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Research report

Stress increases the spontaneous release of ACh and may be involved in the generation and maintenance of myofascial trigger points in mouse

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ABSTRACT

An increase in spontaneous neurotransmission may be related to myofascial pain. Sympathetic neurons innervate most of the neuromuscular junction sand are involved in the modulation of synaptic transmission. Therefore, a direct action of stress on acetylcholine release is expected. For this reason, this study aims to evaluate the relationship between stress and spontaneous neurotransmission. Five acute stressors (immobilization, forced swimming, food and water deprivation, social isolation and ultrasound) were tested in 6 weeks adult Swiss male mice. Subsequently, these types of stress were combined to generate a model of chronic stress. The study of ACh release was evaluated before and after the application of stress by intracellular recording of spontaneous neurotransmission (mEPPs). In each one of the stressors, an increase in the frequency of mEPPs was obtained immediately after treatment, which remained elevated for 5 days and thereafter returned to control values after a week. With chronic stress, a much higher increase in the frequency of mEPPs was obtained and it was maintained for 15 days. In summary, stress, both in its acute and chronic forms, increased spontaneous neurotransmission significantly. There is a possibility that chronic stress is related with the genesis or maintenance of myofascial pain.

1. Introduction

Stress is a physiological response to any alteration in the environment and one of the most common underlying causes of ailments in our society. Regardless of the causes, it can occur in an acute or chronic form [1]. Although this physiological phenomenon is essential for the survival of the individual, it is also strongly related to various brain disorders including depression, anxiety, as well as post-traumatic stress disorder [2].

The autonomic nervous system is related to stress in both humans and animals. For example, the spontaneous muscle electrical activity in the muscles recorded by electromyography is affected by changes in autonomic activity induced by stressful situations [3,4]. Several studies have addressed this issue in muscles in vivo, ex vivo, in neuronal cultures or in brain slices. Furthermore, Shen and Colonese [5] recorded spontaneous electrical activity in the central nervous system of rats in vivo using a multielectrode array.

Some authors have demonstrated that spontaneous muscle electrical activity can be inhibited by injecting botulinum toxin, demonstrating its relationship with spontaneous ACh release [6]. Other authors like Khan et al. [7] have obtained images demonstrating sympathetic input at the neuromuscular junction, which may explain why sympathectomized muscles decreased the compound evoked potentials and also why the use of sympathomimetics increased them. The set of results obtained by Khan and collaborators demonstrated that the autonomic nervous system is crucial for the maintenance and functioning of the neuromuscular synapse. Later, Rodrigues et al. [8] demonstrated the involvement of sympathetic innervation in both evoked and spontaneous neurotransmission.

Because of the afore mentioned scientific evidence, a model of skeletal neuromuscular synapse that involves the necessary participation of sympathetic terminals has been proposed [9]. In other words, it is hypothesized that sympathetic modulation generates a cascade of cellular and molecular events that affect neuromuscular neurotransmission. This phenomenon could be related with the pathophysiology of myofascial pain, which is one of the most prevalent forms of muscle pain i today's society. Its clinical picture is associated with tense bands in the affected muscle that contain palpable and pressuresensitive points known as myofascial trigger points (MTrPs) [10]. At the histological level, in the muscle fiber, they appear in the form of a contraction knot in

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the area close to a synaptic contact [11–13].

A MTrP contains several of these contraction nodes, having a characteristic electromyographic signal called plaque noise (a form of spontaneous electrical activity) that can be obtained in these muscle areas [14]. Moreover, spontaneous electrical activity has been associated with chronic muscle and myofascial pain in both horses [15] and human [16]. End plate noise has been associated with spontaneous ACh release for decades [17] and it has been suggested that it comes from a functionally altered motor end plate.

Currently, the most plausible pathophysiological theory about the generation of MTrPs involves an increase in the spontaneous release of ACh [10]. More recently, a strong increase in spontaneous ACh release was obtained in an animal model of MTrP [11]. According to this theory there are various factors that may be responsible for the activation of a MTrP. Some factors are direct, such as muscle function overload, while others are indirect, such as visceral or joint pathologies. Stress is included in this second group. In this work, the spontaneous ACh release is evaluated under various stressful situations and its temporary permanence.

2. Materials and methods

The mice were cared for in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, following the European Community's Council Directive (2010/63/EU) and the Spanish Royal Decree 53/2013 for the humane treatment of laboratory animals. The Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 11337) reviewed and approved all experiments on animals. The experiments were performed on young adult Swiss male mice (6 weeks, Charles River, L'Arbresle, France): 170 animals to apply stressful procedures and 104 control animals for each procedure. Mice were habituated to the facility for at least 1 week prior to studies and were housed in groups of four, with sawdust bedding and ad libitum access to water and food throughout. The animals' room temperature was maintained at 22 ± 2 °C, a relative humidity of 50 ± 10%, and a 12 h light/dark automatic light cycle.

2.1. Electrophysiology: intracellular recordings

Animals were deeply anesthetized with isoflurane before being euthanized by exsanguination. The *levator auris longus* was excised and dissected on a Sylgard-coated Petri dish containing (in mM) – NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 15, glucose 11 – and bubbled continuously with 95% O2, 5% CO2, which flowed into the Petri dish to superfuse the muscle preparation. The overflow was evacuated by suction. The solution was not bubbled directly in the Petri dish in order to minimize vibration during electrophysiological recording. Temperature and humidity were set to 26 °C and 50%, respectively. The bath temperature was monitored during the experiments (23.4 ºC ± 1.7, Digital Thermometer TMP 812, Letica, Barcelona, Spain). Intracellular recordings (miniature end plate potentials, MEPPs) were performed with conventional glass microelectrodes filled with 3 M KCl (resistance: 20–40 MΩ). Recording electrodes were connected to an amplifier (Tecktronics, AMS02, Oregon, USA), and 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. The signals were digitized (DIGIDATA 1322 A Interface, Axon Instruments Inc., Weatherford, TX, USA), stored and computer analyzed. The software Axoscope 10.0 (Axon Instruments Inc.) was used for data acquisition and analysis.

