BDNF-TRKB SIGNALING AND THE DEVELOPMENT OF SOMATOSENSORY NEURONS

By

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Abstract

Primary sensory neurons detect diverse types of stimuli from both external and internal environments and convey sensory information to the central nervous system. Neurotrophin signaling plays essential roles in many aspects of primary sensory neuron development, including survival, axonal outgrowth, target innervation, subtype differentiation, and synapse formation. Despite a large number of studies over the past several decades, mechanisms underlying the spatial and temporal control of neurotrophin signaling as well as the functions of any particular neurotrophin during many developmental processes remain elusive. Here, I focused on two developmental events. The first one is the sexually dimorphic development of sensory innervation of the mouse mammary gland. Brain-derived neurotrophic factor (BDNF), emanating from mammary mesenchyme and signaling through its receptor TrkB on sensory axons is required for establishing mammary gland sensory innervation of both sexes at early developmental stages. Prior to male gland regression, androgens promote mammary mesenchymal expression of a truncated form of TrkB in males, which prevents BDNF-TrkB signaling in sensory axons leading to a rapid loss of mammary gland innervation independently of apoptosis. Thus, sex hormone regulation of a neurotrophic factor signal leads to sexually dimorphic axonal growth and maintenance, and generation of a sex-specific neural circuit. My second project focused on organization and formation of the mouse Meissner corpuscle, a mechanosensory end organ located in the glabrous skin of mammals. I found that each Meissner corpuscle is innervated by at least two molecularly distinct, large caliber sensory fibers: One fiber type emanates from a “TrkB neuron”, and the other from an “early Ret neuron”. The terminal arborizations of TrkB neurons exhibit a tiled
pattern. During development, Schwann cells are intimately associated with TrkB neurons in dermal papillae, and BDNF, derived from skin epidermal cells, signaling through TrkB is essential for Meissner corpuscle formation. The absolute requirement of TrkB neurons for Meissner corpuscle formation suggests the existence of neuron-glia interaction signals unique to this neuronal population that orchestrate corpuscle development. Overall, my findings show that temporally and spatially regulated neurotrophin signals derived from peripheral targets direct coordinative development of sensory neurons and the end organs they innervate.
Advisor: David D. Ginty, Ph.D.
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Table of contents

Abstract .......................................................................................................................... ii

Acknowledgements ..................................................................................................... v

Table of contents ........................................................................................................ vi

List of figures ............................................................................................................... vii

Chapter 1. Primary sensory neurons and their development ...................................... 1

Chapter 2. Sexually dimorphic BDNF signaling directs sensory innervation ........... 13

Chapter 3. Organization and development of sensory innervation of ...................... 75

of the mouse mammary gland.

Materials and Methods ............................................................................................. 107

References ................................................................................................................... 114

Curriculum Vitae ......................................................................................................... 132
List of figures

Fig. 1.1 Morphology of cutaneous mechanoreceptors.

Fig. 2.1 Mouse mammary gland development and its sensory innervation.

Fig. 2.2 Mammary gland sensory innervation develops a sexually dimorphic pattern prior to male gland regression.

Fig. 2.3 Quantification of neuronal fibers projecting to the rudimentary mammary gland.

Fig. 2.4 Neuronal fibers innervating the E13 rudimentary mammary glands emanate from sensory ganglia.

Fig. 2.5 Sexually dimorphic mammary gland innervation is independent of apoptotic cell death.

Fig. 2.6 The sexually dimorphic pattern of mammary gland sensory innervation is dependent on androgen receptor activation.

Fig. 2.7 BDNF is expressed in developing mouse mammary glands.

Fig. 2.8 BDNF–TrkB signaling is required for sensory innervation of the female mammary gland at E13.

Fig. 2.9 Neurons expressing TrkB at E13 innervate the mammary gland ductal tree structure but not the nipple in postnatal female mice.

Fig. 2.10 TrkB signaling is required at E13 for maintaining sensory innervation of female rudimentary mammary glands.
Fig. 2.11  BDNF is expressed in mammary mesenchyme of both male and female at late E13.

Fig. 2.12  BDNF-TrkB signaling is required for initial axonal projections to both male and female mammary glands.

Fig. 2.13  TrkB is expressed in the male mammary mesenchyme at E13.

Fig. 2.14  TrkB expressed in male mammary mesenchyme at E13 is the truncated (T1) form.

Fig. 2.15  TrkB expression in mammary mesenchyme is dependent on androgen receptor activation.

Fig. 2.16  TrkB expression in the female mammary mesenchyme after testosterone exposure.

Fig. 2.17  Assessment of TrkBEC antibody specificity.

Fig. 2.18  Quantification of mammary mesenchymal TrkB expression levels.

Fig. 2.19  The residual TrkB detected in mammary mesenchymal cells of male TrkB.T1 mutant embryos lacks the TrkB.T1 specific c-terminus.

Fig. 2.20 and Fig. 2.21  Mammary mesenchymal TrkB expression controls formation of the sexually dimorphic pattern of mammary gland innervation prior to male gland regression.
Fig. 2.22  Schematic illustration of the cellular and molecular mechanism by which sexually dimorphic mammary gland sensory innervation is generated.

Fig. 3.1  Meissner corpuscles are innervated by myelinated afferent fibers from molecularly distinct populations of DRG neurons.

Fig. 3.2  A subset of DRG sensory neurons expressing TrkB during development innervate Meissner corpuscles.

Fig. 3.3  TrkB neurons and early Ret neurons are two distinct sensory populations innervating Meissner corpuscles.

Fig. 3.4  Terminal arborizations of TrkB neurons and early Ret neurons in the pad regions of mouse glabrous skin.

Fig. 3.5  BDNF expressed in epithelial cells is essential for Meissner corpuscle formation.

Fig. 3.6  TrkB is required in sensory neurons, but not in glia cells, for Meissner corpuscle formation.

Fig. 3.7  Schwann cells are associated with TrkB-expressing fibers in dermal papillae during development.
Chapter 1. Primary sensory neurons and their development

1.1. Morphology and classification of primary sensory neurons

As one of five basic sensory systems, the somatosensory system may be responsible for the most diverse types of sensation, including nociception (pain), thermoception (temperature), tactioception (touch), and proprioception (body position and muscle strength). The visceral sensory system, which is responsible for sensing internal body status, is often considered a separate sensory system from the somatosensory system. But since primary visceral sensory neurons share many similarities with primary somatic sensory neurons, they are often discussed together. Primary somatosensory neurons (primary sensory neurons for brief), which respond to external and internal stimuli, are located either in dorsal root ganglia (DRG) or in cranial sensory ganglia. These neurons are pseudounipolar cells, with one axonal branch that projects to the periphery, interacting directly with peripheral organs, e.g. skin, muscles, and internal organs, and the other axonal branch projecting centrally, synapsing upon second order neurons in spinal cord or brainstem nuclei.

Primary sensory neurons are heterogeneous with regards to morphology, physiological property and function. One way of classifying primary sensory neurons is based on the conduction velocities of the axonal action potentials. Aα (also called type I afferents) and Aβ fibers exhibit the fastest conduction velocities. These fibers are associated with large-diameter neurons and are heavily myelinated by Schwann cells. Aδ fibers emanate from intermediate-diameter neurons; they are thinly myelinated and have axonal conduction velocities that are substantially slower than Aα/β fibers but faster than C fibers. C fibers
have the slowest conduction velocities and emanate from small-diameter unmyelinated neurons (Willis, 2004).

Cutaneous sensory neurons, which give rise to peripheral axons that innervate skin, include several different subclasses that are responsible for sensing different modalities, such as pain, temperature, itch, and touch. Cutaneous nociceptors respond to noxious stimuli (mechanical, thermal, or chemical) and mediate pain sensation. These neurons give rise mainly to C and Aδ fibers, although a small population has Aβ fibers (Djouhri and Lawson, 2004). There are two main types of cutaneous nociceptors: peptidergic nociceptors, which express peptides including calcitonin gene-related peptide and substance P, and non-peptidergic nociceptors, which does not express these peptides and have binding sites for the lectin, IB4 (Woolf and Ma, 2007). Cutaneous thermal receptors include both cold receptors and warm receptors; these neurons largely overlap with nociceptors, although some only respond to temperature in the innocuous range. A number of Transient receptor potential (TRP) ion channels, such as TRPV1, TRPV2, TRPM8 and TRPA1, are activated (open) within a certain range of temperature, and thus serve as the molecular mediators of thermal sensation (Caterina, 2007; Schepers and Ringkamp, 2010). The existence of itch-specific receptors has been a long debate, and they were considered to be a subpopulation of C-fiber nociceptors. Histamine is the best-characterized mediator of itch response, and histamine receptors have been detected in small diameter DRG neurons (Han et al., 2006). Recently, a small subset of DRG neurons has been identified as itch-specific receptors. These neurons express the Mas-related G protein-coupled receptors, which can be directly activated by Chloroquine (a
non-histamine itch inducing agent), and have been shown to mediate histamine-independent itch response (Liu et al., 2009).

Light, or innocuous touch sensations are mediated by cutaneous low-threshold mechanoreceptors (LTMRs), and their axons include Aβ, Aδ and C fibers (Fig. 1.1). In primate glabrous skin (skin lacking hair), four types of Aβ-LTMRs have been identified based on their rates of adaptation to a sustained stimulus and their receptive fields: slowly adapting type I (SAI), slowly adapting type II (SAII), rapidly adapting type I (RAI), and rapidly adapting type II (RAII or PC). The slowly-adapting LTMRs (SAI and SAII) fire action potentials during the entire period of a sustained stimulus while, in contrast, the rapidly-adapting LTMRs (RAI and RAIi) only fire action potentials at the onset and the offset of a stimulus. All four types of glabrous skin LTMRs form specialized terminal sensory organs: these are Meissner corpuscles (RAI), Pacinian corpuscles (RAII), Merkel endings (SAI), and Ruffini endings (SAII) (Hamann, 1995; Iggo and Andres, 1982). The optimal stimuli that elicit typical responses of these glabrous skin LTMRs are: skin movement/low frequency vibration for RAIIs, high frequency vibration for RAIIs, indentation of skin surface for SAI, and stretch of the skin for SAIIs (Abraira and Ginty, 2013). Meissner corpuscles and RAI-LTMRs will be discussed in details in Chapter 3. In mice, only Meissner corpuscles and Merkel endings have been found in glabrous skin. In hairy skin, Aβ RA-LTMRs, Aδ-LTMRs, and C-LTMRs form lanceolate endings on hair follicles. There are three different types of hair follicles in mouse back hairy skin: guard, awl/auchene, and zigzag (Driskell et al., 2009), and each type has a unique combination of these three LTMRs (Li et al., 2011). Merkel endings in hairy skin are associated with guard hairs, and are innervated by SAI-LTMRs (Iggo and Muir, 1969;
Molecular markers and genetic strategies for labeling different LTMRs are becoming available in mouse during the past a few years. In mechanoreceptors innervating hairy skin, C-LTMRs express tyrosine hydroxylase (TH), Aδ-LTMRs express TrkB (neurotrophin receptor, see Chapter 1.2) (Li et al., 2011), and Aβ RA-LTMRs express Ret (receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factors), the transcription factor MafA, and are labeled by GFP in Npy2r-GFP transgenic mice (Bourane et al., 2009; Li et al., 2011; Luo et al., 2009). The SAI-LTMRs innervating Merkel endings associated with guard hairs also express Ret and MafA (Bourane et al., 2009). Compared to hairy skin, less is known about markers that label LTMRs associated with glabrous skin. Ret is expressed in mechanoreceptors that innervate Meissner corpuscles in mouse glabrous skin (Luo et al., 2009).

Sensory neurons that innervate visceral organs are mainly located in petrosal and nodose ganglia, as well as DRG. Similar to cutaneous sensory neurons, these visceral afferents are classified into different subtypes, including nociceptors, mechanoreceptors, chemoreceptors, and thermoreceptors. Some of these neurons associate with specialized sensory organs, which comprise both neuronal and non-neuronal cellular components, and these include Pacinian corpuscles in the mesentery and connective tissues associated with visceral organs, carotid bodies on the carotid artery, and neuroepithelial bodies in the airway/pulmonary system. Sensory input from visceral organs is not necessary to reach awareness, but is involved in physiological homeostatic regulation and may affect emotional state (Ádám, 1998; Critchley and Harrison, 2013).
1.2. Neurotrophins and development of primary sensory neurons

Neurotrophins are family proteins with essential roles in development and function of the vertebrate nervous system. Four neurotrophins have been identified in mammals; Nerve growth factor (NGF), which was the first neurotrophin described, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, also known as NT-5). These factors are initially synthesized as pro-neurotrophins, which are cleaved to generate mature proteins. Neurotrophins bind to two classes of receptors: the Trk (tropomyosin-receptor kinase) receptors and the p75 neurotrophin receptor (p75NTR). Neurotrophin engagement of these two classes of receptors activates different downstream signaling pathways, resulting in different biological consequences (Bothwell, 1995).

The Trk receptors are tyrosine kinase receptors with specificity for different neurotrophin ligands. NGF specifically activates TrkA, BDNF and NT-4 are specific for TrkB, and NT-3 activates TrkC, as well as TrkA and TrkB but less efficiently. Generally, neurotrophins directly bind to the Trk receptors as dimers and induce dimerization of these receptors. The extracellular domains of Trk receptors consist of leucine rich repeats and immunoglobulin (Ig) domains, and a membrane proximal Ig domain is essential for receptor-ligand interaction (Ultsch et al., 1999). Upon ligand binding, three intracellular tyrosine residues within the autoregulatory loop of the Trk receptors (Y670, Y674 and Y675, corresponding to human TrkA sequence) are phosphorylated, leading to further activation of the receptors and phosphorylation of other intracellular tyrosine residues. Phosphorylation of these tyrosine residues recruits adaptor proteins to bind to the Trk receptors, which couple the receptors with downstream signaling molecules. Two
tyrosine residues are major sites for adaptor protein/effecter binding: Y490 provides a docking site for proteins containing a phosphotyrosine-binding (PTB) motif, and Y785 provides a docking site for proteins containing a src-homology-2 (SH-2) motif. Phosphorylation of Y785 directly recruits phospholipase C (PLC)-γ, and phosphorylation of PLC-γ leads to generation of inositol tris-phosphate (IP3) and diacylglycerol (DAG), and as a consequence activation of multiple kinases, including Ca\(^{2+}\)-calmodulin–regulated protein kinases (CaMKs) and protein kinase C (PKC) isoforms. Phosphorylation of Y490 activates two major downstream pathways: the Ras/ERK and phosphatidylinositol-3-kinase (PI3K)/Akt pathways. Ras/ERK pathway activation leads to stimulation of PI3K and multiple transcriptional factors, such as cAMP-regulated enhancer binding protein (CREB) and serum response factor (SRF), and therefore regulates gene expression. PI3K/Akt pathway activation results in phosphorylation of several proteins involved in regulating cell survival, including BAD, IκB, and the forkhead transcription factor FKHRL1 (Huang and Reichardt, 2003; Kaplan and Miller, 2000).

