Timing of proteasome inhibition as a pharmacologic strategy for prevention of muscle contractures in neonatal brachial plexus injury

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Abstract
Neonatal brachial plexus injury (NBPI) causes disabling and incurable contractures, or limb stiffness, which result from proteasome-mediated protein degradation impairing the longitudinal growth of neonatally denervated muscles. We recently showed in a mouse model that the 20S proteasome inhibitor, bortezomib, prevents contractures after NBPI. Given that contractures uniquely follow neonatal denervation, the current study tests the hypothesis that proteasome inhibition during a finite window of neonatal development can prevent long-term contracture development. Following neonatal forelimb denervation in P5 mice, we first outlined the minimum period for proteasome inhibition to prevent contractures 4 weeks post-NBPI by treating mice with saline or bortezomib for varying durations between P8 and P32. We then compared the ability of varying durations of longer-term proteasome inhibition to prevent contractures at 8 and 12 weeks post-NBPI. Our findings revealed that proteasome inhibition can be delayed 3-4 days after denervation but is required throughout skeletal growth to prevent contractures long term. Furthermore, proteasome inhibition becomes less effective in preventing contractures beyond the neonatal period. These therapeutic effects are primarily associated with bortezomib-induced attenuation of 20S proteasome β1 subunit activity. Our collective results, therefore, demonstrate that temporary neonatal proteasome inhibition is not a viable strategy for preventing contractures long term. Instead, neonatal denervation causes a permanent longitudinal growth deficiency that must be continuously ameliorated during skeletal growth. Additional mechanisms must be explored to minimize the necessary period of proteasome inhibition and reduce the risk of toxicity from long-term treatment.

Keywords
denervation, muscle contracture, muscle length, neonatal brachial plexus injury, proteasome inhibition, protein degradation, sarcomerogenesis

Abbreviations: BPBI, brachial plexus birth injury; DIC, differential interference contrast; DICOM, digital imaging and communications in medicine; I-κB, inhibitor of NF-κB; MicroCT, micro computed tomography; NBPI, neonatal brachial plexus injury; NF-κB, nuclear factor-κB; P, postnatal; PBS, phosphate-buffered saline; ROM, range of motion; [Gly14]-HN, [Gly14]-Humanin G.

Qingnian Goh and Sia Nikolaou contributed equally to this work.
1 | INTRODUCTION

Brachial plexus birth injury (BPBI) is the most common birth injury and the most common cause of pediatric upper limb paralysis.¹ This injury to the nerves that innervate the upper limb occurs in 1-3/1000 live births, and leads to permanent deficits in 20%-40% of affected children.²³ In these children, the initial nerve injury leads to the secondary formation of debilitating muscle contractures, which dramatically reduce joint range of motion (ROM) and limit functional use, leading to dysfunction and deformity of the upper limbs.¹ Unfortunately, current treatments of orthopedic surgery and physical therapy are not effective in restoring joint motion and muscle function once the contractures have developed. Specifically, existing treatment strategies do not address the contracture pathophysiology, and can worsen function by further weakening abnormal muscles.⁵⁷ Therefore, it is imperative to develop novel, effective therapeutic approaches that target-specific mechanisms of contracture pathophysiology.

