MECHANISMS OF STRUCTURAL PLASTICITY IN MATURE SENSORY AXONS: ROLE OF BMP4 IN FEMALE REPRODUCTIVE TRACT

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ABSTRACT

Structural changes in sensory axons are associated with many peripheral nerve disorders. Degenerative loss or excessive sprouting of axons are hallmarks of sensory neuropathies or hyperinnervating pain syndromes respectively. While much is known about mechanisms of developmental axon growth or regenerative outgrowth following nerve injury, there is little information about mechanisms that can induce plasticity in intact adult axons. Lack of model systems, where extensive plasticity can be predictably induced under physiological conditions, has been a fundamental impediment in studying sensory neuroplasticity mechanisms in adult.

The female reproductive tract presents a highly tractable model where sensory axons cyclically undergo extensive plasticity under the influence of estrogen. In rat, high estrogen levels induce reduction in vaginal sensory nerve density, and low estrogen conditions promote sprouting leading to hyperinnervation. We used this model to explore potential factors that can initiate spontaneous plasticity in intact sensory axons. We found that estrogen downregulates expression of Bone Morphogenetic Protein 4 (BMP4) in the vaginal submucosal smooth muscle cells which are associated with most innervation in the vagina. Thus under low estrogen conditions BMP4 expression increased in the smooth muscle, and the resulting trophic stimulus induced sprouting in associated nociceptive sensory axons. Activated Smad1 in nuclei of retrogradely labeled dorsal root ganglion neurons innervating the vagina confirmed BMP4 signaling under low estrogen conditions. When BMP4 was overexpressed in the vaginal submucosal smooth muscle by lentiviral transduction, elevated nerve density prevailed even under high blood estrogen levels.

To identify the signaling mechanism by which BMP4 regulates sensory plasticity downstream from phospho-Smad1, we used the 50B11 sensory neuronal cell line. Receptor

expression and outgrowth responses of the 50B11 cells to neurotrophins and growth factors resembled closely that of primary sensory neurons in culture, providing a reliable assay system. BMP4 treatment induced upregulation of Inhibitor of DNA binding 2 (Id2), a known downstream target of Smad1, in 50B11 cells; and Id2 overexpression induced increased neurite outgrowth. However, Id2 knockdown failed to abrogate BMP4-mediated increase of outgrowth in primary neurons, suggesting alternative mechanisms. We identified that BMP4 downregulates methyl CpG-binding protein 2 (MeCP2), a methylated-DNA binding protein and an epigenetic modifier and global transcriptional repressor, which is highly expressed in neurons. MeCP2 downregulation can prompt a global transcriptional upregulation inducing multiple genes, a situation characteristic of axonal growth initiation. Lentiviral knockdown of MeCP2 resulted in significant elevation of outgrowth in primary sensory neurons that matched growth induced by BMP4.

Thus, we conclude that BMP4 is a factor capable of inducing plasticity in adult sensory axons. Smad1 activation by BMP4 induces sensory outgrowth by downregulating MeCP2 expression. These findings have significant impact on understanding of genetic and epigenetic mechanisms of adult peripheral neuroplasticity. It will also have strong implications in considering therapeutic measures in sensory neuroplasticity-associated disorders. The findings are particularly important in female pelvic pain syndromes like vulvodynia that are associated with excessive nerve sprouting under low estrogen conditions.

DEDICATION

This work is dedicated to my mother Mrs. Bejoyasree Bhattacherjee

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LIST OF ABBREVIATIONS (used extensively in this document)

ANGII – Angiotensin II

AT2 – Angiotensin II receptor type 2

BDNF – Brain Derived Neurotrophic Factor

BMP- Bone Morphogenetic Protein

BMP4 – Bone Morphogenetic Protein 4

CFA – Complete Freund's Adjuvant

CGRP – Calcitonin Gene Related Peptide

CMV – Cytomegalovirus

CNS – Central Nervous System

DRG – Dorsal Root Ganglia

EGFP – Enhanced Green Fluorescent Protein

FGF – Fibroblast Growth Factor

FRT – Female Reproductive Tract

GDNF – Glial cell line Derived Neurotrophic Factor

GFP – Green Fluorescent Protein

HDAC – Histone Deacetylase

HLH – Helix-loop-Helix (protein/transcription factor)

bHLH – Basic Helix-loop-Helix (protein/transcription factor)

ir – immunoreactive or immunoreactivity

Id – Inhibitor of DNA (also called Inhibitor of Differentiation)

Id2 – Inhibitor of DNA 2

MeCP2 – Methyl CpG-binding Protein 2

NGF – Nerve Growth Factor

PGP9.5 – Protein Gene Product 9.5

PNS –Peripheral Nervous System

SP – Substance P

TF – Transcription Factor

TGFβ – Transformaing Growth Factor Beta

TH – Tyrosin Hydroxylase

VAChT – Vesicular Acetylcholine Transporter

VEGF- Vascular Endothelial Growth Factor

Chapter1. GENERAL INTRODUCTION

Background

The peripheral nervous system (PNS) retains significant potential for structural plasticity even in the adult. Throughout life, changes in sensory and autonomic axon density and distribution occur in several organs and tissue microenvironments. While triggered mostly by functional needs in physiological state, such changes are also signature of forthcoming pathological conditions. Pathologies are either associated with degenerative loss of axons as seen in various neurpopathies, or with abnormal sprouting of axons causing tissue hyperinnervation, as most commonly observed in various pain syndromes. This is unlike the central nervous system (CNS), where most connections are hardwired early in life, with little scope of modification or regeneration in the adult. Thus, studying fundamental mechanisms of adult peripheral axon plasticity is crucial for understanding the development, progress and potential remedies of a wide range of PNS disorders.

A major impediment in studying plasticity of the adult PNS has been the lack of suitable animal models where plasticity can be induced in a predictable and possibly reversible fashion, under physiological conditions and studied through the course of its development. This would provide a more thorough understanding of the mechanism that can help in protecting, preserving and rejuvenating innervation to even prevent onset of pathology. In most diseases structural changes in axons occur over a protracted period of time and the initiating factors often disappear by the time the symptoms manifest. While such changes initiate in intact adult axons, most of our current understanding of adult neuroplasticity is reliant upon nerve injury models.

In the female reproductive tract, nerve density changes rapidly, robustly and reversibly with changes in blood levels of estrogen, cyclically throughout reproductive life (Ting et al.,

2004). Dysregulation of nerve density can cause vulvo-vaginal hyperinnervation resulting in pelvic pain syndromes, a set of disorders that prevail in nearly 16% women in the United States alone and cost upto $1.5 billion annually, with no effective cure known (Masheb et al., 2000; Goldstein and Burrows, 2008). In the present study we explored this highly tractable model in rat to find how plasticity is spontaneously induced in mature sensory axons of the vagina in absence of any injury, under physiological conditions.

Peripheral neuroplasticity and disease

Several peripheral neurological disorders are associated with change in the local density of axon terminals (number of terminals per unit area) leading to either hypo- or hyper-innervation. Sensory deficits due to degenerative axonal loss induced by numerous conditions including diabetes, chemotherapeutic drugs, alcoholism, injury or idiopathic conditions are common examples of hypo-innervation disorders (Walsh and McLeod, 1970; Behse et al., 1977; McLeod, 1995; Jaggi and Singh, 2012).

Hyperinnervation, on the other hand, is mainly associated with disorders of hyperactivity or hypersensitivity. Sympathetic hyperinnervation of the heart induced by nerve growth factor (NGF) release from infiltrating immune cells following myocardial infarction leads to subsequent hyperexcitability and increased risk of congestive heart failure (Hasan et al., 2006). Sensory hyperinnervation induced by systemic disorders like vitamin D deficiency leads to chronic musculoskeletal pain (Tague et al., 2011). Increased density of sensory nociceptive fibers is also a hallmark of several chronic and acute painful conditions. Diseases like Achilles tendonitis, chronic knee pain, degenerative disc disease, appendicitis, endometriosis, mastodynia, vulvodynia etc. are characterized by marked nociceptive hyperinnervation at the site of pain and

hypersensitivity (Di Sebastiano et al., 1995; Brown et al., 1997; Sanchis-Alfonso and Rosello-Sastre, 2000; Tympanidis et al., 2003; Gopinath et al., 2005; Schubert et al., 2005; Sandkuhler, 2009). In standard rodent models of inflammatory pain like planter injection of carrageenan or complete Freund's adjuvant, marked hyperinnervation at the injection site was observed which associated with significantly reduced pain thresholds even days after the injection (Chakrabarty et al., 2011). The higher number of branches might contribute not only to summation of locally evoked depolarization potentials but also to elevated release of pro-inflammatory neuropeptides from peptidergic axons seen at high density at these sites. However, factors that can induce plasticity in an adult uninjured axon are poorly defined and mechanisms behind most of these neuroplasticity associated disorders remain unknown.

Estrogen and neuroplasticity

Estrogen has profound effects on the structure and function of the nervous system. Initially it was believed that estrogen affects only hypothalamus and parts of the brain associated with reproductive function or behavior (Harris and Naftolin, 1970; Perez et al., 1990); but subsequently it was shown to affect several other brain regions including hippocampus, cerebral cortex, preoptic area, dorsal raphe, nucleus accumbens, limbic system and many others (Patchev et al., 1995; McEwen, 2002). Indeed, estrogen receptors express extensively in neurons of both the CNS and PNS (Shughrue et al., 1997; Patrone et al., 1999; Mitra et al., 2003). In the CNS estrogen induces structural plasticity like dendritic growth in some parts and functional plasticity like potentiation or receptor expression in some others (McEwen et al., 2001; Sakamoto et al., 2003; Cooke and Woolley, 2005; Cardona-Rossinyol et al., 2013). Beyond functional roles, a prominent neuroprotective role of estrogen has also unveiled over the years (Alonso and Gonzalez, 2012; Shao et al., 2012; Cardona-Rossinyol et al., 2013).

The effect of estrogen in the PNS was described subsequently. In dissociated culture, sensory neurons of the DRG express ERα and respond to estrogen by increased neurite outgrowth (Blacklock et al., 2005; Chakrabarty et al., 2008). *In vivo* CGRP innervation associated with arterioles undergoes increased sprouting with increase in estrogen (Blacklock et al., 2004). CGRP innervation of the mammary gland also increases with rise of estrogen levels (Blacklock and Smith, 2004). Interestingly, in the periphery, interaction of the axon with the target also plays a vital role in plasticity decisions and innervation is crafted according to the target's functional need. Often the target can be estrogen responsive and plasticity could be driven predominantly by the effect of estrogen on the target. In the reproductive tract estrogen induces changes in secretory properties of the target tissue to induce plasticity of the innervating axons. In the uterus effect of estrogen on the myometrium lowers density of associated sympathetic innervation (Zoubina and Smith, 2000; Zoubina et al., 2001). In the vagina lowering levels of estrogen alters trophic properties of submucosal smooth muscle to induce sprouting of the sensory nociceptive and autonomic nerve fibers (Ting et al., 2004). In postmenopausal women, vaginal innervation density may increase due to low levels of estrogen. Local vaginal application of topical estrogen can reduce this innervation density (Griebling et al., 2011), suggesting that a local effect of estrogen on target tissue induces neuroplasticity.

The rodent reproductive tract as a model for study of neuroplasticity

The female reproductive tract is a highly estrogen-responsive organ. It undergoes extensive structural and functional changes under the influence of reproductive steroid hormones, particularly estrogen, cyclically through the reproductive life. With high estrogen levels there is increase in tissue mass, circulation, secretion and other vital functional properties of the reproductive tract. Not surprisingly, this initiates change in the innervation of the tissue on

both structural and functional demand. The female reproductive tract therefore provides a very effective model for studying target-nerve interaction leading to active neuroplasticity under physiological conditions in the adult peripheral nervous system.

In rodents, the female reproductive tract is comprised of the uteri, the cervix and the vagina. The two uterine horns begin from the oviducts and connect at the cervix that leads to the vagina (which opens to the exterior through the vulva) (Fig. 1). Each segment of the tract has unique histological characteristics besides their distinct functional role. The sensory innervation to the different parts of the tract comes from the thoraco-lumber (for uterus) and lumbo-sacral (for cervix and vagina) dorsal root ganglia. The sympathetic nerve supply comes from the equivalent levels of the sympathetic paravertebral ganglia; and the parasympathetic supply comes from the paravertebral and paracervical ganglia.

With increased estrogen levels there is rapid degeneration of sympathetic nerves in the uterus, which is reversed with decreased estrogen levels; no other nerve populations undergo change (Zoubina and Smith, 2000; Zoubina et al., 2001; Latini et al., 2008). In cervix, there is no change of innervation with changing levels of estrogen. While in the vagina, a fall in estrogen levels induces sprouting of sensory nociceptive, sympathetic and parasympathetic nerve fibers. Ovariectomy, which removes most systemic estrogen and simulates surgical menopause, induces marked vaginal hyperinnervation and hypersensitivity. However, this is reduced to normal when estrogen is supplemented exogenously by a subcutaneous slow-releasing pellet implant (Berkley et al., 1993; Ting et al., 2004). Although the entire female reproductive tract is highly estrogen responsive, these observations indicate unique target-nerve interactions at different levels, driven perhaps by distinct functional needs and achieved via unique estrogen response of the local microenvironment.

The change in sympathetic innervation of the uterus has been explained by changes in levels of BDNF (brain derived neurotrophic factor), neurotrimin and semaphorin 3F (matrix proteins) in the target uterine myometrium (smooth muscle layer of uterus) under the influence of estrogen (Krizsan-Agbas et al., 2003; Krizsan-Agbas et al., 2008; Richeri et al., 2011). However, these mechanisms fail to explain neuroplasticity induced in the vagina where sensory nociceptive fibers are affected, unlike in the uterus. (Ting et al., 2004).

The sensory innervation of the vagina majorly comprise of unmyelinated sensory nociceptive fibers that originate mainly from small-medium diameter neurons of the L6-S1 DRG (Berkley et al., 1993). The vaginal tissue consists of three main layers from outside inwards: i) the outer serous tissue comprising mainly of collagen, matrix and connective tissue cells, ii) the middle muscular layer (often referred to as the submucosa) comprising of circular smooth muscle and iii) the inner layer of a thick pseudo-stratified epithelium (often referred to as the mucosa) that lines the inner lumen. Sensory innervation in the tissue is primarily associated with the submucous smooth muscle layer. There is hardly any innervation to the epithelium.

While vulvo-vaginal hyperinnervation under low estrogen conditions has been associated with various pelvic pain syndromes in women, mechanisms stimulating nociceptor sprouting in the vagina and several other pain syndromes remain unknown. In this study we adopted vaginal neuroplasticity in rat as a model to identify potential factors and mechanisms capable of inducing outgrowth in adult sensory axons.

Female Pelvic Pain Syndromes: association with estrogen and hyperinnervation

Pelvic pain syndromes are a set of disorders in women characterized by acute or chronic pain in pelvic, vaginal and vulvar areas. Vulvodynia and vulvar vestibulitis (also known as

vestibulodynia or provoked vulvodynia) are two most prevalent forms of the disease (Bachmann et al., 2006; Goldstein and Burrows, 2008). These pain syndromes manifest through multiple symptoms including intense pain in the vulvo-vaginal areas, burning, itching, rawness, erythema, hyperalgesia, persistent discomfort and dyspareunia (painful intercourse) that can persist for days (Masheb et al., 2000; Haefner et al., 2005).

Vulvodynia (also known as generalized/dysesthetic/ essential/idiopathic vulvodynia) is characterized by spontaneous pain symptoms occurring at multiple locations or all over the vulvo-vaginal region Symptoms erupt suddenly without any provocation and can last for days. This syndrome is more prevalent peri- or post-menopausal women and is associated with low blood estrogen levels.(Bachmann and Rosen, 2006; Bachmann et al., 2006; Reed, 2006; Goldstein and Burrows, 2008).

Vulvar vestibulitis (also known as vestibulodynia) on the other hand, is a form of 'provoked' vulvodynia where symptoms are restricted primarily around the vulvar vestibule and can be induced severely by even slight touch, pressure or friction. This form of vulvodynia is more prevalent in younger women and is associated with estrogen receptor dysfunction, which is can be idiopathic or often resulting from high progesterone (birth control pills) usage in early life. In all its forms vulvodynia results in severe physical & mental distress, a compromised sexual life and an immense loss of productivity (Eva et al., 2003; Bohm-Starke et al., 2004; Haefner et al., 2005; Bachmann et al., 2006; Reed, 2006; Goldstein and Burrows, 2008).

The prevalence of female pelvic pain syndromes is high with about 16% of the adult female population affected by some form or the other, in the United States alone. It is a persistent health issue with no effective cure (Harlow and Stewart, 2003; Haefner et al., 2005). The disease

mechanism being unknown management has relied mostly on physical therapy, tricyclic antidepressants, personal counseling or even local application of gabapentin (Ben-David and Friedman, 1999; Goldstein et al., 2005). However, these offer only limited benefits. Hormone replacement therapy to replenish estrogen in post-menopausal women could provide some relief but it has a host of associated risks and side effects.

Finally, study of biopsy samples from patients of vulvar vestibulitis revealed remarkable sensory nociceptive hyper-innervation at the sites of pain (Bohm-Starke et al., 1999; Tympanidis et al., 2003). Surgical excision of these hyperinnervated portions of the vestibule resulted in alleviation of pain (Goetsch, 1996). Related models in rodents showed that ovariectomy (simulating menopause surgically) can induce marked vaginal hyperalgesia and hyperinnervation in rat. Both pain and hyperinnervation subsided with estrogen supplementation (Bradshaw et al., 1999; Bradshaw and Berkley, 2002; Ting et al., 2004). Thus pelvic pain syndromes are now believed to be associated with hyperinnervation due to impaired estrogen signaling but what induces neuroplasticity under low estrogen conditions remain unknown. It appears that change in estrogen levels alters the trophic environment of the vagina inducing nerve sprouting. While several trophic factors can be present, Bone Morphogenetic Protein 4 is an important member intrinsic to the vaginal tissue that critical not only in the early morphogenesis but also in final maturation of the organ in adult (Cai, 2009).

Bone Morphogenetic Protein signaling

Bone Morphogenetic Proteins or BMPs are members of the Transforming Growth Factor beta (TGFβ) family of proteins that associate with functions like growth, maturation, proliferation, differentiation and cellular migration. Initially discovered for their role in bone

formation, BMPs have wide range of effects on multiple organs and cell types, affecting varied aspects of tissue development from embryogenesis to tumorogenesis. Hence they have been referred (classified) time to time as morphogens, growth factors or cytokines, in context specific manner (Dimitriou and Giannoudis, 2005; Gazzerro and Canalis, 2006).

BMPs are small secreted proteins, acting usually as dimers, expressed as early as embryonic gastrulation and exert their effects locally through paracrine/autocrine mechanisms. About 20 different BMP members have been identified and characterized over time. BMPs act through cell surface serine/threonine kinase receptor heterodimers usually comprising the type I (which could be BMPRIA or BMPRIB variant) and type II (BMPRII). BMP ligands bind to the type II receptor which then heterodimerizes with and phosphorylates the type I receptor subunit inducing the kinase activity of its cytoplasmic domain (Nohe et al., 2004; Gazzerro and Canalis, 2006). This domain phosphorylates cytoplasmic proteins called Smads that carry the message to the nucleus to regulate gene expression (Miyazono et al., 2010) (Fig. 2).

Smad-s are a family of proteins involved in transcriptional regulation, especially downstream of TGFβ signals (Miyazawa et al., 2002) (Fig. 2). These ~500 amino acid proteins are conserved through evolution and derive their name on the basis of homology with Sma and Mad proteins respectively in *C. elegans* and *Drosophila*. Smads have two conserved domains called the mad homology (MH) domains: MH1 at amino terminal and MH2 at carboxy terminal. MH1 can bind DNA directly, while MH2 plays the role of binding other proteins essential for gene regulation (Massague et al., 2005). In mammals there are 8 different Smads. Of these Smad1, 5 and 8 (called R-Smads) propagate signal from the membrane in response to BMPs. They are phosphorylated by the kinase activity of the Type I receptor that initiates binding to Smad4 (called Co-Smad) in cytoplasm and the complex is transported to the nucleus

(independent of importins, by direct interaction with nucleoporins) (Miyazono, 1999; Massague et al., 2005) (Fig. 2). In the nucleus, the complex can directly bind DNA or interact with several transcription factors (eg. Runx-2, Menin, Yin Yang1, Hoxc8 etc.) or act in association with other transcriptional coactivators/repressors (eg. CPB/SnoN) (Miyazono, 1999; Nohe et al., 2004; Massague et al., 2005). While Smad1/5/8 exclusively transduce BMP signals, other TGFβ members signal through Smad2 and Smad3 but share the common Co-Smad (Smad4). Two other Smads, Smad6 and 7, known as I-Smads or inhibitory Smads help maintain dynamic equilibrium when required, through negative feedback loops. Phosphorylation and nuclear migration of R-Smads is a classic evidence/marker of canonical BMP signal transduction. In the nucleus the Smad complex themselves regulate transcription and also induce other transcription factors that regulate gene expression (Miyazono, 1999; Miyazawa et al., 2002; Nohe et al., 2004).

BMP4: Importance and early effects on peripheral nervous system

The functions of BMPs in development are indispensable. BMP4 is one of the most important members of the family which is needed as early as embryonic gastrulation. The ~116 amino acid protein is secreted from the extraembryonic tissues and in conjunction with nodal signaling induces the formation of the primitive streak (Gazzerro and Canalis, 2006). It is eventually secreted from the dorsal notochord and helps establish dorso-ventral axis acting in concert with sonic hedgehog secreted from the ventral notochord. Gene knockouts in mouse for most BMPs are lethal either *in utero* or right after birth. For example, BMP2 knockouts are lost at E7.5-10.5 (amnion/chorion and cardiac malformations) and BMP7 knockout is lethal at (skeletal patterning defect: ribs, skull and hind limbs). However, one of the strongest effects is induced by the BMP4 knockout and embryos are lost as early as E6.5-9.5 with no mesoderm development (Dimitriou and Giannoudis, 2005; Gazzerro and Canalis, 2006).

BMP4 acts as one of the major BMPs regulating events in nervous system from early development inducing neural crest migration, phenotype specification, target innervation and several other functions. Interplay of noggin (an endogenous peptide BMP antagonist) and BMP4 produced in diametrically opposite sources generates a gradient of BMP4 in the dorsal tube that triggers the delamination of the neural crest (Sela-Donenfeld and Kalcheim, 1999a; Gazzerro and Canalis, 2006). Neural crest migration ultimately leads to the formation of the cranial ganglia, dorsal root ganglia, the sympathetic ganglia, the parasympathetic and enteric nervous system (Burian et al., 2000). In the developing peripheral ganglia BMP4 plays a continued role in maturation, target innervation and phenotype specification. It is observed that target derived BMP4 is necessary for the trigeminal sensory ganglia neurons to form specific face maps (Hodge et al., 2007). Target derived BMP4 is also obligatory for the formation of cutaneous sensory nerves (Honig et al., 2005). In the chick hind limb exogenous BMP4 alone was sufficient to generate cutaneous innervation after removal of ectoderm which supplies an ensemble of additional positive and repulsive cues besides BMP4, under normal conditions (Honig et al., 2005).

BMP signaling also contributes to phenotype specification of peripheral sensory neurons. Skin-derived BMP signal is essential for peptidergic neurons of the DRG to initiate expression of CGRP and SP (Hall et al., 1997). Cutaneous innervation in the rat hind limb arrives around E16-17 (Mirnics and Koerber, 1995) and neuropeptides first appear in the innervating lumbar DRG neurons around E17-18 (Kessler and Black, 1981; Kudo and Yamada, 1985; Marti et al., 1987). . Furthermore, stimulation of early embryonic neurons with BMP4 *in vitro* induces expression of these neuropeptides (Hall et al., 1997; Hall et al., 2002). Thus BMP4 has exquisite effects on the peripheral sensory innervation from embryo through neonate, but little is known about its

potential influence on the adult PNS. Evidence in brain indicate that BMP4 could still be an active regulator of nerve growth and plasticity in the adult (Mikawa et al., 2006). Although multiple target tissues continue expressing BMP4 in the adult organism and many coincide with sites of active neuroplasticity, plausible regulatory effects of BMP4 on peripheral nerves remain to be investigated (Cao et al., 2000; Hasan et al., 2006; Chen et al., 2010; Pachori et al., 2010; Scott and Fryer, 2012; Scott et al., 2013).

Initiating outgrowth in adult peripheral neurons requires transcriptional reactivation

Axonal growth is a combinatorial outcome of various subcellular activities like signal processing, protein synthesis, axonal protein transport, dynamic cytoskeletal changes and many others. In the final stages of development, as a neuron matures terminally and its axon contacts the target, these active processes involved in outgrowth are shut down (Chen et al., 2007b). Much of this is achieved by permanent alterations in gene expression. It is therefore believed that to bring an adult neuron out of its quiescence and rekindle outgrowth, a specific transcriptional reactivation program is essential.

Models of axonal injury in the adult peripheral sensory neurons provide evidence of massive transcriptional reactivation (Smith and Skene, 1997; Goldberg, 2003). Inflicting a mechanical injury induces upregulation of a host of factors in the peripheral neurons starting as early as 24h and continuing beyond 7days (Mechaly et al., 2006; Szpara et al., 2007). A significant percentage of genes induced overlap with those active during developmental axon growth (Mechaly et al., 2006; Patodia and Raivich, 2012a). A majority of these include transcription factors, for example, the activating transcription factor 3 (ATF3), the AP-1 family of transcription factors (eg. c-Jun, Jun D), cAMP response element binding protein (CREB),

signal transducer and activator activator of transcription (STAT3), Oct6, Sox11, SnoN, E47, to name a few. In fact at least 39 transcription factors (of 26 families) have been implicated in response to axonal injury (Moore and Goldberg, 2011; Patodia and Raivich, 2012a). *In vivo* knockouts of most of these transcription factors dramatically impair regeneration indicating their critical requirement for outgrowth (Patodia and Raivich, 2012b). Cumulatively, they orchestrate signaling and gene expression to direct subcellular events towards successful outgrowth.

While these findings substantiate the necessity of a large-scale transcriptional reactivation for neurite outgrowth, it is unknown how such activation occurs when outgrowth initiates in undamaged axons (especially under physiological conditions). Few of the signaling triggers activated by the stress of injury (like cytokines, immunogens, growth factors etc.) are likely to be available in absence of injury. What mechanism does the neuron rely upon to drive the multitude of subcellular processes necessary? The present study attempts to answer parts of this question by investigating physiological plasticity in the female reproductive tract. The findings might have strong implications not only in understanding plasticity mechanisms in mature axons, but also in therapeutic design for peripheral nerve disorders associated with abnormal loss or gain of axons.

FIGURES

Figure 1.

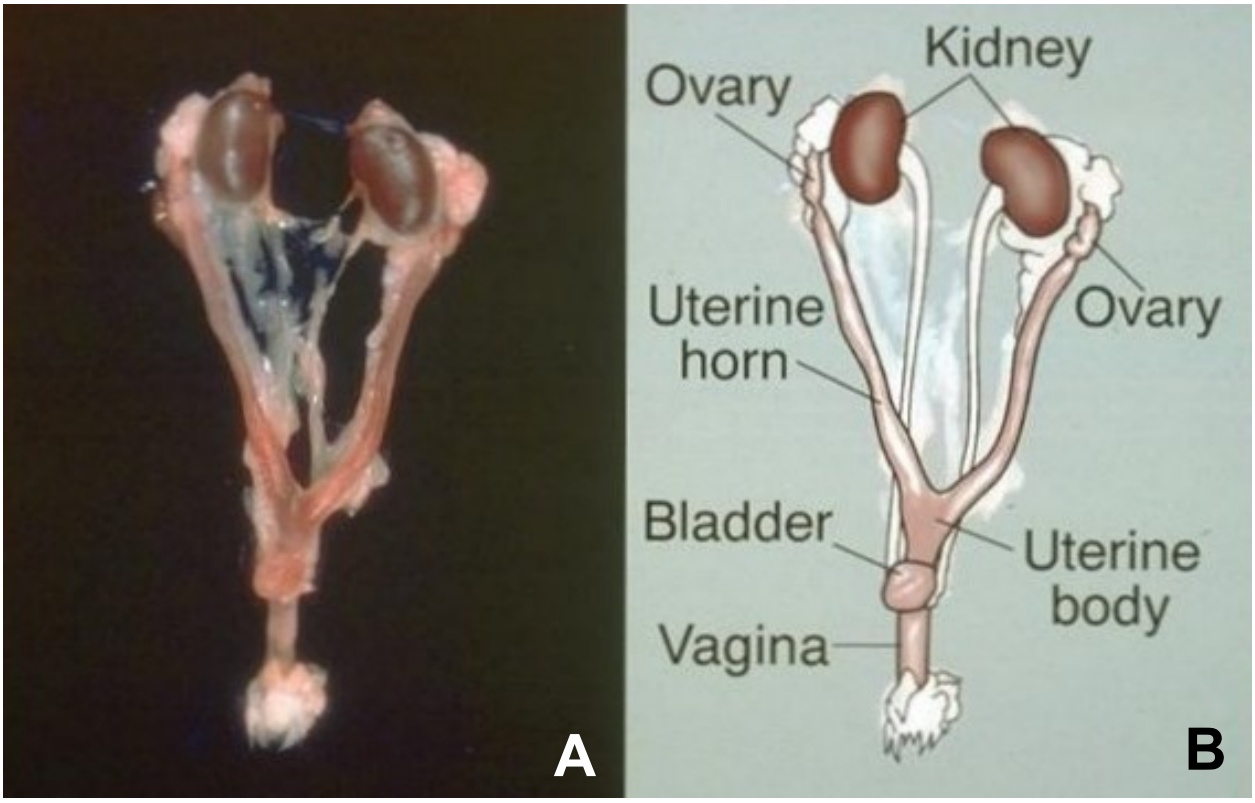


Figure 1. The female reproductive system of rat. **A.** Photograph of a gross dissected and isolated female urino-genital system from an adult (6-8 weeks old rat). **B.** Line drawing representation of structures in A delineating the individual organs. Labels indicate the different parts of the reproductive tract including the uterine horns, uterine body and the vagina. Cervix is located under the urinary bladder. *(Picture adapted and modified from: Dept. of Environmental Health and Safety, University of Cincinnati, ehs.uc.edu)*

Figure 2.

