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World No-Tobacco: effects of tobacco and nicotine on the brain

Edited by
Massimo Grilli, Vijayapandi Pandy, Gilda Angela Neves and Graciela Noemí Balerio

Coordinated by
Isis N. O. Souza

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World No-Tobacco: effects of tobacco and nicotine on the brain

Topic editors
Massimo Grilli — University of Genoa, Italy
Vijayapandi Pandy — MIT World Peace University, India
Gilda Angela Neves — Federal University of Rio de Janeiro, Brazil
Graciela Noemí Balerio — University of Buenos Aires, Argentina

Topic coordinator
Isis N. O. Souza — Federal University of Rio de Janeiro, Brazil

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EDITED AND REVIEWED BY
Nadine Kabbani,
George Mason University, United States

*CORRESPONDENCE
Gilda Angela Neves,
ganeves@icb.ufrj.br
Isis Nem de Oliveira Souza,
isis.souza@icb.ufrj.br

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Editorial: World No-Tobacco: effects of tobacco and nicotine on the brain

Isis Nem de Oliveira Souza[1]*, Graciela N. Balerio[2], Vijayapandi Pandy[3], Massimo Grilli[4] and Gilda Angela Neves[1]*

[1]Laboratório de Farmacologia Molecular, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Instituto de Investigaciones Farmacológicas (ININFA-CONICET) and Cátedra de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [3]Department of Pharmaceutical Sciences, School of Health Sciences and Technology, Dr. Vishwanath Karad, MIT World Peace University, Pune, India, [4]Department of Pharmacy, University of Genoa, Genoa, Italy

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Editorial on the Research Topic
World No-Tobacco: effects of tobacco and nicotine on the brain

Recent evidence places the earliest human tobacco use around 12,300 years ago (Duke et al., 2021). Among plants with psychoactive compounds, tobacco, and by default nicotine, is arguably the most entrenched in human history, affecting several aspects such as health, culture, and social relations (Castaldelli-Maia et al., 2016). Tobacco smoking remains the world's leading preventable cause of death, responsible for approximately 8 million deaths per year (Reitsma et al., 2021). On World No-Tobacco Day, we are advancing our understanding of tobacco and nicotine use and its impact on human health from various perspectives, with the potential to improve public health.

Significant gaps remain in our understanding of sex differences in the development of tobacco and nicotine addiction and relapse (Kcomt et al., 2022; Davis et al., 2023). Chellian et al. investigated the operant response to nicotine and the development of addiction in adult rats of both sexes. They also evaluated whether a period of enforced abstinence affected nicotine-seeking behavior. The results showed that nicotine intake was higher in females than in males when they were given prolonged daily access to the drug, highlighting the need for more individualized approaches to tobacco cessation.

Indeed, tobacco withdrawal effects are severe enough to halt cessation efforts and require specialized help. With a success rate of around 7% (Méndez et al., 2022), understanding the mechanisms nicotine affects the reward system is fundamental to developing better strategies. Using fMRI, (Conti et al.) showed that midbrain reward-related responses are blunted in a British cohort of habitual smokers. Interestingly, they also found more pronounced abnormalities in short-term young smokers, highlighting the importance of understanding nicotine effects at different points of the life span and its long-term compensatory mechanisms for the development of comprehensive and effective smoking cessation strategies.

Recently, the most heated debate on nicotine use concerns new delivery systems. While the media freely depicts e-cigarette or vaping use by adolescents and young adults, emerging evidence suggests that their use during critical periods is associated with long-term

consequences (Yuan et al., 2015). Happer et al. examined the associations between recent nicotine and tobacco product (NTP) use, primarily e-cigarettes, and bilateral hippocampal volume estimates in a sample of adolescents and young adults. Results showed that greater NTP use predicted larger hippocampal volumes but relatively lower memory scores than non-users. These findings suggest that early NTP exposure may alter typical brain-behavior relationships underlying learning and memory. Interestingly, when these findings were examined in the context of cannabis co-use, no interaction between NTP and cannabis was found. However, other studies have shown how one drug exposure can influence the response to another (Laviolette, 2021; Gonçalves et al., 2023). In this context, (Carreño et al.) investigated sex- and genotype-dependent effects of nicotine-induced methamphetamine self-administration in adolescent rats. They focused on a single-nucleotide polymorphism of the α6 nAChR subunit gene, which is well associated with higher cigarette smoking, adolescent drug experimentation, nicotine dependence, and unsuccessful quit attempts (Carreño et al., 2024). Their findings suggest functional changes in α6 nAChRs in brain regions associated with reward influenced by the CHRNA6 genotype, sex, and drug treatment. These findings provide new insights for future prevention and intervention strategies for nicotine addiction.

In addition to e-cigarettes, other unique delivery systems include nicotine pouches, which promise to be less harmful to lung health and aid in smoking cessation (Pluym et al., 2024). Mallock-Ohnesorg et al. evaluated the acute effects of different brands and doses of nicotine pouches in a German cohort of cigarette smokers. Although all pouches successfully reduced cigarette cravings, they were associated with much higher and faster nicotine intake and changes in cardiovascular parameters. These findings emphasize the urgent need for better regulation of new nicotine-releasing products to ensure their safety and effectiveness for tobacco cessation.

Returning to the topic of early life tobacco exposure, Proud et al. evaluated prenatal nicotine exposure using an *in vitro* approach. Their research confirms that nicotine exposure has not only acute but also long-term outcomes on neurogenesis and molecular markers of neural identity, mood disorders, and excitatory/inhibitory balance. Their study demonstrates how sophisticated *in vitro* approaches can contribute to neurodevelopmental research on nicotine exposure and how this exposure can be detrimental, even if not direct. Furthermore, non-smokers can still be affected by tobacco exposure through second-hand smoke (SHS), also known as passive or environmental tobacco smoke exposure.

SHS increases the risk of nine health outcomes, including ischemic heart disease, stroke, diabetes, and lung cancer. Although smoking rates have gradually declined over the past 50 years, ~37% of the world's population is still exposed to smoke emitted from the combustion of tobacco end-products or exhaled by smokers, with higher rates of exposure reported in women and children compared with men (Flor et al., 2024). In their review, Kisby and Raber address the Research Topic of tobacco exposure from the perspective of pathological risk, including that induced by SHS.

Alongside direct deleterious effects, nicotine use is implicated in many co-morbidities, although causal or consequential roles are rarely described (CDC-OSH: National Center for Chronic Disease Prevention and Health Promotion Office on Smoking and Health, 2014). People living with HIV are predisposed to an increased risk of developing inflammatory disorders such as HIV-associated neurocognitive disorder (HAND). Moreover, tobacco use has been observed to exacerbate further the risk of neurocognitive symptoms resulting from HIV-associated neuroinflammation (Chang et al., 2020). Indeed, there is a well-established body of literature linking HIV-1 to NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome signaling in both the periphery and the CNS. Keane and Swartz review tobacco and nicotine effects on HAND neurobiology, including effects on cognition, inflammation, viral latency, and blood-brain barrier integrity. The authors propose the NLRP3 inflammasome as a potential common pathway through which HIV-1 and nicotine may promote neuroinflammation in HIV patients.

Finally, while nicotine is predominantly recognized as the addictive component of tobacco and is linked to various smoking-related diseases, it also possesses cognitive-enhancing and anti-inflammatory properties, suggesting therapeutic potential for several conditions (Valentine and Sofuoglu, 2018; Zhang et al., 2022). The review by Cao et al. explores this dual nature of nicotine, providing a concise overview of nicotine´s physiochemical properties and pharmacology, including insights into its receptors. The discussion includes its toxic effects, which are categorized into five groups: cancer, cardiovascular, respiratory, reproductive, and others. Potential drug development applications are divided into nervous and immune interventions. All these contributions rendered a comprehensive Research Topic, and we firmly believe the readers will find this a unique and valuable reference for the state of the art in the field.

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EDITED BY
Gilda Angela Neves,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Teresa Dalla Costa,
Federal University of Rio Grande do Sul, Brazil
Valeria Lallai,
University of California, Irvine, United States

*CORRESPONDENCE
Nadja Mallock-Ohnesorg,
Mallock-Ohnesorg@med.uni-frankfurt.de

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Small pouches, but high nicotine doses—nicotine delivery and acute effects after use of tobacco-free nicotine pouches

Nadja Mallock-Ohnesorg[1,2]*, Andrea Rabenstein[3], Yvonne Stoll[3], Marcus Gertzen[4], Benedikt Rieder[3], Sebastian Malke[1], Nestor Burgmann[3], Peter Laux[1], Elke Pieper[1], Thomas Schulz[1], Klaas Franzen[5,6], Andreas Luch[1] and Tobias Rüther[3]

[1]Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany, [2]Institute of Legal Medicine, Goethe-University Frankfurt, Frankfurt/Main, Germany, [3]Department of Psychiatry and Psychotherapy, LMU University Hospital, LMU Munich, Munich, Germany, [4]Department of Psychiatry, Psychotherapy and Psychosomatics, Faculty of Medicine, University of Augsburg, Augsburg, Germany, [5]Medical Clinic III, Campus Lübeck, University Hospital Schleswig-Holstein, Lübeck, Germany, [6]Airway Research Center North, Member of the German Center for Lung Research (DZL), Großhansdorf, Germany

Tobacco-free nicotine pouches are new nicotine products for oral consumption. They can contain very high nicotine amounts that have not been addressed with clinical studies yet. Thus, nicotine delivery, effects on craving, and side effects were assessed using pouches with up to 30 mg nicotine. In this single-center, five-arm, crossover study, 15 regular cigarette smokers consumed tobacco-free nicotine pouches from different brands with 6, 20, and 30 mg for 20 min. Comparators were nicotine-free pouches and tobacco cigarettes. At baseline and predefined time points over a study period of 240 min, plasma nicotine concentrations, effects on cigarette craving, and side effects were assessed. Cardiovascular parameters including arterial stiffness were measured using a MobilOGraph. Consumption of 30 mg nicotine pouches has led to a higher nicotine uptake compared with the cigarette (C_{max}: 29.4 vs 15.2 ng/mL; AUC: 45.7 vs 22.1 ng/mL × h). Nicotine uptake in the acute phase was rapid during use of the 30 mg pouch and cigarette. Extraction rate of nicotine differed between pouches. Use of all products has reduced acute cigarette craving, even the nicotine-free pouch. During consumption of the cigarette and the pouches with 20 and 30 mg, heart rate increased about 27, 12, and 25 bpm, respectively. Parameters for arterial stiffness were elevated and all pouches have induced mouth irritations. The pouches with 30 mg nicotine had overall the strongest side effects and may induce addiction. As craving was also reduced by products with less nicotine, it is questionable whether such high nicotine contents should be allowed on the market. A limit of nicotine content is warranted. The nicotine release rate varies across products and needs to be known to estimate the nicotine delivery.

KEYWORDS

nicotine pouches, nicotine delivery, pharmacokinetics, craving reduction, cardiovascular effects, arterial stiffness, high nicotine doses

1 Introduction

Smoking increases the risk for several serious diseases such as lung cancer, cardiovascular disease, and chronic obstructive pulmonary disease (National Center for Chronic Disease Prevention and Health Promotion US Office on Smoking and Health, 2014). The combustion of tobacco and the inhalation of the smoke is the primary cause for these smoking-related diseases (National Center for Chronic Disease Prevention and Health Promotion US Office on Smoking and Health, 2014). Cigarette smoke contains not only nicotine but more than 6,500 compounds, some of them hazardous or potentially hazardous (Rodgman and Perfetti, 2013). During the last 5–10 years, several companies introduced tobacco-free nicotine pouches into the United States and many European countries (Robichaud et al., 2020; Mallock et al., 2024). These pouches contain cellulose, nicotine salts, flavors, and acid regulators but no tobacco-leaf material at all (Robichaud et al., 2020; Mallock-Ohnesorg et al., 2023). The pouches are used for durations from 20 to 60 min between lips and gum (Prasad et al., 2022). Nicotine is released and absorbed by the buccal mucosa. A study by one manufacturer analyzed four brands for several compounds such as formaldehyde, acrolein, 1,3-butadiene, benzene, nornitrosonicotine (NNN), and 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Azzopardi et al., 2022a). The concentrations for all these compounds were under the limit of detection (Azzopardi et al., 2022a). In a recent study, the German Federal Institute for Risk Assessment investigated 44 brands of nicotine-containing pouches, covering a range from 1.79 to 47.5 mg nicotine per pouch. Also tobacco-specific nitrosamines were analyzed in these samples, 24 of 44 showed NNN and three of 44 showed NNK above the detection limits of 0.12 ng per pouch (Mallock et al., 2024).

The pharmacokinetic properties are crucial for the development of nicotine addiction (Henningfield and Keenan, 1993; Guimarães et al., 2021). While the highly addictive cigarettes lead to a rapid uptake of nicotine into the blood and consequently into the brain, smoking cessation products such as nicotine patches or gums show a much slower uptake of nicotine (Henningfield and Keenan, 1993; Guimarães et al., 2021). An important question regarding nicotine pouches is whether their pharmacokinetic properties resemble nicotine gums or cigarettes. Up to now, several studies investigated the pharmacokinetic properties of tobacco-free pouches (Lunell et al., 2020; Rensch et al., 2021; Azzopardi et al., 2022b; Chapman et al., 2022; Liu et al., 2022; McEwan et al., 2022). Most studies have been performed by or in association with manufacturers of nicotine pouches, often tobacco companies. Many studies used pouches with nicotine concentrations between 4 and 10 mg per pouch. The application time ranged from 20 to 60 min. Four of six studies compared the pharmacokinetics of pouches with the consumption of a cigarette (Rensch et al., 2021; Chapman et al., 2022; Liu et al., 2022; McEwan et al., 2022) and only four studies included more than one nicotine dose allowing an estimation of dose-dependency (Lunell et al., 2020; Chapman et al., 2022; Liu et al., 2022; McEwan et al., 2022). None of these studies has investigated the pharmacokinetic properties of nicotine pouches with very high nicotine doses.

This five-arm, crossover study was designed to address the data gap on pharmacokinetics of nicotine pouches with high nicotine contents. The primary aim of the study was the comparison of blood plasma nicotine levels following the application of one of four commercially available products, three nicotine containing pouch brands (6, 20, and 30 mg nicotine per pouch), one brand without any nicotine and a cigarette comparator to assess any differences in the nicotine pharmacokinetic profiles over 4 h. The secondary aim of this study was to assess smoking urges and side effects with a focus on cardiovascular effects and local effects at the application site. The findings will help to understand effects of high dose nicotine pouches on addiction and craving reduction in contrast to lower dose products.

2 Materials and methods

2.1 Aim, study products and ethics

Aim of the study was to assess nicotine uptake, subjective effects, and side effects after use of nicotine pouches with medium to high nicotine contents. Nicotine concentration of venous plasma was determined to assess nicotine uptake in the acute phase (i.e., the first 5 min of consumption) and over the course of 4 h. This single-center, crossover study was conducted with the following five arms:

a. Nicotine-free pouches with mint aroma (Swedish Match, Stockholm Schweden)
b. 6 mg Nicotine pouches with mint aroma (Imperial Brands plc., Bristol, United Kingdom)
c. 20 mg Nicotine pouches with mint aroma (British American Tobacco, London, United Kingdom)
d. 30 mg Nicotine pouches with mint aroma (Fedrs Sp. Z.o.o, Warsaw, Poland)
e. Own-brand tobacco cigarette (different brands).

Pouches were kept between upper lip and gum for 20 min. Products were purchased in October 2021 from online shops. The nicotine strengths mentioned in the manuscript refer to the declared nicotine contents not the analyzed contents. The study was approved by the ethics committee of the LMU Munich (project number 21-0814) and performed in accordance with the principles of the Declaration of Helsinki in the currently valid version. It was registered at the DRKS (DRKS00026244). Informed consent was obtained from all participants before participation in the study. Four hypotheses were tested:

- Nicotine delivery of the studied pouches, measured as C_{max} (maximum plasma concentration) and AUC (area under the plasma-time curve), increases in a dose-dependent manner.
- Use of the pouch with 30 mg nicotine leads to a similarly high plasma nicotine concentration as tobacco cigarettes (C_{max} approximately 15–20 ng/mL).
- In contrast to pouches with 6 mg nicotine, use of pouches with high (20 mg and 30 mg) nicotine doses reduces acute craving for a cigarette comparably to smoking of one cigarette.
- Side effects of pouch use, including cardiovascular effects (i.e., heart rate, arterial stiffness), increase in a dose-dependent manner.

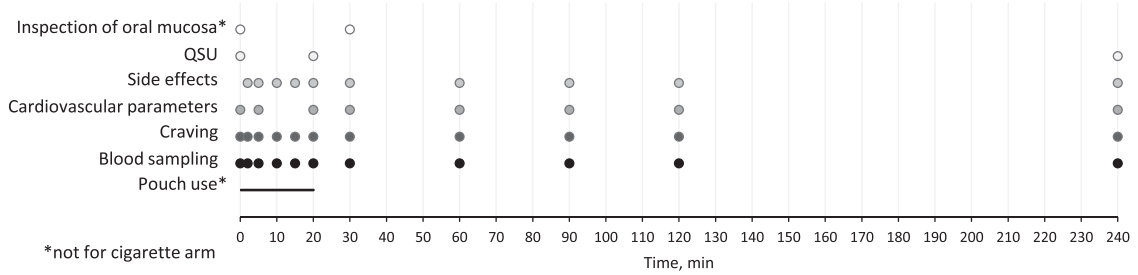

FIGURE 1
Study design with measurements and their time points.

2.2 Participants

The single-center, five-arm, crossover study included 15 active smokers. Recruitment of participants took place via advertisement (social media, LMU intranet, LMU newsletter). Enrolled participants that fulfilled inclusion and exclusion criteria gave written informed consent. Inclusion criteria were: Age between 18 and 55 years, active smoking for at least 5 years with more than 10 cigarettes per day, 12 h of abstinence from any nicotine product prior to testing, CO levels < 5 ppm (in the expiratory air analyzed using a micro-smokerlyzer; Bedfont Scientific Ltd., Anif, Austria) and plasma nicotine concentration at baseline < 10 ng/mL to verify abstinence from cigarettes and other nicotine products, and the ability to give consent. Exclusion criteria were: Age under 18 or over 55 years, use of other nicotine products (e.g., nicotine pouches, snus, e-cigarettes) more often than once a week, acute psychiatric illness according to ICD-10/DSM IV or other serious psychiatric disorders, acute suicidality, pregnancy, breastfeeding, current abuse of drugs, medication, or alcohol, malignant cancer in the past 5 years, serious internal illness, especially cardiovascular diseases, such as manifest arterial hypertension, severe heart disease (DCM, history of heart attack), pacemaker implantation, respiratory diseases (e.g., respiratory failure, asthma, COPD), and severe active infectious disease.

2.3 Study design

The study was conducted from September 2021 to May 2022 at LMU University Hospital in Munich, Germany with six visits per participant. At the first visit, participants were screened for inclusion and exclusion criteria, sociodemographic data and smoking behavior in the past 30 days were inquired, and physical dependence for cigarettes was assessed using the Fagerström Test for Cigarette Dependence (FTCD) (Heatherton et al., 1991). The following visits were study days. While at the first study day, all participants consumed their own-brand cigarette, the order of pouches (with 0, 6, 20, or 30 mg nicotine) used at study days 2–5 was randomized. Prior to study days, participants were asked to stay abstinent from any nicotine product for at least 12 h. The time line at study days with the measurements taken is presented in Figure 1.

Participants were instructed either to smoke one of their own-brand cigarettes as they usually do or to place the pouch between upper lip and gum and keep it for 20 min without chewing or sucking it. 30 min before, during, and 30 min after pouch use, participants were asked not to use chewing gum, eat any food, drink more than small amounts of water, and brush their teeth. Over the whole observation period of 240 min, participants were asked not to use any other further nicotine product or consume food or beverages containing caffeine, mint, or licorice. The abstinence was considered to avoid possible interferences with nicotine metabolism, subjective effects (e.g., head buzz), or cardiovascular effects (Benowitz et al., 2009; Deutch et al., 2019).

2.4 Measurements

Venous blood was sampled using peripheral venous Safety Multifly cannulas and S-Monovettes (Sarstedt AG & Co. KG, Nümbrecht, Germany) at baseline and at 2, 5, 10, 15, 20, 30, 60, 90, 120, and 240 min and was cooled until preparation for quantitative nicotine analysis as described below. Smoking urges were assessed at baseline, at 20 and at 240 min using the German version of the Questionnaire on Smoking Urges (QSU-G) (Müller et al., 2001). Acute craving after a cigarette was asked to be rated ("I now feel the urge for a cigarette") at baseline and at 2, 5, 10, 15, 20, 30, 60, 90, 120, and 240 min on a seven-point Likert scale from 1 (not at all true) to 7 (completely true).

Cardiovascular parameters were measured using a Mobil-O-Graph (I.E.M. GmbH, Stollberg, Germany) at baseline and at 2, 5, 20, 30, 60, 90, 120, and 240 min. The parameters heart rate, peripheral and central blood pressure, augmentation index adjusted at HR 75 bpm (AIX@75), and total peripheral resistance/vascular resistance (TVR) were measured and calculated with the Mobil-O-Graph software version HMS CS 4.2 (I.E.M. GmbH, Stollberg, Germany). The instrument and measurement procedure are described elsewhere (Wassertheurer et al., 2010; Weber et al., 2011; Hauck et al., 2023).

The side effects head buzz, mouth or throat irritations, lightheadedness, dizziness, cold hands or feet, palpitations, headache, perspiration, nausea, and urge to vomit were assessed on a numeric rating scale (NRS) from 0 (no effect) to 10 (strongest effect) at 2, 5, 10, 15, 20, 30, 60, 90, 120, and 240 min. Salivation was inquired on a scale from 0 = lowest salivation (dry mouth) over 5 = normal salivation to 10 = highest salivation (hypersalivation). At study days at which a pouch was used, the oral mucosa was inspected for redness or ulceration at baseline and at 30 min.

2.5 Analysis of nicotine, cotinine, and trans-3′-hydroxycotinine from blood samples

Whole blood was centrifuged (1,500 g, 10 min, 4°C) and 10 µL internal standard mix (500 ng/mL nicotine-d_3, cotinine-d_3, hydroxycotinine-d_3 in acetonitrile) was added to 990 µL blood plasma. Samples were stored at LMU University Hospital in Munich at −80°C and shipped to BfR in Berlin on dry ice. A previously described validated method was used for the quantification of nicotine, cotinine, and trans-3′-hydroxycotinine (hydroxycotinine) from plasma using protein precipitation and liquid chromatography—tandem mass spectrometry (LC-MS/MS) with a matrix-matched calibration (Mallock et al., 2021).

2.6 Determination of nicotine extraction from pouches

After removal, pouches were individually wrapped and stored at −20°C before they were shipped on dry ice to BfR in Berlin, Germany. Method for nicotine content determination was modified from a previously described method (Mallock et al., 2024). Pouches were weighted and placed into an Erlenmeyer flask with stopper followed by a liquid-liquid extraction using 10 mL ultra-pure water, 5 mL sodium hydroxide solution (2 M), and 20 mL n-hexane with the internal standard n-hexadecane (0.5 g/L) for 75 min at 350 rpm (orbital shaker GFL 3005, Lauda-GFL, Lauda-Königshofen, Germany). Of the organic phase, 2 µL were injected into the GC/FID system (G1530A series from Agilent Technologies/Hewlett Packard, Agilent Technologies, Waldbronn, Germany) and analyzed as described in the Supplementary Material.

2.7 Pharmacokinetic (PK) parameters and statistics

For calculation of the half-life ($t_{1/2}$), the elimination rate constant (i.e., the slope of the terminal elimination phase) was determined using the last two nicotine plasma concentrations (at 120 and 240 min). The plasma nicotine concentrations determined at baseline and the individual elimination rate constant were used to determine the residual nicotine concentration at the subsequent nicotine sampling times. These values were then subtracted from the subsequent nicotine levels before PK parameters were calculated. Areas under the plasma concentration-time curve (AUC) were calculated with the linear trapezoid rule. The highest plasma nicotine concentration per curve was used as C_{max} and the according time point for t_{max}. Delays in blood sampling were noted and considered when the individual AUCs were calculated. Relative bioavailability (F_{rel}) between two pouches was calculated using the following equation with the analyzed nicotine content as dose (D):

$$F_{rel} = 100 \cdot \frac{AUC_{\text{Pouch 1}} \cdot D_{\text{Pouch 2}}}{AUC_{\text{Pouch 2}} \cdot D_{\text{Pouch 1}}}$$

Nicotine metabolite ratio (NMR) was calculated by dividing hydroxycotinine by cotinine plasma concentrations at baseline at the first study day. NMR can be used as a surrogate for CYP 2A6 activity (Dempsey et al., 2004; Allenby et al., 2016) with low values (NMR < 0.31) for slow metabolizers and higher values (NMR > 0.31) for normal or rapid metabolizers (Lerman et al., 2015). Median and interquartile ratios (IQR) were calculated for participant characteristics including NMR; for t_{max}, median and range. For AUC and C_{max}, geometric means and coefficients of variation (CV) were calculated. Arithmetic means and 95% confidence intervals (CI) were used for mean plasma curves. Statistical Package for Social Sciences (SPSS) version 26.0 was used for statistical analysis. Two-sided paired t-tests were applied to evaluate differences between groups. For C_{max} and AUC, lognormal values were used. Baseline mean values were used as statistical references for the cardiovascular parameters blood pressure, heart rate, and arterial stiffness parameters. Cardiovascular parameters were tested for normal distribution by Kolmogorov-Smirnov tests and a two-way repeated measures ANOVA based on baseline measurements was used to estimate for an interaction between the product used and time. To individually analyze differences at the various time points in between the study arms, ANOVA is used.

3 Results

3.1 Participants

Of the 18 recruited participants, three dropped out without completion of all study arms. The characteristics of the 15 participants that completed the study are summarized in Table 1. Individual characteristics are presented in Supplementary Table 1. Participants had a low to moderate physical cigarette dependence as measured with the FTCD. Eleven participants had an NMR > 0.31 and were classified as normal/rapid metabolizers; four were classified as slow metabolizers (participants 5, 7, 11, and 15). The participants were daily smokers who smoked about 12 cigarettes per day.

3.2 Nicotine delivery and nicotine extraction from pouches

Mean plasma nicotine curves during and after consumption of the study products are presented in Figure 2A. Individual plasma nicotine curves are displayed in Supplementary Figure 1 and the individual nicotine concentrations are shown in Supplementary Tables 2–6. Consumption of nicotine-free pouches did not result in a nicotine uptake. A magnification of the acute phase, meaning the first minutes of consumption, is shown in Figure 2B. Rise of plasma nicotine levels was fastest for 30 mg nicotine pouches and tobacco cigarettes compared with the other tested products.

The PK parameters C_{max}, t_{max}, $AUC_{0-240min}$, and $t_{1/2}$ are summarized in Table 2 for the nicotine-containing products. The parameters C_{max} and $AUC_{0-240min}$ increased in the order 6 mg nicotine pouch < 20 mg nicotine pouch < tobacco cigarette < 30 mg nicotine pouch with statistically significant differences between all nicotine pouches (C_{max}: all p-values < 0.0001; $AUC_{0-240min}$: all p-values < 0.0001). In comparison with the tobacco cigarette, p-values for C_{max} were 0.0000001, 0.001,

TABLE 1 Participant characteristics including smoking behavior in the past 30 days.

Age, median (IQR)	30 (24–40)
Sex, female, *n* (%)	8 (53%)
Sex, male, *n* (%)	7 (47%)
Height in cm, mean (SD)	171.8 ± 6.0
Weight in kg, mean (SD)	78.1 ± 17.5
Body mass index (BMI), mean (SD)	26.4 ± 5.5
Fagerström Test for Cigarette Dependence (FTCD), median (IQR)	4 (3–5)
Nicotine Metabolite Ratio (NMR), median (IQR)	0.44 (0.31–0.63)
Number of days cigarettes were smoked within the last 30 days, median (IQR)	30 (30–30)
Number of cigarettes smoked on a day with smoking, median (IQR)	12 (12–15)

IQR, interquartile ratio; SD, standard deviation.

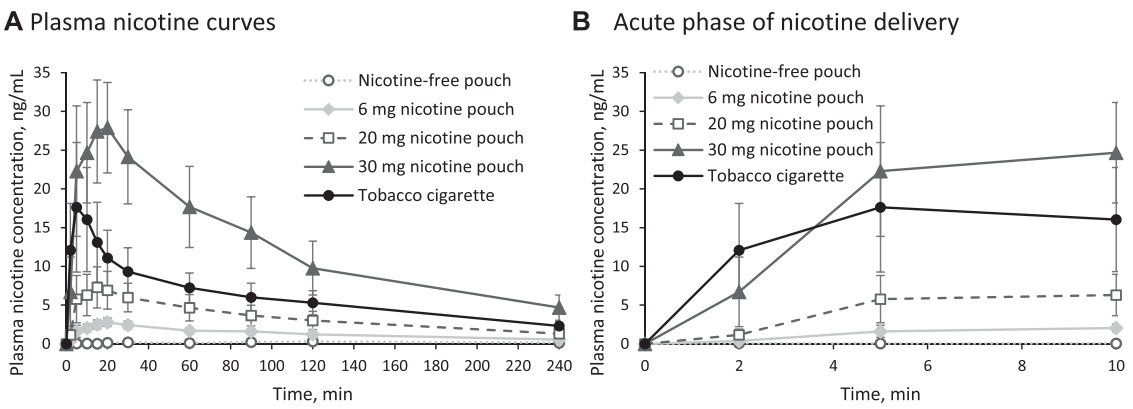

FIGURE 2
(A) Plasma nicotine curves for the five study arms and **(B)** magnification of the acute phase (arithmetic means and 95% confidence interval).

TABLE 2 Summary of relevant pharmacokinetic parameters for the nicotine containing study products and mean extraction rate of nicotine from pouches.

Product	C_{max} (ng/mL)	t_{max} (min)	$AUC_{0-240min}$ (ng/mL × h)	$t_{1/2}$ (h)	Nicotine extraction (%)
6 mg nicotine pouch	2.8 (39%)	20 (5–36)	4.9 (65%)	2.4 (1.2)	38%
20 mg nicotine pouch	7.1 (72%)	15 (5–60)	11.6 (77%)	2.2 (1.5)	24%
30 mg nicotine pouch	29.4 (58%)	15 (5–30)	45.7 (60%)	1.8 (0.6)	52%
Tobacco cigarette	15.2 (111%)	5 (2–15)	22.1 (59%)	2.3 (1.4)	—

C_{max} and AUC: Geometric mean and coefficient of variation (CV%); t_{max}: Median and range; $t_{1/2}$: arithmetic mean and standard deviation.

and 0.0007 for pouches with 6, 20, and 30 mg nicotine, respectively. For $AUC_{0-240min}$, these values were 0.0000001, 0.001, and 0.0002. With tobacco cigarettes, t_{max} was reached the fastest in line with the shorter consumption duration. The half time $t_{½}$ of nicotine was calculated showing no differences between study arms.

Nicotine content of unused nicotine pouches were analyzed with GC/FID. Analyzed nicotine contents were 4.8 ± 0.4 mg, 16.3 ± 3.1 mg, and 27.1 ± 0.2 mg for the nicotine pouches declared with 6, 20, and 30 mg, respectively. Remaining nicotine in used pouches was analyzed (see Supplementary Table 7) and rates of nicotine extraction were calculated. Mean nicotine extraction rates differed between pouches as shown in Table 2. Considering the analyzed total content and the remaining nicotine contents, mean nicotine doses extracted were 1.8 ± 0.8 mg, 4.7 ± 3.5 mg, and 14.1 ± 3.0 mg for the pouches with a nominal nicotine content of 6, 20, and 30 mg, respectively. Relative bioavailability in relation to the 6 mg (analyzed 4.8 mg)

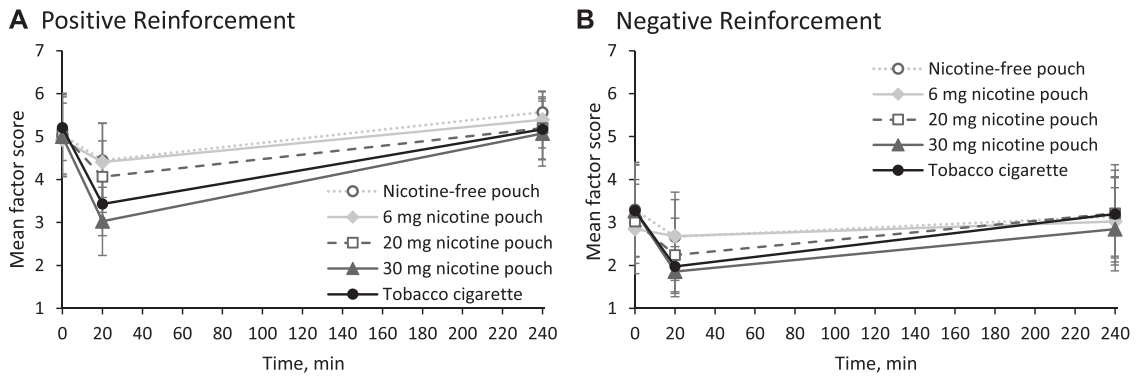

FIGURE 3
Mean scores and 95% confidence intervals for **(A)** factor 1 (positive reinforcement) and **(B)** factor 2 (negative reinforcement) of urges to smoke.

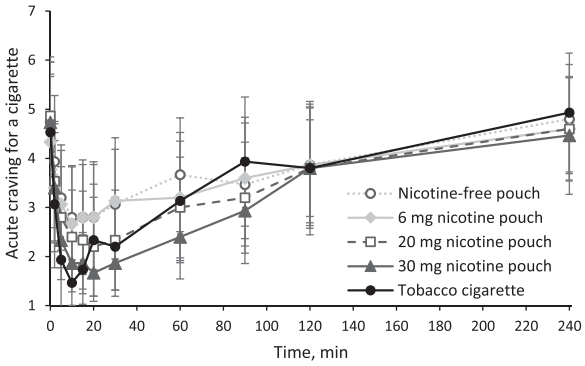

FIGURE 4
Acute craving for a cigarette inquired with a single question ("I now feel the urge for a cigarette") answered on a scale from 1 (lowest) to 7 (highest) as mean scores and 95% confidence intervals.

pouch was 70% for the 20 mg (analyzed 16.3 mg) pouch and 165% for the 30 mg (analyzed 27.1 mg) pouch.

3.3 Effects on craving

Effects on smoking urges were measured using the QSU-G at baseline, at 20 min, and at 240 min as presented in Figure 3. Positive reinforcement is described by factor 1 (Figure 3A) and reflects, for example, the anticipation of positives effects from smoking. Mean score for factor 1 was significantly reduced by tobacco cigarette and by the pouches with 20 and 30 mg nicotine at 20 min ($p < 0.01$). Negative reinforcement, described by factor 2 (Figure 3B), reflects, for example, the anticipation of relief from withdrawal. Mean score for factor 2 was significantly reduced by tobacco cigarette and by the pouches with 20 and 30 mg nicotine at 20 min ($p < 0.05$).

Additionally, acute cigarette craving was inquired with a single question ("I now feel the urge for a cigarette") at each time point of blood sampling as shown in Figure 4. All products, even the nicotine-free pouch, led to a statistically significant reduction of acute craving for a cigarette (all p values < 0.05, see Supplementary Material). In agreement with the earlier time point for t_{max}, reduction was fastest for the tobacco cigarette. Difference in craving reduction was statistically significant between nicotine-free pouch and 30 mg nicotine pouch ($p = 0.04$). Individual ratings for acute craving are presented in Supplementary Tables 8–12.

3.4 Cardiovascular effects and arterial stiffness

Changes in heart rate are displayed in Figure 5. Increases in heart rate occurred in the beginning of product use (5 min) for the 20 and 30 mg nicotine pouches, tobacco cigarettes, and slightly for the 6 mg nicotine pouches. Increases were the strongest for the 30 mg pouches and tobacco cigarettes. Effects on systolic and diastolic blood pressure were minor for all study arms as summarized in the Supplementary Tables 13–15.

Besides peripheral and central blood pressure, the parameters of arterial stiffness, augmentation index adjusted at HR 75 bpm (AIX@

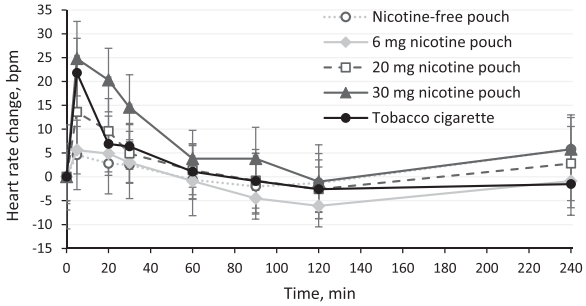

FIGURE 5
Changes in heart rate (mean and 95% confidence interval).

75) and total peripheral resistance/vascular resistance (TVR), were measured at baseline and at 5, 20, 30, 60, 90, 120, and 240 min. Besides peripheral systolic blood pressure, there was a significant increase also in central systolic blood pressure in the study arms using pouches with high nicotine concentrations and tobacco cigarette in the acute phase, the beginning of product consumption. Further, there was a significant increase in the parameters of arterial vascular stiffness. AIX@75 and TVR were significantly elevated during consumption of the high nicotine pouches (20 and 30 mg) and tobacco cigarette. Results are shown in the Supplementary Tables 16, 17.

3.5 Other side effects

No serious adverse effects were reported. One participant who used the 30 mg nicotine pouch experienced circulation problems with mild symptoms. By employing general measures such as elevating the legs, the participant's condition normalized within a few minutes and no serious adverse event was reported.. Strong mouth irritations were reported in the first 10 min of use of the 30 mg nicotine pouch (Figure 6A). Use of the other pouches resulted in medium mouth irritations, regardless of the nicotine strength, while cigarette smoking did not have such an effect. Reported head buzz (i.e., the feeling of a slight intoxication) peaked in the cigarette arm at 2 min and in the pouch arms at 5 min. Cigarette smoking and use of the 30 mg pouch led to a head buzz with medium effect size. None of the other inquired side effects (throat irritations, lightheadedness, dizziness, cold hands or feet, palpitations, headache, perspiration, nausea, and urge to vomit) was rated higher than 3 out of 10 at any time point as summarized in Supplementary Table 18. For salivation, effects in both directions (less salivation or more salivation than usual) were monitored. No increased salivation was observed and a slightly drier mouth was reported, especially in the tobacco cigarette arm (see Supplementary Table 19).

The oral mucosa was inspected before and 10 minutes after pouch use for redness or ulceration. A slightly increased redness was revealed in 1, 4, 3, and 5 cases after use of the pouches with 0, 6, 20, and 30 mg nicotine, respectively. An ulceration was visible in one case after use of the 6 mg pouch. In the remaining cases, appearance of the oral mucosa did not change compared with the inspection at baseline.

4 Discussion

In the landscape of alternative nicotine delivery systems (ANDS), nicotine pouches are among the most recent products. Where available, they are mostly not adequately addressed with specific regulations regarding, for example, the nicotine content (Duren et al., 2023). Scientific knowledge on these products is scarce and was predominantly generated by the manufacturers of the products. In previously published clinical trials, only pouches up to 10 mg nicotine were studied (Lunell et al., 2020; Rensch et al., 2021; Azzopardi et al., 2022b; Chapman et al., 2022; Liu et al., 2022; McEwan et al., 2022). However, much higher nicotine contents are available, up to 50 mg were previously reported (Mallock et al., 2024). The herein presented clinical trial is the first to report nicotine pharmacokinetics, subjective effects, and side effects from use of nicotine pouches with 20 and 30 mg. Additionally, this work is the first to include nicotine-free oral pouches as a control. Nicotine content of the pouches were analyzed with GC/FID and the pouches only contained between 80% and 90% of the declared nicotine content. For brevity, the declared nicotine content is used throughout this manuscript.

An overview over results from previous pharmacokinetic studies is given in Table 3 including highest studied nicotine strength, duration of use, highest achieved mean C_{max}, and highest achieved mean AUC. Mean C_{max} after 20 min of use of the 30 mg nicotine pouch was much higher compared with the highest mean C_{max} values achieved in one of the other studies after the use of pouches with up to 10 mg nicotine regardless of the use duration. In three studies, the participants kept the products for 60 min in their oral cavities (Lunell et al., 2020; Azzopardi et al., 2022b; McEwan et al., 2022), which is three times the use duration compared with the presented study. A use duration of 20 min was chosen for two reasons: Firstly, a market survey conducted by a product manufacturer has revealed that the preferred duration for holding nicotine pouches in the oral cavity ranges from 5 to 20 min in Germany (Prasad et al., 2022). The higher the nicotine strength of the pouch, the shorter was the preferred hold time (Prasad et al.,

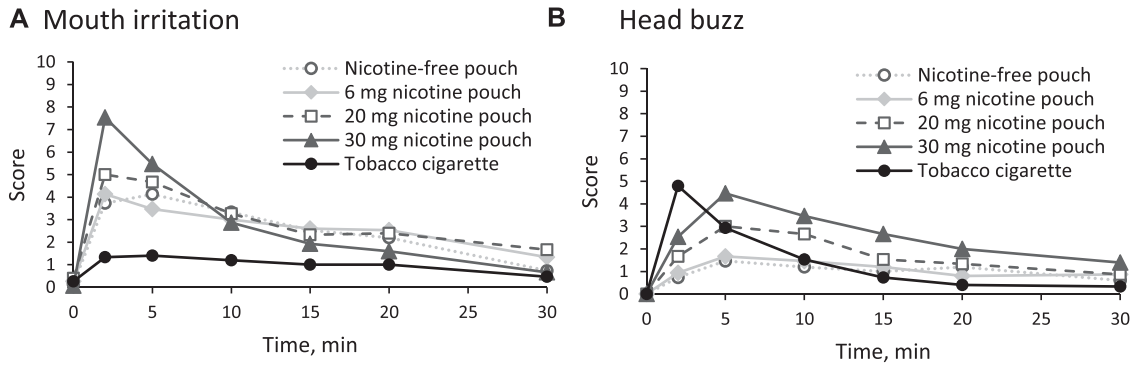

FIGURE 6
Reported side effects **(A)** mouth irritation and **(B)** head buzz in the first 30 min on a numeric rating scale (NRS) from 0 (no effect) to 10 (strongest effect).

TABLE 3 Overview over published clinical data on nicotine pouch pharmacokinetics.

Study (name, year)	Highest studied nicotine strength (mg)	Duration of use (min)	Highest mean C_{max}	Highest mean AUC_{0-t}	Last blood sampling time point (t) (h)
Presented study	30	20	29.4 ng/mL	45.7 ng/mL × h	4
Lunell et al. (2020)	8	60	18.5 ng/mL	58.4 ng/mL × h	6
McEwan et al. (2022)	10	60	18.4 ng/mL	53.7 ng/mL × h	6
Rensch et al. (2021)	4	30	12.1 ng/mL	19.5 ng/mL × h	3
Chapman et al. (2022)	10	20	7.9 ng/mL	18.4 ng/mL × h	8
Azzopardi et al. (2022b)	4	60	8.3 ng/mL	29.7 ng/mL × h	12
Liu et al. (2022)	8	30	14.5 ng/mL	24.0 ng/mL × h	3

2022). Secondly, as no clinical data for the use of pouches with high nicotine concentrations were available, it was unclear how participants would react to such a high nicotine dose. Accordingly, the duration time was kept relatively short and nicotine doses above 30 mg were not included. However, as seen in Table 2, participants only extracted 52% (14 ± 3.0 mg) of the nicotine contained in the 30 mg pouches and even less from the other pouches. It is possible that with a longer use duration, more nicotine would have been extracted potentially leading to increased side effects. In two other studies, nicotine extraction rates have been analyzed, both with use durations of 60 min. Lunell et al. have reported mean extraction rates of 56%, 59%, and 50% for pouches with 3, 6, and 8 mg nicotine, respectively (Lunell et al., 2020). In the study by Azzopardi et al., a mean of 62% was extracted from the 4 mg pouch (Azzopardi et al., 2022b). The total delivered nicotine, represented by the AUC, depends on the administration time of nicotine. Accordingly, the AUC was higher in two studies with a lower nicotine dose but a longer duration of use (Table 3). It should be noted that the last blood sampling time point (t) has a large influence on the AUC_{0-t} and that it varied across the studies mentioned in Table 3. Thus, the different AUC_{0-t} values should be compared with caution.

In the presented study, mean C_{max} and AUC were significantly higher after use of the 30 mg nicotine pouches compared with tobacco cigarettes. In addition to nicotine delivery, assessing the nicotine flush during the acute phase of consumption is crucial for evaluating the product's risk. As seen in Figure 2B, use of the 30 mg nicotine pouch has led to a similarly fast nicotine uptake compared with cigarette smoking. The rate at which nicotine concentration increases in the bloodstream and, consequently, in the brain is associated with the activation of the reward system and the product's addictive potential (Henningfield and Keenan, 1993). The highly addictive nature of the tobacco cigarette can serve as a reference point. A similarly rapid nicotine uptake during the acute phase may suggest a comparable level of addictiveness for the alternative product. However, addictiveness of a product cannot be ruled out only by demonstrating a slower nicotine uptake. Tobacco dependence is complex and involves many behavioral factors as for instance (sensory) conditioning or social learning (Brandon et al., 2004; Eissenberg, 2004; Glautier, 2004).

It should also be noted that inhaling cigarette smoke results in a more rapid increase in nicotine levels in arterial blood compared to venous blood (Henningfield et al., 1993). Nicotine is transported to the brain by arterial blood. Therefore, venous blood concentrations are a poorer surrogate for post smoking nicotine concentrations in the brain than arterial samples. Due to the buccal resorption, the distribution between arterial and venous blood in the early phase of nicotine pouch use is likely to be different to cigarette use. Thus, it remains to be studied how venous plasma concentrations translate to nicotine levels in the brain in the context of nicotine pouch consumption. As an approximation for nicotine effects in the brain, the participants were asked to rate the sensation of a head buzz at different time points. During cigarette smoking, the peak sensation was achieved immediately at the first assessment point after 2 min. During use of the 30 mg nicotine pouch, head buzz peaked after 5 min much before t_{max}. The peak effect sizes of head buzz during cigarette or 30 mg nicotine pouch consumption were comparable.

Another aspect related to the nicotine delivery is the reduction of craving for a cigarette. All participants were regular smokers with mild to moderate addiction according to their FTCD score. Nicotine products that efficiently reduce craving in concert with a markedly lower exposure to harmful chemicals can be beneficial for an addicted smoker who is unable to overcome nicotine use (Kozlowski and Abrams, 2016; Hatsukami and Carroll, 2020). While the toxicity of cigarette smoke, which contains over a hundred highly toxic chemicals, has been extensively studied, much less is known about nicotine pouches. However, when considering recent independent studies (Mallock-Ohnesorg et al., 2023; Mallock et al., 2024), it can be expected that nicotine pouches lead to a substantially lower exposure to toxicants compared to cigarette smoke. All tested products, including the nicotine-free pouch, have significantly reduced acute cravings for a cigarette. During cigarette smoking and consumption of the 20 and 30 mg nicotine pouches, the lowest mean score for acute craving was with 20 min shortly after t_{max} at 15 min. Consumption of the pouches with 0 and 6 mg reduced craving for a cigarette in the first 10 minutes. This underlines that factors such as sensory cues or expectation of reward play a role in reducing craving by these oral products. The acute craving increased most rapidly in the cigarette arm following the initial satisfaction. Craving reduction was not statistically different between the tobacco cigarette and both pouches with high nicotine contents, 20 and 30 mg. Additional to the question on acute craving for a cigarette, the QSU was answered at three time points, at baseline, at 20 min, and at 240 min. With the QSU, effects on positive reinforcement factors (e.g., expectation of a positive effect from smoking) and negative reinforcement factors (e.g., expectation of relief from withdrawal symptoms) for cigarette smoking were measured with multiple items. Both factors of smoking urges were reduced by the tobacco cigarette and the nicotine pouches with high nicotine contents, 20 and 30 mg. As participants did not answer the QSU at 10 min, early effects in craving reduction by the 0 and 6 mg pouches with the single-item measurement were not assessed.

In terms of side effects, cardiovascular effects were expected to be triggered by nicotine. Nicotine stimulates the acetylcholine-receptors causing reaction in the central nervous and vegetative nerval system with a consecutive increasing heart rate and blood pressure (Benowitz and Burbank, 2016). Indeed, in the early phase of consumption, 30 mg nicotine pouches and tobacco cigarettes led with an increase of approximately 25 bpm to similarly strong rises of heart rate. The 20 mg nicotine pouches led to a lower rise, while the 6 mg pouch increased heart rate only slightly and the nicotine-free pouches did not affect heart rate significantly. Only two of the previously published clinical studies have monitored cardiovascular effects of nicotine pouch use (Lunell et al., 2020; Chapman et al., 2022). Chapman et al. have not reported any changes in heart rate or blood pressure after use of a 10 mg nicotine pouch with a C_{max} of 7.9 ng/mL (Chapman et al., 2022). Lunell et al. have described an increase of 10.5 bpm after 60 min of use of the 6 mg nicotine pouch with a C_{max} of 14.7 ng/mL (Lunell et al., 2020). This underlines the dose-dependency of the acute effects of nicotine on heart rate from nicotine pouches.

In addition to heart rate and blood pressure, parameters to measure arterial stiffness (AIX@75, TVR) were assessed. Significant effects were found especially for the high dose pouches as well as the combustible cigarette at the first time point of measurement, at 5 min. Since our study has only a relatively short follow-up period of 240 min, long-term statements can only be formulated speculatively. Most of the variations in central and peripheral blood pressure, heart rate, and arterial stiffness parameters can be attributed to nicotine. Cigarette smoking is associated with a substantially increased risk of cardiovascular disease and mortality (Benowitz and Liakoni, 2022). The main contributors are not nicotine but combustion products that induce chronic inflammation and cardiovascular dysfunction (Benowitz and Liakoni, 2022). Users of snus, a type of oral smokeless tobacco, do not show an increased risk of cardiovascular disease compared to never smokers, but the risk for fatal outcomes is elevated (Benowitz and Liakoni, 2022). Looking at Sweden where the male population predominantly uses snus rather than cigarettes, the cardiovascular health in men has improved over the last decades compared with other developed countries or with Swedish women (Foulds et al., 2003). As nicotine pouches are a very similar product as snus, it is likely that they also pose a much lower risk for cardiovascular events than cigarette smoking does. However, considering the acute effects on parameters reflecting arterial stiffness, an increased risk for arterial hypertension, atherosclerosis, or myocardial infarction especially for consumers with an already existing cardiovascular disease is possible.

Besides cardiovascular effects, local effects were of special interest. The oral mucosa was inspected 10 minutes after the pouches were removed. In some cases, an increased redness was visible and in one case, after use of the 6 mg pouch, an ulceration was detected. Moderate mouth irritation was reported by the participants at the beginning of consumption of the pouches with 0, 6, and 20 mg nicotine. The 30 mg nicotine pouches induced a strong mouth irritation, while the mouth irritation during smoking was low. This suggests a tendency that nicotine may contribute to local effects. However, as the pouches with no nicotine or low amounts also induced local adverse effects, other substances are involved. This is in line with an *in vitro* toxicity study of nicotine pouch extracts in oral fibroblasts in which cytotoxic effects were found to be independent from the nicotine dose (Rinaldi et al., 2023). Results of another *in vitro* cytotoxicity study in gingival epithelial cells also indicate that nicotine pouches can have adverse local effects (Shaikh et al., 2022).

One industry study has compared nicotine deliveries of nicotine pouches with similar nicotine contents (8–10 mg) but from different brands (McEwan et al., 2022). Their findings suggest that there is no direct correlation between the nicotine content of pouches and the nicotine delivered to the bloodstream. Relative bioavailability (regarding C_{max}) in relation to the product with the lowest release ranged from 137% to 245% (McEwan et al., 2022). Results of the herein presented study also speak against a linear relationship between nicotine delivery and nicotine content in the pouch, visible in the relative bioavailability ranging from 70% to 165%. Also, the C_{max} does not increase proportionally with the nicotine content. This is likely due to different nicotine releases from the pouches. The variability in residual nicotine proportions post-use indicates that different products release varying percentages of their total nicotine content. This varied from 24% for the 20 mg nicotine pouch to 52% for the 30 mg nicotine pouch. Lunell et al. have investigated three different nicotine strengths from the same brand and the C_{max} increase was almost linear in relation to the nicotine content (Lunell et al., 2020). *In vitro* nicotine release studies confirm that nicotine pouches can have different release rates and different release profiles depending on the formulation (Aldeek et al., 2021). This should be considered when interpreting the results from the 6 and 20 mg nicotine pouch arms of this study. The 20 mg nicotine pouch chosen for this study happened to only release 24% of its nicotine under the given use conditions. With a different formulation, nicotine pouches can release a higher percentage of nicotine. This means that higher plasma nicotine levels are possible during use of other 20 mg nicotine pouches.

5 Limitations

The participants of this study were not experienced users of oral tobacco/nicotine products. Experienced users of such products may have responded with different subjective effects or may have used the products differently. Although oral products do not allow the same degrees of freedom in terms of use as products for inhalation do (i.e., multiple puffing parameters), some parameters (e.g., the insalivation of the pouches) can be adjusted to manipulate nicotine release. However, enlisting regular users was not possible due to the low prevalence of regular oral tobacco/nicotine product use in Germany. The number of participants was too low for an in-depth statistical analysis investigating potential influences of participant characteristics, e.g., the metabolizer status. Another limitation is that the study only investigated a limited selection of products with its five study arms. Additionally, nicotine contents even higher than the 30 mg used in this study are available. Considering the great variability of nicotine release rates, more data on nicotine deliveries of pouches with high nicotine contents (i.e., 20 mg nicotine and more) is needed. All products come from different brands. This design was chosen in order to cover different formulations and subsequently different nicotine releases. Consequently, the results of this study including differences in subjective effects and side effects may have been affected by other constituents than nicotine as well.

6 Conclusion

The presented study is the first to investigate the use of nicotine pouches with high nicotine contents of up to 30 mg. The nicotine delivery of the herein used 30 mg nicotine pouches exceeded the nicotine delivery of a tobacco cigarette. Overall, these pouches with 30 mg nicotine had the strongest side effects. This study is also the first to include nicotine-free pouches and thus has a control arm with a product that comes close to a placebo. It was shown that use of nicotine-free pouches reduced cigarette craving and induced side effects such as mouth irritation. Consequently, other factors, for example, sensory aspects or expectations of reward, play an important role. Considering the craving reduction by pouches with no or low nicotine content, it is questioned whether nicotine pouches with high nicotine contents such as 30 mg are needed to provide addicted smokers with an alternative for cigarettes. The presented data also demonstrate that knowledge of only the nicotine content is not enough to estimate the nicotine delivery of a product. Further research, e.g., long-term use studies, are needed to clarify the nicotine content that is actually needed to provide an appropriate alternative for smokers. Ideally, it is as low as possible to reduce addictive potential and cardiovascular side effects. Whether nicotine pouches can pose an alternative for cigarette smoking is possible but yet unclear. However, the presented data suggest that nicotine pouches with very high nicotine doses are likely to induce addiction. Therefore, it is advisable that the nicotine content of pouches is limited and more information such as nicotine release rate are available to allow consumers to make informed choices.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics committee of the LMU Munich (project number 21-0814). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

NM-O: Conceptualization, Methodology, Project administration, Visualization, Writing–original draft. AR: Conceptualization, Methodology, Project administration, Writing–review and editing. YS: Investigation, Methodology, Visualization, Writing–review and editing. MG: Conceptualization, Writing–review and editing. BR: Investigation, Writing–review and editing. SM: Investigation, Writing–review and editing. NB: Formal Analysis, Writing–review and editing. PL: Funding acquisition, Supervision, Writing–review and editing.

EP: Conceptualization, Writing–review and editing. TS: Conceptualization, Methodology, Writing–original draft. KF: Formal Analysis, Methodology, Writing–review and editing. AL: Conceptualization, Writing–review and editing, Funding acquisition, Supervision. TR: Conceptualization, Writing–review and editing, Methodology, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1392027/full#supplementary-material

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EDITED BY
Gilda Angela Neves,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Yen-Chu Chen,
University of California, Irvine, United States
Kabirullah Lutfy,
Western University of Health Sciences, United States

*CORRESPONDENCE
A. A. Conti,
aldo.conti@kcl.ac.uk

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Blunted midbrain reward activation during smoking withdrawal: a preliminary study

A. A. Conti[1,2]*, S. Tolomeo[3], A. Baldacchino[2] and J. D. Steele[4]

[1]Department of Child and Adolescent Psychiatry, Institute of Psychiatry, Psychology and Neuroscience, King's College London, London, United Kingdom, [2]Population and Behavioural Science Division, School of Medicine, University of St Andrews, St. Andrews, United Kingdom, [3]Institute of High Performance Computing, Agency for Science, Technology and Research, Singapore, Singapore, [4]Division of Imaging Science and Technology, Medical School, University of Dundee, Dundee, United Kingdom

Introduction: Tobacco smoking is the leading preventable cause of death, causing more than six million deaths annually worldwide, mainly due to cardiovascular disease and cancer. Many habitual smokers try to stop smoking but only about 7% are successful, despite widespread knowledge of the risks. Development of addiction to a range of substances is associated with progressive blunting of brain reward responses and sensitisation of stress responses, as described by the allostasis theory of addiction. There is pre-clinical evidence from rodents for a dramatic decrease in brain reward function during nicotine withdrawal.

Methods: Here we tested the hypothesis that habitual smokers would also exhibit blunted reward function during nicotine withdrawal using a decision-making task and fMRI.

Results: Our findings supported this hypothesis, with midbrain reward-related responses particularly blunted. We also tested the hypothesis that smokers with a longer duration of smoking would have more pronounced abnormalities. Contrary to expectations, we found that a shorter duration of smoking in younger smokers was associated with the most marked abnormalities, with blunted midbrain reward related activation including the dopaminergic ventral tegmental area.

Discussion: Given the substantial mortality associated with smoking, and the small percent of people who manage to achieve sustained abstinence, further translational studies on nicotine addiction mechanisms are indicated.

KEYWORDS

functional magnetic resonance imaging, Positive Valence System, Negative Valence System, smoking, nicotine withdrawal, nicotine, midbrain, reward

Introduction

Tobacco smoking is the leading preventable cause of death, with more than six million deaths annually worldwide; on average, smokers lose 10 years of life compared to people who have never smoked (Kondo et al., 2019). Smoking-related deaths are predominately caused by cancer and cardiovascular disease, with the latter causing one-third of all smoking-related deaths (Kondo et al., 2019). Lung cancer is the leading cause of cancer-related deaths, and local lung cancer mortality largely follows the local geographical tobacco smoking prevalence (Barta et al., 2019).

Knowledge of smoking-related cancer and cardiovascular risks is widespread in society worldwide. Many habitual tobacco smokers attempt to permanently stop, but only

approximately 7% are successful (Pierce, 2022), and the majority of relapses occur within the first few days of attempting to stop smoking. Therefore, knowledge of substantial risks is not enough to make most people stop smoking. Whilst some people mistakenly regard continued smoking as a free choice, the National Health Service (NHS) reports that the main reason people continue to smoke tobacco is because of the addictive nature of nicotine present in tobacco products, such as cigarettes (https://www.nhs.uk/conditions/stop-smoking-treatments/).

Considerable pre-clinical evidence from studies on animals shows that development of addiction to a range of substances, including alcohol and opioids, is associated with progressive blunting of brain reward responses and increasing sensitisation of stress responses (Koob, 2013). Whilst nicotine is less effective as a positive reinforcer than other drugs of abuse in non-dependent animals, nicotine withdrawal symptoms in dependent humans include low mood, anxiety, anger/irritability, and craving (Epping-Jordan et al., 1998; Conti et al., 2020), contributing to the addictive properties of nicotine (Markou et al., 1998). Notably, nicotine withdrawal in rodents has been reported to be associated with a "dramatic decrease" in brain reward function, which lasted for 4 days (Epping-Jordan et al., 1998). The study conducted on rodents used an invasive method to measure relative reward thresholds involving implanted electrodes (Epping-Jordan et al., 1998). In this study, our aim was to test for changes in the reward threshold in humans using a functional magnetic resonance imaging (fMRI)-based non-invasive measurement of reward function.

Ellison reported that in rodents, major addictive stimulants such as cocaine and amphetamine cause degeneration in the fasciculus retroflexus, which carries much of the descending inhibitory control from the forebrain via the lateral habenula to midbrain dopaminergic and serotonergic neurons, linked to drug-related behavioural changes (Ellison, 2002). Nicotine, at plasma concentrations relevant to human smokers, was also found to cause an "extraordinary selective" degeneration of cholinergic fasciculus retroflexus tracts, specifically from the medial habenula, which projects to the midbrain dopaminergic ventral tegmental area (VTA), implying a link between addiction caused by major stimulant drugs and addiction caused by nicotine (Ellison, 2002). Decreased reward function during nicotine withdrawal in rodents is comparable to decreased reward function caused by major drugs that cause dependency, which may be an important factor contributing to relapse to tobacco use in humans (Epping-Jordan et al., 1998).

Various factors affect the impact of habitual smoking. Nicotine is more problematic for younger smokers as the brain is still undergoing significant developmental change. Yuan et al. (2015) identified persistent acetylcholine receptor upregulation and greater acetylcholine disruption in adolescent rodents than in adult rodents. The duration of habitual tobacco smoking is another relevant factor, which is important with regard to carcinogenesis and adverse effects on the cardiovascular system. However, the effect of a longer versus shorter duration of habitual smoking on the reward system is largely unknown, and studies investigating the age at the onset of smoking or nicotine exposure may be confounded by the duration of exposure effects. Pre-clinical studies on rodents tend to be of very short duration compared to the decades of habitual smoking that occurs in humans.

Allostasis theories have been developed from invasive pre-clinical models of human addictions, but similar evidence from invasive studies on humans is not available for ethical reasons, although lower striatal dopamine D2 receptor availability in smokers has been reported using molecular imaging (Wiers et al., 2017). We described a non-invasive fMRI-based approach aligned with the Research Domain Criteria (RDoC) (Insel et al., 2010) focusing on the Positive Valence System (PVS) and Negative Valence System (NVS), using it to test the allostasis theory-derived hypothesis for human binge alcohol drinkers (Tolomeo et al., 2020) and long-term abstinent, former opioid-dependent, patients (Tolomeo et al., 2022).

Here, we used our fMRI-based approach to test our first hypothesis that nicotine-dependent humans experiencing nicotine withdrawal exhibit blunted PVS reward function and elevated NVS function, consistent with allostasis theory predictions. Our second hypothesis was that a longer duration of habitual smoking would be associated with more pronounced negative effects on the reward system. It should be noted that we did not test hypotheses about smoking-related *cues* (McClernon et al., 2005; Englemann et al., 2012). Instead, we tested for hypothesised abnormalities of brain reward and aversion responses to nonsmoking-related *outcomes*, so our study design and analyses reflected this. We also performed exploratory analyses to investigate whether a longer duration of habitual smoking would result in more severe negative mood symptoms (e.g., anger, depression, anxiety, and anhedonia) and tobacco craving for smokers experiencing nicotine withdrawal.

Materials and methods

Participants

Ethical approval for the study was granted by the London Bromley Research Ethics Committee (REC) (REC Reference Number: 19/LO/1176) and the University of St. Andrews Teaching and Research Ethics Committee (UTREC) (UTREC Approval Code: MD14516). Twenty-seven tobacco smokers and 24 matched nonsmoker controls were recruited across the south-east region of Scotland between October 2019 and March 2020 (Conti and Baldacchino, 2021). A group of younger smokers (mean age, 21 years; mean years of habitual smoking, 6 years) and younger nonsmoking controls and a group of older smokers (mean age, 36 years; mean years of habitual smoking, 18 years) and older nonsmoking controls were recruited and matched for the age at the onset of habitual tobacco smoking and use of alcohol. The age at the onset of regular smoking was defined as the age at which participants started smoking ≥5 tobacco cigarettes per day. Smoker participants had to smoke ≥10 cigarettes per day for 2 or more years to be included in the study. Controls had to be lifetime nonsmokers. For inclusion in the study, smokers needed to present a carbon monoxide (CO) level ≥10 ppm and a salivary cotinine level >20 ng/mL, while nonsmokers needed to present a CO level ≤4 ppm and a salivary cotinine level of <20 ng/mL. The presence of illicit substances in participants was tested using urine drug analysis. Participants who were positive for illicit substances were excluded from the study, with the exception of occasional cannabis users (≤2 joints per week). Participants with a significant current and/or previous history of psychiatric and/or neurological illnesses were excluded. Sociodemographic and smoking characteristics of participants are given in Tables 1, 2.

TABLE 1 Participant details.

Session 1					
	Younger smokers	Older smokers	Younger nonsmokers	Older nonsmokers	Significance
Sociodemographic characteristics					
N	15	13	14	10	
Age in years (SD)	21.13 (2.23)	36.23 (4.22)	21.57 (1.86)	38.40 (6.56)	Older smokers > younger smokers = $p < 0.001$
					Older smokers > younger nonsmokers = $p < 0.001$
					Older nonsmokers > younger smokers = $p < 0.001$
					Older nonsmokers > younger nonsmokers = $p < 0.001$
Sex (%)	60.0% females	7.70% females	64.29% females	20.0% females	Younger female smokers > older female smokers = $p < 0.01$
	40.0% males	92.30% males	35.71% males	80% males	Younger female nonsmokers > older female nonsmokers = $p < 0.05$
Tobacco smoking characteristics					
Cigarettes smoked x day	13.50 (3.58)	16.80 (3.92)	N/A	N/A	Older smokers > younger smokers = $p < 0.05$
FTND	4.46 (1.50)	5.69 (1.25)	N/A	N/A	Older smokers > younger smokers = $p < 0.05$
Years of smoking	6.10 (3.55)	18.84 (7.10)	N/A	N/A	Older smokers > younger smokers = $p< 0.001$
Pack years	5.40 (3.77)	16.23 (8.02)	N/A	N/A	Older smokers > younger smokers = $p < 0.05$
Age at the onset of regular smoking (years)	15.16 (2.50)	17.38 (3.99)	N/A	N/A	$p > 0.05$
CO level	18.73 (6.09)	25.53 (10.98)	1.14 (0.53)	1.40 (0.51)	Older smokers > younger nonsmokers = $p < 0.001$
					Older smokers > younger nonsmokers = $p < 0.001$
					Older smokers > younger nonsmokers = $p < 0.001$
					Older smokers > younger nonsmokers = $p < 0.001$
Other substance use characteristics					
Units of alcohol consumed x day	0.93 (1.16)	0.38 (0.86)	0.14 (0.36)	0.45 (0.79)	$p > 0.05$
n Cannabis smokers	2	2	N/A	N/A	$p > 0.05$

Note: Data are presented as the mean and standard deviation (SD) or in percentages (%). Sig1 = significance at $p < 0.05$ in the two-tailed *t*-test. %, percentage; n, number of participants; CO, carbon monoxide; SD, standard deviation; FTND, Fagerström Test for Nicotine Dependence (0–2 = very low dependence, 2–4 = low dependence, 5 = medium dependence, and 6 or more = high dependence).

Procedures

Participants needed to attend two experimental sessions on 2 separate days. The first session was conducted at the University of St. Andrews School of Medicine and encompassed both screening and experimental procedures. Specifically, both smoker and nonsmoker participants undertook a CO breath test, a cotinine saliva test, and urine drug analysis in addition to other screening assessments described previously (Conti and Baldacchino, 2021; Conti and Baldacchino, 2022). Regarding experimental procedures, smokers completed the abbreviated Profile of Mood States (POMS), the Snaith–Hamilton Pleasure Scale (SHAPS), and the Brief Questionnaire of Smoking Urges (QSU). Furthermore, both smoker and nonsmoker participants performed neurocognitive

TABLE 2 Participant details.

