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RESEARCH ARTICLE

Study of oral aniracetam in C57BL/6J mice without pre-existing cognitive impairments [version 1; referees: 2 approved with reservations]

Conner D. Reynolds[1,2], Taylor S. Jefferson[2], Meagan Volquardsen[2], Ashvini Pandian[2], Gregory D. Smith[3], Andrew J. Holley[2], Joaquin N. Lugo [iD][2,3]

[1]Texas College of Osteopathic Medicine, University of North Texas Health Science Center, Forth Worth, Texas, USA
[2]Department of Psychology and Neuroscience, Baylor University, Waco, Texas, USA
[3]Institute of Biomedical Studies, Baylor University, Waco, Texas, USA

Abstract

Background: The piracetam analog, aniracetam, has recently received attention for its cognition enhancing potential, with minimal reported side effects. Previous studies report the drug to be effective in both human and non-human models with pre-existing cognitive dysfunction, but few studies have evaluated its efficacy in healthy subjects. A previous study performed in our laboratory found no cognitive enhancing effects of oral aniracetam administration 1-hour prior to behavioral testing in naïve C57BL/6J mice.
Methods: The current study aims to further evaluate this drug by administration of aniracetam 30 minutes prior to testing in order to optimize any cognitive enhancing effects. In this study, all naïve C57BL/6J mice were tested in tasks of delayed fear conditioning, novel object recognition, rotarod, open field, elevated plus maze, and marble burying.
Results: Across all tasks, animals in the treatment group failed to show enhanced learning when compared to controls.
Conclusions: These results provide further evidence suggesting that aniracetam conveys no therapeutic benefit to subjects without pre-existing cognitive dysfunction.

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1 **Marie-H Monfils**, University of Texas at Austin, USA

   Laura Agee, University of Texas at Austin, USA

2 **Stephan G. Anagnostaras** [iD], University of California, San Diego, USA

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Corresponding author: Joaquin N. Lugo (joaquin_lugo@baylor.edu)

Author roles: Reynolds CD: Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Jefferson TS**: Investigation, Methodology, Writing – Review & Editing; **Volquardsen M**: Investigation, Methodology, Writing – Review & Editing; **Pandian A**: Investigation, Writing – Review & Editing; **Smith GD**: Investigation, Methodology, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Lugo JN**: Conceptualization, Formal Analysis, Investigation, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Introduction

In the 1970s, pharmacologist Cornelius Giurgea coined the term *nootropics* to describe a novel group of compounds capable of enhancing cognitive processes, intersynaptic communication, and the exchange of information between cerebral hemispheres. These compounds can be divided into five primary categories: cholinergic agonists, psychostimulants, piracetam compounds, hormones & essential nutrients, and agonists of cerebral blood flow[1]. Initial interest in these compounds was limited to reversing the cognitive impairments in subjects with neurological damage or age-related decline. This investigation led to the development of a variety of neuroenhancing compounds, showing promise for cognitive restoration following epilepsy[2], traumatic brain injury[3], cerebral vascular accident[4], Alzheimer's disease[5], and dementia[6]. Nootropics have also been investigated in the treatment of many neurodevelopmental disorders, such as autism[7], ADHD[8], and schizophrenia[9].

Recently, there has been increasing prevalence of nootropic use among otherwise healthy subjects aiming to enhance academic performance, particularly college populations. According to recent population-based studies, the overall incidence of non-medicinal prescription psychostimulant use within the college student population is 4.1%–10.8% over the past year, and 6.4%–19.6% during their lifetime[10–14]. However, misuse of these medications can be dangerous, as psychostimulant toxicity has been linked to cardiac dysrhythmia, myocardial infarction, psychosis, and sudden death[15,16].

The piracetam analog, aniracetam, has recently received attention due to its potential for cognitive enhancement associated with minimal reported side effects[17]. In previous studies, aniracetam has been shown to enhance excitatory post synaptic potentials[18], reduce glutamatergic receptor desensitization[18], increase EPSC decay time[19], and augment long-term potentiation in the hippocampus[20]. Although the definitive mechanism of this compound is unclear, some evidence suggests that it acts as a reversible positive allosteric modulator of AMPA receptors[21]. Aniracetam has proven effective in both human[22] and non-human[23–28] models of cognitive dysfunction. However, few studies have evaluated its efficacy in healthy subjects without cognitive impairment.