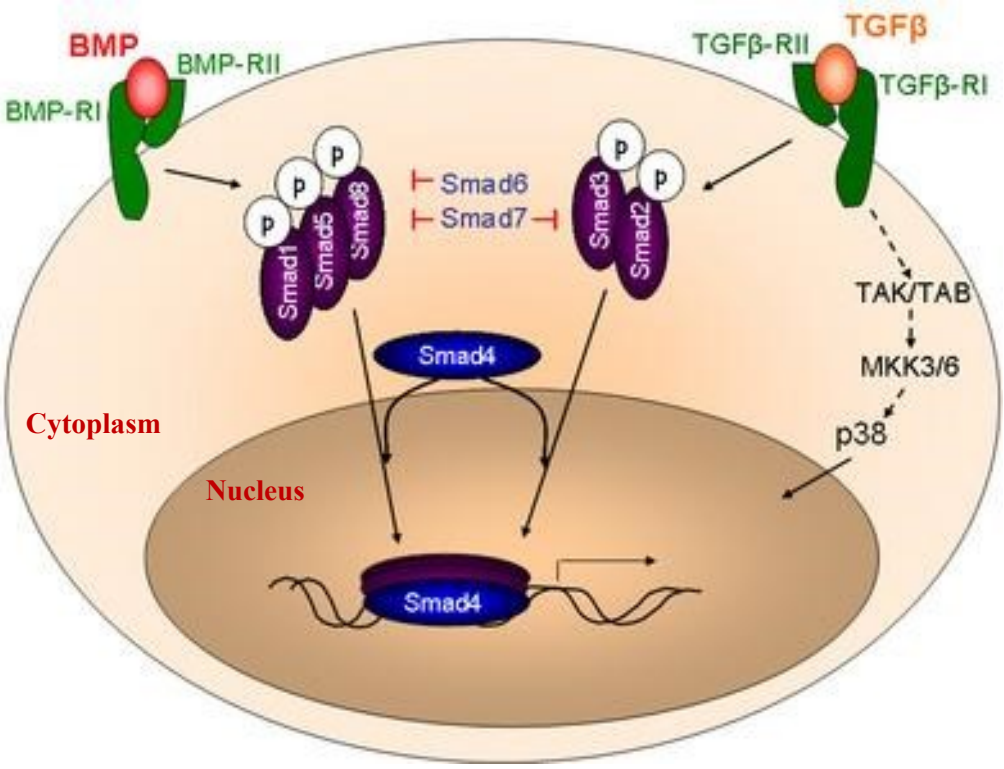

BMP
BMP-RII
BMP-RI
TGFβ-RII
TGFβ
TGFβ-RI
P
Smad1
Smad5
Smad8
Smad6
Smad7
Smad3
Smad2
TAK/TAB
MKK3/6
p38
Smad4
Cytoplasm
Nucleus
Smad4

Figure 2. TGFβ and BMP cellular signaling mechanisms. BMP family of proteins act by binding the cell surface receptor heterodimer of Type I and Type II receptors (also known as BMPR1 and BMPR2). Receptor activation leads to phosphorylation of Smad1/5/8 proteins by the cytoplasmic tail of BMPR1 which complexes with Smad4 and travels to the nucleus to bind Smad-binding domains of promoters in DNA to regulate gene expression. TGFβ works through a similar mechanism but with different set of receptors (TGFβRI and II) and different Smads (Smad2 and 3) but the same co-Smad, Smad4. TGFβ might also elicit p38 MAP kinase signals downstream from the receptor. Smad6 and 7 are inhibitory Smads that help balance signaling through feedback loops of inhibition when necessary. *(Adapted and modified from: www.humpath.com)*

Objective and Specific Aims:

Structural plasticity of mature sensory axons is associated with several peripheral nervous system disorders. Most of our knowledge of sensory axonal plasticity in adult is derived from study of nerve injury models, but mechanisms capable of inducing plasticity in intact axons remain largely unknown. Understanding of these mechanisms is crucial in conceiving curative approaches for several peripheral neuropathies associated with degenerative axon loss, as well as hyperinnervating pain syndromes. The broad objective of the present study was to explore mechanisms of structural plasticity in the intact sensory axons of the adult peripheral nervous system. In the rat vaginal tract, sensory axon density changes spontaneously under physiological conditions, throughout adult reproductive life with change in blood estrogen levels; low estrogen levels stimulate sensory axonal sprouting. We adopted this model system to study neuroplasticity mechanisms of intact sensory axons. We approached the problem through the following three specific aims:

Aim 1: Identifying potential target derived factors in vagina that regulate sensory axon plasticity.

Aim 2: Identifying a suitable high throughput assay system for studying signaling mechanisms in sensory neurons.

Aim 3: Identifying the downstream signaling mechanism that induces axon plasticity in adult sensory neurons.

The findings related to Specific Aims 1, 2 and 3 are reported in Chapters 2, 3 and 4 respectively.

Chapter 2. Bone Morphogenetic Protein 4 Mediates Estrogen-Regulated Sensory Axon Plasticity in the Adult Female Reproductive Tract

ABSTRACT

Peripheral axons are structurally plastic even in the adult, and altered axon density is implicated in many disorders and pain syndromes. However, mechanisms responsible for peripheral axon remodeling are poorly understood. Physiological plasticity is characteristic of the female reproductive tract: vaginal sensory innervation density is low under high estrogen conditions such as term pregnancy, while density is high in low-estrogen conditions such as menopause. We exploited this system in rats to identify factors responsible for adult peripheral neuroplasticity. Calcitonin gene-related peptide-immunoreactive sensory innervation is distributed primarily within the vaginal submucosa. Submucosal smooth muscle cells express Bone Morphogenetic Protein 4 (BMP4). With low estrogen, BMP4 expression was elevated indicating negative regulation by this hormone. Vaginal smooth muscle cells induced robust neurite outgrowth by co-cultured dorsal root ganglion neurons, which was prevented by neutralizing BMP4 with noggin or anti-BMP4. Estrogen also prevented axon outgrowth, and this was reversed by exogenous BMP4. Nuclear accumulation of phosphorylated Smad1, a primary transcription factor for BMP4 signaling, was high in vagina-projecting sensory neurons following ovariectomy, and reduced by estrogen. BMP4 regulation of innervation was confirmed *in vivo* using lentiviral transduction to overexpress BMP4 in an estrogen-independent manner. Submucosal regions with high virally induced BMP4 expression had high innervation density despite elevated estrogen. These findings show that BMP4, an important factor in early nervous system development and regeneration after injury, is a critical mediator of adult physiological plasticity as well. Altered BMP4 expression may therefore contribute to sensory hyperinnervation, a hallmark of several pain disorders including vulvodynia.

INTRODUCTION

Peripheral target organs can undergo significant changes in innervation associated with both adaptive and maladaptive events. Certain pathophysiological conditions such as neuropathies have long been associated with reductions in axon numbers (Lauria et al., 2005), while more recent studies show that increased innervation density (hyperinnervation) can also contribute to pathological conditions such as sudden cardiac death after myocardial ischemia (Cao et al., 2000; Hasan et al., 2006), musculo-skeletal pain in vitamin D deficiency (Tague et al., 2011) and inflammatory pain (Bohm-Starke et al., 1999; Chakrabarty et al., 2011). These findings underscore the malleability of peripheral axons under pathophysiological conditions in the adult organism.

Changes in peripheral innervation also occur physiologically in the mature organism as it adapts to demands incurred as a result of normal activities. This is particularly true for targets that must make substantial and sometimes rapid adjustments in structure and function. Perhaps some of the most vivid examples are those associated with female reproductive functions. For example, the uterus undergoes rapid denervation coincident with the sexually receptive phase of the estrous cycle, which is thought to be important in providing an environment conducive to implantation of the conceptus. Innervation is again depleted during pregnancy, contributing to uterine quiescence prior to hormonally regulated parturition (Latini et al., 2008), followed by reinnervation (Brauer, 2008) . Similarly, vaginal innervation is reduced at term pregnancy, which may facilitate delivery by diminishing vaginal smooth muscle tone and promoting maternal well-being by reducing numbers of local pain-sensing terminals (Liao and Smith, 2011).

Estrogen appears to be a dominant factor driving reproductive tract neuroplasticity. Vaginal innervation by presumptive nociceptor axons containing calcitonin gene-related peptide (CGRP)-immunoreactivity (-ir) is increased in rats under low estrogen conditions following ovariectomy (an animal model of menopause), and reduced by elevating serum estrogen by exogenous administration of 17 β-estradiol (Ting et al., 2004; Liao and Smith, 2011). In humans, systemic estrogen replacement therapy also reduces vaginal innervation density in post-menopausal women, and this effect is more robust when estrogen is applied topically (Griebling et al., 2011) implying a direct effect on vaginal tissue. It therefore appears that declining estrogen levels are associated with increased outgrowth by vaginal nociceptor axons.

Axon outgrowth in the adult is believed to require reactivation of certain transcriptional programs that are typically quiescent in mature neurons. For example, the transcription factor Smad1 is normally expressed at very low levels in intact dorsal root ganglion sensory neurons, but is markedly upregulated in association with regeneration after axonal injury (Zou et al., 2009). Smad1 is regulated by Bone Morphogenetic Protein 4 (BMP4), a member of the TGFβ superfamily of cytokines/morphogens (Miyazono et al., 2010). A possible role for the BMP4/Smad1 signaling pathway in reproductive tract axon remodeling is supported by reports that BMP4 is synthesized by vaginal tissues (Cai, 2009) and negatively regulated by estrogen (Chen et al., 2009). In the present study, we investigated whether BMP4 mediates estrogen-driven plasticity of peripheral sensory innervation.

MATERIALS AND METHODS

All animal protocols and procedures were approved by the Kansas University Medical Center's Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Immunohistochemistry

Female Sprague Dawley rats were surgically ovariectomized (OVX) at 8 wks of age according to established methods (Zoubina et al., 2001). After 7 days, rats (n=4/group) received neck scruff subcutaneous implants of silastic tubing (inner/outer diameter = 1.575/3.175 mm, length = 20 mm, volume =170µl) containing sesame oil vehicle with or without 180µg/ml 17β estradiol (Sigma) (Strom et al., 2008), which raises serum estrogen levels in OVX rats from <15 pg/ml to about 80 pg/ml (high physiological levels similar to estrus and term pregnancy). Seven days later, animals were euthanized by thoracotomy under isofluorene anesthesia, and vagina and dorsal root ganglia (DRG) harvested and fixed by overnight immersion in Zamboni's fixative at 4˚C and cryosectioned at 10 µm as described previously (Ting et al., 2004; Tague and Smith, 2011). In groups used to analyze pSmad1 expression, rats received a single subcutaneous injection of 10µg/kg of 17β-estradiol which produces a rapid rise in serum estrogen (Zoubina et al., 2001) or sesame oil vehicle (n=3/group), and 24h later L6-S1 DRGs removed, fixed in neutral buffered formalin, and cryosectoned.

Sections were immunostained overnight for peripherin (1:400, rabbit antiserum; 1:500, chicken antiserum, Millipore), Protein Gene Product 9.5 (PGP9.5, 1:1000, rabbit antiserum, Serotech), tyrosine hydroxylase (TH,1:200, rabbit antiserum, Millipore), calcitonin gene-related peptide (CGRP,1:400, sheep antiserum, Biomol), Enhanced Green Fluorescent Protein (EGFP,

1:750, chicken antiserum, Aves), BMP4 (1:100, mouse monoclonal antibody, Millipore), Bone Morphogenetic Protein Receptor type 1A (BMPR1A, 1:100, rabbit antiserum, Santa Cruz Biotech), Bone Morphogenetic Protein Receptor type 2 (BMPR2, 1:100, goat antiserum, Santa Cruz Biotech), α-smooth muscle actin (Cy3 conjugated mouse antiserum, Sigma), and Phosphorylated Smad1 (pSmad1, 1:200, rabbit monoclonal antibody, Cell Signaling Technologies). Secondary antibodies included donkey anti-rabbit Cy3 at 1:200 (Jackson Immuno-Research), donkey anti-mouse Cy3 (1:200, Jackson Immuno-Research), donkey anti-rabbit Alexa 488 (1:750, Invitrogen), donkey anti-rabbit Alexa 647 (1:500, Invitrogen), donkey anti-chicken Dylight 488 (1:750 Invitrogen), donkey anti-goat Cy3 (1:200 Jackson Immuno-Research), and goat anti-rabbit Cy3 (1:200 Jackson Immuno-Research), donkey anti-sheep Cy3 (1:200 Jackson Immuno-Research). Antibodies have been previously characterized by our laboratory and others (Abir et al., 2008; Clarke et al., 2010; Tague et al., 2011; Tague and Smith, 2011) and include omissions of primary and secondary antibodies and preabsorption of anti-BMP4 with recombinant mouse BMP4 at 500ng/ml for 45min prior to staining.

Retrograde Tracing

Four days following OVX, rats were anesthetized by isoflurane inhalation. Using a Hamilton syringe and 30 gauge needle, recombinant Cholera toxin-B (CTB) conjugated to Alexa 488 (2µg/µl in PBS, Life Technologies) was injected submucosally at four sites equidistant along the vaginal circumference (2.5 µl/site; 10 µl total) at mid-vaginal level. Two days following CTB injection, rats received either a single subcutaneous injection of 10µg/kg 17β-estradiol (which produces a rapid rise in serum estrogen, (Zoubina et al., 2001) or sesame oil vehicle (n=5/group). Twenty-four h later, rats were deeply anesthetized with ketamine-xylazine and perfused transcardially with heparinized PBS followed by ice cold 4% formaldehyde in

PBS. L6, S1 and S2 DRGs, which are reported to give rise to vaginal innervation (Berkley et al., 1993), were harvested and cryosectioned at 14 μ and immunostained for BMPR1A, BMPR2 and pSmad1, and co-stained for peripherin. Randomized images from 6-8 sections per animal were acquired and percentages of labeled neurons expressing specific markers assessed in approximately 200 retrogradely labeled neurons per rat.

Flow cytometry

L6 to S2 DRG were harvested aseptically from 8 week old female rats, diced in ice-cold L15 medium with 3% glucose, incubated in 1mg/ml collagenase and 2.5 U/ml dispase (Worthington) in HBSS for 40mins, and triturated using fire-polished glass pipettes in L15-glucose with 10%FBS. Cells were filtered through a 100μm mesh and centrifuged through a percoll gradient column to remove debris. The washed pellet was resuspended, fixed in cold 2% formaldehyde for 1h, and washed in PBST on ice. Cells were immunostained for 1h for peripherin (detected with donkey anti-chicken Alexa 488) and BMPR1A (donkey anti-rabbit Cy3) and fixed in 1% formaldehyde and washed. Fluorescence activated cell sorting was conducted using a BD Aria system, with cells categorized based on their fluorescence intensity in arbitrary units.

PCR and immunoblots

Effects of estrogen on BMP4 gene expression were assessed in vaginal tissue *ex vivo*. Vaginas from 8 week old OVX rats were cut along the length of the lumen, everted, and the mucosal surface gently scraped to remove the epithelium. Vaginal wall explants ($1mm^3$) containing smooth muscle and adventitia were cultured in separate wells of a 24 well plate in DMEM/F12 medium for 24h with or without 20nM 17β estradiol, and homogenized in Trizol (Invitrogen) for RNA isolation. cDNA was synthesized from 1μg of total RNA using iScript

Reverse Transcriptase kit (Biorad). Semi-quantitative RT-PCR was performed for 30 cycles with 55°C annealing temperature using primers for BMP4 (Forward: GAGCCTTTCCAGCAAGTTTGTTC; Reverse: GTTCTCCAGATGTTCTTCGTGATG; Product size: 487bp) and GAPDH (Forward: CTCTACCCACGGCAAGTT; Reverse: CTCAGCACCAGCATCACC; Product size: 120bp) and products separated on a 1% agarose gel for densitometric analysis.

To assess BMP4 protein levels, western blots were performed from tissue collected from OVX rats with or without estrogen supplementation *in vivo*, as described above. Vaginal submucosa was homogenized in RIPA buffer, and 40µg total protein per well separated on a 12% polyacrylamide gel, transfered to a PVDF membrane and probed with anti-BMP4 (mouse monoclonal antibody, Millipore,1:500) and reprobed for beta-actin (Sigma, 1:1000). For evaluating BMP4 overexpression via lentiviral transduction, 24h conditioned medium was collected from stably infected 293T cells. From equal volumes of conditioned medium total protein was precipitated by TCA-acetone. Equal amounts of resuspended protein were used for immunoblot and probed for BMP4 as described above.

Neuron and smooth muscle cultures

L6 to S2 DRGs from 5-week-old were harvested as above. To assess neurite outgrowth in response to BMP4, neurons equivalent to one half of a DRG per well were plated onto collagen-precoated 24 well plates (BD Biosciences) in serum-free Neurobasal A medium (Gibco, Life Technologies) with B27 supplement (Gibco, Life Technologies) and 10 or 50 ng/ml mouse recombinant BMP4 (Peprotech) and cultured for 48h, with neurite quantification as described below. For confocal imaging, some neuronal cultures were grown in defined medium for 48h on

laminin/poly D-lysine (Gibco, Life Technologies/Sigma-Aldrich) coated glass coverslips, fixed in 4% PFA, and immunostained for BMPR1A, BMPR2 and peripherin.

For neuron-smooth muscle co-cultures, vaginal tissue from 8 week old rats was harvested in ice-cold L15 medium and stripped of epithelium with a sterile scalpel blade. Portions of smooth muscle were dissected, diced, and immersed in L15 medium containing 2mg/ml collagenase (Sigma-Aldrich) on ice for 30min. Tissue was incubated at 37˚C for 30 min and then triturated using fire-polished glass pipettes while adding medium containing 10% FBS to neutralize the collagenase. The suspension was passed through 100μm mesh to remove debris, centrifuged at 300g for 7min, and the pellet resuspended in DMEM-10%FBS. Cells were plated onto a collagen-coated 35mm dishes (BD Bioscience), incubated at 37^{o}C in 5% CO_2 for 48h, and then divided. To control for non-specific interactions between neurons and co-cultured cells, in some experiments we substituted rat embryonic fibroblasts cultured at greater than 7 passages for smooth muscle cells.

L6 to S2 DRG from female rats aged 35 d were harvested aseptically and dissociated as described above. Neurons equivalent to one half ganglion per well were plated onto confluent layers of smooth muscle cells grown on collagen-coated 48-well plates (BD Biosciences). Cells were co-cultured in the presence or absence of different amendments including 17-β estradiol (20nM), noggin (250ng/ml, Peprotech), anti-BMP4 (500ng/ml, Peprotech), and BMP4 (10ng/ml, Peprotech). After 48h, co-cultures were fixed with 4% formaldehyde and washed with PBS. Cultures were immunostained for peripherin and in some cases for alpha smooth muscle actin.

In some experiments, smooth muscle cells were prepared as above and initially grown in transwells (Corning) for 24h. These were then transferred to 24-well plates in which dissociated

DRG neurons had been seeded on collagen. Transwell co-cultures were maintained for 48h in the presence or absence of anti-BMP4, fixed and immunostained as above.

To quantify neurite outgrowth, 6-8 random images per well of peripherin expressing neurons were captured using a 20X objective (412 x 330 μm field size). Neurite area was measured using a stereological grid (AnalySis version 3.2; Soft Imaging System GmBH, Müenster, Germany) superimposed over each image as described previously (Chakrabarty et al., 2008). Numbers of line intersections overlying stained neurites were divided by total intersections within the field and multiplied by total field area. Neurite area was divided by the number of neurons with at least one neurite within the field and all values in a well averaged and presented as neurite area per neuron.

Lentivirus production and injections

Full-length cDNA of rat BMP4 downstream to a CMV promoter was cloned into the FG12 lentiviral vector (Addgene) between *XbaI* and *PacI* restriction sites. The vector carries an EGFP reporter under a ubiquitin-C promoter. Third generation lentiviral packaging with pMDLg/RRE, pRSV-Rev, and pMD2.G (Addgene) was used to synthesize lentiviral particles for BMP4 and EGFP-only vectors. 293T cells were plated in 6 well plates at 60-80% confluence and transfected with the vector carrying the gene of interest or EGFP alone and all the 3 packaging plasmids using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium (Gibco). Medium was changed 12h later with OptiMEM-5%FBS and supernatant collected every 24h for 2-3 days. Viral particles were concentrated from the supernatant by ultracentrifugation in sealed tubes for 2h at 35000 rpm and resuspended in sterile PBS. Lentiviral titers were measured using the Lentivirus qPCR titer kit (Applied Biological Materials).

OVX rats were anesthetized with ketamine/xylazine (70/6 mg per kg) and injected intravaginally using a 30 gauge needle attached by polyethylene tubing to a Hamilton syringe. Injections of lentivirus expressing BMP4 (n=4) or EGFP alone (n=4) were made at four intravaginal sites (corresponding to 3, 6, 9 and 12 o'clock), and a total volume of 10 µl containing $1x10^8$ viral particles was deposited into submucosal regions corresponding to the location of the vaginal smooth muscle. At the time of injection, rats were implanted with capsules containing sesame oil with or without 17β- estradiol (Ting et al., 2004; Strom et al., 2008). Seven days after injections and implantations, rats were euthanized and vaginal tissues harvested and fixed in Zamboni's solution. Tissues were sectioned at 14 µm and immunostained for PGP 9.5, peripherin, CGRP, TH, BMP4 or EGFP. Innervation density was measured in images captured from regions corresponding to the injection sites as identified by BMP4 and EGFP expression, and from adjacent non-injected sites which did not show virally transduced gene expression. Eight to fourteen images were captured from injection and from adjacent sites for each animal, and axonal area within each 412 x 330 µm field was determined by thresholding (AnalySis Software) and averaged (Ting et al., 2004).

Statistical analysis

All values are presented as mean ± SEM. Comparisons between groups were made using Student's t-test, one-way ANOVA, or two-way ANOVA with post-hoc comparisons by Student Newman-Kuels tests for normally distributed data, and Mann-Whitney rank sum test for non-parametric analysis. Paired t-tests were used to compare innervation densities in injected and non-injected sites within the same animals in the lentiviral studies. Differences were considered significant at $P \leq 0.05$.

RESULTS

BMP4 is expressed in adult rat vagina

BMP 4 is an essential morphogen regulating vaginal development and is expressed in the early postnatal period (Cai, 2009). However, BMP4 expression has not been described in mature vagina. The vagina of the 8-week old rat consists of thickened mucosal epithelium surrounded by connective tissue containing a distinct smooth muscle layer (Fig. 1A). Immunostaining showed abundant BMP4 labeling largely confined to the smooth muscle layer (Fig. 1B), which was eliminated by antibody preabsorption with recombinant BMP4 protein (Fig. 1C). The smooth muscle layer also contains presumptive nociceptor innervation (Ting et al., 2004), and immunostaining revealed many axons immunoreactive for peripherin (Fig. 1B), an intermediate filament protein localized to axons and somata of small to medium diameter neurons, including dorsal root ganglion nociceptors (Ferri et al., 1990); (Goldstein et al., 1991) (Fornaro et al., 2008).

Estrogen downregulates BMP4 expression in vaginal smooth muscle

Vaginal cells, including smooth muscle cells, express estrogen receptors that influence structure and function by altering gene expression (MacLean et al., 1990; Forsberg, 1995; Mowa and Iwanaga, 2000; Giraldi et al., 2002). To assess effects of estrogen on BMP4 expression in mature rat vaginal tissue, we performed RT-PCR on vaginal submucosal explants cultured for 24h in presence or absence of 20nM estrogen. Semi-quantitative RT-PCR showed that, relative to GAPDH expression, estrogen downregulated BMP4 mRNA in the vaginal submucosal smooth muscle (Fig.1D).

We evaluated changes in BMP4 protein content in 8 week old OVX rats implanted with 17β estradiol-releasing capsules for 7 days. In western blot analysis, vaginal BMP4 protein

levels normalized to β-actin were reduced in animals receiving estrogen as compared to vehicle controls (Fig.1E), indicating that estrogen reduces both BMP4 mRNA and protein.

Adult DRG neurons express BMP receptors

BMP4 acts by binding to cell surface receptors that are typically heterodimers consisting of BMPR2 and BMPR1A or BMPR1B. Cervical and thoracic DRG sensory neurons express BMPR2 and predominantly BMPR1A (but not BMPR1B) during development (Zhang et al., 1998), but their presence in more caudal ganglia and persistence into adulthood are unclear. To assess BMP receptor presence and distribution, we performed Fluorescence Activated Cell Sorting (FACS) of 8-week old rat dissociated DRGs from L6-S2, which innervate the rat vagina (Berkley et al., 1993). FACS revealed a substantial cell population with intense fluorescence for both peripherin and BMPR1A (Fig. 2A, P6), confirming the presence of this receptor in the peripherin-immunoreactive population of neurons. A second population of peripherin positive neurons showed lower levels of BMPR1A expression (P4). Additional cells showed low peripherin and low to high BMPR1A-ir (P8, P7).

We examined DRG sections to further assess BMP4 receptor localization. BMPR1A-ir was observed primarily in peripherin expressing neurons, with smaller numbers of peripherin-positive neurons lacking BMPR1A-ir and a few peripherin negative, large diameter neurons expressing the receptor (Fig. 2B-D); preliminary studies confirmed a paucity of BMPR1B staining (not shown). Immunostaining for BMPR2 showed that this co-receptor was also enriched in peripherin immunoreactive neurons (Fig. 2E-G). BMPR2-ir was observed additionally in large diameter neurons, although the signal intensity was typically less than that of peripherin positive neurons.

We assessed the distribution of BMP4 receptors within peripherin-positive neurons in culture (Fig. 2H, I). Both BMPR1A and BMPR2 showed strong immunostaining throughout the soma and axons (Fig. 2J, K).

To confirm that small-diameter neurons projecting to the vagina express BMPR1A and BMPR2 receptors, we immunostained sections of DRGs 3d following CTB injection of the vaginal submucosa in OVX rats with or without estrogen supplementation. CTB-labeled neurons were present bilaterally in L6-S2 DRGs (Fig. 3A, B). Analysis of peripherin-immunostained sections revealed that 61±3% of CTB-labeled neurons were immunoreactive for this small-diameter neuron marker (Fig. 3C, D). BMPR1A-ir was associated with many CTB-labeled cells (Fig. 3E); quantitative analysis revealed that more than two thirds of vagina-projecting neurons expressed BMPR1A (70$\pm$4% in OVX, 67$\pm$4% after estrogen); most of these were also peripherin positive (Fig. 3G; 63$\pm$3% in OVX, 54$\pm$12% after estrogen). BMPR2-ir also was observed in a majority CTB-labeled neurons (Fig. 3F; 81$\pm$3% in OVX, 71$\pm$7% after estrogen), with most of these immunoreactive for peripherin (Fig. 3H; 64$\pm$4% in OVX, 64$\pm$4% after estrogen). Estrogen status did not influence BMPR expression.

BMP4 mediates smooth muscle-induced sensory neurite outgrowth

Our findings show that DRG sensory neurons, including those projecting to the vagina, express BMP4 receptors, and that vaginal target cells synthesize BMP4 more robustly in low-estrogen conditions. Accordingly, reduced BMP4 protein levels could contribute to vaginal axon pruning that occurs when estrogen levels are elevated, as in term pregnancy (Liao and Smith, 2011) and following exogenous hormone replacement (Ting et al., 2004; Griebling et al., 2011).

We tested the responsiveness to BMP4 of primary sensory neurons plated on collagen in defined media. The addition of10ng/ml recombinant BMP4 increased axon outgrowth relative to

that in control media (Fig. 4A); outgrowth was not increased further at 50ng/ml. To delineate whether BMP4 plays a functional role in target regulation of sensory innervation, we cultured dissociated DRG neurons atop near-confluent vaginal smooth muscle cells. Individual neurons elaborated neurite arbors that made multiple contacts with smooth muscle cells (Fig. 4B). We first determined whether members of the BMP family contribute to smooth muscle-induced sprouting by treating cultures with noggin, an endogenous inhibitor of BMP4 (McMahon et al., 1998; Sela-Donenfeld and Kalcheim, 1999b). Noggin at concentrations of 250ng/ml appeared to decrease neurite outgrowth relative to control cultures at 48 hrs (Fig. 4C, D), and this was confirmed by quantifying neurite area per viable neuron, where noggin reduced outgrowth by 38% (Fig. 4E). When adult DRG neurons were cultured under the same conditions but in the absence of smooth muscle cells, noggin failed to alter neurite outgrowth (data not shown). These findings implicate BMPs in smooth muscle cell mediated sensory neuronal sprouting.

While noggin is an effective endogenous inhibitor of BMP4, it can bind other members of the BMP family of proteins. To ensure that reduced neurite outgrowth observed with noggin is due to BMP4 neutralization, we used a function neutralizing antibody directed specifically against BMP4. Relative to control cultures (Fig. 4F), BMP4 antibody reduced neurite outgrowth by 32% (Fig.4G, H) but did not affect outgrowth by neurons cultured in the absence of smooth muscle (data not shown).

