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# BMP7-Induced Dendritic Growth in Sympathetic Neurons Requires p75<sup>NTR</sup> Signaling

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**ABSTRACT:** Dendritic morphology is a critical determinant of neuronal connectivity, and in postganglionic sympathetic neurons, tonic activity correlates directly with the size of the dendritic arbor. Thus, identifying signaling mechanisms that regulate dendritic arborization of sympathetic neurons is important to understanding how functional neural circuitry is established and maintained in the sympathetic nervous system. Bone morphogenetic proteins (BMPs) promote dendritic growth in sympathetic neurons; however, downstream signaling events that link BMP receptor activation to dendritic growth are poorly characterized. We previously reported that BMP7 upregulates p75<sup>NTR</sup> mRNA in cultured sympathetic neurons. This receptor is implicated in controlling dendritic growth in central neurons but whether p75<sup>NTR</sup> regulates dendritic growth in peripheral neurons is not known. Here, we demonstrate that BMP7 increases p75<sup>NTR</sup> protein in cultured sympathetic neurons, and this effect is blocked by pharmacologic inhibition of signaling via BMP type I receptor. BMP7

does not trigger dendritic growth in sympathetic neurons dissociated from superior cervical ganglia (SCG) of p75<sup>NTR</sup> nullizygous mice, and overexpression of p75<sup>NTR</sup> in p75<sup>NTR</sup>−/− neurons is sufficient to cause dendritic growth even in the absence of BMP7. Morphometric analyses of SCG from wild-type versus p75<sup>NTR</sup> nullizygous mice at 3, 6, and 12 to 16 weeks of age indicated that genetic deletion of p75<sup>NTR</sup> does not prevent dendritic growth but does stunt dendritic maturation in sympathetic neurons. These data support the hypotheses that p75<sup>NTR</sup> is involved in downstream signaling events that mediate BMP7-induced dendritic growth in sympathetic neurons, and suggest that p75<sup>NTR</sup> signaling positively modulates dendritic complexity in sympathetic neurons *in vivo*. © 2016 Wiley Periodicals, Inc. *Develop Neurobiol* 00: 000–000, 2016

**Keywords:** BMP7; dendrite; neuronal connectivity; sympathetic neuron; p75NTR

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## INTRODUCTION

Dendrites represent the primary site of synapse formation in the vertebrate nervous system (Purpura, 1967; Purves, 1988; Sejnowski, 1997), therefore, identifying growth factors and mechanisms that regulate the development of dendrites is critical to understanding how neuronal connectivity is controlled during development. Sympathetic neurons provide a well-characterized model for studying dendrite development (Lein et al., 2009). Sympathetic neurons dissociated from rat superior cervical ganglia (SCG) and grown in the absence of serum or ganglionic glia extend a single functional axon, but fail to form dendrites (Bruckenstein and Higgins, 1988). However, the addition of bone morphogenetic proteins (BMPs) triggers these neurons to extend multiple processes that express the morphological, cytoskeletal, and ultrastructural characteristics of dendrites without altering axonal growth or cell survival (Lein et al., 1995, 1996; Guo et al., 1998; Beck et al., 2001). The size of the BMP-induced dendritic arbor in cultured neurons is comparable to that of sympathetic neurons *in vivo*, suggesting that BMPs regulate the full complement of signaling molecules necessary for dendritic growth.

The signaling events that mediate BMP-induced dendritic growth in sympathetic neurons are poorly understood. NGF is not sufficient to trigger dendritic growth in cultured sympathetic neurons, but it is required for BMP stimulation of dendrite formation in cultured sympathetic neurons (Lein et al., 1995, 2007). This observation suggests that neurotrophin signaling may be involved in BMP-induced dendritic growth. The canonical SMAD signaling pathway has also been implicated in BMP-induced dendritic growth *in vitro* (Guo et al., 2001); however, the signaling molecules downstream of or parallel to SMAD activation have yet to be identified. To identify potential downstream mediators of BMP signaling, we performed a microarray screen to identify genes differentially regulated by BMPs in cultured sympathetic neurons (Garred et al., 2011). The p75 neurotrophin receptor (p75<sup>NTR</sup>) was significantly upregulated within 24 h of exposure to BMP7. This receptor is a member of the tumor necrosis factor (TNF) receptor family (Huang and Reichardt, 2001) that interacts with three distinct receptor classes, including the TrkA receptor that binds NGF (Barker, 2004). p75<sup>NTR</sup> has been implicated in the control of dendritic growth in several types of CNS neurons (Carter et al., 2003; Gascon et al., 2005; Salama-Cohen et al., 2005, 2006; Zagrebelsky et al., 2005)

but whether it is also involved in regulating dendritic growth in peripheral neurons is unknown. These observations coupled with evidence that NGF signaling is necessary for BMP-induced dendritic growth in sympathetic neurons (Lein et al., 1995, 2007), led us to test the hypothesis that BMP7-induced dendritic growth in sympathetic neurons requires p75<sup>NTR</sup> signaling. Our findings implicate p75<sup>NTR</sup> as a signaling molecule linking BMPs to dendritic growth in cultured sympathetic neurons, and demonstrate that p75<sup>NTR</sup> modulates dendritic maturation in SCG *in vivo*.

## METHODS

### Materials

Recombinant human BMP-7 was provided by Creative Biomolecules (now known as Curis, Cambridge, MA). A polyclonal antibody (pAb) that specifically binds p75<sup>NTR</sup> was a generous gift from Dr. Philip Barker (University of British Columbia, Vancouver, British Columbia, Canada) and was used in all experiments involving p75 Ab. A recombinant adenovirus vector that expresses wildtype p75<sup>NTR</sup> with a C-terminal V5 tag was kindly provided by Dr. Costanza Emanuelli (Bristol Heart Institute, University of Bristol, UK), and its construction and characterization was previously described (Caporali et al., 2008). DMH-1 (4-[6-[4-(1-Methylethoxy)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline), a specific pharmacologic inhibitor of ALK2, the activin receptor type 1 (Hao et al., 2010), was purchased from Tocris Bioscience (Minneapolis, MN).

