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Reactive Oxygen Species are Important for Promoting BMP-induced Dendritic Growth in Rat Embryonic Sympathetic Neurons

Abstract

Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease and the process of aging in humans are associated with changes in the neuronal morphology, specifically the retraction of dendrites. The purpose of this research study was to investigate the role of reactive oxygen species (ROS) in sympathetic neurons and to determine whether ROS are primarily harmful or beneficial to dendritic growth. ROS are types of free radicals found in cells, and they include molecules such as hydrogen peroxide, hydroxyl, and superoxide radicals. In large quantities, ROS can cause damage to DNA and kill cells, but recent research has shown that ROS production is necessary for non-cytotoxic and/or host defense functions. In this study, we first examined the effects of the antioxidants diphenylene iodinium (DPI) and nordihydroguaiaretic acid (NGA) on BMP-7 induced dendritic growth in cultures of sympathetic neurons from 21 day old rat embryos. MitoSOX Red and MitoTracker Green FM stains were then used to measure the superoxide concentration in BMP-7 treated neurons directly. In addition, since ROS are known to be produced during cellular respiration, we tested the amount and rate of oxygen consumption in neurons treated with BMP-7 using the Seahorse XF24 Analyzer. Our data suggest that ROS are produced in BMP-7 treated sympathetic neurons and are important to dendritic growth at low physiologic levels. Furthermore, different antioxidants can inhibit BMP-7 induced dendritic growth, indicating that though antioxidants are important for protective effects against excess ROS production, high levels of antioxidants may have undue damage to neurons in the form of decreased dendrite number and decreased dendritic arbor.

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Introduction

Many neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease and the process of aging in humans are associated with changes in the neuronal morphology. Most of the studies on changes in neurons during these diseases have focused on axonal loss and cell death. However, it is clear that the process of aging and neurodegeneration is associated with retraction of dendrites. Therefore, understanding the process of dendritic growth and retraction is important for a better knowledge of neuronal development and injury.

A family of proteins called bone morphogenetic proteins (BMP) is known to specifically induce dendritic growth in different types of neurons in the central and peripheral nervous systems. First named for their ability to induce bone formation, BMPs are secreted signaling molecules of the TGF-β super family and have been shown to affect key developmental processes in the central and peripheral nervous systems important effects in early embryonic development of many organs (Hogan, 1996). BMP-7 was the first protein found to initiate dendritic growth in sympathetic neurons (Lein et al., 1995), and it belongs to the 60A subgroup, which also contains BMP-5, BMP-6/Vr-1, BMP-8a/OP-2, BMP-8b, and Drosophila 60A (Massague, 2000). This subgroup is specifically known to stimulate dendritic growth in cultured sympathetic neurons from adult or perinatal ganglia, regardless of axonal growth.

The primary signal transduction pathway of BMP-7 induced dendritic growth involves the translocation of members of the Smad protein family from the cytoplasm to the nucleus (Guo et al., 2000). BMPs are known to cause the phosphorylation of Smad1 and Smad4, and the nuclear translocation of the Smad1/Smad4 and Smad4/Smad5 complexes. This phosphorylation and following nuclear translocation of Smads is thought to be necessary for BMP signaling and dendritic growth (Heldin et al., 1997, Guo et al., 2000).

Though BMPs are needed for induction of dendritic growth, BMPs do not solely determine how many dendrites a neuron puts out. Sympathetic neurons in vivo are seen to have more complexity in terms of the number of their dendrites and the size of their dendritic arbor than the same neurons grown on a culture dish. This suggests that other molecules are interacting with the BMP pathway to influence dendritic growth. Studies have shown that cytokines, interferons, neurotrophic factors, and retinoic acid can alter BMP induced dendritic growth in sympathetic neurons. In this study, we are exploring another class of molecules, namely, reactive oxygen species, for their effects on dendritic growth.

Reactive oxygen species (ROS) are types of free radicals found in cells, and they include molecules such as hydrogen peroxide, hydroxyl, and superoxide radicals. They are formed primarily in response to injury and inflammatory cytokines, in complexes I and III of the mitochondrial electron transport chain of cellular respiration, and by NADPH oxidases at the plasma and endomembranes (Meier et al., 2001, Mandal et al., 2011). In large quantities, ROS can cause damage to DNA and kill cells. Phagocytic immune cells have long been known to activate NADPH oxidase complexes to produce ROS, which in turn have cytotoxic effects on any pathogen the immune cell might engulf (Veal et al., 2007). However, recent evidence has shown that NADPH oxidases are not found solely in phagocytic immune cells, suggesting that ROS production is necessary for non-cytotoxic and/or host defense functions in other cell types. These functions include cell signaling, hormone biosynthesis, oxygen sensing, extra-cellular matrix cross linking, and fertilization as well as contributing to pathways downstream from growth factors (Geiszt et al, 2004, Schreck et al., 1991). This led to the question of whether or not ROS were important for modulating BMP induced dendritic growth.

The purpose of this research study is to investigate the role of reactive oxygen species in sympathetic neurons and to determine whether ROS are primarily harmful or beneficial to dendritic growth. Our data suggest that ROS are produced in BMP-7 treated sympathetic neurons and are important to dendritic growth at low physiologic levels. Furthermore, different antioxidants can inhibit BMP-7 induced dendritic growth, indicating that though they antioxidants are important for protective effects against excess ROS production, high levels of antioxidants may have undue damage to neurons in the form of decreased dendrite number and decreased dendritic arbor.

Materials and Methods

Materials

BMP-7 was obtained from Creative Biomolecules. NGA (nordihydroguaiaretic acid) and DPI (diphenylene iodinium) were obtained from Sigma. MAP2 monoclonal antibody was obtained from Sternberger Monoclonals. The Phospho-Smad-1 antibody was obtained from Millipore. The MitoSOX Red mitochondrial superoxide indicator (M36008) and MitoTracker Green FM (M-7514) stains, Alexa Fluor secondary fluorescent dyes, and DMEM and F12 base media were purchased from Invitrogen. DMEM and F12 base media were also purchased from Gibco. 2,4-dinitrophenol (DNP) was obtained from Sigma.

Tissue Culture

Sympathetic neurons were dissociated from the superior cervical ganglia (SCG) of E21 perinatal rats according to previously described methods (Higgins, 1991). Cells were plated on glass coverslips pretreated with poly-D-lysine (BD Biosciences, 100 µg/ml) and treated with cytosine-β-D arabinoside (Ara-C) at 1 µM for 48 hours. Cultures were maintained in a 1:1 F12 and DMEM media containing 0.5 mg/ml BSA, 5.5 µg/ml transferin, 38.7 nM selenium, 1.4 mM L-glutamine, 10 µg/ml insulin, and 0.1 µg/ml NGF.

Morphological Analyses

Dendritic growth was assessed in neuronal cultures immunostained with an antibody against MAP2 protein, a microtubule associated protein predominantly present in dendrites (Lein et al 1995). In this process, neuronal cultures were fixed in 4% paraformaldehyde for 10 min, permeablized with 0.1% triton X − 100 in PBS for 5 min, blocked with 5% BSA for 20 min, and then incubated with MAP2 as the primary antibody (1:5000 dilution in 5% BSA) overnight at 4ºC. Antigens were then visualized using a fluorescent secondary antibody (1:1000 dilution in 5% BSA). Dendritic length and dendritic arbor were quantified using the Image J and SPOT image analysis systems. The dendritic arbor of a cell was specifically quantified by summing the lengths of all the dendrites and dendrite branches of the cell.

Cellular distribution of Smad1 was visualized by microscopy after cultures had been immunostained as in the process described above, save with Phospho-Smad-1 (1:100 dilution in 5% BSA), a rabbit polyclonal antibody directed against Smad1 as the primary antibody.

Seahorse XF24 Analyzer

The Seahorse XF24 Analyzer was used as per manufacturer's instructions to measure the oxygen consumption rate (OCR) of sympathetic neurons from E21 rat pups. Prior to being run in the machine, the neurons were incubated in unbuffered DMEM media with 5 mM glucose and 2 mM L-glutamine for 1 hour at 37º C, and during the run 60 µM of 2,4-dinitrophenol (DNP) were injected. DNP is an electron transport chain uncoupler, allowing the neurons to consume oxygen without strict regulation.

Results

Antioxidants inhibit BMP-7 induced dendritic growth

Sympathetic neurons from the superior cervical ganglia of 21 day rat embryos were cultured in the absence of serum and other nonneuronal cells such as glia. As it has been previously observed (Bruckenstein and Higgins, 1988), it was seen that sympathetic neurons did not extend dendrites under these conditions. Dendritic growth was observed when the neurons were treated with a maximal concentration of BMP-7 (50 ng/ml) for between 3 and 5 days, between which BMP-7 induced dendritic growth is very similar. The neurons were then treated with various concentrations of two antioxidants, diphenylene iodinium (DPI) and nordihydroguaiaretic acid (NGA). DPI in an irreversible inhibitor of NADPH oxidase, and NGA helps to upregulate cellular mechanisms for neutralizing free radicals. Lower concentrations of DPI than NGA were used due to DPI having a lower effective concentration than NGA.

Sympathetic neurons grown in control media have round cell bodies and do not extend dendrites, as seen in Figure 1A. Partial axonal staining was seen on the control neurons, for axons can be distinguished from dendrites do their uniform thickness and lack of tapering. Neurons grown in the presence of BMP-7, however, extend many dendrites. The cell bodies of BMP-7 treated cells tend to be less round and more triangular than controls, and the dendrites exhibit a large degree of branching, as seen in Figures 1B. When the neurons were treated with antioxidants in addition to BMP-7, the number of dendrites was seen to decrease relative to the BMP-7 treated cells. DPI added in addition to BMP-7 caused dendrite number to decrease and the dendrites to visibly become shorter in comparison to the BMP-7 neurons (Figure 1D). When treated with various concentrations of NGA, the dendrite number dropped as well relative to BMP-7 treated neurons (Figure C).

The dendrite number and size of dendritic arbor put out by each neuron treatment group was then quantified. Measuring the dendritic arbor gives a more complete picture of the morphology of the neuron and how the morphology of the neuron changes under different conditions, which the dendrite number alone cannot give. The dendritic arbor takes into account not only dendrite number, but dendrite length and branching.

