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GTS-21 attenuates loss of body mass, muscle mass and function in rats having systemic inflammation with and without disuse atrophy

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Abstract

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declare that they have no conflict of interest.

Research involving Animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This animal study was approved by the institutional review board (Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital, #2010N000168).

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Muscle changes of critical illness are attributed to systemic inflammatory responses and disuse atrophy. GTS-21 (3-(2,4-dimethoxy-benzylidene)anabaseine), also known as DMBX-A) is a synthetic derivative of the natural product anabaseine that acts as an agonist at $\alpha 7$ -acetylcholine receptors ($\alpha 7$ nAChRs). Hypothesis tested was that modulation of inflammation by agonist GTS-21 (10 mg/kg b.i.d. intraperitoneally) will attenuate body weight (BW) and muscle changes. Systemic inflammation or sham inflammation was produced in 125 rats by *C.parvum* (C.p.) or saline injection on days 0/4/8. 74 rats had one immobilized-limb producing disuse atrophy. GTS-21 effects on BW, tibialis muscle mass (TMM) and function were assessed on day 12. Systemically methemoglobin levels increased 26-fold with C.p. ($p < 0.001$) and decreased significantly ($p < 0.033$) with GTS-21. Control BW increased ($+30 \pm 9$ g, mean $\pm$ SD) at day 12, but decreased with C.p., and superimposed disuse ($p = 0.005$). GTS-21 attenuated BW loss in C.p. ($p = 0.005$). Compared to Controls, TMM decreased with C.p. (0.43 ± 0.06 g to 0.26 ± 0.03 g) and with superimposed disuse (0.18 ± 0.04 g); GTS-21 ameliorated TMM loss to 0.32 ± 0.04 g (no disuse, $p = 0.028$) and to 0.22 ± 0.03 g (with disuse, $p = 0.004$). Tetanic tensions decreased with C.p. or disuse and GTS-21 attenuated tension decrease in animals with disuse ($p = 0.006$) and in animals with C.p. and disuse ($p = 0.029$). C.p.-induced 11-fold increased muscle $\alpha 3\beta 1\gamma$ nAChR expression was decreased by $>60\%$ with GTS-21 treatment. In conclusion, GTS-21 modulates systemic inflammation, evidenced by both decreased methemoglobin levels, and decrease of $\alpha 7$ nAChR expression, and mitigates inflammation-mediated loss of BW, TMM, fiber size and function.

Keywords

3-(2,4-dimethoxybenzylidene)anabaseine; Critical Care; DMXBA; Muscular Disorders, Atrophic; Muscle Weakness

INTRODUCTION

Critical illness, particularly when associated with immobilization in bed or decreased mobility leads to muscle wasting and muscle weakness (MW) [9, 12, 13, 42, 57] occurring in 32–100% of hospital patients [37, 39, 58]. This pathological entity has varying names including critical illness polyneuropathy, critical illness myopathy and/or critical illness polyneuromyopathy [58, 64]. The short-term complications of MW include dependence on respirators with a higher risk for ventilator-associated pneumonia, deep vein thrombosis with pulmonary embolism, and/or decubitus ulcers. The inflammation-associated MW can be further compounded by disuse atrophy because of immobilization in bed, application of bandages and plaster casts and/or decreased motor activity due to illness [22, 23]. The functional neuromuscular disability after critical illness can persist even at 5 years after the initial insult [20, 21, 53]. Current therapeutic maneuvers used for prevention of MW, including better nutrition, physical therapy, and anabolic drug therapy (oxandralone, insulin, metformin, etc.), do not completely reverse the muscle changes and disability during and after their hospitalization [4, 49]. Despite all or some of these interventions, MW continues to occur in critical illness. Early mobilization within 72 hours seems to be the only effective intervention so far [55, 56]. Early mobilization, however, is not possible in all critically ill patients because of factors related to the disease itself.

A concomitant pathologic feature of critical illness is the presence of systemic inflammatory responses with cytokine release leading to increased protein breakdown and decreased protein synthesis [9, 38, 65, 66]. A pathognomic biochemical feature in MW conditions is the presence of a denervation-like state, evidenced by the up-regulation of the fetal (a.k.a. immature) nicotinic acetylcholine receptors (nAChRs) isoform on the muscle membrane [45].