For each Trk receptor, different isoforms generated by alternative splicing events exist. Differences in the extracellular domains among isoforms affect the ability of certain neurotrophins to activate their cognate receptors (Clary and Reichardt, 1994; Strohmaier et al., 1996). Moreover, for both TrkB and TrkC, there are isoforms lacking the intracellular tyrosine kinase domain. Expression of these truncated receptor isoforms can lead to dominant negative inhibition of the full-length receptors by forming heterodimers or by sequestering their corresponding ligands (Biffo et al., 1995; Eide et al., 1996).
Moreover, ligand engagement of these truncated isoforms may directly activate intercellular signaling cascades (Baxter et al., 1997; Hapner et al., 1998).

Neurotrophin-Trk signaling plays key roles in many aspects of the primary sensory neuron development, including sensory neuron survival, axonal outgrowth, cell type differentiation, target innervation, and synapse formation. The primary sensory neurons are mainly derived from neural crest cells, although cranial sensory neurons are derived from a mixture of neural crest and neurogenic placodes. The initial neurogenesis and sensory lineage acquisition appears independent of neurotrophin signaling. Two transcription factors, neurogenin-1 (NGN-1) and neurogenin-2 (NGN-2), are essential for these steps, and are required during two different waves of neurogenesis. The first wave, which requires NGN-2, generates approximately 4% of the final DRG population, which are mostly intermediate to large diameter neurons and express TrkB and/or TrkC either transiently or permanently. The second wave, which requires NGN-1, generates about 91% of the final DRG neuronal population, and includes small diameter TrkA+ neurons, as well as intermediate and large TrkB/TrkC neurons. A third wave of neurogenesis, from the cells of boundary cap, produces about 5% of the final DRG population of exclusively small diameter TrkA+ neurons. Coincident with neurogenesis, these primary sensory neurons acquire their initial sublineages, and correspondingly express different Trk receptors (Ma et al., 1999; Marmigere and Ernfors, 2007).

Since the seminal experiments of Hamburger, Levi-Montalcini and their colleagues revealing an essential function of NGF in supporting nociceptive sensory and sympathetic neuron survival and axonal outgrowth, abundant evidence, especially done through analyses of mice with deletion of genes encoding neurotrophins and their
receptors, have shown that each neurotrophin supports survival of distinct subsets of sensory neurons (reviewed in Huang and Reichardt, 2001). In DRG and trigeminal ganglia, NGF-TrkA signaling is essential for nociceptive and thermoceptive neurons (Crowley et al., 1994; Smeyne et al., 1994), whereas NT3-TrkC signaling is essential for proprioceptive neurons (Ernfors et al., 1994; Farinas et al., 1994). Mice with targeted deletion of genes encoding these neurotrophins and their receptors exhibit nearly complete loss of the respective sensory populations. In BDNF as well as TrkB knock-out mice, there is also substantial neuronal loss in these ganglia, with deficits in mechanosensation (Carroll et al., 1998; Jones et al., 1994; Silos-Santiago et al., 1997). Although NT-4 deletion does not lead to significant cell loss early postnatally, NT4 is required for maintenance or survival of D-hair receptors (Aδ-LTMRs) in the adult mouse (Stucky et al., 1998). In nodose-petrosal ganglia, which contain sensory neurons innervating visceral organs, TrkB deletion leads to ~95% neuronal loss, and BDNF and NT4 knock-out mice each exhibit ~50% loss (Brady et al., 1999; Conover et al., 1995). Neurotrophins are believed to be derived from final or intermediate targets, and the neurotrophin-Trk complexes are retrogradely transported to cell bodies to induce pro-survival signaling cascades. One prominent idea is that limited amount of neurotrophins ensure proper match of sensory neuron number and target tissue requirements.

Besides survival, sensory neuron differentiation, target innervation, and synapse formation are also dependent on neurotrophin signaling. Analyses on neurotrophin or neurotrophin receptor knock-out mice, in conjunction with deletion of the pro-apoptotic gene Bax to eliminate cell loss, revealed a number of developmental deficits of sensory neurons independent of neuronal cell death. For example, in TrkA-/-;Bax-/- mice,
cutaneous innervation of small diameter nociceptive neurons is affected, and expression of many nociceptor specific genes, including CGRP, SP, is absent (Patel et al., 2000). Likewise, in NT3-/-;Bax-/- mice, proprioceptors fail to form monosynaptic connections with motorneurons and also fail to innervate their target muscles (Patel et al., 2003). Interestingly, in mice in which the TrkA locus is replaced by TrkC, a subset of the small diameter neurons switch their fate to proprioceptors (Moqrich et al., 2004), suggesting that neurotrophin-Trk signaling is capable of inducing sensory neurons to adopt particular sensory neuron fates.

p75NTR is a distant family member of the tumor necrosis factor receptor family, sharing a structural similarity of conserved “death” domain in its intracellular domain. Neurontrophin engagement of p75NTR activates a unique set of downstream signaling pathways, including activating NFκB, Jun kinase, and generating ceramide (Chao, 2003). In addition to direct activation of intracellular signaling pathways, p75NTR also modulates neurotrophin-Trk interactions (Barrett, 2000; Huang and Reichardt, 2003). Functions of p75NTR during sensory neuron development are rather complicated. In vitro studies indicate that p75NTR promotes sensory neuron death at embryonic stage in the absence of NGF, but facilitates NGF dependent survival early postnatally (Barrett and Bartlett, 1994). In vivo, p75NTR null mice exhibit fewer sensory neurons and defective peripheral sensory innervation (Bergmann et al., 1997; Lee et al., 1992).

It has been over 50 years since the first neurotrophin was discovered, and a huge number of studies have advanced our understanding of the signaling and function of these growth factors and their receptors. Despite this, there is still much to discover, especially for many biological events, how neurotrophin signaling is regulated and how these signaling
events interact with others to achieve proper nervous system wiring are waiting to be uncovered. For my thesis research, I focused on two developmental processes. The first one is development of sensory innervation of the mouse mammary gland, and I investigated how BDNF-TrkB signaling contributes to generation of a sexually dimorphic sensory innervation pattern (Chapter 2). The second one is formation of mouse Meissner corpuscles, and for this I investigated the organization of sensory neurons that innervate Meissner corpuscles and how the formation of this sensory end organ depends on neurotrophin signaling (Chapter 3). My findings show that neurotrophins acting in the periphery orchestrate key developmental processes that underlie the exquisite organization between sensory neurons and the end organs they innervate.
Fig. 1.1  Morphology of cutaneous mechanoreceptors. Schematic diagram showing mechanoreceptors in glabrous skin (left) and hairy skin (right) (diagram from Abraria and Ginty, 2013). In primate glabrous skin, four types of mechanoreceptors forming specialized sensory terminal organs have been identified. Merkel cells are present in the basal layer of the epidermis, and associated with SAI-LTMRs. Meissner corpuscles, located in the dermal papillae, consist of lamellar cells (specialized Schwann cells) and RAI-LTMR terminals. Ruffini endings are localized in the deep dermis and are spindle-shaped cylinders composed of layers of perineural tissue. They are associated with SAIII-LTMRs. Pacinian corpuscles, also located in deep dermis, are of their characteristic onion-shape morphology, and one Pacinian corpuscle is innervated by a single RAI-LTMR. In mouse hairy skin, three types of hair follicles are identified: they are guard, awl/auchene, and zigzag. Guard hairs are associated with touch domes at the apex and Aβ RA-LTMR longitudinal lanceolate endings at the base. Same as in the glabrous skin, Merkel cells in the touch dome are also innervated by SAI-LTMRs. Awl/auchene hairs are triply innervated by Aβ, Aδ, and C-LTMR longitudinal lanceolate endings. Zigzag hair follicles are innervated by both Aδ and C-LTMR longitudinal lanceolate endings. In addition, all three types of hair follicles are innervated by circumferential lanceolate endings whose physiological properties are unknown.
Fig. 1.1
Chapter 2. Sexually dimorphic BDNF signaling directs sensory innervation of the mouse mammary gland.

2.1. Sexually dimorphic development of the vertebrate nervous system

Sexually dimorphic body structures require unique neural circuits to control gender-specific physiological functions and behaviors. In vertebrate nervous systems, significant sexual dimorphisms have been documented in many different regions, from the cerebral cortex to spinal cord motor neurons. The gonadal steroid hormones, such as estrogen and testosterone, are essential for sexually dimorphic neural circuit development and sex-specific behaviors. These hormones regulate various aspects of neural development and function, including neuronal survival and migration, axonal guidance, synaptogenesis, and neurotransmitter plasticity (Forger, 2006; Morris et al., 2004; Simerly, 2002; Wu and Shah, 2011).

The first demonstration in vertebrates showing that sex hormones regulate brain development was made in 1950s. Prenatal exposure of female guinea pig embryos to testosterone leads to a permanent effect on the female reproductive behaviors in adulthood, whereas exposing in adult does not have the same effect on these behaviors (Phoenix et al., 1959). Later studies, mostly in rats and mice, revealed that prenatal exposure of testosterone masculinizes brain structures, especially the limbic-hypothalamus pathways, which participate in the neural control of reproduction (reviewed in Simerly, 2002). There are several brain regions along this pathway that have been found to be sexually dimorphic, including the medial preoptic nucleus (MPN) (Dodson and Gorski, 1993; Gorski et al., 1978), the anteroventral periventricular nucleus
The AVPV (Simerly, 1991; Sumida et al., 1993), the bed nuclei of the stria terminalis (BNST) (Guillamon et al., 1988), and the posterodorsal medial amygdala (MePD) (Mizukami et al., 1983). Besides the AVPV, which is much larger in females compared to males, the other three brain regions are all bigger in the male compared to the female. The sexual dimorphism of cell number of these brain nuclei is due to hormone modulation of programmed cell death in a cell type specific manner, increasing cell death in the AVPV and decreasing cell death in the other three regions, although how this is achieved is unknown. The responsible hormone, testosterone, is aromatized into estrogen in the brain and conveys its effects via estrogen receptors (Chung et al., 2000; Davis et al., 1996; Dodson and Gorski, 1993; Murakami and Arai, 1989; Sumida et al., 1993; Wu et al., 2009). Besides influencing cell death, steroid hormones also regulate axonal growth and guidance, and synapse formation (Hutton et al., 1998; Toran-Allerand, 1976; Woolley and McEwen, 1992).

Outside of the brain, extensive studies have been done focusing on the spinal nucleus of the bulbocavernosus (SNB). These motor neurons in the spinal cord innervate three groups of muscles, two of which are associated with male penis and atrophy in the female during postnatal development (Cihak et al., 1970). In adult rats, the number of motor neurons in the male SNB is more than three-fold greater than that in the female SNB (Breedlove and Arnold, 1980). As with sexually dimorphic development of brain nuclei, this difference in the SNB is the result of testosterone regulation of apoptotic cell death. But in contrast to the brain, the effect of testosterone on SNB development relies exclusively on androgen receptors (Nordeen et al., 1985). Testosterone exposure does
not directly support SNB motor neuron survival; it primarily prevents death of the target muscles, and then secondarily spares SNB neurons from apoptosis (Freeman et al., 1996).

In the peripheral nervous system, sexual dimorphism has also been observed, mainly in the autonomic nervous system. Preganglionic sympathetic neurons, sympathetic and parasympathetic ganglionic neurons all exhibit much higher numbers in males than in females in the rat (Greenwood et al., 1985; Nadelhaft and McKenna, 1987; Wright, 1987). Exposure of neonatal rats to either estradiol or testosterone prevents cell death in superior cervical ganglia, suggesting a similar mechanism to that which is employed in generating sexual dimorphism of brain nuclei (Wright and Smolen, 1985, 1987). So far, there is no evidence for sexual dimorphism in the sensory components in the peripheral nervous system.
2.2. Androgen dependent axon pruning results in a sexually dimorphic pattern of sensory innervation of mouse rudimentary mammary glands.

2.2.1. Sensory innervation of mouse rudimentary mammary glands develops a sexually dimorphic pattern prior to male gland regression.

While sex differences in neuron numbers are observed in several discrete regions of the vertebrate nervous system, little is known about the mechanisms underlie differential assembly of neural circuits associated with sexually dimorphic organs. Here, I focused on one sexually dimorphic organ, the mammary gland, and investigated how development of neuronal innervation coordinates with sexually dimorphic mammary gland organogenesis. In mice, the initial stages of mammary gland development are virtually identical in males and females, until embryonic day 13 (E13, appearance of vaginal plug = E0). Then, in response to androgens, which are secreted by the developing male gonads, the male mammary glands begin to regress such that sexual dimorphism of the gland is first observable at E14 (Fig. 2.1A) (Dunbar et al., 1999; Robinson, 2007). By birth, each of the ten mammary glands of the female mouse contains a clearly defined nipple and an elaborate ductal tree that is densely innervated by sensory neurons while, in contrast, male glands are barely detectable (Fig. 2.1B). To visualize neuronal fibers associated with rudimentary mammary glands at different embryonic stages, immunostaining was performed using an antibody against the neuron-specific class III β-tubulin (Tuj1). At late E12, the male and female mammary rudiments are innervated by a comparable number of Tuj1+ fibers (Fig. 2.2, quantification method: Fig. 2.3). In the female, the number of fibers associated with mammary rudiments is dramatically increased during the subsequent 24 hours. These Tuj1+ fibers associated
with mammary rudiments at this stage are from sensory neurons, most likely emanating from dorsal root ganglia (DRG) (Fig. 2.4). In contrast, neuronal innervation of male mammary rudiments increases until early E13 but then it is rapidly lost during the subsequent 8 hours. As a result, by late E13, few if any fibers are associated with male mammary rudiments (Fig. 2.2). Likewise, at late E14, when male mammary rudiments are in regression, no fibers are observed associated with the structure (Fig. 2.2). Thus, sensory neuron innervation of mouse mammary glands initially develops in parallel in males and females, and then a sexually dimorphic pattern is generated prior to male gland regression, resulting from a continued growth of axons associated with female glands and a concomitant loss of axons associated with male glands.

2.2.2. The sexually dimorphic pattern of mammary gland sensory innervation is a result of male-specific axon pruning process.