The mean amplitude (mV) per fiber was calculated and corrected for nonlinear summation [18] assuming a membrane potential of – 80 mV. The muscle fiber recording was discarded when the resting potential was less than − 45 mV. We studied about 15 fibers per muscle and for each type of experiment usually 3 muscles from control animals and 5 muscles from stressed mice. The MEPP frequency was recorded for 100 s from several neuromuscular junctions and the values were averaged. The experimental unit is recording from the muscle fiber/-neuromuscular junction.

2.2. Stress procedures

The experimental design includes a series of control animals for each procedure and period.

1. Ultrasounds. The ultrasonic device used was Weitech, (Wavre, Belgium). Mice were exposed to 50 dB ultrasound at a frequency of 20–25 kHz. To avoid habituation of the mice to ultrasound, these were applied randomly in a rotation of approximately every 15–30 s. The animals were exposed in 2 sets of experiments, one for 3 h and the other for 24 h. After exposure to ultrasound, electrophysiological recordings were taken immediately, after 24 h, 48 h, 5 days and 7 days.
2. Deprivation of food and water for 24 h in a standard cage. The food and water were simply removed in the cage in which the animals usually lived. Electrophysiological recordings were taken immediately after 24 h of food and water deprivation. After the procedure, food and water were restored. Then, after 24 h, 48 h, 5 days and 7 days, electrophysiological recordings were made.
3. Immobilization. The stress was applied immobilizing the animal for one hour at a room temperature of 26ºC. A standard cage, designed and validated for this purpose, was used (10 cm long, 3 cm high and a diameter of 3.5 cm (Universal animal restrainer, Bel-Art H46400-0001, Sigma-Aldrich®, MERCK, Darmstadt). Intracellular recordings were made immediately after the time of immobilization and at 5 days. Another experimental group applied 1 h of immobilization daily for 5 consecutive days The intracellular recordings were made on the fifth day.
4. Social isolation. The animals were placed in a metabolic cage (TECNIPLAST® cage: 320 cm2 surface and 14 cm high) for 24 h. One mouse per cage. Once the procedure was completed, the animals were returned to their usual cages. Intracellular recordings were made immediately, 24 h, 48 h, 5 days and 7 days after isolation.
5. Forced swim test (FST). This procedure consists of introducing the animal into a cylindrical tube with water at 21ºC approved for this purpose (40 cm high and 20 cm in diameter) and letting it swim for 6 min [19]. The forced swim test is an acute stressor that involves a psychological component and a motor stimulus such as swimming. The psychological component is activated basically with the imposition of swimming for a short period of time. Similarly, a cold-water temperature of 21ºC potentiates this stressor. Other psychological stressors related to the FST technique are movement restriction because of the limited space or through the repetitive application of cold. To rule out the action of cold water on spontaneous neurotransmission, habituation experiments were performed. The habituation to the aquatic environment was first carried out with a water temperature of 37ºC and with insufficient water to force the animal to swim. Thus, the first day the mouse was introduced into the tube, with little water (2–3 cm high) at a temperature of 37 °C for 6 min. In this way the mouse does not have to swim, but the environment and the water are simulated. After 24 h, the process is repeated, but with the water at 21ºC for 6 min. In addition, the mouse gets used to cold water at 21ºC without swimming. Once the habituation has been applied, after 24 h, the experiment is carried out: the tube is filled with water at 21ºC and the mouse is introduced for a total of 6 min. Electrophysiology was performed immediately after the procedure, at 24 h, 48 h, 5 days, and 7 days later. Some animals stopped swimming abruptly and floated. Those animals were discarded for the study.
6. Exposure to chronic stress. The stressors described above have been combined as follows (see Fig. 1): 24 h of ultrasound followed by another 24 h of rest under normal housing conditions, 24 h of food and water deprivation followed by another 24 h of rest, 1 h of

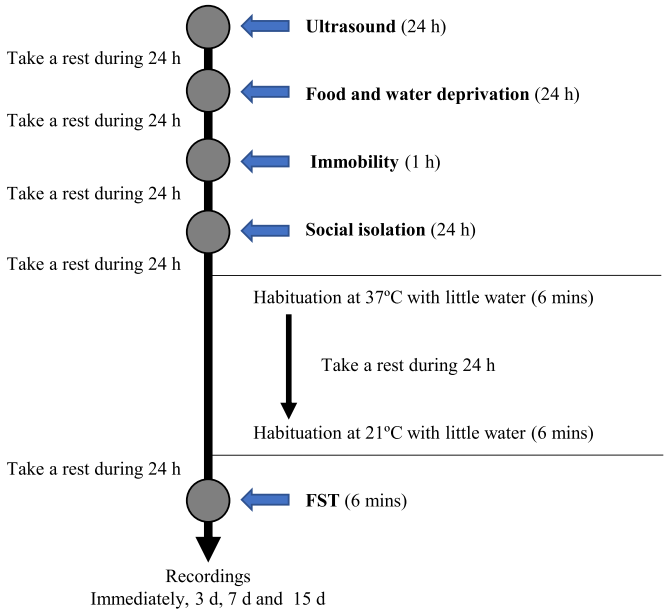

Fig. 1. Procedure for chronic stress. First, twenty-four hours of ultrasound followed by another twenty-four hours of rest under normal housing conditions. Second, twenty-four hours of food and water deprivation followed by another twenty-four hours of rest. Third, one hour of immobilization followed by twenty-four hours of rest under normal housing conditions. Fourth, twenty-four hours of social isolation followed by another day of rest in normal housing conditions. Fifth, six minutes of habituation to water at 37ºC followed by 24 h of rest under normal housing conditions. Six minutes of habituation to water at 21ºC followed by 24 h of rest under normal housing conditions. After FST of 6 min with water at 21ºC followed by 24 h of rest under normal housing conditions. Intracellular recordings were made immediately at the end of the chronic stress period and 3 days, 7 days and 15 days later.

