Cotinine reduces depressive-like behavior, working memory deficits, and synaptic loss associated with chronic stress in mice

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HIGHLIGHTS

- Cotinine reduces depressive-like behavior and memory loss in the restrained mice.
- Cotinine increases synaptic density in the stressed mouse brains.
- Cotinine inhibits glycogen synthase kinase 3β in the restrained mouse brains.

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ABSTRACT

Chronic stress underlies and/or exacerbates many psychiatric conditions and often results in memory impairment as well as depressive symptoms. Such afflicted individuals use tobacco more than the general population and this has been suggested as a form of self-medication. Cotinine, the predominant metabolite of nicotine, may underlie such behavior as it has been shown to ameliorate anxiety and memory loss in animal models. In this study, we sought to investigate the effects of cotinine on working memory and depressive-like behavior in mice subjected to prolonged restraint. Cotinine-treated mice displayed better performance than vehicle-treated cohorts on the working memory task, the radial arm water maze test. In addition, with or without chronic stress exposure, cotinine-treated mice engaged in fewer depressive-like behaviors as assessed using the tail suspension and Porsolt’s forced swim tests. These antidepressant and nootropic effects of cotinine were associated with an increase in the synaptophysin expression, a commonly used marker of synaptic density, in the hippocampus as well as the prefrontal and entorhinal cortices of restrained mice. The beneficial effects of cotinine in preventing various consequences of chronic stress were underscored by the inhibition of the glycogen synthase kinase 3β in the hippocampus and prefrontal cortex. Taken together, our results show for the first time that cotinine reduces the negative effects of stress on mood, memory, and the synapse.

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1. Introduction

Memory and emotion are governed by homeostatic plasticity processes, which are susceptible to psychological stress. Chronic stress can induce deficits ranging from transient mnemonic and/or emotional disturbances to more pervasive memory and/or mood dysfunctions, resulting in various depressive and anxiety disorders such as post-traumatic stress disorder (PTSD) and major depression, among others [1,2]. Stress disorders such as PTSD are often accompanied with volumetric reductions of hippocampus [3,4] as well as associated limbic cortices such as prefrontal cortex (PFC)
[5]. In addition, alterations in limbic circuitry [6,7] as well as insults to hypothalamic–pituitary–adrenal (HPA) axis function have been reported [8–10].

While incapable of fully encapsulating the wide-ranging sequelae of chronic stress in humans, animal models have been useful in elucidating many of the underlying links between psychological stress, memory, and depressive-like behavior [11,12]. In rodents, it has been shown that many of the stress-induced pathological behaviors are accompanied by morphological and neurochemical changes in the limbic system [12,13]. For instance, chronic stress induces reversible dendritic atrophy in CA3 pyramidal cells [12,14]. Chronic-stress models have also been shown to diminish or alter synapse morphology [15,16] and complexity [17], as well as dendrite morphology and length [18,19] in CA1 pyramidal neurons. Chronic stress-induced atrophy of the granule cells in dentate gyrus (DG) has also been reported [19,20]. Other limbic regions, for instance the PFC, are also implicated. Chronic stress induces pyramidal dendritic retraction within the medial PFC [21] as well as disruptions ofafferent pathways from PFC to EC [22] and efferent projections from limbic regions to PFC [23]. Because these limbic regions and PFC are heavily implicated in emotional regulation and memory [24,25], the normalization of their function is a fundamental goal for new therapies to restore working memory and improve mood in individuals subjected to chronic stress.

Restraint stress (RS), is a model of chronic stress in rodents that consistently leads to neuronal remodeling of limbic regions such as PFC [21] and hippocampal CA1 [15,16] and CA3 [12,14] neuronal cells. RS also induces working memory impairment [26,27], anxiety, and depressive-like behavior [28]. These abnormalities mimic those observed in humans exposed to chronic stress or acute stress [29,30].

Cotinine, a tobacco-derived nootropic compound, is a weak agonist of the nicotinic acetylcholine receptors (nAChRs) [31]. However, it has recently been suggested that cotinine may act as a positive allosteric modulator (PAM) of the α7nAChR [32,33]. Also, cotinine acts as agonists of both α4β2 and α3β6/α2 nAChR subtypes in the caudate region leading to dopamine release [34]. In addition to the nAChRs, it has been suggested that cotinine may target other potential receptors [35,36] via activation of the serotergenic (5-HT) and dopaminergic systems [37] and/or other yet-to-be characterized receptors.

Despite growing preclinical evidence, few clinical studies have investigated the effect of cotinine on memory or emotion. One study showed that cotinine-treated abstinent smokers self-reported a reduction of anxiety, though whether this effect was due to an interaction with nicotine dependence is unclear as this report did not include non-smokers [38]. In an animal model, systemic long-term cotinine administration at doses as much as ten times higher than those achieved by smoking tobacco reduced anxiety and facilitated the extinction of a contextual fear memory [39] and this latter finding was replicated following hippocampal cannulae injection of cotinine [40]. Furthermore, cotinine positively influences learning, memory [33,41–43], attention, executive function, and impulsive behavior in various animal models [41,44]. Moreover, cotinine delays the progression of memory loss in a mouse model of Alzheimer's disease (AD) [45]. Because of its ideal pharmacodynamics and pharmacokinetic properties [44,46] as well as positive safety profile in humans [46,47], cotinine has been regarded as a viable therapeutic agent to treat the symptoms or delay the progression of many psychiatric and neurological conditions [33,48,49].

