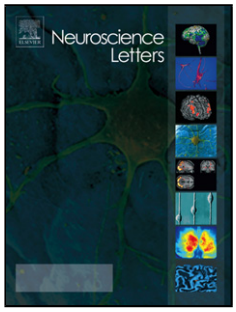

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Endocytosis contributes to BMP2-induced Smad signalling and neuronal growth.

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Highlights

- Endocytosis contributes to BMP2-induced Smad signalling and neuronal growth.
- Dynasore attenuates canonical BMPR-Smad 1/5/8 signalling following BMP2 treatment.
- Dynamin-dependent endocytosis inhibition delays BMP2-activation of Smad signalling.
- Dynasore inhibits BMP2-induced increases of SH-SY5Y neurite length and branching.

Abstract

Bone morphogenetic protein 2 (BMP2) is a neurotrophic factor which induces the growth of midbrain dopaminergic (DA) neurons *in vitro* and *in vivo*, and its neurotrophic effects have been shown to be dependent on activation of BMP receptors (BMPRs) and Smad 1/5/8 signalling. However, the precise intracellular cascades that regulate BMP2-BMPR-Smad-signalling-induced neurite growth remain unknown. Endocytosis has been shown to regulate Smad 1/5/8 signalling and differentiation induced by BMPs. However, these studies were carried out in non-neural cells. Indeed, there are scant reports regarding the role of endocytosis in BMP-Smad signalling in neurons. To address this, and to further characterise the mechanisms regulating the neurotrophic effects of BMP2, the present study examined the role of dynamin-dependent endocytosis in BMP2-induced Smad signalling and neurite growth in the SH-SY5Y neuronal cell line. The activation, temporal kinetics and magnitude of Smad 1/5/8 signalling induced by BMP2 were significantly attenuated by dynasore-mediated inhibition of endocytosis in SH-SY5Y cells. Furthermore, BMP2-induced increases in neurite length and neurite branching in SH-SY5Y cells were significantly reduced following inhibition of dynamin-dependent endocytosis using dynasore. This study demonstrates that BMP2-induced Smad signalling and neurite growth is regulated by dynamin-dependent endocytosis in a model of human midbrain dopaminergic neurons.

[1] **Abbreviations:** BMP(s) - bone morphogenetic protein(s); BMPR(s) - BMP receptor(s); DA – dopaminergic; DIV – day(s) *in vitro*; PD - Parkinson's disease ; VM - ventral midbrain.

Keywords: Endocytosis; BMP2; dynamin-dependent; Smad signalling; neurite growth.

Introduction

Bone morphogenetic proteins (BMPs)[1] are essential for the specification, differentiation and survival of a variety of neuronal populations during nervous system development [7]. BMPs have also emerged as regulators of the development of ventral midbrain (VM) dopaminergic (DA) neurons [9], a subpopulation of which progressively degenerates resulting in the motor

symptoms seen in Parkinson's disease (PD) [15]. Indeed, BMP2 has been shown to have potent neurotrophic effects on VM DA neurons *in vitro* [6, 12, 23] and *in vivo* [4]. Additionally, using the SH-SY5Y neuronal cell line and embryonic rat VM primary cultures, we have shown that BMP2-induced DA neurite growth is dependent on canonical Smad signalling [6, 8]. In this pathway, BMPs bind to a complex of BMP receptor (BMPR) type I and type II. BMPRI subsequently phosphorylates Smad 1/5/8 transcription factors which bind to Smad4, and this phosphorylated Smad complex then translocates to the nucleus to modulate target gene expression [17, 24]. However, the precise molecular cascades that regulate BMP2-BMPR-Smad induced neurotrophic effects in VM DA neurons remain to be fully characterised.

