CPS49-induced neurotoxicity does not cause limb patterning anomalies in developing chicken embryos

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Abstract

Thalidomide notoriously caused severe birth defects, particularly to the limbs, in those exposed *in utero* following maternal use of the drug to treat morning sickness. How the drug caused these birth defects remains unclear. Many theories have been proposed including actions on the forming blood vessels. However, thalidomide survivors also have altered nerve patterns and the drug is known for its neurotoxic actions in adults following prolonged use. We have previously shown that CPS49, an antiangiogenic analog of thalidomide, causes a range of limb malformations in a time sensitive manner in chicken embryos. Here we investigated if CPS49 also is neurotoxic and if effects on nerve development impact upon limb development. We found that CPS49 is neurotoxic, just like thalidomide, and can cause some neuronal loss but only in late developing chicken limbs when the limb is already innervated. However, CPS49 exposure does not cause defects in limb size when added to late developing chicken limbs. In contrast, in early limb buds which are not innervated, CPS49 exposure affects limb area significantly. To investigate in more detail the role of neurotoxicity and its impact on chicken limb development we inhibited nerve innervation at a range of developmental timepoints through using β-bungarotoxin. We found that neuronal inhibition or ablation before, during or after limb outgrowth and innervation does not result in obvious limb cartilage patterning or number changes. We conclude that while CPS49 is neurotoxic, given the late innervation of the developing limb, and that neuronal inhibition/ablation throughout limb development does not cause similar limb patterning anomalies to those seen in thalidomide survivors, nerve defects are not the primary underlying cause of the severe limb patterning defects induced by CPS49/thalidomide.

Introduction

In the late 1950s and early 1960s, thalidomide, a non-addictive, non-barbiturate sedative, was prescribed to pregnant mothers to treat morning sickness (Vargesson, 2013; Vargesson, 2015). Embryonic exposure to thalidomide in a short time-sensitive window resulted in over 10000 children worldwide being born with a range of birth defects including severe and debilitating limb defects, the most common being phocomelia (loss of proximal elements) (Smithells and Newman, 1992; Vargesson, 2009). Thalidomide also is a potent antiangiogenic and anti-inflammatory drug, that more recently has been shown to be effective in the treatment of multiple myeloma, ENL (a side-effect of leprosy) and a wide range of other conditions including Behcets disease, Crohns disease, HIV and graft-versus-host disorders (Vargesson, 2015). However, the drug carries severe side effects, such as teratogenesis, following embryonic exposure, and peripheral neuropathy following long term use in adults. Further understanding of the molecular and morphological action of this drug will aid in uncovering newer, safer alternatives. This has begun to be addressed recently through studying the pharmacological properties of analogs or breakdown products of the drug experimentally and clinically (Beedie et al., 2015; Beedie et al., 2016; Mahony et al., 2013; Richardson et al., 2010). Our previous studies of an antiangiogenic thalidomide analog, CPS49, demonstrated the drug leads to widespread loss of blood vessels throughout the limb, when applied at the time of rapid limb growth (Hamburger and Hamilton (HH) St17-19 in the chicken embryo), and disrupts the actin cytoskeleton of endothelial cells *in-vitro* (Therapontos et al., 2009). These vessel defects precede increased cell death, changes in expression patterns of signalling pathways vital for normal limb development and loss of proximal/medial tissue and structures (Therapontos et al., 2009; Vargesson, 2009). These findings point towards the antiangiogenic properties of thalidomide being responsible for the drug's teratogenic activity. Aside from missing proximal/medial limb skeletal elements, thalidomide survivors also have disrupted neurological patterns (McCredie et al., 1984). Consequently, it has been proposed that neurological damage caused by the drug could contribute to thalidomide's damaging effects upon the embryo, including the developing limb (McCredie and McBride, 1973). In agreement with this idea, we have demonstrated that thalidomide

has a direct neurotoxic action on developing neurites (Mahony et al., 2013). However, developing chicken limbs form with normal cartilage patterns following loss of nerves before or during limb outgrowth occurs (Edom-Vovard et al., 2002; Harsum et al., 2001; Martin and Lewis, 1989; Strecker and Stephens, 1983; Swanson, 1985; Swanson and Lewis, 1982;). Moreover, disrupted nerve patterning in developing mouse limbs following loss of nerve guidance cues has no obvious impact on limb patterning, but does alter bone density and length (Fukuda et al., 2013; Tomlinson et al., 2016). Thus, the role of neurotoxicity in thalidomide-induced limb embryopathy is currently unclear.