Sexually dimorphic development of neuronal populations in both the central nervous system and peripheral nervous system has been linked to differential regulation of apoptotic cell death (Chapter 2.1). Therefore, I tested whether the male-specific axonal loss from rudimentary mammary glands is the result of apoptotic neuronal death or, alternatively, axon pruning, by analyzing Bax-deficient embryos in which apoptotic cell death in sensory ganglia is completely eliminated (Patel et al., 2000). In Bax/-/- embryos, mammary gland innervation at late E13 exhibits a sexually dimorphic pattern, which is quantitatively indistinguishable from that seen in wildtype embryos (Fig. 2.5). This indicates that formation of sexually dimorphic mammary gland innervation is not due to apoptotic loss of male sensory neurons; rather, it is a result of sensory axon withdrawal from the male gland beginning at early E13.
2.2.3. Male-specific sensory axon pruning is dependent on androgen receptor activation.

To assess whether sensory axon pruning from the male mammary rudiments and thus the formation of sexually dimorphic mammary gland innervation is androgen dependent or not, mammary gland innervation in embryos exposed to the androgen receptor antagonist flutamide, testosterone propionate, the non-aromatizable androgen dihydrotestosterone (DHT), and in androgen-insensitive (Tfm) mice (Lyon and Hawkes, 1970) was analyzed. Remarkably, in flutamide treated embryos, sensory fibers projecting to the male mammary rudiments fail to be pruned, and quantification of fiber density shows no difference between the two sexes at late E13 (Fig. 2.6A, C). Similarly, the amount of mammary gland innervation in Tfm male embryos is comparable to both flutamide treated male embryos and wildtype female embryos at late E13 (Fig. 2.6B). Conversely, in both testosterone and DHT treated embryos, sensory fibers projecting to female mammary rudiments are lost within 8 hours (Fig. 2.6A, D and E). Thus, androgen receptor activation is both necessary and sufficient for pruning of sensory axons and thus generation of the sexually dimorphic pattern of mammary gland sensory innervation prior to male gland regression.
Fig. 2.1 Mouse mammary gland development and its sensory innervation. (A) Schematic diagram showing development of the mouse mammary gland at embryonic stages (diagram from Robinson, 2007). Around embryonic day 10 (E10) milk line (orange) is formed as thickening of the ectoderm on both sides of the trunk. At E11.5, the milk line breaks up to individual placodes (orange), and mammary mesenchyme (blue) begins to be formed. In the following days, the placodes sink deeper and become bulb shape rudiments, and mammary mesenchyme is organized into centric layers around the rudiments. Till E13.5, male and female mammary rudiments are morphologically identical; however, in response to androgen, male mammary rudiments begin to regress after E13.5, whereas female mammary rudiments continue to develop. In the female, begin at E15.5, mammary epithelium pushes through mammary mesenchyme and grows towards fat pad. At E18.5, it branches into small ductal system, and the nipple is formed by specialized epithelial cells. (B) Mammary glands were dissected from a P0 Advillin-hPLAP female mouse in which placenta alkaline phosphatase is expressed exclusively in primary sensory neurons (Hasegawa et al., 2007). The ductal tree structure is revealed by Carmine staining (left panel), and sensory innervation of the mammary gland is shown by alkaline phosphatase histochemistry (right panel). This experiment was done two times with similar results.
Fig. 2.1

A

B

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Fig. 2.2 Mammary gland sensory innervation develops a sexually dimorphic pattern prior to male gland regression. (A) Tuj1 (yellow) and TO-PRO-3 (blue, nucleic acid stain) staining of mammary gland sections of embryos at different stages. This staining was used in all subsequent mammary gland innervation analyses unless otherwise specified. Mammary rudiments first appear as thickening of the epithelium at late E11, and then become bud-shape at early E12. Note that neuronal fibers reach mammary rudiments after early E12, and the sexually dimorphic pattern appears between early E13 and late E13. (B) Quantification of mammary gland innervation (n≥5 embryos for each bar). All statistical analyses shown in this chapter (except Fig. 2.8) were done using two-way ANOVA with a Bonferroni post hoc test. Shown are the means ± s.e.m., *p<0.05, **p<0.01, ***p<0.001, n.s. not significant. Scale bar: 50µm.
**Fig. 2.2**

**A**

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**B**

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*Fiber Density (%)*

- Male: n.s.
- Female: ***

---

*Fiber Density (%)*

- Late E12: n.s.
- Early E13: **
- Late E13: ***
Fig 2.3  Quantification of neuronal fibers projecting to the rudimentary mammary gland. For all experiments, images were processed and analyzed using ImageJ software. (A) Mammary gland cross sections were stained with anti-Tuj1 and TO-PRO-3. The edge of the rudimentary mammary gland was determined by TO-PRO-3 staining, and a band with the indicated thickness (X) surrounding the gland was drawn automatically with ImageJ. The total number of Tuj1 positive pixels within this band was counted, divided by the total number of pixels occupied by the entire band area, and this number was multiplied by 100% to obtain the measurement of fiber density. (B) Average fiber densities of different band thicknesses (20 rudimentary mammary glands from 4 late E13 female embryos, mean ± s.e.m.). Since the fiber density peaks for measurements using the 15 µm band, all subsequent quantifications presented in this study use 15 µm as the band thickness.
Fig. 2.3

A

Fiber density (%) = \frac{\text{Area of Tuj1 staining within the band}}{\text{Total area of X µm band surrounding the mammary rudiment}} \times 100\%

B

Graph showing fiber density (%) vs. band thickness (µm).
Fig. 2.4 Neuronal fibers innervating the E13 rudimentary mammary glands emanate from sensory ganglia. The Avil<sup>Cre</sup> mouse line was crossed with a Cre recombinase dependent Rosa<sup>26<sub>LSLtdTomato</sub></sup> reporter line (Madisen et al., 2010) to specifically label sensory neurons. E13 female Avil<sup>Cre/+</sup>; Rosa<sup>26<sub>LSLtdTomato/+</sub></sup> embryos were analyzed, and cross-sections of dorsal root ganglia (DRG), sympathetic ganglia (SG), nodose ganglia (NG), and rudimentary mammary glands were stained with anti-DsRed (to detect tdTomato) and anti-Tuj1. There are many tdTomato<sup>+</sup> neurons in the DRG while very few neurons are found to express tdTomato in the NG. Labeled neurons were not observed in the SG (a section through the superior cervical ganglion (SCG) is shown in the figure). All Tuj1<sup>+</sup> fibers surrounding rudimentary mammary glands are tdTomato positive, indicating that they are from sensory neurons, most likely DRG sensory neurons. This experiment was done two times with similar results. Scale bar: 50µm.
Fig. 2.4

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**Legend:**
- TD: tdTomato
- SCG: SCG
- NG: NG
Fig. 2.5  Sexually dimorphic mammary gland innervation is independent of apoptotic cell death.  (A) Tuj1 and TO-PRO-3 staining of mammary gland sections of wildtype and Bax-/- embryos.  Scale bar: 50µm.  (B) Quantification of mammary gland innervation of wildtype and Bax-/- embryos (n≥3 embryos for each bar).
Fig. 2.5

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B

- **Male**
- **Female**

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n.s.
Fig. 2.6  The sexually dimorphic pattern of mammary gland sensory innervation is dependent on androgen receptor activation. (A) Mammary gland innervation of embryos treated with vehicle, flutamide, testosterone propionate (8 hrs), and DHT (8hrs). The vehicle group shown here is the control for flutamide treatments; controls for the testosterone and DHT treatments are similar (not shown). Each experiment was done at least three times. (B) Mammary gland innervation of heterozygous Tfm female, hemizygous Tfm male, and wildtype male embryos at late E13. This experiment was done two times with similar results. (C-E) Quantification of mammary gland innervation (n≥3 embryos for each bar). The vehicle group for the DHT treatments is the same vehicle group for the testosterone treatments. Scale bar: 50μm.
Fig. 2.6

A

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2.3. Sensory innervation of mouse rudimentary mammary glands requires BDNF-TrkB signaling.

2.3.1. BDNF-TrkB signaling plays essential roles in establishing mammary gland sensory innervation.

The sexually dimorphic pattern of mammary gland sensory innervation results from extension of new sensory fibers into female glands and a coincident pruning of fibers from male glands. This suggests that signaling pathways essential for promoting and maintaining axonal projections to the female mammary rudiments are either absent or disrupted in the male at E13, presumably due to androgen receptor activation. Therefore, to determine the mechanisms by which androgen receptor activation controls the sexually dimorphic mammary gland innervation pattern, I first sought to identify signaling events that mediate development of mammary gland sensory innervation in the female. Neurotrophins play key roles in primary sensory neuron development (Chapter 1.2), and expression of neurotrophins and their receptors has been linked to hormone regulation (Miranda et al., 1994; Murphy et al., 1998; Segal and Murphy, 2001; Simerly, 2002). By whole-mount X-gal staining on mice with β-galactosidase gene (LacZ) knocked into each neurotrphin loci, the expression patterns of each of the four neurotrophins during mammary gland development were assessed. Brain-derived neurotrophic factor (BDNF) emerged as a candidate for controlling sensory innervation of the mammary gland because whole-mount X-gal staining of BDNF<sub>lacZ</sub> knock-in embryos (Gorski et al., 2003) showed that BDNF is robustly expressed in rudimentary mammary glands beginning at E12 whereas the other three neurotrophins are not (Fig. 2.7). Staining of female E13 embryo sections showed that BDNF is expressed in mammary mesenchymal cells, not in
mammary epithelial cells (Fig. 2.8A, inset). To determine whether the BDNF receptor TrkB is expressed in sensory neurons that innervate female mammary rudiments, TrkB<sup>GFP</sup> knock-in mice (Li et al., 2011) were analyzed. In these mice, virtually all Tuj1<sup>+</sup> fibers projecting to female mammary rudiments at E13 are GFP positive (Fig. 2.8B). Experiments using a TrkB antibody that recognizes the extracellular domain of TrkB (TrkB<sup>ECD</sup> antibody) confirmed that TrkB protein is indeed present on most or all axon terminals surrounding female glands at E13 (Fig. 2.13A). Furthermore, a TrkB<sup>CreERT2</sup> knock-in mouse line was used to permanently label sensory neurons that express TrkB at E13. Rosa26<sup>Lsl-tdTomato</sup> reporter mice were crossed with TrkB<sup>CreERT2</sup> mice and treated with tamoxifen at E13. In double heterozygous progenies, postnatally, I observed that labeled axons innervate the ductal tree structure, but not the nipple, of female mammary glands (Fig. 2.9). Thus, BDNF is expressed in mesenchymal cells of the developing mammary gland at a time when axons of TrkB<sup>+</sup> sensory neurons, which provide sensory innervation to the postnatal mammary ductal tree, innervate their target field.

To ask whether BDNF–TrkB signaling is necessary for establishing sensory innervation of the female mammary gland, I assessed the extent of mammary gland innervation in mouse lines harboring targeted mutations of either <i>Bdnf</i> or <i>TrkB</i>. Female embryos with a homozygous null mutation in <i>Bdnf</i> exhibit very few sensory fibers associated with the mammary rudiments at E13 (Fig. 2.8C, D). Moreover, both <i>TrkB</i> null and <i>TrkB</i> conditional mutant embryos in which <i>TrkB</i> is ablated exclusively in primary sensory neurons show a dramatic reduction in female mammary gland innervation at E13, essentially recapitulating the phenotype observed in <i>Bdnf</i> null embryos (Fig. 2.8E-H).
Thus, BDNF–TrkB signaling is required in sensory neurons for innervation of female mammary glands at E13.

2.3.2. BDNF-TrkB signaling is required at E13 for maintaining sensory innervation of female rudimentary mammary glands.

The sexually dimorphic pattern of mammary gland sensory innervation is established within an 8 hour time period, between early E13 and late E13 (Fig. 2.2), suggesting that disruption of BDNF or other axonal growth and maintenance signals in the male during this time window accounts for axon pruning and the differences between male and female innervation patterns. If this is true, then disruption of BDNF–TrkB signaling in the female at E13 for several hours would lead to a male-like sensory innervation pattern. To test this idea, a chemical-genetic strategy that enables precise temporal inhibition of TrkB signaling in vivo was used. TrkBF616A knock-in mice harbor a single amino acid substitution in the TrkB ATP binding pocket that allows specific and potent inhibition of endogenous TrkB kinase signaling by the small molecule inhibitor 1NMPP1 (Chen et al., 2005). 1NMPP1 does not block the catalytic activity of wild-type TrkB receptors. Either 1NMPP1 or vehicle control was administered to pregnant mothers for 5 hours, and embryos were collected at late E13 for mammary gland innervation analysis. Remarkably, mammary gland innervation was dramatically reduced in 1NMPP1 treated TrkBF616A/F616A female embryos, compared to vehicle-treated TrkBF616A/F616A females (Fig. 2.10). 1NMPP1 treatment of wildtype females was without effect, confirming that 1NMPP1 specifically blocks mutant TrkB receptors generated by the TrkBF616A allele, but not an off-target site, to cause this effect (Fig. 2.10). Taken together, BDNF–TrkB signaling is required in sensory neurons for establishment and maintenance of mammary
gland innervation, and disrupting BDNF–TrkB signaling in the female at E13 leads to rapid axon pruning and a male-type pattern of mammary gland sensory innervation.
Fig. 2.7  **BDNF is expressed in developing mouse mammary glands.**  (A) Whole-mount X-gal staining of E13 female \(NGF^{\text{LacZ}/+}, BDNF^{\text{LacZ}/+}, NT-3^{\text{LacZ}/+}\) (Farinas et al., 1994), and \(NT-4^{\text{LacZ}/+}\) embryos. Staining associated with E13 mammary glands is only detected in \(BDNF^{\text{LacZ}/+}\) embryos. Arrows: rudimentary mammary glands (five on each side of the body; glands #1 and #5 are behind the limbs and therefore not seen in this image). Staining of each mouse line was done at least two times with similar results. (B) Whole-mount X-gal staining of \(BDNF^{\text{LacZ}/+}\) embryos at E11 and E12. LacZ expression is associated with the developing mammary glands beginning at E12. Staining was done for mice at each time point at least two times with similar results.
**Fig. 2.7**

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### B

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36
Fig. 2.8  BDNF–TrkB signaling is required for sensory innervation of the female mammary gland at E13.  (A) Whole-mount X-gal staining of a late E13 female $BDNF^{LacZ/+}$ embryo (Gorski et al., 2003).  Inset: cross section of one mammary gland.  Arrows: rudimentary mammary glands (five on each side of the body, glands #1 and #5 are located behind the limbs).  (B) Co-staining of GFP, Tuj1 and TO-PRO-3 of a late E13 female $TrkB^{GFP/+}$ mammary gland section.  (C-H) Mammary gland innervation of late E13 female wildtype (WT), $BDNF^{LacZ/LacZ}$ (null), $TrkB^{GFP/GFP}$ (null), $TrkB^{ff}$, and $TrkB^{ff}; Avil^{Cre/+}$ embryos (the $Avil^{Cre/+}$ mouse is a sensory neuron-specific Cre driver mouse line (da Silva et al., 2011).  Statistical analyses in this figure were done using a student t-test, n≥3 embryos for each bar).  Scale bar: 50µm.
Fig. 2.8

