

# **Expression and function of Wnt, TNF and TGF- $\beta$ superfamily receptors in developing neurons**

**A thesis submitted to Cardiff University**

**for the degree of PhD**

**2009**

**Dânia Sofia da Silva Teixeira**

**Cardiff School of Biosciences**

**Cardiff University**

UMI Number: U584392

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U584392

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

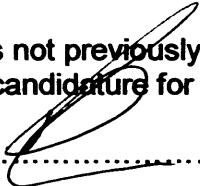
**NOTICE OF SUBMISSION OF THESIS FORM:  
POSTGRADUATE RESEARCH**



**APPENDIX 1:  
Specimen layout for Thesis Summary and Declaration/Statements page to be included in a Thesis**

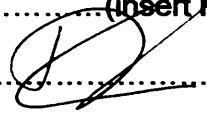
**DECLARATION**

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed  ..... (candidate)      Date 25/11/09 .....

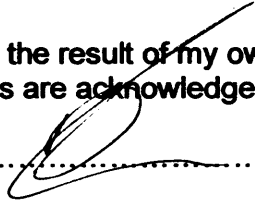
**STATEMENT 1**

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD ..... (insert MCh, MD, MPhil, PhD etc, as appropriate)

Signed  ..... (candidate)      Date 25/11/09 .....

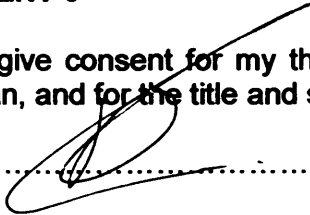
**STATEMENT 2**

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

Signed  ..... (candidate)      Date 25/11/09 .....

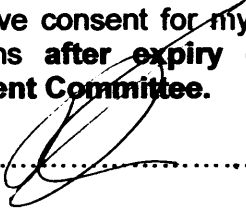
**STATEMENT 3**

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed  ..... (candidate)      Date 25/11/09 .....

**STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS**

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans after expiry of a bar on access previously approved by the Graduate Development Committee.

Signed  ..... (candidate)      Date 25/11/09 .....

## **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- $\beta$  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- $\beta$  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**  
**GDNF – Glial derived neurotrophic factor**  
**GSK-3 $\beta$  – Glycogen synthase kinase 3 $\beta$**   
**HBSS – Hanks balanced salt solution**  
**HGF – Hepatocyte growth factor**  
**HVEM – Herpes virus entry mediator**  
**IKK – Inhibitor  $\kappa$ B 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- $\kappa$ B – 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**  
**PLC $\gamma$  – 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**  
**TGF $\beta$  – Transforming growth factor  $\beta$**   
**TH – Tyrosine hydroxylase**  
**TNF – Tumor necrosis factor**  
**TNFSF- Tumor necrosis factor superfamily**  
**TNFSFR – Tumor necrosis factor superfamily receptor**  
**TNF $\alpha$  – Tumor necrosis factor  $\alpha$**

**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**

# Index

<b>1. General introduction</b>	<b>1</b>
<b>1.1 A brief overview of neuronal development</b>	<b>2</b>
1.1.1 Fertilization to Gastrulation	2
1.1.2 Neural tube formation	3
1.1.3 Neuronal migration	6
1.1.4 Synaptogenesis	8
<b>1.2 Organization and development of the peripheral nervous system</b>	<b>9</b>
1.2.1 Introduction	9
1.2.2 Organization of the somatic PNS	10
1.2.3 Organization of the autonomic PNS	12
1.2.4 Development of the peripheral nervous system	15
<b>1.3 Regulation of neuronal survival in the PNS</b>	<b>16</b>
1.3.1 Introduction	16
1.3.2 Neurotrophic theory	17
1.3.3 Neurotrophic requirements of selected peripheral ganglia	19
1.3.4 NGF	21
1.3.5 BDNF	21
1.3.6 Trk receptors	22
1.3.7 p75	23
<b>1.4 Regulation of neurite outgrowth</b>	<b>23</b>
1.4.1 Axon extension and guidance	23
1.4.2 The role of neurotrophic factors in regulating neurite growth from PNS neurons	24
1.4.3 Intracellular signaling pathways downstream of neurotrophic factors regulating neuronal survival and neurite outgrowth	24
<b>1.5 Wnt Signaling</b>	<b>26</b>
1.5.1 Introduction	26
1.5.2 Wnt signaling pathways	27

1.5.3 Wnt signaling in neuronal development	29
1.5.4 Conclusions	33
1.6 Transforming growth factor $\beta$ (TGF $\beta$ ) superfamily	33
1.6.1 Prototype of the TGF $\beta$ precursor	33
1.6.2 Diversity of ligands	34
1.6.3 The DVR subfamily	34
1.6.4 TGF $\beta$ superfamily receptors	37
1.6.5 The Smads	39
1.7 The tumor necrosis factor (TNF) superfamily	40
1.7.1 TNF ligands	41
1.7.2 TNF receptors	43
1.7.3 TNF and TNFR interactions and cell signaling	44
1.7.5 Structural and functional footprints of the TNF/TNFR superfamily	46
1.7.6 Conclusions	47
1.8. NF- $\kappa$ B signaling	47
2. Material and methods	49
2.1 Introduction	50
2.2 Mouse maintenance	50
2.3 Preparation of dissection and culture media	50
2.3.1 Dissection media (L-15)	50
2.3.2 Washing media (F-12)	51
2.3.3 Culture media (F-14)	51
2.4 Preparation of Tungsten needles	51
2.5 Dissections	52
2.5.1 Embryonic ages	52
2.5.2 Post-natal mice	52
2.6 RNA extraction	52
2.6.1 Background	52
2.6.2 Method	53
2.7 Reverse Transcriptase	53
2.7.1 Background	53
2.7.2 Method	54

<b>2.8 Real time PCR:</b>	<b>54</b>
2.8.1 Background	54
<b>2.9 Cell culture</b>	<b>60</b>
2.9.1 Preparation of dishes	60
2.9.2 Dissection of the trigeminal, nodose and superior cervical ganglia	60
2.9.3 Dissociation of the ganglia	61
2.9.4 Quantification of neuronal survival	61
2.9.5 Quantification of neurite outgrowth	62
<b>2.10 Transfections</b>	<b>62</b>
2.10.1 Plasmid preparation	62
2.10.2 Preparation of the gold microcarriers for transfection	63
2.10.3 Ballistic transfection	63
2.10.4 Electroporation	64
<b>2.11 Immunocytochemistry</b>	<b>64</b>
<b>2.12 Histology</b>	<b>65</b>
2.12.1 Frozen sections	66
2.12.2 Wax sections	66
2.12.3 SCG total counts	67
<b>2.13 Measurement of sympathetic innervation in target tissues</b>	<b>67</b>
<b>2.14 Western Blotting</b>	<b>67</b>
2.14.1 Preparation of culture extracts	67
2.14.2 Western Blotting	68
<b>2.15 siRNA</b>	<b>69</b>
2.15.1 Background	69
2.15.2 Method	69
<b>2.16 Statistical analysis</b>	<b>70</b>
<b>3. RT-QPCR based screening for the expression of Wnt, TGF <math>\beta</math> and TNF superfamily mRNAs in the developing PNS</b>	<b>71</b>
3.1 Polymerase Chain Reaction	72
3.2 Primer design	74
3.3 Real-time PCR	75
3.4 Experimental design	80

<b>3.5 Results/ Discussion</b>	<b>83</b>
3.5.1 Wnt superfamily	84
3.5.2 TGF- $\beta$ superfamily	96
3.5.3 TNF superfamily	110
<b>4. Growth differentiation factor 5 is a target derived growth factor that is required for correct target innervation by sympathetic neurons <i>in vivo</i>.</b>	<b>135</b>
4.1 Introduction	136
4.2 Results	138
4.3 Discussion	159
<b>5. Role of TNFRSF members BCMA and TACI in regulating the neurotrophin survival responses of developing sensory and sympathetic neurons</b>	<b>162</b>
5.1 Introduction	163
5.2 Results	164
5.3 Discussion	190
<b>6. References</b>	<b>195</b>



## Figure Index

Figure 1: Neural tube formation.....	4
Figure 2: Early embryonic CNS.....	5
Figure 3: Defects in neuronal migration.....	7
Figure 4: synaptic structure. ....	9
Figure 5: Cranial nerves. ....	11
Figure 6: Dorsal root ganglion.....	12
Figure 7: Sympathetic and parasympathetic nervous systems.....	14
Figure 8: The molecular basis of apoptosis. ....	17
Figure 9: The three main branches of the Wnt signaling pathway.....	29
Figure 10: BMP signaling through Smad proteins.....	39
Figure 11: The TNF superfamily .....	41
Figure 12: Cellular signaling pathways leading to activation of the main responses by members of the TNF superfamily .....	45
Figure 13: summary of the signaling pathways downstream of BAFF receptor.....	46
Figure 14: The PCR principle. ....	73
Figure 15: Real-time PCR Program graphs. ....	79
Figure 16: Expression profiles for FZD1 and FZD2.....	85
Figure 17: Expression profiles for FZD3 and FZD4.....	85
Figure 18: Expression profiles for FZD5 and FZD6.....	88
Figure 19: Expression profiles for FZD7 and FZD9.....	88
Figure 20: Expression profiles for LRP5 and LRP6. ....	91
Figure 21: Expression profiles for DKK and Ryk.....	91
Figure 22: Expression profile of Kremen1. ....	92
Figure 23: Expression profiles of Alk1 and Alk2. ....	97
Figure 24: Expression profiles of Alk3 and Alk4. ....	97
Figure 25: Expression profiles of Alk5 and Alk6. ....	101
Figure 26: Expression profiles of Alk7 and Noggin. ....	101
Figure 27: Expression profiles of BMPII and TGF $\beta$ -II. .T.....	105
Figure 28: Expression profiles of ActR2a and ActR2b. ....	105
Figure 29: Expression profile of GDF5.....	109
Figure 30: Expression profiles of DR6 and TRAIL. ....	113
Figure 31: Expression profiles of CD30 and DR5. ....	113
Figure 32: Expression profiles of Fas and CD27.....	114
Figure 33: Expression profiles of 4-1BB and OX40.....	114
Figure 34: Expression profiles of OPG and TACI. ....	118

Figure 35: Expression profiles of CD40 and Fn14.....	118
Figure 36: Expression profiles of HVEM and BCMA.....	123
Figure 37: Expression profiles of EDAR and XEDAR.....	123
Figure 38: Expression profiles of TROY and mSOB. ....	126
Figure 39: Expression profiles of BAFF-R and DR3.....	126
Figure 40: Expression profiles of APRIL and TNF- $\alpha$ . ....	129
Figure 41: Expression profiles of BAFF and Tweak. ....	129
Figure 42: Expression profiles of LT $\beta$ -R and TL1A. ....	130
Figure 43: Expression of BMPRs in the developing SCG.....	138
Figure 44: Expression of BMPR-Ib in axons and growth cones.....	139
Figure 45: Expression of GDF-5 in SCG compared to SCG target fields.....	141
Figure 46: Expression of GDF5 in the SCG target tissues. ....	142
Figure 47: GDF5 protein is present in the SCG.....	145
Figure 48: Effect of GDF-5 on sympathetic neuron survival. ....	145
Figure 49: GDF5 dose response.....	147
Figure 50: Effect of GDF-5 on sympathetic neurite growth.....	148
Figure 51: Noggin blocks effects of GDF-5 on neurite growth. ....	149
Figure 52: GDF5 activates Smad pathway in sympathetic neurons.....	151
Figure 53: Brachypod mice show peripheral skeletal malformations .....	151
Figure 54: Effect of brachypod mutation on sympathetic axon growth in vitro. ....	153
Figure 55: Effect of brachypod mutation on sympathetic innervation of the nasal mucosa. ....	154
Figure 56: Effect of brachypod mutation on sympathetic innervation of the SMG.....	155
Figure 57: Reduction in innervation is not due to a loss of neurons or a loss of TH Expression. ..	156
Figure 58: Expression of BCMA, TACI, BAFF and APRIL in the developing SCG.....	164
Figure 59: Expression of BCMA, APRIL, BAFF and TACI in axons and growth cones. ....	167
Figure 60: Expression of BCMA in the SCG ganglion.....	168
Figure 61: Expression of BCMA and APRIL in the SCG compared with target fields.....	168
Figure 62: Expression of BCMA and APRIL in the nasal mucosa.....	169
Figure 63: Expression of BCMA and APRIL in the SMG.....	170
Figure 64 : Effect of blocking BCMA,BAFF,APRIL and TACI on sympathetic neuron survival. 172	
Figure 65: Effect of NGF dose response in sympathetic neurons.....	173
Figure 66: Effect of SiRNA transfections in sympathetic neurons survival.....	176
Figure 67: Effect of knocking down BCMA gene with SiRNA in sympathetic neurons. ....	176
Figure 68: Effect of blocking BCMA on sympathetic neurite growth. ....	179
Figure 69: Expression of BCMA, TACI, BAFF and APRIL in the developing Nodose ganglion. 181	
Figure 70: Expression of BCMA, APRIL, BAFF and TACI in axons and growth cones.....	183
Figure 71: Anti-BCMA dose response.....	185
Figure 72: Effect of BDNF dose response in Nodose neurons.....	185
Figure 73: Effect of blocking BCMA, BAFF, APRIL and TACI on sensory neuron survival.....	186
Figure 74: Effect of blocking BCMA on sensory neurite growth.....	188

## **Acknowledgments**

**Many thanks to Professor Alun Davies, for the supervision and support over the last few years. Thanks for your advice and permanent good nature.**

**Thanks to Dr. Gerard O’Keeffe and Dr. Sean Wyatt for having the time to help me with techniques or discuss experiments in a patient, enthusiastic and friendly manner.**

**To all members of the Davies and Wyatt’s labs, past and present, that helped create a relaxed working environment and were always supportive inside and outside the lab.**

**To all my friends in Portugal who always encouraged me follow my dreams and never give up.**

**To my “abnormal” family, to whom I could not done this without a special thank you.**

# **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 an inner cell mass and outer cell mass or trophoblast. The inner cell mass develops into the embryo, while the trophoblast becomes the placenta. The beginning of gastrulation in mice is evidenced by the formation of a transient structure called the 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/ $\beta$ -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).

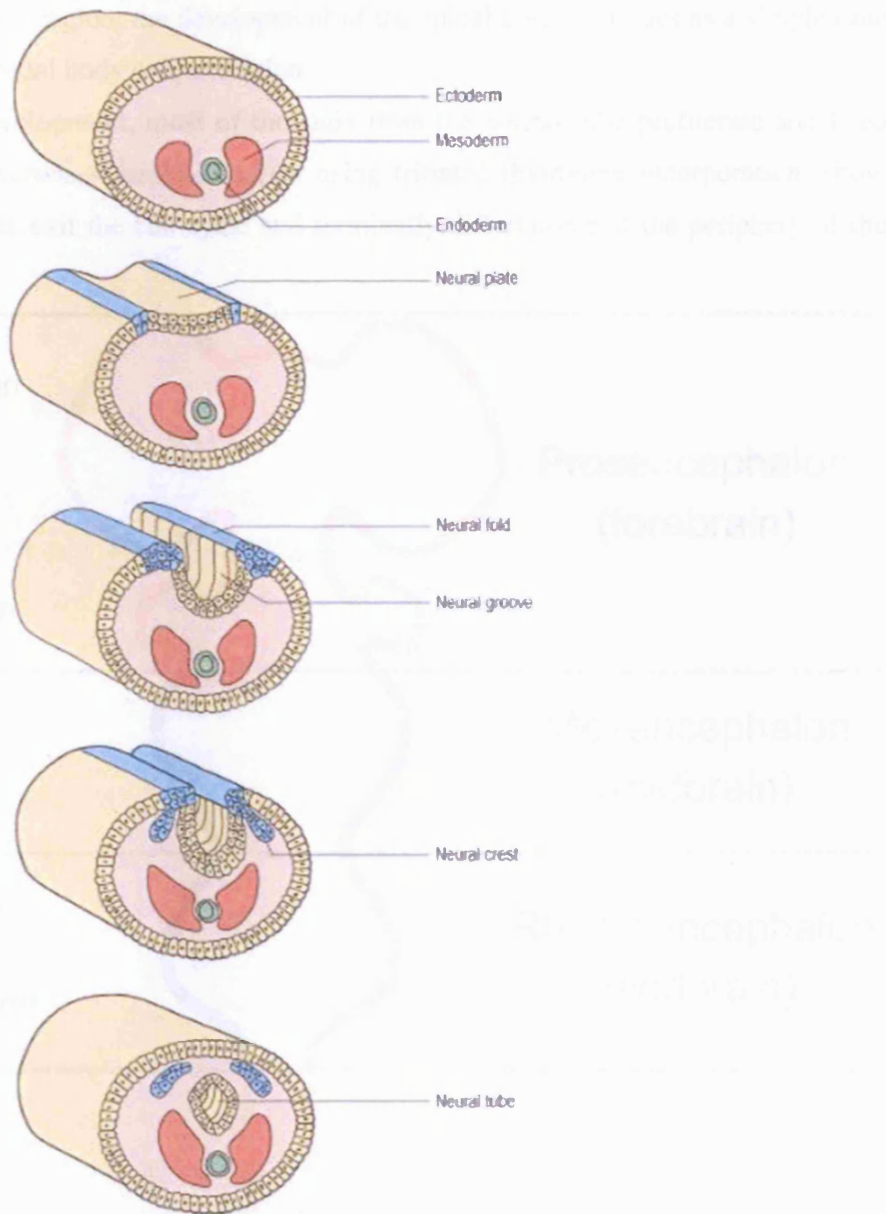
### **1.1.2 Neural tube formation**

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 myelomeningocele (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).

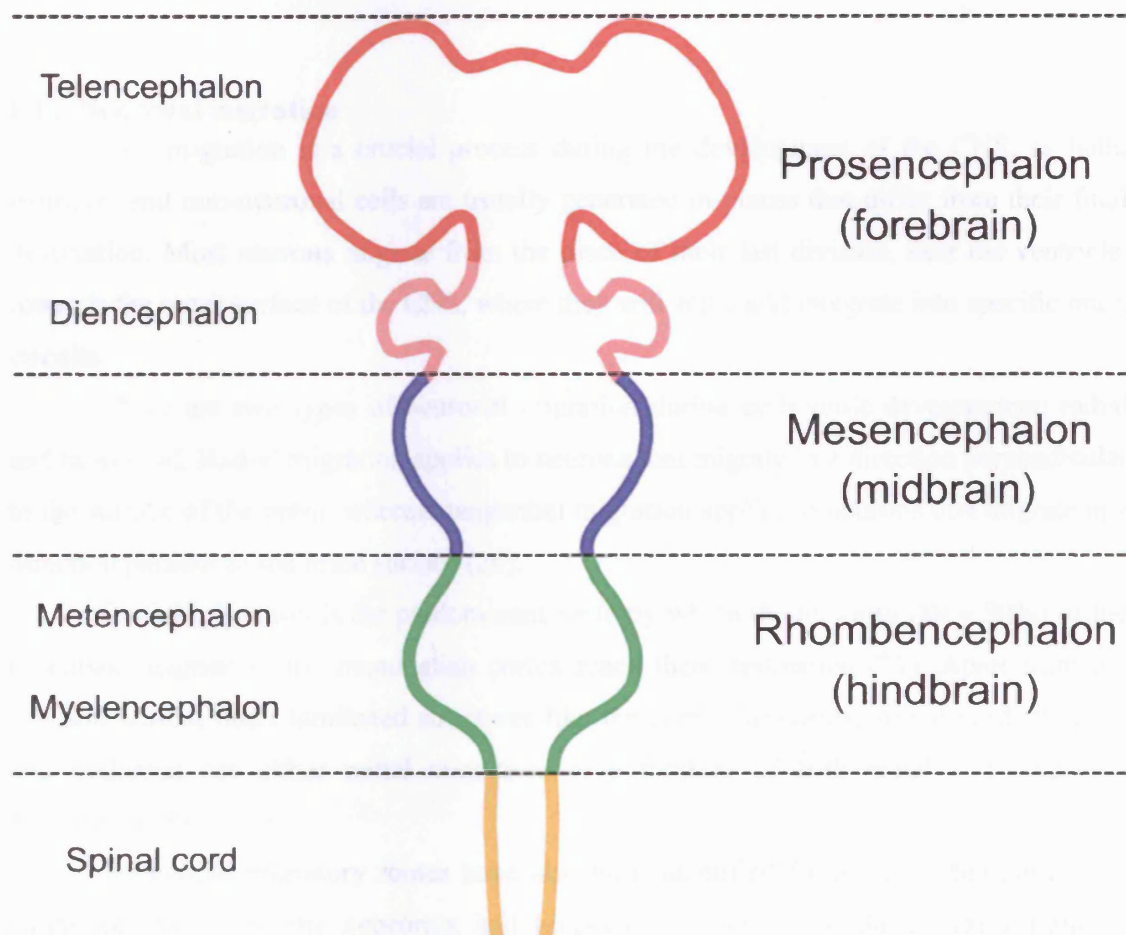


**Figure 1:** Neural tube formation. The neural tube is formed as a flat neural plate that folds toward the midline until it closes to form the neural tube. (www.studentconsult.com)

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.

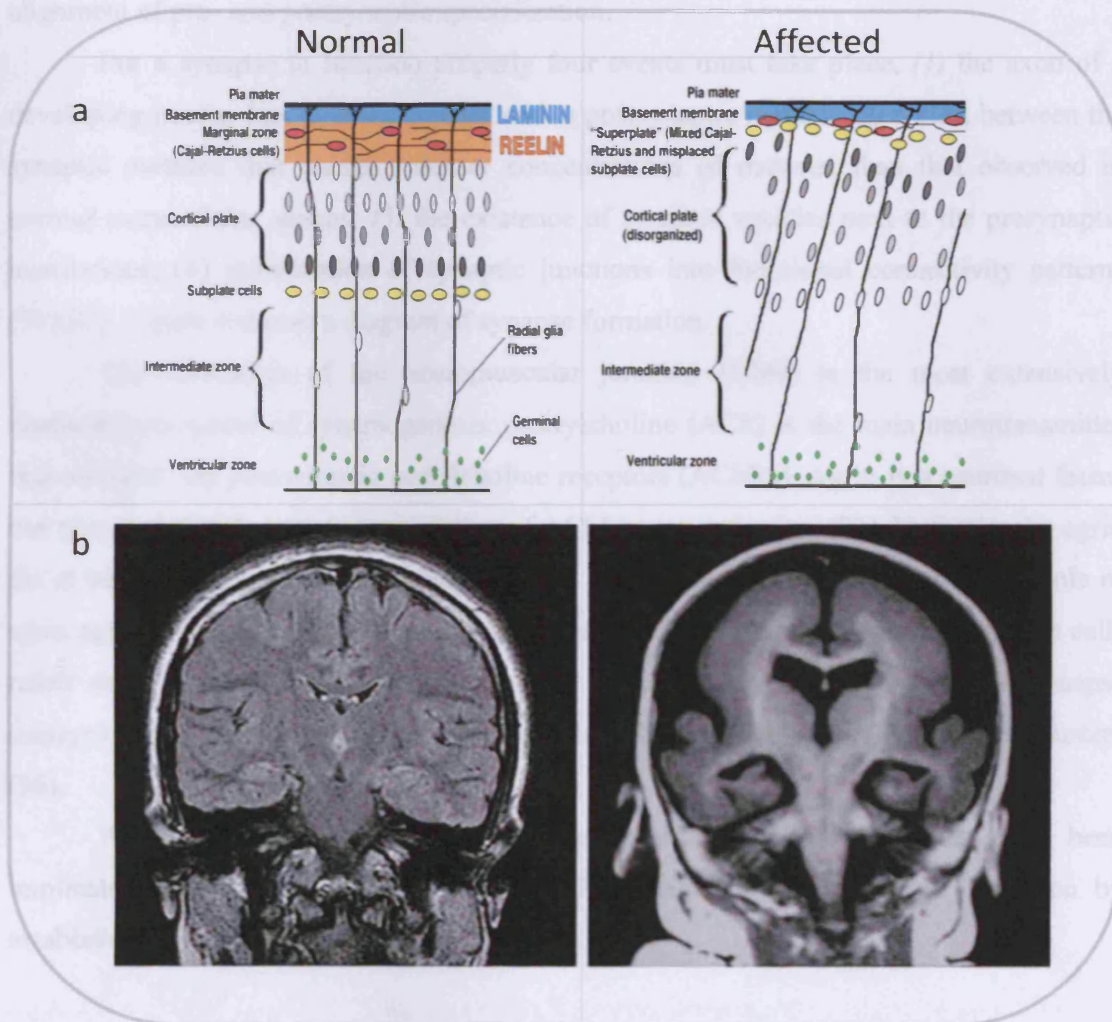
There are two types of neuronal migration during embryonic development: radial and tangential. Radial migration applies to neurons that migrate in a direction perpendicular to the surface of the brain, whereas tangential migration applies to neurons that migrate in a direction parallel to the brain surface (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. Lissencephalic individuals have a smooth cortex lacking the characteristic sulci and gyri observed in the normal brain. (30)

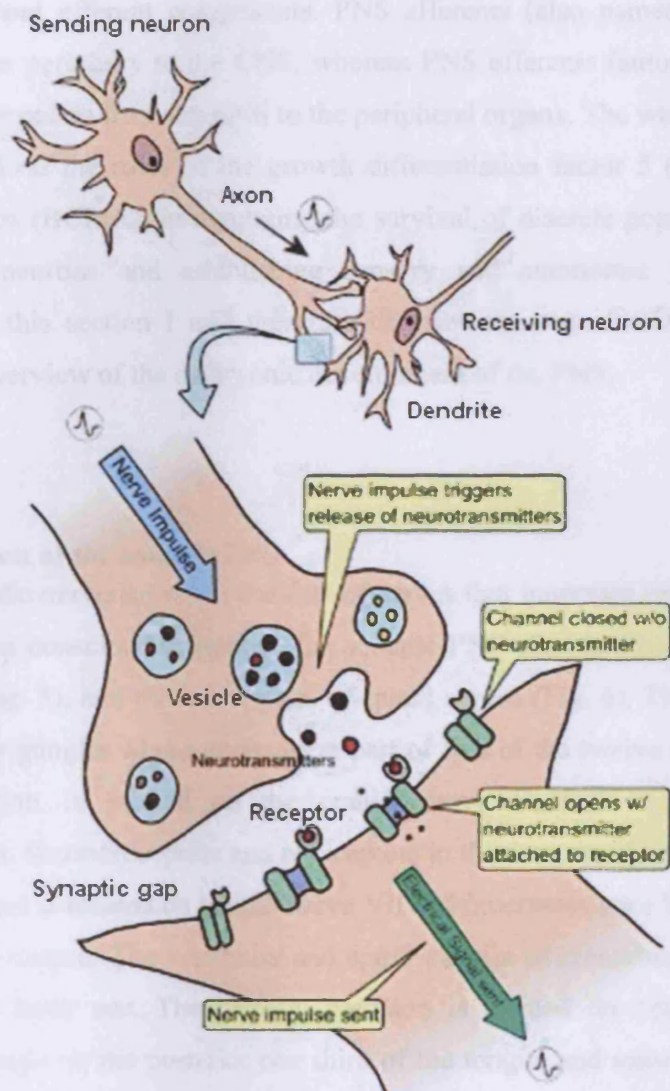
#### **1.1.4 Synaptogenesis**

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

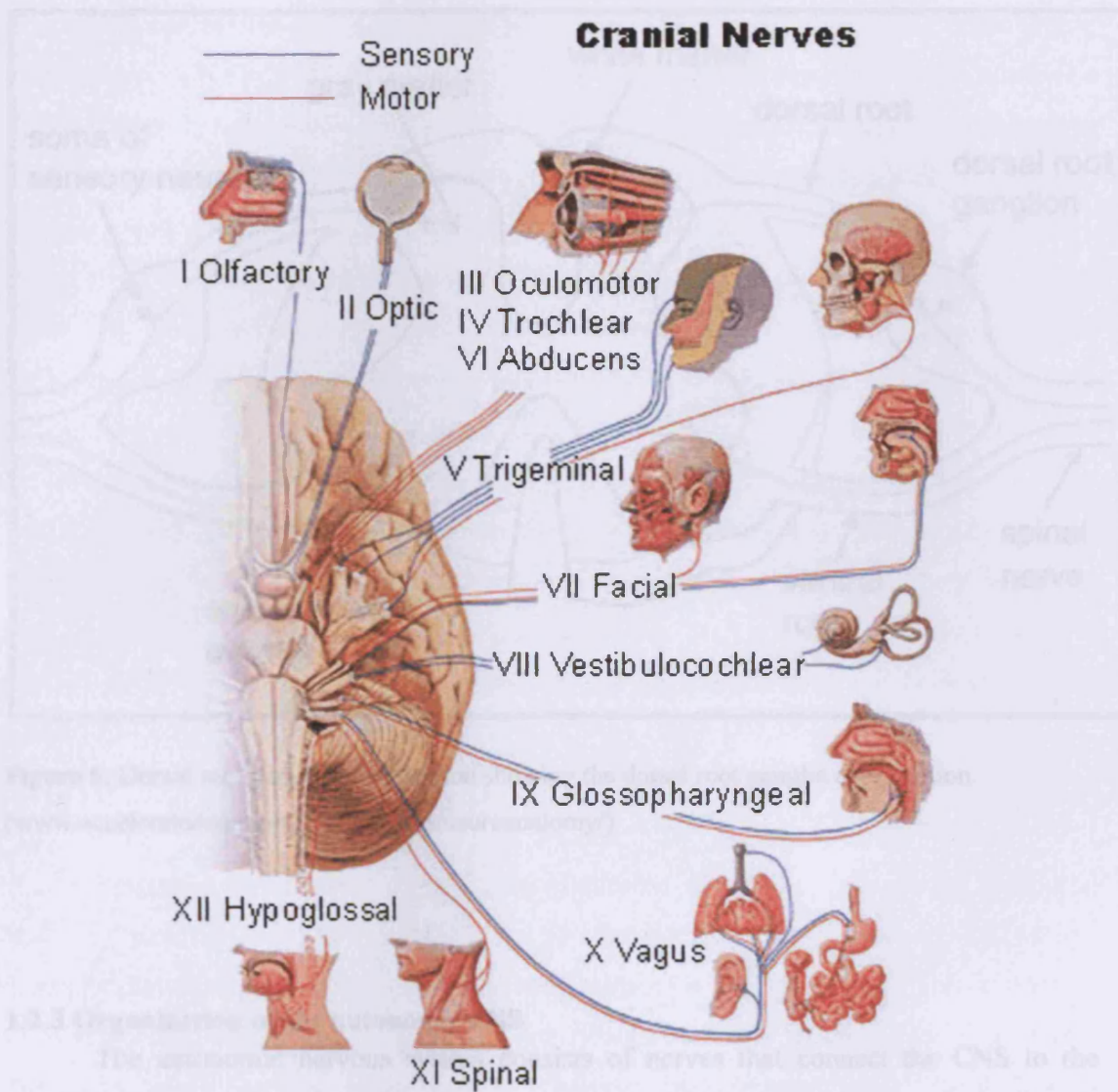
into an afferent and efferent components. PNS afferents (also named sensory), convey impulses from the periphery to the CNS, whereas PNS efferents (autonomic and somatic motor) transmit impulses from the CNS to the peripheral organs. The work described in this thesis is centered on the roles of the growth 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**

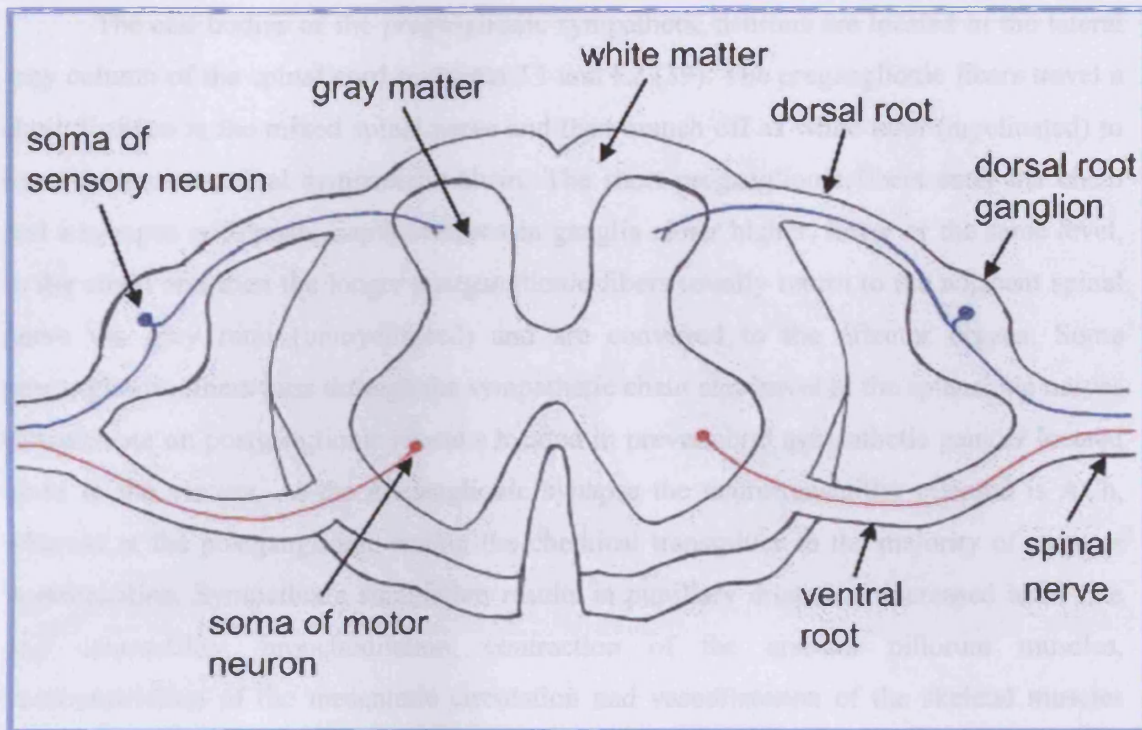
The somatic nervous system consists of nerves that innervate the skin and muscles, and is involved in conscious activities. The somatic PNS signals through twelve pairs of cranial nerves (Fig. 5), and thirty one pairs of spinal nerves (Fig. 6). There are seven pairs of cranial sensory ganglia whose axons 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](http://www.web-books.com))



**Figure 6:** Dorsal root ganglion. Illustration showing the dorsal root ganglia organization.

([www.acceleratedcure.org/msresources/neuroanatomy/](http://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.

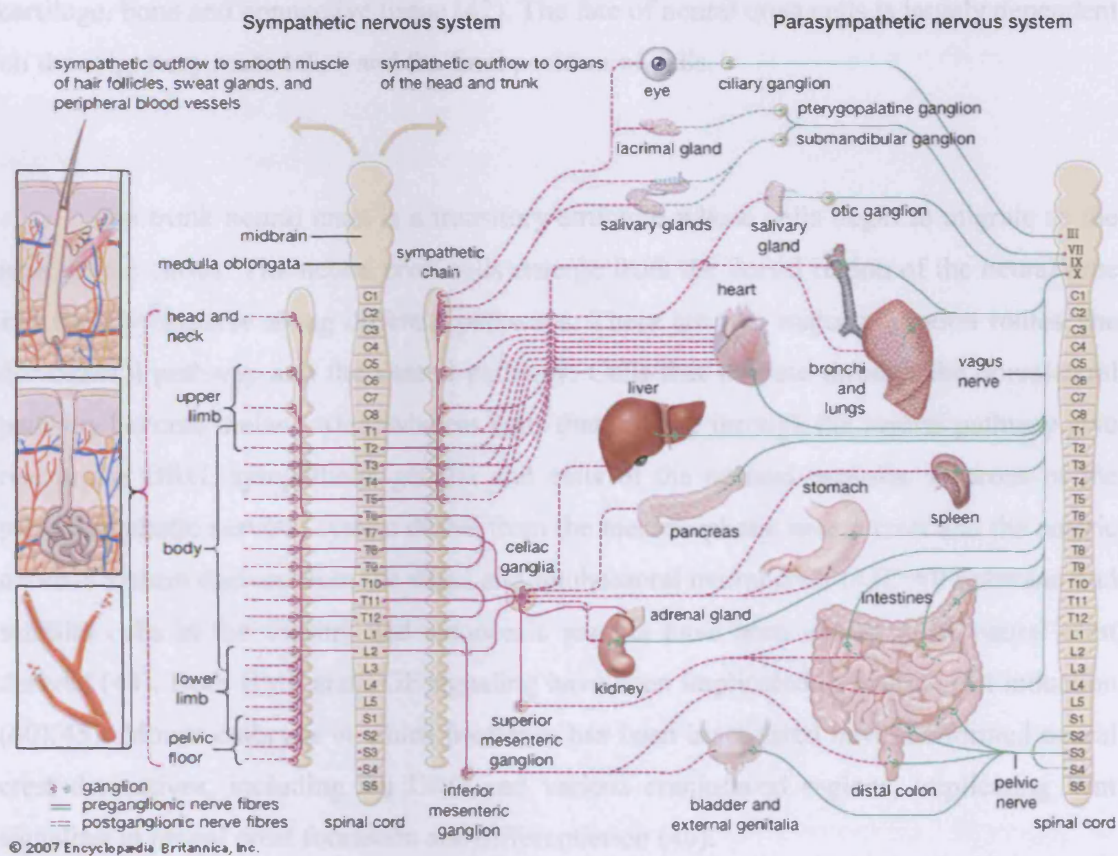
The cell bodies of the preganglionic sympathetic neurons are located in the lateral grey column of the spinal cord segments T1 and L2 (39). The preganglionic fibers travel a short distance in the mixed spinal nerve and then branch off as white rami (myelinated) to enter the paravertebral sympathetic 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.



1.2.4 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  $\beta$ -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**

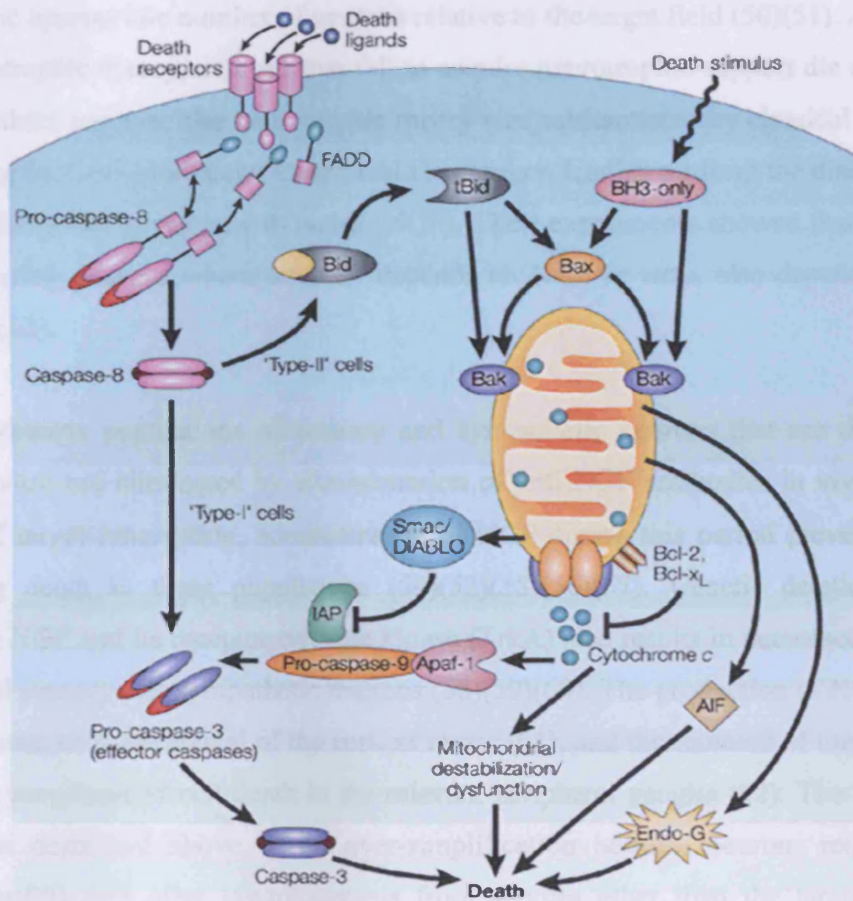
### **1.3.1 Introduction**

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<sup>-/-</sup> 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 in 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  $\beta$  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<sup>-/-</sup> 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<sup>-/-</sup> mice can be prevented in NGF<sup>-/-</sup>/BAX<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice overlap with those lost in the knockout mice of the primary neurotrophin ligand. TrkA<sup>-/-</sup> mice have decreased

neuronal number in trigeminal and sympathetic ganglia (59).  $TrkB^{-/-}$  mice lose 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, ephrins, 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<sup>-/-</sup>/TrkA<sup>-/-</sup> 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 PLC $\gamma$  (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 clathrin-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 PLC $\gamma$ 1 (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/phosphatidylinositol 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 shown to be enough to both sustain the survival of (187)(188)(189)(190) and enhance neurite growth from some 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 ubiquitin 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)  $\gamma$ 1 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/ $\beta$ -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  $\beta$ -catenin-mediated transcriptional activation. Activated DVL induces the disassembly of a complex consisting of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) and  $\beta$ -catenin. In the absence of Wnts, GSK3 $\beta$  phosphorylates  $\beta$ -catenin, which targets it for degradation via the proteasome. Wnt binding to FZD receptors results in GSK3 $\beta$  inhibition, which leads to an increase in the levels of  $\beta$ -catenin in the cytoplasm.  $\beta$ -catenin then translocates to the nucleus and forms a complex with the T-cell specific transcription factor (TCF). This  $\beta$ -catenin/TCF complex then 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).

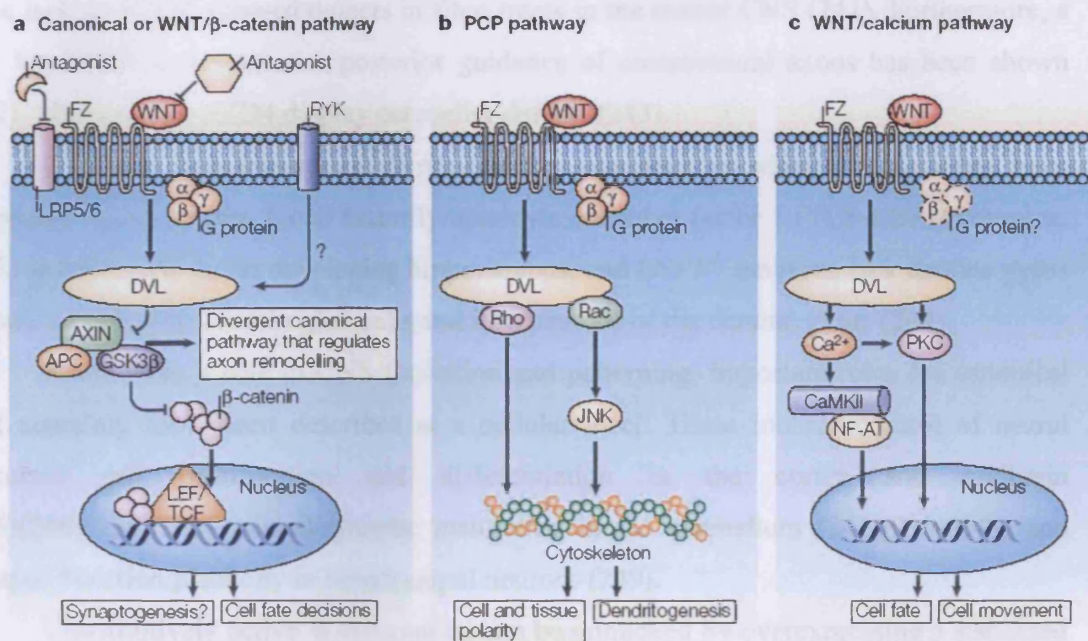
In *Drosophila melanogaster*, activated FZD signals to DVL, which, in turn, activates the small GTPase RhoA and c-Jun amino (N)-terminal kinase (JNK) (221).

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<sup>-/-</sup>* 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  $\beta$ -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  $\beta$ -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 form of  $\beta$ -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  $\beta$ -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. Though this role of  $\beta$ -catenin in neuronal plasticity is closely associated with its role in cell to cell adhesion, there is evidence that the pool of  $\beta$ -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 an 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  $\beta$  ( $A\beta$ ) 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 GSK3 $\beta$  activity (258), recent evidence indicates that at least a component of LiCl's neuroprotective effects is mediated through inhibition of GSK3 $\beta$  and consequent activation of the canonical Wnt pathway by  $\beta$ -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 GSK3 $\beta$  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 A $\beta$ -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<sup>-/-</sup> 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 $\beta$ (TGF $\beta$ ) superfamily**

The transforming growth factor  $\beta$  (TGF $\beta$ ) 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 TGF $\beta$  may stimulate or inhibit cell proliferation depending on whether the target is a fibroblast or a keratinocyte (277). In mammary epithelial cells TGF $\beta$  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 TGF $\beta$  superfamily, TGF $\beta$  was initially discovered in the medium of transformed mouse fibroblasts (279)(280), where it inhibits tumor formation. Subsequent studies have shown that TGF $\beta$  is a tumor suppressor and a potent inhibitor of cell proliferation (for review see (281). These studies have shown that TGF $\beta$  may have potential as a therapeutic agent for cancer therapy (for reviews see (281)(282)(283).

#### **1.6.1 Prototype of the TGF $\beta$ precursor**

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

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

Despite the diversity of TGF $\beta$  members in the animal kingdom all members of the TGF $\beta$  superfamily are structurally related. TGF $\beta$  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 TGF $\beta$  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 TGF $\beta$  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 TGF $\beta$ -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 TGF $\beta$  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 TGF $\beta$  superfamily except for BMP1, which does not have the C terminal sequence of the TGF $\beta$  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).

TGF $\beta$  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 TGF $\beta$ superfamily receptors**

The TGF $\beta$  superfamily of ligands binds to and initiate signal transduction through a family of transmembrane serine/threonine kinases – the TGF $\beta$  receptor superfamily (327). Based on their structural and functional properties, the TGF $\beta$  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 TGF $\beta$  (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 heterotetramers 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 TGF $\beta$  (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 TGF $\beta$  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 TGF $\beta$  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 TGF $\beta$  type I receptors (AR-Smads). Smad4 is the only common-partner Smad (co-Smad) in mammals that is shared by both BMP and TGF $\beta$  activin signaling pathways. Smad6 and Smad7 function as inhibitory Smads (I-Smads) (335).