### Animals

All procedures involving animals were performed according to protocols approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University (Portland, OR) or the University of California, Davis (Davis, CA). Timed-pregnant Holtzman rats purchased from Harlan (Indianapolis, IN) or timed pregnant Sprague-Dawley rats purchased from Charles Rivers (Hollister, CA) were housed individually in standard plastic cages with Alpha-Dri bedding (Shepherd Specialty Papers, Watertown, TN) in a temperature (22 ± 2°C) controlled room on a 12 h reverse light-dark cycle. Food and water were provided *ad libitum*. Dams and pups were humanely euthanized prior to harvesting of SCG from the pups for culture; no experimental manipulations were performed prior to euthanasia.

Wild-type C57BL/6J and p75<sup>NTR</sup> knockout mice (B6.129S4-*Ngfr*<sup>tm1Jae/J</sup>) were obtained from the Jackson Laboratory (Bar Harbor, Maine). The B6.129S4-*Ngfr*<sup>tm1Jae/J</sup> mice, which contain two mutated exon III alleles preventing the expression of functional p75<sup>NTR</sup> (Siao et al., 2012), were backcrossed to the C57BL/6J background. The p75<sup>NTR</sup><sup>-/-</sup> mice were genotyped upon arrival at Oregon

Health and Science University (Portland, OR) to confirm the presence of the p75<sup>NTR</sup><sup>-/-</sup> mutation and a C57BL/6J genetic background, and a colony was maintained using homozygous breeder pairs, with additional genotyping every 6 months. All mice were kept on a 12 h:12 h light dark cycle with *ad libitum* access to food and water. Age and gender-matched male and female mice were used for all experiments.

### Culture and Expression of cDNA in Sympathetic Neurons

Sympathetic neurons were dissociated from the SCG of perinatal (embryonic day 20–postnatal day 1) Holtzman or Sprague-Dawley rats or postnatal (postnatal day 1–5) mouse pups (p75<sup>NTR</sup><sup>+/+</sup> or p75<sup>NTR</sup><sup>-/-</sup>) as previously described (Higgins et al., 1991). Equivalent results were obtained with cultures derived from either rat strain (data not shown). Briefly, isolated SCG were enzymatically dissociated in collagenase (1 mg/mL, Worthington Biochemical, Lakewood NJ) and dispase (5 mg/mL, Roche, Basel, Switzerland), triturated and plated onto glass coverslips (Bellco Glass, Vineland, NJ) precoated with poly-D-lysine (100 µg/mL, Sigma, St. Louis, MO) at a density of 75 neurons/mm<sup>2</sup>. Cultures were maintained in a serum-free C2 medium (50:50 DMEM and F12 supplemented with 1.4 mM L-glutamine, 10 µg/mL insulin, 5.5 µg/mL transferrin, 38.7 nM selenium; Gibco, Waltham, MA; and 0.5 mg/mL fatty acid free bovine serum albumin, Calbiochem, San Diego, CA) containing β-NGF (100 ng/mL, Harlan Bioproducts, Indianapolis, IN). To eliminate all non-neuronal cells, the antimitotic cytosine-β-D-arabinoside (1 µM, Sigma) was added to the medium of all cultures for 48 h beginning 24 h after plating. BMP7 (50 ng/mL) was added to the culture medium on the 5th or 6th day *in vitro* (DIV) and replenished at every medium change (every 2 to 3 days). Cultures of p75<sup>-/-</sup> neurons were infected on DIV 5 with adenoviral vector at a final titer of 2 × 10<sup>6</sup> plaque forming units (pfu)/mL. On DIV 6, the culture medium was changed and neurons were exposed to BMP7 (50 ng/mL) for 3 days to initiate dendritic growth. Infection efficiencies ranged from 70 to 80%. Potential adverse effects of adenovirus infection on cell viability were determined using calcein-AM (0.5 µM, Invitrogen, Carlsbad, CA) and propidium iodide (2.5 µM, Sigma-Aldrich, St. Louis, MO), which label live and dead cells, respectively.

### Analyses of p75<sup>NTR</sup> Expression in Cultured Rat Neurons

p75<sup>NTR</sup> expression was quantified by western blotting. Cultures were lysed with lysis buffer (10 mM Tris, 150 mM NaCl, 1% glycerol supplemented with 1× protease inhibitor mixture [Calbiochem, San Diego, CA], 1 mM PMSF and 1 mM sodium vanadate). Equal amounts of protein from each lysate, as determined using the Bradford assay (BioRad Laboratories, Hercules, CA), were separated by SDS-PAGE (12%), transferred to polyvinylidene difluoride

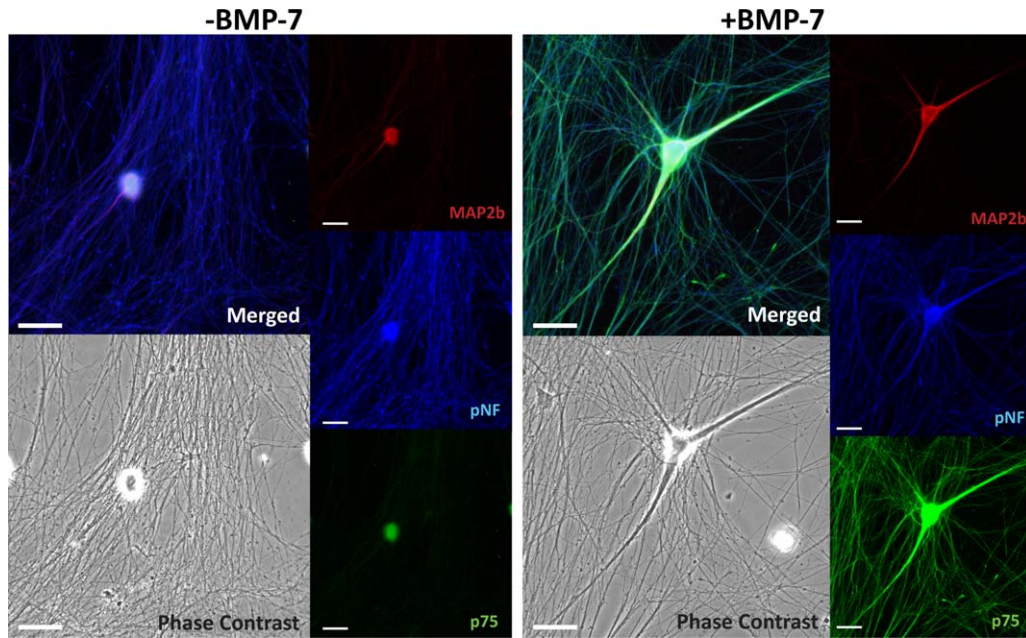
membranes, and then probed with antibodies specific for p75<sup>NTR</sup> and α-tubulin (Sigma Chemical Company, Saint Louis, MO) used at 1:2000 and 1:10,000 dilutions, respectively. To visualize antigen-antibody complexes, blots were reacted with Infrared Dye-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA), and immunoreactive bands quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

