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E-cigarette aerosols containing nicotine modulate nicotinic acetylcholine receptors and astroglial glutamate transporters in mesocorticolimbic brain regions of chronically exposed mice

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Abstract

Nicotine exposure increases the release of glutamate in part through stimulatory effects on pre-synaptic nicotinic acetylcholine receptors (nAChRs). To assess the impact of chronic electronic (e)-cigarette use on these drug dependence pathways, we exposed C57BL/6 mice to three types of

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AUTOR Statement

FA participated in study design and conceptualization, drafted and revised the manuscript, performed western blot and qPCR assays, collected the data. LCEA conceptualized and designed the study, critically revised the manuscript for intellectual content, and approved the final version of the manuscript. AMH and HA performed protein and mRNA expression studies, collected the data and helped with the editing of manuscript. JS and AM performed the animal study with electronic cigarettes containing nicotine and collected the animal brains and helped with the editing of manuscript. AH and ITS performed the UPLC-MS/MS technique, collected the data and helped with the editing of manuscript. YS conceptualized and designed the study, critically revised the manuscript for intellectual content, and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

inhalant exposures for 3 months; 1) e-cigarette aerosol generated from liquids containing nicotine (ECN), 2) e-cigarette aerosol generated from liquids containing vehicle chemicals without nicotine (Veh), and 3) air only (AC). We investigated the effects of daily e-cigarette exposure on protein levels of α7 nAChR and α4/β2 nAChR, gene expression and protein levels of astroglial glutamate transporters, including glutamate transporter-1 (GLT-1) and cystine/glutamate antiporter (xCT), in the frontal cortex (FC), striatum (STR) and hippocampus (HIP). We found that chronic inhalation of ECN increased α4/β2 nAChR in all brain regions, and increased α7 nAChR expression in the FC and STR. The total GLT-1 relative mRNA and protein expression were decreased in the STR. Moreover, GLT-1 isoforms (GLT-1a and GLT-1b) were downregulated in the STR in ECN group. However, inhalation of e-cigarette aerosol downregulated xCT expression in STR and HIP compared to AC and Veh groups. ECN group had increased brain-derived neurotrophic factor in the STR compared to control groups. Finally, mass spectrometry detected high concentrations of the nicotine metabolite cotinine in the FC and STR in ECN group. This work demonstrates that chronic inhalation of nicotine within e-cigarette aerosols significantly alters the expression of nAChRs and astroglial glutamate transporters in specific mesocorticolimbic brain regions.

Keywords

E-cigarettes; Nicotine; α4/β2 nAChR; α-7 nAChR; GLT-1; xCT

Introduction

Emerging evidence indicates that the use of electronic (e) cigarettes is becoming common worldwide [1–3]. Although tobacco companies and cigarette smokers claim that e-cigarettes are a safe alternative to tobacco, harmful effects associated with e-cigarette exposure have been found [4–7]. Recent studies found that e-cigarette exposure induced initiating effects of cancer as well as DNA damage [6, 8, 9]. In addition, addictive behavioral effects have been found in subjects exposed to e-cigarettes chronically [10–12]. For instance, e-cigarette dependence occurred following long-term use of e-cigarettes [11, 12]. Passive exposure to e-cigarettes increases the desire to consume e-cigarettes or conventional cigarettes in smokers [10]. However, nicotine seeking behavior was found significantly higher in female animal model compared with males [13]. With the surge in adolescent use of e-cigarettes, and no highly successful treatments for e-cigarette addiction existing for this at-risk population, it is warranted to investigate the molecular pathways that could be pharmacologically targeted to attenuate the use of e-cigarettes.

The release of glutamate and dopamine after exposure to nicotine is modulated by nicotinic acetylcholine receptors (nAChRs) in the mesocorticolimbic areas [14–17]. The α7 subunit of nAChR is mainly expressed in glutamatergic neurons, while α4/β2 nAChR is mainly expressed in the GABAergic and dopaminergic neurons respectively [18–20]. It is important to note that targeting α7 nAChR or α4/β2 nAChR has been found to reduce the effects of nicotine on glutamate or dopamine release [17, 21]. Recently, we found that six months exposure to e-cigarettes upregulated α7 nAChR in the frontal cortex (FC) and striatum (STR) of female CD1 mice [22]. Thus, changes in functions or protein expression of these subtypes of nAChRs might affect the glutamate/dopamine homeostasis after chronic

exposure to e-cigarettes. This effect might contribute to the development of nicotine dependence. A previous study used two mouse models (CD-1 and C57BL/6 mice) and exposed them to e-cigarettes vapors-containing nicotine for 3 and 6 months [23]. This study found that circulating profibrotic and proinflammatory proteins were elevated in these two models. Kidney fibrosis was also observed in the two mouse models. These findings suggest that these two animal models exhibit similar pathophysiological changes after long-term exposure to e-cigarette vapors-containing nicotine.

The glutamatergic system is known to be involved in the reinforcement of nicotine effects. Glutamate transporter-1 (GLT-1) was found to remove the majority of extracellular levels of glutamate into astrocytes [24, 25]. Downregulation in GLT-1 and consequently increase in the total extracellular concentration of glutamate have been observed in animals exposed to drugs of abuse [26, 27]. GLT-1 is present in different isoforms in the brain; GLT-1a is expressed in neurons and astrocytes, while GLT-1b is localized in astrocytes [28, 29]. Although six-month exposure of e-cigarette aerosols reduced GLT-1 expression in the STR [22], it is unknown whether chronic use of e-cigarettes-containing nicotine affect GLT-1 isoforms in the mesocorticolimbic system. In addition to GLT-1, xCT is another astroglial glutamate transporter that showed ability to regulate the homeostasis of glutamate through exchange intracellular glutamate for extracellular cystine [30]. Six month exposure to e-cigarette aerosols reduced xCT protein expression in the STR and HIP [22]. However, the effect of exposure to nicotine on the gene expression of GLT-1 and xCT has not been elucidated.

Cotinine, a major metabolite of nicotine, is widely used as an indicator of smoking in humans [31, 32]. Cotinine has a longer half-life compared to nicotine, thus it is a biomarker that can be found in urine, plasma and the brain in subjects exposed to nicotine [22, 32–34]. Cotinine has been found to modulate nAChRs in the brain of animal models [35, 36]. In the present study, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify the concentration of cotinine in two critical brain areas, FC and STR, in mice exposed to e-cigarette aerosols for three months, thus confirming the systemic delivery of nicotine via our established murine e-cigarette aerosol inhalation system.

Changes in the expression of nicotinic receptors and glutamate transporters in the mesocorticolimbic system have been observed in animal developed nicotine dependence [37, 38] and after short withdrawal [39]. Modulation of these targets attenuated nicotine-seeking behaviors [40, 41]. In this study, we investigated the effects of daily exposure to E-cigarette aerosol-containing nicotine (ECN) for three months on protein expression of α4/β2 nAChR and α-7 nAChR as well as GLT-1 isoforms. We extended our findings to investigate the mRNA expression of GLT-1 and xCT in the FC, STR and HIP in female C57BL/6J mice. Finally, we determined the expression of activity-regulated cytoskeleton-associated protein (ARC) and brain-derived neurotrophic factor (BDNF) in the STR to investigate whether ECN affects neuronal plasticity. Our study provides supportive information about the effects of long term exposure to ECN on nicotinic receptors, glutamatergic transporters, ARC and BDNF.

Materials and Methods

E-cigarettes

E-cigarette cartomizers (tanks; 2.4 ohm, plastic, refillable) as well as the ingredients of e-liquids including nicotine, propylene glycol, and vegetable glycerin were acquired online from Xtreme Vaping. Preparation of e-liquids was performed in our laboratory by adding 24 mg/mL nicotine to a mixture of 50% propylene glycol and 50% vegetable glycerin. This mixture is widely used in e-cigarettes brands and have been studied in previous pre-clinical studies [22, 42]. E-cigarette batteries (280 mAh fixed, automatic, rechargeable, stainless steel) were bought online from FastTech. Briefly, a standard tank-containing e-liquids (1.8 Ω) was connected to a battery (lithium ion) at 3.4 V. Every 20 seconds, heating coil within e-liquid was activated for 4 seconds for vaporization (activation of e-cigarette). E-cigarette aerosol was generated, at the same time, using 2 L/min negative pressure to draw the e-liquid through the atomizer into the nose-only chambers.