immobilization followed by 24 h of rest under normal housing conditions, 24 h of social isolation followed by another 24 h of rest under normal housing conditions, 6 min of habituation to water at 37ºC followed by 24 h of rest under normal housing conditions, 6 min of habituation to water at 21ºC followed by 24 h of rest under normal housing conditions, 6 min FST with water at 21ºC followed by 24 h rest under normal housing conditions. Intracellular recordings were taken immediately at the end of the combined stress and 3 days, 7 days and 15 days later.

2.3. Statistical procedure

The values are expressed as "percentage of change". This is defined as: [experimental value / control value] X 100, Data were analyzed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as mean ± SEM, considering the 95% CI. Normality was assessed by Shapiro–Wilk test. We used the two tailed Welch's t-test for unpaired values because our variances were not equal. This test was chosen as it is more conservative than the ordinary t-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Ultrasounds

After 3 h of exposure to ultrasound, no changes were obtained in the frequency of mEPPs with respect to the control values (% variation with respect to control: 113.07 ± 5.90, N = 45 records from 3 control animals and N = 51 records from 5 stressed mice, P = 0.2973, Fig. 2). Neither at 24 h (% of variation with respect to control: 118.17 ± 5.28, N = 45 records from 3 control animals and N = 53 records from 5 stressed mice, P = 0.1418) nor at 48 h (% of variation with respect to control: 95.62 ± 1.28, N = 45 records from 3 control animals and N = 50 records from 5 stressed mice, P = 0.6899).

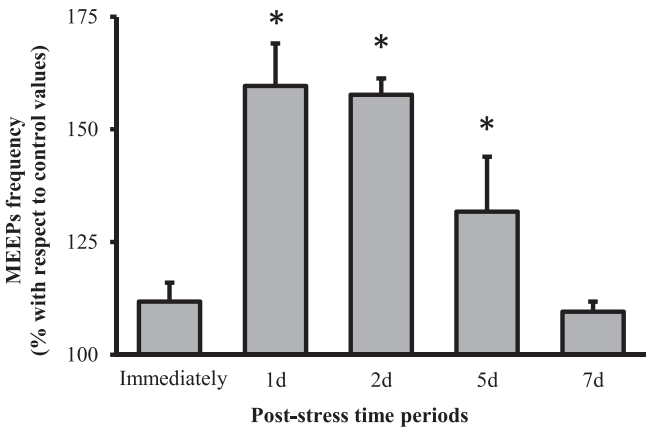

Fig. 2. Ultrasound exposure for 24 h. The records were made immediately (% variation with respect to control: 111.76 ± 4.25, N = 45 records from 3 control animals and N = 57 records from 5 stressed mice, P = 0.3276), 24 h (% variation with respect to control: 159.66 ± 9.39, N = 45 records from 3 control animals and N = 57 records from 5 stressed mice, P < 0.0001), 48 h (% variation with respect to control: 157.67 ± 3.65, N = 45 records from 3 control animals and N = 59 records from stressed mice, P < 0.0001), 5 days (% variation with respect to control: 131.72 ± 12.2, N = 45 records from 3 control animals and N = 61 records from 5 stressed mice, P < 0.0001) and 7 days (% variation with respect to control: 109.56 ± 2.21, N = 45 records from 3 control animals and N = 66 records from 5 stressed mice, P = 0, 3989) after applying ultrasound. Values above 100 indicate the increase in the frequency of mEPPs. Y-axis: percentage of experimental value with respect to the control. X-axis: time after treatment. *P < 0.05.

To achieve the stressful effect of ultrasound, the exposure time was increased to 24 h. Immediately after exposure to ultrasound, the frequency of MEPPs did not change either (11% variation, P = 0.3276). On the other hand, 24 h after the application of ultrasound, the frequency of mEPPs increased by 60% (P < 0.0001), which was maintained during the 48 h to decrease at 5 days post-exposure and achieve control values at 7 days (9% variation P = 0.3989).

The mean amplitude of the mEPPs did not vary significantly in any recorded period (the maximum% variation was: 88.03 ± 8.00, P > 0.05). There were no variations in the membrane potential either (the maximum% variation was: 97.98 ± 0.68, P > 0.05).

3.2. Deprivation of food and water for 24 h in a standard cage

The records were taken after fasting was imposed on the animals for 24 h (Fig. 3). The response in the frequency of the mEPPs was not immediate (9% variation with respect to control values, P = 0.5368), but it appeared after 24 h of the application of stress (54%, P = 0.0015). This increase remained high at 48 h (44%, P = 0.0014). At 5 days post treatment it was still elevated (25%, P = 0.0491). After one week, the frequency of appearance of mEPP returned to control values (6% variation, P = 0.6741).

On the other hand, the mean amplitude of the mEPPs remained unalterable in all the experiments to the control (% of maximum variation recorded was: 19.45 ± 2.30, P > 0.05). On the other hand, the membrane potential did not show significant variations in any of the post-treatment periods compared to the control (% of maximum variation recorded was: 10.18 ± 1.09, P > 0.05).