To examine the subcellular localization of p75<sup>NTR</sup>, 12 DIV sympathetic neurons were fixed in 4% paraformaldehyde and 0.12 M sucrose in phosphate buffered saline (PBS, 0.1 M phosphate, pH 7.4, 150 mM NaCl), blocked with 2% goat serum albumin and 5% bovine serum albumin in PBS, reacted with p75<sup>NTR</sup> pAb diluted 1:10,000 in blocking buffer, and visualized using Alexa Fluor 488-conjugated secondary antisera (Molecular Probes, Eugene, OR) diluted 1:500 in blocking buffer. The specificity of this p75<sup>NTR</sup> mAb was demonstrated in earlier studies (Lachance et al., 1997). To visualize colocalization of p75 with dendrites and axons, neurons were also reacted with mAb that recognizes the dendrite-selective cytoskeletal protein microtubule-associated protein 2 (MAP2: Synaptic Systems, Goettingen, Germany) as well as a mAb that recognizes the phosphorylated form of neurofilaments (p-NF: Sternberger Immunocytochemicals, Baltimore, MD), which is selectively localized to neuronal somata and axons (Lein and Higgins, 1989). These were both diluted 1:1000 in blocking buffer, and visualized using Alexa Fluor 568-conjugated secondary antisera (Invitrogen, Grand Island, NY) diluted 1:1000 in blocking buffer, and Alexa Fluor 647-conjugated secondary antisera (Invitrogen) diluted 1:500 in blocking buffer, respectively. Fluorescent and phase contrast images were acquired using a Nikon Eclipse E400 microscope equipped with a cooled CCD camera (SPOT RT Monochrome) using the same exposure time across all samples.

### Quantification of Dendritic Growth in Cultured Mouse Neurons

To visualize and distinguish dendrites from axons, cultures were fixed with 4% paraformaldehyde and co-reacted with mAb specific for MAP2 to identify dendrites and mAb for phosphorylated neurofilaments to identify axons as previously described (Lein et al., 2007). In cultures infected with adenoviral vector, neurons were dual labeled using anti-MAP2 mAb and polyclonal antibodies that react with V5 (Molecular Probes) to identify neurons successfully transfected with the adenovirus. The number of primary dendrites, total dendritic length and number of dendritic branch points were quantified per neuron in digitized images of MAP2 immunopositive neurons by individuals blinded to the experimental condition. Image acquisition and morphometric analyses were performed using SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI). Approximately 10 neurons per coverslip were analyzed from six coverslips per treatment. Experiments were replicated three times using cultures derived from three





**Figure 1** BMP7 increases p75<sup>NTR</sup> expression in sympathetic neurons cultured from rat SCG. Representative fluorescence and corresponding phase contrast micrographs illustrating the subcellular localization of p75<sup>NTR</sup> in rat sympathetic neurons grown in the absence (left) or presence (right) of BMP7 (50 ng/mL) from DIV5 through DIV12. On DIV12, cultures were immunostained for MAP2b (red), phosphorylated neurofilament (blue) and p75<sup>NTR</sup> (green). The individual immunoreactivities are shown to the right of the merged image. Similar observations were made in 10 fields from three of three independent cultures per experimental condition. Bar = 50  $\mu$ m.

independent dissections. Statistically significant differences between treatment groups were identified by ANOVA using a  $p$  value of 0.05.

### Quantification of Dendritic Growth in Intact SCG

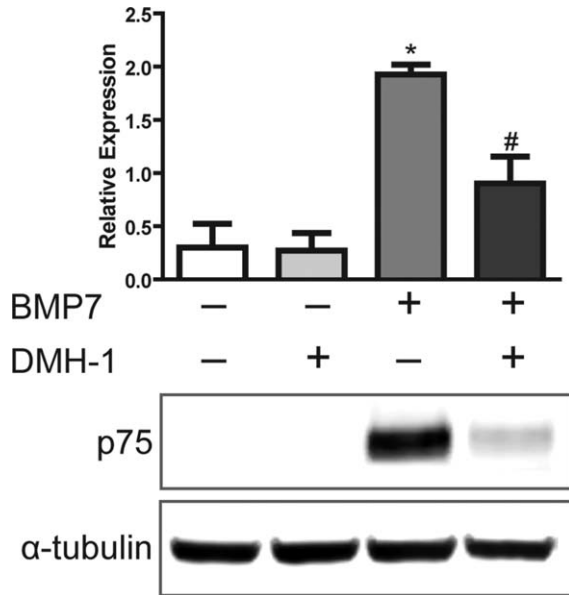
To quantify dendritic arborization of sympathetic neurons *in vivo*, SCG were harvested from euthanized, nonperfused wildtype and p75<sup>NTR</sup><sup>-/-</sup> mice at postnatal days 21, 42, and >84, e.g., at 3, 6 and >12 (12–16) weeks of age, immediately fixed and stored in 4% paraformaldehyde in 0.1 M sodium phosphate buffer at 4°C for up to 4 weeks. Individual neurons in fixed SCG were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI; Molecular Probes, Eugene, Oregon) using a ballistic delivery system known as Diolistics (Grutzendler et al., 2003), as previously described (Kim et al., 2009). Neurons were selected for morphometric analysis if a tungsten particle was located within the cell body of the neuron and the most distal aspects of the dendritic arbor were labeled as evidenced by tapering of fluorescence to very fine tips of dendritic processes. Three-dimensional images were reconstructed using Voxx II software (MSI Global, New Taipei City, Taiwan) and then compressed into two-dimensional images for morphometric analyses of dendritic lengths and somal diameter using NIH ImageJ v.1.33 by individuals

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blinded to the experimental treatment. Statistically significant differences between treatment groups were determined using Student's  $t$ -test at  $p < 0.05$ .

