The role of glucocorticoid receptor signaling in metabolic disease: a matter of time and sex

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Sexual dimorphism in transcriptional and functional glucocorticoid effects on skeletal muscle

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ABSTRACT

Muscle atrophy is a common problem in patients with increased glucocorticoid exposure, but it is unclear whether this response differs between males and females, and if so, why. In this study, we evaluated glucocorticoid-induced muscle atrophy in mouse models of increased corticosterone exposure and synthetic glucocorticoid treatment. We found that increased corticosterone exposure specifically reduced female grip strength, but that muscle mass was suppressed in both sexes. The skeletal muscle transcriptional responses to elevated corticosterone were generally much more pronounced and widespread in male mice. Upon synthetic glucocorticoid treatment, we found a reduction in grip strength for both male and female mice, but muscle atrophy in female mice was more sensitive compared to males. To evaluate the role of androgens, we repeated synthetic glucocorticoid treatment in chemically-castrated male mice. We found that androgen depletion and glucocorticoid treatment additively reduce muscle mass, but no interaction effects. Altogether, we show sex differences in response to glucocorticoids in skeletal muscle, and although differences in androgen levels may in part contribute to this. Further studies are warranted to fully delineate the mechanism behind these sex-specific effects. We believe that this study will contribute to a better understanding of the sex differences in muscle atrophy in patients with elevated glucocorticoid exposure.

INTRODUCTION

Muscle atrophy is the wasting or loss of muscle tissue and significantly reduces the quality of life and increases mortality [1-3]. Muscle atrophy is observed in multiple diseases including cancer, diabetes, sepsis and renal failure, but also upon synthetic glucocorticoid (GC) treatment. GC-induced muscle atrophy is prevalent, and is mostly the result of high dose and the sustained usage of GCs or increased endogenous GC levels [4, 5]. Muscle atrophy and reduced muscle function were observed at different doses and treatment regimens with the synthetic glucocorticoids dexamethasone and prednisolone [6-9]. Another commonly used synthetic glucocorticoid is betamethasone [10], which is prescribed for a range of inflammatory diseases at a wide range of doses [11].

Skeletal muscle is composed of different types of muscle fibers, including the slow/oxidative type 1 fibers and fast/glycolytic type 2 fibers [12, 13]. Type 1 fibers have an oxidative capacity and contain more myoglobin and mitochondria, are important primarily to muscle endurance, and have higher resistance to fatigue as compared with type 2 fibers [14]. Type 2 fibers are predominantly glycolytic and can be subdivided in several subtypes including type 2A and type 2B fibers. Type 2A fibers have a fast contraction velocity and are less prone to fatigue compared to type 2B fibers. Type 2B fibers are the largest fiber type and generate ATP by anaerobic metabolic processes when maximum power is required [15]. Muscle types are characterized by a distinct mixture of fiber types [16], and a changes in muscle function and atrophy generally often also involve a re-distribution in muscle fiber type composition [17].

Total muscle mass is regulated by many endocrine factors, including anabolic factors such as androgens and catabolic factors such as GCs [18, 19]. GCs negatively regulate muscle mass directly and via interference with anabolic pathways, and this results in a loss of protein and a reduction of muscle fiber number and density [20, 21]. Upon GC exposure, the ubiquitin-proteasome system is activated in skeletal muscle, which plays a major role in myofibrillar protein degradation [22]. Muscle atrophy F-box (*atrogin-1*) and muscle ring finger 1 (*MurF-1*) are two muscle-specific ubiquitin ligases of which expression is increased under atrophy-inducing conditions, and these so-called atrogenes play a critical role in muscle atrophy [23, 24]. Krüppel-like transcription factor (*Klf15*) is a pivotal factor in skeletal muscle, and was shown to directly regulate the expression of the *atrogin-1* and *MurF-1* atrogenes [25] but is also involved in muscle endurance [26].

Many processes that are influenced by GC exposure are known to be sexually dimorphic [27, 28], possibly explained by differences in sex hormone levels. Synthetic glucocorticoid treatment influences sex hormone levels, i.e. lowers the testosterone level in male rats, while it increases testosterone levels in female

rats [29]. In humans, glucocorticoids response in muscle function can be sexually dimorphic, but it is unclear to what extent androgens play a role in such effects. In this study, we investigated the effects of corticosterone and synthetic glucocorticoid treatment on muscle atrophy and function in male and female mice. We found that male and female muscle responded differently to glucocorticoid exposure at a transcriptomic and functional level, and that androgen signaling may in part contribute to these differences.

METHODS

Animals

All animal experiments were approved by the ethical committee of Leiden University Medical Center (functional cohorts) or Erasmus MC (RNA-sequencing cohort). Mice were purchased from Charles Rivers Laboratories and group housed in conventional cages with a 12-hour:12-hour light:dark cycle and had ad libitum access to water and RM3 chow diet (Special Diet Services, Essex, UK). Male and female C57BL/6J mice aged 8-10 weeks were used.

Animal experiments

To test muscle sensitivity to corticosterone treatment, male (N=8/group) and female mice (N=8/group) were implanted subcutaneously with either a corticosterone-pellet (20 mg corticosterone and 80 mg cholesterol) or a vehicle-pellet (100 mg cholesterol) in the neck region [30, 31], and mice were followed for 14 days. Corticosterone and vehicle pellets were synthesized at Leiden University Medical Center.

To study sex differences in sensitivity to synthetic glucocorticoid betamethasone treatment, male (N=4/group) and female mice (N=6/group) were intraperitoneally injected with 3 mg/kg betamethasone, 25 mg/kg betamethasone, or PBS (vehicle) daily for 14 days. The dose of betamethasone was based on previous muscle atrophy studies with dexamethasone [32], which has approximately the same potency as compared to betamethasone.

To investigate the role of androgen signaling in glucocorticoid-induced muscle atrophy, male mice were chemically castrated using a subcutaneous injection with 25 mg/kg degarelix (MedChemExpress), which is a GnRH antagonist that blocks LH and FSH release and results in diminished testosterone levels [33]. Intact and chemically-castrated mice were intraperitoneally injected daily with 3 mg/kg betamethasone or vehicle (PBS) for 14 days (N=8/group).

Body Weight, Body Composition, Grip Strength and Grid Hanging Measurement

All cohorts were subjected to several functional tests and measurements to assess body weight, body composition and muscle function. Body weight, body composition (EchoMRI-100-analyzer) and grip strength were measured twice a week and grid hanging was measured once a week, and all functional measurements were performed between 3-6 hours after lights-on. Grip strength of the forelimb was measured using a grid attached to an isometric force transducer (Chatillon, Columbus Instruments 080529). The force transducer records the maximum force that is required to break the mouse's grip from the mesh surface. In total, we recorded five sets of measurements, each consisting of three pulls and with a resting period of at least one minute between them. The three highest values obtained were averaged. Overall muscle function was assessed with the four limbs hanging test, the mouse was placed on a grid, which was turned upside down, 15 cm above a cage filled with soft bedding. This test was performed weekly with a maximum of three attempts per session from which the best performance was used. Maximum allowed hanging time was 600 seconds. At the end of the experiments, mice were killed by CO2 asphyxiation (between 3-6 hours after lights-on) and several muscle types were isolated, weighed and frozen in liquid nitrogen for further processing.