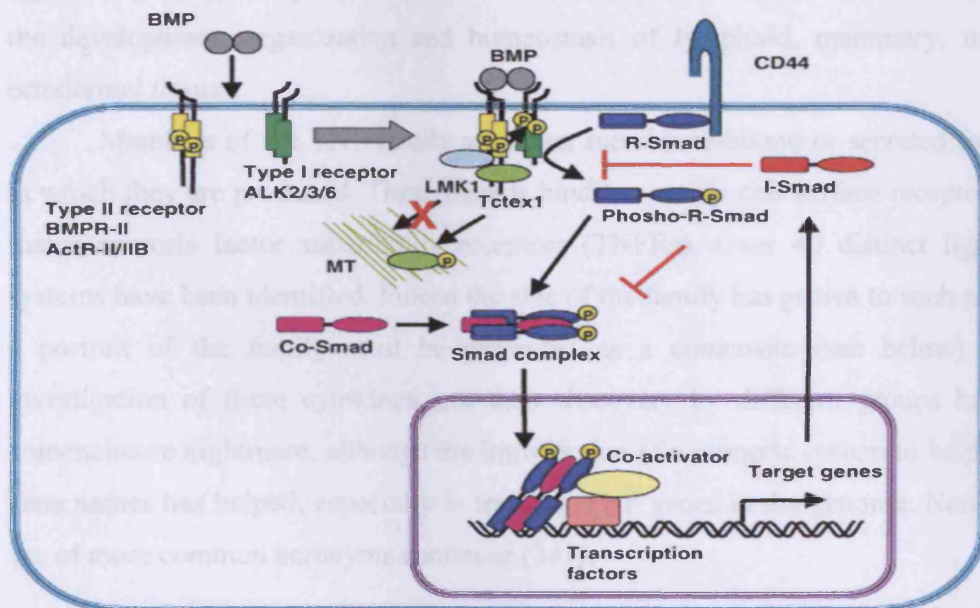


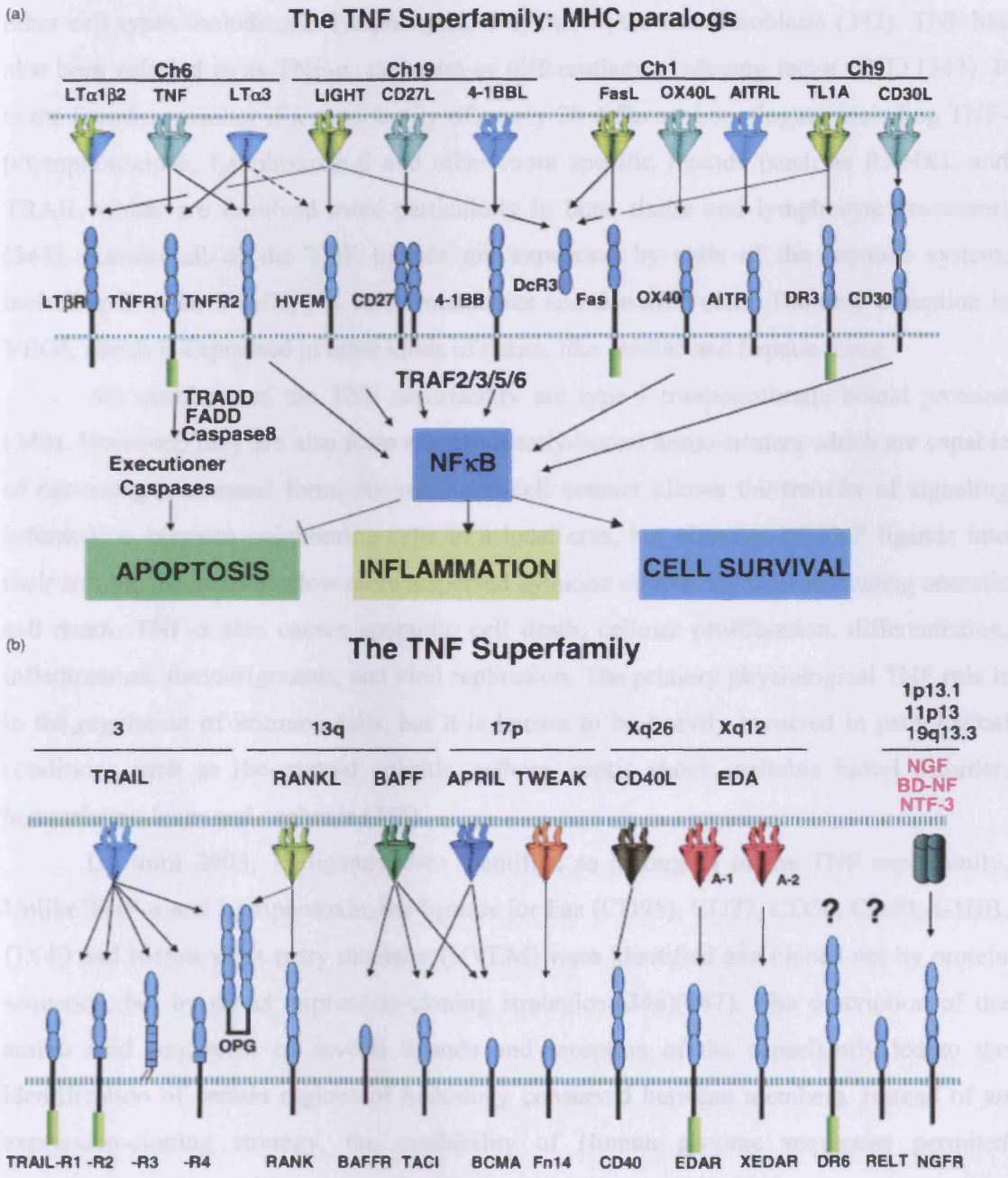
Figure 10: BMP signaling through Smad proteins. (335)

## **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- $\alpha$  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- $\alpha$ , cachectin or differentiation inducing factor (DIF) (343). It is the founder member of a superfamily of nearly 20 different homologues including TNF- $\beta$ /lymphotoxin- $\alpha$ , lymphotoxin- $\beta$  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 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- $\alpha$  also causes apoptotic cell death, cellular proliferation, differentiation, inflammation, tumorigenesis, 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- $\alpha$  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 permitted 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- $\kappa$ B (NF- $\kappa$ B) ligand (RANKL), also known as TRANCE (TNF-related activation-induced cytokine) or 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  $\beta$ -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- $\alpha$ , 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 not 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- $\alpha$  only binds two of these receptors, TNFR1 and TNFR2. TNF- $\alpha$  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).

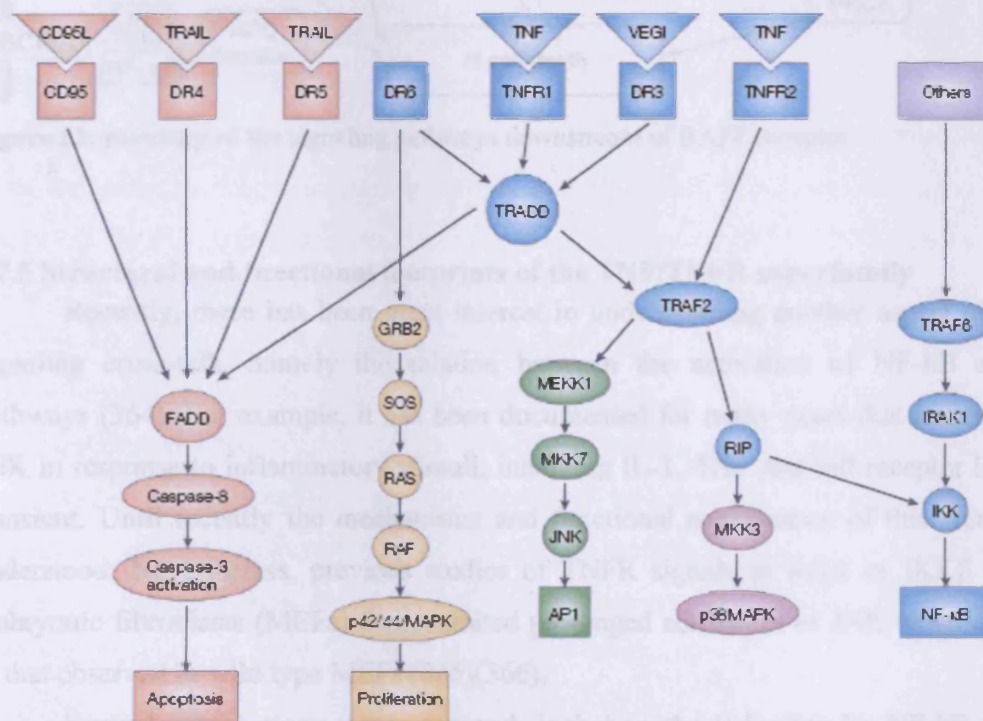
A number of conditions have been identified which shed light on the physiological role of some of these receptors. For example, mutations in the extracellular domain of TNFR1 have been shown to suppress the secretion of the receptor leading to a family of dominantly inherited autoinflammatory syndromes (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- $\alpha$ , perhaps because its receptors are expressed by all cell types. This fact might also account for the non-specific toxicity of TNF- $\alpha$ . 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

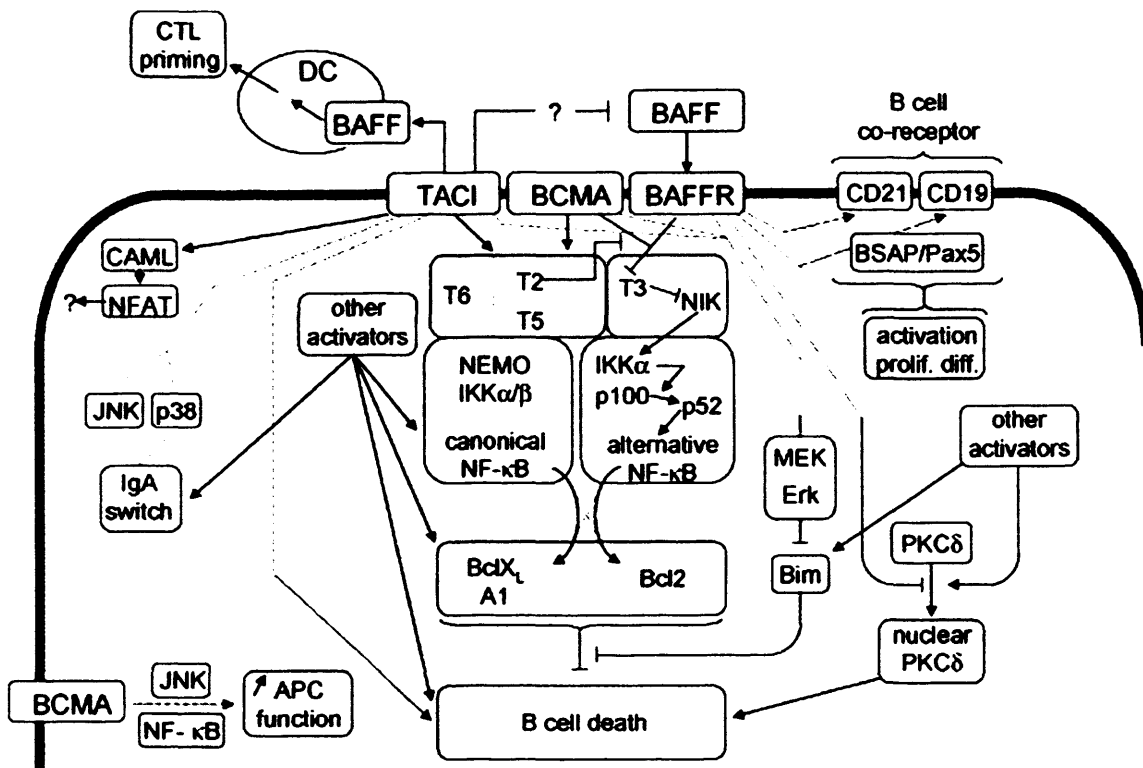
OPG variant that has two death domains, but lacks a transmembrane domain, however, has also been shown to function as a ligand through as unknown receptor (363).

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- $\kappa$ B, 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-κB 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-κB 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-κB 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- $\kappa$ B 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- $\kappa$ B 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 do 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- $\kappa$ B signaling**

NF- $\kappa$ B was first described as a nuclear factor that, when activated by bacterial lipopolysaccharides binds to the enhancer region of the gene encoding the  $\kappa$  light chain of antibodies in B cells (371). Five NF- $\kappa$ B family members have been cloned and characterized, c-Rel, NF $\kappa$ B1 (p50/p105), NF $\kappa$ B2 (p52/p100), RelA (p65) and RelB. All members of the NF- $\kappa$ B family contain a characteristic Rel homology domain (RHD)

involved in DNA binding, dimerization and interaction with inhibitory proteins called I $\kappa$ B (372). All NF- $\kappa$ B proteins form either homo or heterodimers, NF- $\kappa$ B being the original name for the p50-p65 heterodimer, which is the most abundant form in the nervous system. NF- $\kappa$ B 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- $\kappa$ B may be involved in synaptic plasticity. NF- $\kappa$ B signaling is also regulated during development and plays a role in proliferation and migration of early neurons (377)(382)(383). NF- $\kappa$ B promotes the survival of cortical neurons, cerebellar granule cells and sympathetic and sensory neurons (164)(377)(384)(385)(386)(387). Inhibiting NF- $\kappa$ B decreased neurite growth and arborization in the neonatal somatosensory cortex and nodose ganglion (388). Increased NF- $\kappa$ B activity has been demonstrated in brain tissue from Alzheimer's disease patients (389)(390)(391).

Recently, NF- $\kappa$ B signaling has been shown 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- $\kappa$ B (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 libitum*.

GDF5<sup>-/-</sup> mice were obtained from overnight matings of GDF5<sup>+/-</sup> mice (resulting from a mating between GDF5<sup>-/-</sup> 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 the 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<sub>2</sub> 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 denaturing guanidine-thiocyanate-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 glycolysis 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  $2 \times 2$  times as much, after 3 cycles -  $2 \times 2 \times 2$  times as much or  $8 (2^3)$  times as much, after 4 cycles  $2 \times 2 \times 2 \times 2$  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 plateau 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 tetrachlorofluorescein (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<sub>2</sub> (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
<b>FZD1</b>	F	NM_021457	GCT GTC CCT GTG GCT TCC	54.8°C	90.6°C
	R		CGC TAT CGG TCG GTT ACT G		
<b>FZD2</b>	F	NM_020510	TCA GAG GAC GGT TAT CGC	52.2°C	87.8°C
	R		CCA GGT GAG GGA CAG AAT C		
<b>FZD3</b>	F	NM_021458	TTC TGA ACC ATT ATG ACC AAC	52.6°C	89.0°C
	R		ACG GGA CAC CAA ACA TCT C		
<b>FZD4</b>	F	NM_008055	ATG TTA TCG GAG TTT GGT CTA	50.4°C	84.9°C
	R		GCA TTC TGG AGG TTC ATT AGG		
<b>FZD5</b>	F	NM_022721	CAC ACC CAC TCT ACA ACA AG	56.3°C	93.5°C
	R		GGA GAT GAA GCA CAG CAC A		
<b>FZD6</b>	F	NM_008056	GCT ACT TTG CGA CTG TGA TAG	52.1°C	87.0°C
	R		AAC CTA CCA TCT TAC CTG CTG		
<b>FZD7</b>	F	NM_008057	TCG CTT CTA CCA CAG ACT CAG	55.9°C	91.5°C
	R		ACC TCC AAA TAA CTT CTC ACT TCC		
<b>FZD9</b>	F	XM_284144	GCT CTC TTC CAT ATC CGC AAA	53.5°C	87.4°C
	R		ATC AGC AGA CAA TGA CGC AGG TG		
<b>LRP5</b>	F	NM_008513	AGT TCT CAG CCC ATC CTT G	57.2°C	94.5°C
	R		CAC TCT GGT CAG CAC ACT C		

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

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

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

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

<b>4-1BB</b>	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
<b>Ox40</b>	F R	X85214	GCT TGG CGT TGA CTG TGT TC GGT GGC GGG TCT GCT TTC	55.1°C	89.9°C
<b>OPG</b>	F R	AF053713	CCC TTG CCC TGA CCA CTC TAA CGC CCT TCC TCA CAC TC	55.3°C	90.7°C
<b>TACI</b>	F R	AF257673	GTG ACA GAG GCT TGC GAC CGT GTA TCC CGA GCG AAC	56.2°C	92.6°C
<b>RELT</b>	F R	AK088621	ATT GCC ATC GTT CCT GTC GCA TTC TCT TTC TTC TCT GTC	54.8°C	92°C
<b>CD40</b>	F R	M83312	GCA CCG CAG AAT CAG ACA C GCC ATC TCC ATA ACT CCA AAG C	53.7°C	88.4°C
<b>Fn14</b>	F R	AF156164	CTG GAG AAG ATG CCG CCG AGA ATG AAT GAA TGG ACG ACG AG	55.6°C	90.9°C
<b>HVEM</b>	F R	AY264405	AGT TCC TTG TGG GAG ACG AG CCG CAG GGC AGA CAC TTG	56.3°C	56.3°C
<b>BCMA</b>	F R	NM_011608	TGA CCA GTT CAG TGA AAG G GGG TTC ATC TTC CTC AGC	51.6°C	87.1°C
<b>EDAR</b>	F R	AF160502	GAC AGC AAC ACC CAC AAA G AGC GAC AGC AGA CAT AGC	54.2°C	90.1°C
<b>XEDAR</b>	F R	AK034909	AAG ACA GTG GAG GAG GAT TC AGG ATG GAG TTA AGT GGT TTG	50.8°C	85.7°C
<b>TROY</b>	F R	AK083283	TGT GTG CCC TGC GGA GAC GCT GGA GAC GGT GGA GGA G	54°C	87.5°C
<b>mSOB</b>	F R	AY165625	AAT CAT ACT CCT TCA ACT GTC GCC ATT ATC TTT CCC TGT G	50.7°C	86.7°C
<b>BAFF-R</b>	F R	Ak008142	CCT CCG CTC AAA GAA GAT G CCT CCA CTG CTG CTA TTG	56.1°C	86.8°C
<b>DR3</b>	F R	AF329969	CCA GGT GCT TCT AGG AGT CG' GCC AGT CTG TGG TGA GGC	55.5°C	90.8°C
<b>TNF-<math>\alpha</math></b>	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 950 $\mu$ l HBSS (Gibco, Invitrogen) containing 50 $\mu$ l 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:

<b>Developmental age</b>	<b>Time in 0.05% trypsin (min)</b>
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 10 $\mu$ l 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<sup>2</sup> grid consisting of 36 squares each of 2mm<sup>2</sup>. 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 an 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 were 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 1.6 $\mu$ m gold particles were suspended in 100 $\mu$ l of 50mmol/l spermidine and 2 $\mu$ g of pYFP together with the 10 $\mu$ g of target plasmid or 10 $\mu$ g of an empty plasmid (pcDNA3.1). The gold particles were precipitated with 100 $\mu$ l of 2M CaCl<sub>2</sub>, washed three times with 100% ethanol, resuspended in 1.2ml of 100% ethanol, resuspended in 1.2ml of 100% ethanol plus 0.01 mg/ml polyvinylpyrrolidone 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 50 $\mu$ l 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<sub>2</sub> 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 70 $\mu$ m 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 then 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<sub>2</sub> 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 1 hour 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
ActinR1B	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 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<sub>2</sub>O 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<sub>2</sub>O for 5min, crystal fast violet for 5min, H<sub>2</sub>O 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<sup>-/-</sup> and GDF5<sup>+/-</sup> 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<sup>-/-</sup> and GDF5<sup>+/-</sup> 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 1 hour at room temperature. Sections were then 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- $\beta$ -tubulin (1:10,000, Chemicon) in 1% BSA in PBS-T for 1 hour 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 1 hour 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 then 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 were 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 were selected and the bacteria grown for 2 hours in LB media. The DNA was extracted using mini-prep® Qiagen kit, and sent to be sequenced. The DNA sequenced was then 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  $\pm$  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 $\beta$ 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- $\beta$  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- $\beta$  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.

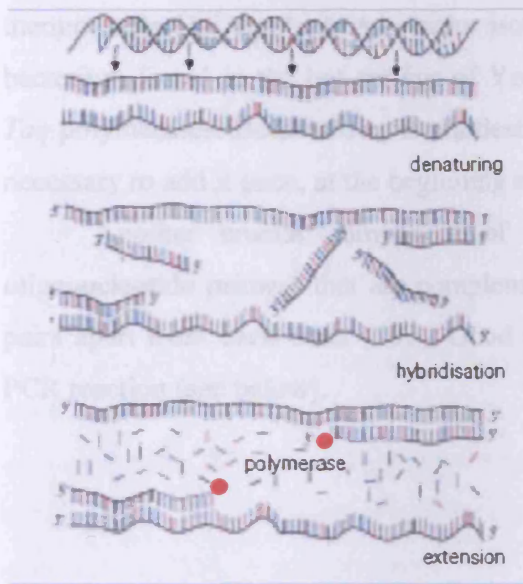
The basic principle of PCR is very simple. As the name implies, it is a chain reaction. One molecule of DNA originates two copies, then four, then eight and so forth. PCR can increase the copy number of specific nucleic acid sequences of 100 to 2000 base pairs in length by  $10^6$  fold in an in vitro 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 thermophilic bacteria, and this rapidly adds further nucleotides that are complimentary in sequence to the original template DNA to the

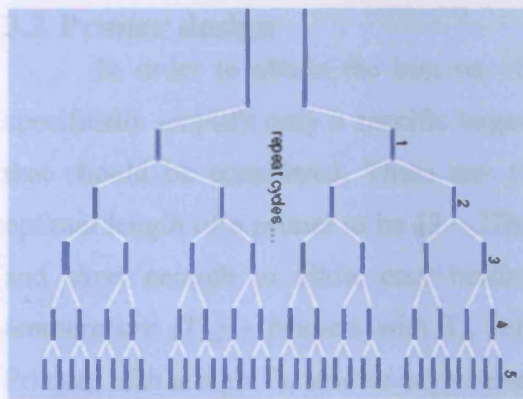
developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments that are not fully complementary will be broken. This process is known as *extension*.

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 thermophilic 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 fluorescence 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 exquisitely 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<sub>2</sub> 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.

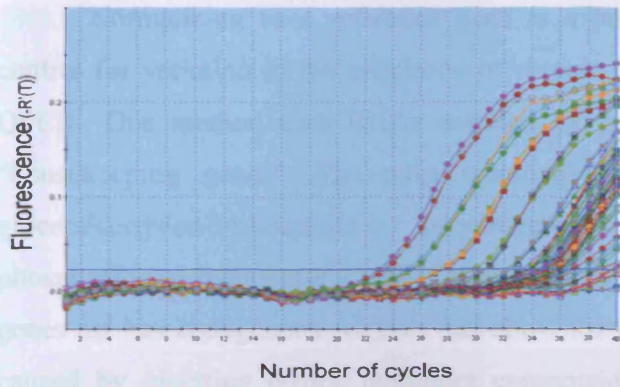
Each set of QPCR reactions were set up with unknown samples and several consecutive dilutions of the standard curve cDNA (1/1, 1/4, 1/16, 1/64). For accuracy, the 1/1 standard cDNA was serially diluted using a blank solution which consisted of the same reagents as the RT reaction but with water added 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

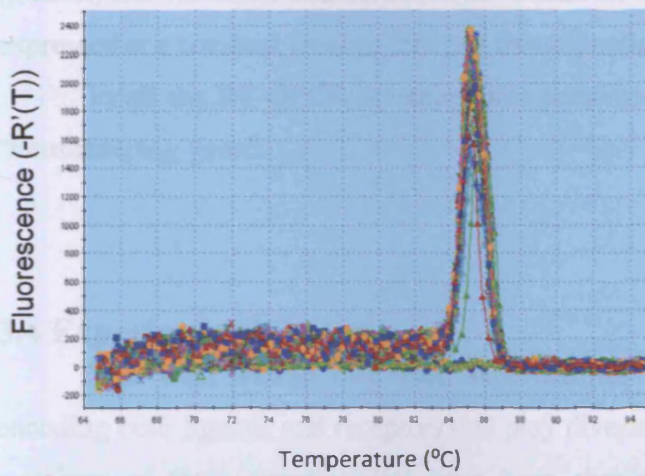


showing Ct (threshold line, above which the program starts to quantify the DNA) versus cycle number. The linear regression of the standard curve should have an RSq ( $R^2$ ) value as close to 1 as possible. The coefficient of determination  $R^2$  is the proportion of variability in a data set, in the 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

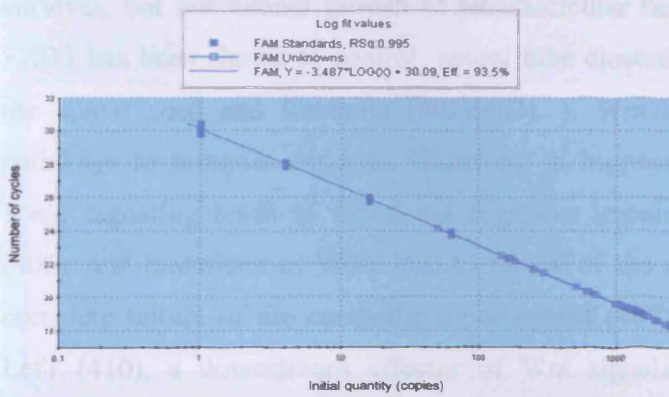
(A)



(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  $\beta$ -actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT1) and 18S ribosomal RNA (399). These housekeeping genes act as 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 ( $\beta$ -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- $\beta$  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- $\beta$  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- $\kappa$ B 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 shown 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- $\beta$ s. TNF- $\alpha$ , has been the most extensively studied and has been shown to be expressed in many types of cells including glial cells and neurons. TNF- $\alpha$  regulates responses to NGF, promoting neural cell survival in neuroblastoma cells (421). TNF- $\alpha$  pre-treatment provides tolerance to ischemia (422). TNF- $\alpha$  increases cellular proliferation in the adult cortex (423). In contrast, a pro-death effect of TNF- $\alpha$  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  $\beta$ -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- $\beta$  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- $\beta$  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- $\beta$  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.

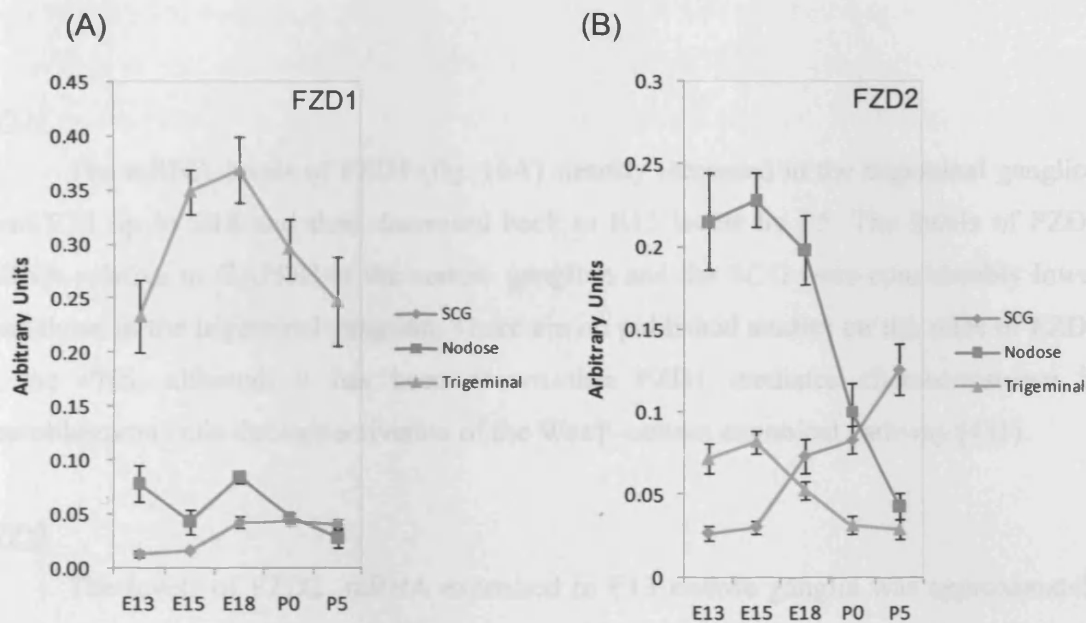
### **3.5 Results/ Discussion**

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- $\beta$  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 TGF $\beta$  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- $\beta$  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.

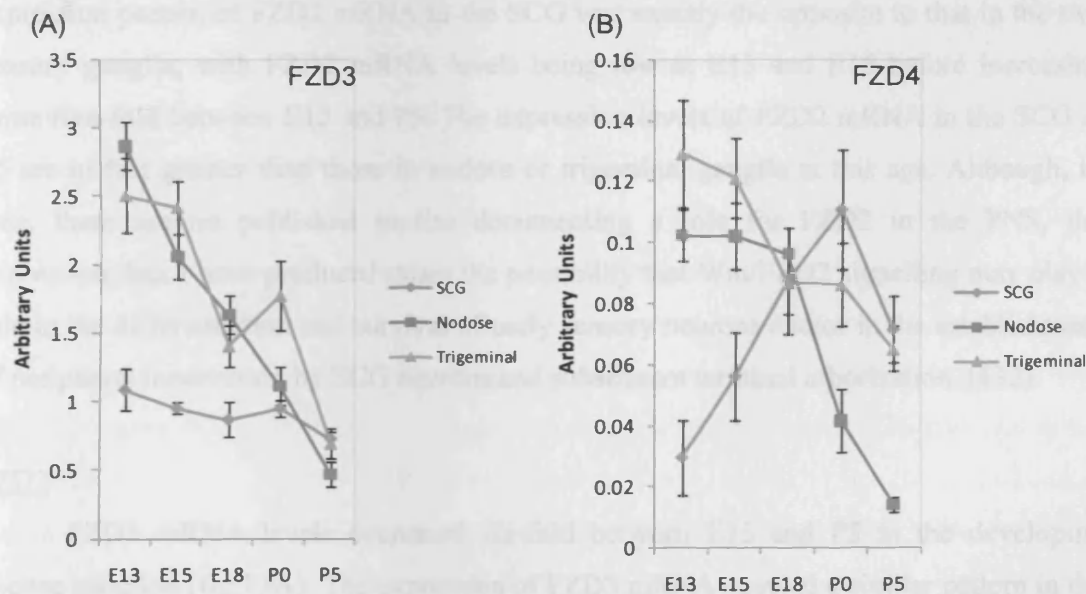
### **3.5.1 Wnt superfamily**

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 are 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 chemoresistance in neuroblastoma cells through activation of the Wnt/ $\beta$ -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

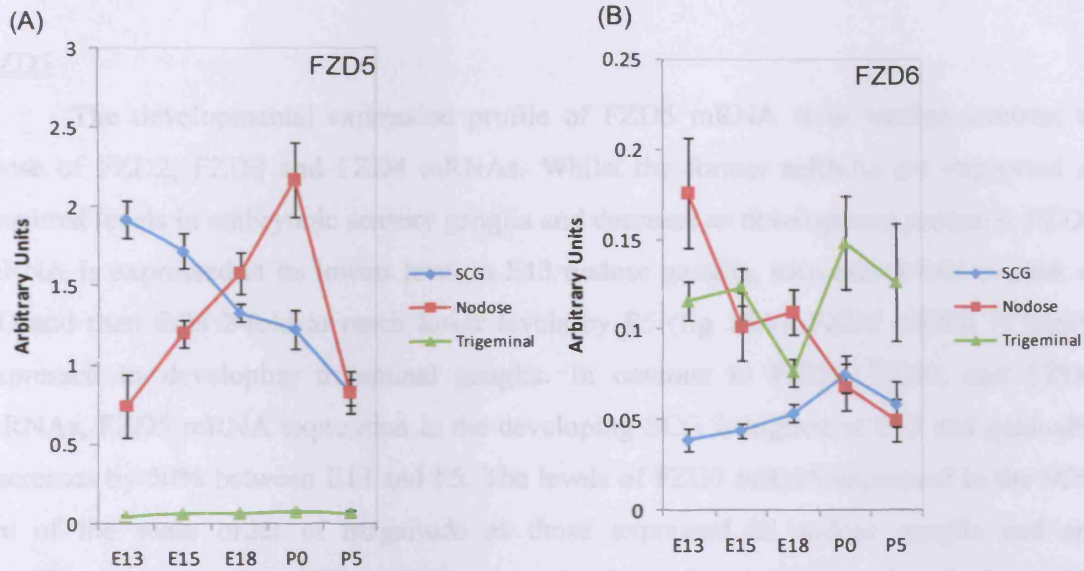
FZD3 mRNA levels decreased six-fold between E13 and P5 in the developing nodose ganglion (fig.17A). The expression of FZD3 mRNA showed a similar pattern in the developing trigeminal ganglion, although the developmental decrease was only four-fold and was not entirely linear. In contrast, the expression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

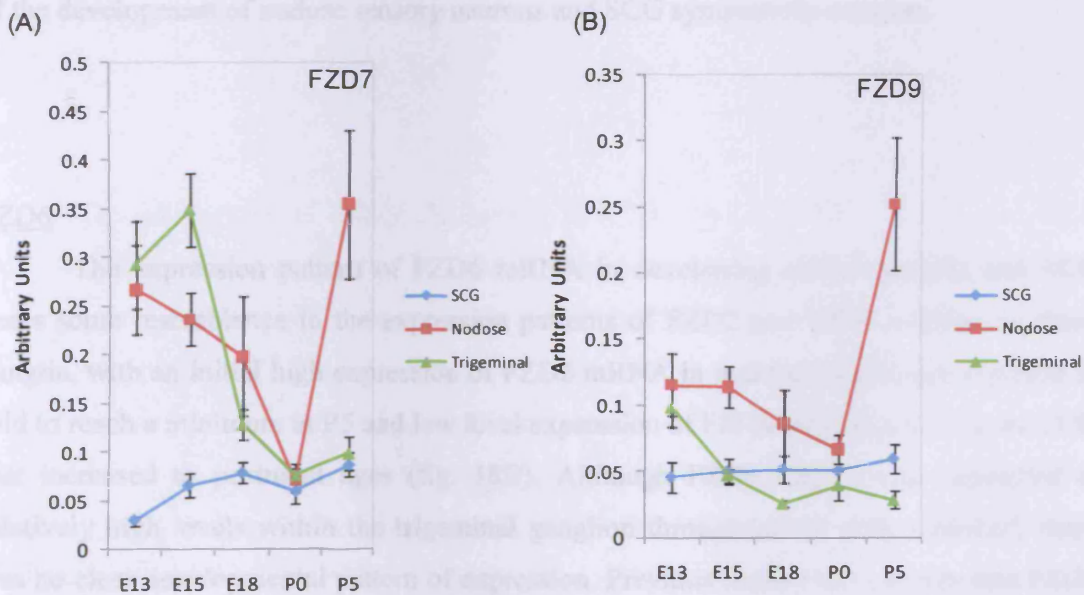
#### FZD4

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<sup>-/-</sup> 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

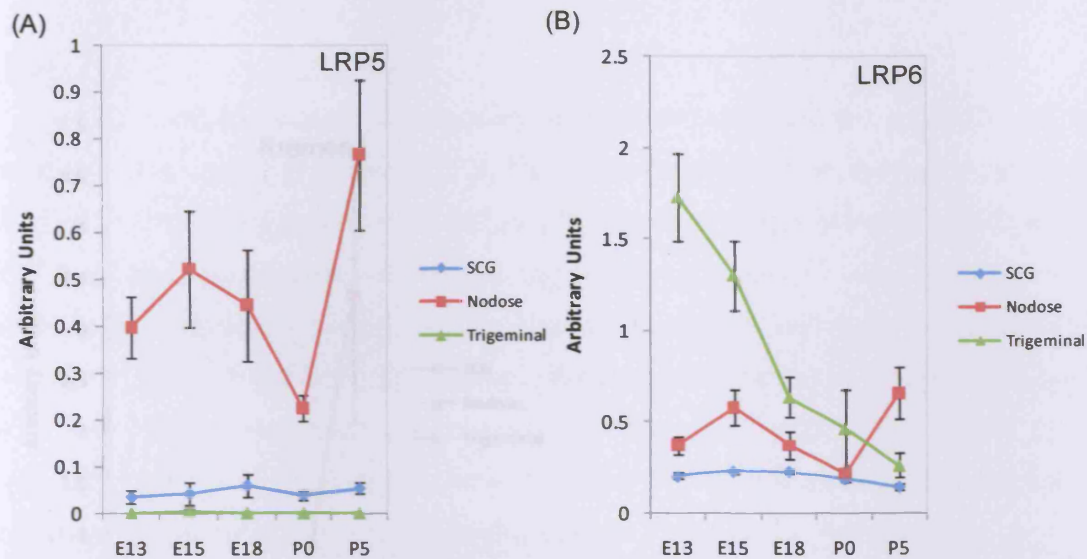
The expression pattern of FZD6 mRNA in developing nodose ganglia and SCG bears some resemblance to the expression patterns of FZD2 and FZD4 mRNAs in these ganglia, with an initial high expression of FZD6 mRNA in nodose ganglia that declined 3-fold to reach a minimum at P5 and low level expression of 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

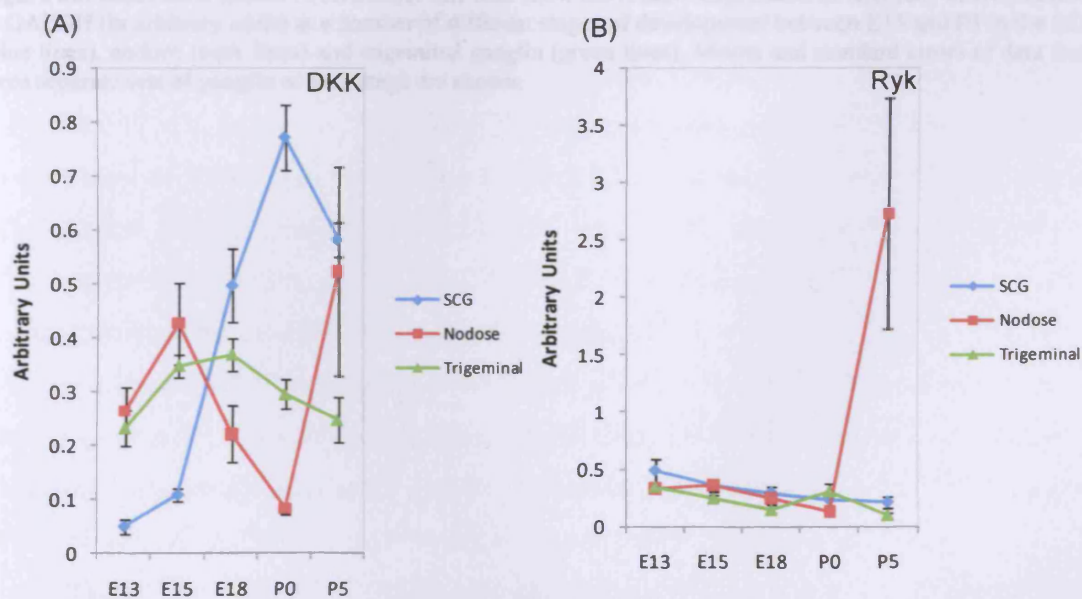
The developmental screening results for FZD7 mRNA (fig. 19A) showed that the expression levels of this mRNA were very low in E13 SCG, but thereafter gradually increased with age to be some 3-fold higher by P5. Early embryonic trigeminal ganglia expressed significantly more FZD7 mRNA than SCG neurons 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).

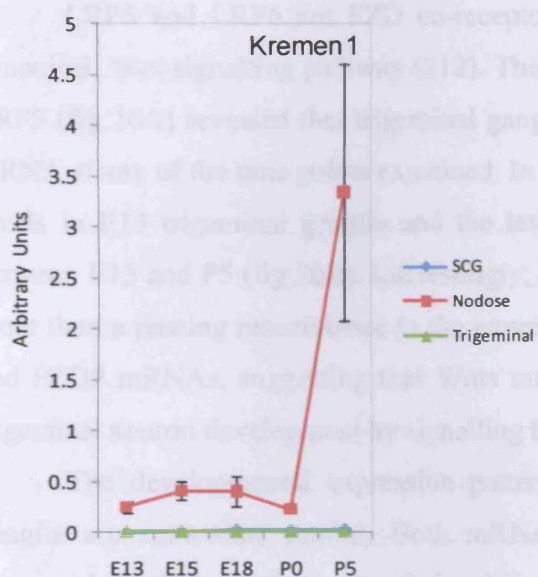


**Figure 20:** Expression profiles for LRP5 and LRP6. The data show the relative expression of (A) LRP5 and (B) LRP6 mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green 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  $\beta$ -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.

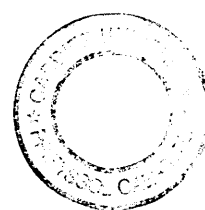
### Ryk

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

Kamitoy 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

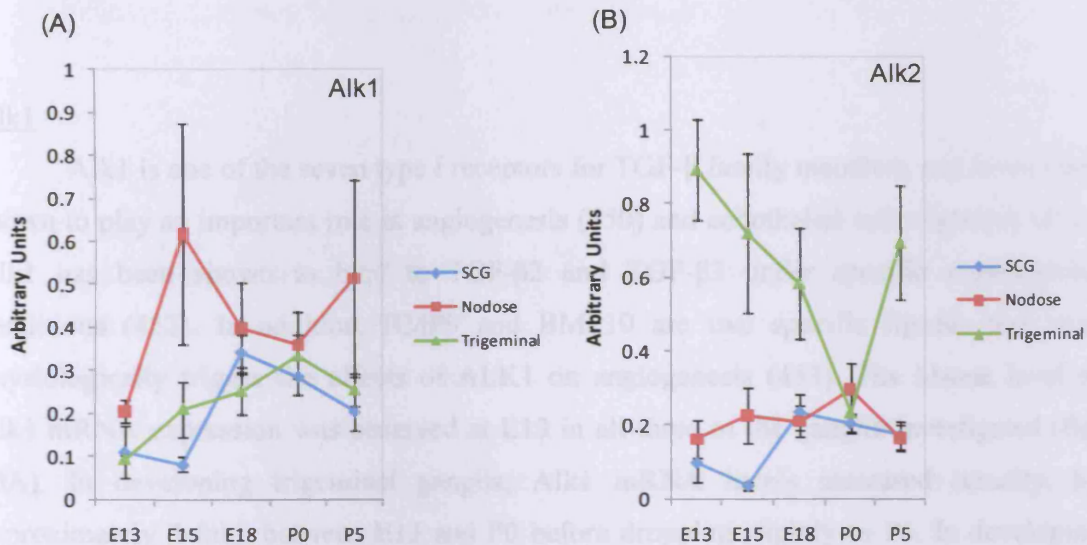


activation of this Wnt/ $\beta$ -catenin pathway (447). Kremen 1 mRNA was virtually undetectable in developing SCG and trigeminal neurons at any of the ages studied (fig. 22). Although Kremen 1 mRNA was expressed at low, constant levels in the nodose ganglion between E13 and P0, it increased dramatically 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.

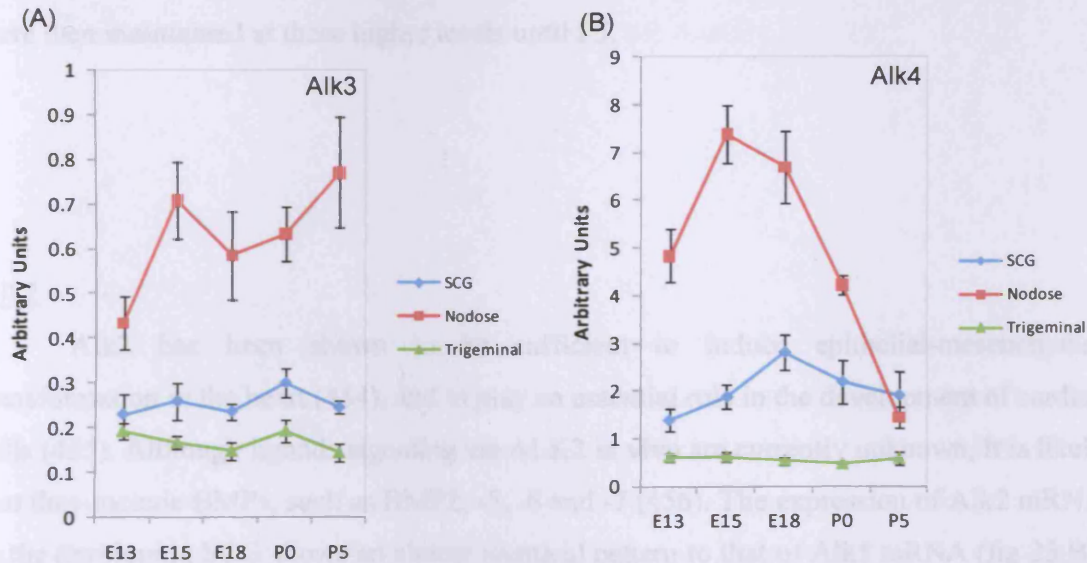
### **3.5.2 TGF- $\beta$ superfamily**

TGF $\beta$  superfamily ligands include the TGF $\beta$ , 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 TGF $\beta$  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- $\beta$  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- $\beta$ 2 and TGF- $\beta$ 3 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.

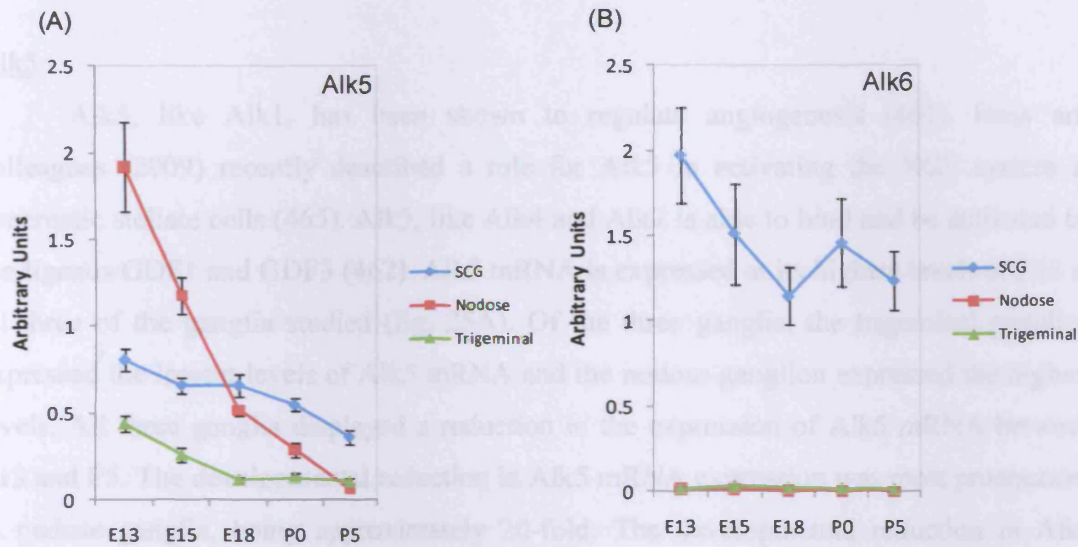
### Alk3

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.