To further investigate the role of neurotoxicity in inducing limb defects we have analysed the effect of CPS49 on chicken limb innervation and development at a range of developmental timepoints; before, during and after limb initiation and outgrowth. We have further investigated the role nerves play in mediating correct limb development by applying β-bungarotoxin, a potent neurotoxin from snakes that prevents the neurotransmission of signals along the nerves permanently, to ablate nerves from the developing limb at different developmental timepoints. Our findings demonstrate that CPS49 is neurotoxic *in-vitro*, but induces only minor changes in nerve-patterning in CPS49 treated chicken embryo limbs. Moreover, through using β-bungarotoxin at different developmental timepoints we found that, unlike CPS49 treatment, loss of nerves prior to, during or after limb initiation, does not result in the range of limb defects associated with CPS49 administration to chicken embryos. These findings demonstrate that (i) the neurotoxic actions of CPS49 are not responsible for the severe limb patterning malformations the drug causes; (ii) nerve inhibition/ablation before, during and after limb outgrowth in the chicken embryo does not result in loss of limb elements.

Materials and Methods

Chicken Embryos

Fertilised White Leghorn chicken embryos (Henry Stewart, Herefordshire, UK) were incubated at 37°C. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992). Compounds were dissolved in DMSO (Sigma Aldrich) and diluted in prewarmed DMEM (Sigma Aldrich) to give a final DMSO concentration of 0.1%. 100µl of the drug solution was applied to the upper

half of the embryos, as described previously (Therapontos et al., 2009). Right (treated) forelimbs from treated embryos were compared to either contralateral limbs or right forelimbs from stage matched DMSO control embryos or right forelimbs from stage matched untreated controls.

Cartilage staining

Embryos where incubated until day 7/8 and fixed in 5% trichloroacetic acid overnight, rinsed in 70% alcohol for 5 min then washed twice in acid alcohol (1% concentrated Hydrochloric acid in 70% alcohol) for 10min. Embryos were stained with Alcian blue (0.1% in acid alcohol) for 6hr, rinsed in acid alcohol overnight, dehydrated in ethanol, cleared in methyl salicylate (Sigma Aldrich), and photographed.

Immunohistochemistry

Wholemount Immunohistochemistry was based on previously published protocols (Vargesson and Laufer, 2001). Briefly, embryos were dissected, then incubated in dents fix overnight, followed by dents bleach overnight. Embryos were washed 3 x 1hr in 100 % methanol, followed by 3x 1hr PBS washes. Embryos were incubated in 1° antibody (3A10, 1:20; Developmental Studies Hybridoma Bank) overnight, in a solution of 5% goat serum and 20% DMSO in PBS. The next day, the tissue was washed in 5x 1hr PBS, followed by incubation with 2° antibody (goat antirabbit-Cy3, 1:1000; Jackson Immunoresearch) in 5% donkey serum and 20% DMSO in PBS. The next day tissue was washed in PBS 5x 1hr, followed by 3x 100% methanol washes. The tissue was cleared in Benzyl Alcohol/Benzyl Benzoate, and imaged.

Retinal growth cone explant cultures

Experiments were performed using E14.5 C57BL/6J embryos from an inhouse breeding colony. Noon on the day a vaginal plug as found was considered E0.5. Retinal explants were prepared as described previously (Erskine et al., 2011) and cultured in a 1:1 mixture of bovine dermis and rat tail collagen (BD Biosciences) overnight in 0.1% DMSO or drugs dissolved in serum free medium (DMEM/F12 (Life Technologies)) containing 1% BSA and ITS supplement (Sigma-Aldrich). Cultures were fixed with 4 % PFA for 1hr at room temperature, and washed with PBS for 4x 30 min. Cultures were blocked with 10% NGS/ 0.2% triton/ PBS for 90 min and incubated overnight at 4°C with anti–β-Tubulin (1:500; Sigma-Aldrich) in blocking

solution. Cultures were washed in PBS for 8x30 min washes, incubated overnight at 4°C with goat anti-mouse-IgG-Cy3 (1:2000; Jackson Immunoresearch) in 1% NGS/PBS, followed by 8x30 min PBS washes. Images were captured using a Nikon DS5 camera attached to a Nikon SMZ1500 microscope. Image J was used to quantify total axon outgrowth as described previously (Erskine et al., 2011). Results are the mean (± s.e.m.) from at least 3 independent experiments for each condition. Statistical comparisons were made using ANOVA.

