Expression and function of Wnt, TNF and TGF-β superfamily receptors in developing neurons

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Abstract

In the developing peripheral nervous system, neuronal survival and axonal growth are regulated by a variety of factors, the most extensively studied of which are secreted neurotrophic factors. To identify additional candidate molecules involved in regulating these aspects of neuronal development, I carried out a PCR screen for the expression of transcripts encoding receptors and selected ligands of three important superfamilies ligands and receptors (Wnt, TGF-β and TNF) over a broad range of developmental ages in three experimentally tractable populations of PNS neurons of mice (superior cervical ganglion, nodose ganglion and trigeminal ganglion). From these screening results I selected one member of the TGF-β superfamily (growth differentiation factor 5, GDF5), and one of the TNF receptor superfamily (B Cell Maturation Antigen, BCMA).

These studies have revealed that GDF5 is synthesized in sympathetic targets and promotes sympathetic axon growth and branching independently of NGF when these axons are ramifying in their distal targets. Mice lacking GDF5 have major deficiencies in sympathetic innervation without loss of neurons, indicating that target-derived GDF5 is essential for establishing appropriate tissue innervation during development.

Function blocking antibodies against BCMA and one of its ligands, BAFF, markedly impair the ability of NGF to promote the survival of cultured sympathetic neurons from the SCG ganglion at the postnatal age of P0.

Similarly, blocking the functions of BCMA and its other ligand, TACI, also leads to impaired survival, but at the earlier age of E15. These findings reveal novel regulators of axonal growth and neuronal survival in developing peripheral nervous system.

List of Abbreviations

AchR – Acetylcholine receptor

ActR-2a – Activin like kinase receptor 2a

ActR-2b – Activin like kinase receptor 2b

AD – Alzheimer's disease

Akt – Ak transforming/protein kinase B

Alk – activin receptor-like kinase

ANOVA – Analysis of variance

APC – Adenomatosis polysis coli

APP – Amyloid precursor protein

BAFF – B-cell-activator factor

Bcl-2 – B cell lymphoma

BCMA – B cell maturation antigen

BDNF – Brain derived neurotrophic factor

BMP –Bone morphogenetic protein

BMPR – Bone morphogenetic protein receptor

CamKII – Calcium/calmodulin-dependent kinase II

CNS – Central nervous system

CNTF – Cilliary neurotrophic factor

CRD – Cysteine-rich domain

CT-1 – Cardiotrophin-1

DIF – Differentiation inducing factor

DKK1 – Dickkopf 1

DR4 – Death receptor 4

DR5 – Death receptor 5

DR6 – Death receptor 6

DRG – Dorsal root ganglion

DVL – Disheveled

DVR – Decapentaplegic-Vg-related family

ERK – Extracellular signal regulated kinase

FGF –Fibroblast growth factor

FZD – Frizzled

GDF 5 – Growth differentiation factor 5

GFP – Green fluorescent protein

GNDF – Glial derived neurotrophic factor

GSK-3β – Glycogen synthase kinase 3β

HBSS – Hanks balanced salt solution

HGF – Hepatocyte growth factor

HVEM – Herpes virus entry mediator

IKK – Inhibitor kB kinase

IL-6 – Interleukin 6

JNK – c-Jun amino (N)-terminal kinase

Kremen 1 – Kringle-containing transmembrane protein 1

Kremen 2 – Kringle-containing transmembrane protein 2

LIF – Leukemia inhibitory factor

LRP5 – Lipoprotein receptor-related protein 5

LRP6 – Lipoprotein receptor-related protein 6

LT – Lymphotoxin

LTP – Long term potentiation

LTP – Long term potentiation

MAPK – Mitogen activated protein kinase

MSP – Macropage stimulatory protein

MuSK – Muscle specific kinase

NEMO – Nuclear factor kappa B essential modifier

NF-κB – Nuclear factor kappa B

NGF – Nerve growth factor

NMJ – Neuromuscular junction

NM – Nasal Mucosa

NPC – Neural precursor cell

NT-3 – Neurotrophin 3

NT4/5 – Neurotrophin 4/5

NT-6 – Neurotrophin 6

NT-7 – Neurotrophin 7

NTD – Neural tube defects

OPG – Osteoprotegerin

OSM – Oncostatin M

p75 – Low affinity neurotrophin receptor

PBS – Phosphate buffered saline

PCD – Programmed cell death

PCP – Planar cell polarity

PHD – Proline hydroxylase domain

PI-3K – Phosphatidyl inositol 3 kinase

PKC – Protein kinase C

PLAD – Pre-ligand-binding assembly domain

PLCγ – Phospho lipase C gamme

PNS – Peripheral nervous system

PS – Primitive streak

RANKL – Receptor activator of NF-kB ligand

Rapsyn – Receptor associated protein of the synapse

RELT – Receptor expressed in lymphoid tissues

RYK – Tyrosine kinase related receptor

SCG – Superior cervical ganglion

SHH – Sonic Hedgehog

SMG – Submandibular gland

TACI – Transmembrane activator and cyclophilin ligand interactor

TCF – T cell specific transcription factor

TCF-LEF – T cell factor/limphocyte enhancer factor 1 complex

TGFβ – Transforming growth factor β

TH – Tyrosine hydroxylase

TNF – Tumor necrosis factor

TNFSF- Tumor necrosis factor superfamily

TNFSFR – Tumor necrosis factor superfamily receptor

TNFα – Tumor necrosis factor α

TRAIL – TNF-related apoptosis-inducing ligand

TRANCE –TNF-related activation-induced cytokine

Trk – Tropomyosin related kinase

VEGF – Vascular endothelial growth factor

VEGI – Vascular endothelial cell-growth inhibitor

WG – Wingless

YFP – Yellow fluorescent protein

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1. General introduction

1.1 A brief overview of neuronal development

1.1.1 Fertilization to Gastrulation

Embryonic development in vertebrates starts with the fertilization of the ovum, which is then called the zygote. The zygote undergoes several divisions until it becomes a mass of undifferentiated cells, the morula. A fluid filled cavity develops in the dividing ball of cells, converting the morula into the blastocyst. This is divided into a inner cell mass and outer cell mass or trophoblast. The inner call mass develops into the embryo, while the trophoblast becomes the placenta. The beginning of gastrulation in mice is evidenced by formation of a transient structure called primitive streak that will later form the posterior end of the embryo (1).

Gastrulation uses a set of morphogenetic movements coupled with cell proliferation to convert an embryo with two germ layers into one with three layers, namely, ectoderm, mesoderm and endoderm (2). These three layers will give rise to the adult body.

Once induced, endoderm forms an epithelial sheet that undergoes specification to distinct regions known as foregut, midgut and hindgut (3). This specification is controlled by factors secreted by surrounding mesoderm-derived tissues, for instance, FGF and BMP4 together have been shown to induce hepatic fate in activin-induced endoderm (4), whereas retinoic acid combined with inhibition of Sonic Hedgehog (SHH) resulted in specification to a pancreatic fate (5).

The hematopoietic, vascular, cardiac, and skeletal muscle lineages derive from subpopulations of mesoderm induced in a defined temporal pattern.

Many studies have been performed to try to understand which are the mechanisms responsible for the induction of the hematopoietic mesoderm, concluding that a combination of Wnt, activin/Nodal and BMP are essential for it (6)(7). Several studies suggested that transient inhibition of Wnt/β-catenin signaling during mesoderm induction is essential for the induction of the cardiac mesoderm (8)(9).

The neural lineages of the skin are derived from ectoderm, which is induced from epiblast cells in the anterior region of the embryo. Inhibition of BMP, Wnt and activin/nodal signaling prevents neuroectodermal induction, which is consistent with

studies showing that these pathways are not active in the region of ectoderm induction in the early embryo (10)(11)(12).

1.1.2 Neural tube formation

Neurulation is the process of forming the neural tube, which becomes the brain and spinal cord. The formation of the neural tube starts in early embryogenesis as the ectoderm thickens, and its cells increase in height, undergo pseudostratification and begin to express unique molecular markers (13). The neural plate induction is mediated by inhibition of BMPs and Wnt signaling pathways (14).

Once the neural plate is formed, it undergoes elongation by convergent extension, a process in which laterally placed cells move toward and are intercalated into the midline (15)(16)(17). After the neural plate induction, its lateral borders elevate into neural folds, and these folds move toward the midline to fuse. After elevation has occurred, bending of the neural folds happens in two steps, furrowing and folding. Furrowing consists in the formation of median hinge (MH) and lateral hinge points (LHP). Folding occurs around these hinge points, with that involving the MHP resulting in elevation of the folds, and that around LHPs producing convergence of the folds (18)(19).

Once the neural folds meet in the midline, they undergo fusion. Cell processes are extended from one to another, and cell surface coats, consisting of glycoproteins, are deposited at regions of fusion (20). These surface coats act as a glue to hold the folds in place until more permanent cell to cell contacts are established. Closure of the neural tube starts near the junction of the hindbrain and spinal cord, and then continues in a "zipper-like" fashion cranially and caudally (21). While joining, some of the cells of the neural fold separate laterally from the others to form the neural crest between the neural plate and the surface non-neuronal ectoderm (22). See figure 1 for details.

If the neural tube fails to close, it can cause several birth defects referred to as neural tube defects (NTDs). In humans the most common NTDs are anencephaly (a fatal failure of rostral neural tube to close) and myelomeningocoele (failure of the vertebral neural tube fusion). To date, many null mice have been generated which display NTDs, suggesting that there is a range of factors that can trigger NTDs (23).

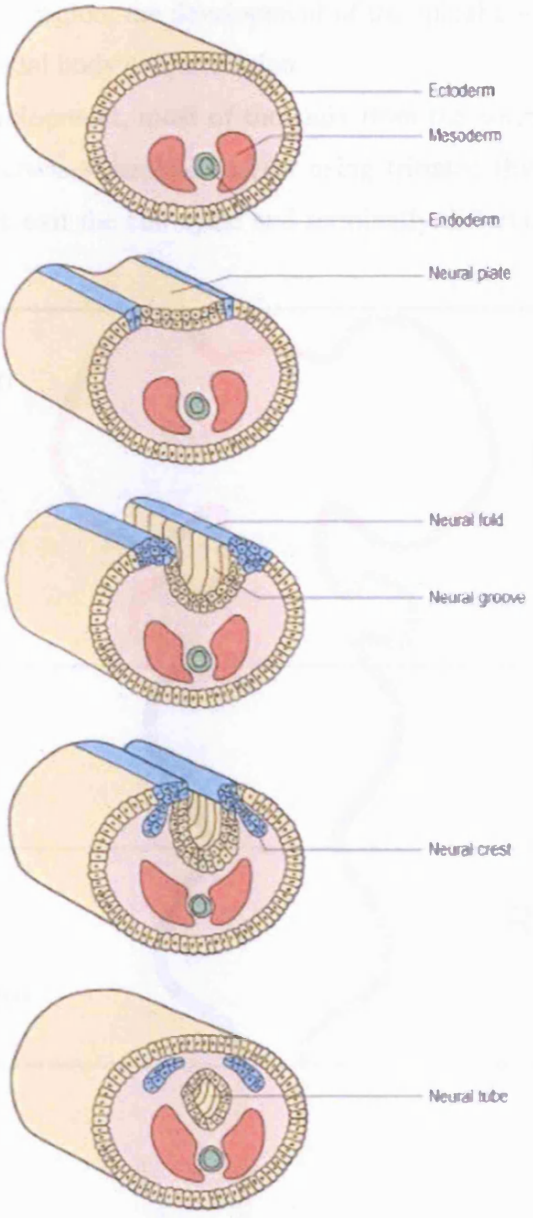


Figure 1: Neural tube formation. The neural tube is formed as a flat neural plate that folds toward the midline until it closes to form the neural tube. (www.studentconsult.com)

Regionalization of the neural tube happens in parallel to the changes in its shape. At the anterior cephalic region where the brain will form, the wall of the neural tube grows, and undergoes a kind of swelling, that defines the various brain compartments (Fig. 2).

Caudally to the brain region, the development of the spinal cord continues as a simple tube that elongates to caudal body axis extension.

In early development, most of the cells from the neural tube proliferate and keep stem-cell like properties. Classical studies using tritiated thymidine incorporation, show that these cells later exit the cell cycle and terminally differentiate at the periphery of the neural tube (24).

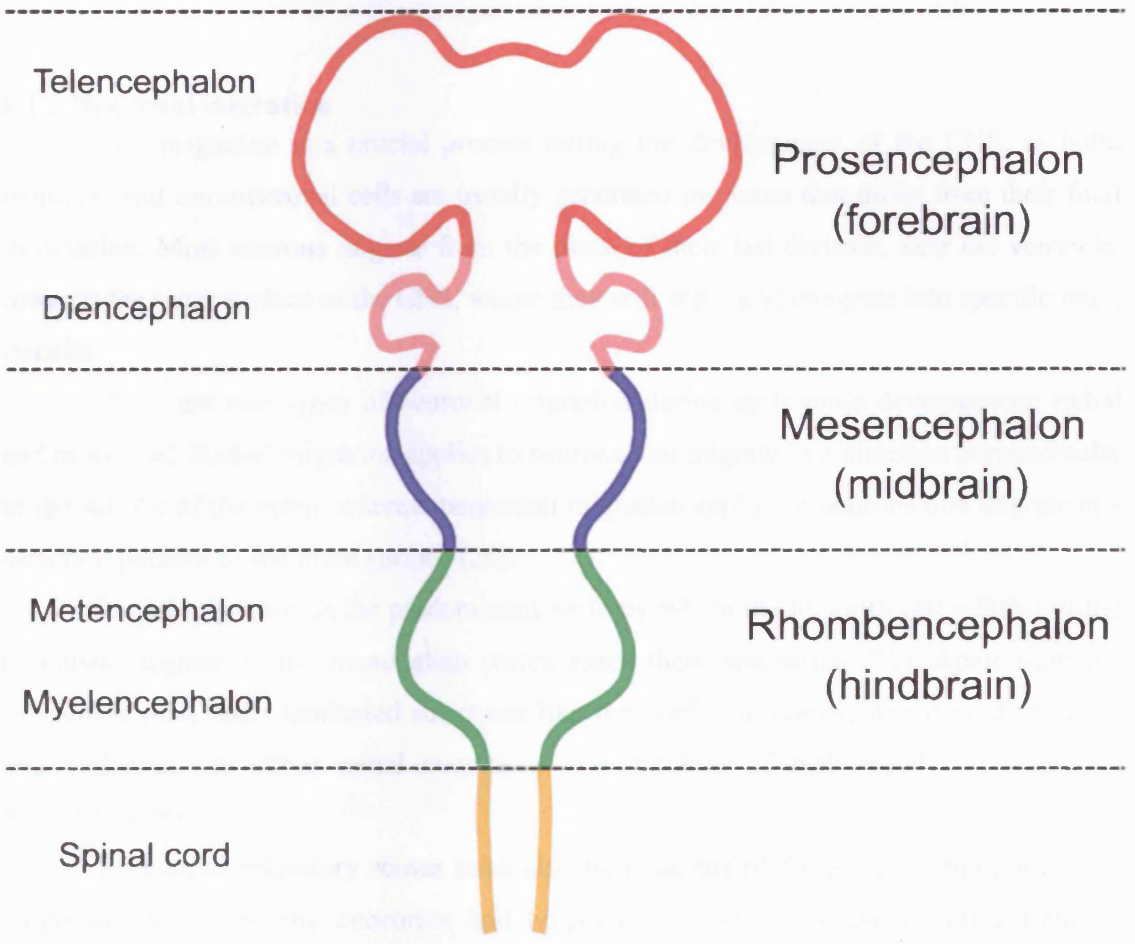


Figure 2: Early embryonic CNS. Drawing showing the neural tube and vesicles. Proliferation and migration of progenitor cells in the walls of the neural tube will give rise to the forebrain, midbrain, hindbrain and spinal cord. (http://blog.vitonis.com)

As mentioned above, the proliferating cells in the neural tube will give rise to the CNS. In order for this event to occur, these cells require cellular and molecular events that

control the patterning of the neural tube along its dorsal and ventral axis. The initial position of these cells in the neural tube defines their exposure to specific signals (morphogens). The best studied morphogens are the Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs), Sonic Hedgehog (SHH) and Wnt pathway (for review see (25)).

1.1.3 Neuronal migration

Cell migration is a crucial process during the development of the CNS, as both, neuronal and non-neuronal cells are usually generated in places that differ from their final destination. Most neurons migrate from the place of their last division, near the ventricle, towards the outer surface of the CNS, where they will settle and integrate into specific brain circuits.

There are two types of neuronal migration during embryonic development: radial and tangential. Radial migration applies to neurons that migrate in a direction perpendicular to the surface of the brain, whereas tangential migration applies to neurons that migrate in a direction parallel to the brain surface (26).

Radial migration is the predominant route by which the majority (80 – 90%) of the precursor neurons in the mammalian cortex reach their destination (27). Apart from the cerebral cortex, other laminated structures like the cerebellar cortex, spinal cord, striatum and thalamus use either radial migration, or a mixture of both radial and tangential migration (28).

Tangential migratory routes have also been identified from the medial ganglionic eminence (MGE) to the neocortex and hippocampus, and from the lateral ganglionic eminence (LGE) to the olfactory bulb.

Defects in neuronal migration can lead to severe problems. Studies in null mice for *reeler* – the gene involved in cell adhesion, show abnormal corticogenesis (Fig. 3a); whereas in null mice for *weaver*, granule cell precursors in the cerebellar cortex fail to migrate along glial fibers and die in ectopic positions (29). In Humans, abnormalities of neural migration cause a range of disorders, most notably lissencephaly, caused by the

disruption in both radial and tangential migration and is characterized by a cortex that has only four layers and a brain that appears smooth with an absence of gyri and sulci (30).

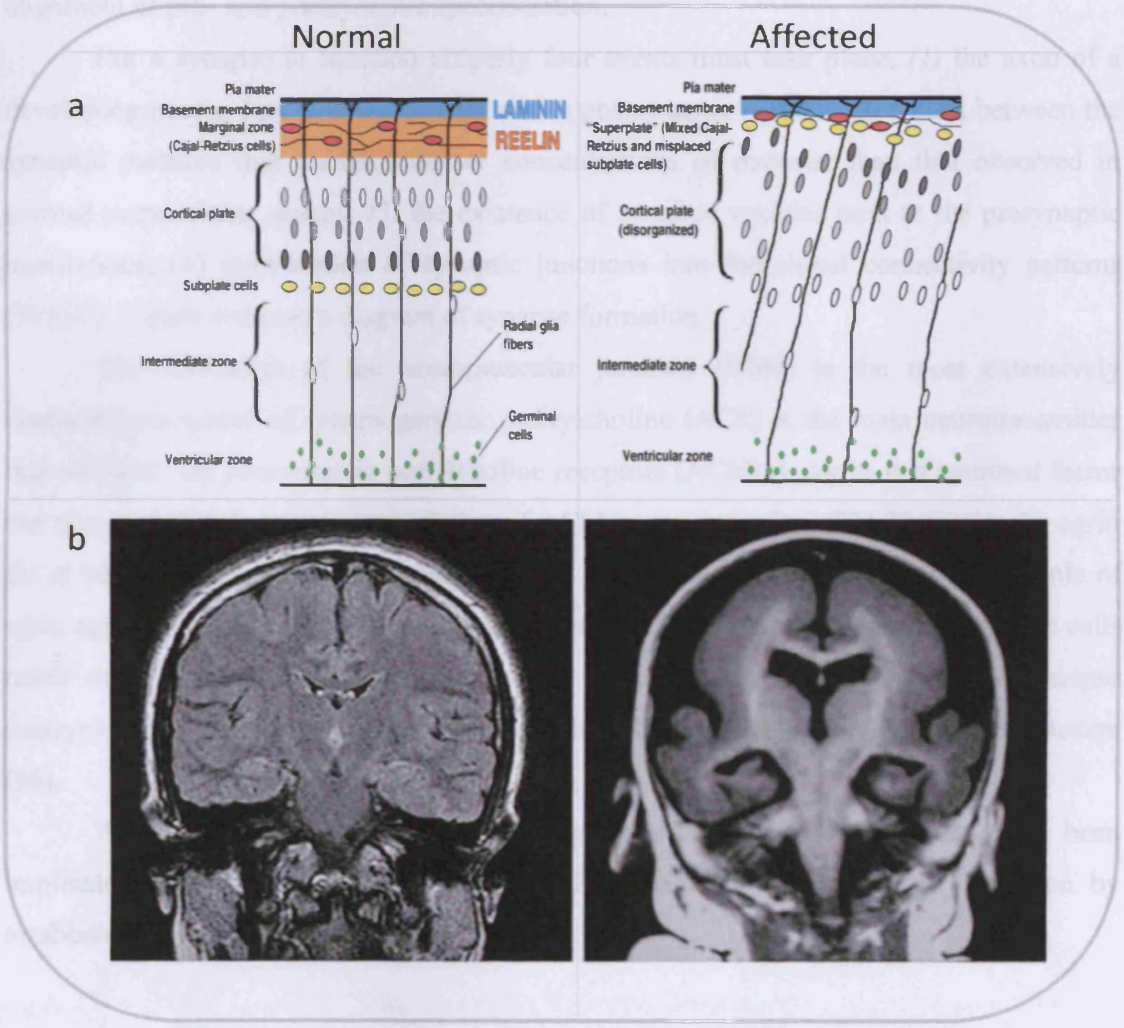


Figure 3: Defects in neuronal migration. (a) Diagram shows corticogenesis in a normal and *reeler* mice. In the normal mouse, the first neurons to take their place are the subplate neurons (yellow). Next are the cortical plate neurons (black), which migrate past the subplate level. In the *reeler* mouse note the disorganized cellular layers, oblique angles of radial glia fibers. (b) Normal and Lissencephalic brain. Lessencephalic individuals have a smooth cortex lacking the characteristic sulci and gyri observed in the normal brain. (30)

1.1.4 Synaptogenesis

Synaptogenesis involves the formation of a neurotransmitter release site in the presynaptic neuron and a receptive field at its postsynaptic partner(s), and the precise alignment of pre- and postsynaptic specialization.

For a synapse to function properly four events must take place, *(1)* the axon of a developing neuron has to make contact with a postsynaptic partner; *(2)* a cleft between the synaptic partners that contains denser concentrations of material than that observed in normal extracellular spaces; *(3)* the existence of synaptic vesicles next to the presynaptic membranes; (4) stabilization of synaptic junctions into functional connectivity patterns (31)(32). Figure 4 shows a diagram of synapse formation.

The formation of the neuromuscular junction (NMJ) is the most extensively characterized model of synaptogenesis. Acetylcholine (ACh) is the main neurotransmitter that activates the postsynaptic acetylcholine receptors (AChRs). Agrin is a neuronal factor that plays a key role in the accumulation of AChR at synaptic sites (33). Null mice for agrin die at birth because neurotransmitter function is disrupted (34). The physiological role of agrin appears to be to prevent the dispersion of AChR clusters on developing muscle cells rather than inducing initial clustered pattern (35). Receptor associated protein synapse (rapsyn) forms complexes with AChRs which are required for the formation of clusters (36).

Another postsynaptic protein, muscle specific kinase (MuSK) has also been implicated in receptor clustering in vivo (37). MuSK regulates synaptic formation by establishing the postsynaptic density (38).

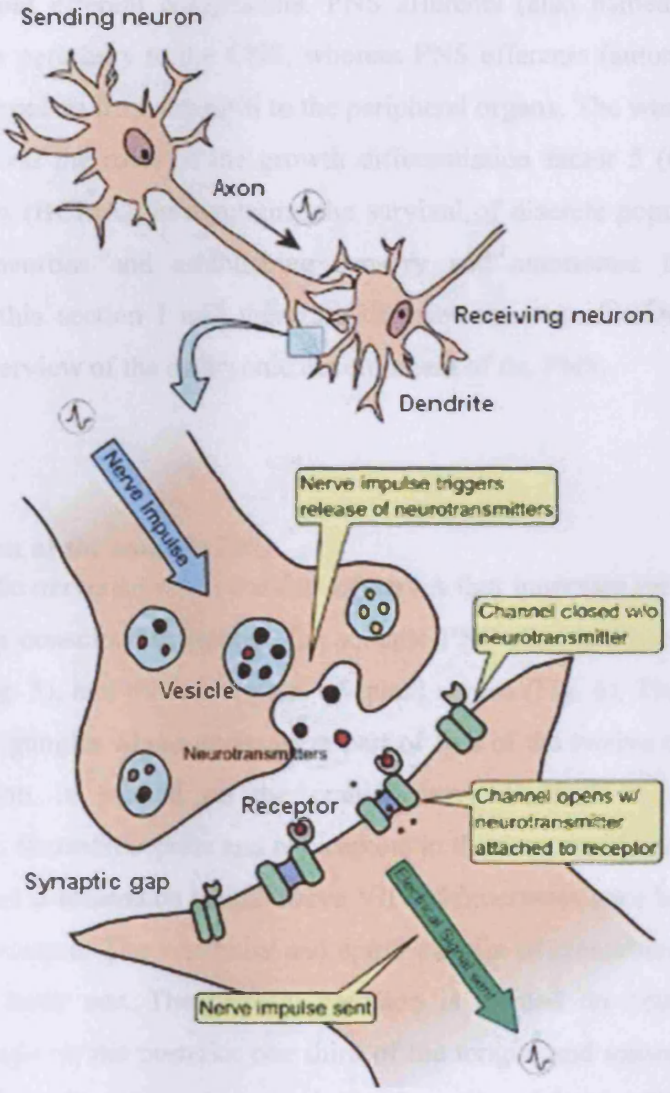


Figure 4: synaptic structure. Diagram showing a synapse between two neurons. Neurotransmitters contained within synaptic vesicles are released from the presynaptic cell, traverse the synaptic cleft and bind to receptors on the postsynaptic membrane. (http://universe-review.ca/R10-16-ANS.htm)

1.2 Organization and development of the peripheral nervous system

1.2.1 Introduction

The CNS which develops from the neural tube (as described above), includes the brain and spinal cord. The peripheral nervous system (PNS) consists of nerves and ganglia that provide communication between the CNS and the periphery. The PNS is subdivided

into an afferent and efferent components. PNS afferents (also named sensory), convey impulses from the periphery to the CNS, whereas PNS efferents (autonomic and somatic motor) transmit impulses from the CNS to the peripheral organs. The work described in this thesis is centered on the roles of the growth differentiation factor 5 (GDF5) and B cell maturation antigen (BCMA), in regulating the survival of discrete populations of sensory and autonomic neurons and establishing sensory and autonomic innervation during development. In this section I will therefore describe the organization of the PNS and provide a brief overview of the embryonic development of the PNS.

1.2.2 Organization of the somatic PNS

The somatic nervous system consists of nerves that innervate the skin and muscles, and is involved in conscious activities. The somatic PNS signals through twelve pairs of cranial nerves (Fig. 5), and thirty one pairs of spinal nerves (Fig. 6). There are seven pairs of cranial sensory ganglia whose axons form part of five of the twelve cranial nerves. The trigeminal ganglion is located on the cranial nerve V, and its neurons innervate mechanoreceptors, thermoreceptors and nociceptors in the face, nasal and oral cavities. The geniculate ganglion is located on cranial nerve VII and innervates taste buds on the anterior two thirds of the tongue. The vestibular and spiral ganglia of cranial nerve VIII innervate hair cells in the inner ear. The petrosal ganglion is located on cranial nerve IX and innervates taste buds on the posterior one third of the tongue and sensory receptors in the carotid body. The superior glossopharyngeal ganglion of cranial nerve IX innervates part of the skin of the pinna and tympanic membrane. The jugular and nodose ganglia of cranial nerve X innervate various sensory targets in the pharynx, thorax and abdomen.

Each spinal nerve is connected to the spinal cord by a dorsal root consisting of sensory fibers and a ventral root of motor fibers. The cell bodies of the sensory neurons of spinal nerves are located in the dorsal root ganglia (DRG), and the motor-neuron cell bodies are located in the ventral grey matter. The two roots join to form the spinal nerve just before the nerve leaves the vertebral column via an intervertebral foramen. Each spinal nerve subsequently divides in dorsal and ventral rami, consisting of mixed sensory and motor fibers. See Figure 6 for details.

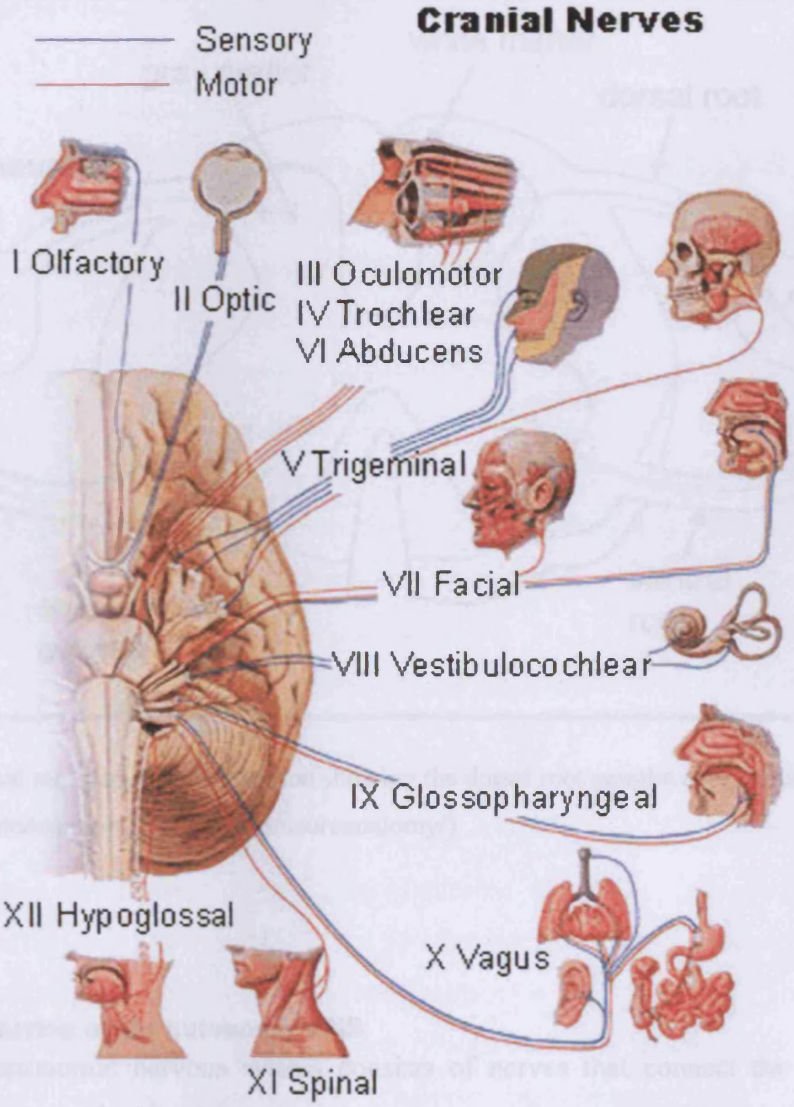


Figure 5: Cranial nerves. Illustration showing the roots of the twelve cranial nerves and the main tissues they innervate. (www.web-books.com)

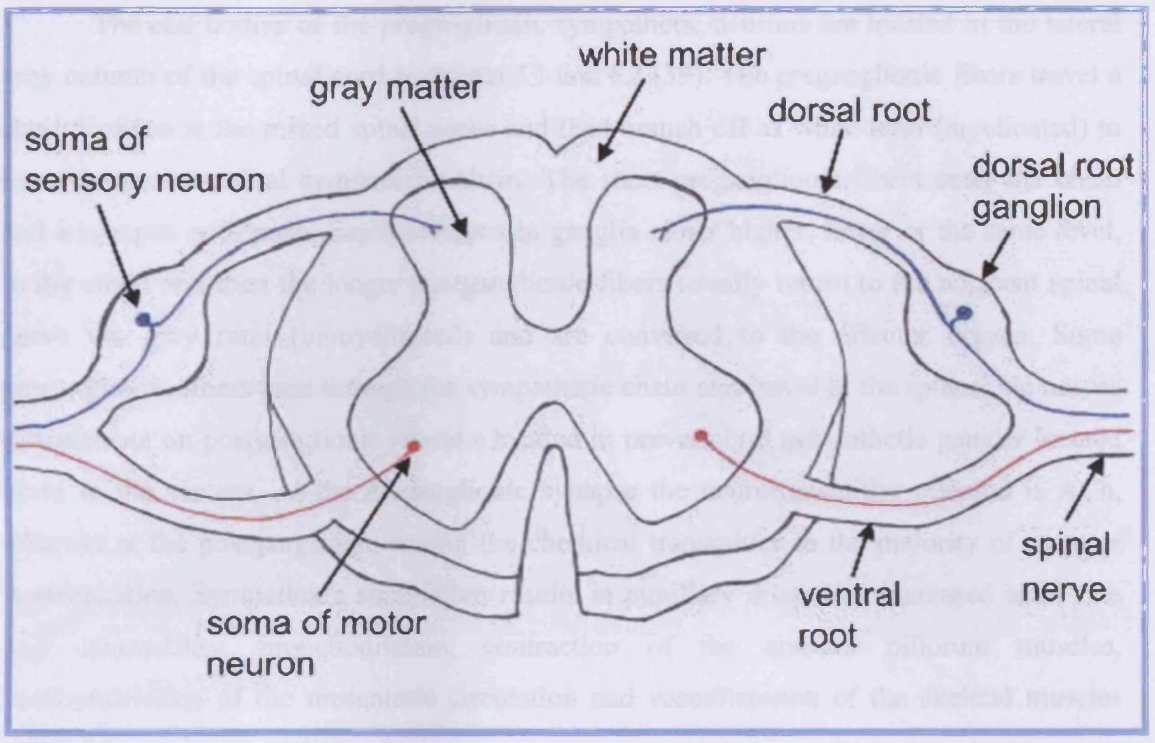


Figure 6: Dorsal root ganglion. Illustration showing the dorsal root ganglia organization. (www.acceleratedcure.org/msresources/neuroanatomy/)

1.2.3 Organization of the autonomic PNS

The autonomic nervous system consists of nerves that connect the CNS to the viscera, various glands, blood vessels and the arrector pilorum muscles of hair follicles. It mediates unconscious activities. The autonomic nervous system can be subdivided into three divisions: the sympathetic nervous system, parasympathetic nervous system and the enteric nervous system.

The sympathetic nervous system prepares the organism for "fight or flight", whereas the parasympathetic system is responsible for "rest and digest". In order to respond to emergency situations, the hypothalamus activates the sympathetic nervous system, resulting in an increase of the sympathetic outflow to the heart and other viscera, peripheral vasculature, sweat glands, and arrector pilorum muscles. In contrast, the parasympathetic system maintains basal heart rate, respiration, and metabolism under normal conditions.

The cell bodies of the preganglionic sympathetic neurons are located in the lateral grey column of the spinal cord segments T1 and L2 (39). The preganglionic fibers travel a short distance in the mixed spinal nerve and then branch off as white rami (myelinated) to enter the paravertebral sympathetic chain. The short preganglionic fibers enter the chain and a synapse with postsynaptic neurons in ganglia either higher, lower or the same level, in the chain and then the longer postganglionic fibers usually return to the adjacent spinal nerve via grey rami (unmyelinated) and are conveyed to the effector organs. Some preganglionic fibers pass through the sympathetic chain and travel in the splanchnic nerves to terminate on postganglionic neurons located in prevertebral sympathetic ganglia located close to the viscera. At the preganglionic synapse the neurotransmitter released is ACh, whereas at the postganglionic ending the chemical transmitter in the majority of cases is noradrenaline. Sympathetic stimulation results in pupillary dilatation, increased heart rate and contractility, bronchodilation, contraction of the arrector pillorum muscles, vasoconstriction of the mesenteric circulation and vasodilatation of the skeletal muscles arterioles.

Preganglionic outflow of the parasympathetic nervous system arises from cell bodies of preganglionic parasympathetic nuclei related to the cranial nerves III, VII, IX and X and in the lateral grey column of sacral spinal segments (S2, S3 and S4). This is referred to as the cranio-sacral outflow. Preganglionic fibers synapse in ganglia close to or within the organ they innervate, giving rise to relatively short postganglionic fibers. Cranial postganglionic parasympathetic ganglia related to the cranial nerves III, VII and IX promote pupillary constriction, visual accommodation and salivary gland secretion, whereas the terminal ganglia of cranial nerve X innervates the heart, lungs, stomach, upper intestine and ureter. The sacral parasympathetic outflow innervates the distal colon, rectum, bladder and sexual organs.

Physiologically, the parasympathetic system reduces heart rate and blood pressure, and facilitates digestion and consequently nutrient absorption. The neurotransmitter at both pre and postganglionic synapses in the parasympathetic system is ACh.

The enteric nervous system controls the coordinated muscular contractions and secretion of glands along the gastrointestinal tract. The neurons and their processes are located predominantly in two plexuses: the submucosal plexus innervates the mucosa and regulates secretion; and the myenteric plexus innervates the circular and longitudinal smooth muscle layers and regulates motility.

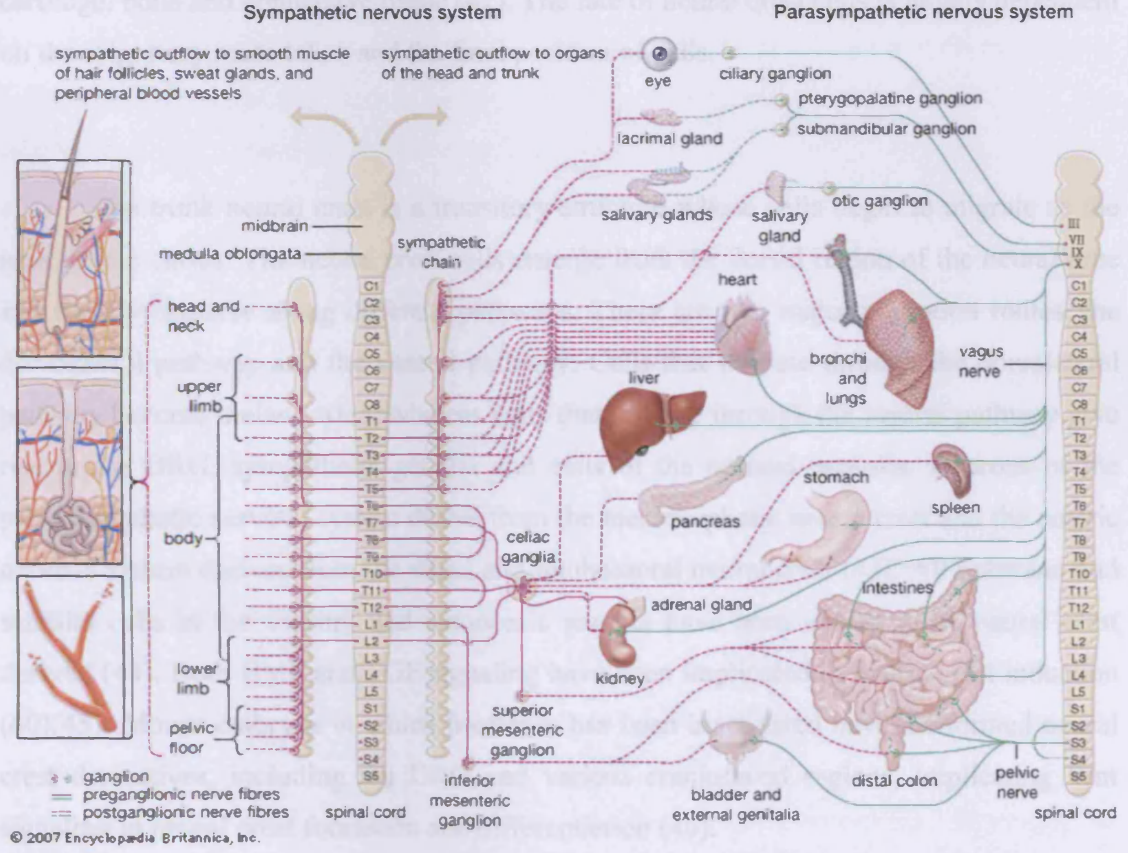


Figure 7: Sympathetic and parasympathetic nervous systems. Illustration showing the organizations of the sympathetic (left) and parasympathetic (right) nervous systems and some peripheral target tissues of each system. (www.britannica.com)

1.2.4 Development of the peripheral nervous system

With exception of certain cranial sensory neurons, all neurons and glial cells of the peripheral nervous system are derived from the neural crest.

The neural crest originates from the neural folds after interactions between the neural plate and the presumptive epidermis (40)(41). Migration and differentiation of neural crest cells from the dorsal neural tube give rise to a wide variety of cell types including peripheral nervous system neurons, Schwann cells, satellite cells, melanocytes, facial cartilage, bone and connective tissue (42). The fate of neural crest cells is largely dependent on the migratory route taken and the final position of cells.

The trunk neural crest is a transitory structure whose cells begin to migrate as the neural tube closes. The neural crest cells emerge from the dorsal region of the neural tube and rapidly disperse along different pathways. There are two major migration routes, the dorsolateral pathway and the ventral pathway. Cells that migrate through the dorsolateral pathway become melanocytes, whereas cells that migrate through the ventral pathway give rise to the DRG, sympathetic ganglia and cells of the adrenal medulla. Neurons of the parasympathetic nervous system derive from the mesencephalic neural crest and the enteric nervous system derives from the vagal and lumbosacral neural crest (43). All Schwann and satellite cells in the sensory and autonomic ganglia have been shown to be neural crest derived (44). Both BMP and FGF signaling have been implicated in neural crest induction (40)(45). Mouse embryos in which β-catenin has been inactivated have malformed neural crest derivatives, including the DRG and various craniofacial regions, implicating Wnt signaling in neural crest formation and differentiation (46).

Neurogenic placodes are focal thickenings of embryonic cranial ectoderm that are induced by signals from the surrounding cranial tissue. These placodes can be further divided in dorsolateral and epibranchial, based in their position in the developing head. The dorsolateral placodes give rise to the neurons of the vestibular ganglia and ventrolateral part of the trigeminal ganglia, whereas the epibranchial placodes give rise to the neurons of the nodose, petrosal and geniculate ganglia.

1.3 Regulation of neuronal survival in the PNS

1.3.1 Introduction

Apoptosis or programmed cell death (PCD) has long been shown to be crucial for the correct development of the nervous system. During development between 20 and 80% of neurons generated in various populations undergo PCD.

The occurrence of PCD in the early development of the nervous system has been reported in a large spectrum of organisms, from *C. elegans* to mouse. A common feature between species is that it happens within proliferative or newly post-mitotic cells, and it is usually among the earliest cell deaths during development.

All pathways to apoptosis converge on the activation of caspases (which are proteases that coordinate the efficient dismantling of doomed cells). These pathways are governed mainly (though not exclusively) by the Bcl-2 family of proteins (47). For details see figure 8. Two distinct pathways, both resulting in caspase activation, can be distinguished depending on whether or not the Bcl-2 family of proteins is involved and by which caspases are decisive for cell death. They are (1) *the intrinsic pathway* – activated by developmental cues or cytotoxic insults, such as growth factor deprivation, viruses, or stress, and is strictly controlled by Bcl-2 family of proteins; (2) *the extrinsic pathway* – triggered by ligation to death receptors where two distinct intracellular pathways can be engaged. "Type I cells" pathway is activated if there are sufficient levels of caspase 8, or "type II cells" pathway when a low level of caspase 8, without any involvement of the Bcl-2 family (48).

The Bcl-2 family is usually grouped into three classes of proteins depending on their action: *(1)* those that inhibit apoptosis (Bcl-2, Bcl-XL, Bcl-W, Bcl-B and A1); *(2)* those that promote apoptosis (BAX, BAK and BOK); *(3)* Bcl-2 homology domain (BH-3)-only proteins (BAD, BIK, BID, HRK, BIM, NOXA and PUMA) (49).

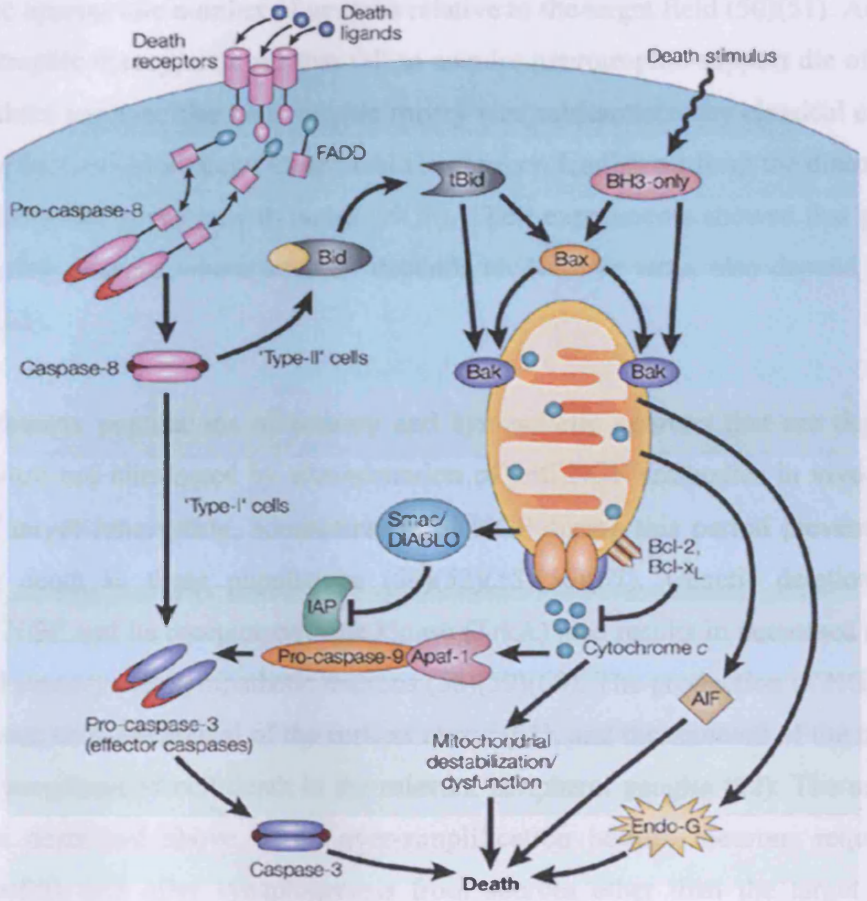


Figure 8: The molecular basis of apoptosis. Diagram showing the intracellular machinery that regulates and executes apoptosis. (48)

Neuronal apoptosis is regulated by a tightly controlled balance of surviving-promoting neurotrophic factors and cell death promoting signals. As an example, nerve growth factor (NGF) binding to TrKA activates pro-survival pathways such as PI-3 kinase, Akt and MEK/MAPK. This will be further explored in a later section of the introduction.

1.3.2 Neurotrophic theory

During the normal development of the nervous system, neurons are generated in excess. A proportion undergo a phase of cell death shortly after axons reach their targets, eliminating unnecessary neurons and neurons with inappropriate synaptic connections,

leaving the appropriate number of neurons relative to the target field (50)(51). According to the neurotrophic theory, neurons that fail to acquire neurotrophic support die of apoptosis, and the others survive. The neurotrophic theory was substantiated by classical experiments carried out by Levi-Montalcini Cohen and Hamburger, leading on from the discovery of the first neurotrophin, nerve growth factor (NGF). Their experiments showed that populations of developing neurons whose survival depends on NGF *in vitro*, also depend of NGF *in vivo* (52)(53).

Whereas populations of sensory and sympathetic neurons that are dependent on NGF in vitro are eliminated by administration of anti-NGF antibodies in vivo during the period of target innervation, administration of NGF during this period prevents naturally occurring death in these populations (54)(52)(55)(56)(57). Genetic deletion of genes encoding NGF and its receptor tyrosine kinase (TrkA) also results in decreased numbers of peripheral sensory and sympathetic neurons (58)(59)(60). The production of NGF begins in target tissues with the arrival of the earliest axons (61), and the removal of the target tissue increases programmed cell death in the relevant peripheral ganglia (62). The neurotrophic theory, as described above, is an over-simplification because neurons require trophic support before and after synaptogenesis from sources other than the target field (51). Furthermore, many populations of neurons switch their dependence from one neurotrophic factor to another at certain stages of development (63)(64). In vitro studies have shown that populations of PNS neurons survive independently of the neurotrophic factors at the stage when axons begin to sprout. For several populations of neurons, the duration of the neurotrophic factor independence is proportional to the distance and time needed for axons to grow toward and reach their targets (65)(66).

Since the discovery of NGF, much research has been done attempting to identify additional neurotrophic factors. Brain-derived neurotrophic factor (BDNF) was the second neurotrophic factor to be purified (67). Since then, several other neurotrophic factors have been identified. The neurotrophin family of proteins includes nerve growth factor (NGF), brain-derived growth factor (BDNF) neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) (among the vertebrates, the latter two

neurotrophins are only found in fish). The neurotrophins are structurally and functionally related and signal through two classes of receptors: the high-affinity tyrosine kinase receptors of the Trk family (TrkA, TrkB and TrkC) and a common low-affinity receptor, p75. Several other families of neurotrophic factors have been identified to promote survival of various neuronal populations at various stages of development. These include the glial cell-derived neurotrophic factor (GDNF) family (GDNF, neurturin, artemin and persephin), the neurotrophic cytokines [ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotrophin-1 (CT-1) and interleukin-6 (IL-6)] and the related cytokines hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP) (63)(68) (69)(70).

In addition to regulating neuronal survival during development, neurotrophins have a role in diverse processes from cellular proliferation, differentiation and process outgrowth to synaptic plasticity (71)(72)(73)(74)(75). The original research described in this thesis has a bearing on the role of neurotrophins in the regulation of neuronal survival and process outgrowth during the development of specific populations of the peripheral sensory and sympathetic neurons. Although the individual neurotrophic requirements of PNS neurons during development have been described, the downstream signaling events mediating neuronal survival and neurite outgrowth are only beginning to be elucidated. I will therefore briefly describe the neurotrophic requirements of the relevant peripheral ganglia, followed by an introduction to the biology of the neurotrophic factors and their receptors.

1.3.3 Neurotrophic requirements of selected peripheral ganglia

The studies described in this thesis were performed on sensory neurons of the mouse nodose ganglion and trigeminal ganglion, and on the sympathetic neurons of the superior cervical ganglion (SCG).

In vitro survival of nodose neurons has been shown to depend mainly on BDNF support and to a lesser extent NT-3 and NT-4 (66)(76)(77). Null mice for BDNF, NT-3 and NT-4 show increased cell death in the nodose ganglion (78)(79)(80)(81). Early in development, the survival of a small subpopulation of nodose neurons is supported by NGF

in vitro. This observation was further supported by the slight decrease in the number of neurons in the nodose ganglion in $NGF^{-/-}$ mice (82). Subpopulations of the nodose neurons respond to a variety of other neurotrophic factors, notably neurotrophic cytokines CNTF, LIF OSM and CT-1 (83). The in vitro survival of neonatal nodose neurons is equally well supported by both BDNF and these neurotrophic cytokines.

Trigeminal neurons have been shown to switch their dependence between neurotrophins at different stages of development. Early trigeminal neurons are dependent on both NT-3 and BDNF for survival but by embryonic day 12 (E12) most neurons become NGF-dependent (84). The switch in neurotrophin response is correlated with a change in the appropriate Trk receptor expression and a switch of neurotrophin from BDNF to NGF in the target tissue (64)(84)(85)(86).

The sympathetic neurons of the SCG originate from precursor cells in the neural crest. During their development, SCG neurons respond to a variety of neurotrophic factors, and the *in vitro* survival of SCG neurons can be supported by a wide range of neurotrophic factors between early embryonic and early adult life. Artemin promotes sympathetic neuroblast proliferation and hepatocyte growth factor (HGF) promotes differentiation of sympathetic neuroblasts into postmitotic sympathetic neurons (73)(87)(88). SCG neurons respond to NGF at E14 and remain dependent on NGF for survival throughout the rest of their embryonic life (89). By birth and into early adulthood the survival of SCG neurons *in vitro* can be supported by both NGF and NT-3. The dependence of SCG neurons on NGF and/or NT-3 correlates with expression of TrkA and not TrkC during and after the period of naturally occurring death (90). Late postnatal SCG neurons also respond to CNTF, LIF and HGF *in vitro* (91)(92).

Cell culture systems, described herein, were used to analyze neuronal survival and/or process outgrowth in embryonic or postnatal neurons from the trigeminal (supported by NGF), nodose (supported by BDNF) and SCG (supported by NGF) ganglia. The biology of these neurotrophic factors and their associated receptor systems is introduced in the upcoming sections.

1.3.4 NGF

NGF, the founder member of the neurotrophic family, was first described the 1950's, after its isolation from mouse salivary glands (93)(94). In the subsequent years researchers identified the amino acid sequence of NGF (95) and its 3D structure (96). All NGF is synthesized in its precursor form, i.e, pro-NGF. It is then enzymatically cleaved, to give rise to mature NGF. Mature NGF is a homodimer consisting of two 118 amino acid subunits, each containing a cysteine rich motif and two anti-parallel β strands (95)(96)(97).

NGF has been shown to be expressed in many regions of the CNS, such as neocortex, basal forebrain and hippocampus (98). The targets of NGF-dependent neurons also secrete NGF, including the heart, submandibular gland and whisker pads (61)(99). Several non-neuronal cells also express NGF, namely cells from the immune, reproductive, endocrine and cardio-vascular system, although the function of NGF in these tissues is poorly understood (100). $NGF^{-/-}$ mice lose substantial numbers of sensory and sympathetic neurons, during development, demonstrating the crucial role of NGF signaling during development (58). More than regulating neuronal survival, NGF is required for terminal innervation of target tissues. The excessive death of sensory neurons in $NGF^{-/-}$ mice can be prevented in $NGF^{-/-}/BAX^{-/-}$ mice (101). These double knockout mice have reduced peripheral innervation, suggesting that NGF signaling regulates the extent of terminal branching in target tissues (101). In this way, NGF has been shown to play a crucial role in establishing the innervation of distal sympathetic targets (102).

1.3.5 BDNF

BDNF was identified in 1982, becoming the second member of the neurotrophin family. Just like NGF, BDNF was reported to be a target-derived factor and to promote neuronal survival and neurite outgrowth during development (67). BDNF is synthesized as a precursor and then cleaved to form mature, biologically active BDNF (103). BDNF shares approximately 50% of its sequence with NGF (67)

BDNF expression is widespread in the CNS including cerebral cortex, hippocampus, cerebellum, basal forebrain, striatum, hypothalamus and brain stem (104).

BDNF has been implicated in several aspects of development including cell proliferation, learning and memory (105). BDNF has been shown to play an important role in long term potentiation (LTP) (106)(107)(108). BDNF has emerged as a major regulator of synaptic plasticity and key target in neurological diseases (109).

BDNF is also expressed in certain populations of peripheral sensory ganglia and in target tissues of BDNF-dependent neurons, including the heart, skin, muscle and lung (84)(110)(111). BDNF promotes survival of placode derived trigeminal, nodose, petrosal, geniculate and vestibular neurons during development (112)(113)(114)(115). $BDNF^{-/-}$ mice die perinatally and display severe defects in the populations of sensory neurons that depend on BDNF for survival (116)(117). BDNF has been implicated in neurite outgrowth in vestibular, DRG, sympathetic and nodose neurons (118)(119)(120)(121)(122) (123)(124)(125) of the PNS and in neurite outgrowth and maintenance of cortical and hippocampal processes (126)(127)(128)(129)(130)(131)(132)(133).