To confirm that the effects of the BMP4 function neutralizing antibody are dependent upon BMP4 synthesis by co-cultured cells, we grew DRG neurons with rat embryonic fibroblasts which are not believed to synthesize BMP4. The addition of anti-BMP4 to the culture medium did not alter neurite outgrowth ($6381\pm482\mu m^2$ control vs. $6210\pm675\mu m^2$ anti-BMP4).

BMP4 is a secreted protein that influences its targets by way of diffusion. To ensure that BMP4 is acting as a secreted factor and not through any direct physical interaction between neuron and muscle, smooth muscle cells were cultured in transwell inserts that allow diffusible factors to reach neurons growing at the bottom of the culture well but prevent direct interactions. The addition of BMP4 neutralizing antibody to the culture medium was as effective in inhibiting outgrowth in these transwell co-cultures as it was when neurons and smooth muscle were in direct contact (Fig. 4H).

Estrogen modulates smooth muscle-mediated axon growth *in vitro* via BMP4

Protein and RNA measurements show that estrogen reduces vaginal BMP4 expression. To assess whether estrogen modulates smooth muscle-induced outgrowth by way of BMP4, we grew vaginal smooth muscle cells in culture for 72 h in the presence or absence of 17β estradiol. Adult DRG neurons were then plated over the near-confluent cultures in estrogen-free medium. Measurement of axon length per neuron showed that outgrowth at 48 h was significantly lower in the estrogen-pretreated muscle cell cultures as compared to the untreated cultures (Fig. 5A, B, D).

We reasoned that if reduced outgrowth in estrogen-pretreated cultures is due to the reduction in BMP4 protein, then we should be able to reverse it by adding exogenous BMP4 to the estrogen pretreated cultures. We performed smooth muscle-DRG neuronal co-cultures as above and added recombinant BMP4 to the medium in one estrogen pretreated group at the time of neuronal plating. Forty-eight h after adding BMP4 to cultures pretreated with estrogen, outgrowth was increased significantly relative to cultures pretreated with estrogen alone, and was statistically comparable to that seen in co-cultures where smooth muscle cells were not treated with estrogen (Fig. 5C, D).

Estrogen-modulated axon plasticity is associated with changes in DRG pSmad1

BMP4 binds to cell surface receptors that induce phosphorylation of cytoplasmic Smad1. pSmad1 travels to nucleus to regulate transcription factors mediating axonal outgrowth (Zou et al., 2009). If estrogen regulates axonal outgrowth by way of BMP4 signaling, then nuclear pSmad1 localization should be reduced when vaginal sensory innervation is lower. We investigated pSmad1 expression in L6-S2 DRGs which originate vaginal innervation. Under low-estrogen conditions in OVX rats, many pSmad1 positive nuclei were evident (Fig. 6A). 24h after estrogen injection, pSmad1 immunoreactive nuclei were much less frequent (Fig. 6B), with quantitative analysis showing an 85% reduction of pSmad1 labeled nuclei (Fig. 6C). Double immunostaining showed that many pSmad1 positive nuclei were present in peripherin expressing neurons (Fig. 6D).

To confirm that global changes in pSmad1 expression also occur in vagina-projecting neurons, we assessed pSmad1-ir in retrogradely labeled L6-S2 DRG neurons (Fig. 6E, G). In OVX rats, nearly all CTB-labeled peripherin positive neurons displayed pSmad1-ir (Fig. 6F); about half of the peripherin-negative CTB-labeled neurons also contained pSmad1-ir. Following estrogen administration, numbers of pSmad1, CTB-labeled, peripherin-positive neurons (Fig. 6H) were reduced by approximately 60% (Fig. 6I); non-peripherin-ir CTB labeled neurons showed a smaller reduction of about 20% (Fig. 6I).

Local viral expression of BMP4 prevents estrogen-induced nerve depletion *in vivo*

These findings are consistent with the hypothesis that vaginal innervation density is regulated in part by estrogen's suppression of BMP4 synthesis. If so, then sustained BMP4 synthesis despite estrogen elevation should prevent this reduction in innervation. We used a lentiviral delivery system to drive BMP4 gene expression by the estrogen-independent CMV

promoter. The vector also expresses EGFP reporter under the UbiC promoter. Competence of the lentiviral agent was confirmed by assessing BMP4 gene and protein expression in transduced 293T cells (Fig. 7A, B).

OVX rats received vaginal submucosal lentiviral injections and were concurrently implanted with vehicle- or estrogen-releasing capsules and maintained for 7d. To identify regions that had incorporated viral gene expression, tissue was visualized using fluorescent stereomicroscopy and the vagina was cut transversely through the region showing the greatest EGFP fluorescence (Fig. 7C). Cryosections through this region showed EGFP expression in individual cells within the vaginal smooth muscle layer (Fig. 7D). In OVX rats injected with the BMP4 vector and receiving estrogen, smooth muscle showed elevated BMP4-ir (Fig. 7E) relative to rats receiving the EGFP vector alone (Fig. 7F) and as compared to regions adjacent to the injection sites.

We next determined whether sustained BMP4 expression prevents the reduction in vaginal innervation that occurs normally with estrogen elevation. OVX rats show reduced vaginal PGP9.5 immunoreactive innervation after 7 d of estrogen supplementation (Ting et al., 2004). Assessment of tissues adjacent to sites injected with lentivirus carrying BMP4 or EGFP constructs was similar to that described previously. Injection of lentivirus containing the EGFP vector alone also did not affect innervation as assessed using PGP9.5-ir (Fig. 8A, C) or peripherin (Fig. 8D). Sites injected with lentivirus carrying the BMP4 construct exhibited innervation patterns similar to controls but did not show the reduced innervation density normally seen after estrogen administration (Fig. 8B, C, D).

We investigated whether the effects of BMP4 are selective for different populations of vaginal axons. Putative peptidergic nociceptors were identified by immunostaining for CGRP (Lawson, 1992). Axon density was similar in uninjected and EGFP-vector-injected tissues but was increased in tissues receiving BMP4 lentivirus (Fig. 9A, B, C). In contrast, using TH-ir as a marker for sympathetic axons, we found no differences in innervation density among any of the sampled regions (Fig. 9D).

DISCUSSION

BMP4 regulates development of many smooth muscle targets by promoting differentiation, organization, and maturation of smooth muscle cells. Typically, BMP4 from adjacent epithelium, endothelium or fibroblasts acts in a paracrine fashion to regulate smooth muscle development (Frank et al., 2005; Cai, 2009; Wang et al., 2009; Tasian et al., 2010). Our findings show that in the adult, vaginal smooth muscle cells themselves synthesize BMP4, raising the possibility that BMP4 may also act in an autocrine fashion. Indeed, a recent report suggests that BMP4 exerts an autocrine effect to promote myotube formation by C2C12 cells (Umemoto et al., 2011). Hence, BMP4 may have roles in regulating smooth muscle integrity and function beyond the developmental period.

Smooth muscle cells are generally well innervated by peripheral axons and, accordingly, secrete a number of proteins that potentially influence axon growth and integrity. These include neurotrophins, extracellular matrix components, growth factors and cytokines (Weintraub et al., 1996; Knox et al., 2001; Gerthoffer and Singer, 2002; Krizsan-Agbas et al., 2003; Krizsan-Agbas et al., 2008). However, members of the BMP family of proteins have not been identified previously as regulators of peripheral innervation in the adult. In early development, BMP4 is critical for peripheral neuron migration and maturation, including formation of trigeminal

sensory facial maps and phenotype specification of DRG neurons (Hall et al., 2002; Guha et al., 2004; Hodge et al., 2007). The present study shows that BMP4 mediates peripheral sensory axon plasticity in the adult as well. DRG neurons express BMP4 receptors, and both smooth muscle-derived and exogenous BMP4 exert similar effects in stimulating sensory (but not sympathetic) axon outgrowth. While BMP4 is likely to induce outgrowth by interacting with axonal receptors proximate to sites of target synthesis, we did not directly demonstrate BMP4 receptors on terminals and therefore cannot rule out effects on other regions of the axon or soma which uniformly express these receptors. It is noteworthy that other members of the TGFβ family of proteins are known to interact with growth factors such as NGF to influence sensory neuron phenotype (Ai et al., 1999; Hall et al., 2002); hence the ultimate effects of BMP4 *in vivo* will clearly be determined by combinatorial influences that occur in conjunction with other local regulatory molecules. Nonetheless, our findings that DRG axon outgrowth is enhanced when BMP4 levels are increased in defined culture conditions, as well as when they are selectively increased by viral transduction *in vivo*, lend strong support to the idea that BMP4 acts directly as a pro-neuritogenic factor for at least some types of peripheral neurons in the mature organism.

In developing tissues, many factors regulate BMP4 expression including FGF3, vitamin A, and the wnt and notch signaling pathways (Baker et al., 1999; Endo et al., 2003; Baleato et al., 2005). Much less is known about the regulation of BMP4 synthesis in mature tissues. Our findings show that estrogen is an important factor in determining BMP4 expression in adult smooth muscle. Hence, both BMP4 mRNA and protein levels are decreased when vaginal tissue is cultured in the presence of estrogen. Because these studies were conducted *ex vivo*, we can exclude the possibility that estrogen acts indirectly by altering other hormonal systems. Similarly, since vaginal smooth muscle cells possess estrogen receptors, this is most likely a

direct effect on the smooth muscle (although we cannot exclude paracrine effects by other estrogen-responsive cells present in the explanted tissue). Consistent with a direct effect of estrogen, *in silico* analysis reveals several putative EREs upstream of the BMP4 gene (data not shown). As illustrated by our findings at 24h *ex vivo* and 7 day *in vivo*, estrogen has both short-term and sustained regulatory effects on BMP4 expression. In support of the idea that estrogen is a negative regulator of BMP4 expression, a recent report shows that BMP4 levels are elevated in prostate gland stromal cells of the estrogen receptor alpha knockout mouse (Chen et al., 2009). It therefore appears that estrogen can act to suppress BMP4 synthesis in multiple mature tissues types.

A striking feature of the female reproductive tract is the extensive and relatively rapid degree to which innervation remodeling occurs in conjunction with varying reproductive states. Our findings are consistent with the hypothesis that BMP4 plays a major role in this remodeling. Dispersed DRG neuron-vaginal smooth muscle co-cultures showed that estrogen's ability to diminish axon sprouting is fully reversed by addition of exogenous BMP4, supporting the idea that this cytokine is responsible for promoting outgrowth under low-estrogen conditions. This was illustrated most vividly in *in vivo* lentiviral transduction studies where smooth muscle cells containing the estrogen-independent CMV-promoter-driven BMP4 transcript showed high levels of sensory innervation whereas regions immediately adjacent that had only native, estrogen-dependent BMP4 expression showed markedly reduced innervation. Therefore, diminished BMP4 expression accounts, at least in part, for reduced vaginal innervation density when estrogen levels rise.

Several lines of evidence show that BMP-Smad signaling plays a crucial role in nerve regeneration following injury. Analysis of the transcriptome of DRG neurons following distal

axon injury showed that Smad1 is strongly upregulated during regeneration, and that this transcription factor is obligatory, as siRNA knockdown abrogated regenerative outgrowth (Zou et al., 2009). The importance of BMP induction of Smad1 in axon regeneration has recently been shown in studies of DRG neurons following spinal cord injury. Central DRG axons, which normally show minimal regenerative capacity, displayed more robust outgrowth through the lesion when BMP4 was overexpressed in neurons through viral transduction (Parikh et al., 2011). In contrast, blocking BMP signaling by administering noggin or ablating the BMP co-receptor, RMGb, inhibited DRG neuronal outgrowth following axotomy (Ma et al., 2011). Therefore, BMPs acting through the Smad1 pathway are among a number of different cytokines (Dubovy, 2011) that are implicated in the initiation of axon regeneration following injury.

We show here that BMP4/Smad1 signaling is also critical in determining target innervation under physiological conditions. In lumbar DRGs giving rise to vaginal innervation, low estrogen levels following ovariectomy were accompanied by high vaginal innervation density (presumably a result of axon sprouting), and levels of nuclear pSmad1 were high in peripherin expressing neurons including those that project to the vagina. While possible contributions of non-canonical signaling mechanisms (Sanchez-Camacho and Bovolenta, 2009) cannot be ruled out, these findings support the hypothesis that smooth muscle-derived BMP4 acts on DRG peripherin-positive neurons to induce Smad1 phosphorylation and nuclear translocation as a prelude to terminal sensory axon outgrowth in the estrogen-deprived vagina.

Alterations in estrogen status are associated with changes in vaginal autonomic as well as sensory innervation in both rats and humans (Ting et al., 2004; Griebling et al., 2011). While lentiviral BMP4 overexpression mimicked the effect of ovariectomy on CGRP immunoreactive sensory axons, TH expressing sympathetic axons were unaffected. This implies that, although

both sensory and autonomic neuronal progenitors are responsive to BMP4 in early development (Guha et al., 2004; Liu et al., 2005), BMP4 influences only sensory innervation density in the mature organism. It is unclear why mature sympathetic neurons are not responsive, but it seems likely that other estrogen-regulated factors are involved in achieving the full repertoire of vaginal axon remodeling. Estrogen is known to influence the expression of trophic proteins (Bjorling et al., 2002), as well as factors shown to be repulsive to sympathetic axons (Krizsan-Agbas et al., 2003; Krizsan-Agbas et al., 2008; Richeri et al., 2011). Hence, while our findings show that BMP4 signaling is necessary and sufficient to achieve high levels of sensory CGRP innervation, it is clear that estrogen-mediated regulation of reproductive tract innervation involves complex interactions at the level of the target and neuron.

Abnormal sensory innervation is a hallmark feature of some female pain syndromes. Vulvodynia, characterized by abnormal sensitivity of the genital tissues, affects some 16% of the adult female population (Harlow and Stewart, 2003), and the 'provoked' form of this disorder is associated with marked hyperinnervation of the vaginal vestibule by CGRP immunoreactive axons (Bohm-Starke et al., 1999; Tympanidis et al., 2003) and impaired estrogen receptor signaling (Eva et al., 2003); excising the hyperinnervated region frequently alleviates the pain (Goetsch, 1996). 'Spontaneous' vulvodynia selectively affects perimenopausal women, where estrogen levels are low and vaginal hyperinnervation also occurs (Griebling et al., 2011). Although the present study does not directly demonstrate that BMP4 elevation leads to hypersensitivity, several lines of evidence support this concept: i) Smad signaling pathways activated by BMP4 and related ligands are implicated in regulating DRG neuron excitability and pain sensitivity (Jeub et al., 2011); ii) our findings show that BMP4 selectively promotes sprouting of axons with a peripherin/CGRP-positive phenotype corresponding to small diameter

nociceptors (Bove and Light, 1995; Goldstein et al., 1996), and axons with this phenotype appear to be obligatory for detecting some types of noxious stimuli (McCoy et al., 2012); iii) the proliferation of these axons in OVX comports well with the previously documented hypersensitivity to vaginal distension that occurs under this low-estrogen condition (Bradshaw and Berkley, 2002; Ting et al., 2004); and iv) proliferation of peripheral axons of comparable phenotype is now documented as a common feature of many painful conditions (Di Sebastiano et al., 1995; Brown et al., 1997; Schubert et al., 2005; Tague et al., 2011). While additional research is required to fully define the interrelationships among innervation, estrogen and pain sensitivity, BMP4 clearly represents a potent estrogen-regulated determinant of peripheral sensory innervation.

FIGURES

Figure 1.

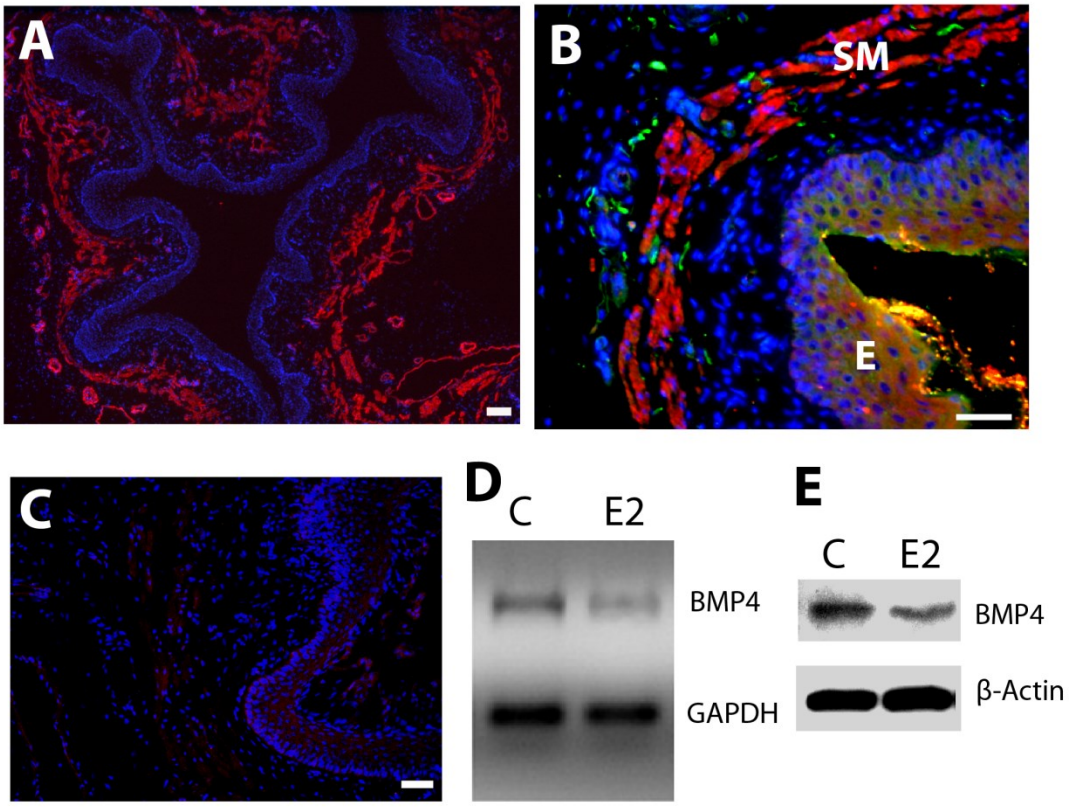


Figure 1. BMP4 expression and regulation in the adult rat vagina. **A.** Low magnification image shows gross histology of the vagina in cross section. Alpha-smooth muscle actin-immunoreactivity (ir) reveals the submucosal smooth muscle layer (red), which lies beneath the epithelium demarcated by the dense aggregation of DAPI-stained nuclei (blue). **B.** BMP4-ir (red) is expressed almost exclusively within smooth muscle (SM) lying beneath the epithelium (E) and this corresponds to the location of most peripherin positive axons (green). **C.** Preabsorption of the BMP4 antibody with an excess of mouse recombinant BMP4 eliminated staining of vaginal tissues. Bars=50μm. **D.** Submucosal vaginal tissue explants cultured for 24h with 20 nM estrogen (E2) show reduced BMP4 gene expression by RT-PCR relative to GAPDH as compared to untreated controls (C). **E.** *In vivo* treatment of ovariectomized rats for 7 d with estrogen also reduced BMP4 protein levels relative to β-actin as assessed by immunoblot.

Figure 2.

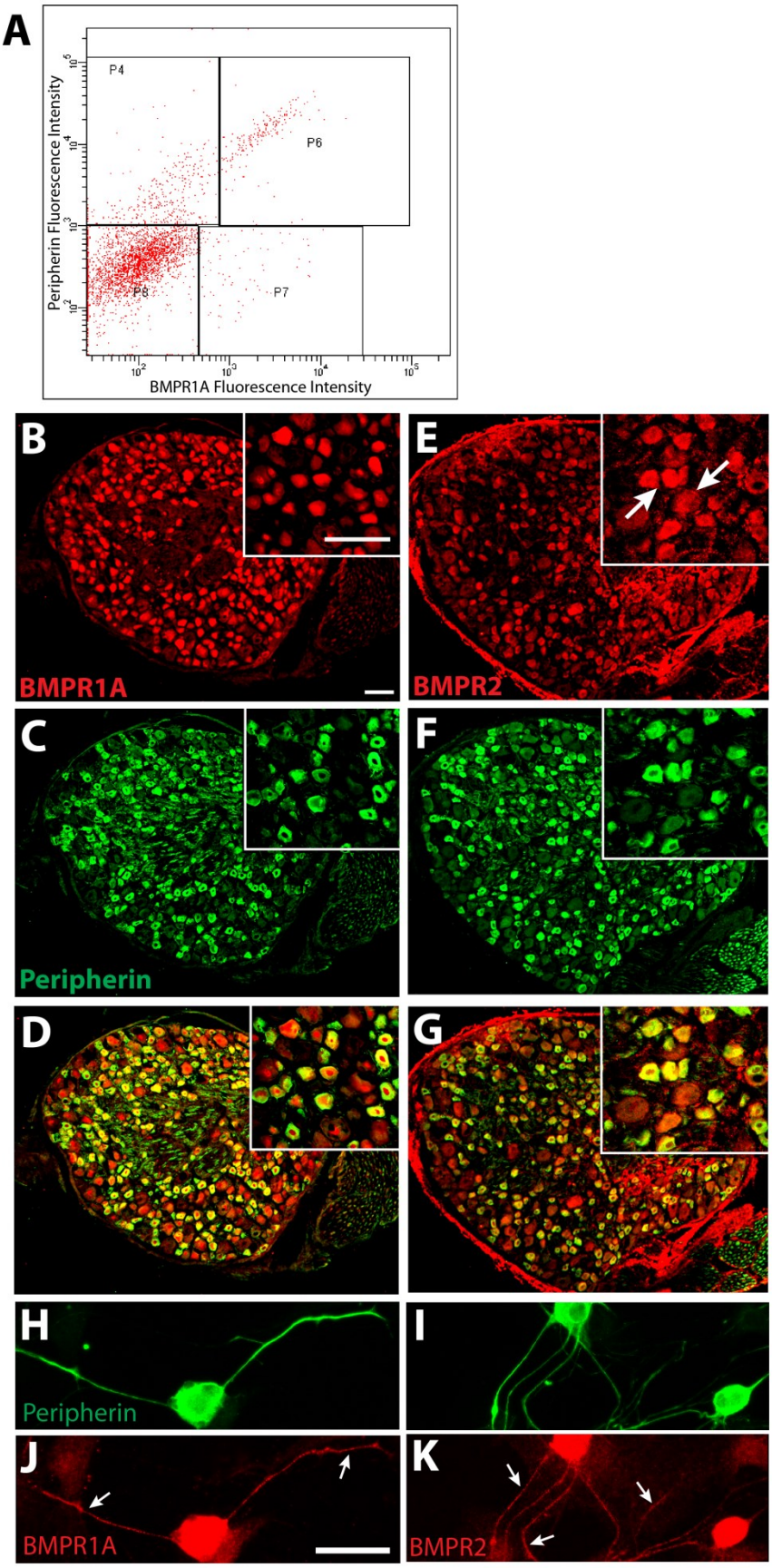

A
P4
P6
P8
P7
Peripherin Fluorescence Intensity
BMPR1A Fluorescence Intensity
B
BMPR1A
E
BMPR2
C
Peripherin
F
D
G
H
Peripherin
I
J
BMPR1A
K
BMPR2

Figure 2. BMP receptors are expressed in L6 to S2 sensory neurons. **A.** Fluorescence activated cell sorting of dissociated DRG cells immunolabeled for BMPR1A (Cy3) and peripherin (Alexa 488) reveal high levels of BMPR1A fluorescence intensity in cells displaying high peripherin fluorescence intensity (P6). Other populations include peripherin-negative cells with high BMPR1A (P7), peripherin-positive cells with low BMPR1A expression (P4), and cells negative for either marker (P8). **B.** Immunostaining of an L6 DRG section shows BMPR1A protein is widely distributed; inset is a higher power image showing BMPR1A localized predominantly within neurons. **C.** Distribution of peripherin immunostaining in the section shown in B. **D.** A merged image shows that BMPR1A-ir is present predominantly in peripherin-positive neurons. **E.** BMPR2 immunostaining shows wider localization across different neuronal subtypes. **F.** Immunostaining reveals peripherin expressing DRG neurons in E. **G.** Most peripherin-positive neurons show BMPR2 immunoreactivity. **H, I**. Peripherin immunoreactive neurons in dissociated DRG culture. Immunostaining for BMPR1A (**J**) and BMPR2 (**K**) show that both receptors are present throughout the axons (arrows) as well as the cell body. Scale bar in all panels and insets = 50 μm.

Figure 3.

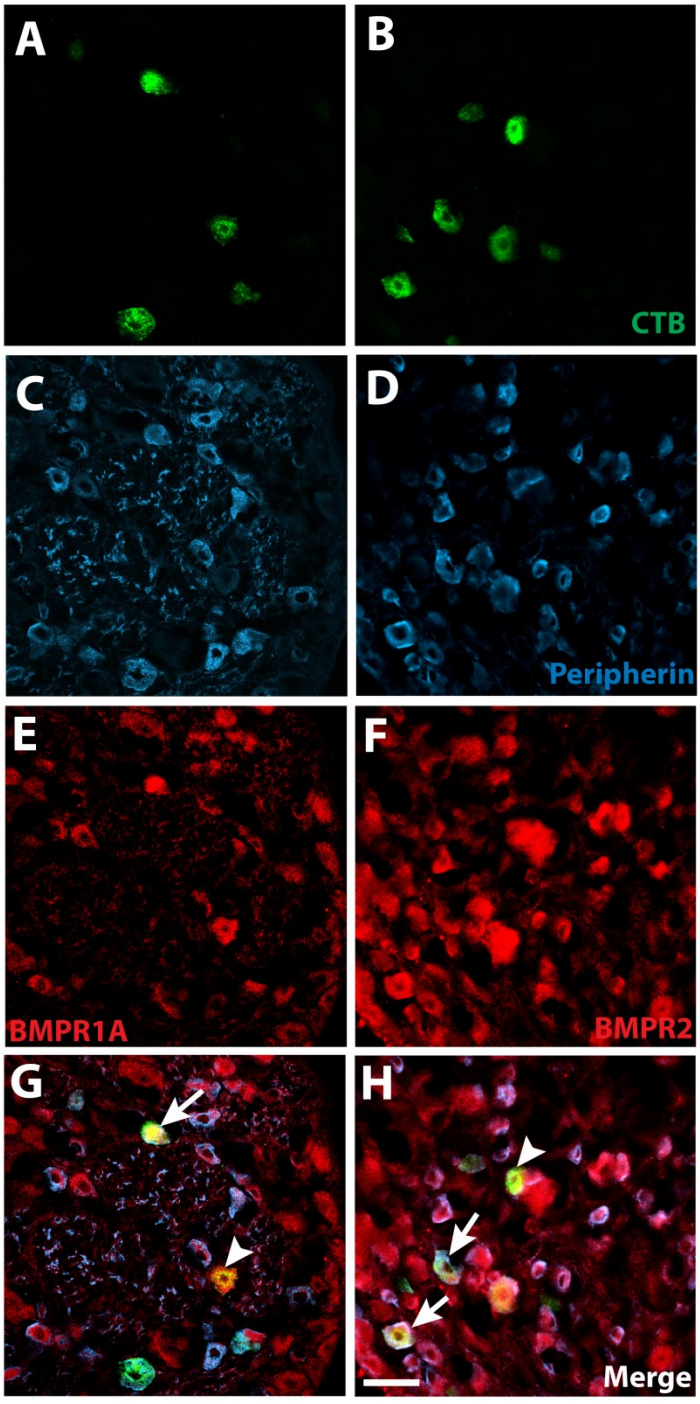


Figure 3. Vagina-projecting neurons express BMP4 receptors. **A, B.** Neurons projecting specifically to the vaginal submucosa were labeled following Alexa-488 conjugated recombinant Cholera Toxin-B injection (CTB), as shown here in an S1 dorsal root ganglion. **C, D.** Retrogradely labeled neurons frequently display peripherin immunoreactivity. **E, F**. Vagina-projecting neurons exhibit immunoreactivity for both BMPR1A and BMPR2. **G, H**. Arrows indicate CTB-labeled, peripherin positive neurons expressing BMPR. Arrowheads indicate CTB-labeled, peripherin negative neurons expressing BMPR. Scale bar in H = 50μm in all panels.

Figure 4.