A

B

C

D

E

F

G

H

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Fig. 2.9 Neurons expressing TrkB at E13 innervate the mammary gland ductal tree structure but not the nipple in postnatal female mice. The TrkB<sup>CreERT2</sup> mouse line was crossed with a Rosa<sub>26</sub><sup>LSLtdTomato</sup> reporter line and the pregnant females were treated with 4mg tamoxifen at E13 (11am) by oral gavage. TrkB<sup>CreERT2/+</sup>; Rosa<sub>26</sub><sup>LSLtdTomato/+</sup> females were sacrificed at P3, and isolated mammary glands with nipples and surrounding skin were obtained. Cross-sections of mammary glands were stained with anti-DsRed and TO-PRO-3 (sections shown here are from two different mammary glands). All tdTomato positive fibers associated with the mammary glands innervate the ductal structure, but not the nipples (arrow points to the nipple region). This experiment was done two times with similar results. Scale bar: 50µm.
Fig. 2.9

**P3 TrkB^{CreERT2/4, LSLtdTomato/+}**
Fig. 2.10  TrkB signaling is required at E13 for maintaining sensory innervation of female rudimentary mammary glands.  (A) Wildtype and \(TrkB^{F616A/F616A}\) female embryos were exposed to either vehicle or 1NMPP1 or 5hrs, and were immediately collected after treatments at late E13. Sections were stained with anti-Tuj1 and TO-PRO-3. Scale bar: 50µm.  (B) Quantification of mammary gland innervation.  \(N\geq3\) embryos for each bar.
**Fig. 2.10**

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B  

![Graph showing fiber density comparison between WT and \( TrkB^{F616A/F616A} \) under Vehicle and 1NMPP1 conditions.](image)

- **Vehicle**
- **1NMPP1**

**Fiber Density (%)**

- WT: 8.5 ± 1.2
- \( TrkB^{F616A/F616A} \): 4.0 ± 0.5

Significance levels:
- ***p < 0.001
- **p < 0.01
2.4. Androgen-dependent TrkB.T1 expression in male mammary mesenchyme accounts for the sexually dimorphic sensory innervation pattern of mouse mammary glands.

2.4.1. TrkB.T1 is expressed in mammary mesenchymal cells in the male at E13

I next asked whether BDNF–TrkB signaling is absent or disrupted in the male at E13, thus accounting for the androgen dependent sexually dimorphic pattern of mammary gland sensory innervation. The androgen receptor is expressed in both male and female mammary mesenchymal cells but not in DRG sensory neurons at E13 (Fig. 2.11A and Fig. 2.13), suggesting that the site of androgen action is the rudimentary mammary gland itself. Firstly, the level of BDNF in mammary mesenchymal cells of both males and females was assessed. Whole mount X-gal staining and X-gal staining of mammary gland cross-sections of \( BDNF^{LacZ^+} \) embryos shows that BDNF expression in the male mammary mesenchyme is comparable to that of the female at late E13 (Fig. 2.11B).

Next, I examined TrkB expression in both males and females by immunostaining mammary gland sections using the TrkB\(^{ECD}\) antibody. At late E12, as in the female, all Tuj1\(^+\) fibers projecting to male mammary rudiments express TrkB, and deletion of \( TrkB \) in male mice results in near complete loss of mammary gland sensory innervation at this stage (Fig. 2.12). At late E13, few if any TrkB\(^+\) sensory fibers are associated with male glands. However, surprisingly, male mammary mesenchymal cells robustly express TrkB at this stage, as detected both by TrkB\(^{ECD}\) staining and GFP staining of \( TrkB^{GFP^+} \) embryos (Fig. 2.13). This pattern of TrkB expression in the male mammary mesenchyme is not observed in female glands at the same stage nor is it seen in the glands of either sex one day earlier, at E12 (Fig. 2.13 and Fig. 2.12). These findings raise the possibility that
BDNF is sequestered by male mammary mesenchymal cells at E13, thereby preventing BDNF–TrkB signaling in sensory fibers associated with male mammary rudiments, leading to pruning of axons in the male and the sexually dimorphic mammary gland innervation pattern.

This model was tested by exploring the nature of TrkB expression in the male mammary mesenchyme, its androgen dependence, and the requirement of mesenchymal TrkB expression for generating the male pattern of mammary gland sensory innervation. Thus far, two isoforms of TrkB have been described in mice: a full-length form and a truncated form (also called TrkB.T1). These two forms are generated through alternative splicing of the same gene; the full-length form contains the intracellular tyrosine kinase domain required for canonical receptor tyrosine kinase signaling and BDNF-dependent axonal growth (Atwal et al., 2000), whereas TrkB.T1 lacks the tyrosine kinase domain, and instead has a unique, short, C-terminal intracellular domain (Klein et al., 1990; Klein et al., 1989). Both TrkB forms bind to BDNF and become internalized together with ligand (Biffo et al., 1995; Fryer et al., 1997), demonstrating their ability to sequester BDNF. To determine which form(s) of TrkB is expressed in male mammary mesenchymal cells at E13, immunohistochemistry using a TrkB.T1 specific antibody that recognizes the C-terminal intracellular domain of TrkB.T1 was performed. This experiment revealed that TrkB.T1 is robustly expressed in the male mammary mesenchyme at this stage while, in contrast, it is undetectable in the female gland (Fig. 2.14A). In complementary experiments, in situ hybridizations using two different probes on sections of both male and female glands were performed. A probe that detects only full-length TrkB failed to detect a transcript in mammary mesenchymal cells of either sex, whereas a probe that
detects both full-length and truncated TrkB detected a strong signal in mammary mesenchymal cells of male embryos but not female embryos (Fig. 2.14B). These findings indicate that sexually dimorphic expression of TrkB in mammary mesenchymal cells at E13 is the result of differential transcription of the TrkB gene and expression of the truncated form of TrkB exclusively in the male gland.

2.4.2. TrkB.T1 expression in mammary mesenchymal cells is dependent on androgen receptor activation.

Since mammary mesenchymal cells of both sexes express the androgen receptor at E13 (Fig. 2.13B), I next asked whether TrkB expression in male mammary mesenchymal cells is androgen dependent. Double immunostaining for GFP and androgen receptor on mammary gland sections of TrkB$^{GFP/+}$ knock-in embryos revealed that the TrkB expressing cells surrounding the male mammary rudiment are indeed a major subset of androgen-expressing mammary mesenchymal cells (Fig. 2.13B). Next, mammary gland sections of either flutamide or testosterone propionate treated embryos were immunostained using the TrkB$^{ECD}$ antibody. Strikingly, in flutamide treated male embryos, as in untreated female embryos, mammary mesenchymal cells do not express TrkB, and the male mammary rudiments are innervated by many TrkB-expressing neuronal fibers. Conversely, in testosterone treated female embryos, TrkB expression is detected in mammary mesenchymal cells, and TrkB-expressing neuronal fibers are absent (Fig. 2.15A). Experiments that assessed GFP levels in mammary mesenchymal cells of TrkB$^{GFP/+}$ embryos treated with flutamide are consistent with the TrkB$^{ECD}$ antibody labeling experiments and indicate that androgens control TrkB expression through regulation of TrkB gene transcription in mammary mesenchymal cells (Fig. 2.15B).
Moreover, the increase of TrkB expression in the female mammary mesenchyme following testosterone treatment is temporally coincident with the loss of sensory innervation of female rudimentary glands (Fig. 2.16). Thus, truncated TrkB transcription in the mammary mesenchyme is androgen dependent and inversely related to the amount of TrkB⁺ sensory fibers associated with the rudimentary gland, supporting a model in which androgen-dependent truncated TrkB expression in the male mammary mesenchyme controls generation of the sexually dimorphic pattern of mammary gland innervation.

2.4.3. TrkB.T1 expression in male mammary mesenchyme is required for generation of the sexually dimorphic pattern of mammary gland sensory innervation.

To determine whether the truncated form of TrkB expressed in the male mammary mesenchyme at E13 plays an essential role in the sexually dimorphic patterning of mammary gland innervation, I analyzed mammary gland innervation of TrkB.T1 mutant mice in which the exon encoding the C-terminal residues unique to TrkB.T1 is missing (Dorsey et al., 2006). These mice do not express TrkB.T1 but they do express a normal level of full-length TrkB. Since TrkB.T1 is not expressed in DRG neurons at E13 (data not shown), experiments using the TrkB.T1 mutant mice allowed us to address the role of TrkB.T1 without directly affecting BDNF–TrkB signaling in DRG neurons themselves. I first examined mammary mesenchymal TrkB expression in male TrkB.T1 mutant embryos by immunostaining using the TrkB ECD antibody (specificity was confirmed (Fig. 2.17)) and quantifying immunofluorescence signals in the mammary mesenchyme. The analysis revealed a significant reduction, but not a complete loss, of TrkB expression in mammary mesenchyme of male TrkB.T1 mutant embryos at late E13, as compared to
wildtype males (Fig. 2.18). Although the residual TrkB protein found in *TrkB.T1* mutant embryos lacks the TrkB.T1-specific C-terminus (Fig. 2.19), it is likely capable of associating with BDNF because it does contain the extracellular domain of TrkB. Quantification of mammary gland innervation revealed a significant increase in fiber density in *TrkB.T1* mutant males compared with wildtype males at late E13, and no significant difference between *TrkB.T1* mutant females and wildtype females (Fig. 2.20 and Fig. 2.21A). Moreover, since the amount of residual TrkB varies between glands and embryos, I also assessed the relationship between the amount of residual TrkB expression and relative fiber density for individual glands from several *TrkB.T1* mutant males (Fig. 21B, C). This analysis revealed a strong inverse correlation between the amount of residual mesenchymal TrkB observed in individual mammary glands of *TrkB.T1* mutant males and the number of sensory fibers associated with these glands (Fig. 2.21C; correlation coefficient: $r=-0.6499$, $p<0.0001$). Glands from *TrkB.T1* mutant males with little or undetectable residual TrkB exhibit relatively high densities of sensory innervation, whereas glands with high residual TrkB exhibit low densities of sensory innervation (Fig. 2.20A). Taken together, androgen-dependent expression of the truncated form of TrkB in male mammary mesenchyme is required for generating the male-type pattern of mammary gland sensory innervation at late E13.
Fig. 2.11  BDNF is expressed in mammary mesenchyme of both male and female at late E13.  (A) An E13 male DRG section was stained with an androgen receptor antibody (AR), and co-stained with anti-TrkB$^{ECD}$ and TO-PRO-3.  A similar pattern was observed in sections of female DRGs (not shown).  The androgen receptor is not expressed in DRG neurons at E13.  Scale bar: 50µm.  (B) X-gal staining of late E13 male and female $BDNF^{LacZ/+}$ embryos.  BDNF expression level in mammary mesenchyme is comparable between male and female.  All experiments were done at least three times.
### Fig. 2.11

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#### B

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Fig. 2.12  BDNF-TrkB signaling is required for initial axonal projections to both male and female mammary glands.  (A) Embryos were collected at late E12 and sections of both male and female rudimentary mammary glands were stained with anti-TrkB^{ECD}, anti-Tuj1 and TO-PRO-3.  (B) Mammary gland innervation of wildtype, TrkB^{GFP/GFP}, and BDNF^{LacZ/LacZ} embryos at late E12 (stained with anti-Tuj1 and TO-PRO-3).  These experiments were done two times with similar results.  Scale bar: 50µm.
Fig. 2.12

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Scale bar: 100 μm
Fig. 2.13  TrkB is expressed in the male mammary mesenchyme at E13.  (A) Mammary gland sections of late E13 male and female embryos were stained with anti-TrkB\textsuperscript{ECD}, anti-Tuj1 and TO-PRO-3.  Scale bar: 50\textmu m. (B) TrkB\textsuperscript{GFP/+} embryos were obtained at late E13, and sections of both male and female rudimentary mammary glands were stained with anti-GFP, anti-AR, and TO-PRO-3.  Pictures were taken by confocal microscopy by acquiring 1-\mu m optical images through the middle of the sections.  Note that GFP is expressed in androgen receptor expressing mammary mesenchymal cells in the male but not the female.  This experiment was done three times with similar results. Scale bar: 50\textmu m for original pictures, 25 \mu m for the cropped panels.
**Fig. 2.13**

### A

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Fig. 2.14  TrkB expressed in male mammary mesenchyme at E13 is the truncated (T1) form.  (A) Mammary gland sections of late E13 male and female embryos were stained with a truncated form-specific TrkB antibody (TrkB.T1) and TO-PRO-3. Staining observed in the female section is due to non-specific antibody binding to blood cells.  Scale bar: 50µm.  (B) In situ hybridization of mammary gland sections of male and female late E13 embryos using two different TrkB probes.  The probe that detects full-length TrkB recognizes the 3’ UTR of full-length TrkB transcript; the probe that detects both forms recognizes sequences encoding the extracellular domain of TrkB.  Note that there is no full-length transcript detected either in male or female mammary mesenchyme at this stage.  All experiments were done at least three times with similar findings.
Fig. 2.14

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Fig. 2.15 TrkB expression in mammary mesenchyme is dependent on androgen receptor activation. (A) Mammary gland sections of late E13 male and female embryos exposed to vehicle, flutamide, or testosterone propionate (8 hrs) were stained with anti-TrkBEC, anti-Tuj1, and TO-PRO-3. These experiments were done at least three times with similar findings. (B) Pregnant dams crossed with TrkBGFP/+ males were treated with flutamide beginning at late E12. Embryos were collected at late E13, and mammary gland sections were stained with anti-GFP, anti-Tuj1, and TO-PRO-3. Note that GFP expression was not detected in mammary mesenchymal cells of the flutamide treated male embryos, which suggests that Androgens regulate mesenchymal TrkB expression at the transcriptional level. This experiment was done two times with similar results. Scale bar: 50µm.
Fig. 2.15

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Fig. 2.16  TrkB expression in the female mammary mesenchyme after testosterone exposure. TrkB$^{ECD}$ and Tuj1 staining of mammary gland sections from female embryos that were treated with testosterone for the indicated times. All embryos were collected at late E13. In the quantification plot, each dot represents the average fiber density of the ten mammary glands of one embryo (red lines: means ± standard deviation, statistical analysis was done using one-way ANOVA, and data for each time point is compared with the control group). Scale bar: 50µm.
Fig. 2.16

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<td>8hrs</td>
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Fiber Density (%)

- Vehicle
- 2.5hrs
- 5hrs
- 8hrs

Testosterone exposure time (hrs)

- n.s.
- ***

TrkBECD Tuj1 TO-PRO-3
Fig. 2.17  **Assessment of TrkB^{ECD} antibody specificity.** Mammary gland sections of late E13 *TrkB^{GFP/GFP} and wildtype male embryos were immunostained using the TrkB^{ECD} antibody and TO-PRO-3 or the GFP antibody and TO-PRO-3. This experiment was done two times with similar results. Scale bar: 50µm.
Fig. 2.17

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Fig. 2.18 Quantification of mammary mesenchymal TrkB expression levels. (A) All images were taken using a Zeiss LSM700 Laser scanning confocal microscope. The scanning gain was determined by adjusting the fluorescence intensity of TrkB\textsuperscript{ECD} staining of neuronal fibers to saturation. All images were processed using ImageJ software. The threshold was determined using the default setting in ImageJ, and the average mammary mesenchymal TrkB\textsuperscript{ECD} staining fluorescence intensity (mean $F_{\text{TrkB}}$) is calculated as the average fluorescence intensity of over-threshold pixels (mean $F_{\text{TrkB,ot}}$, avoiding TrkB and Tuj1 double positive pixels) within the 15μm band minus the average background fluorescence intensity (mean $F_{\text{background}}$, within the yellow square in the image shown).