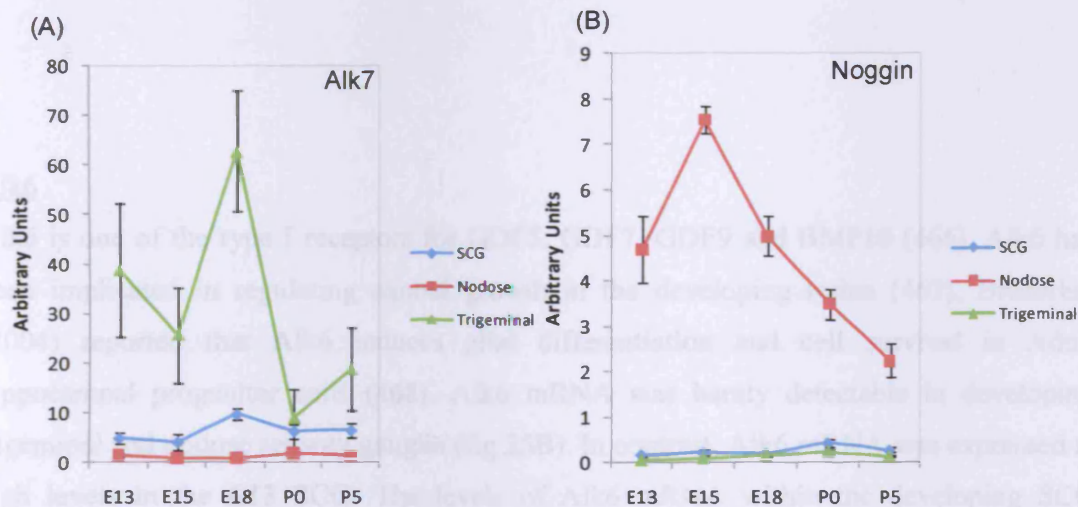
### Alk4

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

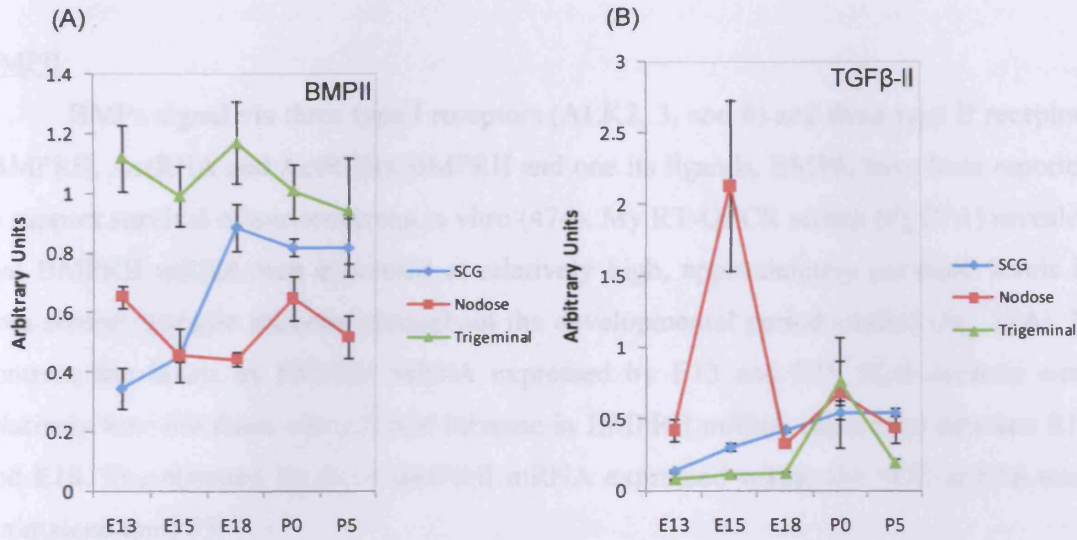
Alk7 is structurally similar to Alk4 and Alk5, and binds the same ligands, GDF1 and GDF3. It has also been reported to be able to bind GDF8, activins and nodals (469). Carlsson and colleagues (2009) have recently linked Alk7 to metabolic disease (470). In contrast to the other Alk receptors that bind 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

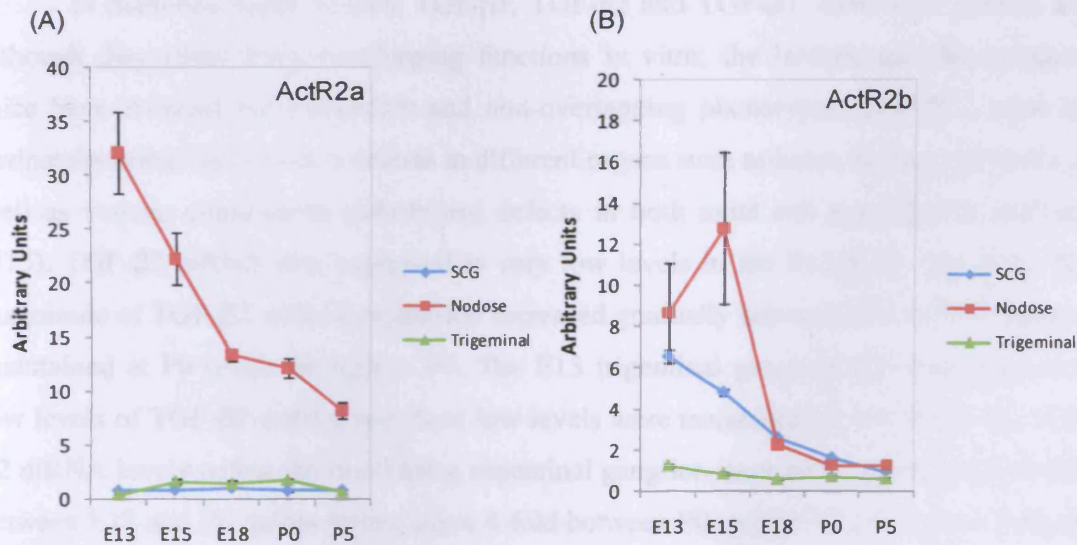
Noggin is a small glycoprotein originally characterized as a secretory molecule derived from Spermann organizer during the amphibian development (471). During mammalian embryogenesis, noggin is expressed in the node, notochord, dorsal somite, condensing cartilage, and immature chondrocytes and regulates 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- $\beta$  signal transduction by binding to TGF- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$  family signal transduction, the high levels and distinct pattern of expression of its mRNA in the developing nodose ganglion suggests that TGF- $\beta$  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.

## BMPRII

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 motoneurons 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- $\beta$ 2

In mammals three TGF $\beta$ s, TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, 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- $\beta$ 2<sup>-/-</sup> 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- $\beta$ 2 mRNA was expressed at very low levels in the E13 SCG (fig 27B). The magnitude of TGF- $\beta$ 2 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- $\beta$ 2 mRNA and these low levels were maintained at E15 and E18. TGF- $\beta$ 2 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- $\beta$ 2 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- $\beta$ 2 mRNA expressed within nodose ganglia between E13 and E15 and this increase was promptly reversed between E15 and E18. These lower levels of TGF- $\beta$  mRNA expression at E18 were maintained between E18 and P5. The dramatic peaks in TGF- $\beta$ 2 mRNA expression in trigeminal and nodose ganglia, at P0 and E15, respectively,

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

### Activin receptors

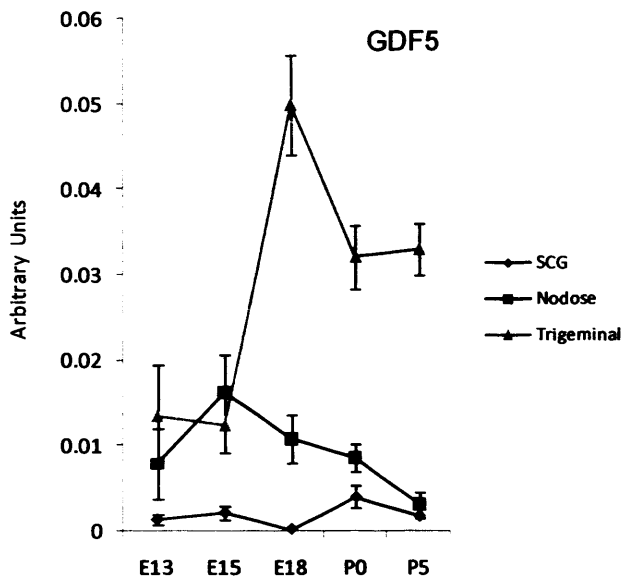
Activins have a wide range of biological effects on cell growth and differentiation. Activins bind directly to activin receptor type 2a (ActR2a) or 2b (ActR2b). ActR2b<sup>-/-</sup> mice display various endocrine pancreas-related abnormalities, including islet hypoplasia and glucose intolerance, demonstrating the crucial role of ActR2b in the regulation of pancreas development (476). ActR2a<sup>-/-</sup> 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, TNF $\alpha$ , LT $\alpha$ , LT $\beta$  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- $\kappa$ B 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.

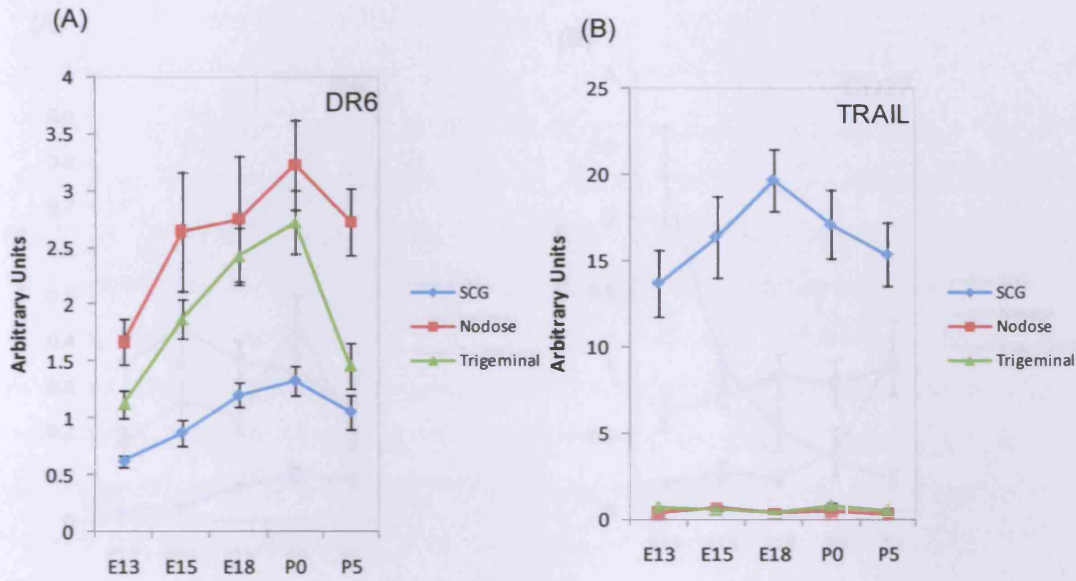
### CD30

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

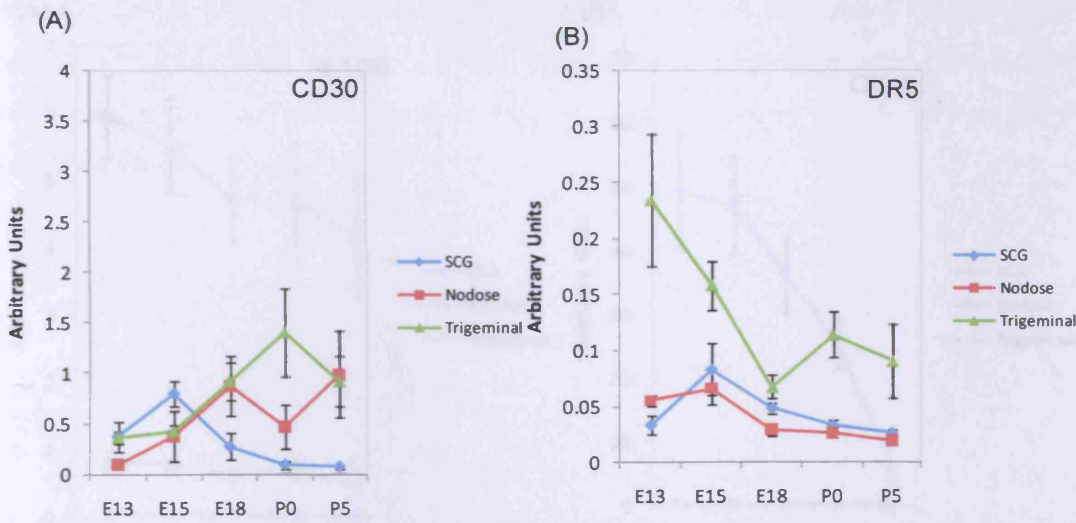
excessive cell death of T-cells (485). CD30 mRNA was expressed at very low levels within E13 nodose ganglia (fig. 31A). However, the levels of this mRNA within nodose ganglia increased 8-fold between E13 and E18. After a 35% decrease in expression between E18 and P0, CD30 mRNA levels increased 2-fold 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

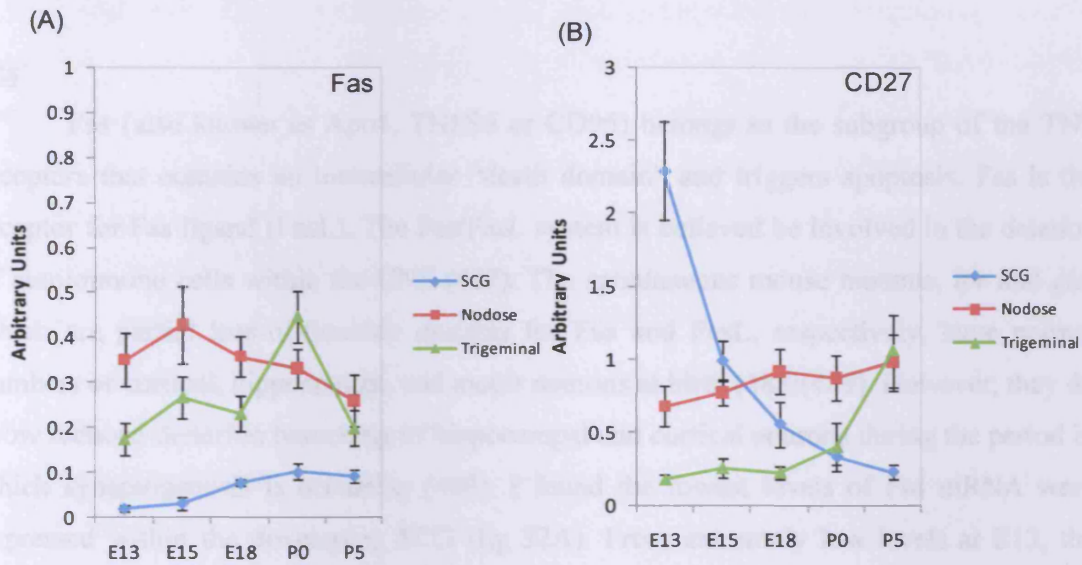
Death receptor (DR) 5 (TNFRSF10B) is one of the receptors for TRAIL. Death receptors and their ligands are expressed in the developing nervous system, and many recent reports confirm their ability to trigger death of embryonic neurons and glia *in vitro* (486). However, with the exception of earlier reports concerning p75<sup>NTR</sup>, 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.



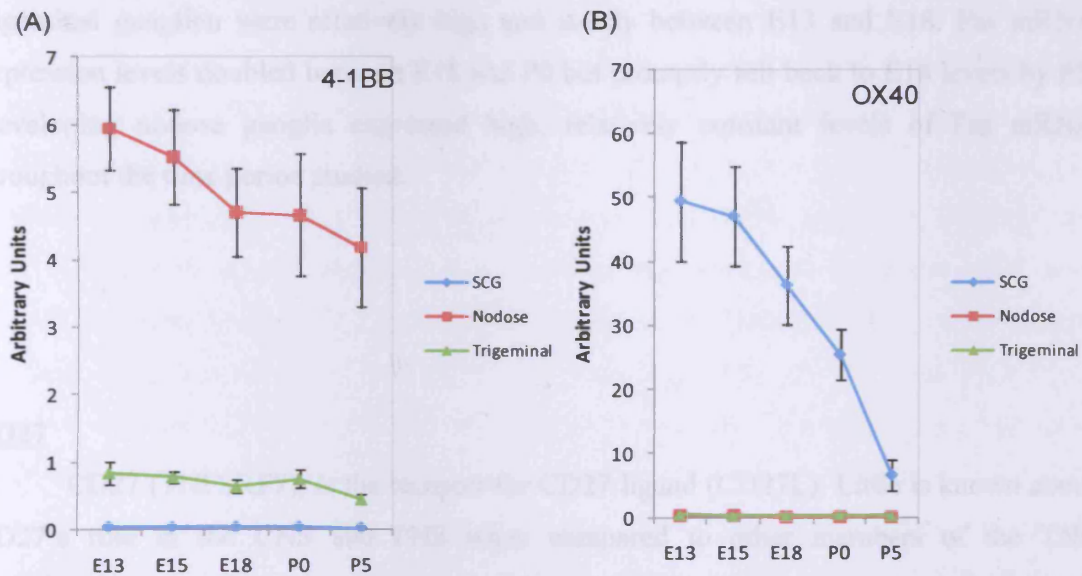
**Figure 30:** Expression profiles of DR6 and TRAIL. The data show the relative expression of DR6 and TRAIL mRNA relative to GAPDH (in arbitrary units) at a number of different stages of development between E13 and P5 in the SCG (blue lines), nodose (pink lines) and trigeminal ganglia (green lines). 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 to 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 this increase is statistically significant.

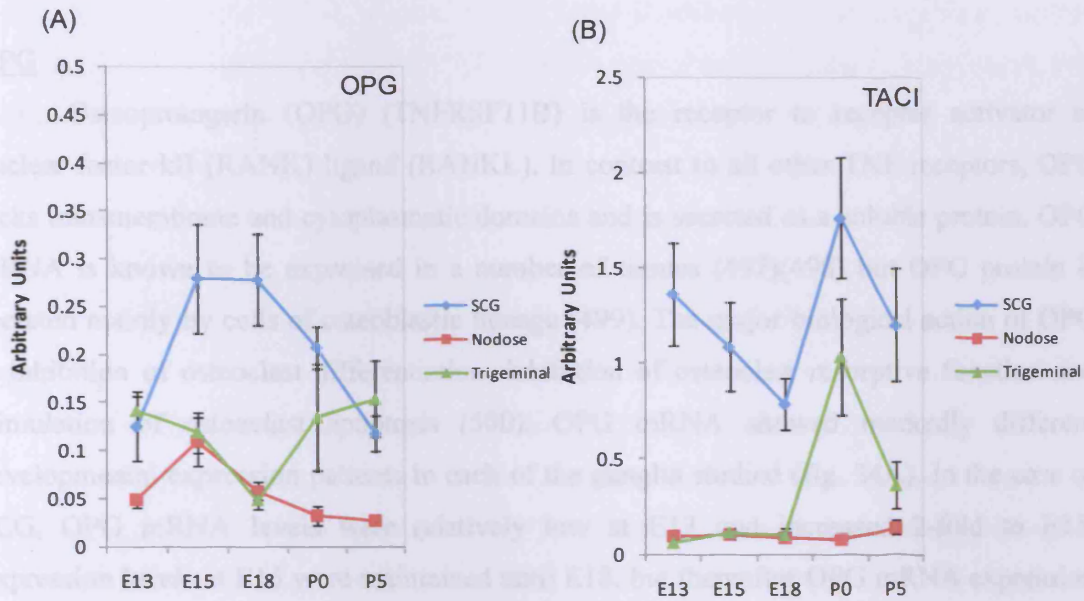
#### 4-1BB

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-1 $\alpha$  (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.

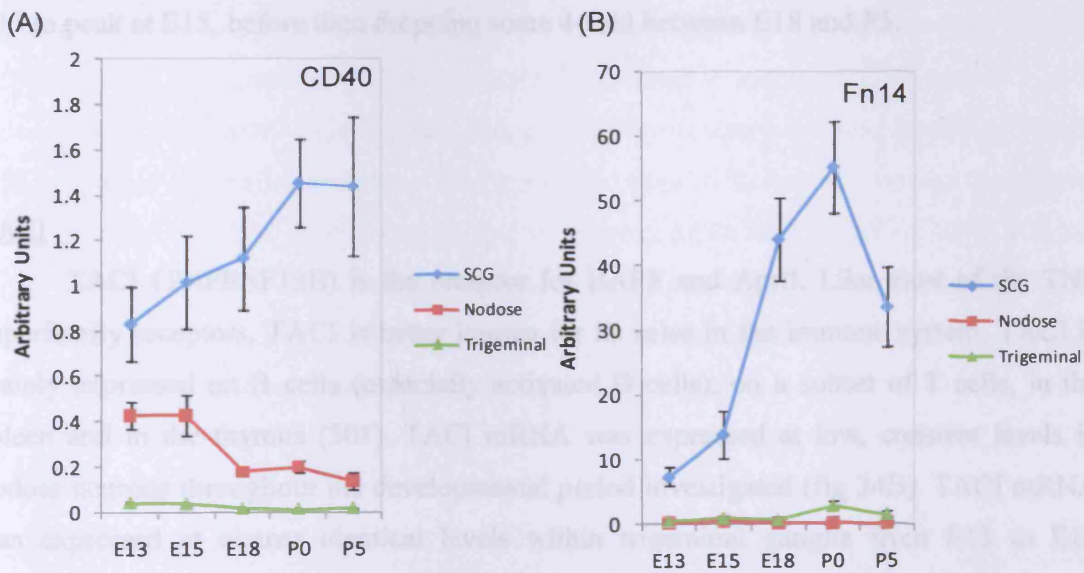
## OX40

OX40 (also known as TNFRSF4 or CD134) is the receptor for OX40 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<sup>+</sup>T cells and a subset of CD8<sup>+</sup> 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 TAC1. The data show the relative expression of OPG and TAC1 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- $\kappa$ B (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 TAC1 mRNA at E13 fell approximately 40% to E18, after which there was a 2-fold increase in TAC1 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<sup>+</sup> 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- $\alpha$ , 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).

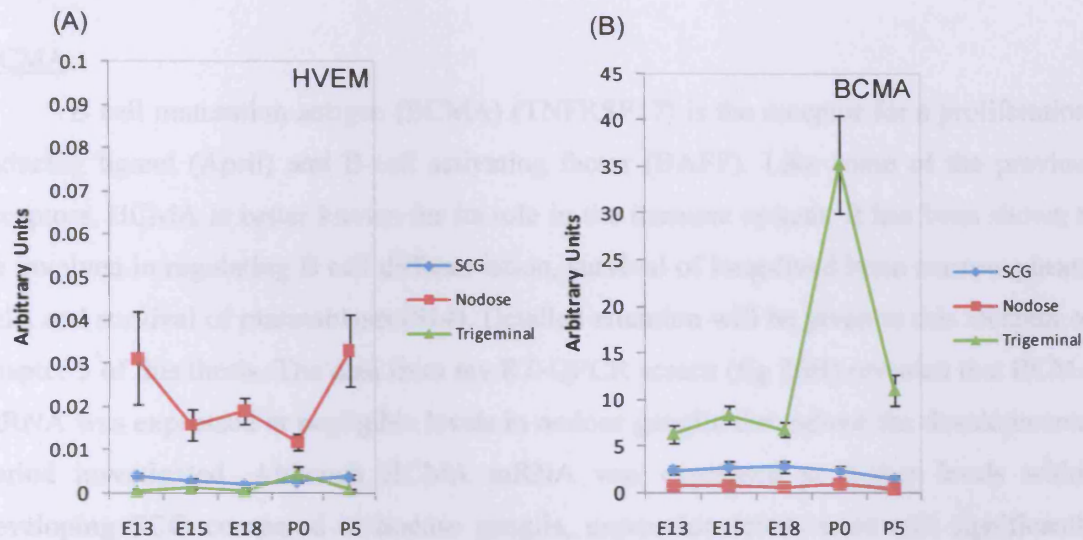
Nodose ganglia did not express appreciable levels of Fn14 mRNA at any developmental time point studied (fig 35B). Similarly, trigeminal ganglia did not express significant levels of Fn14 mRNA between E13 and E18. Although there was an approximately 10-fold increase in the levels of Fn14 mRNA expressed 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

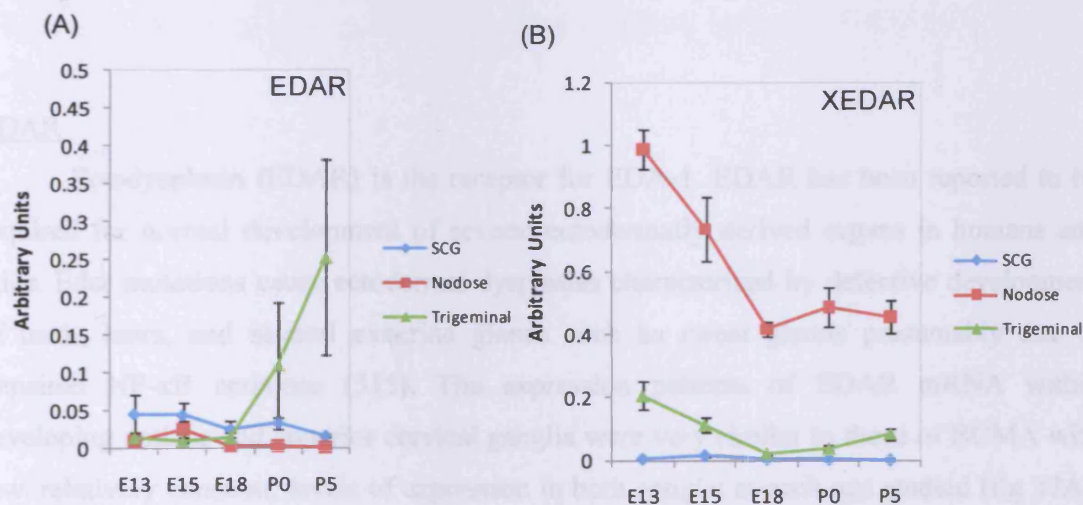
Herpes virus entry mediator (HVEM, TNFRSF14) is the receptor for lymphotoxin-related inducible ligand (LIGHT). HVEM was identified as a cellular mediator of herpes simplex virus (HSV) entry. Binding of HSV viral envelope glycoprotein D to this receptor protein has been shown to be part of the viral entry 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- $\beta$  (LT- $\beta$ ). 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- $\kappa$ B 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.

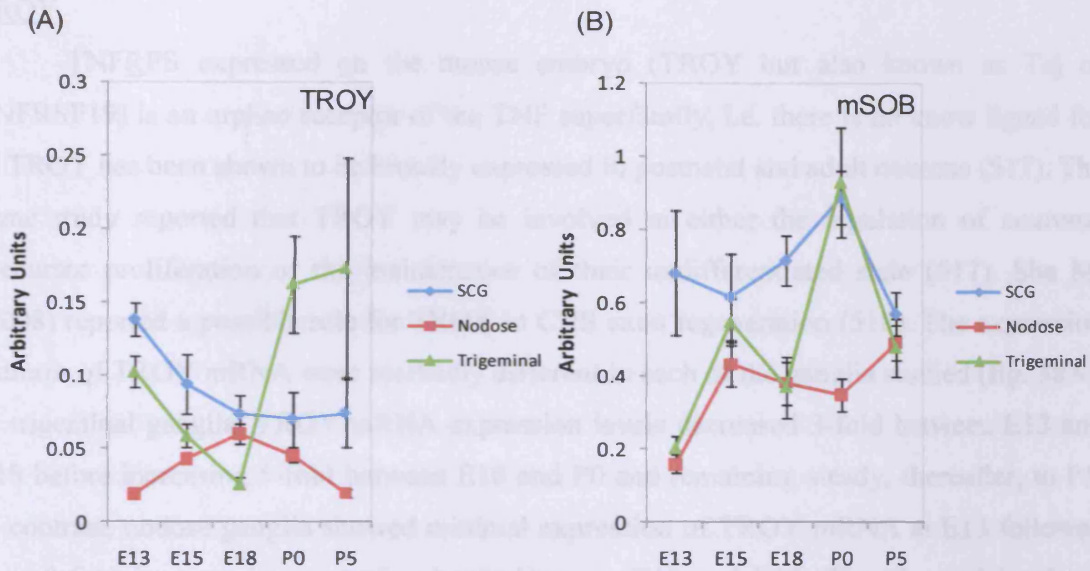
## EDAR

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- $\kappa$ B 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.

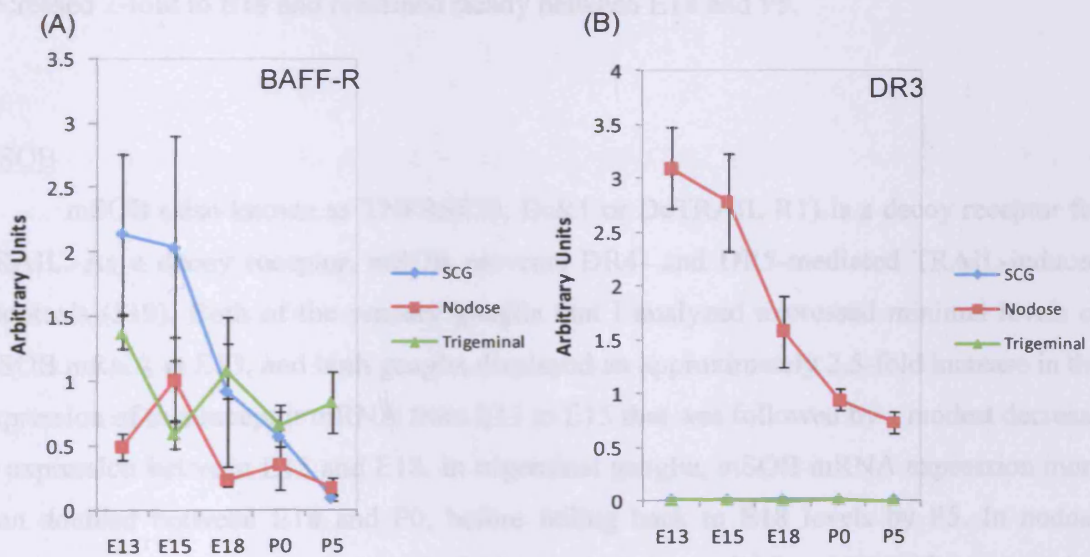
## XEDAR

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 known 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

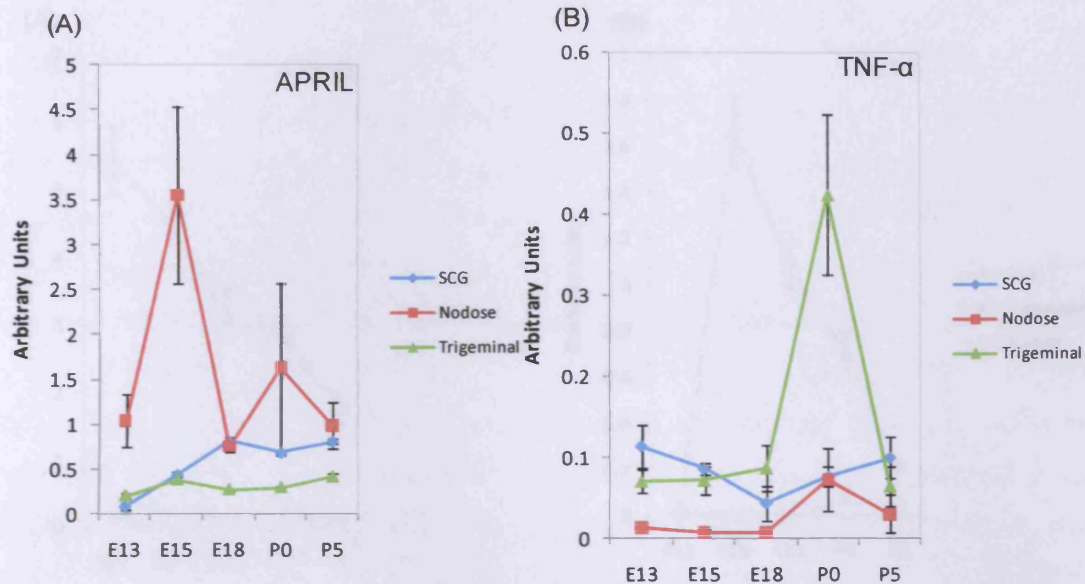
mSOB (also known as TNFRSF23, DcR1 or DcTRAIL R1) is a decoy receptor for TRAIL. As a decoy receptor, mSOB prevents DR4- and DR5-mediated TRAIL-induced apoptosis (519). Both of the sensory ganglia that I analyzed expressed minimal levels of mSOB mRNA at E13, and both ganglia displayed an approximately 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

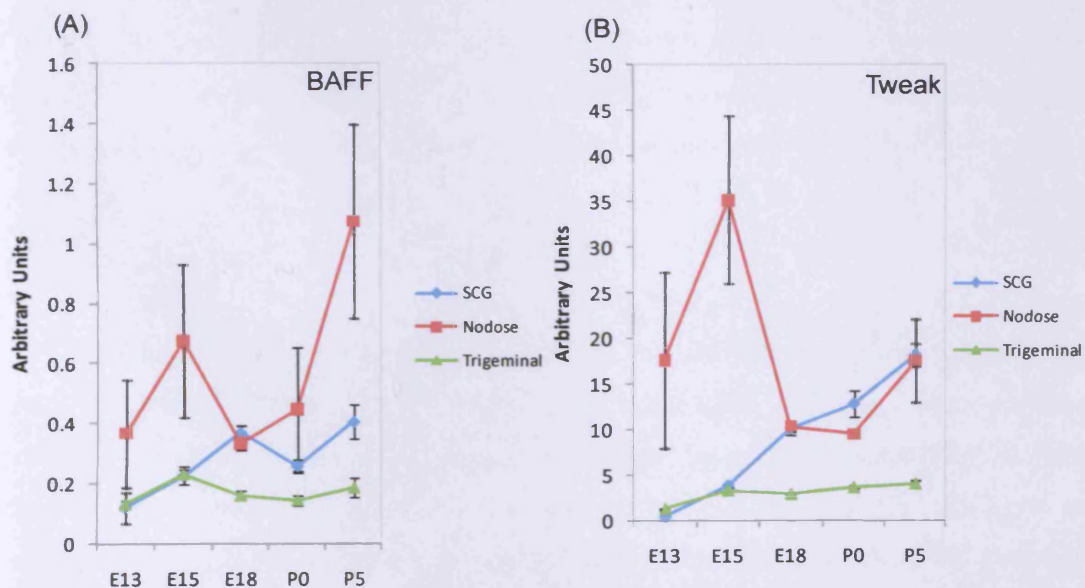
B cell activating factor receptor (BAFF-R, TNFRSF13C) is the receptor for B cell activating factor (BAFF). Like many other members of the TNF superfamily, BAFF-R has been well studied in the immune system, specially its role in regulating mature B cell and survival and maintenance (520). In BAFF-R<sup>-/-</sup> mice, B cells develop normally up to the stage of IgM<sup>+</sup> 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

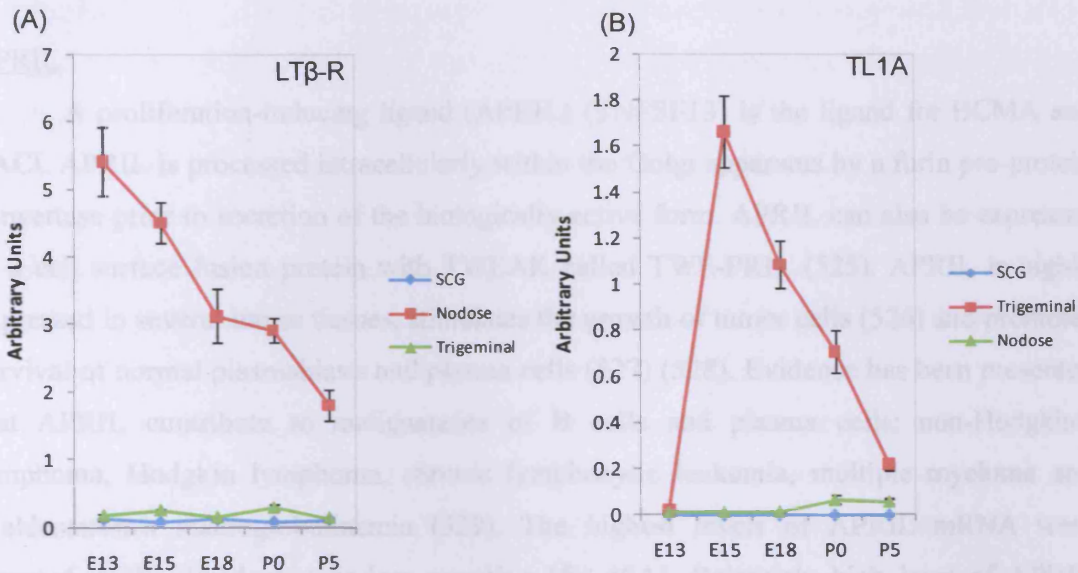
Death receptor (DR) 3 (also known as TNFRSF25, TRAMP, LARD or WSL-1) is the receptor for TL1A. DR3 has been reported to be expressed mainly in T lymphocytes (522). DR3 acts by binding to the adaptor molecule TRADD, which in turn endows DR3 with dual-signaling capacity to either activate NF- $\kappa$ B 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- $\alpha$ . The data show the relative expression of APRIL and TNF- $\alpha$  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- $\alpha$

TNF- $\alpha$  (TNFSF1A) is the ligand for TNFR1 and TNFR2. TNF- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  is functionally important in the development of sensory neurons. SCG express moderately high levels of TNF- $\alpha$  mRNA at E13 and expression levels fall approximately 65% between E13 and E18. After E18, the expression levels of TNF- $\alpha$  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.

### LT $\beta$

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

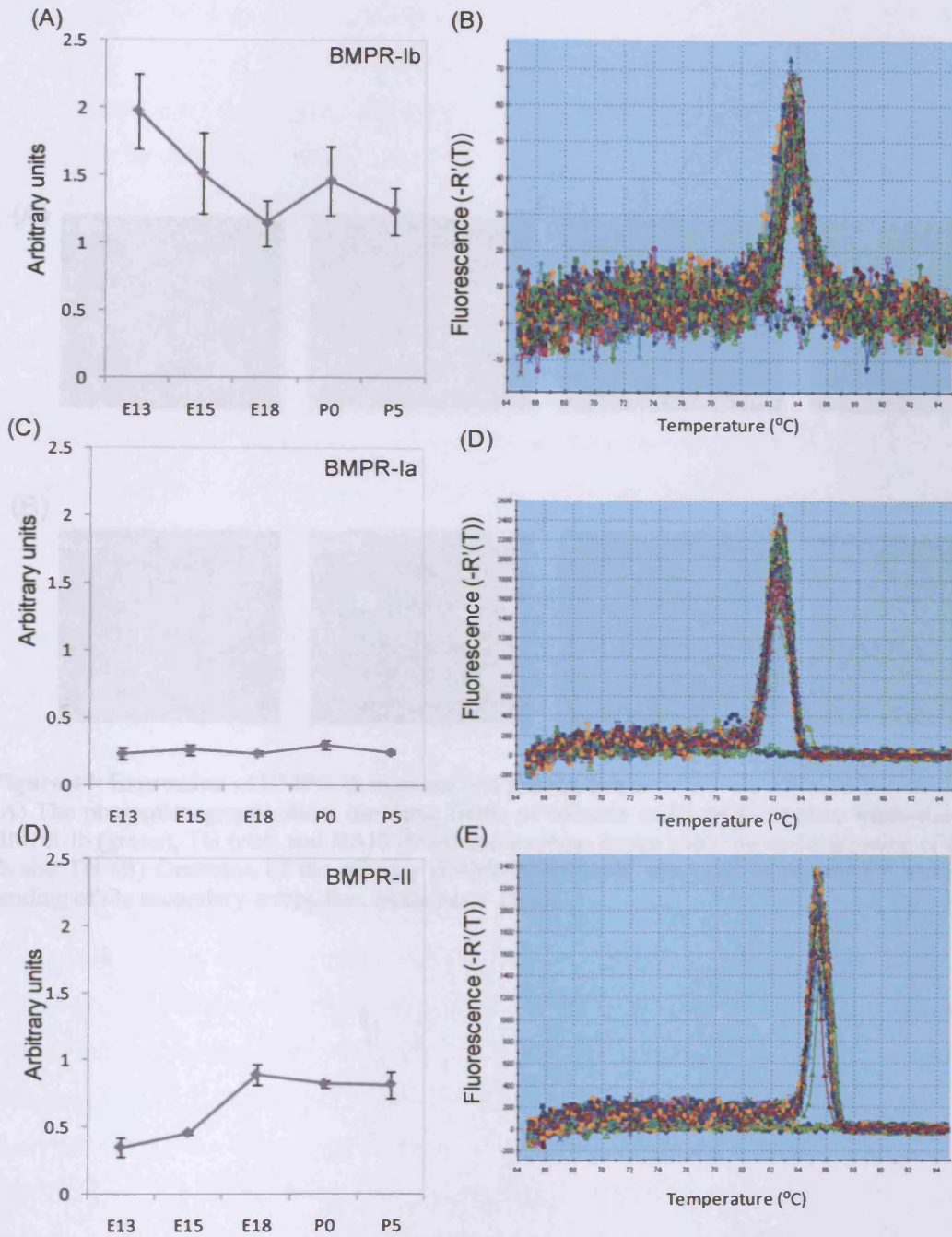
Nerve growth factor (NGF) is the paradigmatic target-derived neurotrophic factor on which the foundations of neurotrophic theory are based(51) In the developing peripheral nervous system, postganglionic sympathetic neurons and the majority of sensory neurons depend for their survival on a supply of NGF 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 TGF $\beta$  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

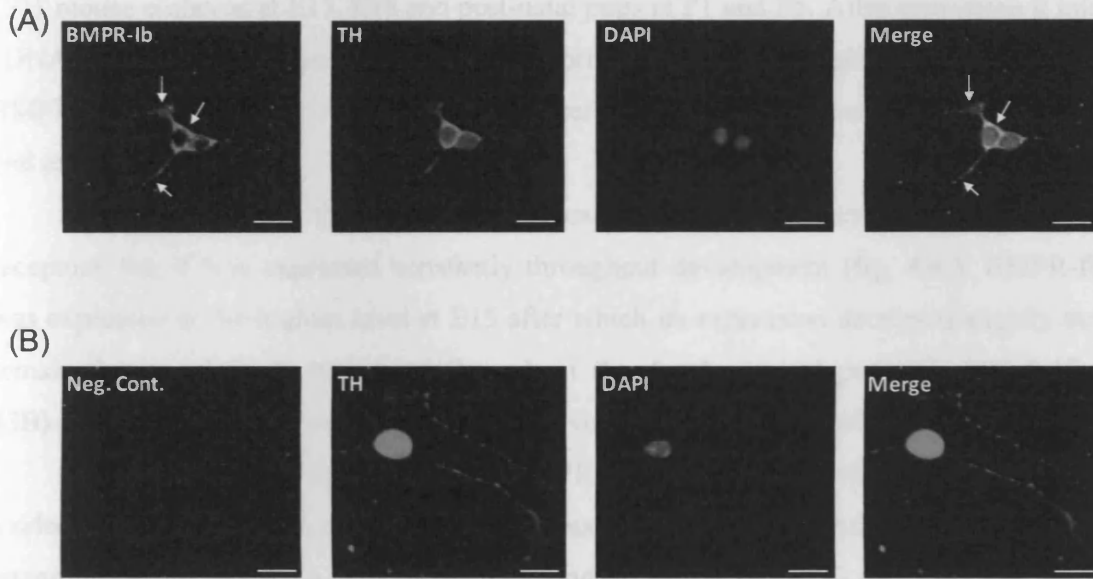
neurotrophic factor for developing SCG neurons that selectively promotes axonal growth and branching and is essential for establishing sympathetic innervation in vivo, but does not promote and is not required for sympathetic neuron survival.

## 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.

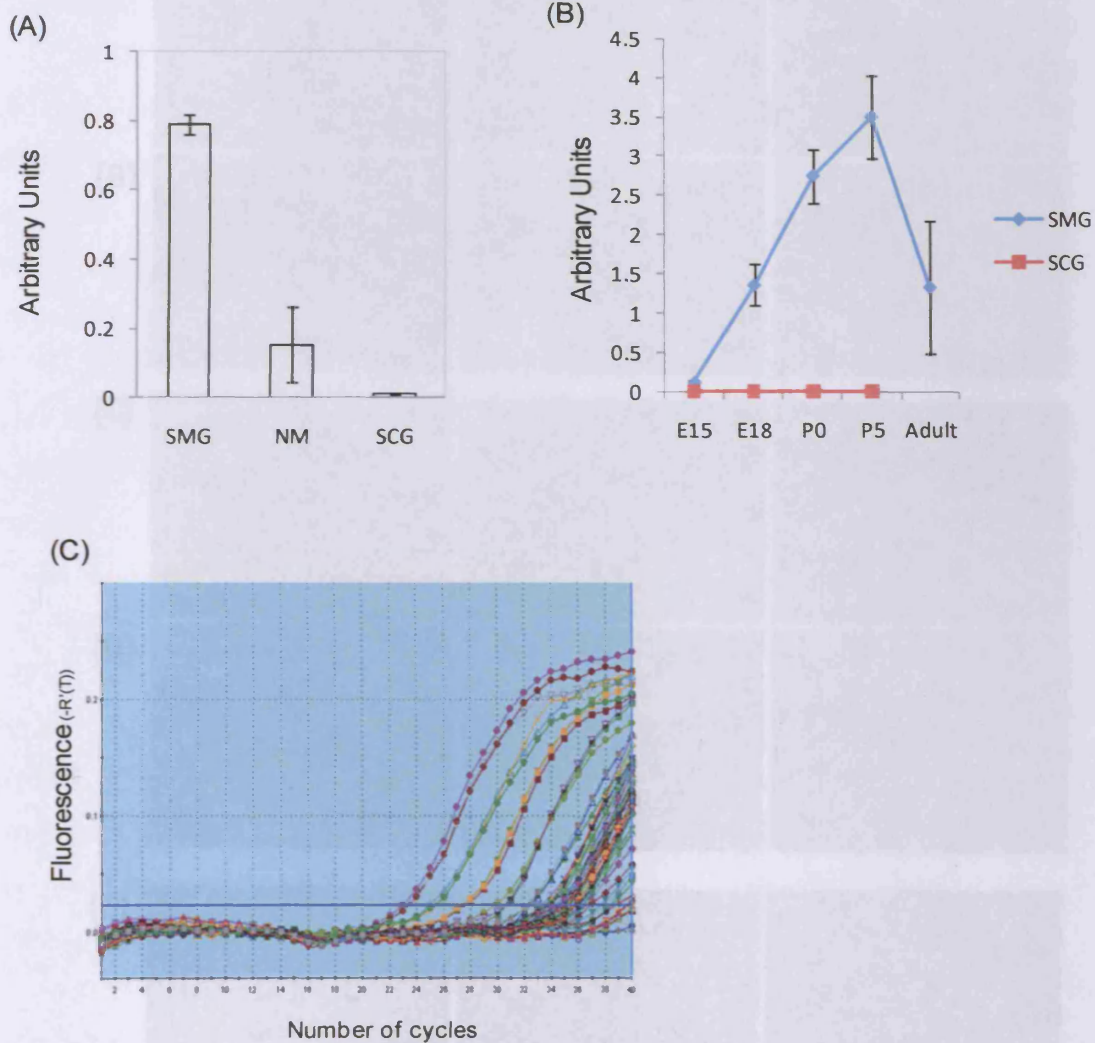
(A) The photomicrographs show the same fields of cultures of P1 SCG neurons triple-stained for BMPR-Ib (green), TH (red) and DAPI (blue) and a merge image showing co-localisation of BMPR-Ib and TH (B) Omission of the primary BMPR-Ib antibody was used to control for non-specific binding of the secondary antibodies. Scale bar = 10 $\mu$ m.

## **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).

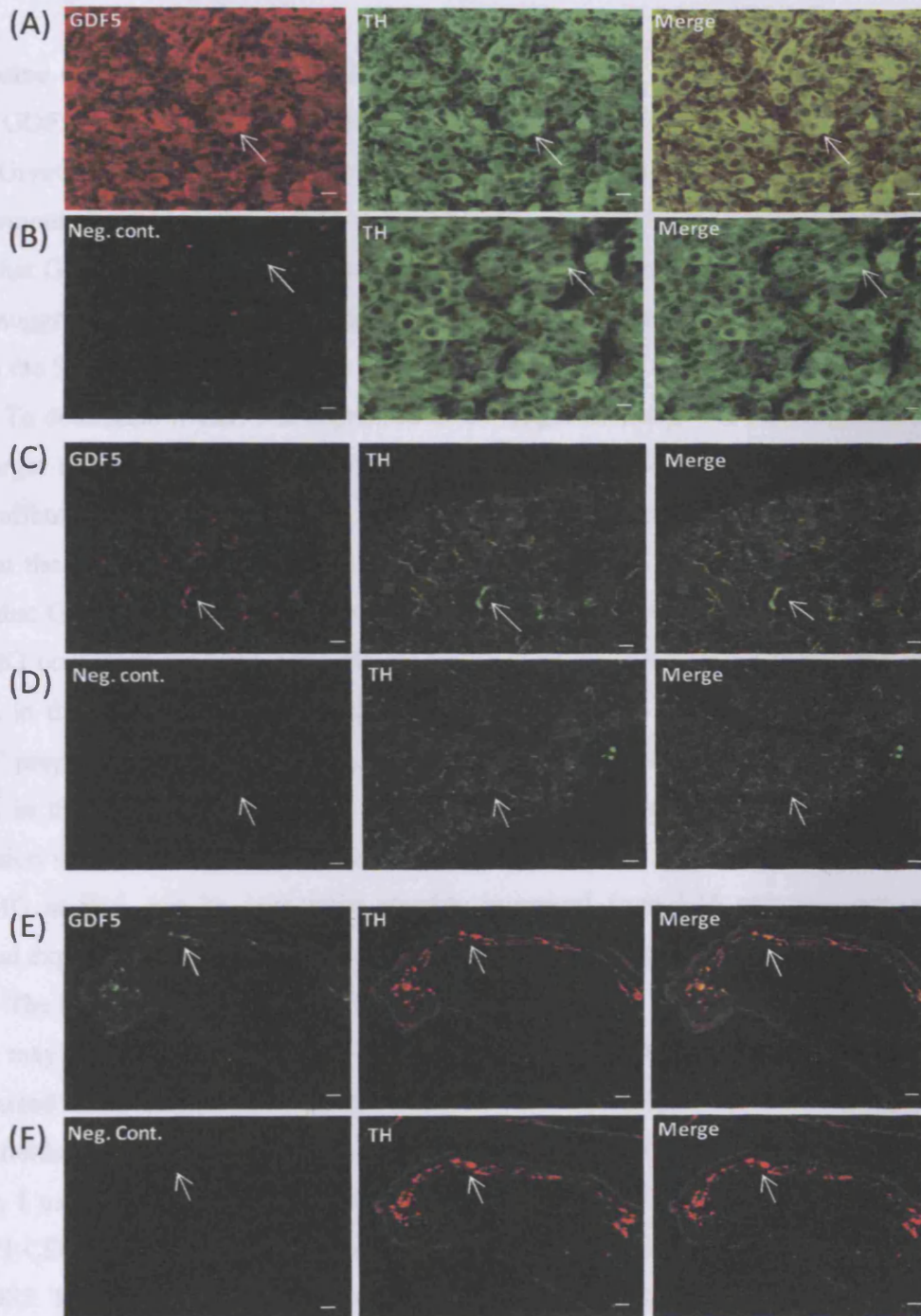
My results show that BMPR-Ia was expressed at the lowest level of all three receptors, but it was expressed constantly throughout development (fig. 43C). BMPR-Ib was expressed at the highest level at E15 after which its expression decreased slightly but remained at a relatively high level throughout 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 = 10 $\mu$ m.