Through a murine model of neonatal brachial plexus injury (NBPI) pioneered in our laboratory, we previously demonstrated that contractures result from impaired longitudinal growth of muscle denervated during a critical window of neonatal development.⁸⁻¹¹ The contribution of impaired longitudinal muscle growth to contracture formation has been replicated by others using similar rodent models,¹²,¹³ and is further supported by clinical studies in infants and children.¹⁴,¹⁵ While the regulation of muscle length and its contribution to skeletal muscle growth/development are not well characterized, prior studies have posited a role for muscle stem cells (satellite cells) in governing the length of a muscle.¹⁶⁻¹⁸ To scrutinize the role of satellite cells in denervation-induced muscle growth deficits, we utilized a transgenic mouse line to inhibit satellite cell fusion,¹⁹,²⁰ thereby blocking subsequent addition of myonuclei to growing multinucleated myofibers (myonuclear accretion).²¹ Intriguingly, we discovered that complete neonatal denervation does not impair myonuclear accretion, and that neonatal inhibition of myonuclear accretion does not impair longitudinal muscle growth.²² These findings rule out a role for myonuclear accretion in longitudinal muscle growth and contractures. Instead, we observed elevated levels of protein degradation in the denervated muscles, specifically characterized by increased 20S proteasome activity.²² The association of proteasome-mediated protein degradation with impaired longitudinal muscle growth thus establishes a critical mechanistic insight into contracture pathophysiology and, importantly, offers a prospective target for treatment strategies. With pharmacologic inhibition of protein degradation using an FDA-approved proteasome inhibitor, bortezomib,²³,²⁴ we successfully prevented contractures in our NBPI model.²² The correction of the contracture phenotype was accompanied by an attenuation in proteasome-mediated protein degradation and rescue of functional muscle length. This discovery, therefore, represents a novel and exciting breakthrough as the first ever pharmacologic strategy to prevent contractures by targeting an underlying molecular mechanism. However, due to potential cumulative toxicity from prolonged treatment with a proteasome inhibitor, we must elucidate the timing of delivery for proteasome inhibition before translating this strategy to children.

First, we must determine when the treatment has to begin in order to be effective at preventing contractures, as delaying treatment would be clinically beneficial. Many children with BPBI have mild nerve injuries that spontaneously recover and do not go on to cause contractures.² Thus, in these children, proteasome inhibition would not be warranted. Unfortunately, such transient injuries cannot be distinguished at birth from more permanent injuries. These transient injuries can only be identified by clinically observing neurologic recovery over time, with recovery by 4 weeks of age indicating mild injury without risk of musculoskeletal complications.² It would, therefore, be ideal to delay treatment until approximately 4 weeks of age in infants, when the need for treatment has been identified. We have previously shown in neonatal mice that axonal regeneration from early repair of the brachial plexus injured at postnatal (P) day 5 (equivalent to stage of birth in humans)⁵ prevents the development of contractures at P33 (equivalent to completion of neonatal muscle development in humans).¹¹,²⁵ This finding indicates that skeletal muscle can resume growth after a brief period of neonatal denervation, and thus, a delay between NBPI and initiation of proteasome inhibition treatment may still prevent contractures.

Next, we must delineate how long proteasome inhibition is required in order to prevent contractures throughout skeletal growth. Clinically, muscle contractures do not occur in denervated muscle when the onset of denervation is outside the neonatal period of development.²⁶ We have replicated this observation in our mouse model, demonstrating that brachial plexus injury does not induce contractures when performed at P28.¹⁴ These findings suggest the presence of a critical window of neonatal susceptibility to denervation. Specifically, it is, therefore, possible that longitudinal muscle growth is sensitive to increased protein degradation only during this critical neonatal window, and consequently, proteasome inhibition would be required solely during a finite neonatal period. Ideally, treatment during this neonatal window would optimally provide long-term contracture prevention, as opposed to merely delaying the onset of contractures until treatment cessation. Theoretically, the prevention of contractures must be effective at least until recovery of innervation, either by spontaneous recovery of an axonotmetic injury or by nerve reconstruction, both of which in children take several months.²⁷,²⁸

In the current study, we employed our mouse model of NBPI with varying durations of bortezomib treatment to
determine the minimum developmental time period during which proteasome inhibition is required to permanently prevent contractures, and to test the hypothesis that temporary inhibition of protein degradation during a critical neonatal period leads to long-term preservation of muscle growth and prevention of contractures.

2 | MATERIALS AND METHODS

2.1 | NBPI surgical model

Muscle contractures were induced using a murine model of denervation as previously described. Briefly, unilateral, global (C5-T1), postganglionic NBPIs were surgically created in P5 CD-1 wild-type mice (Charles River) by extraforaminal nerve root excision under isoflurane anesthesia. Mice were subsequently returned to their mums and housed in standard cages on a 12-hour light/12-hour dark cycle, with nutrition and activity ad libitum. To ensure permanent deficits were elicited and eliminate any potential confounding effects of reinnervation, we validated deficits in motor function both postoperatively and prior to sacrifice. Mice that displayed preserved or recovered movement in the denervated limb were excluded from the study.

