Pharmacology, Biochemistry and Behavior 181 (2019) 1–8

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Prolonged exposure of rats to varenicline increases anxiety and alters serotonergic system, but has no effect on memory

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ARTICLE INFO

Keywords:
Varenicline
Anxiety
Memory
Cholinergic receptors
Central nervous system

ABSTRACT

Varenicline is a drug used for smoking addiction cessation treatment and acts as a partial agonist of nicotinic cholinergic receptors. Recent clinical trial data support use of varenicline for treatment of conditions/addictions that are not related to smoking cessation. Considering the importance of this issue and the need for new studies on its effects, especially on behavior, more studies using animal models are necessary. Thus, the aim of this study was to evaluate the effects of prolonged exposure to varenicline in anxiety-like behavior and memory, as well as in cerebral neurochemistry of rats. Male rats received three different doses of varenicline: 0.03 (therapeutic dose for humans), 0.1 and 0.3 mg/kg orally (gavage) for 30 days. Animal behavior was analyzed through open field, elevated plus maze, light/dark box, social interaction, Barnes maze and novel object recognition tests. Neurotransmitter levels and their metabolites in different brain structures (hippocampus, striatum and frontal cortex) were measured. Results showed that prolonged exposure of rats to varenicline: 1) did not interfere in motor activity, but caused an anxiogenic effect on elevated plus maze, light/dark box and social interaction testes; 2) did not alter memory; and 3) promoted alterations on serotoninergic system in the striatum and frontal cortex. In conclusion, compilation of the data indicates that prolonged exposure of rats to varenicline promoted anxiogenic effects and alteration in serotonergic system, which corroborated behavioral findings.

1. Introduction

Varenicline is a synthetic chemical substance produced by cytosine alkaloid from *Cytisus laburnum* L. and it's clinically used for smoking cessation treatment (Cahill et al., 2012; Crunelle et al., 2009; Mihalak et al., 2006). This substance has a higher rate of long-term smoking cessation than other methods, such as nicotine replacement (patch, nicotine gum and spray) and related medications (bupropion and cytisine) (Nocente et al., 2013; King et al., 2011).

Varenicline binds to nicotinic cholinergic receptors in the central nervous system. Among the cholinergic receptors, varenicline has high affinity for types α4β2, α3β4 and α7 (Arias et al., 2015; Rollema et al., 2010). It acts as a partial agonist of α4β2 and α3β4 receptors and as a full agonist of α7 receptors (Crunelle et al., 2009). In its role as a partial agonist, varenicline acts via two mechanisms in order to exert its therapeutic effects: 1) it binds and activates nicotinic cholinergic

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https://doi.org/10.1016/j.pbb.2019.03.009
Received 5 October 2018; Received in revised form 26 March 2019; Accepted 29 March 2019
Available online 01 April 2019
0091-3057/

receptors, which causes the release of dopamine, similarly to actions of nicotine, but with a diminished response; this agonistic effect ameliorates the intensity of withdrawal syndrome; 2) it acts as a competitive inhibitor of nicotine, therefore preventing binding of the later to nicotinic cholinergic receptors and avoiding addictive reinforcing effects of continuous nicotine use (antagonistic effect) (Iida et al., 2012; Jorenby et al., 2006).

Several studies exploring other effects of varenicline are currently being held. Based on its mildly rewarding mechanism of action involving release of dopamine in the dorsal striatum and nucleus accumbens, researchers have indicated the possibility of varenicline treatment for dependency on other addictive drugs (Faessel et al., 2010; Turner et al., 2011). Furthermore, reduced numbers of dopaminergic receptors D2 and D3 were found to be a common phenotype of susceptible individuals towards increased risk of developing drug addiction (Nader et al., 2006). Varenicline might exert beneficial effects by increasing the availability of D2 and D3 (Crunelle et al., 2009). Studies performed by Steensland et al. (2007) and Ericson et al. (2009) have shown that varenicline could reduce alcohol consumption through minimization of dopamine release at the brain reward center. In addition, preliminary data presented by Hooten and Warner (2015) suggest that varenicline was able to decrease withdrawal symptoms caused by cessation of several recreational drugs.

Varenicline has also been described as having effects on memory and cognition. Preclinical studies performed by Patterson et al. (2009) showed that this drug was able to improve the attention and working memory of patients after only three days of nicotine withdrawal. The mechanism by which varenicline affects memory and learning is not yet elucidated. The central hypothesis for such effects takes into consideration the release of presynaptic neurotransmitters, especially gamma-aminobutyric acid – GABA (Rollema et al., 2011).