Most recently the *de novo* expression of another isoform, the $\alpha 7$ nAChR (consisting of 5 homomeric $\alpha 7$ subunits only) has been described in muscle wasting conditions [32, 41, 43, 60]. The expression of $\alpha 7$ nAChRs in normal mature muscle is minimal. In contrast to muscle expression of $\alpha 7$ nAChRs only in pathologic states, the $\alpha 7$ nAChRs are constitutively expressed in macrophages and other circulating leucocytes [25, 40, 44, 62]. During inflammation, however, these $\alpha 7$ nAChRs are further up-regulated *in vitro* in macrophages [30] and *in vivo* in burned patients [51]. Several studies have documented anti-inflammatory properties when $\alpha 7$ nAChRs are stimulated by agonists. Exogenous nicotine, and endogenous acetylcholine or choline have been the prototypical $\alpha 7$ nAChRs agonists, but all of them have non-specific effects on other receptors too. The systemic administration of GTS-21, a highly selective $\alpha 7$ nAChR agonist [28, 61], *in vivo* decreased cytokine release and/or inflammation [26] and resulted in improved function in inflamed pathologic organs (e.g. lungs [35]) or survival [30, 31]. GTS-21 (which is the laboratory name of 3-(2,4-dimethoxy-benzylidene)anabaseine) also known as DMBX-A is a synthetic derivative of the natural toxin anabaseine produced by nemertines [27]. It binds to both the $\alpha 4\beta 2$ and $\alpha 7$ subtypes, but activates only the $\alpha 7$ to any significant extent [2, 48]. It has been clinically tested to decrease systemic inflammatory responses in a phase II clinical trial [34], as well as in schizophrenic, Alzheimer's disease and attention-deficit hyperactivity disorder subjects [11, 29, 33, 50]. Disuse atrophy *per se* leads to inflammation and *de novo* expression of $\alpha 7$ nAChRs in the wasting muscles, despite absence of systemic inflammation [32, 41]. The ability of the $\alpha 7$ nAChR agonist GTS-21 to mitigate bacteria-induced systemic inflammatory responses and the skeletal muscle changes associated with it have not yet been tested.

We have previously described a model of systemic inflammation or disuse alone, and their combination (double-hit model) in rats mimicking critical illness induced muscle disuse atrophy [9, 12, 57]. In this model, systemic inflammation was produced by injection of *Corynebacterium parvum* (C.p.) [1, 8, 10, 46], and disuse of one leg was produced by pinning [9, 12, 57]. The advantages of this model are: (1) systemic inflammation can be maintained over a longer time period [1, 63] without inducing sepsis with a much higher mortality like other models (e.g. endotoxemia or caecal ligation) [3, 52] and (2) the nerve-muscle composition stays intact in comparison to denervation models mimicking an intensive care patient much better. The inflammation produced by C.p. was evidenced by increased methemoglobin (MetHb) levels (a surrogate for increased nitric oxide-levels), as well as increased $\alpha 1$ -acid glycoprotein levels (an acute phase reactant protein), and decreased platelet and erythrocyte counts [9, 63]. Thus, there is *a priori* evidence that systemic inflammation and/or muscle wasting (disuse, sepsis and denervation) upregulate $\alpha 7$ nAChRs both in muscle and leucocytes [30, 32, 41, 59]. Based on this evidence, the hypothesis tested in this study was that these upregulated $\alpha 7$ nAChRs could be used to decrease systemic and local muscle inflammation, and therefore reverse the systemic

inflammation-and/or disuse-induced muscle changes in the previously described rat model [9, 57].

MATERIALS AND METHODS

Animal Model and Study Design

After approval by the institutional review board (Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital, #2010N000168), 125 male Sprague-Dawley rats (Taconic Farms Inc, Germantown, NY, USA, 151–175g) were acclimated to the standard conditions of our animal facility with free access to chow and water for 7 days. The previously described model, consisting of 4 groups, was used [9]: (1) sham-immobilization with saline (sham-inflammation), (2) immobilization with saline, (3) sham-immobilization with C.p. (inflammation) and (4) immobilization with C.p. Systemic inflammation was induced by intravenous injection of 56 mg/kg whole cell preparation of heat-inactivated C.p. (Roche, Penzberg, Germany) via dorsalis penis vein during anesthesia at days 0, 4, and 8 as described previously [1, 8–10, 46, 57]. We use methemoglobin measurements as surrogate marker for the severity of inflammation, since methemoglobin correlates with the upregulated nitric oxide concentration and acute phase proteins [1, 9, 10, 57, 63]. Substitute animals (n=17) had to be added to the inflammation groups because of higher mortality (Figure 1). Injection of 0.9% normal saline solution (sham-inflammation) served as control.

To induce disuse atrophy of one hind limb was immobilized for a period of 12 days by pinning of knee and ankle joints at 90 degrees using 1.0-mm Kirschner wires (immobilization). After a skin incision at each joint the calcaneus, tibia, and femur were drilled and the joints were pinned with the knee in flexion and ankle in dorsiflexion [9, 12, 57]. Sham-Immobilization was achieved by insertion of the Kirschner wires into the bone, and the wire removed immediately thereafter (sham-immobilization). The contralateral leg of the immobilized and sham-immobilized animals on which no surgery was performed in saline groups were used to provide naïve control values.