1.3.6 Trk receptors

The Trk receptors are receptors tyrosine kinases that primarily regulate growth, differentiation and programmed cell death of neurons in both the peripheral and central nervous systems. These receptors were first described in 1986 as the product of the *trk* oncogene, rather than for their trophic actions on neuronal cells (134)(135). Only a few years later, researchers gathered enough evidence to show that the biological role of the Trk tyrosine protein kinase was to serve as signaling receptor for NGF (136)(137)(138)(139). Subsequent studies showed Trk tyrosine kinase was a member of a small family of receptors that included TrkB (140)(141)(142), and TrkC (143). TrkB mediates the biological activity of both BDNF (141)(144)(145) and NT-4 (146)(147)(148), and TrkC is the primary receptor for NT-3 (149). There is also some physiologically relevant, lower affinity binding between NT-3 and TrkA and TrkB and between NT-4/5 and TrkA (147)(144)(145)(146).

All peripheral neurons express Trk receptors, with the exception of parasympathetic ciliary neurons (86)(150)(140)(151)(152)(143).

In general, the populations of neurons that are lost in $Trk^{-/-}$ mice overlap with those lost in the knockout mice of the primary neurotrophin ligand. $TrkA^{-/-}$ mice have decreased

neuronal number in trigeminal and sympathetic ganglia (59). $TrkB^{-/-}$ mice loose a substantial proportion of trigeminal and nodose neurons (153), while $TrkC^{-/-}$ mice have decreased numbers of myelinated axons in the dorsal roots accompanied by the loss of a subpopulation of DRG neurons (154).

1.3.7 p75

p75 was discovered and identified as an NGF receptor in 1986 (155), and was subsequently shown to be able to bind all neurotrophins (156). p75 is expressed both in the CNS and PNS as well as non-neuronal cells and its expression increases after trauma (157). p75 has been shown to modify the function of Trk receptors (157), to bind to pro-neurotrophins in a complex with a co-receptor called sortilin to elicit apoptosis (158)(159)(160), and can therefore either promote (161)(162)(163) or inhibit (164) cell death.

1.4 Regulation of neurite outgrowth

1.4.1 Axon extension and guidance

Neuronal connections form during embryonic development when each individual differentiating neuron sends out an axon, tipped at its leading edge by the growth cone, which migrates through the embryonic environment to its synaptic targets. Observations of developing axonal projections in vivo have revealed that the great majority of axons extend to the vicinity of their appropriate target regions in a highly stereotyped and directed manner, making very few mistakes (165).

The first axons that develop navigate through an axon-free environment when the embryo is still relatively small, but most axons face an expanding environment crisscrossed by a scaffold of earlier projecting axons (166) Axons respond to coordinate actions of four types of guidance cues: attractive and repulsive, which can be either short or long-range (167). Many of these attractive and/or repulsive cues have been identified and include semaphorins, netrins, slits, epherins, morphogens and associated receptor systems (168)(169).

1.4.2 The role of neurotrophic factors in regulating neurite growth from PNS neurons

The role of neurotrophic factors in regulation of neurite outgrowth *in vitro* has been well established over many years (102)(170)(120)(171)(172)(173)(174). The role of neurotrophic factors in neurite outgrowth *in vivo* has been much more difficult to ascertain, however, because the effects of neurotrophic factors on neuronal survival have impeded efforts to investigate the requirement of neurotrophic factors for axon extension and peripheral target innervation. The separation of survival effects from effects on neurite outgrowth was achieved by studying $NGF^{-/-}/TrkA^{-/-}$ double knockout (101). BAX deletion prevents programmed cell death in developing peripheral neurons (175), so crossing these double knockout mice provided an ideal framework for analyzing the growth-promoting effects of NGF/TrkA signaling *in vitro* and *in vivo* (101). Further work has shown that peripheral axons can be induced to grow towards ectopic sources of neurotrophic factors, a process that can be reversed by application of function-blocking antibodies to NGF, BDNF or NT-3 (119). Furthermore, in vivo analysis of the neurotrophin-overexpressing mice has revealed that increased levels of NGF or NT-3 in the brain, pancreas, heart or skin leads to increased axonal growth in sensory and sympathetic neurons in these tissues (81)(176)(177)(178).

More recently, Kuruvilla et al, have reported that NGF and NT-3 control sympathetic development through differential control of TrkA internalization and retrograde signaling. NT-3 signals via TrkA to promote outgrowth but not survival, whereas NGF controls both survival and outgrowth (170).

1.4.3 Intracellular signaling pathways downstream of neurotrophic factors regulating neuronal survival and neurite outgrowth

Trk receptors are activated through ligand-mediated dimerisation followed by phosphorylation of several conserved cytoplasmic tyrosine residues, providing docking sites for adaptor molecules and enzymes mediating intracellular signaling pathways (74)(153)(154)(155). Neurotrophin signaling through Trk receptors leads to the activation of many intracellular signaling molecules such as Ras, PI3-K and PLCγ (156). Neurotrophin signaling in the cell soma and nucleus is crucial for neuronal survival (52)(157). However, especially in the PNS, the source of neurotrophic factor may be at

quite a distance from the soma. These observations prompted investigations into the mechanisms of retrograde signal transduction after ligand engagement. Ligand engagement stimulates internalization of Trk receptors through clatherin-coated pits and by macropinocytosis in cell surface ruffles (158)(159). After internalization, neurotrophins are localized with the Trk receptors in endosomes that also possess activated signaling intermediates, such as Shc and PLCγ1 (160). NGF-TrkA internalization and retrograde transport to the soma is required for transmission of the neurotrophin survival signal (161)(162). Recent data describing NGF-promoted neuronal survival without NGF internalization (163) and the lack of retrograde signaling of NT-3/TrkA in sympathetic neurons have added layers of complexity to neurotrophin signaling cascades.

Once neurotrophins bind to their respective Trk receptors, they are known to activate protein kinase A (PKA), Ras/phosphotidyl inositol 3'-phosphate-kinase (PI3-K)/Akt, (hereafter referred as PI3-K/Akt) and Ras/Raf/mitogen-activated protein kinase (MAPK) and MAPK/extracellular signal-regulated kinase (ERK) (hereafter referred as MAPK/ERK) signaling pathways (179)(180)(181)(182)(183)(184)(185)(186).

Activation of PI3-K/Akt or MAPK/ERK signaling pathways in the absence of neurotrophins has been show to be enough to both sustain the survival of (187)(188)(189)(190) and enhance neurite growth fromsome neuronal populations (191).

Many studies have shown the link between PI3K/Akt pathway and cell survival downstream of growth factors, oncogenes and cell stress. Akt promotes cell survival when it blocks the function of pro-apoptotic proteins, like the Bcl-2 homology domain 3 (BH3)-only proteins, preventing them from binding to and subsequently activating pro-survival Bcl-2 protein members. Akt can also inhibit the expression of BH3-only proteins by blocking transcription factors like FOXO and p53, or even by blocking MDM2, an E3 uniquitin ligase that triggers p53 degradation. Akt activation by any of the previous routes prevents the processing of pro-caspase9, preventing apoptosis (192).

The role of the MAPK cascade in neurite outgrowth has been extensively studied in the PC12 neuronal cell line, and to a lesser extent in primary neurons.

Following differentiation of naive PC12 cells to a sympathetic neuron like cell, neurite outgrowth stimulated by NGF is not dependent on the MAPK cascade (Sano and

Kitajima, 1998). Thus, activation of the MAPK cascade appears to be a *permissive* signal involved in making naive PC12 cells *competent* to extend neurites in response to a growth factor stimulus.

Binding of NGF to TrkA results in phosphorylation of cytoplasmic tyrosine residues on the cytoplasmic domains of these receptors, and phosphorylation of these residues further activates the receptor. Therefore, MAPK/ERK, PI3-K/Akt and phospholipase C (PLC) γ1 are activated (193)(194)(195)(196).

BDNF and NT4/5 have also been shown to activate PI3-K and MAPK pathways to promote neuronal survival (184)(197)(198)(199).

1.5 Wnt Signaling

1.5.1 Introduction

In 1982, Nusse and Varmus reported the identification of a proto-oncogene *Wnt1* (originally called *Int-1*), as a signaling molecule involved in the development of mammary tumors (200). Several years later, Wnt1 and Wingless (WG), its *Drosophila melanogaster* orthologue, emerged as key morphogens that regulate the embryonic body plan (201)(202). It is now well established that Wnt proteins are important mediators of intracellular communication, and that signaling by members of the Wnt family of molecules is crucial for normal embryonic development in various systems including the nervous system.

Wnts have been shown to regulate diverse cellular processes, including cell proliferation and fate, cell polarity and movement, and programmed cell death (203). They can influence tissue organization and growth by functioning locally, in an autocrine manner, and can also act at a distance, by generating a gradient across a tissue. Studies in various developmental systems have contributed to our extensive understanding of Wnt signaling, and have shed new light on the mechanisms through which cells interpret their environment.

1.5.2 Wnt signaling pathways

Wnts are secreted glycoproteins with an unusual post-translational modification, a palmitoylation (covalent attachment of fatty acids that enhances the hydrophobicity of proteins and contributes to their membrane association.) at a conserved cysteine residue that is essential for their function. The current model proposes that Wnts signal through their Frizzled receptors (FZD), which consist of seven-pass transmembrane proteins (204). The Wnt-FZD receptor complex activates Dishevelled (DVL), a cytoplasmic scaffold protein that brings together components of the pathway for efficient transduction (205). There are many Wnts and FZD receptors in the genome of a number of species, for example, 19 *wnt* and 9 *fzd* genes have been identified in the mouse genome. Recent studies show that Wnts can also signal through the tyrosine kinase related receptor RYK (206), which interacts with FZD and DVL (207)(208). Downstream of DVL, the Wnt pathway diverges into at least three branches: the canonical or Wnt/β-catenin pathway, the planar cell polarity (PCP) pathway and the Wnt/Calcium pathway. Evidence is emerging that these main pathways also bifurcate leading to a daunting array of signaling branches in response to Wnt ligands (209)(210).

In the canonical pathway (see fig 8a), Wnts control early cell fate decisions through the regulation of gene expression by inducing β-catenin-mediated transcriptional activation. Activated DVL induces the disassembly of a complex consisting of Axin, adenomatosis polysis coli (APC), glycogen synthase kinase 3β (GSK3β) and β-catenin. In the absence of Wnts, GSK3β phosphorylates β-catenin, which targets it for degradation via the proteosome. Wnt binding to FZD receptors results in GSK3β inhibition, which leads to an increase in the levels of β-catenin in the cytoplasm. β-catenin then translocates to the nucleus and forms a complex with the T-cell specific transcription factor (TCF). This β-catenin/TCF complex than activates the transcription of target genes (211).

The interaction of the FZD proteins with their co-receptors, low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6, which are proteins containing a single transmembrane domain, is specifically required for signaling through the canonical pathway (212).

Complex regulation of LRP5 and LRP6 occurs at the cell surface that can modulate Wnt signaling through the canonical pathway. For example, LRP5/6 can interact with

Dickkopf 1 (DKK1), a secreted Wnt antagonist (213)(214) which prevents the formation of the ternary complex between Wnt, FZD and LRP, and consequently, activation of the canonical pathway (215)(216)(217). Another level of control is achieved through the interaction of DKK1 with the kringle-containing transmembrane protein 1 (kremen1) and kremen2 (218). Binding of DKK1 to kremen1 or kremen2 results in the removal of the DKK-LRP complex from the membrane and inhibition of the canonical pathway (218). LRP5/6 can also activate the canonical pathway by binding to the intracellular axis inhibitor Axin, and inducing its degradation (213)(219).

The second branch of the Wnt pathway is known as PCP pathway (see fig 8b) (220). This pathway controls the orthogonal polarity of cells within an epithelium (221), the convergent extension movements during gastrulation (222) and the orientation of cochlear cells in the inner ear (223).

In *Drosophila melanogaster*, activated FZD signals to DVL, which, in turn, activates the small GTPase RhoA and c-Jun amino (N)-terminal kinase (JNK) (221).

In vertebrates, DVL can signal through both Rho and Rac1 (another small GTPase) to regulate convergent extension movements during gastrulation (224). This role for the Rho GTPases and JNK pathways indicates that both the actin cytoskeleton and microtubules are likely to be affected by activation of the PCP pathway. However, it should be noted that despite its name, in some cases the PCP pathway may affect cell behavior without changing cell polarity (203).

The third branch of the Wnt pathway is the Wnt/Calcium pathway (see fig 8c). The Wnt/Calcium pathway induces a ventral fate while it antagonizes a dorsal fate in early *Xenopus laevis* embryos (225). This pathway also regulates cell movement during gastrulation and heart development (226)(227). Binding of specific Wnts to FZD receptors leads to DVL activation which induces calcium influx, activation of protein kinase C (PKC) and calcium/calmodulin dependent protein kinase II (CaMKII) (226)(227)(228). The role of this pathway in other developmental systems is yet to be established.

More recently, novel components of the Wnt pathway have been described. Seven-pass transmembrane receptors usually signal through trimeric G proteins (a family of proteins involved in second messenger cascades). They are so-called because of their signaling mechanism which uses the exchange of guanosine diphosphate (GDP) for

guanosine triphosphate (GTP) as a general molecular "switch" function to regulate cell processes) (229)(230). Pharmacological and overexpression studies have indicated that G proteins are involved in Wnt signaling (231)(229). Recent loss-of-function studies also showed a clear role for G proteins in Wnt signaling. Furthermore, studies in *Drosophila melanogaster* have shown that G proteins are required for the canonical and PCP pathways (230). These new findings indicate that G proteins are likely to have a crucial role in Wnt-FZD signaling in vertebrates (for detailed reviews on Wnt signaling see (232)(233)(234).

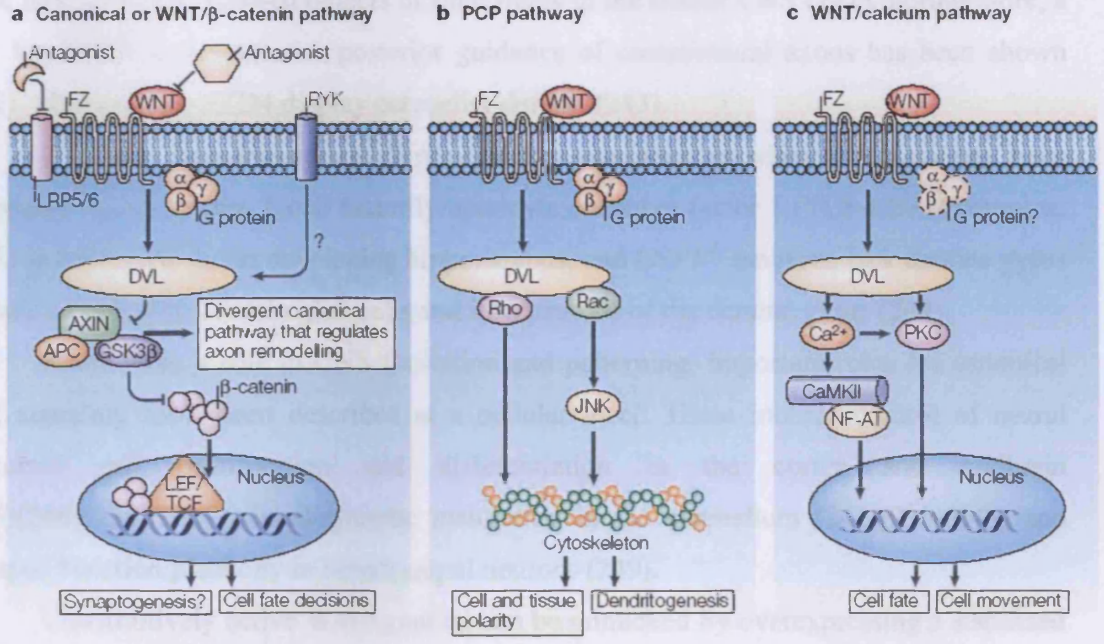


Figure 9: The three main branches of the Wnt signaling pathway. (235)

1.5.3 Wnt signaling in neuronal development

In recent years it has emerged that Wnt proteins are key regulators in embryogenesis and, in particular, during CNS development.

In mammals, the role of canonical Wnt signaling in developmental processes has been investigated principally by knocking down individual Wnt genes, FZD receptors or LRP co-receptors. Results from these studies have shown that Wnt signaling plays an important role during nervous system development. $Wnt1^{-/-}$ mice have a significant loss of neurons in the cerebellum and midbrain (235). Furthermore, in support of these findings, Wnts have been shown to be crucial for the development of midbrain dopaminergic

neurons (236). Further studies have supported these initial findings as $Wnt3a^{-/-}$ mice display significant reductions in neural crest derivatives and a loss of hippocampal neurons (237)(238). Hall and colleagues (2000) showed that $Wnt7a^{-/-}$ have a delayed maturation of synapses in the cerebellum (239). This conclusion was supported by a subsequent study by Hirabayashi and colleagues (2004) suggesting that Wnt7a may promote neural differentiation (240).

Mice lacking various FZD genes also show selective defects in CNS development. Mice lacking FZD3 showed defects in fibre tracts in the rostral CNS (241). Furthermore, a role for FZD3 in the anterior posterior guidance of commissural axons has been shown (242). Mice lacking FZD4 display cerebellar defects (243).

Amongst the downstream Wnt signaling components whose function has been addressed *in vivo*, is the T cell factor/lymphocyte enhancer factor 1 (TCF-LEF1) complex. LEF1 is expressed in the developing hippocampus, and $LEF1^{-/-}$ embryos lack dentate gyrus granule cells but do contain glial cells and interneurons of the dentate gyrus (244).

Aside from a role in CNS formation and patterning, important roles for canonical Wnt signaling have been described at a cellular level. These include control of neural precursor cell proliferation and differentiation in the cortex and midbrain (245)(246)(236)(240) axonal/synaptic maturation in the cerebellum (247)(239)(248) and synaptic function/plasticity in hippocampal neurons (249).

Constitutively active Wnt signaling can be mimicked by overexpressing a stabilized form of β-catenin, that cannot be degraded. Using this approach, the effects of constitutive Wnt signaling in neuronal precursors and in differentiated, mature neurons could be addressed. Overexpression of a stabilized form of β-catenin from the nestin promoter (a neuronal stem-cell specific promoter) demonstrated a role for canonical Wnt signaling in the proliferation of the cortical NPC pool (245). Conversely, stimulation of canonical Wnt signaling by overexpressing either a stabilized for of β-catenin or Wnt7a in maturing NPCs promotes the differentiation process (246)(240).

These data indicate that Wnt signaling can promote NPC self-renewal or NPC differentiation depending on the stage of maturation of neuronal precursors. In fully differentiated, mature neurons constitutively active Wnt signaling does not result in detectable abnormalities (250). In the cerebellum, Wnt signaling can promote axonal

remodeling and synaptic differentiation (247)(251)(239), with Wnt7a being proposed as a synaptogenic factor (251). The recently acknowledged importance of β-catenin as a modulator of synaptic strength in response to depolarization (249) has paved the way for an understanding of the role of Wnt signaling in neuronal plasticity, which is thought to be instrumental in higher brain functions such as memory and cognition. Thought this role of β-catenin in neuronal plasticity is closely associated with its role in cell to cell adhesion, there is evidence that the pool of β-catenin involved in neuronal plasticity is also controlled by Wnt signaling (252)(249)(253). Indeed, it has been shown that activation of the canonical Wnt signaling pathway causes membrane depolarization in neurons (249) suggesting that Wnts play a role in the function and plasticity of mature, differentiated neurons. Additionally, evidence exists that one outcome of canonical Wnt signaling is the stabilization of axonal microtubules (254), an event which would be consistent with the role of Wnts in the modulation of neuronal plasticity. Accumulating evidence therefore points to canonical Wnt signaling as a key player in the development and patterning of the CNS and in NPCs proliferation, in their differentiation and in the maturation, homeostasis and function in mature neurons.

Although it is clear that Wnt signaling plays as important role during the normal development of the nervous system, it has also been shown that deregulation of Wnt signaling can be associated with pathological brain conditions, such as Alzheimer's disease.

The processing of the amyloid precursor protein (APP), the precursor of the amyloid β (Aβ) peptide involved in Alzheimer's disease, can be modulated by Wnt signaling (255). Evidence also exists that implicates deregulation of canonical Wnt signaling in the ontogeny of many CNS tumors and of neurodegenerative diseases. LiCl has been well characterized as an agent capable of mimicking Wnt activity (256)(257). In fact one of the earliest, albeit indirect, pieces of evidence implicating Wnt signaling in neuronal degeneration comes from the neuroprotective activity of LiCl in virtually all models of neurodegeneration in vitro and in vivo (258). Although the effects of LiCl are complex and are not limited to inhibition of GSK3β activity (258), recent evidence indicates that at least a component of LiCl's neuroprotective effects is mediated through inhibition of GSK3β and consequent activation of the canonical Wnt pathway by β-catenin stabilization (259)(260). One proposed theory is that neurodegeneration is associated with decreased

Wnt signaling (261)(262). Support for this theory came when it was shown that overexpression of GSK3β caused neurodegeneration and neuronal death, both in vivo and in vitro (263)(264)(261). Furthermore, the neuroprotective effects of LiCl could be reproduced by addition of Wnt to the culture medium. For example Wnts protect against Aβ-mediated neurotoxicity, which is used as a model of neurodegenerative events occurring in AD (265)(266)(267).

The observation that constitutive activation of Wnt signaling in NPCs or in mature neurons is not per se sufficient to result in tumor formation, supports the notion that multiple mutagenic events are required to induce oncogenic transformation of CNS cells. The Wnt family members can be subdivided into three distinct groups based on their "transforming ability": the high transforming, the intermediately transforming and those that fail to induce transformation (268)(269). The initial theory that Wnts were only tumorigenic is no longer true as it has been reported that some Wnts are able to act as tumors suppressors or affect the signaling of others, dependent on the cell type. Liang (2003) showed that Wnt5a acts as a tumor suppressor, as $Wnt5a^{-/-}$ mice develop lymphoid malignancies (270). However data exist which contradict this claims, Weeraratna (2002) showed that increased Wnt5a expression increased the motility and invasion of melanoma cells and was correlated to higher grade tumors (271).

Howng and colleagues (2002) analyzed the expression of a panel of four Wnt genes (*wnt1*, *wnt5a*, *wnt10* and *wnt13*) in a subset of brain tumor samples (from astrocytic tumors, meningiomata and adenomata). Wnt1 was described as a highly transforming member. Wnt5a was associated with different processes: proliferation of breast tumor cells, motility and invasion of melanoma cells, a negative regulator of Wnt1 or as a tumor suppressor. Wnt10b and Wnt13 (or Wnt2b) were suggested to direct cell-growth regulation during development. The outcome of the study showed that Wnt5a, Wnt10b and Wnt13 were found in most brain tumors and Wnt1 was shown only in few (4/45). Wnt expression pattern analysis of some brain tumor cell lines showed increased Wnt14 (or Wnt9a) and Wnt10a mRNA levels (272)(273). Wnt8a and Wnt8b mRNAs were undetectable in brain tumor cell lines, but present in NT2 cells of cells were not differentiated into neuronal cells, suggesting that Wnt8a and Wnt8b might play key roles in embryonic tumor development (274).

1.5.4 Conclusions

Although much is known regarding function of Wnts and Wnt signaling during the development of the CNS, we know very little about their roles during the development of the PNS. The roles of Wnt signaling in neuronal survival and neurite growth in the CNS suggests that Wnt might possibly play roles in the development of the PNS. However, in order to identify which Wnts and cognate receptors that are relevant, I carried out screens to identify components of the Wnt family that are expressed in developing sensory and sympathetic ganglia of the PNS, in order to begin understanding these signaling pathways during PNS development.

1.6 Transforming growth factor β (TGFβ) superfamily

The transforming growth factor β (TGFβ) family of cell signaling active polypeptides have attracted much attention because their ability to control cellular functions that regulate embryo development and tissues homeostasis from nematodes to mammals (for reviews see, (275)(276). For example, in the same healing wound TGFβ may stimulate or inhibit cell proliferation depending on whether the target is a fibroblast or a keratinocyte (277). In mammary epithelial cells TGFβ will cause arrest or metastatic behavior depending on the level of oncogenic Ras activity present in the cell (278).

As a founder member of the TGFβ superfamily, TGFβ was initially discovered in the medium of transformed mouse fibroblasts (279)(280), where it inhibits tumor formation. Subsequent studies have shown that TGFβ is a tumor suppressor and a potent inhibitor of cell proliferation (for review see (281). These studies have shown that TGFβ may have potential as a therapeutic agent for cancer therapy (for reviews see (281)(282)(283).

1.6.1 Prototype of the TGFβ precursor

The TGFβ superfamily is divided into subfamilies based on sequence similarities. These subfamilies include the TGFβs (type 1-3), the bone morphogenetic proteins (BMPs), the growth differentiation factors (GDFs), the Nodals, the Activins, the Anti-Müllerian

hormone, and may other structurally related factors in vertebrates, insects and nematodes (276).

Despite the diversity of TGFβ members in the animal kingdom all members if the TGFβ superfamily are structurally related. TGFβ family members signal by binding to two types of serine-threonine kinase receptors, type I and type II receptors (284)(285). Upon ligand binding, a tetrameric complex is formed, consisting of two type I and two type II receptors: this induces phosphorylation of the cytoplasmic domain of the type I receptor by the type II receptor. This activates an intracellular signaling cascade which culminates in the activation of Smad proteins, causing them to translocate into the nucleus and affect transcription (286). BMPs can signal through any of several type II receptors and either of two type I receptors, BMPR-Ia or BMPR-Ib (287)(288).

1.6.2 Diversity of ligands

The TGFβ superfamily includes a large variety of multifunctional locally acting polypeptides. Until 2004, no less than 45 members have been identified in humans and most of them appear to have orthologous representatives in the vertebrate lineage. The initial discovery that the decapentaplegic (Dpp) locus in *Drosophila melanogaster* harbors a gene encoding a protein exhibiting significant sequence homology with vertebrate TGFβ members indicates of an early origin of this superfamily within the animal kingdom (289)(290).

The genes that encode the BMPs and the Vg-related proteins are mammalian members of a group of TGFβ-related genes, designated the DVR family, that includes the Dpp gene of *D. melanogaster* and Vg1 gene of *X .laevis*. Members of the DVR (decapentaplegic-Vg-related) family have been implicated in diverse processes during development, particularly in epithelial-mesenchymal interactions (291)

1.6.3 The DVR subfamily

The largest subfamily of the TGFβ superfamily is the DVR subfamily of related proteins. The DVR subfamily is made up of BMPs and growth differentiation factors (GDFs). These proteins play a crucial role during developmental organogenesis in

delivering positional information in both vertebrates and invertebrates. They are also involved in the development of hard as well as soft tissue (290).

1.6.3.1 Bone morphogenetic proteins

The BMP ligands are subdivided into groups, based on their structural homologies. Thus, BMP2 and BMP4 belong to the Dpp subgroup; BMP5, BMP6 and BMP7 belong to the 60A subgroup.

Although initially discovered for their ability to induce bone formation, BMPs have been found to play diverse roles in the development of many tissues, including the nervous system (292)(293), heart (294)(295), gut (296)(297) and kidney (298)(299). They are members of the TGFβ superfamily except for BMP1, which does not have the C terminal sequence of the TGFβ superfamily (276)

In the nervous system, BMPs act at different stages of neural development and in different regions of the CNS to regulate cell fate, proliferation and differentiation (287). The first involvement of BMPs in the development of the CNS is in mediating an early embryonic cell fate decision that determines whether cells will form neural or non-neural ectoderm. The CNS initially develops from the dorsomedial region of the embryonic ectoderm, a process that requires the active expression of BMP signals. Experiments in frog embryos provided the first clues to the role of BMPs in this fate decision. The dorsal side of the frog embryo normally becomes neural tissue, whereas the ventral side forms ventral tissues, such as the body wall and blood. Classic experiments by Spemann and Mangold showed that transplantation of tissue from the dorsal side of the early embryo to the ventral side caused the formation of neural tissue and suppression of non-neural fates (300)(301). Genetic studies in zebrafish and mice also highlight the importance of BMP signaling in ventral tissue formation (blood and central body wall/gut) and the suppression of BMP signaling by endogenous antagonists in the formation of neural tissue. Although the single knockouts of noggin and chordin do not produce a phenotype that is revealing in terms of the neural/ectodermal fate decision, mutations in both genes lead to the loss of anterior head structures (302). It is speculated that the loss of head structures is due to the loss of this early inductive event. Molecular embryology and genetic experiments therefore

provide convincing evidence for a contest between BMP and their antagonists in the induction of neural versus ectodermal fate (301).

Once neural induction has been completed, BMPs can influence cell fate decisions within the CNS. It has been shown that BMPs cause apoptosis in early CNS precursor cells (303), neural differentiation in midgestation CNS precursors (304)(305), and glial differentiation in late embryonic or adult CNS precursors (306).

TGFβ and BMP signaling has been implicated in retinal neurogenesis, RGC differentiation, and axonal pathfinding. The BMP receptor BMPR-Ib/Alk6 is preferentially expressed in ventral retina during embryonic development and is required for normal axon pathfinding of ventral RGCs at the optic nerve head (307). Lönn and colleagues (2005) has shown that stimulating PC12 cells with NGF plus BMP4 substantially increases neurite outgrowth compared to NGF alone (308).

Previous studies have shown that BMPs do not affect the sustained NGF-induced phosphorylation of ERK1/2 (309), considered to be a prerequisite for neural differentiation (194). A remaining question is where the NGF and BMP activated pathways converge to enhance neuronal differentiation and neurite outgrowth. An obvious possibility is that these parallel pathways cooperate to regulate transcription of effector genes participating in the differentiation process (308).

1.6.3.2 The growth differentiation factors

The growth differentiation factors (GDFs) are a subfamily of the highly conserved class of BMP molecules known to play a variety of roles in the musculoskeletal system. As discussed by Storm (2004), molecules in any given BMP subfamily share 74-92% of their mature C-terminal signaling region amino acid sequence, whereas members of different subfamilies share only 40-60%. On the basis of their high degree of homology in the signaling domains of GDF5, 6 and 7 (80-86% identity with the exception of the glycine-rich sequence of 26 amino acids in GDF7), these three molecules have been grouped together in their own subclass within BMP family (310).

Members of the BMP and GDF subfamilies play critical roles in skeletal development (311). GDF5 (also known as cartilage-derived morphogenetic protein 1 or

BMP14) is one of these molecules that has received particular interest, because naturally occurring mutations in the mouse and humans *gdf5* gene results in defects in the appendicular skeleton. Heterozygous mutations in the *gdf5* gene cause brachydactyly type C (312), characterized by underdevelopment or absence of the phalanges and metacarpals. Homozygous mutations in *gdf5* result in more severe conditions known as brachypodism in mice (310) and the chondrodysplasias Grebe type I (CGT) and Hunter-Thompson type (CHTT) in Humans (313)(314). These conditions are characterized by pronounced shortening of the skeletal elements, with more severe effects distally and the loss of one or more joints. Interestingly, although no functional GDF5 protein is made in CGT and CHTT patients, no neurological impairments have yet been reported in these patients.

In the developing chick limb, GDF5 is expressed in the condensing mesenchyme of cartilage elements prior to joint formation and within the developing joint capsule at a later stage (310)(315). GDF5 has been found to be involved in the development of skeletal elements, in part by accelerating the initial steps of chondrogenesis by increasing cell adhesion, and later in control of chondrocyte proliferation and differentiation (316)(317)(318)(319). GDF5 signals through the BMPR-Ib receptor to induce mesenchymal condensation, but other receptors are necessary for its role in differentiation and proliferation of chondrocytes. It has recently been reported that GDF5-induced chondrogenesis depends on the receptor Ror2, a tyrosine kinase receptor (320).

GDF5 has also been shown to play roles in tendon and ligament formation (321) (322), tooth formation (323), morphogenesis of joints (324) and angiogenesis (325). One potential clinical application of GDF5 may be to improve the healing of articular cartilage, which heals poorly following damage caused by, for example, arthritis (326).

1.6.4 TGFβ superfamily receptors

The TGFβ superfamily of ligands binds to and initiate signal transduction through a family of transmembrane serine/threonine kinases – the TGFβ receptor superfamily (327). Based on their structural and functional properties, the TGFβ receptor family is divided into two subfamilies consisting of type I and type II receptors. These receptors cluster separately in two distinct polyphyletic groups. Type I receptors form a group that is related to, but excludes type II, and vice-versa. Within each sub-group, receptors are clustered according

to the type of ligand they bind to. Thus, by implication, type I and type II receptors are divided in three categories: binding Activins, BMP and TGFβ (290).

1.6.4.1 Type I receptors

Type I receptors are not crucial in determining pathway-specificity in regard to ligands. Their affinity for ligand is usually relatively weak and lacks specificity. While type I receptors specifically bind ligand bound type II they also easily form heterotetracomplexes in which different subtype I receptors can aggregate together (328)(329)(330).

There are seven type I receptors designated as activin receptor-like kinases (Alks) (331). They include the type I receptors to TGFβ (Alk5 and Alk1), Activins (Alk4), several BMPs/GDFs (Alk3, Alk6 and Alk2) and Nodal (Alk7) (332)(333)(334).

1.6.4.2 Ligand specific type II receptors

The specificity of the response by TGFβ superfamily of ligands is determined by their specific binding to type II receptors. In mammals five type II receptors have been identified(286), including activin type II and type IIB (ActR2 and ActR2b, respectively) receptors, BMP type II receptor and AMH type II receptor (290).

The serine/threonine kinase domains of type II receptors is constitutively active and phosphorylate Gly-Ser (GS) domains in the type I receptors upon ligand binding leading to the activation of the type I receptor kinases (335).

During BMP signaling, ligand binding results in a hetero-multimer formation between type I and type II BMP receptors (336). Within the hetero-multimer, type II receptors then phosphorylate type I in a Gly/Ser rich domain located immediately upstream of the kinase domain. This phosphorylation leads to activation of the kinase activity of type I receptors and allows them to phosphorylate downstream targets such as Smads (336)(337).

1.6.5 The Smads

The activated type I receptor signals to a subset of intracellular protein mediators: the Smad family. Smad family members were originally identified through genetic screen on flies (*mad drosophila* mutants) and worms (*sma caenorhabditis* mutants). Subsequently, the family quickly increased to include eight mammalian orthologous counterparts, now known as the Smads (290).

After ligands and receptors, the central importance of these major components of the TGFβ superfamily signaling pathway is to convert and integrate the type I receptor phosphorylation state into gene expression and regulation (290).

Of the eight Smad proteins identified in mammals, Smad1, Smad5 and Smad8 are receptor-regulated Smads (R-Smads) activated by BMP type I receptors (BR-Smads) whereas Smad2 and Smad3 are activated by activin and TGFβ type I receptors (AR-Smads). Smad4 is the only common-partner Smad (co-Smad) in mammals that is shared by both BMP and TGFβ activin signaling pathways. Smad6 and Smad7 function as inhibitory Smads (I-Smads) (335).

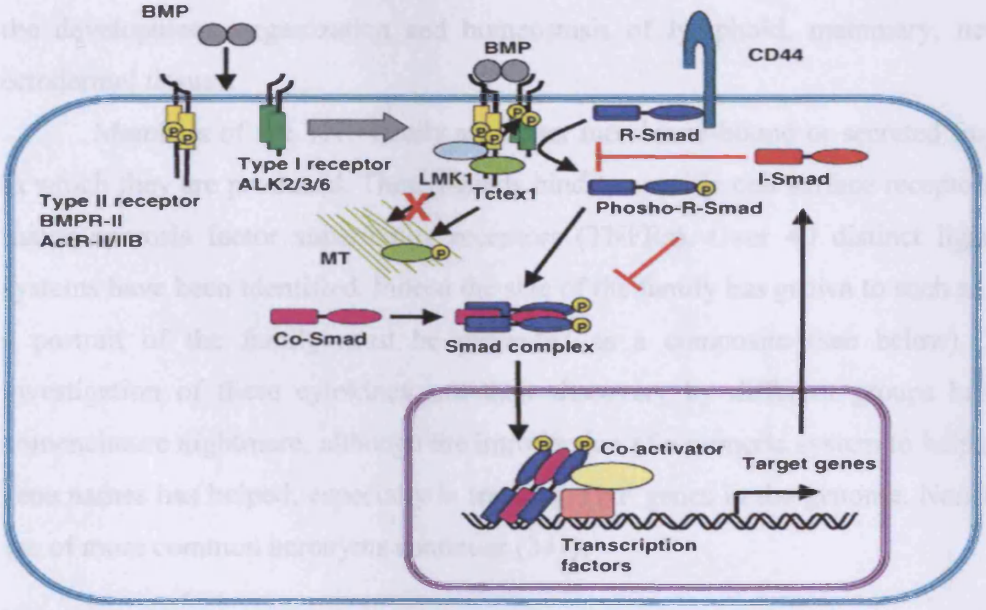


Figure 10: BMP signaling through Smad proteins. (335)

1.7 The tumor necrosis factor (TNF) superfamily

The term "tumor necrosis factor" was coined when it was shown that tumor masses which became contaminated by bacterial infection would, on occasion regress and disappear. It was thought that the bacteria were releasing a factor which made the tumor cells undergo necrosis and die. This factor was termed "tumor necrosis factor" or TNF (338). It was not until more recent advances in immunology that it became clear that antigens from the bacterial invader caused the release of the patient's own TNF, which then caused tumor regression. The hunt was on to isolate and purify this TNF, in the hope that it could be used as a "magic therapy" to control cancer growth and persistence. Furthermore, the isolation of this TNF would also provide insight into understanding the ways a cell dies. The first TNFs to be identified were TNF-α and lymphotoxin (LT). It was discovered that TNF and LT were secreted from macrophages and lymphocytes and were capable of lysing many cell types, including some tumor cells (339)(340).

Today, the TNF superfamily is a large family of related ligands which activate signaling pathways important to cell survival, death and differentiation. They orchestrate the development, organization and homeostasis of lymphoid, mammary, neuronal and ectodermal tissues.

Members of the TNF family are either membrane-bound or secreted from the cells in which they are produced. These ligands bind to specific cell surface receptors known as tumor necrosis factor superfamily receptors (TNFRs). Over 40 distinct ligand-receptor systems have been identified. Indeed the size of the family has grown to such an extent that a portrait of the family must be presented as a composite (see below). The active investigation of these cytokines and their discovery by different groups has created a nomenclature nightmare, although the introduction of a numeric system to help standardize gene names has helped, especially in tracking TNF genes in the genome. Nonetheless, the use of more common acronyms continues (341).

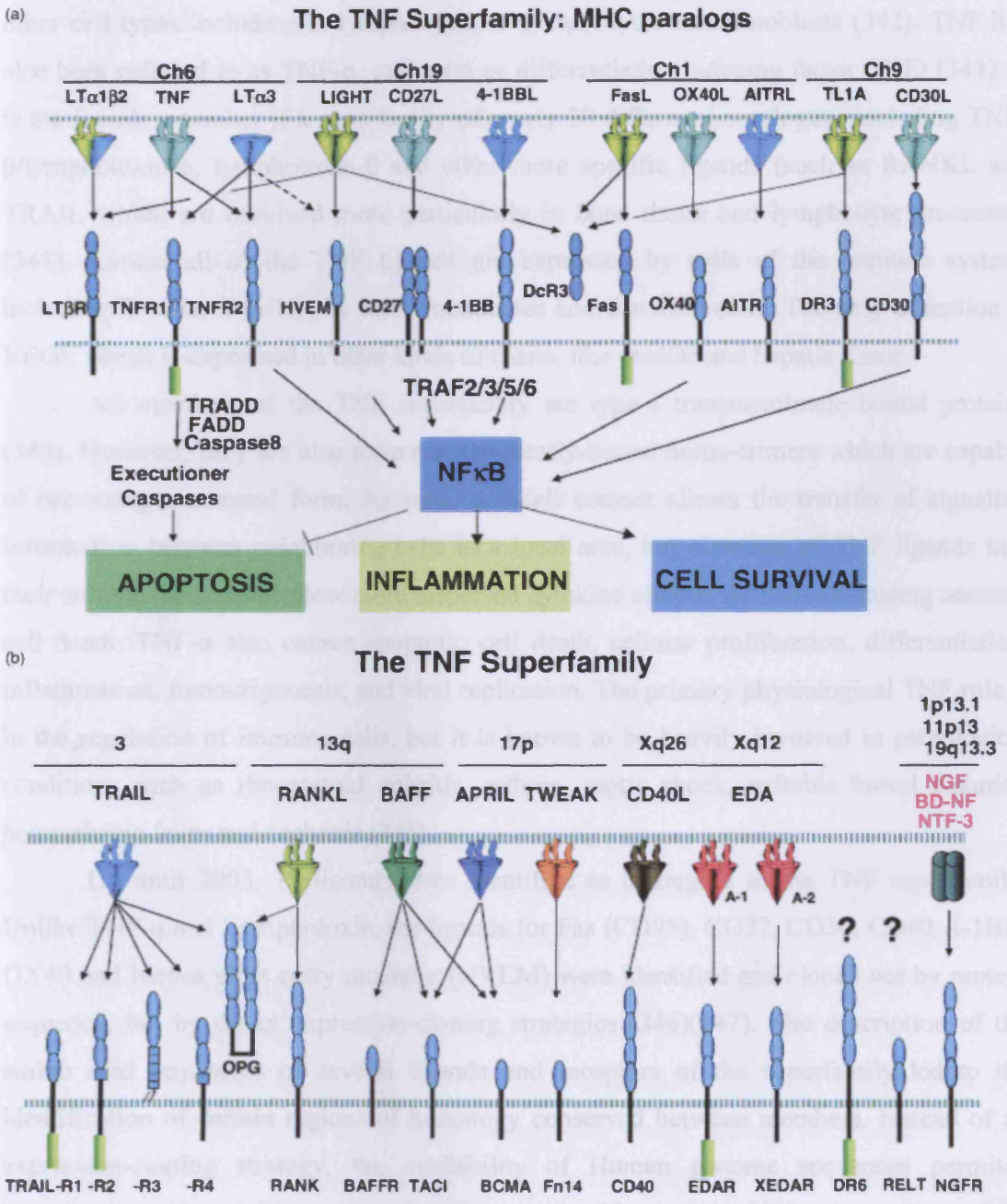


Figure 11: The TNF superfamily. (341)

1.7.1 TNF ligands

Biochemically isolated in 1984, TNF has since been found to be a pleiotropic cytokine produced mostly by activated macrophages and monocytes, but also by many

other cell types including B lymphocytes, T lymphocytes and fibroblasts (342). TNF has also been referred to as TNF-α, cachectin or differentiation inducing factor (DIF) (343). It is the founder member if a superfamily of nearly 20 different homologues including TNF-β/lymphotoxin-α, lymphotoxin-β and other more specific ligands (such as RANKL and TRAIL which are involved more particularly in bone tissue and lymphocyte processes) (344). Almost all of the TNF ligands are expressed by cells of the immune system, including B cells, T cells, NK cells, monocytes and dendritic cells. The only exception is VEGI, which is expressed in other kinds of tissue, like cardiac and hepatic tissue.

All members of the TNF superfamily are type I transmembrane bound proteins (343). However, they are also form non-covalently-bound homo-trimers which are capable of becoming a secreted form. As such, cell-cell contact allows the transfer of signaling information between neighboring cells in a local area, but cleavage of TNF ligands into their soluble forms may allow more dispersed cytokine effects. As well as causing necrotic cell death, TNF-α also causes apoptotic cell death, cellular proliferation, differentiation, inflammation, tumourigenesis, and viral replication. The primary physiological TNF role is in the regulation of immune cells, but it is known to be heavily involved in pathological conditions such as rheumatoid arthritis, asthma, septic shock, irritable bowel disorder, hemorrhagic fever and cachexia (345).

Up until 2003, 19 ligands were identified as belonging to the TNF superfamily. Unlike TNF-α and Lymphotoxin, the ligands for Fas (CD95), CD27, CD30, CD40, 4-1BB, OX40 and herpes virus entry mediator (HVEM) were identified and cloned not by protein sequence, but by direct expression-cloning strategies (346)(347). The description of the amino acid sequences of several ligands and receptors of the superfamily led to the identification of certain regions of homology conserved between members. Instead of an expression-cloning strategy, the availability of Human genome sequences permited homology searches using conserved regions to identify several additional members of the TNF superfamily. TNF-related apoptosis-inducing ligand (TRAIL) also known as APO2L, was identified independently by two different groups, followed by the identification of receptor activator of nuclear factor-kB (NF-kB) ligand (RANKL), also known as TRANCE (TNF-related activation-induced cytokine) pr osteoprotegerin ligand (OPGL) (348)(349), vascular endothelial cell-growth inhibitor (VEGI) also known as TLIA (350)(351), and B-

cell-activator factor (BAFF) (347)(352) also known as β-lymphocyte stimulator (BLYS), all by similar approaches. In many cases, because numerous groups discovered the same cytokine independently and simultaneously, numerous names exist for each TNF family member.

An intriguing feature of TNF superfamily ligands is that when certain ligands are secreted, they inhibit the function of the ligand-receptor complex. For example, whereas the membrane-bound CD95 ligand (CD95L) kills Human peripheral-blood T cells, soluble CD95L inhibits this killing effect (353). The fact that some soluble ligands act as agonists whereas others act as antagonists is intriguing. Furthermore, the transmembrane expression of most of the TNF superfamily members indicated that they are meant to act locally. Only under non-physiological conditions, when these ligands are released, do they prove harmful. Perhaps the best example is TNF-α, which when released binds to various cell types to mediate various diseases, such as brain ischemia (354), and steatohepatitis (a type of liver disease) (355).

1.7.2 TNF receptors

The TNFRs are an ever increasingly superfamily of proteins that consist of at least 41 members characterized by their extracellular sequence homology. The TNFRs are expressed by a wide variety of cells. For example, no cell type in the body as yet been found that does no express TNFR1 (also known as p55TNFR, p60, CD120a, TNFRSF1a), whereas TNFR2 (also known as p75TNFR, p80, CD120b, TNFRSF1b) is mainly expressed in endothelial cells and cells of the immune system (356)(357).

The more well known members of the TNFR family include the TNF receptors (TNFR1 and TNFR2), Fas, CD40, the low-affinity nerve growth factor receptor p75, TRAIL receptors, RANK and death decoy receptors (344)(358). Many of these receptors are known by multiple names and are activated by specific ligands. TNF-α only binds two of these receptors, TNFR1 and TNFR2. TNF-α achieves all its different physiological and pathological effects by its binding to TNFR1 and TNFR2 (359). Both, TNFR1 and TNFR2 contain an extracellular pre-ligand-binding assembly domain (PLAD) that pre-complexes receptors and encourages them to trimerize particularly upon activation by TNF ligand (360). The PLAD region is required for the assembly of TNFR complexes that bind TNF

and mediate signaling. Other members of the TNFR superfamily, including TRAIL receptor 1 (TRAILR1) and CD40, show similar homotypic associations. TNFRs and related receptors seem to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding (361).

In general, TNFR superfamily members are characterized by the presence of a cysteine-rich domain (CRD) in the extracellular portion. These include four decoy receptors known as DcR1, DcR2, DcR3 and OPG, which reduce the ligand signaling. The TNFR superfamily members are broadly classified in two groups: those containing a "death domain" in their C-terminus and those that do not. The death domain is a region of approximately 80 amino acids residues first identified independently by two different groups in the cytoplasmic domain of CD95 and the TNFRs; their deletion abolishes ligand-induced apoptosis (361).

A number of conditions have been identified which shed light on the physiological role of some of these receptors. For example, mutations in the extracellular domain of TNFR1 have been shown to suppress the secretion of the receptor leading to a family of dominantly inherited autoinflammatory syndromes (359).

1.7.3 TNF and TNFR interactions and cell signaling

As the most extensively studied member of TNF superfamily, numerous physiological roles have been assigned to TNF-α, perhaps because its receptors are expressed by all cell types. This fact might also account for the non-specific toxicity of TNF-α. Whereas most TNF superfamily ligands bind to a single receptor, others bind to more than one. For example, TRAIL binds to as many as five receptors (DR4, DR5, DcR1, DcR2 and OPG), whereas BAFF binds to three receptors: transmembrane activator and cyclophilin ligand interactor (TACI), B-cell maturation antigen (BCMA) and BAFFR. Why there are several receptors for the same ligand is unclear. Both homomeric and heteromeric complexes of DR4 and DR5 have been described leading to the recruitment of downstream cell signaling intermediates (356)(357). In the case of TRAIL, although DR4 and DR5 transduce most signals, DcR2 transduces partial signals only (362). Through sequestration of the ligand DcR1, DcR2, DcR3 and OPG have been shown to inhibit cell signaling. An

OPG variant that has two death domains, but lacks a transmembrane domain, however, has also been shown to function as a ligand through as unknown receptor (363).

After binding to the receptor, members of the TNF superfamily mediate a variety of cellular responses including apoptosis (such as TNF, LT, CD95L, TRAIL, VEGI, TWEAK and LIGHT), survival (such as RANKL and BAFF), differentiation (such as TNF, RANKL and DR6) or proliferation (such as TNF, CD27L, CD30L, CD40L, OX40L, 4-1BBL, APRIL and BAFF) through the activation of several signaling pathways involving NF-kB, JNK, p42/p44 MAPK and p38 MAPK. Signaling through TNF and CD95 has been a paradigm for most other members of the TNF superfamily. None of the receptors of the mammalian TNF superfamily has any enzymatic activity. By using the yeast two-hybrid system for protein-protein interaction, co-precipitation and dominant negative approaches, several proteins that mediate TNF signaling have been identified (fig 11) (361).

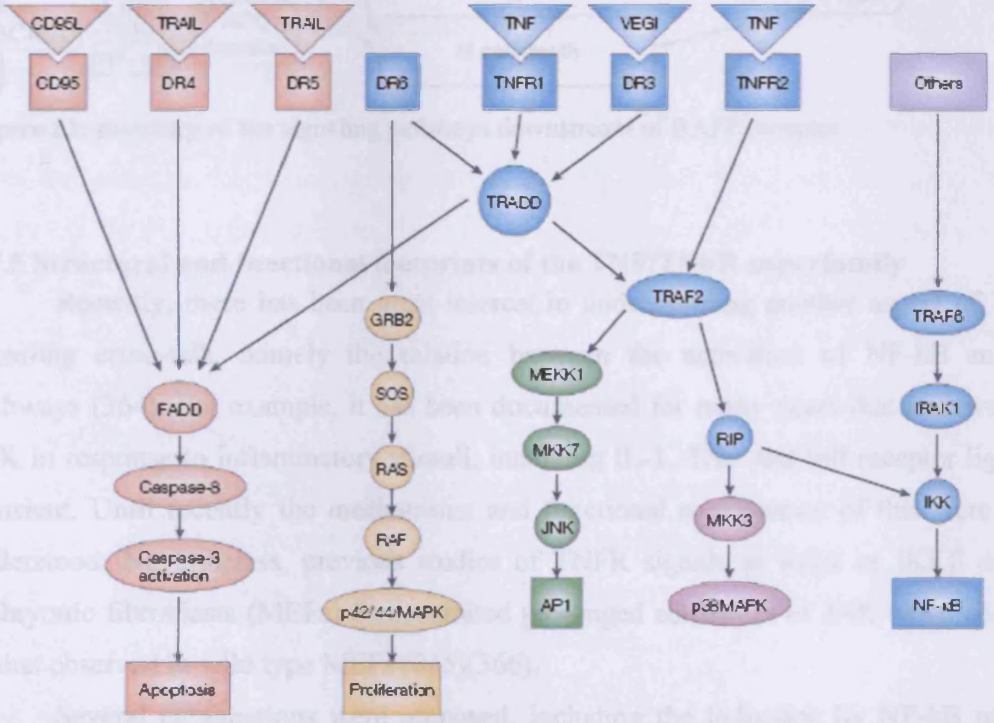


Figure 12: Cellular signaling pathways leading to activation of the main responses by members of the TNF superfamily. (361)

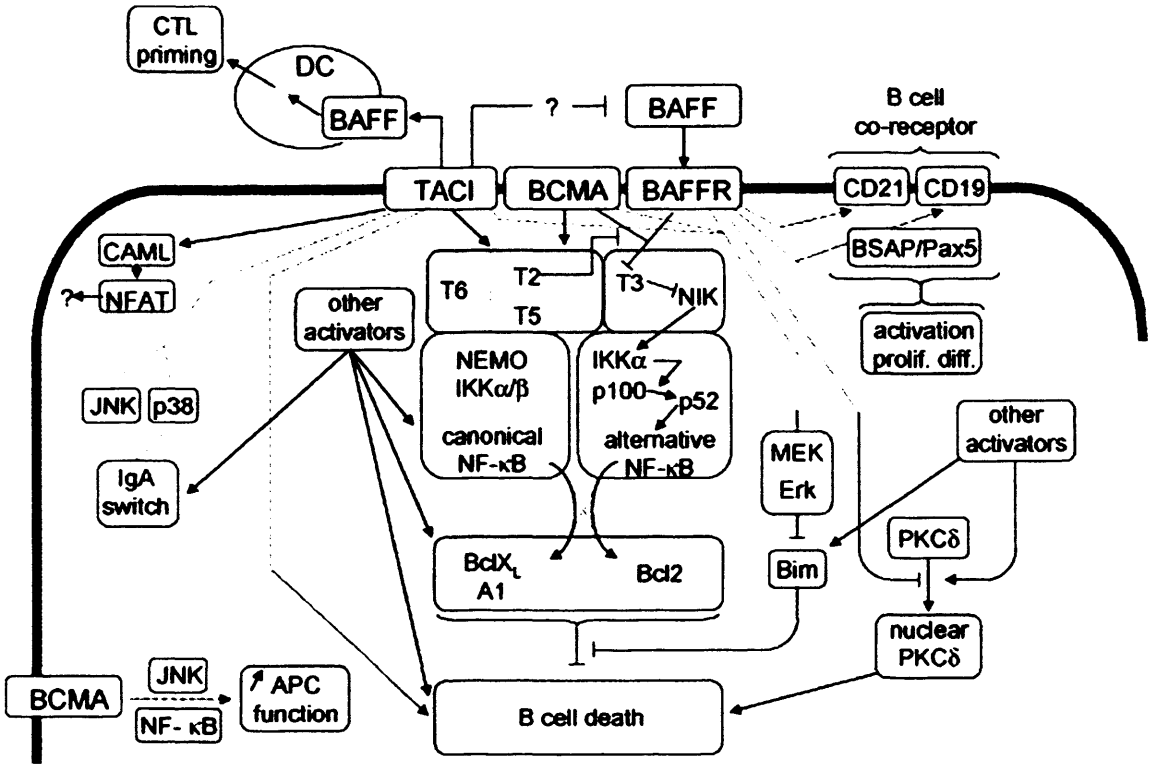


Figure 13: summary of the signaling pathways downstream of BAFF receptor.

1.7.5 Structural and functional footprints of the TNF/TNFR superfamily

Recently, there has been great interest in understanding another aspect of TNFR1 signaling cross-talk, namely the relation between the activation of NF-kB and JNK pathways (364). For example, it has been documented for many years that inactivation of JNK in response to inflammatory stimuli, including IL-1, TNF and toll receptor ligands is transient. Until recently the mechanisms and functional significance of this were poorly understood. Nevertheless, previous studies of TNFR signals in RelA or IKKβ deficient embryonic fibroblasts (MEFs) demonstrated prolonged activation of JNK when compared to that observed in wild type MEFs (365)(366).