### RESULTS

We previously demonstrated that BMP7 upregulates expression of p75<sup>NTR</sup> mRNA in sympathetic neurons (Garred et al., 2011), and that NGF signaling is necessary for BMP-induced dendritic growth (Lein et al., 1995, 2007). Thus, to test the hypothesis that p75<sup>NTR</sup> signaling is involved in BMP-induced dendrite growth, we first determined whether BMP7 also increases p75<sup>NTR</sup> expression at the protein level. Treatment with BMP7 significantly increased p75<sup>NTR</sup> immunoreactivity relative to sister cultures maintained in the absence of BMP7 (Fig. 1). Within the soma, immunoreactivity for p75<sup>NTR</sup> is observed in neurons grown either in the absence or presence of BMP7. Treatment with BMP7 appears to increase the intensity of somal p75<sup>NTR</sup> immunostaining; however, it is not obvious whether this is a true increase or simply an artifact of the increase in soma size typically observed in cultures treated with exogenous BMP7 (Lein et al., 1995; Majdazari et al., 2013). In contrast,



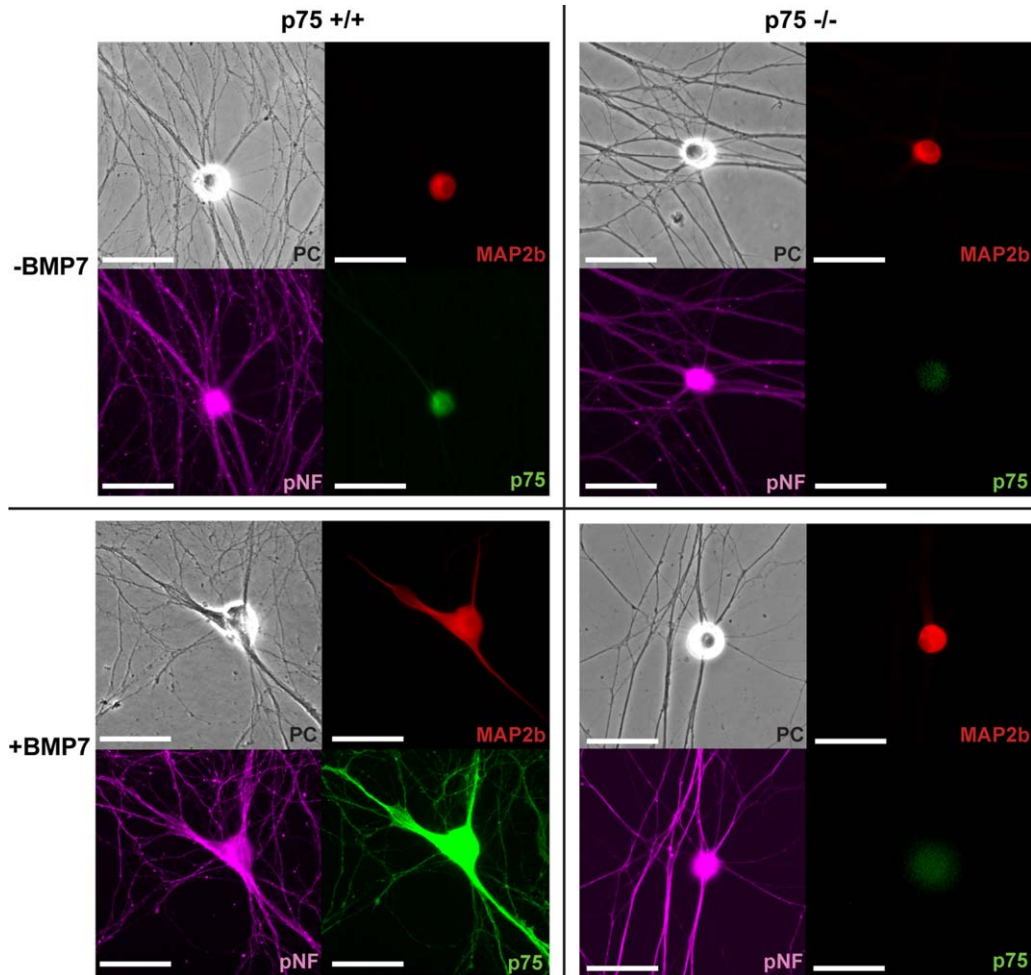
**Figure 2** BMP7-induced p75 expression is blocked by selective pharmacological inhibition of the BMP7 receptor ALK2. Representative western blots and densitometric analyses of cell lysates from cultured sympathetic neurons demonstrate that treatment with BMP7 (50 ng/mL for 48 h) increases p75<sup>NTR</sup> expression. This effect is significantly reduced in cultures pretreated with DMH-1 (final concentration of 5  $\mu$ M in culture medium) to block activation of ALK2. Densitometric values for p75<sup>NTR</sup> immunoreactive bands are normalized to those of  $\alpha$ -tubulin immunoreactive bands in the same sample (mean  $\pm$  SD;  $n = 3$  blots from cultures derived from three independent dissections; \*Significantly different from cultures grown in the absence of BMP7 at  $p < 0.01$ ; #significantly different from BMP7-treated cultures grown in the absence of DMH-1 at  $p < 0.01$  (one-way ANOVA with *post hoc* Tukey's test).