Treatment of sympathetic neurons with DPI plus BMP-7 shows a dose dependent drop in the number of dendrites put out and the dendritic arbor size in comparison to neurons treated with BMP-7 (Figure 2A). Neurons treated with DPI plus BMP-7 had significant dramatic decreases in dendrite number compared to BMP cells when the DPI concentration increased above 30 nM (A). At concentrations of 30 nM, 100 nM, 300 nM, and 1 μM of DPI the dendrite numbers were only 76%, 26%, 16%, and 13% respectively compared to the neurons treated with BMP-7 alone (Figure 2C). Concerning dendritic arbor, neurons treated with DPI at 30 nM plus

BMP-7 and 1 μM plus BMP-7 had a much smaller arbor when compared to BMP-7 cells (Figure 2B), the arbors being 56% and 41% respectively of that of BMP-7 treated neurons (Figure 2D). This indicates that while the dendrites of DPI treated cells might numerically be similar to BMP-7 treated cells, they are morphologically much different, being shorter with less branching.

Neurons treated with NGA plus BMP-7 also show a dose dependent drop in the number of dendrites and the dendritic arbor size. NGA treated neurons had fewer dendrites per neuron compared to BMP-7 treated neurons (Figure 3A), though the drop in number of dendrites was not as seen with DPI plus BMP-7. At concentration of 1 μM, 10 μM, and 100 μM of NGA the dendrite numbers were 95%, 87%, and 76% respectively compared to the neurons treated with BMP-7 alone, the last two percentages being statistically significant (Figure 3C). However, the dendritic arbor sizes of NGA treated neurons were a much smaller percentage of the BMP-7 dendritic arbors. Though NGA 100 μM treated neurons had a dendrite number 76% of the dendrite number seen in BMP-7 treated cells, the dendritic arbor was only 29% of the dendritic arbor of the BMP-7 treated neurons. This is a more dramatic drop, indicating that while dendrites in NGA (100 μM) plus BMP-7 cells might numerically be similar to BMP-7 treated cells, they are morphologically much different, being shorter with less branching. This trend of a greater percentage drop in dendritic arbor size compared to dendrite number was seen in all concentrations of NGA.

In addition, sympathetic neurons were treated with a combination of DPI, NGA, and BMP-7 to determine if the collective effects of the antioxidants caused a greater decrease in dendrite number and dendritic arbor size compared to the neurons treated with one antioxidant and BMP-7. DPI and NGA together plus BMP-7 (Figure 4E) did not appear to alter the number or length of dendrites qualitatively compared to equivalent concentrations of DPI plus BMP-7

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(Figure 4D) and NGA plus BMP-7 (Figure 4C), though the dendrite number and length appeared to be different from BMP-7 treated cells. The neurons treated with the aforementioned combination did show a significant decrease in both dendrite number and dendritic arbor size compared to neurons treated with BMP-7 alone, as seen in Figure 5. However, the decreases were not statistically significant to the neurons treated with either DPI and BMP-7 or NGA and BMP-7.

NGA does not affect the nuclear translocation of Smad proteins

The inhibition of dendritic growth by NGA and DPI suggested that there was an interaction between BMP signaling pathway and the antioxidant pathways. Since BMPs predominantly signal via nuclear translocation of Smad proteins (Guo et al. 2001), sympathetic neurons were stained with a Phospho-Smad-1 antibody, which tags Smad1 protein. Neurons exposed to control media showed Smad staining in the cytoplasm, with no staining in the nucleus. This was similar to neurons treated with NGA alone at 100 µM as evidenced by the red cytoplasmic staining and the dark, unstained nucleus of the cell (Figure 5A, B). Neurons treated with NGA plus BMP-7, however, showed nuclear staining (Figure 5C), indicating that BMP-mediated Smad1 translocation was unaffected by the presence of NGA.

Sympathetic neurons did not show increased ROS production in response to BMP-7

Treatment of sympathetic neurons with DPI or NGA along with dendritic growth factor BMP-7 resulted in a net decrease of dendrite number and size of dendritic arbor. The

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commonality between DPI and NGA is their inhibition of ROS. To further support this evidence, we examined the presence of ROS in BMP-7 treated neurons.

A direct way to measure the presence of ROS is through the MitoSOX Red stain, which causes cells to fluoresce red in the presence of superoxides. MitoSOX Red is commonly used for detection of superoxides at toxic levels. The MitoSOX Red stain for the presence of superoxides was used for sympathetic neurons which had been treated with control medium or a maximal dose of BMP-7 for 2 hours and 2 days. MitoTracker Green FM was used to visualize the mitochondria, which are major sites of superoxide production. A neuronal cell is producing superoxides if the cell fluoresces green with MitoTracker Green FM and then also fluoresces red with MitoSOX Red. If a neuronal cell is not producing superoxides, it should still fluoresce green with MitoTracker Green FM but not fluoresce red with MitoSOX Red. At 2 hours, the control cells show no production of superoxides (Figure 6A), while few BMP-7 treated neurons show production of superoxides (Figure 6C). When the BMP-7 treatment time was extended to 2 days, the BMP-7 treated neurons (Figure 6G) looked similar to the control neurons (Figure 6E).

BMP-7 treatment leads to a change in metabolic oxygen consumption in neurons

Since MitoSOX is used as an indicator to measure ROS at high levels, it was possible that the dye did not have the sensitivity to measure physiologic levels of ROS. Hence, an indirect measure of ROS production was used to address if BMP treated cells may be producing increased levels of ROS. Studies have shown that increase in metabolism leads to an increase in the levels of ROS within the cells. Recent studies have correlated the increase in oxygen

consumption rate measured using the Seahorse XF24 analyzer with increased ROS production (Wen et al., 2011). Therefore, sympathetic neurons treated with BMP-7 were examined for an increase in oxygen consumption, which is an indicator for increased metabolism. The results in Figure 7 show that when sympathetic neurons were treated with a maximal dose of BMP-7, their oxygen consumption rate and thus rate of metabolism increases compared to controls. Control sympathetic neurons consumed oxygen at an average rate of 22 pMoles/min, whereas cells treated with BMP 7 at 24 and 48 hours consumed oxygen at average rates of 39 and 48 pMoles/min respectively, indicating that BMP treated cells had a greater baseline metabolism rate compared to control cells. Uncoupling the electron transport chain with DNP resulted in a much greater increase in oxygen consumption with BMP-7 compared to controls. At 24 and 48 hours, the oxygen consumption rate of cells treated with BMP-7 jumped to 62 and 65 pMoles/min respectively, while the control neurons consumed oxygen at a lower average rate of 40 pMoles/min. BMP-7 treated cells were not significantly different from control cells following a 30 min and 2 hour treatment with BMP-7, suggesting that the increase in metabolism was later than Smad translocation to the nucleus. Given that ROS are byproducts of cellular respiration, an increased rate of metabolism indicates that ROS production must be increased as well. This provides an indirect evidence of ROS production in BMP treated neurons.

A different way to measure ROS production is by measuring cell metabolism, which indirectly proves the presence of ROS. The results in Figure 7 show that when E21 sympathetic neurons were treated with a maximal dose of BMP-7, their oxygen consumption rate and thus rate of metabolism increases compared to controls. The BMP-7 treated cells were initially consuming more oxygen than the controls, and when DNP was added between time measurements 3 and 4, the BMP-7 cells continued to consume more oxygen compared to the

controls. Given that ROS are byproducts of cellular respiration, an increased rate of metabolism indicates that ROS production must be increased as well. This provides indirect evidence of ROS production in BMP-7 treated neurons.

Discussion

Our data indicate that antioxidants can inhibit BMP-7 induced dendritic growth in a dose dependent manner, suggesting that ROS act as intermediates in the signaling cascade that ultimately leads to dendritic growth. This inhibitory effect by antioxidants was dendrite specific. The antioxidants DPI and NGA caused a dose dependent decrease in BMP-7 induced dendrite and dendritic arbor growth. DPI was a stronger dendritic growth inhibitor compared to NGA, and this is expected to be due to the functions of the antioxidants. DPI is an irreversible inhibitor of NADPH oxidase, one of the primary production pathways for ROS. NGA upregulates cellular mechanisms for neutralizing radicals. Blocking ROS production from the start with DPI appears to be a more effective way at inhibiting BMP-7 function than eliminating ROS with NGA once they have already been generated. So while NGA can neutralize ROS, it cannot directly prevent their production. Future studies with other classes of common antioxidants—such as Vitamins C and E—will indicate if all types of antioxidants have similar effects to those shown by DPI and NGA is this study as well as give a more complete picture about how BMP-7 induced dendritic growth is regulated

While the average number of dendrites and the average size of the dendritic arbor in BMP-7 treated neurons varied between different experiments (Figures 2 and 3), the same general trends were seen. The average number of dendrites and the average size of the dendritic arbor

were always lower in neurons that had been treated with antioxidants plus BMP-7 compared to neurons treated with BMP-7 alone. As well, the dendrite number and dendritic arbor size decreased as the antioxidant concentration increased. Variability in the number of dendrites and size of dendritic arbor induced to grow by BMP-7 can be due to factors such as cell plate density, maturity of rat pups upon dissection, and intrinsic differences between superior cervical ganglia in individual rat pups.

The idea behind the DPI and NGA combination study was that in blocking both a major source of production and source of neutralization of ROS, BMP-7 induced dendritic growth would be inhibited to a much greater extent than just by DPI and NGA alone. Neurons treated with a combination of DPI, NGA, and BMP-7 did have significantly lower dendrite numbers and dendritic arbor sizes compared to BMP-7 treated neurons. However, the two antioxidants did not appear to have a significant effect on dendritic growth compared to neurons treated with DPI plus BMP-7 and NGA plus BMP-7, suggesting that the two antioxidants do not have a great synergistic effect.

BMPs are known to cause the phosphorylation of Smad1 and Smad4 and the nuclear translocation of the Smad1/Smad4 and Smad4/Smad5 complexes. This phosphorylation and following nuclear translocation is thought to be necessary for BMP signaling and dendritic growth (Heldin et al., 1997, Guo et al., 2000). Sympathetic neurons treated with control media or the antioxidant NGA alone did not show Smad1 nuclear translocation and did not extend any dendrites, while neurons treated with NGA plus BMP-7 did show Smad1 nuclear translocation and did extend dendrites (Figure 5). The fact that NGA did not block Smad1 translocation in BMP-7 treated cells indicates that NGA's negative effects on the BMP-7 pathway occur further downstream from the site of Smad1 translocation.

Sympathetic neurons were stained with MitoSOX Red and MitoTracker Green FM in order to directly determine if ROS were being produced in the form of superoxides when BMP-7 was present. After 2 hours, the control neurons did not show any superoxide production. The BMP-7 treated neurons showed superoxide production, but in a low number of cells (Figures 6C and 6D). This low superoxide production is likely related to the timing of the translocation of Smad1—when sympathetic neurons are treated with BMP-7, it takes up to two hours for Smad1 to translocate to the nucleus, and then up to 24 hours for the neurons to start putting out their first dendrites (Guo et al., 2000). Only when Smad1 has translocated to the nucleus is BMP-7 induced dendritic growth seen.

