The role of APRIL in the development of the peripheral nervous system

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Contents

Abstract

Critical to the development of the peripheral nervous system is the regulation of the growth and survival of neurons during the phase of target field innervation. Neurons extend axons towards their target tissues and branch extensively forming complete functional synaptic connections by the end of development.

This study has revealed the first known role for the tumor necrosis superfamily (TNF) member a proliferation inducing ligand (APRIL) in the development of the nervous system. Neurons of the mouse superior cervical ganglion (SCG) were chosen for study as an excellent model of peripheral development. These neurons can be easily dissected and cultured to study their growth and survival under controlled experimental conditions. The target fields innervated by this population of neurons are clearly defined, offering the opportunity to study final target field innervation, meaning this population offers excellent experimental potential.

This study demonstrated for the first time that the mRNA transcripts for the TNF superfamily member APRIL and its receptors B cell maturation antigen (BCMA) and transmembrane activator and cycophylin interactor (TACI) are expressed in the SCG and target fields during the development of these neurons at a time when they are innervating their peripheral targets. Investigation of protein expression demonstrated that these neurons express this ligand and its receptors, localised to the cells themselves as opposed to projections innervating distal target fields.

Functional analysis of the role of APRIL, BCMA and TACI on the growth and survival of SCG neurons *in vitro* demonstrated that, in the

presence of nerve growth factor (NGF) the principal neurotrophin acting on these cells, either treatment with recombinant APRIL or a function blocking antibody to APRIL, enhance the growth of these neurons independent of survival. This effect is clear over a wide developmental window from embryonic day 16 to post natal day three. The ability of recombinant APRIL to enhance this growth requires the presence of a putative heparin sulfate binding domain. In addition to the activity of these factors, function blocking antibodies against the receptors BCMA and TACI also enhance NGF promoted neurite growth as do soluble recombinant forms of the receptors. Interaction with APRIL signalling acts to promote the growth of SCG neurons in culture. This enhancement of growth requires the activity of mitogen-activated protein kinase kinase and Phosphatidylinositide 3-kinase.

Neurons cultured from APRIL null mice grow larger than their wild type counterparts in the presence of NGF. Two target fields innervated by these neurons, the submandibular salivary gland and nasal mucosa are hyperinnervated at post natal day five in APRIL null mice. This phenotype is not evident by post natal day 10 and so points to a transient, but significant, increase in target field innervation.

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Chapter 1

Introduction

An introduction to neuronal development

Introduction

The development of the vertebrate nervous system requires a multitude of highly coordinated mechanisms to function spatially and temporally throughout with the end goal of producing an operative nervous system. The focus of this thesis is centred on neurons that are born, migrate to a final destination within the developing animal and extend processes towards target regions to produce functional synapses and thus a working network. The introduction that follows will give an overview of the major stages involved in neuronal development focusing on the peripheral nervous system (PNS) including regulation of neurite outgrowth and the survival of peripheral neurons. Following this the tumour necrosis factor superfamily (TNFSF) will be introduced with a more detailed examination of its members, a proliferation inducing ligand (APRIL), B cell maturation antigen (BCMA), transmembrane activator and cyclophilin ligand interactor (TACI), B cell activator factor (BAFF) and B cell activator factor receptor (BAFFR). These members are the main focus of research contained within this thesis.

The origins of the nervous system

The earliest stages of vertebrate embryonic development follow the fertilisation of an ovum giving rise to a zygote. Subsequent stages of cell division form a morula and eventually a blastocyst, defined by

the presence of a fluid filled cavity. From this early developmental structure the inner mass of cells will go on to form the embryo in its entirety while the outer cell mass, termed the trophoblast, will generate the placenta and thus support the embryo. The formation of a transient primitive streak evidences the beginnings of gastrulation, the conversion of a dual layered embryo into a three layered one [1].

The three germ layers of an embryo post gastrulation are the ectoderm, mesoderm and endoderm and it is from these layers that all tissues of the adult vertebrate will arise [2]. Conversion to this state from a bi-layer structure comprises a mixture of movement and proliferation. It is the ectoderm that will eventually give rise to the entire vertebrate nervous system in addition to the epidermis [3]. During the process of neural induction, ectodermal cells become elongated and can subsequently be termed neural plate cells.

Early experimental evidence in amphibians successfully identified a structure termed the 'Spemann's Organiser' located within the dorsal blastopore lip of the embryo. A secondary neural axis can be established through transplantation of this tissue into the ventral area of a second embryo[4, 5].

Within the organiser itself, several molecular mechanisms instruct ectodermal cells to take on a neuronal fate. Bone morphogenic proteins (BMPs) are key players in these mechanisms and it has been demonstrated that BMP4 acts to inhibit the establishment of a neural fate and thus promotes epidermal differentiation of the ectoderm[6]. The proteins noggin[7], follistatin[8] and chordin[9] are all inhibitors of BMPs and thus their action prevents the action of BMP4 allowing ectodermal cells to assume a neuronal fate. This simplified view

forms the default model of neural induction but the involvement of other BMP inhibitors including cerberus[10], gremlin[11] and ogon/sizzled[12] adds additional layers of complexity to the system, while the actions of other proteins such as Fibroblast growth factor (FGF), wingless (Wnt) as well as calcium are also involved in the process of neural induction and formation of the neural plate[13].

Formation of the neural tube

The central nervous system (CNS) including the brain and spinal cord form from an embryonic structure termed the neural tube. This structure results through initial thickening of the ectoderm as its cells grow in height and undergo pseudostratification[14]. Following on from the formation of the neural plate is a process called convergent extension which involves laterally located cells moving towards the midline[15, 16]. Regulation by members of the non-canonical Wnt pathway add a layer of control to this process[17]. Lateral borders begin to elevate forming neural folds and these converge at the midline to finally fuse forming a hollow tube partially regulated by Sonic hedgehog[18]. Fusion proceeds rostrally and caudally leaving open ends called neuropores[19].

Initial fusion is maintained by process extension and glycoprotein deposition before permanent cell-cell contacts are established[20]. Not all cells of the neural fold remain as part of the neural tube; instead lateral separation gives rise to a layer of cells located between the neural plate and surface ectoderm called the neural crest[21] (see figure 1).

While the neural tube begins as a more homogenous structure regionalisation soon begins to divide the tube into sub-regions that will go on to develop different structures of the adult CNS. Anterior cephalic portions grow and swell to form primordial brain regions while caudal regions remain more tube like and go on to establish the spinal cord[22].

In the early stages of development, cells located within the neural tube undergo proliferation and eventually exit the cell cycle to become terminally differentiated in peripheral regions of the neural tube.

Exposure to a variety of morphogens along the dorsal ventral axis of the neural tube including BMPs, FGFs, sonic hedgehog and Wnt determine the patterning of the neural tube [23].

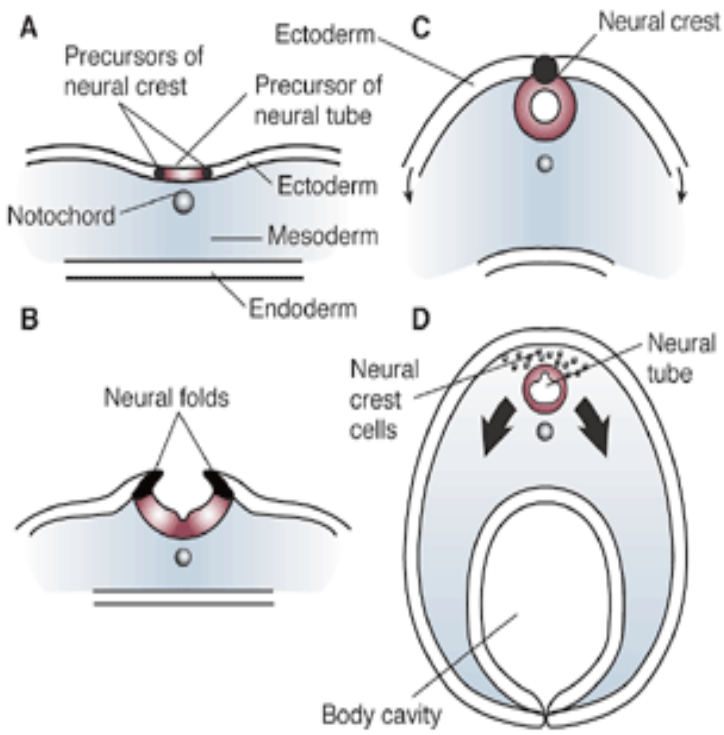


Figure 1: Schematic representation of neural tube formation

Formation of the neural tube (cross view). Early in an embryo's development, a strip of specialized cells called the notochord (A) induces the cells of the ectoderm directly above it to become the primitive nervous system (i.e., neuroepithelium). The neuroepithelium then wrinkles and folds over (B). As the tips of the folds fuse together, a hollow tube (i.e., the neural tube) forms (C) the precursor of the brain and spinal cord. Meanwhile, the ectoderm and endoderm continue to curve around and fuse beneath the embryo to create the body cavity, completing the transformation of the embryo from a flattened disk to a three-dimensional body. Cells originating from the fused tips of the neuroectoderm (i.e., neural crest cells) migrate to various locations throughout the embryo, where they will initiate the development of diverse body structures (D) [24].

Neurogenesis

Within the neural tube bi-polar neuroepithelial cells extend from the pial surface through to the ventricular surface. The nuclei of these cells travel back and forth between the two surfaces by interkinetic nuclear migration[25]. While synthesising new DNA at the G1 phase of the cell cycle nuclei can be observed moving towards the pial surface while those that have continued into G2 journey back to the ventricular surface[26]. Upon arrival at the ventricular zone their cyclical journey is completed and these cells undergo division, giving rise to daughter cells. This results in the beginning of a new cycle of proliferation or the accumulation in the intermediate zone developing the swellings of the neural tube destined to become regions of the adult brain (see figure 2).

The earliest cells to withdraw from the cell cycle and thus end proliferation can be found in the hindbrain before those of the spinal cord and ventral mesencephalon. Those cells destined to make up the cortex and cerebellum withdraw later on in development.

The action of cyclin kinase inhibitors on cyclin kinases in addition to the inhibitory action of retinoblastoma protein on EF2 family transcription factors are examples of some regulators of cell cycle termination and thus the beginnings of post-mitotic migration and terminal differentiation[27].

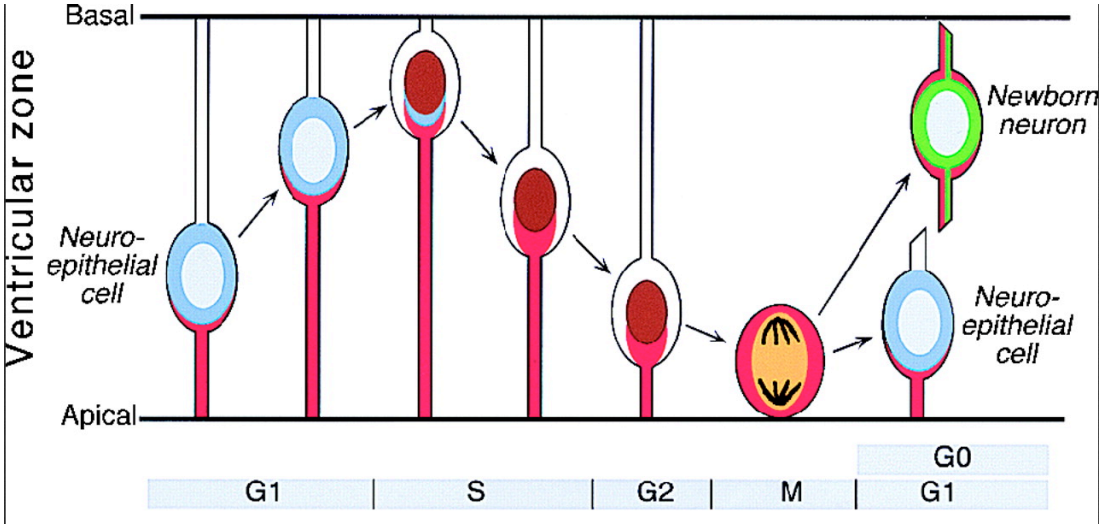


Figure 2: Schematic representation of interkinetic nuclear migration

During neuroepithelial cell division, interkinetic nuclear migration allows the cells to divide unrestricted while maintaining a dense packing. During G1 the cell nucleus migrates to the basal side of the cell and remains there for S phase and migrates to the apical side for G2 phase and undergoes cytokinesis at the apical surface. This migration requires the help of microtubules and actin filaments [28].

Neuronal migration in the CNS

The birth of both neuronal and non-neuronal cells within the CNS often takes place distant from their final location within the adult nervous system. As such migration from an initial site towards the outer surface where specific brain circuits are formed comprises a critical phase of neuronal development.

The vast majority of young neurons within the cerebral cortex travel to their final sites within the CNS by radial migration perpendicular to the surface[29]. Here radial glial cells, extending processes from the pial to ventricular surface of the neural tube, provide a framework along which neurons can migrate[30]. In other brain regions a mixture of radial and tangential migration parallel to the outer surface allow neurons to travel to the correct location and begin establishing neural circuitry[31].

The utilisation of tangential migration has been demonstrated between the medial ganglionic eminence to the neocortex and hippocampus in addition to the olfactory bulb from the lateral ganglionic eminence.

The critical importance of neuronal migration in the formation of a functional nervous system can be demonstrated in several transgenic models. Mice lacking the cell adhesion gene *reeler* demonstrate abnormal development of the cortex, while in another model lacking *weaver,* the cerebellum shows a reduced number of granular cells following incomplete migration via glial fibres and neuronal cell death[32].

Synaptogenesis

Following successful migration and axon extension, neurons go on to establish neurotransmitter release sites aligned with the receptive field of its respective postsynaptic partner. The establishment of these synapses are the foundations of neural connectivity within networks of both neuronal and non-neuronal cells.

The process begins with the extending axon reaching its target postsynaptic partner, be it a neuron, non-neuronal cell or target field. Upon arrival at such a site a synaptic cleft forms between the two interacting partners containing a denser concentration of material. Synaptic vesicles containing neurotransmitters form on the side of the presynaptic membrane and finally a stable synaptic junction is formed allowing the functional communication of signals from presynaptic neuron to post synaptic partner[33, 34].

Signals are transduced through the release of neurotransmitter particles into the synaptic cleft through fusion of their vesicles with the presynaptic membrane upon arrival of an action potential. Diffusion across the cleft results in binding to postsynaptic receptors either stimulating or inhibiting electrical activity of the postsynaptic cell.

Synapses are formed over a large time frame of neuronal development from embryonic to postnatal ages and are by no means consistent. Their continual formation and elimination are intimately involved in adult brain functions from learning to memory.

A well characterised example of synaptogenesis is that of the neuromuscular junction. The neurotransmitter acetylcholine (ACh) acts upon postsynaptic ACh receptors which are clustered at the

postsynaptic membrane through regulation by agrin[35, 36]. This protein acts to retain postsynaptic clusters of ACh receptors and thus establish the site of a synapse with the help of receptor associated protein synapse (rapsyn)[37] (see figure 3).

The protein muscle specific kinase (MuSK) acts to establish the post synaptic density, the site of post synaptic receptor clustering[38, 39].

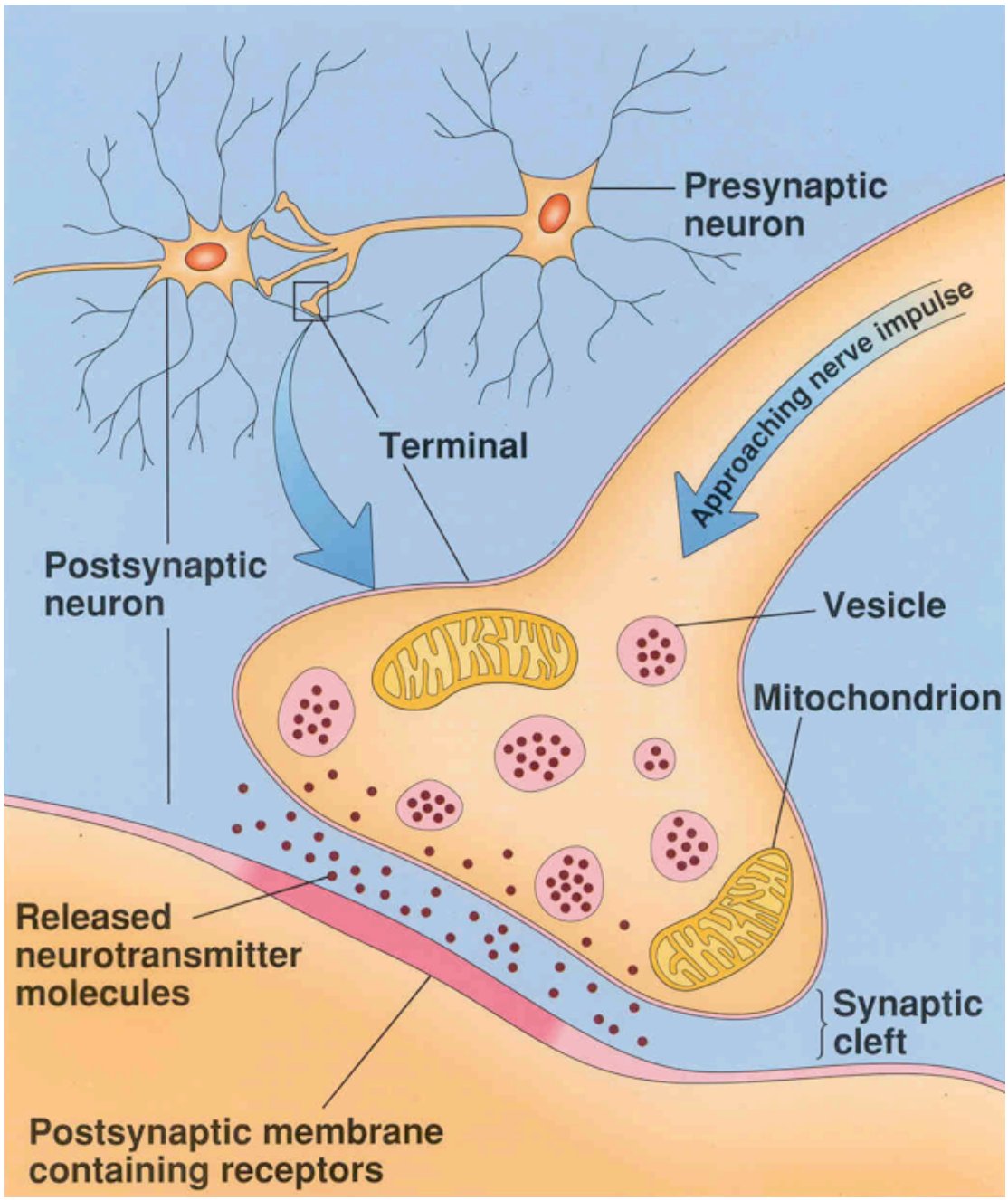


Figure 3: Structure and basic function of a synapse

The arrival of a nerve impulse at the terminal of a presynaptic process results in the fusion of synaptic vesicles with the presynaptic membrane. These vesicles contain neurotransmitters which, upon fusion, are released into the synaptic cleft. The passive diffusion of these neurotransmitters across the cleft leads to an interaction with receptors of the postsynaptic membrane of another neuron. Neurotransmitters can either excite the postsynaptic neuron leading to the propagation of an action potential or inhibit such an event [open source image].

Development of the peripheral nervous system

Introduction

Much of the work within this thesis focuses on the peripheral nervous system (PNS) and the development of its neurons. Thus the PNS will be discussed in greater detail.

It is the role of the PNS within the developed animal to link the CNS with its periphery and all of the sensory and effector components of a complex developed animal. Two main divisions split the PNS into somatic and autonomic components. The somatic sensory PNS is made up of neurons of the dorsal root and cranial sensory ganglia while the autonomic PNS consists of the sympathetic, parasympathetic and enteric subdivisions.

It is the role of sensory afferents to relay information from peripheral regions to the CNS for processing while autonomic and somatic motor components do the opposite and relay signals from the CNS to targets within the periphery.

The work contained within this thesis explores novel roles for the TNFSF members APRIL and BAFF and their receptors BCMA, TACI and BAFFR in the growth and survival of sympathetic and sensory neurons both *in vitro* and *in vivo.* The mechanisms regulating PNS development from axonal growth and neuronal survival to final target field innervation will be further explored.

Organisation of the somatic PNS

The somatic nervous system is the division associated with voluntary control of the body via skeletal muscle and the nerve fibres that innervate these muscles, joints and skin. Signals are conveyed through a system of 12 pairs of cranial nerves and an additional 31 pairs of spinal nerves. Five of these cranial nerve pairs comprise axons arising in seven cranial sensory ganglia.

Found on cranial nerve V, the trigeminal ganglion projects axons to innervate the face along with oral and nasal cavities relaying sensory information from mechanoreceptors, thermoreceptors and nociceptors. Sensory information gathered by taste buds on the anterior portion of the tongue are relayed via axons of the geniculate ganglion of cranial nerve VII while it is projections of the vestibular and spiral ganglia of cranial nerve VIII that innervate hair cells of the inner ear. Sensory information from the posterior remainder of the tongue travel down axons of the petrosal ganglion of cranial nerve IX, as well as information from the carotid body. Afferent projections of the superior glossopharyngeal ganglion of cranial nerve IX innervate skin of the pinna and tympanic membrane and finally the role of sensory afferents of the jugular and nodose ganglion found along cranial nerve X is to innervate sensory targets of the pharynx, thorax and abdomen.

The 31 pairs of spinal nerves that also make up the somatic PNS each connect to the spinal cord via a dorsal root comprising sensory fibres and a ventral root of motor fibres. The cell bodies that project these sensory fibres can be seen in multiple dorsal root ganglia (DRG), while those that project motor fibres can be found in the ventral grey

matter. Just prior to projecting forth from the vertebral column, through an intervertebral foramen, each pair of dorsal and ventral root join into one of the spinal nerves while each nerve goes on to divide into dorsal and ventral rami, a mixture of sensory and motor fibres projecting proximally to their targets of innervation.

Organisation of the autonomic PNS

Mediating unconscious activity and control, nerves of the autonomic nervous system link the CNS to viscera, glands, blood vessels and the arrector pili muscles of hair follicles. The three subdivisions of the autonomic nervous system are the sympathetic, parasympathetic and enteric systems.

The sympathetic and parasympathetic systems can be seen to balance each other and thus regulate homeostatic control over many critical body systems. In heightened danger a flight or fight response, mediated by enhanced sympathetic activity regulated by the hypothalamus, results in an increase in sympathetic activity of the heart, viscera, peripheral vascalature, sweat glands and arrector pili muscles. Opposing this side of the autonomic nervous system, the parasympathetic branch acts to mediate basal levels of heart rate, respiration and metabolism under more normal external conditions.

Within the lateral grey column of the spinal cord specifically segments T1 and L2 cell bodies project preganglionic sympathetic fibres which form part of the mixed spinal nerve[40]. After travelling in this form, fibres branch off as myelinated white rami to enter the paravertebral sympathetic chain. These preganglionic fibres synapse with postsynaptic neurons which extend projections via

unmyelinated grey rami back to adjacent spinal nerves before extending peripherally to their targets of innervation. A subdivision of the preganglionic fibres bypass any postganglionic neurons within the paravertebral sympathetic chain and project out via the splanchnic nerves to synapse with postganglionic neurons of prevertebral sympathetic ganglia found closer to the viscera. The transmission of information from preganglionic fibres to postganglionic neurons utilises the neurotransmitter acetylcholine (ACh) while at the terminals of postganglionic fibres the predominant neurotransmitter found is noradrenaline. The end result of enhanced sympathetic activity is dilation of the pupils, increased heart rate and contractility, bronchodilation as well as contraction of arrector pili muscles, vasoconstriction of mesenteric vessels and vasodilation of skeletal muscle vessels.

Parasympathetic preganglionic fibres originate from cell bodies of the preganglionic parasympathetic nuclei of cranial nerves III, VII, IX and X but also within the lateral grey column of sacral spinal regions S2-4. This combination of fibres extending from both cranial and sacral origins is referred to as cranial-sacral outflow. Unlike their sympathetic counterparts preganglionic parasympathetic fibres extend much further to synapse with ganglia located much closer to their respective targets. The result of this is much shorter postganglionic fibres in comparison with the sympathetic branch of the autonomic nervous system. The parasympathetic ganglia associating with cranial nerves III, VII and IX act on the pupils to promote pupillary constriction in addition to visual accommodation and enhanced salivary gland secretion. The heart, lungs, stomach, upper intestine and ureter receive parasympathetic innervation from

the terminal ganglia of cranial nerve X. Fibres originating from sacral sections of the spine project to targets including the distal colon, rectum, bladder and gonads.

In opposition to the sympathetic nervous system, parasympathetic activity promotes a reduction in heart rate and blood pressure while enhancing digestive activity. Both preganglionic and postganglionic axons utilise ACh as their neurotransmitter.

The third and final division of the autonomic nervous system is the enteric nervous system. Neurons originating in the submucosal plexus innervate gut mucosa regulating secretion, while those of the myenteric plexus project to circular and longitudinal smooth muscle, again of the gut, controlling motility. The enteric nervous system works to coordinate and regulate both muscular activity and glandular secretions of the gastrointestinal tract (see figure 4).

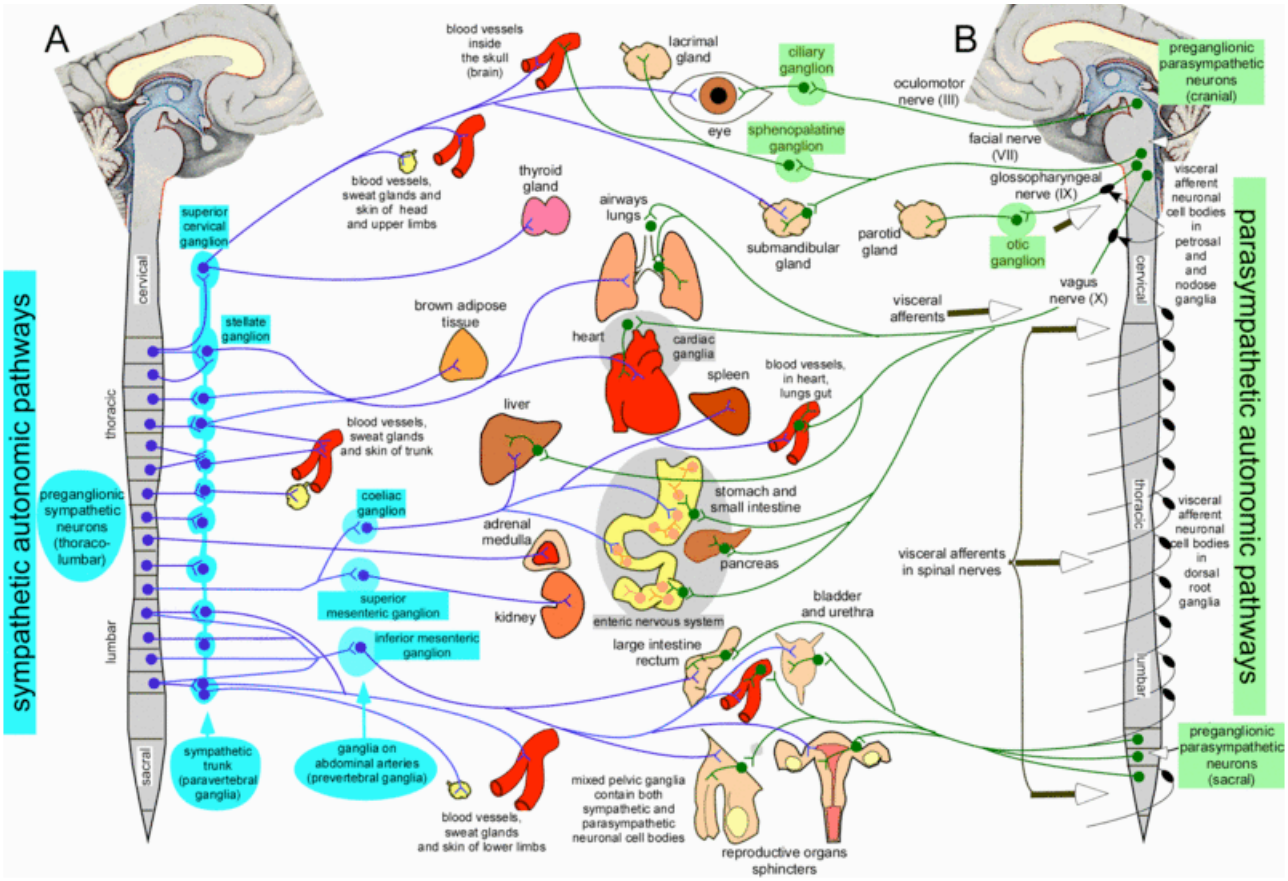


Figure 4: Sympathetic and parasympathetic nervous system

Summary of sympathetic (A) and parasympathetic (B) autonomic neural outflows from the central nervous system. Preganglionic sympathetic fibres project to sympathetic ganglia who in turn extend processed distally to target fields of innervation. In the case of the adrenal medulla, sympathetic innervation is provided directly by preganglionic fibers. Parasympathetic projections also innervate distal target fields opposing the actions of the sympathetic branch. A number of these target fields can be seen above including the fibers and ganglia that innervate them [Blessing and Gibbins (2008), Scholarpedia, 3(7):2787].

Origins of the peripheral nervous system

Neurons of the peripheral nervous system, as well as glial cells, are derived from the neural crest[41, 42]. The exceptions to this are some populations of cranial sensory neurons. The final fate of cells of the neural crest depends heavily on their chosen routes of migration and final positioning within the developing animal. Migration from the neural crest results in cells of the PNS as well as Schwann cells, satellite cells in addition to melanocytes, cartilage of the face, bone and connective tissue.

The neural crest itself is transient in nature and migration of cells is already underway by the time neural tube fusion is complete. Through dorsolateral migration neural crest cells go on to become melanocytes while those that migrate via a ventral route form DRG neurons, sympathetic ganglia and cells of the adrenal medulla. The parasympathetic branch of the autonomic nervous system arises from the mesencephalic neural crest while their enteric counterparts arise from the vagal and lumbar crest[43].

Wnt signalling is a critical component of neural crest formation and differentiation with both BMPs and FGF involvement[41, 44]. Mouse models expressing inactivated β-catenin show abnormalities of the neural crest and display an abnormal DRG formation[45].

The cranial sensory neurons that are not derived from the neural crest instead arise from an additional structure termed neurogenic placodes. Neurons of the nodose, petrosal and geniculate ganglia develop from structures called the epibranchial placodes while neurons of the vestibular ganglia and ventrolateral trigeminal ganglia

arise from dorsolateral placodes. These placodes result from focal thickenings of the cranial ectoderm.

Neuronal survival in the PNS

Introduction

A critical phase of development of the nervous system is a window of cell death in which up to 80% of some neuronal populations die in a process termed programmed cell death (PCD). This process takes place in many neuronal populations shortly after the completion of proliferation or within proliferating populations.

Cells within this phase of development may undergo apoptosis coordinated by a cascade of proteases called caspases. These proteases are tightly regulated by members of the B-cell lymphoma-2 (Bcl-2) family[46]. This intrinsic signalling pathway can be activated by a number of cues from DNA damage to factor deprivation and programmed developmental signals. Extrinsic caspase activation can be initiated independently of the Bcl-2 family through death receptor signalling[47].

Three subgroups of the Bcl-2 family exist being pro-apoptotic, anti-apoptotic or to regulate anti-apoptotic members enhancing apoptotic activity. Pro-apoptotic members include BAX, BAK and BOK while anti-apoptotic members include Bcl-2 itself, Bcl-X_L and Bcl-W. Members that regulate the anti-apoptotic members include BAD, BAK, BIM and NOXA[48, 49].

As an example, the action of BAX a pro-apoptotic factor is to firstly permeabilise the mitochondrial membranes within the cell in question, releasing cytochrome c and DIABLO. It is in part the binding of cytochrome c to apoptotic protease-activating factor 1 (APAF-1) to

form an apoptosome that goes on to activate caspase 9 leading to apoptosis of the cell[50, 51] (see figure 5).

Within the developing nervous system PCD is tightly controlled to ensure adequate neurons remain to form functional neuronal circuitry and peripheral target field innervation. A myriad of both pro and anti-survival factors act upon developing neurons to ensure this is the result. The binding of nerve growth factor (NGF) to its high affinity nerve growth factor receptor (TrkA) initiates the activation PI-3 kinase, Akt and MEK signalling to inhibit pro-apoptotic proteins such as BAD[52].

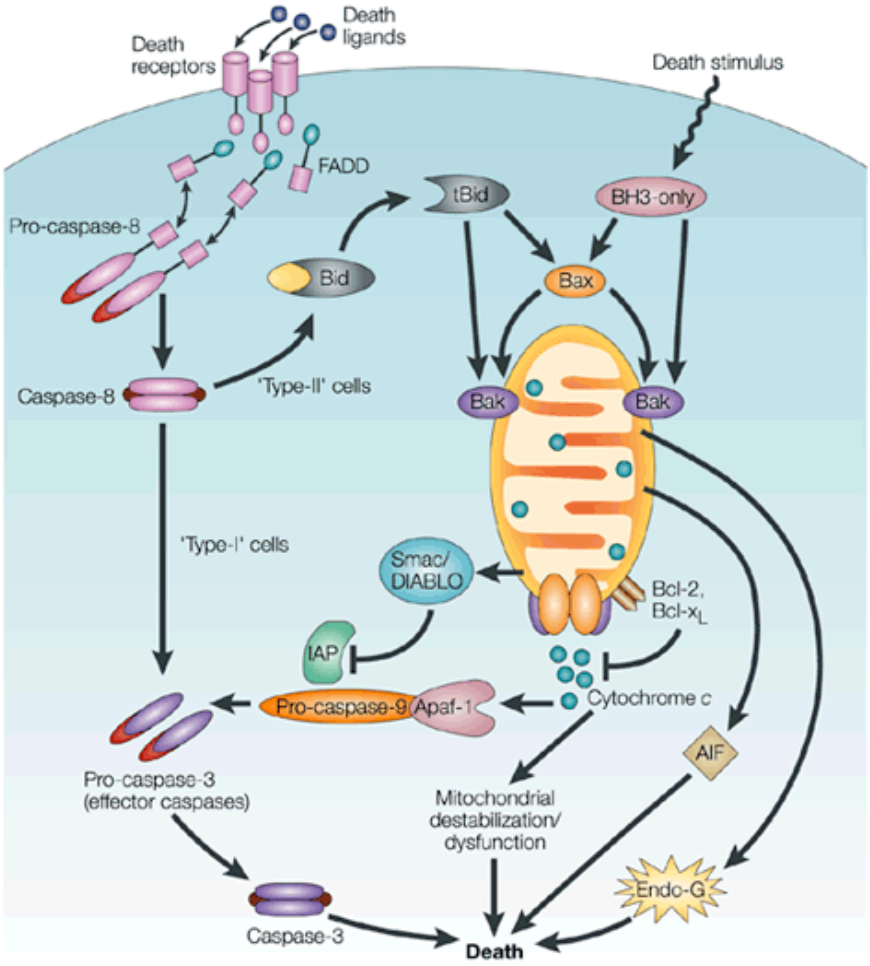


Figure 5: Diagrammatic representation of apoptosis.

The core intracellular machinery that regulates and executes apoptosis in vertebrates comprises families of proteins that interact with each other and with intracellular organelles. In many cases, information from the environment is relayed to a central 'apoptosis rheostat' at the mitochondrion by stimulus-specific induction, modification or movement of pro-apoptotic 'BH3 domain-only' members of the Bcl-2 family. Once 'activated', these proteins are thought to facilitate, through many mechanisms, the assembly of pro-apoptotic 'multi-domain' members of the Bcl-2 family into heterodimeric units or 'pores' in the outer mitochondrial membrane. This causes the release of numerous 'apoptogenic' factors from the mitochondria into the cytosol through a process that can be blocked by anti-apoptotic multi-domain Bcl-2 family[53].

Neurotrophic theory

Early neuronal development is characterised by an excess of neurons and it is by the process of PCD that ensures neurons that are unnecessary, or form incorrect synaptic connections, are purged from the system[54-56]. This phase of cell death occurs after neurons begin to innervate their target fields and acts to ensure suitable numbers of neurons innervate the correct appropriate targets in the PNS. Target fields of peripheral innervation express a controlled amount of specific neurotrophins and innervating neurons thus compete for this limited supply. Without adequate trophic support neurons undergo apoptosis.

The first neurotrophin to be discovered was NGF[57] along with its critical role in survival of neurons during target field innervation. This was established through the use of anti-NGF antibodies and the use of NGF itself. The action of anti-NGF both *in vitro* and *in vivo* on sympathetic neurons dependent on this neurotrophin for survival causes an increase in cell death while the addition of NGF has the opposite effect and eliminates the phase of cell death that occurs under normal development[57-59].

Following on from these experiments animal models that lack NGF or its receptor TrkA display a severe reduction in sympathetic and sensory populations of neurons[60-62]. Production of NGF is localised to specific sites of target field innervation and removal of these prior to the completion of innervation results in a loss or neurons within the ganglia extending axons towards those targets[63].

It is not simply the case, however, that populations of neurons compete for specific neurotrophins within a small window of development. Indeed many populations display affinities for different neurotrophins through various stages of development and source the factors from sites other than target fields[64-66]. Periods of neurotrophic independent survival exist in most populations of PNS neurons and these portions of growth usually correlate to the absolute distance different populations of neurons have to travel to reach a target field and thus their main source of neurotrophic support[67, 68].

While NGF was the first neurotrophin to be discovered many other examples have since been characterised. Brain-derived neurotrophic factor (BDNF)[69], neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) have been characterised in vertebrates while neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) can be found in fish.

Two classes of receptor mediate neurotrophin signalling and these are the high affinity tyrosine kinase receptors (TrkA, TrkB and TrkC) in addition to the common low affinity receptor p75. While these neurotrophins and their receptors regulate neuronal survival additional families of neurotrophins also exist. Glial cell derived neurotrophic factor (GDNF) family members GDNF, neurturin, artemin and persephin also support survival plus the neurotrophic cytokines, ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), interleukin-6 (IL-6), cardiotrophin-1 (CT-1), oncostatin-M (OSM) and hepatocyte growth factor (HGF) to name some other examples[70-74].

Neurotrophic requirements of the PNS

The experimental models explored in this thesis focus mainly on neurons of the superior cervical ganglion, trigeminal ganglion and nodose ganglion. As such the differing neurotrophic requirements of these will be further explored in terms of neuronal survival.

The SCG derives from cells of the neural crest and respond to a variety of neurotrophins throughout development. It is the action of artemin that promotes the proliferation of early SCG neuroblasts while HGF signals the differentiation of these proliferating neuroblasts into post mitotic sympathetic neurons[75-77]. From embryonic day 14 through to postnatal development, SCG neurons are dependent on NGF for survival[78] and they can be maintained in culture through the addition of NGF to media. Early postnatal SCG neurons can be maintained *in vitro* by NT-3 while the ability of both NGF and NT-3 to promote survival within this time period is mediated by expression of TrkA[79]. In cell culture, later postnatal SCG neurons can be supported by the addition of CNTF, LIF and HGF[80, 81].

The sensory trigeminal neurons show a dependence on BDNF and NT-3 at early embryonic ages but, like SCG neurons, develop an NGF dependency from E12 onwards[82]. This switch in neurotrophin preference correlates with an increase in NGF expression in trigeminal target tissues as well as a switch to TrkA expression on the majority of trigeminal neurons themselves[66, 82-84].

The sensory nodose neuron responds throughout development to BDNF, however, early neurons can also respond to NT-3 and NT-4[68, 85, 86]. A minority of early nodose neurons can also support

survival through a presence of NGF[87]. Subpopulations of nodose neurons throughout development can also respond to CNTF, LIF, OSM and CT-1[88].

NGF

Synthesis of NGF begins with the production of its precursor pro-NGF. Enzymatic cleavage releases a homodimer comprising two 118 amino acid subunits containing a cysteine rich motif and two anti-parallel β strands[89-93].

NGF expression within the developing nervous system can be found within both the PNS and CNS. Within the developing brain the neocortex, basal forebrain and hippocampus are all examples of CNS regions expressing NGF[94]. In the PNS it is the main targets of innervation that express this neurotrophin including the submandibular gland (SMG), pineal gland, iris, nasal mucosa, heart and whisker pads[95]. NGF expression is also not unique to the nervous system with members of the immune system, reproductive system and endocrine system also displaying NGF production[96]. NGF null mice display a considerable loss of both sympathetic and sensory neuronal populations highlighting the critical role of this neurotrophin in the survival of these populations[97].

