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Research report

Cotinine enhances the extinction of contextual fear memory and reduces anxiety after fear conditioning

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ABSTRACT

Posttraumatic stress disorder (PTSD) is an anxiety disorder triggered by traumatic events. Symptoms include anxiety, depression and deficits in fear memory extinction (FE). PTSD patients show a higher prevalence of cigarette smoking than the general population. The present study investigated the effects of cotinine, a tobacco-derived compound, over anxiety and contextual fear memory after fear conditioning (FC) in mice, a model for inducing PTSD-like symptoms. Two-month-old C57BL/6J mice were separated into three experimental groups. These groups were used to investigate the effect of pretreatment with cotinine on contextual fear memory and posttreatment on extinction and stability or retrievability of the fear memory. Also, changes induced by cotinine on the expression of extracellular signal-regulated kinase (ERK)1/2 were assessed after extinction in the hippocampus. An increase in anxiety and corticosterone levels were found after fear conditioning. Cotinine did not affect corticosterone levels but enhanced the extinction of contextual fear, decreased anxiety and the stability and/or retrievability of contextual fear memory. Cotinine-treated mice showed higher levels of the active forms of ERK1/2 than vehicle-treated mice after FC. This evidence suggests that cotinine is a potential new pharmacological treatment to reduce symptoms in individuals with PTSD.

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1. Introduction

Posttraumatic stress disorder (PTSD) results from exposure to traumatic events that are perceived as danger of injury or death and is characterized by symptoms that include flashbacks, nightmares and intrusive thoughts predominantly derived from a failure to extinguish trauma memories [1]. Anxiety and abnormal fear responses, triggered by reminders of trauma, result from a failure in fear extinction and represent one of the more troublesome characteristics of PTSD. These symptoms correlate with the overactivation of the hypothalamus–pituitary–adrenal (HPA) axis [2], the dysregulation of several neurotransmitter systems and functional and structural changes in brain regions such as the amygdala, hippocampus and prefrontal cortex [3,4]. This dysregulation of the HPA axis is considered a key factor inducing such changes in the brain [2]. For example, a decrease in the baseline levels of cortisol in PTSD has been postulated [5]. This decrease has been attributed to an enhanced sensitivity of the glucocorticoid negative feedback loop at the pituitary level after chronic exposure to high levels of corticosteroids [6]. However, according to a reported meta-analysis, low cortisol levels in the serum or plasma of PTSD patients are found only under certain circumstances and must be influenced by factors including gender and history of abuse as well as the measurement methods used [7].

Individuals with PTSD experience a pronounced deficit in fear memory extinction that is commonly resistant to current treatments. Selective-serotonin-reuptake-inhibitors (SSRIs) such as paroxetin and sertraline are currently the first-line treatments for PTSD [8,9]. However, these drugs are ineffective for many individuals [10]. Thus, new pharmacological therapies are needed to be used alone or in conjunction with psychotherapy to favor memory extinction. Consequently, many laboratories are investigating new drugs that facilitate extinction in patients with PTSD [11–13].

Abbreviations: ANOVA, analysis of variance; CS, conditioned stimulus; US, unconditioned stimulus; ERK, extracellular signal-regulated kinase; EPM, elevated plus maze; FE, fear memory extinction; HPA, hypothalamus–pituitary–adrenal; h, hours; min, minutes; OF, open field; PBS, phosphate buffered saline; PTSD, posttraumatic stress disorder; RT, room temperature; s, seconds; TBS, Tris-buffered saline; TBST, TBS with 0.1% Tween 20; SSRIs, selective serotonin reuptake inhibitors.