Recent clinical trial data support varenicline use as an effective and generally well-tolerated therapy for smoking cessation in healthy adult smokers and embolden expansion of its use for non-smoking related clinical treatments. Considering the importance of this issue and the need for new studies on its effects, especially on behavior, more studies using animal models are necessary. Thus, in the present study, we have used rats to evaluate anxiety-like and memory-related behaviors. We further aimed to evaluate the effects of varenicline's prolonged exposure on cerebral neurochemistry.

2. Methods

2.1. Animals

Male Wistar rats (90 days old) were obtained from the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo. All rats were housed in 43 × 23 × 16 polycarbonate cages and maintained in specific conditions of constant 12-hr light/dark cycles (light: 06:00–18:00), controlled temperature (22 ± 2 °C) and free access to food and water (Zaccarelli-Magalhães et al., 2018).

All animal manipulations followed the Ethical Principles of Animal Research that were adopted by the Ethics Committee on the Use of Animals by the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol no. 3304041214) and by the Presbyterian Mackenzie University (protocol no. 132/11/2015).

2.2. Drug and treatment

Varenicline tartrate was purchased as the commercial product Champix® (Pfizer) and dissolved in water. The rats received 0.03 (comparable to therapeutic dosage for humans), 0.1 and 0.3 mg/kg of varenicline (King et al., 2011; Wecker et al., 2013; Goutier et al., 2013) or drinking water (control group) by gavage in volumes that did not exceed 1.0 ml/kg body weight. This route of administration was chosen since it resembles exposure of individuals to varenicline under normal treatment conditions.

The rats were divided into 4 groups: 3 experimental groups that received different doses of varenicline (0.03, 0.1 or 0.3 mg/kg) and a control group that received water by gavage daily for 30 consecutive days. The administration of varenicline or water occurred daily in the morning, between 9 and 10 am, and the behavioral tests were conducted 1 h after drug administration. All equipment used for behavioral assessment were cleaned with 5% ethanol solution before each test. Control and experimental rats were intermixed for all measured observations.

2.3. Experiment 1: evaluation of anxiety-like behavior

Twenty-eight rats were divided into four experimental groups (n = 7 animals/group), which were in turn treated with varenicline or water by gavage daily for 30 consecutive days. The rats were exposed to the open field test and the elevated plus maze on the 28th day of treatment. The dark/light box test was performed on the 29th day of treatment and the social interaction test on the 30th day of treatment.

The open field test was conducted in a round arena as described by Sandini et al. (2014). Hand-operated counters were used to score locomotion frequency (the number of floor sections covered), rearing frequency (the number of times the animal stood on its hind legs) and grooming frequency (the number of times the animal touched its forepaws to the head and snout). A chronometer was used to measure the duration of immobility (the total time in seconds without spontaneous movement).

The elevated plus maze test was conducted as described by Sandini et al. (2015). Hand-operated counters were used to score the frequency of entries in the open arms and in the closed arms, and a timer was used to measure the time spent in the open and close arms.

The light/dark box test was performed as described by Kumar et al. (2003). A timer was used to measure the latency in the dark compartment and the time spent in the light and/or dark compartments.

The social interaction test was performed by observing the behavior of a pair of rats placed in the open field arena. The tested rats were from the same experimental group and the weight variation did not exceed 20 g. Initially, each rat was placed individually in the open field for 10 min for apparatus habituation. On the second day, the pair of animals was placed in the open field for 10 min for habituation in the presence of the testing partner. On the third day, the test itself was performed, when the pair was placed again in the open field and their behavior was observed for 10 min. The total time of social interaction was evaluated. Social interaction behaviors taken into account consisted of sniffing, chasing and passing the other rat.

2.4. Experiment 2: evaluation of memory and cognition

Twenty-eight rats were divided into four experimental groups (n = 7 animals/group) that were either treated with varenicline or water by gavage daily for 30 consecutive days. The rats were submitted to the Barnes maze and the novel object recognition tests.

The Barnes maze test was conducted as described by Sandini et al. (2018). Each rat was submitted to 4 days of training and 1 day of test (fifth day), going through the apparatus in the morning (between 10:00 and 12:00 a.m.) and in the afternoon (between 2:00 and 4:00 p.m.). The parameters evaluated were latency (seconds) to enter the escape box and number of errors (number of openings the rat sniffed before finding the escape box). Observations were made from the 24th to 28th days of treatment.