The four groups were further divided into two subgroups, depending on the pharmacological therapy received: A) saline as placebo, or B) 10mg/kg GTS-21, administered i.p. twice a day. GTS-21 was provided by William Kem (University of Florida, Gainesville, FL., chemical purity >99%). GTS-21, due to its weak light sensitivity, was prepared with sterile saline 0.9% in a dark room using only yellow light. The preparation was adjusted to a pH of 7.35, stored in syringes in the darkroom, transported in a light protected container to the animal room, and injected with lights switched off using a small yellow lamp as illumination.

The combination of perturbations described above resulted in the 8 groups (Figure 1). For the immobilization procedure and injections of C.p./saline on day 0, 4, and 8, the animals were anesthetized with isoflurane in a Plexiglas chamber. After induction of anesthesia, the head of the animal was placed in a chamber with a continuous flow of isoflurane while the animal was breathing spontaneously. At day 12 after the perturbations (induction of inflammation, immobilization or sham procedures), the Kirschner wires were removed (from

the immobilized animals) and neuromuscular function studies performed in all animals. For the neuromuscular functional studies at day 12, anesthesia was induced with pentobarbital i.p., and maintained with empirical doses of 10–20 mg/kg given every 10–20 minutes based on withdrawal response to toe clamping. After loss of consciousness, the rats were tracheotomized and mechanically ventilated with air. The jugular vein was cannulated as access for administration of fluids. A cannula in the carotid artery was used to measure arterial blood pressure and for blood for gas analyses.

Neuromuscular Function Test

Before the neuromuscular experiments, ventilation was adjusted to maintain a PaCO₂ between 35 and 45 mm Hg, PaO₂ of > 90 mm Hg, and pH between 7.36 and 7.44. Whenever necessary, base deficit was corrected with 1mM sodium bicarbonate to values between –3 and 3 mM. Blood glucose levels were controlled between 80–130 mg/dl. Heart rate and mean arterial pressure were continuously monitored to ensure stable hemodynamic conditions throughout the experiment. Rectal temperature was controlled between 36.8°C and 37.2°C by adjusting the heat generated from the experiment table and with a heat lamp, if needed. Nerve-induced neuromuscular function was assessed by evoked mechanomyography [9, 12, 57]. The functional measures included single twitch tension and peak tetanic tension at 50 Hz stimulation. Following these functional studies, the animals were euthanized by exsanguination after high dose of pentobarbital (200mg/kg).

Ex vivo Analyses

α7AChR expression: The cranial tibialis muscle on each side was dissected out, weighed, snap frozen in liquid nitrogen, and stored airtight at –80°C for western blot analysis. For Western Blot of α7AChRs expression, each tibialis muscle was homogenized in homogenization buffer (Sigma-Aldrich Chemie GmbH, Munich, Germany) containing protease inhibitor (Sigma) as described previously [41], using a tissue lyser (TissueLyser LT, Qiagen, Hilden, Germany). The samples were centrifuged, and aliquots of the supernatant containing equal amounts of protein (by Bradford Assay) were subjected to TGX Stain-Free™ Gels (Bio-Rad Laboratories GmbH, Munich, Germany), and then immunoblotting was performed [30]. Equal amounts of protein (40 µg) per lane were subjected to NuPAGE and then blotted onto PVDF membrane (Bio-Rad Laboratories GmbH, Munich, Germany). The membrane was blocked by Roti®-Block (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Anti-α7AChR (dilution 1:2.500, ab24644, Abcam, Cambridge, UK) was used as primary antibody. The membranes were incubated in horseradish peroxidase–conjugated goat anti-rat secondary antibody (NA935V, GE Healthcare, Amersham, UK) for 60 min (dilution 1:10.000). The specific proteins were detected by Bio-Rad Molecular Imager® ChemiDoc™ XRS+ (Bio-Rad Laboratories GmbH, Munich, Germany) and analyzed with ImageLab (Bio-Rad Laboratories GmbH, Munich, Germany) using total protein normalization using stain-free technology [14–16, 54].

Immunohistochemistry: After dissection of TA, muscles were snap-frozen in melting isopentane to prevent freeze artefacts. Fixed with OCT (Surgipath FSC 22 Clear, Leica, Wetzlar, Germany) muscles were cut using a cryotome (Kryostat HM 560-V, Thermo Scientific GmbH, Schwerte, Germany) in 10 µm and mounted on glass slides (Superfrost

