J Appl Physiol 126: 160–169, 2019.
First published October 11, 2018; doi:10.1152/japplphysiol.00248.2018.

RESEARCH ARTICLE

Experimental myofascial trigger point creation in rodents

Ramon Margalef,[1] Marc Sisquella,[1] Marc Bosque,[1] Clara Romeu,[1] Orlando Mayoral,[2] Sonia Monterde,[3] Mercedes Priego,[1] Rafael Guerra-Perez,[4] Nicolau Ortiz,[5] Josep Tomàs,[1] and Manel M. Santafe[1]

[1]Unit of Histology and Neurobiology, Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Rovira i Virgili University, Reus, Spain; [2]Physical Therapy Unit, Hospital Provincial de Toledo, Toledo, Spain; [3]Unit of Physiotherapy, Department of Medicine and Surgery, Faculty of Medicine and Health Sciences, Rovira i Virgili University, Centre Tecnològic de Nutrició i Salut Avinguda Universitat, Reus, Spain; [4]RG Clinic, Madrid, Spain; and [5]Neurology Section, Department of Medicine, Sant Joan University Hospital, Reus, Spain

Submitted 19 March 2018; accepted in final form 3 October 2018

Margalef R, Sisquella M, Bosque M, Romeu C, Mayoral O, Monterde S, Priego M, Guerra-Perez R, Ortiz N, Tomàs J, Santafe MM. Experimental myofascial trigger point creation in rodents. *J Appl Physiol* 126: 160–169, 2019. First published October 11, 2018; doi:10.1152/japplphysiol.00248.2018.—Myofascial pain syndrome is one of the most common forms of muscle pain. In this syndrome, pain is originated by the so-called trigger points, which consists of a set of palpable contraction knots in the muscle. It has been proposed that a high, spontaneous neurotransmission may be involved in the generation of these contraction knots. To confirm this hypothesis, we exposed mouse muscles to an anticholinesterasic agent to increase the neurotransmision in the synaptic cleft in two different conditions, in vivo and ex vivo experiments. Using intracellular recordings, a sharp increase in the spontaneous neurotransmission in the levator auris longus muscle and a lower increase in the diaphragm muscle could be seen. Likewise, electromyography recordings reveal an elevated endplate noise in gastrocnemius muscle of treated animals. These changes are associated with structural changes such as abundant neuromuscular contracted zones observed by rhodaminated α-bungarotoxin and the presence of abundant glycosaminoglycans around the contraction knots, as shown by Alcian PAS staining. In a second set of experiments, we aimed at demonstrating that the increases in the neurotransmission reproduced most of the clinical signs associated to a trigger point. We exposed rats to the anticholinesterase agent neostigmine, and 30 min afterward we observed the presence of palpable taut bands, the echocardiographic presence of contraction knots, and local twitch responses upon needle stimulation. In summary, we demonstrated that increased neurotransmission induced trigger points in both rats and mice, as evidenced by glycosaminoglycans around the contraction zones as a novel hallmark of this pathology.

NEW & NOTEWORTHY In rodents, when neostigmine was injected subcutaneously, the neuromuscular neurotransmission increased, and several changes can be observed: an elevated endplate noise compared with normal endplate noise, as evidenced by electromyographyc recording; many muscular fibers with contraction knots (narrower sarcomeres and locally thickened muscle fiber) surrounded by infiltration of connective tissue like glycosaminoglycans molecules; and palpable taut bands and local twitch responses upon needle stimulation. Several of these signs are also observed in humans with muscle pain.

Address for reprint requests and other correspondence: M. M. Santafe, Unit of Histology and Neurobiology, Dept. of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Rovira i Virgili University, Carrer St. Llorenç num 21, 43201-Reus, Spain (e-mail: manuel.santafe@urv.cat).

animal model; endplate noise; myofascial pain syndrome; myofascial trigger point; spontaneous acetylcholine release

INTRODUCTION

Myofascial pain syndrome (MPS) is one of the most frequent causes of muscular pain observed clinically (reviewed in Ref. 16). MPS is a collection of known sensory, motor, and autonomic symptoms caused by myofascial trigger points (MTrPs) (9, 29). An MTrP is an hyperirritable area in a skeletal muscle associated with a palpable and tender nodule located in a taut band of muscle. This taut band consists of a group of muscle fibers of increased consistency and with an abnormal state of tension. MPS is very common in humans, and it is estimated that almost everyone might suffer at least one episode during his life (16).

Electromyography (EMG) allows clinicians to confirm the existence of MTrPs. Electromyographically, MTrPs can be diagnosed by the finding of a characteristic recording called endplate noise, which corresponds to the spontaneous release of acetylcholine (ACh) in the motor endplates of some muscular fibers (8, 17). According to Hong and Simons (12), an MTrP is composed of multiple active loci from where this characteristic EMG recording can be obtained. Furthermore, the existence of elongated hypoechoic regions in the area of MTrPs can be revealed by ultrasound diagnosis (30). Currently, the most accepted theory on the pathophysiology of myofascial trigger point involves an altered or abnormal neurotransmitter release, and this starts a cascade of events ultimately forming a MTrPs (27). It has been experimentally demonstrated that a maintained increase (from 20 to 30 min) of ACh in the synaptic cleft generates a myocyte membrane potential with insufficient values to "trigger" an action potential but enough to allow an increase in Na^{2+} in the subsynaptic cytoplasm (15). This increase in sodium permeates the sarcotubular system locally and depolarizes the system, which induces a progressive efflux of Ca^{2+}. This calcium will locally activate contraction. Finally, a disk of contraction is built in the subsynaptic area, which constitutes the palpable nodule previously discussed as myofascial trigger points. An integrated hypothesis proposes that shortening of sarcomeres in knot contractions causes a shortening of the muscle fiber causing tau band (27). On the other hand, Shah and colleagues (25, 26), using a sophisticated technique of microanalysis in patients,

demonstrated the existence of substances associated with pain and inflammation. Specifically an elevated concentration of protons, SP, calcitonin gene-related peptide, bradykinin, TNFα, IL-1β, IL-6, IL-8, 5-HT and norepinephrine were found in an area of MTrPs located in the upper trapezius. Despite these few works, MPS pathophysiology has been poorly studied.

Simons and Stolov (28) performed a biopsy and obtained the first images of contraction knots from a palpable nodule located in a taut band in the gracillis muscle of dogs. Many years later, Mense et al. (21) obtained disks of contraction similar to those of MTrPs in the gastrocnemius muscle of the rat by means of an electrically induced contractile activity added to the treatment with the anticholinesterase agent DHP. Nevertheless, they also obtained undesirable muscle injuries that Simons and Stolov (28) had not described.

More recently, Shah and colleagues (25, 26), using a sophisticated technique of microanalysis in patients, demonstrated the existence of high concentrations of substances associated with pain and inflammation in an area of MTrPs. Despite these few works, the pathophysiology of MPS remains poorly studied.

We have generated multiple contraction knots clearly identified by microscopy and multiple active loci identified by electrophysiology techniques in mice. Myofascial trigger points were generated by increasing the ACh in the synaptic cleft by means of anticholinesterasic agents injected subcutaneously. This model reproduced the related signs of MTrPs described in the literature and would become an important tool to better understand the pathophysiology of MPS.

MATERIALS AND METHODS

Animals

Experiments were performed on adult young male Swiss mice (45–50 days postnatal; $n = 139$) and Sprague-Dawley rats (60 to 70 days post-natal, $n = 18$; Charles River, L'Arbresle, France). Animals were euthanized by exsanguination under anesthesia. The animals were cared for in accordance with the guidelines of the European Community's Council Directive of November 24, 1986 (86/609/EEC) and the Spanish Royal Decree 53/2013 for the humane treatment of laboratory animals. This study was approved by the Ethics Committee of the Rovira i Virgili University.

