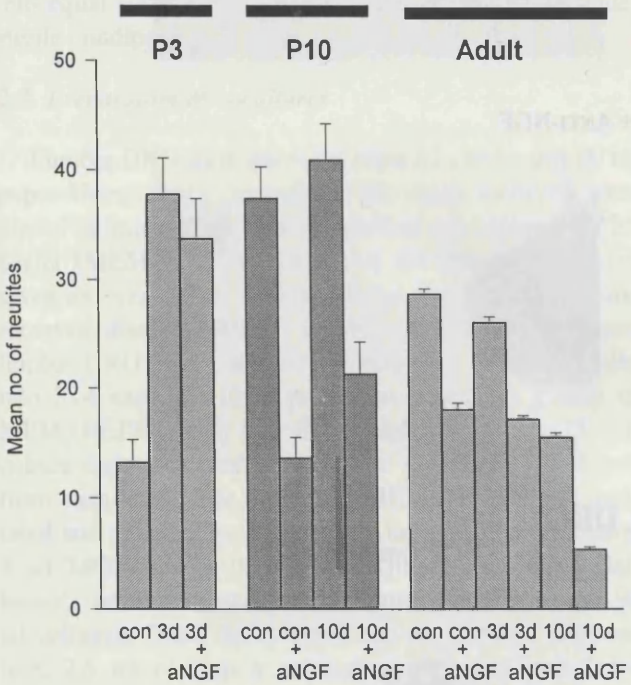

Fig. 2. Bar chart showing the mean total neurite outgrowth from E7 chick dorsal root ganglia when cocultured with (1) P3 skin in the following experiments: normal P3 skin (con); P3 skin that had been wounded 3 days earlier at birth (3d); and wounded P3 skin in the presence of anti-NGF (3d + aNGF); (2) P10 skin in the following experiments: normal P10 skin (con); normal P10 skin in the presence of anti-NGF (con + aNGF); P10 skin that had been wounded 10 days earlier at birth (10d); and wounded P10 skin in the presence of anti-NGF (10d + aNGF); (3) adult skin in the following experiments: normal adult skin (con); normal adult skin in the presence of anti-NGF (con + aNGF); adult skin that had been wounded 3 days earlier (3d); 3 day wounded adult skin in the presence of anti-NGF (3d + aNGF); adult skin that had been wounded 10 days earlier (10d); 10 day wounded adult skin in the presence of anti-NGF (10d + aNGF).

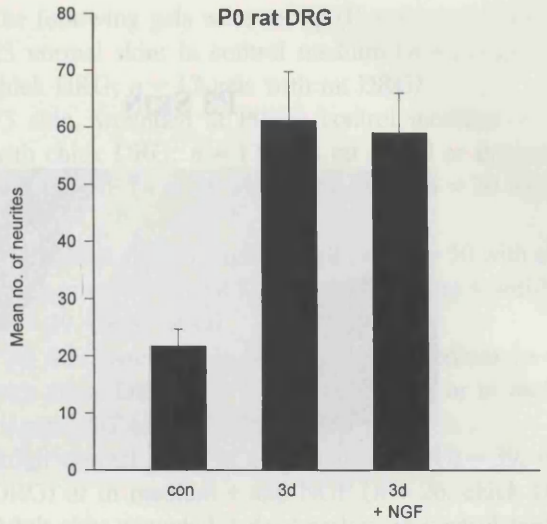

Fig. 3. Bar chart showing the mean neurite outgrowth from P0 rat dorsal root ganglia when cocultured with (a) normal P3 skin, (b) P3 skin that had been wounded 3 days earlier at birth, and (c) wounded P3 skin in the presence of anti-NGF.

P0 rat DRG neurons showed slightly more neurite outgrowth than E7 chick neurons when co-cultured with normal P3 skin. The mean total number of neurites was 21.71 ± 3.0 ($n = 17$), with no sign of directional growth (Fig. 3).