Plus, Thermo Scientific GmbH, Schwerte Germany). Section were stored at -80°C until staining. Before staining, sections were fixed for 5 min in acetone-isopropanol 1:1 solution -20° . Prior to staining, unspecific antibody binding was blocked by 10% bovine serum albumin in phosphate buffered saline/tween20 (PBS/T) with pH 7.4 for 60 min. Subsequently, specific epitopes were detected by using the following primary antibodies: myosin heavy chain slow (M8421, Sigma, Germany) and myosin heavy chain fast (M4276, Sigma, Germany). Therefore, the antibodies were diluted 1:1000 in PBS/T buffer and incubated over night at 4°C . Goat-anti-mouse antibody (1070-05, SouthernBiotech, Birmingham, USA) was used as secondary antibody diluted in PBS/T 1:5000 for one hour at room temperature. This was followed by DAB-staining with DAKO Liquid DAB+ Substrate chromogen System (K3467, DAKO, Glostrup, Denmark) with DAB+ Chromogen (3,3'-daiminobenzidine in chromogen solution) using 30 droplets DAB in 10ml substrate buffer, staining for 5 min. Two minutes with Mayer's hematoxylin (Roth, Germany) was used for counterstaining thereafter. Finally, sections were rinsed in aqua dest., dehydrated in ascending ethanol dilutions (70% 96%, 100% for 5 min each), dipped in xylol and covered with Dako Mounting Medium (Dako, CA, USA).

Morphometric measurement: All histological analyses were executed blinded as well as in a random order. Additionally, measurements were done twice by two independent researchers. Histological images were acquired using Axio Imager microscope (Zeiss, GER) and calibrated AxioVision SE64 Rel. 4.9 software tool (Zeiss, Germany). To quantify the total mean of muscle fiber cross section area (μm^2), 20 fibers of three different sections were analyzed in the H&E as well as myosin heavy chain (slow/fast) stains. Mean fiber areas were quantified manually by using AxioVision measurement tool and computed by Excel 2016 (Microsoft, USA).

Statistical Analyses

Three hypotheses were tested for the variables that were recorded in each group: 1) effect of therapy, 2) effect of inflammation, and 3) effect of mobility. For this purpose, data were subjected to univariate analyses of variance with the factors (1) therapy (placebo vs. GTS-21), (2) inflammation (saline vs. C.p.), and (3) mobility (sham-immobilization vs. immobilization), as well as all possible interaction terms. If the respective main effect or interaction term proved to be significant, *post hoc* analyses were hierarchically performed. The probability error was set at 0.05. Due to the positively skewed distribution, the arbitrary values of $\alpha 7\text{nAChRs}$ expression were transformed into logarithmic scale. Statistical analyses were performed using IBM SPSS Statistics 25.0 (SPSS, Chicago, IL), figures were created with STATA 14 (Stata Corp LP, College Station, TX).

RESULTS

The different perturbations in the four groups were performed in rats totaling 125. However, only 92 were used in the final analyses because of mortality before and on Day 12 during neuromuscular function tests (Figure 1).

Systemic effects on MetHb levels and body weight and response to GTS-21

The MetHb levels as a fraction of total hemoglobin (percent) were almost undetectable in all saline groups ($0.3 \pm 0.1\%$) regardless of presence or absence of immobilization.

Consequently, GTS-21 had no influence on MetHb levels in saline groups. MetHb increased 26-fold on average in C.p. animals ($p < 0.001$) with a 32-fold increase when compared to placebo subgroups. GTS-21 significantly decreased the elevated MetHb levels seen in C.p. treated with saline to 19-fold ($p = 0.049$, Figure 2). Immobilization did not affect MetHb levels.

Over the 12-day period, the body weights decreased significantly with C.p. alone and with immobilization and GTS-21 mitigated these changes (Figure 3). Rats receiving saline or GTS-21 alone without C.p. and disuse (Controls) gained body weight significantly ($+30 \pm 9\text{g}$) or GTS-21 ($+31 \pm 18\text{g}$), respectively, compared to Day 0. With C.p. alone, the body weight at day 12 significantly decreased ($16 \pm 20\text{g}$ on average, $p < 0.001$) relative to body weights of saline groups. Compared to saline treatment, GTS-21 prevented the loss of body weight that occurred with inflammation ($-23 \pm 17\text{g}$ vs. $-9 \pm 20\text{g}$, $p = 0.011$, Figure 3).