2.2 | Bortezomib treatment

To inhibit protein degradation, mice were treated with the 20S proteasome inhibitor bortezomib at 0.3 mg/kg body weight via intraperitoneal injections every other day, a protocol that optimally minimizes mortality. For determination of the critical neonatal window, bortezomib treatment was started between P8 (the earliest start point that minimizes death) and P10, and continued till P32. Mice were subsequently assessed at P33, which marks the completion of neonatal muscle development and a time point at which contractures are reliably established in our model. Once the optimal start time for preventing contractures was identified (P8), the earliest cessation time before P33 was ascertained by stopping treatment between P26 and P32 and assessing mice at P33. For prolonged proteasome inhibition, mice were assessed during postnatal development at P61 following treatment from P8 to P32 or P8 to P60. Similarly, mice were assessed at P89 upon skeletal maturity following treatment from P8 to P60 or P8 to P88. [Gly14]-Humanin G ([Gly14]-HN, Sigma-Aldrich #H6161) was coadministered at 1 µg along with bortezomib to limit toxicity. Saline (Baxter) was injected in separate litters of mice, as controls for all experiments at all-time points. All control and experimental groups in this study were randomized by litter, with all treatments administered at noon and in the respective cages. Daily body weights were recorded for all mice to monitor for signs of toxicity.

2.3 | Assessment of contractures

At 4, 8, or 12 weeks post-NBPI (P33, P61, and P89, respectively), mice were euthanized by CO2 asphyxiation. Post sacrifice, images of bilateral elbows and shoulders were captured at maximum passive extension and external rotation, respectively, with blinding to treatment group. Elbow flexion and shoulder internal rotation contractures were subsequently calculated by measuring and comparing elbow and shoulder ROM in AxioVision (Zeiss) using a validated digital photography technique as previously described, also blinded to treatment groups. Representative images of forelimbs shown within each figure have been processed to reflect comparable levels of sharpness, brightness, and contrast for illustrative purposes, although no image manipulation was performed prior to measurements.

2.4 | Whole muscle collection and preparation

Following image photography, bilateral biceps muscles were then dissected and snap frozen, before processing for proteasome activity. The remaining forelimbs were harvested and positioned on cork at 90° elbow flexion, imaged by digital x-ray for humerus length, and fixed in 10% of formalin. Brachialis muscles were subsequently removed, soaked in 25% of Lugol solution (Sigma-Aldrich #32922) overnight, and imaged by micro computed tomography (MicroCT; Inveon, Siemens) at 20 µm resolution for assessment of whole muscle size (cross sectional area and volume). Muscles were then recovered by soaking in PBS overnight at 4°C, digested in 15% of sulfuric acid for 30 minutes to obtain muscle bundles, and imaged for sarcomere length.

2.5 | Muscle length

The true functional length of a whole muscle is defined by the total number of sarcomeres in series, which cannot be measured directly due to various morphological constraints. Instead, we determined the relative functional length of the brachialis muscles on the denervated and contralateral control limbs by measuring the average sarcomere length at 90° elbow flexion. With both limbs positioned symmetrically, elongated (overstretched) sarcomeres on one side would indicate fewer sarcomeres in series, or shorter functional whole muscle length, since the fewer sarcomeres a muscle has in
series, the more each sarcomere has to stretch to accommodate any given position.

Thus, following formalin fixation of bilateral forelimbs at 90° of elbow flexion (to prevent sarcomere relaxation with muscle removal), brachialis muscles were removed, digested, and dissected into muscle bundles for imaging with differential interference contrast (DIC) microscopy at 40x on a Nikon Ti-E SpectraX widefield microscope. A total of six images from different muscle bundles was acquired per muscle. Average sarcomere length was subsequently determined by measuring a series of 10 sarcomeres from each of the six images in AxioVision software with blinding to treatment groups. Representative images of sarcomeres shown within each figure have been cropped to identical sizes, and processed to reflect comparable levels of sharpness, brightness, and contrast for illustrative purposes. No image manipulation was performed prior to measurements.

2.6 | Humerus length and whole muscle size

Humerus lengths were determined from the digital x-rays by using AxioVision software to measure the distance between the proximal humerus physis to the distal articular surface. Whole muscle cross-sectional area and muscle volume were obtained by processing the MicroCT scans into digital imaging and communications in medicine (DICOM) images with Fiji programs (Segmentation Editor and 3D Viewer, respectively). All measurements were performed with blinding to treatment groups.