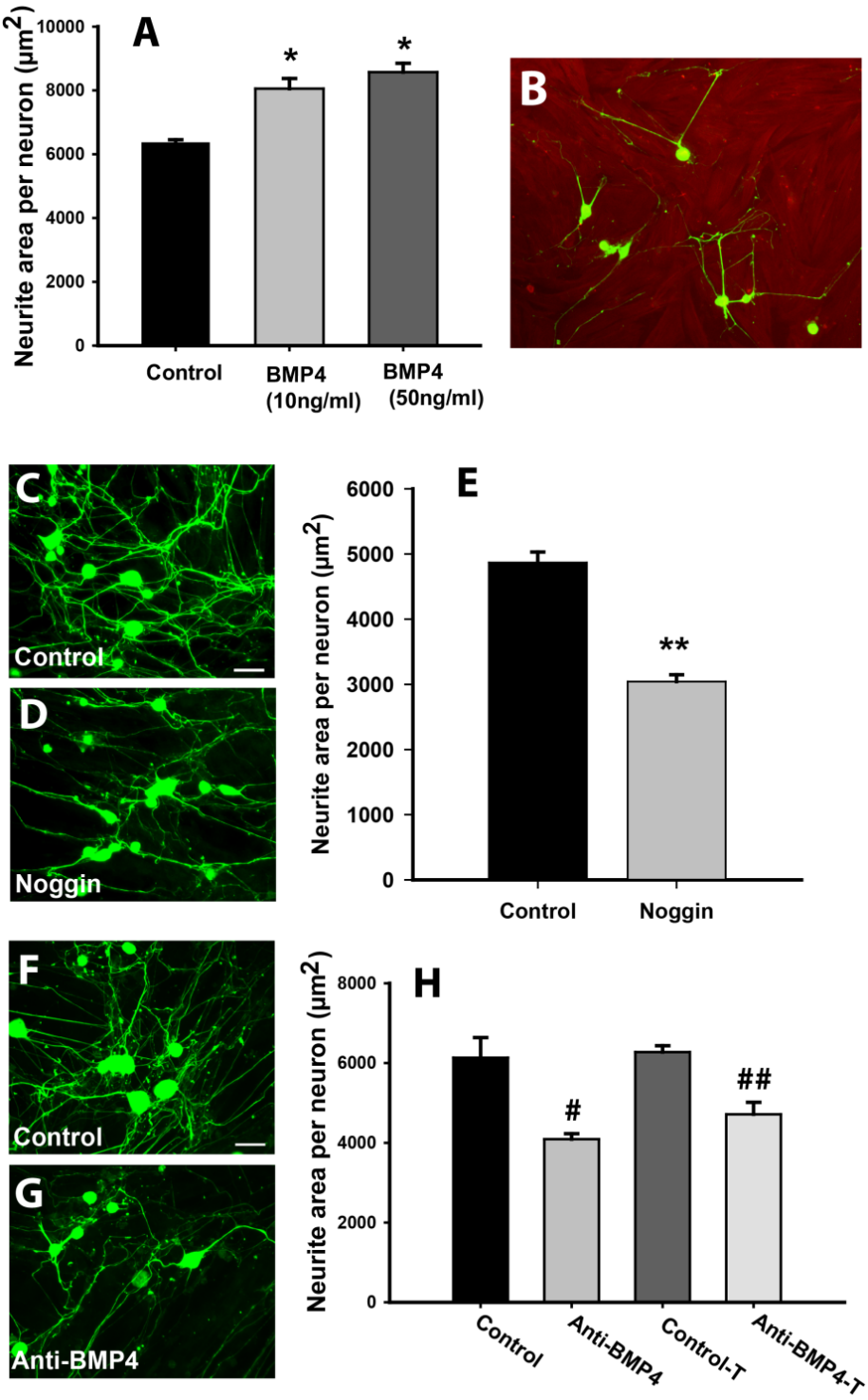

A
Neurite area per neuron (μm2)
10000
8000
6000
4000
2000
0
*
*
Control
BMP4 (10ng/ml)
BMP4 (50ng/ml)
B
C
Control
D
Noggin
E
6000
5000
4000
3000
2000
1000
0
**
Control
Noggin
F
Control
G
Anti-BMP4
H
8000
6000
4000
2000
0
#
##
Control
Anti-BMP4
Control-T
Anti-BMP4-T

Figure 4. Target-derived BMP4 promotes sensory neurite outgrowth. **A.** Dissociated dorsal root ganglion neurons grown on collagen coated plates showed increased neurite outgrowth in the presence of 10ng/ml or 50ng/ml BMP4 (**P*≤*0.007*). **B.** Vaginal smooth muscle cells immunostained for α-smooth muscle actin (red) were grown on collagen-coated plastic. Dorsal root ganglion neurons immunostained for peripherin (green) were plated on smooth muscle cells, and most extended neurites that made contacts with muscle cells at 48h. **C.** Vehicle treated co-cultures immunostained for peripherin showed many neurites. **D**. Co-cultures treated with noggin (250ng/ml) had fewer neurites. **E.** Quantitative analysis confirmed a reduction in neurite outgrowth per neuron in noggin treated co-cultures (***P=0.001*). **F.** Neurites elaborated by neurons grown directly on smooth muscle co-cultures (Control) are reduced by the addition of a BMP4 function-neutralizing antibody *(#P=0.005* **G, H**). Anti-BMP4 was equally effective in reducing axon outgrowth when smooth muscle cells were grown in transwell inserts (**H**, Control-T, Anti-BMP4-T, *##P=0.004*). Bars in C and F = 50 μm.

Figure 5.

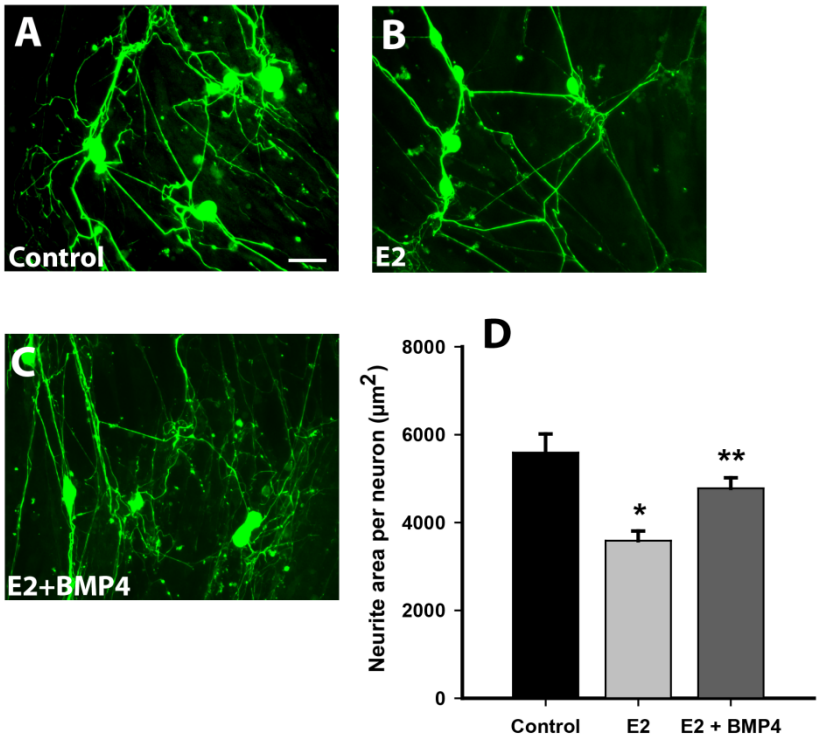

A
Control
B
E2
C
E2+BMP4
D
Neurite area per neuron (μm2)
8000
6000
4000
2000
0
*
**
Control
E2
E2 + BMP4

Figure 5. Neurite outgrowth in co-cultures is downregulated by estrogen and rescued by exogenous BMP4. **A.** Smooth muscle cells (unstained) were grown for 72 hours, on which dissociated sensory neurons (green) were cultured for 48h (bar = 50 μm in A-C). **B.** When smooth muscle cells were grown in the presence of $2x10^{-8}$ M 17β estradiol, neurite outgrowth appeared to be reduced. **C.** Addition of 10 ng/ml recombinant BMP4 appeared to increase numbers of neurites. **D.** Quantitative analysis showed that neurite outgrowth per neuron was reduced by estrogen (E2) pretreatment (**P= 0.001 vs. Control)*, and the decrease was abrogated by BMP4 (*#P=0.004 vs. E2*). There was no significant difference between Control and E2+BMP4.

Figure 6.

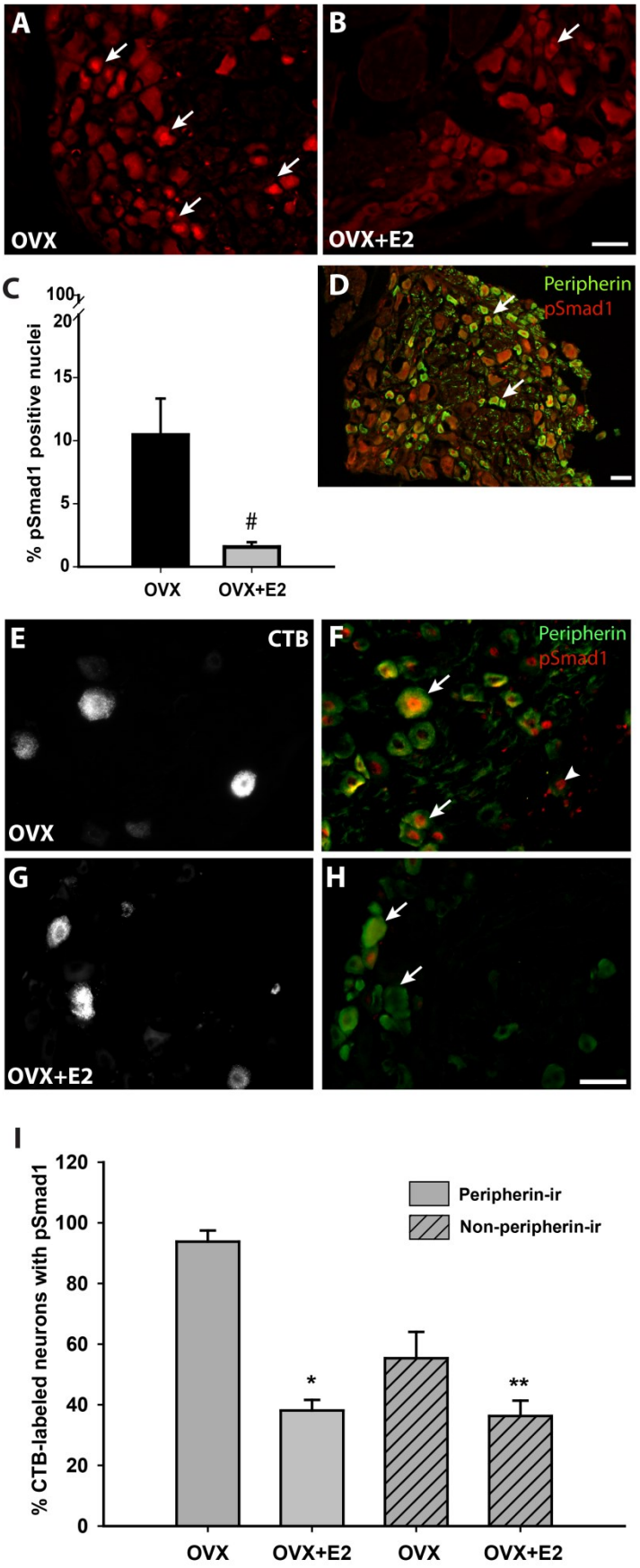

A
OVX
B
OVX+E2
C
% pSmad1 positive nuclei
100
20
15
10
5
0
#
OVX
OVX+E2
D
Peripherin
pSmad1
E
CTB
OVX
F
Peripherin
pSmad1
G
OVX+E2
H
I
% CTB-labeled neurons with pSmad1
120
100
80
60
40
20
0
Peripherin-ir
Non-peripherin-ir
*
**
OVX
OVX+E2
OVX
OVX+E2

Figure 6. pSmad1 expression in dorsal root ganglion neurons is reduced after estrogen administration. **A.** In vehicle-injected ovariectomized (OVX) rats, many nuclei show high levels of pSmad1-ir (arrows). **B.** 24h following estrogen administration (OVX+E2), pSmad1-positive nuclei were observed infrequently. **C**. Quantitative analysis confirms that numbers of pSmad1-positive nuclei are markedly reduced by estrogen administration (#*P=0.038*). **D.** Double-immunostaining for peripherin (green) and pSmad1 (red) show that positive nuclei are frequently present in peripherin positive neurons in the OVX rat. **E.** Vaginal submucosal injection of CTB resulted in retrograde labeling of DRG neurons of OVX rats. **F.** Several CTB labeled neurons in this low-estrogen condition (E) display nuclear pSmad1. Arrows points to peripherin positive CTB labeled neurons and arrowhead shows a peripherin negative CTB neuron. **G.** Vaginal CTB injection also labeled DRG neurons in OVX rats receiving estrogen. **H.** In presence of estrogen, CTB labeled neurons (G) in both peripherin positive (arrow) and negative (arrowhead) groups were frequently pSmad1 negative. **I.** In CTB-labeled neurons, pSmad1immunoreactive nuclei occurred more frequently in peripherin positive as compared to peripherin negative neurons (*P=0.004 by 2-way anova*). Both populations responded to estrogen with a reduction in the percentage of pSmad1 expressing nuclei (*****$P<0.001$, ******$P=0.034$ by Student Newman-Kuels tests). However, the decline was much stronger in the peripherin positive population (*P=0.007*). All images from the L6 and S1 DRG. Scale bars = 50 μm.

Figure 7.

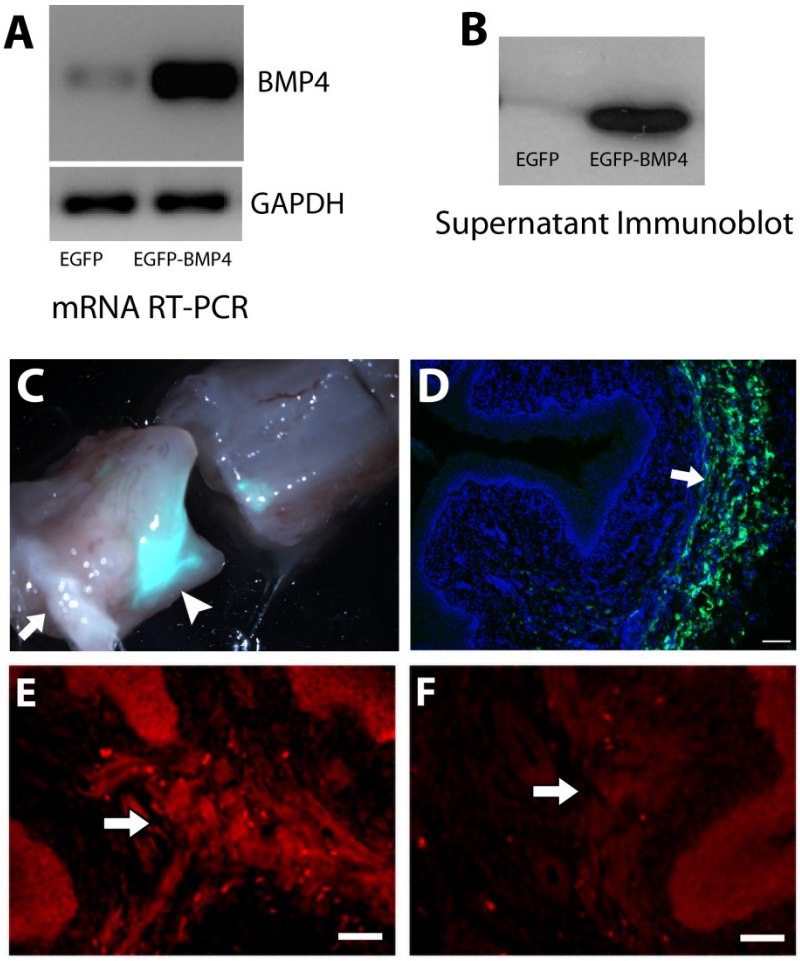
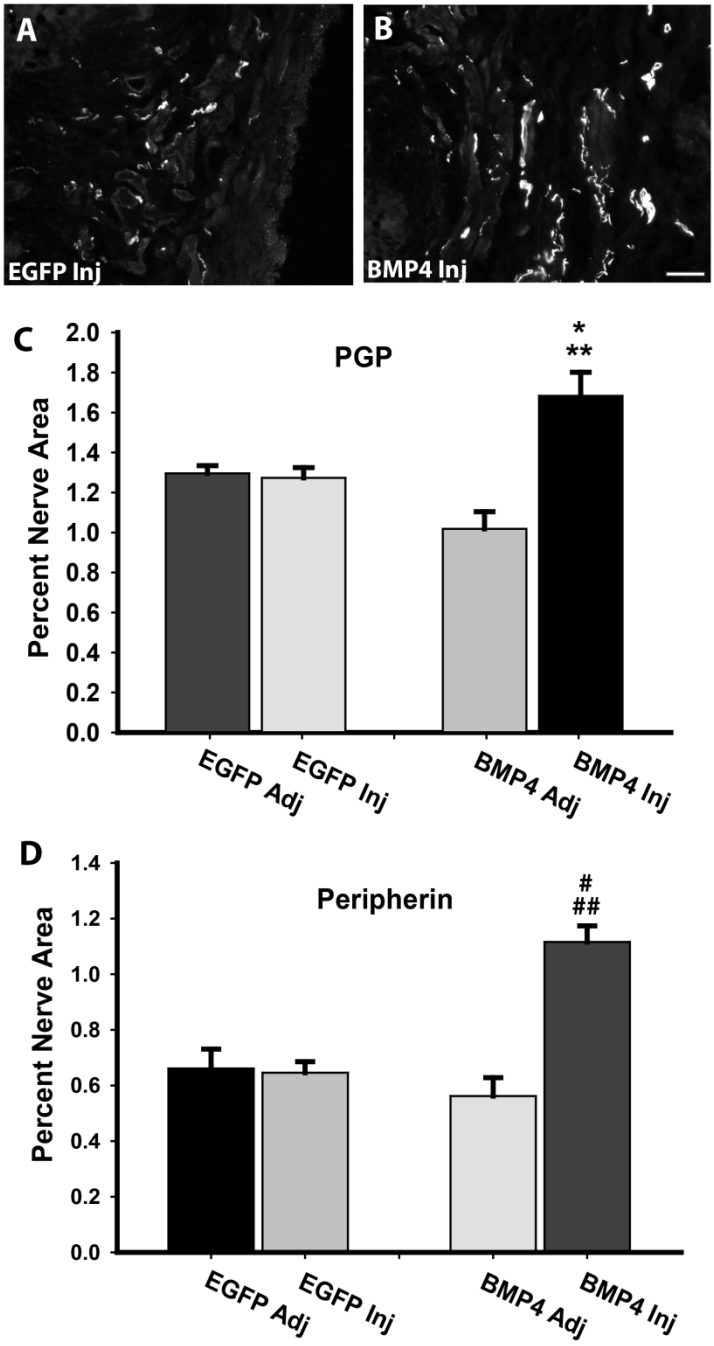

A
BMP4
GAPDH
EGFP
EGFP-BMP4
mRNA RT-PCR
B
EGFP
EGFP-BMP4
Supernatant Immunoblot
C
D
E
F

Figure 7. Lentiviral injection regulates BMP4 expression in vaginal smooth muscle. **A.** 293T cells overexpress BMP4 as assessed by RT-PCR 48 h after transduction with viral particles containing vectors for EGFP-BMP4 expression compared to those with only EGFP expressing control. GAPDH was used as a housekeeping control gene. **B.** Immunoblot showing abundant amounts of secreted BMP4 protein in conditioned media of 293T cells transduced with EGFP-BMP4 compared to EGFP alone. **C.** Bright field and epifluorescence overlay of the whole vagina (cut transversely) shows intense EFGP fluorescence at the injection site (arrowhead) at 7d. Arrow indicates the vaginal orifice. **D.** Overlay of DAPI staining and EGFP in a section of the specimen shown in C. Arrow indicates abundant EGFP expression in the smooth muscle layer (Scale bar = 200μm). **E.** Immunostaining for BMP4 shows robust fluorescence in vaginal smooth muscle (arrow) of an estrogen-treated OVX rat transduced with EGFP-BMP4 at 7d. **F.** Estrogen-treated rats receiving control virus with EGFP alone showed very low levels of BMP4 immunostaining. Scale bars in E and F = 50μm.

Figure 8.


A
EGFP Inj
B
BMP4 Inj
C
PGP
Percent Nerve Area
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1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
EGFP Adj
EGFP Inj
BMP4 Adj
BMP4 Inj
D
Peripherin
Percent Nerve Area
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
##
EGFP Adj
EGFP Inj
BMP4 Adj
BMP4 Inj

Figure 8. BMP4 overexpression preserves vaginal innervation at high estrogen. **A.** Immunostaining for PGP9.5 at the site of injection 7d following injection of lentivirus driving expression of only EGFP (EGFP-Inj) showed low innervation density typical of OVX rats receiving estrogen supplementation. **B.** PGP positive innervation density at sites injected with lentivirus driving BMP4 expression (BMP4-Inj) was considerably higher, characteristic of low-estrogen OVX rats (scale bar = 50μm). **C.** Injection of EGFP lentivirus did not alter innervation density relative to uninjected adjacent tissues (EGFP Adj), or to innervation at sites adjacent to injection with the BMP4 lentivirus (BMP4 Adj). Innervation density at sites receiving BMP4 lentivirus was significantly greater than that of other sites (**P=0.019* for BMP4 Inj vs. EGFP Inj, and ***P=0.026* for BMP4 Inj vs. BMP4 Adj). **D.** Staining for peripherin confirmed that changes noted with PGP9.5 also occur within this axonal population (#*P≤0.001* for BMP4 Inj vs. EGFP Inj, and ##*P≤0.001* for BMP4 Inj vs. BMP4 Adj).

Figure 9.

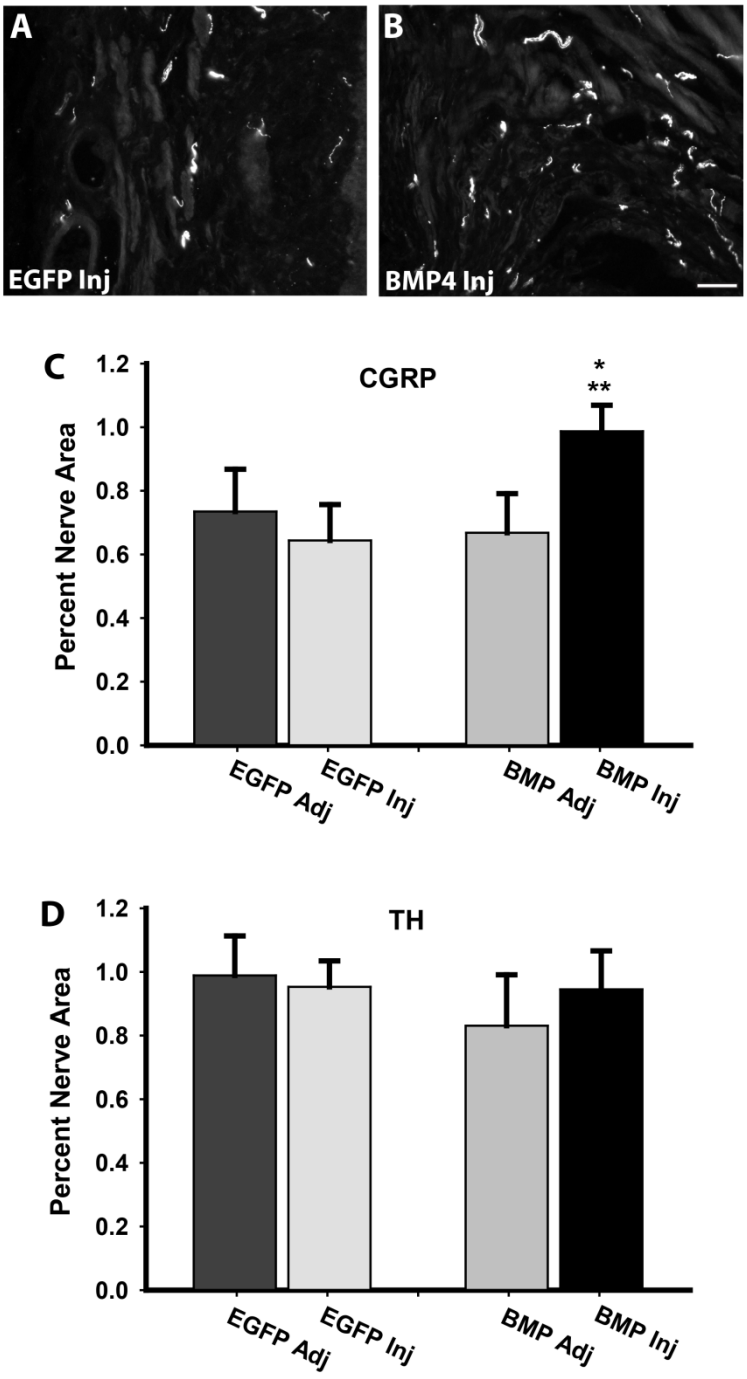

A
EGFP Inj
B
BMP4 Inj
C
CGRP
*
**
Percent Nerve Area
1.2
1.0
0.8
0.6
0.4
0.2
0.0
EGFP Adj
EGFP Inj
BMP Adj
BMP Inj
D
TH
Percent Nerve Area
1.2
1.0
0.8
0.6
0.4
0.2
0.0
EGFP Adj
EGFP Inj
BMP Adj
BMP Inj

Fig. 9. BMP4 over-expression selectively affects sensory innervation density. **A.** In OVX rats receiving estrogen and injected with EGFP lentivirus (EGFP Inj) sensory axons immunoreactive for calcitonin gene-related peptide (CGRP) were depleted. **B**. Sites injected with the BMP4 lentivirus (BMP4 Inj) showed higher levels of CGRP immunoreactive innervation. Scale Bar = 50μm. **C.** Quantitative analysis confirmed that CGRP axon density in tissue receiving BMP4 lentiviral injections was greater than that of tissue adjacent to the injection site (BMP Adj, **P=0.012*) and to tissue receiving EGFP lentivirus (EGFP Inj, ***P=0.049*). **D.** In contrast, injection with BMP4 lentivirus did not affect densities of axons expressing the sympathetic axon marker tyrosine hydroxylase (TH).

Chapter 3. Trophic factor and hormonal regulation of neurite outgrowth in sensory neuron-like 50B11cells

ABSTRACT

Sensory axon integrity and regenerative capacity are important considerations in understanding neuropathological conditions characterized by hyper- or insensitivity. However, our knowledge of mechanisms regulating axon outgrowth are limited by an absence of suitable high-throughput assay systems employing cells that are easily grown and faithfully display phenotypes similar to primary nociceptive neurons. While several model cell lines for dorsal root ganglion neurons have been proposed, most have not accurately captured the sensory neuronal phenotype, particularly with regard to their ability to rapidly extend axons in culture. The 50B11 cell line generated from rat embryonic dorsal root ganglion neurons offers a promising model for screening assays. These cells have been partially characterized and shown to express cytoskeletal proteins and genes encoding ion channels and neurotrophin receptors in common with sensory nociceptor neurons. In the present study we further characterized 50B11 cell phenotypes and quantified axon outgrowth induced by neurotrophic and hormonal factors.

50B11 cells express cytoplasmic proteins characteristic of small, unmyelinated sensory neurons including the microtubular protein beta-3 tubulin, the intermediate filament protein peripherin (a marker of unmyelinated neurons), and the pan-neuronal ubiquitin hydrolase, PGP9.5. In these short-term cultures only PGP9.5 immunoreactivity was uniformly distributed throughout soma and axons, and therefore presented the best means for visualizing the entire axon arbor. All cells co-express both NGF and GDNF receptors and addition of these ligands increased neurite length. 50B11 cells also showed immunoreactivity for the estrogen receptor-α and the angiotensin receptor type II, and both 17-β estradiol and angiotensin II increased outgrowth by differentiated cells.

50B11 cells show many features in common with primary unmyelinated nociceptor neurons. They are responsive to the axonal growth-promoting actions of classical neurotrophic factors as well as hormonal modulators. Coupled with their ease of culture and predictable differentiation, 50B11 cells represent a promising cell line on which to base assays that more clearly reveal mechanisms regulating axon outgrowth and integrity which may give rise to new therapeutic targets.

INTRODUCTION

The raison d'etre of the nervous system is to acquire, transmit and process information. Peripheral sensory innervation is critical in transducing environmental information necessary for awareness and protection of the organism. Pathological loss of peripheral axons of sensory dorsal root ganglion (DRG) neurons underlies common forms of neuropathy affecting many clinical populations including diabetics and cancer survivors who received chemotherapy (Younger, 2004; Jaggi and Singh, 2012). Similarly, abnormal proliferation of DRG sensory axons occurs in many chronic inflammatory pain syndromes (Chakrabarty et al., 2011; Tague et al., 2011). Thus, appropriate structural geometry of peripheral axons appears to be critical for ensuring optimal sensory function.

Factors regulating DRG target innervation are incompletely understood. A reason for our limited understanding is a lack of robust assay systems. Primary DRG cultures have long provided the main means for assessing factors regulating axon outgrowth *in vitro*. However, primary cultures are limited by low throughput, cellular heterogeneity, and tedious preparation protocols. Attempts to simulate sensory outgrowth *in vitro* have included PC12 cells and neuroblastomas (Radio et al., 2008; Radio and Mundy, 2008), but with limited success. Immortalized cell lines generated from rat, mouse and human DRGs have included the F11 cell line which fused mouse hybridoma with rat embryonic DRG neurons (Platika et al., 1985a; Platika et al., 1985b), the ND lines generated by fusing neonatal mouse DRG cells with neuroblastoma cells (Wood et al., 1990) and the HD10.6 lines derived by incorporating a tetracycline-inducible *v-myc* oncogene into 3rd trimester human embryonic DRG neurons (Raymon et al., 1999). While these lines have been useful in analyzing sensory neuron electrophysiology, signaling and biochemistry (Wood et al., 1990; Fan et al., 1992; Faravelli et

al., 1996), none display axonal morphologies similar to those of primary cultures, thus limiting their use in studying axonogenesis.

Recently, Höke and colleagues created the 50B11 cell line by electroporating E14.5 rat primary DRG neurons to incorporate the SV40 large T-antigen and human telomerase reverse transcriptase. While these cells remain largely undifferentiated under standard culture conditions, in the presence of forskolin a significant proportion differentiate and undergo axonogenesis reminiscent of primary DRG neurons (Chen et al., 2007a). These cells express other features in common with small diameter, nociceptor neurons including gene expression for some neurotrophin receptors and voltage-gated ion channels (Chen et al., 2007a). Given their similarities to primary DRG neurons, 50B11 cells may hold promise as a model for studying axon growth. However, DRG axonogenesis involves intricate interactions among trophic, tropic and modulatory factors acting on multiple receptors to regulate cytoskeletal structural protein expression, and it remains unclear how closely 50B11 cells replicate outgrowth regulatory mechanisms in primary neurons. In the present study, we show that differentiated 50B11 cells display phenotypes and responses to growth factors that are highly similar to those of DRG neurons.

