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# Regulation of Dendritogenesis in Sympathetic Neurons, Autonomic Nervous System

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Chapter 5

# Regulation of Dendritogenesis in Sympathetic Neurons

Vidya Chandrasekaran and Pamela J. Lein

Additional information is available at the end of the chapter

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**Abstract**

In postganglionic sympathetic neurons, the size of the dendritic arbor determines presynaptic convergence, which correlates with tonic activity, and aberrant dendritic morphology is associated with disease. There is, therefore, great interest in understanding how dendritic morphology is regulated in these neurons. Early studies established a role for target-derived nerve growth factor (NGF) in regulating the size of the dendritic arbor of sympathetic neurons *in vivo*. However, *in vitro* studies revealed that even in the presence of optimal concentrations of NGF, rat sympathetic neurons cultured in the absence of serum or non-neuronal cells survive and elaborate extensive axonal arbors, but fail to form dendrites. Subsequently, it was discovered that bone morphogenetic proteins (BMPs) trigger cultured sympathetic neurons to extend a dendritic arbor comparable to that of their *in vivo* counterparts. The goals of this chapter are to: (i) summarize these early experiments; (ii) discuss evidence substantiating a role for BMPs in glial-induced dendritic growth *in vitro* and regulation of dendritic growth *in vivo*; (iii) review what is known about the molecular mechanisms by which NGF, BMPs and other factors influence dendritic arborization of sympathetic neurons; and (iv) identify key data gaps in understanding of how dendrites are regulated in sympathetic neurons.

**Keywords:** afferent input, BMPs, dendrites, neuronal polarity, NGF, p75, reactive oxygen species (ROS), Rit, Smad, STAT, sympathetic neurons, target-derived factors

## 1. Introduction

Differences in dendritic morphology between neurons are a striking feature of the vertebrate nervous system with important functional implications. The shape of dendrites influences the propagation and integration of postsynaptic potentials [1], and determines presynaptic

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convergence [2, 3]. These observations coupled with evidence that aberrant dendritic structure is strongly associated with neurologic disease [4, 5] have generated significant interest in understanding how dendrites are regulated.

Postganglionic sympathetic neurons are a well-characterized model for studying dendrite development and plasticity [6]. The dendritic arbor of these neurons is relatively complex with an average of two to six primary dendrites, depending on the animal species, and multiple orders of branching [7]. In postganglionic sympathetic neurons, the size of the dendritic arbor correlates with not only the number and pattern of synaptic inputs [3, 8], but also tonic activity [8, 9]. As is true of central neurons, aberrant morphology of sympathetic neuron dendrites is associated with disease. For example, dendritic hypertropy of sympathetic neurons in stellate and superior cervical ganglia (SCG) is observed in the spontaneously hypertensive rat [10, 11], and is thought to contribute to the pathogenesis of hypertension in this model [11]. Therapeutic intervention with statins not only decreases sympathetic activity and normalizes blood pressure in the spontaneously hypertensive rat [12], but also decreases dendritic arborization of both stellate and SCG neurons [13].

In this chapter, we will review what is known about the molecular and cellular mechanisms that regulate dendrites in postganglionic sympathetic neurons, and identify key data gaps.

## 2. Early studies of dendritic growth in sympathetic neurons

The majority of dendritic growth in postganglionic sympathetic neurons occurs during the postnatal period; however, dendrites continue to grow into adulthood [14–16], and *in situ* imaging of mature sympathetic neurons has demonstrated that their dendritic arbors continually grow and retract throughout life [17, 18]. In the rat, neonatal deafferentation has negligible effect on dendritic growth in SCG neurons throughout the first month of life [19–21], indicating that dendritic growth in sympathetic neurons does not require afferent input. In contrast, target tissues strongly influence dendritic growth in these neurons. Experimentally reducing target size causes dendritic arbors of SCG neurons to be smaller than normal; conversely, increasing the target size significantly increases the size of the dendritic arbor [16]. The influence of target is further illustrated by observations that sympathetic neurons within the same ganglion that project to different targets exhibit varying dendritic morphologies [18, 19].

The effect of target tissues on dendritic growth in sympathetic neurons is mediated, at least in part, by nerve growth factor (NGF) [22–25]. Separation of neurons from target tissues by axonal ligation causes dendritic atrophy in the few neurons that survive, and this effect is attenuated by systemic administration of NGF [26, 27]. However, exogenous NGF reverses axotomy-induced dendritic retraction by <50%, even though cell survival is completely rescued [27], indicating that additional target-derived factors are needed to fully account for the effects of target on dendritic growth. Consistent with this conclusion, the dendritic complexity of axotomized sympathetic neurons recovers to control levels upon ganglion cell reinnervation of the periphery [28]. *In vitro* studies further suggest that factors in addition to NGF are

required for dendritic growth in sympathetic neurons. When grown in low-density cultures in the absence of serum or non-neuronal cells, but in the presence of optimal concentrations of NGF, sympathetic neurons form an axon but no dendrites [29, 30].