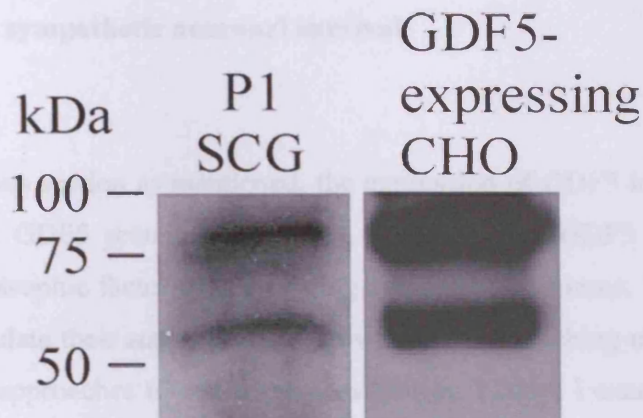
### **Expression of GDF5 in the developing SCG and targets**

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).

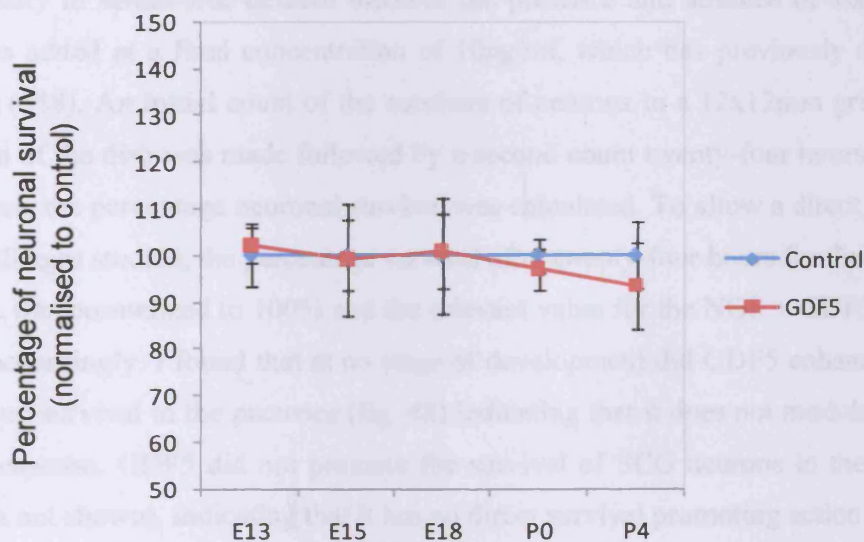
The high expression of GDF5 mRNA in the SMG but not in the SCG suggested that GDF5 may act in a similar way to NGF developing sympathetic neurons, i.e, being synthesized in the target fields of these neurons and be retrogradely transported from the target fields to the neuron cell bodies of the SCG (546). 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.

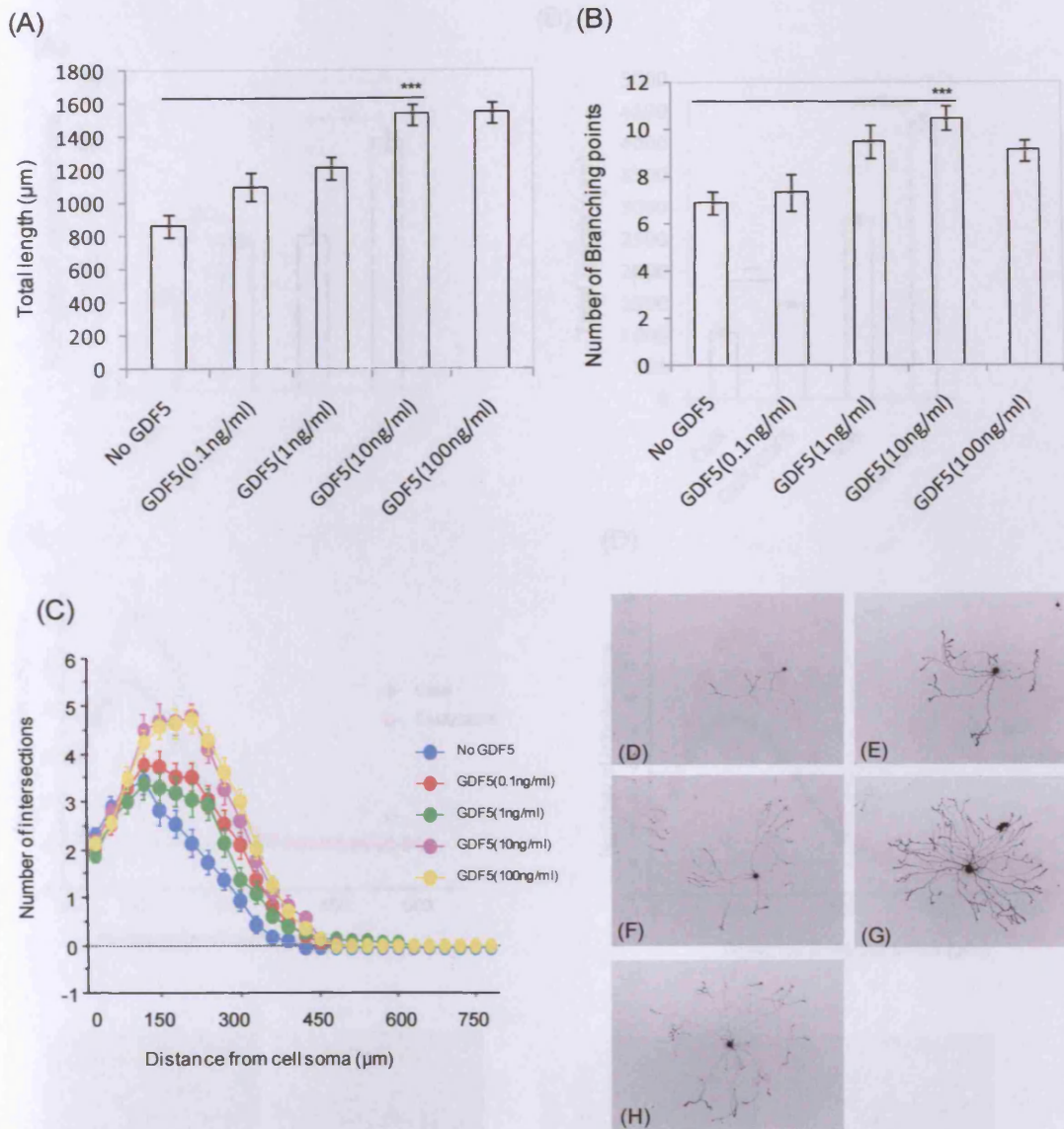
Means and standard errors of data from three separate sets of ganglia at each age are shown.

## **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.

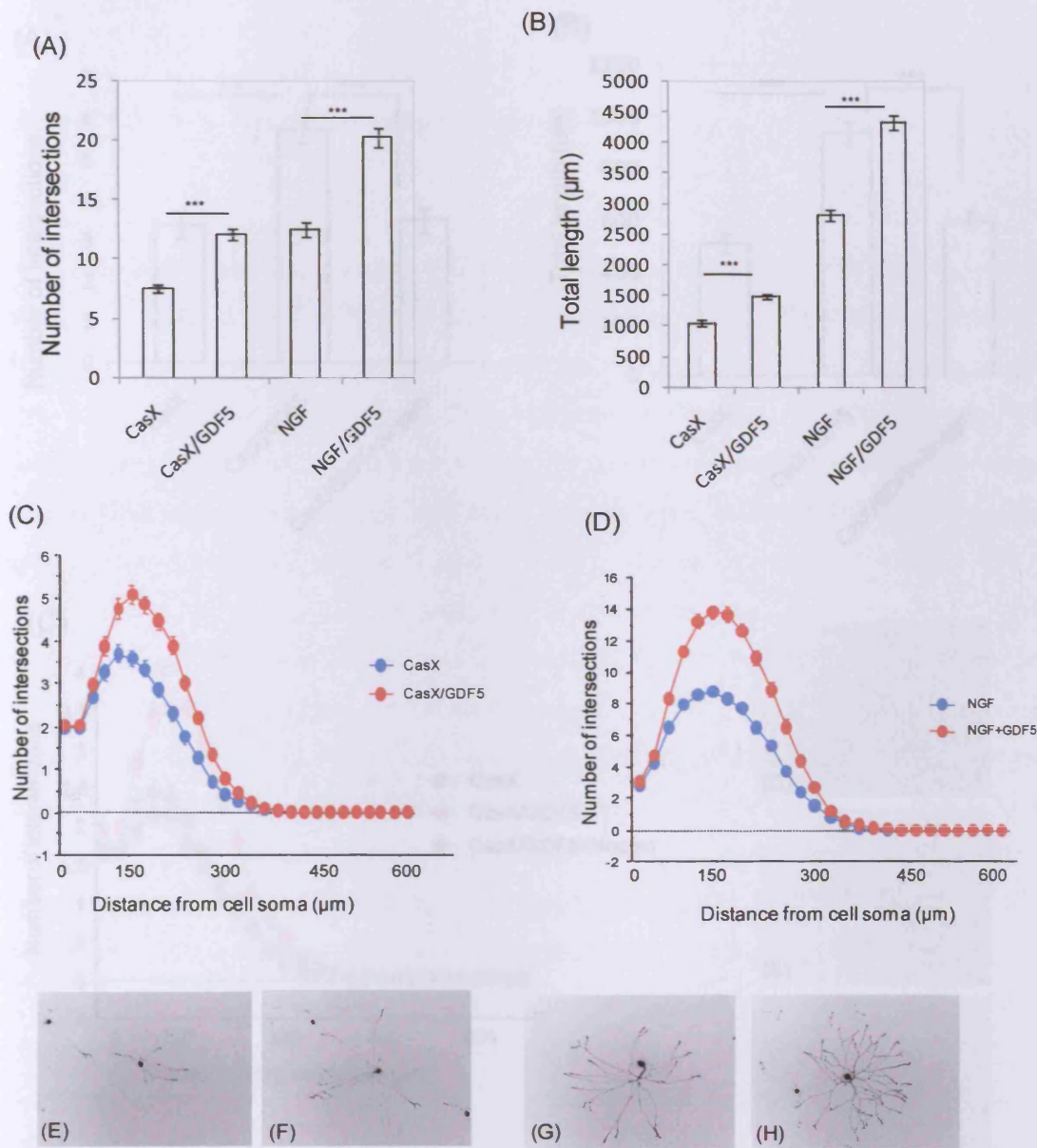




**Figure 49: GDF5 dose response.**

Branching (A), length (B) and shall 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.

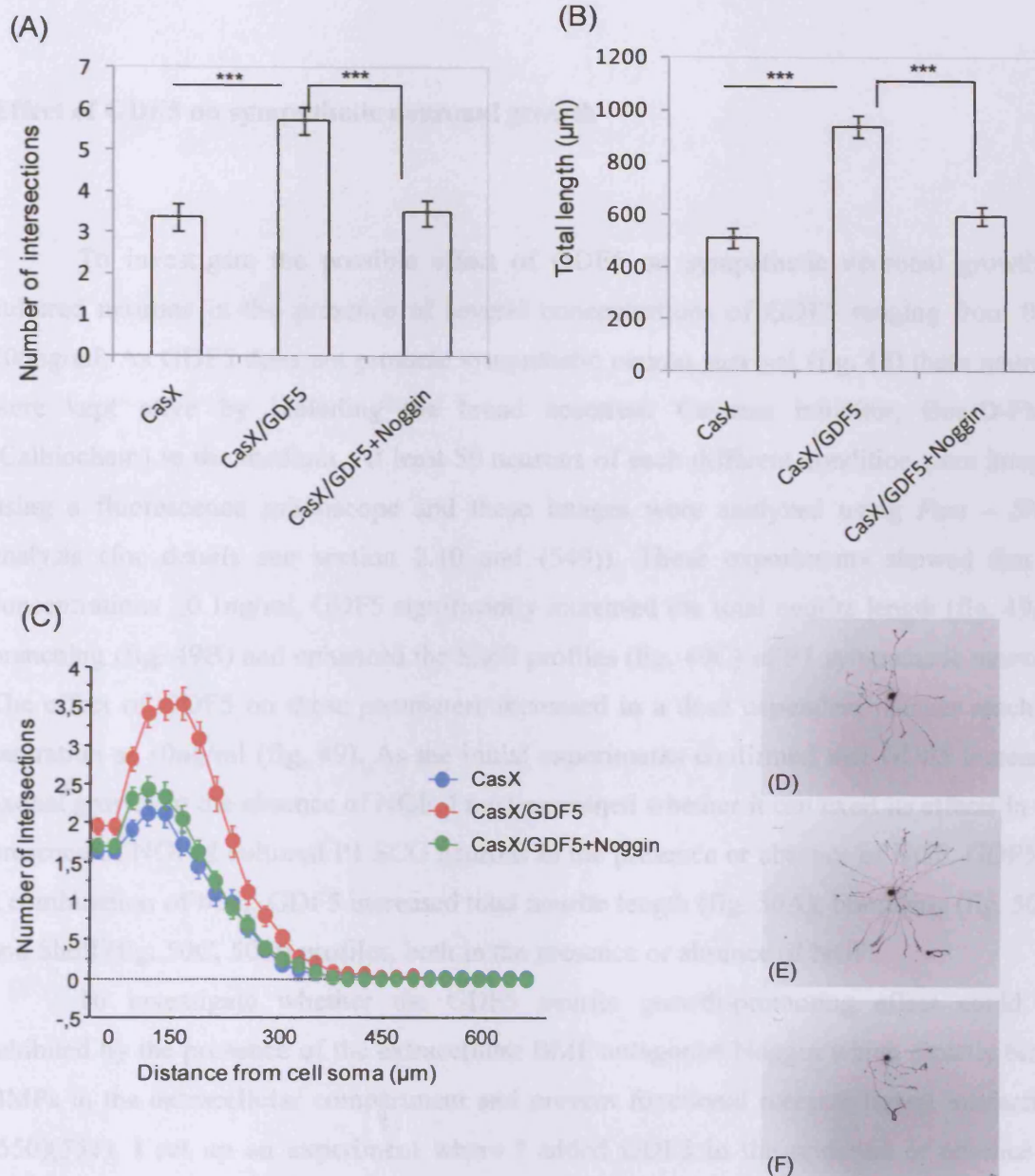
Data from at least 150 neurons in each condition from three independent experiments are shown (\*\*\*) indicates  $p < 0.001$ , statistical comparison with control, one-way ANOVA with Fisher's *post hoc*.



**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.

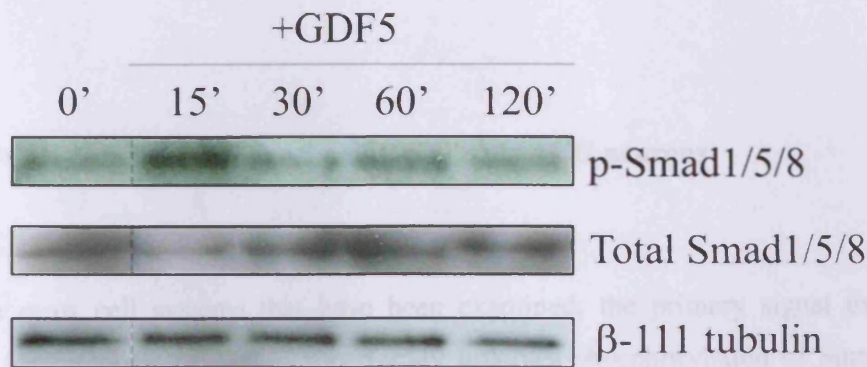
Data from at least 150 neurons in each condition from three independent experiments are shown (\*\*\*) indicates  $p < 0.001$ , statistical comparison with control, one-way ANOVA with Fisher's *post hoc*).



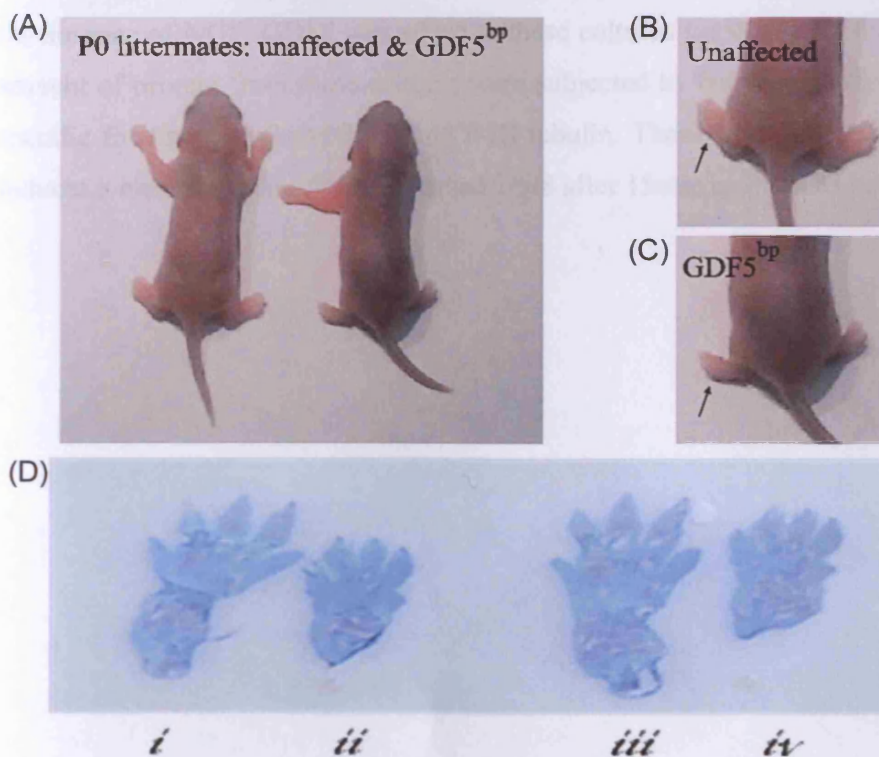
## **Effect of GDF5 on sympathetic neuronal growth**

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  $\geq 0.1$ ng/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.



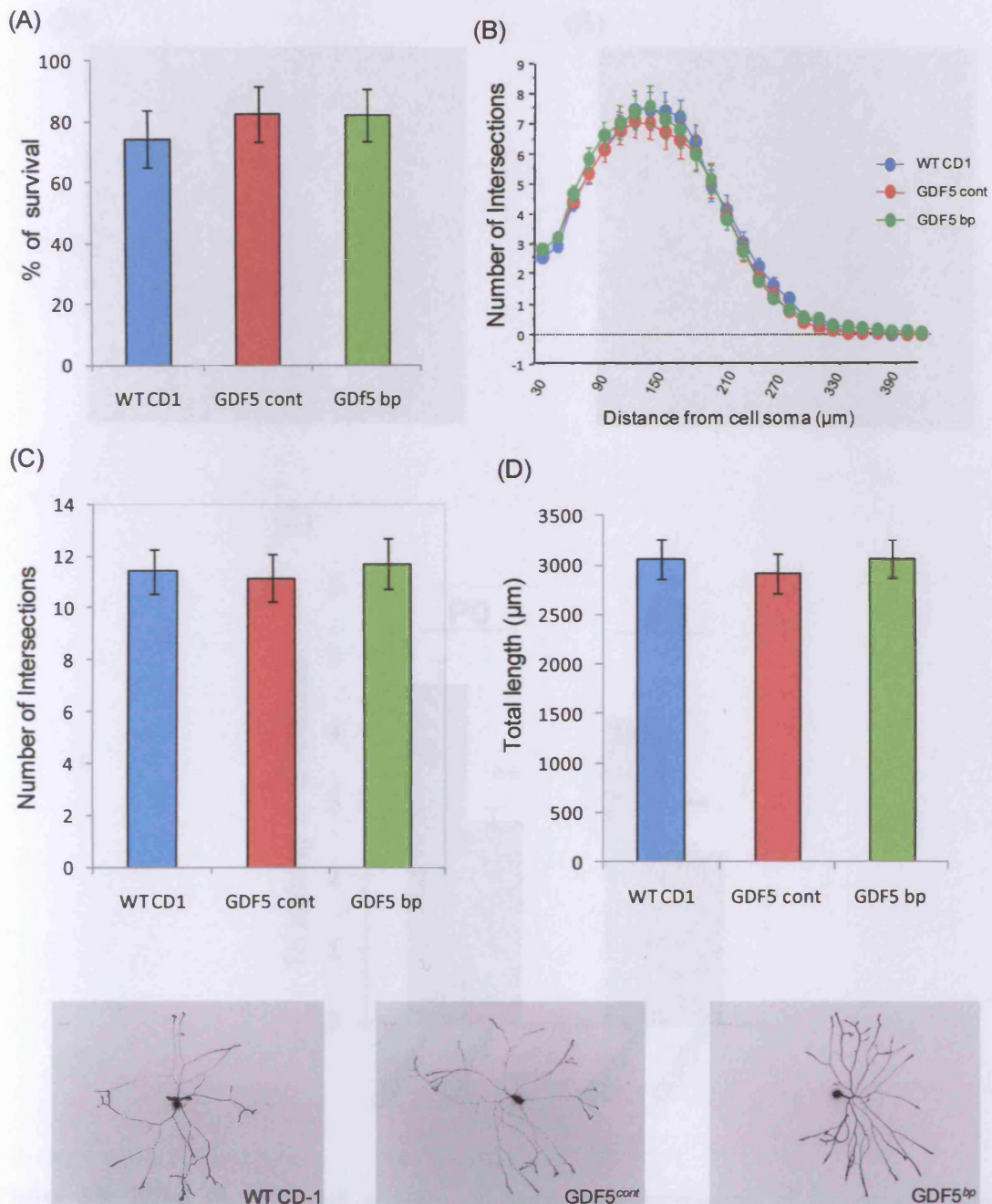
**Figure 52:** GDF5 activates Smad pathway in sympathetic neurons. Western blots showing phospho- Smad 1/5/8, total Smad 1/5/8 and β-III tubulin in SCG neurons from CD1 mice grown overnight in medium containing Boc-D-FMK before exposure to 10ng/ml GDF5 (time ranging from 0 to 120min). Results show activation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



**Figure 53:** Brachypod mice show peripheral skeletal malformations. Picture of P0 GDF5<sup>cont</sup> and GDF5<sup>bp</sup> 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<sup>bp</sup> (*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- $\beta$  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  $\beta$ -III tubulin. These experiments showed that GDF5 induces a clear phosphorylation of Smad 1/5/8 after 15min in P1 SCG neurons (fig. 52).

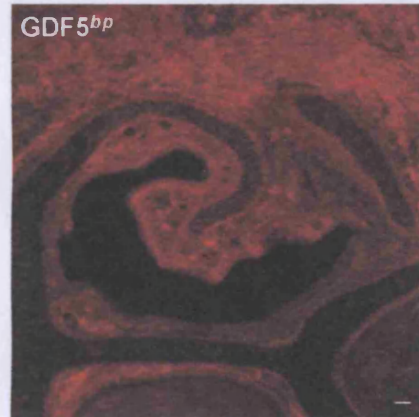


**Figure 54:** Effect of brachypod mutation on sympathetic axon growth in vitro. The data show (A) survival, (B) shell analysis, (C) branching and (D) total length of P0 SCG neurons, grown overnight in the presence of NGF. Data from at least 150 neurons in each condition from three independent experiments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

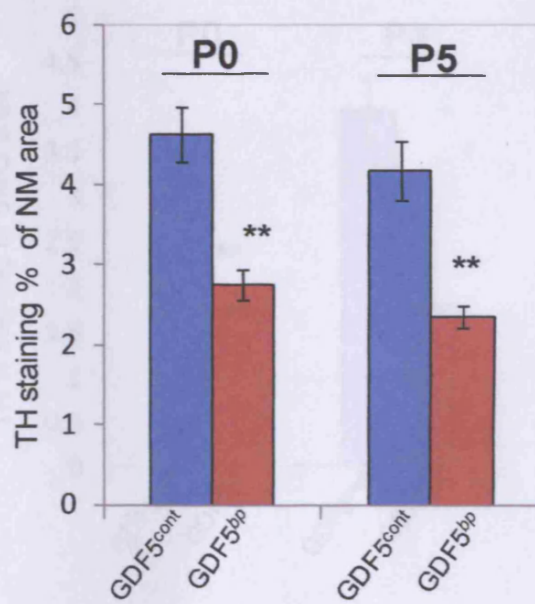
(A)



(B)

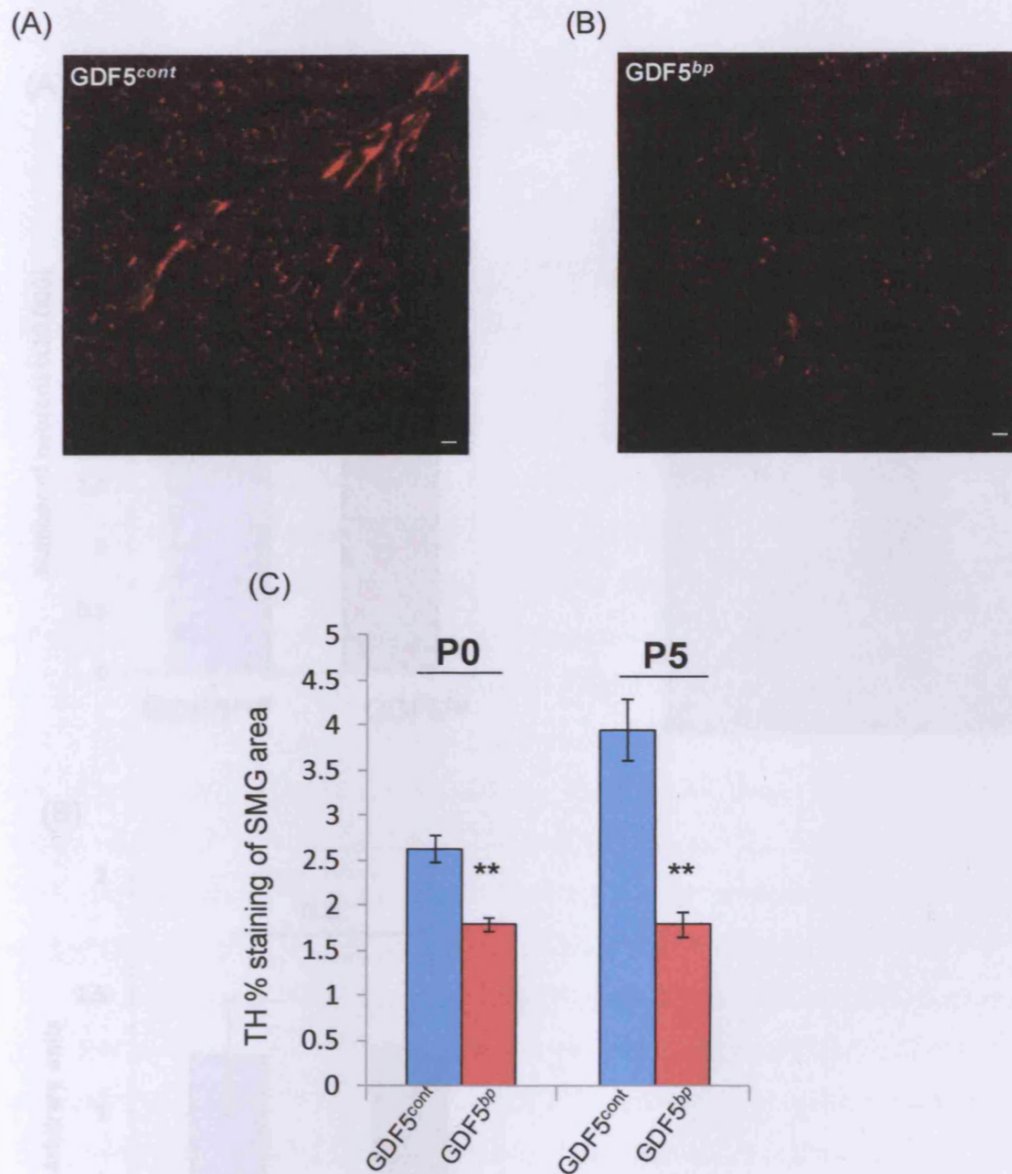


(C)



**Figure 55:** Effect of brachypod mutation on sympathetic innervation of the nasal mucosa. Sympathetic innervation density in GDF5<sup>bp</sup> and GDF5<sup>cont</sup> 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<sup>cont</sup>, 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<sup>cont</sup> (A) and GDF5<sup>bp</sup> (B).





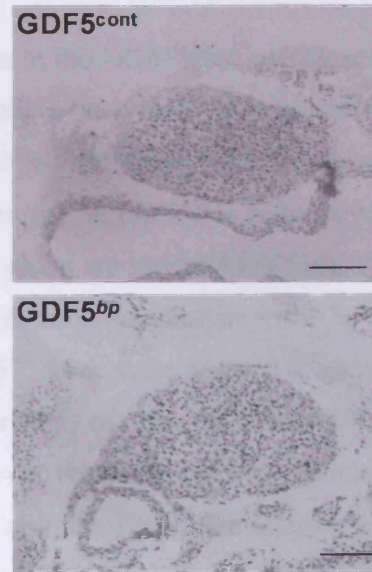
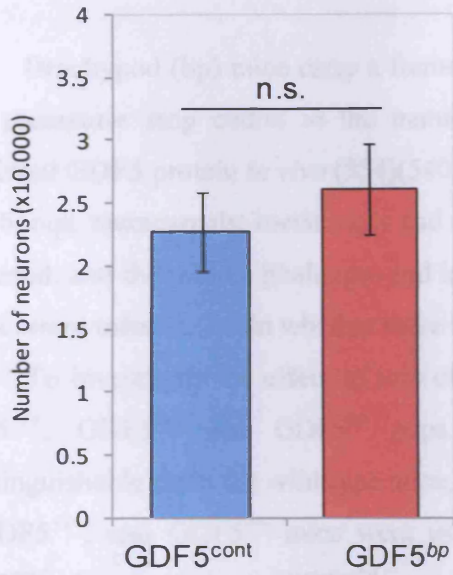
**Figure 56:** Effect of brachypod mutation on sympathetic innervation of the SMG.

Sympathetic innervation density in GDF5<sup>bp</sup> and GDF5<sup>cont</sup> mice. Bar chart showing the percentage area of selected SMG stained for tyrosine hydroxylase in P0 and P5 mice.

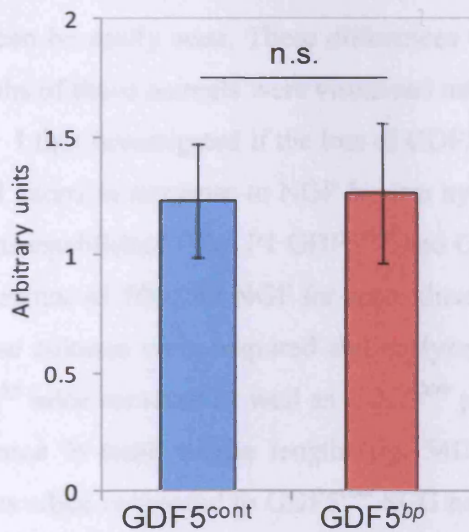
(\*\* indicates  $p < 0.001$ , statistical comparison with GDF5<sup>cont</sup>, ANOVA with Fisher's *post hoc*  $n = 3$  per genotype). Scale bar = 10  $\mu$ m

The photomicrographs show typical tyrosine hydroxylase stained sections of SMG of GDF5<sup>cont</sup> (A) and GDF5<sup>bp</sup> (B).

(A)



(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<sup>bp</sup> and GDF5<sup>cont</sup> littermates. (B) The level of tyrosine hydroxylase mRNA relative to GAPDH mRNA (arbitrary units) in P0 SCG dissected from GDF5<sup>bp</sup> and GDF5<sup>cont</sup> littermates is shown.

Mean  $\pm$  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<sup>+/-</sup> mice were crossed to generate GDF5<sup>+/+</sup>, GDF5<sup>+/-</sup> and GDF5<sup>bp</sup> pups. Because carriers of the bp mutation are indistinguishable from the wild-type mice, and cannot be conveniently genotyped a mixture of GDF5<sup>+/+</sup>, and GDF5<sup>+/-</sup> mice were used as control animals (hereafter referred to as GDF5<sup>cont</sup>). In mixed litters, GDF5<sup>bp</sup> 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<sup>bp</sup> (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<sup>cont</sup> and GDF5<sup>bp</sup> 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<sup>bp</sup> mice survived as well as GDF5<sup>cont</sup> pups (fig. 54A) and P1 GDF5<sup>bp</sup> neurons had no difference in total neurite length (fig. 54D), branching (fig. 54C) and Sholl (fig. 54B) profiles when compared to GDF5<sup>cont</sup> SCG neurons. This suggests, perhaps not surprisingly, that neurons from GDF5<sup>bp</sup> 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<sup>cont</sup> and GDF5<sup>bp</sup> 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<sup>bp</sup>* 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<sup>bp</sup>* mice does not impact on NGF retrograde survival signaling of the innervating neurons. In *NT3<sup>-/-</sup>* 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- $\alpha$ 2, 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<sup>bp</sup>* 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.

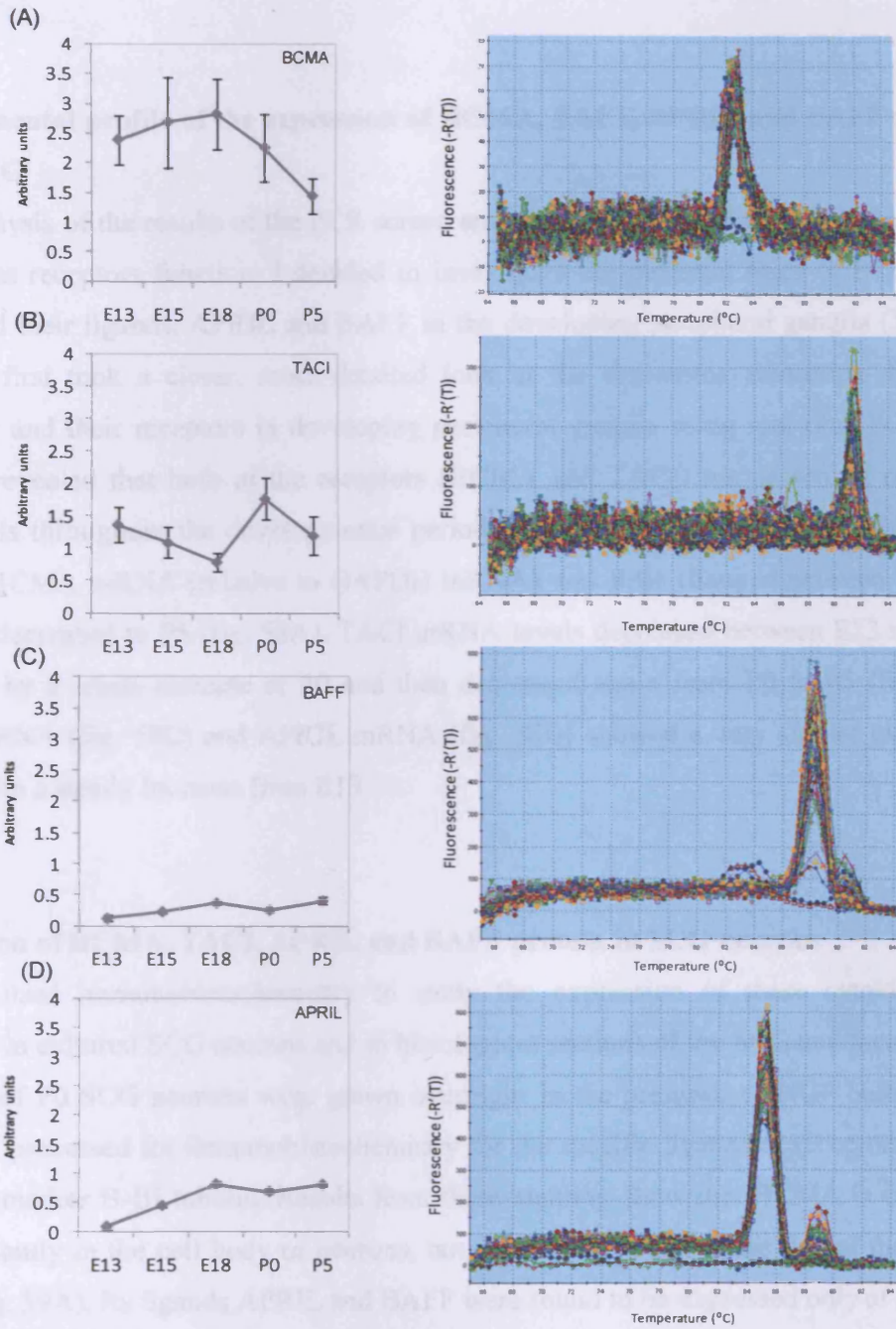
**5. Role of TNFRSF members BCMA and TACI in regulating the neurotrophin survival responses of developing sensory and sympathetic neurons**

---

## **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**

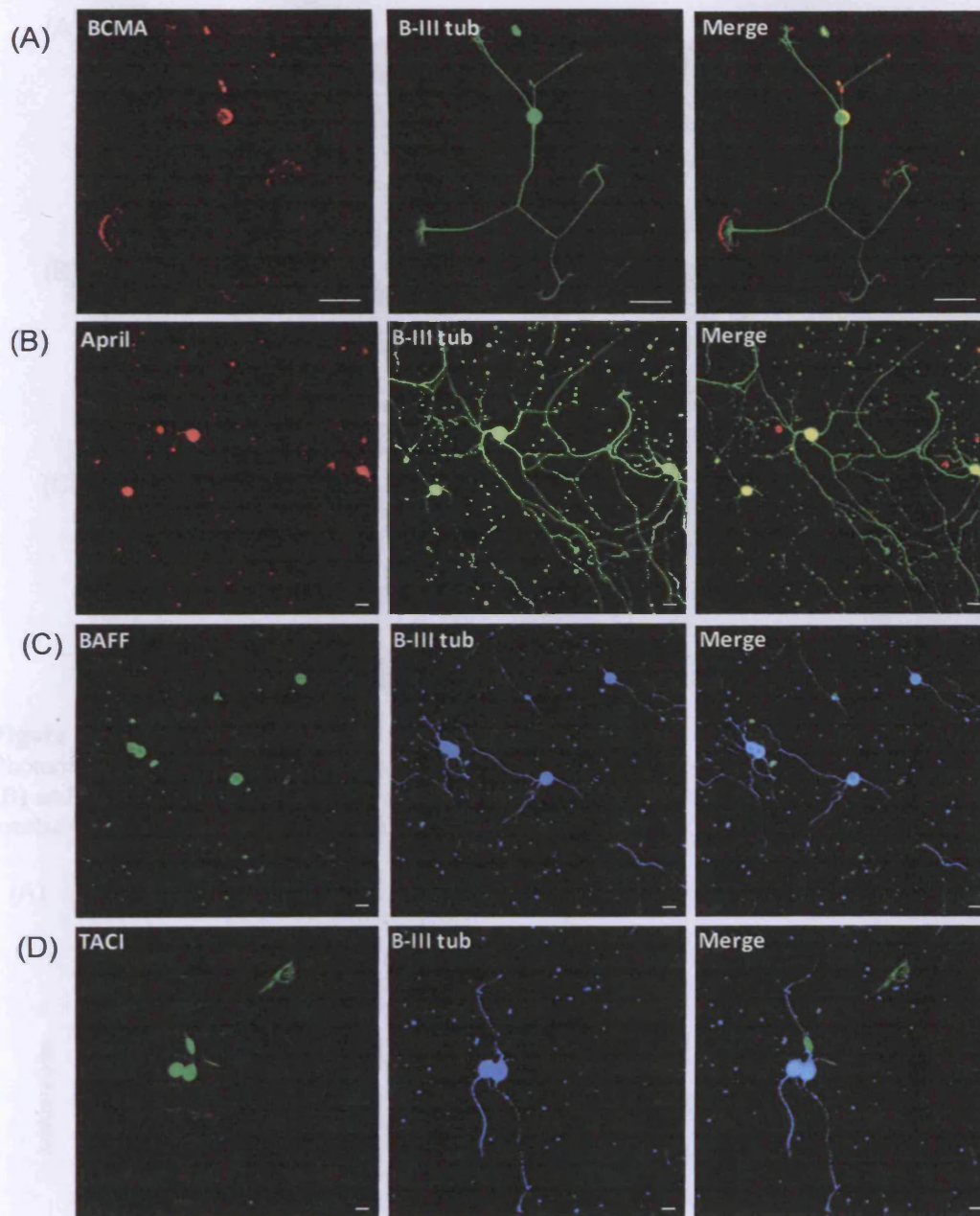
From analysis of the results of the PCR screen and a consideration of the available tools to manipulate receptors function, I decided to investigate the potential roles of BCMA and TACI and their ligands, APRIL and BAFF in the developing peripheral ganglia (514). To do so, I first took a closer, more 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**

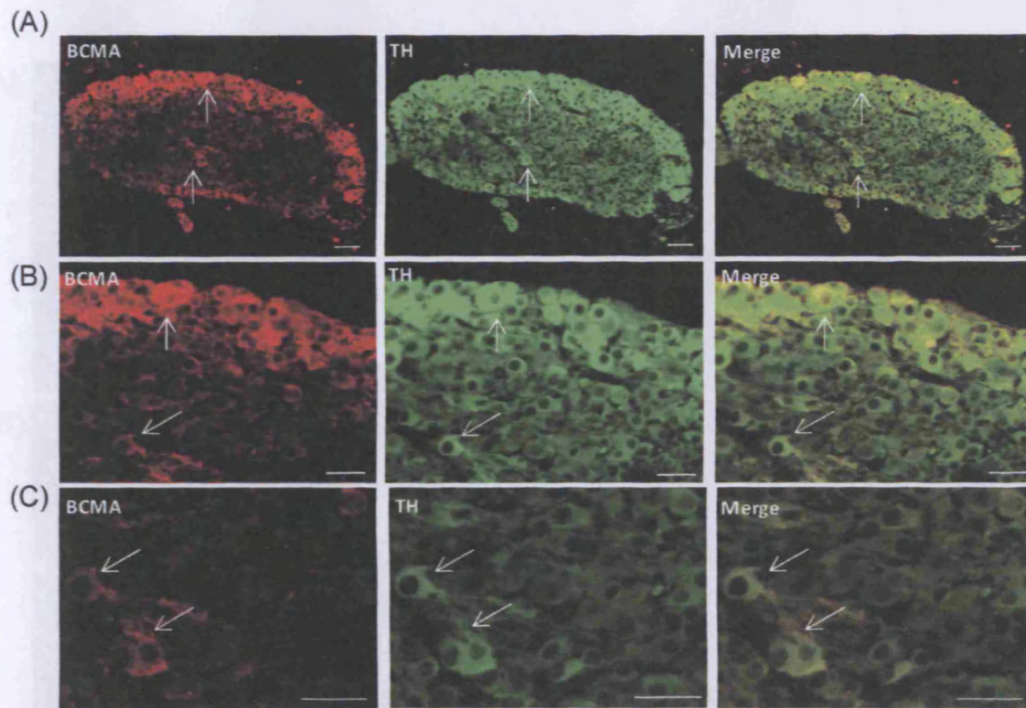
I used immunohistochemistry to study the expression of these cytokines and receptors in cultured SCG neurons and in histological sections of the SCG and their targets. Cultures of P0 SCG neurons were grown overnight in the presence of NGF before being fixed and processed for immunohistochemistry for 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



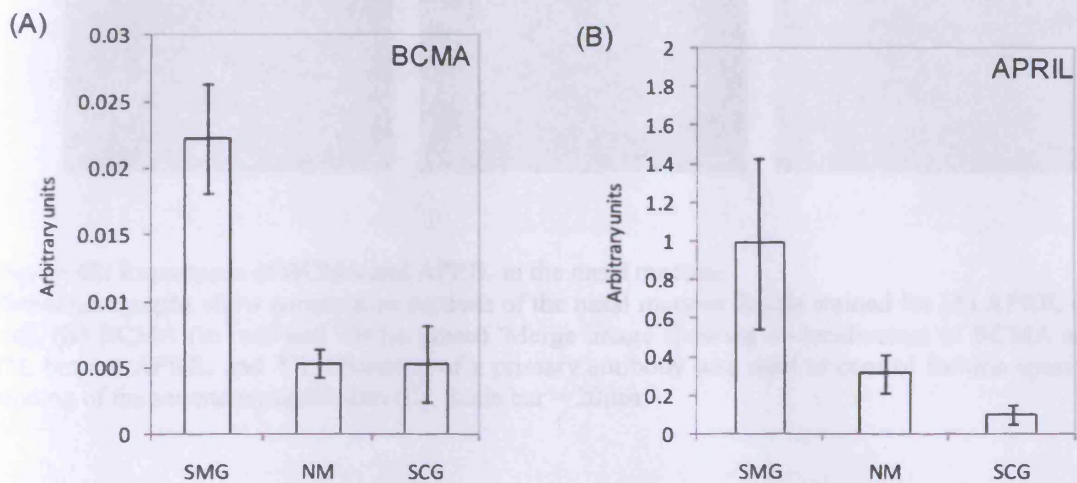
raises the possibility that it could interact with any of its ligands should they be expressed in the intermediate or distal target of these neurons (discussed later). Considering the relatively high expression of BCMA throughout the developmental period studied, and its expression in cultured P0 SCG 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 colocalization of these proteins with the marker B-III tubulin. Scale bar = 10 $\mu$ m