The novel object recognition test was conducted as described by Antunes and Biala (2012). Briefly, the test was conducted during 3 days, from the 28th to 30th day of treatment. On the first day each rat was placed alone in the open field arena for 5 min (habituation phase). On the second day each rat was placed in the open field with two identical

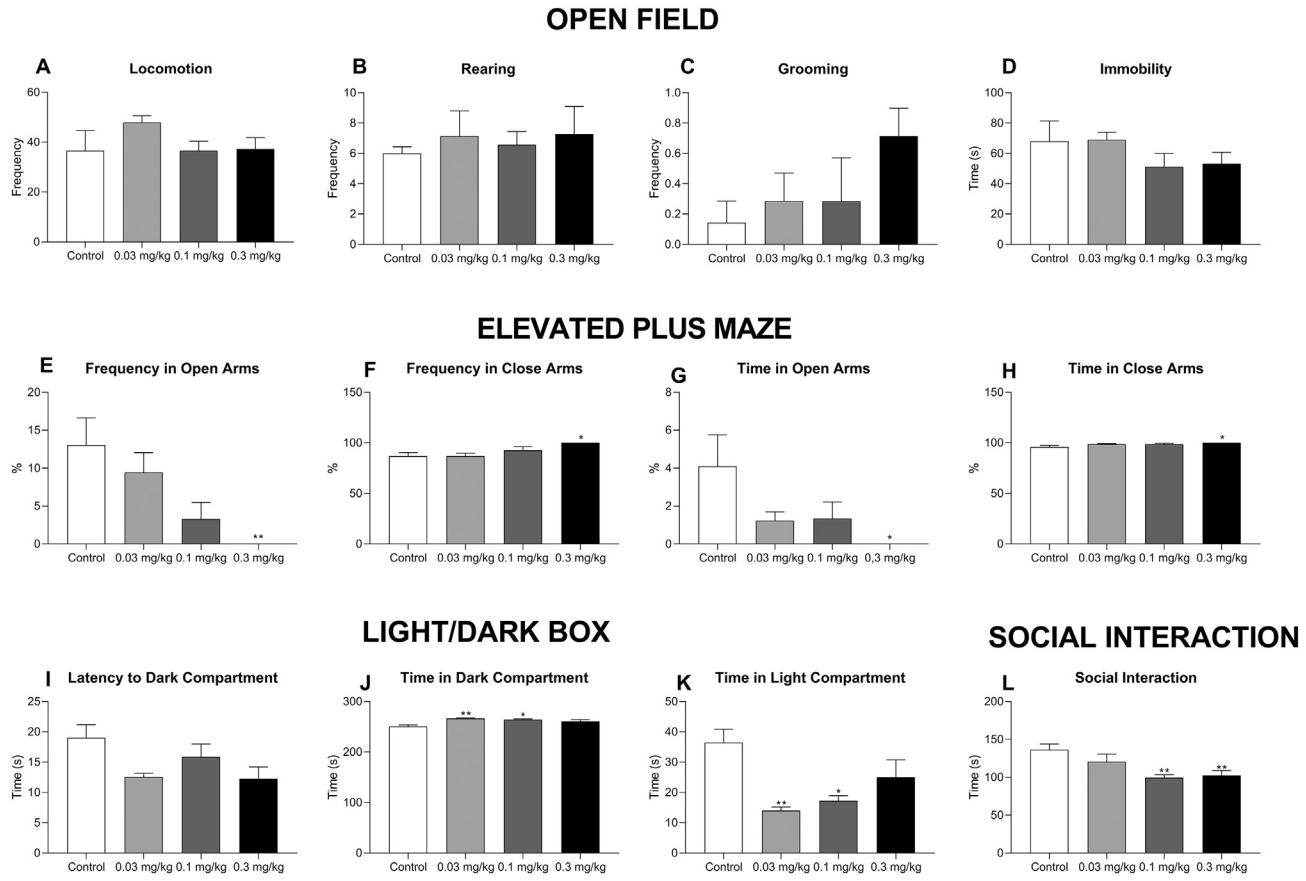

Fig. 1. Evaluation of anxiety-like behavior in rats that received varenicline (0.03, 0.1 or 0.3 mg/kg) or water (control) by gavage for 30 days. Means and respective standard errors are displayed. (A–D) parameters evaluated in the open field test, (E–H) parameters evaluated in the elevated plus maze test, (I–K) parameters evaluated in the light/dark box test, and (L) total time of social interaction. $n = 7$ animals/group.
$^{*}p < 0.05$, $^{**}p < 0.01$, (A–D; I–L) one-way ANOVA followed by Dunnett's post-hoc and (E–H) Kruskal-Wallis followed by the Dunn's post-hoc.

objects for 5 min (familiarization phase). On the third day each rat was placed again in the open field for 5 min, but one of the objects from the familiarization phase was exchanged for a novel object, completely different from the one used for familiarization on the day before. Time spent exploring the novel and the familiar object (it was considered exploration the behaviors of sniffing and/or thigmotaxis) was measured using a timer. The preference index was calculated by taking the ratio between time exploring the novel object over the total time spent exploring both objects.

2.5. Experiment 3: evaluation of cerebral neurochemistry

Thirty-two rats were subdivided into four experimental groups ($n = 8$ animals/group) that were either treated with varenicline or given water (control group) by gavage daily for 30 consecutive days. On the 30th day of treatment, 1 h after the varenicline administration, rats were submitted to euthanasia by decapitation in order to obtain respective brain structures as described below for determination of neurotransmitters levels as well as their metabolites.