there is a striking difference in p75<sup>NTR</sup> immunoreactivity in the neurites of cultures grown in the absence versus the presence of BMP7. Neurons grown in the absence of BMP7 extend a robust axonal plexus but few to no dendrites, as demonstrated in both the phase contrast micrograph and fluorescence micrograph of this same field showing immunoreactivity for phosphorylated neurofilaments and MAP2b, which are selective for axons and dendrites, respectively [see also (Lein et al., 1995)]. Consistent with previous studies (Lein et al., 1995; Majdazari et al., 2013), treatment with BMP7 increased the number, length and branching of MAP2b-immunoreactive dendritic processes (Fig. 1). In the cultures grown in the absence of BMP7, axonal processes exhibit negligible p75<sup>NTR</sup> immunoreactivity (Fig. 1). In contrast, in cultures treated with BMP7, both axonal and dendritic processes exhibit robust p75<sup>NTR</sup> immunoreactivity, which

appears continuous throughout the cytoplasm of both process types (Fig. 1). Western blot analyses confirmed that treatment with BMP7 increased protein levels of p75<sup>NTR</sup> (Fig. 2). Pretreatment of cultures with DMH-1, a selective inhibitor of the intracellular kinase domain of ALK2 (Hao et al., 2010), the predominant BMP type I receptor for BMP7, effectively blocked the induction of p75<sup>NTR</sup> by BMP7 (Fig. 2).

We next asked whether p75<sup>NTR</sup> was required for BMP7-induced dendrite formation, using sympathetic neurons cultured from mice lacking p75<sup>NTR</sup> (p75<sup>-/-</sup>) and congenic wildtype mice (p75<sup>+/+</sup>). In the absence of BMP, very few neurons expressed any MAP2 immunopositive processes that met the morphological criteria for dendrites regardless of p75<sup>NTR</sup> genotype [Figs. 3 and 4(A)]. Similarly, p75<sup>NTR</sup> genotype had no effect on neuronal cell size [Fig. 4(D)] or the density of the axonal plexus as assessed by phase contrast microscopy and immunoreactivity for phosphorylated neurofilaments (Fig. 3). Treatment of p75<sup>+/+</sup> neurons with BMP7 for 5 days significantly increased the number, length and branching complexity of dendrites relative to cultures that were maintained in the absence of BMP7 (Figs. 3 and 4). In contrast, treating p75<sup>-/-</sup> neurons with BMP7 for 5 days did not alter the number or branching complexity but decreased the length of MAP2 immunopositive processes [Fig. 4(B)]. To further elucidate the role of p75<sup>NTR</sup> in dendrite extension, we infected p75<sup>-/-</sup> sympathetic neurons with an adenovirus expressing p75<sup>NTR</sup> (Caporali et al., 2008), which restores ligand-induced responses and can cause ligand-independent activation of the receptor (Roux et al., 2001). Overexpression of p75<sup>NTR</sup> in p75<sup>-/-</sup> neurons was sufficient to increase the number, length and branching complexity of dendrites relative to p75<sup>+/+</sup> and p75<sup>-/-</sup> neurons grown in the absence of BMP7 [Fig. 4(A-C)]. Addition of BMP7 to p75<sup>-/-</sup> neurons infected with the p75<sup>NTR</sup> adenoviral vector did not further increase dendrite number or branching complexity [Fig. 4(A,C)] but did enhance dendritic length relative to control [Fig. 4(B)]. Thus, overexpression of p75<sup>NTR</sup> in cells that lack the receptor phenocopies the effect of BMP7 on dendrite growth and partially restores responsiveness to the dendrite promoting activity of BMP7. Although BMP7 and p75<sup>NTR</sup> genotype had significant effects on dendrite morphology, they had no effect on cell soma size [Fig. 4(D)].

Since p75<sup>NTR</sup> signaling was required for BMP-stimulated dendrite branching and elongation *in vitro*, we asked whether dendritic morphology would be altered in sympathetic neurons of mice with a genetic deletion of p75<sup>NTR</sup> compared to congenic wildtype mice. Superior cervical ganglia were isolated from 3-,