3.1.2. P3 skin wounded at P0

In contrast to normal skin, chick DRG neurons cocultured with P3 skin that had been wounded 3 days earlier at P0 showed a dramatic increase in total neurite outgrowth compared with normal P3 skin, with the mean total neurite number of 37.8 ± 3.3 ($n = 44$) (Fig. 2). Most neurites exceeded 600 μm in length and in many cases appeared to grow over the surface or penetrate the skin (Fig. 1b). Although consistent orientation of the skin samples was difficult to achieve in all cocultures, a marked orientation of neurite outgrowth was apparent. Growth in the quadrant towards the skin (16.1 ± 1.6 neurites) was more than twice that in the quadrant away (7.3 ± 1.3 neurites) which was similar to the growth in the two parallel quadrants (7.2 ± 0.9 and 7.2 ± 1.1) (Fig. 4). Neurites in these lateral quadrants, however, showed no consistent tendency to turn towards the skin, outgrowth being essentially radial but more densely concentrated in one quadrant.

Rat P0 DRG neurons also showed a marked increase, of almost 3-fold, in neurite outgrowth in the presence of P3

skin that had been wounded at birth. The mean total neurite number was 61.2 ± 8.6 ($n = 17$) (Fig. 3). There was no sign of directional growth.

3.1.3. Normal P10 skin

Total neurite outgrowth from the chick DRG cocultured with normal P10 skin was much greater than that with normal P3 skin, showing a similar density and pattern of growth to that observed with P3 wounded skin. The mean total neurite number was 37.4 ± 2.9 ($n = 59$), almost three times greater than normal P3 skin (Fig. 1d and Fig. 2). Normal P10 skin also consistently induced more extensive neurite outgrowth in the quadrant towards the skin (15.5 ± 1.5 neurites) than in the quadrant away (6.6 ± 0.8) or parallel (7.9 ± 0.9 and 7.4 ± 0.8) (Fig. 5). Neurites in all quadrants reached comparable lengths, many greater than 500 μm, and many neurites in the quadrant towards the skin reached and grew over its surface.

Similar results were obtained with P0 rat DRG, where the total neurite outgrowth with normal P10 skin was 42.7 ± 7.6 ($n = 11$), which was significantly greater (nearly 2-fold) than the growth with P3 skin. Again there was no directional growth.

3.1.4. P10 skin 10 days after wounding at P0

Total chick DRG neurite outgrowth with cocultures of skin wounded 10 days earlier at birth (40.9 ± 3.3 neurites, $n = 64$) was not significantly different from outgrowth with P10 normal skin (Fig. 1e, Fig. 2). As with normal P10 skin, directional growth was obvious (Fig. 5). Neurite growth in the quadrant towards the skin (15.7 ± 1.4 neurites) was nearly twice that in the quadrants away from and parallel to the skin (8.8 ± 1.0, 7.1 ± 0.6 and 9.2 ± 1.3 neurites respectively). Neurite lengths were again comparable in all quadrants.

A similar result was obtained with P0 rat DRG. Total neurite outgrowth with wounded 10 day old skin was 53.7 ± 7.2 which is not significantly different from growth in the presence of P10 unwounded skin.

3.2. Cocultures of neonatal skin: medium + anti-NGF

3.2.1. P3 skin wounded at P0 + anti-NGF

Neurite outgrowth from chick DRG cocultured with P3 wounded rat skin was not significantly altered in the presence of α-NGF (Fig. 1c). The mean total number of neurites in the presence of α-NGF was 33.7 ± 3.7 ($n = 24$) which is not significantly different from the mean number in control medium of 37.8 ± 3.3 (Fig. 2). This indicates that there is an NGF-independent neurite stimulating effect from the wounded skin. Directional growth was also not inhibited by the antibody (Fig. 4), with total neurite number in the quadrant towards the skin (17 ± 2.5) being nearly three times more numerous than in either of the other three quadrants (5.0 ± 0.8, 5.4 ± 0.9 and 6.3 ± 1.3 neurites respectively). There was also no consistent effect on neurite length.

A similar result was obtained with rat P0 DRG, where in the presence of α-NGF, wounded P3 skin produced the same total neurite outgrowth, 59.1 ± 7.0 ($n = 20$) as was seen in control medium (61.2 ± 6.1) (Fig. 3).

