Charles & Mary Latham Fund - Final Report (Medical)

Results & Budget Status Report

Final Report: Grant Information

Due Date

September 30, 2018

Organization Name

Howard University

Principal Researcher

Dr. Ozra Dehkordi

Study Title

Neurochemical Profile of Menthol Activated Cells in the Nicotine Reward-Addiction Circuitry

Grant Purpose

Project Summary (250 words or less)
Menthol cigarette use is prevalent among African-American smokers and may contribute to health-related disparities in this population. However, the data regarding the molecular mechanism through which menthol acts to enhance nicotine addiction is limited. Menthol, a widely used cooling-anesthetic and flavoring agent in tobacco and other nicotine delivery systems, regulates sensory transduction by activating TRPM8 and TRPA1 channels located specifically in sensory neurons. In addition to its peripheral sensory impact, recent studies have demonstrated the presence of menthol in the CNS after in vivo exposure. This implies that menthol may also impact nicotine addiction through central mechanisms. Menthol has been shown to modulate the function and levels of nicotinic acetylcholine receptors (nAChRs) and to stimulate GABAA receptors and enhance GABAergic transmission in the spinal cord and hippocampal neurons. However, the neuroanatomical and neurochemical profile of the CNS neurons targeted by peripheral and/or direct central effects of menthol is not known. Thus, in the present study in mice we utilize c-Fos immunohistochemical technique to identify the anatomical location of menthol-induced c-Fos activated cells in the addiction circuitry. Immunohistochemical labeling will then be performed to identify which of the menthol activated neurons in nicotine reward- addiction sites are dopaminergic or GABAergic and which contains GABAergic receptors (GABAA) and/or nAChRs (Î±4 and Î±7). Quantitative and qualitative receptor binding and autoradiography will be performed to determine whether menthol alone or in combination with nicotine differentially changes the density of nAChRs and GABAA receptors on VTA and NAcc.

Statement of Problem

African Americans have disproportionately higher rates of smoking-related morbidity and mortality than white smokers (USD-HHS, 1998; 2, 9,30). This disparity is believed to be partly associated with higher rates of menthol cigarette smoking among African American smokers compared to white smokers (approximately 70% of African Americans vs. 30% whites) (US-FDA-TPSAC, 2). Menthol, a widely used flavoring tobacco additive, and now also an additive in many other nicotine delivery systems (e-cigarettes, vaporizers, etc.) increases the ease of smoking by acting peripherally on transient receptor potential M8 channels (TRPM8) (79) and transient potential A1 channels (TRPA1) (45) of sensory neurons. Through these receptors, menthol masks the harshness of nicotine and provides a cooling sensation that appeals to many smokers. In addition to facilitating their smoking behavior, individuals that smoke menthol cigarettes have greater difficulty in quitting smoking (US-FDA-TPSAC, 2011). This implies that menthol may enhance the addictive properties of nicotine. Recent studies have demonstrated that in addition to its peripheral sensory impact, menthol may have direct effects on CNS to modulate the activity of

nicotinic acetylcholine receptors (nAChRs) and GABAA receptors at various brain regions. In-vivo and in-vitro studies have shown that menthol allosterically regulates the function and expression of nAChRs, including the Î ±4Î²2-, Î±7-, and Î±3Î²4-containing nAChRs (3, 31,32, 77). Menthol also enhances GABAA receptor-mediated currents in midbrain periaqueductal gray neurons (40, 87). nAChRs and GABAergic receptors are expressed by dopaminergic, GABAergic, glutamatergic and cholinergic neurons in the ventral tegmental area (VTA) and nucleus accumbens (NAcc) (7, 85, 89). How menthol's modulation or direct activation of these receptor types can bring about biochemical changes in reward-related structures that could enhance nicotine addiction and reduce tobacco cessation rates is not known. The present study was therefore designed to fill in this knowledge gap by 1) identifying the neuroanatomical and neurochemical profile of neurons in the reward-addiction circuitry that are activated by menthol and 2) identifying how chronic exposure to menthol modulates the nAChRs and GABAA receptors in this circuitry. Thus, in the present study we hypothesize that 1) the dopaminergic and GABAergic cells of the VTA and NAcc are targets of acute menthol in the CNS, 2) acute menthol activates the neurons of the reward-addiction circuitry through modulation of nAChRs and GABAergic receptors and 3) chronic exposure to menthol modulates the expression of nAChRs and GABAergic receptors in VTA and NAcc. We will test our hypotheses by the following two specific aims:

Specific Aims

Aim 1. To identify the anatomical and neurochemical profile of neurons activated by acute intra-peritoneal (I.P) injection of menthol in mice. Our working hypotheses are that 1) dopaminergic and GABAergic neurons will be activated by menthol and that both GABAA and nAChRs located primarily in dopaminergic and GABAergic and other menthol activated cells will be modulated by acute menthol.

Approach: The menthol-activated cells will be identified by immunohistochemical localization of menthol-induced c-Fos expression. Sequential double and triple immunohistochemical labeling and genetically modified mice model (GAD67-GFP knock-in mice), combined with laser scanning confocal and fluorescence microscopy will then be performed to identify the subpopulation of menthol-stimulated c-Fos expressing cells in VTA and NAcc that are dopaminergic or GABAergic as well as those that express positive immunoreactivity for GABAA receptors and/or immunoreactivity for Î±4- and /or Î±7-containing nAChRs.

Aim 2. To determine whether chronic exposure to menthol alters the number of nAChRs and/or GABAergic receptors on VTA and NAcc regions of the reward circuitry. Our working hypothesis
is that chronic menthol will modulate the level of expression of nAChRs and GABAA receptors.

Approach: We will utilize quantitative and qualitative receptor binding and autoradiography in the control mice and mice exposed to chronic menthol alone and/or menthol plus nicotine to determine whether menthol changes the density of nAChRs and/or GABAA receptors on VTA and NAcc.

Research Strategy: Significance

Several studies have suggested that menthol, a commonly used tobacco additive in cigarettes facilitates smoke initiation and nicotine addiction (33,52). However, little is still known regarding the anatomical and molecular components involved in the reward-addiction pathways that make it so difficult for smokers of mentholated cigarette to quit, why they have a higher relapse rate, and why it may be easier for naÃ¯ve individuals to get "hooked" faster when they begin smoking mentholated cigarettes than non-mentholated ones (25, 29, 43, 59, 69-70). This is of particular significance for the African American population of which more than 70% smoke mentholated cigarettes (US-FDA-TPSAC, 2011, 41,76). This makes menthol, a seemingly "inert" additive, a discriminating deadly partner to tobacco and a health disparity concern. Moreover, menthol has also become an important additive to new nicotine delivery systems such as e-cigarettes and hookahs, and this will have enormous added impact on public health worldwide. What we know about the pharmacological effects of menthol is that it activates TRPM8 and TRPA1 ion channels of sensory neurons to produce analgesic and cooling sensations that mask the harshness of nicotine and promotes inhalation of tobacco smoke (45,79). Menthol also alters nicotine metabolism and increases nicotine bioavailability (1,5). More recently, menthol has been found to serve as a negative allosteric modulator of Î±4Î²2, Î±7 and Î±3Î²4 nAChR subtypes (3, 31). What we don't know about menthol is the neuroanatomical and neurochemical profiles of cells in the reward-addiction pathways that are impacted by menthol either through sensory inputs or directly by menthol's stimulation of receptors in these sites. We also don't know if GABAergic receptors, which are present in the reward-addiction sites (49,60,78) are also targets of menthol, although evidence suggests that they are modulated by menthol elsewhere (8, 24,87). We are now focusing on how menthol may be affecting the nicotine reward-addiction circuitry through determining the neuroanatomical and neurochemical components that are either indirectly impacted by the peripheral exposure to menthol through sensory inputs or directly affected by menthol's effects on receptors in the CNS reward-addiction pathways. Thus in the present study in mice , we hypothesize that menthol impacts cigarette smoking behavior via interaction with nAChRs and GABAA receptors in ventral tegmental area (VTA) and nucleus accumbens (NAcc), two important structures of the reward-addiction pathways, and that chronic menthol

causes a change in the density of nAChRs and GABAergic receptors that leads to further dysregulation of the brain reward-addiction circuitry. We will test this hypothesis by immunohistochemical identification of CNS neurons that are activated by menthol and by sequential double and triple labeling of the activated cells to determine their chemical profiles and to verify the expression of nAChRs and/or GABAergic receptors by these neurons (Specific Aim1). Receptor binding and autoradiography studies will be used to determine how chronic menthol alone or in combination with nicotine modifies the expression levels of nAChRs, and GABAergic receptors in reward addiction structures (Specific Aim 2).