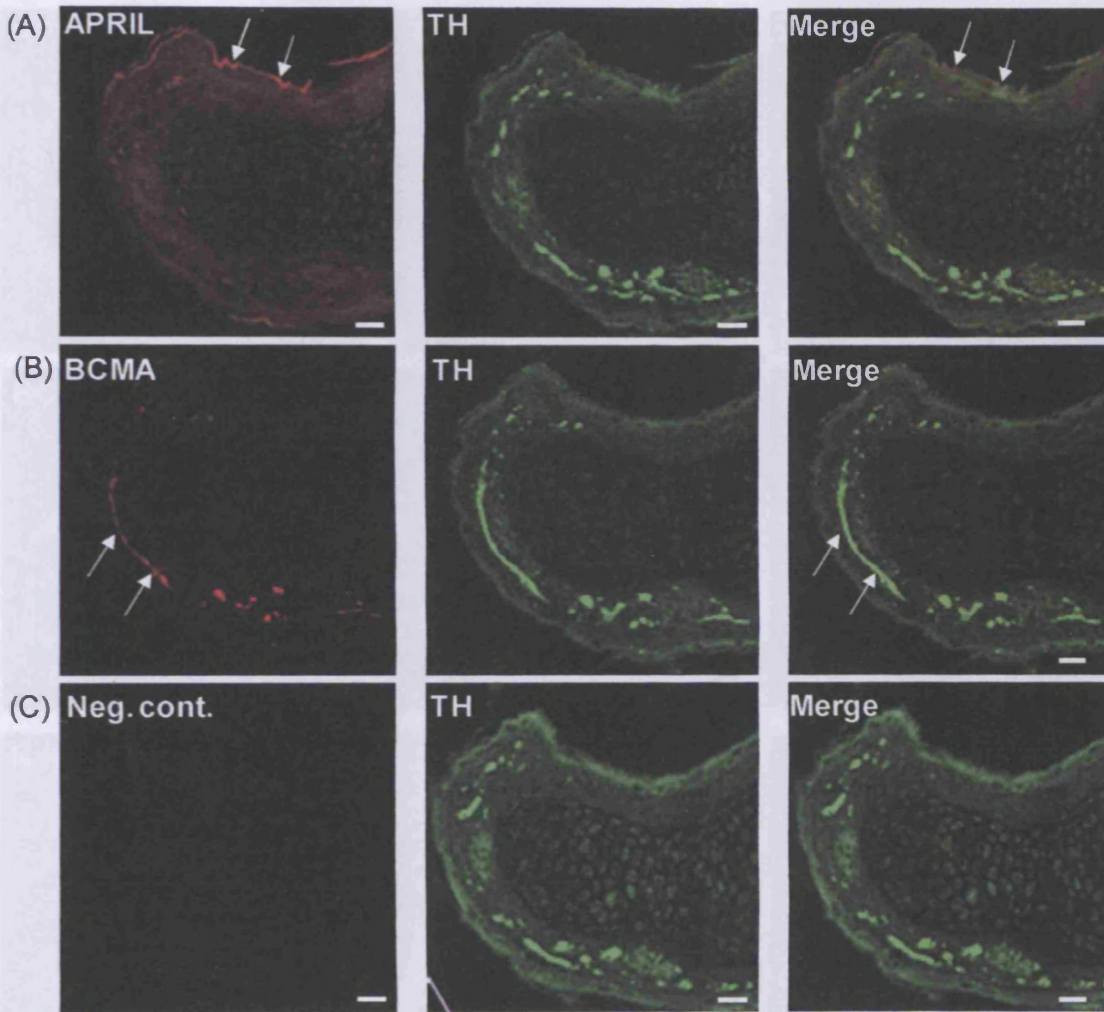


**Figure 60:** Expression of BCMA in the SCG ganglion. Photomicrographs show consecutive sections of the entire SCG (A), the outer part of the ganglion (B) and centre part (C) double stained for BCMA (red) and TH (green). Merge image showing colocalization of BCMA and TH. Scale bar =10μm

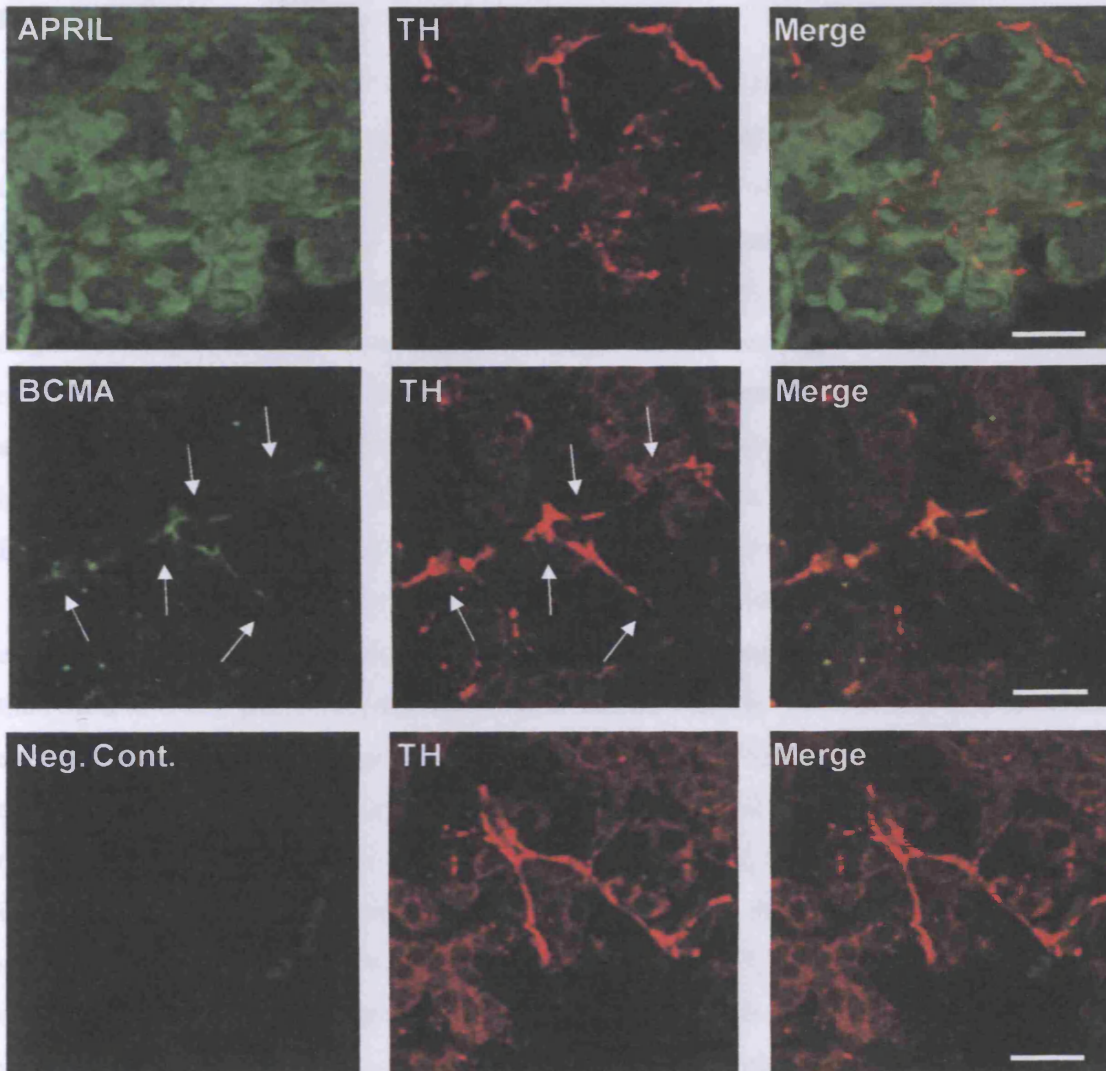


**Figure 61:** Expression of BCMA and APRIL in the SCG compared with target fields. The data show the mRNA level of (A) BCMA and (B) APRIL in the submandibular gland (SMG), nasal mucosa (NM) and SCG relative to GAPDH in CD-1 mice. Means and standard errors of data from three separate sets of each tissue 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 = 20 $\mu$ m



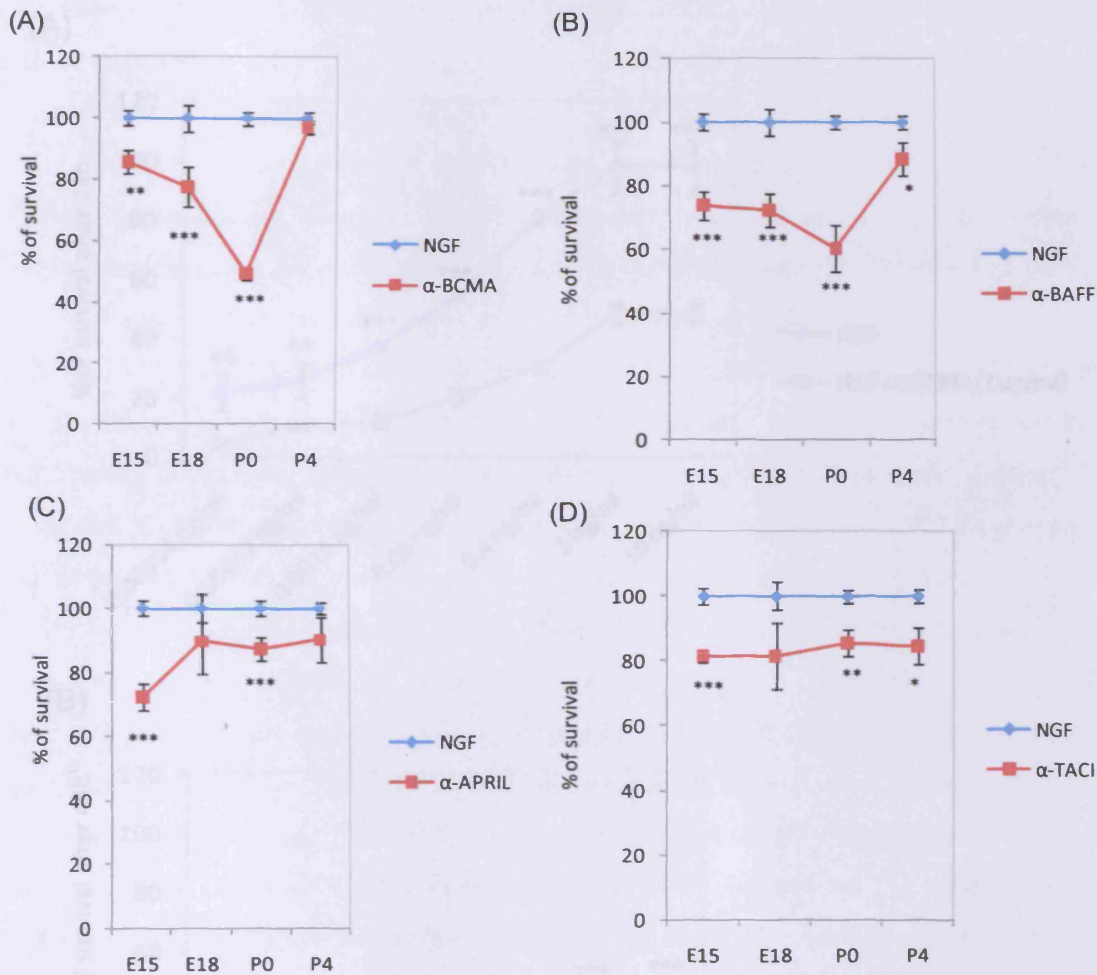
**Figure 63:** Expression of BCMA and APRIL in the SMG.

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 = 20 $\mu$ m

### **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.

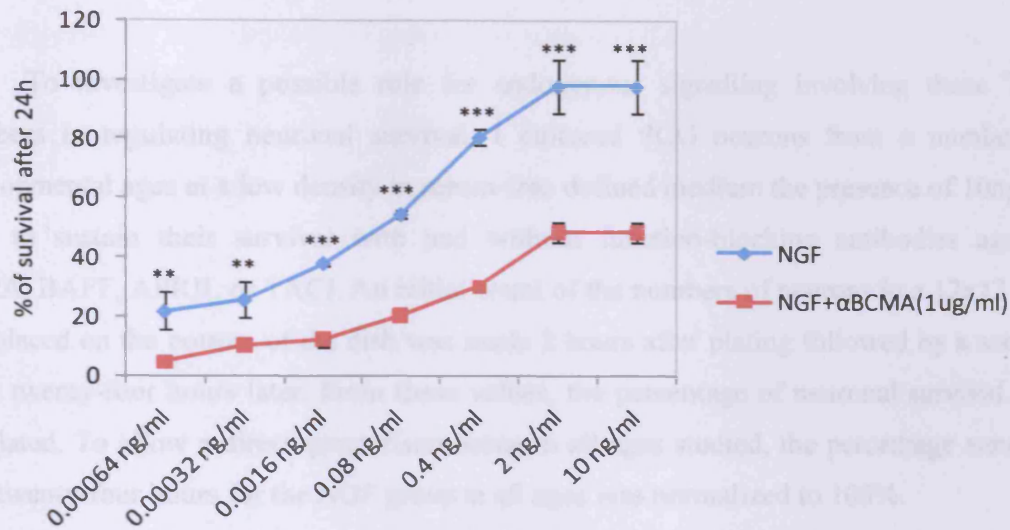
I used immunohistochemistry to confirm that BCMA and APRIL proteins are expressed in the targets and determine their localization. I prepared histological sections of the NM and SMG of P0 animals, and double stained them for BCMA and TH, or APRIL and TH. In the NM, BCMA and TH were co-localized (fig. 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).



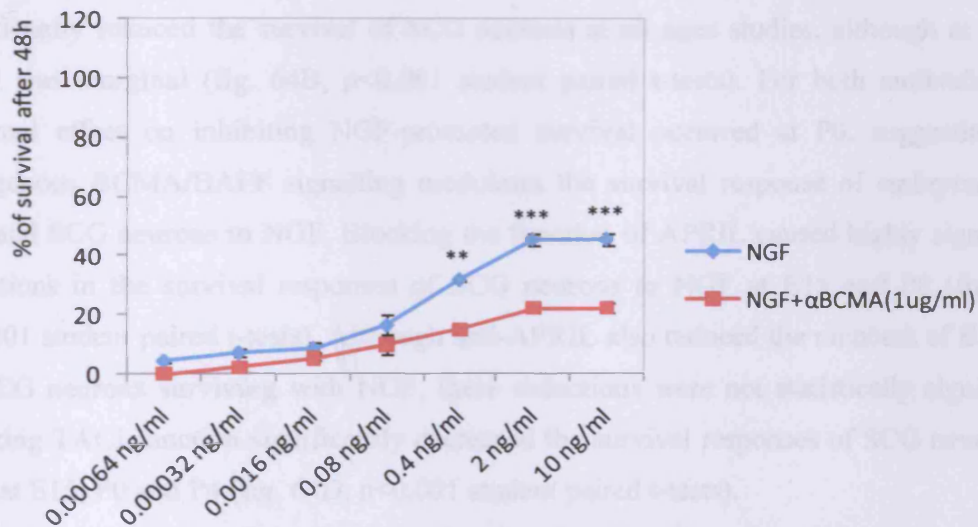
**Figure 64 :** Effect of blocking BCMA, BAFF, APRIL and TACI on sympathetic neuron survival. The data show SCG neurons time course survival (24h) comparison between neurons grown only with 10ng/ml of NGF (control normalized to 100%) and function blocking antibodies for the TNF family members. (A) 1 $\mu$ l/ml of  $\alpha$ -BCMA, (B) 10 $\mu$ g/ml of  $\alpha$ -BAFF, (C) 5 $\mu$ g/m of  $\alpha$ -APRIL and (D) 10 $\mu$ g/m of  $\alpha$ -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*).



(A)



(B)



**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*.



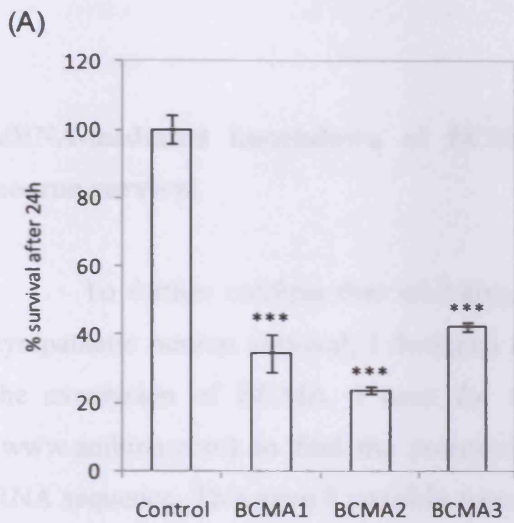
## **The effects of BCMA, TACI and their ligands APRIL and BAFF on sympathetic neuron survival**

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%.

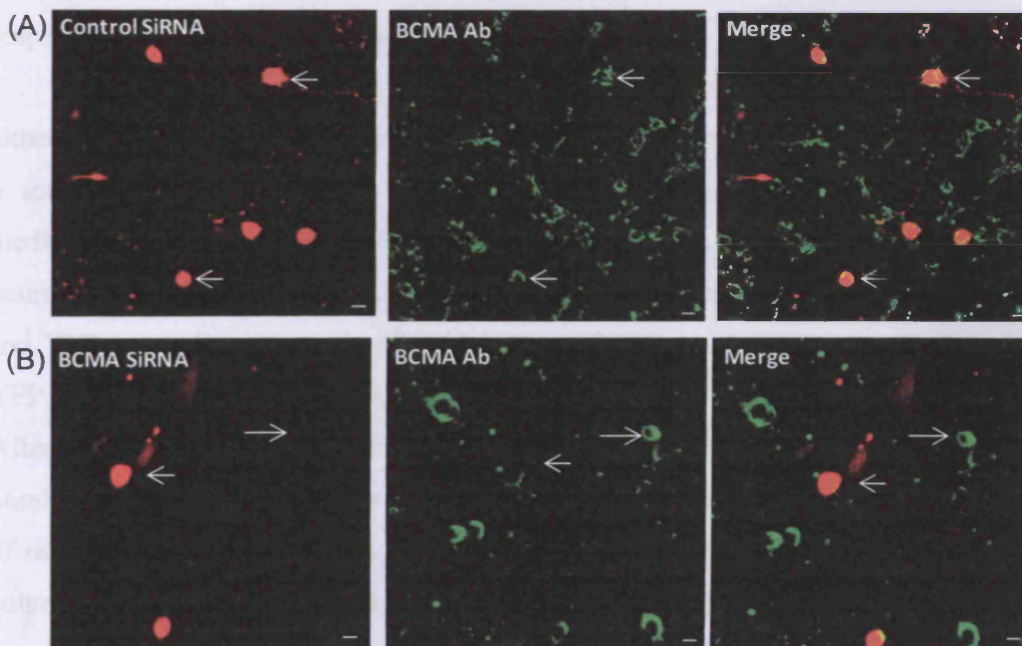
These experiments showed that BCMA function blocking antibodies significantly reduced the survival of cultured sympathetic neurons at E15, E18 and P0, but not P4 (fig. 64A,  $p < 0.001$  student paired t-tests). Similarly, a function blocking BAFF antibody significantly reduced the survival of SCG neurons 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 = 20 $\mu$ m.

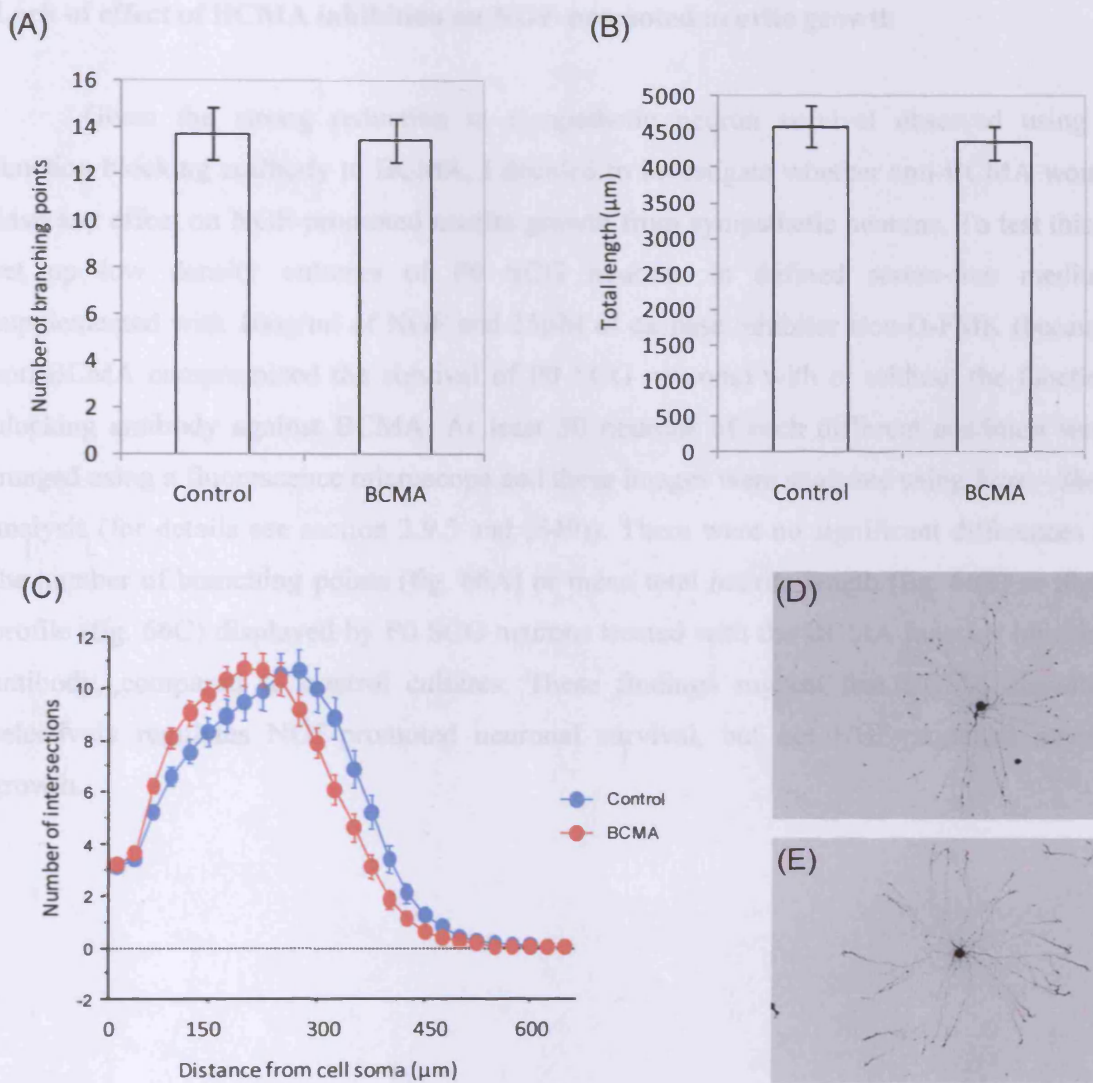
## **siRNA-mediated knockdown of BCMA receptor expression reduces sympathetic neuron survival**

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](http://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 *pSilencer*<sup>TM</sup> 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).

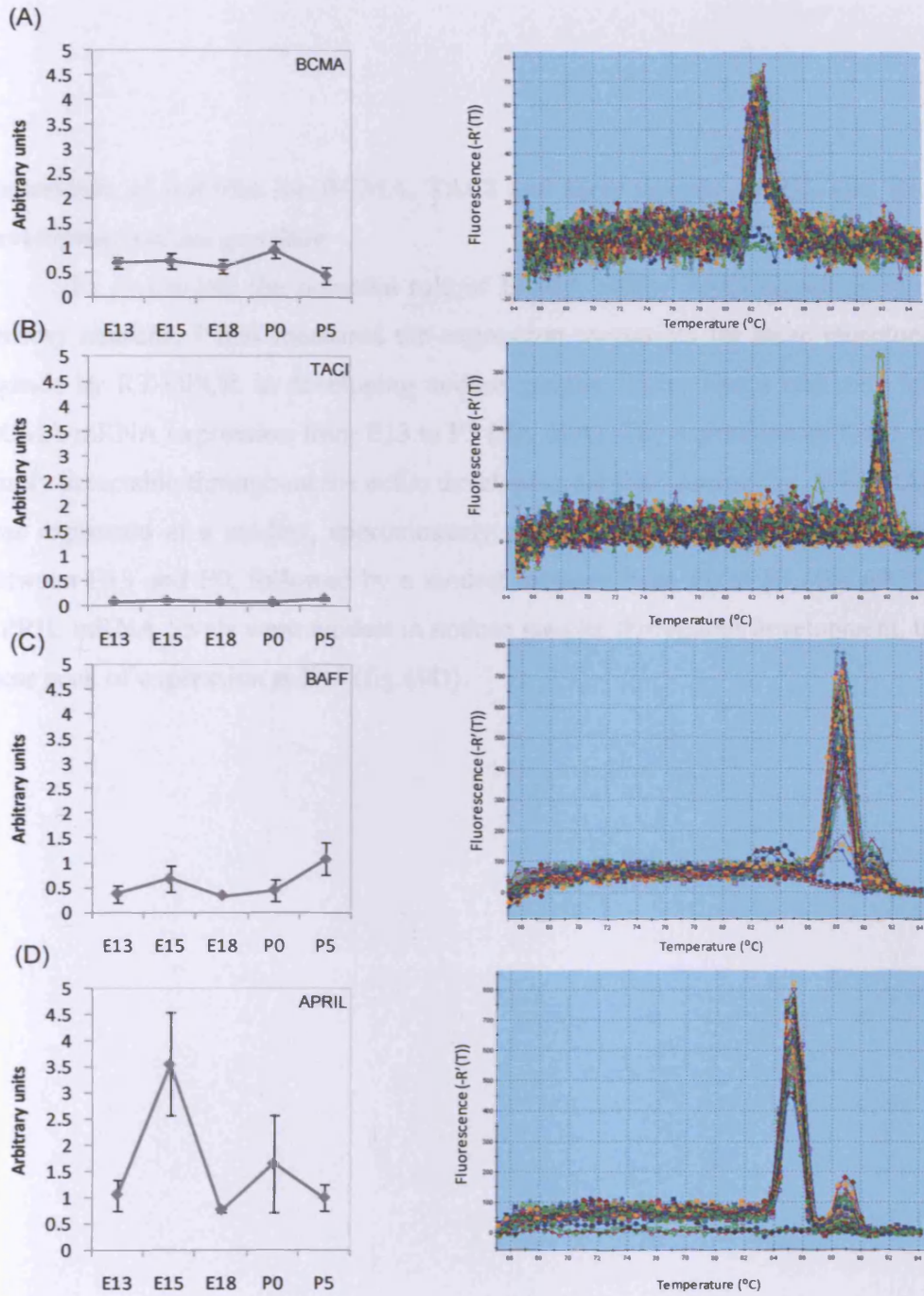


**Figure 68:** Effect of blocking BCMA on sympathetic neurite growth. Branching (A), Length (B) and sholl analysis (C) of the neurite arbors of P0 SCG neurons grown overnight with Caspase inhibitors and NGF (Control) or Caspase inhibitors, NGF and BCMA. (D) and (E) show illustrative draws of neuronal 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 25 $\mu$ M 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.



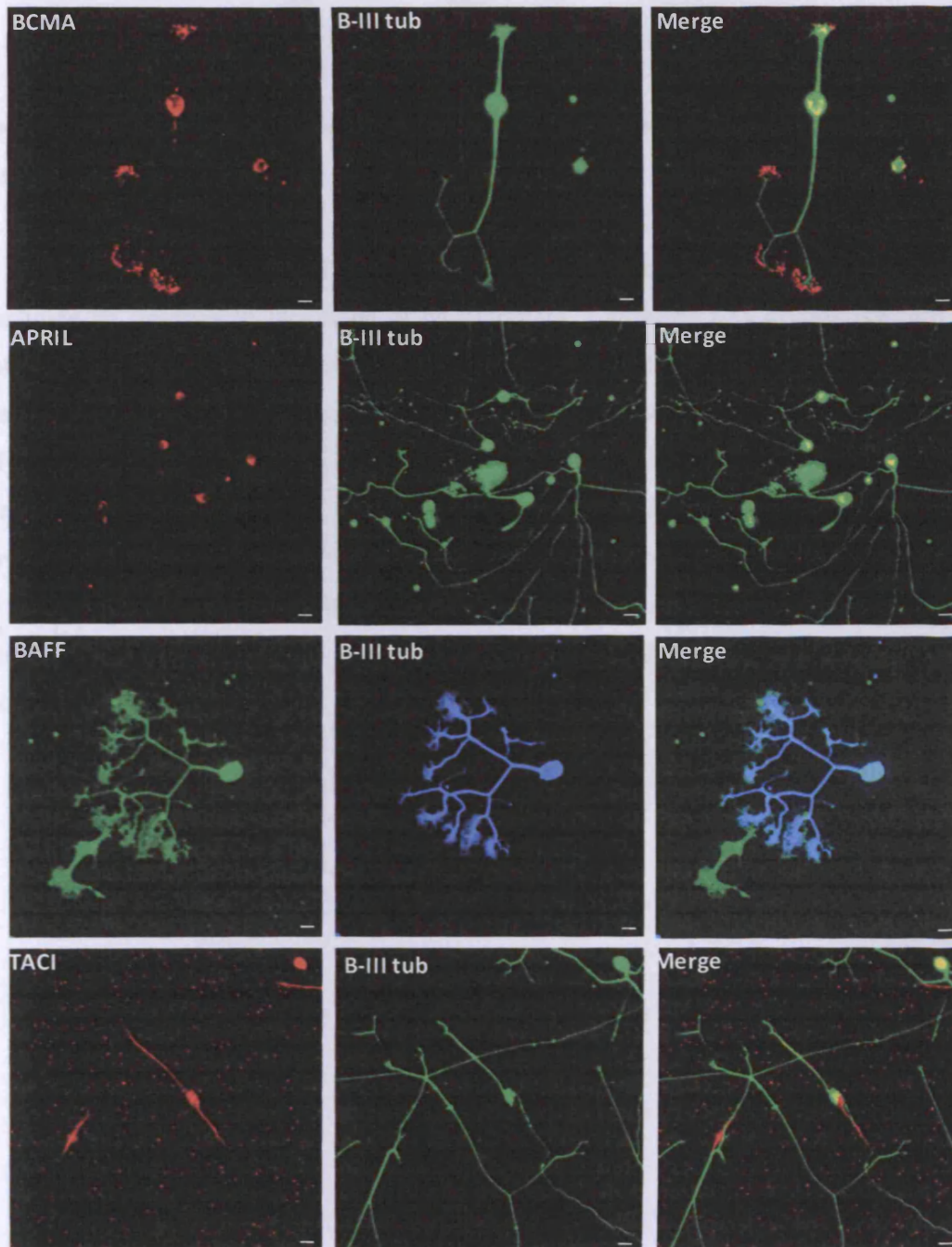


**Figure 69:** Expression of BCMA, TACI, BAFF and APRIL in the developing Node ganglion. The data show the relative expression of (A) BCMA, (B) TACI, (C) BAFF and (D) APRIL relative to GAPDH mRNA (in arbitrary units) and the respective Real time PCR melting curves (showing primer specificity in E13, E15, E18, P0 and P5 Node. 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**

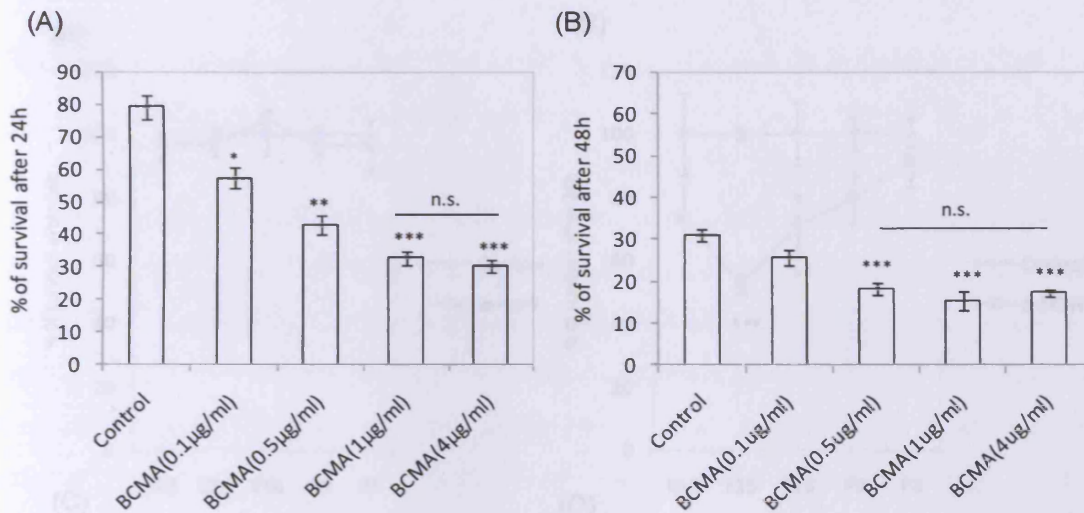
To investigate the potential role of BCMA and/or TACI signalling in developing sensory neurons, I first measured the expression transcripts for these receptors and their ligands by RT-QPCR in developing nodose ganglia. There was a relatively low level of BCMA mRNA expression from E13 to P5 (fig. 69A). 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 = 10 $\mu$ m.

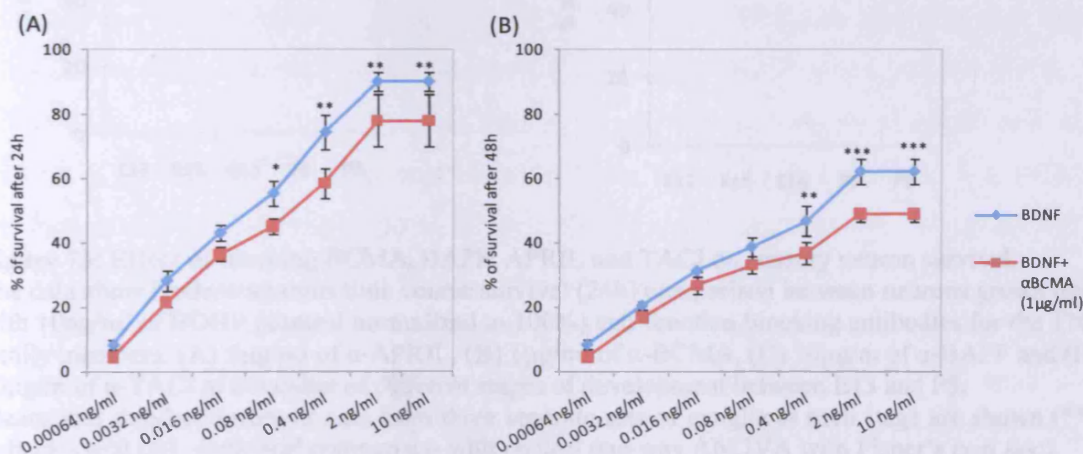
### **Expression of BCMA, TACI, and their ligands APRIL and BAFF in cultured nodose neurons**

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  $\beta$ -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.

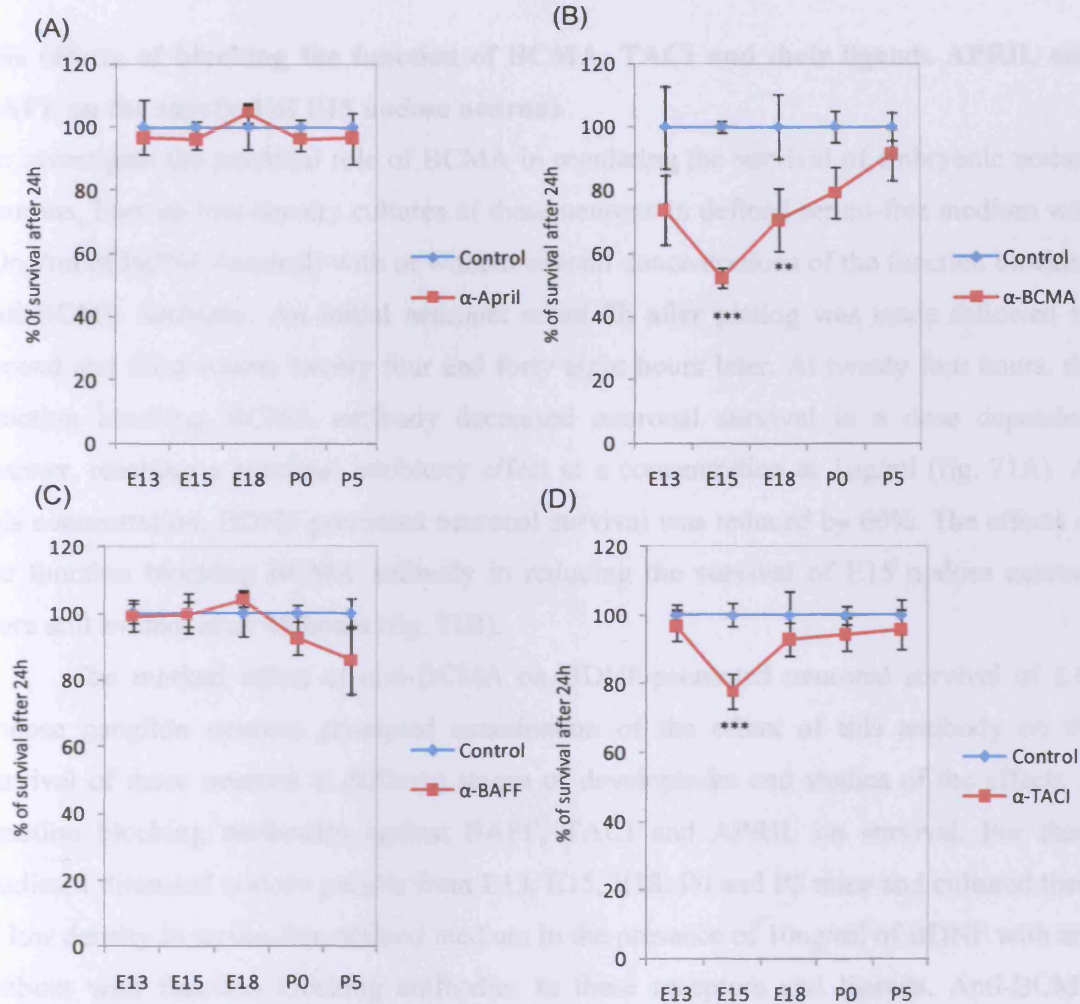
The data show E15 nodose neurons grown overnight with 10ng/ml BDNF (control) plus several concentrations of BCMA ranging from 1 to 4µg/ml of BCMA. Data from three independent experiments are shown. Statistical comparison with control, one-way ANOVA with Fisher's *post hoc*. \*\*\* indicates  $p < 0.001$



**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 without 1µg/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) 5 $\mu$ g/ml of  $\alpha$ -APRIL, (B) 1 $\mu$ g/ml of  $\alpha$ -BCMA, (C) 10 $\mu$ g/m of  $\alpha$ -BAFF and (D) 10 $\mu$ g/m of  $\alpha$ -TACI at a number of different stages of development between E13 and P5.

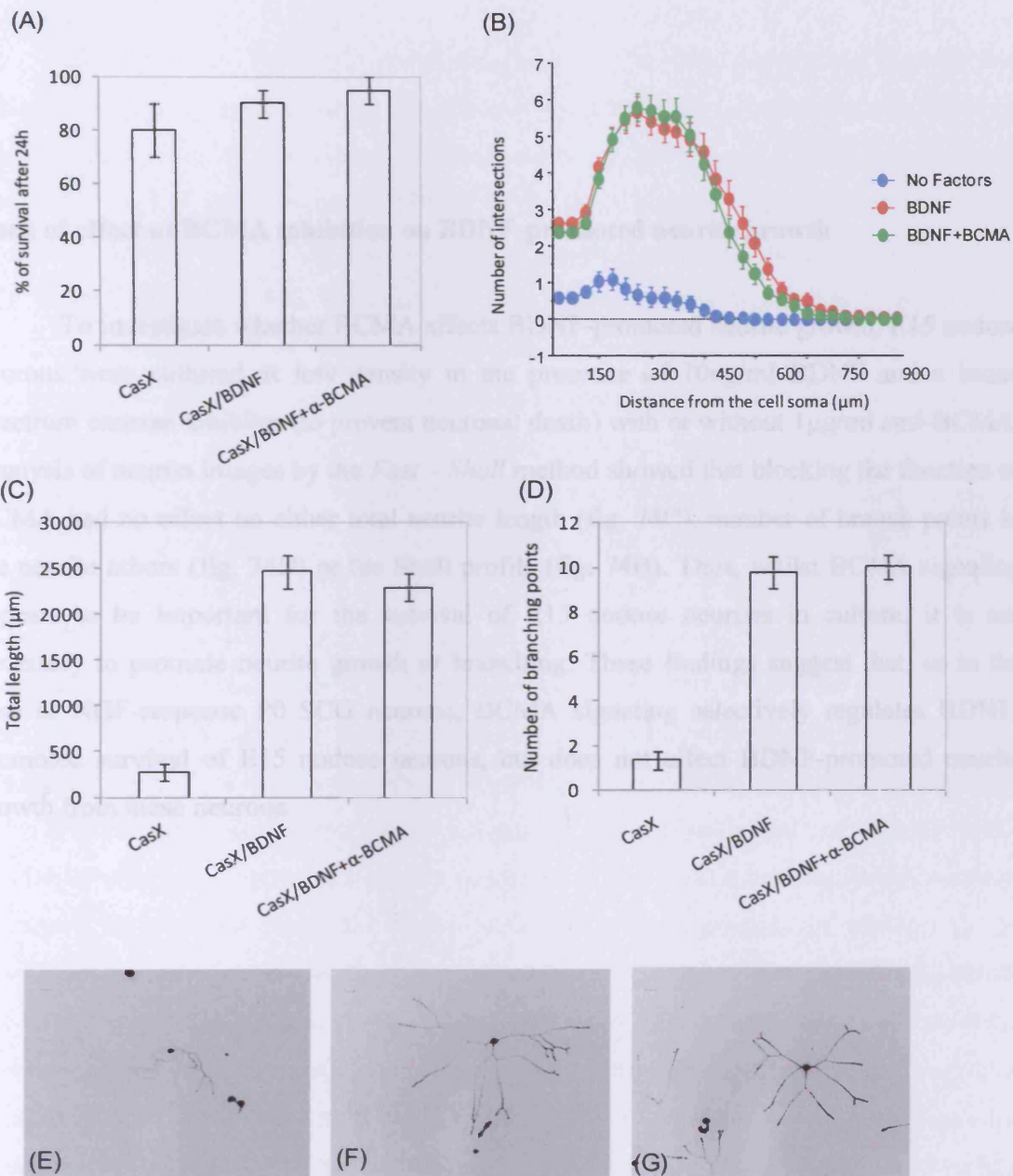
Means and standard errors of data from three separate sets of ganglia at each stage are shown (\*\*\*) indicates  $p < 0.001$ , statistical comparison with control one-way ANOVA with Fisher's *post hoc*.



### **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 1 $\mu$ g/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 1 $\mu$ g/ml of  $\alpha$ -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 1 $\mu$ g/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 strikingly 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- $\kappa$ B 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- $\kappa$ B 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 particularly 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.

## **6. References**

---

1. *Mouse gastrulation: the formation of a mammalian body plan.* Tam PP, Behringer RR. 1997, *Mech Dev*, pp. 68(1-2):3-25.
2. *Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development.* Murry CE, Keller G. 2008, *Cell*, pp. 132(4):661-80.
3. *Vertebrate endoderm development.* Wells JM, Melton DA. 1999, *Annu Rev Cell Dev Biol.* , pp. 15:393-410.
4. *BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm.* Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G. 2006, *Nat Biotechnol.* , pp. 24(11):1402-11.
5. *Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells.* D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. 2006, *Nat Biotechnol.* , pp. 24(11):1392-401.
6. *A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells.* Park C, Afrikanova I, Chung YS, Zhang WJ, Arentson E, Fong Gh G, Rosendahl A, Choi K. 2004, *Development*, pp. 131, 2749-2762.
7. *Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood.* Nostro MC, Cheng X, Keller GM, Gadue P. 2008, *Cell Stem Cell.*, pp. 2(1):60-71.
8. *Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis.* Naito AT, Shiojima I, Akazawa H, Hidaka K, Morisaki T, Kikuchi A, Komuro I. 2006, *Proc Natl Acad Sci U S A.* , pp. 103(52):19812-7.
9. *Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells.* Ueno S, Weidinger G, Osugi T, Kohn AD, Golob JL, Pabon L, Reinecke H, Moon RT, Murry CE. 2007, *Proc Natl Acad Sci U S A.* , pp. 104(23):9685-90.
10. *Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation.* Aubert J, Dunstan H, Chambers I, Smith A. 2002, *Nat Biotechnol.*, pp. 20(12):1240-5.
11. *Development of definitive endoderm from embryonic stem cells in culture.* Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. 2004, *Development*, pp. 131(7):1651-62.
12. *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Ying QL, Stavridis M, Griffiths D, Li M, Smith A. 2003, *Nat Biotechnol.*, pp. 21(2):183-6.
13. *Neurulation: coming to closure.* Smith JL, Schoenwolf GC. 1997, *Trends Neurosci.* , pp. 20(11):510-7.
14. *Neural induction.* Harland R. 2000, *Curr Opin Genet Dev.* , pp. 10(4):357-62.
15. *Roles of neuroepithelial cell rearrangement and division in shaping of the avian neural plate.* Schoenwolf GC, Alvarez IS. 1989, *Development.* , pp. 106(3):427-39.
16. *Planar induction of convergence and extension of the neural plate by the organizer of Xenopus.* Keller R, Shih J, Sater AK, Moreno C. 1992, *Dev Dyn.* , pp. 193(3):218-34.
17. *Mechanisms of convergence and extension by cell intercalation.* Keller R, Davidson L, Edlund A, Elul T, Ezin M, Shook D, Skoglund P. 2000, *Philos Trans R Soc Lond B Biol Sci.* , pp. 355(1399):897-922.
18. *Quantitative analyses of changes in cell shapes during bending of the avian neural plate.* Schoenwolf GC, Franks MV. 1984, *Dev Biol.*, pp. 105(2):257-72.

19. *Towards a cellular and molecular understanding of neurulation.* **Colas JF, Schoenwolf GC.** 2001, *Dev Dyn.*, pp. 221(2):117-45.
20. *Distribution of surface coat material on fusing neural folds of mouse embryos during neurulation.* **Sadler TW.** 1978, *Anat Rec.*, pp. 191(3):345-9.
21. *The two sites of fusion of the neural folds and the two neuropores in the human embryo.* **O'Rahilly R, Müller F.** 2002, *Teratology*, pp. 65(4):162-70.
22. *A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick.* **Weston J.A.** 1963, *Dev Biol.*, pp. 6:279-310.
23. *Neurulation in the cranial region—normal and abnormal.* **Copp AJ.** 2005, *J Anat.*, pp. 207(5):623-35.
24. *analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography.* **Fujita S.** 1964, *J Comp Neurol*, pp. 122:311-27.
25. *Morphogens in motion: growth control of the neural tube.* **Cayuso J., Martí E.** 2005, *J Neurobiol*, pp. 64(4):376-87.
26. *Neuronal migration: unraveling the molecular pathway with humans, mice, and a fungus.* **Keays DA.** 2007, *Mamm Genome*, pp. 18(6-7):425-30.
27. *Central nervous system neuronal migration.* **Hatten ME.** 1999, *Annu Rev Neurosci*, pp. 22:511-39.
28. *Patterns of neuronal migration in the embryonic cortex.* **Kriegstein AR, Noctor SC.** 2004, *Trends Neurosci*, pp. 27(7):392-9.
29. *Neuronal differentiation rescued by implantation of Weaver granule cell precursors into wild-type cerebellar cortex.* **Gao WQ, Hatten ME.,** 1993, *Science*, pp. 260(5106):367-9.
30. *Lissencephaly and other malformations of cortical development: 1995 update.* **Dobyns WB, Truwit CL.,** 1995, *Neuropediatrics*, pp. 26(3):132-47.
31. *Fine structure of synaptogenesis in the vertebrate central nervous system.* **Vaughn JE.** 1989, *Synapse*, pp. 3(3):255-85.
32. *The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis.* **Cline H, Haas K.** 2008, *J Physiol*, pp. 586(6):1509-17.
33. *The agrin hypothesis.* **McMahan UJ.** 1990, *Cold Spring Harb Symp Quant Biol*, pp. 55:407-18.
34. *Defective neuromuscular synaptogenesis in agrin-deficient mutant mice.* **Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR.** 1996, *Cell*, pp. 85(4):525-35.
35. *Development of the neuromuscular junction.* **Witzemann V.** 2006, *Cell Tissue Res*, pp. 326(2):263-71.
36. *Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice.* **Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP.** 1995, *Nature*, pp. 377(6546):232-6.
37. *The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo.* **DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD.** 1996, *Cell*, pp. 85(4):501-12.
38. *Mechanisms of vertebrate synaptogenesis.* **Waites CL, Craig AM, Garner CC.** 2005, *Annu Rev Neurosci.*, pp. 28:251-74.
39. *Functional anatomy of the autonomic nervous system.* **Shields RW Jr.** 1993, *J Clin Neurophysiol*, pp. 10(1):2-13.
40. *Origins of the avian neural crest: the role of neural plate-epidermal interactions.* **Selleck MA,**



- Bronner-Fraser M.** 1995, *Development*, pp. 121(2):525-38.
41. *Neural crest formation in Xenopus laevis: mechanisms of Xslug induction.* **Mancilla A, Mayor R.** 1997, *Dev Biol*, pp. 177(2):580-9.
42. **Le Douarin NM.** *The neural crest.* Cambridge : University Press, 1982.
43. *Neural crest cell plasticity and its limits.* **Le Douarin NM, Creuzet S, Couly G, Dupin E.** 2004, *Development*, pp. 131(19):4637-50.
44. *Contributions of placodal and neural crest cells to avian cranial peripheral ganglia.* **D'Amico-Martel A, Noden DM.** 1983, *Am J Anat*, pp. 166(4):445-68.
45. *Neural crest induction in Xenopus: evidence for a two-signal model.* **LaBonne C, Bronner-Fraser M.** 1998, *Development*, pp. 125(13):2403-14.
46. *Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development.* **Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, Sommer L, Boussadia O, Kemler R.** 2001, *Development*, pp. 128(8):1253-64.
47. *The Bcl-2 family: roles in cell survival and oncogenesis.* **Cory S, Huang DC, Adams JM.,** 2003, *Oncogene*, pp. 22(53):8590-607.
48. *The BCL-2 protein family: opposing activities that mediate cell death.* **Youle RJ, Strasser A.,** 2008, *Nat Rev Mol Cell Biol*, pp. 9(1):47-59.
49. *Bcl-2-family proteins and hematologic malignancies: history and future prospects.* **Reed JC.** 2008, *lood*, pp. 111(7):3322-30.
50. *Tracking neurotrophin function.* **Davies AM.** 1994, *Nature*, pp. 368(6468):193-4.
51. *Regulation of neuronal survival and death by extracellular signals during development.* **Davies AM.** 2003, *EMBO J*, pp. 22(11):2537-45.
52. *The nerve growth factor 35 years later.* **Levi-Montalcini R.** 1987, *Science*, pp. 237(4819):1154-62.
53. *Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions.* **Hamburger V, Levi-Montalcini R.,** 1949, *J Exp Zool.*, pp. 111(3):457-501.
54. *Nerve growth factor.* **Levi-Montalcini R, Angeletti PU.,** 1968, *Physiol Rev*, pp. 48(3):534-69.
55. *Central nervous system and peripheral nerve growth factor provide trophic support critical to mature sensory neuronal survival.* **Johnson EM Jr, Yip HK.,** 1985, *Nature*, pp. 314(6013):751-2.
56. *Effects of antibodies to nerve growth factor on intrauterine development of derivatives of cranial neural crest and placode in the guinea pig.* **Pearson J, Johnson EM, Brandeis L.,** 1983, *Dev Biol*, pp. 96(1):32-6.
57. *Neurite-promoting activities for embryonic spinal neurons and their developmental changes in the chick.* **Henderson CE, Huchet M, Changeux JP.,** 1984, *Dev Biol*, pp. 104(2):336-47.
58. *Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons.* **Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD, et al.,** 1994, *Cell*, pp. 76(6):1001-11.
59. *Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene.* **Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M.,** 1994, *Nature*, pp. 368(6468):246-9.
60. *Fetal NGF augmentation preserves excess trigeminal ganglion cells and interrupts whisker-related pattern formation.* **Henderson TA, Johnson EM Jr, Osborne PA, Jacquin MF.,** 1994, *J Neurosci*, pp. 14(5 Pt 2):3389-403.