In this study, we utilized RS to investigate cotinine's effect on the cognitive and emotional deficits associated with chronic-stress exposure, i.e. spatial and working memory impairment and depressive-like behavior. In addition, we sought to determine cotinine's effects on synaptic density in various limbic regions by immunolabeling the synaptic marker, synaptophysin. Finally, we investigated the effect of cotinine on the expression of glycogen synthase kinase 3 β (GSK3β) in brains of mice exposed or not to chronic restraint stress.

2. Materials and methods

2.1. Animals

Male C57Bl/6J mice at ∼3 months of age (The Jackson Laboratories, Bar Harbor, ME), weighing 25–35 g were maintained on a 12-h light/dark cycle with ad libitum access to food and water and at a regulated room temperature (25 ± 1°C). Mice were group housed and habituated to environmental conditions for 7 days. All protocols were approved by the Institutional Animal Care and Use Committee of the Bay Pines Veterans Affairs Healthcare System and followed the National Institutes of Health standards.

2.2. Drugs

Cotinine ((5S)-1-methyl-5-(3-pyridyl) pyrroolidin–2-one; Sigma–Aldrich, St. Louis, MO) was prepared by dissolving the powdered compound in sterile phosphate-buffered saline (PBS, pH 7.4). Based on treatment level assignment, mice were orally administered vehicle (PBS, pH 7.4) or cotinine per group assignment (below) via gavage each day at approximately 16:00 EST. The first gavage treatment occurred at least 7 days prior to any experimentation and mice in each group were continuously gavaged daily until the end of each timeline described below. Cotinine was administered via gavage because it has been established that about 80% of cotinine is absorbed with peak plasma concentration averaging 45 min when given orally [50,51]. Cotinine readily crosses the blood–brain barrier [52] at a rate of about 43–61 μg/g/day based on the analysis of unidirectional influx in rat [53]. The dosage of cotinine chosen for this study (5 mg/kg) was based on dose–response curves included in previous studies in our laboratory showing that this was the smallest yet most efficacious dose tested improving anxiety, fear extinction [39] and depressive-like behavior after RS (1 h/day for 14 days; unpublished data).

2.3. Experimental design

Following 7 days of acclimation to the vivarium and housing conditions, mice were divided into 5 cohorts (described below) and randomly assigned to treatment groups (vehicle or cotinine 5 mg/kg). For all mice, drug administration occurred daily until euthanasia, including a 7-day pretreatment period wherein no other experimental procedures were conducted.

The first cohort (C–1) was used to investigate the effects of cotinine in RS-exposed mice across an array of behavioral and neurochemical assessments (described in greater detail below). This cohort consisted of 3 experimental groups: (1) vehicle-treated, non-RS mice (NS + Veh, n = 5), (2) vehicle-treated RS mice (RS + Veh, n = 8), and (3) cotinine-treated RS mice (RS + Cot 5, n = 8). Following the cessation of the RS period (day 35), all mice were tested in open field (OF; day 37), radial arm water maze (RAWM; days 38–47), tail suspension test (TST; day 49) and Porsolt’s forced swim test (PT; day 50). Daily treatments continued from 7 days prior to RS until euthanasia (day 51). A timeline depicting the experimental protocol for C–1 can be seen in Fig. 1A.

An additional cohort of mice (C–2) was used to elucidate the effects of cotinine in the RAWM in non-RS (NS) treatment groups (NS + Veh, n = 8; NS + Cot 5, n = 8). Mice in C–2 were exposed to identical conditions as NS mice in C–1 but were not exposed to OF and were sacrificed following RAWM testing (day 47) and
brains collected for neurochemical analysis. A timeline depicting the experimental protocol for C-2 can be seen in Fig. 1A.

To ascertain the effects of cotinine on depressive-like behavior in NS mice not previously exposed to RAWM, two additional cohorts (C-3 and C-4) of NS mice (NS + Veh, n = 8; NS + Cot 5, n = 8) were treated for 7 days and exposed to TST or PT, respectively. Time-lines depicting the experimental protocols for C-3 and C-4 can be seen in Fig. 2A.

A final cohort (C-5) was included to determine the synaptic densities of mice exposed to RS immediately following stress exposure. Two groups (RS + Veh, n = 7; RS + Cot, n = 8) were exposed to an identical timeline as RS-exposed mice from C-1 but were euthanized 24h following the cessation of the RS procedure. A timeline depicting the experimental protocol for C-5 can be seen in Fig. 4A.