Research Strategy: Innovation

The present study is innovative in that it uses multidisciplinary anatomical and pharmacological techniques in a genetically modified mice model (GAD67-GFP knock-in mice) to identify the neurochemical and neuroanatomical components of the reward circuitry that are impacted by acute and chronic menthol exposure (expected outcome). Identification of this circuitry will provide new knowledge about the mechanism(s) through which menthol in mentholated cigarettes and other nicotine delivery systems modulates the activity of dopaminergic cells of the VTA to enhance nicotine addiction. The results will have a significant impact on future clinical outcomes by providing new targets for the development of drugs and/or treatment programs directed primarily for smokers of mentholated cigarettes that are known to have lower cessation rates in standardized treatment programs.

Research Strategy: Approach

Specific Aim 1: Approach Immunohistochemical studies:: The menthol-activated cells will be identified by immunohistochemical localization of menthol-induced c-Fos expression. Sequential double and triple immunohistochemical labeling and genetically modified mice model (GAD67-GFP knock-in mice), combined with laser scanning confocal and fluorescence microscopy will then be performed to identify the subpopulation of menthol-stimulated c-Fos expressing cells in VTA and NAcc that are dopaminergic or GABAergic as well as those that express positive immunoreactivity for GABAA receptors and/or immunoreactivity for Î±4- and /or Î±7-containing nAChRs.These studies will be performed by Dr. Ozra Dehkordi, The P.I in this project.

Specific Aim 2: Approach: We will utilize quantitative and qualitative receptor binding and autoradiography in the control mice and mice exposed to chronic menthol alone and/or menthol plus nicotine to determine whether menthol changes the density of nAChRs and/or GABAA receptors on VTA and NAcc. These studies will be performed by Dr. Martha, Davila-Garcia, The Co- PI in this project

Research Design and Methods

RESEARCH DESIGN AND METHODS:

Specific Aim 1

Rationale:1.

1.To identify the cells of the reward-addiction circuitry that are activated by I.P. injection of menthol;

2. To evaluate the expression of nAChRs (Î±4Î²2, Î±7) and GABAA receptor proteins by menthol- activated cells

3. To determine whether dopaminergic and/or GABAergic cells of the VTA and NAcc are the primary targets of menthol in CNS

In these studies, adult mice (2-3 month-old) weighing 20-25 g will be used. Animals (30), will be treated, by I.P injection of vehicle (40% saline and 60% ethanol), and/or menthol (1 and 2 mg/kg Sigma Aldrich). Two hrs. after I.P injection of the vehicle and/or the menthol, the mice will be anesthetized with isoflurane (5%) and perfused transcardially with saline, followed by 1-2% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. After perfusion, the brains will be post- fixed in 1-2% paraformaldehyde for one hr. and then cryoprotected in a 30% sucrose solution for a minimum of 2 days. Transverse sections of the brain will be cut at 40 Î¼m using a Bright OTF Cryostat and will be stored in 0.5% sodium azide in 0.1 M PB (pH 7.4). Immunohistochemical procedures will be performed using free floating sections as follows: Briefly, 1-in-5 series of brain sections extending from bregma -- 5.41 to bregma 2.33 mm (Paxinos and Franklin 1997) will be rinsed three times in 0.1 M phosphate buffered saline (PBS) at pH 7.4 and processed for immunohistochemical labeling according to our previously described protocols.