Session 2 (fMRI)					
	Younger smokers	Older smokers	Younger nonsmokers	Older nonsmokers	Significance
Sociodemographic characteristics					
n	12	11	10	9	
Age in years (SD)	21.50 (2.27)	36.27 (4.60)	21.90 (2.13)	38.55 (6.94)	Older smokers > younger smokers = $p < 0.001$
					Older smokers > younger nonsmokers = $p < 0.001$
					Older nonsmokers > younger smokers = $p < 0.001$
					Older nonsmokers > younger nonsmokers = $p < 0.001$
Sex (%)	66.66% females	9.10% females	60.0% females	22.22% females	Younger female smokers > older female smokers = $p < 0.01$
	33.33% males	90.90% males	40.0% males	77.78% males	
Tobacco smoking characteristics					
Cigarettes smoked x day	13.79 (3.85)	17.36 (3.99)	N/A	N/A	Older smokers > younger smokers = $p < 0.05$
FTND	4.50 (1.56)	5.81 (1.25)	N/A	N/A	Older smokers > younger smokers = $p < 0.05$
Years of smoking	6.83 (3.56)	18.63 (7.74)	N/A	N/A	Older smokers > younger smokers = $p< 0.001$
Pack years	6.25 (3.69)	16.72 (8.68)	N/A	N/A	Older smokers > younger smokers = $p < 0.001$
Age at the onset of regular smoking (years)	15.20 (2.79)	17.45 (4.34)	N/A	N/A	$p > 0.05$
CO level	3.00 (1.70)	6.63 (2.54)	N/A	N/A	Older smokers > younger smokers = $p < 0.001$
Other substance use characteristics					
Units of alcohol consumed x day	0.75 (0.86)	0.36 (0.92)	0.20 (0.42)	0.22 (0.36)	$p > 0.05$
n cannabis smokers	2	2	N/A	N/A	$p > 0.05$

Note: Data are presented as the mean and standard deviation (SD) or in percentages (%). Sig1 = significance at $p < 0.05$ in the two-tailed *t*-test. %, percentage; n, number of participants; CO, carbon monoxide; SD, standard deviation; FTND, Fagerström Test for Nicotine Dependence (0–2 = very low dependence, 2–4 = low dependence, 5 = medium dependence, and 6 or more = high dependence).

tests that have been reported previously (Conti and Baldacchino, 2021), but those are outside the scope of this paper. Smokers were instructed to smoke as they wished (*ad libitum* smoking) prior to attending the first experimental session.

The second experimental session was conducted at Ninewells Hospital, Dundee. Smoker participants attended the session in a nicotine-withdrawal state. Specifically, smoker participants were instructed to stop smoking the night before the scanning session at 22:00. Nicotine withdrawal was verified through a CO breath test by utilising a cut-off value of ≤9 ppm in accordance with the Society for Research on Nicotine and Tobacco (SNRT) subcommittee on biochemical verification (Benowitz et al., 2020). The mean hours of smoking withdrawal at the time of scanning were 14.5 h (SD = 1.78). This session involved an fMRI procedure for both smoker and nonsmoker participants. Smoker participants were also asked to complete the POMS, SHAPS, and QSU prior to scanning.

Measures

Abbreviated POMS

The abbreviated POMS is a self-report psychological rating scale designed to measure transient mood states across different mood constructs including tension–anxiety, anger–hostility, and depression–dejection (McNair and Loor, 1971). The POMS has been widely utilised to investigate changes in mood states resulting from nicotine withdrawal (Heffner et al., 2011; Conti et al., 2020). The abbreviated version of the POMS constitutes of 40 items. Participants needed to rate each item on a 5-point Likert scale

ranging from 0 (not at all) to 4 (extremely). Scores were computed for each mood construct in addition to a total POMS score. The psychometric properties of the abbreviated POMS scale were investigated by Grove and Prapavessis (1992), showing acceptable validity and high reliability with a mean coefficient of 0.80.

Snaith–Hamilton Pleasure Scale

The SHAPS is a 14-item scale designed by Snaith et al. (1995) to measure the ability to experience pleasure from naturally rewarding stimuli (hedonic capacity) during a determined period of time. As stated by Borsini et al. (2020), anhedonia is characterised by deficits in three reward-processing subtypes: reward liking, reward wanting, and reward learning.

The scale does not utilise the Likert scoring method; it has two options: the "disagree" option scores 1 point, while the "agree" option scores 0 point. Thus, participants needed to rate each item on a 4-point scale: "strongly disagree" (1), "disagree" (1), "agree" (0), and "strongly agree" (0). The total score ranges from 0 to 14 (Snaith et al., 1995). A score >2 indicates an "abnormal" hedonic capacity (Snaith et al., 1995). Nakonezny et al. (2010) explored the psychometric properties of the scale, revealing high validity and excellent internal consistency with a coefficient of 0.91.

Brief Questionnaire of Smoking Urges

The Brief Questionnaire of Smoking Urges (QSU-Brief) (Cox et al., 2001) is the 10-item version of the QSU instrument developed by Tiffany and Drobes (1991) to measure the magnitude of smoking urges/craving emerging during nicotine withdrawal. As underlined by Cox et al. (2001), the QSU-Brief assesses the multidimensional nature of craving by measuring two distinct factors: "Factor 1 represents a strong desire and intention to smoke, with smoking perceived as rewarding for active smokers, while Factor 2 reflects an anticipation of relief from negative affect and an urgent desire to smoke" (Cox et al., 2001, p.13). Participants needed to rate each item through a 7-point Likert scale ranging from 1 (strongly disagree) to 7 (strongly agree). Several studies investigated the psychometric properties of the QSU-Brief, revealing good internal reliability and validity (Toll et al., 2006; Littel et al., 2011).

Neuroimaging

For each participant, functional whole-brain images were acquired using a 3T Siemens Tim Trio scanner at the Clinical Research Centre, Ninewells Hospital, Dundee. A total of 37 slices were obtained per volume, with an echo-planar imaging sequence comprising a repetition time (TR) of 2.5 s, echo time (TE) of 30 ms, flip angle of 90°, field of view of 22.4 cm, 64 × 64 matrix, and a voxel size of 3.5 × 3.5 × 3.5 mm. Images were pre-processed using statistical parametric mapping (SPM12) (Friston, 1994), which comprised realignment to the initial scan in each participant's time series. The mean realigned image was calculated and then used to determine the spatial normalisation transformations, which were applied to the realigned images smoothed using an 8-mm FWHM Gaussian kernel.

fMRI paradigm

Figure 1 shows the reward–gain and loss avoidance instrumental learning task used during fMRI (Tolomeo et al., 2020; Tolomeo et al., 2022). Before scanning, all participants had a brief training session on the task on a PC, which used different stimuli from those used in the scanner. The task had three possible outcomes: rewarding ("win"), aversive ("lose"), and neither win or lose ("nothing"). Participants were informed that the aim of the task was to maximise winning and avoid losing points ("vouchers') as much as possible: win–gain trials had the possible outcomes "win" or "nothing," and loss–avoidance trials had the possible outcomes "lose" or "nothing." One pair of fractal images were associated with each type of outcome (win or lose), and the association between a given pair of fractal images and outcomes was randomised across participants. The probability of win/loss fractal pairs had a fixed high probability (70%) and a fixed low probability (30%). Each session had 90 trials, with each session lasting 13 min in total and four sessions per subject. The reward–gain and loss avoidance trials were presented in a pseudo-random order.

Statistical analysis

Factorial ANOVAs were utilised to compare younger smokers, older smokers, younger nonsmokers, and older nonsmokers in relation to age, daily alcohol usage, and tobacco smoking variables (n° cigarettes smoked per day, years of smoking, Fagerström Test for Nicotine Dependence [FTND] scores, and age at the onset of regular smoking) for both experimental sessions. Chi-squared (χ^2) tests of associations were instead utilised to investigate differences in relation to biological sex and n° of occasional cannabis users between the four groups of participants. Independent-sample *t*-tests and chi-squared (χ^2) tests of associations were utilised to compare all smokers (younger + older groups) against all nonsmokers (younger + older groups) in relation to age, biological sex, and units of alcohol consumed per day as per our previous paper (Conti and Baldacchino, 2021).

Mixed ANOVAs were utilised to investigate whether a longer duration of habitual smoking resulted in more severe negative moods and tobacco-craving symptoms for smokers experiencing nicotine withdrawal. Particularly, the smoker group (younger vs. older) was inserted as a between-subject factor, while the smoking condition (*ad libitum* smoking vs. nicotine withdrawal) was inserted as a within-subject factor. Main effect comparisons were conducted with Bonferroni adjustment to control for type-1 error. The significance level was set at $p < 0.05$. SPSS v. 28 (SPSS Inc., United States) was utilised for this part of the analysis.

For fMRI event-related, random-effects analyses, data on each subject were analysed separately (first-level analyses) before summary "beta" images were tested at a group level (second-level analyses). First-level within-subject analyses focused on the feedback event in the reward–gain ("win" or "nothing") and loss–avoidance ("loss" or "nothing") trials. For

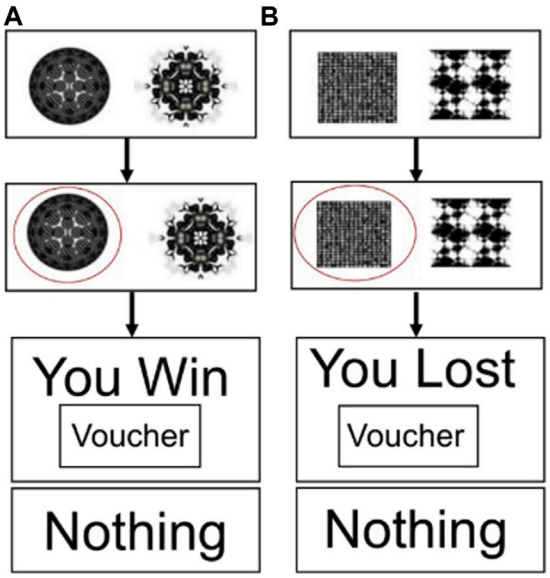

FIGURE 1
Decision-making task used during fMRI. **(A)** Reward–gain trials and **(B)** loss–avoidance trials.

second-level between-subject analyses, summary "beta" images from the first-level analyses used one-group and two-group *t*-tests. For voxel-based analyses, significance was defined as $p < 0.05$ at a whole-brain, family-wise error-corrected level, comprising a simultaneous requirement for a voxel threshold ($p < 0.05$) and a minimum cluster extent (120 voxels) identified using the popular Monte Carlo method (Slotnick et al., 2003). In Figure 2, significance as $p < 0.05$ cluster corrected is indicated by the presence of any colour. Further interpretation is not possible because a cluster-based significance correction method does not allow inferences about the relative significance of any coloured region, although SPM shows lighter colours where voxel t-values are numerically larger.

To test for any specific change in VTA reward-related activity, we used an *a priori* defined volume of interest defined by two previous studies (Gu et al., 2010; Hadley et al., 2014), comprising a 6-mm-diameter sphere centred at (0, −16, −7). A binary mask was created using MarsBaR (Brett et al., 2006; Brett et al., 2002) for this volume, and SPM12 was used to calculate the mean whitened and filtered beta values for each subject. The null hypothesis of no difference was then tested using JASP (https://jasp-stats.org/).

Results

Subjects

The sociodemographic and smoking characteristics of participants are given in Tables 1, 2. Ten participants (five smokers and five nonsmokers) dropped out from the study before attending session 2. Two smoker participants were excluded from the study prior to session 2 due to failure to maintain nicotine withdrawal as objectively verified by the CO breath test; two participants (one smoker and one nonsmoker) dropped out due to the burden of attending an fMRI session, while the remaining six participants (four nonsmokers and two smokers) did not attend session 2 due to COVID-19 restrictions.

During both sessions (session 1 and session 2), comparison of the younger group of smokers with younger nonsmokers and older smokers with older nonsmokers revealed no significant differences in age ($p > 0.05$). Similarly, no significant biological sex differences were identified between young smokers and young nonsmokers and between older smokers and older nonsmokers ($p > 0.05$). However, the % of females in the young smoker and young nonsmoker groups was significantly higher than that in the older smoker and older nonsmoker groups ($p < 0.05$).

Younger and older smokers had no statistically significant differences regarding the age at the onset of regular smoking ($p > 0.05$). Furthermore, there were no statistically significant differences between younger and older smokers regarding the units of alcohol consumed per day and the number of occasional cannabis users ($p > 0.05$). No statistically significant differences in alcohol consumption were detected when comparing older smokers with older nonsmokers ($p > 0.05$). The average ages of the younger and older groups of smokers and nonsmokers were significantly different ($p < 0.001$), and the older group of smokers reported longer years of smoking than the younger group ($p < 0.001$). Furthermore, significant differences were identified between the younger group of smokers and the older group of smokers in relation to number of cigarettes smoked daily, pack years, and severity of nicotine dependence (as assessed by the FTND) ($p < 0.05$). Specifically, the older group of smokers reported a greater number of cigarettes smoked daily, higher pack years, and more severe nicotine dependence than the younger group of smokers.

No significant differences were detected when comparing all smokers with all nonsmokers in relation to age, biological sex, and units of alcohol consumed per day during both experimental sessions as reported in our previous study (Conti and Baldacchino, 2021).

fMRI paradigm

Behavioural analyses

There were no significant differences for rewards gained and losses avoided between groups. This meant that the groups were well-balanced with regard to their behaviour during fMRI, which is important for interpreting fMRI results.

Positive valence system

For the nonsmoker control groups combined, using a one-group *t*-test, there was significant reward-related activation in the midbrain (−4, −20, −12), $t = 4.14$, and bilateral nucleus accumbens (NAC) (−4, 0, −6), $t = 3.59$ (14, 10, −4) and $t = 3.12$, as shown in Figure 2. This is consistent with many previous independent studies (Johnston et al., 2015; Gradin et al., 2011). Comparing the younger and older nonsmoker control groups combined, with the younger and older smoking groups combined, using a two-group *t*-test showed that there was blunted reward-related activation in the midbrain (−6, −20, −12), $t = 3.03$, and significant blunted reward-related activation in the bilateral nucleus accumbens (−10, 4, −8), $t = 2.50$ (14, 0, −12) and $t = 2.7$ of smokers. Comparing only younger nonsmoker controls matched to younger smokers, there was significant blunted reward-related activation in the midbrain

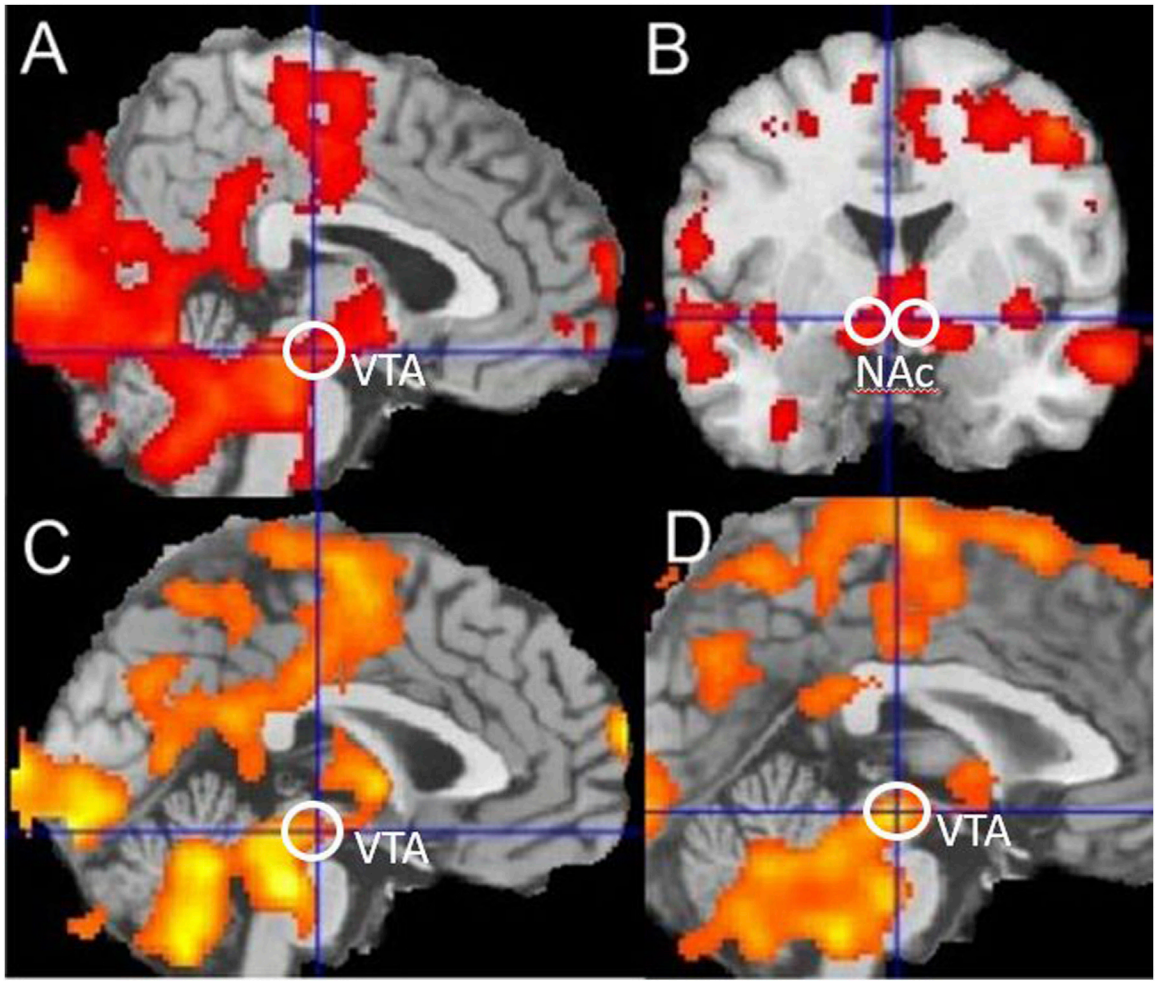

FIGURE 2
Reward-related activation in smokers and controls. **(A,B)** Reward-linked activation for all nonsmoker controls, **(C)** blunted reward-related activation in all smokers during withdrawal compared to nonsmoker controls, and **(D)** blunted reward-related activation in younger smokers during withdrawal compared to younger nonsmoker controls. Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAc) regions are indicated. Significance as $p < 0.05$ corrected is indicated by the presence of any colour in the figure.

(−2, −22, −10), t = 3.43, and significant blunted reward-related activation in the nucleus accumbens (−10, 6, −6), t = 3.14, of younger smokers. Comparing younger nonsmokers to older nonsmokers showed that there was reward-related activation in the nucleus accumbens (−10, 8, 0), t = 2.76, and no difference in VTA activation. Comparing only older smokers to older nonsmoker controls showed that there was no significant difference in reward-related activations. Using the VTA volume of interest, significantly blunted reward-related activation was identified (t = 2.2, df = 18, p = 0.04) for younger smokers compared to matched younger nonsmoker controls.

Negative valence system

Regarding loss-related activations, there were no significant differences when comparing groups.

Exploratory analyses—mood and craving measures

Regarding anhedonia (SHAPS scores), there was no statistically significant interaction between the smoker groups (younger smokers vs. older smokers) and smoking conditions (*ad libitum* smoking vs. nicotine withdrawal) [F(1, 21) = 0.20, p = 0.65, partial η2 = 0.01]. However, the main effect of smoking conditions showed a statistically significant difference in the mean SHAPS scores between *ad libitum* smoking and nicotine withdrawal [F(1, 21) = 10.18, p < .005, partial η2 = 0.32]. Similarly, no statistically significant interaction was identified between the smoker groups and smoking conditions regarding anxiety [F(1, 21) = 0.16, p = 0.69, partial η2 = 0.00], anger [F(1, 21) = 0.30, p = 0.59, partial η2 = 0.01], depression [F(1, 21) = 3.03, p = 0.09, partial η2 = 0.12], and the total POMS score [F(1, 21) = 0.01, p = 0.89, partial η2 = 0.00]. Nonetheless, the main effect of smoking conditions showed a statistically significant difference in mean anxiety [F(1, 21) = 20.66, p < .001, partial η2 = 0.49], anger [F(1, 21) = 22.56, p < .001, partial η2 = 0.51], depression [F(1, 21) = 4.72, p < 0.05, partial η2 = 0.18], and total POMS scores [F(1, 21) = 17.26, p < 0.001, partial η2 = 0.45] between *ad libitum* smoking and nicotine withdrawal. These results suggest that the combined groups of smokers experienced an increase in negative mood states including

anxiety, anger, depression, and anhedonia when they were nicotine-deprived for 14.5 h (mean) during experimental session 2.

No statistically significant interaction was identified between the smoker groups and smoking conditions regarding QSU factor 1 [$F(1, 21) = 2.97$, $p = 0.09$, partial $\eta 2 = 0.12$] and QSU factor 2 [$F(1, 21) = 0.47$, $p = 0.49$, partial $\eta 2 = 0.02$]. As per the previously reported mood measures, the main effect of smoking conditions showed a statistically significant difference in mean QSU factor 1 scores [$F(1, 21) = 36.80$, $p < 0.001$, partial $\eta 2 = 0.63$] and QSU factor 2 scores [$F(1, 21) = 44.47$, $p < 0.001$, partial $\eta 2 = 0.67$] between *ad libitum* smoking and nicotine withdrawal. Therefore, these results suggest that the combined groups of smokers experienced a surge in tobacco cravings and a strong desire to smoke to a) experience the pleasurable/rewarding effects of smoking (QSU factor 1) and b) relieve negative mood symptoms (QSU factor 2) while they were nicotine-deprived for 14.5 h (Mean) during experimental session 2.

Discussion

Based on the pre-clinical allostasis theory proposed by Koob (2013) and the investigation of nicotine-dependent rodents during nicotine withdrawal (Epping-Jordan et al., 1998), we tested the hypothesis that nicotine-dependent humans would exhibit blunted PVS function (blunted reward-linked signals) and increased NVS function (increased loss-linked signals) during nicotine withdrawal. Our results supported the hypothesis that nicotine-dependent humans exhibit blunted PVS function during nicotine withdrawal (14.5 mean h since the last smoked cigarette). However, we did not find evidence for elevated NVS responses during nicotine withdrawal. We also tested whether a longer duration of smoking was associated with more pronounced abnormalities. Contrary to predictions, we found more pronounced reward signal blunting in younger smokers with a shorter smoking history. Exploratory analyses showed that the combined groups of smokers experienced an increase in negative mood states (anhedonia, depression, anxiety, and anger) and craving symptoms during nicotine withdrawal. However, older habitual smokers with a longer smoking history (and a higher level of nicotine dependence) did not experience more severe withdrawal symptoms compared to younger smokers.

The primary objective of the present study was to test whether there was evidence for blunting of the reward system of human habitual tobacco smokers during withdrawal, as predicted by a pre-clinical rodent study on nicotine withdrawal (Epping-Jordan et al., 1998). In that study, rodent reward thresholds were measured using intracranial self-stimulation (ICSS), an instrumental reward learning paradigm, whereby rodents learn to continuously respond (e.g., by lever pressing) to electrical stimulation of electrodes implanted in a region such as the posterior–lateral hypothalamus (Epping-Jordan et al., 1998), which is a major part of the reward system in all animals. ICSS is an established method for testing reward system function in all animals including humans (Rolls, 1975). It should be noted that ICSS brain stimulation is electrical and not by infusion of nicotine; the procedure did not involve cues for lever pressing to deliver ICSS outcomes, and functional connectivity between different rodent brain regions was not measured (Epping-Jordan et al., 1998).

For the present study, we used an instrumental reward learning and loss avoidance learning task that we have used for studies on depressive illnesses (Johnston et al., 2015), opioid dependency (Gradin et al., 2014; Tolomeo et al., 2022), and binge alcohol drinking (Tolomeo et al., 2020; 2023). Subjects had to learn to choose between two pairs of nonsmoking-related visual stimuli to maximise rewards and avoid losses. The events of interest were the times when the subjects learnt the outcomes of their decisions, e.g., "you win" or "nothing." As described in a series of previous studies, this contrast provides a non-invasive measure of brain reward function (Steele et al., 2007; Gradin et al., 2014; Johnston et al., 2015; Tolomeo et al., 2022). It is important to note that the reward signal at the outcome time is different from the signal at the time the pairs of stimuli are presented, and a choice has to be made, the latter being the expected reward value signal (Tolomeo et al., 2023). Similarly, studies measuring brain responses to already learned drug cues (e.g., McClernon et al., 2005) test hypotheses different from those tested here (Tolomeo et al., 2023). Additionally, fMRI brain connectivity studies test different hypotheses and measure different brain signals.

Preclinical studies (Koob and Schulkin, 2019) and human addiction studies using positron emission tomography (Wiers et al., 2017; Goldstein and Volkow, 2002) have provided considerable evidence that addiction to a variety of substances in animals and humans involves a shift from positive reinforcement to negative reinforcement. The RDoC were designed to link subjective symptoms to specific brain functions and can also facilitate forward and reverse translation between preclinical studies on animals and non-invasive studies on humans (Tolomeo et al., 2020). We have used this approach to test for blunted PVS brain responses and elevated NVS brain responses in alcohol binge drinkers (Tolomeo et al., 2020) and long-term abstinent, former opioid-dependent patients (Tolomeo et al., 2022), reporting results consistent with predictions.

Abstinence syndromes in dependent rodents have been observed after stopping alcohol, opiates, other sedatives, and nicotine (Epping-Jordan et al., 1998). In the case of nicotine, this occurs spontaneously and can also be precipitated by nicotinic receptor antagonists, reversed by nicotine administration (Epping-Jordan et al., 1998). Subcutaneous nicotine administration to rodents was used to maintain stable plasma nicotine concentrations, comparable to those reported for human tobacco smokers consuming 30 cigarettes per day, and thresholds of electrical ICSS to the rodent posterior lateral hypothalamus were measured. During rodent nicotine withdrawal, significant elevations of reward thresholds were reported, corresponding to blunted reward function, peaking at 6–8 h after withdrawal, with reward thresholds exceeding 140% baseline values (Epping-Jordan et al., 1998). Consistent with this, we found blunted reward function in humans and a diminished capacity to experience natural rewarding stimuli (i.e., anhedonia as measured by the SHAPS) during nicotine withdrawal (14.5 h).

In the subgroup analysis, contrary to expectations, we found more pronounced reward system blunting in habitual younger tobacco smokers, which implies a shorter duration of exposure to

nicotine, and these marked reward system abnormalities could then be due to increased neurotoxic effects of nicotine at a younger age, as has been noted in animal studies (Yuan et al., 2015). However, this interpretation appears less likely as the older and younger groups of smokers were well-matched for the age at the onset of smoking.

Blunting of reward-linked activation in a broad midbrain region included a VTA volume defined by two independent studies, one reporting significant VTA functional connectivity decreased in chronic cocaine users (Gu et al., 2010) and the other reporting reduced VTA connectivity in schizophrenic patients, which was normalised with the response to antipsychotic medication (Hadley et al., 2014). Tobacco smoking is very common in people experiencing schizophrenia, and Ellison argued that the highly specific nicotine-induced degeneration of the fasciculus retroflexus projection to the VTA dopaminergic neurons could interact with the vulnerability to develop schizophrenia and other severe neuropsychiatric illnesses (Ellison, 2002). Consistent with this, we found marked blunting of midbrain reward-related activation in the younger group of smokers in a region that included the VTA.

The results of the exploratory analyses are in line with previous meta-analytic evidence showing an increase in negative mood symptoms during acute nicotine withdrawal (within the first 24 h after smoking cessation) (Conti et al., 2020). Smokers also experienced a surge in cravings to relieve negative mood symptoms (QSU factor 1) and to experience the pleasurable effect of smoking (QSU factor 2). However, no group-by-time interactions were identified regarding these mood and craving symptoms. This may be related to the small sample size as the minimum number of subjects needed to detect group-by-time interactions should be four-fold the number of subjects needed to detect main effects (Heo and Leon, 2010; Guo et al., 2013). Indeed, the small sample size of this study may be considered its main limitation. However, it is important to underline that the present work is a preliminary study, and future research is warranted to replicate these results in a larger number of participants. It should also be noted that the recruitment procedures for this study were hampered by the COVID-19 pandemic, which also posed a challenge for matching the smoker and nonsmoker groups. While the smoker groups were well-matched to the nonsmoker control groups (younger smokers vs. younger nonsmokers, older smokers vs. older nonsmokers, and combined groups of smokers vs. combined groups of nonsmokers), both younger groups of smokers and nonsmokers had more female participants in comparison to older groups of smokers and nonsmokers. It is well known that female smokers experience more severe withdrawal symptoms and have more difficulties in quitting smoking compared to male smokers (e.g., Bjornson et al., 1995; Rojas et al., 1998; Conti et al., 2020). Furthermore, neuroimaging studies have reported sex differences pertaining to the structure and the functional connectivity of the smokers' frontostriatal system (McCarthy et al., 2019; Lin et al., 2020). Therefore, the possibility that the fMRI findings could have been influenced by the greater percentage of women in the group with younger smokers cannot be excluded.

Another important limitation is the lack of a group with young smokers with a longer smoking history and a group with older smokers with a shorter smoking history. The inclusion and comparisons of these groups, in addition to the comparison between younger smokers with a shorter smoking history and younger smokers with a longer smoking history, would have helped determine whether the blunting of the reward system identified in the current preliminary study is, indeed, independent from the duration of tobacco consumption. For this reason, the findings of this preliminary study should be considered with caution, and the inclusion of these groups, in addition to a pre-withdrawal fMRI session in a nicotine-satiated state, is warranted for future confirmatory studies recruiting a larger sample size.

Despite the above limitations, the numbers of subjects were sufficient to reject the null hypothesis of no blunting in reward function for all smokers and specifically for the younger group of smokers. The limited study size affects the interpretation of null findings. It is possible that a larger study would also find evidence for blunted reward function in older smokers and an increased NVS response during nicotine abstinence. It would not, however, contradict the main findings of this study.

The main strengths of our work are its translation design allowing specific non-invasive tests of allostasis theory, in particular predictions based on findings from invasive ICSS studies on rodents during nicotine withdrawal. To the best of our knowledge, this is the first study in the literature that tested whether the neurobiological effects, as described by Koob's allostasis theory, in rodents also occur in humans during nicotine withdrawal. Notably, the literature pertaining to neuroimaging on nicotine withdrawal is limited to smoking and reward cue-reactivity studies (for a meta-analysis, see Lin et al., 2021) and functional connectivity studies (Faulkner et al., 2018; Ghahremani et al., 2021). Another strength of this study consists in the stringent inclusion and exclusion criteria and that objective tests (urine analysis, saliva cotinine test, and CO breath test) were utilised to assess the smoking and other substance use status of participants in addition to nicotine withdrawal.

In conclusion, consistent with preclinical studies on rodents, we found evidence for blunted midbrain reward function in humans during nicotine withdrawal, which was more marked in younger smokers with a shorter smoking history. Further human–animal translational studies on different stages of nicotine use and withdrawal are indicated to better understand the additive and neurotoxic effects of nicotine, investigating the different effects of the duration of smoking and age at the onset of smoking, aimed at the development of better treatments for nicotine addiction to reduce its substantial associated mortality and morbidity.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the National Health Service (NHS) London Bromley Research Ethics Committee (REC) (REC Reference Number: 19/LO/1176) and the University of

St. Andrews Teaching and Research Ethics Committee (UTREC) (UTREC Approval Code: MD14516). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AC: conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, validation, visualisation, writing–original draft, and writing–review and editing. ST: conceptualisation, formal analysis, investigation, methodology, project administration, software, validation, visualisation, writing–original draft, and writing–review and editing. AB: conceptualisation, funding acquisition, methodology, project administration, resources, supervision, validation, and writing–review and editing. JS: conceptualisation, formal analysis, investigation, methodology, project administration, software, supervision, validation, visualisation, writing–original draft, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Vijayapandi Pandy,
MIT World Peace University, India

REVIEWED BY
Marsida Kallupi,
University of California, San Diego, United States
Maw Shin Sim,
University of Malaya, Malaysia

*CORRESPONDENCE
Diana Carreño,
ldcarreno@gmail.com

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Sub-chronic nicotine exposure influences methamphetamine self-administration and dopamine overflow in a sex-and genotype-dependent manner in humanized *CHRNA6* 3′-UTR SNP (rs2304297) adolescent rats

Diana Carreño[1]*, Antonella Facundo[2], Anjelica Cardenas[3] and Shahrdad Lotfipour[1,2,4]

[1]Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, CA, United States, [2]Department of Emergency Medicine, University of California, Irvine, Irvine, CA, United States, [3]Department of Radiation Oncology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, United States, [4]Department of Pathology and Laboratory Medicine, University of California, Irvine, Irvine, CA, United States

Introduction: The rewarding effects of drugs of abuse are associated with the dopaminergic system in the limbic circuitry. Nicotine exposure during adolescence is linked to increased use of drugs of abuse with nicotine and methamphetamine (METH) commonly used together. Nicotine acts on neuronal nicotinic acetylcholine receptor (nAChR) systems, critical for reward processing and drug reinforcement, while METH leads to a higher dopamine (DA) efflux in brain reward regions. A human single nucleotide polymorphism (SNP) in the 3′-untranslated region (UTR) of the α6 nicotinic receptor subunit gene (*CHRNA6*, rs2304297), has been linked with tobacco/nicotine and general substance use during adolescence. Using CRISPR-Cas9 genomic engineering, our lab recapitulated the *CHRNA6* 3′UTR^{C123G} SNP, generating α6^{CC} and α6^{GG} allele carriers in Sprague Dawley rats. We hypothesized the *CHRNA6* 3′UTR^{C123G} SNP would sex- and genotype-dependently enhance nicotine-induced METH self-administration as well as nicotine-induced DA overflow in the nucleus accumbens shell of adolescent α6^{GG} and α6^{CC} carriers.

Methods: Adolescent male and female rats underwent a 4-day sub-chronic, low-dose (0.03 mg/kg/0.1 mL, x2) nicotine pretreatment paradigm to assess intravenous METH (0.02 mg/kg/0.1 mL) self-administration as well as nicotine- and METH (0.02 mg/kg/0.1 mL)-induced DA overflow in the nucleus accumbens shell (NAcS) using *in vivo* microdialysis coupled with high-performance liquid-chromatography-electrochemical detection (HPLC-ECD).

Results: Nicotine pretreatment sex- and genotype-dependently enhanced subsequent METH self-administration in adolescent *CHRNA6* 3′UTR^{C123G} SNP rats. Further nicotine and METH-induced DA overflow is observed in α6^{CC} females as compared to α6^{GG} females, with METH-induced DA overflow enhanced in α6^{GG} males when compared to α6^{CC} males.

Conclusion: These findings demonstrate that the *CHRNA6* 3′-UTR^{C123G} SNP can sex- and genotype-dependently impact adolescent nicotine-induced effects on METH self-administration and stimulant-induced DA overflow in reward regions of the brain.

KEYWORDS

electronic cigarettes, addiction, developing brain, microdialysis, tobacco, co-morbid drug use

1 Introduction

Adolescence is a critical period of development characterized by heightened susceptibility to environmental influences, including substance use. Nicotine and methamphetamine (METH) are two commonly abused substances that can have profound, long lasting consequences on the developing brain. Adolescents predominantly favor electronic cigarettes as their primary choice among tobacco products (Gorukanti et al., 2017). In 2023, the Annual National Youth Tobacco Survey reported more than one in four of current youth e-cigarette users use an e-cigarette product every day (Birdsey et al., 2023). Most e-cigarettes contain nicotine, a highly addictive substance that can harm the developing brain of adolescents (Goriounova and Mansvelder, 2012; Yuan et al., 2015). Adolescent exposure to nicotine may lead to the use of other tobacco products and drugs of abuse and amplify common underlying risk factors, i.e., genetic predisposition, peer influence, socioeconomic status and family history (McCabe et al., 2018; Berry et al., 2019; Ren and Lotfipour, 2019).

The neurobiological actions of nicotine are similar to other psychomotor stimulants including cocaine and METH (Laviolette and van der Kooy, 2004). Nicotine binds to nAChRs, leading to increased dopamine (DA) release and neurotransmitter modulation, resulting in enhanced alertness, reward and potential dependence (Di Chiara and Imperato, 1988; Albuquerque et al., 2009; Dani, 2015). METH is a potent analog of the stimulant, amphetamine. METH increases DA levels by inhibiting the reuptake and promoting release of DA, causing intense euphoria, heightened alertness, and potential neurotoxicity with chronic use (Rothman et al., 2001; Elkashef et al., 2008; Rusyniak, 2011; Ferrucci et al., 2019). It has been hypothesized that nicotine addiction is mediated by DA release in the mesolimbic system via local action at somatodendritic sites in the ventral tegmental area (VTA) (Di Chiara and Imperato, 1988; Nisell et al., 1994). Indeed, injections of 6-hydroxydopamine, a neurotoxin used to selectively destroy dopaminergic and noradrenergic neurons, into the nucleus accumbens (NAc) blocked nicotine self-administration in adult rats (Corrigall et al., 1994). *In vitro* studies have shown that METH evokes greater efflux of DA via the dopamine transporter (DAT) than its counterpart, amphetamine (Goodwin et al., 2009). *In vivo* studies have shown that nicotine pretreatment enhances the acquisition of METH self-administration and increases its intake in adolescent male, but not female rats (Dao et al., 2011; Cardenas and Lotfipour, 2022). Additionally, repeated administration of METH and nicotine in mice produced locomotor sensitization effects and a symmetrical cross-sensitization (Kuriban, 1999).

During adolescence, the α6 nAChR subunit reaches peak mRNA expression in dopaminergic cell bodies in the VTA and substantia nigra (SNg) (Azam et al., 2007). Moreover, the α6 nAChR subunit is localized in the mesolimbic and nigrostriatal DA pathway (Le Novère et al., 2002; Azam et al., 2007; Jackson et al., 2009) which may indicate a role of α6*-containing nAChRs in nicotine-induced behaviors and DA release (*denotes other associated nAChR subunits) (Le Novère et al., 2002; Azam et al., 2007; Pons et al., 2008; Jackson et al., 2009; Zoli et al., 2015). *In vivo* studies have demonstrated the α6* nAChRs have been shown to be involved in nicotine-induced locomotion (Drenan et al., 2008; Cardenas et al., 2022), nicotine self-administration and drug-seeking behavior (Pons et al., 2008; Brunzell et al., 2010; Carreño and Lotfipour, 2023; Carreño et al., 2024). Nevertheless, there is a scarcity of studies that examine the impact of α6* nAChRs in adolescent nicotine-induced behaviors. Despite this gap in research, it is important to note a robust correlation between tobacco/nicotine intake during adolescence and subsequent METH use (Brecht et al., 2007; Russell et al., 2008). Repeated self-administration of METH upregulated the *CHRNA6* mRNA in the VTA of adult male rats (Bosch et al., 2015). Given these results, exploring how nicotine affects METH self-administration and the underlying mechanisms for how nicotine and METH exposure impact DA overflow in the NAc shell in adolescent rodent lines carrying a human *CHRNA6* 3′-UTR SNP becomes crucial.

To model adolescent initiation of nicotine smoking behavior in rats, a low-dose 4-day intravenous nicotine pretreatment paradigm was used. Previously, this paradigm has shown nicotine-induced acquisition of subsequent cocaine, fentanyl, METH, and alcohol in adolescent rats. (McQuown et al., 2007; Dao et al., 2011; Linker et al., 2020; Cardenas et al., 2021). Following nicotine treatment, animals underwent 2-h operant intravenous METH self-administration over 5-days (Dao et al., 2011; Cardenas and Lotfipour, 2022). Given our prior results that nicotine exposure can sex- and age-dependently enhance METH self-administration (Cardenas and Lotfipour, 2022), our current studies evaluated the combined interactions in our *CHRNA6* 3′UTR SNP rat lines in order to test the hypothesis that α6*-containing nAChRs mechanisms influence these relationships. In parallel, we used the same 4-day sub-chronic paradigm to evaluate nicotine- and METH-induced DA overflow in the NAc shell in adolescent male and female *CHNRA6* 3′-UTR SNP rats. Our recent studies illustrate that adolescent nicotine reinstatement behavior predicts DA tissue levels in *CHRNA6* 3′UTR SNP rat lines providing support for our hypothesis (Carreño et al., 2024). To better understand mechanisms underlying our observed effects, male and female adolescent rats (PN 31) underwent *in vivo* analytical quantification of neurotransmitters collected from the interstitial fluid from the NAc shell. DA, and its metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) from the

extracellular space were measured by high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) methods. Our studies test the hypothesis that sex- and genotype-dependent effects will be observed for nicotine-induced METH self-administration and drug-induced DA overflow in adolescent *CHRNA6* 3′-UTR SNP rats. The objective of this study is to fill critical gaps in understanding how nicotine exposure during adolescence and the *CHRNA6* 3′-UTR SNP influences METH self-administration and associated neurobiological changes. By exploring sex- and genotype-dependent effects, we gain insights into the complex interplay between genetics, sex, and co-substance use in adolescents, thereby informing future strategies for the prevention and intervention of substance abuse disorders.

2 Methods

2.1 Animals

Male and female, *CHRNA6*3′-UTR SNP knock-in rats were developed and bred in house as previously described (Cardenas et al., 2022; Carreño and Lotfipour, 2023). Juveniles were weaned at postnatal day (PN 21), separated by sex, and handled for 3 days prior to experimentation. Rats were group-housed in a controlled 12-h light–dark cycle in an AAALAC-accredited vivarium. Food and water were available *ad libitum* except when indicated. Animals were weighed daily to ensure the maintenance of normal growth. To avoid potential litter effects, one pup per litter per experimental group was used for data collection. Animals were allocated to experimental groups using a random sequence generator. The study was conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine (UCI) (protocol: AUP-21-022, 4 February 2021). UCI's IACUC has Animal Welfare Assurance #D16-00259 (#A3416.01) approval on file with the Office of Laboratory Animal Welfare at NIH and is fully accredited by AAALAC, International. *CHRNA6* 3′UTR SNP rat strains (SD-*CHRNA6*^{em1Slot} 984, SD-*CHRNA6*^{em2Slot} 985) have been donated to the Rat Resource and Research Center (RRRC).

2.2 Drugs

Nicotine tartrate (Glentham Life Sciences, Corsham, Wiltshire, United Kingdom) was calculated as a base, dissolved in saline, with a final pH of 7.2–7.4. Methamphetamine (METH) (National Institute of Drug Abuse (NIDA), Bethesda, MD) was dissolved in saline and filtered via 0.22 μm sterile filters (VWR). Equithesin (Pentobarbital and Chloral Hydrate (Sigma Aldrich, St. Louis, MO, United States), propylene glycol, ethanol and magnesium sulfate) is made in the laboratory, similar to our prior studies (Carreño and Lotfipour, 2023; Carreño et al., 2024). Carprofen (Zoetis, Parsippany, NJ, United States) was diluted in saline. Nicotine, Equithesin, and carprofen were filtered via 0.22 μm sterile filters (VWR, Radnor, PA, United States). Propofol (Medvet, Mettawa, IL, United States) in a 5 mg/kg, intravenous (i.v.) was administered to test for catheter patency.

2.3 Surgical implantation of intravenous catheter

Catheter construction and implantation was done as described previously (Belluzzi et al., 2005). Animals were anesthetized with Equithesin (0.0035 mL/g body weight), and a chronic catheter was surgically implanted into the right external jugular vein. The catheter was passed subcutaneously from the animal's back to the jugular vein where the tubing was inserted. The cannula assembly was affixed to the animal's back and sealed to maintain an unobstructed system. Wound closures were made with wound clips, antiseptic ointment was applied to the incisions, and carprofen (5 mg/kg, subcutaneous) was injected to prevent infection. The animals were kept in a warm cage for post-surgical observation until they emerged from anesthesia. Catheter patency was tested for rapid (5–10 s) anesthesia by infusion of propofol (5 mg/kg) before and after completion of self-administration and *in vivo* microdialysis experiments (Figure 1).

2.4 Nicotine pretreatment

Starting on PN 28 rats were administered nicotine (2 × 0.03 mg/kg/0.1 mL, i.v.) or saline injections spaced 1 minute apart daily for 4 days (Figure 1A). The dual injection of 0.03 mg/kg nicotine daily over 4 days was chosen to model early initiation of nicotine use during adolescence (Cardenas et al., 2021), models prior studies using the same paradigm (Cardenas et al., 2021), and reduced receptor desensitization and toxic effects (McQuown et al., 2007). Furthermore, the 0.6 mg/kg dose is the optimal dose regularly chosen in nicotine self-administration studies, is equivalent to 1-2 cigarettes worth of nicotine/day and produces 30 ng/mL peak plasma levels in adolescent and adult rats (Cao et al., 2007; Matta et al., 2007).

2.5 METH self-administration

One day after nicotine pretreatment, animals were tested in 28 × 25 × 30 cm self-administration chambers with two nose pokes holes for 5 days (PN 32–36) (Figure 1A). Adolescent animals underwent a 2-h nose poke session on a fixed ratio 1 (FR1) schedule to administer METH (0.02 mg/kg/infusion (inf)) (Dao et al., 2011; Cardenas and Lotfipour, 2021) with a time-out period of 20 s signaled by a cue light over the reinforced hole. During this time-out period, animals were restricted from receiving another reinforced response (inf) and the house light was turned off. Nose poke of the nonreinforced hole was scored but did not result in a signal or inf. To determine catheter patency, after the last intravenous self-administration session, rats were administered propofol, a rapid anesthetic (0.05 mL for adolescents, 0.1 mL for adults, i.v.). Propofol negative animals were removed from the study analyses.

2.6 *Surgery, in vivo* microdialysis, and HPLC-ECD

2.6.1 Stereotaxic surgery

Following the placement of the i.v. Catheter, animals designated for microdialysis underwent the implantation of a

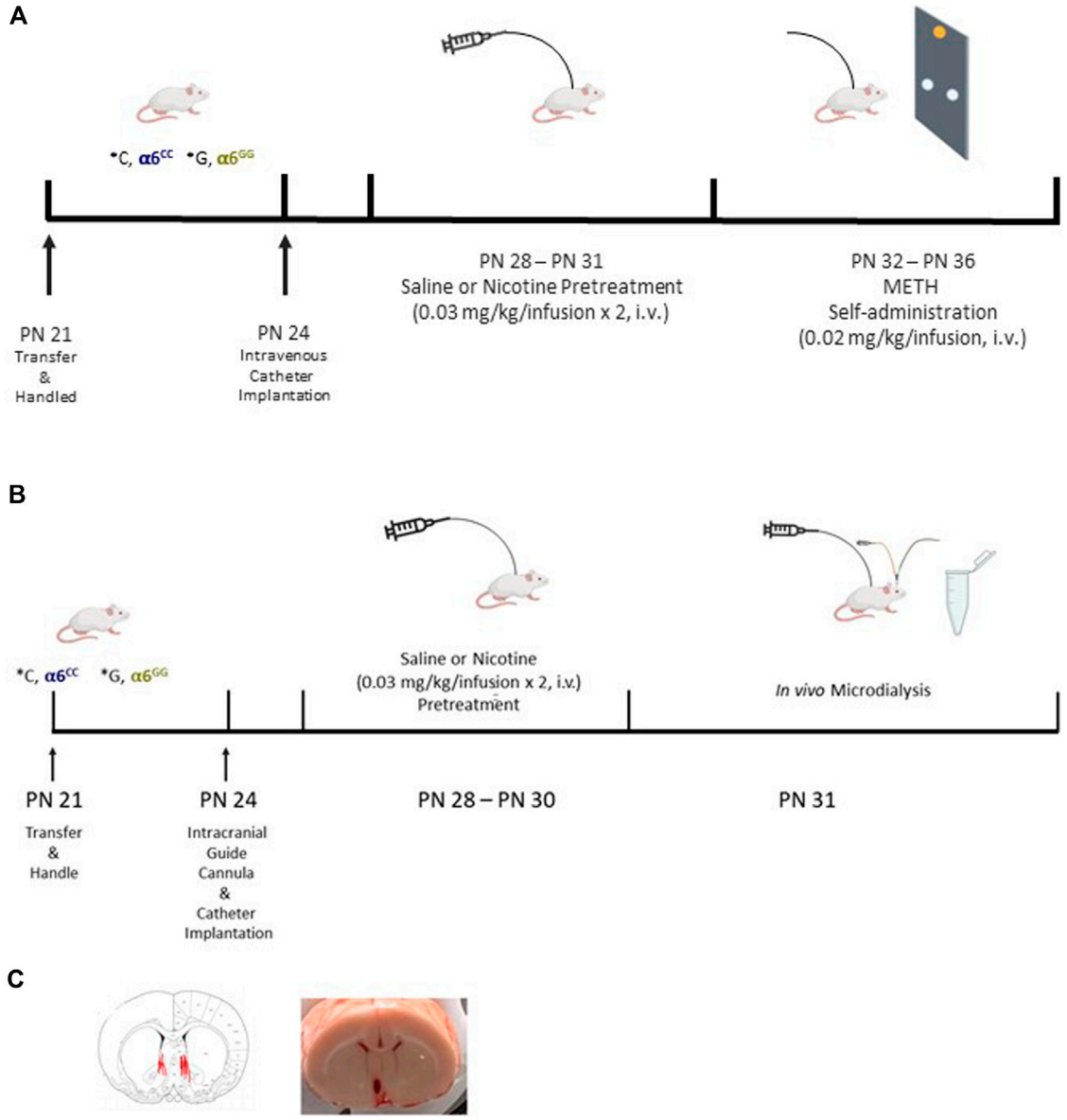

FIGURE 1
Experimental Design in the *CHRNA6* 3′UTR SNP Knock-In. Male and female *CHRNA6* 3′-UTR SNP knock-in rats were bred in-house and underwent a series of procedures including nicotine pretreatment and METH self-administration **(A)**. *In vivo* microdialysis was performed to assess dopamine and its metabolites after drug administration **(B)**. Probe placement confirmation in male and female *CHRNA6* 3′-UTR SNP knock-in rats **(C)**.

cranial guide cannula (PN 24). Animals were placed in a stereotaxic frame (Stoelting Co., Chicago, Il, United States), their skulls revealed and drilled to expose the dura. A chronic guide cannula (Bioanalytical Systems, Inc., West Lafayette, IN) was stereotaxically implanted 2.0 mm above the target area, fixed to the skull with acrylic dental cement, and sealed with a dummy cannula. Anatomical coordinates for adolescent animals (PN 28-29) were determined from the adult atlas (Paxinos and Watson, 1989), then empirically identified in a preliminary experiment with histological confirmation. The following guide cannula coordinates were measured from the dura; nucleus accumbens shell AP, + 2.1 mm; ML, ± 0.6 mm; DV,-6 mm.

2.6.2 *In Vivo* microdialysis procedure

Animals were given 3 days to recover after surgeries with daily handling after surgeries and catheters flushed daily. Beginning on PN 28, the animals received either saline (acute condition) or nicotine (sub-chronic condition) pretreatment (PN 28-31, Figure 1A). Intravenous catheter patency was tested by propofol 1 day before the experiment. On the experimental day (PN 31), the dummy cannula was replaced with a 2 mm microdialysis probe with a 30 kDa cut-off membrane (MD-2200; Bioanalytical Systems, Inc., West Lafayette, IN). The probe membrane extended 2 mm beyond the tip of the guide cannula. The quality of probes was tested *in vitro* before the experiment with an average recovery of 10%

(data not shown). Microdialysis was carried out under a free-moving condition in Culex NxT with Raturn–Multi Animal (Bioanalytical Systems, Inc., West Lafayette, IN), with the probe continuously perfused with artificial cerebrospinal fluid (Ringers Solution 147 mM NaCl, 2.2 mM $CaCl_2$, 4 mM KCl) at a constant flow rate of 1.1 mL/min delivered by a Empis infusion pump (CX-300; Bioanalytical Systems, Inc., West Lafayette, IN) (Figure 1B). Dialysate was collected into a Honeycomb refrigerated fraction collector (MD-1201; Bioanalytical Systems, Inc., West Lafayette, IN) that maintained the samples at 4°C. After 4-h perfusion to establish an equilibration between the probes' internal and external environment, baseline samples were collected every 20 min for 60 min. When DA levels reached a stable baseline, animals were given two 0.100 mL injections (i.v.) of saline, 1 min apart. After 100 min, nicotine (0.03 mg/kg/0.100 mL injection, i.v.) was injected twice at a 1 min interval and samples were collected for another 200 min. After 220 min, METH (0.02 mg/kg/0.100 mL injection, i.v.) was injected twice at 1 min interval and samples were collected for another 100 min (Figure 1). DA and its metabolite levels were quantified by HPLC-ECD. The position of microdialysis probes was verified histologically and mapped onto relevant atlas sections (Paxinos and Watson, 1986) (Figure 1C).

2.6.3 HPLC-ECD

Microdialysate samples (20 µL) were automatically injected by an ESA 542 refrigerated auto-sampler onto a 150 × 3 mm ODS C18 column (ESA Inc., Chelmsford MA) connected to an ESA 580 HPLC pump. The column was kept at 35°C and perfused by MD-TM mobile phase (ESA, Chelmsford, MA) at a rate of 0.6 mL/min. DA and metabolite levels were determined by an electrochemical ESA 5600 detector with an ESA 5011 microdialysis cell with the dominant potential of 160 mV. The sensitivity of the detector is 500 fg. Measurements were analyzed using CoulArray for Windows Software 2.0 (ESA Inc., Chelmsford, MA, United States). Standard curves were generated with catecholamine (ThermoScientific, Wlatham, MA), DOPAC, and HVA (Sigma-Aldrich, St Louis, MO) standards, and levels in experimental samples were determined from the curve and expressed as ng/20 µL injection (Equation 1), as there were no significant differences in probe recovery. Basal levels of DA and its metabolites were determined by averaging the samples before nicotine injection. Nicotine- and METH-induced changes in DA and DA metabolite levels were expressed as area under the curve (AUC) given by Equation 1 below.

$$\begin{aligned} AUC = &\left(\textbf{Drug Time Point 1} - \textbf{Average Baseline}\right) \\ &+ \left(\textbf{Drug Time Point 2} - \textbf{Average Baseline}\right) \\ &+ \left(\textbf{Drug Time Point 3} - \textbf{Average Baseline}\right) \end{aligned} \quad (1)$$

2.7 Statistics

Data were analyzed with JMP statistical analysis software (SAS Institute, Cary, NC). A total of 186 rats were used in our studies. Animals that did not exhibit anesthesia from propofol were removed from the analysis (n = 41). Further exclusions were applied to animals that fell outside the boundaries defined by predetermined box and whisker plots for each group (n = 6 for METH self-administration and n = 8 for microdialysis). The 5-day METH acquisition mean response data over days were analyzed by a repeated measure five-way analysis of variance (ANOVA) for sex × genotype × pretreatment × response × day, with a repeated measure on day and response. Microdialysate samples AUC were analyzed by a four-way ANOVA for sex x genotype x pretreatment (acute vs. sub-chronic) x drug (nicotine, METH, saline) with repeated measures for drug. Significant main effects or interactions were further tested by *t*-test with the Bonferroni adjustment for multiple comparisons.

3 Results

3.1 Nicotine pretreatment enhances subsequent METH self-administration in the *CHRNA6* 3′-UTR SNP knock-in rats in a sex- and genotype-dependent manner

To determine whether the human SNP in the 3′-UTR of the *CHRNA6* gene influences nicotine-induced METH self-administration, we evaluated mean response data over time. Overall ANOVA showed a main effect for reinforced/nonreinforced response [$F_{1,72}$ = 41.86, p = 0.0001] with sex × reinforced/nonreinforced [$F_{1,72}$ = 6.58, p = 0.012], pretreatment × reinforced/nonreinforced [$F_{1,72}$ = 7.05, p = 0.0098], genotype × sex × pretreatment × reinforced/nonreinforced [$F_{1,72}$ = 4.35, p = 0.04], response × day [$F_{4,288}$ = 7.48, p = 0.0001], response × day × sex × pretreatment [$F_{4,288}$ = 2.44, p = 0.047] and day × genotype [$F_{4,288}$ = 2.47, p = 0.045] interactive effects. Since we identified interactions for every measure, we separated the data by genotype, sex, pretreatment, response, and day (Figures 2A–D). For females, nicotine-treated $\alpha6^{CC}$ and saline-treated $\alpha6^{GG}$ animals acquired and maintained METH self-administration on days 1–5 as measured by preference for reinforced over non-reinforced nose pokes (p < 0.05, Figures 2A, B). Conversely, saline-treated $\alpha6^{CC}$ female rats demonstrated preference for reinforced over nonreinforced nose poke on day 1 of METH self-administration (p = 0.023) whereas nicotine-treated $\alpha6^{GG}$ female rats acquired METH self-administration on days 4–5 (p < 0.05, Figures 2A, B). Nicotine-treated males responded for METH significantly more than saline-treated males on days 2–4, independent of genotype (p < 0.05, Figures 2C, D). Taken together, our findings demonstrate that adolescent sub-chronic nicotine exposure influences the acquisition and maintenance of METH in a sex-and genotype-dependent manner.

3.2 Nicotine pretreatment impacts drug-induced DA overflow in a sex- and genotype-dependent manner in the *CHRNA6* 3′-UTR SNP rats

The effects of nicotine- and METH-induced DA overflow in the NAc shell, were assessed in male and female adolescent (PN 31)

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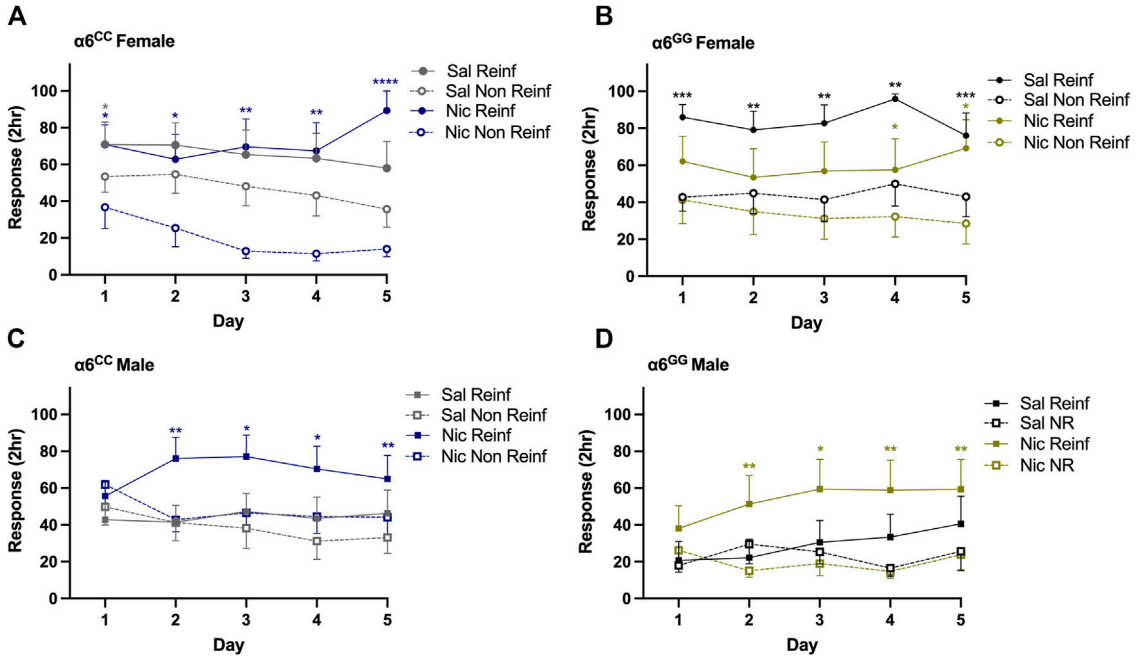

FIGURE 2
Sex- and genotype-dependent nicotine-induced METH self-administration in male and female *CHRNA6* 3′-UTR SNP rats. METH self-administration mean 2-h responses ±SEM across 5 days in **(A)** $\alpha6^{CC}$ **(B)** $\alpha6^{GG}$ females, **(C)** $\alpha6^{CC}$ and **(D)** $\alpha6^{GG}$ males. Nicotine-treated $\alpha6^{CC}$ and saline-treated $\alpha6^{GG}$ females maintain consistent METH self-administration, saline-pretreated $\alpha6^{CC}$ females initially prefer reinforced responding. Nicotine-treated males show sustained METH self-administration from days 2–5, independent of genotype. Open circles represent female data; open and closed squares represent male data. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ Reinf versus Non Reinf responses; n = 9/12/group. METH = methamphetamine, Reinf = Reinforced, Non Reinf = Non-Reinforced, Sal = Saline (grey/black), Nic = Nicotine (blue/gold).

CHRNA6 3′-UTR SNP rats. An overall ANOVA for DA overflow showed a between main effect for pretreatment [$F_{1,46} = 5.9267$, $p = 0.0189$], as well as between interactive effects for genotype x sex [$F_{1,46} = 7.3049$, $p = 0.0096$], genotype x sex x pretreatment [$F_{1,46} = 8.6829$, $p = 0.0050$]. In addition, we observed a within main effect for drug [$F_{2,92} = 12.1493$], as well as within interactive effects for drug x genotype x sex [$F_{2,92} = 7.3632$, $p = 0.0011$], drug x pretreatment [$F_{2,92} = 4.9620$, $p = 0.0090$], and drug x genotype x sex x pretreatment [$F_{1,92} = 6.2993$, $p = 0.0027$]. Thus, we evaluated these parameters separately. Nicotine and METH induced DA overflow is enhanced in $\alpha6^{CC}$ females as compared to $\alpha6^{GG}$ females ($p < 0.05$), primarily in acute condition (Figures 3A–D). METH induced DA overflow is enhanced in $\alpha6^{GG}$ males in the acute condition, but not sub-chronic condition. No variation in genotype were noted for nicotine effects in both acute and sub-chronic conditions in males. (Figures 3E–H).

We assessed DA metabolites, DOPAC and HVA, to determine their change in release in acute and sub-chronic condition. For DOPAC, a between main effect for pretreatment [$F_{1,52} = 4.6560$, $p = 0.0356$], in addition a between interactive effects for genotype x sex [$F_{1,52} = 13.2859$, $p = 0.0006$] and genotype x pretreatment [$F_{1,52} = 12.0334$, $p = 0.0011$] were observed. A 4-way multivariate ANOVA revealed drug x genotype x sex x genotype [$F_{2,104} = 4.4833$, $p = 0.0136$] interactive effects (Figures 4A–H). Data were separated by sex, genotype and pretreatment. In the acute condition, $\alpha6^{CC}$ females displayed higher DOPAC overflow for saline and nicotine for the acute condition as compared to $\alpha6^{GG}$ females ($p < 0.001$), this distinction was not evident in males (Figures 4A–F). In the sub-chronic condition, $\alpha6^{GG}$ males exhibited a greater METH-induced DOPAC overflow compared to $\alpha6^{CC}$ males ($p < 0.001$) (Figures 4G, H). HVA, an extracellular DA metabolite showed a between main effect for genotype [$F_{1,47} = 12.4396$, $p = 0.0010$], and between interactive effects for genotype x sex [$F_{1,47} = 19.5262$, $p = 0.001$], genotype x pretreatment [$F_{1,47} = 7.2047$, $p = 0.01$] and genotype x sex x pretreatment [$F_{1,47} = 4.0431$, $p = 0.0501$]. A 4-way multivariate ANOVA revealed drug x sex x genotype × pretreatment interaction [$F_{2,98} = 3.7283$, $p = 0.0313$]. Data were separated for sex, genotype and pretreatment. Nicotine and METH altered HVA overflow in saline- and nicotine-treated $\alpha6^{CC}$ females as compared to $\alpha6^{GG}$ females, these effects were not observed in males (Figures 5A–H).

4 Discussion

In the present investigation, the impact of the *CHRNA6* 3′-UTR SNP on nicotine-induced METH self-administration and the overflow of DA in the NAc shell revealed multiple interactions, indicating an intricate interrelationship involving sex, pretreatment, and genotype. Notably, nicotine-treated $\alpha6^{CC}$ and saline-treated $\alpha6^{GG}$ females exhibited METH self-administration over several days. $\alpha6^{CC}$ females when pretreated with saline (and not nicotine) initially showed a preference for reinforced versus non-reinforced responding for Day 1 METH self-administration. Nicotine-treated males, regardless of genotype, exhibited METH self-administration on days 2–5. These results highlight the sex- and genotype-dependent effects of adolescent sub-chronic nicotine

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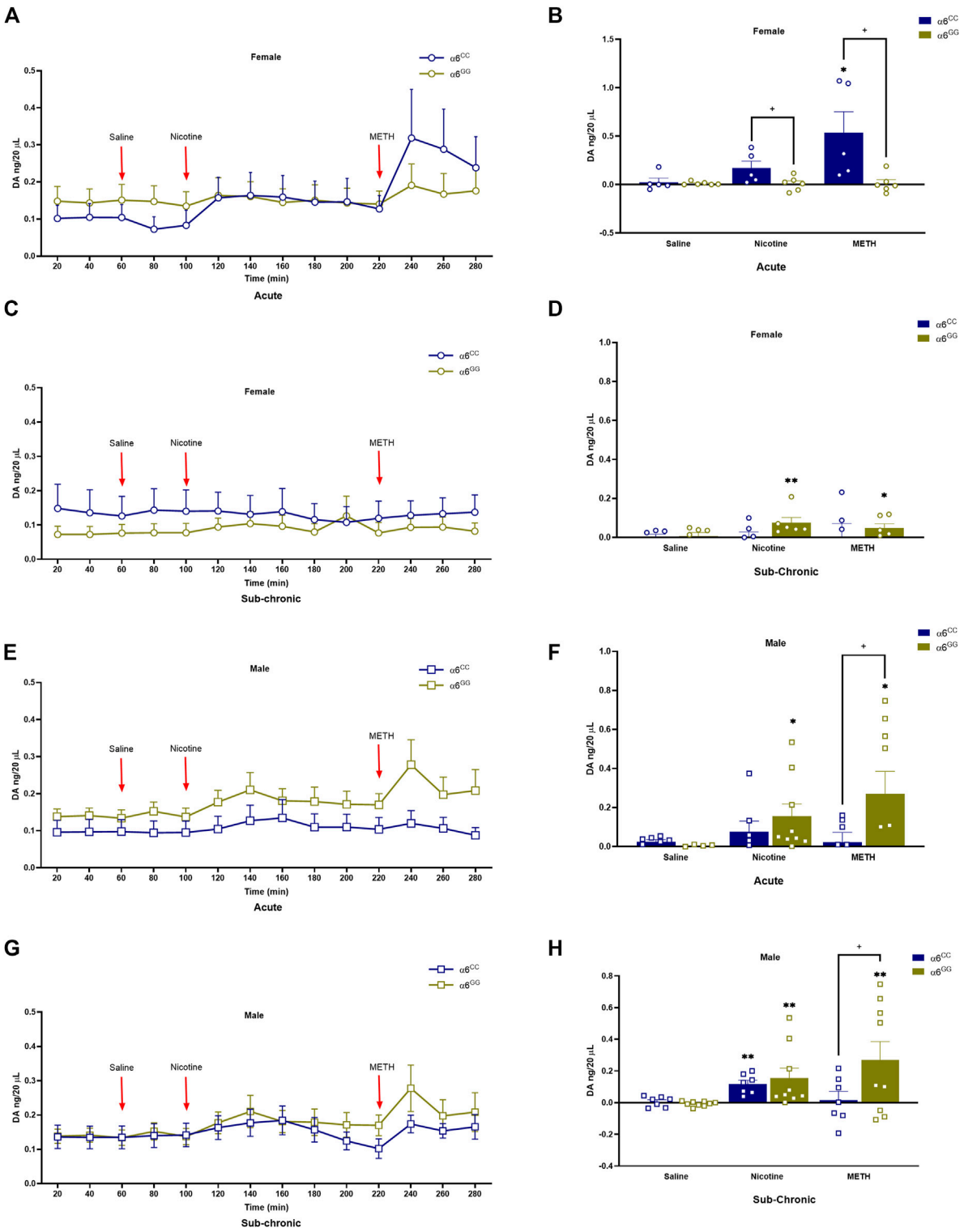

FIGURE 3
DA levels in male and female in *CHRNA6* 3′-UTR SNP Rats. The effects of nicotine- and METH-induced DA overflow in the NAc shell, were assessed in male and female adolescent (PN 31) *CHRNA6* 3′-UTR SNP rats. Female **(A)** acute timeline and **(B)** AUC **(C)** sub-chronic timeline and **(D)** AUC for saline, nicotine, and METH; Male **(E)** acute timeline and **(F)** AUC **(G)** sub-chronic timeline and **(H)** AUC for saline, nicotine, and METH. Nicotine and METH induce greater DA overflow in $\alpha6^{CC}$ females than $\alpha6^{GG}$ females in the acute condition, while $\alpha6^{GG}$ males show enhanced METH-induced DA overflow only acute condition. No significant genotype differences are noted in nicotine-induced DA overflow in males under either acute or sub-chronic conditions.$^{**}p < 0.01$ vs. saline; $^{*}p < 0.05$ vs. saline; $+p < 0.05$ $\alpha6^{CC}$ vs. $\alpha6^{GG}$. AUC = area under the curve METH = Methamphetamine N = 5-12/group.