Endocytosis of transmembrane receptors, such as BMPRs, regulates their availability at the cell membrane, and also attenuates signal transduction [25]. Receptor endocytosis occurs via either a clathrin-mediated or caveolae-mediated mechanism, both of which are dependent on dynamin [11, 14]. The internalized receptors are then recycled to the plasma membrane, degraded in lysosomes, or alternatively employ the endosome as a signalling platform, in which downstream components are presented for further activation [14]. BMPRs initiate distinct intracellular cascades depending on their endocytic route, membrane localization and mode of oligomerization [5]. Hartung et al. (2006) showed that the phosphorylation of Smad 1/5/8 by BMPRI is induced at the plasma membrane, while continuation of Smad signalling can occur, via endosomes, following endocytosis of the BMPRs in the C2C12 cell line [5]. Furthermore, inhibition of endocytosis has been shown to affect BMP-induced differentiation of osteoblasts [10, 22]. More recently, dynamin-dependent endocytosis was shown to serve as a regulatory mechanism which fine-tunes Smad signalling [19]. Endocytosis therefore regulates canonical Smad signalling, and attenuates the differentiation-inducing effects of BMPs. However, at present, there are scant reports regarding the requirement for endocytosis in BMP-Smad signalling in neurons. To address this we assessed the contribution of dynamin-dependent endocytosis to BMP2-induced canonical Smad signalling and neurite outgrowth in the SH-SY5Y neuronal cell line.

Materials and Methods

Cell Culture

SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Sigma), supplemented with 10% foetal calf serum (Sigma), 100nM L-Glutamine (Sigma),

100U/ml Penicillin/Streptomycin (Sigma), in a humidified atmosphere containing 5% CO_2 at 37˚C. Where indicated, the cells were treated with 200 ng/ml of recombinant human BMP2 (R&D Systems), and pre-treated (30 min prior to BMP2 application) with 40 μM of Dynasore (Sigma). Dynasore treatment was removed 4 h post BMP-application. To test Smad pathway activation, cells were treated for 0, 5, 30 or 60 min. For the neurite growth assay, cells were treated daily for 4 DIV and then labelled with the vital fluorescent dye calcein-AM (1:500; Invitrogen), by incubation at 37°C for 30 min. To assess the dynamin-dependent endocytosis inhibition efficiency of dynasore, SH-SY5Y cells were incubated in 30 μg/ml of Alexa594-transferrin (Invitrogen), as previously described in a separate study [10].

Immunocytochemistry and Measurement of Cellular Morphology

Immunocytochemistry and measurement of cellular morphology was performed as previously described [8]. Cultures were incubated in the following antibodies: BMPRII (1:200; R&D Systems), BMPRIb (1:200; R&D Systems), Smad 1/5/8 (1:200; Cell Signalling) or phopsho-Smad 1/5/8 (1:200; Cell Signalling).

Statistical Analysis

Unpaired Student's t-test or one-way ANOVA with a *post hoc* Tukey's test or simple linear regression were performed and considered significant when $p<0.05$.

Results

Dynasore inhibits dynamin-dependent endocytosis in SH-SY5Y cells

Firstly, immunocytochemistry was used to demonstrate that SH-SY5Y cells express both type I and type II BMPRs (Fig. 1a), as well as Smad 1/5/8 transcription factors (Fig. 1b), and can therefore initiate BMPR-Smad 1/5/8 signalling in response to BMP ligands. To determine if BMP-BMPR-Smad signalling is regulated by endocytosis in SH-SY5Y cells, the small molecule inhibitor dynasore, which specifically interferes with dynamin-dependent endocytosis [16], was used. To test the ability of dynasore to inhibit endocytosis, uptake of fluorescently-labelled transferrin was examined as a measurement of endocytosis [3]. Uptake of Alexa594-transferrin was inhibited following treatment with dynasore for 2 h (Fig. 2a-c). Dynasore treatment did not affect cell numbers compared to the control (Fig. 2d). These findings demonstrate that dynasore inhibits endocytosis in SH-SY5Y cells.