Briefly, tissues will first be processed for immunohistochemical labeling of menthol induced c-Fos IR cells. After the detection of c-Fos, tissues will be washed in PBS and processed for labeling of nAChRs, GABAA receptors, GABAergic (GAD67, GAD65 immunoreactive) and dopaminergic (tyrosine hydroxylase immunoreactive) cells. (see

our previous publication for details)

Specific Aim 2
Receptor binding and autoradiography: The purpose for these studies is to determine whether menthol alone or in combination with nicotine will change the density of nAChR (Î±4- and Î±7-nAChRs) or GABA receptors (GABAA) on GABAergic and putative dopaminergic expressing neurons of the VTA and NAcc

Measuring Nicotinic Receptor Levels by Receptor Binding and Autoradiography: The sections will be preincubated three times for 5 min in Tris--HCl buffer (pH 7.4), then binding will be carried, at room temperature with the same buffer containing 80pM [125I]-epibatidine (Perkin Elmer, Akron, OH) for 4 hrs or at 37oC with 500pM [125I]-Î±-bungarotoxin (Perkin Elmer, Akron, OH) for 40 min. Nonspecific binding will be determined in adjacent sections in the presence of 300 Î¼M (âˆ’)nicotine hydrogen tartrate in both cases. After incubation, sections will be rinsed twice for 5 min each in ice-cold buffer, then dipped briefly into distilled water. After air drying, the sections will be dipped in NTB film emulsion (Kodak, Rochester, NY), (ARC, St. Louis, MO) for a predetermined time (24hrs to 10 days). A slide similarly treated will contain a strip of [125I] and one of [14C] standards. Quantitative densitometric analysis of binding will be done using the MCID imaging system (Ontario, Canada). The VTA and NAcc will be identified according to the atlas of Paxinos and Franklin (1997). To determine specific binding, nonspecific binding (NSB) will be performed in parallel in adjacent sections incubated with the addition of 300uM nicotine bitartrae. Specific binding will be determined as the difference betweeen total binding sections and adjacent NSB sections in the equivalent anatomical region.
Measuring GABA Receptor Levels by Receptor Binding and Autoradiography: GABAergic receptors will be labeled with 50mM [3H]-muscimol, GABAA selective agonist using the following procedures. Sections will be rinsed twice for 15 min at 4oC in 50 mM Tris/Citrate buffer (pH 7.0). To assess total binding, sections will be incubated 60 min at 4Â°C in 50 mM Tris/Citrate (pH 7.0) plus 50 nM [3H]-muscimol (Perkin Elmer, Akron, OH) in the presence of baclofen. Nonspecific binding will be assessed by incubation with the tritiated ligand plus 100 Î¼M unlabeled GABA (RBI, Natick, MA). After five 2-sec rinses in ice-cold 50 mMTris/Citrate (pH 7.0), sections will be dipped once in ice-cold dH2O. All sections will be air dried and stored or dipped into NTB film emulsion (Kodak, Rochester, NY) along with standardized autoradiographic microscales (ARC, St. Louis, MO), and stored at 4Â°C for up to four weeks. The films will then be developed, fixed, and air-dried. Slides will be stained with cresylecht violet or thionin for anatomical identification. Density of receptor binding in the VTA and NAcc will be quantified using MCID imaging (Ontario, Canada).

Challenges
Immunohistochemical Studies:
The c-fos gene and its product c-Fos protein have been used as cellular markers to identify activated neurons within the CNS. This gene is rapidly and transiently expressed within the cell nucleus following cell activation with different stimuli including oxygen deprivation and CO2/H+ elevation This technique has also been used to detect menthol-induced activated cells in brainstem after application of menthol to nasal mucosa). However, this technique has the following limitations, which will be taken into consideration when evaluating the data obtained in these studies. The nonspecific expression of c-Fos will be addressed through appropriate experimental design and appropriate control experiments, as stated in the aforementioned paragraphs in the methods.. We expect that some difficulties may arise from double and/or triple-labeling of c-Fos with nAChRs, GABAA and/or GABAB receptors, TH and GAD65 because of cross-reactivity of the chemical reagents that are used for detecting these proteins. However, our experience has shown that choosing the appropriate primary and secondary antibodies, blocking sera, and titrating the concentrations of these chemicals properly will alleviate these problems. The other difficulties anticipated in all immunohistochemical studies, including the proposed studies, is the auto fluorescence and high background staining that may occur in some labeling. This problem can be corrected through adjustment of the concentration of antibodies and other chemicals used in the immunohistochemical staining. The selection of secondary antibodies with different excitation and emission wavelengths, choice of appropriate blocking agents, and duration of the blocking time can also be adjusted to prevent nonspecific background staining.