In a previous study, our laboratory evaluated whether daily oral administration of aniracetam (50 mg/kg) 1-hour prior to testing could improve cognitive performance in naïve C57BL/6J mice[29]. Through a series of behavioral tasks, we observed that aniracetam did not improve spatial learning, fear learning, or motor learning. Further investigation of aniracetam pharmacokinetics suggested that peak serum levels are achieved approximately 30 minutes following oral administration[30]. In light of this evidence, the current study aims to further evaluate aniracetam's effects by administering aniracetam 30 minutes prior to testing, in order to optimize any cognitive enhancing effects. If aniracetam is truly a cognitive enhancer, we hypothesized that treated mice would display significantly greater learning and memory compared to controls.

Materials and methods

Experimental design

Twenty-four C57BL/6J male mice were generated at Baylor University for use in this study. The strain was originally purchased from Jackson Labs and bred at Baylor University. All mice were independently housed in a vivarium, where environmental conditions were controlled to an ambient temperature of 22°C with 12-hour light/12-hour dark diurnal cycles. All mice were also given *ad libitum* access to food and water. No health concerns were found during the courses of the experiments in this study. There were no adverse effects on the mice during the studies, and every effort was made to ameliorate any discomfort.

After reaching approximately 2 months of age, all mice were randomized to receive either one dose of aniracetam (100mg/kg) (1-[4-methoxybenzoyl)]-2-pyrrolidinone) (Shanghai Suyong Biotechnologies Inc., China), or an identical placebo by oral administration in a gelatin-based suspension 30 minutes prior to behavioral testing. Aniracetam or placebo was administered prior to each behavioral test. This route of administration was selected in order to mimic the typical mode of aniracetam consumption used in humans. During the double blind phase, all mice were subjected to a battery of behavioral tests by designated experimenters blinded to treatment group assignments. All procedures were conducted in compliance with Baylor University Institutional Animal Care and Use Committee, as well as the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. All protocols were approved by the Baylor University Animal Care and Use Committee (Animal Assurance Number A3948-01).

Fear conditioning

A two-day delayed fear conditioning protocol was used to assess amygdala-dependent learning. For this procedure, we used a 26cm x 22cm x 18cm operant chamber, composed of two clear acrylic sides and two metal sides, a metal grid floor capable of receiving an electric shock, an interior light providing constant luminescence (2 lux), and a speaker. The operant chamber was then placed inside of a sound attenuated isolation cubicle (Coulburn Instruments, Allentown, PA, USA) in order to control for external light and sound contamination. During all phases of this task, learning was assessed by the degree of freezing, as it is the most reliable measure of fear memory in mice. All testing was recorded and measured by automatic video tracking software, with visual confirmation of conditioned stimulus (CS) and unconditioned stimulus (US) presentations by the designated experimenter.

Novel object

For this procedure, we used a 40cm x 40cm x30cm clear acrylic open top box. This task was performed in an isolated room controlled for light levels, temperature, and background noise. Prior to testing, all mice were habituated to the arena without any objects for 20 minutes. During the first phase of testing, the two identical objects were placed on opposite sides of the apparatus and interactions with each were measured over a 10-minute period. During the second phase of testing, both objects were removed and replaced with the original object and a novel object. These were placed on

opposite sides of the arena and interactions with each were measured over a 10-minute period. All trials were video recorded and manually scored by the designated experimenter after testing.

Rotarod
The rotarod task was used to assess cerebellar motor coordination and learning. For this procedure, we used a rotating rod (Series 8 Rotarod; IITC Inc., Woodland Hills, CA, USA) which gradually accelerated from 5rpm to 40rpm. All mice were subjected to two 5 minute trials, with a 60 minute ITI, across 4 days of testing. The designated experimenter was responsible for monitoring and recording the length of time in which mice could hold onto the rotating rod before falling. This task was performed in an isolated room controlled for light levels, temperature, and background noise.

Open field
The open field task was used to assess locomotion and anxiety. For this procedure, we used a 40cm x 40cm x 30cm clear acrylic box. This task was performed in an isolated room controlling for light levels, temperature, and background noise. All mice were placed in the center of the apparatus and allowed to explore for 10 minutes. Total time spent in the inner and outer regions were recorded and measured via Fusion optical recording system. Time spent in the outer and inner regions of the field was examined. A greater amount of time spent in the outer region is associated with anxious behavior.