Trk receptors

The ability of neurotrophins to enhance survival and process outgrowth and cell survival is mediated by Trk receptors in both the

PNS and CNS[98-101]. The effects of different neurotrophins are mediated by the expression of different Trk receptors. TrkA is the receptor that binds NGF while TrkB[102-104] associates with BDNF[105-107] and NT-4[108-110]. TrkC[111] acts as the primary mediator for NT-3[112] while TrkA and TrkB can also weakly interact with this neurotrophin. TrkA has the ability to weakly associate with NT-4/5[109].

Parasympathetic ciliary neurons represent a single example of peripheral neurons that do not express Trk receptors demonstrating their wide involvement in PNS development[113-115].

Following binding of neurotrophins, Trk receptors undergo ligand mediated dimerisation and autophosphorylation of multiple conserved cytoplasmic tyrosine residues[116-120]. It is this tyrosine phosphorylation that provides sites for interaction for multiple adaptor molecules and enzymes that begin intracellular signalling cascades. Activation of Ras, PI3-K and PLCγ are examples of intracellular signalling mediators that interact with phosphorylated Trk receptors[121].

As the predominant source of neurotrophin during development is target field derived, binding to the Trk receptors is often distant from the cell soma. Thus ligand mediated dimerisation results in internalisation through clathrin-coated pits and macropinocytosis[122, 123]. Retrograde transport of signalling endosomes containing active Trk receptor and signalling intermediates such as PLC-γ1 relay target mediated NGF signalling to the nucleus[124-126].

It is an interesting observation that mice models lacking Trk receptors suffer a loss of specific neuronal populations similar to

those witnessed in neurotrophin null models. TrkA null mice lose neurons similar to NGF null mice such as sympathetic populations[61] while TrkB mice lose large numbers of nodose neurons similar to BDNF null mice[127].

p75

The low affinity neurotrophin receptor p75[128] binds all neurotrophins[129] and is expressed both in the PNS and CNS in addition to numerous non-neuronal cells. Expression of this receptor modifies the function of Trk receptors[121] in addition to binding proNGF through interaction with a co-receptor sortilin. This interaction can promote apoptosis[130] or inhibit cell death[131].

Neuronal outgrowth in the PNS

Axon extension and guidance

The basis of a functional nervous system is for each neuron to synapse with an appropriate target and thus form functional neuronal circuitry. For the majority of neurons a single axon is extended from the cell soma to reaching a specific target in order to deliver a signal, while multiple dendrites receive signals from other neurons. At the advancing end of the axon is a structure termed the growth cone. This conical broadening of the leading end provides both the movement capabilities and directional control that allows the axon to advance to its target. While a newly elaborating neuron extends multiple homogenous processes each tipped with a growth cone, it is a single projection that will become the axon and can be defined by the expression of axon specific proteins. Through specific polarised intracellular signalling axons and dendrites are defined and attempt to synapse with differing targets integrating the neuron into its network. A highly magnified view of a growth cone reveals fine fibres, the filopodia and lammellipodia that extend outwards and assess the surrounding tissue environment. In addition to this the activity of a vast array of soluble chemotactic factors over differing diffusion gradients provides highly specific directional control of the projecting axon through its growth cone by modulating cytoskeletal turnover and stability. Intracellular signal regulation components such as the GTPases Rho and Rac modulate the turnover of actin

filaments within the growth cone itself and thus help determine direction of movement[132].

During their journey to a specific target, projecting axons require the presence of a substratum, be it with extracellular matrix components or the surface of other cells. Through the process of contact guidance, axons following a common path will group into fascicles[133, 134]. Groups of membrane glycoproteins mediate cell-cell adhesion including the immunoglobulin superfamily member N-CAM and Ca2+ -dependent cadherin family member N-cadherin allowing advancing growth cones to follow the path of fellow projecting axons[135]. These adhesion molecules are expressed on not only neurons and their growth cones but also glial cells and muscle cells, helping facilitate the projection of axons of both the central and peripheral nervous systems[136].

An additional regulation of growth cone motility and direction consists of extracellular matrix composition with substratum including laminin promoting extension[137] while chondroitin sulfate proteoglycans negatively regulate outgrowth[138].

A combination of chemotactic factors have the ability to steer axons very precisely in some cases across the entire organism. A clear example of this ability is how neurons originating in the spinal cord of vertebrates are guided first ventrally towards the floor plate of the neural tube before crossing it and projecting longitudinally towards the brain, never again crossing the floor plate. Floor plate secreted *netrin* firstly attracts the growth cones guiding the axon ventrally via its transmembrane receptor DCC[139]. Neurons lacking this receptor do not experience attraction to the floor plate while the presence of Unc-5C, an alternative netrin receptor has the opposite effect and

repulses neurons. Once the neurons have crossed the floor plate an alteration in gene expression confers slit sensitivity, a repulsive cue secreted from the floor plate that prevents axons from travelling back over the floor plate towards the source of netrin[140]. Dorsal projection is prevented by a newly active repulsion to semaphorins forcing the growth cones to track along the floor plate towards the brain[141].

Neurotrophic factors in the regulation of neurite outgrowth

Initial investigation into the roles of neurotrophins in the growth of PNS neurites focused on *in vitro* culture and evidence is well established[142-146]. To study the effects of neurotrophins on neurite growth *in vivo* presented a major problem as these factors play such a crucial role in the survival of populations of PNS neurons. Through crossing transgenic lines of NGF/TrkA double knockouts with BAX knockout animals the effects of NGF and its receptor TrkA on neurite outgrowth could be elucidated without apoptotic loss of NGF responsive neurons[147, 148]. Exposing cultured neurons to a defined, distant source of neurotrophin has demonstrated that peripheral axons *in vitro* are induced to grow towards such a source, while this effect can be eliminated through the application of function blocking antibodies against the neurotrophin in question[149]. Further evidence for the ability of neurotrophins to enhance neuronal outgrowth can be seen in transgenic models over-expressing NGF or NT-3. Here, target organs of peripheral innervation such as the skin, heart and pancreas display significantly

higher levels of sensory and sympathetic target field innervation[150-152].

This simplified model of peripheral development becomes more intricate with the observation that different neurotrophins can elicit differing effects throughout development based on differential control of Trk mediated signalling. In sympathetic development NT-3 signalling through TrkA acts to promote the growth of neurites but does so independent of survival. NGF signalling through TrkA, however, regulates both outgrowth and survival simultaneously and it is through control of internalisation and retrograde transport that these differences are seen[142].

Intracellular signalling mediating neurotrophic action on PNS development

Following neurotrophin binding Trk receptors have the ability to activate a number of intracellular signalling cascades that transduce receptor binding to cellular effect. Activated Trk receptors can enhance protein kinase A (PKA), Ras/phosphotidyl inositol 3'-phosphate-kinase (PI3-K)/Akt, Ras/Raf/mitogen-activated protein kinase (MAPK) and MAPK/extracellular-regulated kinase (ERK) signalling[153-160].

In certain neuronal populations it can be demonstrated that PI3K/Akt or MAPK/ERK can enhance the survival of the neurons in addition to promoting neurite outgrowth, all in the absence of neurotrophin[161-164].

The activity of PI3K/Akt signalling in terms of survival promotion is well characterised following activation by growth factors, oncogenes and cell stress. Survival enhancement stems from an inhibition of pro-apoptotic proteins such as Bcl-2 homology domain 3 (BH3)-only proteins. A subsequent inability of BH3-only proteins to bind Bcl-2 family members leads to increased activity of these anti-apoptotic factors. In addition to blocking the activity of BH3-only proteins, positive PI3K/Akt activity can down regulate its expression through the blockade of transcription factors including FOXO and p53. By preventing the cellular processing of pro-caspase 9 PI3K/Akt can prevent apoptosis by multiple routes[165].

The role of MAPK signalling in neurite outgrowth has primarily been investigated in PC12 cells. Following differentiation into a sympathetic like neuronal cell, NGF promoted neurite outgrowth

does not require MAPK activity to be effective. The cascade appears to have a role instead in making naïve PC12 cells competent to extend neurites following an application of growth factor[166, 167].

The activation of TrkA by NGF and the subsequent auto phosphorylation of cytoplasmic tyrosine residues leads to the activation of MAPK/ERK, PI3K/Akt and PLC[167-170]. Additionally both BDNF and NT4/5 have been shown to activate PI3K and MAKP signalling via Trk receptor binding[171-173] (see figure 6).

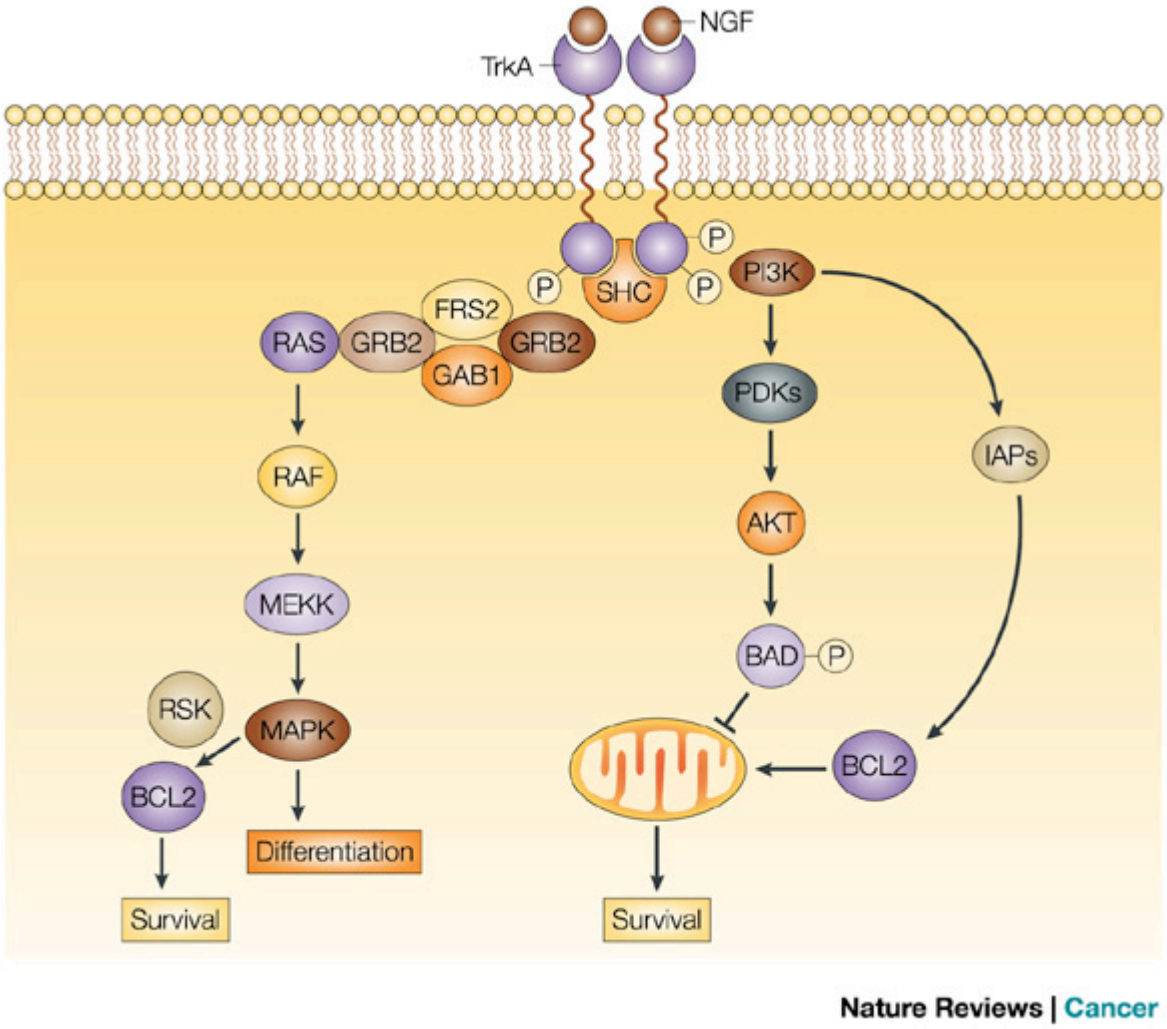


Figure 6: Signalling downstream of Trk receptors.

Binding of nerve growth factor (NGF) to the ligand-binding domain of TrkA leads to TrkA autophosphorylation and activation of various signalling cascades. Proteins that are thought to interact directly with the Trk intracellular domain are SHC, PLCγ1, SH2B and IAPs, some of which are shown here. Binding of a ligand to TrkA can also trigger the RAS signalling pathway, leading to survival and differentiation, and an alternative survival signalling pathway through phosphatylinositol 3-kinase (PI3K).

[Brodeur, *Nature Reviews Cancer* **3**, 203-216 2003].

The tumor necrosis factor superfamily (TNFSF)

Introduction

The first member of the TNFSF to be discovered was TNF-α closely followed by lymphotoxin (LT). The name tumor necrosis factor (TNF) arose from the observation that bacterial infection of tumour masses could lead to a regression of the tumour[174]. The prevailing opinion was that a factor, released by the bacteria, was able to cause this regression. It eventually became clear that TNF was in fact released from the patient's own cells, as part of an immune response to the bacterial infection. Experimental studies into the early members TNF-α and LT demonstrated their ability to lyse many different cell types as well as showing that both macrophages and lymphocytes secreted these factors[175, 176].

Today it is understood that the TNFSF is a large group of ligands which play major roles in a wide range of cellular processes from survival to differentiation. This wide range of effects are also present in a broad set of tissue groups including lymphoid, mammary, neuronal and ectodermal tissues.

Ligands of this superfamily exist in two forms, as membrane bound or soluble secreted factors. The ligands bind to and elicit effects via a group of receptors termed tumor necrosis factor superfamily receptors (TNFSFRs) and the family of ligands and receptors now contains more than 40 members[177].

TNFSF ligands

TNF is an example of a pleiotropic cytokine produced most abundantly by macrophages and monocytes but also a much broader group of cells from B lymphocytes, T lymphocytes to fibroblasts[178]. Since the discovery of TNF, up to nearly 20 other TNFSF ligands have been characterised. Most members can be seen throughout the immune system expressed by B cells, T cells, natural killer cells, monocytes and dendritic cells. The exception to the rule is VEGI which can instead be found in both cardiac and hepatic tissue[179].

Members of the TNFSF are type II transmembrane proteins which can form non-covalently bound homotrimers suitable for secretion from the cell[180]. Due to this TNFSF ligands are able to signal in a paracrine fashion with close neighbours or in an endocrine manner following cleavage and secretion.

From its initially characterised role in necrotic cell death, TNF-α has also been demonstrated to play a role in a wide variety of cellular processes from apoptosis to proliferation, differentiation, inflammation and tumourogenesis. Outside of immune regulation, TNF mediated signalling plays a role in many pathologies including inflammatory rheumatoid arthritis, hemorrhagic fevers, irritable bowel syndrome, septic shock and asthma[181].

Following the discovery of TNF and LT, direct expression-cloning methods expanded the family to include several new members such as Fas, CD27, CD30, CD40, 4-1BB, 0X40 AND herpes virus entry mediator (HVEM)[182, 183]. Later discoveries following human genome sequencing include TNF-related apoptosis-inducing ligand

(TRAIL), receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), vascular endothelial cell-growth inhibitor (VEGI) and B-cell activator (BAFF)[184-186].

Members of the TNFSF often appear with differing nomenclature following simultaneous discovery by multiple groups and some examples include an alternative name for BAFF, of β-lymphocyte stimulator (BLYS), RANKL referred to as either TNF-related activation-induced cytokine (TRAIL) or osteoprotegerin ligand (OPG).

Activity of TNF members can often depend on their condition, being either membrane bound or secreted. Membrane bound CD95 acts locally to kill peripheral blood T cells, however, secretion of the same ligand inhibits this killing effect. In contrast it is soluble TNF-α binding to cells distant from the site of secretion that can lead to conditions such as brain ischemia. Thus some soluble TNF ligands actually act as antagonists or agonists to their membrane bound forms[187].

TNF receptors

Like their ligand counterparts TNFSFRs are a large group of receptors that are often characterised by extracellular sequence homology. These receptors are widely expressed and TNFR1 can be found on all cells of the body. In contrast TNFR2 can be found expressed broadly in cells of the immune system as well as endothelial cells[188].

As with the ligands, most members of the TNFSFRs have multiple names and nomenclature can be complex. Members include the receptors for TNF, TNFR1 and TNFR2, Fas, CD40 and p75[189]. Activation is usually by specific ligands as in the case with TNF activating its two receptors. These receptors contain an extracellular pre-ligand binding assembly domain or PLAD whose role is to pre-complex receptors and encourage trimerisation[190]. This PLAD domain mediated trimerisation allows for subsequent ligand binding and activation. Similar mechanisms can be seen with TRAIL receptor 1 (TRAIL1) and CD40 and thus it seems that TNF receptors prefer to function as preformed complexes as opposed to monomeric receptors that oligomerise following binding of a ligand[191].

Receptors of the TNF superfamily can be further characterised by the presence of a cysteine rich domain (CRD) within their extracellular component. These domains include four decoy receptors DcR1, DcR2, DcR3 and OPG which are capable of reducing ligand signalling following receptor binding (see figure 7).

Receptors of the superfamily can be divided into those that contain a C-terminal death domain and those that lack such a domain. This 80 amino acid region confers an ability to induce apoptosis to the receptor while experimental deletion abolishes such an ability[191].

Figure 7: Schematic representation of the TNF superfamily

All ligands, except LT-α and VEGI, are type II transmembrane proteins with a carboxy-terminal extracellular domain, an amino-terminal intracellular domain and a single transmembrane domain. The C-terminal extracellular domain is responsible for binding to the receptor. Most members of the TNF superfamily are released from the cell surface by proteolysis through distinct proteases. TNF receptors (TNFRs) are characterized as type I transmembrane proteins (extracellular N-terminus and intracellular C-terminus). Because they lack a signal peptide sequence, BCMA, TACI, BAFFR and XDAR belong to type III transmembrane protein group. Osteoprotegerin and DCR3 lack a transmembrane domain and are therefore secreted as soluble proteins. The numbers on the left represent the number of amino acids in the cytoplasmic domain of the receptor [Aggarwal, *Nature Reviews Immunology* **3**, 745-756 2003].

TNF and TNFR mediated cell signalling

TNFα remains the most widely studied member of the TNF superfamily and as a result has the widest range of physiological roles assigned to it. Its wide range of effects might also be due to the prevalence of expression of TNFR1 and TNFR2 throughout the body. While certain members of the family act in a single ligand receptor system such as 4-1BBL and 4-1BB, many others act as part of a larger group with TRAIL, for example, binding up to five different receptors including DR4, DR5, DcR1, DcR2 and OPG. BAFF is a good example of another more complex system as it can bind three receptors TACI, BCMA and BAFFR[188].

Further complexity can be added as both TNFSF ligands and their receptors do not necessarily act as homotrimers but can also heterotrimerise, as in the case of DR4 and DR5 as well as BAFF and APRIL, leading to the recruitment of different cell signalling intermediaries or altering receptor binding affinities[188].

The activity of certain ligands depends heavily on cell surface expression of competing receptors as is the case of TRAIL. Binding to DR4 and DR5 transduces a signal, however, binding to DcR2 can only partially transduce a signal[192]. The ability of DcR1, DcR2, DcR3 and OPG to sequester TRAIL inhibits cell signalling via DR4 and DR5 that would otherwise take place. It is also interesting to note that a functional OPG has been reported to act as a ligand through an, as yet unknown receptor, and this form differs from the norm by the presence of an additional death domain[193].

Following ligand binding TNFSF members show an ability to both promote and reduce survival of target cells. Pro-apoptotic members

include TNF, LT, CD95L, TRAIL, VEGI, TWEAK and LIGHT. Both RANKL and BAFF have been reported to enhance survival while members TNF, CD27L, CD30L, CD40L, OX40L, 4-1BBL, APRIL and BAFF have all been reported to play a role in proliferation. Activation of a number of signalling cascades mediate these responses including NF-Kb, JNK, P42/44 MAPK and p38 MAPK[194] (see figure 8).

Figure 8: Cellular signalling pathways downstream of TNF superfamily members

Tumour-necrosis factor receptor 1 (TNFR1) binds to TNFR-associated death domain (TRADD) protein. Through its death domain, TNFR1 sequentially recruits TRADD, TNFR-associated factor 2 (TRAF2), receptor-interacting protein (RIP) and inhibitor of nuclear factor-κB (NF-κB) kinase (IKK), leading to the activation of NF-κB; and the recruitment of TRADD, FAS-associated death domain (FADD) and caspase-8, leads to the activation of caspase-3, which in turn induces apoptosis. CD95, death receptor 4 (DR4) and DR5 activate apoptosis through sequential binding of FADD to caspase-8 and caspase. Besides TNFR1, DR3 and DR6 are also known to bind TRADD and mediate the activation of apoptosis, NF-κB and JUN N-terminal kinase (JNK). TNFR1 activates JNK through the sequential recruitment of TRAF2, MAP/ERK kinase kinase 1 (MEKK1) and MAPK kinase 7 (MKK7)[194].

The TNF superfamily in the nervous system

Recent work has demonstrated that TNFSF proteins modulate neurite growth from developing neurons. It has been shown that both soluble GITRL and FasL are able to enhance the growth of neurons[195, 196]. The opposite has also been demonstrated with LIGHT, TNF and RANKL all reducing neurite growth[197, 198]. Reverse signalling via membrane integrated TNF has also recently been shown to promote sympathetic axon growth and tissue innervation[199].

This study focuses on the TNF member APRIL (A Proliferation-Inducing Ligand, TNFSF13), a recently discovered member of the TNFSF that was first characterized for its ability to promote tumour growth[200], but is best characterised for its role in regulating lymphocyte survival and activation[201]. The initial discovery by Hahne et al came though a public database search based on an optimal alignment with currently known TNF superfamily members at the time. Several candidate clones were found coding for a unique, novel TNF-α–related ligand which was termed APRIL (a proliferation-inducing ligand). Two of these cDNA clones (AA292358 and AA292304) contained full-length sequences (1.5 and 1.7 kb, respectively) encoding a protein of 250 amino acids, with a predicted cytoplasmic domain of 28 amino acids, a hydrophobic transmembrane region, and an extracellular domain of 201 amino acids. The absence of a signal peptide suggested that APRIL was a type II membrane protein which is typical of the members of the TNF ligand family. The single N-linked glycosylation site (N124) predicted for this protein lies within the first of several β strands which are

folded into an antiparallel β sandwich structure. The sequence of the extracellular domain of APRIL showed highest homology with FasL (21% amino acid identity), TNF-α (20%), and LTβ (18%), followed by TRAIL, TWEAK, and TRANCE (15%).

APRIL has also been implicated in the aetiology and/or maintenance of a variety of autoimmune diseases[202]. In addition to its synthesis by a variety of cells of the immune system[203], APRIL has also been detected in adipocytes, keratinocytes and osteoclasts[204-206]. APRIL is unusual among the TNFSF in not being expressed at the cell surface as a membrane-anchored protein, but is processed in the Golgi apparatus by a furin-convertase enzyme to generate a biologically active, secreted protein[207]. An endogenous hybrid mRNA encodes TWE-PRIL, a functional cell surface TWEAK-APRIL fusion protein[208].

APRIL binds two TNFRSF members, BCMA (B-Cell Maturation Antigen, TNFRSF17) and TACI (transmembrane activator and cyclophilin ligand, TNFRSF13B)[209], and can also bind to the proteoglycan syndecan-1[200]. A closely related TNFSF member, BAFF (B cell activating factor, TNFSF13B) also binds to BCMA and TACI and to another TNFRSF member BAFFR (BAFF receptor, TNFRSF13C)[210]. However, whereas APRIL binds strongly to BCMA and moderately to TACI, BAFF binds weakly to BCMA and strongly to TACI and BAFFR[211].

The gene encoding APRIL is located on chromosome 17p13 near to the TNF superfamily member TWEAK. The gene itself comprises 6 exons transcribed as three alternately spliced mRNA of 1.8, 2.1 and 2.4 kb, encoding a final 250-amino-acid-protein. The mature, soluble form of APRIL exists as a 63-kDa non-covalent trimer. The crystal

structure of murine APRIL is similar to that of the human ligand, a compact trimeric ligand[212].

While BAFF exists as a membrane anchored protein, released by proteolytic cleavage, as with many TNF ligands, APRIL exists solely as a soluble ligand. The functional cell surface TWEAK-APRIL fusion protein TWEPRIL arises from a hybrid mRNA transcript of APRIL and TWEAK. The protein comprises the cytoplasmic and transmembrane portions of TWEAK fused to the carboxyl-terminal TNF domain of APRIL. Studies into the role of this form of APRIL are few and far between and little is known of its function. It is proposed that TWEPRIL might act as a membrane bound form of the usually soluble APRIL, offering cell surface presentation of the ligand to nearby cells expressing the receptors for this ligand[212, 213]. While APRIL is known to exist as a non-covalent homotrimer, heterotrimerisation with BAFF can also occur. TWEPRIL is able to bind both BCMA and TACI while the APRIL/BAFF heterotrimers appear to only bind to TACI[212].

The three receptors BCMA, TACI and BAFFR are structurally unusual for TNFRSF members. Having only a single cysteine-rich domain and only a partial domain in the case of BAFFR, each receptor reacts with a single ligand subunit. Other TNFRSF members have multiple domains and are able to interact with the boundary between two ligand subunits[214].

Heparan sulfate proteoglycans have been identified as novel binding partners of APRIL. The basic sequence QKQKKQ towards the amino terminus of mature APRIL is responsible for this binding and interaction between this domain and the proteoglycan can be blocked by heparin[215]. The ligand is capable of simultaneous

binding to both its receptors and heparin sulfate proteoglycans. The purpose of this interaction is unclear with it being proposed that haemopoetic cells expressing heparin sulfate proteoglycans could accumulate APRIL to trigger autocrine growth through BCMA and TACI[216]. Other TNF members including FasL and CD40L require higher order oligomerisation to function effectively and proteoglycan binding may facilitate this for APRIL. The interactions between APRIL, BAFF and their receptors in addition to some of the effects are depicted in figure 9. In addition the role of APRIL in the development of a number of different cell types can be seen in figure 10.

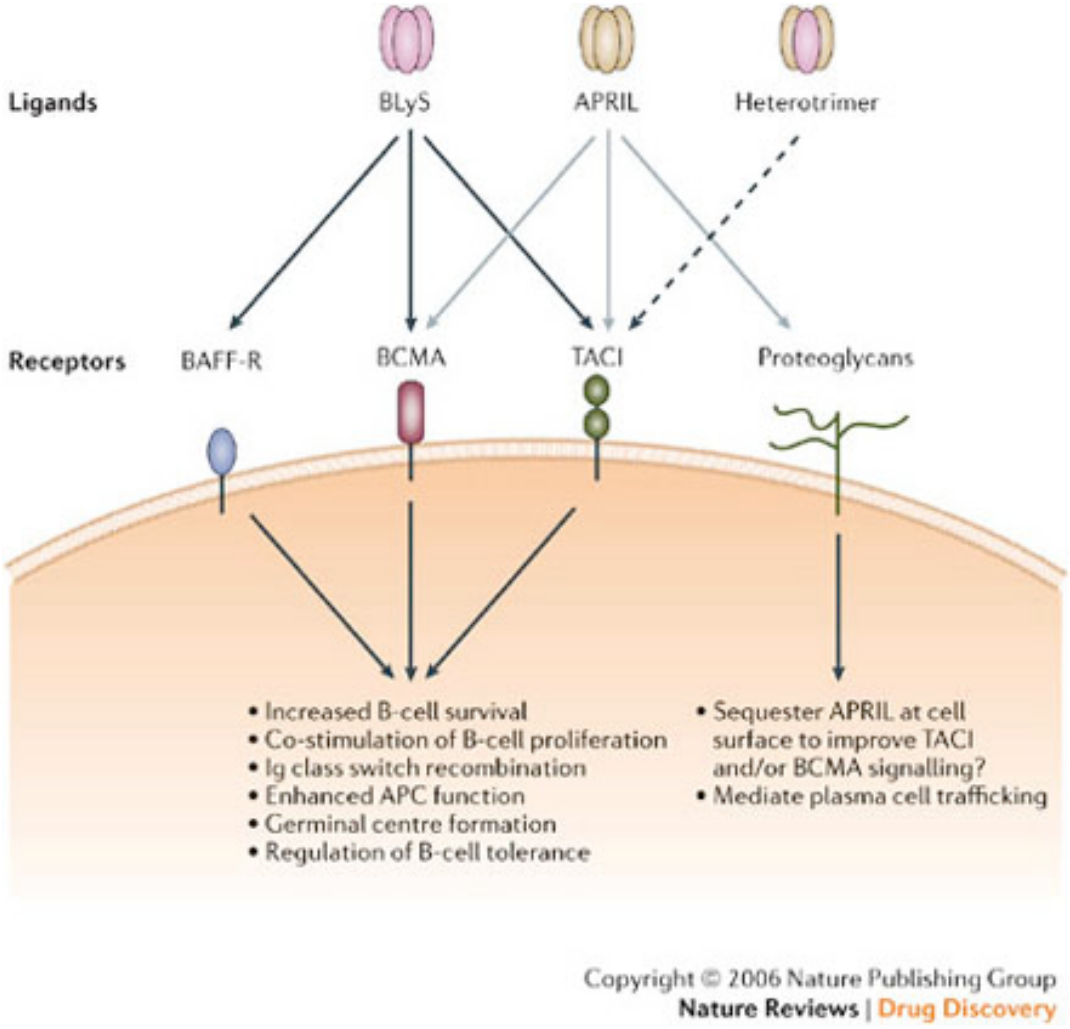


Figure 9: Interactions of APRIL and BAFF with their receptors.

The interactions between the soluble ligands B lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) and their receptors expressed on B cells are shown, along with some of the key functions mediated by these associations. The interaction (dashed line) between BLyS/APRIL heterotrimers and transmembrane activator and CAML interactor (TACI) is postulated because TACI-immunoglobulin (TACI-Ig), but not the B-cell maturation antigen-Ig (BCMA-Ig) or BAFF-R-Ig soluble receptors, have been shown to bind heterotrimers. Not shown: BLyS also exists as a type II transmembrane protein that is cleaved by a furin-like protease to release the soluble form. TWE-PRIL is hypothesized to bind to TACI and BCMA, but this has not yet been demonstrated. APC, antigen-presenting cell.

[Dillon *et al. Nature Reviews Drug Discovery* advance online publication;
published online 10 February 2006 | doi:10.1038/nrd1982]

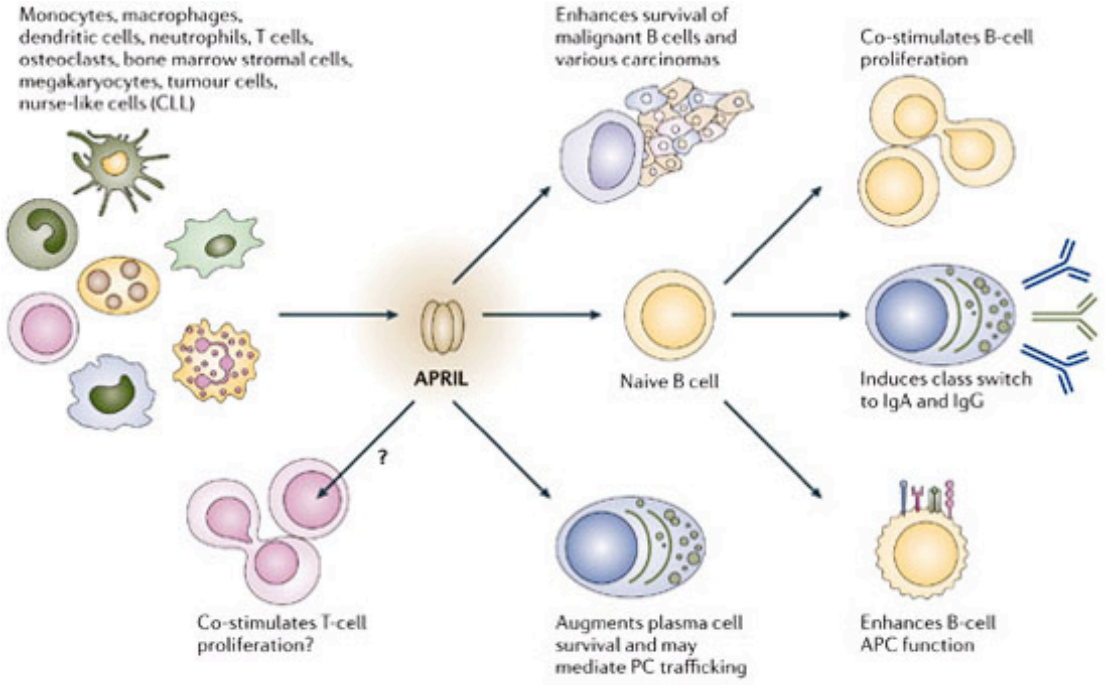


Figure 10: The actions of APRIL on a variety of cell types.

Produced and secreted by a wide variety of cell types (listed upper left), APRIL acts on both normal and malignant B cells to enhance survival, proliferation, APC function and class switch recombination at various stages of B-cell development. APRIL also seems to enhance the survival and proliferation of various carcinomas and, like BLyS, can co-stimulate T cells under certain conditions. APC, antigen-presenting cell; APRIL, a proliferation-inducing ligand; BLyS, B lymphocyte stimulator.

[Dillon *et al. Nature Reviews Drug Discovery* advance online publication;
published online 10 February 2006 | doi:10.1038/nrd1982]

Project aims

The work contained within this thesis aims to investigate a novel role of APRIL and its receptors in the development of the peripheral nervous system. The mouse superior cervical ganglion has been chosen as a model of this development offering defined, dissectible populations of the neurons themselves and their target fields.

Initial work aims to identify the presence of mRNA transcripts and protein expression for these TNF superfamily members and their spatial and temporal distribution within the developing SCG and representative target fields. Such information gives an initial insight into developmental points of interest and offers an indication of where and when to pursue subsequent functional analysis.

Following the expression analysis is a functional study aiming to identify the activity of APRIL signalling *in vitro.* Work focuses on the role of this ligand and its receptors in the growth and survival of SCG neurons in culture and what potential intracellular mechanisms mediate any phenotype.

The study aims to expand on *in vitro* functional data through the use of APRIL null mice. By comparing the development of the SCG and target fields innervated by neurons of this ganglion between wild type and knockout littermates, it is possible to investigate the *in vivo* relevance of APRIL signalling in the development of the sympathetic nervous system.

Chapter 2

Materials and Methods

Materials and Methods

Introduction

The research described within this thesis utilises a combination of both *in vitro* and *in vivo* techniques to assess both neuronal survival and outgrowth in the PNS. The in vitro methods are both well established and widely used as tools for quantifying neuronal survival and outgrowth and the mechanisms that regulate them. Where interesting observations have been noted the use of appropriate transgenic models and *in vivo* observations of neuronal number and target field innervation have been used to ascertain the relevance of *in vitro* observations in the development of the vertebrate peripheral nervous system.

Animal maintenance

The majority of research was conducted on tissues obtained from CD1 and C57BL/6 mice (*Mus musculus*). Mice were housed in a 12 hour light-dark cycle with access to food and water *ad libitum*. Breeding and housing was approved by the Cardiff University Ethical Review Board and was performed within the guidelines of the Home Office Animals (Scientific Procedures) Act, 1986.

Both embryonic and post natal wild-type mice were obtained from timed matings of CD1 mice.

APRIL$^{-/-}$ were obtained by mating APRIL$^{+/-}$ mice overnight with confirmation of breeding being the presence of a vaginal plug. The period of gestation was considered to be embryonic day (E) 1.

Preparation of media

Dissection media (L-15)

In an autoclaved 1L bottle, a single 500ml bottle of L-15 Medium (Leibovitz) without L-glutamine (Sigma-Aldrich) was combined with penicillin (60µg/ml) and streptomycin (100µg/ml) and the final mixture was filter sterilised using a 0.2µm bottle top filter (Nalgene). The complete dissection media was then stored at 4^0C for up to four weeks.

Wash media (F-12)

In an autoclaved 1L bottle a single 500ml bottle of Ham's Nutrient Mixture F12 (Sigma-Aldrich) was combined with 25ml heat inactivated horse serum (Sigma-Aldrich), penicillin (60µg/ml) and streptomycin (100µg/ml) and the final mixture was filter sterilised using a 0.2µm bottle top filter (Nalgene). The complete wash media was then stored at 4^0C for up to four weeks.

Culture media (F-14)

In an autoclaved 1L bottle 294mg of sodium hydrogen carbonate was added to 250ml distilled water (Lonza). After dissolving, 25ml was removed and discarded. 25ml 10x stock F-14 (Sigma-Aldrich) was added in addition to penicillin (60µg/ml), streptomycin (100µg/ml), 2.5ml 200mM Glutamax (Life Technologies) and 5.5ml albumax comprising Albumax I (Life technologies), progesterone (60µg/ml), putrescine (16µg/ml), L-thyroxine (400ng/ml), sodium selenite (38ng/ml) and tri-iodothyronine (340ng/ml). The final mixture was

filter sterilised using a 0.2µm bottle top filter (Nalgene). The complete culture media was then stored at 4^0C for up to four weeks.

Preparation of dissection needles

Fine needles for embryonic dissection and the cleaning of dissected tissue were made and sharpened as per requirement. Two lengths of 0.5mm tungsten wire were bent at a 100^o angle 3cm from their end. These blunt needles were electrolytically sharpened in a 2M KOH solution using a variable 3-12V AC power supply. Needles were sharpened to a fine elongated point before mounting with a needle holder (interfocus). Prior to each use needles were immersed in 70% ethanol and flamed.

Dissections

Prior to each dissection all instruments were immersed in 70% ethanol prior to flaming. A laminar flow hood provided a clean environment for all dissection and culture work and this was cleaned with 70% ethanol prior to and after each use.

Embryonic dissection

Pregnant mothers were collected at the required day of gestation and sacrificed by CO_2 asphyxiation followed by confirmation of death by cervical dislocation. The abdomen was thoroughly cleaned with 70% ethanol before embryos were removed by laparotomy and stored in a Petri dish (Greiner) containing L-15 media.

The embryos were removed from their individual sacs and cleaned in a new petri dish (Greiner) containing fresh L-15. Before use the exact embryonic stage was confirmed by criteria set by Theiler. A stage selector can be seen at http://www.emouseatlas.org/emap/ema/home.html
Dissection of all sympathetic and sensory ganglia and target tissues used forceps and scissors with final cleaning of tissue using tungsten needles. At embryonic ages preceding E15 all dissection used tungsten needles.

Post-natal dissection

Mice at all post natal ages up to P10 were sacrificed by decapitation while those older than P10 were sacrificed by CO_2 asphyxiation followed by confirmation of death by cervical dislocation.

Real Time PCR

RNA extraction

To collect high quality RNA for use in real time PCR tissue was firstly dissected and suspended in an RNA stabilisation solution (Sigma) before being stored at -80^{0}C. Total RNA of samples was collected using a RNeasy extraction kit (Qiagen) following the manufacturers protocol.

Reverse transcription

RNA was reverse transcribed for one hour at 47^0C with an affinity script reverse transcriptase kit (Agilent). RNA was incubated in 20µl volumes with the appropriate kit buffer supplemented with 5mM dNTP mix (Agilent), 10µM random hexamers (Fermentas) and affinity script reverse transcriptase as per manufacturer's instructions. Following reverse transcription cDNA was immediately stored at 4^0C. In parallel to all experimental samples a set of controls lacking the reverse transcriptase was set up. This control checks for genomic contamination of DNA in downstream applications.

Real time PCR

Real time PCR was carried out using a Brilliant QPCR kit (Agilent) as per manufacturer's instructions. Each individual reaction was prepared using 2µl cDNA while a no template control contained no cDNA. The reference dye ROX (1 in 500) was added to each master mix to control for differences in reaction volume. A single reaction contained 10µl 2x master mix, 1µl 7.5µM forward and reverse primer and 0.8µl probe at 10µM.

