Bone Morphogenetic Protein 7 Regulates Sensory Neurogenesis

by

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Abstract

Background: The trigeminal ganglion (TG) is a cluster of neuron bodies in the cranium that supplies the face and skull with sensory innervation. In the mouse, neurons of the TG supply the whisker pad that is important for spatial navigation and intraspecific communication. Specialized cells in the TG can detect tactile stimuli, positional stimuli, and pain. The origin of these neurons is thought to be the neural crest and the neuroectoderm. Previous studies have established that early neurogenesis requires the activation of neurogenin 1 and 2 (Ngn1/2), early growth response protein 2 (Egr2) and brain-specific homeobox protein 3a (Brn3a) to specify neural crest cells to sensory neurons. Subsequently, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3/NT4), produced by target tissues, are required for nerve subtype specification, target innervation, and survival. Recently, bone morphogenetic proteins (Bmps) have been implicated to participate in these processes by retrograde signaling. Bmps are secreted peptides that are important for cell survival, differentiation, and homeostasis. Currently, it is not known which or how Bmps regulate sensory nerve development. Here we demonstrate an altered sensory phenotype in mice carrying a complete and neural crest-specific (Wnt1-cre) deletion of Bmp7.

Methods: Embryos were harvested from mice carrying either a complete deletion ($Bmp7^{\Delta/\Delta}$) or a conditional deletion of Bmp7 in the neural crest ($Bmp7^{fl/fl}$ Wnt1-cre) and characterized using histological, immunohistochemical, and gene expression analysis (qPCR) of various sensory neuronal receptors, peptides, and transcription factors (TrkA, TrpV1, CGRP, Runx1, Ret for nociceptive neurons; TrkB, TrkC, Runx3, Ret for mechano-and proprioceptive neurons). The whisker pads of adult mice carrying a neural crest-specific deletion of Bmp7 were tested with a 15 psi air puff current to determine whether molecular differences result in physiological changes.

Results: The trigeminal ganglion in embryos carrying a full and conditional deletion of Bmp7 is morphologically altered, appearing to be smaller and slightly misshapen in comparison to wild-type littermates. In addition, qPCR analysis shows mRNA differences in some receptors, pain associated peptides, and transcription factors (TrkA, Trpv1, Ret, CGRP, Runx1), indicative of an

increased nociceptor phenotype or a delay in neuronal development. At 15psi air pressures, conditional knockout mice were less mechanosensitive and more pain sensitive.

Conclusion: Bmp7 is important for peripheral nerve development. Gene expression analysis reveals that sensory neurons of mutants may have an increased nociceptor phenotype or may be delayed in development. Immunohistochemical analyses established that the TGs are smaller and misshapen in mutants. Air puff assays indicate that Wnt1-cre mutants have an inhibited mechano-response, but a hyperalgesic response. Future experiments will elucidate the mechanisms by which these changes occur.

Preface

This thesis is an original work by Peter Sabiri. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Genetic Analysis of Tissue Patterning during Orofacial and Tooth Formation", No. AUP00001149, July 16, 2014

A Psalm of David:

[13] For You formed my inward parts;
You knitted me together in my mother's womb.
[14] I praise You, for I am fearfully and wonderfully made.
Wonderful are Your works;
my soul knows it very well.
[15] My frame was not hidden from You,
when I was being made in secret,
intricately woven in the depths of the earth.
[16] Your eyes saw my unformed substance;
in Your book were written, every one of them,
the days that were formed for me,
when as yet there was none of them.

Psalm 139: 13-16, The Holy Bible, English Standard Version

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Table of Contents

List of Tables and Figures viii

Abbreviations x

Chapter I: Introduction 1

Chapter 1.1: Anatomy of the Trigeminal Ganglion 2

Chapter 1.2: Origin and Migration of the Neural Crest 3

Chapter 1.3: Neural Crest Cell Differentiation to Sensory Neurons 6

Chapter 1.4: Neural Crest Cells Differentiate to Schwann Cell Precursors (SCPs) and Their Derivatives 9

Chapter 1.5: Neurotrophin Signaling 10

Chapter 1.6: Evidence for Retrograde Signaling of Neurotrophins 11

Chapter 1.7: Neurotrophins Regulate Sensory Nerve Development 12

Chapter 1.8: BMP signaling 14

Chapter 1.9: Bmps as Regulators of Nerve Development 16

Chapter 2: Aims and Hypothesis 18

Chapter 3: Materials and Methods 20

Chapter 4: Results 25

Chapter 5: Discussion 57

Conclusion 68

Chapter 6: Future Directions 69

Bibliography 72

List of Tables and Figures

Chapter 1 Table:

Table 1.1: Summary of sensory nerve subtypes and the receptors, peptides, and transcription factors that they express……………………………………………………………………………8

Chapter 3 Tables:

Table 3.1: Sequences of Forward and Reverse Primers used for qPCR…………………………22

Table 3.2: Air puff Assay Scoring Rubric……………………………………………………………..24

Chapter 1 Figures:

Figure 1.1: Induction of the neural crest……………………………………………………………..5

Figure 1.2: Summary of nerve subtype specification…………………………………………………9

Figure 1.3: Summary of neurotrophin signaling cascade……………………………………….....11

Figure 1.4: Summary of Bmp signaling cascade……………………………………………………15

Chapter 4 Figures:

Figure 4.1: Paraffinograf showing serial sagittal sections of E14.5 Bmp7 deficient and wild-type embryos……………………………………………………………………………………………..26

Figure 4.2: Bmp7$^{wt/lacZ}$ embryos at various developmental stages showing Bmp7 expression in the meninges…………………………………………………………………………………………27

Figure 4.3: E14.5 Bmp7$^{wt/lacZ}$ embryos showing Bmp7 expression in the epithelium and vibrissae follicles……………………………………………………………………………………………...28

Figure 4.4: Gene expression profile of isolated E14.5 Bmp7+/+ and Bmp7-/- trigeminal Ganglia…………………………………………………………………………...…………29

Figure 4.5: E12.5 trigeminal ganglia showing TrkA expression……………………………………31

Figure 4.6: E12.5 trigeminal ganglia showing TrkC expression……………………………………32

Figure 4.7: E12.5 trigeminal ganglia showing p75ntr expression………………………………….33

Figure 4.8: E13.5 trigeminal ganglia showing TrkA expression……………………………………34

Figure 4.9: E13.5 trigeminal ganglia showing TrkC expression……………………………………35

Figure 4.10: E13.5 trigeminal ganglia showing p75ntr expression………………………………...36

Figure 4.11: E14.5 trigeminal ganglia showing TrkA expression…………………………………..37

Figure 4.12: E14.5 trigeminal ganglia showing TrkB expression…………………………………..38

Figure 4.13: E14.5 trigeminal ganglia showing p75ntr expression………………………………...39

Figure 4.14: E15.5 trigeminal ganglia showing TrkA expression…………………………………..40

Figure 4.15: E15.5 trigeminal ganglia showing TrkC expression…………………………………..41

Figure 4.16: E15.5 trigeminal ganglia showing p75ntr expression………………………………...42

Figure 4.17: E12.5 trigeminal ganglia showing pSmad expression………………………………..44

Figure 4.18: E13.5 trigeminal ganglia showing pSmad expression………………………………..45

Figure 4.19: E15.5 trigeminal ganglia showing CCas3 expression………………………………..47

Figure 4.20: Gene expression profile of isolated E14.5 $Bmp7^{fl/fl}$ and $Bmp7^{fl/fl}$ Wnt1-cre trigeminal ganglia……………………………………………………………………48

Figure 4.21: E15.5 trigeminal ganglia showing TrkA expression…………………………………..50

Figure 4.22: E15.5 trigeminal ganglia showing TrkB expression…………………………………..51

Figure 4.23: E15.5 trigeminal ganglia showing TrkC expression…………………………………..52

Figure 4.24: E15.5 trigeminal ganglia showing p75ntr expression………………………………...53

Figure 4.25: Air puff assay on wild-type and conditional knockout mice……………………..…..55

Figure 4.26: Wnt1-cre mice demonstrate a bimodal response to an air puff stimulus……..…...56

Chapter 5 Figures:

Figure 5.1: Organization of mechano- and nociceptive neurons in the skin…………………….…..60

Figure 5.2: The circuit in the spinal nucleus of the TG……………………………………….……..64

Figure 5.3: Neuron activity in response to the air puff stimulus in wild-type mice…………..……65

Figure 5.4: Neuron activity in response to the air puff stimulus in neural crest conditional knockout mice………………………………..…………………………………………………..…..66

Abbreviations

AM-A-fiber mechanoreceptors

BDNF-Brain derived neurotrophic factor

bHLH-Basic helix-loop-helix

Bmp-Bone morphogenetic protein

Brn3a-Brain-specific homeobox protein 3a

Cash1-Chicken achaete-scute homolog-1

CCas3-Cleaved caspase 3

Cdc42-Cell division control protein 42

CGRP-Calcitonin gene related peptide

cKO-Conditional Knockout

CNC-Cranial neural crest

CSF-Cerebrospinal fluid

DAPI-4',6-diamidino-2-phenylindole

DREZ-Dorsal root entry zone

DRG-Dorsal root ganglia

ECM-Extracellular matrix

EMT-Epithelial to mesenchymal transition

Fgf-Fibroblast growth factor

FoxD3-Forkhead box D3

GI-Gastrointestinal

HER3/ErB3-Human epidermal growth factor receptor 3

HGFR/c-Met-Hepatocyte growth factor receptor

HMG-High mobility group

Hmx1-Homeobox protein H6

5-HT- 5-hydroxytryptamine / Serotonin

IB4-Isolection B4

Krox20/Egr2-Early growth response protein 2

Lef1-Lymphoid enhancer binding factor 1

LTMRs-Low threshold mechanoreceptors

MAPK1/ERK1- Mitogen activated protein kinase/Extracellular signal regulated kinase 1

MAPK2/ERK2- Mitogen activated protein kinase/Extracellular signal regulated kinase 2

Mash1/Arcl-1-Mammalian achaete scute homolog-1

MMPs-Matrix metalloproteinases

Mrgpr-Mas-related G-protein coupled receptors

NCC-Neural crest cell

NF200/NFH-Neurofilament 200

NGF-Nerve growth factor

Ngn1-Neurogenin 1

Ngn2-Neurogenin 2

Nrg1-Neuregulin 1

NSE promoter-Neuron specific enolase promoter

NT3-Neurotrophin 3

NT4-Neurotrophin 4

OC1-Onecut1

OC2-Onecut2

PFA-Paraformaldehyde

Phox2a-Paired mesoderm homeobox protein 2A

Phox2b-Paired mesoderm homeobox protein 2B

PI3K-Phosphatidylinositol 3 kinase

PLC-Phospholipase C

$p75^{ntr}$-p75 neurotrophin receptor

Psi-Pounds per square inch

Ret-Rearranged during transformation

RGM-Repulsive guidance molecule

Runx1-Runt-related transcription factor 1

Runx3-Runt-related transcription factor 3

SCP-Schwann cell precursor

Shox2-Short stature homeobox 2

Smad-Mothers against decapentaplegic

Sox10- SRY related HMG box 10

SRY- Sex determining region Y

SubP-Substance P

SVZ-Subventricular zone

Tbx3-T box protein 3

TG-Trigeminal ganglion

TGF-β-Transforming growth factor beta

TH-Tyrosine hydroxylase

TIMPS-Tissue inhibitors of matrix metalloproteinases

TrkA-Tropomyosin receptor kinase A

TrkB-Tropomyosin receptor kinase B

TrkC-Tropomyosin receptor kinase C

Troma1-Cytokeratin 8/18

VPM- Ventral posteromedial

V1-Opthalmic branch of the trigeminal ganglion

V2-Maxillary branch of the trigeminal ganglion

V3-Mandibular branch of the trigeminal ganglion

Wnt-Wingless related integration site

Chapter I: Introduction

Chapter 1.1: Anatomy of the Trigeminal Ganglion

The trigeminal ganglion (TG), also known as cranial nerve V, is a cluster of neurons in the somatic sensory nervous system that gives rise to the major sensory nerves of the head. Analogous to the dorsal root ganglia (DRGs) of the body, the trigeminal ganglion transmits afferent information from the head and face to the somatosensory cortex enabling mammals to sense head and muscle position, mechanical stimuli, pain, and changes in temperature. It is a vital sensor that allows vertebrates to interact with their environment and it coordinates, along with motor neurons, the mastication cycle. The TG consists of three main branches: the ophthalmic division (V1), the maxillary division (V2) and the mandibular division (V3). The ophthalmic branch exits the cranial vault via the superior orbital fissure and innervates the nasal cavity, the forehead, the eyelid and the cornea. The maxillary branch exits via foramen rotundum and innervates the maxillary teeth, the nose, the palate, and the cheeks. Importantly, in rodents, it also innervates the vibrissae which are important for spatial perception and communication. Last, the mandibular branch, which exits through foramen ovale, innervates the lower gums, teeth, and tongue. Unlike the other divisions, the mandibular branch also supplies some muscles with motor fibers (the muscles of mastication (the masseter, the lateral and medial pterygoids, and the temporalis) and the muscles of the first branchial arch (tensor tympani, tensor veli palatini, and anterior digastric muscles)). However, the somas of these motor neurons are in the trigeminal motor nucleus located in the midbrain.

The anatomical location of the trigeminal ganglion is particularly interesting. Unlike the DRGs, it is encased in a cerebrospinal fluid-containing subarachnoidal pouch termed Meckel's cave (or the trigeminal cave) that sits on the petrous part of the temporal bone and the greater wing of the sphenoid bone inside the cranial vault. Just like the brain and spinal cord, it too receives nutrients and other factors from the cerebrospinal fluid, adding another layer of complexity to its development and function. The blood supply to the TG comes from the internal carotid artery and is drained by the cavernous venous plexus which contains V1 and V2.

The major cell types of the TG are first order pseudo-unipolar sensory neurons and

Schwann cells, and these can interact with surrounding fibroblasts and meningeal cells. The neurons of the TG are thought to originate from both neuroectoderm and neural crest, while Schwann cells are thought to originate strictly from neural crest. However, this is still debated and genetic lineage tracing experiments are needed to verify these findings.

The first order sensory neurons of the TG synapse onto second order neurons located in one of three sensory nuclei in the midbrain, pons, and medulla oblongata, while first order motor neurons have their somas in the motor nucleus located ventromedial to the principal sensory nucleus. The names of the sensory nuclei from superior to inferior starting from the midbrain are the following: the mesencephalic nucleus, the principal sensory nucleus, and the spinal nucleus. The mesencephalic nucleus contains large diameter proprioceptive and mechanosensory neurons that coordinate the mastication cycle and the jaw-jerk reflex with neurons of the motor nucleus. Neural crest transplant experiments from embryonic mesencephalon between quail donors and duck hosts' fore-, mid-, and hindbrain grafts support the hypothesis that the mesencephalic nucleus is neural crest cell derived[1]. The other nuclei also receive sensory inputs both from large and small diameter neurons and, like the mesencephalic nucleus, they project inputs to various parts of the thalamus and cerebellum. However, unlike the mesencephalic nucleus, the other nuclei are derived from neuroectoderm. For further reading, refer to *The Mouse Nervous System* by Charles Watson, an excellent book about mouse neuroanatomy[2].

Chapter 1.2: Origin and Migration of the Neural Crest

The vertebrate head consists of highly diversified and evolutionary conserved structures that fit together like pieces of a puzzle to house all the special sensory organs and to protect the brain. The anterior aspect of the head, including most of the tissues of the orofacial region, the base of the skull, and parts of the trigeminal ganglion are cranial neural crest (CNC) cell derived. CNC cells are multipotent stem cells that arise from the ectoderm of the dorsal neural tube and play a fundamental role in craniofacial patterning and development. Shortly after their induction from the neural tube by bone morphogenetic proteins (Bmps)[3,4], wingless related integration sites

(Wnts)[5], and fibroblast growth factors (Fgfs)[6,7], CNC cells undergo an epithelial to mesenchymal transition (EMT). EMT consists of changes in cell adhesion properties, cytoskeletal rearrangements, and loss of cell-cell contacts. CNC cells then break through the extracellular matrix and migrate along discrete pathways to their final destinations.

The induction of CNC cells begins at the neural plate border shortly after gastrulation, when Bmps, Wnts, and Fgfs activate neural plate border specifier genes. Although these three signaling pathways are common in all organisms studied, the source of the inducing Bmps, Wnts, and Fgfs varies between species. In *Xenopus laevis*, both Wnts and Fgfs originate from intermediate mesoderm, as do Bmp antagonists chordin and noggin, while Bmps are already expressed in the neural plate border (which will later become the neural folds)[3]. During induction, chordin[3], noggin[8], and other Bmp antagonists maintain intermediate levels of Bmps in the overlaying neural plate border, while Wnt[3] and Fgf[7] activity increases, leading to CNC induction. Later, Bmp antagonists are down-regulated while Wnt and Fgf signaling continues, leading to CNC cell maintenance and further specification[3]. Hence, intermediate levels of Bmps and high levels of Wnts and Fgfs are required for CNC induction, while high levels of Bmps and high levels of Wnts and Fgfs are required for CNC cell maintenance. Below is a figure showing the induction of the neural crest.

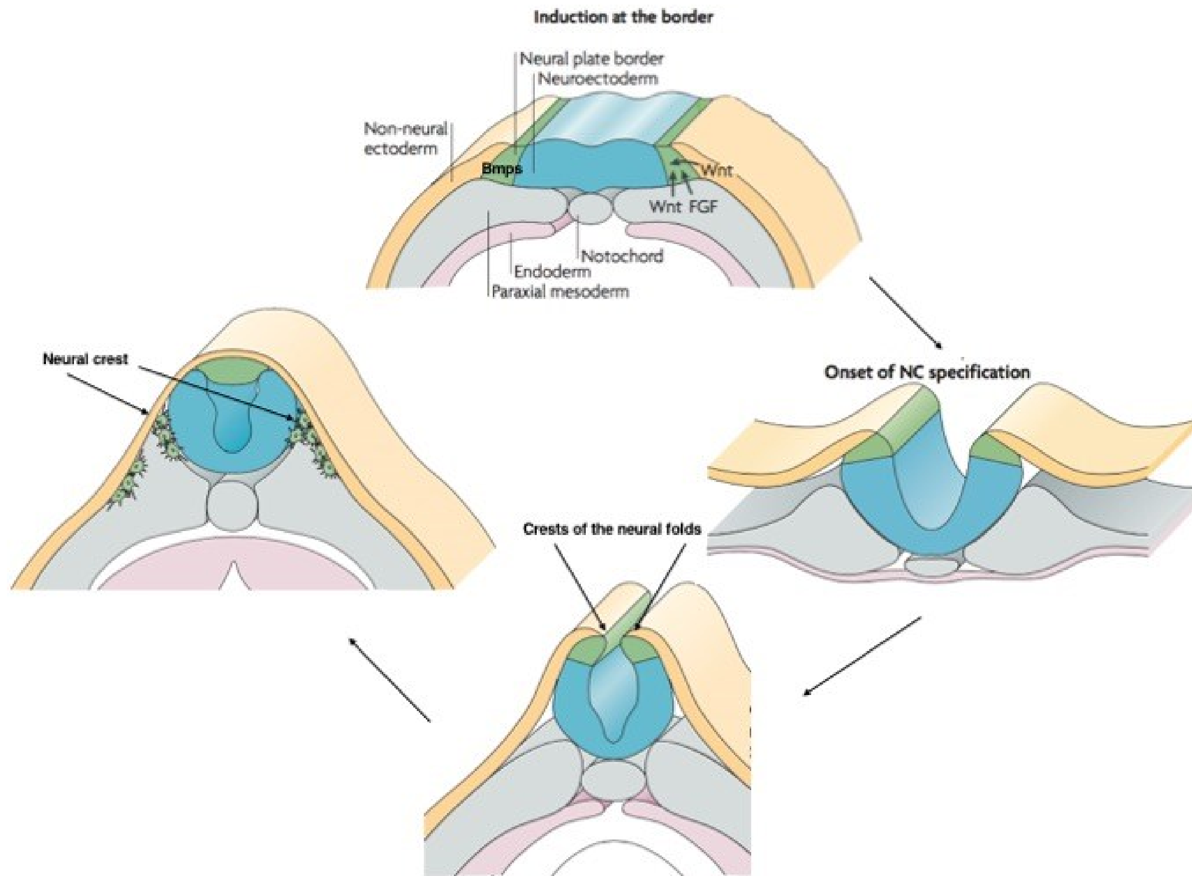

Figure 1.1[9]: Induction of the neural crest, modified from Sauka-Spengler, T., & Bronner-Fraser, M. (2008)

Once induction takes place, several transcription factors, termed neural crest specifiers, are upregulated. Neural crest specifiers such SRY-related HMG box 10 (Sox10)[10], Snail2[11], and forkhead box D3 (FoxD3)[12] will give neural crest cells the ability to delaminate from the neural folds and begin an epithelial to mesenchymal transition (EMT).

EMT is characterized by changes to cell adhesion molecules, cytoskeletal rearrangements, and loss and establishment of new cell-cell contacts. Ultimately, cells lose their apical-basal cell polarity, change from a columnar to a star shaped morphology with filopodia, and become mobile and invasive. Changes to cell adhesion are characterized by downregulation of E-cadherin and N-cadherin (type I cadherins) mediated by Snail transcription factors[13] leading to the loss of

epithelial cell polarity, followed by upregulation of cadherin-7[14] (type II cadherins) and gain of mobility[15]. Cytoskeletal changes affect actin filaments, intermediate filaments, and microtubules. Cells undergoing EMT downregulate cytokeratins in favor of another intermediate filament, vimentin[16,17]. Additionally, integrins that link the extracellular matrix (ECM) to the cell's actin filaments and are necessary for migration[18] are also modified. Upon acquiring these and other changes, neural crest cells gain migratory ability and delaminate from the neural tube.

Following delamination, guidance cues such as sema3F[19] and ephrin ligands[20] direct CNC cells along organized paths in the frontal eminence, maxillary processes and mandibular arch to reach their cranial targets. To move to their targets, CNC cells need to remodel the ECM with an appropriate balance of matrix metalloproteinases (MMPs)[21] and tissue inhibitors of matrix metalloproteinases (TIMPS). Once CNC cells reach their destination, they differentiate to form cartilage, bone, teeth, nerves, glia, and melanocytes.

Chapter 1.3: Neural Crest Cell Differentiation to Sensory Neurons

Sensory neurogenesis of some cranial and all dorsal root ganglia in the mouse begins shortly after neural crest cells (NCCs) begin migrating from the neural tube between E8.5 and E10[22]. Sensory neurons of the DRGs and the TGs formed from three consecutive waves of differentiating neural crest cells can be distinguished by the transcription factors they express.

In the first wave of neurogenesis, E9.5 Sox10+ *migrating NCCs* begin expressing the basic helix-loop-helix (bHLH) transcription factor neurogenin 2 (Ngn2)[23] and become committed to a sensory neuronal fate. Once these neural precursors reach the future position of the DRG, they begin expressing the POU-homeodomain transcription factor brain-specific homeobox protein 3a (Brn3a)[24] and downregulate Sox10. Next, at E10.5-11.5, neuroblasts of the first wave express runt-related transcription factor 3 (Runx3), and by E12.5 its expression co-localizes with neurotrophin tropomyosin receptor kinase C (TrkC)[25], forming large proprioceptive pioneering neurons (Runx3+/TrkC+). By the time the mouse reaches E14.5, the large diameter Runx3+/TrkC+ neurons will give rise to two distinct populations. The first will continue to express

Runx3+/TrkC+ and will remain large proprioceptors, while the second will downregulate Runx3 and express neurotrophin tropomyosin receptor kinase B (TrkB) and become mechanoreceptors[23]. Hence, Runx3+/TrkC+ neurons will give rise to Runx3+/TrkC+ Aα-proprioceptors (Ia muscle spindle, and Ib Golgi tendon afferents)[25] and TrkB+/TrkC+, TrkB+, TrkB+/Ret+, Ret+[26] Aβ-low-threshold mechanoreceptors (LTMRs). Aα-fibers will be myelinated and will have large axon fiber diameters and very high action potential conduction velocities. Aβ-fibers will also be myelinated but will have medium axon fiber diameters and medium action potential conduction velocities.