3.2.2. P10 skin with and without wounds made at P0 + anti-NGF (n = 36 and 29 gels)

Neurite outgrowth in chick DRG was considerably reduced in both P10 normal and P10 wounded skin cocultures in the presence of anti-NGF (Fig. 1f). Total mean neurite number induced by P10 normal skin was substantially reduced to 13.6 ± 1.9 ($n = 36$) in the presence of antibody compared to the value in normal medium of 37.4 ± 2.9 (Fig. 2). Directional growth was reduced but still present with 5.2 ± 1.0, 2.9 ± 0.6, 3.1 ± 0.7 and 2.4 ± 0.4 neurites towards, away from and parallel to the skin, respectively (Fig. 5).

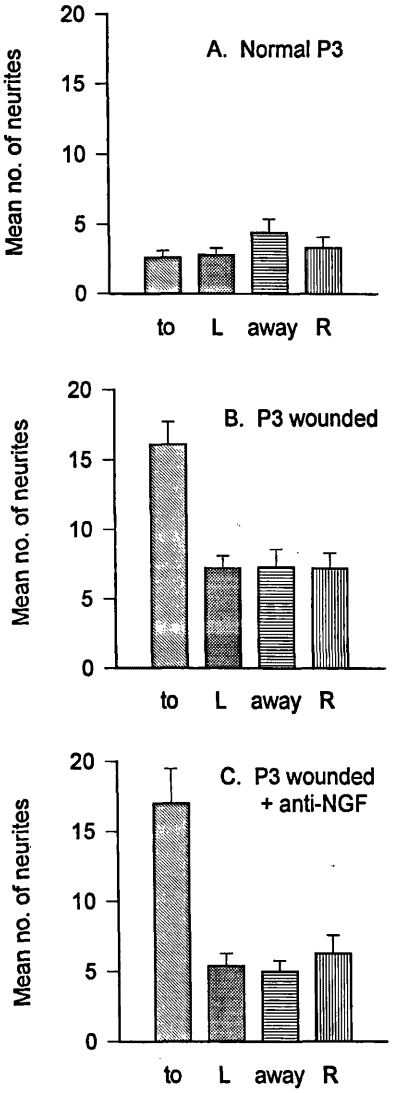

Fig. 4. Bar chart showing the mean neurite outgrowth from E7 chick dorsal root ganglia when cocultured with (a) normal P3 skin, (b) P3 skin that had been wounded 3 days earlier at birth, and (c) wounded P3 skin in the presence of anti-NGF. The neurite outgrowth has been divided into four quadrants representing growth towards, away from and parallel to the skin explant.

In cocultures with P10 skin wounded 10 days previously, neurite outgrowth was also substantially affected by the presence of anti-NGF, being reduced to 21.3 ± 3.0 ($n = 29$) neurites from the observed 40.9 ± 3.3 in normal medium (Fig. 2). This reduced value is, however, significantly greater than the 13.6 ± 1.9 neurites observed with normal 10 day skin in the presence of anti-NGF, indicating a residual NGF-independent activity from neonatally wounded skin after 10 days. Directional growth was still present and significantly more neurites grew towards the skin (7.8 ± 1.2) than in any of the other three quadrants (4.5 ± 1.1, 4.0 ± 0.8 and 5.1 ± 0.9 neurites away from and parallel, respectively) (Fig. 5).

3.3. Cocultures of adult skin: control medium

3.3.1. Normal adult skin

Total neurite outgrowth from DRG cocultures with normal adult skin was extensive (mean number of neurites, 28.7 ± 0.45, $n = 39$), although significantly less than those in cocultures with P10 normal skin (37.4 ± 2.9) (Fig. 2). No directional growth was apparent, with a mean of 7.6 ± 1.2 neurites in the quadrant towards the skin compared with means of 8.7 ± 1.3, 5.1 ± 0.7 and 7.4 ± 1.5 respectively in the other three quadrants (Fig. 6).