Photography and analysis

Photography was performed using a Nikon SMZ1500 fluorescent stereomicroscope with a Nikon DS-5 digital camera. Images were prepared and analysed using Adobe Photoshop and Image J.

Results

Neurotoxicity of CPS49 in-vitro

To determine if CPS49 is neurotoxic to developing neurons we used an established mouse retinal explant outgrowth assay (Erskine et al., 2011; Mahony et al., 2013). E14.5 retinas from C57BL/6J mice were dissected and the explants cultured for 18hr in a collagen gel with control (DMSO) or CPS49 containing medium, fixed, stained for neuron-specific β-tubulin, and the extent of axon outgrowth quantified. We found that compared to the DMSO control CPS49 inhibited neurite outgrowth in a dose-dependent manner (Fig. 1A-E). At 1µg/ml CPS49 had no significant effect on axon outgrowth (Fig. 1A) but at 5µg/ml and 10µg/ml induced a significant decrease in the extent of axon outgrowth (Fig. 1B-D). At 40µg/ml CPS49 resulted in complete loss of outgrowth and, possibly, death of the explants (n=11/12). These findings demonstrate that CPS49, as with thalidomide, is neurotoxic *in vitro*.

Nerve innervation of the developing limb occurs from HH St23/4 and is complete by HH St31

Next, we examined the extent of limb innervation throughout limb development and correlated this with the neurotoxic properties of CPS49 using the *in vivo* chicken embryo model. First, we established the normal innervation pattern throughout limb development using immunofluorescence labelling with antibodies specific for

neurofilaments. We found that innervation of the limb does not begin until after HH St23/24 (E4), which is approximately 1.5days after the limb bud has formed and started to grow out from the flank (Fig. 2B). Up to this point the developing limb bud is aneural (Fig. 2A). Innervation then occurs rapidly and by HH St27/28 (E6.5) innervation can be seen throughout the proximal and up to medial part of the limb (Fig. 2C). By HH St31 (E10) axons are present throughout the limb and extending into the distal handplate (Fig. 2D).

Neurotoxicity of CPS49 in vivo

To investigate the effect of CPS49 upon limb neuronal innervation, we treated chicken embryos at a range of developmental timepoints over the upper forelimb. Following 24hr drug incubation periods, nerve outgrowth was analysed by staining embryos with antibodies against neurofilaments.

In embryos treated at HH St17/18 in which limb outgrowth has just begun and the limb is aneural, application of CPS49 had no significant impact on nerve growth into the limb (Fig. 3). At 24hrs after drug application, developing nerves had extended a small distance into the proximal part of the limb in both the control and CPS49 treated embryos (Fig. 3A). However, overall limb area was decreased in CPS49 treated embryos compared to the controls (Fig. 3B, C). These findings demonstrate that defects in limb growth occur before developing neurites have entered the limb, and in the absence of obvious defects on initial neurite outgrowth.

We next applied CPS49 to HH St27-28 embryos, when nerves are present throughout the limb (Fig. 2C), and quantified changes in axon length, distal nerve area and the limb area 3hr and 24hr after drug application (Fig. 4). Distal axon area was quantified by measuring total area of the distal end of the radialis profundus nerve, which is undergoing dynamic growth at this developmental time point (Turney et al., 2003). At 3hr after application no significant difference was found between axon length, distal nerve area and limb area between the control and CPS49 treated limbs (Fig. 4A-A`, B-B`; E, F, G). At 24hr after drug application, CPS49 induced a small but significant decrease in distal axon area but had no significant effect on axon length (Fig. 4C-C`, D-D`, E, F). In agreement with our previous findings demonstrating a time-sensitive window for CPS49-induced limb defects (Therapontos et al., 2009), total limb area was not altered significantly following

CPS49 application at this time point, despite some defects in nerve growth (Fig. 4G). Our findings demonstrate that CPS49 exerts small but significant neurotoxic effects *in vivo* but only when applied at a stage of development when nerves are already present and established within the limb. Normal limb growth can occur despite these defects in neuronal patterning.