Several explanations were proposed, including the induction by NF-kB of XIAP and GADD45b which in turn negatively regulated kinase pathways upstream of JNK; according to this model, expression of these negative regulators would be absent in RelA or IKKβ deficient cells. However, further analysis of cells deficient in XIAP and GADD45b failed to demonstrate anomalies of JNK activation (367)(368), and so alternative mechanisms were sought. Attempts to establish a relation between NF-kB activation and

the activity of specific phosphatases which regulate MAPK activation, including JNK, proved to be more fruitful. Recent published data have demonstrated unambiguously that the missing link is the induction by TNF of reactive oxygen species (ROS) (369). In NF-kB deficient cells there is a massive accumulation of hydrogen peroxide, increased oxidative stress, and inactivation of phosphatases through oxidation of a critical cysteine residue in their catalytic domain. When taken in conjunction with the interactions between NF-kB and the caspase pathway, we can now begin to think of acute and chronic TNF responses as being rather distinct at biochemical level (370).

1.7.6 Conclusions

A bewildering array of proteins interact with TNF receptors. Whether this is the tip of the iceberg is unknown, but advances in molecular biology have driven our understanding of TNF accessible signaling pathways. There is no doubt much is still to be learnt about the molecular mechanisms of TNFR1 and TNFR2, with many advances coming from studies on related receptors and ligands. The signaling processes are vastly more complicated than was first imagined. Clearly, much has been learned about TNF receptors and their signal transduction patterns since the first published reviews just over a decade ago. This is due in part to technological improvements in research, but it has also been driven by the quest to understand how TNF and its related cytokines can signal for such a range of assorted cellular actions. It is intriguing how one ligand acting at two receptors can generate such a variety of cellular responses. It is probable that the TNF receptors to not always act in exactly the same way each time they are activated. The subtle differences in expression patterns of associated machinery and the modulation of the proteins it interacts with in its immediate environment, will account for much of the observed signaling diversity in different cell types and tissues.

1.8. NF-kB signaling

NF-kB was first described as a **n**uclear **f**actor that, when activated by bacterial lipopolysaccharides binds to the enhancer region of the gene encoding the **k** light chain of antibodies in **B** cells (371). Five NF-kB family members have been cloned and characterized, c-Rel, NFkB1 (p50/p105), NFkB2 (p52/p100), RelA (p65) and RelB. All members of the NF-kB family contain a characteristic Rel homology domain (RHD)

involved in DNA binding, dimerization and interaction with inhibitory proteins called IkB (372). All NF-kB proteins form either homo or hereodimers, NF-kB being the original name for the p50-p65 heterodimer, which is the most abundant form in the nervous system. NF-kB is widely expressed throughout the nervous system (373)(374)(375)(376)(377)(378) and has been implicated in plasticity, learning and memory, development and neurodegenerative disease. p65 is located distally in dendrites and basal synaptic input has been shown to stimulate retrograde transport of p65, in association with dynein/dynactin to the nucleus (379)(380)(381). The relationship between synaptic activity and nuclear translocation of p65 implies that NF-kB may be involved in synaptic plasticity. NF-kB signaling is also regulated during development and plays a role in proliferation and migration of early neurons (377)(382)(383). NF-kB promotes the survival of cortical neurons, cerebellar granule cells and sympathetic and sensory neurons (164)(377)(384) (385)(386)(387). Inhibiting NF-kB decreased neurite growth and arborization in the neonatal somatosensory cortex and nodose ganglion (388). Increased NF-kB activity has been demonstrated in brain tissue from Alzheimer's disease patients (389)(390)(391).

Recently, NF-kB signaling has been show to inhibit as well as promote neurite growth, depending on the phosphorylation status of the p65 subunit. Phosphorylation at serine 536 confers a profound neurite growth-inhibitory effect on activated NF-kB (Gutierrez et al., 2008).

2. Material and methods

2.1 Introduction

The research described in this thesis uses a variety of *in vitro* and *in vivo* protocols to analyze neuronal survival and axonal growth in wild-type and various transgenic animals. The *in vitro* protocols developed in this lab are well established, widely used and serve as a powerful tool to investigate the mechanisms of neuronal survival and neurite outgrowth. In certain cases, *in vitro* observations were substantiated by using *in vivo* quantification of neuronal number and target field innervation density.

2.2 Mouse maintenance

Animal rights were respected according to the regulations of the Home Office Animals (Scientific Procedures) Act, ASPA, 1986.

Embryonic and postnatal wild-type mice were obtained from timed matings of CD1 mice. Adult CD1 mice were fed with rodent global diet pellets (Harlan) and given water *ad libidum.*

$GDF5^{-/-}$ mice were obtained from overnight matings of $GDF5^{+/-}$ mice (resulting from a mating between $GDF5^{-/-}$ and CD1). Breeding was confirmed by the presence of a vaginal plug and the period of gestation was considered to be embryonic day (E) 1. Animals were fed with rodent global diet pellets (Harlan) and given water *ad libitum.*

2.3 Preparation of dissection and culture media

2.3.1 Dissection media (L-15)

In an autoclaved conical flask 1L of distilled water was added to 13.90g of L-15 powder (Gibco). The flask was placed on a magnetic stirrer to dissolve all the powder, and supplemented with penicillin (60mg/L) and streptomycin (100mg/mL). pH was adjusted to 7.2 – 7.4. The supplemented L-15 medium was then filter sterilized using a 0.2μm bottle top filter (Nalgene) and stored at 4°C.

2.3.2 Washing media (F-12)

In an autoclaved conical flask 1L of distilled water was added to 10.63g of F-12 powder (Gibco). The flask was placed on a magnetic stirrer to dissolve all the powder, and filter sterilized using a 0.2μm bottle top filter (Nalgene). 225ml of filtered media was mixed with 25ml horse serum (Gibco) and supplemented with Penicillin (60mg/L) and streptomycin (100mg/L). The media was re-filtered and stored at 4°C.

2.3.3 Culture media (F-14)

In an autoclaved bottle, 500mg of Sodium Hydrogen Carbonate was added to 250ml of distilled water. The bottle was placed on a magnetic stirrer to dissolve all the powder, and 25ml were discarded and replaced with 10X stock F-14 (JRH Biosciences) solution previously supplemented with penicillin (60mg/l) and streptomycin (100mg/ml). To complete the media, 2.5ml of a 200mM glutamine (Gibco, Invitrogen) (2mM final) solution and 5.5ml of an Albumax I solution containing Albumax I (Gibco, Invitrogen), progesterone (60μg/ml), putrescine (16μg/ml), L-thyroxine (400ng/ml), sodium selenite (38ng/ml) and tri-iodothyronine (340ng/ml) (all Sigma) were added. Media was finally filter sterilized using a 0.2μm bottle top filter (Nalgene) and stored at 4°C.

2.4 Preparation of Tungsten needles

Two pieces of 0.5mm tungsten wire were placed in a needle holder (interfocus). The top 3cm of the wire were bent to a 90° angle and electrolytically sharpened in 1M KOH with a 3-12 V AC current passed through the solution until de desired shape was obtained. Tungsten needles were sterilized using ethanol and a Bunsen burner flame before and after each use.

2.5 Dissections

All instruments used for dissection were washed before use and flamed in 70% ethanol previous to use.

All dissections were carried out in a sterile environment inside a laminar flow unit which was previously wiped with 70% ethanol in order to prevent contamination.

2.5.1 Embryonic ages

Pregnant females were killed by carbon dioxide asphyxiation followed by cervical dislocation at 13, 15, and 18 days of gestation. The female abdomen was swabbed with 70% alcohol and then embryos were isolated by laparotomy and placed in a Petri dish (Greiner) containing L-15.

Embryos were carefully removed from the embryonic sac and placed in a new Petri dish (Greiner) containing fresh L-15 medium. The precise stage of development was determined by the criteria of Theiler (http://genex.hgu.mrc.ac.uk/Databases/Anatomy).

Dissection of the nodose, SCG and trigeminal ganglia was carried out using forceps and scissors (E15 and E18) followed by tungsten needles to remove adherent connective tissue; or for younger ages (E13) tungsten needles were used for the whole dissection.

2.5.2 Post-natal mice

When post-natal mice pups were required, mice were sacrificed by CO_2 asphyxiation followed by decapitation with a pair of sharp scissors.

Dissection of the peripheral ganglia was carried out using forceps and scissors followed by tungsten needles to remove adherent connective tissue.

2.6 RNA extraction

2.6.1 Background

Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments, including, RT-PCR, in vitro translation and cDNA library construction. To be successful, however, the RNA isolation

procedure should include some important steps both before and after the actual RNA purification.

This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100μg of RNA longer than 200 bases to bind to the kit silica membrane. Samples are the first lysed and homogenized in the presence of a highly denaturating guanidine-thiocynate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA.

RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analysis such as quantitative RT-PCR, such as TaqMan®, and other nucleic acid-based technologies.

2.6.2 Method

Total RNA from the unprocessed ganglia was extracted using RNeasy ® Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was measured spectrophotometrically. Briefly, Qiagen protocol involved dissociation of the ganglia in RLT buffer (provided in the kit) with 1% β-mercaptoethanol through a 26gauge needle, washes in RW1 and RPE buffer (provided in the kit), DNase1 incubation (Qiagen, UK) and elution in RNase-free water (provided in the kit).

2.7 Reverse Transcriptase

2.7.1 Background

The reverse transcriptase-polymerase chain reaction (RT-PCR) technique is a highly sensitive method for detecting and characterizing RNA molecules in small samples of cells or body fluids. It employs reverse transcription of the target RNA followed by PCR amplification of its cDNA. Widely used primers for cDNA synthesis are oligo (dT), random hexanucleotides or a sequence-specific antisense oligonucleotide primer.

2.7.2 Method

The RNA was reverse transcribed for 1 hr at 37°C with StrataScript reverse transcriptase (Stratagene) in a 40 µl reaction containing the manufacturer's buffer supplemented with 5mM dNTPs (Stratagene) and 10 µM random hexamers (Amersham).). cDNA was synthesized from 3µL of total RNA extracted from the peripheral ganglia at different stages, 4µL of Stratascript 10x buffer (Stratagene), 2µL of 100mM dNTPs (Stratagene), 2µL of random hexamers (Amersham Biosciences), 28.6µL of distilled water and 0.38µL of RT enzyme (Stratagene), and kept at 37°C for 90minutes. After this incubation period, samples were stored at -20°C until required. Together with the reverse transcription reaction of the samples, a negative control was created. This negative control contained 4µl of distilled instead of RNA, and undergone the same conditions as the samples. This negative control is also used to create the standard curve for the real time PCR.

2.8 Real time PCR:

2.8.1 Background

PCR is a method that allows logarithmic amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification.

PCR is the most sensitive method and can discriminate closely related mRNAs. It is technically simple but, it is difficult to get truly quantitative results using conventional PCR. In contrast to regular reverse transcriptase-PCR and analysis by agarose gels, real-time PCR gives quantitative results. An additional advantage of real-time PCR is the relative ease and convenience of use compared to some older methods.

The most accurate quantitative result from real time PCR is to compare the experimental sample with the reference gene. The reference gene is to be used as a loading control (or

internal standard) and should have various features, such as: having the same copy number in all cells; be expressed in all cells, and have a medium copy number, to permit more accurate comparison. The reference gene used in this study was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

GAPDH is an enzyme that catalyzes the sixth step of glycolisis and thus serves to break down glucose for energy and carbon molecules.

The amount of DNA theoretically doubles with every cycle of PCR. After each cycle, the amount of DNA is twice what it was before, so after two cycles there is 2 X 2 times as much, after 3 cycles - 2 X 2 X 2 times as much or 8 (2^3) times as much, after 4 cycles 2 X 2 X 2 X 2 times as much or 16 times (2^4) as much. Thus, after N cycles we shall have 2^N times the amount of starting template. As the reaction cannot go on forever, and it eventually tails off and reaches a plateau phase.

In most reactions the amplification in the earlier parts of the PCR cycles cannot be detected because the changes are too small. Eventually, in the last few cycles the experimental sample rises above the baseline being exponentially amplified until it reaches the plateu phase, when there is nothing left to be amplified.

In real-time PCR using SYBR green binding to amplified cDNA, we are simply measuring the fluorescence increase as the dye binds to the increasing amount of DNA in the reaction tube. We hope that this increase in fluorescence is coming from the DNA that we wish to measure but some of the signal could come from DNA other than that which we are trying to amplify. The way to make sure that the correct fragments were amplified is checking the products' melting curve.

TaqMan real-time PCR is one of the two types of quantitative PCR methods. Unlike the SYBR Green method, TaqMan uses a fluorogenic probe which is a single stranded oligonucleotide of 20-26 nucleotides and is designed to bind only the DNA sequence between the two PCR primers. Therefore only specific PCR product can generate fluorescent signal in TaqMan PCR. To do TaqMan PCR, besides reagents required for regular PCR, additional things required are two PCR primers with a preferred product size of 50-150 bp, a probe with a fluorescent reporter or fluorophore such as 6-carboxyfluorescein (FAM) and tetrachlorofluorescin (TET) and quencher such as tetramethylrhodamine (TAMRA) covalently attached to its 5' and 3' ends, respectively.

The standard curve for the real time PCR was prepared with E15 limb cDNA which was synthesized using the same method for the samples as described on section 2.7. This standard curve consisted on consecutive dilutions from 1 to 1/64 which were diluted in a "blank" RT reaction mixture (see section 2.7 for details)

Each real-time PCR reaction was prepared using 2.5µL of cDNA, 2.5µL of Brilliant QPCR core buffer (Stratagene), 1.5µL of 3mM $MgCl_2$ (Stratagene), 0.5µL of primers, 0.4µL of (1/500) reference dye (Stratagene), 0.25µL (1/1000) Sybr Green® (Invitrogen), 1µL of 20mM dNTPs (Stratagene), 15.8µL of distilled water and 0.33µL of Hot Taq® enzyme (Stratagene).

The following table shows the primer set for each gene and the respective annealing and melting temperatures.

Gene	Primer	Locus	Sequence (5' to 3')	Annealing temperature	Melting temperature
FZD1	F R	NM_021457	GCT GTC CCT GTG GCT TCC CGC TAT CGG TCG GTT ACT G	54.8°C	90.6°C
FZD2	F R	NM_020510	TCA GAG GAC GGT TAT CGC CCA GGT GAG GGA CAG AAT C	52.2°C	87.8°C
FZD3	F R	NM_021458	TTC TGA ACC ATT ATG ACC AAC ACG GGA CAC CAA ACA TCT C	52.6°C	89.0°C
FZD4	F R	NM_008055	ATG TTA TCG GAG TTT GGT CTA GCA TTC TGG AGG TTC ATT AGG	50.4°C	84.9°C
FZD5	F R	NM_022721	CAC ACC CAC TCT ACA ACA AG GGA GAT GAA GCA CAG CAC A	56.3°C	93.5°C
FZD6	F R	NM_008056	GCT ACT TTG CGA CTG TGA TAG AAC CTA CCA TCT TAC CTG CTG	52.1°C	87.0°C
FZD7	F R	NM_008057	TCG CTT CTA CCA CAG ACT CAG ACC TCC AAA TAA CTT CTC ACT TCC	55.9°C	91.5°C
FZD9	F R	XM_284144	GCT CTC TTC CAT ATC CGC AAA ATC AGC AGA CAA TGA CGC AGG TG	53.5°C	87.4°C
LRP5	F R	NM_008513	AGT TCT CAG CCC ATC CTT G CAC TCT GGT CAG CAC ACT C	57.2°C	94.5°C

LRP6	F R	NM_008514	CTA CTC CCT GAA TGC TGA CAA C GCT GTG GAT AGG AAG GAT GAT G	53.5°C	88.3°C
Ryk	F R	NM_013649	AAG TCC AAG GTT GAA TAT AAG C GAC CCG AAA CAC TGA TAA AG	50.3°C	85.7°C
Kremen1	F R	NM_032396	CAC ATA CAG ATC GGT TTC C GAT GAC ACA TGG TAG TTG G	52.5°C	89.4°C
Kremen2	F R	NM_028416	GCA TCT ATG AAG TGT CTG TGG GAA GGC ACG GAG TAG GTT G	56.2°C	93.0°C
Dkk	F R	NM_010051	GGT GTG CTA CAT TGC CTT G CCG ACT ATT CCA TTT CTC CTT AC	48.3°C	81.7°C

Gene	**Primer**	**Locus**	**Sequence (5' to 3')**	**Annealing temperature**	**Melting temperature**
Alk 3	F R	NM_009758	TGC TGT CAT CAT CTG TTG TC GGA AAT CTG CCC TGG AAT AG	49.0°C	81.8°C
Alk 6	F R	NM_007560	GCA TTA AAC ACC AGC AAA GC GCA CAT AGA AGG AAC AAG CC	51.4°C	86.2°C
BMP II	F R	NM_007561	CGC TTG GAC TCA TCT ACT G TCT GCT TCT CTC TGG ACA C	50.7°C	86.0°C
GDF 5	F R	NM_008109	GAC ACT CAG ACT GAG GAG AAA GC TCA ATG ACA CCA CAG AGA AAG GC	57.8°C	92.8°C
Alk 7	F R	XM_194020	AAG AAG AAC CGT GCG AAG G AGT GGT GAA TGG AAC AGG ATA G	48.7°C	81.8°C
Alk 5	F R	NM_009370	GCA TCT GTC TAA GTG GAA ACG AAC TTC AAG CAC ATC TGG GTA GGA G	50.1°C	82.8°C
Alk 4	F R	NM_007395	CCT GGC TGT GGG TGG TGA TTT G ACT GAA CTA TGT GCG TTT GAG TCT GG	51.1°C	82.4°C
Alk 2	F R	NM_007394	ACT GTT CAC ACC ACA AAG AC CGA AAG ATA CGC AGA GAG C	53.3°C	89.2°C
Alk 1	F R	NM_009612	GGT AGA GTG TGT GGG AAA GG CAG GAC TGC TCA TCT CGT G	53.4°C	88.7°C

ActR2b	F R	NM_007397	TGA ACG ACT TTG TGG CTG TG CCC TTG AGG TAA TCC GTG AG	55.3°C	91.4°C
ActR2a	F R	NM_007396	TTG AGT CAC TTT AAC ATC CAT AC ACA GAG ACA ACG CAC AGT	48.7°C	83.0°C
TGFβRII	F R	AF4066755	CGC CAA CAA CAT CAA CCA CAA C CCA CGG TCT CAA ACT GCT CTG	56.5°C	91.4°C
Noggin	F R	NM_008711	CGA GAT CAA AGG GCT GGA G AGA CTT GGA TGG CTT ACA CAC	57.1°C	94.0°C

Gene	Primer	Locus	Sequence (5' to 3')	Annealing temperature	Melting temperature
DR6	F R	AK0 43 823	GAT TCC AAC TCT ACT GCC TGT G GGT CCT ATT AGT GCC TTC CTT C '	51.2°C	85.1°C
LTβ-R	F R	U29173	CTG TCG CTG GTC CTC TTT CTG AAA TGT GGG TCG GCT CTT GG	56.9°C	92.4°C
Light	F R	NM_019418	CCT TCA GTG TTT GTG GTG GAT GG GCG TTG GCT CCT GTA AGA TGT G	58.5°C	93.8°C
TL1A	F R	NM_177371	CAA AGT GTC AGT CAT CAA ATC C ACT CCT TGT GGC ATC TAC C	47.9°C	81.4°C
April	F R	NM_023517	GGT GGT ATC TCG GGA AGG GCG GAG AAA GGC TAA GTT T	51.1°C	86.4°C
Tweak	F R	NM_011614	CGC TAC GAC CGC CAG ATT G GGT AGA CAG CCT TTC CCT CAT'	51.8°C	85.6°C
LTβ	F R	NM_008518	CTA CAG GAG AGG GAA GAC C CGG ACA TCA CGA TTC ACA C	51.9°C	87.5°C
BAFF	F R	NM_033622	GGG AAA GCC GTC AGC GAA AG AAG GTT GCG GTG CGT GTT G	55.3°C	89.5°C
CD30	F R	NM_009401	GAA GAA TAT GAC CTT GGA GC CCC GTG GAC AAT GGA GAG	53.7°C	90.7°C
DR5	F R	BC065141	AGT AGT GCT GCT GAT TGG AG TGT TGT GGT TAG AGT CAT TTG TC	52.9°C	88.1°C
Fas	F R	NM_007987	GAC TTC TAC TGC GAT TCT C GCG ATT TCT GGG ACT TTG	51.3°C	87.7°C
CD27	F R	XM_284241	ATA GCC AGC GGT CAT CCC ATA G GCA CCC AGG ACG AAG ATA AGA	53.5°C	86.8°C

			AAC		
4-1BB	F R	NM_011612	TCT TCA TTA CTC TCC TGT TCT CTG CTC ATA GCC TCC TCC TCC TC	51.8°C	86.3°C
Ox40	F R	X85214	GCT TGG CGT TGA CTG TGT TC GGT GGC GGG TCT GCT TTC	55.1°C	89.9°C
OPG	F R	AF053713	CCC TTG CCC TGA CCA CTC TAA CGC CCT TCC TCA CAC TC	55.3°C	90.7°C
TACI	F R	AF257673	GTG ACA GAG GCT TGC GAC CGT GTA TCC CGA GCG AAC	56.2°C	92.6°C
RELT	F R	AK088621	ATT GCC ATC GTT CCT GTC GCA TTC TCT TTC TTC TCT GTC	54.8°C	92°C
CD40	F R	M83312	GCA CCG CAG AAT CAG ACA C GCC ATC TCC ATA ACT CCA AAG C	53.7°C	88.4°C
Fn14	F R	AF156164	CTG GAG AAG ATG CCG CCG AGA ATG AAT GAA TGG ACG ACG AG	55.6°C	90.9°C
HVEM	F R	AY264405	AGT TCC TTG TGG GAG ACG AG CCG CAG GGC AGA CAC TTG	56.3°C	56.3°C
BCMA	F R	NM_011608	TGA CCA GTT CAG TGA AAG G GGG TTC ATC TTC CTC AGC	51.6°C	87.1°C
EDAR	F R	AF160502	GAC AGC AAC ACC CAC AAA G AGC GAC AGC AGA CAT AGC	54.2°C	90.1°C
XEDAR	F R	AK034909	AAG ACA GTG GAG GAG GAT TC AGG ATG GAG TTA AGT GGT TTG	50.8°C	85.7°C
TROY	F R	AK083283	TGT GTG CCC TGC GGA GAC GCT GGA GAC GGT GGA GGA G	54°C	87.5°C
mSOB	F R	AY165625	AAT CAT ACT CCT TCA ACT GTC GCC ATT ATC TTT CCC TGT G	50.7°C	86.7°C
BAFF-R	F R	Ak008142	CCT CCG CTC AAA GAA GAT G CCT CCA CTG CTG CTA TTG	56.1°C	86.8°C
DR3	F R	AF329969	CCA GGT GCT TCT AGG AGT CG' GCC AGT CTG TGG TGA GGC	55.5°C	90.8°C
TNF-α	F R	NM_013693	CCA GTC TGT GTC CTT CTA AC CCT GAC CAC TCT CCC TTT G	50°C	89.1°C

2.9 Cell culture

All *in vitro* work was carried out in a sterile laminar flow cabinet using strictly aseptic techniques. Every experiment was repeated at least 3 times with at least three separate dishes per experiment.

2.9.1 Preparation of dishes

Neurons were cultured on a laminin/poly-ornithine substratum. Dishes were prepared by adding 2ml of 500mg/L poly-DL-ornithine (Sigma) / borate solution to 35mm tissue culture dishes (Greiner) and left overnight at room temperature. The poly-ornithine solution was aspirated after 24 hours and the dishes were washed three times with sterile distilled water before being allowed to air dry in a laminar flow hood for 2 hours. 50 – 150µl of a 20mg/ml solution of laminin (Sigma) in Hank's Balance Salt Solution (HBSS) (Gibco, Invitrogen) was added to the centre of each dish. The dishes with laminin solution were then placed in an incubator at 37°C for 2 hours. After dissection and dissociation, neurons were plated onto the prepared dishes once the excess laminin had been removed by aspiration.

2.9.2 Dissection of the trigeminal, nodose and superior cervical ganglia

The trigeminal, nodose and superior cervical ganglia were removed from embryonic or postnatal pups as follows, using standard sterile technique, in a laminar flow hood.
The top of the skull and underlying forebrain was removed and the head was cut in half along the sagittal plane. The jugular foramen was opened up by deflection of the occipital bone using watchmaker's forceps, revealing the nodose and superior cervical ganglia at the mouth of the foramen. The nodose ganglion was identifiable due to its spherical appearance and prominent vagus nerve attached to its distal aspect (Davies book chapter). The SCG is a more elongated structure lying above the carotid artery, attached caudally to the sympathetic chain (Davies book chapter). The trigeminal ganglion was dissected from neonatal mice by removing the top of the skull and forebrain in a plane above the eyes and

whisker pads. Trigeminal ganglia were found bilaterally on the base of the skull and identified by their elongated appearance.

2.9.3 Dissociation of the ganglia

After dissection, ganglia were added to 950µl HBSS (Gibco, Invitrogen) containing 50µl of 0.05% trypsin (Worthington). The trypsin-HBSS mixture was then incubated at 37°C for a period of time suitable for that developmental stage as it follows:

Developmental age	Time in 0.05% trypsin (min)
E13	5
E15	10
E18	20
P0	25
P5	30

The trypsin-HBSS mixture was then aspirated and the ganglia were washed twice with 10ml of F-12 (Gibco, Invitrogen) (see appendix A) to inactivate any residual trypsin. Ganglia were then gently triturated using a fire-polished siliconized glass pipette to provide a dissociated cell suspension.

After dissociation, a sample of 10µl was analyzed under the microscope in order to estimate the required numbers of neurons that would be plated. For low density cultures, an average of 100 to 300 cells was plated per 35mm dish. Neurons were grown in defined F-14 serum-free medium (see appendix A) in 35mm tissue culture dishes in the presence of either NGF (for SCG and trigeminal neurons) or BDNF (for nodose neurons) at concentrations ranging from 0.01ng/ml to 100ng/ml where indicated. All exogenous factors were added at the time of plating.

2.9.4 Quantification of neuronal survival

To quantify the percentage neuronal survival under different experimental conditions, a standard graticule for examining each culture dish was constructed from the

base of a 900mm plastic Petri dish, where a scalpel blade was used to inscribe a 12 x 12mm^2 grid consisting of 36 squares each of 2mm^2. The numbers of cells attached to the bottom of the culture dish were counted_2 hours after plating. The number of neurons surviving in this grid was counted again 24h (and in some cases 48h) later. The number of surviving neurons in the grid at these time points was expressed as a percentage of the initial count.

2.9.5 Quantification of neurite outgrowth

Twenty four hours after plating, 3µl of the vital dye Calcein AM (Invitrogen) were added to each dish and incubated for 15min. This dye is up-taken by healthy cells and metabolised to a fluorescent dye, allowing visualisation of the cell soma and associated axonal architecture. Following this 15min incubation, the media containing calcein was removed and replaced with fresh F-14 medium. Greater than 40 images of individual neurons were taken using and inverted fluorescence microscope (Zeiss). For this, concentric digitally generated rings, 30 µm apart were centered on the cell soma, and the number of neurites intersecting each ring was counted (392)

2.10 Transfections

2.10.1 Plasmid preparation

Competent *E. coli* H107 (Promega) cells was transferred from -80°C and thawed on ice. To 100µl of these bacteria, 5 to 20ng of plasmid DNA was added in a volume of 1 to 5µl and incubated on ice for 30min. Competent *E. coli* were heat shocked at 42°C for 30sec and returned to ice for 2min before adding 800µl LB broth (see appendix A). The bacteria were set shaking at 37°C at 200rpm for 45min. 100µl of transformation reaction was subsequently spread onto selective LB/agar plates and incubated overnight at 42°C. The following day, a single colony was selected and added to 100ml LB media in a sterile bottle and incubated shaking overnight at 37°C at 200rpm in a shaking incubator. The following

day, LB containing the bacteria was then centrifuged for 10min at 6000rpm. Plasmid DNA was isolated from the resulting bacterial pellet using a plasmid midi kit (Qiagen) according to the manufacturer's protocol. The concentration of plasmid DNA was then measured spectrophotometrically.

2.10.2 Preparation of the gold microcarriers for transfection

Ballistic transfection was carried out by shooting gold microcarriers coated with plasmid DNA and YFP into dissociated neurons. Gold particle cartridges were prepared using the manufacturer's protocol (Bio-rad). Briefly, 20mg of 1.6μm gold particles were suspended in 100μl of 50mmol/l spermidine and 2μg of pYFP together with the 10μg of target plasmid or 10μg of an empty plasmid (pcDNA3.1). The gold particles were precipitated with 100μl of 2M $CaCl_2$, washed three times with 100% ethanol, ressuspended in 1.2ml of 100% ethanol, ressuspended in 1.2ml of 100% ethanol plus 0.01 mg/ml polyvinylpirrolidone and loaded into Teflon tubing. The gold particles were then thoroughly dried and stored at 4°C for up to 30 days.

2.10.3 Ballistic transfection

Gold microcarriers coated with the relevant plasmid DNA and YFP were shot into dissociated neurons using a hand-held gene gun (Helios Gene-gun, BioRad Hercules, CA USA). Approximately 1000 to 3000 neurons were plated in a 50μl droplet of defined F-14 medium in the centre of a 35 mm diameter tissue culture dish that had been pre-coated with poly-ornithine (Sigma) and laminin (Sigma). Neurons were incubated at 37°C in a humidified 5% CO_2 incubator for 2 hours to allow the cells to attach. The medium was removed from the dish just prior to transfection. The coated gold particles were shot into the cultured neurons with the gun pressurized at 200psi. A 70μm nylon mesh screen was placed between the gun and the culture to protect the cells from the shock wave. After transfection, 2ml of F-14 containing the appropriate neurotrophic factor were added and cells returned to the incubator at 37°C overnight.

2.10.4 Electroporation

Plasmids for BCMA were electroporated into dissociated neurons using a Digital Bio® microporator. Approximately 300 to 500 neurons were dissociated in 12µl of electrolytic buffer. To the dissociated neurons 1µg of YFP and 1µg of plasmid were added, and electroporated three times at 1500volts with a 10ms width. Neurons were than plated in a 50µl droplet of defined F-14 medium in the centre of a 35 mm diameter tissue culture dish that had been pre-coated with poly-ornithine (Sigma) and laminin (Sigma). Neurons were incubated at 37°C in a humidified 5% CO_2 incubator overnight.

2.11 Immunocytochemistry

To visualize protein expression in dissociated neurons, after 24 hours in culture, neurons were fixed with ice-cold methanol for 10min at room temperature. After washing with phosphate-buffered saline (PBS), cells were incubated in a PBS-based blocking buffer containing 0.1% Triton X and 5% of albumin bovine serum albumin (BSA) for 1hour at room temperature. The cells were incubated with primary antibodies diluted in the respective blocking buffer overnight at 4°C (for dilutions of antibodies see below). Cells were incubated with the appropriate secondary antibodies (anti-mouse, anti-rabbit, anti-goat and/or anti-rat conjugated to either FITC, TRITC or Cy5; Alexa Fluor 1:500) for 1 hour at room temperature, and then washed three times with PBS. To visualise all cells present in the cultures, nuclei were stained using DAPI (Invitrogen; 50ng/ml).

The following primary antibodies and dilutions were used:

Antibody name	Antibody type	Company	Dilution
BCMA	Mouse monoclonal	R&D systems	1:200
ActivinR1B	Mouse monoclonal	R&D systems	1:1000
β-III Tubulin	Mouse monoclonal	R&D systems	1:1000
Alk1	Mouse polyclonal	R&D systems	1:500
Alpha Tubulin	Rat monoclonal	Serotec	1:200
MAP2a	Mouse monoclonal	Sigma-Aldrich	1:100

Tyrosine hydroxylase (TH)	Rabbit polyclonal	Chemicon	1:250
GDF5	Mouse monoclonal	R&D systems	1:250
BMPR-IB	Mouse monoclonal	R&D systems	1:250
TACI	Mouse polyclonal	R&D systems	1:200
BAFF	Mouse polyclonal	R&D systems	1:200
April	Rabbit polyclonal	R&D systems	1:200

2.12 Histology

Histology is the study of tissue. To examine the tissue components, it is necessary to process the tissue. The steps of processing involve fixation, sectioning, and visualization.

Fixation involves processing the tissue to prevent its decomposition; cell metabolism is stopped. It is usually, done before sectioning. The most common fixative is Formalin, a 37% aqueous solution of formaldehyde, which is either used on its own or in combination with other chemicals. Formaldehyde reacts with the amino groups of proteins, and thus preserves the general structure of the cell and extracellular components.

To allow a specimen to be examined, it must be thinly sliced. Tissue can be frozen and sectioned at a low temperature, using a cryostat with a razor blade knife. The fresh or fixed tissue is frozen to a "chuck" which is placed in a holder in a refrigerated chamber (-20°C). When a wheel is turned, the holder advances towards the blade by the required width of the tissue; if 7 um thick sections are wanted, the holder moves 7 um. During this process, the holder also moves up and down, which brings the edge of the tissue down on the top of the blade.

Another way of sectioning requires a microtome. A microtome works similarly to a cryostat but does not require cold. Instead, the tissue is fixed, dehydrated and completely permeated with paraffin. It is then embedded in a paraffin block. This block is stuck to a chuck, and then placed in a holder, which is progressed by a rotating wheel.

When observing a freshly fixed section of tissue through a microscope, very little contrast is observed between adjacent regions of tissue which may have vastly different composition and function. As a result, individual cells or parts of cells are difficult or impossible to

visualize. To solve this problem, staining and imaging techniques allow different cell types, or structural or molecular components, to be preferentially visualized. Stains can also be used in combination on a single tissue sample. When a stain is dissolved in ethanol, all of the water in the tissue must be replaced by ethanol before the staining can take place.

2.12.1 Frozen sections

After dissection, the tissues were fixed for 48h in formalin at 4°C and then transferred to a 20% sucrose solution in order to cryo-protect them. Tissues were stored in sucrose at 4°C. Prior to the sectioning, tissues were embedded in OCT and let to freeze inside the cryostat (Leica). 20µm sections were cut and placed directly onto to Superfrost-plus slides, and let to dry overnight

2.12.2 Wax sections

After dissection, the tissues were fixed for up to 1 week in formalin at 4°C, and then transferred to a 10% EDTA solution for 1 week to decalcify the tissue. The 10% EDTA was changed every 3 days. To begin the embedding process, tissues were then washed twice in dH_2O for 30min then dehydrated in 75% ethanol overnight. Tissues were transferred to two 96% ethanol solutions during 15min , followed by two 100% ethanol solutions and (1/1) ethanol/chloroform solution each for 15min before being incubated in 100% chloroform overnight. Tissues were then incubated in liquid wax twice for 1 hour and new wax overnight at 58°C. Tissues were finally embedded in wax before sectioning. Each third section of 5µm was attached to slides and incubated in a 65°C oven overnight.

For cresyl fast violet (CFV) staining, slides were washed in 100% Xylene solution for 5min each, 100% ethanol for 5min, 96% ethanol for 5min, 74% ethanol for 5min, H_2O for 5min, crystal fast violet for 5min, H_2O for 5min, 74% ethanol for 5min, 2 solutions of 96% ethanol for 5min each, 2 solutions of 100% ethanol for 5min each, 2 solutions of 100% Xylene for 5min each and let to dry. The slides were mounted and coverslipped using the permanent mount DPX.

2.12.3 SCG total counts

Pieces of tissue containing the SCG from P0 and P5 GDF5$^{-/-}$ and GDF5$^{+/-}$ mice were treated according to the description on section 2.12.2. The slides were then placed under the microscope and images acquired. Images were then analyzed using a Matlab® application to predict the total number of neurons present in the SCG ganglion.

2.13 Measurement of sympathetic innervation in target tissues

SMG and Nasal mucosa from P0 and P5 GDF5$^{-/-}$ and GDF5$^{+/-}$ mice were treated according to the description on section 2.12.1. Next day the sections were blocked by incubation in PBS containing 5% horse serum (Gibco, Invitrogen) and 0.1% Triton X for 1hour at room temperature. Sections were the incubated with rabbit anti-TH polyclonal antibody (Chemicon) diluted in the blocking buffer overnight at 4°C. Following the sections were washed thoroughly in PBS to remove unbound primary antibody. Sections were then incubated in the appropriate secondary antibody for 1h at room temperature, and then washed three times with PBS. Slides were mounted in a 50% glycerol solution in PBS before being coverslipped.

Images were acquired in a confocal microscope (Zeiss), and further analyzed using Adobe Photoshop® software.

2.14 Western Blotting

2.14.1 Preparation of culture extracts

E15 Nodose ganglia were dissected and dissociated according to previous sections (2.5.1 and 2.9.2, respectively). Neurons were plated in a high density culture in a 96 well plate (Greiner) previously treated with laminin/poly-ornithine solution (section 2.9.1) and incubated overnight in the presence of Caspase Inhibitors. The following day BDNF was added to the wells for the indicated times. Culture medium was removed, 50µl of RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 10% Glycerol, 1% Triton X-100, 1mM EDTA

and 100μg/ml PMSF) were added and the cell extract was kept in ice for 1hour. Insoluble debris was removed by centrifugation t 10000rpm for 10min, and the supernatant stored in an eppendorf tube at -80°C.

2.14.2 Western Blotting

Protein extracts were analysed by sodium dodecyl sulfate (SDS) polyacrylamide gels, following the protocol of Laemmli (1970) with modifications.

A 10% or 12% SDS-polyacrylamide separating minigel with a 5% stacking gel was used (Bio-Rad, Mini-PROTEAN® 3 Cell). Equal amounts of protein extract and sample buffer were added in an eppendorf tube and boiled for 5min before being loaded in the gel. Biotinylated molecular weight markers (Amersham), diluted 1:10 according to the manufacturer's instructions were loaded onto the gels to confirm molecular weights. The gel was assembled and run for 20min at 80V followed by 45min at 140V.

Proteins were blotted to polyvinyl-idene difluoride (PVDF) membrane (Amersham). Electrophoretic transfer was carried out at a constant current of 100V for 1hour in transfer buffer according to the manufacturer's instructions.

After transfection, the membrane was blocked for 1hour in 5% w/v milk in 10mM phosphate buffered saline (PBS) with 1% tween-20 (PBS-T) at room temperature with gentle agitation on an orbital shaker.

The blocking solution was removed and the membrane rinsed twice in PBS-T before being incubated at 4°C overnight with the primary antibody (in 1% BSA in PBS-T)

The membranes were then washed (5 x 10 min) in PBS-T and incubated at room temperature in the adequate secondary antibody in 1% BSA in PBS-T for 1hour at room temperature with gentle agitation on an orbital shaker.

Following washing as above, visualization of the antibody complexes was carried out using ECL-Plus (Amersham) according to the manufacturer's instructions. ECL-plus reagent was applied to the blot for 1min, and then drained from the membrane. The membrane was sandwiched between two sheets of acetate and was placed in an autoradiography cassette and exposed to Hyperfilm™ (Amersham). The film was developed and fixed manually (Kodak GBX developing and fixing solution, Sigma). After

visualization of the antibody complexes had been completed, the blot was stripped of all primary antibodies and incubated in mouse anti-β-tubulin (1:10,000, Chemicon) in 1% BSA in PBS-T for 1hour at room temperature. The blot was then washed as described above and incubated in anti-mouse secondary antibody in 1% BSA in PBS-T for 1hour at room temperature. After washing as described above, visualization of the antibody complexes was carried out as above.

2.15 siRNA

2.15.1 Background

Small interfering RNA (siRNA) is also known as short interfering or silencing RNA. siRNA constitutes a new level of gene regulation in eukaryotic cells. It is based on the observation that the presence of double stranded RNA in a cell eliminates the expression of a gene having the same sequence, whereas expression of other unrelated genes are left undisturbed. siRNA usually consists of a 21 nucleotide long double-stranded RNA molecule. Recently, many constructs have been engineered for in vivo expression of siRNA upon transient or stable transfer into mammalian cells. In order to obtain the best possible siRNA, some the following characteristics should be considered **(1)** the double stranded nature of siRNA makes it relatively resistant to ribonucleases, even when based on natural nucleotide chemistry; **(2)** siRNAs are not immunogenic and do not trigger an immune response; **(3)** siRNA is at least 100 times more potent than conventional antisense-based drugs; **(4)** siRNA has an unprecedented high specificity for the gene that has a perfect match to the siRNA sequence.

2.15.2 Method

siRNA against BCMA was designed using ambion's online siRNA designer tool. The mRNA sequence of the gene of interest obtained from PubMed was used as key to

obtain the possible target sequences. The following sequences were chosen as the siRNA targets:

BCMA_1:AACTGCTTTGCAAAGTGTCAT;

BCMA_2:AAGCCAACTCACACTAGATAA;

BCMA_3:AACTCACACTAGATAATGAGC;

The siRNAs were than blasted in order to determine whether they shared any significance with other genes. The targets with lower known homology were used to design the respective 21 nucleotide sequence. The two single stranded sequences where then annealed to form a double-strand siRNA according to manufacturer's guidelines.

Bacteria were transformed as described in section 2.10. Next day five individual colonies where selected and the bacteria grown for 2hours in LB media. The DNA was extracted using mini-prep® Qiagen kit, and sent to be sequenced. The DNA sequenced was than analyzed using Bioedit program to assure the presence of the insert. The plasmid DNA from the selected colonies was used to transform new bacteria as described in section 2.10 and once purified, the plasmids were electroporated into neurons using procedure described in section 2.10.4

2.16 Statistical analysis

Data are presented as means ± SEM, and statistical significance was determined by student's T-test or, if there were more than two sets of data to compare, the one-way analysis of variance (ANOVA) with Fisher's post hoc test was used. The level of significance accepted was $P<0.05$. For neurite growth analysis, between 40 and 70 neurons were sampled per condition and each experiment was repeated at least three times. All other studies presented were performed on litter mate-controls and were repeated using at least three separate litters and the error calculated using the average values obtained from each repeat.

3. RT-QPCR based screening for the expression of Wnt, TGF β and TNF superfamily mRNAs in the developing PNS

Real Time Polymerase Chain Reaction (PCR) amplification of reverse transcribed mRNA (RT-PCR) has proved to be a very useful method for quantifying developmental changes in mRNA expression. Making use of this method, I screened for the expression of transcripts encoding several receptors and ligands of the Wnt, TGF-β and TNF superfamilies in developing mouse nodose and trigeminal sensory neurons and sympathetic neurons of the SCG. The aim of the work described in this chapter was to characterize the developmental expression patterns of receptors and ligands in these experimentally tractable populations of neurons in order to identify potential ligands of the Wnt, TGF-β and TNF superfamilies that may play an important role in promoting the survival of and/or target field innervation by developing neurons.

3.1 Polymerase Chain Reaction

PCR was first described in 1987 (392). In the PCR procedure, trace amounts of DNA can be quickly and repeatedly copied to produce a quantity sufficient to investigate using conventional molecular biological methods.

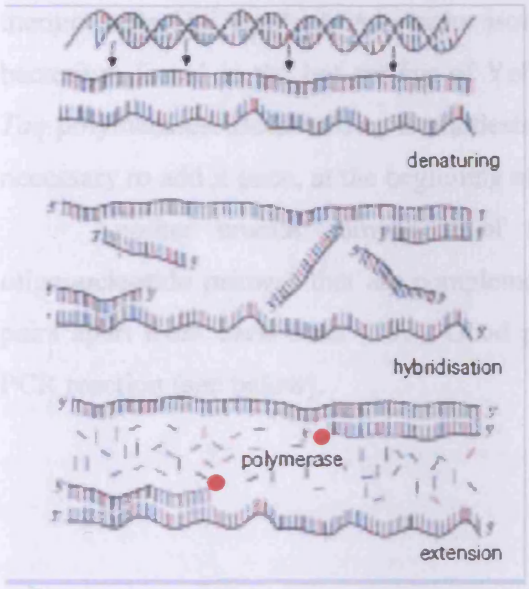
The basic principle of PCR is very simple. As the name implies, it is a chain reaction. One molecule of DNA originates two copies, then four, then eight and so forth. PCR can increase the copy number of specific nucleic acid sequences of 100 to 2000 base pairs in length by 10^6 fold in an in vitro reaction of only a few hours in duration (393).

PCR consists of repeated cycles, each of which consists of three steps: (1) the reaction mix, that contains the DNA template to be amplified as well as oligonucleotide primers, nucleotides and thermostable DNA polymerase, is heated to 95°C, leading to separation of the strands of DNA. This process is known as ***denaturation***; (2) Lowering the temperature of the reaction mix allows the primers to bind to the DNA template re-forming a short region of double stranded DNA. The polymerase then slowly begins to attach additional nucleotides to the 3' end of the primer that are complimentary in sequence to the DNA template, thereby increasing the length of double stranded DNA and thus strengthening the bond between primers and the DNA template. This process is called ***annealing***; (3) The temperature of the reaction mix increases to 72°C, the ideal temperature for DNA polymerases derived from thermophylic bacteria, and this rapidly adds further nucleotides that are complimentary in sequence to the original template DNA to the

developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments that are not fully complementary will be broken. This process is known as ***extension***.

Each of these three steps is repeated on average between 20 to 40 times per reaction. Whilst this is the basic method for a PCR reaction, each reaction is individual and may require optimization of several parameters.

(A)



(B)

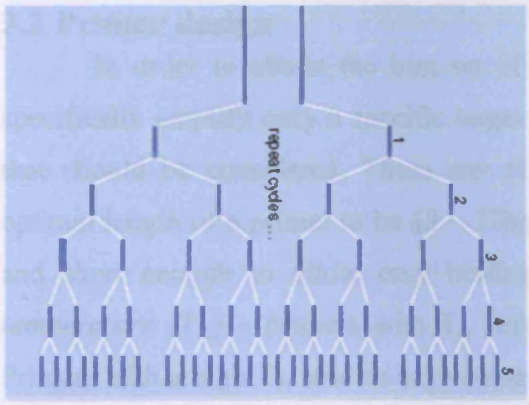


Figure 14: The PCR principle. (A) Steps required for amplification of a DNA sequence by PCR. A cycle of PCR consists of denaturation, annealing or hybridization and extension. (B) Schematic representation of the method, from one molecule of DNA template to hundreds

When the PCR method was first invented DNA polymerase had to be added freshly during the annealing step of each cycle (because the high temperature reached during extension and denaturation destroyed it), causing the reaction to be very time-consuming. In modern PCR procedures, however, thermostable DNA polymerases are used The first thermostable DNA polymerases were isolated from *Thermus aquaticus*, a thermophylic bacterium found in the hot springs of Yellowstone National Park in the USA, and called *Taq* polymerases. Because *Taq* is not destroyed by the high temperature of PCR, it is only necessary to add it once, at the beginning of the reaction (394).

Another crucial component of a PCR reaction is the design of the two oligonucleotide primers that are complementary to target sequences several hundred base pairs apart from each other (393). Good primer design is essential to the success of any PCR reaction (see below).

3.2 Primer design

In order to obtain the best set of primers, i.e., those primers that anneal to and specifically amplify only a specific target sequence, there are a number of crucial details that should be considered. These are: (1) ***primer length*** – it's generally accepted the optimal length of a primer to be 18 – 22bp. This is long enough to ensure high specificity, and short enough to allow easy binding of the primer to the template; (2) ***melting temperature*** (T_m) – primers with T_m between 52-58°C usually produce the best results. Primers with a high T_m tend to have secondary annealing, leading to non-specific products in the PCR reaction; (3) ***annealing temperature*** (T_a) – primers that require a high T_a usually lead to insufficient primer-template hybridization resulting in low PCR product yield; primers requiring a low T_a may also amplify non-specific products due to mismatches; (4) ***GC content*** – GC content should be between 40-60%; if the GC content is

too high, requires a high T_a, if the GC content is too low it leads to a low T_m; (5) ***GC clamp*** – where possible primers should be designed to include G or C bases in the last five bases from the 3'end of the primer helps to promote specific binding due to the strength of the GC bond; (6) ***secondary structures*** – secondary structures like hairpins, self dimers and cross dimers, should be avoided because they lead to poor product yield; (7) ***repeats*** – a repeat is a di-nucleotide occurring consecutive times like ATATATAT, di-nucleotides repeats should not be greater than four; (8) runs – long runs of a single base should be kept in numbers below five, as they lead to mismatches.(395)(396)(397)(398)

3.3 Real-time PCR

As previously mentioned, PCR can be used to amplify a specific DNA segment for subsequent purification and manipulation, a process known as qualitative PCR. PCR can also be used to quantify the levels of a specific template or "target" DNA/cDNA that was present in a mixed DNA/cDNA sample prior to PCR amplification, a technique known as quantitative PCR. Many protocols have been developed over the years for quantitative PCR, but presently the most flexible approach is known as real-time PCR or QPCR.

QPCR monitors the fluorescence emitted by accumulating PCR products at a set point in each PCR cycle. The intensity of fluoresence in each sample is proportional to the concentration of amplified "target" DNA present, which in turn is proportional to the pre-amplification concentration of the specific "target" DNA. In its simplest form, QPCR measures the fluorescence produced by the incorporation of intercalating fluorescent dyes into amplified double-stranded DNA. Such dyes only fluoresce strongly in the presence of an appropriate excitatory light source when they incorporate themselves into double-stranded DNA. The most commonly used intercalating dye for QPCR is SYBR Green. In some cases, to ensure higher specificity of amplification, fluorescence is produced in response to accumulating "target" DNA by incorporating dual-labeled hybridization probes or dual-labeled Molecular Beacon probes into the PCR reaction mix,. The data presented in this chapter was mostly obtained using RT-QPCR with SYBR green incorporation. One

advantage of using SYBR Green is that it is exquisitively sensitive and can detect extremely low copy number DNAs and cDNAs. Another advantage of using this dye is that only unmodified oligonucleotide primers are required for PCR amplification, which facilitates primer design and reduces cost. However, SYBR Green does not discriminate between the amplification products of the correct "target" DNA and the non-specific amplification of related sequences and/or primer artifacts. Whilst the amplification of the correct product can be verified retrospectively following the PCR reaction by melting curve analysis of PCR products, PCR reactions can require extensive optimization of several parameters, like primer concentration, annealing temperature and $MgCl_2$ concentration, in order to produce melting curves with a single sharp peak of the correct melting temperature. For some "target" DNA templates, the reliable production of such a melting curve peak proves to be impossible even after trying several primer sets. In these rare cases, I used Molecular Beacon probes to confirm the amplification of specific target DNAs during QPCR reactions.

Molecular beacons are single-stranded oligonucleotide hybridization probes (essentially they are modified primers) that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a sequence in the "target" DNA that is being amplified, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore is covalently linked to the end of one arm and a quencher molecule is covalently linked to the end of the other arm. Molecular beacons do not fluoresce when they are free in solution and the stem loop structure is intact. However, when they hybridize to a nucleic acid strand containing a sequence complementary to the probe sequence they undergo a conformational change that separates the fluorophore from the quencher molecule enabling them to fluoresce brightly. Like SYBR Green, the amount of fluorescence is directly proportional to the amount of PCR product being formed. Whilst Molecular Beacon probes are highly specific and guarantee that the correct "target" DNA sequence is being amplified and quantified, they are less sensitive than SYBR green, difficult to design and expensive.

Whichever method is used (SYBR Green or molecular beacons), QPCR remains a quantitative method for measuring the levels of cDNAs that have been reverse transcribed from mRNA. The fluorescence produced in each reaction is constantly monitored, and data

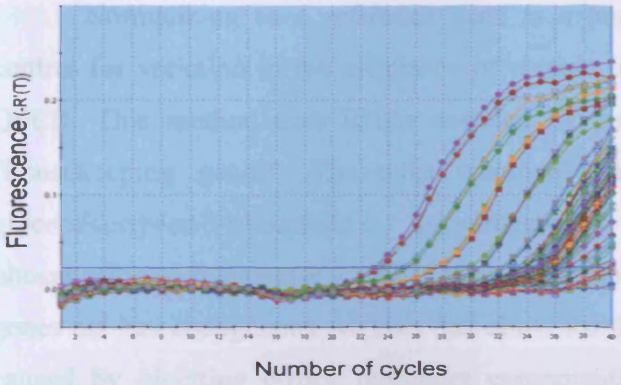
stored in a computer attached to the thermocycler. The principal behind the quantification lies in the inverse correlation between cycle number and product concentration. As a rule, the lower the cycle number required for the sample to pass threshold, the higher the initial concentration of the relevant cDNA in the sample, and vice-versa. In order to directly compare the levels of a specific reverse transcribed cDNA, I generated a dilution series or standard curve of cDNA that had been reverse transcribed from a tissue known to express high levels of the mRNA under investigation. In this way it was possible to directly compare cDNA levels between unknown samples, by comparing them to the standard curve.

Each set of QPCR reactions were set up with unknown samples and several consecutive dilutions of the standard curve cDNA (1/1, 1/4, 1/16, 1/64). For accuracy, the 1/1 standard cDNA was serially diluted using a blank solution which consisted of the same reagents as the RT reaction but with water added instead of RNA (for details see Section 2.7.2). If the standards were diluted in water, a linear regression would not be obtained, the reason being that reagents in the RT mastermix (most notably the RT enzyme and Random Hexamers) are known to inhibit the real time PCR reaction. Therefore, if the standards were diluted in water the efficiency of the reaction would increase slightly with every dilution.