Muscles

Animals were anesthetized with 2% tribromoethanol (0.15 ml/10 g body weight ip) and euthanized by exsanguination while deeply anesthetized. The levator auris longus (LAL; fast-twitch muscle) and diaphragm (slow-twitch muscle) with their nerve supply were excised and dissected on a Sylgard-coated Petri dish containing normal Ringer solution and continuously bubbled with 95% O_2-5% CO_2. Both the LAL and diaphragm were used for the electrophysiological study. The gastrocnemius muscles were used for electromyography and ultrasonographic recordings (Table 1).

Anticholinesterasic Exposure

Studies done by injecting drugs into the live animal (in toto) ensure a physiological environment in terms of oxygen, hormones, vitamin trace elements, and other supplies provided by the blood, but we do not know the exact dose of anticholinesterase that reaches every muscle. On the other hand, studies in the recording chamber (ex vivo) allow us to control the dose of exposure, but the physiological solution (Ringer) used, despite being a good saline solution, does not bring vitamins, hormones, or other ions in blood. Finally, to overcome these limitations, we decided to include both experimental approaches.

We increased ACh in the synaptic cleft in two different experimental conditions of exposure to anticholinesterasic agents: in vivo and ex vivo experiments. To choose the best drug and the most effective dose at inhibiting acetylcholinesterase, we tested several anticholinesterase agents: three classic drugs such as neostigmine methyl sulfate (NTG; 0.1 mg NTG/kg body wt; Sigma), pyridostigmine (PTG; 0.1 mg PTG/kg body wt; Sigma), and rivastigmine (RTG; 0.1mg RTG/kg body wt; and Fasciculin II (FII; 350 nM, Sigma), the last of which is the most recent generation of anticholinesterase.

For in vivo experiments, NTG, PTG, or RTG were injected subcutaneously (thoracolumbar area) into adult male Swiss mice. To ensure the correct drug administration, we expected the appearance of a cholinergic syndrome within 30 min posttreatment. Cholinergic syndrome is characterized by generalized tremor (clearly seen in the extremities), increased salivation, and tearing. If the cholinergic syndrome did not appear, the animal was discarded for the study. We have chosen a treatment with subcutaneous neostigmine (whole animal) rather than intramuscular treatment to prevent from the needle injuries that could interfere with the results.

The muscles were exposed to anticholineserasic in ex vivo experiments for intracellular recordings exclusively (see below). The muscles were normally excised and dissected on a Sylgard-coated Petri dish containing normal Ringer solution that was continuously bubbled with 95% O_2-5% CO_2. Then, the anticholineserasic (NTG or FII) was added to the bath for 60 min before the recording started.

Table 1. *Methodological approach*

		Time After Neostigmine Treatment					
Animal (Technique)	Muscle	30 min	1 h	3 h	24 h	48 h	72 h
Mouse							
Intracellular recordings	LAL/diaphragm	■	■	□	■	■	□
Methylene Blue	LAL	□	■	□	■	□	□
PAS/Alzian Blue	LAL	■	□	□	■	□	■
Histochemical	LAL	■	□	□	■	■	□
Electromyography	Gastrocnemius	■	□	■	■	□	□
Rat							
Intracellular recordings	LAL	■	□	□	□	□	□
PAS/Alzian Blue	LAL	■	□	□	□	□	□
Electromyography	Gastrocnemius	■	□	□	■	□	□
Ultrasound	Gastrocnemius	■	□	□	■	□	□

Several techniques were performed on the muscles shown at the indicated time periods after neostigmine exposure. ■, Technique performed; □, no technique performed.

Electrophysiology: Intracellular Recordings

Spontaneous miniature endplate potentials (mEPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (20–40 MΩ resistance). Records were rejected if the membrane potential (Vm) was less than −50 mV or if it fell by >5 mV during the recording period.

The recording electrodes were connected to an amplifier (Tecktronics, AMS02). A distant Ag-AgCl electrode connected to the bath solution via an Agar bridge (Agar 3.5% in 137 mM NaCl) was used as reference. MEPPs were digitized (DIGIDATA 1200 Interface; Axon Instruments), stored, and analyzed by computer. We used Axoscope 10.2 (Axon Instruments) for data acquisition and analysis. The MEPP frequency was recorded for 100 s from ≥15 different neuromuscular junctions, and the values were averaged. The mean amplitude (mV) per fiber was calculated and corrected for nonlinear summation (19) assuming a membrane potential of −80 mV.

In ex vivo experiments, we proceeded as follows. First, we recorded mEPPs from ≥15 different control neuromuscular junctions. Then we incubated the muscle with the anticholineserasic (NTG or FII) for 1 h, and we recorded mEPPs from ≥15 different neuromuscular junctions.

For in vivo experiments, we proceeded as follows: NTG, PTG, or RTG was injected subcutaneously in adult male Swiss mice; when the acute cholinergic syndrome disappeared (30 min), the sample (LAL or diaphragm) was normally excised and dissected as explained above, and mEPPs were recorded from ≥15 different neuromuscular junctions.

Histological Techniques

Methylene blue. Whole LAL muscles were removed and exposed to a 1% methylene blue dissolved in 1% borax for 2 min. Subsequently, the samples were washed with distilled water for three steps of two minutes each. Finally, we proceeded to dehydration and mounting with epoxy resin.

Periodic acid Schiff/Alcian Blue staining. The samples were not fixed, and they were directly labeled. In this manner, the cellular membrane is quite preserved, and the extracellular glycosaminoglycans (GAGs) are better labeled. The LAL muscles were normally excised and dissected on a Sylgard-coated Petri-dish containing normal Ringer solution. The muscles maintained in the Sylgard-coated Petri-dish were directly labeled with Alcian Blue 8GX and 3% acetic acid for 15 min. Subsequently, the samples were washed with distilled water and oxidized in periodic acid for 2 min. Then the samples were placed in Coleman's Schiff's Reagent for 10 min. Later, the muscles were washed in water for 3 min and placed in 10% sodium metabilsulfite. Initially, the samples were directly mounted in glycerin because the label colors can be seen better than when the samples are dehydrated. To preserve the samples for a long time, after the analyses we proceeded to dehydration and mounting with epoxy resin.

Immunochemical technique. Whole LAL muscles were removed and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 45 min at room temperature (~22°C). The LALs were labeled for postsynaptic nicotinic acetylcholine receptors with tetramethyl rhodamine isothiocyanate (TRITC)-α-BTX (1:800 dissolution; Molecular Probes,). Muscles were mounted in Mowiol with *p*-phenylenediamine (Sigma). A morphometric study of the image was made to evaluate the area for each synaptic contact with the Nis Elements (Nikon). Finally, the frequency distribution analysis of the measured synaptic areas was performed.

Endplate Noise Recordings

The needle EMG (nEMG) records were always obtained from an anesthetized animal at a controlled room temperature between 22 and 25.8°C. The muscle used for this study was the gastrocnemius because it is easy to access and is suitable for the study. Results from intracellular recording experiments determined that the neostigmine was the most effective anticholinesterasic in terms of increasing the frequency of mEPPs. For this reason, endplate noise experiments were performed only with neostigmine.

Registers were obtained with an electromyography system (Medelec Mystro plus, GR20) with a monopolar EMG needle (Natus Manufacturing). The needle is slowly inserted into the muscle and, once inside, is moved to record in all directions. The muscle was divided into 12 areas (Fig. 1*A*) to cover all the muscle and to avoid registering the same endplate noise twice. The recording needle is introduced into the gastrocnemius until a change in the noise can be heard. Electromyography screen is then studied, and if it is correct (without alternating current, artifacts, etc.) endplate noise is recorded. The number of areas with endplate noise (≤12; see Fig. 1*A*) was recorded. During the time recording endplate noise, we count the number of potential per second that have appeared. That is the frequency and is expressed in Hz.