Following decapitation, the rats' brains were removed and placed on dry ice for preservation. Entire brain structures corresponding to the hippocampus, striatum and frontal cortex were collected in order to maximize the amount of biological material. The collection procedure was performed quickly, not exceeding 3 min of total time. The structures were placed in Eppendorf tubes on dry ice for rapid freezing and were stored for up to 20 days at a temperature of −80 °C.

Continuation of experiments consisted of homogenization of collected structures in 0.1 M perchloric acid ($ClHO_4$) containing 3,4-dihydroxybenzylamine (DHBA - internal standard) using a high-frequency sonicator. After homogenization, the structures were left overnight in a refrigerator at 10 °C for precipitation of proteins and nucleic acids. Next, samples were centrifuged at 10,000 rpm (Eppendorf®-5804 R centrifuge), at 4 °C for 30 min. Supernatants were removed and placed in Eppendorf tubes to be stored at −80 °C until further analytical quantification.

Measurements of dopamine (DA) and its metabolite [4,4-dihydroxyphenylacetic acid (DOPAC)], serotonin (5HT) and its metabolite [5-hydroindole, 3-acetic acid (5HIAA)] and GABA were obtained. Turnover rates were calculated by taking the ratios of metabolites over neurotransmitters:

The monoamines (dopamine and serotonin) and their metabolites analysis were analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-ED). A Shimadzu Model 20A chromatograph with a C-18 column (Shimpak) with line filter and an Antec Decade electrochemical detector were used. Each sample was run for 24 min and the detection limit ranged from 0.25 to 1 ng for all analyses.

GABA analysis was performed using high-performance liquid chromatography with a diode array detector (HPLC-DAD). A Shimadzu with a chromatographic column (ACE 3 C18-300®, 150 × 3.0 mm) coupled to a PDA (photo diode array) detector, set at a wavelength of 254 nm, was used. The analytical run was performed in up to 46 min and the detection limit was 20 ng for all analytes.

2.6. Statistical analyses

Data analysis was performed using software GraphPad Prism 6 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The Bartlett

test was used to verify homoscedasticity of the data. One-way ANOVA test was used for the open field, light/dark box, social interaction, novel object recognition tests and neurochemical evaluation, followed by the Dunnett test. Kruskal-Wallis test followed by the Dunn's test was used for the elevated plus maze experiment and for determination of preference index in the novel object recognition test. For the Barnes maze test, the two-way ANOVA with repeated measures was used, followed by the Bonferroni post-test. Results were expressed as the means ± standard errors or as median and their respective limits and the differences among the groups were considered statistically significant at $p < 0.05$.

3. Results

3.1. Experiment 1: evaluation of anxiety-like behavior

In the open field test, one-way ANOVA showed no significant differences in all parameters tested: locomotion ($F(3,24) = 1.128$, $p > 0.05$), rearing ($F(3,24) = 0.1959$, $p > 0.05$), grooming ($F(3,24) = 1.440$, $p > 0.05$) and immobility ($F(3,24) = 1.047$, $p > 0.05$) – Fig. 1A–D.

In the elevated plus maze test, Kruskal-Wallis showed significant differences between the groups in regards to frequency in open arms ($F(3,24) = 5.698$, $p < 0.01$), frequency in closed arms ($F(3,24) = 4.659$, $p < 0.05$), time in open arms ($F(3,24) = 3.194$, $p < 0.05$) and time in closed arms ($F(3,24) = 3.194$, $p < 0.05$). Dunn's post-hoc test showed that rats which had received 0.3 mg/kg of varenicline correlated with a decrease in frequency ($p < 0.01$) and time ($p < 0.05$) spent in the open arms and an increase in frequency ($p < 0.05$) and time ($p < 0.05$) spent in the closed arms compared with control group – Fig. 1E–H.

For the light/dark box test, one-way ANOVA showed significant differences between groups related to time spent in the dark compartment ($F(3,24) = 7.905$, $p < 0.001$) compared to time spent in the light compartment ($F(3,24) = 6.850$, $p < 0.01$), but did not show significant differences in the latency associated to the dark compartment ($F(3,24) = 3.009$, $p > 0.05$). Dunnett's post-hoc test showed that animals from the 0.03 and 0.1 mg/kg groups had an increase in time spent in the dark compartment ($p < 0.01$ and $p < 0.05$ respectively) and a decrease in time spent in the light compartment ($p < 0.01$ and $p < 0.05$ respectively) when compared to the control groups – Fig. 1I–K.