The activation, temporal kinetics and magnitude of BMP2-induced Smad 1/5/8 signalling in SH-SY5Y neuronal cells are regulated by dynamin-dependent endocytosis

We next investigated the effect of dynasore on the activation, temporal kinetics and magnitude of Smad signalling induced by BMP2 in SH-SY5Y cells. Smad transcription factors are phosphorylated by type I and type II BMPRs and translocate to the nucleus, following activation of BMPR2 and BMPRIb by BMP2 (Fig. 3a). To examine this, cells were treated with BMP2 for 5, 30 and 60 min in the presence or absence of dynasore. Linear regression showed that BMP2 significantly increased p-Smad 1/5/8 levels over time (r^2=0.71, p=0.0006) (Fig. 3b). However, 30 min pre-treatment with dynasore prior to the addition of BMP2 delayed the increase in phospho-Smad 1/5/8 levels. Significant increases in phospho-Smad 1/5/8 levels were only detected after 30 min, rather than from 5 min onwards (Fig. 3d, e). Dynasore pre-treatment also significantly reduced the magnitude of BMP2-induced Smad signalling at 60 min (Fig. 3d, e). Taken together, these data demonstrate that activation of Smad signalling by BMP2 is regulated by dynamin-dependent endocytosis.

BMP2-induced neurite growth of SH-SY5Y neuronal cells is significantly reduced by inhibition of dynamin-dependent endocytosis

As BMP2 induces neurite growth via activation of BMPRs and Smad 1/5/8 transcription factors [8], we next examined whether dynasore inhibited BMP-induced neurite growth. SH-SY5Y cells were treated with BMP2 daily before being stained with the vital fluorescent dye calcein at 4 DIV, to allow visualisation of the neurites. To inhibit dynamin-dependent endocytosis, dynasore was added 30 min prior to the addition of BMP2. Dynasore treatment was maintained for the initial 4 h of BMP-stimulation, as longer-term dynasore treatment caused cell death (data not shown). Treatment with BMP2 resulted in a significant increase in the total length of neurites and axonal branching of the SH-SY5Y cells (Fig. 4). Similarly, SH-SY5Y cells treated with dynasore and BMP2 had significantly longer neurites when compared to the control (Fig. 4a, b). However, dynasore treatment significantly inhibited BMP2-induced axonal branching of SH-SY5Y cells (Fig. 4a, c). Furthermore, dynasore treatment significantly reduced the BMP2-induced increase in neurite length (Fig. 4a, b). Collectively, these findings show that dynamin-dependent endocytosis plays a role in BMP2-induced neurite growth.

Discussion

BMP2 is a neurotrophic factor that regulates the neurite growth of VM DA neurons *in vitro* and *in vivo* [4, 6, 12, 23], and this effect has been shown to be dependent on BMPRIb activation of canonical Smad signalling [6, 8]. The present study aimed to build on these findings by investigating the contribution of endocytosis to the regulation of BMP2-induced Smad signalling and neurite growth in the human SH-SY5Y neuronal cell line, a widely used model of human midbrain DA neurons [2, 8, 26, 28].

In order to achieve this, we inhibited dynamin-dependent endocytosis using the small molecule dynasore [16], an approach which was validated by measuring tranferrin uptake into the SH-SY5Y cells. We showed that Smad 1/5/8 signalling is still activated by BMP2 when dynamin-dependent endocytosis is inhibited, a finding also observed in non-neuronal cells [10, 19]. However, dynasore treatment delayed the onset and reduced the magnitude of BMP2-induced Smad 1/5/8 signalling, which was shown by measuring the nuclear accumulation of phosphorylated Smad transcription factors. These data suggest that efficient phosphorylation and/or nuclear translocation of Smad 1/5/8, in response to BMP2, is regulated by dynamin-dependent endocytosis in SH-SY5Y neuronal cells. In support of these findings, Heining et al. (2011) showed that inhibition of endocytosis delays and reduces BMP2-induced Smad phosphorylation in the C2C12 cell line [10], while Paarmann et al. (2016) have shown that endocytosis serves as a regulatory mechanism which fine-tunes Smad signalling. Dynamin is crucial for fission of vesicles prior to their release from the plasma membrane [11, 14], which means that dynasore prevents the detachment of vesicles from membranes, leading to an accumulation of vesicles at the membrane. Such a disturbance to the localization of BMPRs may contribute to the delay and reduction in BMP2-induced phospho-Smad 1/5/8 nuclear accumulation observed in neuronal cells in this study. However, it is unclear whether the dynasore-mediated reduction in the nuclear accumulation of phospho-Smad 1/5/8 induced by BMP2 is due to a reduction in the levels of Smad phosphorylation at the membrane, or to a disruption of the translocation of phosphorylated Smads into the nucleus. To address this, the mechanisms which regulate BMP2-induced Smad-BMPR dissociation following phosphorylation, and the involvement of endocytosis in this process, need to be investigated.