Mouse inhalation of e-cigarette aerosols

Female C57BL/6 mice, 6–8 weeks old, were purchased from Harlan and were exposed via the SciReq inExpose system as recently described [22]. Nose-only e-cigarette aerosol exposure was used to focus effects on the respiratory system. Mice were divided into three exposed groups; 1) E-cigarette aerosol-containing nicotine (ECN). 2) E-cigarette aerosol with vehicle chemicals only (Veh). 3) Air-only control (AC) group. All groups were placed in the same restraints for 12 seconds/minute, for 60 minutes/day, five days/week, for three months as described in previous study [23]. E-liquids contain 24 mg/mL nicotine in a solution containing 1/1 ratio of vegetable glycerin and propylene glycol. This composition is used in several commercial brands. We here exposed the mice to e-cigarette vapors-containing nicotine for 1 hour/day, 5 days/week, for three months. This methodology was also used in previous study that tested e-cigarette nicotine vapor exposure for 6-month [22], and showed an average plasma cotinine concentration of 243 +/− 14 ng/mL. This is in accordance to a clinical study that showed blood cotinine levels of 250–300 ng/mL in human cigarette smokers [43]. Active smokers were found to have average serum cotinine levels at range of 250–300 ng/mL [44], however, heavy smokers might have serum cotinine of 500–800 ng/mL. After each exposure session, mice were recovered for 30 minutes in pre-warmed cages. Two hours after the final exposure, mice were euthanized using terminal intracardiac bleed, under general anesthesia (ketamine and xylazine). All animals were used in accord with the NIH guidelines for animal use under protocols approved by the IACUC committee at the University of California, San Diego and San Diego VA health system.

Brain Tissue Harvesting

Brains were extracted and immediately kept at −80°C. Stereotaxic coordinates from the Mouse Brain Atlas [45] was used to indicate the interesting brain regions (FC, STR and HIP). The isolated brain tissues were stored in liquid nitrogen at −80°C for protein and mRNA expression testing assays as well as LC-MS/MS analysis.

Quantitative PCR (qPCR) assay for detection of GLT-1, and xCT mRNA expression

The mRNA expression of astroglial glutamate transporters for all tissue samples was investigated using real-time quantitative PCR (RT-PCR) assay as described previously [46]. TRizol reagent (Invitrogen# 15596–018) was used to isolate the total RNAs from the FC, STR, and HIP tissues. Reverse transcription (RT) was performed using commercially available cDNA synthesis kit (Thermo Scientific, cat#AB-1453/A). Appropriate forward and reverse primers for the genes of interest were bought from Invitrogen (Table 1.). A reaction mixture-containing primers, SYBR Green (BIORAD, #170–8882) and cDNA samples were used for iCycler real time PCR testing system (Bio-Rad laboratories, München, Germany). As described in a previous study [47], the $2^{-\Delta\Delta CT}$ method was performed to calculate and analyze the amount of mRNA for each sample based on threshold cycle number (CT). This relative amount of mRNA for the genes of interest was compared between each group. The values of ΔCT were calculated by subtracting the average values of CT for the control gene (GAPDH) from the average values of CT for the nicotinic receptors and astroglial glutamate transporters genes. To calculate ΔΔCT, the mean ΔCT value of AC group was subtracted from the mean values of ΔCT for the Veh and ECN groups. The ΔΔCT values for each group were further analyzed by $2^{-\Delta\Delta CT}$ to obtain the relative fold change from control for each gene of interest. The values of fold change were shown as mean ± SEM. One percent of agarose gel was used to detect the bands of the mRNA data. Images of the bands were taken by a digital camera (Canon) connected to a UV light apparatus (PhotoDoc-It UVP 50 Imaging System).

Western blot assay for detection of α-4 nAChR, β-2 nAChR, α-7 nAChR, GLT-1, GLT-1a, GLT-1b, and xCT protein expression

The changes in protein expression of α-4 nAChR, β-2 nAChR, α-7 nAChR, GLT-1,and xCT in the FC, STR and HIP tissues were investigated using western blot assay as described previously [37, 48]. Homogenization of brain samples of FC, STR and HIP tissues were performed in a specific lysis buffer-containing protease inhibitors. Bio-rad DC (detergent compatible) protein assay (Bio-Rad, Hercules, CA, USA) was used to detect the total protein in each brain sample. After protein quantification, 10–20% polyacrylamide gels were loaded with similar amounts of protein from each tissue samples for electrophoretic separation. The transfer of protein into PVDF membranes was performed using a transfer apparatus equipment. Different concentrations of milk (5% for α-4/β-2/α-7 nAChRs) and (3% for GLT-1, GLT-1 isoforms, xCT and GAPDH) in Tris-buffered saline with Tween-20 (TBST) were used to block PVDF membranes at room temperature for 30 minutes. Subsequently, the primary antibodies: rabbit anti-α-4 nAChR (1:1000 Abcam), rabbit anti-β-2 nAChR (1:1000 Thermo Fisher Scientific), rabbit anti-α-7 nAChR (1:500 Abcam), guinea pig anti-GLT-1 (1:5000 Millipore), rabbit anti-GLT-1a (1:5000), rabbit anti-GLT-1b (1:5000), rabbit anti-xCT (1:1000 Abcam), rabbit anti-BDNF (1:5000 Millipore) and rabbit anti-ARC (1:5000 Millipore) were incubated with the membranes at 4°C overnight. To control the loading of proteins, mouse anti-GAPDH (1:5000 Millipore) was used. On the second day, membranes were washed five times with TBST and followed by 30 minute-blocking with 3% milk in TBST at room temperature. Horseradish peroxidase-labeled (HRP) secondary antibodies (anti-rabbit IgG, anti-guinea pig IgG, or anti-mouse IgG) at ratio of 1:3000 were incubated with the membranes for 90 minutes. Membranes were then further washed with TBST

followed by drying in a Whitman paper. Commercial available kits (Super Signal West Pico, Pierce Inc.), chemiluminescence, was used and applied to the membranes to detect the proteins of interest. Radiographic films (HyBlot CL Film Thermo Fisher Scientific) were exposed to the membranes and these films were developed using an SRX-101A processor. MCID software was used to express the digitized image bands of proteins (α-4 nAChR, β-2 nAChR, α-7 nAChR, GLT-1, GLT-1a, GLT-1b, xCT and GAPDH) in numbers. The values obtained from ECN or Veh were normalized to the matched group of AC animals (100%) to determine whether ECN or Veh induce changes in the expression for the proteins of interest in the FC, STR and HIP as described in previous studies [46, 49].

Quantitation of Cotinine Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for quantification of cotinine in the FC and STR

Brain samples, FC and STR, were spiked with an acidic solution containing a mixture of deuterated internal standards, homogenized, extracted using a solid phase resin, and analyzed using LC-MS/MS via multiple reaction monitoring (MRM) to quantitate cotinine. Treatment duration, route, conditions, and identity of samples were blinded to the LC-MS experimenter. Briefly, FC and STR tissue (1–30 mg [n = 5–7]) was weighed and transferred to a 1.5 mL RINO tube (Next Advance Inc.) followed by addition of dH2O (200 μL, 0.05% formic acid [FA]) containing the following internal standards; dopamine [1–13C] (750 nM), serotonin-d4 (250 nM), nicotine-d4 (250 nM), and cotinine-d3 (250 nM) and diluted in ammonium acetate (200 μL at 10 mM). The solution was homogenized with The Bullet Blender Storm (Next Advance Inc.) for 1 min (speed 8) followed by centrifugation at 14,000 rpm for 1 min. The supernatant was collected and applied to a solid phase extraction resin (Evolute Express WCX cartridge; size = 30 mg/1 mL [Biotage]) using a Vacmaster manifold (Biotage). Cartridges were washed with ammonium acetate (500 μL at 50 mM), gravity flow for 5 min followed by application of vacuum for 1 min. Cartridges were then washed with isopropyl alcohol (IPA) (125 μL), gravity flow for 5 min followed by application of vacuum for 1 min. Cartridges were then washed with dichloromethane (DCM) (500 μL), gravity flow for 5 min followed by application of vacuum for 5 min. Analytes were eluted with dH2O: IPA (85:15) +0.1 % FA (125 μL). 50 μL of the elutant was spiked with 20 μL of the IPA wash (cotinine elutes in IPA wash). Samples were then analyzed by LC-MS/MS via a Shimadzu Nexera XR UPLC coupled with a Shimadzu 8050 triple quadrupole mass spectrometer. The instrument was optimized for MRM transitions using analytical grade standards of dopamine, dopamine [1–13C], serotonin, serotonin-d4, nicotine, nicotine-d4, cotinine, cotinine-d3, and (Sigma Aldrich and Cambridge Isotopes). UPLC utilized a Kinetex-core column (Phenomenex): 2.6 μm HILIC 100 Å, 100 × 4.6 mm. UPLC Method: aqueous ammonium formate (5 mM, solvent A); acetonitrile: dH2O (9:1) (solvent B); flow rate 0.4 mL/min, Gradient: t = 0 min, 50 % B; t = 1.5 min, 50 % B; t = 2 min, 5 % B; t = 6 min, 5 % B; t = 6.1 min, 50 % B. Stop time 10 min. The resulting total ion chromatograms (TIC) for each MRM event demonstrated parent ion transition with two or three authentic fragment ions and were integrated to determine the area under the curve (AUC) of each analyte in each sample. Quantitation was carried out using a calibration curve (Figure 8D) and the ratio of the AUC of each analyte peak with the corresponding internal standard (AUCCot/AUCCotd3). The calibration curve was prepared in blank sample of matrix (homogenized brain tissue) spiked with varying concentrations of each analyte (0.02 μM,

0.08 μM, 0.2 μM, 0.8 μM, 2 μM) and a fixed concentration of internal standard (0.25 μM for all except dopamine [750 μM) with solid phase extraction as described above.