*In vitro* studies have further revealed that the initiation and maintenance of dendritic growth in sympathetic neurons is regulated by trophic interactions. Addition of serum to NGF-containing medium stimulates sympathetic neurons to form dendrites, although under these culture conditions, the dendritic arbor is significantly less complex than is observed *in vivo* [31]. In contrast, co-culture with ganglionic glial cells causes these neurons to form a dendritic arbor comparable to that of their *in vivo* counterparts [32]. Subsequently, it was discovered that the addition of bone morphogenetic proteins (BMPs) to the culture medium similarly triggers sympathetic neurons to form a complex dendritic arbor [29, 33]. Multiple BMP family members have been shown to stimulate dendritic growth in cultured sympathetic neurons, including BMPs 2, 4, 5, 6, 7 and 60A; however, this activity appears to be restricted to the dpp and 60A BMP subfamilies since BMP-3 and other members of the TGFβ superfamily, including TGFβ1, TGFβ2, TGFβ3, activin A, inhibin, and GDNF, have no effect on dendritic growth in cultured sympathetic neurons [29, 33, 34].

The trophic actions of BMPs are specific to dendritic growth in that BMPs do not support cell survival, nor do they enhance axonal growth in cultured sympathetic neurons [29]. Consistent with observations of dendritic growth in sympathetic neurons *in vivo*, the dendrite-promoting activity of BMP-7 is independent of synaptic or electrical activity [35], but is modulated by NGF [29, 36]. Importantly, the dendritic arbor induced by BMPs in cultured sympathetic neurons is comparable to that of their *in vivo* counterparts with respect to not only size and complexity, but also accumulation and post-translational modification of dendrite-specific cytoskeletal and membrane proteins, exclusion of axonal proteins, transport of select mRNA, and formation of synaptic contacts of the appropriate polarity [29, 34, 35]. These observations indicate that BMPs selectively induce the execution of a developmental program in sympathetic neurons that controls both quantitative and qualitative aspects of dendritic growth.

These observations suggest that BMPs mediate the effects of ganglionic glia and target tissues on dendritic growth in sympathetic neurons. Immunocytochemical and *in situ* hybridization studies indicate that the spatiotemporal expression of BMPs 5, −6, and −7 in rat SCG is consistent with a role in the initial stages of dendritogenesis [33, 37]. *In vitro*, both SCG glia and neurons express BMP mRNA and protein when grown in the absence or presence of each other [37]. However, co-culture of sympathetic neurons with ganglionic glia markedly increases BMP protein coincident with a significant decrease in levels of the soluble BMP antagonists, follistatin and noggin [37]. Functional assays indicate that glial-induced dendritic growth is significantly reduced by BMP-7 antibodies and completely blocked by exogenous noggin and follistatin [37]. Collectively, these data suggest a model in which glia influence the rapid perinatal expansion of the dendritic arbor in sympathetic neurons by increasing BMP activity via modulation of the balance between BMPs and their antagonists. Whether this model holds true *in vivo* has yet to be tested.

The question of whether BMPs also contribute to target effects on dendritic growth has yet to be addressed experimentally. Sympathetic targets, including the eye, heart, lung, kidney, and

blood vessels, express significant levels of BMPs during embryonic development, throughout the postnatal period, and into adulthood [38–40]. Thus, target tissues may be a source of BMPs to sympathetic neurons not only during initial expansion of the dendritic arbor, but also in the maintenance and remodeling of dendritic arbors that continues throughout the life of the animal.

## 3. Signaling pathways that regulate dendritic growth in sympathetic neurons

Research over the past few decades has provided insights into the signaling pathways and molecular mechanisms that control dendritic growth in sympathetic neurons. As discussed in the preceding section, BMPs and NGF play predominant roles in the initiation and maintenance of dendrites in these autonomic neurons. While the importance of these growth factors as regulators of dendritic growth in sympathetic neurons is well established, the downstream effectors that link BMP and NGF to increased dendritic growth are not fully understood. In this section, we will discuss the signaling pathways activated by these growth factors, the evidence implicating downstream effectors of BMPs and NGF in dendritic regulation, and the identification of factors that interact with these signaling pathways to alter their influence on the dendritic arborization of sympathetic neurons.