3.3.2. Adult skin 3 days and 10 days after wounding

Cocultures of adult skin 3 days after wounding showed no increase in total neurite number (mean total, 26.1 ± 0.51 neurites, $n = 36$) (Fig. 2) compared with normal skin but significant directional growth was apparent, with a mean total neurite number in the quadrant towards the skin of 12.2 ± 1.7 compared with 5.2 ± 0.8, 5.6 ± 0.8 and 5.3 ± 0.9 away from and parallel to the skin, respectively (Fig. 6). In cocultures with adult skin 10 days after wounding the total neurite outgrowth was significantly reduced (mean total 15.5 ± 0.41 neurites, $n = 38$, Fig. 2) compared with that in cocultures of normal adult skin and there was no indication of directional growth (4.7 ± 0.8, 5.4 ± 1.0 and 3.7 ± 0.8 and 3.8 ± 0.7 neurites in quadrants towards, away from and parallel to skin respectively) (Fig. 6).

3.4. Cocultures of adult skin: medium + anti-NGF

3.4.1. Normal adult skin + anti-NGF

Anti-NGF in the culture medium reduced the mean total number of neurites produced by normal adult skin by 37% (18.0 ± 0.62 total neurites ($n = 26$ gels) compared to 28.7 ± 0.45) (Fig. 2) in cocultures of adult skin in control medium. As in control medium, no directional growth was apparent (Fig. 6).

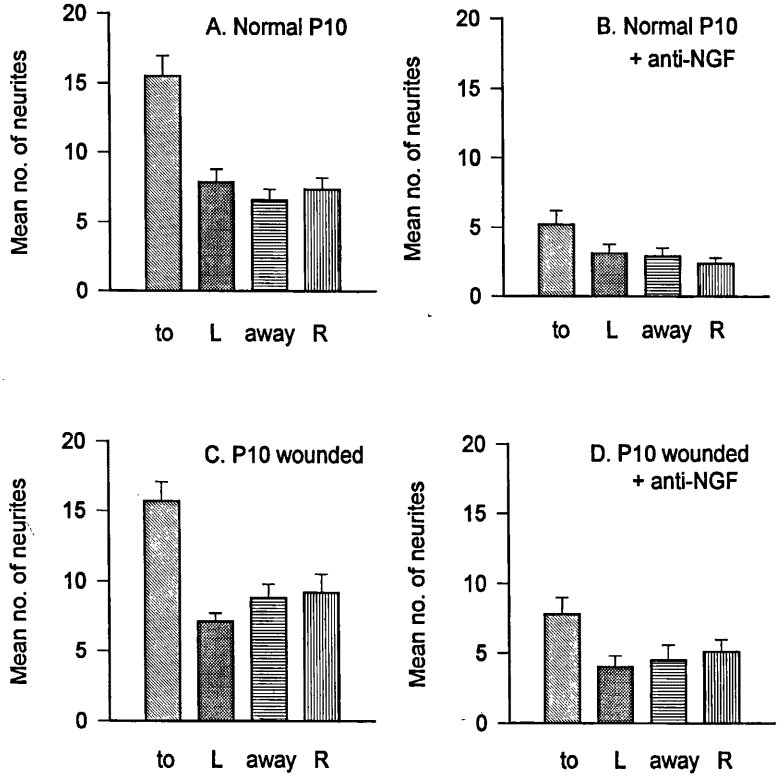

Fig. 5. Bar chart showing the mean neurite outgrowth from E7 chick dorsal root ganglia when cocultured with (a) normal P10 skin, (b) normal P10 skin in the presence of anti-NGF, (c) P10 skin that had been wounded 10 days earlier at birth, and (d) wounded P10 skin in the presence of anti-NGF. The neurite outgrowth has been divided into four quadrants representing growth towards, away from and parallel to the skin explant.