Statistical analyses

One-way ANOVA followed by Newman-Keuls post hoc test was used to analyze mRNA and western blot as well as LC-MS/MS data between AC, Veh and ECN groups. The expression of immunoblot bands were represented as 100% of AC group as performed previously [50–55]. All statistical analysis tests were shown as a $p<0.05$ level of significance.

Results

Effects of exposure to e-cigarette aerosols on protein expression of α-4 nAChR in the FC, STR and HIP

We investigated the effects of chronic e-cigarette use on the protein expression of α-4 nAChR in the FC [$F (2, 12) = 4.309$ ($p = 0.0389$)], STR [$F (2, 12) = 5.268$ ($p = 0.0228$)] and HIP [$F (2, 12) = 8.072$ ($p = 0.0060$)] brain regions. One way ANOVA showed significant differences in the expression of α-4 nAChR among AC, Veh and ECN groups in the FC, STR and HIP. As compared to Veh group, Newman Keuls post-hoc multiple comparisons test revealed a significant increase in α-4 nAChR protein expression in all studied brain areas in ECN group (Fig. 1B).

Effects of exposure to e-cigarette aerosols on protein expression of β-2 nAChR in the FC, STR and HIP

We further investigated the protein expression of β-2 nAChR after three months of exposure to e-cigarette aerosols. Statistical analysis showed significant differences in β-2 nAChR mRNA and protein expression among AC, Veh and ECN groups in the FC [$F (2, 12) = 5.188$ ($p = 0.0238$)], STR [$F (2, 12) = 9.879$ ($p = 0.0029$)] and HIP [$F (2, 12) = 4.276$ ($p = 0.0396$)]. Newman-Keuls multiple comparison test revealed that three-month inhalation of e-cigarette aerosols increased β-2 nAChR protein expression in ECN group compared to AC group in all studied brain areas (Fig. 2B).

Effects of exposure to e-cigarette aerosols on protein expression of α-7 nAChR in the FC, STR and HIP

Statistical analysis revealed significant changes in the protein expression of α-7 nAChR among AC, Veh and ECN groups in the FC [$F (2, 12) = 8.975$ ($p = 0.0041$)] and STR [$F (2, 12) = 6.288$ ($p = 0.0136$)] but not in the HIP [$F (2, 12) = 0.7276$ ($p = 0.5032$)]. Statistical analysis showed that α-7 nAChR protein expression was significantly increased in ECN group compared to AC and Veh groups in both FC and STR (Fig. 3B).

Effects of exposure to e-cigarette aerosols on mRNA and protein expression of GLT-1 in the FC, STR and HIP

The effects of nicotine containing e-cigarette aerosol exposure on GLT-1 mRNA and protein expression in the FC, STR and HIP were investigated. One way ANOVA followed by Newman-Keuls analysis did not show any significant changes in GLT-1 mRNA expression

in the FC [F (2, 12) = 0.4825 (p = 0.6287)] and HIP [F (2, 12) = 0.38941 (p = 0.6857)] (Fig. 4B&J) and GLT-1 protein expression in both FC [F (2, 12) = 0.4556 (p = 0.6446)] and HIP [F (2, 12) = 0.208 (p = 0.8152)] (Fig. 4D&L) among all three groups. However, one way ANOVA indicated a significant difference in the striatal GLT-1 mRNA [F (2, 12) = 4.523 (p = 0.0344)] and protein [F (2, 12) = 5.462 (p = 0.0206)] expression in the STR among the groups. Newman-Keuls test showed that three-month exposure to ECN reduced striatal GLT-1 mRNA and protein expression compared to AC and Veh groups (Fig. 4F&H).

Effects of exposure to e-cigarette aerosols on protein expression of GLT-1 isoforms in the FC, STR and HIP

We further determined the expression of GLT-1 isoforms in the FC, STR and HIP in AC, Veh and ECN groups. One-way ANOVA analysis indicated non-significant differences in GLT-1a expression in the FC [F (2, 12) = 1.024 (p = 0.3884)] and HIP [F (2, 12) = 0.691 (p = 0.5200)] as well as GLT-1b expression in the FC [F (2, 12) = 0.00713 (p = 0.9929)] and HIP [F (2, 12) = 0.432 (p = 0.6591)] among the three groups. However, the statistical analysis showed significant changes in the expression of GLT-1a [F (2, 12) = 7.536 (p = 0.0076)] and GLT-1b [F (2, 12) = 6.247 (p = 0.0138)] among AC, Veh and ECN groups in the STR. Newman-Keuls multiple comparison analysis showed a significant reduction in GLT-1a and GLT-1b expression in ECN compared to the control groups in the STR (Fig. 5F&H) but not in the FC (Fig. 5B&D) or HIP (Fig. 5J&L).

Effects of exposure to e-cigarette aerosols on mRNA and protein expression of xCT in the FC, STR and HIP

xCT mRNA and protein expression following three-month inhalation of ECN have been determined in the FC, STR and HIP. One-way ANOVA revealed that there are significant differences in xCT mRNA and protein expression among AC, Veh and ECN groups in the STR [F (2, 12) = 4.646 (p = 0.0321) for mRNA expression] and [F (2, 12) = 5.097 (p = 0.0250) for protein expression] and HIP [F (2, 12) = 7.914 (p = 0.0064) for mRNA expression] and [F (2, 12) = 7.479 (p = 0.0078) for protein expression] but not in the FC [F (2, 12) = 0.0983 (p = 0.9071) for mRNA expression] and [F (2, 12) = 1.140 (p = 0.3520) for protein expression]. Newman-Keuls multiple comparison test showed significant decrease in xCT mRNA expression in the ECN in the STR compared to both AC and Veh groups (Fig. 6F) as well as in the HIP (Fig. 6J) compared to Veh group. The statistical analysis also revealed a significant reduction in xCT protein expression in the STR and HIP in ECN group compared to Veh and AC groups (Fig. 6H&L). There were no significant changes in xCT mRNA and protein expression in the FC between all groups (Fig. 6B&D).

Effects of exposure to e-cigarette aerosols on protein expression of BDNF and ARC in the STR

Since we found changes in the gene and protein expression of GLT-1 and xCT after three-month exposure to e-cigarette aerosols, we explored the effects of inhalation of these aerosols on the expression of BDNF and ARC in the STR. One-way ANOVA revealed significant changes in BDNF protein expression among AC, Veh and ECN groups in the STR [F (2, 12) = 5.084 (p = 0.0252)]. The analysis did not show any significant changes in the ARC expression among AC, Veh and ECN groups in the STR [F (2, 12) = 0.8401 (p =

0.4555)] (Fig. 7B). Newman-Keuls multiple comparison test revealed marked increase in BDNF protein expression in the ECN in the STR compared to both AC and Veh groups (Fig. 7B).

Determination of cotinine concentrations in the FC and STR

Quantitative LC-MS technique was used to detect cotinine concentrations in the FC and STR based on a standard calibration curve, which represents the area under the curve relationship of the labeled isotope of cotinine analytical standard (Cotinne-d^3) at a specific concentration. We found that there is significant differences in the cotinine concentrations among AC, Veh and ECN groups in the FC [$F(2, 12) = 8.756$, $p = 0.0039$] and STR [$F(2, 12) = 5.074$, $p = 0.0220$]. Statistical analysis revealed that chronic inhalation of nicotine with e-cigarette aerosols was associated with significant cotinine concentrations in both FC and STR as compared to both AC and Veh groups (Fig. 8C).