3.3. Immobilization

As shown in Fig. 4, after 1 h of immobilization, the mEPPS frequency increased by 25% (P = 0.027) immediately compared to the control

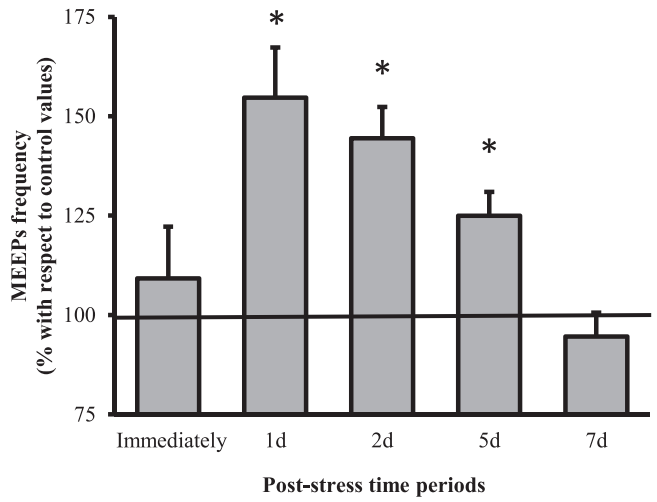

Fig. 3. Food and water deprivation for 24 h. Intracellular recordings were evaluated im-mediately (% variation with respect to control: 109.23 ± 12.98, N = 45 records from 3 control animals and N = 67 records from 5 stressed mice, P = 0.5368), 24 h (% variation with respect to control: 154.67 ± 12.6, N = 45 records from 3 control animals and N = 66 records from 5 stressed mice, P = 0.0015), 48 h (% variation with respect to control: 144.44 ± 7.91, N = 45 records from 3 control animals and N = 67 records from 5 stressed mice, P = 0.0014), 5 days (% variation with respect to control: 124.97 ± 6, N = 45 records from 3 control animals and N = 59 records from 5 stressed mice, P = 0.0491) and 7 days (% variation with respect to control: 94.62 ± 5.97, N = 45 records from 3 control animals and N = 51 records from 5 stressed mice, P = 0.6741) after the specific stress. Values above 100 indicate the increase in the frequency of mEPPs. Y-axis: percentage of experimental value with respect to the control. *P < 0.05.

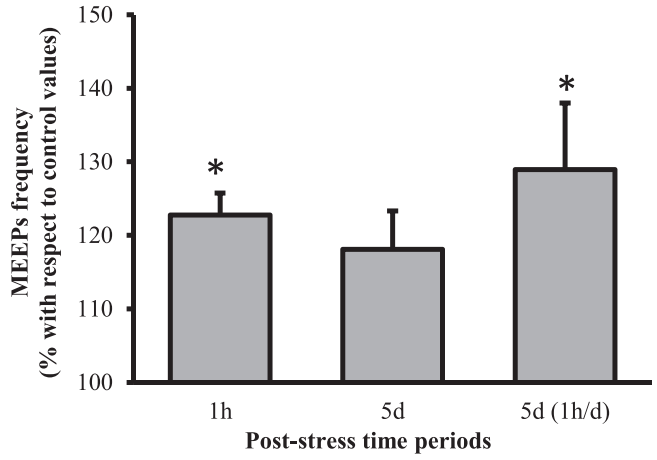

Fig. 4. Immobilization. Electrophysiological recordings were made at the first hour (% of variation with respect to control: 122.74 ± 3.00, N = 45 records from 3 control animals and N = 80 records from stressed 5 mice, P = 0.027) and at 5 days (% of variation with respect to control: 118.1 ± 5.2, N = 45 records from 3 control animals and N = 53 records from 5 stressed mice, P = 0.142) after immobilization for a single hour. An immobilization of 1 h was also carried out for consecutive days (5d 1 h / d) (% variation with respect to control: 128.94 ± 9.05, N = 45 records from 3 control animals and N = 64 records from stressed 5 mice, P = 0.045). Values greater than 100 indicate the increase produced. Y-axis: percentage of experimental value with respect to the control. * P < 0.05.

values. At 5 days post-treatment, the values had already normalized (P = 0.142).

Surprisingly when the immobilization of 1 h was repeated for 5 consecutive days, an increase in mEPPs was obtained similar to those obtained in a single exposure (28%, P = 0.045).

The amplitude of the mEPPS did not vary significantly at any time (% of maximum variation recorded was: 9.54 ± 1.64, P > 0.05). With respect to the membrane potential, something similar happened (% of maximum variation recorded was: 12.29 ± 4.04, P > 0.05).

3.4. Social isolation

Mice were socially isolated for a period of 24 h (Fig. 5). Immediately after the procedure, the frequency of appearance of mEPPs increased slightly but not significantly (20%, P = 0.0871). After 24 h, a significant increase was obtained (30%, P = 0.0136) that was maintained at 48 h (40%, P = 0.0154) until the fifth post-treatment day (26%, P = 0, 0218). One week after applying the procedure, the mEPPS frequency returned to the control values (93%, P = 0.6393). On the other hand, no changes were detected in the mean amplitude of the mEPPs in any of the periods studied (% of maximum variation recorded was: 14.96 ± 6.96, P > 0.05). Neither were changes in the membrane potential obtained in any of the evaluated post-treatment times (% of maximum variation recorded was: 3.51 ± 3.01%, P > 0.05).