Elevated plus maze
The elevated plus maze task was used to assess levels of anxiety. For this procedure, we used a maze constructed of four white acrylic arms raised 40cm from the floor. All arms were 30cm long x 5cm wide. Two opposing arms were enclosed (walls, 15cm tall) and two opposing arms were left open. During this task, mice were placed in an open arm near the center of the maze and were allowed to explore for 10 minutes. Total distance and time spent in open versus closed arms was recorded by Noldus motion-tracking software (Ethovision, Netherlands). Video recordings were also manually scored by designated experimenters for additional behavioral observations, such as number of rearings in the open versus closed arms and number of head dips in the open arms. A greater amount of time spent in the closed arms versus open arms indicates higher levels of anxiety.

Marble burying
The marble burying task was used to examine repetitive behavior. For this procedure clean home cages were filled with approximately 2–3cm of bedding and twenty black glass marbles were assembled into four evenly spaced columns of five rows. All mice were then placed into the testing cage in front of the array of marbles for 30 minutes. Several measurements of the percentage of the marble buried (50, 75, 100 and completely buried) was recorded by the designated experimenter. The measurement of 100% refers to a marble that is buried to its entire height with some bedding covering, but still in view of the experimenter, while completely buried marbles refers to those not in view of the experimenter. The increased marble burying reflects a higher tendency towards repetitive behavior.

Statistical analysis
All behavioral data with a single measurement was analyzed using an independent samples t-test. The Independent samples t-test was used to analyze behavior in the open field, for day 2 of fear conditioning (fear memory), and for novel object recognition. All behavioral data with repeated measures were analyzed using a two-way Analysis of Variance (ANOVA), with experimental group as the independent factor and the trials or block number as the repeated factor. The two-way analyses were performed on rotarod data and on data from day 1 of fear conditioning (acquisition of fear learning). All data were analyzed using SPSS 20.0 (IBM, USA) or GraphPad Prism 7 software (La Jolla, CA). Values are shown as mean ± S.E.M. for each group.

Results
Aniracetam does not suppress anxiety levels or enhance overall activity
In the elevated plus maze task, we found no significant differences in time spent in the open $t(1,22) = 0.63$, $p = 0.53$; center $t(1,22) = 0.04$, $p = 0.23$; or closed arms $t(1,22) = 0.42$, $p = 0.67$ (Figure 1A). Similar results were found in the frequency of arm entries, with no difference in number of arm entries into the open arms $t(1,22) = 0.69$, $p = 0.49$; center $t(1,22) = 0.39$, $p = 0.69$; or closed arms $t(1,22) = 0.05$, $p = 0.96$ (Figure 1B).

In the open field task, we found no significant differences between the groups in total distance moved in the 10 minute trial $t(1,22) = 0.90$, $p = 0.37$ (Figure 1C). There were also no significant differences observed in stereotypy time $t(1,22) = 1.45$, $p = 0.16$. (Figure 1D) Together, these results suggest that aniracetam has no effect on locomotion or anxiety.

Aniracetam does not enhance motor learning
Across 8 rotarod trials, we did not observe any main effect between groups (F $(1,22) = 0.4073$, $p = 0.5299$) (Figure 2). However, there was a main effect of learning across multiple trials (F $(7, 154) = 11.97$; $p < 0.0001$), indicating that motor learning had occurred within both groups. These results suggest that aniracetam has no cognitive enhancing effect on motor learning.

Aniracetam does not affect repetitive behavior
In the marble burying task, we found no significant differences in performance when measured at: 50% $t(1,22) = 1.18$, $p = 0.24$; 75% t$(1,22) = 0.76$, $p = 0.45$; 100% t$(1,22) = 0.50$, $p = 0.61$; or at the completely buried level $t(1,22) = 0.05$, $p = 0.95$ (Figure 3). These results suggest that aniracetam has no effect on repetitive behavior.