RNA Isolation and RT-qPCR Analysis

Total RNA was isolated by using Tripure (Roche) according to the manufacturer's instructions. RNA concentration was measured by NanoDrop spectrophotometer (Thermo Fisher). Total RNA was diluted into 1 µg for reverse transcription using M-MLV reverse-transcriptase (Promega). cDNA (4 ng) was used per 10 µl RT-qPCR reaction, and each qPCR reaction contained 1 µl primers (0.5 µl forward and 0.5 µl reverse of each) and 5 µl SYBR green supermix (Bio-Rad) using a Bio-Rad CFX96. GAPDH was used as housekeeping gene. Primer sequences: MurF-1 Fwd: TGTGCAAGGAACAGAAGAC; Rev: CCAGCATGGAGATGCAGTTA; Atrogin-1 Fwd: TTGGATGAGAAAAGCGGCAG; Rev: TACAGTATCCATGGCGCTCC; Klf15 Fwd: AAATGCACTTTCCCAGGCTG; Rev: CGGTGCCTTGACAACTCATC; Gapdh Fwd: GGGGCTGGCATTGCTCTCAA; Rev: TTGCTCAGTGTCCTTGCTGGGG.

RNA Sequencing

To study the corticosterone-induced transcriptome in quadriceps muscle, male and female mice were subcutaneously implanted in the neck region with slow-release pellets containing corticosterone (50 mg corticosterone and 50 mg cholesterol; N=6 per sex) or vehicle (100 mg cholesterol; N=6 per sex) (corticosterone and vehicle pellets were synthesized at Leiden University Medical Center). After 14 days, mice were fasted for 5 hours and killed by cardiac puncture under isoflurane anaesthesia (28). Quadriceps muscle was collected and homogenized in Tripure using a Kimble pellet pestle followed by a phase-

separation with chloroform. Total RNA was isolated using the RNeasy kit according to manufacturer's instructions (Qiagen 74104). RNA quality was ensured (RNA Integrity number > 7.0 and 28/18s ratio > 1.0) using the RNA 6000 Nano kit bioanalyser (Agilent). Stranded mRNA libraries were constructed and 100bp paired-end bulk RNA-sequencing was performed at BGI Genomics (Hong Kong, China) on the DNBseq platform. Over 20 million reads were sequenced per sample. RNA sequencing data has been deposited in NCBI's Gene Expression Omnibus (GEO series accession number GSE202787).

RNA Sequencing Data Analysis

The RNA-seq pipeline (version 4.1.0), published as part of BioWDL, was used for read quality control, alignment and quantification. BioWDL contains the main sequencing analysis pipelines and workflows developed at Leiden University Medical Center by the sequencing analysis support core with code being accessible at https://biowdl.github.io/.

Quality control was performed using FastQC and MultiQC. Reads were aligned to Mus Musculus genome version 10 (mm10) using STAR (version 2.7.3a). Tool settings used were: '–runThreadN' '4' '–outSAMunmapped' 'Within KeepPairs' '–twopassMode' 'Basic'. The gene-read quantification was performed using HTSeq-count (version 0.12.4). Tool settings used were: '–order' 'pos' '–stranded' 'reverse'. Uniquely assigned reads were mapped to known genes based on Ensembl release 97 of mm10. HTSeq-count output files were merged into a count matrix per experiment as input for differential gene expression analysis.

DEseq2 (version 1.29.4) was used for normalization of the count data (median of ratio's method) and identification of differentially expressed genes. For the differential expression analysis, all genes which were expressed in a minimum of four replicates with >20 normalized counts for at least one of the groups were selected. This resulted in 13,049 genes that were included in the analysis. Pair-wise comparisons of groups within experiments were analysed and a false discovery rate adjusted p-value of 0.01 and a log2FC <-1 or >1 was used as a cut-off for detection of differential gene expression. Principal component analysis was performed using DEseq2 and heatmaps of scaled, normalized counts were made with pheatmap (version 1.0.12). Gene ontology (GO) term enrichment analysis was performed with the ViSEAGO package (version 1.4.0), using fisher's exact test with 0.01 as a significance cut-off.

Histology and Immunofluorescence Microscopy

Muscles were isolated and frozen in liquid nitrogen-cooled isopentane. Samples were stored at -80˚C until further processing. Gastrocnemius tissue was cryo-sectioned (8 µm thick) using a cryostat (Leica CM3050S). Cryosections were first stained with rabbit anti-laminin (1:100, Abcam) for 3 hours. After washing with

PBS/Tween, sections were stained with secondary goat-anti-rabbit antibodies conjugated to Alexa Fluor-647 (1:1000, Abcam). Sections were incubated overnight at 4°C with a mixture of the following fiber-type specific fluorophore-conjugated primary antibodies (Molecular Probes, Life Technologies): BA-D5 conjugated to Alexa Fluor 350 (1:400; type 1), SC-71 conjugated to Alexa Fluor 488 (1:800; type 2B), and BF-F3 conjugated to Alexa Fluor 594 (1:600; type 2A). A Zeiss Axio Observer A1 microscope was used for imaging. Area quantification and representative pictures were acquired via ZEN 2 software.

Image Quantification

Carl Zeiss Image format were converted to multichannel TIFF files, and image processing was performed in Fiji. Mean fluorescence intensity (MFI) was recorded from each myofiber object using a modified Muscle J macro [34]. In brief, tissue masks from the laminin staining were created to determine muscle regions for quantification. The masks were then manually corrected, removing technical artifacts such as tissue folds. To improve the myofiber segmentation outputs, a classifier was trained in the Ilastik pixel classification algorithm [35]. All the masked laminin images were processed followed by myofiber segmentations defined as the region of interest. Mean MFI and geometrical properties were recorded for each myofiber. As the laminin segmentation was automated, non-myofiber objects were removed by implementing a percentile filtering for the pixel-classification on the object boundary, pixel-classification in the interior of the object, cross-sectional area and the circularity values. After the filtering step, MFI values for each of the three MyHC isoforms were scaled per myofiber as previously described [36]. MFI values for each vehicle group were normalized as 1 transformed (natural logarithm) and a myofiber-based MFI analysis was carried out in R (version 3.5.1).

Statistical Analysis

Statistical analyses were performed with SPSS (version 25) and GraphPad Prism version 9.0.1. The following statistical analyses were used: ANOVA with Turkey multi-comparison according to different variables (including 1-way ANOVA for one variable, 2-way ANOVA for two variables) and unpaired Student t-test. All data are presented as means ± SEM.

RESULTS

Elevated corticosterone exposure causes muscle atrophy in male and female mice, but specifically decreases grip strength in female mice

To identify the effects of elevated corticosterone exposure, male and female mice were subcutaneously implanted with a vehicle or corticosterone slow-release pellet. Total body weight was not altered in male and female mice up to two weeks during chronic corticosterone exposure (**Fig. 1A**). Elevated corticosterone exposure did significantly decrease total lean mass (**Fig. 1B**) and increased fat mass in both male and female mice (**Fig. 1C**). To investigate the effect of this excess corticosterone exposure on muscle function, we performed grip strength and grid hanging measurements. We found that upon excessive corticosterone exposure grip strength was only decreased in female mice, but not in male mice (**Fig. 1D**). Grid hanging time was not influenced in neither male nor female mice (**Fig. 1E**).