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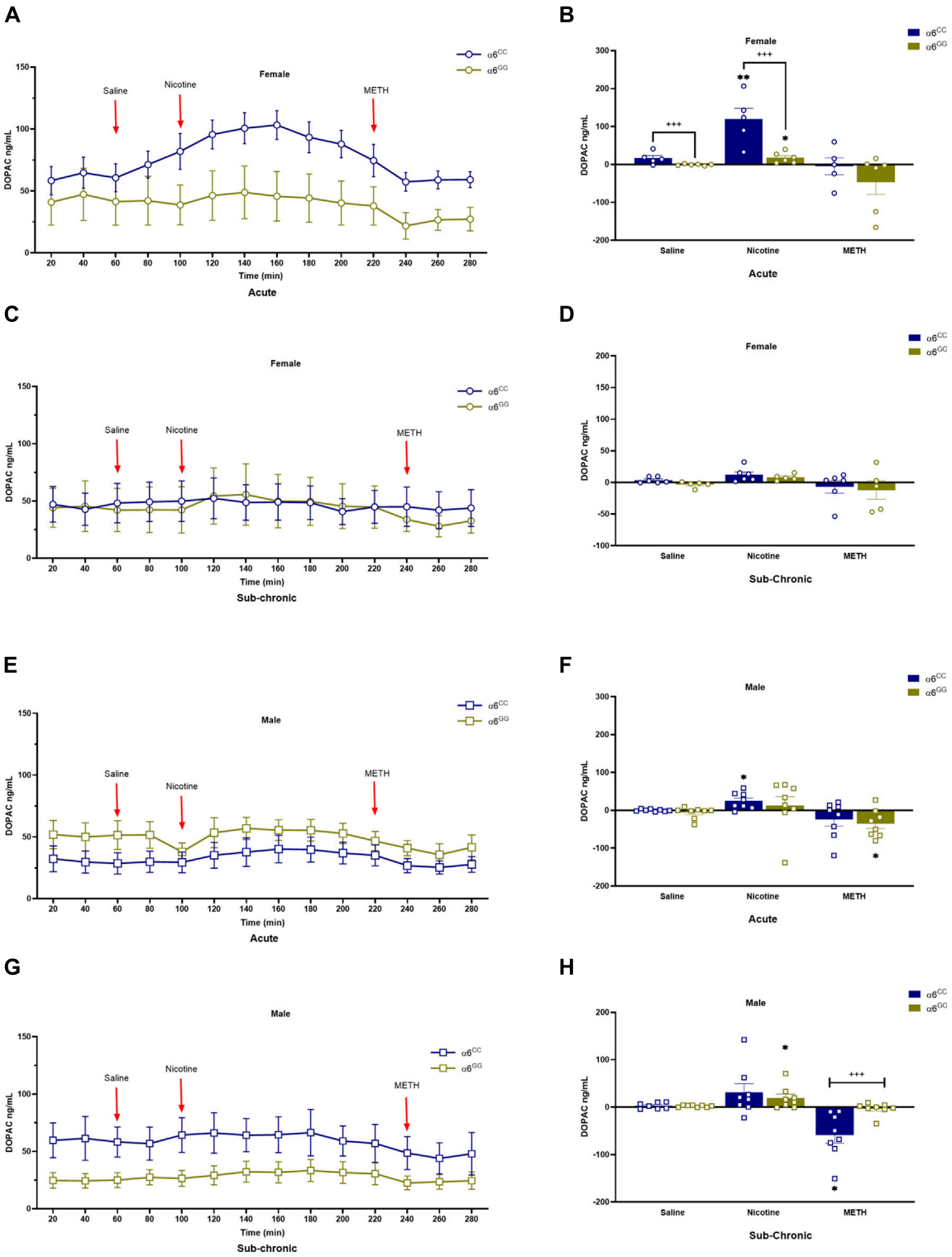

FIGURE 4
DOPAC levels in male and female in *CHRNA6* 3′-UTR SNP Rats. Female **(A)** acute timeline and **(B)** AUC **(C)** sub-chronic timeline and **(D)** AUC for saline, nicotine, and METH; Male **(E)** acute timeline and **(F)** AUC **(G)** sub-chronic timeline and **(H)** AUC for saline, nicotine, and METH. $\alpha6^{CC}$ females exhibit higher DOPAC levels than $\alpha6^{GG}$ females in response to saline and nicotine pretreatments, indicating genotype-specific DA metabolism in females. In males, this effect emerges under sub-chronic METH treatment, with $\alpha6^{GG}$ males showing higher DOPAC levels than $\alpha6^{CC}$ males. During acute conditions, $\alpha6^{CC}$ females display significantly higher DOPAC overflow for both saline and nicotine compared to $\alpha6^{GG}$ females ($p < 0.001$), a disparity not seen in males. Sub-chronic nicotine-treated $\alpha6^{GG}$ males exhibit greater METH-induced DOPAC overflow than $\alpha6^{CC}$ males. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. saline; $^{+++}p < 0.001$ $\alpha6^{CC}$ vs. $\alpha6^{GG}$. AUC = area under the curve; METH = Methamphetamine N = 5-12/group.

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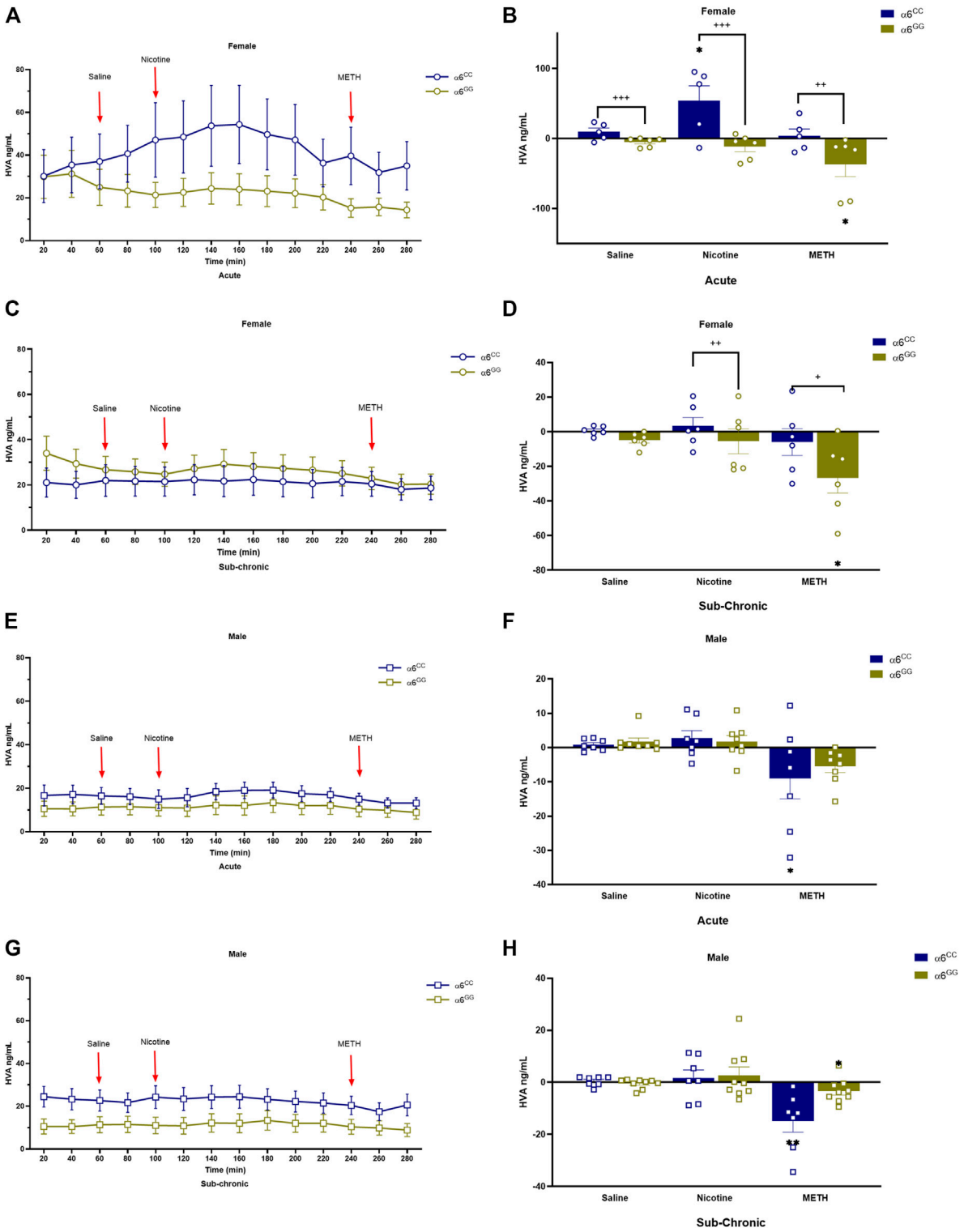

FIGURE 5
Figure 6 HVA levels in male and female in *CHRNA6* 3′-UTR SNP Rats. Female **(A)** acute timeline and **(B)** AUC **(C)** sub-chronic timeline and **(D)** AUC for Sal, Nic, and METH; Male **(E)** acute timeline and **(F)** AUC **(G)** sub-chronic timeline and **(H)** AUC for saline, nicotine, and METH. Saline- and nicotine-treated $\alpha6^{CC}$ females show altered HVA overflow compared to $\alpha6^{GG}$ females, a difference not observed in males $^{**}p < 0.01$, $^{*}p < 0.05$ vs. saline; $^{+++}p < 0.001$ $\alpha6^{CC}$ vs. $\alpha6^{GG}$. AUC = area under the curve, METH = Methamphetamine N = 5-12/group.

exposure on the acquisition and maintenance of METH self-administration in rats with the human *CHRNA6* 3′-UTR SNP. Moreover, nicotine- and METH-induced DA overflow are enhanced in $\alpha6^{CC}$ females as compared to $\alpha6^{GG}$ females primarily in acute condition whereas $\alpha6^{GG}$ males showed enhanced METH-induced DA overflow in acute, but not sub-chronic condition. No genotype differences were observed for males in either acute- or sub-chronic condition for nicotine-induced DA overflow. Taken together, these findings illustrate the complex interplay of sex, genotype, and pretreatment in influencing the effects of nicotine and METH on DA dynamics and self-administration behavior.

4.1 The influence of nicotine pre-treatment on METH self-administration in the *CHRNA6* 3′-UTR knock-in rats

Previous behavioral studies in rodents with the *CHRNA6* 3′-UTR SNP knock-in have shown that $\alpha6^{CC}$ females and $\alpha6^{GG}$ males displayed nicotine-induced enhanced locomotor and anxiolytic behavior when compared to their saline-treated counterparts (Cardenas et al., 2022). In addition, nicotine-induced locomotion and anxiolytic behavior in $\alpha6^{CC}$ females was significantly higher than $\alpha6^{GG}$ females. In contrast, nicotine-induced anxiolytic behavior in males was significantly higher in $\alpha6^{GG}$ as compared to $\alpha6^{CC}$ males (Cardenas et al., 2022). In the current study, we illustrate additional sex- and genotype-dependent adolescent nicotine exposure effects on subsequent METH self-administration. Nicotine pretreatment enhanced self-administration for METH in $\alpha6^{CC}$ female rats. In contrast, saline-, but not nicotine-treated, $\alpha6^{GG}$ females show self-administration for METH. In comparison, Sprague Dawley WT adolescent females did not exhibit increased METH responsiveness, whether or not they received nicotine pretreatment (Cardenas and Lotfipour, 2021). In male adolescents, prior nicotine exposure increased subsequent METH self-administration, its maintenance, and intake, independent of *CHRNA6* 3′-UTR SNP genotype. *CHRNA6* 3′-UTR SNP knock-in adolescent male rat data replicates adolescent WT male nicotine-induced enhancement of METH self-administration (Dao et al., 2011; Cardenas and Lotfipour, 2022). Sex and nicotine effects have been observed for METH and amphetamine self-administration as well as stimulant locomotor sensitization which may be further impacted by genetics (Collins et al., 2004; Collins and Izenwasser, 2004; Harmony et al., 2020). A potential mechanism for developmental nicotine exposure effects on subsequent METH self-administration is regulation of α6* nAChRs. A bidirectional effect of sex on transcriptional regulation of α6 nAChR mRNA expression and nicotine behaviors has been observed though protein kinase C epsilon in adult mice (Moen et al., 2021). However, we do not observe baseline or nicotine-induced α6 nAChR subunit mRNA differences in the *CHRNA6* 3′-UTR SNP knock-in rats (Cardenas et al., 2022). Whether the *CHRNA6* 3′-UTR SNP impact the translation of the α6 nAChR subunit protein needs to be evaluated in future studies. However, we are limited by lack of α6 specific antibodies available (Cardenas et al., 2020). Either way, our current findings suggest functional changes of α6 nAChRs in reward regions of the brain based on the *CHRNA6* 3′-UTR SNP genotype, sex, and drug treatment effects observed in our studies. These findings emphasize the critical role of using translational models to understand the complex interplay of sex, genotype, and pretreatment in influencing nicotine and METH effects. This approach can help in developing more targeted and effective interventions for substance use disorders. While our current studies have focused on drug reinforcement and neurotransmitter overflow, future studies could evaluate the effects of natural rewards as well in the *CHRNA6* 3′UTR SNP rodent line. Indeed, we have previously illustrated that our adolescent humanized *CHRNA6* 3′UTR SNP rats do not differ based on natural food rewards (Carreño and Lotfipour, 2023). Further, prior studies in wild type Sprague Dawley rats have illustrated that the 4-day nicotine pretreatment paradigm has minimal impacts on sucrose taking behavior, with no impacts on extinction and reinstatement during adolescents or adults, which is different than what is observed for stimulants (Mojica et al., 2014). For these reasons, we have not assessed sucrose reinforcement, extinction and reinstatement in our studies. Nevertheless, future studies could evaluate this to confirm whether similar results would be observed in the *CHRNA6* 3′UTR SNP rats. Furthermore, future studies should investigate sex-, genotype-, and nicotine-dependent α6* nicotinic receptor protein expression via binding assays in tissue of humanized *CHRNA6* 3-UTR SNP rats.

4.2 Nicotine pretreatments effects on drug-induced DA overflow are impacted in a sex- and genotype-dependent manner in *CHRNA6* 3′-UTR SNP rats

We have previously shown sex, age and genotype difference of DA tissue levels in adolescent, adult and nicotine-seeking *CHRNA6* 3′-UTR SNP knock-in rats (Carreño et al., 2024). Post-reinstatement, male $\alpha6^{GG}$ rats show suppressed DA levels in the NAc shell compared to baseline (Carreño et al., 2024). The present study emphasizes the distinctions of DA transmission in the *CHRNA6* 3′-UTR SNP knock-in adolescent female and male rats of nicotine and METH. In the acute condition, $\alpha6^{CC}$ females demonstrated heighten DA overflow by nicotine- and METH as compared to the $\alpha6^{GG}$ females. In contrast, $\alpha6^{GG}$ males exhibited greater METH-induced DA overflow when compared to $\alpha6^{CC}$ males in the acute, but not sub-chronic condition, which may suggest tolerance effects. No genotype differences were observed for nicotine-induced DA overflow in the acute or sub-chronic condition in adolescent males. While there is nicotine-induced DA overflow among adolescent $\alpha6^{CC}$ and $\alpha6^{GG}$ males, there is no genotype difference in animals pretreated with saline or nicotine. However, we observe a genotype difference for METH-induced DA overflow in adolescent male saline-pretreated $\alpha6^{CC}$ and $\alpha6^{GG}$ rats. In contrast, we observe a more pronounced genotype difference in saline pretreated $\alpha6^{CC}$ females for both nicotine- and METH-induced DA release when compared to $\alpha6^{GG}$ females. Notably, $\alpha6^{CC}$ females exhibit greater DA overflow in response to nicotine and METH compared to $\alpha6^{GG}$ females particularly in the saline pretreatment condition. These results are in accordance with a previous study reporting acute nicotine increases DA transmission while long-term nicotine exposure reduces the release of DA in the NAc (Perez et al., 2012). In addition, the significant main and interactive effects of drugs indicate that nicotine and METH differentially influence DA overflow and its metabolites depending on genotype, sex, and pretreatment.

There is a significant difference in DOPAC levels based on genotype and sex, with $\alpha6^{CC}$ females showing higher levels of DOPAC in response to saline and nicotine pretreatments compared to $\alpha6^{GG}$ females, suggesting that genotype influences

DA metabolism differently in females. In males, this genotype effect is not evident under acute conditions but becomes apparent under sub-chronic conditions with METH treatment, where $\alpha6^{GG}$ males exhibit higher DOPAC levels than $\alpha6^{CC}$ males. In the acute condition, $\alpha6^{CC}$ females exhibit higher DOPAC overflow for saline and nicotine compared to $\alpha6^{GG}$ females ($p < 0.001$), a difference not observed in males, while in the sub-chronic condition, $\alpha6^{GG}$ males show greater METH-induced DOPAC overflow compared to $\alpha6^{CC}$ males ($p < 0.001$). DOPAC levels suggest that the DAT, which is responsible for reuptake of DA from the synapse, may be influenced by genotype, sex, and drug exposure. Higher DOPAC levels in response to saline and nicotine pretreatments in $\alpha6^{CC}$ females compared to $\alpha6^{GG}$ females could indicate differences in DA reuptake or metabolism related to the DAT. Similarly, the differences observed in males under sub-chronic conditions with METH treatment suggest a potential role of the DA transporter in mediating these effects. Furthermore, the analysis of HVA levels revealed significant main and interactive effects influenced by genotype, sex, and pretreatment, as well as their interactions with drugs. Specifically, HVA overflow was altered in saline- and nicotine-treated $\alpha6^{CC}$ females compared to $\alpha6^{GG}$ females, an effect not observed in males. These results suggest that DA metabolism to DOPAC and HVA in the brain varies depending on the genetic background, sex, and prior exposure to substances like nicotine in female rats. The variations in HVA levels further suggest that the DA transporter may play a role in modulating DA metabolism in response to genetic and environmental factors. The altered HVA overflow in saline- and nicotine-pretreated $\alpha6^{CC}$ females compared to $\alpha6^{GG}$ females, specifically, could indicate differences in DA reuptake or metabolism that are influenced by the DAT. This variability in DA metabolism could potentially have implications for understanding how these factors contribute to differences in behavior or susceptibility to certain conditions. Future research that centers on the DA transporter has the potential to reveal additional insights into its function in these processes and its possible impact on behavior and vulnerability to specific conditions. Such studies could expand on these discoveries to develop a more thorough comprehension of how genetic and environmental factors influence DA metabolism and how these influences contribute to behavior and susceptibility to certain conditions.

Some potential limitations of our studies include the lack of assessment of drug-induced neurotransmitter release during METH self-administration. Given the technical difficulty of coupling *in vivo* microdialysis with METH self-administration, we have instead used a method that provides a potential readout of what our self-administration studies could provide if neurotransmitter release was simultaneously assessed during behavior. Given that contingent as compared with non-contingent delivery of drugs could differentially impact drug-induced neurotransmitter release, future studies could aim to couple these techniques with either *in vivo* microdialysis and/or fiber photometry to better understand the temporal dynamics of drug-induced neurotransmitter release and behavior. Further, our self-administration studies focus primarily on reinforcement behavior to METH at a fixed ratio one schedule of reinforcement. Thus, future studies could look at different schedules of reinforcement (e.g., fixed ratio 2-5), extended access and/or progressive ratio schedules of reinforcement, as well as extinction or reinstatement schedules of reinforcement, in order to determine how our findings impact other parameters of METH self-administration. Finally, while our studies focus on drug associated rewards, we could also evaluate whether our results impact natural rewards. While this is unlikely, given prior results related to this in wild type rats (Mojica et al., 2014) and the *CHRNA6* SNP rats (Carreño and Lotfipour, 2023; Carreño et al., 2024), it would be an important question to assess in *CHRNA6* 3′UTR SNP rats in order to confirm that drug, not natural, rewards are what are most impacted by adolescent nicotine exposure.

5 Conclusion

The study investigated the effects of adolescent nicotine exposure on METH self-administration and DA overflow in the NAc shell of *CHRNA6* 3′-UTR SNP knock-in rats. The findings revealed significant interactions between sex, genotype, and nicotine pretreatment in modulating drug-induced DA overflow, metabolites, and METH self-administration in these rats. Nicotine pretreatment enhanced discrimination for reinforced non-reinforced responding for METH self-administration in $\alpha6^{CC}$ females rats, whereas saline-treated, but not nicotine-treated $\alpha6^{GG}$ females showed this type of discrimination for METH self-administration. Additionally, in male adolescents, prior nicotine exposure increased subsequent METH self-administration, maintenance, and intake, independent of the *CHRNA6* 3′-UTR SNP genotype. Female $\alpha6^{CC}$ rats showed higher DA overflow in response to nicotine and METH compared to $\alpha6^{GG}$ females. Male $\alpha6^{GG}$ rats exhibited higher METH-induced DA overflow than $\alpha6^{CC}$ males under sub-chronic pretreatment conditions, $\alpha6^{CC}$ females had higher DOPAC levels after saline and nicotine pretreatments than $\alpha6^{GG}$ females, indicating genotype influences DA metabolism differently in females. Significant differences in HVA levels were found based on genotype, sex, and pretreatment, with altered HVA overflow in saline- and nicotine pretreated $\alpha6^{CC}$ females compared to $\alpha6^{GG}$ females. In males, this effect appeared under sub-chronic METH conditions. The study suggests that the dopamine transporter plays a crucial role in modulating DA metabolism, with implications for behavior and susceptibility to conditions based on genetic and environmental factors. Understanding the complex interactions between sex, genotype, and nicotine exposure in modulating drug-induced behaviors and DA overflow can contribute to the development of more effective prevention and intervention strategies in the future. This can pave the way for more customized combined treatments such as pharmacotherapy, psychotherapy, and education to counter or lessen addiction behavior. Unlike conventional addiction methods that often follow a one-size-fits-all model with little consideration of a person's genetics, personalized approaches can significantly improve treatment outcomes by tailoring interventions to an individual's genetic profile and specific needs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine (protocol: AUP-21-022, 4 February 2021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DC: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. AF: Data curation, Formal Analysis, Funding acquisition, Investigation, Writing–review and editing. AC: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Writing–review and editing. SL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Massimo Grilli,
University of Genoa, Italy

REVIEWED BY
Qinghe Meng,
Upstate Medical University, United States
Ana Domi,
University of Gothenburg, Sweden

*CORRESPONDENCE
Jin Zhang,
zhangjin_tobacco@163.com
Lin Li,
ifelli@xmu.edu.cn
Shun Zhou,
tobacco_shun@163.com

†These authors have contributed equally to this work and share first authorship

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The double-edged nature of nicotine: toxicities and therapeutic potentials

Yun Cao[1†], Jiali Sun[2†], Xiaofeng Wang[1], Xiaoyu Zhang[1], Huijuan Tian[1], Lingling Huang[3], Ze Huang[2], Yaping Zhang[1,4], Jin Zhang[1*], Lin Li[2*] and Shun Zhou[1,4*]

[1]Key Laboratory of Combustion & Pyrolysis Study of CNTC, China Tobacco Anhui Industrial Co., Ltd., Hefei, China, [2]The Institute of Flexible Electronics (IFE, Future Technologies), Xiamen University, Xiamen, China, [3]Department of Obstetrics, Women and Children's Hospital, School of Medicine, Xiamen University, Xiamen, China, [4]Key Laboratory of Aerosol Analysis Regulation and Biological Effects of Anhui Province, China Tobacco Anhui Industrial Co., Ltd., Hefei, China

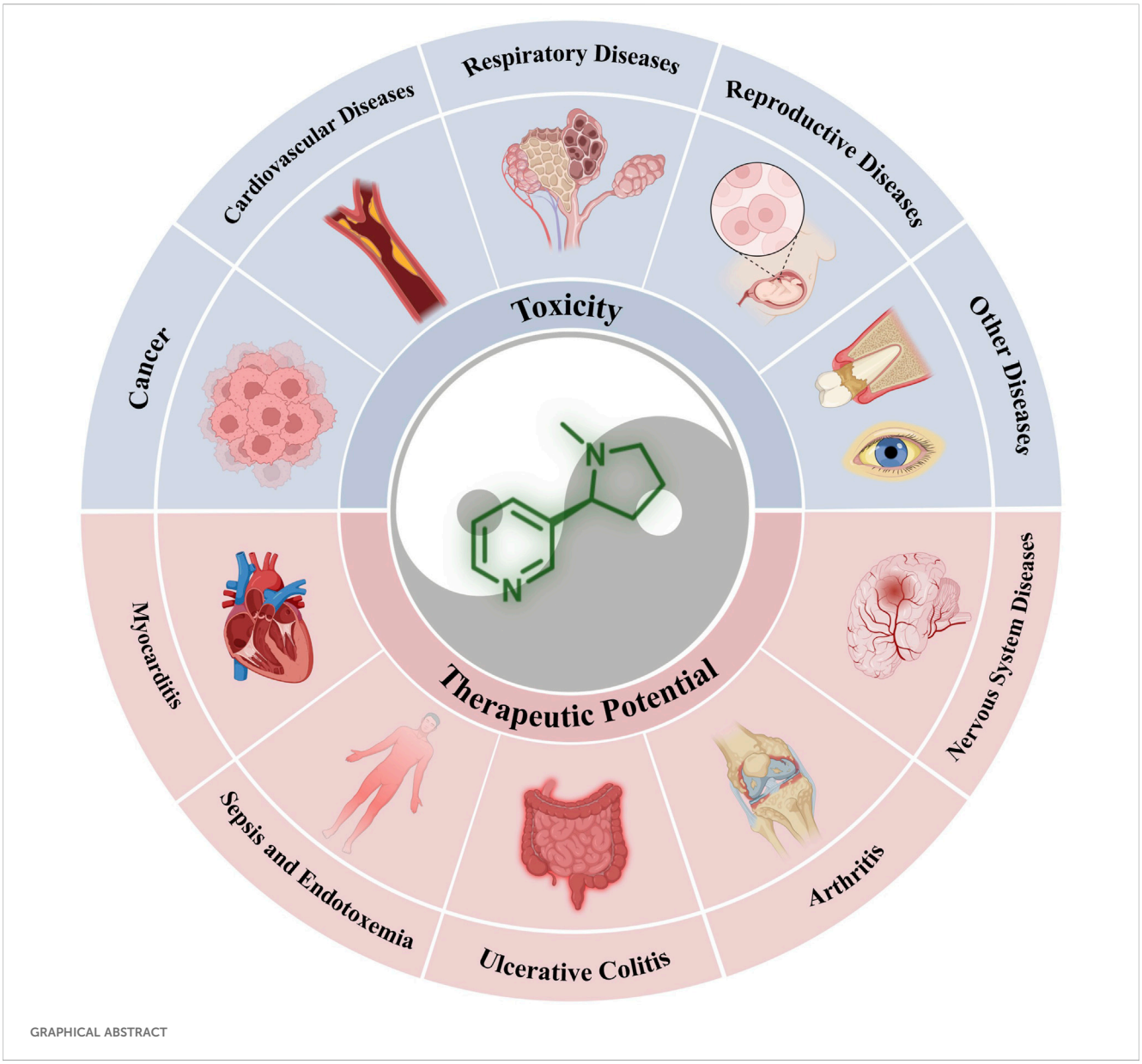
Nicotine is the primary addictive component of cigarette smoke and is associated with various smoking-related diseases. However, recent research has revealed its broader cognitive-enhancing and anti-inflammatory properties, suggesting its potential therapeutic applications in several conditions. This review aims to examine the double-edged nature of nicotine, encompassing its positive and negative effects. We provide a concise overview of the physiochemical properties and pharmacology of nicotine, including insights into nicotine receptors. Therefore, the article is divided into two main sections: toxicity and therapeutic potential. We comprehensively explored nicotine-related diseases, focusing on specific signaling pathways and the underlying mechanisms that contribute to its effects. Furthermore, we addressed the current research challenges and future development perspectives. This review aims to inspire future researchers to explore the full medical potential of nicotine, which holds significant promise for the clinical management of specific diseases.

KEYWORDS

nicotine, double-edged effect, toxicity, therapeutic potential, pharmacodynamics

1 Introduction

Nicotine, an alkaloid naturally occurring in plants of the nightshade family, is a controversial molecule within the tobacco research community (Xu and Deng, 2004). However, it has notably garnered recognition as a therapeutic agent with the potential to treat various diseases. Nicotine has a double-edged nature and significantly affects the occurrence and progression of various diseases when introduced into the human body. For instance, nicotine binds to nicotinic acetylcholine receptors (nAChRs) in cancer development. It activates multiple downstream pathways, contributing to the regulation of cancer cell proliferation, phenotypic transformation, and cell migration, thereby damaging the human body (Xu and Deng, 2004). However, nicotine can interact with nAChR on immune cells in diseases such as sepsis and endotoxemia, reducing the damage caused by inflammatory cytokines and excessive immune responses, thus playing a beneficial role (Jull et al., 2001; van Westerloo et al., 2005). Therefore, it becomes crucial to differentiate the effects of nicotine from those of other toxic substances in



cigarettes to fully explore its medicinal potential. This article objectively assesses the toxicity and therapeutic value of nicotine and offers a valuable reference for researchers.

This review focuses on the dual nature of nicotine, examining both its positive and negative characteristics. First, we offer a concise introduction to the physicochemical properties of nicotine and its crucial receptors on cell membranes. Subsequently, its toxic effects and therapeutic potential have been discussed. We categorized the diseases associated with nicotine toxicity into five groups: cancers, cardiovascular diseases, respiratory system diseases, reproductive system diseases, and other diseases. In the therapeutic section, we categorized the relevant diseases as nervous and immune system diseases, considering the wide-ranging cognitive-enhancing and anti-inflammatory properties of nicotine. Finally, we reflect on the current challenges and discuss potential future research directions.

2 Nicotine and its physiochemical properties

Nicotine (3-(1-methyl-2-pyrrolidinyl)-pyridine) chemically consists of a pyridine and pyrrolidine ring (Figure 1A). It is an important psychoactive substance and an addictive component of tobacco (Hussain et al., 2018).

Nicotine's absorption is extremely fast and unmatched by any product used in nicotine replacement therapy (Benowitz et al., 1988). As an amphipathic organic base, when the pH value of the surrounding environment is appropriate, nicotine (pKa = 7.9) can directly pass through the cell membrane to alter mitochondrial respiration and the production of cell signaling molecules (Malińska et al., 2019). For example, nicotine is ionized and hydrophilic at low pH; therefore, it cannot pass through cell membranes. However, in the blood (pH = 7.4), 31% of nicotine is non-ionized and lipophilic, making it easy to cross the cell membrane and thus affecting the cell

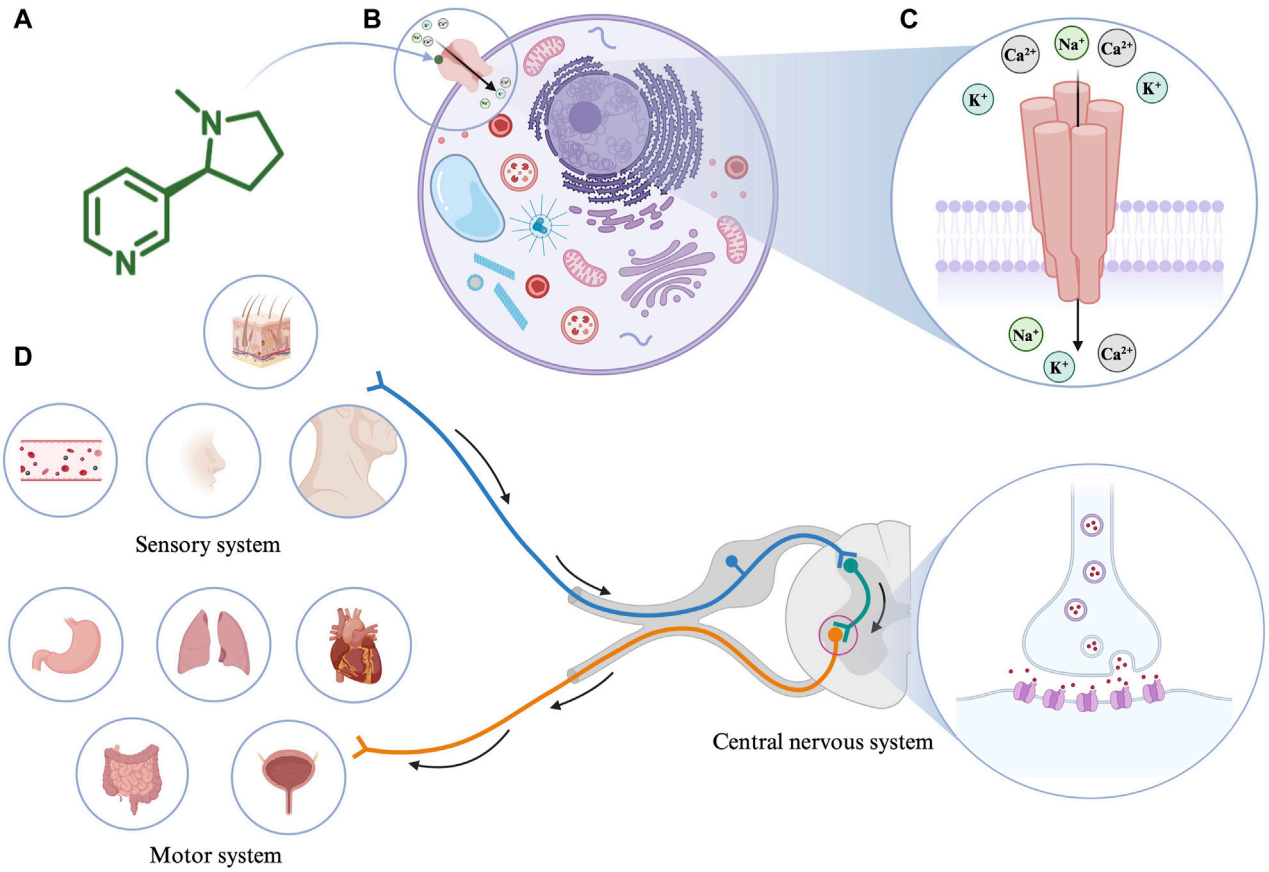


FIGURE 1
(A) The molecular structure of nicotine. **(B)** Interaction of nicotine with cells. **(C)** The structure of nicotinic acetylcholine receptor (nAChR). **(D)** The pharmacological effects of nicotine. Created with BioRender.com.

signal cascade (Yildiz, 2004). In the human body, the metabolic half-life of nicotine is 2 h. Consequently, 10%–20% of nicotine is directly excreted in the urine, and most of the remaining nicotine is metabolized by cytochrome P450 2A6 in the liver. Therefore, nicotine and its related products should be used cautiously in case of impaired liver function.

In addition, different nicotine elimination rates cause differences in plasma nicotine levels. Nicotine clearance rates can vary up to four times between individuals; therefore, different individuals will have different plasma and body nicotine levels when ingesting the same amount of nicotine. In addition, because nicotine is primarily metabolized in the liver, changes in hepatic blood flow, including exercise and meals, can affect the rate of nicotine elimination. Among these, meal consumption has been shown to affect blood nicotine levels 30 min after a meal. Therefore, pay particular attention to dietary effects when using nicotine products is essential.

In 1905, John Langley proposed that nicotine acts by binding to receptors (Langley, 1905). Notably, nAChRs, as the most critical nicotine acceptors, have been researched for a long time. As an agonist of nAChR, nicotine mediates various cellular processes by binding to nAChRs (Figure 1B). nAChRs are located in the plasma membrane and comprise five subunits that form homologous or heterologous pentamer. The subunits are organized around a central pore in the membrane (Figure 1C). They are expressed in the central and peripheral nervous systems (neuronal nAChRs), muscles (muscle nAChRs), and other tissues. Neuronal nAChRs include homopentamers composed of five identical α7, α8, or α9 subunits and heteropentamers comprising five α2-α6 or α10 subunits combined with β2-β4 subunits. Muscle nAChRs include heteropentamers composed of α1 subunits combined with β1, γ, δ, or ε subunits (Schuller, 2009). Different isoforms often play different roles in regulating physiological processes. When an agonist such as nicotine binds to nAChRs, the conformation of the receptor changes, the central ion channel opens, and cations, such as Na^+, K^+, and Ca^{2+}, enter the cell from the outside (Gopalakrishnan et al., 1995; Lindstrom et al., 1996). The precise regulatory process of nAChR keeps it throughout the evolutionary process, among which α7nAChR and α4β2nAChR occupy dominant positions in the mammalian brain (Le Novère and Changeux, 1995). A variety of receptor agonists have been developed, such as the α4β2* nAChR agonists ABT-418, ispronicline and ABT-089 as well as the α7nAChR agonist GTS-21 (Sarter et al., 2009; Mei et al., 2018). Presently, most of the biological effects of nicotine are based on the research on α7nAChR and α4β2nAChR receptors. Nicotine appears to bind with higher affinity to α4β2nAChR than to α7nAChR (Gotti et al., 1997). The higher affinity results in long-term inactivation or desensitization of

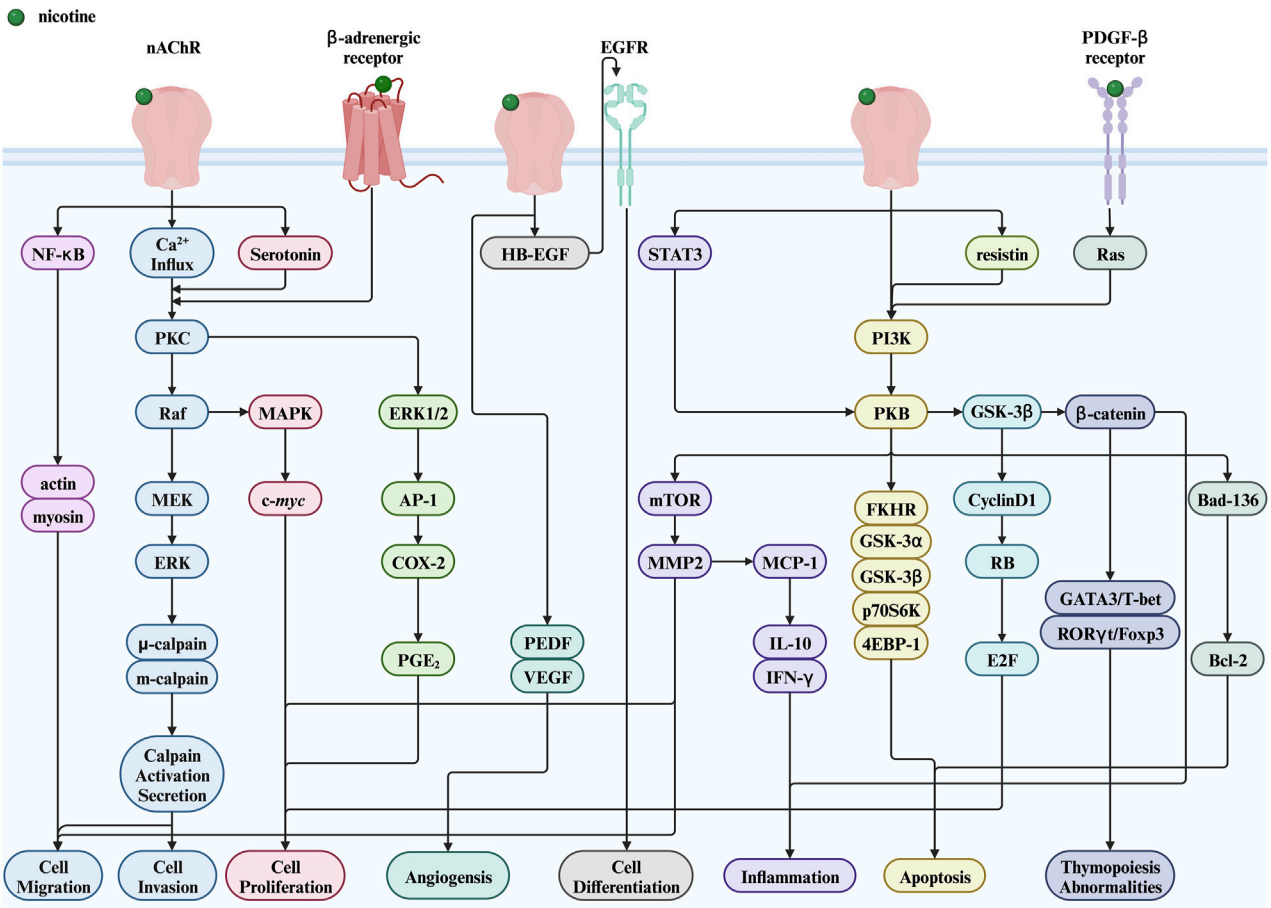


FIGURE 2
Nicotine toxicity and its related signaling pathways. Created with BioRender.com. (Abbreviations: MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol-3-kinase; NF-κB, nuclear factor kappa-B; PKB, protein kinase B; GSK-3α, glycogen synthase kinase-3 alpha; GSK-3β, glycogen synthase kinase-3 beta; p70S6K, p70 ribosomal protein S6 kinase; 4EBP-1, a binding protein for eukaryotic translation initiation factor 4E; FKHR, a member of the forkhead transcription factor family; HB-EGF, heparin binding-epidermal growth factor; EGFR, epidermal growth factor receptor; PGE2, prostaglandin E2; STAT3, signal transducer and activator of transcription 3; RB, retinoblastoma protein; PEDF, pigment epithelium-derived factor; nAChR, nicotinic acetylcholine receptor; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; COX-2, cyclooxygenase-2; mTOR, mechanistic target of rapamycin; MMP2, matrix metalloproteinase 2; MCP-1, monocyte chemoattractant protein-1; IL-10, interleukin-10; IFN-γ, interferon-γ; GSK-3β, glycogen synthase kinase-3 beta; E2F, early two factor).

the α4β2nAChR when exposed to nicotine levels comparable to those in light or heavy smokers; however, the sensitivity of the α7nAChR is barely affected (Kawai and Berg, 2001).

The pharmacological effects of nicotine on the body are mainly categorized as the motor, sensory, and central nervous system responses (Figure 1D). In the motor system, low doses of nicotine activate the sympathetic ganglion cells. For example, nicotine acts on the heart, blood vessels, bladder, and gastrointestinal tract by stimulating the paraspinal sympathetic ganglia while increasing blood glucose levels and metabolic rates. Conversely, high-dose nicotine treatment causes these effects to disappear. Furthermore, nicotine causes twitching in skeletal muscles by acting on the motor endplates. Based on animal experiments, many effects of nicotine on the sensory system are associated with chemoreceptors. Low doses of nicotine initiate various bodily reflexes, including increased breathing rate and depth, vasoconstriction, increased heart rate, and increased blood pressure, by stimulating chemoreceptors in the carotid artery and aorta. In addition, low concentrations of nicotine can stimulate skin receptors through axonal reflexes, causing sweat secretion and hair erection (Comroe, 1960). In the central nervous system, nicotine acts mainly by binding to the presynaptic nAChR receptors. For example, nicotine promotes dopamine metabolism in the mesolimbic and nigrostriatal neurons by stimulating the presynaptic nAChR in dopaminergic neurons (Balfour, 1994).

3 Toxicities: the harmful effects of nicotine on the human body

This section focuses on the toxic nature of nicotine and introduces several diseases closely associated with nicotine use. Figure 2 shows a complete map of the molecular mechanisms that regulate various genes, transcription factors, and proteins involved in the pathogenic effects of nicotine. The descriptions explain the following five different types of diseases in detail.

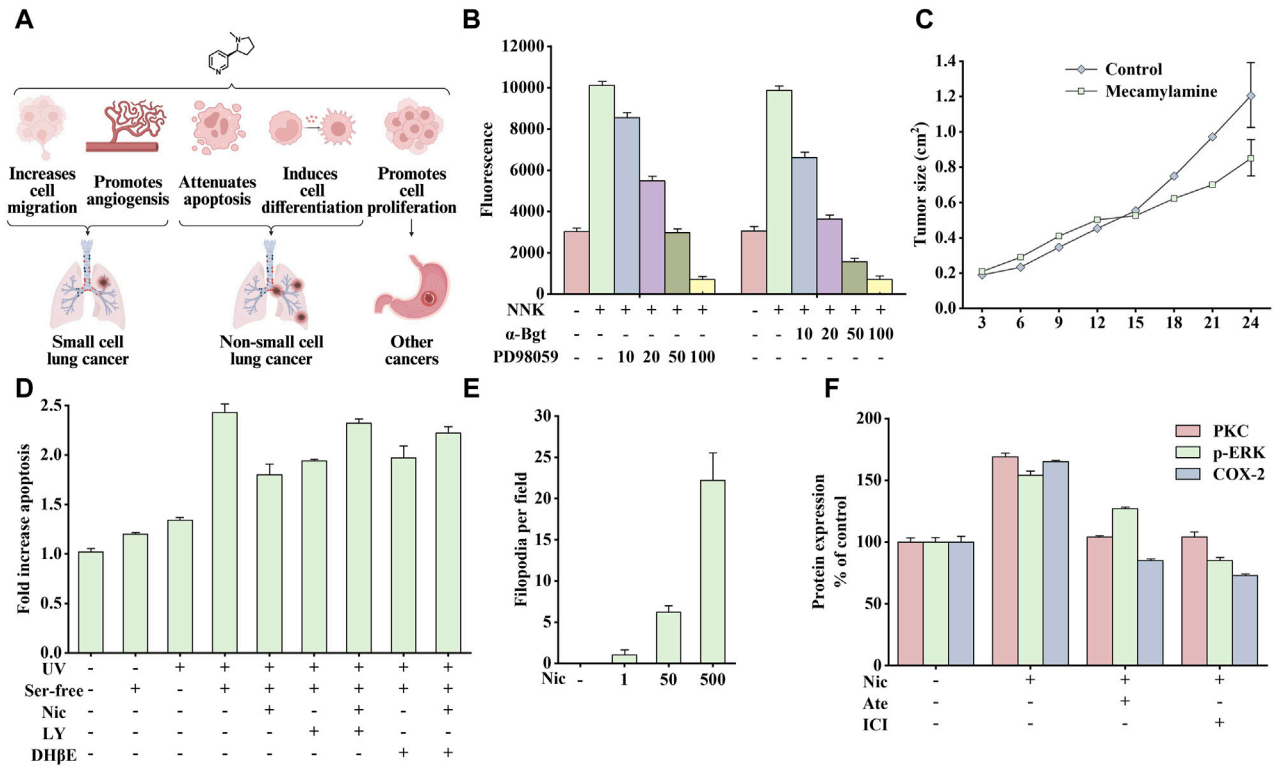


FIGURE 3
Effects of nicotine or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on cancer and related data graphs. **(A)** The role of nicotine in cancer development. Created with BioRender.com. **(B)** The migration of cells under different treatment conditions. Reprinted (adapted) with permission from Ref. (Xu and Deng, 2004). Copyright 2004, Elsevier Inc. **(C)** The tumor growth of Lewis lung tumor model under mecamylamine treatment. Reprinted (adapted) with permission from Ref. (Heeschen et al., 2002). Copyright 2002, American Society for Clinical Investigation. **(D)** The apoptosis of cells under different treatment conditions. Reprinted (adapted) with permission from Ref. (West et al., 2003). Copyright 2003, American Society for Clinical Investigation. **(E)** The phenotypical changes of cells under nicotine treatment. Reprinted (adapted) with permission from Ref. (Martinez-Garcia et al., 2008). Copyright 2008, Elsevier Inc. **(F)** Protein kinase C (PKC), p-extracellular signal-regulated kinase (ERK), and cyclooxygenase-2 (COX-2) protein expression in nicotine, atenolol, and ICI 118551 treatment. Reprinted (adapted) with permission from Ref. (Shin et al., 2007). Copyright 2006, Oxford University Press. (Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; α-Bgt, α-bungarotoxin; Ser-free, serum-free; Nic, nicotine; LY, LY294002; DHβE, dihydro-β-erythroidine; Ate, atenolol; ICI, ICI 118551).

3.1 Cancers

Cancer is one of the most widely discussed and recognized issues in biology and medicine. Its essence is the uncontrolled growth, division, and reproduction of a particular part of the cells in the human body, which destroys the body's normal physiological functions, causes organ failure, and eventually leads to death. Studies have shown that nicotine is crucial in cancer induction, as shown in Figure 3A It promotes cell proliferation, inhibits apoptosis, and promotes phenotypic transformation, cell migration, and angiogenesis (Schuller, 2009).

3.1.1 Small cell lung cancer

Small cell lung cancer (SCLC) is a typical neuroendocrine lung cancer. The influence of smoking on SCLC is particularly crucial. Notably, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nitrosation product of nicotine, has been identified as a potent carcinogen in tobacco smoke, and its affinity for α7nAChR is much higher than that of nicotine (Schuller and Orloff, 1998). Studies have shown that nicotine or NNK stimulates the release of autocrine growth factors serotonin and mammalian bombesin in a dose-dependent manner by binding to α7nAChRs (Cattaneo et al., 1993), which subsequently activates downstream protein kinase C (PKC)/Raf-1/mitogen-activated protein kinase (MAPK)/c-*Myc* mitogenic signaling pathway and promotes the growth of SCLC cell subpopulations *in vitro* (Jull et al., 2001). In addition, nicotine stimulates the release of serotonin and the proliferation of SCLC *in vitro*, which can be blocked by α-bungarotoxin (α-Bgt), indicating that α7nAChR is critical in the process of nicotine absorption, promoting the secretion of related endogenous neurotransmitters and mitosis (Codignola et al., 1994).

In addition, smoking promotes tumor production and induces cancer cell metastasis. The main members of the calpain family, μ-calpain and m-calpain, are widely expressed in SCLC cells. NNK activates ERK1 and ERK2 through α7nAChR/Ca^{2+} Influx/PKC/Raf/MAPK/ERK kinase (MEK) to phosphorylate μ-calpain and m-calpain, leading to calpain activation and secretion and promoting the migration and spread of cancer cells *in vivo*. As shown in Figure 3B, NNK significantly enhanced cell migration, which was blocked by PD98059 (inhibiting ERK1/2 activation) and α-Bgt, indicating that calpain is vital in this process (Xu and Deng, 2004). In addition, nicotine also promotes angiogenesis in various environments, such as inflammation, ischemia, and tumors, and this process is mainly mediated by α7nAChR. The stimulation of

angiogenesis by α7nAChR is completely dependent on the phosphatidylinositol-3-kinase (PI3K)/MAPK/nuclear factor kappa-B (NF-κB) pathway and partially on vascular endothelial growth factor (VEGF). Therefore, pharmacological inhibition or genetic interference with α7nAChR expression can significantly reduce ischemia- and inflammation-induced angiogenesis. Similarly, inhibiting nAChR activity by mecamylamine can reduce angiogenesis and inhibit tumor growth (Figure 3C) (Heeschen et al., 2002).

3.1.2 Non-small cell lung cancer

Non-small cell lung cancers include squamous cell carcinoma, lung adenocarcinoma, and large-cell carcinoma. Here, smoking is also a critical environmental factor. Squamous cell carcinoma mainly develops from bronchial epithelial cell lesions. In contrast, most lung adenocarcinomas arise from bronchiolar epithelial and alveolar type II cell lesions, in which both homomeric α7nAChRs and heteromeric α4β2nAChRs are present. However, exposure to nicotine concentrations comparable to those in smokers results in long-term inactivation of α4β2nAChRs, whereas α7nAChRs are largely unaffected (Kawai and Berg, 2001).

Studies on the above cells have shown that nicotine or NNK can rapidly activate PI3K/PKB (protein kinase B) to phosphorylate various downstream substrates, such as glycogen synthase kinase-3 alpha (GSK-3α), glycogen synthase kinase-3 beta (GSK-3β), p70 ribosomal protein S6 kinase (p70S6K), a binding protein for eukaryotic translation initiation factor 4E (4EBP-1), and a member of the forkhead transcription factor family (FKHR), ultimately inhibiting epithelial cell apoptosis. The above signal is mediated by nAChRs containing α3/α4 subunits (Brognard et al., 2001; West et al., 2003). As shown in Figure 3D, nicotine (10 μM) could prevent apoptosis induced by UV irradiation and serum-free culture. However, pretreatment with PI3K inhibitor LY294002 or α3/α4nAChR antagonist dihydro-β-erythroidine (DHβE) could reduce the anti-apoptotic effect of nicotine. Therefore, developing drugs that target the α3nAChR/PI3K/PKB signaling pathway may be promising for cancer treatment.

In addition, nicotine inhibits apoptosis induced by chemotherapeutic drugs. In three groups of human non-small cell lung cancers cells (A549, H23, and H1299), nicotine upregulated the X-linked inhibitor of apoptosis protein and survivin to inhibit apoptosis induced by gemcitabine, cisplatin, and taxol, which also depends on PKB regulation. When X-linked inhibitor of apoptosis protein and survivin are depleted, the inhibitory effect on apoptosis is lost (Dasgupta et al., 2006a). In another experiment, nicotine reduced serum deprivation or chemotherapy-induced apoptosis, which depends on NF-κB activation (Tsurutani et al., 2005).

In addition to inhibiting apoptosis, nicotine can promote cell proliferation through α7nAChR/β-arrestin/Src/Rb-Raf-1 pathway. Blocking either β-arrestin or Rb-Raf-1 interaction inhibits nicotine-induced proliferation (Dasgupta et al., 2006b). Consequently, nicotine can promote tumor development by inducing epithelial cell transformation. A study on normal bronchial epithelial cells showed that repeated stimulation with nicotine induces cells to differentiate into a neuron-like phenotype and secrete various neural cell adhesion molecules, thus enhancing intercellular adhesion after differentiation. Figure 3E shows the number of filamentous filopodia produced in normal human bronchial epithelial cells treated with different doses of nicotine. This process is associated with nicotine-mediated shedding of heparin binding epidermal growth factor (HB-EGF) and phosphorylation of the epidermal growth factor receptor (EGFR); while adding AG1478 (an inhibitor of EGFR tyrosine phosphorylation) can inhibit this effect (Martinez-Garcia et al., 2008).

3.1.3 Other cancers

In addition to lung cancer, the harmful effects of nicotine on other cancers cannot be disregarded. Pancreatic ductal adenocarcinoma is an aggressive cancer closely associated with smoking, in which β-adrenergic receptors may be crucial. As a β1-and β2-AR agonist, NNK promotes the proliferation of pancreatic cancer cells in a hamster model. In immortalized human pancreatic cells, NNK activates β-adrenergic receptors, causing intracellular cyclic adenosine monophosphate (cAMP) accumulation and downstream phosphorylation of mitogen-activated protein kinase ERK1/2 to promote cell proliferation, which is inhibited by beta-blockers (propranolol), adenylyl cyclase inhibitors (SQ 22536) and Erk inhibitors (PD98059) (Askari et al., 2005). In the above experiment, NNK stimulated the proliferation through the cAMP/PKA/cAMP response element binding protein signaling cascade. However, γ-aminobutyric acid inhibits the isoproterenol-induced cAMP signaling through the γ-aminobutyric acid receptor. Therefore, stimulating γ-aminobutyric acid receptors may be useful in treating pancreatic cancer (Schuller et al., 2008). Further, nicotine also promotes pancreatic cancer development by stimulating the release of epinephrine and norepinephrine from the adrenal medulla (Li and Forsberg, 1996). Studies have shown that the migration and invasiveness of colon, prostate, and breast cancers are associated with β-adrenergic activity (Masur et al., 2001; Drell et al., 2003; Palm et al., 2006).

A study using HT-29 cells identified the mechanism that nicotine promotes the growth of colon cancer cells. Nicotine increases the expression of catecholamine synthases tyrosine hydroxylase (TH), dopamine-β-hydroxylase, and phenylethanolamine N-methyltransferase in a dose-dependent manner, thereby promoting adrenaline synthesis. Adrenaline stimulates colon cancer cell proliferation by acting on adrenoceptors, and α7nAChRs are involved in this process. Methyllycaconitinee, an α7nAChR antagonist, reversed the stimulatory effects of nicotine on cell proliferation, TH and dopamine-β-hydroxylase expression, and adrenaline production (Wong et al., 2007).

β-adrenergic receptors were also crucial in researching gastric cancer development with nicotine use. In Shin's study, nicotine stimulated β-adrenergic receptors and subsequently activated the downstream PKC/ERK1/2/activator protein-1 (AP-1)/cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) signaling cascade to promote gastric cancer cell growth and proliferation (Shin et al., 2007). As shown in Figure 3F, atenolol and ICI 118551 (β1 and β2-AR antagonists, respectively) reversed PKC expression, ERK1/2 phosphorylation, and COX-2 upregulation induced by nicotine. In another study, by orthotopically transplanting gastric cancer cells into the stomach wall of nude mice, the tumor area was found to be significantly larger than that in the control group after

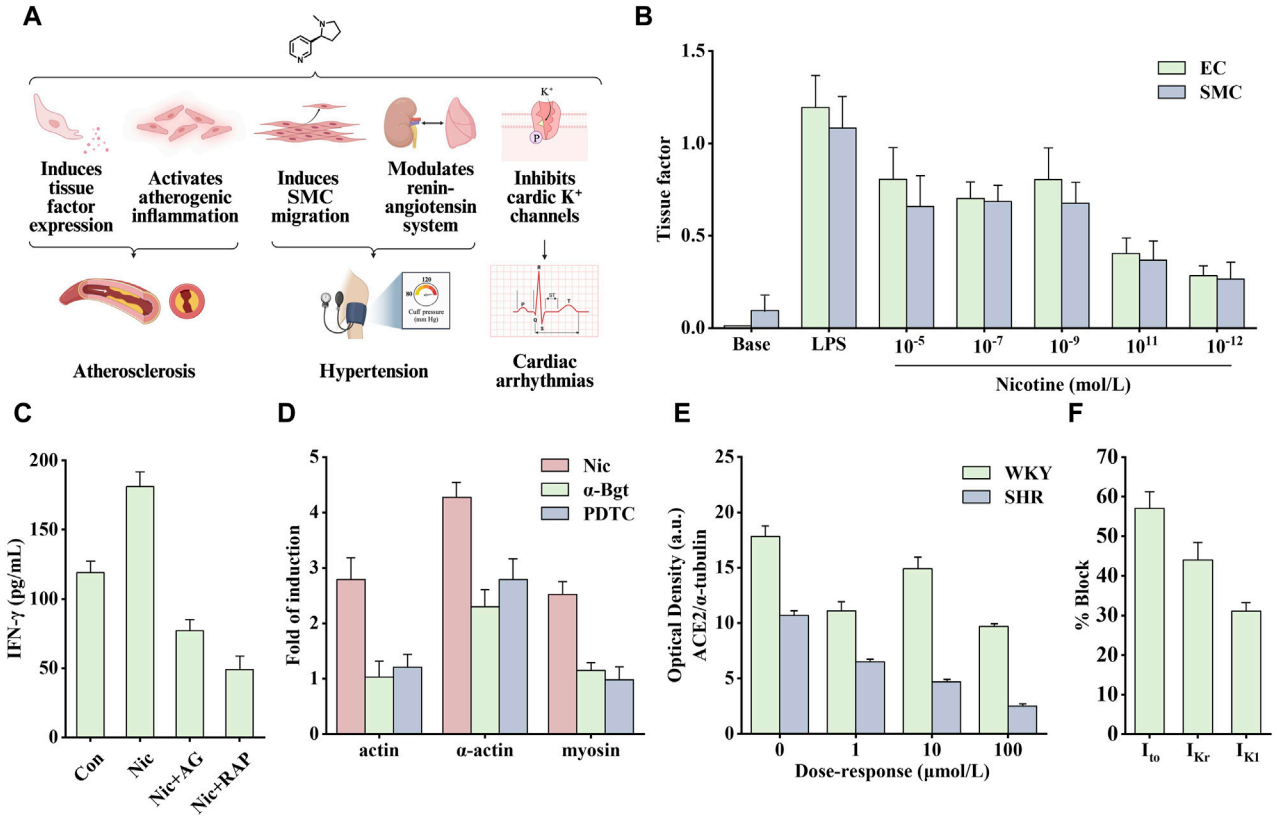


FIGURE 4
Effects of nicotine on cardiovascular diseases and related data graphs. **(A)** The effects of nicotine on cardiovascular diseases. Created with BioRender.com. **(B)** The tissue factor in endothelial cells (EC) and smooth muscle cells (SMC) under lipopolysaccharide (LPS) or nicotine treatment. Reprinted (adapted) with permission from Ref. (Cirillo et al., 2006). Copyright 2006, Elsevier Inc. **(C)** Interferon-γ (IFN-γ) released by cells under nicotine, AG490, and rapamycin treatment. Reprinted (adapted) with permission from Ref. (Xu et al., 2019). Copyright 2006, Xu et al. **(D)** Cell cytoskeletal protein expression under nicotine, α-bungarotoxin, and PDTC treatment. Reprinted (adapted) with permission from Ref. (Wang et al., 2013). Copyright 2012, Elsevier Ltd. **(E)** Angiotensin-converting enzyme 2 (ACE2) protein expression in spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rats under nicotine treatment. Reprinted (adapted) with permission from Ref. (Ferrari et al., 2007). Copyright 2007, Springer Nature. **(F)** K+ currents in ventricular myocytes under nicotine treatment. Reprinted (adapted) with permission from Ref. (Wang et al., 1999). Copyright 1999, Elsevier Inc. (Abbreviations: SMCs, smooth muscle cells; Nic, nicotine; AG, AG490; RAP, rapamycin; α-Bgt, α-bungarotoxin; Ito, transient outward K+ current; IKr, delayed rectifier K+ current; IK1, inward rectifier K+ current).

3 months of nicotine treatment; which was associated with the ERK/COX-2/VEGF signaling pathway (Shin et al., 2004).

3.2 Cardiovascular diseases

Presently, cardiovascular diseases have become the leading cause of death in developed and developing countries, and the global death toll increased from 12.1 million in 1990 to 18.6 million in 2019 (Roth et al., 2020). As the population ages, the death toll is also expected to rise.

Cardiovascular diseases affect the heart and blood vessels (arteries, veins, and capillaries). They can be divided into acute and chronic diseases and are generally associated with arteriosclerosis. Notably, several causes of cardiovascular disease include poor eating habits, lack of exercise, smoking, and drinking. Among them, smoking is considered the main preventable factor (Roth et al., 2020). As the main particle phase component of smoke produced by tobacco combustion (Centner et al., 2020), the pathogenic effect of nicotine has also been extensively studied (Figure 4A).

3.2.1 Cardiovascular diseases

Atherosclerosis mainly occurs in the coronary arteries, cerebral arteries, and aorta and is the pathological basis of many cardiovascular diseases. After the endothelial cells are damaged, they capture adhesion molecules, attract lymphocytes and monocytes to infiltrate the arterial wall and induce inflammation. White blood cells, fat, and cholesterol floating in the blood vessels are then deposited, resulting in plaque formation, stiffness, and thickening of the arterial inner wall (Tabas et al., 2015). Once the plaque ruptures, platelets accumulate at the top, plugging the already narrowed lumen and restricting oxygen and nutrient uptake by the associated cells. If this process occurs in the blood vessels of the heart, it causes myocardial infarction and death of heart muscle cells that do not receive nutrients. Stroke occurs when an embolism blocks the blood flow to the brain. During this process, there is increased proliferation and migration of vascular smooth muscle

cells (VSMCs). Nicotine is involved in almost all stages of atherosclerosis occurrence and development.

Nicotine promotes the secretion of various cytokines, such as basic fibroblast growth factor, transforming growth factor beta, VEGF, and platelet-derived growth factor (PDGF), stimulating the vascular growth process (Grozio et al., 2007). A study on VSMCs found that nicotine modulates aortic production of basic fibroblast growth factor and transforming growth factor beta, which is critical in developing neointimal fibroplasia (Cucina et al., 2000). In addition, Carty et al. found that in human smooth muscle cells (SMCs), nicotine and its metabolite cotinine promoted the production and secretion of basic fibroblast growth factor and upregulated the expression of several matrix metalloproteinases (MMPs), such as collagenase-1, stromelysin-1 and gelatinase A, which are crucial in cell migration (Carty et al., 1996). When exposed to nicotine, the expression of tissue factor, a small molecular glycoprotein involved in the regulation of blood coagulation, hemostasis, and thrombus formation, is significantly increased in endothelial cells (ECs) and SMCs (Figure 4B). This process is regulated by the transcription factor NF-κB (Cirillo et al., 2006). Tissue factor forms a tissue factor-Vlla complex with coagulation factor Vlla to activate factors IX and X to generate thrombin (Toschi et al., 1997). To sum up, nicotine regulates the secretion of various growth factors and promotes the development of atherosclerosis.

Therefore, the role of inflammation in disease development should not be underestimated. Nicotine binds to nAChR to promote the opening of ion channels and regulate intracellular Na^+, K^+, Ca^{2+}, and other ion concentrations, thereby activating different signaling cascade pathways and promoting atherosclerotic inflammation. Among them, the signal transducer and activator of transcription 3 (STAT3) is an important transcription factor involved in regulating various extracellular signals associated with cell growth and inflammation. After STAT3 activation, it enters the nucleus to promote the transcription of target genes. Studies have shown that there is a direct interaction between STAT3 and α1nAChR. After knocking out α1nAChR, the upregulation of p-STAT3, p-PKB, and p-mechanistic target of rapamycin (mTOR) induced by nicotine *in vitro* was significantly reduced, whereas the effect was opposite when α1nAChR was overexpressed (Xu et al., 2019). Staining for activated STAT3 in sections of human atherosclerotic lesions showed that the nuclei of cells in areas of inflammation stained strongly, in contrast to those of cells in areas with little or no inflammation. Endothelial STAT3 knock-out mice have smaller atherosclerotic lesions, and this inhibition may involve the PKB/mTOR/MMP2 signaling pathway. As shown in Figure 4C, nicotine upregulated levels of the pro-inflammatory factor interferon-γ (IFN-γ), which was inhibited by AG490 (STAT3 inhibitor) and rapamycin (mTOR inhibitor) (Gharavi et al., 2007; Xu et al., 2019). In another study on VSMCs, nicotine upregulated the production of reactive oxygen species (ROS). It activated the pattern recognition NOD-like receptor thermal protein domain associated protein 3 (NLRP3). NLRP3 activation leads to elevated C-reactive protein levels, an inflammatory cytokine that induces vascular inflammation. In addition, pro-caspase-1 formed inflammasomes by combining with NLRP3, simultaneously releasing inflammatory factors interleukin (IL)-18 and IL-1β, thus aggravating the inflammatory response (Hecker et al., 2015; Yao et al., 2019). By treating human aortic endothelial cells with nicotine, the above signaling pathway was found to mediate pyroptosis, and the nicotine-NLRP3-apoptosis-associated speck-like protein containing CARD (ASC)-pyroptosis pathway was activated by ROS (Wu et al., 2018).