The area of mesenchymal TrkB\textsuperscript{ECD} staining ($S_{\text{TrkB}}$) is defined as the area of over-threshold pixels of TrkB\textsuperscript{ECD} staining ($S_{\text{TrkB,ot}}$) minus the area of over-threshold pixels of Tuj1 staining ($S_{\text{Tuj1,ot}}$) within the 15 μm band. The total mesenchymal TrkB level ($\sum F_{\text{TrkB}}$) is defined as the mean $F_{\text{TrkB}}$ multiplied by $S_{\text{TrkB}}$. (B) Quantification of mammary mesenchymal TrkB level in wildtype and TrkB.T1 mutant male embryos at late E13. N≥5embryos.
mean $F_{\text{TrkB}} = \text{mean } F_{\text{TrkB} \text{Bot}} - \text{mean } F_{\text{background}}$

$S_{\text{TrkB}} = S_{\text{TrkB} \text{Bot}} - S_{\text{Tuj1} \text{tot}}$

$\Sigma F_{\text{TrkB}} = \text{mean } F_{\text{TrkB}} \times S_{\text{TrkB}}$
Fig. 2.19  The residual TrkB detected in mammary mesenchymal cells of male \textit{TrkB.T1} mutant embryos lacks the TrkB.T1 specific c-terminus. Mammary gland sections of late E13 wildtype and \textit{TrkB.T1} mutant males were stained using the truncated form-specific TrkB antibody, the TrkB\textsuperscript{ECD} antibody and TO-PRO-3. The residual TrkB expressed in the mutant and recognized by the TrkB\textsuperscript{ECD} antibody cannot be detected by the truncated form-specific TrkB antibody. This experiment was done two times with similar results. Scale bar: 50µm.
### Fig. 2.19

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Fig. 2.20 and Fig. 2.21 Mammary mesenchymal TrkB expression controls formation of the sexually dimorphic pattern of mammary gland innervation prior to male gland regression.

Fig. 2.20. (A-B) Mammary mesenchymal TrkB expression and mammary gland innervation of wildtype and *TrkB.T1* mutant male (A) and female (B) embryos. Sections were stained with anti-TrkB<sup>ECD</sup>, anti-Tuj1, and TO-PRO-3. Middle panels in A shows a male mammary gland with relatively low mesenchymal TrkB expression. Right panels in A shows a male mammary gland with relatively high mesenchymal TrkB expression. Scale bar: 50µm.

Fig. 2.21. (A) Quantification of mammary gland innervation of wildtype and *TrkB.T1* mutant embryos at late E13 (n≥7 embryos for each bar). (B-C) The relationship between the total mammary mesenchymal TrkB level and the relative fiber density of individual mammary glands of wildtype and *TrkB.T1* mutant male embryos. The amount of mammary gland innervation of *TrkB.T1* mutant male embryos is inversely correlated with residual mesenchymal TrkB expression level. Relative fiber density: fiber density of individual mammary glands divided by the average fiber density of the corresponding female mammary glands at the same position along the anterior-posterior axis at late E13. Each data point represents one mammary gland from a male embryo (38 glands from 5 wildtype mice, and 65 glands from 8 *TrkB.T1* mutant mice).
Fig. 2.21

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Fiber Density (%)

B

WT Male

Relative fiber density vs. $\Sigma F_{TrkB}$ (X10^4)

$P=0.0016$

$r=-0.4893$

C

TrkB.T1 mutant Male

Relative fiber density vs. $\Sigma F_{TrkB}$ (X10^4)

$P<0.0001$

$r=-0.6499$
2.5. Discussion I

This study describes a sexually dimorphic pattern of developing mouse mammary gland sensory innervation and the mechanism by which it is established. BDNF produced in both male and female mammary glands promotes initial ingrowth and maintenance of TrkB-dependent sensory fibers. Beginning at E13, androgens acting upon receptors in male mammary mesenchymal cells trigger robust expression of the truncated form of TrkB, which neutralizes BDNF–TrkB signaling, leading to rapid withdrawal of fibers from male glands. Thus, a sex hormone-dependent switch in BDNF–TrkB signaling generates the sexually dimorphic pattern of rudimentary mammary gland innervation (Fig. 2.22).

In this study, I found that BDNF-TrkB signaling is required for maintaining sensory innervation of the mammary gland at E13, and inhibition of this signaling pathway leads to pruning of sensory axons associated with the mammary gland within several hours. This fast pruning process suggests that a repulsive mechanism might be involved. BDNF, possibly pro-BDNF, signaling through p75NTR has been implicated in promoting local axon elimination of sympathetic neurons by inhibiting NGF-TrkA signaling that is essential for axon maintenance (Singh et al., 2008). Pro-BDNF, which is likely to behave similar to pro-NGF, has a higher binding affinity to p75NTR compared to TrkB receptors (Lee et al., 2001). Therefore, one possible repulsive mechanism is that TrkB.T1 selectively sequesters mature BDNF and pro-BDNF activates p75NTR on sensory axons to induce axon pruning. However, preliminary results from analyses on p75NTR−/− embryos shows that female mammary glands of the mutant embryos exhibit much less innervation compared to the wildtype female at E13, suggesting a role of p75NTR in
supporting axonal projections instead of inducing axon pruning. Semaphorin ligands and their receptors, neuropilins and plexins, have also been implicated in axon pruning processes both in the central nervous system and the peripheral nervous system (Vanderhaeghen and Cheng, 2010). Interestingly, preliminary analyses on *neuropilin-1* (*npn-1*) mutant embryos (*npn-1*-*sema-* mice, in which semaphorin-Npn-1 binding is disrupted in homozygous mutants (Gu et al., 2003)) shows that more sensory fibers innervate the male mammary gland in mutant embryos than in wildtype embryos. Moreover, sensory axons that innervate female mammary glands at E13 express Npn-1, as observed by antibody staining. These findings suggest that semaphorin-Npn-1 signaling may play a role in the male-specific axon pruning process and generation of the sexually dimorphic pattern of mammary gland sensory innervation.

Despite the fact that most past studies on sexually dimorphic structures of the nervous system are focused on the sizes of different neuronal populations, there are few studies that have paid attention to neuronal connectivity. Sengelaud and Arnold have shown that the early projections of the SNB to their target musculature in males and females are comparable prenatally, and their study suggested that androgens may be involved in stabilization of the peripheral projections of the SNB (Sengelaub and Arnold, 1986). Moreover, projections from the BNST to the AVPV region of the brain exhibit a sexually dimorphic pattern, the formation of which is regulated in a target dependent manner (Hutton et al., 1998; Ibanez et al., 2001). More recently, Yang et al. showed that progesterone receptor expressing neurons in the ventral medial hypothalamus send many more projections to AVPV in the female compared to that in the male (Yang et al., 2013).

Since neurotrophic factors and their receptors are dynamically expressed throughout the
nervous system and are required for development and maintenance of myriad neural circuits, it would be interesting to investigate if a similar mechanism as described in this study is also applied to generation of sexually dimorphic patterns of neural connectivity in other parts of the nervous system.

Stimulation of sensory nerve endings in the nipple by infant suckling induces oxytocin release from the posterior pituitary, and oxytocin acting on the mammary gland leads to milk ejection. This process is called the milk letdown reflex. Prior studies have shown that sensory innervation of the nipple is required for this milk letdown reflex (Lincoln and Paisley, 1982). However, the function of ductal tree innervation during this reflex is unclear. In this study, with genetic labeling, I found that TrkB-expressing sensory neurons projecting to rudimentary mammary glands at E13 innervate the ductal tree structure, but not the nipple, in postnatal females. As TrkB-expressing sensory neurons projecting to hairy skin are low-threshold mechanoreceptors (Li et al., 2011), the TrkB-expressing sensory neurons that innervate the mammary gland may be ductal mechanoreceptors. A few studies have suggested that activation of ductal mechanoreceptors antagonizes catecholamine mediated inhibition of milk yield upon suckling (Mena et al., 1995; Morales et al., 2001). It would be interesting to assess the physiological properties of these mammary gland innervating TrkB neurons and test if they play any role in modulating milk production. Moreover, dynamic regulation of circulating factors, such as glucocorticoids, progesterone, and prolactin, is essential for mammary development, lactation, and post-lactational mammary gland involution (Bachelot and Binart, 2007; Watson and Kreuzaler, 2011). A neural pathway that conveys sensory information from the gland to the central nervous system might be
involved in regulating production of these factors during lactation and involution. Determining the existence and exploring the nature of such neuro-endocrine interactions would provide important insights to our understanding of mammary gland function and health.
Fig. 2.22 Schematic illustration of the cellular and molecular mechanism by which sexually dimorphic mammary gland sensory innervation is generated. BDNF produced in both male and female mammary mesenchymal cells promotes initial ingrowth and maintenance of TrkB-dependent sensory fibers. Then, beginning at E13, androgens released by male gonads and acting upon receptors in male mammary mesenchymal cells trigger robust expression of the truncated form of TrkB, which neutralizes BDNF–TrkB signaling, leading to rapid pruning of sensory fibers associated with male glands. Thus, a sex hormone-dependent switch in BDNF–TrkB signaling generates the sexually dimorphic pattern of rudimentary mammary gland sensory innervation.
Fig. 2.22

Late E12
Female
Epithelium
Mammary mesenchyme
secreted BDNF
sequestered BDNF

Late E13
TrkB.T1
TrkB-expressing sensory fibers

Late E14
Male
Androgens

Female
Epithelium
Mammary mesenchyme
secreted BDNF
sequestered BDNF

Male
Androgens

TrkB.T1
Chapter 3. Organization and development of sensory innervation of mouse Meissner corpuscles.

3.1. Structure and physiology of Meissner corpuscles.

The Meissner corpuscle is a type of cutaneous sensory terminal organ, first described in primate glabrous skin in the 1850s by Meissner and Wagner. Similar structures were identified in other mammalian species, including cat and rodents (the structure was called Krause’s end bulb in cats). Meissner corpuscles are primarily located within dermal papillae (extensions of the dermis into the epidermis, between epidermal ridges) in glabrous skin. In human, they are most abundant in finger tips and to a lesser extent in palms and plantar feet (Bolton et al., 1966). These structures are believed to be mechanosensory end organs involved in light touch sensation.

Both histological and ultra-structural analyses in several species have revealed that each Meissner corpuscle is composed of multiple sensory afferent fibers and specialized Schwann cells, and it is sometimes partially encapsulated by perineural cells (Cauna, 1956a, b; Cauna and Ross, 1960; Chouchkov, 1973; Ide, 1976; Iggo and Andres, 1982; Pare et al., 2002). The sensory afferent fibers supplying Meissner corpuscles are mainly myelinated fibers originating from large and intermediate diameter sensory neurons. These fibers enter Meissner corpuscles, lose their myelin sheath before or shortly after entering, and terminate with enlarged and flattened terminals. In addition, unmyelinated C-fibers bypass or terminate within Meissner corpuscles, and Pare et al. showed that in monkeys these C-fibers include both peptidergic and non-peptidergic subtypes (Cauna, 1956a; Ide, 1976; Pare et al., 2001). The Schwann cells associated with Meissner
corpuscles are specialized, with many cytoplasmic processes forming lamellae interwoven with neuronal terminals, and are therefore called lamellar cells. Collagen fibers are also observed within interlamellar spaces, and tonofibrils of the epidermal cells merge with collagen fibers to form connections between the basal epidermis and the corpuscle (Andres and von During, 1973; Cauna and Ross, 1960). Moreover, in mice, by ultra-structural section analyses, gap junctions are observed between axonal terminals and lamellar cells and between lamellar cell processes (Ide et al., 1985; Yoshida et al., 1989). These structural features are likely responsible for the transmission of mechanical force on the skin surface to electrical activity on the sensory afferent axons.

Developmental processes of Meissner corpuscles are similar between primates and rodents (Ide, 1977; Renehan and Munger, 1990; Saxod, 1996). In the mouse, at 18 days of gestation, extending neurites approach the epidermis and a few of them penetrate the basal lamina. One day after birth, the number of intraepidermal neurites increases, and Schwann cells are observed associated with epidermal cells. Four days after birth, Schwann cells invade the epidermis, and begin to form cytoplasmic lamellae. By 8 days after birth, both neurites and Schwann cells begin to show characteristic features of Meissner corpuscles. By 20-25 days after birth, Meissner corpuscles have attained their mature, adult form. In primates, these steps of maturation begin in the third trimester of gestation and are complete prior to birth. Studies done with genetically manipulated mouse lines indicate that Meissner corpuscle development is dependent on BDNF-TrkB signaling. Mice lacking TrkB or BDNF fail to develop Meissner corpuscles postnatally; however, mice lacking NT-4, another TrkB ligand, exhibit normal Meissner corpuscle formation (Gonzalez-Martinez et al., 2005; Gonzalez-Martinez et al., 2004). Furthermore,
overexpression of either BDNF or NT-4 in the skin of transgenic mouse lines leads to an increase in both the number and size of Meissner corpuscles (Krimm et al., 2006; LeMaster et al., 1999). While these findings implicate BDNF–TrkB signaling during the formation of Meissner corpuscles, the cellular source of BDNF and its site of action during corpuscle formation remain unknown.