β-bungarotoxin exposure inhibits limb innervation but does not cause obvious cartilage pattern changes

We next investigated if loss of nerves within the limb disrupts the final proximo-distal limb pattern. For these experiments we used β-bungarotoxin, a potent nerve inhibitor (Chiappinelli et al., 1981; Rugulo et al., 1986). We applied β-bungarotoxin to HH St18-19 embryos (when the nerves are absent from the limb; Fig. 2A), St23-24 (when nerves have just entered the proximal limb bid; Fig. 2B), and St27-28 embryos (when nerves are detected up to the medial region; Fig. 2C). Embryos were treated with β-bungarotoxin then fixed 24hr or 48hr later and nerve growth and limb area analysed. Treatment for 24hr or 48hr resulted in a significant decrease in nerve projection within the limb but no change in limb area, when compared to the control embryos at either 24hr or 48hr (Fig. 5A-F`). Typically, aneural limbs resulted following 24hr treatment at all timepoints (Fig. 5A, C, E), with some small projections seen proximally after 48hr treatment at HH St18-19 and HH St23-24 (Fig. 5A, C, E; n=4/5) which could be due to the neurotoxin effect wearing off and allowing some reinnervation.

To assess the impact on limb patterning in aneural limbs we next examined limb cartilage patterning in embryos treated with β-bungarotoxin at HH St15, St17, St20, St23-24 or St27-28, incubated until E7 or E8, and fixed and stained with alcian blue to label cartilage. At all timepoints treatment with β-bungarotoxin had no significant effect on limb patterning which appeared indistinguishable from control limbs. Moreover, quantification of the total limb length and the lengths of the humerus, radius, ulna and handplate demonstrated no significant difference between aneural β-bungarotoxin-treated limbs and control limbs (Fig. 6A, B). These findings demonstrate that loss of nerves throughout the limb before, during or after limb outgrowth has occurred has no substantial effects on the final proximo-distal cartilage pattern or growth of the limb.

Discussion

We have shown previously that CPS49 is potently anti-angiogenic, affecting blood vessel development in chicken embryos within 2hrs of exposure and causing a range of limb defects in a time sensitive manner (Therapontos et al., 2009). However, thalidomide is also neurotoxic, but whether this contributes to the limb defects induced by the drug remains controversial. We have found that CPS49, like thalidomide is neurotoxic *in-vitro*. However, using chicken embryos as a model we have demonstrated that limb outgrowth occurs initially in the absence of nerves and that application of CPS49 at these early time points (HH St17-18) impairs limb growth in the absence of any obvious impact on nerve growth and patterning. In contrast, when applied at HH St27-28 when nerves are present up to the medial part of the limb, CPS49 causes subtle decreases in neuronal outgrowth, in distal parts of the limb, but limbs are not reduced in area (Fig. 5F, G), and no apparent cartilage pattern loss was observed (Fig. 6A). Thus, although CPS49 has neurotoxic actions, given the late innervation of the limb bud (Fig. 2) and that CPS49 induces defects in limb growth before innervation has occurred, neurotoxicity cannot explain the teratogenic effect of CPS49 on the developing limb.

To determine if a loss of nerves contributes to changes in the final proximo-distal cartilage pattern or additional defects associated with thalidomide embryopathy, β-bungarotoxin was applied at a range of stages. We found no evidence that nerve inhibition at any of the timepoints we tested causes phocomelic-like defects. Not only do aneural limbs have normal limb area (Fig. 5), they also have a normal proximo-distal cartilage pattern (Fig. 6). By directly ablating or inhibiting nerves from the limb over a range of developmental timepoints we have shown that loss of nerves does not cause the typical CPS49 mediated limb defects (Therapontos et al., 2009).