The experiments proceeded as follows: *1*) a control nEMG recording in the left gastrocnemius, *2*) subcutaneous injection of NTG, *3*) the animal showing cholinergic syndrome, *4*) nEMG examination of the right gastrocnemius muscle (see Fig. 1*B*).

Ultrasonography

Muscular ultrasonography was performed in rats with an ultrasound (LOGIQ E R7; General Electric) and a transducer (General

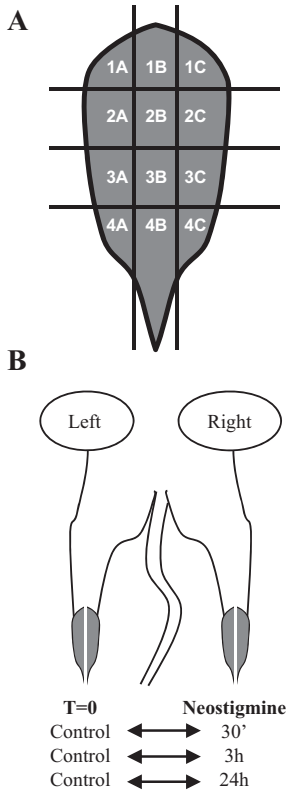

Fig. 1. Endplate noise. *A*: scheme of the gastrocnemius muscle showing the 12 virtual areas used for registration to ensure no repetitions or absences. *B*: scheme of the protocol used for recording the endplate noise. Before treatment, the left gastrocnemius was recorded. Then, the animal was treated with subcutaneous neostigmine, and 30 min, 3 h, or 24 h later the right gastrocnemius examination was performed. In all cases, to minimize interindividual variability, the values obtained in the right (exposed to neostigmine) and the left gastrocnemius (control) were compared.

Electric, L10-22-RS). Ultrasound needle within tissues is identified as a bright line that penetrates tissue or only as a reflection of the tip. If a sudden and very localized movement when the needle is inserted into the muscle is seen, it is considered to be a local twitch response. If the needle insertion shows only the tip deforming the tissue while penetrating, it is considered that the local twitch response has not occurred. First MTrPs and local twitch response to dry needling muscles were assessed in left gastrocnemius before treatment. Then, rats were treated with neostigmine, and 30 min later the right gastrocnemius muscles were palpated for MTrPs and dry needled during ultrasonographic monitoring. In both cases, the dry needling procedure was applied using a solid filament needle and with the multiple insertion technique usually employed to elicit local twitch responses in a muscle with MTrPs.

Lists of Chemicals and Equipment

Mice were supplied by Charles River Laboratories (L'Arbresle, France). Tribromoethanol and other salts were supplied by Sigma Aldrich (Steinheim, Germany). Sylgard was supplied by Dow Corning. Anticholinesterase agents neostigmine (NTG), piridostigmine (PTG), rivastigmine (RTG), and fasciculin II (FII) were supplied Sigma-Aldrich, stereoscopic magnifying glass (EMT) was from Meiji Techno (Tokyo, Japan), monopolar EMG needles were from Natus Manufacturing (Galway, Ireland), micromanipulator was from Prior (Cambridge, UK), Medelec Mystro was from Teca Medelec (London, UK), and ultrasound and transducer were from General Electric (Boston, MA).

Statistical Procedure

Values are expressed as means ± SE. The values are expressed as "percentage of change." This is defined as [experimental value/control value] × 100. We used the two-tailed Welch's *t*-test for unpaired values because our variances were not equal. We prefer this test because it is more conservative than the ordinary *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Electrophysiology: Intracellular Recordings

To choose the best drug and the most effective dose at inhibiting acetylcholinesterase, we tested several anticholinesterase agents. Two muscles, the diaphragm as a slow-twitch muscle and LAL as a fast-twitch muscle, were also chosen (see Fig. 2*A*). To visualize the muscle fibers and localize the possible NMJ requires flat, thin, and transparent muscles. These muscles are very thin and easy to manipulate for electrophysiological studies.

The results obtained in the diaphragm were globally moderate compared with those obtained in the LAL muscle (Fig. 2*B*). The higher increase in the frequency of mEPPs was obtained when the diaphragm was exposed to neostigmine (NTG) in the recording chamber (ex vivo experiments; ~200% increase). Twenty-four hours after a single subcutaneous injection of NTG (0.1 mg NTG/kg body wt), no significant changes in frequency were produced (variation of experimental values with respect to control values: 2.88% ± 4.77; $n = 45$

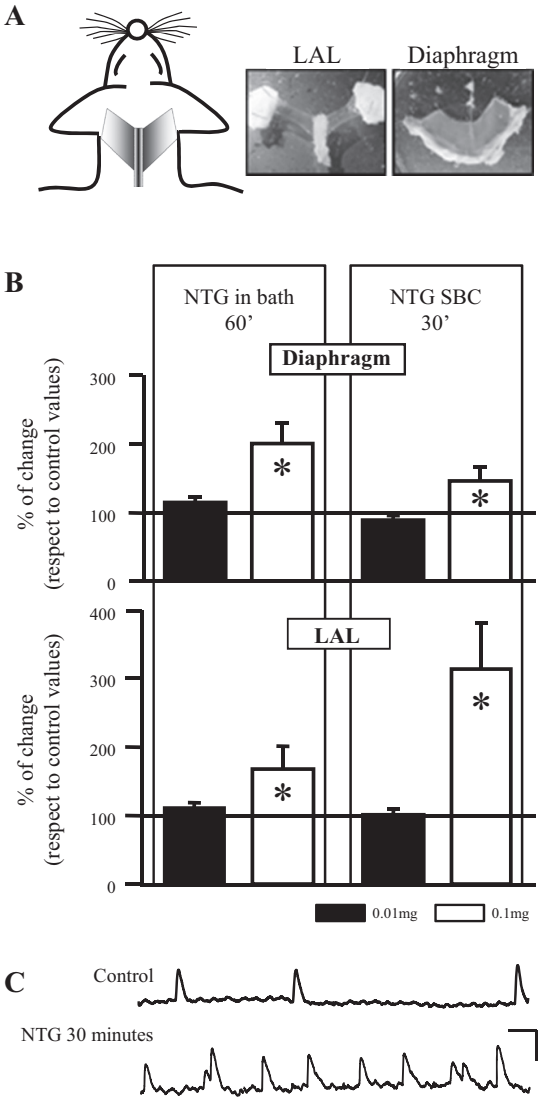

Fig. 2. Intracellular recordings [miniature endplate potentials (mEPPs)]. *A*: scheme of the anatomic location of levator auris longus (LAL) muscle (*left*). Photograph of the LAL muscle (*middle*) and right hemidiaphragm (*right*). Note that these muscles are almost transparent because they are extremely thin. *B*: histograms at *left* show the increases in mEPP frequency in ex vivo experiments; the samples were exposed to neostigmine (NTG) in a chamber of recording. Histograms at *right* show in vivo experiments; the muscles were exposed to NTG by subcutaneous injection (SBC) to the animal in toto. Two doses were used: 0.01 mg (black bars) and 0.1 mg (open bars) of NTG/kg body weight. Note that NTG (0.1 mg NTG/kg) is most effective for in toto experiments and in LAL muscles. *$P < 0.05$; hemidiaphragm ex vivo experiments: 0.1 mg of NTG, $P = 0.0068$, 62° of freedom, $n = 45$ synapses from 5 animals; hemidiaphragm ex vivo experiments: 0.01 mg of NTG, $P = 0.3034$, 117° of freedom, $n = 90$ synapses from 10 animals; hemidiaphragm in toto experiments: $n = 45$ synapses from 7 animals treated with 0.1 mg of NTG, $n = 45$ synapses from 6 control animals, $P = 0.0201$, 76° of freedom; hemidiaphragm in toto experiments: $n = 45$ synapses from 5 animals treated with 0.01 mg of NTG, $n = 45$ synapses from 6 control animals, $P = 0.3884$, 70° of freedom; LAL ex vivo experiments: 0.1 mg of NTG, $P = 0.0007$, 78° of freedom, $n = 45$ synapses from 5 animals; LAL ex vivo experiments: 0.01 mg of NTG, $P = 0.3826$, 132° of freedom, $n = 90$ synapses from 10 animals; LAL in toto experiments: $n = 36$ synapses from 6 animals treated with 0.1 mg of NTG, $n = 66$ synapses from 8 control animals, $P < 0.0001$, 100° of freedom; LAL in toto experiments: $n = 45$ synapses from 5 animals treated with 0.01 mg of NTG, $n = 45$ synapses from 6 control animals, $P = 0.9707$, 85° of freedom. *C*: examples of intracellular recordings from a control muscle (*top*) and a muscle exposed to NTG subcutaneously in toto (*bottom*); note the increased frequency of mEPPs. Vertical bar, 1 mV; horizontal bar, 25 ms.

synapses from 3 treated animals, $n = 45$ synapses from 6 control animals; $P = 0.6115$; 70° of freedom).