Most of what we know about the physiological properties and functions of Meissner corpuscles stems from neurophysiological and psychophysiological studies of primates (including humans) and the cat. It is well accepted that Aβ low-threshold mechanoreceptors (LTMRs) innervate Meissner corpuscles and convey light touch information from the corpuscle to the CNS. These low-threshold mechanosensory fibers (LTMRs) have small receptive fields with distinct borders, usually circle or oval in shape, and they are rapidly-adapting (RA, also called FAI or QA in studies done with primates and human); They only fire action potentials at the onset and offset of stimulation (Iggo and Ogawa, 1977; Johansson and Vallbo, 1980; Knibestol, 1973; Lindblom, 1965; Vallbo and Johansson, 1984). These fibers respond to indentation of the skin, and are well activated by repetitive stimuli, optimally tuned at around 30Hz (in monkey hand) (Talbot et al., 1968). Under low-intensity stimulation, the Meissner corpuscle Aβ RA-LTMR fibers are sensitive to stimulus position, whereas under high-intensity stimuli, their responses encode velocity (Looft, 1996). Microneuronographical studies have shown that in humans activation of these Aβ RA-LTMRs is associated with perceptions of tapping or flutter on the skin, depending on the rate of stimulation (Macefield et al., 1990; Torebjork et al., 1987). The Meissner corpuscle Aβ RA-LTMRs are also implicated in the detection sudden slip between the skin and an object held in the hand and thus the
control of grip strength (Johansson and Westling, 1984; Macefield et al., 1996; Srinivasan et al., 1990).
3.2. Identification and characterization of two distinct populations of DRG neurons innervating mouse Meissner corpuscles.

3.2.1. Meissner corpuscle are innervated by myelinated afferent fibers of molecularly distinct populations of DRG sensory neurons

Immunohistological studies done by Pare et al. suggested that a single Meissner corpuscle in monkey finger pads is innervated by myelinated afferent fibers that possibly emanating from multiple sensory neurons (Pare et al., 2002). However, there is so far no direct evidence to prove or disprove this idea in any animal model. To directly address this issue, I analyzed mice in which Meissner corpuscle innervating neurons were permanently labeled with a fluorescent reporter protein. Previous work done by Luo et al. showed that a subset of large-diameter DRG neurons called “early Ret neurons”, which express Ret beginning at early embryonic stages, innervate Meissner corpuscles in mouse glabrous skin (Luo et al., 2009). Using a similar strategy, I crossed Ret<sup>CreERT2</sup> mice with Rosa<sub>26</sub>L<sup>SL-tdTomato</sup> mice (Madisen et al., 2010), and treated the mice with tamoxifen daily, from E10.5 to E13.5, to permanently label the “early Ret neurons” with tdTomato. Glabrous skin sections of these labeled mice were stained with both a neurofilament heavy chain (NFH) antibody and an S100 antibody to visualize the myelinated fibers and lamellar cells associated with Meissner corpuscles. Consistent with previous findings, the tdTomato<sup>+</sup> fibers in Ret<sup>CreERT2</sup>.Rosa<sub>26</sub>L<sup>SL-tdTomato</sup> mice project to glabrous skin and innervate Meissner corpuscles (Fig. 3.1A). Remarkably, majority of the dermal papillae that contain tdTomato<sup>+</sup> fiber(s) also contain NFH<sup>+</sup>/tdTomato<sup>−</sup> fiber(s), and these NFH<sup>+</sup>/tdTomato<sup>−</sup> terminals are intermingled with tdTomato<sup>+</sup> terminals (Fig. 3.1B). This
result shows that a substantial number of Meissner corpuscles are innervated by myelinated afferent fibers from at least two sensory neurons.

Since efficiency of the labeling strategy used for the aforementioned experiments was relatively low, I further analyzed Ret<sup>CFP/+</sup> mice, in which the coding determinants of cyan fluorescent protein (CFP) were knocked into the Ret locus. Since early Ret neurons express Ret postnatally, sensory fibers that are NFH<sup>+</sup>/CFP<sup>+</sup> and project into dermal papillae represent the early Ret neurons innervating Meissner corpuscles. Morphologically, NFH<sup>+</sup>/CFP<sup>+</sup> fibers are much thicker than non-peptidergic C fibers (CFP<sup>+</sup>/NFH<sup>-</sup>) and have enlarged terminals within dermal papillae (Fig. 3.1C). In these mice, at P20, >60% of NFH<sup>+</sup>/CFP<sup>+</sup> fibers within dermal papillae are accompanied with NFH<sup>+</sup>/CFP<sup>-</sup> fibers. Thus, the majority of Meissner corpuscles are innervated by myelinated afferents emanating from multiple neurons, and there are at least two molecular distinct neuronal populations. One is the early Ret population, and the other does not express Ret at P20.

3.2.2. Identification of a second population of myelinated afferent fibers innervating Meissner corpuscles.

Prior studies have shown that BDNF-TrkB signaling plays essential roles in Meissner corpuscle formation in mice (Gonzalez-Martinez et al., 2005; Gonzalez-Martinez et al., 2004). To test the possibility that the NFH<sup>+</sup>/CFP<sup>-</sup> Meissner corpuscle innervating neuronal population expresses TrkB during development, TrkB<sup>GFP</sup> knock-in mice were analyzed at different developmental stages. As early as E15.5, there are GFP<sup>+</sup> neuronal fibers project to palmoplantar pads and digital pads, and postnatally, GFP<sup>+</sup> fibers were
observed in dermal papillae in glabrous skin (Fig. 3.2A, B). However, at P20, GFP+ neuronal fibers in dermal papillae were difficult to find, in part because many epidermal and dermal cells express TrkB at this age (date not shown). In adult mice, no GFP+ fibers were detected in glabrous skin (Fig. 3.2C).

One possibility is that the TrkB-expressing Meissner corpuscle neurons express TrkB only transiently, during development, while a second possibility is that TrkB-expressing neurons are present during development and die at postnatal times. To distinguish between these possibilities, $TrkB^{CreERT2}$ knock-in mice were crossed with $Rosa26^{LSL-tdTomato}$ reporter mice and double heterozygous progenies were treated with tamoxifen between E15.5 and P5 to permanently label this neuronal population. In adults, tdTomato labeled fibers that project into dermal papillae in glabrous skin and terminate within S100+ corpuscle structures were observed (Fig. 3.2D). Co-labeling with NFH and myelin basic protein (MBP) antibodies showed that these fibers are NFH positive (some lose NFH immunoreactivity at axonal endings) and myelinated (Fig. 3.2E, E’, and F).

To test if the TrkB-positive neurons and the early Ret neurons that innervate Meissner corpuscles are the same neurons, I next crossed $TrkB^{CreERT2/+}; Rosa26^{LSL-tdTomato}$ mice with $Ret^{CFP/+}$ mice to obtain triple heterozygous progeny. In these mice, the TrkB population expresses tdTomato following tamoxifen treatment, while the early Ret population expresses both CFP and NFH. At P20, both tdTomato+ and CFP+/NFH+ fibers were observed within glabrous skin dermal papillae. These fibers do not overlap; rather, their terminals are intermingled as they terminate within corpuscles (Fig. 3.3A). Our preliminary quantifications showed that 76% of the dermal papillae with tdTomato+ terminals contain CFP+/NFH+ terminals (forelimbs: 87.5%, hindlimbs: 53.1%).
A screening of transgenic mouse lines generated by Gene Expression Nervous System Atlas (GENSAT) project previously found that the \(Npy2r-GFP\) BAC transgenic line specifically labels A\(\beta\) RA-LTMRs in DRGs. Further analysis revealed that in Npy2r-GFP mice GFP-expressing DRG neurons innervate a subset of Meissner corpuscles in glabrous skin (Fig. 3.3B). As an alternate test of the idea that the TrkB population and the early Ret population are distinct populations of Meissner corpuscle innervating neurons, both \(TrkB^{CreERT2/+};Rosa26^{LSL-tdTomato/+};Npy2r-GFP\) and \(Ret^{CreERT2/+};Rosa26^{LSL-tdTomato/+};Npy2r-GFP\) mice were generated and analyzed. Following tamoxifen treatments at appropriate ages, either the TrkB population or the early Ret population is permanently labeled. In \(TrkB^{CreERT2/+};Rosa26^{LSL-tdTomato/+};Npy2r-GFP\) mice, all Npy2r-GFP\(^+\) fibers projecting into dermal papillae are tdTomato\(^+\), suggesting that Npy2r-GFP\(^+\) neurons projecting to glabrous skin are a subset of the TrkB population. In contrast, in \(Ret^{CreERT2/+};Rosa26^{LSL-tdTomato/+};Npy2r-GFP\) mice, none of the Npy2r-GFP\(^+\) fibers projecting into dermal papillae are tdTomato\(^+\) (Fig. 3.3C). These results are consistent with our findings and indicate that the TrkB population and the early Ret population are molecularly distinct neuronal populations innervating Meissner corpuscles.

3.2.3. Terminal arborizations of TrkB neurons and early Ret neurons in the pad regions of mouse glabrous skin.

Since two distinct myelinated sensory neuronal subtypes that innervate Meissner corpuscles were identified, I next analyzed the terminal arborization patterns of each population. By crossing \(TrkB^{CreERT2}\) and \(Ret^{CreERT2}\) mouse lines with a \(Rosa26^{IAP}\) mouse line (Badea et al., 2009), and treating the \(TrkB^{CreERT2};Rosa26^{IAP}\) and \(TrkB^{CreERT2};Rosa26^{IAP}\) progenies with a low dose of tamoxifen, the two populations of
Meissner corpuscle neurons were sparsely labeled. With AP staining of whole-mount preparations of glabrous skin, terminal arborizations of individual labeled fibers of each population were traced and measured (Fig. 3.4A, B). Each TrkB neuron gives rise to $8.69 \pm 0.41$ terminals ($6.60 \pm 0.51$ for forelimbs, and $9.27 \pm 0.42$ for hindlimbs), whereas each early Ret neuron produces $7.93 \pm 0.81$ terminals ($7.56 \pm 0.85$ for forelimbs, and $8.50 \pm 1.65$ for hindlimbs). Although the average number of terminals for these two populations is comparable, the variability of terminal numbers for early Ret fibers is higher than that of TrkB fibers (Fig. 3.4C, D). Moreover, compared to terminal arborizations of TrkB fibers, which are concentrated with all terminals close to each other, the terminal arborizations of early Ret fibers are more disorganized and spread out.

I next asked whether a single Meissner corpuscle is innervated by more than one TrkB neuron. For this, $TrkB^{CreERT2+/+}\cdot Rosa26^{LSL-tdTomato/LSL-YFP}$ mice were generated, and in these mice TrkB fibers express either tdTomato, yellow fluorescent protein (YFP), or both YFP and tdTomato following tamoxifen treatment. By tracing individual terminals with different combinations on serial sections of glabrous skin, I found that virtually all dermal papillae contain one terminal, or terminals with one combination (out of 690 dermal papillae with labeled terminals of 3 mice, only 3 contain terminals with two combinations). Moreover, terminals with the same reporter tend to be located in adjacent dermal papillae (Fig. 3.4E). In previous AP whole-mount staining experiments, there are a few cases in $TrkB^{CreERT2; Rosa26^{IAP}+/+}$ mice in which two fibers project to the same palmpiblantar or digital pads in glabrous skin. In these cases, the two terminal arborizations occupy separate spaces; even if the terminals were found to be very close to each other, they have sharp boundaries (Fig. 3.4F). These findings indicate that the
TrkB fiber terminals in each dermal papilla are from a single TrkB sensory neuron and that their terminal arborizations have distinct borders, in other words, they are tiled.
Fig. 3.1  Meissner corpuscles are innervated by myelinated afferent fibers from molecularly distinct populations of DRG neurons.  (A-B) Forelimb palmar pad sections of P20 $Ret^{CreERT2/+}\cdot Rosa26^{LSL-tdTomato/+}$ mice were stained with anti-S100 (A), anti-NFH (B) and TO-PRO-3 (nucleic acid staining), and tdTomato expression was detected with endogenous fluorescence.  Tamoxifen was given to the moms by oral gavage at E10.5 to E11.5 to induce tdTomato expression.  (C) A hindlimb digital pad section of a P50 $Ret^{CFP/+}$ mouse was stained with anti-NFH and anti-GFP (detect CFP expression).  Yellow arrow: CFP+/NFH+ early Ret fiber.  Green arrow: CFP+/NFH- non-peptidergic C-fiber.  Red arrowheads: NFH+/CFP- fibers.  Scale bar: 25 µm.
Fig. 3.1

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Fig. 3.2 A subset of DRG sensory neurons expressing TrkB during development innervate Meissner corpuscles. (A-C) Co-staining of GFP (green) and TO-PRO-3 (blue) on Forelimb palmar pad sections of $TrkB^{GFP/+}$ mice at different developmental stages. (D) A hindlimb digital pad section of a P50 $TrkB^{CreERT2/+;Rosa26^{LSL-tdTomato/+}}$ mouse treated with tamoxifen at E16.5 (deliver to the mom by oral gavage, 1mg). The section was co-stained with anti-DsRed (detect tdTomato), anti-S100 and TO-PRO-3. (E) A hindlimb digital pad section of the same mouse as in D, co-stained with anti-DsRed, anti-NFH and TO-PRO-3. Pannels in E’ are enlarged views of region cropped in E. (F) A hindlimb plantar pad section of a P20 $TrkB^{CreERT2/+;Rosa26^{LSL-tdTomato/+}}$ mouse treated with tamoxifen at P1, co-stained with anti-DsRed, anti-MBP and To-PRO-3. Scale bar: 50µm.
Fig. 3.2

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dtTomato

S100 TO-PRO-3

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tdTomato NFH TO-PRO-3

tdTomato

NFH

Merge

tdTomato

S100 TO-PRO-3

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Fig. 3.3  TrkB neurons and early Ret neurons are two distinct sensory populations innervating Meissner corpuscles.  (A) A hindlimb digital pad section of a P50 \( \text{TrkB}^{\text{CreERT2/+}; \text{Rosa26}^{\text{LSL-tdTomato/+}; \text{Ret}^{\text{CFP/+}}}} \) mouse treated with tamoxifen at P5, co-stained with anti-DsRed, anti-GFP and anti-NFH. Scale bar: 10\( \mu \text{m} \). (B) A forelimb palmar pad section of a P20 \( \text{Npy2r-GFP} \) mouse was stained with anti-GFP, anti-S100 and TO-PRO-3. Scale bar: 50\( \mu \text{m} \). (C) Glabrous skin sections of a P20 \( \text{TrkB}^{\text{CreERT2/+}; \text{Rosa26}^{\text{LSL-tdTomato/+}; \text{Npy2r-GFP}}} \) mouse (upper panels, forelimb digital pad) and a P20 \( \text{Ret}^{\text{CreERT2/+}; \text{Rosa26}^{\text{LSL-tdTomato/+}; \text{Npy2r-GFP}}} \) mouse (lower panel, forelimb palmar pad) were stained with anti-DsRed, anti-GFP and TO-PRO3. The \( \text{TrkB}^{\text{CreERT2/+}; \text{Rosa26}^{\text{LSL-tdTomato/+}; \text{Npy2r-GFP}}} \) mouse was treated with tamoxifen at P5, and the \( \text{Ret}^{\text{CreERT2/+}; \text{Rosa26}^{\text{LSL-tdTomato/+}; \text{Npy2r-GFP}}} \) mouse was treated with tamoxifen at E10.5. Scale bar: 25\( \mu \text{m} \).
Fig. 3.3