Aniracetam does not enhance associative fear memory
On the first day of fear conditioning, mice were placed into an operant chamber where multiple tone and foot shocks were administered. We observed no main effect of group (F $(1, 19) = 0.1048$; $p = 0.7497$) or interaction between groups (F $(4, 76) = 0.5453$; $p = 0.7029$) (Figure 4A). However, there was a main effect of learning across multiple trials (F $(4, 76) = 42.35$ $p < 0.0001$), indicating that fear learning had occurred within both groups. On the second conditioning day mice were placed into the same operant conditioning chamber with novel context. Upon
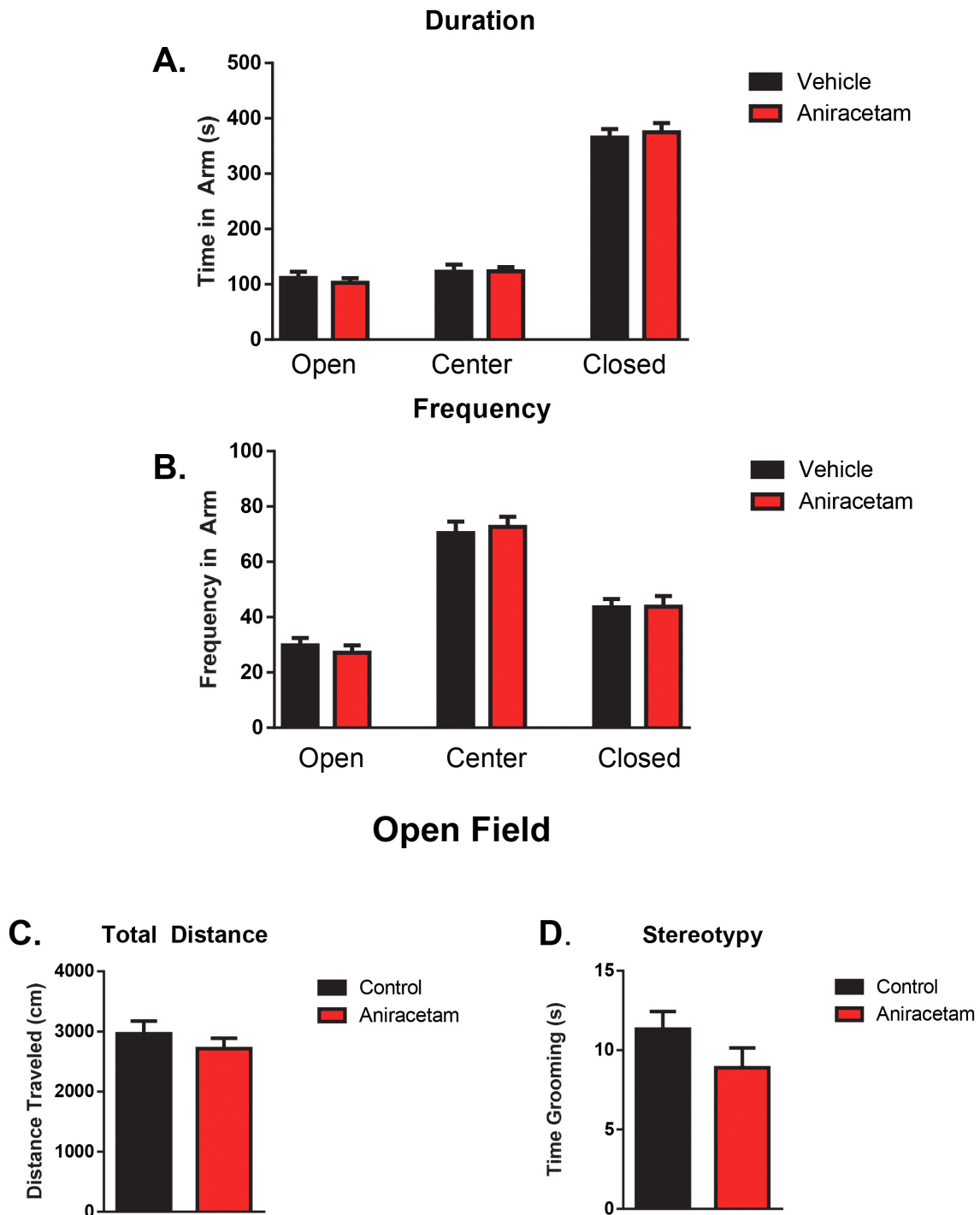
Duration

A.

Frequency

B.