After 2 days of BMP-7 treatment, however, the BMP-7 treated neurons looked similar to the controls. There are number of possible reasons for the inability to detect ROS production in sympathetic neurons. The first possibility is that the MitoSOX indicator was not sensitive to detect physiologic levels of ROS. Most of the studies using this indicator examine ROS at toxic levels. The other possibility is that the sympathetic neurons produce other types of reactive species besides superoxide radicals. Since MitoSOX indicator only detects superoxide radicals, the indicator would not be able to detect other radicals such as peroxide, hydroxyl and nitrous oxide radicals. The third possibility is that the ROS production is transient to trigger a signaling pathway, but is not present in the sympathetic neurons at the time point of measurement.

Though BMP treated neurons did not show an increase in ROS production, there was a significant increase in oxygen consumption in BMP-7 treated neurons compared to control cells, providing indirect evidence of ROS production. If BMP-7 treated neurons are putting out processes and the cell body shape is becoming less round compared to controls (Figures 1A and 1B), the neurons should be ramping up their metabolism in order to grow. This requires an

increase in oxygen consumption rate (OCR), and by extension and increase in ROS as electrons move through the electron transport chain during cellular respiration. An OCR increase was found to occur in BMP-7 treated cells when DNP was added to them, thus uncoupling complexes in the electron transport chain (Figure 7). This is the first time that BMP-7 treated sympathetic neurons have been shown to increase their metabolism. A significant difference was not observed in neurons treated with BMP-7 for 24 hours or 48 hours, suggesting that at some time after BMP-7 exposure the OCR and ROS production rate hits a plateau. Garred et al. (2011) recently discovered that BMP-7 specifically regulates 56 unique genes at 6 hours and 185 unique genes after 24 hours in sympathetic neurons. Among these genes, genes that are known to correspond to metabolic functions were found to be significantly upregulated. Our results show physiologically what Garred et al. (2011) indicate is happening genetically during BMP-7 induced dendritic growth.

Many neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are characterized by neuronal apoptosis induced by oxidative stress, which is believed to be caused by overproduction of ROS (Zhao 2009). To counter the oxidative stress, antioxidants are seen as potential treatments for these diseases due to their neuroprotective properties (Zhao 2009, Simonyi et al., 2010). Though antioxidants such as vitamin E have not been found to benefit Alzheimer's or Parkinson's patients (Brewer 2010, Thomas 2009), antioxidants such as Co-enzyme Q10 found in the electron transport chain have shown promise in early clinical trials (Thomas 2009). Our data provide new insights into neuronal injury and treatment of neurodegenerative disease. BMPs are known to be protective against neuronal injury and apoptosis (Yabe et al., 2002), and since ROS seem to be involved in BMP signaling they may be part of cell protection mechanisms as well at low physiological levels. Though antioxidants are

important for protective effects against excess ROS production, high levels of antioxidants may have undue damage to neurons in the form of decreased dendrite number and decreased dendritic arbor.

In summation, this study demonstrates that ROS at low physiologic levels are likely nontoxic to sympathetic neurons and are involved in BMP-7 signaling. As well, the data suggest that ROS are present during BMP-7 induced dendritic growth as sympathetic neurons increase their metabolism.

Figure 1: Antioxidants qualitatively reduce dendrite number in BMP-7 treated neurons

Cultures of sympathetic neurons from E21 rat pups were treated with BMP-7 (50 ng/ml) (B), NGA at 100 μM plus BMP-7 (C), and DPI 1μM (D). The cultures were then immunostained with MAP2 antibody (1:1000 dilution in 5% BSA) and visualized by microscopy under UV light with a secondary fluorescent antibody. All photographs were taken at 20 X magnification. Neurons treated with various concentrations of NGA or DPI alone looked similar to those treated with control media.

(A) Control

Axonal Staining

Cell Body
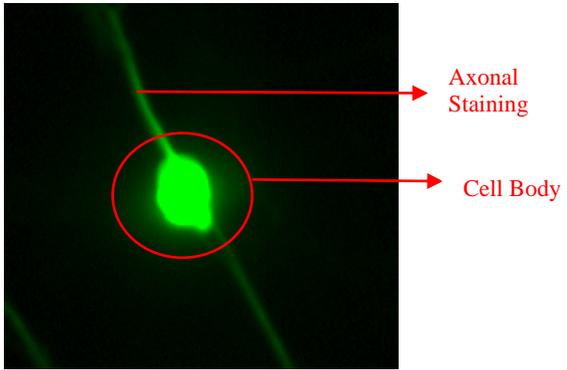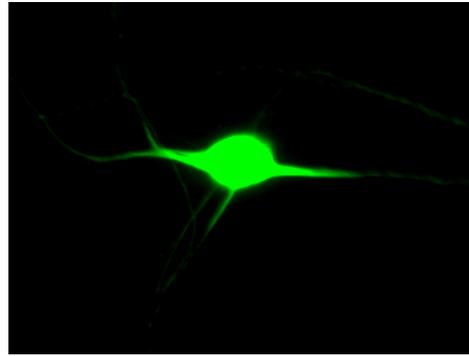
(C) NGA 100 μM + BMP-7
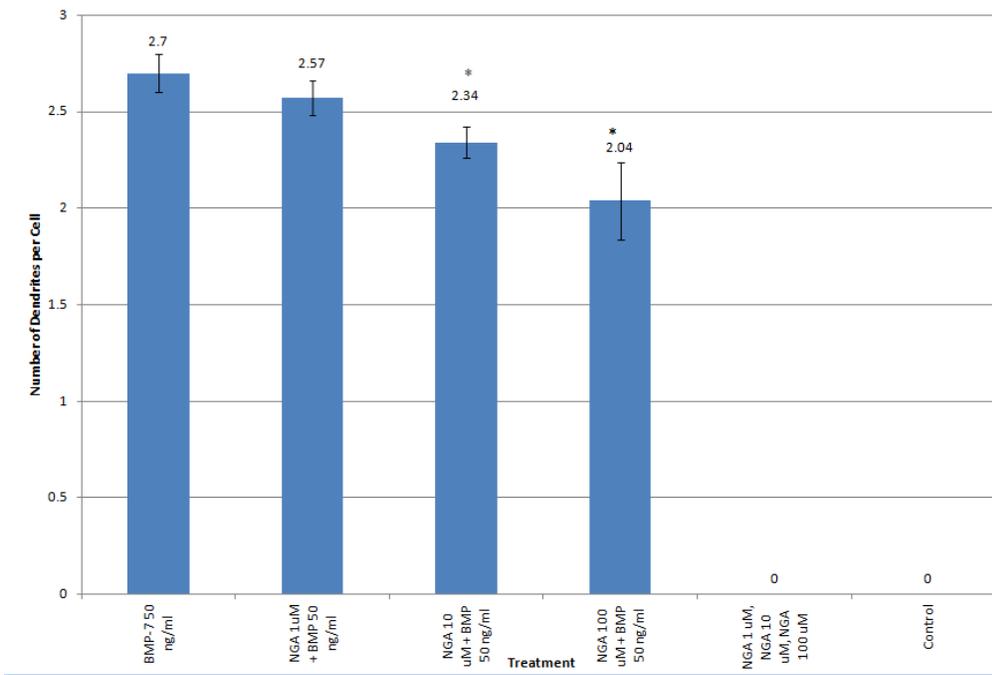

(B) BMP-7 50 ng/ml

Dendrites

(D) DPI 1 μM + BMP-7

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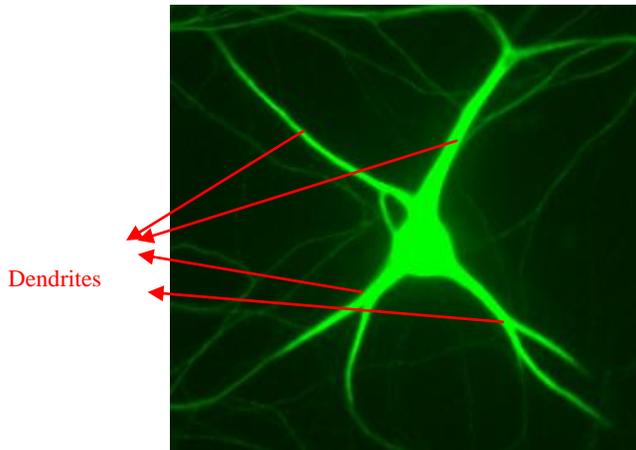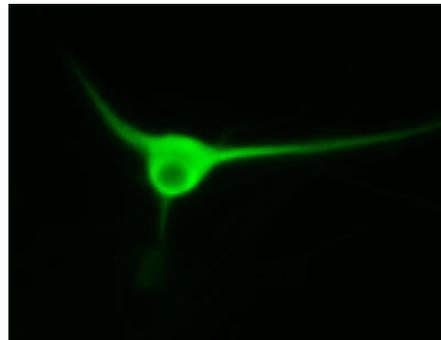
Figure 2: Effect of DPI on BMP-7 induced dendritic growth

Cultures of sympathetic neurons from E21 rat pups were treated with either control medium, BMP-7 (50 ng/ml), DPI (100 nM, 300 nM, 1 μM), or DPI plus BMP-7 for one day. Since, DPI is an irreversible inhibitor it was removed from the medium by repeated rinsing with the control medium. These neurons were then maintained in control medium or in BMP-7 (50 ng/ml) for additional 3 days. The neurons were then immunostained with the MAP2 antibody against dendritic proteins, and the number of dendrites per cell was subsequently measured. The changes in the number of dendrites/cell are shown in (A) and the changes to the dendritic arbor are graphed in (B). The data are expressed as number of dendrites /cell (A, N ≈ 100) and dendritic arbor size per cell (B, N ≈ 50). The average number of dendrites and average dendritic arbor size were of the DPI treated neurons were also calculated as a percentage of the neurons put out by a neuron treated with BMP-7 alone. (C) is the percentage of the neuron numbers and (D) is the percentage of the dendritic arbor size. These graphs consolidate the data from (A) and (B), and each data point percentage is relative to the BMP-7 group from the experiment it was originally calculated from, since BMP-7 induced dendritic growth can vary among cultures.

*$p \leq 0.05$ by Student T-test as compared to cultures treated with BMP-7.