Classical studies have investigated the impact of the loss of nerves on the final cartilage pattern using chick limb tissue transplants, and demonstrated a reduction in overall skeletal length (Hamburger and Waugh, 1940). More recently, studies have shown that aneural limbs in chicken embryos have normal cartilage patterns and also demonstrated that innervation of the limb is a relatively late event (Edom-Vovard et al., 2002; Harsum et al., 2001; Martin and Lewis, 1989; Strecker and Stephens, 1983; Swanson, 1985; Swanson and Lewis, 1982). Aneural limbs in rats and tadpoles have reduced cartilage element length and cross sectional area (Dietz,

1989; Edoff et al., 1997), possibly due to a reduction in osteoblast proliferation and differentiation, due to a lack of stimulatory neuropeptides normally secreted from the nerves (Edoff et al., 1997). However, we found that β-bungarotoxin treatment applied at a range of developmental timepoints before, during and after chicken limb outgrowth and innervation, eliminated nerves from the limb but did not give significant reductions in overall or individual skeletal length or induce limb element patterning changes/loss in chicken embryos. Moreover, we have confirmed previous findings that innervation of the developing limb is a late event with no nerves in the limb until at least HH St23/24 (Harsum et al., 2001; Martin and Lewis, 1989; Swanson and Lewis, 1982). The loss of nerves within our assay may likely give a small reduction in element lengths had we incubated embryos until a later timepoint. This is particularly relevant given recent findings detailing the extensive neuroinnervation of bone and the role of nerves in bone metabolism (Masi, 2012). Further support for this hypothesis comes from the findings in mice that loss of Sema3a or TrkA, both involved in axon guidance, results in loss of bone density and bone length inhibition, but crucially not changes in the pattern of the elements (Fukada et al., 2013; Tomlinson, et al., 2016). Thus, neurotoxicity, seems to affect bone length and density, rather than the patterning, number and order of the cartilage condensations.

Innervation of the developing chicken limb occurs 1.5days after limb initiation and outgrowth, and around the time the cartilage condensations for the future long bones occurs. We have shown that loss of limb innervation does not result in obvious cartilage pattern loss. However, we cannot exclude that nerve defects could exacerbate limb defects/damage already caused by thalidomide/CPS49 through causing misinnervation of remaining bones and, consequently, reduced bone density or length (Masi, 2012; Fukada et al., 2013; Tomlinson et al., 2016).

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Author Contributions

CM, SM, AJR, S-L B, WDF, LE, NV - Acquisition of data; data analysis and interpretation; approval of article.

LRF – Acquisition of data.

CM, LE, NV – Writing manuscript.

MG, WDF – Supply of reagents.

NV – Concept/design and direction of study.

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Figure Legends

Figure 1 CPS49 is neurotoxic *in vitro*
Explants of retinas from E14.5 mice embryos were cultured with DMSO/CPS49 and after 18hrs, fixed and stained with an anti-β-tubulin antibody and the area of neurite outgrowth was analysed. (**A-D**) neurite outgrowth from retinal explants following 0.1 % DMSO or CPS49 treatment. (**E**) Quantification of neurite outgrowth. Statistical significance was analysed using one way Anova. Data are mean±SEM. Scale bar, 600μm. White arrowheads indicate areas of neurite outgrowth in control (A) to compare with neurite outgrowth in treated explants (B-D). NS, not significant ($p>0.05$). ****, $p<0.001$. Numbers on bars of graph indicate number of explants analysed for each condition.

Figure 2 Chicken embryo limbs are innervated by neuronal projections after HH St23/24
Chicken embryos at HH St 18-19, 23-24, 27-28 and 31 were fixed and stained for a marker of neurofilaments. (**A**) HH St18-19 forelimb, (**B**) HH St23-24 forelimb, (**C**) HH St27-28 forelimb. (B) HH St31 forelimb. Scale bar, 300μm.

Figure 3 CPS49 is not neurotoxic *in vivo* when applied at HH st17-18
Chicken embryos at HH St17-18 were treated with CPS49 (100 μg/ml), then fixed after 24hr and stained for a maker of neurofilaments. CPS49 was applied over the right upper limb. (**A**) Nerve outgrowth in the forelimb in control or CPS49 treated embryos. (**B, C**) Quantification of axon length (**B**) and limb area (**C**). Data are mean±SEM. Statistical significance was analysed using Student`s T-Test. NS, $p>0.05$. *, $p<0.05$. Scale bar, 100μm. Numbers (n) analysed are indicated in each panel (A)