61. *Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor.* Davies AM, Bandtlow C, Heumann R, Korsching S, Rohrer H, Thoenen H., 1987, Nature, pp. 326(6111):353-8.
62. *Peripheral target regulation of the development and survival of spinal sensory and motor neurons in the chick embryo.* Calderó J, Prevetie D, Mei X, Oakley RA, Li L, Milligan C, Houenou L, Burek M, Oppenheim RW. 1998, J neurosci, pp. 18(1):356-70.
63. *Neurotrophic factors. Switching neurotrophin dependence.* Davies AM. 1994, Curr Biol, pp. 4(3):273-6.
64. *Neurotrophin switching: where does it stand?* Davies AM. 1997, Curr Opin Neurobiol, pp. 7(1):110-8.
65. *Intrinsic differences in the growth rate of early nerve fibres related to target distance.* Davies AM. 1989, Nature, pp. 337(6207):553-5.
66. *The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation.* Vogel KS, Davies AM., 1991, Neuron, pp. 7(5):819-30.
67. *Purification of a new neurotrophic factor from mammalian brain.* Barde YA, Edgar D, Thoenen H., 1982, EMBO J, pp. 1(5):549-53.
68. *Physiology of the neurotrophins.* Lewin GR, Barde YA., 1996, Annu Rev Neurosci. , pp. 19:289-317.
69. *Hepatocyte growth factor, a versatile signal for developing neurons.* Maina F, Klein R., 1999, Nat Neurosci, pp. 2(3):213-7.
70. *The GDNF family: signalling, biological functions and therapeutic value.* Airaksinen MS, Saarma M., 2002, Nat Rev Neurosci , pp. 3(5):383-94.
71. *Neurotrophins: neurotrophic modulation of neurite growth.* Davies AM. 2000, Curr Biol, pp. 10(5):R198-200.
72. *Neurotrophins and activity-dependent plasticity.* Thoenen H. 2000, Prog Brain Res, pp. 128:183-91.
73. *Multiple roles for hepatocyte growth factor in sympathetic neuron development.* Maina F, Hilton MC, Andres R, Wyatt S, Klein R, Davies AM. 1998, Neuron, pp. 20(5):835-46.
74. *Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system.* Bibel M, Barde YA., 2000, Genes Dev, pp. 14(23):2919-37.
75. *Cytokines, growth factors and sprouting at the neuromuscular junction.* English AW. 2003, J Neurocytol, pp. 32(5-8):943-60.
76. *The response of chick sensory neurons to brain-derived neurotrophic factor.* Davies AM, Thoenen H, Barde YA., 1986, J Neurosci, pp. 6(7):1897-904.
77. *Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons.* Davies AM, Horton A, Burton LE, Schmelzer C, Vandlen R, Rosenthal A. 1993, J Neurosci, pp. 13(11):4961-7.
78. *Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4.* Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, McClain J, Pan L, Helgren M, Ip NY, Boland P, et al. 1995, Nature, pp. 375(6528):235-8.
79. *Sensory but not motor neuron deficits in mice lacking NT4 and BDNF.* Liu X, Ernfors P, Wu H, Jaenisch R. 1995, Nature, pp. 375(6528):238-41.
80. *Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing.* Erickson JT, Conover JC, Borday V,

**Champagnat J, Barbacid M, Yancopoulos G, Katz DM.** 1996, *J Neurosci*, pp. 16(17):5361-71.

81. *Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 complement and cooperate with each other sequentially during visceral neuron development.* **ElShamy WM, Ernfors P.**, 1997, *J Neurosci*, pp. 17(22):8667-75.

82. *In vivo survival requirement of a subset of nodose ganglion neurons for nerve growth factor.* **Forgie A, Kuehnel F, Wyatt S, Davies AM.**, 2000, *Eur J Neurosci*, pp. 12(2):670-6.

83. *Cytokines promote the survival of mouse cranial sensory neurones at different developmental stages.* **Horton AR, Barlett PF, Pennica D, Davies AM.** 1998, *Eur J Neurosci*, pp. 10(2):673-9.

84. *Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons.* **Buchman VL, Davies AM.**, 1993, *Development*, pp. 118(3):989-1001.

85. *Regulation of expression of mRNAs encoding the nerve growth factor receptors p75 and trkA in developing sensory neurons.* **Wyatt S, Davies AM.**, 1993, *Development*, pp. 119(3):635-48.

86. *Expression and function of TrkB variants in developing sensory neurons.* **Ninkina N, Adu J, Fischer A, Piñón LG, Buchman VL, Davies AM.** 1996, *EMBO J*, pp. 15(23):6385-93.

87. *Multiple effects of artemin on sympathetic neurone generation, survival and growth.* **Andres R, Forgie A, Wyatt S, Chen Q, de Sauvage FJ, Davies AM.** 2001, *Development*, pp. 128(19):3685-95.

88. *Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFR $\alpha$ 3-RET receptor complex.* **Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson EM Jr, Milbrandt J.** 1998, *Neuron*, pp. 21(6):1291-302.

89. *Regulation of nerve growth factor receptor gene expression in sympathetic neurons during development.* **Wyatt S, Davies AM.**, 1995, *J Cell Biol*, pp. 130(6):1435-46.

90. *Sympathetic neuron survival and TrkA expression in NT3-deficient mouse embryos.* **Wyatt S, Piñón LG, Ernfors P, Davies AM.**, 1997, *EMBO J*, pp. 16(11):3115-23.

91. *Postnatal development of survival responsiveness in rat sympathetic neurons to leukemia inhibitory factor and ciliary neurotrophic factor.* **Kotzbauer PT, Lampe PA, Estus S, Milbrandt J, Johnson EM Jr.** 1994, *Neuron*, pp. 12(4):763-73.

92. *HGF promotes survival and growth of maturing sympathetic neurons by PI-3 kinase- and MAP kinase-dependent mechanisms.* **Thompson J, Dolcet X, Hilton M, Tolcos M, Davies AM.**, 2004, *Mol Cell Neurosci*, pp. 27(4):441-52.

93. *A nerve growth-stimulating factor isolated from sarcomas 37 and 180.* **Cohen S, Levi-Montalcini R, Hamburger V.**, 1954, *Proc Natl Acad Sci U S A.*, pp. 40(10):1014-8.

94. *In vitro and in vivo effects of a nerve growth-stimulating agents isolated from snake venom.* **Levi-Montalcini R, Cohen S.**, 1956, *Proc Natl Acad Sci U S A.*, pp. 42(9):695-9.

95. *Nerve Growth Factor from Mouse Submaxillary Gland: Amino Acid Sequence.* **Angeletti RH, Bradshaw RA.**, 1971, *Proc Natl Acad Sci U S A*, pp. 68(10): 2417-2420.

96. *New protein fold revealed by a 2.3-Å resolution crystal structure of nerve growth factor.* **McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL.** 1991, *Nature*, pp. 354(6352):411-4.

97. *Nerve growth factor signaling, neuroprotection, and neural repair.* **Sofroniew MV, Howe CL, Mobley WC.**, 2001, *Annu Rev Neurosci*, pp. 24:1217-81.

98. *NGF effects on developing forebrain cholinergic neurons are regionally specific.*

- Johnston MV, Rutkowski JL, Wainer BH, Long JB, Mobley WC.,** 1987, *Neurochem Res*, pp. 12(11):985-94.
99. *Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues.* **Heumann R, Korsching S, Scott J, Thoenen H.,** 1984, *EMBO J*, pp. 3(13):3183-9.
100. *Pleiotropic functions of neurotrophins in development.* **Tessarollo L.** 1998, *Cytokine Growth Factor Rev*, pp. 9(2):125-37.
101. *Development of sensory neurons in the absence of NGF/TrkA signaling in vivo.* **Patel TD, Jackman A, Rice FL, Kucera J, Snider WD.,** 2000, *Neuron*, pp. 25(2):345-57.
102. *Heterogeneous requirement of NGF for sympathetic target innervation in vivo.* **Glebova NO, Ginty DD.,** 2004, *J Neurosci.* , pp. 24(3):743-51.
103. *Regulation of cell survival by secreted proneurotrophins.* **Lee R, Kermani P, Teng KK, Hempstead BL.,** 2001, *Science*, pp. 294(5548):1945-8.
104. *Immunohistochemical localization of brain-derived neurotrophic factor in adult rat brain.* **Kawamoto Y, Nakamura S, Nakano S, Oka N, Akiguchi I, Kimura J.,** 1996, *Neuroscience*, pp. 74(4):1209-26.
105. *Physiology of BDNF: focus on hypothalamic function.* **Tapia-Arancibia L, Rage F, Givalois L, Arancibia S.** 2004, *Front Neuroendocrinol*, pp. 25(2):77-107.
106. *Neurotrophin-evoked rapid excitation through TrkB receptors.* **Kafitz KW, Rose CR, Thoenen H, Konnerth A.,** 1999, *Nature*, pp. 401(6756):918-21.
107. *Neurotrophins and hippocampal synaptic transmission and plasticity.* **Lu B, Chow A.,** 1999, *J Neurosci Res*, pp. 58(1):76-87.
108. *BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis.* **Bramham CR, Messaoudi E.,** 2005, *Prog Neurobiol*, pp. 76(2):99-125.
109. *BDNF and the diseased nervous system: a delicate balance between adaptive and pathological processes of gene regulation.* **Hu Y, Russek SJ.,** 2008, *J Neurochem*, pp. 105(1):1-17.
110. *NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression.* **Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM, Yancopoulos GD.** 1990, *Neuron*, pp. 5(4):501-9.
111. *Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons.* **Schecterson LC, Bothwell M.** 1992, *Neuron*, pp. 9(3):449-63.
112. *Differences and similarities in the neurotrophic growth factor requirements of sensory neurons derived from neural crest and neural placode.* **Lindsay RM, Barde YA, Davies AM, Rohrer H.,** 1985, *J Cell Sci Suppl*, pp. 3:115-29.
113. *Different factors from the central nervous system and periphery regulate the survival of sensory neurones.* **Davies AM, Thoenen H, Barde YA.,** 1986, *Nature*, pp. 319(6053):497-9.
114. *Brain derived neurotrophic factor.* **Barde YA, Davies AM, Johnson JE, Lindsay RM, Thoenen H.,** 1987, *Prog Brain Res*, pp. 71:185-9.
115. *Brain-derived neurotrophic factor prevents neuronal death in vivo.* **Hofer MM, Barde YA.,** 1988, *Nature*, pp. 331(6153):261-2.
116. *Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development.* **Jones KR, Fariñas I, Backus C, Reichardt LF.,** 1994, *Cell*, pp. 76(6):989-99.
117. *Mice lacking brain-derived neurotrophic factor develop with sensory deficits.* **Ernfors P, Lee KF, Jaenisch R.,** 1994, *Nature*, pp. 368(6467):147-50.

118. *Axonal outgrowth from adult mouse nodose ganglia in vitro is stimulated by neurotrophin-4 in a Trk receptor and mitogen-activated protein kinase-dependent way.* Wiklund P, Ekström PA., 2000, *J Neurobiol*, pp. 45(3):142-51.
119. *Neurotrophins are required for nerve growth during development.* Tucker KL, Meyer M, Barde YA. 2001, *Nat Neurosci*, pp. 4(1):29-37.
120. *Neurotrophins support the development of diverse sensory axon morphologies.* Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD., 1999, *J Neurosci*, pp. 19(3):1038-48.
121. *Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation.* Kohn J, Aloyz RS, Toma JG, Haak-Frendscho M, Miller FD., 1999, *J Neurosci*, pp. 19(13):5393-408.
122. *Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons.* Paves H, Saarma M., 1997, *Cell Tissue Res*, pp. 290(2):285-97.
123. *Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons.* Postigo A, Calella AM, Fritzscht B, Knipper M, Katz D, Eilers A, Schimmang T, Lewin GR, Klein R, Minichiello L. 2002, *Genes Dev*, pp. 16(5):633-45.
124. *Developing inner ear sensory neurons require TrkB and TrkC receptors for innervation of their peripheral targets.* Schimmang T, Minichiello L, Vazquez E, San Jose I, Giraldez F, Klein R, Represa J. 1995, *Development*, pp. 121(10):3381-91.
125. *The role of neurotrophic factors in regulating the development of inner ear innervation.* Fritzscht B, Silos-Santiago I, Bianchi LM, Fariñas I. 1997, *Trends Neurosci*, pp. 20(4):159-64.
126. *Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth.* McAllister AK, Katz LC, Lo DC., 1997, *Neuron*, pp. 18(5):767-78.
127. *Accelerated dendritic development of rat cortical pyramidal cells and interneurons after biolistic transfection with BDNF and NT4/5.* Wirth MJ, Brun A, Grabert J, Patz S, Wahle P. 2003, *Development*, pp. 130(23):5827-38.
128. *Inhibitory but not excitatory cortical neurons require presynaptic brain-derived neurotrophic factor for dendritic development, as revealed by chimera cell culture.* Kohara K, Kitamura A, Adachi N, Nishida M, Itami C, Nakamura S, Tsumoto T. 2003, *J Neurosci*, pp. 23(14):6123-31.
129. *Brain-derived neurotrophic factor mediates activity-dependent dendritic growth in nonpyramidal neocortical interneurons in developing organotypic cultures.* Jin X, Hu H, Mathers PH, Agmon A., 2003, *J Neurosci*, pp. 23(13):5662-73.
130. *Contact-associated neurite outgrowth and branching of immature cortical interneurons.* Sang Q, Tan SS., 2003, *Cereb Cortex*, pp. 13(6):677-83.
131. *BDNF release from single cells elicits local dendritic growth in nearby neurons.* Horch HW, Katz LC. 2002, *Nat Neurosci*, pp. 5(11):1177-84.
132. *Destabilization of cortical dendrites and spines by BDNF.* Horch HW, Krüttgen A, Portbury SD, Katz LC. 1999, *Neuron*, pp. 23(2):353-64.
133. *The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB.* Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF. 2000, *J Neurosci*, pp. 20(18):6888-97.
134. *A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences.* Martin-Zanca D, Hughes SH, Barbacid M. 1986, *Nature*, pp. 319(6056):743-8.
135. *Molecular and biochemical characterization of the human trk proto-oncogene.* Martin-Zanca

- D, Oskam R, Mitra G, Copeland T, Barbacid M. 1989, *Mol Cell Biol.*, pp. 9(1):24-33.
136. *Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF.* Kaplan DR, Martin-Zanca D, Parada LF. 1991, *Nature*, pp. 350(6314):158-60.
137. *The trk proto-oncogene product: a signal transducing receptor for nerve growth factor.* Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. 1991, *Science*, pp. 252(5005):554-8.
138. *The trk proto-oncogene encodes a receptor for nerve growth factor.* Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. 1991, *Cell*, pp. 65(1):189-97.
139. *High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor.* Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. 1991, *Nature*, pp. 350(6320):678-83.
140. *trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development.* Klein R, Parada LF, Coulier F, Barbacid M. 1989, *EMBO J*, pp. 8(12):3701-9.
141. *The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain.* Klein R, Conway D, Parada LF, Barbacid M. 1990, *Cell*, pp. 61(4):647-56.
142. *trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors.* Middlemas DS, Lindberg RA, Hunter T. 1991, *Mol Cell Biol.*, pp. 11(1):143-53.
143. *Developmental expression of trkC, the neurotrophin-3 receptor, in the mammalian nervous system.* Lamballe F, Smeyne RJ, Barbacid M. 1994, *J Neurosci*, pp. 14(1):14-28.
144. *The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor.* Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, et al. 1991, *Cell*, pp. 65(5):895-903.
145. *trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor.* Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, et al. 1991, *Cell*, pp. 65(5):885-93.
146. *Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB.* Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A. 1991, *Neuron*, pp. 7(5):857-66.
147. *The trkB tyrosine protein kinase is a receptor for neurotrophin-4.* Klein R, Lamballe F, Bryant S, Barbacid M. 1992, *Neuron*, pp. 8(5):947-56.
148. *Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity.* Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa R 3rd, Squinto SP, et al. 1992, *Proc Natl Acad Sci U S A.*, pp. 89(7):3060-4.
149. *trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3.* Lamballe F, Klein R, Barbacid M. 1991, *Cell*, pp. 66(5):967-79.
150. *trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues.* Tessarollo L, Tsoulfas P, Martin-Zanca D, Gilbert DJ, Jenkins NA, Copeland NG, Parada LF. 1993, *Development*, pp. 118(2):463-75.
151. *Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development.* Martin-Zanca D, Barbacid M, Parada LF. 1990, *Genes Dev.*, pp. 4(5):683-94.
152. *Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation in utero.* Carroll SL, Silos-Santiago I, Frese SE,

- Ruit KG, Milbrandt J, Snider WD. 1992, *Neuron*, pp. 9(4):779-88.
153. *Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death.* Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M., 1993, *Cell*, pp. 75(1):113-22.
154. *Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements.* Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M. 1994, *Nature*, pp. 368(6468):249-51.
155. *Expression and structure of the human NGF receptor.* Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M., 1986, *Cell*, pp. ;47(4):545-54.
156. *Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor.* Rodríguez-Tébar A, Dechant G, Götz R, Barde YA. 1992, *EMBO J*, pp. 11(3):917-22.
157. *Neurotrophin signaling through the p75 neurotrophin receptor.* Roux PP, Barker PA. 2002, *Prog Neurobiol.* , pp. 67(3):203-33.
158. *Regulation of cell survival by secreted proneurotrophins.* Lee R, Kermani P, Teng KK, Hempstead BL. 2001, *Science*, pp. 294(5548):1945-8.
159. *Sortilin is essential for proNGF-induced neuronal cell death.* Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM. 2004, *Nature*, pp. 427(6977):843-8.
160. *ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin.* Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL. 2005, *J Neurosci*, pp. 25(22):5455-63.
161. *Induction of apoptosis by the low-affinity NGF receptor.* Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LL, Bredesen DE. 1993, *Science*, pp. 261(5119):345-8.
162. *The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development.* Barrett GL, Bartlett PF. 1994, *Proc Natl Acad Sci U S A.* , pp. 91(14):6501-5.
163. *Nerve growth factor: two receptors, multiple functions.* Frade JM, Barde YA., 1998, *Bioessays.* , pp. 20(2):137-45.
164. *p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor.* Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM., 1999, *Mol Cell Neurosci.* , pp. 14(1):28-40.
165. *Developmental mechanisms that generate precise patterns of neuronal connectivity.* Goodman CS, Shatz CJ., 1993, *Cell*, pp. 72 Suppl:77-98.
166. *Guidance of neuronal growth cones: selective fasciculation in the grasshopper embryo.* Raper JA, Bastiani MJ, Goodman CS., 1983, *Cold Spring Harb Symp Quant Biol.* , pp. 48 Pt 2:587-98.
167. *Growth cone behavior on gradients of substratum bound laminin.* McKenna MP, Raper JA., 1988, *Dev Biol.* , pp. 130(1):232-6.
168. *Axon guidance: receptor complexes and signaling mechanisms.* Grunwald IC, Klein R., 2002, *Curr Opin Neurobiol*, pp. 12(3):250-9.
169. *Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance.* Charron F, Tessier-Lavigne M. 2005, *Development*, pp. 132(10):2251-62.
170. *A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling.* Kuruvilla R, Zweifel

- LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD. 2004, *Cell*, pp. 118(2):243-55.
171. *Development of sympathetic neurons in compartmentalized cultures. II Local control of neurite growth by nerve growth factor.* Campenot RB. 1982, *Dev Biol*, pp. 93(1):1-12.
172. *Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor.* Gundersen RW, Barrett JN., 1979, *Science*, pp. 206(4422):1079-80.
173. *cAMP-induced switching in turning direction of nerve growth cones.* Song HJ, Ming GL, Poo MM., 1997, *Nature*, pp. 388(6639):275-9.
174. *Subplate neurons: a missing link among neurotrophins, activity, and ocular dominance plasticity?* McAllister AK. 1999, *Proc Natl Acad Sci U S A.* , pp. 96(24):13600-2.
175. *BAX is required for neuronal death after trophic factor deprivation and during development.* Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ., 1996, *Neuron*, pp. 17(3):401-11.
176. *Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system.* Albers KM, Wright DE, Davis BM., 1994, *J Neurosci.* , pp. 14(3 Pt 2):1422-32.
177. *Cutaneous overexpression of NT-3 increases sensory and sympathetic neuron number and enhances touch dome and hair follicle innervation.* Albers KM, Perrone TN, Goodness TP, Jones ME, Green MA, Davis BM., 1996, *J Cell Biol.* , pp. 134(2):487-97.
178. *Expression of NGF in sympathetic neurons leads to excessive axon outgrowth from ganglia but decreased terminal innervation within tissues.* Hoyle GW, Mercer EH, Palmiter RD, Brinster RL., 1993, *Neuron*, pp. 10(6):1019-34.
179. *Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase.* Hetman M, Kanning K, Cavanaugh JE, Xia Z., 1999, *J Biol Chem.* , pp. 274(32):22569-80.
180. *The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase.* Atwal JK, Massie B, Miller FD, Kaplan DR. 2000, *Neuron*, pp. 27(2):265-77.
181. *Brain-derived neurotrophic factor-mediated neuroprotection of adult rat retinal ganglion cells in vivo does not exclusively depend on phosphatidylinositol-3'-kinase/protein kinase B signaling.* Klöcker N, Kermer P, Weishaupt JH, Labes M, Ankerhold R, Bähr M. 2000, *J Neurosci*, pp. 20(18):6962-7.
182. *Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway.* Brunet A, Datta SR, Greenberg ME., 2001, *Curr Opin Neurobiol.* , pp. 11(3):297-305.
183. *Neurotrophins use the Erk5 pathway to mediate a retrograde survival response.* Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA., 2001, *Nat Neurosci.* , pp. 4(10):981-8.
184. *Brain-derived neurotrophic factor prevents axotomized retinal ganglion cell death through MAPK and PI3K signaling pathways.* Nakazawa T, Tamai M, Mori N. 2002, *Invest Ophthalmol Vis Sci.* , pp. 43(10):3319-26.
185. *Neurotrophins elevate cAMP to reach a threshold required to overcome inhibition by MAG through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase.* Gao Y, Nikulina E, Mellado W, Filbin MT. 2003, *J Neurosci.* , pp. 23(37):11770-7.
186. *Characterization of a neurotrophin signaling mechanism that mediates neuron survival in a temporally specific pattern.* Shalizi A, Lehtinen M, Gaudilliere B, Donovan N, Han J, Konishi Y, Bonni A. 2003, *J Neurosci.*, pp. 23(19):7326-36.
187. *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Datta SR, Dudek H, Tao X, Masters S, Fu H,



- Gotoh Y, Greenberg ME. 1997, *Cell*, pp. 91(2):231-41.
188. *Evidence for and against a pivotal role of PI 3-kinase in a neuronal cell survival pathway.* Williams EJ, Doherty P., 1999, *Mol Cell Neurosci.* , pp. 13(4):272-80.
189. *Regulation of cell death protease caspase-9 by phosphorylation.* Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. 1998, *Science*, pp. 282(5392):1318-21.
190. *Cyclic AMP elevation is sufficient to promote the survival of spinal motor neurons in vitro.* Hanson MG Jr, Shen S, Wiemelt AP, McMorris FA, Barres BA. 1998, *J Neurosci.* , pp. 18(18):7361-71.
191. *Distinct neurite outgrowth signaling pathways converge on ERK activation.* Perron JC, Bixby JL. 1999, *Mol Cell Neurosci.* , pp. 13(5):362-78.
192. *AKT/PKB signaling: navigating downstream.* Manning BD, Cantley LC., s.l.: Cell, 2007, Vols. 129(7):1261-74.
193. *Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor.* Yao R, Cooper GM., 1995, *Science*, pp. 267(5206):2003-6.
194. *Neurotrophin signal transduction in the nervous system.* Kaplan DR, Miller FD., 2000, *Curr Opin Neurobiol.* , pp. 10(3):381-91.
195. *The synergistic effects of NGF and IGF-1 on neurite growth in adult sensory neurons: convergence on the PI 3-kinase signaling pathway.* Jones DM, Tucker BA, Rahimtula M, Mearow KM. 2003, *J Neurochem.*, pp. 86(5):1116-28.
196. *The MAP kinase pathway is upstream of the activation of GSK3beta that enables it to phosphorylate MAP1B and contributes to the stimulation of axon growth.* Goold RG, Gordon-Weeks PR. 2005, *Mol Cell Neurosci.* , pp. 28(3):524-34.
197. *Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway.* Skaper SD, Floreani M, Negro A, Facci L, Giusti P. 1998, *J Neurochem.* , pp. 70(5):1859-68.
198. *Activation of phosphatidylinositol 3-kinase, but not extracellular-regulated kinases, is necessary to mediate brain-derived neurotrophic factor-induced motoneuron survival.* Dolcet X, Egea J, Soler RM, Martin-Zanca D, Comella JX. 1999, *J Neurochem.* , pp. 73(2):521-31.
199. *Early BDNF, NT-3, and NT-4 signaling events.* Yuen EC, Mobley WC. 1999, *Exp Neurol.* , pp. 159(1):297-308.
200. *Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome.* Nusse R, Varmus HE., 1982, *Cell*, pp. 31(1):99-109.
201. *Localization of transcripts from the wingless gene in whole Drosophila embryos.* Baker NE. 1988, *Development*, pp. 103(2):289-98.
202. *Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite.* McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP. 1998, *Genes Dev.*, pp. 12(10):1438-52.
203. *Retrograde signalling at the synapse: a role for Wnt proteins.* Salinas PC. 2005, *Biochem Soc Trans.* , pp. 33(Pt 6):1295-8.
204. *A new member of the frizzled family from Drosophila functions as a Wingless receptor.* Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. 1996, *Nature*, pp. 382(6588):225-30.
205. *Runnin' with the Dvl: proteins that associate with Dsh/Dvl and their significance to Wnt signal transduction.* Wharton KA Jr. 2003, *Dev Biol.* , pp. 253(1):1-17.

206. *Wnt-mediated axon guidance via the Drosophila Derailed receptor.* Yoshikawa S, McKinnon RD, Kokel M, Thomas JB. 2003, Nature, pp. 422(6932):583-8.
207. *Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth.* Lu W, Yamamoto V, Ortega B, Baltimore D. 2004, Cell, pp. 119(1):97-108.
208. *C. elegans LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling.* Inoue T, Oz HS, Wiland D, Gharib S, Deshpande R, Hill RJ, Katz WS, Sternberg PW. 2004, Cell, pp. 118(6):795-806.
209. *Dishevelled-1 regulates microtubule stability: a new function mediated by glycogen synthase kinase-3beta.* Krylova O, Messenger MJ, Salinas PC. 2000, J Cell Biol, p. J Cell Biol.
210. *A divergent canonical WNT-signaling pathway regulates microtubule dynamics: dishevelled signals locally to stabilize microtubules.* Ciani L, Krylova O, Smalley MJ, Dale TC, Salinas PC. 2004, J Cell Biol, pp. 164(2):243-53.
211. *The Wnt signaling pathway in development and disease.* Logan CY, Nusse R., 2004, Annu Rev Cell Dev Biol., pp. 20:781-810.
212. *Wnt signaling in disease and in development.* Nusse R. 2005, Cell Res, pp. 15(1):28-32.
213. *Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway.* Mao J, Wang J, Liu B, Pan W, Farr GH 3rd, Flynn C, Yuan H, Takada S, Kimelman D, Li L, Wu D. 2001, Mol Cell., pp. 7(4):801-9.
214. *Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow.* Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. 2001, Nat Cell Biol., pp. 3(7):683-6.
215. *LDL-receptor-related proteins in Wnt signal transduction.* Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. 2000, Nature, pp. 407(6803):530-5.
216. *arrow encodes an LDL-receptor-related protein essential for Wingless signalling.* Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, Vaizel-Ohayon D, Schejter E, Tomlinson A, DiNardo S. 2000, Nature, pp. 407(6803):527-30.
217. *An LDL-receptor-related protein mediates Wnt signalling in mice.* Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC., 2000, Nature, pp. 407(6803):535-8.
218. *Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling.* Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, Glinka A, Niehrs C. 2002, Nature, pp. 417(6889):664-7.
219. *Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity.* Tolwinski NS, Wehrli M, Rives A, Erdeniz N, DiNardo S, Wieschaus E. 2003, Dev Cell, pp. 4(3):407-18.
220. *New aspects of Wnt signaling pathways in higher vertebrates.* Huelsken J, Birchmeier W., 2001, Curr Opin Genet Dev., pp. 11(5):547-53.
221. *The role of RhoA in tissue polarity and Frizzled signalling.* Strutt DI, Weber U, Mlodzik M. 1997, Nature, pp. 387(6630):292-5.
222. *Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation.* Heisenberg CP, Tada M, Rauch GJ, Saude L, Concha ML, Geisler R, Stemple DL, Smith JC, Wilson SW. 2000, Nature, pp. 405(6782):76-81.
223. *Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea.* Dabdoub A, Donohue MJ, Brennan A, Wolf V, Montcouquiol M, Sassoon DA, Hsieh JC, Rubin JS, Salinas PC, Kelley MW. 2003, Development, pp. 130(11):2375-84.

224. *Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation.* Habas R, Dawid IB, He X. 2003, Genes Dev. , pp. 17(2):295-309.
225. *The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos.* Saneyoshi T, Kume S, Amasaki Y, Mikoshiba K. 2002, Nature, pp. 417(6886):295-9.
226. *Zebrafish prickles, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements.* Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. 2003, Curr Biol. , pp. 13(8):680-5.
227. *Dishevelled activates Ca<sup>2+</sup> flux, PKC, and CamKII in vertebrate embryos.* Sheldahl LC, Slusarski DC, Pandur P, Miller JR, Kühl M, Moon RT. 2003, J Cell Biol. , pp. 161(4):769-77.
228. *The Wnt/Ca<sup>2+</sup> pathway: a new vertebrate Wnt signaling pathway takes shape.* Kühl M, Sheldahl LC, Park M, Miller JR, Moon RT. 2000, Trends Genet. , pp. 16(7):279-83.
229. *G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway.* Liu T, DeCostanzo AJ, Liu X, Wang Hy , Hallagan S, Moon RT, Malbon CC. 2001, Science, pp. 292(5522):1718-22.
230. *Trimeric G protein-dependent frizzled signaling in Drosophila.* Katanaev VL, Ponzelli R, Sémériva M, Tomlinson A. 2005, Cell, pp. 120(1):111-22.
231. *Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling.* Slusarski DC, Corces VG, Moon RT. 1997, Nature, pp. 390(6658):410-3.
232. *Balancing cell adhesion and Wnt signaling, the key role of beta-catenin.* Brembeck FH, Rosário M, Birchmeier W. 2006, Curr Opin Genet Dev. , pp. 16(1):51-9.
233. *Two sides of the same coin: Wnt signaling in neurodegeneration and neuro-oncology.* Caricasole A, Bakker A, Copani A, Nicoletti F, Gaviraghi G, Terstappen GC. 2005, Biosci Rep. , pp. 25(5-6):309-27.
234. *Axonal growth: where neurotrophins meet Wnts.* Arévalo JC, Chao MV., 2005, Curr Opin Cell Biol. , pp. 17(2):112-5.
235. *The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain.* McMahon AP, Bradley A., 1990, Cell, pp. 62(6):1073-85.
236. *Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a.* Castelo-Branco G, Wagner J, Rodriguez FJ, Kele J, Sousa K, Rawal N, Pasolli HA, Fuchs E, Kitajewski J, Arenas E. 2003, Proc Natl Acad Sci U S A. , pp. 100(22):12747-52.
237. *Wnt signalling required for expansion of neural crest and CNS progenitors.* Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S. 1997, Nature, pp. 389(6654):966-70.
238. *A local Wnt-3a signal is required for development of the mammalian hippocampus.* Lee SM, Tole S, Grove E, McMahon AP. 2000, Development, pp. 127(3):457-67.
239. *Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling.* Hall AC, Lucas FR, Salinas PC. 2000, Cell, pp. 100(5):525-35.
240. *The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells.* Hirabayashi Y, Itoh Y, Tabata H, Nakajima K, Akiyama T, Masuyama N, Gotoh Y. 2004, Development, pp. 131(12):2791-801.
241. *Frizzled-3 is required for the development of major fiber tracts in the rostral CNS.* Wang Y, Thekdi N, Smallwood PM, Macke JP, Nathans J. 2002, J Neurosci. , pp. 22(19):8563-73.
242. *Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling.* Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, Wang

- Y, Nathans J, Tessier-Lavigne M, Zou Y. 2003, Science, pp. 302(5652):1984-8.
243. *Progressive cerebellar, auditory, and esophageal dysfunction caused by targeted disruption of the frizzled-4 gene.* Wang Y, Huso D, Cahill H, Ryugo D, Nathans J. 2001, J Neurosci. , pp. 21(13):4761-71.
244. *Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1.* Galceran J, Miyashita-Lin EM, Devaney E, Rubenstein JL, Grosschedl R. 2000, Development, pp. 127(3):469-82.
245. *Regulation of cerebral cortical size by control of cell cycle exit in neural precursors.* Chenn A, Walsh CA., 2002, Science, pp. 297(5580):365-9.
246. *beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system.* Zechner D, Fujita Y, Hülsken J, Müller T, Walther I, Taketo MM, Crenshaw EB 3rd, Birchmeier W, Birchmeier C. 2003, Dev Biol. , pp. 258(2):406-18.
247. *WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons.* Lucas FR, Salinas PC., 1997, Dev Biol. , pp. 192(1):31-44.
248. *Synaptogenesis: Wnt and TGF-beta take centre stage.* Salinas PC. 2003, Curr Biol. , pp. 13(2):R60-2.
249. *Beta-catenin is critical for dendritic morphogenesis.* Yu X, Malenka RC. 2003, Nat Neurosci. , pp. 6(11):1169-77.
250. *Expression of stabilized beta-catenin in differentiated neurons of transgenic mice does not result in tumor formation.* Kratz JE, Stearns D, Huso DL, Slunt HH, Price DL, Borchelt DR, Eberhart CG. 2002, BMC Cancer. , p. 2:33.
251. *Wnt factors in axonal remodelling and synaptogenesis.* Salinas PC. 1999, Biochem Soc Symp. , pp. 65:101-9.
252. *Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function.* Murase S, Mosser E, Schuman EM. 2002, Neuron, pp. 35(1):91-105.
253. *Convergence of Wnt, beta-catenin, and cadherin pathways.* Nelson WJ, Nusse R. 2004, Science, pp. 303(5663):1483-7.
254. *A divergent canonical WNT-signaling pathway regulates microtubule dynamics: dishevelled signals locally to stabilize microtubules.* Ciani L, Krylova O, Smalley MJ, Dale TC, Salinas PC. 2004, J Cell Biol. , pp. 164(2):243-53.
255. *Dishevelled regulates the metabolism of amyloid precursor protein via protein kinase C/mitogen-activated protein kinase and c-Jun terminal kinase.* Mudher A, Chapman S, Richardson J, Asuni A, Gibb G, Pollard C, Killick R, Iqbal T, Raymond L, Varndell I, Sheppard P, Makoff A, Gower E, Soden PE, Lewis P, Murphy M, Golde TE, Rupniak HT, Anderton BH, Lovestone S. 2001, J Neurosci., pp. 21(14):4987-95.
256. *A molecular mechanism for the effect of lithium on development.* Klein PS, Melton DA. 1996, Proc Natl Acad Sci U S A. , pp. 93(16):8455-9.
257. *Activation of the Wnt signaling pathway: a molecular mechanism for lithium action.* Hedgepeth CM, Conrad LJ, Zhang J, Huang HC, Lee VM, Klein PS. 1997, Dev Biol. , pp. 185(1):82-91.
258. *Neuroprotective effects of lithium in cultured cells and animal models of diseases.* Chuang DM, Chen RW, Chalecka-Franaszek E, Ren M, Hashimoto R, Senatorov V, Kanai H, Hough C, Hiroi T, Leeds P. 2002, Bipolar Disord. , pp. 4(2):129-36.
259. *Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3.* Zhang F, Phiel CJ, Spece L, Gurvich N, Klein PS. 2003, J Biol Chem. , pp. 278(35):33067-77.

260. *Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium.* O'Brien WT, Harper AD, Jové F, Woodgett JR, Maretto S, Piccolo S, Klein PS. 2004, J Neurosci., pp. 24(30):6791-8.
261. *Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice.* Lucas JJ, Hernández F, Gómez-Ramos P, Morán MA, Hen R, Avila J. 2001, EMBO J. , pp. 20(1-2):27-39.
262. *Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain.* Caricasole A, Copani A, Caraci F, Aronica E, Rozemuller AJ, Caruso A, Storto M, Gaviraghi G, Terstappen GC, Nicoletti F. 2004, J Neurosci. , pp. 24(26):6021-7.
263. *The role of GSK3beta in regulating neuronal differentiation in Xenopus laevis.* Marcus EA, Kintner C, Harris W. 1998, Mol Cell Neurosci., pp. 12(4-5):269-80.
264. *Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal.* Hetman M, Cavanaugh JE, Kimelman D, Xia Z. 2000, J Neurosci. , pp. 20(7):2567-74.
265. *Wnt signaling involvement in beta-amyloid-dependent neurodegeneration.* Inestrosa N, De Ferrari GV, Garrido JL, Alvarez A, Olivares GH, Barría MI, Bronfman M, Chacón MA. 2002, Neurochem Int. , pp. 41(5):341-4.
266. *Wnt-3a overcomes beta-amyloid toxicity in rat hippocampal neurons.* Alvarez AR, Godoy JA, Mullendorff K, Olivares GH, Bronfman M, Inestrosa NC. 2004, Exp Cell Res., pp. 297(1):186-96.
267. *Trolox and 17beta-estradiol protect against amyloid beta-peptide neurotoxicity by a mechanism that involves modulation of the Wnt signaling pathway.* Quintanilla RA, Muñoz FJ, Metcalfe MJ, Hitschfeld M, Olivares G, Godoy JA, Inestrosa NC. 2005, J Biol Chem. , pp. 280(12):11615-25.
268. *The mouse Wnt-1 gene can act via a paracrine mechanism in transformation of mammary epithelial cells.* Jue SF, Bradley RS, Rudnicki JA, Varmus HE, Brown AM. 1992, Mol Cell Biol. , pp. 12(1):321-8.
269. *Differential transformation of mammary epithelial cells by Wnt genes.* Wong GT, Gavin BJ, McMahon AP. 1994, Mol Cell Biol. , pp. 14(9):6278-86.
270. *Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue.* Liang H, Chen Q, Coles AH, Anderson SJ, Pihan G, Bradley A, Gerstein R, Jurecic R, Jones SN. 2003, Cancer Cell. , pp. 4(5):349-60.
271. *Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma.* Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM. 2002, Cancer Cell., pp. 1(3):279-88.
272. *Expression of WNT10A in human cancer.* Kirikoshi H, Inoue S, Sekihara H, Katoh M. 2001, Int J Oncol. , pp. 19(5):997-1001.
273. *Expression of WNT14 and WNT14B mRNAs in human cancer, up-regulation of WNT14 by IFNgamma and up-regulation of WNT14B by beta-estradiol.* Kirikoshi H, Sekihara H, Katoh M. 2001, Int J Oncol. , pp. 19(6):1221-5.
274. *Expression and regulation of WNT8A and WNT8B mRNAs in human tumor cell lines: up-regulation of WNT8B mRNA by beta-estradiol in MCF-7 cells, and down-regulation of WNT8A and WNT8B mRNAs by retinoic acid in NT2 cells.* Saitoh T, Mine T, Katoh M. 2002, Int J Oncol. , pp. 20(5):999-1003.
275. *TGF-beta signalling from cell membrane to nucleus through SMAD proteins.* Heldin CH, Miyazono K, ten Dijke P. 1997, Nature, pp. 390(6659):465-71.

276. *Transcriptional control by the TGF-beta/Smad signaling system.* Massagué J, Wotton D. 2000, EMBO J. , pp. 19(8):1745-54.
277. *Bidirectional regulation of macrophage function by TGF-beta.* Ashcroft GS. 1999, Microbes Infect. , pp. 1(15):1275-82.
278. *TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells.* Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E., 1996, Genes Dev., pp. 10(19):2462-77.
279. *Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction.* Roberts AB, Lamb LC, Newton DL, Sporn MB, De Larco JE, Todaro GJ. 1980, Proc Natl Acad Sci U S A., pp. 77(6):3494-8.
280. *Transforming growth factor production by chemically transformed cells.* Moses HL, Branum EL, Proper JA, Robinson RA. 1981, Cancer Res. , pp. 41(7):2842-8.
281. *Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer.* Siegel PM, Massagué J. 2003, Nat Rev Cancer. , pp. 3(11):807-21.
282. *The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer.* Mishra L, Shetty K, Tang Y, Stuart A, Byers SW. 2005, Oncogene , pp. 24(37):5775-89.
283. *Inhibition of TGFbeta signaling in cancer therapy.* Arteaga CL. 2006, Curr Opin Genet Dev. , pp. 16(1):30-7.
284. *TGFbeta signaling: receptors, transducers, and Mad proteins.* Massagué J. 1996, Cell, pp. 85(7):947-50.
285. *Bone morphogenetic protein receptors.* Yamashita H, Ten Dijke P, Heldin CH, Miyazono K. 1996, Bone, pp. 19(6):569-74.
286. *Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins.* Miyazono K. 1999, Bone, pp. 25(1):91-3.
287. *Bone morphogenetic proteins in the nervous system.* Mehler MF, Mabie PC, Zhang D, Kessler JA. 1997, Trends Neurosci. , pp. 20(7):309-17.
288. *Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5.* Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, Yamashita H, Enomoto S, Miyazono K. 1996, J Biol Chem. , pp. 271(35):21345-52.
289. *A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family.* Padgett RW, St Johnston RD, Gelbart WM. 1987, Nature, pp. 325(6099):81-4.
290. *Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans.* Herpin A, Lelong C, Favrel P. 2004, Dev Comp Immunol., pp. 28(5):461-85.
291. *The TGF-beta-related DVR gene family in mammalian development.* Lyons KM, Jones CM, Hogan BL. 1992, Ciba Found Symp. , pp. 165:219-30.
292. *Generation of cerebellar granule neurons in vivo by transplantation of BMP-treated neural progenitor cells.* Alder J, Lee KJ, Jessell TM, Hatten ME. 1999, Nat Neurosci. , pp. 2(6):535-40.
293. *Genetic analyses demonstrate that bone morphogenetic protein signaling is required for embryonic cerebellar development.* Qin L, Wine-Lee L, Ahn KJ, Crenshaw EB 3rd. 2006, J Neurosci. , pp. 26(7):1896-905.
294. *Bone morphogenetic protein-2 acts synergistically with transforming growth factor-beta3 during endothelial-mesenchymal transformation in the developing chick heart.* Yamagishi T, Nakajima Y, Miyazono K, Nakamura H. 1999, J Cell Physiol. , pp. 180(1):35-45.