In the second wave of sensory nerve formation, NCCs expressing Sox10 during migration will have a high rate of proliferation and *once they reach the DRG*, will express neurogenin 1 (Ngn1) and Brn3a[27]. However, unlike the Ngn2 wave of neurogenesis, by E14.5, these neuroblasts will give rise to runt-related transcription factor 1 and the neurotrophin tropomyosin receptor kinase A (Runx1+/TrkA+)[28] small diameter neurons as well as Runx3+/TrkC+ proprioceptors[23]. The Runx1+/TrkA+ neural precursors will give rise to two populations of nociceptive/thermal neurons postnatally. The one will be peptidergic, while the other non-peptidergic. The peptidergic population will be Runx1-/TrkA+ and Ret+ or Ret- and will express calcitonin gene-related peptide (CGRP), substance P (SubP) or other peptides. This population will develop to peptidergic C-fibers which are unmyelinated, have small axon diameters, slow action potentials and express peripherin. The second population that will arise will be Runx1+/TrkA+/Ret+, and these neurons will be non-peptidergic but will express glutamate as their neurotransmitter. This population will consist of Aδ-fibers and C-fibers. The Aδ-fibers will be sparsely myelinated, have small axon diameters, have moderate action potential velocities, and will express both neurofilament 200 (NF200/NFH) and peripherin. The non-peptidergic C-fibers in this group will be unmyelinated, small diameter neurons with slow action potential velocities and will be isolectin B4 positive[29] (IB4+) and peripherin positive. Aδ-fibers are associated with acute sharp pain and respond to painful mechanical or temperature stimuli. There are two well-known types, A-fiber mechanoreceptors (AM; free nerve endings) and D-hair afferents which are like LTMRs. C-fibers are associated with a dull chronic inflammatory pain and respond to painful mechanical, temperature, and chemical stimuli. In summary, Aδ-fibers express NF200, and

peripherin, along with C-fiber associated receptors, peptides, and transcription factors. C-fibers do not express NF200, but do express peripherin, and can be either glutamatergic (IB4+) or peptidergic (CGRP+).

Finally, the last wave of neurogenesis arises from boundary cap cells, derivatives of NCCs that are transiently present at the entry point of sensory roots, just outside the neural tube in an area called the dorsal root entry zone (DREZ)[30]. These neural precursors express Sox10 and the zinc finger transcription factor Krox20 (Egr2), and will follow the same developmental cascade as Ngn1+ second wave neuroblasts, but will only contribute to Runx1+/TrkA+ nerve types[31]. Although the focus in this thesis has been on the development of neurons, the same Sox10+ NCCs can give rise to Schwann cells if they receive another set of differentiation factors. Below is a table and a figure that summarizes the various nerve subtypes:

Neuron Subtype	Axon Diameter	Myelination	Relative Action Potential Conduction Velocity	Receptors, Peptides, and Transcription Factors Expressed
Aα-proprioceptors	Large	Myelinated	Very high action potential velocity	Runx3+/TrkC+/NF200+
Aβ-LTMRs	Medium	Myelinated	High action potential velocity	TrkB+/TrkC+ TrkB+ TrkB+/Ret+ Ret+ NF200+
Aδ-fibers	Small	Lightly myelinated	Medium action potential velocity	Runx1+/TrkA+/Ret+ peripherin+/NF200+ Runx1-/TrkA+/Ret+/CGRP+
C-fibers non-peptidergic	Small	Unmyelinated	Slow action potential velocity	Runx1+/TrkA+/Ret+ peripherin+/IB4+ Glutamatergic or other non-peptide neurotransmitter
C-fibers peptidergic	Small	Unmyelinated	Slow action potential velocity	Runx1-/TrkA+/Ret+ Runx1-/TrkA+/Ret- CGRP+ or Substance P+ or other peptides

Table 1.1: Summary of sensory nerve subtypes and the receptors, peptides, and transcription factors that they express

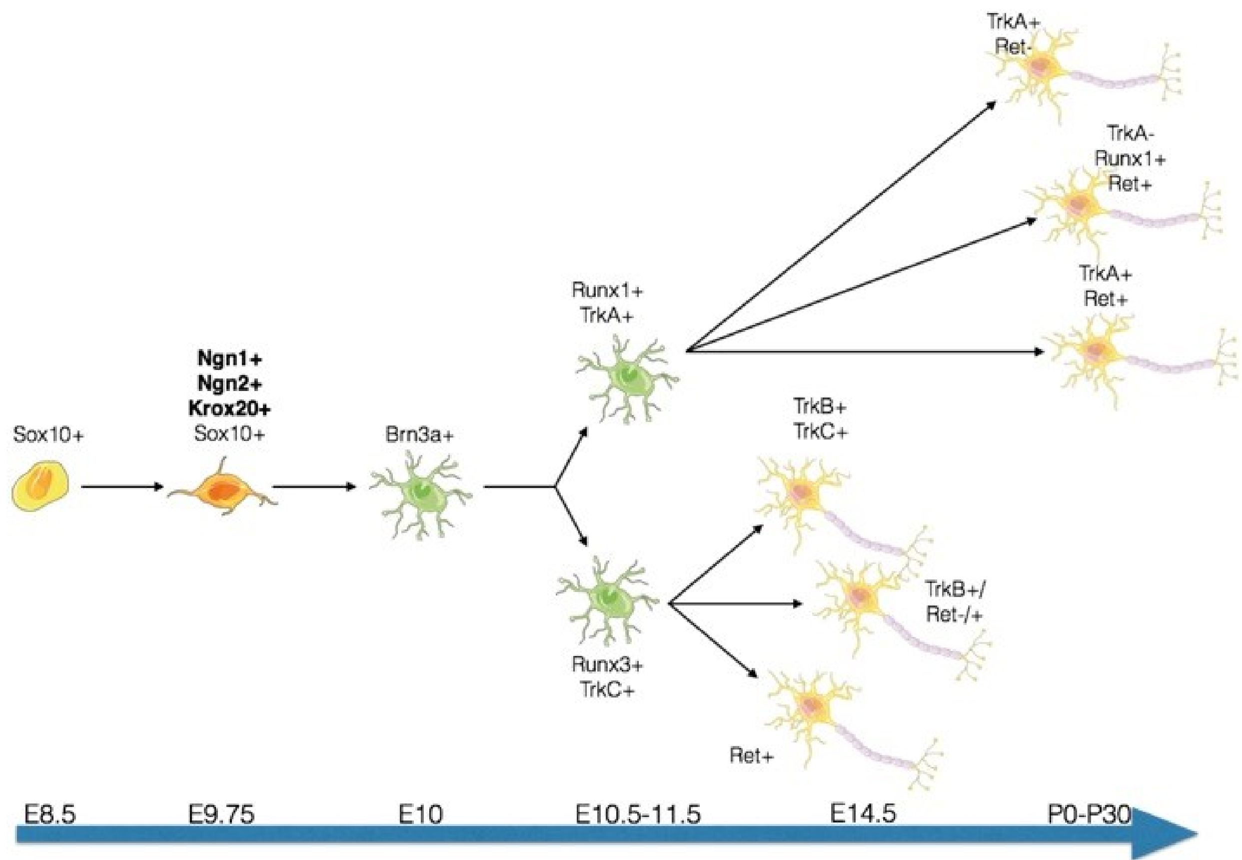

Figure 1.2[32,33]: Summary of sensory nerve subtype specification modified from Marmigère, F., & Ernfors, P. (2007)

Chapter 1.4: Neural Crest Cells Differentiate to Schwann Cell Precursors (SCPs) and Their Derivatives

Although some neural crest cells can give rise to neurons, others termed Schwann cell precursors (SCPs), migrate along axonal paths, express Sox10 and p75 and have the potential to differentiate to Schwann cells, as well as melanocytes[34], fibroblasts[35], odontoblasts[36], and parasympathetic ganglia[37]. SCPs are present at the border of developing DRGs[27] and nerve axons, but do not acquire a neuronal fate. Why some differentiate while others remain undifferentiated near neurons remains a mystery. A few factors are known to regulate SCPs

survival and differentiation. Neuregulin 1(Nrg1) produced by neurons is necessary for SCP survival, as mice lacking its receptor ErbB3 (HER3) have less SCPs than controls[38]. Nrg1 has been demonstrated to promote a glial fate in migrating neural crest while inhibiting neuronal development[39]. Conversely, Bmps promote a neuronal fate and inhibit glial differentiation[40]. Adameyko et al. have found that the transcription factor homeobox protein H6 (Hmx1) is expressed in some SCPs in the DRG and that its downregulation leads to a loss of neuronal cell fate and promotion of a melanocyte fate[34]. However, a detailed understanding of all the factors affecting SCP survival, migration and differentiation is still lacking. Other factors that are important for Schwann cell and nerve development are the neurotrophins.

Chapter 1.5: Neurotrophin Signaling

Neurotrophins are secreted growth factors that participate in the development, differentiation, and survival of neurons, both in the peripheral and in the central nervous system. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) are produced as pro-peptides and can be cleaved intracellularly by enzymes or secreted in their pro-forms[41]. Extracellularly, they form dimers and bind to tropomyosin receptor kinases (Trks) and the low-affinity p75 neurotrophin receptor ($p75^{ntr}$). Both mature and propeptide forms can activate the receptors[42] to achieve different goals; however, each neurotrophin preferentially activates one receptor type. NGF activates TrkA[43], BDNF[44] and NT4[45] activate TrkB, and NT3 activates TrkC[46]. All neurotrophins weakly bind the p75 neurotrophin receptor[47]. Even though one neurotrophin binds preferentially to a particular Trk receptor, it can still bind to the other receptors, and ligand affinities for a particular receptor can be modulated by expression of different neurotrophin receptors. As an example, NT3 has been hypothesized to bind TrkA[48] and TrkB[44] receptors in addition to TrkC, and the $p75^{ntr}$ can functionally interact with TrkA to increase its affinity for NGF[49]. Like the neurotrophins, the receptors consist of two monomer subunits that dimerize when ligands bind. The receptors then undergo an autophosphorylation event that enables them to activate downstream effectors such as phospholipase C (PLC)[50], phosphatidylinositol 3 kinase (PI3K)[51], mitogen-activated protein kinase (MAPK)[52], and many

others. Below is a figure that summarizes the different downstream neurotrophin signaling pathways which are important for neuronal survival, death, and differentiation.

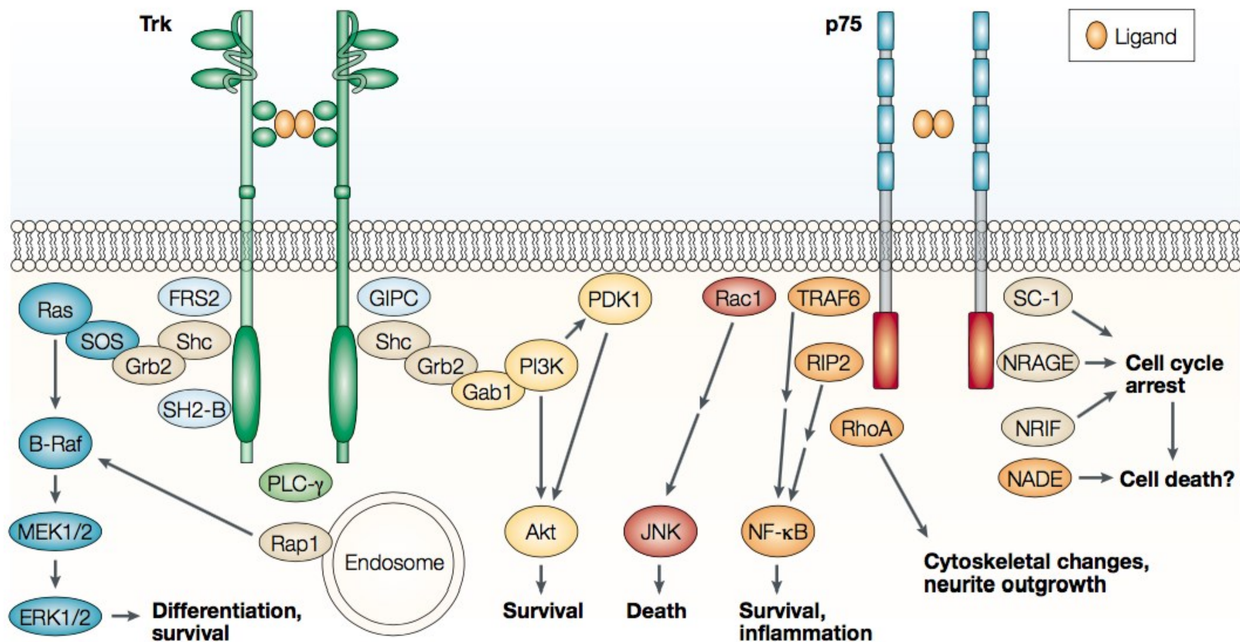

Figure 1.3[53]: Summary of neurotrophin signaling cascade adapted by permission from Macmillan Publishers Ltd: Chao, M. V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nature Reviews Neuroscience, 4(4), 299-309

Chapter 1.6: Evidence for Retrograde Signaling of Neurotrophins

In 1988, Dale Purves suggested the neurotrophin factor hypothesis to explain the large number of neuronal death observed in developing embryos[54]. He postulated that neuronal targets produce survival factors in limiting amounts and that neurons must compete for these factors to survive. Neurons that are most fit will innervate their target fields first and avoid apoptosis by obtaining survival factors, while those that are not fit will not receive enough survival signals and undergo programmed cell death. Today, a growing wealth of evidence supports this hypothesis. Neurotrophins derived from target tissues are known to mediate neuronal survival through retrograde signaling.

Retrograde signaling is a long-distance communication phenomenon between neurons and other cells. In retrograde signaling, neurons take up molecules from their target fields and transport them backwards until they reach their cell bodies some distance away. The molecules then signal neurons to survive and differentiate through the various signaling cascades discussed above. The most well-established hypothesis for explaining retrograde signaling is the endosome signaling model[55]. In this model, once target-derived neurotrophins bind Trk receptors at the nerve terminal, the ligand-receptor complexes are endocytosed and transported to the cell body. In 2001, Howe and colleagues demonstrated internalization of the NGF-TrkA complex in clathrin-coated pits and downstream activation of the Erk1/2 pathway[56]. In another study, Bhattacharyya and colleagues used gold immunoelectron microscopy to show association of Trk receptors with vesicles, and immunoprecipitation to demonstrate activated NGF-TrkA ligand-receptor complexes in these vesicles[57]. Some studies have also demonstrated association of Trk receptors with dynein motor proteins[58]. Together, these and other investigations prove that neurotrophins and their receptors can be internalized and retrogradely transported to activate downstream targets important for neuronal development. However, this does not mean that retrograde signaling must be mediated through endosomes. One study points to a different model of retrograde signaling that does not involve internalization of neurotrophins or their receptors in endosomes. In an elegant experiment, MacInnis and Campenot applied NGF conjugated microspheres (which cannot be internalized) to distal axons in order demonstrate neuronal survival without endosomal signaling[59]. Hence, neurotrophins can signal via endosomes or other unknown retrograde signaling mechanisms.

Chapter 1.7: Neurotrophins Regulate Sensory Nerve Development

Many knockout and overexpression studies of the neurotrophins and their receptors implicate them in retrograde signaling and sensory nerve development.

When either NGF or TrkA are completely deleted in mice, all nociceptors, both unmyelinated C-fibers and myelinated Aδ-fibers are absent from mouse mystacial whisker pads[60]. Heterozygous knockouts of NGF also show a perinatal loss of nociceptors and sympathetic

neurons[61]. In a study where anti-NGF serum was administered to rats from postnatal day 1 to five-week-old adults, high-threshold mechanoreceptors (Aδ-fibers; A-fiber mechanoreceptors (AM)) were depleted, while a type of low-threshold mechanoreceptor called D-hair afferents were present in larger numbers[62]. In NGF overexpression experiments, CGRP and substance P unmyelinated axons increased along with sympathetic innervation to the skin[63,64]. A similar conditional overexpression experiment of NGF in the skin, using the keratin 14 promotor, demonstrated increased survival of C- and Aδ-fibers. Furthermore, in that same study, the authors found that the thermal response property of neurons increased while the mechanosensory property decreased[65]. Taken together, it can be concluded that skin-derived NGF, possibly produced by keratinocytes, melanocytes, and mast cells is important for nociceptor and sympathetic nerve development as well as target innervation. When levels of NGF are increased, more peptidergic unmyelinated neurons develop over every other subtype. Furthermore, in the absence of NGF, other neurotrophins and signaling molecules instruct neurons to take on a different fate, perhaps to become D-afferents (Aδ-LTMRs).

Neurotrophin 3 is expressed in the mesenchyme of E11.5 and E12.5 embryos[66] by epithelial-mesencymal interactions involving Wnt signaling from the epithelium[67,68]. NT3 seems to mediate survival of early Ngn2/Ngn1 derived TrkA, TrkB, and TrkC neurons. Supporting this is the loss of TrkA, TrkB, and TrkC neurons in NT3 mutants shortly after they are generated[69]. Mice lacking NT3 display severe limb movement defects and loss of proprioceptive neurons (TrkC+) and the targets they innervate (Golgi tendon organs and muscle spindles), but display no noticeable difference in nociceptive neuron populations (the AM type)[70]. Similarly, TrkC deficient mutants display an identical phenotype[71]. NT3 is also important for the development of slow adapting mechanoreceptors and Merkel cells. A study combining electrophysiological recordings with genetic manipulation demonstrated a substantial reduction of Merkel cells (quinacrine+ / or Troma1[72] cells) and their associated Aβ-slow-adapting fibers. Furthermore, there was also a reduction in D-hair afferents, but not A-fiber mechanoreceptors[73]. Therefore, NT3 is important for the development of proprioceptors, their Golgi tendon organs, and their muscle spindles. In addition, NT3 is important for the generation of LTMRs including slow-adapting fibers and their Merkel cell targets, as well as D-hair afferents belonging to the Aδ group.

Brain-derived neurotrophic factor activates TrkB receptors and is important for the development as well as the maintenance of mechanosensory neurons. In mouse DRGs, it activates about 30% of intermediate to large diameter neurons[74]. In postnatal life, mechanosensors require BDNF to maintain their sensitivity. BDNF deficient and haploinsufficient mice demonstrate attenuated mechanical activation of slow adapting LTMRs as evidenced by electrophysiological recordings and Von Frey experiments[75]. This attenuation can be rescued by administration of recombinant BDNF, supporting the notion that it maintains sensitivity of Aβ-slow-adapting fibers in the adult. In conclusion, BDNF is important for the development of mechanosensory neurons as well as their maintenance in adult mice. NT4 serves a similar function to BDNF and will not be discussed. Recently, BDNF had been demonstrated to participate with retrograde Bmp signaling to influence nerve development[76].

Chapter 1.8: BMP signaling[77]

Bone morphogenetic proteins (Bmps) are secreted peptides that belong to the transforming growth factor beta (TGF-ß) family of ligands. They are synthesized as propeptides and form homodimers or heterodimers with other Bmps. After being cleaved, they bind complexes consisting of type I serine kinase receptors (Alk1, Alk2, Alk3 or Alk6) and type II receptors (BmprII, ActRII, ActRIIb). Binding of receptors by Bmps leads to the transphosphorylation of type I receptors by type II receptors and the subsequent phosphorylation of Smads 1/5/8 by the type I receptor. Next, Smad 4 complexes with Smad 1/5/8 and together they enter the nucleus and modify transcription. Activated receptors can also mediate signaling via non-Bmp specific transduction pathways involving MAPK, Akt, and Rho GTPases. Regulation of Bmps occurs at several levels. First, in the extracellular space, antagonists like Noggin, Chordin, Gremlin and Twisted gastrulation bind Bmps and prevent ligand-receptor interactions. Second, receptors such as BAMBI may bind Bmps and Type II receptors, but do not phosphorylate Smads. Other receptors belonging to the dragon family (RGMs) recruit more Type II receptors and hence amplify signaling. Lastly, intracellular regulation occurs through inhibitory Smads (Smad6, Smad7). Refer to the figure below:

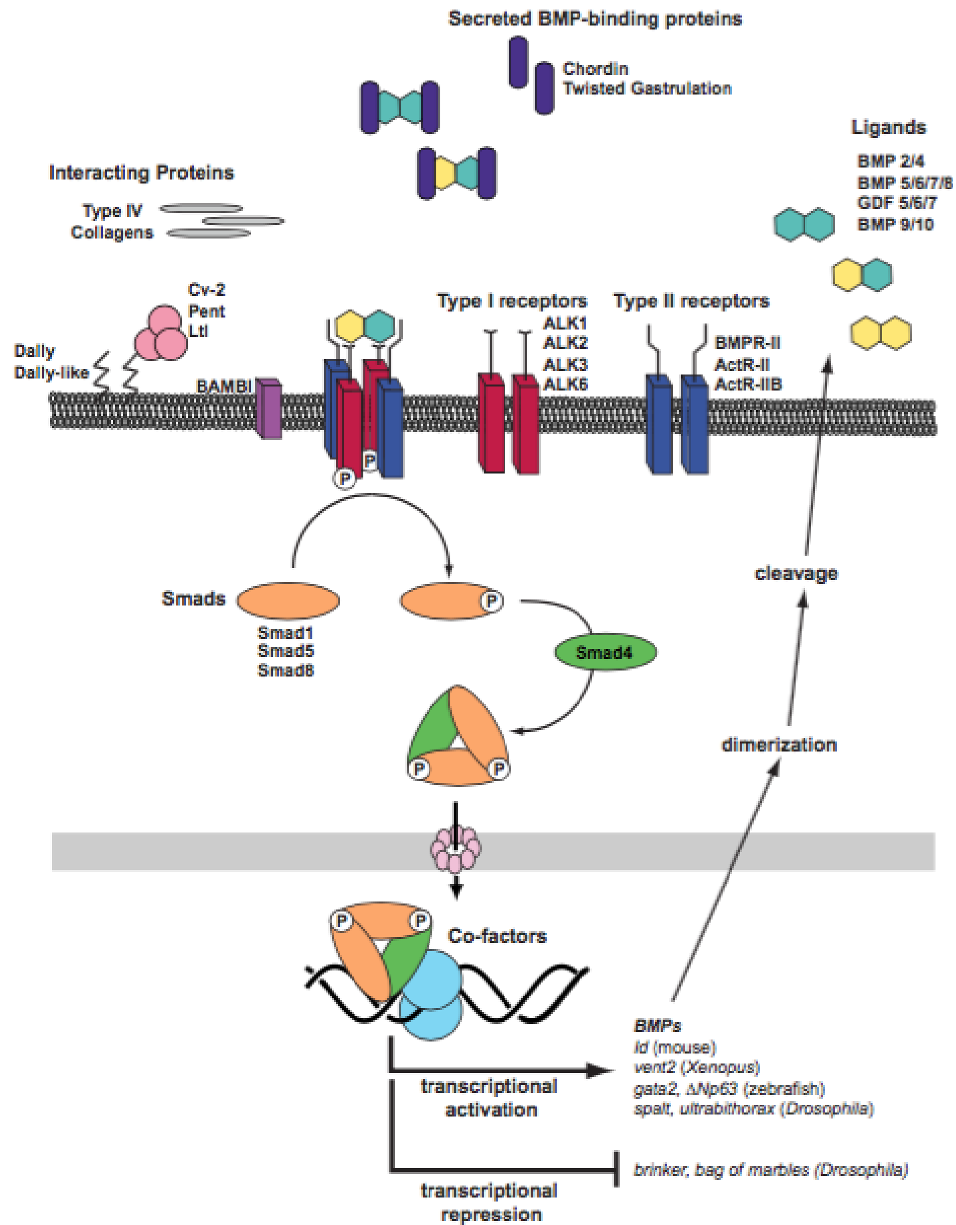

Figure 1.4[78]: Summary of Smad mediated Bmp signaling cascade adapter from Ramel, M. C., & Hill, C. S. (2012). Spatial regulation of BMP activity. FEBS letters, 586(14), 1929-1941 under the Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported (CC BY-NC-ND 3.0) licence

Chapter 1.9: Bmps as Regulators of Nerve Development

Starting in the 1990s, many studies began to point out a role for Bmps in nerve development. Today, this role has been widely expanded to include various aspects of central-, sensory peripheral-, and enteric nervous system development.