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Fig. 3.4 Terminal arborizations of TrkB neurons and early Ret neurons in the pad regions of mouse glabrous skin. (A-B) Whole-mount AP staining of forelimb glabrous skin of a TrkB\textsuperscript{CreERT2/+;Rosa26 \textit{iAP}/+} mouse (A) and a Ret\textsuperscript{CreERT2/+;Rosa26 \textit{iAP}/+} mouse (B). In A, 1mg tamoxifen was delivered to the mom at E14.5 to avoid AP expression in dermal and epidermal cells. Tamoxifen treatment between E12.5 to E14.5 labels the same Meissner corpuscle innervating neurons as treated later (data not shown). In B, 2mg tamoxifen was delivered to the mom at E12.5. Upper panels show the terminal arborizations of a single labeled fiber. Lower panels show the same region with a different focal plane, and red arrow points to the branching site of that fiber. Scale bar: 250µm. (C) Quantifications of numbers of terminals from single TrkB fibers or single early Ret fibers (23 TrkB fibers from 4 mice, and 15 early Ret fibers from 4 mice). (D) Quantification of the variations of terminal numbers for each population. (E) TrkB\textsuperscript{CreERT2/+;Rosa26 \textit{LSL-tdTomato}/\textit{LSL-YFP}} mice were treated with tamoxifen at E12.5 and E13.5. Serial sections of glabrous skin of these mice were cut, and stained with anti-DsRed, anti-GFP and TO-PRO-3. Three adjacent sections are shown here. Y: fibers express both tdTomato and YFP; R: fibers express tdTomato only; G: fibers express YFP only. Y’ and R’: fibers without terminals on the sections. Scale bar: 50µm. (F) Whole-mount AP staining of hindlimb glabrous skin of a TrkB\textsuperscript{CreERT2/+;Rosa26 \textit{iAP}/+} mouse. There are two fibers project to this plantar pad, and terminals of either fiber were traced and marked with red dots or yellow stars, shown on the right panel. There is a sharp boundary between the terminal arborizations of these two fibers (dashed line). Scale bar: 100 µm. All statistical analyses shown in this figure and subsequent figures in this
chapter were done using student t-test. Shown are the means ± s.e.m., *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \), n.s. not significant.
Fig. 3.4

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n.s.

+ *
3.3. Differential dependence of mouse Meissner corpuscle development on the two populations of innervating neurons.

3.3.1. BDNF expressed in the epidermis is required for Meissner corpuscle formation.

Meissner corpuscles are absent in mouse glabrous skin of both BDNF and TrkB null mice (Gonzalez-Martinez et al., 2005; Gonzalez-Martinez et al., 2004). But yet, how BDNF-TrkB signaling controls this developmental process is not known. Using BDNF\textsuperscript{lacZ} knock-in mice (Gorski et al., 2003), I first analyzed BDNF expression patterns at different developmental stages. BDNF is expressed in DRG neurons as well as mesodermal tissues at embryonic stages (Fig. 3.5A, and data not shown), and after E15.5 BDNF is expressed in epithelial cells in the pad regions of glabrous skin (Fig. 3.5B-D). To identify the source of BDNF that is responsible for Meissner corpuscle formation, I analyzed BDNF conditional knock-out mice using either Wnt1Cre or Keratin5-Cre (K5Cre) driver lines (Danielian et al., 1998; Gorski et al., 2003; Ramirez et al., 2004). In Wnt1Cre;BDNF\textsuperscript{f/f} mice, in which BDNF expression is eliminated in both DRG sensory neurons and Schwann cells, the abundance of Meissner corpuscles is comparable to control littermates (Fig. 3.5E, F). In contrast, in K5Cre;BDNF\textsuperscript{f/f} mice, in which BDNF expression is eliminated in skin epithelial cells, the number of Meissner corpuscles is dramatically reduced in both forelimb and hindlimb glabrous skin (Fig. 3.5G-I). These findings indicate that BDNF secreted from epithelial cells of the skin is essential for Meissner corpuscle formation.

3.3.2. TrkB is required in sensory neurons, but not in glia cells, for Meissner corpuscle formation.
Next, I investigated the identity of cell type in which TrkB is required for Meissner corpuscle formation. A \textit{TrkB} conditional line was crossed with \textit{Advillin-Cre (AvilCre)} mice to selectively eliminate TrkB expression in primary sensory neurons (da Silva et al., 2011; Liu et al., 2012). In \textit{AvilCre;TrkB^{f/f}} mice, S100 staining shows that virtually no Meissner corpuscles are present in glabrous skin (Fig. 3.6A, B and I). To exclude the possibility that in sensory neuron specific TrkB conditional knock-out mice Meissner corpuscles are formed but fail to express S100 protein, toluidine/methylene blue staining on semi-thin sections of the glabrous skin of both control and \textit{AvilCre;TrkB^{f/f}} mice was done. Consistent with findings using S100 staining, no Schwann cells were observed in dermal papillae of adult \textit{AvilCre;TrkB^{f/f}} mice (Fig. 3.6C, D). Similar results were found in mice at P12, a time when Meissner corpuscles are not yet mature (Fig. 3.6E, F). This finding further indicates that the absence of Meissner corpuscles in \textit{AvilCre;TrkB^{f/f}} mice is a consequence of a developmental deficit, not a maintenance deficit. Moreover, to test whether TrkB is required in Schwann cells for Meissner corpuscle formation, I generated \textit{DhhCre;TrkB^{f/f}} mice in which TrkB expression is specifically eliminated in Schwann cells (Jaegle et al., 2003). Meissner corpuscles are formed in comparable numbers in glabrous skin of control and \textit{DhhCre;TrkB^{f/f}} mice (Fig. 3.6G, H and J). Thus, TrkB is required in sensory neurons, but not in lamellar cells, for Meissner corpuscle formation.

Interestingly, NFH\(^{+}\) fibers are present in some of the dermal papillae in glabrous skin of \textit{AvilCre;TrkB^{f/f}} mice at P14, and on semi-thin sections of these mice at P12, I also found large caliber neuronal fibers within dermal papillae structures in the mutant mice (Fig. 3.6F). However, after P20, NFH\(^{+}\) myelinated fibers are rarely seen within dermal papillae of \textit{AvilCre;TrkB^{f/f}} animals (Fig. 3.6D and data not shown). These findings
suggest that, when TrkB expression is eliminated in all sensory neurons, a subset of myelinated sensory axons project to the dermal papillae initially but they fail to maintain their projections in the dermal papillae at later stages. Since only the TrkB neurons innervating Meissner corpuscles expresses TrkB during development, it is likely that the axons present in the dermal papillae of AvilCre;TrkB<sup>f/f</sup> mice before P20 are from the early Ret population. Indeed, I observed that, in AvilCre;TrkB<sup>f/f</sup>;Ret<sup>CFP/+</sup> mice, the majority of the NFH<sup>+</sup> fibers present in glabrous skin dermal papillae at P14 are CFP<sup>+</sup> (Fig. 3.6K). Thus, the projections of “TrkB neurons” lacking TrkB are severely disrupted, and these projections are necessary for Meissner corpuscle formation. These findings further indicate that projections of early Ret fibers into the dermal papillae are insufficient for Meissner corpuscle formation.

3.3.3. Schwann cells are associated with TrkB neurons during their migration into dermal papillae prior to differentiation into lamellar cells.

Since the TrkB fibers located in dermal papillae are essential for Meissner corpuscle formation, I assessed the relationship between Schwann cells and TrkB fibers at early postnatal stages. DhhCre;TrkB<sup>CFP/+</sup>;Rosa26<sup>LSL-tdTomato/+</sup> mice were generated and analyzed at P5, before Schwann cells differentiate into lamellar cells. At this stage, GFP<sup>+</sup> fibers project into dermal papillae and Schwann cells, which are tdTomato<sup>+</sup>, are also present in dermal papillae. Remarkably, the neurite tips of virtually all GFP<sup>+</sup> fibers in dermal papillae are associated with tdTomato<sup>+</sup> Schwann cell(s) (Fig. 3.7). This result suggests that TrkB fibers may be required for the migration of Schwann cells to the apex of dermal papillae where they differentiate into the lamellar cells of the corpuscle.
Fig. 3.5  BDNF expressed in epithelial cells is essential for Meissner corpuscle formation. (A) X-gal staining of a DRG section of an E15.5 \( BDNF^{lucZ/+} \) embryo. (B) Whole-mount X-gal staining of a forelimb of an E16.5 \( BDNF^{lucZ/+} \) embryo. (C-D) X-gal staining of forelimb palmar pad sections of \( BDNF^{lucZ/+} \) mice at E15.5 and P12. (E-H) Hindlimb plantar pad sections were stained with anti-S100 to reveal Meissner corpuscles. The number of Meissner corpuscles in glabrous skin of control and \( Wnt1Cre;BDNF^{f/f} \) mice are comparable, and this comparison was done with two pairs of control and \( Wnt1Cre;BDNF^{f/f} \) mice. Scale bar: 50µm. (I) Quantifications of the number of Meissner corpuscles in all hindlimb plantar pads of control and \( K5Cre;BDNF^{f/f} \) mice at P20 (N=3 mice for each bar). Serial sections were cut throughout the whole plantar region and Meissner corpuscles were identified by S100 staining and counted on all sections.
Fig. 3.5

A

B

C

D

E15.5

E16.5

E15.5

P12

E

F

G

H

Wnt1Cre;BDNF+/+  
K5Cre;BDNFfg/

Wnt1Cre;BDNFfg/

K5Cre;BDNFfg/

I

Number of MCs (all plantar pads)

Control

K5Cre;BDNFfg/

**
Fig. 3.6  TrkB is required in sensory neurons, but not in glia cells, for Meissner corpuscle formation.  

(A, B) S100 staining of hindlimb plantar pad sections of control and AvilCre;TrkBff mice at P14. Scale bar: 50µm.  

(C-F) Semi-thin sections (0.5µm) of hindlimb plantar pads of control and TrkBff; AvilCre mice were cut parallel to skin surface. In C and D, mice were sacrificed at P60, and staining was done with methylene blue. Red arrowheads: cytoplasmic processes of lamellar cells; black arrows: nerve terminals (densely stained). In E and F, mice were sacrificed at P12, and staining was done with toluidine blue. Red arrowheads: pre-mature corpuscle, white arrows: large caliber nerve fibers.  

(G, H) S100 staining of hindlimb plantar pad sections of control and DhhCre;TrkBff mice at P20. Scale bar: 50µm.  

(I) Quantification of the number of Meissner corpuscles in control and AvilCre;TrkBff mice at one to two months of age (N=3 mice for each bar). Meissner corpuscles in the top two plantar pads of each hindlimb were counted.  

(J) Quantification of the number of Meissner corpuscles in control and DhhCre;TrkBff mice at P20 (N=3 mice for each bar). Meissner corpuscles in the bottom two palmar pads of each forelimb were counted.  

(K) A forelimb palmar pad section of a AvilCre;TrkBff;RetCFP/+ mouse was stained with anti-GFP and anti-NFH. Scale bar: 50 µm.
Fig. 3.6

**A**
AvilCre;TrkB^+/+

**B**
AvilCre;TrkB^+/+

**G**
DhhCre;TrkB^+/+

**H**
DhhCre;TrkB^+/+

**I**
Graph showing number of MCs (top two plantar pads) with significant difference (** ***

**J**
Graph showing number of MCs (bottom two palmar pads) with no significant difference (n.s.)

**K**
Merge images of GFP, NFH, and Merge.
Fig. 3.7 Schwann cells are associated with TrkB-expressing fibers in dermal papillae during development. Upper panels: A forelimb palmar pad section of a $Dhh^{Cre};TrkB^{GFP/+};Rosa26^{1SL-tdTomato/+}$ mouse, stained with anti-GFP, and tdTomato expression was detected by endogenous fluorescence. For each GFP$^+$ fiber in dermal papillae, there are Schwann cell(s) associated with its tip. Scale bar: 50µm. Lower panels: enlarged views of the region cropped in
3.4. Discussion II

In this study, I identified two molecularly distinct DRG neuronal populations with myelinated axons that innervate Meissner corpuscles of the mouse. Peripheral projections of the two neuronal populations exhibit different terminal arborization patterns in glabrous skin. During development, one population, which expresses TrkB and depends on skin epidermis-derived BDNF, is necessary for Meissner corpuscle formation. The other population, which expresses Ret, is not sufficient for inducing lamellar cell formation and Meissner corpuscle maturation. This study elucidates the organization of myelinated sensory afferents associated with Meissner corpuscles and reveals the differential contributions of distinct sensory neuron subpopulations during formation of a mechanosensory end organ.

Prior structural analyses have described four different types of nerve endings from large caliber fibers in human Meissner corpuscles (Cauna, 1956a). In contrast, electrophysiological recordings have only revealed one type of myelinated fiber supplying Meissner corpuscles: the Aβ RA-LTMRs. Here, I have identified two molecularly distinct populations that innervate Meissner corpuscles in the mouse, and they are both myelinated and thus likely to be Aβ subtypes with fast conduction velocity. Interestingly, in TrkB\textsuperscript{CreERT2/+};Rosa26\textsuperscript{LSL-tdTomato/+};Ret\textsuperscript{CFP/+} mice, in addition to tdTomato\textsuperscript+ and CFP\textsuperscript+/NFH\textsuperscript+ fibers, some dermal papillae (more prominent in forelimbs) may contain a third type of fiber that is NFH\textsuperscript+/tdTomato\textsuperscript−/CFP\textsuperscript−. This suggests the possibility of a third population of myelinated fibers innervating mouse Meissner corpuscles. What is the function of multiple sensory neurons with distinct molecular identities innervating a single Meissner corpuscle? One possibility is that different neuronal populations have
distinct terminal arborization patterns and central connections. Therefore, activating distinct sensory neurons by a given stimulus may lead to recruitment of multiple neural pathways in the central nervous system that are involved in generating different perceptual and behavioral consequences. Another possibility is that molecularly distinct populations exhibit different physiological properties, including their response thresholds and tuning properties, adaptation properties, and conduction velocities. Thus, as a result, the distinct sensory neurons could encode unique features of the stimulus. These two possibilities are not mutually exclusive. To understand how these two populations of neurons respond to different physiological stimuli on the skin surface, electrophysiological recordings of DRG neurons with intact peripheral terminals will be required (McIlwrath et al., 2007; Woodbury et al., 2001). The present study reveals useful genetic labeling strategies for future work identifying the two populations of Meissner corpuscle neurons for electrophysiological assessment, as well as for further anatomical analyses. Moreover, the present work suggests new genetic strategies that could be used to either activate or silence each of the two populations of Meissner corpuscle innervating neurons during behavioral tasks to assess the physiological functions of each population.