295. *Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field.* **Rivera-Feliciano J, Tabin CJ.**, 2006, *Dev Biol.* , pp. 295(2):580-8.
296. *Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut.* **Roberts DJ, Johnson RL, Burke AC, Nelson CE, Morgan BA, Tabin C.** 1995, *Development*, pp. 121(10):3163-74.
297. *Roles of BMP signaling and Nkx2.5 in patterning at the chick midgut-foregut boundary.* **Smith DM, Nielsen C, Tabin CJ, Roberts DJ.** 2000, *Development*, pp. 127(17):3671-81.
298. *BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis.* **Piscione TD, Yager TD, Gupta IR, Grinfeld B, Pei Y, Attisano L, Wrana JL, Rosenblum ND.** 1997, *Am J Physiol.* , pp. 273(6 Pt 2):F961-75.
299. *BMP4 substitutes for loss of BMP7 during kidney development.* **Oxburgh L, Dudley AT, Godin RE, Koonce CH, Islam A, Anderson DC, Bikoff EK, Robertson EJ.** 2005, *Dev Biol.* , pp. 286(2):637-46.
300. *Induction of embryonic primordia by implantation of organizers from a different species. 1923.* **Spemann H, Mangold H.** 2001, *Int J Dev Biol.* , pp. 45(1):13-38.
301. *Bone morphogenetic protein signalling and vertebrate nervous system development.* **Liu A, Niswander LA.**, 2005, *Nat Rev Neurosci.* , pp. 6(12):945-54.
302. *The organizer factors Chordin and Noggin are required for mouse forebrain development.* **Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J, De Robertis EM.** 2000, *Nature*, pp. 403(6770):658-61.
303. *Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development.* **Furuta Y, Piston DW, Hogan BL.** 1997, *Development*, pp. 124(11):2203-12.
304. *Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP.* **Li W, Cogswell CA, LoTurco JJ.** 1998, *J Neurosci.* , pp. 18(21):8853-62.
305. *Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate.* **Mehler MF, Mabie PC, Zhu G, Gokhan S, Kessler JA.** 2000, *Dev Neurosci.* , pp. 22(1-2):74-85.
306. *Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells.* **Gross RE, Mehler MF, Mabie PC, Zang Z, Santschi L, Kessler JA.** 1996, *Neuron*, pp. 17(4):595-606.
307. *Bone morphogenetic proteins promote neurite outgrowth in retinal ganglion cells.* **Kerrison JB, Lewis RN, Otteson DC, Zack DJ.** 2005, *Mol Vis.* , pp. 11:208-15.
308. *BMP enhances transcriptional responses to NGF during PC12 cell differentiation.* **Lönn P, Zaia K, Israelsson C, Althini S, Usoskin D, Kylberg A, Ebendal T.** 2005, *Neurochem Res.* , pp. 30(6-7):753-65.
309. *Bone morphogenetic protein signalling in NGF-stimulated PC12 cells.* **Althini S, Usoskin D, Kylberg A, ten Dijke P, Ebendal T.** 2003, *Biochem Biophys Res Commun.* , pp. 307(3):632-9.
310. *Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily.* **Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ.** 1994, *Nature*, pp. 368(6472):639-43.
311. *BMP signaling and HOX transcription factors in limb development.* **Li X, Cao X.** 2003, *Front Biosci.* , pp. 8:s805-12.
312. *Mutations in CDMP1 cause autosomal dominant brachydactyly type C.* **Polinkovsky A, Robin NH, Thomas JT, Irons M, Lynn A, Goodman FR, Reardon W, Kant SG, Brunner HG, van der Burgt I, Chitayat D, McGaughran**

- J, Donnai D, Luyten FP, Warman ML.** 1997, *Nat Genet.*, pp. 17(1):18-9.
313. *A human chondrodysplasia due to a mutation in a TGF-beta superfamily member.* **Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP.** 1996, *Nat Genet.* , pp. 12(3):315-7.
314. *Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1.* **Thomas JT, Kilpatrick MW, Lin K, Erlacher L, Lembessis P, Costa T, Tsipouras P, Luyten FP.** 1997, *Nat Genet.*, pp. 17(1):58-64.
315. *BMP/GDF-signalling interactions during synovial joint development.* **Francis-West PH, Parish J, Lee K, Archer CW.** 1999, *Cell Tissue Res.*, pp. 296(1):111-9.
316. *Recombinant human growth/differentiation factor 5 stimulates mesenchyme aggregation and chondrogenesis responsible for the skeletal development of limbs.* **Hötten GC, Matsumoto T, Kimura M, Bechtold RF, Kron R, Ohara T, Tanaka H, Satoh Y, Okazaki M, Shirai T, Pan H, Kawai S, Pohl JS, Kudo A.** 1996, *Growth Factors.* , pp. 13(1-2):65-74.
317. *Mechanisms of GDF-5 action during skeletal development.* **Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten FP, Archer CW.** 1999, *Development*, pp. 126(6):1305-15.
318. *p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells.* **Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K, Makishima F.** 1999, *Exp Cell Res.*, pp. 250(2):351-63.
319. *Growth/differentiation factor-5 (GDF-5) and skeletal development.* **Buxton P, Edwards C, Archer CW, Francis-West P.** 2001, *J Bone Joint Surg Am.* , pp. 83-A Suppl 1(Pt 1):S23-30.
320. *Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2.* **Sammar M, Stricker S, Schwabe GC, Sieber C, Hartung A, Hanke M, Oishi I, Pohl J, Minami Y, Sebald W, Mundlos S, Knaus P.** 2004, *Genes Cells.* , pp. 9(12):1227-38.
321. *Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family.* **Wolfman NM, Hattersley G, Cox K, Celeste AJ, Nelson R, Yamaji N, Dube JL, DiBlasio-Smith E, Nove J, Song JJ, Wozney JM, Rosen V.** 1997, *J Clin Invest.* , pp. 100(2):321-30.
322. *Enhanced tendon healing with GDF 5 and 6.* **Aspenberg P, Forslund C.** 1999, *Acta Orthop Scand.* , pp. 70(1):51-4.
323. *Gene expression of growth and differentiation factors-5, -6, and -7 in developing bovine tooth at the root forming stage.* **Morotome Y, Goseki-Sone M, Ishikawa I, Oida S.** 1998, *Biochem Biophys Res Commun.* , pp. 244(1):85-90.
324. *Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family.* **Storm EE, Kingsley DM.** 1996, *Development*, pp. 122(12):3969-79.
325. *Growth/differentiation factor-5 induces angiogenesis in vivo.* **Yamashita H, Shimizu A, Kato M, Nishitoh H, Ichijo H, Hanyu A, Morita I, Kimura M, Makishima F, Miyazono K.** 1997, *Exp Cell Res.* , pp. 235(1):218-26.
326. *Bone morphogenetic proteins in the development and healing of synovial joints.* **Edwards CJ, Francis-West PH.** 2001, *Semin Arthritis Rheum.* , pp. 31(1):33-42.
327. *Expression cloning of an activin receptor, a predicted transmembrane serine kinase.* **Mathews LS, Vale WW.** 1991, *Cell*, pp. 65(6):973-82.
328. *Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors.* **Gilboa L, Nohe A, Geissendörfer T, Sebald W, Henis YI, Knaus P.** 2000, *Mol Biol Cell.* , pp. 11(3):1023-35.



329. *Requirement of Bmpr1a for Müllerian duct regression during male sexual development.* **Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR.** 2002, *Nat Genet.* , pp. 32(3):408-10.
330. *Specificity of TGFbeta signaling is conferred by distinct type I receptors and their associated SMAD proteins in Caenorhabditis elegans.* **Krishna S, Maduzia LL, Padgett RW.** 1999, *Development*, pp. 126(2):251-60.
331. *Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity.* **ten Dijke P, Ichijo H, Franzén P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K.** 1993, *Oncogene.* , pp. 8(10):2879-87.
332. *TGF-beta signal transduction.* **Massagué J.** 1998, *Annu Rev Biochem.*, pp. 67:753-91.
333. *Assignment of transforming growth factor beta1 and beta3 and a third new ligand to the type I receptor ALK-1.* **Lux A, Attisano L, Marchuk DA.** 1999, *J Biol Chem.* , pp. 274(15):9984-92.
334. *Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms.* **Yeo C, Whitman M.,** 2001, *Mol Cell.* , pp. 7(5):949-57.
335. *BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk.* **Miyazono K, Maeda S, Imamura T.** 2005, *Cytokine Growth Factor Rev.*, pp. 16(3):251-63.
336. *Mechanism of activation of the TGF-beta receptor.* **Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J.** 1994, *Nature*, pp. 370(6488):341-7.
337. *Smads and early developmental signaling by the TGFbeta superfamily.* **Whitman M.** 1998, *Genes Dev.* , pp. 12(16):2445-62.
338. *Tumor necrosis factor (TNF).* **Old LJ.** 1985, *Science*, pp. 230(4726):630-2.
339. *An endotoxin-induced serum factor that causes necrosis of tumors.* **Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B.** 1975, *Proc Natl Acad Sci U S A.* , pp. 72(9):3666-70.
340. *Lymphocyte in vitro cytotoxicity: specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells.* **Granger GA, Shacks SJ, Williams TW, Kolb WP.** 1969, *Nature*, pp. 221(5186):1155-7.
341. *The TNF superfamily.* **Ware CF.** 2003, *Cytokine Growth Factor Rev.*, pp. 14(3-4):181-4.
342. *TNF alpha and the TNF receptor superfamily: structure-function relationship(s).* **Idriss HT, Naismith JH.** 2000, *Microsc Res Tech.* , pp. 50(3):184-95.
343. *Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-kappaB.* **Aggarwal BB.** 2000, *Ann Rheum Dis.* , pp. 59 Suppl 1:i6-16.
344. *Signal transduction by tumour necrosis factor and tumour necrosis factor related ligands and their receptors.* **Darnay BG, Aggarwal BB.,** 1999, *Ann Rheum Dis.*, pp. 58 Suppl 1:i2-i13.
345. *The TNF and TNF receptor superfamilies: integrating mammalian biology.* **Locksley RM, Killeen N, Lenardo MJ.** 2001, *Cell*, pp. 104(4):487-501.
346. *The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death.* **Smith CA, Farrah T, Goodwin RG.,** 1994, *Cell*, pp. 76(6):959-62.
347. *Human tumor-infiltrating lymphocytes transfected with tumor necrosis factor gene could augment cytotoxicity to autologous tumor cells.* **Itoh Y, Kohgo Y, Watanabe N, Kanisawa Y, Sakamaki S, Takahashi M, Hirayama Y, Ono H, Himeno T, Niitsu Y.** 1991, *Jpn J Cancer Res.* , pp. 82(11):1203-6.
348. *Kinase cascades regulating entry into apoptosis.* **Anderson P.** 1997, *Microbiol Mol Biol Rev.* , pp. 61(1):33-46.

349. *Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.* Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998, *Cell*, pp. 93(2):165-76.
350. *VEGI, a new member of the TNF family activates nuclear factor-kappa B and c-Jun N-terminal kinase and modulates cell growth.* Haridas V, Shrivastava A, Su J, Yu GL, Ni J, Liu D, Chen SF, Ni Y, Ruben SM, Gentz R, Aggarwal BB. 1999, *Oncogene*, pp. 18(47):6496-504.
351. *TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator.* Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P. 2002, *Immunity*, pp. 16(3):479-92.
352. *BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth.* Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, Ambrose C, Lawton P, Bixler S, Acha-Orbea H, Valmori D, Romero P, Werner-Favre C, Zubler RH, Browning JL, Tschopp J. 1999, *J Exp Med.*, pp. 189(11):1747-56.
353. *Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing.* Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S. 1997, *J Exp Med.*, pp. 186(12):2045-50.
354. *Translational event mediates differential production of tumor necrosis factor-alpha in hyaluronan-stimulated microglia and macrophages.* Wang MJ, Kuo JS, Lee WW, Huang HY, Chen WF, Lin SZ. 2006, *J Neurochem.*, pp. 97(3):857-71.
355. *NF-kappaB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis.* Dela Peña A, Leclercq I, Field J, George J, Jones B, Farrell G. 2005, *Gastroenterology*, pp. 129(5):1663-74.
356. *TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB.* Schneider P, Thome M, Burns K, Bodmer JL, Hofmann K, Kataoka T, Holler N, Tschopp J. 1997, *Immunity*, pp. 7(6):831-6.
357. *Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5.* Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. 2000, *Immunity*, pp. 12(6):611-20.
358. *Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling.* Inoue J, Ishida T, Tsukamoto N, Kobayashi N, Naito A, Azuma S, Yamamoto T. 2000, *Exp Cell Res.*, pp. 254(1):14-24.
359. *TNF receptor subtype signalling: differences and cellular consequences.* MacEwan DJ. 2002, *Cell Signal*, pp. 14(6):477-92.
360. *A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling.* Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. 2000, *Science*, pp. 288(5475):2351-4.
361. *Signalling pathways of the TNF superfamily: a double-edged sword.* Aggarwal BB. 2003, *Nat Rev Immunol.*, pp. 3(9):745-56.
362. *Targeting death and decoy receptors of the tumour-necrosis factor superfamily.* Ashkenazi A. 2002, *Nat Rev Cancer*, pp. 2(6):420-30.
363. *TR1, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferation and inhibits osteoclastogenesis and bone resorption.* Kwon BS, Wang S, Udagawa N, Haridas V, Lee ZH, Kim KK, Oh KO, Greene J, Li Y, Su J, Gentz R, Aggarwal BB, Ni J. 1998, *FASEB J.*, pp. 12(10):845-54.
364. *Linking JNK signaling to NF-kappaB: a key to survival.* Papa S, Zazzeroni F, Pham CG,

- Bubici C, Franzoso G. 2004, *J Cell Sci.*, pp. 117(Pt 22):5197-208.
365. *Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling.* De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R, Franzoso G. 2001, *Nature*, pp. 414(6861):308-13.
366. *Inhibition of JNK activation through NF-kappaB target genes.* Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. 2001, *Nature*, pp. 414(6861):313-7.
367. *Cell signalling: cell survival and a Gadd45-factor deficiency.* Amanullah A, Azam N, Balliet A, Hollander C, Hoffman B, Fornace A, Liebermann D. 2003, *Nature*, p. 424(6950):741.
368. *To be, or not to be: NF-kappaB is the answer—role of Rel/NF-kappaB in the regulation of apoptosis.* Kucharczak J, Simmons MJ, Fan Y, Gélinas C. 2003, *Oncogene*, pp. 22(56):8961-82.
369. *Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases.* Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. 2005, *Cell*, pp. 120(5):649-61.
370. *What does tumour necrosis factor excess do to the immune system long term?* Clark J, Vagenas P, Panesar M, Cope AP. 2005, *Ann Rheum Dis.*, pp. 64 Suppl 4:iv70-6.
371. *Multiple nuclear factors interact with the immunoglobulin enhancer sequences.* Sen R, Baltimore D. 1986, *Cell*, pp. 46(5):705-16.
372. *The NF-kappa B and I kappa B proteins: new discoveries and insights.* Baldwin AS Jr. 1996, *Annu Rev Immunol.*, pp. 14:649-83.
373. *A brain-specific transcription activator.* Korner M, Rattner A, Mauxion F, Sen R, Citri Y. 1989, *Neuron*, pp. 3(5):563-72.
374. *Brain synapses contain inducible forms of the transcription factor NF-kappa B.* Kaltschmidt C, Kaltschmidt B, Baeuerle PA. 1993, *Mech Dev*, pp. 43(2-3):135-47.
375. *Constitutive NF-kappa B activity in neurons.* Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeuerle PA. 1994, *Mol Cell Biol.*, pp. 14(6):3981-92.
376. *NF-kappa B: a crucial transcription factor for glial and neuronal cell function.* O'Neill LA, Kaltschmidt C., 1997, *Trends Neurosci.*, pp. 20(6):252-8.
377. *Constitutive nuclear factor-kappa B activity is required for central neuron survival.* Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA. 2002, *J Neurosci.*, pp. 22(19):8466-75.
378. *Apoptosis in cerebellar granule neurons is associated with reduced interaction between CREB-binding protein and NF-kappaB.* Yalcin A, Koulich E, Mohamed S, Liu L, D'Mello SR. 2003, *J Neurochem.*, pp. 84(2):397-408.
379. *Retrograde transport of transcription factor NF-kappa B in living neurons.* Wellmann H, Kaltschmidt B, Kaltschmidt C. 2001, *J Biol Chem.*, pp. 276(15):11821-9.
380. *NF-kappa B functions in synaptic signaling and behavior.* Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D. 2003, *Nat Neurosci.*, pp. 6(10):1072-8.
381. *Transcription factor NF-kappaB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons.* Mikenberg I, Widera D, Kaus A, Kaltschmidt B, Kaltschmidt C. 2007, *PLoS ONE.*, p. 2(7):e589.
382. *NF-kappa B-like factors in the murine brain. Developmentally-regulated and tissue-specific expression.* Bakalkin GYa, Yakovleva T, Terenius L. 1993, *Brain Res Mol Brain Res.*, pp. 20(1-2):137-46.
383. *Potential role of NF-kappaB in adult neural stem cells: the underrated steersman?* Widera D,

- Mikenberg I, Kaltschmidt B, Kaltschmidt C.** 2006, *Int J Dev Neurosci.*, pp. 24(2-3):91-102.
384. *Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons.* **Maggirwar SB, Sarmiere PD, Dewhurst S, Freeman RS.** 1998, *J Neurosci.*, pp. 18(24):10356-65.
385. *Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons.* **Middleton G, Hamanoue M, Enokido Y, Wyatt S, Pennica D, Jaffray E, Hay RT, Davies AM.** 2000, *J Cell Biol.*, pp. 148(2):325-32.
386. *Inhibition of nuclear factor kappa B (NFkappaB) activity induces nerve growth factor-resistant apoptosis in PC12 cells.* **Tagliatela G, Robinson R, Perez-Polo JR.** 1997, *J Neurosci Res.*, pp. 47(2):155-62.
387. *Characterization of the molecular events following impairment of NF-kappaB-driven transcription in neurons.* **Chiarugi A.** 2002, *Brain Res Mol Brain Res.*, pp. 109(1-2):179-88.
388. *NF-kappaB signalling regulates the growth of neural processes in the developing PNS and CNS.* **Gutierrez H, Hale VA, Dolcet X, Davies A.** 2005, *Development.*, pp. 132(7):1713-26.
389. *Enhancement of immunoreactivity for NF-kappa B in human cerebral infarctions.* **Terai K, Matsuo A, McGeer EG, McGeer PL.** 1996, *Brain Res.*, pp. 739(1-2):343-9.
390. *Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease.* **Kaltschmidt B, Uherek M, Volk B, Baeuerle PA, Kaltschmidt C.** 1997, *Proc Natl Acad Sci U S A.*, pp. 94(6):2642-7.
391. *Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease.* **Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M, Faucheux BA, Agid Y, Hirsch EC.** 1997, *Proc Natl Acad Sci U S A.*, pp. 94(14):7531-6.
392. *Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction.* **Mullis KB, Faloona, FA.** 1987, *Methods Enzymol.*, pp. 155:335-350.
393. *Nucleic acid amplification in vitro: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection.* **Guatelli JC, Gingeras TR, Richman DD.** 1989, *Clin Microbiol Rev.*, pp. 2(2):217-26.
394. *Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase.* **Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA.** 1988, *Science*, pp. 239(4839):487-91.
395. *A critical review of PCR primer design algorithms and cross-hybridization case study.* **Burpo, FJ.** 2001, *Biochemistry* 218, pp. 1-12.
396. *Optimization of the annealing temperature for DNA amplification in vitro.* **Rychlik W, Spencer WJ, Rhoads RE.** 1990, *Nucleic Acids Res.*, pp. 18(21):6409-12.
397. *A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics.* **JR., John Santalucia.** 1998, *Proc. Natl. Acad. Sci. USA*, pp. Vol. 95, pp. 1460-1465.
398. *A computer program for selection of oligonucleotide primers for polymerase chain reactions.* **Lowe T, Sharefkin J, Yang SQ, Dieffenbach CW.** 1990, *Nucleic Acids Res.*, pp. 18(7):1757-61.
399. *Real-time RT-PCR normalisation; strategies and considerations.* **Huggett J, Dheda K, Bustin S, Zumla A.** 2005, *Genes Immun*, pp. 6(4):279-84.
400. *Quantitative real-time RT-PCR—a perspective.* **Bustin SA, Benes V, Nolan T, Pfaffl MW.** 2005, *J Mol Endocrinol*, pp. 34(3):597-601.
401. *An essential role for Frizzled5 in neuronal survival in the parafascicular nucleus of the*

- thalamus*. Liu C, Wang Y, Smallwood PM, Nathans J. 2008, J Neurosci, pp. 28(22):5641-53.
402. *Frizzled-3 is required for the development of major fiber tracts in the rostral CNS*. Wang Y, Thekdi N, Smallwood PM, Macke JP, Nathans J. 2002, J Neurosci, pp. 22(19):8563-73.
403. *Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling*. Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, Wang Y, Nathans J, Tessier-Lavigne M, Zou Y. 2003, Science, pp. 302(5652):1984-8.
404. *Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development*. Rosso SB, Sussman D, Wynshaw-Boris A, Salinas PC. 2005, Nat Neurosci, pp. 8(1):34-42.
405. *Increased expression of Wnt-1 in schizophrenic brains*. Miyaoka T, Seno H, Ishino H. 1999, Schizophr Res, pp. 38(1):1-6.
406. *Mfrp, a gene encoding a frizzled related protein, is mutated in the mouse retinal degeneration 6*. Kameya S, Hawes NL, Chang B, Heckenlively JR, Naggert JK, Nishina PM. 2002, Hum Mol Genet, pp. 11(16):1879-86.
407. *The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain*. McMahon AP, Bradley A. 1990, Cell, pp. 62(6):1073-85.
408. *Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development*. Thomas KR, Capecchi MR. 1990, Nature, pp. 346(6287):847-50.
409. *A local Wnt-3a signal is required for development of the mammalian hippocampus*. Lee SM, Tole S, Grove E, McMahon AP. 2000, Development, pp. 127(3):457-67.
410. *Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1*. Galceran J, Miyashita-Lin EM, Devaney E, Rubenstein JL, Grosschedl R. 2000, Development, pp. 127(3):469-82.
411. *Wnt signalling required for expansion of neural crest and CNS progenitors*. Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S. 1997, Nature, pp. 389(6654):966-70.
412. *Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system*. Dickinson ME, Krumlauf R, McMahon AP. 1994, Development, pp. 120(6):1453-71.
413. *The bone morphogenetic protein type Ib receptor is a major mediator of glial differentiation and cell survival in adult hippocampal progenitor cell culture*. Brederlau A, Faigle R, Elmi M, Zarebski A, Sjöberg S, Fujii M, Miyazono K, Funa K. 2004, Mol Biol Cell, pp. 15(8):3863-75.
414. *TGF- $\beta$ 1 activates two distinct type I receptors in neurons: implications for neuronal NF- $\kappa$ B signaling*. König HG, Kögel D, Rami A, Prehn JH. 2005, J Cell Biol, pp. 168(7):1077-86.
415. *Defective ALK5 signaling in the neural crest leads to increased postmigratory neural crest cell apoptosis and severe outflow tract defects*. Wang J, Nagy A, Larsson J, Dudas M, Sucov HM, Kaartinen V. 2006, BMC Dev Biol, p. 6:51.
416. *Alternative neural crest cell fates are instructively promoted by TGF $\beta$  superfamily members*. Shah NM, Groves AK, Anderson DJ. 1996, Cell, pp. 85(3):331-43.
417. *Culture conditions determine the prevalence of bipolar and monopolar neurons in cultures of dissociated spiral ganglion*. Whitlon DS, Grover M, Tristano J, Williams T, Coulson MT. 2007, Neuroscience, pp. 146(2):833-40.
418. *Osteogenic protein-1 induces dendritic growth in rat sympathetic neurons*. Lein P, Johnson M, Guo X, Rueger D, Higgins D. 1995, Neuron, pp. 15(3):597-605.
419. *Bone morphogenetic protein-7 enhances dendritic growth and receptivity to innervation in cultured hippocampal neurons*. Withers GS, Higgins D, Charette M, Banker G. 2000, Eur J Neurosci, pp. 12(1):106-16.

420. *Noggin antagonizes BMP signaling to create a niche for adult neurogenesis.* Lim DA, Tramontin AD, Trevejo JM, Herrera DG, García-Verdugo JM, Alvarez-Buylla A. 2000, *Neuron*, pp. 28(3):713-26.
421. *Tumor Necrosis Factor {alpha} Regulates Responses to Nerve Growth Factor, Promoting Neural Cell Survival but Suppressing Differentiation of Neuroblastoma Cells.* Takei Y, Laskey R., 2008, *Mol Biol Cell*, pp. 19(3):855-64.
422. *TNF-alpha pretreatment induces protective effects against focal cerebral ischemia in mice.* Nawashiro H, Tasaki K, Ruetzler CA, Hallenbeck JM. 1997, *J Cereb Blood Flow Metab*, pp. 17(5):483-90.
423. *Tumor necrosis factor-alpha modulates the proliferation of neural progenitors in the subventricular/ventricular zone of adult rat brain.* Wu JP, Kuo JS, Liu YL, Tzeng SF. 2000, *Neurosci Lett.* , pp. 292(3):203-6.
424. *TNFalpha contributes to the death of NGF-dependent neurons during development.* Barker V, Middleton G, Davey F, Davies AM. 2001, *Nat Neurosci*, pp. 4(12):1194-8.
425. *NGF-promoted axon growth and target innervation requires GITRL-GITR signaling.* O'Keeffe GW, Gutierrez H, Pandolfi PP, Riccardi C, Davies AM. 2008, *Nat Neurosci*, pp. 11(2):135-42.
426. *Neutralization of TRAIL death pathway protects human neuronal cell line from beta-amyloid toxicity.* Cantarella G, Uberti D, Carsana T, Lombardo G, Bernardini R, Memo M. 2003, *Cell Death Diffe*, pp. 10(1):134-41.
427. *The role of TNF related apoptosis-inducing ligand in neurodegenerative diseases.* Huang Y, Erdmann N, Peng H, Zhao Y, Zheng J. 2005, *Cell Mol Immunol*, pp. 2(2):113-22.
428. *Molecular regulation of CD40 gene expression in macrophages and microglia.* Benveniste EN, Nguyen VT, Wesemann DR. 2004, *Brain Behav Immun*, pp. 18(1):7-12.
429. *Role of WNT signaling in normal and malignant hematopoiesis.* Khan NI, Bendall LJ., 2006, *Histol Histopathol.*, pp. 21(7):761-74.
430. *Mutant Frizzled 4 associated with vitreoretinopathy traps wild-type Frizzled in the endoplasmic reticulum by oligomerization.* Kaykas A, Yang-Snyder J, Héroux M, Shah KV, Bouvier M, Moon RT. 2004, *Nat Cell Biol.* , pp. 6(1):52-8.
431. *The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway.* Flahaut M, Meier R, Coulon A, Nardou KA, Niggli FK, Martinet D, Beckmann JS, Joseph JM, Mühlethaler-Mottet A, Gross N *Oncogene*. Jun11;28(23) 2245-56, 2009.
432. *Expression of Wnt genes and frizzled 1 and 2 receptors in normal breast epithelium and infiltrating breast carcinoma.* Milovanovic T, Planutis K, Nguyen A, Marsh JL, Lin F, Hope C, Holcombe RF. *Int J Oncol*. Nov;25(5):1337-42., 2004.
433. *A role for frizzled 3 in neural crest development.* Deardorff MA, Tan C, Saint-Jeannet JP, Klein PS. 2001, *Development.*, pp. 128(19):3655-63.
434. *The human frizzled-3 (FZD3) gene on chromosome 8p21, a receptor gene for Wnt ligands, is associated with the susceptibility to schizophrenia.* Katsu T, Ujike H, Nakano T, Tanaka Y, Nomura A, Nakata K, Takaki M, Sakai A, Uchida N, Imamura T, Kuroda S. 2003, *Neurosci Lett.* , pp. 353(1):53-6.
435. *Progressive cerebellar, auditory, and esophageal dysfunction caused by targeted disruption of the frizzled-4 gene.* Wang Y, Huso D, Cahill H, Ryugo D, Nathans J. *J. Neurosci*. Jul 1;21(13):4761-71, 2001.
436. *An essential role for Frizzled5 in neuronal survival in the parafascicular nucleus of the thalamus.* Liu C, Wang Y, Smallwood PM, Nathans J. *J Neurosci*. May 28;28(22):5641-53, 2008.

437. *Frizzled6 controls hair patterning in mice.* Guo N, Hawkins C, Nathans J. *Proc Natl Acad Sci U S A.* Jun 22;101(25):9277-81, 2004.
438. *The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells.* Wang Y, Guo N, Nathans J. *J Neurosci.* Feb 22;26(8):2147-56, 2006.
439. *Variable FZD7 expression in colorectal cancers indicates regulation by the tumour microenvironment.* Vincan E, Flanagan DJ, Pouliot N, Brabletz T, Spaderna S. *Dev Dyn.* Aug 4., 2009.
440. *Frizzled9 protein is regionally expressed in the developing medial cortical wall and the cells derived from this region.* Zhao C, Pleasure SJ. *Brain Res Dev Brain Res.* Jun 9;157(1):93-7, 2005.
441. *Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6.* Semenov MV, Tamai K, Brott BK, Kühl M, Sokol S, He X. 2001, *Curr Biol.* , pp. 11(12):951-61.
442. *Kremen proteins interact with Dickkopf1 to regulate anteroposterior CNS patterning.* Davidson G, Mao B, del Barco Barrantes I, Niehrs C. 2002, *Development*, pp. 129(24):5587-96.
443. *Expression of receptor tyrosine kinase RYK in developing rat central nervous system.* Kamitori K, Machide M, Osumi N, Kohsaka S. 1999, *Brain Res Dev Brain Res.* , pp. 114(1):149-60.
444. *Cell-type-specific expression of protein tyrosine kinase-related receptor RYK in the central nervous system of the rat.* Kamitori K, Machide M, Tomita K, Nakafuku M, Kohsaka S. 2002, *Brain Res Mol Brain Res.* , pp. 104(2):255-66.
445. *Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth.* Lu W, Yamamoto V, Ortega B, Baltimore D. 2004, *Cell*, pp. 119(1):97-108.
446. *Ryk: a novel Wnt receptor regulating axon pathfinding.* Keeble TR, Cooper HM., 2006, *Int J Biochem Cell Biol.* , pp. 38(12):2011-7.
447. *The functions and possible significance of Kremen as the gatekeeper of Wnt signalling in development and pathology.* Nakamura T, Nakamura T, Matsumoto K. *J Cell Mol Med.* Apr;12(2):391-408, 2008.
448. *Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators.* Balemans W, Van Hul W., 2002, *Dev Biol.* , pp. 250(2):231-50.
449. *DRAGON, a bone morphogenetic protein co-receptor.* Samad TA, Rebbapragada A, Bell E, Zhang Y, Sidis Y, Jeong SJ, Campagna JA, Perusini S, Fabrizio DA, Schneyer AL, Lin HY, Brivanlou AH, Attisano L, Woolf CJ. 2005, *J Biol Chem.*, pp. 280(14):14122-9.
450. *Controlling the angiogenic switch: a balance between two distinct TGF- $\beta$  receptor signaling pathways.* Goumans MJ, Lebrin F, Valdimarsdottir G. *Trends Cardiovasc Med.* 13(7):301-7, 2003.
451. *Activin receptor-like kinase 1 inhibits human microvascular endothelial cell migration: potential roles for JNK and ERK.* David L, Mallet C, Vailhé B, Lamouille S, Feige JJ, Bailly S. *J Cell Physiol.* 213(2):484-9, 2007.
452. *Truncated activin type II receptors inhibit bioactivity by the formation of heteromeric complexes with activin type I receptors.* De Winter JP, De Vries CJ, Van Achterberg TA, Ameerun RF, Feijen A, Sugino H, De Waele P, Huylebroeck D, Verschuere K, Van Den Eijden-Van Raaij AJ. *Exp Cell Res.* 1;224(2):323-34, 1996.
453. *Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells.* David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S. *Blood.* 1;109(5):1953-61, 2007.
454. *Activin receptor-like kinase 2 and Smad6 regulate epithelial-mesenchymal transformation*

- during cardiac valve formation. **Desgrosellier JS, Mundell NA, McDonnell MA, Moses HL, Barnett JV.** *Dev Biol.* 280(1):201-10, 2005.
455. *Cardiac outflow tract defects in mice lacking ALK2 in neural crest cells.* **Kaartinen V, Dudas M, Nagy A, Sridurongrit S, Lu MM, Epstein JA.** *Development.* 131(14):3481-90, 2004.
456. *Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart.* **Wang J, Sridurongrit S, Dudas M, Thomas P, Nagy A, Schneider MD, Epstein JA, Kaartinen V.** *dev Biol.* 286(1):299-310, 2005.
457. *Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3T3 cells.* **Koenig BB, Cook JS, Wolsing DH, Ting J, Tiesman JP, Correa PE, Olson CA, Pecquet AL, Ventura F, Grant RA, et al.** 1994, *Mol Cell Biol.*, pp. 14(9):5961-74.
458. *Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4.* **ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K.** *J Biol Chem.* 269(25):16985-8, 1994.
459. *Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2.* **Macías-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL.** *J Biol Chem.* 273(40):25628-36, 1998.
460. *Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation.* **Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T.** *J Cell Sci.* 112 ( Pt 20):3519-27, 1999.
461. *Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5.* **Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, Yamashita H, Enomoto S, Miyazono K.** 1996, *J Biol Chem.*, pp. 271(35):21345-52.
462. *Distinct and cooperative roles of mammalian Vgl homologs GDF1 and GDF3 during early embryonic development.* **Andersson O, Bertolino P, Ibáñez CF.** *Dev Biol.* 311(2):500-11, 2007.
463. *Developmental analysis of activin-like kinase receptor-4 (ALK4) expression in Xenopus laevis.* **Chen Y, Whitaker LL, Ramsdell AF.** *Dev Dyn.* 232(2):393-8, 2005.
464. *ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2.* **Park SO, Lee YJ, Seki T, Hong KH, Fliess N, Jiang Z, Park A, Wu X, Kaartinen V, Roman BL, Oh SP.** *Blood.* 111(2):633-42, 2008.
465. *Transforming growth factor-beta induces nerve growth factor expression in pancreatic stellate cells by activation of the ALK-5 pathway.* **Haas SL, Fitzner B, Jaster R, Wiercinska E, Gaitantzi H, Jesenowski R, Lohr JM, Singer MV, Dooley S, Breitkopf K.** *Growth Factors.* 28:1, 2009.
466. *Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands.* **Mazerbourg S, Hsueh AJ.** *Hum Reprod Update.* 12(4):373-83, 2006.
467. *BMP receptor 1b is required for axon guidance and cell survival in the developing retina.* **Liu J, Wilson S, Reh T.,** 2003, *Dev Biol*, pp. 256(1):34-48.
468. *The bone morphogenetic protein type Ib receptor is a major mediator of glial differentiation and cell survival in adult hippocampal progenitor cell culture.* **Brederlau A, Faigle R, Elmi M, Zarebski A, Sjöberg S, Fujii M, Miyazono K, Funa K.** 2004, *Mol Biol Cell.*, pp. 15(8):3863-75.
469. *Signal transduction pathway through activin receptors as a therapeutic target of musculoskeletal diseases and cancer.* **Tsuchida K, Nakatani M, Uezumi A, Murakami T, Cui X.** *Endocr J.* 55(1):11-21, 2008.



470. *ALK7 expression is specific for adipose tissue, reduced in obesity and correlates to factors implicated in metabolic disease.* Carlsson LM, Jacobson P, Walley A, Froguel P, Sjöström L, Svensson PA, Sjöholm K. *Biochem Biophys Res Commun.* 382(2):309-14, 2009.
471. *Neural induction by the secreted polypeptide noggin.* Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopoulos GD, Harland RM. 1993, *Science*, pp. 262(5134):713-8.
472. *Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton.* Brunet LJ, McMahon JA, McMahon AP, Harland RM. 1998, *Science*, pp. 280(5368):1455-7.
473. *Identification of mammalian noggin and its expression in the adult nervous system.* Valenzuela DM, Economides AN, Rojas E, Lamb TM, Nuñez L, Jones P, Lippman NY, Espinosa R 3rd, Brannan CI, Gilbert DJ, et al. 1995, *J Neurosci.*, pp. 15(9):6077-84.
474. *BMP6 is axonally transported by motoneurons and supports their survival in vitro.* Wang PY, Koishi K, McLennan IS. *Mol Cell Neurosci.* 34(4):653-61, 2007.
475. *TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes.* Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. *Development.* 124(13):2659-70, 1997.
476. *Activin receptor patterning of foregut organogenesis.* Kim SK, Hebrok M, Li E, Oh SP, Schrewe H, Harmon EB, Lee JS, Melton DA. *Genes Dev.* 14(15):1866-71, 2000.
477. *Impaired male sexual behavior in activin receptor type II knockout mice.* Ma X, Reyna A, Mani SK, Matzuk MM, Kumar TR. *Biol reprod.* 73(6):1182-9, 2005.
478. *GDF-5 deficiency in mice delays Achilles tendon healing.* Chhabra A, Tsou D, Clark RT, Gaschen V, Hunziker EB, Mikic BJ. *Orthop Res.* 21(5):826-35, 2003.
479. *Growth/differentiation factor 5 enhances chondrocyte maturation.* Coleman CM, Tuan RS. *Dev Dyn.* 228(2):208-16, 2003.
480. *Effects of growth/differentiation factor-5 on human periodontal ligament cells.* Nakamura T, Yamamoto M, Tamura M, Izumi Y. *J Periodontol Res.* 38(6):597-605, 2003.
481. *APP binds DR6 to trigger axon pruning and neuron death via distinct caspases.* Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. *Nature.* 457(7232):981-9, 2009.
482. *Molecular crosstalk between TRAIL and natural antioxidants in the treatment of cancer.* Rushworth SA, Micheau O. *Br J Pharmacol.* 157(7):1186-8, 2009.
483. *A novel role of CD30L/CD30 signaling by T-T cell interaction in Th1 response against mycobacterial infection.* Tang C, Yamada H, Shibata K, Muta H, Wajjwalku W, Podack ER, Yoshikai Y. *J Immunol.* 181(9):6316-27, 2008.
484. *Structure and expression of murine CD30 and its role in cytokine production.* Bowen MA, Lee RK, Miragliotta G, Nam SY, Podack ER. *J Immunol.* 156(2):442-9, 1996.
485. *Tumor necrosis factor/tumor necrosis factor receptor family members that positively regulate immunity.* So T, Lee SW, Croft M. *Int J Hematol.* 83(1):1-11, 2006.
486. *Signaling by death receptors in the nervous system.* Haase G, Pettmann B, Raoul C, Henderson CE. *Curr Opin Neurobiol.* 18(3):284-91, 2008.
487. *Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-alpha, or IFN-gamma.* Choi C, Park JY, Lee J, Lim JH, Shin EC, Ahn YS, Kim CH, Kim SJ, Kim JD, Choi IS, Choi IH. *J Immunol.* 162(4):1889-95, 1999.

488. *Fas/tumor necrosis factor receptor death signaling is required for axotomy-induced death of motoneurons in vivo.* Ugolini G, Raoul C, Ferri A, Haenggeli C, Yamamoto Y, Salaün D, Henderson CE, Kato AC, Pettmann B, Hueber AO. *J Neurosci.* 23(24):8526-31, 2003.
489. *Control of neuronal branching by the death receptor CD95 (Fas/Apo-1).* Zuliani C, Kleber S, Klussmann S, Wenger T, Kenzelmann M, Schreglmann N, Martinez A, del Rio JA, Soriano E, Vodrazka P, Kuner R, Groene HJ, Herr I, Krammer PH, Martin-Villalba A. *Cell Death Differ.* 13(1):31-40, 2006.
490. *Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas.* Gruss HJ, Dower SK. *Blood* 85(12):3378-404, 1995.
491. *CD137 (ILA/4-1BB), expressed by primary human monocytes, induces monocyte activation and apoptosis of B lymphocytes.* Kienzle G, von Kempis J *Int Immunol.* 12(1):73-82, 2000.
492. *ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages.* Schwarz H, Valbracht J, Tuckwell J, von Kempis J, Lotz M. *Blood.* 85(4):1043-52, 1995.
493. *Costimulation of CD28- T lymphocytes by 4-1BB ligand.* DeBenedette MA, Shahinian A, Mak TW, Watts TH. *J Immunol.* 158(2):551-9., 1995.
494. *Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses.* Gramaglia I, Weinberg AD, Lemon M, Croft M. *J Immunol.* 161(12):6510-7, 1998.
495. *Expression and function of OX40 ligand on human dendritic cells.* Ohshima Y, Tanaka Y, Tozawa H, Takahashi Y, Maliszewski C, Delespesse G *J Immunol.* 159(8):3838-48, 1997.
496. *CD134 plays a crucial role in the pathogenesis of EAE and is upregulated in the CNS of patients with multiple sclerosis.* Carboni S, Aboul-Enein F, Waltzinger C, Killeen N, Lassmann H, Peña-Rossi C. *J Neuroimmunol.* 145(1-2):1-11, 2003.
497. *Osteoprotegerin: a novel secreted protein involved in the regulation of bone density.* Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P. *Cell.* 89(2):309-19, 1997.
498. *Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL.* Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. *Proc Natl Acad Sci U S A.* 5(7):3597-602, 1998.
499. *Effects of different magnitudes of mechanical strain on Osteoblasts in vitro.* Tang L, Lin Z, Li YM. *Biochem Biophys Res Commun.* 344(1):122-8, 2006.
500. *Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice.* Oshiro T, Shiotani A, Shibasaki Y, Sasaki T. *Anat Rec.* 266(4):218-25, 2002.
501. *NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily.* von Bülow GU, Bram RJ. *Science.* 278(5335):138-41, 1997.
502. *CD40 and CD154 in cell-mediated immunity.* Grewal IS, Flavell RA. 1998, *Annu Rev Immunol.*, pp. 16:111-35.
503. *CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis.* Gerritse K, Laman JD, Noelle RJ, Aruffo A, Ledbetter JA, Boersma WJ, Claassen E. 1996, *Proc Natl Acad Sci U S A.* , pp. 93(6):2499-504.
504. *Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation.* Tan J, Town T, Paris D, Mori T, Suo Z,

- Crawford F, Mattson MP, Flavell RA, Mullan M. 1999, *Science*, pp. 286(5448):2352-5.
505. *Induction of cyclooxygenase-2 in reactive glial cells by the CD40 pathway: relevance to amyotrophic lateral sclerosis.* Okuno T, Nakatsuji Y, Kumanogoh A, Koguchi K, Moriya M, Fujimura H, Kikutani H, Sakoda S. 2004, *J Neurochem.* , pp. 91(2):404-12.
506. *Ligation of CD40 stimulates the induction of nitric-oxide synthase in microglial cells.* Jana M, Liu X, Koka S, Ghosh S, Petro TM, Pahan K. 2001, *J Biol Chem.* , pp. 276(48):44527-33.
507. *The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration.* Meighan-Mantha RL, Hsu DK, Guo Y, Brown SA, Feng SL, Peifley KA, Alberts GF, Copeland NG, Gilbert DJ, Jenkins NA, Richards CM, Winkles JA. 1999, *J Biol Chem.* , pp. 274(46):33166-76.
508. *Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells.* Harada N, Nakayama M, Nakano H, Fukuchi Y, Yagita H, Okumura K. 2002, *Biochem Biophys Res Commun.*, pp. 299(3):488-93.
509. *TWEAK induces angiogenesis and proliferation of endothelial cells.* Lynch CN, Wang YC, Lund JK, Chen YW, Leal JA, Wiley SR. 1999, *J Biol Chem.* , pp. 274(13):8455-9.
510. *The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors.* Tran NL, McDonough WS, Donohue PJ, Winkles JA, Berens TJ, Ross KR, Hoelzinger DB, Beaudry C, Coons SW, Berens ME. 2003, *Am J Pathol.* , pp. 162(4):1313-21.
511. *Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth.* Tanabe K, Bonilla I, Winkles JA, Strittmatter SM. 2003, *J Neurosci.* , pp. 23(29):9675-86.
512. *Unconventional ligand activation of herpesvirus entry mediator signals cell survival.* Cheung TC, Steinberg MW, Osborne LM, Macauley MG, Fukuyama S, Sanjo H, D'Souza C, Norris PS, Pfeffer K, Murphy KM, Kronenberg M, Spear PG, Ware CF. *Proc Natl Acad Sci U S A.* 106(15):6244-9, 2009.
513. *Developmental regulation of sensory neurite growth by the tumor necrosis factor superfamily member LIGHT.* Gavalda N, Gutierrez H, Davies AM. *J Neurosci.* 29(6):1599-607, 2009.
514. *BAFF, APRIL and their receptors: structure, function and signaling.* Bossen C, Schneider P., 2006, *Semin Immunol.*, pp. 18(5):263-75.
515. *Ectodysplasin signaling in development.* Mikkola ML, Thesleff I. *Cytokine Growth Factor Rev.* 14(3-4):211-24, 2003.
516. *Myodegeneration in EDA-A2 transgenic mice is prevented by XEDAR deficiency.* Newton K, French DM, Yan M, Frantz GD, Dixit VM. 2004, *Mol Cell Biol.* , pp. 24(4):1608-13.
517. *Expression of a member of tumor necrosis factor receptor superfamily, TROY, in the developing mouse brain.* Hisaoka T, Morikawa Y, Kitamura T, Senba E. *Brain Res Dev Brain Res.* 143(1):105-9, 2003.
518. *Troy/Taj and its role in CNS axon regeneration.* Mi S. *Cytokine Growth Factor Rev.* 19(3-4):245-51, 2008.
519. *Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2.* Mérino D, Lalaoui N, Morizot A, Schneider P, Solary E, Micheau O. *Mol Cell Biol.* 6(19):7046-55, 2006.
520. *Crucial role for BAFF-BAFF-R signaling in the survival and maintenance of mature B cells.* Rauch M, Tussiwand R, Bosco N, Rolink AG. *PLoS One.* 4(5):e5456, 2009.
521. *TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology.* Sasaki Y,

- Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M. *J Immunol.* 173(4):2245-52, 2004.
522. *LARD: a new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing.* Screamon GR, Xu XN, Olsen AL, Cowper AE, Tan R, McMichael AJ, Bell JL. *Proc Natl Acad Sci U S A.* 94(9):4615-9, 1997.
523. *Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95.* Chinnaiyan AM, O'Rourke K, Yu GL, Lyons RH, Garg M, Duan DR, Xing L, Gentz R, Ni J, Dixit VM. *Science.* 274(5289):990-2, 1996.
- 524.. *TL1A-induced NF-kappaB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells.* Wen L, Zhuang L, Luo X, Wei P *J Biol Chem.* 278(40):39251-8, 2003.
- 525.. *An endogenous hybrid mRNA encodes TWEAK-PRIL, a functional cell surface TWEAK-APRIL fusion protein.* Pradet-Balade B, Medema JP, López-Fraga M, Lozano JC, Kolfschoten GM, Picard A, Martínez-A C, Garcia-Sanz JA, Hahne M *EMBO J.* 21(21):5711-20, 2002.
526. *APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth.* Hahne M, Kataoka T, Schröter M, Hofmann K, Irmeler M, Bodmer JL, Schneider P, Bornand T, Holler N, French LE, Sordat B, Rimoldi D, Tschopp J. *J Exp Med.* 88(6):1185-90, 1998.
527. *APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells.* Belnoue E, Pihlgren M, McGaha TL, Tougne C, Rochat AF, Bossen C, Schneider P, Huard B, Lambert PH, Siegrist CA. *Blood.* 111(5):2755-64, 2008.
528. *APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa.* Huard B, McKee T, Bosshard C, Durual S, Matthes T, Myit S, Donze O, Frossard C, Chizzolini C, Favre C, Zubler R, Guyot JP, Schneider P, Roosnek E. *J Clin Invest.* 118(8):2887-95, 2008.
529. *APRIL is overexpressed in cancer: link with tumor progression.* Moreaux J, Veyrune JL, De Vos J, Klein B. *BMC Cancer.* 9:83, 2009.
- 530.. *TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease.* McCoy MK, Tansey MG *J Neuroinflammation.* 5:45, 2008.
531. *Involvement of tumor necrosis factor alpha in hippocampal development and function.* Golan H, Levav T, Mendelsohn A, Huleihel M. *Cereb cortex.* 14(1):97-105, 2004.
532. *Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations.* Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, Tschopp J, Browning JL. *J exp Med.* 190(11):1697-710, 1999.
533. *TWEAK and the central nervous system.* Yepes M. *Mol Neurobiol.* 35(3):255-65, 2007.
534. *TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator.* Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P. *Immunity.* 16(3):479-92, 2002.
- 535.. *Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons.* Ye H, Kuruvilla R, Zweifel LS, Ginty DD *Neuron.* 39(1):57-68, 2003.
536. *NGF and the local control of nerve terminal growth.* Campenot RB. 1994, *J Neurobiol.* , pp. 25(6):599-611.
537. *Directed expression of NGF to pancreatic beta cells in transgenic mice leads to selective hyperinnervation of the islets.* Edwards RH, Rutter WJ, Hanahan D. *Cell.* 58(1):161-70, 1989.
538. *Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons.* Honma Y, Araki T, Gianino S, Bruce A,

- Heuckeroth R, Johnson E, Milbrandt J.** 2002, *Neuron*, pp. 35(2):267-82.
539. *Endothelins are vascular-derived axonal guidance cues for developing sympathetic neurons.* **Makita T, Sucov HM, Garipey CE, Yanagisawa M, Ginty DD.** *Nature*. 452(7188):759-63, 2008.
540. *Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family.* **Storm EE, Kingsley DM.** 1996, *Development*, pp. 122(12):3969-79.
541. *The role of growth/differentiation factor 5 (GDF5) in the induction and survival of midbrain dopaminergic neurones: relevance to Parkinson's disease treatment.* **Sullivan AM, O'Keeffe GW.,** 2005, *J Anat.* , pp. 207(3):219-26.
542. *Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development.* **Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, Kozak CA, Reddi AH, Moos M Jr.** 1994, *J Biol Chem.* , pp. 269(45):28227-34.
543. *Bone morphogenetic proteins regulate interdigital cell death in the avian embryo.* **Merino R, Gañán Y, Macias D, Rodríguez-León J, Hurlé JM.** 1999, *Ann N Y Acad Sci.* , pp. 887:120-32.
544. *The anatomy and development of brachypodism in the mouse.* **Grunenberg H, Lee AJ.,** 1973, *J Embryol Exp Morphol*, pp. 30:119-41.
545. *Growth and survival signals controlling sympathetic nervous system development.* **Glebova NO, Ginty DD.** 2005, *Annu Rev Neurosci.* , pp. 28:191-222.
546. *NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway.* **Howe CL, Valletta JS, Rusnak AS, Mobley WC.** 2001, *Neuron*, pp. 32(5):801-14.
547. *Expression of growth differentiation factor-5 in the developing and adult rat brain.* **O'Keeffe GW, Hanke M, Pohl J, Sullivan AM.** 2004, *Brain Res Dev Brain Res.* , pp. 151(1-2):199-202.
548. *Effects of growth/differentiation factor 5 on the survival and morphology of embryonic rat midbrain dopaminergic neurones in vitro.* **O'Keeffe GW, Dockery P, Sullivan AM.** 2004, *J Neurocytol.* , pp. 33(5):479-88.
549. *A fast and accurate procedure for deriving the Sholl profile in quantitative studies of neuronal morphology.* **Gutierrez H, Davies AM.** 2007, *J Neurosci Methods*, pp. 163(1):24-30.
550. *Bone morphogenetic proteins and their antagonists.* **Gazzerro E, Canalis E.** 2006, *Rev Endocr Metab Disord*, pp. 7(1-2):51-65.
551. *Intracellular BMP signaling regulation in vertebrates: pathway or network?* **von Bubnoff A, Cho KW.** 2001, *Dev Biol.* , pp. 239(1):1-14.
552. *TGF-beta signaling: a tale of two responses.* **Rahimi RA, Leof EB.** 2007, *J Cell Biochem.* , pp. 102(3):593-608.
553. *Upregulation of ID protein by growth and differentiation factor 5 (GDF5) through a smad-dependent and MAPK-independent pathway in HUVMC.* **Chen X, Zankl A, Niroomand F, Liu Z, Katus HA, Jahn L, Tiefenbacher C.** 2006, *J Mol Cell Cardiol*, pp. 41(1):26-33.
554. *Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily.* **Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ.** 1994, *Nature*, pp. 368(6472):639-43.
555. *NGF-promoted axon growth and target innervation requires GITRL-GITR signaling.* **O'Keeffe GW, Gutierrez H, Pandolfi PP, Riccardi C, Davies AM.** 2008, *Nat Neurosci.* , pp. 11(2):135-42.
556. *Low-affinity nerve growth factor receptor p75NTR immunoreactivity in the myocardium with sympathetic hyperinnervation.* **Zhou S, Cao JM, Swissa M, Gonzalez-Gomez I, Chang CM,**

- Chien K, Miyauchi Y, Fu KJ, Yi J, Asotra K, Karagueuzian HS, Fishbein MC, Chen PS, Chen LS. *J Cardiovasc Electrophysiol.* 15(4):430-7, 2004.
557. *Role of nerve growth factor in the development of rat sympathetic neurons in vitro. I. Survival, growth, and differentiation of catecholamine production.* Chun LL, Patterson PH. *J Cell Biol.* 75(3):694-704, 1977.
558. *NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors.* Francis N, Farinas I, Brennan C, Rivas-Plata K, Backus C, Reichardt L, Landis S. 1999, *Dev Biol.*, pp. 210(2):411-27.
559. *Role of PI 3-kinase, Akt and Bcl-2-related proteins in sustaining the survival of neurotrophic factor-independent adult sympathetic neurons.* Orike N, Middleton G, Borthwick E, Buchman V, Cowen T, Davies AM. *J Cell Biol.* 154(5):995-1005, 2001.
560. *Regulation of axonal and dendritic growth by the extracellular calcium-sensing receptor.* Vizard TN, O'Keefe GW, Gutierrez H, Kos CH, Riccardi D, Davies AM. 2008, *Nat Neurosci.*, pp. 11(3):285-91.
561. *Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse.* Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson EM Jr *Exp Neurol.* 158(2):504-28, 1999.
562. *Sympathetic cholinergic target innervation requires GDNF family receptor GFR alpha 2.* Hiltunen PH, Airaksinen MS. *Mol Cell Neurosci.* 26(3):450-7, 2004.
563. *Trophic and protective effects of growth/differentiation factor 5, a member of the transforming growth factor-beta superfamily, on midbrain dopaminergic neurons.* Kriegstein K, Suter-Crazzolara C, Hötten G, Pohl J, Unsicker K. 1995, *J Neurosci Res.*, pp. 42(5):724-32.
564. *Signalling pathways of the TNF superfamily: a double-edged sword.* Aggarwal, BB. 2003, *Nat Rev Immunol*, pp. 3(9):745-56.
565. *BAFF: a fundamental survival factor for B cells.* Mackay F, Browning JL., 2002, *Nat Rev Immunol*, pp. 2(7):465-75.
566. *BAFF AND APRIL: a tutorial on B cell survival.* Mackay F, Schneider P, Rennert P, Browning J., 2003, *Annu Rev Immunol*, pp. 21:231-64.
567. *B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling.* Mackay F, Silveira PA, Brink R., 2007, *Curr Opin Immunol*, pp. 19(3):327-36.
568. *Peripheral target regulation of dendritic geometry in the rat superior cervical ganglion.* Voyvodic JT. 1989, *J Neurosci.*, pp. 9(6):1997-2010.
569. *Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs.* Willis DE, van Niekerk EA, Sasaki Y, Mesngon M, Merianda TT, Williams GG, Kendall M, Smith DS, Bassell GJ, Twiss JL. *J Cell Biol.* 178(6):965-80, 2007.
570. *Identification and characterization of nuclear factor kappaB binding sites in the murine bcl-x promoter.* Glasgow JN, Wood T, Perez-Polo JR. 2000, *J Neurochem.*, pp. 75(4):1377-89.
571. *Regulation of A1/Bfl-1 expression in peripheral splenic B cells.* Trescol-Biémont MC, Vershelde C, Cottalorda A, Bonnefoy-Bérard N. 2004, *Biochimie.*, pp. 86(4-5):287-94.
572. *Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses.* Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR. 1994, *Neuron*, pp. 12(3):691-705.
573. *Neurotrophins: roles in neuronal development and function.* Huang EJ, Reichardt LF. 2001, *Annu Rev Neurosci.*, pp. 24:677-736.