2.7 | Proteasome activity

To determine the magnitude of proteasome inhibition, we assayed for proteasome activity with blinding to treatment groups as previously described. Briefly, bilateral biceps muscles were homogenized in 20 mM of Tris-HCl, pH 7.2, 0.1 mM of EDTA, 1 mM of 2-mercaptoethanol, 5 mM of ATP, 20% of glycerol, and 0.04% of Nonidet P-40. Following centrifugation, protein concentration was determined using the Pierce 660 nm protein assay kit (Thermo Scientific #22662). The caspase-like activity of the 20S proteasome β-1 catalytic subunit and chymotrypsin-like activity of the β-5 catalytic subunit were assayed with 25 μg total protein per muscle, through detection of the 7-amino-4-methylcoumarin (AMC) labeled fluorogenic peptide substrates Z-LLE-AMC (S-230, Boston Biochem) and Suc-LLVY-AMC (S-280, Boston Biochem), respectively. Endpoint fluorescence was measured at 380 nm/460 nm on a SpectraMax M5 microplate reader ( Molecular Devices), and relative fluorescence units were then calculated per μg protein.

2.8 | Statistics

Prism 8 software (GraphPad) was used for statistical analysis. For all continuous data, outliers were detected a priori by Grubbs' test and excluded. All data were subsequently tested for normality with the Shapiro-Wilk test. Normally distributed data and were compared with two-tailed Student's t test, paired where parameters were compared between forelimbs (NBPI vs contralateral) in individual animals, and unpaired when parameters were compared between animals. Non-normally distributed data were compared using Mann-Whitney U-tests for unpaired data or Wilcoxon signed rank tests for paired analyses where parameters were compared between forelimbs (NBPI vs contralateral). All data are presented as mean ± SD. The degree of significance between data sets is depicted as follows: *P < .05, **P < .01, ****P < .0001. A priori power analyses based on prior work were performed for the phenotypic variables of contracture severity, determining that 6 mice per group were required for at least 80% power to detect a 10° difference in contractures and a 0.2 μm difference in sarcomere lengths between experimental conditions.

2.9 | Ethical statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mice were handled according to approved institutional animal care and use committee (IACUC) protocols (#2017-0084) of the Cincinnati Children’s Hospital Medical Center. All surgeries were performed under isoflurane anesthesia, and every effort was made to minimize suffering.

3 | Results

3.1 | Proteasome inhibition is required throughout neonatal development to prevent contractures

To identify the minimum window of time required for neonatal proteasome inhibition to prevent contractures, postnatal (P) day 5 mice underwent unilateral surgical excision of the brachial plexus (C5-T1) to induce NBPI, and were treated with 0.3 mg/kg bortezomib intraperitoneally every other day for varying durations between P8 and P32. We began by iteratively delaying the start of treatment by daily increments (Figure 1A). Delaying treatment beyond P9 (4 days post-NBPI) led to a reduction in the efficacy of contracture prevention at both the elbow and shoulder at 4 weeks post-surgery (P33) (Figure 1B-D). Similarly, brachialis sarcomere overstretch, a measure of
**FIGURE 1**  Optimal start point for neonatal proteasome inhibition. A, Schematic depiction of the strategy to identify the optimal start time for bortezomib treatment in neonatal mice following NBPI at P5. B-D, Delaying proteasome inhibition beyond P9 resulted in less efficacious prevention of both elbow and shoulder contractures at 4 weeks post-NBPI. (E), (F) whereas preservation of sarcomere length in the brachialis muscle was most effective with bortezomib treatment starting at P8. Data are presented as mean ± SD. In (C) and (D), contracture severity is calculated as the difference in passive elbow extension and shoulder rotation between the denervated (NBPI) side and the contralateral control side (n = 8-19 mice). In (F), sarcomere length on the NBPI side is normalized to the contralateral side, with a normal sarcomere length ratio of 1.0, and any value over 1.0 indicating sarcomere overstretch on the NBPI side, and thus, functional muscle shortening (n = 8-23 mice). Statistical analyses: (C), (D), (F) unpaired two-tailed Student's t tests. *P < .05, **P < .01, ***P < .001, ****P < .0001. All samples represent independent biological data points. Scale bar: 10 µm
**FIGURE 2** Optimal endpoint for neonatal proteasome inhibition. A, Schematic depiction of the strategy to discern the appropriate endpoint for bortezomib treatment during neonatal muscle development. B-D, Cessation of proteasome inhibition before P32 resulted in less efficacious improvements in both elbow and shoulder contracture severity, (E), (F) as well as brachialis sarcomere length. Data are presented as mean ± SD. In (C) and (D), contracture severity is calculated as the difference in passive elbow extension and shoulder rotation between the denervated (NBPI) side and the contralateral control side (n = 6-16 mice). In (F), sarcomere length on the NBPI side is normalized to the contralateral side, with a normal sarcomere length ratio of 1.0, and any value over 1.0 indicating sarcomere overstretch on the NBPI side, and thus, functional muscle shortening (n = 6-10 mice). Statistical analyses: (C), (D), (F) unpaired two-tailed Student's t tests. *P < .05, **P < .01, ***P < .001, ****P < .0001. All samples represent independent biological data points. Scale bar: 10 µm
inadequate functional muscle length,22 progressively worsened with treatments starting past P8 (Figure 1E,F). These data demonstrate that Bortezomib treatment can be effective at rescuing longitudinal muscle growth and preventing contractures even if begun following a 3-4 day delay in mice, corresponding to a 3-4 week delay in human infants.