Number of Dendrites per Cell Treated with BMP-7 50 ng/ml and BMP-7 + DPI

(A)
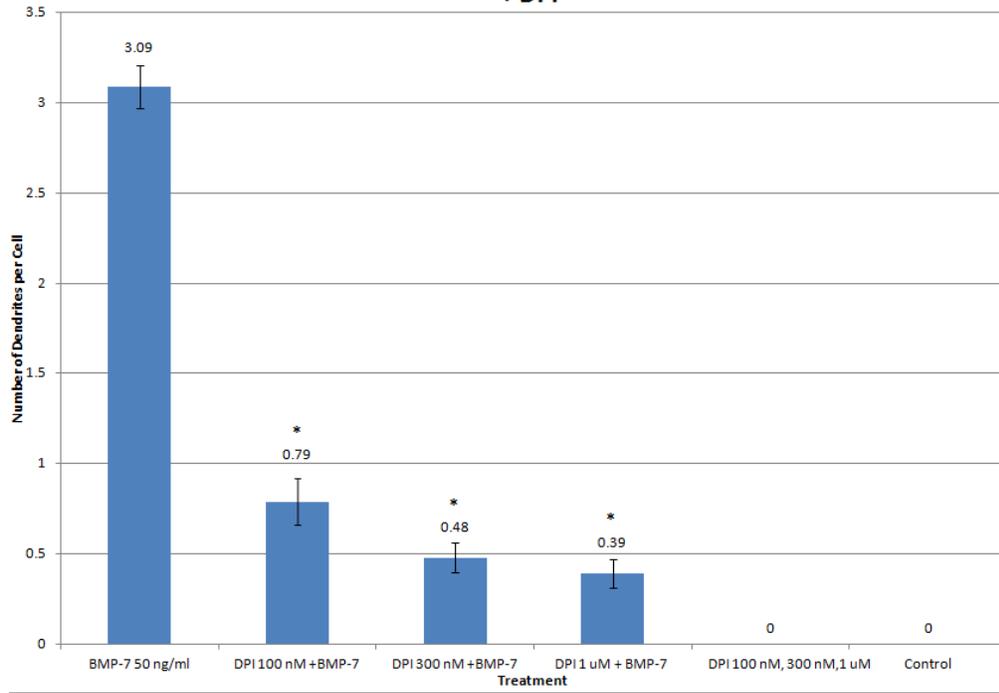
Average Dendritc Arbor of BMP-7 and DPI + BMP-7 Treated SCG
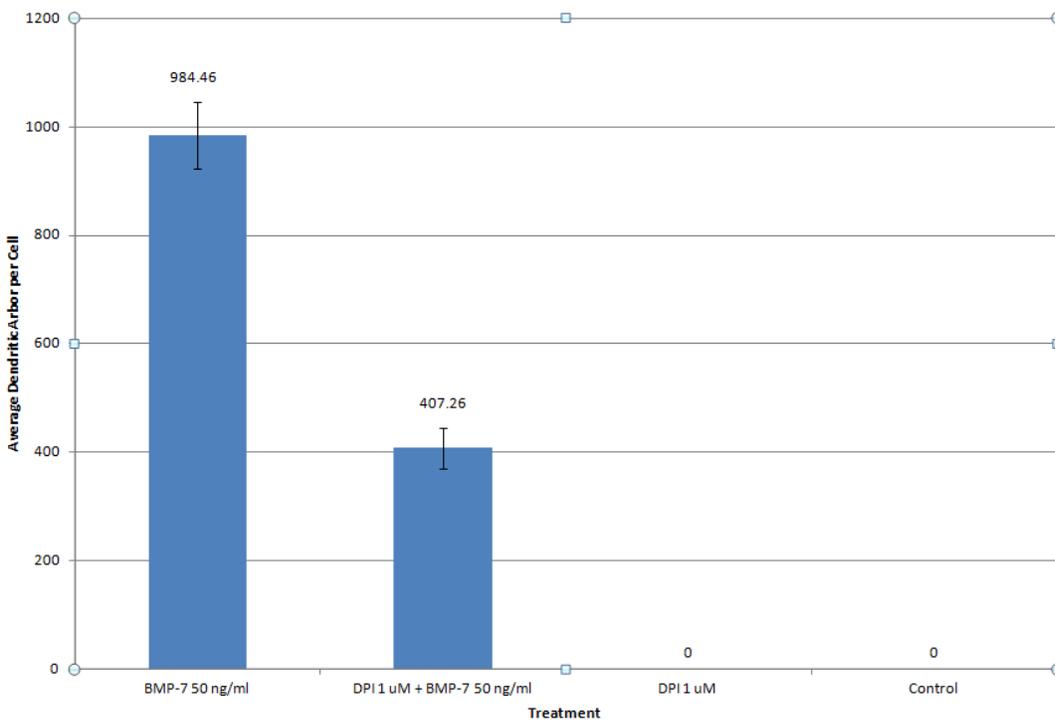
(B)

Percentage comparison of the effect of DPI on BMP-7 induced dendritic growth
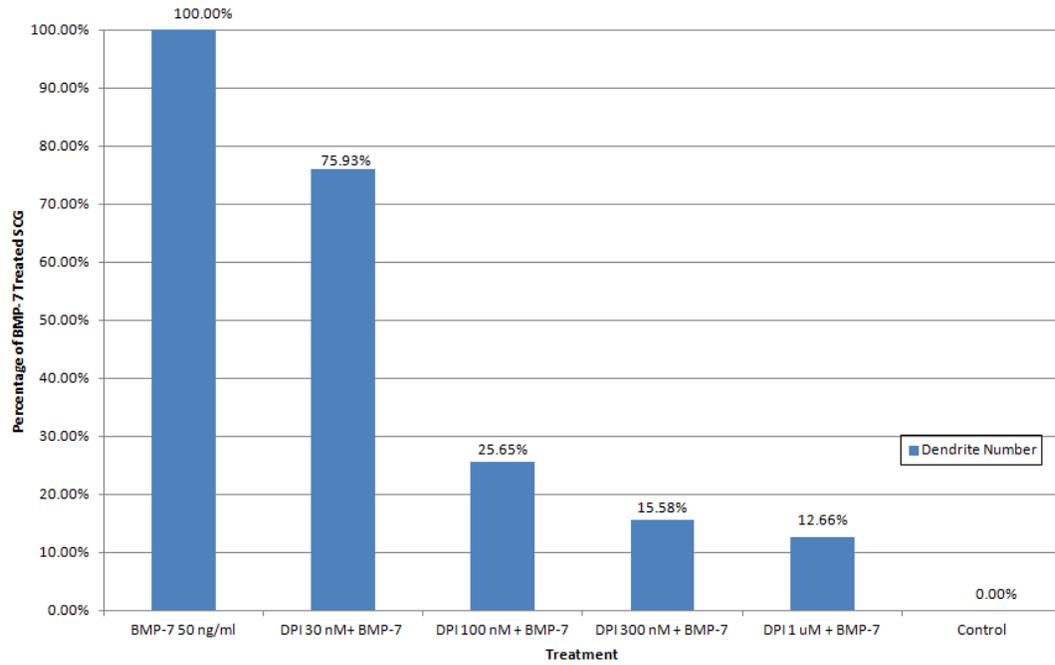
(C)

Percentage comparison of the effect of DPI on BMP-7 induced dendritic arbor
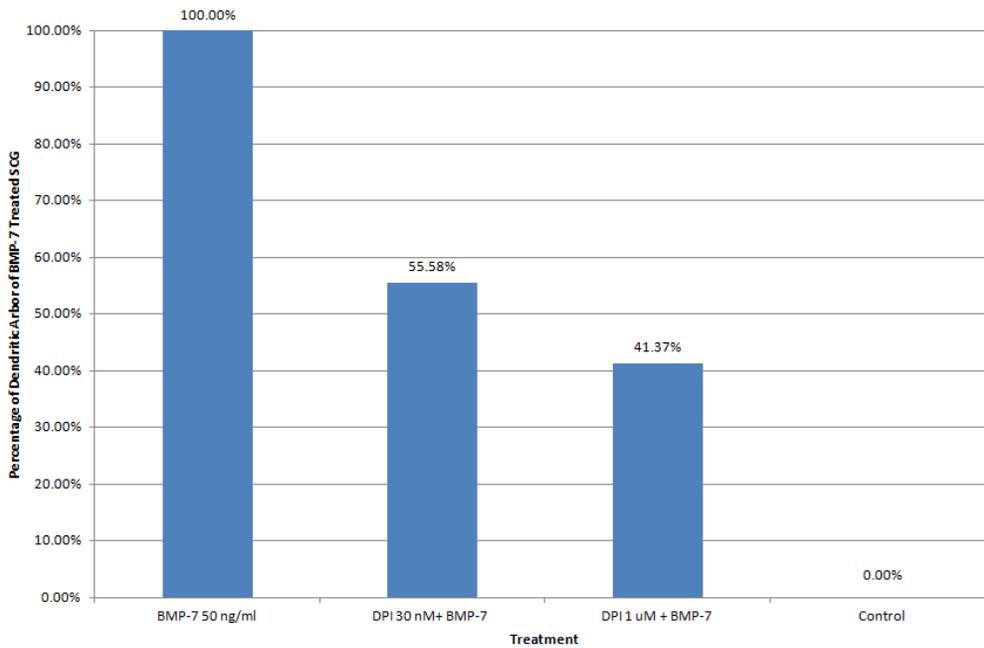
(D)

Figure 3: Effect of NGA on BMP-7 induced dendritic growth

Cultures of sympathetic neurons from E21 rat pups were treated with either control medium, BMP-7 (50 ng/ml), NGA (1 µM, 10 µM, 100 µM), or NGA plus BMP-7 for 5 days. The cultures were then immunostained with MAP2 antibody and the number of dendrites per cell was subsequently measured. The changes in the number of dendrites/cell are shown in (A) and the changes to the dendritic arbor are graphed in (B). The data are expressed as number of dendrites /cell (A, N ≈ 105) and dendritic arbor size per cell (B, N ≈ 50). The average number of dendrites and average dendritic arbor size were of the NGA treated neurons were also calculated as a percentage of the neurons put out by a neuron treated with BMP-7 alone (C). This graph consolidate the data from (A) and (B), and each data point percentage is relative to the BMP-7 group from the experiment it was originally calculated from, since BMP-7 induced dendritic growth can vary among cultures.

*$p \leq 0.05$ by Student T-test as compared to cultures treated with BMP-7.