The highest increase in the frequency of mEPPs (~300%) was obtained in the LAL muscle 30 min after a subcutaneous injection of the NTG (0.1 mg NTG/kg body wt; see Fig. 2, *B* and *C*). In the ex vivo experiments, the frequency of mEPPs was also increased (Fig. 2*B*). Consistently with the results obtained from the diaphragm, when the LAL muscle was extracted 24 or 48 h after treatment (0.1 mg NTG/kg body wt, repeating every 12 h), the frequency of mEPPs did not change (%variation when the LAL muscle was extracted 24 h after treatment respect to control values: 2.88 ± 5.52, $P = 0.6395$, 94° of freedom; $n = 66$ synapses from 7 control animals, $n = 45$ synapses from 3 treated muscles; %variation when the LAL muscle was extracted 48 h after treatment respect to control values: 15.48 ± 4.77, $P = 0.1816$, 92° of freedom, $n = 66$ synapses from 7 control animals, $n = 36$ synapses from 3 treated muscles). On the other hand, after a single subcutaneous injection of NTG (0.1 mg NTG/kg body wt), the mEPPs frequency remained high only for the first day, whereas 48 h after the frequency, values of spontaneous events returned to normal values (%change: 16.80 ± 4.55, $P = 0.6032$, 68° of freedom, $n = 30$ synapses from 2 treated muscles, $n = 66$ synapses from 7 control animals). It is important to note that a low dose of NTG (0.01 mg NTG/kg body weight, Fig. 2B, black bars) was completely ineffective in this regard.

Because NTG is a reversible anticholinesterasic drug (ki = 1.02 mM) used for ~40 yr, we also tried the selective and irreversible inhibitor of the acetylcholinesterase fasciculin II (FII; 350 nM; ki = 0.33mM), which is more potent than NTG. Ex vivo experiments were performed in LAL muscle, and mEPP frequency did not change (%change: 11.42 ± 8.32, $P = 0.5369$, 88°of freedom, $n = 45$ synapses from 3 muscles), but the mEPP amplitude was strongly increased (%change: 201.29 ± 10.96; $P = 0.0001$, 100° of freedom, $n = 45$ synapses from 3 muscles). These results showed that FII is an anticholinesterase agent that induced a lower increase in the frequency of mEPPs than NTG.

Moreover, some experiments were made in toto with pyridostigmine (PTG; 0.1 mg PTG/kg body wet). Pyridostigmine is an anticholinesterasic from the same family as NTG but had the lowest carbamylation rate of the studied substances. We found that pyridostigmine had no effect on the frequency of mEPPs (%change: 4.31 ± 0.70, $P = 0.6758$, 44°of freedom, $n = 66$ synapses from 3 treated muscles, $n = 66$ synapses from 7 control animals). To study the contribution of a central effect of anticholinesterase drugs, we also performed some experiments with rivastigmine (RTG; 1 mg RTG/kg body wt). Rivastigmine is also an anticholinesterasic agent from the family of NTG that readily enters the central nervous system, but its effects on peripheral tissue or muscles are considered negligible. We observed that RTG had no effect on the frequency of mEPPs (0.1mg RTG/kg body wt; %change: 21.38 ± 4.84; $P = 0.3748$, 89° of freedom, $n = 66$ synapses from 3 treated muscles, $n = 66$ synapses from 7 control animals).

In summary, the subcutaneous treatment with the anticholinesterasic neostigmine at a single dose of 0.1 mg/kg was the most effective in terms of increasing the frequency of mEPPs, and the optimal results were obtained in LAL muscle.

Morphological Study

Methylene blue. We were interested in a long-term effect of NTG; however, this drug is a reversible anticholinesterasic inhibitor. For this reason, we first planned experiments for repeated dosing every 12 h (0.1 mg NTG/kg body wt). Strikingly, the repeated treatment resulted in muscle damage within the first 24 h, whereas a single subcutaneous dose of NTG did not produce any muscular damage, and we decided to use a single subcutaneous injection of NTG (Fig. 3).

Periodic acid Schiff-Alcian technique. The fixers (for example, formaldehyde or paraformaldehyde) produced a hypercontraction of the muscle fibers, and the contraction knots appeared blurred. Accordingly, this technique was performed without fixation, with the additional advantage that the cell membranes were preserved, and only extracellular glycosaminoglycans (GAGs) could be seen.

After 30 min of NTG subcutaneous injection (0.1 mg NTG/kg body wt) and working at low magnification, intramus-

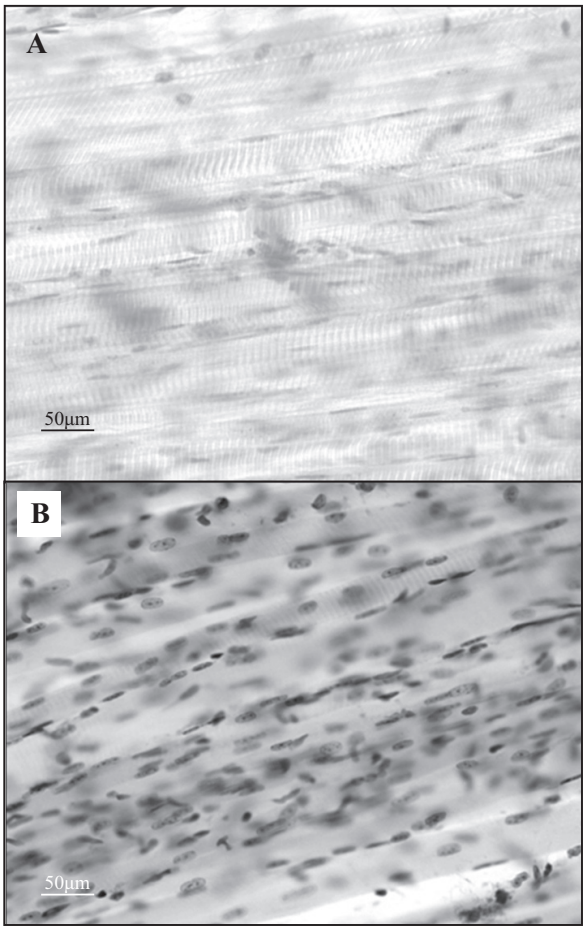

Fig. 3. Muscle injury from repetitive treatment with neostigmine. *A*: levator auris longus (LAL) muscle 60 min after treatment with neostigmine (NTG). This muscle is completely normal in shape, and we can observe the usual sarcomeres without inflammatory cells. *B*: LAL 24 h after 2 injections of NTG administered every 12 h. The inflammatory reaction is observed as a large number of nuclei between muscle fibers. The sarcomeres are almost indistinguishable. In all cases, the dose of neostigmine was 0.1 mg/kg body wt.