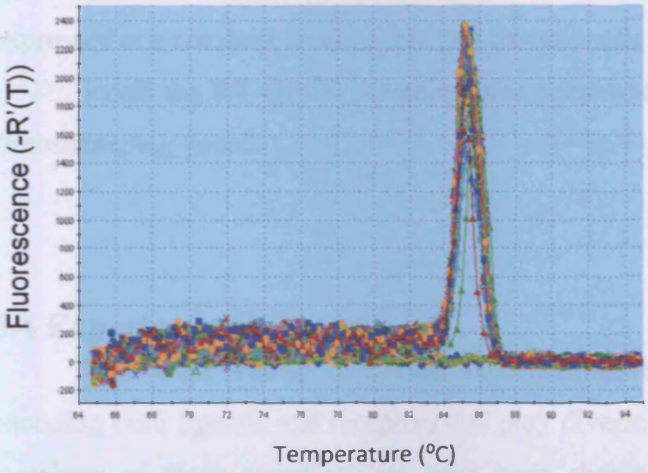
As mentioned above, all data from each real-time reaction was stored in a computer attached to the thermocycler. I used the MX3000P® thermocycler and software (Stratagene). At the end of the reaction, the MX3000P® allows the user to view several parameters of the reaction in a graphical format. These parameters include the following. (a) Amplification plots showing the fluorescence versus cycle number for each sample well. They resemble a sigmoidal curve that is easily recognized if samples have been amplified. (b) A melting curve showing the rate of change of fluorescence versus temperature, where all samples should show a sharp peak for the calculated T_m. This graph is particularly important as it allows the user to know if the amplified product is the desired one or not. Each set of primers generates a specific product with a particular T_m, so this graph should one have one sharp peak at the right temperature. (c) A standard curve

showing Ct (threshold line, above which the program starts to quantify the DNA) versus cycle number. The linear regression of the standard curve should have an RSq (R^2) value as close to 1 as possible. The coefficient of determination R^2 is the proportion of variability in a data set, in the case of linear regression, R^2 is simply the square of a correlation coefficient which indicates the strength and direction of a linear relationship between random variables. The correlation is 1 in the case of an increasing linear relationship, −1 in the case of a decreasing linear relationship, and some value in between in all other cases, indicating the degree of linear dependence between the variables. Rsq values <0.95 were deemed unsatisfactory and results were not calculated from these data sets). The efficiency of the reaction was calculated from the slope of the standard curve by the software. 100% efficiency corresponds to a doubling of the product in the end of each cycle. For most reactions efficiencies of 80-100% were obtained after optimization. In cases where reactions generated false products or primer artifacts, as determined by the melting curve, the efficiency of the reaction was often seen to be over 100%. In these cases data was rejected

(A)



(B)



(C)

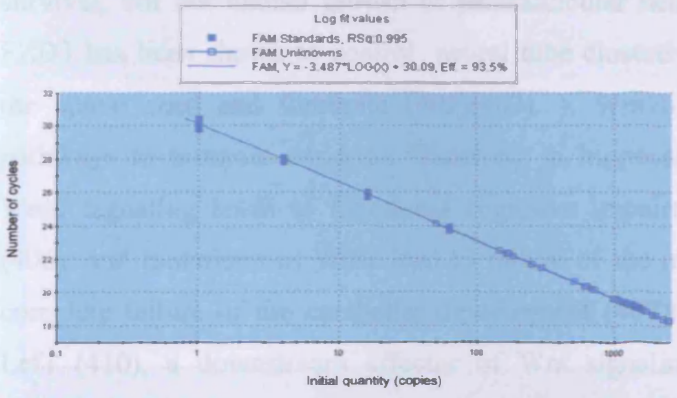


Figure 15: Real-time PCR Program graphs. (A) Amplification plots, (B) melting curve, (C) Standard curve

Normalizing to a reference gene is a popular and simple method to internally control for variation in the efficiency of reverse transcription and pipetting errors in RT-QPCR. This method uses RNAs encoded by genes that have been collectively called "housekeeping genes". The most commonly used reference genes include β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT1) and 18S ribosomal RNA (399). These housekeeping genes act has endogenous controls that allow for the correction of experimental variations caused by pipetting errors, inhibitory compounds, RT efficiency or quality of starting material (400). Due to their key roles in metabolism (GAPDH and HPRT 1), cytoskeleton (β-actin) and ribosome structure (18S ribosomal RNA) it is generally assumed that they are expressed at a constant level in different tissues, cells or experimental treatments

In all the RT-QPCR screening data presented in this chapter, I used GAPDH as a "housekeeping gene".

3.4 Experimental design

The Wnt, TGF-β and TNF superfamilies are large groups of related proteins encoding both ligands and receptors that play diverse roles in many different tissues. Some members of these superfamilies have been shown to play important roles within the nervous system. For example, the Wnt receptor, FZD5 has been implicated in promoting survival, but not axonal growth of parafascicular neurons of the thalamus (401), whilst FZD3 has been shown to control neural tube closure and axonal growth and guidance in the spinal cord and forebrain (402)(403).). Wnt7b and disheveled activate signaling pathways to promote dendritic branching in hippocampal neurons (404). Alterations in Wnt1 signaling leads to functional cognitive impairment (405) and retinal degradation (406), and mutations in Wnt1 lead to failure of the midbrain development and partial or complete failure of the cerebellar development (407)(408). Mutations in Wnt3a (409) or Lef1 (410), a downstream effector of Wnt signaling block the normal expansion of hippocampal precursors. Moreover, the phenotype of Wnt1/Wnt3a double knock-out mice shows that these two Wnts act redundantly to expand the number of dorsal neural crest precursors along the length of the spinal cord (411). Consistent with these loss-of-function

phenotypes, ectopic expression of Wnt1 in the developing spinal cord causes a local increase in cell proliferation (412).

In the TGF-β superfamily studies have shown that BMPR-Ib is an important mediator of glial differentiation and cell survival in hippocampal progenitor cells (413). Overexpression of constitutively active Alk1 induces NF-kB activation and subsequent enhanced neuroprotection in rat hippocampal neurons (414); whilst Alk5 has been reported to play an essential role in the survival of post migratory cardiac neural crest cells (415). BMP2 has been show to induce neurogenesis in neural crest stem cells (416). Although BMP4 has been shown to have no effect on promoting/reducing survival in neurons cultured from spiral ganglion, but it decreases the neurite length in the same cultures (417). BMP7 has been shown to promote dendritic growth in sympathetic neurons (418), and hippocampal neurons (419). The BMP antagonist, noggin has been shown to be able to inhibit BMP signaling and promote neuronal differentiation of subventricular zone neurons (420);

There is less known about the functions of various TNF superfamily members in the nervous system compared to Wnts and TGF-βs. TNF-α, has been the most extensively studied and has been shown to be expressed in many types of cells including glial cells and neurons. TNF-α regulates responses to NGF, promoting neural cell survival in neuroblastoma cells (421). TNF-α pre-treatment provides tolerance to ischemia (422). TNF-α increases cellular proliferation in the adult cortex (423). In contrast, a pro-death effect of TNF-α has been demonstrated in cultures embryonic sympathetic and trigeminal sensory neurons (424)

Recently GITR and its ligand GITRL have been shown to promote neurite growth from sympathetic neurons (425) and TRAIL has been shown to be expressed in macrophages and induced in neurons by β-amyloid peptide (426). Induced TRAIL may cause apoptosis of brain cells as well as virally infected cells (427). It has also been suggested that aberrant expression of CD40 and its ligand CD154 has been linked to neurodegenerative diseases such as multiple sclerosis or Alzheimer's (428).

As can be seen from above, members of Wnt, TGF-β and TNF superfamilies play multifunctional roles during nervous system development. However, the "transcriptome", for ligands and receptors of these families within the developing peripheral nervous system, i.e., those genes that are transcribed in the developing peripheral nervous system, is largely unknown. Here I have used a RT-QPCR based screening approach to document which mRNAs are expressed in the SCG, trigeminal and nodose ganglia at a number of embryonic (E13, E15, E18) and post-natal (P0, P5) ages. These studies have revealed complex patterns of gene expression for each superfamily that show ganglion-specific and age-specific differences. The developmental mRNA expression patterns that have emerged for receptors and ligands within each ganglion suggests that some members of the Wnt, TGF-β and TNF superfamilies may play important roles in regulating the development of certain neuronal populations. From this information, one member of the TNF superfamily and one member of the TGF-β superfamily were chosen, based on their expression patterns, for further functional analysis. The results of this functional analysis are reported in Chapters 4 and 5.

3.5 Results/ Discussion

In this chapter I describe a RT-QPCR based screen to determine the developmental expression patterns of the ligands and receptors that comprise the Wnt TGF-β and TNF superfamilies in the developing SCG and nodose and trigeminal sensory ganglia. This was carried out in order to identify new candidate signalling molecules involved in regulating either neuronal survival and/or the growth of neuronal processes in the developing PNS. What is clear from this screen is that the mRNA expression patterns of members of the Wnt, TNF and TGFβ superfamilies in developing ganglia of the PNS are very diverse and show distinct temporal changes in expression levels. An exhaustive discussion of the biological function of each of these receptors and ligands is beyond the scope of this thesis. Instead, I will discuss the relevance of my findings in relation to what is already known about the functions of these molecules in the nervous system. One important caveat about my data that must be born in mind when interpreting the physiological relevance of the mRNA expression patterns that I describe is that RT-QPCR cannot give information as to the site of expression of mRNAs within developing sensory and sympathetic ganglia. Wnt TGF-β and TNF superfamily mRNAs, and subsequently proteins, may be expressed in neurons, non-neuronal cells or both and the exact site of expression will dictate the biological functions of these ligands and receptors within the developing PNS.

3.5.1 Wnt superfamily

The Wnt signalling pathway plays a crucial role in cell fate determination, survival, proliferation and movement in variety of tissues. Abnormalities in the WNT signalling pathway have been implicated in a number of diseases, most notably cancer (429).

Frizzleds (FZD) are cell surface receptors for Wnt proteins that belong to a class of seven-pass transmembrane receptors (204). There are ten known members of the FZD gene family in humans and nine in mice. Little is known about the specificity or affinity of FZDs for individual WNTs but there is likely to be some redundancy because there as twice as many WNTs as FZDs (430).

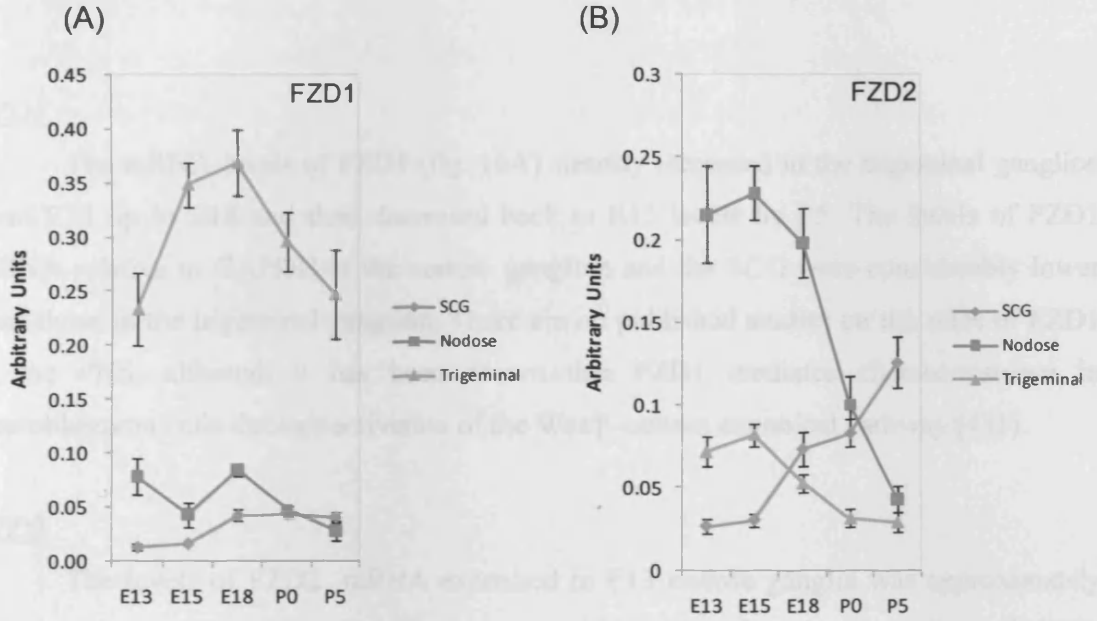


Figure 16: Expression profiles for FZD1 and FZD2. The data show the relative expression of (A) FZD1 and (B) FZD2 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown

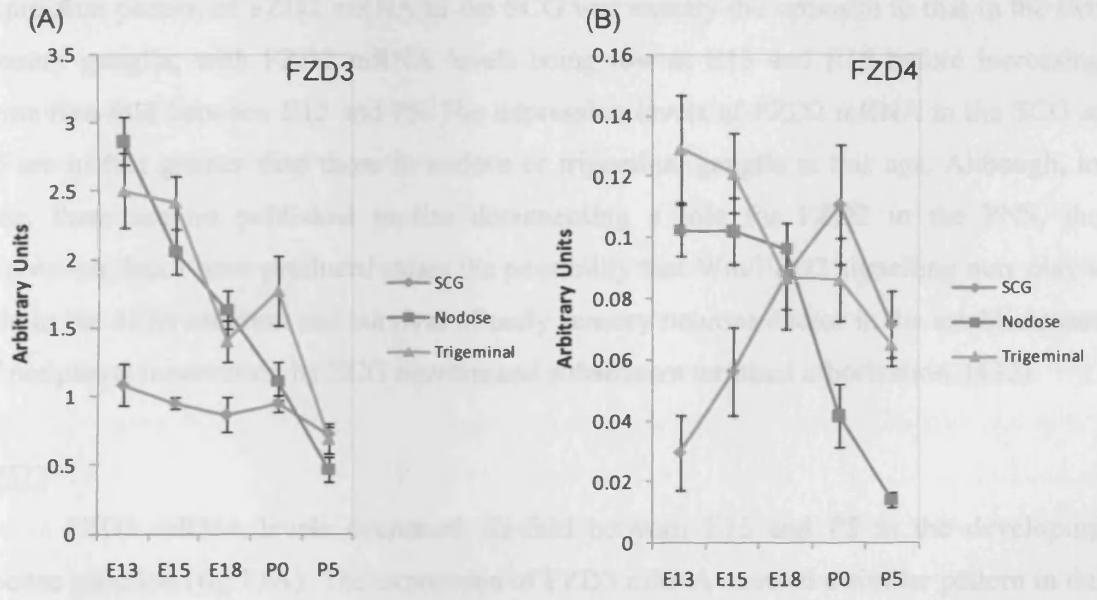


Figure 17: Expression profiles for FZD3 and FZD4. The data show the relative expression of (A) FZD3 and (B) FZD4 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown

FZD1

The mRNA levels of FZD1 (fig. 16A) steadily increased in the trigeminal ganglion from E13 up to E18 and then decreased back to E13 levels by P5. The levels of FZD1 mRNA relative to GAPDH in the nodose ganglion and the SCG were considerably lower than those in the trigeminal ganglion. There are no published studies on the roles of FZD1 in the PNS, although it has been shown that FZD1 mediates chemoresistence in neuroblastoma cells through activation of the Wnt/β-catenin canonical pathway (431).

FZD2

The levels of FZD2 mRNA expressed in E13 nodose ganglia was approximately three- and ten-fold higher than that expressed by E13 trigeminal ganglia and SCG, respectively (fig. 16B). The developmental expression pattern of FZD2 mRNA was similar in both nodose and trigeminal ganglia, with mRNA levels being highest at E13 and E15 and dropping thereafter to much lower levels by P5. Interestingly, the developmental expression pattern of FZD2 mRNA in the SCG was exactly the opposite to that in the two sensory ganglia, with FZD2 mRNA levels being low at E13 and E15 before increasing some five-fold between E15 and P5. The expression levels of FZD2 mRNA in the SCG at P5 are in fact greater than those in nodose or trigeminal ganglia at this age. Although, to date, there are no published studies documenting a role for FZD2 in the PNS, the expression data I have produced raises the possibility that Wnt/FZD2 signalling may play a role in the differentiation and survival of early sensory neurons and/or in the establishment of peripheral innervation by SCG neurons and subsequent terminal arborization. (432).

FZD3

FZD3 mRNA levels decreased six-fold between E13 and P5 in the developing nodose ganglion (fig.17A). The expression of FZD3 mRNA showed a similar pattern in the developing trigeminal ganglion, although the developmental decrease was only four-fold and was not entirely linear. In contrast, the expression of FZD3 mRNA was maintained at almost constant, relatively low levels, between E13 and P5 in the developing SCG.

Lyuksyutova and colleagues (2003) suggested that an interaction between Wnt4 and FZD3 contributes to the caudal-rostral projection of commissural axons in the ascending somato-sensory pathway (403). FZD3 is also implicated in the formation of several major axon tracts within the forebrain (402). Deardorff and Wang, (2001; 2002) have shown that FZD3 mediates Wnt signalling in the formation of the neural crest in the developing Xenopus embryo, as well as in the development of major fibre tracts in the rostral CNS (433)(402). suggesting that disruption of *frizzled 3* may result in neurodevelopmental abnormalities (434). As in the case of FZD2, my data would seem to suggest that Wnt/FZD3 signalling could play a role in regulating some aspects of early sensory neuron development.

FZD4

As in the case of FZD2 mRNA, FZD4 mRNA is expressed at the highest levels in sensory ganglia during the embryonic period and its expression decreases markedly by P5 (fig. 17B). Whilst the developmental decrease in FZD4 mRNA expression is only approximately 2-fold in the trigeminal ganglion, it is more than 5-fold in the nodose ganglion. The expression pattern of FZD4 mRNA also bears a resemblance to that of FZD2 mRNA in the developing SCG, with the lowest levels being present at E13 and expression increasing thereafter. However, whereas FZD2 mRNA expression peaked at P5 in SCG, FZD4 mRNA expression peaked at P0 and fell 30% from P0 and P5.

Loss of function studies have shown that FZD4 plays a crucial role during development. $FZD4^{-/-}$ animals present three distinct defects: (1) progressive cerebellar degeneration associated with severe ataxia, (2) absence of a skeletal muscle sheath around the lower esophagus associated with progressive esophageal distension and dysfunction, and (3) progressive deafness caused by a defect in the peripheral auditory system that is not associated with a loss of either hair cells or other auditory neurons (435). Once again, as in the case of FZD2 and FZD3, my data would seem to suggest that Wnt/FZD4 signalling could play a role in regulating some aspects of early sensory neuron development. My data also raises the possibility that Wnt/FZD4 signalling modulates target field innervation by developing late embryonic and neonatal SCG neurons.

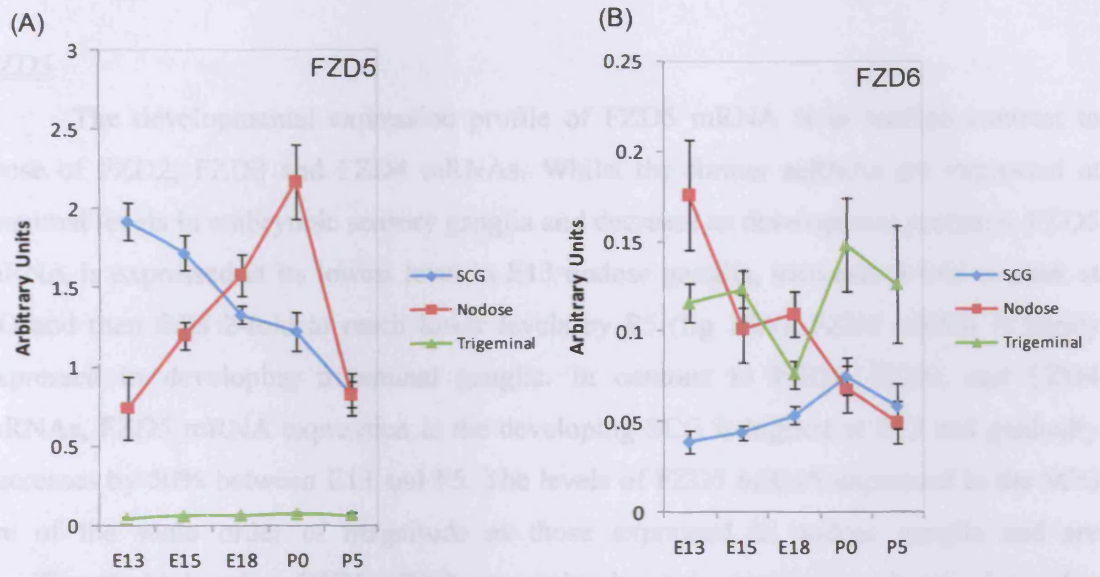


Figure 18: Expression profiles for FZD5 and FZD6. . The data show the relative expression of (A) FZD5 and (B) FZD6 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown

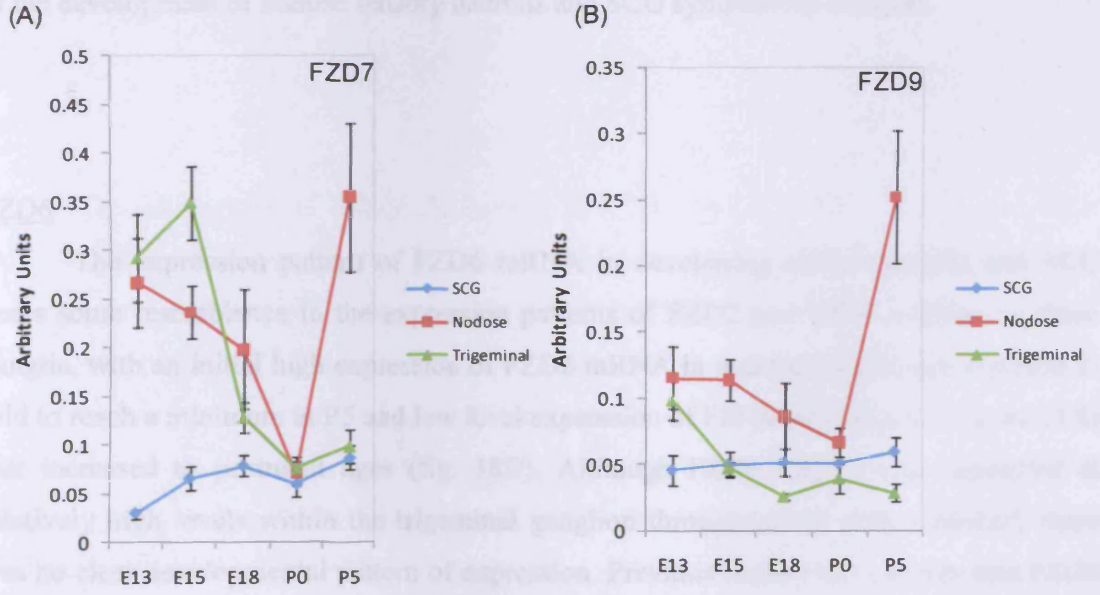


Figure 19: Expression profiles for FZD7 and FZD9. The data show the relative expression of (A) FZD7 and (B) FZD9 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown

FZD5

The developmental expression profile of FZD5 mRNA is in marked contrast to those of FZD2, FZD3 and FZD4 mRNAs. Whilst the former mRNAs are expressed at maximal levels in embryonic sensory ganglia and decrease as development proceeds, FZD5 mRNA is expressed at its lowest level in E13 nodose ganglia, increases 3-fold to peak at PO and then falls 2-fold to reach lower levels by P5 (fig 18A). FZD5 mRNA is barely expressed in developing trigeminal ganglia. In contrast to FZD1, FZD2, and FZD4 mRNAs, FZD5 mRNA expression in the developing SCG is highest at E13 and gradually decreases by 50% between E13 and P5. The levels of FZD5 mRNA expressed in the SCG are of the same order of magnitude as those expressed in nodose ganglia and are significantly higher than FZD5 mRNA expression levels in developing trigeminal ganglia. Liu and colleagues (2008) have shown that FZD5 is required continuously and in a cell autonomous manner for the survival of adult parafascicular nucleus neurons of the thalamus (436). My data is the first indication that Wnt/FZD5 signalling may mediate some aspects of the development of nodose sensory neurons and SCG sympathetic neurons.

FZD6

The expression pattern of FZD6 mRNA in developing nodose ganglia and SCG bears some resemblance to the expression patterns of FZD2 and FZD4 mRNAs in these ganglia, with an initial high expression of FZD6 mRNA in nodose ganglia that declined 3-fold to reach a minimum at P5 and low level expression of FZD6 mRNA at E13 in the SCG that increased to postnatal ages (fig. 18B). Although FZD6 mRNA was expressed at relatively high levels within the trigeminal ganglion throughout the period studied, there was no clear developmental pattern of expression. Previous studies have shown that FZD6 controls the hair patterning of the skin (437), and together with FZD3, controls neural tube closure and the planar orientation of hair bundles on a subset of auditory and vestibular sensory cells (438)

FZD7

The developmental screening results for FZD7 mRNA (fig. 19A) showed that the expression levels of this mRNA were very low in E13 SCG, but thereafter gradually increased with age to be some 3-fold higher by P5. Early embryonic trigeminal ganglia expressed significantly more FZD7 mRNA than SCG neurons of any age. After peaking at E15, the magnitude of FZD7 mRNA expression fell by 80% to P0 within this ganglia and expression was maintained at these lower levels at P5. Although the pattern of FZD7 mRNA expression was similar in nodose neurons compared to trigeminal neurons between E13 and P0, with peak levels of expression at early ages falling to much lower levels by P0, there was a dramatic 6-fold increase in the levels of FZD7 mRNA expressed within nodose ganglia between P0 and P5. Little is known about the functions of FZD7 in either the CNS or PNS. (439). However, the striking expression patterns of FZD7 mRNA expression within immature sensory neurons may indicate that Wnt/FZD7 signalling has a role to play in sensory neuron development.

FZD9

The developmental expression pattern of FZD9 mRNA within nodose ganglia is similar to that of FZD7 mRNA (fig. 19B). FZD9 mRNA levels were highest at E13 and E15, dropped between E15 and P0 and then increased greatly between P0 and P5. In trigeminal ganglia, FZD9 mRNA was also expressed at maximal levels at E13. FZD9 mRNA levels within developing trigeminal ganglia decreased approximately 3-fold between E13 and E18 and remained relatively stable thereafter. Developing SCG expressed relatively low, stable levels of FZD9 mRNA throughout the period studied. The only published literature linking FZD9 to the nervous system is a study showing its expression in hippocampus, astrocytes and dentate gyrus of adult mice (440).

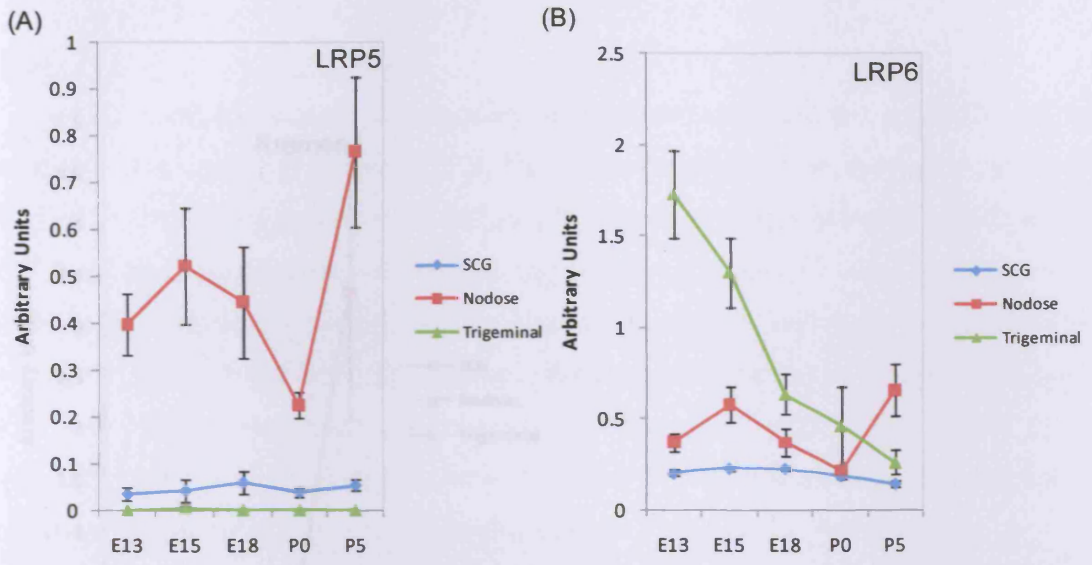


Figure 20: Expression profiles for LRP5 and LRP6. The data show the relative expression of (A) LRP5 and (B) LRP6 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

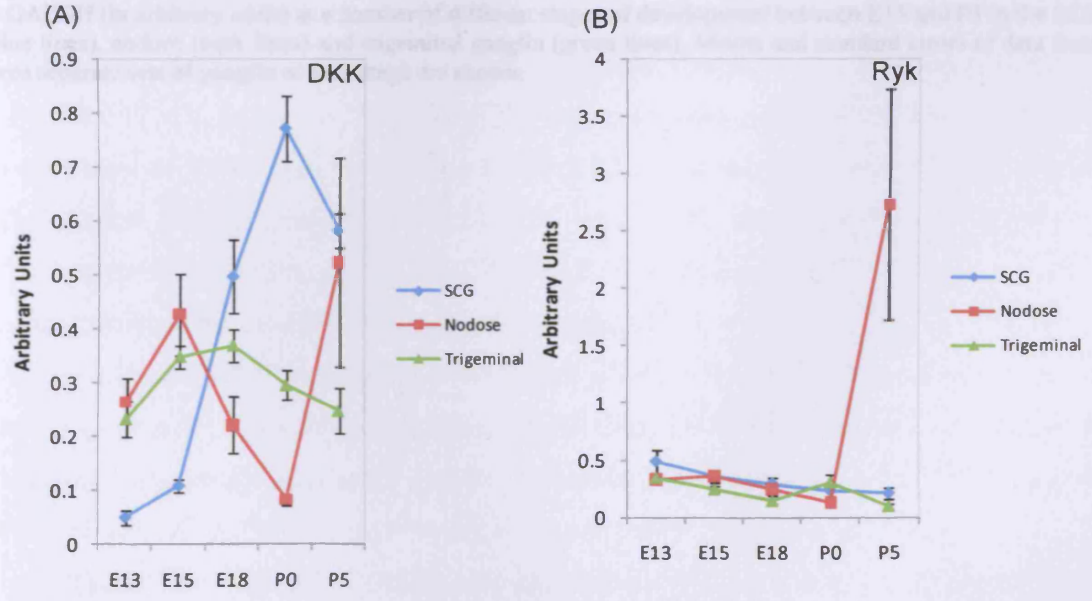


Figure 21: Expression profiles for DKK and Ryk. The data show the relative expression of (A) DKK and (B) Ryk mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown

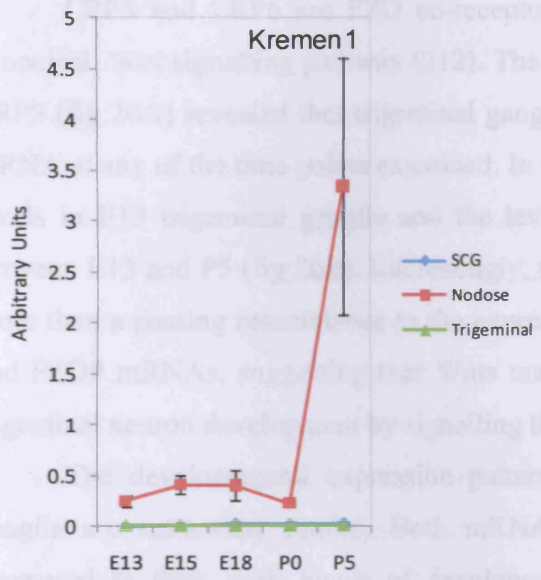


Figure 22: Expression profile of Kremen1. The data show the relative expression of Kremen1 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

LRPs

LRP5 and LRP6 are FZD co-receptors that are necessary for activation of the canonical Wnt signalling pathway (212). The developmental mRNA expression screen for LRP5 (fig 20A) revealed that trigeminal ganglia expressed barely detectable levels of this mRNA at any of the time points examined. In contrast, LRP6 mRNA was expressed at high levels in E13 trigeminal ganglia and the level of expression decreased 7-fold thereafter between E13 and P5 (fig 20B). Interestingly, the expression pattern of LRP6 mRNA bears more than a passing resemblance to the expression patterns of FZD2, FZD3, FZD4, FZD7 and FZD9 mRNAs, suggesting that Wnts may play a role in regulating some aspects of trigeminal neuron development by signalling through the canonical Wnt pathway.

The developmental expression patterns of LRP5 and LRP6 mRNAs in nodose ganglia are somewhat similar. Both mRNAs are expressed at relatively high levels, compared to their peak levels of developmental expression at E13 and both show a moderate increase in expression between E13 and E15. LRP5 and LRP6 mRNA expression levels within nodose ganglia decrease by approximately 60% between E15 and P0 and the levels of both mRNAs increase substantially between P0 and P5. The relatively high levels of LRP5 and LRP6 mRNAs between E13 and E18 raise the possibility that Wnts can signal via FZD2, FZD3, FZD4 or FZD6 down the canonical pathway to affect nodose neurons development at these ages. LRP5 and LRP6 mRNA expression patterns within nodose ganglia most closely resemble those of FZD7 and FZD9, suggesting that complexes of Wnts and these two FZDs along with LRP5 or LRP6 may play a role in modulating nodose neuron development throughout the period investigated.

LRP5 and LRP6 mRNAs were expressed at relatively low, approximately constant, levels between E13 and P5 in developing SCG (figs. 20 A and 20B), suggesting that if Wnt/FZD signalling does play a role in regulating some aspects of SCG neuron development, it is unlikely to involve signalling via the canonical Wnt pathway.

Tamai and colleagues (2000) reported that in *Xenopus* embryos, LRP6 activated Wnt-FZD signalling, and induced Wnt responsive genes, dorsal axis duplication and neural crest formation (215). In addition, a dominant-negative LRP6 mutant lacking the carboxyl intracellular domain blocked Wnt/FZD signalling, but not Dishevelled or β-catenin

activation, and inhibited dorsal axis duplication and neural crest development (215). These authors concluded that LRP6 functions upstream of DVL in Wnt-responding cells and synergizes with either Wnts or FZDs to form a ternary Wnt/FZD/LRP6 functional signalling complex.

DKK1

Dickkopf 1 (DKK1) is a secreted ligand for LRP5 and LRP6 that, by inhibiting the formation of a ternary complex between Wnt, FZD and LRP, prevents activation of the canonical Wnt signalling pathway (214)(218)(441). but not other Wnt based signalling pathways (216)(441). In addition to LRPs, Dkk1 interacts with another recently identified receptor class, the transmembrane proteins Kremen1 and Kremen2, to synergistically inhibit LRP6 (218)(442). Kremen proteins and Dkk1 interact to regulate anteroposterior CNS patterning and this interaction is necessary for anterior neural development (442).

DKK1 mRNA shows a striking expression pattern within developing SCG (fig. 21A). Like FZD2, FZD4, FZD6 and FZD7 mRNAs, DKK1 was expressed at its lowest levels in E13 SCG and the level of expression increased as SCG aged. The increase in DKK1 mRNA expression levels between E13 and P0 was of the order of 15-fold, suggesting that DKK1 has an important role in the developing SCG over this time, a period when SCG neurons are dependent on neurotrophic factors for survival and are innervating their peripheral targets. In accordance with the expression patterns of LRP5 and LRP6 mRNAs within the developing SCG, my data on DKK1 mRNA expression suggests that if Wnts do interact with FZD2, FZD4, FZD6 and FZD7 to modulate the development of SCG neurons in the perinatal period, then it is unlikely that signalling is via the canonical Wnt signalling pathway. The expression of DKK1 mRNA within the developing trigeminal ganglion resembled that of FZD1 mRNA with relatively high levels of mRNA at E15 increasing by around 50% to E18 before gradually falling back down to E13 levels by P5 (fig. 21A). DKK1 mRNA expression in the developing nodose ganglion showed a similar pattern to LRP5 mRNA expression with a modest increase in expression levels between E13 and E15, a decrease in the levels of expression between E15 and P0 and a significant

increase in the expression of DKK1 mRNA between P0 and P5. In the case of DKK1 mRNA this postnatal increase in expression was approximately 7-fold. The similarities between LRP5, LRP6 and DKK1 mRNA expression patterns suggests that Wnt/FZD signalling via the canonical pathway is under tight developmental control in the nodose ganglion.

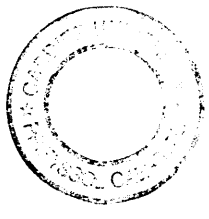
Ryk

Ryk (related to tyrosine (Y) kinase), is a type I transmembrane glycoprotein that binds Wnt and forms a Wnt receptor complex with frizzled. RT-QPCR analysis of Ryk mRNA expression revealed that the levels of this mRNA were extremely low and relatively constant in developing SCG and trigeminal ganglia over the period analyzed (fig. 21B). Whilst this was also generally the case in the developing nodose ganglion, there was a dramatic, approximately 20-fold, increase in Ryk mRNA expression within the nodose ganglion between P0 and P5

Kamitory and colleagues (1999) reported Ryk's distribution in the developing cerebral cortex of rats, and the results of that study showed that Ryk is highly expressed in neural progenitor cells in the ventricular zone and in mature neurons in the cortical plate, however, hardly any expression was detected in migrating neurons in the intermediate zone (443)(444). Furthermore, silencing Ryk results in misrouting of cranial motor nerves, suggesting that Ryk can contribute to Wnt-mediated axon guidance in vertebrates (445). Keeble (2006) showed that Ryk is a key guidance receptor in the establishment of the corpus callosum, and that Wnt5 is a chemorepulsive ligand for Ryk (446).

Kremen1

Kringle containing transmembrane protein 1 (Kremen 1), is a high affinity receptor for DKK1 and cooperates with it to inhibit LRP signaling and the canonical Wnt signaling pathway (218)(442). In fact, it has been suggested that Kremen 1 acts as a gatekeeper for



activation of this Wnt/β-catenin pathway (447). Kremen 1 mRNA was virtually undectable in developing SCG and trigeminal neurons at any of the ages studied (fig. 22). Although Kremen 1 mRNA was expressed at low, constant levels in the nodose ganglion between E13 and P0, it increased dramatically by around 20-fold between P0 and P5 in a similar manner to mRNAs of the other regulators of Wnt/FZD signaling efficacy, LRP5, DKK1 and Ryk. The large increase in expression of FZD7, FZD9, LRP5, DKK1, Ryk and Kremen 1 mRNAs in nodose ganglia between P0 and P5 strongly suggests that either FZD7/Wnt and/or FZD9/Wnt signalling is playing an important role in the developing nodose ganglion at this time.

3.5.2 TGF-β superfamily

TGFβ superfamily ligands include the TGFβ, bone morphogenetic protein (BMP), growth and differentiation factor (GDF) and nodal-related families. These families play a pleiotropic role in vertebrate development, influencing diverse cellular functions in many cell systems, including cell specification, differentiation, proliferation, patterning, and migration (448). These functions require the tight control of ligand production, ensuring a highly ordered spatio-temporal distribution and specific activation, via receptor complexes of particular intracellular signalling pathways (449).

In this chapter I report the initial real time PCR screen for the expression of a large number of TGFβ receptor superfamily members and selected ligands in the trigeminal, nodose and SCG ganglia at stages of development ranging from E13 to P5.

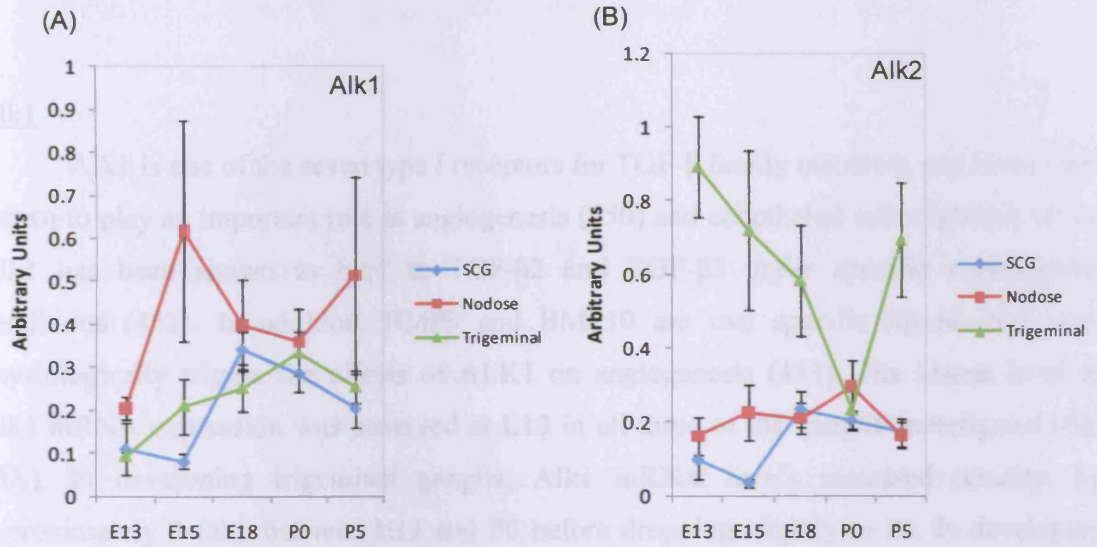


Figure 23: Expression profiles of Alk1 and Alk2. The data show the relative expression of Alk1 and Alk2 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

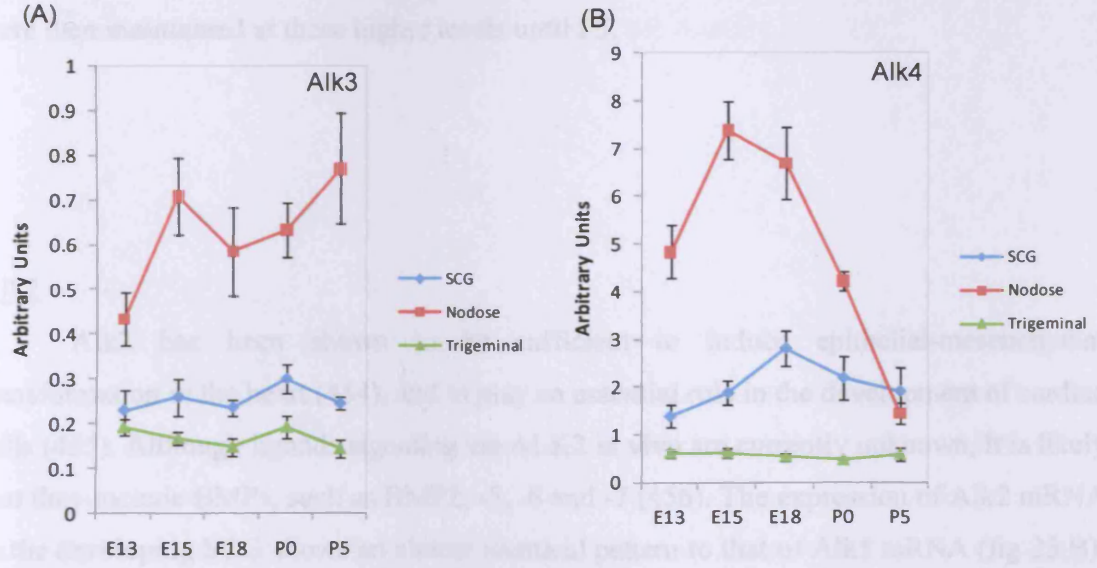


Figure 24: Expression profiles of Alk3 and Alk4. The data show the relative expression of Alk3 and Alk4 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

Alk1

Alk1 is one of the seven type I receptors for TGF-β family members, and it has been shown to play an important role in angiogenesis (450) and endothelial cell migration (451). Alk1 has been shown to bind to TGF-β2 and TGF-β3 under specific experimental conditions (452). In addition, BMP9 and BMP10 are two specific ligands that may physiologically trigger the effects of ALK1 on angiogenesis (453). The lowest level of Alk1 mRNA expression was observed at E13 in all three of the ganglia investigated (fig. 23A). In developing trigeminal ganglia, Alk1 mRNA levels increased steadily, by approximately 3-fold, between E13 and P0 before dropping slightly to P5. In developing SCG, Alk1 mRNA levels remained steady between E13 and E15, increased 3-fold between E15 and E18 and then dropped by approximately 30% between E18 and P5. The expression pattern of Alk1 mRNA within developing nodose ganglia was a little erratic with large standard errors of the mean. In general, Alk1 mRNA levels increased from E13 to E15 and were then maintained at these higher levels until P5.

Alk2

Alk2 has been shown to be sufficient to induce epithelial-mesenchymal transformation in the heart (454), and to play an essential role in the development of cardiac cells (455). Although ligands signaling via ALK2 in vivo are currently unknown, it is likely that they include BMPs, such as BMP2, -5, -6 and -7 (456). The expression of Alk2 mRNA in the developing SCG shows an almost identical pattern to that of Alk1 mRNA (fig 23 B). Alk2 mRNA is expressed at low, approximately constant, levels within developing nodose ganglia. In developing trigeminal ganglia, Alk2 mRNA is maximally expressed at E13 and levels fall approximately 4-fold from this age to P0. Postnatally, there is an increase in the

levels of Alk2 mRNA expressed within trigeminal ganglia, so that by P5 there is approximately 3-fold more Alk2 mRNA expressed than at P0.

Alk3

Alk3 has been shown to be the receptor for BMP2 (457), BMP4 (458), BMP7 (459), weakly to BMP6 (460) and GDF5 (461) with low affinity. The results of the developmental RT-QPCR screen for Alk3 mRNA revealed that this mRNA showed an almost identical pattern of expression in developing trigeminal ganglia and SCG (fig 24A). In both cases, Alk3 mRNA levels were relatively low and unchanged throughout the developmental period analyzed. Alk3 mRNA was expressed at significantly higher levels in the developing nodose ganglion compared to trigeminal ganglia and SCG. The expression pattern of Alk3 mRNA in nodose ganglia was not dissimilar to that of Alk1 mRNA. In particular, both mRNAs were expressed at their lowest levels in E13 nodose ganglia, both mRNAs increased their expression levels significantly between E13 and E15 and Alk1 and Alk3 mRNAs were both retained at these increased levels between E15 and P5.

Alk4

Alk4 is the receptor for GDF1 and GDF3 (462) that functions to modulate fundamental embryological events (463). Alk4 mRNA is expressed at low constant levels within trigeminal ganglia during the developmental period studied (fig. 24B). Alk4 mRNA is expressed at higher levels in the developing SCG compared to trigeminal ganglia. In the SCG, the lowest levels of Alk4 mRNA are expressed at E13 and expression levels steadily increase from E13, by approximately 75%, to peak at E18. There is a gradual 40% drop in the level of Alk4 mRNA expression in the SCG between E18 and P5. Amongst the ganglia analyzed, the highest levels of Alk4 mRNA, by far, were expressed in the nodose ganglion. The levels of Alk4 mRNA expressed by E13 nodose ganglia were almost 10-fold and 4-

fold higher than in E13 SCG and trigeminal ganglia, respectively. Alk4 mRNA expression levels increased approximately 50% between E13 and 15 and then decreased slightly to E18. There was an approximately 6-fold reduction in the amount of Alk4 mRNA expressed by nodose ganglia between E18 and P5. The highest levels of Alk4 mRNA within nodose ganglia correspond to a period when nodose neurons are going through a period of programmed cell death regulated by the availability of target field derived neurotrophic factors (51). This observation raises the possibility that Alk4 may play some role in mediating this process.

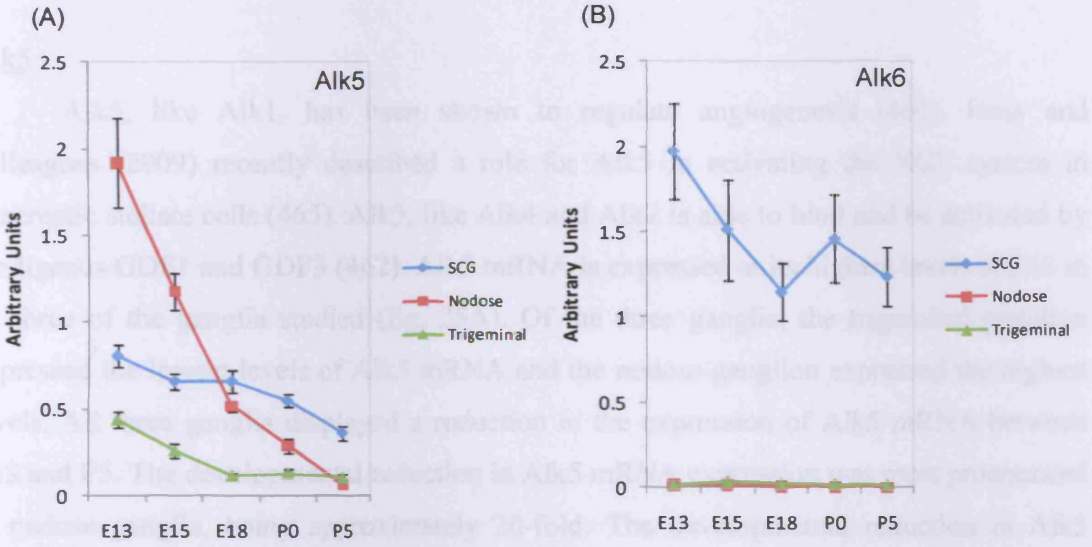


Figure 25: Expression profiles of Alk5 and Alk6. The data show the relative expression of Alk5 and Alk6 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

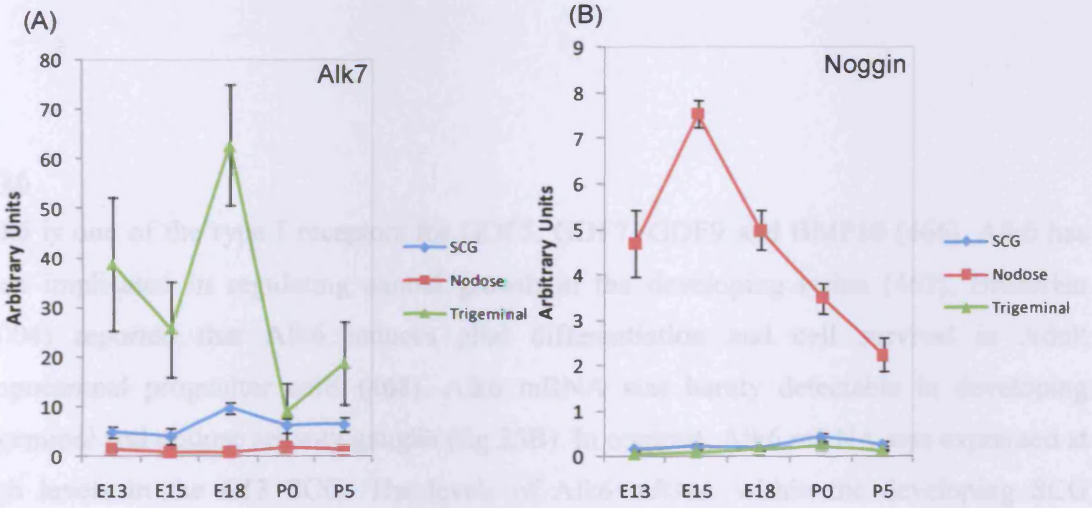


Figure 26: Expression profiles of Alk7 and Noggin. The data show the relative expression of Alk7 and Noggin mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

Alk5

Alk5, like Alk1, has been shown to regulate angiogenesis (464). Hass and colleagues (2009) recently described a role for Alk5 in activating the NGF system in pancreatic stellate cells (465). Alk5, like Alk4 and Alk7 is able to bind and be activated by the ligands GDF1 and GDF3 (462). Alk5 mRNA is expressed at its highest levels at E13 in all three of the ganglia studied (fig. 25A). Of the three ganglia, the trigeminal ganglion expressed the lowest levels of Alk5 mRNA and the nodose ganglion expressed the highest levels. All three ganglia displayed a reduction in the expression of Alk5 mRNA between E13 and P5. The developmental reduction in Alk5 mRNA expression was most pronounced in nodose ganglia, being approximately 20-fold. The developmental reduction in Alk5 mRNA expression was approximately 2-fold in SCG and 4-fold in trigeminal ganglia. The expression patterns of Alk5 mRNA suggest that Alk5 may regulate some aspect of neuronal differentiation in each of the three ganglia studied.

Alk6

Alk6 is one of the type I receptors for GDF5, GDF7, GDF9 and BMP10 (466). Alk6 has been implicated in regulating axonal growth in the developing retina (467). Brederlau (2004) reported that Alk6 induces glial differentiation and cell survival in Adult hippocampal progenitor cells (468). Alk6 mRNA was barely detectable in developing trigeminal and nodose sensory ganglia (fig 25B). In contrast, Alk6 mRNA was expressed at high levels in the E13 SCG. The levels of Alk6 mRNA within the developing SCG decreased approximately 90% between E13 and E18 and remained relatively constant thereafter. The restriction of Alk6 mRNA expression to developing SCG and the high level of expression within this ganglion may indicate that this receptor plays some role in mediating the differentiation and/or survival of, or target innervation by, developing SCG neurons.

Alk7

Alk7 is structurally similar to Alk4 and Alk5, and binds the same ligands, GDF1 and GDF3. It has also been reported to be able to bind GDF8, activins and nodals (469). Carlsson and colleagues (2009) have recently linked Alk7 to metabolic disease (470). In contrast to the other Alk receptors that bind GD1 and GDF3, nodose ganglia expressed negligible levels of Alk7 mRNA (fig. 26A). The expression pattern of Alk7 mRNA in developing SCG was similar to that of Alk4 mRNA, increasing by approximately 75% between E13 and E18 and then decreasing by approximately 40% between E18 and P5. The expression of Alk6 mRNA within developing trigeminal ganglia was somewhat erratic with large standard error of the mean values at each age. The levels of Alk7 mRNA expressed by E13 and E15 trigeminal ganglia are high and approximately equal. Expression increases 50% from E15 to peak at E18 before falling 5-fold to P0 and remaining approximately constant thereafter.

Noggin

Noggin is a small glycoprotein originally characterized as a secretory molecule derived from Spermann organizer during the amphibian development (471). During mammalian embryogenesis, noggin is expressed in the node, notochord, dorsal somite, condensing cartilage, and immature chondrocytes and regulates patterning of the neural tube and somites (472)(202) Mammalian noggin plays an important role in early neural tube formation and in the adult nervous system (473). Noggin inhibits TGF-β signal transduction by binding to TGF-β family ligands and preventing them from binding to their corresponding receptors. Noggin has a key role in neural induction by inhibiting BMP4, along with other TGF-β signaling inhibitors such as chordin and follistatin (473)(202).

My data (fig 26B) revealed that noggin mRNA was barely expressed within developing trigeminal ganglia and SCG. However E13 nodose neurons expressed

significant levels of the mRNA for this TGF-β signaling inhibitor. Noggin mRNA levels increased approximately 50% between E13 and E15 in the developing nodose ganglion before falling 3-fold between E15 and P5. Since noggin is such an important regulator of TGF-β family signal transduction, the high levels and distinct pattern of expression of its mRNA in the developing nodose ganglion suggests that TGF-β family ligands play an important role in the development of nodose neurons.