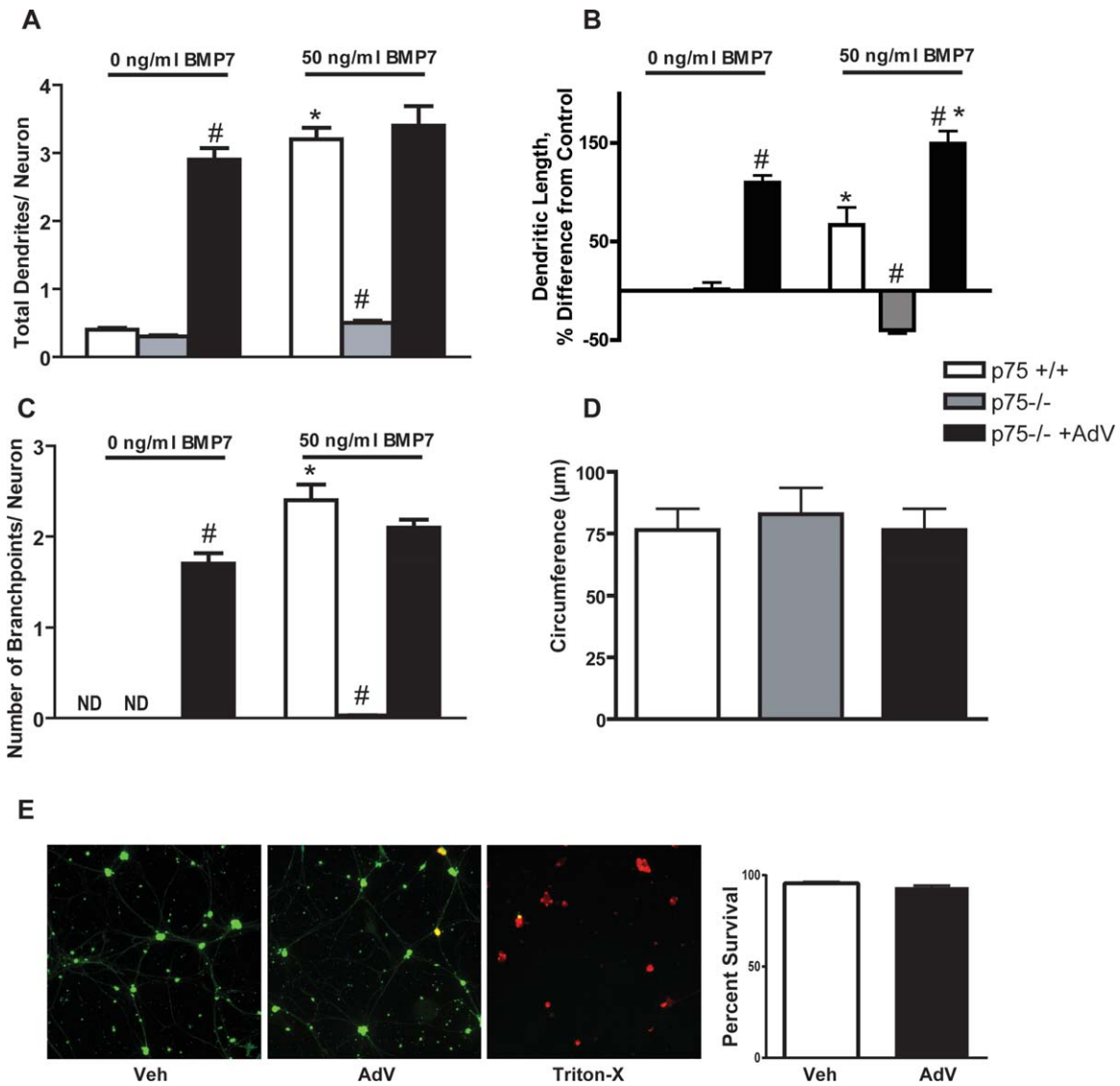
**Figure 3** BMP-induced dendritic growth is attenuated in sympathetic neurons cultured from  $p75^{\text{NTR}}$  nullizygous mice. Representative fluorescence photomicrographs illustrating MAP2 immunoreactivity in sympathetic neurons derived from the SCG of wild-type ( $p75^{+/+}$ ) or  $p75^{\text{NTR}}$  nullizygous ( $p75^{-/-}$ ) mice (left and right, respectively) grown in the absence (top) or presence (bottom) of BMP7 (50 ng/mL) from DIV 6 through 10. At DIV 10, cultures were fixed with 4% paraformaldehyde and triple-labeled for MAP2 (red), a dendrite-selective cytoskeletal protein, phosphorylated neurofilaments (pNF, magenta), an axon-selective cytoskeletal protein, and p75 (green). Staining intensity in cultures derived from  $p75^{-/-}$  mice reacted with mAb specific for p75 was not significantly different from secondary only controls (data not shown). Bar = 50  $\mu\text{m}$ .

6-, and 12 to 16 (>12) weeks old  $p75^{+/+}$  and  $p75^{-/-}$  mice, and individual neurons were labeled by Diolistics [Fig. 5(A)]. Quantification of dendritic arbors revealed that total dendritic length per neuron was significantly reduced in  $p75^{-/-}$  neurons at 6 and >12 weeks, but not at 3 weeks [Fig. 5(B)]. Total number of branch points, however, were significantly reduced at 3 weeks but not at 6 or >12 weeks [Fig. 5(C)]. Circumference of the neuronal cell body did not differ significantly between  $p75^{+/+}$  and  $p75^{-/-}$  neurons at any age. Examining the changes over time within each genotype we observed that in wildtype ganglia the number of dendrite branch points increased

between 6 and >12 weeks and dendrites were lengthened between 3 and >12 weeks of age. In contrast, the number of branch points increased between 3 and >12 weeks of age in  $p75^{-/-}$  ganglia, while overall length was unchanged. This is consistent with a delay in dendrite maturation in sympathetic neurons due to the lack of  $p75^{\text{NTR}}$ .