3.5. Forced swim test (FST)

First, the animals were accustomed to space and contact with water by conducting experiments with little water at 37 °C. In the records obtained immediately afterwards, no changes were obtained in the frequency of mEPPs with respect to the control values (% variation: 96.79 ± 6.17, N = 45 records from 3 control animals and N = 62 records from 5 stressed mice, P = 0.793). The record was repeated at 24 h (% of variation with respect to control: 89.65 ± 5.40, N = 45 records from 3 control animals and N = 55 records from 5 stressed mice, P = 0.403) and 48 h (% of variation with respect to control: 95.20

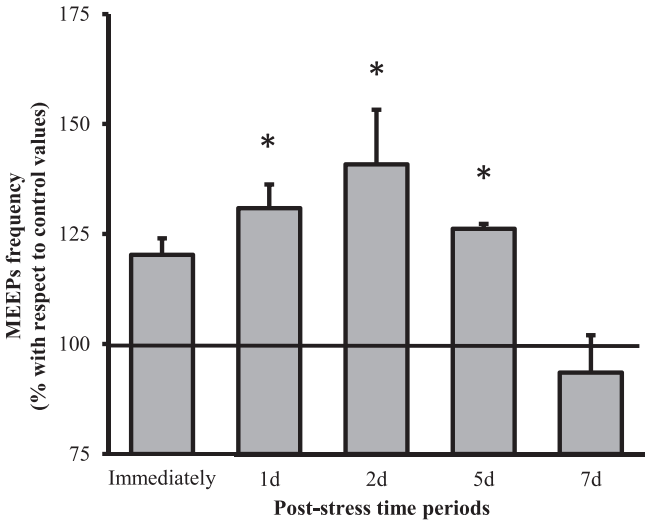

Fig. 5. Social isolation. After one day of social isolation, the records were made immediately (% variation with respect to control: 120.24 ± 3.80, N = 45 records from 3 control animals and N = 59 records from 5 stressed mice, P = 0.0871), 24 h (% variation with respect to control: 130.86 ± 5.38, N = 45 records from 3 control animals and N = 61 records from 5 stressed mice, P = 0.0136), 48 h (% variation with respect to control: 140.85 ± 12.37, N = 45 records from 3 control animals and N = 67 records from 5 stressed mice, P = 0.0154), 5 days (% variation with respect to control: 126.19 ± 1.11, N = 45 records from 3 control animals and N = 48 records from 5 stressed mice, P = 0.0218) and 7 days later (% variation with respect to control: 93.47 ± 8.51, N = 45 records from 3 control animals and N = 69 records from 5 stressed mice, P = 0.6393).Values greater than 100 indicate the increase produced. Y-axis: percentage of experimental value with respect to the control. * P < 0.05.

± 4.58, N = 45 records from 3 control animals and N = 56 records from 5 stressed mice, P = 0.684) after the application of stress, waiting for a possible late reaction, but a null effect was obtained at that temperature. There were no changes either in the mean amplitude of the mEPPs (% of maximum variation recorded was: 8.54 ± 7.01, P > 0.05), nor in the membrane potential (% of maximum variation recorded was: 9.91 ± 8.96, P > 0.05) in none of the post-exposure periods.

Once the animals had been adapted to the aqueous medium and the habitat, the animal was exposed to water at 21ºC. The first step was to place the mouse in the container with little water (2–3 cm) at 37ºC for 6 min. After 24 h, the same procedure was carried out, but this time with water at 21ºC for 6 min. The records taken immediately after the procedure showed a significant increase in the frequency of mEPPs (% variation with respect to control: 137.11 ± 3.13, N = 45 records from 3 control animals and N = 40 records from 5 stressed mice, P = 0.0019), but at 24 h later, the frequency was already normalized (% variation with respect to control: 2.08 ± 7.18, N = 45 records from 3 control animals and N = 57 records from 5 stressed mice, P = 0.877). The mean amplitude of the mEPPS did not show significant differences with respect to the control values (% of maximum variation recorded was: 9.32 ± 2.19, P > 0.05). The membrane potential did not show significant changes either (% of the maximum variation recorded was: 7.09 ± 1.70, P > 0.05).

Once it was established that the temperature induces only transient changes, the FST was carried out at 21ºC with the container full of water for 6 min (see Fig. 6). The frequency of mEPPs did not increase immediately (20% variation, P = 0.109) and only increased at 24 h (36%, P = 0.0029). These results indicate that habituation has not significantly interfered with the temperature of 21ºC in the results obtained. After 24 h, a gradual increase in frequency was recorded until 48 h (69% increase, P < 0.0001). Subsequently, it remained elevated until 5 days after the application of the FST (52% increase, P = 0.0128). At 7 days post exposure, control values were already registered (17% variation with respect to control, P = 0.1192). The mean amplitude of the mEPPS did not change in any case (% of maximum variation recorded was: 19.02 ± 1.64%, P > 0.05), neither did the membrane potential (% of maximum variation recorded was: 15.12 ± 1.99, P > 0.05).

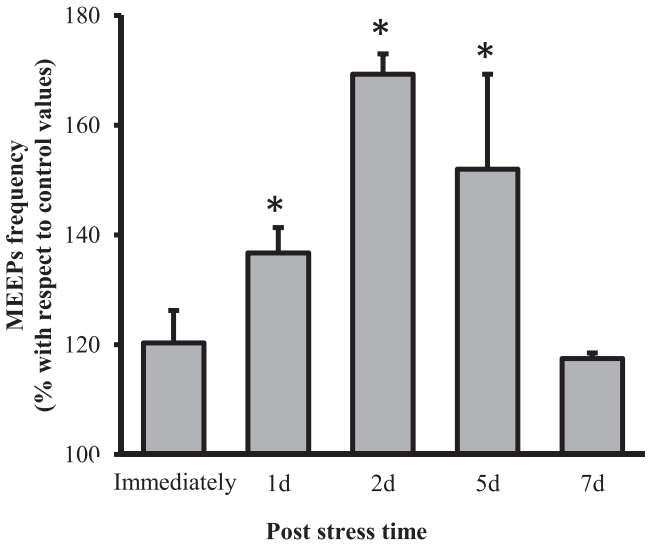

Fig. 6. Forced Swimming test. Electrophysiological recordings were made immediately (% variation with respect to control: 120.32 ± 5.93, N = 45 records from 3 control animals and N = 55 records from 5 stressed mice, P = 0.109), 24 h (% with respect to control: 136.76 ± 4.65, N = 45 records from 3 control animals and N = 59 records from 5 stressed mice, P = 0.0029), 48 h (% variation with respect to control: 169.31 ± 3.73, N = 45 records from 3 control animals and N = 63 records from 5 stressed mice, P < 0.0001), 5 days (% variation with respect to control: 152.00 ± 17.30, N = 45 records from 3 control animals and N = 57 records from 5 mice, P = 0.0128) and 7 days (% variation with respect to control: 117.50 ± 1.02, N = 45 records from 3 control animals and N = 55 records from 5 stressed mice, P = 0.1192) after a stress through FST without support on the ground for 6 min at 21ºC.Values greater than 100 indicate the increase produced. Y-axis: percentage of experimental value with respect to the control. * P < 0.05.