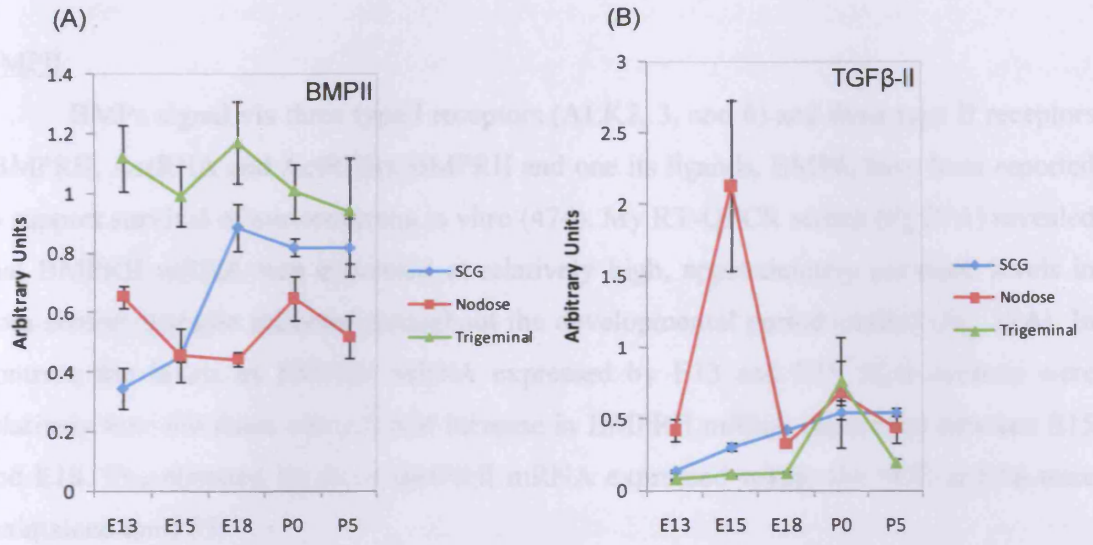


Figure 27: Expression profiles of BMPII and TGFβ-II. .The data show the relative expression of BMPII and TGFβ-II mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

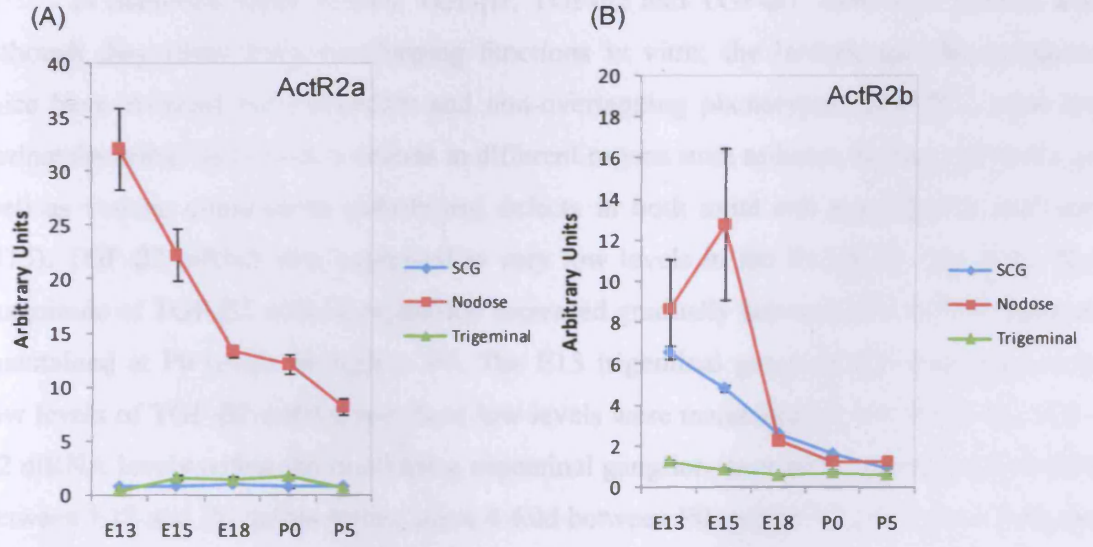


Figure 28: Expression profiles of ActR2a and ActR2b. The data show the relative expression of ActR2a and ActR2b mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

BMPII

BMPs signal via three type I receptors (ALK2, 3, and 6) and three type II receptors (BMPRII, ActRIIA and ActRIIB). BMPRII and one its ligands, BMP6, have been reported to support survival of motorneurons in vitro (474). My RT-QPCR screen (fig 27A) revealed that BMPRII mRNA was expressed at relatively high, approximately constant, levels in both sensory ganglia analyzed throughout the developmental period studied (fig. 27A). In contrast, the levels of BMPRII mRNA expressed by E13 and E15 SCG neurons were relatively low and there was a 2-fold increase in BMPRII mRNA expression between E15 and E18. The elevated levels of BMPRII mRNA expressed within the SCG at E18 were maintained until P5.

TGF-β2

In mammals three TGFβs, TGF-β1, TGF-β2 and TGF-β3, have been cloned, and although they often show overlapping functions in vitro, the isoform-specific knockout mice have revealed non-redundant and non-overlapping phenotypes. TGF-$\beta2^{-/-}$ mice are perinatally lethal and develop defects in different organs such as heart, kidney and testis, as well as various craniofacial defects and defects in both axial and appendicular skeleton (475). TGF-β2 mRNA was expressed at very low levels in the E13 SCG (fig 27B). The magnitude of TGF-β2 mRNA expression increased gradually between E13 and P0 and was maintained at P0 levels through to P5. The E13 trigeminal ganglion also expressed very low levels of TGF-β2 mRNA and these low levels were maintained at E15 and E18. TGF-β2 mRNA levels within the developing trigeminal ganglion increased approximately 6-fold between E18 and P0 before falling some 4-fold between P0 and P5. In accordance with the situation in SCG and trigeminal ganglia, TGF-β2 mRNA was expressed at relatively low levels, in relation to peak expression, in E13 nodose ganglia. There was a five-fold increase in the amount of TGF-β2 mRNA expressed within nodose ganglia between E13 and E15 and this increase was promptly reversed between E15 and E18. These lower levels of TGF-β mRNA expression at E18 were maintained between E18 and P5. The dramatic peaks in TGF-β2 mRNA expression in trigeminal and nodose ganglia, at P0 and E15, respectively,

would seem to suggest that this TGF-β family ligand plays a defined role within these developing sensory ganglia at these ages by functioning in an autocrine or paracrine manner.

Activin receptors

Activins have a wide range of biological effects on cell growth and differentiation. Activins bind directly to activin receptor type 2a (ActR2a) or 2b (ActR2b). ActR2b$^{-/-}$ mice display various endocrine pancreas-related abnormalities, including islet hypoplasia and glucose intolerance, demonstrating the crucial role of ActR2b in the regulation of pancreas development (476). ActR2a$^{-/-}$ male mice display multiple reproductive behavioral deficits, including delayed initiation of copulation, reduced mount, and intromission frequencies, and increased mount, intromission, and ejaculation latencies (477). My RT-QPCR screen revealed that ActR2a mRNA was expressed at relatively low levels in developing SCG and trigeminal ganglia throughout the developmental time frame investigated, suggesting that ActR2a does not play a role in any aspect of SCG or trigeminal neurons development (fig. 28A). In contrast, very high levels of ActR2a mRNA were detected in E13 nodose ganglia. There was a gradual, almost 4-fold, drop in the levels of ActR2a mRNA expressed in the developing nodose ganglion between E13 and P5, suggesting that ActR2a may play a role in the differentiation, early maturation or pioneer neuron target innervation of nodose neurons.

As in the case of ActR2a mRNA expression, ActR2b mRNA was expressed at relatively low, constant levels in trigeminal ganglia throughout the period studied (fig 28B). E13 SCG neurons expressed relatively high levels of ActR2b mRNA and these high levels of mRNA expression gradually decreased, approximately 6-fold, between E13 and P5, suggesting that ActR2b may be involved in modulating SCG neuron differentiation, early maturation and initial target field innervation. High levels of ActR2b mRNA expression within E13 nodose ganglia increased even further over the next two embryonic days to reach a peak at E15. There was a 6-fold decrease in the amount of ActR2b mRNA expressed within nodose ganglia between E15 and E18 and a further 50% decrease in the

expression of this mRNA between E18 and P5. The dramatic expression profile of ActR2b mRNA within developing nodose ganglia may indicate a role for this receptor in regulating neuronal differentiation in this ganglion.

GDF5

GDF5 binds specifically to BMPR1b, BMPR2 and ActR2a receptors, forming a heterodimeric complex that leads phosphorylation of smad1, 5, 8 and their translocation to the nucleus. GDF5 was initially identified as a growth factor involved in skeletal development and joint morphogenesis in humans and mice and has been shown to play a crucial role in the morphogenesis of tendons, ligaments and bones (478)(479)(480). GDF5 mRNA was expressed at very low, approximately constant, levels in the developing SCG throughout the period examined (fig. 29) suggesting that it does not function in an autocrine or paracrine manner within the developing SCG to regulate neuronal development. At E13, nodose ganglia expressed almost 10-fold more GDF5 mRNA than SCG of the same age. These relatively high levels of GDF5 mRNA were increased 2-fold between E13 and E15 in the developing nodose ganglion. However, over the next 10 days the expression of GDF5 mRNA within nodose ganglia decreased steadily by 6-fold to reach its lowest level at P5. Trigeminal ganglia expressed similar levels of GDF5 mRNA compared to nodose ganglia at E13 and these levels remained unchanged at E15. There was an abrupt, 4-fold, increase in the expression of GDF5 mRNA between E15 and E18 followed by a 40% decrease between E18 and P0. The reduced levels of GDF5 mRNA found in the trigeminal ganglion at P0 compared to E18 were maintained until P5. The high level of GDF5 mRNA expressed within trigeminal ganglia in the perinatal period suggests that this ligand may act in an autocrine or paracrine manner within this ganglion to regulate some aspect of neuronal or glial development. GDF5 will be the subject of chapter 4 of this thesis.

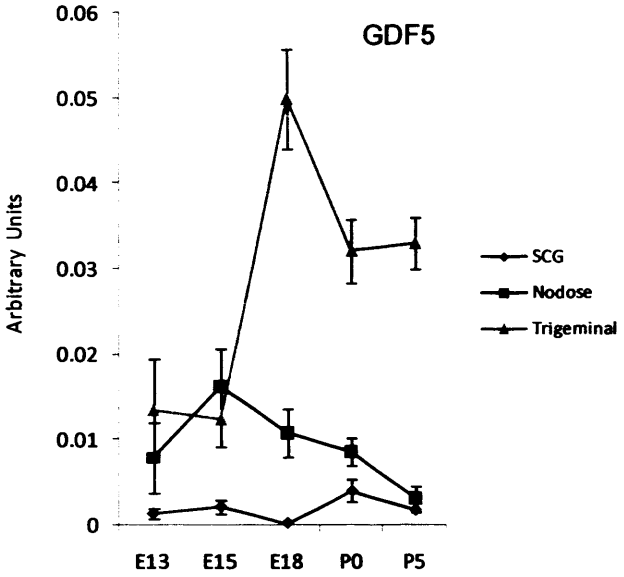


Figure 29: Expression profile of GDF5. The data show the relative expression of GDF5 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

3.5.3 TNF superfamily

Members of the tumour necrosis factor (TNF)/TNF-receptor (TNFR) superfamily are key regulators in the functioning of the immune system. They coordinate the immune response at multiple levels. For example, TNFα, LTα, LTβ and RANKL provide signals required for lymphoid neogenesis, In addition, CD27, OX40, 4-1BB and CD30 deliver co-stimulatory signals to augment immune responses, whilst pro-apoptotic members, such as TNF, CD95L and TRAIL, may contribute to the termination of the response. The biological function of individual family members has been examined in studies using mice over-expressing or lacking the gene in question. A growing appreciation of the molecular basis of signalling pathways activated by TNFRs has provided a framework for better understanding of the biology of this expanding family. However, although the rapid and robust activation of NF-kB and MAPK pathways is typical of acute TNF-R engagement, the molecular basis of sustained receptor signalling remains a mystery, in spite of its relevance to chronic inflammatory and immune responses (370).

DR6

Death receptor (DR) 6 (TNFRSF21), is broadly expressed by developing neurons, and is required for normal cell body death and axonal pruning both *in vivo* and after trophic-factor deprivation *in vitro* (481). DR6 is activated locally by an inactive surface ligand(s) that is released in an active form after trophic-factor deprivation, this ligand has been recently identified as being the beta-amyloid precursor protein (APP) (481). Amongst all the TNF superfamily receptor and ligand mRNAs that I screened, DR6 was the only mRNA that showed a similar developmental expression pattern in trigeminal, nodose and superior cervical ganglia (fig 30A). In all three ganglia, the lowest levels of DR6 mRNA were found at E13 and the expression of this mRNA increased gradually from E13 to peak at P0. The magnitude of increased DR6 mRNA expression between E13 and P0 was

approximately 2-fold in each of the ganglia studied. After peaking at P0, DR6 mRNA levels decreased in all three ganglia to reach lower levels at P5. The developmental expression of DR6 mRNA in the peripheral ganglia studied appears to coincide with the timing and extent of naturally occurring neuronal cell death (51), suggesting that DR6 may play a role in this process.

TRAIL

The endogenous protein tumor necrosis factor receptor apoptosis-inducing ligand (TRAIL) (also known as TNFRSF22, Apo2L or SOBa), has been shown to induce apoptosis in a wide variety of transformed and cancer cells, but not normal healthy cells. Therefore, TRAIL is considered to be a tumour-selective, apoptosis-inducing cytokine (482). Given the size of the TNF superfamily, some ligands have several receptors and vice-versa. TRAIL has four receptors: Death Receptors 4 (DR4) and 5, and Decoy Receptors 1 (DCR1) and 2. TRAIL mRNA was expressed at extremely low, probably non-functional, levels in both sensory ganglia throughout the developmental period studied (fig 30B). However, developing SCG expressed significant amounts of TRAIL mRNA at all ages. TRAIL mRNA expression within the developing SCG appeared to increase around 30% between E13 and E18 and then decrease back down to E13 levels between E18 and P5. However, given the large standard error of the mean value at each age, it may be that expression levels of TRAIL mRNA within SCG are in fact constant throughout the developmental period analyzed.

CD30

CD30, (also known as CD153, TNFSF8) is a 40-kDa type II membrane associated glycoprotein expressed on CD4, Th1 and Th2 cells, macrophages, dendritic cells (DC) and B cells (483). CD30 has only one ligand, CD30L, which has been reported to be expressed preferentially by activated or memory Th2 cells but not by resting B or T cells (484). CD30L/CD30 signaling has been reported to support activation and survival, and prevent

excessive cell death of T-cells (485). CD30 mRNA was expressed at very low levels within E13 nodose ganglia (fig. 31A). However, the levels of this mRNA within nodose ganglia increased 8-fold between E13 and E18. After a 35% decrease in expression between E18 and P0, CD30 mRNA levels increased 2-fold after P0 to peak at P5. The lowest levels of CD30 mRNA were also found at E13 in the trigeminal ganglion. The expression of CD30 mRNA in E15 trigeminal ganglia remained at the same levels as E13, however, CD30 mRNA expression increased 3-fold between E15 and P0, before falling 30% between P0 and P5. In the developing SCG, CD30 mRNA was expressed at relatively modest levels at E13 and increased approximately 2-fold between this age and E15. There was an 8-fold drop in the levels of CD30 mRNA expressed in the developing SCG between E15 and P5.

DR5

Death receptor (DR) 5 (TNFRSF10B) is one the receptors for TRAIL. Death receptors and their ligands are expressed in the developing nervous system, and many recent reports confirm their ability to trigger death of embryonic neurons and glia *in vitro* (486). However, with the exception of earlier reports concerning $p75^{NTR}$, there is still relatively little evidence that death receptors play a role in nervous system development *in vivo*. Of the three ganglia that I studied, the highest levels of DR5 mRNA were expressed in the trigeminal ganglion (fig. 31B). Peak expression of DR5 mRNA in the trigeminal ganglion occurred at E13, and levels of this mRNA fell approximately 4-fold between E13 and E18. The amount of DR5 mRNA expressed by trigeminal ganglia increased by 75% between E18 and P0 and thereafter remained fairly constant between P0 and P5. Nodose ganglia expressed several fold-lower levels of DR5 mRNA than trigeminal ganglia. At E13 this difference was almost 5-fold. DR5 mRNA levels within developing nodose ganglia remained constant between E13 and E15, but fell by 50% between E15 and E18. The reduced levels of DR5 mRNA expressed within the nodose ganglion at E18 were maintained until P5. The low levels of DR5 mRNA expressed within E13 SCG increased 2-fold between E13 and E15. After E15, DR5 mRNA expression within the SCG gradually fell, by approximately 60%, to reach a minimum at P5.

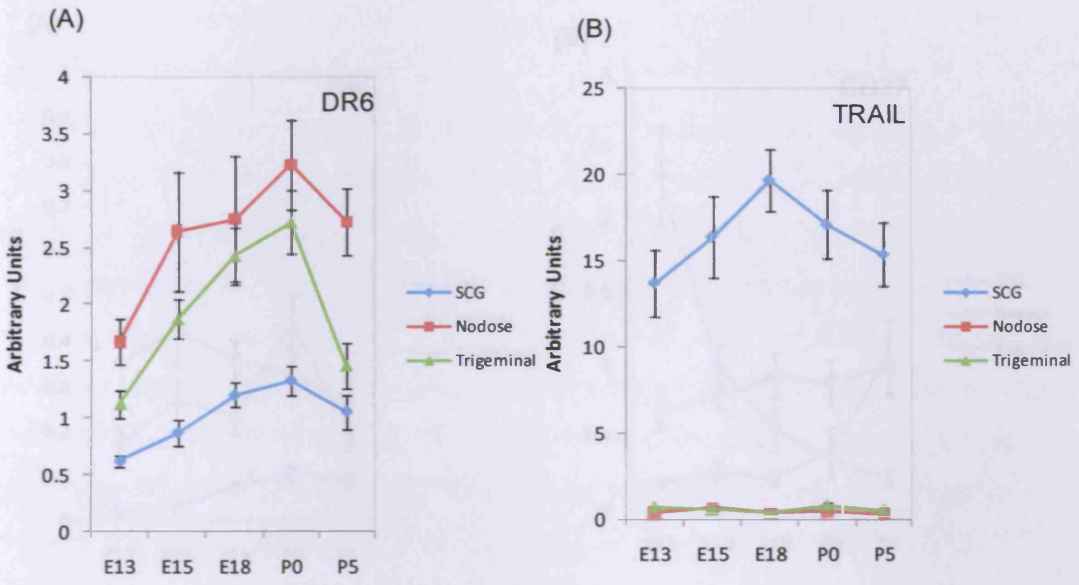


Figure 30: Expression profiles of DR6 and TRAIL. The data show the relative expression of DR6 and TRAIL mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

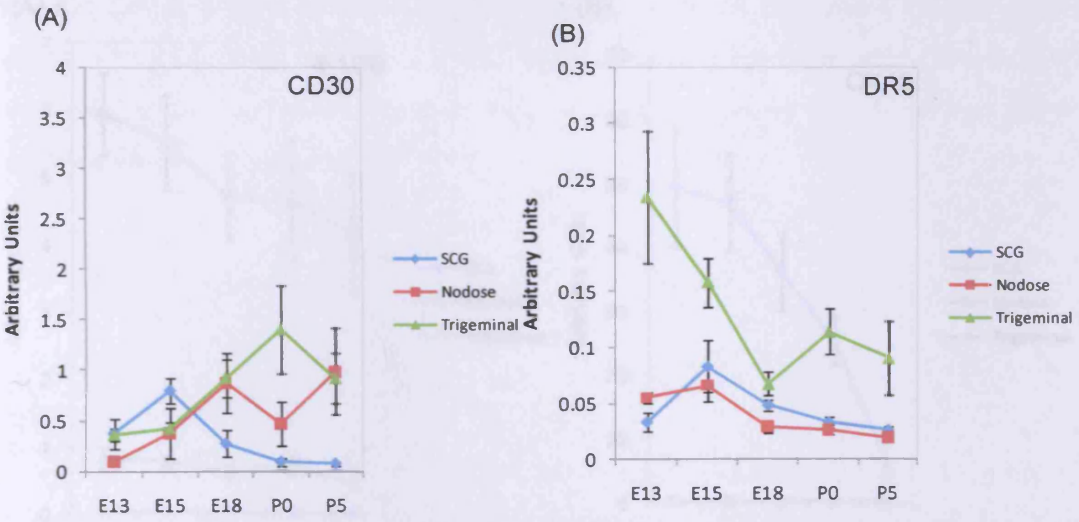


Figure 31: Expression profiles of CD30 and DR5. The data show the relative expression of CD30 and DR5 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

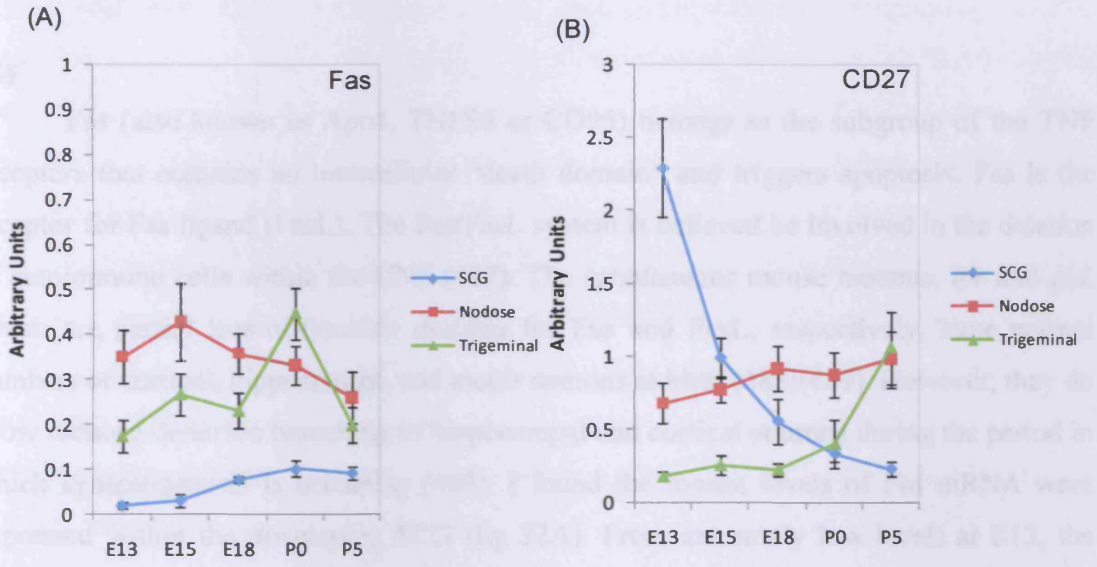


Figure 32: Expression profiles of Fas and CD27. The data show the relative expression of Fas and CD27 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

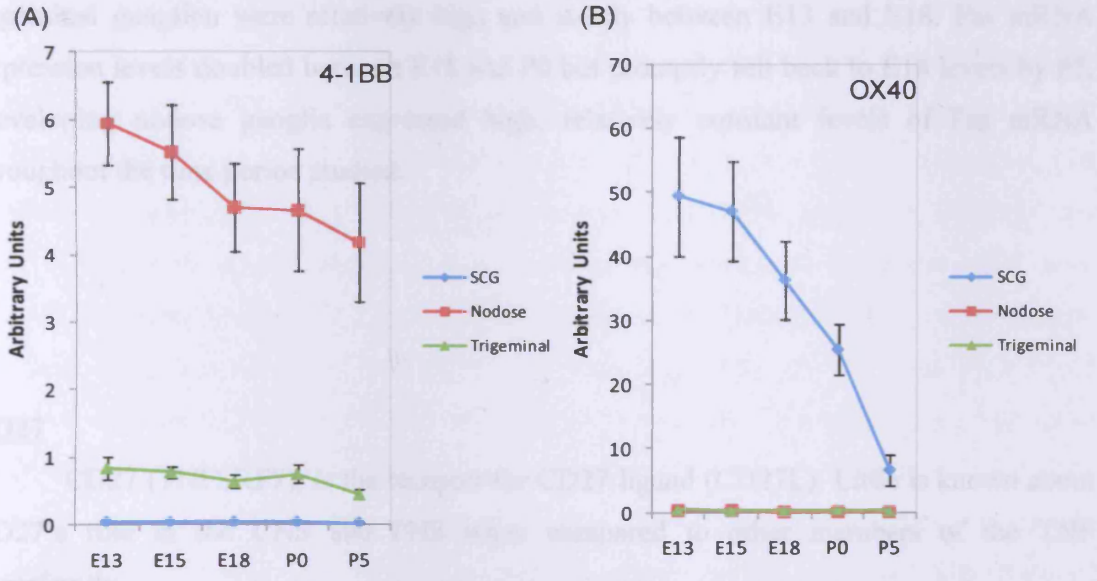


Figure 33: Expression profiles of 4-1BB and OX40. The data show the relative expression of 4-1BB and OX40 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

Fas

Fas (also known as Apo1, TNFS6 or CD95) belongs to the subgroup of the TNF receptors that contains an intracellular "death domain" and triggers apoptosis. Fas is the receptor for Fas ligand (FasL). The Fas/FasL system is believed be involved in the deletion of autoimmune cells within the CNS (487). The spontaneous mouse mutants, *lpr* and *gld*, which are partial loss-of-function mutants for Fas and FasL, respectively, have normal numbers of cortical, hippocampal, and motor neurons at birth (488)(489). However, they do show reduced dendritic branching of hippocampal and cortical neurons during the period in which synaptogenesis is occurring (489). I found the lowest levels of Fas mRNA were expressed within the developing SCG (fig 32A). From extremely low levels at E13, the amount of Fas mRNA expressed by developing SCG increased by approximately 5-fold to P0 and these increased levels were maintained until P5. However, even at postnatal ages Fas mRNA levels in the SCG were significantly lower than those expressed within either sensory ganglia at any time point studied. The expression levels of Fas mRNA within the trigeminal ganglion were relatively high and steady between E13 and E18. Fas mRNA expression levels doubled between E18 and P0 but promptly fell back to E18 levels by P5. Developing nodose ganglia expressed high, relatively constant levels of Fas mRNA throughout the time period studied.

CD27

CD27 (TNFSRF7), is the receptor for CD27 ligand (CD27L). Little is known about CD27's role in the CNS and PNS when compared to other members of the TNF superfamily.

CD27 is expressed by medullary thymocytes, most peripheral blood T cells, a subset of mature B cells, and NK cell. The biologic functions of CD27L include a co-stimulatory signal for T-cell proliferation, generation of cytotoxic T cells, and enhanced cytokine secretion (490). The expression pattern of CD27 mRNA within developing SCG is

striking (fig. 32B). CD27 is expressed at very high levels within this ganglion at E13 and thereafter expression drops precipitously, in a logarithmic manner, to be some 12-fold lower at P5. This expression pattern would seem to suggest that CD27 plays some role in the differentiation and early maturation of SCG neurons. In marked contrast, CD27 mRNA is expressed at very low levels in the E13 trigeminal ganglion and these low levels are maintained between E13 and E18. From E18, CD27 mRNA expression within developing trigeminal ganglia increases 5-fold to reach a maximum at P5. The expression pattern of CD27 mRNA within developing nodose ganglia is less interesting than those of trigeminal and superior cervical ganglia. Moderately high levels of CD27 mRNA in E13 nodose ganglia steadily increase by around 25% to reach a maximum at P5, although it is unlikely that his increase is statistically significant.

4-1BB

4-1BB (also known as TNFRSF9, CD137) is the receptor for 4-1BB ligand (4-1BBL). Just like CD27, most studies involving 4-1BB and its ligand have been performed in the immune system. 4-1BB is expressed by activated T lymphocytes and monocytes and expression in primary cells is strictly activation dependent (491). Its mRNA, however, can also be detected in nonlymphoid cells such as epithelial and hepatoma cells after stimulation with IL-1α (492). 4-1BBL is present on activated B cells, macrophages, myeloid cells, and dendritic cells (493). My data (fig 33A) revealed that the nodose ganglion had the highest levels of 4-1BB mRNA expression. The expression levels of 4-1BB mRNA were highest at E13 in the nodose ganglion and decreased by some 30% between this age and P5. The expression pattern of 4-1BB mRNA within developing trigeminal ganglia was very similar to that in nodose ganglia, although the levels of expression at each age were approximately 6-fold lower. 4-1BB mRNA was virtually undetectable within developing SCG.

OX40

OX40 (also known as TNFRSF4 or CD134) is the receptor forOX40 ligand (OX40L). OX40 was initially identified as a T-cell activation marker and after cloning shown to be a member of the TNF superfamily with co-stimulatory function (494). OX40 is expressed on activated $CD4^+$T cells and a subset of $CD8^+$ T, OX40L has been found to be expressed by activated T cells, B cells, dendritic cells, microglial cells and endothelial cells (495). OX40 has been shown to be involved in the pathogenesis of encephalomyelitis and to be up regulated in MS (496). OX40 mRNA was barely present in either of the developing sensory ganglia I analyzed (fig 33B), seemingly ruling out a role for OX40L in the development of cranial sensory neurons. In stark contrast OX40 mRNA was expressed at very high levels within embryonic SCG. Peak expression of OX40 mRNA in SCG occurred at E13 and E15. After E15, the expression of this mRNA decreased greatly so that P5 SCG expressed 9-fold less OX40 mRNA than E15 SCG. My data raises the possibility that OX40 may play a role in the differentiation and early maturation of SCG neurons.

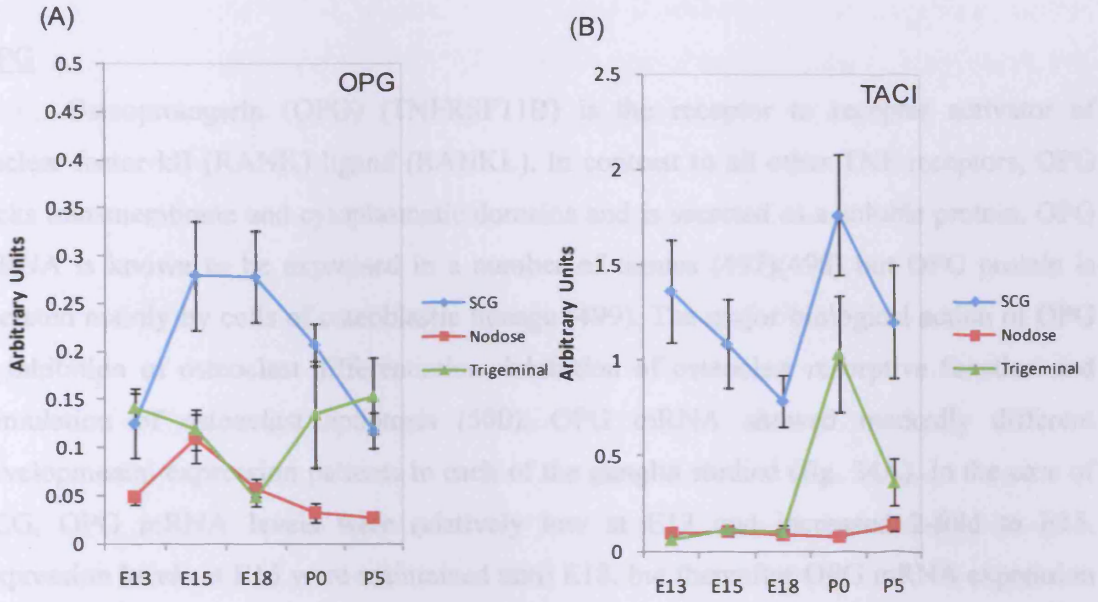


Figure 34: Expression profiles of OPG and TACI. The data show the relative expression of OPG and TACI mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

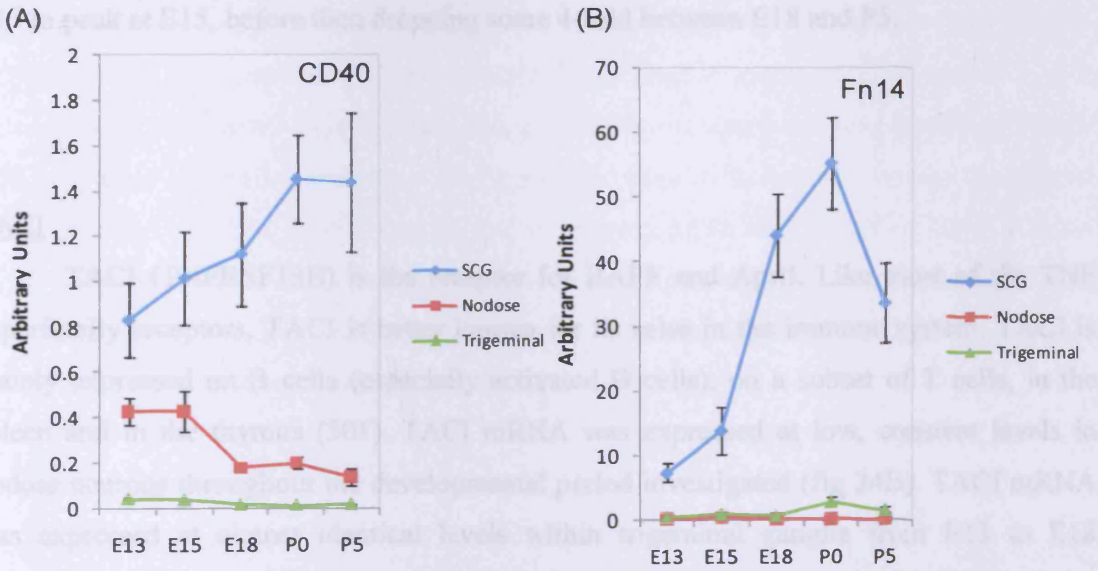


Figure 35: Expression profiles of CD40 and Fn14. The data show the relative expression of CD40 and Fn14 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

OPG

Osteoprotegerin (OPG) (TNFRSF11B) is the receptor to receptor activator of nuclear factor-kB (RANK) ligand (RANKL). In contrast to all other TNF receptors, OPG lacks transmembrane and cytoplasmatic domains and is secreted as a soluble protein. OPG mRNA is known to be expressed in a number of tissues (497)(498) but OPG protein is secreted mainly by cells of osteoblastic lineage (499). The major biological action of OPG is inhibition of osteoclast differentiation, inhibition of osteoclast resorptive function and stimulation of osteoclast apoptosis (500). OPG mRNA showed markedly different developmental expression patterns in each of the ganglia studied (fig. 34A). In the case of SCG, OPG mRNA levels were relatively low at E13 and increased 2-fold to E15. Expression levels at E15 were maintained until E18, but thereafter OPG mRNA expression decreased 2–fold to P5. OPG mRNA displayed a "V" shaped developmental expression pattern within trigeminal ganglia, whereby relatively high levels of expression at E13 fell 3-fold to E18 before increasing 3-fold between E18 and P5. Nodose ganglia expressed relatively low levels of OPG mRNA at E13. OPG mRNA expression increased 2-fold from E13 to peak at E15, before then dropping some 4-fold between E18 and P5.

TACI

TACI (TNFRSF13B) is the receptor for BAFF and April. Like most of the TNF superfamily receptors, TACI is better known for its roles in the immune system. TACI is mainly expressed on B cells (especially activated B cells), on a subset of T cells, in the spleen and in the thymus (501). TACI mRNA was expressed at low, constant levels in nodose neurons throughout the developmental period investigated (fig 34B). TACI mRNA was expressed at almost identical levels within trigeminal ganglia from E13 to E18 compared to those in the nodose ganglia during this period. Interestingly, there was an abrupt, 10-fold, increase in the amount of TACI mRNA expressed within trigeminal ganglia between E18 and P0 and an almost as dramatic, 5-fold, decrease in TAC1 mRNA expression between P0 and P5. This dramatic peak in TAC1 mRNA expression would

seem to suggest that BAFF and/or APRIL play a crucial role in trigeminal neuron development in the perinatal period. TAC1 mRNA also showed quite a striking expression pattern within developing SCG. High level expression of TACI mRNA at E13 fell approximately 40% to E18, after which there was a 2-fold increase in TACI mRNA expression to P0 followed by a modest decrease in expression to P5.

CD40

CD40 (also known as CD154 or TNFSF5) is the receptor for CD40 ligand (CD40L). CD40 is expressed in both immune and non-immune cell types (502). CD40L is expressed mainly on activated $CD4^+$ T lymphocytes. CD40/CD40L has been shown to play a crucial role in cellular and humoral immune responses. In the CNS, CD40 expression is associated with MS lesions, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) (503)(504)(505). Whether CD40 expression is causative or reactive to these disease processes is unknown. CD40 has been also shown to enhance inflammation through production of TNF-α, NO, and COX-2 (504)(506)(505). CD40 mRNA was not expressed at significant levels in the developing trigeminal ganglion (fig 35A). Within nodose ganglia, CD40 mRNA was expressed at moderately high, similar levels at E13 and E15. The amount of CD40 mRNA expressed by nodose ganglia decreased 2.5-fold between E15 and E18 and these reduced levels were maintained between E18 and P5. By far the highest levels of CD40 mRNA were found in the developing SCG. The levels of CD40 mRNA expressed within E13 SCG were twice that expressed by E13 nodose ganglia and expression steadily increased, by approximately 80%, between E13 and P0. The elevated levels of CD40 mRNA within P0 SCG were maintained at P5.

Fn14

FGF-inducible 14-kDa protein (Fn14 but also known as TNFRSF12A or TWEAKR) is the receptor for TNF-like weak inducer of apoptosis (TWEAK). Fn14 mRNA is found in many tissues, but surface expression of Fn14 protein has been described only for fibroblasts (507), endothelial (508)(509), glioma (510) and neuronal cells (511).

Nodose ganglia did not express appreciable levels of Fn14 mRNA at any developmental time point studied (fig 35B). Similarly, trigeminal ganglia did not express significant levels of Fn14 mRNA between E13 and E18. Although there was an approximately 10-fold increase in the levels of Fn14 mRNA expressed by trigeminal ganglia between E18 and P0, the magnitude of Fn14 mRNA expression was still dramatically below that found in developing SCG. The expression pattern of Fn14 mRNA within developing SCG was striking. At E13, SCG expressed approximately 50-fold higher levels of this mRNA than sensory ganglia of the equivalent age. The high levels of Fn14 mRNA within SCG increased even further, by 7-fold, between E13 and P0 and thereafter fell approximately 40% between P0 and P5. The expression pattern of Fn14 mRNA within the developing SCG bears a striking resemblance to that of NGF receptor, trkA (51), suggesting that Fn14/TWEAK signaling may play a role in regulating the survival of and/or target field innervation by developing SCG neurons.

HVEM

Herpes virus entry mediator (HVEM, TNFRSF14) is the receptor for lymphotoxin-related inducible ligand (LIGHT). HVEM was identified as a cellular mediator of herpes simplex virus (HSV) entry. Binding of HSV viral envelope glycoprotein D to this receptor protein has been shown to be part of the viral entry mechanism. Within the immune system, HVEM activates both stimulatory and inhibitory pathways, serving as a molecular switch by engaging two distinct classes of molecules: the TNF-related cytokines and the members of the Ig superfamily. HVEM engages the canonical TNF-related ligands LIGHT and lymphotoxin-β (LT-β). LIGHT initiates a strong co-stimulatory signal, promoting inflammation and enhancing immune responses. By contrast, HVEM engagement of the Ig superfamily members B and T lymphocyte attenuator (BTLA) and CD160 activates inhibitory signaling in lymphoid cells (512).

HVEM mRNA was expressed at very low levels in developing trigeminal and superior cervical ganglia compared to its expression in nodose ganglia (fig 36A). Relatively

high levels of HVEM mRNA within E13 nodose ganglia decreased 2-fold between E13 and E15. This lower level of expression was maintained until P0, after which HVEM mRNA expression increased 2.5-fold to P5. In accordance with the relatively high level of HVEM mRNA expression within developing nodose ganglia compared to trigeminal ganglia and SCG, recent work from our lab has shown that LIGHT/HVEM signaling negatively regulates neurite growth from developing nodose neurons via NF-kB inhibition (513).

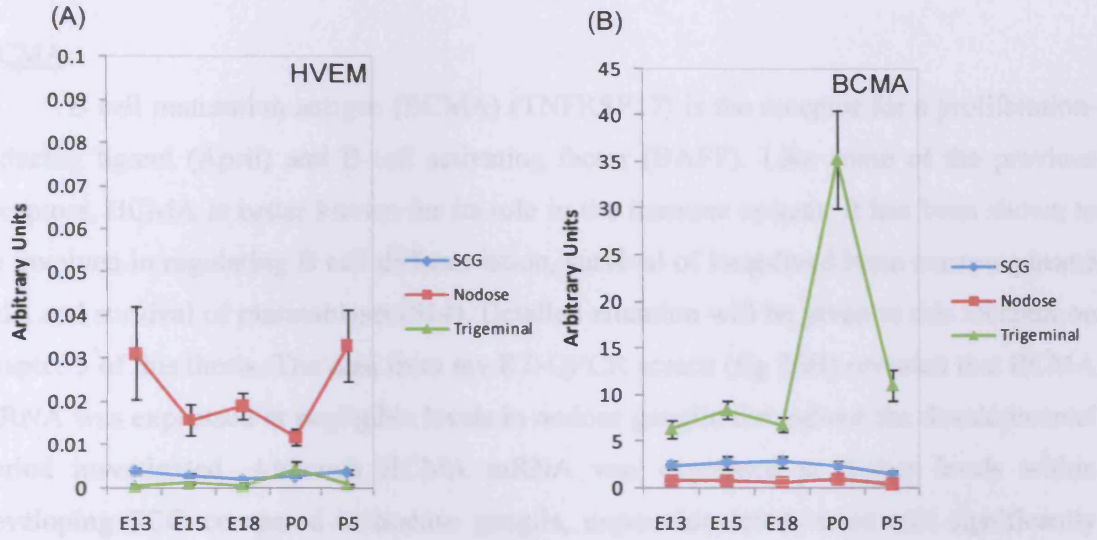


Figure 36: Expression profiles of HVEM and BCMA. The data show the relative expression of HVEM and BCMA mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

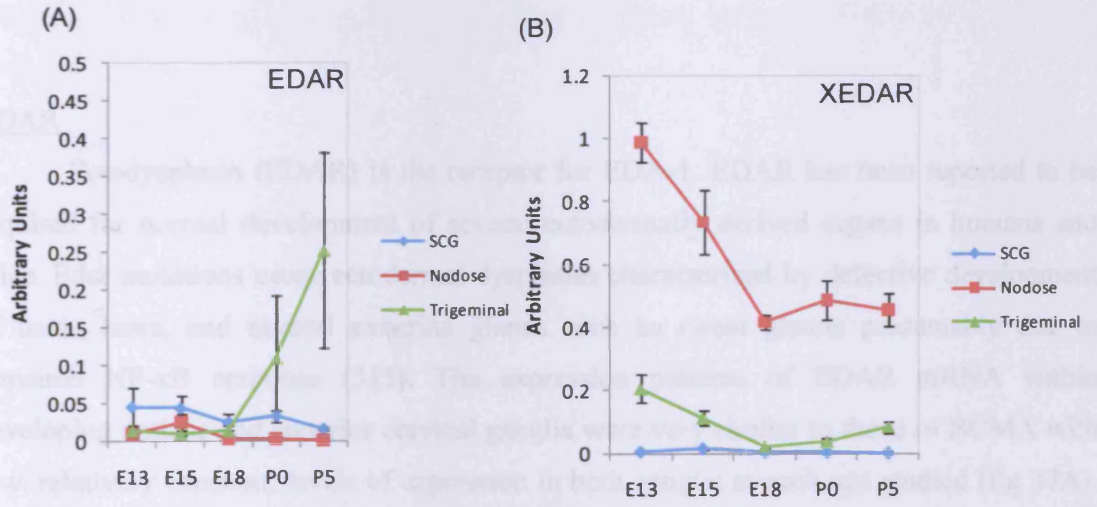


Figure 37: Expression profiles of EDAR and XEDAR. The data show the relative expression of EDAR and XEDAR mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

BCMA

B cell maturation antigen (BCMA) (TNFRSF17) is the receptor for a proliferation-inducing ligand (April) and B-cell activating factor (BAFF). Like some of the previous receptors, BCMA is better known for its role in the immune system. It has been shown to be involved in regulating B cell differentiation, survival of long-lived bone marrow plasma cells and survival of plasmablasts (514). Detailed attention will be given to this receptor on chapter 5 of this thesis. The data from my RT-QPCR screen (fig 36B) revealed that BCMA mRNA was expressed at negligible levels in nodose ganglia throughout the developmental period investigated. Although BCMA mRNA was expressed at higher levels within developing SCG compared to nodose ganglia, expression levels were still significantly lower than in developing trigeminal ganglia. At E13, BCMA mRNA was expressed at 3-fold higher levels in trigeminal ganglia than at any age in nodose ganglia and this level was maintained at E15 and E18. There was a dramatic, almost 7-fold, increase in BCMA mRNA levels between E18 and P0 which was almost completely reversed between P0 and P5.

EDAR

Ectodysplasin (EDAR) is the receptor for EDA-1. EDAR has been reported to be required for normal development of several ectodermally derived organs in humans and mice. Edar mutations cause ectodermal dysplasias characterized by defective development of teeth, hairs, and several exocrine glands such as sweat glands presumably due to impaired NF-κB response (515). The expression patterns of EDAR mRNA within developing nodose and superior cervical ganglia were very similar to those of BCMA with low, relatively constant, levels of expression in both ganglia at each age studied (fig 37A). EDAR mRNA was expressed at minimal levels within E13, E15 and E18 trigeminal ganglia. However, there appeared to be a large increase in the amount of EDAR mRNA expressed by trigeminal ganglia between E18 and P0 and thereafter between P0 and P5, although the large standard error of the mean values at P0 and P5 questions the validity of this data.

XEDAR

X-linked ectodysplasin receptor (XEDAR) is a receptor for EDA-A2. EDA-A2, like EDA-1, has been implicated in the development of ectoderm-derived organs (516). According to the same study, XEDAR is dispensable for normal mouse development. However, a potential role for XEDAR in skeletal muscle homeostasis was revealed by EDA-A2 transgenic mice, which developed multifocal myodegeneration that was dependent on expression of XEDAR. XEDAR mRNA was barely detectable in the developing SCG throughout the period I studied (fig 37B). Maximal levels of XEDAR mRNA were expressed at E13 in trigeminal and nodose ganglia, although in the latter the levels of XEDAR mRNA were 5-fold higher. Both ganglia displayed a significant reduction in XEDAR mRNA expression between E13 and E18. In the case of nodose ganglia the magnitude of this reduction was 2.5-fold, whereas for trigeminal ganglia it was 10-fold. XEDAR mRNA levels remained constant in nodose ganglia between E18 and P5, whereas in trigeminal ganglia the levels of this mRNA increased 4-fold over the same period.

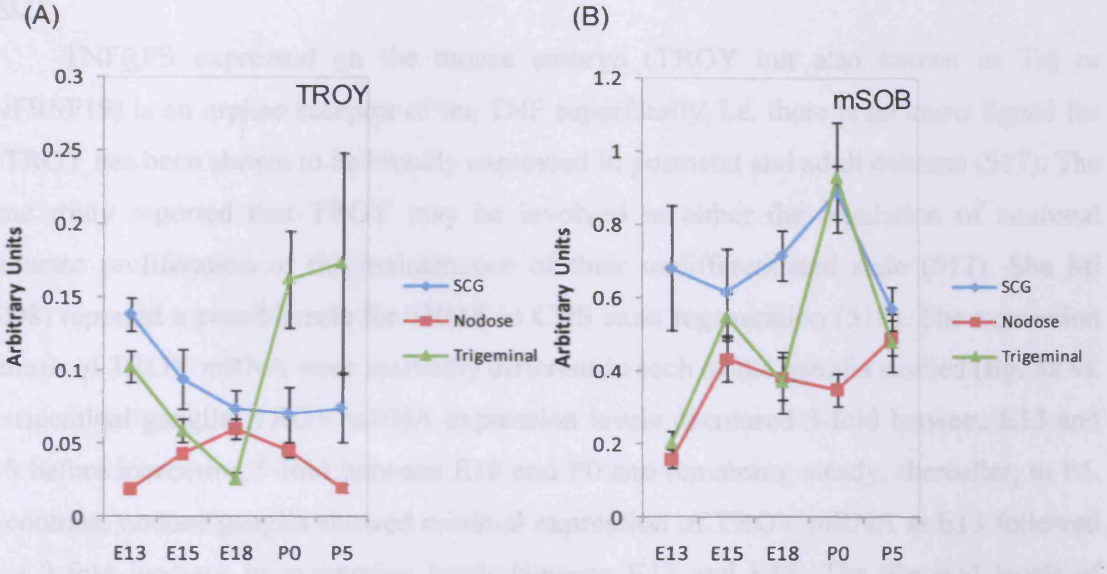


Figure 38: Expression profiles of TROY and mSOB. The data show the relative expression of TROY and mSOB mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

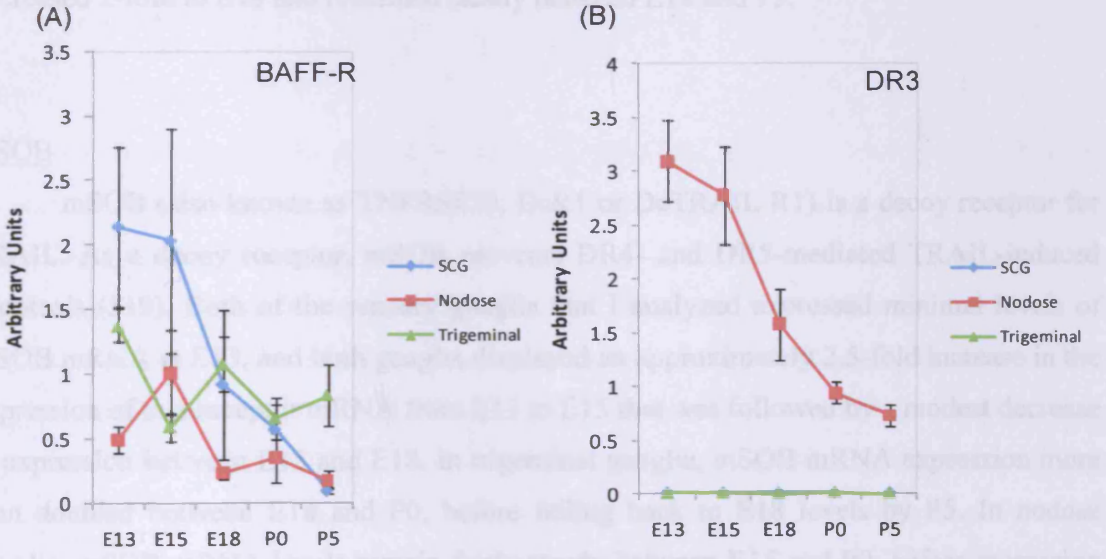


Figure 39: Expression profiles of BAFF-R and DR3. The data show the relative expression of BAFF-R and DR3 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

TROY

TNFRFS expressed on the mouse embryo (TROY but also known as Taj or TNFRSF19) is an orphan receptor of the TNF superfamily, i.e. there is no know ligand for it. TROY has been shown to be broadly expressed in postnatal and adult neurons (517). The same study reported that TROY may be involved in either the regulation of neuronal precursor proliferation or the maintenance of their undifferentiated state (517). Sha Mi (2008) reported a possible role for TROY in CNS axon regeneration (518). The expression patterns of TROY mRNA were markedly different in each of the ganglia studied (fig. 38A). In trigeminal ganglia, TROY mRNA expression levels decreased 3-fold between E13 and E18 before increasing 5-fold between E18 and P0 and remaining steady, thereafter, to P5. In contrast, nodose ganglia showed minimal expression of TROY mRNA at E13 followed by a 3-fold increase in expression levels between E13 and E18. The elevated levels of TROY mRNA in the E18 nodose ganglia were not maintained as development proceeded, rather expression of this mRNA decreased by 3-fold between E18 and P5. Highest levels of TROY mRNA expression occurred in the developing SCG at E13. Thereafter, expression decreased 2-fold to E18 and remained steady between E18 and P5.

mSOB

mSOB (also known as TNFRSF23, DcR1 or DcTRAIL R1) is a decoy receptor for TRAIL. As a decoy receptor, mSOB prevents DR4- and DR5-mediated TRAIL-induced apoptosis (519). Both of the sensory ganglia that I analyzed expressed minimal levels of mSOB mRNA at E13, and both ganglia displayed an approximately 2.5-fold increase in the expression of this receptor mRNA from E13 to E15 that was followed by a modest decrease in expression between E15 and E18. In trigeminal ganglia, mSOB mRNA expression more than doubled between E18 and P0, before falling back to E18 levels by P5. In nodose ganglia, mSOB mRNA levels remain fairly steady between E15 and P0, before increasing by 50% between P0 and P5. The developing SCG expressed the highest levels of mSOB mRNA during the embryonic period. However, after reaching a peak of expression at P0, mSOB mRNA levels decreased 30% between P0 and P5.

BAFF-R

B cell activating factor receptor (BAFF-R, TNFRSF13C) is the receptor for B cell activating factor (BAFF). Like many other members of the TNF superfamily, BAFF-R has been well studied in the immune system, specially its role in regulating mature B cell and survival and maintenance (520). In BAFF-$R^{-/-}$ mice, B cells develop normally up to the stage of IgM^{+} immature/transitional B cells, but cannot complete maturation in the spleen, as BAFF/BAFF-R–dependent survival signals are missing (521). I found that the highest levels of BAFF-R mRNA were expressed by the E13 and E15 SCG (fig. 39A). After E15, BAFF-R mRNA levels within the developing SCG fell by 15-fold to reach a minimum at P5. BAFF-R mRNA showed an erratic pattern of expression in developing trigeminal ganglia with moderate levels of this mRNA being expressed throughout the period studied. E13 nodose ganglia expressed relatively low levels of BAFF-R mRNA that increased 2-fold to reach a peak at E15. After E15, the expression of this receptor mRNA decreased 4-fold to E18 and remained relatively stable thereafter.

DR3

Death receptor (DR) 3 (also known as TNFRSF25, TRAMP, LARD or WSL-1) is the receptor for TL1A. DR3 has been reported to be expressed mainly in T lymphocytes (522). DR3 acts by binding to the adaptor molecule TRADD, which in turn endows DR3 with dual-signaling capacity to either activate NF-kB and MAP-kinase signaling or alternatively trigger caspase activation and apoptosis (523)(522)(524). DR3 mRNA was expressed at barely detectable levels throughout the period E13 to P5 in both SCG and trigeminal ganglia (fig. 39B). However, DR3 mRNA displayed a striking expression pattern within developing nodose ganglia. Levels of this mRNA were highest at E13 and E15 and fell approximately 4-fold between E15 and P5.