2.4. Chronic restraint stress

For those mice in C-1 and C-5 to be exposed to chronic stress, mice were restrained for 6h a day for 21 days as previously described [54]. Briefly, mice were allowed to tunnel via modified plastic funnel (DecapiCone, Braintree Scientific, Braintree, MA) into a 50 ml conical transparent plastic centrifuge tube with screw sealing cap (Corning Inc., NY, NY) containing non-protruding perforations in the walls and ends of the tube for ventilation. Each tube was fixed to the base of an empty mouse cage with scotch tape to disallow movement. During the restraint period, mice were kept in individual cages under periodic supervision with no access to food or water. During the restraint period, NS mice in C-1 and C-2 were removed from vivarium each day and transported to procedure room in conditions identical to RS mice but remained in their home cages.

2.5. Behavioral tests

2.5.1. Locomotor activity

We have previously shown that cotinine treatment does not affect locomotor activity in the OF in non-stressed and acute stressed conditions [39]. Here, we sought to determine whether cotinine induced changes in locomotor activity in mice exposed to highly stressful conditions. Two days following the cessation of the RS period, mice in C-1 were placed in a randomly assigned corner of an uncovered square arena (40 cm × 40 cm × 35 cm) under bright lighting (≈80 lux) and allowed to freely explore for 25 min while observed via closed-circuit television feed (CCTV) from an adjacent room. Total distance traveled and mean velocity were quantified via a video tracking software (ANY-Maze, Stoelting, Wood Dale, IL).

2.5.2. Radial arm water maze (RAWM, spatial and working memory)

For mice in C-1 and C-2, the RAWM task of spatial and working memory was performed as described [55]. Briefly, a plastic insert was placed into a 100 cm diameter circular pool to create 6 radially distributed arms emanating from a central circular swim area as described [56]. At the arms' ends were fixed an assortment of 2- and 3-D visual cues of various size, complexity and distance from the pool (6-total including the uniformed experimenter). For each of 9 days, a different randomly selected arm contained a submerged escape platform (9 cm diameter; ≈1 cm deep), excluding the arm at the experimenter’s position. Each day, the remaining 5 arms were randomly assigned as the start arm for each of 5 trials (T1–T5). T1–T4 (acquisition trials) were conducted with only a 30-s “consolidation” period between them, whereas T5 (retention trial) occurred 30 min following T4. Between T4 and T5 mice were dried via hand towel and returned to their home cages. During each 60-s trial, the mice were free to explore in search of the submerged platform, but upon making an erroneous decision were gently relocated via tail without removal from water to the start arm. If 60-s lapsed without success, mice were gently ushered to the platform via tail but nose first. Upon each acquisition trial’s end, either by guidance to or successfully finding the platform, each mouse was given a 30-s rest during the “consolidation” period. Latency and number of erroneous choices per trial were scored. Errors were defined as (1) entering completely and reaching the end of an incorrect arm, (2) entering at least 25% of but departing an incorrect arm, (3) making no choice for longer than 10 s, and (4) swimming into the correct arm but leaving without finding the platform.

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**Fig. 1.** Cotinine improved working memory performance after prolonged restraint stress in mice. Mice in C-1 and C-2 (n = 5–8/group; A) began treatment with vehicle (Veh) or cotinine 5 mg/kg (Cot 5) that continued daily throughout the experiment. One week later, mice in chronic stress groups were subjected to restraint stress (RS; 6h daily for 21 days) and subsequently all groups were behaviorally tested. Cotinine significantly improved working memory performance regardless of the presence of stress. In the final block of the radial arm water maze (RAWM; days 8–9) cotinine-treated mice make lower number of errors in the trial 5 of the than vehicle-treated mice (p = 0.0002; B) and latency times to find the submerged platform (p = 0.00216; C). PT, Porsolt’s test; OF, open field; TST, tail suspension test. *, only mice exposed to RS were tested in the OF test.
2.5.3. **Depressive-like behavior: tail suspension and Porsolt’s forced swim tests**

To identify the influence of cotinine on depressive-like behavior in RS and NS conditions, mice were tested using TST [57] and PT [58]. Both of these instruments have a highly similar degree of construct validity [59], and the consistency in results across both instruments drastically reduces the likelihood that observed reductions in immobile postures were due to procedure-specific effects.

Mice were tested using a commercial TST device (Stoelting) as previously described [57]. Briefly, mice were hung from a mounted blunt hook via a strip of masking tape (20 cm × 1.9 cm) with their nose positioned ≈150 mm from padded chamber floor for 6 min. Behavior was monitored and recorded using closed-circuit television monitors (CCTV) by experimenters from an adjacent room. Immobility, defined as the summation of time a mouse does not struggle to escape was quantified and averaged across two observers blind to treatment groups.

For PT, mice were placed in an inescapable plexiglas cylindrical tank (40 cm high × 20 cm in diameter w/depth of 30 cm, Stoelting) filled with tap water (25 ± 2 °C) for 5 min and behavior was recorded via CCTV and monitored from an adjacent room. Immobility, considered the absence of mobility other than the minimal necessary for the mouse to stay afloat, was scored and averaged by two observers blind to treatment groups.