3.6. Exposure to chronic stress

Immediately after the application of the chronic stress procedure, a significant increase in the frequency of mEPPs by 50% (P < 0.0001) was obtained (Fig. 7). This increase exceeds any of the procedures tested in isolation. On day 3 after the procedure, the increase was almost double over control values (P < 0.0001). The strongest increase was obtained on day 7 post-procedure (115% increase compared to control, P < 0.0001). After 15 days of the procedure, the values were still elevated when compared to control (65% increase with respect to control, P < 0.0001). On the other hand, the mean amplitude of the mEPPs did not change significantly in any period studied (% of maximum variation recorded was: 16.04 ± 3.81, P > 0.05). No changes were detected in the membrane potential (% of maximum variation recorded was: 7.05 ± 3.09, P > 0.05).

4. Discussion

In each of the stressors, an increase in the frequency of mEPPs was obtained after the first day of treatment, which remained high until the following days and, generally, normalized after one week. Chronic stress caused the greatest increase in the frequency of mEPPs and this was maintained for a much longer period.

Recent studies reported a site for sympathetic varicosities very close to the neuromuscular junctions [7,8]. Sympathetic innervation shows the presence of endogenous catecholamines near the neuromuscular junctions and its involvement in the modulation of synaptic transmission. A significant decrease in the amplitude, frequency and duration of the miniature end plate potentials (MEPPs) as well as the amplitude of

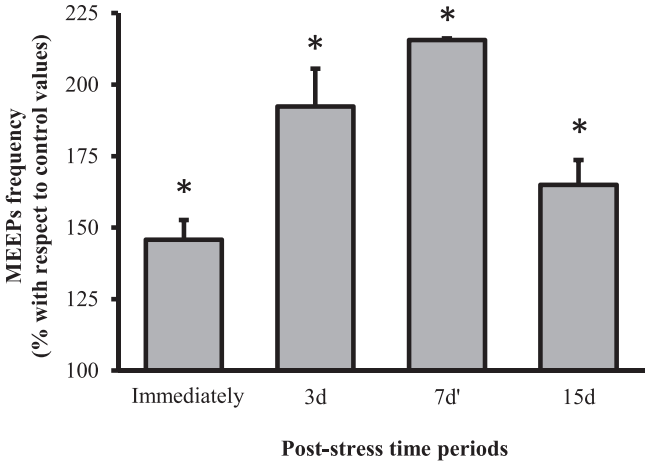

Fig. 7. Chronic stress. Electrophysiological recordings were made immediately (% variation with respect to control: 145.78 ± 6.86, N = 45 records from 3 control animals and N = 50 records from 5 stressed mice, P < 0.0001), 3 days (% variation with respect to control: 192.39 ± 13, 15, N = 45 records from 3 control animals and N = 57 records from 5 stressed mice, P < 0.0001), 7 days (% variation with respect to control: 215.58 ± 0.62, N = 45 records from 3 control animals and N = 37 records from 5 stressed mice, P < 0.0001) and at 15 days (% of variation with respect to control: 164.99 ± 8.66, N = 45 records from 3 control animals and N = 53 records from 5 stressed mice, P < 0.0001) after the application of the procedure. Values greater than 100 indicate the increase produced. Y-axis: percentage of experimental value with respect to the control. * P < 0.05.

the evoked potentials (EPP) and the quantum content was obtained by sympathectomy [8]. Therefore, the results obtained in this study may be directly related to this sympathetic innervation of the neuromuscular junctions.

Alterations in postsynaptic receptors can influence the size of mEPPs [20]. In previous studies, AChR aggregation has been associated with sympathetic innervation of the NMJ, establishing that sympathetic denervation causes a decrease in AChR and the amplitude of MEPPs and EPPs [8]. However, there are no studies evaluating the amplitude of MEPPs, EPPs or AChR aggregation after sympathetic stimulation. In the present study, no changes were obtained in the amplitude of the mEPPs, neither during the application of each stressor procedure nor in any post-treatment period. Therefore, it can be concluded that the stressor procedures do not affect the structural integrity or functionality of the postsynaptic AChRs of muscle fibers.

On the other hand, chronic stress can injure neurons [21] and lead to the appearance of inflammatory cells [22]. This implies a change in the membrane potential to even higher positive values. For this reason, the membrane potential has been analyzed and has not been modified in any record related to stress. Therefore, the results obtained in this study do not support the concept of a classic lesion of sarcoplasm disruption.

As expected, the duration of exposure to a stressor appears to be an important factor in increasing the frequency of mEPPs. For example, the application of ultrasound is only stressful for 24 h. In stressful procedures of longer duration, such as food and water deprivation or social isolation, a greater increase in the frequency of mEPPs is induced. However, the FTS maintained during 6 min causes a stimulus on the frequency of the mEPPs that lasts for five days. Therefore, the intensity of the stressor stimulus is also important. Stressors, which are often multidimensional, produce not only immediate actions but also long-term effects secondary to the primary stressor (see, for example, Anisman and Merali, [23]). While some stressful effects may decrease over time (eg, guilt), the effects of other stressors may increase (eg, loss of social support). In addition, the stress response itself can be a stressor in such a way that time can be a factor that enhances its effects.