Figure 4 CPS49 is neurotoxic when applied at HH St27-28
Chicken embryos at HH St27-28 were treated with CPS49 (100 μg/ml), then fixed at 3hr or 24hr and stained for a maker of neurofilaments. CPS49 was applied over the right forelimb. (**A-D`**) Nerve outgrowth in the forelimb in control or CPS49 treated embryos. (**E-G**) Quantification of proximo-distal axon protrusion (distance from body wall to most distal axonal projection; (**E**), area of the distal end of the radialis profundus nerve (**F**) and total limb area (**G**) in control and CPS49 treated limbs. Statistical significance was analysed using Student`s T-Test. Data are mean±SEM. NS, not significant, ($p>0.05$). *, $p<0.05$. Scale bar, 300μm. Numbers on graph bars indicate N numbers analysed.

Figure 5 Neural ablation does not change limb size
Chicken embryos at HH St 18-19, 23-24, or 27-28 were treated with β-bungarotoxin, and incubated for 24hr or 48hr. Innervation was visualised using 3A10 anti-neurofilament anti-body staining. The limb area and length of the most distal neuronal projection was measured. Embryos treated with β-bungarotoxin at HH St18-19 (**A-E`**), HH St23-24 embryos (**F-J`**) and HH St27-28 embryos (**K-O`**) showed reductions in nerve length at 24hr, and following 48hr incubation, with no change in limb area, when compared to the control embryos. In contrast to treatments at HH St18-19 and HH St23-24 where some recovery of neuronal projections in proximal

parts of the limb were found following 48hr incubation, treatment at HH St27 were aneural with no recovery of neuronal projections observed. Statistical significance was analysed using Student`s T-Test. Data are mean±SEM. Numbers (n) analysed are indicated in each panel (A, C, E). in each panel, refers to N number. NS= p>0.05, *= p<0.01, ****= p<0.001. Scale bar, 300μm.

Figure 6 Aneural limbs have normal cartilage pattern

Control or β-bungarotoxin treated chicken embryos incubated until day 7 (HH St30) or day 8 (HH St31). Embryos were then cartilage stained and imaged to examine proximal to distal elements. (**A**) Control and β-bungarotoxin treated embryos treated at a variety of timepoints gave normal cartilage pattern. (**B**) Measurement of proximal to distal skeletal elements normalized to control limbs showed no decrease in aneural limb length relative to the average measurements from DMSO or contralateral control limbs. Data are mean±SD. Numbers (n) analysed are indicated in each panel (A). Scale bar, 300μm.

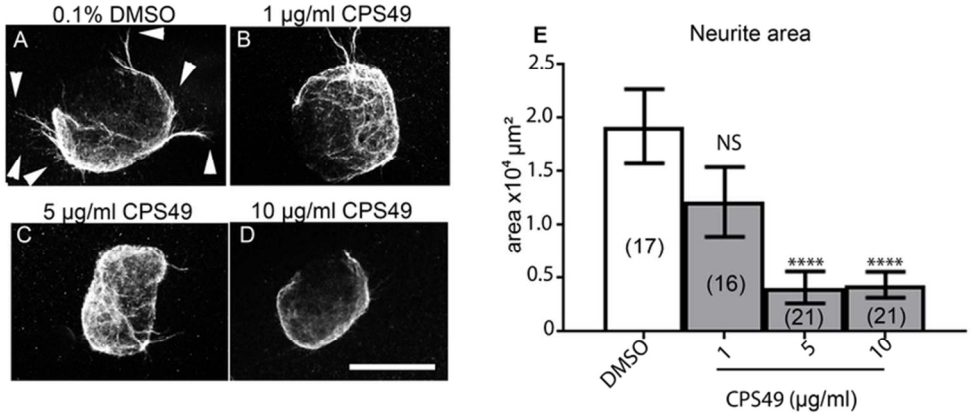

Figure 1

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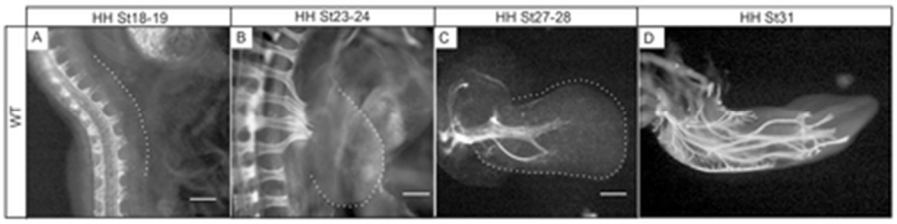