In the social interaction test, one-way ANOVA showed significant differences between groups ($F(3,24) = 5.435$, $p < 0.01$). Dunnett's post-hoc test indicated that animals which were exposed to 0.1 and 0.3 mg/kg of varenicline had decreased social interaction time when compared to control group ($p < 0.01$) – Fig. 1L.

3.2. Experiment 2: evaluation of memory and cognition

In the Barnes maze test, which measures the number of errors each animal makes before finding the escape box, two-way ANOVA with repeated measurements did not show significant differences between treatments ($F(3,18) = 0.7394$, $p > 0.05$), but showed significance in time differences ($F(4,24) = 10.24$, $p < 0.0001$) without interaction ($F(12,72) = 0.3908$, $p > 0.05$). Regarding the latency to finding the escape box, two-way ANOVA with repeated measurements did not show significant differences between treatments ($F(3,18) = 0.5569$, $p > 0.05$), but showed significant differences in time ($F(4,24) = 34.59$, $p < 0.0001$) without interaction ($F(12,72) = 0.5454$, $p > 0,05$) – Fig. 2A–B.

In the novel object recognition test, one-way ANOVA showed significant differences between the groups in time spent exploring the new object ($F(3,24) = 13.93$, $p < 0.0001$) and in time exploring the familiar object ($F(3,24) = 8.199$, $p < 0.001$). Dunnett's post-hoc indicated that animals exposed to all doses of varenicline had a decrease in time exploring the new object ($p < 0.0001$, $p < 0.0001$ and $p < 0.001$ respectively) and a decrease in time exploring the familiar object ($p < 0.05$, $p < 0.05$ and $p < 0.001$ respectively) – Fig. 2C and D. Regarding the preference index, the Kruskal-Wallis test showed no significant differences between groups ($p > 0,05$).

3.3. Experiment 3: evaluation of cerebral neurochemistry

For the neurochemical evaluation, one-way ANOVA showed significant differences in all structures analyzed. Regarding the striatum, one-way ANOVA showed significant differences in the levels of 5HT ($F(3,28) = 2.179$, $p < 0.05$), 5HIAA ($F(3,28) = 2.576$, $p < 0.05$) and GABA ($F(3,28) = 3.037$, $p < 0.05$). Dunnett's post-hoc test indicated that animals which received 0.03 mg/kg of varenicline had increased GABA levels ($p < 0.05$) and rats which received 0.3 mg/kg of varenicline had decreased levels of 5HT ($p < 0.05$) and 5HIAA ($p < 0.05$) when compared to control group levels– Table 1.

Regarding the hippocampus, one-way ANOVA showed significant differences only in GABA levels ($F(3,28) = 3.012$, $p < 0.05$). Dunnett's post-hoc indicated that animals exposed to 0.3 mg/kg of varenicline had decreased GABA levels ($p < 0.05$) when compared to the control group – Table 1.

One-way ANOVA analysis of frontal cortex neurochemical levels showed significant differences in 5HIAA/5HT turnover ($F(3,28) = 4.551$, $p < 0.05$). Dunnett's post-hoc indicated that animals exposed to the highest dose had increased 5HIAA/5HT turnover ($p < 0.01$) when compared to the control group – Table 1.

4. Discussion

In the present study, we have used a rat animal model to evaluate the effects of the typical therapeutic dose of varenicline used in humans (0.03 mg/kg) as well as a range of doses (0.1 and 0.3 mg/kg) that were administered via oral route (gavage). Our experimental design followed protocol guidelines for toxicity evaluation, which recommend testing of at least three different doses and a route of administration that is comparable to usage by individuals under normal conditions of treatment (OECD, 2008).

The aims of our study focused on the evaluation of varenicline prolonged exposure in regards to: (a) anxiety-like behavior and memory, and (b) relevant neurochemical components that modulate these behaviors.

Anxiety is defined as an adaptive response to threatening or dangerous situations and is triggered by different neurochemical changes in the body (Judd et al., 1985). Several animal models are available for evaluation of anxiety-like behavior, which show homologous behaviors to those presented by patients with anxiety disorder (Kumar et al., 2003).

The scientific literature does not have a consensus regarding varenicline being classified as an anxiolytic or as an anxiogenic drug. Turner et al. (2010) evaluated the effects of acute and chronic administration of nicotine and varenicline in three mice models of anxiety utilizing elevated zero-maze, marble-burying test and novelty-induced hypophagia test. The group showed that acute administration of varenicline had anxiolytic effects in marble-burying tests and novelty-induced hypophagia tests. Turner et al. (2011) evaluated anxiety-like behavior in mice through the marble-burying test and found an anxiolytic effect related to varenicline's usage. In a systematic review, Thomas et al. (2015) conducted a meta-analysis in order to determine the risk of neuropsychiatric adverse events in published controlled trials of varenicline. Results showed that treatment with the maximum dose of varenicline (1 mg twice daily) was associated with 25% reduction in the risk of anxiety.