We subsequently examined whether dynamin-dependent endocytosis regulates BMP2-induced neurite growth. Dynasore pre-treatment significantly inhibited BMP2-induced increases in the total length of neurites and axonal branching of SH-SY5Y cells. This result is not surprising, considering that inhibition of dynamin-dependent endocytosis affects the activation, kinetics and magnitude of BMP2-induced Smad 1/5/8 signalling, which is known

to drive SH-SY5Y neurite growth [8]. Collectively, these data indicate a requirement for dynamin-dependent endocytosis in BMP2-induced neurite growth in SH-SY5Y cells. In support of the conclusion that endocytosis regulates BMP2-Smad-induced neuronal growth, BMP-Smad-induced synaptic growth at the *Drosophila* neuromuscular junction has been shown to be negatively regulated by a dynamin-dependent endocytosis inhibitor Nwk [18]. Moreover, BMP2-induced osteoblastic differentiation of C2C12 cells is also attenuated following early and short-term inhibition of dynamin-dependent endocytosis, using a dynasore treatment paradigm similar to this study [10]. Importantly, dynasore also regulates cholesterol in plasma membranes in a dynamin-independent manner [21], which should be considered when interpreting the findings of studies employing dynasore. Thus, dynamin-dependent endocytosis appears to contribute to BMP-induced differentiation of both neuronal and non-neuronal cells.

BMP2 has been shown to be internalised by endocytosis in C2C12 cells [1, 13, 20, 27]. Interestingly, endocytosis of BMP2-BMPR occurs immediately after formation of the ligand-receptor complex, with no further increase in BMP2-BMPR endocytosis occurring until after 30 min [1]. This is due to an initial BMPR saturation by the ligand, which is overcome following BMPR recycling to the plasma membrane [1]. Hartung et al. (2006) showed that the phosphorylation of Smad 1/5/8 by BMPRI is induced at the plasma membrane, while propagation of Smad signalling can occur following endocytosis of the BMPRs, where the endosome acts as a platform for continued BMPR-Smad signalling [5]. In this study, BMP2-induced Smad signalling levels increased from 5 to 30 min, and from 30 to 60 min. Taking the findings of Hartung et al. (2006) and Alborzinia et al. (2013) into account, Smad phosphorylation at the plasma membrane may account for the levels observed at 5 min, while the significant increase at 30 min may be due to continuation/propagation of Smad signalling via endosomes following endocytosis of the BMP2-BMPR complex. The increase in BMP2-BMPR endocytosis after 30 min of treatment would then contribute to the further increase in BMP2-induced nuclear phosho-Smad 1/5/8 levels at 60 min. In this case, dual-temporal inhibition of endocytosis by dynasore, first at 0 min (which delays Smad signalling) and then again at 30 min, causes the significant reduction in Smad signalling levels observed at 60 min following BMP2 treatment in SH-SY5Y cells. It would be worthwhile to examine whether this reduction in nuclear phosho-Smad 1/5/8 levels is sustained at later time-points. Based on the literature, we hypothesise that BMP2-induced Smad signalling levels would remain significantly inhibited at later time points, when BMP2 endocytosis continues to increase [1]. Indeed, Alborzinia et al. (2013) found that while saturation of BMPR binding