Discussion

In the present study, the effects of chronic exposure to e-cigarette aerosols-containing nicotine on the expression of nicotinic receptor and astroglial glutamate transporters were investigated in the FC, STR and HIP. We found that nicotine-containing e-cigarette aerosols induced upregulatory effects on nicotinic receptors in mesocorticolimbic brain areas. Additionally, chronic inhalation of e-cigarette aerosols reduced astroglial glutamate transporters mRNA and proteins expression in specific brain areas. Cotinine levels within the mesocorticolimbic brain regions demonstrate effective delivery of nicotine within e-cigarette aerosols to the systemic circulation via our established murine exposure system.

Nicotinic receptors are distributed in pre and post-synaptic neurons in the brain and the stimulation of these receptors have been found to induce dopamine and glutamate release [14–16]. Studies found that chronic subcutaneous administration of nicotine as well as intravenous infusion of nicotine increased α4/β2 nAChR expression in the central reward areas [56, 57]. In this study, we found that three-month inhalation of e-cigarette aerosols-containing nicotine upregulated α4/β2 nAChR in the FC, STR and HIP as well as α7 nAChR in the FC and STR. It is important to note that chronic exposure to e-cigarette aerosols and tobacco smoke increased the binding sites of α4/β2 nAChR in the cortex, nucleus accumbens (NAc) and hippocampus of mice [58]. These findings were supported by previous studies showing that exposure to nicotine increased α4/β2 nAChR protein expression in the cortex [39, 57] and bindings in midbrain, prefrontal cortex and striatum [39]. Moreover, six month exposure to e-cigarette aerosols induced upregulatory effects on α7 nAChR in the FC and STR in female CD1 mice [22]. Although there were no changes in α4/β2 nAChR binding in the hippocampus of rats after chronic exposure to mainstream cigarette smoke [59], a previous study found that chronic exposure to either tobacco smoke or e-cigarette aerosols increased hippocampal α4/β2 nAChR in BALB/cJ mice [58]. These findings along with prior studies indicate that nicotine containing e-cigarette aerosols has upregulatory effects on the nicotinic receptors in the mesocorticolimbic areas.

Glutamate homeostasis is regulated by astroglial glutamate transporters including GLT-1 and xCT [24] and the expression of these transporters are decreased in the NAc following

exposure to ethanol, cocaine and nicotine [46, 49, 60, 61]. Chronic inhalation or intravenous self-administration of nicotine reduced GLT-1 and xCT expression in the brain areas in the mesocorticolimbic system [22, 61]. A recent study from our laboratory reported that e-cigarette aerosol exposure for six month reduced GLT-1 in the STR and xCT in the STR and HIP [22]. We reported here that e-cigarettes reduced mRNA and protein expression of GLT-1 in the STR and xCT in the STR and HIP. Our data suggests that nicotine affects the mRNA level of these transporters and consequently the number of glial transporters in the astrocytes. A previous study from our laboratory reported that chronic exposure to drugs of abuse such as cocaine decreased the relative mRNA level of GLT-1 and xCT in the NAc [46]. These data indicate that chronic exposure to nicotine suppresses mRNA of astroglial glutamate transporters and consequently reduced the expression of these astroglial proteins in the surface of astrocytes. Thus, nicotine containing e-cigarettes might reduce the expression of astroglial glutamate transporters in the STR and HIP through transcription mechanisms. Additionally, we found that inhalation of aerosol-containing nicotine induced downregulatory effects in GLT-1 isoforms (GLT-1a and GLT-1b) only in the STR. This suggests that chronic exposure to nicotine reduces GLT-1 and its isoforms in the astrocytes.

Our results suggest that the upregulatory effects of nicotine containing e-cigarettes on nicotinic receptors may lead to a form of synaptic plasticity involving long term potentiation [For review [62]]. Studies found that BDNF and ARC are essential in the development of synaptic plasticity [63, 64]. Since we found alterations in nicotinic receptors and glutamatergic system mainly in the STR, we determined the expression of both BDNF and ARC in the STR after three-month inhalation of nicotine-containing e-cigarette aerosols. In our study, we found that nicotine exposed mice had higher BDNF expression as compared to AC and Veh groups. Increased glutamate release was suggested as a key player for the increase in BDNF expression in the STR in animals developed nicotine-seeking [For review see [65]]. This increase in the expression of BDNF may be a neuroadaptation feedback for synaptic plasticity. This indicates that changes in striatal nicotinic receptors and glutamatergic system might lead synaptic plasticity in animals exposed to abused drugs such as nicotine for long-term period. Although a slight increase in the Arc expression was found in the STR of ECN group, our analysis did not reveal any significant differences between all groups. However, study from others demonstrated that exposure to nicotine cues has been associated with increase in mRNA Arc expression in the lateral prefrontal cortex using northern blot analysis [66]. Age and brain regions are considered key factors to regulate the effects of nicotine on the expression of Arc in the forebrain [67]. Further studies are warranted to determine the involvement of Arc on nicotine-induced neuroplasticity in different brain areas.

In our study, LC-MS/MS shows significant concentrations of cotinine in the FC and STR which indicates the validity of our computerized inhalations exposure system for nicotine delivery into the brains of animals through inhalation of e-cigarette aerosols [For review see [68]]. This also indicates that nicotine is metabolized into cotinine and the metabolite is accumulated in the mesocorticolimbic brain regions. Since the same mesh restraints were used with AC, Veh and ECN groups, the exposure to the aerosols of e-cigarettes in the environment (fume hood) might lead to very low concentrations of cotinine in the STR of both control groups. The low concentrations of nicotine and cotinine have been detected in

the FC of AC group in our previous six-month study, and this is because AC group was placed in the same mesh restraints as experimental group (e-cigarette-containing nicotine group) [22].

Conclusion and future directions

Our work reveals that a clinically and physiological exposure to nicotine through e-cigarette aerosol exposure induced changes in the expression of nicotinic receptors and astroglial glutamate transporters in the mesocorticolimbic system. These alterations may mediate the initiation and development of nicotine dependence. Moreover, these effects might lead to neuronal plasticity and synaptic neuroadaptation. Further research is required to study the correlation between the alterations in nicotinic receptors and glutamate transporters with glutamate/dopamine homeostasis in the mesocorticolimbic brain reward regions. Further investigations are needed to study the interactions between glutamatergic system and nicotinic receptors in animal exposed to nicotine. For instance, future work may investigate the expression of astroglial glutamate transporters in the brain of nAChR knockout mice.

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Abbreviations:

AC	air control
ARC	activity-regulated cytoskeleton-associated protein
α-7 nAChR	alpha-7 nicotinic acetylcholine receptor
α4/β2 nAChR	alpha 4/beta 2 nAChR
BDNF	brain-derived neurotrophic factor
e-cigarette	electronic cigarette
Veh	e-cigarette vehicle control
ECN	e-cigarette aerosol-containing nicotine
FC	frontal cortex
GLAST	glutamate/aspartate transporter
GLT-1	Glutamate transporter-1
HIP	hippocampus

NAc	nucleus accumbens
PFC	prefrontal cortex
STR	striatum
VTA	ventral tegmental area
xCT	cystine/glutamate antiporter

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Highlights

- E-cigarette-nicotine exposure for 3 months upregulated α4/β2 nAChR in FC, STR and HIP.
- E-cigarette-nicotine exposure for 3 months upregulated α7 nAChR in FC and STR.
- E-cigarette-nicotine exposure for 3 months downregulated GLT-1 and GLT-1 isoforms in STR.
- E-cigarette vapors-nicotine exposure for 3 months downregulated xCT in STR and HIP.
- E-cigarette-nicotine exposure for 3 months increased in BDNF in STR.