Receptor Binding and autoradiography:
Because these studies will be performed in freshly-cut tissues, we do not know how well the sections will allow us to do immunohistochemical labelling. So, sections will be divided in all cases into three groups. The first group will

be directed only to autoradiography, a second group that will be postfixed and immunocytostained for ACh, GLU, GABA or dopaminergic markers. A third group will use GAD67 knock-in mice to identify GABAergic neurons, and photographed by microscopy before the sections are subjected to the binding and autoradiography protocol. This is to make sure that the binding protocol itself does not affect their re-identification. A subset of the sections used for binding will be dipped in film emulsion instead of being exposed to film. This is because we are not sure if the liquid film will interfere with the double labelling (Immunostaining for TH and immunofluorescence for GAD67-GFP).

Future Plans
The long-term goal of the proposed studies is to implement anatomical, behavioral neurochemical, genetic and radiological studies to answer why it is so difficult for smokers of mentholated cigarette to quit, why they have a higher relapse rate, and why it may be easier for naÃ¯ve individuals to get "hooked" faster when they begin smoking mentholated cigarettes than non-mentholated ones. Therefore, during the first phase of this project (first 8 months) both specific Aims 1 and 2 will be addressed and the preliminary data obtained will be used in second phase for development and submission of an R15, R21 or R01 grant application to NIH.

Budget Breakdown
1. Antibodies: $ 4200.00
2. Radioligand reagents: $ 4000.00
3. Secondary antibodies, blocking serums: $ 1700.00
4. Tyramide signal amplification kits: $ 1339.0
5. Microscope slides and mounting media: $ 1261
6. Osmotic mini-pumps: $ 500.0
7. Animals $ 2000.0
Total: $ 15000.0

Grant Amount

7500.0000

Final Report: Results

Please describe your study and the research methods used. (500 word limit)

Menthol cigarette use is prevalent among African-American smokers and may contribute to health-related disparities in this population. However, the data regarding the molecular mechanism through which menthol acts to enhance nicotine addiction is limited. Menthol, a widely used cooling-anesthetic and flavoring agent regulates sensory transduction by activating TRPM8 and TRPA1 channels located specifically in sensory neurons. In addition to its peripheral sensory impact, recent studies have demonstrated the presence of menthol in the CNS after in vivo exposure. This implies that menthol may impact nicotine addiction through central mechanisms. Menthol has been shown to modulate the function and levels of nicotinic acetylcholine receptors (nAChRs) and to stimulate GABAA receptors and enhance GABAergic transmission in the spinal cord and hippocampal neurons. However, the neuroanatomical and neurochemical profile of the CNS neurons targeted by peripheral and/or direct central effects of menthol is not known. Thus, in the present study in mice we hypothesized that 1) the dopaminergic and GABAergic cells of the ventral tegmental area (VTA) and nucleus accumbens (Acb) are targets of acute menthol in the CNS, 2) acute menthol activates the neurons of the reward-addiction circuitry through modulation of nAChRs and GABAergic receptors and 3) chronic exposure to menthol modulates the expression of nAChRs and GABAergic receptors in reward addiction circuitry. The menthol-activated cells were identified by immunohistochemical localization of menthol-induced c-Fos expression. Sequential double and triple immunohistochemical labeling technique combined with fluorescence microscopy were used to identify the anatomical location of menthol-stimulated c-Fos expressing cells with respect to dopaminergic or GABAergic cells of reward addiction circuitry. Experiments are underway if menthol activated cells express positive immunoreactivity for GABAA and/or immunoreactivity for Î±4-containing nAChRs and /or Î±7-containing nAChRs.

Is your study complete? If still ongoing, please indicate when you expect it to be completed. (300 word limit)

We have completed a component of Specific Aim 1. Experiments are underway to complete the first objective. These experiments include double and triple immunohistochemical studies to determine if menthol-induced c-Fos activated cells in the VTA-Acb pathway express nAChRs and GABAA receptors. If the funds provided by Charles & Mary Latham Fund (Ella O. Latham Trust) allows, we also intend to perform receptor binding and autoradiography studies to determine if chronic menthol changes the expression of nAChRs and GABAA receptors in VTA and Acb.