Endothelial dysfunction and altered vascular smooth muscle function are involved in the progression of atherosclerosis. Reduced NO production is a typical feature of endothelial dysfunction. Nicotine causes significant structural and functional changes in the aorta. The combination of nicotine and nAChR promoted the activation of ERK1/2 and activated NF-κB, which stimulated the synthesis of intercellular adhesion molecule one and vascular cell adhesion molecule 1 (Egleton et al., 2009). In addition, nicotine can also stimulate macrophages to secrete tumor necrosis factor alpha (TNF-α) and IL-1β, increase the expression of human EC adhesion molecules, and lead to increased ahesion of monocytes to human umbilical vein ECs (Wang Y. et al., 2004), which may be one of the causes of EC disorders. In addition, Wang et al. found that nicotine-induced autophagy promoted VSMC phenotypic transformation and is partially mediated through the nAChRs/ROS/NF-κB signaling pathway (Wang Y. et al., 2004). NF-κB was also involved in the upregulation of nicotine-induced cytoskeletal proteins through α7nAChR. As shown in Figure 4D, the α7nAChR inhibitor α-Bgt and the NF-κB inhibitor PDTC significantly inhibited the expression of nicotine-induced cytoskeletal proteins, including actin, α-actin, and myosin (Wang et al., 2013). Another study showed that nicotine activated the death-associated protein kinase 3/AMP-activated protein kinase (AMPK) signaling cascade through receptors in VSMCs, thereby inducing endoplasmic reticulum stress-related protein expression and VSMC differentiation (Li K. X. et al., 2019).

3.2.2 Hypertension and cardiac arrhythmias

In addition to atherosclerosis, it seems that nicotine is more than just a bystander of hypertension or arrhythmias. Nicotine alters vascular tone by modulating the release of NO, bradykinin, and leukotrienes (Kuhlmann et al., 2005). In addition, nicotine acts as a sympathomimetic agent to increase blood pressure by stimulating the release of catecholamines (Benowitz, 2001). Angiotensin-converting enzyme 2 (ACE2) can convert angiotensin II to angiotensin (1–7) to promote vasodilation and inhibit proliferation. Nicotine inhibits this process by reducing ACE2 expression, indirectly leading to increased blood pressure. As shown in Figure 4E, nicotine dose-dependently reduced the optical density of ACE2 protein in cultured neurons of spontaneously hypertensive and Wistar Kyoto rats (Ferrari et al., 2007).

Arrhythmias include atrial and fibrillation, ventricular fibrillation, and tachycardia. Nicotine can induce arrhythmias by increasing the automaticity of the sinoatrial node and accelerating conduction through the atrioventricular node (Benowitz and Gourlay, 1997), which may involve multiple factors. Studies have shown that nicotine increases catecholamine release by stimulating β1 receptors and stimulates sympathetic nerve activity to increase heart rate and blood pressure through direct peripheral and centrally mediated effects (Shinozaki et al., 2008). In addition, the increase in heart rate may be associated with a decreased vagal tone (Benowitz and Gourlay, 1997). The cellular mechanism involved may be

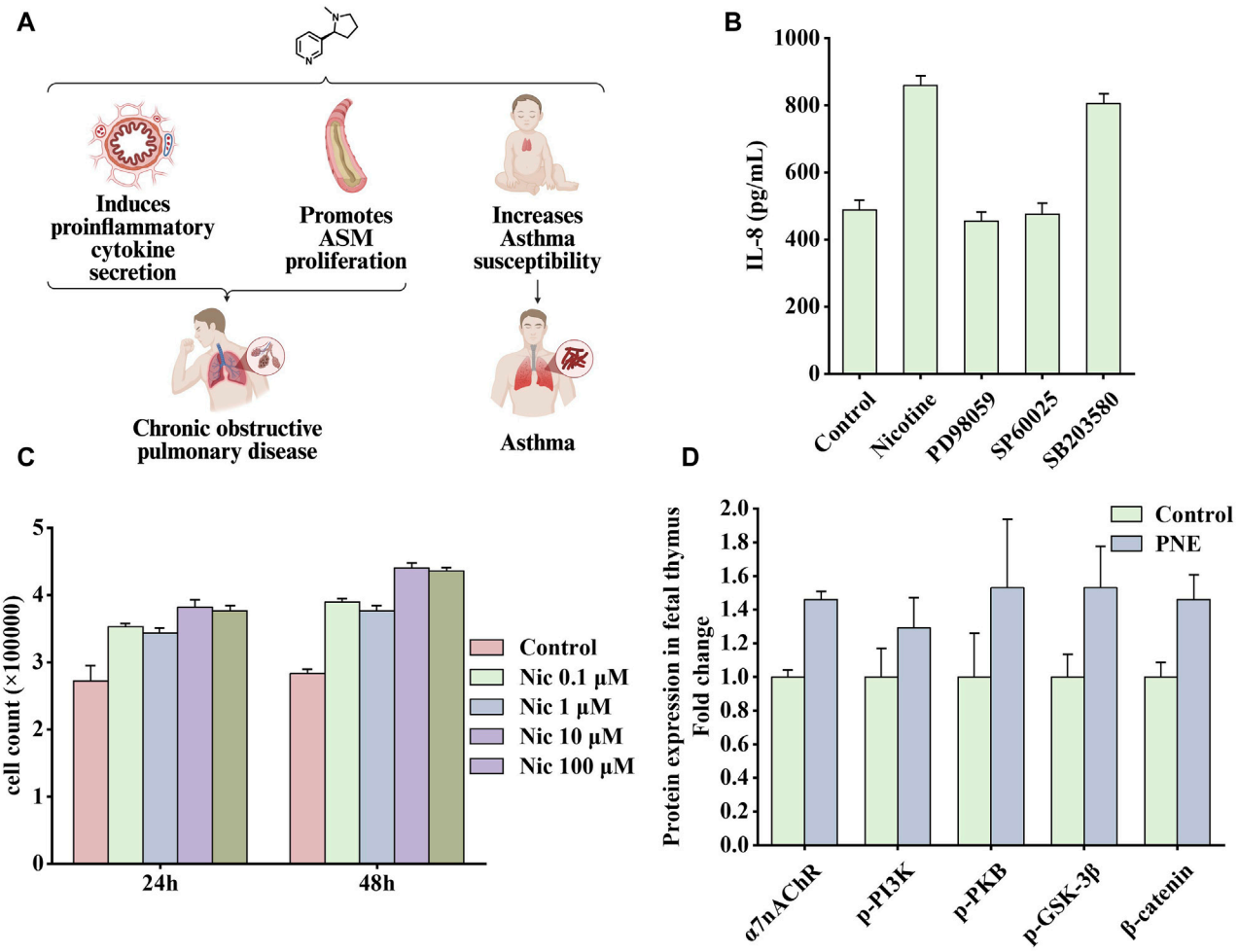


FIGURE 5
Effects of nicotine on respiratory diseases and related data graphs. **(A)** The effects of nicotine on respiratory diseases. Created with BioRender.com. **(B)** Interleukin-8 (IL-8) protein concentration in cells under nicotine, PD98059, SP60025, and SB203580 treatment. Reprinted (adapted) with permission from Ref. (Tsai et al., 2006). Copyright, 2006, Mary Ann Liebert, Inc. **(C)** Cell numbers under nicotine treatment. Reprinted (adapted) with permission from (He et al., 2014). Copyright, 2014, Public Library Science. **(D)** Effects of prenatal nicotine exposure (PNE) on protein expression of α7 nicotinic acetylcholine receptor (α7nAChR), p-phosphatidylinositol-3-kinase (PI3K), p-protein kinase B (PKB), p-glycogen synthase kinase three beta (GSK-3β), and β-catenin during fetal thymopoiesis. Reprinted (adapted) with permission from Ref. (Wen et al., 2022). Copyright, 2022, Elsevier Inc. (Abbreviations: ASM, airway smooth muscle; Nic, nicotine).

related to the function of nicotine in prolonging action potential and depolarizing membrane potential. As a non-specific blocker of K^+ channel, nicotine directly inhibits cardiac K^+ channels to block various types of K^+ currents, including transient outward K^+ current (Ito), delayed rectifier K^+ current (I_{Kr}), and inward rectifier K^+ current (I_{K1}) (Figure 4F) (Wang et al., 1999). Therefore, nicotine likely causes arrhythmias and heart problems by inhibiting cardiac K^+ channels.

3.3 Respiratory diseases

Respiratory diseases are common and frequently occurring conditions. The primary lesions are located in the trachea, bronchi, lungs, and chest cavity. Patients with mild symptoms often experience cough, chest pain, and altered breathing. Patients with severe symptoms experience dyspnea, hypoxia, and even death due to respiratory failure. Two diseases are highlighted in this summary: chronic obstructive pulmonary disease (COPD) and tuberculosis, in which smoking is an important pathogenic environmental factor. Figure 5A shows the effects of nicotine on these diseases.

3.3.1 Chronic obstructive pulmonary disease

COPD is a classic lung disease characterized by persistent airflow limitation. It is often associated with an increased chronic inflammatory response of the airways and lungs to noxious particles or gases, ultimately leading to accelerated aging of the lungs. It includes chronic obstructive bronchitis (narrowing of the small airways) and emphysema (damage to the alveoli). In the lungs of healthy individuals, alveoli attach to the small airways to keep them open, whereas in patients with COPD, the peripheral wall of the bronchioles is thickened, small airways are narrowed, alveoli are damaged, and mucus production increases (Christenson et al.,

2022). Smoking is the main risk factor for developing COPD. Therefore, understanding its physiological mechanism is essential for the treatment and prevention of the disease.

Smoking causes small airway remodeling. In a guinea pig model, exposure to cigarette smoke produced symptoms similar to small airway remodeling, such as increased airway resistance, decreased airflow, and air trapping (Wright et al., 2007). A study showed that nicotine promoted the proliferation of human airway smooth muscle cells, and this process was associated with the α5nAChR-mediated Ca^{2+} influx dependent on transient receptor potential canonicals (TRPCs) channels. Knocking out the TRPC3 gene attenuated nicotine-induced $Ca2^{+}$ influx and cell proliferation effects (Jiang et al., 2019). Another study explored the impact of nicotine addiction on the expression of 1800 genes using microarray bioinformatics analysis. Through integration, several overexpressed genes were found to be involved in the MAPK pathway, among which ERK1/2 and c-Jun N-terminal kinase (JNK) were more likely to function (Tsai et al., 2006). In addition, the MAPK pathway mediates IL-8 production. As an important pro-inflammatory factor, IL-8 may be associated with lung inflammation and tumorigenesis. As shown in Figure 5B, nicotine increased the synthesis of IL-8, which was inhibited by PD98059 (ERK 1/2 inhibitor) or SP600125 (JNK inhibitor); however, the inhibitor of ribonuclease P protein subunit p38 (p38) SB203580 had little effect on this process. Another characteristic of COPD is increased airway smooth muscle mass, in which PKB plays an important role. PKB, also known as protein kinase B, as an essential signaling hub, participates in various signaling pathways related to cell proliferation, apoptosis, and migration (Liang and Slingerland, 2003). In a vitro rat airway smooth muscle cell model, nicotine bound to nAChRs and subsequently activated the PI3K/PKB/GSK-3β/cyclin D1/retinoblastoma protein (RB)/early two factor (E2F) signaling cascade, ultimately leading to a significant increase in DNA synthesis and cell number. As shown in Figure 5C, nicotine treatment at concentrations of 0.1, 1, 10, and 100 μM significantly increased the number of cells (He et al., 2014). In addition, studies have shown that patients with COPD have a significantly increased risk of developing SCLC (Purdue et al., 2007). In this experiment, mice with COPD were more prone to develop neuroendocrine tumors after exposure to nicotine, whereas healthy mice in the control group had no tumors (Schuller et al., 1995).

3.3.2 Asthma

Asthma is an inflammatory disease of the lungs characterized by hyperresponsiveness and reversible obstruction of the large and small conducting airways. Globally, more than 300 million individuals are affected by asthma (Holgate et al., 2015).

In several animal studies, Holgate et al. found that prenatal exposure to nicotine increased the probability of asthma in the offspring. This may be associated with the downregulation of homeostatic lung mesenchymal peroxisome proliferator-activated receptor γ (PPARγ) signaling, which mediates paracrine communication of specific molecules between the alveolar epithelium and interstitium. Nicotine treatment in pregnant rats showed that PPARγ was downregulated, and the expression of mesenchymal markers of tracheal contraction response and airway contraction was significantly increased. However, the PPARγ agonist, rosiglitazone, could effectively block the above changes (Holgate et al., 2015). In addition, nicotine promotes the trans differentiation of alveolar interstitial fibroblasts to myofibroblasts. Alveolar interstitial fibroblasts convert to a phenotype detrimental to alveolar homeostasis, resulting in damage to developing alveoli and lung injury. However, upregulation of PPARγ can block this transformation (Krebs et al., 2010). The above experiments suggest that PPARγ may serve as a novel target for developing asthma drugs.

Another study identified signaling pathways associated with asthma susceptibility in mice (Wen et al., 2022). By administering 3 mg/kg/day of nicotine to establish a prenatal nicotine exposure (PNE) mouse model, this study showed that PNE impaired fetal thymus and postnatal $CD4^{+}$ T cell development. Figure 5D shows that during fetal thymogenesis, nicotine treatment upregulated the expression of α7nAChR and subsequently increased the phosphorylation level of downstream PI3K/PKB/GSK-3β signaling molecules, which inhibited the process in which GSK3β degraded β-catenin and increased the levels of β-catenin. In addition, it seems that increased β-catenin level causes the fetal thymus to shape a Th2/Th17 bias-generating gene expression pattern during generation, characterized by higher expression levels of GATA3/T-bet and RORγt/Foxp3, thus leading to thymopoiesis abnormalities.

3.4 Reproductive system diseases

Studies have shown that tobacco exposure before and during pregnancy can lead to adverse outcomes, such as reduced fertility and increased maternal, fetal, and infant morbidity and mortality, further aggravating the adverse effects on the mother and offspring, in which nicotine, the main ingredient of tobacco, seems to play an important role (National Center for Chronic Disease et al., 2014). Figure 6A shows some influence of nicotine on preterm birth and sudden infant death syndrome.

3.4.1 Preterm birth

Several studies have suggested that nicotine is contributed to the increased risk of preterm birth associated with smoking. Schuller et al. found that smoking increased the risk of preterm birth due to premature rupture of membranes and bleeding in late pregnancy (Kyrklund-Blomberg et al., 2005). Günther et al. found that all smoking groups had the higher preterm birth rate compared to nonsmokers and the majority of preterm infants were male except for very heavy smokers (≥22 cigarettes/day) (Figure 6B). Women who used snus and continued smoking had an increased risk of preterm birth compared with women who had quit smoking, as tested using a multiple logistic regression model. The nicotine content in snus is similar to that absorbed from cigarettes but contains no other ingredients, clarifying the role of nicotine in inducing preterm birth (Baba et al., 2012). A subsequent study recommended a reduction in nicotine use during pregnancy and showed that snus was associated with preterm birth. The study also demonstrated that smoking cessation before antenatal appointments was not associated with an increased risk of preterm birth (Dahlin et al., 2016).

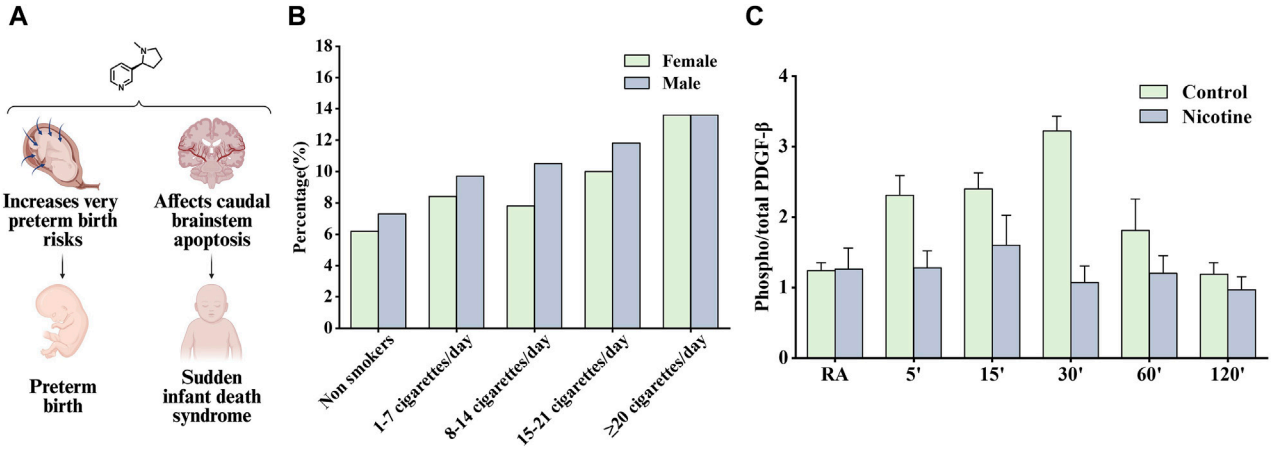


FIGURE 6
Effects of nicotine on reproductive diseases and related data graphs. **(A)** The effects of nicotine on reproductive system diseases. Created with BioRender.com. **(B)** Impact of fetal gender in combination with maternal smoking on preterm birth. Reprinted (adapted) with permission from Ref. (Günther et al., 2021). Copyright, 2020, Cambridge University Press. **(C)** Effects of prenatal nicotine exposure (PNE) on the phosphorylation of platelet-derived growth factor (PDGF)-β receptor during hypoxia. Reprinted (adapted) with permission from Ref (Simakajornboon et al., 2010). Copyright, 2010, Elsevier Ireland Ltd. (Abbreviations: RA, room air).

3.4.2 Sudden infant death syndrome

Sudden infant death syndrome is an important cause of death in infants aged 1 month to 1 year; however, its etiology is unknown and may be associated with impaired cardiorespiratory control and arousal responses, but perinatal exposure to cigarette smoke has been listed as a risk factor (Mitchell and Milerad, 2006). Studies have shown that nicotine can accumulate in breast milk, placenta, and amniotic fluid, continue to have adverse effects on fetuses and newborns, and may involve the interaction of cellular mechanisms such as oxidative stress, inflammation, and endoplasmic reticulum stress (Wong et al., 2015). Infants who died of sudden infant death syndrome often experienced severe bradycardia and apnea, and Huang et al. considered the above responses as cardiorespiratory hyperresponses to hypoxia or hypercapnia. Subsequent findings in hypoxic/hypercapnic mice show that prenatal exposure to nicotine recruits excitatory neurotransmission to cardiac vagal neurons, leading to heart rate changes (Huang et al., 2005). In another study, α4nAChRs in the preBötzinger Complex were found to regulate glutamatergic neurotransmitter transmission and respiratory rate, and activating α4nAChRs on sublingual (XII) motor neurons was reported to increase respiratory rate, which may also be the pharmacological basis of nicotine contributing to sudden infant death syndrome development (Shao and Feldman, 2009). However, Simakajornboon et al. suggested a different point of view that the hypoxic ventilatory response may be associated with the PDGF-β receptor in the caudal brainstem and its downstream anti-apoptotic cascade. In a rat model, prenatal nicotine exposure attenuated the phosphorylation of the PDGF-β receptor (Figure 6C). Subsequently, it activated the Ras/PI3K/PKB/Bad-136/Bal-2/RB/E2F signaling cascade during hypoxia in the developing rat caudal brainstem, which is thought to increase caudal brainstem cell apoptosis and the vulnerability of neuronal cells in the respiratory control zone (Simakajornboon et al., 2010).

3.5 Periodontitis and age-related macular degeneration

In addition to its involvement in the development of aforementioned diseases, nicotine plays a pro-inflammatory role in many other diseases, such as periodontitis and age-related macular degeneration (AMD). Figure 7A shows some effects of nicotine on periodontitis and AMD.

3.5.1 Periodontitis

Periodontitis, the leading cause of tooth loss in adults, is the result of dysbiosis of the oral microbiota, which interacts with host defense mechanisms (Kinane et al., 2017).

Inflammatory response is critical in disease development. Phospholipase D (PLD), including two subtypes, PLD1 and PLD2, is a signal transduction enzyme. It is expressed in almost all mammalian tissues and involves in various physiological functions, including lipid degradation, cell proliferation, cell differentiation, and immune response process (Locati et al., 2001; Jenkins and Frohman, 2005). Notably, compared with healthy controls and non-smoking patients, mRNA expression of PLD1 and PLD2 was significantly increased in smoking patients, which is consistent with the upregulation in HPDLC stimulated by nicotine and lipopolysaccharide (LPS) *in vitro* (Shin et al., 2015). In addition, inflammatory factors TNF-α and IL-1β can significantly upregulate the expression of PLD, and perhaps the overall increase in inflammatory response is responsible for promoting PLD expression. NO, PGE2, IL-1β, TNF-α, and IL-8 are critical in the occurrence and development of periodontitis (Agarwal et al., 1995). Therefore, blocking the expression of PLD can inhibit nicotine and LPS-induced changes in the abovementioned factors, which is consistent with the findings of previous studies. As shown in Figure 7B, PLD1 and PLD2 siRNA attenuates nicotine and LPS-induced TNF-α expression (Sethu et al., 2010; Kang et al., 2013; Sethu, 2013).

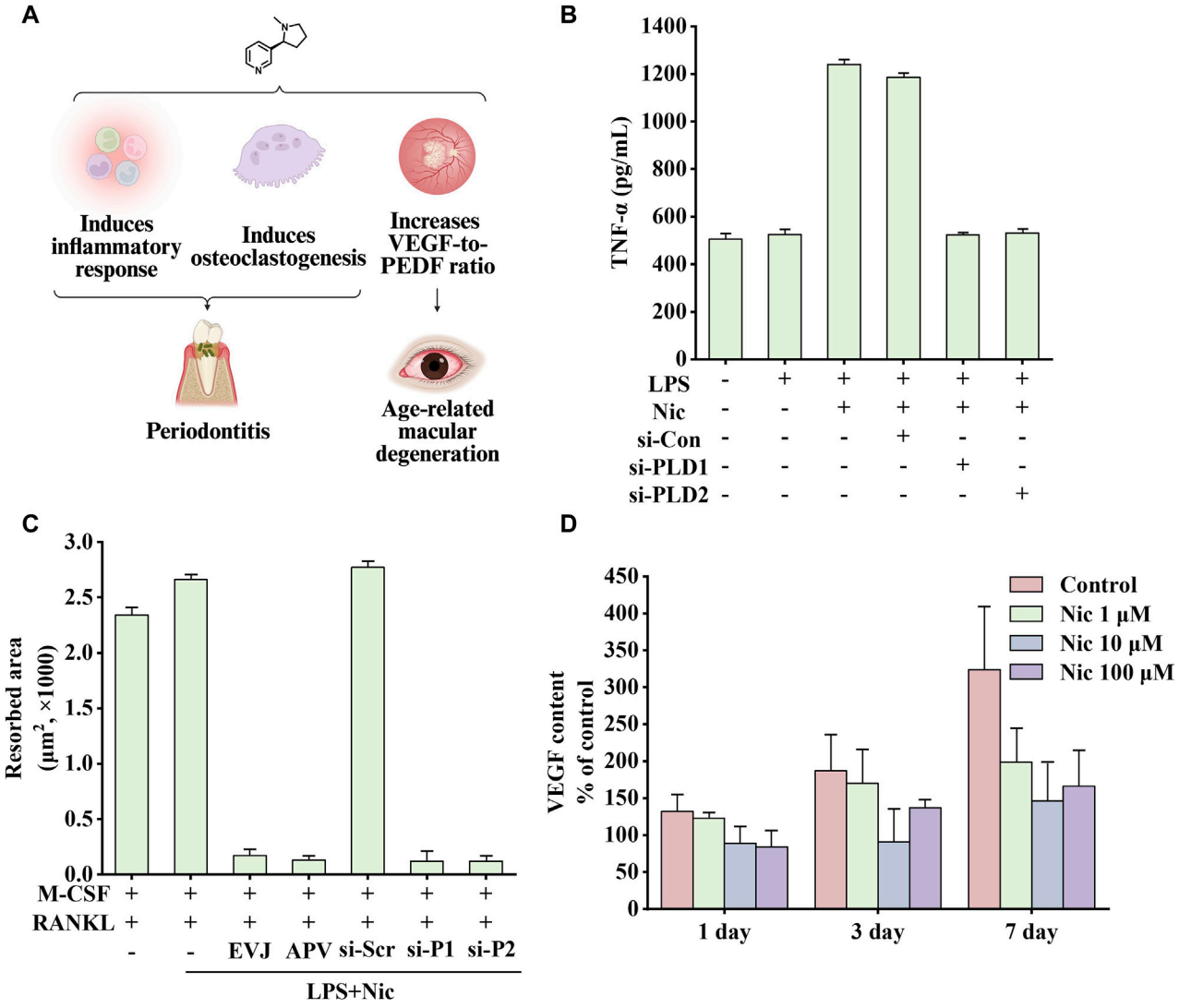


FIGURE 7
Effects of nicotine on periodontitis and age-related macular degeneration (AMD) and related data graphs. **(A)** The effects of nicotine on periodontitis and AMD. Created with BioRender.com. **(B)** Secretion of tumor necrosis factor-alpha (TNF-α) in cells under different treatments. Reprinted (adapted) with permission from Ref. (Shin et al., 2015). Copyright, 2015, Wiley-VCH. **(C)** The area of osteoclast-induced resorption under different treatments. Reprinted (adapted) with permission from Ref. (Shin et al., 2015). Copyright, 2015, Wiley-VCH. **(D)** Vascular endothelial growth factor (VEGF) content in cells under nicotine treatment. Reprinted (adapted) with permission from Ref. (Klettner et al., 2012). Copyright, 2012, Springer Nature. (Abbreviations: PEDF, pigment epithelium-derived factor; LPS, lipopolysaccharide; Nic, nicotine; si-Con, Control siRNA; si-PLD1, phospholipase D1 (PLD1) siRNA; si-PLD2, phospholipase D1 (PLD2) siRNA; RANKL, receptor activator of nuclear factor kappa-B ligand).

In Kang's study, nicotine increased β-catenin levels and inflammation through the resistin/PI3K/PKB/GSK3β signaling cascade. In addition, by injecting nicotine at a dose of 0.7 mg/kg in rats for 30 days, Li et al. found that the above treatment would reduce the levels of bone alkaline phosphatase and osteocalcin, increase the expression of TNF-α and COX-2, and increase alveolar bone loss (Li et al., 2017). In addition, nicotine and LPS activate the PI3K/PKC/MAPK pathway to promote the expression of inflammatory factors and mediate the development of osteoclasts through the NF-κB/c-Fos/NFCTc1 signaling pathway in human periodontal ligament cells. Figure 7C shows that nicotine increased the area of osteoclast-induced absorption pits, which were eliminated by EVJ, APV, PLD1 siRNA, and PLD2 siRNA (inhibiting PLD isoforms) (Shin et al., 2015).

As a key regulator in osteoarthritis (OA) development, hypoxia-inducible factor-2α (HIF-2α) was increased in periodontal ligament cells of patients with periodontitis. Bae et al. found that combined treatment with nicotine and LPS induced the production of NO and PGE2, and upregulated the expression of inducible nitric oxide synthase (iNOS), COX-2 protein in human periodontal ligament cells. Besides, the mRNA expression of various pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-11, and IL-17 was also upregulated. In addition, the expression of collagenases (MMP1, MMP8, and MMP13) and gelatinases (MMP2 and MMP9) was upregulated, and various cytokines, including PKB, Janus kinase 2 (JAK2), STAT3, ERK, and JNK-MAPK, were activated. Moreover, an increase in the number of osteoclasts and osteoclast-specific genes was also observed. These effects were attenuated by HIF-2α

inhibitor or HIF-2α siRNA. Therefore, inhibiting HIF-2α inhibits inflammatory cytokines and blocks osteoclast differentiation (Bae et al., 2015). Interestingly, another study by Cho et al. showed that inhibition or silencing of peptidyl-prolyl cis/trans isomerase NIMA-interacting protein 1 (PIN1) played a similar role. Inhibiting PIN1 by juglone or the knockdown of PIN1 gene expression by siRNA attenuated nicotine and LPS-induced PGE2, NO production, COX-2 and iNOS expression, NF-κB activation, increased osteoclast number, and osteoclast-specific gene expression, whereas overexpression of PINI enhanced these effects (Cho et al., 2015).

3.5.2 Age-related macular degeneration

AMD is the leading cause of vision loss in people aged >55 years in developed countries and is expected to affect 288 million people worldwide by 2040. The pathology of AMD is characterized by massive accumulation of extracellular deposits that form drusen (Fleckenstein et al., 2021).

Smoking is a high-risk environmental factor for AMD. Dysregulation of VEGF and pigment epithelium-derived factor (PEDF) expression, two substances that promote and inhibit angiogenesis respectively, may lead to choroidal neovascularization and further development, causing vision loss. In a non-transformed human retinal pigment epithelium cell line, nicotine upregulated VEGF expression and downregulated PEDF expression through nAChRs. In the rat retinal pigment epithelium, nicotine upregulated the expression of VEGF and PEDF. By altering the ratio of the two growth factors, nicotine may play a role in the development of AMD (Pons and Marin-Castaño, 2011). Interestingly, nicotine treatment showed completely opposite results on VEGF expression in Klettner's study, which may be related to different animal models. As shown in Figure 7D, perfused organ cultures of retina/retinal pigment epithelium/choroid treated with different concentrations of nicotine showed varying degrees of reduction in VEGF expression. Among them, 10 μM nicotine treatment had the most significant effect on inhibiting VEGF expression. Another study showed that on exposing mice to nicotine, VEGF, PDGF, or a combination of one of these factors with nicotine, nicotine increased choroidal neovascularization size and vascularity, especially in aged mice. This effect was blocked by hexamethonium, a non-specific nicotinic receptor antagonist. In addition, the growth of choroidal vascular SMCs was significantly increased after exposure to the combined treatment with PDGF and nicotine (Suñer et al., 2004). Taken together, nicotine may increase the size and severity of choroidal neovascularization in mouse models by enhancing the PDGF-mediated proliferation of choroidal SMCs.

3.5.3 Diabetes

Diabetes is a chronic metabolic disease and one of the top ten causes of death in adults. Its development trend is getting more and more fierce and the number of cases worldwide has reached 425 million in 2017 (Saeedi et al., 2019). Diabetes includes three types, type 1 diabetes, type 2 diabetes, and gestational diabetes, of which type 2 diabetes accounts for more than 90% of all cases. The pathological features of type 2 diabetes are impaired insulin secretion, insulin resistance, or both (DeFronzo et al., 2015).

Epidemiological studies have shown that smokers are much more likely to develop diabetes than non-smokers. Studies have shown that nicotine-fed rats have increased circulating levels of glucagon and insulin, and are also accompanied by symptoms of glucose homeostasis disorders (Duncan et al., 2019). Among them, adrenaline plays a key role. By analyzing skeletal muscle biopsy samples from smokers and non-smokers, Bergman et al. found that smokers had increased Ser636 phosphorylation of IRS-1 and reduced PPAR-γ expression, resulting in reduced insulin sensitivity (Caligiuri and Kenny, 2021), which is consistent with the conclusion that nicotine aggravates insulin resistance in patients with type 2 diabetes and healthy smokers (Chen et al., 2023). In addition, patients with type 2 diabetes seem to metabolize nicotine faster, resulting in greater smoking and longer smoking time and further endangering human health (Keith et al., 2019).

Nicotine often enters the body through smoking; therefore, the parts that come into direct contact with tobacco smoke are often more prone to diseases such as periodontitis and lung cancer. In addition, nicotine has subtle effects on blood vessels, including damaging ECs, triggering inflammation, and eventually causing vascular obstruction. Excessive nicotine exposure from tobacco can increase the risk of preterm birth in pregnant women. Therefore, smokers may need to pay more attention to the health of relevant body parts and reduce tobacco intake appropriately. Table 1 lists the diseases associated with nicotine, experimental models used, nicotine doses, administration methods, influencing factors, and the effects of nicotine.

4 Pharmacodynamics: the beneficial effects of nicotine on the human body

Despite its adverse effects leading to the development of various diseases, nicotine exhibits potential therapeutic and pharmacological benefits in some diseases. This section highlights the therapeutic potential of nicotine, mainly focusing on two aspects: nervous and immune system diseases. Figure 8 illustrates the molecular mechanisms underlying the positive roles of nicotine in related diseases, including regulating various genes, transcription factors, and proteins. This figure helps readers more intuitively understand the therapeutic effect of nicotine in related diseases.

4.1 Nervous system diseases

Nicotine can extensively improve cognition, which has long attracted the interest of researchers. This section introduces the therapeutic effects of nicotine on Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia, attention-deficit/hyperactivity disorder (ADHD), and major depressive disorder (MDD). The associated therapeutic effects are shown in Figure 9A.

4.1.1 Alzheimer's disease

AD is a typical neurodegenerative disorder. In the early stages of the disease, it often manifests as learning and memory decline and mild language and movement impairments. As the disease progresses, patients eventually lose the ability to live independently and remain in bed for a long time until death. (Alan et al., 2023).

AD is marked by the deposition of extracellular amyloid beta (Aβ) and the aggregation of tubulin tau in neurons (Knopman et al.,

TABLE 1 Overview of the toxic effects of nicotine in different diseases.

Diseases	Model	Nicotine dosage	Administration	Factors	Effect	Ref.
Small cell lung cancer	Human SCLC cell lines (GLC8, NCI-N592 and NCI-H-69)	100 nM	*In vitro*	Serotonin	Stimulates serotonin release and SCLC proliferation	Codignola et al. (1994)
Non-small cell lung cancer	H157 and H1703 cells	100 nM	*In vitro*	Cyclin D1, PKB	Increases proliferation	Tsurutani et al. (2005)
Colon adenocarcinoma	HT-29 cells	10 nM, 100 nM,1,000 nM	*In vitro*	α7nAChR, TH, adrenaline	Stimulates proliferation and adrenaline production	Wong et al. (2007)
Gastric tumor	Athymic nude mice implanted with AGS	50 or 200 mg/mL in drinking water	Oral	ERK, COX-2, VEGF	Promotes gastric tumor growth and neovascularization	Shin et al. (2004)
Atherosclerosis	Primary human umbilical vein ECs and SMCs	1 μM	*In vitro*	α7nAChR, NF-κB	Induces SMC cytoskeleton protein up-expression	Wang et al. (2013)
Atherosclerosis	Human aortic endothelial cells	0.1 μM, 1 μM	*In vitro*	NLRP3, ASC, caspase-1, IL-1β, IL-18	Activates NLRP3-ASC inflammasome and pyroptosis	Wu et al. (2018)
Preterm birth	776,836 live singleton births in Sweden from 1999 to 2009	Snuff	Sniff	—	Increases the risk of preterm birth	Baba et al. (2012)
Sudden infant death syndrome	Adult female rats on gestation	2.1 mg/d	Osmotic minipump	Hypoxia/ hypercapnia	Elicits an increase in excitatory neurotransmission to cardiac vagal neurons	Huang et al. (2005)
Chronic obstructive pulmonary disease	Human airway smooth muscle cells	0.1 mM, 1 mM, 5 mM, 10 mM, 50 mM	*In vitro*	α5nAChR, TRPC3	Promotes proliferation	Jiang et al. (2019)
Tuberculosis	Prenatal exposure	3 mg/kg	Administeration	α7nAChR, PI3K, PKB, β-catenin	Induces β-catenin level increase and thymopoiesis abnormalities	Wen et al. (2022)
Periodontitis	Human periodontal ligament cells	LPS (1 μg/mL) and nicotine (5 mM)	*In vitro*	HIF-2α, NO, PGE_2, TRAP	Stimulates inflammatory response and osteoclastic differentiation	Bae et al. (2015)
Periodontitis	Human periodontal ligament cells	LPS (1 μg/mL) and nicotine (5 mM)	*In vitro*	PIN1, NO, PGE_2, COX-2, NF-κB	Stimulates inflammatory response and osteoclastic differentiation	Cho et al. (2015)
Age-Related Macular Degeneration	C57BL/6 mice treated with laser	100 μg/mL in drinking water	Oral	PDGF, MMP2	Increases size and vascularity of choroidal neovascularization	Suñer et al. (2004)

Abbreviations: ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3-kinase; NF-κB, nuclear factor kappa-B; VEGF, vascular endothelial growth factor; PKB, protein kinase B; TH, tyrosine hydroxylase; AP-1, activator protein-1; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; EC, endothelial cells; SMC, smooth muscle cells; LPS, lipopolysaccharide; PDGF, platelet-derived growth factor; ASC, apoptosis-associated speck-like protein containing CARD; HIF-2α, hypoxia-inducible factor-2α; PIN1, peptidyl-prolyl cis/trans isomerase NIMA-interacting protein 1; SCLC, small cell lung cancer; AGS, a poorly differentiated human gastric adeno-carcinoma cell line; NLRP3, NOD-like receptor thermal protein domain associated protein 3; TRPC3, transient receptor potential canonical 3; TRAP, tartrate-resistant acid phosphatase; MMP2, matrix metalloproteinase 2.

2021). Among them, Aβ is produced in the cracking process of amyloid precursor protein (APP) and further accumulates to form amyloid fibers, causing neurotoxicity. In addition, α7nAChR has a strong affinity for Aβ, which can promote Aβ to enter cells through endocytosis. Lysis occurs when neurons are overburdened, and Aβ is released extracellularly, where further accumulation creates plaques (Ma and Qian, 2019).

Epidemiological studies have shown a significant negative correlation between smoking and AD incidence (Fratiglioni and Wang, 2000). In a 4-week transdermal nicotine treatment for patients with AD, nicotine significantly improved the patient's attention performance (White and Levin, 1999). In another study of APP (V717I) transgenic mice, nicotine treatment reduced the accumulation of insoluble Aβ in the cortex and hippocampus, as shown in Figure 9B, which is associated with the MAPK/NF-κB/c-*myc* pathway mediated by α7nAChR. In addition, there appears to be a connection between cell cycle-related proteins and neuronal loss. Nicotine regulates the cell cycle and apoptotic processes to reduce neuronal loss by decreasing the mRNA and protein levels of cyclin D1 and CDK4 and the pro-apoptotic factors Bax and caspase-3 (Liu et al., 2007).

Another study published by Inestrosa et al. pointed out that nicotine prevents Aβ-induced neuronal synapse damage through the α7nAChR/PI3K pathway. In addition, Wnt/β-catenin signaling may be crucial in neuroprotection. The Wnt signaling pathway can regulate synaptic transmission and plasticity, and increased β-catenin expression can improve the structure of dendrites. Activating Wnt can promote α7nAChR expression, and nicotine prevents Aβ-induced β-catenin reduction through α7nAChR. In addition, nicotine has been shown to improve memory in APP/

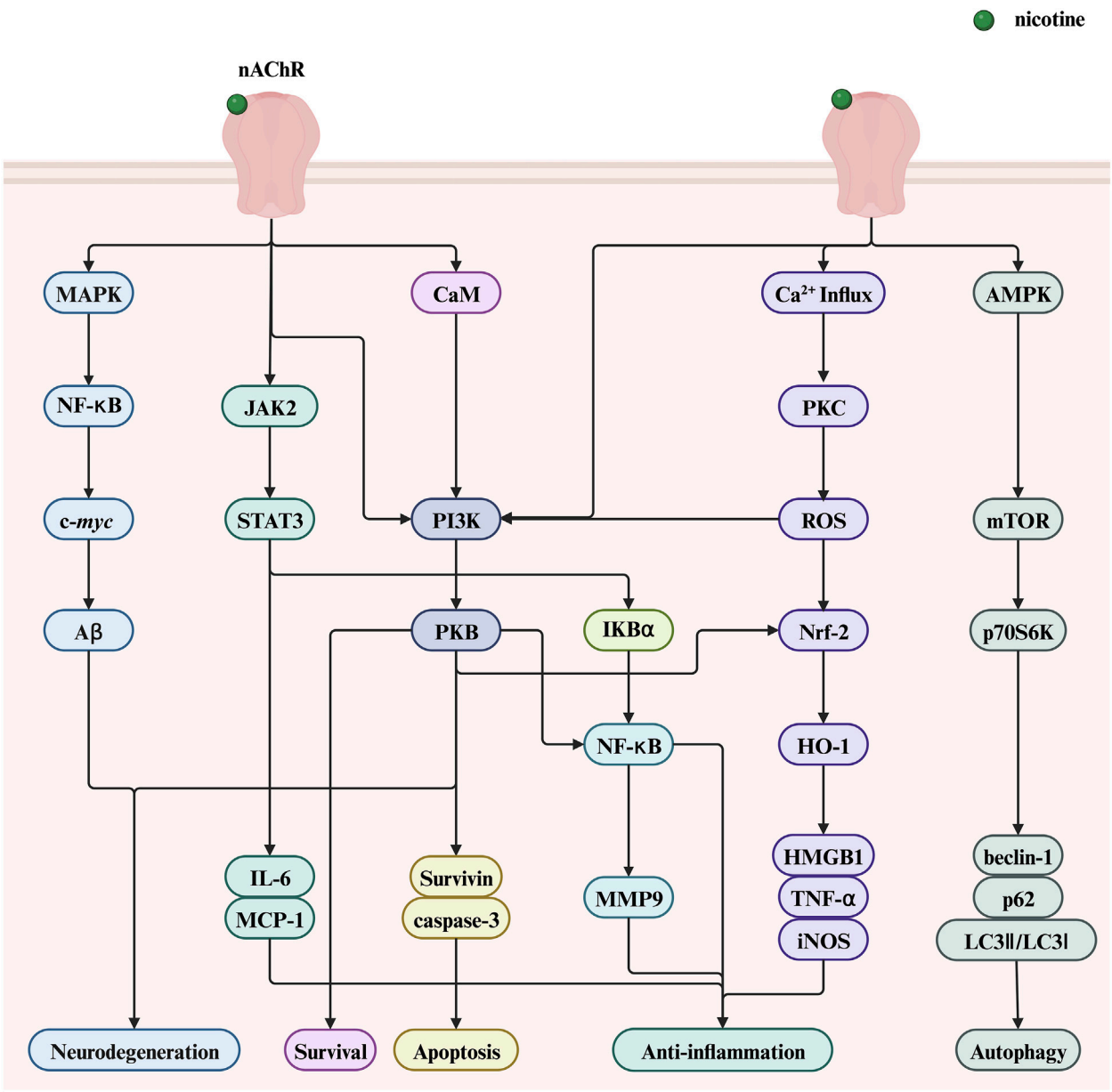


FIGURE 8
The therapeutic potential of nicotine and related signaling pathways. Created with BioRender.com. (Abbreviations: PI3K, phosphatidylinositol-3-kinase; NF-κB, nuclear factor kappa-B; PKB, serine/threonine kinase; STAT3, signal transducer and activator of transcription 3; ROS, reactive oxygen species; AMPK, AMP-activated protein kinase; JAK2, Janus kinase 2; CaM, calmodulin; IκBα, nuclear factor kappa B alpha; MCP-1, monocyte chemoattractant protein-1; HMGB1, high-mobility group box 1; HO-1, heme oxygenase-1; DSS, dextran sodium sulfate; nAChR, nicotinic acetylcholine receptor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; Aβ, amyloid beta; IL-6, interleukin-6; MMP9, matrix metalloproteinase 9; PKC, protein kinase C; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related Factor 2; TNF-α, tumor necrosis factor-alpha; mTOR, mechanistic target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase).

PS1 transgenic mice (Inestrosa et al., 2013; Inestrosa and Varela-Nallar, 2014).

4.1.2 Parkinson's disease

PD is the second most prevalent neurodegenerative disorder, affecting 2%–3% of people >65 years of age. Patients often present with motor symptoms such as bradykinesia, rigidity, resting tremor, and persistent cognitive, autonomic, and mood disturbances. Neuropathological features include striatal dopamine deficiency due to loss of substantia nigra dopaminergic neurons and extensive intracellular α-synuclein aggregates (Dickson et al., 2009). Neuronal damage is associated with multiple factors such as α-synuclein aggregates, mitochondrial dysfunction, and neuroinflammation (Poewe et al., 2017b).

Studies have shown a significant negative correlation between PD incidence and smoking, coffee consumption, and drinking alcohol (Nicoletti et al., 2010). Further research showed that nicotine can protect animal models of PD from nigrostriatal

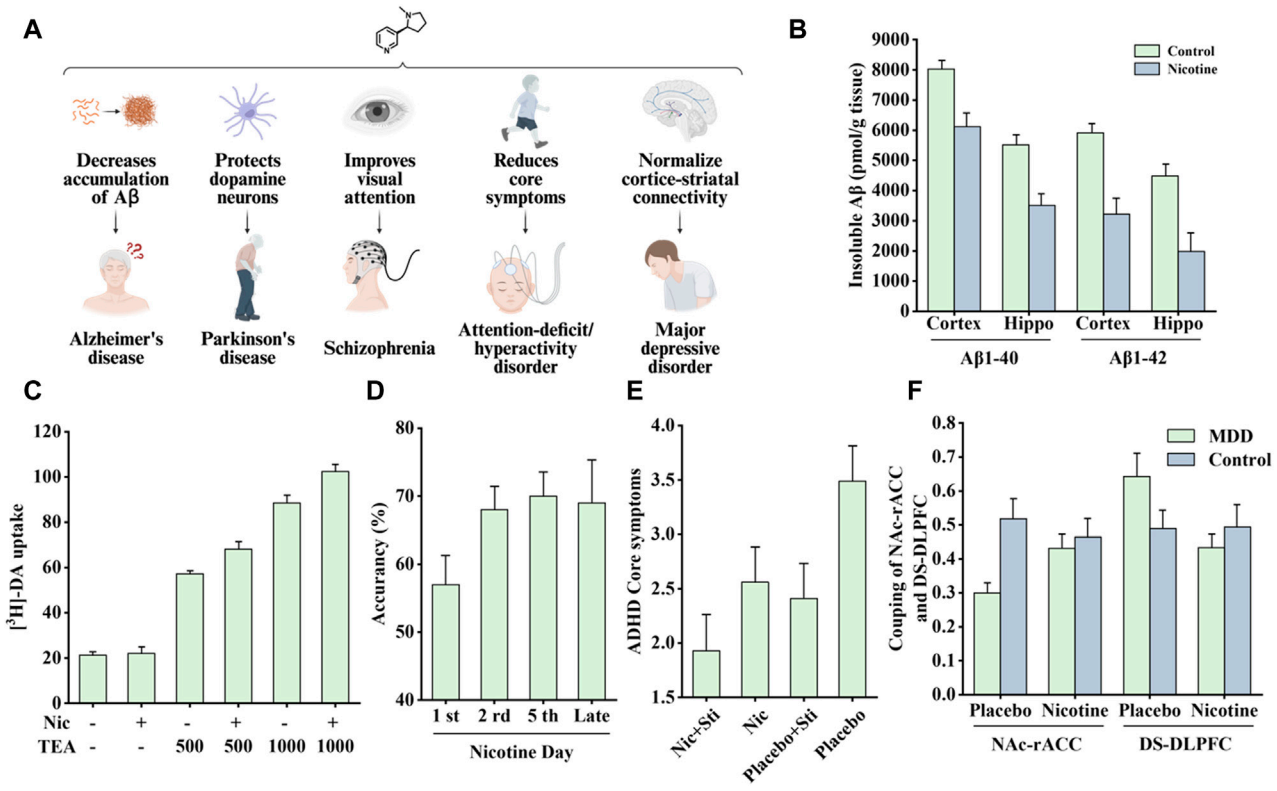


FIGURE 9
Effects of nicotine on nervous system diseases and related data graphs. **(A)** The effects of nicotine on the nervous system. Created with BioRender.com. **(B)** The level of insoluble amyloid beta (Aβ) in the cortex and hippocampus of transgenic mice under nicotine treatment. Reprinted (adapted) with permission from Ref. (Liu et al., 2007). Copyright 2007, Wiley-VCH. **(C)** Dopamine cell survival under nicotine and tetraethyl ammonium (TEA) treatment. Reprinted (adapted) with permission from Ref. (Toulorge et al., 2011). Copyright, 2011, Wiley-VCH. **(D)** Visual attention improvement in rats under nicotine treatment. Reprinted (adapted) with permission from Ref. (Bueno-Junior et al., 2017). Copyright, 2017, Springer Nature. **(E)** The average attention-deficit/hyperactivity disorder (ADHD) core symptoms under different treatments. Reprinted (adapted) with permission from Ref. (Gehricke et al., 2006). Copyright, 2006, Oxford University Press. **(F)** The coupling of nucleus accumbens-anterior cingulate cortex (NAc-rACC) and dorsal striatum-dorsolateral prefrontal cortex (DS-DLPFC) in the major depressive disorder (MDD) and healthy control groups under nicotine treatment and placebo. Reprinted (adapted) with permission from Ref. (Janes et al., 2018). Copyright, 2018, Springer Nature. (Abbreviations: Hippo, hippocampus; Nic, nicotine; DA, dopamine; Sti, stimulants).

damage. In rats, administering nicotine before injury effectively reduced striatal dopamine loss (Costa et al., 2001), and in MPTP-treated monkeys, chronic oral nicotine decreased dopamine turnover, enhanced synaptic plasticity, and improved substantia nigra function after striatal injury (Quik et al., 2006), but this protective effect only occurred before striatal injury. This indicated that nicotine can only exert a protective effect on striatal injury rather than a repairing effect (Huang et al., 2009).

Nicotine-mediated neuroprotection also involves nAChRs and is subsequently divided into calcium-dependent and calcium-independent pathways (Quik et al., 2012). The calcium-dependent pathway involves an increase in intracellular Ca^{2+} concentration, which may be mediated by nAChR or other membrane channels, thereby activating different downstream signaling channels to exert neuroprotective effects. In a study by Toulorge et al., nicotine promoted cell survival by activating downstream calmodulin (CaM)/PI3K/PKB-dependent signaling. As shown in Figure 9C, in the presence of K^+-channel blocker tetraethyl ammonium (TEA), nicotine treatment exerted more significant neuronal protective effects (Toulorge et al., 2011). The calcium-independent pathways involve the JAK2-STAT3 signaling cascade. Blocking downstream inhibitor of nuclear factor kappa B alpha (IκBα) phosphorylation and NF-κB translocation can reduce neuronal inflammation, which depends on α4β2nAChR (Hosur and Loring, 2011).

As reported by Holmes et al., nicotine selectively affected the non-smokers' controlled semantic processing that is impaired in PD patients in a cognitive test, which may be attributed to nicotine's enhancement of expectation or inhibition mechanism (Holmes et al., 2011). Villafane et al. found that chronic high-dose transdermal nicotine treatment in five PD patients resulted in improved motor scores and reduced dopaminergic responses, but with side effects such as nausea and vomiting (Villafane et al., 2007). Itti et al. studied six PD patients who received nicotine therapy for 1 year and found that motor function improved at 3 months, but the improvement was less sustained after 1 year. Dopamine transporter imaging showed that the density of striatal neurons in the treated patients was relatively stable, indicating that nicotine has pharmacological and neuroprotective effects (Itti et al., 2009). PD patients often have symptoms of low blood pressure. DiFrancisco et al. found that patients' systolic blood pressure would increase within 10 min after taking 4 mg nicotine gum and remain elevated

within 90 min by studying 10 subjects (DiFrancisco-Donoghue et al., 2019). Another study on two PD patients found that nicotine gum and patch treatment improved the patients' motor delay and confusion (Fagerström et al., 1994). Nicotine gum is rapidly absorbed and is expected to become a new type of treatment. Although nicotine has shown some value in the treatment of PD, it seems that larger-scale population trials are needed to confirm its application if to be widely promoted.

4.1.3 Schizophrenia

Schizophrenia is a serious mental illness classified into three categories: positive, negative, and cognitive. Positive symptoms are often accompanied by delusions, hallucinations, and behavioral disturbances; negative symptoms are often accompanied by depression, anhedonia, and social withdrawal; and cognitive symptoms mainly manifest as cognitive dysfunction (Kahn et al., 2015). Presently, the etiology of the disease remains unclear, and patients mainly rely on drug therapy. Drug therapy can improve positive symptoms but cannot effectively relieve negative and cognitive symptoms (Stępnicki et al., 2018).

Smoking is a high-risk factor for patients with schizophrenia; however, the biological mechanisms underlying this association remain unclear. Liu et al. investigated the relationship between alterations in intrinsic brain activity associated with schizophrenia pathology and nicotine addiction. The authors found that smoking reversed intrinsic brain activity in the right striatum and prefrontal cortex, consistent with the pharmacological theory of schizophrenia. Furthermore, addictive effects are independent of the disease (Liu et al., 2018). Interestingly, Whitton et al. found that dopamine D2 receptor antagonists reduced the reward-enhancing effects of nicotine and were associated with increased smoking rates in patients, which explains why patients taking potent dopamine D2 receptor antagonists often exhibit more symptoms of nicotine dependence (Whitton et al., 2019).

Smucny et al. examined the effect of nicotine on the connectivity within a ventral attention network. When patients performed a selective attention task, the connectivity between the ventral parietal cortex seeds and the inferior frontal gyrus decreased, and nicotine increased this connectivity (Smucny et al., 2016). Another study found that in a mouse model, nicotine treatment increased gamma oscillations in the prefrontal cortex, which was associated with enhanced visual attention. As shown in Figure 9D, nicotine exposure increased visual attention accuracy in rats (Bueno-Junior et al., 2017).

The prefrontal cortex is fundamental to higher cognitive processes and regulated by nAChRs. A genome-wide association study found that single-nucleotide polymorphisms (SNPs) in the CHRNA5 gene encoding the α5nAChR subunit were associated with an increased risk of smoking and schizophrenia. In mice expressing α5-SNP and α5-knockout mice, interneuron inhibition of layer II/III pyramidal neurons increases, activity reduces, and frontal function declines. However, chronic nicotine administration modulates layer II/III inhibition circuitry through nAChR, reversing the above effects (Koukouli et al., 2017). A subsequent study showed that these prefrontal cortex circuit dynamics involved changes in the structure of active-state stability and that changes in amplitude were associated with reduced cone firing rates in α5 SNP mice. In addition, this experiment demonstrated that nicotine induced the desensitization and upregulation of β2nAChR on somatostatin interneurons but not the activation of α5nAChR on vasoactive intestinal polypeptide interneurons, which explains why the activity of α5 SNP mice normalized after nicotine treatment. In addition, the study showed that nicotine withdrawal may exacerbate SNP-induced frontal hypocreasia (Rooy et al., 2021).

4.1.4 Attention-deficit/hyperactivity disorder

ADHD is a common neurodevelopmental disorder caused by multiple genetic and environmental factors. Typical features include inattention and hyperactive impulsiveness, which affect approximately 5% of children and adolescents worldwide, with huge financial costs and family stress. For now, medication remains an effective way to reduce ADHD symptoms (Faraone et al., 2015).

Molecular genetic studies have shown that susceptibility to ADHD is associated with three genes: D4 dopamine receptor, D2 dopamine receptor, and dopamine transporter genes. Neurological deficits in children include executive function and working memory deficits, which may be associated with dysfunction in the frontal lobar cortex (Faraone and Biederman, 1998).

Nicotine has been shown to improve attentional performance in this disorder. In a rat model, nicotine administration improved working memory in the radial-brachial maze, an effect associated with α4β2nAChR and α7nAChR in the ventral hippocampus and basolateral amygdala. Local infusion of α4β2nAChR and α7nAChR antagonists induced working memory deficits, whereas ventral hippocampal α4β2nAChR blockade-induced working memory deficits were reversed by systemic nicotine treatment (Levin, 2002). In addition, patients with ADHD appear to have a higher risk of smoking and failure to quit, perhaps because of the "self-medication hypothesis." Accordingly, patients' active or continued exposure to cigarettes is attributed to nicotine in tobacco products that can supplement the lack of dopamine in the cortico-striatal pathway, thereby relieving symptoms. In addition, the nicotine analogs varenicline and bupropion improved ADHD-related symptoms and also supported the above hypothesis (Taylor et al., 2022). Nicotine patches and stimulant medications, alone or in combination, have been found to reduce concentration difficulties and core symptoms in patients with ADHD (Figure 9E) (Gehricke et al., 2006). Another acute nicotine treatment in patients with ADHD revealed that these patients had improved recognition memory, increased delay tolerance, and a corresponding reduction in reaction time in a stop-signal task (Potter and Newhouse, 2008). A recent study pointed out that nicotine improved two pathways involving the VTA; one normalized abnormal activity in animal models of ADHD, and the other induced atypical brain responses in animals with ADHD (Poirier et al., 2017a).

4.1.5 Major depressive disorder

MDD is a mental illness often accompanied by depression, loss of interest, cognitive impairment, fatigue, difficulty in sleeping, loss of appetite, and other symptoms. The incidence rate in women is generally higher than that in men, and genetic factors and childhood abuse can increase the incidence rate (Seedat et al., 2009; Li et al., 2016). The disease is associated with altered brain volume in the

hippocampus, altered function of the cognitive control and affective-salience networks, and disturbances of the hypothalamic-pituitary-adrenal axis and immune system (Otte et al., 2016).

MDD and nicotine dependence are highly comorbid, and their causal link remains unclear. Markou et al. believe that it may be associated with changes in the function of neurotransmitters in limbic brain structures (Markou et al., 1998). Cardenas et al. further speculated that the two may be associated with the dysfunctional dopaminergic brain reward system. However, nicotine treatment did not change the brain's stimulus response to d-amphetamine, and the severity of depression was highly correlated with this reward effect (Cardenas et al., 2002). A clinical and preclinical study on nicotine and depression showed that nicotine may share some of the properties of antidepressants, whereas some antidepressants are also effective smoking cessation agents; therefore, MDD and nicotine dependence may share some related neuronal substrates (Laje et al., 2001; Dome et al., 2010). In a recent study, nicotine reportedly improved neurobiological dysfunction in the cortico-striatal circuit associated with MDD. Specifically, in patients with MDD, connectivity between the nucleus accumbens (NAc) and the rostral anterior cingulate cortex (rACC) is reduced, whereas connectivity between the dorsal striatum (DS) and dorsolateral prefrontal cortex (DLPFC) is increased. Acute nicotine treatment normalized these pathways to the levels observed in healthy controls (Figure 9F).

Notably, the effect of nicotine on NAc-rACC connectivity was associated with anhedonia, a network implicated in rewarding effects (Janes et al., 2018). An electroencephalogram study involving patients with MDD showed decreased activation of the left and right frontal cortices, in which the left frontal cortex was associated with positive emotion regulation. Nicotine treatment normalized these changes, including a modest increase in right hemisphere alpha1 amplitude and reduced left-biased alpha1 amplitude asymmetry (Jaworska et al., 2011).

4.2 Immune system diseases

Inflammation involves multiple genes and signaling pathways. In addition, it promotes the occurrence and development of various diseases to varying degrees. Nicotine plays an active role in various immune disorders because of its broad anti-inflammatory properties. This section describes the therapeutic effects of nicotine in rheumatoid arthritis (RA), OA, sepsis, endotoxemia, ulcerative colitis (UC), and myocarditis. The related effects are shown in Figure 10A, which may be associated with the different models, concentrations, durations, and corresponding tissues and organs.

4.2.1 Arthritis

Arthritis refers to any condition affecting the joints, including joint pain and stiffness, often accompanied by redness, warmth, and swelling. RA and OA are two typical joint diseases (March et al., 2014).

RA is a chronic inflammatory disease characterized by synovial inflammation, cartilage destruction, and bone erosion. It is an autoimmune disease; when the peptide undergoes a post-translational modification process of citrullination, the presentation of the newly generated peptide by the antigen-presenting cell activates the immune system and induces the production of autoantibodies, mainly immunoglobulin G (rheumatoid factor and anti-citrullinated protein antibodies). Subsequently, fibroblast-like synoviocytes (FLSs) and antigen-presenting cells are activated to produce inflammatory factors, leading to synovial inflammation, whereas a sustained immune response eventually leads to cartilage degeneration and bone erosion (Schellekens et al., 1998; Smolen et al., 2018).

The most typically used animal model of RA is the collagen-treated DBA/1 mouse, which often exhibits cartilage damage, cell infiltration, and bone destruction similar to human RA symptoms. Li's study showed that nicotine inhibited the secretion of IL-6 and monocyte chemoattractant protein-1 (MCP-1) in RA-FLSs through the α7nAChR/JAK2/STAT3 pathway to exert anti-inflammatory effects (Li et al., 2015). The pro-inflammatory cytokine IL-6 regulates T lymphocytes to produce osteoclast factors and inflammation-induced bone marrow osteoclast differentiation (Wong et al., 2006). However, the chemokine MCP-1 regulates the RA process by recruiting monocytes (Ogata et al., 1997). As shown in Figure 10B, STAT3-specific small interfering RNA transfection (STAT3-siRNA) leads to a significant decrease in STAT3 expression, while leading to an overall anti-inflammatory degree of nicotine (inhibition of TNF-α-induced IL-6 increase) reduction compared with that in the control group (Con-siRNA). The higher the dose, the weaker the anti-inflammatory effect of nicotine. Therefore, the anti-inflammatory effects of nicotine require STAT3 activation.

Cholinergic anti-inflammatory pathways play a crucial protective role in disease development. The vagus nerve's efferent activity promotes the release of acetylcholine from various organs, which inhibits the release of pro-inflammatory cytokines by binding to receptors on the surface of macrophages (Tracey, 2002). Similar to acetylcholine, nicotine can exert anti-inflammatory effects by inhibiting the release of inflammatory factors through the vagus nerve. In Maanen's study, nicotine (400 mg/kg, intraperitoneal injection) administration for 7 days reduced bone degradation and TNF-α expression in the synovial tissue, and this process may be associated with the specific effect of the vagus nerve on α7nAChR, because cutting the vagus nerve led to the deteriorating condition of mice (van Maanen et al., 2009). In another experiment, nicotine (0.1, 1, and 10 μM) decreased the expression of IL-6 and IL-8 in TNF-α-induced FLSs and inhibited the translocation of NF-κB from the cytoplasm to nucleus (Zhou et al., 2012). In addition, the combined treatment with thymol (50 mg/kg) and nicotine (1.25 mg/kg) showed a better therapeutic effect and led to a decreased expression of rheumatoid factor, myeloperoxidase, and IL-1 (Golbahari and Abtahi Froushani, 2019).

Multiple risk factors are responsible for OA development, and its prevalence increases sharply with age, with a higher incidence in women. OA affects the entire joint, including articular cartilage, subchondral bone, and synovium. Chondrocytes cause changes in the cartilage matrix. When chondrocytes cannot repair the damaged cartilage matrix, they secrete matrix-degrading enzymes, ROS, cytokines, and chemokines, which further trigger synovial inflammation and lead to cartilage degeneration (Martel-Pelletier et al., 2016).

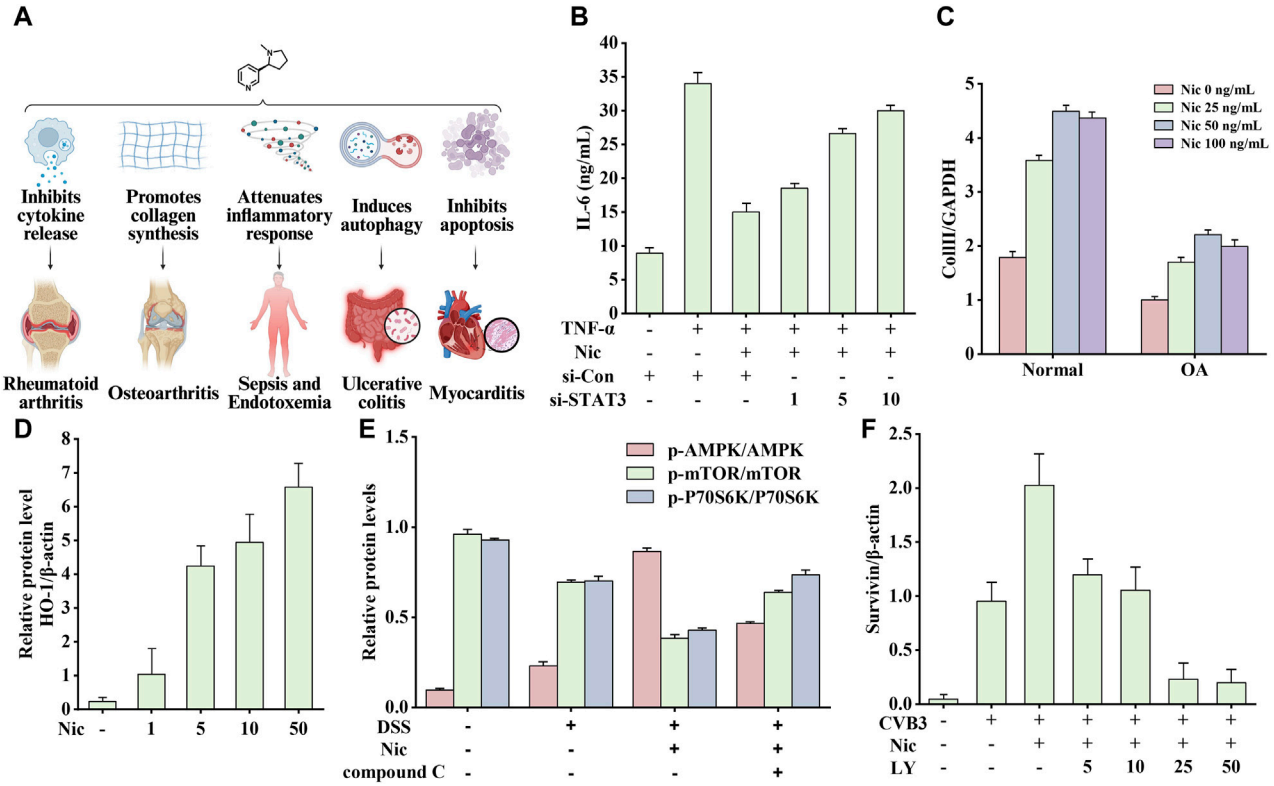


FIGURE 10
Effects of nicotine on immune system diseases and related data graphs. **(A)** The effects of nicotine on immune system diseases. Created with BioRender.com. **(B)** Interleukin-6 (IL-6) protein concentration in culture supernatants under different treatments. Reprinted (adapted) with permission from Ref. (Li et al., 2015). Copyright 2015, Springer Nature. **(C)** The expressed mRNA of type II collagen (Coll II in cells under nicotine treatment. Reprinted (adapted) with permission from Ref. (Ying et al., 2012a). Copyright 2012, Springer Nature. **(D)** The expression of heme oxygenase-1 (HO-1) in macrophages under nicotine treatment. Reprinted (adapted) with permission from Ref. (Tsoyi et al., 2011). Copyright 2011, Mary Ann Liebert, Inc. **(E)** The relative protein levels of p-AMP-activated protein kinase (AMPK), p-mechanistic target of rapamycin (mTOR), and p-p70 ribosomal protein S6 kinase (p70S6K) in cells under dextran sodium sulfate (DSS), nicotine, and compound C treatment. Reprinted (adapted) with permission from Ref. (Gao et al., 2020). Copyright 2020, Elsevier B.V. **(F)** Survivin expression in cells under nicotine, coxsackievirus B3 (CVB3), and LY294002 treatment. Reprinted (adapted) with permission from Ref. (Li P. et al., 2019). Copyright 2019, Springer Nature. (Abbreviations: TNF-α, tumor necrosis factor-alpha; Nic, nicotine; si-Con, Control siRNA; si-STAT3, STAT3 siRNA; OA, osteoarthritis; LY, LY294002).

