Inhibition of Inflammation with Celastrol Fails to Improve Muscle Function in Dysferlin-deficient A/J Mice

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Running Title: Effect of celastrol on dysferlin-deficient muscle

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Key words: Muscular dystrophy, skeletal muscle, dysferlin, celastrol, NF- κ B, and inflammation.

Abstract

The dysferlin-deficient A/J mouse strain represents a homologous model for limb-girdle muscular dystrophy 2B. We evaluated the disease phenotype in 10 month old A/J mice compared to two dysferlin-sufficient, C57BL/6 and A/JOlaHsd, mouse lines to determine which functional end-points are sufficiently sensitive to define the disease phenotype for use in preclinical studies in the A/J strain. A/J mice had significantly lower open field behavioral activity (horizontal activity, total distance, movement time and vertical activity) when compared to C57BL/6 and A/JOlaHsd mice. Both A/J and A/JOlaHsd mice showed decreases in latency to fall with rotarod compared to C57BL/6. No changes were detected in grip strength, force measurements or motor coordination between these three groups. Furthermore, we have found that A/J muscle shows significantly increased levels of the pro-inflammatory cytokine TNF- α when compared to C57BL/6 mice, indicating an activation of NF- κ B signaling as part of the inflammatory response in dysferlin-deficient muscle. Therefore, we assessed the effect of celastrol (a potent NF- κ B inhibitor) on the disease phenotype in female A/J mice. Celastrol treatment for four months significantly reduced the inflammation in A/J muscle; however, it had no beneficial effect in improving muscle function, as assessed by grip strength, open field activity, and *in vitro* force contraction. In fact, celastrol treated mice showed a decrease in body mass, hindlimb grip strength and maximal EDL force. These findings suggest that inhibition of inflammation alone may not be sufficient to improve the muscle disease phenotype in dysferlin-deficient mice and may require combination therapies that target membrane stability to achieve a functional improvement in skeletal muscle.

Introduction

Dysferlinopathies are autosomal recessive muscle disorders caused by mutations in the dysferlin (*DYSF*) gene (1-3). Predominant phenotypes associated with mutations in the *DYSF* gene include limb girdle muscular dystrophy 2B (LGMD2B) and Miyoshi myopathy (MM). Both LGMD2B and MM are slow progressing, late-onset dysferlinopathies; LGMD2B patients present with proximal muscle weakness, while MM patients initially show weakness in distal muscles. Histologically, muscle biopsies show myofiber degeneration as well as significant inflammation, including accumulation of macrophages, CD8⁺ T cells, and CD4⁺ T cells (4). Currently, several genetic approaches are being tested in the hope of attaining curative treatment for dysferlinopathies, including exon skipping and trans-splicing; however, these techniques are in their infancy and will take time to move to human clinical trials (5-8). Even if these therapies are successful, it is likely that they will not be completely curative because of differential correction in various muscles. Genetic alteration to dysferlin could have effects on specific protein interaction sites in the Dysferlin protein. Therefore, the use of palliative therapeutic approaches using pharmacological and immunological agents to modulate dysferlin-associated pathology would be a more effective and useful approach either alone or in combination with other therapies.

Animal models of human disease play an important role in understanding disease pathogenesis, identification of drug targets and evaluation of therapeutic efficacy of drugs in preclinical trials. Therefore, characterization of disease phenotypes in these animal models would help us better interpret the results of preclinical trials. Several dysferlin mouse strains exist; these include: Bl/a/J, A/J, C57BL/6J-Chr6^{A/J}/NaJ, SJL/J, and B10.SJL-Dysfim/AwaJ. Of these, the A/J and SJL/J strains have spontaneous mutations in the dysferlin gene (9-15). More

specifically, A/J mice have an ETn retrotransposon inserted into intron 4 of the dysferlin gene and develop histological evidence of muscular dystrophy after 4 to 5 months of age. These mice show slow progression of disease pathology with persistent muscle weakness (9). By 14 months of age, the muscles of A/J mice show marked inflammatory and fatty infiltration and significant muscle degeneration. The A/J strain is one of the commercially available models used to understand pathogenesis of dysferlin deficiency (9).