Other studies have showed that varenicline triggered anxiety symptoms as a side effect of treatment. Our data is in accordance with a review by Parker (2016), which described that varenicline is one of

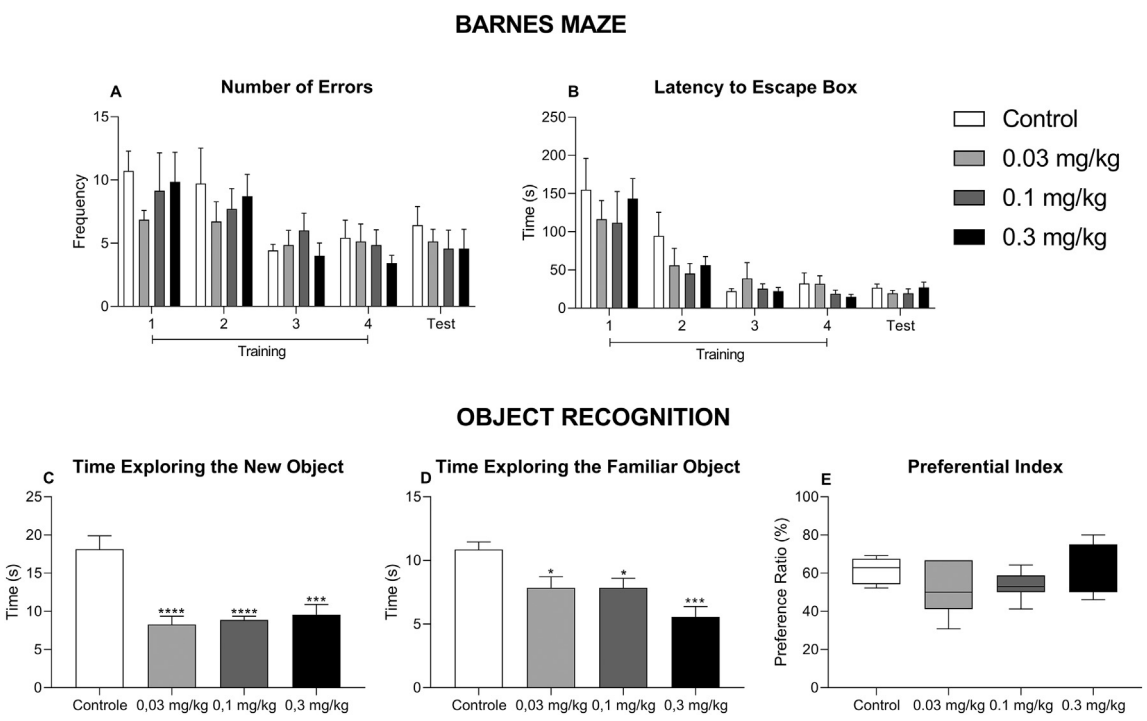

Fig. 2. Evaluation of memory and cognition in rats that received varenicline (0.03, 0.1 or 0.3 mg/kg) or water (control) by gavage for 30 days in the open field test. Means and respective standard errors are displayed. (A–B) parameters evaluated in the Barnes maze test and (C–E) parameters evaluated in object recognition test. $n = 7$ animals/group.
*$p < 0.05$, ***p < 0.001, ***p < 0.0001, (A–B) two-way ANOVA with repeated measures followed by Bonferroni post-hoc, (C–D) one-way ANOVA followed by Dunnett's post-hoc and (E) Kruskal-Wallis followed by the Dunn's post-hoc.

Table 1
Evaluation of cerebral neurochemistry in rats that received varenicline (0.03, 0.1 or 0.3 mg/kg) or water (control) by gavage for 30 days. Means and respective standard errors are displayed. $n = 8$ animals/group.