Open Field

C. **Total Distance**

D. **Stereotypy**

Figure 1. Aniracetam pretreatment does not change performance on the elevated plus maze or open field tasks. (**A**) In the elevated plus maze test, an independent measures t-test revealed no significant differences in time spent in the open arms $t(1,22) = 0.63$, $p = 0.53$; center $t(1,22) = 0.04$, $p = 0.23$; or closed arms $t(1,22) = 0.42$, $p = 0.67$. (**B**) There were also no significant differences in the number of entries into the open arms $t(1,22) = 0.69$, $p = 0.49$; center area $t(1,22) = 0.39$, $p = 0.69$; or closed arms $t(1,22) = 0.05$, $p = 0.96$. (**C**) In the open field test, an independent measures t-test revealed no significant differences between groups in total distance moved $t(1,22) = 0.90$, $p = 0.37$, (**D**) or stereotypy time $t(1,22) = 1.45$, $p = 0.16$.
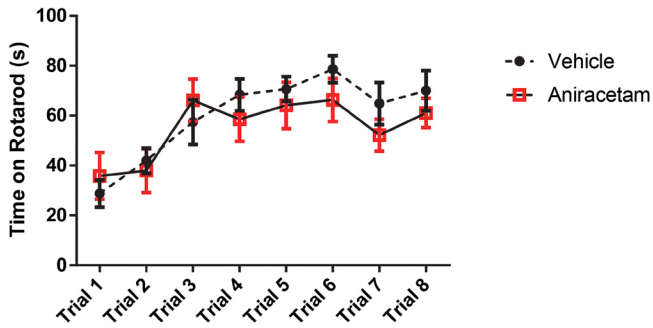
Figure 2. Aniracetam pretreatment does not change performance on the rotarod task. Across 8 trials, there was no main effect between groups ($F_{(1,22)} = 0.4073$, $p = 0.5299$). However, there was a main effect of learning across multiple trials ($F_{(7, 154)} = 11.97$; $p<0.0001$), indicating that motor learning had occurred within both groups.
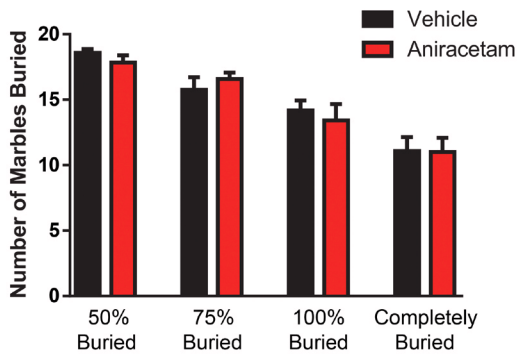

Figure 3. Aniracetam pretreatment does not change performance on the marble burying task. Independent measures t-tests revealed no significant differences in the animal's performance in marble burying when measured at: 50% $t_{(1,22)} = 1.18$, $p = 0.24$; 75% $t_{(1,22)} = 0.76$, $p = 0.45$; 100% $t_{(1,22)} = 0.50$, $p = 0.61$; or at the level of completely buried $t_{(1,22)} = 0.05$, $p = 0.95$.

presentation of the tone both groups displayed increased fear, however there was no significant difference in the freezing behavior expressed between treated and control mice $t_{(1,19)} = 1.013$; $p = 0.3238$ (Figure 4B). These results suggest that aniracetam treatment has no cognitive enhancing effect on associative fear learning.