MATERIALS AND METHODS

Cell Culture, differentiation and treatments

50B11 cells were a gift from Dr. Ahmet Hoke of the Johns Hopkins University. Cells were plated onto plastic in 6 or 24 well tissue culture plates in Neurobasal medium (Life Technologies, Gibco) supplemented with FBS (Sigma-Aldrich), B27 (Life Technologies), glucose (Fisher) and glutamine (Sigma-Aldrich), as described previously (Chen et al., 2007a). Cells were plated at different densities including at low density to facilitate visualization of individual neuronal axon arbors, thus ensuring accurate measurements of neurite length. 24h after plating cells were differentiated by the adding forskolin (Sigma-Aldrich, 75 μM final concentration) to the medium. Based on observations by Chen *et al.* (Chen et al., 2007a) and our preliminary studies, we concluded that neuronal phenotype was most stable between about 20 and 36h post-forskolin, and treatment protocols were designed to be completed within this time frame.

Seventeen hours after initiating forskolin-induced differentiation, cells were treated with nerve growth factor (NGF, 50ng/ml recombinant mNGF, Peprotech), glial cell line-derived neurotrophic factor (GDNF, 50ng/ml recombinant hGDNF, Peprotech), estrogen (17β-estradiol, 20 nM, Sigma-Aldrich) or angiotensin II (ANGII, 100 nM, Sigma-Aldrich). Cells were maintained for 20h and fixed with 4% paraformaldehyde.

Immunostaining

Cells were washed after fixation and incubated in blocking solution containing 1% BSA (Sigma-Aldrich), 5% normal donkey serum (Millipore), in phosphate buffered saline (Sigma-Aldrich) containing 0.3% Triton X-100 (Sigma-Aldrich) for 1h at room temperature. They were

immunostained for PGP9.5 (1:700, rabbit antiserum, Serotec), βIII-tubulin (1:400, mouse antiserum, Millipore), peripherin (1:200, chicken antiserum Millipore), TrkA (1:200, rabbit antisera, Millipore), GFR alpha1 (1:200, goat antiserum, R&D Systems), GFR alpha2 (1:200, goat antiserum, R&D Systems), estrogen receptor (ER) alpha (1:200, rabbit antiserum, Santa Cruz Biotech), and ANGII receptor type 2 (AT2, 1:200, rabbit antiserum, Alamone Labs). Donkey IgG (1:200 to 1:400, Jackson Immunoresearch) tagged with Cy3 or Alexa 488 was directed against host primary antibodies. All antibodies were diluted in phosphate buffered saline containing 0.3% Triton X-100 and 5% normal donkey serum. Primary and secondary antibody specificities have been confirmed using antibody preabsorption and omission controls in our lab and others (Baloh et al., 1998; Barker-Gibb et al., 2001; Chakrabarty et al., 2008; Chakrabarty et al., 2011; Tague et al., 2011).

Quantitation of neurite outgrowth

Individual neurons with distinct neurite arbors were visible in low-density cultures. For each treatment group in each experiment, 75-100 individual neurons were imaged. Neurons were counted from randomly collected images (about 15-20 per well). Single neurons with minimal or no overlap of neurite arbors with adjacent cells were analyzed for neurite outgrowth using NIH ImageJ software with the NeuronJ Plugin. The distances from the soma perimeter to the tips of each axon extension were measured by tracing the arbors; data were expressed as the summed length of outgrowth within the arbor and as the maximum length of the longest axon. Treatment effects were compared by Mann-Whitney rank sum tests.

RESULTS

Characterization of axonal markers in differentiated 50B11 cells

Following forskolin differentiation, 50B11 cells acquire many phenotypic features characteristic of DRG neurons, including expression of the cytoskeletal proteins βIII-tubulin and the hypophosphorylated form of neurofilament H in axon-like processes (Chen et al., 2007a). We further characterized these cells to more fully assess their content of other cytoskeletal proteins typical of DRG neurons.

50B11 cells immunostained 20h after forskolin confirmed the presence of βIII-tubulin, which was most intense in the cell body and fainter in the axons (Fig. 1A). PGP9.5 is a ubiquitin hydrolase expressed in all intact axons (Lundberg et al., 1988) and has been used extensively for staining nerve fibers both *in vitro* and *in vivo* (Wilkinson et al., 1989). PGP9.5 immunostaining was intense within the cell body, but was also bright throughout the axons, allowing us to resolve much finer details of the processes (Fig.1B). PGP9.5 showed consistent colocalization with βIII-tubulin within differentiated cells (Fig. 1C) and with lower expression in undifferentiated 50B11 cells.

Peripherin is a type III intermediate filament protein that is a selective marker for unmyelinated axons *in vivo* and *in vitro* (Fuchs and Weber, 1994; Fornaro et al., 2008). Immunostaining of differentiated 50B11 cells showed expression that was largely restricted to the soma (Fig. 1D). Peripherin expression was weak in undifferentiated cells.

When stained for PGP9.5, cultures plated at densities allowing identification of individual axonal arborizations revealed a range of morphologies, with cells generally extending

2 or 3 neurites (Fig. 1B) but occasionally as many as 4 or more as described previously (Chen et al., 2007a).

Receptor expression in 50B11 cells

Differentiated 50B11 cells resemble small diameter nociceptive DRG sensory neurons and express mRNA for trkA, p75, c-RET, and GFR alpha1 (Chen et al., 2007a). *In* vivo, DRG nociceptive neurons are often categorized into distinct subclasses based on expression of NGF or GDNF-family receptors (Molliver and Snider, 1997; Molliver et al., 1997). We performed immunocytochemistry to localize trkA, GFR alpha1 and GFR alpha2 receptor subtypes in these cells. Differentiated 50B11 cells express all these receptor subtypes (Fig. 2A, B, E, F). However, co-localization showed that all cells express both the NGF and GDNF-family receptors (Fig. 2C & G) and cannot be subdivided based on unique receptor expression.

In addition to neurotrophins, other agents such as hormones affect axon outgrowth in primary DRG neuron cultures. ANGII for example enhances DRG axon growth by signaling through the AT2 receptor (Chakrabarty et al., 2008). AT2 expression in DRG neurons is upregulated by estrogen acting on ER alpha, which also increases axonal growth (Chakrabarty et al., 2008). We investigated expression of AT2 and ER alpha in differentiated 50B11 cells. At 24h after forskolin-induced differentiation, 50B11 cells showed AT2 receptor immunoreactivity throughout the soma extending into the neurites (Fig. 2D); undifferentiated cells weakly expressed AT2. Differentiated 50B11 cells also showed prominent expression of ER alpha which was localized to the cell nucleus; ER alpha expression was low or absent in undifferentiated cells (Fig. 2H).

NGF and GDNF promote axon outgrowth in 50B11 cells

50B11 cells express neurotrophin receptors, and their gene expression is regulated by NGF and GDNF (Chen et al., 2007a). We quantified axon outgrowth in response to these neurotrophins. Twenty h following NGF treatment, total neurite outgrowth was increased by about 21% (Fig. 3A, $p \leq 0.001$), while the length of the longest neurite was increased by about 44% (Fig. 4A, $p \leq 0.001$). GDNF also increased outgrowth by 23% and 51%, respectively (Figs. 3B, 4B, $p \leq 0.001$). The increased outgrowth in response to both neurotrophic factors was statistically comparable.

Angiotensin II and estrogen promote axon growth in 50B11 cells

AT2 receptor activation promotes axon growth by DRG nociceptor neurons *in vivo* and *in vitro* (Chakrabarty et al., 2008) and 50B11 cells express receptors for both AT2 and estradiol, which upregulates AT2. Addition of ANGII at concentrations optimal for increasing outgrowth in primary DRG cultures (Chakrabarty et al., 2008) increased total arbor length by 27% (Fig. 3C, $p \leq 0.001$) and length of the longest neurite by 28% (Fig. 4C, $p \leq 0.001$). 50B11 cells also responded to E2 by increasing total arbor length by about 15% (Fig. 3D, $p \leq 0.001$) and length of the longest neurite by 25% (Fig. 4D, $p \leq 0.001$) at a concentration optimal for DRG neurite outgrowth (Chakrabarty et al., 2008). The effects of both E2 and ANGII on total outgrowth and longest axon length were comparable. Similarly, both agents produced increases in overall outgrowth that were comparable to those induced by NGF and GDNF. However, neurotrophins were more effective than ANGII in augmenting maximum axon length (NGF vs ANGII, $p=0.03$; GDNF vs ANGII, $p=0.002$); while NGF was comparable to E2 in increasing maximum axon length, GDNF was more effective than E2 ($p=0.004$).

DISCUSSION

The utility of the 50B11 cells in studying neurodegeneration has been previously noted (Chen et al., 2007a; Melli and Hoke, 2009) and the present study extends these findings to demonstrate their potential in studying axonal outgrowth. Axon growth and maintenance are dependent upon specific cytoskeletal proteins, many of which can be used as selective markers. βIII tubulin is one of the most extensively used neuron-specific markers for axon/neurite staining and quantification of primary neurons both *in vitro* and *in vivo* (Feit et al., 1971; Guo et al., 2010; Guo et al., 2011). In differentiated 50B11 cells, we observed intense βIII-tubulin immunostaining within the cell body and proximal neurites but less intense staining in the more distal regions of the neurite. It is unclear if this is because βIII-tubulin distribution in 50B11 cells differs from that of primary neurons or is attributable to the culture conditions. Thus, the post-treatment interval for working with stably differentiated 50B11 cells is comparatively short, and since βIII-tubulin populates the axon relatively late as compared to actin, it is possible that microtubule content had not yet reached its maximum when our samples were prepared.

Peripherin immunoreactivity was also abundant in the cell body, providing further evidence that the 50B11 cells display a phenotype consistent with sensory nociceptors. However, fluorescence intensity was insufficient to fully elucidate axons. This may again reflect culture duration; cytoskeletal integration of this intermediate filament protein lags behind tubulin in growing DRG axons, and peripherin is a relatively late arrival to distal regenerating axons (Goldstein et al., 1991).

In contrast, differentiated 50B11 cells showed high levels of PGP9.5 throughout the soma, axons and terminal neurites. PGP 9.5 (ubiquitin carboxy-terminal hydrolase L1) is a

neuron-specific ubiquitin hydrolase and a member of a large family of deubiquinating enzymes (Day and Thompson, 2010). Although specific functions of this protein are not fully clear, it is believed to play a role in cleavage of either ubiquitin-protein bonds or C-terminal extensions of ubiquitin itself. The high turnover rate for protein in neuronal cells, and particularly in the growing tips of neurites, actively involves the ubiquitin-proteasome pathway (Tai and Schuman, 2008) which has been implicated in a number of neuronal functions including outgrowth, synaptic transmission, and plasticity (Yi and Ehlers, 2007). Indeed, PGP9.5 comprises some 5-10% of the cytoplasmic protein in primary neurons (Day and Thompson, 2010), and apparently high levels are also retained in 50B11 cell, including within the terminal neurites. Accordingly, not only do 50B11 cells express this neuron-specific protein at high levels, but it is a useful marker for visualizing neurite extension by differentiated cells.

In postnatal mouse DRG it is possible to detect distinct populations of small-diameter neurons based on their neurotrophin dependencies; some are dependent upon NGF for survival, while others require members of the GDNF family of proteins (Priestley et al., 2002). Chen *et al.* showed it was possible to detect mRNA encoding NGF and GDNF receptors from differentiated 50B11 cell cultures (Chen et al., 2007a), and our immunofluorescence studies show that 50B11 cells synthesize NGF and GDNF receptor proteins. However, we also show that both receptors are co-expressed in differentiated 50B11 cells. This may not be altogether surprising given that 50B11 cells were derived from d14.5 embryonic DRG neurons. At this stage of development, migration of neural crest cells forming the DRG is largely complete and the cells have undergone preliminary differentiation (Marmigere and Ernfors, 2007). All small neurons at this stage are positive for trkA. Subsequently, under the influence of the Runx1 transcription factor, some cells lose trkA and acquire Ret expression, with these 2 populations

differentiating into mature 'peptidergic' (i.e., calcitonin gene-related peptide-positive) and 'non-peptidergic' (isolectin IB4-positive) populations (Marmigere and Ernfors, 2007). It appears that, in the process of immortalizing embryonic DRG neurons, the normal developmental program may have been altered such that all resulting cells ultimately express both receptor types. It should be noted that mRNAs encoding NGF and GDNF receptors are widely colocalized in DRG neurons of the adult rat (Kashiba et al., 2003) and our findings suggest that differentiated 50B11 cells also show a neurotrophin receptor profile that may reflect responsiveness to both families of neurotrophic proteins.

A question unanswered is whether DRG neurons expressing both NGF and GDNF receptors can respond to both types of neurotrophic factors. Our studies examining axonal architecture show that, at least in differentiated 50B11 cells, both pathways are capable of inducing axon outgrowth. Thus, both NGF and GDNF increased total arbor length and the length of the longest axon to comparable extents.

Many factors in addition to classical neurotrophins influence axonal growth. In the case of DRG nociceptor neurons, there is accumulating evidence that these cells can be strongly influenced by local or systemic hormonal factors. For example, sustained elevation of plasma estrogen in vivo results in increased sensory nociceptor innervation of the skin, mesentery, and mammary gland in rats (Blacklock et al., 2004; Blacklock and Smith, 2004). Estrogen acts on nociceptor ER alpha to increase levels of AT2 mRNA and protein. AT2 mediates the neuritogenic effects of the hormone ANGII, which is derived from autocrine or paracrine sources (Chakrabarty et al., 2008). Our findings show that differentiated 50B11 cells, like DRG nociceptors neurons, express both AT2 and ER alpha receptors, consistent with potential hormonal modulation of these cells.

The AT2 receptor and ER-alpha appear to function comparably in both 50B11 cells and DRG nociceptors neurons. Hence, peripherin-positive DRG neurons show enhanced neurite outgrowth when primary cultures are treated for 48-72h with either estrogen or ANGII, and the present studies show that both agents are equally effective in inducing 50B11 cell neurite outgrowth. It is noteworthy that neither ANGII nor estrogen promoted outgrowth to the extent obtained with either NGF or GDNF. In contrast, in primary neonatal DRG cultures, estrogen was more effective in eliciting outgrowth than was NGF (Blacklock et al., 2005). It is unclear if this is due to intrinsic differences between 50B11 cells and DRG neurons, or if 50B11 cells, which derive from much younger neurons, retain a greater sensitivity to NGF which has been downregulated in the neonatal neurons. Nonetheless, it is clear that 50B11 cells respond to hormones and neurotrophic factors in a manner that is qualitatively comparable to that of native DRG nociceptors neurons.

Thus from the present study, some significant conclusions can be drawn. Alterations in peripheral sensory innervation are strongly associated with many pathological conditions, such as peripheral neuropathies where sensory axons are lost (McLeod, 1995; Low and Cheng, 2005; Luo and O'Leary, 2005; Saxena and Caroni, 2007), and chronic inflammatory pain syndromes characterized by nociceptor axon proliferation (Di Sebastiano et al., 1995; Masheb et al., 2000; Alfredson et al., 2003; Tympanidis et al., 2003; Schubert et al., 2005). Therefore, signaling pathways responsible for regulating nociceptor axonal architecture are attractive targets for therapeutic intervention to either increase or decrease outgrowth as appropriate. To date, the intracellular signaling proteins and their interactions remain incompletely understood, and we lack effective tools to modify outgrowth, in part because of the difficulty in conducting high-throughput screening on primary neuronal cultures. We show here that 50B11 cells constitute a

robust assay system for assessing neurite outgrowth in vitro. Moreover, these cells respond to trophic factors and hormones in a manner that is largely indistinguishable from DRG neurons. These findings provide strong validation for the idea that differentiated 50B11 cells provide a useful tool for investigating mechanisms regulating nociceptor axon outgrowth.

FIGURES

Figure 1.

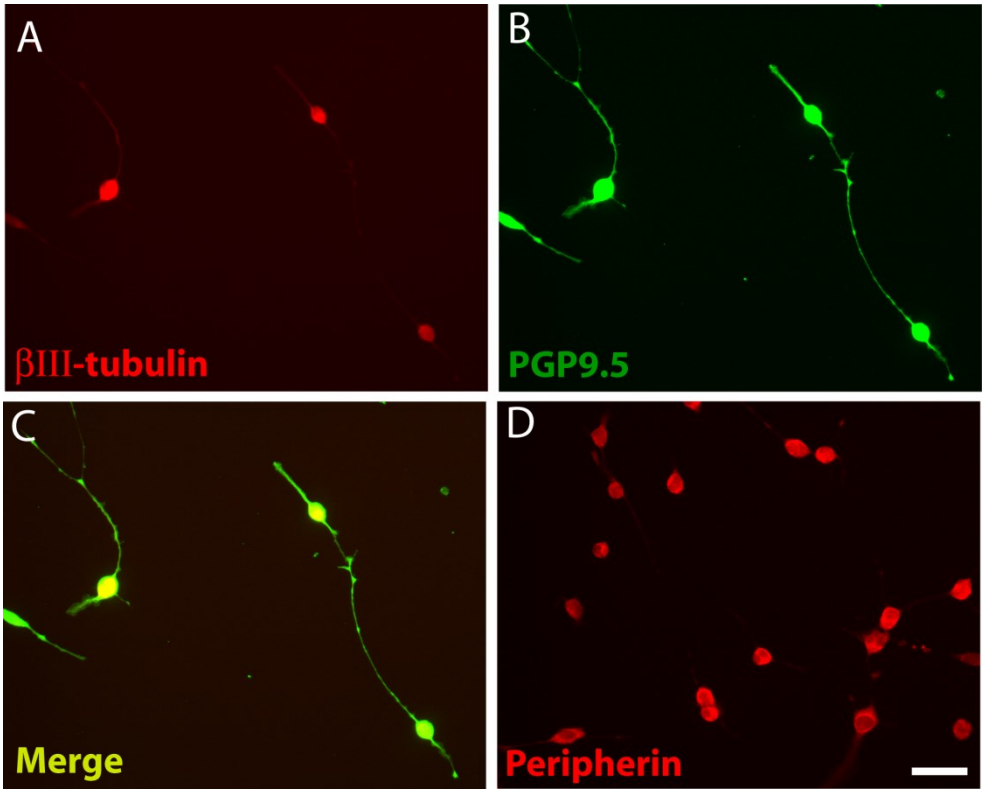


Figure 1. Differentiated 50B11 cells show cytoplasmic proteins characteristic of DRG neurons. **A.** Staining for βIII-tubulin is prominent in the cell body but diminished in distal processes. **B.** PGP9.5 immunostaining delineates both soma and axons in good detail. **C.** An overlay of A and B shows colocalization of these neuronal markers. **D.** Peripherin immunohistochemistry shows strong expression in the soma but little in processes. Bar in E=50μm for all panels.

Figure 2.

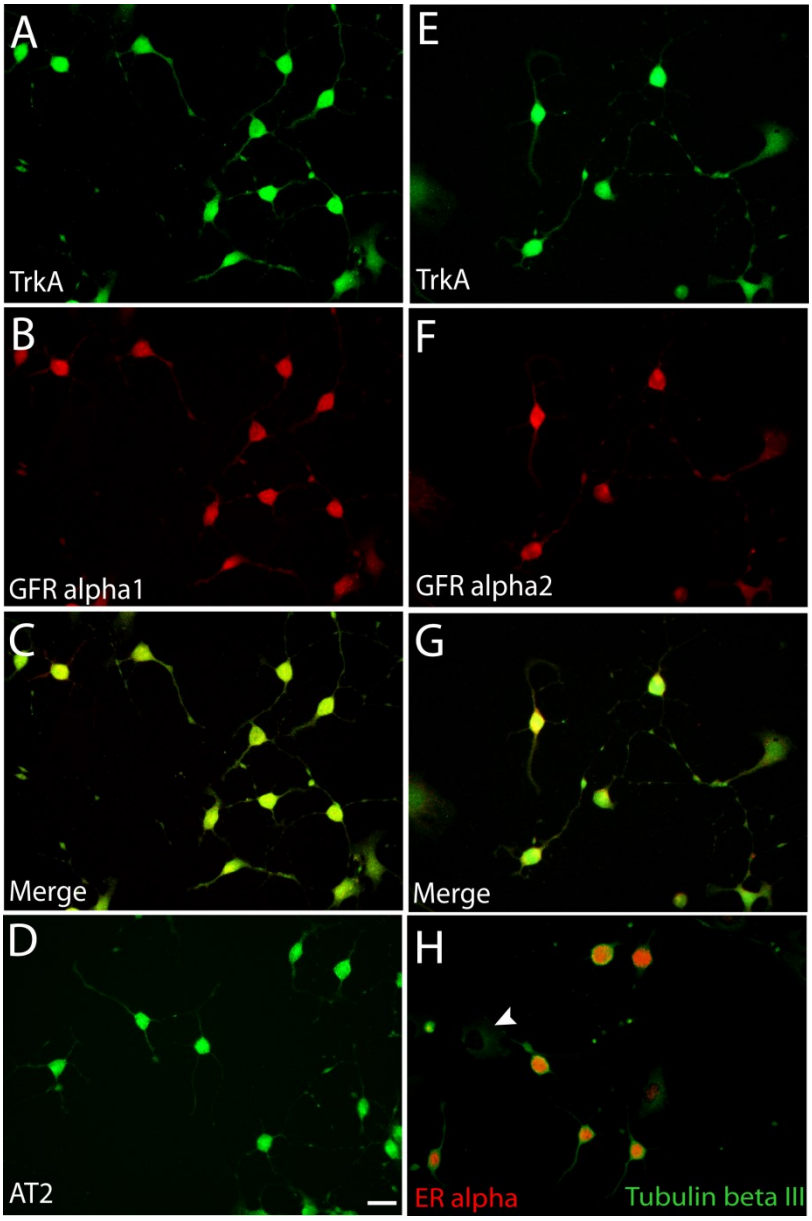

A
TrkA
E
TrkA
B
GFR alpha1
F
GFR alpha2
C
Merge
G
Merge
D
AT2
H
ER alpha
Tubulin beta III

Figure 2. Expression of growth factor receptors by 50B11 cells. Cells were differentiated for 20h and then fixed and stained for receptor proteins. **A.** All cells showing neuron-like morphologies after differentiation showed immunostaining for the NGF receptor, trkA. **B.** Cells also showed strong immunoreactivity for the GDNF receptor, GFR alpha1. **C.** A merged image shows that all differentiated neuron-like cells express both trkA and GFR alpha1. **D.** Differentiated cells also show immunoreactivity for the ANGII receptor, AT2. **E.** TrkA staining of differentiated cells. **F.** Immunostaining of the same field as E shows cells uniformly expressing GFR alpha2. **G.** A merged image of E and F shows colocalization of trkA and GRF alpha2. **H.** Differentiated, tubulin beta III positive cells display predominantly nuclear immunoreactivity for estrogen receptor alpha whereas undifferentiated cells display little or no ER alpha. Bar in D =50μm for all panels.

Figure 3.

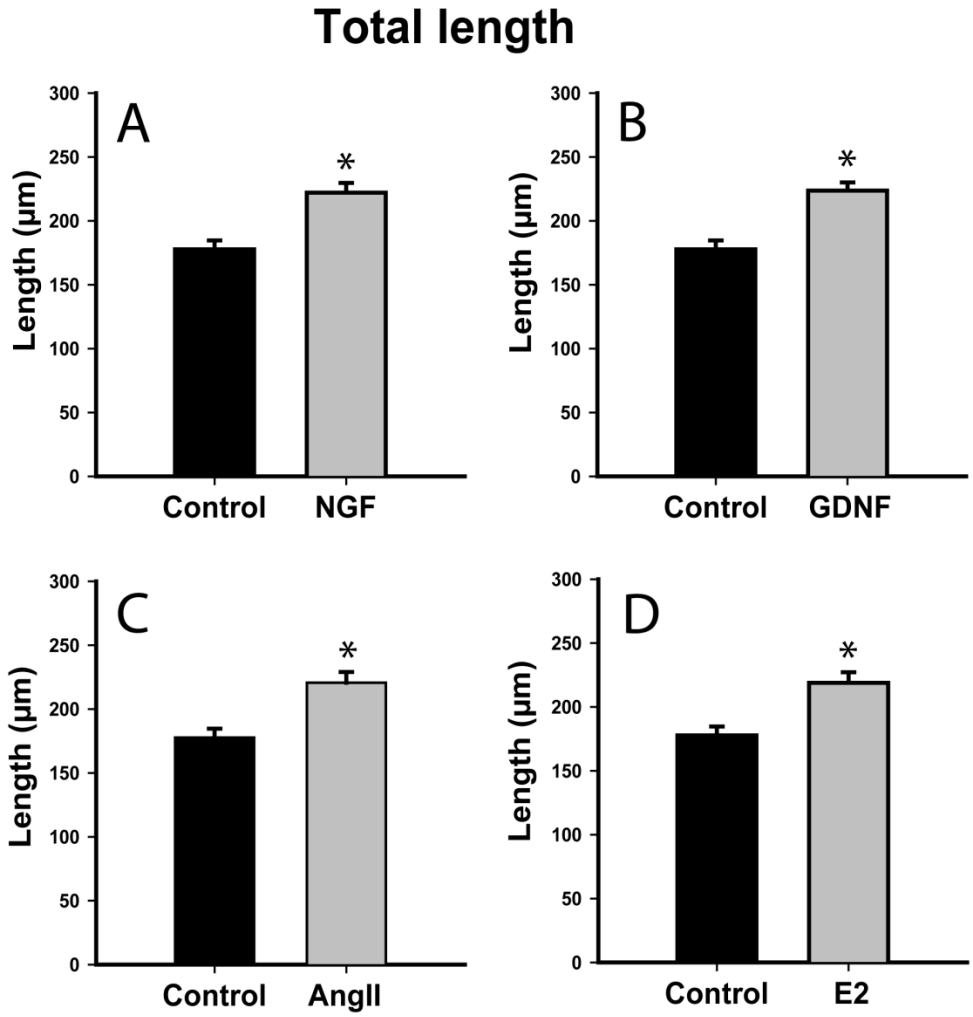

Total length
A
B
C
D
Length (µm)
300
250
200
150
100
50
0
*
Control
NGF
GDNF
AngII
E2

Figure 3. Both neurotrophic and hormonal factors increase total neurite length in differentiated 50B11 cells. Total length of all processes emerging from each differentiated neuron 20h after treatment were summed and compared to untreated controls. **A.** Treatment with 50 ng/ml Nerve Growth Factor (NGF) increased axon length. **B.** Treatment with 50 ng/ml Glial Derived Neurotrophic Factor (GDNF) also increased total outgrowth from 50B11 cells to an extent similar to NGF. **C.** Angiotensin II (AngII) at 100mM concentration increased total neurite length. **D.** Estrogen (E2) at a concentration of 20nM also increased total axon arbor length. *$P \leq 0.001$ vs. Control.

Figure 4.