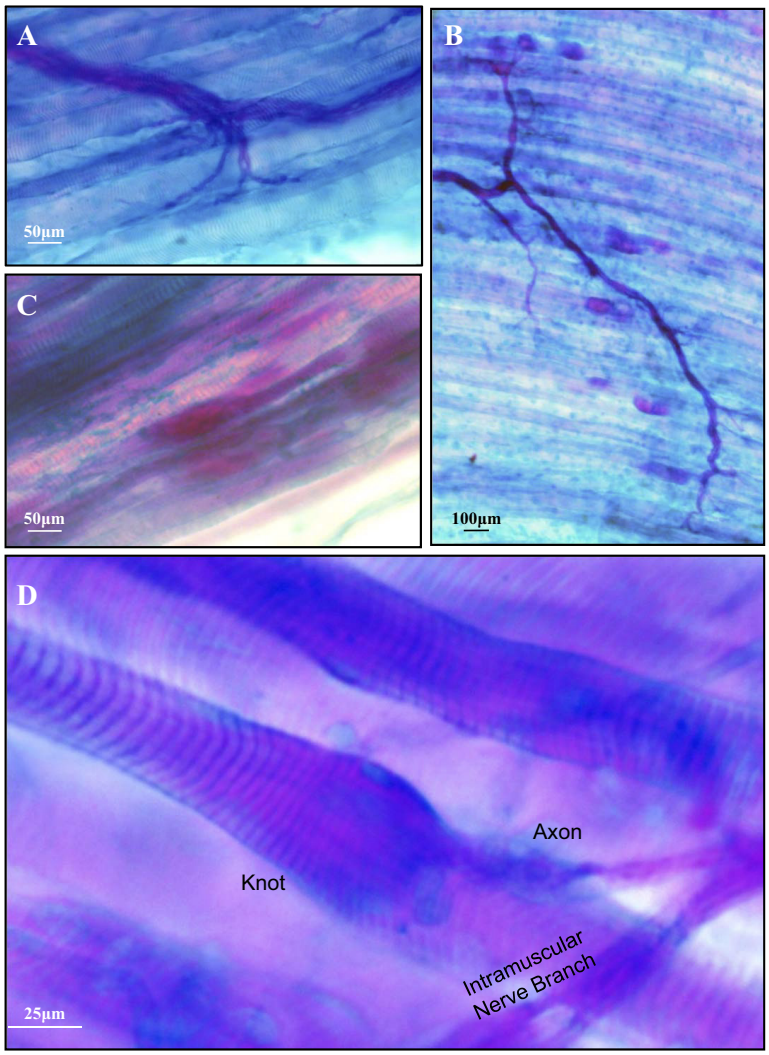

Fig. 4. Periodic acid Schiff (PAS)-Alcian technique. *A*: control muscle; note that there are no knots of contraction. *B*: PAS-positive knots of contraction surrounding an intramuscular nerve branch 30 min after a subcutaneous administration of neostigmine. *C*: 72 h after a subcutaneous injection of neostigmine, some knots of contraction are still present. *D*: details of a knot of contraction. A thickening of the muscle fiber is identified by more tight sarcomeres. The axon innervating the fiber is clearly identified. Pink color indicates the existence of neutral glycosaminoglycans (GAGs), and bluish color indicates the presence of acidic GAGs 30 min after a subcutaneous administration of neostigmine. All images have been obtained from the levator auris longus (LAL) muscle.

cular nerves surrounded by nodules as grapes could be identified (see Fig. 4*A*). Using high magnification, a local thickening of the muscle fiber could be observed showing shortened sarcomeres; occasionally, it could even be seen how an axon reached a node (see Fig. 4*B*). These nodular images are compatible with those previously described as contraction knots by Simons (29). Moreover, this large increase can be seen with some pink coloration indicating the presence of neutral GAGs and a bluish color indicating the presence of acid GAGs. After a single subcutaneous injection of NTG, the frequency of contraction knots decreases progressively within 24 to 48 h. At 3 days posttreatment, the contraction knots are scarce (see Fig. 4*C*), and 4 days after treatment the contraction knots have completely disappeared.

Histochemical techniques. Although in Fig. 4*B* we can see an axon reaching the contraction knot, it is necessary to demonstrate a direct relationship between contraction knots and neuromuscular synapses.

The rhodaminated α-bungarotoxin is a toxin that labels acetylcholine receptors (AChR). The AChR are in the postsynaptic component on the muscle fiber membrane. This technique shows an image of the postsynaptic component under the axon. As shown in the control (Fig. 5*A*) between axonal branches, there are spaces (arrowheads), whereas these spaces disappear in NTG images (Fig. 5*B*, arrowheads). NTG causes the knot of contraction immediately below the postsynaptic membrane, and thus the image shown by the technique of the rhodaminated α-bungarotoxin is more compact without spaces (Fig. 5*B*, arrowheads). Initially, we performed a simple count according to the morphology, and it was found that in controls 10% of the muscles showed a contracted morphology at synaptic contacts (11.65% ± 3.22, $n = 412$ control synapses evaluated). However, 30 min after NTG treatment, 30% of the muscles displayed a contracted morphology at synaptic contacts (27.10 ± 6.94%; $n = 676$ treated muscle synapses evaluated; treated synapse values with respect to control synapse values: $P = 0.0446$, 834°of freedom). It is important to relate the contraction knots to the neuromuscular synapses. However, the visual simple count is a subjective parameter and might show variations, depending on the experimenter. For this

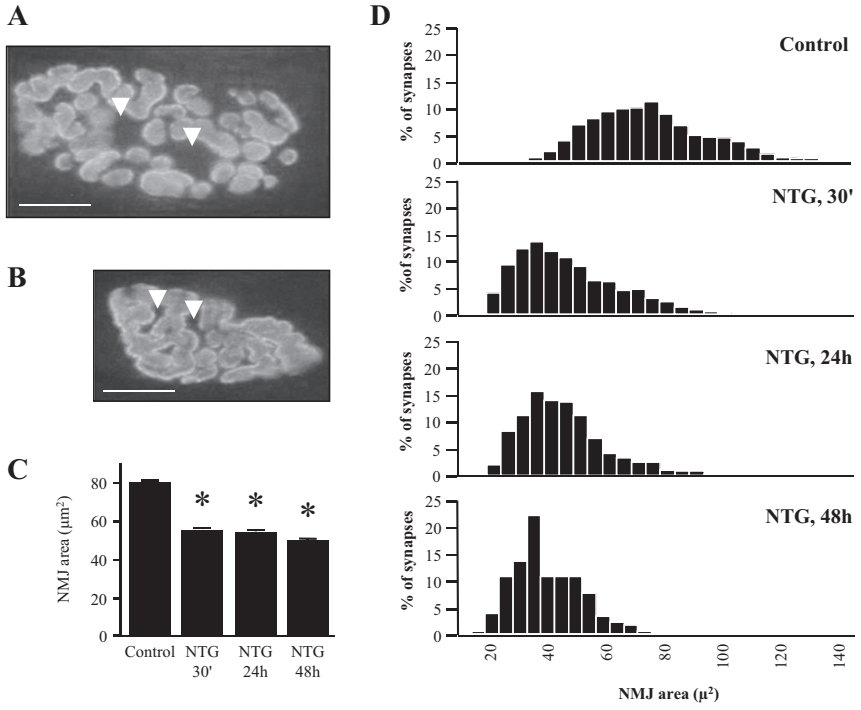

Fig. 5. Postsynaptic study. *A*: example of a normal synaptic contact (scale bar, 5 μm). *B*: example illustrating contractured synapses; because knots of contraction are under the synaptic areas of each muscular fiber, the shapes of synapses are also contractured (scale bar, 5 μm). Note how the mark-free spaces are lower in *B* (arrowheads). *C*: histogram shows the results of morphometric analyses of postsynaptic areas (*$P < 0.05$, treated values with respect to control values; no. of control synapses analyzed = 444; no. of synapses analyzed after 30 min = 780, $P = 0.0001$, 946° of freedom; no. of synapses analyzed after 24 h = 477, $P = 0.0002$, 687° of freedom; no. of synapses analyzed after 48 h = 182, $P = 0.0001$, 542° of freedom). Note that there is a significant reduction in the treated postsynaptic area. *D*: distribution of synaptic areas from control and treated animals with neostigmine (NTG). Note that overall the curves from treated muscles shift to the left, indicating reduced synaptic areas. No. of synapses analyzed: control, 444; after 30 min, 780; after 24 h, 477; after 48 h, 182. All the images have been obtained in the levator auris longus (LAL) muscle.

reason, a morphometric study of synaptic areas was conducted, and a significant reduction in the synaptic area of muscles treated with NTG was found (Fig. 5*C*). These results were processed to study the distribution of frequencies of the synaptic areas measured, and the typically Gaussian curves were obtained (Fig. 5*D*). The curve shifts to the left in muscles treated with neostigmine. Thus, synaptic contacts after being exposed to NTG are globally contracted, confirming that contraction knots are in the subsynaptic area.