Number of Dendrites per Cell Treated with BMP-7 and BMP-7 + NGA

(A)

Average Dendritic Arbor per Cell Treated with BMP-7 and BMP-7 + NGA

(B)

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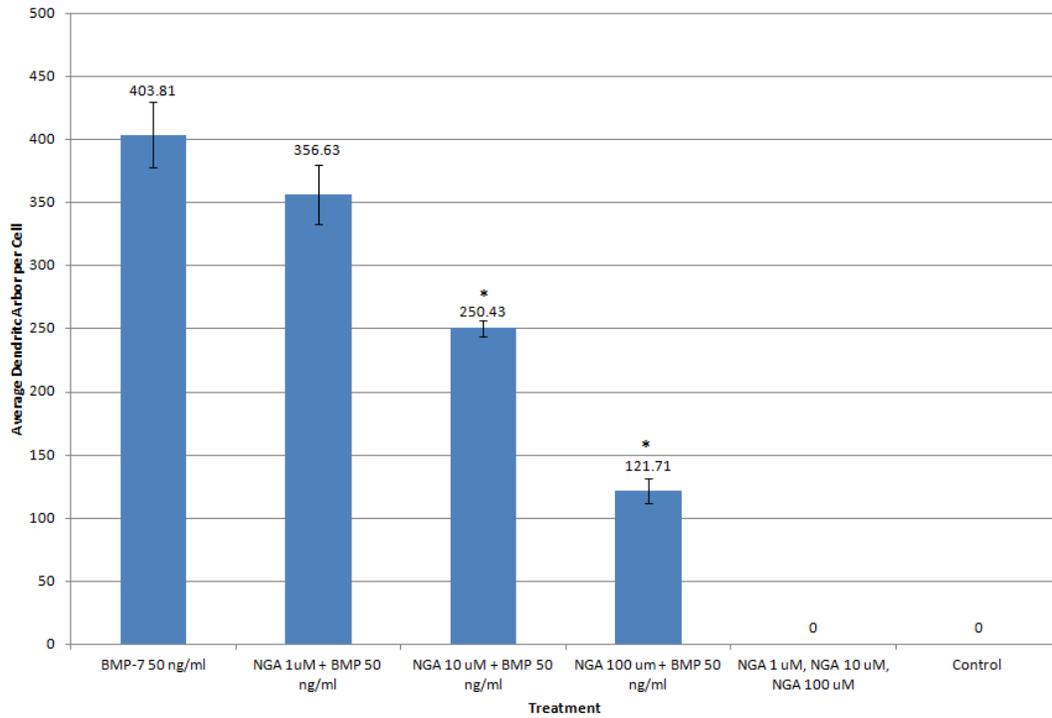
Percentage comparison of the effect of NGA on BMP-7 induced dendritic growth and dendritic arbor size
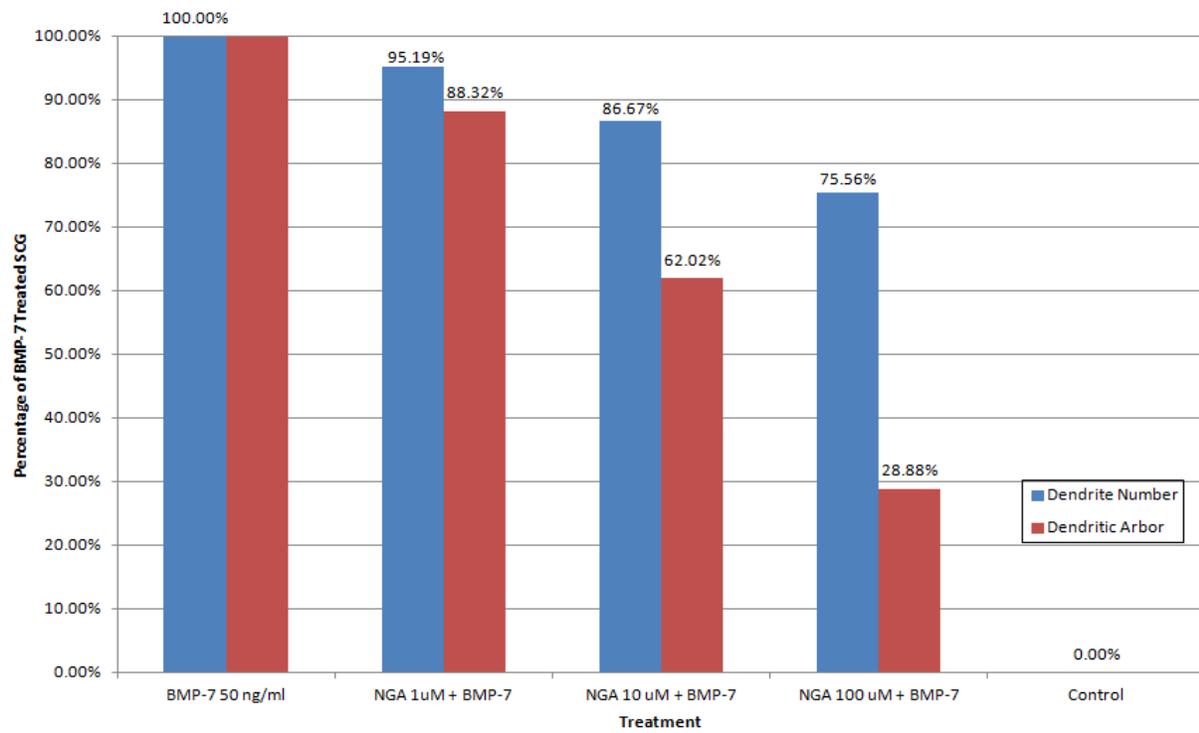
(C)

Figure 4: A combination of DPI and NGA qualitatively reduce dendrite number in BMP-7 treated neurons

Cultures of sympathetic neurons from E21 rat pups were treated with BMP-7 (50 ng/ml) (B), NGA plus BMP-7 (10 µM) (C), DPI plus BMP-7 (30 nM) (D), and DPI and NGA plus BMP-7 (E). The controls are seen in (A). The cultures were then immunostained with MAP2 antibody (1:1000 dilution in 5% BSA) and visualized by microscopy under UV light with a secondary fluorescent antibody. All photographs were taken at 20 X magnification. Neurons treated with various concentrations of DPI, NGA, and DPI plus NGA alone looked similar to those treated with control media.
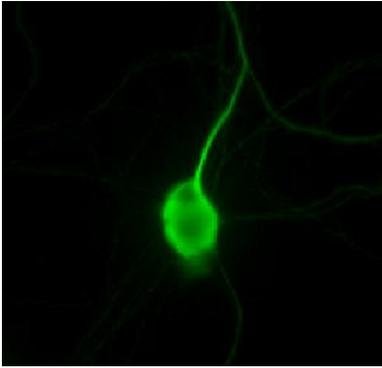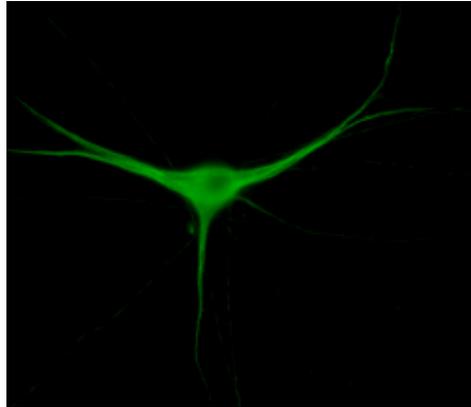
(A) Control

(C) NGA 10 μM + BMP-7
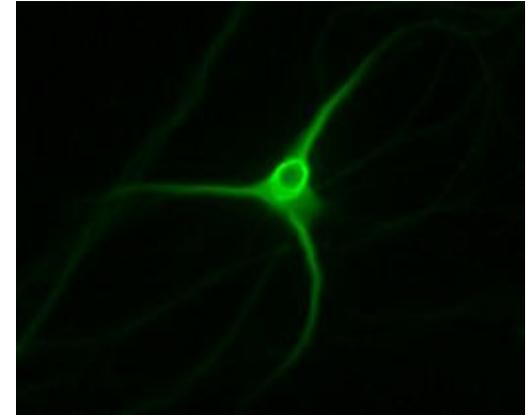
(E) DPI 30 nM + NGA 10 μM + BMP-7
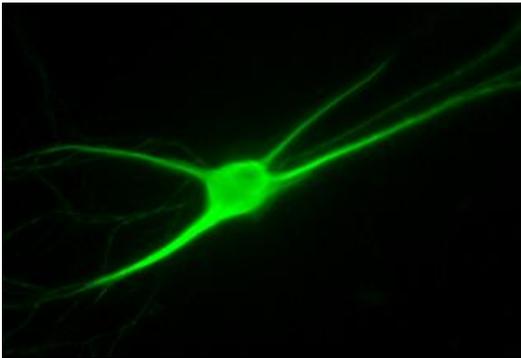
(B) BMP-7 50 ng/ml
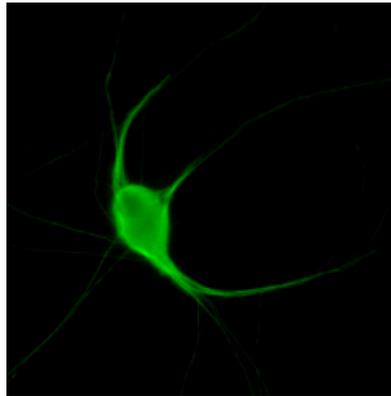
(D) DPI 30 nM + BMP-7

Figure 5: Effect of a combination of NGA and DPI on BMP-7 induced dendritic growth

Cultures of sympathetic neurons from E21 rat pups were treated with either control medium, BMP-7 (50 ng/ml), DPI (30 nM), NGA (10 µM), DPI plus NGA, or DPI and NGA and BMP-7 all together for 3 days. The cultures were then immunostained with the MAP2 antibody, and the number of dendrites per cell was subsequently measured. The changes in the number of dendrites/cell are shown in (A) and the changes to the dendritic arbor are graphed in (B). The data are expressed as number of dendrites per cell (A, N ≈ 36) and dendritic arbor size per cell (B, N ≈ 35).

*p≤0.05 by Student T-test as compared to cultures treated with BMP-7.