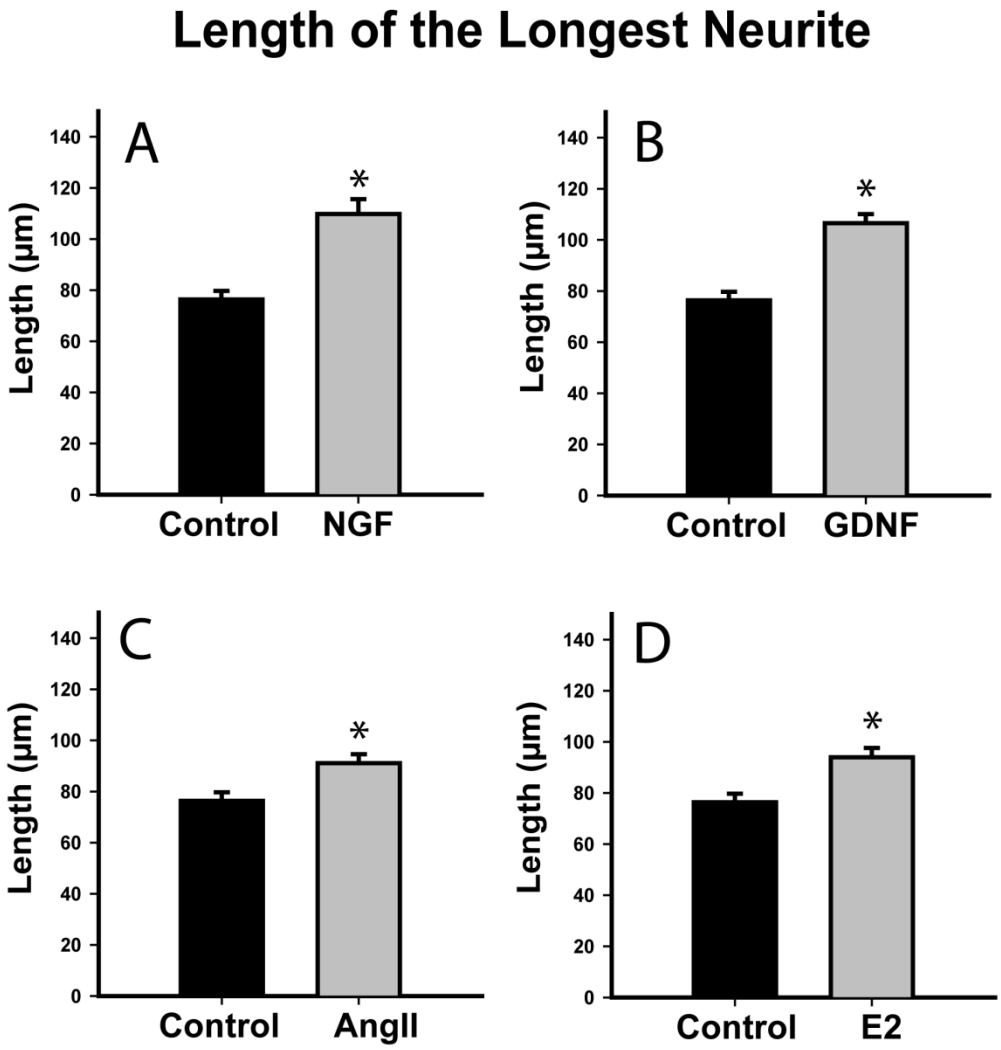

Length of the Longest Neurite
A
B
C
D
Length (µm)
0
20
40
60
80
100
120
140
*
Control
NGF
GDNF
AngII
E2

Figure 4. Neurotrophins and hormones increase length of the longest neurite in differentiated 50B11 cells. The length of the longest neurite from each cell was measured 20h after treatment. **A.** NGF (50 ng/ml) increased maximum neurite length. **B.** GDNF (50ng/ml) also increased axonal length. **C.** AngiotensinII (AngII, 100mM) increased maximum neurite length. **D.** Estrogen (E2, 20nM) induced significantly longer axons in treated neurons as compared to controls. *P≤0.001 vs. Control

Chapter 4. Mechanisms of Bone Morphogenetic Protein 4 mediated sensory axon plasticity

ABSTRACT

Bone Morphogenetic Protein 4 (BMP4) can induce axonal growth in adult peripheral sensory neurons of the dorsal root ganglia (DRG) not only following injury, but also under physiological conditions. Outgrowth is associated with phosphorylation and nuclear translocation of Smad1. We investigated the signaling mechanism by which BMP4 induces outgrowth. BMP4 can activate the helix-loop-helix transcription factor Inhibitor of DNA-2 (Id2) downstream of Smad1, which has been previously indicated in axonal growth-regulation in developing central nervous system (CNS). We found that BMP4 and also several other trophic factors upregulated Id2 in the 50B11 sensory neuronal cell line preceding onset of outgrowth. Overexpression of Id2 in 50B11 cells significantly increased neurite outgrowth even in basal media in absence of any trophic factors. Lentiviral knockdown of Id2 in dissociated DRG neurons decreased outgrowth significantly. However, Id2 knockdown failed to completely abrogate outgrowth induced by BMP4 indicating possible involvement of other BMP4-induced factors. We found that Methyl CpG binding Protein 2 (MeCP2), a methylated-DNA binding protein and a global transcriptional repressor, known to highly express in neurons, is downregulated by BMP4 in 50B11 cells. From separate studies, MeCP2 is also known to downregulate Id2 transcription. It is widely accepted that induction of outgrowth in a mature quiescent adult neuron is associated with transcriptional reactivation of a large number of factors otherwise dormant since development. We observed that MeCP2 is highly expressed in DRG neurons of adult rats both *in vitro* and *in vivo*. BMP4 downregulated MeCP2 expression levels in primary neurons when treated *in vitro*. When MeCP2 was knocked down by lentiviral shRNA delivery, neurite outgrowth in primary DRG culture increased dramatically. BMP4 treatment of MeCP2 knocked down neurons failed to elicit any further increase of outgrowth. Thus BMP4 potentially induces axonal growth in the adult sensory

neuron by downregulating MeCP2 to bring about a global transcriptional upregulation inducing multiple genes essential for rekindling outgrowth.

INTRODUCTION

In order to start growing its axons again, a mature adult neuron needs to reactivate specific transcriptional programs, much of which has stayed dormant since after development (Mechaly et al., 2006; Patodia and Raivich, 2012b, a). Such transcriptional rekindling can be achieved by inflicting a mechanical axonal injury. Peripheral axon injury activates a host of signaling molecules and several transcription factors (TFs). Activating transcription factor 3, AP1 family of transcription factors (c-Jun/Jun D), cAMP response element binding protein (CREB), STAT3 etc. are examples of few transcription factors that activate (phosphorylate) and translocate to the nucleus upon axotomy (Patodia and Raivich, 2012a). These TFs induce genes essential for axonal growth, membrane trafficking, cell adhesion, cytoskeletal dynamics etc. that are critical for elongation (Mechaly et al., 2006; Szpara et al., 2007).

However, in parts of the peripheral nervous system, spontaneous structural changes in axons continue to occur through adult life even in absence of any injury. Plasticity and axon growth is often driven by local functional requirements (eg. different parts of the female reproductive tract, mammary gland, vascular system etc.) or disease state (e.g., musculo-skeletal hyperinnervation in vitamin D deficiency, hyperinnervating pain syndromes, inflammatory pain etc.) in specific tissues or organs (Tympanidis et al., 2003; Blacklock et al., 2004; Blacklock and Smith, 2004; Ting et al., 2004; Chakrabarty et al., 2011; Tague et al., 2011). Although traumatic injury has been the most popular model to study axon growth in adult neurons, innervation changes in most human diseases ensue in undamaged axons, often under physiological

conditions. However, little is known about the cellular mechanisms that initiate outgrowth in intact peripheral axons.

It has only been recently known that Smad1 is a unique transcription factor that can modulate outgrowth not only following injury, but also in intact peripheral sensory axons under physiological conditions. Smad1 is activated by BMP4 and induces outgrowth in axotomized neurons *in vitro* and *in vivo* (Zou et al., 2009; Parikh et al., 2011). We recently found that BMP4 can induce outgrowth via Smad1 activation, in intact sensory axons. In the female reproductive tract, sensory axons sprouted with increase in BMP4 levels in a Smad1 dependent manner to increase innervation density in the vagina (Bhattacherjee et al., 2013). While knockdown of Smad1 abrogates outgrowth, it remains unknown what events in the nucleus, downstream of Smad1 activation, leads to outgrowth initiation (Zou et al., 2009).

In non-neural cells, Smad1 can induce Id (Inhibitor of DNA binding) family of transcription factors comprising of four members from Id1 through Id4 (Miyazawa et al., 2002). In the developing central nervous system, level of Id2 is very high in the maturing neuronal cell during its phase of active axon extension (Lasorella et al., 2006). Id2 is rapidly degraded as the axon reaches target and extension ceases (Jackson, 2006; Lasorella et al., 2006). Does Smad1 recapitulate this developmental phenomenon in the peripheral neurons to induce outgrowth via Id2 upregulation? Growing evidence suggests that Smad1 is capable of regulating a wide landscape of transcriptional networks by inducing multiple TFs downstream (Massague et al., 2005; Miyazono et al., 2010). Delicate balance of these regulatory networks is often involved in major decisions such as fate determination of differentiating cells (Massague et al., 2005). In this study we investigated the mechanism by which BMP4 induces sensory axon outgrowth downstream of Smad1 activation.

MATERIALS AND METHODS

All animal protocols and procedures were approved by the Kansas University Medical Center's Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Culture and treatment of 50B11 cells

50B11 cells were cultured in 24 well plates using previously described defined media () at about 50-60% density and differentiated with 75μm forskolin 24h after plating. At 17h following onset of differentiation cells were treated respectively with: 20nM 17β estradiol (Sigma-Aldrich), 100nM Angiotensin II (Sigma-Aldrich), 50ng/ml recombinant mouse Nerve Growth Factor (NGF, Peprotech), 50ng/ml recombinant mouse BMP4 (Peprotech). Cells were washed and harvested for RNA isolation at 6h and 12h time points.

RNA isolation and PCR

For RNA isolation from cells cultured in 24 well plates, media was removed and 500μl Trizol (Invitrogen) was added to each well and mixed by pipetting up and down few times. RNA was isolated from this lysate following the standard chloroform/ isopropanol extraction method. cDNA was synthesized from 1μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen) using random primers (Invitrogen), dNTP (Fermentas) according to the manufacturer's protocol. Quantitative RT-PCR was performed using iQ SYBR Supermix (BioRad). Primers used (5'-3'): Id2 - (Forward: AGAGACCTGGACAGAACC; Reverse: CGCATTTATTTAGCCACAGAG); GAPDH (Forward: CTCTACCCACGGCAAGTT; Reverse: CTCAGCACCAGCATCACC).

Immunoblots

To assess protein levels of Id2 and MeCP2, cell lysates from 50B11 cells and primary dissociated DRG cultures with relevant treatments or overexpression/knockdown were harvested in ice-cold RIPA lysis buffer. About 50-75µl of lysis buffer was used for each well of 24 well plate. About 15-40µg total protein is loaded per well and separated on a 12% or 4-12% polyacrylamide gel, transferred to a PVDF membrane and probed with anti-Id2 (rabbit polyclonal antibody, Sigma,1:500) or anti-MeCP2 (rabbit monoclonal antibody, Cell Signaling Tech, 1:1000) and reprobed for beta-actin (Sigma, 1:1000) or GAPDH (Millipore, 1:1000).

Cloning and Transfection

For Id2 overexpression, full length rat Id2 cDNA was cloned into the pcDNA3 (Invitrogen) expression vector. Rat trophoblast stem cells express high Id2 levels. Total RNA from trophoblast stem cell culture (a kind gift from Ms. Damayanti Chakraborty of Soares Lab at KUMC) was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) using manufacturer's protocol. Full length Id2 from this cDNA was PCR cloned into the pcDNA3 expression vector between the *BamHI* and *XbaI* restriction sites. Fast Digest enzymes (Fermentas) were used for restriction of the vector and insert and ligation was performed for 1h at room temperature using T4 DNA ligase (Fermentas). Competent *E. coli* (NEB Turbo® from New England Biolabs or XL10Gold from Agilent) were transformed with ligation mix, cultured at 37^{o}C for an hour (in SOC or NZY broth respectively) with shaking and plated on ampicillin plates (50-100µg/ml) for overnight incubation at 37^{o}C. Isolated colonies were picked and cultured next day and PCR screened. Minipreps were done for clones with inserts and DNA was sequenced using primers for the CMV promoter to confirm correct insertion and sequence of Id2.

The prepared plasmid was then transfected into 50B11 cells that had been split and plated day before, using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The control group was transfected with the empty pcDNA3 vector. Transfection media (Opti-MEM, Invitrogen with 5% FBS, Sigma) was replaced after 6-8h with complete 50B11 culture medium as described before. After 24h fresh medium was added to the cells with geneticin (Gibco, Life Technologies) selection at doses of 200, 400, 600 and 1000mg/ml. It takes 24-48 h for selection to occur. Cells with the highest compatible dose were selected (600mg/ml) and medium was changed with equivalent dose of the antibiotic and maintained. The selected cells were subsequently used in experiments with geneticin free defined 50B11 culture media.

Lentivirus production and transduction

Five to seven candidate shRNAs, commercially available from Sigma in the pLKO1 lentiviral vector backbone (from Addgene, with puromycin selection cassette) directed against mouse or human genes (Id2 or MeCP2) were first selected for each gene on the basis of 100% sequence homology with the rat counterparts. Using these lentiviral particles were synthesized 239T cells and 50B11 cells were infected with these as well as the scrambled shRNA control in the same vector. After puromycin selection, percent gene knockdowns were assessed at the RNA and protein levels. The shRNA with the highest efficiency of knockdown was selected for study. This shRNA was then cloned out by PCR from the pLKO1 vector along with its promoter and cloned into the FG12 lentiviral vector (Addgene) between *XbaI* and *PacI* restriction sites. The vector carries an EGFP reporter under a ubiquitin-C promoter. Third generation lentiviral packaging with pMDLg/RRE, pRSV-Rev, and pMD2.G (Addgene) was used to synthesize lentiviral particles for BMP4 and EGFP-only vectors. 293T cells were plated in 6 well plates at 60-80% confluence and transfected with the vector carrying the gene of interest or EGFP alone

and all the 3 packaging plasmids using Lipofectamine 2000 (Invitrogen, Life Technologies) in Opti-MEM medium (Gibco, Life Technologies). Medium was changed 12h later with OptiMEM-5%FBS and supernatant collected every 24h for 2-3 days. This supernatant was used for transducing primary neuronal cultures plated in 24 well plates with 80µl per well in 500µl medium. If necessary, viral particles were concentrated from the supernatant by ultracentrifugation in sealed tubes for 2h at 35000 rpm and resuspended in sterile PBS. Lentiviral titers were measured using the Lentivirus qPCR titer kit (Applied Biological Materials).

Primary neuronal culture

Lumbosacral DRGs from 5 to 6-week-old female rats were harvested and dissociated following previously described protocols. Briefly, the ganglia were isolated and placed in ice-cold L15 medium containing 3% glucose. They were chopped into pieces and incubated at 37^{o}C in 1% collagenase (Sigma-Aldrich) and 2.4u dispase (Worthington) in HBSS (Sigma-Aldrich). After 40mins, enzyme was removed and DRG chunks were neutralized with L-15/10% FBS and triturated using fire-polished glass pipettes to complete dissociation. The suspension was passed through 100µm nylon strainer (BD Biosciences) and then centrifuged through a percoll (Sigma-Aldrich) column to remove undissociated chunks and myelin debris. The pellet was washed in L15-FBS to remove any percoll and resuspended in complete Neurobasal A medium (Gibco) supplemented with B27 (Gibco), glutamine (Sigma-Aldrich), Primocin (Invivogen) and 10% FBS as described before. Neurons were plated at a low density onto laminin/poly-D lysine pre-coated 24 well plates (BD Biosciences) in 500µl media per well. Neurons to be transduced with lentivirus were directly plated with polybrene (Sigma-Aldrich) and viral supernatant was added after 1h. After 24h medium was changed to serum-free Neurobasal A containing glutamine and B27, now supplemented with uridine and fluorodeoxy-uridine (Sigma-Aldrich) to inhibit glial

proliferation (1ml medium/well). In treatment groups, 50 ng/ml mouse recombinant BMP4 (Peprotech) was added during this medium change at 24h. After 48h from plating, cultures were fixed with 4% formaldehyde and washed with PBS. Cultures were immunostained for markers like tubulin βIII, peripherin, GFP.

To quantify neurite outgrowth, about 80-100 EGFP positive isolated neurons were randomly imaged from 3-4 wells per treatment group using a 10X objective. Total length of outgrowth per neuron was quantified by tracing using the NeuronJ application module of NIH ImageJ.

Immunohistochemistry

Dissociated primary neuronal cultures and 50B11 cells were fixed with 4% PFA at the end of culture for 20mins. Cells were then washed with PBS blocked in host serum and subjected to primary antibody incubation overnight (further details below). For staining of the dorsal root ganglia animals were euthanized by thoracotomy under isofluorane anesthesia and lumbo-sacral dorsal root ganglia (DRG) harvested and fixed by overnight immersion in 4% PFA at 4˚C and cryosectioned at 14 μm as described previously (Chapter 2).

Cultures and sections were blocked in 5% primary host serum with 1% BSA in PBST and immunostained overnight for peripherin (1:400, rabbit antiserum; 1:500, chicken antiserum, Millipore), Tubulin βIII (1:400, mouse antiserum, Chemicon/Millipore), calcitonin gene-related peptide (CGRP,1:400, sheep antiserum, Biomol), Enhanced Green Fluorescent Protein (EGFP, 1:750, chicken antiserum, Aves), Methyl CpG binding Protein 2 (MeCP2, 1:200, rabbit monoclonal antibody, Cell Signaling Technologies), Id2 (1:200, rabbit antiserum, Sigma-Aldrich). Secondary antibodies included donkey anti-rabbit Cy3 at 1:200 (Jackson Immuno-

Research), donkey anti-mouse Cy3 (1:200, Jackson Immuno-Research), donkey anti-rabbit Alexa 488 (1:750, Invitrogen), donkey anti-rabbit Alexa 647 (1:500, Invitrogen), donkey anti-chicken Dylight 488 (1:750 Invitrogen), donkey anti-goat Cy3 (1:200 Jackson Immuno-Research), and donkey anti-sheep Cy3 (1:200 Jackson Immuno-Research). Antibodies have been previously characterized by our laboratory and others (Abir et al., 2008; Clarke et al., 2010; Tague et al., 2011; Tague and Smith, 2011) and include omissions of primary and secondary antibodies.

Statistical analysis

All values are presented as mean ± SEM. Comparisons between groups were made using Student's t-test, one-way ANOVA, or two way ANOVA with post-hoc comparisons by Student Newman-Kuels tests for normally distributed data, and Mann-Whitney rank sum test for non-parametric analysis. Paired t-tests were used to compare innervation densities in injected and non-injected sites within the same animals in the lentiviral studies. Differences were considered significant at $P \leq 0.05$.

RESULTS

Multiple growth promoting factors induce transient Id2 upregulation in 50B11 cells

50B11 cells are a neuronal cell line generated from 14.5d embryonic rat DRG neurons (Chen et al., 2007a). We and others (Chen et al., 2007a; Melli and Hoke, 2009) have previously shown that multiple factors, including both canonical and non-canonical neurotrophic factors, induce increased neurite outgrowth in these cells. The responses closely represent that of primary DRG neurons *in vitro*. We treated differentiated 50B11 cells with BMP4 to see whether it

induced upregulation of Id2. Cells were also treated with a set of other growth factors known to promote outgrowth, to investigate potential association of Id2 with neurite growth. Total RNAs were isolated from control and different treatment groups 6h following treatment and Id2 expression was assessed by qRT-PCR. Treatment of 50B11 cells with BMP4 induced upregulation of Id2 levels by about 3 folds compared to control (Fig.1). Other growth promoters like estrogen (E2), angiotensin II (AngII) and nerve growth factor (NGF) induced increase in relative Id2 levels by almost 2 fold (Fig.1). These indicate a potential role of Id2 in neurite growth, requiring transient upregulation of the transcription factor at the time outgrowth initiation.

Overexpression of Id2 in 50B11 cells induced increased neurite outgrowth

To determine the effect of Id2 on neurite outgrowth and neurite length, we overexpressed Id2 in 50B11 cells. Full length rat Id2 cDNA was reverse transcribed from rat trophoblast stem cell (known to express high Id2 levels) total RNA and PCR cloned into the pcDNA3 expression vector (Fig. 2A). In pcDNA3 gene expression is driven by the CMV promoter and the vector contains a neomycin/geneticin selection cassette. 50B11 cells were transfected either with pcDNA3 containing the full length Id2 cDNA or with the empty vector for control and were both selected at the similar dose (1mg/ml) of geneticin (starting at 48h from transfection; selection was started at 400μg/ml and then gradually increased). Elevated expression of Id2 was observed in 50B11 cells transfected with the cDNA (~5 fold mRNA increase, data not shown) compared to the empty pcDNA3 transfection (Fig. 2B). Upon forskolin (a plant derived diterpene compound that is a potent inducer of adenylyl cyclase that can increase intracellular levels of cAMP) induced differentiation, the Id2 overexpressing cells extended longer neurites than the control cells (Fig. 2C). The basal total length of outgrowth per neuron increased significantly in

presence of high Id2. BMP4 at 50ng/ml induced significantly elevated outgrowth in control cells (Fig. 2D). In Id2-overexpressing cells total outgrowth was not elevated any further with BMP4 treatment (Fig. 2D).

Knockdown of Id2 in primary DRG neuron *in vitro* downregulated neurite outgrowth

To determine the necessity of Id2 in outgrowth we then performed shRNA mediated Id2 gene knockdown in primary DRG neurons *in vitro* using lentiviral delivery. Candidate shRNAs were screened to find the highest efficiency of knockdown. Screening was performed in the 50B11 cells with delivery of the shRNA in the pLKO1 vector backbone with puromycin selection.

A shRNA with 88% efficiency of knockdown was selected (Fig. 3B). This sequence was cloned into the FG12 lentiviral vector backbone which expresses EGFP but has no antibiotic selection (Fig. 3A). Primary DRG neuronal cultures prepared from young adult rats (about 40d old) were infected with lentivirus carrying expression of either scrambled shRNA for control or shRNA for Id2 knockdown at the time of plating (Fig. 4A-F). Culture medium was changed to fresh basal media 24h after plating. Cells were fixed after another 24h (i.e. 48h from plating) and stained for tubulin-βIII. About 80-100 EGFP expressing neurons per group were imaged randomly from 3 replicate wells and length of total outgrowth was estimated by tracing using the NeuronJ application module of the NIH ImageJ program (Fig. 4 A-F). Results showed that downregulation of Id2 induced a significant reduction of basal outgrowth in DRG neurons in culture (Fig. 4G).

Id2 knockdown failed to abrogate BMP4 induced increase in neuronal outgrowth

To validate the hypothesis that BMP4 induced outgrowth in DRG neurons is mediated by Id2, we treated Id2-knockdown neurons with BMP4. A similar protocol for culture, virus infection and fixation was followed as above. Treatment was given at the time of medium change, 24h after plating and virus infection. In control (with scrambled shRNA) BMP4 induced significant increase of total neurite outgrowth per neuron. In the Id2 knockdown group BMP4 also induced a significant increase of neurite outgrowth, which is slightly lower than the effect on the scrambled group, but is not significantly different (Fig. 5).

DRG neurons show high levels of MeCP2 expression

Our findings so far indicate that BMP4 induces increase in Id2 expression as well as increased neurite outgrowth in sensory neurons. While Id2 may contribute to BMP4 mediated outgrowth, it appears that there could be additional factors induced downstream of Smad1 that are required for successful outgrowth and can compensate for the absence of Id2. Furthermore, when activated, Smad1 can induce multiple factors as observed in other cells (rather than functioning through a single transcription factor) (Massague et al., 2005).

Methyl CpG-binding Protein2 (MeCP2) is a DNA binding protein that can bind to methylated DNA, usually found heavily in gene promoters and act as a structural and function modifier of chromatin (Meehan et al., 1992; Nan et al., 1993; Becker et al., 2013). In association with other co-repressors, MeCP2 can act as a global repressor affecting expression of multiple genes at the same time (Nan et al., 1997; Nan et al., 1998a; Chandler et al., 1999). Recently, MeCP2 has been shown to regulate expression of all members of the Id family of genes in CNS, including Id2 (Peddada et al., 2006). Also, it is observed, that cells receiving high levels of

BMP4 signals, like embryonic stem cells, show relatively low expression of MeCP2. Expression of MeCP2 is very high in neurons of the adult CNS (Kaufmann et al., 2005). To investigate whether MeCP2 could be a potential downstream effector of BMP4 signaling, we first analyzed expression of the protein in sensory neurons of the DRG.

Lumbar DRGs of the L4-L6 levels from adult rats aged between 40-60 days were isolated. The ganglia were fixed overnight in 4% PFA at 4^{o}C and sectioned and stained them for MeCP2. Images showed very strong nuclear expression of MeCP2 in the DRG neurons (Fig. 6A, D). Expression could also be detected in some non-neuronal cells of glial and vascular lineage but at significantly lower levels (Fig. 6A, D). The sections were stained for peripherin, to localize the small diameter neurons, a subpopulation affected most strongly by BMP4/Smad1 signaling in reproductive tract (Fig. 6B). All peripherin labeled cells show strong expression of MeCP2 (Fig. 6B,E,F). High MeCP2 expression was observed also in dissociated DRG neurons in culture. Peripherin positive neurons that express CGRP (subpopulation affected in vaginal plasticity) retained high levels of MeCP2 *in vitro* (Fig. 6G-J). Expression levels were low in non-neuronal cells (Fig. 6I, J).

BMP4 can regulate MeCP2 expression in peripheral sensory neurons

MeCP2 is a global transcriptional repressor in neurons (Nan et al., 1997). Downregualtion of MeCP2 can therefore induce a global upregulation of gene expression, simultaneously turning on multiple genes (a phenomenon commonly observed preceding onset of axon regrowth) (Costigan et al., 2002). We tested whether BMP4 can regulate expression of MeCP2.

First, 50B11 cells were treated with 50ng/ml BMP4 17h after differentiation as described before and cell lysates were collected at 6h and 12h time points for mRNA isolation. Comparisons with qPCR showed that BMP4 treatment induced progressive decline in MeCP2 mRNA levels at 6 and 12h (Fig. 7A). We then treated dissociated primary DRG cultures with BMP4 in a similar fashion and harvested total protein at 12h time point. BMP4 downregulated the expression of MeCP2 (Fig. 7B).

MeCP2 knockdown in primary sensory neurons increased neurite outgrowth

We next asked whether change in levels of MeCP2 can affect outgrowth in sensory neurons. We tested whether MeCP2 knockdown impacts outgrowth in dissociated DRG neurons. A similar strategy was adopted as described before. Lentiviral shRNAs in the pLKO1 vector backbone were screened in 50B11 cells and one with 85% knockdown was selected and cloned into the EGFP expressing FG12 backbone. Dissociated neuronal cultures were plated with polybrene and an hour later infected with lentiviral supernatants carrying shRNA for scrambled control and MeCP2 knockdown respectively in two groups. At 48h after plating cells were fixed and stained for tubulin-βIII. Immunostaining for MeCP2 showed a marked reduction in the levels of thee protein in the EGFP stained cells containing knockdown, compared to adjacent uninfected neurons (Fig. 8). Neurite counting in EGFP expressing cells showed that MeCP2 knockdown significantly decreased total outgrowth per neuron (Fig. 9A-C).

BMP4 treatment failed to induce further upregulation of neurite outgrowth in MeCP2 knocked down neurons

BMP4 treatment induced significant increase of outgrowth in the scrambled shRNA transduced neurons (Fig. 9C). However, treating MeCP2 knocked down cells with BMP4

produced no further increase of outgrowth compared to the untreated knocked down cells (Fig. 9C).

DISCUSSION

In this study we described a potential mechanism by which BMP4 can induce axonal outgrowth in adult sensory neurons. By phosphorylating and activating the Smad1 transcription factor, BMP4 can elicit outgrowth not only under injury but also in uninjured axons under physiological conditions *in vivo*. Id2 is one of the downstream targets of pSmad1 (Miyazawa et al., 2002; Massague et al., 2005). We found that BMP4 substantially increases Id2 levels in the DRG neuronal cell line 50B11. Not only so, multiple other axon growth-promoting factors transiently increased Id2 levels.

It is widely believed that regeneration recapitulates development, at least partially (Patodia and Raivich, 2012a). In the developing hippocampus or cerebral cortex, as a neuroblast matures into a neuron, escalating levels of Id2 induce vigorous axon extension. As maturation & target innervation is completed, growth ceases, and Id2 is rapidly degraded (Jackson, 2006; Lasorella et al., 2006). Id2 is a member of the helix-loop-helix (HLH) family of transcription factors which unlike the basic-HLH (b-HLH) transcription factors lack the basic domain (Powell and Jarman, 2008). Id2 can heterodimerize competitively with ubiquitously expressed bHLH TFs like E47 or E12, inhibiting the more tissue specific bHLH TFs (Aronheim et al., 1993; Powell and Jarman, 2008). In the mature neuron, heterodimers of pro-neural bHLH TFs (like NeuroD (s), neurogenins, Mash (s), etc.) and the non-tissue-specific bHLHs (like E47) bind to E-box elements of gene promoters of pro-neural factors and axon growth inhibitory factors that cumulatively induce maturation and resist further axon growth (Kabos et al., 2002; Jackson,

2006). But if Id2 levels are increased, it competes with bHLHs to bind and sequester E47. The resulting heterodimer fails to bind DNA and pro-neural bHLHs are ubiquitinylated and degraded (Iavarone and Lasorella, 2006; Powell and Jarman, 2008). This can potentially revert the neuron to a development-like state, thus activating outgrowth. This possibility is supported by the finding that overexpression of Id2 in 50B11 cells enhanced neurite outgrowth under basal conditions that was comparable to growth induced by trophic factor treatments, particularly BMP4. Furthermore, the downregulation of outgrowth in primary neurons with Id2 knockdown confirmed a role of the protein in adult axon outgrowth.

The 50B11 cells provided a great deal of strength and convenience in the present study. It helped fulfill a serious dearth of cell lines for neurons, especially in the PNS. These cells not only retain most essential properties of the DRG neurons, but also more than 70-80% cells in culture differentiate to form neurons. This provided a much more enriched model to study neuronal expression of proteins like Id2 that are substantially present in most non-neuronal cells which comprise the majority population in a primary DRG culture. Furthermore, the cells provided an excellent tool to test and validate genetic modifications specifically in rat sensory neuron, especially in shRNA screening, which was hitherto unimaginable.

The findings that BMP4 increases Id2 levels in sensory neurons and Id2 can activate neurite growth, led us to hypothesize that BMP4 may induce outgrowth via Id2. However, while knockdown of Id2 inhibited outgrowth, it failed to fully abrogate BMP4's effect. This raises the possibility that BMP4 may act further upstream in signaling cascade engaging multiple factors (and possibly few redundant ones) to bring about its effect cumulatively. Over nearly past two decades several studies have discovered multiple proteins and TFs to have critical regulatory impact on regenerative outgrowth following axon injury (Mechaly et al., 2006; Szpara et al.,

2007). Significant contribution of TFs like ATF3, AP1s, CREB, STAT3 etc. towards successful regeneration have been well documented by both *in vitro* and *in vivo* evidences (Patodia and Raivich, 2012a). Genetic manipulations of these proteins have significantly downregulated growth following injury (Patodia and Raivich, 2012a). Thus axon growth *in vivo* is a multi-factorial event driven by the combined effect of all these different factors required to activate several critical processes including protein synthesis & transport, cytoskeletal dynamics and cell adhesion.