In ascertaining the cotinine-induced influence over depressive-like behavior in mice not exposed to chronic stress, we needed to ensure there were no residual stress effects associated with RAWM testing that might influence depressive-like behavior. Therefore, mice exposed only to the drug administration for 7 days were tested with TST or PT (C-3 and C-4, respectively).

2.6. **Western blot and immunohistochemical analyses**

2.6.1. **Tissue preparation**

Tissue was processed according to our previously described methods [39,43,60,61]. Animals from C-1 and C-2 were anesthetized with isofluorane and euthanized by transcardial perfusion with ice-cold PBS. Brains were then quickly removed, dissected, and
stored at −80 °C for subsequent Western blot analyses. For mice in C-5, 24 h following the cessation of the RS period, animals were anesthetized with isofluorane and transeardially perfused with PBS and 4% paraformaldehyde. Brains were then dissected and kept in 4% paraformaldehyde for subsequent immunohistochemical analysis.

2.6.2. Western blot analysis

Tissues from animals in C-1 and C-2 were analyzed by Western blot as previously described [43]. Briefly, previously sectioned brains (see above) were removed from −80 °C and region-of-interest (PFC; hippocampal formation) dissected on a dry ice. Dissected regions were then homogenized by sonication in cold cell lyses buffer containing phosphatase inhibitors (Cell Signaling Technology, Danvers, MA), supplemented with 1 mM phenylmethanesulfonylfluoride (Sigma–Aldrich) and a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Brain extracts were then incubated on ice for 30 min and centrifuged at 20,000 × g for 30 min at 4 °C. Protein concentrations of supernatants were measured using the Bio–Rad protein assay (Bio–Rad, Hercules, CA) and equal amounts of protein were separated by gradient (4–20%) SDS–PAGE then transferred to nitrocellulose membranes (BA83, 0.2 µm, Bio–Rad). The membranes were blocked in TBS with 0.1% Tween 20 (TBST, Bio–Rad) containing 10% skim milk for 45 min and incubated in TBST with primary antibodies overnight at 4 °C. These include polyclonal antibodies directed against phospho-GSK3β (Serine 9) and total GSK3β (all from Cell Signaling Technology). Further, a mAb against synaptophysin (BD Biosciences, San Jose, CA) was used to detect synaptic density and a mAb against β-tubulin (1:10,000; Promega, Madison, WI) was used to control protein sample loading and transfer efficiency. Following wash, membranes incubated with LI-COR’s goat anti-mouse IRDye secondary antibodies (LI-COR Biosciences, Lincoln, NE) for 1 h. After final wash, images were acquired using an Odyssey Infrared Imaging System (LI-COR Biosciences) and analyzed using NIH Image J software.

2.6.3. Immunohistochemistry

For animals in C-5, tissues were routinely processed in paraffin blocks and later divided into 5 coronal sections (per set) with a 100-µm interval and a thickness of 5-µm for each brain region (CA1, CA3, and DG regions of the hippocampus, and EC: bregma −2.92 to −3.64 mm) [62]. Immunohistochemical staining was performed according to the manufacturer’s protocol using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) coupled with the diaminobenzidine reaction. A synaptophysin monoclonal antibody (clone SY38, 1:10, Dako, Carpinteria, CA) was used as a primary antibody. Using additional sets of sections, normal mouse serum (isotype control) or PBS was used instead of primary or secondary antibody or ABC reagent as negative controls.

2.6.4. Immunohistochemical image quantification

Quantitative image analysis was done based on previously validated methods [56]. Images were acquired as digitized tagged-image format files to retain maximum resolution using a BX60 microscope with an attached CCD camera system (DP-70, Olympus, Tokyo, Japan), and digital images were routed into a Windows PC for quantitative analyses using SimplePCI software (Hamamatsu Photonics). For the platform, we captured images of five 5-µm sections through each anatomic region of interest (hippocampal regions and EC) based on anatomical criteria defined by Franklin and Paxinos [62] and a threshold optical density was obtained that discriminated staining from background. Each anatomic region of interest was manually edited to eliminate artifacts. To evaluate synaptophysin immunoreactivity (IR), images were converted to gray scale and the average optical density of positive signals from each image was quantified in hippocampus (CA1, CA3, and DG sub-regions) and EC as a relative number from zero (white) to 255 (black) and expressed as mean intensity of synaptophysin IR. Each analysis was done by a single examiner blinded to sample identities.

2.7. Statistical analyses

For analyses of all measures where two cohorts were included, the group means and standard error of each vehicle-treated, NS groups were first compared to ensure differences between cohorts were not attributable to unpredictable confounding factors and cohorts could be combined in subsequent analyses. Therefore, for all applicable comparisons (RAWM, TST and PT), vehicle-treated, NS mice were pooled and differences between experimental groups were compared using a 2 (stress) × 2 (treatment), multifactorial analysis of variance (ANOVA). For the RAWM test, a multifactorial, 2 (stress) × 2 (treatment) × 9 (day) repeated measures ANOVA was first conducted followed by analyses of pooled days using the aforementioned 2 × 2 ANOVA. The weight analysis was performed using a 2 (stress) × 3 (period) repeated measures ANOVA. For Western blot data and immunohistochemical tests, comparisons using three groups were performed using a one-way ANOVA followed by a Tukey or Tukey–Kramer post hoc analyses. For comparisons with only two groups (OF, Western blot, Immunohistochemistry), the Student’s t-test was used. For all tests, α = 0.05.