In several of the procedures used in this study, the animals have been accustomed to situations different from those common in their daily lives. Habituation is one of the simplest forms of non-associative learning, and it occurs when an organism reduces or ends up suppressing its response to a continuous and persistent stimulus [24]. The reduction of the response can last minutes, hours, or days, depending on the type of stimulus performed, and also on the experiment that is being developed [25]. Mice have an innate preference for new stimuli, so no positive or negative reinforcement or excessively long training programs are necessary [26]. In the present study, no positive or negative reinforcement was given to mice during habituation.

4.1. Continuous exposure to ultrasound

After 24 h of continuous exposure to ultrasound, the frequency of mEPPs in-creased and was maintained during the 48 h period to decrease at 5 days post-exposure and achieve control values at 7 days. The mouse hears at a much higher frequency (5–50 kHz) than humans (20 Hz-20 kHz) [27]. Surprisingly, some of the common animal facility noises can be stressful to mice, which can have adverse effects on behavioral experiments. For example, Cheng et al. observed decreased learning in mice subjected to noise pollution from the animal facility [28]. In short, chronic exposure to ultrasound over variable periods turns out to be a powerful stressor in rodents [29]. This form of stress is often used to induce depression in animals. For example, Pavlov and colleagues showed that a 3-week exposure to alternating ultrasound frequencies (20–45 kHz) can induce depression in murine [30]. Furthermore, exposure to high frequency ultrasound can also cause social defeat, pain, aversion or fear [31]. For this reason, in this study we have chosen to use an exposure to an alternate frequency lower than that which causes depression (maximum of 20–25 kH). This frequency corresponds to a murine vocalization referring to a “negative emotional state” [32].

Prolonged ultrasound exposure periods, for example during 3 weeks, are common for causing anxiety and depression [33]. To avoid this, in the present study exposures of only 3 h were initially performed. However, since no results were obtained, the ultrasound exposure period was increased to 24 h. This time of exposure to ultrasound is a stressor without depressive effects.

4.2. Food and water deprivation

After 24 h of food and water deprivation, the frequency of mEPPs did not change immediately, but after the first day it increased markedly. This increase was maintained for 5 days.

Food "restriction" and "deprivation" are protocols with different responses. Some authors, such as Corton and Brown-Borg [34], propose restriction as a generator of adaptive homeostatic metabolic responses. Instead, deprivation generates much more pronounced metabolic reactions. In this sense, prolonged fasting in rodents can cause a loss of body weight, which is totally contrary to the comfort of the animal [35]. The most widely used protocols use food deprivation periods of 24 and 48 h. Abstaining from food for a period of 48 h can lead to a loss of body weight by up to 20% [36]. In this sense, the European Parliament guidelines on the protection of animals used for scientific purposes establish that 24-hour food deprivation in rodents is defined as “mild” and for a period of 48 h it is defined as “moderate” (Directive 2010/63 / EU). On the other hand, Malatová and Ahlers [37] evaluated the plasma corticosterone level in normal fed rats, fasting for 24 and 48 h. These authors obtained insignificant changes in a 24-hour fast, but after a 48-hour deprivation period, the changes were notable. It can be suggested that the food deprivation protocol applied in the present study is adequate to generate stress without interference from undesirable effects.

The greatest risk of the water withdrawal is dehydration. The degree of dehydration developed after 24 h without water depends on the animal species [38]. For example, in rodents, periods of deprivation of 24 h or less did not alter the amounts of compensatory intake compared to those that did have water, but if the period of deprivation is longer, significant increases are based on increased consumption [39]. In the experiments developed in the present study, water deprivation in mice lasted only 24 h and no compensatory increase in intake was observed. In summary, dehydration as the mechanism of action in increasing ACh release can be ruled out.

4.3. Immobilization

With immobilization, the frequency of mEPPs increased discretely both immediately and after 5 days of evaluation. With the idea of generating more powerful stress and results, the mice were subjected to 1 h of immobilization each day for 5 consecutive days. Surprisingly, the results obtained in this second immobilization methodology were identical to those obtained with the animals subjected to a single 1 h of immobilization. It is evident that a habituation phenomenon occurred: the animals were only stressed during the first few hours, and the rest of the time it was redundant, they were not stressed.

Immobilization is a widely used and easily applied stressor. "Immobilization" and "restriction of movement" are different procedures. Immobilization is a stressful stimulus of both physical and psychological nature and is therefore more intense than restriction of movement [40]. In this work immobilization has been applied. Most of the immobilizing procedures used in stress research in mice are cylindrical tubes like those used in the present work [41]. Although there are also studies that reported the presence of psychological stress in mice with mobility restriction [42]. In summary, there is only a slight increase in spontaneous neurotransmission despite the use of a physical and psychological stressor.

4.4. Social isolation

The frequency of mEPP did not change immediately after the fifth day of social isolation in metabolic cages. At 24 h after the procedure was applied, a late increase occurred that was maintained up to 5 days after exposure to social isolation.

Metabolic cages contain several structural conditions that are aversive to animals. For example, these cages require the use of mesh flooring, therefore, there is no substrate for rodents to make their nests or elements of environmental enrichment and a space that is one third less the inhabitable area. In addition to this is social isolation. Numerous studies show that all of these factors elicit negative behavioral and somatic responses in rodents. In this sense, Kalliokoski et al. [43] when evaluating acute oxidative stress together with clinical signs, concluded that the mice are not capable of adapting or acclimatizing to this type of cage. In addition, Kalliokoski et al. found that the use of metabolic cages for 3 weeks led to an increase in the general metabolism in rodents. For this reason, in the present work, there was 24-hour exposure to the metabolic cage: this time frame is long enough to generate changes in neurotransmission, and short enough not to generate undesirable oxidative changes. On the other hand, periods longer than 24 h cause anxiety or depression [44].