It has been unclear whether similar signaling intermediates and TFs are involved in spontaneous outgrowth when there is no injury. Several active components generated at an injury site like cytokines, chemokines, growth factors etc. are essential to trigger the signaling intermediates of regenerative growth (Makwana and Raivich, 2005). However, what drives such multifactorial gene regulation in absence of the inflammatory cocktail has remained a baffling question. Downregulation of MeCP2 through Smad1 signaling offers a competent explanation. As a global regulator of gene expression, MeCP2 has strong influence on the neuronal transcriptome (Nan et al., 1997; Kaufmann et al., 2005; Guy et al., 2011). Discovered first as a methyl binding protein, MeCP2 is now well established as a strong regulator of neuronal gene expression (Meehan et al., 1992; Chandler et al., 1999; Kaufmann et al., 2005; Guy et al., 2011). Methylation of cytosine in the CpG dinucleotides is highly common in promoter regions of genes (Deaton and Bird, 2011). Binding to these methylated promoter regions MeCP2 helps recruit additional corepressors and Histone Deacetylases (HDACs), all of which repress gene expression (Nan et al., 1998b; Kaufmann et al., 2005). We found MeCP2 is extremely abundant in the sensory neuronal nuclei (like that of most CNS neurons) and thus a downregulation of MeCP2 may remove repression and lead to a global gene activation. More recent studies show that the

high neuronal expression levels of MeCP2 almost equal a 1:1 ratio with histone octamers (Skene et al., 2010). This indicates a more generalized role of the protein in chromatin packaging (and global chromatin state) rather than methyl binding alone, and hence a stronger implication in global gene regulation. Although there are some candidate genes identified as targets of MeCP2 regulation like FKBP5, sgk1 and sult1a1, the evolving consensus from findings in developing brain indicates that MeCP2 acts on a genome wide scale (often in activation-dependent manner) (Skene et al., 2010; Della Ragione et al., 2012; Tochiki et al., 2012). Whether in the sensory neuron MeCP2 downregulation invokes specific groups of genes to activate growth remains a question for future investigation using global gene expression analysis in MeCP2 knockdown.

It is unknown whether BMP4 can regulate MeCP2 in non-neuronal cells. However, the phenotypic/functional outcomes of BMP4 treatment have dramatic cell fate-determining effects on several cell types (eg. stem cells, ES cells, bone cells, bone marrow progenitors etc.), which is associated with a global regulation of gene expression state. Thus, our findings open up the possibility of a new mechanism of downstream signaling from BMP4. Several questions including localization of BMP response elements (Smad binding regions) in MeCP2 promoter as evidence of direct vs. indirect regulation remain to be explored.

MeCP2 is abundantly expressed in almost all neurons of brain. Expression is low in the embryo, but progressively increases in the neonate, reaching a final maximum in adult (Gonzales and LaSalle, 2010; Guy et al., 2011). Ever since the discovery of MeCP2 mutation as the cause of Rett's Syndrome (an Autism Spectrum Disorder), a huge wealth of literature has accumulated on role of the protein in neurons of the developing as well as adult brain (Kaufmann et al., 2005). However, little is known about its expression or role in the PNS. While we found strong expression of the protein in DRG neurons and its critical role in outgrowth, very recent reports

also indicate a functional role in pain perception. In states of inflammatory pain induced by planter CFA injection, levels of MeCP2 decreased in both the spinal cord superficial horn and the DRG (Tochiki et al., 2012). Expression also decreased in DRG with a spared nerve injury (Tochiki et al., 2012). Since axotomy can initiate inflammation, is unclear whether axotomy or inflammation or both can cause MeCP2 downregulation. Furthermore, hyperinnervation at inflammation site has also been implicated as a cause for hyperalgesia. The downregulation of MeCP2 might also be possibly induced by axonal sprouting at the inflammation site. Further studies using knockout animal models should help find answers for many such questions.

In humans, mutations of MeCP2 leads to Rett's syndrome, an intellectual disability disorder categorized under the autism spectrum disorders (ASDs). Babies develop normally until about 18months of age, beyond which mental and locomotor disabilities ensue concurrently with increasing levels and activity of MeCP2 in brain (Kaufmann et al., 2005). However, much has been speculated about impact on peripheral neurons of these individuals. There is controversy about the validity of some earlier reports of altered sensitivity and pain perception due to less stringent data collection criteria. Intellectual disabilities of the subjects present a major challenge in effective data collection, which is often reliant on the care givers. In a recent study that carefully avoided most of these pitfalls, it was revealed that there increased threshold and delayed response to pain stimuli in these patients. Both delayed and decreased response to injection, fall, trauma, burns was reported (Downs et al., 2010). Thus the findings of this study indicate that significant epigenetic regulations continue to occur in the adult sensory neuron and further research is needed for a complete understanding of its role in maintenance of the structure and function of the peripheral nerve.

FIGURES

Figure 1.

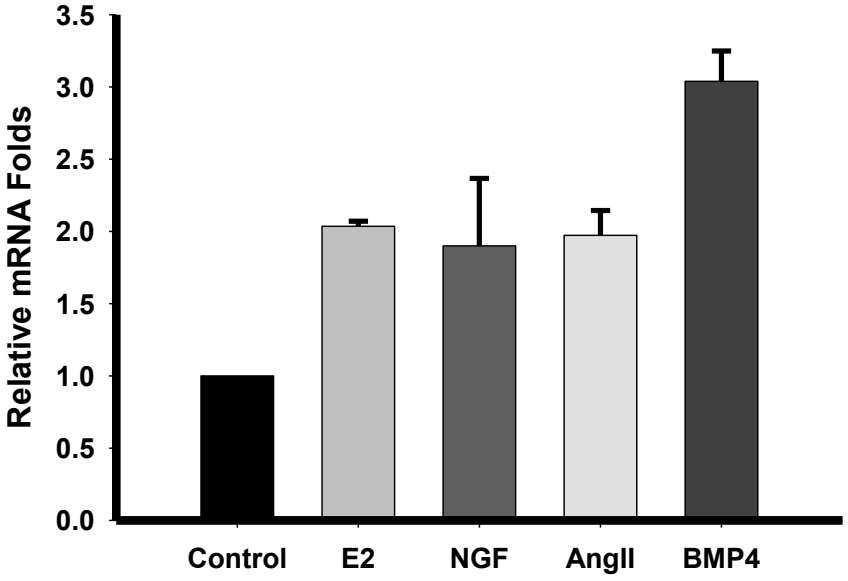
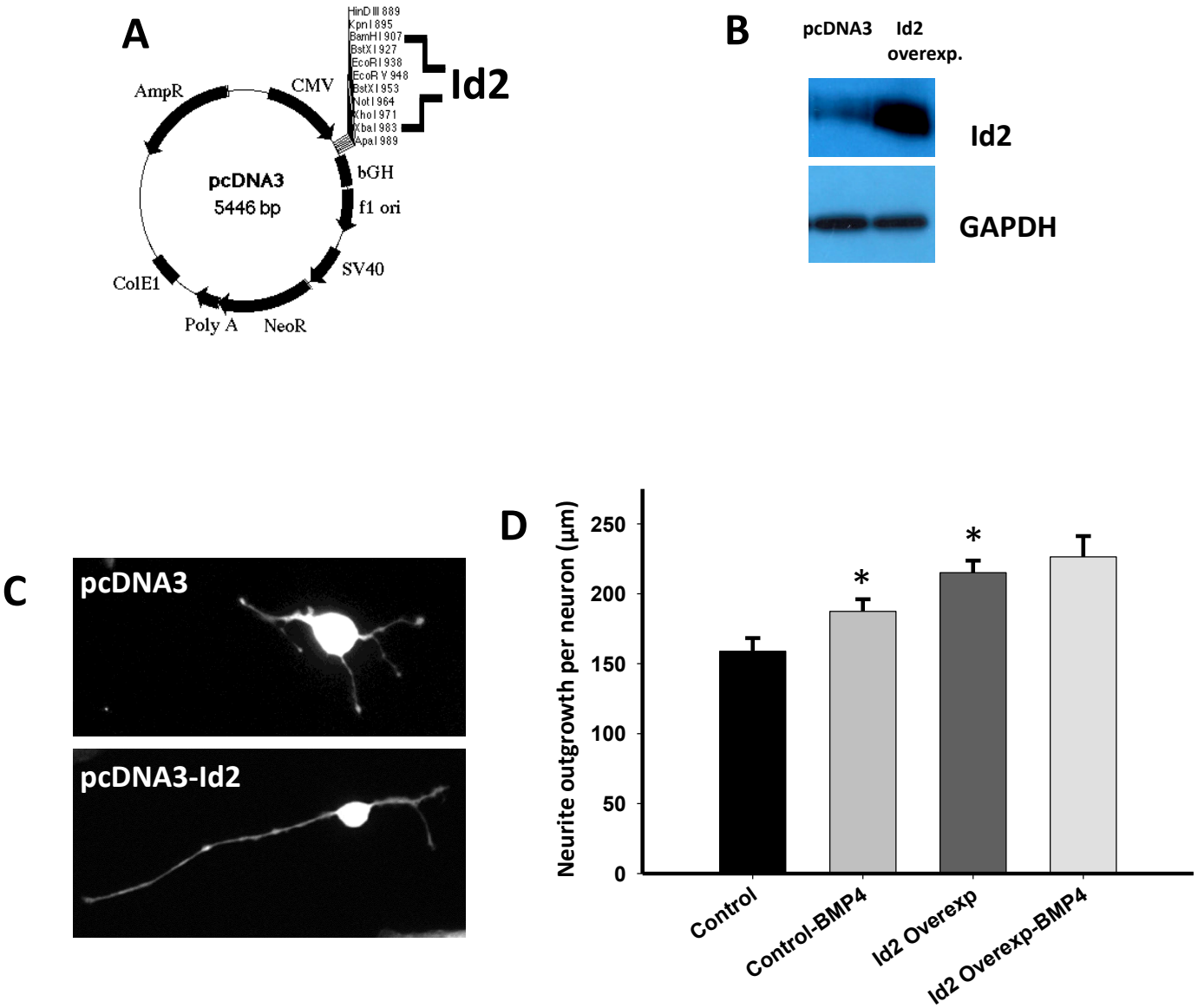


Figure 1. Multiple growth factors induce Id2 upregulation in 50B11 cells. Relative expression of Id2 mRNA collected 6h after treatment of differentiated 50B11 cells with estrogen (E2, at 20nM), nerve growth factor (NGF, at 50ng/ml), angiotensin II (AngII, at 100mM) and bone morphogenetic protein 4 (BMP4, at 50ng/ml).

Figure 2.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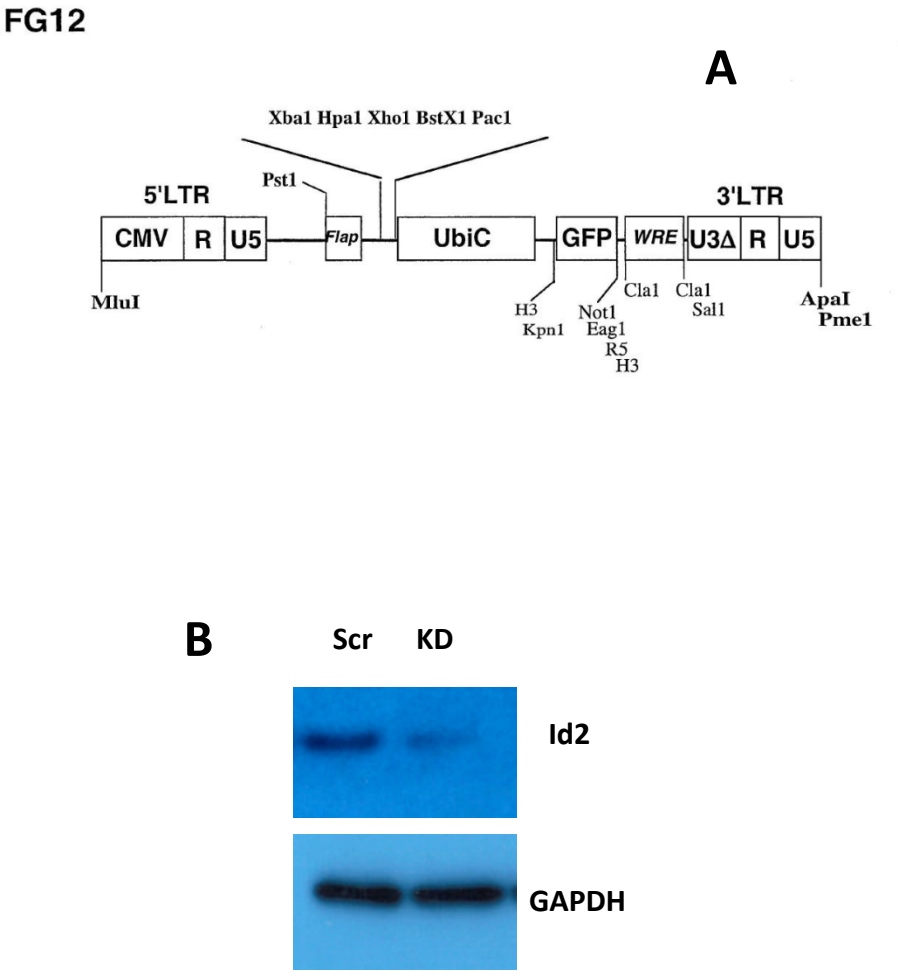
Kpn I 895
BamH I 907
BstX I 927
EcoR I 938
EcoR V 948
BstX I 953
Not I 964
Xho I 971
Xba I 983
Apa I 989
Id2
AmpR
CMV
pcDNA3
5446 bp
bGH
f1 ori
SV40
ColE1
Poly A
NeoR
B
pcDNA3
Id2 overexp.
Id2
GAPDH
C
pcDNA3
pcDNA3-Id2
D
Neurite outgrowth per neuron (µm)
0
50
100
150
200
250
*
*
Control
Control-BMP4
Id2 Overexp
Id2 Overexp-BMP4

Figure 2. Id2 overexpression induced increased neurite outgrowth in 50B11 cells. **A.** Full length Id2 cDNA was expressed in the pcDNA3 mammalian expression vector between the BamHI and XbaI restriction sites. **B.** Immunoblot showing Id2 overexpression in 50B11 cells transfected with vector in A. GAPDH was used as an internal control. (RNA level showed 5 fold increase) **C.** Differentiated 50B11 cells containing pcDNA3-Id2 overexpression showed increased neurite outgrowth than cells transfected with empty pcDNA3. **D.** Quantification shows that Id2 overexpression increased total neurite outgrowth per neuron significantly compared to untreated as well as BMP4 treated control cells that are transfected with empty pcDNA3. (*$p<0.05$).

Figure 3.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Pst1
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3'LTR
CMV
R
U5
Flap
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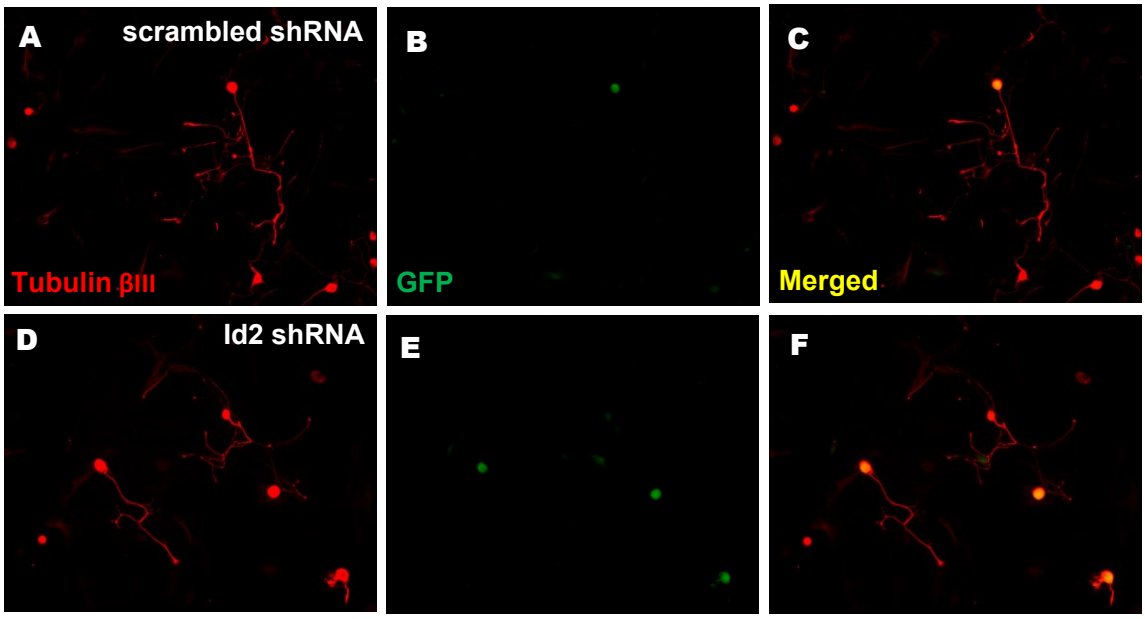
WRE
U3Δ
R
U5
MluI
H3
Kpn1
Not1
Eag1
R5
H3
Cla1
Cla1
Sal1
ApaI
Pme1
B
Scr
KD
Id2
GAPDH

Figure 3. Id2 knockdown in using lentiviral vector. **A.** The FG12 lentiviral vector. The shRNA against Id2 along with a CMV promoter was cloned between the XbaI and PacI sites. **B.** Lentiviral transduction of the Id2 shRNA induced a strong knockdown of Id2 in 50B11 cells. GAPDH was used as the internal control.

Figure 4.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Tubulin βIII
B
GFP
C
Merged
D
Id2 shRNA
E
F

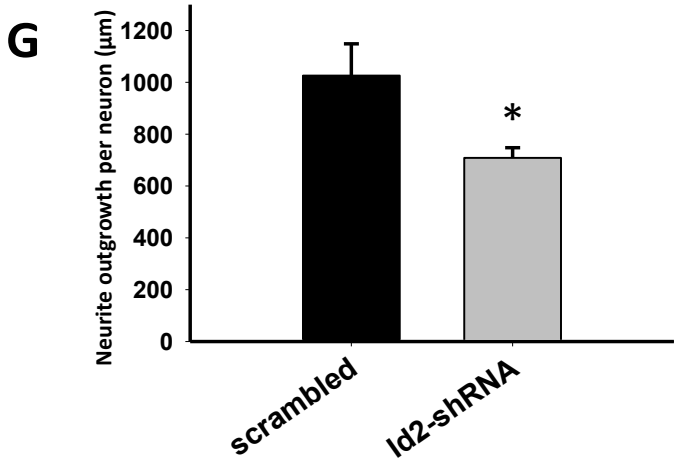

G
Neurite outgrowth per neuron (µm)
1200
1000
800
600
400
200
0
*
scrambled
Id2-shRNA

Figure 4. Id2 knockdown downregulated neurite outgrowth in primary DRG neurons. **A.** TubulinβIII staining in a control neuron transfected with scrambled shRNA. **B.** GFP labeling in the same neuron in A confirms viral transduction. **C.** A merge of A and B. **D.** TubulinβIII staining in a control neuron transfected with shRNA for Id2 knockdown. **E.** GFP labeling in the same neuron in D confirms viral transduction. **F.** Merge of D and E.**G.** Estimation of total neurite length per neuron showed that Id2 knock down significantly downregulated neurite outgrowth. (*$p<0.05$)

Figure 5.

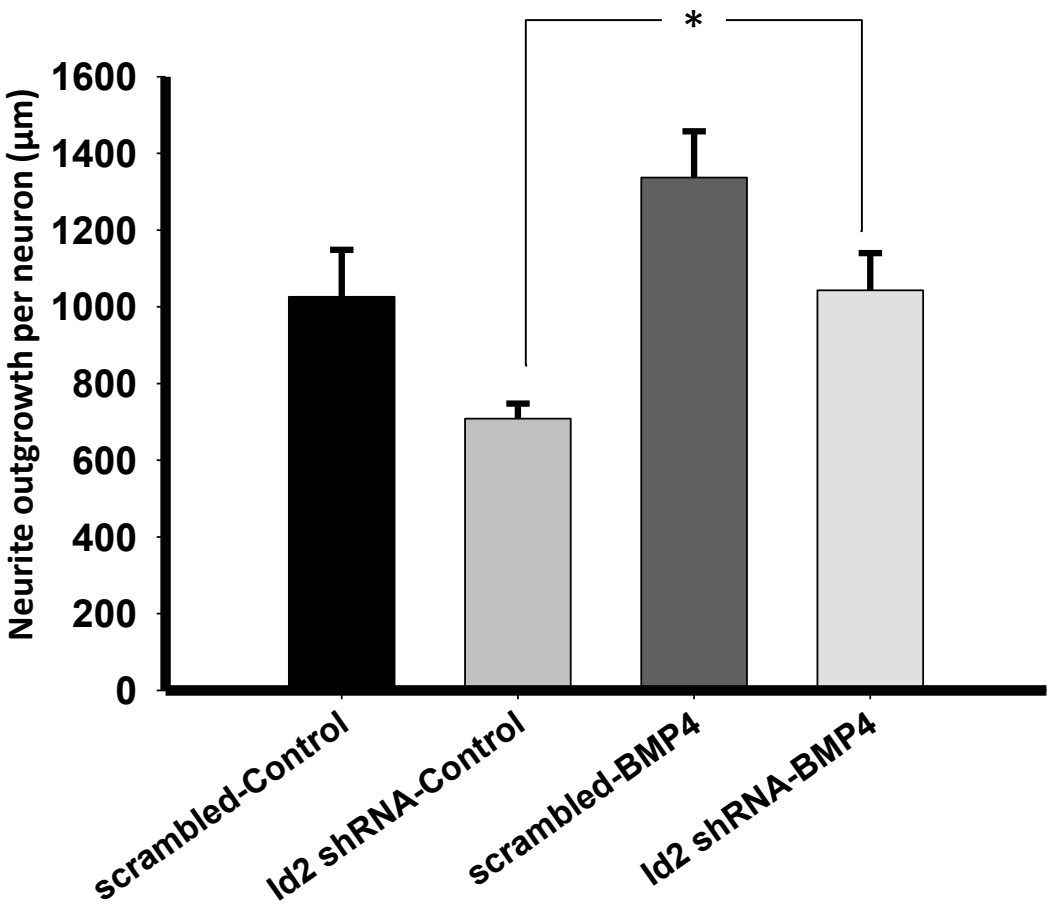

*
Neurite outgrowth per neuron (µm)
1600
1400
1200
1000
800
600
400
200
0
scrambled-Control
Id2 shRNA-Control
scrambled-BMP4
Id2 shRNA-BMP4

Figure 5. Quantification of neurite outgrowth in Id2 knock down primary DRG neurons *in vitro* following BMP4 treatment. In untreated groups, total neurite outgrowth per neuron was lower in the Id2 knock down cells compared to the scrambled shRNA transduced cells. After 24h following 50ng/ml BMP4 treatment, outgrowth significantly increased in the Id2 knock down neurons (*$p<0.05$).

Figure 7.

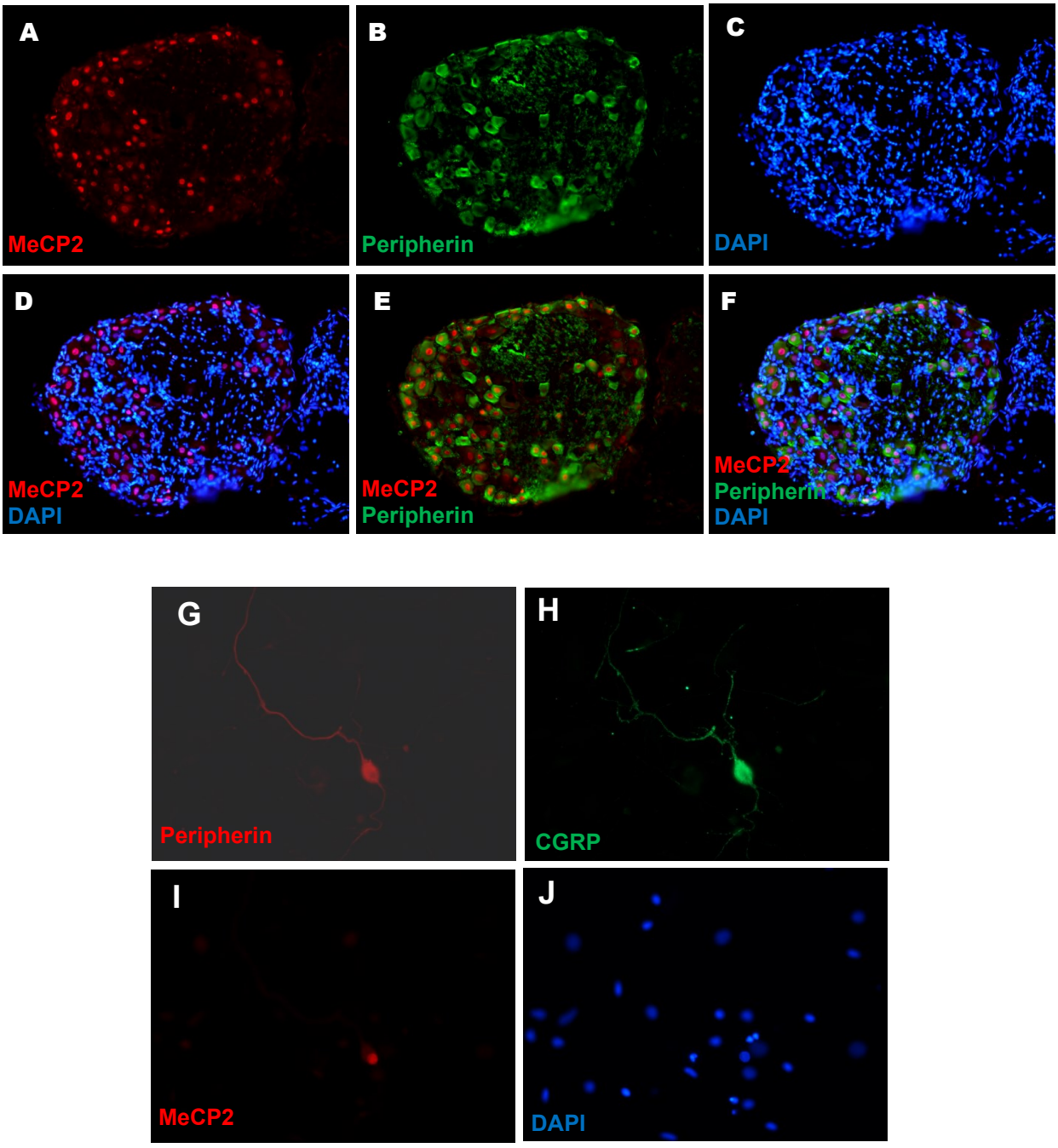
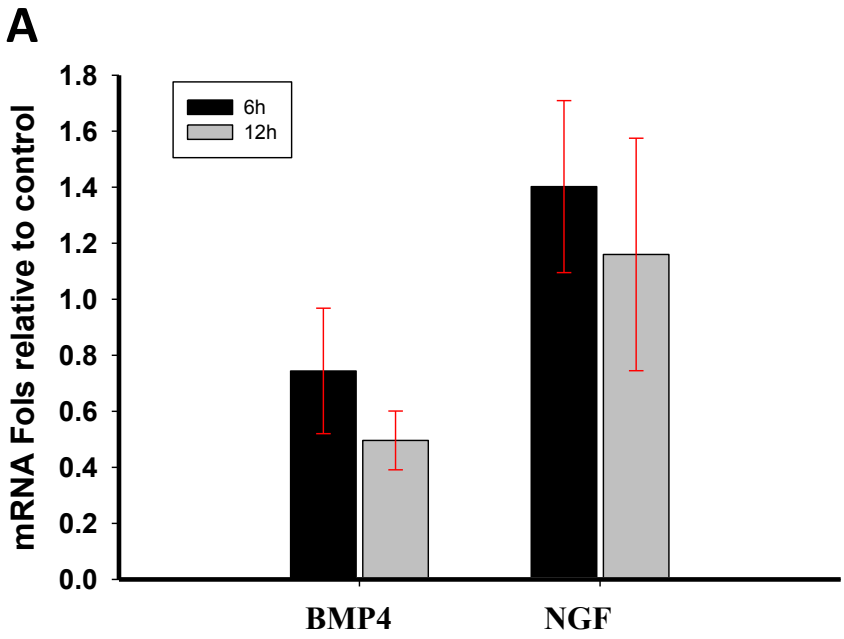

A
MeCP2
B
Peripherin
C
DAPI
D
MeCP2
DAPI
E
MeCP2
Peripherin
F
MeCP2
Peripherin
DAPI
G
Peripherin
H
CGRP
I
MeCP2
J
DAPI

Figure 6. MeCP2 is strongly expressed in adult DRG neurons. **A.** MeCP2 labeling in cross section of a L6 DRG. **B.** Peripherin counter staining in the same section. **C.** DAPI counter staining in the DRG section. **D.** Merge of A and C showing co-localization of MeCP2 in the neuronal nuclei. **E.** Merge of A and B shows that MeCP2 is strongly expressed in all peripherin positive neurons. **F.** Merge of A, B and C. **G.** A peripherin expressing adult DRG neuron in culture. **H.** The neuron in G also expresses CGRP. **I.** The neuron shows strong nuclear MeCP2 expression in culture. **J.** DAPI counter-staining in the same visual field showing non-neuronal cells in the vicinity that express little or no MeCP2.

Figure 7.


A
mRNA Fols relative to control
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6h
12h
BMP4
NGF

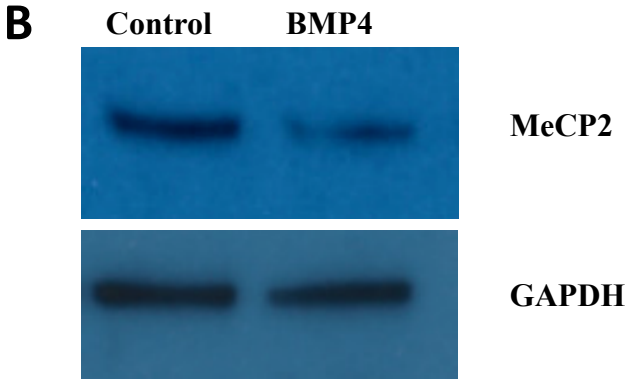

B
Control
BMP4
MeCP2
GAPDH

Figure 7. BMP4 downregulates MeCP2 expression in primary sensory neurons. **A.** Relative mRNA folds (compared to untreated control) of MeCP2 in 50B11 cells at 6h and 12h following BMP4 (50ng/ml) and NGF (50ng/ml) treatments. **B.** Representative immunoblot showing downregulation of MeCP2 protein in primary DRG neuronal culture 12h following treatment with 50ng/ml BMP4. GAPDH was used as an internal control.