C.p. and/or Immobilization-induced Muscle Changes and Response to GTS-21

Maintenance of the immobilization for 12 days (without C.p.) consistently decreased tibialis weight in placebo groups ($p < 0.001$, Figure 4A) as well as twitch tension (72 ± 16 vs. $146 \pm 7\text{ N}$, $p < 0.0001$) and tetanic tension (163 ± 11 vs. 469 ± 41 , $p < 0.0001$). Tibialis muscle mass was also significantly decreased by inflammation ($p < 0.001$), but even more with their combination (inflammation $\times$ immobilization: $p < 0.001$). Therapy with GTS-21 b.i.d. significantly ameliorated the effects in all respective groups ($p = 0.003$). Post-hoc analysis in animals with sham-immobilization ($p = 0.044$), immobilization ($p = 0.019$) as well as in C.p. injected animals ($p < 0.001$) revealed a significant effect of GTS-21 therapy vs. placebo. GTS-21 was able to increase tibialis mass in C.p. groups without (from $0.26 \pm 0.03\text{g}$ to $0.32 \pm 0.04\text{g}$, $p = 0.028$) and with disuse superimposed (from $0.18 \pm 0.04\text{g}$ to $0.22 \pm 0.03\text{g}$, $p = 0.004$). Although all three main factors tested (therapy, inflammation, mobility) were significant in single twitch, post-hoc analysis showed no significant therapy effect (data not shown). Maximum tetanic tensions were significantly decreased by inflammation ($p < 0.001$) or immobilization ($p < 0.001$), and even more by their combination (inflammation $\times$ immobilization: $p < 0.001$). Post-hoc analysis in animals with immobilization revealed a significant effect of GTS-21 therapy vs. placebo ($p = 0.006$). Furthermore, GTS-21 significantly ameliorated the negative effects on peak tetanic contraction in all respective groups ($p = 0.035$) and in the inflammation with immobilization group ($1.35 \pm 0.51\text{ N}$ vs. $1.81 \pm 0.42\text{ N}$, $p = 0.029$, Figure 4B).

H&E staining of muscle tissue demonstrated a significant effect for all factors (inflammation $p < 0.001$, mobility $p < 0.001$ and therapy $p = 0.010$) including the interaction of inflammation and mobility ($p = 0.001$) on fiber size (Figure 5A). The muscle fiber type area was influenced significantly by all three factors as well: Slow myosin fiber area as well as fast myosin fiber area were significantly decreased by inflammation ($p = 0.012$, $p < 0.001$), mobility ($p = 0.003$, $p < 0.001$) and therapy ($p = 0.004$, n.s. for main factor, but mobility $\times$ therapy ($p = 0.003$) and inflammation $\times$ therapy ($p = 0.029$)), respectively. Posthoc analysis

in slow muscle fiber area showed a significant increase by GTS-21 vs. placebo in groups with saline ($1184 \pm 50 \mu\text{m}^2$ vs. $1013 \pm 61 \mu\text{m}^2$, $p = 0.031$) and inflammation ($1037 \pm 46 \mu\text{m}^2$ vs. $879 \pm 56 \mu\text{m}^2$, $p = 0.041$, Figure 5B). In fast fiber area, post-hoc analysis in of GTS-21 therapy vs. placebo revealed a significant increase of fiber size in immobilized animals ($1159 \pm 235 \mu\text{m}^2$ vs. $993 \pm 294 \mu\text{m}^2$, $p = 0.003$) and in C.p. animals with disuse superimposed ($1064 \pm 58 \mu\text{m}^2$ vs. $718 \pm 89 \mu\text{m}^2$, $p = 0.005$, Figure 5C).

$\alpha 7\text{nAChR}$ expression was low in rats without C.p. independent of immobilization in placebo treated animals (0.19 ± 0.19 arbitrary units). In rats with C.p., the $\alpha 7\text{nAChR}$ expression was significantly increased 11-fold ($p < 0.001$). GTS-21 significantly ameliorated $\alpha 7\text{nAChR}$ expression in the C.p. groups by more than 60% (1.6 ± 1.2 vs. 0.6 ± 0.3 arbitrary units, $p = 0.028$, Figure 6).

DISCUSSION

Indicative of the presence of systemic inflammation, this study demonstrates that all C.p.-injected groups had significant elevations of MetHb levels because of increased expression of inducible nitric oxides [19, 47]. GTS-21 decreased MetHb levels compared to their respective Controls. This study further demonstrates that immobilization alone reduced body weight, however, inflammation alone had a significantly greater effect. The superimposition of immobilization and inflammation further aggravated the body weight loss. In contrast, treating rats having inflammation and immobilization with GTS-21 maintained their body weight relative to day 0. A notable finding is that GTS-21 consistently attenuated tibialis muscle mass loss seen with sham-inflammation alone, immobilization alone, with inflammation alone and when inflammation was combined with immobilization (Figure 4A). The most salient finding is that GTS-21 also mitigated the loss of maximal tetanic tension generating capacity (functional capacity) in the animals with immobilization and in animals with inflammation and superimposed immobilization. Although single twitch tensions did not show differences in post-hoc analysis for therapy, the tibialis muscle mass changes with GTS-21 was associated with statistically significant improvement in maximal tetanic tension particularly when inflammation was present with immobilization. Parenthetically one might add tetanic tension and not single twitch measurements reflect the ability to perform repetitive work.