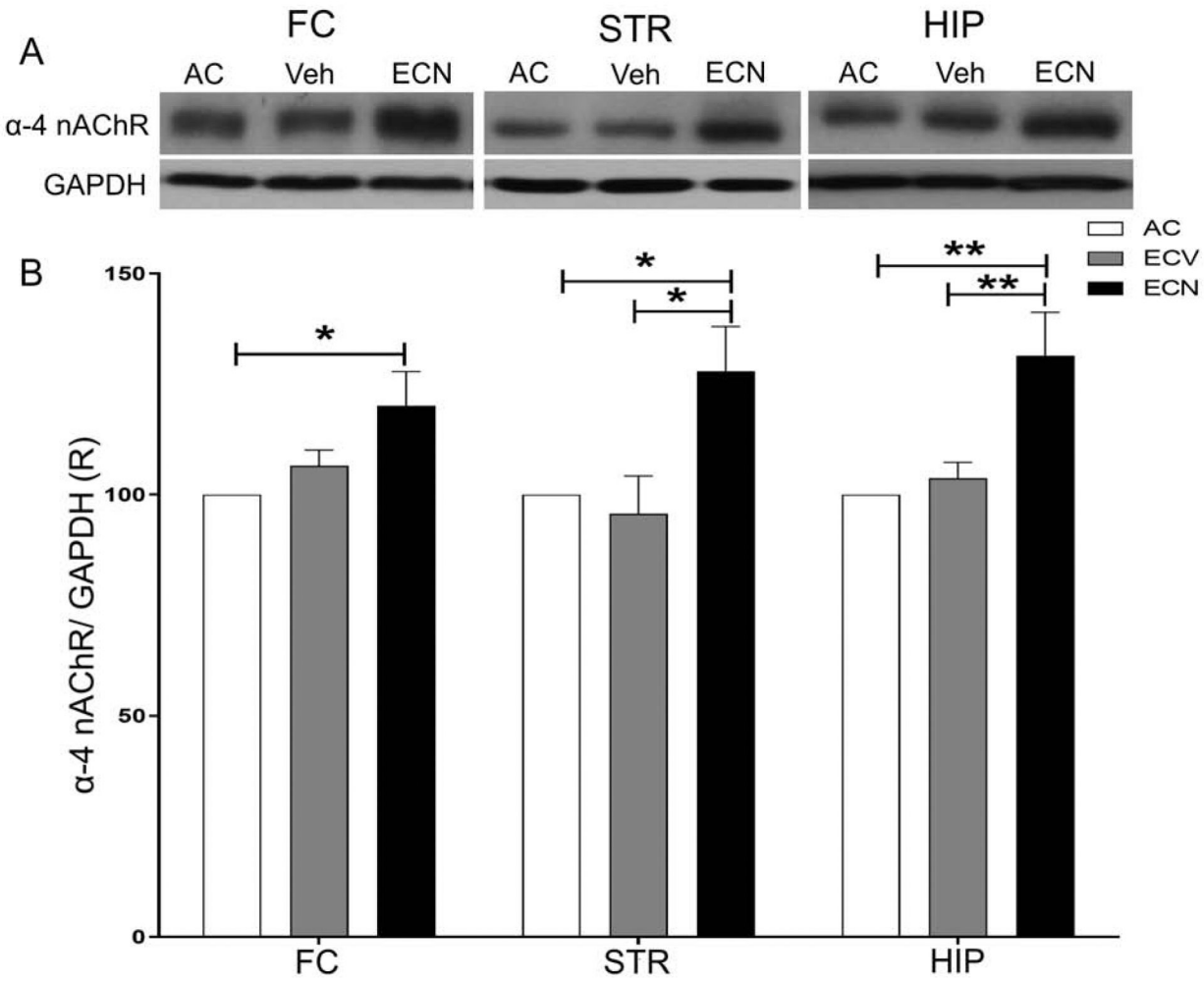

Fig. 1.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on α-4 nAChR relative (R) protein expression in the FC, STR and HIP. **A)** Expression of α-4 nAChR and GAPDH (loading control) blots in the FC, STR and HIP. B) One-way ANOVA followed by Newman-Keuls analysis revealed a significant increase in α-4 nAChR protein expression in ECN as compared to AC in all studied brain areas as well as Veh groups in the STR and HIP. Values are represented as mean ± SEM (*$p<0.05$, **$p<0.01$), (n=5 for each group).

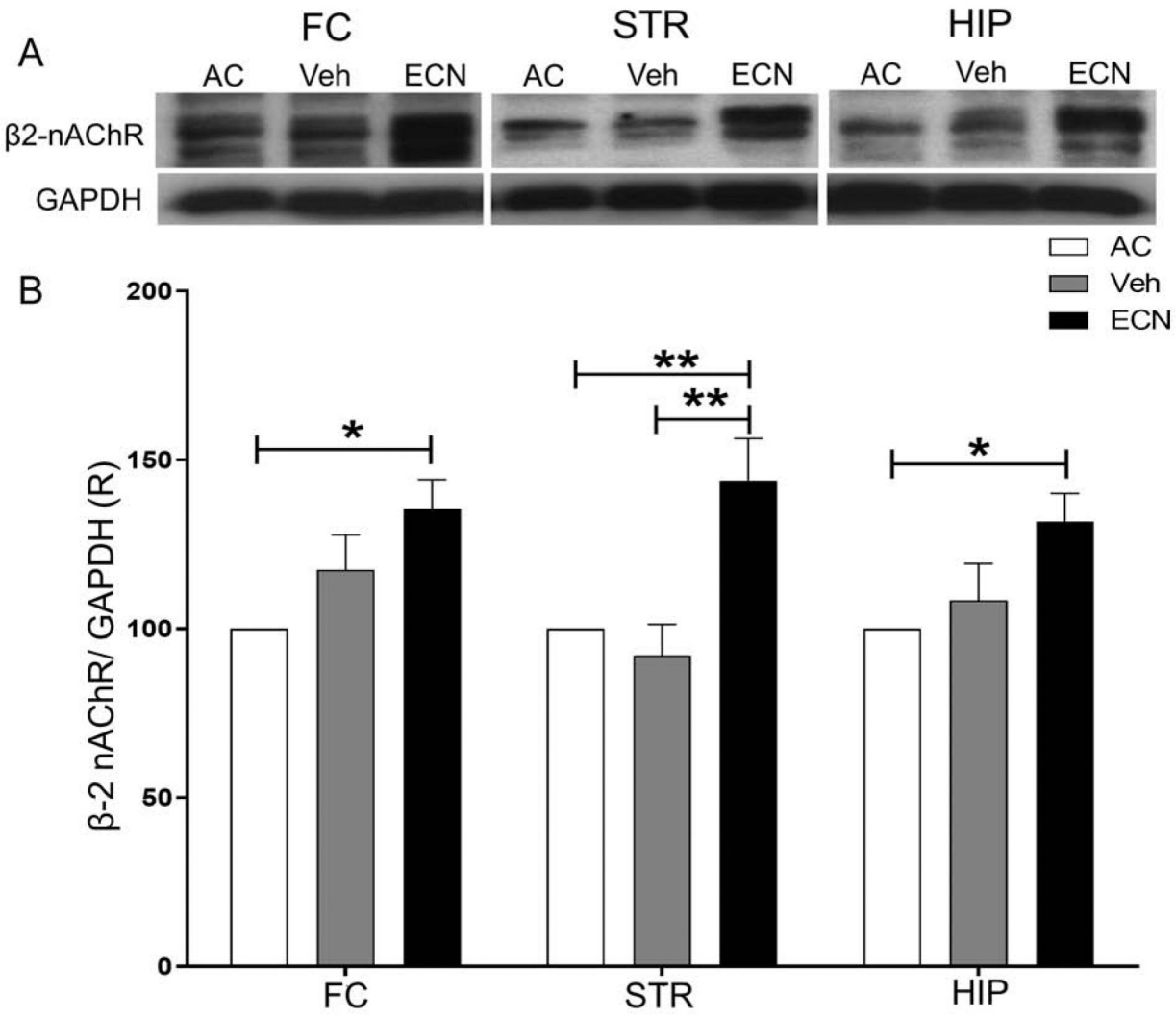

Fig. 2.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on β-2 nAChR relative (R) protein expression in the FC, STR and HIP. A) Expression of β-2 nAChR and GAPDH (loading control) blots in the FC, STR and HIP. B) One-way ANOVA followed by Newman-Keuls analysis revealed a significant increase in β-2 nAChR protein expression in ECN as compared to AC in all studied brain areas as well as Veh groups in the STR. Values are represented as mean ± SEM (*$p<0.05$, **$p<0.01$), (n=5 for each group).