3. Results

3.1. Cotinine treatment improved working memory impairment in stressed and non-stressed mice

It is well known that RS induces changes in cognitive abilities in rodents [27]. To investigate the effect of cotinine in spatial and working memory after chronic stress, mice were tested in the RAWM test (Fig. 1). The results of the overall performance in the RAWM test show that cotinine improved working memory performance in both RS and control groups (Fig. 1B and C). A multifactorial ANOVA (2 × 2 × 9; stress × treatment × days) on erroneous decisions in the delayed trial 5 (TS) revealed a significant main effect of stress (F(1,30) = 10.227; p = 0.003) thus demonstrating that RS mice (regardless of treatment) made more erroneous choices in the RAWM test than controls (not shown). Furthermore, there was a significant effect of treatment (F(1,30) = 15.602; p = 0.005), as well as days (F(8,223) = 2.433; p = 0.046), with an interaction between the two (F(8,223) = 2.604; p = 0.035). However, there was no interaction between treatment and stress, regardless of days (F(1,30) = 1.819; p = 0.180). Therefore, cotinine improved the accuracy of locating the platform in both RS and NS groups.

The data of latency times to find the platform were also tested using a multifactorial ANOVA (2 × 2 × 9; stress × treatment × days), which revealed a significant effect of treatment (F(1,30) = 5.813; p = 0.022), and days (F(8,223) = 3.901; p = 0.005), with an interaction between the two (F(8,223) = 2.861; p = 0.023). The statistical analysis did not reveal a significant effect of stress over the latency to find the hidden platform (F(1,30) = 0.055; p = 0.816). Therefore, cotinine increased the speed to find the platform regardless of the presence of stress.

During the final block of testing (days 8–9), a 2 × 2 ANOVA (treatment × stress; Fig. 1B) revealed a significant effect of treatment (F(1,30) = 17.35; p = 0.0002) but not stress (F(1,30) = 1.951; p = 0.1728). Furthermore, a 2 × 2 ANOVA of latencies during the final block (treatment × stress; Fig. 1C) similarly revealed a significant effect of treatment (F(1,30) = 5.876; p = 0.0216) but not stress (F(1,30) = 0.5017; p = 0.4842). Therefore, during the final block of testing, results
demonstrate that cotinine has a positive effect on working-memory regardless of chronic stress exposure.

3.2. Effect of cotinine on locomotor activity after chronic RS

To assess changes on stress-induced increases of locomotor activity induced by cotinine, total distance moved and velocity in the OF test were analyzed from mice subjected to RS in C-1. Student’s t-tests reveal that cotinine-treated RS mice ambulated less ($t = 2.30, p = 0.04$) and more slowly ($t = 2.30, p = 0.04$) than vehicle-treated cohorts. Pertinent descriptive statistics are shown Table 1.

3.3. Effect of cotinine on the weight of mice exposed to RS

We have previously shown that cotinine treatment does not influence weight gain in non-stressed and acute stress conditions [39]. To determine if cotinine influences weight in highly stressful events, the weights prior to, during, and following RS from mice in C-1 were analyzed using a multifactorial ANOVA (2 × 3; treatment × time). This analysis showed that overall, cotinine did not influence weight gain, regardless of length of treatment or subject to RS ($F_{(1,31)} = 0.31; p = 0.59$; Table 1).

3.4. Cotinine significantly reduced depressive-like behavior in stressed and non-stressed mice

The effect of cotinine on depressive-like behavior in NS and RS conditions was tested in mice from C-1, C-3 and C-4 (Fig. 2). In the TST test, a two-way ANOVA showed that there were significant main effects of stress ($F_{(1,126)} = 8.78, p = 0.0064$) and treatment ($F_{(1,126)} = 20.44, p < 0.0001$) but with no interaction ($F_{(1,126)} = 3.165, p = 0.0869$). The data shows that cotinine treatment markedly reduced immobility times in the TST regardless of stress (Fig. 2B). This finding was replicated in the PT (Fig. 2C). A two-way ANOVA analysis of immobility in the PT revealed that there were significant differences in immobility induced by stress ($F_{(1,25)} = 87.263, p < 0.0001$) and treatment ($F_{(1,25)} = 32, p < 0.0001$) with no interaction found ($F_{(1,25)} = 0.0135, p = 0.9086$). Thus, cotinine treatment reduces depressive-like behavior associated with the TST and PT in mice regardless of the presence and effects of stress.