### 3.1. BMP signaling

BMPs mediate their cellular effects by binding to a heteromeric receptor complex of transmembrane serine/threonine kinas receptor subunits comprised of a type I receptor [BMP type I receptor A (BMPR1A), which is also known as activin receptor-like kinase-3 (ALK-3) or BMP type I receptor B (BMPRIB, also known as ALK-6)], and a type II receptor [BMP type II receptor (BMPRII), activin type II receptor (ActRII), or activin type IIB receptor (ActRIIB)] [41, 42]. Ligand binding causes type II receptors to phosphorylate type I receptors, which then phosphorylate Smads 1, 5, and/or 8, also known as receptor Smads (R-Smads). Phosphorylated R-Smads complex with Smad 4, triggering translocation of the Smad complex to the nucleus to regulate gene transcription [42, 43]. Studies in *Smad* knockout animals and *Smad* deficient cells suggest that BMPs can also signal through Smad-independent pathways via activation of mitogen-activated protein kinase (MAPK), c-jun amino terminal kinase (JNK), p38-MAPK, phosphoinositol-3-kinase (PI3K), LIM kinase 1 (LIMK1) or small GTPases [43–46].

BMP signaling pathways are active in sympathetic ganglia during developmental periods corresponding to the initiation, extension and maintenance of dendrites. Quantitative PCR, *in situ* hybridization, and immunocytochemistry studies have confirmed the presence of mRNA and protein for BMPRIA, BMPRIB, ActRII and BMPRII in neurons of mouse SCG starting as early as embryonic day 14, and persisting into postnatal development and even adulthood [47, 48]. A role for BMPRIA in regulating dendritic growth in sympathetic neurons *in vivo* was confirmed in a BMPRIA conditional knockout mouse generated by crossing a *Dbhi-Cre* mouse line to a *BmprIa* floxed line [49]. *Dbhi-Cre* is expressed in sympathetic ganglia around E10.5, thus, *BmprIa* expression is knocked out after neuronal specification, but before initiation of dendritic growth. The loss of BMPRIA expression in sympathetic ganglia significantly

diminished but did not completely block dendritic growth *in vivo* [49]. At postnatal day 3, the number of primary dendrites and the size of the dendritic arbor were not significantly different in sympathetic neurons of SCG from *BmprIa* conditional knockout mice *vs*. SCG neurons from congenic wildtype controls. At later developmental times, however, dendritic arborization was significantly decreased in SCG neurons of *BmprIa* knockout vs. wildtype mice. These data suggest that, *in vivo*, BMP signaling is required for the maintenance of dendrites, but not for the initiation and early growth of dendrites in sympathetic neurons.

Several caveats of the *in vivo* study suggest that the lack of effect of *BmprIa* knockout on early stages of dendritic growth *in vivo* may not be discrepant with *in vitro* data showing that BMPs are necessary and sufficient for induction of dendritic growth in sympathetic neurons [29, 49]. First, while the authors confirmed that *BmprIa* mRNA was not generated in the SCG after embryonic day 11.5, they did not examine BMPRIA protein levels in the SCG of knockout animals. Thus, the possibility that BMPRIA protein was still present during early stages of dendritic growth cannot be ruled out. Moreover, recent biochemical studies have shown that BMPRs cycle between various cellular compartments, and the association of the receptors with endocytosis machinery is required for Smad-mediated signal transduction [50]. Thus, additional studies are needed to characterize BMPR turnover and changes in receptor localization in wildtype *vs*. *BMPR* knockouts. Another, perhaps more likely, explanation is that sympathetic neurons in the *BmprIa* knockout mice express other type I receptors, such as the activin receptors, that may be activated by BMPs to trigger dendritic growth at early developmental stages. Further studies targeting other BMPRs are warranted to fully elucidate the role of BMP signaling in dendritogenesis *in vivo*.

### *3.1.1. Smad-dependent transcriptional regulation of dendritic growth*

Canonical BMP signaling involves the Smad family of transcription factors. Immunocytochemical analyses of primary rat SCG neurons have demonstrated that Smad 1/5/8 translocates to the nucleus within 20 minutes of exposure to BMP-7, with maximal nuclear translocation observed within 2 hours of adding BMP-7 to the culture medium. Transfection with a Smad1 dominant negative mutant, Smad1 (3SA), blocked BMP-7-induced dendritic growth in primary sympathetic neurons [51], indicating that Smad 1 activation is necessary for induction of dendritic growth by BMP-7. In contrast to the *BmprIa* conditional knockouts, conditional knockdown of *Smad4* in sympathetic neurons, generated by crossing *Dbhi-Cre* mice to *Smad4* floxed mice, increased dendritic length and the size of the dendritic arbor in SCG neurons [49], suggesting that Smad 4 may play a role in limiting dendritic growth *in vivo*.