Number of Dendrites per Cell Treated with BMP-7, NGA + BMP-7, DPI + BMP-7, and NGA + DPI + BMP-7

(A)
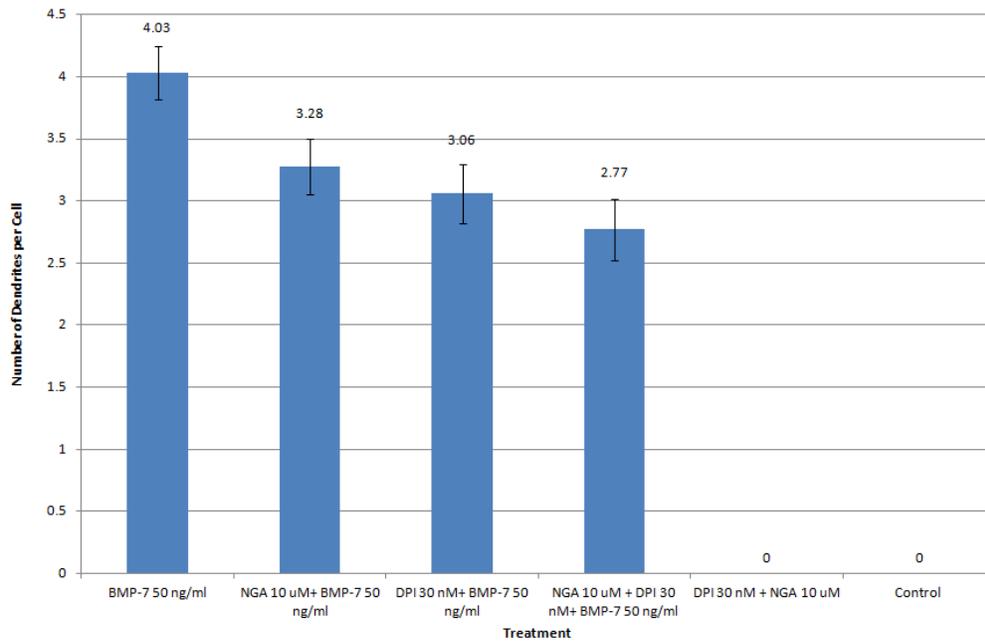
Average Dendritic Arbor of Cells Treated with BMP-7, NGA + BMP-7, DPI + BMP-7, and NGA + DPI + BMP-7
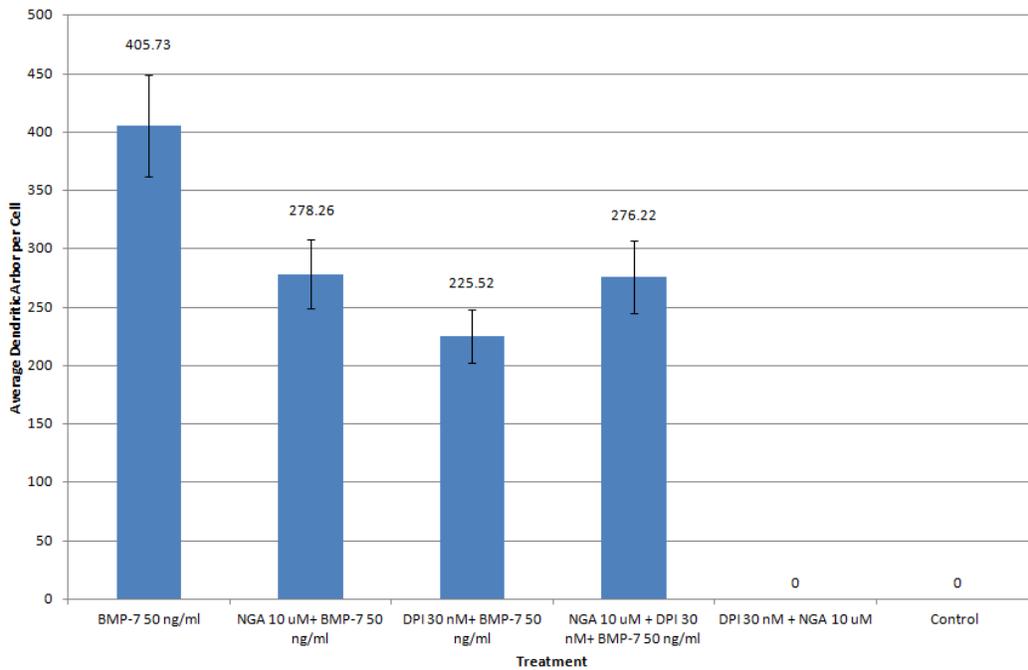
(B)

*Figure 5: Smad1 translocation in neurons treated with BMP-7 and NGA*

Cultures of sympathetic neurons from E21 rat pups were grown on media and then treated with (A) control medium, (B) NGA (100 μM), or (C) NGA (100 μM) plus BMP-7 (50 ng/ml) for 2 hours and then immunostained with a rabbit antibody to Smad1. When seen at 20X magnification, the control and NGA treated cells have staining in the cytoplasm while the nucleus remains dark and unstained. The NGA plus BMP-7 cells have staining predominately in the nucleus.
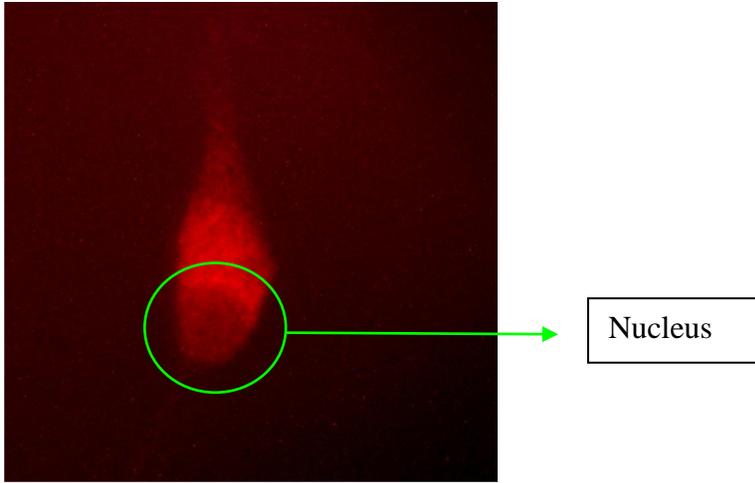
(A)
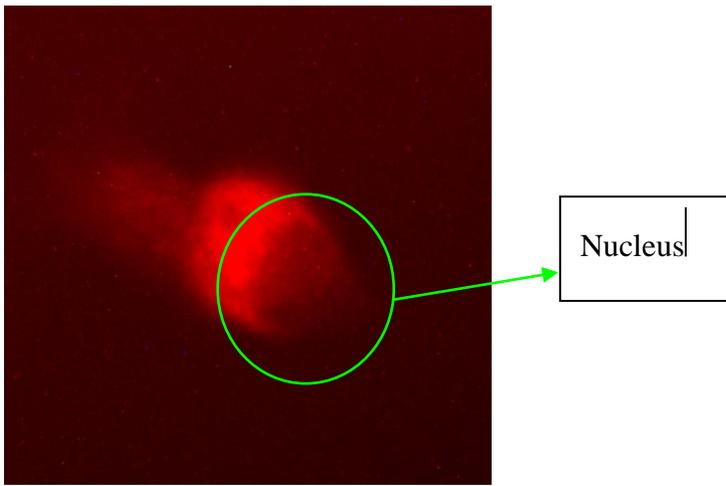
(B)
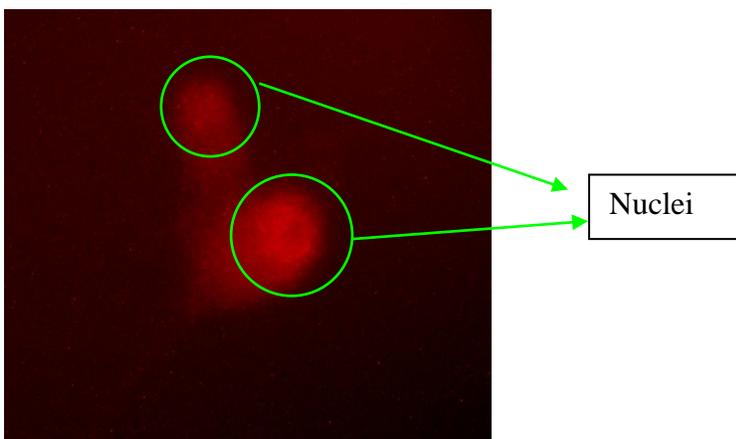
(C)

Figure 6: MitoSOX Red mitochondrial superoxide indicator and MitoTracker Green FM mitochondrial stain indicate that there is superoxide production in BMP-7 treated neurons