Figure 8.

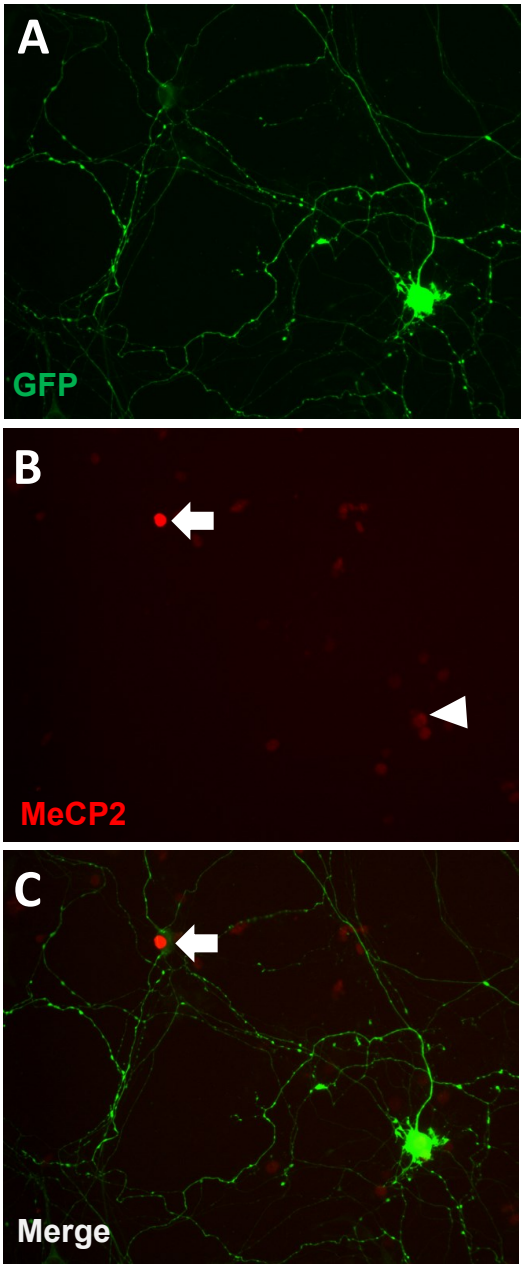
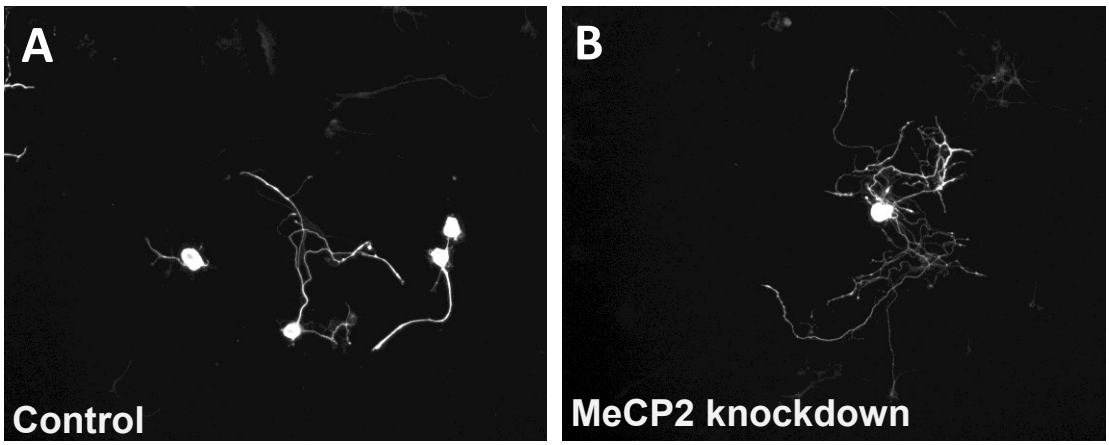


Figure 8. Lentiviral shRNA delivery effectively knocks down MeCP2 in primary sensory neurons of DRG in culture. **A.** GFP staining shows a DRG neuron in culture transduced with lentivirus, 48h following infection. **B.** MeCP2 staining in the same visual field shows high expression in an non-transduced neuron (arrow) and significantly reduced expression (arrowhead) in the virus transduced GFP-expressing neuron. **C.** Merge of A and B. Arrow shows the nucleus of the un-transduced neuron with high levels of MeCP2.

Figure 9.


A
Control
B
MeCP2 knockdown

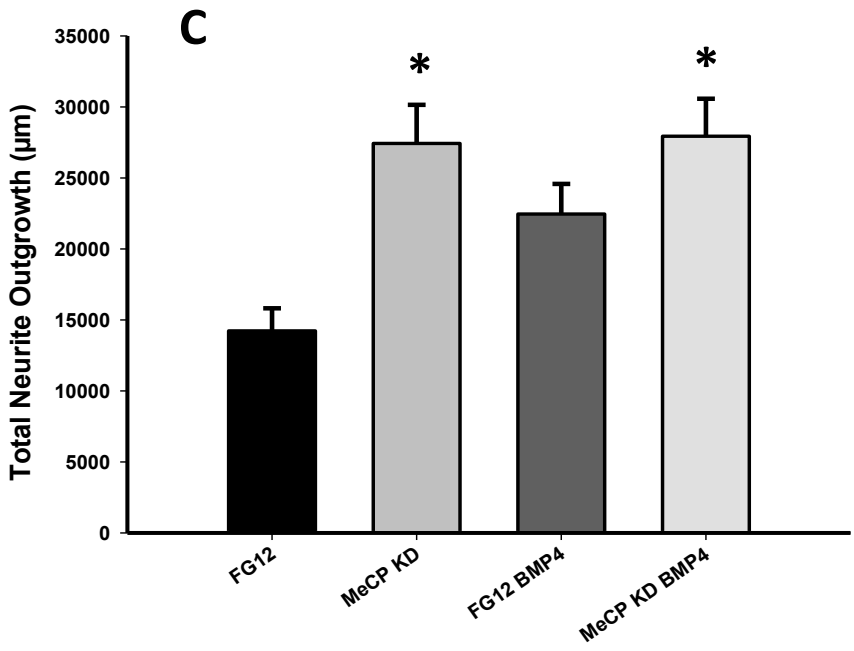

C
Total Neurite Outgrowth (µm)
35000
30000
25000
20000
15000
10000
5000
0
*
*
FG12
MeCP KD
FG12 BMP4
MeCP KD BMP4

Figure 9. MeCP2 knockdown induces robust increase of neurite outgrowth in primary DRG neurons. **A.** Untreated neurons transduced with scrambled shRNA stained for tubulinβIII showing the basal level of outgrowth in culture. **B.** Untreated neuron transduced with MeCP2 knockout shows enhanced outgrowth under basal conditions. **C.** Comparison of neurite outgrowth per neuron in following MeCP2 knockdown, with or without 50ng/ml BMP4 treatment. MeCP2 knockdown significantly increases neurite outgrowth (MeCP KD) compared to scrambled transduced (FG12). BMP4 treatment of the scrambled group also increases outgrowth (FG12 BMP4). But BMP4 treatment fails to induce further increase in outgrowth in MeCP2 knock down (MeCP KD vs. MeCP KD BMP4). *(*$p<0.05$).*

Chapter 5. GENERAL DISCUSSION

Structural plasticity of peripheral nerves and its mechanisms have been studied for several years now. While much has been known about progress and mechanisms of regenerative outgrowth following injury, there was hardly any information about what drives spontaneous growth in adult axons in physiological states. The present study for the first time identifies BMP4 as a potential target-derived factor that can induce sensory outgrowth even in physiological conditions, by inducing a global transcriptional upregulation via genetic and epigenetic mechanisms. The female reproductive tract provided a highly tractable model system to study induction of plasticity under physiological conditions. And understanding of the mechanism provided information that can also be of high value in therapeutic design for potentiating axon growth as well as for ameliorating hyperinnervating pain conditions, particularly female pelvic pain syndromes.

The persistent role of morphogenic growth factors in adult nervous system

The present study demonstrates how growth factors that play critical role during embryonic morphogenesis (empirically referred to as morphogens) may have persistent effect on the mature nervous system. In the vagina, BMP4 not only persists beyond development to fulfill maturation, but continues to modulate innervation in the tissue through adult reproductive life (Chapter 2, (Bhattacherjee et al., 2013)). BMP4 expression is sustained in many other tissues in the adult organism like heart, lung, liver, pancreas, skin, bone and vascular endothelium that are richly innervated by peripheral axons (Pachori et al.; Sato et al., 1999; Sorescu et al., 2003; Frank et al., 2005; Hua and Sarvetnick, 2007; Maegdefrau et al., 2009; Le Bras et al., 2010; Pegorier et al., 2010; Do et al., 2012; Singh et al., 2012). Neuroplasticity has been reported in many of these organs under physiological or pathological conditions (Sternini and Brecha, 1986; Hasan et al., 2006; Cheng et al., 2010; Cui et al., 2010). From our current findings it is plausible

that BMP4 could be a potential regulator of plasticity in these cases. Future studies are required to confirm these possibilities. BMPs are also expressed by activated immune cells like macrophage (Champagne et al., 2002; Honda et al., 2006). Although inflammation can be associated with hyperinnervation, BMP signaling in immune cells has only been considered for an autocrine function for the cell's fate transition so far (Hong et al., 2009; Millot et al., 2010; Chakrabarty et al., 2011). In light of the present study, concurrence of hyperinnervation and activated immune cell localization (which stain positive for BMP4, our preliminary studies) at inflammation sites warrant further investigation of BMP4's role in inflammatory hyperinnervation as well.

Like BMP4, there is a plethora of other morphogens including other BMP members, TGFβ members, Wnts, Frizzled, Sonic hedgehog, netrins (Tabata and Takei, 2004) that play critical roles in development of the nervous system (Rogers and Schier, 2011). In the embryo, concentration gradients of these morphogens set up along the path can translate navigating instruction for the progressing growth cone, leading to organization of the topographic mapping of innervation (Sanchez-Camacho et al., 2005; Miyashita et al., 2009; Rogers and Schier, 2011). Growing evidence indicate that many of these morphogens may regulate plasticity in the adult nervous system as well. Wnts, TGFβ, netrins have been shown to govern axon growth following injury (Zou, 2004; Park et al., 2007; Shim and Ming, 2010; Hellal et al., 2011). Expression of one or more of these proteins are also recorded in adult tissues like skin, bone, heart, lung, kidney, liver, skeletal muscle (Jakowlew et al., 1991; Zeisberg et al., 2005; Breitkopf et al., 2006; Wetzel et al., 2006; Chen et al., 2010; Pachori et al., 2010; Ye et al., 2010; Kluk et al., 2012; Singh et al., 2012). Many of these comprise sites for potential or active neuroplasticity

and/or functional changes like pain (Hasan et al., 2006; Miyashita et al., 2009; Chen et al., 2010; Ye et al., 2010; Chakrabarty et al., 2011; Lee et al., 2012).

Interestingly, besides their independent action, many morphogens can collaborate with each other or different growth factors for effective and often tissue/cell specific outcomes. For example, while Wnts can partner with TGFβ in cellular adherence, tumorogenesis or metastasis, netrins collaborate with slit and wnt to help axons choose their axis of migration (Attisano and Labbe, 2004; Bovolenta et al., 2008; Killeen and Sybingco, 2008; Lopez-Rios et al., 2008; Feigenson et al., 2011). On the other hand, BMP signaling can collaborate with NGF to promote PC12 cell differentiation; with VEGF to stimulate endothelial cell migration and vasculogenesis; with Wnts to prevent oligodendrocyte maturation and induce cell lineage regulation, to mention a few (Deckers et al., 2002; Lonn et al., 2005; Holderfield and Hughes, 2008; Suzuki et al., 2008; Sharov et al., 2009; Feigenson et al., 2011; Trompouki et al., 2011; van Meeteren et al., 2012).

However, in vagina BMP4 alone was sufficient to drive plasticity; additional support from neurotrophins like NGF did not seem additive or necessary for sensory sprouting. It is not known whether BMP4 plays a role in the genesis of innervation and axon distribution in the vagina during embryonic and early postnatal development; also, what the state of BMP4 expression is before the animal begins cycling. Lack of suitable transgenic animal model is a major impediment. BMP4 null animals are lethal in utero as early as gastrulation (Gazzerro and Canalis, 2006) and lack of knowledge of unique promoters prevents generating conditional knockouts in vaginal submucosal smooth muscle. Due to their critical role in the embryo, it is a common problem with transgenic manipulations of most morphogens. Histological studies along developmental time lines coupled with *in vivo* viral gene modifications might help finding answers to some questions of these kinds.

Thus, while the present study reinstates the persistent importance of embryonic morphogens as growth factors in adult PNS, further research is needed to reveal the full repertoire of factors participating in adult neuroplasticity (for understanding of mechanisms and innovation in therapeutics).

50B11 cells: A new tool for reliable and efficient assays in sensory neuronal research

Studying cell signaling mechanisms in neurons has always been challenging owing to lack of suitable cell lines. In peripheral neurons, biochemical/signaling mechanisms have mostly been interpreted from experiments in non-neuronal cell lines or neuroblastomas and assessing phenotypic or structural outcomes have relied solely on PC12 line (adrenal medullar carcinoma, not even neuronally derived) (Greene and Tischler, 1976; Shastry et al., 2001). Some DRG neuronal lines generated intermittently in mouse or human have not only suffered from identity drifting in culture over multiple passages, but also failed to provide neuronal morphological phenotype (Platika et al., 1985a; Platika et al., 1985b; Wood et al., 1990; Fan et al., 1992; Raymon et al., 1999). The 50B11 cells for the first time presented both genotypic and phenotypic identity with primary sensory neurons, thus providing scope for more accurate results and interpretations. Owing to its complete neuron-like morphology and similar outgrowth dynamics, the cell signaling events due to treatments or genetic modifications directly translated to outgrowth changes and relevant interpretations could be drawn. For example, transcription factors like Smad1 and Id2 are not neuron specific, but rather widely prevalent with diverse effects on different cell types. Without the 50B11 line, several experiments performed in the present study would have therefore been difficult to interpret, or even conceive. Furthermore, the tools for genetic manipulations like overexpression or knockdown vectors could be validated

with much higher confidence in this rat cell line before transferring them to neurons in primary culture.

Demonstrating majority of the characteristics of unmyelinated small diameter primary DRG neurons, the 50B11 cells have provided a powerful tool in study of sensory neuron biology spanning area like plasticity, degeneration, inflammation and pain. Apart from the typical neurotrophin and growth factor receptors the cells express key markers like TRPV1 and also show characteristic electrical properties (Chen et al., 2007a). However, further characterization demonstrating expression of other growth factor receptors, other TRP-channels, markers and ion channels would open opportunities of even broader application of this cell line as an assay system. Presently up to 80% cells in culture differentiate with forskolin treatment. Subcloning and selectively propagating a mixture of several highly differentiating subclones would be an ideal future goal for even further refinement of the assay. Genetic modifications can also be considered with constitutive (or inducible) overexpression of adenylate cyclase that can impart sustained differentiated neuronal morphology without a need for forskolin treatment. The cells hold great potential for generating overexpression and knockdown lines of various genes and studying their interactions. Cumulatively, these possibilities along with our findings put forward the 50B11 cells a powerful tool for high throughput studies that can bypass the inherent variability and heterogeneity of primary DRG cultures, giving sensory neuron research a whole new direction in future.

BMP4 signaling via Smad1 can have a broad influence on gene expression

BMP4 mediated sensory outgrowth in neurons is associated with the activation of the Smad1 transcription factor (Chapter 2, (Bhattacherjee et al., 2013)). With the help of 50B11 cells

we found that BMP4 can upregulate Id2 levels in DRG neurons (Chapter 4). While the Id family (Id1 to Id4) comprises a principal target of Smad1 (Miyazono, 1999; Massague et al., 2005), Id2 is selectively increased in developing CNS neurons (in cortex, cerebellum, hippocampus) during the phase of active axon growth (Jackson, 2006; Lasorella et al., 2006). However, Id2 knockout mice show no major changes in brain histology or behavior, except for slight reductions in size of cortex and olfactory bulbs (Yokota et al., 2001). While overexpression of Id2 could potentiate *in vitro* outgrowth (Chapter 4) and adenoviral overexpression can improve regeneration following spinal cord injury (Yu et al., 2011), knocking down the protein failed to abrogate BMP4-mediated outgrowth. These cumulatively suggested a capability of Smad1 to induce outgrowth by regulating a broader umbrella of factors than Id2 alone.

Growing evidence indicates that Smad1/5/8 is a powerful transcription factor that can regulate multiple other transcription factors downstream to modulate gene regulation rather than individual regulation of candidate genes (Massague et al., 2005; Fei et al., 2010). About 5% of the mouse genome (about 1500 genes) is committed to transcription factors (TFs), of which 1444 are expressed at some stage in sensory neurons through development (Gray et al., 2004; Yusuf et al., 2012). While few TFs can act independently, most are parts of hierarchical patterns where one transcription factor can regulate multiple others downstream forming an organized network, to tightly control gene expression (Ocone and Sanguinetti, 2011). Tissue or cell-specific fine tuning at different levels of such hierarchies can generate unique gene expression patterns (Ocone and Sanguinetti, 2011). Smads are TFs located at the core of one of the most primitive signaling pathways, the TGFβ/BMP. Conserved from flies through vertebrates, the diverse effects of this signaling pathway on cellular growth, division, differentiation, morphology, metabolism etc. across different tissue types is evidence of the powerful and multifactorial

influence of Smad signaling (Massague et al., 2005). Thus Smads are master regulators of hierarchical transcriptional networks that can induce cell-specific effects acting via different downstream set of targets (Massague et al., 2005).

Regulation of the 'stemness' (pluripotency) of stem cells by inducing Smad1/5/8 is a hallmark of the multifactorial effect of BMP4 (Zhang and Li, 2005). A recent study mapped Smad target genes genome wide in response to BMP signaling using embryonic stem (ES) cells as a model system. Promoter occupancy for Smad1/5 and Smad4 using ChIP sequencing (Chromatin Immunoprecipitation Sequencing) indicated regulation of multiple genes including a host of other transcription factors, cell surface proteins, ankyrins, ion channels, etc. (Fei et al., 2010). However, as ES cells differentiated forming embryoid bodies, regulation of some of these factors changed differentially (Fei et al., 2010). Thus, while Smad1 is capable of simultaneously regulating multiple subcellular processes its action may be customized tissue specifically (Massague et al., 2005).

Our findings showed that BMP4 can regulate MeCP2 in sensory neurons to regulate outgrowth (Chapter 4). As a global repressor of gene expression, downregulation of this protein may globally upregulate multiple genes with potential role in axon growth. Although expression of MeCP2 is highest in neurons as compared to any other cell in the body (Kishi and Macklis, 2005), it remains to be evaluated whether BMP4 regulates this protein selectively in neurons. There is no precedence of BMP4 mediated regulation of MeCP2 in other cells. In neurons, Chromatin Immunoprecipitation experiments would be essential in future to confirm whether Smad1 directly regulates MeCP2 by binding its promoter or indirectly through other factor (s). Although knockdown of MeCP2 mimics the effect of BMP4 on neurite outgrowth, it remains to be determined whether MeCP2 (especially given the multifactorial effect of Smad1 in non-

neuronal cells) is the only mediator delegated by BMP signaling to execute the effect on outgrowth.

Interestingly, in CNS neurons outgrowth is arrested in a maturing neuroblast as Id2 levels begin declining (Jackson, 2006) and beyond this point as a neuroblast matures, MeCP2 levels increase (Kaufmann et al., 2005; Kishi and Macklis, 2005). These corroborate well with our findings in the sensory neuron showing that increase of Id2, but decrease of MeCP2 are associated with potentiation of outgrowth.

MeCP2 and the epigenetic regulation of axon growth

The downregulation of MeCP2 by BMP4 can induce epigenetic changes in the sensory neuron. In the past couple decade science has gained a vivid understanding of the epigenetic mechanisms in eukaryotic cells. Epigenetically, gene regulation is achieved by modifying chromatin (the nucleosomal histone-DNA complex) structurally and/or functionally, with no change in the genetic code encrypted in DNA (Bird, 2007; Kim et al., 2009). Footprints like cytosine methylation marks on DNA or modification of nucleosomal histone tails (lysine acetylation, methylation etc.) associated with particular genes form common landmarks for epigenetic modifiers to locate their binding target (Berger, 2002; Bird, 2007; Kim et al., 2009). These modifiers, usually proteins, bind and modify chromatin structure to alter accessibility of transcription factors or can further recruit additional co-activators or co-repressors (to the chromatin) to establish (or embellish) their influence (Bird, 2007; Kim et al., 2009). The MeCP2 is one such modifier expressed heavily in neurons and bind methylated DNA with high affinity (Meehan et al., 1992; Guy et al., 2011). While selective Smad1/5 and 4 binding to large groups of genes associated with rich H3K27 trimethylated and H3K4 trimethylated bivalent histone H3

(i.e. trimethylation at 27th and 4th K or lysine residue, signature of active or repressive epigenetic marks respectively) has been demonstrated, direct regulation of epigenetic modifiers by BMP4 has not been reported before (Fei et al., 2010). Thus our findings not only define a novel mechanism of neuronal outgrowth regulation, but also reveal a new mechanism of BMP4 mediated epigenetic regulation.

Heavy methylation of the cytosine in CpG dinucleotides of gene promoters are signatures of low-expressing or silenced genes (Miranda and Jones, 2007). MeCP2, through its methyl-binding domain, binds to DNA in these regions across the genome and recruits Histone Deacetylases (HDACs) apart from other co-repressors and adaptor proteins (Nan et al., 1998b). The complex promotes deacetylation in tails of histones associated with the genes which allows the histones to wrap DNA more tightly resulting in repression/silencing (Nan et al., 1998b; Kishi and Macklis, 2005). Thus removing MeCP2 will potentiate gene expression globally, an outcome conducive for neuronal outgrowth.

However, neuronal expression of MeCP2 is much higher than would be expected for selectively binding some methylated promoters alone. In sensory neurons too we detected levels comparable to CNS neurons. Some recent studies revealed that the protein not only expresses at stoichiometrically equal proportion to that of histone octamers, but can even compete with histone H1 to bind the linker DNA of nucleosome (Skene et al., 2010; Thambirajah et al., 2012). Diversifying MeCP2 actions even further, some reports show that it may participate in transcriptional activation in special cases (Chahrour et al., 2008). A very recent study documented MeCP2 binding to methyl-cytosine and 5-hydroxy methyl cytosine with equal affinity, even in the intragenic regions in DNA, specifically in neurons (Mellen et al., 2012). While there appears to be several confounding factors in describing the effects of MeCP2 on

neuronal transcriptome, the emerging consensus indicates that it likely regulates neuronal gene expression by globally altering chromatin state rather than specific target gene regulations (Skene et al., 2010; Della Ragione et al., 2012).

Presence and appropriate regulation of MeCP2 levels is essential for neuronal process formation. While MeCP2 knockdown in adult sensory neurons boosted outgrowth (Chapter 4), the role of the protein in developing sensory neurons remain unknown. In CNS, which has been explored extensively for MeCP2 function, knockout resulted in increased cell density but reduced cell size and neurite growth leading to reduced thickness of neocortex in null mice (Kishi and Macklis, 2005). Overexpression of MeCP2 in embryonic cortical neurons *in vitro* as well as transgenic overexpression induced formation of longer neurites and axons (Jugloff et al., 2005; Taylor and Doshi, 2012). Although MeCP2 knockdown in adult CNS neurons *in vitro* is deemed to be neuroprotective, no result on the effect on outgrowth has been reported (Russell et al., 2007; Dastidar et al., 2012). Therefore, while MeCP2 apparently plays contrasting roles in outgrowth in CNS and PNS, the studies conducted so far involved neurons from the two systems in different developmental stages (i.e. embryonic loss vs. adult knockdown respectively). Further research is essential to determine whether such differences are cell-type (i.e. CNS or PNS neuron) intrinsic or attributable to the cell's developmental state. While outcomes in either direction would be groundbreaking in further understanding of MeCP2's role in outgrowth, standing evidences substantiate that a fine balance of MeCP2 level is essential to maintain optimal transcription in a neuron initiating axon growth.

Importantly, the functions performed by cortical neurons are different from those delegated by the primary sensory neurons. Accordingly, the sensory neurons that transduce information as primitive as pain, somatic sensation and kinesthesia are hardwired into the

circuit/function much early in development. On the contrary, the cortical neurons require adaptive inputs for substantial period in early life as it continues to make essential synaptic interconnections. In the developing brain the expression and influence of MeCP2 is low and gradually increases, peaking at about 12-18 months of age (in human) (Kaufmann et al., 2005; Kishi and Macklis, 2005; Guy et al., 2011). This time point coincides with the onset of psychomotor symptoms in patients of Rett's syndrome who have MeCP2 mutation. Through these early days of postnatal development, MeCP2 is believed to undergo experience-dependent regulation and phosphorylation (Cohen et al., 2011) that potentially wires the long term connections of the neuron and prepares its transcriptional profile based upon the signals coming in from the environment. While comparable levels of MeCP2 expression is detected in adult sensory neurons, it remains unknown whether similar adaptive adjustments occur through early development, concurrently with the rise of sensory perceptions, or even potentially sustain through later life to define or refine sensory modalities. Supportive evidence from recent findings of elevated pain thresholds in Rett's syndrome patients, decreased spinal and DRG MeCP2 in inflammatory pain and our findings on MeCP2 in plasticity give way to strong hypotheses for further studies in this direction (Downs et al., 2010; Tochiki et al., 2012). A complete study of the epigenetic landscape governing structure and function in the PNS holds promise not only for better mechanistic understanding of plasticity and perception, but also for advanced therapeutic design for disease states.

Clinical Implications

Our findings in the present study have valuable implications in several aspects of human disease. The results have direct and most substantial implication in women's health. Pelvic pain syndromes like vulvodynia and vulvar vestibulitis are neurogenic pain syndromes associated

with genital CGRP hyperinnervation (Bohm-Starke et al., 1999; Tympanidis et al., 2003). These unmyelinated fibers not only contribute directly to transduction of nociceptive pain, they can also induce local CGRP release by antidromic conduction. CGRP induces vasodialation, immune cell infiltration, mast cell degranulation, protein extravasation etc. to bring about pain and hyperalgesia (Sato et al., 2000; Sandkuhler, 2009). Although local anti-inflammatory drugs are sometimes used as a treatment, observed benefits have been low with this approach in vulvodynia. Due to lack of understanding of the mechanism of hyperinnervation, surgical excision of the hyperinnervated vulvar tissue remained as the only option for permanent cure (Goetsch, 1996; Goetsch et al., 2010). We found that under low estrogen conditions BMP4 induces selective proliferation of the CGRP fibers in the vaginal submucosa acting via the BMPR1A and BMPR2 receptor complex (Chapter 2, (Bhattacherjee et al., 2013)). On the basis of this, pharmacological, biologic, as well as vector mediated therapeutic strategies can be explored.

Dorsomorphin is a recently discovered small molecule inhibitor of BMP signaling. It selectively inhibits BMP type I receptor and blocks BMP-mediated Smad1/5/8 phosphorylation. As a small molecule, dorsomorphin has high tissue penetration capability and bioavailability (Chapter 2, (Hao et al., 2008; Yu et al., 2008; Bhattacherjee et al., 2013)) making it a good candidate for local therapeutic application. The molecule is already being used in animal models *in vitro* and *in vivo* for regulation of stem cell fate, and can be explored for pharmacological inhibition of BMP4 mediated nerve sprouting in vulvodynia via both local and systemic delivery methods (Hao et al., 2008; Yu et al., 2008; Hao et al., 2010).

While small molecules are still being explored largely in basic science laboratories, biologics like recombinant proteins are already in clinical application. For example recombinant

BMP2, BMP7 and the BMP-inhibitor noggin are approved and regularly used in orthopedic surgeries like bone reconstruction, spinal fusion and related procedures as slow release implants embedded in collagen matrix (Boden et al., 2002; Govender et al., 2002; Dimitriou and Giannoudis, 2005; Dimitriou et al., 2005). Local vulvar implantation of recombinant BMP antagonists can therefore be a promising direction to pursue in therapeutics. Furthermore, given our current findings that BMP4 can induce outgrowth in adult sensory axons, a similar strategy for recombinant BMP4 therapy might also be considered in neuropathies associated with degenerative sensory nerve loss.

With rapid advances in gene therapy and success of some early clinical trials, recombinant protein therapy could be successfully replaced by local genetic modifications (Alexander et al., 2007; Tani et al., 2011). In the vulvo-vaginal tissue, that is easily accessible by non-invasive methods, local gene delivery can induce permanent cure by knocking down factors like BMP4 that induce hyperinnervation. While our present study showed highly successful gene delivery using viral techniques in rodent, both viral and chemical delivery methods can be explored in human for long term cure in future.

Eventually, targeting the molecular machinery involved like Smad1 or MeCP2 might render the most control over adult sensory neuroplasticity in therapeutic development. However, more research is needed in this direction to first establish functional *in vivo* animal models. Since HDACs are essential partners for MeCP2 mediated gene repression, HDAC inhibitors (that have already entered clinical use) can also be explored as a pharmacological intervention to activate global gene expression for axon growth (Mei et al., 2004; Shabason et al., 2010).

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