limits the initial BMP2 uptake within the first hour of treatment, more binding sites subsequently appear at the plasma membrane, enabling an increase in BMP2 endocytosis over time [1]. In addition to this, dorsomorphin, a small molecular inhibitor of BMPRI [29], significantly reduces BMP2-BMPR endocytosis for up to 8 h [1]. Such a mechanism may have contributed to dorsomorphin-induced inhibition of BMP2-induced activation of the Smad 1/5/8 signalling pathway at 15 and 60 min in SH-SY5Y cells, which was observed in our previous study [8]. Furthermore, dorsomorphin also inhibited BMP2-induced neurite growth in SH-SY5Y cells [8]. This may have been due, at least in part, to dorsomorphin-mediated short-term inhibition of BMP2-BMPR endocytosis [1]. In the current study, early and short-term inhibition of endocytosis (treatment with dynasore for initial 4 h) was sufficient to significantly inhibit the neurite-growth-promoting effects of BMP2.

Dynamin-dependent endocytosis thus appears to be required during the initial phase of BMP2 stimulation in order for this factor to exert its maximal effect on neurite outgrowth in SH-SY5Y cells. It is interesting that an initial, short-term, and dynasore-mediated inhibition of endocytosis results in a significant reduction in SH-SY5Y neurite growth at 4 DIV following BMP2 treatment. We have previously shown that BMP2 induces significant increases in Smad signalling levels up to 2h after treatment in SH-SY5Y cells [8], and that these levels largely return to baseline ~6h-8h after treatment (unpublished observations). Furthermore, BMP-induced increases in neurite growth at 4 DIV is entirely dependent on Smad signalling (Smad4 siRNAs prevent this effect). Thus, this early, short-term and transient Smad activation window appears to be critical for longer-term neurite growth. It may be the case that interfering with a critical early window of Smad activation disrupts the long-term differentiation-inducing effects of BMPs that are observed at later time points. In support of this, BMP2-induced osteoblastic differentiation of C2C12 cells was also attenuated following an initial, early and short-term inhibition of dynamin-dependent endocytosis [10]. In the Heining et al. (2011) study, two classes of BMP2-induced genes, termed endocytosis-dependent and endocytosis-independent genes, were also indentified. It may be the case that genes which contribute to BMP2-induced neurite growth are endocytosis-dependent. A characterisation of the molecular changes that are induced by BMPs in neurons, and their role in neurite growth, is an important question for further research.

The present study is the first to investigate endocytic regulation of BMP-BMPR-Smad signalling in mammalian neuronal cells. Given the essential physiological function of BMP-Smad signalling during nervous system development [7], it would be interesting to investigate the contribution of endocytic regulation to BMP-Smad signalling-controlled

nervous system developmental processes. Furthermore, dynamin-dependent endocytosis should also be investigated for its potential to enhance the DA neurotrophic effects of BMP-BMPR-Smad signalling in preclinical models of PD.

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Figure Legends

Figure 1: SH-SY5Y cells express type I and type II BMP receptors and Smad 1/5/8 transcription factors. Representative photomicrographs of SH-SY5Y cells

immunocytochemically stained for (a) the BMPRs, BMPRIb and BMPRII, or (b) Smads 1/5/8, and counterstained with DAPI (blue). Scale bar = 100 μm.