In the central nervous system, Bmps participate in various developmental processes. They can regulate both nerve and glia formation depending on the spatiotemporal context. On the one hand, Bmp7 expressed in the choroid plexus can flow into the cerebrospinal fluid (CSF)[79] and regulate Ngn2-dependent nerve development from progenitors in the ventricular and subventricular zones (SVZ) of E14.5 mouse cortices[80]. On the other hand, its expression in the meninges promotes radial glial cell attachment to the basement membrane and glial cell survival[80]. In a similar manner, Bmp7 expressing meninges have been demonstrated to promote neurogenesis in the dentate gyrus by regulating Lef1 expression[81] to enhance Wnt signaling[82,83]. Bmps in avian models have also been demonstrated to regulate neurogenesis in the hindbrain[84]. Conversely, in *in vitro* studies of cultured SVZ, Bmps have been demonstrated to promote astrocyte differentiation[85]. Further genetic studies of Bmps and their antagonists in the central nervous system will help clarify these issues and provide further insight.

In the peripheral nervous system, the role of Bmps in nerve development starts early with the induction of the neural crest, where they first influence neural crest differentiation and delamination from the neural tube. As discussed in Chapter 1.2, Bmps along with Wnts and Fgfs are necessary for this process[3,86]. Second, once neural crest cells are induced, Bmps maintain their multipotency, self-renewal, and proliferation by regulating *Id* transcription factors in combination with Wnt signaling[87,88,89,90]. Higher levels of Bmps with Wnts maintain stem cell character, while low levels of Bmps with continued Wnt expression promote neuronal differentiation. Third, Bmps are also expressed in migrating neural crest cells[91]. Several experiments have demonstrated a Bmp2-dependent activation of PI3K, the small GTPase Cdc42[92], as well as p38 MAPK[93] for cytoskeletal rearrangements promoting migration. Fourth, Bmps 2 and 4 inhibit the differentiation of Schwann cells but favor the development of neurons[40] from neural crest cells. Additionally, varying levels of Bmps are important for specifying the type

of neuron formed. In high doses, Bmps favor the development of autonomic neurons, while more limited amounts favor sensory nerve development[94]. Concerning autonomic neurons, a high dose of Bmps (Bmp2/4/7) produced by the dorsal aorta (near the developing sympathetic chain ganglia)[95] drives the expression of numerous autonomic specifier genes *Mash1*[96], *Phox2a*[97], *Phox2b*[98], and *Cash1*[99] both in neural crest cultures and *in vivo* avian models. Concerning sensory neurons, new evidence points to a novel role for Bmps as nerve organizers, and as modulators of neurotrophin signaling. Hodge and colleagues have demonstrated that target-derived Bmp4 regulates the expression and spatial organization of *Onecut1 (OC1), Onecut2 (OC2), H6 Family Homeobox 1 (Hmx1), and T Box Protein 3 (Tbx3)* transcription factors in the mouse trigeminal ganglion. Additionally, they showed that application of Bmp4 with neurotrophins produces a different spatial organizing effect than if either Bmp4 or neurotrophins alone are added to nerve cultures[100]. These findings demonstrate that distinct face maps of the trigeminal ganglion possess molecular differences that can be regulated by both Bmp and neurotrophin expressing targets. A 2012 study by Ji and Jaffrey corroborated these findings by solving an intracellular mechanism implicating Bmp4 and BDNF. In this experiment, Ji and Jaffrey isolated E13.5 mouse trigeminal neurons and grew them in micro-fluid chambers that physically isolate the nerve cell bodies from their axons. Using this model, they demonstrated that target-derived BDNF induces translation of axon localized Smad1/5/8 transcripts, and that target-derived endosomal Bmp4 phosphorylates Smad1/5/8 and produces transcriptional changes[76]. In addition to this study, many others also find that Bmp receptors and ligands can be internalized in endosomes to mediate signaling[101,102,103]. Taken together, these investigations establish a convergence point between Bmp and neurotrophin signaling and suggest that Bmps can also participate in endosomal retrograde signaling with neurotrophins to fine-tune nerve development. Therefore, Bmps participate in the development of both sensory and autonomic ganglia.

Chapter 2: Aims and Hypothesis

Bmp7$^{\Delta/\Delta}$ mice present with many craniofacial defects such as cleft lip and cleft palate[104], anophthalmia[105], various tooth defects[106], and appear to have delayed target innervation (Kozlova and Graf, unpublished).

A previous study has established the expression pattern of Bmp7 in various embryonic craniofacial structures including, the nasal process and its epithelium, the mesenchyme and the epithelium of the maxillary process, maxillary hair follicles and vibrissae, and the exit zone of the trigeminal root[106]. Furthermore, another study found a similar expression pattern of Bmp type I and type II receptors in late embryos using *in situ* hybridization[107]. Together these results indicate that Bmp7, as well as its receptors, are expressed in regions of the trigeminal ganglion and its target fields. However, to date, it is not known how, when, or where Bmp7 is required for sensory nerve development. The purpose of this investigation was to determine a role for Bmp7 in sensory neurogenesis. First, to study the expression pattern of Bmp7, we used Bmp7$^{wt/LacZ}$ mice. Second, we compared the trigeminal ganglia of Bmp7$^{\Delta/\Delta}$ mice with wild-type littermates to identify a molecular phenotype. Third, we began investigating mesenchyme-derived Bmp7 signaling using Bmp7$^{fl/fl}$ Wnt1-cre mice, that conditionally delete Bmp7 in the neural crest.

We hypothesize that Bmp7 is a regulator of sensory neurogenesis because it is expressed in regions of the trigeminal ganglion as wells as its target fields throughout different stages of development.

Chapter 3: Materials and Methods

Animals

All animal protocols were performed under the University of Alberta's Animal Care and Use Committees in line with the Canadian Council on Animal Care Guidelines (AUP 1149). All mice lines were backcrossed for more than 10 generations to the C57Bl/6J background. $Bmp7^{tm1.1Dgra}$ ($Bmp7^{fl/fl}$) mice were crossed to Tg(Wnt1-cre)11Rth (Wnt1-cre) to induce neural crest-specific deletion of Bmp7 ($Bmp7^{fl/fl}$ Wnt1-cre). $Bmp7^{tm2Rob}$ ($Bmp7^{lacZ}$ reporter mice) were used to detect *Bmp7* expression. Mice embryos deficient for *Bmp7* and carrying the LacZ reporter were generated by crossing heterozygous parents ($Bmp7^{wt/LacZ}$ females with $Bmp7^{wt/LacZ}$ males). Neural crest conditional knockouts were generated by mating $Bmp7^{fl/fl}$ females with $Bmp7^{fl/fl}$ Wnt1-cre (+) males. Timed matings determined embryonic ages. Adult mice were placed together in the evening, and the following day, if the female had a vaginal plug, she was considered 0.5 days pregnant.

LacZ Staining

$Bmp7^{wt/LacZ}$ embryo heads were dissected in 4ºC 1X PBS and were incubated in X-gal solution containing a 1mg/mL X-gal substrate. Heads were then fixed overnight in 4% PFA at 4ºC. The following day they were washed with cold 1X PBS, dehydrated in 50% ethanol for 20 minutes and processed for embedding in paraffin wax the next day.

RNA extraction and qPCR

Trigeminal ganglia were isolated from E14.5 embryos and flash frozen in liquid nitrogen to be stored in a -80ºC freezer. RNA was extracted using Trizol and Chloroform (both Thermofisher Scientific) and using the Thermofisher Scientific GeneJet RNA purification kit. 110ng of RNA was then reverse transcribed to cDNA using Applied Biosystems reverse transcription kit. Quantitative PCR was then performed using the KAPA SYBR FAST system with the appropriate primer pairs (See tables below for primer sequences). Acidic ribosomal phosphoprotein (36B4) was used as a control and as a reference gene to calculate ΔC_q ($Cq_{candidate\ gene}-Cq_{36B4}$). Amplification of each gene was performed in duplicates or triplicates. The plate was sealed with an adhesive cover (Thermofisher Scientific) and was spun at 1500 rpm for 4 minutes

and then analyzed by a Biorad CFX96 Touch Real Time PCR Detection System.

Gene	Forward Primer	Reverse Primer
Bmp7	GTGGCAGAAAACAGCAGC	GGGCAGTGAGAGACTTAG
36B4	GTGTGTCTGCAGATCGGGTA	CAGATGGATCAGCCAGGAAG
Nav1.7	TGGATTCCCTTCGTTCACAGA	GTCGCAGATACATCCTCTTGTTT
Nav1.8	AAGACAAAACCCTCTTGCCA	TTTGGTCAACTGTGTGTGCAT
Nav1.9	TACGGGCAAGTTCTGGGATA	TATGAGAGGGTCCAAGACGG
TrkA	GCCTAACCATCGTGAAGAGTG	CCAACGCATTGGAGGACAGAT
TrkB	TCCCAGAGTTCAGCTCACAG	GTTGGCGAGACATTCCAAGT
TrkC	GCTCCCTCACCCAATTCTCT	CCAGTACTTCCGTCAGGGTC
p75ntr	CTGGGTGCTGGGTGTTGT	ACACTGAGCGCCAGTTACG
NF200	GCTTTCTGTAAGCGGCAATC	ACCAGGACGCTATTCAGCAG
Runx1	TTTCAAGGTACTCCTGCCTGA	CAGTGAGAAGGACCAGAGACT
Runx3	CTGGCCACTTGGTTCTTCAT	CTTCAAGGTGGTGGCCCT
Ret	CCAGCTGGCTGGTCTACATA	GAAGCTGATTTTGCTCCTGC
TrpV1	AACCAGGGCAAAGTTCTTCC	CATCATCAACGAGGACCCAG
CGRP	GAACTTCAGGAAGCCCATGA	AAGAGTCACCGCTTCGCA

Table 3.1: Sequences of Forward and Reverse Primers used for qPCR

Histological Preparation and Immunofluorescence

Embryo heads were dissected in cold 1X PBS and fixed overnight in 4% PFA. The following day, they were washed with cold 1X PBS and processed through a series of increasingly concentrated ethanol, then xylene and paraffin wax. The following day heads were embedded in paraffin blocks and were cut on an 820 Spencer Microtome to generate 7µm thick sections. Blocks and sections were stored at room temperature.

For immunofluorescence, sections were incubated for 2-3 minutes in a 60ºC oven, then were deparaffinized in xylene, and rehydrated in an ethanol gradient. Boiling sections achieved antigen retrieval in pH 6 10mM sodium citrate buffer. Once cooled and washed with 1X PBS, slides were blocked using a 10% serum of the secondary antibody's host. Sections were then incubated overnight at 4ºC in the following antibody solutions: Tuj1 (1:1000, Biolegends); NFM (1:2000, Developmental Studies Hybridoma Bank, clone 2H3); TrkA (1:400, Reichardt lab, University of California, SF); TrkB (1:200, Cell Signaling); TrkC (1:800, Cell Signaling); p75ntr (1:1000, Cell Signaling); CCas3 (1:300, Cell Signaling). The next day, slides were incubated in the following secondary antibodies: Anti-rabbit Alexa 647 (1:1000, Molecular Probes), Anti-mouse IgG2a Alexa 555 (1:1000, Molecular Probes).

Fluorescent images were taken on an Olympus IX53 inverted microscope and analyzed by Fiji software. The microscope settings were kept constant for both wild-type and mutant sections. However, brightness and contrast settings were altered by Fiji to make sections visually comparable.

Air puff Assay

This assay measures behaviors of mice in response to a 15psi air puff directed at their whisker pad. Previous experiments have assessed nociceptive sensitivity in mice using a similar protocol[108,109]. Mice could move freely in a small cage made of wire mesh and transparent acrylic (3.5 x 1.75 x 1.5 inch). Before testing, mice were acclimatized for 5 minutes in these cages every second day for 3 days while the experimenter sat quietly near them. When testing began, 15psi (controlled by PicoSpritzer II) air puffs were applied at 90º to the whisker pad. Air was delivered

through a blunted 18-gauge needle tip. Three series of 4 air puffs were applied to each whisker pad for a total of 12 stimuli/whisker pad. Behaviors were recorded by two Canon Vixia HF R500 camcorders placed on each side of the cage. Videos were then analyzed in real time, and behaviors were scored for quantification. Below is a table of behaviors and their assigned scores.

linear point Assignment	Response to 15 psi air puff
1	**No response**
2	**Brisk withdrawal of the head from the probe or attacking the probe**
3	**Unilateral or bilateral forepaw swipes down the snout or whiskers**
4	**Continuous uni/bi-lateral forepaw swipes down the snout or whiskers**

Table 3.2: Air puff Assay Scoring Rubric

The number of each specific behavior observed was tallied and divided by the total number of behavioral responses for each mouse to generate a ratio.

Statistical Analysis

No statistical test was conducted on the qPCR data because only one or two biological repeats existed. The error bars on the qPCR plots represent the standard deviation from the average Cq value of two or more technical replicates.

A one-way ANOVA was conducted to measure statistical significance for the air puff assay. Behaviors were assumed to follow a normal distribution, and the test was conducted to compare the ratios of each behavior for each mouse. See Figure 4.26 scatter plot.

Chapter 4: Results

Bmp7 deficient E14.5 embryos have smaller and slightly misshaped trigeminal ganglia in comparison to wild-types

We first examined the size and morphology of the TG in Bmp7$^{-/-}$ and Bmp7$^{+/+}$ embryos for gross anatomical disturbances that might be indicative of major molecular pathologies. To do this, we bred Bmp7$^{wt/lacZ}$ mice and dissected embryos that were 14.5 embryonic days old. We then fixed their heads in 4% PFA and embedded them in paraffin. Next, we cut 7μm serial sagittal sections through the entire depth of the TG, aligned comparable mutant and wild-type sections, and imaged them using a light microscope. We found that E14.5 Bmp7 deficient embryos consistently have smaller and more spherical TGs than their corresponding controls (see Figure 4.1).

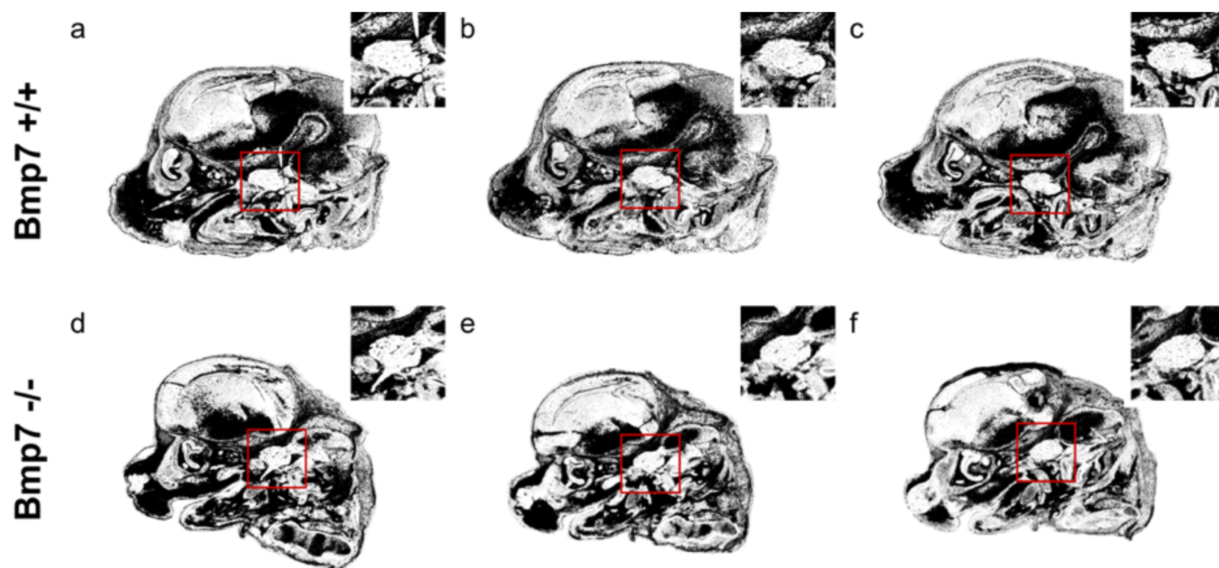

Figure 4.1: Paraffinograf showing serial sagittal sections of E14.5 Bmp7 deficient and wild-type embryos. Mutant TGs are smaller and more spherical in shape. Scale bar represents 1mm.

Bmp7 is expressed in the meninges throughout development and is maintained in adulthood

Next, we wanted to determine where *Bmp7* was expressed. We used Bmp7$^{wt/lacZ}$ mice that report on Bmp7 transcription to identify its localization in tissues. We isolated embryos of various

stages (E13.5-E18.5), and after removing the skin and outer meningeal covering near the temporal region, we incubated them in LacZ staining solution. We found that *Bmp7* is expressed in the meninges as early as E13.5 and is maintained throughout development (Figure 4.2).

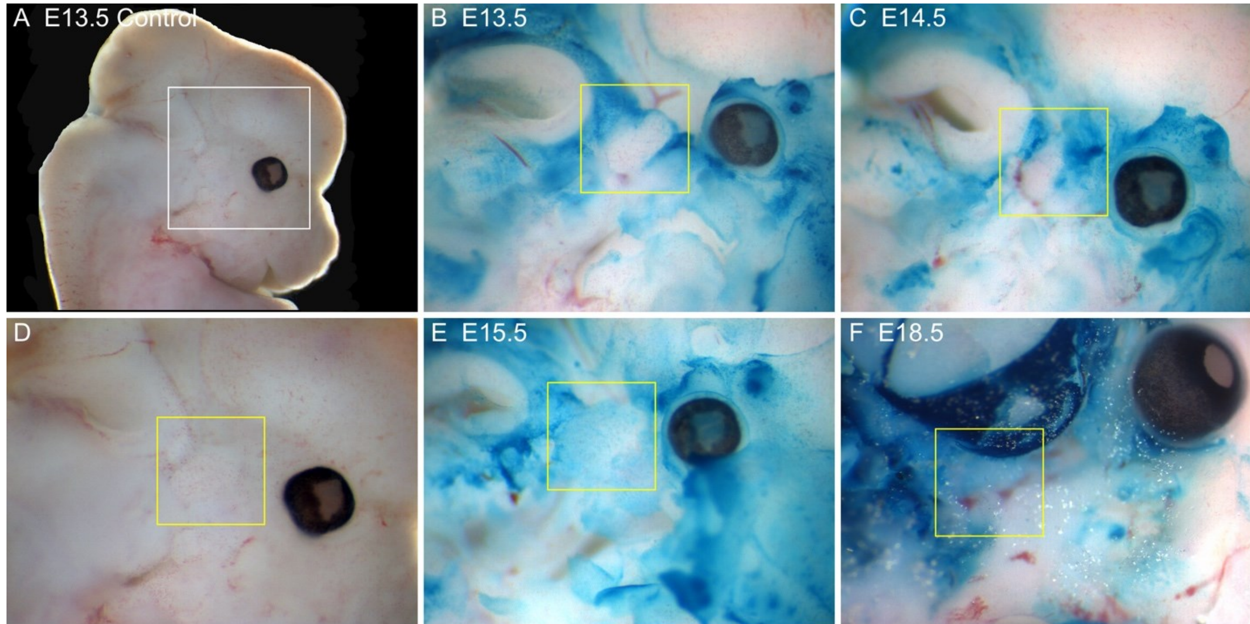

Figure 4.2: Bmp7$^{wt/lacZ}$ embryos at various developmental stages showing Bmp7 expression in the meninges. A and D show a wild-type Bmp7 embryo used as a control for the lacZ stain. The skin has been removed to expose the TGs. D is the magnification of the white square shown in A. Yellow squares in B-F show TGs.

Bmp7 is expressed in the epithelium and vibrissae follicles of E14.5 embryo

Using the same reporter mice described earlier, and following a similar protocol, we looked for Bmp7 expression in sites that are innervated by the trigeminal ganglion. The rationale for this was that Bmp7 could be important for target innervation or retrograde signaling. We found Bmp7 expression in the epithelium and vibrissae of E14.5 embryos (Figure 4.3).

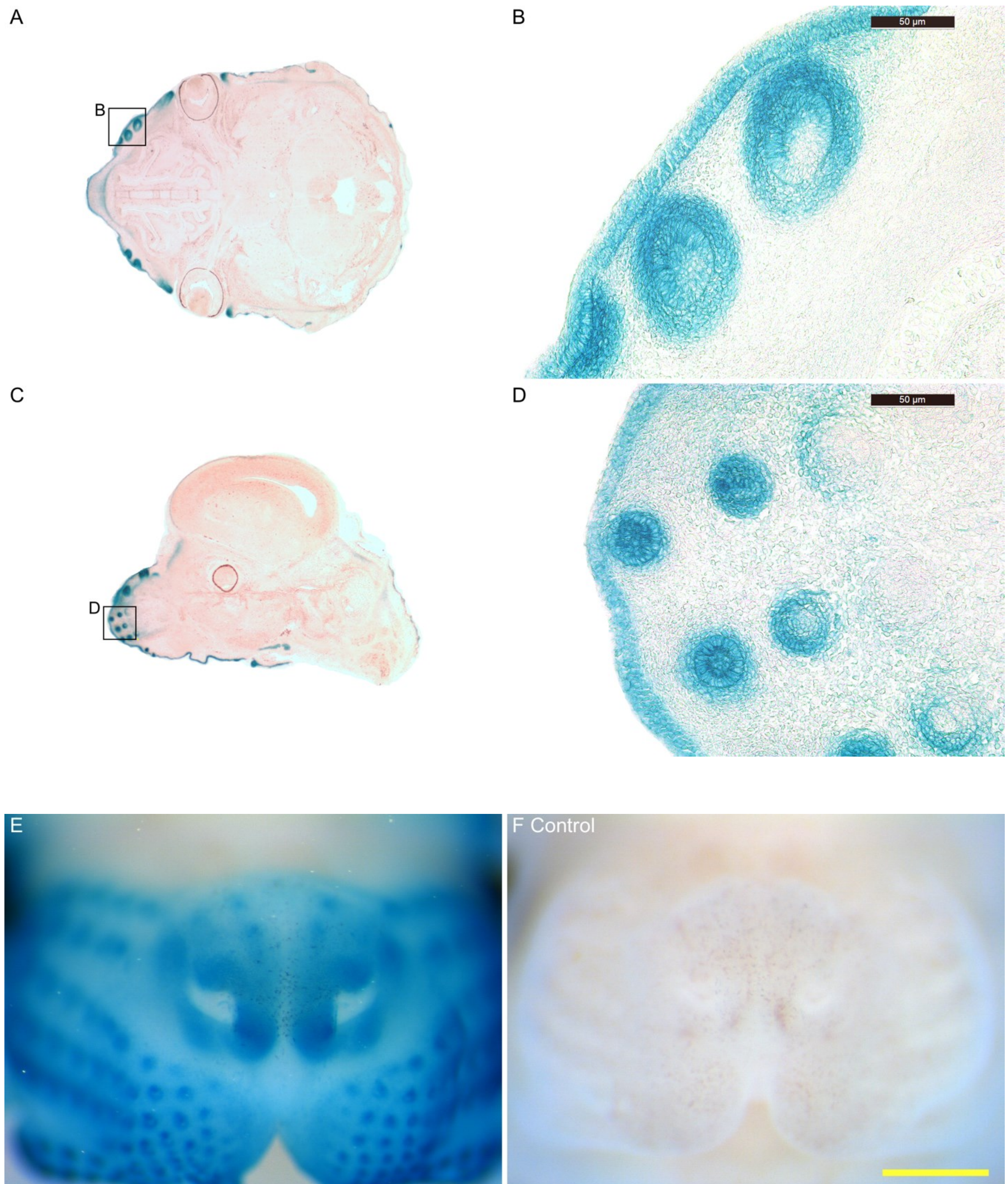

Figure 4.3: E14.5 Bmp7$^{wt/lacZ}$ embryos showing Bmp7 expression in the epithelium and vibrissae follicles. A-D are paraffin sections, while E-F are whole mount maxillary regions. B and D are magnified images of A and C respectively. F shows a Bmp7$^{wt/wt}$ as a control for the lacZ stain. The scale bar in F represents 200µm.

Trigeminal ganglia of E14.5 Bmp7$^{-/-}$ embryos maybe showing greater nociceptor character than their wild-type counterparts

Next, after finding out that the size and shape of the trigeminal ganglion was different in Bmp7 mutants, we wanted to examine the genetic profile of sensory neurons in the trigeminal ganglia of both wild-type and mutant embryos. We first isolated E14.5 TGs, flash froze them in liquid nitrogen, extracted RNA, converted the RNA into cDNA and then performed two qPCRs from two independent litters. We chose to look at nerve subtype associated transcription factors, receptors, and peptides to learn about the genetic and cellular makeup of the TG. We found that TrkA, TrpV1 (capsaicin receptor), CGRP (calcitonin gene-related peptide), and Ret (rearranged during transformation) expression (not shown on this qPCR) were increased in Bmp7 mutants (see Figure 4.4 for a representative qPCR).