The presence of inflammatory infiltrates in dysferlin-deficient muscle suggests their prominent role in muscle pathology. Recent reports indicate that pro-inflammatory nuclear factor κ B (NF- κ B) is upregulated in dysferlin-deficient myotubes (16). Celastrol is a triterpene derived from the plant *Tripterygium wilfordii*, the “thunder god vine,” and has been used in traditional Chinese medicine for its anti-inflammatory effects. Celastrol has been shown to suppress I κ B phosphorylation and NF- κ B activation by inhibiting TAK1 activation (17). We have previously developed a muscle-based NF- κ B inhibitor screening assay and shown that celastrol is one of the powerful inhibitors of NF- κ B under this system (18). Based on this data, inhibition of NF- κ B anti-inflammatory agents such as celastrol to suppress immune activation in dysferlin-deficient muscle could ameliorate the disease pathology. In this study, the phenotypes of dysferlin-deficient A/J mice were compared to dysferlin-sufficient C57BL/6 and A/JOlaHsd mice using well-established functional and behavioral assessments. Using these assays, we also tested the effect of celastrol on muscle disease phenotype in dysferlin-deficient A/J mice.

Materials and Methods

Animals

All mice were handled according to local institutional animal care and use committee guidelines. For the phenotyping experiments examining the differences in functional and behavioral measures between normal and diseased mice, A/JOlaHsd (n=7) were purchased from Harlan laboratories (Indianapolis, IN). A/J (n=8) and C57BL/6 (n=12) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All mice examined in phenotyping experiments were 9 months old and groups contained the following numbers of males and females: A/JOlaHsd (n=7, 3 females & 4 males), A/J (n=8, 4 females & 4 males) and C57BL/6 (n=12, 6 females & 6 males). For pre-clinical evaluation of celastrol, 10 month old female A/J mice (n=20) were purchased from the Jackson Laboratories. All mice were housed in an individually vented cage system with a 12-h light-dark cycle and received free access to standard mouse chow and water. **Administration of celastrol**

Female A/J mice were randomly separated into two groups, untreated (n=7) and celastrol-treated (n=13). The untreated group received untreated water, whereas the treatment group received water with celastrol. The drug was administered in the drinking water for 4 months at a concentration of 8.6 µg/mL. Celastrol-supplemented water was replaced three times a week, and water consumption was closely monitored throughout the trial. Body weight as well as functional and behavioral measures were monitored at 14 months of age and are described in detail below. At the end of the trial, mice were euthanized, and various muscle tissues were collected for histological evaluations. *In vitro* muscle force was also monitored to assess the effect of the drug on muscle function.

Functional Tests

Functional and behavioral tests were performed to monitor the disease phenotype and to assess the effect of celastrol treatment, as described previously (15, 19). Grip strength was assessed for both the fore- and hindlimbs, and the data are expressed as kg force/kg body weight (KGF/kg). Motor coordination (Rotarod) was monitored as described previously (15). The length of time that each mouse stayed on the rod was recorded as latency to fall (sec), and the mean values obtained for each group were compared. Open-field behavioral activity was measured using an open-field Digiscan apparatus (Omnitech Electronics, Columbus, Ohio), as described previously (15, 19). Data were collected every 10 min over a period of one hour each day over four consecutive days. Total distance (cm), movement time (seconds), rest time (seconds), vertical activity (units), and horizontal activity (units) were expressed as mean $\pm$ SEM.

***In vitro* force measurement**

EDL muscles of the right hindlimbs were removed from anesthetized mice, and muscle mass was recorded (mg). Maximal force and the specific force produced by the EDL muscle were monitored as described previously (15, 19). The maximal force generated by the EDL was measured and represented as milliNewtons (mN). The specific force was assessed by dividing the maximal force by the cross-sectional area of the muscle and expressed in kN/m^2 (20) and expressed as mean $\pm$ SEM.

Measurement of Pro-inflammatory transcripts in A/J Mice

The expression levels of NF- κ B target genes were determined using qPCR. Quadriceps tissues were obtained from 10- to 12-month-old A/J and C57BL/6 mice (n=3/group). Total RNA was isolated using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. cDNA was synthesized using a Life Technologies High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies Cat#4374966). qPCR was performed

using an Applied Biosystems 7900HT Real-Time PCR machine. TaqMan probe sets TNF- α (Mm00443260_g1), MCP1 (Mm00441242_m1), IL6, HPRT, and 18S (4319413E) were purchased from Applied Biosystems. Relative induction was calculated using the $\Delta\Delta C_t$ method, with 18S or HPRT as the internal reference gene. Fold changes were expressed as means $\pm$ SEM and statistical analysis was performed on the raw delta C_t values using the non-parametric Wilcoxon rank sum test.