Nicotine tends to positively affect OA, in which α7nAChR plays a key role. In a rat model of early OA, nicotine (50 mg/mL in drinking water) promoted matrix production, ameliorated cartilage destruction, decreased TNF-α levels in the serum and synovial tissue, and upregulated the expression of α7nAChR in synovial tissue, showing promising therapeutic potential (Gu et al., 2015). In the same year, Liu et al. conducted a more in-depth exploration and found that nicotine (10 μM) significantly attenuated joint degeneration and p38, Erk1/2, and JNK MAPK activation of chondrocytes in monosodium iodoacetate-treated mice, all of which were reversed by α7nAChR antagonist methyllycaconitine (Liu et al., 2015). Elevated MMP9 expression is a sign of OA joint inflammation. Nicotine inhibits LPS-induced NF-κB translocation through the α7nAChR/PI3K/PKB pathway, thereby reducing MMP9 gene expression. In addition, mice cartilage degeneration and mechanical allodynia were reduced (Teng et al., 2019).

The imbalance between autophagy and apoptosis in chondrocytes may accelerate cartilage degeneration, and the expression of α7nAChR was found to be downregulated in the knee articular cartilage tissue of patients, leading to mTOR phosphorylation and increased possibility of apoptosis. Nicotine-activated α7nAChRs/mTOR signaling pathway alleviates pain and cartilage degeneration and regulates the balance between apoptosis and autophagy (Liu et al., 2021). Furthermore, as shown in Figure 10C, nicotine promoted type II collagen expression in chondrocytes isolated from normal humans and patients with OA in a dose-dependent manner. OA chondrocytes were expressed less than normal cells (Ying et al., 2012a). The authors also found that appropriate nicotine concentrations enhanced the cartilage differentiation ability of bone marrow stromal stem cells (Ying et al., 2012b).

4.2.2 Sepsis and endotoxemia

Sepsis typically results from a dysregulated systemic inflammatory and immune response to infection, ultimately leading to organ damage. The exact process is not fully understood but mainly includes two phases: inflammatory outbreaks and immunosuppression. An inflammatory outbreak occurs mainly when infection-derived microorganisms are recognized, simultaneously triggering multiple signaling cascades to release multiple inflammatory cytokines, leading to vasodilation, tissue damage, and multiorgan failure. In the next stage, patients

show chronic suppression of the innate and adaptive immune systems, resulting in severe leukocyte apoptosis (Hotchkiss et al., 2016).

In high-income countries, 31.5 million cases and 5.3 million deaths are reported annually (Hotchkiss et al., 2016). In multiple sepsis and experimental models, nicotine improved survival and attenuated multiple organ dysfunction under different conditions. This suggests that nicotine could potentially be developed as a drug for treating sepsis.

Cholinergic pathways are involved in nicotine-mediated protection. The vagus nerve is critical in regulating innate immune responses to bacterial infections. Septic peritonitis was induced in mice by intraperitoneal injection of live *Escherichia coli*, and the subsequent effects were observed by transection of the vagus nerve or nicotine treatment. Nicotine preconditioning reduces cytokine release during septic peritonitis, independent of vagal integrity. Further research showed that neutrophil influx, pro-inflammatory cytokine levels, and liver damage increased after vagus nerve-cutting treatment, whereas nicotine preconditioning reversed these effects (van Westerloo et al., 2005).

The toll-like receptor (TLR) is involved in initiating inflammatory responses. In a mouse model of sepsis established by cecal ligation and puncture, intraperitoneal injection of nicotine attenuated the increase in TLR4 protein and gene expression. In addition, the regulation of TLR4 gene expression in macrophages determines the strength and timing of the response to endotoxins, among which the myeloid-specific factor PU.1 serves as an important transcription factor. Nicotine inhibited PU.1-binding activity in macrophages via α7nAChR, and PU.1 inhibition was observed at 6 h after cecal ligation and puncture to coincide with a reduced TLR4 mRNA expression (Pedchenko et al., 2005). Based on the above results, it can be reasonably speculated that nicotine-regulated TLR4 transcription is mediated by PU.1.

High-mobility group box 1 (HMGB1) is a lethal cytokine in the cecal ligation and puncture-induced sepsis model. In Konstantin's study, nicotine was found to exert anti-inflammatory effects through the α7nAChR/Ca2+ influx/PKC/ROS/PI3K/PKB/nuclear factor erythroid 2-related Factor 2 (Nrf2) pathway by inducing macrophages to produce heme oxygenase-1 (HO-1), thereby decreasing the release of HMGB1, TNF-α, and iNOS, which ultimately increases survival in mice with sepsis. As shown in Figure 10D, nicotine dose-dependently increased the relative protein level of HO-1 (Kim et al., 2014). In another experiment, nicotine inhibited the release of HMGB1 from macrophages induced by endotoxin or TNF-α by activating cholinergic signaling pathways, prevented NF-κB pathway activation, and improved the survival rate in experimental models of sepsis. A positive treatment effect can still be observed after disease onset. In addition, nicotine (400 μg/kg, intraperitoneal injection) alleviates clinical manifestations such as lethargy, diarrhea, piloerection, and curling up and prevents hypothermia and decreased hematocrit caused by endotoxemia (Wang H. et al., 2004).

Nicotine has also been shown to be beneficial in treating endotoxemia. Wistar rats were injected with LPS (5 mg/kg body weight) to establish an animal model of endotoxemia; the expression of TNF-α and IL-6 increased, and nicotine treatment could significantly inhibit these increased expressions. In addition, nicotine treatment (0.1 mg/kg) inhibited the increase in plasma alanine aminotransferase levels and restored diamine oxidase activity. Among them, α7nAChR was also involved in these anti-inflammatory and protective effects (Zhou et al., 2011).

Overall, nicotine appeared to improve sepsis and endotoxemia. As two fatal diseases, nicotine can be considered for emergency patients requiring life-saving care in the absence of specific drugs. However, the side effects of nicotine on the human body should be minimized.

4.2.3 Ulcerative colitis

Recently, the annual incidence of UC has increased. UC is a typical chronic inflammatory bowel disease that extends from the rectum to the distal colon, where a long-term and persistent inflammatory response in the innermost intestinal mucosa develops, eventually leading to ulcers and bloody diarrhea (Kobayashi et al., 2020).

In patients with UC, the diversity of the microbiota and thickness of the mucus layer are reduced, the synthesis of tight junction proteins and the pore-forming protein claudin two is reduced, the barrier is impaired, and further development can lead to barrier breakdown. Subsequently, many microbes cross the epithelial barrier, activate macrophages and antigen-presenting cells, and attract neutrophils. Activated neutrophils form neutrophil extracellular traps that cause mucosal damage. Monocytes bind to and infiltrate the adhesion molecules expressed by the vascular endothelium and mature into macrophages. Macrophages secrete various cytokines, such as TNF, IL-12, and IL-6, which polarize T helper cells and promote further UC development (Kobayashi et al., 2020).

Generally, nicotine is beneficial for treating UC. In particular, transdermal nicotine patches or nicotine enemas used as therapeutic agents in patients have been shown to improve colitis histology and global clinical scores (Abdrakhmanova et al., 2010). Among these, 6 mg of oral and transdermal nicotine is well tolerated and represents the highest therapeutic dose used in clinical practice, with a low risk of adverse reactions in humans (Ingram et al., 2004).

The most typically used animal model of UC is the dextran sodium sulfate (DSS)-induced mouse or rat model, often accompanied by symptoms such as rectal bleeding, diarrhea, and weight loss. In C57BL/6J mice treated with 3% DSS, nicotine (0.1 mg/mL) administration through drinking water attenuated DSS-induced increases in mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), vascular cell adhesion molecule 1, and leukocyte recruitment, and disease activity index and histological scores were also attenuated. Recruiting leukocytes to the inflamed colon is essential to UC disease progression. These results may suggest that nicotine treatment can improve colitis by inhibiting MAdCAM-1 expression on inflamed colonic microvessels (Maruta et al., 2018). In another study, nicotine (10 μg/kg/day, gavage) modulated autophagy through the AMPK-mTOR-P70S6K signaling pathway to enhance the expression of LC3II/LC3I and beclin-1 and decrease the p62 protein level, which also reduced the disease activity score, body weight, histological damage score, and colon levels of inflammatory factors.

As shown in Figure 10E, nicotine treatment increased p-AMPK levels and decreased p-mTOR and p-P70S6K levels, and this effect was attenuated by compound C (AMPK inhibitor). Therefore, drugs targeting AMPK are expected to help treat UC. In addition, nicotine

increased the expression of LC3II/LC3I and beclin-1 but decreased p62 protein levels, all associated with nicotine-promoted autophagy (Gao et al., 2020). In BALB/C mice, nicotine reduced the number and size of the colonic tumors associated with chronic colitis through the IL-6/Stat3/miR-21 signaling pathway. In addition, nicotine also attenuated colonic severity of inflammation (Hayashi et al., 2014).

Early reports mentioned that patients with UC had worsening disease after quitting smoking and improved after resuming smoking (Lakhan and Kirchgessner, 2011). In clinical practice, patients with moderate UC achieved relief after taking nicotine gum (Wolf and Lashner, 2002), and transdermal nicotine-assisted methotrexate enema therapy was significantly superior to combined oral and rectal methotrexate therapy (Guslandi et al., 2002). Although transdermal nicotine shows potential for the treatment of UC, it is often associated with side effects such as nausea, dizziness, and headache. While added to conventional treatment as an adjunctive treatment, nicotine enemas have shown almost no side effects (Green et al., 1997).

4.2.4 Myocarditis

Myocarditis may be accompanied by various symptoms, ranging from dyspnea or chest pain to severe cardiogenic shock or death and dilated cardiomyopathy with sequelae of chronic heart failure. Myocarditis is usually caused by common viral infections; in specific cases, it can also be caused by other pathogens, toxic or hypersensitive drug reactions, or sarcoidosis. Currently, specific treatments for myocarditis are lacking (Cooper, 2009).

The cholinergic anti-inflammatory pathway effectively protects the myocardium from viral infections. When pathogens invade the body, damaged tissues produce and release inflammatory factors that act on the afferent sensory nerve of the solitary nucleus, and the efferent vagus nerve is activated to release acetylcholine. Subsequently, acetylcholine binds to α7nAChR on the surface of inflammatory cells, inhibits the synthesis and release of pro-inflammatory factors, reduces inflammatory responses, and prevents tissue damage and death (Oke and Tracey, 2009).

In a coxsackievirus B3 (CVB3) murine myocarditis model, nicotine activated α7nAChR, increased STAT3 phosphorylation, reduced the expression of TNF-α and IL-6, and attenuated the damage due to viral myocarditis. Conversely, methyllycaconitine exerted an effect exactly opposite to that of nicotine (Cheng et al., 2014). To further study the dose-related effects of nicotine in mice with viral myocarditis, Li et al. administered 0.1, 0.2, or 0.4 mg/kg thrice daily for seven or 14 consecutive days and found that the mice survived a dose-dependent increase in TNF-α, IL-1β, IL-6, and IL-17A mRNA expression and protein levels, decreased myocardial inflammation, and improved left ventricular function (Li-Sha et al., 2015).

Interestingly, these nicotine-mediated changes were independent of vagal integrity. Right cervical vagotomy was shown to inhibit the cholinergic anti-inflammatory pathway, aggravate cardiomyopathy, and impair left ventricular function, and activation of the cholinergic pathway by nicotine treatment could reverse these changes; this process was dependent on α7nAChR (Li-Sha et al., 2017). Furthermore, cardiomyocyte apoptosis is critical for the development of CVB3-induced myocarditis. In another study, nicotine inhibited apoptosis by mediating the anti-apoptotic protein survivin and caspase-3 through the α3β4nAChR/PI3K/PKB pathway. As shown in Figure 10F, adding the PI3K inhibitor LY294002 decreased survivin levels, which worsened ventricular systolic function and reduced survival in mice compared with that in the nicotine control group. In addition, nicotinic agonists reduced CVB3 replication in a dose-dependent manner *in vitro*, suggesting that nAChRs may mediate a protective mechanism in myocarditis (Li P. et al., 2019).

Nicotine has been associated with positive effects on cognition and inflammation, which may benefit individuals with neurological and immune system disorders. As a stimulant, nicotine can bind to the acetylcholine receptors on neurons to promote the release of dopamine and alleviate various neurological diseases. Anti-inflammatory effects against some diseases asre associated with the cholinergic anti-inflammatory pathway. Nicotine reduces the release of various inflammatory cytokines by binding to the macrophage surface receptors. Table 2 summarizes the positive effects of nicotine on the abovementioned diseases, its dosage, and administration.

5 Discussion

Nicotine, a well-known component of cigarettes, is associated with numerous health risks. However, recent research has suggested that nicotine may have beneficial effects, as smokers exhibit lower rates of certain diseases that appear to be associated with nicotine. Here, we provided a comprehensive review of nicotine by discussing its role in various diseases and exploring its toxicity and therapeutic potential.

The effects of nicotine associated with different diseases demonstrate its double-edged characteristics, which may be influenced by diverse factors, including pathophysiological mechanisms, tissue and cell responses, treatment duration, and dosage. Research indicates that the impact of nicotine on animals and humans is primarily mediated via nAChRs. However, it is crucial to recognize that many studies have been limited to the cellular level, potentially differing from the overall effects in the body. Further research, including clinical studies, is necessary to fully understand the therapeutic potential of nicotine in various diseases. In addition, when considering the clinical use of nicotine, safety concerns associated with its complex pharmacological effects, addictive nature, and human tolerance must be considered carefully.

As a known addictive substance, it must be considered that nicotine should be avoided from preventive use despite it shows positive effects in a variety of diseases. Besides, the control of related patches and preparations should be more stringent. In addition, treatment decisions involving patients susceptible to nicotine addiction should be more cautious in clinical applications. Last but most least, there is an urgent need to improve the awareness of the patient population.

There have been numerous reports on the mechanisms of action of nicotine in specific diseases; however, the complexity and diversity of the molecular signals involved make it challenging to cover all regulatory processes clearly and accurately. Notably, many studies have focused on specific molecular signaling pathways associated with nicotine and particular diseases, sometimes leading to conflicting conclusions. Mental diseases such as

TABLE 2 The overview of nicotine's therapeutic potential in different diseases.

Diseases	Model	Nicotine dosage	Administration	Factors	Effect	Ref.
Alzheimer's disease	APP (V717I) transgenic mice	200 μg/mL in drinking water	Oral	MAPK, NF-κB, c-*myc*, α7nAChR	Decreases accumulation of β-amyloid	Liu et al. (2007)
Parkinson's disease	Rat midbrain cultures	10 μM	*In vitro*	α7nAChR, cytosolic Ca^{2+}	Affords neuroprotection to dopamine neurons	Toulorge et al. (2011)
Attention deficit hyperactivity disorder	ADHD combined type adults	7 mg nicotine patch	Affixes to the skin	—	Improves the Stop Signal Reaction Time measure	Potter and Newhouse (2008)
Major depressive disorder	Non-smokers with and without MDD	2 mg nicotine lozenge	Dissolves in the mouth	Normalizes NAc-rACC and DS–DLPFC connectivity	Normalizes cortico-striatal connectivity	Janes et al. (2018)
Rheumatoid arthritis	TNF-α stimulated RA-FLSs	10 μM	*In vitro*	JAK2, STAT3	Downregulates production of IL-6 and MCP-1	Li et al. (2015)
Rheumatoid arthritis	Mice with collagen-induced arthritis	50 μg/mL in drinking water	Oral	α7nAChR, TNF-α	Inhibits bone degradation and reduces TNF-α expression	van Maanen et al. (2009)
Osteoarthritis	C57BL/6J mice treated with MIA	0.5 or 1 mg/kg	Intraperitoneal injection	α7nAChR-PI3K-PKB-NF-κB-MMP9	Suppress MIA-induced cartilage degradation	Teng et al. (2019)
Osteoarthritis	Male Sprague-Dawley rats treated with MIA	1 mg/kg	Intraperitoneal injection	p38-ERK-JNK, α7nAChR	Alleviates MIA-induced joint degradation	Liu et al. (2015)
Ulcerative colitis	C57BL/6J mice treated with 3% DSS	100 μg/mL in drinking water	Oral	MAdCAM-1	Attenuate leukocyte recruitment	Maruta et al. (2018)
Ulcerative colitis	C57BL/6 mice treated with 3% DSS	10 μg/kg per day	Gavage	AMPK-mTOR-p70S6K	Regulates autophagy and improves colitis	Gao et al. (2020)
Sepsis and Endotoxemia	Mice treated with cecal ligation and puncture	400 μg/kg	Intraperitoneal injection	α7nAChR-PI3K-PKB	Attenuates organ failure and suppresses inflammatory cytokines	Kim et al. (2014)
Sepsis and Endotoxemia	RAW 264.7 cells	10 μM	*In vitro*	PI3K, PKB, Nrf2	Upregulates HO-1 and provides anti-inflammatory action	Tsoyi et al. (2011)
Myocarditis	CVB3-infected neonatal rat cardiomyocytes	1 μM	*In vitro*	α3β4nAChR, PI3K, PKB	Protects cardiomyocytes from CVB3-induced apoptosis	Li et al. (2019b)

Abbreviations: MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3-kinase; NF-κB, nuclear factor kappa-B; PKB, protein kinase B; STAT3, signal transducer and activator of transcription 3; AMPK, AMP-activated protein kinase; JNK, c-Jun N-terminal kinase; JAK2, Janus kinase 2; ADHD, attention-deficit/hyperactivity disorder; MDD, major depressive disorder; APP, amyloid precursor protein; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; MCP-1, monocyte chemoattractant protein-1; heme oxygenase-1; DSS, dextran sodium sulfate; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; CVB3, coxsackievirus B3; NAc, nucleus accumbens; rACC, rostral anterior cingulate cortex; DS, dorsal striatum; DLPFC, dorsolateral prefrontal cortex; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; MIA, monosodium iodoacetate; α7nAChR, α7 nicotinic acetylcholine receptor; NF-κB, nuclear factor kappa-B; MMP9, matrix metalloproteinase nine; p38, ribonuclease P Protein Subunit p38; ERK, extracellular signal-regulated kinase; p70S6K, p70 ribosomal protein S6 kinase; mTOR, mechanistic target of rapamycin; Nrf2, nuclear factor erythroid 2-related Factor 2; HO-1, heme oxygenase-1; α3β4nAChR, α3β4 nicotinic acetylcholine receptor.

schizophrenia and MDD are particularly complex due to their unclear pathogenesis, indicating that the pharmacological effects of nicotine in these conditions are still in the exploratory stage. Deeper and more comprehensive research is eagerly anticipated in the future.

Choosing an appropriate method of administration for disease treatment can have a significant impact, ensuring that nicotine does not accumulate in healthy organs. Techniques such as direct injection and atomized administration can achieve a lower dosage and a quicker onset of effects. Inhalation therapy, which involves delivering drugs directly to the airways, has proven effective in treating asthma and COPD and may also benefit individuals with dysphagia and tremors, such as patients with PD. To ensure an effective drug dose in the bronchi and subsequent absorption into the bloodstream, drug preparations with a high proportion of fine particles and consistent and accurate dosing of the active substance are necessary (Sorino et al., 2020).

Moreover, designing precisely targeted drugs for different diseases may be a promising approach to address these issues. Recent studies have explored the synthesis of fully active nanomedicines for targeted cancer treatment (Fang et al., 2023). By incorporating an appropriate targeting group into nicotine, controlled drug release and direct drug enrichment can be achieved, thereby minimizing undesired drug accumulation in nontargeted cells and organs. Further investigation is encouraged to explore this approach and potentially facilitate the clinical use of nicotine in a more controlled and effective manner.

In conclusion, nicotine is widely recognized for its harmful effects; however, recent research has shed light on its potential therapeutic benefits in certain diseases. Consequently, it is eesential

to approach nicotine with caution and conduct comprehensive research to fully understand its effects and ensure its safe and effective clinical use. A deeper understanding of the mechanisms of action of nicotine in different diseases and the development of targeted drug delivery systems can pave the way for its use as a medicinal agent.

Author contributions

YC: Conceptualization, Writing–original draft. JS: Validation, Writing–review and editing. XW: Writing–review and editing. XZ: Validation, Writing–review and editing. HT: Validation, Writing–original draft. LH: Resources, Visualization, Writing–original draft. ZH: Visualization, Writing–original draft. YZ: Resources, Writing–original draft. JZ: Supervision, Writing–review and editing. LL: Supervision, Writing–review and editing. SZ: Supervision, Validation, Writing–review and editing.

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Conflict of interest

Authors YC, XW, XZ, HT, YZ, JZ, and SZ were employed China Tobacco Anhui Industrial Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

nAChRs	nicotinic acetylcholine receptors
SCLC	small cell lung cancer
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PKC	protein kinase C
MAPK	mitogen-activated protein kinase
α-Bgt	α-bungarotoxin
ERK	extracellular signal-regulated kinase
MEK	MAPK/ERK kinase
PI3K	phosphatidylinositol-3-kinase
NF-κB	nuclear factor kappa-B
VEGF	vascular endothelial growth factor
PKB	protein kinase B
GSK-3α	glycogen synthase kinase-3 alpha
GSK-3β	glycogen synthase kinase-3 beta
p70S6K	p70 ribosomal protein S6 kinase
4EBP-1	a binding protein for eukaryotic translation initiation factor 4E
FKHR	a member of the forkhead transcription factor family
DHβE	dihydro-β-erythroidine
HB-EGF	heparin binding-epidermal growth factor
EGFR	epidermal growth factor receptor
cAMP	cyclic adenosine monophosphate
TH	tyrosine hydroxylase
AP-1	activator protein-1
COX-2	cyclooxygenase-2
PGE2	prostaglandin E2
ECs	endothelial cells
SMCs	smooth muscle cells
LPS	lipopolysaccharide
ACE2	angiotensin-converting enzyme 2
VSMC	vascular smooth muscle cells
PDGF	platelet-derived growth factor
MMPs	matrix metalloproteinases
STAT3	signal transducer and activator of transcription 3
ROS	reactive oxygen species
NLRP3	NOD-like receptor thermal protein domain associated protein 3
IL	interleukin
ASC	apoptosis-associated speck-like protein containing CARD
AMPK	AMP-activated protein kinase
COPD	chronic obstructive pulmonary disease
PNE	prenatal nicotine exposure
TRPCs	transient receptor potential canonicals
JNK	c-Jun N-terminal kinase
RB	retinoblastoma protein
PPARγ	peroxisome proliferator-activated receptor γ
AMD	age-related macular degeneration
PLD	phospholipase D
OA	osteoarthritis
HIF-2α	hypoxia-inducible factor-2α
JAK2	Janus kinase 2
PIN1	peptidyl-prolyl cis/trans isomerase NIMA-interacting protein 1
PEDF	pigment epithelium-derived factor
AD	Alzheimer's disease
PD	Parkinson's disease
ADHD	attention-deficit/hyperactivity disorder
MDD	major depressive disorder
TEA	tetraethyl ammonium
Aβ	amyloid beta
APP	amyloid precursor protein
CaM	calmodulin
IκBα	nuclear factor kappa B alpha
SNP	single-nucleotide polymorphisms
NAc	nucleus accumbens
rACC	rostral anterior cingulate cortex
DS	dorsal striatum
DLPFC	dorsolateral prefrontal cortex
UC	ulcerative colitis
RA	rheumatoid arthritis
FLS	fibroblast-like synoviocytes
MCP-1	monocyte chemoattractant protein-1
TLR	toll-like receptor
HMGB1	high-mobility group box 1
HO-1	heme oxygenase-1
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
CVB3	coxsackievirus B3
IFN-γ	interferon-γ
TNF-α	tumor necrosis factor-alpha
mTOR	mechanistic target of rapamycin
iNOS	inducible nitric oxide synthase
E2F	early two factor.

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EDITED BY
Massimo Grilli,
University of Genoa, Italy

REVIEWED BY
Yu Tang,
Gabaeron, Inc., United States
Mary-Louise Risher,
Marshall University, United States

*CORRESPONDENCE
Joanna Jacobus
✉ jjacobus@health.ucsd.edu

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Nicotine use during late adolescence and young adulthood is associated with changes in hippocampal volume and memory performance

Joseph P. Happer[1], Kelly E. Courtney[1], Rachel E. Baca[1], Gianna Andrade[1], Courtney Thompson[1], Qian Shen[2], Thomas T. Liu[3,4] and Joanna Jacobus[1]*

[1]Department of Psychiatry, University of California, San Diego, San Diego, CA, United States, [2]Department of Neurosciences, University of California, San Diego, San Diego, CA, United States, [3]Center for Functional MRI, University of California, San Diego, San Diego, CA, United States, [4]Department of Radiology, University of California, San Diego, San Diego, CA, United States

Background: With the advent of electronic nicotine delivery systems, the use of nicotine and tobacco products (NTPs) among adolescents and young adults remains high in the US. Use of e-cigarettes additionally elevates the risk of problematic use of other substances like cannabis, which is often co-used with NTPs. However, their effects on brain health, particularly the hippocampus, and cognition during this neurodevelopmental period are poorly understood.

Methods: Healthy late adolescents/young adults ($N = 223$) ages 16–22 completed a structural MRI to examine right and left hippocampal volumes. Memory was assessed with the NIH Toolbox Picture Sequence Memory Test (PSMT) and Rey Auditory Verbal Learning Test (RAVLT). Cumulative 6-month NTP and cannabis episodes were assessed and modeled continuously on hippocampal volumes. Participants were then grouped based on 6-month NTP use to examine relationships with the hippocampus and memory: current users (CU) endorsed weekly or greater use; light/abstinent users (LU) endorsed less than weekly; and never users (NU).

Results: NTP use predicted larger hippocampal volumes bilaterally while cannabis use had no impact nor interacted with NTP use. For memory, larger left hippocampal volumes were positively associated with PSMT performance, RAVLT total learning, short delay and long delay recall for the NU group. In contrast, there was a negative relationship between hippocampal volumes and performances for LU and CU groups. No differences were detected between NTP-using groups.

Conclusion: These results suggest that the hippocampus is sensitive to NTP exposure during late adolescence/young adulthood and may alter typical hippocampal morphometry in addition to brain-behavior relationships underlying learning and memory processes.

KEYWORDS

hippocampus, memory, nicotine, vaping, adolescence, young adult

1 Introduction

Rates of nicotine/tobacco-related product (NTP) use remain high among U.S. adolescents and young adults despite declining use of traditional combustible cigarettes (Gentzke, 2019). With the advent of electronic nicotine delivery systems, vaping has dramatically increased over the past several years (Johnston et al., 2020). Roughly 21% of 12th graders and approximately 14% of young adults reported e-cigarette usage within the past month in recent studies (Miech et al., 2023; Patrick et al., 2023). Vaping allows for easy consumption across the day often leading to increased use and intensity (Cerdá et al., 2020; Vogel et al., 2020). This is particularly concerning among adolescents who may be more vulnerable to nicotine dependence even after minimal exposure (DiFranza et al., 2000, 2007; Lin et al., 2022). Use of e-cigarettes has also been associated with increased risk for problematic use of other substances such as cannabis (Cobb et al., 2018; Fadus et al., 2019), which is also commonly co-used with NTPs (Knapp et al., 2019; Moustafa et al., 2022). Indeed, with reports of NTP and cannabis co-use among late adolescents and young adults ranging anywhere from 21% to over 50% in the past month (Cobb et al., 2018; Dunbar et al., 2020; McCauley et al., 2024), understanding the effects of NTPs in the context of cannabis use is essential, particularly as state and local laws may influence patterns of co-use (Wang et al., 2016).

Adolescence is a critical period of neurodevelopment that extends into early adulthood, marked by significant changes in gray matter tissue. This phase involves the maturation of cortical and subcortical brain structures through processes including synaptic pruning and cortical thinning (Spear, 2013; Wierenga et al., 2014). Use of substances such as nicotine during this period may alter these developmental trajectories (Akkermans et al., 2017; Chaarani et al., 2019). Studies using structural magnetic resonance imaging (MRI) to examine brain health in nicotine-using adolescents/young adults have noted that nicotine use in younger populations has been associated with changes in cortical regions, such as reduced thickness in the medial prefrontal, insular, parahippocampal, and temporal regions (Li et al., 2015; Dai et al., 2022; Hernandez Mejia et al., 2024). Alterations in subcortical regions have also been noted, including smaller amygdala and thalamus volumes and larger striatum volumes (Li et al., 2015; Yu et al., 2018). These changes in adolescent brain structure have been observed even after minimal nicotine exposure (Chaarani et al., 2019), and animal models suggest that low, intermittent doses of nicotine during adolescence can have a lasting impact on brain health and functioning that continues later into adulthood (Trauth et al., 2000; Abreu-Villaça et al., 2003; Oliveira-da-Silva et al., 2009; Leslie, 2020). However, few studies have examined the structural integrity of the hippocampus in NTP-using adolescents and young adults, and those that have report smaller volumes in NTP users (Harper et al., 2023) or no differences (Filbey et al., 2015) compared to non-users. Notably, these studies were conducted in young adults who primarily smoked traditional combustible cigarettes, which likely result in greater toxic exposure compared to the e-cigarettes more commonly used today (Rubinstein et al., 2018; Marques et al., 2021; Lin et al., 2022; Wade et al., 2022).

Within neurobiological models of substance use and addiction, the hippocampus is heavily implicated in the development and maintenance of substance use disorders (Volkow et al., 2019) as it modulates reinforcement learning and episodic memory of rewarding stimuli (Subramaniyan and Dani, 2015), and these processes may be especially heightened in adolescents (Yuan et al., 2015). Adolescents and young adults who regularly use NTPs have demonstrated impairments on measures of hippocampal-dependent processes, such as working memory, verbal memory, and attention compared to their non-using counterparts (Jacobsen et al., 2005, 2007a,b,c; Fried et al., 2006; Colby et al., 2010; Filbey et al., 2015; Treur et al., 2015; Wade et al., 2021; Dai et al., 2022). Functional neuroimaging studies have similarly found alterations in hippocampal and parahippocampal activation elicited by working memory (Jacobsen et al., 2007a,b) and smoking-related cues (Rubinstein et al., 2011; Chen et al., 2018) in NTP-using adolescents. Importantly, nicotine binds to nicotinic acetylcholine receptors (nAChRs), which are found in abundance throughout the hippocampus (Zeid et al., 2018), and stimulation of these nAChRs may enhance synaptic connections with other regions involved in addiction such as the nucleus accumbens (Subramaniyan and Dani, 2015). Hippocampal structure (Lorenzetti et al., 2019) and functioning (Filbey et al., 2015; Scott et al., 2018; Jacobus et al., 2019) are also impacted in cannabis-using adolescents as endocannabinoid receptors are also widely distributed in the hippocampus (Mechoulam and Parker, 2013); yet, few studies have examined how cannabis-related hippocampal alterations may also be modulated by nicotine co-use (Filbey et al., 2015). Thus, it is important to consider the impact of NTP use on hippocampal integrity in the context of cannabis co-use, given the high prevalence of co-use among adolescents/young adults (Cobb et al., 2018).

In light of these considerations, the aim of the current study was to examine the associations between recent NTP use, primarily in the form of e-cigarette use, and bilateral hippocampal volume estimates in a sample of adolescent and young adults aged 16–22. Additionally, it was investigated whether cannabis use mediates the relationship between NTP use and hippocampal volume estimates in this age group. Relationships between nicotine use, hippocampus volumes, and performances on measures of verbal and non-verbal learning and memory were also investigated. It was hypothesized that greater cumulative NTP use would be associated with smaller hippocampal volumes, and nicotine would negatively impact hippocampal-based memory assessments.

2 Methods

2.1 Participants and procedures

Two hundred and twenty-three participants ages 16–22 were recruited as part of a study on the effects of nicotine and cannabis co-use on brain structure and function during adolescence/young adulthood. As previously reported (Courtney et al., 2020, 2022), participants were recruited via flyers posted physically and electronically at schools, community colleges, four-year universities, and social media sites targeting San Diego County. Initial recruitment was stratified based on use of NTP, cannabis products, or both during the previous 6-month period to ensure variability in NTP and cannabis use. For the purposes of examining the relationship between hippocampal volume and memory performances, participants were categorized into three groups based on 6-month NTP frequency alone: Current Users (CU) who endorsed ≥26 NTP use episodes (~at least weekly); Light/Abstinent Users (LU) who endorsed <26 use

episodes (< weekly use); and Never Users (NU) who endorsed having never used NTP in their lifetime. NTP use was defined as the use of any combination of electronic cigarettes (e.g., vape pens, e-hookah), combustible cigarettes, hookah with tobacco, tobacco pipe, cigars (including blunts, spliffs), snus, smokeless tobacco, chew, snuff, and/or nicotine replacement.

Exclusion criteria included >10 lifetime episodes of illicit substance use; lifetime DSM-5 psychiatric diagnoses other than tobacco and/or cannabis use disorder; acute influence of cannabis or alcohol use at study visit; use of any psychoactive medications; major medical problems; MRI contraindications; or history of prenatal substance exposure or developmental disability.

Participants completed a single 4-h session consisting of a battery of interviews, self-report assessments covering demographic information, mental health, substance use, and neurocognitive functioning, which was followed by an MRI session. Before beginning the study session, all participants gave written informed consent (≥18 years old) or parental consent and participant assent (<18 years old). Participants were asked to refrain from using cannabis and alcohol 12 h prior to the appointment, which was confirmed with oral fluid, urine, and breathalyzer. Urine samples were used to confirm abstinence from illicit substances. Participants abstained from caffeine for at least 30 min prior to MRI scanning. They were not required to abstain from NTP use to avoid nicotine withdrawal effects during testing. Time of last NTP use was documented. All procedures were approved by the University of California, San Diego Human Research Protections Program.

2.2 Measures

Demographic data (e.g., age, sex at birth, race/ethnicity, education) were obtained from a psychosocial interview. To assess quantity and frequency of NTP and cannabis use, the Customary Drinking and Drug Use Record structured interview (Brown et al., 1998) was used, including a modification to include additional nicotine and cannabis questions (Jacobus et al., 2018; Karoly et al., 2019a,b). Past 6 months and lifetime use were measured in terms of independent episodes, allowing for multiple uses to be reported within a single day (e.g., first thing in the morning, again before bed). Participants were asked to provide additional details related to each substance reported including age at first use and onset of regular (weekly) use. Alcohol use was queried for the previous 30 days. For this study, individuals who reported having never used a substance (i.e., NTP, cannabis, alcohol) were recorded as having zero episodes during the respective timeframes.

2.3 Memory assessment

As part of a comprehensive neurocognitive battery, participants completed the Picture Sequence Memory Test (PSMT) from the National Institutes of Health (NIH) toolbox cognition battery (Weintraub et al., 2013) and the Rey Auditory Verbal Learning Task (RAVLT, Schmidt, 1996). For this study, the primary test of interest from the NIH toolbox was the PSMT; however, participants completed all seven cognitive tasks of the toolbox battery, which were later used to compute crystalized composite scores (derived from Picture Vocabulary and Oral Reading tests). The PSMT is a measure of episodic memory where participants had to recall a sequence of delayed pictures. The task was completed using the NIH Toolbox app on 3rd generation iPad Air devices (10.5 in). Participants were seated upright and used their dominant index finger to make each response. To prevent participants from inadvertently skipping through instructions, a one-second touch-and-hold button was required to advance to the next task. Population-adjusted scores, which adjusted for age, sex, race, ethnicity, and education level, were used in the present analyses.

For the RAVLT, participants complete five learning trials during which they were read a list of 15 words and asked to recall the list at the end of each trial. A new, second list was read, and then participants were asked to again recall the original list after this short delay. After 30 min, they were again asked to recall the original list. Total raw score over all learning trials, short delay recall, and long delay recall were examined.

2.4 Imaging acquisition and processing

Participants were scanned on a 3.0 Tesla GE Discovery MR750 scanner with a 32-channel receive head coil at the UCSD Center for Functional MRI. A high-resolution T1-weighted anatomical fast spoiled gradient echo (FSPGR) scan was acquired with TI/TE/TR = 1060/2/2500 ms, 256×256 matrix, flip angle = 8°, FOV = 256 mm, 1.0 mm^3 voxels. Brain images for each participant were spatially normalized, field-bias corrected, and segmented using the Freesurfer pipeline (version 6.0, Fischl et al., 2002, 2004). Right and left hippocampal volumes and an estimate of total brain volume ("BrainSegVolNotVent") were extracted for analyses.

2.5 Data analyses

2.5.1 Hippocampal volume

Data analyses were conducted using R (v4.3.2). Changes in bilateral hippocampal volumes were examined using linear regressions that modeled cumulative 6-month NTP use episodes, cumulative 6-month cannabis use episodes, and their interaction as continuous variables, while controlling for demographic factors (i.e., age, sex), past 30-day alcohol use, and estimated total brain volume as covariates in the model. Follow-up analyses explored potential moderators of this relationship including age of NTP initiation and recency of NTP use within those who reported lifetime NTP use. Combustible cigarette usage was similarly explored as a possible moderator given the potential for greater toxic exposure and addiction severity (Rubinstein et al., 2018; Marques et al., 2021; Lin et al., 2022; Wade et al., 2022).

The large range of both cannabis and NTP cumulative use episodes raises the possibility of a single or several data points having significant leverage on the models (Belsley et al., 2005). If a substance use variable was significant within the model, the variable was examined for influential points using DFBETAS. Highly influential points were defined as those whose DFBETAS were above the calculated threshold ($2 \div \sqrt{n}$) of 0.135 (Belsley et al., 2005). Models were then rerun without those data points.

2.5.2 Hippocampal volume and memory performance

Participants were then grouped based on NTP status as described above (i.e., Current Users [CU], Light/Abstinent Users [LU], and Never Users [NU]) to examine relationships with hippocampal volume and memory. Group characteristics were compared using ANOVA and chi-square tests for continuous and categorical variables, respectively. The contribution of NTP group status, left and right hippocampal volumes, and their interaction to demographically-adjusted T-scores from the PSMT were examined using individual linear regressions for each hemisphere, controlling for NIH toolbox crystalized composite scores, 6-month cannabis use, and 30-day alcohol use, which were modeled as continuous covariates in the model. Raw RAVLT Total Learning, Short Delay, and Long Delay were similarly examined as outcomes with individual linear regressions, controlling for age, sex, crystalized composite score, cannabis use, and alcohol use as covariates in the model. A statistically significant threshold of $p < 0.05$ was set for all analyses.

3 Results

3.1 Participants

The final sample ($N = 223$) was roughly split on sex at birth (54% male) and approximately 50% self-identified as White (see Table 1). Between groups comparisons indicated that relative to both NTP use groups, NU were younger (p's < 0.001), had fewer years of education (p's < 0.01), fewer alcohol use episodes over the past 30 days (p's < 0.0001), and fewer cannabis use episodes over the past 6 months (p's < 0.0001). Additionally, NU had more females compared to CU ($\chi^2 = 6.8$, $p = 0.009$), while there were no differences in sex between CU and LU ($p > 0.07$). The NTP groups were found to differ only on NTP use episodes in the past 6 months ($t = -5.3$, $p < 0.0001$) and days since last NTP use ($t = 4.5$, $p < 0.001$), as anticipated.

3.2 Hippocampal volume estimates

Regression models examined the linear contributions of cumulative NTP and cannabis use episodes and their interaction on bilateral hippocampal volumes, controlling for age, sex, past 30-day alcohol use, and estimated brain volume. The overall models were significant for both left, $F(7,213) = 27.5$, $p < 0.0001$, $R^2 = 0.46$, and right hippocampal volumes, $F(7,213) = 24.4$, $p < 0.0001$, $R^2 = 0.43$. As seen in Figure 1, results indicated that greater NTP use episodes in the past 6 months predicted larger hippocampal brain volumes bilaterally (Left: $B = 0.036$, $t = 2.4$, $p = 0.017$; Right: $B = 0.042$, $t = 2.6$, $p = 0.011$), while cannabis use episodes had no significant impact (p's > 0.2) nor was there an interaction between NTP and cannabis use (p's > 0.1). Age, alcohol use, and sex were not significant covariates for either model (p's > 0.2); however, estimated brain volume was a significant covariate for both (Left: $B = 0.002$, $t = 9.8$, $p < 0.0001$; Right: $B = 0.002$, $t = 9.4$, $p < 0.0001$). NTP age of initiation and recency of use were then explored as possible moderators within participants who reported lifetime NTP use. Neither significantly influenced the relationship between NTP use and hippocampal volumes (age of initiation: p's > 0.08; recency: p's > 0.3). Likewise, the possible contribution of combustible cigarette usage was explored in follow-up analyses; however, no significant association with hippocampal volume or NTP status by combustible product use interaction on volume was detected (p's > 0.4).

TABLE 1 Sample demographics and characteristics.

	Never Users[a] N = 67[1]	Light/Abstinent Users[b] N = 64[1]	Current Users[c] N = 92[1]	p-value[2]
Age	18.8 (±1.7)[b,c]	19.8 (±1.5)[a]	19.8 (±1.5)[a]	<0.001
% Male	29 (43%)[c]	32 (50%)	59 (64%)[a]	0.026
Race/Ethnicity				
% White	29 (43%)	32 (50%)	51 (55%)	0.11
% Hispanic	28 (42%)	28 (44%)	29 (32%)	0.2
Education (Years Completed)	12.6 (±1.7)[b,c]	13.3 (±1.3)[a]	13.3 (±1.4)[a]	0.003
NIH Toolbox Crystalized Composite (Age-Corrected)	106 (±15)	108 (±11)	105 (±11)	0.3
Alcohol Use Previous 30 Days	2.0 (±3.6)[b,c]	5.6 (±4.8)[a]	6.7 (±5.5)[a]	<0.001
Cannabis Use Episodes Previous 6 Months	29 (±69)[b,c]	167 (±186)[a]	185 (±258)[a]	<0.001
Days since last cannabis use	26 (±43)	10 (±21)	51 (±153)	0.073
Nicotine use episodes previous 6 months		4 (±6)	1,512 (±2,278)	<0.001
Age of onset of nicotine use		16.58 (±2.24)	16.55 (±1.88)	>0.9
Years of nicotine use		3.17 (±2.60)	3.29 (±1.93)	0.7
Days since last nicotine use		253 (±538)	3 (±6)	<0.001
Number of cigarettes previous 6 months		3 (±3)	125 (±521)	0.3

Superscript letters denote significant group differences ($p < 0.05$). [1]Mean (±SD); n (%). [2]One-way ANOVA; Pearson's Chi-squared test.

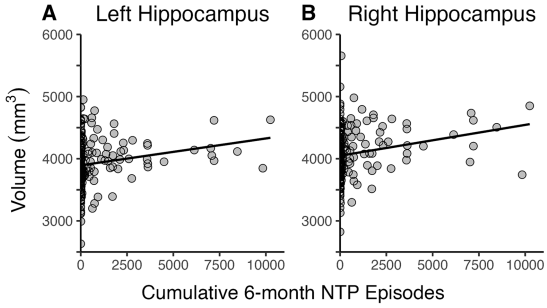

FIGURE 1
Scatterplots depicting relationship between cumulative 6-month NTP use and bilateral hippocampal volumes. Greater cumulative 6-month nicotine and tobacco product (NTP) use was associated with larger left **(A)** and right **(B)** hippocampal volumes.

Data from four participants was found to be highly influential for both the left and right hippocampi models with DFBETAS exceeding the threshold of 0.135. Models were rerun without these data points, and the results indicated that NTP use was still a significant predictor of bilateral hippocampal volumes (p's < 0.05).

3.3 Relationship between hippocampal volume, NTP use, and memory performances

3.3.1 NIH toolbox PSMT

Individual linear regression models assessed the relationship between left and right hippocampus volumes and NTP group status on PSMT performance, controlling for crystalized composite score, past 6-month cumulative cannabis use, and past 30-day cumulative alcohol use. Significant Hippocampus Volume × NTP group interactions were found for both the Left, $F(2,208) = 5.3$, $p = 0.006$, and Right, $F(2,208) = 3.2$, $p = 0.044$, hippocampi (see Figure 2A; right not shown). Follow-up analyses indicated larger hippocampal volumes were positively associated with PSMT specifically for the NU group (Left: $B = 0.012$, $t = 2.9$, $p = 0.004$; Right: $B = 0.009$, $t = 2.3$, $p = 0.023$) while significant negative relationships were observed for LU (Left: $B = -0.019$, $t = -3.2$, $p = 0.002$; Right: $B = -0.014$, $t = -2.3$, $p = 0.020$) and CU groups, though the right was only at trend level (Left: $B = -0.012$, $t = -2.1$, $p = 0.035$; Right: $B = -0.010$, $t = -2.0$, $p = 0.051$). LU and CU groups did not differ from one another ($p > 0.2$). Crystalized composite scores were a significant covariate (Left: $B = 0.233$, $t = 2.6$, $p = 0.009$; Right: $B = 0.240$, $t = 2.7$, $p = 0.008$), but neither alcohol nor cannabis use were significant (p's > 0.3).

3.3.2 RAVLT learning, short delay, and long delay

3.3.2.1 Verbal learning

Models indicated a significant Left Hippocampus Volume x NTP group interaction, $F(2,205) = 3.2$, $p = 0.043$, while a Right × NTP group interaction was not significant ($p > 0.3$). Follow-up analyses revealed that larger left hippocampal volume was a significant predictor of greater verbal learning ($B = 0.006$, $t = 2.2$, $p = 0.030$) for NU (Figure 2B). In contrast, larger left hippocampal volumes were associated with worse verbal learning for LU ($B = -0.008$, $t = -2.2$, $p = 0.031$) and CU ($B = -0.007$, $t = -2.1$, $p = 0.036$). No differences were detected between LU and CU ($p > 0.8$). This was after accounting for crystalized composite score ($B = 0.411$, $t = 17.7$, $p < 0.001$), biological sex (female as reference group: $B = -2.4$, $t = -2.0$, $p = 0.046$), and 6-month cannabis use episodes ($B = -0.010$, $t = -3.5$, $p < 0.001$). Age and alcohol use were not significant contributors (p's > 0.09).

3.3.2.2 Short delay recall

Hippocampal volume differentially contributed to verbal recall after a short delay depending on NTP group as indicated by a significant Left Hippocampus x NTP group interaction, $F(2,205) = 4.4$, $p = 0.013$. The Right Hippocampus × NTP group interaction was not significant ($p > 0.4$). For the NU group, larger left hippocampal volumes predicted greater verbal recall ($B = 0.001$, $t = 1.9$, $p = 0.056$; Figure 2C), while the larger left hippocampal volume was associated with worse performance for the LU group ($B = -0.003$, $t = -2.9$, $p = 0.004$). For the CU group, a significant difference was detected relative to the LU group ($B = 0.001$, $t = 2.1$, $p = 0.036$), while no differences were observed compared to the NU group ($p > 0.3$). Crystalized composite ($B = 0.088$, $t = 3.2$, $p = 0.002$) and cannabis use ($B = -0.002$, $t = -2.8$, $p = 0.005$) were significant covariates, while age, sex, and alcohol use were not (p's > 0.07).

3.3.2.3 Long delay recall

Finally, a similar significant Left Hippocampus × NTP group interaction was found for the number of words recalled after a long delay, $F(2,205) = 3.2$, $p = 0.044$, while no interaction was detected for the Right ($p > 0.1$). Follow-up analyses indicated the NU group again exhibited a positive relationship between hippocampal volume and the words recalled ($B = 0.002$, $t = 2.0$, $p = 0.052$; Figure 2D). In contrast, larger hippocampal volumes negatively predicted performance for LU ($B = -0.003$, $t = -2.4$, $p = 0.018$) with a similar trend for CU ($B = -0.002$, $t = -1.8$, $p = 0.073$). No differences were observed between CU and LU ($p > 0.4$). Crystalized composite ($B = 0.101$, $t = 3.3$, $p = 0.001$) and cannabis use ($B = -0.003$, $t = -2.9$, $p = 0.004$) were significant covariates, while age, sex, and alcohol use were not (p's > 0.1).

4 Discussion

In the present study, we examined associations between cumulative 6-month NTP use and bilateral hippocampal volume in the context of cumulative 6-month cannabis use in a sample of late adolescents/young adults. The results indicated that greater NTP use predicted *larger* bilateral hippocampal volumes, while co-use of cannabis had no significant impact. We then examined whether NTP use at the group level modulated the relationship between hippocampal volumes and measures of learning and memory. We found differential associations between brain and cognitive performances such that larger hippocampal volumes, particularly left, were associated with better learning and memory for individuals who had never used NTPs, while larger volumes for adolescents/young adults who had used NTPs were linked with relatively lower memory scores.

Contrary to our hypothesis, cumulative NTP use was associated with *larger* hippocampal volumes. Previous studies examining structural differences in combustible tobacco-using adolescents and

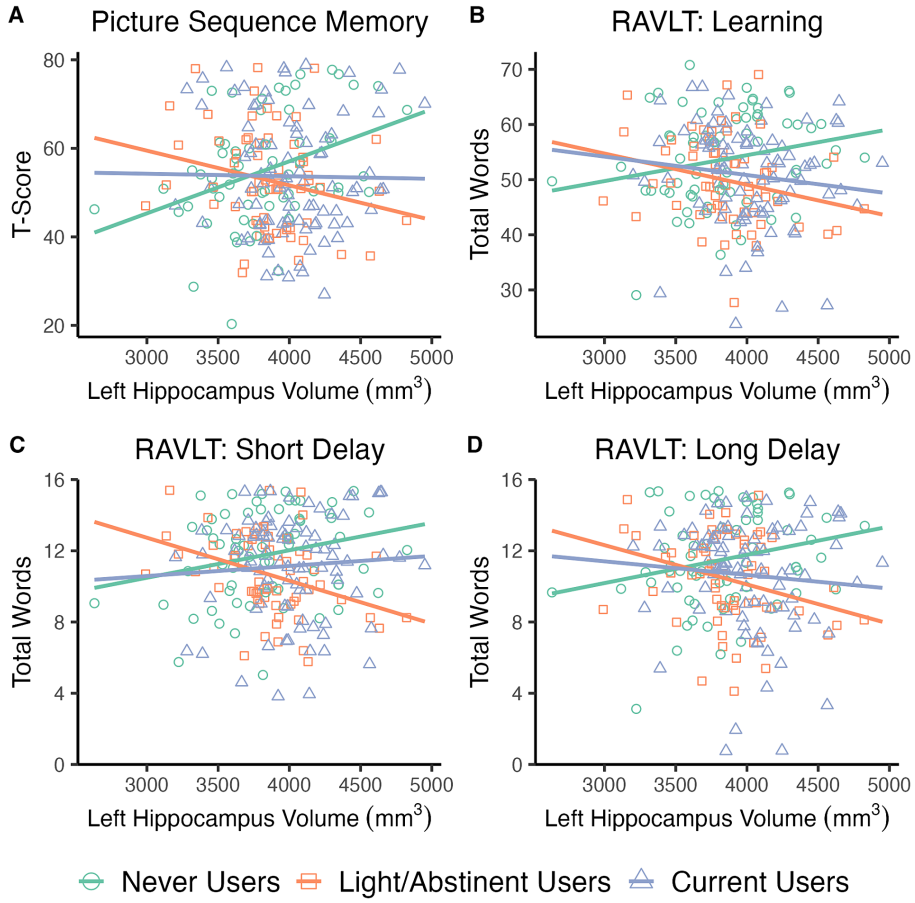

FIGURE 2
Scatterplots depicting relationship between nicotine group, hippocampal volume, and memory performance. Larger left hippocampal volumes predicted better performance for the Never Users on Picture Sequence Memory Test **(A)**, Rey Auditory Verbal Learning Test (RAVLT) total words learned across all 5 trials **(B)**, RAVLT short delay recall **(C)**, and RAVLT long delay recall **(D)**. In contrast, larger left hippocampal volumes were associated with worse performance for the Light/Abstinent Users across all measures. Larger left hippocampal volumes similarly predicted worse performance for Current Users for the Picture Sequence Memory Test, RAVLT total words, and a trend for long delay. Current Users did not differ from Never Users on short delay recall.

young adults have found *smaller* volumes (Harper et al., 2023) or no differences (Filbey et al., 2015) compared to non-using controls. Notably, however, these studies were with participants that were generally older and in their early- to mid-20s compared to the present study where the average age was ~19. Not only could these studies represent a different point in neurodevelopment (Spear, 2013), being older provides the opportunity for more years of nicotine use that could then be associated with greater cumulative effects (Leslie, 2020). Finally, use of traditional combustible cigarettes may have the potential for greater toxic exposure and addiction severity (Rubinstein et al., 2018; Marques et al., 2021; Lin et al., 2022; Wade et al., 2022). While use of combustible cigarettes did not moderate the relationship between NTP use and hippocampal volumes in the present study, the vast majority of our 16-22-year-old participants were e-cigarette users as compared to previous studies in which the samples were primary combustible users. Overall, differences in sample characteristics between the present study and those that have previously examined hippocampal volumes could provide insight into more subtle effects of NTP use, e-cigarette use in particular, during late adolescent/young adult neurodevelopment.

Nicotine exerts its effects on the brain by binding to nAChRs, which are abundant in the hippocampus (Zeid et al., 2018). Nicotinic activation of nAChRs can affect neuroimmune function by modifying microglia activity (Mahajan et al., 2021). Microglia are essential for synaptic pruning and cortical refinement during neurodevelopment (Paolicelli et al., 2011). In this context, the increased hippocampal volumes observed in the present study may represent nicotine-induced alterations in developmental trajectories, although prospective studies are needed to establish the directionality of effects. The impact on microglia may also contribute to nicotine's neurotoxic effects on neuronal structure and integrity (Mahajan et al., 2021). In animal models of adolescent nicotine exposure, nicotine was associated with an increase in biomarkers indicative of decreased cell numbers but also with markers of increased cell size within the hippocampus such that gross anatomical weight of the region remained the same (Trauth et al., 2000; Abreu-Villaça et al., 2003; Oliveira-da-Silva et al., 2009). Notably, these changes occurred not only at blood plasma nicotine levels like could be found in current smokers but even at levels similar to brief intermittent exposure, highlighting that the adolescent brain is uniquely sensitive to nicotine.

In the present study, larger hippocampal volumes, particularly left, predicted worse verbal learning and memory performances for adolescents/young adults who had used NTPs (i.e., both Light/Abstinent Users and Current Users), while larger volumes were associated with better performance for the Never User group. Verbal learning and memory has largely been demonstrated to be preferentially subserved by the left hippocampus (Frisk and Milner, 1990; Lee et al., 2002; Pryor and Veselis, 2006; Sweatt, 2010), while more spatially-oriented memory with the right (Burgess et al., 2002). In this context, left hippocampal volumes being associated with better performance on the RAVLT for NTP Never Users is consistent with the broader literature. Moreover, a similar pattern was observed for bilateral hippocampal volumes predicting better NIH PSMT performance for Never Users with it additionally tapping into spatially memory. Importantly, the general pattern of relationships between hippocampal volumes and memory performances suggests that the morphological changes within the hippocampus associated with even light nicotine use may confer a functional disadvantage. NTP use during adolescence/young adulthood has consistently been linked to impairments on hippocampal-related cognitive processes (Jacobsen et al., 2005, 2007a,b; Colby et al., 2010; Filbey et al., 2015; Treur et al., 2015; Wade et al., 2021; Dai et al., 2022) as well as alterations in hippocampal activation (Jacobsen et al., 2007a,b; Rubinstein et al., 2011; Chen et al., 2018). As noted, nicotine has neurotoxic effects on neuronal structure and integrity within the hippocampus (Trauth et al., 2000; Abreu-Villaça et al., 2003; Oliveira-da-Silva et al., 2009). Animal studies have further demonstrated that adolescent nicotine exposure impacts neuritic projections and glial densities within the hippocampus (Abreu-Villaça et al., 2003; Oliveira-da-Silva et al., 2009), which could impact neuronal connections within the hippocampus and with other regions leading to functional detriment. Indeed, several recent human DTI studies have found nicotine use during adolescence/young adulthood to be associated with alterations in white matter morphometry (Courtney et al., 2022; Wallace et al., 2024). Notably, one such finding was within the fornix, a major output tract of the hippocampus, which could contribute to overall neural inefficiency during memory and learning. Overall, nicotine use during adolescence/young adulthood appears to have a negative relationship with hippocampal morphology with potential implications for downstream behavior.

Nicotine is commonly co-used with cannabis (Knapp et al., 2019; Moustafa et al., 2022), and we sought to examine the impact of nicotine use in this context. However, we found no relationship between cannabis use and hippocampal volumes nor was there an interaction between nicotine and cannabis use. This is contrary to expectation as meta-analyses suggest that regular cannabis use may be associated with reductions in hippocampal volume (Lorenzetti et al., 2019). However, this could represent opposing effects within individuals who moderately use both nicotine and cannabis (Courtney et al., 2022). That is, nicotine could be associated with relative increases in hippocampal volume while cannabis could be associated with decreases. As the majority of the present sample used both cannabis and nicotine at least minimally, these countering effects could obscure any potential interactions, particularly at lower levels of use. Consistent with previous studies (Scott et al., 2018; Jacobus et al., 2019), we did find cannabis use to be associated with poorer performance on measures of verbal learning and memory.

The results and conclusions of this study must be considered within its limitations. The study was cross-sectional in design, which limits the ability to make causal interpretations related to nicotine use. Differences in hippocampal brain volumes could have existed prior to nicotine initiation. Though beyond the scope of this study, there is growing evidence that multigenerational substance use may contribute to brain development and influence baseline morphometry (Cservenka, 2016; Henderson et al., 2018; Gonçalves et al., 2024). Therefore, large, longitudinal studies such as the Adolescent Brain Cognitive Development (ABCD) Study (Volkow et al., 2018) that examine and follow adolescents prior to and after initiation of nicotine use will be essential for determining relationships between nicotine and brain health as well as the contributions of transgenerational substance use to developmental trajectories. Additionally, while alcohol was controlled for in this study and was not a significant covariate, alcohol could still have an impact on brain development, including the hippocampus (Jacobus and Tapert, 2013; Squeglia et al., 2014; Tapert and Eberson-Shumate, 2022). Large studies such as ABCD will be well-powered to assess the impact of polysubstance use (e.g., co-use of alcohol with NTPs and cannabis) on brain morphometry. We also failed to find any relationship between hippocampal volumes and cannabis use, contrary to the extant literature (Lorenzetti et al., 2019), although a large percentage of our sample report use of both cannabis and nicotine. Thus, our findings may not align with existing studies that focus on individuals who engage in single substance cannabis use only.

In sum, the present study examined changes in hippocampal morphometry and function associated with NTP use in a sample of adolescent/young adults. The results indicate that greater nicotine use predicted increased bilateral hippocampal volume which could represent alterations in neurodevelopmental trajectories. Importantly, larger volumes in adolescents/young adults who had ever used NTPs were associated with worse performance on cognitive processes dependent on hippocampal integrity. While these findings were examined in the context of cannabis co-use, no interaction between NTP and cannabis nor an effect of cannabis alone was detected, though this could suggest opposing effects of the two substances. Greater understanding of the impact of nicotine use on brain health during this vulnerable developmental period are necessary for guiding public health policy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by University of California, San Diego Human Research Protections Program. The studies were conducted in accordance with the local legislation and

institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JH: Formal analysis, Writing – original draft, Writing – review & editing. KC: Methodology, Writing – review & editing. RB: Data curation, Investigation, Writing – review & editing. GA: Data curation, Investigation, Writing – review & editing. CT: Data curation, Investigation, Writing – review & editing. QS: Data curation, Writing – review & editing. TL: Writing – review & editing. JJ: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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EDITED BY
Richard Lowell Bell,
Indiana University Bloomington, United States

REVIEWED BY
Penelope Truman,
Massey University, New Zealand
Karine Guillem,
Centre National de la Recherche Scientifique (CNRS), France
Zheng-Ming Ding,
The Pennsylvania State University, United States

*CORRESPONDENCE
Adriaan W. Bruijnzeel,
awbruijn@ufl.edu

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Sex differences in nicotine intake and relapse behavior in nicotine-dependent adult wistar rats

Ranjithkumar Chellian, Azin Behnood-Rod and Adriaan W. Bruijnzeel*

Department of Psychiatry, University of Florida, Gainesville, FL, United States

Introduction: Tobacco use is highly addictive and the leading cause of premature mortality in the world. Long-access nicotine self-administration procedures in rats closely model human smoking behavior. However, significant gaps remain in our understanding of sex differences in the development of dependence and relapse in adult rats.

Methods: In the present study, we investigated operant responding for both nicotine and saline and the development of dependence in adult rats of both sexes. The rats had daily access to nicotine or saline for 6 h per day, 7 days per week. Dependence was assessed by evaluating precipitated and spontaneous somatic withdrawal signs, measuring locomotor activity in the small open field test, and assessing anxiety-like behavior in the large open field and elevated plus maze test. The sucrose preference test was used to determine if cessation of nicotine intake leads to anhedonia. It was also investigated if a period of forced abstinence affects nicotine-seeking behavior.

Results: This study showed that nicotine intake is higher in females than in males when given daily long access to nicotine. Daily nicotine self-administration led to more precipitated and spontaneous somatic withdrawal signs compared to saline self-administration, with no sex differences observed. In addition, cessation of nicotine intake led to a similar increase in activity in both males and females in the small open field test. However, cessation of nicotine intake did not increase anxiety-like behavior or cause anhedonia in either males or females. A time course analysis revealed that the nicotinic acetylcholine receptor antagonist mecamylamine affected nicotine intake differently in males and females, increasing intake in males and decreasing intake in females. Three weeks of forced abstinence led to an increase in nicotine and saline-seeking behavior. The rats exhibited more nicotine than saline seeking, and the females displayed more nicotine seeking than the males.

Discussion: The present findings demonstrate that females self-administer more nicotine and display more nicotine-seeking behavior than males. Furthermore, there were no sex differences in somatic withdrawal signs or activity during

abstinence from nicotine. This work underscores the importance of considering sex differences across various aspects of addiction, including intake and relapse, when developing novel treatments for tobacco use disorder.

KEYWORDS

smoking, nicotine, self-administration, sex, dependence, withdrawal, drug seeking

1 Introduction

Tobacco use disorder is characterized by a loss of control over smoking, a strong desire to smoke, craving for cigarettes, and withdrawal signs upon a smoking cessation attempt (American Psychiatric Association, 2013). Worldwide, there are about 1.3 billion people who use tobacco products, and eighty percent of them live in low- and middle-income countries (WHO, 2021). Smoking is the leading preventable cause of disease and death in the world. Smoking increases the risk for a wide range of diseases, including chronic obstructive pulmonary disease, cardiovascular disease, cancer, and Alzheimer's disease (Ott et al., 1998; Ambrose and Barua, 2004; Sasco et al., 2004; Forey et al., 2011). Furthermore, smoking induces changes in the brain that increase the risk for psychiatric disorders, including depression (Bruijnzeel et al., 2011; Bruijnzeel, 2012a). Although the use of electronic cigarettes is on the rise, smoking remains the primary method of nicotine consumption (Cornelius et al., 2020). There is an urgent need for new animal models that allow the evaluation of potential smoking cessation treatments to develop novel therapies for tobacco-use disorder. Because in many countries, more than thirty percent of smokers are female, it is imperative to evaluate whether there are sex differences in nicotine intake, the development of dependence, and relapse in animal models of tobacco use disorder (Statista, 2021).

Nicotine is highly addictive and the primary psychoactive compound in tobacco and e-liquid that sustains smoking and vaping (Stolerman and Jarvis, 1995; Kinnunen et al., 2019). Animal models have been developed to study both the positive and negative reinforcing properties of nicotine. Nicotine induces mild euphoria and cognitive enhancement, which significantly contributes to the initiation and maintenance of smoking (Wesnes and Warburton, 1983; Pomerleau and Pomerleau, 1992). The negative reinforcing properties of nicotine, including anhedonia, anxiety, and craving, also play a pivotal role in the maintenance of smoking and contribute to relapse following abstinence (Bruijnzeel, 2012b). The methods for intravenous nicotine self-administration in rats were established during the late 1970s (Lang et al., 1977; Hanson et al., 1979). Some of the first studies showed that blockade of nicotinic acetylcholine receptors (nAChRs) and dopamine D1 receptors decreases the reinforcing properties of nicotine in rats with short access (1 h/day) to nicotine (Corrigall and Coen, 1989; 1991). Blockade of nAChRs and dopamine D1 receptors also prevents the development of nicotine-induced place preference in rats (Fudala et al., 1985; Acquas et al., 1989). Furthermore, the United States Food and Drug Administration (FDA)-approved smoking cessation drugs bupropion and varenicline decrease nicotine intake in rats with short access to nicotine (Bruijnzeel and Markou, 2003; O'Connor et al., 2010). The negative reinforcing properties of nicotine have been mainly investigated in rats that received nicotine noncontingently via minipumps. Cessation of noncontingent nicotine administration leads to somatic withdrawal signs, anhedonia, hyperalgesia, and cognitive impairments (Shoaib and Bizarro, 2005; Rylkova et al., 2008; Bruijnzeel et al., 2009; Yohn et al., 2014; Bagdas et al., 2018). Treatment with bupropion and varenicline diminishes somatic and affective withdrawal signs associated with the cessation of noncontingent nicotine administration (Cryan et al., 2003; Igari et al., 2013).

Adaptations induced by drug self-administration may more closely align with those observed in drug users than those induced by noncontingent drug administration (Markou et al., 1999; Jacobs et al., 2003; Kuhn et al., 2019; Schweppe et al., 2020). Therefore, there is growing interest in studying the development of dependence in animals that self-administer nicotine. Paterson and Markou compared the development of dependence in rats with short (1 h/day, 5 and 7 days/week) and long (6 h/day, 7 days/week) access to nicotine (Paterson and Markou, 2004). This study showed that animals with daily access to nicotine (1 and 6 h) developed dependence but not those that self-administered nicotine only 5 days per week. On a similar note, the nAChRs antagonist mecamylamine precipitated somatic withdrawal signs in rats with daily access but not in those that did not have daily access to nicotine. Thus, daily nicotine intake is critical for the development of dependence in rodents. The dose of nicotine also plays a role in the development of nicotine dependence. O'Dell et al. (2007) demonstrated that, in rats with daily long access to nicotine, those self-administering 0.06 mg/kg/inf of nicotine had higher levels of nicotine intake and displayed more mecamylamine-precipitated withdrawal signs compared to those self-administering 0.015 mg/kg/inf of nicotine. It is unlikely that doses higher than 0.06 mg/kg/inf lead to more severe dependence in rats, as nicotine intake only slightly increases when the dose is increased above 0.06 mg/kg/inf (Donny E. et al., 2000; O'Dell and Koob, 2007). These findings indicate that rats are most likely to develop dependence when they have daily access to nicotine and self-administer the 0.06 mg/kg/inf dose or slightly higher doses.