Please describe the results of your study and how they compare to your anticipated outcomes. (500 word limit)

Menthol-induced activated cells in CNS:
One of the objectives of the present study was to identify the neuroanatomical and neurochemical profile of CNS neurons that are activated by intraperitoneal (IP) injection of menthol (100mg/kg) and to determine whether GABAergic and dopaminergic cells of the nicotine reward-addiction circuitry are targets of menthol in the CNS. Our preliminary studies using c-Fos immunohistochemical technique have demonstrated that acute IP administration of menthol stimulates neurons at multiple sites of the CNS including several structures previously shown to be activated by nicotine. At the pontine medullary junction, menthol activated cells were detected at areas corresponding to locus coeruleus (LC) and laterodorsal tegmental nucleus (LDTg), both areas are known to send projections to VTA. Scattered c-Fos expression was also observed in areas that overlaps rostral ventral tegmental area (RVTA). Other areas strongly stimulated by menthol included Acb and bed nucleus of stria terminalis (ST).

Neuroanatomical location of menthol-induced c-Fos immunoreactive cells with respect to dopaminergic cells of the nicotine reward-addiction circuitry:
In most caudal regions of VTA only sparse number of cells were activated by menthol and activated cells were distinct from dopaminergic cells at these sites. In the RVTA, moderate number of menthol activated cells were seen at areas overlapping retromamillary nucleus (RM) and at sites that were ventral to the dopaminergic cells of VTA. None of the dopaminergic cells were activated by menthol at these sites.

Is there future research or additional work expected as a result of your findings? (300 word limit)

In future studies we would like to determine the neurochemical nature of the cells in the Acb and VTA that are activated by menthol. We would then like to identify if these cells contain nAChRs or GABA receptors. Once we determine the nature of these cell types we would also like to perform functional and pharmacological studies to understand how the activation of these cells affects the reward-addiction pathways.

Please share any scholarly activities related to this grant including abstracts, presentations, publications, etc. (500 word limit)

We are in the process of preparing an abstract to be submitted to the Society of Nicotine and Tobacco Research (SNTR) in September, 2018 and present our findings at their meeting in February 2019.

Final Report: Budget Status Report

Please outline how the grant funds were spent. What balance (if any) remains? (500 word limit)

All of the funds so far used to address the first set of experiments for the first objective were from previous residual University funds. We have reserved all funds provided by Charles & Mary Latham Fund (Ella O. Latham Trust) to complete next set of immunohistochemical studies to determine if menthol-activated cells in CNS express nAChRs and GABAA receptors. Because the funds received were reduced from those requested, we hope that we have sufficient funds to address a component of the second objective which involves using receptor

binding and autoradiography to determine if chronic menthol administration changes the level of expression of nAChRs and GABAA receptors in reward-addiction pathways. These last studies have been slightly delayed due to closure of Dr. Davila-Garciaâ€™s lab because of critical damage due to water pipes bursting last winter. Many of the damaged labs remains closed and it is expected that they will be opened by August 18.

If funds remain, please indicate how and when they will be spent (remembering that funds must be utilized within 2 years). (500 world limit)

As stated in the answer to question number 6. We will utilize the Charles & Mary Latham Fund (Ella O. Latham Trust) funds to complete the first objective of the present study and to obtain preliminary data for the second objective which includes receptor binding and autoradiography.

Attachments

Title	File Name
Other	Preliminary Data.pdf

PRELIMINARY DATA:

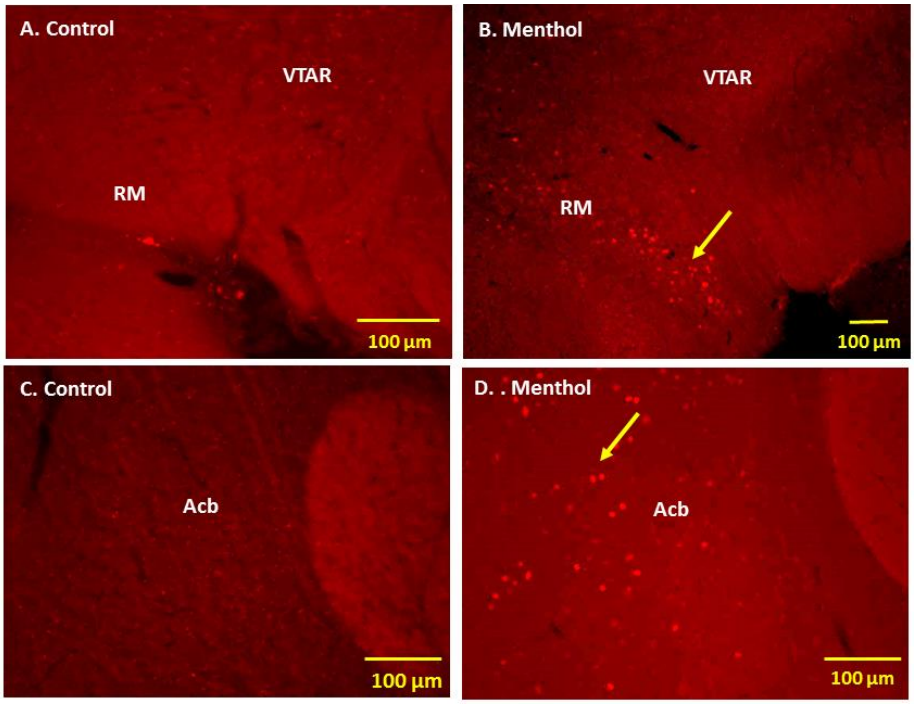

Figure 1: Fluorescent microscopy images demonstrating the location of menthol- induced c-Fos activated cells in and Acb. Panels A and C: Control data demonstrating physiological saline (PS) -induced c-Fos activated cells of VTA and Acb respectively. Panels B and D shows menthol induced c-Fos activated cells of VTA and Acb respectively. Abbreviations: RM=retromamillary nucleus, VTAR= rostral ventral tegmental area, Acb=Nucleus accumbens.

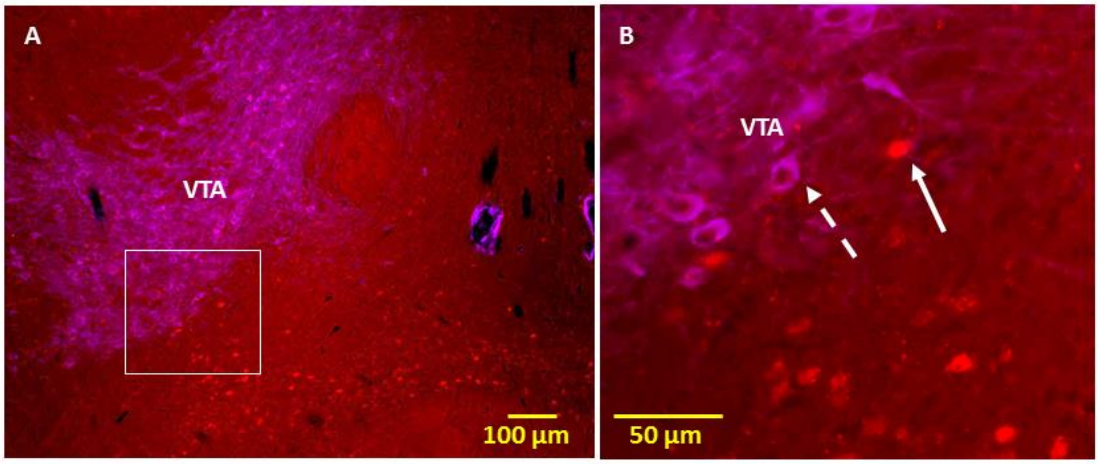

Figure 2: Double immunofluorescence microscopy images demonstrating the location of menthol-induced c-Fos immunoreactive (IR) neurons with respect to tyrosine hydroxylase (TH) IR dopaminergic cells of ventral tegmental area (VTA). Panels A and B: Low and high-power magnification showing menthol-induced c-Fos IR cells with respect to TH IR cells of VTA.