Having established P8 as the optimal start of treatment for effective contracture prevention and preservation of functional muscle length, we next ceased treatment prior to P32 in iterative 2-day increments to discern the earliest effective terminal date (Figure 2A). Any cessation of bortezomib treatment before P32 led to a reduction in the efficacy of contracture prevention at both the elbow and shoulder at 4 weeks post-NBPI (P33) (Figure 2B-D), as well as less effective prevention of sarcomere elongation in the brachialis (Figure 2E,F). Contrary to our hypothesis, these results indicate that proteasome inhibition is required throughout the majority of neonatal muscle development to successfully prevent contractures.

### 3.2 Neonatal proteasome inhibition does not prevent long-term contracture formation

Having established the critical neonatal window for successful contracture prevention, we next sought to determine if temporary proteasome inhibition during this neonatal period is sufficient to prevent contractures at later stages of development. P5 mice were treated after NBPI with 0.3 mg/kg bortezomib either P8-P32 or P8-P60 (Figure 3A). At 8 weeks post-NBPI (P61), neonatal proteasome inhibition alone (P8-P32) failed to permanently prevent contracture formation, as elbow and shoulder contractures were observed to be similar between saline controls and bortezomib treatment (Figure 3B-D). These contractures were accompanied by overstretched sarcomeres in the brachialis, indicating a reduction in functional muscle length (Figure 3E,F). In contrast, continuous bortezomib treatment over 8 weeks was effective in reducing the severity of the elbow flexion contracture, although with less effect at the shoulder (Figure 3B-D). Additionally, continuous bortezomib treatment resulted in an improvement in functional length of the brachialis (Figure 3E,F). These findings demonstrate that improvements in contracture severity with transient neonatal proteasome inhibition are lost after proteasome inhibition is stopped, and instead, that chronic proteasome inhibition throughout development is necessary for long-term contracture prevention.

### 3.3 Chronic proteasome inhibition partially prevents contractures at skeletal maturity

To confirm that continuous proteasome inhibition throughout development is required for effective prevention of contractures after skeletal muscle maturity, P5 mice were treated 0.3 mg/kg bortezomib P8-P60 or P8-P88 (Figure 4A). At 12 weeks post-surgery (P89), substantial ameliorations in elbow and shoulder contracture severity were observed only with continuous bortezomib treatment throughout this period (Figure 4B-D). Indeed, prior improvements over 8 continuous weeks of treatment (Figure 3B-D) were not sustained 4 weeks later if treatment was discontinued at 8 weeks. Similarly, after 12 weeks post-NBPI, brachialis functional length was only improved with continuous bortezomib treatment, as cessation of treatment after 8 weeks nullified prior improvements in muscle length (Figure 4E,F). Therefore, these findings establish the necessity for chronic proteasome inhibition in reducing contracture severity and preserving muscle length beyond skeletal maturity.