In previous studies during disuse atrophy [32, 41] and during denervation and sepsis [60], the up-regulation of $\alpha 7\text{nAChRs}$ has been documented. The present study documents that systemic inflammation leading to MW also results in marked up-regulation of $\alpha 7\text{nAChRs}$ in muscle (Figure 6). When we previously characterized the used double-hit model, the changes observed included increases in MetHb levels, and acute phase reactant protein responses, and decrease of red blood cell and platelet counts [9, 57]. Two recent studies demonstrate that after injury to muscle, the $\alpha 7\text{nAChRs}$ are up-regulated (with a peak around the 7th-9th day after the impact). It was reasoned that $\alpha 7\text{nAChRs}$ are involved in muscle regeneration by modulating inflammatory reaction and fibroblast activity [7, 59]. Experimental evidence confirming this hypothesis, however, was not provided.

The utility of GTS-21 to decrease systemic inflammatory responses has been tested in a phase II clinical trial [34], where GTS-21 could safely be used in healthy non-smoking volunteers. Although higher plasma concentration of GTS-21 significantly correlated with lower blood cytokine levels, there was no significant difference between GTS-21 versus placebo. The ineffectiveness of GTS-21 to control cytokines was probably related to the oral dosing regimen, since the low bioavailability of orally administered GTS-21 resulted in relatively low blood concentrations (mean, 13 ng/ml at time of LPS administration). GTS-21 has previously been administered orally to healthy, schizophrenic, Alzheimer's disease and attention-deficit hyperactivity disorder subjects in clinical tests [11, 29, 33, 50]. These human clinical studies are indeed important in that no serious side effects have been documented, paving the way for more studies with GTS-21 using alternative routes of administration. In our study, a dose of 10mg/kg b.i.d. demonstrated especially beneficial effects evidenced by the attenuation of inflammation alone-induced and inflammation with disuse-induced biochemical, physiological and functional changes. These findings suggest that the $\alpha 7$ nAChRs could be used to advantage to mitigate the inflammatory responses and its consequences on body weight, muscle mass and force if initiated at the beginning of a perturbation and continued during prolonged systemic inflammation.

Our dose of 10mg/kg GTS-21 did not completely reverse the body weight and muscle changes. Treatment for longer period than 12 days may have most likely reversed muscle mass faster than untreated controls. The pharmacokinetics, however, of GTS-21 in critically illness of rodents is unknown. Some drugs have a faster clearance in critical illness [5, 17, 18]. Thus, alternative therapeutic modalities such as smaller, more frequent doses and/or for longer periods may result in complete reversal of changes.

GTS-21 down-regulated $\alpha 7$ nAChR expression in tibialis muscle in all groups, especially in inflammation animals by more than 60% compared to Controls. This down-regulation probably suggests decreased inflammation in muscle, consistent with a previous *in vitro* finding in macrophages [30] and this study that inflammation up-regulates $\alpha 7$ nAChR expression. The down-regulation was concomitantly associated with mitigation of the inflammation-induced biochemical and body mass and muscle changes including force. This begs the question whether the site of action of GTS-21 is via actions on $\alpha 7$ nAChRs on circulating cells or on muscle itself. Future studies with depletion of cells are warranted as which of the $\alpha 7$ nAChRs (muscle vs. macrophages) play a more predominant role. These studies could include the use of muscle specific $\alpha 7$ nAChR knockout mice (currently not available) or by a depletion of macrophages *in vivo* [24].

Our study has some limitations. First, we did not measure cytokines levels as inflammation markers, but only methemoglobin as previously [9, 57]. Second, our study is descriptive and does not provide mechanistic data to establish a direct link of GTS-21 induced $\alpha 7$ nAChR reduction on improvements in muscle mass and function. Third, in order to have a clinically relevant model, e.g. a longer duration of immobilization and inflammation, as well as clinically relevant endpoints, we performed an *in vivo* study. This results in a higher variability because of the individual responses of the different animals. In summary, most previous studies focused on altering disease-induced muscle changes to reverse MW of critical illness. This current study is different in that it attempts control systemic and

possibly local inflammation in muscle to reverse or mitigate the disease-associated changes. Using a novel molecular target, namely the $\alpha 7$ nAChRs, we confirmed our hypothesis that modulation of inflammatory responses via $\alpha 7$ nAChRs by use of GTS-21 can indeed attenuate body mass, muscle mass, and/or tension loss when inflammation is present alone and with immobilization. The current study also adds to the evidence that the $\alpha 7$ nAChR is a novel target that might be the subject of future studies concerned with inflammation and organ dysfunction [30, 35]. These findings are indeed encouraging in view of the recent clinical trials on $\alpha 7$ nAChR modulators in humans to control lipopolysaccharide-induced inflammatory responses [36] and safe use of $\alpha 7$ nAChR agonists to treat neuro-inflammation and schizophrenia [6]. Our findings could have direct applications to the bedside after validations of some of the questions that have been raised by us.

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Fig. 1.
Flow Chart

Fig. 2.