A comprehensive analyses of Smad-dependent dendritic growth in sympathetic neurons provided evidence for early transcriptional regulation of dendritic growth downstream of BMP-7 [52]. In neuronal cell cultures from embryonic rat SCG, BMP-7-induced dendritic growth could be blocked by pharmacologic inhibition of transcription with actinomycin-D when the inhibitor was added within the first 24 hours of BMP-7 exposure but not when it was added after 48 hours of BMP-7 exposure. Microarray analyses identified over 250 genes that were differentially regulated by BMP-7 within the first 24 hours after adding BMP-7 to the culture medium. Of these, 56 mRNAs were altered within the first 6 hours and 185 mRNAs were differentially regulated at 24 hours after BMP exposure [52]. Many of the differentially regulated genes were linked to signaling pathways previously implicated in dendritogenesis in other

neuronal cell types or neuronal morphogenesis and/or axonal guidance, such as BMP, Notch, integrin, Wnt, and NGF signaling molecules. However, the functional relevance of most of these genes to dendritic growth in sympathetic neurons has yet to be determined. Moreover, recent reports of limited correlation between transcriptome and proteome analysis in yeast, plants and mice [53, 54], suggest that in order to generate a more complete understanding of the molecular pathways that link BMPs to dendritic growth in sympathetic neurons, detailed proteome analyses are needed to complement the existing transcriptomic dataset.

One gene identified as being strongly upregulated by BMP-7 in primary sympathetic neurons, the gene encoding the p75 neurotrophin receptor ($p75^{NTR}$) [52], has been evaluated for a role in BMP-induced dendritic growth. A member of the tumor necrosis factor (TNF) receptor family, $p75^{NTR}$ regulates diverse neurobiological processes, including axonal growth, synaptic plasticity, dendritic growth in central neurons, and neuronal cell death [55–61]. $p75^{NTR}$ binds diverse ligands to mediate its effects, including NGF, other neurotrophins, myelin-derived polypeptides, such as myelin-associated glycoprotein (MAG) or Nogo, and β-amyloid peptide [60, 62, 63]. In cultured embryonic rat SCG neurons, $p75^{NTR}$ mRNA and protein expression are significantly upregulated within 24 hours of exposure to BMP-7 [52, 64], and pharmacologic inhibition of signaling via BMPRI prevents induction of $p75^{NTR}$ protein expression in primary sympathetic neurons exposed to BMP-7 [64]. Functional studies revealed that BMP7 does not trigger dendritic growth in primary sympathetic neurons derived from SCG of $p75^{NTR}$ knockout mice; conversely, ligand-independent activation of $p75^{NTR}$ via overexpression of a $p75^{NTR}$ cDNA construct in $p75^{NTR-/-}$ neurons [65], phenocopies the dendrite-promoting effects of BMP-7 [64]. Morphometric analyses of SCG from wildtype *vs*. $p75^{NTR}$ knockout mice at 3, 6 and 12–16 weeks of age indicated that genetic deletion of $p75^{NTR}$ does not prevent dendritic growth, but does significantly stunt dendritic maturation in sympathetic neurons. These data support the hypotheses that $p75^{NTR}$ is involved in downstream signaling events that mediate BMP7-induced dendritic growth in sympathetic neurons, and suggest that $p75^{NTR}$ signaling positively modulates dendritic complexity in sympathetic neurons *in vivo*.

An outstanding question regarding $p75^{NTR}$ effects on dendritic growth in sympathetic neurons is the identity of ligand(s) and co-receptor(s) that $p75^{NTR}$ interacts with to mediate BMP-induced dendritic growth. Several lines of evidence argue against a direct interaction between $p75^{NTR}$ and the BMP receptor complex: (*i*) inhibiting transcription blocks both BMP-induced dendritic growth and upregulation of $p75^{NTR}$ mRNA [52]; (*ii*) pharmacologic blockade of BMPRI signaling blocks $p75^{NTR}$ induction [64]; and (*iii*) overexpressing $p75^{NTR}$ in cultured $p75^{NTR-/-}$ neurons induces dendritic growth even in the absence of BMP-7 [64]. NGF is a potential activating ligand, and NGF is required for BMP-induced dendritic growth [29, 36]. However, the observation that $p75^{NTR}$ interactions with the primary receptor for NGF, TrkA, serve to limit synapse formation in sympathetic neurons [66], argues against $p75^{NTR}$-TrkA interactions enhancing dendritic development. Likewise, pro-neurotrophin activation of the $p75^{NTR}$-sortilin complex serves to promote sympathetic axon degeneration and cell death [67], suggesting that this interaction is also unlikely to stimulate dendritic growth. The identity of the $p75^{NTR}$ ligand that mediates BMP-7-induced dendritic growth remains an outstanding question.