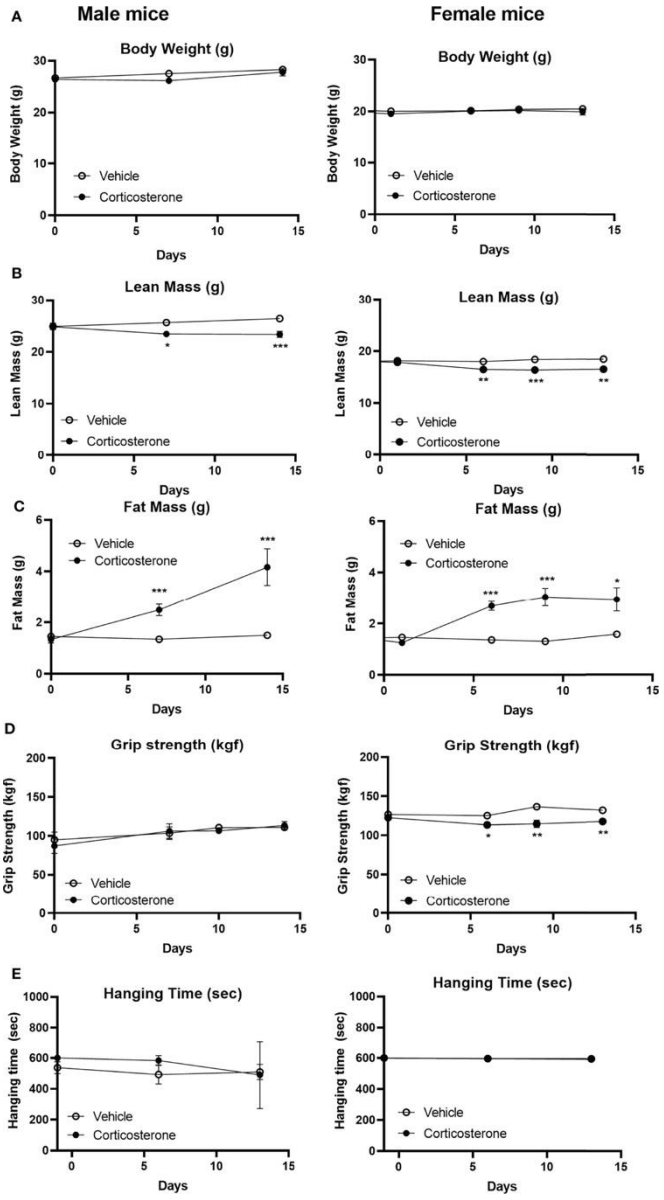

Fig. 1 Corticosterone treatment specifically decreases muscle function in female mice. The effect of corticosterone treatment on (A) total body mass, (B) lean mass, (C) fat mass, (D) fore limb grip strength and (E) grid hanging in male and female C57BL/6J mice. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using a two-way ANOVA.

To investigate muscle atrophy, we collected 3 different muscle types in male and female mice upon elevated corticosterone exposure. We found reduced muscle weights of gastrocnemius (**Fig. 2A**), extensor digitorum longus (EDL) (**Fig. 2B**) and tibialis anterior (TA) (**Fig. 2C**) upon excess corticosterone exposure, similarly in male and female mice albeit not significant for male EDL, indicative of muscle atrophy in both sexes. Overall, the results show that excessive

corticosterone exposure has sexual dimorphic effects on grip strength, but not on other functional parameters.

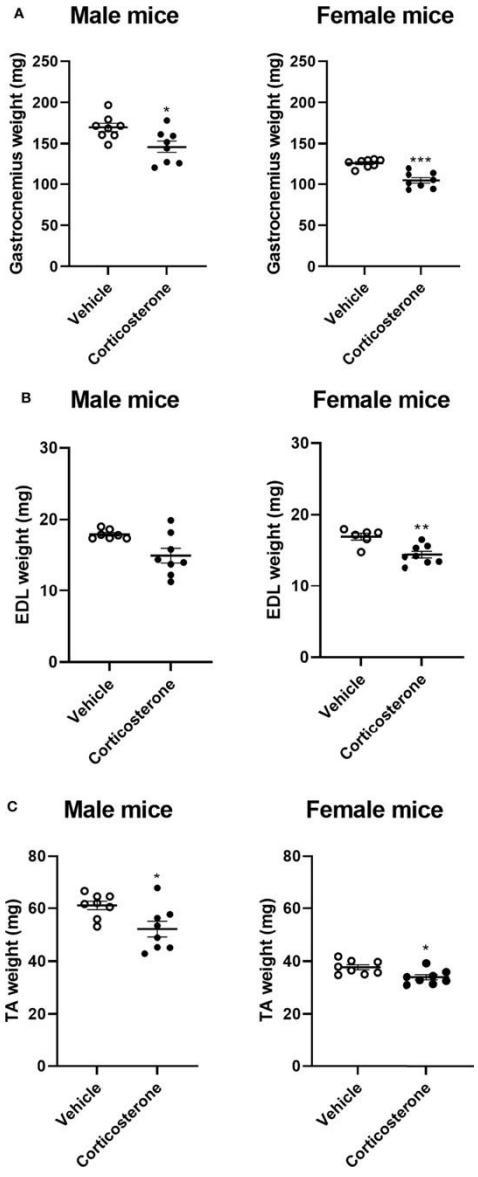

Fig. 2 Corticosterone treatment causes muscle atrophy in male and female mice. The effect of corticosterone treatment on tissue weight of (A) gastrocnemius, (B) extensor digitorum longus (EDL) and (C) tibialis anterior (TA) of male and female C57BL/6J mice. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using an unpaired Student's t-test.

Elevated corticosterone exposure has sexually dimorphic effects on transcription in quadriceps muscle

We next investigated the transcriptional effects of elevated corticosterone exposure in both male and female mice. We choose the quadriceps muscle for RNA-sequencing analysis, as this muscle type is representative for human

muscle [37] and showed muscle atrophy in mice after corticosterone exposure (data not shown). We identified 1817 differentially expressed genes upon corticosterone exposure that were shared between male and female mice, while 3576 genes were specifically regulated in male mice and 2002 genes were specifically regulated in female mice (**Fig. 3A**). Principal component analysis showed that biological replicates of corticosterone-treated male mice clustered closely together, while female mice exhibited considerable variation in muscle transcriptome as response to corticosterone exposure (**Fig. 3B**). Further scrutiny confirmed the considerable overlap in corticosterone-regulated genes between sexes as well as the sex-specific effects (**Fig. 3C**). Heatmap representation generated 4 clusters of corticosterone-regulated genes, comprising of genes similarly regulated between sexes (upper two gene clusters) and sex-specific effects (lower two gene clusters) (**Fig. 3D**).