Furthermore, the degree of contracture prevention decreased with prolonged exposure to bortezomib (Figure 5A), as improvements in elbow and shoulder contracture severity were greatest after 4 weeks of neonatal proteasome inhibition, but became less prominent after 8 and 12 weeks of continuous treatment (Figure 5B,C). Likewise, the preservation of sarcomere length in brachialis muscles becomes less efficacious after the 4-week neonatal period (Figure 5D). Taken together, our findings suggest that proteasome inhibition is both necessary and less efficacious beyond the neonatal period to prevent long-term contractures and preserve functional muscle length.

This diminished efficacy may be attributed to the emergence of joint defects with long-term denervation. In particular, we observed elbow arthrosis, deformity, and dislocation in denervated limbs of saline-treated mice, arising between 8 and 12 weeks post-NBPI (Figure 5—Supplemental Figure S1A-C). These observations, although not the primary aim of this study, indicate that continuous proteasome inhibition does not protect against long-term bone defects despite improving contractures. Such bone abnormalities may be in part driven by contractures that are only partially prevented, by accumulated trauma on an insensate limb, or by direct effects of bone denervation. Regardless, they may confound the contracture phenotype at the elbow, thereby potentially accounting for the variability in elbow extension contracture severity after 12 weeks of surgery (Figure 5C). Indeed, when analyzing saline-treated mice from all time points in this study (Figure 5—Supplemental Figure S1D), we observed a reduction in elbow contracture severity concomitant with an exacerbation of shoulder contractures between 4 and 12 weeks of denervation (Figure 5—Supplemental Figure S1E,F). These results represent a subtle shift in the muscle contracture phenotype with prolonged denervation.

A potential concern with proteasome inhibition, especially during a prolonged administration, is its deleterious effects on skeletal growth.32-34 As sexual dimorphism in body and muscle mass occurs in mice during postnatal development,35...
we analyzed markers of skeletal growth according to individual sexes. With our treatment regimen, we observed that continuous bortezomib treatment attenuated body weight and impaired bone growth of the humerus in male mice by 3%-7% during postnatal development at 8 weeks post-NBPI, with no such effect on female mice (Figure 5—Supplemental Figure S2A-C). Importantly, despite preserving longitudinal muscle growth, continuous proteasome inhibition impeded skeletal muscle hypertrophy across both sexes, as cross-sectional area and volume of non-denervated brachialis muscles in bortezomib-treated mice were attenuated by 8%-17% at 8 and 12 weeks post-NBPI (Figure 5—Supplemental Figure S2A-C).
S2D-G). While we had previously observed that neonatal proteasome inhibition does not alter whole muscle cross-sectional area or volume, either positively or negatively, our results here further indicate that continuous proteasome inhibition beyond the neonatal period is detrimental to overall developmental muscle growth. Along with the inability of continuous proteasome inhibition to protect against bone deformities associated with long-term denervation, these growth detriments suggest that chronic proteasome inhibition alone is not a viable pharmacologic strategy for preventing contractures beyond neonatal development.

3.4 | **Chronic bortezomib treatment is associated with decreased β1 proteasome activity**

As a potent proteasome inhibitor, the use of bortezomib is approved by the FDA in treatments against multiple myeloma and mantle cell lymphoma. In these disease models, bortezomib has been reported to preferentially bind to the β5 and β1 subunits of the 20S catalytic core complex of the 26S proteasome. In our prior investigations, we demonstrated that bortezomib does indeed suppress the denervation-induced elevations in proteasome activity in skeletal muscles after 2 weeks of continuous treatment (P5-P18), through attenuation of the β5 subunit activity. To further decipher potential mechanistic insights by which chronic proteasome inhibition reduces contracture severity, we assayed for proteasome activity in both denervated and non-operated forelimbs after 4, 8, and 12 weeks of continuous bortezomib treatment (Figure 6A). Surprisingly, β5 subunit activity of the 20S catalytic core complex, the primary binding site for bortezomib, was not blunted at any of these time points (Figure 6B). Instead, continuous bortezomib treatment inhibited both denervation-induced and global β1 subunit activity throughout neonatal and postnatal development by ~20%-25%, and ~40%-60%, respectively (Figure 6C). The magnitude of bortezomib-induced reduction in β1 proteasome activity of denervated muscles decreased over time, and was accompanied by an unexpected decrease in proteasome activity of non-treated muscles beyond 4 weeks of denervation (~30%). As the diminishing attenuation in β1 proteasome activity corresponded to the gradual decline in contracture prevention with prolonged bortezomib treatment (Figure 5B,C), it could account for the diminishing effectiveness of proteasome inhibition in preventing contractures beyond the neonatal period. Overall, these findings reveal intriguing insights into the dynamics between protein degradation and the development of contractures.