Aniracetam does not enhance novel object recognition

During the initial phase of testing, object preference was measured for identical objects. There was no significant preference towards the left or right object between treated $t_{(1,22)} = 1.333$, $p = 0.20$ or control group mice $t_{(1,22)} = 0.1583$, $p = 0.88$ (Figure 5A). During the second phase of testing, object preference was measured between a familiar and novel object. There was a significant preference towards the novel object in both treated $t_{(1,22)} = 4.968$, $p < 0.0001$ and control mice $t_{(1,22)} = 3.776$, $p < 0.001$. However, there were no differences in preference between the groups in the novel object condition $t_{(1,22)} = 0.6112$, $p = 0.5474$ (Figure 5B).
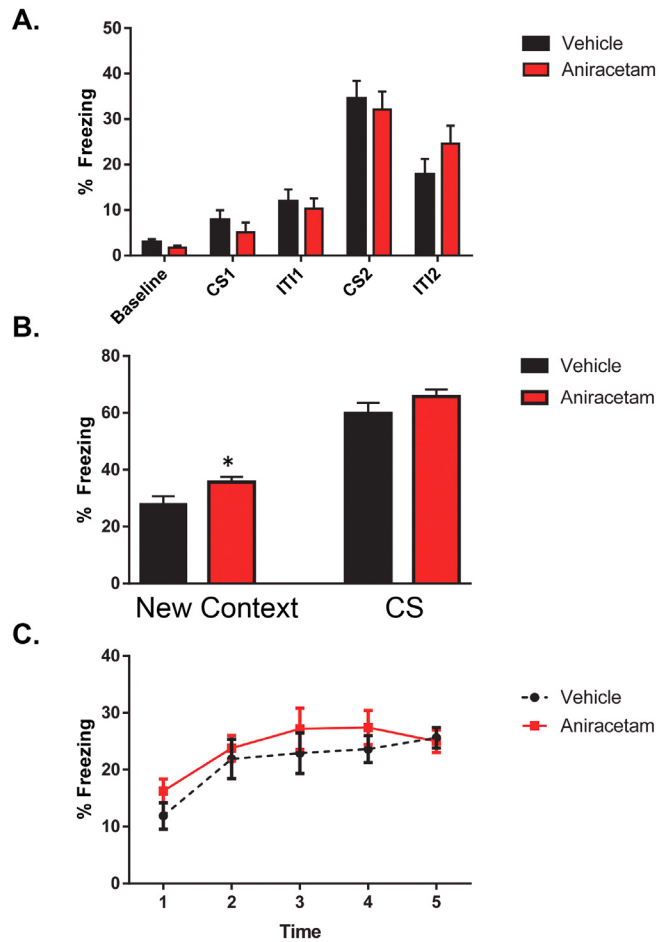

Figure 4. Aniracetam pretreatment does not change performance on the delayed fear conditioning task. (**A**) On the first day of testing, mice were placed into the operant chamber and 2 minutes of baseline activity levels were recorded. This was followed by a 30 second conditioned stimulus (CS) tone (80dB white noise), a 2 second unconditioned stimulus (US) shock (0.85mA), and a 2-minute inter-trial interval (ITI). Another identical CS-US pairing was then presented and followed with a 30 second ITI. Following multiple foot shock and tone presentations, a two-way ANOVA test indicated no main effect of group ($F_{(1, 19)} = 0.1048$; $p = 0.7497$) or interaction between groups ($F_{(4, 76)} = 0.5453$; $p = 0.7029$). (**B**) The second day of testing consisted of two trials. During the first trial, mice were placed back into the original operant chamber for 5 minutes and baseline activity was recorded. Before the second trial the operant chamber was modified with a foam pad under an acrylic square to cover the metal floor grid, an acrylic wall placed diagonally across that halved the space into triangular form, and 1mL of pure vanilla extract (Adam's Extracts, USA) placed beneath the floor. These changes to the tactile, spatial, and olfactory contexts of the chamber were made in order to prevent context dependent learning. During the second trial, mice were placed into the contextually modified operant chamber and 3 minutes of the trial baseline activity was monitored. This was followed by a 3-minute period of CS tone presentation (80dB white noise). Upon presentation of the tone within novel context, an independent measures t-test indicated no significant difference in the freezing behavior exhibited between groups in novel context $t_{(1,19)}=1.013$; $p=0.3238$.
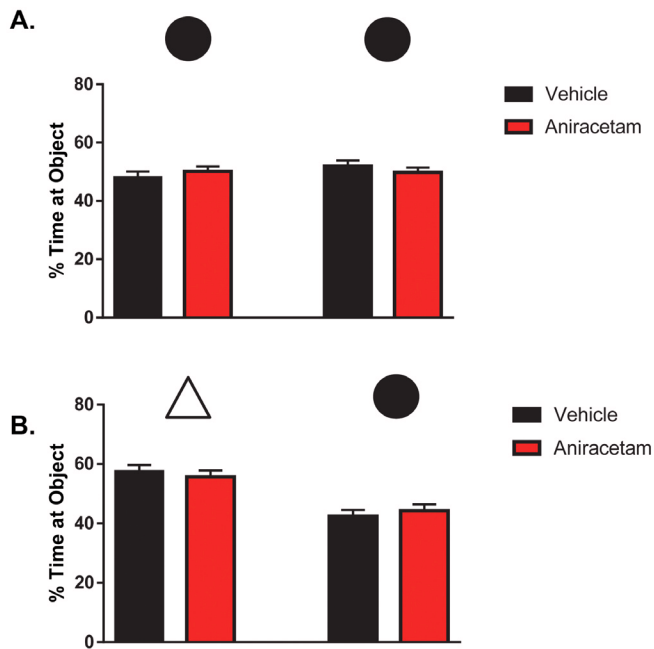
Figure 5. Aniracetam pretreatment does not change performance on the novel object recognition task. (**A**) Independent measures t-tests indicated no significant preference towards the left or right object between treated $t(1,22)$ = 1.333, p = 0.20 or control group mice $t(1,22)$ = 0.1583, p = 0.88. (**B**) An independent measures t-test indicated no differences in preference between groups in the novel object condition $t(1,22)$ = 0.6112, p = 0.5474.