	Control	Varenicline		
		0.03 mg/kg	0.1 mg/kg	0.3 mg/kg
Hippocampus				
DA	113.1 ± 23.80	107.3 ± 19.32	97.25 ± 18.61	86.15 ± 7.61
DOPAC	23.29 ± 4.360	24.66 ± 4.550	22.49 ± 3.639	13.92 ± 2.096
DOPAC/DA	0.2221 ± 0.035	0.2173 ± 0.036	0.2144 ± 0.017	0.1556 ± 0.022
5HT	1621 ± 77.59	1802 ± 116.6	1686 ± 51.76	1906 ± 89.14
5HIAA	1169 ± 48.59	1184 ± 39.31	1209 ± 26.74	1254 ± 47.60
5HIAA/5HT	0.7204 ± 0.026	0.6666 ± 0.030	0.6941 ± 0.027	0.6885 ± 0.031
GABA	37.59 ± 2.718	34.14 ± 2.389	34.72 ± 1.293	29.18 ± 1.188*
Striatum				
DA	15,530 ± 1622	14,057 ± 802.9	12,399 ± 821.4	16,186 ± 887.2
DOPAC	1340 ± 143.1	1229 ± 72.10	1145 ± 84.63	1408 ± 66.76
DOPAC/DA	0.08488 ± 0.0020	0.0830 ± 0.0026	0.09288 ± 0.0031	0.08813 ± 0.0017
5HT	1981 ± 51.74	1867 ± 136.4	1689 ± 108.2	1718 ± 31.95*
5HIAA	1557 ± 28.48	1415 ± 71.05	1388 ± 77.78	1353 ± 20.70*
5HIAA/5HT	0.7738 ± 0.01460	0.7715 ± 0.02747	0.8256 ± 0.03207	0.7760 ± 0.02385
GABA	26.12 ± 1.031	31.60 ± 1.521*	30.60 ± 1.917	29.38 ± 0.6528
Frontal cortex				
DA	202.6 ± 33.19	215.8 ± 10.52	187.4 ± 19.28	206.1 ± 16.65
DOPAC	32.67 ± 7.82	41.78 ± 4.59	45.32 ± 8.20	45.83 ± 10.16
DOPAC/DA	0.1644 ± 0.02	0.1916 ± 0.02	0.2190 ± 0.02	0.2221 ± 0.04
5HT	2687 ± 243.0	2766 ± 211.0	3052 ± 374.9	2506 ± 238.7
5HIAA	878.3 ± 81.65	912.6 ± 26.38	972.4 ± 108.4	960.8 ± 39.97
5HIAA/5HT	0.2958 ± 0.008	0.3414 ± 0.023	0.3186 ± 0.135	0.4078 ± 0.035**
GABA	32.34 ± 2.508	33.59 ± 2.032	30.47 ± 2.295	30.91 ± 2.114

DA = dopamine, DOPAC = 4,4-dihydroxyphenylacetic acid, 5HT = serotonin, 5HIAA = 5-hydroindole, 3-acetic acid and GABA = gamma-aminobutyric acid.
* $p < 0.05$.
** $p < 0.01$, one-way ANOVA followed by Dunnett's post-hoc.

many pharmacotherapeutic drugs that present psychiatric adverse reactions, including changes in behavior, anxiety, agitation, aggression and depressed mood. Furthermore, an anxiogenic effect is described by Medicines and Healthcare Regulatory Agency of United Kingdom (2018) as a common adverse effect reported by patients treated with varenicline. Harrison-Woolrych and Ashton (2011) in their cohort study showed an association between varenicline and psychiatric adverse events, including anxiety disorder. The latter described anxiety disorders as a clinical subgroup of adverse effects, which included anxiety, worsening of anxiety, irritability, panic, stress reaction and restlessness.

Our results corroborate the data found in the above-mentioned studies. In order to evaluate anxiety-like behavior, we employed the open field, the elevated plus maze, the light/dark box and the social interaction tests. Compilation of our data show increased anxiety-like behavior in animals treated with all doses of varenicline, except in the open field test, which is a model for evaluating general activity and is not specific for anxiety. Therefore, the open field test was not sensitive enough to point out the results observed by the other specifics tests.

The second part of this study encompassed the evaluation of memory and learning. Several neurotransmitters are involved in these processes, including acetylcholine and GABA. The cholinergic system plays an important role in memory and learning, especially taking into consideration its association with the hippocampus, amygdala and striatum regions of the brain (Gold, 2003). Thus, cholinergic antagonists are used to induce amnesia in humans and animals, whereas cholinergic agonists have a beneficial effect on memory (Baratti et al., 2009). Varenicline, as a partial agonist of cholinergic receptors, could promote some beneficial effects on memory.

Another hypothesis states that varenicline could act on memory and learning through GABA, since nicotinic cholinergic receptors are present in large quantities in GABAergic neurons in the hippocampus and prefrontal cortex. The association of varenicline with nicotinic receptors would increase the release of GABA in these brain structures that are very important for such processes (DuBois et al., 2013; Bird and Burgess, 2008).

We have used two tests to evaluate memory and learning: the Barnes maze and the novel object recognition tests. The Barnes maze is based on the animal's ability to learn and remember the location of an escape box using visual cues and evaluating spatial memory (Harrison et al., 2006). The novel object recognition test is used to evaluate the spatial declarative memory of the animals (Dix and Aggleton, 1999). In rodents, this test may be related to the hippocampus and the Perirhinal cortex, two cerebral areas that show higher density of nicotinic cholinergic receptors (Broadbent et al., 2009; Winters et al., 2010). The habituation on this test is essential to evaluate the short-term memory and not the animal anxiety-like behavior. Since the animal is in a familiar environment, there is a tendency for further exploration of the new object (Antunes and Biala, 2012).