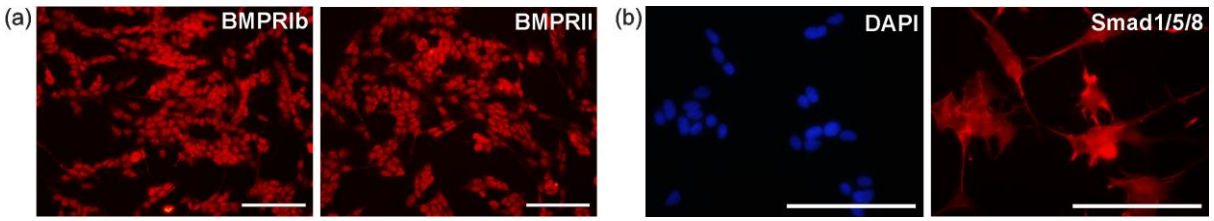

Figure 2: Dynasore inhibits dynamin-dependent endocytosis in SH-SY5Y cells. (a) Low power and (b) high power representative photomicrographs of Alexa594-transferrin immunofluorescence (red) in control and 40 μM dynasore-treated SH-SY5Y cells counter stained with DAPI (blue). (c) Relative immunofluorescence intensity of Alexa594-transferrin and (d) total cell number in cultures of SH-SY5Y cells treated with 40 μM dynasore or DMSO as a control (*** P < 0.001 vs. control; Student's *t*-test; 20 fields for each group per experiment. N = 3). Data are expressed as mean ± SEM. Scale bar = 100 μm in (a) and 10 μm in (b) respectively.

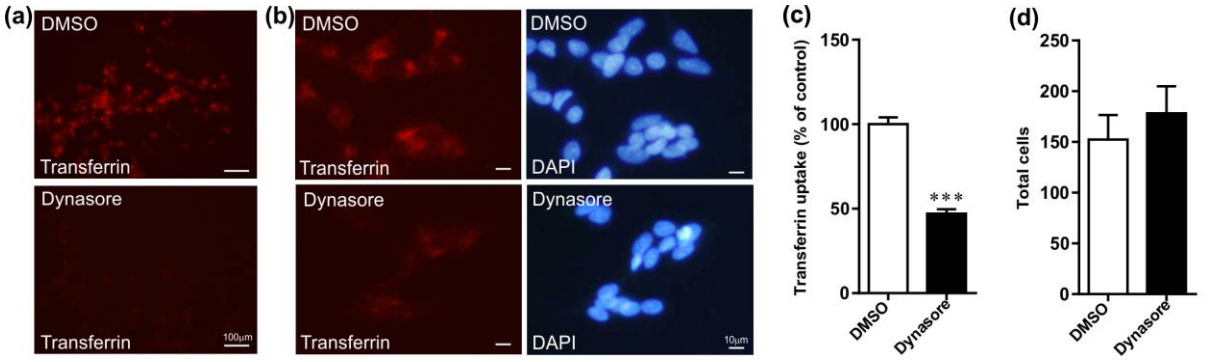

Figure 3: Dynasore impairs BMP2-induced Smad1/5/8 phosphorylation in SH-SY5Y cells. (a) Schematic representation showing that BMP2-activated BMPR signalling results in increased phosphorylation of Smad 1/5/8 (phospho-Smad 1/5/8), immunocytochemistry for which is shown in SH-SY5Y cells in the representative photomicrographs in (d). (b) Linear regression showing a significant correlation between time of exposure to BMP2 and nuclear phospho-Smad 1/5/8 levels. (c) Relative intensity of phospho-Smad immunofluorescence at 0 (control), 5, 30, and 60 min after treatment with BMP2 with or without 30 minute pre-treatment with 40 μM dynasore (*** P < 0.001 vs. Cont (0 min);; $$$ P < 0.001; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. (d) Representative photomicrographs of BMP2-treated SH-SY5Y

cells, with or without 40 μM dynasore treatment, and immunocytochemically stained for phospho-Smad 1/5/8 at 60 min. Scale bar = 100 μm.