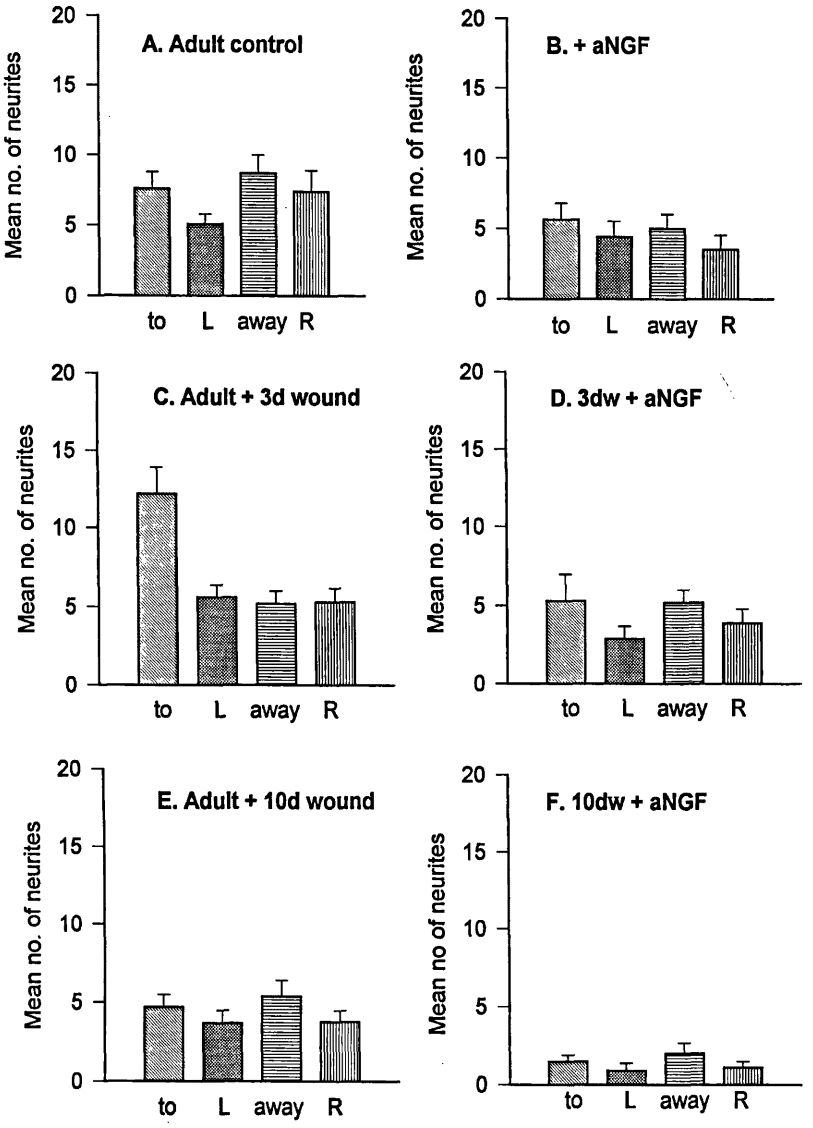

Fig. 6. Bar chart showing the mean neurite outgrowth from E7 chick dorsal root ganglia when cocultured with (a) normal adult skin, (b) normal adult skin in the presence of anti-NGF, (c) adult skin that had been wounded 3 days earlier, (d) 3 day wounded adult skin in the presence of anti-NGF, (e) adult skin that had been wounded 10 days earlier, and (f) 10 day wounded adult skin in the presence of anti-NGF. The neurite outgrowth has been divided into four quadrants representing growth towards, away from and parallel to the skin explant.

3.4.2. Adult skin 3 days and 10 days after wounding + anti-NGF

Total neurite number in cocultures of adult skin 3 days after wounding were reduced by a comparable amount to normal skin, i.e. 35%, when cocultured in the presence of anti-NGF (i.e. from 26.1 ± 0.51 in normal medium to 17.1 ± 0.38 in anti-NGF, $n = 32$) (Fig. 2). The directional growth seen in these cocultures with normal medium was abolished in the presence of anti-NGF, with equal numbers of neurites in the quadrants towards (5.3 ± 1.1) and away from (5.2 ± 1.2) the skin (Fig. 6).

Cocultures with adult skin 10 days after wounding showed a more dramatic reduction of 65% in neurite outgrowth in the presence of anti-NGF (from 15.5 ± 0.41 to 5.4 ± 0.23, $n = 23$) (Fig. 2). As in these cocultures in control medium, there was no indication of directional growth (Fig. 6).

3.5. Assessment of medium + anti-NGF

Alongside the cocultures, chick DRG explants were set up in collagen gels without skin samples either in control medium + 10 ng/ml NGF or in this medium with additional anti-NGF in dilutions of 1/500 or 1/1000. Measurement of the area of neurite outgrowth (see Section 2) showed that the reduction of NGF induced neurite outgrowth effected by anti-NGF under the conditions of these experiments ranged between 75–95%.