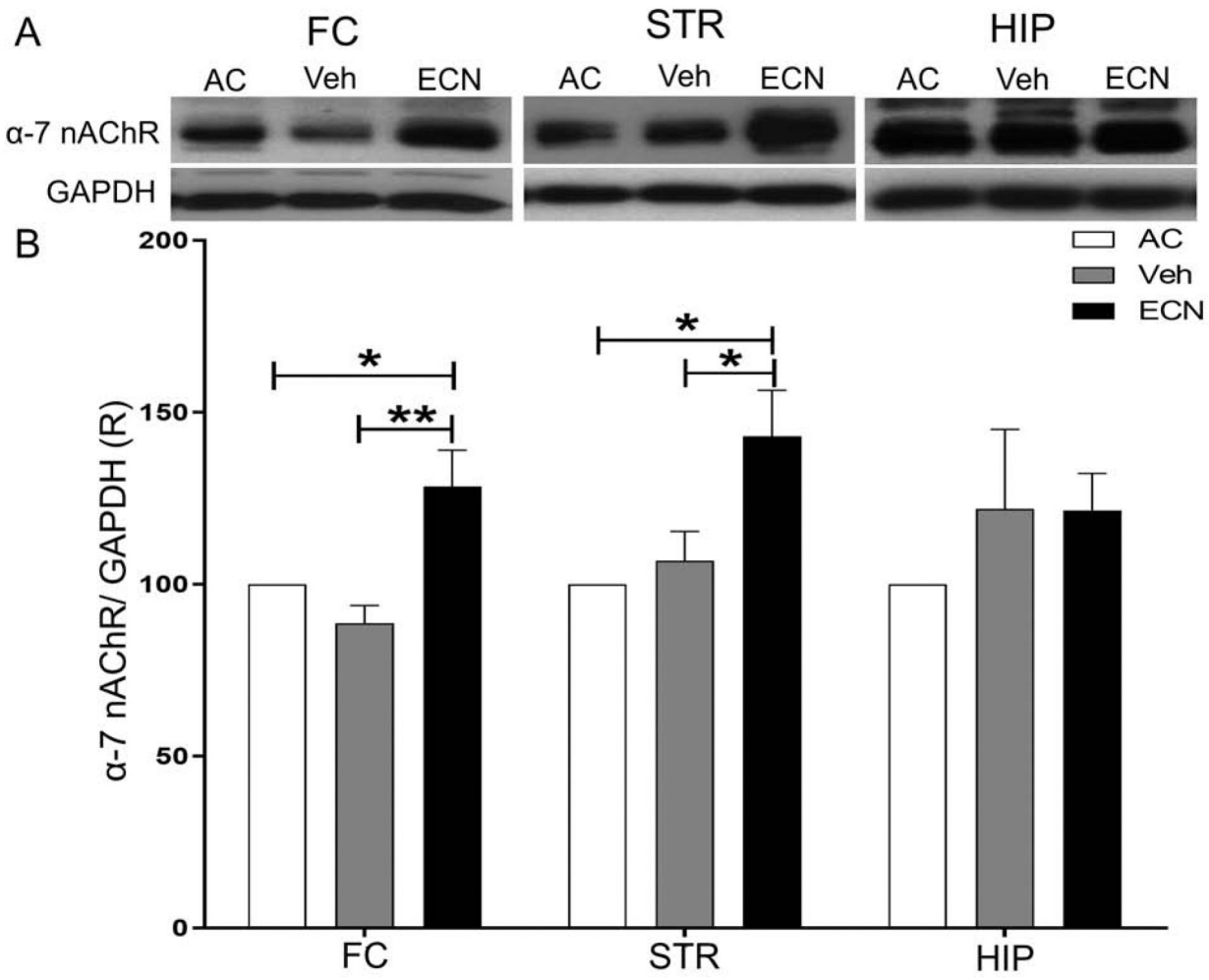

Fig. 3.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on α-7 nAChR relative (R) protein expression in the FC, STR and HIP. A) Expression of α-7 nAChR and GAPDH (loading control) blots in the FC, STR and HIP. B) One-way ANOVA followed by Newman-Keuls analysis revealed a significant increase in α-7 nAChR protein expression in ECN as compared to AC and Veh groups in the FC and STR but not in the HIP. Values are represented as mean ± SEM (*p<0.05, **p<0.01), (n=5 for each group).

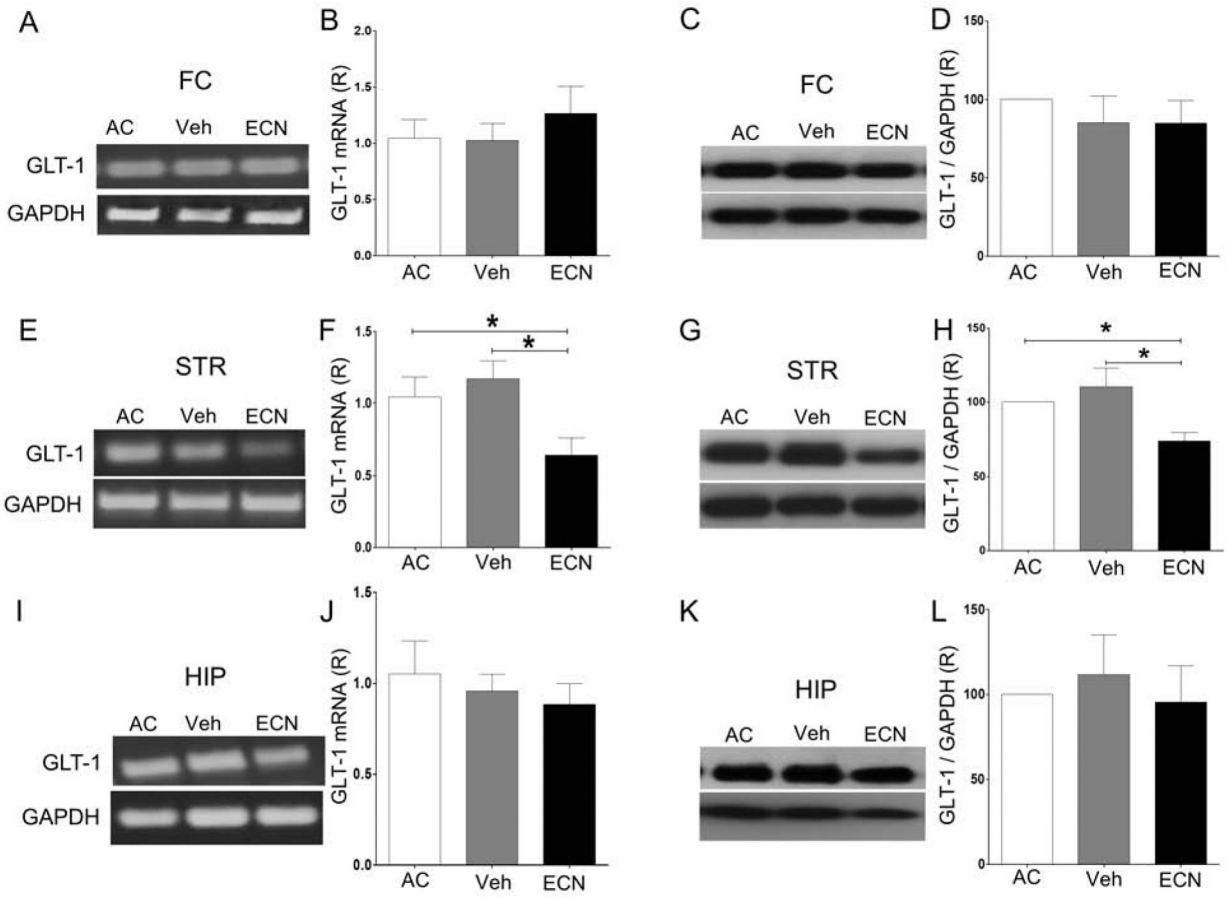

Fig. 4.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on GLT-1 relative (R) mRNA and protein expression in the FC, STR and HIP. A) Expression of GLT-1 and GAPDH (loading control) transcript (RT-PCR) in the A) FC, E) STR and I) HIP. Using one-way ANOVA followed by Newman-Keuls analysis B) There were no significant changes in GLT-1 mRNA expression in ECN as compared to the control groups in the FC. F) A significant decrease in GLT-1 mRNA expression in ECN as compared to the control groups in the STR. J) There were no significant changes in GLT-1 mRNA expression in ECN as compared to the control groups in the HIP. Western blot bands for GLT-1 and GAPDH (loading control) in the C) FC, G) STR and K) HIP. Using one-way ANOVA followed by Newman-Keuls analysis D) There were no significant changes in GLT-1 protein expression in ECN as compared the control groups in the FC. H) A significant decrease in GLT-1 protein expression in ECN as compared to the control groups in the STR. L) There were no significant changes in GLT-1 protein expression in ECN as compared to the control groups in the HIP. Values are represented as mean ± SEM (*$p<0.05$), (n=5 for each group).