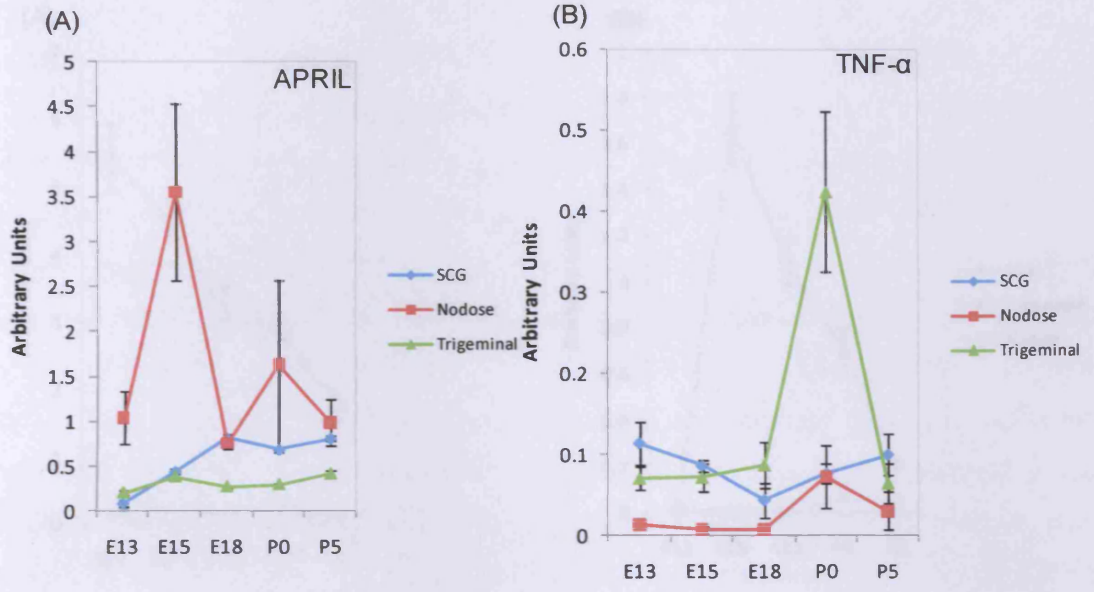


Figure 40: Expression profiles of APRIL and TNF-α. The data show the relative expression of APRIL and TNF-α mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

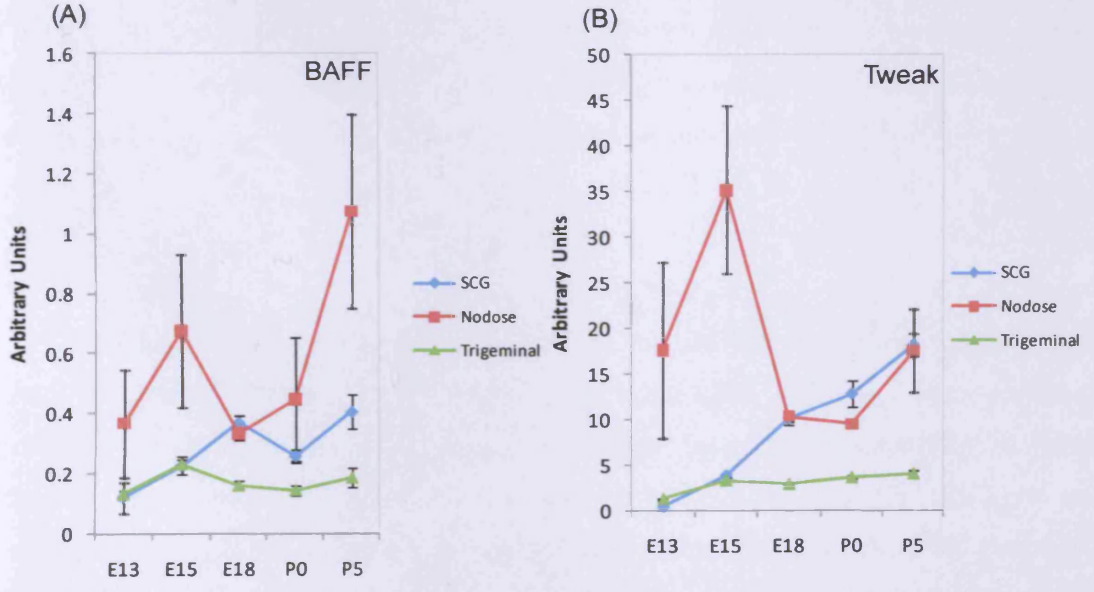


Figure 41: Expression profiles of BAFF and Tweak. The data show the relative expression of BAFF and Tweak mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

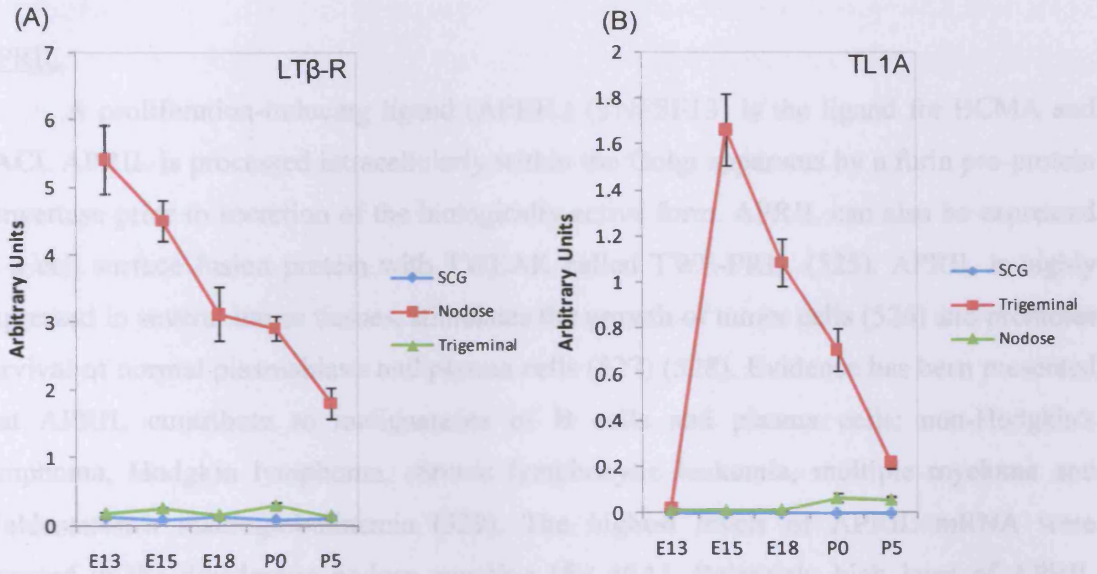


Figure 42: Expression profiles of LTβ-R and TL1A. The data show the relative expression of LTβ-R and TL1A mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). Means and standard errors of data from three separate sets of ganglia at each stage are shown.

APRIL

A proliferation-inducing ligand (APRIL) (TNFSF13) is the ligand for BCMA and TACI. APRIL is processed intracellularly within the Golgi apparatus by a furin pro-protein convertase prior to secretion of the biologically active form. APRIL can also be expressed as a cell surface fusion protein with TWEAK called TWE-PRIL (525). APRIL is highly expressed in several tumor tissues, stimulates the growth of tumor cells (526) and promotes survival of normal plasmablasts and plasma cells (527) (528). Evidence has been presented that APRIL contribute to malignancies of B cells and plasma cells: non-Hodgkin's lymphoma, Hodgkin lymphoma, chronic lymphocytic leukemia, multiple myeloma and Waldenstrom's macroglobulinemia (529). The highest levels of APRIL mRNA were detected in the developing nodose ganglion (fig 40A). Relatively high level of APRIL mRNA at E13 increased 3.5-fold to peak at E15, before falling back to E13 levels by E18. APRIL mRNA levels within the nodose ganglion almost doubled between E18 and P0 before once again decreasing from P0 to P5. APRIL mRNA was expressed at very low levels in E13 SCG and trigeminal ganglia. In the case of trigeminal ganglia, the expression of APRIL mRNA remained at fairly constant levels between E13 and P5. In the developing SCG, APRIL mRNA expression increased 3-fold between E13 and E18, fell by 25% between E18 and P0 and then increased by 30% between P0 and P5.

TNF-α

TNF-α (TNFSF1A) is the ligand for TNFR1 and TNFR2. TNF-α signaling has been shown to have several important functions within the CNS including: injury-mediated microglial and astrocyte activation; regulation of blood brain barrier permeability; febrile responses; regulating glutamatergic transmission; modulating synaptic plasticity and scaling (530). TNFR1 is expressed in most cell types, and can be activated by binding of either soluble TNF (solTNF) or transmembrane TNF (tmTNF), with a preference for solTNF. TNFR2 is expressed primarily by microglia and endothelial cells and is preferentially activated by tmTNF. TNF-α appears to regulate hippocampal neuronal development as TNF-deficient mice display accelerated maturation of the dentate gyrus and

smaller dendritic trees in the hippocampus (531). TNF-α mRNA was expressed at very low levels in nodose ganglia at E13, E15 and E18 (fig. 40B). However, the expression levels of this mRNA within nodose ganglia increased 7-fold between E18 and P0, before halving between P0 and P5. E13 trigeminal ganglia expressed 3.5-fold more TNF-α mRNA than nodose ganglia of the same age. Like nodose ganglia, the expression levels of this mRNA remained constant within developing trigeminal ganglia between E13 and E18, before increasing 4-fold to reach a peak at P0. The expression levels of TNF-α mRNA within trigeminal ganglia decreased 5-fold between P0 and P5, mirroring the expression pattern of this mRNA in nodose ganglia. The similarity in expression patterns between nodose and trigeminal sensory ganglia suggests that TNF-α is functionally important in the development of sensory neurons. SCG express moderately high levels of TNF-α mRNA at E13 and expression levels fall approximately 65% between E13 and E18. After E18, the expression levels of TNF-α mRNA within SCG double to P5.

BAFF

B cell activation factor (BAFF) (also known as TNFSF13B, Blys, TALL-1 or THANK) is the ligand for BCMA, TACI and BAFF-R. BAFF has been reported to be critical for maintaining the development and homeostasis of normal B-cells (352). BAFF acts as a potent B-cell growth factor and co-stimulator of immunoglobulin (Ig) production. Transgenic overexpression of BAFF in mice results in elevated numbers of mature B cells, and development of autoimmune-like manifestations reminiscent of systemic lupus erythematosus (532). BAFF mRNA was expressed at the highest levels in developing nodose ganglia (fig 41A). From relatively high levels of expression at E13, BAFF mRNA levels in the nodose ganglion increased by 70% to peak at E15. Thereafter, BAFF mRNA expression decreased to E13 levels between E15 and E18, before increasing 3-fold between E18 and P5. E13 trigeminal ganglia expressed 3-fold lower levels of BAFF mRNA compared to E13 nodose ganglia. The expression of this mRNA within trigeminal ganglia doubled from E13 to peak at E15 and then decreased by 40% between E15 and E18. These reduced levels of BAFF mRNA were maintained in the trigeminal ganglion until P5. E13 SCG expressed identical levels of BAFF mRNA to those expressed by E13 trigeminal

ganglia. After E13, BAFF mRNA levels within the developing SCG increased 2.5-fold to peak at E18. Thereafter, mRNA levels decreased slightly between E18 and P0 before being restored to E18 levels by P5.

TWEAK

TNF-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) is the ligand for Fn14. Studies have shown expression of TWEAK in the CNS, and reported that in response to a variety of stimuli, including cerebral ischemia, there is an increase in TWEAK and Fn14 expression in perivascular astrocytes, microglia, endothelial cells, and neurons with subsequent increase in the permeability of the blood-brain barrier (BBB) and cell death (533). TWEAK mRNA was expressed at low, almost constant, levels within trigeminal ganglia over the time period investigated (fig. 41B). E13 nodose ganglia expressed high levels of TWEAK mRNA that increased even further to peak at E15. After E15, TWEAK mRNA levels within nodose ganglia decreased by 3.5-fold to E18, remained constant between E18 and P0 and then increased by 75% between P0 and P5. Interestingly, TWEAK mRNA was expressed in a similar pattern to the mRNA of its receptor, Fn14, in the developing SCG (figs. 41B and 35B). TWEAK mRNA was virtually undetectable in the E13 SCG but its expression levels increased almost 30-fold between E13 and P0, the age of peak expression of Fn14 mRNA. Unlike Fn14 mRNA, the expression levels of TWEAK mRNA increased further between P0 and P5.

LTβ

Lymphotoxin β (LTβ) (TNFSF3) is the ligand for LTβ-R. Little is known about this ligand when compared to other members of the TNF superfamily. Cannela and colleagues (1997) reported expression of LTβ in different glial cell types in the CNS. My developmental RT-QPCR screen (fig 42A) showed very low and practically constant LTβ mRNA levels in the SCG and trigeminal ganglia throughout the developmental period studied. In contrast, LTβ mRNA was expressed at very high levels in the nodose ganglion

at E13. From E13 to P5, the expression levels of this mRNA within nodose ganglia steadily fell by approximately 3-fold.

TL1A

TNF ligand related molecule 1(TL1A, TNFSF15, VEGI) is the ligand for DR3 and the decoy receptor (DcR) 3. TL1A is expressed primarily in endothelial cells. DR3 is expressed mainly on lymphocytes, and its interaction with TL1A can provide a costimulatory signal for the activation of T cells (534). TL1A mRNA was virtually undetectable in developing SCG throughout the period studied (fig. 42B). Although TL1A mRNA was also virtually undetectable in the developing nodose ganglion from E13 to E18, its expression levels increased 6-fold between E18 and P0, a level that was maintained until P5. Despite the increase in the expression of TL1A mRNA from E18, the levels of this mRNA expressed in postnatal nodose ganglia are probably still too low to be functional. TL1A mRNA displays a dramatic expression pattern in developing trigeminal ganglia. Extremely low levels of expression at E13 increase more than 50-fold to peak at E15. From E15, the levels of TL1A mRNA expressed within developing trigeminal decreases by 8-fold to P5. The high levels of TL1A mRNA expressed within E15, E18 and P0 trigeminal ganglia is somewhat curious, since trigeminal ganglia do not appear to express functional levels of DR3 mRNA (fig. 39B) and hence it is unlikely that TL1A will be able to act in a paracrine or autocrine manner to regulate any aspects of neuronal or glial cell development within this ganglia.

4. Growth differentiation factor 5 is a target derived growth factor that is required for correct target innervation by sympathetic neurons *in vivo*.

4.1 Introduction

Nerve growth factor (NGF) is the paradigmatic target-derived neurotrophic factor on which the foundations of neurotrophic theory are based(51) In the developing peripheral nervous system, postganglionic sympathetic neurons and the majority of sensory neurons depend for their survival on a supply of NGF that is synthesized in their targets and conveyed by retrograde axonal transport to their perikarya where it promotes survival signalling (52)(535). Target-derived NGF not only governs the number of sympathetic and sensory neurons that survive a phase of programmed cell death that occurs shortly after axons reach their targets, but also acts locally on axon terminals to promote growth and branching within target tissues(536)(537). While several extracellular signals are known to promote the growth of sympathetic axons and guide them to their targets *in vivo*, such as artemin, NT3 and endothelins (538)(170)(539), NGF is the only *bone fide* target-derived factor shown to be crucial for the axons of NGF-dependent sympathetic and sensory neurons to grow and ramify within their distal target organs and tissues *in vivo*(102)(101).

The PCR screen reported in the previous chapter has identified several potentially novel regulators of sympathetic neuron development, including members of the TGFβ superfamily. In particular, I found high levels of expression of BMPR-Ib and BMPR-II, which form a high affinity complex for GDF5. This factor has been shown to play roles in tendon ligament formation (321)(322) tooth formation (323) morphogenesis of the joints (540) angiogenesis (325), and survival of midbrain dopaminergic neurons (541). GDF5 is mostly expressed at sites of cartilage differentiation in developing limbs, where it may function as a signal for chondrogenesis (310)(542). It has been shown that overexpression of GDF5 increases the length and width of skeletal elements with fused joints (317)(543). Brachypodism (bp) mice carry a functional mutation for the GDF5 gene. Bp mice exhibit a reduction in the length of the limb bones, metacarpals, metatarsals and proximal phalanges, in particular being severely shortened, and the middle phalanges and interphalangeal joints are absent (310)(540)(544).

The *in vitro* and *in vivo* studies of GDF5 in the well-characterized mouse SCG model system reported in this chapter have established that GDF5 is a novel, target-derived

neurotrophic factor for developing SCG neurons that selectively promotes axonal growth and branching and is essential for establishing sympathetic innervation in vivo, but does not promote and is not required for sympathetic neuron survival.

4.2 Results

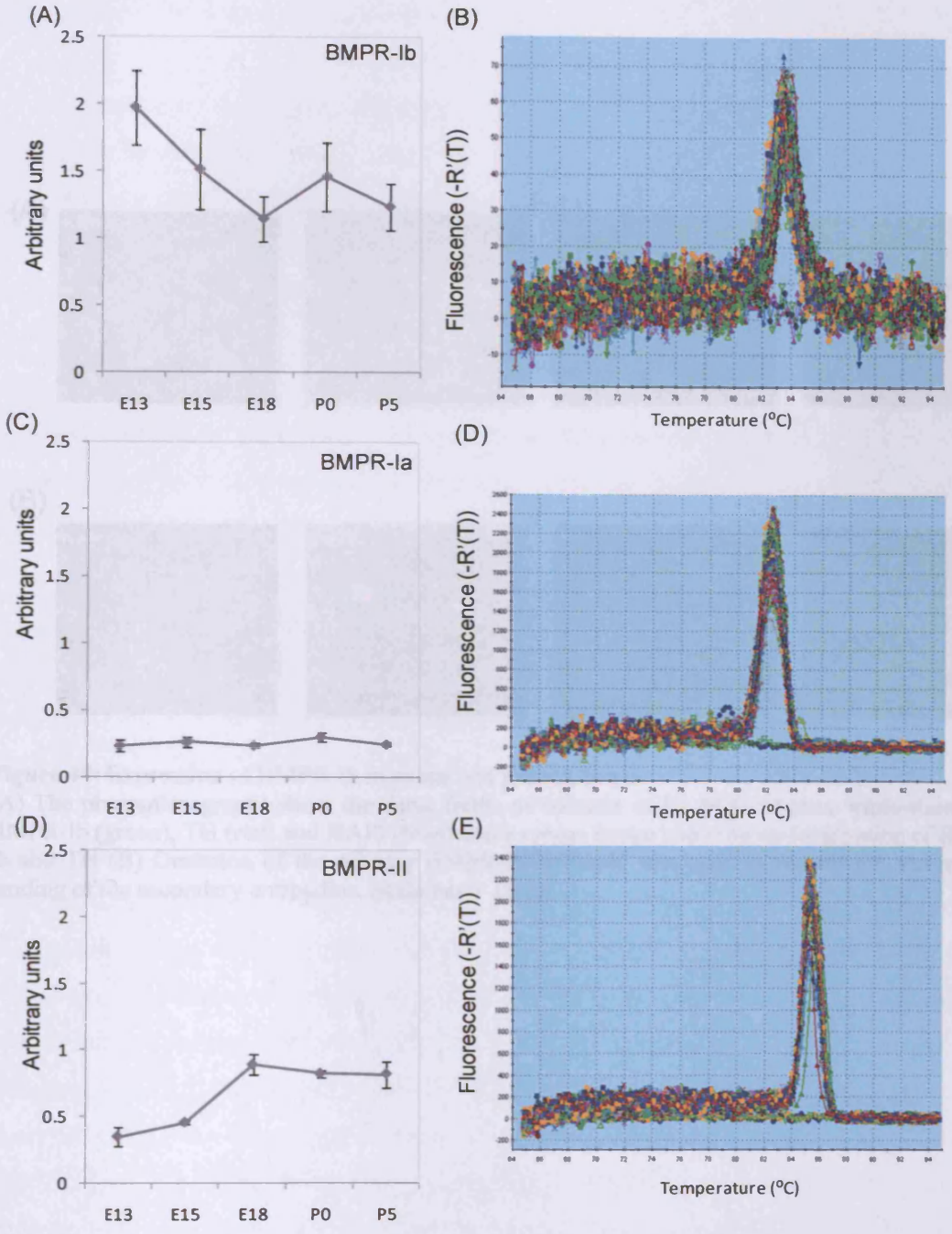


Figure 43: Expression of BMPRs in the developing SCG.
The data show the relative expression of (A) BMPR-Ib, (B) BMPR-Ia and (C) BMPR-II mRNA relative to GAPDH mRNA (in arbitrary units) and the respective Real time PCR melting curves (showing primer specificity) in E13, E15, E18, P0 and P5 SCG. Means and standard errors of data from three separate sets of ganglia at each age are shown.

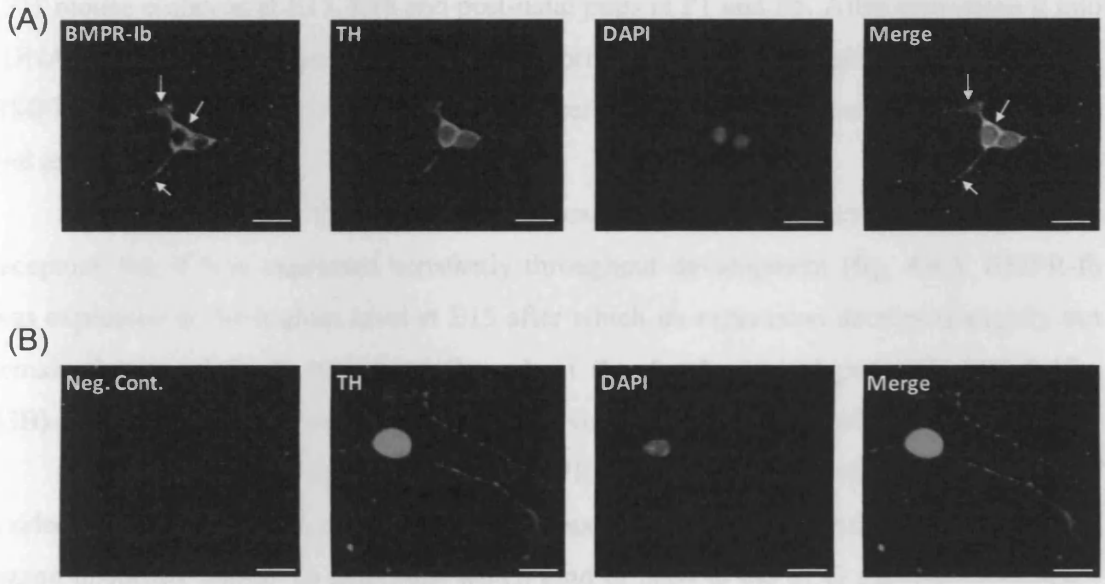


Figure 44: Expression of BMPR-Ib in axons and growth cones.
(A) The photomicrographs show the same fields of cultures of P1 SCG neurons triple-stained for BMPR-Ib (green), TH (red) and DAPI (blue) and a merge image showing co-localisation of BMPR-Ib and TH (B) Omission of the primary BMPR-Ib antibody was used to control for non-specific binding of the secondary antibodies. Scale bar = 10μm.

Expression of BMPRs in the developing SCG

I started by quantifying mRNA levels for the BMP receptors in the SCG at intervals throughout development by real time PCR. Briefly, RNA was extracted from the SCG of CD1 mouse embryos at E15, E18 and post-natal pups at P1 and P5. After converting it into cDNA, real time PCR was carried out using primers specifically designed for BMPR-II, BMPR-Ia and BMPR-Ib (for details on sequences and annealing temperatures see materials and methods section 2.8).

My results show that BMPR-Ia was expressed at the lowest level of all three receptors, but it was expressed constantly throughout development (fig. 43C). BMPR-Ib was expressed at the highest level at E15 after which its expression decreased slightly but remained at a relatively high level throughout the developmental period I studied (fig. 43B). BMPR-II was expressed at a consistent level at all ages examined (fig. 43A).

The high level of expression of the BMPR-Ib receptor, along with the availability of a selective ligand (GDF5), gave me the tools to examine the function of this receptor and its ligand in further detail. To determine which kind of cells in the SCG express BMPR-Ib, I used immunohistochemistry to identify this receptor in dissociated SCG cultures from P1 mice. These cultures were grown overnight in medium supplemented with NGF before being fixed and processed for triple immunohistochemistry. In these preparations neurons were positively identified with antibodies to TH, and cell nuclei were labelled with the fluorescent nuclear marker DAPI. BMPR-Ib was expressed on the cell soma of all neurons (fig. 44A). Furthermore I found strong BMPR-Ib expression on developing axons and growth cones, raising the possibility that growing axons may be able to respond to endogenous GDF5 that might be expressed en-route to or within their target tissues. Non-neuronal cells showed no expression of BMPR-Ib (data not shown). To control the specificity of the BMPR-Ib antibody, a negative control was used in which the BMPR-Ib antibody was omitted from the procedure. In these preparations no staining was evident (fig. 44B), indicating the specificity of the BMPR-Ib staining shown in fig. 44A.

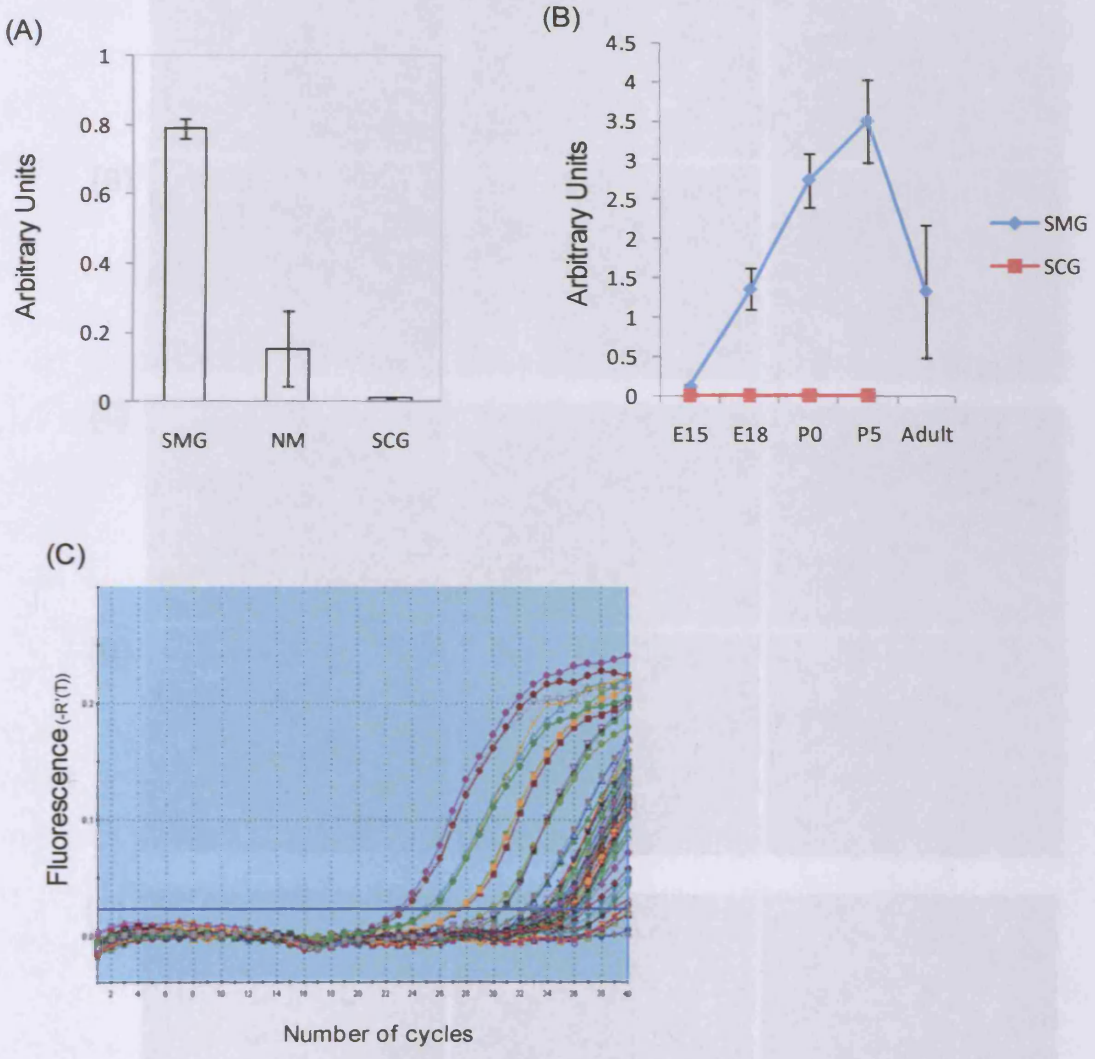


Figure 45: Expression of GDF-5 in SCG compared to SCG target fields.
(A) The data show the level of GDF-5 mRNA in the submandibular gland (SMG), nasal mucosa (NM) and SCG relative to GAPDH mRNA at P0 in wild-type CD-1 mice. (B) Data show the relative expression of GDF5 in SMG (blue) compared with SCG (red). (C) Data shows amplifications plots for GDF5 real time PCR of the SMG time course. Means and standard errors of data from three separate sets of ganglia at each age are shown.

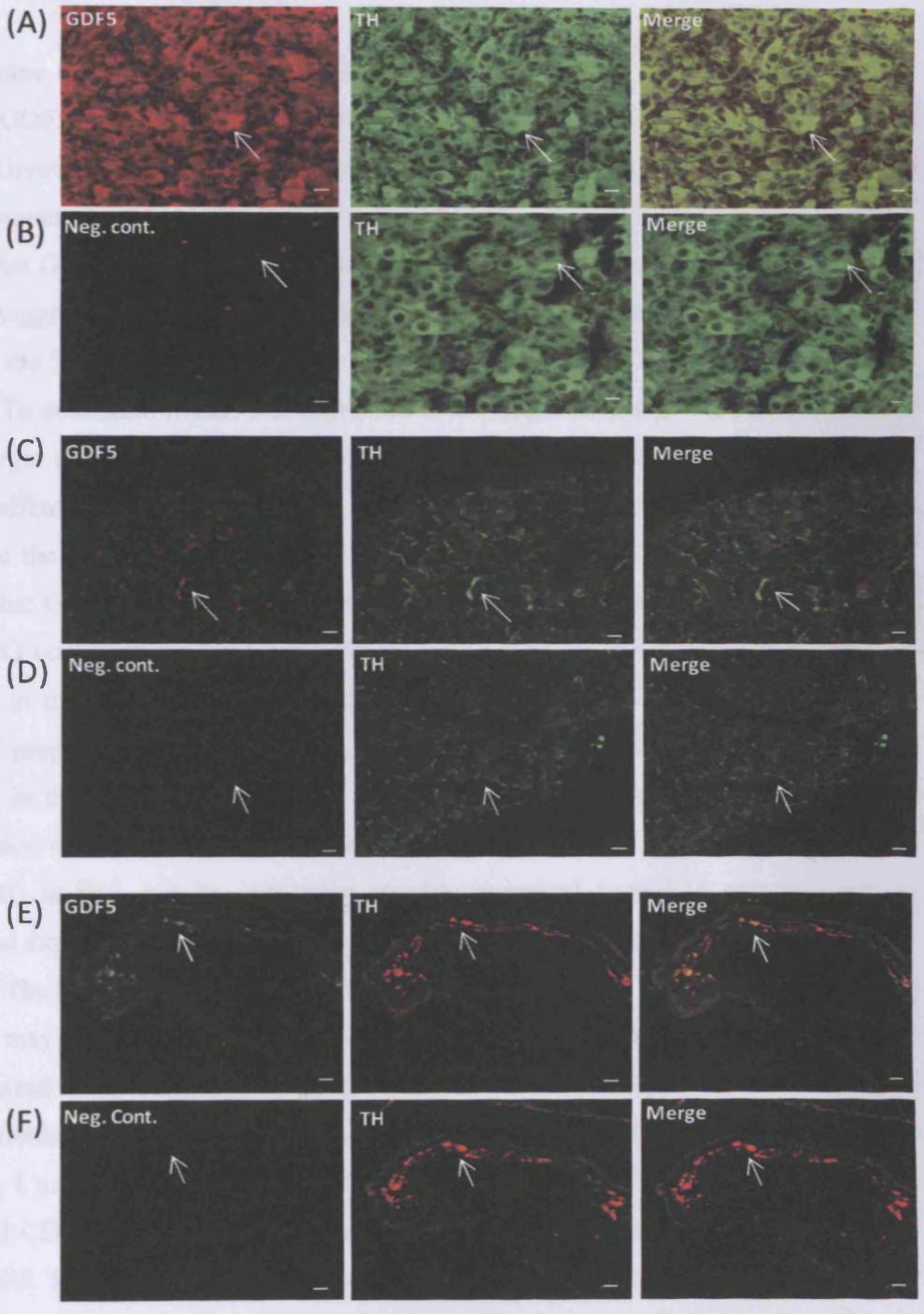


Figure 46: Expression of GDF5 in the SCG target tissues.
Photomicrographs show consecutive sections of SCG (A), SMG (C) and NM (E) double stained for GDF5 (red in (A) and (C) , green in (E)) and TH (green in (A) and (C) , red in (E)) . Merge image showing co-localization of GDF5 and TH. Omission of the primary GDF5 antibody was used to control for non-specific binding of the secondary antibodies (B, D, F). Scale bar = 10μm.

Expression of GDF5 in the developing SCG and targets

GDF5 has been shown to be a selective ligand for BMPR-Ib but not BMPR-Ia (461). Given this information, and the high expression of BMPR-Ib in the SCG during development, I used real time PCR to quantify the level of GDF5 mRNA in the SCG. I found that GDF5 mRNA was only just above the detection limit of real time PCR program, which suggests GDF5 is either not synthesized at all or synthesized at an extremely low level in the SCG (fig. 45A, 45B).

To determine if GDF5 is expressed in the target tissues of the SCG, I dissected two such target tissues that receive a dense sympathetic innervation from the SCG (545), the submandibular gland (SMG) and the nasal mucosa (NM). I carried out a direct comparison between the expression of GDF5 in the P0 SCG, SMG and NM by real time PCR, and found that GDF5 was clearly expressed in these targets with about a 7 fold higher level in the SMG compared with the NM relative to GAPDH mRNA whereas the level of GDF5 mRNA in the SCG was barely detectable (fig. 45A). Due to the difficulty of obtaining a "clean" preparation of nasal mucosa, I focused on characterizing the expression of GDF5 mRNA in the SMG. I carried out a real time PCR experiment looking at GDF5 mRNA expression in the SCG and SMG over a range of ages. GDF5 mRNA was not expressed in the SMG at E15, but its expression steadily increased from E15 onwards, reaching its maximal expression at P5 and then decreased in adulthood (fig. 45B).

The high expression of GDF5 mRNA in the SMG but not in the SCG suggested that GDF5 may act in a similar way to NGF developing sympathetic neurons, i.e, being synthesized in the target fields of these neurons and be retrogradely transported from the target fields to the neuron cell bodies of the SCG (546). To investigate this possibility further, I used two approaches. Firstly I dissected the SMG, SCG and the nasal mucosa from P1 CD1 mice and processed them for immunohistochemical analysis using antibodies for GDF5. To show the innervating sympathetic axons, I also. stained the tissue for tyrosine hydroxylase (TH – marker for noradrenergic neurons). For all immunohistochemistry, a negative control was included to control non-specific binding of the primary GDF5 antibody.

In the nasal mucosa, I found strong expression of GDF5 protein in the nasal epithelium (fig. 46E), in the region innervated by TH-positive axons (fig.46F). Furthermore, I also found strong expression of GDF5 in SMG (fig. 45C). Although I found no significant amount of GDF5 mRNA in the SCG itself, I examined the SCG by immunohistochemistry. Interestingly I found that GDF5 was abundantly present in the SCG at P5 and co-localized with TH (fig. 46A). Sections from which the GDF5 primary antibody was omitted from the staining procedure, showed little or no background staining, indicating the specificity of the primary antibody (fig. 46B, 46D, 46F). To confirm the presence of GDF5 protein in the SCG, I used a different antibody against GDF5 (MP-52), which has previously been shown to recognize both precursor and mature forms of GDF5 by western blot (547). In extracts of P0 SCG analyzed by western blot, this antibody recognized two high molecular isoforms of GDF5 which correspond to those in a cell extract of GDF5 expressing CHO cells, which were used as a positive control (fig. 47). This finding agrees with the immunohistochemical data showing the presence of GDF5 protein in the SCG. These findings when taken in conjunction with the real time PCR data examining the expression of GDF5 mRNA, suggest that GDF5 is synthesized in the target fields of the SCG neurons, but not in the neurons themselves, and is taken up by the axons of the innervating neurons and conveyed by retrograde transport from the targets to the cell bodies of the neurons in the SCG.

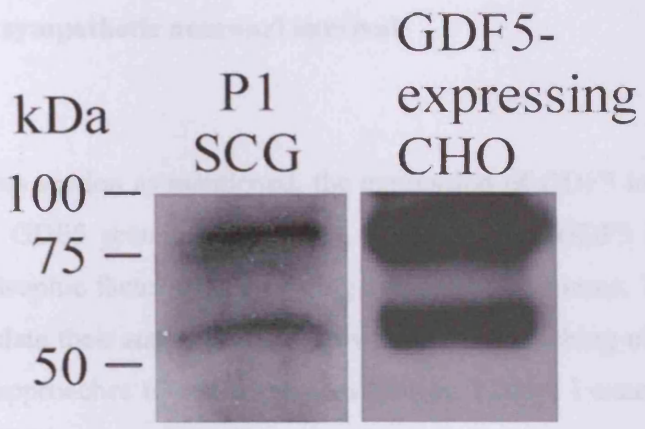


Figure 47: GDF5 protein is present in the SCG.
P0 SCG extracts grown overnight and further analyzed by western blot, showing the presence of two high molecular isoforms of GDF5 that correspond to those in a cell extract of GDF5 expressing Chinese Hamster Ovary (CHO) cell line.

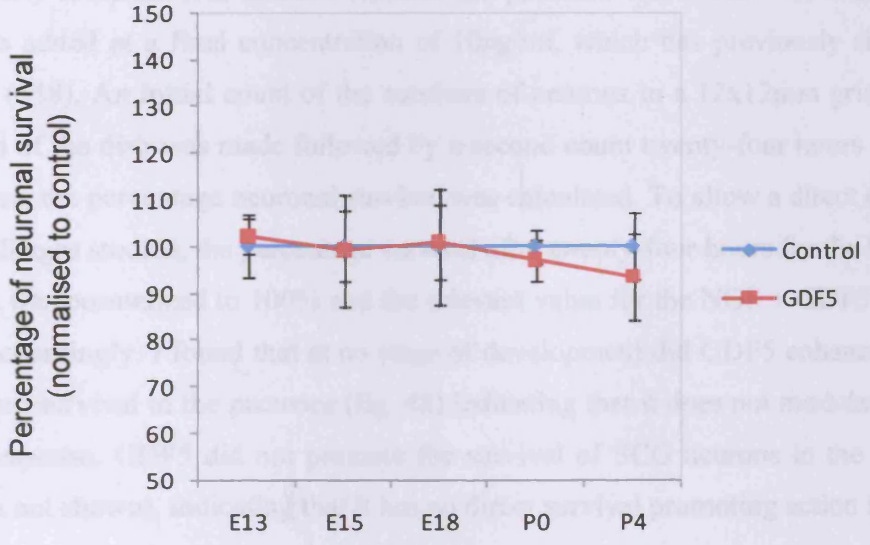


Figure 48: Effect of GDF-5 on sympathetic neuron survival.
The data show SCG neurons time course survival (24h) comparison between neurons grown only with 10ng/ml of NGF (control normalized to 100%) and 10ng/ml of NGF plus 10ng/ml plus 10ng/ml of GDF5 at a number of different stages of development between E13 and P4.
Means and standard errors of data from three separate sets of ganglia at each age are shown.

Effect of GDF-5 on sympathetic neuronal survival

In the previous section as mentioned, the expression of GDF5 in SCG target fields and the presence of GDF5 protein in the SCG, suggested that GDF5 may function as a target-derived neurotrophic factor for developing sympathetic neurons. This implies that it may potentially regulate their survival or the growth and/or branching of the axons in their targets. I used two approaches to test these possibilities. Firstly, I examined the effect of adding exogenous GDF5 on sympathetic neuronal survival and axon growth in cultures of P1 SCG neurons and secondly, carried out comparative studies of neuronal survival and sympathetic innervation density in wild type mice and GDF-5 deficient mice.

To investigate the possible effect of GDF5 on sympathetic neuron survival, I cultured SCG neurons from a number of developmental ages (E13, E15, E18, P0 and P4) at a low density in serum-free defined medium the presence and absence of 10ng/ml NGF. GDF5 was added at a final concentration of 10ng/ml, which has previously shown to be saturating (548). An initial count of the numbers of neurons in a 12x12mm grid placed on the bottom of the dish was made followed by a second count twenty-four hours later. From these values, the percentage neuronal survival was calculated. To allow a direct comparison between all ages studied, the percentage survival after twenty-four hours for the NGF group at all ages was normalized to 100% and the relevant value for the NGF + GDF5 group was adjusted accordingly. I found that at no stage of development did GDF5 enhance or inhibit SCG neuron survival in the presence (fig. 48) indicating that it does not modulate the NGF survival response. GDF5 did not promote the survival of SCG neurons in the absence of NGF (data not shown), indicating that it has no direct survival promoting action itself.

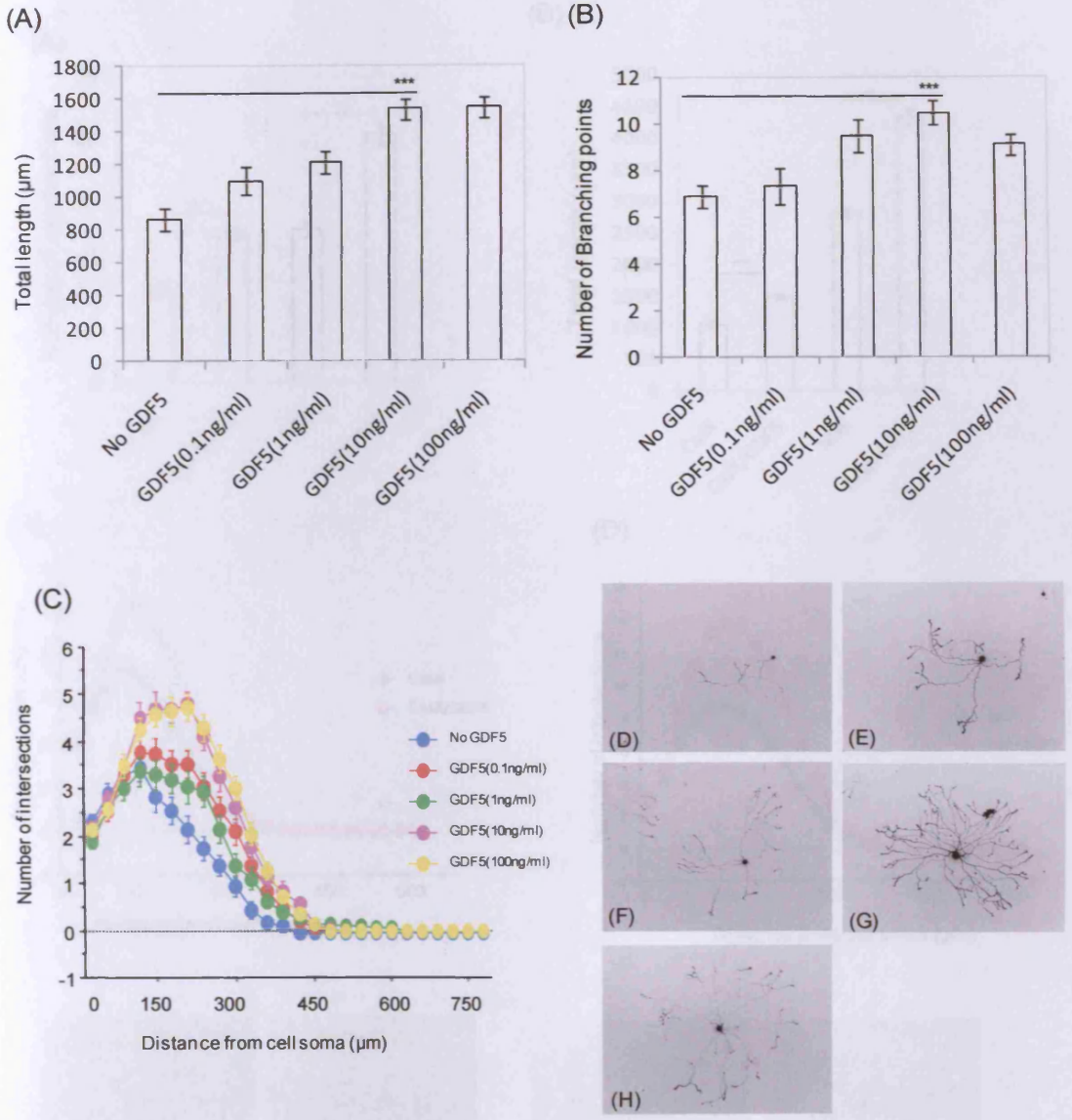


Figure 49: GDF5 dose response.
Branching (A), length (B) and sholl analysis (C) of the neurite arbors of P0 SCG neurons grown overnight with Caspase inhibitors, plus or minus several concentrations of GDF5 ranging from 0 to 100ng/ml. (D) to (H) show illustrative drawings of neuronal growth in the different experimental conditions.
Data from at least 150 neurons in each condition from three independent experiments are shown (*** indicates $p<0.001$, statistical comparison with control, one-way ANOVA with Fisher's *post hoc*).

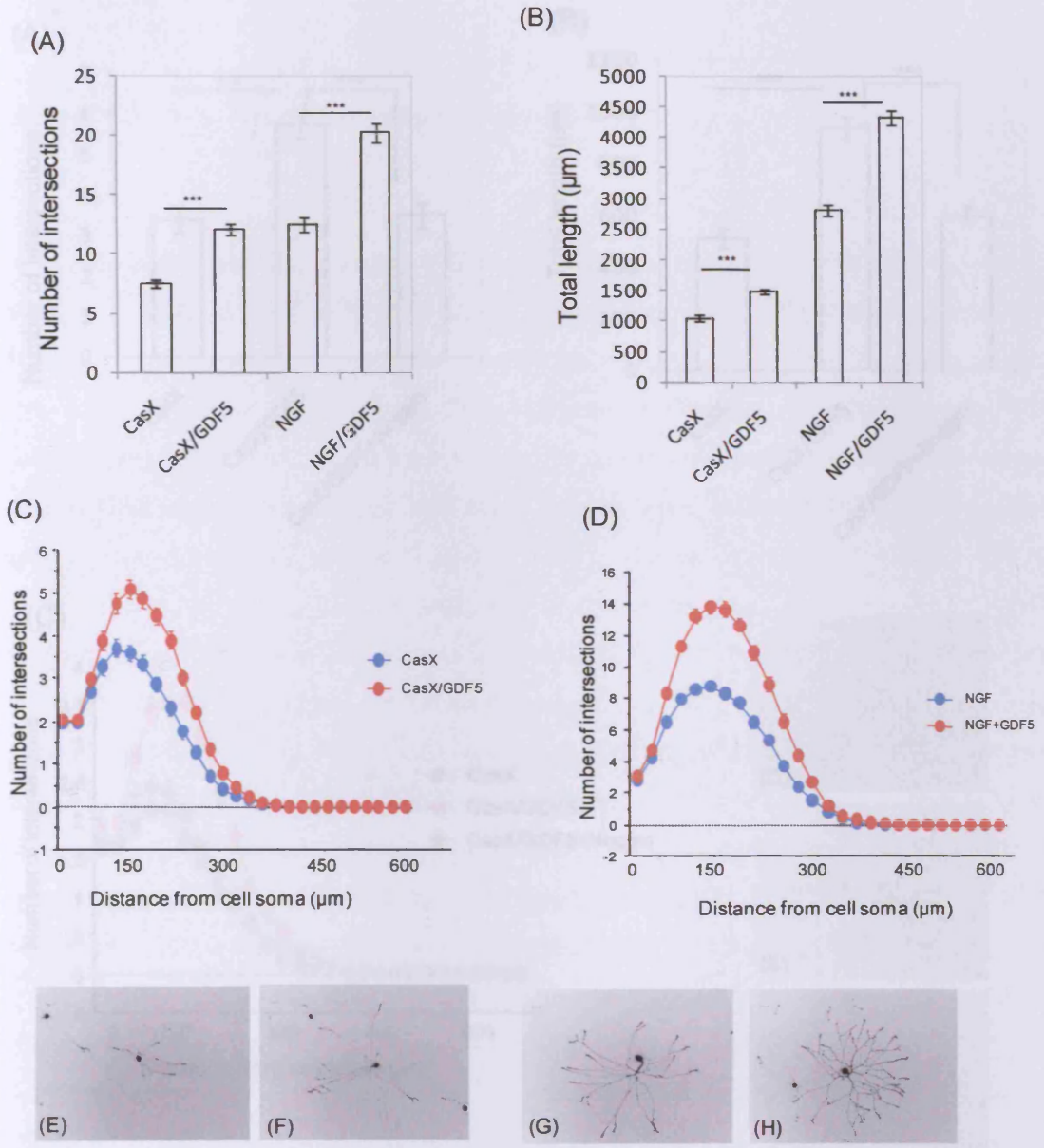


Figure 50: Effect of GDF-5 on sympathetic neurite growth.
Branching (A), length (B) and sholl analysis (C,D) of the neurite arbors of P0 SCG neurons grown overnight with Caspase inhibitors or NGF plus or minus GDF5. (E) to (H) show illustrative drawings of neuronal growth in the different experimental conditions.
Data from at least 150 neurons in each condition from three independent experiments are shown (*** indicates $p<0.001$, statistical comparison with control, one-way ANOVA with Fisher's *post hoc*).

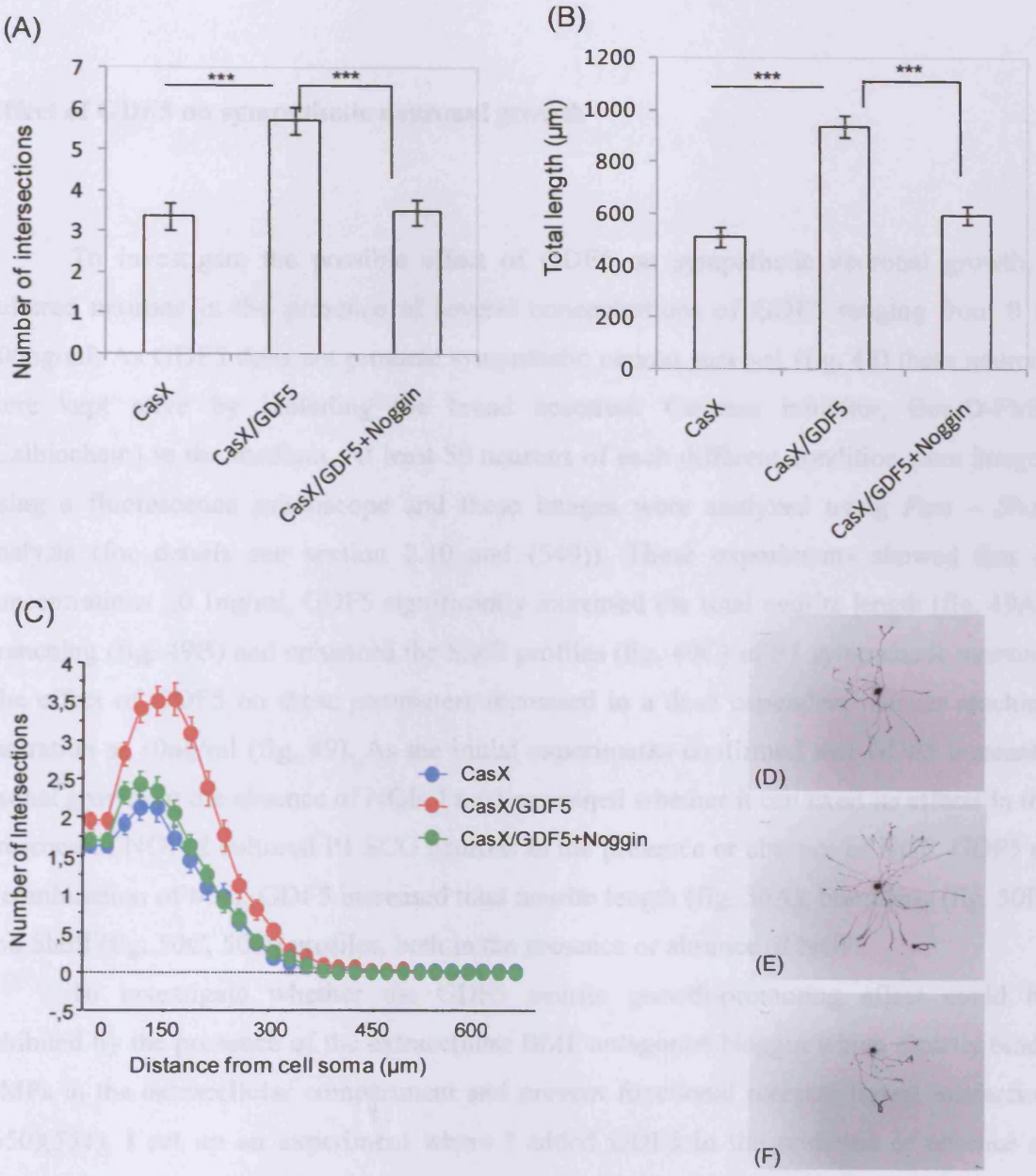


Figure 51: Noggin blocks effects of GDF-5 on neurite growth.
Branching (A), length (B) and sholl analysis (C) of the neurite arbors of P0 SCG neurons grown overnight with NGF, NGF+GDF5, and NGF + Noggin. (D) to (F) show illustrative drawings of neuronal growth in the different experimental conditions.
Data from at least 150 neurons in each condition from three independent experiments are shown (*** indicates $p<0.001$, statistical comparison with control, one-way ANOVA with Fisher's *post hoc*).

Effect of GDF5 on sympathetic neuronal growth

To investigate the possible effect of GDF5 on sympathetic neuronal growth, I cultured neurons in the presence of several concentrations of GDF5 ranging from 0 to 100ng/ml. As GDF5 does not promote sympathetic neuron survival (fig. 48) these neurons were kept alive by including the broad spectrum Caspase inhibitor, Boc-D-FMK (Calbiochem) in the medium. At least 50 neurons of each different condition were imaged using a fluorescence microscope and these images were analyzed using *Fast – Sholl* analysis (for details see section 2.10 and (549)). These experiments showed that at concentrations ≥0.1ng/ml, GDF5 significantly increased the total neurite length (fig. 49A), branching (fig. 49B) and enhanced the Sholl profiles (fig. 49C) of P1 sympathetic neurons. The effect of GDF5 on these parameters increased in a dose dependent manner reaching saturation at 10ng/ml (fig. 49). As the initial experiments confirmed that GDF5 increased axonal growth in the absence of NGF, I next examined whether it can exert its effects in the presence of NGF. I cultured P1 SCG neurons in the presence or absence of NGF, GDF5 or a combination of both. GDF5 increased total neurite length (fig. 50A), branching (fig. 50B) and Sholl (fig. 50C, 50D) profiles, both in the presence or absence of NGF.

To investigate whether the GDF5 neurite growth-promoting effect could be inhibited by the presence of the extracellular BMP antagonist Noggin which directly binds BMPs in the extracellular compartment and prevent functional receptor/ligand interaction (550)(551), I set up an experiment where I added GDF5 in the presence or absence of Noggin in cultures of P1 SCG neurons. Analysis 24h later revealed that Noggin, presumably by binding to GDF5, inhibited the effects of GDF5 on neurite length (fig. 51A), branching (fig. 51B) and Sholl (fig. 51C) profiles in P1 SCG neurons.

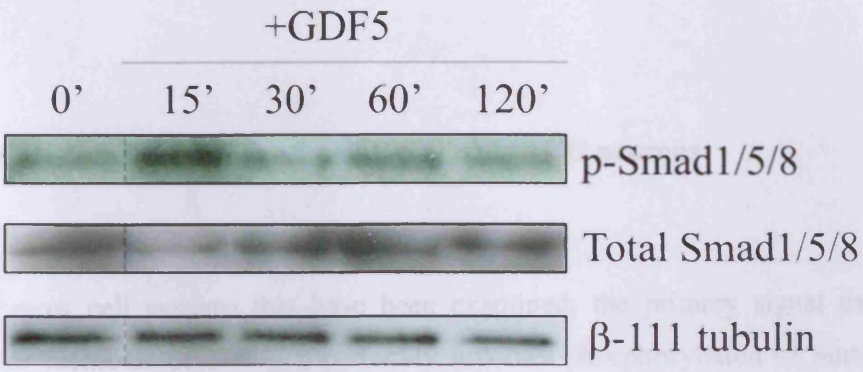


Figure 52: GDF5 activates Smad pathway in sympathetic neurons.
Western blots showing phospho- Smad 1/5/8, total Smad 1/5/8 and β-III tubulin in SCG neurons from CD1 mice grown overnight in medium containing Boc-D-FMK before exposure to 10ng/ml GDF5 (time ranging from 0 to 120min). Results show activation of phospho-Smad 1/5/8 after 15min exposure.