In the present study, double labeling experiments reveal that each Meissner corpuscle is innervated by only one TrkB neuron and the terminal arborizations of each TrkB neuron occupies its own, distinct territory. Thus, the TrkB neuron projections are tiled. It would be interesting to follow development of TrkB neuron arborizations to see how this tiled pattern is achieved. Moreover, whether the terminal arborizations of early Ret neurons are also tiled is worthy of further investigation. Since a single Meissner corpuscle
receives innervation by both TrkB and early Ret neurons, the territory occupied by
terminal arborization of a single TrkB neuron surely overlaps, at least to a certain degree,
with the territory occupied by the terminal arborizations of one or more early Ret neurons.
This phenomenon is similar to that observed for the dendritic organization of
multidendritic sensory neurons in Drosophila: cells within the same morphological or
functional class innervate the body wall with distinct, non-overlapping territorial domains,
whereas the dendritic domains of cells in different classes overlap extensively (Grueber et
al., 2002; Grueber and Sagasti, 2010). The organization of sensory neurons innervating
Meissner corpuscles may serve as a vertebrate model for studying the molecular basis of
homotypic and heterotypic axonal tiling.

What are the respective roles of TrkB neurons and early Ret neurons for Meissner
corpuscle formation? During neonatal development, Schwann cells are associated with
TrkB fibers in dermal papillae, and TrkB fibers are necessary for Meissner corpuscle
formation. In addition, my preliminary findings indicate that, in sensory neuron specific
Ret knock-out mice, the early Ret fibers are missing from dermal papillae, but Meissner
corpuscles are properly formed in these mutant mice. This finding suggests that TrkB
fibers are both necessary and sufficient to induce Meissner corpuscle formation.
Moreover, these findings suggest that certain neural-glial interactions or perhaps signals
specifically derived from TrkB fibers that enable corpuscle formation. In previous ultra-
structural studies, it was observed that axons, Schwann cells, and basal epidermal cells
are always intimately associated in the developing Meissner corpuscle, suggesting a
contribution of basal epidermal cells to corpuscle formation (Ide, 1977; Renehan and
Munger, 1990). One possible function of TrkB fibers is to facilitate and maintain close
interactions between Schwann cells and basal epidermal cells, and BDNF derived from the epidermis may promote the intercellular associations within this complex. The identification of signals emanating from TrkB neurons that promote lamellar cell maturation and Meissner corpuscle formation would provide insights into our understanding of the neuron-glia-skin interactions that underlie the formation of mechanosensory end organs.
Materials and Methods

Embryo stages

The day on which the vaginal plug was observed after setting up matings was counted as embryonic day 0. Stages of embryos were further confirmed by checking embryo morphology and limb development after dissection (Kaufman, 1992). For Chapter 2, embryos were collected at two different times at a given embryonic day: 9-10am was considered as early in the day, and 4-5pm was considered late in the day.

Genotype protocol for determining sex

Primers for genotyping the Zfy1 gene are: 5’-ACATGGAGAGCCACAAGCTAACCA-3’ and 5’-CTTTCTTGTGCAGACGCAAGCATGT-3’. The size of the PCR product from male embryos is 468bp.

Mouse lines

The androgen-insensitive Tfm, AvilCre, BDNF^{LacZ}, DhhCre; K5Cre; Npy2r-GFP; Ret^{CFP}, Ret^{CreERT2}, Rosa26^{LAP}, Rosa26^{Lsl-Tomato}, Rosa26^{Lsl-YFP}, TrkB.T1, TrkB^{GFP} (previously referred as TrkB^{tauEGFP}), and Wnt1Cre mice have been described previously (Badea et al., 2009; da Silva et al., 2011; Danielian et al., 1998; Dorsey et al., 2006; Gorski et al., 2003; Jaegle et al., 2003; Li et al., 2011; Luo et al., 2009; Lyon and Hawkes, 1970; Madisen et al., 2010; Ramirez et al., 2004; Uesaka et al., 2008). The targeting vector used to generate the NGF^{LacZ} knock-in allele and the embryonic stem (ES) cells used to generate the NT-4^{LacZ} knock-in allele were obtained from EUCOMM. Generation of mice harboring the TrkB^{CreERT2} allele has been described in dissertation of Dr. Michael Rutlin.
The $TrkB^f$ and $TrkB^{F616A}$ mice were modified versions of the TrkB chemical-genetic mice described previously (Chen et al., 2005). For the $TrkB^f$ allele, a 3.2 kb sequence containing exon 15 and a FRT-Neo-FRT cassette was flanked by two loxP sites. For the $TrkB^{F616A}$ allele, the same 3.2 kb sequence containing exon 15, but lacking loxP sites, harbors the F616A mutation. Homologous recombination for both targeting constructs was performed using 129.1 mouse strain embryonic stem (ES) cells. ES cell clones that exhibited homologous recombination were screened by PCR, and results were confirmed by Southern blotting. Correctly targeted ES clones were injected into C57BL/6 blastocysts, which were then introduced into pseudopregnant females. Heterozygous mice were generated by crossing chimeric mice with C57BL/6 mice, and these mice were subsequently crossed with mice expressing FlpE recombinase in germ cells to excise the Neo cassette. Both lines were backcrossed and maintained on a C57BL/6 background. Mice were genotyped using PCR reactions with following primers: 5’-GGGCTTGAGAAGAGGGCAAAAGGGTTGCTCAG-3’ and 5’-GTTGGTCACCAGCAGAACACTCGACTCAC-3’. The sizes of PCR products of the wildtype, $TrkB^f$, and $TrkB^{F616A}$ alleles are 280bp, 430bp, and 350bp, respectively.

**Drug treatments**

Flutamide (Sigma F9397) was dissolved in DMSO to make a 500mg/ml stock, and 20µl of this stock solution or DMSO diluted with 500µl sunflower seed oil was delivered to pregnant females by oral gavage at E12 (5-6pm) and E13 (10-11am). Testosterone propionate (Sigma T1875) was dissolved in sunflower seed oil to make a 20mg/ml solution, and 50µl of this solution or sunflower seed oil was delivered to the pregnant females by subcutaneous injections. DHT was dissolved in ethanol to make a 50mg/ml
solution, right before use 20µl of this solution was mixed well with 60µl sunflower seed oil, and vacuum centrifuged to evaporate the ethanol before subcutaneous injections.

For 1NMPP1/TrkB^{F616A} chemical-genetic experiments, the TrkB^{F616A} inhibitor 1NMPP1 was dissolved in DMSO to make a 200mM stock, and 2.5µl of the 1NMPP1 stock solution or DMSO was diluted into 100µl of injection solution (0.9% NaCl, 2.5% Tween-20) for subcutaneous injections. For oral gavage administration, 0.5µl 1NMPP1 stock solution or DMSO was diluted into 20ul of injection solution and then further diluted into 500µl water. Treatments were given every 1.5hr, four times, beginning at E13 (11am). The inhibitor 1NMPP1 was synthesized by Aurora Analytics, LLC, as previously described (Bishop et al., 2000).

Tamoxifen (Toronto Research Chemicals) was dissolved in 100% ethanol to make a 20mg/ml stock solution. For embryonic stage delivery, 50-150µl stock solution was diluted with sunflower seed oil at 1:3 dilution, vortexed for 5-10min, and vacuum centrifuged for 20min. Working solution was delivered to pregnant mothers by oral gavage. To label early Ret population with high efficiency, 2mg of tamoxifen was given each day to pregnant mothers at E10.5 and E11.5 for forelimb labeling, and at E12.5 and E13.5 for hindlimb labeling. To label TrkB population (in Chapter 3) with high efficiency, tamoxifen was given to pregnant mothers either at E12.5 and E13.5 or at E15.5 and E16.5, 2mg for each day. TrkB population can also be specifically labeled by delivering tamoxifen to early postnatal pups. For treating early postnatal pups, 5µl (for P1 pups) or 10-25µl (for P5 pups) tamoxifen stock solution was diluted with 50µl or 100µl sunflower seed oil and mixed well by vortexing. Working solution was administrated to pups by intraperitoneal injection. For sparse labeling experiments using
*Rosa<sup>IAP</sup>* reporter, 1mg tamoxifen was given to pregnant mother at E14.5 to label TrkB population, and 2mg tamoxifen was given to pregnant mother at E12.5 to label early Ret population.

**Immunohistochemistry**

Embryos and early postnatal tissues (under P10) were fixed in PBS containing paraformaldehyde (PFA, 4%) at 4°C for 2 hours to overnight. P3 mammary glands were dissected together with fat pads and adjacent skin, fixed in 4% PFA in PBS at 4°C for 1-2hrs. Mice older than P14 were perfused with 4% PFA in PBS, spinal cords and distal limbs were dissected from the perfused mice. Spinal cords were post-fixed with 4% PFA in PBS at 4°C for 1-2hrs. Distal limbs were further processed to expose the internal surface of glabrous skin and then post-fixed with 4% PFA in PBS at 4°C for 4-6hrs. All tissues were cryoprotected in 30% sucrose in PBS at 4°C. Tissues were embedded in OCT (Tissue Tek), frozen at -20°C, and sectioned at 20µm. Sections were dried overnight at room temperature, washed with PBS and blocked with 5% normal serum (goat or donkey) in 0.1% PBST (0.1% Triton X-100 in PBS) for 30min, followed by primary antibody incubation (diluted in blocking solution) overnight at 4°C. The next day, sections were washed with 0.1% PBST, and incubated with secondary antibodies diluted in blocking solution for 1-2hrs, washed with 0.1% PBST again, and mounted with fluoromount-G (Southern Biothech). Pictures were taken using a Carl Zeiss LSM700 laser scanning microscope.

Primary antibodies used for immunohistochemistry were: rabbit anti-Androgen receptor (Invitrogen, 1:300), rabbit anti-DsRed (Clontech, 1:500), goat anti-mCherry (Sicgen,
1:500), chicken anti-GFP (Aves Lab, 1:500), rabbit anti-GFP (Invitrogen, 1:500), goat anti-GFP (US Biological 1:500), rat anti-MBP (Millipore, 1:500), rabbit anti-NFH (Sigma, 1:1000), chicken anti-NFH (Aves Lab, 1:500), rabbit anti-S100 (Dako, 1:300), goat anti-TrkB ECD (R&D, 1:100), rabbit anti-TrkB.T1 (Santa Cruz, 1:50), rabbit anti-Tuj1 (Covance, 1:1000). The nucleic acid stain TO-PRO-3 (Invitrogen 1:500) was applied along with secondary antibody incubation during immunohistochemistry.

**In situ hybridization**

Protocols for *in situ* hybridization were described previously (Luo et al., 2007). Embryos were fixed in 4% PFA in PBS at 4°C for overnight, and cryoprotected in 30% sucrose in PBS at 4°C. Tissues were embedded in OCT (Tissue Tek) and frozen at -20°C. Tissue sections were cut at 14-20µm and then dried at room temperature for 1hr. The probe for full-length *TrkB* was generated to detect an 842bp sequence in the 3’UTR of full-length *TrkB* mRNA (NM_001025074.1). Primers for generating this probe construct were: 5’-ATGAGCGAGACAGAGACAAGCCAT-3’ and 5’-ACCTTGGAATGAAACCACTCTCCC-3’. The probe for both forms of TrkB was generated to detect an 853bp sequence in the coding region for the extracellular domain of TrkB. Primers for generating this probe construct were: 5’-AATGAGAGCAGCAAGAACATGCCC-3’ and 5’-GCCAAACTTGGAATGTCTCGCCAA-3’. Probes were labeled with Dioxygenin (DIG).

**X-gal staining**
Embryos and glabrous skin were fixed with a glutaraldehyde solution (0.2% glutaraldehyde, 2mM MgCl₂ in PBS) overnight at 4°C. For whole-mount staining, fixed embryos or skin were washed with detergent rinse buffer (0.01% sodium deoxycholate, 0.02% NP40, 2mM MgCl₂ in phosphate buffer, pH 7.4), and stained with staining buffer (0.01% sodium deoxycholate, 0.02% NP40, 2mM MgCl₂, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 1mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside in phosphate buffer, pH 7.4) at room temperature. After staining, embryos were dehydrated sequentially with 50% methanol, 80% methanol, and 100% methanol. For staining on sections, 12µm sections were cut and dried for several hours at room temperature. The staining procedure was the same as described for whole-mount staining. After staining, sections were fixed overnight at 4°C in 4% PFA in PBS, and mounted with fluoromount-G.

Carmine staining

Whole mount carmine staining was carried out by procedures described previously (Mueller et al., 2002). P0 mammary glands were dissected with fat pads intact, and fixed in 4% PFA in PBS at 4°C for 1hr. To reveal the gland ductal tree structure, fixed mammary glands were stained with carmine aluminum (Sigma C1022) solution overnight at room temperature, dehydrated with 50%, 75%, 95%, 100% ethanol, and cleared in xylene.

Alkaline phosphatase histochemistry

Placental alkaline phosphatase (PLAP) staining was carried out by procedures described previously (Liu et al., 2007). P0 mammary glands were dissected with fat pads intact,
and fixed in 4% PFA in PBS at 4°C for 1hr. P20 mice were perfused with 4% PFA in PBS, and glabrous skin was dissected and post-fixed with 4% PFA in PBS for 4hrs. After fixation, tissues were washed with PBS, and incubated with PBS at 65-68 °C for 2-3 hrs. To detect the PLAP signal, tissues were stained with BCIP/NBT (Roche) solution (diluted in 0.1M Tris pH 9.5, 0.1M NaCl, 50mM MgCl₂, 0.1% Tween-20 solution) overnight at room temperature, followed by post-fixation in 4% PFA in PBS overnight at 4°C, dehydrated with 50%, 80%, 100% methanol, and cleared in BABB.
References


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**Advisor:** Dr. Shi-Qiang Wang, Ph.D.

**Research Grant:** the President Fund, Peking University

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**Publications**


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**Presentations at Scientific Meetings**


**Liu, Y.,** Rutlin, M., Ginty, D.D. TrkB-expressing DRG neurons projecting to glabrous skin during development are a novel neuronal population responsible for Meissner corpuscle formation. *Poster Presentation.* Society of Neuroscience Annual Meeting. San Diego, CA, October 2010

Teaching Services

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