4 | **DISCUSSION**

Treatments for muscle contractures stemming from pediatric neuromuscular dysfunction such as NBPI and cerebral palsy have traditionally focused on palliative mechanical solutions. Without addressing the underlying pathophysiology of contracture development, the outcomes of such strategies have been modest at best, and contractures remain a rate limiting factor of physical function in these disorders. Driven by the need to better understand contracture pathophysiology to prevent contractures, we have recently identified increased proteasome-mediated protein degradation as a causative mechanism for impaired longitudinal muscle growth and contracture formation following NBPI. This discovery has advanced the possibility of using proteasome inhibition as a potential treatment strategy in preventing contractures. In this current study, we delineate the timing of proteasome inhibition required to prevent contractures, identifying several key findings. First, the onset of treatment can be delayed as much as 3 days following NBPI in mice, corresponding developmentally to an approximate 3-4 week delay in humans infants. This finding is critical as the determination of a permanent brachial plexus injury in a child is based on failure to recover active motion within the first 3-4 weeks of life. If proteasome inhibition can be delayed by 3-4 weeks without losing efficacy at preventing contractures, then only children with permanent paralysis, and thus, at risk of developing contractures, will need to be treated. In addition, we have...
discovered that proteasome inhibition must be continued beyond the neonatal period in order to prevent contractures at skeletal maturity. This suggests that there is not a finite neonatal window during which treatment is necessary, but rather, that denervation causes a permanent deficiency of muscle growth that must be continuously ameliorated during musculoskeletal development. Furthermore, proteasome inhibition gradually loses its efficacy in preventing contractures after the neonatal period, and prolonged exposure is detrimental to postnatal skeletal growth in muscle cross-sectional area and
FIGURE 4 Chronic proteasome inhibition is required throughout skeletal muscle development to prevent contractures after skeletal maturity. A, Schematic depiction of saline or bortezomib treatment during postnatal muscle development prior to skeletal maturity (P8-P60), or beyond skeletal maturity (P8-P88), and assessment of function at 12 weeks post-NBPI in separate groups of mice. B-D, Representative forelimbs images, and quantitation of contracture severity showed improvements in both elbow and shoulder contractures at 12 weeks post-NBPI only with continuous proteasome inhibition for 12 weeks. D, Representative DIC images of sarcomeres and quantitation of sarcomere length revealed that continuous proteasome inhibition is required to prevent sarcomere elongation in brachialis muscles 12 weeks post-NBPI. In (C) and (D), contracture severity is calculated as the difference in passive elbow extension and shoulder rotation between the denervated (NBPI) side and the contralateral control side (n = 10-19 mice). In (F), sarcomere length on the NBPI side is normalized to the contralateral side, with a normal sarcomere length ratio of 1.0, and any value over 1.0 indicating sarcomere overstretch on the NBPI side, and thus, functional muscle shortening (n = 10-19 mice). Data are presented as mean ± SD. Statistical analyses: (C), (D), (F) unpaired two-tailed Student’s t tests. *P < .05, **P < .01, ***P < .001. All samples represent independent biological data points. Scale bar: 10 µm
Figure 5  Protopase inhibition is required but insufficient for preventing contractures beyond neonatal development. A, Schematic depiction of continuous bortezomib treatment and assessment of function at 4, 8, or 12 weeks post-NBPI. B-D, Despite the necessity for chronic proteasome inhibition throughout postnatal muscle development (8 weeks post-NBPI) and beyond skeletal maturity (12 weeks post-NBPI), reductions in both elbow and shoulder contracture severity, as well as sarcomere elongation, became less effective after the neonatal period (4 weeks post-NBPI). In (B) and (C), contracture severity is calculated as the difference in passive elbow extension and shoulder rotation between the denervated (NBPI) side and the contralateral control side, and then, the mean contracture severity among saline-treated animals is set to 1.0 to allow normalization of the treatment groups across time points (n = 9-19 mice). In (D), sarcomere length on the NBPI side is normalized to the contralateral side to generate a sarcomere length ratio, and then, the mean sarcomere length ratio among saline-treated animals is set to 1.0 to allow normalization of the treatment groups across time points, where reduced sarcomere overstretch indicates improved muscle length (n = 8-19 mice). Data are analyzed as fold change over corresponding saline controls. Statistical analyses: (B), (C), (D) unpaired two-tailed Student’s t tests. *P < .05, **P < .01, ***P < .001, ****P < .0001. All samples represent independent biological data points.
protein turnover, is insufficient to prevent substantial levels of protein degradation. Instead, simultaneous inhibition of the chymotrypsin-like site with the caspase-like β1 site, or with the trypsin-like β2 site, is required to prevent significant protein degradation. Given our findings of reduced β1 activity in the absence of any decrease in β5 activity with prolonged bortezomib treatment, we are inclined to believe that bortezomib acts upstream of the complex regulatory process.
network leading to sarcomerogenesis, as opposed to directly suppressing the degradation of sarcomeric proteins. A potential upstream target is the pro-inflammatory transcription factor, nuclear factor-kB (NF-kB). Here, bortezomib has been shown to stabilize the inhibitor I-kB by preventing its proteasomal degradation and the subsequent translocation of NF-kB to the nucleus of cancer cells, thereby inhibiting the NF-kB pathway. As NF-kB signaling has been implicated in denervation-induced muscle atrophy, attenuation of this regulatory pathway may serve as mechanism by which bortezomib regulates sarcomerogenesis. To conclusively elucidate the function of bortezomib in preventing contractures and validate its potential as a pharmacologic strategy, future efforts should thoroughly interrogate the mechanistic links between denervation, proteasome activity, and sarcomerogenesis. In particular, it is valuable to discern the dynamics between the catalytic activities of the 20S subunits and longitudinal muscle growth in denervated muscles. As other classes of proteasome inhibitors, such as Carfilzomib and Marizomib, bind to different proteolytic sites of the 20S catalytic core complex including the trypsin-like β2 subunit, they provide a unique opportunity to further dissect the contributions of the different proteolytic sites to sarcomerogenesis and muscle length.