Similarly, the downstream effector molecule(s) that link $p75^{NTR}$ to increased dendritic arborization remain to be determined. Key candidates include the Rho GTPases. Rho GTPases function as central regulators of dendritic morphology, linking extracellular signals to changes

in the dendritic actin cytoskeleton [68, 69]. $p75^{NTR}$ has been shown to interact with RhoA in the yeast two-hybrid system [70]. In cultured rat sympathetic neurons, exposure to BMP-7 increases the levels of the GTP-bound form of RhoA, but not GTP-Rac1 or GTP-Cdc42, as determined by a GTP pull down assay, and triggers RhoA translocation from the cytoplasm to the membrane [13]. The observation that BMP-7-induced dendritic growth in primary sympathetic neurons requires RhoA activation [13], suggests a model in which BMP-7 sequentially activates BMPRIA, Smad 1/5/8, $p75^{NTR}$, and then RhoA to induce dendritic growth in sympathetic neurons.

### 3.2. Signaling pathways that interact with Smad signaling to modulate BMP-induced dendritic growth

The shape of the dendritic arbor of developing sympathetic neurons is determined by interactions between positive and negative regulators of dendritic growth. A number of signaling pathways have been shown to interact with BMP signaling to modulate the number of dendrites, total dendritic length and dendritic branching in sympathetic neurons. In this section, we will review known positive and negative regulators of Smad signaling that impact BMP-induced dendritogenesis in sympathetic neurons.

#### *3.2.1. Positive regulators of Smad signaling that enhance dendritic growth*

*Proteasome-mediated signaling.* The ubiquitin-proteasome pathway is unique in that it is one of the few signaling pathways identified thus far whose interactions with BMP signaling enhance dendritic arborization in sympathetic neurons. In the nervous system, proteasome-mediated protein degradation pathways are important for self-renewal of neurons, axonal growth, vesicle release, receptor turnover, signal transduction and synaptic plasticity [71–73]. The ubiquitin-proteasome pathway has been implicated in regulating dendritic growth in the mammalian central nervous system, with multiple E3 ubiquitin ligases, including Anaphase promoting complex (APC), cullin RING-type E3 ubiquitin ligases (CUL) and neuronal precursor cell expressed and developmentally downregulated protein (Nedd4), determined to be necessary for the extension and maintenance of the dendritic arbor of central neurons [71, 74–76].

Biochemical studies have shown that R-Smads interact with components of the proteasome complex, as well as enzymes and proteins involved in the ubiquitination-deubiquitination of proteins, in many systems, and the ability of Smads to regulate transcription is dependent on association with the proteasome complex in various cell types [77, 78]. Interactions between BMP signaling molecules and the proteasome pathway have been reported in perinatal rat sympathetic neuronal cultures prior to dendritic growth induction by BMP-7 [51]. In this study, interactions between Smad1 and multiple proteasome components were confirmed using a yeast two-hybrid assay, and pharmacologic inhibition of proteasome activity by lactacystin and ALLN (N-acetyl-Leu-Leu-norleucinal) selectively blocked BMP-7-induced dendritic growth in primary sympathetic neurons in the absence of any effect on axonal growth [51]. These proteasome inhibitors also suppressed Smad-mediated transcriptional regulation in a biochemical assay using a Tlx -luciferase construct transfected into P19 cells [51]. One caveat of this study is that although there was clearly a functional interaction between BMP signaling and the proteasome pathway in the context of dendritic growth, a biochemical

interaction between Smads and proteasomes were not demonstrated in primary sympathetic neurons. Further studies are necessary to fully understand the genetic and biochemical interactions between Smads and ubiquitin-proteasome pathway during dendritogenesis in sympathetic neurons.