Endplate noise recordings. The electromyographic record (EMG) from a neuromuscular junction is called "endplate noise." The EMG representation of endplate noise is an increase in the anfractuosity of the baseline (it is more circuitous and rough than habitual). Endplate noise corresponds to the spontaneous release of ACh in several muscular fibers (see Fig. 6*A*) (7, 16, 29). In our experiments, the frequency of mEPPs generated by a neuromuscular junction is on average one event per second. However, endplate noise is ~50–80 events per second. This means that the monopolar EMG needle is registering ~50–80 different synapses.

To illustrate this, Fig. 6*B* shows three records: no noise, low level of noise (few events), and a high level of noise (many events). The number of areas with endplate noise increases immediately after treatment with NTG and continues to increase for 24 h (Fig. 6*C*). Event frequency does not significantly increase immediately in treated or control animals. After 3 h, the treatment group shows increases in event frequency (Fig. 6*D*).

Muscular ultrasonography. The mouse is an extremely small animal to perform muscular ultrasound or find palpable MTrPs or palpable taut bands. For this reason, we performed some experiments in rats with subcutaneous NTG to confirm that MTrPs signs appeared. As we had previously observed in mice, 30 min after the injection of NTG, contraction knots could be seen by periodic acid Schiff (PAS)-Alcian technique, the frequency of mEPPs (%change: 212.35 ± 28.55, $P = 0.0001$, 88° of freedom, $n = 45$ synapses from 3 LAL-treated muscles, 3 rats), and the number of areas with endplate noise increased (%change: 150.87 ± 5.37, $P = 0.0329$, 2° of freedom, 3 gastrocnemius treated from 3 rats). The number of areas with endplate noise continued to increase for 24 h (%change: 180.33 ± 8.75, $P = 0.0489$, 2° of freedom, 3 gastrocnemius treated from 3 rats).

Thirty minutes after the injection of NTG, taut bands could be palpated in gastrocnemius. Five expert physiotherapists identified taut bands in six rats on 3 different days (2 rats each day) at both 30 min and 24 h after NTG treatment. Taut bands were palpated in all treated gastrocnemius. Nevertheless, the nodular characteristic often felt when palpating human MTrPs could never be felt by palpation in this model. To manipulate the rats without risk to physiotherapists or stress to the animals, the rats were sedated with a low dose of TBE. Unfortunately, this procedure prevented us from knowing whether the animals experienced pain.

After the taut band was identified, dry needling was performed with multiple rapid insertions of a solid filament needle, and palpable local twitch responses were elicited in the right gastrocnemius. These local twitch responses could not be seen but could be identified by palpation. To get more objective evidence of these twitches, the dry needling procedure was applied with diagnostic ultrasound monitoring, and several local twitch responses were shown only in treated animals (Supplemental Videos S1 and S2; Supplemental Material for this article can be found on the *Journal of Applied Physiology* website). Moreover, before treatment with NTG, no taut band was palpated, and no local twitch response was elicited with

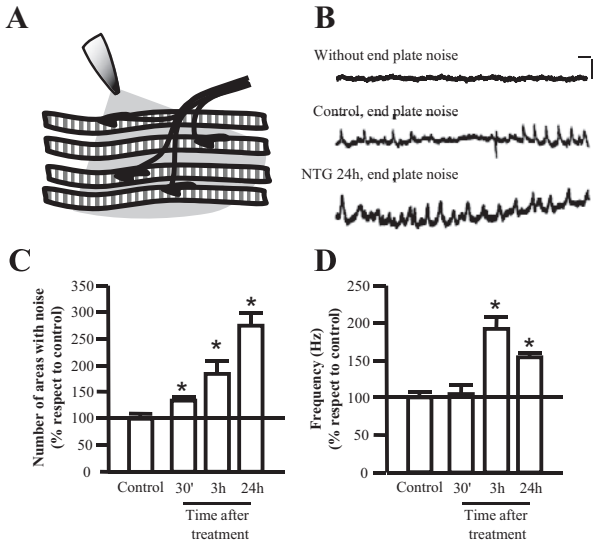

Fig. 6. Endplate noise. *A*: electromyographic recording scheme. The recording electrode reaches the vicinity of an area of neuromuscular synapses (black). A concentric needle covers a recorded field (gray) from several synapses. *B*: examples of electromyographic recordings. Note a significant increase in the no. of events in the gastrocnemius treated with neostigmine (NTG; 3 records show a lapse of time of 100 ms). Vertical bar, 50 μV; horizontal bar, 25 ms. *C*: no. of areas with endplate noise in gastrocnemius; in treated animals the no. of areas with endplate noise increased compared with their respective controls from the first 30 min to 24 h. Thirty minutes after treatment compared with control values, $P = 0.0001$, 16° of freedom, $n = 12$ animals; Three hours after treatment compared with control values, $P = 0.0011$, 13° of freedom, $n = 12$ animals; 24 h after treatment compared with control values, $P = 0.0001$, 12° of freedom, $n = 12$ animals. *D*: each area with endplate noise was analyzed by quantifying the no. of events/s (Hz). Thirty minutes after treatment with NTG, new areas with endplate noise appear, but these areas initially do not show an increase in the frequency of events. The increase in the frequency of events appears 3 h later. Thirty minutes after treatment compared with control values, $P = 0.7143$, 11° of freedom, $n = 12$ animals; Three hours after treatment compared with control values, $P = 0.0001$, 11° of freedom, $n = 12$ animals; 24 h after treatment compared with control values, $P = 0.0001$, 14° of freedom, $n = 12$ animals. *$P < 0.05$, experimental values compared with control values.

dry needling in left gastrocnemius. In the same animals, 30 min after NTG treatment, taut bands could be palpated, and multiple local twitch responses could be elicited by dry needling in the right limbs.

DISCUSSION

Our results show that a sustained increase of ACh in the synaptic cleft induces contraction knots in the subsynaptic area as well as an increase of the endplate noise and mEPPs frequency. These contraction knots are surrounded by abundant glycosaminoglycans (GAGs). Presumably, in human muscles, both the whole of the contraction knots together with accumulated glycosaminoglycans could contribute to the nodular appearance often felt when myofascial trigger points (MTrPs) are palpated. Unfortunately, in our animal model we could not feel such a nodular characteristic in the palpated taut bands, probably because of the small size of muscles and the limitations of palpation in them.

The integrated hypothesis is one of the most widely accepted pathophysiological explanations of myofascial pain syndrome (MPS) (10, 29). According to this hypothesis, an excessive secretion of ACh initiates a cascade of events leading to the formation of contraction knots. Thus, artificially increasing ACh in the synaptic cleft, the contraction knots should be obtained in animals. To increase the ACh in the synaptic cleft, we tried several anticholinesterasics. The best results were obtained with a single subcutaneous injection of NTG administered at doses commonly used with therapeutic purposes in humans (3, 5, 15). We also explored two types of muscles: a slow-twitch muscle (diaphragm) and a fast-twitch muscle [levator auris longus (LAL)]. After treatment with neostigmine, spontaneous ACh release is more important in LAL muscle than in the diaphragm muscle. It is possible that neostigmine is more effective in phasic muscles such as LAL than in tonics such as the diaphragm.