Figure 2

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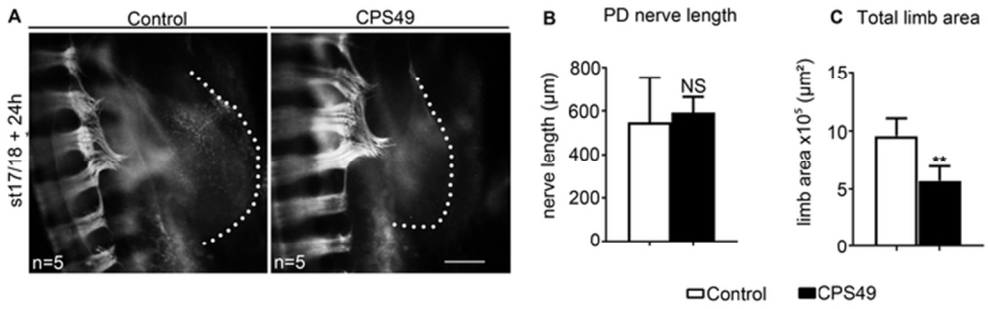

Figure 3

62x20mm (300 x 300 DPI)

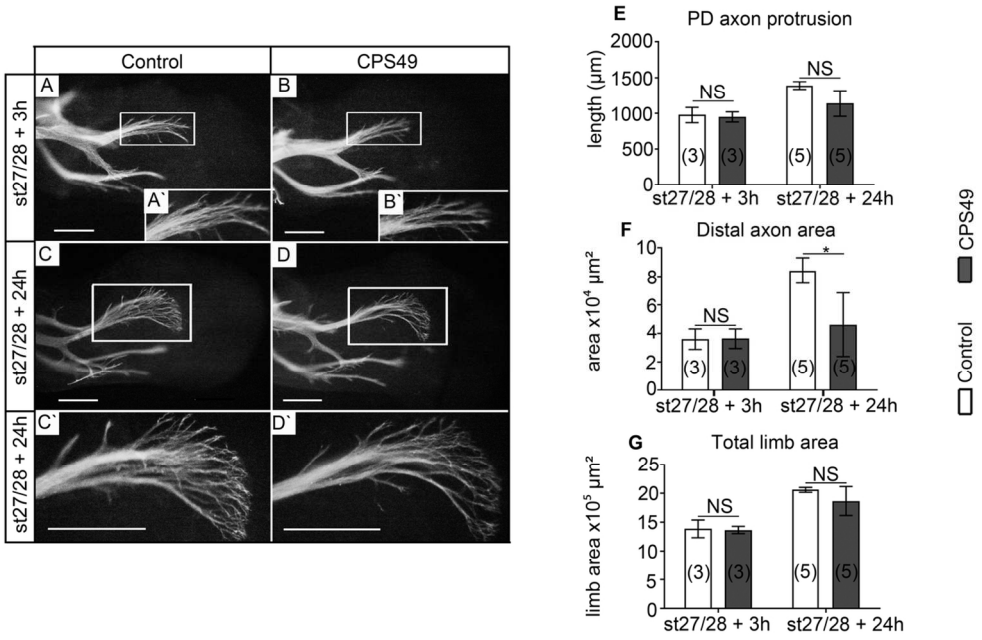

Figure 4

119x77mm (300 x 300 DPI)