*Signaling mediated by reactive oxygen species (ROS).* Under physiological conditions, ROS are generated by the mitochondrial electron transport chain or as a consequence of the activity of NADPH oxidase (NOX) [79–81]. While the deleterious effects of ROS at high concentrations are well documented, over the past decade, there is growing appreciation of the beneficial role of ROS at physiological concentrations [80–84], and of the importance of the NOX family of enzymes in regulating many aspects of neuronal development including neurogenesis, neurite outgrowth and synaptic plasticity [80, 83, 85]. NOX2 is expressed in neonatal rat SCG neuronal cell cultures [86], and exposure of cultured embryonic SCG neurons to BMP-7 increases the expression of NOX2 [87]. Pharmacologic inhibition or siRNA knockdown of NOX2 significantly decreases BMP-7-induced dendritic growth in primary sympathetic neurons, an effect that is also observed in cultures co-exposed to BMP-7 and any of three mechanistically and structurally distinct antioxidants that block ROS generation [87]. Antioxidants block BMP-induced dendritic growth downstream of Smad signaling since BMP-induced nuclear translocation of Smads is unaffected by antioxidant treatment [87].

Collectively, these data support the hypothesis that ROS are involved in the downstream signaling events that mediate BMP7-induced dendritic growth, and suggest that ROS-mediated signaling positively modulates dendritic complexity in sympathetic neurons. One caveat of this study, however, is that while BMP-7 was observed to increase NOX2 levels and oxygen consumption in sympathetic neurons, increased ROS levels were not detected in sympathetic neurons exposed to BMP-7. Likely, this reflects the fact that physiologic BMP signaling generates levels of ROS that are below the detection threshold for standard ROS detection assays. Further work is needed to determine whether these *in vitro* observations translate to a role for ROS in regulating dendritic growth in sympathetic ganglia *in vivo*.

### *3.2.2. Negative regulators of SMAD signaling that influence dendritic growth*

*STAT signaling*. The interferons (IFN) and the neuropoietic cytokines, which include interleukins (IL), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), signal through the activation and nuclear translocation of signal transducers and activators of transcription (STAT) proteins, which then regulate transcription of target genes [88]. Previous studies have shown that cytokines that signal through STATs *vs*. members of the TGFβ superfamily of signaling molecules tend to exert opposing effects on cellular function [89–91]. Similar antagonistic interactions between STAT-dependent cytokine signaling pathways and the BMP signaling pathways have been documented during dendritogenesis in perinatal sympathetic neurons. In primary perinatal sympathetic neurons, addition of LIF, CNTF or gamma-IFN (IFNγ) significantly decreased the number of primary dendrites and total dendritic length in cultures exposed to BMP-7, but had no effect on axonal growth or cell viability [92–94]. In addition, all three cytokines triggered retraction of preexisting dendrites [92, 94], suggesting a role for them in the dendritic retraction observed following neuronal injury. Addition of LIF, CNTF or IFNγ to primary sympathetic neurons resulted in nuclear translocation of STAT, and transfection of a dominant negative STAT1 construct blocked

IFNγ-mediated dendritic retraction [92, 94]. These data provide evidence that neuropoietic cytokines negatively regulate dendritic growth in perinatal sympathetic neurons via activation of STAT proteins. However, many questions remain, including the mechanism by which nuclear translocation of STAT proteins limits the activity of SMAD proteins, and the downstream targets of STAT and SMAD signaling during dendritogenesis.

*Mitogen-activated protein kinase (MAPK) signaling*. MAPK family members, including extracellular signal-regulated kinase (ERK), p38, and c-Jun $NH_2$-terminal kinase (JNK), are serine–threonine kinases that function as downstream effectors of many extracellular signaling molecules, including NGF, epidermal growth factor (EGF), and fibroblast growth factor (FGF) [95, 96]. The activation of MAPK regulates gene expression to alter various cellular activities such as cell division, growth, survival and cell death [95, 96]. Previous studies in *Xenopus* embryos and cells such as radial glia, astrocytes, hippocampal neurons, and PC12 cells, have demonstrated interplay between Smad and MAPK signaling pathways, and shown that phosphorylation of the linker on the Smad protein by MAPK is necessary for coordinating the cellular effects of BMPs and other growth factors [43, 97–99]. Interplay between BMP and MAPK signaling pathways is also observed during dendritogenesis in rat and mouse sympathetic neurons. Pharmacologic inhibition of MAPK signaling with PD98059 or transfection of a dominant negative MEK1 or ERK2 mutant increases the number of dendrites and total dendritic arbor induced following BMP-7 exposure, whereas overexpression of MEK1 decreases BMP-7 induced dendritic growth [100]. Inhibition of MAPK signaling in primary sympathetic neurons also increases Smad nuclear accumulation following BMP-7 exposure, and FGF was identified as one of the ligands responsible for activating the ERK signaling pathway in these neurons [100]. These findings suggest that, in perinatal sympathetic neurons, FGF activates the MAPK pathway, which inhibits the nuclear accumulation of SMADs, thereby limiting BMP-induced dendritic growth.