The following table shows the primer sets and Taqman probes used in this study:

Name	Sequence
m_BCMA_Taq_F1	TGA CCA GTT CAG TGA AAG G
m_BCMA_Taq_R1	GGG TTC ATC TTC CTC AGC
m_BCMA_taqman1	FAM-CGT ACA CGG TGC TCT GGA TCT TCT T-BHQ1
m_TACi_Taq_F1	CTC AAG GAA ATC CTG TGT

m_TACi_Taq_R1	GAA TTT GCA GAA GTC TGT AC
m_TACi_taqman_1	FAM-CGC TGG CTC CTC TGG CTG-BHQ1
M_Baff_taq_F1	TGA ATC TGA TCC AAA CCA
M_Baff_taq_R1	CGT TTC TCA TAA CTC ACA T
M_Baff_taqman_1	FAM-ATA ACA GAC AGC CAC AAC CGA AG-BHQ1
M_BaffR_taq_F1	AAT TAG CGG CTG GAG AAA T
M_BaffR_taq_R1	CAT TCT GGG AAT CAA ACT CT
M_BaffR_taqman_1	FAM-ATC CTC TGG AAT CAC AGT AAG CG-BHQ1
m_April_Taq_F5	CTG TCC TTC CTA GAT AAT G
m_April_Taq_R5	CTA GTG ACA CTC TGA CAC
m_April_probe_5	FAM-CAC CAA ATT CTC CTG AGG CT-BHQ1
m_Fn14_Taq_F1	ACT CGT CGT CCA TTC ATT
m_Fn14_Taq_R1	CTC TAA GCC CAG TCC TTG
m_Fn14_probe_1	FAM-TGA ATC ACC ACC TCG CCC-BHQ1
m_Tweak_Taq_F1	CTT GCT CTT CTT TAA CAT CC
m_Tweak_Taq_R1	GAT AAG TAG GGG CTT TGG
m_Tweak_probe_1	FAM-CCA CCA CAA CTA TCC ACC TCA C-BHQ1
TWEPRIL_TaqLF	ATT CTC AGC CAC AGC AGC
TWEPRIL_TaqLR	TTC GCC CCA TCC TTC CAG
M-Twepril_**LNA** probe 1	FAM-CCA **G**GA **C**AT **C**AG **G**AC TCT-BHQ1
m_gapdh_Taq_F1	GAG AAA CCT GCC AAG TAT G
m_gapdh_Taq_R1	GGA GTT GCT GTT GAA GTC
m_gapdh_probe_1	FAM-AGA CAA CCT GGT CCT CAG TGT-BHQ1
m_SDHA_Taq_F1	GGA ACA CTC CAA AAA CAG
m_SDHA_Taq_R1	CCA CAG CAT CAA ATT CAT
m_SDHA_probe_1	FAM-CCT GCG GCT TTC ACT TCT CT-BHQ1
m_trkA_Taq_F1	CTG TGT CCA TCA CAT CAA
m_trkA_Taq_R1	GAA GGT TGT AGC ACT CAG
m_trkA-probe_1	FAM-CGC CAG GAC ATC ATT CTC AAG T-BHQ1
m_NGF_taq_F	AAA CGG AGA CTC CAC TCA CC

m_NGF_taq_R	GTC CTG TTG AAA GGG ATT GTA CC
m_NGF_taqman	FAM-TGT TCA GCA CCC AGC CTC CAC CCA-BHQ1
m_p75NTR_Taq_F1	ACC AGA GGG AGA GAA ACT
m_p75NTR_Taq_R1	GCA GGC TAC TGT AGA GGT
m_p75NTR_probe_1	FAM-ACA GCG ACA GCG GCA TCT-BHQ1

The PCR cycle profile was set up according to the manufacturer's instructions. Parameters included a 3 minute 95^0C step followed by 40x cycles of 10 seconds at 95^0C followed by 35 seconds at 60^0C.
All samples were normalised to the geometric mean of two housekeeping genes succinate dehydrogenase and GAPDH.

Cell culture

All culture work took place in a laminar flow hood following aseptic technique in order to minimise infection risk. All surfaces were cleaned prior to and after completion of work with 70% ethanol. Cell culture experiments were repeated a minimum of three times from individual dissections.

Preparation of culture dishes

All neurons cultured in this thesis were grown on a laminin/poly-ornithine substratum. 35mm dishes (Greiner) and 35mm dishes with four wells (Greiner) were initially coated with 1ml 500mg/L poly-DL-ornithine (Sigma) in a borate solution. These dishes were left overnight at room temperature. After this period, the dishes were washed twice with sterile distilled water to remove the poly-DL-ornithine solution before being air dried inside a laminar flow hood

for a minimum of one hour. Dishes prepared this way were stored up to one week at room temperature.

Four hours prior to use, 35mm dishes were coated with 150µl of 20mg/ml laminin (Sigma) in Hank's Balanced Salt Solution (HBSS) (Life technologies). For four well dishes 80µl of laminin was added to each well. The dishes were subsequently incubated at 37^0C for four hours to build up a suitable substratum for cell culture.
At the point of plating cells excess laminin was removed by aspiration immediately prior to the application of cell suspension to prevent drying and damage to the substratum.

Dissection of sympathetic and sensory ganglia

The dissection of ganglia for cell culture took place in a laminar flow hood following aseptic technique.
To access the superior cervical and trigeminal ganglia from postnatal pups and embryonic ages older than E15 a sagittal cut was used to bisect the head. The brain was subsequently removed to reveal the elongated structure of the trigeminal ganglion located bilaterally on the base of the skull.
To access the SCG forceps were used to open the jugular foramen and remove the occipital bone. Revealed below is the SCG lying above the carotid artery and attached caudally to the sympathetic chain.
Both of these ganglia were removed using forceps and excess connective tissue removed using tungsten needles. Upon cleaning ganglia were transferred to a 15ml Falcon tube (Greiner) in HBSS (Life Technologies) for subsequent processing.

Dissociation to single cell suspension

Following dissection ganglia were suspended in 950µl HBSS (life technologies) containing 50µl 0.05% trypsin (Worthington). This mixture was maintained at 37^0C in a water bath for a time period relative to developmental age of the ganglia. Too long in trypsin results in poor viability of cells while an insufficient treatment leads to difficulty in dissociation of tissue and subsequent damage to cells. Respective times of trypsin digestion were as follows:

Developmental Age	Time in Trypsin (min)
E13	10
E15	15
E18	25
P0	30
P5	40

After trypsin digestion the ganglia were first washed twice in 10ml F-12 wash media to remove and inactivate the trypsin. Following the two washes a 200µl bevelled tip pipette (Starlab) was used to gently triturate the ganglia resulting in a single cell suspension. At this stage cells were inspected under a phase contrast microscope to ensure only healthy dissociated neurons were used for cell culture. The presence of remaining intact small processes emanating from cell soma along with a healthy brown colouring and intact cell membrane indicate viable neurons. An estimate of cell number was also made at this stage to help determine correct plating densities for subsequent analysis.

For low density cell culture suitable for analysis of neurite outgrowth and survival 100 to 150 neurons were plated per well of a four well 35mm dish or up to 300 neurons plated over a single 35mm dish (Greiner).

Neurons were diluted in an appropriate volume of F-14 culture media prior to plating to achieve these densities and treated with common exogenous factors at this point. Cell suspension was separated into 1.5ml microcentrifuge tubes (Greiner) for the addition of condition specific exogenous factors prior to plating. After successful plating of neurons in their appropriate media, cells were cultured at 37^0C with 5% CO_2 in a humidified incubator.

Many cultures include the presence of NGF to promote survival and outgrowth at ages when these processes are critically dependent on the presence of this neurotrophin. A concentration of 1ng/ml promoted maximal survival over 25 hours in culture and is thus the preferred concentration for all experiments where used unless otherwise stated.

Quantification of neuronal survival

For quantification of cell survival by counting each 35mm culture dish or each individual well of a four well dish were counted using a graticule for reference. The graticule consisted of a 12x12mm^2 grid inscribed onto a 900mm plastic Petri dish (Greiner). This provided a 36 square grid in which all live healthy cells could be counted using a phase contrast microscope. All healthy cells with intact cell membranes were counted. At two hours post plating a random selection of wells or 35mm dishes were counted to provide an initial neuronal cell number figure. Following the desired length in culture

every dish or well was again counted to give the number of cells alive after that time period. Survival data represent the percentage of cells that survived the culture period relative to the initial plating number.

Quantification of neurite growth

After the required time in culture, neurons destined for analysis of growth needed to be visualised. To each four well dish 1ml of pre warmed F-14 culture media was added containing 1.5µl calcein acetoxymethyl ester (Life Technologies). For 35mm dishes this factor was added directly to the culture media. Cells were then incubated for an additional 30 minutes to allow for uptake of this factor and processing to a fluorescent dye. If more than six dishes were to be analysed this process would be split to avoid over exposure of cells to calcein acetoxymethyl ester and subsequent cell death. After this 30 minute period cells were individually imaged using an inverted fluorescence microscope (Zeiss). If plated at low density neurons could be imaged individually with minimal overlap of processes.

For each experimental condition 60 images were collected covering all areas of each dish and thus best representing the populations cultured within.

Unless otherwise stated, neurons were cultured in the presence of a pan caspase OPH inhibitor. Q-VD-OPh (quinolyl-valyl-O-methylaspartyl-[-2, 6-difluorophenoxy]-methyl ketone) is a cell permeable, irreversible, broad-spectrum Caspase Inhibitor. It is effective in preventing apoptosis mediated by three major apoptotic pathways, caspase 9/3, caspase 8/10 and caspase 12. Replacement of the fluoromethyl ketone (fmk) with a carboxy terminal O-Phenoxy group significantly reduces toxicity, even at high levels. This ensured

consistency of survival of cells in culture reducing the possibility that differences in growth observed was instead a result of reduced survival in a particular condition.

Analysis of neurite growth on subsequent images used Sholl analysis to quantify the number of neurites intersecting concentric rings of ever increasing size centred on the cell soma. For E16 neurons through to post natal ages Sholl analysis used 25 rings set 30μm apart. The analysis provided a graphical plot of the number of neurites intersecting each ring as distance from the cell soma increased in addition to information of total neurite length and total number of branching points per neuron.

Immunocytochemistry

To visualise the expression of proteins by cells in culture immunocytochemistry was the technique of choice. Cells were cultured for the required period before media was washed away with 1ml pre warmed PBS per dish. Application of 4% PFA followed for 15 minutes at room temperature to fix the cells and their processes. After successful fixation the PFA was removed by three subsequent washes with PBS for two minutes each. Permeabilisation of neurons was coupled with blocking of non specific antibody binding through a one hour block. A 1ml solution containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 dissolved in PBS by vortexing was applied to each dish and left at room temperature for 30 minutes to one hour. Following the block all subsequent antibody applications used a solution made by diluting this blocking buffer five fold in PBS (antibody buffer).

Primary antibodies diluted to their desired concentration in antibody buffer were applied to the dishes and incubated overnight at 4^0C. For primary antibodies used and relevant dilutions see the table below:

Antibody	Species	Dilution	Company	Type
APRIL	Rabbit	1:200	abcam	Polyclonal IgG
BCMA	Rabbit	1:200	abcam	Polyclonal IgG
TACI	Rabbit	1:200	abcam	Polyclonal IgG
B-III tubulin	Mouse	1:1000	R and D Systems	
Tyrosine Hydroxylase	Mouse	1:500	Millipore	

Following primary antibody incubation cells were washed three times in PBS for five minutes each before application of secondary antibodies diluted in antibody buffer. Secondary antibodies were incubated at room temperature for one hour in the dark. For relevant secondary antibodies and dilutions see the table below:

Antibody	Species	Dilution	Company
Alexafluor 546 anti-mouse	Goat	1:500	abcam
Alexafluor 546 anti-rabbit	Goat	1:500	abcam
Anti-rabbit	Goat	1:500	Vector

biotin			laboratories
Anti-mouse biotin	Goat	1:500	Vector laboratories

After secondary antibody application cells were washed a further three times in PBS for five minutes each. If a biotin conjugated secondary was used cells were incubated with Cy2 conjugated streptavidin for 30 minutes at room temperature diluted 1:500 in antibody buffer.

After this final application and a final three washes in PBS for five minutes each, cells were suspended in PBS and stored in the dark at 4^0C until imaging.

Visualisation of cells took place using a laser scanning confocal microscope (Zeiss) operated according to manufacturer's instructions.

Immunohistochemistry

Frozen sections

Tissue destined for immunohistochemistry by frozen sectioning was first dissected at the relevant age and fixed in 4% PFA for an appropriate time period at 4^0C. Smaller tissue fragments including ganglia and dissected targets were fixed for four to six hours while larger intact regions were left overnight in fixative.

Following fixation, tissue was cryoprotected by immersion in 30% sucrose for 48 hours then transferred to a mould containing OCT

mounting reagent (Leica) and frozen at -80^{0}C. Frozen tissue was stored wrapped in tin foil at -80^{0}C until sectioning.

Sectioning was performed using a cryostat (Leica) and tissue blocks were mounted on the microtome for one hour prior to cutting to acclimatise to the temperature of the cryostat. Target tissues for subsequent immunostaining were sectioned at 16µm while ganglia were sectioned at 8µm. Individual sections were mounted in rows on Xtra adhesive slides (Leica) that electrostatically bond the tissue to the slide surface. Use of these slides significantly reduced loss of sections during subsequent stages. Once dried for two hours slides could be stored frozen at -80^{0}C until immunolabelling began.

For antibody based staining of frozen tissue slides were first thawed before being washed once with PBS to remove residual OCT mounting medium. A 1ml solution containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 dissolved in PBS was subsequently applied for one hour at room temperature to block non-specific antibody binding and to permeabilise tissue. Following this blocking step tissue slides were incubated with primary antibody diluted in antibody buffer (five-fold diluted blocking buffer in PBS). 150µl of this solution was added to each slide and covered with a section of parafilm to prevent drying. The antibody was incubated overnight at 4^{0}C.

The next day slides were washed three times in PBS for five minutes to remove residual primary antibody before incubation with 500µl secondary antibody dilution. Secondary antibody was also diluted in antibody buffer and incubated for one hour at room temperature. This solution was then removed by a further three PBS washes for five minutes each. If a biotin conjugated secondary antibody was

used then Cy2 conjugated streptavidin was added at a 1:500 dilution in antibody buffer for 30 minutes at room temperature.

Following a further three PBS washes for five minutes each all sections were counterstained with the nuclear marker TOTO-3 Iodide diluted 1:10000 in PBS and applied to the slides for 10 minutes. A final three PBS washes for five minutes each was followed by mounting of the slides with a glass coverslip glued in place with VectorShield (Vector labs) hard mount that contains an anti-fade reagent.

Slides were left to dry overnight at room temperature in the dark before storage at 4^0C in the dark. All images were acquired using a confocal laser scanning microscope (Zeiss). TOTO-3 Iodide emits a signal in the far red range and for the purpose of clarity of images this signal was false coloured blue for all images using this reagent. ImageJ software was used to split each image into its separate colour channels and export the images as TIFF files.

Measurement of sympathetic target field innervation

To quantify the numbers of sympathetic fibres innervating defined target tissues, these target tissues were fixed, stained and imaged as explained above. A primary antibody against tyrosine hydroxylase was used followed by a biotin conjugated secondary antibody and Cy2 conjugated streptavidin. Use of a biotin/streptavidin system enhances the signal of positive stain giving a large difference in contrast between positively stained fibres and background. This contrast gives a more reliable quantification than use of images with either a low fluorescent signal or high background.

Following image acquisition analysis took place in Adobe Photoshop. Images were first converted to greyscale thus giving a visual representation of the intensity of stain ranging on an individual pixel scale from 0 to 255. 0 represents pure black background while 255 represents pure white and a maximum intensity of TH stain. Using a representative histogram of each image the percentage of pixels in a defined area from 127 to 255 was recorded giving a quantification of TH positive stain.

A user defined area of each image was quantified. For SMG tissue an edge had to be visible and the section had to contain a minimum level of TH fibres. For NM tissue a defined fold was imaged from beginning to end on a rostral-caudal plane. By only quantifying these defined areas the analysis can be standardised between animals.

Cell counts of ganglia

Following sectioning at 8μm, tissue of SCG ganglia was blocked and stained with TOTO-3 Iodide. Images were acquired using a laser scanning microscope (Zeiss) using a 40x water immersion objective. The number of nuclei in a randomly selected field from each tissue section were counted in ImageJ [Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012].

Wholemount immunostaining

Wholemount immunostaining of submandibular gland and quantification of target-field innervation was performed as follows. The submandibular glands of APRIL$^{+/+}$ and APRIL $^{-/-}$ littermates were dissected at P10 and fixed in 4% paraformaldehyde for a minimum of 24 hours. The tissue was dehydrated in 50% methanol for 1 h at room temperature and 80% methanol for a further 1 h. Endogenous peroxidase activity was quenched by placing tissue in a solution of 80% methanol, 17% DMSO and 3% H_2O_2 overnight at 4 °C. Tissue was rehydrated by placing in 50% methanol for 1 h, 30% methanol for 1 h, PBS for 1 h, all at room temperature, and was blocked overnight at 4 °C with 4% BSA containing 1% Triton X-100 in PBS. The tissue was then incubated with polyclonal antibody to tyrosine hydroxylase (1:200, Millipore) in blocking solution for 72 h at 4 °C. After washing three times for 2 h in 1% Triton X-100 in PBS at room temperature, the tissue was kept at 4 °C overnight in a fourth wash before being incubated with rabbit, HRP-conjugated antibody (1:300, Promega) in blocking solution at 4 °C overnight. The tissue was then washed for 2 h at room temperature with PBS containing 1% Triton X-100. Tyrosine hydroxylase-positive fibers were visualized by DAB-HRP staining: the tissue was incubated with 1× DAB for 20 min at room temperature and then with 1× DAB containing 0.006% H_2O_2 for 2–5 min to develop the staining. After washing with PBS, the tissue was and incubated at 4 °C overnight in PBS. BABB (one part benzyl alcohol: two parts benzyl benzoate) was used as a clearing solution. The tissue was placed in 50% methanol for 10 min, washed 3 times with 100% methanol (once for 30 min and twice for 15 min) at room

temperature and was incubated in 50% BABB for 5 min before being placed in BABB. To compare the extent of sympathetic nerve branching near the gland hilus, a modified line-intercept method was used. Using ImageJ, a grid of 24 squares (4 × 6 squares) of side length 158 μm per square was aligned in a standard orientation next to the hilus of each gland. The number of fiber bundles intersecting the sides of squares in the grid was scored blind for the glands from each animal. Fiber density was estimated using the formula $\pi DI/2$, where D is the interline interval (158) and I the mean number of intersections along one side of each square in the grid. The data are expressed as a percentage of the mean data for wild-type mice.

APRIL transgenic mice

The use of APRIL knockout mice made up a significant portion of this study. Mice were supplied Genentech Inc., South San Francisco, California and produced by Varfolomeev et al, 2004. An APRIL targeting vector was first constructed based on the TNLOX1-3 vector by replacing 2.5 kb of the APRIL gene, encompassing the first and all five downstream exons, with a PGK-*neo*^{r} cassette. The construct contained two DNA stretches derived from the mouse genome: a 3.1 kb fragment encompassing the sixth and seventh exons of TWEAK and part of exon one of APRIL, placed 5' of the *neo* cassette, and a 4.1 kb fragment encompassing the first and second SMT3IP1 exons placed 3' of the PGK-*neo*^{r} cassette. TWEAK and SMT3IP1 flank the APRIL genomic locus. R1 embryonic stem cells were transfected with the linear vector by electroporation and G418- resistant clones screened for successful recombination by Southern blot analysis.

Two APRIL$^{-/-}$ lines were microinjected into C57BL/6 blastocytes. Germline transmission in mice generated through crossing chimeric males with C57BL/6 females was confirmed by a two-step PCR. APRIL null mice were subsequently backcrossed five times onto a C57BL/6 background.

The APRIL targeting neomycin cassette inactivates both the secreted form of APRIL in addition to the membrane bound form TWEPRIL. Gene deficiency was proven by genomic PCR screening and fluorescence-activated cell sorting using an anti-mouse APRIL antibody. Both mRNA expression and protein translation of APRIL was abolished in splenocytes isolated from *APRIL*-deficient mice. The mRNA expression of both TWEAK and SMT3IP1 was also tested to ensure that the APRIL targeting cassette did not affect the expression of these genes. Both were stably expressed in mice deficient for APRIL. For experimental litters used in this thesis, heterozygous animals were crossed resulting in litters of mixed genotype. As such wild type and knockout littermates could be compared experimentally.

Statistical analysis

Data are presented as mean values ± standard error of mean (s.e.m.). Data with greater than 10 samples per condition were first tested for normality of distribution by D'Agostino-Pearson omnibus normality test. If data did not differ significantly from a Gaussian distribution means were compared with an unequal variance t test with Welch's correction. For data containing more than two conditions means were compared by one-way ANOVA followed by individual

comparisons by Tukey's multiple comparisons test or multiple comparisons with control by Dunnett's multiple comparisons test.
If data deviated significantly from a Gaussian distribution the non-parametric Mann-Whitney test was used to compare distributions. For data containing more than two conditions means were compared by Kruskal-Wallis test followed by individual comparisons by Dunn's multiple comparisons test.

Chapter 3

Expression analysis of TNF members in the developing PNS

Introduction

The novel findings that multiple members of the TNF superfamily and their receptors play a part in the development of the peripheral nervous system raised the intriguing possibility that other members may have a part to play. As previously stated It has been shown that both soluble GITRL and FasL are able to enhance the growth of neurons[195, 196] while it has also been demonstrated that LIGHT, TNF and RANKL all reduce neurite growth[197, 198]. These demonstrate that members of the TNF superfamily are critical regulators of neurite growth acting to either enhance the process or to reduce it.

The next chapter of this thesis sets out with the aim of investigating if other members of the TNF superfamily and their receptors are expressed by peripheral neurons and/or their target fields of innervation. APRIL and BAFF, two ligands of the TNF superfamily sharing a high degree of homology are known to play significant roles in the development of a number of cell types in the body in addition to acting on malignant B cells to enhance their survival. The first step in the investigation of their role in the development of the peripheral nervous system has to be to determine their presence and abundance. Knowledge of where and when these ligands and their receptors can be found in cells of the developing nervous system will offer the first indication of any novel role in addition to aiding in the design and optimisation of experiments to ascertain any functional role.

The superior cervical ganglion was chosen as a source of sympathetic neurons to investigate the expression of APRIL, BAFF and their receptors for a number of reasons. These neurons offer an

ideal model for investigating the development of the peripheral nervous system as they are relatively easy to isolate and subsequently establish cultures of high homogeneity. In cultures established from the SCG the vast majority of cells are neurons and thus it makes a good model for investigating the role of novel factors in the growth and survival of neurons *in vitro*. In addition to this the target fields that receive innervation by SCG neurons are distinct and well defined. This offers the chance to investigate the role of novel factors in final target field innervation and build up a more complete view of their actions in the development of the peripheral nervous system. Neurons from the SCG can be cultured for less than 24 hours before quantification of neuronal growth and survival can take place. All in all the SCG offers an excellent model for the study of the development of the peripheral nervous system and so was selected as the prime candidate for investigation within this thesis.

To begin the study into the potential novel role of APRIL and BAFF in the development of the peripheral nervous system I set out to answer the hypothesis that these ligands and their receptors are expressed by neurons of the SCG and their target fields in the period of development associated with neuronal survival, outgrowth and final target field innervation.

Expression of TNF members of interest in the developing SCG and its peripheral target fields

To approach the hypothesis that APRIL, BAFF and their receptors are expressed by SCG neurons and their target fields a detailed look at their spatial and temporal distribution was required.

Initial investigation into the potential role of APRIL and BAFF as well as their receptors BCMA, TACI and BAFFR used a real time PCR based approach to quantify the relative expression of these members over a developmental time course from embryonic day 13 through to adulthood. This period covers the entire developmental period of interest including the period of neuronal call death, process extension and final target innervation. An understanding of mRNA levels within SCG neurons or their target fields offers a first insight into a possible role in the development of these neurons. An understanding of the level of expression acts as an indicator of points of interest for further investigation. The presence of mRNA transcripts indicates an ideal time to further investigate the presence of a protein and if present, to further investigate any functional role. It is therefore imperative to understand when the genes encoding APRIL, BAFF and their receptors are expressed as this gives a strong clue as to when to investigate the presence of a translated protein and its function. The aim of this screen was to firstly determine if transcripts for these proteins were detectable in the developing SCG and secondly to plot changes in expression in each gene over this time period.

The expression of APRIL mRNA was sub divided into APRIL and TWEPRIL through use of specific primer and Taqman probes for each to distinguish between transcripts encoding the soluble and

membrane bound form of the ligand. In addition to APRIL and BAFF and their receptors the expression of TWEAK and Fn14, its receptor, was also investigated due to the APRIL variant TWEPRIL comprising the intracellular and transmembrane portion of TWEAK.

A minimum of four pairs of ganglia were collected for each age and stored in an RNA stabilisation solution at-80^{0}C. Following RNA extraction and reverse transcription real time PCR was carried out using Taqman probes and primers (specific primers and probes are detailed in materials and methods chapter 2).

Expression of APRIL begins at a low level at E13 and rises to its highest expression by E15 (Fig. 11A). The ligand remains expressed throughout the development of the animal into adulthood. Levels of expression fall from E15 to P3 but show a small rise between this age and P10.

TWEPRIL expression was not detectable at E13 but is detectable by E15 rising to a peak of expression at P0. This ligand then remains expressed throughout development into adulthood (Fig. 11B).

Both receptors BCMA and TACI show a rise in expression throughout development with first detection visible at E18 in both cases. Expression of BCMA continues to rise to a peak once the animal is fully mature while TACI expression reaches a maximal level by P3 and remains constant into older ages (Fig. 11C-D).

BAFF and its receptor BAFFR are both also detectable in the developing SCG. BAFF transcripts can be identified as early as E15 and continue on a steady rise throughout development to peak in adulthood (Fig. 12A). BAFFR on the other hand rises to peak expression at P5 and then declines sharply by the end of development (Fig. 12B).

TWEAK expression becomes detectable from E15 and reaches a peak at E18 (Fig. 12C). This ligand is then stably expressed into adulthood. Its receptor Fn14 cannot be quantified at E13 but is consistently expressed throughout the rest of development declining as the mouse fully matures (Fig. 12D).

In addition to the developing SCG, the levels of APRIL and TWEPRIL expression were assayed in several peripheral target fields of innervation at P5. This age was selected for investigation as the target fields are receiving large numbers of innervating fibres at this stage. This explores the possibility that these TNF members may act as a target derived factor when sympathetic fibres are innervating. If any of the TNF members under investigation act as a target field derived factor on incoming SCG fibres their expression at this age is of great interest. Similar to the SCG time course, four of each target region were dissected and stored in an RNA stabilisation solution at-80^0C. Following RNA extraction and reverse transcription real time PCR was carried out using Taqman probes and primers (specific primers and probes are detailed in materials and methods chapter 2). Expression of APRIL and TWEPRIL could be seen in all sympathetic target fields investigated including the nasal mucosa, submandibular gland, Iris and pineal (Fig. 13). It could also be found in two additional targets, the heart and sensory innervated whisker pads. Expression of APRIL was higher in sympathetic targets of innervation relative to the heart and whisker pads. Highest levels of expression at P5 were seen in the SMG and pineal. TWEPRIL in contrast showed greatest level of expression in the pineal. The nasal mucosa, submandibular gland, Iris and pineal were selected as these receive innervation from the SCG. The heart and whisker pads instead

receive innervation by sensory fibres and were included as a comparison.

These results indicate that mRNA encoding APRIL and BAFF, their receptors and other members of the TNF superfamily are expressed throughout development of the SCG and APRIL can be detected in target fields of peripheral innervation. Such expression warrants an investigation into the presence of functional proteins in these locations.

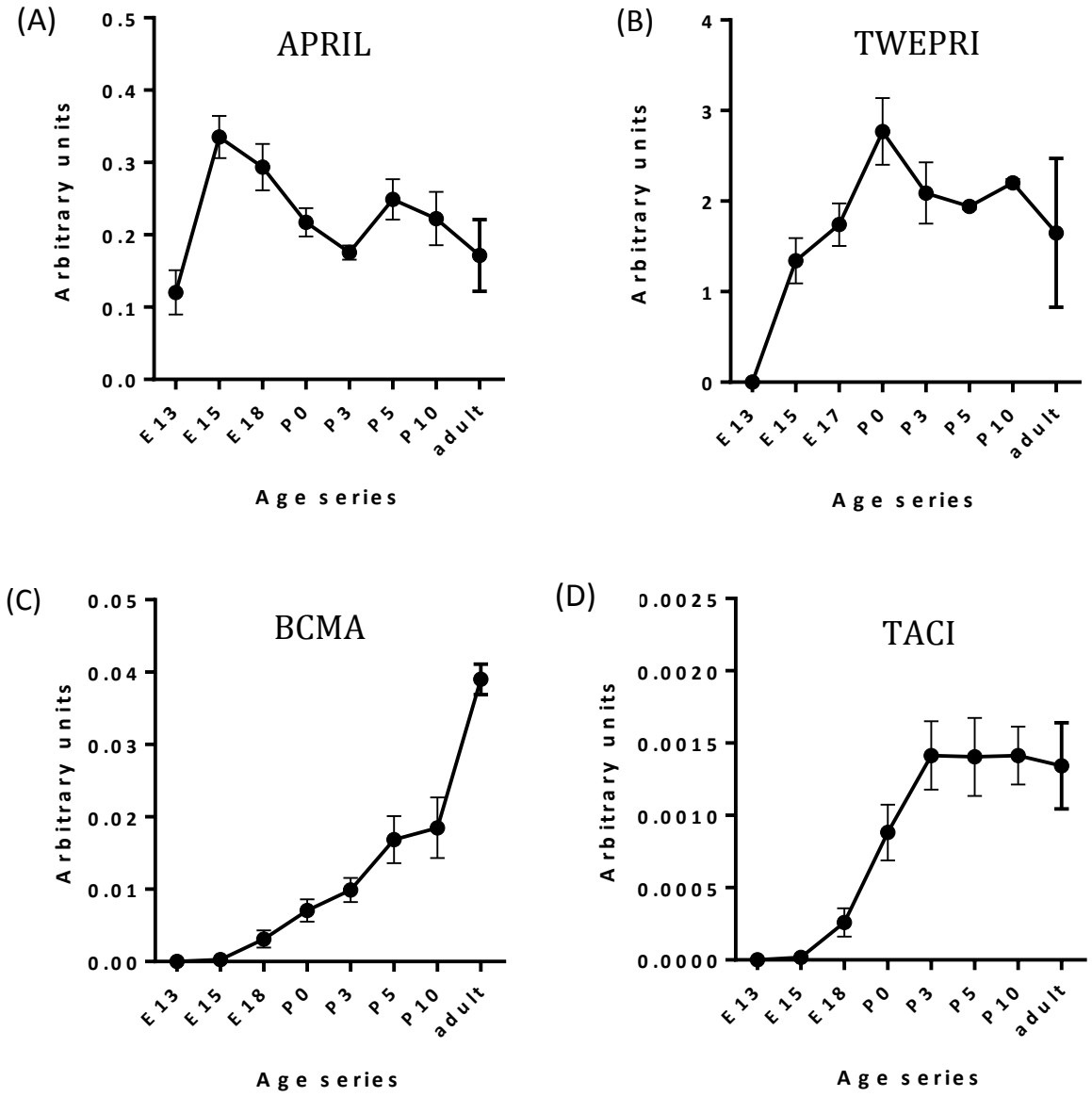


Figure 11: Relative expression of APRIL, TWEPRIL, BCMA and TACI mRNA from E13 to adult in the SCG (A-D) Expression profiles for APRIL, TWEPRIL, BCMA and TACI mRNA in a variety of developmental ages of SCG ganglia. (A) APRIL (B) TWEPRIL (C) BCMA (D) TACI expression relative to GAPDH and SDHA in the developing SCG. The data shown represent the mean ± s.e.m of data compiled from four pairs of ganglia per age.

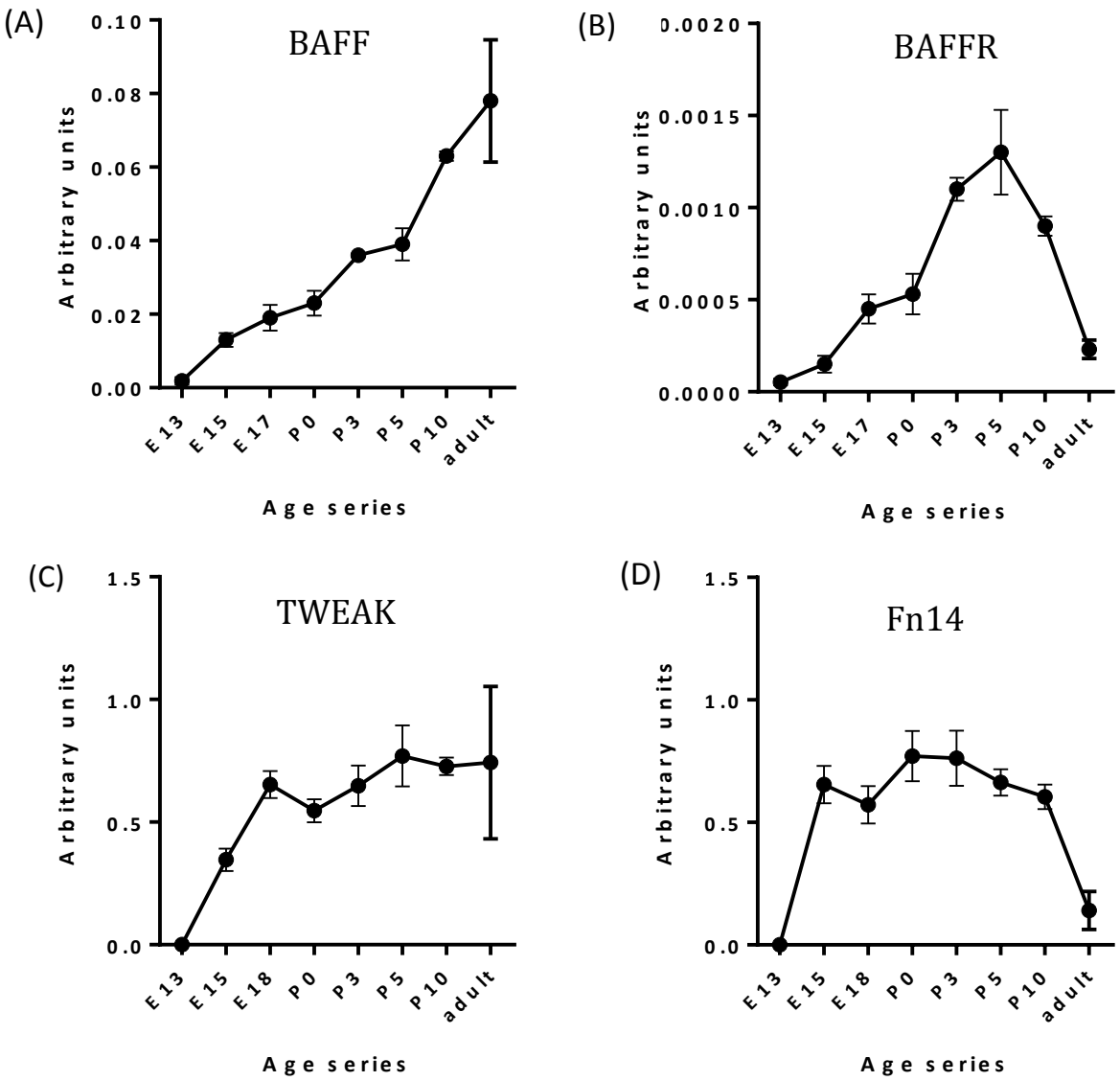


Figure 12: Relative expression of BAFF, BAFFR, TWEAK and Fn14 mRNA from E13 to adult in the SCG (A-D) Expression profiles for BAFF, BAFFR, TWEAK and Fn14 mRNA in a variety of developmental ages of SCG ganglia. (A) BAFF (B) BAFFR (C) TWEAK (D) Fn14 expression relative to GAPDH and SDHA in the developing SCG. The data shown represent the mean ± s.e.m of data compiled from four pairs of ganglia per age.

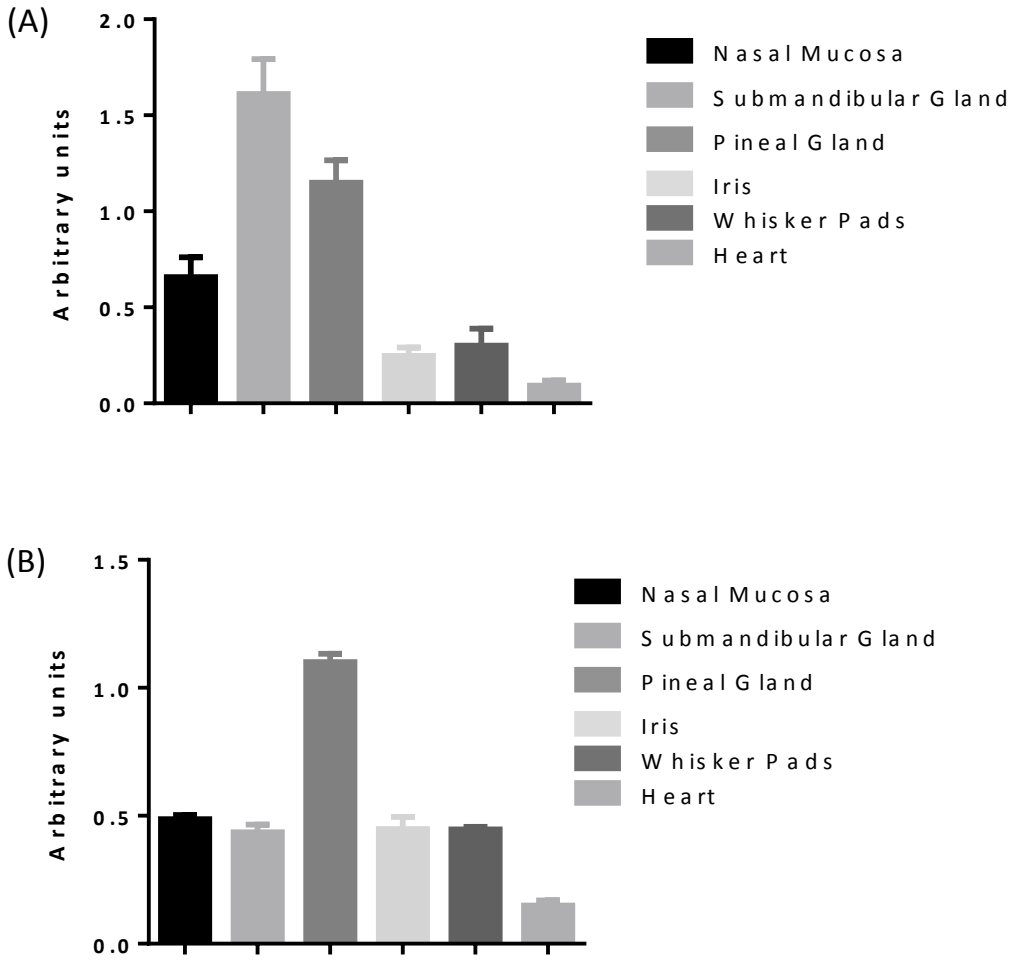


Figure 13: Relative expression of APRIL and TEWPRIL mRNA in P5 target fields of peripheral innervation (A-B) Expression profiles for APRIL and TWEPRIL mRNA in P5 target tissues. (A) APRIL (B) TWEPRIL expression relative to GAPDH and SDHA in the developing SCG. The data shown represent the mean ± s.e.m of data compiled from four targets.

Expression of APRIL, BCMA and TACI in cultured P0 SCG neurons

Following the discovery that mRNA encoding APRIL and its receptors were both expressed throughout development of the SCG and in its target fields of innervation the expression of these proteins was investigated using immunocytochemistry. This technique allowed for the localisation of protein to be identified and thus to see if the expression data translate to the presence of a protein *in vitro* and what the sub-cellular localisation of any protein actually is.

Dissociated P0 SCG cultures were established and these neurons were grown in the presence of NGF at 1ng/ml for the duration of culture to promote survival and neurite outgrowth. After 16 hours in culture the neurons were fixed in 4% PFA and stained with primary antibodies against APRIL, BCMA and TACI. These antibodies were identified by use of a biotin/strepdavidin secondary system. Neurons were identified by counterstaining with the microtubule protein B-III tubulin. All images were acquired using a confocal laser scanning microscope. The APRIL antibody is derived from the internal region of APRIL and therefore identifies APRIL prior to proteolytic cleavage and release as a soluble factor and does not recognise TWEPRIL as its internal region corresponds to TWEAK. Antibodies against BCMA and TACI are derived from synthetic peptides corresponding to human sequences of these receptors.