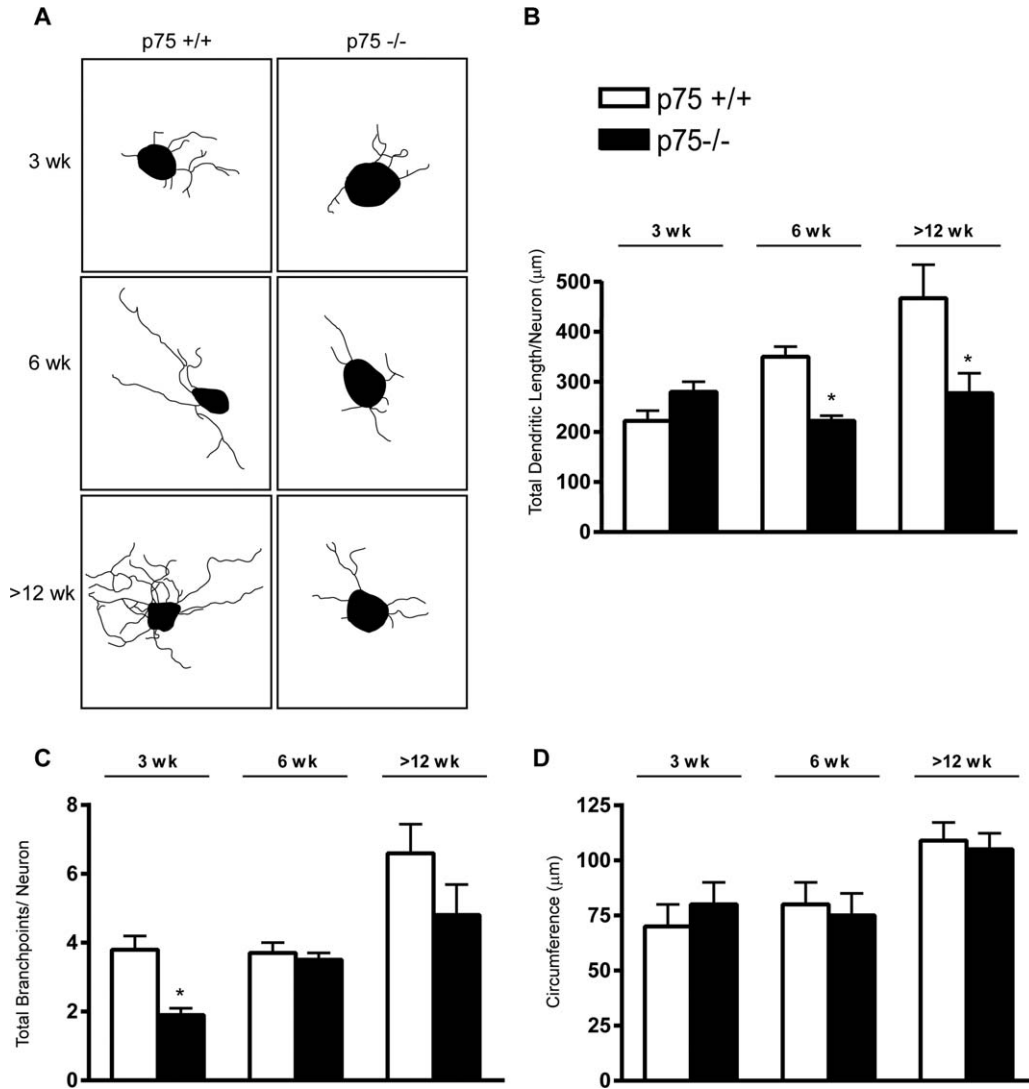
## DISCUSSION

This study provides three lines of evidence to support the hypothesis that  $p75^{\text{NTR}}$  is involved in downstream



**Figure 4** *p75<sup>NTR</sup>* signaling is required for BMP-induced dendritic growth in cultured sympathetic neurons. Sympathetic neurons were dissociated from SCG of *p75<sup>+/+</sup>* mice (white bars) or *p75<sup>-/-</sup>* mice (grey bars) and grown in the absence or presence of BMP7 (50 ng/mL) from DIV 6 through 9. A subset of cultures derived from *p75<sup>-/-</sup>* mice were infected with adenoviral vector expressing *p75<sup>NTR</sup>* tagged with V5 (AdV) on DIV 5 (black bars). At the end of the treatment period, cultures were fixed and immunostained for MAP2b to visualize dendrites and for V5 to identify neurons infected with the *p75<sup>NTR</sup>* adenoviral vector. Dendritic morphology was quantified with respect to the number of primary dendrites (A), the percent change in total dendritic length from control, which was the average for *p75<sup>+/+</sup>* neurons cultured in the absence of BMP7 (B) and the number of branch points (C) per neuron. The circumference of the neuronal cell body (*aka* soma) was measured as an indicator of the trophic status of the neuron (D). Data are presented as the mean  $\pm$  SEM ( $n = 40$  to  $60$  neurons per experimental condition). \*Significantly different from cultures of the same genotype grown in the absence of BMP7 at  $p < 0.01$ ; #significantly different from other genotypes in the same BMP7 treatment group at  $p < 0.01$  (one-way ANOVA with *post hoc* Tukey's test). ND, none detected. (E) The effect of infection with AdV on cell viability was determined by quantifying cellular uptake of calcein-AM (green) and propidium iodide (red) fluorescent dyes in vehicle-treated and ADV-infected cultures. Treatment with Triton-X served as an all-dead control.





**Figure 5** Dendritic growth is altered in SCG of p75<sup>NTR</sup> nullizygous mice. SCG harvested from p75<sup>+/+</sup> mice (white bars) or p75<sup>-/-</sup> mice (black bars) at 3, 6, and >12 (12–16) weeks (wk) of age were immediately post-fixed in 4% paraformaldehyde. A subset of neurons within each ganglia were labeled using Diolistics and their dendritic arbors visualized by confocal microscopy. (A) Representative wire images of the three-dimensional reconstructed confocal images. Wire images were used to quantify dendritic morphology with respect to total dendritic length (B) and number of branch points (C) per neuron. The circumference of the neuronal cell body was measured as an indicator of the trophic status of the neuron (D). Data are presented as the mean  $\pm$  SEM ( $n = 10$  neurons per experimental condition from at least five animals per genotype). Significantly different from p75<sup>+/+</sup> at the same age at  $p < 0.05$  (Student's  $t$ -test).

signaling events that mediate BMP7-induced dendritic growth in sympathetic neurons. First, BMP7 upregulates p75<sup>NTR</sup> at the protein level, an effect that is blocked by DMH-1, a selective inhibitor of the BMP type 1 receptor ALK2 that blocks Smad phosphorylation downstream of BMP receptor 1 activation (Hao et al., 2010). These findings suggest that the canonical Smad signaling pathway mediates BMP-induced p75<sup>NTR</sup> expression. Second, cultured sympathetic

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neurons derived from p75<sup>-/-</sup> mice do not respond to the dendrite-promoting activity of BMP7. Conversely, overexpressing p75<sup>NTR</sup>, which causes ligand-independent activation of the receptor (Roux et al., 2001), phenocopies the effects of BMP7 on dendritic growth. Third, dendritic arborization of neurons in the SCG was significantly stunted in p75<sup>-/-</sup> mice relative to age and sex-matched WT mice. Both *in vitro* and *in vivo* studies suggest that

decreased dendritic growth is not due to adverse effects of genetic deletion of p75<sup>NTR</sup> on neuronal cell viability since the somatal circumference of sympathetic neurons did not vary as a function of genotype. Collectively, these findings demonstrate that p75<sup>NTR</sup> signaling plays a functional role in BMP-induced dendritic growth.