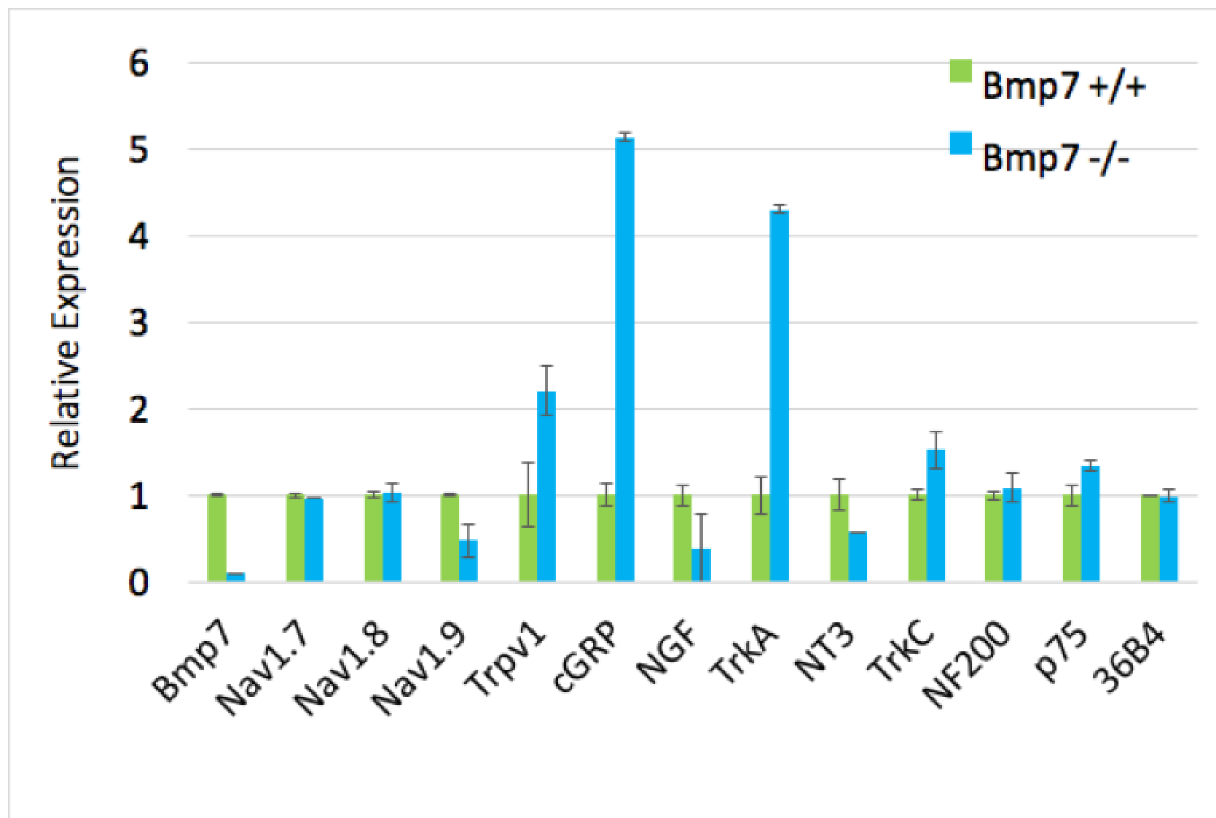

Figure 4.4: Gene expression profile of isolated E14.5 Bmp7+/+ and Bmp7-/- trigeminal ganglia. Bmp7$^{\Delta/\Delta}$ TGs show increased TrkA, TrpV1 and CGRP expression corresponding to a greater nociceptive neuronal identity. The error bars represent the standard deviation from the average Cq value. This experiment was repeated twice on embryos from two separate litters. The above plot represents the data from one of those litters.

Trigeminal ganglia of Bmp7 deficient mutants are smaller and have a different shape throughout various stages of development (E12.5-E15.5). However, no obvious difference in neurotrophin receptor expression exists

Next, using the qPCR data as a guide, we investigated protein expression by immunofluorescence. We looked to see if Trk receptors were differentially organized, or if their overall expression was altered in $Bmp7^{-/-}$ mutants. After isolating embryos ranging in age from E12.5 to E15.5, we fixed them in 4% PFA, embedded them sagittally in paraffin, and cut serial sections throughout the entire depth of the TG. Then, after aligning wild-type and mutant sections, we used antibodies against neurotrophin receptors (TrkA, TrkB, TrkC, p75ntr). We cannot point out any obvious differences in neurotrophin receptor expression within the TG at these developmental stages, but we conclude that the overall size of the TG is smaller in mutants and has a less elongated and more elliptical shape (see Figures 4.5-4.16).

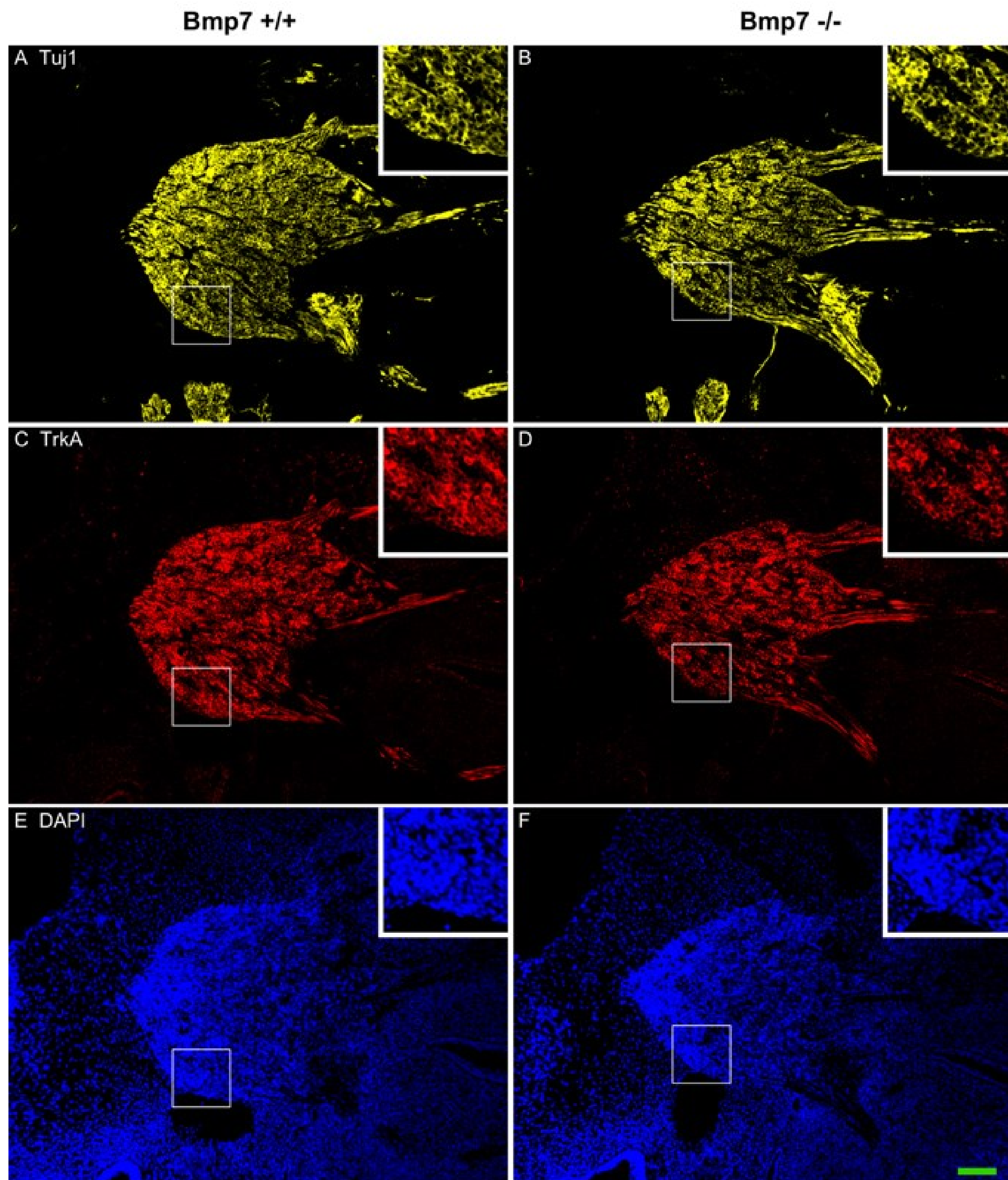

Figure 4.5: E12.5 trigeminal ganglia showing TrkA expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

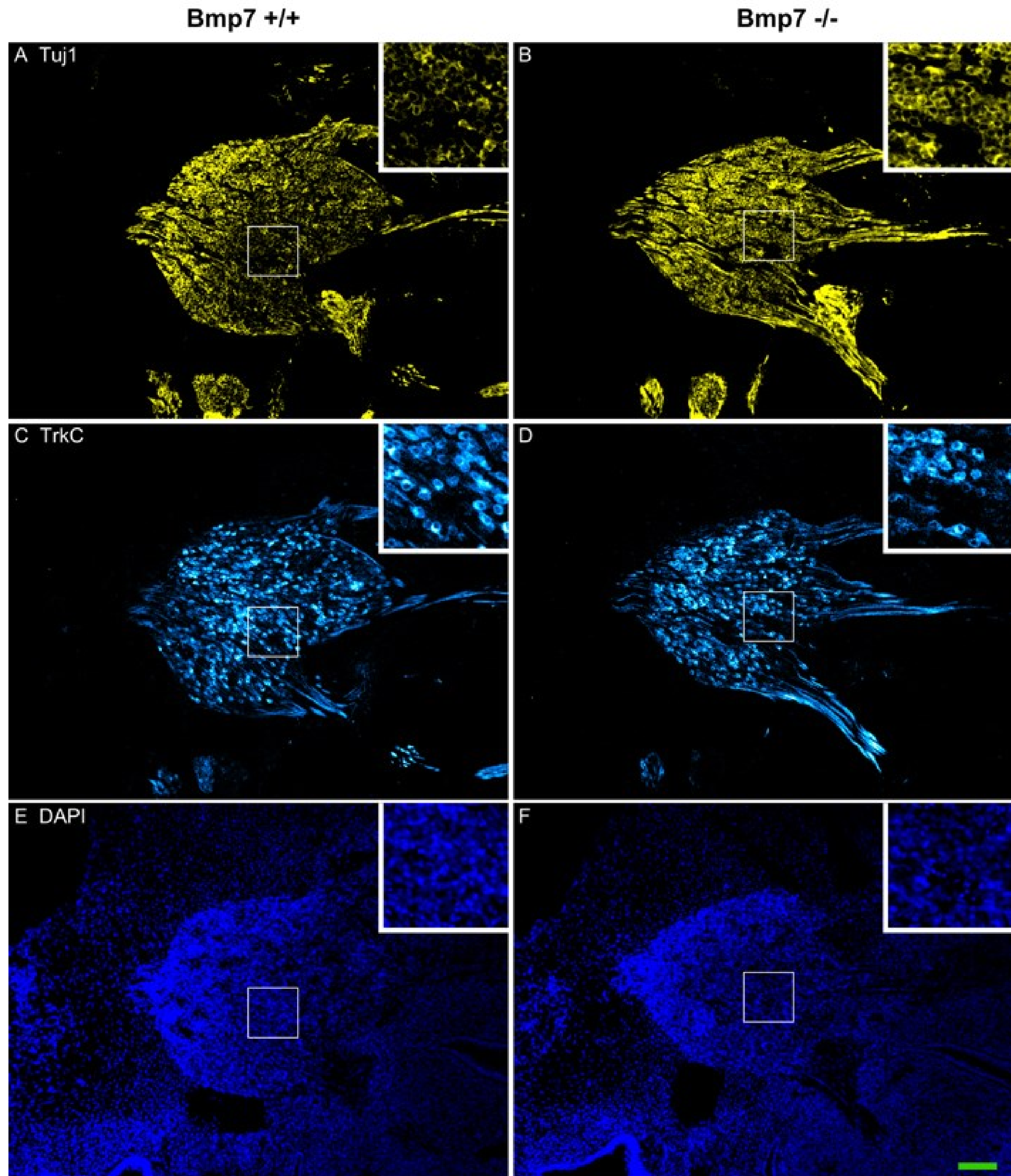

Figure 4.6: E12.5 trigeminal ganglia showing TrkC expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

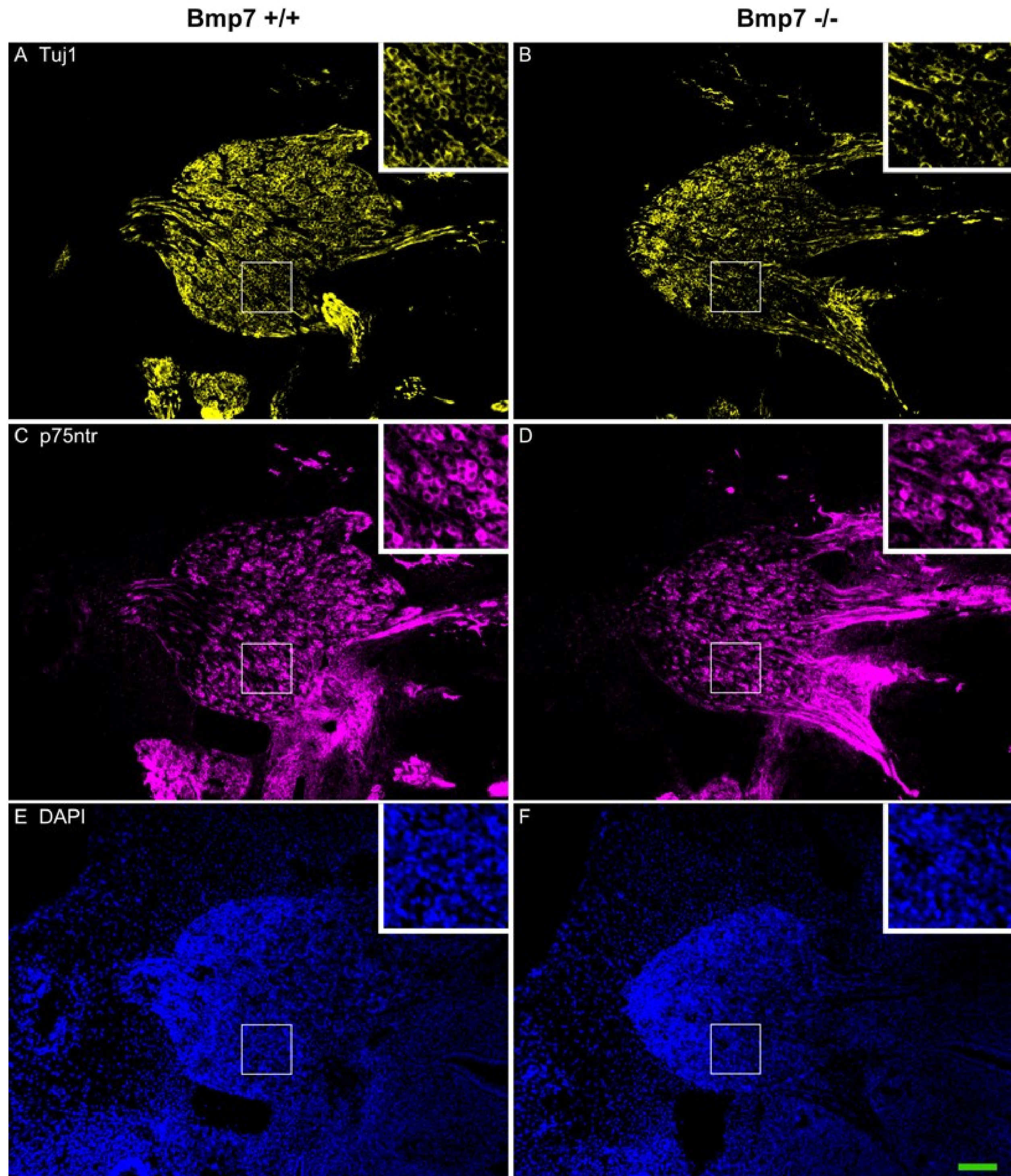

Figure 4.7: E12.5 trigeminal ganglia showing p75ntr expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

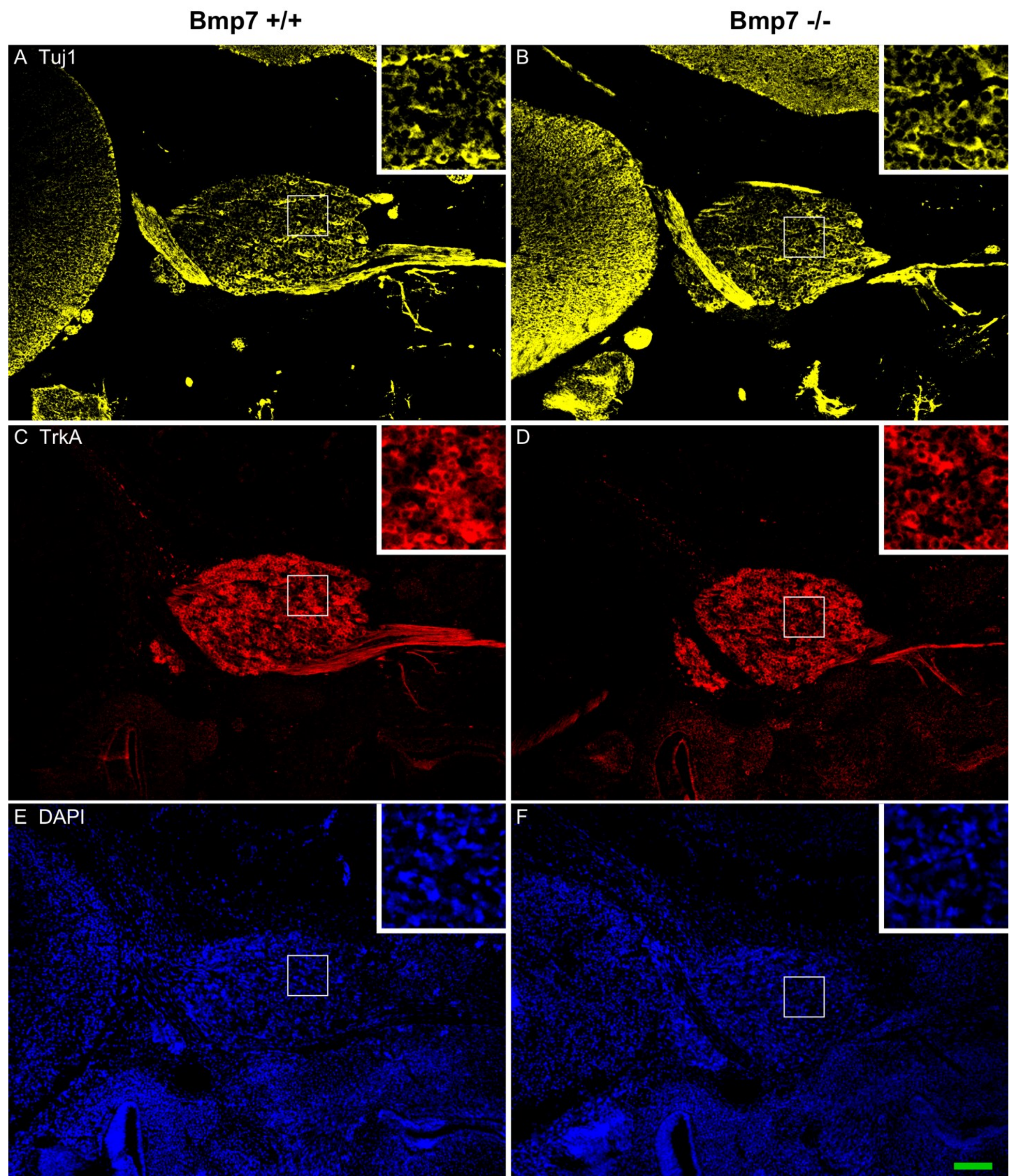

Figure 4.8: E13.5 trigeminal ganglia showing TrkA expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

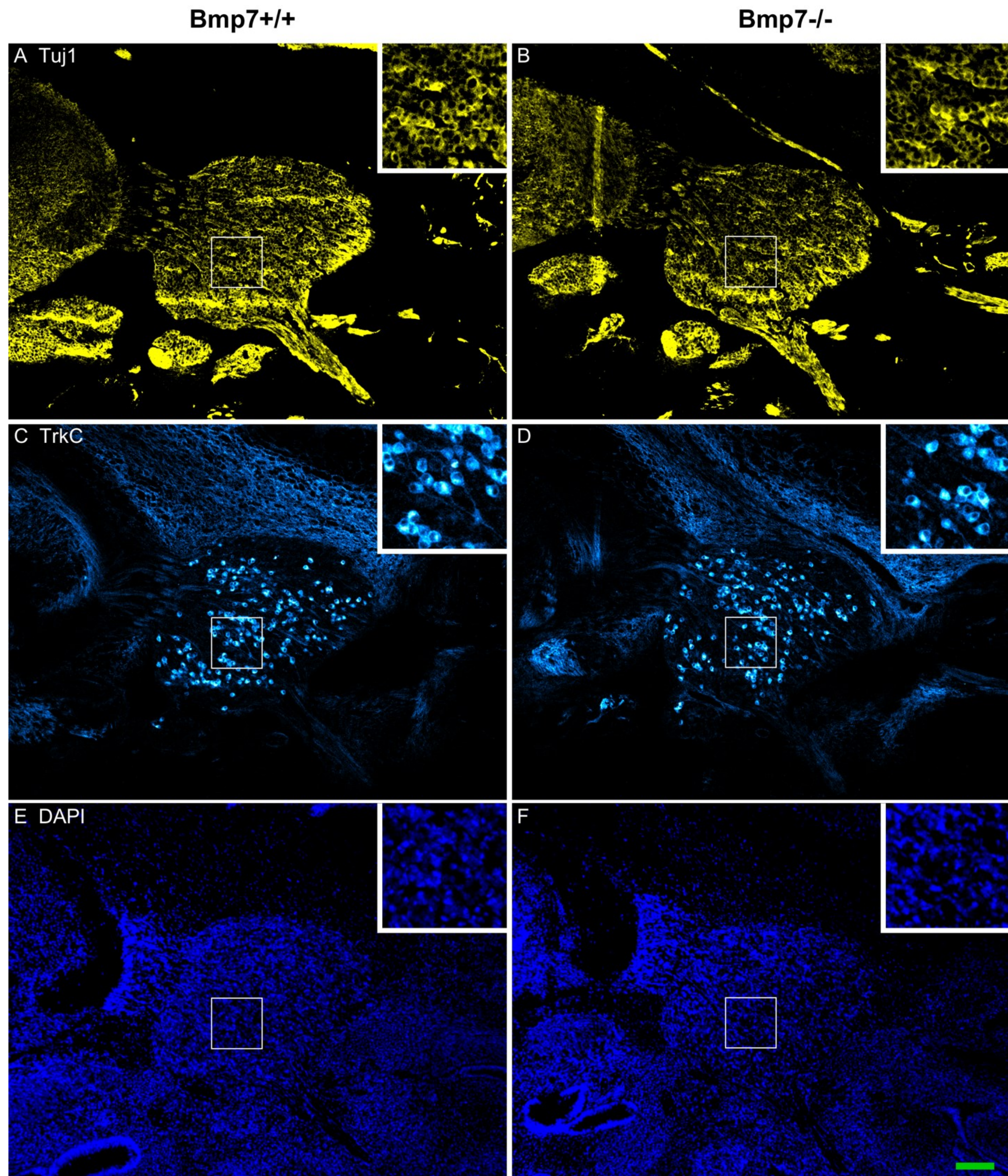

Figure 4.9: E13.5 trigeminal ganglia showing TrkC expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