Cultures of sympathetic neurons form E21 rat pups were treated with control medium or BMP-7 (50 ng/ml) for 2 hours or 2 days. The neurons were then stained with MitoSOX Red and MitoTracker Green FM and imaged using an Olympus Confocal microscope at 20X magnification. MitoSOX Red fluoresces red in the presence of superoxides, and MitoTracker Green FM stains mitochondria with green fluorescence. (A) MitoSOX Red stain for control cells at 2 hours (B) MitoTracker Green FM stain for control cells at 2 hours (C) MitoSOX Red stain for BMP-7 treated cells at 2 hours (D) MitoTracker Green FM stain for BMP-7 treated cells at 2 hours (E) MitoSOX Red stain for control cells at 2 days (F) MitoTracker Green FM stain for control cells at 2 days (G) MitoSOX Red stain for BMP-7 treated cells at 2 days (H) MitoTracker Green FM stain for BMP-7 treated cells at 2 days.
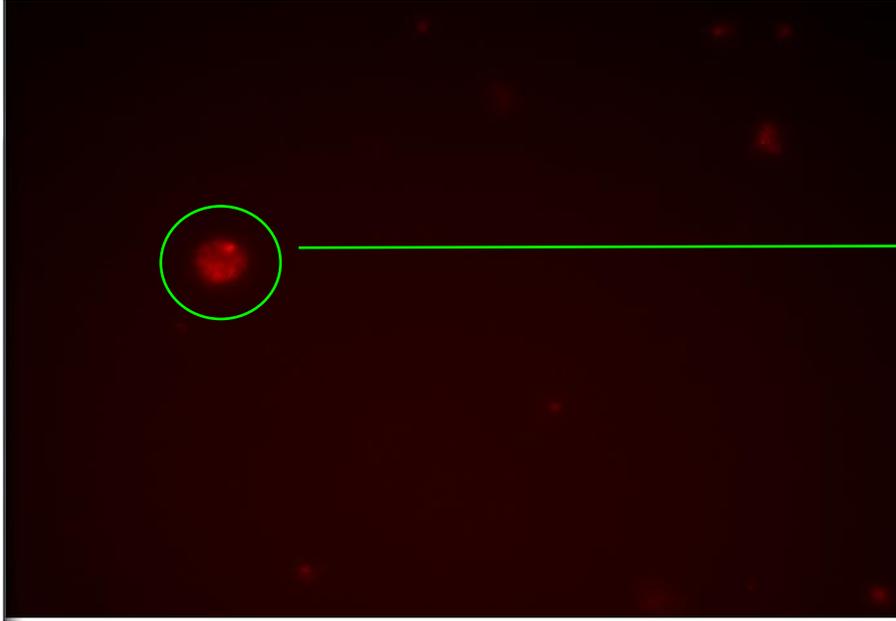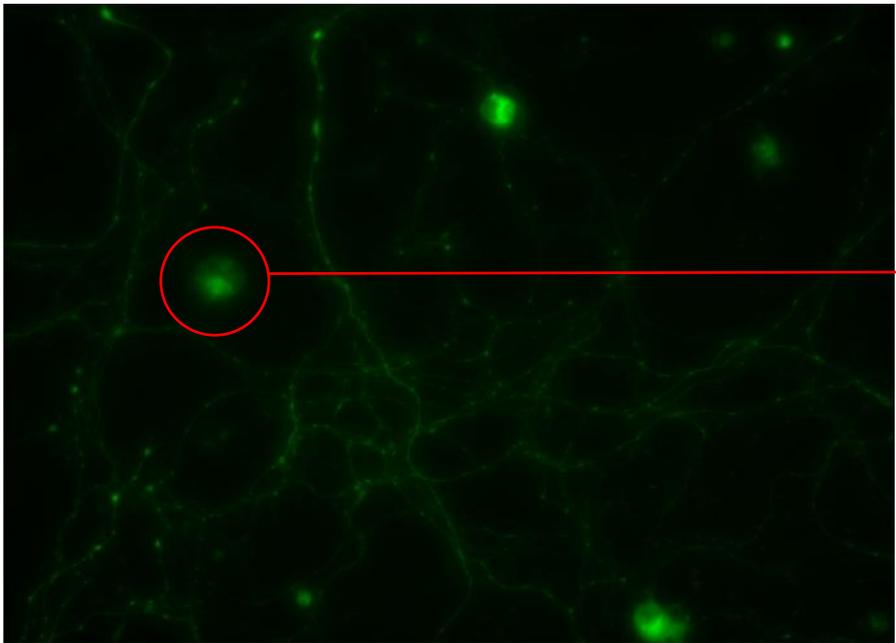
(A)
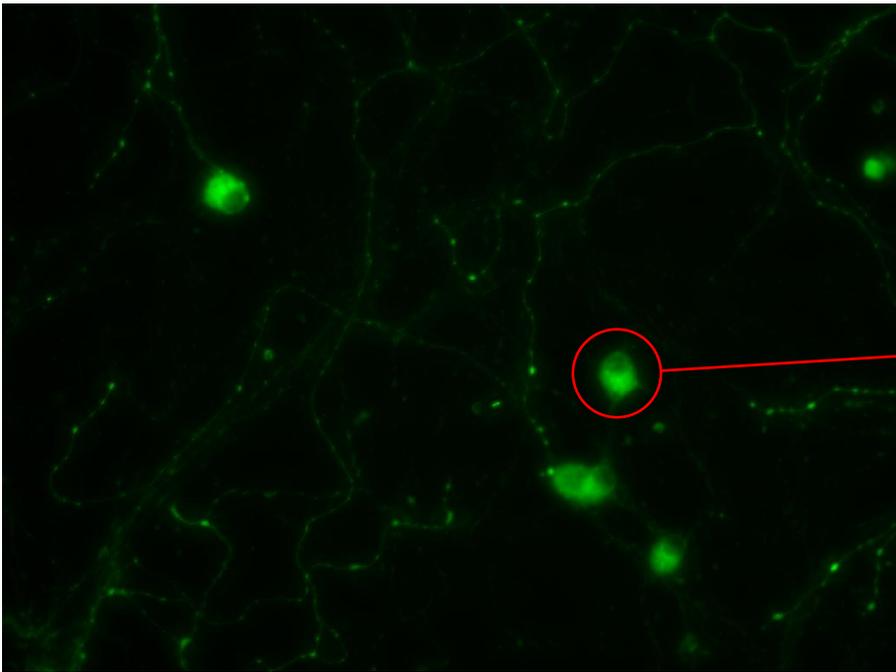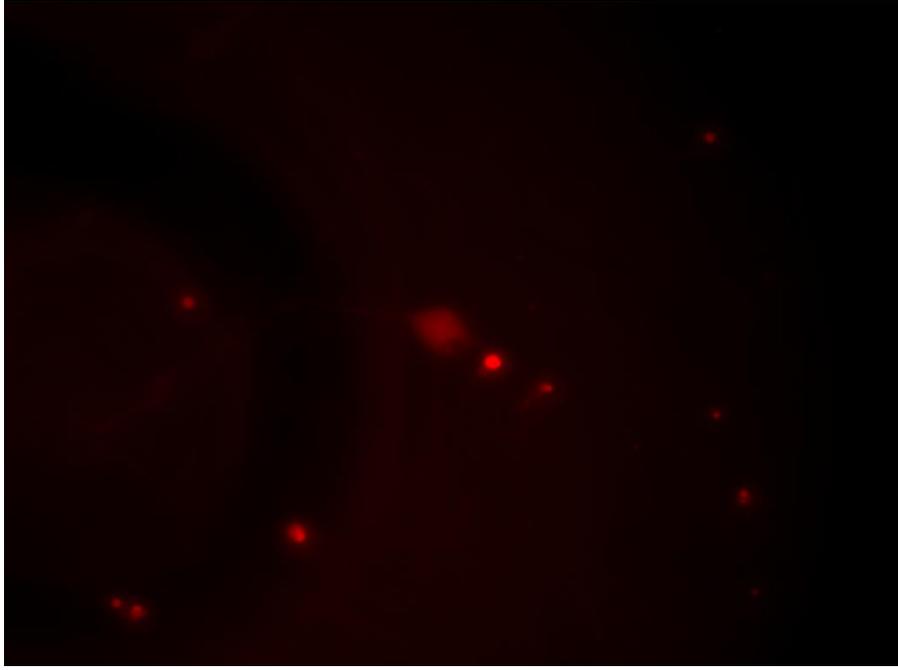

Cell body stained for mitochondria

(B)

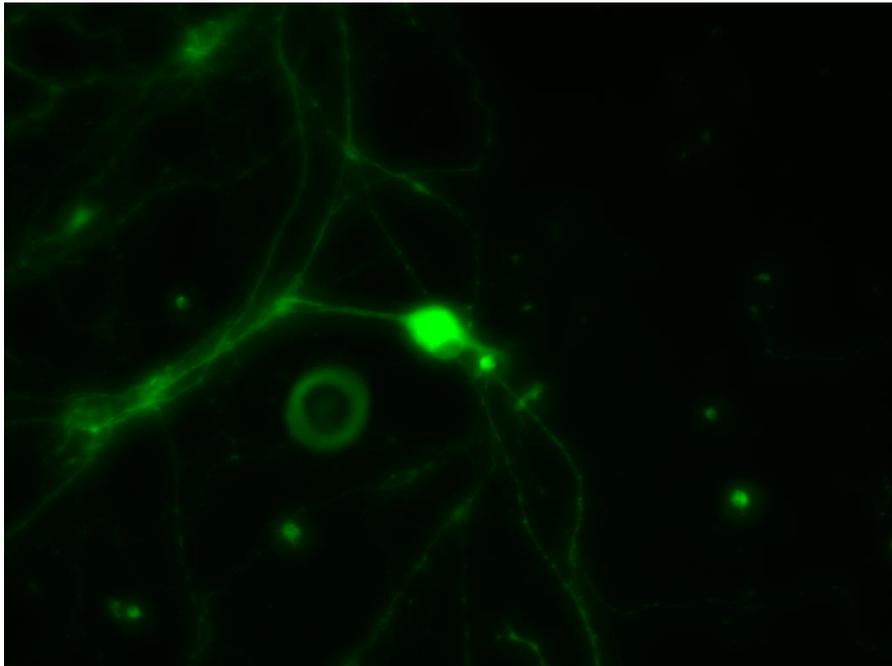Cell body stained for superoxides

(C)

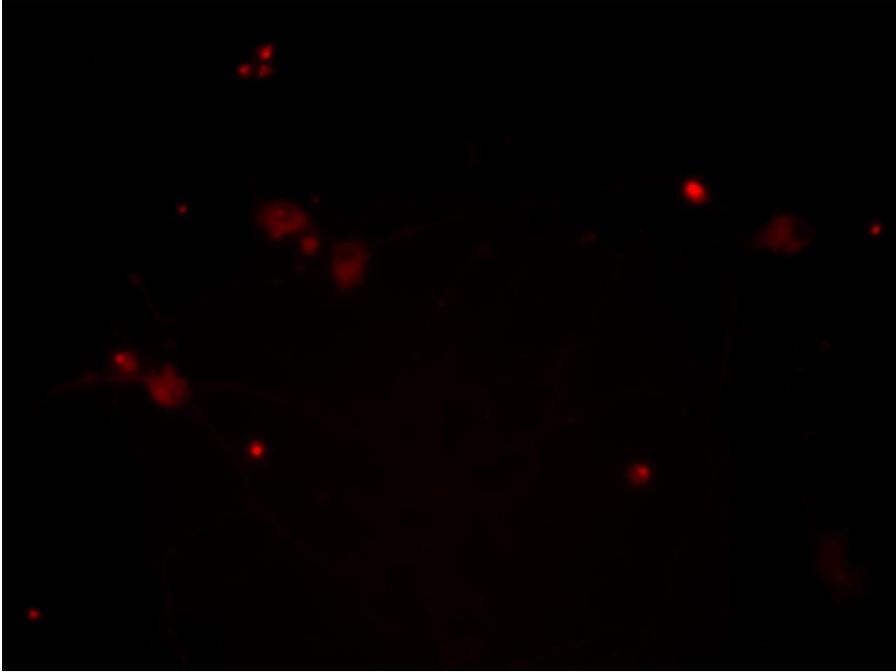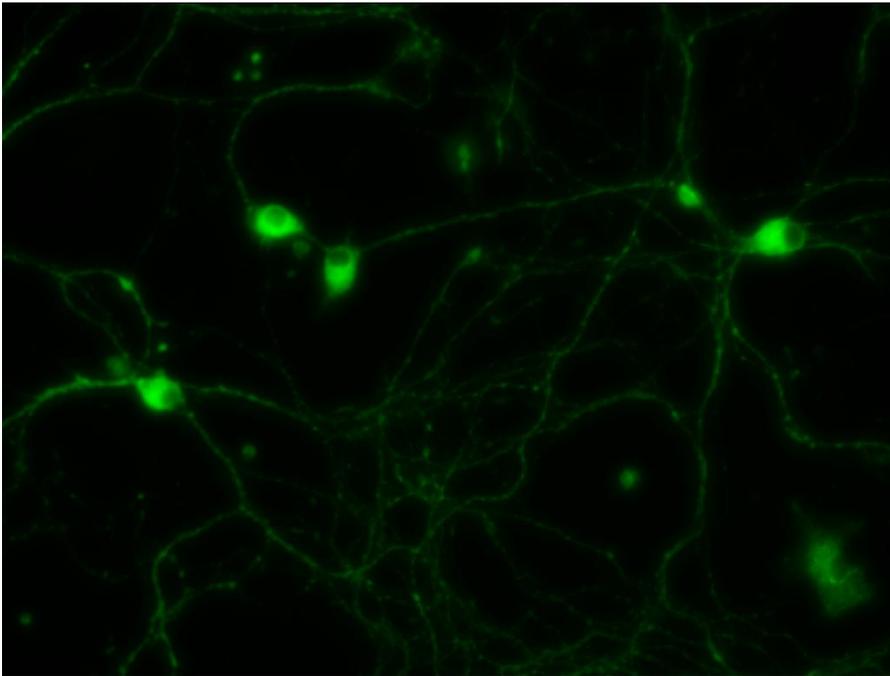Cell body stained for mitochondria.