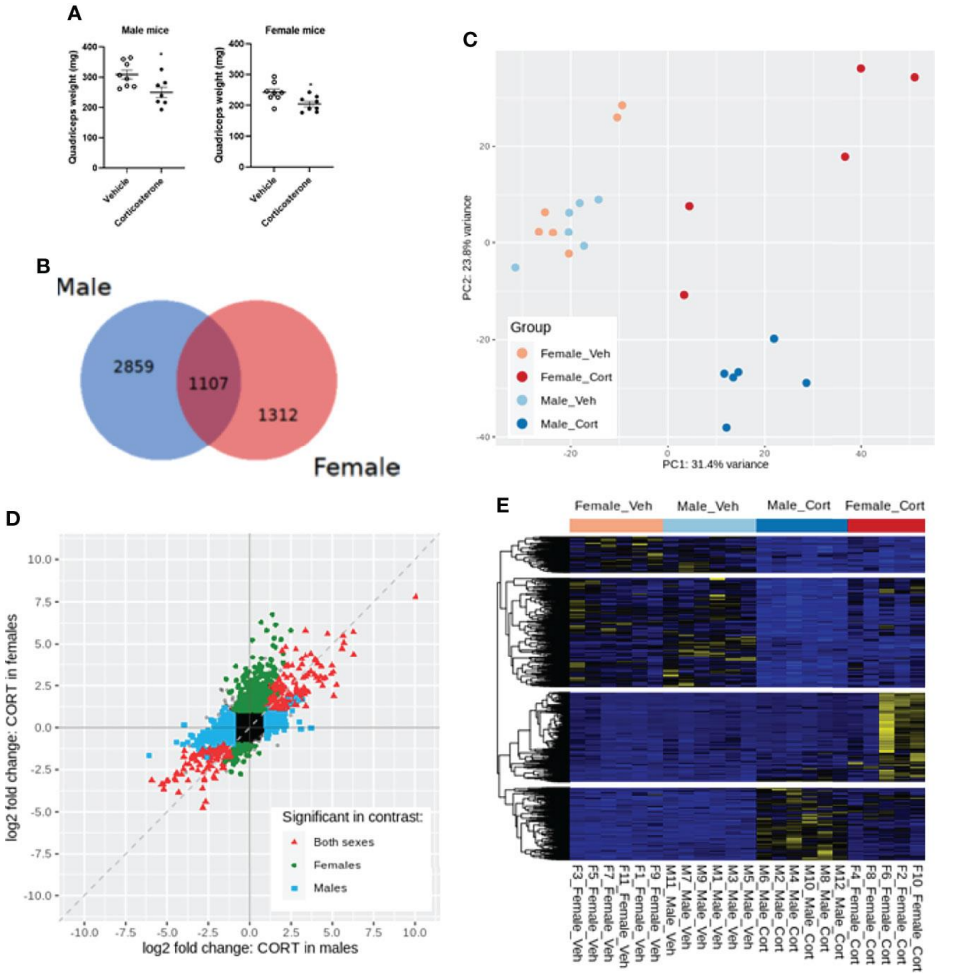

Fig. 3 Transcriptome profiling of the quadriceps in male and female mice after corticosterone treatment. (A) The effect of corticosterone treatment on muscle weight of the quadriceps. (B) Venn-diagram representing sex-specific and shared differentially expressed genes upon corticosterone treatment. (C) Principal component analysis of vehicle- and corticosterone-treated male and female C57BL/6J mice. (D) Fold change-fold change plot comparing significant changes in corticosterone-treated male and female mice. Male-specific differentially expressed genes are shown in blue, female-specific genes in green, and genes differentially expressed in both sexes in red. © Heatmap showing all genes regulated by corticosterone. A blue color code represents low expression, a yellow color code high expression. N=6 mice/group for all groups except female-Cort (N=5).

When evaluating expression of specific genes, we first plotted expression of genes that encode for the superfamily of nuclear steroid receptors. We found that the *Nr3c1* gene (encoding for the glucocorticoid receptor) was significantly downregulated by corticosterone exposure in female but not male mice, while *Nr3c2* (mineralocorticoid receptor) was downregulated in both male and female quadriceps muscle, albeit only significant in male mice (**Suppl. Table 1** and **Suppl. Fig. 1A**). *Nr3c3* (progesterone receptor) and *Esr2* (estrogen receptor-β) were not detected in quadriceps muscle, while the *Nr3c4* (androgen receptor) was not significantly changed by corticosterone exposure in both male and female mice and *Esr1* (estrogen receptor-α) was strongly downregulated in the quadriceps muscle of both male and female mice (**Suppl. Table 1** and **Suppl. Fig. 1A**). We next looked at classical GR target genes, including *Fkbp5, Gilz* (*Tsc22d3*), *Per1*, *Sgk1* and *Zbtb16,* as proxies for GR activity. All evaluated GR target genes were upregulated stronger in male mice as compared to female mice (**Suppl. Table 2** and **Suppl. Fig. 1B**). In an attempt to better understand the sex-specific effect of corticosterone exposure on muscle function (grip strength), we performed a go-term analysis. Comparison of differentially expressed genes after corticosterone showed many pathways that were specifically regulated in male mice, including the muscle atrophy pathway [25, 38], with 37.5% genes differentially expressed in male mice (p>0.01) and 0% in female mice (p=1.00) (**Suppl. Table 3**). Male-specific regulated genes involved in atrophy included Klf15 and its downstream ubiquitin ligases *MurF-1* (*Trim63*) and *atrogin-1* (*Fbxo32*) (**Suppl. Table 4** and **Suppl. Fig. 1C**). Other factors involved in ubiquitination, including *UBC*, *Ube4b* and *Usp14*, were not influenced by corticosterone exposure in male and female mice. Other noteworthy sex differences in corticosterone response was the stronger upregulation of the *FoxO1, -3* and *-4* transcription factors in male as compared to female mice. There were no clear effects of corticosterone on several proteasome subunits, and factors related to autophagy (*Bnip3, LC3*) were similarly upregulated in male and female mice (**Suppl. Table 4** and **Suppl. Fig. 1C**). Collectively our data show many transcriptomic similarities and differences between male and female quadriceps muscle in response to excess corticosterone, with several noteworthy sexually dimorphic effects including atrophy-related genes.

Daily betamethasone treatment similarly decreases grip strength in both sexes, while female mice are more sensitive to muscle atrophy

To evaluate the effects of synthetic glucocorticoid treatment on muscle function, male and female mice were injected daily with 3 or 25 mg/kg betamethasone for a period of 2 weeks. In both sexes, body weight was non-significantly decreased by daily betamethasone treatment (**Fig. 4A**). Treatment with 25 mg/kg betamethasone significantly decreased lean mass of male mice, while treatment with both 3 and 25 mg/kg betamethasone decreased lean mass in female mice (**Fig. 4B**). Fat mass was similarly increased in both male and female mice (**Fig. 4C**). In both male and female mice, treatment with 25 mg/kg betamethasone significantly reduced grip strength (**Fig. 4D**). Treatment with 3 mg/kg betamethasone transiently decreased grip strength in male mice, while reduced grip strength in female mice was only observed after 14 days of treatment (**Fig. 4D**). Grid hanging performance was not significantly altered in male nor female mice after betamethasone treatment, although male mice tended to perform better upon treatment with 25 mg/kg betamethasone (**Fig. 4E**).