Histological evaluations

Quadriceps muscles collected in formalin were used for hematoxylin and eosin (H&E) staining. The muscles were sent to Histoserv, Inc (Germantown, MD) for staining. Digital images were taken at 20X magnification on an Olympus BX61 bright-field microscope and processed using Image J (NIH), with additional threshold plug-ins to process jpeg images. Pixels corresponding to non-muscle areas were highlighted and normalized to the total pixel area of the tissue image. Based on these values, we derived the muscle area and expressed the results as percentages. To quantify inflammation, stained sections were viewed using bright-field microscopy, and inflammatory foci per section were counted at 40X magnification. Inflammatory foci were defined as a group of 10 blue infiltrating nuclei in the muscle tissue. Entire tissue sections were quantified in a blinded fashion. Results were expressed as means $\pm$ SEM.

Echocardiography

Echocardiography was performed as described previously (21-23). Qualitative and quantitative measurements were made offline using analytic software (VisualSonics, Toronto, Ontario, Canada). Electrocardiogram kHz-based visualization (EKV) software analysis produced offline reconstruction for simulated 250- to 1000-Hz static and cine-loop images.

Echocardiography measurements included vessel diameters, ventricular chamber size, and blood flow velocities across the atrioventricular and semilunar valves. Modified parasternal long-axis EKV loops were also used to measure ejection fraction (EF) via Simpson's method. M-mode images were used to measure left ventricular (LV) chamber sizes and wall thicknesses. Percent shortening fraction was calculated from M-mode measurements using the leading edge-to-leading edge method via the formula: % SF= [LV internal diameter (diastole) [LVID (d)] – LV internal diameter (systole)]/ [LVID (d)].

Statistical analysis

For phenotyping experiments, normality of each outcome was tested with the Shapiro-Wilk normality test. Comparisons of normally distributed outcomes between strains used one-way analysis of variance (ANOVA) models. For those ANOVA models with a statistically significant overall p-value, *post-hoc* pair-wise comparisons were performed and the resulting p-values adjusted for multiple comparisons using the Sidak method. Comparisons of non-normally distributed outcomes between strains used non-parametric Kruskal-Wallis tests. Those outcomes with a statistically significant Kruskal-Wallis p-value were further analyzed in a pair-wise fashion using Wilcoxon rank sum tests. Resulting p-values were again adjusted for multiple comparisons. For celastrol treatments, all analyses compared means between celastrol and untreated groups using t-tests.

Results

Dysferlin-deficient A/J mice exhibits a deficit in behavioral activity measures.

Evaluation of dysferlin-deficient A/J mice and dysferlin-sufficient C57BL/6 and A/JolaHsd mice strains showed no significant differences in body mass between A/J, A/JolaHsd

and C57BL/6 mice strains (Table 1). A/J mice performed significantly worse than both C57BL/6 and A/JOl^aHsd mice in behavioral activity measures such as horizontal activity, total distance, movement number, rest time and vertical activity (Table 1). Overall, A/J mice demonstrated approximately 50% less activity than both the control strains tested. Both A/J and A/JOl^aHsd mice showed decreased motor coordination, as evidenced by latency-to-fall assessments on the Rotarod apparatus compared to C57BL/6 suggesting strain differences in motor coordination. Grip strength measurements and *in vitro* force measurements using EDL muscle showed no differences between the strains tested (Table 1), suggesting dysferlin deficiency manifests in lowered activity between these groups.

Proinflammatory cytokine TNF- α increased in dysferlin-deficient muscle

To assess the proinflammatory status of the skeletal muscle at this age, we evaluated the expression of TNF, IL-6, and MCP at the mRNA level. TNF α mRNA expression was increased in the dysferlin-deficient A/J muscle when compared to the C57BL/6 muscle (Figure 1). The A/J muscle demonstrated a 3-fold increase in TNF- α transcript expression when compared to 18S and HPRT expression. The relative expression levels of the MCP-1 and IL-6 transcripts did not change between the A/J and C57BL/6 muscles (data not shown).