(D)

(E)

(F)

(G)

(H)

Figure 7: Seahorse XF24 Analyzer shows that neurons treated with BMP-7 have a larger amount and higher rate of oxygen consumption than controls

Neurons were grown at high density on a 24-well plate and treated with control media or BMP-7 (50 ng/ml) for either 24 or 48 hours. The oxygen consumption was analyzed in these neurons using the XF24 Analyzer from Seahorse Biosciences. Following the third baseline measurement, 60 μM DNP was injected into the wells and the remaining 3 measurements were taken.

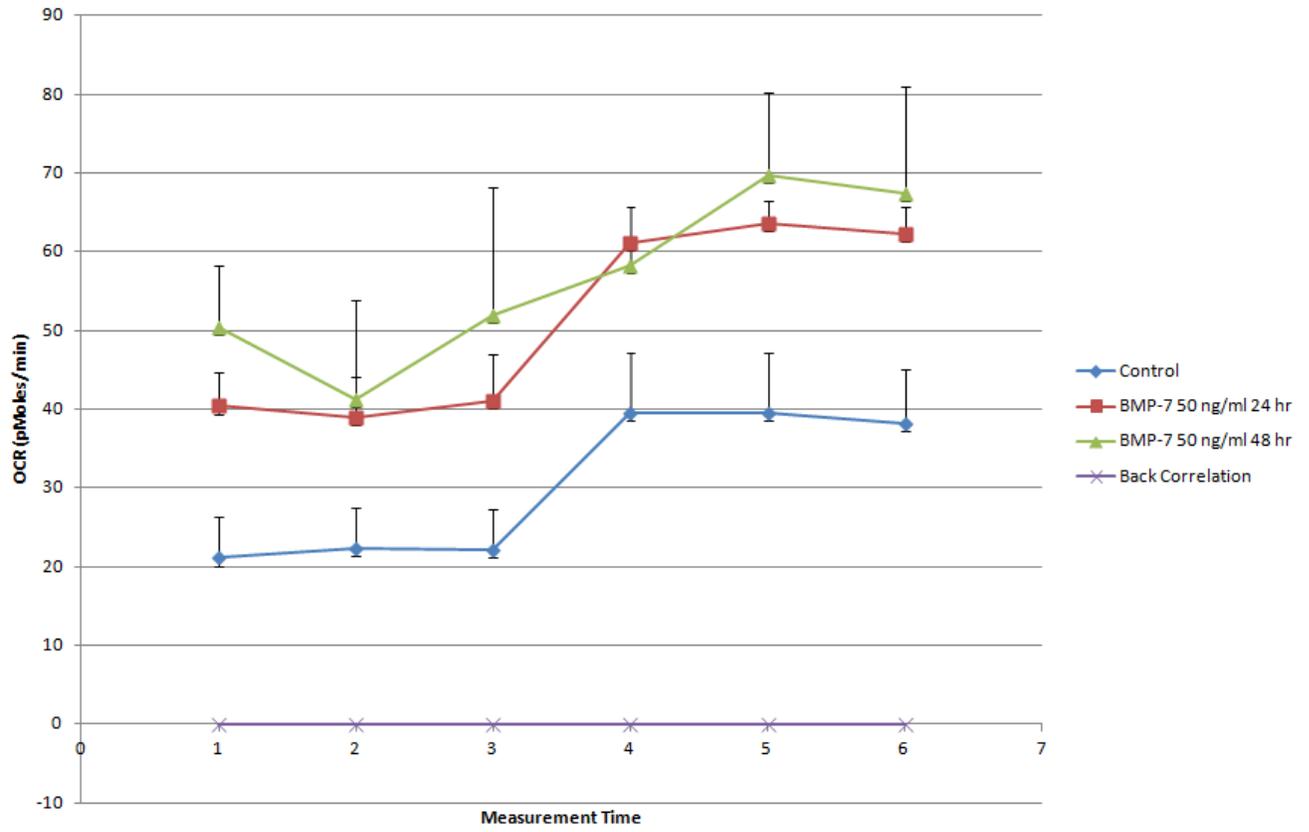Average Oxygen Consumption Rate (OCR) of BMP-7 (50 ng/ml) Treated SCG as Measured by the Seahorse XF24 Analyzer

Figure 7

References

Beck, H.N., Drahushuk K., Jacoby D.B., Higgins D., Lein P.J. Bone morphogenetic protein-5 (BMP-5) promotes dendritic growth in cultured sympathetic neurons. BMC Neuroscience (2001) 2:12. Epub 2001 Sep 11.

Brewer, G. Why Vitamin E Therapy Fails for Treatment of Alzheimer's Disease. Journal of Alzheimer's Disease (2010) 19:27–30.

Bruckenstein D.A., Higgins D. Morphological differentiation of embryonic rat sympathetic neurons in tissue culture: I. Conditions under which neurons form axons but not dendrites. Dev. Biol. (1988) 128:324-336.

Garred M. M., Wang M. M., Guo X., Harrington C., Lein P. J. Transcriptional Responses of Cultured Rat Sympathetic neurons during BMP7-Induced Dendritic Growth. PLoS One. (2011) 6(7):e21754. Epub 2011 Jul 13.

Geiszt M., Leto T.L. The Mox family of NAD(P)H oxidases: host defense and beyond. J. Biol. Chem (2004) 279:51715-51718.

Guo X., Lin Y., Horbinski C., Brahushuk K.M., Kim, I.J., Kaplan P.L., Lein P., Wang T., Higgins D. Dendritic Growth Induced by BMP-7 Requires Smad1 and Proteasome Activity. J. Neurobiol. (2001) 2:120-30.

Heldin, C.H., Miyazono K., ten Dijke, P. TGF-b signaling from cell membrane to nucleus through SMAD proteins. Nature (1997) 390:465-571.

Higgins D., Lein P., Osterhout D., Johnson M.I. Tissue culture of autonomic neurons. In: *Culturing Nerve Cells*. Banker G, Goslin K, eds. (1991) Cambridge, MA: MIT Press 1991177-205.

Hong, Y.H., Peng H.B., La Fata V., Liao J.K. Hydrogen peroxide-mediated transcriptional induction of macrophage colony-stimulating factor by TGF-beta1. J. Immunol. (1997) 159(5):2418-2423.

Hogan B.L. Bone morphogenetic proteins in development. Curr Opin Genet Dev. (1996) 4:432-8.

Lein P., Johnson M., Guo X., Rueger D., Higgins D. Osteogenic protein-1 induces dendritic growth in ray sympathetic neurons. Neuron (1995) 15:597-605.

Mandal, C.C., Ganapahty S., Gorin Y., Mahadev K., Block K., Abboud H.E., Harris, S.E., Ghosh-Choudhury G., Chosh-Choudhury N. Reactive oxygen species derived from

Nox4 mediate *BMP2* gene transcription and osteoblast differentiation. Biochem. J. (2011) 433:393-402.

Massague J. How cells read TGF-β signals. Nature Rev. Mol. Cell. Biol. (2000) 1:169-178.

Meier B., Cross A.R., Hancock J.T., Kaup F.J., Jones O.T.G. Identification of a superoxide-generating NADPH oxidase system in human fibroblasts. Biochem. J. (1991) 275:241-245.

McAllister, A.K. Cellular and Molecular Mechanisms of Dendrite Growth. Cereb. Cortex (2000) 10:963-973.

Myazono K., Kamiya Y., Morikawa M. Bone morphogenetic protein receptors and signal transduction. J. Biochem. (2010) 147(1):35-51.

Schreck R., Reiber P., Bauerle P.A. Reactive oxygen intermediates as apparently widely used messenger in the activation of NF-kappa B transcription factor and HIV-1. EMBO J (1991) 10:2247-2258.

Simonyi A., He Y., Sheng W., Sun A.Y., Wood W.G., Weisman G.A., Sun G.Y. Targeting NADPH Oxidase and Phospholipases A2 in Alzheimer's Disease. Mol Neurolbiol (2010) 41:73-86.

Thomas, B. Parkinson's Disease: From Molecular Pathways in Disease to Therapeutic

Approaches. Antioxidants and Redox Signaling (2009) 11:2077-2082.

Veal E.A, Day A.M., Morgan B.A. Hydrogen peroxide sensing and signaling. Mol. Cell (2007)

26:1-14.

Wen Y., Li W., Poteet E.C., Xie L., Tan C., Yan L.J., Ju X., Liu R., Qian H., Marvin M. A.,

Goldberg M.S., She H., Mao Z., Simpkins J.W., and Yang S.HAlternative Mitochondrial

Electron Transfer as a Novel Strategy for Neuroprotection. (2011) J. Bio. Chem.

286:18:16504-16515.

Yabe T., Samuels I., Schwartz J.P. Bone morphogenetic proteins BMP-6 and BMP-7 have

differential effects on survival and neurite outgrowth of cerebellar granule cell neurons.

J. Neurosci Res (2002) 68:161–168.

Zhao, B. Natural Antioxidants Protect Neurons in Alzheimer's Disease and Parkinson's Disease.

Neurochem Res (2009) 34:630–638.