Neurons at P0 were selected for immunocytochemistry as this age is in the period of development when these cells are innervating their target fields and are undergoing NGF promoted growth and survival. In culture P0 SCG neurons express both APRIL (Fig. 14) and its receptors BCMA (Fig. 15) and TACI (Fig. 16). Merged images show

that expression of these proteins co-localises with the neuronal marker B-III tubulin. Expression of all three proteins can be seen throughout the cultured neuron most strongly visible at the cell soma. Higher magnification of the soma and processes are included and these clearly identify the expression of APRIL, BCMA and TACI throughout the cultured neurons.

In all staining's a set of single labelled and no primary control cultures were set up. Before acquiring final images all primary antibodies had to show a similar staining pattern in isolation to avoid the possibility that signal cross over affects conclusions drawn from the experiment. In addition the no primary controls had to demonstrate that secondary antibody binding was specific. If any of these two controls failed the experiment was not used.

The presence of the ligand APRIL and its receptors BCMA and TACI can therefore be seen in P0 neurons cultured in the presence of NGF. This confirms that mRNA detected in the developmental screen for these TNF members are translated and produce a protein.

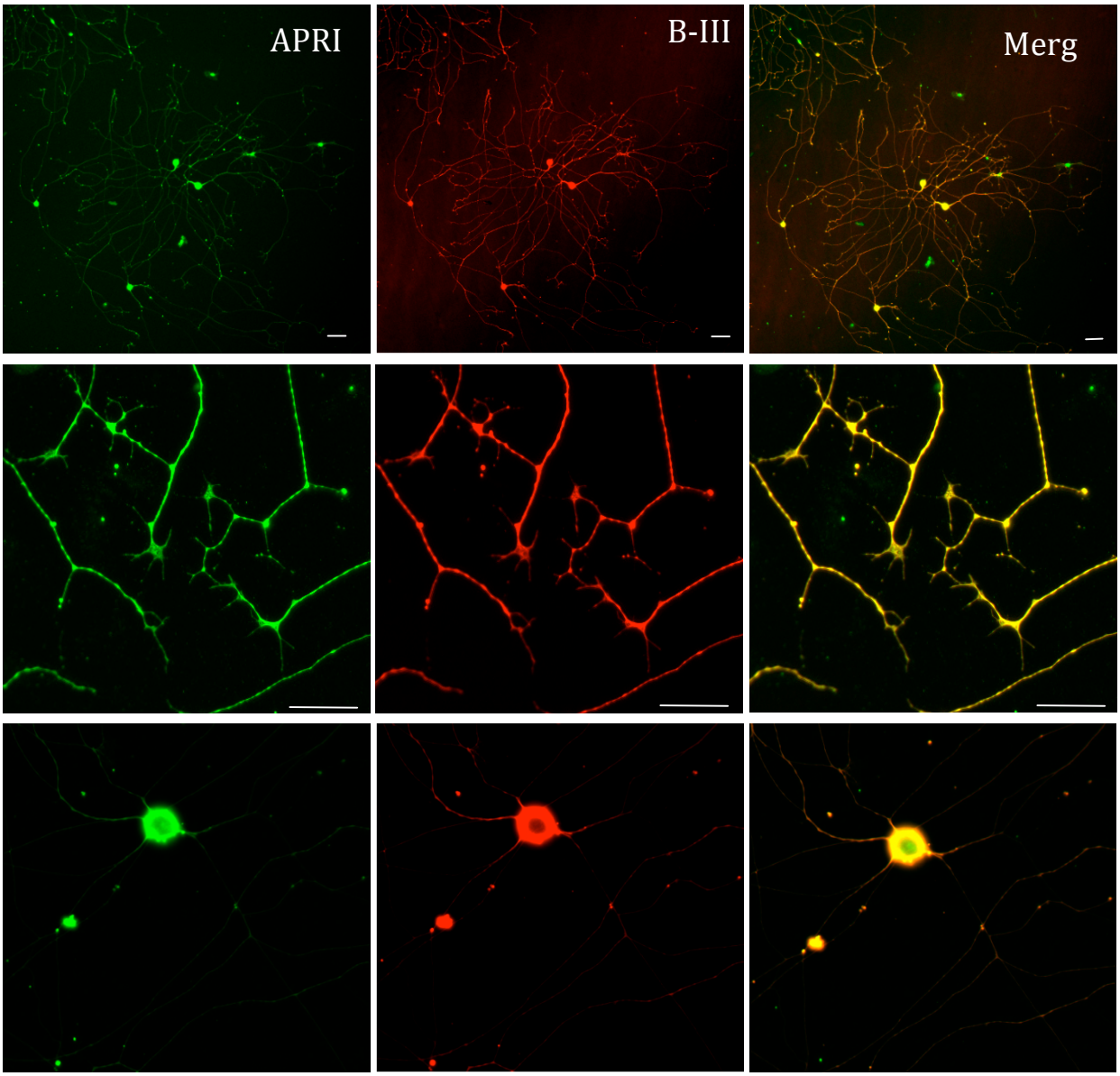


Figure 14: Expression of APRIL and B-III tubulin in cultured P0 SCG neurons Expression of APRIL and B-III tubulin in P0 SCG neurons cultured for 16 hours in the presence of 1ng/ml NGF. APRIL, B-III tubulin and a merge showing co-localisation are depicted. Scale bar represents 20µm.

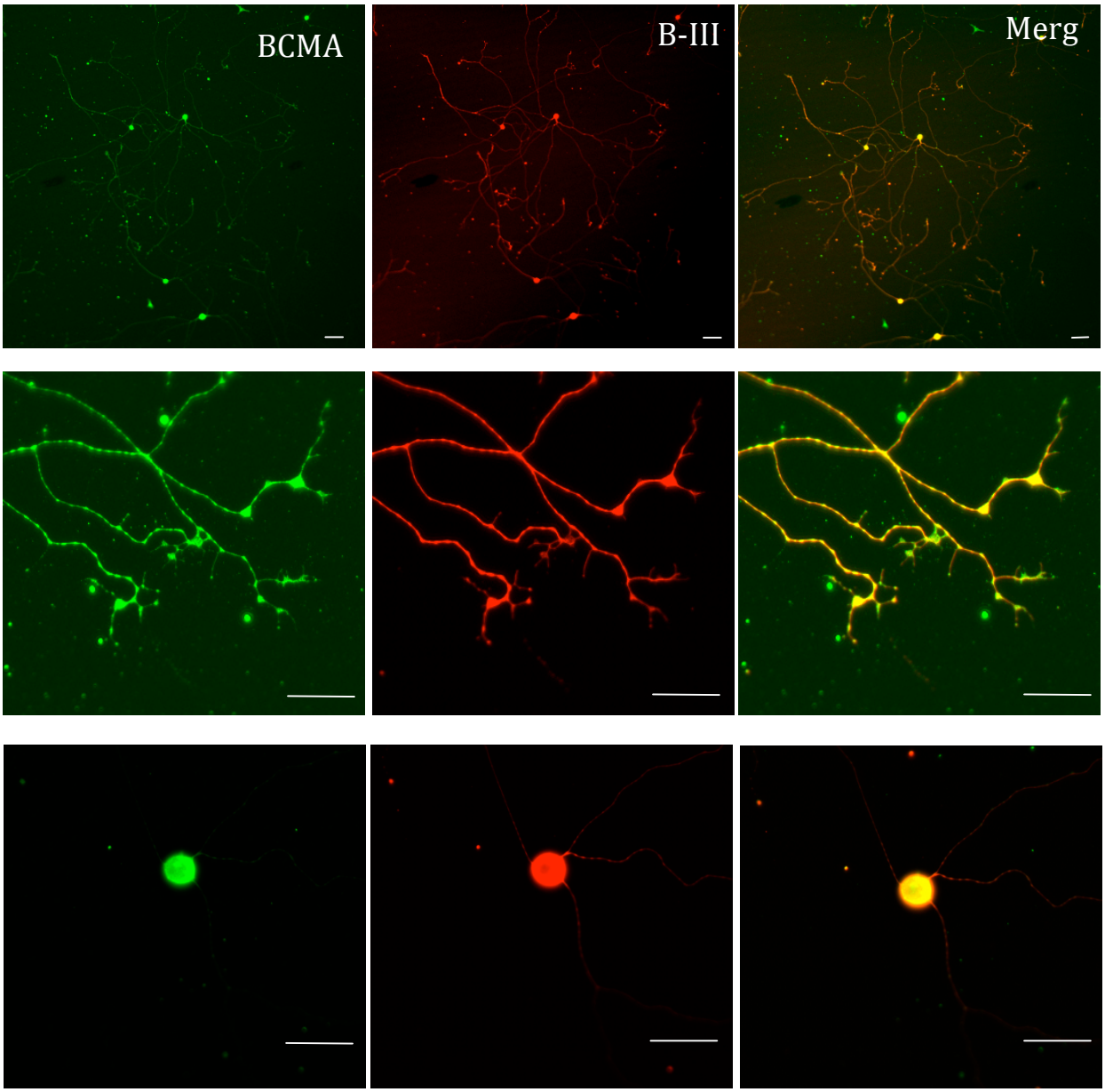


Figure 15: Expression of BCMA and B-III tubulin in cultured P0 SCG neurons Expression of BCMA and B-III tubulin in P0 SCG neurons cultured for 16 hours in the presence of 1ng/ml NGF. BCMA, B-III tubulin and a merge showing co-localisation are depicted. Scale bar represents 20µm.

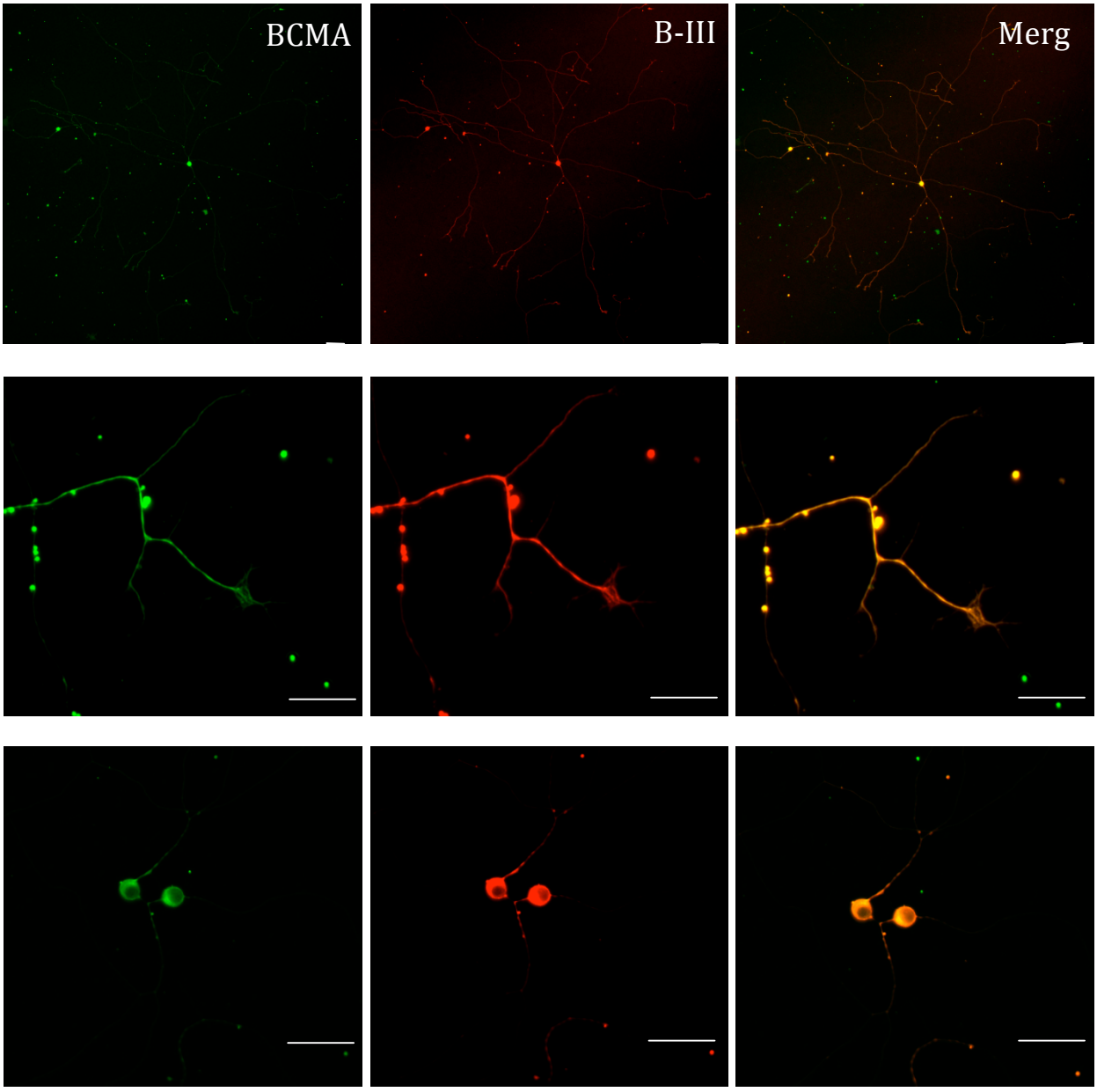


Figure 16: Expression of TACI and B-III tubulin in cultured P0 SCG neurons Expression of TACI and B-III tubulin in P0 SCG neurons cultured for 16 hours in the presence of 1ng/ml NGF. TACI, B-III tubulin and a merge showing co-localisation are depicted. Scale bar represents 20µm.

Expression of APRIL, BCMA and TACI by immunohistochemistry

Now that we know APRIL, BCMA and TACI are expressed in cultured neurons, within the SCG throughout a wide developmental window and within several target fields of peripheral innervation, immunohistochemistry was used to localise the target field expression of these proteins as well as their expression within the developing SCG. The submandibular gland was chosen for analysis at P0, P5 and P10 covering a broad range of the developmental time window within which sympathetic neurons are ramifying in these targets. Ganglia were imaged at E16, E18, P0 P5 and P10 to cover much of the range in which expression of mRNA was identified by real time PCR.

Submandibular glands of P0, P5 and P10 mice were collected and fixed in 4% PFA. Up to 10 individual ganglia were dissected and cleaned with tungsten needles prior to fixation in 4% PFA. Following cryoprotection these targets were individually sectioned at 16µm while ganglia were sectioned at 8µm. Tissue was stained with primary antibodies against either APRIL, BCMA or TACI. All sections were also stained with a primary antibody against tyrosine hydroxylase to identify the presence of sympathetic fibres within these tissues. Finally the sections were counterstained with TOTO-3 Iodide to identify nuclei. Images were acquired using a confocal laser scanning microscope. The APRIL antibody is derived from the internal region of APRIL and therefore identifies APRIL prior to proteolytic cleavage and release as a soluble factor and does not recognise TWEPRIL as its internal region corresponds to TWEAK. Antibodies against BCMA and TACI are derived from synthetic

peptides corresponding to human sequences of these receptors. Each figure identifies expression of the TNF member in question (red), tyrosine hydroxylase nerve fibres (green), cell nuclei (blue), a merge of red and green channels and finally a merge of all three channels.

APRIL, BCMA and TACI are detectable by immunohistochemistry in SMG's from P0 through to P10 (Fig. 20-22). Their presence can often be identified in the regions innervated by tyrosine hydroxylase positive fibres, however, little evidence of co-localisation is apparent. It would appear that while expression of APRIL and its receptors is evident in the submandibular gland these proteins are not present on TH positive fibres innervating the tissue.

In contrast to expression in target tissue, APRIL, BCMA and TACI are all evident within cells of the SCG from E16 through to P10 (Fig. 17-19). Highest expression is seen in cell soma at the later ages P5 and P10 although images for all ages show the presence of these proteins. Interestingly, as with the targets, when tyrosine hydroxylase positive sympathetic fibres are visible these do not co-localise with either the ligands or its receptors. The vast majority of cells within the ganglia appear to express APRIL, BCMA and TACI and therefore it is reasonable to conclude that these cells co-express the ligand and both its receptors. It is therefore more apparent that expression of APRIL, BCMA and TACI in is localised to the cell soma of SCG neurons. As the APRIL antibody used only identifies internal APRIL evidence of the protein in these sections corresponds to APRIL that is synthesised prior to proteolytic cleavage and does not include either TWEPRIL or APRIL released as a soluble factor.

The apparent lack of co-localisation between the TNF members and tyrosine hydroxylase points to an absence of interaction between the

target field and innervating fibres. Instead, expression by SCG neurons in the ganglia itself points to APRIL signalling taking place locally within the SCG. This raises the possibility of autocrine or paracrine signalling taking place.

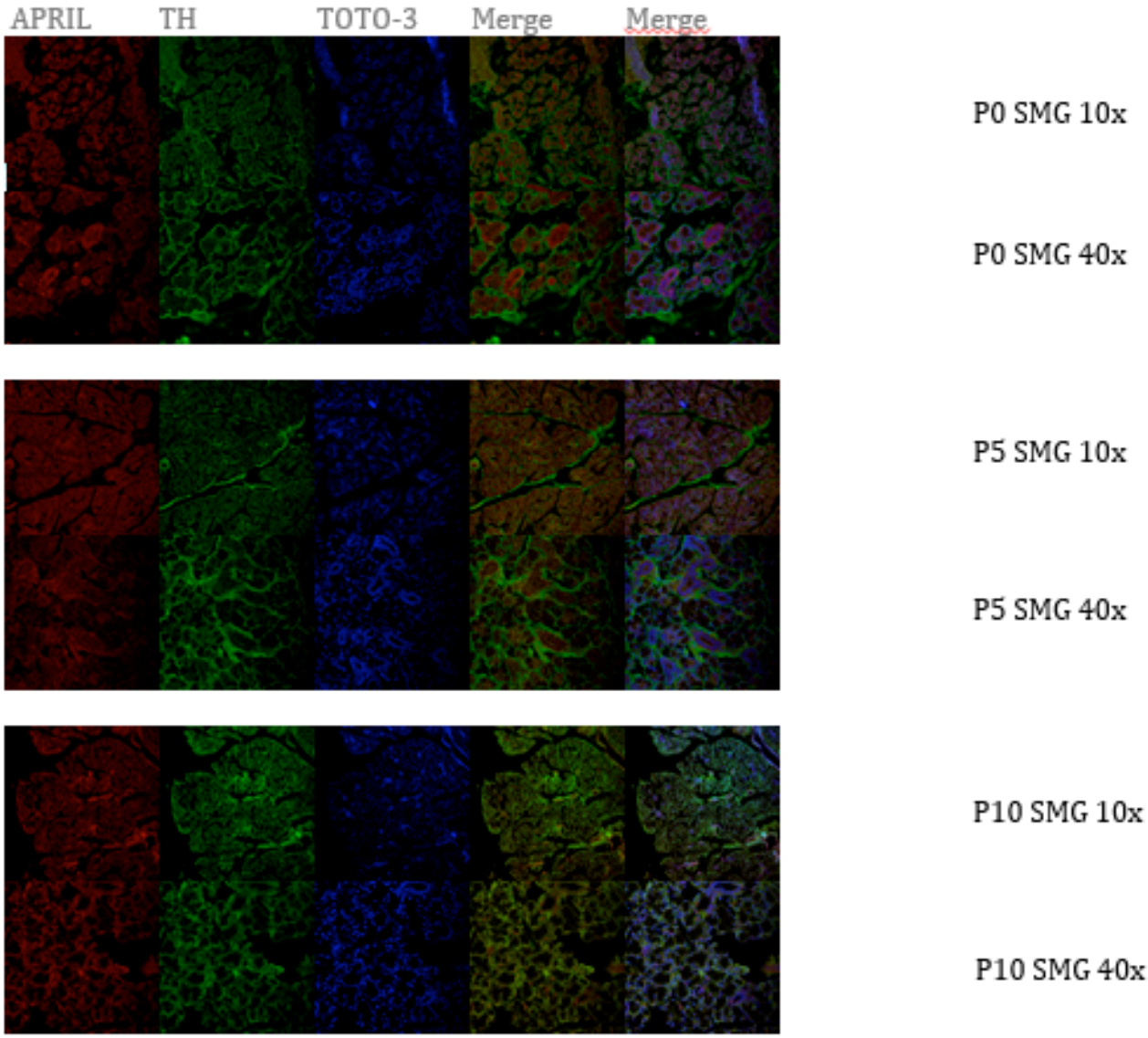


Figure 17: Expression of APRIL and Tyrosine hydroxylase in the developing SMG

Expression of APRIL and Tyrosine hydroxylase in P0, P5 and P10 SMG. APRIL, Tyrosine hydroxylase, TOTO-3 and two merges showing co-localisation are depicted.

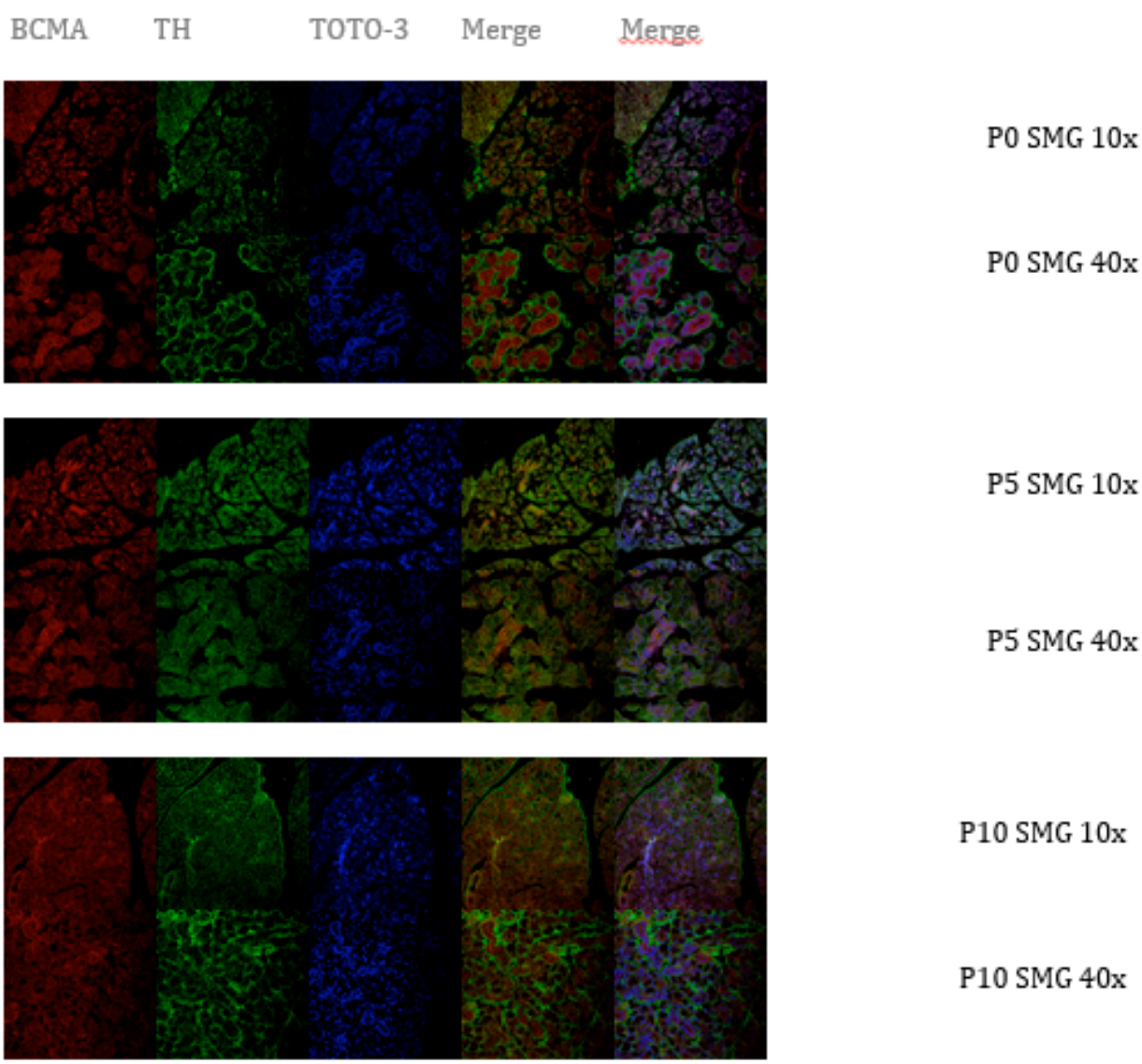


Figure 18: Expression of BCMA and Tyrosine hydroxylase in the developing SMG

Expression of BCMA and Tyrosine hydroxylase in P0, P5 and P10 SMG. BCMA, Tyrosine hydroxylase, TOTO-3 and two merges showing co-localisation are depicted.

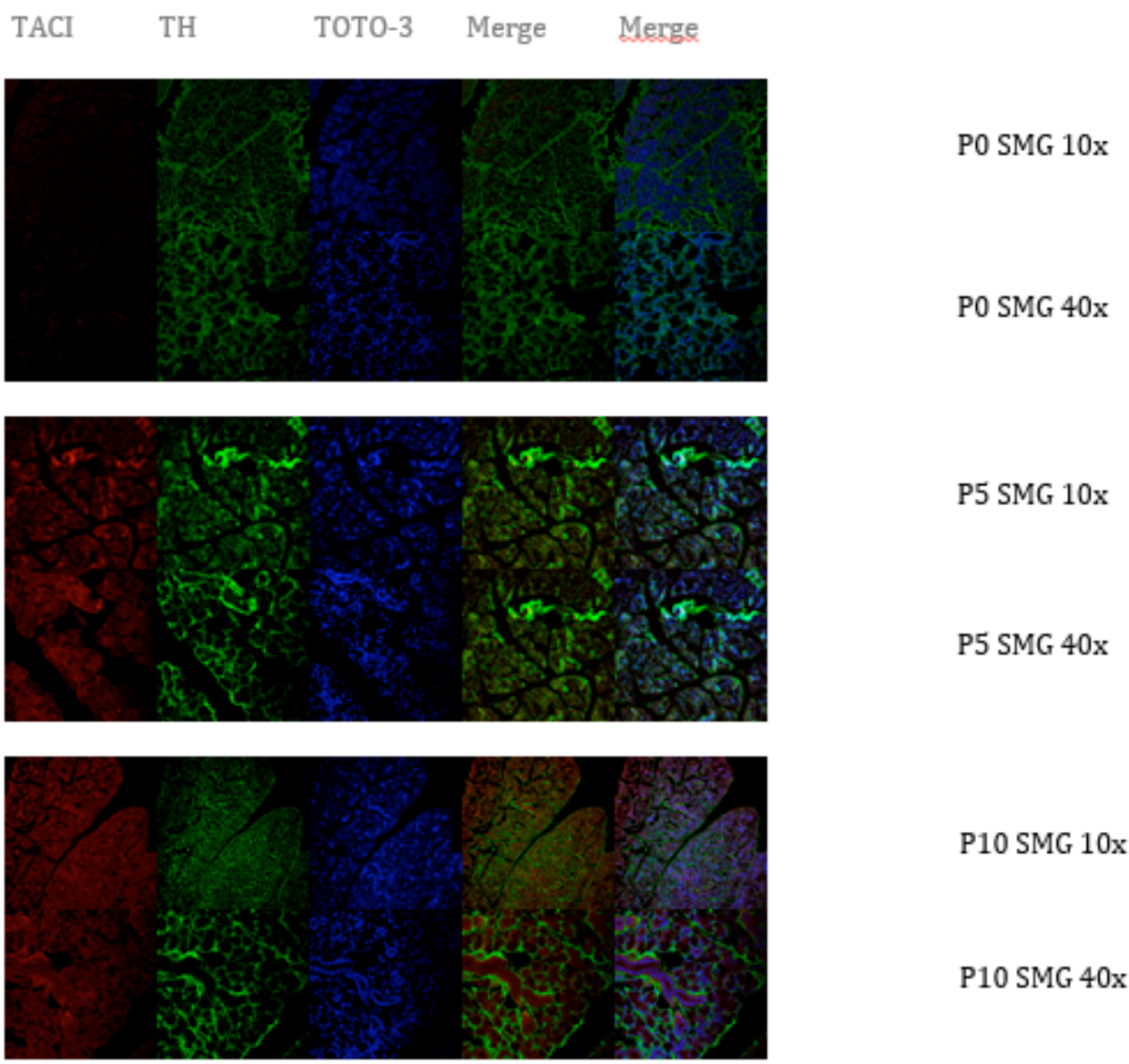


Figure 19: Expression of TACI and Tyrosine hydroxylase in the developing SMG

Expression of TACI and Tyrosine hydroxylase in P0, P5 and P10 SMG. TACI, Tyrosine hydroxylase, TOTO-3 and two merges showing co-localisation are depicted.

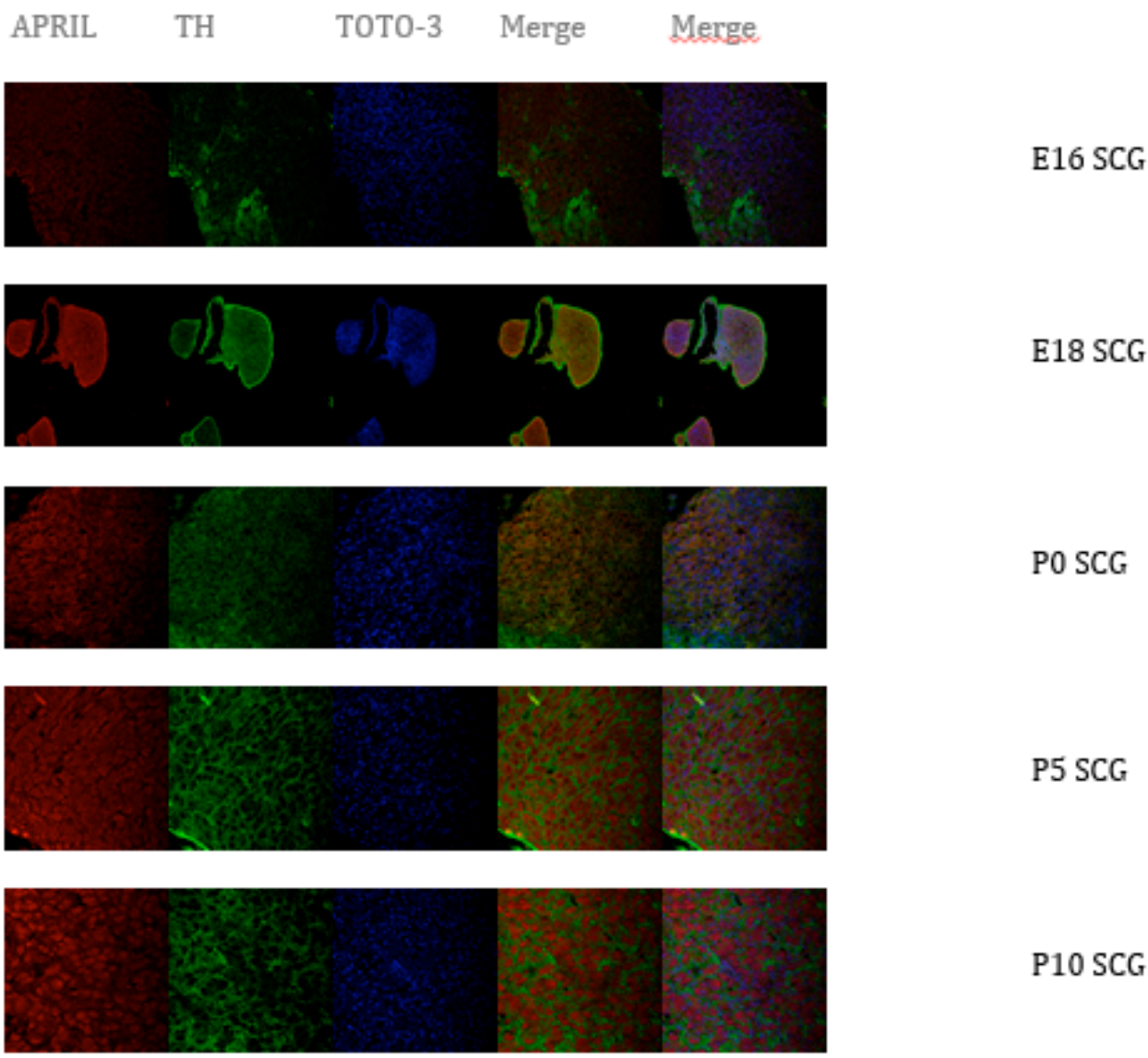


Figure 20: Expression of APRIL and Tyrosine hydroxylase in the developing SCG

Expression of APRIL and Tyrosine hydroxylase in E16, E18, P0, P5 and P10 SCG. APRIL, Tyrosine hydroxylase, TOTO-3 and two merges showing co-localisation are depicted.

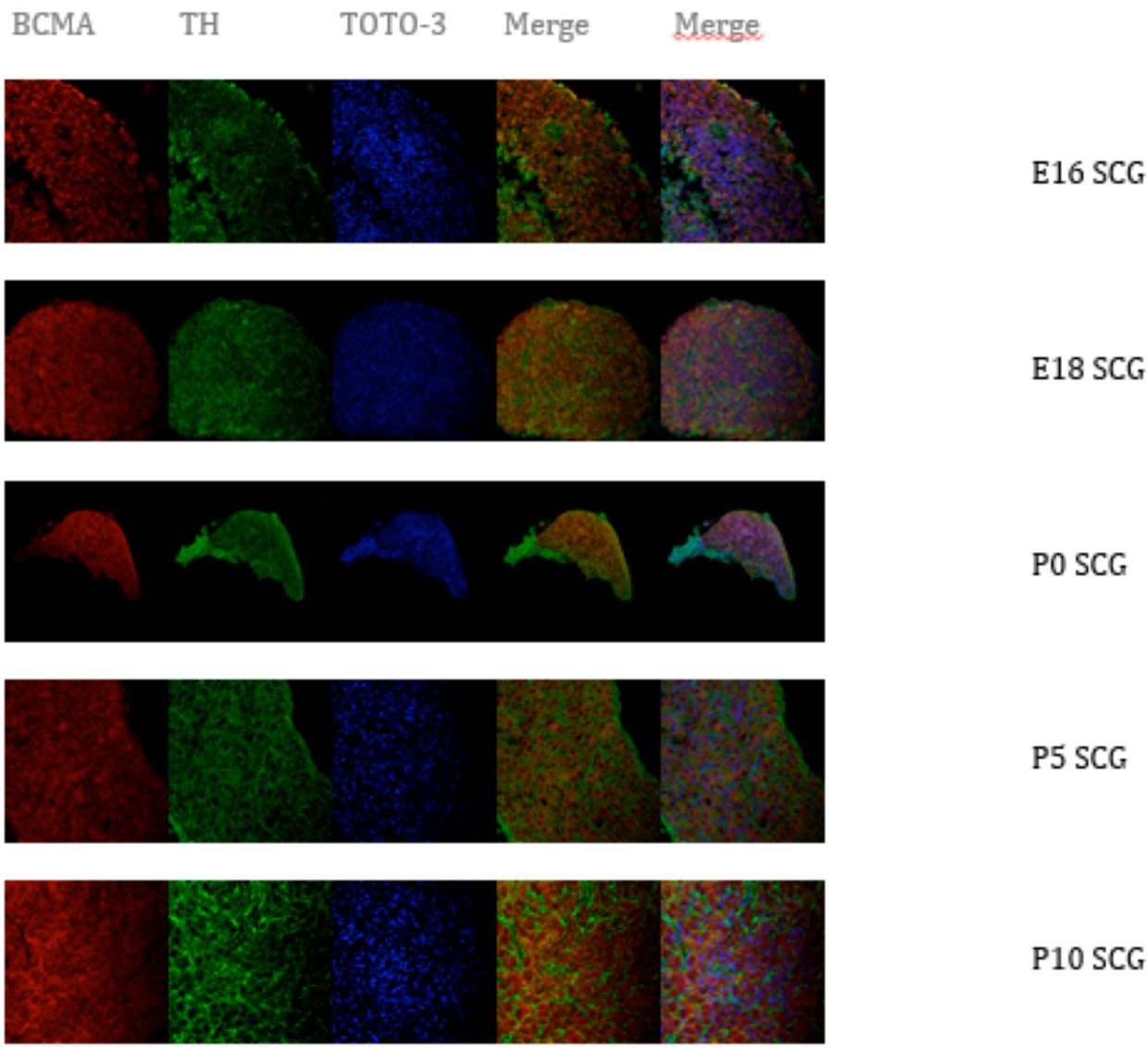


Figure 21: Expression of BCMA and Tyrosine hydroxylase in the developing SCG
Expression of BCMA and Tyrosine hydroxylase in E16, E18, P0, P5 and P10 SCG. BCMA, Tyrosine hydroxylase, TOTO-3 and two merges showing co-localisation are depicted.

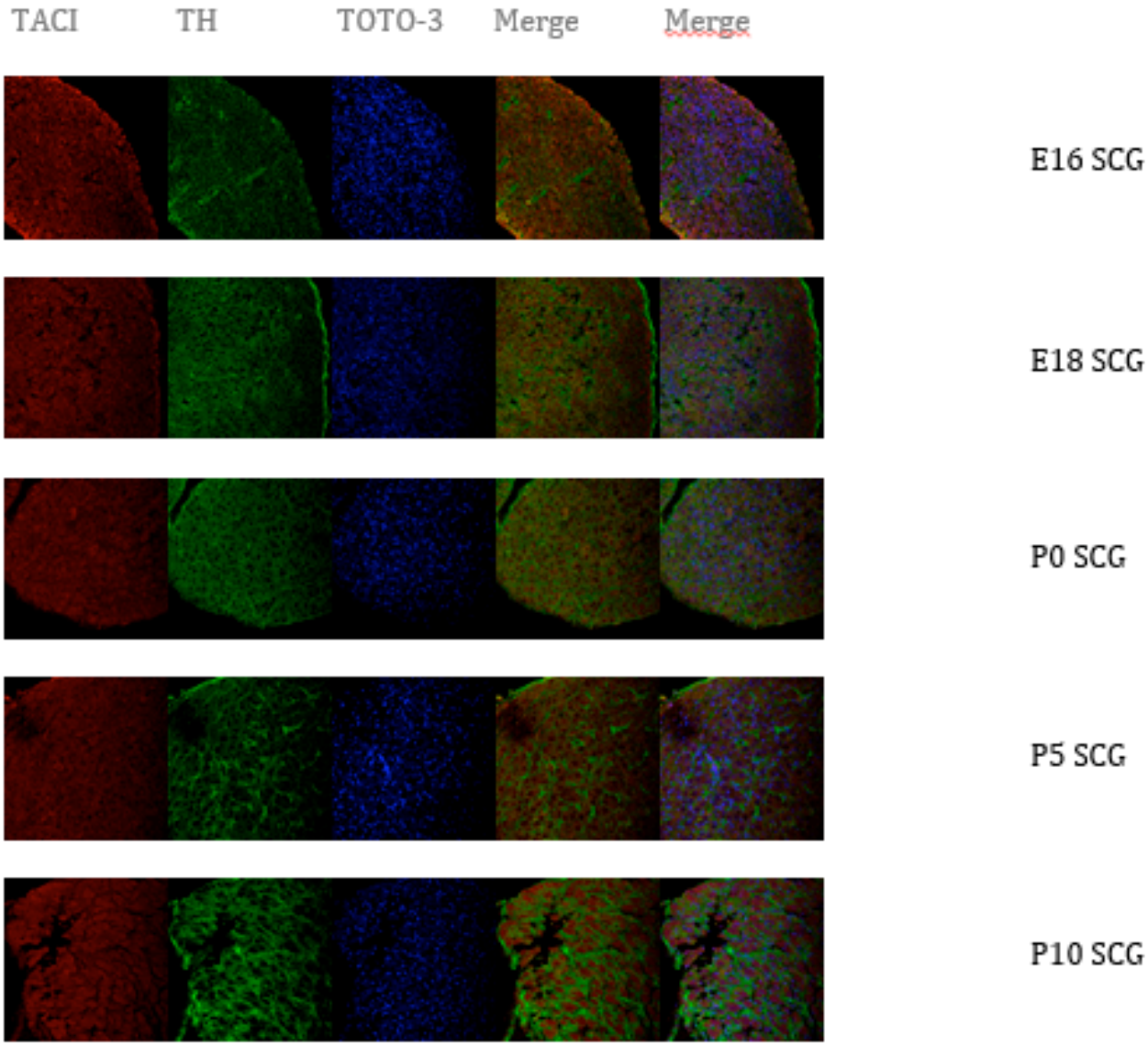


Figure 22: Expression of TACI and Tyrosine hydroxylase in the developing SCG

Expression of TACI and Tyrosine hydroxylase in E16, E18, P0, P5 and P10 SCG. TACI, Tyrosine hydroxylase, TOTO-3 and two merges showing co-localisation are depicted.