Previous studies demonstrated that daily access to nicotine leads to dependence in adult male rats (for a review on this topic, see (Chellian et al., 2022a)). However, there remain many gaps in our understanding of the development of dependence and relapse in adult animals that self-administer nicotine. For example, it is not known whether there are sex differences in nicotine intake in animals with long access to nicotine, and if the development of nicotine dependence follows the same trajectory in male and female rats. Furthermore, important control groups that self-administered saline were not included in prior studies. Therefore, in the present study, we investigated operant responding for both nicotine and saline and the development of dependence in adult male and female rats. The rats had daily access to nicotine or saline for 6 h per day, 7 days per week. The development of dependence was investigated

by assessing precipitated and spontaneous somatic withdrawal signs and measuring anxiety-like behavior in the large open field and elevated plus maze test (Rylkova et al., 2008; Bauzo and Bruijnzeel, 2012; Knight et al., 2021). Furthermore, the sucrose preference test was conducted to determine if cessation of nicotine intake leads to anhedonia (Primo et al., 2023). At the end of the study, it was investigated if a period of forced abstinence affects nicotine and saline seeking behavior. The present study showed that nicotine intake and nicotine seeking is higher in females than males with daily long access to nicotine. Furthermore, nicotine self-administration led to the development of dependence, as indicated by somatic withdrawal signs, but no sex differences were observed. In addition, cessation of nicotine intake increased activity in the small open field test, but it did not lead to an increase in anxiety-like behavior or anhedonia in the males and the females.

2 Materials and methods

2.1 Animals

Adult male (200–250 g, 8–9 weeks of age; N = 28) and female (175–225 g, 8–9 weeks of age; N = 28) Wistar rats were purchased from Charles River (Raleigh, NC). The rats were housed with a rat of the same sex in a climate-controlled vivarium on a reversed 12 h light-dark cycle (light off at 7 a.m.). The rats were handled for 2–3 min per day for several days before the food training sessions. During the food training period, the rats were singly housed and remained singly housed for the rest of the study. Prior to the onset of the studies, food was available *ad libitum* in the home cage. During the food training, and the nicotine and saline self-administration sessions, the rats were fed 90–95 percent of their *ad libitum* food intake (males: 23 g, females: 19 g). A mild level of food restriction facilitates food training and nicotine self-administration in rats (Donny et al., 1998; Garcia et al., 2014). Water was available *ad libitum* throughout the study except for 1 day when the rats had only access to two bottles with a 2% w/v sucrose solution (see sucrose preference test for details). The experimental protocols were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC). All experiments were performed in accordance with relevant IACUC guidelines and regulations and in compliance with ARRIVE guidelines 2.0 (Animal Research: Reporting of *In Vivo* Experiments).

2.2 Drugs

For intravenous self-administration, (−)-nicotine hydrogen tartrate (NIDA Drug Supply Program) was dissolved in sterile saline (0.9% sodium chloride), and the pH was adjusted to 7.2 ± 0.2 using 1 M NaOH. The rats self-administered 0.03 or 0.06 mg/kg/inf of nicotine in a volume of 0.1 mL/inf. Nicotine doses are expressed as base. For drug treatments, (−)-nicotine hydrogen tartrate and mecamylamine hydrochloride (NIDA Drug Supply Program) were dissolved in sterile saline and administered subcutaneously (SC) in a volume of 1 mL/kg body weight. The nicotine dose is expressed as base and the mecamylamine dose is expressed as salt.

2.3 Experimental design

All rats in this study underwent identical procedures and successive tests, with the only difference being that the control groups self-administered saline, while the experimental groups self-administered nicotine. A schematic overview of the experimental design is depicted in Figure 1. Before the catheter surgeries, rats underwent 10 days of food training. After 5 days of food training, the rats were singly housed and remained so for the duration of the study. Upon completion of the training, the rats were prepared with catheters in the jugular vein. The catheter surgery was followed by a 7-day recovery period. The male (N = 28) and female (N = 28) rats were each divided into two self-administration groups: nicotine (N = 14/sex) and saline (N = 14/sex). The rats initially self-administered nicotine or saline for five 1-h baseline sessions. This was followed by 36 days of daily 6-h long access self-administration sessions. To investigate the development of dependence during this period, precipitated somatic withdrawal signs were examined by administering mecamylamine after the 15th, 22nd, and 29th self-administration session. After thirty-six self-administration sessions, there was a 14-day forced abstinence period. To further investigate the development of dependence, spontaneous withdrawal was examined during this forced abstinence period. Spontaneous somatic withdrawal signs were recorded 16–17 h after the last self-administration session. Immediately after counting somatic signs, anxiety-like behavior was measured in the elevated plus-maze test, and then locomotor activity was assessed in the small open field test. Furthermore, 48 h after the last nicotine and saline self-administration session, anxiety-like behavior was measured in the large open field test. To determine if the cessation of nicotine self-administration leads to anhedonia, the sucrose preference test was conducted from withdrawal day 3 to day 8. Subsequently, it was investigated whether a 14-day forced abstinence period affects nicotine and saline intake. After the abstinence period, the rats were allowed to self-administer nicotine and saline for 6 days. After these six self-administration sessions, the effects of mecamylamine and nicotine treatment on nicotine and saline self-administration was investigated. This was followed by a 21-day forced abstinence period, after which nicotine and saline-seeking behavior was investigated. Nicotine and saline-seeking behavior were investigated during a 6-h session. The rats were fed 90–95 percent of their *ad libitum* food intake during food training (Weeks 1–3, Figure 1), the first self-administration period (Weeks 5–11), and the second self-administration period (Weeks 13–15). The rats were fed *ad libitum* before the onset of food training, during the period when the catheter surgeries were conducted (Weeks 3–5), and during the first (Weeks 11–13) and second (Weeks 15–18) forced abstinence period.

2.4 Food training

Rats were trained to press a lever for food pellets in operant chambers that were placed in sound- and light-attenuated cubicles (Med Associates, St. Albans, VT). Responding on the active lever resulted in the delivery of a food pellet (45 mg, F0299, Bio-Serv, Frenchtown, NJ), and responding on the inactive lever was recorded but did not have scheduled consequences. Food delivery was paired

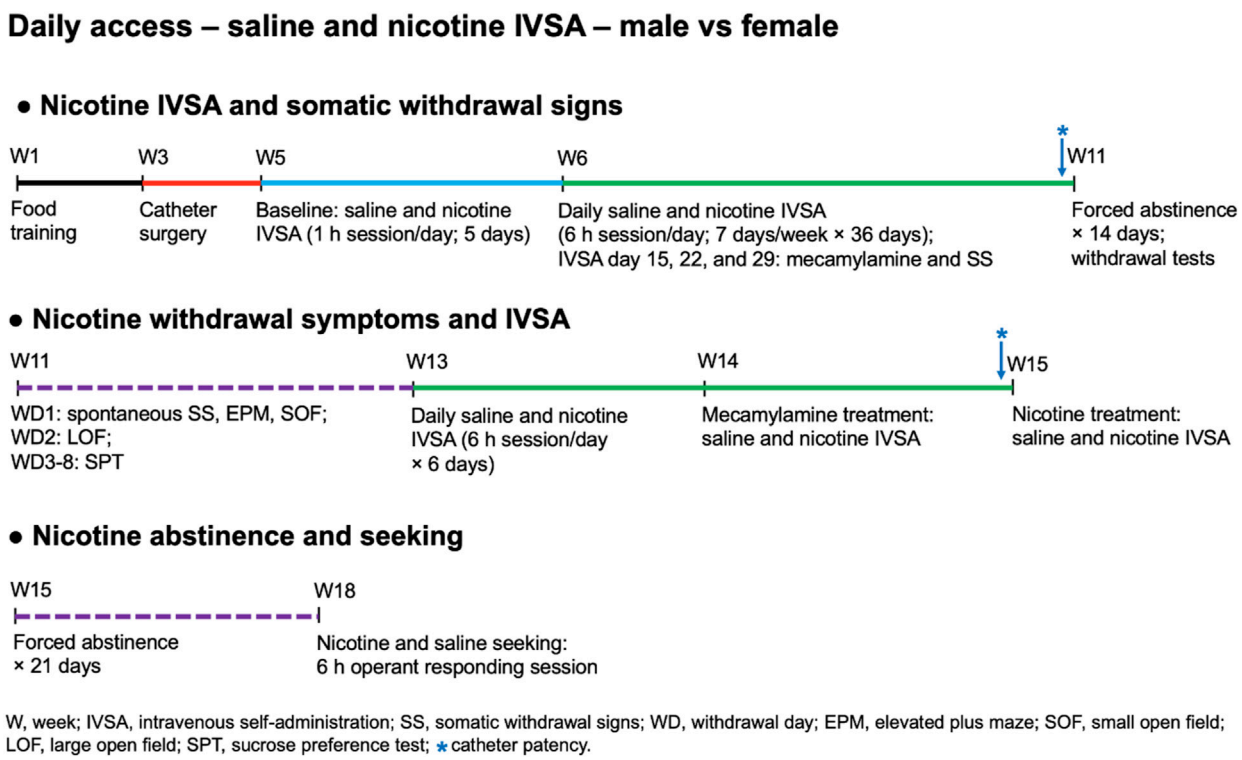

FIGURE 1
Schematic overview of the experiment. Adult male and female Wistar rats were trained to respond for food pellets and were then prepared with IV catheters. The rats were allowed to self-administer saline or nicotine in five 1-h baseline sessions and then the rats self-administer saline or nicotine in 6-h sessions/day for 36 days. Mecamylamine-precipitated somatic withdrawal signs were assessed after the 15th, 22nd, and 29th nicotine and saline self-administration sessions. Spontaneous somatic withdrawal signs, locomotor activity, and affective withdrawal symptoms (anxiety and anhedonia) were determined during forced abstinence period. Fourteen days after nicotine withdrawal, the effects of forced abstinence on nicotine and saline self-administration were investigated. After six nicotine and saline self-administration sessions, the effects of mecamylamine and nicotine treatment on nicotine and saline self-administration were investigated. After completing the last nicotine and saline self-administration sessions, the rats were housed in the vivarium for 21 days and the effects of forced abstinence on nicotine and saline seeking behavior were investigated.

with a cue light, which remained illuminated throughout the time-out (TO) period. The food training sessions were conducted for 10 days. Instrumental training started under a fixed ratio 1 (FR1) time-out 1 s (TO1s) reinforcement schedule, and the rats remained on this schedule for 5 days (30 min sessions per day). After the fifth food training session, the rats were singly housed and remained so for the rest of the study. On day 6, the time-out period was increased to 10 s. The rats were allowed to respond for food pellets under the FR1-TO10s schedule (20 min sessions) for 5 days. Both levers were retracted during the 10 s time-out period.

2.5 Intravenous catheter implantation

The catheters were implanted as described before (Chellian et al., 2021b; Chellian et al., 2021c; Chellian et al., 2022b). The rats were anesthetized with an isoflurane-oxygen vapor mixture (1%–3%) and prepared with a catheter in the right jugular vein. The catheters consisted of polyurethane tubing (length 10 cm, inner diameter 0.64 mm, outer diameter 1.0 mm, model 3Fr, Instech Laboratories, Plymouth Meeting, PA). The right jugular vein was isolated, and the catheter was inserted 2.9 cm for males and 2.5 cm for females. The tubing was then tunneled subcutaneously and connected to a vascular access button (Instech Laboratories, Plymouth Meeting, PA). The button was exteriorized through a 1-cm incision between the scapulae. During the 7-day recovery period, the rats received daily infusions of the antibiotic Gentamycin (4 mg/kg, IV, Sigma-Aldrich, St. Louis, MO). A sterile heparin solution (0.1 mL, 50 U/mL) was flushed through the catheter before and after administering the antibiotic and after nicotine self-administration. After flushing the catheter, 0.05 mL of a sterile heparin/glycerol lock solution (500 U/mL, Instech Laboratories, Plymouth Meeting, PA) was infused into the catheter. The animals received carprofen (5 mg/kg, SC) daily for 72 h after the surgery. Three days before the start of the nicotine self-administration sessions, the rats were allowed to respond for food pellets under the FR1-TO10s schedule (one 20-min session). Catheter patency was evaluated with Brevital at the end of the first and second self-administration period (Figure 1). The catheters were tested by infusing 0.2 mL of the ultra-short-acting barbiturate Brevital (1% methohexital sodium). Rats with patent catheters displayed a sudden loss of muscle tone. If the rats did not respond to Brevital, their self-administration data were excluded from the analysis. Six rats (2 male nicotine, 2 female saline, and 2 female nicotine rats) did not respond to Brevital during the first test, and therefore, their data were not included in the study. Four rats (1 male saline, 2 male nicotine, and 1 female nicotine) did not respond to Brevital during the second test and their data were excluded after week 12 (Figure 1).

2.6 Baseline and thirty-six daily nicotine and saline self-administration sessions

Male (N = 28) and female (N = 28) rats were each divided into two self-administration groups: nicotine (N = 14/sex) and saline (N = 14/sex). Animals in the control groups self-administered saline and all self-administration procedures were the same for the saline groups and the nicotine groups. Briefly, the rats were allowed to self-administer nicotine and saline for five daily 1-h baseline sessions. During the first three sessions (days 1–3), the rats self-administered saline and 0.03 mg/kg/inf of nicotine under an FR1-TO10s schedule. During the following two sessions (days 4 and 5) the rats self-administered saline and 0.06 mg/kg/inf of nicotine under an FR1-TO60s schedule. Rats that self-administer 0.06 mg/kg/inf of nicotine have a higher level of nicotine intake and more somatic withdrawal signs compared to rats that self-administered 0.03 mg/kg/inf of nicotine (Shoaib and Stolerman, 1999; Chaudhri et al., 2005). High doses of nicotine can cause seizures in rodents (Hanson, 1979; Corrigall and Coen, 1989; Damaj et al., 1999). To prevent seizures, the time-out period was increased from 10 to 60 s when the dose of nicotine was increased from 0.03 to 0.06 mg/kg/inf. Total nicotine intake over a 1-h nicotine self-administration period is not affected by the time-out period (10–60 s)(Corrigall and Coen, 1989). During the first day that the rats received the 0.03 or 0.06 mg/kg/inf dose, nicotine intake was limited to prevent aversive effects (i.e., seizures and dysphoria). The maximum number of infusions was set to 20 on the first day that the rats received the 0.03 mg/kg/inf of nicotine dose and to 10 on the first day that the rats received the 0.06 mg/kg/inf dose. On these days, saline infusions were limited to a similar degree as the nicotine infusions. After five self-administration sessions, rats in the saline group continued to self-administer saline, and rats in the nicotine group continued to self-administer nicotine (0.06 mg/kg/inf) daily for 36 days under an FR1-TO60s schedule in 6-h sessions. Responding on the active lever resulted in the delivery of a nicotine or a saline infusion (0.1 mL infused over a 6.5-s period). Active lever presses include both effective active lever presses, which turn on the pump and cue light, and ineffective active lever presses, which have no scheduled consequences (Kosten et al., 2004; Flagel et al., 2010). The ineffective active lever presses occur immediately after an active lever press and before the lever has been fully retracted. The initiation of the delivery of an infusion was paired with a cue light, which remained illuminated throughout the time-out period. Responding on the inactive lever was recorded but did not have scheduled consequences. The active and inactive levers were retracted during the time-out period. During the 6-h nicotine and saline self-administration sessions, the rats had access to water in the operant chambers. On day 36, the rats received *ad libitum* food in their home cage after completing the self-administration sessions.

2.7 Mecamylamine-precipitated somatic withdrawal signs

Somatic signs were observed in a transparent Plexiglas observation chamber (25 cm × 25 cm x 46 cm) with 1 cm of corncob bedding as described previously (Rylkova et al., 2008; Yamada et al., 2010). The rats were habituated to the observation chambers for 5 min per day on 3 consecutive days before the somatic withdrawal test. The rats received mecamylamine injections (2 mg/kg, SC) immediately after the 15th, 22nd, and 29th nicotine and saline self-administration sessions. Ten minutes after the mecamylamine injections the rats were placed in the observation chamber and somatic withdrawal signs were recorded for 10 min. The following somatic withdrawal signs were recorded: body shakes, head shakes, chews, teeth chattering, cheek tremors, gasps, writhes, ptosis, genital licks, foot licks, and yawns. Ptosis was counted once per minute if present continuously. Somatic signs were observed in a quiet, brightly lit room. The total number of somatic signs was the sum of the individual occurrences.

2.8 Spontaneous nicotine withdrawal

2.8.1 Withdrawal day 1: spontaneous somatic withdrawal signs, elevated plus maze test, and small open field test

Spontaneous somatic withdrawal signs were recorded 16–17 h after the last nicotine and saline self-administration session. Somatic signs were observed for 20 min in a transparent Plexiglas observation chamber (Rylkova et al., 2008; Yamada et al., 2010). After recording the somatic withdrawal signs, anxiety-like behavior was measured in the elevated plus-maze test for 5 min. The elevated plus-maze test is used to measure anxiety-like behavior in rodents and was performed as described in our previous work (Knight et al., 2021; Bruijnzeel et al., 2022). The elevated plus maze apparatus (Coulbourn Instruments, Holliston, MA) consisted of two closed arms (i.e., with black walls, 50 cm × 10 cm × 30 cm; Length × Width × Height, L × W × H) and two open arms (i.e., without walls; 50 cm × 10 cm; L × W). The open and closed arms were connected by a central platform, and the open arms had 0.5 cm tall ledges to prevent the rats from falling off. The open arms were placed opposite of each other, and the maze was elevated 55 cm above the floor on acrylic legs. At the beginning of each test, the rats were placed in the central area facing an open arm and were allowed to explore the apparatus for 5 min. The rats were recorded with a camera mounted above the maze, and the test was conducted in a quiet, dimly lit room (100 lux). The open-arm and closed-arm duration, the number of open and closed-arm entries, and total distance traveled were determined automatically (center-point detection) using EthoVision XT 11.5 software (Noldus Information Technology, Leesburg, VA). The percentage of open arm entries (open arm entries/total arm entries) and percentage time on the open arms (open arm time/total time on the arms) were calculated. Heatmaps were produced with the EthoVision heatmap generator. The apparatus was cleaned with a Nolvasan solution (chlorhexidine diacetate) between tests.

After the elevated plus-maze test, locomotor activity was measured in the small open field for 10 min. The small open-field test (SOF) was conducted as described before (Qi et al., 2016; Bruijnzeel et al., 2022). The small open field test was conducted to assess locomotor activity, rearing, and stereotypies. These motor behaviors were measured using an automated animal activity cage system (VersaMax Animal Activity Monitoring System, AccuScan Instruments, Columbus, OH, United States). Horizontal beam breaks and total distance traveled reflect locomotor activity and vertical beam breaks reflect rearing. The distance traveled is

dependent on the path of the animal in the open field and is considered a better indicator of locomotor activity than horizontal beam breaks. Repeated interruptions of the same beam are a measure of stereotypies (stereotypy count) (Calma et al., 2021). The setup consisted of four animal activity cages made of clear acrylic (40 cm × 40 cm × 30 cm; L × W × H), with 16 equally spaced (2.5 cm) infrared beams across the length and width of the cage. The beams were located 2 cm above the cage floor (horizontal activity beams). An additional set of 16 infrared beams were located 14 cm above the cage floor (vertical activity beams). All beams were connected to a VersaMax analyzer, which sent information to a computer that displayed beam data through Windows-based software (VersaDat software). The small open field test was conducted in a dark room, and the cages were cleaned with a Nolvasan solution between animals. At the beginning of each test, the rats were placed in the center of the small open field, and activity was measured.

2.8.2 Withdrawal day 2: large open field test

The large open field test was conducted 48 h after the last nicotine and saline self-administration session. The large open field test is used to assess locomotor activity and anxiety-like behavior. The test was conducted for 10 min in a dimly lit room (75 lux), as described previously (Knight et al., 2021). The large open field apparatus consisted of a large arena measuring 120 × 120 × 60 cm (L × W × H). The arena was made of black high-density polyethylene panels that were fastened together and placed on a plastic bottom plate (Faulkner Plastics, Miami, FL). The rats' behavior was recorded with a camera mounted above the arena and analyzed with EthoVision XT 11.5 software (Noldus Information Technology, Leesburg, VA). The large open field was divided into three zones: an outside zone (20 cm wide), a middle zone (20 cm wide), and a center zone (40 × 40 cm; L × W). The following behaviors were analyzed: total distance traveled, distance traveled in each zone (outside, middle, and center), number of entries into each zone, and latency to enter the middle and center zone. Rats avoid open spaces and therefore spend most of their time in the outside zone. Rats spend more time in the middle zone compared to the center zone, suggesting that the middle zone is less aversive than the center zone (Hernandez et al., 2021; Knight et al., 2021). Large open field heatmaps were produced with the EthoVision heatmap generator. The large open field was cleaned between rats with a Nolvasan solution.

2.8.3 Withdrawal days 3–8: sucrose preference test

During the first withdrawal day, the rats were habituated to a pair of 180 mL Kaytee Chew Proof water bottles. The bottles were placed on top of the home cage and served as the only fluid source. During the second withdrawal day, the rats were habituated to two bottles containing a 2% (w/v) sucrose solution. These bottles were also placed on top of the home cage, and they were the sole source of fluid. Sucrose preference was measured for 5 days during nicotine withdrawal. The test was done using a two-bottle choice procedure, as described in our previous work (Bruijnzeel et al., 2019; Chellian et al., 2020). The sucrose solution (2%, w/v) was prepared daily with autoclaved water. One bottle with water and one bottle with a sucrose solution (2%, w/v) were placed on top of the home cage. The bottles were switched (left/right position) daily to reduce the side bias. The weight of each bottle was recorded before and after the 24-h choice test. The difference in bottle weights was used to measure water and sucrose intake. Sucrose preference (percentage) was calculated using the following formula: (sucrose intake/total fluid intake) × 100. After the sucrose preference test, the bottles were removed and water was available in the home cage.

2.9 Forced abstinence and nicotine intake

During the 2 weeks of forced abstinence, the rats were housed in the vivarium and were handled twice a week. After the 2-week forced abstinence period, the rats were allowed to self-administer nicotine (0.06 mg/kg/inf) and saline daily for 6 days under an FR1-TO60s schedule in 6-h sessions. During the 6-h self-administration sessions, the rats had access to water in the operant chambers.

2.10 Mecamylamine treatment, nicotine treatment, and nicotine intake

After six nicotine and saline self-administration sessions, the effects of mecamylamine on saline and nicotine self-administration (0.06 mg/kg/inf; FR1-TO60s schedule, 6 h) were investigated. Mecamylamine (0, 2 mg/kg, SC) was administered according to a Latin square design 10 min before the self-administration sessions. Seventy-two h after the last mecamylamine treatment, the effects of nicotine treatment on saline and nicotine self-administration (0.06 mg/kg/inf; FR1-TO60s schedule, 6 h) in rats was investigated. Nicotine (0, 0.4 mg/kg, SC) was administered according to a Latin square design 10 min before the self-administration sessions. Twenty-four and 48 h after each mecamylamine or nicotine treatment, saline and nicotine self-administration sessions were conducted without any drug treatment. Mecamylamine and nicotine treatment doses were based on one of our previous studies, which showed an effect on nicotine self-administration in rats 10 min after treatment but not at 24 and 48 h after treatment (Chellian et al., 2024). During the 6-h self-administration sessions, the rats had access to water in the operant chambers.

2.11 Forced abstinence and seeking behavior

During the 3 weeks of forced abstinence, the rats were housed in the vivarium and were handled twice a week. During the seeking tests, the rats were placed in the same operant chambers where they previously self-administered nicotine and saline. The operant sessions were conducted for 6 h under an FR1-TO60s schedule, without attaching the tethers to the vascular access buttons. Responding on the active lever turned on the pump for a 6.5-s period (saline or nicotine was not infused) and a cue light above the right lever, which remained illuminated throughout the time-out period. Responding on the inactive lever was recorded but did not have scheduled consequences. The active and inactive levers were retracted during the time-out period. Both active lever responses and effective active lever responses served as measures of seeking behavior. Active lever presses include both effective active lever presses, which turn on the pump and cue light, and ineffective active

lever presses, which have no scheduled consequences (Kosten et al., 2004; Flagel et al., 2010). The ineffective active lever presses occur immediately after an active lever press and before the lever has been fully retracted. Following an effective active lever press, rats are exposed to the same cues (pump noise and visual cues) as they would be during a regular self-administration session but no nicotine or saline is infused. During the 6-h sessions, the rats had access to water in the operant chambers.

2.12 Statistics

Baseline (5 sessions) and long access (36 sessions) nicotine and saline self-administration data were analyzed using two- or three-way ANOVAs with hours, days, and session as within-subjects factors, and IVSA group and sex as between-subjects factors. Somatic withdrawal scores were analyzed with two- or three-way ANOVAs, with mecamylamine treatment and days as within-subjects factors, and IVSA group and sex as between-subjects factors. The effects of sex and nicotine self-administration on behavior in the elevated plus maze test, small open field, and large open field were analyzed using two- or three-way ANOVAs with IVSA group and sex as between-subjects factors. The effects of nicotine abstinence on sucrose preference were analyzed using three-way ANOVAs with days as a within-subjects factor, and IVSA group and sex as between-subjects factors. The effects of forced abstinence on nicotine intake and seeking were investigated using two- or three-way ANOVAs, with IVSA group and sex as between-subjects factors, and hours, days, and session as within-subjects factors. The effects of mecamylamine and nicotine treatment on nicotine intake were investigated using two- or three-way ANOVAs, with IVSA group and sex as between-subjects factors, and mecamylamine treatment, nicotine treatment, and hours as within-subjects factors. For all statistical analyses, significant interaction effects found in the ANOVAs were followed by Bonferroni's *post hoc* tests to determine which groups differed. *p*-values less than or equal to 0.05 were considered significant. Data were analyzed with SPSS Statistics version 29 and GraphPad Prism version 10.1.2. The figures were generated using GraphPad Prism version 10.1.2. Large open field and elevated plus maze heatmaps were produced with the EthoVision heatmap generator.

3 Results

3.1 Baseline and thirty-six daily nicotine and saline self-administration sessions

3.1.1 Week 5: baseline nicotine and saline self-administration

During the 1-h baseline self-administration sessions, infusions and active lever responses were higher for the rats that self-administered saline than for those that self-administered nicotine. Nicotine intake was the same in the males and the females during the baseline sessions (see Supplementary File S1 for results; Supplementary Figures S1A–H).

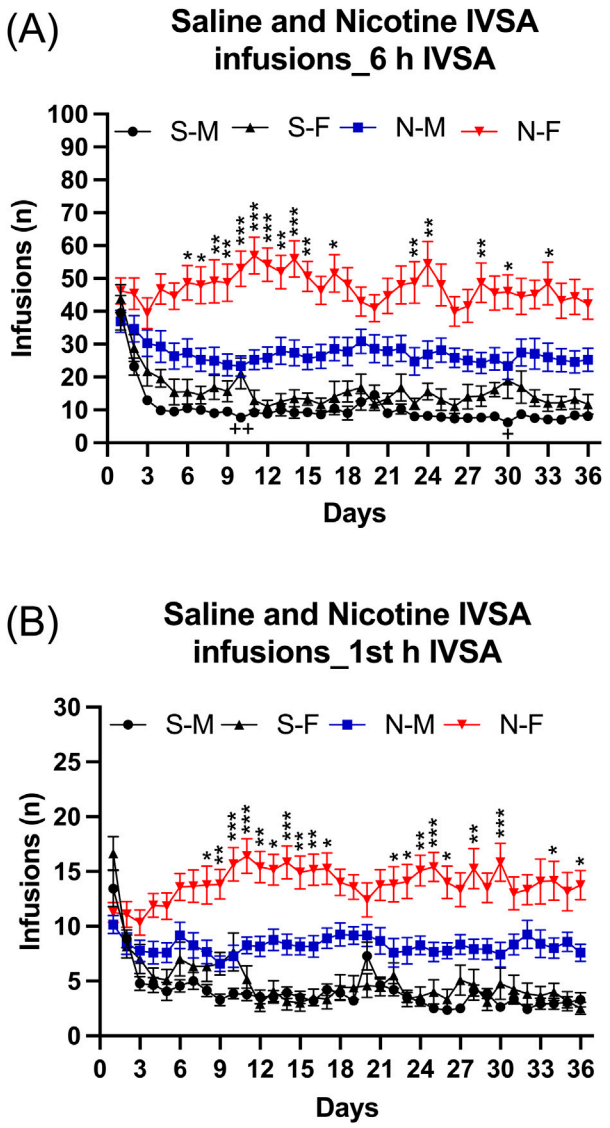

FIGURE 2
Daily 6-h nicotine and saline self-administration in male and female rats. The rats self-administered nicotine (0.06 mg/kg/inf) and saline in 6-h self-administration sessions for 36 days. Infusions **(A)** in 6-h nicotine and saline self-administration sessions. First hour infusions **(B)** in nicotine and saline self-administration groups. Asterisks indicate more infusions (nicotine intake) in the females that self-administered nicotine than in the males that self-administered nicotine on the same test day. Plus signs indicate more infusions in the females that self-administered saline than in the males that self-administered saline on the same test day. *, + $p < 0.05$; **, ++ $p < 0.01$; ***$p < 0.001$. Abbreviations and group size: S-M, saline IVSA-male (N = 14); N-M, nicotine IVSA-male (N = 12); S-F, saline IVSA-female (N = 12); N-F, nicotine IVSA-female (N = 12). Data are expressed as means ± SEM.

3.1.2 Week 6–11: long access nicotine and saline self-administration

Infusions: Infusions were higher for the rats that self-administered nicotine than for those that self-administered saline (Figure 2A: IVSA group F1,46 = 62.478, $p < 0.001$). Infusions were much higher for the females that self-administered nicotine than for the males that self-administered nicotine and there was also a small

sex difference in the saline groups (Figure 2A: Sex F1,46 = 18.03, $p < 0.001$; Sex × IVSA group F1,46 = 5.86, $p < 0.05$). The *post hoc* tests revealed that nicotine infusions (intake) in the female nicotine group were higher from day 6 compared to the male nicotine group (Figure 2A). However, on only 2 days (day 10 and 30) were infusions higher in the female saline group than in the male saline group (Figure 2A). In addition, the *post hoc* tests showed that the males and females in the nicotine group had more infusions than the males and females in the saline group (Supplementary Table S1). Infusions were stable in the females that self-administered nicotine but decreased in all other groups (Figure 2A; Supplementary Table S2: Days F35, 1610 = 11.547, $p < 0.001$; Days × Sex F35,1610 = 2.624, $p < 0.001$; Days × IVSA group F35,1610 = 6.602, $p < 0.001$; Days × Sex × IVSA group F35, 1610 = 2.128, $p < 0.001$).

Active lever: Active lever responses were higher for the rats that self-administered nicotine than for those that self-administered saline (see Supplementary File S2 for results; Supplementary Figure S2A; Supplementary Table S1, S2).

Inactive lever: Inactive lever responses declined more in the rats that self-administered saline than in the rats that self-administered nicotine and stabilized at a lower level in the saline rats than in the nicotine rats (see Supplementary File S2 for results; Supplementary Figure S2B; Supplementary Table S1, S2).

3.1.2.1 Long access first hour infusions

During the first hour of access, rats had more nicotine than saline infusions (Figure 2B, IVSA group F1,46 = 68.433, $p < 0.01$). Furthermore, the female rats had more nicotine infusions compared to the males, but there was no sex difference in saline infusions (Sex F1,46 = 16.967, $p < 0.001$; Sex × IVSA group F1,46 = 9.447, $p < 0.05$). The *post hoc* tests showed that nicotine infusions (intake) in the female group were significantly higher from day 8 than in the males (Figure 2B). In addition, the *post hoc* tests showed that the males and females in the nicotine group had more infusions than males and females in the saline group (Supplementary Table S1). The number of infusions decreased over time in rats with access to saline, but remained stable in rats with access to nicotine (Supplementary Table S2, Days F35,1610 = 7.925, $p < 0.001$; Days × Sex F35,1610 = 2.0, $p < 0.001$; Days × IVSA group F35,1610 = 11.88, $p < 0.001$). In all groups, there was a decrease in infusions except for the nicotine females in which the number of infusions increased (Supplementary Table S2, Days × Sex × IVSA group F35,1610 = 2.226, $p < 0.001$).

3.1.2.2 Long access first and last session

Infusions: Rats that self-administered nicotine had more infusions responses compared to those that self-administered saline (Figure 3A: IVSA group F1,46 = 13.643, $p < 0.001$). Additionally, female rats had more infusions than male rats (Figure 3 As: Sex F1,46 = 7.042, $p < 0.05$; Sex × IVSA group F1, 46 = 2.039, NS). The number of infusions decreased from the first to the last session, with the greatest decrease in the saline group (Figure 3A: Session F1, 46 = 84.927, $p < 0.001$; Session × Sex F1, 46 = 0.637, NS; Session × IVSA group F1,46 = 31.016, $p < 0.001$; Session × Sex × IVSA group F1, 46 = 0.973, NS). The *post hoc* showed that both the male and the female rats in the saline group had fewer infusions during the last session compared to the first session (Figure 3A).

Active lever: Rats that self-administered nicotine had more infusions and active lever responses compared to those that self-administered saline (see Supplementary File S3 for results; Supplementary Figure S3A).

Inactive lever: Responding on the inactive lever decreased more from the first to the last session in the saline rats than in the nicotine rats (see Supplementary File S3 for results; Supplementary Figure S3B).

3.1.2.3 Time course analysis, first day compared to last self-administration day

Saline self-administration group: Saline infusions were higher during the first session than the last session and they decreased over time (1–6 h) (Figure 3B, Session F1, 24 = 92.888, $p < 0.01$; Hours F5,120 = 45.335, $p < 0.001$). There were no effects of sex on saline infusions (Sex F1, 24 = 0.842, NS; Session × Sex F1, 24 = 0.015, NS; Hours × Sex F5, 120 = 0.688, NS). Furthermore, saline infusions were lower in both males and females during the last session compared to the first session (Session × Hours F5, 120 = 27.475, $p < 0.001$; Session × Hours × Sex F5, 120 = 2.457, $p < 0.05$). The *post hoc* analysis showed that, compared to the same time points during the first session, saline infusions in males and females were lower from the first hour to the fifth hour of the last session (Figure 3B). In addition, the *post hoc* analysis revealed that for both males and females, saline infusions were lower from the second to the sixth hour compared to the first hour during the first session. However, the *post hoc* showed that only for males the saline infusions were lower during the third and fourth hour compared to the first hour during the last sessions (Figure 3B).

Nicotine self-administration group: Nicotine infusions (intake) were higher during the first than the last session and it decreased over time (1–6 h) (Figure 3C, Session F1, 22 = 8.458, $p < 0.01$; Hours F5, 110 = 64.7, $p < 0.001$). Females self-administered more nicotine than the males (Sex F1, 22 = 7.434, $p < 0.05$; Session × Sex F1, 22 = 2.025, NS; Hours × Sex F5, 110 = 1.737, NS). Furthermore, nicotine infusions were lower in males during the last session compared to the first session, but in the females, it remained the same during the first and last sessions (Session × Hours F5, 110 = 3.22, $p < 0.01$; Session × Hours × Sex F5, 110 = 3.994, $p < 0.01$). The *post hoc* analysis showed that, compared to the same time points during the first session, nicotine infusions in males were lower during the first hour and the third to fifth hour of the last session (Figure 3C). In addition, the *post hoc* showed that for both males and females nicotine infusions were lower from the second to the sixth hour compared to the first hour during both the first and last sessions (Figure 3C).

3.2 Week 6–11: mecamylamine-precipitated somatic withdrawal signs

Treatment with mecamylamine induced more somatic withdrawal signs in the rats that self-administered nicotine than in those that self-administered saline (Figure 4A, IVSA group F1, 46 = 9.086, $p < 0.01$). Furthermore, the number of somatic withdrawal signs increased over time and this effect was greater in the nicotine group than in the saline group (Figure 4A, Days F2, 92 = 97.894, $p < 0.001$; Sex F1, 46 = 3.267, NS; Days × Sex F2, 92 = 11.936, $p < 0.001$; Days × IVSA group F2, 92 = 3.779, $p < 0.05$; Sex × IVSA group F1, 46 = 0.478, NS; Days × Sex × IVSA group F2, 92 = 2.042, NS).

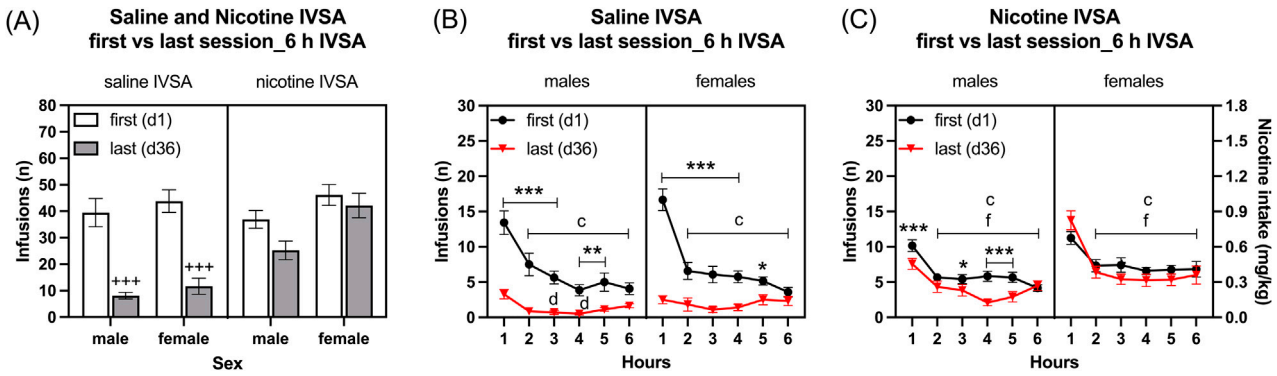

FIGURE 3
Comparison of the first and last nicotine and saline self-administration session in male and female rats. The rats self-administered saline and nicotine 0.06 mg/kg/inf in 6-h self-administration sessions for 36 days and compared their first (day 1) and last (day 36) self-administration sessions. Infusions **(A)** in 6-h nicotine and saline self-administration sessions. Time course of saline infusions **(B)** and nicotine infusions **(C)** during 6-h nicotine and saline self-administration sessions. Plus signs indicate a significant decrease in infusions in the last session compared to the first session with the same self-administration group and same sex. Asterisks indicate a significant difference in infusions between the first and last session in rats of the same sex, and at the same time point. Letter c indicates fewer infusions compared with first hour infusions in the first session with the same sex. Letters d and f indicate lower infusions compared with first hour infusions in the last session with the same sex. *, d $p < 0.05$; **$p < 0.01$; +++, ***, c, f $p < 0.001$. Saline IVSA-male (N = 14), nicotine IVSA-male (N = 12), saline IVSA-female (N = 12), and nicotine IVSA-female (N = 12). Data are expressed as means ± SEM.

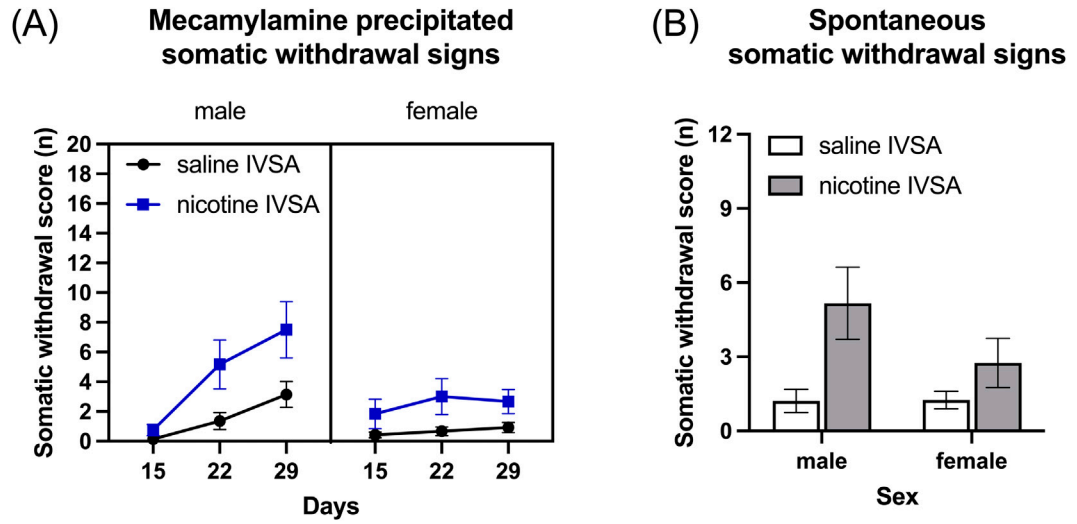

FIGURE 4
Mecamylamine-precipitated somatic withdrawal signs and spontaneous somatic withdrawal signs in male and female rats. Somatic withdrawal signs were recorded after mecamylamine administration **(A)** and after cessation of nicotine intake **(B)**. Saline IVSA-male (N = 14), nicotine IVSA-male (N = 12), saline IVSA-female (N = 12), and nicotine IVSA-female (N = 12). Data are expressed as means ± SEM.

3.3 Week 11: spontaneous nicotine withdrawal

3.3.1 Withdrawal day 1: spontaneous somatic withdrawal signs

Somatic withdrawal signs were assessed 16–17 h after the last self-administration session. The rats that self-administered nicotine displayed more somatic withdrawal signs than the rats that self-administered saline and there was no effect of sex (Figure 4B, Sex F1, 46 = 1.732, NS; IVSA group F1, 46 = 9.081, $p < 0.01$; Sex × IVSA group F1, 46 = 1.837, NS).

3.3.2 Withdrawal day 1: elevated plus maze test

Nicotine abstinence did not increase anxiety-like behavior in either males or females (see Supplementary File S4 for results; Supplementary Figure S4). In comparison to the males, the females displayed less anxiety-like behavior in the elevated plus maze test (see Supplementary File S4 for results; Supplementary Figure S4).

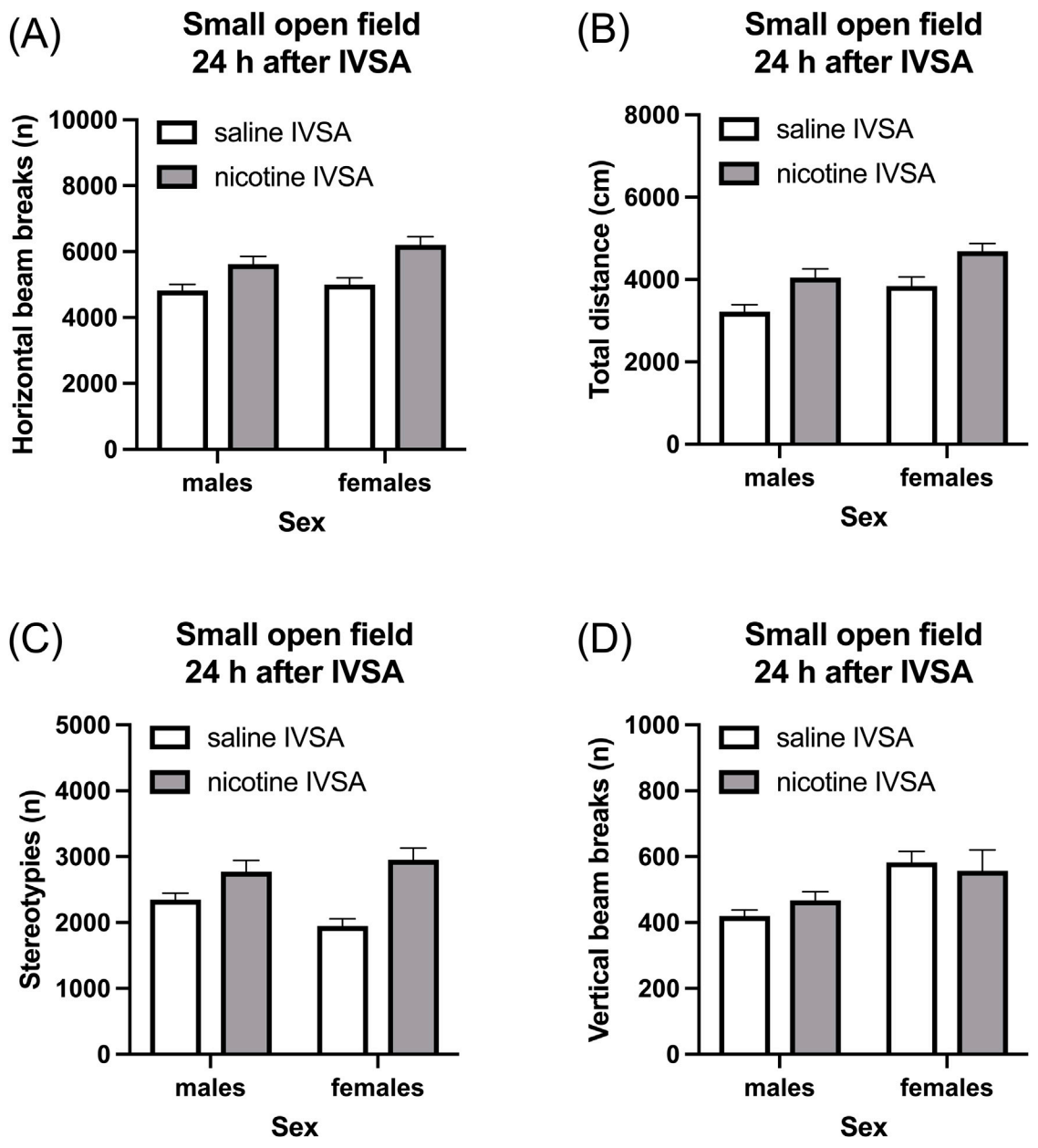

FIGURE 5
Nicotine withdrawal and locomotor activity in male and female rats. Nicotine withdrawal increases locomotor activity **(A, B)** and stereotypies **(C)** in the small open field test. Nicotine withdrawal does not affect the vertical beam breaks in the small open field test **(D)**. Saline IVSA-male (N = 14), nicotine IVSA-male (N = 12), saline IVSA-female (N = 12), and nicotine IVSA-female (N = 12). Data are expressed as means ± SEM.

Heatmaps for both males and females in the nicotine and saline groups from the elevated plus-maze test are presented in Supplementary Figure S5.

3.3.3 Withdrawal day 1: small open field test

Nicotine abstinence led to an increase in horizontal beam breaks (Figure 5A, IVSA group F1, 46 = 21.095, $p < 0.001$; Sex × IVSA group F1,46 = 0.817, NS), total distance traveled (Figure 5B, IVSA group F1,46 = 18.387, $p < 0.001$; Sex × IVSA group F1, 46 = 0.001, NS), and stereotypies (Figure 5C, IVSA group F1, 46 = 25.431, $p < 0.001$; Sex × IVSA group F1, 46 = 4.229, NS) and this was not affected by the sex of the rats. Nicotine abstinence did not affect vertical beam breaks (Figure 5D, IVSA group F1, 46 = 0.081, NS; Sex × IVSA group F1, 46 = 0.919, NS). Compared to the males, the females traveled a greater distance (Figure 5B, Sex F1, 46 = 10.495, $p < 0.01$) and had more vertical beam breaks (Figure 5D, Sex F1, 46 = 11.018, $p < 0.01$). There was no sex difference in horizontal beam breaks (Figure 5A, Sex F1, 46 = 3.059, NS) and stereotypies (Figure 5C, Sex F1, 46 = 0.597, NS).

3.3.4 Withdrawal day 2: large open field test

Nicotine withdrawal did not increase anxiety-like behavior in either males or females (see Supplementary File S5 for results; Supplementary Figure S6). The females displayed less anxiety-like

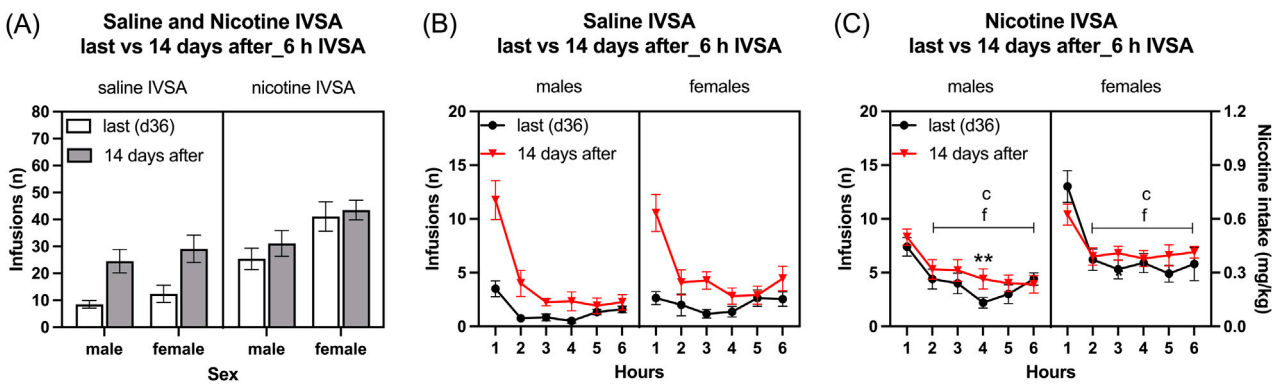

FIGURE 6
Nicotine and saline self-administration immediately after forced abstinence in male and female rats. Fourteen days after last self-administration (day 36), the rats self-administered saline and nicotine 0.06 mg/kg/inf in 6-h self-administration sessions. Infusions **(A)** in 6-h nicotine and saline self-administration sessions. Time course of saline infusions **(B)** and nicotine infusions **(C)** during 6-h nicotine and saline self-administration sessions. Asterisks indicate more infusions after the abstinence period in rats in the same sex and at the same time point. Letter c indicates fewer infusions compared with first-hour infusions in the last session within the same sex. Letters f indicates fewer infusions compared with first-hour infusions after the abstinence period (14 days after session) within the same sex. $^{**}p < 0.01$; c, f $p < 0.001$. Saline IVSA-male (N = 12); nicotine IVSA-male (N = 10); saline IVSA-female (N = 11); nicotine IVSA-female (N = 10). Data are expressed as means ± SEM.

behavior than the males in the large open field test (see Supplementary File S5 for results; Supplementary Figure S6). Heatmaps for both males and females in the nicotine and saline groups from the large open field test are presented in Supplementary Figure S7.

3.3.5 Withdrawal days 3–8: sucrose preference test

Nicotine abstinence did not affect sucrose preference in either males or females (see Supplementary File S6 for results; Supplementary Figure S8).

3.4 Week 13: forced abstinence and nicotine intake

3.4.1 Last self-administration day compared to first one after forced abstinence

Infusions: The period of abstinence led to an increase in the number of infusions and this increase was greater in the rats that self-administered saline than in those that self-administered nicotine (Figure 6A: Session F1, 39 = 28.832, $p < 0.001$; IVSA group F1, 39 = 20.783, $p < 0.001$; Session × IVSA group F1,39 = 10.491, $p < 0.01$). The female rats had more infusions than the males (Figure 6A: Sex F1, 39 = 6.252, $p < 0.05$). The effect of sex on infusions was unaffected by the abstinence period, as well as by whether nicotine or saline was being self-administered (Figure 6A: Session × Sex F1, 39 = 0.114, NS; Sex × IVSA group F1, 39 = 1.806, NS; Session × Sex × IVSA group F1, 39 = 0.281, NS).

Active lever: The period of abstinence led to an increase in the number of active lever presses and this increase was greater in the rats that self-administered saline than in those that self-administered nicotine (see Supplementary File S7.1 for results; Supplementary Figure S9A)

Inactive lever: The rats that self-administered nicotine had more inactive lever responses than the rats that self-administered saline (see Supplementary File S7.1 for results; Supplementary Figure S9B)

3.4.2 Time course analysis, last self-administration day compared to first one after forced abstinence

Saline self-administration group: Saline infusions were higher after the abstinence period than the last session and decreased over time (1–6 h) (Figure 6B, Session F1, 21 = 34.887, $p < 0.001$; Hours F5,105 = 39.55, $p < 0.001$; Session × Hours F5, 105 = 17.166, $p < 0.001$). There was no sex difference in saline infusions before and after the abstinence period (Sex F1, 21 = 0.88, NS; Session × Sex F1, 21 = 0.017, NS; Hours × Sex F5, 105 = 2.097, NS; Session × Hours × Sex F5,105 = 0.684, NS).

Nicotine self-administration group: Nicotine infusions (intake) were higher in female rats compared to males (Figure 6C, Sex F1, 18 = 5.692, $p < 0.05$). There was no significant difference in nicotine intake before and after the abstinence period (Session F1, 18 = 2.54, NS; Session × Sex F1, 18 = 0.422, NS). Nicotine infusions decreased over time, and this decrease was dependent on the sex of the rats (Hours F5, 90 = 57.595, $p < 0.001$; Hours × Sex F5,90 = 3.057, $p < 0.05$; Session × Hours F5, 90 = 2.121, NS; Session × Hours × Sex F5, 90 = 2.413, $p < 0.05$). The *post hoc* showed that nicotine infusions decreased over time in all groups. Furthermore, the *post hoc* showed that the nicotine infusions were higher during the fourth hour in the nicotine group after the 14-day abstinence period than before the abstinence period (Figure 6C).

3.4.3 Self-administration after forced abstinence, six self-administration sessions

Infusions: The females had more infusions than the males (Figure 7A: Sex F1,39 = 13.859, $p < 0.001$). Furthermore, infusions were higher for the rats that self-administered nicotine than for those that self-administered saline (Figure 7A: IVSA group F1,39 = 32.433, $p < 0.01$; Sex × IVSA group F1,39 = 0.317, NS). Infusions decreased over time, and this effect was greatest in the males that self-administered saline (Figure 7A: Days F5, 195 = 26.077, $p < 0.001$; Days × Sex F5,195 = 2.259, $p = 0.058$; Days × IVSA group F5, 195 = 4.662, $p < 0.001$; Days × Sex × IVSA group F5, 195 = 2.182, $p = 0.058$). The *post hoc* tests showed that

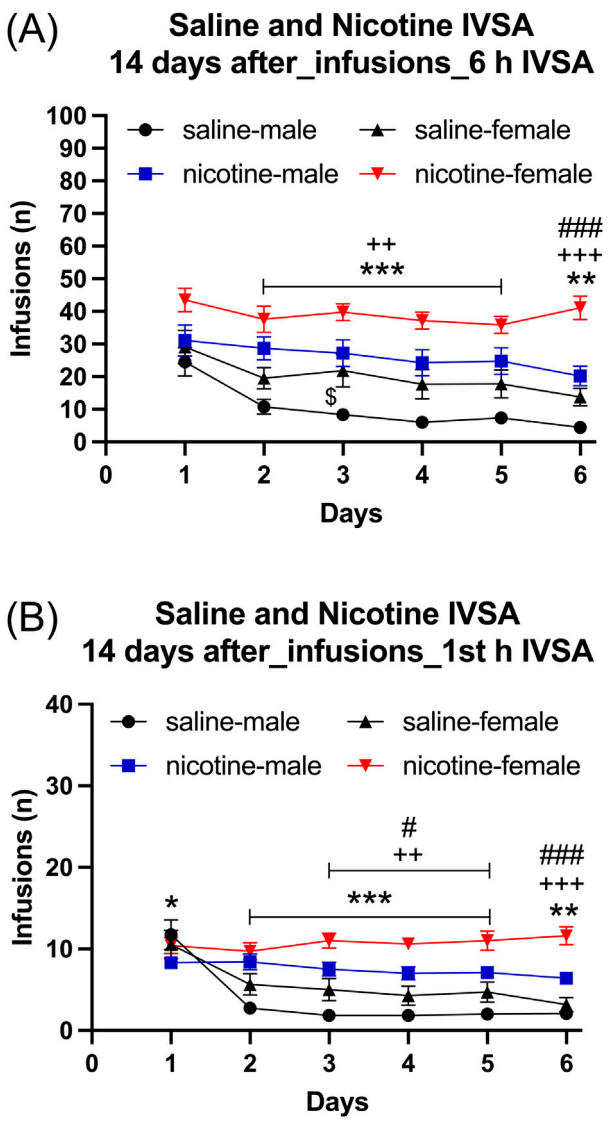

FIGURE 7
Daily nicotine and saline self-administration after forced abstinence in male and female rats. After a 14-day abstinence period, the rats self-administered saline and 0.06 mg/kg/inf of nicotine in 6-h self-administration sessions for 6 days. Infusions **(A)** in 6-h nicotine and saline self-administration sessions. First hour infusions **(B)** in nicotine and saline self-administration groups. Asterisks indicate more infusions in the nicotine group males than in the saline group males on the same day **(A, B)**. Plus signs indicate more infusions in the nicotine group females than in the saline group females at the same day **(A, B)**. Dollar signs indicate more infusions in the saline group females than in the saline group males on the same day **(A)**. Pound sign indicate more nicotine infusions in females than in males that self-administered nicotine on the same day **(A, B)**. *, $, #$p < 0.05$; **, ++ $p <$ 0.01; ***, +++, ###$p < 0.001$. Saline IVSA-male (N = 12); nicotine IVSA-male (N = 10); saline IVSA-female (N = 11); nicotine IVSA-female (N = 10). Data are expressed as means ± SEM.

infusions were higher in the nicotine group than in the saline group (Figure 7A). Furthermore, females in both the nicotine and saline group had more infusions compared to the males (Figure 7A).

Active lever: The active lever responses were higher for the rats that self-administered nicotine than for those that self-administered saline (see Supplementary File S7.2 for results; Supplementary Figure S10A)

Inactive lever: The rats that self-administered saline had fewer inactive lever responses than the rats that self-administered nicotine (see Supplementary File S7.2 for results; Supplementary Figure S10B)

3.4.4 Self-administration after forced abstinence, first hour infusions during six self-administration sessions

Female rats had more infusions than male rats (Figure 7B, Sex F1, 39 = 10.067, $p < 0.01$). Additionally, rats that self-administered nicotine had a higher number of infusions compared to those that self-administered saline (IVSA group F1, 39 = 30.416, $p < 0.001$; Sex × IVSA group F1, 39 = 0.769, NS). There was a decrease in the number of infusions over time, with the most noticeable reduction observed in male rats that self-administered saline (Days F5, 195 = 25.687, $p < 0.001$; Days × Sex F5, 195 = 2.895, $p < 0.05$; Days × IVSA group F5, 195 = 21.698, $p < 0.001$; Days × Sex × IVSA group F5, 90 = 2.383, $p < 0.05$). The *post hoc* test showed that the rats in the nicotine group had a higher number of infusions than the rats in the saline group (Figure 7B). Furthermore, the females had more nicotine, but not saline, infusions compared to the males (Figure 7B).

3.5 Week 14: mecamylamine treatment and self-administration

Infusions: Infusions were higher in females than in males, and they were higher in rats that self-administered nicotine compared to those that self-administered saline (Figure 8A: Sex F1, 39 = 6.484, $p < 0.05$; IVSA group F1, 39 = 27.691, $p < 0.001$). Mecamylamine decreased the number of infusions in the females that self-administered nicotine. However, mecamylamine increased the number of infusions in the females that self-administered saline, while not affecting infusions in the males (Figure 8A: Mecamylamine treatment F1, 39 = 0.008, NS; Mecamylamine treatment × Sex F1, 39 = 0.546, NS; Mecamylamine treatment × IVSA group F1, 39 = 3.294, NS; Sex × IVSA group F1, 39 = 0.242, NS; Mecamylamine treatment × Sex × IVSA group F1, 39 = 8.455, $p < 0.01$). The *post hoc* analysis showed that infusions were higher in mecamylamine-treated females that self-administered saline than in saline-treated females that self-administered saline (Figure 8A).

Active lever: Mecamylamine decreased the active lever responses in the females that self-administered nicotine. However, it increased the active lever responses in the females that self-administered saline, while not affecting active lever responses in the males (see Supplementary File S8 for results; Supplementary Figure S11A).

Inactive lever: Mecamylamine treatment increased inactive lever responses and this parameter was not affected by the sex of the rats (see Supplementary File S8 for results; Supplementary Figure S11B).

3.5.1 Time course analysis, mecamylamine treatment and self-administration

Saline self-administration group: Mecamylamine administration did not affect the saline infusions in both males and females (Figure 8B, Mecamylamine treatment F1, 21 = 2.994, NS; Mecamylamine treatment × Sex F1, 21 = 3.892, NS;

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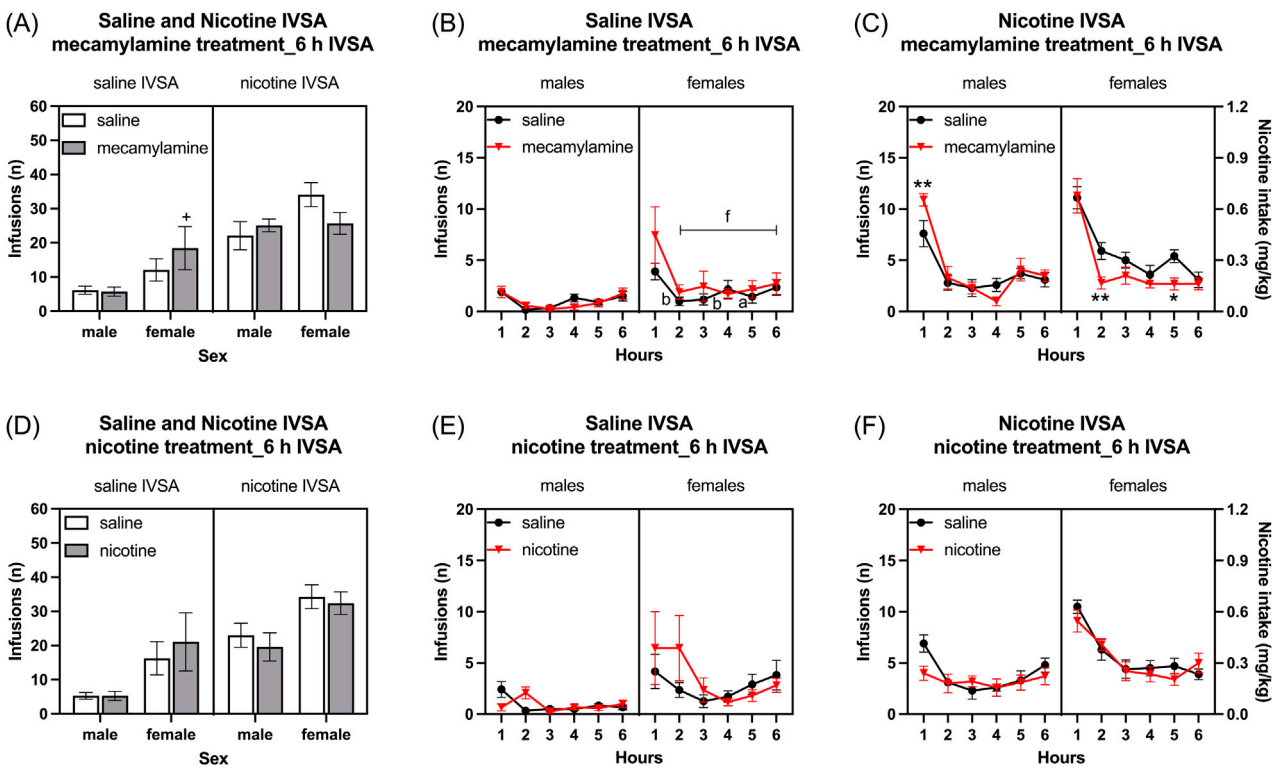

FIGURE 8
Effects of mecamylamine and nicotine treatment on nicotine and saline self-administration in male and female rats. Infusions **(A)** in 6-h nicotine and saline self-administration sessions after mecamylamine treatment. Time course of saline infusions **(B)** and nicotine infusions **(C)** during 6-h nicotine and saline self-administration sessions after mecamylamine treatment. Infusions **(D)** in 6-h nicotine and saline self-administration sessions after nicotine treatment. Time course of saline infusions **(E)** and nicotine infusions **(F)** during 6-h nicotine and saline self-administration sessions after nicotine treatment. The plus signs indicate more infusions in rats treated with mecamylamine compared to those treated with the vehicle, within the same self-administration group and sex. Asterisks indicate a difference in infusions between rats treated with mecamylamine and vehicle within the same sex, and time point. Letters a and b indicate lower infusions compared with first hour infusions in vehicle-treated rats with the same sex. Letter f indicates lower infusions compared with first hour infusions in mecamylamine-treated rats with the same sex. +, *, a $p < 0.05$; **, b $p < 0.01$; f $p < 0.001$. Saline IVSA-male (N = 12), nicotine IVSA-male (N = 10), saline IVSA-female (N = 11), and nicotine IVSA-female (N = 10). Data are expressed as means ± SEM.

Mecamylamine treatment × Hours F5, 105 = 2.102, NS; Mecamylamine treatment × Hours × Sex F5, 105 = 1.322, NS). There was no sex difference in saline infusions (Sex F1, 21 = 3.964, NS). However, saline infusions decreased over time only in females (Hours F5, 105 = 11.037, $p < 0.001$; Hours × Sex F5, 105 = 2.926, $p < 0.05$). The *post hoc* showed that in females, saline infusions were lower from the second hour in both the saline and mecamylamine treatment groups (Figure 8B).

Nicotine self-administration group: Treatment with mecamylamine differently affected nicotine infusions (intake) in females and males (Figure 8C, Mecamylamine treatment F1, 18 = 0.99, NS; Mecamylamine treatment × Sex F1, 18 = 4.413, $p < 0.05$). The *post hoc* analysis showed that treatment with mecamylamine increased nicotine infusions in the males and decreased nicotine intake in the females (Figure 8C). There was no sex difference in nicotine infusions (Sex F1, 18 = 2.789, NS). However, nicotine infusions decreased over time in both males and females (Hours F5, 90 = 62.916, $p < 0.001$; Hours × Sex F5, 90 = 1.787, NS). Moreover, the effect of mecamylamine on nicotine infusions was dependent on the time point (Mecamylamine treatment × Hours F5, 90 = 3.611, $p < 0.01$; Mecamylamine treatment × Hours × Sex F5, 90 = 1.796, NS).

3.6 Week 15: nicotine treatment and self-administration

Infusions: The administration of nicotine 10 minutes before the self-administration sessions did not affect the number of infusions (Figure 8D,: Nicotine treatment F1, 39 = 0.005, NS; Nicotine treatment × Sex F1, 39 = 0.777, NS; Nicotine treatment × IVSA group F1, 39 = 1.992, NS; Nicotine treatment × Sex × IVSA group F1, 39 = 0.214, NS). The female rats had a higher number of infusions compared to the males (Figure 8D: Sex F1, 39 = 10.476, $p < 0.01$). Furthermore, the rats that self-administered nicotine had more than the rats that self-administered saline and this was not affected by the sex of the rats (Figure 8D: IVSA group F1, 39 = 15.224, $p < 0.001$; Sex × IVSA group F1,39 = 0.031, NS).

Active lever: The administration of nicotine 10 minutes before the self-administration sessions did not affect the number of active lever responses (see Supplementary File S9 for results; Supplementary Figure S11C).

Inactive lever: Treatment with nicotine or the sex of the rats did not affect inactive lever responses (see Supplementary File S9 for results; Supplementary Figure S11D).