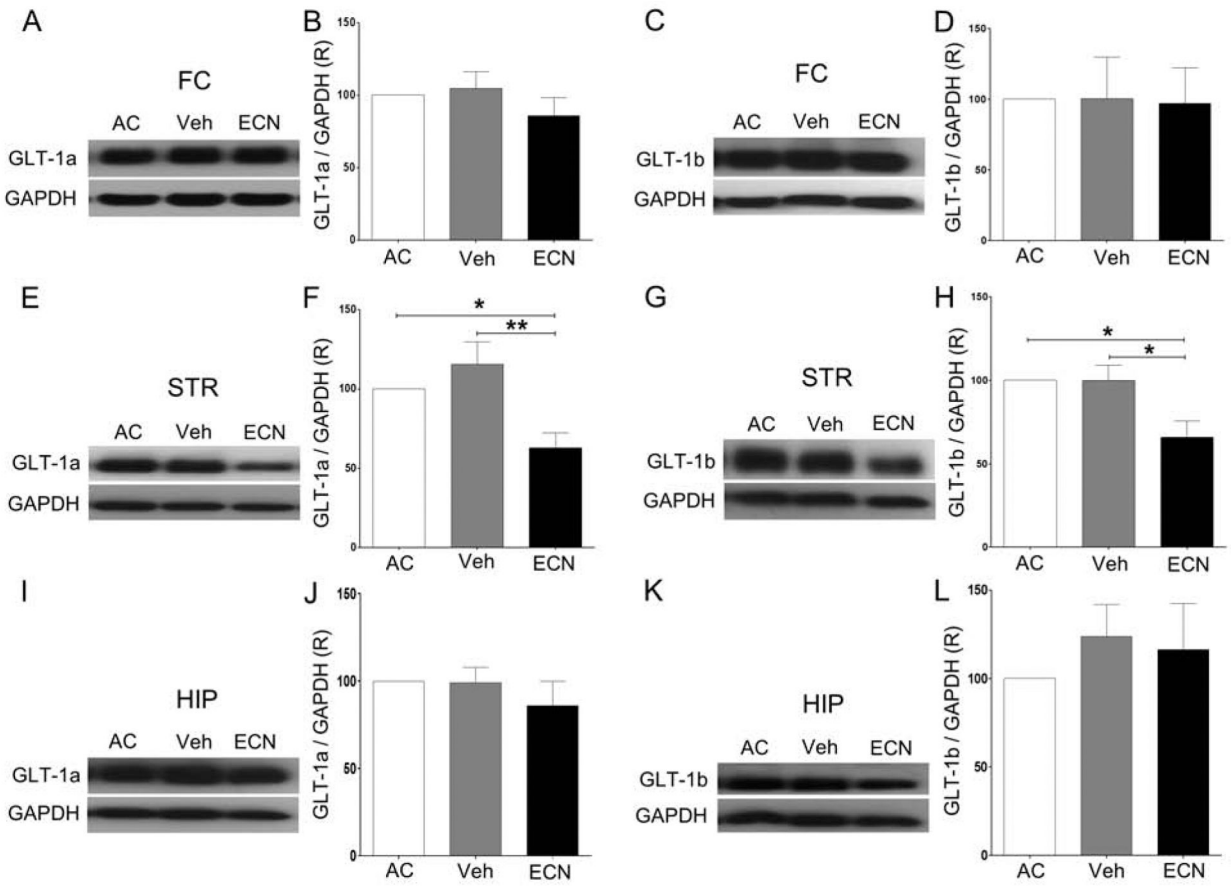

Fig. 5.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on GLT-1 isoforms relative (R) protein expression in the FC, STR and HIP. A) Western blot bands for GLT-1a and GAPDH (loading control) in the A) FC, E) STR and I) HIP. Using one-way ANOVA followed by Newman-Keuls analysis. B) There were no significant changes in GLT-1a protein expression in ECN as compared to control groups in the FC. F) A significant decrease in GLT-1a protein expression in ECN as compared to the control groups in the STR. J) There were no significant changes in GLT-1a protein expression in ECN as compared to the control groups in the HIP. Western blot bands for GLT-1b and GAPDH (loading control) in the C) FC, G) STR and K) HIP. Using one-way ANOVA followed by Newman-Keuls analysis. D) There were no significant changes in GLT-1b protein expression in ECN as compared the control groups in the FC. H) A significant decrease in GLT-1b protein expression in ECN as compared to the control groups in the STR. L) There were no significant changes in GLT-1b protein expression in ECN as compared to the control groups in the HIP. Values are represented as mean ± SEM (*$p<0.05$, **$p<0.01$), (n=5 for each group).

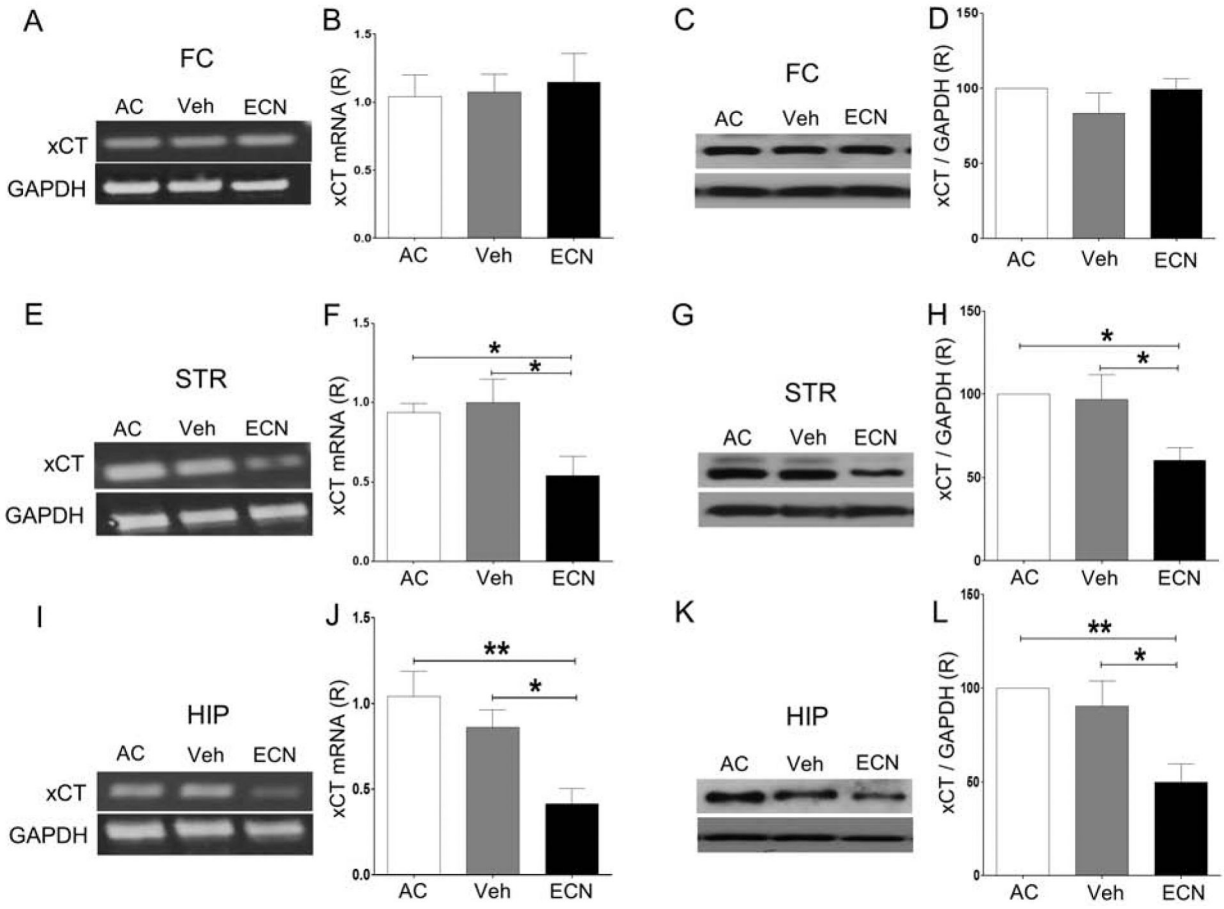

Fig. 6.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on xCT relative (R) mRNA and protein expression in the FC, STR and HIP. A) Expression of xCT and GAPDH (loading control) transcript (RT-PCR) in the A) FC, E) STR and I) HIP. Using one-way ANOVA followed by Newman-Keuls analysis. B) There were no significant changes in xCT mRNA expression in ECN as compared to the control groups in the FC. F) A significant decrease in xCT mRNA expression in ECN as compared to the control groups in the STR. J) A significant decrease in xCT mRNA expression in ECN as compared to the control groups in the HIP. Western blot bands for xCT and GAPDH (loading control) in the C) FC, G) STR and K) HIP. Using one-way ANOVA followed by Newman-Keuls analysis D) There were **no** significant changes in xCT protein expression in ECN as compared the control groups in the FC. H) A significant decrease in xCT protein expression in ECN as compared to the control groups in the STR. L) A significant decrease in xCT protein expression in ECN as compared to the control groups in the HIP. Values are represented as mean ± SEM (*$p<0.05$), (n=5 for each group).