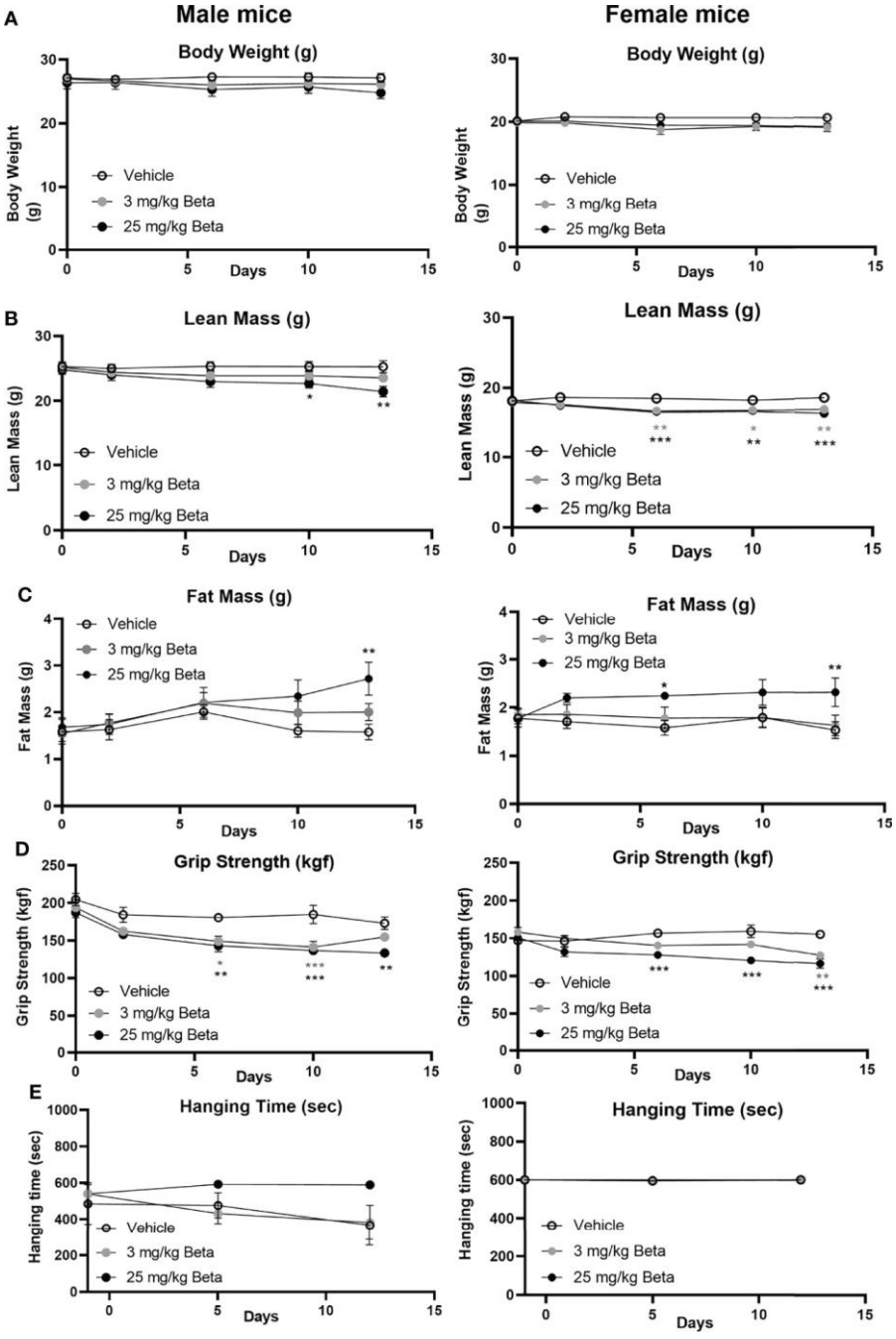

Fig. 4 Daily betamethasone treatment decreases muscle function in male and female mice. The effect of daily treatment with 3 or 25 mg/kg betamethasone on (A) total body mass, (B) lean mass, (C) fat mass, (D) fore limb grip strength, and © grid hanging time in male and female C57BL/6J mice. N=4 male mice/group, N=6 female mice/group. *p<0.05 vs.

Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using a one-way ANOVA.

To investigate the effect of daily betamethasone treatment on muscle atrophy, we collected 5 different muscle types. Gastrocnemius weight of female mice was significantly decreased after 3 and 25 mg/kg betamethasone treatment, whereas male mice only showed a decrease in muscle weight upon 25 mg/kg betamethasone treatment (**Fig. 5A**). Similar patterns were observed for the muscle weights of EDL (**Fig. 5B**) and TA (**Fig. 5C**). In the glucocorticoid-resistant soleus muscle, we did not observe any significant effect of betamethasone treatment on muscle weight in both male and female mice (**Fig. 5D**). Consistent with the patterns observed on muscle weight, gene expression analysis of atrophy-related genes in the gastrocnemius muscle revealed a possible decrease of *Klf15*, *atrogin-1* and *MurF-1* in male mice treated with 3 mg/kg betamethasone, but increased expression after 25 mg/kg betamethasone expression (**Fig. 5E-G**). Female mice showed an upregulation of *atrogin-1* and *MurF-1* in gastrocnemius muscle after 25 mg/kg betamethasone treatment (**Fig. 5F-G**).

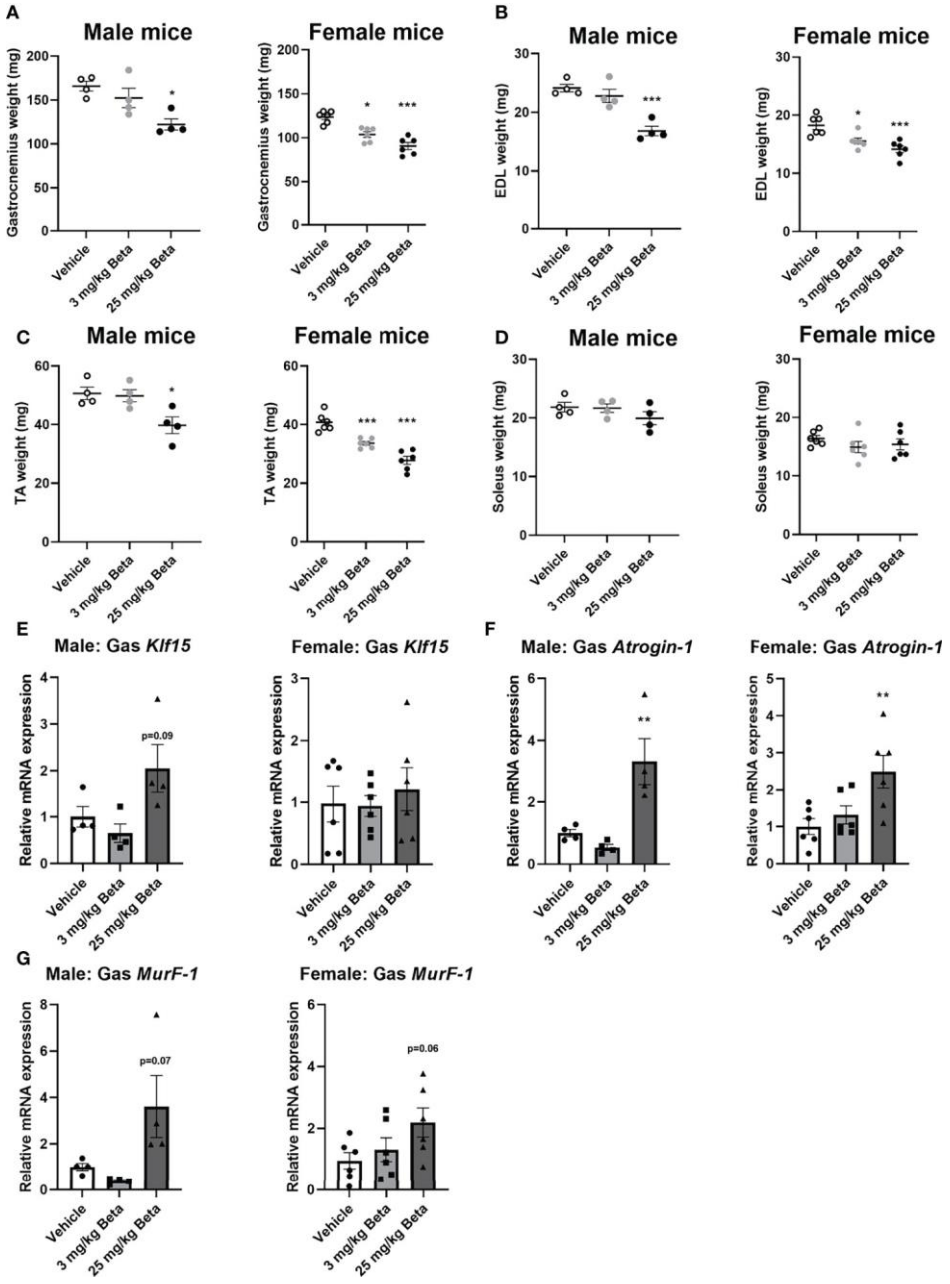

Fig. 5 Female mice exhibit muscle atrophy at lower doses of daily betamethasone treatment as compared to male mice. The effect of daily treatment with 3 or 25 mg/kg betamethasone on tissue weight of (A) gastrocnemius, (B) extensor digitorum longus (EDL), (C) tibialis anterior (TA) and (D) soleus. The effect of 3 or 25 mg/kg betamethasone treatment on gene expression in gastrocnemius muscle of © Klf15, (F) atrogin-1 and (G) MurF-1. N=4

male mice/group, N=6 female mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using a one-way ANOVA.