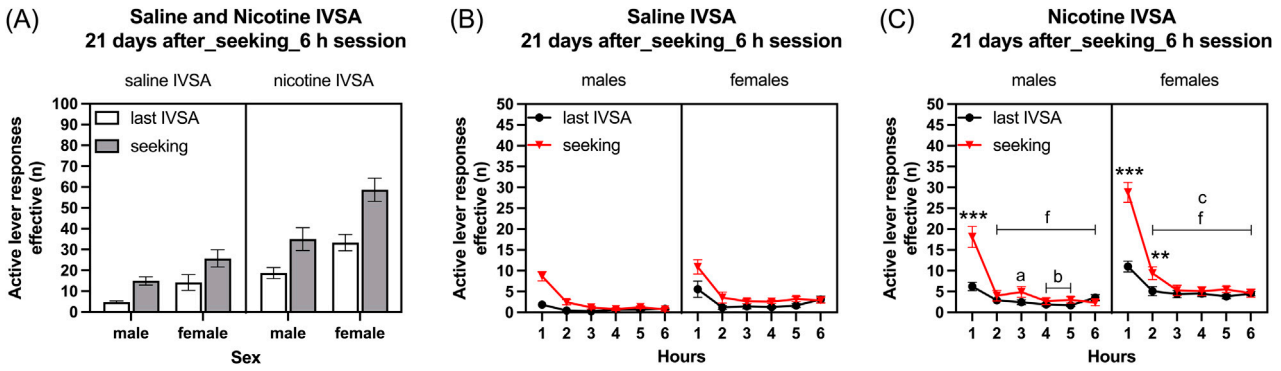

FIGURE 9
Nicotine and saline seeking behavior after forced abstinence in male and female rats. Twenty-one days after last self-administration session, the rats were placed in the operant chamber and the lever responses were recorded in a 6-h session. Effective active lever responses **(A)** in 6-h sessions. Time course of effective active lever responses in saline group **(B)** and nicotine group **(C)** rats during 6-h sessions. Asterisks indicate a significant difference in effective active lever responses between the seeking session and last self-administration session within the same sex and time point. The letters a, b, and c indicate fewer effective active lever responses compared with the first-hour time point in the last session within the same sex. The letter f indicates fewer effective active lever responses compared with first-hour effective active lever responses in the seeking session within the same sex. a $p < 0.05$; **, b $p < 0.01$; ***, c, f $p < 0.001$. Saline IVSA-male (N = 12); nicotine IVSA-male (N = 9); saline IVSA-female (N = 11); nicotine IVSA-female (N = 10). Data are expressed as means ± SEM.

3.6.1 Time course analysis, nicotine treatment and self-administration

Saline self-administration group: Saline infusions were higher in females than males (Figure 8E, Sex F1, 21 = 4.935, $p < 0.05$). Nicotine treatment affects the saline infusions in both males and females only at the beginning of the self-administration period (Figure 8E, Nicotine treatment F1, 21 = 0.678, NS; Nicotine treatment × Sex F1, 21 = 0.678, NS; Nicotine treatment × Hours F5, 105 = 3.105, $p < 0.05$; Nicotine treatment × Hours × Sex F5, 105 = 2.086, NS). Saline infusions decreased over time and there was no effect of sex (Hours F5, 105 = 3.618, $p < 0.01$; Hours × Sex F5, 105 = 1.193, NS).

Nicotine self-administration group: Nicotine infusions were higher in the females than the males (Figure 8F, Sex F1, 18 = 6.367, $p < 0.05$). Nicotine infusions decreased over time and this effect was greater in the females than the males (Hours F5, 90 = 39.32, $p < 0.001$; Hours × Sex F5, 90 = 9.761, <0.001). Treatment with nicotine slightly decreased nicotine infusions at the beginning of the session and increased infusions towards the end of the session (Figure 8F, Nicotine treatment F1, 18 = 2.06, NS; Nicotine treatment × Hours F5, 90 = 2.929, $p < 0.05$; Nicotine treatment × Sex F1, 18 = 0.165, NS; Nicotine treatment × Hours × Sex F5, 90 = 1.691, NS).

3.7 Week 18: forced abstinence and nicotine- and saline-seeking behavior

3.7.1 Last self-administration day compared to seeking session after forced abstinence

Effective active lever: Rats that self-administered nicotine had more effective active lever responses than those self-administering saline (Figure 9A, IVSA group F1, 38 = 40.139, $p < 0.001$). After a period of abstinence, effective active lever presses increased more in rats with a history of nicotine self-administration compared to those with a history of saline self-administration (Session F1, 38 = 1112.318, $p < 0.001$; Session × IVSA group F1, 38 = 11.063, $p < 0.001$). Additionally, the females had more effective active lever presses than the males (Sex F1, 38 = 18.599, $p < 0.001$; Session × Sex F1, 38 = 2.988, NS; Sex × IVSA group F1,38 = 1.753, NS; Session × Sex × IVSA group F1,38 = 1.681, NS).

Active lever: Following a period of abstinence, active lever presses increased, and this effect was greater in the rats with a history of nicotine self-administration than in the rats with a history of saline self-administration (see Supplementary File S10 for results; Supplementary Figure S12A).

Inactive lever: The inactive lever presses increased after abstinence in the rats with a history of nicotine self-administration and saline self-administration (see Supplementary File S10 for results; Supplementary Figure S12B).

3.7.2 Time course analysis, last self-administration day compared to seeking session after forced abstinence

Saline self-administration group: Effective active lever presses decreased over time and the females had more effective active lever presses than the males (Figure 9B, Hours F5, 105 = 48.894, $p < 0.001$; Sex F1, 21 = 7.087, $p < 0.05$). Effective active lever responses were higher after the abstinence period in rats of both sexes (Figure 9B, Session F1, 21, 58.602, $p < 0.001$; Session × Sex F1, 21, 0.207, NS). Over time, there was a decrease in effective active lever responses, and this decrease was more significant during the seeking session compared to the final self-administration session (Hours × Sex F5, 105 = 1.63, NS; Hours × Session F5, 105 = 13.088, $p < 0.001$; Hours × Session × Sex F5, 105 = 0.582, NS).

Nicotine self-administration group: Effective active lever presses decreased over time and the females had more effective active lever presses than the males (Figure 9C, Hours F5, 85 = 98.353, $p < 0.001$; Sex F1, 17 = 10.548, $p < 0.01$). Over time, the decrease in effective active lever responses was more pronounced in females than in males, and the decrease in responses was larger during the seeking session compared to the final self-administration session (Figure 9C, Hours × Sex F5, 85 = 5.82, $p < 0.001$; Hours × Session F5, 85 =

46.744, $p < 0.001$). Effective active lever responses were higher after the abstinence period in rats of both sexes (Session F1, 17, 54.649, $p < 0.001$; Session × Sex F1, 17, 2.579, NS). Despite the initial strong increase in effective active lever responses in females following the abstinence period, there was a rapid decline in effective active lever responses after the first hour of access to the operant chambers (Hours × Session × Sex F5, 85 = 2.592, $p < 0.05$). The *post hoc* showed that effective active lever responses were higher during the first hour in males and first and second hour in females compared to the last self-administration session. Additionally, effective active lever responses decreased over time in all groups (Figure 9C).

4 Discussion

These studies aimed to determine whether there are sex differences in nicotine intake, affective and somatic withdrawal signs, and relapse parameters in rats. Specifically, we investigated whether male and female rats with long access to nicotine or saline display differences in nicotine intake (infusions), somatic withdrawal signs, activity levels, anxiety-like behavior, anhedonia, and sensitivity to nicotine and mecamylamine. Additionally, we assessed whether there are sex differences in nicotine and saline intake, as well as in nicotine and saline intake after forced abstinence, and seeking behavior following a period of forced abstinence. Our findings revealed that female rats self-administer more nicotine than male rats. However, there was no sex difference in precipitated somatic withdrawal signs. In addition, following the cessation of nicotine self-administration, both male and female rats displayed spontaneous somatic withdrawal signs and were hyperactive in the small open field, with no significant sex differences observed. However, cessation of nicotine intake did not lead to an increase in anxiety-like behavior in the large open field test or in the elevated plus maze test. Additionally, cessation of nicotine intake did not cause anhedonia, as indicated by the absence of a difference in sucrose preference between the rats that self-administered nicotine or saline. A period of forced abstinence increased saline, but not nicotine, intake in the males and the females. Mecamylamine differently affected nicotine intake in the males and the females. In males, mecamylamine treatment increased nicotine intake, whereas in females, mecamylamine decreased nicotine intake. Operant responding for nicotine was not affected by a period of forced abstinence. However, nicotine seeking was increased after a period of forced abstinence. The females displayed more nicotine seeking than the males after the abstinence period. Taken together, these findings indicate that there are significant sex differences in nicotine intake, the effects of mecamylamine on nicotine intake, and nicotine seeking following a period of forced abstinence.

In the present study, we investigated sex differences in nicotine intake during both five 1-h and thirty-six 6-h self-administration sessions. During the initial five 1-h nicotine self-administration sessions (3 sessions with 0.03 mg/kg/inf of nicotine and 2 sessions with 0.06 mg/kg/inf of nicotine), there was no sex difference in nicotine intake. Contrary to the findings of this current study, in our previous work with adult rats, we found that nicotine intake was higher in females than males when the rats had access to 0.03 mg/kg/inf for 1 h per day (Chellian et al., 2021b; Chellian et al., 2021c). However, these studies differed from the present study in which the rats were trained to respond for food pellets before the nicotine self-administration sessions and then self-administered 0.03 mg/kg/inf of nicotine for 3 days. In one of the prior studies the sex differences in nicotine intake were only observed after the first 10 days of nicotine self-administration (Chellian et al., 2021b). In the other study, the rats did not receive any food training and the spontaneous acquisition of nicotine intake was investigated (Chellian et al., 2021c). Several other studies also reported no sex differences in nicotine intake when the rats have short access (1–2 h/day) to 0.03 mg/kg/inf of nicotine (Donny E. C. et al., 2000; Chaudhri et al., 2005; Feltenstein et al., 2012).

In the present study, there was no sex difference in nicotine intake during the two short access sessions where the rats had access to 0.06 mg/kg/inf of nicotine. However, nicotine intake was higher in the females than in the males when the rats had long access to 0.06 mg/kg/inf of nicotine. In line with the current study, one of our previous studies, in which the rats self-administered 0.06 mg/kg/inf of nicotine for 1 h per day, also did not detect a sex difference in nicotine intake (Chellian et al., 2023). Other studies reported that nicotine intake is higher in females than males with long access (23-h sessions) to 0.06 mg/kg/inf of nicotine (Grebenstein et al., 2013; Flores et al., 2016). A recent meta-analysis with 20 studies concluded that female rats self-administer more nicotine than male rats (Flores et al., 2019). This meta-analysis also showed that sex differences in nicotine self-administration are greater in animals with long than shorter access periods. This finding aligns with our observation that while no sex difference in nicotine intake was observed when the rats self-administered 0.06 mg/kg/inf of nicotine for 1 h a day, a robust significant sex difference was observed when the rats self-administered the same dose for 6 h per day (Chellian et al., 2023).

In the present study, the rats had a higher level of operant responding for nicotine than for saline. This corroborates previous studies that demonstrated that operant responding for nicotine is higher than for saline (Sanchez et al., 2014; Jin et al., 2020; Chellian et al., 2021b). These findings support the hypothesis that nicotine is a potent reinforcer in rodents (Stolerman and Jarvis, 1995; Balfour, 2008). In the present study, we also determined sex difference in the self-administration of saline. Interestingly, the females responded more for saline than the males. This higher level of responding for saline was observed during the first ten long access self-administration sessions and again during the second half of the 36-day self-administration period. This higher level of responding for saline in the females is not in line with prior studies that reported that there are no sex differences in the self-administration of saline (Sanchez et al., 2014; Johansen and McFadden, 2017). Several potential factors could explain the higher level of operant responding for saline in the female rats. Firstly, females are more active than males, which could potentially lead to more responding for saline (Knight et al., 2021). Secondly, prior to the saline self-administration sessions, the rats were trained to respond for food pellets and delivery of the food pellets was paired with a visual cue. Female rats are more responsive to cues associated with food rewards than males (Grimm et al., 2022). Therefore, it might be possible that the females responded more for saline because of their heightened responsiveness to cues paired with the delivery of food pellets.

In this study, we assessed both precipitated and spontaneous somatic withdrawal signs in male and female rats. Extensive evidence indicates that noncontingent exposure to nicotine via injections, minipumps, or tobacco smoke leads to the development of dependence, as indicated by spontaneous and precipitated somatic withdrawal signs (Epping-Jordan et al., 1998; Skjei and Markou, 2003; Small et al., 2010; Chellian et al., 2021a). The development of dependence has also been investigated in male rats that self-administer nicotine under long access conditions (Paterson and Markou, 2004; O'Dell et al., 2007; O'Dell and Koob, 2007; Cohen et al., 2012; Cohen et al., 2015). These studies indicate that both precipitated and spontaneous somatic withdrawal signs can be observed in rats with long access to nicotine (Chellian et al., 2022a). Our study expands on this work by examining precipitated and spontaneous withdrawal signs in both male and female rats and control rats that self-administer saline. Our findings show that rats that self-administer nicotine have more precipitated and spontaneous somatic withdrawal signs compared to those that self-administer saline, and there are no sex differences in precipitated and spontaneous somatic withdrawal signs. Interestingly, we also found that the number of precipitated somatic withdrawal signs in the nicotine group increased over time. This finding aligns with our previous study, which showed that mecamylamine-precipitated somatic withdrawal signs increase over time in rats exposed to tobacco smoke (Chellian et al., 2020). This pattern of results suggests that rats gradually develop nicotine dependence (Malin et al., 1992).

The rats were tested in the small open field test to determine if cessation of nicotine self-administration affects locomotor activity. Cessation of nicotine intake led to an increase in horizontal beam breaks, locomotor activity, and stereotypies in both males and females. The observation that cessation of nicotine self-administration increases locomotor activity aligns with the observation that removal of nicotine pumps leads to an increase in locomotor activity in male mice (Kota et al., 2007). However, the same group reported that removal of the nicotine pumps did not lead to an increase in locomotor activity in female mice (Kota et al., 2008). Numerous other studies reported that cessation of nicotine administration does not affect or decreases locomotor activity in rats and mice (Isola et al., 1999; Hamilton et al., 2009; Malin and Goyarzu, 2009). Interestingly, restlessness is a common symptom of smoking cessation in humans (Hughes and Hatsukami, 1986). Notably, in a recent study, restlessness (26.82%) was the second most commonly reported withdrawal symptom, following urges to smoke (34.01%) (Cui et al., 2023). The observed increase in locomotor activity in both male and female rats after cessation of nicotine intake could potentially serve as an animal model for studying the restlessness experienced during a smoking cessation attempt in humans.

To investigate whether cessation of nicotine self-administration leads to an increase in anxiety-like behavior, the rats were tested in the elevated plus maze test and in the large open field. Cessation of nicotine self-administration did not increase anxiety-like behavior in these tests. These outcomes were somewhat unexpected, as some previous studies reported that cessation of nicotine self-administration increases anxiety-like behavior. For example, cessation of long access (23 h) nicotine self-administration increases anxiety-like behavior in rats in the elevated plus maze test (Cohen et al., 2015). Similarly, cessation of noncontingent nicotine administration has been shown to increase anxiety-like behavior in the elevated plus maze test in both rats and mice (Pandey et al., 2001; Kotagale et al., 2015; Bagosi et al., 2016). However, it should be noted that other studies did not report an increase in anxiety-like behavior after the cessation of noncontingent nicotine administration or nicotine self-administration (Grabus et al., 2005; Biała et al., 2014; Zaniewska et al., 2021). Given these discrepancies in the effects of nicotine cessation on anxiety-like behavior, further research is needed to identify the specific factors that contribute to anxiety-like behavior following the cessation of nicotine administration.

In the present study, the females displayed less anxiety-like behavior than the males in the elevated plus maze, as indicated by a higher percentage of time on the open arms and a higher percentage of open arm entries. Furthermore, the females displayed less anxiety-like behavior in the large open field test, as indicated by a shorter latency to enter the center of the field and a longer duration spent in the center. These observations are in line with our previous work, in which we showed that females display less anxiety-like behavior than males in the large open field test and the elevated plus maze test (Knight et al., 2021).

In this study, we also investigated the effects of a period of forced abstinence on the self-administration of nicotine and saline. We found that the self-administration of saline, but not nicotine, increased after the period of forced abstinence. Several studies have investigated nicotine intake after a period of abstinence, but these studies did not include female rats (George et al., 2007; O'Dell and Koob, 2007). A study by George et al. (2007) demonstrated that rats, when given 23-h access to nicotine at a dose of 0.03 mg/kg/inf, have increased nicotine intake following a 3-day abstinence period (George et al., 2007). O'Dell and Koob (2007) also investigated the abstinence effect in rats (O'Dell and Koob, 2007). In their study, the rats were allowed to self-administer either saline or a gradually increasing dose of nicotine (0.015 mg/kg/inf to 0.09 mg/kg/inf). The rats had access to each nicotine dose for four 23-h sessions, with a 2-day break between each dose. Nicotine intake was highest during the initial self-administration session and subsequently declined. The rats stopped responding for saline after the first two self-administration sessions. There are some similarities and differences between the outcomes of the present study and those of previous studies that investigated the effects of forced abstinence on nicotine intake. In the present study, we found that nicotine intake was highest during the first session after the abstinence period and then gradually declined. Previous studies that investigated the effects of abstinence on nicotine intake reported a similar pattern with the highest nicotine intake in the first session after abstinence, followed by a gradual decline (George et al., 2007; O'Dell and Koob, 2007). However, we did not observe an increase in nicotine intake after the abstinence period as reported previously (George et al., 2007). There are several potential differences between the present study and the study by George et al. (2007) that might account for this discrepancy. Firstly, the nicotine dose was 0.06 mg/kg/inf in the present study and 0.03 mg/kg/inf in the study by George et al. (2007). Nicotine intake is higher when the rats have access to the 0.06 mg/kg/inf dose compared to when they have access to the lower 0.03 mg/kg/inf dose (Shoaib and Stolerman, 1999; Cohen et al., 2015). Other

differences between the present study and the study by George et al. (2007) that might account for the differences in nicotine intake after abstinence include the duration of the self-administration sessions (23 h in their study *versus* 6 h in our study) and the duration of the abstinence period (3 days in their study *versus* 14 days in our study).

Previous work has shown that rats respond for saline when the delivery of saline is paired with a visual cue (Jin et al., 2020; Tapia et al., 2022; Stringfield et al., 2023). It was interesting to note that in the present study there was a robust increase in the intake of saline, but not nicotine, in the males and females after the forced abstinence period. This may suggest that the period of abstinence enhances the reinforcing properties of environmental cues, such as the visual cue paired with saline, which in turn increases responding for saline. It is also possible that the period of abstinence increased the reinforcing properties of the visual cue paired with nicotine, but this did not further increase nicotine intake because high doses of nicotine are aversive (Risinger and Oakes, 1995; Igari et al., 2013). It has been suggested that nicotine intake leads to satiety, and that after this satiety point has been reached, the motivation to self-administer nicotine decreases (Fowler and Kenny, 2014). Nicotine intake is rewarding up to the point of satiety, and intake beyond this point gradually becomes more aversive. It is possible that the period of forced abstinence does not change the satiety point and therefore abstinence does not lead to an increase in nicotine intake.

In this study, we also investigated the effects of 3 weeks of forced abstinence on nicotine and saline seeking. The abstinence period led to an increase in nicotine and saline seeking. A previous study suggested that nicotine and saline-seeking behavior is only observed in rats that received food-training prior to the self-administration sessions (Clemens et al., 2010). In addition, they found that food training results in similar levels of nicotine and saline seeking. This suggests that seeking behavior is primarily associated with food acting as a reinforcer (Clemens et al., 2010). However, in our study, the rats with a prior history of food training displayed significantly more nicotine-seeking than saline-seeking behavior. Therefore, our findings indicate that nicotine also acts as a reinforcer for seeking behavior. In our study, the females displayed more nicotine seeking than the males. This work builds upon prior research that investigated nicotine seeking after abstinence but did not include females or saline self-administration groups (Markou et al., 2018; Domi et al., 2023). We are unaware of any studies that investigated sex differences in nicotine seeking after a period of forced abstinence. However, sex differences in nicotine seeking have been investigated in adult rats following the extinction of nicotine-seeking behavior (Feltenstein et al., 2012). Interestingly, Feltenstein et al. (2012) did not find sex differences in cue, nicotine, or yohimbine-induced reinstatement of nicotine seeking. In contrast, in our current study, we found sex differences in nicotine seeking after a period of abstinence with no extinction training. Therefore, these findings suggest that sex differences in nicotine seeking might be observed after a period of abstinence without extinction training but not after abstinence with extinction training. Another difference between our study and the study by Feltenstein et al. (2012) where no sex differences were observed, is the strain of rats used. Feltenstein et al. (2012) used adult Sprague-Dawley rats, whereas for our study we used adult Wistar rats. Sex differences in drug seeking have been more thoroughly investigated in rats with a history of cocaine intake rather than nicotine intake. Several studies with cocaine have reported that, following forced abstinence with or without extinction training, females exhibit more cocaine seeking than males (Kerstetter et al., 2008; Nicolas et al., 2019; Corbett et al., 2021).

We also investigated the effects of nicotine and mecamylamine treatment on the self-administration of nicotine and saline. We investigated the effects of nicotine and mecamylamine on total nicotine intake over a 6-h self-administration period. Nicotine treatment did not affect operant responding for nicotine in either males or females over the 6-h self-administration period. Similarly, mecamylamine treatment did not affect nicotine self-administration in males over the same period. In a previous study, we also found that mecamylamine did not affect nicotine intake over a 6-h self-administration period in male rats (Chellian et al., 2024). In the present study, mecamylamine did, however, decrease nicotine intake in females over the 6-h self-administration period. Several studies have investigated the effects of mecamylamine on nicotine self-administration in male rats. These studies show that mecamylamine decreases nicotine intake in male rats with short or long access to nicotine (Corrigall and Coen, 1989; Paterson and Markou, 2004; DeNoble and Mele, 2006). The findings are not in line with our studies, which showed that mecamylamine did not affect nicotine intake in males over a 6-h self-administration period. This discrepancy might be due to the fact that we used a higher dose of nicotine (0.06 mg/kg/inf *versus* 0.03 mg/kg/inf) combined with a longer self-administration period (8 weeks) compared to previous studies (Corrigall and Coen, 1989; Paterson and Markou, 2004; DeNoble and Mele, 2006).

Given the short half-lives (t1/2) of nicotine (t1/2, 1 h) and mecamylamine (t1/2, 1.2 h) in rats, we also examined the time-course effects of nicotine and mecamylamine on the self-administration of nicotine and saline (Miller et al., 1977; Debruyne et al., 2003). Treatment with nicotine decreased nicotine intake at the beginning of the self-administration session. However, mecamylamine treatment increased nicotine intake during the first hour of access in the males. In a prior study, we found that nicotine decreased and mecamylamine increased first-hour nicotine intake in males with long access to nicotine (Chellian et al., 2024). These findings are in line with clinical studies demonstrating that treatment with nicotine decreases smoking and treatment with mecamylamine increases smoking (Pomerleau et al., 1987; Benowitz and Jacob, 1990; Rose et al., 2001). Interestingly, in the present study, mecamylamine treatment did not increase first-hour nicotine intake in the females. However, mecamylamine decreased nicotine intake in the females during the second and fifth hour of access. These findings indicate that mecamylamine differently affects nicotine intake in males and females. Nicotine treatment slightly decreased nicotine intake at the beginning of the session and increased intake towards the end of the session. This effect of nicotine treatment could be attributed to its locomotor depressant and stimulant effects. Previous studies have shown that nicotine treatment in drug-naïve mice and rats initially

has locomotor depressant effects, followed by an increase in locomotor activity (Clarke and Kumar, 1983; Clarke, 1990; Stolerman et al., 1995; Weiss et al., 2007). Overall, our study suggests that mecamylamine has different effects in males and females.

The main goal of the present studies was to examine sex differences in nicotine intake, affective and somatic withdrawal signs, and nicotine intake after a period of forced abstinence. Overall, our findings indicate that there are significant sex differences across most of the investigated parameters related to nicotine intake, effects of mecamylamine on nicotine intake, and relapse. We discovered that female rats had higher nicotine intake during long access sessions. Furthermore, the females displayed more nicotine seeking than the males after the forced abstinence period. Mecamylamine precipitated more somatic withdrawal signs in the rats that self-administered nicotine compared to those that self-administered saline, and there was no sex difference. However, mecamylamine increased nicotine intake in males and decreased nicotine intake in females. We also found that cessation of nicotine self-administration led to spontaneous somatic withdrawal signs and an increase in locomotor activity, but no sex differences were observed in these parameters. Cessation of nicotine self-administration did not increase anxiety-like behavior or cause anhedonia in the males or the females. Furthermore, a period of forced abstinence did not lead to an increase in nicotine self-administration in the males or the females. These findings provide critical insights into the sex differences in nicotine addiction and aid in the development of more sex-specific therapeutic interventions for smoking cessation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by University of Florida Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RC: Conceptualization, Formal Analysis, Investigation, Visualization, Writing–review and editing. AB-R: Investigation, Project administration, Writing–review and editing. AB: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1415219/full#supplementary-material

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EDITED BY
Massimo Grilli,
University of Genoa, Italy

REVIEWED BY
David Tattersall,
GlaxoSmithKline, United States
Debashis Dutta,
University of Nebraska Medical Center, United States
Joseph Bryant,
University of Maryland, United States

*CORRESPONDENCE
Talia H. Swartz,
talia.swartz@mssm.edu

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The impacts of tobacco and nicotine on HIV-1 infection, inflammation, and the blood-brain barrier in the central nervous system

Aislinn M. Keane[1] and Talia H. Swartz*[,2]

[1]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2]Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, United States

Human immunodeficiency virus (HIV-1) remains a persistent global health crisis. Even while successfully virologically suppressed, people with HIV (PWH) experience a higher risk for inflammatory disorders such as HIV-associated neurocognitive disorder (HAND). Tobacco use puts PWH at higher risk for neurocognitive symptoms resulting from HIV-associated neuroinflammation. The NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome has been implicated as a driver of HIV-associated inflammation, including HAND. Nicotine, the psychoactive component of tobacco smoke, has also been shown to signal through the NLRP3 inflammasome and modulate inflammatory signaling in the CNS. Here, we explore the impacts of nicotine and tobacco on the complex neurobiology of HAND, including effects on cognition, inflammation, viral latency, and blood-brain barrier integrity. We outline nicotine's role in the establishment of active and latent infection in the brain and posit the NLRP3 inflammasome as a common pathway by which HIV-1 and nicotine promote neuroinflammation in PWH.

KEYWORDS

HIV-1, NLRP3 inflammasome, neuroinflammation, nicotine, blood-brain barrier (BBB)

Introduction

Human immunodeficiency virus (HIV-1) affects more than 39 million people worldwide and remains incurable despite major advances in antiretroviral therapy (ART). Although patient life expectancy and quality of life has dramatically improved over the past few decades, People with HIV (PWH) are still at greater risk for several comorbid conditions due to viral persistence in latent reservoirs and associated low-level chronic inflammation (Chun et al., 2010; Collora and Ho, 2022; Global, 2024; Lee et al., 2020; Siliciano et al., 2003; White et al., 2022). These include a variety of conditions related to aging, including cardiovascular disease, osteoporosis, cancer, and neurocognitive disorders (Aberg, 2012; Guaraldi et al., 2011; Heaton et al., 2010; Kaplan-Lewis et al., 2017; Kearns et al., 2017). PWH also tend to develop these conditions earlier than the

Abbreviations: PWH, People with HIV; BBB, Blood brain barrier; nAChR, nicotinic acetylcholine receptor; HAND, HIV-associated neurocognitive disorder; ART, antiretroviral therapy.

general population (Aberg, 2012; Kaplan-Lewis et al., 2017). Chronic inflammation due to persistent infection in viral reservoirs such as the central nervous system (CNS) is thought to be the primary driver of this increased risk (Desplats et al., 2013; Sonti et al., 2021).

HIV-associated neurocognitive Disorder (HAND) is a common HIV-associated condition affecting 20%–50% of PWH (Heaton et al., 2010). HAND is characterized by increased neuroinflammation, blood-brain barrier (BBB) breakdown, metabolic dysfunction, and measurable cognitive impairment (Saylor et al., 2016). Drug use, including smoking, is a risk factor for the development of HAND in PWH (Saylor et al., 2016). Tobacco users with HIV experience poorer outcomes, including increased risk of mortality and virologic rebound and poorer response to cART (Han et al., 2018). Because of the high prevalence of tobacco use in PWH and its known detrimental effects, unraveling its role in neuroinflammation will provide much-needed insights into mechanisms and possible therapeutic strategies for PWH.

Biology of HAND: Chronic inflammation in the CNS

People with HIV are more likely to exhibit markers of inflammation even when viral levels are suppressed below detection by long-term ART treatments (Aberg, 2012; Deeks, 2011; Sieg et al., 2021). In virologically suppressed individuals, replication persists in tissues where ART has limited penetrance (Kulpa and Chomont, 2024). Despite ART, this leads to persistent immune activation, senescence, and an increased systemic inflammatory milieu (Aberg, 2012; Deeks, 2011). The brain is a viral reservoir with unique characteristics due to its immune-privileged status and thus presents a distinct set of challenges for a sterilizing cure. Although improved access to ART has greatly improved the quality of life and life expectancy for PWH, even complete viral suppression under ART treatment does not eliminate the cognitive symptoms associated with HIV infection of the brain (Heaton et al., 2010). HAND is defined clinically as a spectrum of disorders ranging from Asymptomatic Neurocognitive Impairment (ANI) to Mild Neurocognitive Disorder (MND) to HIV-associated Dementia (HAD), the most severe presentation (Heaton et al., 2011). Clinical studies have shown that biomarkers associated with CNS inflammation in blood plasma and cerebrospinal fluid (CSF) are more prevalent during acute HIV infection and decline over time and with ART treatment (Longino et al., 2022). As access to earlier ART intervention improves, rates of the more severe forms of HAND have been declining. Still, it is estimated that between 20% and 50% of PWH experience some form of neurocognitive impairment due to chronic HIV infection (Heaton et al., 2010).

Neuroinflammation in the HIV-1 infected CNS is characterized by BBB dysfunction, immune cell infiltration, and infection and inflammatory signaling of resident CNS cells, particularly microglia (Khanal et al., 2021; Saylor et al., 2016; Sreeram et al., 2022). Infected microglia compose the bulk of the viral reservoir in the CNS, and HIV is seeded into the brain in the very early stages of infection, primarily by infected monocytes, which migrate across the blood-brain barrier (Davis et al., 1992; Kahn and Walker, 1998; Longino et al., 2022; Valcour et al., 2012). Damage to the blood-brain barrier is a key aspect of many neuroinflammatory conditions, and chronic neuroinflammation from HIV infection is no exception. BBB dysfunction persists even in the presence of ART and during chronic infection (Kulpa and Chomont, 2024). Brain microvascular endothelial cells (BMVECs), pericytes, and astrocytes, are all dysregulated by the presence of virus in the CNS (Andersson et al., 2001; Eugenin et al., 2011; Leibrand et al., 2017; Osborne et al., 2020; Piekna-Przybylska et al., 2019). Endothelial cells are prone to dysfunction and death due to direct interaction with viral proteins and exposure to inflammatory cytokines released by infected cells (Andersson et al., 2001; Lee et al., 2004; Leibrand et al., 2017). Dysregulation of endothelial tight junction proteins such as claudin-5, occludin, and ZO-1 leads to increased BBB permeability and may facilitate infiltration of peripheral immune cells to the CNS (Boven et al., 2000; Chaudhuri et al., 2008; Dallasta et al., 1999; Eugenin et al., 2011). Decreased pericytes coverage of the endothelium and infected or dysregulated astrocytes have also been shown to contribute to barrier dysfunction in the HIV-infected brain (Piekna-Przybylska et al., 2019; Pla-Tenorio et al., 2023; Valdebenito et al., 2021). Although HIV can both cross and dysregulate the BBB, BBB penetrance remains a complex problem for the efficacy of antiretroviral drugs. The viral reservoir in the brain is formed by a combination of this challenge for ART delivery and the development of a population of latently infected cells, primarily microglia, which harbor HIV provirus (Osborne et al., 2020).

As the cell population most susceptible to HIV infection in the brain, microglia form the bulk of the latent reservoir (Wallet et al., 2019). An established literature shows a relationship between HIV-1 proteins, neuronal death, and microglial activation. Transgenic mouse studies show that viral proteins such as Tat and gp120 are produced by infected cells and have neurotoxic properties (Leibrand et al., 2017). Additionally, infected human microglia become reactive and release pro-inflammatory cytokines such as TNFα, CCL2, IL-1β, IL-6, and CCL5 (Alvarez-Carbonell et al., 2019). Infected microglia are also prone to mitochondrial dysfunction and overproduction of ROS (Alvarez-Carbonell et al., 2019; Borrajo et al., 2021).

Recent models suggest that cyclical reactivation of latent provirus in microglia may cause the ongoing cascade of inflammation in HAND (Sreeram et al., 2022). The susceptibility of microglial cells to latent infection is thought to be tied to the activation state at the time of infection, with more quiescent cells favoring a latent status than reactive or activated cells (Sreeram et al., 2022; Wallet et al., 2019). Activation of the transcription factor NF-κB or IRF3 in response to an inflammatory stimulus can reactivate viral replication in a latently infected human microglial cell line (Alvarez-Carbonell et al., 2017). Thus, inflammatory stimuli such as damaged neurons or drug exposures can potentiate latency reversal and promote the chronic inflammation characteristic of HAND (Alvarez-Carbonell et al., 2019). We will further explore the role of NLRP3 inflammasome signaling and nicotine exposures in driving this complex inflammatory cascade.

HIV and the NLRP inflamamsome

The NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome, which consists of NLRP3, ASC, and pro-caspase-

1, has been implicated in HAND and other HIV-associated inflammatory pathologies. Upon activation by intracellular pathogens and binding of Damage-associated Molecular Patterns (DAMP) to PRRs (Pattern Recognition Receptors), the inflammasome activates caspase-1, leading to the release of pro-inflammatory cytokines (IL-1β and Il-18) and pyroptosis, an inflammatory form of cell death known to contribute to inflammation in PWH (Doitsh et al., 2014; Katuri et al., 2019; Mamik and Power, 2017). HIV infection in T-cells may lead to incomplete transcription and viral fragments, triggering PRRs and leading to NLRP3 activation, inflammatory cytokine release, and cell death (Doitsh et al., 2014). In myeloid cells, NLRP3 activation and inflammatory cytokine release are associated with many HIV-associated inflammatory processes, such as atherosclerosis (Mao et al., 2021; Mullis and Swartz, 2020).

Animal studies in the CNS have shown that the NLRP3 inflammasome can be activated in microglia in response to viral proteins such as Tat, Vpr, and gp120, resulting in inflammatory cytokine release and neuroinflammation (Chivero et al., 2017; He et al., 2020). HIV infection was shown to promote IL-1β release in primary human microglia (Walsh et al., 2014). Furthermore, NLRP3 inflammasome inhibitors have shown a therapeutic effect in a mouse HAND model (He et al., 2020; Mamik and Power, 2017).

Alongside microglia-induced inflammatory signaling, the NLRP3 inflammasome is also implicated in BBB dysregulation. HIV-1 dysregulates the BBB through a variety of mechanisms, from direct interaction of brain microvascular endothelial cells (BMVECs) with viral proteins to inflammatory cytokine release (Il-6, TNF-a, IL-1β) from infected microglia and infiltrating monocytes in the CNS (Atluri et al., 2015; Caligaris et al., 2021). Studies indicate that HIV readily establishes latency in microglia and that microglial activation by NLRP3-associated inflammatory cytokines such as TNF-α and IL-1β may contribute to viral emergence from proviral latency, contributing to the chronic inflammation of HAND (Alvarez-Carbonell et al., 2017; Alvarez-Carbonell et al., 2019; Ko et al., 2019; Li and Barres, 2018; Plaza-Jennings et al., 2022; Sreeram et al., 2022).

Nicotine and inflammation

Nicotine and its effects in the context of HAND are particularly of interest due to the high prevalence of smoking among PWH and the heightened health risks experienced by PWH who smoke. The established literature points to tobacco use as contributing to inflammation in a variety of contexts, both inside and outside of the CNS. However, its pro-inflammatory and anti-inflammatory effects can be disease and context dependent. Nicotine signals through the Nicotinic Acetylcholine receptor family (nAChRs), which are involved in cholinergic signaling and reward stimulus (de Kloet et al., 2015). They are also widely expressed in various cell types, including monocytes, macrophages, and microglia (Richter and Grau, 2023; Suzuki et al., 2006; Zoli et al., 2018). In non-excitable cells, signaling is primarily mediated through the influx of calcium ions directly through the ion-gated channel of the receptor (Zoli et al., 2018). In different non-neuronal cell types, signaling can be ionically or non-ionically driven and trigger downstream signaling via various pathways (Sorimachi et al., 1994; Zia et al., 2000; Zoli et al., 2018).

The general pro- and anti-inflammatory properties of nicotine are complex and context-dependent, with factors such as cell type, receptor type, and disease context playing important roles. In many studies, nicotine exhibits anti-inflammatory properties due to cholinergic signaling in neurons and non-neuronal cells (Zoli et al., 2018). However, the literature consistently links nicotine and NLRP3 inflammasome activation, especially in myeloid-derived cell types. There is a well-established relationship between nicotine and NLRP3-driven atherosclerosis progression (Duan et al., 2021; Mullis and Swartz, 2020; Wu et al., 2018; Xu et al., 2021). One study demonstrated that nicotine promotes NLRP3 inflammasome activation in peripheral myeloid cells, driving atherosclerosis progression due to inflammatory cytokine release in macrophages and monocytes (Mao et al., 2021). Nicotine was also shown to exacerbate proliferation and cell migration in lung adenocarcinoma via a5-nAChR and NLRP3 signaling (Jia et al., 2022).

In the context of the CNS, nicotine exerts direct pro-inflammatory effects on both microglia and the vasculature of the BBB. Exposure to NNKs (nicotine-derived nitrosamine ketones, a nicotine-derived metabolite) increased ROS and inflammatory cytokine release in mouse microglia (Ghosh et al., 2009). Tobacco use is generally associated with BBB disruption, CNS oxidative stress, and decreased cognitive performance in clinical studies (Ande et al., 2013; Ghosh et al., 2009; Louboutin and Strayer, 2014; Mazzone et al., 2010). BBB damage due to nicotine exposure is primarily linked to mitochondrial dysfunction, ROS production, and release of inflammatory cytokines such as TNFa and IL-6, which can trigger endothelial dysfunction by dysregulation of tight junction proteins such as claudin-5 and occludin (Hossain et al., 2011; Hutamekalin et al., 2008; Kousik et al., 2012; Manda, Mittapalli, Geldenhuys, et al., 2010; Pimentel et al., 2020). Additionally, Zhang et al. have linked the NLRP3 inflammasome directly to nicotine-induced endothelial barrier dysfunction and hyperpermeability via the release of HMGB1 (Zhang et al., 2019).

HIV and nicotine in HAND

While nicotine and HIV alone both contribute to neuroinflammation and BBB dysfunction, in PWH who smoke, additive effects have been observed on neuroinflammation and decreased cognitive performance (Bryant et al., 2013; Chang et al., 2020). These clinical findings are supported by studies in HIV-1 transgenic rat models, where nicotine promotes overexpression of immune-related genes and inflammatory cytokine expression (Royal et al., 2018; Yang et al., 2016). Additionally, Delgado-Velez et al. showed that gp120 exposure can directly upregulate the expression of a7-nAChRs in peripheral immune cells (Delgado-Vélez et al., 2015). In addition to promoting their upregulation, the viral protein gp120 can bind to the α7 receptors. α7-nAChR signaling promoted amyloid-beta accumulation in an HIV-gp120 mouse model (Liu et al., 2017). While certain animal studies show that nicotine alone increases cognitive performance in behavioral tests, this improvement is ameliorated in HIV-1 Tg rats (Nizri et al., 2009; Revathikumar

et al., 2016; Royal et al., 2018; Yang et al., 2016). Along a similar vein, α7-nAChRs were found to be upregulated in the monocytes of women with HIV, but greater receptor abundance did not result in a protective anti-inflammatory effect when MDMs were treated with LPS (Delgado-Vélez et al., 2015). These findings, when taken together, suggest that the presence of HIV may alter the cholinergic anti-inflammatory response via the α7-nAChRs, dampening or even reversing the anti-inflammatory effects of nicotine alone.

It is important to consider that in people who smoke cigarettes, nicotine is always accompanied by the thousands of other compounds present in tobacco smoke. Tobacco smoke and nicotine alone have been shown to have differing effects on inflammation, and their respective contributions to HIV associated neuroinflammation are not yet clearly delineated. Tobacco smoke is almost universally pro-inflammatory in both pre-clinical and clinical studies. Cigarette smoke condensate increases apoptosis, viral replication, and oxidative stress in human monocytes (Rao et al., 2016). Tobacco smoke extract similarly increases viral replication in bronchial epithelial cells and alveolar macrophages (Abbud et al., 1995; Chinnapaiyan et al., 2018). In clinical studies, PWH who smoke exhibit higher levels of inflammatory markers, increased viral load, and increased risk of HAND compared to non-smokers (Han et al., 2018; Valiathan et al., 2014; Wojna et al., 2007). As mentioned above, nicotine can induce anti-inflammatory cholinergic signaling through the α7 nAChR and can be neuroprotective in certain contexts, including in some HIV-1 Tg rat studies (Cao et al., 2013; Cao et al., 2016). However, chronic nicotine exposure is known to cause BBB disruption, a key component of HAND pathology (Feldman and Anderson, 2013). Furthermore, there is evidence that nicotine treatment promotes HIV infection in human microglia (Rock et al., 2008). Further studies with both nicotine and tobacco smoke are needed to unravel their roles in the context of HAND.

In addition to the many compounds present in tobacco smoke, antiretroviral drug treatments and other substance use in PWH add another layer of complexity to the neuroinflammatory context of the HIV-infected CNS. Many studies have shown that earlier intervention with ART can significantly reduce the risk of more severe forms of HAND in PWH (Brew, 2004; Sacktor et al., 2002). However, as outlined earlier, infection and inflammation persist in the brain despite the presence of ART. As reviewed elsewhere, certain antiretroviral drugs can have neurotoxic effects through mechanisms such as oxidative stress and mitochondrial dysfunction, and balancing this neurotoxicity against viral suppression and penetrance of the CNS reservoir remains an important clinical challenge (Shah et al., 2016; Yuan and Kaul, 2021). In PWH who smoke tobacco, there is the additional factor of drug-drug interactions between nicotine and ARTs (reviewed by Ghura et al., 2020). Nicotine has been shown to affect ART metabolism directly and can impact drug delivery by compromising BBB integrity (Kumar et al., 2015; Manda, Mittapalli, Bohn, et al., 2010; Manda, Mittapalli, Geldenhuys, et al., 2010; Pal et al., 2011). Animal model studies with the protease inhibitor saquinavir demonstrate that nicotine-induced BBB compromise may facilitate entry of ART into the CNS, but nicotine and saquinavir cause additive oxidative stress in the brain endothelium leading to dysregulation of Notch-4 and ZO-1 (Manda, Mittapalli, Bohn, et al., 2010; Manda, Mittapalli, Geldenhuys, et al., 2010). Balancing ART toxicity with BBB penetrance remains an important clinical problem, as long-term exposure to certain ARTs has been associated with neurovascular toxicity (Bertrand et al., 2021). Thus, although nicotine's ability to disrupt the BBB may lead to greater drug penetrance, it is likely that the additive impacts on BBB dysfunction and subsequent inflammation and immune infiltration of the CNS represent an overall detrimental outcome (Ahmed et al., 2018; Bertrand et al., 2021). ART has also been observed to increase nicotine metabolism in PWH (Ashare et al., 2019; Earla et al., 2014). ARTs may also impact nicotine signaling by acting on nicotinic receptors. For example, the protease inhibitor indinavir was demonstrated to impact cholinergic signaling by inhibiting the α7-nAChR activity (Ekins et al., 2017). More investigation is necessary to understand the specific interactions between various ARTs and nicotine to inform best practices for the treatment of PWH.

As outlined here, the NLRP3 inflammasome pathway is a common mechanism by which HIV and nicotine promote neuroinflammation. More studies are needed to fully understand how HIV and nicotine interact to promote inflammation, BBB dysregulation, and viral reactivation in the CNS.

Discussion

Chronic inflammation leads to increased risks for comorbid disorders in PWH, even in the presence of ART (Chun et al., 2010; Collora and Ho, 2022; Lee et al., 2020; Siliciano et al., 2003; White et al., 2022). In the brain, this manifests as an increased risk for neuroinflammation and cognitive impairment classified under the family of neurocognitive disorders known as HAND (Heaton et al., 2011; Saylor et al., 2016). The persistence of HIV in viral reservoirs such as the CNS is a primary driver of this chronic inflammation (Desplats et al., 2013; Sonti et al., 2021). The use of substances such as tobacco increases the risk of comorbidities for PWH and further contributes to HIV-associated inflammatory pathologies (Han et al., 2018).

There is a well-established literature linking HIV-1 with NLRP3 inflammasome signaling both in the periphery and in the CNS. Studies have shown that HIV can stimulate the inflammasome through the binding of viral fragments and proteins to PRRs and through the activation of purinergic receptors (Doitsh et al., 2014; Freeman and Swartz, 2020; Swartz et al., 2015). In the brain, HIV drives NLRP3-associated inflammation primarily through infected and activated microglia, which contribute to the inflammatory environment by releasing neurotoxic factors and pro-inflammatory cytokines (Chivero et al., 2017; He et al., 2020; Walsh et al., 2014). HIV also drives BBB damage through several mechanisms, including NLRP3-driven dysregulation of endothelial cells (Atluri et al., 2015; Caligaris et al., 2021).

NLRP3 inflammasome activation, a key feature of neuroinflammation and BBB dysregulation, is influenced by both HIV and nicotine. Like HIV infection, nicotine is known to drive endothelial dysfunction by promoting ROS, mitochondrial dysfunction, and NLRP3 inflammasome activation (Ghosh et al., 2009; Hossain et al., 2011; Hutamekalin et al., 2008; Kousik et al.,

2012; Manda, Mittapalli, Bohn, et al., 2010; Pimentel et al., 2020; Zhang et al., 2019). There is evidence that nicotine in tobacco enhances viral replication in microglia and macrophages and has been shown to cause activation of these resident immune cells and compromise the BBB (Ghosh et al., 2009; Manda, Mittapalli, Geldenhuys, et al., 2010; Pimentel et al., 2020; Rock et al., 2008). Taken together, the literature suggests that the combined effect of HIV and nicotine-driven NLRP3 activation plays a key role in the heightened neuroinflammation and cognitive symptoms observed in PWH who smoke.

Further investigation is necessary to understand the mechanism of HIV and nicotine's interactions in the CNS and the precise role of NLRP3 in driving their combined effects. Furthermore, there are many critical unanswered questions surrounding the impact of substance use on the establishment and reactivation of viral latency in the CNS. Atluri et al. suggest that nicotine increases the risk of viral latency establishment due to the upregulation of HDAC2 in a neuronal cell line (Atluri et al., 2014). In this model, the transcriptional repressor HDAC2 is synergistically upregulated by HIV and nicotine, leading to more compact chromatin organization, reduced gene transcription, and increased latent infection. Treatment with the HDAC inhibitor vorinostat reversed this effect and reactivated latent virus. However, is important to note that the biological relevance of this study is limited, as neurons are not typically infected with HIV-1 and do not form a significant portion of the CNS viral reservoir. In a murine macrophage model of atherosclerosis, another histone deacetylase, HDAC6, was shown to promote nicotine-mediated inflammation and pyroptosis via deacetylation of p65, and activation of NF-kB and NLRP3 transcription (Xu et al., 2021). HIV-1 is known to integrate itself into transcriptionally active regions of the genome located in regions of open chromatin (Schröder et al., 2002). It relies on host cell machinery and activation states to regulate its latency, favoring activated over quiescent states in both microglia and $CD4^+$ T-cells (Mbonye and Karn, 2017; Sreeram et al., 2022; Wallet et al., 2019). All of this considered, it seems likely that nicotine may play a role in regulating viral latency in microglia by promoting activation of NF-kB and NLRP3 associated genes, leading to transcription of latent provirus. However, nicotine's specific role in viral latency formation and maintenance in the CNS remains largely unexplored in the literature, and thorough investigation of its impacts on latent infection in microglia is especially necessary.

There are additionally many open questions regarding poly-substance use in PWH and the impacts of combined drug use on cognitive impairment in these patients. As previously reviewed by our group, cannabis use is largely associated with protective effects against inflammation in PWH, and CB2R signaling has been linked to reduced HIV-1 infection and NLRP3 inflammasome activation (Min et al., 2023). Further study is needed to better understand the interactions between the cannabinoid system, nicotine, and NLRP3 and the impacts of multiple drug exposures on neuroinflammation in PWH. Investigating these questions will provide valuable insights into the mechanism of nicotine's impact on the pathogenesis of HIV-1-associated neurodegeneration, informing possibilities for future therapeutic development.

Author contributions

AK: Conceptualization, Data curation, Formal Analysis, Investigation, Visualization, Writing–original draft, Writing–review and editing. TS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Gilda Angela Neves,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Deirdre M. McCarthy,
Florida State University, United States
Pablo Trindade,
Federal University of Rio de Janeiro, Brazil

*CORRESPONDENCE
Steven R. Laviolette,
steven.laviolette@schulich.uwo.ca

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Chronic nicotine exposure induces molecular and transcriptomic endophenotypes associated with mood and anxiety disorders in a cerebral organoid neurodevelopmental model

Emma K. Proud[1,2,3], Mar Rodríguez-Ruiz[1,2,3], Dana M. Gummerson[1,2,3], Sebastian Vanin[4], Daniel B. Hardy[4,5,6], Walter J. Rushlow[1,2,7] and Steven R. Laviolette[1,2,3,7]*

[1]Addiction Research Group, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada, [2]Department of Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada, [3]St. Jopsephs Health Care, Lawson Health Research Institute, London, ON, Canada, [4]Department of Physiology and Pharmacology, Western University, London, ON, Canada, [5]Department of Obstetrics and Gynecology, Western University, London, ON, Canada, [6]St. Josephs Healthcare, Children's Health Research Institute, London, ON, Canada, [7]Department of Psychiatry, University of Western Ontario, London, ON, Canada

Introduction: Prenatal nicotine exposure (PNE) from maternal smoking disrupts regulatory processes vital to fetal development. These changes result in long-term behavioral impairments, including mood and anxiety disorders, that manifest later in life. However, the relationship underlying PNE, and the underpinnings of mood and anxiety molecular and transcriptomic phenotypes remains elusive.

Methods: To model nicotine exposure during prenatal development, our study used human cerebral organoids that were chronically exposed to nicotine and collected for molecular analyses.

Results: Short-term, nicotine altered molecular markers of neural identity, mood and anxiety disorders and those involved in maintaining the excitatory/inhibitory (E/I) balance in the cortex. RNA sequencing further revealed transcriptomic changes in genes pertaining to embryonic development, neurogenesis, and DNA binding. Long-term, mature organoids demonstrated similar disruptions in E/I balance, decreased expression of neural identity markers, and altered dopamine receptor expression.

Discussion: Collectively, our results demonstrate that nicotine-induced alterations occur acutely and persist at later stages of development. These findings validate an *in vitro* model of PNE to better comprehend the emergence of neuropsychiatric molecular and transcriptomic endophenotypes resulting from gestational nicotine exposure.

KEYWORDS

prenatal development, prenatal nicotine exposure, anxiety, depression, human cerebral organoids

Introduction

Rates of cigarette smoking have declined substantially in recent years. However, the use of electronic nicotine delivery systems such as vapes and e-cigarettes has been increasing exponentially, including in women of reproductive age (Brooks and Henderson, 2021; Obisesan et al., 2020). These trends are alarming given that nicotine has been reported to disrupt several regulatory processes vital to healthy fetal development (Dwyer et al., 2009). As a result, prenatal nicotine exposure (PNE) has been linked to numerous physical and emotional disturbances that persist later into the life of the offspring (Blood-Siegfried and Rende, 2010; Dwyer et al., 2009).

Nicotinic acetylcholine receptors (nAchRs) are critical in facilitating key aspects of prenatal neurodevelopment such as neurogenesis, cell survival, apoptosis, and axonal and synaptic growth (Ekblad et al., 2010; Smith et al., 2010). Cholinergic signaling plays a crucial role in coordinating brain maturation and premature or chronic overactivation of nAchRs during this critical period of neurodevelopment interferes with these regulatory processes (Dwyer et al., 2009; Moylan et al., 2013). This produces detrimental changes in nAchR distribution, sensitivity, and neurotransmitter functions, which can lay the foundation for future mood and anxiety disorders (Dwyer et al., 2009; Laviolette, 2021; Mahar et al., 2012; Sailer et al., 2019). Specifically, the most abundant nAchRs in the cortex implicated in mood and anxiety disorders are the α_7 and $\alpha_4\beta_2$ subunits. Nicotine also modulates the release and signaling of several neurotransmitter systems within the central nervous system, such as glutamate, γ-aminobutyric acid (GABA) and dopamine (Mahar et al., 2012; Moylan et al., 2013; Sailer et al., 2019). For example, α_7 nAchRs located presynaptically on glutamatergic neurons indirectly mediate the release of dopamine from neighboring neurons, which alters dopamine signaling and excitatory/inhibitory (E/I) balance in the cortex (Livingstone et al., 2010). Prenatal neurogenesis is a precise series of synchronized events during which the brain is tremendously vulnerable to external environmental stimuli, like nicotine exposure (Ross et al., 2015). This exposure produces perpetual alterations in neuronal cytoarchitecture and brain circuity of the fetus in brain regions vital for emotional regulation, such as the prefrontal cortex (PFC), with adverse neurobehavioral outcomes persisting into adulthood (Aoyama et al., 2016; Blood-Siegfried and Rende, 2010; Dwyer et al., 2019; Laviolette, 2021; Mahar et al., 2012; Minatoya et al., 2019; Moylan et al., 2015; Sailer et al., 2019; Smith et al., 2010).

Rodent models have provided considerable insights in identifying biomarkers associated with both mood and anxiety-like behaviors and developmental nicotine exposure (Hudson et al., 2021; Jobson et al., 2019; Polli et al., 2020; Slawecki et al., 2003; Smith et al., 2006; Vaglenova et al., 2004). Alterations in these biomarkers include changes in the expression of α_7 and $\alpha_7\beta_2$ nAchRs, dopamine 1 (D1R) and dopamine 2 (D2R) receptors (Hudson et al., 2021; Jobson et al., 2019; Mineur et al., 2011; Philip et al., 2010). Additional studies have also identified aberrant GABAergic and glutamate signaling as underlying factors in major-depressive disorder (MDD) and anxiety-related psychopathology. For example, cortical levels of glutamic acid decarboxylase (GAD67), GABA transporter type-1 (GAT-1), parvalbumin (PV) interneurons, N-methyl-D-aspartate and metabotropic (mGLUR) receptors are reportedly altered in *postmortem* and human imaging studies of patients with these disorders (Duman et al., 2019; Feyissa et al., 2010; Hashimoto, 2009; Hasler et al., 2007; Karolewicz et al., 2010; Rajkowska et al., 2007). Similarly, numerous differentially expressed genes (DEGs) involving GABAergic and glutamatergic neurotransmission have been reported in transcriptomic studies of patients with MDD, which further reinforces the association between altered E/I balance in the cortex and mood and anxiety disorders (Choudary et al., 2005; Klempan et al., 2009; Mehta et al., 2010; Sequeira et al., 2007; Sequeira et al., 2009). Furthermore, there is evidence of upregulation of genes encoding for proteins that facilitate transcription and translation in mood disorders, which is useful for understanding the genetic basis of molecular biomarkers and the pathophysiology of anxiety and depression (Iwamoto et al., 2004; Mehta et al., 2010).

Due to the paucity of access to fetal brain tissue, most of our knowledge regarding PNE, and the emergence of mood and anxiety disorders comes from clinical populations and animal models (Blood-Siegfried and Rende, 2010; Corrêa et al., 2022; Dwyer et al., 2009; Ekblad et al., 2010; Mahar et al., 2012; Minatoya et al., 2019; Moylan et al., 2013; Sailer et al., 2019; Smith et al., 2010). Although valuable, these models have many limitations due to confounding variables and species-specific differences in brain development, respectively. To bridge the gap between animal and two-dimensional *in vitro* models, three-dimensional cerebral organoids are proposed as an effective preclinical platform to recapitulate aspects of the developing human brain, in this case, in conjunction with nicotine exposure (Centeno et al., 2018; Kim et al., 2020; Lancaster et al., 2013). Previous organoid studies investigating the effect of nicotine exposure on prenatal brain development exist, but have not taken the emergence of mood and anxiety molecular endophenotypes into consideration (Notaras et al., 2021; Wang et al., 2018).

The present study aimed to validate an *in vitro* cerebral organoid model of PNE, to characterize how nicotine exposure early in pregnancy can lead to aberrant neurodevelopmental events and the emergence of molecular biomarkers of mood and anxiety disorders. We employed the use of immunofluorescence (IF), quantitative polymerase chain reaction (qPCR) and RNA sequencing (RNA-Seq) to identify different categories of biomolecules underlying how nicotine exposure contributes to molecular endophenotypes observed in mood and anxiety disorders' pathologies. We report that nicotine altered established molecular markers pertaining to neural identity, mood and anxiety disorders and those involved in maintaining E/I balance in the cortex, with some of these effects persisting into later stages of development. Changes at the transcriptomic level were also reported. Observing these alterations in tandem validate our PNE model and further illuminate how the molecular mechanisms underlying nicotine exposure can alter human cortical brain development and dysregulate molecular pathways associated with mood and anxiety disorders.

Materials and methods

iPSC maintenance and organoid generation

Three human, control, induced pluripotent stem cell (iPSC) lines were acquired from deposits made to the National Institute of Mental Health Repository and Genomics Resource center and

obtained from RUCDR Infinite Biologics. Each cell line (MH0185865, MH0185983 and MH0185984) originated from healthy patients without any history of neuropsychiatric disorders. The three iPSC cell lines included two males, aged 29, and one female, aged 24. They were chosen due to their age proximity and shared Caucasian background. The iPSC lines were maintained in hypoxic conditions (4% oxygen) until organoid generation and cultured concurrently to minimize variations from individual culturing practices. Briefly, for approximately 2 weeks, iPSCs were maintained in mTeSR™1 medium (StemCell, 85850) in Matrigel® (Corning, 354277) coated plates and cells were passaged using Gentle Disassociation Reagent (StemCell, 07174) if colonies were roughly 70% confluent. To improve the success of cortical differentiation, for 4 days before starting the organoid protocol, the iPSCs were pretreated with mTeSR™1 containing different growth factors as previously described by Watanabe et al. (2019): bone morphogenetic protein 4 [(final) = 0.1 ng/mL], transforming growth factor beta-1 [(final) = 0.1 ng/mL], transforming growth factor beta-3 [(final) = 1 ng/mL] and activin-A [(final) = 10 ng/mL]. The intended starting confluency of the cell lines was approximately 70%.

Cerebral organoids were generated using the STEMdiff™ Cerebral Organoid Kit (StemCell, 08570) and the protocol was derived from Lancaster et al. (2013). On day 0 of organoid culture [embryoid body (EB) formation], iPSCs were lifted using Gentle Disassociation Reagent and gently triturated to create a single-cell suspension. Following centrifugation and resuspension, 100 µL of cell suspension (9,000 cells/well) was plated in each well of a 96-well round-bottom ultra-low attachment plate (Corning, 7007) with EB Formation Medium containing 10 µM rho-kinase inhibitor (StemCell, 72302). On days 2 and 4, 100 µL of EB Formation Medium was added to each well and the plate was incubated at 37°C until organoid induction on day 5.

On day 5, EBs were transferred to a 24-well ultra-low attachment plate (Corning, 3473), containing Induction Medium, that was pretreated with AggreWell™ Rinsing Solution (StemCell, 07010). The plates were incubated at 37°C for 48 h until organoid expansion on day 7.

On day 7, organoids were transferred to an Organoid Embedding Sheet (StemCell, 08579) and cold Matrigel was added dropwise onto each EB. To polymerize the Matrigel, the plate was placed in the incubator at 37°C for 30 min. Upon removing the plate from the incubator, EBs were washed from the Embedding Surface into 6-well plates containing Expansion Medium and the plates were incubated at 37°C for 3 days until organoid maturation on day 10.

On day 10, all Expansion Medium was removed and replaced with 3 mL/well of Maturation Medium. The plates of organoids were placed on an orbital shaker and incubated in normoxic conditions at 37°C. Following day 10, Maturation Medium was changed every 3 days, except for days organoids received nicotine treatment (Figure 1). Beginning on day 48 (until approximately 4 months, 1 mL of Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2 (R&D Systems, 3536-005-02) was added to each bottle of Maturation Medium to support organoid development and maturation. Following the four-month mark, the organoids received regular Maturation Medium until their final collection day.

Nicotine treatment

Beginning on day 28, organoids were cultured in Maturation Medium with no nicotine [vehicle (VEH)], or a Maturation Medium treated with 0.1, 1 or 10 µM of nicotine for 14 days. The doses and the timing of nicotine treatment were based on studies conducted by Wang et al. (2018) and Notaras et al. (2021) to mimic physiological relevancy and organoid viability. The selected doses fell within the range of average serum concentrations previously reported in pharmacokinetic studies of cigarette smoking and/or nicotine replacement therapies (Deveaugh-Geiss et al., 2010; Massadeh et al., 2009; Oncken et al., 1997; Russell et al., 1980). Additional considerations influencing the selected developmental window of exposure included the active period of neurogenesis, timing of nAchR expression and recapitulating human brain development at early/mid gestation (Lancaster et al., 2013; Wang et al., 2018; Daviaud et al., 2019). To prepare the nicotine, a 100 µmol stock was created by dissolving nicotine hydrogen tartrate (Sigma, N5260) in sterile distilled water and 1 mL aliquots were kept at −20°C. On each day of nicotine treatment, an aliquot was thawed and diluted in Maturation Medium to reach the desired treatment concentrations. The Maturation Medium from all wells was aspirated, VEH or nicotine-treated medium was added to each respective well and the plate was incubated at 37°C for 24 h until the next drug treatment. Medium changes occurred daily for 14 days (the last treatment was on day 41). The organoids were collected for various histological and molecular techniques at day 42 (D42) and remained in culture until their final collection at day 180 (D180; Figure 1). Due to the prospective separation into the wells prior to nicotine treatment and the organoids being grown under the same experimental conditions, no specific criteria were used to select the organoids for each treatment. However, organoids that appeared similar in shape and size between conditions were selected for each experiment.

Histology and immunofluorescence

The organoids were incubated in 4% paraformaldehyde, cryopreserved in 30% sucrose, embedded in 7.5% gelatin (Sigma, G2500) in embedding molds and cryosectioned at 20 µm. Tissue sections were stained with hematoxylin & eosin or used for IF. Hematoxylin & eosin staining was performed to confirm successful morphological development and neuronal induction (data not shown). Following sample preparation, slides were selected for staining and send to Pathology at Robarts Research Institute (Western University, Ontario, Canada). The slides were imaged using a brightfield microscope (Nikon H600L). Images were acquired with a 10x magnification and at 2880 × 2048 resolution.

Slides selected for IF were washed three times for 10 min in phosphate-buffered saline with 0.1% Tween®20 (PBS-T; Sigma, P9416) to completely remove the gelatin surrounding the organoids and an ImmEdge™ hydrophobic PAP pen (Vector, H-4000) was used to circle each organoid on the slide. The organoids were blocked in 5% normal donkey serum (Millipore, S30) in PBS-T in a humidified chamber for 1 h at room temperature. Primary antibodies were prepared in 5% donkey serum in PBS-T according to the recommended dilutions. Primary antibodies included α_7

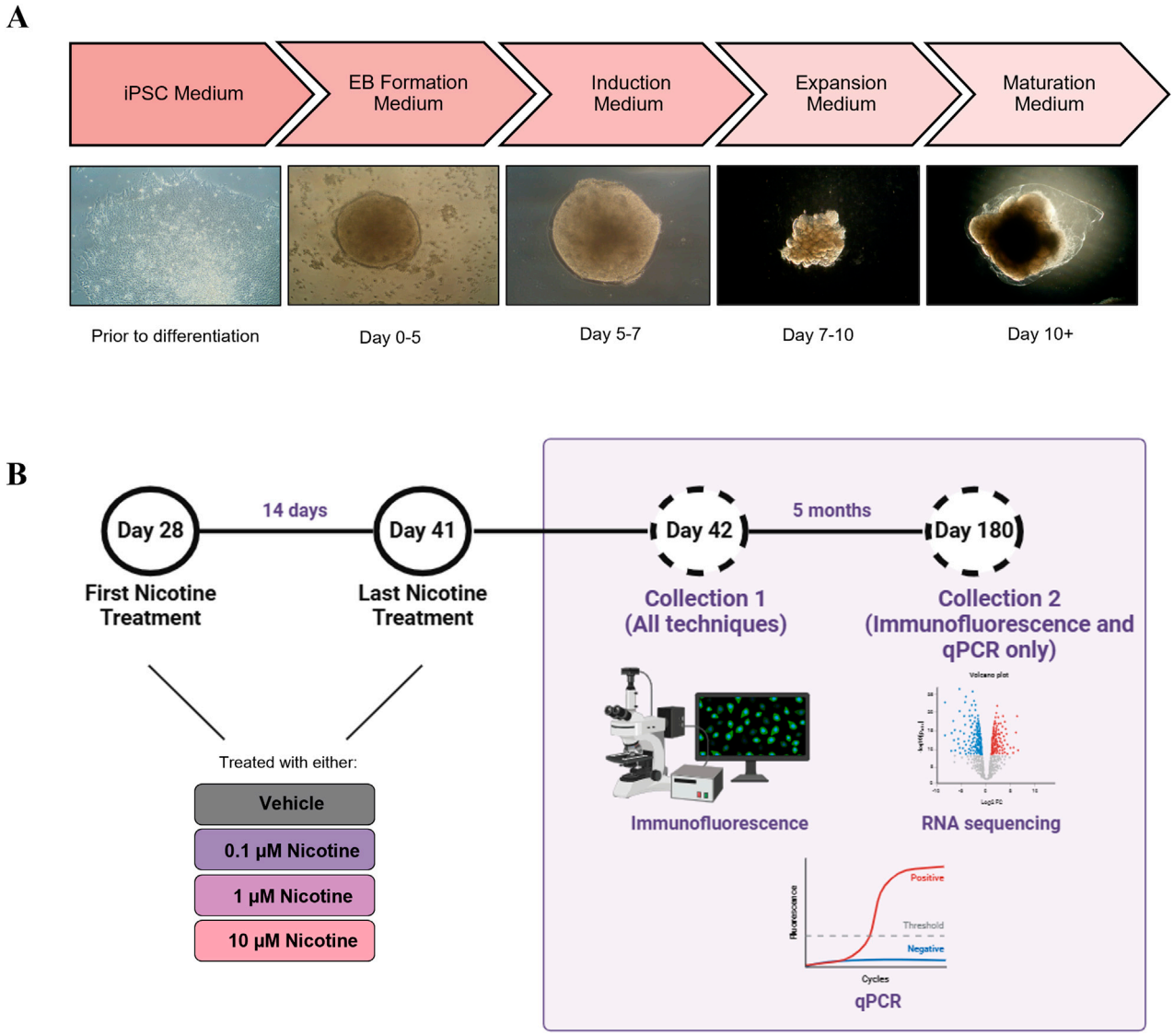

FIGURE 1
(A) Description of the cerebral organoid culture system and representative microscopic images. The iPSCs were maintained in culture for 2 weeks prior to organoid generation. On day 0, the iPSCs were cultured in an EB Formation Medium, and aggregated to form EBs. On day 5, the EBs were cultured in an Induction Medium which induced them to a neural fate. On day 7, the organoids were embedded in Matrigel and cultured in an Expansion Medium to allow expansion of the neuroepithelium. On day 10, the organoids were cultured in Maturation Medium and placed on an orbital shaker. Aside from nicotine treatment, when the medium was changed daily, the Maturation Medium was changed once every 3 days. **(B)** Details of nicotine treatment and organoid collection. Beginning on D28, the organoids were treated daily with VEH medium or medium containing one of three doses of nicotine, for 14 days. Following nicotine exposure, on D42 and at D180, the organoids were collected for IF, RNA-Seq and qPCR. Figure made using BioRender.

nAchR (rabbit, Alomone labs ANC-007, 1:50), α_4 nAchR (mouse, Santa Cruz sc-74519, 1:50), β_2 nAchR (goat, Abcam ab189174, 1:100), D1R (rabbit, Abcam ab40653, 1:50), D2R (mouse, Sigma-Aldrich MABN53, 1:200), mGLUR2/3 (rabbit, Sigma-Aldrich 06–676, 1:50), GAD67 (mouse, Sigma-Aldrich mab5406, 1:50), Ki67 (rabbit, Abcam ab15581, 1:500), PROX1 (mouse, Sigma-Aldrich mab5654, 1:100), FZD9 (goat, Abcam ab110886, 1:100), FGFR1 (rabbit, Abcam ab0646, 1:200), CCasp3 (rabbit, Cell signaling 9661, 1:200), CTIP2 (rat, abcam ab18465, 1:100), CDH13 (goat, Novus Biologicals, AF3264, 1:200), MAP2 (mouse, Sigma-Aldrich mab3418, 1:100), NR2B (goat, Novus Biologicals NB100-41097, 1:50), GAT-1 (rabbit, Rockland Immunochemicals 612-401-D56, 1:100) and PV (mouse, Sigma-Aldrich P3088, 1:50). For negative controls, the organoids remained covered with the blocking solution and received no primary antibody. The slides were incubated overnight at 4°C in humidified chambers. The slides were washed three times for 10 min in PBS-T, secondary antibodies were prepared in 5% donkey serum in PBS-T and sections were incubated at room temperature for 2 h in a humidified chamber covered from light. Secondary antibodies included rabbit (Alexa Fluor 488, A32790), mouse (Alexa Fluor 568, A10037), goat (Alexa Fluor 647, A21447) and rat (Alexa Fluor 647, A78947; Alexa Fluor 488, A21208). All secondary antibodies were used at a 1:250 dilution and obtained from Invitrogen. The slides were washed three times for

TABLE 1 qPCR primer sequences.