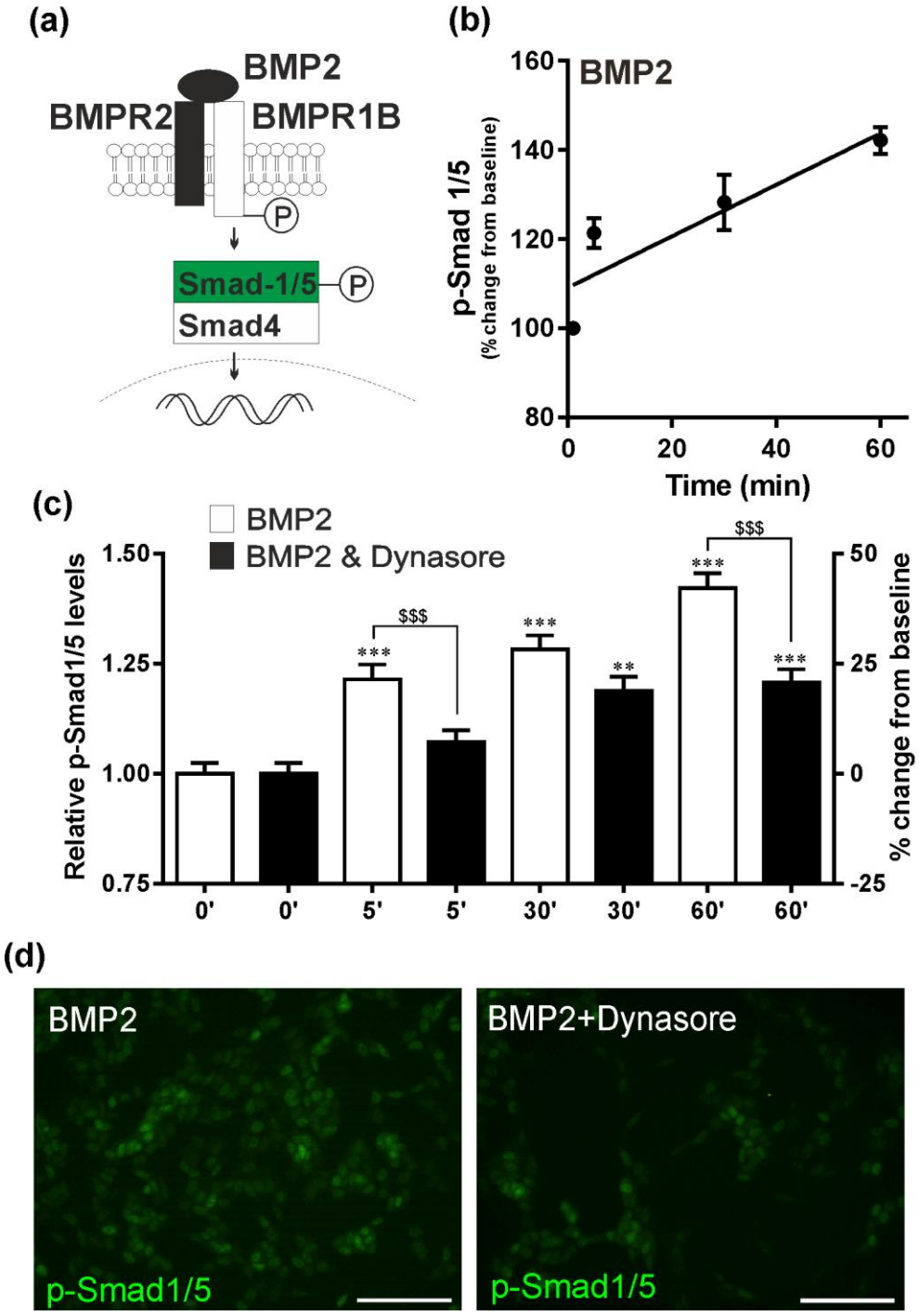

Figure 4: Dynasore impairs BMP2-induced neurite growth in SH-SY5Y cells. (a) Representative photomicrographs, (b) average neurite length and (c) number of axon branches per field in BMP2-treated SH-SY5Y cells with or without 40 μM dynasore, stained with the vital fluorescent dye Calcein after 4 DIV. Scale bar = 50 μm. (* $P < 0.05$ and **** $P < 0.0001$ vs Cont; $^{\$\$\$}$ $P < 0.0001$; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment; N = 3 experiments). Data are expressed as mean ± SEM.