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A greater incidence of cigarette smoking has been found among individuals diagnosed with PTSD than the general population [14–17]. It has been proposed that smoking can be a form of self-medication to reduce PTSD symptoms among individuals with PTSD [18]. In the search for new therapies to reduce PTSD-symptoms, we investigated the effect of cotinine, the main metabolite of nicotine, on anxiety and the consolidation, stability and extinction of contextual fear memory after fear conditioning (FC) in mice. We studied cotinine because of its unique pharmacological properties, including its capacity to enhance memory [19] and serotonin availability in the brain [20,21]. Cotinine, which accumulates in the body as a result of tobacco exposure [22], crosses the blood–brain barrier and has a long half-life (19–24 hours (h)). In humans, cotinine has no reported toxic or cardiovascular effects at doses in plasma 10-times higher than the ones reached by heavy smoking [23,24].

Fear conditioning (FC) is a commonly used model to study fear and anxiety behavior that involves the presentation of a neutral conditioned stimulus (CS; context) with an aversive unconditioned stimulus (US; electric shock). After training, the CS alone elicits a conditioned fear response such as freezing behavior. This fear response is influenced by the extent of fear memory processes of acquisition, consolidation, retrieval and stability. The extinction of fear (FE) is a learned reduction of fear responses after repetitive exposures to the CS (context) that is no longer paired with the aversive US [25].

To investigate the effect of cotinine on contextual fear, we used a mouse model of associative memory. This model has been largely used to investigate the effects of drugs over contextual fear responses that are triggered by environments acting as reminders of an aversive stimulus as well as to identify signaling factors mediating FE [26–29]. We used FC to investigate the effect of cotinine on anxiety and fear responses. We examined the effect of an 8-day pretreatment with cotinine on contextual fear memory acquisition as tested measuring short-term retention of contextual fear memory (1 h after FC), initial consolidation of the fear memory as tested measuring retention 24 h after conditioning and long-lasting anxiety 6 days after FC. In addition, we studied the effect of post-treatment with cotinine on fear extinction triggered by extinction trials consisting in repeated exposure to the CS. We also studied the effect of posttreatment with cotinine on the stability of the contextual fear memory or reconsolidation of the fear memory after a single reexposure to the CS during the retention test. Several of these studies have shown that FE is associated with changes in the cerebral activity of several protein kinases including the extracellular signal-regulated kinase (ERK)1/2 [26] and its upstream activator PI3K [30]. An upregulation of the active forms of ERK1/2 (phospho-ERKs) after contextual FC and extinction has been observed in the hippocampus [26,30], a region playing a central role in modulating contextual FC and extinction [3,31]. More significantly, it has been shown that inhibition of ERK activity immediately after memory retrieval blocked contextual memory extinction [30]. Thus, based on this evidence, we also investigated the effect of cotinine on the activation of ERK1/2 in the hippocampus during fear memory extinction.

Our results suggest that the ability of cotinine to reduce anxiety may underlie the high incidence of tobacco smoking in individuals suffering from anxiety disorders and therefore represents a possible new therapeutic agent to be investigated for pharmacological treatment of these disorders.

2. Materials and methods

2.1. Animals

Two-month-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), weighing 25–30 g were maintained at a controlled temperature (25 ± 1 °C) on a 12-h light/dark cycle (light on at 07:00 h) with *ad libitum* access to food and water. Initially, mice were group housed and habituated to housing conditions for 7 days. The behavioral analysis was performed with mice between 8 and 12 weeks of age that were individually housed by investigators blind to the treatment of mice. All protocols were previously approved by the Institutional Animal Care and Use Committees of the University of South Florida and Bay Pines Veterans Affairs Healthcare System and followed the National Institutes of Health standards.

2.2. Cotinine treatments

Cotinine ((5*S*)-1-methyl-5-(3-pyridyl) pyrrolidin-2-one) (Sigma–Aldrich Corporation, St. Louis, MO, USA) was prepared by dissolving the powdered compound in sterile phosphate buffered saline (PBS). Mice (n = 7–8/group) were treated with vehicle, cotinine (1 mg/kg), or cotinine (5 mg/kg) via gavage per random assignment. The schedule of treatments used varied by protocol assignment.