In recent years, several authors have been looking for an image of contraction knots (13, 14, 21). Mense et al. (21) experimentally increased the ACh in the synaptic cleft using a well-known ACh inhibitor. The anticholinesterase agent employed is also a fluorochrome [diisopropylfluorophosphate (DFP)], which allows the morphological study of the neuromuscular junctions at the same time. However, no subsynaptic contraction knots were observed. These authors, suspecting that the chosen anticholinesterase agent was not enough, decided to impose additional neurotransmission by electrical stimulation of the muscles treated with DFP. This combination of an anticholinesterase agent and electrical stimulation caused undesirable muscle damage. In our model, we were able to induce subsynaptic contraction knots by the single administration of the potent anticholinesterase agent neostigmine. Moreover, the thin and flat LAL muscle that we used did not require further manipulations to apply microscopy and electrophysiological techniques (1).

Huang and coworkers (13, 14), looking for an image of contraction knots, created a rat model of myofascial trigger points (MTrPs) by crushing of the vastus medialis muscle of the hindlimb through repeated contusions. The electrophysiological recordings showed high frequency of endplate noise and polyphasic spikes. However, the histological study of their model did not show contraction knots but indirect signs of MTrPs as large muscle fibers with circular or elliptical cross-sectional shapes. By contrast, the animal model described in the present paper shows both true images of contraction knots and endplate noise recordings.

When AChE is inhibited, ACh is not hydrolyzed, and therefore, it accumulates in the synaptic cleft. An increase of ACh in the synaptic cleft causes an increase in the secretion of more ACh (23, 24). Briefly, ACh secreted by the axon activates synaptic metabotropic autoreceptors in the axonal membrane. These receptors are types 1 and 2 muscarinic receptors (mAChR). The synchronous activation of the two mAChR enhances the release of ACh via activation of protein kinases C and A (23). Moreover, during neurotransmission, adenosine compounds are also released. We have described that these adenosine compounds are also involved in modulating the release of ACh (24). Thus, using NTG will increase the mEPPs frequency by several mechanisms.

Intracellular recording of mEPPs is not directly associated with any disease. However, using electromyography in patients with MPS, it is relatively easy to find endplate noise (see for example, Ref. 8), which is classically referred to as mEPP (18).

Endplate noise recording seems pathognomonic of MPS (8). It seems clear that the endplate noise arises largely from the mEPPs, and probably several synapses are involved (8, 17, 18, 31). For this reason, it is important to assess the frequency of endplate noise events. In our experiments, the mEPP frequency increases during the 1st day, as the areas and the frequency of endplate noise events are also increased. Because endplate noise is the electromyographic expression of the spontaneous neurotransmission, it seems reasonable that both endplate noise and mEPP frequency are increased.

The analysis of the electron microscopy images of contraction knots obtained with NTG at therapeutic doses (3, 5, 15) shows that there is always an abnormally enlarged space between adjacent muscle fibers, and sometimes the presence of fibroblasts is evident. The diameter increase of the muscle fiber at the contraction knot is ~1 μm. Therefore, thousands of muscle fibers with localized contraction knots would be needed to achieve a palpable MTrP in humans. When a MTrP is palpated into a muscle, if it is associated with clinical evidence it is called active, and if it is not associated with clinical evidence, it is called latent. These observations suggest that in the MTrP areas, other factors could also contribute to the palpable nodule in MTrPs. Moreover, the immediate milieu surrounding active MTrP is characterized by a significant increase in the levels of protons, nociceptive, and proinflammatory substances compared with latent MTrPs and normal muscle tissue (25, 26). These substances may sensitize muscle nociceptors, causing muscle hyperalgesia. In turn, fibroblasts, insulted by the low pH and nociceptive substances, would synthesize GAGs (6), which are accumulated in the vicinity of the contraction knots. GAGs are highly hygroscopic and absorb and retain liquid, increasing the volume of the contraction knots zone, which could contribute to increase the size of the MTrP, making it more readily palpable. In our model, we demonstrated the presence of GAGs by PAS-Alcian staining. Although there have been no palpable differences in size, it has been shown by ultrasound imaging that the size of MTrPs is dependent on its degree of activity, with active MTrPs being larger than latent MTrPs (2). Moreover, GAGs can trap substances (see, for example, Ref. 21), such as the nociceptive and sensitizing substances discussed above. These phenomena associated with GAGs could partially explain the release effect commonly described when manual compression and massage techniques are applied (see, for example, Ref. 9) and could add to their mechanisms a hypothetical drainage effect of the MTrP area by squeezing the liquid from the GAGs, reducing its volume and removing nociceptive substances, thereby decreasing pain. Thus, the presence of GAGs in our model brings new insights into the pathophysiology of the MTrP and provides some reasonable arguments for the beneficial therapeutic effects often observed after manual treatments.

One of the most commonly employed techniquea in the treatment of MTrPs is dry needling (DN) (4). DN consists of the use of the mechanical stimuli of a needle to either eliminate or inactivate the MTrP for which the elicitation of local twitch responses has proven to be therapeutically useful (11). The local twitch response is pathognomonic of myofascial trigger point (29). In our experiments with rats, we have found both taut bands, which are identified by several expert examiners, and local twitch responses, which are clearly documented by diagnostic ultrasound imaging.

After a single subcutaneous injection of NTG, contraction knots can be seen only from 30 min to 48 h. At 3 days posttreatment, contraction knots are already scarce, and at 4 days they cannot be further observed. Forty-eight hours after the neostigmine injection, mEPP frequency returns to control values. This means that contraction knots persist for some hours after spontaneous neurotransmission is already normalized.

Several factors seem to be implicated in the onset of MPS. For instance, MTrPs are thought to be formed in response to overload, such as different types of forced or maintained contractions (7). Other mechanisms of increased or altered muscle demands include prolonged muscle contraction, such as postural inadequacies in the workplace, proximal nerve compression and the resultant muscle spasm, and posttrauma (29). Moreover, MTrPs are more common in conditions of psychological stress (20). These stress-related triggers are also factors that contribute to the perpetuation of MPS. It is generally accepted that in the absence of perpetuating factors, MTrPs could disappear without treatment when no muscle overload is imposed (29). This might be the reason why the contraction knots induced by NTG injection could disappear in ~3 days. Further research is needed to test the hypothesis that contraction knots could last longer if some kind of perpetuating factor was applied to the muscle harboring them.

Limitations of this model are related mainly to the small size of rats and mice that prevents the palpation of the MTrPs. In this model, only the taut band can be palpated in rats. Moreover, the duration of MPs in humans lasts for more than 1 wk; however, our animal model lasts only 3 days, which can be related to the absence of perpetuating factors.

In conclusion, the animal model of MTrPs created by a single subcutaneous injection of neostigmine includes morphological characteristics (contraction knots) electrophysiological signs (mEPP frequency and endplate noise) as well as clinical signs (taut bands and local twitch response elicitation by needling) of the MTrPs. Moreover, we showed for the first time the accumulation of extracellular GAGs, which could contribute to the pathophysiology and the clinical signs of this entity.

ACKNOWLEDGMENTS

We greatly appreciate the suggestions of Dr. M. T. Colomina in the draft of this article. We thank PRIM Fisioterapia for supplying the diagnostic ultrasound device used in this study.