Discussion

The data contained within this chapter provide novel evidence for the expression of APRIL and BAFF and their receptors BCMA, TACI and BAFFR in the developing peripheral nervous system. The vast majority of published data investigating TNF superfamily members characterises their roles in the immune system. It is well established that these factors play major roles in B-cell function regulating processes as diverse as homeostasis, differentiation, function and survival.

Real time PCR was used to examine the developmental profiles of mRNAs in the SCG from embryonic day 13 through to adulthood. These profiles demonstrated that APRIL and BAFF along with their receptors are expressed by cells of the developing SCG. Expression of all these mRNAs were at their lowest levels at the earliest stage tested, embryonic day 13. As developmental age increases towards birth APRIL, BAFF and TWEAK ligand expression all rise while the same can be said for their receptors. This trend points to a possibly functional role in the development of the SCG.

In corroboration of the real time PCR data cultured P0 SCG neurons grown in the presence of NGF express the proteins APRIL, BCMA and TACI. The prevalent site of expression appears to be the soma of neurons in culture, however, both the ligand and its two receptors can be seen throughout neurites up to the distal ends.

Expression of APRIL and its cell surface form TWEPRIL were also investigated in target tissues of peripheral innervation by real time

PCR. Here both forms of the ligand are expressed in a variety of sympathetic innervation targets including the submandibular glands, nasal mucosa, iris and pineal gland. APRIL is expressed at higher levels in the sympathetic targets relative to the heart and whisker pads, a site of innervation by the trigeminal ganglion. TWEPRIL expression appears greatest in the pineal gland while APRIL can be seen at higher levels in the submandibular gland and pineal relative to other targets.

The presence of these mRNA transcripts within dissected target tissues provides little information in terms of a link with the peripheral neurons that are innervating them. To address this immunohistochemistry was used to determine the localisation of APRIL, BCMA and TACI within two representative targets of sympathetic innervation. The submandibular gland and nasal mucosa were chosen and within these targets the location of APRIL and its receptors relative to sympathetic nerve fibres was examined. While the TNF ligand and both its receptors were found expressed within these tissues in accordance with the real time PCR data little evidence of co-localisation with tyrosine hydroxylase nerve fibres was available. These proteins were expressed by cells intimately associated with extending sympathetic fibres but were not present on the fibres themselves.

As cultured neurons demonstrated highest levels of APRIL expression in the soma the same being true for the receptors sections of the SCG were examined for expression of APRIL, BCMA and TACI. Over the course of embryonic development from E16 through to post

natal day five these TNF members can be found within the developing ganglia. Expression generally rises towards the postnatal ages again corroborating the real time PCR data. Where tyrosine hydroxylase sympathetic fibres are visible no co-localisation with APRIL, BCMA or TACI can be seen matching the target field expression findings.

It would therefore appear that neurons of the SCG themselves co-express APRIL, BCMA and TACI throughout embryonic development at least from E16 onwards. This expression rises towards birth and post natal development at a time when these neurons are extending axons towards target tissues and elaborating dendrites within them. As such these TNF members present themselves as worthy of further investigation into possible functional roles in the development of the sympathetic nervous system.

The functional role of APRIL and its receptors *in vitro*

Introduction

Within the latter stages of development of the sympathetic nervous system neurons extend their axons towards peripheral targets resulting in final target field innervation. This process is intimately controlled by neurotrophins, in the case of sympathetic neurons, nerve growth factor (NGF). Both a majority of postganglionic sympathetic as well as a number of sensory neurons require this factor to promote their survival and their ability to innervate target fields. As such, target derived NGF, through retrograde transport to the cell soma is critical to the proper development of the peripheral sympathetic and sensory nervous systems.

Multiple factors including artemin, NT3 and endothelins have been shown to aid in the guidance of axons towards their targets *in vivo.* Target derived NGF has been demonstrated extensively *in vivo* and *in vitro* to not only play a major role in promoting the growth of axons to their targets but to also enable those that make it to the correct target to ramify and survive the spell of apoptotic cell death that ensures adequate numbers of neurons innervate the correct target fields.

The previous chapter identified that the TNF members APRIL and BAFF along with their receptors BCMA, TACI and BAFFR are expressed in both neurons of the sympathetic cervical ganglion and the trigeminal ganglion throughout late embryonic and postnatal development during the time that these neurons are extending axons towards target tissues and innervating these same locations. Experiments contained within this chapter therefore set out with the aim of establishing whether these factors play any role in the growth and survival of sympathetic SCG and sensory TRI in vitro.

Primary cell culture derived from neurons at various stages of growth from E16 through to P3 were established and the activity of APRIL, BAFF and their receptors modulated through the use of recombinant forms of the ligands and function blocking antibodies as well as soluble forms of the receptors. Analysis of the growth and survival of these neurons in culture aimed to identify the role of these factors *in vitro.*

Effect of recombinant APRIL on NGF promoted sympathetic neurite outgrowth in the absence of NGF

Due to the prevalence in expression of APRIL and its receptors by sympathetic SCG neurons, initial *in vitro* investigation set out to determine if these TNF members played any role in the growth of SCG neurons in culture. By exposing neurons in culture to a recombinant form of APRIL and quantifying the growth of these neurons it is possible to determine if the recombinant protein has an effect on the growth of these neurons.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were exposed to 100ng/ml recombinant APRIL. Recombinant APRIL used in this study is Human embryonic kidney cell, HEK293-derived (R&D) and forms non-covalent homotrimers. The biological activity of the recombinant ligand was measured in a cell proliferation assay using anti-IgM stimulated mouse B cells by the manufacturer.
The ED50 for this effect is typically 5-25 ng/mL in the presence of goat anti-mouse IgM. Knowing this 100ng/ml was selected as a starting point for investigation in culture. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different

occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using the Mann-Whitney U test.

The application of 100ng/ml recombinant APRIL for the duration of short term culture caused a significant increase in both the total length (Fig.23B) and number of branching points (Fig.23C) of P0 SCG neurons in the presence of NGF. This strengthens the case that APRIL plays a functional role in the growth of SCG neurons. The recombinant form of APRIL forms homotrimers like the natural protein in vivo and thus an effect with this recombinant ligand is a strong indicator that the role of APRIL in the growth of SCG neurons warrants further investigation.

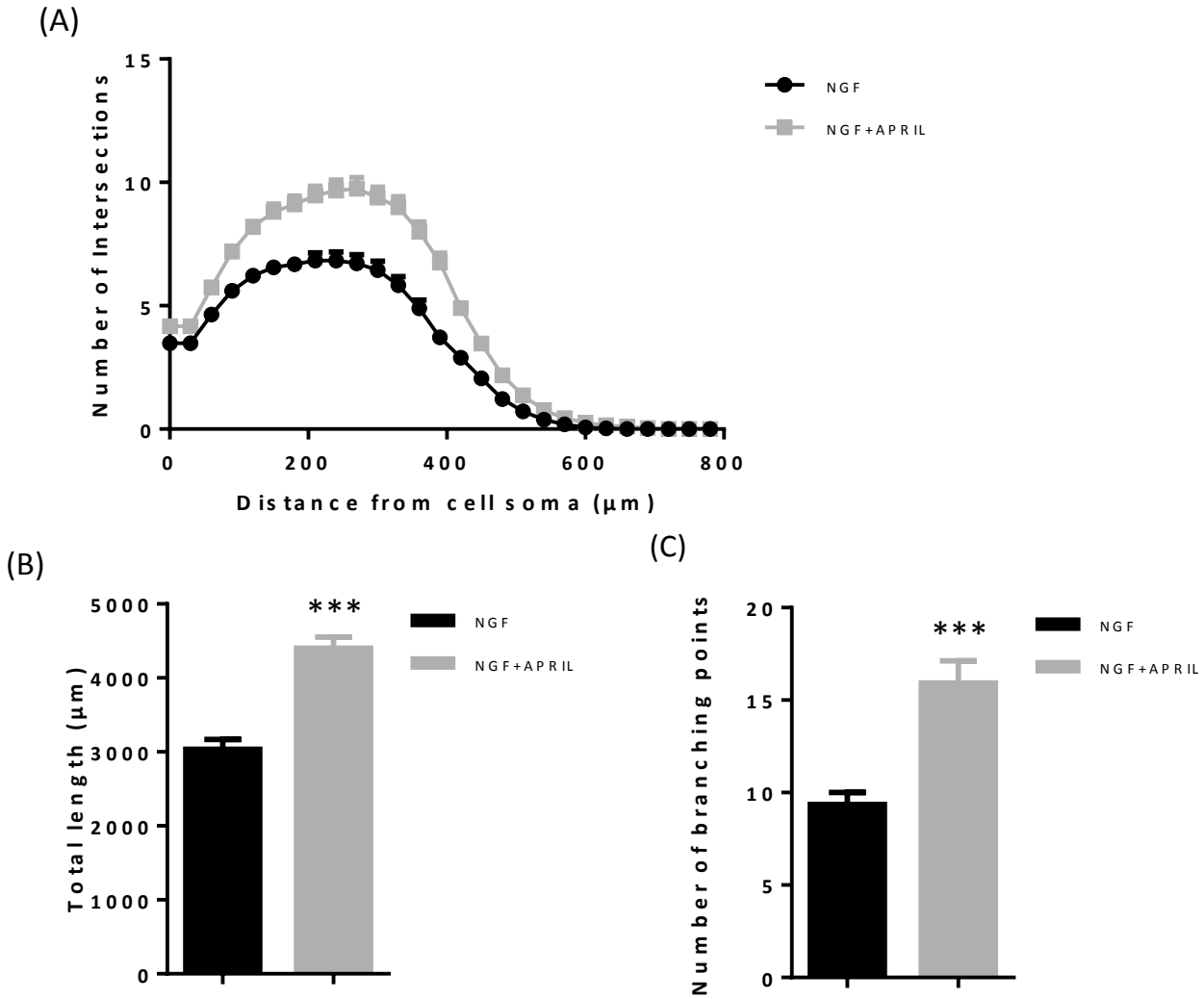


Figure 23: APRIL enhances neurite growth from cultured SCG neurons in the presence of NGF (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) Bar chart of neurite length (C) Bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Mann-Whitney U test).

Effect of recombinant APRIL on sympathetic neurite outgrowth

Following the observation that recombinant APRIL enhanced neurite outgrowth from P0 SCG neurons in the presence of NGF an experiment was conducted to ascertain if this effect happens independently of NGF signalling or if NGF promoted growth is critical for the actions of APRIL. This information is important as at P0 neurons critically depend on NGF for both growth and survival. It is important to know is the effects of recombinant APRIL are also dependent on the presence of NGF or if its growth promoting effects are self-regulating.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to a pan caspase OPH Inhibitor at 10μM. Due to an absence of NGF at a time of development when SCG neurons are critically dependent on this neurotrophin for survival the presence of a broad spectrum caspase inhibitor ensures the neurons do not undergo apoptosis for the duration of culture and thus neurite growth independent of NGF could be assayed. In addition to this control condition a separate set of neurons were exposed to 100ng/ml recombinant APRIL. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the Fast Sholl technique followed by statistical comparison to control using the Mann-Whitney U test.

The application of 100ng/ml recombinant APRIL for the duration of short term culture resulted in no significant difference in either neurite length (Fig.24B) or branching (Fig.24C) in comparison with control. In the absence of NGF promoted neurite outgrowth recombinant APRIL demonstrated no ability to affect the complexity of P0 SCG neurons.

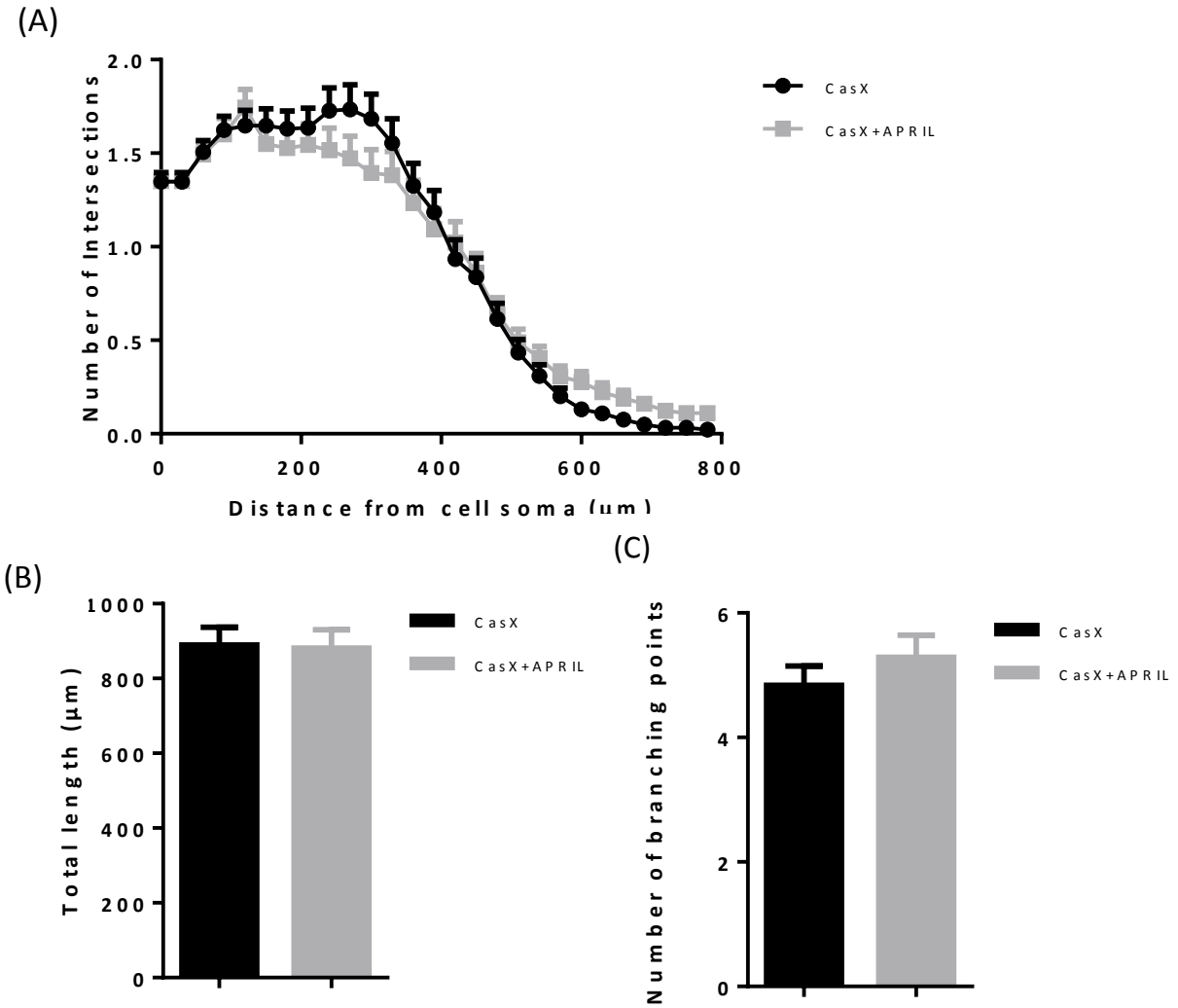


Figure 24: APRIL does not enhance growth in the absence NGF *in vitro* (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor (control) or pan caspase OPH Inhibitor and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (statistical comparison with control, Mann-Whitney U test).

Recombinant APRIL enhances sympathetic neurite outgrowth over a broad dose range

With the observation that recombinant APRIL selectively enhances NGF promoted neurite outgrowth from P0 SCG neurons *in vitro* it became important to know over what range of doses this effect could be observed and if indeed APRIL can enhance NGF promoted growth at physiologically relevant concentrations.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition, separate sets of neurons were exposed to varying concentrations recombinant APRIL from 1ng/ml up to 1000ng/ml. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

The application recombinant APRIL at all experimental doses assayed caused a significant increase in both the total length (Fig.25B) and number of branching points (Fig.25C) of P0 SCG neurons in the presence of NGF. Recombinant APRIL therefore displays an ability to enhance NGF promoted neurite outgrowth at concentrations at least as low as 1ng/ml. The maximal increase in

length and branching in comparison with control occurred with the 100ng/ml concentration of recombinant APRIL while a tenfold increase to 1000ng/ml reduced the enhancement significantly, although this higher dose still enhances NGF promoted neurite outgrowth above control level. This experiment demonstrates that recombinant APRIL enhances the growth of SCG neurons at concentrations that are physiologically relevant and so this functional role may be of relevance to the development of the mouse *in vivo*.

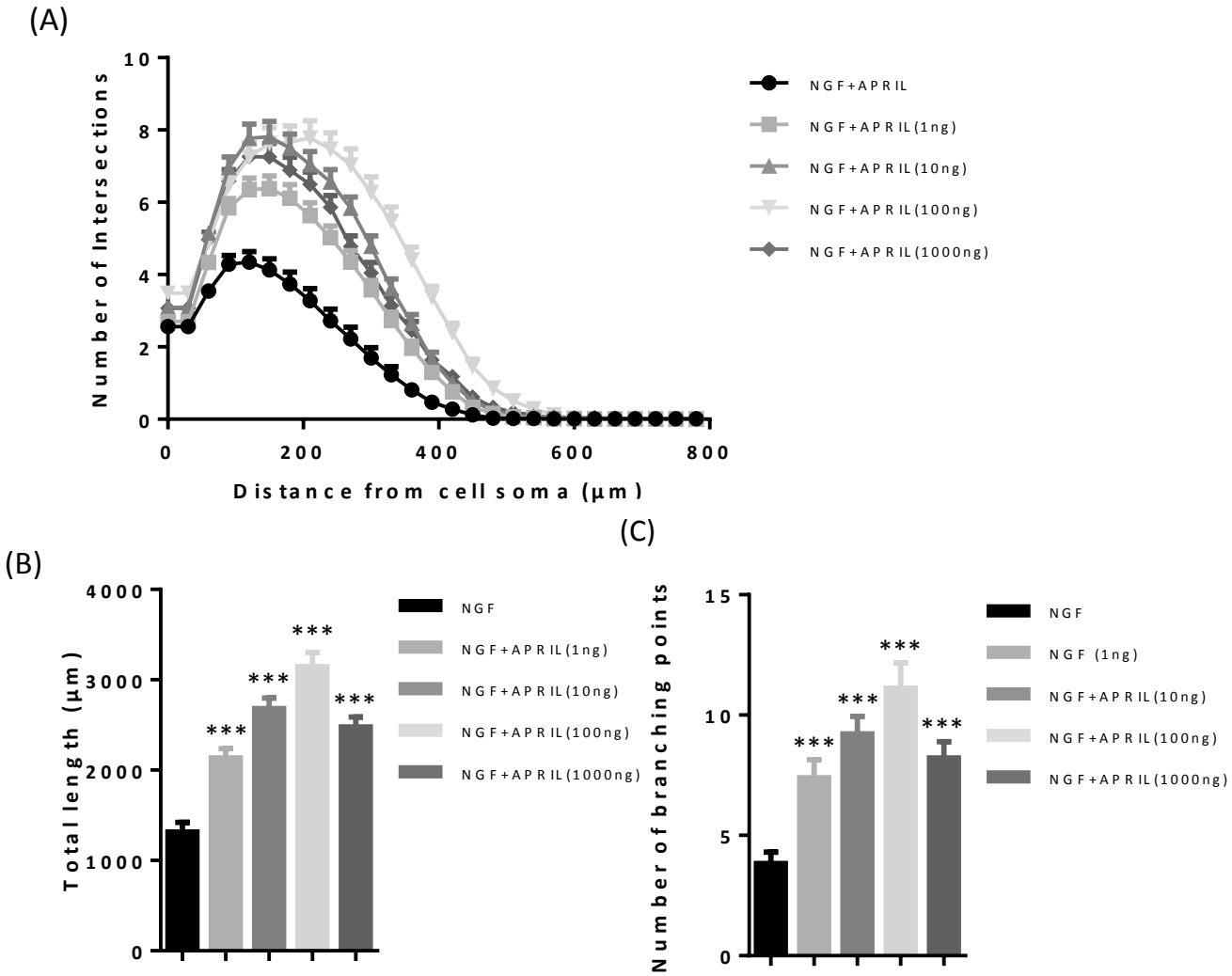


Figure 25: APRIL enhances neurite growth over a range of doses (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF. Neurons were exposed to a range of recombinant APRIL concentrations from 1ng to 1000ng while control neurons received no recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Recombinant APRIL and its function blocking counterpart enhance sympathetic neurite outgrowth

Due to the ability of recombinant APRIL to enhance NGF promoted axonal growth taken together with the observations that SCG neurons express APRIL and its receptors BCMA and TACI an experiment was undertaken to investigate the possibility of autocrine signalling at the cell surface or paracrine signalling between neighbouring neurons. This involved the use of a function blocking antibody against APRIL. If expression of APRIL in culture by the neurons themselves acts to enhance neurite growth the application of the function blocking antibody would block this signal.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were exposed to 100ng/ml recombinant APRIL or 3.2μg/ml aAPRIL function blocking antibody. The antibody used is a mouse monoclonal raised against Human embryonic kidney cell, HEK293-derived recombinant APRIL (R&D). Its function blocking capabilities were confirmed by the manufacturer by its ability to neutralize APRIL/TNFSF13 induced proliferation in mouse B cells. The Neutralization Dose (ND50) is typically 0.15-0.75ug/mL in the presence of 100 ng/mL Recombinant Human APRIL/TNFSF13. The dose selected for use was used to exceed the ND50 specified by the manufacturer. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope.

Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the Fast Sholl technique followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Both the application of 100ng/ml recombinant APRIL and the function blocking antibody against APRIL caused a significant increase in both the total length (Fig.26B) and number of branching points (Fig.26C) of P0 SCG neurons in the presence of NGF. While the action of recombinant APRIL acts to repeat earlier observations the activity of the function blocking antibody raises a number of interesting questions. It does not appear to be the case that a pro-growth autocrine or paracrine signalling loop is blocked by this antibody. Either blockade of endogenous APRIL activity enhances growth or the monoclonal antibody is acting in a manner that requires further investigation.

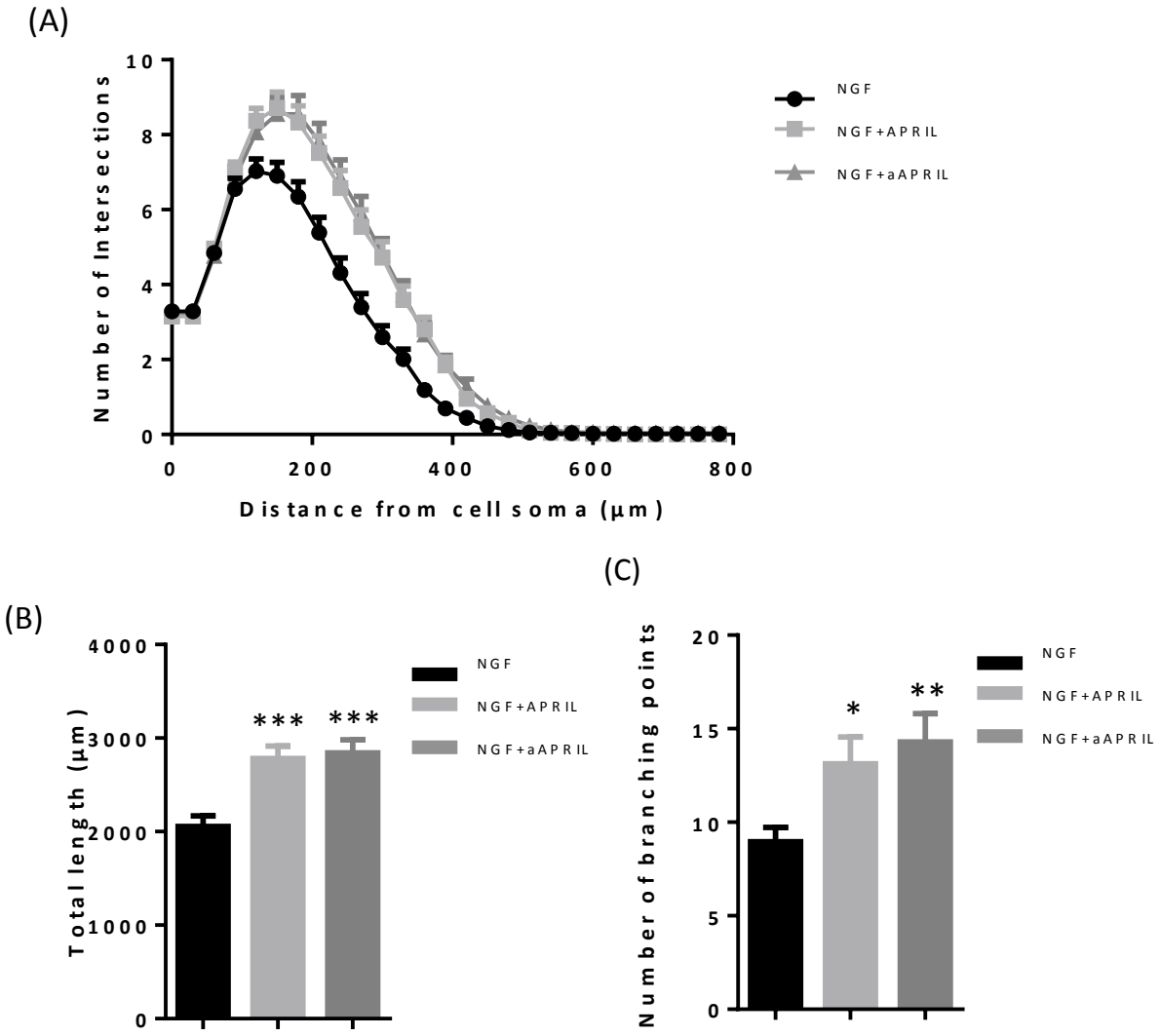


Figure 26: APRIL and aAPRIL enhance neurite growth from cultured SCG neurons
(A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control), 100ng/ml recombinant APRIL or 3.2µg/ml aAPRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (* indicates P <0.05, ** indicates P <0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Recombinant APRIL and its function-blocking counterpart enhance sympathetic neurite outgrowth over a variety of developmental ages in the SCG

Sympathetic neurite outgrowth and target field innervation begins during embryonic development and continues post birth. As both the application of recombinant APRIL and a function blocking antibody against APRIL enhances NGF promoted growth an experiment was established to ascertain over what developmental time period this effect is significant.

SCG ganglia of embryonic day 16 and 18 plus post natal day 0 and 3 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were exposed to 100ng/ml recombinant APRIL or 3.2μg/ml function blocking antibody aAPRIL. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Either the application of 100ng/ml recombinant APRIL or the function blocking antibody against APRIL caused a significant increase in the total length (Fig.27-30B) and number of branching points (Fig.27-30C) of SCG neurons in the presence of NGF cultured

from embryonic day 16 through to post natal day three. Therefore, modulating APRIL activity enhances NGF promoted outgrowth over a broad developmental range throughout the time these neurons are extending axons towards target tissues.

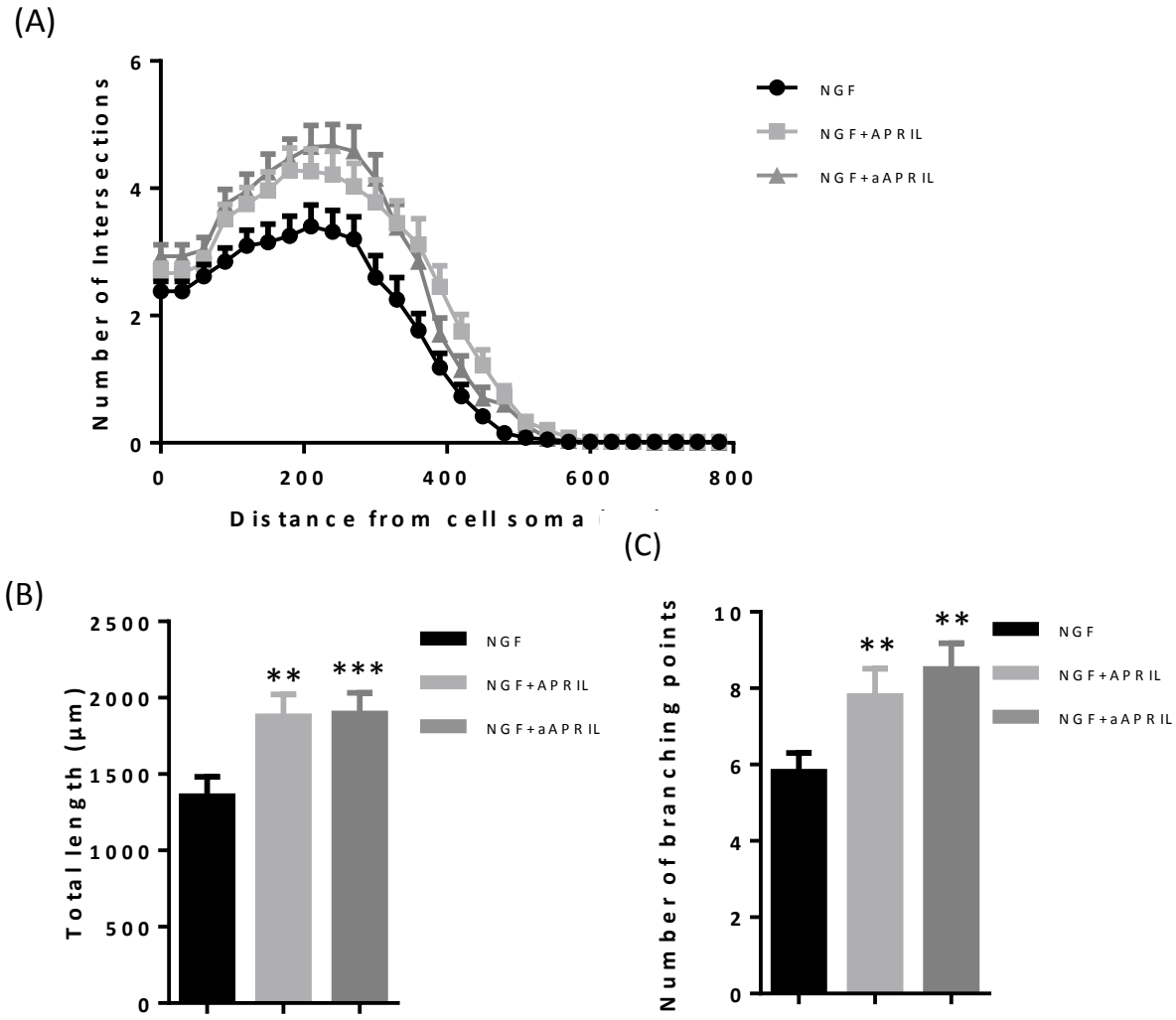


Figure 27: APRIL enhances neurite growth from cultured E16 SCG neurons (A-C) Neurite growth from E16 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (** indicates P<0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

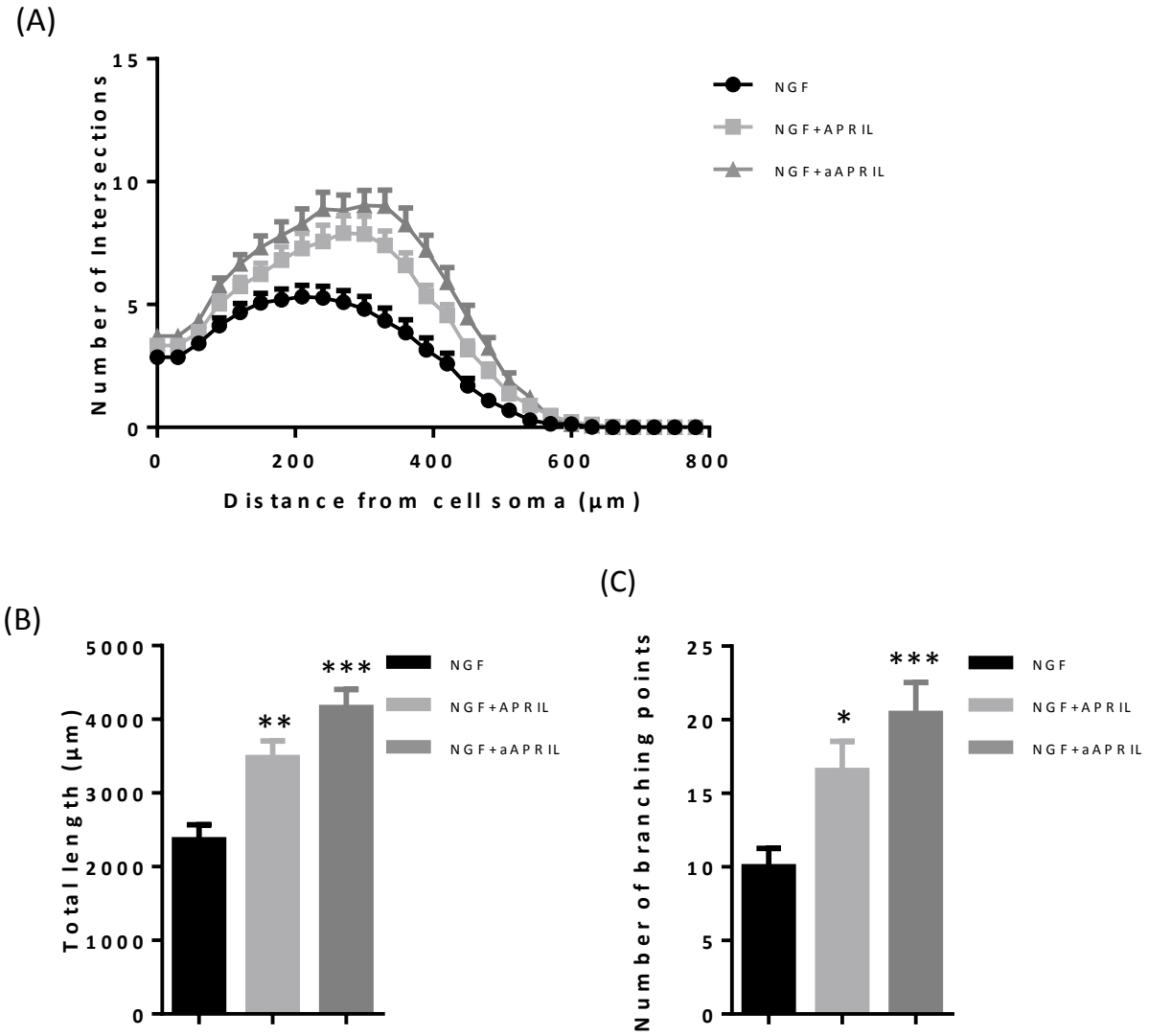


Figure 28: APRIL enhances neurite growth from cultured E18 SCG neurons (A-C) Neurite growth from E18 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (* indicates P <0.05, ** indicates P <0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

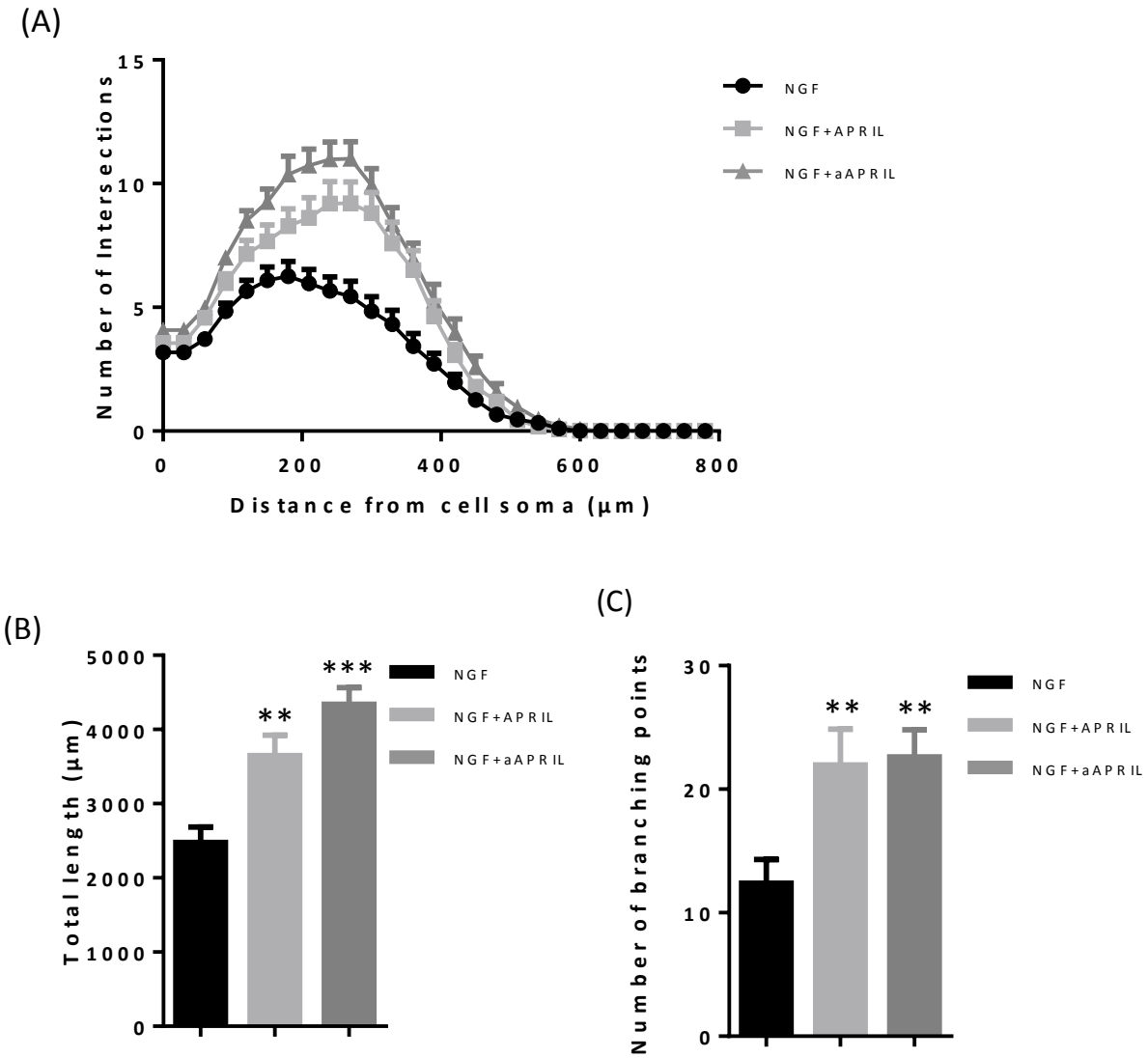


Figure 29: APRIL enhances neurite growth from cultured P0 SCG neurons (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (**indicates P<0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

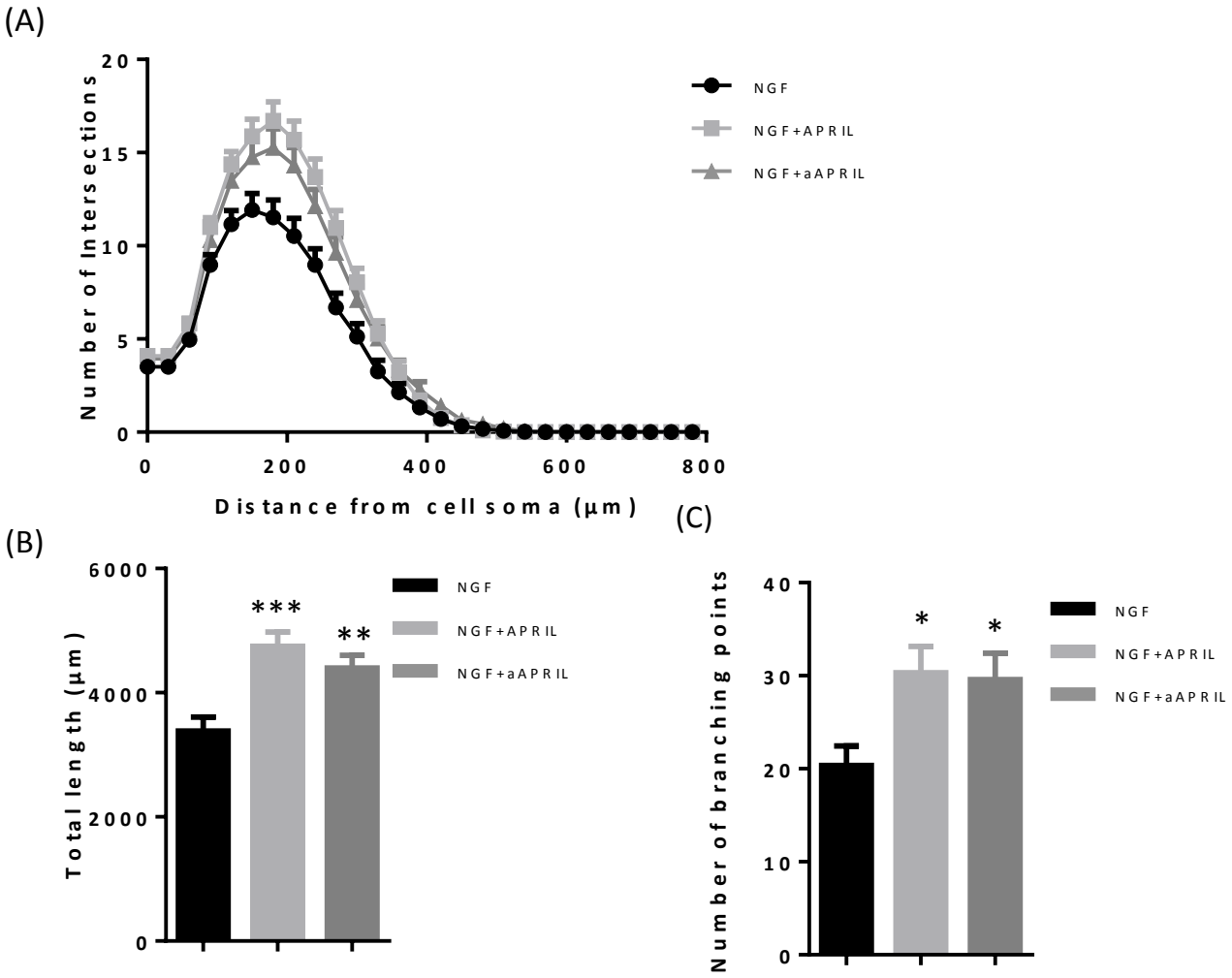


Figure 30: APRIL enhances neurite growth from cultured P3 SCG neurons (A-C) Neurite growth from P3 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (* indicates P <0.05, ** indicates P <0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Recombinant APRIL and its function-blocking counterpart do not enhance trigeminal sensory neurite outgrowth over a variety of developmental ages

It has been observed that recombinant APRIL and function blocking aAPRIL have the ability to enhance NGF promoted neurite outgrowth from embryonic day 16 through to P3 in populations of sympathetic neurons. A course was set up to discover if the case was the same in a sensory population of neurons established from the trigeminal ganglia. The experiment was designed to ascertain if the effects of APRIL signalling are isolated to sympathetic development or might occupy a broader developmental role in the development of other populations of peripheral neurons.