2.2.1. Protocol 1

To study the effect of treatment with cotinine on the acquisition and retention of contextual fear memory, mice were treated daily with vehicle (PBS) or cotinine (1 mg/kg or 5 mg/kg) for 8 days before behavioral training and continuously throughout the protocol until euthanasia. Mice were weighed the first and last days of testing to investigate any effect of treatments or behavioral tasks on weight gain. On the last day of pretreatment (day 8), mice were tested for anxiety levels using the elevated plus maze (EPM) and open field (OF) tests.

After pretreatment, mice were trained for contextual FC and later tested for the retention of contextual fear, 1 h (short-term fear retention) or 24 h after FC. The retention of contextual fear memory was assessed by determining the freezing behavior after re-exposure to the conditioned context. After the short-term retention test, blood was collected to perform plasma corticosterone analysis. To prevent any confounding effect between the retention tests, the analysis of short-term fear retention was performed with a different cohort of mice than the one used to investigate fear memory retention 24 h after FC. Also, to control for fear or anxiety responses in the mice not due to contextual FC, we used two control groups. The first control group included vehicle-treated mice that were shocked and immediately removed from the conditioning chamber (immediate shock). The second control group consisted of mice that were exposed to the chamber (context) for the same period of time as the conditioned mice but were not shocked.

The following day after the 24 h retention test, mice were tested for anxiety using the elevated plus maze and open field tests, respectively. In addition, 6 days after the 24 h retention test, sensorimotor abilities and pain sensitivity were tested using the rotarod and hot plate tests, respectively. Once behavioral testing concluded, mice were euthanized by cervical dislocation and the brains collected and stored at −80 °C for neurochemical analysis.

2.2.2. Protocol 2

To assess the effect of cotinine on the extinction of contextual fear memory, elicited by the repeated exposure to the context (extinction trials), mice were randomly assigned to a treatment group and then, in absence of any pharmacological treatment, underwent a single FC training trial. Immediately after the retention test and daily during the ensuing extinction trials (days 2–6) as well as continuously until euthanasia (day 9), mice were treated with vehicle or cotinine (1 mg/kg or 5 mg/kg). Mice were euthanized by cervical dislocation and the brains collected for neurochemical analyses.

2.2.3. Protocol 3

This protocol was designed to investigate the effect of posttreatment with cotinine on the stability and/or retrievability of contextual fear memory after a single event of fear memory retrieval but in absence of active extinction trials. Mice were trained for FC, and immediately after the retention test (day 2) started with vehicle (PBS) or cotinine (5 mg/kg) treatments via gavage until the end of the experiment. After the retention test, mice were treated but none re-exposed to the chamber until day 10, when freezing behavior was determined.

2.3. Contextual fear conditioning

Contextual FC was performed in a conditioning chamber surrounded by a sound-attenuating box (67 cm × 53 cm × 55 cm) (Harvard Apparatus, Holliston, MA, USA). To provide background white noise (72 dB), a single computer fan was installed in one of the sides of the sound-attenuating chamber. The conditioning chamber (25 cm × 25 cm × 25 cm) contains on one side a speaker and on the opposite side has a 24-V light. The chamber has a 36-bar insulated shock grid floor. After each use, it was cleaned with 70% ethanol and dried. Sensory perception of the shock was determined through threshold assessment. Mice were placed in the conditioning chamber and permitted to explore for 2 minutes (min) before the onset of a discrete tone (a sound lasting 30 seconds (s) at 4000 Hz and 90 dB). In the last 2 s of this 30 s period, mice received a foot shock of 1 mA. After the shock, mice were kept in the conditioning chamber for an additional 150 s and then placed back in their cages. Freezing behavior is defined as the absence of all movement except for that necessitated by breathing for 2 s. Freezing behavior was expressed as percentage of time spent freezing as assessed using the Freezing software (Harvard Apparatus).