GRANTS

This work is funded by a grant from Instituto de Salud Carlos III, Acción Estratégica en Salud (MMS, PI13/02084), and from the Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the Generalitat de Catalunya (MB, 2016 FI-B 00909).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.M., M.S., M.B., C.R., S.M., M.P., R.G.-P., and N.O. performed experiments; R.M., M.B., C.R., S.M., M.P., R.G.-P., N.O., J.T., and M.M.S. interpreted results of experiments; R.M., M.S., M.B., C.R., O.M., S.M., M.P., R.G.-P., N.O., J.T., and M.M.S. approved final version of manuscript; M.S., M.B., C.R., S.M., M.P., N.O., and J.T. analyzed data; M.S. and M.M.S. prepared figures; O.M. and M.M.S. drafted manuscript; O.M. and M.M.S. edited and revised manuscript.

REFERENCES

1. **Angaut-Petit D, Molgo J, Connold AL, Faille L.** The levator auris longus muscle of the mouse: a convenient preparation for studies of short- and long-term presynaptic effects of drugs or toxins. *Neurosci Lett* 82: 83–88, 1987. doi:10.1016/0304-3940(87)90175-3.
2. **Ballyns JJ, Shah JP, Hammond J, Gebreab T, Gerber LH, Sikdar S.** Objective sonographic measures for characterizing myofascial trigger points associated with cervical pain. *J Ultrasound Med* 30: 1331–1340, 2011. doi:10.7863/jum.2011.30.10.1331.
3. **Blackman JG, Hopkins WG, Milne RJ, Peterson DW.** Supercontracture at endplates of mammalian muscle fibers caused by decamethonium and related agonists. *Proc Univ Otago Med Sch* 56: 71–72, 1978.
4. **Dommerholt J, Grieve R, Hooks T, Layton M.** A critical overview of the current myofascial pain literature - October 2015. *J Bodyw Mov Ther* 19: 736–746, 2015. doi:10.1016/j.jbmt.2015.09.003.
5. **Duxson MJ, Vrbová G.** Inhibition of acetylcholinesterase accelerates axon terminal withdrawal at the developing rat neuromuscular junction. *J Neurocytol* 14: 337–363, 1985. doi:10.1007/BF01217751.
6. **Fraser JR, Laurent TC, Laurent UB.** Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med* 242: 27–33, 1997. doi:10.1046/j.1365-2796.1997.00170.x.
7. **Gerwin RD, Dommerholt J, Shah JP.** An expansion of Simons' integrated hypothesis of trigger point formation. *Curr Pain Headache Rep* 8: 468–475, 2004. doi:10.1007/s11916-004-0069-x.
8. **Gerwin RD, Shannon S, Hong CZ, Hubbard D, Gevirtz R.** Interrater reliability in myofascial trigger point examination. *Pain* 69: 65–73, 1997. doi:10.1016/S0304-3959(96)03248-4.
9. **Hanten WP, Olson SL, Butts NL, Nowicki AL.** Effectiveness of a home program of ischemic pressure followed by sustained stretch for treatment of myofascial trigger points. *Phys Ther* 80: 997–1003, 2000.
10. **Harden RN, Bruehl SP, Gass S, Niemiec C, Barbick B.** Signs and symptoms of the myofascial pain syndrome: a national survey of pain management providers. *Clin J Pain* 16: 64–72, 2000. doi:10.1097/00002508-200003000-00010.
11. **Hong CZ.** Lidocaine injection versus dry needling to myofascial trigger point. The importance of the local twitch response. *Am J Phys Med Rehabil* 73: 256–263, 1994. doi:10.1097/00002060-199407000-00006.
12. **Hong CZ, Simons DG.** Pathophysiologic and electrophysiologic mechanisms of myofascial trigger points. *Arch Phys Med Rehabil* 79: 863–872, 1998. doi:10.1016/S0003-9993(98)90371-9.
13. **Huang QM, Lv JJ, Ruanshi QM, Liu L.** Spontaneous electrical activities at myofascial trigger points at different stages of recovery from injury in a rat model. *Acupunct Med* 33: 319–324, 2015. [Erratum in *Acupunct Med* 33: 434, 2015.] doi:10.1136/acupmed-2014-010666.
14. **Huang QM, Ye G, Zhao ZY, Lv JJ, Tang L.** Myoelectrical activity and muscle morphology in a rat model of myofascial trigger points induced by blunt trauma to the vastus medialis. *Acupunct Med* 31: 65–73, 2013. doi:10.1136/acupmed-2012-010129.
15. **Hudson CS, Rash JE, Tiedt TN, Albuquerque EX.** Neostigmine-induced alterations at the mammalian neuromuscular junction. II. Ultrastructure. *J Pharmacol Exp Ther* 205: 340–356, 1978.
16. **Jafri MS.** Mechanisms of Myofascial Pain. *Int Sch Res Notices* 2014: pii: 523924, 2014. doi:10.1155/2014/523924.
17. **Kimura J.** Electrodiagnosis in diseases of nerve and muscle: principles and practice. In: *Techniques to Assess Muscle Function*, edited by Kimura J. Oxford, UK: Oxford University Press, 2001.
18. **Liley AW.** An investigation of spontaneous activity at the neuromuscular junction of the rat. *J Physiol* 132: 650–666, 1956. doi:10.1113/jphysiol.1956.sp005555.
19. **McLachlan EM, Martin AR.** Non-linear summation of end-plate potentials in the frog and mouse. *J Physiol* 311: 307–324, 1981. doi:10.1113/jphysiol.1981.sp013586.
20. **McNulty WH, Gevirtz RN, Hubbard DR, Berkoff GM.** Needle electromyographic evaluation of trigger point response to a psychological stressor. *Psychophysiology* 31: 313–316, 1994. doi:10.1111/j.1469-8986.1994.tb02220.x.
21. **Mense S, Simons DG, Hoheisel U, Quenzer B.** Lesions of rat skeletal muscle after local block of acetylcholinesterase and neuromuscular stimulation. *J Appl Physiol (1985)* 94: 2494–2501, 2003. doi:10.1152/japplphysiol.00727.2002.
23. **Santafé MM, Lanuza MA, Garcia N, Tomàs J.** Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. *Eur J Neurosci* 23: 2048–2056, 2006. doi:10.1111/j.1460-9568.2006.04753.x.
24. **Santafe MM, Priego M, Obis T, Garcia N, Tomàs M, Lanuza MA, Tomàs J.** Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. *Eur J Neurosci* 42: 1775–1787, 2015. doi:10.1111/ejn.12922.
25. **Shah JP, Gilliams EA.** Uncovering the biochemical milieu of myofascial trigger points using in vivo microdialysis: an application of muscle pain concepts to myofascial pain syndrome. *J Bodyw Mov Ther* 12: 371–384, 2008. doi:10.1016/j.jbmt.2008.06.006.
26. **Shah JP, Phillips TM, Danoff JV, Gerber LH.** An in vivo microanalytical technique for measuring the local biochemical milieu of human skeletal muscle. *J Appl Physiol (1985)* 99: 1977–1984, 2005. doi:10.1152/japplphysiol.00419.2005.
27. **Simons DG.** Review of enigmatic MTrPs as a common cause of enigmatic musculoskeletal pain and dysfunction. *J Electromyogr Kinesiol* 14: 95–107, 2004. doi:10.1016/j.jelekin.2003.09.018.
28. **Simons DG, Stolov WC.** Microscopic features and transient contraction of palpable bands in canine muscle. *Am J Phys Med* 55: 65–88, 1976.
29. **Simons DG, Travell JG, Simons LS.** *Myofascial Pain and Dysfunction: The Trigger Point Manual*. Philadelphia, PA: Lippincott William & Wilkins, 1999.
30. **Turo D, Otto P, Shah JP, Heimur J, Gebreab T, Zaazhoa M, Armstrong K, Gerber LH, Sikdar S.** Ultrasonic characterization of the upper trapezius muscle in patients with chronic neck pain. *Ultrason Imaging* 35: 173–187, 2013. doi:10.1177/0161734612472408.
31. **Wiederholt WC.** "End-plate noise" in electromyography. *Neurology* 20: 214–224, 1970. doi:10.1212/WNL.20.3.214.