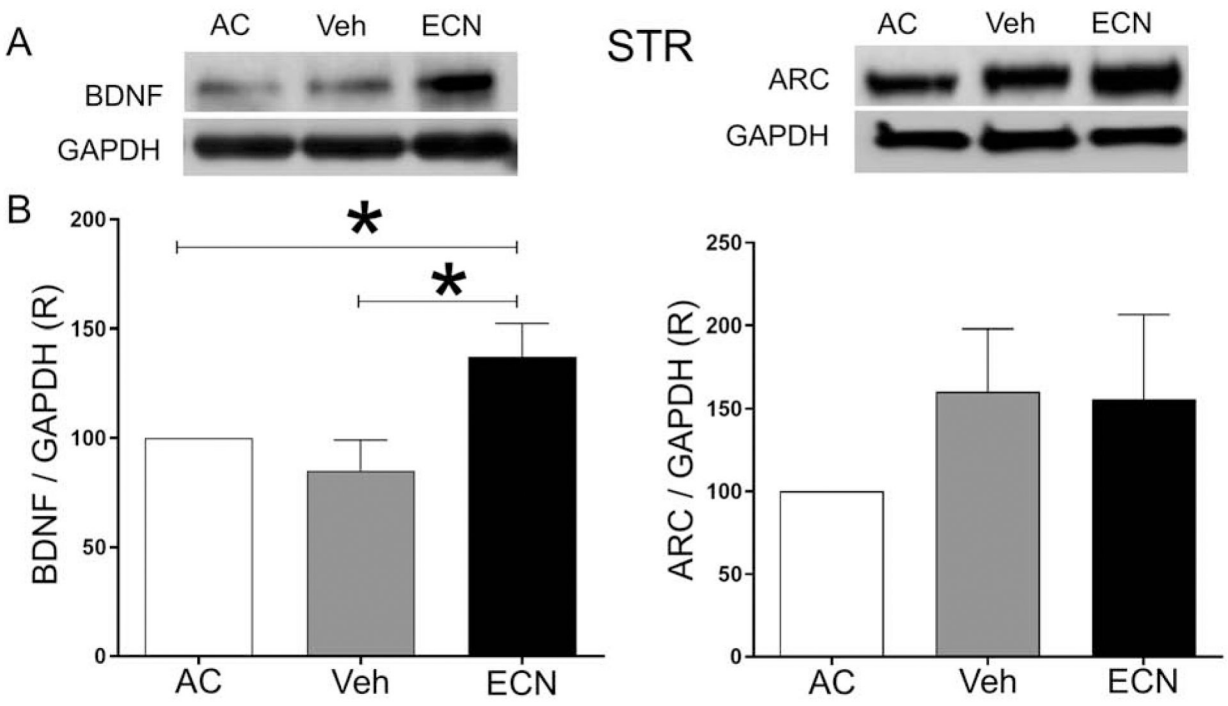

Fig. 7.

Effects of three-month inhalation of e-cigarette aerosols containing nicotine on BDNF and ARC relative (R) protein expression in the STR. A) Expression of BDNF, ARC and GAPDH (loading control) blots in the STR. B) One-way ANOVA followed by Newman-Keuls analysis revealed a significant increase in BDNF protein expression in ECN as compared to AC and Veh groups in the STR (left panel), while the analysis did not reveal any significant changes in ARC expression between the three groups (right panel). Values are represented as mean ± SEM (*$p<0.05$), (n=5 for each group).

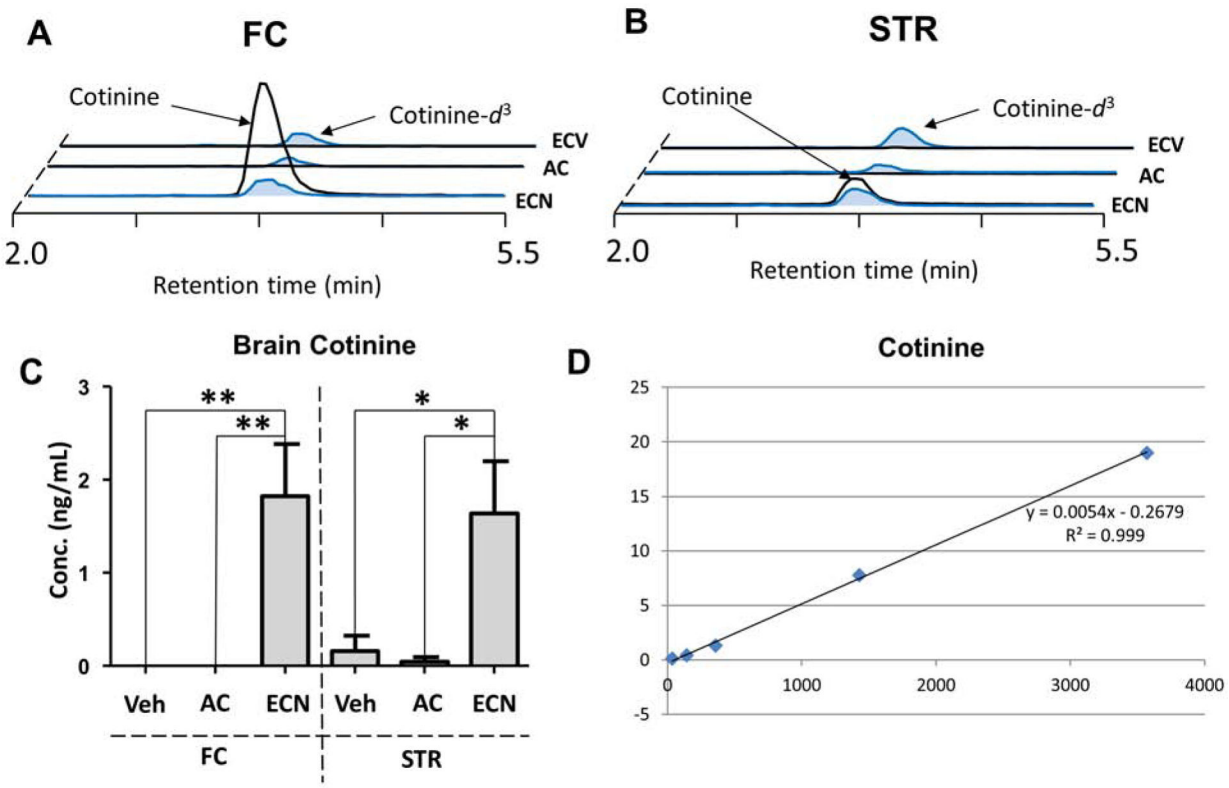

Fig. 8.

LC-MS/MS analysis of cotinine in the FC and STR tissue samples. A) Representative chromatograms of each group for FC brain samples. B) Representative chromatograms for each group for STR brain samples. C) Quantitation based on the ratio of the AUC of the MRM transitions of cotinine and cotinine-d3 relative to a calibration curve. D) Cotinine calibration curve. Concentrations were calculated after normalizing for tissue weight (assuming tissue/plasma density = 1). Values of ECN group are represented as mean ± SEM and compared to AC and Veh groups using one-way ANOVA analysis with Newman-Keuls post-hoc test. Values are represented as mean ± SEM (*p<0.05, **p<0.01), (n=5 for each group).

Table 1:

shows primer sequence for each gene in mice animal model. These primer sequences are GLT-1 primer sequence from [69]. xCT primer sequence from [70]. GAPDH primer sequence from [71].

Gene	Primer	Sequence
GLT-1	Forward primer	5’-AGCCGTGGCAGCCATCTTCATAGC-3’
	Reverse primer	5’-ATGTCTTCGTGCATTCGGTGTTGGG-3’
xCT	Forward primer	5’-AAGTGGTTCAGACGATTATCAG-3’
	Reverse primer	5’-AAGAAACGTGGTAGAGGAATG-3’
GAPDH	Forward primer	5’-GGGTGGAGCCAAACGGGTC-3’
	Reverse primer	5’-GGAGTTGCTGTTGAAGTCGCA-3’