To further investigate the effect of betamethasone treatment on muscle in male and female mice, we analysed gastrocnemius myofiber composition. As expected, gastrocnemius muscles of vehicle-treated male mice were comprised of relatively little type 1 fibers and type 2A fibers, and relatively many type 2B fibers in (Fig. 6A-B). In contrast, vehicle-treated female mice had relatively many type 2A fibers (Fig. 6C-D). Betamethasone treatment of male mice significantly increased type 2A myofibers (Fig. 6B). In female mice, type 1 and type 2A myofiber composition was unaffected by betamethasone treatment, while type 2B tended to be decreased after daily betamethasone treatment (Fig. 6D). Collectively, these functional data suggest that female mice are more sensitive to betamethasone-induced muscle atrophy, with similar effects on grip strength between sexes.

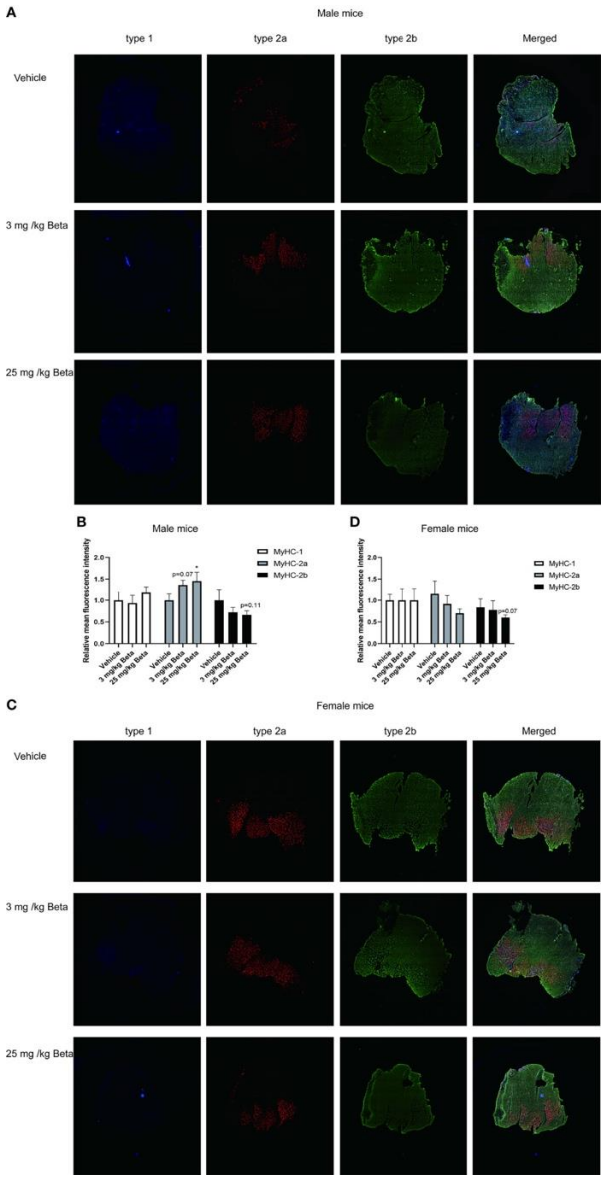

Fig. 6 Daily betamethasone treatment increases abundance of type 2A myofibers in male mice. Histological analysis of gastrocnemius muscle for type 1, type 2A and type 2B myofibers. (A) Representative images of myofiber staining in gastrocnemius muscle of male mice and (B) Relative mean fluorescence intensity for individual myofiber isoforms. N=3 mice/group. (C) Representative images of myofiber staining in gastrocnemius muscle of female mice and (D) Relative mean fluorescence intensity for individual myofiber isoforms. N=3 mice/group. Type-1=Blue; Type-2a=Red; Type-2b=Green. *p<0.05 vs. Vehicle.

Daily betamethasone treatment and chemical castration similarly and additively cause muscle atrophy

To explore the underlying mechanism of male-female differences in glucocorticoid response in muscle function, we investigated a possible contribution of androgen signalling [30]. To this end, we chemically castrated male mice using the GnRH antagonist degarelix, and intact and chemically-castrated male mice were subsequently injected daily with 3 mg/kg betamethasone for 2 weeks. During this treatment period, chemical castration on itself did not significantly influence total body weight and lean body mass, but seemed to potentiate the effect of betamethasone treatment on body weight and lean mass (**Fig. 7A-B**). Fat mass appeared to transiently decrease upon chemical castration, but was not influenced by betamethasone treatment (**Fig. 7C**). Chemical castration on its own decreased grip strength, and additional betamethasone treatment did not further influence this (**Fig. 7D**).

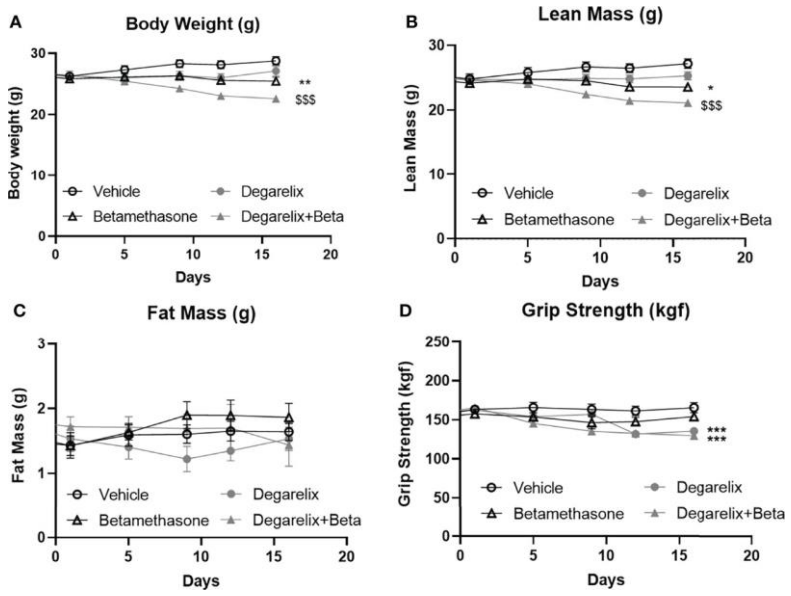

Fig. 7 Daily betamethasone treatment reduces total body weight and lean mass in intact and chemically-castrated male mice. The effect of daily treatment with 3 mg/kg betamethasone in chemically-castrated mice and intact mice on (A) total body mass, (B) lean mass, (C) fat mass, and (D) fore limb grip strength. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle, $$$ p<0.001 vs degarelix. Statistical significance was calculated using a two-way ANOVA.

As expected, post-mortem analysis of glucocorticoid and androgen-responsive tissues showed decreased adrenal weight after betamethasone treatment but no effect of chemical castration (Fig. 8A), and diminished seminal vesicle weight after chemical castration (Fig. 8B). Analysis of muscle tissue showed that

chemical castration alone significantly decreased gastrocnemius, EDL and TA, but not soleus weight (Fig. 8C-F). Treatment with 3 mg/kg daily betamethasone reduced gastrocnemius weight in intact male mice, and further reduced muscle weight in chemically-castrated mice (Fig. 8D). Similar observations were found for EDL and TA muscle weight, for which betamethasone treatment further decreased muscle weight of chemically-castrated mice (Fig. 8E-F). Expression analysis revealed that in gastrocnemius muscle, degarelix treatment induced the expression of *Klf15*, an effect that was lowered by betamethasone treatment (Fig. 8G). In line with our previous experiment, 3 mg/kg betamethasone treatment lowered *MurF-1* expression, which was unaffected by chemical castration (Fig. 8H). Similarly as in gastrocnemius, degarelix treatment induced *Klf15* in tibialis anterior muscle, while *MurF-1* expression was unaltered (Fig. 8I-J). Collectively, these data suggest that androgen depletion and glucocorticoid treatment both have separate effects on muscle atrophy, and that combined intervention has additive effects.