Effect of chronic celestrol treatment on dysferlin-deficient muscle

Since we found evidence of inflammation, we treated the mice with the known anti-inflammatory compound celestrol for 4 months and evaluated the phenotypes of the treated and untreated mice.

Body weight and functional assessments

Celestrol treatment significantly reduced the body weight but did not show an effect on the individual muscle or organ weights (gastrocnemius, soleus, heart, and spleen) when

compared to untreated A/J mice (Table 2). Celestrol treated mice also showed significantly decreased hindlimb grip strength, however, no differences were observed in the normalized grip strength, open-field behavioral measurements or motor coordination between the groups after drug treatment (Table 2).

In vitro force measurements

Celestrol treatment significantly decreased the EDL muscle mass 27% in A/J mice when compared to the untreated group (Figure 2A). Notably, celestrol treatment significantly reduced maximal force 25.2% (Figure 2B). However, no significant differences in specific force were found between the treated and untreated groups (Figure 2C).

Histopathology

Hematoxylin and eosin staining of muscle sections revealed replacement of muscle tissue by adipose tissue, muscle fiber regeneration, inflammatory infiltration, and centralized nuclei in untreated A/J mice (Figure 3 A,D). Celestrol treatment significantly decreased the inflammatory foci when compared to untreated A/J mice (Figure 3C). A/J mice had approximately 10% non-muscle area at 14 months of age (Figure 3 A, B, D, E). Treatment with celestrol did not significantly affect the percentage of muscle area when compared to that of the untreated mice (Figure 3 D-F). The values for percentage of muscle area (mean $\pm$ SEM) for the treated and untreated mice were 91.34 ± 1.61 and 88.5 ± 2.74 , respectively.

Echocardiography

In order to evaluate cardiac function, we monitored left ventricular shortening and ejection fraction. The results indicated that celestrol-treated mice showed lower shortening and ejection fraction values than did the untreated mice; however, these differences were not statistically significant. Left ventricular shortening fraction values in the celestrol-treated and

untreated A/J mice were 35.60 ± 3.95 and 45.51 ± 6.20 , respectively (mean $\pm$ SEM). Similarly, the percent ejection fraction values for the celastrol-treated and untreated A/J mice were 65.44 ± 5.85 and 76.54 ± 6.42 , respectively (mean $\pm$ SEM).

Discussion

Evaluation of outcome measurements in preclinical trials is important for the development of standard testing procedures for new pharmacological agents used for the treatment of muscle myopathies. Our group recently reported the characterization of SJL/J mouse phenotypes (15). This study was presented as an initial step toward the development of standard operating procedures for preclinical testing in dysferlin-deficient animal models (15).

The current study has assessed the phenotypic characteristics of dysferlin-deficient A/J mice, focusing on behavioral, functional, and histological measurements. A/J mice performed poorly on behavioral activity measures compared to dysferlin-sufficient C57BL/6 and A/JolaHsd mice, suggesting an underlying pathology in the A/J mice. Celastrol treatment did not prevent disease progression or improve muscle function in the dysferlin-deficient A/J mice.

Dysferlin-deficient A/J and SJL/J mice exhibit both similarities and differences. When comparing the two strains, we observe differences in body weight, grip strength, and Rotarod performance but similarities in behavioral outcome measures and *in vitro* force. A/J mice are similar in body mass and grip strength to C57BL/6 mice. SJL/J mice, however, weighed less and had increased grip strength compared to C57BL/6 mice (15). Rotarod assessment for motor coordination showed a decrease in A/J mice and no difference in SJL/J mice when compared to C57BL/6 mice. SJL/J mice performed similarly to A/J mice in open-field behavioral measurements when compared to C57BL/6 mice as control. The maximal and specific force of the EDL for the A/J mice were not changed; however the SJL/J had significantly lower force

than that of age-matched C57BL/6 mice [14]. These differences can be attributed to the age of each strain at the time of assessment, as it is known that disease onset in SJL/J mice occurs earlier, and has a different rate of progression than in A/J mice. It is possible that these differences could be resolved by assessing the A/J and SJL/J mice at the ages at which the disease pathology is more comparable. We need to carefully interpret our comparative data between A/J and C57BL/6 mice because some of these differences in dysferlin-deficient and -sufficient mice could be due to strain differences, and others could be due to the disease. We have addressed this in this study and utilized an additional control strain, A/JO1aHsd mice, which have been shown to express normal levels of dysferlin (3). Comparison studies showed that most of the endpoints, with the exception of rotarod, are similar between older C57BL/6 and A/J wild type control strains, suggesting that the decrease in rotarod performance in A/J is likely due to strain differences and decrease in open field activity.