3.5. Cotinine activated the GSK3β pathway in PFC and hippocampus of RS mice

It has been shown that GSK3β activity is involved in the neuronal changes associated with depressive-like behavior [63-66]. We investigated the effect of cotinine on the expression of the inactive form of GSK3β, phospho-GSK3β (Ser9) in PFC and hippocampus of mice in C-1 and C-2. In PFC of C-1 mice, a one-way ANOVA revealed significant difference between treatment groups ($F_{(2,9)} = 5.057, p = 0.0337$; Fig. 2D). Unexpectedly, vehicle-treated NS mice showed levels of phospho-GSK3β similar to vehicle-treated RS mice as indicated by a Tukey-Kramer post hoc test. However, cotinine-treated RS mice showed significantly higher levels of the inactive form of GSK3β than the vehicle-treated RS mice (Fig. 2D). In NS mice of C-2, cotinine treatment significantly elevated PFC levels of phospho-GSK3β ($t = 3.753; p = 0.0056$; Fig. 2F). In the hippocampus of C-1 mice, the differences in GSK3β expression were not statistically significant between groups ($F_{(2,17)} = 1.144, p = 0.3418$; Fig. 2E). In the absence of RS, cotinine-treated mice in C-2 showed higher levels of phospho-GSK3β when compared to vehicle-treated NS mice in hippocampus. However, these differences were not statistically significant ($t = 1.066; p = 0.3$).

3.6. Cotinine increased the synaptic density in hippocampus, EC, and PFC of chronic stress mice

To further investigate the effect of cotinine in synaptic density after RS, we assessed synaptophysin expression in brains on mice from C-1 and C-2 (Fig. 3). First, the expression of synaptophysin in PFC and hippocampus was assessed by Western blot (Fig. 3A and B). A one-way ANOVA of synaptophysin expression data in PFC of C-1 revealed significant differences between treatment groups ($F_{(2,18)} = 10.87, p = 0.0008$; Fig. 3A). The post hoc Tukey-Kramer test showed that synaptophysin expression in the PFC of C-1 mice was no different between vehicle-treated NS and RS mice ($p = 0.05$). However, cotinine-treated RS mice showed significantly higher levels of synaptophysin than both vehicle-treated NS ($p < 0.01$) and RS mice ($p < 0.01$). Moreover, cotinine-treated NS mice from C-2 did not show significantly higher levels of synaptophysin than vehicle-treated RS mice in PFC ($t = 0.6073; p = 0.56$) (Fig. 3C). In the hippocampus of C-1 mice, however no significant difference in synaptophysin expression was found between groups (ANOVA, $F_{(2,16)} = 0.28, p = 0.7539$; Fig. 3B). Also, in the absence of RS (C-2), cotinine-treated NS mice showed similar levels of synaptophysin when compared to vehicle-treated RS mice ($t = 1.964; p = 0.106$) (Fig. 3D).

To better define the effect of cotinine on the expression of synaptophysin in hippocampus of RS mice, two additional cohorts of mice were treated, exposed to RS, and immediately euthanized in absence of behavioral testing. Then, brains were dissected and processed for immunohistochemical analysis of synaptophysin IR. The immunolabeling data was compared between groups using Student’s t-test. This analysis showed that cotinine-treated RS mice displayed significantly greater levels of synaptic density throughout several brain regions (Fig. 4C–F). Higher levels of synaptophysin expression were found in CA1 ($t = 6.986; p < 0.0001$; Fig. 4C), CA3 ($t = 6.077; p < 0.0001$; Fig. 4D), DG ($t = 4.147; p = 0.0011$; Fig. 4E), and EC ($t = 8.486; p < 0.0001$; Fig. 4F) from the cotinine-treated RS mice.

4. Discussion

Chronic stress is associated with the development of several psychiatric conditions leading to cognitive and emotional impairments such as major depression and PTSD [1,2]. Treatment options to mitigate these effects are limited in efficacy and may help only a small proportion of those affected, leading many of the afflicted to search for relief elsewhere, perhaps in tobacco, and/or other addictive substances or behaviors [67]. Here, we provide evidence supporting the therapeutic value of cotinine in treating depression and memory deficits in individuals with psychiatric disorders triggered by stress. Chronic, systemic cotinine treatment administered prior to and following chronic stress mitigated many of the deleterious effects of prolonged restraint stress (RS), including working memory impairment and depressive-like behavior. Furthermore, cotinine increased the synaptic densities in various hippocampal and limbic cortices of restrained mice. As expected, we also confirmed that cotinine produces nootropic effects on non-stressed mice. However, we provide novel findings that cotinine reduces depressive-like behavior in chronic stress and non-stressed conditions. Finally, we observed a cotinine-induced increase of the inactive form of cerebral GSK3β. This protein kinase is a key signaling factor controlling mood and neuronal repair [68,69].

4.1. The effects of cotinine on emotional behavior in mice exposed to chronic stress

We have recently demonstrated that cotinine attenuated anxiety-like behavior following an acute stressor in mice [39]
Fig. 3. Cotinine increased synaptic density after chronic restraint stress in mice. In restraint stress (RS) mice from C-1 (n = 5–8/group), cotinine significantly increased the levels of synaptophysin in PFC (p = 0.0008; A) but not hippocampus (p = 0.7539; B). No changes were observed in non-restraint stress exposed (NS) mice (C-2; n = 8/group) in either PFC (p = 0.560; C) or hippocampus (p = 0.106; D). Plots represent synaptophysin immunoreactivity (IR) as tested by Western blot. The IR data was normalized against tubulin levels and expressed as percentage of control values. Cot 5, cotinine (5 mg/kg); ***p < 0.001; ns, not statistically significant; Veh, vehicle.