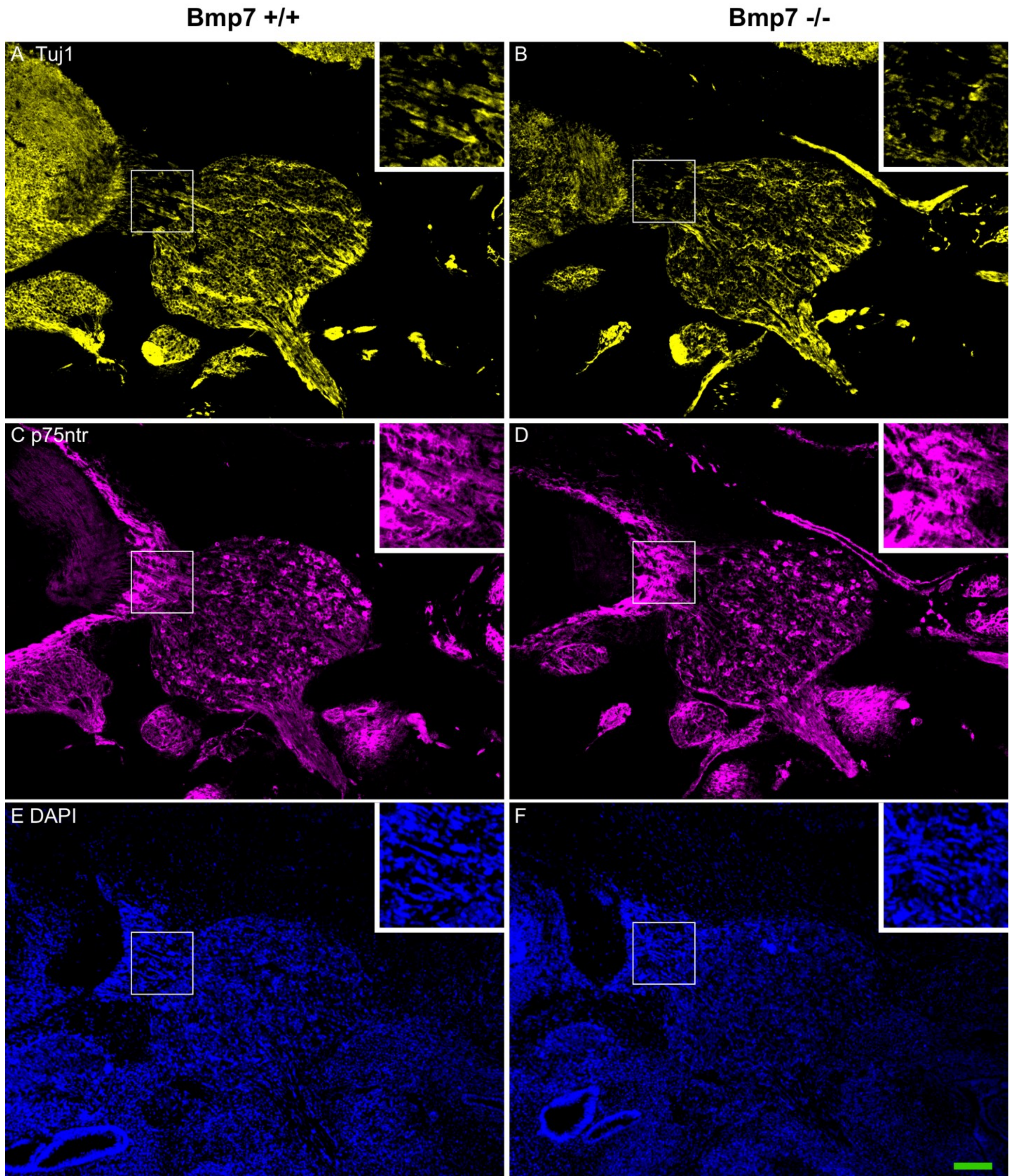

Figure 4.10: E13.5 trigeminal ganglia showing p75ntr expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

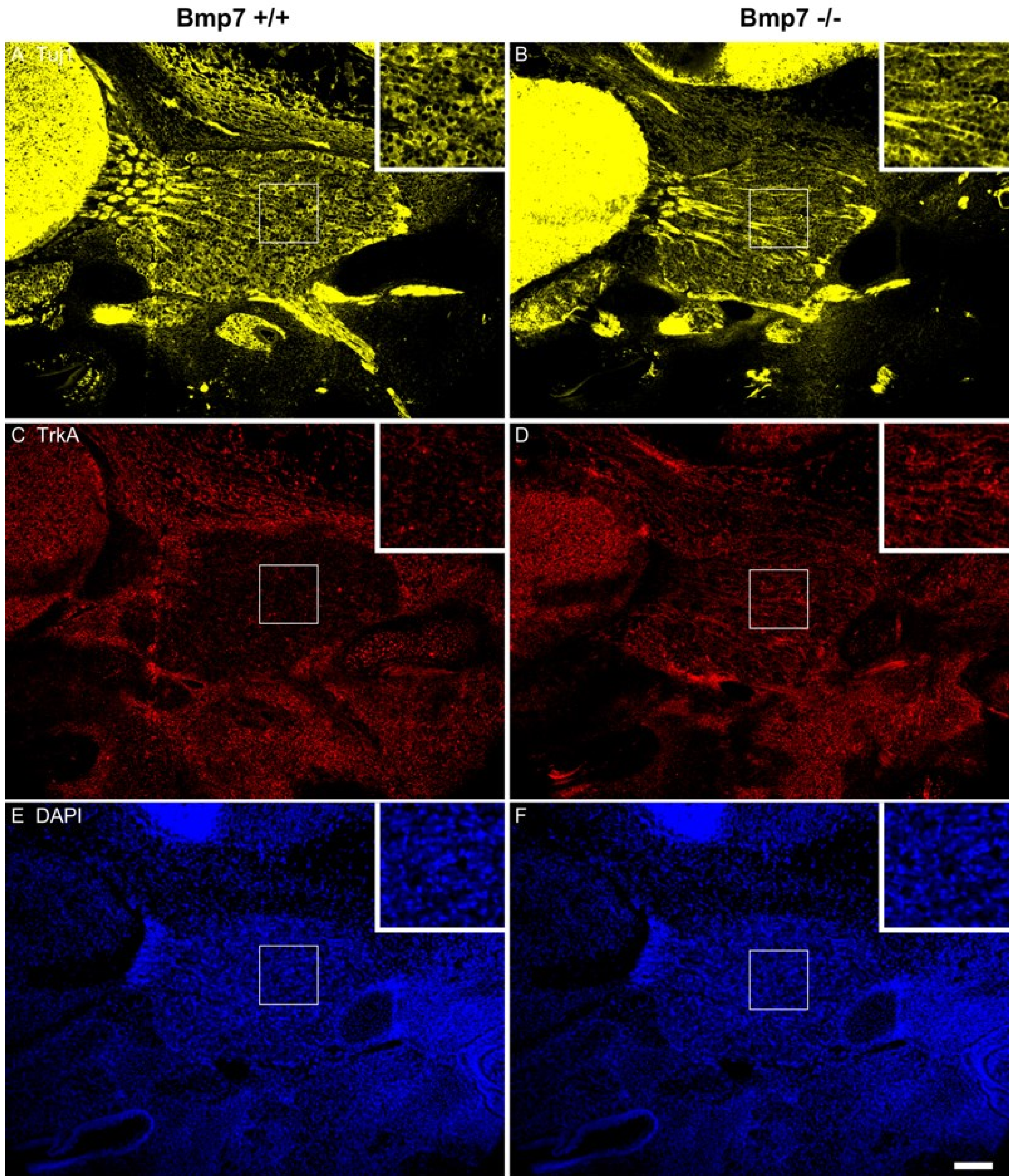

Figure 4.11: E14.5 trigeminal ganglia showing TrkA expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

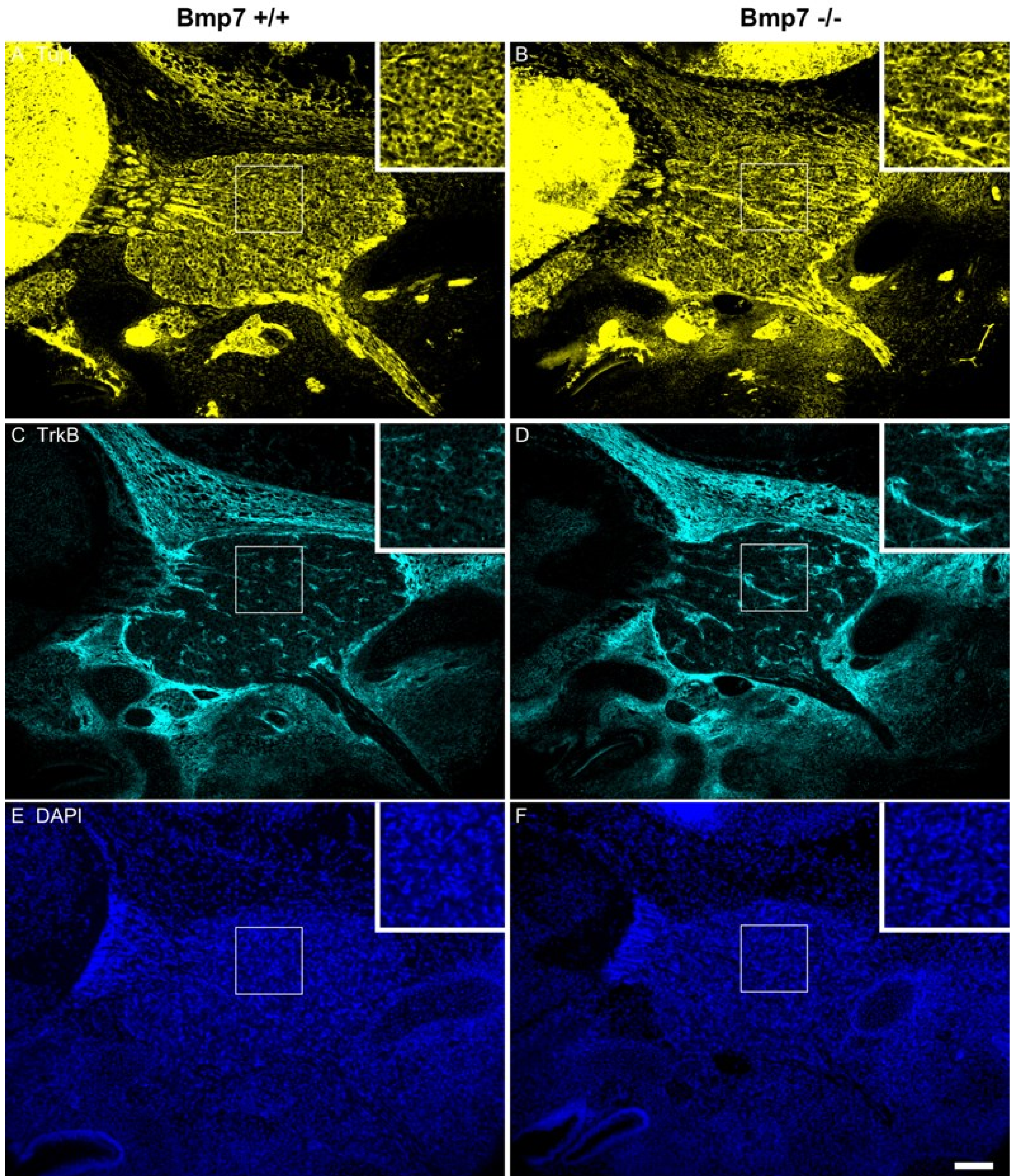

Figure 4.12: E14.5 trigeminal ganglia showing TrkB expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

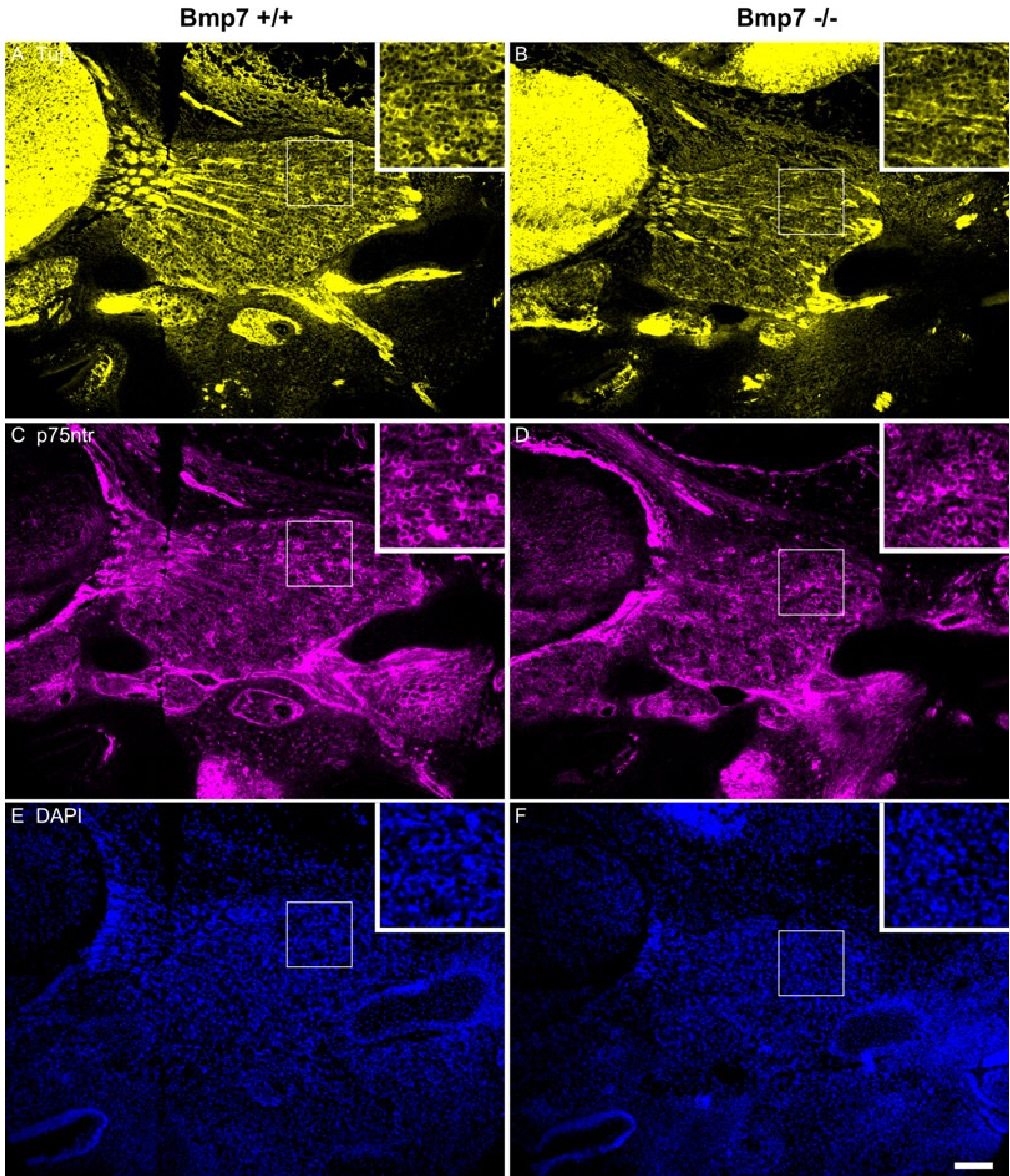

Figure 4.13: E14.5 trigeminal ganglia showing p75ntr expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

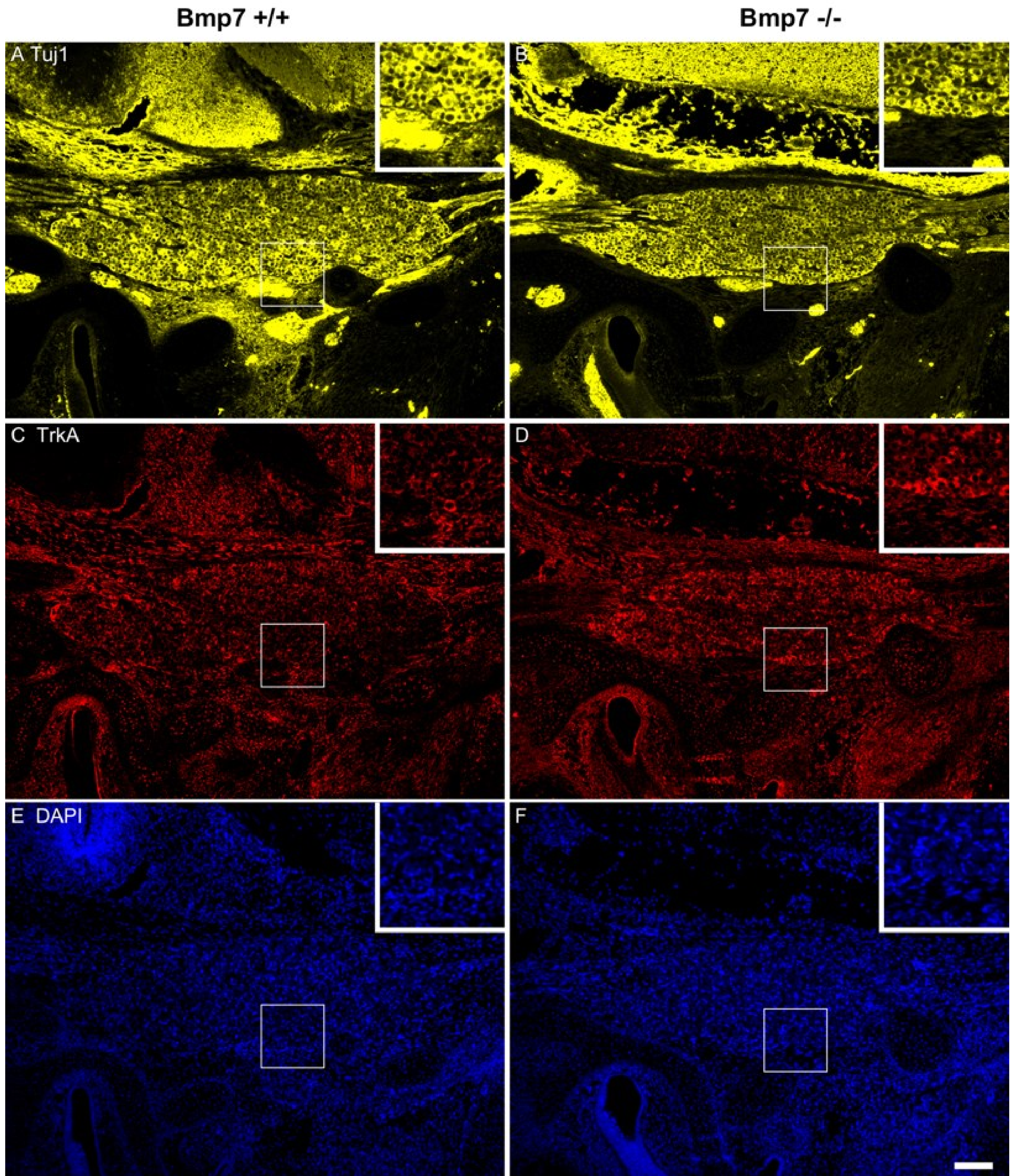

Figure 4.14: E15.5 trigeminal ganglia showing TrkA expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

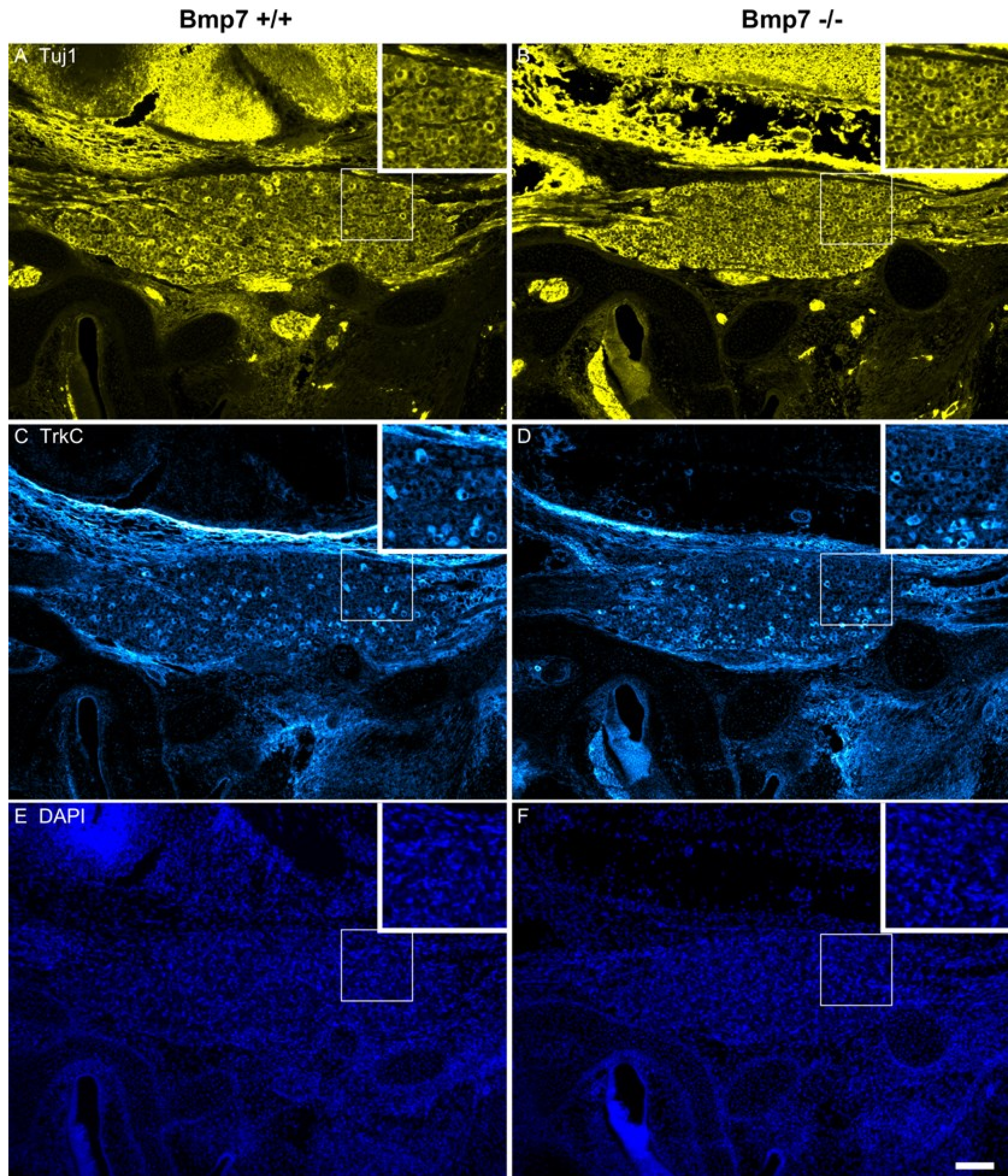

Figure 4.15: E15.5 trigeminal ganglia showing TrkC expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

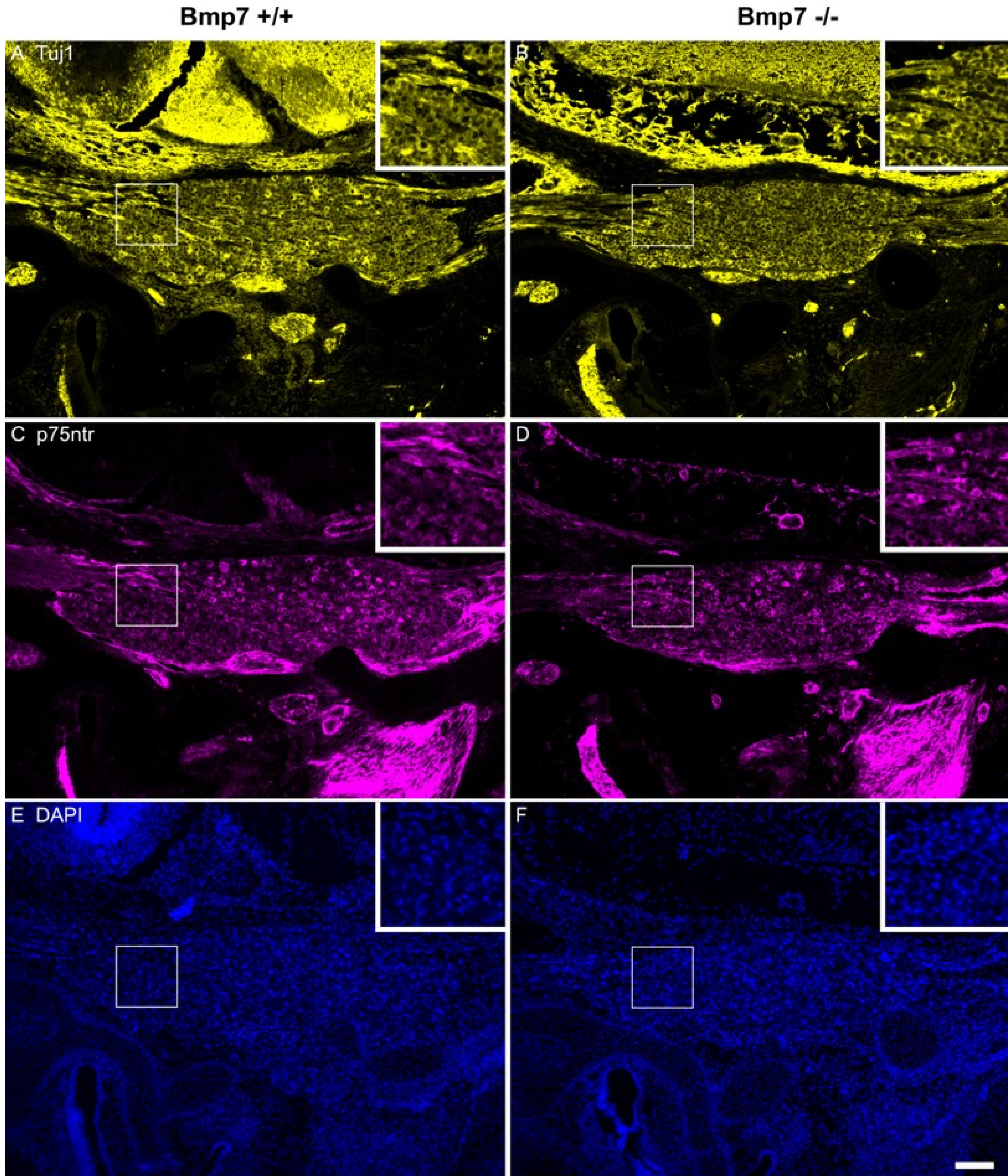

Figure 4.16: E15.5 trigeminal ganglia showing p75ntr expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

Phospho-Smad activity in trigeminal ganglia of E12.5 and E13.5 Bmp7$^{-/-}$ embryos persists, perhaps suggesting the involvement of other Bmps

Next, we examined phospho-Smad1/5 activity in E12.5 and E13.5 trigeminal ganglia. Smad 1/5/8 is a downstream intracellular protein in the Bmp signaling cascade that is phosphorylated when Bmps bind their receptors. It then translocates into the nucleus, where it regulates transcription of various genes. By examining phosphorylated Smad, we hoped to determine differences in downstream signaling and pSmad transcriptional activity between wild-type and mutant TGs. We did not find any obvious change in pSmad expression (Figures 4.17-4.18; on the next two pages).