Trigeminal ganglia of embryonic day 16 and 18 plus post natal day 0 and 3 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were exposed to 100ng/ml recombinant APRIL or 3.2μg/ml function blocking antibody aAPRIL. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before being imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Either the application of 100ng/ml recombinant APRIL or the function blocking antibody against APRIL caused no significant change in both the total length (Fig.31-34B) and number of branching points (Fig.31-34C) of trigeminal neurons in the presence of NGF cultured from embryonic day 16 through to post natal day 3. APRIL and aAPRIL do not affect the outgrowth of these sensory neurons *in vitro*. It therefore appears that the role of APRIL signalling in the development of the peripheral nervous system is specific to certain subpopulations of neurons.

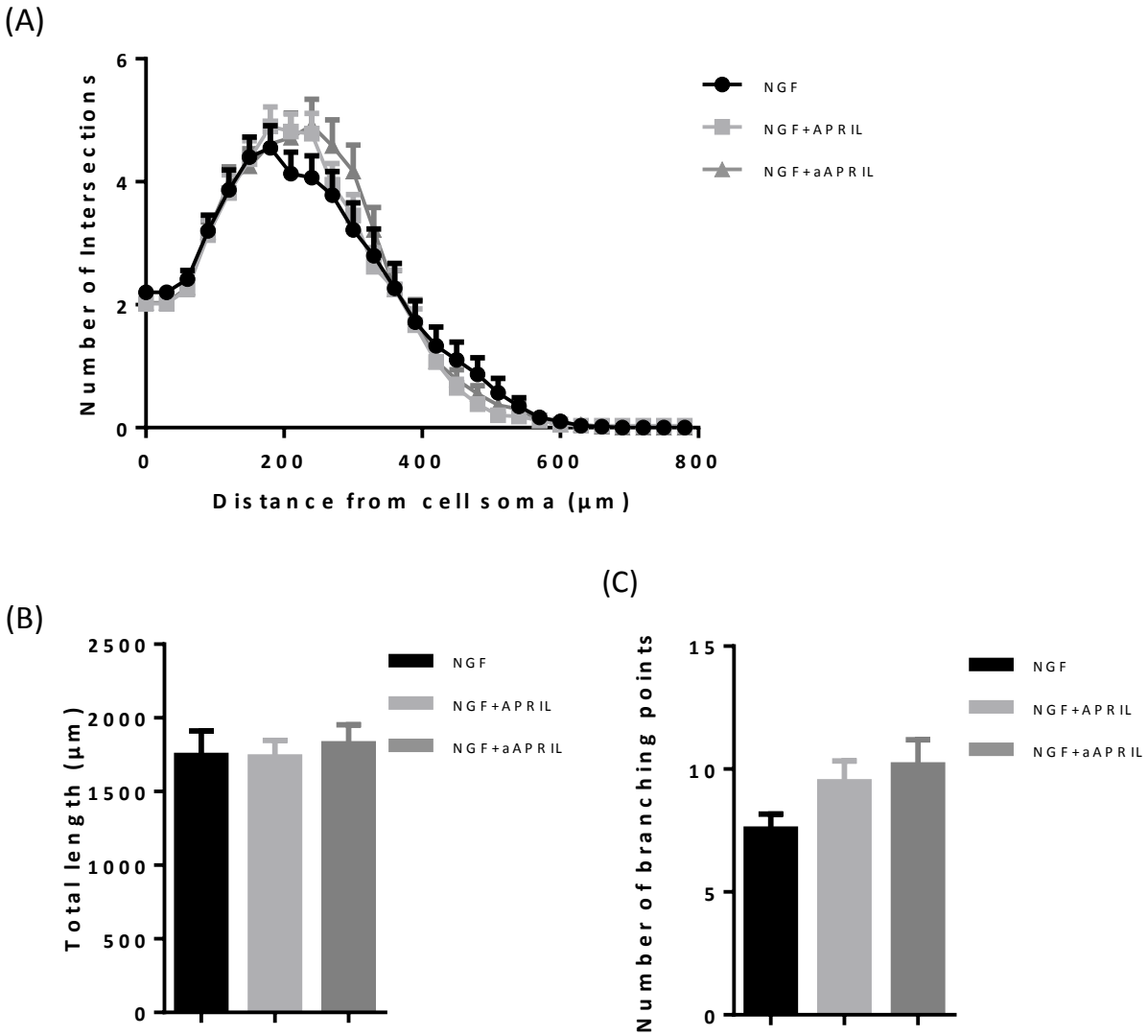


Figure 31: APRIL does not enhance neurite growth from cultured E16 trigeminal neurons (A-C) Neurite growth from E16 TRI neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

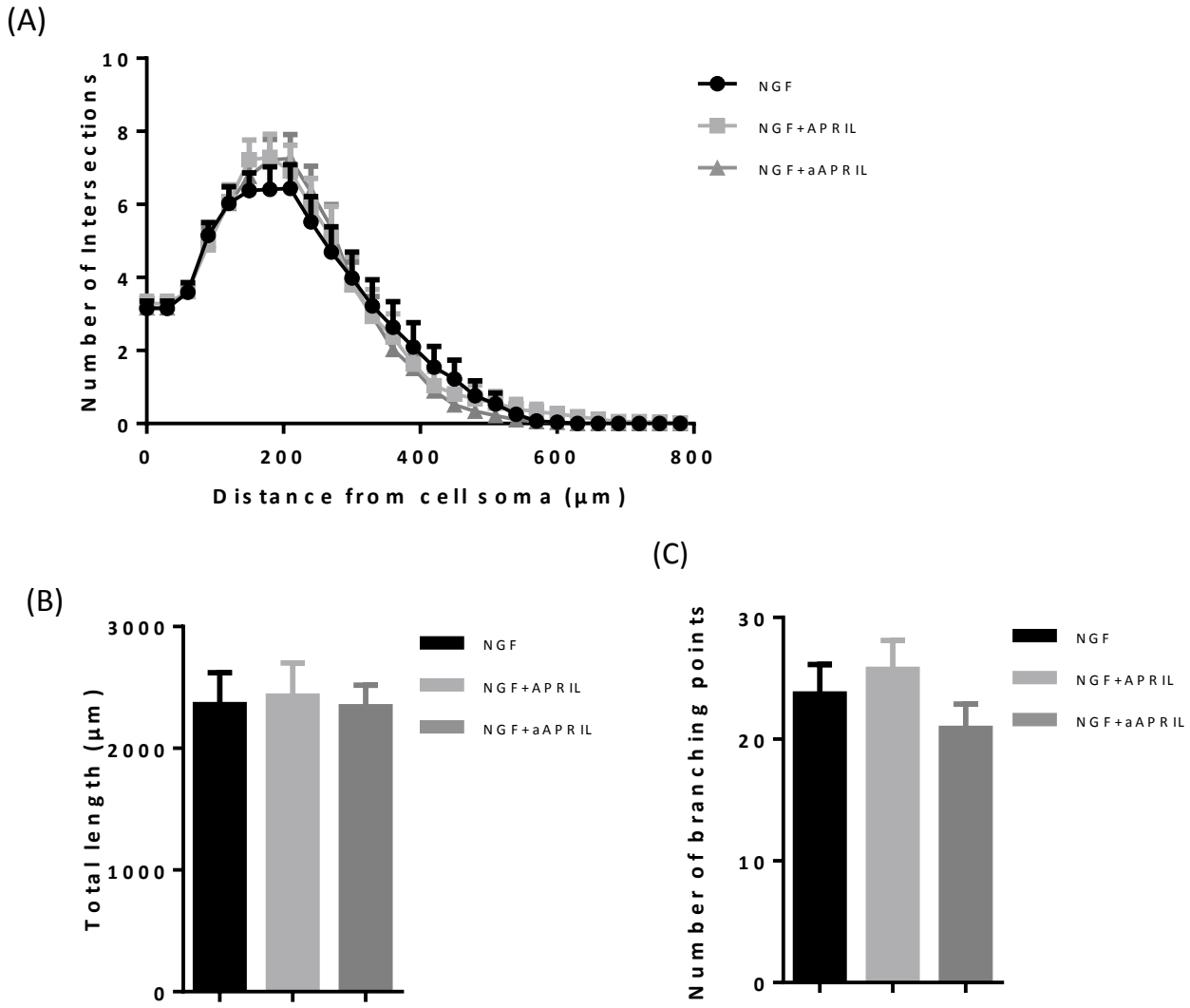


Figure 32: APRIL does not enhance neurite growth from cultured E18 trigeminal neurons (A-C) Neurite growth from E18 TRI neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

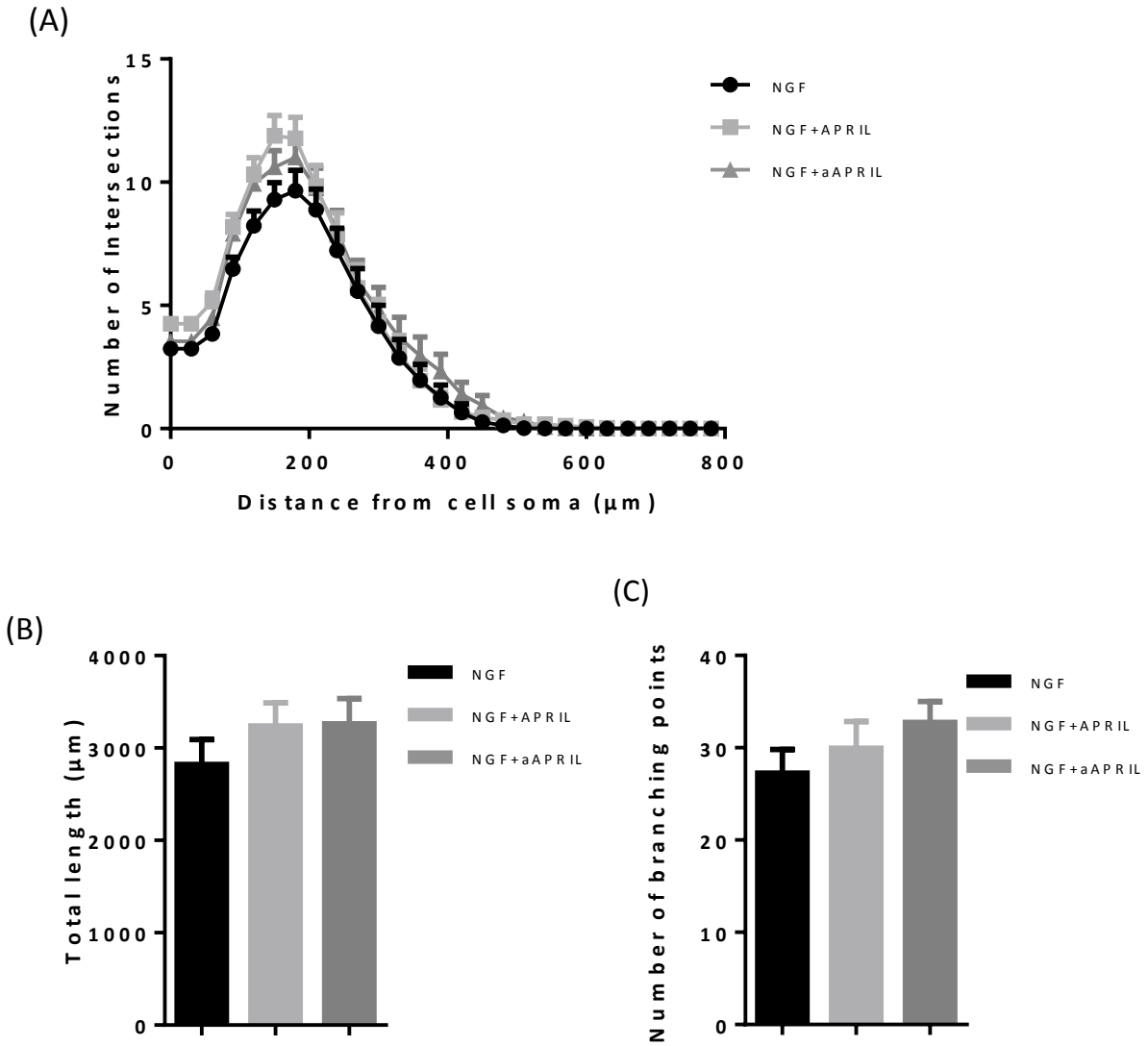


Figure 33: APRIL does not enhance neurite growth from cultured P0 trigeminal neurons (A-C) Neurite growth from P0 TRI neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

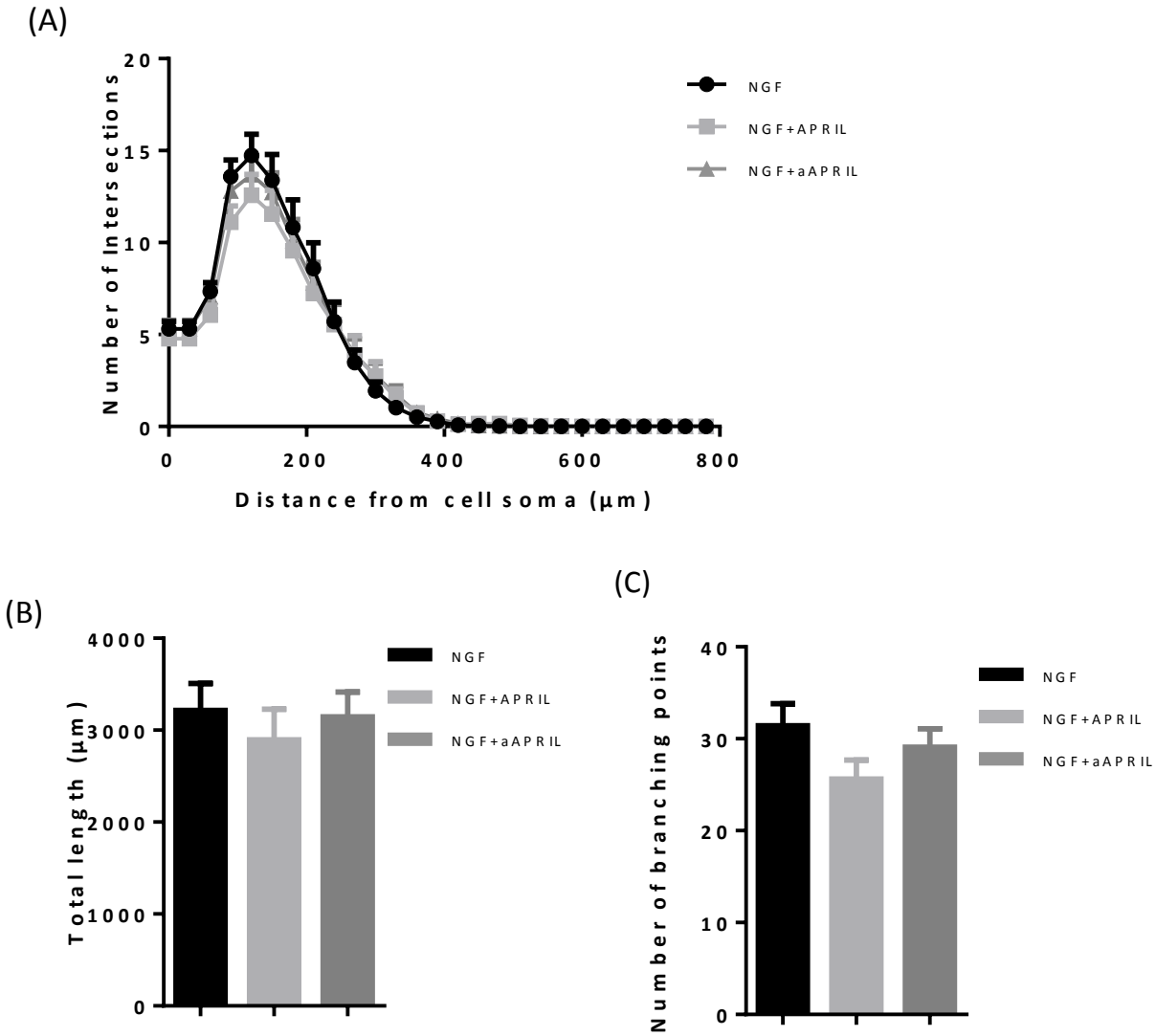


Figure 34: APRIL does not enhance neurite growth from cultured P3 trigeminal neurons (A-C) Neurite growth from P3 TRI neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) or pan caspase OPH Inhibitor, 1ng/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Recombinant APRIL enhances sympathetic neurite outgrowth in a sub saturating dose of NGF

In all previous cultures containing NGF the neurotrophin was present at 1ng/ml. The aim of this experiment was to discover if the effect of recombinant APRIL on NGF-promoted growth differed if the concentration of NGF was altered. At 1ng/ml the ability of NGF to promote survival and growth has saturated and is far above physiological levels of expression. Investigating the effects of recombinant APRIL at lower levels of NGF will confirm if the observations previously seen occur in the presence of physiologically relevant concentrations of neurotrophin.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 10pg/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were exposed to 100ng/ml recombinant APRIL. The presence of the pan caspase inhibitor acted to ensure adequate neuronal survival due to the lower concentration of NGF in a time period when SCG neurons depend on this neurotrophin for survival. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using the Mann-Whitney U test.

The application of 100ng/ml recombinant APRIL to cultures of P0 SCG neurons grown in 10pg/ml of NGF significantly increased NGF promoted length (Fig. 35B) and branching (Fig. 35C). The significance of this result is that recombinant APRIL demonstrates an ability to enhance neurite growth in the presence of NGF at a physiologically relevant level. 10pg/ml NGF promotes 50% survival of SCG neurons cultured for 24h while a tenfold reduction does not promote survival (Fig 39). At 10pg/ml the ability of NGF to promote survival is not saturating. That the magnitude of growth increase seen in neurons treated with APRIL is less than seen in cultures treated with a higher concentration of NGF is interesting. It could be the case that APRIL enhances NGF promoted neurite growth and as such increasing the concentration of NGF increases the level of growth enhancement in the presence of APRIL.

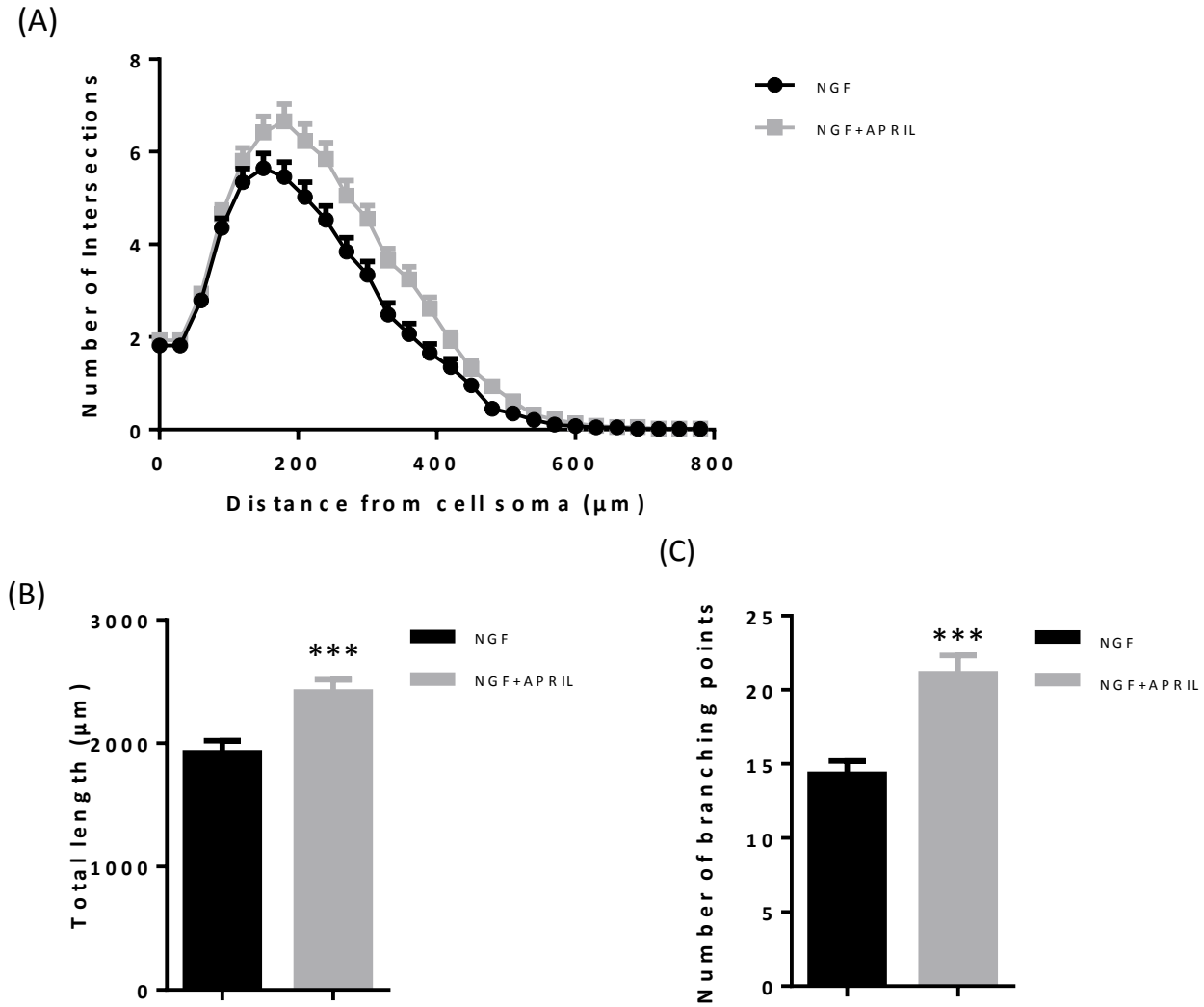


Figure 35: APRIL enhances neurite growth from cultured SCG neurons in a sub saturating dose of NGF (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. The neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 10pg/ml NGF (control) or pan caspase OPH Inhibitor, 10pg/ml NGF and 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Mann-Whitney U test).

Recombinant APRIL enhanced NGF promoted growth required heparin sulfate binding *in vitro*

APRIL contains a binding domain for heparin sulfate however its function is poorly understood. To ascertain whether the ability of APRIL to bind heparin sulfate is required for *in vitro* function a form of recombinant ligand lacking a heparin sulfate binding domain, APRIL (H98) was used. The use of this recombinant protein explores the relevance of the interaction between APRIL and heparin sulfate proteoglycan in its ability to enhance neurite outgrowth in the SCG.
SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were exposed to either 100ng/ml recombinant APRIL or 100ng/ml recombinant APRIL (H98). APRIL (H98) has the same structure as the standard recombinant ligand used and forms non-covalent homotrimers. The recombinant (H98) ligand also retains the ability to bind BCMA and TACI, however, the putative binding domain for heparin sulfate proteoglycans has been removed. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using Kruskal-Wallis test followed by Dunn's multiple comparisons test.

The application of 100ng/ml recombinant APRIL to cultures of P0 SCG neurons enhanced NGF promoted neurite length (Fig. 36B) and branching(Fig. 36C), however, APRIL(H98) with its lack of heparin sulfate binding domain did not enhance NGF promoted neurite outgrowth. The lack of neurite growth enhancement when treated with APRIL lacking the ability to interact with heparin sulfate proteoglycans points to a significant role of this interaction in the ability of APRIL to regulate NGF promoted growth *in vitro.* While the nature of the requirement is not known, without proteoglycan involvement, APRIL cannot enhance neurite outgrowth.

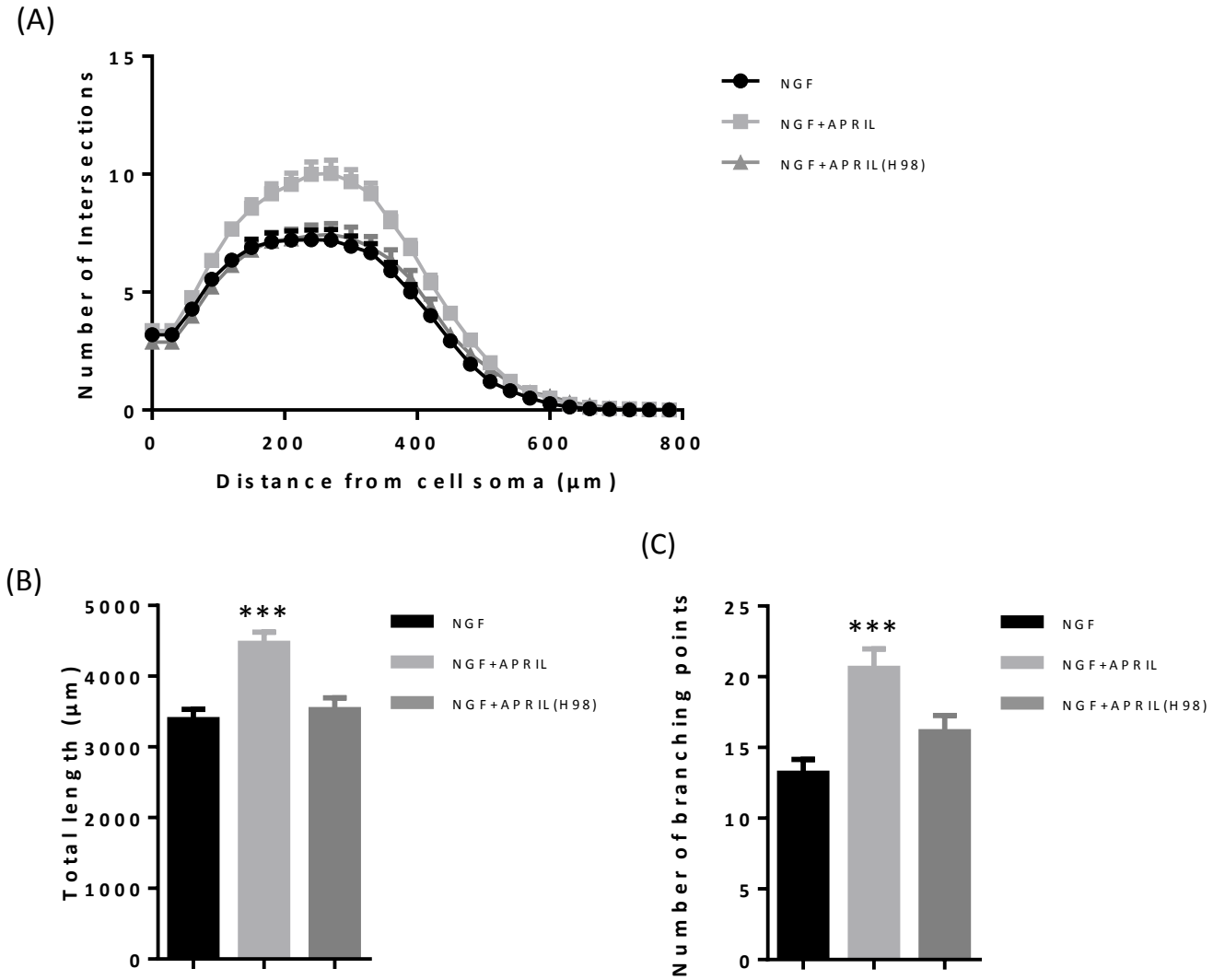


Figure 36: APRIL enhanced neurite growth requires a putative heparin sulfate binding domain (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control), and either 100ng/ml recombinant APRIL or 100ng/ml recombinant APRIL(H98). Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Functional blockade of APRIL or its receptors enhanced NGF promoted growth

Following the observation that APRIL, in addition to a function blocking antibody against APRIL, enhances NGF promoted neurite growth the function of the receptors BCMA and TACI were investigated using function blocking antibodies that prevent APRIL binding. The functional blockade of APRIL receptors and any resulting effect on neurite growth is required to better understand the mechanism by which APRIL signalling is acting *in vitro* and why both treatment with recombinant ligand and blockade of this same ligand both increase growth.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were treated with either function blocking antibodies antibodies aBCMA (aB, 3.2μg/ml), aTACI (aT, 2μg/ml), aAPRIL (aA, 3μg/ml), or a combination of aBCMA and aTACI in the presence or absence of 100ng/ml recombinant APRIL. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons

test. Mouse myeloma cell line NS0 derived recombinant human BCMA was used to prepare the aBCMA while the manufacturer determined in a functional ELISA, 0.2-0.8μg/mL of this antibody will block 50% of the binding of 500 ng/mL of recombinant human APRIL to immobilized recombinant human BCMA coated at 1 μg/mL (100 μL/well). At 5 μg/mL, this antibody will block >90% of the binding (R&D). Mouse myeloma cell line NS0 derived recombinant human TACI/TNFRSF13B was used to prepare aTACI while the manufacturer determined in a functional ELISA, 0.03-0.12μg/mL of this antibody will block 50% of the binding of 50 ng/mL of Recombinant Human BAFF/BLyS/TNFSF13B to immobilized Recombinant Human TACI/TNFRSF13B Fc Chimera coated at 1μg/mL (100μL/well). At 10 μg/mL, this antibody will block >90% of the binding (R&D).

The functional blockade of ligand binding to the receptors BCMA and TACI leads to a significant increase in NGF promoted neurite outgrowth at P0 (Fig. 37). The addition of recombinant APRIL and its function blocking antibody once again also significantly increases neurite growth in the presence of NGF. A combination of aBCMA and aTACI shows no additive effect while the application of recombinant APRIL in tandem with receptor blockade also demonstrates no additive enhancement of NGF promoted growth. This data points to endogenous APRIL signalling acting to diminish growth, with blockade enhancing outgrowth. The action of recombinant APRIL acting to increase neurite outgrowth needs further investigation to ascertain why its actions to not align with those of the function blocking reagents.

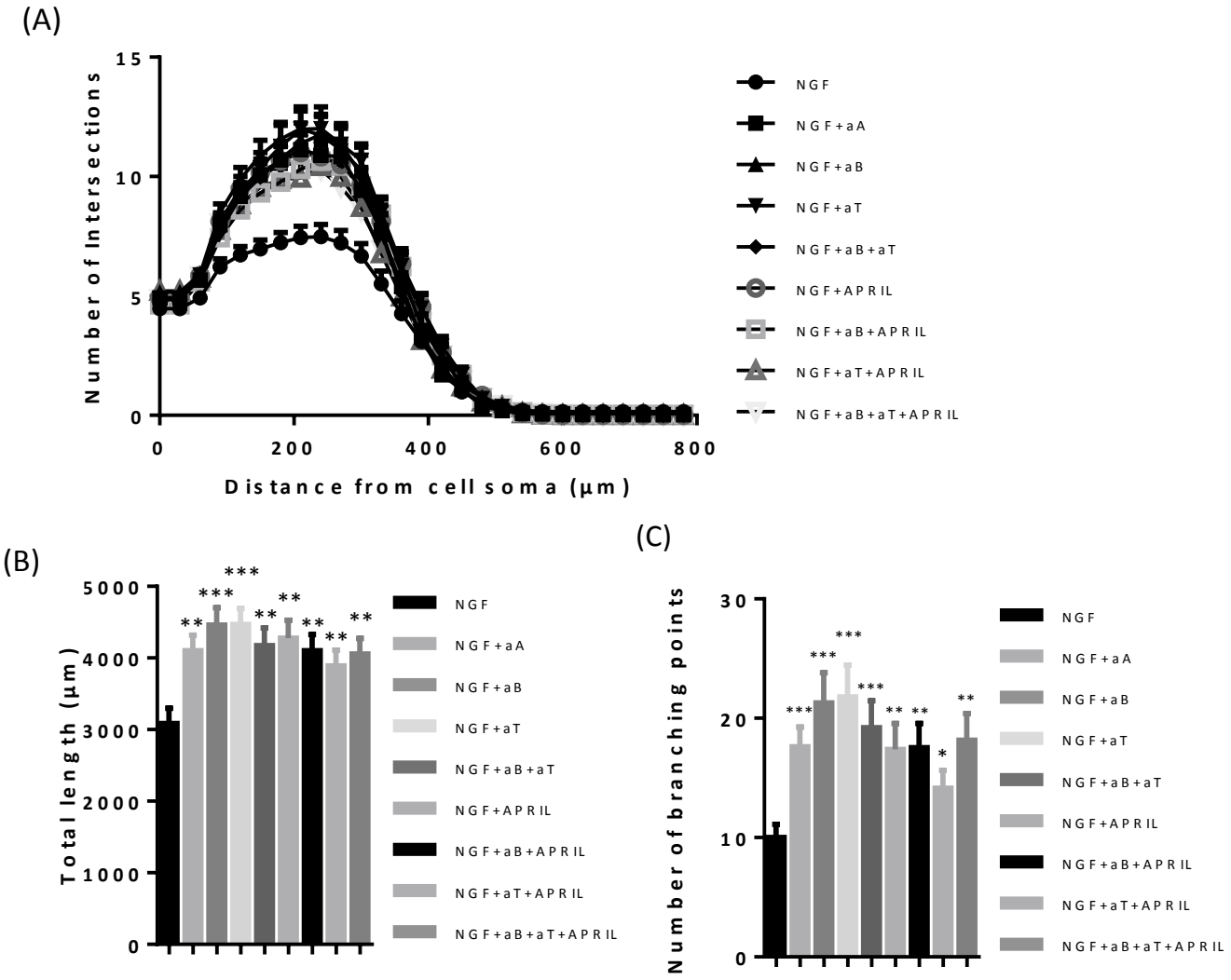


Figure 37: Blockade of APRIL and its receptors enhances neurite growth from cultured SCG neurons (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) Other neurons were treated with either function blocking antibodies aBCMA (aB, 3.2µg/ml), aTACI (aT, 2µg/ml), aAPRIL (aA, 3µg/ml), a combination of aBCMA and aTACI (aC) in the presence or absence of 100ng/ml recombinant APRIL. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (* indicates P <0.05, ** indicates P <0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

While recombinant APRIL enhances NGF promoted growth in P0 SCG neurons recombinant BAFF has no effect

Following the observations that APRIL enhances NGF promoted neurite growth at P0 in SCG neurons the function recombinant BAFF at this developmental time was tested. As BAFF shares a number of common receptors with APRIL, can form heterotrimers with APRIL itself and shares sequence homology it is important to investigate its potential role in neurite growth at the time when APRIL exhibits its effect.

SCG ganglia of post natal day 0 mice were dissected and neurons plated at low density. Short term cultures were established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition a separate set of neurons were treated with either 100ng/ml recombinant APRIL or 100ng/ml recombinant BAFF. Recombinant BAFF used was Mouse myeloma cell line, NS0-derived and its biological activity measured in a cell proliferation assay using anti-IgM stimulated mouse B cells. The ED_{50} for this effect is typically 0.1-0.5ng/mL in the presence of goat anti-mouse IgM μ chain (R&D). The presence of the pan caspase inhibitor acted to ensure consistent neuronal survival. Following 16 hours of culture the neurons were treated with Calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the Fast Sholl technique

followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

While recombinant APRIL acted to enhance NGF promoted growth an equivalent dose of recombinant BAFF did not enhance NGF promoted growth in P0 SCG neurons (Fig. 38). While APRIL enhances the growth of P0 SCG neurons the potential role of BAFF at this particular age does not appear to be related with neurite growth. Its interactions with APRIL and the receptors BCMA and TACI will need to be further investigated.

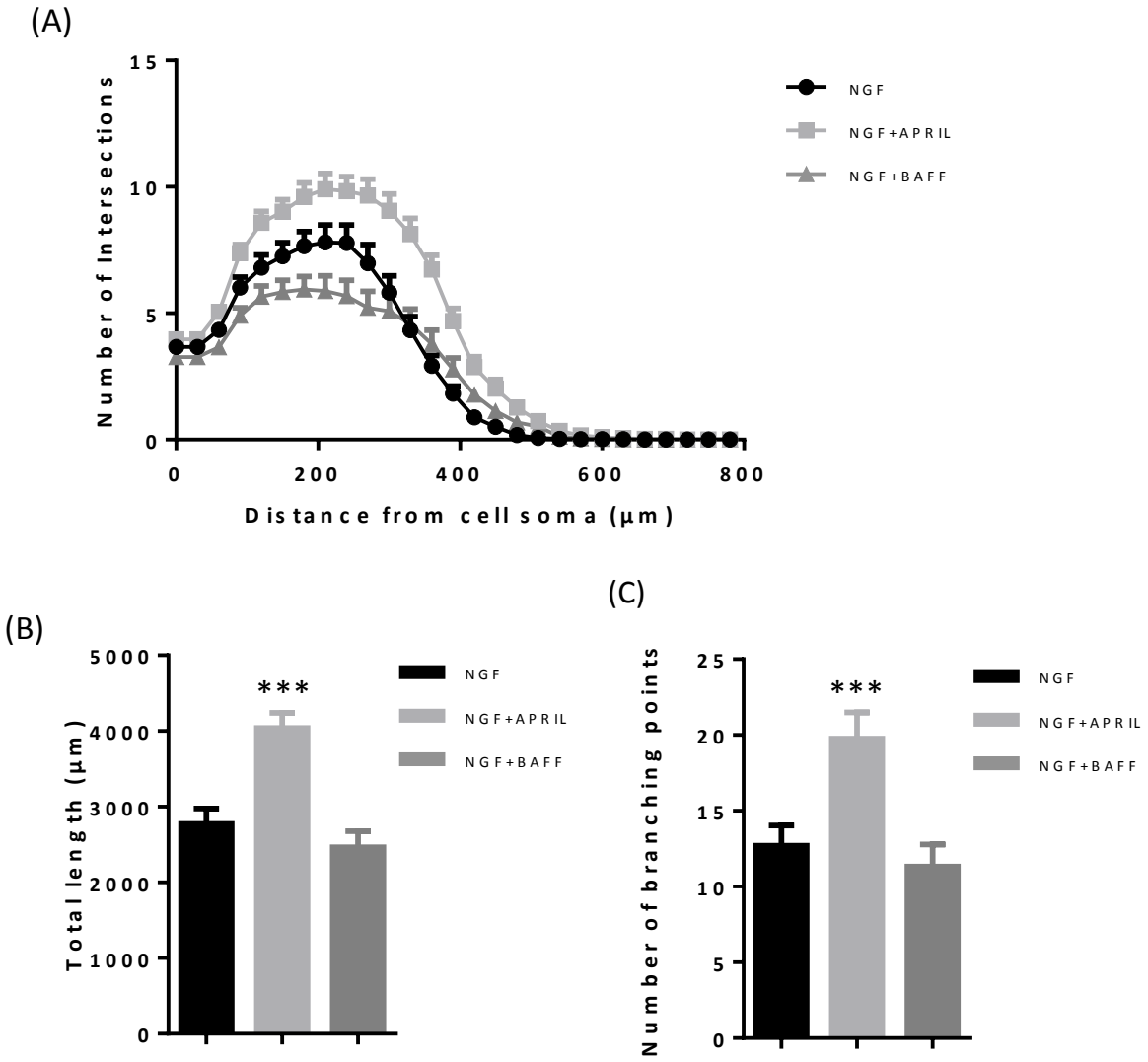


Figure 38: Recombinant APRIL enhances NGF promoted growth while recombinant BAFF has no effect (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) other neurons were treated with either 100ng/ml recombinant APRIL or 100ng/ml recombinant BAFF. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Soluble BCMA and TACI enhance the growth of P0 SCG neurons while soluble BAFFR has no effect

As both recombinant APRIL and function blocking antibodies against its receptors enhance NGF promoted growth of P0 SCG neurons an additional approach was implemented to test if soluble BCMA, TACI and BAFFR in the form of Fc chimeras had any effect on the growth of sympathetic neurons in culture. The use of soluble forms of the receptor test two possibilities. Firstly, these soluble receptors can act to block endogenous signalling by acting as decoy receptors blocking the interaction between APRIL and cell surface BCMA and TACI in addition to BAFF binding BAFFR. Secondly, soluble receptors can bind to cell surface ligand and initiate a reverse signal as in the case of TNFαreverse signalling in sympathetic neuronal growth(199). If APRIL reverse signalling is acting to inhibit neurite growth, blockade of receptors, aAPRIL and the use of recombinant APRIL would all interfere with such a signalling mechanism to increase growth. The soluble receptors offer an additional way to explore this hypothesis as binding of these to cell surface APRIL may activate the reverse signal to inhibit growth.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and grown for 16 hours. At the point of plating, neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. In addition to this control condition neurons were treated with either BCMA receptor chimera (BCMA Fc, 50ng/ml), TACI receptor chimera (TACI Fc, 50ng/ml) or BAFFR chimera (BAFFR Fc, 50ng/ml). All chimeras are Mouse myeloma cell line NS0 derived and form disulphide-linked

homodimers. The activity of BCMA Fc was measured by its ability to inhibit APRIL-mediated proliferation of anti-IgM stimulated mouse B cells, TACI and BAFFR Fc by their ability to inhibit BAFF-mediated proliferation of anti-IgM stimulated mouse B cells (R&D). Following 16 hours of culture the neurons were treated with Calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the Fast Sholl technique followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Soluble forms of BCMA and TACI enhance the growth of P0 SCG neurons in the presence of NGF while soluble BAFFR does not (Fig. 39). The activity of BAFFR Fc is in line with the observation that recombinant BAFF does effect neurite growth (Fig. 38). It appears that BAFF and its specific receptor BAFFR do not play a functional role in the growth of PO SCG neurons. Soluble BCMA and TACI significantly increased neurite growth again implicating these receptors in NGF promoted growth. As to whether the soluble receptors activate endogenous reverse signalling or merely block forward signalling more needs to be done to determine this.