Fig. 4. Immunohistochemical analysis of the effect of Cotinine on synaptic density after chronic restraint stress in mice. One week after arrival, mice from cohort 5 (n = 7–8/group; A) began treatment with cotinine (5 mg/kg; gray bars) or vehicle (black bars) which continued daily for 7 days and throughout the restraint stress (RS; 6 h daily for 21 days) period. 24 h after RS, mice were euthanized and brains tissues were analyzed for the expression of synaptophysin by immunohistochemical analysis. (B) The micrograph left depicts the increase in synaptophysin immunoreactivity (IR) in CA1 and CA3 regions of hippocampus, dentate gyrus (DG), and entorhinal cortex (EC; scale bar = 30 μm). The plots to the right represent synaptophysin IR in CA1 (p < 0.0001; C) and CA3 (p < 0.0001; D), and DG (p = 0.0011; E) regions of hippocampus and EC (p < 0.0001; F) in mice subjected to RS treated with vehicle (black bars) or cotinine 5 mg/kg (gray bars). ****p < 0.0001; **p < 0.01.
and also improved both learning and memory in AD model mice [43]. However, nothing has been reported regarding cotinine’s influence on mood or cognitive abilities in prolonged or elevated salience-of-stress conditions. Our results indicate that pre- and concurrent treatment with cotinine significantly reduces the levels of depressive-like behavior in non-stressed and chronically restrained mice. This was evidenced across the two most commonly used tests for antidepressant activity, TST and PT [59]. To our knowledge, this is the first evidence of the antidepressant effects of cotinine in stressed or non-stressed conditions.

Because TST and PT are inherently reliant on measures of mobility, the cotinine-induced reduction of immobility in these tests could be interpreted as a cotinine-induced sensitization of locomotor activity. However, previous evidence suggests that cotinine does not influence OF ambulation in non-stressed or acute stress conditions [39]. Here, we show that following prolonged RS, cotinine-treated mice ambulated less than vehicle-treated cohorts in the OF test, though this was greater than mice not subjected to RS (Table 1). Therefore, our findings of a cotinine-induced reduction in immobile postures in both TST and PT tests cannot be explained by a cotinine-induced change in locomotor behavior in the OF test. Since stress has been shown to increase the locomotor response [70], we postulate that the observed cotinine-induced decrease in ambulation in the OF tests likely reflects a mitigation of the effect of the chronic stress exposure. Taken together, our results demonstrate that cotinine treatment reduces depressive-like behavior in chronically stressed and non-stressed conditions.

The fact that cotinine, a tobacco-derived compound, decreased depressive-like behavior is in line with the hypothesis that tobacco is used to improve mood balance [67,71,72]. The vast literature supports this view through self-reports and clinical observations, which indicate high correlations between nicotine dependence and psychiatric conditions characterized by depressive behavior [67,72,73]. Growing interest into the therapeutic potential of cotinine is continually providing support for the hypothesis that cotinine likely underlies many of the benefits associated with nicotine use [33,41,42,44], including improved attention, learning, and executive functions [74,75].

4.2. Cotinine protects from working memory loss and influences synaptic density after stress

To date, numerous studies have suggested that cotinine improves learning and memory abilities, though little is known about its effects under highly stressful conditions. Early evidence demonstrated an enhancement induced by cotinine in animal performance across various learning and memory tasks [36,76]. More recently, reports have shown a cotinine-induced improvement in working memory, attention and executive function in various animal models of conditions such as schizophrenia, AD and aging [41–44]. Here, we show that cotinine enhanced the spatial and working memory performance in both RS and NS wild-type C57BL/6j mice in the RAWM test. Our data shows that cotinine’s nootropic activity extends to chronic stress conditions. This evidence supports the notion that cotinine is a versatile general memory enhancer, with the potential to protect against the deleterious effects of stress over cognitive abilities. Whether these nootropic effects are due to a preventative or a dynamic restorative effect remains to be elucidated.

Synaptogenesis is a dynamic process, which inherently influences neuronal connectivity and brain functioning including cognitive abilities and mood. Because stress has negative effects on neurite outgrowth and synapse formation [77], the resulting neuronal changes are thought to affect behavior and are considered to play an important role in psychiatric disorders [78].

It has long been shown that repetitive RS, in addition to inducing working memory deficits [79], provokes atrophy of neurites and reduces the number of synaptic sites in the hippocampus and other limbic regions of the brain [21]. Synaptophysin is found in synaptic vesicles and is the most widely used marker for the determination of synaptic density [80]. To determine whether the positive effects of cotinine on behavior were accompanied by an increase of synaptic density in the brain, we investigated the effect of cotinine on the expression of synaptophysin in brains of RS mice. We found that cotinine-treatment resulted in a significant increase in synaptophysin expression in PFC of RS mice, though such an increase was not detectable in the hippocampus of those animals.