Although the age of onset and rate of disease progression differ in the A/J and SJL/J mice, muscle tissue from both strains is marked by mononuclear cell infiltration, consistent with an inflammatory response. This finding suggests a prominent role for inflammation in the muscle pathology. In addition, observations have been made in dysferlin-deficient muscle derived from both LGMD2B patients and in dysferlin-deficient mouse muscle, including: 1) enhanced phagocytic activity in dysferlin-deficient monocytes (24); 2) increased expression of rho family GTPases (RhoA, Cdc42, and Rac1) that are involved in monocyte phagocytosis (24, 25); 3) the presence of markers for dendritic T-cell and macrophage activation in dysferlin-deficient muscle; 4) central component (C3) enhances muscle injury (26), which suggests that there is an over-aggressive inflammatory response in the affected muscle (24). It was recently reported that pro-inflammatory NF- κ B signaling is upregulated in dysferlin-deficient myotubes and that the

intrinsic activation of NF- κ B and myogenic processes are connected in dysferlin-deficient muscle cells. Additionally, muscle specific transgenic expression of dysferlin in dysferlin-null mice rescues the muscle phenotype indicating the inflammatory response seen in this model is caused by the absence of dysferlin. Further studies showed that bone marrow transplantation reconstituted dysferlin expression in peripheral blood but not in skeletal muscle, and inflammatory infiltrates were present in the muscles of both treated and untreated mice - confirming the previous observation that the absence of dysferlin produces an inflammatory response in the tissue (3). The importance of the inflammatory response is further supported by *in vitro* evidence that celastrol (a potent NF- κ B inhibitor) reduces NF- κ B activation and improved myogenesis in dysferlin-deficient cultures (16). In addition, we have now shown an upregulation of TNF- α in dysferlin-deficient A/J mice when compared to C57BL/6 mice. Because TNF- α is regulated by NF- κ B, this upregulation is an indication of the NF- κ B pathway's involvement in dysferlin-deficient myopathy. Therefore, we treated A/J mice with celastrol, a potent anti-inflammatory agent that has been found to be effective *in vivo* in models of autoimmune arthritis, amyotrophic lateral sclerosis, and Alzheimer's disease (27-30). Baudy *et al.* generated a dose-response curve with celastrol on C2C12 myoblasts and myotubes from the nanomolar range to 10 μ M and found that its inhibitory potency for NF- κ B inhibition peaks at around 0.1 μ M (18).

To our knowledge, no earlier preclinical trials of celastrol have been conducted in A/J mice to determine the effective dose range. In this study, animals received an average of 5mg/kg/day of celastrol in the drinking water. We found that, celastrol treatment showed no beneficial effect on dysferlin-deficient A/J muscle. Celastrol treatment significantly reduced the body weight of the A/J mice when compared to the untreated mice. Because body weight can

affect grip strength, grip strength assessments were normalized to body weight. A/J mice treated with celastrol showed increased hindlimb grip strength when compared to untreated A/J mice, although this significance was not present when normalizing the data to body weight. In all behavioral assessments, A/J mice treated with celastrol showed no differences from untreated A/J mice, and there were no improvements in motor coordination. No significant difference was found in muscle weight between the celastrol-treated and untreated A/J mice, and treatment with celastrol decreased the maximal strength of the EDL muscle, while having no effect on the specific force.

While both treated and untreated A/J mice had muscle tissue that exhibited inflammation, regenerating fibers, and large sections of adipose tissue, celastrol treatment appeared to reduce the severity of this pathology. Treatment with celastrol significantly decreased the number of inflammatory foci in the muscle of A/J mice when compared to untreated A/J mice. A similar reduction in inflammation upon treatment with celastrol was reported earlier in a rat model of adjuvant-induced arthritis (AIA) (28). Another study reported a significant reduction in the chemokines RANTES, MCP-1, MIP1a, and GRO/KC and suppression of autoimmune arthritis in a rat model after treatment with celastrol (31).