4.5. Forced swim test (FST)

The forced swim test is an acute stressor that involves a psychological component and a motor stimulus such as swimming. The imposition of swimming for a short period of time activates, for example, the sympathetic system [45]. In addition, the use of a cold-water temperature of 21ºC, further enhances this stressor [46]. The relationship between stress and cold is widely documented, either in combination with other stressors, such as immobilization [47] or through the repetitive application of cold without restriction of movement [48].

In the present work, habituation to the aquatic environment was carried out first with a water temperature of 37ºC and with insufficient water to force the animal to swim. The process was then repeated with the water at 21 °C. This habituation did not cause any change in the frequency of the MEPPs. Thus, the results obtained do not respond to other possible stressors such as: cold water [48], neophobia [49], reduced space [40], etc. In the bibliography we can find other authors such as Zhong et al. who also tested with habituation before carrying out their experiments with FST [50]. Specifically, these authors carried out habituation by keeping the animals swimming for a longer period than in the present study (15 min).

Once habituation was applied, the animals were placed in cold water at 21ºC for 6 min. Some animals abruptly stopped swimming and floated. These animals were discarded for the study since they did not follow the same procedure as the rest of the animals. In addition, in these animals it is described that the FST can generate an undesirable depression [21].

4.6. Exposure to chronic stress

In order to maintain a prolonged ACh increase, the different acute stress protocols were combined. With a combination of different stressors, spontaneous neurotransmission increased immediately post-procedure, reaching the highest values of all procedures (~200% increase) already at 3 days and remaining up to 15 days. Stressor sequencing is important as unpredictable stressor exposure induces even more stress in mice [51].

In the design of the sequence of stressors, the FST was arranged last to detect animals that enter depression [52]. In addition, 24 h of rest or recovery were applied between the different stressor procedures so that the animal recovered, sometimes even physically, which makes it capable of facing a new stressor. For example, food deprivation for a period of 48 h can cause weight loss in mice [36].

4.7. Relationship between stress and myofascial trigger points

It has been widely demonstrated that an increase in the spontaneous release of ACh is involved in the pathophysiology of MTrPs [10,11,53]. Initially, Simons described the spontaneous release of ACh as the starter of a cascade of events leading to the development of myofascial trigger points [10] More recently, Gerwin provided information that, for the first time, places the muscle fiber at the center of pathophysiology [53]. This new approach also involves an increase in the spontaneous release of ACh.

The β-adrenergic sympathetic nervous system (SNP) plays an important role in upregulating the effect of ACh on the NMJ in MTrPs, as demonstrated by the inhibitory effect of β-adrenergic inhibitors, such as phentolamine on EPN [7,8]. Stress excites the PNS [9], which justifies the increase in spontaneous ACh release obtained in this study. On the other hand, SNP activation can increase cytosolic calcium concentration [54], which can indirectly cause muscle damage. However, we did not find signs suggestive of muscle injury. Membrane potentials are maintained in all forms of stress studied, suggesting that there is no structural loss of the sarcolemma.

Gerwin proposes the participation of the calcitonin-gene-related-peptide (CGRP) in the pathophysiology of the MTrP. [53]. CGRP is co-released with ACh from the presynaptic motor component and has the effect of increasing ACh levels at the motor end plate [55]. For this reason, Gerwin has proposed that CGRP may be involved in the MTrPs. In the present study we have described a strong increase in the spontaneous release of ACh produced by stress. Therefore, stress can also increase the release of this CGRP pathway, entering a potentiation circle that can contribute to the pathophysiology of MTrPs. Additionally, the same ACh can activate muscarinic autoreceptors of the presynaptic motor terminals, further enhancing the spontaneous secretion of ACh [56]. The CGRP and the muscarinic auto receptors generate a cycle that potentiate the spontaneous release of ACh. The ACh Increased in the synaptic cleft activates repeatedly postsynaptic nicotinic ACh receptors (nAChRs). Activation of nAChRs opens ionotropic sodium channels. Spontaneous neurotransmission rarely achieves sufficient synchrony to displace the membrane sufficiently to trigger a muscle action potential. However, spontaneous neurotransmission allows enough sodium to enter the subsynaptic sarcoplasm to depolarize the cisterns of the sarcotubular system. Enough calcium flows out of the cisterns to activate the tropomyosin and anchor the myosin heads and create localized contraction: subsynaptic contraction knocks. In the new pathophysiological model proposed recently by Gerwin [53], they involve metabotropic muscarinic receptors that modulate the release of ACh molecules into the synaptic space, and postsynaptic ionotropic sodium and potassium channels that control the entry of calcium into the muscle cytosol. Gerwin considers these mechanisms to be protective of ACh released into the synaptic gap and to inhibit presynaptic release of ACh from the MNT and postsynaptic activation of nAChRs that modulate ACh through a transsynaptic feedback loop. He considers that the failure of this type of feedback mechanisms are crucial in the development of MTrPs. We have found that stress modulates the release of ACh and can also modulate the previously mentioned mechanisms in the generation of MTrPs.

5. Conclusions

In summary, acute stress increases spontaneous neurotransmission moderately and late (24–48 h), which is maintained for less than a week. Similarly, chronic stress increases spontaneous neurotransmission overtime in a sustainable manner. It can be proposed that chronic stress may be involved in the genesis or maintenance of myofascial trigger points.

Conflicts of interest

The authors declare no conflict of interest.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Research Committee of the Universitat Rovira i Virgili (Generalitat de Catalunya, Departament d'Acció Climàtica, Alimentació i Agenda Rural, Direcció General de Polítiques Ambientals i Medi Natura) (protocol code 11337, approval on 12/01/2022 13).

Informed consent statement

"Not applicable."

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Data availability

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

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