In addition to modulating BMP effects on dendritic arborization via activation of MAPK signaling, FGFs regulate neuronal differentiation via the integrative nuclear FGFR1 signaling (INFS) pathway [101]. FGFR1 is expressed in the nucleus of adult rat SCG neurons following axotomy [102]. Nuclear localization of FGFR1 is also increased in perinatal rat sympathetic neurons following exposure to BMP-7, and transfection of a mutant FGFR1 receptor inhibits FGFR1 nuclear localization and decreases the dendritic response to BMP-7 [103]. These data suggest that the INFS-mediated FGF signaling pathway functions downstream of BMP signaling to limit BMP-induced dendritogenesis in sympathetic neurons.

Rit GTPase, a member of the small GTPase family, has also been shown to activate ERK1/2 in primary sympathetic neurons, and the transfection of dominant negative (dn) Rit or constitutively active (ca) Rit were observed to increase or decrease BMP-induced dendritic growth, respectively [36]. Rit GTPase also negatively modulates dendritic growth as a downstream target of IFNγ signaling as demonstrated by inhibition of IFNγ-mediated dendritic retraction in primary sympathetic neurons transfected with dnRit constructs [104]. Addition of IFNγ to cultures of pheochromocytoma cells, which are often used as a model for sympathetic neurons, increased levels of GTP-Rit, and transfection of dnRit inhibited IFNγ-induced activation of p38 MAPK [104]. These observations suggest that a novel Rit-p38 MAP kinase signaling pathway functions in parallel with the canonical JAK–STAT signaling pathway to mediate IFNγ-induced dendritic retraction. Collectively, these studies provide evidence for crosstalk

between BMP signaling and MAPK signaling during dendritogenesis in sympathetic neurons, and suggest that in contrast to its effects in central neurons, MAPK signaling functions as a negative regulator of BMP-induced dendritic growth in sympathetic neurons.

*Retinoic acid signaling.* In the developing nervous system, BMPs and retinoic acid work synergistically to regulate neural tube patterning and specification of neuronal identity [105–108]. Similarly, interplay between BMP and retinoic acid signaling have been documented during dendritogenesis in sympathetic neurons; however, their interaction in this latter context is antagonistic [106]. Genes required for synthesis of retinoic acid are expressed in cultured perinatal rat SCG neurons, and retinoids synthesized by SCG explants were able to activate transcriptional reporters in retinoic acid-responsive F9 cells [106]. The addition of retinoic acid, or specific retinoic acid receptor and retinoid X receptor agonists, to the medium of cultured sympathetic neurons inhibited BMP-induced dendritic growth without altering axonal growth or cell viability [106]. What is not known, however, is the point of convergence between these two pathways in the context of dendritic growth. Further studies are also needed to determine whether retinoic acid regulates the dendritic arborization of sympathetic neurons *in vivo*.

*Signaling by pituitary adenylate cyclase (PACAP) and vasoactive intestinal peptide (VIP).* PACAP is a member of the secretin/VIP family of peptides that regulates the development of cells within the sympathoadrenal lineage [109]. In sympathetic neurons, PACAP regulates cell survival, proliferation, and catecholamine secretion, and release of PACAP and VIP from preganglionic neurons stimulates depolarization of sympathetic neurons [109]. In cultured perinatal rat sympathetic neurons, PACAP38 and VIP decrease BMP-induced dendritic growth, as evidenced by a decrease in the percentage of cells with dendrites, number of dendrites per neuron, and size of the dendritic arbor [110]. Using receptor specific antagonists and antibodies against phosphorylated cyclic AMP response element binding (CREB) protein, the PACAP response was shown to be mediated by $PAC_1$ receptor activation, which results in the nuclear accumulation of phospho-CREB. Inhibition of adenylate cyclase activity by SQ22526 overcomes the inhibitory effects of PACAP on dendritic growth, and agents that increase cAMP levels, such as forskolin, inhibit BMP-induced dendritic growth [110]. These data suggest that peptides released by preganglionic nerves modulate dendritic growth in postganglionic sympathetic neurons by a cAMP-dependent mechanism.