4. Discussion

The results show that skin wounded at birth releases neurotrophic factors that stimulate sensory neurite outgrowth in vitro even when NGF activity is blocked. While the majority of the experiments were performed using

chick DRG as a sensitive assay, the main results were confirmed in the newborn rat DRG. It therefore seems reasonable to assume that these same factors are involved in the sensory hyperinnervation response of rat sensory neurons in vivo following neonatal skin wounding [34]. The NGF independence of the neurite outgrowth induced from sensory neurons when co-cultured with neonatally wounded skin was initially surprising in view of the known upregulation of NGF that follows neonatal skin wounding [7] and the role of NGF in collateral sprouting of sensory nerves [11,12,28]. Although increased exposure to NGF through experimental inflammation or local injections in the adult leads to upregulation of neuropeptides CGRP and substance P in sensory neurons [22,25,37] and mechanical and thermal hyperalgesia [3,24,27,37], it appears not be a primary factor in the sprouting of sensory neurons triggered by neonatally wounded skin. This is supported by recent in vivo studies where systemic anti-NGF treatment has been found to have no effect upon the sensory hyperinnervation of neonatal skin wounds (Reynolds and Fitzgerald, in preparation). Neonatally wounded skin must therefore release other factors in addition to and independent of NGF that are not a feature of the adult wounding response.

Skin wounds will cause release of a very large number of growth factors and cytokines to be released from macrophages and other inflammatory cells many of which may be candidates for the observed sprouting response. BDNF, NT3, NT4 and GDNF have also been identified in skin [17,19,36] and whereas these neurotrophins have largely been studied in relation to their ability to promote the survival of classes of large diameter sensory neurons [1,6,15,20,21], they are also upregulated in a number of experimental models of peripheral nerve damage [16,35]. Furthermore, different and largely non-overlapping populations of DRG neurons show developmentally regulated expression of mRNA for the different neurotrophin receptors and 25% of these neurons have no detectable known receptor mRNA [29,38]. Levels of both trkA and trkB in the DRG decrease 2-fold over the postnatal period[1,14,5]. Neurotrophic function is known to regulate and be regulated by cytokines, components of the immune system [32]. A functional trkA receptor is found on monocytes and macrophages, lymphocytes and basophils and synthesis of NGF is regulated by a cytokine cascade including interleukin-1 and TNF-α. Furthermore the activities of cytokines change with development [26] and differences in the inflammatory reaction and cytokine profile in the skin with development have been observed [4]. Interleukin-6, interleukin-1β, TNF-α, LIF in sensory nerves are all developmentally regulated and upregulated following peripheral axotomy [8,30,31]. This range of developmental changes in receptors and their ligands may well underlie the hyperinnervation response in neonatal skin wounds, which the in vitro model will allow us to investigate further.

The NGF independent factors released by neonatal wounded skin were easily detected at 3 days following the wounding and less so by 10 days. Normal skin at 10 days of age contains high levels of constitutive NGF which are released into the medium but when this was neutralised with anti-NGF, wounded skin at 10 days still stimulated significantly more neurite outgrowth than normal 10 day skin. This was not true in the adult where wounded skin was no more effective at stimulating neurite outgrowth than normal skin with or without the presence of anti-NGF. The detection of a background release of NGF from normal skin at 10 days of age and to a lesser extent in the adult is entirely consistent with previously reported changes in postnatal NGF levels in normal skin [7]. NGF is first detected in rat hindlimb skin at E16, coinciding with the arrival of sensory axons [7,9] and levels stay fairly constant until postnatal days 10–14 when a substantial rise is observed, peaking at P(postnatal day)21 and then falling again at P30 to adult levels. The cause of this postnatal change is not known but may be due to reduced uptake by maturing sensory neurons which are no longer dependent on NGF for survival but now require lower levels for maintenance [23]. The source of this NGF is also not clear although a number of cell types including skin fibroblasts and epidermal cells synthesize NGF under cytokine regulation [18] and could be responsible for the upregulation.

In conclusion, a skin wound performed at birth results in the continued release of a non-NGF neurotrophic factor for at least 10 days. This does not occur following wounding in adult skin and is an example of the fundamentally different responses observed in developing and mature sensory nerves in response to injury.

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