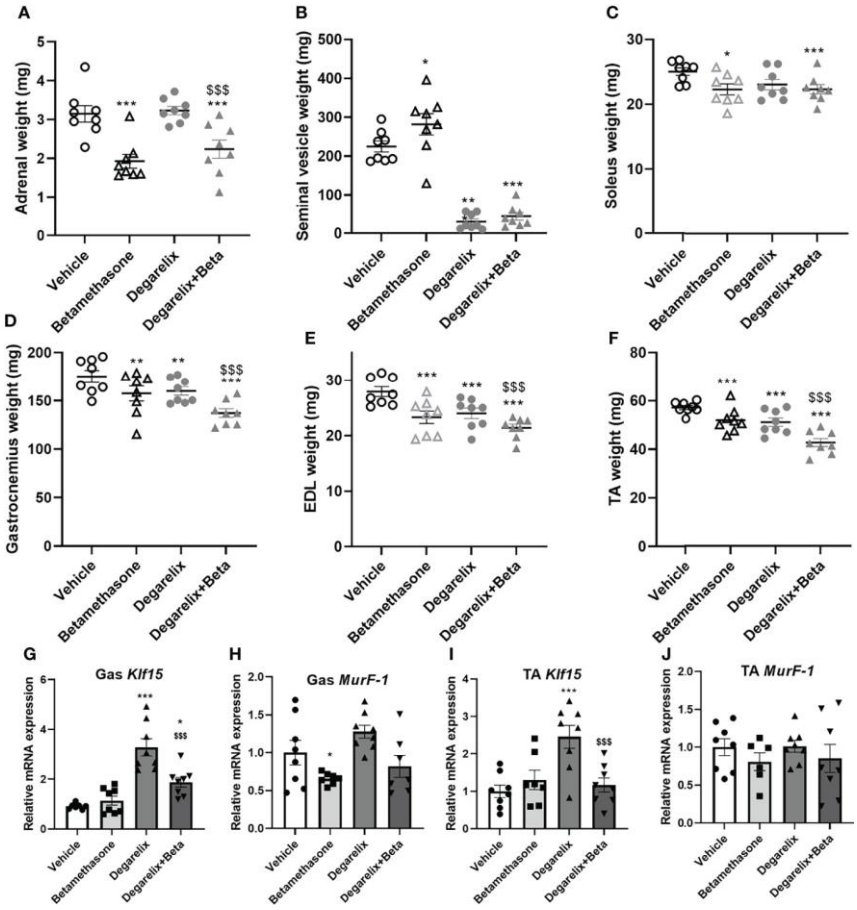

Fig. 8 Chemical castration and daily betamethasone treatment additively decrease muscle weight in male mice. The effect of daily treatment with 3 mg/kg betamethasone on intact and chemically-castrated male mice on weight of the (A) adrenal gland, (B) seminal vesicle, (C) soleus, (D) gastrocnemius muscle, (E) extensor digitorum longus (EDL) and (F) tibialis anterior (TA). The effect of daily treatment with 3 mg/kg betamethasone on intact and chemically-castrated male mice on expression of (G) Klf15 in gastrocnemius, (H) MurF-1 in gastrocnemius, (I) Klf15 expression in TA, and (J) MurF-1 expression in TA. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle, $$$ p<0.001 vs degarelix. Statistical significance was calculated using a two-way ANOVA.

DISCUSSION

In this study, we set out to investigate sexual dimorphism in glucocorticoid-induced muscle dysfunction. Muscle dysfunction as a result of elevated glucocorticoid exposure is common in patients with hypercortisolism but is also frequently observed during synthetic glucocorticoid treatment regimens. Although the magnitude of this problem in clinical practice is evident, to our knowledge no studies exist to study sex differences and the role of androgen sex hormones in glucocorticoid-induced muscle dysfunction. In our present study, we found that elevated corticosterone exposure similarly causes muscle atrophy in male and female mice, based on the analysis of five different types of skeletal muscle. Despite similar atrophy-inducing effects in both sexes by corticosterone, only female mice exhibited a decreased grip strength, while male mice were unaffected by this. We performed an extensive transcriptomic analysis of male and female quadriceps muscle after corticosterone exposure in an attempt to capture the similarities and differences between sexes. Overall, we observed more differentially expressed genes in male mice as compared to female mice (3576 male-specific genes versus 2002 female-specific genes after corticosterone treatment). This finding was also evident when looking at several classic GR-target genes (e.g. *Gilz*, *Per1* and *Sgk1*) that were found stronger regulated in male mice as compared to female mice. It is interesting to note that in contrary to the results above, the response to fasting shows greater induction of GR-regulated genes in female gastrocnemius muscle as compared to male [39]. The response to glucocorticoid/GR-induced transcription thus appears context-dependent but likely also muscle fiber type-dependent. In corticosterone-treated female mice, we found a large in-group variation in transcriptomic response (as represented in the PCA analysis and heatmap), possibly related to different stages of the estrous cycle at which tissues were collected for which we did not stratify.

Gene ontology analysis revealed muscle atrophy amongst the main sexually dimorphic pathways, and indeed male-specific upregulations of atrophy-related genes were found for *Klf15*, *atrogin-1* and *MurF-1*, amongst several others. Despite that the transcriptional response to corticosterone in quadriceps muscle was thus stronger in male mice (including genes related to muscle atrophy), this did not yield stronger atrophy-induction in males, and decreased grip strength was even specific for female mice. It is likely that different pathways contribute to muscle atrophy in male and female muscle, and in addition to direct catabolic effects also antagonism of anabolic pathways can underlie decreased muscle mass by glucocorticoids. It should also be noted that the transcriptomic analysis was performed after 14 days of elevated corticosterone exposure – a timeframe that allows adaptation in tissue response - and transcriptional effects after acute corticosterone treatment may differ. We also observed that reduced muscle

mass does not necessarily influence muscle function (grip strength), but it should be noted that we analyzed muscle weight in the back limps, while the functional test evaluated forelimb muscle strength. We do expect that the muscle atrophy upon elevated corticosterone that we observed in back limps is representative for most muscles in the mouse. A notable exception to this is the soleus muscle – previously reported to be largely resistant to glucocorticoid-induced atrophy [25]. Also in our studies the soleus was largely unaffected by synthetic glucocorticoid treatment in both male and female mice, and this lack of response in the soleus muscle is likely attributed to low GR expression levels.

For synthetic glucocorticoid treatment with betamethasone, we show that both male and female mice exhibited reduced grip strength – with both doses that were tested in this study. We found that male mice are less sensitive to betamethasone-induced muscle atrophy – and treatment with 25 mg/kg/day was required to induce atrophy in male mice while 3 mg/kg/day betamethasone induced this in female mice. Differences in sensitivity are not explained by GR expression levels, as these were reported to be similar in male and female gastrocnemius muscle [39]. The analysis of atrophy-related gene expression [40] in gastrocnemius revealed that *Klf15*, *atrogin-1* and *MurF-1* expression were only upregulated after 25 mg/kg betamethasone in male mice, while 3 mg/kg even seemed to reduce expression of these atrogenes. However, also female mice were mostly responsive to the 25 mg/kg dose, while the 3 mg/kg dose had little effect on the expression of the tested atrogenes. We observed that betamethasone induced a transformation of muscle fibers in gastrocnemius muscle [17, 41, 42], with increased type 2A and decreased type 2B fibers in male mice after betamethasone treatment. Such a shift of muscle fiber isoforms was previously associated with a reduction of muscle strength [43, 44]. In female mice, we did not observe a distinct change in type 1 fibers, consistent with the lack of effect on muscle endurance during the grid hanging test.