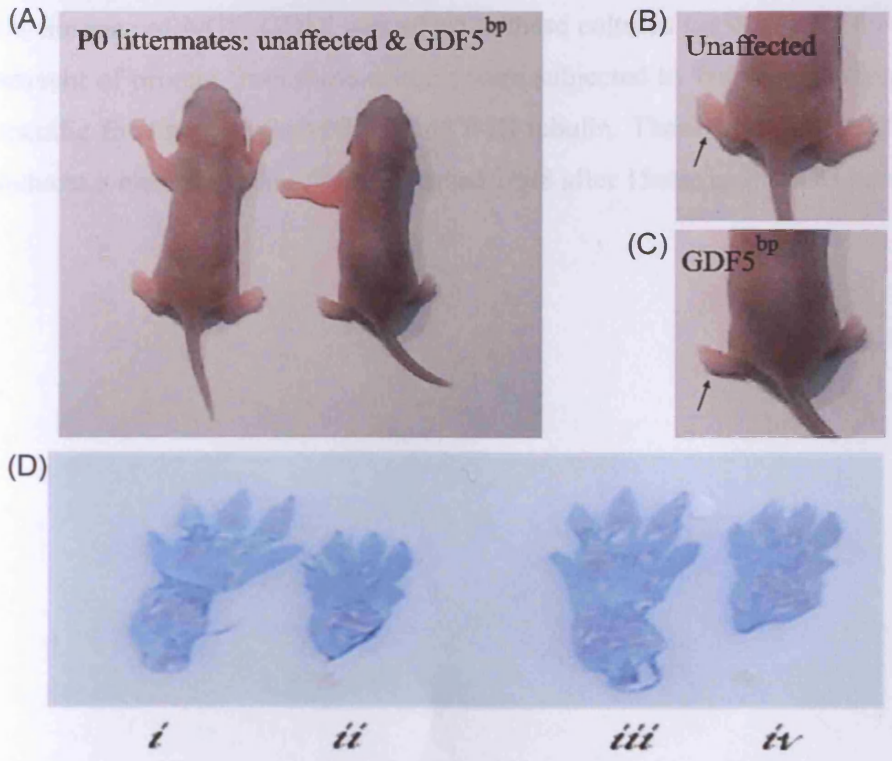


Figure 53: Brachypod mice show peripheral skeletal malformations
Picture of P0 $GDF5^{cont}$ and $GDF5^{bp}$ littermates with obvious skeletal differences. (A) Overall view, (B) and (C) detailed view of the limb and (D) Cresyl Violet staining for unaffected (*i, iii*) and $GDF5^{bp}$ (*ii, iv*) limbs from these animals.

Effect of GDF5 on intracellular Smad signaling in SCG neurons

In most cell systems that have been examined, the primary signal transduction pathway for members of TGF-β superfamily involves phosphorylation of Smad proteins (for review see (552)). GDF5 (like BMPs) has been shown to phosphorylate Smad 1/5/8 upon receptor binding (553). To examine whether GDF5 induces Smad-phosphorylation in SCG neurons, I set up cultures of P1 SCG neurons and grew them overnight in medium containing the broad spectrum Caspase inhibitor. Boc-D-FMK, to keep the neurons alive in the absence of NGF. GDF5 was added to these cultures for 0, 15, 30, 60 or 120min. Equal amount of protein from these cultures were subjected to Western blotting using antibodies specific for Phospho-Smad 1/5/8 and β-III tubulin. These experiments showed that GDF5 induces a clear phosphorylation of Smad 1/5/8 after 15min in P1 SCG neurons (fig. 52).

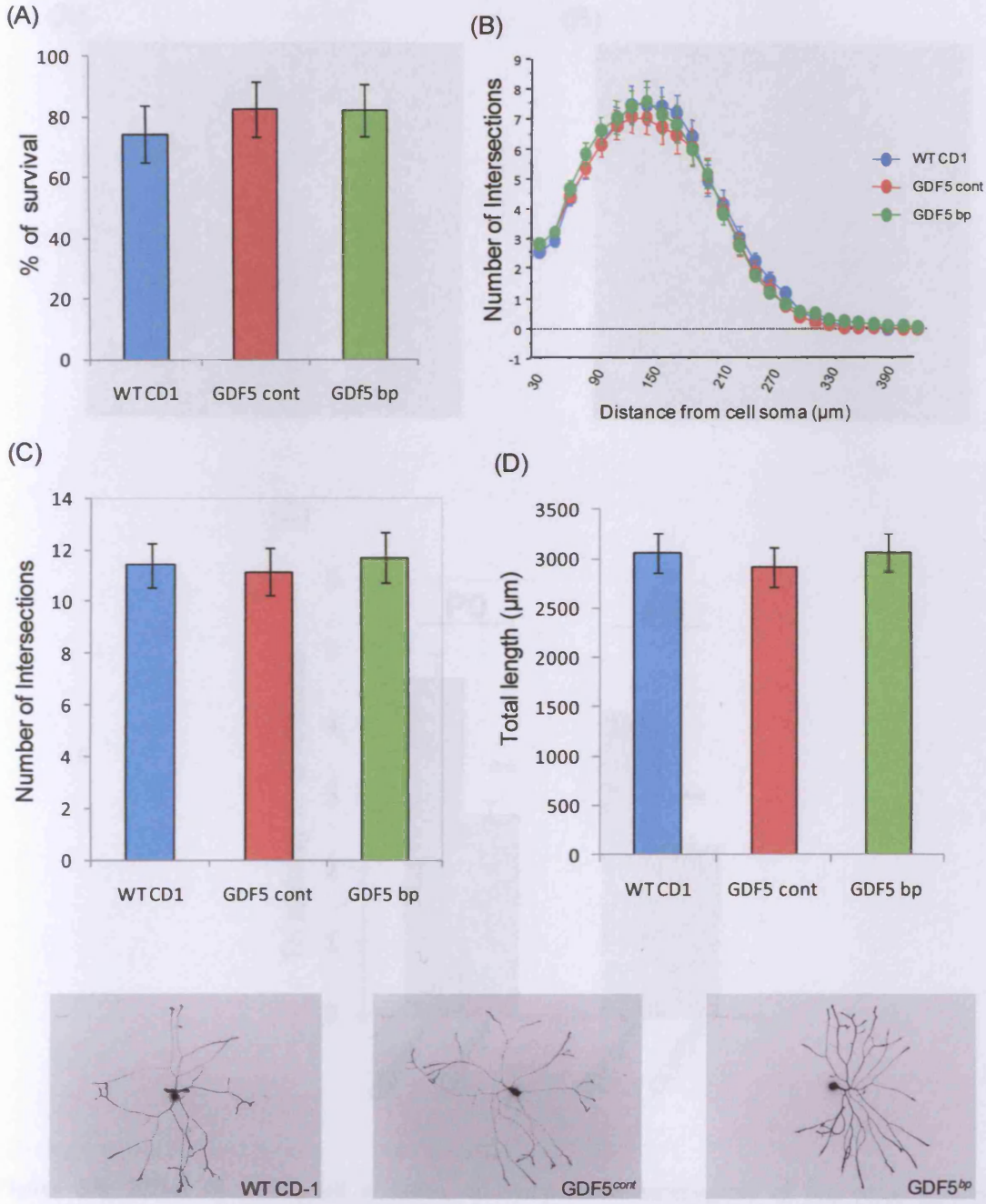


Figure 54: Effect of brachypod mutation on sympathetic axon growth in vitro.
The data show (A) survival, (B) sholl analysis, (C) branching and (D) total length of P0 SCG neurons, grown overnight in the presence of NGF.
Data from at least 150 neurons in each condition from three independent experiments are shown (statistical comparison with control, one-way ANOVA with Fisher's *post hoc*)

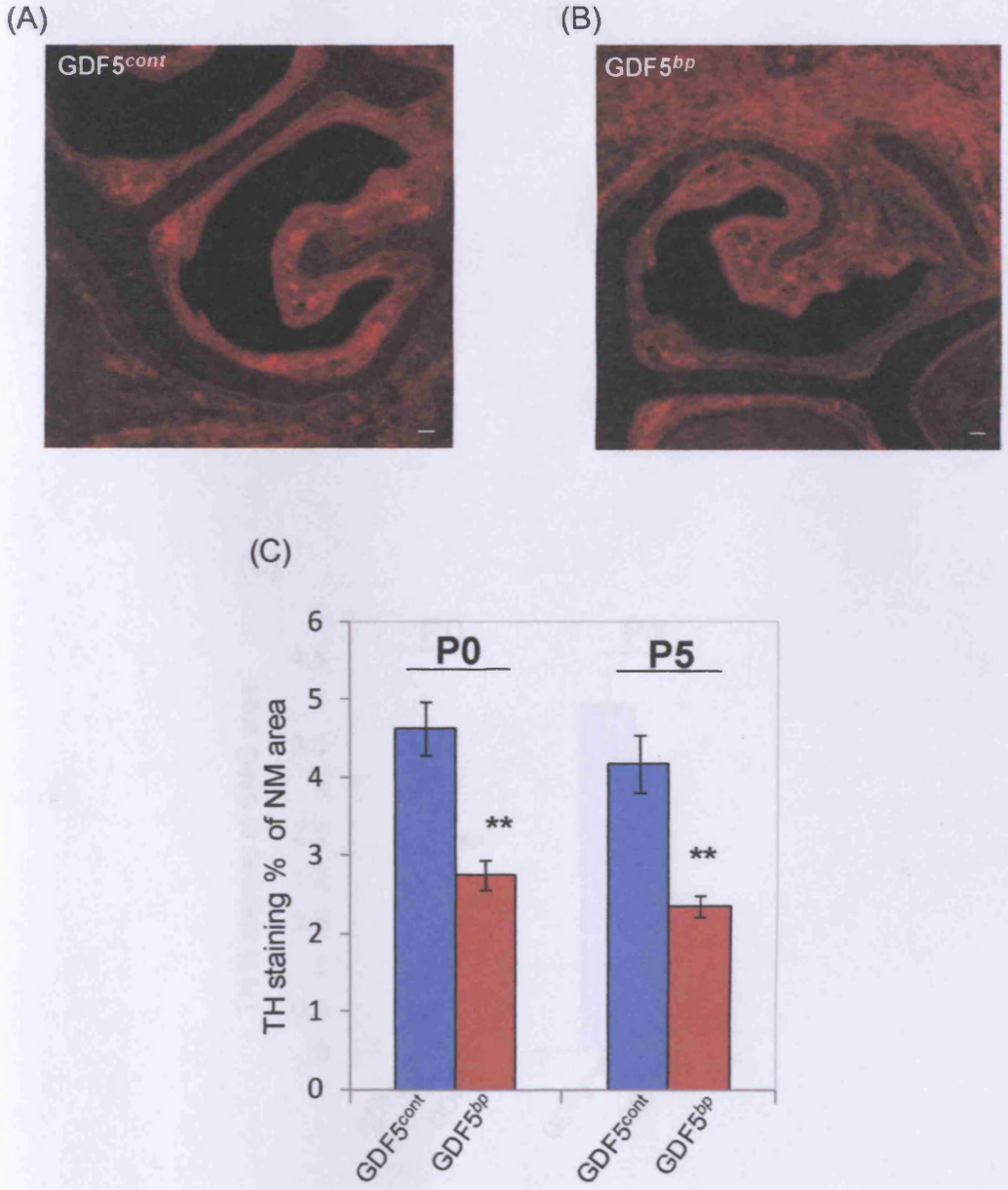


Figure 55: Effect of brachypod mutation on sympathetic innervation of the nasal mucosa. Sympathetic innervation density in $GDF5^{bp}$ and $GDF5^{cont}$ mice. Bar chart showing the percentage area of selected nasal mucosa stained for tyrosine hydroxylase in P0 and P5 mice.
(** indicates $p<0.001$, statistical comparison with $GDF5^{cont}$, ANOVA with Fisher's post hoc n=3 per genotype). Scale bar = 10µm
The photomicrographs show typical tyrosine hydroxylase stained sections of nasal mucosas of $GDF5^{cont}$ (A) and $GDF5^{bp}$ (B).

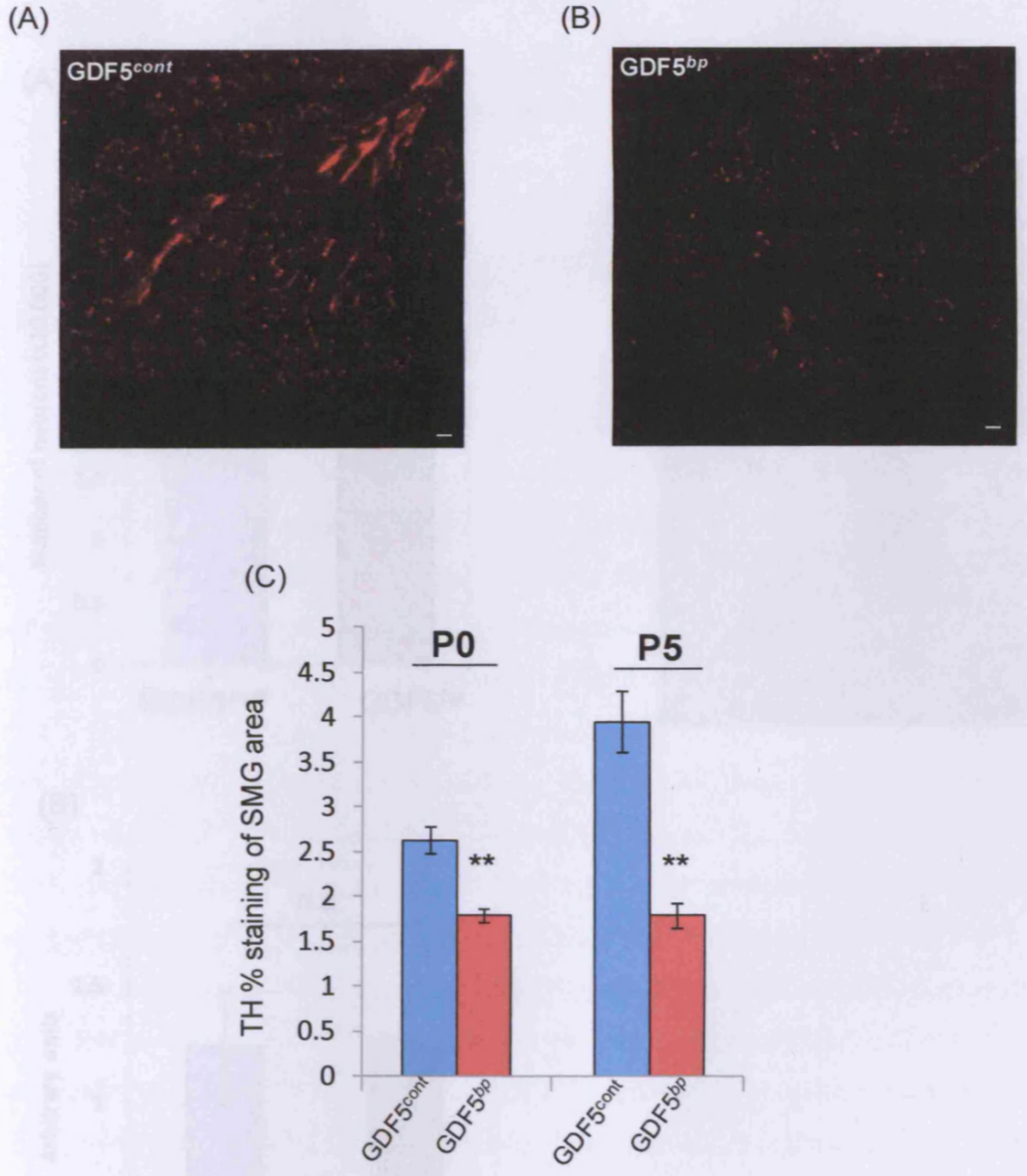


Figure 56: Effect of brachypod mutation on sympathetic innervation of the SMG.
Sympathetic innervation density in GDF5^{bp} and GDF5^{cont} mice. Bar chart showing the percentage area of selected SMG stained for tyrosine hydroxylase in P0 and P5 mice.
(** indicates $p<0.001$, statistical comparison with GDF5^{cont}, ANOVA with Fisher's *post hoc* n=3 per genotype). Scale bar = 10μm
The photomicrographs show typical tyrosine hydroxylase stained sections of SMG of GDF5^{cont} (A) and GDF5^{bp} (B).

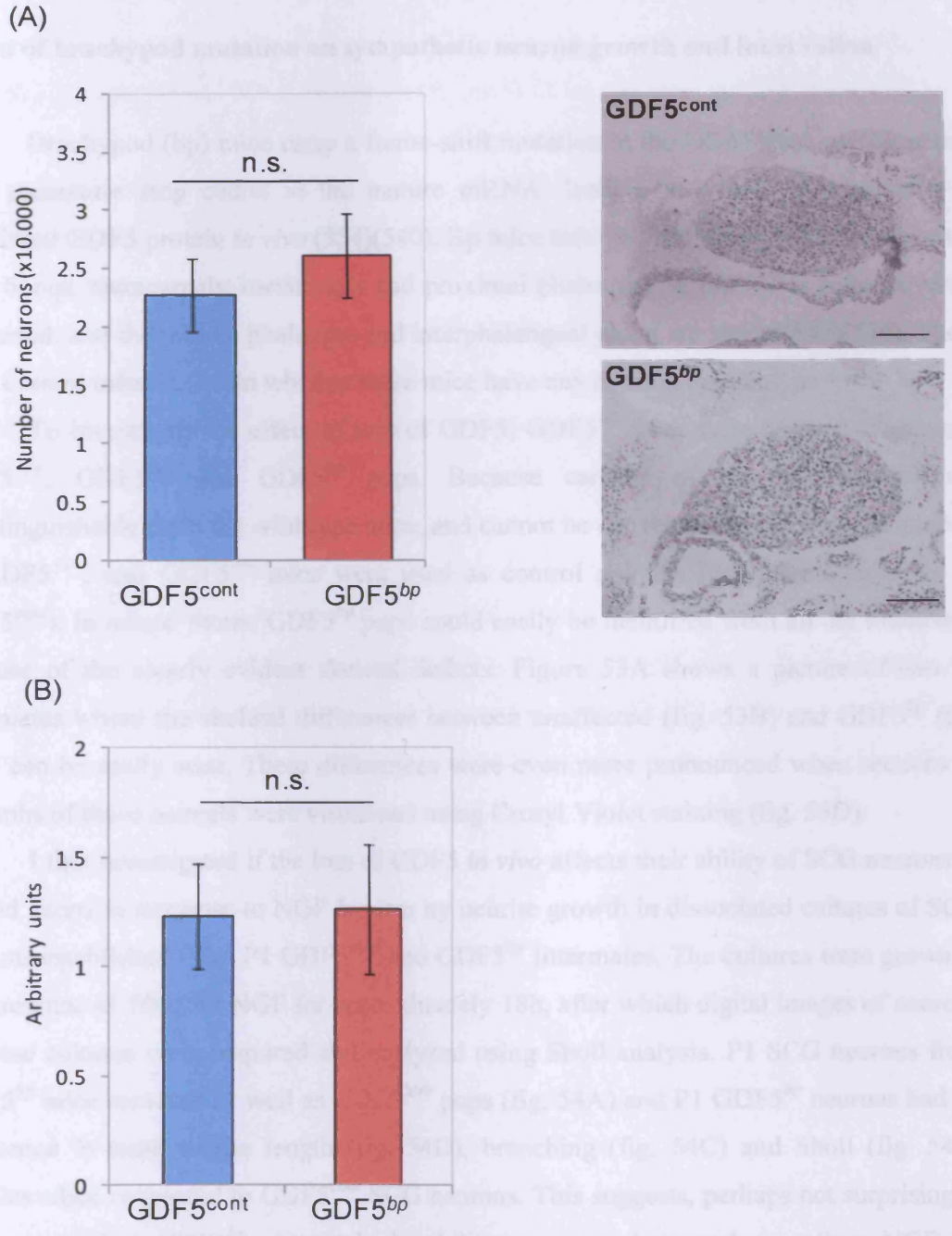


Figure 57: Reduction in innervation is not due to a loss of neurons or a loss of TH Expression.
(A) Estimates of neuronal number in the SCG of P5 GDF5^{bp} and GDF5^{cont} littermates. (B) The level of tyrosine hydroxylase mRNA relative to GAPDH mRNA (arbitrary units) in P0 SCG dissected from GDF5^{bp} and GDF5^{cont} littermates is shown.
Mean ± s.e.m. of data from five mice of each genotype are shown.

Effect of brachypod mutation on sympathetic neuron growth and innervation

Brachypod (bp) mice carry a frame-shift mutation in the GDF5 gene, which results in a premature stop codon in the mature mRNA, leading to a loss of expression of functional GDF5 protein *in vivo* (554)(540). Bp mice exhibit a reduction in the length of the limb bones, metacarpals, metatarsals and proximal phalanges, in particular being severely shortened, and the middle phalanges and interphalangeal joints are absent(540)(554). There is no current information in whether these mice have any neuronal phenotype.

To investigate the effect of loss of GDF5, $GDF5^{+/-}$ mice were crossed to generate $GDF5^{+/+}$, $GDF5^{+/-}$ and $GDF5^{bp}$ pups. Because carriers of the bp mutation are indistinguishable from the wild-type mice, and cannot be conveniently genotyped a mixture of $GDF5^{+/+}$, and $GDF5^{+/-}$ mice were used as control animals (hereafter referred to as $GDF5^{cont}$). In mixed litters, $GDF5^{bp}$ pups could easily be identified from all the littermates because of the clearly evident skeletal defects. Figure 53A shows a picture of two P0 littermates where the skeletal differences between unaffected (fig. 53B) and $GDF5^{bp}$ (fig. 53C) can be easily seen. These differences were even more pronounced when sections of P0 limbs of these animals were visualized using Cresyl Violet staining (fig. 53D).

I first investigated if the loss of GDF5 *in vivo* affects their ability of SCG neurons to extend axons in response to NGF *in vitro* by neurite growth in dissociated cultures of SCG neurons established from P1 $GDF5^{cont}$ and $GDF5^{bp}$ littermates. The cultures were grown in the presence of 10ng/ml NGF for approximately 18h, after which digital images of neurons in these cultures were acquired and analyzed using Sholl analysis. P1 SCG neurons from $GDF5^{bp}$ mice survived as well as $GDF5^{cont}$ pups (fig. 54A) and P1 $GDF5^{bp}$ neurons had no difference in total neurite length (fig. 54D), branching (fig. 54C) and Sholl (fig. 54B) profiles when compared to $GDF5^{cont}$ SCG neurons. This suggests, perhaps not surprisingly, that neurons from $GDF5^{bp}$ mice retain the ability to grow and respond normally to NGF. To determine if the loss of GDF5 *in vivo*, affects sympathetic innervation in SCG target tissues, I compared sympathetic innervation density in $GDF5^{cont}$ and $GDF5^{bp}$ littermates. The nasal mucosa and SMG normally receive a dense sympathetic innervation from the

SCG that can be easily identified and quantified by immunohistochemistry for tyrosine hydroxylase (555). At P0 and P5, there was a very significant reduction in innervation in NM (fig. 55) and SMG (fig. 56), in GDF5^{bp} mice when compared with their littermates ($p<0.001$). Furthermore, these defects in sympathetic innervation were still apparent at P5 (fig. 55 and fig. 56). To rule out the possibility that the reduction in the sympathetic innervation density in GDF5^{bp} mice was due to a down-regulation of tyrosine hydroxylase (TH) expression in SCG neurons, I quantified the TH mRNA levels and TH immunofluorescence in the SCG of GDF5^{cont} and GDF5^{bp} littermates. The results showed no significant differences in the levels of either TH mRNA or TH immunofluorescence in the SCG of these mice (fig. 57B), supporting the idea that the reduction the sympathetic innervation is the result of a reduction in the number or branching of sympathetic axons in the targets.

The reduction in sympathetic innervation density could not only occur as a result of defective axonal growth and branching, but also from a loss of the numbers of innervating neurons in the SCG. To determine if a loss of SCG neurons contributes to the innervation defect, I counted the numbers of neurons in the SCG of GDF5^{cont} and GDF5^{bp} littermates at P5 and found no significant difference in neuronal number (fig. 57A). Taken together these results suggest that GDF5 is required for the correct establishment of sympathetic innervation by SCG neurons, but is not required for survival of these neurons *in vivo*.

4.3 Discussion

I have discovered that GDF5 is a novel target-derived factor for developing sympathetic neurons that promotes growth and branching of their axons and is crucial for the establishment of appropriate sympathetic innervation density *in vivo*. GDF5 acts independently of NGF, the only other target-derived factor shown to be required for the growth and ramification of sympathetic axons in their distal target organs *in vivo* (102), and its effects on sympathetic neurons differ in several important respects from those of NGF. GDF5 promotes axonal growth and branching from SCG neurons by BMPR-IB-dependent activation of Smad transcription factors, a signaling pathway that has no known links with the TrkA-dependent signaling events operative in the growth cones of NGF-responsive neurons that mediate the effects of NGF on axonal growth (556). GDF5 promotes axonal growth from cultured neonatal SCG in the absence of NGF, and in the presence of NGF, GDF5 enhances axonal length and branching beyond that which is achieved with maximally effective concentrations of NGF alone.

In marked contrast to NGF, GDF5 does not promote the survival of SCG neurons *in vitro* and is not required for their survival *in vivo*. The number of SCG neurons in *GDF5*^{bp} mice is not significantly different from that in wild type mice at stages up to P10, well after the period of development during which GDF5 affects sympathetic axon growth *in vitro* and innervation density *in vivo*. Because GDF5 begins to exert its effect on axonal growth and branching after sympathetic axons have reached their target organs and have begun to ramify within these targets under the influence of target-derived NGF, it seems that the subsequent impairment of terminal arborization in the sympathetic targets of *GDF5*^{bp} mice does not impact on NGF retrograde survival signaling of the innervating neurons. In *NT3*$^{-/-}$ mice, however, where sympathetic axons fail to reach their targets in sufficient numbers, many die as a secondary consequence of failure to obtain an adequate supply of NGF from their targets even though NT3 does not initiate retrograde survival signaling itself (170).

GDF5 also contrasts with NGF in exerting its effects on axonal growth and branching during a very narrow developmental window immediately after birth. SCG neurons respond to NGF over a much more protracted period of development, extending

from the time the earliest sympathetic axons reach their targets to well after the phase of programmed cell death (95)(557)(90)(558)(559). Yet, despite its transient action on developing sympathetic neurons, the influence GDF5 is nonetheless crucial for establishing appropriate levels of sympathetic innervation density. Transient activation of two other SCG-expressed receptors during narrow developmental windows has also recently been shown to be essential for the establishment of sympathetic innervation *in vivo*. Constitutive activation of the extracellular calcium sensing receptor CaSR in the relatively hypercalcaemic milieu of the fetus in the immediate prenatal period (560) and autocrine activation of GITR, a member of the TNF receptor superfamily, by its ligand GITRL between P1 and P3 (425) are each necessary for establishing appropriate innervation of SCG target organs and tissues *in vivo*. These recent findings and our current study indicate that superimposed on the extended requirement for NGF signaling, three sequential, transient phases of CaSR, GDF5/BMPR and GITRL/GITR signaling are required for the establishment of appropriate levels of sympathetic innervation in the perinatal period. However, unlike GDF5/BMPR signaling, which promotes axonal growth independently of NGF, neither CaSR nor GITR promote significant axonal growth in the absence of NGF. GITR signaling affects axonal growth by facilitating NGF/TrkA-induced ERK1/ERK2 activation which is required for NGF-promoted axonal growth (425). Later in development, after sympathetic innervation has been established by the action of NGF and other factors, there is evidence that the long-term maintenance of cholinergic sympathetic innervation of sweat glands may become dependent on neurturin. Sweat glands express neurturin mRNA (561), and although the cholinergic sympathetic innervation of these glands develops normally in mice lacking the preferred neurturin receptor GFR-alpha2, this is subsequently lost in these mice in late postnatal development (562).

These findings raise the interesting question of the extent to which GDF5 influences the development of other neurons. Although GDF5 does not affect neurite growth from developing NGF-responsive and BDNF-responsive sensory neurons at several different stages of embryonic and postnatal development, it has been reported to increase the number of dopaminergic neurons and enhance the growth of neurites from these neurons in dissociated cultures of embryonic midbrain cells (563)(547). GDF5 also promotes astrocyte proliferation in these cultures (547), so it is possible that its effects on

dopaminergic neuron differentiation and/or survival may be secondary to the effects of increased number of astrocytes in these cultures. However, since GDF5 is expressed locally within the midbrain at this stage of development (547) it will be important to study dopaminergic neuron development in *GDF5*^{bp} mice.

In summary, I have discovered the first physiological role for GDF5 in the nervous system as a novel target-derived factor that promotes the growth and branching of sympathetic axons independently of NGF during a window of development after these axons have started to ramify in their targets under the influence of target-derived NGF. However, in marked contrast to NGF, GDF5 has no demonstrable role in regulating sympathetic neuronal survival either *in vitro* or *in vivo*. Whereas NGF acts locally on distal axons to promote growth and branching (536), its survival-promoting effects require retrograde transport in signalling endosomes to the cell soma (170). Because the effects of GDF5 on sympathetic axon growth and branching may be dependent on Smad transcriptional activation, it is possible that these effects *in vivo* require retrograde signaling to the cell body. Whether this is indeed the case and how GDF5-promoted Smad activation affects axon growth are intriguing and important questions for future research.

5. Role of TNFRSF members BCMA and TACI in regulating the neurotrophin survival responses of developing sensory and sympathetic neurons

5.1 Introduction

The complexity of nervous system development is astounding. Neurons must be generated in appropriate numbers in different parts of the nervous system and have to extend axons, elaborate dendrites, and form synapses to ensure the exquisite specificity of connectivity necessary for normal function (545). Over the past few decades there has been a great improvement in knowledge about the families of extracellular signals that regulate the generation and survival of neurons and the growth and elaboration of their processes. The main aim of the research carried out in this thesis is to explore the potential roles in the developing nervous system of families of extracellular signaling proteins that have well characterized functions in other organs and tissues. The PCR screens reported in the third chapter of this thesis revealed several interesting expression profiles in developing PNS neurons for members of the TNFRSF with no known function in the nervous system, warranting further functional studies. One such set of receptors, which are the subject of the functional studies reported in this chapter, are BCMA, TACI and BAFFR and their ligands APRIL and BAFF. Whereas BCMA and TACI are receptors for both APRIL and BAFF, BAFFR is a selective receptor for BAFF. The functions of these receptors and ligands have been extensively studied in the immune system, where they play important roles in B cell homeostasis, differentiation, function and survival(359)(564)(565)(566)(567). However, little is known about their role in the nervous system. In this chapter I report the results of studies suggesting that BCMA signaling plays an important roles in facilitating NGF-promoted survival of neonatal sympathetic neurons but not NGF-promoted neurite growth from neurons and facilitating BDNF-promoted survival of embryonic sensory neurons but not BDNF-promoted neurite growth from these neurons.

5.2 Results

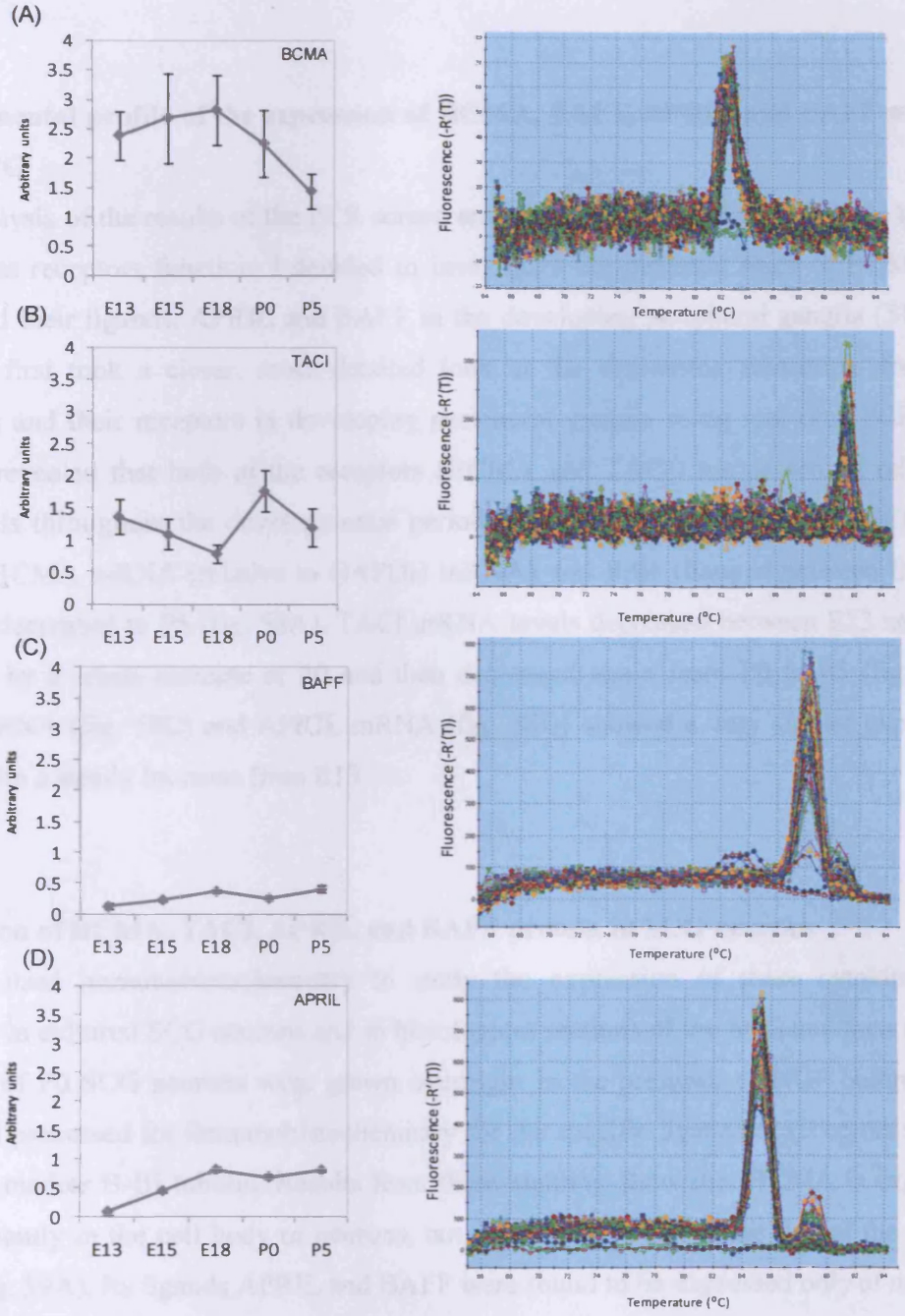


Figure 58: Expression of BCMA, TACI, BAFF and APRIL in the developing SCG.
The data show the relative expression of (A) BCMA, (B) TACI, (C) BAFF and (D) APRIL relative to GAPDH mRNA (in arbitrary units) and the respective Real time PCR melting curves (showing primer specificity in E13, E15, E18, P0 and P5 SCG. Means and standard errors of data from three separate sets of ganglia at each stage are shown

Developmental profile of the expression of BCMA, TACI, APRIL and BAFF mRNAs in the SCG

From analysis of the results of the PCR screen and a consideration of the available tools to manipulate receptors function, I decided to investigate the potential roles of BCMA and TACI and their ligands, APRIL and BAFF in the developing peripheral ganglia (514). To do so, I first took a closer, more detailed look at the expression transcripts for these cytokines and their receptors in developing peripheral ganglia using real time PCR. This analysis revealed that both of the receptors (BCMA and TACI) are present at relatively high levels throughout the developmental period examined (E13 to P5) in the SCG. The level of BCMA mRNA (relative to GAPDH mRNA) was little changed between E13 and E18 and decreased to P5 (fig. 58A). TACI mRNA levels decreased between E13 and E18, followed by a small increase at P0 and then decreased again from P0 to P5 (fig. 58B). BAFF mRNA (fig. 58C) and APRIL mRNA (fig. 58D) showed a very similar expression profile with a steady increase from E13.

Expression of BCMA, TACI, APRIL and BAFF protein in SCG neurons

I used immunohistochemistry to study the expression of these cytokines and receptors in cultured SCG neurons and in histological sections of the SCG and their targets. Cultures of P0 SCG neurons were grown overnight in the presence of NGF before being fixed and processed for immunohistochemistry for the specific ligands or receptors and the neuronal marker B-III tubulin. Results from these staining show that BCMA is expressed predominantly in the cell body of neurons, but it was also found at the tips of the growth cones (fig. 59A). Its ligands APRIL and BAFF were found to be expressed only at neuronal cell bodies (figs. 59B, 59C) and not at growth cones. TACI (fig. 59D) was expressed in the cell bodies of neurons and in non-neuronal cells. The pattern of expression of BCMA, TACI, BAFF and APRIL at the cell body of neurons raises the possibility of autocrine signaling occurring at the cell soma. The expression of BCMA on the tips of growth cones

raises the possibility that it could interact with any of its ligands should they be expressed in the intermediate or distal target of these neurons (discussed later). Considering the relatively high expression of BCMA throughout the developmental period studied, and its expression in cultured P0 SCG neurons, I decided to investigate BCMA protein expression in the SCG ganglion. Sections through the SCG were stained with anti-BCMA and TH. Labeling was clearly observed in the cell bodies of at least a subset of SCG neurons (figs. 60A, B, C).

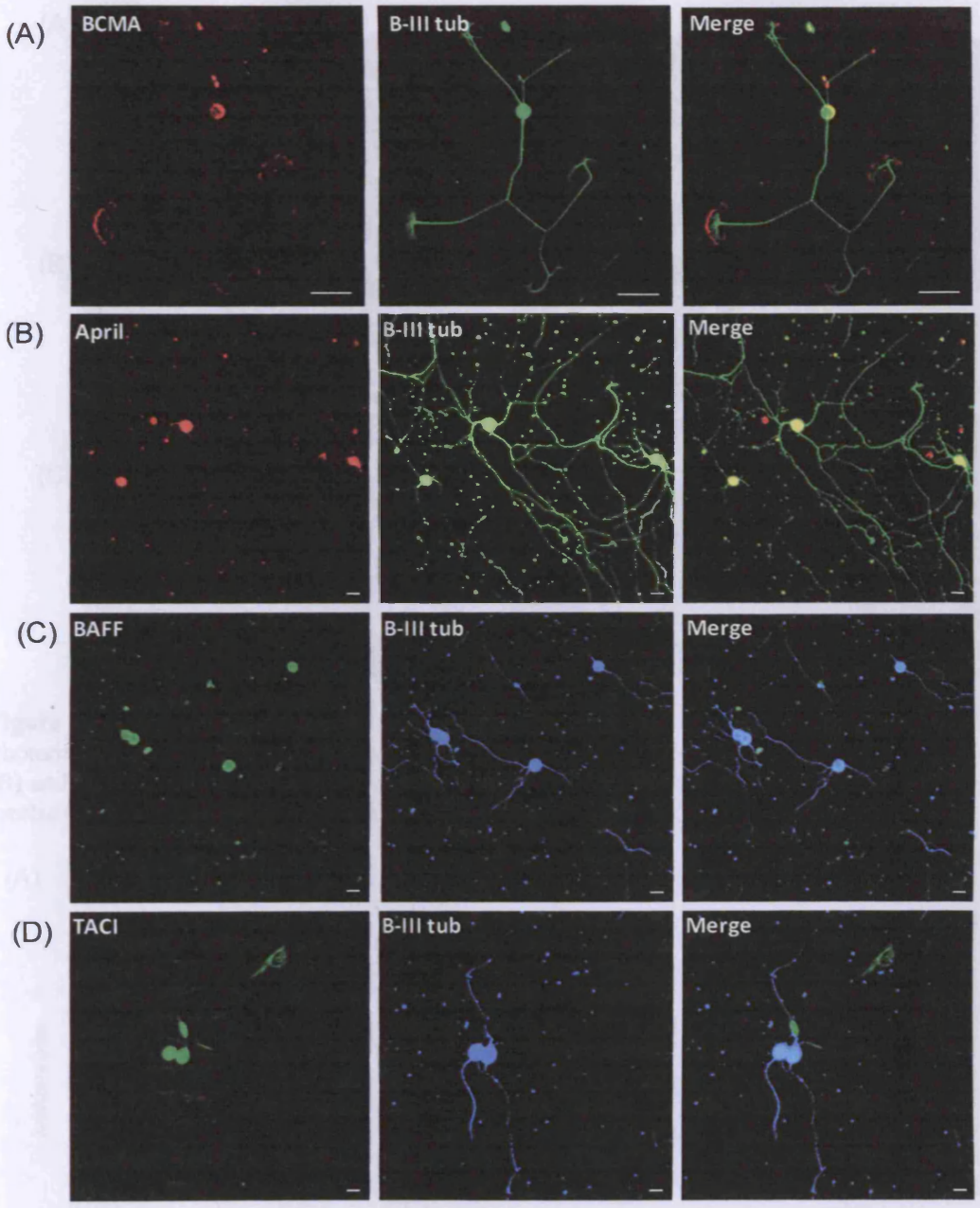


Figure 59: Expression of BCMA, APRIL, BAFF and TACI in axons and growth cones. Photomicrographs show P1 SCG neurons double stained for B-III tubulin (middle column) and (A) BCMA, (B) APRIL, (C) BAFF and (D) TACI, and a merge image (right column) showing co-localization of these proteins with the marker B-III tubulin. Scale bar = 10μm

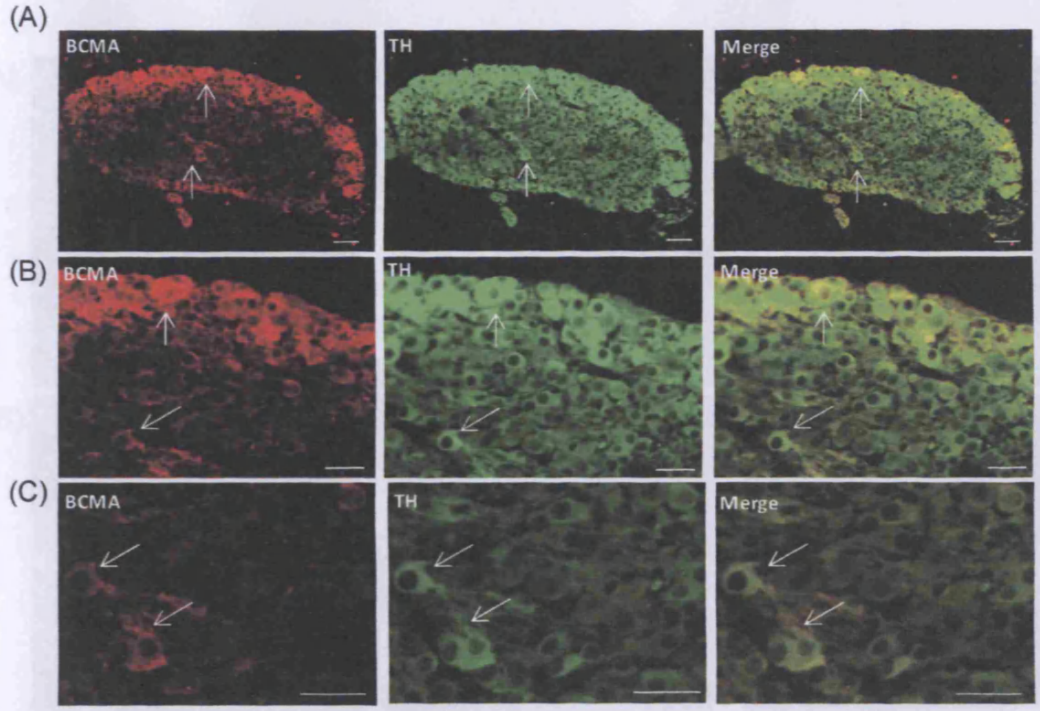


Figure 60: Expression of BCMA in the SCG ganglion.
Photomicrographs show consecutive sections of the entire SCG (A), the outer part of the ganglion (B) and centre part (C) double stained for BCMA (red) and TH (green). Merge image showing co-localization of BCMA and TH. Scale bar =10μm

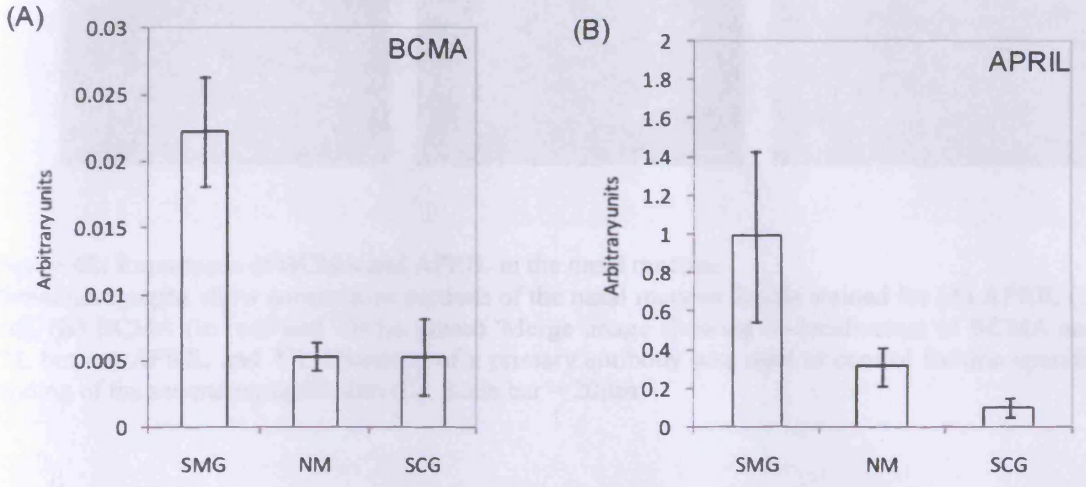


Figure 61: Expression of BCMA and APRIL in the SCG compared with target fields.
The data show the mRNA level of (A) BCMA and (B) APRIL in the submandibular gland (SMG), nasal mucosa (NM) and SCG relative to GAPDH in CD-1 mice.
Means and standard errors of data from three separate sets of each tissue are shown.

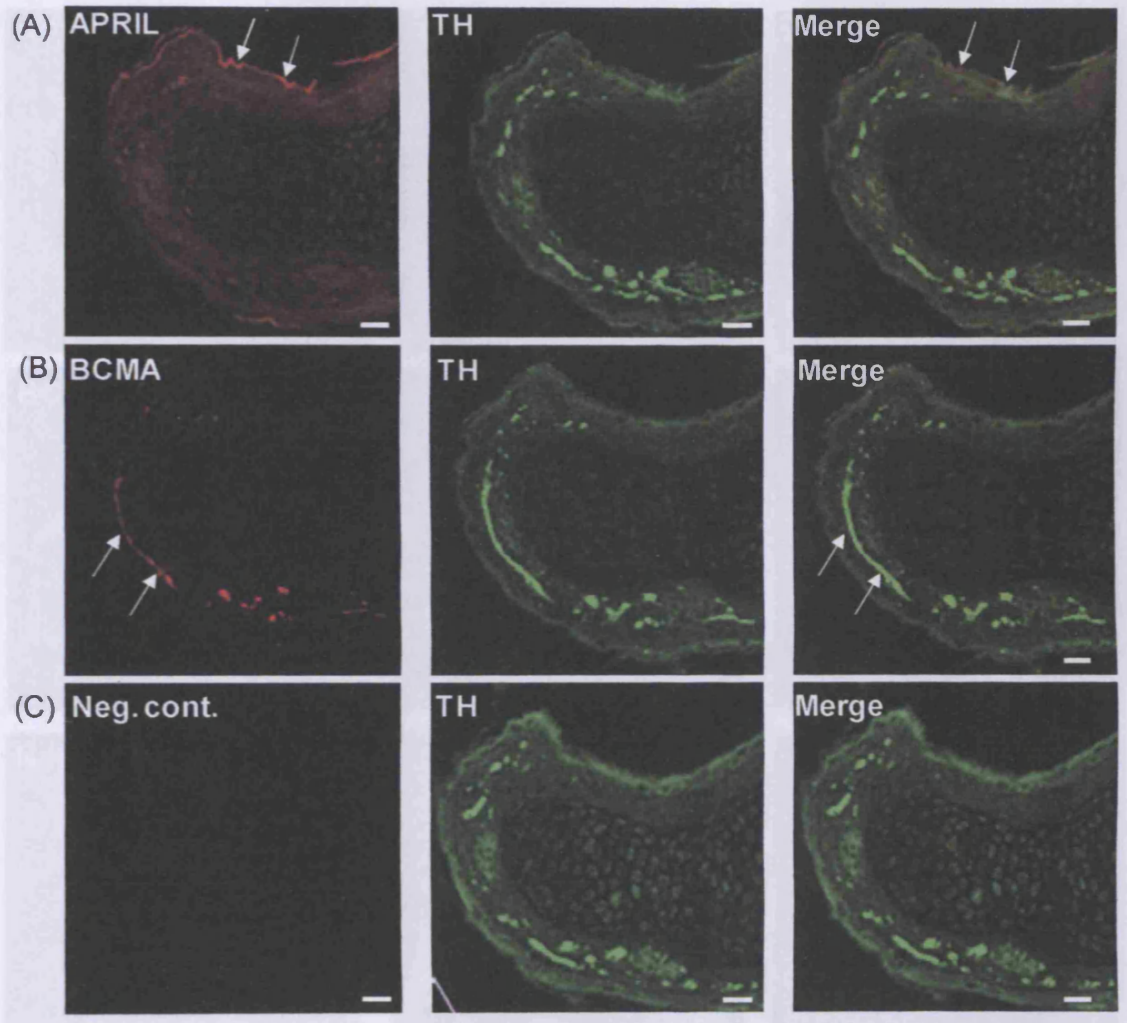


Figure 62: Expression of BCMA and APRIL in the nasal mucosa.
Photomicrographs show consecutive sections of the nasal mucosa double stained for (A) APRIL (in red), (B) BCMA (in red) and TH (in green). Merge image showing co-localization of BCMA and TH, but not APRIL and TH. Omission of a primary antibody was used to control for non-specific binding of the secondary antibodies (C). Scale bar = 20μm

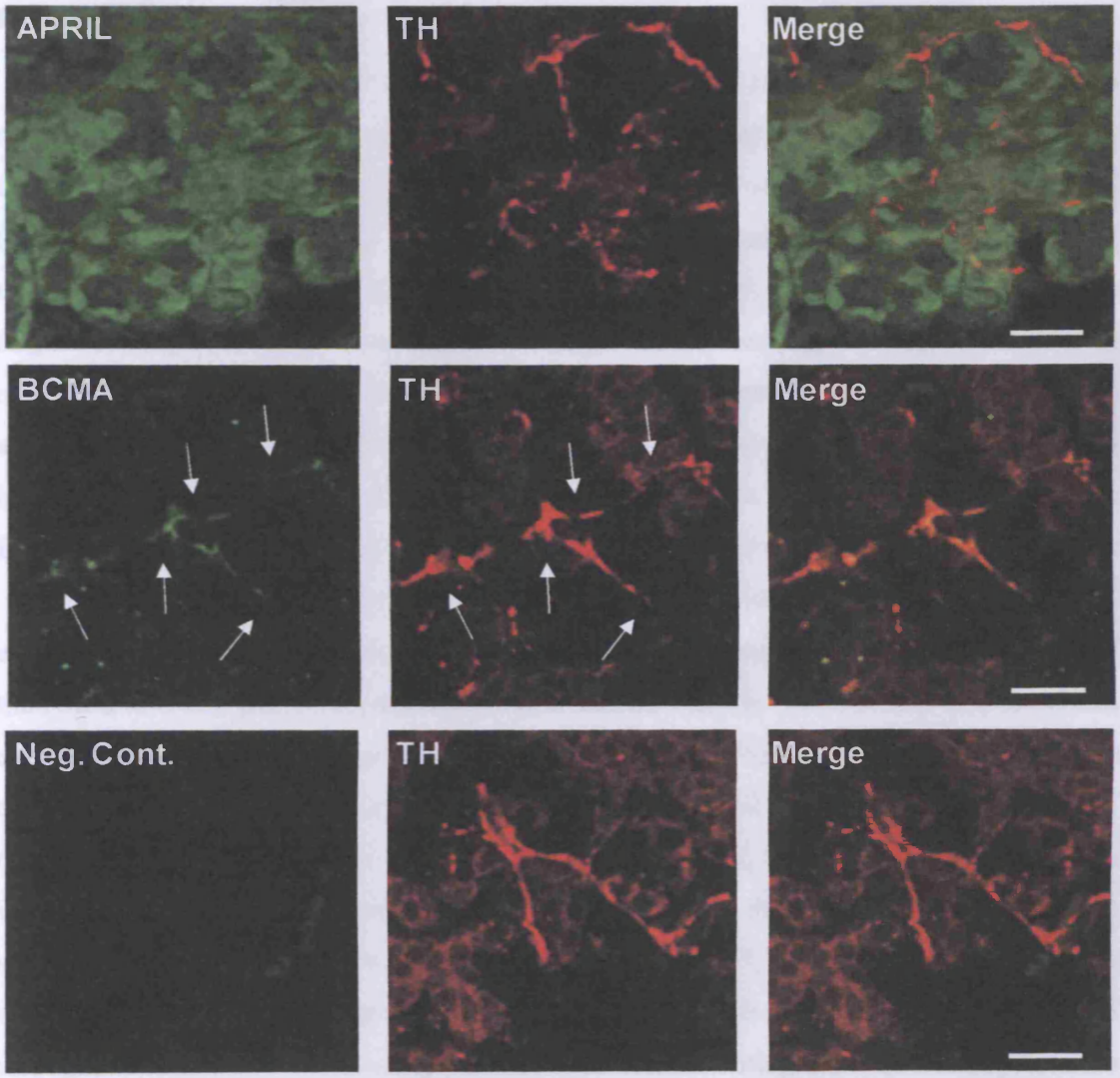


Figure 63: Expression of BCMA and APRIL in the SMG.
Photomicrographs show consecutive sections of the SMG double stained for (A) APRIL (in green), (B) BCMA (in green) and TH (in red). Merge image showing co-localization of both BCMA and April with TH. Omission of a primary antibody was used to control for non-specific binding of the secondary antibodies (C). Scale bar = 20μm

Expression of APRIL mRNA and protein in SCG targets

Because of the prominent expression of BCMA in the growth cones of P0 SCG neurons (see above), I examined the expression of APRIL mRNA in the target tissues that these neurons innervate. For these studies, I dissected the submandibular salivary gland (SMG) and the nasal mucosa (NM) from P0 animals and quantified APRIL mRNA by real time PCR (fig. 61B). These results demonstrated APRIL mRNA expression in both of these target tissues (fig. 61B). Interestingly, these target tissues also expressed appreciable levels of BCMA mRNA (fig. 61A). The SMG had the highest expression of both BCMA mRNA and April mRNA. This may be in fact related to the innervation density, as the SMG has a higher innervation density when compared to the NM (568). In addition, APRIL mRNA, BCMA mRNA was also expressed in the targets of SCG neurons.

I used immunohistochemistry to confirm that BCMA and APRIL proteins are expressed in the targets and determine their localization. I prepared histological sections of the NM and SMG of P0 animals, and double stained them for BCMA and TH, or APRIL and TH. In the NM, BCMA and TH were co-localized (fig. 62B), indicating that BCMA is expressed on the innervating axons. APRIL was expressed in the outer epithelial layer of the NM and did not co-localize with TH (fig. 62A), showing that it is not expressed by the innervating axons, but it is instead expressed by cells in the target tissue. In the SMG sections, BCMA and TH were likewise co-localized (fig. 63B), suggesting BCMA is also expressed in on the innervating axons. APRIL was much more abundantly expressed throughout the tissue and did not co-localize with TH, indicating it is expressed by the cells in the tissue (fig. 63A). In sections of the both NM and SMG no immunoreactivity was observed in the absence of primary antibodies, demonstrating no non-specific binding of the secondary antibodies (fig. 62C, 63C).