574. *TrkA tyrosine residues involved in NGF-induced neurite outgrowth of PC12 cells.* Inagaki N, Thoenen H, Lindholm D. 1995, Eur J Neurosci., pp. 7(6):1125-33.
575. *Early days of the nerve growth factor proteins.* Shooter EM. 2001, Annu Rev Neurosci., pp. 24:601-29.
576. *NGF signals through TrkA to increase clathrin at the plasma membrane and enhance clathrin-mediated membrane trafficking.* Beattie EC, Howe CL, Wilde A, Brodsky FM, Mobley WC. 2000, J Neurosci., pp. 20(19):7325-33.
577. *Pincher, a pinocytotic chaperone for nerve growth factor/TrkA signaling endosomes.* Shao Y, Akmentin W, Toledo-Aral JJ, Rosenbaum J, Valdez G, Cabot JB, Hilbush BS, Halegoua S. 2002, J Cell Biol., pp. 157(4):679-91.
578. *NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway.* Howe CL, Valletta JS, Rusnak AS, Mobley WC. 2001, Neuron, pp. 32(5):801-14.
579. *Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons.* Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD. 1999, Science, pp. 286(5448):2358-61.
580. *Retrograde support of neuronal survival without retrograde transport of nerve growth factor.* MacInnis BL, Campenot RB. 2002, Science, pp. 295(5559):1536-9.
581. *The role of neurotrophins in the developing nervous system.* Davies AM. 1994, J Neurobiol, pp. 25(11):1334-48.
582. *Neural crest-derived proprioceptive neurons express nerve growth factor receptors but are not supported by nerve growth factor in culture.* Davies AM, Lumsden AG, Rohrer H., 1987, Neuroscience, pp. 20(1):37-46.
583. *Wnts and TGF beta in synaptogenesis: old friends signalling at new places.* Packard M, Mathew D, Budnik V. 2003, Nat Rev Neurosci., pp. 4(2):113-20.
584. *Novel regulators of bone formation: molecular clones and activities.* Wozney JM, Rosen V, Celeste AJ, Mitscock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. 1988, Science, pp. 242(4885):1528-34.
585. *TNF receptor subtype signalling: differences and cellular consequences.* DJ., MacEwan. 2002, Cell Signal., pp. 14(6):477-92.
586. *APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth.* Hahne M, Kataoka T, Schröter M, Hofmann K, Irmeler M, Bodmer JL, Schneider P, Bornand T, Holler N, French LE, Sordat B, Rimoldi D, Tschopp J. 1998, J Exp Med., pp. 188(6):1185-90.
587. *NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily.* von Bülow GU, Bram RJ., 1997, Science, pp. 278(5335):138-41.
588. *BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF.* Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, Hession C, Schneider P, Sizing ID, Mullen C, Strauch K, Zafari M, Benjamin CD, Tschopp J, Browning JL, Ambrose C. 2001, Science, pp. 293(5537):2108-11.
589. *Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency.* Yan M, Brady JR, Chan B, Lee WP, Hsu B, Harless S, Cancro M, Grewal IS, Dixit VM. 2001, Curr Biol., pp. 11(19):1547-52.
590. *A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma.* Laâbi Y, Gras MP, Carbone F, Brouet JC, Berger R, Larsen CJ, Tsapis A. 1992, EMBO J., pp. 11(11):3897-904.
591. *Identification of proteoglycans as the APRIL-specific binding partners.* Ingold K, Zumsteg A, Tardivel A, Huard B, Steiner QG, Cachero TG,

- Qiang F, Gorelik L, Kalled SL, Acha-Orbea H, Rennert PD, Tschopp J, Schneider P. 2005, *J Exp Med.* , pp. 201(9):1375-83.
592. *Heparan sulfate proteoglycan binding promotes APRIL-induced tumor cell proliferation.* Hendriks J, Planelles L, de Jong-Odding J, Hardenberg G, Pals ST, Hahne M, Spaargaren M, Medema JP. 2005, *Cell Death Differ.* , pp. 12(6):637-48.
593. *The role of APRIL and BAFF in lymphocyte activation.* Schneider P. 2005, *Curr Opin Immunol.*, pp. 17(3):282-9.
594. *Selective activation of TACI by syndecan-2.* Bischof D, Elsawa SF, Mantchev G, Yoon J, Michels GE, Nilson A, Sutor SL, Platt JL, Ansell SM, von Bulow G, Bram RJ. 2006, *Blood*, pp. 107(8):3235-42.
595. *BCMAP: an integral membrane protein in the Golgi apparatus of human mature B lymphocytes.* Gras MP, Laâbi Y, Linares-Cruz G, Blondel MO, Rigaut JP, Brouet JC, Leca G, Haguenaer-Tsapis R, Tsapis A. 1995, *Int Immunol.* , pp. 7(7):1093-106.
596. *B cell maturation protein is a receptor for the tumor necrosis factor family member TALL-1.* Shu HB, Johnson H., 2000, *Proc Natl Acad Sci U S A.* , pp. 97(16):9156-61.
597. *TNF receptor family member BCMA (B cell maturation) associates with TNF receptor-associated factor (TRAF) 1, TRAF2, and TRAF3 and activates NF-kappa B, elk-1, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase.* Hatzoglou A, Roussel J, Bourgeade MF, Rogier E, Madry C, Inoue J, Devergne O, Tsapis A. 2000, *J Immunol*, pp. 165(3):1322-30.
598. *TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production.* Xu LG, Shu HB., 2002, *J Immunol.*, pp. 169(12):6883-9.
599. *Key molecular contacts promote recognition of the BAFF receptor by TNF receptor-associated factor 3: implications for intracellular signaling regulation.* Ni CZ, Oganessian G, Welsh K, Zhu X, Reed JC, Satterthwait AC, Cheng G, Ely KR. 2004, *J Immunol.* , pp. 173(12):7394-400.
600. *The two NF-kappaB activation pathways and their role in innate and adaptive immunity.* Bonizzi G, Karin M., 2004, *Trends Immunol.* , pp. 25(6):280-8.
601. *TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-kappaB pathway by TRAF-binding TNFRs.* Hauer J, Püschner S, Ramakrishnan P, Simon U, Bongers M, Federle C, Engelmann H. 2005, *Proc Natl Acad Sci U S A.* , pp. 102(8):2874-9.
602. *BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells.* Claudio E, Brown K, Park S, Wang H, Siebenlist U. 2002, *Nat Immunol.* , pp. 3(10):958-65.
603. *IkappaB kinase alpha is essential for mature B cell development and function.* Kaisho T, Takeda K, Tsujimura T, Kawai T, Nomura F, Terada N, Akira S. 2001, *J Exp Med.*, pp. 193(4):417-26.
604. *Requirement for NF-kappaB in osteoclast and B-cell development.* Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonard A, Tran T, Boyce BF, Siebenlist U. 1997, *Genes Dev*, pp. 11(24):3482-96.
605. *NF-kappa B1 p50 is required for BlyS attenuation of apoptosis but dispensable for processing of NF-kappa B2 p100 to p52 in quiescent mature B cells.* Hatada EN, Do RK, Orlofsky A, Liou HC, Prystowsky M, MacLennan IC, Caamano J, Chen-Kiang S. 2003, *J Immunol.* , pp. 171(2):761-8.
606. *Role of NF kappa B activator Act1 in CD40-mediated signaling in epithelial cells.* Qian Y, Zhao Z, Jiang Z, Li X., 2002, *Proc Natl Acad Sci U S A.* , pp. 99(14):9386-91.
607. *Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent*



- immune responses. Xu Y, Cheng G, Baltimore D. 1996, *Immunity*, pp. 5(5):407-15.
608. Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. Liao G, Zhang M, Harhaj EW, Sun SC. 2004, *J Biol Chem.*, pp. 279(25):26243-50.
609. TRAF2 differentially regulates the canonical and noncanonical pathways of NF-kappaB activation in mature B cells. Grech AP, Amesbury M, Chan T, Gardam S, Basten A, Brink R., 2004, *Immunity*, pp. 21(5):629-42.
610. Receptor-specific signaling for both the alternative and the canonical NF-kappaB activation pathways by NF-kappaB-inducing kinase. Ramakrishnan P, Wang W, Wallach D. 2004, *Immunity*, pp. 21(4):477-89.
611. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Chen C, Edelstein LC, Gélinas C. 2000, *Mol Cell Biol.*, pp. 20(8):2687-95.
612. NF- kappa B2/p100 induces Bcl-2 expression. Viatour P, Bentires-Alj M, Chariot A, Derogowski V, de Leval L, Merville MP, Bours V. 2003, *Leukemia*, pp. 17(7):1349-56.
613. The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. Grossmann M, O'Reilly LA, Gugasyan R, Strasser A, Adams JM, Gerondakis S. 2000, *EMBO J.*, pp. 19(23):6351-60.
614. Cutting edge: BlyS enables survival of transitional and mature B cells through distinct mediators. Hsu BL, Harless SM, Lindsley RC, Hilbert DM, Cancro MP. 2002, *J Immunol.*, pp. 168(12):5993-6.
615. Nuclear factor-kappaB activation via tyrosine phosphorylation of inhibitor kappaB-alpha is crucial for ciliary neurotrophic factor-promoted neurite growth from developing neurons. Gallagher D, Gutierrez H, Gavalda N, O'Keefe G, Hay R, Davies AM. s.l.: *J Neurosci.*, 2007, Vols. 27(36):9664-9.
616. Current and future uses of real-time polymerase chain reaction and microarrays in the study of intestinal microbiota, and probiotic use and effectiveness. Carey, Christine M., et al. 2007, *Canadian Journal of Microbiology*, pp. 537-550(14).
617. Detection of sickle cell gene by analysis of amplified DNA sequences. Huang SZ, Sheng M, Zhao JQ, Qiu XK, Zeng YT, Wang QS, He MX, Zhu JM, Liu WP, Li WW. 1989, *Yi Chuan Xue Bao*, pp. 16(6):475-82.
618. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. MA, Sirover. 1999, *Biochim Biophys Acta*, pp. 1432(2):159-84.
619. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Shi Y, Massagué J. 2003, *Cell*, pp. 113(6):685-700.
620. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Siegel PM, Massagué J. 2003, *Nat Rev Cancer*, pp. 3(11):807-21.
621. Two major Smad pathways in TGF-beta superfamily signalling. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. 2002, *Genes Cells*, pp. 7(12):1191-204.
622. TGF-beta signaling in tumor suppression and cancer progression. Derynck R, Akhurst RJ, Balmain A. 2001, *Nat Genet*, pp. 29(2):117-29.
623. Bone morphogenetic proteins. Chen D, Zhao M, Mundy GR. 2004, *Growth Factors*, pp. 22(4):233-41.
624. What MAN1 does to the Smads. TGFbeta/BMP signaling and the nuclear envelope. Bengtsson, L. 2007, *FEBS J*, pp. 274(6):1374-82.
625. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M. 1988, *Cancer Res*, pp. 48(5):1159-61.

626. *Inhibition of matrix metalloproteinases blocks lethal hepatitis and apoptosis induced by tumor necrosis factor and allows safe antitumor therapy.* Wielockx B, Lannoy K, Shapiro SD, Itoh T, Itohara S, Vandekerckhove J, Libert C. 2001, Nat Med, pp. 7(11):1202-8.
627. *Isolated limb perfusion with tumor necrosis factor and melphalan for nonresectable sSewart-Treves lymphangiosarcoma.* Lans TE, de Wilt JH, van Geel AN, Eggermont AM. 2002, Ann Surg Oncol, pp. 9(10):1004-9.
628. *Tube or not tube: remodeling epithelial tissues by branching morphogenesis.* Affolter M, Bellusci S, Itoh N, Shilo B, Thiery JP, Werb Z. 2003, Dev Cell, pp. 4(1):11-8.
629. *Wnt signaling: a common theme in animal development.* Cadigan KM, Nusse R. 1997, Genes Dev, pp. 11(24):3286-305.
630. *Transcriptional control by the TGF-beta/Smad signaling system.* Massagué J, Wotton D. 2000, EMBO J, pp. 19(8):1745-54.
631. *Epithelial-mesenchymal transitions in tumour progression.* Thiery, JP. 2002, Nat Rev Cancer, pp. 2(6):442-54.
632. *Intercellular adhesion, signalling and the cytoskeleton.* Jamora C, Fuchs E. 2002, Nat Cell Biol, pp. 4(4):E101-8.
633. *TNF receptor subtype signalling: differences and cellular consequences.* MacEwan, DJ. 2002, Cell Signal, pp. 14(6):477-92.
634. *BMP7 null mutation in mice: developmental defects in skeleton, kidney, and eye.* Jena N, Martín-Seisdedos C, McCue P, Croce CM. 1997, Exp Cell Res, pp. 230(1):28-37.
635. *The Noggin null mouse phenotype is strain dependent and haploinsufficiency leads to skeletal defects.* Tylzanowski P, Mebis L, Luyten FP. 2006, Dev Dyn, pp. 235(6):1599-607.
636. *WNTs in the vertebrate nervous system: from patterning to neuronal connectivity.* Ciani L, Salinas PC., 2005, Nat Rev Neurosci., pp. 6(5):351-62.
637. *Morphogens as growth cone signalling molecules.* Sánchez-Camacho C, Rodríguez J, Ruiz JM, Trousse F, Bovolenta P. 2005, Brain Res Brain Res Rev. , pp. 49(2):242-52.
638. *Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family.* Brown SD, Twells RC, Hey PJ, Cox RD, Levy ER, Soderman AR, Metzker ML, Caskey CT, Todd JA, Hess JF. 1998, Biochem Biophys Res Commun. , pp. 248(3):879-88.
639. *A new low density lipoprotein receptor related protein, LRP5, is expressed in hepatocytes and adrenal cortex, and recognizes apolipoprotein E.* Kim DH, Inagaki Y, Suzuki T, Ioka RX, Yoshioka SZ, Magoori K, Kang MJ, Cho Y, Nakano AZ, Liu Q, Fujino T, Suzuki H, Sasano H, Yamamoto TT. 1998, J Biochem. , pp. 124(6):1072-6.
640. *Cloning of a novel member of the low-density lipoprotein receptor family.* Hey PJ, Twells RC, Phillips MS, Yusuke Nakagawa , Brown SD, Kawaguchi Y, Cox R, Guochun Xie , Dugan V, Hammond H, Metzker ML, Todd JA, Hess JF. 1998, Gene, pp. 216(1):103-11.
641. *Molecular cloning and characterization of LR3, a novel LDL receptor family protein with mitogenic activity.* Dong Y, Lathrop W, Weaver D, Qiu Q, Cini J, Bertolini D, Chen D. 1998, Biochem Biophys Res Commun. , pp. 251(3):784-90.
642. *Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer.* Leyns L, Bouwmeester T, Kim SH, Piccolo S, De Robertis EM. 1997, Cell, pp. 88(6):747-56.
643. *A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors.* Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. 1997, Proc Natl Acad Sci U S A., pp. 94(7):2859-63.

644. *Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8.* Wang S, Krinks M, Lin K, Luyten FP, Moos M Jr. 1997, *Cell*, pp. 88(6):757-66.
645. *A new secreted protein that binds to Wnt proteins and inhibits their activities.* Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, Nusse R, Dawid IB, Nathans J. 1999, *Nature*, pp. 398(6726):431-6.
646. *Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus.* Glinka A, Wu W, Onichtchouk D, Blumenstock C, Niehrs C. 1997, *Nature*, pp. 389(6650):517-9.
647. *The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals.* Piccolo S, Agius E, Leyns L, Bhattacharyya S, Grunz H, Bouwmeester T, De Robertis EM. 1999, *Nature*, pp. 397(6721):707-10.
648. *The specification of dorsal cell fates in the vertebrate central nervous system.* Lee KJ, Jessell TM., 1999, *Annu Rev Neurosci.*, pp. 22:261-94.
649. *Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm.* Liem KF Jr, Tremml G, Roelink H, Jessell TM. 1995, *Cell*, pp. 82(6):969-79.
650. *Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development.* Lyons KM, Hogan BL, Robertson EJ., 1995, *Mech Dev.*, pp. 50(1):71-83.
651. *BMP-7 influences pattern and growth of the developing hindbrain of mouse embryos.* Arkell R, Beddington RS., 1997, *Development*, pp. 124(1):1-12.
652. *Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos.* Dudley AT, Robertson EJ., 1997, *Dev Dyn.*, pp. 208(3):349-62.
653. *Neural fold formation at newly created boundaries between neural plate and epidermis in the axolotl.* Moury JD, Jacobson AG., 1989, *Dev Biol.*, pp. 133(1):44-57.
654. *Dorsalization of the neural tube by the non-neural ectoderm.* Dickinson ME, Selleck MA, McMahon AP, Bronner-Fraser M. 1995, *Development*, pp. 121(7):2099-106.
655. *A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord.* Liem KF Jr, Tremml G, Jessell TM. 1997, *Cell*, pp. 91(1):127-38.
656. *Involvement of Bone Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse.* Jones CM, Lyons KM, Hogan BL., 1991, *Development*, pp. 111(2):531-42.
657. *Development of bone morphogenetic protein receptors in the nervous system and possible roles in regulating trkC expression.* Zhang D, Mehler MF, Song Q, Kessler JA. 1998, *J Neurosci.*, pp. 18(9):3314-26.
658. *Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4.* ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. 1994, *J Biol Chem.*, pp. 269(25):16985-8.
659. *Identification of a human type II receptor for bone morphogenetic protein-4 that forms differential heteromeric complexes with bone morphogenetic protein type I receptors.* Nohno T, Ishikawa T, Saito T, Hosokawa K, Noji S, Wolsing DH, Rosenbaum JS. 1995, *J Biol Chem.*, pp. 270(38):22522-6.
660. *Cloning and characterization of a human type II receptor for bone morphogenetic proteins.* Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K. 1995, *Proc Natl Acad Sci U S A.*, pp. 92(17):7632-6.
661. *Potentiating interactions between morphogenetic protein and neurotrophic factors*

- in developing neurons.* Bengtsson H, Söderström S, Kylberg A, Charette MF, Ebendal T. 1998, *J Neurosci Res.* , pp. 53(5):559-68.
662. *Bone morphogenetic protein-5 (BMP-5) promotes dendritic growth in cultured sympathetic neurons.* Beck HN, Drahushuk K, Jacoby DB, Higgins D, Lein PJ. 2001, *BMC Neurosci.* , p. 2:12.
663. *BMPs as mediators of roof plate repulsion of commissural neurons.* Augsburger A, Schuchardt A, Hoskins S, Dodd J, Butler S., 1999, *Neuron*, pp. 24(1):127-41.
664. *A role for BMP heterodimers in roof plate-mediated repulsion of commissural axons.* Butler SJ, Dodd J., 2003, *Neuron*, pp. 38(3):389-401.
665. *Glia induce dendritic growth in cultured sympathetic neurons by modulating the balance between bone morphogenetic proteins (BMPs) and BMP antagonists.* Lein PJ, Beck HN, Chandrasekaran V, Gallagher PJ, Chen HL, Lin Y, Guo X, Kaplan PL, Tiedge H, Higgins D. 2002, *J Neurosci.* , pp. 22(23):10377-87.
666. *Bone morphogenetic protein-7 stimulates initial dendritic growth in sympathetic neurons through an intracellular fibroblast growth factor signaling pathway.* Horbinski C, Stachowiak EK, Chandrasekaran V, Miuzukoshi E, Higgins D, Stachowiak MK. 2002, *J Neurochem.* , pp. 80(1):54-63.
667. *The role of bone morphogenetic proteins in sympathetic neuron development.* Rohrer H. 2003, *Drug News Perspect.* , pp. 16(9):589-96.
668. *Target-derived BMP signaling limits sensory neuron number and the extent of peripheral innervation in vivo.* Guha U, Gomes WA, Samanta J, Gupta M, Rice FL, Kessler JA. 2004, *Development.* , pp. 131(5):1175-86.
669. *Specification of catecholaminergic and serotonergic neurons.* Goridis C, Rohrer H. 2002, *Nat Rev Neurosci.* , pp. 3(7):531-41.
670. *Mechanisms and perspectives on differentiation of autonomic neurons.* Howard MJ. 2005, *Dev Biol.* , pp. 277(2):271-86.
671. *Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity.* Baur ST, Mai JJ, Dymecki SM. 2000, *Development*, pp. 127(3):605-19.
672. *TGF- $\beta$ 1 activates two distinct type I receptors in neurons: implications for neuronal NF- $\kappa$ B signaling.* König HG, Kögel D, Rami A, Prehn JH. 2005, *J Cell Biol.* , pp. 168(7):1077-86.
673. *The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor- $\kappa$ B activation.* Brown SA, Richards CM, Hanscom HN, Feng SL, Winkles JA. 2003, *Biochem J.* , pp. 371(Pt 2):395-403.
674. *Fibroblast growth factor-inducible 14 mediates multiple pathways of TWEAK-induced cell death.* Nakayama M, Ishidoh K, Kojima Y, Harada N, Kominami E, Okumura K, Yagita H. 2003, *J Immunol.* , pp. 170(1):341-8.
675. *TWEAK is expressed by glial cells, induces astrocyte proliferation and increases EAE severity.* Desplat-Jégo S, Varriale S, Creidy R, Terra R, Bernard D, Khrestchatsky M, Izui S, Chicheportiche Y, Boucraut J. 2002, *J Neuroimmunol.* , pp. 133(1-2):116-23.
676. *TWEAK stimulation of astrocytes and the proinflammatory consequences.* Saas P, Boucraut J, Walker PR, Quiquerez AL, Billot M, Desplat-Jégo S, Chicheportiche Y, Dietrich PY. 2000, *Glia.*, pp. 32(1):102-7.
677. *Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice.* Cafferty WB, Gardiner NJ, Das P, Qiu J, McMahon SB, Thompson SW. 2004, *J Neurosci.* , pp. 24(18):4432-43.
678. *Expression of interleukin-6 and its receptor in the sciatic nerve and cultured Schwann cells: relation to 18-kD fibroblast growth factor-2.*

- Grothe C, Heese K, Meisinger C, Wewetzer K, Kunz D, Cattini P, Otten U. 2000, *Brain Res.* , pp. 885(2):172-81.
679. *Costimulation of T cells by OX40, 4-1BB, and CD27.* Croft M. 2003, *Cytokine Growth Factor Rev.* , pp. 14(3-4):265-73.
680. *OX40-OX40 ligand interaction through T cell-T cell contact contributes to CD4 T cell longevity.* Soroosh P, Ine S, Sugamura K, Ishii N. 2006, *J Immunol.* , pp. 176(10):5975-87.
681. *Amelioration of experimental autoimmune encephalomyelitis with anti-OX40 ligand monoclonal antibody: a critical role for OX40 ligand in migration, but not development, of pathogenic T cells.* Nohara C, Akiba H, Nakajima A, Inoue A, Koh CS, Ohshima H, Yagita H, Mizuno Y, Okumura K. 2001, *J Immunol.* , pp. 166(3):2108-15.
682. *Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule related to nerve growth factor receptor.* Mallett S, Fossum S, Barclay AN. 1990, *EMBO J.* , pp. 9(4):1063-8.
683. *The tumor necrosis factor ligand and receptor families.* Bazzoni F, Beutler B. 1996, *N Engl J Med.*, pp. 334(26):1717-25.
684. *TNFR1 signalling is critical for the development of demyelination and the limitation of T-cell responses during immune-mediated CNS disease.* Probert L, Eugster HP, Akassoglou K, Bauer J, Frei K, Lassmann H, Fontana A. 2000, *Brain*, pp. 123 ( Pt 10):2005-19.
685. *T cell co-stimulatory molecules other than CD28.* Watts TH, DeBenedette MA. 1999, *Curr Opin Immunol.* , pp. 11(3):286-93.
686. *Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses.* Gramaglia I, Weinberg AD, Lemon M, Croft M. 1998, *J Immunol.* , pp. 161(12):6510-7.
687. *Identification of OX40 ligand and preliminary characterization of its activities on OX40 receptor.* Baum PR, Gayle RB 3rd, Ramsdell F, Srinivasan S, Sorensen RA, Watson ML, Seldin MF, Clifford KN, Grabstein K, Alderson MR, et al. 1994, *Circ Shock.* , pp. 44(1):30-4.
688. *Affinity and kinetics of the interaction between soluble trimeric OX40 ligand, a member of the tumor necrosis factor superfamily, and its receptor OX40 on activated T cells.* Al-Shamkhani A, Mallett S, Brown MH, James W, Barclay AN. 1997, *J Biol Chem.* , pp. 272(8):5275-82.
689. *Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells.* Stüber E, Neurath M, Calderhead D, Fell HP, Strober W. 1995, *Immunity.* , pp. 2(5):507-21.
690. *CD28-independent costimulation of T cells by OX40 ligand and CD70 on activated B cells.* Akiba H, Oshima H, Takeda K, Atsuta M, Nakano H, Nakajima A, Nohara C, Yagita H, Okumura K. 1999, *J Immunol.* , pp. 162(12):7058-66.
691. *Expression and function of OX40 ligand on human dendritic cells.* Ohshima Y, Tanaka Y, Tozawa H, Takahashi Y, Maliszewski C, Delespesse G. 1997, *J Immunol.* , pp. 159(8):3838-48.
692. *The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells.* Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, Imamura S, Uchiyama T. 1996, *J Exp Med.* , pp. 183(5):2185-95.
693. *TNFalpha contributes to the death of NGF-dependent neurons during development.* Barker V, Middleton G, Davey F, Davies AM. 2001, *Nat Neurosci.* , pp. 4(12):1194-8.
694. *Role of CD40 ligand in amyloidosis in transgenic Alzheimer's mice.* Tan J, Town T, Crawford F, Mori T, DelleDonne A, Crescentini R, Obregon D, Flavell RA, Mullan MJ. 2002, *Nat Neurosci.*, pp. 5(12):1288-93.

695. *Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis.* Sun Y, Lin X, Chen HM, Wu Q, Subudhi SK, Chen L, Fu YX. 2002, *J Immunol.*, pp. 168(3):1457-65.
696. *Role of 4-1BB in immune responses.* Vinay DS, Kwon BS., 1998, *Semin Immunol.* , pp. 10(6):481-9.
697. *TL1A-induced NF-kappaB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells.* Wen L, Zhuang L, Luo X, Wei P. 2003, *J Biol Chem.* , pp. 278(40):39251-8.
698. *TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator.* Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P. 2002, *Immunity.* , pp. 16(3):479-92.
699. *Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease.* Bamias G, Martin C 3rd, Marini M, Hoang S, Mishina M, Ross WG, Sachedina MA, Friel CM, Mize J, Bickston SJ, Pizarro TT, Wei P, Cominelli F. 2003, *J Immunol.* , pp. 171(9):4868-74.
700. *Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-gamma, in mucosal inflammation.* Prehn JL, Mehdizadeh S, Landers CJ, Luo X, Cha SC, Wei P, Targan SR. 2004, *Clin Immunol.*, pp. 112(1):66-77.
701. *Role of TL1A and its receptor DR3 in two models of chronic murine ileitis.* Bamias G, Mishina M, Nyce M, Ross WG, Kollias G, Rivera-Nieves J, Pizarro TT, Cominelli F. 2006, *Proc Natl Acad Sci U S A.* , pp. 103(22):8441-6.
702. *Message in a bottle: long-range retrograde signaling in the nervous system.* Ibáñez CF. 2007, *Trends Cell Biol.* , pp. 17(11):519-28.
703. *Cell death during development of the nervous system.* Oppenheim RW. 1991, *Annu Rev Neurosci.* , pp. 14:453-501.
704. *NGF mRNA expression in developing cutaneous epithelium related to innervation density.* Harper S, Davies AM., 1990, *Development*, pp. 110(2):515-9.
705. *The nerve growth factor family.* Barde YA. 1990, *Prog Growth Factor Res.* , pp. 2(4):237-48.
706. *Target-derived neurotrophic factors regulate the death of developing forebrain neurons after a change in their trophic requirements.* Lotto RB, Asavaritikrai P, Vali L, Price DJ., 2001, *J Neurosci.*, pp. 21(11):3904-10.
707. *GDNF is expressed in two forms in many tissues outside the CNS.* Suter-Crazzolara C, Unsicker K., 1994, *Neuroreport.*, pp. 5(18):2486-8.
708. *GDNF is an age-specific survival factor for sensory and autonomic neurons.* Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM. 1995, *Neuron*, pp. 15(4):821-8.
709. *Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons.* Trupp M, Rydén M, Jörnvall H, Funakoshi H, Timmusk T, Arenas E, Ibáñez CF. 1995, *J Cell Biol.* , pp. 130(1):137-48.
710. *Focal expression of glial cell line-derived neurotrophic factor in developing mouse limb bud.* Wright DE, Snider WD., 1996, *Cell Tissue Res.* , pp. 286(2):209-17.
711. *IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life.* Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD. 1997, *Neuron*, pp. 19(4):849-61.
712. *Renal and neuronal abnormalities in mice lacking GDNF.* Moore MW, Klein RD, Farifás I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A. 1996, *Nature*, pp. 382(6586):76-9.

713. *Defects in enteric innervation and kidney development in mice lacking GDNF.* Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. 1996, *Nature*, pp. 382(6586):73-6.
714. *Renal agenesis and the absence of enteric neurons in mice lacking GDNF.* Sánchez MP, Silos-Santiago I, Frisé J, He B, Lira SA, Barbacid M. 1996, *Nature*, pp. 382(6586):70-3.
715. *Biological effects of targeted inactivation of hepatocyte growth factor-like protein in mice.* Bezerra JA, Carrick TL, Degen JL, Witte D, Degen SJ. 1998, *J Clin Invest.* , pp. 101(5):1175-83.
716. *RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP.* Gaudino G, Follenzi A, Naldini L, Collesi C, Santoro M, Gallo KA, Godowski PJ, Comoglio PM. 1994, *EMBO J.* , pp. 13(15):3524-32.
717. *Characterization of the mouse cDNA and gene coding for a hepatocyte growth factor-like protein: expression during development.* Degen SJ, Stuart LA, Han S, Jamison CS. 1991, *Biochemistry.*, pp. 30(40):9781-91.
718. *Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration.* Matsumoto K, Nakamura T., 1997, *Biochem Biophys Res Commun.*, pp. 239(3):639-44.
719. *Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons.* Maina F, Hilton MC, Ponzetto C, Davies AM, Klein R. 1997, *Genes Dev.* , pp. 11(24):3341-50.
720. *Transforming growth factor production by chemically transformed cells.* Moses HL, Branum EL, Proper JA, Robinson RA. 1981, *Cancer Res.* , pp. 41(7):2842-8.
721. *Presence of cartilage-derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement in vitro.* Erlacher L, Ng CK, Ullrich R, Krieger S, Luyten FP., 1998, *Arthritis Rheum.* , pp. 41(2):263-73.
722. *Bone morphogenetic protein signaling is required for maintenance of differentiated phenotype, control of proliferation, and hypertrophy in chondrocytes.* Enomoto-Iwamoto M, Iwamoto M, Mukudai Y, Kawakami Y, Nohno T, Higuchi Y, Takemoto S, Ohuchi H, Noji S, Kurisu K. 1998, *J Cell Biol.* , pp. 140(2):409-18.
723. *Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis.* Dewulf N, Verschuere K, Lonnoy O, Morén A, Grimsby S, Vande Spiegle K, Miyazono K, Huylebroeck D, Ten Dijke P. 1995, *Endocrinology*, pp. 136(6):2652-63.
724. *Background Activity of Neurons of the Superior Cervical Ganglion That Innervate the Submandibular Gland in the Rat.* Maslov VY. 2005, *Neurophysiology*, pp. Vol. 37, No. 1, pp. 26-31.
725. *Evidence for the existence of an outflow of noradrenaline nerve fibres in the ventral roots of the rat spinal cord.* Dahlström A, Fuxe K., 1965, *Experientia.* , pp. 21(7):409-10.
726. *Adrenergic innervation of the nasal mucosa in cat. A histological and physiological study.* Anggård A, Densert O., 1974, *Acta Otolaryngol.* , pp. 78(3-4):232-41.
727. *GFR alpha3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion.* Nishino J, Mochida K, Ohfuji Y, Shimazaki T, Meno C, Ohishi S, Matsuda Y, Fujii H, Saijoh Y, Hamada H. 1999, *Neuron*, pp. 23(4):725-36.
728. *RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons.* Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson EM Jr, Milbrandt J. 2001, *Development*, pp. 128(20):3963-74.
729. *Prenatal and postnatal requirements of NT-3 for sympathetic neuroblast survival and innervation of specific targets.* ElShamy WM,

- Linnarsson S, Lee KF, Jaenisch R, Ernfors P. 1996, *Development*, pp. 122(2):491-500.
730. *Developmental changes in neurite outgrowth responses of dorsal root and sympathetic ganglia to GDNF, neurturin, and artemin.* Yan H, Newgreen DF, Young HM. 2003, *Dev Dyn.*, pp. 227(3):395-401.
731. *Characterization of growth/differentiation factor 5 (GDF-5) as a neurotrophic factor for cultured neurons from chicken dorsal root ganglia.* Farkas LM, Scheuermann S, Pohl J, Unsicker K, Krieglstein K. 1997, *Neurosci Lett.*, pp. 236(2):120-2.
732. *Neuroprotective effects of delayed administration of growth/differentiation factor-5 in the partial lesion model of Parkinson's disease.* Hurley FM, Costello DJ, Sullivan AM. 2004, *Exp Neurol.*, pp. 185(2):281-9.
733. *Bone morphogenetic protein-7 stimulates initial dendritic growth in sympathetic neurons through an intracellular fibroblast growth factor signaling pathway.* Horbinski C, Stachowiak EK, Chandrasekaran V, Miuzukoshi E, Higgins D, Stachowiak MK. 2002, *J Neurochem.*, pp. 80(1):54-63.
734. *Osteogenic protein-1 induces dendritic growth in rat sympathetic neurons.* Lein P, Johnson M, Guo X, Rueger D, Higgins D. 1995, *Neuron*, pp. 15(3):597-605.
735. *OP-1 enhances dendritic growth from cerebral cortical neurons in vitro.* Le Roux P, Behar S, Higgins D, Charette M. 1999, *Exp Neurol.*, pp. 160(1):151-63.
736. *Bone morphogenetic protein-7 enhances dendritic growth and receptivity to innervation in cultured hippocampal neurons.* Withers GS, Higgins D, Charette M, Banker G. 2000, *Eur J Neurosci.*, pp. 12(1):106-16.
737. *Signaling mechanisms underlying dendrite formation.* Miller FD, Kaplan DR. 2003, *Curr Opin Neurobiol.*, pp. 13(3):391-8.
738. *Target-dependent inhibition of sympathetic neuron growth via modulation of a BMP signaling pathway.* Moon JI, Birren SJ., 2008, *Dev Biol.*, pp. 315(2):404-17.
739. *BMP inhibits neurite growth by a mechanism dependent on LIM-kinase.* Matsuura I, Endo M, Hata K, Kubo T, Yamaguchi A, Saeki N, Yamashita T. 2007, *Biochem Biophys Res Commun.*, pp. 360(4):868-73.
740. *Culture conditions determine the prevalence of bipolar and monopolar neurons in cultures of dissociated spiral ganglion.* Whitlon DS, Grover M, Tristano J, Williams T, Coulson MT. 2007, *Neuroscience.*, pp. 146(2):833-40.
741. *Noggin is a negative regulator of neuronal differentiation in developing neocortex.* Li W, LoTurco JJ., 2000, *Dev Neurosci.*, pp. 22(1-2):68-73.
742. *The Smad pathway.* Wrana JL, Attisano L. 2000, *Cytokine Growth Factor Rev.*, pp. 11(1-2):5-13.
743. *Cleft hand/foot: clinical and developmental aspects.* Buss PW. 1994, *J Med Genet.*, pp. 31(9):726-30.
744. *Distal limb malformations: underlying mechanisms and clinical associations.* Sifakis S, Basel D, Ianakiev P, Kilpatrick M, Tsipouras P. 2001, *Clin Genet.*, pp. 60(3):165-72.
745. *Regulation of Dlx5 and Dlx6 gene expression by p63 is involved in EEC and SHFM congenital limb defects.* Lo Iacono N, Mantero S, Chiarelli A, Garcia E, Mills AA, Morasso MI, Costanzo A, Levi G, Guerrini L, Merlo GR. 2008, *Development*, pp. 135(7):1377-88.
746. *PITX2 gain-of-function induced defects in mouse forelimb development.* Holmberg J, Ingner G, Johansson C, Leander P, Hjalt TA. 2008, *BMC Dev Biol.*, p. 8:25.
747. *Abnormal sympathoadrenal development and systemic hypotension in PHD3<sup>-/-</sup> mice.* Bishop T, Gallagher D, Pascual A, Lygate CA, de Bono JP, Nicholls LG, Ortega-Saenz P, Oster H,



- Wijeyekoon B, Sutherland AI, Grosfeld A, Aragonés J, Schneider M, van Geyte K, Teixeira D, Diez-Juan A, Lopez-Barneo J, Channon KM, Maxwell PH, Pugh CW, Davies. 2008, *Mol Cell Biol.* , pp. 28(10):3386-400.
748. *Regulation of neuronal survival and death by extracellular signals during development.* AM., Davies. 2003, *EMBO J*, pp. 22(11):2537-45.
749. *Nerve growth factor enhances neurite arborization of adult sensory neurons; a study in single-cell culture.* Yasuda T, Sobue G, Ito T, Mitsuma T, Takahashi A., 1990, *Brain Res.*, pp. 524(1):54-63.
750. *Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat.* Diamond J, Holmes M, Coughlin M. 1992, *J Neurosci.* , p. *J Neurosci.* .
751. *Localized sources of neurotrophins initiate axon collateral sprouting.* Gallo G, Letourneau PC., 1998, *J Neurosci.*, pp. 18(14):5403-14.
752. *Chemotactic response of nerve fiber elongation to nerve growth factor.* Letourneau PC. 1978, *Dev Biol.*, pp. 66(1):183-96.
753. *Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor.* Gundersen RW, Barrett JN., 1979, *Science*, pp. 206(4422):1079-80.
754. *The trkA receptor mediates growth cone turning toward a localized source of nerve growth factor.* Gallo G, Lefcort FB, Letourneau PC. 1997, *J Neurosci.* , pp. 17(14):5445-54.
755. *Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons.* Paves H, Saarma M., 1997, *Cell Tissue Res.* , pp. 290(2):285-97.
756. *Null mutations for exon III and exon IV of the p75 neurotrophin receptor gene enhance sympathetic sprouting in response to elevated levels of nerve growth factor in transgenic mice.* Dhanoa NK, Krol KM, Jahed A, Crutcher KA, Kawaja MD. 2006, *Exp Neurol.* , pp. 198(2):416-26.
757. *Deletion of Nfl in neurons induces increased axon collateral branching after dorsal root injury.* Romero MI, Lin L, Lush ME, Lei L, Parada LF, Zhu Y. 2007, *J Neurosci.* , pp. 27(8):2124-34.
758. *A conditioning lesion enhances sympathetic neurite outgrowth.* Shoemaker SE, Sachs HH, Vaccariello SA, Zigmond RE. 2005, *Exp Neurol.* , pp. 194(2):432-43.
759. *Comparison of target innervation by sympathetic axons in adult wild type and heterozygous mice for nerve growth factor or its receptor trkA.* Ghasemlou N, Krol KM, Macdonald DR, Kawaja MD. 2004, *J Pineal Res.* , pp. 37(4):230-40.
760. *Morphological alterations in the peripheral and central nervous systems of mice lacking glial cell line-derived neurotrophic factor (GDNF): immunohistochemical studies.* Granholm AC, Srivastava N, Mott JL, Henry S, Henry M, Westphal H, Pichel JG, Shen L, Hoffer BJ. 1997, *J Neurosci.*, pp. 17(3):1168-78.
761. *The influences of p75 neurotrophin receptor and brain-derived neurotrophic factor in the sympathetic innervation of target tissues during murine postnatal development.* Jahed A, Kawaja MD. 2005, *Auton Neurosci.* , pp. 118(1-2):32-42.
762. *Programmed cell death in the developing nervous system.* Burek MJ, Oppenheim RW. 1996, *Brain Pathol*, pp. 6(4):427-46.
763. *Programmed cell death: a missing link is found.* Jacobson MD. 1997, *Trends Cell Biol*, pp. 7(12):467-9.
764. *Cell death in development.* Vaux DL, Korsmeyer SJ., 1999, *Cell*, pp. 96(2):245-54.
765. *Bcl-2 gene family in the nervous system.* Merry DE, Korsmeyer SJ., 1997, *Annu Rev Neurosci.* , pp. 20:245-67.
766. *Caspases: the proteases of the apoptotic pathway.* Nuñez G, Benedict MA, Hu Y,

- Inohara N., 1998, *Oncogene* , pp. 17(25):3237-45.
767. *Apoptosis in the nervous system.* Yuan J, Yankner BA., 2000, *Nature*, pp. 407(6805):802-9.
768. *Isolated limb perfusion in the management of locally advanced extremity soft tissue sarcoma.* Eggermont AM. 2003, *Surg Oncol Clin N Am.*, pp. 12(2):469-83.
769. *An important role for B-cell activation factor and B cells in the pathogenesis of Sjögren's syndrome.* Mackay F, Groom JR, Tangye SG. 2007, *Curr Opin Rheumatol*, pp. 19(5):406-13.
770. *Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase.* López-Fraga M, Fernández R, Albar JP, Hahne M. 2001, *EMBO Rep.* , pp. 2(10):945-51.
771. *BAFF, APRIL, TWE-PRIL: who's who?* Daridon C, Youinou P, Pers JO. 2008, *Autoimmun Rev.* , pp. 7(4):267-71.
772. *To switch or not to switch--the opposing roles of TACI in terminal B cell differentiation.* Salzer U, Jennings S, Grimbacher B. 2007, *Eur J Immunol.* , pp. 37(1):17-20.
773. *Living in context with the survival factor BAFF.* Cancro MP. 2008, *Immunity.*, pp. 28(3):300-1.
774. *TRAF2 and TRAF3 signal adapters act cooperatively to control the maturation and survival signals delivered to B cells by the BAFF receptor.* Gardam S, Sierro F, Basten A, Mackay F, Brink R. 2008, *Immunity*, pp. 28(3):391-401.
775. *Analysis of Ret knockin mice reveals a critical role for IKKs, but not PI 3-K, in neurotrophic factor-induced survival of sympathetic neurons.* Encinas M, Rozen EJ, Dolcet X, Jain S, Comella JX, Milbrandt J, Johnson EM Jr. 2008, *Cell Death Differ.* , pp. 15(9):1510-21.