These results suggest that aniracetam treatment has no cognitive enhancing effect on novel object recognition.

Dataset 1. Raw data for 'Study of oral aniracetam in C57BL/6J mice without pre-existing cognitive impairments.'

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(A) Open field total distance data and stereotypy results for vehicle and aniracetam-treated subjects. (B) Elevated-plus maze mean time and total frequency visits for open, closed, and center arms for vehicle and aniracetam-treated subjects. (C) Marble burying data for marbles buried at 50%, 75%, 100%, and total marbles for vehicle and aniracetam-treated subjects. (D) Delay fear conditioning data for day 1 and day 2 for vehicle and aniracetam-treated subjects. (E) Rotarod data for latency to fall off rotarod for vehicle and aniracetam-treated subjects. (F) Novel object recognition data for phase 1 and phase 2 for vehicle and aniracetam-treated subjects.

Discussion

Although significant progress has been made towards understanding the neuroenhancing effects of aniracetam in subjects with cognitive impairment, there has been little investigation into its therapeutic effects on healthy subjects. In a previous study[29], our laboratory demonstrated that drug treatment in healthy C57BL/6J mice did not produce any significant effects on learning and memory,

anxiety, locomotion, or repetitive behavior. Given the existing body of evidence supporting aniracetam's cognitive enhancing effects, we elected to investigate this substrate further by using a modified drug treatment schedule to ensure peak serum levels during behavioral testing. Through this follow-up investigation, it was demonstrated that aniracetam conveys no significant cognition enhancing effects in healthy subjects.

Our findings are in contrast to a previous study by Rao *et al.*[26], which demonstrated that intrahippocampal aniracetam infusions significantly improved Y-maze performance in healthy rats. A key difference in experimental design between this study and ours is the route of administration. Intrahippocampal drug infusion provides tightly controlled, localized doses by circumventing first-pass metabolism. This method leads to a more accurate assessment of the drug serum levels necessary to achieve a therapeutic effect, but is restricted specifically to animal studies. Oral drug administration typically has a much lower bioavailablity due to hepatic biotransformation, but provides a higher ecological validity. For the purposes of our study, we elected to administer aniracetam orally, as it closely mimics the route of administration used most commonly in human reports. Although it is theoretically possible that cognitive enhancement could be achieved by aniracetam treatment, the therapeutic dose required to achieve this effect may be unrealistic.

Despite any peer-reviewed data of non-medicinal use in humans, our findings contrast many subjective reports from healthy individuals purporting the cognitive enhancing effects of aniracetam and other piracetam-analogs. In a previous study, Corazza *et al.*[31] performed a multilingual qualitative assessment from a range of available online resources subjectively reporting benefits from piracetam use. These authors found that while the drug is used to improve academic and work-related performance, its use is also associated with side effects, such as hallucinations, dysphoria, fatigue, dizziness, memory loss, and headaches. Their findings indicate these side effects may be dose-dependent; however, because both the drug and their manufacturers are currently unregulated, it is impossible to determine an effective dose or therapeutic index in humans.

To our knowledge, both the present and previous studies represent the first empirical evidence of aniracetam treatment by oral administration in healthy subjects.

Because this study closely mimics the drug administration in humans, we can infer that these results should most accurately depict the effects in healthy human subjects. Based on our findings, it can be suggested that non-medicinal and/or recreational use by healthy individuals may have only marginal therapeutic benefit, while the risk of harmful side effects remains.