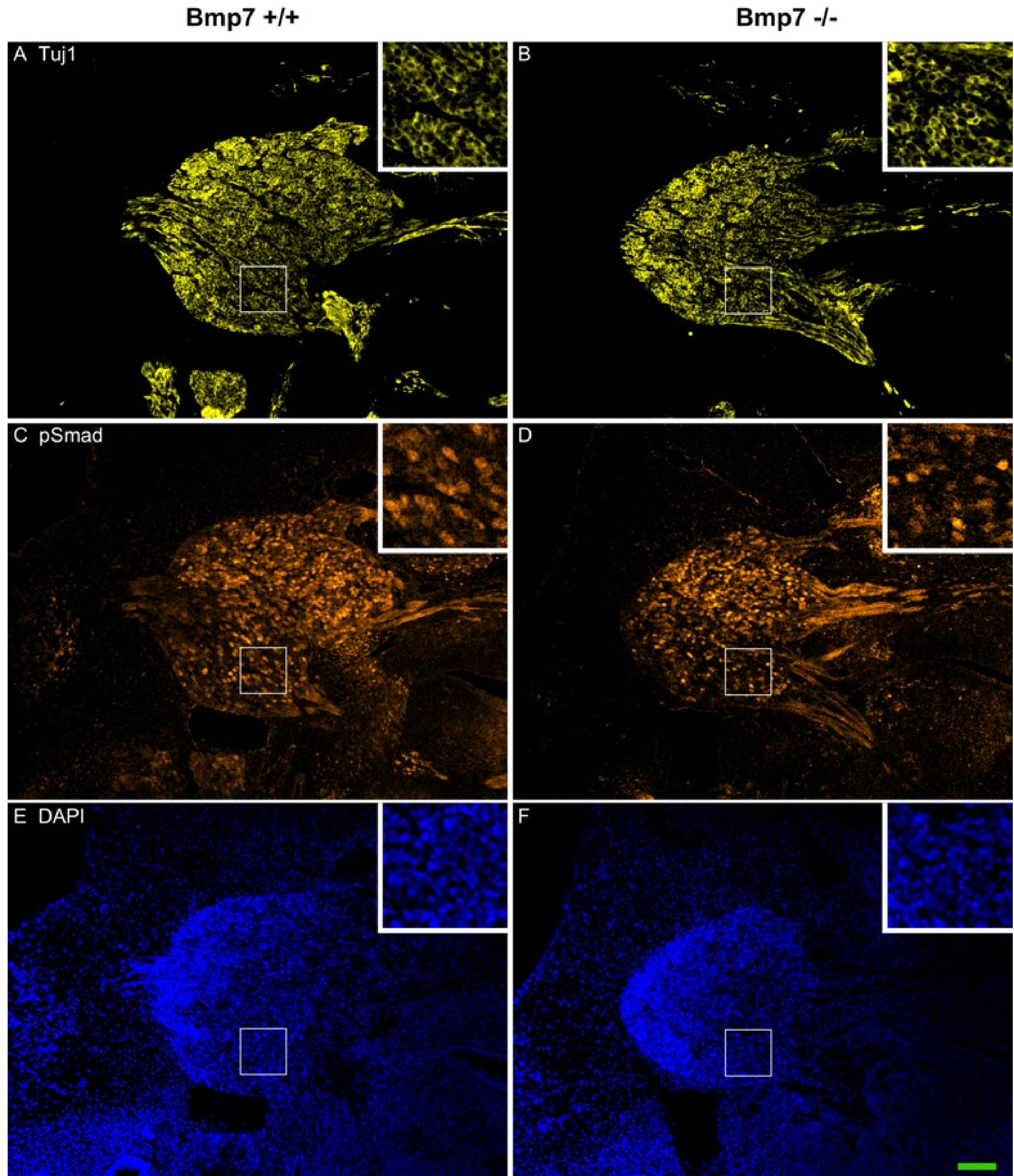

Figure 4.17: E12.5 trigeminal ganglia showing pSmad expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

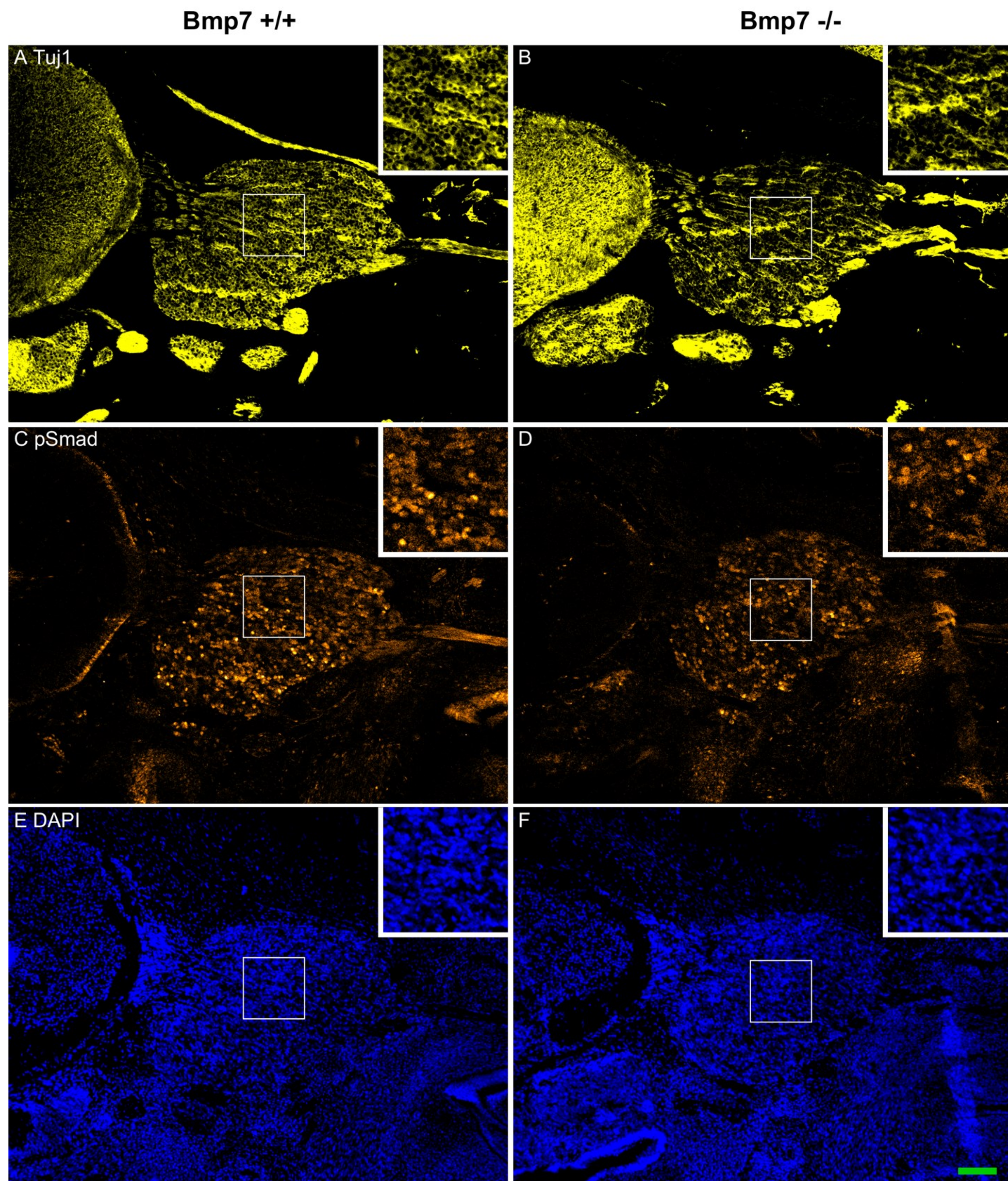

Figure 4.18: E13.5 trigeminal ganglia showing pSmad expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

Cleaved caspase 3 expression is similar in wild-type and mutant trigeminal ganglia at E15.5

Bmp7 from the meninges or trigeminal target fields could be promoting neuronal survival through a non-Smad pathway. So, we next examined neuronal apoptosis in the trigeminal ganglion at E15.5, a time after neurons have reached their peripheral targets and obtained survival factors. We used an antibody against cleaved caspase 3, an active executioner caspase that cleaves proteins during either extrinsic or intrinsic apoptosis. We found that neuronal death was comparable between wild-type and mutant mice as determined by a similar expression pattern of cleaved caspase 3 (see Figure 4.19).

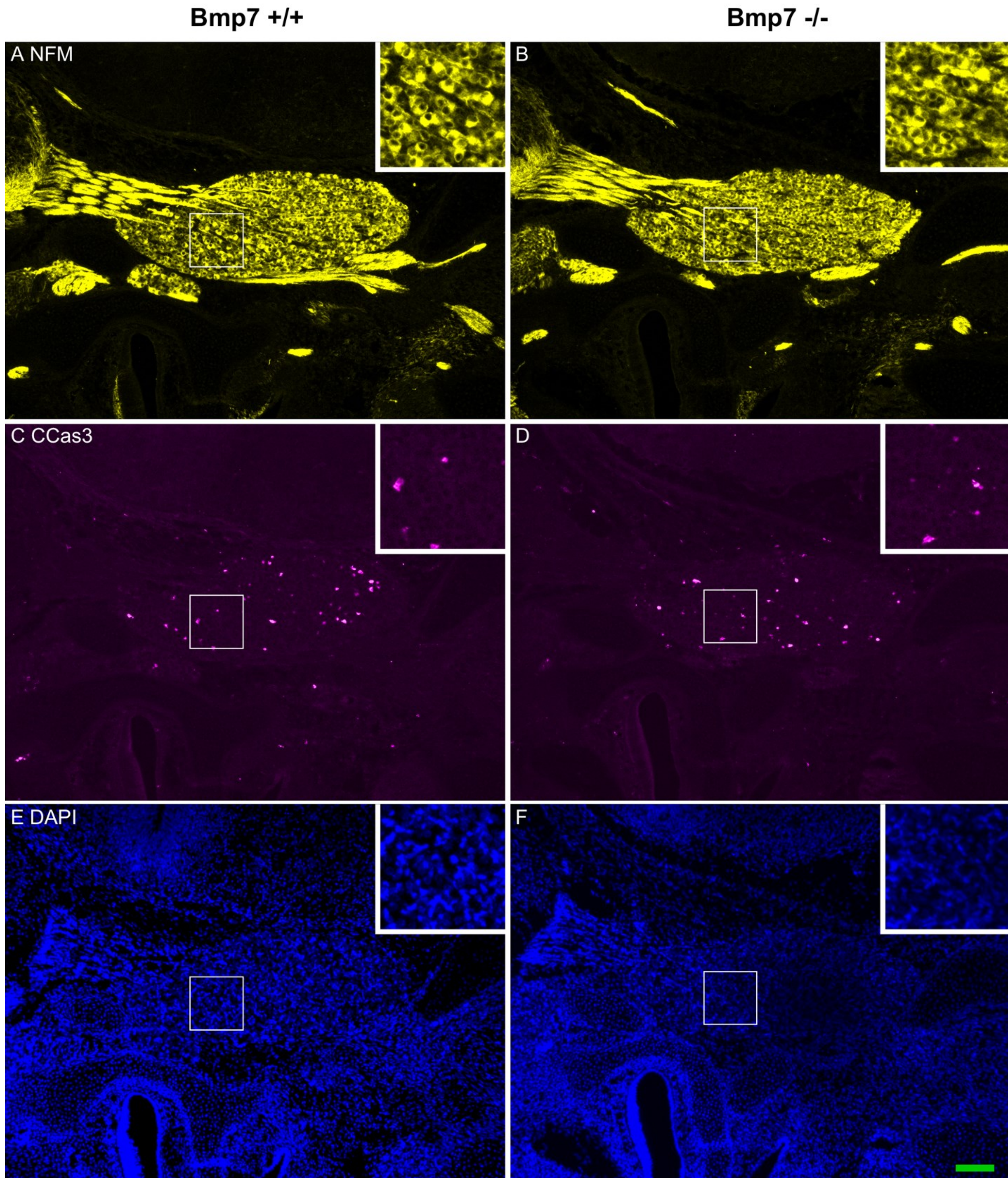

Figure 4.19: E15.5 trigeminal ganglia showing pSmad expression. An antibody against medium chain neurofilament was used to identify the trigeminal ganglion (NFM). DAPI was used to stain DNA. CCas3 is cleaved caspase 3. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

E14.5 Trigeminal ganglia of embryos with a Bmp7 deletion in the neural crest (Wnt1-cre) display greater nociceptor character and more precursor associated molecules than their wild-type counterparts

Because the cellular makeup of the trigeminal ganglion and its targets consists of neural crest-derived cells, and because Bmp7 deficient embryos die at birth, we decided to examine the TGs of mice lacking Bmp7 in the neural crest. We performed a similar qPCR reaction on isolated E14.5 TGs as was described earlier for Bmp7 deficient embryos. We found that in addition to increases in pain associated proteins (TrkA, Runx1, Ret, TrpV1), transcription factors denoting mechanosensory neuronal precursors were also upregulated (Runx3). See Figure 4.20 below.

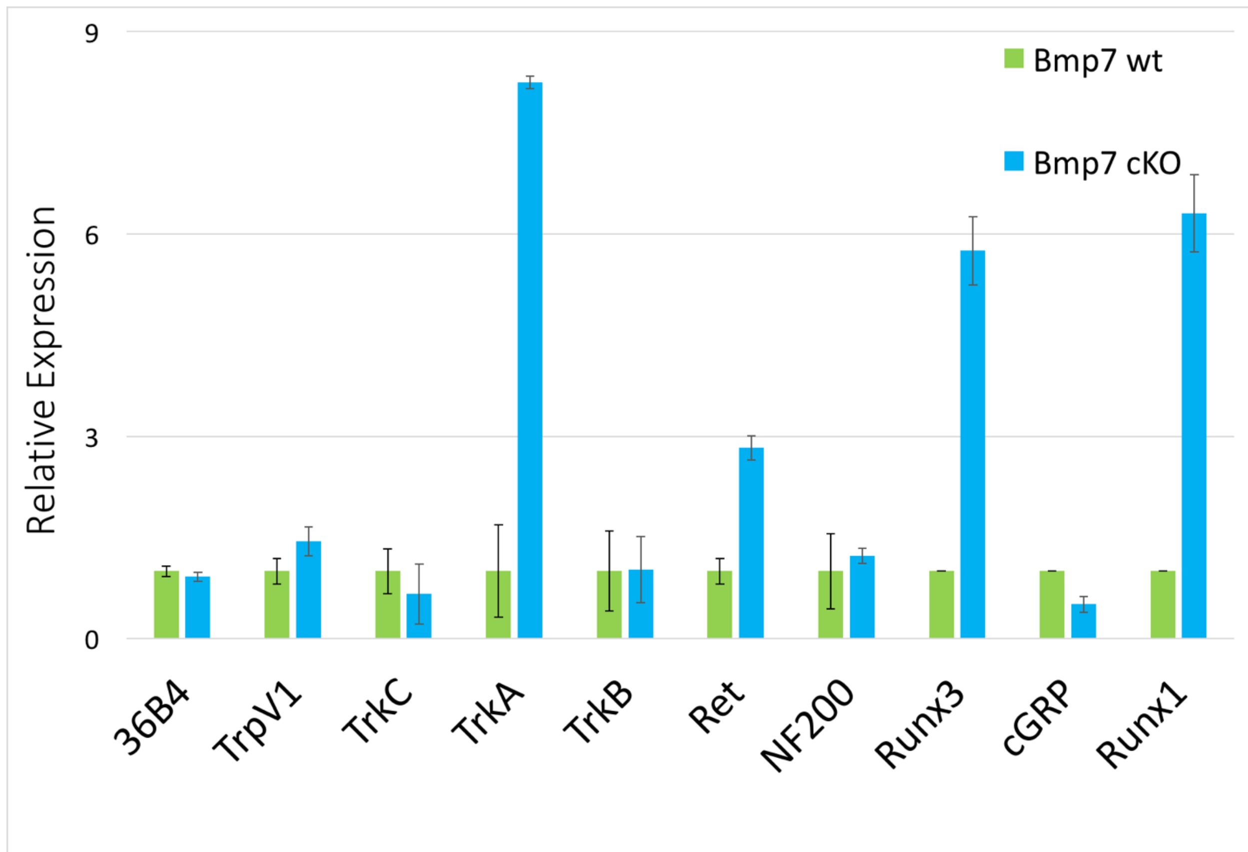

Figure 4.20: Gene expression profile of isolated E14.5 $Bmp7^{fl/fl}$ and $Bmp7^{fl/fl}$ Wnt1-cre trigeminal ganglia. Wnt1-cre TGs show increased TrkA, TrpV1 as well as Ret, and Runx1 expression corresponding to a greater nociceptive neuronal identity. Runx3 is also upregulated in condition mutants showcasing a greater mechanoreceptor precursor identity. Wt: wild-type, cKO:

conditional neural crest knockout. Error bars represent the standard deviation from average Cq values. This experiment was only conducted once.

Embryos with a Bmp7 deletion in the neural crest (Wnt1-cre) also show smaller and misshapen trigeminal ganglia

Following our observations in Bmp7 deficient embryos and the qPCR findings in the Wnt1-cre knockout, we thought that we would observe similar size and shape differences in the trigeminal ganglia Bmp7$^{fl/fl}$ Wnt1-cre mice. Therefore, we examined E15.5 embryos for the same neurotrophin receptors (TrkA, TrkB, TrkC, and p75^{ntr}). We found that conditional mutants have a slightly smaller sized, and different shaped TG than their corresponding wild-type embryos (see Figures 4.21-4.24).

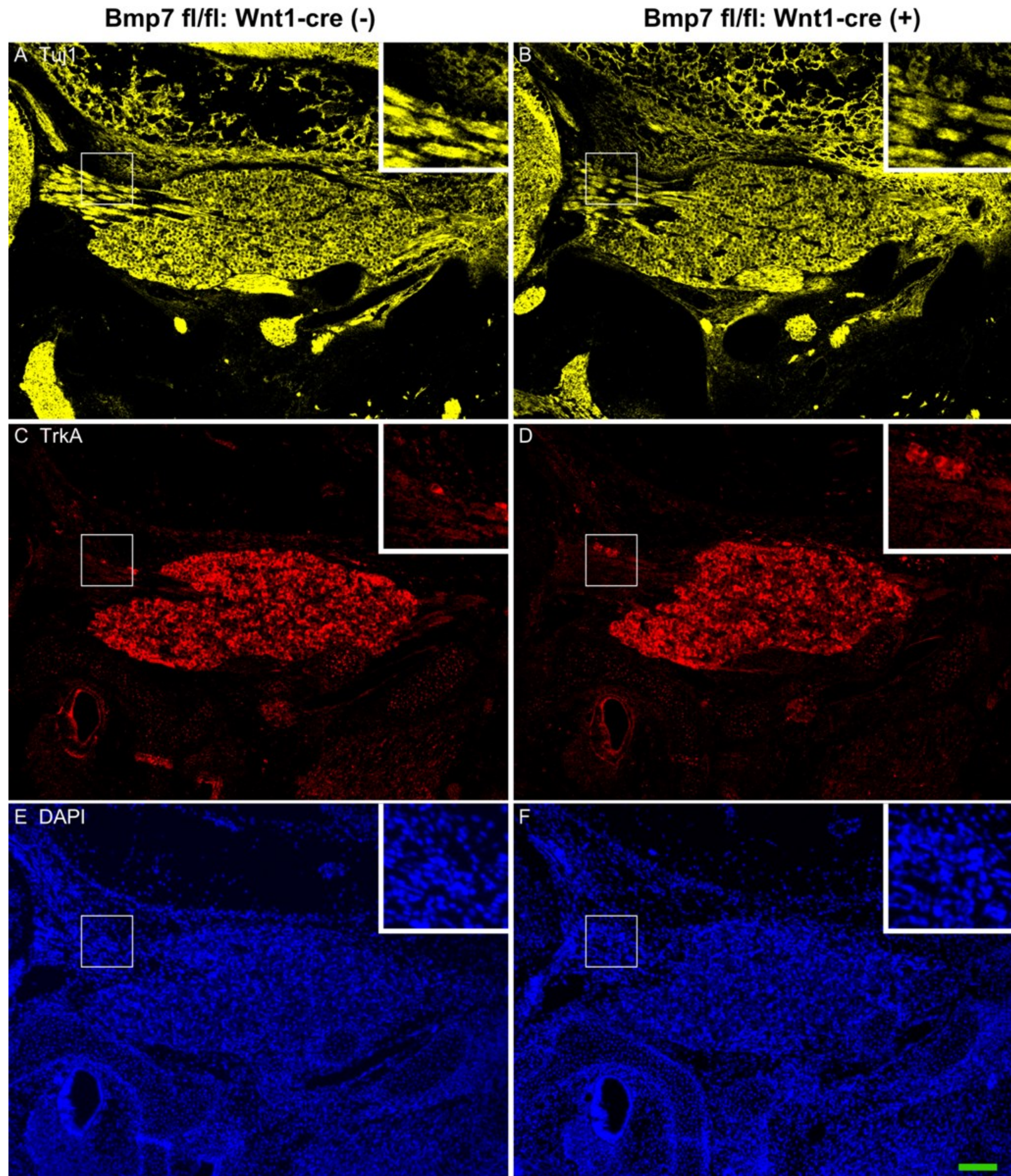

Figure 4.21: E15.5 trigeminal ganglia showing TrkA expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