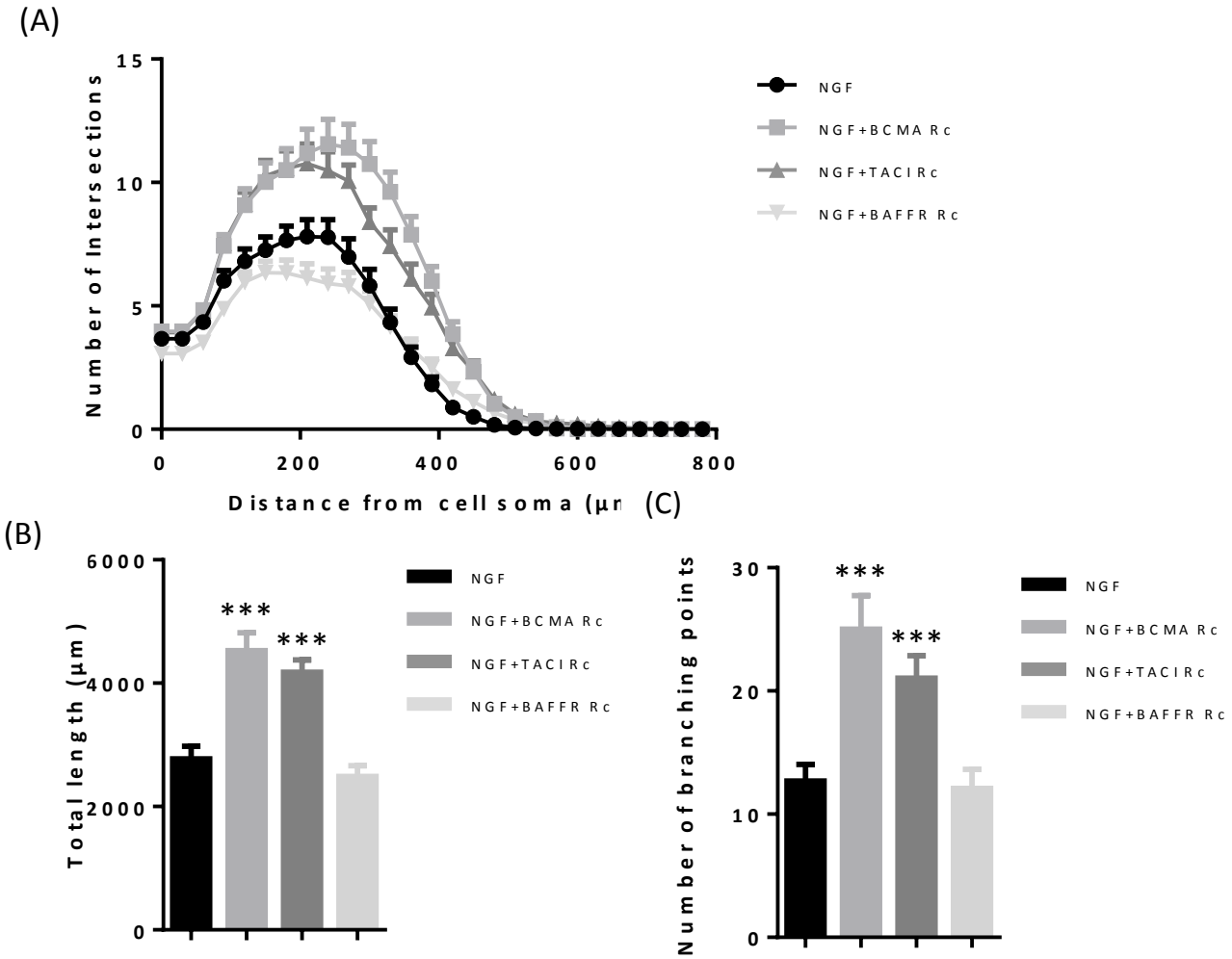


Figure 39: Soluble BCMA and TACI receptors enhance NGF promoted growth of P0 SCG neurons while soluble BAFFR has no effect (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF (control) Other neurons were treated with either BCMA receptor chimera (BCMA Fc, 50ng/ml), TACI receptor chimera (TACI Fc, 50ng/ml) or BAFFR chimera (BAFFR Fc, 50ng/ml). Cells were stained with the fluorescent balcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

The ability of recombinant APRIL to enhance the growth of P0 SCG neurons requires MEK and PI3K signalling

NGF promoted growth is known to enhance a number of intracellular signalling pathways including MAPK and PI3K/Akt. To investigate the potential involvement of these signalling cascades in APRIL enhanced NGF promoted outgrowth specific inhibitors of MEK and PI3K were used. These signalling cascades are known to act downstream of NGF, BCMA and TACI in other systems and so their potential activity in mediating the action of APRIL in neurite growth has been tested.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and cultured for one hour in F-14 media to allow adhesion to the culture surface. After this period the neurons were exposed to NGF at a concentration of 1ng/ml and a pan caspase OPH Inhibitor at 10μM. Neurons were then left for a period of four hours in culture before treatment with an inhibitor against MEK (10μM) or PI3K (10μM). The MEK inhibitor PD98059 has been shown to act in vivo as a highly selective inhibitor of MEK1 activation and the MAP kinase cascade. PD98059 binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf. The PI3K inhibitor LY294002 has been shown to act in vivo as a highly selective inhibitor of phosphatidylinositol 3 (PI3) kinase. When used at a concentration of 50 μM, it specifically abolished PI3 kinase activity but did not inhibit other lipid and protein kinases such as PI4 kinase, PKC, MAP kinase or c-Src. Both inhibitors were tested and supplied by Cell Signalling Technology. The 4 hour time period ensures NGF has adequate time to act and so and addition of

inhibitor does not negatively affect NGF promoted survival or growth. In this way the role of these signalling pathways in the action of APRIL can be isolated form the actions of NGF. This four hour period ensured the blockade of MEK and PI3K signalling does not inhibit NGF promoted outgrowth. After an additional one hour of culture the neurons were treated with 100ng/ml recombinant APRIL where appropriate. As the inhibitors are reconstituted in DMSO an equivalent concentration of DMSO was applied to the control condition (NGF) to ensure all neurons were cultured in a comparable environment. Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Due to the four hour gap between NGF addition to cultures and the application of inhibitors to MAK and PI3K, cultures treated with these factors display similar levels of neurite growth to control. The application of 100ng/ml recombinant APRIL enhances this growth. In those conditions pre-treated with both MEK and PI3K inhibitors APRIL does not significantly increase neurite growth in the presence of NGF (Fig. 40). It is therefore apparent that the activity of these intracellular signalling cascades are required for the action of APRIL to increase the growth of P0 SCG neurons *in vitro.*

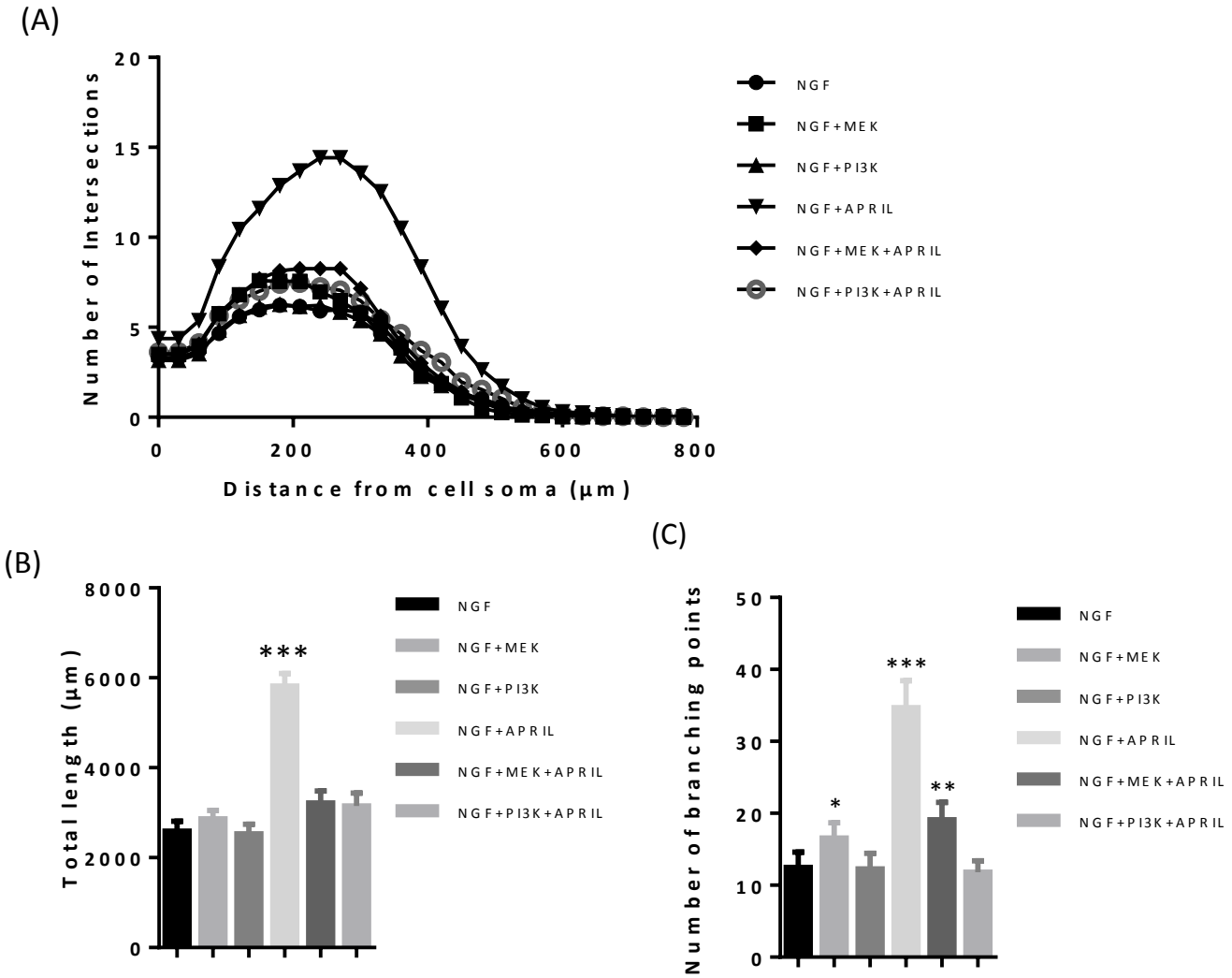


Figure 40: APRIL enhanced neurite growth from cultured SCG neurons requires the action of MEK and PI3K (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. Neurons were plated for one hour before treatment with a pan caspase OPH Inhibitor and 1ng/ml NGF. Following four hours in culture neurons were treated with an inhibitor against MEK (10µM) or PI3K (10µM) and left for a further one hour. Neurons were treated with 100ng/ml recombinant APRIL for a further 10 hours. Cells were stained with the fluorescent Calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) Bar chart of neurite length (C) Bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (* indicates P <0.05, ** indicates P <0.001, *** indicates P <0.0001, statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

APRIL and its receptors do not affect the survival of P0 SCG neurons in the presence or absence of NGF

Throughout this chapter neurite growth has been investigated while the presence of a pan caspase inhibitor ensured a block on any apoptotic signalling. However, the ages investigated occur in a time window of development when neurons of the sympathetic and sensory nervous system undergo programmed cell death. It became critical to investigate if APRIL or BAFF and their receptors played a role in the survival of neurons at the age most studied in addition to their roles in neurite growth.

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and cultured for 16 hours in the presence of a pan caspase OPH Inhibitor or 1ng/ml NGF. Recombinant APRIL or BAFF and aBCMA (aB), aTACI (aT), aAPRIL (aA) or aBAFFR (aBR) were added to cultures both in the presence of NGF or in its absence. The antibodies were from the same source as previously used (Fig. 34) Following 16 hours of culture the cells were counted on a phase contrast microscope. The data shown represent the mean ± s.e.m of data compiled from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

None of the factors tested showed any effect on the survival of cultured P0 SCG neurons either in the presence of NGF or in its absence (Fig. 41). The significance of this is that the role of APRIL and its receptors seems to be in relation to NGF promoted neurite outgrowth independent of NGF promoted survival.

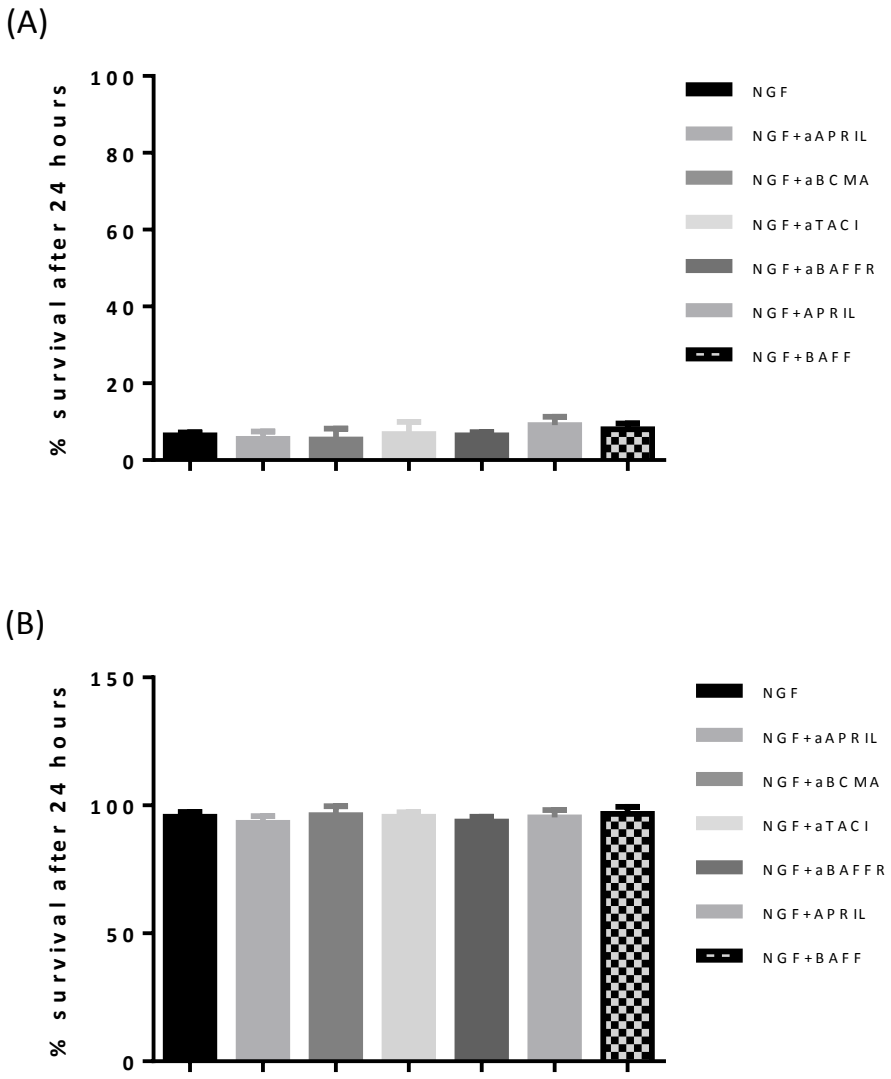


Figure 41: APRIL and BAFF or blockade of their receptors does not affect the survival of cultured P0 SCG neurons in the presence or absence of NGF (A) Neuronal survival of P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of a pan caspase OPH Inhibitor (A) or 1ng/ml NGF (B). Other neurons were treated with either 100ng/ml recombinant APRIL, 100ng/ml recombinant BAFF or function blocking antibodies aBCMA (aB, 3.2µg/ml), aTACI (aT, 2µg/ml), aAPRIL (aA, 3µg/ml), aBAFFR (aBR, 3µg/ml). Cells were counted after 16 hours on a phase contrast microscope. The data shown represent the mean ± s.e.m of data compiled from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test.).

APRIL does not affect NGF promoted survival over a range of NGF concentrations

While APRIL showed no ability to either enhance or diminish survival in either a saturating concentration 1ng/ml NGF or in its complete absence it was further tested over a range of NFG concentrations to investigate the possibility that any effect might require a sub saturating concentration of NGF. Thus a broad range of NGF concentrations was tested from a saturating level (1ng/ml) to concentration where NGF no longer promotes survival *in vitro* (0.1pg/ml).

SCG ganglia of post natal day 0 mice were dissected and low density, short term, cultures established and cultured for 16 hours in the presence of a 100ng/ml recombinant APRIL. Neurons were treated with NGF doses from 0.1pg/ml to 1ng/ml. Following 16 hours of culture the cells were counted on a phase contrast microscope. The data shown represent the mean ± s.e.m of data compiled from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Over this range of NGF concentrations in a 24 hour period survival rates fell from more than 90% to below 10% (Fig. 42). APRIL did not affect the survival of neurons *in vitro* over the wide range of NGF concentrations.

(A)

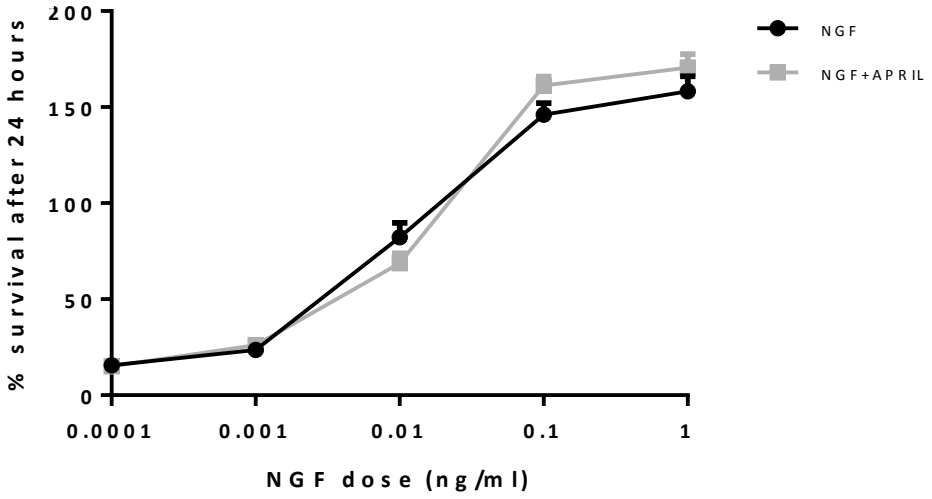


Figure 42: APRIL does not affect NGF promoted survival over a broad range of NGF concentrations (A) Neuronal survival of P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in the presence of 100ng/ml recombinant APRIL and a variety of NGF concentrations from 0.1pg/ml to 1ng/ml NGF. Cells were counted after 16 hours on a phase contrast microscope. The data shown represent the mean ± s.e.m of data compiled from at least three separate experiments (statistical comparison with control, Kruskal-Wallis test followed by Dunn's multiple comparisons test).

Discussion

These data demonstrate the first known role of APRIL signalling within the nervous system. Recombinant APRIL acts to enhance NGF-promoted neurite outgrowth from cultures SCG neurons in a broad developmental window stretching from embryonic day 16 through to post natal day three (Figs 27-30).

The observation that APRIL only enhances neurite growth when cells are cultured in the presence of NGF (Fig 23) and does not affect outgrowth when NGF (Fig 24) is absent raised the possibility that this TNF superfamily ligand acts to regulate NGF promoted growth in sympathetic neurons. This is taking place at a time when these neurons are critically dependent on this neurotrophin for both survival and process extension.

APRIL enhances NGF promoted growth at a dose as small as 1ng/ml and continues to increase growth up to at least 1µg/ml (Fig 25). This demonstrates that the ligand can act to promote growth at physiologically relevant dose *in vitro.*

While the action of recombinant APRIL acting in a homotrimeric form is to enhance NGF promoted neurite outgrowth the same can be said for functional blockade of APRIL signalling. The application of a function blocking aAPRIL antibody to neurons in culture also significantly increases the growth of P0 SCG neurons in the presence of NGF.

While these effects appear contradictory they do show consistency throughout multiple developmental ages. From E16 through to P3 the application of either recombinant APRIL or aAPRIL increases NGF promoted neurite outgrowth Figs 27-30). The same cannot be said in the sensory trigeminal ganglion (Figs 31-34). Here NGF promoted growth remained consistent regardless of the application of either APRIL or aAPRIL. These observations indicate that the role of APRIL in enhancing NGF-promoted growth varies in different populations of neurons. APRIL may have no effect on the growth of sensory trigeminal neurons or any effect may occur over a different developmental window requiring a different dose of recombinant ligand. It is clear that the use of 100ng/ml recombinant APRIL is not sufficient to alter NGF-promoted growth in trigeminal neurons either shortly before birth or for a period after when this same dose causes an enhancement in the growth of SCG neurons.

The effect of APRIL on NGF promoted growth remains even at a subsaturating dose of NGF. At 10pg/ml the neurotrophin no longer maintains maximal survival *in vitro* (Fig 42) and neurons cultured in this concentration show a less marked promotion in growth when treated with recombinant APRIL (Fig 35).

The effect of receptor blockage at P0 matches that of APRIL blockade. Here either aBCMA or aTACI enhance NGF promoted growth *in vitro.* Receptor blocking antibodies added to culture in combination with recombinant APRIL results in a similar enhancement of neurite growth (Fig 37). The same is again true for soluble forms of BCMA and TACI (Fig 39). These factors are another example of how

modulation of APRIL signalling *in vitro* enhances NGF promoted neurite outgrowth. These soluble receptors may be acting in several ways *in vitro*. Addition of soluble BCMA and TACI may act to bind endogenously produced APRIL and block its interaction with endogenous cell surface receptor. In this way, addition of the soluble receptors would block an endogenous APRIL signal. Alternatively, soluble BCMA and TACI may bind to cell surface TWEPRIL and initiate a response in the ligand expressing cell.

This anomaly raised a number of interesting possibilities that merit further future investigation. How the action of a ligand and its opposing function blocking counterpart result in the same experimental phenotype presents an interesting problem. It is known that APRIL requires multimerisation *in vivo* to bind its receptors in a way that activates downstream signalling. Indeed a form of APRIL that lacks the ability to multimerise through heparin sulfate interaction has no effect on neurite growth at P0 (Fig 36). This experiment demonstrates that the ability to bind heparin sulfate is critical to the ability of APRIL to enhance neurite growth.

It is possible that recombinant APRIL binds to cell surface BCMA and/or TACI but is not able to initiate a cellular response. The recombinant form of the ligand purchased for use in these experiments has been shown to enhance B cell proliferation by the manufacturer but its ability to interact with and activate BCMA and TACI on the surface of SCG neurons is not known. The action of the recombinant APRIL may be to bind cell surface BCMA and/or TACI on SCG neurons but not activate the receptors. In this case, binding of

functionally active APRIL produced by the neurons themselves would be interfered with. This proposed action demonstrates a scenario in which the addition of recombinant ligand to cultured neurons in fact blocks endogenous ligand-receptor interaction rather than enhance such a system.

This is one possible explanation for the anomaly presented above. While it is true that traditional APRIL forward signalling through one or both of its receptors may indeed promote neurite growth it has now been demonstrated that TNFα reverse signalling can enhance neurite growth. Reverse signalling involves a receptor, either expressed on the surface of a cell or released as a soluble form, acting functionally as a ligand. The receptor binds to its ligand partner present on a cell and initiates an intracellular response through the ligand. It is simply the reverse of traditional forward signalling in which the traditional receptor and ligand swap position. Interaction between ligand and receptor causes a cell response via the ligand rather than the receptor. In this paradigm APRIL reverse signalling would act to negatively regulate neurite growth and the addition of soluble APRIL would block interaction between receptor and cell surface APRIL thus resulting in increased neurite growth. For this paradigm to hold true the function blocking antibodies and soluble receptors would also be blocking interaction between endogenous receptor and endogenous cell surface TWEPRIL resulting in enhanced neurite growth. TWEPRIL would be the likely candidate for a reverse signalling paradigm as it exists on the cell membrane displaying its receptor binding domain on the extracellular surface. APRIL is only expressed on the Golgi membrane and is proteolytically

cleaved to release a soluble ligand. Due to this, no reverse signal could be initiated via APRIL.

The complexity of this system is further increased by the presence of BAFF and BAFFR. While BAFF's preferred receptor is BAFFR it can also bind to BCMA and TACI. Heterotrimers can form between APRIL and BAFF altering receptor binding affinities and so this system is far from simple. The observation that recombinant BAFF does not affect neurite growth at the time when APRIL exerts its affect *in vitro* coupled with a lack of any observable difference in neurons exposed to soluble BAFFR points to APRIL being the major player in terms of neurite growth at P0 in this group of TNF members.

The role of APRIL and its receptors appear, as far as has been experimentally tested, to affect neurite growth independently of survival. At P0, the most extensively used developmental age in this study, blockade of either BCMA, TACI or BAFF receptor, or the addition of the soluble ligands either promotes or diminishes survival. This is true either in the presence or absence of NGF.

Final culture work in this chapter focused on attempting to understand the intracellular processes that mediate this enhanced neurite growth. The blockade of both MEK and PI3K activity through specific inhibitors abolished the ability of APRIL to enhance neurite growth. These pathways have been extensively shown to mediate neurotrophin promoted neurite growth and the same is true for APRIL and its role in neurite growth.

Future work must aim to uncover the exact mechanism by which APRIL signalling mediates NGF promoted growth. The use of shRNA knockdown of APRIL and its receptors coupled with the expression of dominant negative forms of the receptors could provide more evidence for the mode of their function in SCG culture.

In addition to this the intracellular activity of MEK and PI3K signalling can be further probed in APRIL mediated signalling. Although blockade of these pathways abolished APRIL enhancement of NGF promoted growth can APRIL application increase the activity of MEK and PI3K signalling?

Chapter 5

The functional role of APRIL and its receptors *in vivo*

Introduction

It has been determined that APRIL and its receptors BCMA and TACI play a significant role in NGF promoted outgrowth from SCG neurons *in vitro.* While the exact mechanism remains elusive, experimentally modifying the interaction between the ligand and its receptors leads to an enhancement of growth.

The use of APRIL-deficient mice opens up the possibility of investigating the significance of previous data in the normal development of the mouse and the resulting effects of its abolishment *in vivo.* The APRIL-deficient mice used in this chapter were kindly provided by Avi Ashkenazi and produced by Varfolomeev et al. Deficient mice lack both forms of APRIL, the soluble secreted and membrane bound TWEPRIL. By investigating the development of these mice it is possible to determine if APRIL activity is of physiological importance for the animal.

APRIL-deficient mice demonstrate no embryonic lethality and develop to full adulthood. Female animals are able to litter and raise young as normal. For experiments in this chapter matings of heterozygous animals produce litters of mixed genotype allowed comparison between wild type and APRIL-deficient littermates.

Target field innervation, measured by the number of innervating fibres in defined regions offers the chance to see how a lack of APRIL effects the number of fibres reaching target fields and elaborating dendrites. Quantification of cell number in sympathetic ganglia determines the role of APRIL in the development of an intact ganglia. Finally, cultures established from APRIL-deficient mice offer a chance of further investigating the role of APRIL and its receptors *in vitro.*

NGF promoted growth is enhanced in P0 SCG neurons cultured from APRIL null mice

With the in *vitro* findings that recombinant APRIL enhances NGF promoted growth in P0 SCG, neurons from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were cultured at a similar density and at the same time. The aim of this experiment was to establish if neurons dissected from APRIL mice grew differently than those of their wild type counterparts.

P0 SCG neurons from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were dissected and dissociated separately. Following dissociation, the neurons were suspended in media containing a pan caspase inhibitor and 1ng/ml NGF. The number of neurons in each cell suspension was counted before plating and normalised to each other to ensure the final numbers were comparable between cultures. As neuronal density effects outgrowth comparable cell numbers between conditions is critical to draw correct conclusions. If this was not the case neuronal growth between genotypes would not be fairly comparable

Following 16 hours of culture the neurons were treated with calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the fast Sholl technique followed by statistical comparison to control using Mann-Whitney U test.

Following 16 hours in culture neurons from the SCG of APRIL$^{-/-}$ grew significantly larger than those from APRIL$^{+/+}$ littermates in the

presence of NGF. Those neurons that lacked APRIL displayed an enhancement of NGF promoted growth (Fig. 43). The fact that neurons cultured from APRIL-deficient mice grow larger indicates that APRIL signalling acts to restrict the size of SCG neurons. If APRIL acted to promote growth its abolishment should act to increase growth. This result enhances the argument that APRIL signalling acts to reduce NGF promoted neurite outgrowth and that its blockade therefore enhances growth.

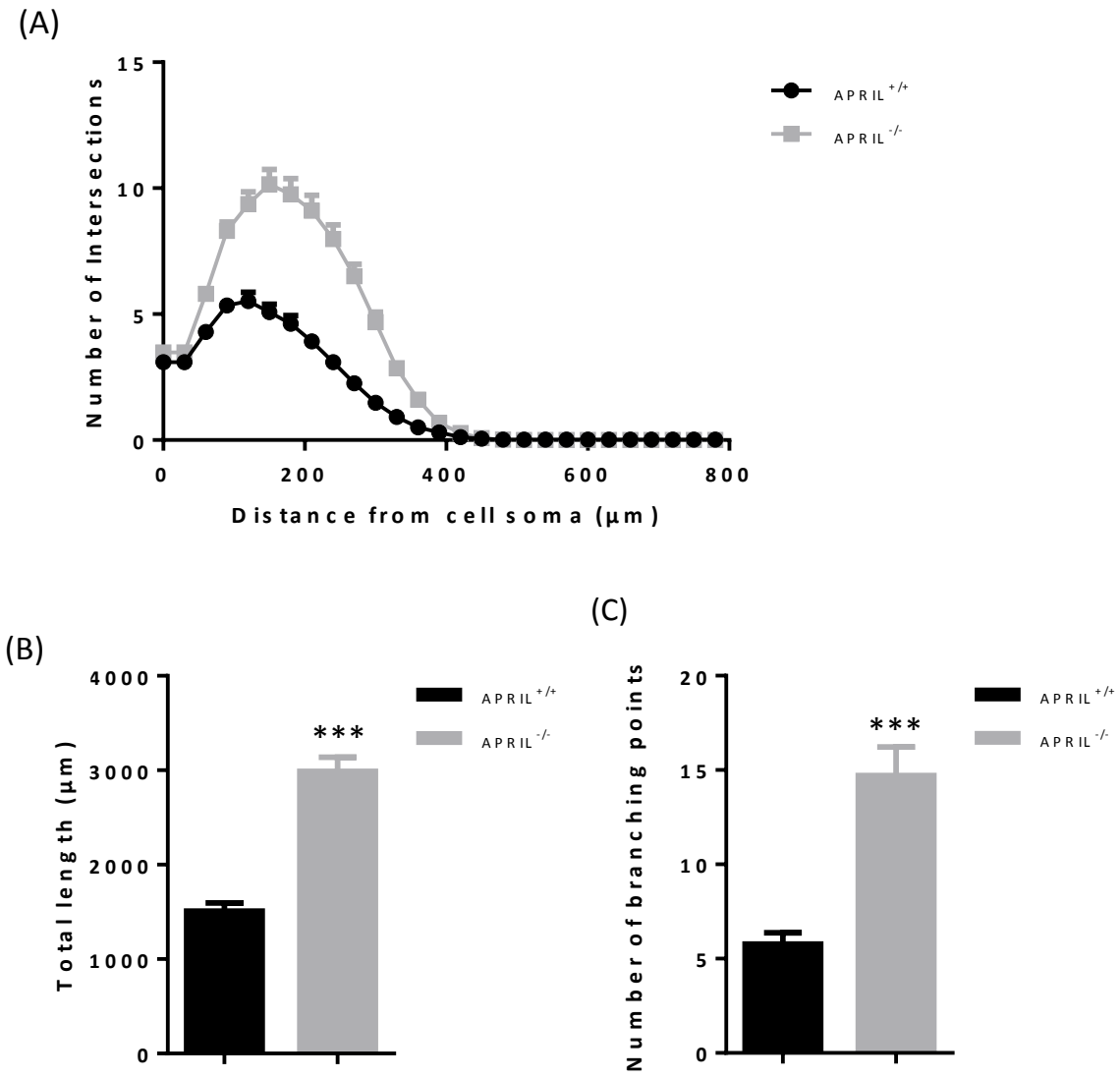


Figure 43: Cultured SCG neurons from APRIL$^{-/-}$ mice exhibit enhanced neurite growth in the presence of NGF (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. Neurons from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were cultured separately for 16 hours in the presence of a pan caspase OPH Inhibitor and 1ng/ml NGF. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (*** indicates P <0.0001, statistical comparison with control, Mann-Whitney U test).

NGF promoted growth is not enhanced in P0 SCG neurons cultured from APRIL null mice in the absence of NGF

While initial data demonstrated that neuritis ofSCG neurons cultured from APRIL$^{-/-}$ mice grow larger than those of wild type littermates in the presence of NGF it was not clear if this effect absolutely required the presence of the neurotrophin. Therefore, outgrowth of neurons from APRIL$^{-/-}$ and APRIL$^{+/+}$ mice was compared in the absence of NGF. P0 SCG neurons from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were dissected and dissociated separately. Following dissociation, the neurons were suspended in media containing a pan caspase inhibitor. The number of neurons in each cell suspension was determined before and normalised before plating to ensure the final numbers were comparable between cultures. If this were not the case neuronal growth between genotypes would not be comparable. The presence of a pan caspase inhibitor prevents apoptotic cell death thus maintaining cell survival in the absence of NGF.

Following 16 hours of culture the neurons were treated with Calcein AM for 30 minutes before the neurons were imaged on a fluorescence microscope. Within each experimental condition a minimum of 60 images were acquired while the experiment was repeated on three different occasions. Imaged analysis used the Fast Sholl technique followed by statistical comparison to control using Mann-Whitney U test.

In the absence of NGF there was no significant difference in the growth of P0 SCG neurons cultured from APRIL$^{-/-}$ and APRIL$^{+/+}$ mice (Fig. 44). This result confirms earlier work that APRIL activity

regulated the activity of NGF promoted growth and does not act independently of the neurotrophin.

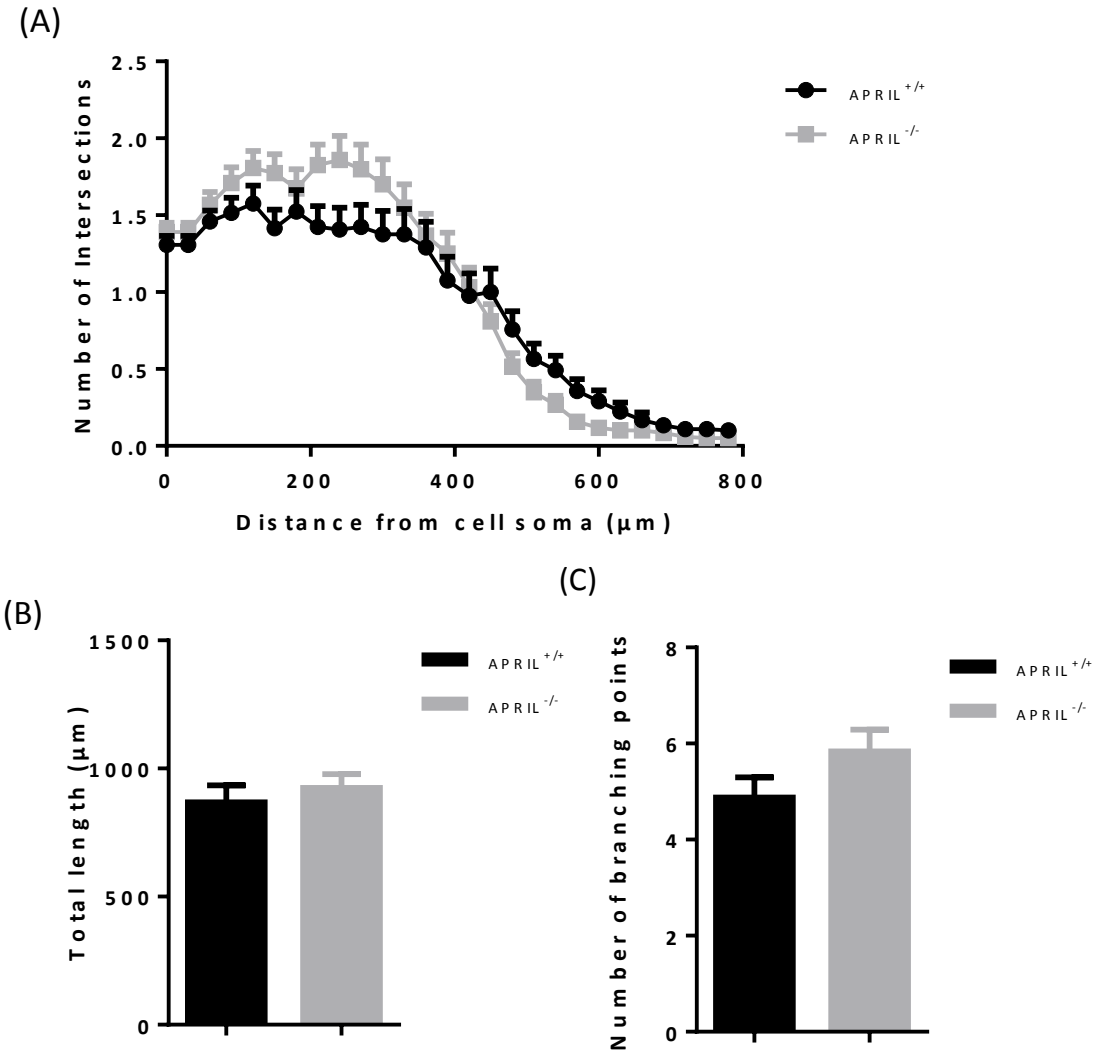


Figure 44: SCG neurons from APRIL$^{-/-}$ and APRIL$^{+/+}$ littermates show similar growth in the absence of NGF (A-C) Neurite growth from P0 SCG neurons after 16 hours *in vitro*. Neurons from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were cultured separately for 16 hours in the presence of a pan caspase OPH Inhibitor. Cells were stained with the fluorescent calcein AM before image acquisition using an inverted microscope (A) Sholl analysis (B) bar chart of neurite length (C) bar chart of number of branching points. The data shown represent the mean ± s.e.m of data compiled from >150 neurons per condition from at least three separate experiments (statistical comparison with control, Mann-Whitney U test).