Our results of the Barnes maze test showed no significant alterations in the number of errors and in the latency to find the escape box, indicating that exposure to varenicline did not improve spatial memory, contrary to expectations. However, such expectations were based on results of previous studies conducted in animals that had previously been sensitized with nicotine. Those studies had found cognitive improvement in spatial learning. Here, we have used naïve animals to evaluate the effects of exposure to varenicline.

Our results of the novel object recognition test showed that the control group performed this test as expected based on observations of more often and longer interaction periods with the new object in comparison to interaction time with the familiar object. This behavior indicated that the familiar object was fixed in the rodents' memory and did not stimulate the exploratory behavior in the same way as the new object. However, all the experimental groups did not present the same behavior, once they did not choose to explore neither the new nor the familiar object. This lack of preference may not be related to memory deficit, but to an increase in anxiety levels (Antunes and Biala, 2012). Anxious animals tend to stay in darker, closed and known places, without moving much (Bourin et al., 2007). The habituation phase of the test aims to minimize or eliminate the initial anxiety variable parameter (Antunes and Biala, 2012). Our results of anxiety-like behavior indicated that all groups treated with varenicline showed increased levels of anxiety, which indicated that these animals, despite habituation, were more anxious and had the exploration behavior inhibited when compared to control rats. Anxiety at a very high level may interfere with memory, as shown by Arbabi et al. (2015), who correlated memory deficit with anxiety in patients in a clinic setting. This may be a possible explanation for the results obtained in this study. Based on these data it is possible to verify that the novel object recognition test was not adequate for evaluation of spatial declarative memory, since the experimental animals did not explore any of the objects.

In the present study, the levels of neurotransmitters and their metabolites in different brain structures (hippocampus, striatum and frontal cortex) were evaluated. These structures were chosen due to their physiologic functions. The hippocampus is responsible for memory and learning, and is part of the limbic system (Fanselow and Dong, 2010). The striatum is part of the reward system of the brain and is related to decision making, therefore it is an important region for behavior of drug addiction (Báez-Mendoza and Schultz, 2013; Do et al., 2012). The frontal cortex has an important function in behavioral regulation, being involved in executive processing of sensory input (Russell et al., 2017).

The above-mentioned brain structures are subdivided into different areas that have distinctive and complex functions, many related to the expression of behavior. However, for this study, it was necessary to combine these different areas and analyze them as a single structure, since the equipment used required a large amount of biological sample to perform the analytical quantification of cerebral neurotransmitters. Sample volume requirement imposed a major limitation for the present study.

Our results showed decrease in serotonin levels as well as its metabolite 5HIAA in the striatum, suggesting that varenicline was able to alter the synthesis or release of this neurotransmitter. Such alterations could, consequently, lead to increased anxiety-like behavior (Handley, 1995; Graeff, 2002; Zangrossi Jr and Graeff, 2014). This result was in agreement with another neurochemical finding which showed an increase in 5HIAA/5HT turnover in the frontal cortex, an indicative of an enzymatic inducing role by varenicline, which may lead to a decrease in availability of serotonin in the synaptic cleft. This is a possible explanation for the results obtained in the behavioral evaluation; however it is not a definitive connection between serotonergic activity and anxiety-like behavior in these animals due to lack of correlation. We shall address this limitation with further studies.

GABA is the main inhibitory neurotransmitter of the nervous system and acts as a memory modulator. GABA's amnesic capacity is known for some time and can be induced by substances such as diazepam (Cahill et al., 1986), which mimics GABA's effects. Further studies, however, suggest that this neurotransmitter plays an important role in conserving memory, since it stimulates protein synthesis in the brain. Protein stimulation allows long-term memory consolidation (Thanapreedwat et al., 2013). It has also been shown that GABA depletion in the prefrontal cortex results in slow working memory (Davis and Squire, 1984; Lew and Tseng, 2014). Our results showed an increase in GABA levels in the lowest dose on the striatum and a decrease in the highest dose in the hippocampus. Along with the literature, our findings are inconclusive in relation to GABA's effects on memory, since there were no behavioral changes observed and the neurochemical alterations were not consistent throughout the experiments.

In conclusion, our data indicate that prolonged exposure of rats to varenicline promoted anxiogenic effects, evidenced by the elevated plus maze, light/dark box and social interaction evaluations. Furthermore, we observed alteration in serotoninergic system, corroborating with

behavioral findings.

Acknowledgements

This work was supported by FAPESP (São Paulo Research Foundation) [grant number 2015/05049-3], CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) [grant number: 305500/2013-9]. This work is part of the first author's master's dissertation on the Graduate Program of Experimental and Compared Pathology, Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo. The authors thank Admilson Aparecido da Cunha for technical assistance in amino acids levels.

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