It is not clear why reduction in muscle inflammation had little or no effect on the behavioral and functional outcomes tested; however, there are several possible explanations. If inflammation is secondary to the progression of disease pathology, a decrease in inflammation may be effective in improving the functional aspects of the disease. It is possible that celastrol did not reduce the inflammation to the level necessary to observe a functional improvement. Alternatively, although celastrol was successful in improving the disease pathology, the functional and behavioral assays used may not be sensitive enough to detect a slight

improvement. A 10% decrease in muscle area at 14 months of age in A/J mice suggests an ongoing disease process. It appears that the muscle is being replaced by fatty tissue; however, further studies that use specific fat staining are required to confirm these findings. Nevertheless, the lack of muscle tissue potentially contributed to the decrease in behavior and mobility from the digiscan analysis in the A/J mice when compared to dysferlin-sufficient C57BL/6 or A/J01aHsd mice.

Next, the echocardiography measurements indicated that celastrol treatment had no effect on cardiac functional parameters. Furthermore, our results indicate that the percent ejection and shortening fractions were within the normal range for both the untreated and celastrol-treated groups of A/J mice ($>60\%$ and $>30\%$, respectively). Earlier reports indicated that normal mice have average shortening fraction of $33.6 \pm 4.8\%$ and an ejection fraction of $54 \pm 11\%$ (22). On the other hand, a previous study reported significant deterioration in cardiac function, as evidenced by % ejection fraction and % fractional shortening in A/J mice at 8 to 10 months of age. However, they also reported that the mice recovered from the cardiac phenotype by 10 to 12 months of age suggesting age specific cardiac defects in the model (32). Such a recovery might be one reason for the lack of cardiac phenotype or an effect of the drug in this study where cardiac assessments were performed at 14 months of age.

These studies indicate celastrol treatment significantly decreased inflammation, with little effect on functional and behavioral assessments; hence, we speculate that the use of alternative anti-inflammatory drugs with additional beneficial properties such as membrane stabilization might be useful in improving the strength and overall function of dysferlin-deficient muscle.

Acknowledgements

Dr. Nagaraju is supported by NIH (5U54HD053177; K26OD011171, P50AR060836-01), Muscular Dystrophy Association, and US Department of Defense (W81XWH-05-1-0616, W81XWH-11-1-0782, W81XWH-11-1-0330).

Figure legends:

Figure 1. TNF-alpha expression in dysferlin-deficient A/J mice

Total messenger RNA was isolated from the quadriceps femoris of 10- to 12-month-old A/J (n=3) and C57BL/6 (n=3) mice. qPCR analysis demonstrated a 3-fold induction of TNF-alpha transcripts (relative to an 18S endogenous mRNA control) in A/J mice as compared to C57BL/6 mice. Mean values were compared using non-parametric Wilcoxon rank-sum test.

*p<0.05. Error bars indicate $\pm$ SEM.

Figure 2. Celastrol treatment reduced the maximal force in A/J mice

A/J mice were either not treated or treated with celastrol for 4 months. *In vitro* muscle function tests were performed on the EDL to determine the effect of celastrol treatment on muscle force. Shown are the graphs for muscle mass (A) (mg), maximal force (B) (mN), and specific force (C) (kN/m^2). Values were as follows for the means: muscle weight – untx 9.1 and celastrol treated 6.6 mg; maximal force – untx 299.3 and 223.8 mN; specific force – untx 220.0 and celastrol treated 219.7 kN/m^2 . Mean values were compared for n=6 mice from each group using Student's t-test. *, p<0.05. Error bars indicate $\pm$ SD.

Figure 3. Celastrol treatment decreases inflammation

A/J mice were treated with celastrol (n=13) or left untreated (n=7) for 4 months. Quadriceps muscles were sectioned stained with hematoxylin and eosin. Inflammatory foci per section were quantified manually at 40X magnification by an experienced researcher in a blinded fashion. Shown are images of muscle histopathology in untreated (A) and celastrol-treated (B) A/J mice at 40X magnification. Also shown is the graph for inflammatory foci per section (C) which was significantly decreased in celastrol treated mice. Images of histopathology in untreated (D) and celastrol-treated (E) A/J mice at 10X are shown with the quantitation of the

non-muscle area (F) which was not different between the treated and untreated groups. Mean values were compared using Student's *t*-test. *, $p < 0.05$. Error bars indicate $\pm$ SEM.