We postulated that the sex difference in glucocorticoid effects on muscle may be related to the relative androgen levels. Androgens are well-known anabolic factors that are involved in muscle physiology, and increased anabolic signaling may protect from glucocorticoid-induced muscle atrophy and dysfunction. To test to what extent androgen signaling contributes to the sex differences, we chemically castrated male mice using GnRH antagonist degarelix. Androgen depletion on its own strongly reduced forelimb grip strength, but betamethasone treatment did not further influence this. Muscle weight of several muscle types was reduced after androgen depletion – likely due to reduced anabolic signaling. In addition, chemical castration of male mice seemed to potentiate the atrophy-inducing effects of low dose betamethasone treatment, but for many muscle types both effects were additive rather than synergistic. Our findings thus cannot rule out separate anabolic and catabolic signaling

pathways, and do not provide direct evidence for crosstalk between these pathways.

Sex-based differences in skeletal muscle physiology are known in humans, including differences in fiber type prevalence which translates to altered performance, endurance and recovery of skeletal muscles [45]. Also in response to glucocorticoids, humans show differences between sexes. In patients with subclinical hypercortisolism, women exhibited lower skeletal muscle mass but men do not [46]. In addition to this, differential expression of 11β-HSD1 is likely to influence local glucocorticoid turnover, and in fact higher 11β-HSD1 expression in women is associated with reduced grip strength [47]. It should be noted that while 11β-HSD1 influences (local) levels of endogenous glucocorticoids, many synthetic glucocorticoids are not a substrate for such enzymatic (in)activation and their activity is thus unlikely to be affected. Also at the receptor level, differences between men and women exist, and polymorphisms in the GR gene were shown to reduce grip strength in male patients with overt hypercortisolism (Cushing's syndrome) [48]. Altogether, clearly many aspects of glucocorticoid response in skeletal muscle are different between men and women. Our studies in mice provide suitable models to further study the mechanisms that underlie these sex-specific effects, and propose that differential androgen levels may in part contribute to these discrepancies.

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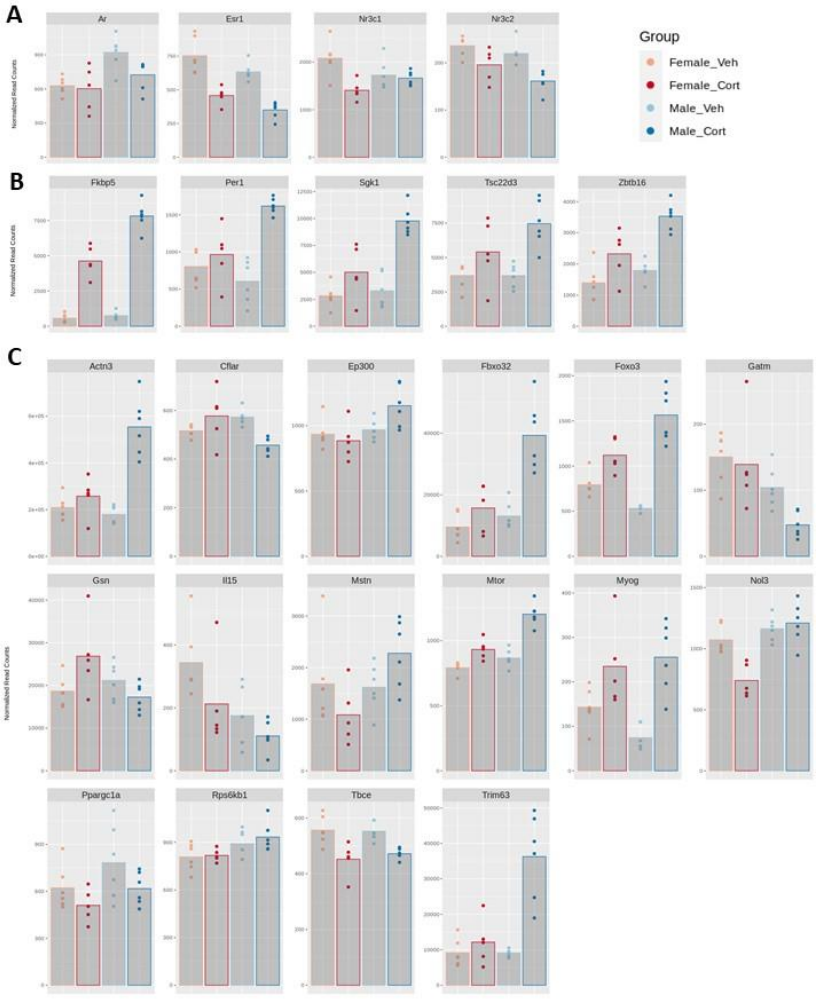
SUPPLEMENT

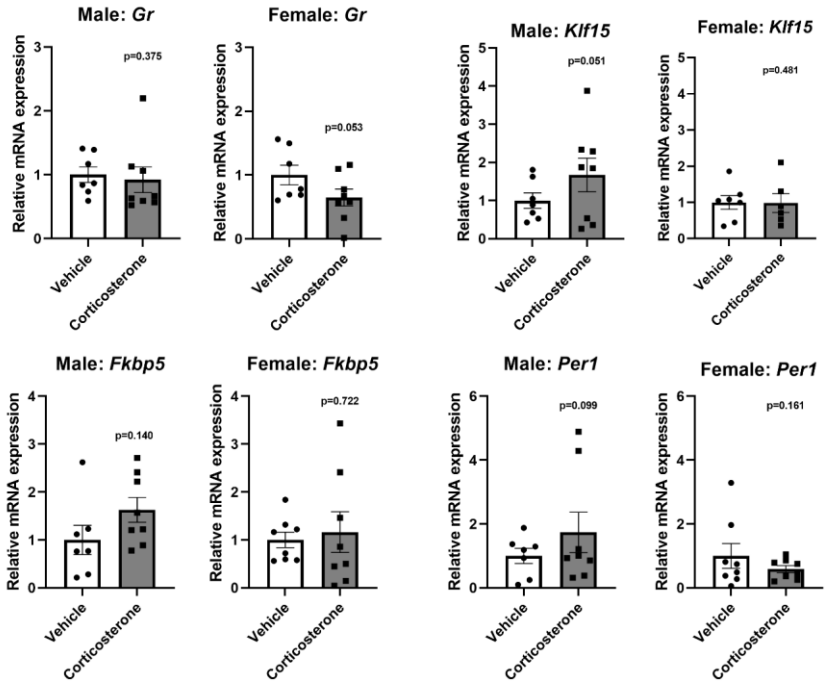
Suppl. Figure 1



Supplementary Fig. 1

Quadriceps RNA sequencing data of selected genes. (A) Normalized read counts for (A) genes encoding for steroid nuclear receptors, (B) classical glucocorticoid receptor-target genes, and (C) genes included in the gene ontology term muscle atrophy.

Suppl. Figure 2



Supplementary Fig. 2

Quadriceps RT-PCR analysis of selected genes. Relative mRNA expression of Gr, Klf15, Fkbp5 and Per1 in quadriceps muscle of male and female mice after corticosterone treatment (20 mg). N=6-8 per group. Statistical significance was calculated using an unpaired students t-test.