Western blot analysis cannot reveal localized changes in synaptic density. Therefore, we used immunohistochemical analysis to determine synaptophysin expression in several brain sub-regions. Since training in the RAWM can induce changes in factors controlling synaptic density such as the brain-derived neurotrophic factor [81], we performed this analysis immediately after RS. Results show that cotinine induced a pronounced increase of synaptic density in EC, DG, and hippocampus (CA1 and CA3 regions). These observed differences in synaptic density likely underlie the improved memory performance after chronic stress. The dendritic remodeling in hippocampus and PFC regions induced by stress is thought to also underlie depressive-like behavior. Thus, we postulate that the observed reductions of stress-induced depressive-like behavior and memory impairment in cotinine-treated animals are due, at least in part, to its enhancing effects on synaptic density. This effect of cotinine is not likely related to its ability to increase 5-HT availability as previous evidence show that SSRI are not able to reduce the stress-induced dendritic atrophy in rats [82]. Therefore, the mechanism(s) through which cotinine exerts its effects on synaptic plasticity still need to be fully elucidated. Since synaptic deficits are associated with numerous neurological and psychiatric conditions leading to memory loss and depression, cotinine may

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Table 1

<table>
<thead>
<tr>
<th>Locomotor activity</th>
<th>Vehicle</th>
<th>Cotinine</th>
<th>t</th>
<th>df</th>
<th>P</th>
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<tbody>
<tr>
<td>Open field</td>
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<td>2.30</td>
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<tr>
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<td>4.22</td>
<td>2.30</td>
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<table>
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<th>F</th>
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<th>P</th>
</tr>
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<td>27.4; 0.61</td>
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<td>0.59</td>
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<table>
<thead>
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<th>Weight</th>
<th>Day</th>
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<th>Cotinine</th>
<th>F</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
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<td>28.2; 0.89</td>
<td>27.5; 0.70</td>
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<tr>
<td>Pre-restraint</td>
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<td>27.9; 0.84</td>
<td>27.4; 0.61</td>
<td></td>
<td></td>
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<tr>
<td>Post-restraint</td>
<td>35</td>
<td>27.8; 0.90</td>
<td>27.2; 0.43</td>
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</tbody>
</table>

* Significantly different (p < 0.05).
potentially be useful in alleviating these deficits in many of these conditions.

4.3. Cotinine's influence over stress-induced depression and memory impairment may involve GSK3β inhibition

GSK3β is a ubiquitous, expressed, serine-threonine protein kinase that has been implicated in many mental health conditions including mood disorders and disease-related cognitive impairments [64,89]. GSK3β is found constitutively active in the brain and its activity is inhibited via phosphorylation by several protein kinases (PKs) including PKA, PKB (Akt), and PKC [68]. Thus, this factor is a common target for many signaling pathways promoting synaptic plasticity and memory abilities. In fact, inhibitors of GSK3α, such as lithium, SSRIs, tricyclics and other serotonin-related antidepressants [63], have been reported to protect and/or restore cognitive functions under several pathological brain conditions (see [64] for review).

Cotinine administration has been shown to contribute to GSK3β inhibition by stimulating the Akt/GSK3β pathway in vitro [84,85] and in vivo [43]. Therefore, we sought to determine the effect of cotinine on the expression of GSK3β in the hippocampus and PFC of mice following prolonged RS. Not surprisingly, cotinine-treatment resulted in an increase in the inactive form GSK3β in RS mice. Consistent with previous findings, these mice also displayed lower levels of depressive-like behavior compared to vehicle-treated RS mice.

The inhibition of GSK3β by cotinine may be mediated by multiple factors including the enhancement of the 5-HT and/or dopamine neurotransmission [86,87]. In fact, GSK3β has recently been shown to mediate both dopaminergic [88] and serotoninergic [89,90] neurotransmission. To better understand these potential mechanisms, further studies are needed to investigate whether cotinine effects are mediated by an increase in 5-HT or dopamine release.

5. Conclusion

Tobacco use is higher in depressed individuals, who may use it as a way to counteract the negative effect of stress. However, tobacco is addictive and associated with serious health problems including cardiopulmonary and cardiovascular diseases [91]. Treatment options for conditions associated with emotional imbalance and cognitive decline are limited. Nicotine, the predominant metabolic product of tobacco, which has been shown to have some beneficial effects on brain functions, is also associated with numerous health problems [92]. Cotinine, the predominant metabolite of nicotine, may represent a new therapeutic option to mitigate the deleterious actions of stress. Cotinine is not addictive, has a positive safety profile and pro-cognitive, antidepressant and anxiolytic activities. Furthermore, cotinine's effects are distinct from those of nicotine and other components of tobacco. In fact, some of nicotine actions are even antagonized by cotinine in humans [93]. Here, we report for the first time evidence that cotinine has anti-depressant effects and prevents stress-induced spatial memory deficits and increases synaptic density in several brain regions in RS mice. Future studies are required to reveal the significance of these findings as they pertain to the human condition.

Conflict of interest

The authors report no potential conflict of interest.

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