Blood methemoglobin (MetHb) levels in rats with each perturbation on day 12 [fold change compared to saline groups]. MetHb fraction of total hemoglobin was significantly increased by C.p. (26-fold average increase, $p < 0.001$) and significantly reduced in all GTS-21 groups ($p = 0.033$). Post-hoc analysis revealed a 40% decrease in C.p. injected animals by therapy (from 32-fold with placebo to 19-fold with GTS-21, ▲, $p = 0.049$). Immobilization did not influence MetHb levels significantly. Sham = Sham-Immobilization, Immob = Immobilization/superimposed disuse, C.p. = systemic inflammation with *Corynebacterium parvum*, Saline = sham-inflammation with saline, GTS = GTS-21 DMBX-A = 3-[(3E)-3-[(2,4-dimethoxyphenyl)methylidene]-5,6-dihydro-4H-pyridin-2-yl]pyridine, bid = *bis in die* = twice a day

Fig. 3.

Body weight changes between day 0 and day 12. The change of body weight comparing day 12 vs. day 0 was significantly decreased by C.p. (-16 ± 20 g, $p < 0.001$). Therapy with 10 mg/kg GTS-21 b.i.d. significantly attenuated body weight loss in the C.p. groups (inflammation $\times$ therapy: $p = 0.005$). Post-hoc analysis revealed in C.p. showed a significant improvement by GTS-21 therapy compared to placebo ($\blacktriangle$, -9 ± 20 g vs. -23 ± 17 g, respectively $p = 0.011$). Immobilization effected body weight change in all respective groups ($p < 0.001$). GTS-21 had a significant effect in post-hoc analysis of therapy compared to placebo in C.p. with disuse superimposed ($\bullet$, from -29 ± 16 g to -14 ± 10 g, respectively, $p = 0.009$)

Sham = Sham-Immobilization, Immob = Immobilization/superimposed disuse, C.p. = systemic inflammation with *Corynebacterium parvum*, Saline = sham-inflammation with saline, GTS = GTS-21 DMBX-A = 3-[(3E)-3-[(2,4-dimethoxyphenyl)methylidene]-5,6-dihydro-4H-pyridin-2-yl]pyridine, bid = *bis in die* = twice a day

Fig. 4.

Muscle changes (mass and force) with disuse, or inflammation alone and their combination without and with GTS-21

(A) Tibialis muscle mass. Gray background area indicates the lower and upper limit of tolerance (i.e., mean $\pm 1.96 \times$ SD of the naïve leg of saline animals). Tibialis muscle mass was significantly decreased by inflammation ($p < 0.001$) or immobilization ($p < 0.001$), but even more with their combination (inflammation $\times$ immobilization: $p < 0.001$). Therapy with GTS-21 b.i.d. significantly ameliorated the effects in all respective groups ($p = 0.003$). Post-hoc analysis in animals with sham-immobilization ($\diamond$, $p = 0.044$) and immobilization ($\blacklozenge$, $p = 0.019$) as well as in C.p. injected animals revealed a significant effect of GTS-21 therapy vs. placebo ($\blacktriangle$, $p < 0.001$). GTS-21 was able to increase tibialis mass in C.p. groups without ($\circ$, from 0.26 ± 0.03 g to 0.32 ± 0.04 g, $p = 0.028$) and with disuse superimposed ($\bullet$, from 0.18 ± 0.04 g to 0.22 ± 0.03 g, $p = 0.004$).

(B) Maximum tetanic tension. Colored area in broad blue indicates the lower and upper limit of tolerance (i.e., mean $\pm 1.96 \times$ SD of the naïve leg of saline animals). Tetanic maximum tensions were significantly decreased by inflammation ($p < 0.001$) or immobilization ($p < 0.001$), but even more with their combination (inflammation $\times$ immobilization: $p < 0.001$). Therapy with GTS-21 significantly ameliorated the effects in all respective groups ($p = 0.035$). Post-hoc analysis in animals with immobilization revealed a significant effect of GTS-21 therapy vs. placebo ($\blacklozenge$, $p = 0.006$). Post-hoc analysis in C.p. injected animals with disuse superimposed revealed a significant effect of GTS-21 therapy vs. placebo ($\bullet$, 1.35 ± 0.51 N vs. 1.81 ± 0.42 Newton, $p = 0.029$).

Sham = Sham-Immobilization, Immob = Immobilization/superimposed disuse, C.p. = systemic inflammation with *Corynebacterium parvum*, Saline = sham-inflammation with saline, GTS = GTS-21 DMBX-A = 3-[(3E)-3-[(2,4-dimethoxyphenyl)methylidene]-5,6-dihydro-4H-pyridin-2-yl]pyridine, bid = *bis in die* = twice a day

Fig. 5.

Immunohistochemistry of tibialis with disuse, or inflammation alone and their combination without and with GTS-21. Values presented as mean $\pm$ SE.