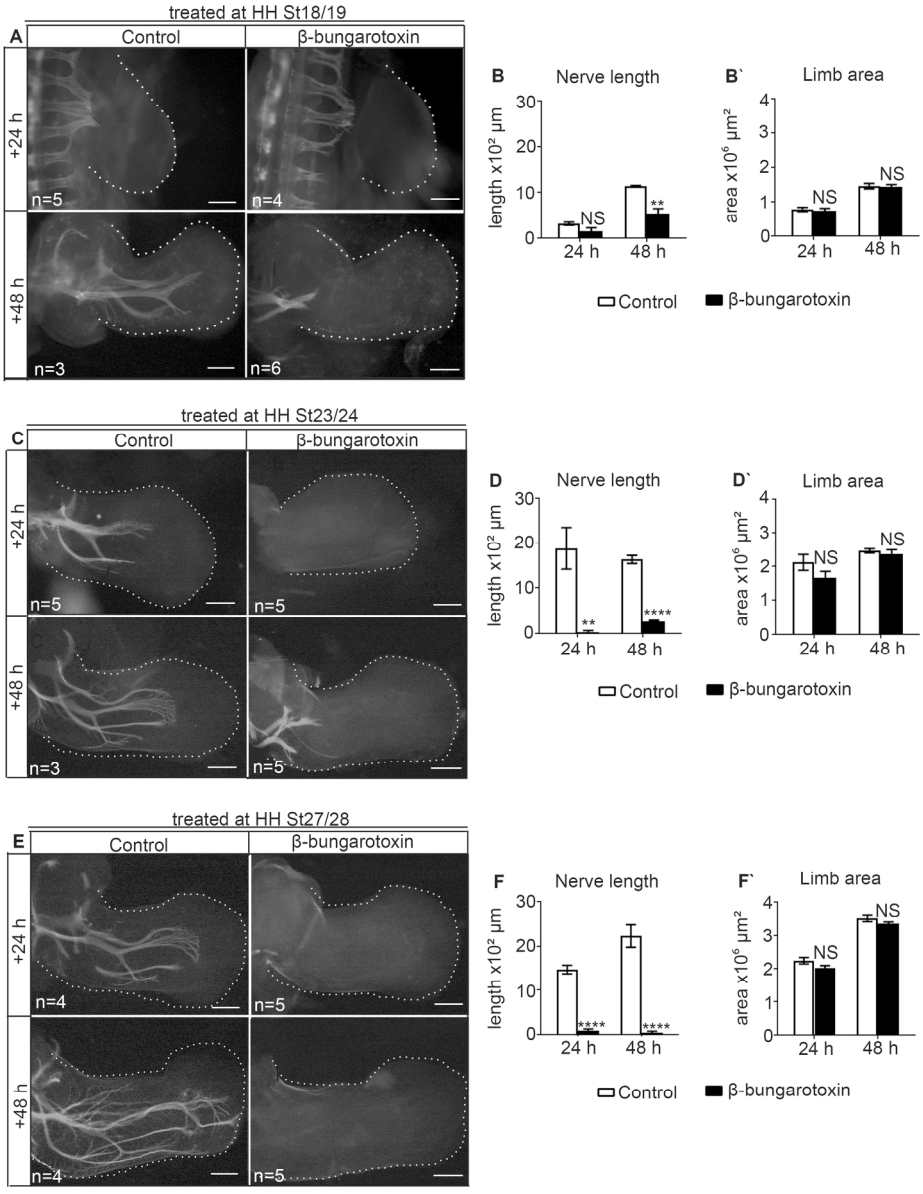

Figure 5

183x233mm (300 x 300 DPI)

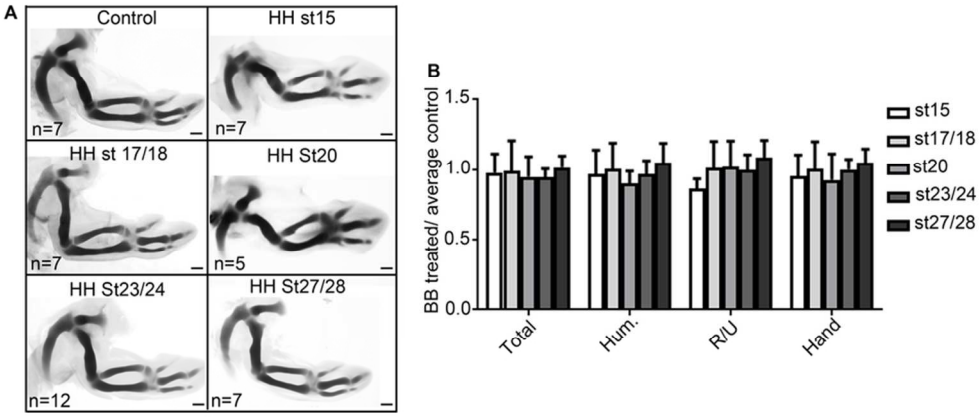

Figure 6

82x35mm (300 x 300 DPI)