The effects of p75<sup>NTR</sup> knockout on dendritic growth *in vivo* were not as robust as that observed *in vitro*. Furthermore, the parameter of dendritic morphology impacted by p75 genotype *in vivo* (dendritic length versus dendritic branching) varied as a function of age. However, at all time points examined (3, 6 and 12-16 weeks old), dendritic arborization was stunted in neurons in the SCG of p75<sup>-/-</sup> mice relative to p75<sup>+/+</sup> mice, suggesting that *in vivo*, p75<sup>NTR</sup> is not necessary for dendrite formation, but is critically important for dendrite maturation. While the reason for the discrepant *in vitro* versus *in vivo* effects of p75<sup>NTR</sup> deletion on dendritic arborization are not known, one possibility is that the neuronal cell cultures are deficient in other signals that drive dendritic growth in the intact ganglia. This is supported by recent observations that inhibition of BMP signaling by conditional knockout of BMP type I receptors in sympathetic neurons blocks BMP-induced dendritic growth *in vitro* and inhibits dendritic maturation but not initial dendritic formation *in vivo* (Majdazari et al., 2013).

Our work extends previous reports that p75<sup>NTR</sup> signaling positively regulates dendritic growth *in vitro* in subventricular zone-derived neuronal progenitors (Gascon et al., 2005) and *in vivo* in Purkinje cells (Carter et al., 2003). In contrast, p75<sup>NTR</sup> has been reported to negatively regulate dendritic complexity in cultured hippocampal neurons (Salama-Cohen et al., 2005, 2006; Zagrebelsky et al., 2005). The discrepancies between our observations and these latter studies likely reflect differences between experimental models with respect to neuronal cell types and the profile of p75<sup>NTR</sup> binding partners and ligands (Barker, 2004) within each system. The identity of ligand(s) and co-receptor(s) that p75<sup>NTR</sup> interacts with to mediate BMP-induced dendritic growth has yet to be determined. Several lines of evidence argue against a direct interaction between p75<sup>NTR</sup> and the BMP receptor complex. First, our earlier study (Garred et al., 2011) determined that inhibiting transcription blocks both BMP-induced dendritic growth and upregulation of p75<sup>NTR</sup> mRNA. Furthermore, in this study we showed that pharmacologic blockade of BMP7 receptor activation blocks p75<sup>NTR</sup> induction and that overexpressing p75<sup>NTR</sup> in cultured p75<sup>-/-</sup> neurons induces dendritic growth in the absence of

BMP7. NGF is a potential activating ligand since NGF is present in our culture medium and we previously demonstrated that NGF is required for BMP-induced dendritic growth (Lein et al., 1995, 2007). However, the observation that p75<sup>NTR</sup> interactions with TrkA in dendrites serves to limit synapse formation (Sharma et al., 2010) argues against a p75<sup>NTR</sup>-TrkA interaction enhancing dendritic development. Likewise, pro-neurotrophin activation of a p75<sup>NTR</sup>-sortilin complex serves to promote sympathetic axon degeneration and cell death (Nykjaer et al., 2004), suggesting that this is also an unlikely mechanism for stimulating dendrite arborization.

Potential effector molecules for p75<sup>NTR</sup> effects on dendritic arborization include the Rho GTPases. Rho GTPases function as central regulators of dendritic morphology, linking extracellular signals to changes in the dendritic actin cytoskeleton (Redmond and Ghosh, 2001; Van Aelst and Cline, 2004). p75<sup>NTR</sup> has been shown to interact with RhoA in the yeast two-hybrid system (Yamashita et al., 1999), and to activate RhoA in sensory neurons of dorsal root ganglia, cortical neurons and cerebellar neurons *in vitro* (Gehler et al., 2004; Harrington et al., 2008; Sun et al., 2012). RhoA activation by p75<sup>NTR</sup> is consistent with the discrepancy of positive *versus* negative modulation of dendritic arborization by p75<sup>NTR</sup> in sympathetic *versus* hippocampal neurons, respectively. Studies of cultured hippocampal and cortical neurons suggest that RhoA activation negatively regulates activity-dependent dendritic growth (Ruchhoeft et al., 1999; Li et al., 2000; Nakayama et al., 2000; Wong et al., 2000; Sin et al., 2002; Ahnert-Hilger et al., 2004), whereas we previously observed that BMP7-induced dendritic growth in sympathetic neurons requires RhoA activation (Kim et al., 2009). Distinguishing between these possibilities is the focus of future work.

These observations are relevant to not only understanding mechanisms that regulate dendritic arborization during development but may also have implications for understanding remodeling of dendritic arbors after injury. For example, extensive remodeling of cardiac sympathetic neurons is observed when the left stellate ganglion is removed from patients with ischemic or non-ischemic heart disease in order to resolve intractable arrhythmias (Ajijola et al., 2012). Studies in dogs suggest that this ganglionic remodeling is correlated with increased transmission within the ganglion, consistent with the larger dendritic arbor and increased synaptic connections (Han et al., 2012). Similarly, the dendritic arbors of cardiac sympathetic neurons are increased after T5 spinal cord transection (Lujan et al., 2012).

Each of these injury paradigms results in elevated cardiac NGF, and retrograde signaling by NGF is thought to contribute to the dendritic arbor changes observed in these pathologies. Thus, p75<sup>NTR</sup> may be involved in dendrite remodeling after injury in addition to its role in sympathetic dendrite development.

In conclusion, these data demonstrate that p75<sup>NTR</sup> signaling contributes to BMP7-induced dendritic growth in cultured sympathetic neurons, and that dendritic arborization in sympathetic ganglia *in vivo* is modulated by p75<sup>NTR</sup> expression. Collectively, these observations extend previous studies demonstrating that p75<sup>NTR</sup> modulates sympathetic neuron survival, axon outgrowth, and synapse formation during development by interacting with an array of co-receptors that allow it to respond to a diverse set of ligands (Lee et al., 1994; Dechant and Barde, 2002). Our findings together with these previous studies highlight a critical role for p75<sup>NTR</sup> in controlling diverse aspects of sympathetic neuron development and maturation.

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