(A) H&E staining. H&E area of muscle fiber was significantly decreased by inflammation ($p < 0.001$) or immobilization ($p < 0.001$), but even more with their combination (inflammation $\times$ immobilization: $p = 0.001$). Therapy with GTS-21 b.i.d. significantly ameliorated the effects on muscle fiber size in all respective groups ($p = 0.010$). Post-hoc analysis of GTS-21 therapy vs. placebo revealed a significant effect in C.p. injected animals revealed a significant effect ($\blacktriangle$, $1036 \pm 46 \mu\text{m}^2$ vs. $826 \pm 61 \mu\text{m}^2$, $p = 0.011$) as well as in immobilized animals ($\blacklozenge$, $991 \pm 205 \mu\text{m}^2$ vs. $781 \pm 220 \mu\text{m}^2$, $p = 0.002$). GTS-21 was able to increase H&E area in C.p. injected animals with disuse superimposed vs. placebo ($\bullet$, $955 \pm 56 \mu\text{m}^2$ vs. $630 \pm 87 \mu\text{m}^2$, $p = 0.007$).

(B) Slow Myosin Fiber area. Slow myosin fiber area was significantly decreased by inflammation ($p = 0.012$) or mobility ($p = 0.003$). Therapy with GTS-21 b.i.d. significantly ameliorated the effects of pinning and C.p. in all respective groups ($p = 0.004$). Post-hoc

analysis in immobilized animals showed a significant increase in slow myosin fiber size with GTS-21 therapy compared to placebo (◆, $p = 0.003$). Post-hoc analysis in inflammation groups showed a significant increase in slow myosin fiber size with GTS-21 therapy compared to placebo in animals with saline (, $1184 \pm 50 \mu\text{m}^2$ vs. $1013 \pm 61 \mu\text{m}^2$, $p = 0.031$) or C.p. injected (▲, $1037 \pm 46 \mu\text{m}^2$ vs. $879 \pm 56 \mu\text{m}^2$, $p = 0.041$). GTS-21 was able to increase slow myosin fiber size in saline animals with disuse (×, $1176 \pm 70 \mu\text{m}^2$ vs. $879 \pm 75 \mu\text{m}^2$, $p = 0.013$).

(C) Fast Myosin Fiber area. Fast myosin fiber area was significantly decreased by inflammation. ($p < 0.001$) or mobility ($p < 0.001$). Main factor therapy was not significant, however, interaction terms of mobility × therapy ($p = 0.003$) and inflammation × therapy ($p = 0.029$) were significant. Post-hoc analysis of GTS-21 therapy vs. placebo revealed a significant effect in immobilized animals (◆, $1159 \pm 235 \mu\text{m}^2$ vs. $993 \pm 294 \mu\text{m}^2$, $p = 0.003$). Post-hoc analysis in C.p. injected animals with disuse superimposed revealed that GTS-21 increased fast myosin fiber area vs. placebo (●, $1064 \pm 58 \mu\text{m}^2$ vs. $718 \pm 89 \mu\text{m}^2$, $p = 0.005$).

Fig. 6.

$\alpha 7$ nAChR expression in the tibialis muscle (arbitrary units).

(A) Gray background area indicates the lower and upper limit of tolerance (i.e., mean $\pm$ 1.96 SD of the naïve leg of saline animals). Disuse alone with no inflammation did not change $\alpha 7$ nAChR expression in muscle. $\alpha 7$ nAChR expression was significantly increased 11-fold by C.p. ($p < 0.001$). Therapy with 10 mg/kg GTS-21 b.i.d. significantly ameliorated the effects in all respective groups ($p < 0.001$). Post-hoc analysis for inflammation revealed a significant effect of therapy in saline groups (, 0.2 ± 0.2 in placebo vs. 0.02 ± 0.02 in GTS-21 [arbitrary units], $p < 0.001$) and C.p. groups (▲, 1.6 ± 1.2 vs. 0.6 ± 0.3 , respectively [arbitrary units], $p = 0.028$). GTS-21 was able to decrease $\alpha 7$ nAChR in saline groups without disuse (+, $p < 0.001$) and with disuse (×, $p = 0.008$) and C.p. groups with disuse superimposed (●, $p = 0.007$).

(B) Representative blots of $\alpha 7$ AChR expression in the tibialis muscle. Blots reveal that, similar to denervation, C.p with and without disuse up-regulates $\alpha 7$ AChR expression in muscle. Sham = Sham-Immobilization, Immob = Immobilization/superimposed disuse, C.p. = systemic inflammation with *Corynebacterium parvum*, Saline = sham-inflammation with saline, GTS = GTS-21 DMBX-A = 3-[(3E)-3-[(2,4-dimethoxyphenyl)methylidene]-5,6-dihydro-4H-pyridin-2-yl]pyridine, bid = *bis in die* = twice a day