In summary, signaling by cytokines, growth factors, small molecules, and peptides, such as retinoic acid, PACAP and VIP, antagonize BMP signaling during dendritogenesis in sympathetic neurons. Most of the relevant data were collected from studies of primary perinatal sympathetic neurons cultured from rodent SCG. While the findings from this model have provided a glimpse into the complexity of the interactions that influence dendritic arborization of these neurons, further studies are required to understand the mechanisms by which these factors interact to regulate dendritic growth, how these pathways are spatially and temporally coordinated to influence dendritic arborization of sympathetic neurons *in vivo*, and to determine their relevance to the human condition.

### 3.3. Other pathways that regulate dendritic growth in sympathetic neurons

As described earlier, NGF is an important regulator of dendritic growth in sympathetic neurons. However, the molecular mechanisms by which NGF regulates dendritic growth are

not well characterized. Early growth response-3 (Egr3), a transcriptional regulator known to be induced by NGF via MAPK signaling, has been identified as a potential downstream regulator of NGF-induced dendritic growth [111]. Sympathetic neurons from a conditional *Egr3* knockout generated using *Dbhi-Cre* have fewer primary dendrites and shorter dendritic arbors compared to sympathetic neurons from congenic wildtype animals. In addition, *Egr3* knockout animals exhibit defects in axonal guidance, and in innervation of autonomic targets [111]. Although, Egr3 had previously been shown to not be required for NGF effects on neuronal survival [112], some neuronal cell death was observed in germline mutants for Egr3. It is, therefore, possible that the dendritic effects of knocking out *Egr3* are secondary to adverse effects on neuronal cell viability or changes in target innervation.

Neuronal depolarization induced by electric field stimulation or the addition of potassium chloride to primary postnatal sympathetic neurons cultured was shown to trigger the formation of dendrites in the presence of NGF that retracted in the absence of neuronal activity [113]. Neuronal depolarization enhanced stability of microtubules and activated calcium calmodulin dependent kinase II (CaMKII) in dendrites. The latter was shown to be causally related to the effects of neuronal depolarization on dendritic growth: pharmacologic inhibition of CaMKII activity using KN62 or mAIP completely blocks activity-dependent dendritic growth in cultured sympathetic neurons [113].

Signaling by integrin-linked kinase (ILK) and glycogen synthase kinase-3β (GSK-3β) have also been shown to be downstream effectors of activity-dependent dendritic growth in postnatal sympathetic neurons [114]. ILK and GSK-3β are serine threonine kinases that are downstream effectors of integrin and neurotrophin signaling [115]. ILK has been shown to phosphorylate and inactivate GSK-3β to regulate NGF-mediated axonal growth [116]. Increased phosphorylation of GSK-3β protein was observed in cultured postnatal rat SCG neurons in response to increased neuronal activity, and inhibition of ILK activity by QLT0254, as well as transfection of dominant negative ILK or siRNA for ILK, blocked activity-dependent dendritic growth in these neurons. Similarly, inhibition of GSK-3β activity using kenpaullone or genetic knockdown of GSK-3β expression increased the number of primary dendrites formed in response to potassium chloride, suggesting that GSK-3β inhibition is necessary for early stages of activity-dependent dendritic growth in sympathetic neurons.

Interestingly, unlike BMP-induced dendritic growth, inhibition of ERK activity inhibited activity-dependent dendritic growth in postnatal sympathetic neurons *in vitro* [113, 114]. Pharmacologic inhibition of ERK by PD98059 blocked activity-dependent dendritic growth, inhibition of GSK-3β increased depolarization-dependent ERK activation, and inhibition of ERK reversed the enhanced dendritic growth observed with GSK-3β inhibition [113, 114]. The reasons for the opposing roles of ERK in BMP-induced vs. activity-dependent dendritic growth remain unexplored.

## 4. The path forward

The experimental evidence clearly implicate NGF, BMP and neuronal activity as positive regulators of dendritic growth in perinatal sympathetic neurons *in vitro*. However, the interaction between these three pathways and their relative contributions to the induction and

maintenance of dendritic growth in sympathetic neurons *in vivo* are not fully understood. Multiple mechanistically and structural diverse signaling molecules have been implicated as negative regulators of dendritic growth *in vitro*. The spatial and temporal regulation of these pathways, how they interact with each other and with positive modulators of dendritic growth to shape dendritic arbors, and their functional significance *in vivo* remain outstanding questions. In addition, further studies are needed to understand the genes and proteins that are regulated downstream of each of these signaling pathways and to identify those that serve as hubs for interactions between multiple signaling pathways to regulate the final dendritic arbor of sympathetic neurons. Finally, a key data gap is the relevance of these observations in experimental models to the human condition.

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## Conflict of interest

The authors declare no conflict of interest.

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