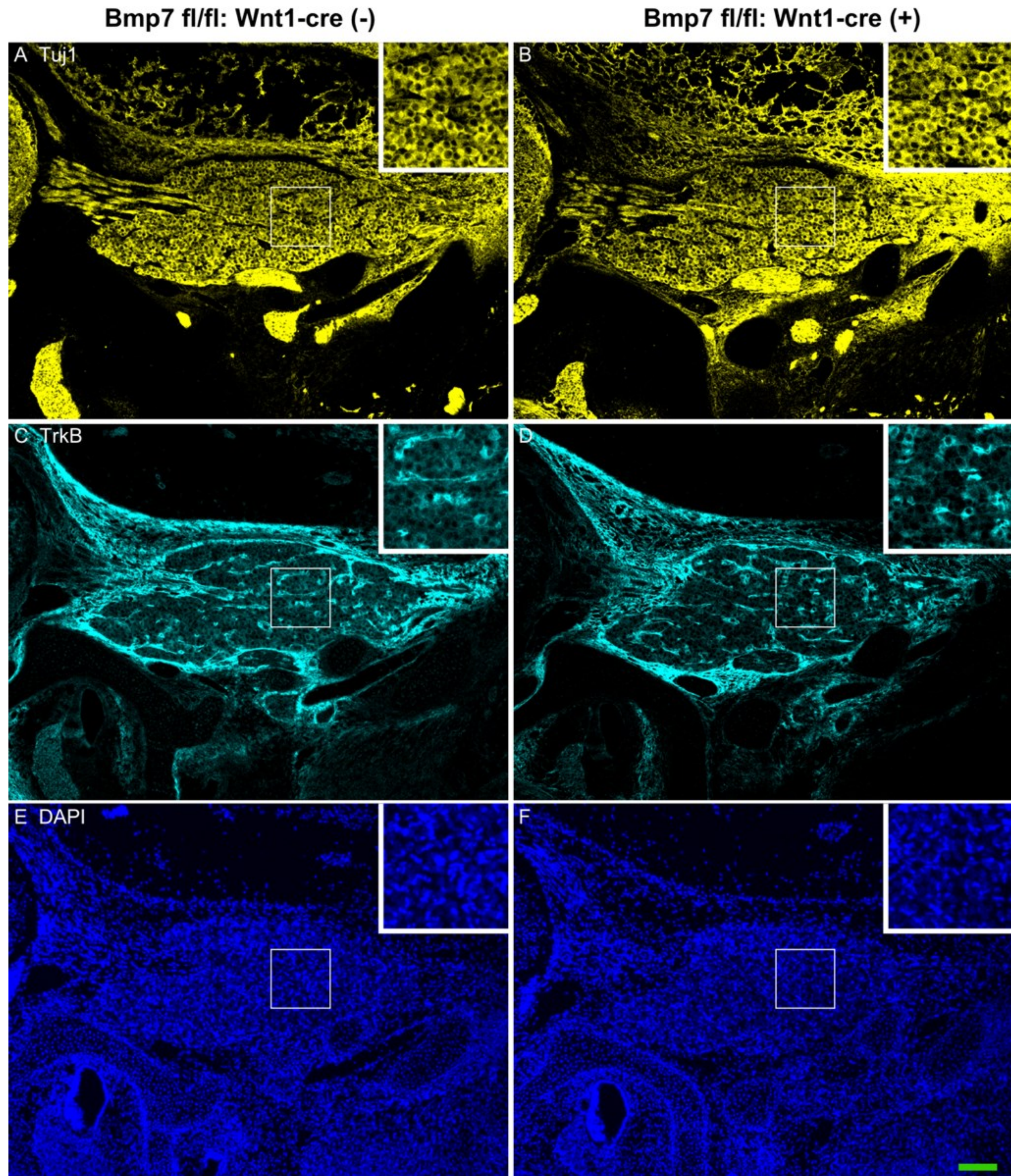

Figure 4.22: E15.5 trigeminal ganglia showing TrkB expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

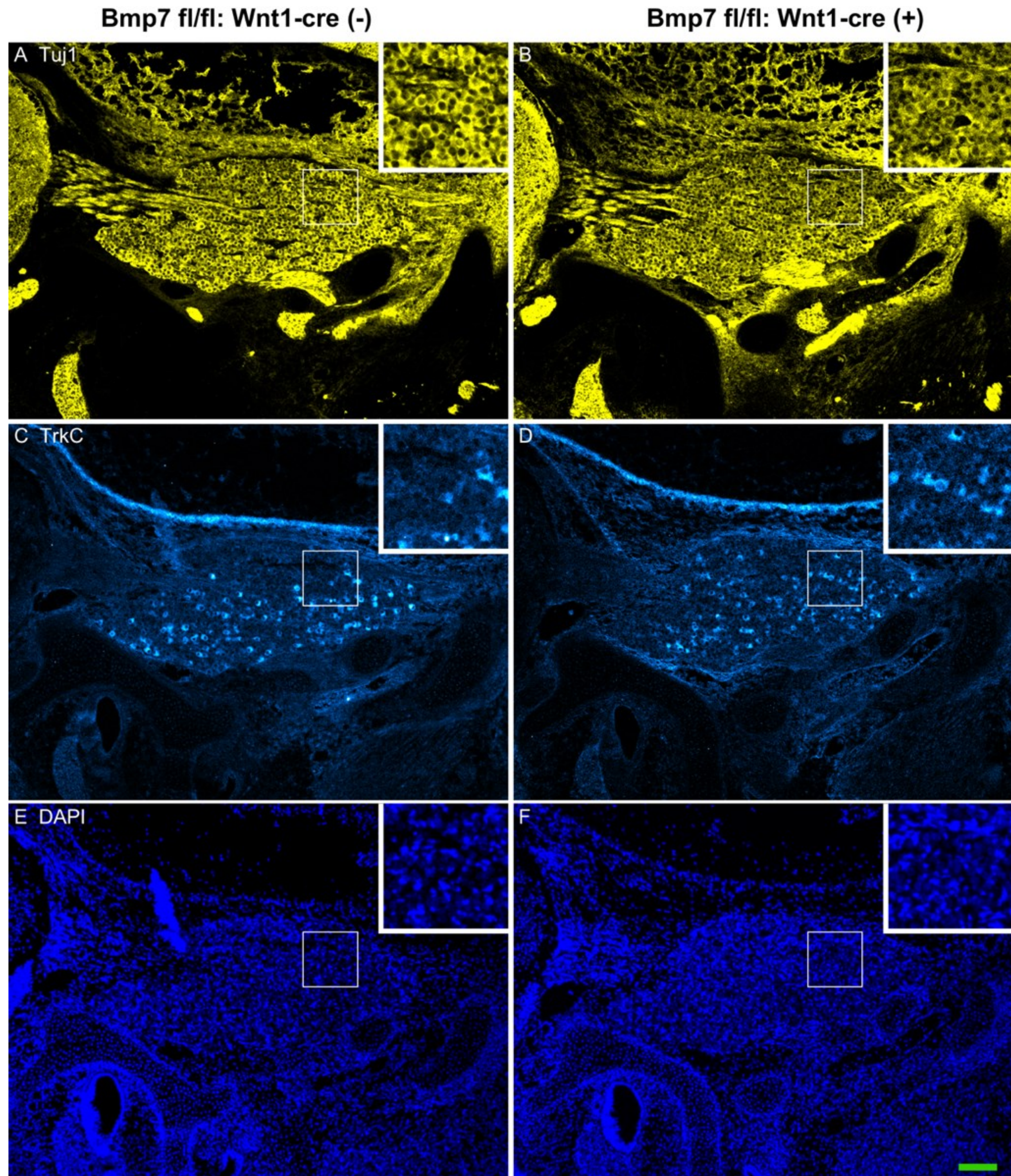

Figure 4.23: E15.5 trigeminal ganglia showing TrkC expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100µm. Brightness and contrast settings for wild-type and mutant sections are different.

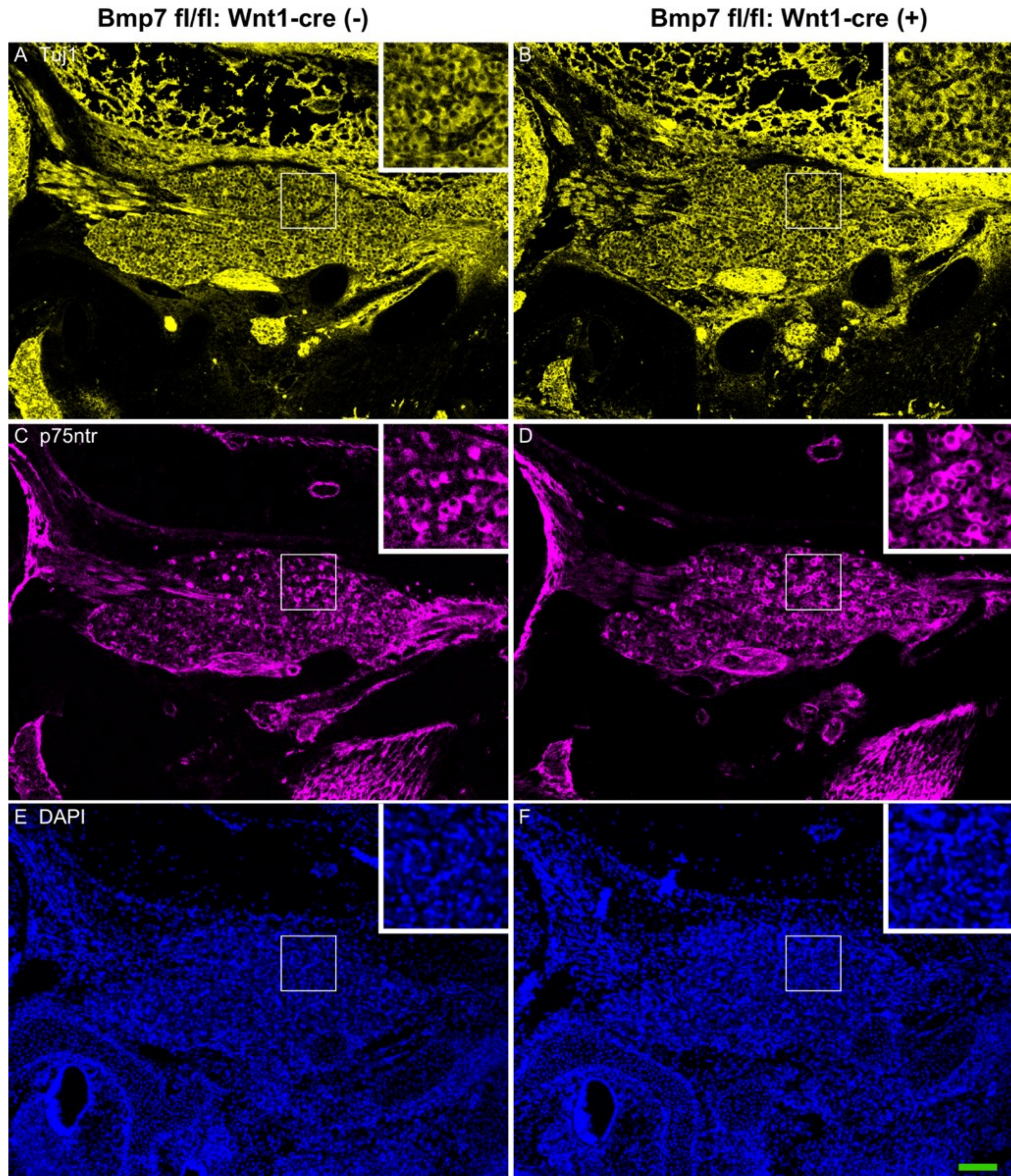

Figure 4.24: E15.5 trigeminal ganglia showing p75ntr expression. An antibody against beta III tubulin, a microtubule subunit expressed in neurons, was used to identify the trigeminal ganglion (Tuj1). DAPI was used to stain DNA. Scale bar is 100μm. Brightness and contrast settings for wild-type and mutant sections are different.

Adult mice with a neural crest deletion of Bmp7 show decreased mechanosensation but increased pain sensitization in the air puff assay

Unlike Bmp7$^{\Delta/\Delta}$ mice which die at P0, conditional knockout mice survive after birth and can have their trigeminal ganglia physiologically tested. Following the observed molecular, size, and shape differences in the TGs of neural crest deleted Bmp7 mice, we hypothesized that adult mice would have altered nerve function. We conducted a physiological assay that consists of applying an air current near the whisker pad of mice and scored their reactions. Figure 4.25 shows the difference in behavioral reactivity of the mice to the 15psi air puff directed at their whiskers. Figure 4.26 shows a scatter plot and radar plot that demonstrates a bimodal response in the mutants.

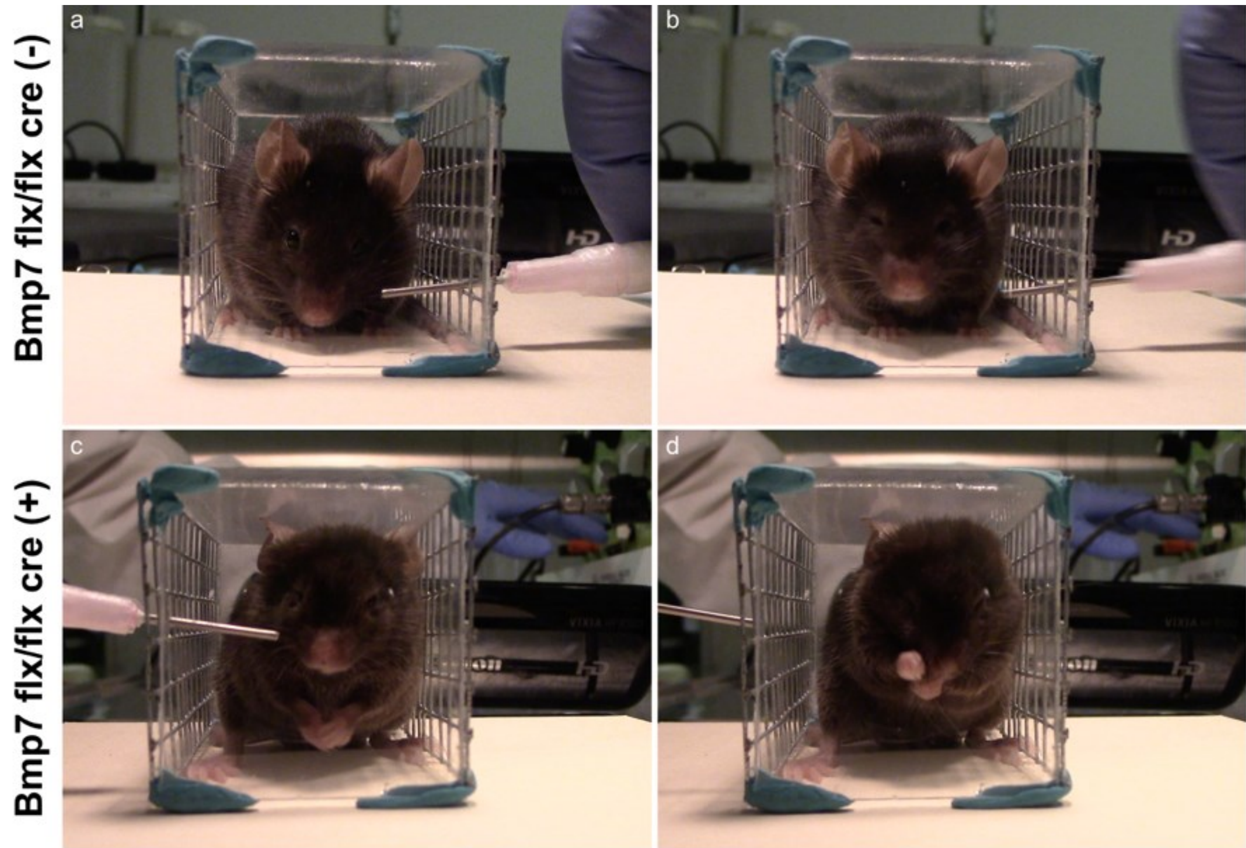

Figure 4.25: Air puff assay on wild-type (a-b) and conditional knockout mice (c-d). In response to the air puff, wild-type mice squint and withdraw their head, while conditional mutant mice either don't respond or wipe their cheeks (d). Five wild-type and four mice were tested.

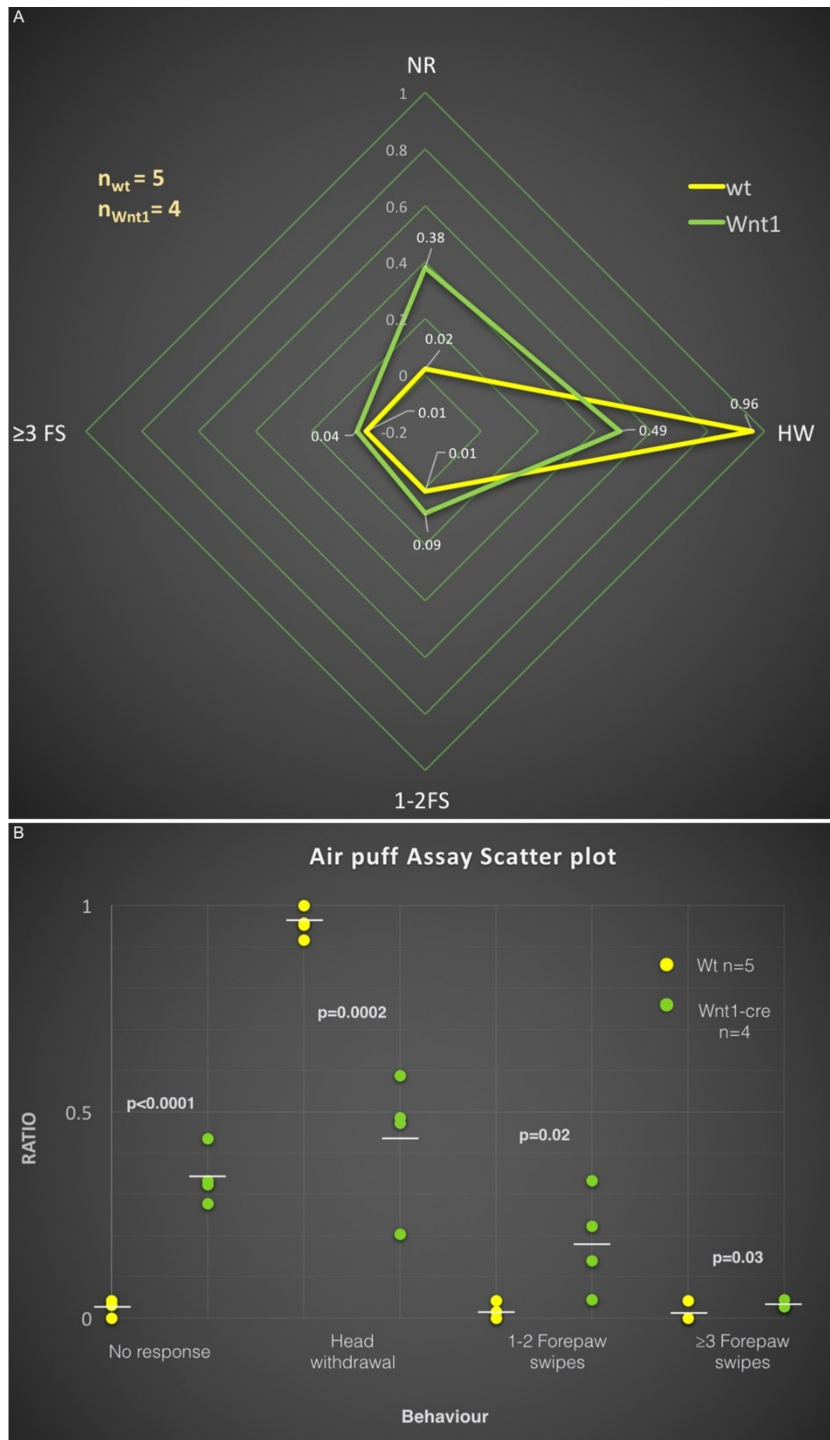

Figure 4.26: Wnt1-cre mice demonstrate a bimodal response to an air puff stimulus. A is a radar plot that demonstrates the average ratio of each behavior for the wild-type and mutant mice (the white horizontal line in the scatter plot below), B is a scatter plot where each point represents a mouse. Wild-type mice are represented by yellow points while mutant mice by the green points. Ratios in the scatter plot were obtained by dividing the occurrence of one behavior by the total number of occurrences of all behaviors for each mouse. NR: no response; HW: head withdrawal; 1-2FS: 1-2 forepaw swipes; ≥3FS: 3 or more forepaw swipes. Five wild-type and four mutant mice were tested. P-values for a one-way ANOVA between each mouse for each behavior are shown above.

Chapter 5: Discussion

Our understanding of sensory neurogenesis and sensory subtype specification has advanced considerably in the last couple of years, particularly because of better genetic tools. However, despite this advancement, there are many gaps in our knowledge. We currently do not know whether the meninges play an important role in the development of the trigeminal ganglion, or if they simply cushion and protect it from physical damage (or even a combination of both). Furthermore, although many studies have shed light onto our understanding of neuron-target cell interactions, we do not know the detailed mechanisms of retrograde signaling by which target cells influence nerve subtype specification. Bmp7 is an interesting extracellular protein that may regulate sensory nerve subtype specification. Mice lacking Bmp7 have a smaller and misshapen TG, Bmp7 is expressed in the meninges and trigeminal target fields, mRNA associated with specific nerve subtypes are altered in Bmp7 deficient and in neural crest deleted Bmp7 mutants (Wnt1-cre), and behavior assays show that Wnt1 mutants are mechano-insensitive and pain hypersensitive. Together, these results demonstrate that Bmp7 is a regulator of sensory nerve development and pave the way for future investigations to identify mechanisms.

First, the trigeminal ganglion of Bmp7 deficient mutants is morphologically altered. It is smaller and slenderer in shape than the trigeminal ganglia of wild-type littermates (Figure 4.1 and 4.5-4.16). At earlier stages of development, such as E12.5, there aren't any ossified bones, and the neural crest-derived meninges surrounding the trigeminal ganglion are not fully formed[110] nor do they express Bmp7[106]. Consequently, the modified morphology of the TG that is observed as early as E12.5 in Bmp7 deficient mutants (Figures 4.15-4.17) must be attributed to an earlier developmental event that requires Bmp7. One of two possibilities exist. Either Bmp7 expressed in early migrating neural crest is important for maintaining a neural precursor niche along with Wnt[87] signaling, or neuroectoderm derived[111] precursors require Bmp7 for expansion and survival. In either case, if Bmp7 is deleted, precursors might not undergo many rounds of self-renewal and instead would exit the cell cycle to differentiate. When Bmp7 was deleted from the neural crest using the Wnt1 promoter, minor morphological alterations to the trigeminal ganglion were observed (Figures 4.21-4.24). It was not possible to examine a neuroectoderm specific deletion of Bmp7, so a non-neural crest source of Bmp7 for precursor expansion and organization cannot

be ruled out. Nonetheless, these results indicate that Bmp7 and neural crest-derived Bmp7 are important for regulating the size and shape of the trigeminal ganglion.

As development progresses, cartilage and bone begin to be formed, and brain development accelerates, resulting in applied forces to the TG that could then influence its morphology. The development of the greater wing of the sphenoid, on which the TG sits and sends forth its branches through foramen, will certainly play an important role in modulating its shape. These phenomena could therefore explain the shape differences observed at later stages (E14.5-E15.5) in both Bmp7$^{\Delta/\Delta}$ and Bmp7$^{fl/fl}$ Wnt1-cre mice.

Second, Bmp7 is expressed in the meninges and tissues innervated by the trigeminal ganglion. Figure 4.2 shows Bmp7 expression in the meninges from E13.5 to E18.5. Furthermore, although not shown, Bmp7 was found to be expressed in the meninges of adult mice long after nerve development ceases. Because Bmp7 expression in the meninges persists even after the completion of nerve differentiation, we conclude that Bmp7 in the meninges is serving a structural, rather than a developmental role. It could be a vital extracellular component that helps organize and maintain the integrity of the meningeal ECM.

Additionally, Bmp7 was also found to be expressed in the epithelium and vibrissae of E13.5-E14.5 embryos (Figure 4.3). Many tactile and pain receptors are housed in these organs and are innervated by the TG. Below is a figure that shows the organization of tactile receptors in the skin.