Table 1: Functional and behavioral assessments between dysferlin-deficient (A/J) and sufficient (A/JO1aHsd and C57BL/6) mice.				
Measurement	A/JO1aHsd (N=7)	A/J (N=8)	C57BL/6J (BL6) (N=12)	Significantly different means
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Body weight (g)*	26.3 (19.6 – 36.1)	24.0 (21.8 – 31.5)	27.8 (21.8 – 33.5)	NONE
GSM – forelimb (KGF)	0.126 $\pm$ 0.011	0.142 $\pm$ 0.022	0.142 $\pm$ 0.014	NONE
GSM – hindlimb (KGF)	0.350 $\pm$ 0.031	0.358 $\pm$ 0.022	0.362 $\pm$ 0.026	NONE
Normalized GSM – forelimb (KGF/kg)	4.94 $\pm$ 0.65	5.47 $\pm$ 0.50	5.37 $\pm$ 1.16	NONE
Normalized GSM – hindlimb (KGF/kg)	13.88 $\pm$ 2.68	14.00 $\pm$ 1.70	13.75 $\pm$ 3.09	NONE
Specific Force (KN/m ²)	241 $\pm$ 30	252 $\pm$ 26	240 $\pm$ 27 [#]	NONE
Horizontal activity (beam interruptions)	896 $\pm$ 251	447 $\pm$ 181	817 $\pm$ 128	AJ+/+ vs. AJ-/- (p=0.001) AJ-/- vs. BL6 (p=0.002)
Total distance* (cm)	187 (70 – 398)	64 (34 – 129)	192 (115- 231)	AJ+/+ vs. AJ-/- (p=0.015) AJ-/- vs. BL6 (p<0.001)
Movement number	46.6 $\pm$ 19.6	22.1 $\pm$ 8.0	43.9 $\pm$ 6.8	AJ+/+ vs. AJ-/- (p=0.011) AJ-/- vs. BL6 (p=0.010)
Rest time* (sec)	582.3 (561.4 – 592.6)	591.2 (586.4 – 595.2)	577.0 (573.0 – 585.8)	AJ+/+ vs. AJ-/- (p=0.011) AJ-/- vs. BL6 (p<0.001)
Vertical activity (beam interruptions)	28 $\pm$ 17	3 $\pm$ 3	22 $\pm$ 7	AJ+/+ vs. AJ-/- (p<0.001) AJ-/- vs. BL6 (p=0.001)
Rotarod*	17 $\pm$ 11	18 $\pm$ 12	35 $\pm$ 7	AJ+/+ vs. BL6 (p=0.022)

* Nonparametric test performed – data expressed at median (range)

[#] For Specific Force - C57BL/6J, N=8; 4 males and 4 females

Table 2. Functional and behavioral parameters in dysferlin-deficient A/J mice treated with celastrol for 4 months.			
Measurement	Celastrol (N=12)	Untreated (N=6)	P-value
Body mass (kg)	0.024 ± 0.003	0.027 ± 0.002	0.039*
GSM – forelimb (KGF)	0.096 ± 0.005	0.095 ± 0.005	0.706
GSM – hindlimb (KGF)	0.143 ± 0.003	0.149 ± 0.004	0.005***
Normalized GSM-forelimb (KGF/kg)	4.089 ± 0.625	3.546 ± 0.300	0.063
Normalized GSM-hindlimb (KGF/kg)	6.098 ± 0.799	5.551 ± 0.375	0.135
Total distance (cm)	109 ± 42	95 ± 21	0.445
Movement time (sec)	13 ± 4	10 ± 6	0.157
Rest time (sec)	587 ± 4	588 ± 2	0.425
Vertical activity (beam interruptions)	13 ± 10	15 ± 7	0.656
Horizontal activity (beam interruptions)	677 ± 128	656 ± 124	0.746
Gastroc. wt. (mg)	66.8 ± 13.5	73.8 ± 11.4	0.292
EDL wt. (mg)	6.9 ± 1.3	8.1 ± 1.1	0.071
Soleus wt. (mg)	6.3 ± 1.3	6.6 ± 1.6	0.767
Heart wt. (mg)	98.5 ± 14.1	102.7 ± 9.5	0.529
Spleen wt. (mg)	65.5 ± 15.7	71.1 ± 8.3	0.426

All analyses compared means between celastrol and untreated groups using t-tests.

*n=6 for celastrol group

Figure 1

Figure 2

Figure 3

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