Survival of P0 SCG neurons from APRIL-/- mice is comparable to those of APRIL+/+ mice *in vitro*

As neuronal arbours from APRIL$^{-/-}$ mice grow larger than those of wild type littermates in the presence of NGF neurons were assayed for survival to determine if this growth effect takes place independently of NGF promoted survival in sympathetic neurons.

P0 SCG neurons from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were dissected and dissociated separately. Following dissociation, the neurons were suspended in F-14 culture media either in the presence or absence of 1ng/ml NGF. The number of neurons in each cell suspension was determined and normalised before plating to ensure the final numbers were comparable between cultures. If this was not the case neuronal survival between genotypes would not be fairly comparable.

Following 16 hours of culture the cells were counted on a phase contrast microscope. The data shown represent the mean ± s.e.m of data compiled from at least three separate experiments (statistical comparison with control, ANOVA followed by Dunnett's multiple comparisons test).

No differences in survival between APRIL$^{+/+}$ and APRIL$^{-/-}$ neurons were observed. Those neurons cultured in media lacking NGF displayed a low level of survival below 10%, while neurons cultured in the presence of NGF survived at an average greater than 80% (Fig. 45). This confirms earlier work that NGF promoted survival is not regulated by APRIL signalling.

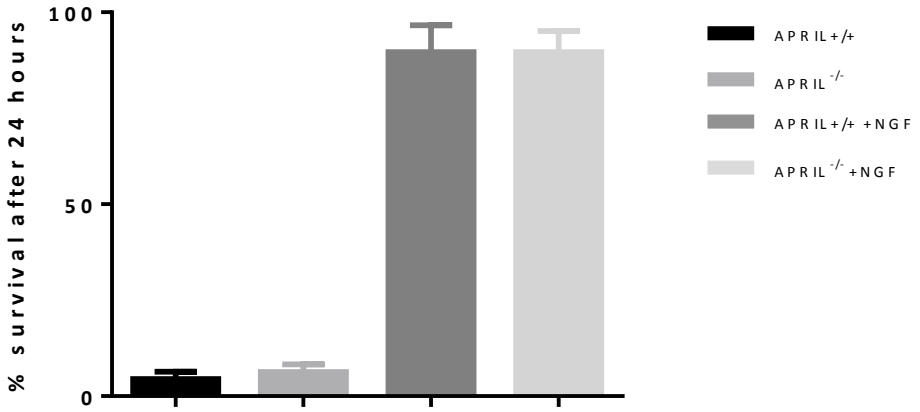


Figure 45: Survival of P0 SCG neurons is not affected in APRIL$^{-/-}$ mice *in vitro*

Neuronal survival of P0 SCG neurons after 16 hours *in vitro*. All neurons were cultured for 16 hours in F-14 culture media or 1ng/ml NGF. Cells were counted after 16 hours on a phase contrast microscope. The data shown represent the mean ± s.e.m of data compiled from at least three separate experiments (statistical comparison with control, ANOVA followed by Dunnett's multiple comparisons test).

Effect of APRIL on sympathetic target field innervation at P5

With the mounting evidence that APRIL plays a role in NGF promoted neurite outgrowth, a transgenic mouse model was employed to investigate whether a loss of APRIL leads to a detectable phenotype in terms of peripheral nervous system development. By analysing the level of target field innervation through immunohistochemistry and the quantification of sympathetic fibres by tyrosine hydroxylase staining in defined targets it could be determined if APRIL signalling has an *in vivo* role in peripheral nervous system development.

Dissected P5 submandibular glands and nasal mucosa from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were first fixed in 4% PFA overnight before cryoprotection in 30% sucrose for up to 48 hours. Following this the target field tissue was sectioned at 16µm. Sections of SMG and NM were stained with a primary antibody against tyrosine hydroxylase to label sympathetic fibres innervating these targets. Following staining the sections were imaged on a Zeiss laser scanning microscope.

For tyrosine hydroxylase quantification the number of TH positive pixels within a defined area of target field was quantified using Adobe Photoshop. Four animals per genotype were used to allow for variability between pups and statistical comparison between genotypes was unpaired t-test with Welch's correction.

Coupled with the analysis of target field innervation by tyrosine hydroxylase immunostaining ganglia from P5 SCG APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were collected and stored in an RNA stabilisation solution before freezing at -80^{0}C for long term storage. Following RNA extraction and reverse transcription, real time PCR was carried

out using Taqman probes and primers specific for TH. The quantification of TH expression within the SCG determines if a difference in target field innervation through an alteration in TH immunoreactivity results from a true change in the number of innervating fibres or instead through an alteration in the expression of TH.

At P5 both the SMG and NM of APRIL$^{-/-}$ mice showed an increased level of tyrosine hydroxylase pixel density (Fig. 46-47). The combination of this observation and the fact that TH expression within the SCG at P5 shows no difference between genotypes (Fig. 48) means that these target fields have an increased number of innervating sympathetic fibres when APRIL is absent. This result further points to APRIL acting as a negative regulator of NGF promoted growth. The abolishment of APRIL increases the number of sympathetic tyrosine hydroxylase fibres reaching the submandibular gland and nasal mucosa by P5.

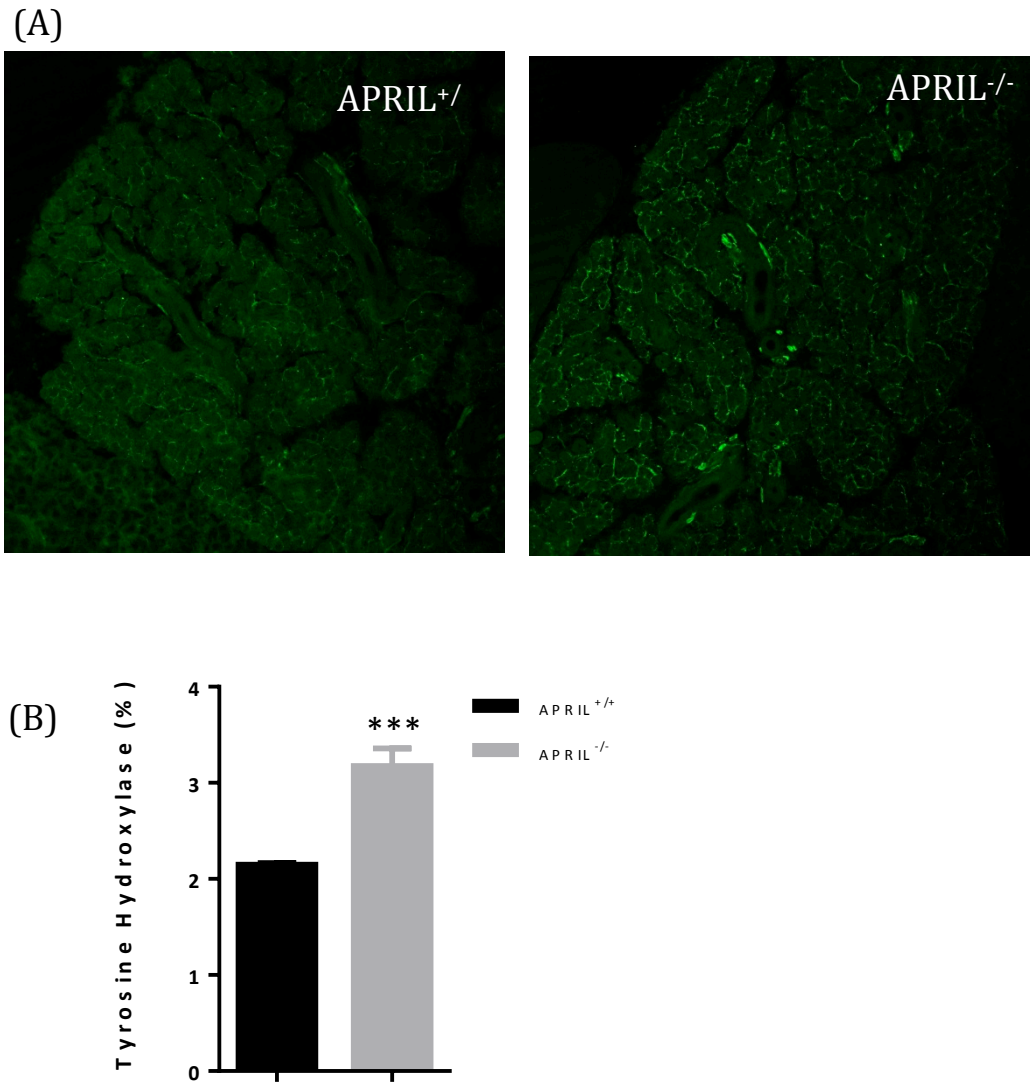
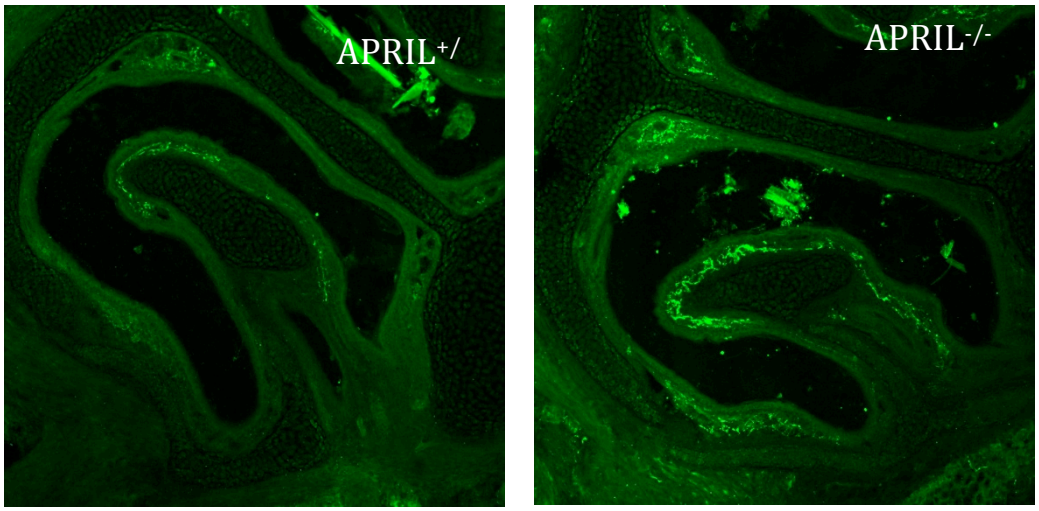


Figure 46: APRIL$^{-/-}$ mice display increased submandibular gland target field innervation at P5

Sympathetic innervation density in P5 SMG of APRIL$^{+/+}$ and APRIL$^{-/-}$. P5 SMG were dissected and fixed in 4% PFA before cryoprotection with 30% sucrose. Following sectioning at 16µm the SMG were stained for tyrosine hydroxylase. Images were acquired using a Zeiss laser scanning microscope. (A) APRIL$^{+/+}$ representative image (B) APRIL$^{-/-}$ representative image (C) tyrosine hydroxylase percentage of defined SMG area. The data shown represent the mean ± s.e.m of data compiled from four animals per genotype (statistical comparison between genotypes, unpaired t-test with Welch's correction).

(A)



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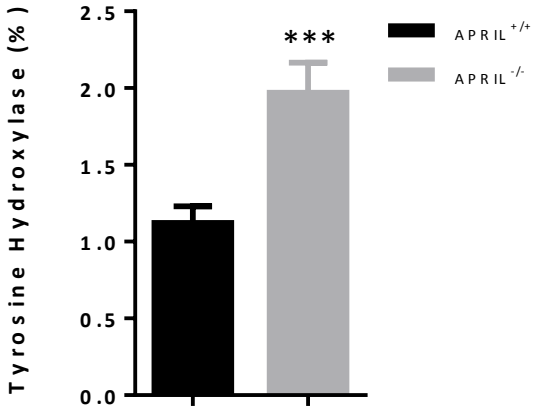


Figure 47: APRIL$^{-/-}$ mice display increased nasal mucosa target field innervation at P5

Sympathetic innervation density in P5 NM of APRIL$^{+/+}$ and APRIL$^{-/-}$. P5 NM were dissected and fixed in 4% PFA before cryoprotection with 30% sucrose. Following sectioning at 16µm the NM were stained for tyrosine hydroxylase. Images were acquired using a Zeiss laser scanning microscope. (A) APRIL$^{+/+}$ representative image (B) APRIL$^{-/-}$ representative image (C) tyrosine hydroxylase percentage of defined SMG area. The data shown represent the mean ± s.e.m of data compiled from four animals per genotype (statistical comparison between genotypes, unpaired t-test with Welch's correction).

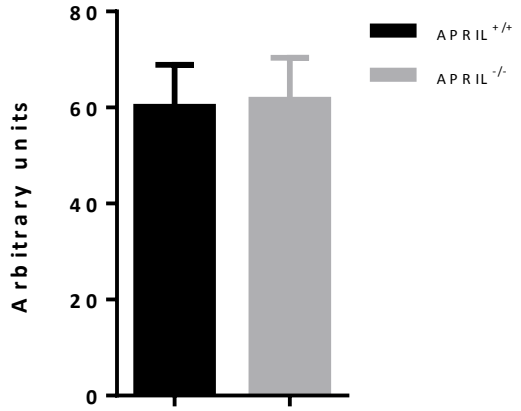


Figure 48: Relative expression of tyrosine hydroxylase in P5 SCG ganglia Expression profile for tyrosine hydroxylase from P5 SCG ganglia of APRIL$^{+/+}$ and APRIL$^{-/-}$ mice. tyrosine hydroxylase expression relative to GAPDH and SDHA in p5 SCG. The data shown represent the mean ± s.e.m of data compiled from four pairs of ganglia per genotype (statistical comparison with control, unpaired t-test with Welch's correction).

Effect of APRIL on sympathetic target field innervation at P10

After observing that APRIL null mice display a greater level of target field innervation at P5 the same quantification was carried out at P10. By quantifying the level of target field innervation at this later time point it can be determined if the increased levels at P5 remain throughout development as target field innervation is nearing completion. In addition to target field innervation a quantification of cell population in the developing ganglia can help determine if a loss of APRIL affects the total numbers of sympathetic neurons within the ganglia itself after their period of NGF dependence for survival.

Dissected P5 SMG and NM as well as SCG from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates were first fixed in 4% PFA overnight before cryoprotection in 30% sucrose for up to 48 hours. Following this the target field tissue was sectioned at 16µm while SCG were sectioned at 8µm. Sections of SMG and NM were stained with a primary antibody against tyrosine hydroxylase to label sympathetic fibres innervating these targets. Ganglia were stained with the nuclear marker TOTO-3 iodide. Following staining the sections were imaged on a Zeiss laser scanning microscope.

For tyrosine hydroxylase quantification the number of TH positive pixels within a defined area of target field was quantified using Adobe Photoshop. For cell counts of the SCG a high magnification image would be used and the number of cell nuclei counted using ImageJ software. Four animals per genotype were used to allow for variability between pups and statistical comparison between genotypes was unpaired t-test with Welch's correction.

Unlike at P5 the SMG and NM of P10 APRIL$^{-/-}$ mice have the same level of tyrosine hydroxylase immunostaining as their wild type littermates (Fig. 49-50). As the level of TH expression within the ganglia at P10 is also consistent between genotypes (Fig. 51) the number of sympathetic innervating fibres at P10 is the same between APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates.

Cell counts of the SCG also determined that the total population of the ganglia is the same between these genotypes and thus a lack of APRIL throughout development to P10 results in a fully formed and populated SCG ganglia (Fig. 52).

Wholemount immunostaining of the P10 submandibular glands of APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates was conducted to provide additional evidence of target field innervation in these mice. The technique uses an alternative quantification and staining protocol to sectioned tissue and thus provides an additional technique for the quantification of sympathetic target innervtion.

Following dissection sub dissection of P10 SMG from immersion fixed APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates the tissue was post fixed in 4% PFA for one hour. Following dehydration, quenching of endogenous peroxidase activity and rehydration tissue was immunostatined for tyrosine hydroxylase with DAB staining. Images were acquired using an Olympus stereomicroscope and the extent of sympathetic nerve branching near the hilus quantified by a modified line-intercept method. Fibre density was estimated and compared between genotypes.

The comparison of fibre density between APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates by wholemount immunostaining revealed no difference between genotypes (Fig. 53). Any increase in sympathetic

innervation density seen at P5 appears to be transitory as the same cannot be seem at P5. While APRIL-deficient mice do display an *in vivo* phenotype, by P10 their sympathetic target fields are developing identically to wild type littermates.

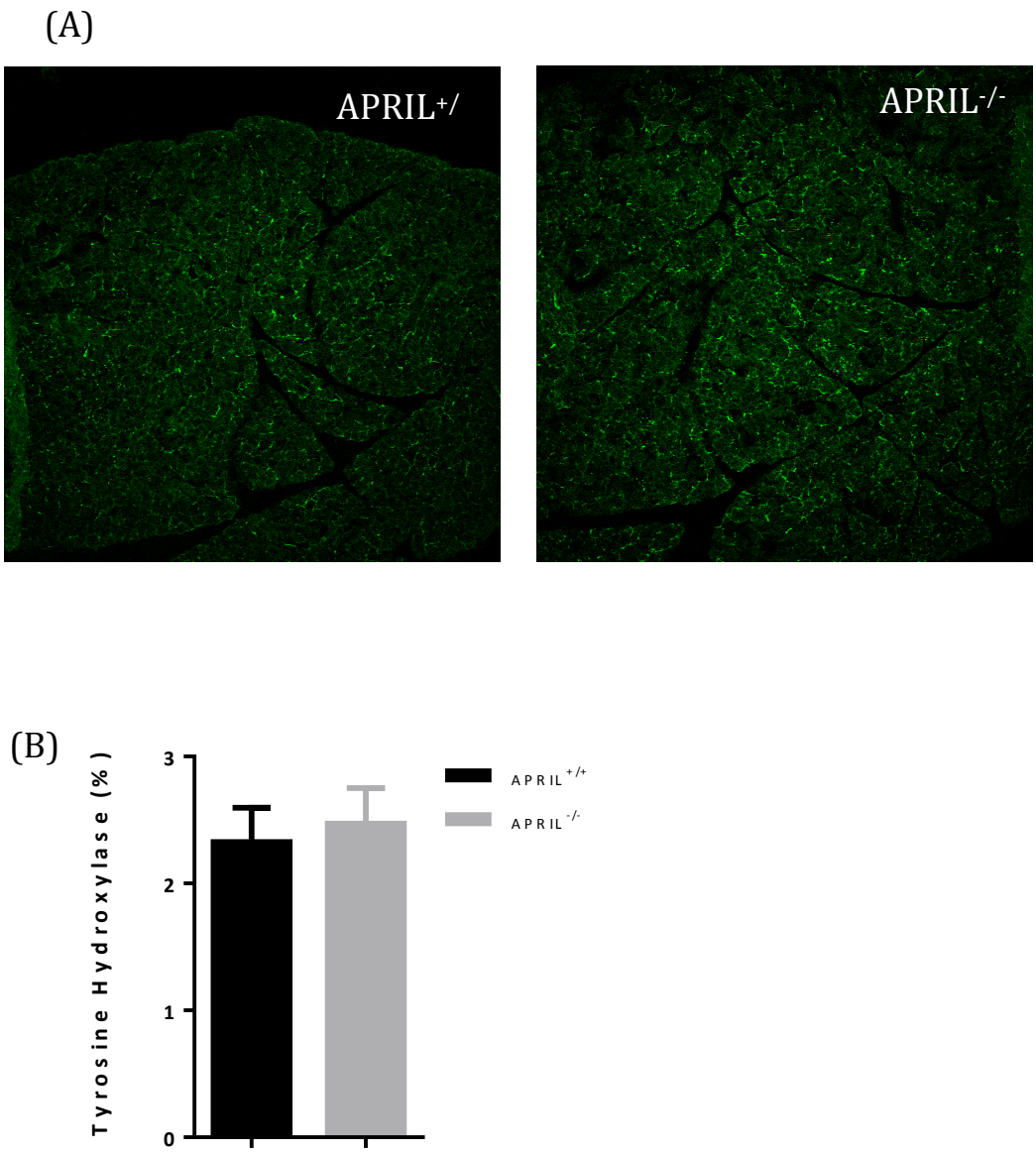
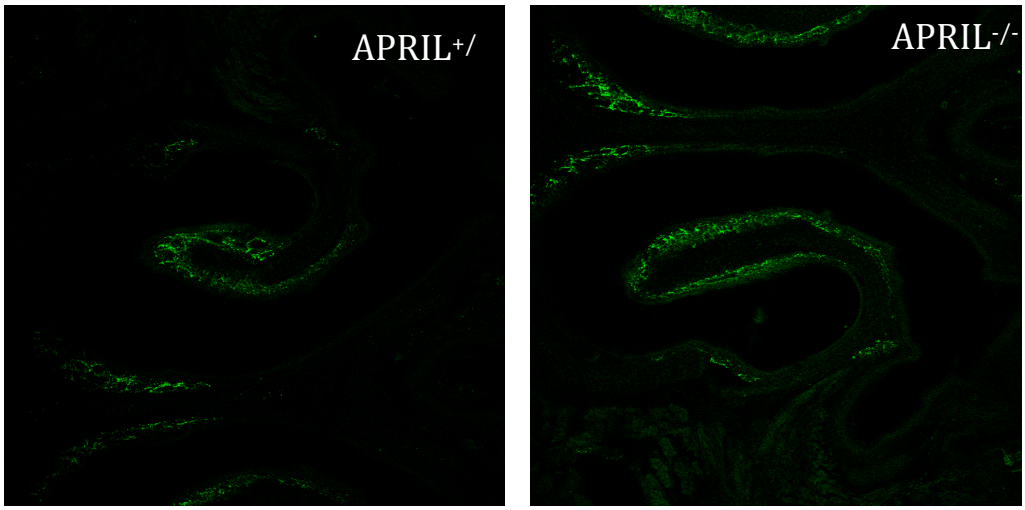


Figure 49: APRIL$^{-/-}$ mice display no increased submandibular gland target field innervation at P10

(A-C) Sympathetic innervation density in P10 SMG of APRIL$^{+/+}$ and APRIL$^{-/-}$. P10 SMG were dissected and fixed in 4% PFA before cryoprotection with 30% sucrose. Following sectioning at 16µm the SMG were stained for tyrosine hydroxylase. Images were acquired using a Zeiss laser scanning microscope. (A) APRIL$^{+/+}$ representative image (B) APRIL$^{-/-}$ representative image (C) tyrosine hydroxylase percentage of defined SMG area. The data shown represent the mean ± s.e.m of data compiled from four animals per genotype (statistical comparison between genotypes, unpaired t-test with Welch's correction).

(A)



(B)

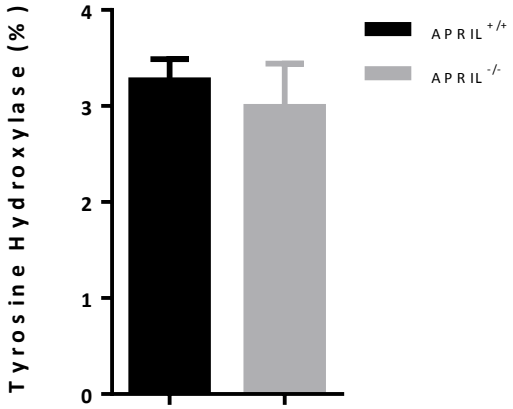


Figure 50: APRIL$^{-/-}$ mice display no increased nasal mucosa target field innervation at P10

Sympathetic innervation density in P10 NM of APRIL$^{+/+}$ and APRIL$^{-/-}$. P10 NM were dissected and fixed in 4% PFA before cryoprotection with 30% sucrose. Following sectioning at 16µm the NM were stained for tyrosine hydroxylase. Images were acquired using a Zeiss laser scanning microscope. (A) APRIL$^{+/+}$ representative image (B) APRIL$^{-/-}$ representative image (C) tyrosine hydroxylase percentage of defined SMG area. The data shown represent the mean ± s.e.m of data compiled from four animals per genotype (statistical comparison between genotypes, unpaired t-test with Welch's correction).

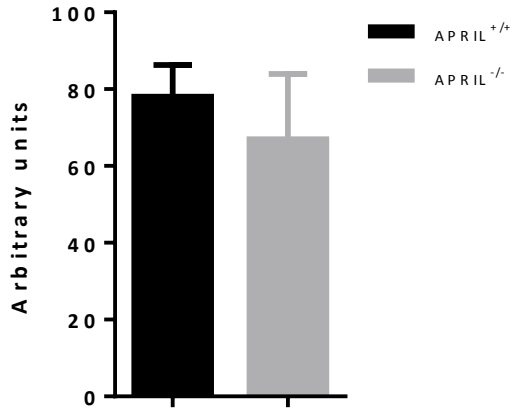
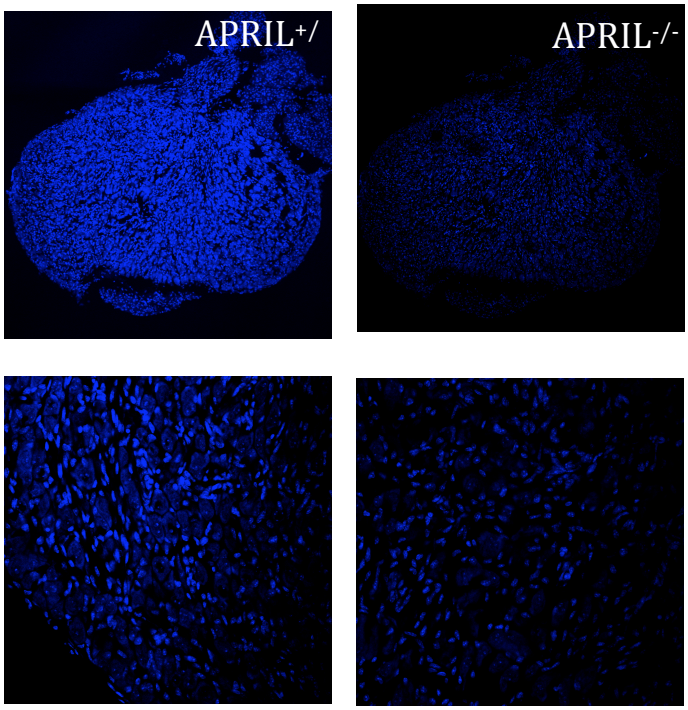


Figure 51: Relative expression of Tyrosine hydroxylase in P10 SCG ganglia Expression profile for tyrosine hydroxylase from P10 SCG ganglia of APRIL$^{+/+}$ and APRIL$^{-/-}$ mice. tyrosine hydroxylase expression relative to GAPDH and SDHA in p5 SCG. The data shown represent the mean ± s.e.m of data compiled from four pairs of ganglia per genotype (statistical comparison with control, unpaired t-test with Welch's correction).

(A)



(B)

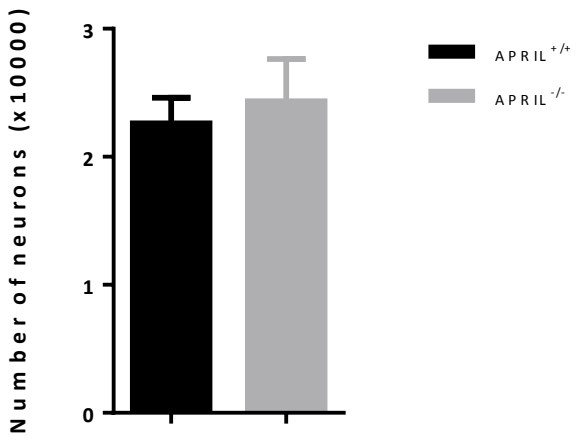
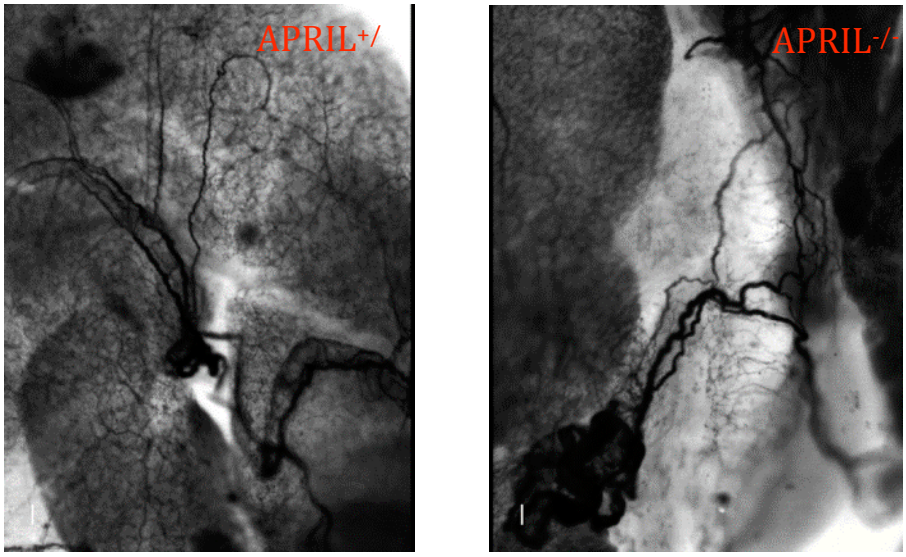


Figure 52: Survival of SCG neurons is not affected in APRIL$^{-/-}$ mice *in vivo*

(A-E) SCG cell population in P10 ganglia. P10 SCG were dissected and fixed in 4% PFA before cryoprotection with 30% sucrose. Following sectioning at 8µm the gamglia were stained for TOTO-3 iodide. Images were acquired using a Zeiss laser scanning microscope. The data shown represent the mean ± s.e.m of data compiled from four animals per genotype (statistical comparison between genotypes, unpaired t-test with Welch's correction).

(A)



(B)

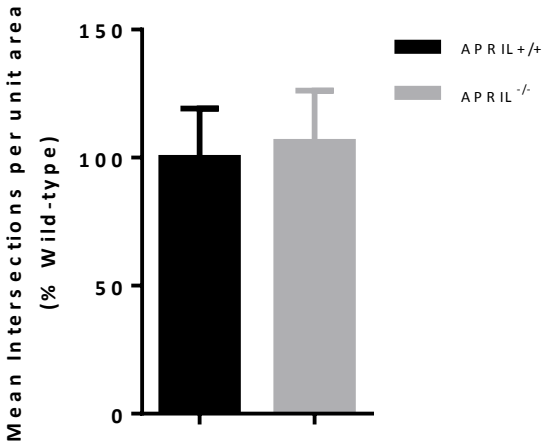


Figure 53: Whole-mount analysis of submandibular gland of *April*-null mice at P10

Whole-mount tyrosine hydroxylase (TH) immunostaining of the submandibular gland. Sympathetic target field innervation is not affected at P10 in mice lacking APRIL. Representative images whole-mount preparations of the submandibular gland hilus of P10 *April*$^{+/+}$ and *April*$^{-/-}$, mice labelled with anti-TH are shown (n=4 animals per genotype). Statistical comparison between genotypes was unequal variance t test with Welch's correction. Scale bar = 100 μm.

APRIL$^{-/-}$ does not alter the Expression of NGF, TrkA and p75 IN P5 SCG or SMG

APRIL null P0 SCG neurons display enhanced NGF promoted outgrowth while the nasal mucosa and submandibular glands of these same mice display greater levels of innervating fibres at P5. A set of P5 SCG ganglia and P5 SMG were collected from APRIL$^{+/+}$ and APRIL$^{-/-}$ littermates to compare the expression of NGF, TrkA and p75 in both sympathetic neurons and their target field and thus identified if enhanced NGF promoted growth results from a modification of NGF signalling.

P5 SCG and SMG were collected and stored in an RNA stabilisation solution before freezing at -80^{0}C for long term storage. Following RNA extraction and reverse transcription, real time PCR was carried out using Taqman probes and primers specific for NGF, TrkA and p75.

Transcripts for NGF, TrkA and p75 were detected in both the SCG and SMG. In both the ganglia and target field tested expression transcripts of the neurotrophin and receptors remained constant between genotypes. No differences in the regulation of NGF, TrkA or p75 expression were therefore apparent in APRIL knockout mice (Fig. 54). While the mRNA levels in question remain constant more needs to be done to investigate protein levels in the target fields.

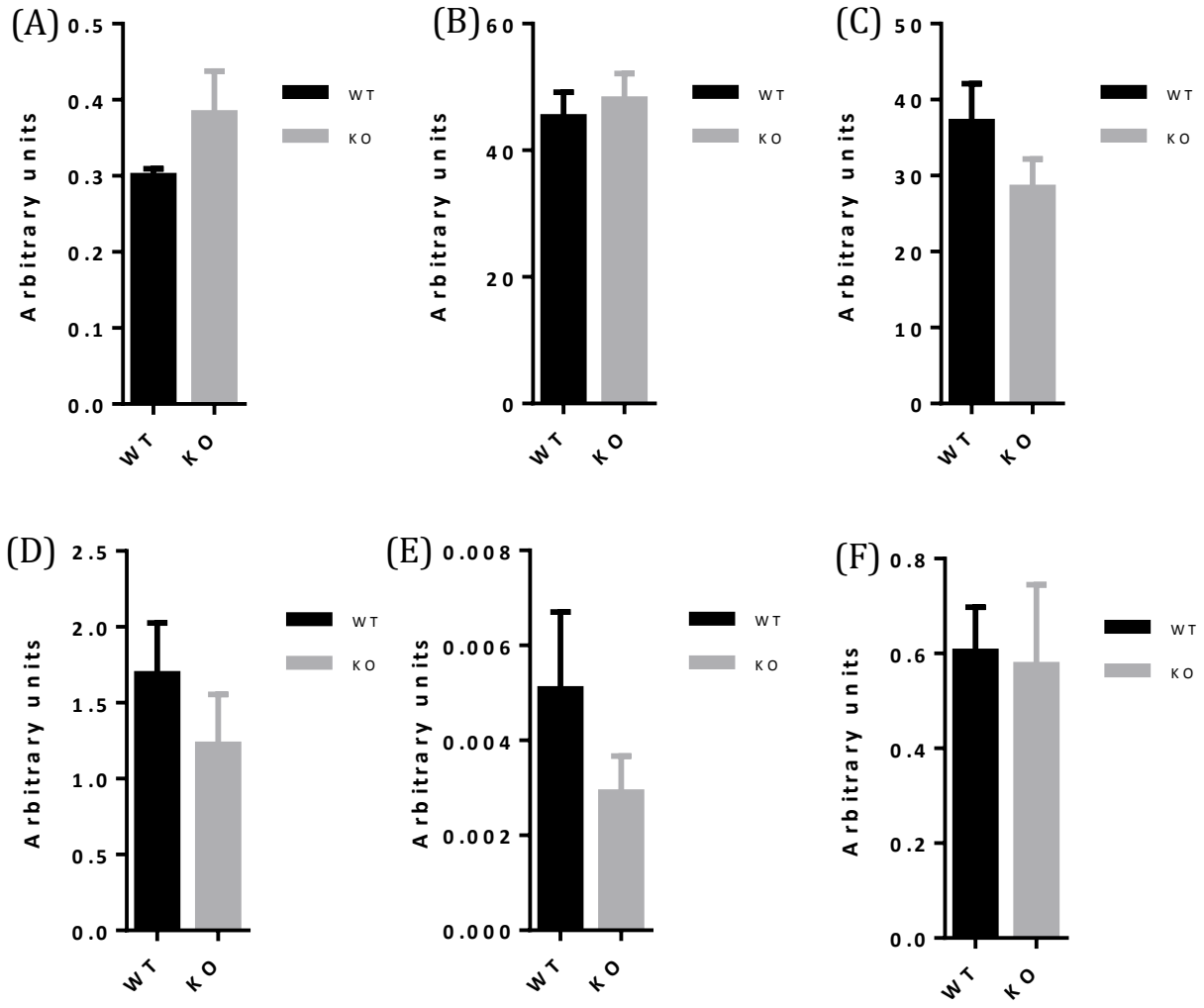


Figure 54: Relative expression of NGF, TrkA and p75 in P5 SCG ganglia and SMG is not effected in APRIL$^{-/-}$ mice (A-F) Expression profiles for NGF, TrkA and p75 from P5 SCG ganglia and SMG of APRIL$^{+/+}$ and APRIL$^{-/-}$ mice. (A) NGF (B) TrkA (C) p75 expression relative to housekeepers in p5 SCG. (D) NGF (F) TrkA (G) p75 expression relative to GAPDH and SDHA in p5 SMG. The data shown represent the mean ± s.e.m of data compiled from four pairs of ganglia per genotype (statistical comparison with control, unpaired t-test with Welch's correction).

Discussion

The role of APRIL signalling in the developing sympathetic nervous system deepens with the novel data presented within this chapter. The aim of this chapter was to use a mouse transgenic model lacking APRIL to assess if the *in vitro* findings have a role in the development of the sympathetic nervous system *in vivo.*

Neurons taken from mice lacking APRIL display a marked increase in NGF promoted outgrowth in culture. This confirms two key points taken from the *in vitro* observations. Firstly the effect of APRIL signalling is indeed to act upon NGF promoted outgrowth and secondly, APRIL signalling appears to be acting to reduce the size of neurons as its abolition here results in growth enhancement.

Again, in agreement with the *in vitro* observations from the last chapter the abolition of APRIL expression plays no role in survival of neurons at P0. These survive in culture at normal levels in the presence of NGF and survival, in the absence of NGF is poor.

Moving on from culture methods the level of sympathetic target field innervation in mice lacking APRIL was investigated to determine if the increased size of neurons in culture translates to an increase in the number of fibres reaching these targets. Interestingly, mice lacking APRIL show a significant increase in target field innervation by sympathetic fibres in both the submandibular gland and nasal mucosa at P5. Later on in development at P10 this increase is no longer detectable either by tyrosine hydroxylase quantification in

frozen sections of target tissue or by wholemount staining. It would appear that a loss of APRIL signalling manifests as a transient increase in sympathetic target field innervation that is not significant come the final stages of the process.

The SCG ganglia are fully formed with a correct cell population by P10 coupled with normal target field innervation by this ganglia. This raises several fascinating possibilities. Firstly, although APRIL signalling has been shown to matter little in terms of neuronal survival when investigated, the cell population of ganglia will have to be quantified earlier in development including at P5. This will confirm whether the increase in target field innervation seen at this age is indeed due to an increase in the number of innervating fibres as opposed to an increased neuronal population in the SCG. Secondly target field innervation quantification by both sectioning and wholemount staining will have to be expanded out from P5 both to younger ages and older ages preceding P10. This will establish over what period the transient increase can be observed.

It will be of utmost importance to investigate whether the transient increase in target field innervation in APRIL null mice at P5 is due to an increase in the number of fibres reaching the target or if it is the rate at which fibres reach the target that is affected. Also the possibility that increased numbers of fibres are lost due to competition for limited supplies of target derived NGF must be explored. Real time PCR analysis shows stable NGF levels in the targets of APRIL null mice while TrkA and p75 are stably expressed by the neurons themselves.

Finally it would be interesting to explore the possibility that NGF regulates APRIL expression in SCG neurons. It would be fascinating to discover if NGF acts to increase expression of APRIL which in turn negatively regulates its own growth promoting effects.

Concluding remarks

The data presented in this thesis provide the first example of a functional role of the TNF superfamily member APRIL and its receptors in the development of the nervous system.

These TNF members are expressed by neurons of the SCG in a developmental window associated with target field innervation. APRIL null mice display a transient hyperinnervation phenotype shortly after birth while blockade of APRIL or its receptors in culture leads to an enhancement of NGF promoted growth.

In opposition to this, the application of recombinant APRIL also enhances neurite growth. Two possible theories could explain this apparent contradiction. Firstly, if the recombinant ligand displays receptor binding properties but is unable to activate the receptor as with endogenous APRIL the factor would merely act to block endogenous signalling as with the other function blocking antibodies used in this study. Secondly, if reverse signalling through membrane bound APRIL is the paradigm of endogenous activity then the application of recombinant APRIL would again block this activity much like a soluble decoy factor.

In both of these theories endogenous APRIL signalling acts to inhibit NGF promoted neurite growth and blockade with a variety of factors or the transgenic model abolishes this inhibition and leads to increased neurite growth and target field innervation.

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