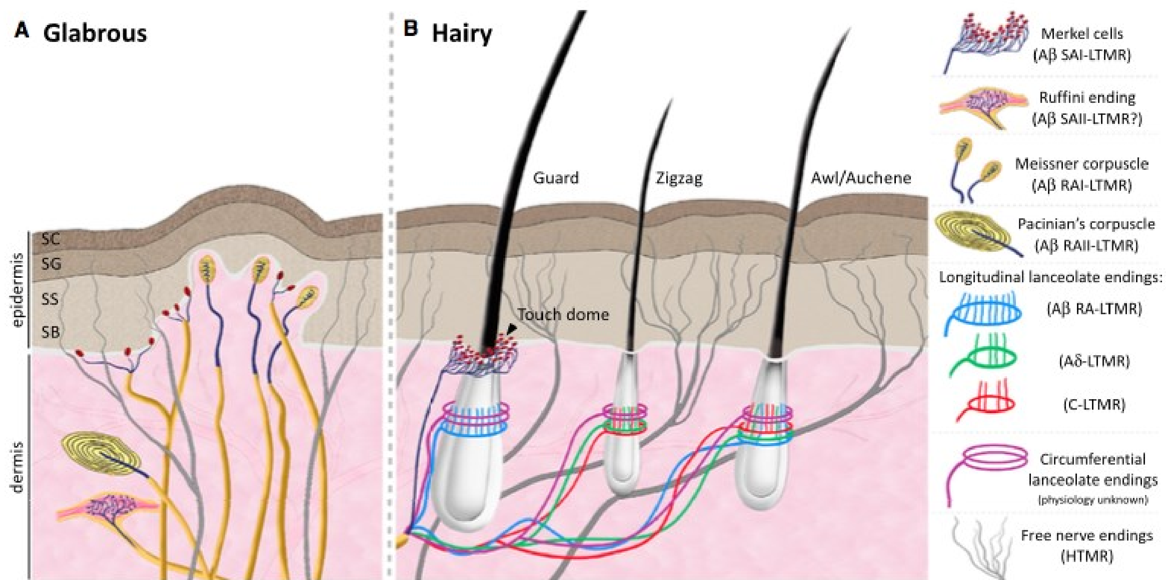

Figure 5.1[112]: Organization of mechano- and nociceptive neurons in the skin. Free nerve endings pass into the epidermis while most mechanoreceptors remain in the dermis reprinted from The sensory neurons of touch. Neuron*, 79(4), Abraira, V. E., & Ginty, D. D., 618-639, (2013) with permission from Elsevier*

Bmp7 from the skin could be modulating nerve subtype specification in the TG by retrograde signaling. As was discussed in Chapter 1.9, Bmps could be functioning with neurotrophins to regulate nerve development. Evidence for this comes from multiple sources that support a Bmp involvement in neurotrophin signaling [76,100,107]. Additionally, Bmp7 is expressed in regions where both NGF[63,113] and NT3[67] are expressed, meaning that it is spatial possible for them to regulate nerve development in concert. Bmp7 signaling could promote a mechanoreceptor phenotype in mesenchyme along with NT3, and a nociceptor phenotype in the epidermis with NGF.

Third, the qPCR data demonstrating relative quantitative gene expression for various nerve subtype associated receptors, transcription factors, and peptides in Figure 4.4 corroborates this hypothesis and points to a role for Bmp7 signaling in nerve specification. In this experiment, trigeminal ganglia of E14.5 embryos from wild-type and mutant mice were isolated and compared for various nerve subtype associated receptors, transcription factors, and peptides. TrkA, CGRP (calcitonin gene-related peptide), and TrpV1(capsaicin receptor; transient receptor potential

cation channel) were all upregulated in the mutant. Because these molecules are associated with peptidergic C-fibers[114] (see intro), a type of nociceptive neuron, the removal of Bmp7 from all cells (including the epidermis and dermis) might favor the development of this population. Interestingly, when Bmp7 is deleted from the neural crest, the qPCR results demonstrate an upregulation of TrkA, TrpV1, Runx1, Ret, and Runx3, but downregulation of CGRP (Figure 4.20). At first, these results seem to be conflicting. The dermis is neural crest cell derived[115] and houses mechanoreceptors. The epidermis is ectoderm derived and houses nociceptors. Maybe when Bmp7 is deleted in the neural crest (Wnt1-cre), and hence the dermis, mechanoreceptors do not differentiate properly and consequently, mechano-precursors in the TG are upregulated (increased Runx3 expression). If this were true, then Bmp7 maybe potentiating the effects of NT3 and BDNF in mechanoreceptor development. Simultaneously, Bmp7 expressed in the epidermis persists and perhaps, along with NGF, favors the development of nociceptors as demonstrated by an upregulation of TrpV1, Runx1, and Ret (Figure 4.20). If this is true, one would expect the opposite result in mice lacking Bmp7 in the epidermis. Mice with an epidermal deletion (K14-cre) of Bmp7 may have an upregulation of mechanoreceptor associated transcripts, while a down regulation of non-peptidergic neuron transcripts. This prediction seems to be validated by behavioral tests showing an increased tactile perception and a decreased heat response in K14 Bmp7 knockouts (Christine Webber, unpublished data). In mice with a complete deletion of Bmp7, Bmp7 is removed from the dermis and the epidermis. Hence, one would expect a decrease in non-peptidergic neurons and mechanoreceptors, but an increase in another population. This population seems to be peptidergic neurons, perhaps C-fibers (Figure 4.4). By removing Bmp7 from both the epidermis and the dermis, peptidergic neuron development may be favored.

Following the qPCR results, we conducted an immunohistochemical analysis of various neurotrophin receptors (TrkA, TrkB, TrkC, p75) in the trigeminal ganglia of E12.5-E15.5 embryos. β-III tubulin was co-labelled with the Trk receptors to identify neurons. No obvious difference in receptor expression or organization could be determined in either Bmp7 deficient (Figures 4.5-4.16) or neural crest-specific mutants (Figures 4.21-4.25). These results seem to conflict with previous qPCR findings. However, there are several possible explanations. First, immunohistochemistry is not a quantitative technique, so one cannot compare relative decreases

or increases in expression unless they are substantial and observable. Second, many transcripts are made in the neuron cell body but are then localized to dendrites, axons, and axon terminals, where they are translated to proteins. This includes neurotrophins[116]. Therefore, protein expression might be increased in axons or axon terminals and not in the somas, where we looked. Third, mRNA transcripts might not be translated to proteins at all because of other regulatory mechanisms that maintain homeostasis. This might be true especially because immunofluorescent data did not show any observable changes in the expression of neurotrophin receptors. Furthermore, we only studied a few stages of nerve development; perhaps protein changes would be seen at later or earlier stages that we did not investigate. β-III tubulin expression was observed in neurons, but could also be seen in the meninges. The meningeal labelling might not be specific and the antibody may need to be verified.

We also performed immunohistochemical analysis for pSmad at E12.5 and E13.5 (4.17-4.18). Phospho-Smad is a downstream effector of the Bmp signaling cascade (see intro). No observable differences in pSmad expression between wild-types and mutants could be detected. However, because pSmad was observed, other Bmps might also be important for nerve development. Once we determined that pSmad expression was similar, we looked to see if apoptosis was greater in TGs lacking Bmp7 and if neuron death of a specifc subtype might account for the nerve subtype differences observed in the qPCRs. Using an antibody against cleaved caspase 3 (an active executioner caspase that cleaves cysteine-aspartic acid peptide bonds during apoptosis), we could not detect any qualitative differences in cell death between wild-type and $Bmp7^{\Delta/\Delta}$ mutants (Figure 4.19). This suggests that more neurons are not dying in mutants, but are rather undergoing a lineage switch. Hence, neuronal precursors are plastic and can be differentiated to different subtypes depending on their developmental context.

Lastly, after finding molecular differences in the gene profiles of both $Bmp7^{\Delta/\Delta}$ and $Bmp7^{fl/fl}$ Wnt1-cre embryos, we wanted to investigate behavioral responses to sensory stimuli in adult mice. Because Bmp7 deficient mice die at birth, we performed the air puff assay on $Bmp7^{fl/fl}$ Wnt1-cre adult mice. The air puff assay is a physiological test that is conducted by applying an air current to the whisker pad of mice and monitoring their responses (Figure 4.25). Mice lacking Bmp7 in the neural crest demonstrate a bimodal response where their behavior shifts from head

withdrawal to either no response or to forepaw swipes, indicating a reduced ability to integrate tactile and painful stimuli (Figure 4.26). If one considers circuits in the medulla, then this change in behavior can be explained by the hypothesis proposed above, that Bmp7 in the epidermis is required for nociceptive nerve development, while Bmp7 in the dermis is required for mechanosensory nerve development. Below is one possible explanation of the observed responses with diagrams.

According to the famous gate theory of pain mechanisms, proposed by Melzack and Wall, painful stimuli can be inhibited by non-painful inputs[117]. For example, pruritus induced by a mosquito bite will seem less painful when we scratch it. The substrates for gate control theory exist. In the pons and medulla, first order mechanosensory neurons (Aβ-fibers) and nociceptive neurons (C-fibers) synapse onto an interneuron and a second order projection neuron via collaterals in the spinal nucleus of the TG. These second order neurons send their axons across the midline (decussate) at the level of the brainstem to join the spinothalamic tract and terminate in the contralateral ventral posteromedial nucleus (VPM) of the thalamus. The second order neurons have a baseline activity. Below is a diagram illustrating the circuit.

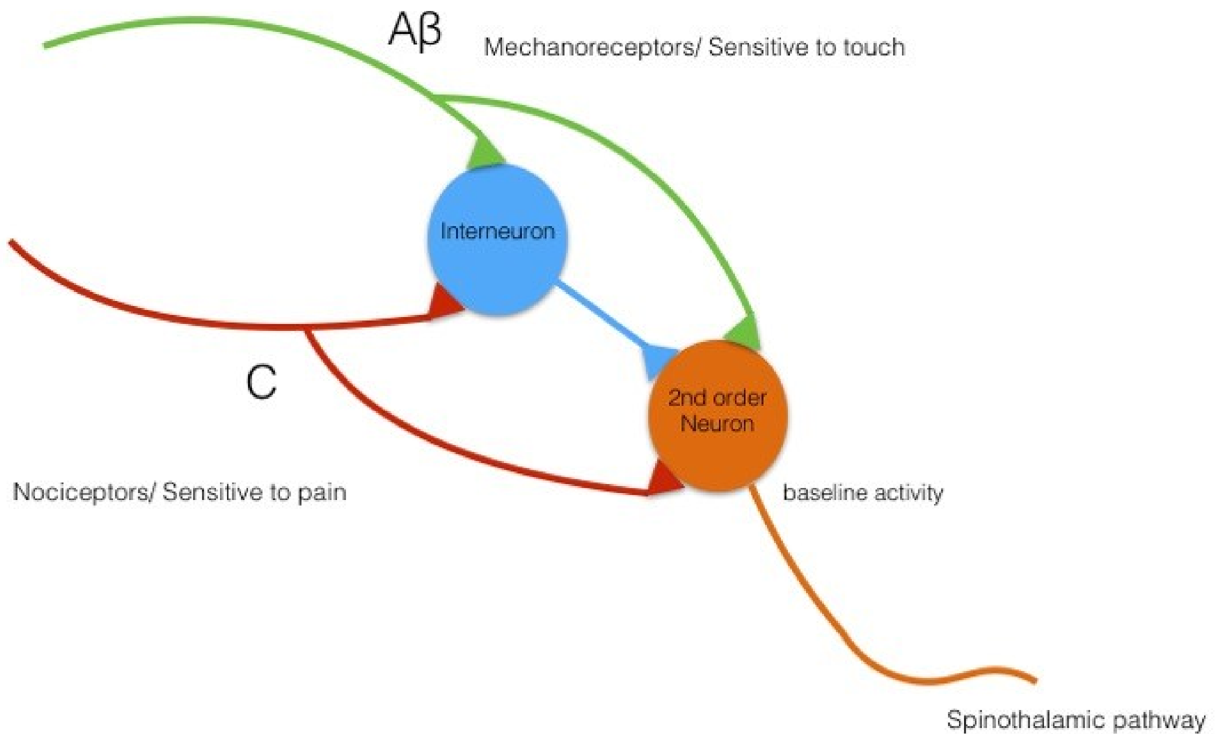

Figure 5.2: The circuit in the spinal nucleus of the TG. The second order neuron has a baseline activity. Positive signs (+) represent activation, while negative signs (-) represent inhibition.

In wild-type mice, the 15-psi air puff might activate most of the Aβ-fibers and a few C-fibers in the whisker pad. The Aβ-fibers might then activate the interneuron more than the C-fibers and the interneuron inhibits the second order neuron (because the inhibitory activity of the interneuron surpasses the activating activity of the C- and Aβ-fibers). Consequently, the mouse withdraws its head but does not swipe its whiskers (possibly mechanical sensitivity but not pain; see Figure 5.3).

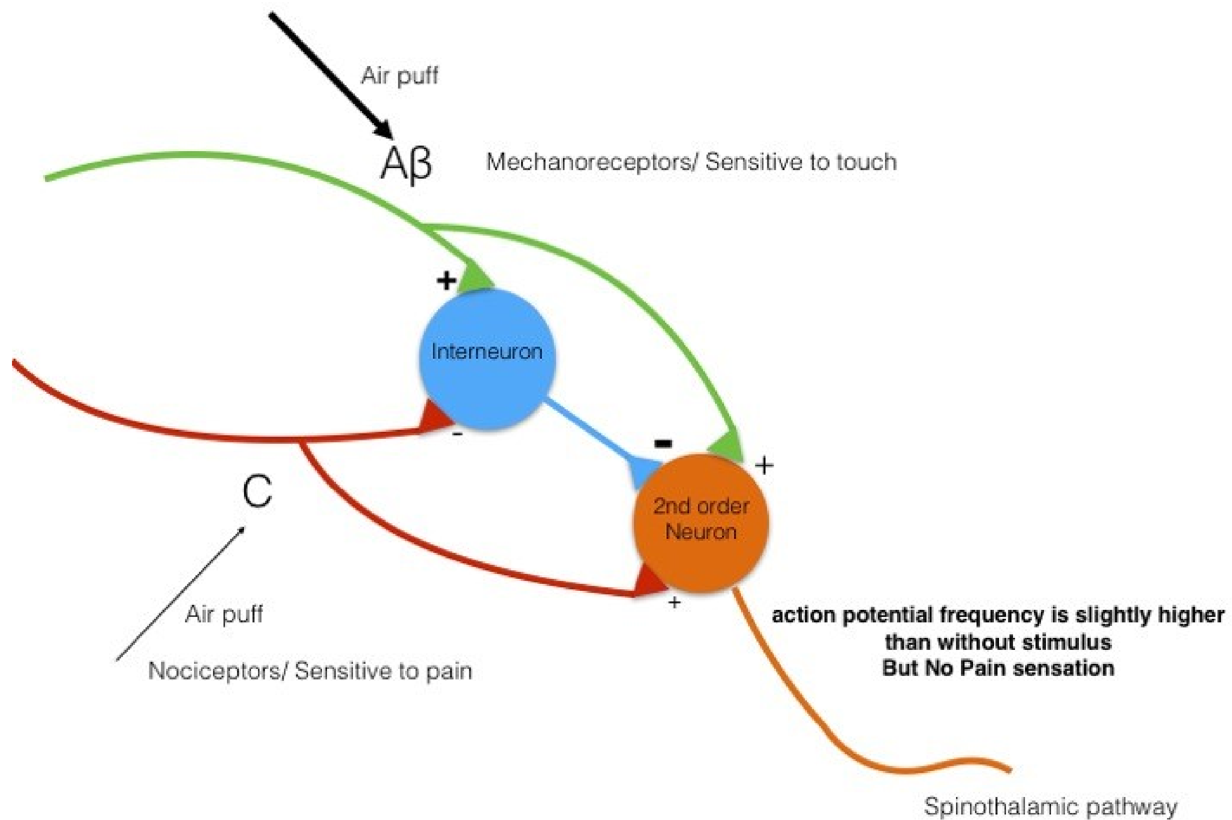

Figure 5.3: Neuron activity in response to the air puff stimulus in wild type mice

Based on my hypothesis, Bmp7 might be required for the proper formation of mechanosensory neurons (Aβ-fibers) in the dermis. Without Bmp7 in the neural crest (dermis) there is a disruption in the development of Aβ-fibers, consequently leading to a change in identity of those neurons and upregulation of nociceptor peptides (TrkA, TrpV1, Runx1, CGRP). In mutant mice, there could be less functional Aβ-fibers and more C-fibers activated in response to this same 15 psi air puff stimulus. Thus, conditional mutants might not always react to this mechanical stimulus (are mechanically less sensitive), and the interneuron may be inhibited leading to an increased probability for the second order neuron to fire. The mouse may then perceive this as a painful stimulus and rather than withdrawing its head, will swipe its whiskers (see Figure 5.4

below). However, Aβ-fibers need to be quantified in the dermis of both wild-type and mutant mice to support the gate control theory explanation of the observed behaviours.

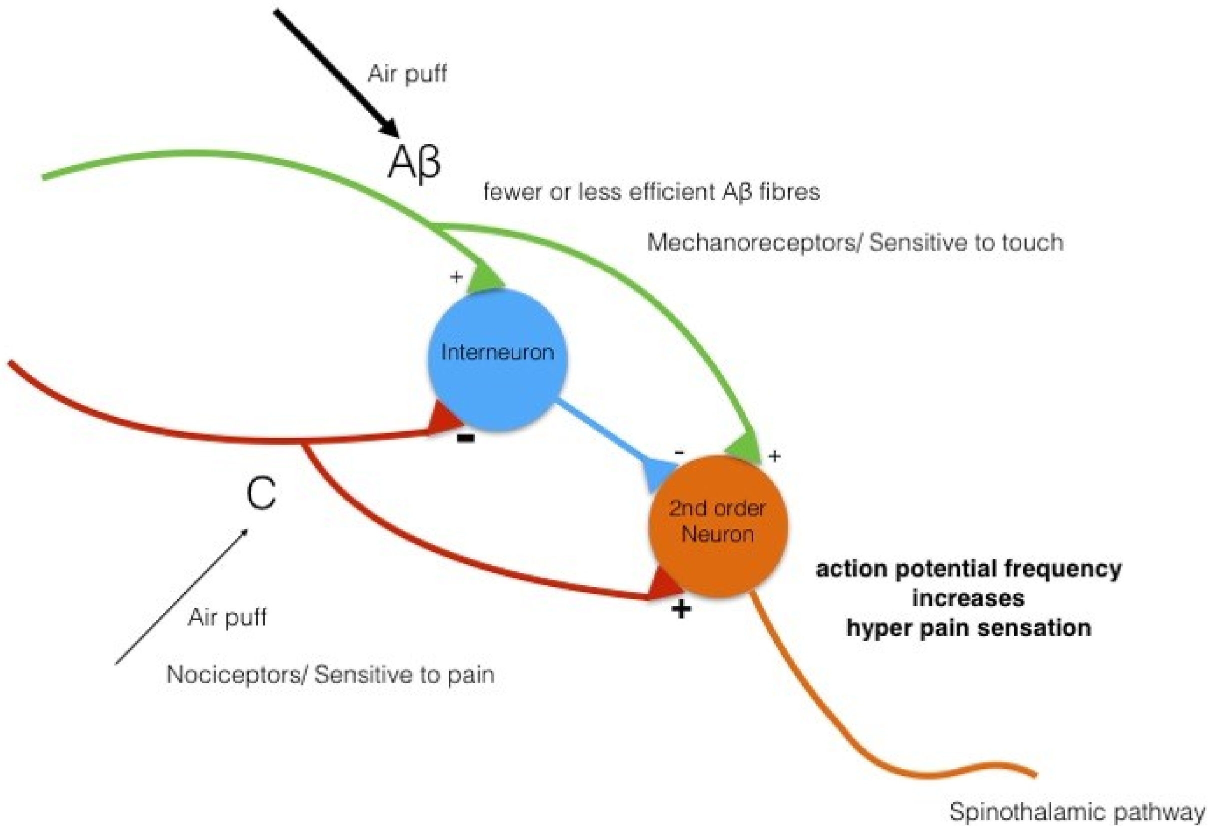

Figure 5.4: Neuron activity in response to the air puff stimulus in neural crest conditional knockout mice

Hence, the behaviour data might support the hypothesis that Bmp7 signaling is important for mechanosensory nerve development in the dermis and nociceptor nerve development in the epidermis.

The discussion above only represents one possible explanation for how Bmp7 signaling could be required for sensory neurogenesis. Alternatively, Bmp7 may be important for initiating the first wave of sensory nerve development with Wnt signaling by coordinating the precise timing of neurogenin 2 expression.

When β-catenin is mutated in mice, neurogenin 2 expression is reduced and peripheral sensory neurogenesis is inhibited[118]. Conversely, when β-catenin is constitutively expressed, neural crest cells express neurogenin 2 ectopically[119]. Together, these findings suggest that Wnt signaling plays a role in neurogenin 2 expression. As mentioned in Chapter 1.9, Bmp7 signaling is also important for the precise timing of neurogenin 2 activation. Neural precursors in the subventricular zone of the developing cortex require Bmp7 to express neurogenin 2 at E14.5[80]. Furthermore, Bmp7 signaling along with Lef1 signaling mediated by Wnts, is important for the development of the dentate gyrus[81,82]. Together these studies point to a possible role for Bmp7 and Wnt signaling in the development of neurons from precursors. Perhaps Bmp7 in combination with Wnt signaling is necessary for coordinating the expression of neurogenin 2, and the appropriate development of early pioneering proprioceptive and nociceptive neurons. In the absence of Bmp7, the qPCR findings presented in Figure 4.20 might then represent a delay in the development of those early neurons (as observed by precursor receptor and transcription factor overexpression). Further evidence supporting this hypothesis comes from genetic, neural crest lineage tracing experiments. When Bmp7 is deleted from all cells, there is a delayed innervation of peripheral tissues by sensory neurons (unpublished data, Graf lab). Further experiments will be needed to explore neurogenin 2 expression in the absence of Bmp7 and whether Bmp and Wnt signaling coordinate the development of the first wave of sensory neurogenesis.

Conclusion

The histological and morphological data combined with the analysis of Bmp7 expression, qPCR gene profiling, immunohistochemistry, and mouse behavioral data point to a role for Bmp7 as a protein that regulates nerve subtype specification. Trigeminal ganglia in Bmp7 mutants are smaller and slenderer. Bmp7 is expressed in the epithelium and vibrissae. TrkA, TrpV1, and CGRP are upregulated in Bmp7$^{\Delta/\Delta}$ mutants, TrkA, TrpV1, Ret, Runx1, and Runx3 are upregulated in neural crest deleted Bmp7 mutants. Adult mice lacking Bmp7 in the neural crest are mechano-insensitive and hyperalgesic. Therefore, Bmp7 signaling may be a regulator of nerve subtype specification.

Chapter 6: Future Directions

The results in Chapter 4 indicate a novel role for Bmps in sensory nerve development and provide insights about cranial sensory nerve subtype specification. To build on these findings, future experiments should first provide additional data supporting nerve subtype specification, and second, answer specific mechanistic questions about how Bmp7 could favor the development of different nerve populations.

Additional data would include more qPCRs on E14.5 to E18.5 Wnt1-cre and K14-cre Bmp7$^{fl/fl}$ trigeminal ganglia that screen for the same receptors, transcription factors, and peptides that were investigated in this study and several others. These would include Runx1, Ret, TrkA, TrpV1, tyrosine hydroxylase (TH), as well as different types of Mas-related G-protein coupled receptors (MrgprA3/B4/D) for non-peptidergic neurons; TrkA, TrpV1, CGRP, SubP and hepatocyte growth factor receptor (HGFR/c-Met) for peptidergic neurons; and Runx3, TrkB, TrkC, Ret, and short stature homeobox 2 (Shox2) for mechano-and proprioceptive neurons.

The qPCR data could then be followed with high-quality confocal immunofluorescence analysis in the skin and mesenchyme of both Wnt1-cre and K14 mutants for various nerve subtype associated receptors and peptides. The use of high-quality confocal imaging should be stressed because nerve subtypes in the skin are spatially segregated and because a conventional fluorescent microscope can not penetrate thicker sections and tissues. Ret antibody should be used to identify non-peptidergic nociceptors in the periphery, anti-CGRP for peptidergic neurons, anti-NF200 for myelinated fibers, anti-Troma1 for Merkel cells and other antibodies as associated subtype proteins become discovered.

Furthermore, one can employ electrophysiological approaches which have been demonstrated to reliably identify different nerve subtypes to investigate differences between Wnt1-cre and K14 mutants. Behavioral assays such as the Von Frey, Air puff assay, Hargraves and others can complement these studies by helping to elucidate which nerve subtypes are affected and how physiological responses to stimuli are alteioured in mutant mice.

Lastly, to investigate mechanisms of Bmp7 signaling in nerve development, one could use neuronal cell cultures. Micro-fluid chambers could be used to separate neuronal bodies

from axons and study retrograde transport and signaling cascades using pharmacological agents that inhibit various processes.

The combined research approach would provide strong evidence for Bmp signaling in nerve development and new insight that could be employed for studying the role of Bmp signaling in other neural and non-neural tissues.

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