Data availability

Dataset 1: Raw data for 'Study of oral aniracetam in C57BL/6J mice without pre-existing cognitive impairments.' (A) Open field total distance data and stereotypy results for vehicle and

aniracetam-treated subjects. (B) Elevated-plus maze mean time and total frequency visits for open, closed, and center arms for vehicle and aniracetam-treated subjects. (C) Marble burying data for marbles buried at 50%, 75%, 100%, and total marbles for vehicle and aniracetam-treated subjects. (D) Delay fear conditioning data for day 1 and day 2 for vehicle and aniracetam-treated subjects. (E) Rotarod data for latency to fall off rotarod for vehicle and aniracetam-treated subjects. (F) Novel object recognition data for phase 1 and phase 2 for vehicle and aniracetam-treated subjects. DOI, 10.5256/f1000research.11023.d172542[32]

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Data Source

Open Peer Review

Current Referee Status: ?   ?

Version 1

Referee Report 17 October 2017

Stephan G. Anagnostaras (iD)

Department of Psychology, University of California, San Diego, La Jolla, CA, USA

This is a fairly well-performed study but it has a serious flaw that isn't really addressed, which is that it uses a single dose. Single dose studies are problematic unless that dose is really well established as the appropriate dose, and it's really not very clear here how or why that dose is chosen. Although the dose chosen is maybe in the dose range people tend to take (maybe double the maximum dose people seem to report), pro-cognitive effects are notoriously dose-specific and it is possible they simply have the wrong dose. Rather than the very extensive behavioral approach, why didn't the authors simply run one task (e.g., fear conditioning) using a range of doses? At the very least the authors need to acknowledge this substantial limitation.

The other issues have been mentioned in the other review; in particular, the fear conditioning parameters and scoring methodology are not adequately described.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Dec 2017

Joaquin Lugo, Baylor University, USA

We would like to thank Dr. Stephan G. Anagnostaras for his comments on our paper. We have included discussion on the limitation of the use of a single dose. We agree that this is a substantial limitation of our paper and the reviewer provided a great experimental design to determine an effective dose on cognition. We appreciate the input and plan to use this approach in future studies. We have also included more information on the methods of the fear conditioning test.

Competing Interests: none

Referee Report 12 October 2017

? **Marie-H Monfils** , **Laura Agee**

Department of Psychology, University of Texas at Austin, Austin, TX, USA

Summary

The study was focused on assessing whether healthy (i.e. non-cognitively impaired) mice would display benefits following the administration of the nootropic drug aniracetam. Mice were run through a battery of tests aimed following oral administration of either a vehicle solution or an aniracetam solution. Though the main focus of the paper seemed to be on identifying whether aniracetam administered mice showed improvement in a variety of learning and memory tasks, mice were also assessed on measures of anxiety and repetitive behavior. The aniracetam treated mice did not show any significant improvements on any of the learning and memory tasks compared to controls. In addition, aniracetam treated mice did not display increased anxiety or repetitive behaviors as compared to control mice.

Suggested Changes

1. The title should be rewritten to include information about the outcome measures that are used. As it is, it is not readily clear from the title that this is a behavioral study.

2. The introduction does not provide an explanation for some of the tests that were run. Specifically, there was no mention of anxiety or repetitive behavior being linked with nootropic use. A discussion of this relationship should be included at some point in the introduction.

3. The authors do not mention which light phase the mice were run during. Given that all the outcome measures are behavioral and metabolic factors may influence how the mice process the aniracetam, this is a detail that needs to be included.

4. Whether or not the order of the tests was randomized and the amount of time that was given between each test should be mentioned.

5. The write-up of the fear conditioning methodology is missing a number of important details. The following need to be added: (1) A thorough description of the CS in use (e.g. was it a pure tone or white noise? What was the volume?), (2) the number of CS-US pairings, (3) the intensity and duration of the US, (4) the amount of time the mouse was given to habituate to the cage, and (5) the amount of time between CS presentations.

6. Excitatory post-synaptic current should be written out fully before being abbreviated to EPSC.

7. A description of Figure 4C needs to be added to the caption of Figure 4.

8. A significant difference between the freezing displayed by aniracetam treated rats and control rats is indicated in Figure 4B but the statistics backing up this finding are not included in the results or discussed at any point in the paper.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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