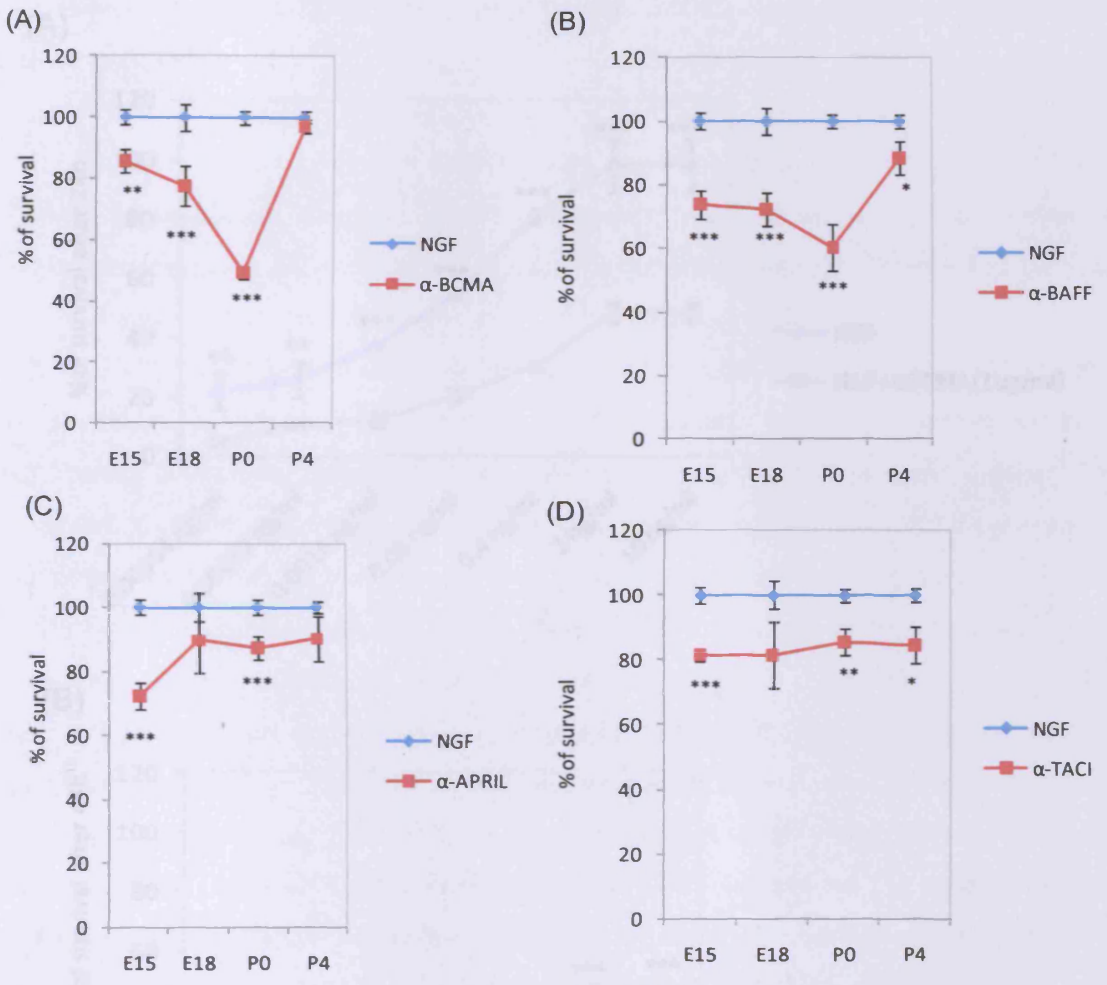


Figure 64 : Effect of blocking BCMA, BAFF, APRIL and TACI on sympathetic neuron survival. The data show SCG neurons time course survival (24h) comparison between neurons grown only with 10ng/ml of NGF (control normalized to 100%) and function blocking antibodies for the TNF family members. (A) 1µl/ml of α-BCMA, (B) 10µg/ml of α-BAFF, (C) 5µg/m of α-APRIL and (D) 10µg/m of α-TACI at a number of different stages of development between E15 and P4.
Means and standard errors of data from three separate sets of ganglia at each stage are shown (*** indicates $p<0.001$, statistical comparison with control one-way ANOVA with Fisher's *post hoc*).

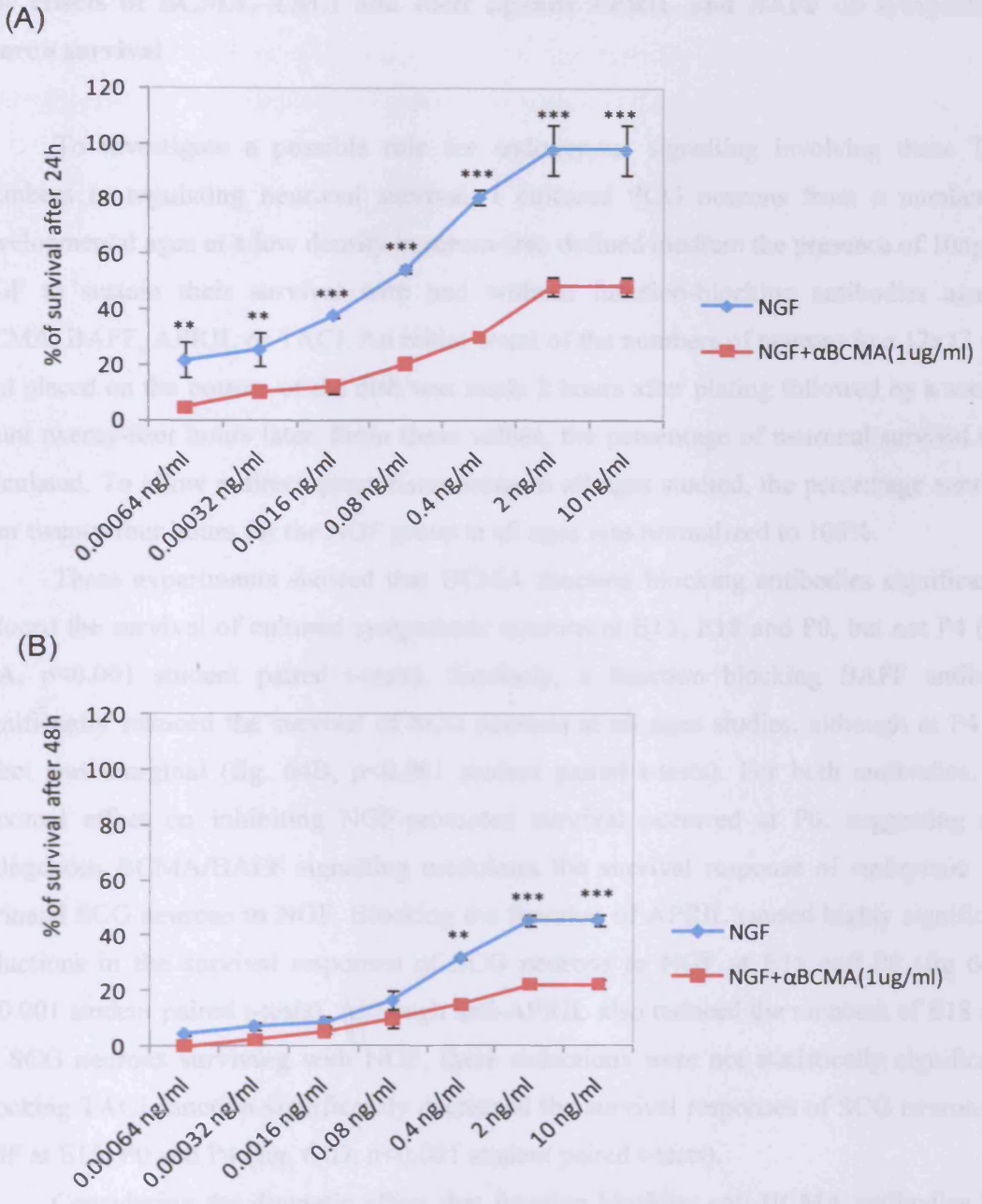


Figure 65: Effect of NGF dose response in sympathetic neurons.
The data show survival at (A) 24h and (B) 48h of P0 SCG neurons grown overnight with several concentrations of NGF ranging from 0.00064 to 10ng/ml (control) plus or minus 1µg/ml of α-BCMA.
Data from three independent experiments are shown. Statistical comparison with control, one-way ANOVA with Fisher's *post hoc*.

The effects of BCMA, TACI and their ligands APRIL and BAFF on sympathetic neuron survival

To investigate a possible role for endogenous signalling involving these TNF members in regulating neuronal survival, I cultured SCG neurons from a number of developmental ages at a low density in serum-free defined medium the presence of 10ng/ml NGF to sustain their survival with and without function-blocking antibodies against BCMA, BAFF, APRIL or TACI. An initial count of the numbers of neurons in a 12x12 mm grid placed on the bottom of the dish was made 2 hours after plating followed by a second count twenty-four hours later. From these values, the percentage of neuronal survival was calculated. To allow a direct comparison between all ages studied, the percentage survival after twenty-four hours for the NGF group at all ages was normalized to 100%.

These experiments showed that BCMA function blocking antibodies significantly reduced the survival of cultured sympathetic neurons at E15, E18 and P0, but not P4 (fig. 64A, $p<0.001$ student paired t-tests). Similarly, a function blocking BAFF antibody significantly reduced the survival of SCG neurons at all ages studies, although at P4 the effect was marginal (fig. 64B, $p<0.001$ student paired t-tests). For both antibodies, the maximal effect on inhibiting NGF-promoted survival occurred at P0, suggesting that endogenous BCMA/BAFF signalling modulates the survival response of embryonic and perinatal SCG neurons to NGF. Blocking the function of APRIL caused highly significant reductions in the survival responses of SCG neurons to NGF at E15 and P0 (fig 64C, $p<0.001$ student paired t-tests). Although anti-APRIL also reduced the numbers of E18 and P4 SCG neurons surviving with NGF, these reductions were not statistically significant. Blocking TACI function significantly decreased the survival responses of SCG neurons to NGF at E15, P0 and P4 (fig. 64D, $p<0.001$ student paired t-tests).

Considering the dramatic effect that function blocking anti-BCMA antibodies had on the survival of P0 SCG neurons, I decided to investigate the effect of these antibodies in neurons cultured with different concentrations of NGF. For this, I set up a P0 SCG low density culture with concentrations of NGF ranging from 0.00064 to 10ng/ml. After 24h incubation (fig. 65A), irrespective of the concentration of NGF used, function blocking anti-BCMA antibodies significantly reduced neuronal survival. After 48h incubation (fig.

65B), few neurons were still surviving in the lowest concentrations of NGF, but at concentrations of 0.4ng/ml NGF and higher, where more than 30% of the neurons were still surviving, anti-BCMA antibodies significantly reduced NGF-promoted neuronal survival.

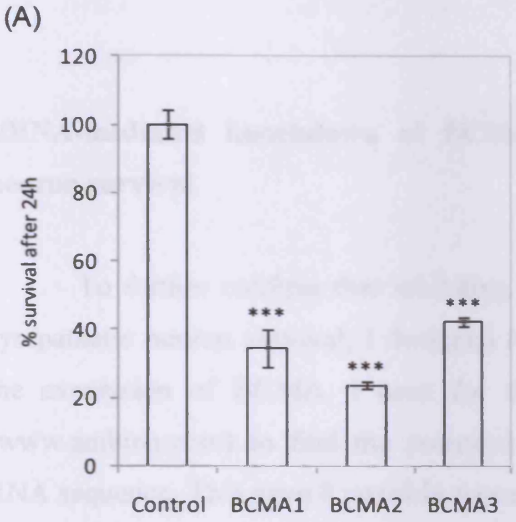


Figure 66: Effect of SiRNA transfections in sympathetic neurons survival.
Data show (A) survival of P0 SCG neurons grown overnight with NGF. Neurons were transfected with a scrambled sequence (control) and three different siRNAs for BCMA. Results show a very significant decrease in survival after 24h. Means and standard errors of data from three independent experiments (*** indicates $p<0.001$, statistical comparison with control one-way ANOVA with Fisher's *post hoc*).

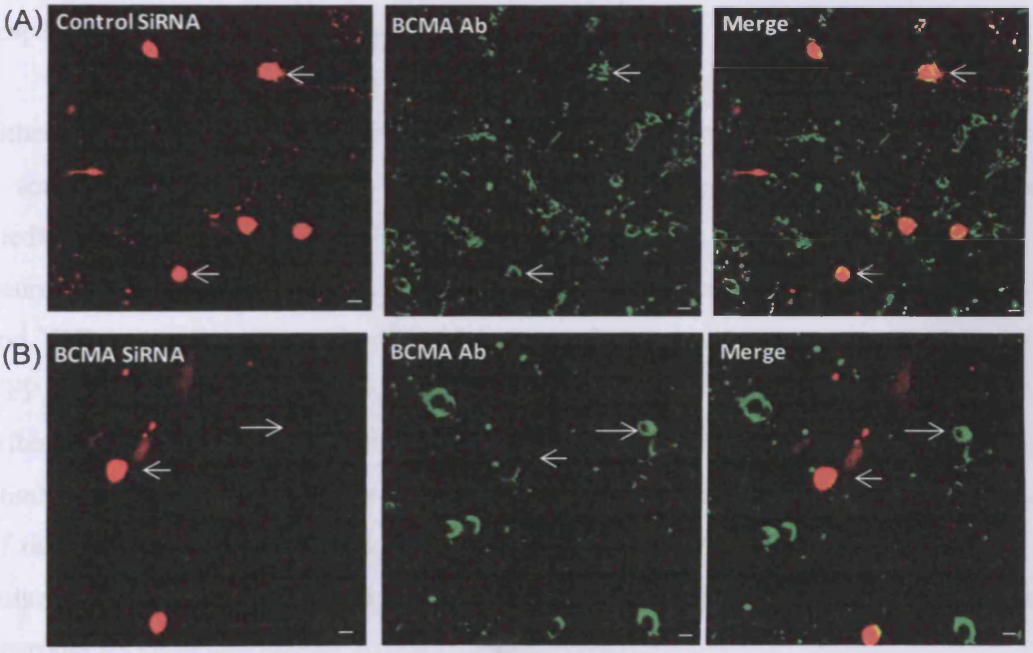


Figure 67: Effect of knocking down BCMA gene with SiRNA in sympathetic neurons. Photomicrographs showing (A) Control SiRNA and (B) BCMA SiRNA in P0 SCG neurons grown overnight with NGF and Caspase inhibitors. Results show BCMA staining in the control transfections but not in the neurons transfected with BCMA SiRNA. Scale bar = 20μm.

siRNA-mediated knockdown of BCMA receptor expression reduces sympathetic neuron survival

To further confirm that inhibiting the function of BCMA impairs NGF-promoted sympathetic neuron survival, I designed and made plasmid based siRNAs to knockdown the expression of BCMA. I used the siRNA designing tool on the Ambion website (www.ambion.com) to find the potential siRNA target sequences based on the BCMA RNA sequence. This gave 8 possible sequences which I blasted on NCBI, and subsequently chose the three with the lowest percentage of similarity to other genes. Based on the target sequence I obtained the siRNA sequence from the same website. Following manufacturer's instructions, I created three vectors expressing these siRNAs using the p*Silencer*^{TM} 4.1-CMV hygro expression vector kit (Ambion), one for each of the target sequences (for details see section 2.10.4). These vectors were then used to transform *E. coli*, and respective plasmid DNA was extracted.

P0 SCG neurons were co-transfected with an RFP expression plasmid together with either a BCMA siRNA expression plasmid or its corresponding control plasmid expressing a scrambled sequence RNA. Transfected neurons were plated in defined serum-free medium with 10ng/ml of NGF and incubated overnight. Separate experiments in which neurons were transfected by electroporation using the Microporator using a mixture of RFP and YFP expression plasmids showed that transfected neurons co-expressed both RFP and YFP, indicating that both plasmids were taken up by the same neurons (data not shown). After 24h, all RFP positive neurons were counted using a fluorescence microscope. The numbers of surviving neurons in control cultures were normalized to 100%, and the number of neurons in the cultures transfected with BCMA siRNA expressed a percentage of the control. Neuronal counts revealed that all three BCMA siRNA's significantly reduced neuronal survival to a similar extent (fig. 66).

To demonstrate that the BCMA siRNA constructs reduced the expression of BCMA protein in transfected neurons, I cultured P0 SCG neurons co-transfected with an RFP expression plasmid and either control or BCMA siRNA in the presence of 10ng/ml of NGF

and caspase inhibitors and immunolabelled them against BCMA. As expected, I found that neurons transfected with the scramble control still expressed BCMA whereas neurons transfected with BCMA siRNA expressed virtually no BCMA, showing that siRNA effectively knocked down the expression of BCMA (fig. 67).

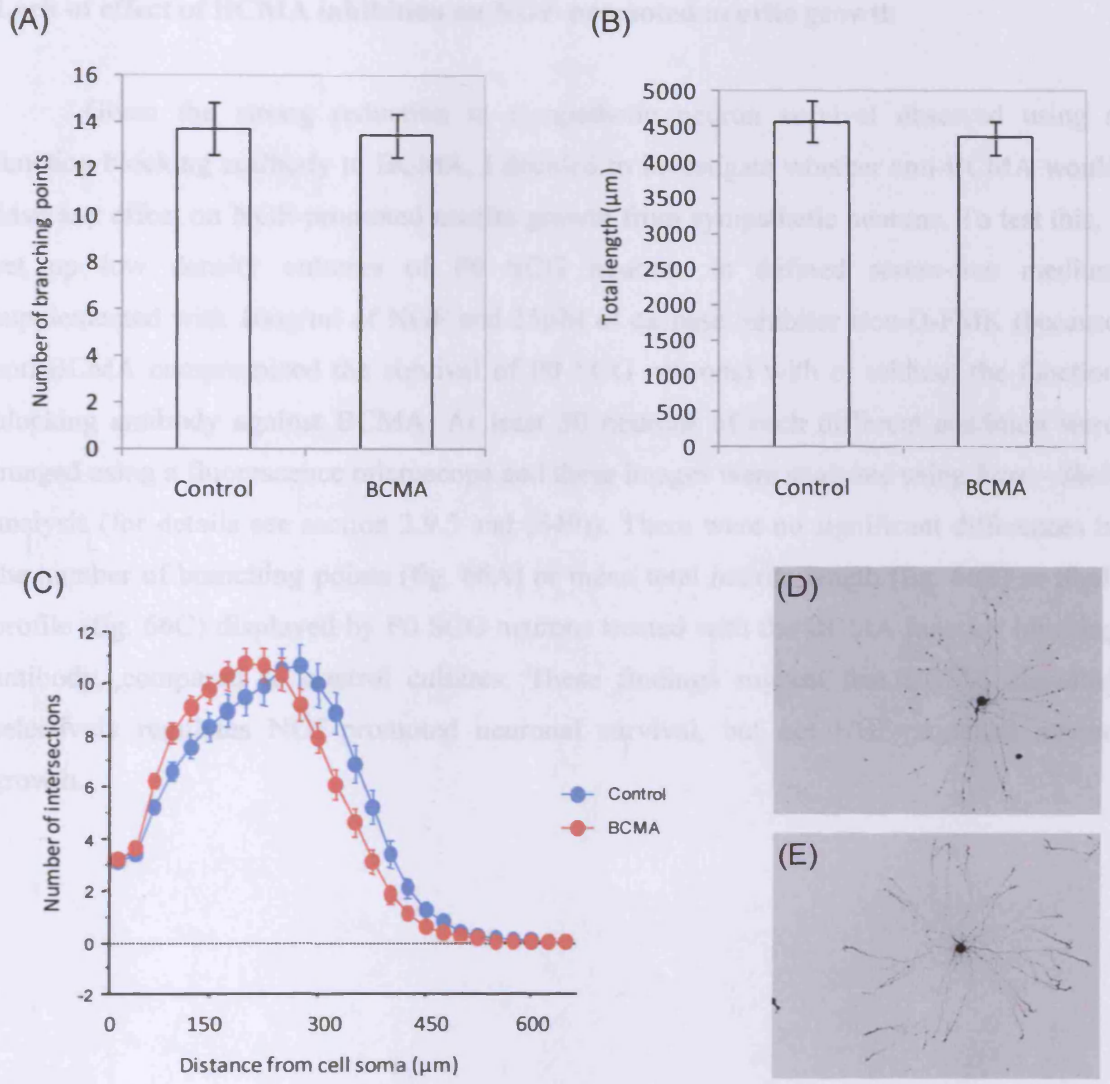


Figure 68: Effect of blocking BCMA on sympathetic neurite growth.
Branching (A), Length (B) and sholl analysis (C) of the neurite arbors of P0 SCG neurons grown overnight with Caspase inhibitors and NGF (Control) or Caspase inhibitors, NGF and BCMA. (D) and (E) show illustrative draws of neuronal growth in the different experimental conditions.
Data from at least 150 neurons in each condition from three independent experiments are shown

Lack of effect of BCMA inhibition on NGF-promoted neurite growth

Given the strong reduction in sympathetic neuron survival observed using a function blocking antibody to BCMA, I decided to investigate whether anti-BCMA would have any effect on NGF-promoted neurite growth from sympathetic neurons. To test this, I set up low density cultures of P0 SCG neurons in defined serum-free medium supplemented with 10ng/ml of NGF and 25μM of caspase inhibitor Boc-D-FMK (because anti-BCMA compromised the survival of P0 SCG neurons) with or without the function blocking antibody against BCMA. At least 50 neurons of each different condition were imaged using a fluorescence microscope and these images were analyzed using *Fast – Sholl* analysis (for details see section 2.9.5 and (549)). There were no significant differences in the number of branching points (fig. 66A) or mean total neurite length (fig. 66B) or Sholl profile (fig. 66C) displayed by P0 SCG neurons treated with the BCMA function blocking antibody, compared to control cultures. These findings suggest that BCMA signaling selectively regulates NGF-promoted neuronal survival, but not NGF-promoted neurite growth.

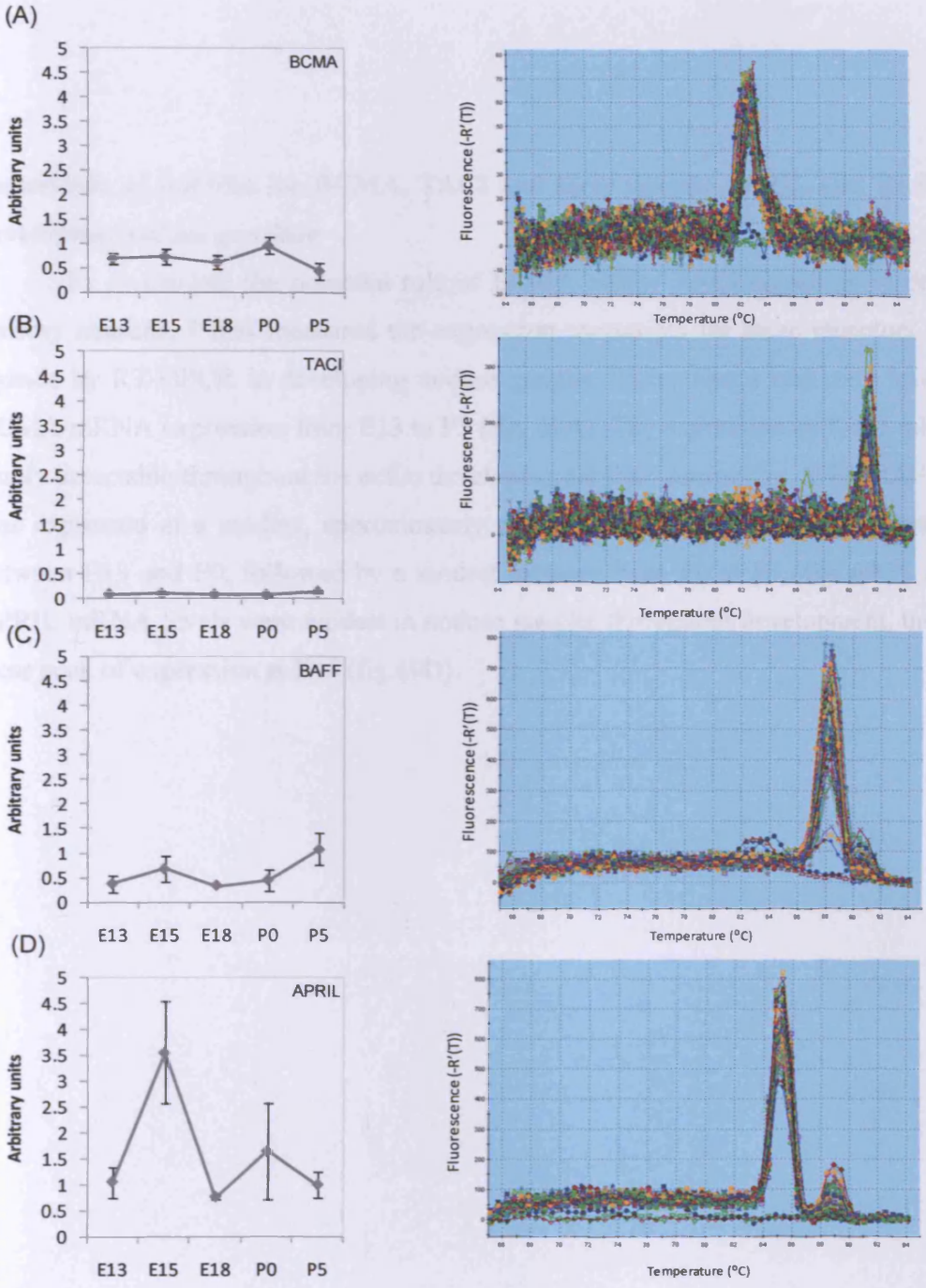


Figure 69: Expression of BCMA, TACI, BAFF and APRIL in the developing Nodose ganglion. The data show the relative expression of (A) BCMA, (B) TACI, (C) BAFF and (D) APRIL relative to GAPDH mRNA (in arbitrary units) and the respective Real time PCR melting curves (showing primer specificity in E13, E15, E18, P0 and P5 Nodose. Means and standard errors of data from three separate sets of ganglia at each stage are shown.

Expression of mRNAs for BCMA, TACI and their ligands APRIL and BAFF in the developing Nodose ganglion

To investigate the potential role of BCMA and/or TACI signalling in developing sensory neurons, I first measured the expression transcripts for these receptors and their ligands by RT-QPCR in developing nodose ganglia. There was a relatively low level of BCMA mRNA expression from E13 to P5 (fig. 69A). The expression of TACI mRNA was barely detectable throughout the entire developmental time course (fig. 69B). BAFF mRNA was expressed at a modest, approximately constant levels in developing nodose ganglia between E13 and P0, followed by a modest increase from P0 to P5 (fig. 69C). Although APRIL mRNA levels were modest in nodose ganglia throughout development, there was a clear peak of expression at E15 (fig.69D).

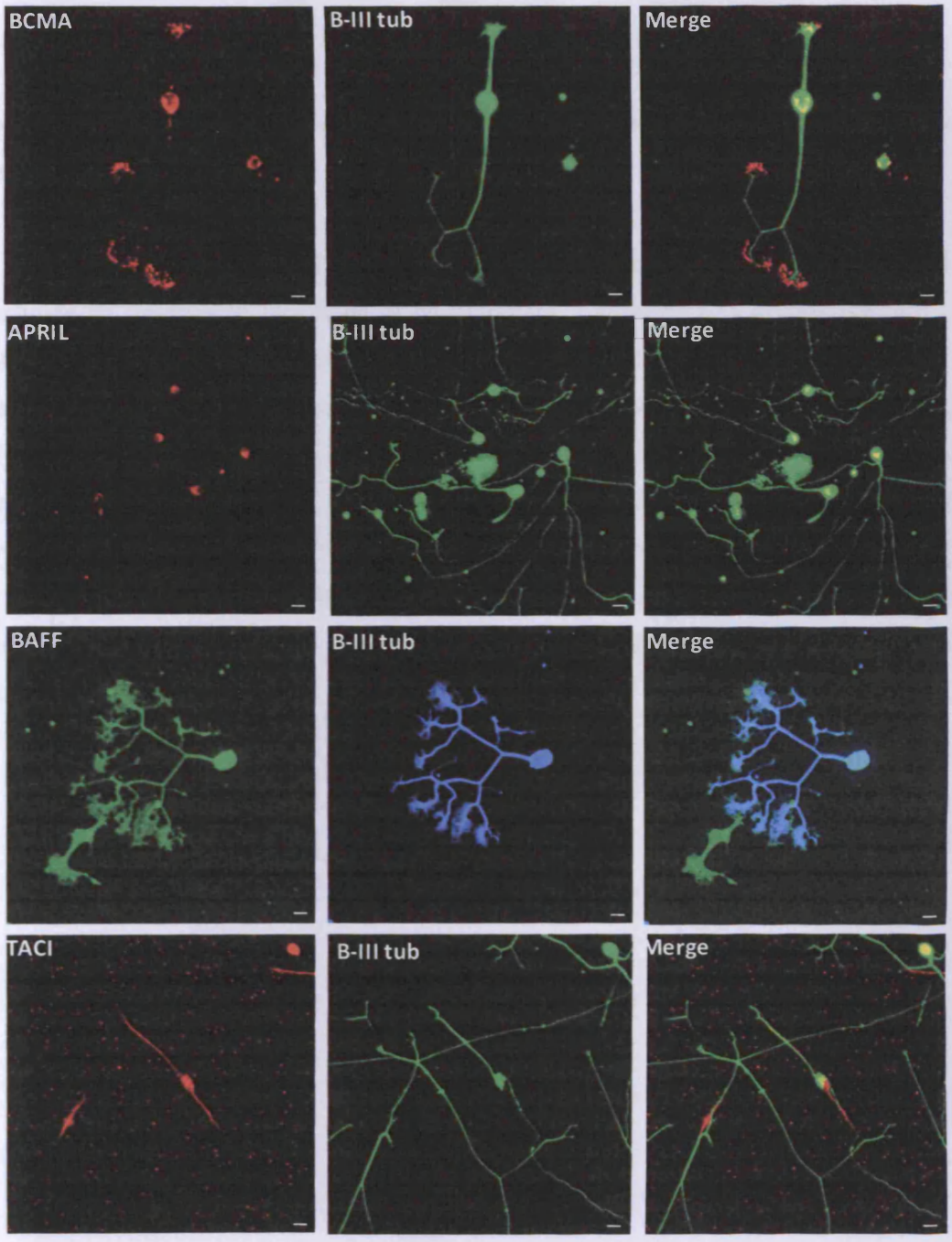


Figure 70: Expression of BCMA, APRIL, BAFF and TACI in axons and growth cones. Photomicrographs show E15 Nodose neurons double stained for B-III tubulin (middle column) and (A) BCMA, (B) APRIL, (C) BAFF and (D) TACI, and a merge image (right column) showing co-localization of these proteins with the marker B-III tubulin. Scale bar = 10μm.

Expression of BCMA, TACI, and their ligands APRIL and BAFF in cultured nodose neurons

Given the high level of APRIL mRNA expression at E15, I decided to further investigate its function at this stage of development. I cultured E15 nodose neurons in a serum-free defined medium in the presence of 10ng/ml of BDNF to support their survival. After 24h, the neurons were fixed and double stained for β-III tubulin and either BCMA, BAFF, TACI or APRIL. BCMA was strongly expressed in the cell bodies and at the tips of their growth cones (fig. 70A). APRIL expression was restricted to the cell bodies of sensory neurons (fig. 70B), and although BAFF mRNA was expressed at extremely low levels in E15 nodose ganglia, BAFF protein was expressed in both neuronal and non-neuronal cells *in vitro* (fig. 70C). TACI was expressed in the cell bodies and processes of most E15 nodose neurons (fig. 70D).

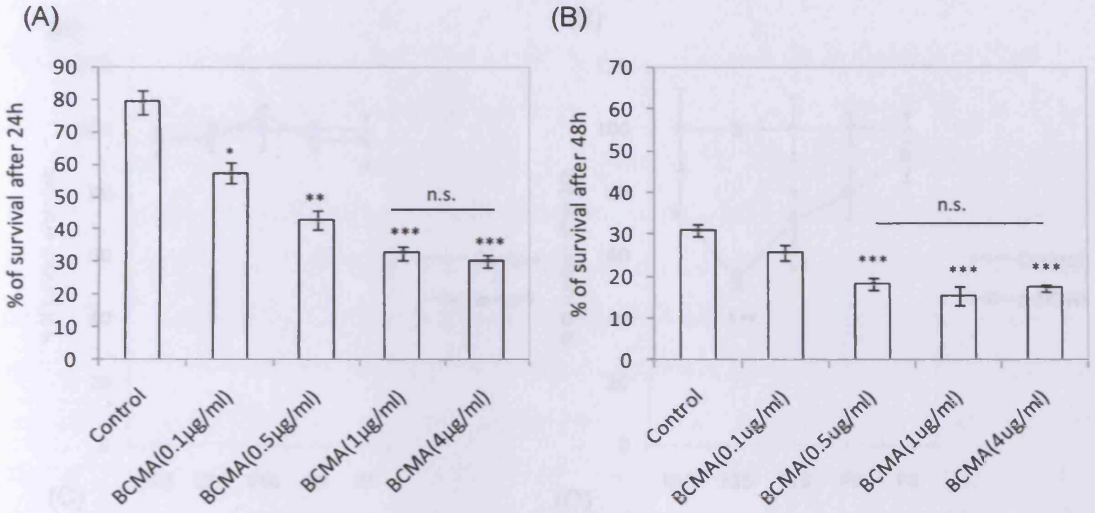


Figure 71: Anti-BCMA dose response.
The data show E15 nodose neurons grown overnight with 10ng/ml BDNF (control) plus several concentrations of BCMA ranging from 1 to 4µg/ml of BCMA
Data from three independent experiments are shown. Statistical comparison with control, one-way ANOVA with Fisher's *post hoc*. *** indicates $p<0.001$

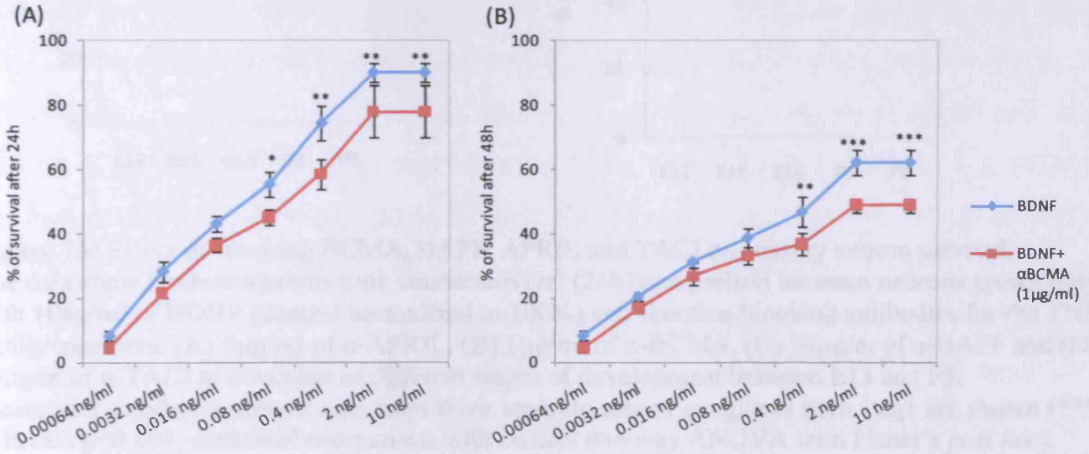


Figure 72: Effect of BDNF dose response in Nodose neurons.
The data show survival at (A) 24h and (B) 48h of E15Nodose neurons grown overnight with several concentrations of BDNF ranging from 0.00064 to 10ng/ml (control) with or without1µg/ml of α-BCMA.
Data from three independent experiments are shown. Statistical comparison with control, one-way ANOVA with Fisher's *post hoc*.

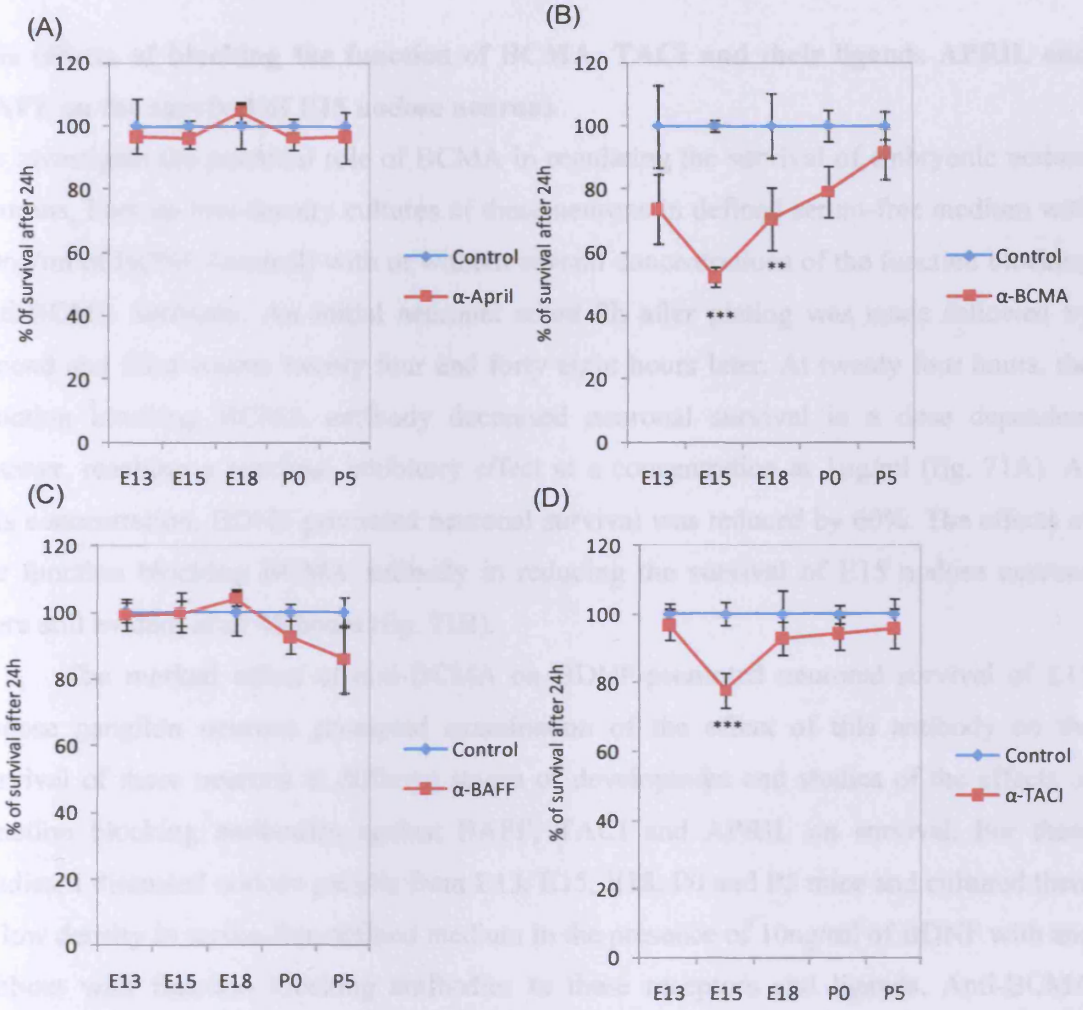


Figure 73: Effect of blocking BCMA, BAFF, APRIL and TACI on sensory neuron survival.
The data show Nodose neurons time course survival (24h) comparison between neurons grown only with 10ng/ml of BDNF (control normalized to 100%) and function blocking antibodies for the TNF family members. (A) 5µg/ml of α-APRIL, (B) 1µg/ml of α-BCMA, (C) 10µg/m of α-BAFF and (D) 10µg/m of α-TACI at a number of different stages of development between E13 and P5.
Means and standard errors of data from three separate sets of ganglia at each stage are shown (*** indicates $p<0.001$, statistical comparison with control one-way ANOVA with Fisher's *post hoc*).

The effects of blocking the function of BCMA, TACI and their ligands APRIL and BAFF on the survival of E15 nodose neurons

To investigate the potential role of BCMA in regulating the survival of embryonic nodose neurons, I set up low-density cultures of these neurons in defined serum-free medium with 10ng/ml of BDNF (control) with or without several concentrations of the function blocking anti-BCMA antibody. An initial neuronal count 2h after plating was made followed by second and third counts twenty four and forty eight hours later. At twenty four hours, the function blocking BCMA antibody decreased neuronal survival in a dose dependent manner, reaching a maximal inhibitory effect at a concentration at 1μg/ml (fig. 71A). At this concentration, BDNF-promoted neuronal survival was reduced by 60%. The effects of the function blocking BCMA antibody in reducing the survival of E15 nodose neurons were still evident after 48 hours (fig. 71B).

The marked effect of anti-BCMA on BDNF-promoted neuronal survival of E15 nodose ganglion neurons prompted examination of the effect of this antibody on the survival of these neurons at different stages of development and studies of the effects of function blocking antibodies against BAFF, TACI and APRIL on survival. For these studies, I dissected nodose ganglia from E13, E15, E18, P0 and P5 mice and cultured them at low density in serum-free defined medium in the presence of 10ng/ml of BDNF with and without with function blocking antibodies to these receptors and ligands. Anti-BCMA reduced BDNF-promoted survival at all ages, although the reduction was only statistically significant at E15 and E18, with the greatest (50% reduction in survival) and most significant ($P<0.001$, student's paired t-test) effect being observed at E15. Anti-TACI also caused a highly significant reduction in BDNF-promoted survival at E15 (25% reduction, $P<0.001$, student's paired t-test), but had no significant effect on neuronal survival at other ages. Surprisingly, the function-blocking antibodies against APRIL (fig. 73A) and BAFF (fig. 73C) did not have any effect on survival of nodose neurons throughout the developmental period studied.

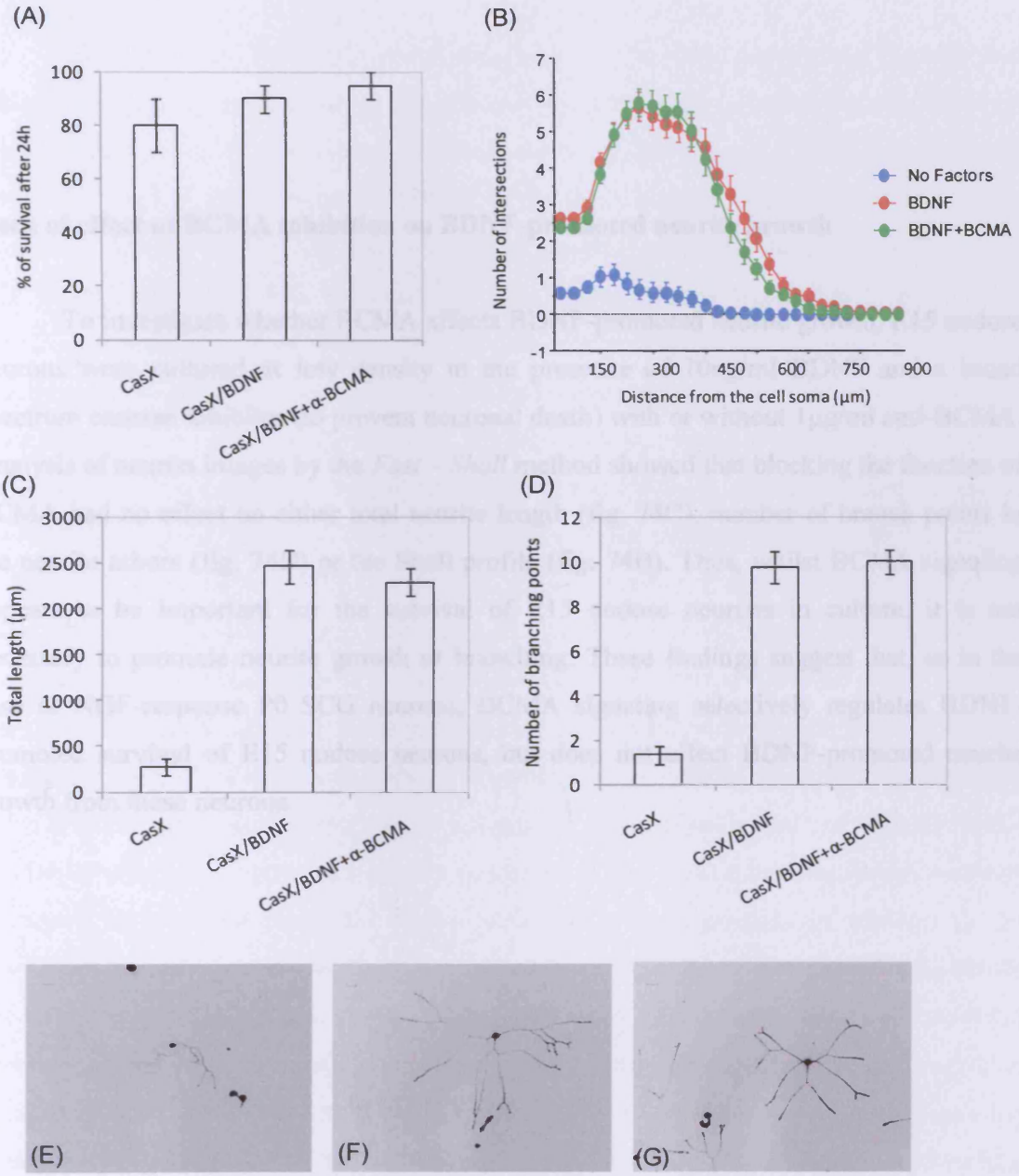


Figure 74: Effect of blocking BCMA on sensory neurite growth.
The data show (A) survival, (B) sholl analysis (C) total length and (D) number of branching points of E15 Nodose neurons, grown overnight in the presence of Caspase inhibitors and 10ng/ml of BDNF plus or minus 1µg/ml of α-BCMA. (E) to (G) show illustrative draws of neuronal growth in the different experimental conditions
Means and standard error of data from three separate sets of ganglia at each stage are shown.

Lack of effect of BCMA inhibition on BDNF-promoted neurite growth

To investigate whether BCMA affects BDNF-promoted neurite growth, E15 nodose neurons were cultured at low density in the presence of 10ng/ml BDNF and a broad spectrum caspase inhibitor (to prevent neuronal death) with or without 1μg/ml anti-BCMA. Analysis of neuron images by the *Fast – Sholl* method showed that blocking the function of BCMA had no effect on either total neurite length (fig. 74C), number of branch points in the neurite arbors (fig. 74D) or the Sholl profile (fig. 74B). Thus, whilst BCMA signaling appears to be important for the survival of E15 nodose neurons in culture, it is not necessary to promote neurite growth or branching. These findings suggest that, as in the case of NGF-response P0 SCG neurons, BCMA signaling selectively regulates BDNF-promoted survival of E15 nodose neurons, but does not affect BDNF-promoted neurite growth from these neurons.

5.3 Discussion

To date, little is known about the roles of the TNF superfamily ligands and receptors in the nervous system. The studies reported in this chapter provide the first evidence for a role for the related TNFRSF members BCMA and TACI in the nervous system. RT-QPCR data indicated that mRNAs for these receptors and their ligands APRIL and BAFF are expressed in the SCG and nodose ganglia throughout the period studied from E13 to P5 (figs. 34B, 36B, 40A and 41A). Interestingly, the higher levels of receptor mRNAs were present in the SCG compared with the nodose ganglia (figs. 34B and 36B), and the nodose ganglia expressed higher levels of the ligand mRNAs than the SCG (figs. 40A and 41A). In accordance with the RT-QPCR data, immunocytochemistry revealed expression of these receptors and ligands in cultured P0 SCG and E15 nodose ganglion neurons. The great majority of neurons within these cultures individually expressed BCMA, TACI, BAFF and APRIL, and while a small number of neurons were not labelled, these findings suggest that these receptors and ligands are co-expressed in the majority of neurons. These findings together with the observation that the cell bodies of the neurons were immunoreactive for both receptors and both ligands raised the possibility of autocrine signalling taking place at the cell body of these neurons. In addition to expression at the cell body, BCMA was striking expressed at the growth cones of both kinds of neurons. TACI and BAFF were also expressed along the processes of E15 nodose neurons. BAFF was also expressed by non-neuronal cells in the SCG and nodose ganglia. In addition to the possibility of autocrine signalling occurring within the ganglia, these expression patterns open up the possibility that APRIL and BAFF may affect some aspect of neuronal development by paracrine signalling within ganglia, non-neuronal cell to neuron paracrine signalling within ganglia and target field cell to axon terminal signalling. To investigate the matter, I used RT-QPCR and immunohistochemistry to determine the expression of APRIL and BCMA in two target fields of the SCG, the submandibular gland (SMG) and the nasal mucosa (NM). Double staining of the NM and SMG with antibodies against BCMA and tyrosine hydroxylase (TH) revealed that BCMA was expressed in the terminal arbors of sympathetic neurons innervating both structures, which is consistent with the expression of BCMA at the terminals of sympathetic neurites in culture. APRIL protein did not co-localise with sympathetic axon in these target tissues, rather it was expressed by target field

cells. RT-QPCR demonstrated that the SMG expressed higher levels of APRIL mRNA than the NM and both target tissues expressed higher levels of APRIL mRNA than the SCG, strengthening the possibility that target field-derived APRIL might affect some aspect of SCG neuron development via interaction with BCMA expressed by innervating neurons. Surprisingly, both SCG target fields investigated also expressed substantial amounts of BCMA mRNA. The origin of this BCMA mRNA is unclear. It may be expressed by target field cells or, alternatively, it may be expressed within the SCG neurons themselves, since it is now becoming clear that the mRNAs of many proteins are anterogradely transported to terminal arbors of axons where they are translated locally (569).

Given the fact that BCMA, TACI and their ligands APRIL and BAFF are known to be involved in regulating cell survival in the immune system (617)(618), I decided to investigate whether the inhibition of these receptors or their ligands would have any effect on sympathetic neuron survival. Inhibiting the function of BCMA, TACI, BAFF and APRIL using function blocking antibodies significantly reduced the NGF-promoted survival of SCG neurons at several stages of development. The greatest inhibitory effects were observed with anti-BCMA and anti-BAFF at P0. Anti-TACI and anti-APRIL also promoted highly significant reductions in NGF-promoted survival at this age, and all anti-ligand and anti-receptor antibodies promoted highly significant reductions in NGF-promoted neuronal survival at both E13 and E18, except anti-TACI at E18. However, by P4, anti-BCMA and anti-APRIL no longer exerted an inhibitory effect on NGF-promoted survival and anti-TACI and anti-BAFF exerted inhibitory effects that were only marginally significant. Dose response analysis of NGF-promoted survival of P0 SCG neurons demonstrated that anti-BCMA reduced the survival of SCG neurons across a broad range of NGF concentrations. To further confirm that endogenous BCMA plays a role in facilitating the NGF-dependent survival of P0 SCG neurons I used several different siRNAs to reduce the expression of BCMA and demonstrated that these siRNAs, but not control siRNAs, greatly reduced the survival of P0 SCG neurons cultured in the presence of NGF. Because the above studies of the effects of function-blocking antibodies and siRNAs were obtained from neurons cultured at very low density, to the point that the vast majority of individual neurons were not touching each other, these findings suggest that autocrine signalling involving the BCMA and TACI receptors and the BAFF and APRIL ligands is required for

NGF-promoted survival of SCG neurons during a developmental window that extends from E13 to P4.

In marked contrast to the inhibitory effect of anti-BCMA on NGF-promoted survival of P0 SCG neurons, anti-BCMA had no effect whatsoever on the extent or pattern of NGF-promoted neurite growth from these neurons. This suggests that endogenous BCMA signaling in these neurons selectively facilitates the survival-promoting actions of NGF but not the neurite growth-promoting effects of NGF. Interestingly, autocrine signaling involving another member of the TNFRSF, GITR, and its ligand GITRL has recently been shown to be selectively required for NGF-promoted neurite growth but not NGF-promoted survival of postnatal SCG neurons(425). Furthermore, in embryonic nodose neurons signaling involving another TNFSF/TNFRSF ligand/receptor pair LIGHT and HVEM has been recently reported to be a negative regulator of BDNF-promoted neurite growth but has no effect on BDNF-promoted neuronal survival. From these two recent studies and the findings reported in this chapter, it is beginning to look as if endogenous signaling involving members of the TNFRSF and their cognate ligands are selective modulators of particular aspects of the cellular responses of developing neurons to neurotrophins. GITRL/GITR facilitates NGF-promoted neurite growth and branching from postnatal SCG neurons by selectively facilitating downstream signaling events from TrkA that are selectively required for neurite growth without activating these pathways directly, namely, facilitating activation of MEK1/MEK2-ERK1/ERK2 by NGF/TrkA (425). In future work, it will be important to ascertain the molecular mechanism by which BCMA facilitates NGF-promoted neuronal survival, and whether it does so by selectively facilitating signalling pathways downstream of TrkA that are required for sustaining neuronal survival. BAFF, for example, has been shown to regulate B cell survival via activation of the NF-kB pathway (570), prevents NGF-mediated survival in sympathetic neurons (571). For this reason, it will be important to ascertain the influence of BCMA and TACI signaling on NF-kB activation in SCG neurons and whether this plays any role in the influence of BCMA and TACI on NGF-promoted survival. Furthermore, it will also be informative to investigate whether the effects of BCMA and TACI signaling in SCG neurons are restricted to NGF-promoted survival or whether they affect the survival of these neurons in response to Artemin or NT-3.

Blocking the function of BCMA and TACI also greatly reduced the survival of E15 nodose neurons cultured with BDNF, suggesting that endogenous signalling by these receptors facilitates the survival response of these neurons to BDNF. As in the case of SCG, blocking BCMA function did not affect neurotrophin-promoted neurite growth. Surprisingly, and in contrast to SCG neurons, function-blocking antibodies to either ligand did not affect neurotrophin-promoted survival of E15 nodose neurons. This raises the question of the ligand or mechanism by which BCMA and TACI are presumably activated in cultured embryonic nodose neurons. It is possible, however, that there is redundancy in the ligand receptor interactions such that blocking a single ligand is insufficient to sufficiently disrupt receptor action by the remaining ligand to the extent that it affects BDNF-promoted neuronal survival. For this reason, it would have been informative to have studied the consequences of treating the neurons with function blocking antibodies to both ligands in combination. Likewise, experiments involving different combinations of function blocking antibodies to receptors and ligands would have been informative in addressing issues of potential redundancy and potential combinatorial effects of BCMA and TACI signalling in sympathetic neurons. Furthermore, had time permitted, it would have been informative to study the effects of exogenous soluble ligands on the survival and growth of neurites from cultured nodose and SCG neurons in the presence and absence of neurotrophins. These experiments would have been particular pertinent given the immunohistochemical findings that suggest that APRIL could potentially act as a target-field derived factor for developing SCG neurons.

Perhaps the most pressing and important further studies will be to investigate the physiological relevance of the in vitro observations reported in this chapter by studying the phenotype of mice lacking *BCMA*, *BAFF*, *APRIL* or *TACI* genes. Does deletion of these genes singularly and/or in combination lead to a reduction in the number of neurons in the SCG and nodose ganglia? If so and there are accompanying reductions in innervation density in target tissues, if neuronal death is prevented by generating double knockout mice with the BAX gene, will innervation density remain unaffected or will there be changes that might be explained by disrupted ligand receptor signalling in the target tissues in the case of *BAX/BCMA* or *BAX/APRIL* double knockout mice compared with *BAX* mice? Furthermore, are other populations of neurons and their projections affected? Clearly, the

novel findings reported in this chapter not only provide evidence for the first role for BCMA and TACI signalling in neurons but also form the basis of an important body of future research.

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