In conclusion, we delineated the necessary timing of proteasome inhibition for preventing contractures after neonatal denervation. We discovered that treatment may be briefly delayed following denervation, but that it is required throughout growth to prevent contractures. Furthermore, its efficacy diminishes over time. Finally, the therapeutic effect of prolonged proteasome inhibition is associated with attenuation of β1 subunit activity of the 20S proteasome. By delineating the timing of proteasome inhibition required to prevent contractures, the current study provides important information to guide translation of a pharmacologic strategy to prevent pediatric muscle contractures through the specific targeting of a causative mechanism. Our findings also establish critical insights into the kinetics of protein degradation, muscle growth, and contractures, with potential implications in other pediatric muscle disorders.

ACKNOWLEDGMENTS
We thank the following entities within Cincinnati Children’s Hospital Medical Center: Jenny Melzer of Veterinary Services for surgical assistance, the Confocal Imaging Core for microscope assistance, and the Millay Laboratory for discussions and feedback. We also thank Sharon Wang from the Preclinical Imaging Core (University of Cincinnati College of Medicine) for MicroCT assistance. This work was supported by grants to RC from the National Institutes of Health (NIH) (R01HD098280-01) and Pediatric Orthopedic Society of North America (POSNA), as well as funding from the Cincinnati Children’s Hospital Division of Orthopedic Surgery and Junior Cooperative Society. The respective funding sources were not involved in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

CONFLICT OF INTEREST
The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS
Q. Goh, S. Nikolaou, and R. Cornwall designed research; Q. Goh, S. Nikolaou, K. Shay-Winkler, M.E. Emmert, and R. Cornwall performed research; Q. Goh, S. Nikolaou, K. Shay-Winkler, M.E. Emmert, and R. Cornwall analyzed data; Q. Goh wrote the paper.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.