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Gene accession #
D1R	gaccttgtctgtactcatctcct	gtcacagttgtctatggtctcag	NM_000794.5
EMX1	cgcaggtgaaggtgtggtt	tccagcttctgccgtttgt	NM_004097.3
EOMES	gtgcccacgtctacctgtg	cctgccctgtttcgtaatgat	NM_001278182.2
FOXG1	aggagggcgagaagaagaac	tgaactcgtagatgccgttg	NM_005249.5
GAD1	gcggaccccaataccactaac	cacaaggcgactcttctcttc	NM_000817.3
GRM2	ccgcattgcacgcatcttc	ggcccgagataagtgccag	NM_000839.5
ISL1	gcggagtgtaatcagtatttgga	gcatttgatcccgtacaacct	NM_002202.3
TBR1	gactcagttcatcgccgtca	tgctagtaccctagccttgc	NM_006593.4

10 min in PBS-T, dried, mounted with FluoroShield™ (Sigma-Aldrich, F6057) containing DAPI and covered with a 1.5 mm coverslips (Fisherbrand, 12542A). The slides were stored at 4°C until imaging.

The organoids were imaged using a Leica SP8 (D42) or Leica STELLARIS5 (D180) microscope. The 40x and 63x magnifications were used on the Leica SP8 and STELLARIS5, respectively. Each treatment (i.e., VEH, and each nicotine dose) had one organoid collected for cryosectioning, per cell line. One section of organoid was selected for each experimental condition, for each combination of labels, and approximately two regions of interest (ROIs) were imaged per organoid. This was performed for each experimental condition, for each combination of labels. Images were taken in a 1024 × 1024 format, at a speed of 400 Hz. For each ROI, multiple optical z-slices were imaged and combined per microscopy image. Six steps ranging from 1.0–1.5 μm each, were taken per image of each ROI used for analysis. This method of imaging resulted in a range of 16–36 ROIs total (i.e., not per organoid) included for quantification, for each combination of labels. Data analysis for each marker of interest was performed using FIJI ImageJ (NIH). Regions of interest were manually selected, and images were normalized to the area by dividing the number of particles by the area (particles by mm^3). Each marker of interest was analyzed at both time points, with nonsignificant data not shown.

qPCR

Total RNA was extracted from whole organoids using the TRIizol (Invitrogen) and chloroform as specified by the manufacturer. Isopropanol was used to precipitate RNA, which was centrifuged to obtain a pellet. The pellet was dissolved in Diethyl pyrocarbonate (DEPC)-treated water. RNA was diluted to 1 μg/μL for reverse-transcriptase with a High-Capacity cDNA RT Kit (Applied Biosystems, 4368814) to make cDNA. The cDNA was diluted to 1:40 in qPCR.

Forward and reverse primers were designed using NCBI Primer Blast, and Harvard PrimerBank and sequences were validated using NIH Nucleotide Blast (Table 1). All primers were ordered from ThermoFisher, and quality was checked by analyzing the melt curves. DEPC blanks and 3 μL of cDNA were loaded into a 384 well plate (VWR, 82006-678) in triplicates. A master mix comprised of DEPC, 2.5 μM forward and reverse primer mix and SensiFAST SYBR (Meridian Bioscience, Bio-98050) was added to each well for a total reaction volume of 8 μL. Bio-Rad CFX384 Real-Time System was used with cyclic conditions set at 95°C for 3 min, followed by 43 cycles of; 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. When the plate was finished running, the quality of the run was checked by examining the cycle quantification (Cq) values and melt curves. Values with a difference of > 0.5 Cq within a triplicate were removed and a Cq average was calculated for each sample. The values obtained for all gene targets of interest were normalized to the geometric means of housekeeping genes *ACTB* and *GAPDH*. *ACTB* and *GAPDH* were determined to be suitable housekeeping genes by using the comparative ΔCq method. The 2-ΔΔCq method was used to calculate the relative fold change of gene expression within the experimental samples. To enhance data transparency, ΔCt values for each primer were calibrated to experimental samples with the lowest transcript abundance (highest Ct value). Relative transcript abundance was then calculated for each primer set as determined by the formula $2^{-\Delta\Delta CT}$, where ΔΔCt was the normalized value. Each target of interest was analyzed at both time points, with nonsignificant data not shown.

RNA-sequencing

Pooled male and female VEH (n = 3) and 0.1 μM nicotine-treated (n = 3) organoids were snap frozen and sent to Genome Quebec (Montreal, Quebec, Canada) for total RNA extraction, library preparation and RNA-Seq. Quality checks were performed by Genome Quebec following extraction and library preparation. The RNA integrity number was used to assess RNA quality and all samples had RNA integrity number scores ≥ 7.0. Paired-end reads (25 million) were sequenced on the Illumina NovaSeq platform. All raw reads were aligned and annotated with the latest ENSEMBL Homo Sapien GRCH38.p13 reference genome using STAR version 2.7.10a with recommended settings. Raw counts were generated using the Rsubread subpackage featureCounts (Liao et al., 2019). Lowly expressed genes were filtered out using a count per million (CPM) cutoff of 0.4 in at least two or more samples. Normalization and differential expression analysis were done using the edgeR package (Chen et al., 2016). Briefly, counts were normalized for both library size and library composition using the trimmed means of the M-values method. Normalized counts were then fit to a gene-wise negative

binomial generalized linear model, and a quasi-likelihood F test was used for DE analysis. To account for multiple testing, *p*-values were adjusted using Benjamini & Hochberg False Discovery Rate (FDR) correction. An FDR cut-off of < 0.05 was used to determine significance. The gprofiler2 (Kolberg et al., 2020) R interface for the web toolset g: Profiler was used to convert ENSEMBL gene IDs to gene symbols, and to perform functional enrichment analysis (i.e., over-representation analysis) on the DEGs from databases of interest. The databases included in the analysis were the Gene Ontology (GO) database, the Reactome database, the TRANSFAC database, the human protein atlas and the WikiPathways database. A g: SCS adjusted *p*-value threshold of < 0.01 was used to determine the significance of the functional enrichment analysis. To examine DEGs between VEH and 0.1 μM nicotine organoids, a heatmap was generated using the pheatmat R package (Hu, 2021). Further analysis of DEGs was completed using VarElect (http://ve.genecards.org). VarElect is an application that permits the analysis of specific DEGs following sequencing and ranks genes that are found to have variants according to specific phenotype-gene associations. To generate this list, VarElect uses information obtained from several databases such as GeneCards® (www.genecards.org), Malacards (www.malacards.org), LifeMap Discovery® (discovery.lifemapsc.com) and Pathcards (pathcards.genecards.org). The list of DEGs was imported into VarElect and three phenotypes of interest were individually searched: nicotine exposure, anxiety, and depression. An annotated list of DEGs associated with each phenotype was generated. This list is formed based on direct (GeneCards) and indirect links (Genecards and Malacards) between the genes and phenotype of interest. The top 20 genes for each phenotype were compared to assess which DEGs were associated across multiple phenotypes.

Statistical analyses

Outliers were removed using Grubbs' test (α = 0.05) and normality was assessed. All results for IF and qPCR were analyzed with one-way analysis of variance (ANOVA) or Kruskal–Wallis if appropriate. Significant ($p < 0.05$) or trending ($p < 0.1$) tests were followed up using Fisher's Least Squares Difference *post hoc* test (α = 0.05). All analyses were performed using GraphPad Prism (version 9.4.1 for Windows) and graphs are presented as mean ± standard error of the mean. For RNA-Seq, to account for multiple testing, *p*-values were adjusted using Benjamini & Hochberg FDR correction. An FDR cut-off of < 0.05 was used to determine significance.

Results

Short-term effects of PNE

Human cerebral organoids demonstrate successful neural induction and features of the developing fetal brain

Successful neural induction was confirmed by IF to characterize VEH organoids at D42 (Figure 2). VEH organoids stained positive for proliferation marker Ki67 (Figure 2A) and regional markers specific to hippocampal and cortical tissue (FZD9 and PROX1; Figures 2B, C). Neural induction was also confirmed by the presence of MAP2 (Figure 2D), which stains the neural cytoskeleton. Additionally, the organoids expressed markers vital to neurodevelopment (FGFR1 and CHD13; Figures 2E, F). These results exhibit that our organoids model aspects of neurogenesis, successful cortical differentiation, and developmental signatures of the fetal brain. Following initial characterization, the impact of nicotine exposure on these neurodevelopmental markers was also analyzed, however there were no significant differences (data not shown).

PNE acutely dysregulates aspects of neurogenesis and alters the expression of neural identity markers in the cortex

Following the 14-day nicotine treatment, there were changes in other markers used to characterize our model (Figure 3A). Indeed, there was a significant increase in cleaved caspase 3 (CCasp3) expression, a marker of apoptotic cell death ($H_{(3,16)} = 9.174$, $p = 0.0271$; Figure 3B). *Post hoc* analysis revealed an increase at 10 μM ($p = 0.0114$), with no effect observed at 0.1 or 1 μM ($p > 0.05$). We also examined deep-layer marker CTIP2, which was significantly increased in nicotine-treated organoids compared to VEH ($H_{(3,16)} = 12.73$, $p = 0.0053$; Figure 3C). The *post hoc* analysis demonstrated a similar result to CCasp3, with a significant increase at 10 μM ($p = 0.0060$) but not at lower doses of nicotine ($p > 0.05$). These findings demonstrate the toxicity of the 10 μM dose and the dose-dependent effect of nicotine on these specific markers. This suggests that these CCasp3 and CTIP2 may be more susceptible to the influences of nicotine at higher doses earlier in development, resulting in increased cell death and increased number of early-born neurons, whereas other developmental markers, such as those in Figure 2, are less sensitive to the effects of nicotine. Due to the observed toxicity at 10 μM, organoid viability was compromised, with limited experimental findings at D42 and no experimental analysis was performed at D180.

PNE has short-term effects on the expression of nAchRs implicated in anxiety and depression

Developmental nicotine exposure has been shown to dysregulate markers that are present in mood and anxiety disorders (Hudson et al., 2021; Jobson et al., 2019; Laviolette, 2021). To further investigate this, we employed IF to assess the impact of nicotine on these biomarkers at the protein level. To understand how nicotine may impact the expression of its target receptor, we looked at nAchR subunits that comprise the most abundant receptors in the cortex and are implicated in anxiety and depression; the α_7, α_4 and β_2 nAchR subunits (Figure 4A). One-way ANOVA revealed that nicotine had no effect on α_7 at any dose ($F_{(3,19)} = 1.095$, $p = 0.3818$; Figure 4B), but there was a significant increase in α_4 ($H_{(3,15)} = 7.424$, $p = 0.0449$) and β_2 ($F_{(3,15)} = 6.706$, $p = 0.0043$) nAchR subunits (Figures 4C, D) in nicotine treated organoids compared to VEH. Follow-up *post hoc* comparisons revealed a marked increase in α_4 at the 1 ($p = 0.0294$) and 10 μM dose ($p = 0.0205$), but not at 0.1 ($p > 0.05$). Similarly, this was also seen in β_2 at these doses ($p = 0.0477$; $p = 0.0006$; $p > 0.05$). Thus, nicotine upregulates the expression of some but not all nAchRs implicated in anxiety and depression at D42.

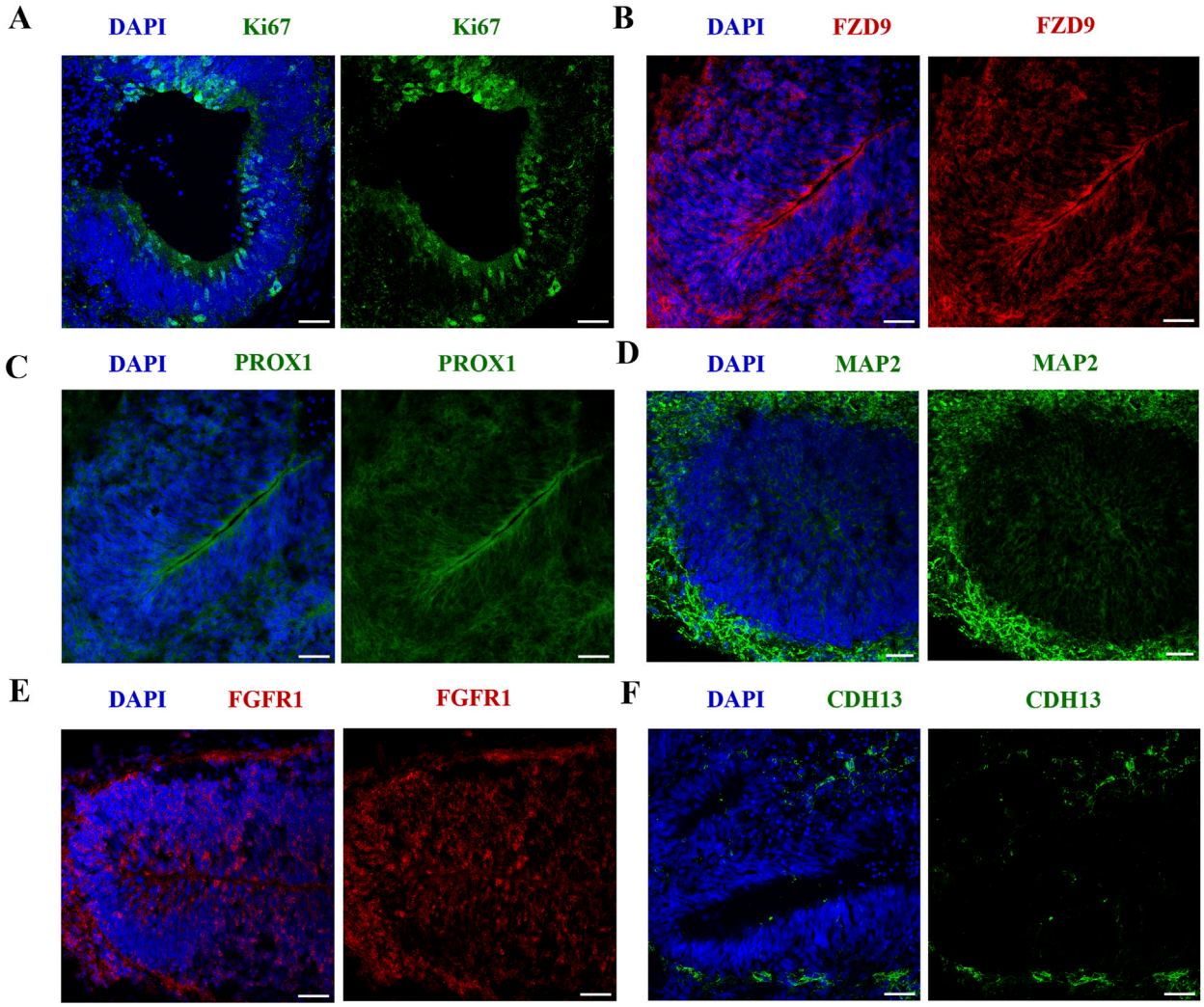

FIGURE 2
iPSC-derived cerebral organoids express markers specific to early brain regionalization and vital to development. Scale bar = 50 μm. **(A–F)** Immunofluorescent images were captured using confocal microscopy. **(A–C)** Staining of VEH organoids was performed for the expression of proliferation marker Ki67 **(A)** and regional markers FZD9 **(B)** and PROX1 **(C)** on D42. **(D–F)** VEH organoids also expressed neuronal marker MAP2 **(D)**, developmental markers FGFR1 **(E)** and CDH13 **(F)**.

PNE acutely perturbs D1R and D2R expression

Since developmental nicotine exposure has been shown to disrupt dopaminergic signaling in brain regions associated with mood and anxiety control (Jobson et al., 2019), we decided to investigate changes in D1R and D2R expression following chronic nicotine exposure (Figure 5A). Following the 14 days of nicotine exposure, one-way ANOVA revealed a significant decrease in D1R receptor expression ($F_{(2,13)} = 5.624$, $p = 0.0174$; Figure 5B), with *post hoc* analysis suggesting this decrease occurred at 0.1 ($p = 0.0061$), with no effect at 1 μM ($p > 0.05$). Compared to VEH, one-way ANOVA indicated nicotine also significantly decreased D2R receptor expression ($F_{(2,13)} = 6.023$, $p = 0.0141$; Figure 5C). Further investigation using *post hoc* analysis exhibited that the decrease occurred at both 0.1 ($p = 0.0077$) and 1 μM ($p = 0.0178$). These results indicate that expression of these dopaminergic subtypes is influenced at lower doses of nicotine exposure, consistent with changes in protein expression seen in mood and anxiety disorders.

PNE disrupts GABAergic markers associated with cortical E/I balance at D42

Finally, altered cortical E/I balance is a hallmark attribute of mood and anxiety disorders (Martin et al., 2020; Hashimoto, 2009) so we investigated the influence of nicotine on various GABAergic markers due to their role in cortical inhibition and neuron excitability (Figure 6A). A one-way ANOVA concluded that compared to VEH, nicotine significantly decreased the expression of GABA transporter GAT-1 ($F_{(3,15)} = 8.778$, $p = 0.0013$; Figure 6B), with *post hoc* analysis revealing a significant decrease at 10 ($p = 0.0004$), not 1 or 0.1 μM ($p > 0.05$). GABAergic alterations were also supported by a trend towards a decrease in PV interneurons in cortical regions of interest ($H_{(3,14)} = 7.277$, $p = 0.0502$; Figure 6C). Due to trending significance, a *post hoc* analysis was conducted and demonstrated a significant decrease in PV at 10 μM ($p = 0.0350$), but not lower doses of nicotine ($p > 0.05$). Finally, there was a trending decrease in levels of the GABA synthesis marker, GAD67 ($F_{(3,16)} =$

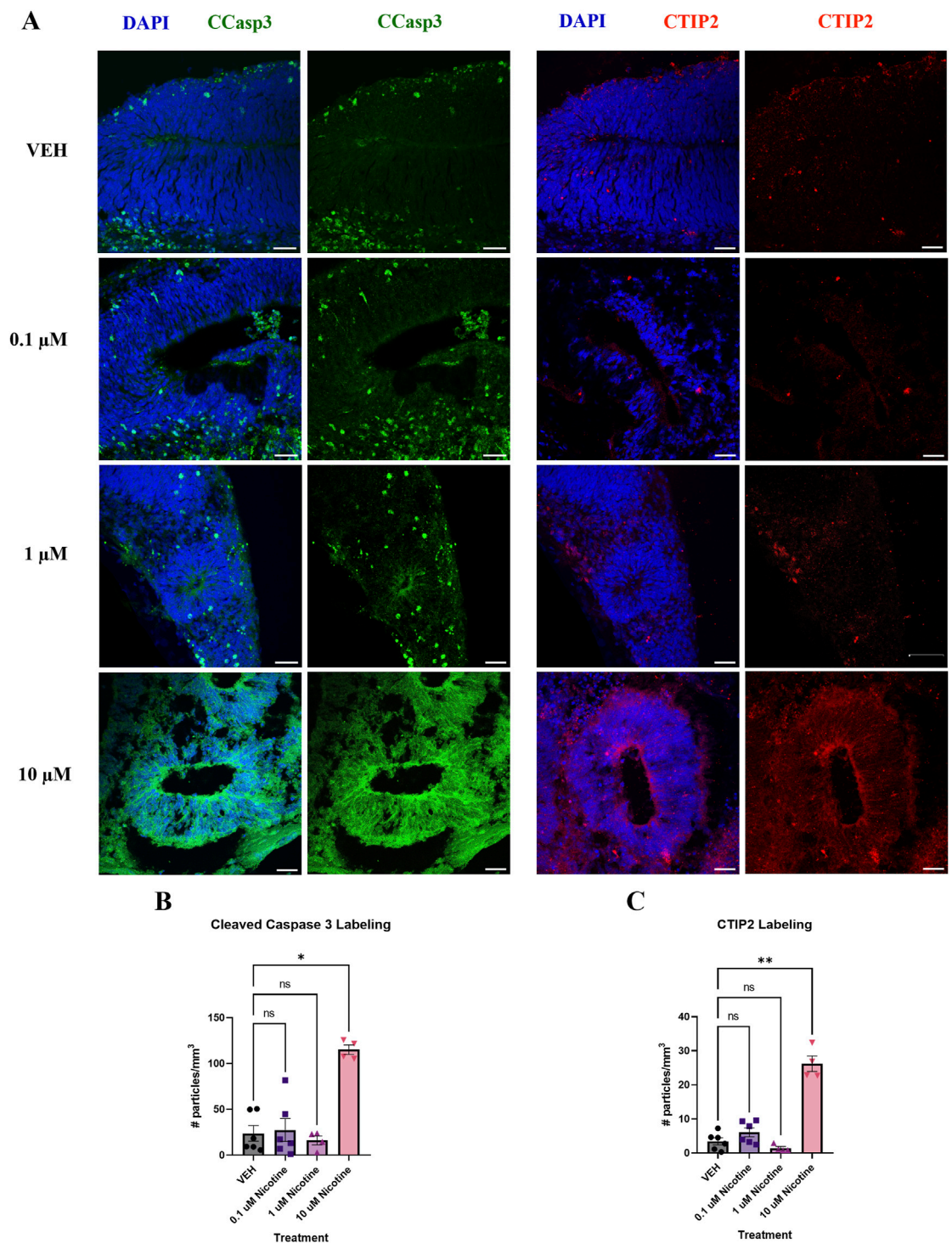

FIGURE 3
Nicotine significantly increases cell death and the number of early-born neurons on D42. **(A)** Immunofluorescent images captured using confocal microscopy of CCasp3 (green) and CTIP2 (red) in brain organoids treated with (0.1, 1 or 10 µM) nicotine or without (VEH) for 14 days. Scale bar = 50 µm. **(B, C)** Quantification of immunofluorescent images by the number of particles per area (mm^3). **(B)** Organoids treated with 10 µM displayed a significant increase in apoptotic cell death, denoted by increased expression of CCasp3. **(C)** Nicotine significantly increased the presence of cortical layer marker CTIP2. Comparisons were made with Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 3 organoids per group; 20 total ROIs per marker, $^{**}p < 0.01$, $^{*}p < 0.05$, trending = $p < 0.1$, ns = not significant, $p > 0.05$. Each data point represents one ROI.

3.150, $p = 0.0540$; Figure 6D), which may suggest altered GABA neurotransmission in our organoids. Analogous to PV, *post hoc* comparisons were performed and revealed a significant deficit in GAD67 at 10 µM ($p = 0.0157$). These results suggest that at the protein level, higher doses of nicotine have a significant effect on GABAergic markers implicated in maintaining cortical E/I balance.

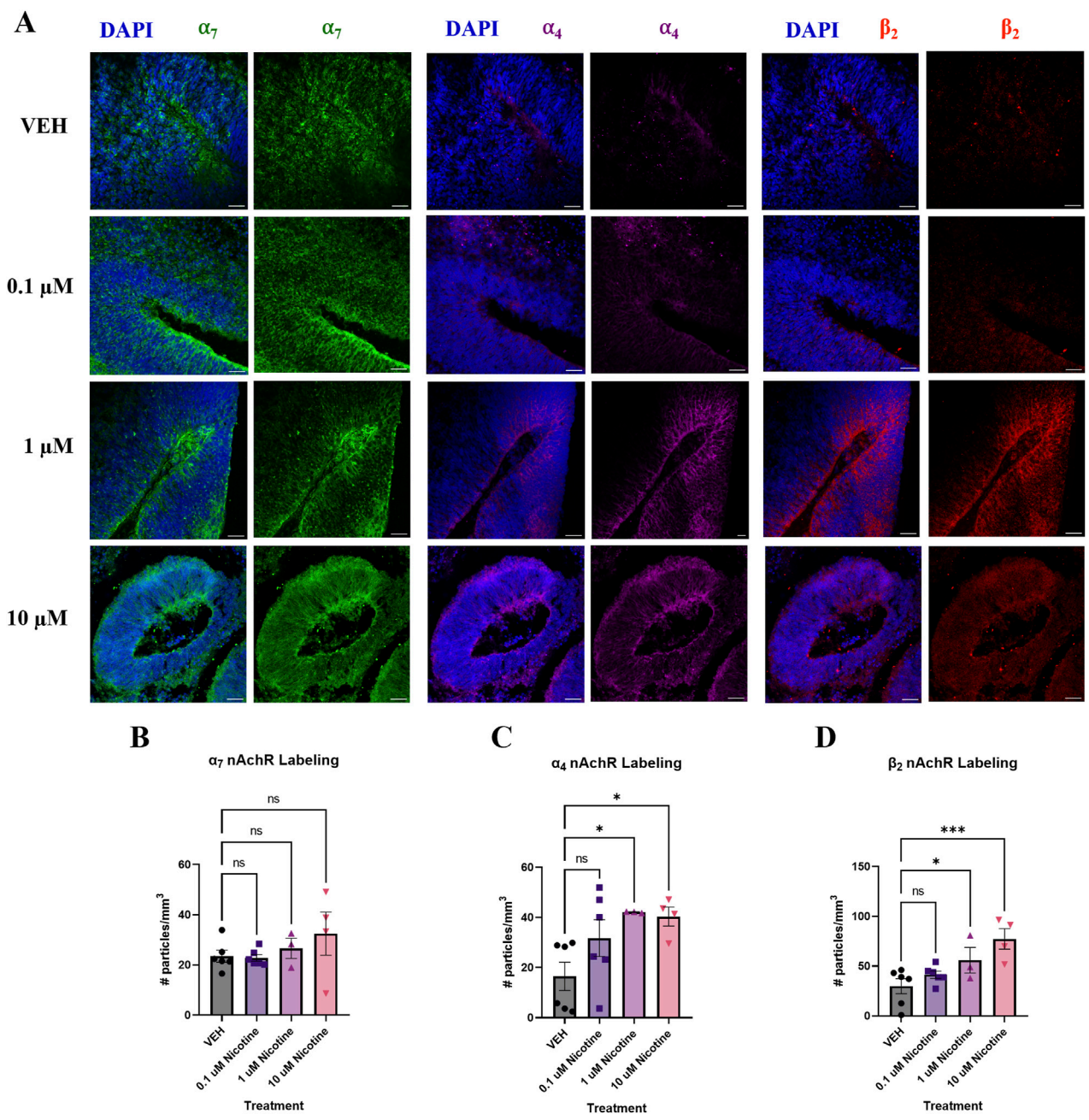

FIGURE 4
Nicotine selectively upregulates certain nAchR populations at D42. **(A)** Immunofluorescent images captured using confocal microscopy of α_7 (green), α_4 (magenta) and β_2 nAchR (red) in brain organoids treated with (0.1, 1 or 10 μM) nicotine or without (VEH) for 14 days. Scale bar = 50 μm. **(B–D)** Quantification of immunofluorescent images by the number of particles per area (mm^3). Nicotine did not affect α_7 nAchR expression **(B)** but significantly increased α_4 **(C)** and β_2 **(D)** compared to VEH organoids. Comparisons were made with one-way ANOVA or Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 3 organoids per group; 18–20 total ROIs per marker, ***$p < 0.001$, *$p < 0.05$, ns = not significant, $p > 0.05$. Each data point represents one ROI.

Nicotine-treated organoids endure significant transcriptomic changes in genes pertaining to nervous system development, neurogenesis and transcription regulation

Following IF analysis of proteins, nicotine-induced alterations were investigated at the transcriptomic level. Previously, chronic nicotine exposure has been associated with changes in markers pertaining to neural identity and forebrain development (Wang et al., 2018). Therefore, qPCR was used to evaluate changes in genes that may play a role in cortical development in conjunction with emotional and behavioral processes (Figure 7). One-way ANOVA suggested a trending increase in *EMX1* ($F_{(2,14)} = 3.716$, $p = 0.0508$; Figure 7A), which is implicated in the formation of the developing cerebral cortex. Due to trending significance, a *post hoc* analysis was performed and revealed a significant increase in *EMX1* at 0.1 ($p = 0.0255$) but not 1 μM ($p > 0.05$). Another gene implicated in the

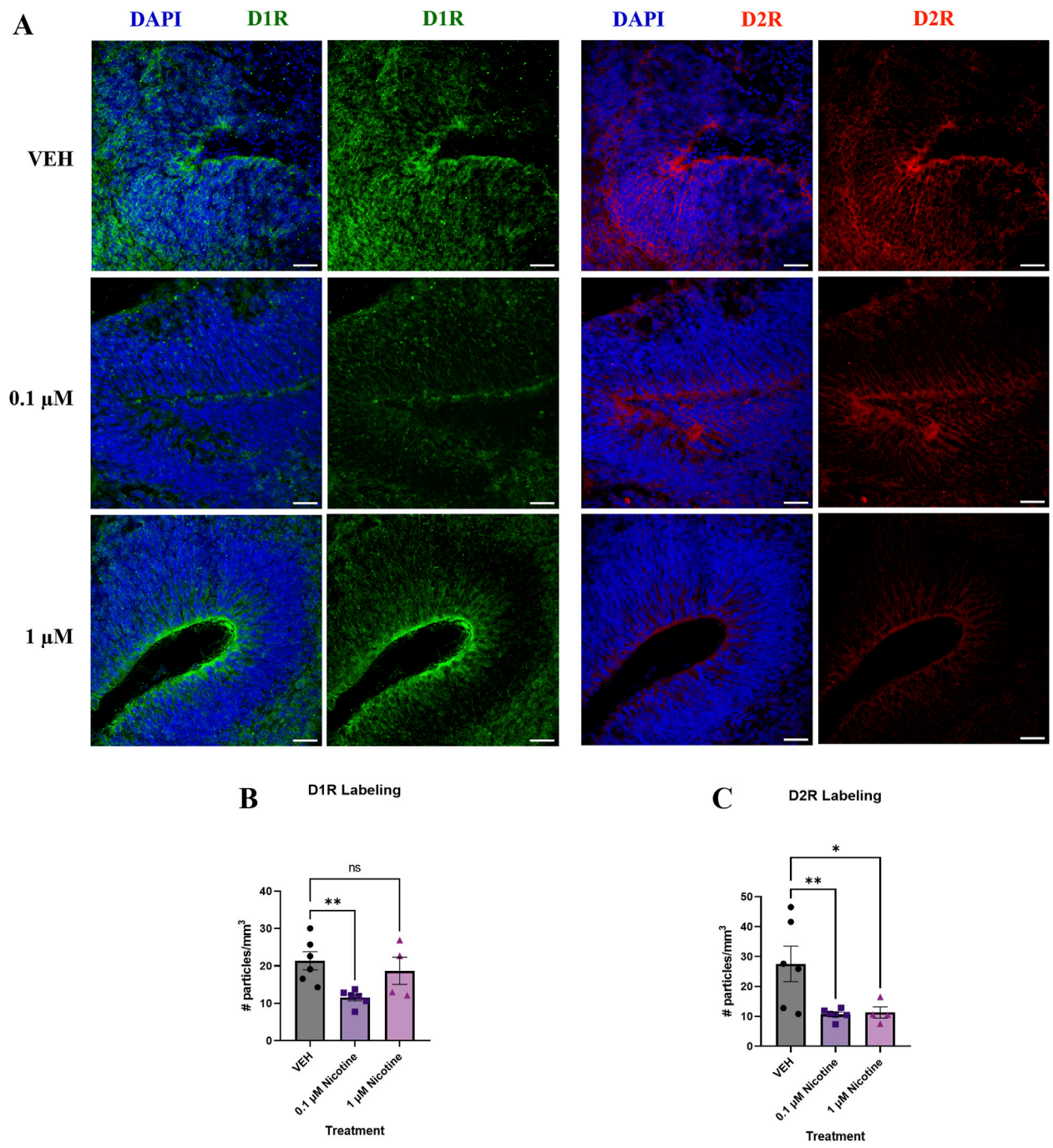

FIGURE 5
Nicotine induces significant alterations in dopaminergic receptors implicated in mood and anxiety disorders at D42. **(A)** Immunofluorescent images captured using confocal microscopy of D1R (green) and D2R (red) brain organoids treated with (0.1 or 1 µM) nicotine or without (VEH) for 14 days. Scale bar = 50 µm. **(B, C)** Quantification of immunofluorescent images by the number of particles per area (mm^3). Compared to VEH, organoids treated with nicotine had significantly decreased levels of D1R at 0.1 µM **(B)** and D2R at 0.1 and 1 µM **(C)**. Comparisons were made with one-way ANOVA or Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 3 organoids per group; 16 total ROIs per marker, $^{**}p < 0.01$, $^{*}p < 0.05$, trending = $p < 0.1$, ns = not significant, $p > 0.05$. Each data point represents one ROI.

maturation of the cortex is *FOXG1*. One-way ANOVA also showed a trending increase in *FOXG1* in nicotine organoids compared to VEH ($F_{(2,13)} = 3.582$, $p = 0.0577$; Figure 7B). Due to trending significance, a *post hoc* analysis was completed and a significant increase in *FOXG1* was shown at 0.1 ($p = 0.0350$) but not 1 µM ($p > 0.05$). The final neural identify marker analyzed was *ISL1*, a gene vital to embryonic brain development. Analysis with one-way ANOVA demonstrated that nicotine organoids had a significant decrease in *ISL1* compared to VEH ($F_{(2,14)} = 3.898$, $p = 0.0451$; Figure 7C). Follow-up with *post hoc* comparisons determined there was a significant decrease at 0.1 ($p = 0.0145$) but not 1 µM ($p > 0.05$). These results demonstrate that genes underlying cortical

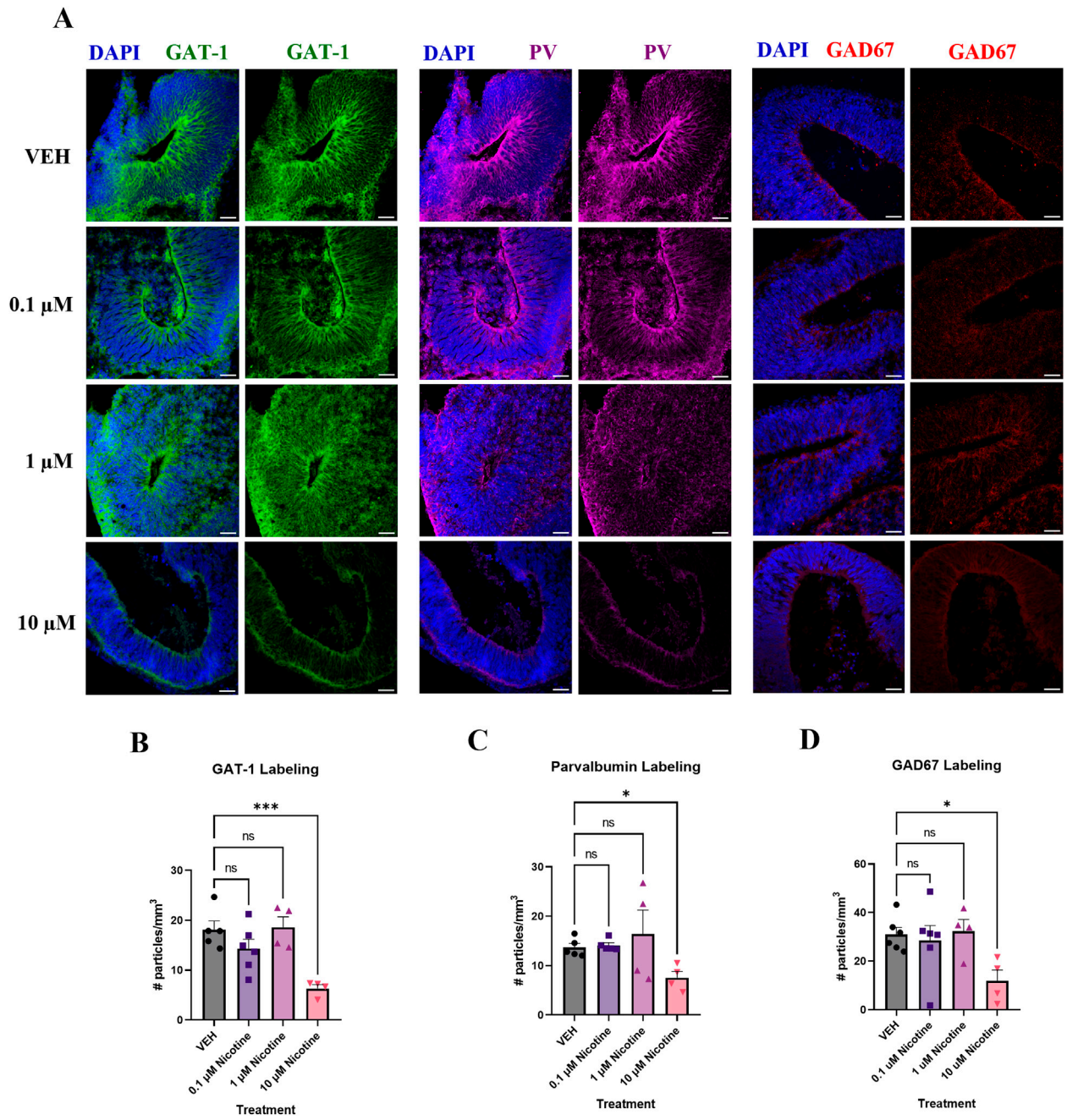

FIGURE 6
Nicotine induces significant deficits in markers vital to GABAergic synthesis, transport and signaling on D42. **(A)** Immunofluorescent images captured using confocal microscopy of GABA transporter GAT-1 (green), interneuron marker PV (magenta) and GABA synthesis marker GAD67 (red) in brain organoids treated with (0.1, 1 or 10 µM) nicotine or without (VEH) for 14 days. Scale bar = 50 µm. **(B–D)** Quantification of immunofluorescent images by the number of particles per area (mm^3). At the 10 µM dose, organoids exhibited a significant decrease in GAT-1 **(B)**, PV **(C)**, and GAD67 **(D)** expression compared to VEH. Comparisons were made with one-way ANOVA or Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 3 organoids per group; 18–20 total ROIs per marker, ***$p < 0.001$, *$p < 0.05$, trending = $p < 0.1$, ns = not significant, $p > 0.05$. Each data point represents one ROI.

development are more sensitive to lower doses of nicotine, which suggests that PNE may dysregulate cortical systems that are vital to emotional regulation.

Both nicotine and mood/anxiety disorders have been known to cause cortical transcriptomic changes in clinical and preclinical studies (Lauterstein et al., 2016; Malki et al., 2015; Semick et al., 2020; Yoshino et al., 2021). Thus, it was of great interest to investigate if similar DEGs and transcriptomic alterations were captured using RNA-Seq in the brain organoids (Figure 8). The 0.1 µM dose was selected due to our D42 qPCR results as there were significant transcriptomic changes at 0.1 µM, but not 1 µM. To gain a sense of sample variation, a multidimensional scale plot was

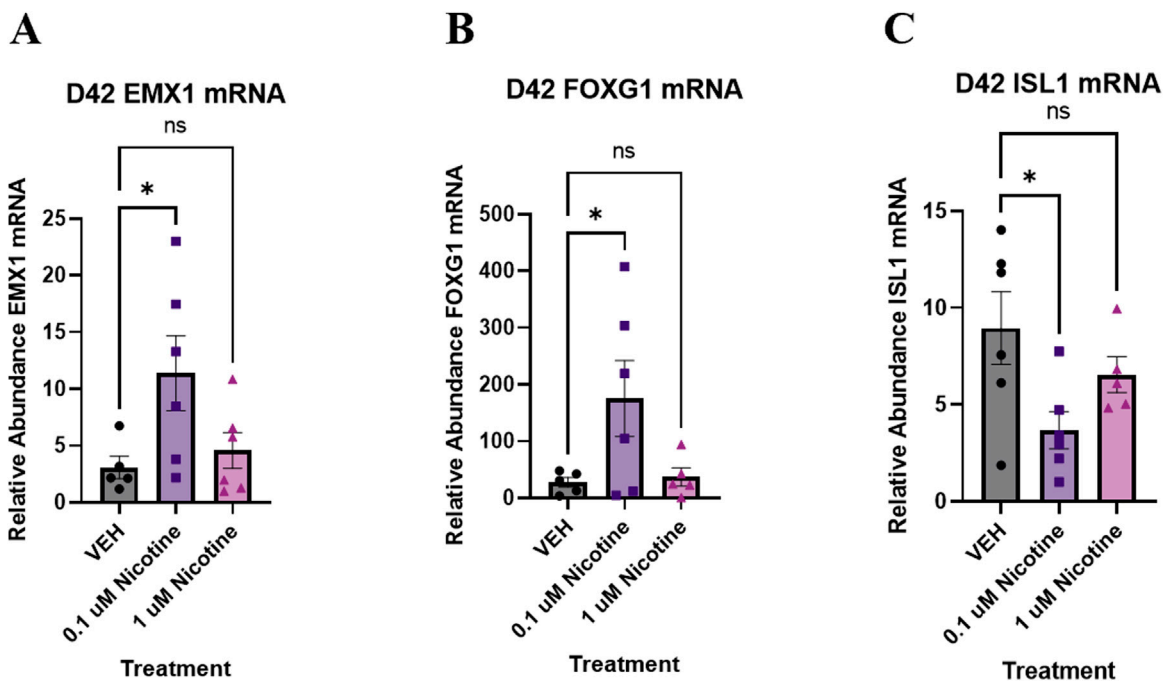

FIGURE 7
Nicotine elicits short-term changes in gene expression of various neural identity markers on D42. **(A–C)** Expression of relative abundance of mRNA of neural identity markers by qPCR in brain organoids exposed to nicotine (0.1, or 1 μM) or without (VEH) for 14 days. Relative mRNA abundance was calculated by normalizing the marker of interest to the geometric means of two housekeeping genes, *GAPDH* and *ACTB*. Organoids demonstrated a trending increase in cortical marker *EMX1* **(A)** and forebrain marker *FOXG1* **(B)**. There is a significant decrease in *ISL1*, a marker of embryonic development **(C)**. Comparisons were made with one-way ANOVA or Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 5–6 organoids per group, *$p < 0.05$, trending = $p < 0.1$, ns = not significant, $p > 0.05$.

created (Figure 8A) and revealed that VEH and nicotine organoids separated more along the *x*-axis (42%) compared to the *y*-axis (27%). This indicates that the treatment groups were more different from each other than they were similar, with VEH grouping towards the top half of the multidimensional scale plot and nicotine organoids at the bottom. The short-term effects of nicotine exposure were examined by quantifying the number of DEGs and it was discovered that there were 91 downregulated genes and 40 upregulated genes when comparing nicotine-treated organoids to VEH (Figure 8B). Of these DEGs, when examining the top 20 (10 most downregulated and upregulated genes sorted by log fold change), upregulated genes include *NEUOROG2, EOMES* and the downregulated gene *CYP26C1* (Table 2). There were also multiple novel transcripts within the top 20 DEGs [denoted NA in Table 2]. Moreover, changes in DEG expression were summarized using a heat map (Figure 8C), which demonstrated that relative gene expression patterns between VEH and nicotine organoids looked quite different. There appears to be a larger number of genes that are transcribed more in the VEH (red) organoids whereas the nicotine organoids have a larger number of genes that are transcribed less (blue). Alternatively, there appears to be a smaller number of genes that are transcribed less in the VEH organoids whereas the nicotine organoids have a smaller number of genes that are transcribed more. To delve deeper into these classifications, GO analysis was conducted to examine specific terms that were enriched within BP and MF categories (Figure 8D). The GO database defines BP as specific physiological or cellular roles carried out by the gene whereas MF describes the molecular activity of a gene but does not provide any spatial information about where these functions occur in the cell. Analysis with GO BP, which had the highest number of counts, reveals terms enriched for several developmental processes (Figure 9). These terms involve organ (p = 1.56e-10), nervous system (p = 1.58e-7), and anatomical structural development (p = 1.53e-8). Likewise, a significant number of terms referring to neurogenesis (p = 1.42e-5) were present, including generation of neurons (p = 3.37e-6), neuronal differentiation (p = 5.62e-6), and cell migration (p = 2.02e-5). Several terms also included various regulatory processes like biosynthetic processes (p = 1.78e-5) and regulation of transcription (p = 2.30e-5). Like the transcription terms within BP, several terms were rereferring to transcription and DNA binding within the GO MF analysis, which further implicates the effect of nicotine on gene transcription (Figure 10). Integrin (p = 0.011), signaling receptor (p = 0.026), and transcription factor binding (p = 2.39e-5) were also principal terms reported in the MF analysis. Ultimately, the GO analysis elucidated that nicotine significantly impacted several BPs and MFs linked to embryonic development, transcription and gene expression.

To follow up the GO analysis, we used VarElect to investigate the genetic overlap and phenotype-gene associations of our DEGs between three phenotypes of interest: nicotine exposure, anxiety,

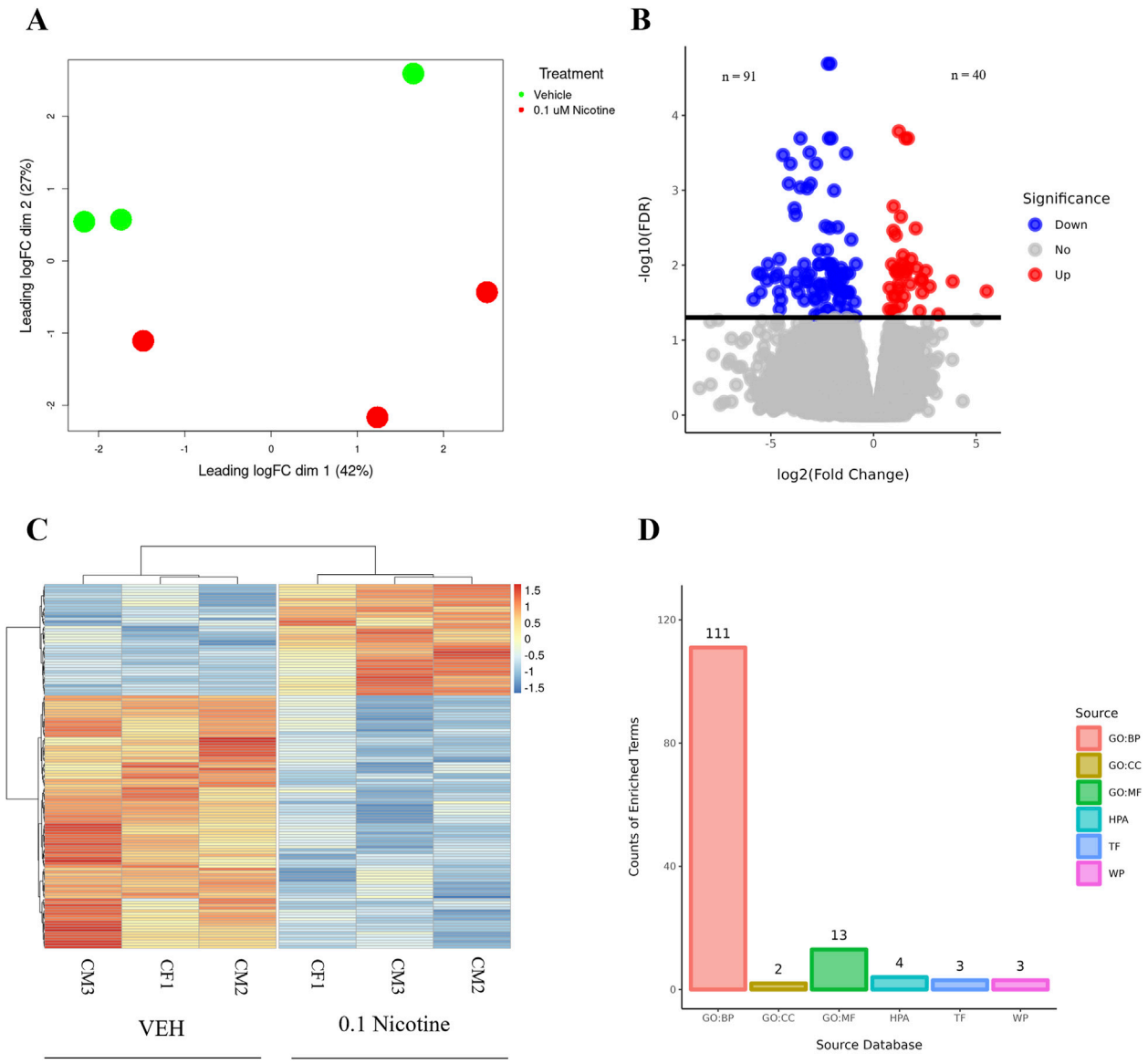

FIGURE 8
RNA-Seq differential gene expression and functional enrichment analysis of D42 VEH and 0.1 μM nicotine-treated organoids. **(A)** Multidimensional scaling plot demonstrating the separation of VEH (green) and 0.1 μM (red) nicotine-treated organoids. **(B)** Volcano plot illustrating the number of DEGs in VEH and 0.1 μM treated organoids. Upregulated genes in red (n = 40), downregulated genes in blue (n = 91). **(C)** Heatmap of log2 transformed normalized CPM values for all DEGs for VEH and 0.1 μM treated organoids. Values were centered, scaled across rows, and clustered using Ward's clustering algorithm. Upregulated genes in red (n = 40), downregulated genes in blue (n = 91), n = 3 per group, FDR ≤ 0.05. **(D)** Overrepresented genes are categorized according to their functional characteristics quantified by the number of genes (counts) within a certain category. The categories originate from certain databases and include, from left to right, gene ontology (GO) biological processes (GO BP; 111), GO cellular component (GO CC; 2), GO molecular function (GO MF; 13), Human Protein Atlas (HPA; 4), Transfac (TF; 3) and Wikipathways (WP; 3).

and depression (Stelzer et al., 2016; Table 3). All DEGs were imported into VarElect and ranked according to the elected phenotype. A list of the top 20 genes for each phenotype was generated. Within the list for each phenotype, five genes were consistent: dopamine transporter (*SLC6A3*), secreted phosphoprotein 1 (*SPP1*), nerve growth factor receptor (*NGFR*), histone deacetylase 9 (*HDAC9*) and insulin-like growth factor 2 (*IGF2*). These results imply that there is a shared genetic overlap between these phenotypes of interest. This may provide more insight as to how PNE and genetic variation within these genes underlie the incidence of mood and anxiety disorders.

Long-term effects of PNE

PNE chronically perturbs proteins related to neuronal differentiation, dopaminergic and glutamatergic receptor expression

PNE has been associated with the emergence of mood and anxiety disorders later in the lives of children exposed to nicotine during pregnancy (Corrêa et al., 2022; Moylan et al., 2015). Therefore, we wanted to examine not only the immediate effects of nicotine but long-term outcomes during the later stages of organoid maturation (D180; Figure 11A). Unlike the

TABLE 2 Top 20 DEGs in VEH and nicotine organoids.

Symbol	Gene	Log fold change	*p*-value	FDR	Significance
ENSG00000145626	*UGT3A1*	−5.85	1.40E-04	0.029	Down
ENSG00000250511	NA	−5.61	3.32E-05	0.013	Down
ENSG00000187553	*CYP26C1*	−5.50	1.01E-04	0.023	Down
ENSG00000279607	NA	−5.44	3.84E-05	0.013	Down
ENSG00000158022	*TRIM63*	−5.20	5.18E-05	0.016	Down
ENSG00000240990	*HOXA11-AS*	−5.13	1.65E-05	0.009	Down
ENSG00000248329	*APELA*	−4.79	4.44E-05	0.014	Down
ENSG00000174407	*MIR1-1HG*	−4.67	3.58E-05	0.013	Down
ENSG00000078399	*HOXA9*	−4.60	9.60E-05	0.023	Down
ENSG00000253293	*HOXA10*	−4.59	1.21E-05	0.008	Down
ENSG00000163508	*EOMES*	2.09	2.28E-05	0.011	Up
ENSG00000112333	*NR2E1*	2.24	2.29E-04	0.041	Up
ENSG00000178403	*NEUROG2*	2.31	4.84E-05	0.015	Up
ENSG00000087510	*TFAP2C*	2.35	5.21E-05	0.016	Up
ENSG00000251621	NA	2.37	1.04E-04	0.023	Up
ENSG00000286232	NA	2.55	2.79E-05	0.012	Up
ENSG00000168453	*HR*	2.73	7.45E-05	0.019	Up
ENSG00000280222	*NA*	3.16	2.55E-04	0.045	Up
ENSG00000280409	*LINC01101*	3.85	5.65E-05	0.016	Up
ENSG00000119614	*VSX2*	5.51	9.31E-05	0.022	Up

Abbreviations: FDR, false discovery rate; NA, novel transcript.

significant increase reported at D42, at D180 we report a significant decrease in CTIP2 expression in nicotine-exposed organoids ($F_{(2,32)}$ = 3.476, p = 0.0431; Figure 11B). *Post hoc* analysis stated a reduction in CTIP2 at both 0.1 (p = 0.0444) and 1 μM (p = 0.0213) in comparison to VEH. Due to the short-term effects of nicotine at D42, we also examined long-term changes in the dopaminergic receptor, D1R. One-way ANOVA also demonstrated a significant increase in D1R expression in nicotine organoids at D180 ($F_{(2,33)}$ = 4.349, p = 0.0211; Figure 11C). *Post hoc* analysis identified an increase at 0.1 (p = 0.0059), but not 1 μM (p > 0.05). In addition to alterations in dopaminergic receptors, further long-term dysregulation was reported in glutamatergic receptors NR2B and mGLUR2/3. At D180, Kruskal–Wallis testing revealed a significant upregulation in NR2B ($H_{(2,28)}$ = 6.136, p = 0.0465; Figure 11D). A *post hoc* was performed and showed an increase, specifically at 0.1 (p = 0.0333), but not 1 μM (p > 0.05). Finally, levels of mGLUR2/3 were significantly increased ($H_{(2,33)}$ = 11.80, p = 0.0027; Figure 11E), with *post hoc* comparisons showing that mGLUR2/3 was elevated at both 0.1 (p = 0.0079) and 1 μM (p = 0.0013) compared to VEH. Altogether, these results signify long-lasting changes in neuronal differentiation, dopaminergic and glutamatergic proteins that persist until later stages of development following chronic nicotine exposure.

PNE has long-term transcriptomic effects on neural and cortical development

To complement our IF results, the last set of experiments analyzed long-term changes in gene expression induced by nicotine exposure. We report that at D180, there was dysregulation in more than one neural identity marker. One-way ANOVA revealed a trend toward significantly decreased *TBR1*, a cortical pre-plate marker ($F_{(2,14)}$ = 3.535, p = 0.0572; Figure 12A). Due to trending significance, a *post hoc* analysis was completed and revealed a significant decrease in *TBR1* at 1 (p = 0.0207) but not 0.1 μM (p > 0.05). Compared to VEH, there was also a significant decrease in neural progenitor marker *EOMES* (also known as *TBR2*; $F_{(2,13)}$ = 4.441, p = 0.0339; Figure 12B). *Post hoc* analysis was completed and revealed a significant decrease in *EOMES* at 0.1 (p = 0.0206) and 1 μM nicotine (p = 0.0236). These results suggest that PNE has an enduring impact on neural and cortical development.

PNE elicits chronic alterations in glutamatergic, gabaergic and dopaminergic markers implicated in mood and anxiety disorders

Lastly, we quantified long-term changes in gene expression in glutamatergic, GABAergic, and dopaminergic markers implicated in mood and anxiety disorders. There was evidence of altered

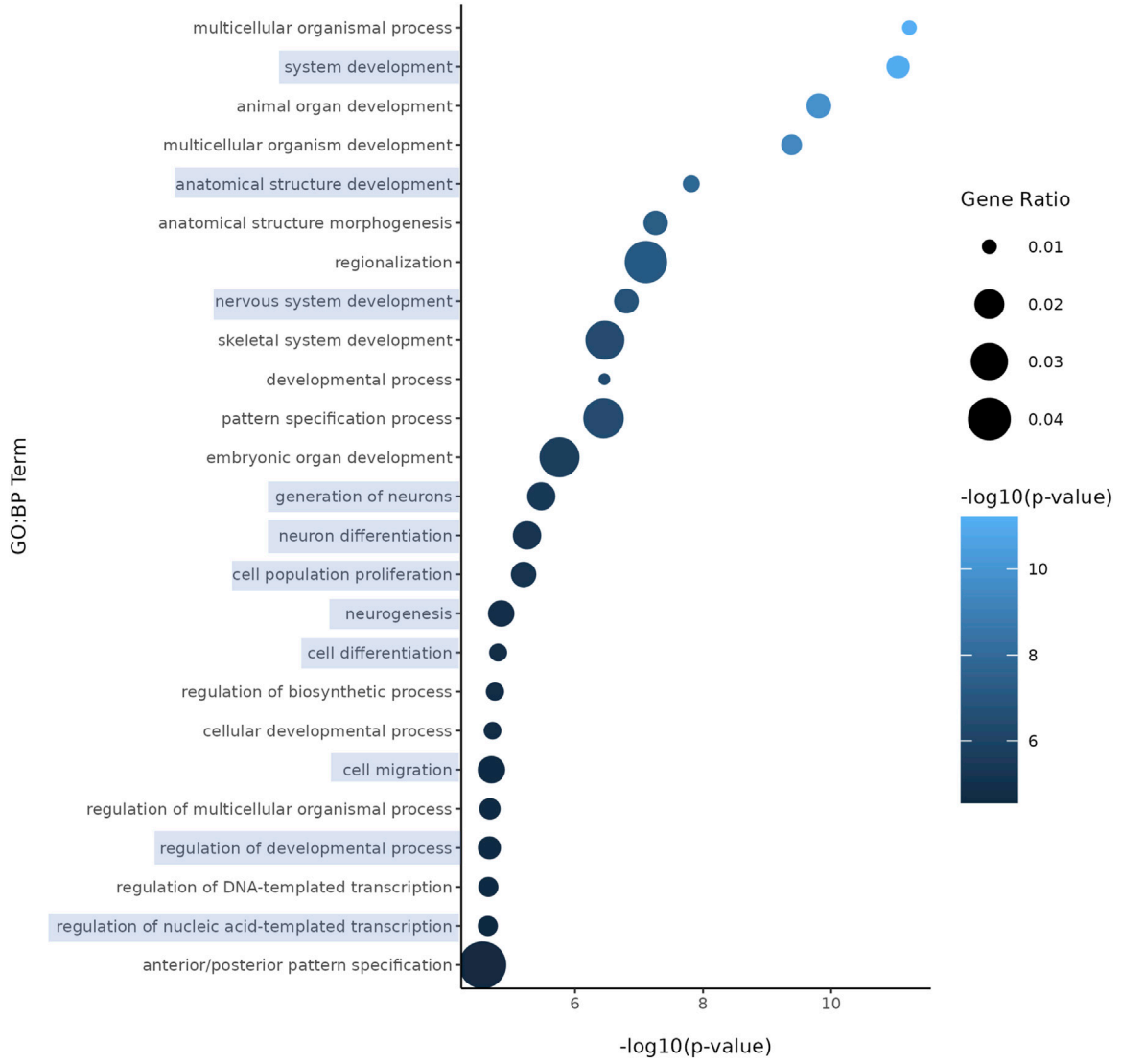

FIGURE 9
Enriched GO terms in D42 VEH and 0.1 μM nicotine-treated organoids. GO BP terms reveal that PNE elicits alterations in genes within processes such as nervous system development, neurogenesis, and other developmental processes. Circle size represents gene ratio and color represents fold change (-log10 transform of the adjusted *p*-value). Blue boxes represent terms of particular interest.

GABAergic and glutamatergic gene expression at D180, with a trending decrease in *GRM2*, the gene for mGLUR2 ($F_{(2,14)}$ = 2.796, p = 0.0951; Figure 12C) and a significant decrease in *GAD1*, the gene for GAD67 ($F_{(2,10)}$ = 14.55, p = 0.0011; Figure 12D). Due to trending significance in *GRM2*, a *post hoc* analysis was completed and revealed a significant decrease at 1 (p = 0.0447) but not 0.1 μM ($p > 0.05$). *Post hoc* comparisons were also performed for *GAD1* and showed a significant decrease at 0.1 μM (p = 0.0007) compared to VEH. There was no significant effect at 1 μM ($p > 0.05$). Consistent with dopaminergic perturbations at D42, one-way ANOVA described a trending decrease in *D1R* at D180 compared to VEH ($F_{(2,14)}$ = 3.256, p = 0.0690; Figure 12E). Since trending significance was reported, *post hoc* comparisons were done and indicated a significant decrease at 1 (p = 0.0231) but not 0.1 μM ($p > 0.05$). These transcriptomic results signify that PNE unremittingly modifies neurotransmitter systems into later stages of neurodevelopment.

Discussion

The association between developmental nicotine exposure and the emergence of mood and anxiety behaviors has been reported in various clinical and preclinical studies (Corrêa et al., 2022; Hudson et al., 2021; Jobson et al., 2019; Moylan et al., 2013; Moylan et al., 2015). However, the use of cerebral organoids to explicitly model PNE, offers a unique human-derived platform to build on prior research findings and bypass existing experimental limitations. Moreover, the relationship between PNE and the development of specific mood and anxiety molecular endophenotypes remains

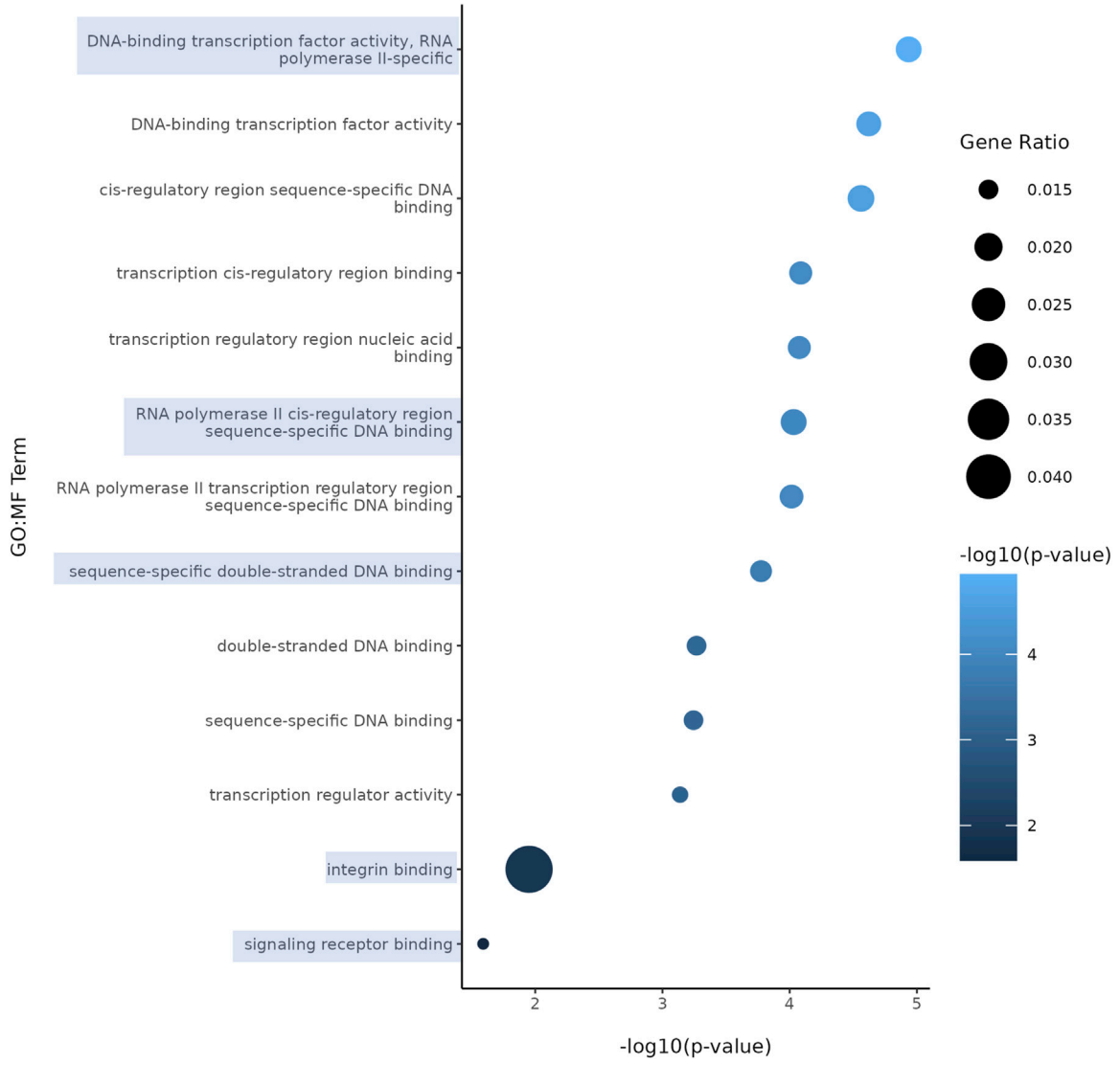

FIGURE 10
Enriched GO terms in D42 VEH and 0.1 μM nicotine-treated organoids. GO MF terms illustrate genes enriched in functions pertaining to DNA, signaling receptor and integrin binding activity. Circle size represents gene ratio and color represents fold change (-log10 transform of the adjusted *p*-value). Blue boxes represent terms of particular interest.

TABLE 3 Common top 20 ranked genes related to nicotine, anxiety and depression phenotypes.

Gene symbol	Description	Rank (1–20) "Nicotine"	Rank (1–20) "Anxiety"	Rank (1–20) "Depression"
SLC6A3	Dopamine transporter	1	1	1
SPP1	Secreted phosphoprotein 1	2	8	17
NGFR	Nerve growth factor receptor	3	13	8
HDAC9	Histone deacetylase 9	6	11	18
IGF2	Insulin-like growth factor 2	9	7	11

elusive and to our knowledge, has yet to be explored in cerebral organoids.

Consistent with previous research, chronic exposure to physiologically relevant doses of nicotine had widespread neuronal, molecular, and transcriptomic effects on our organoids (Notaras et al., 2021; Wang et al., 2018). We report that chronic nicotine exposure (0.1–10 μM) triggered apoptotic cell death and impacted the normal expression of various neural identity markers

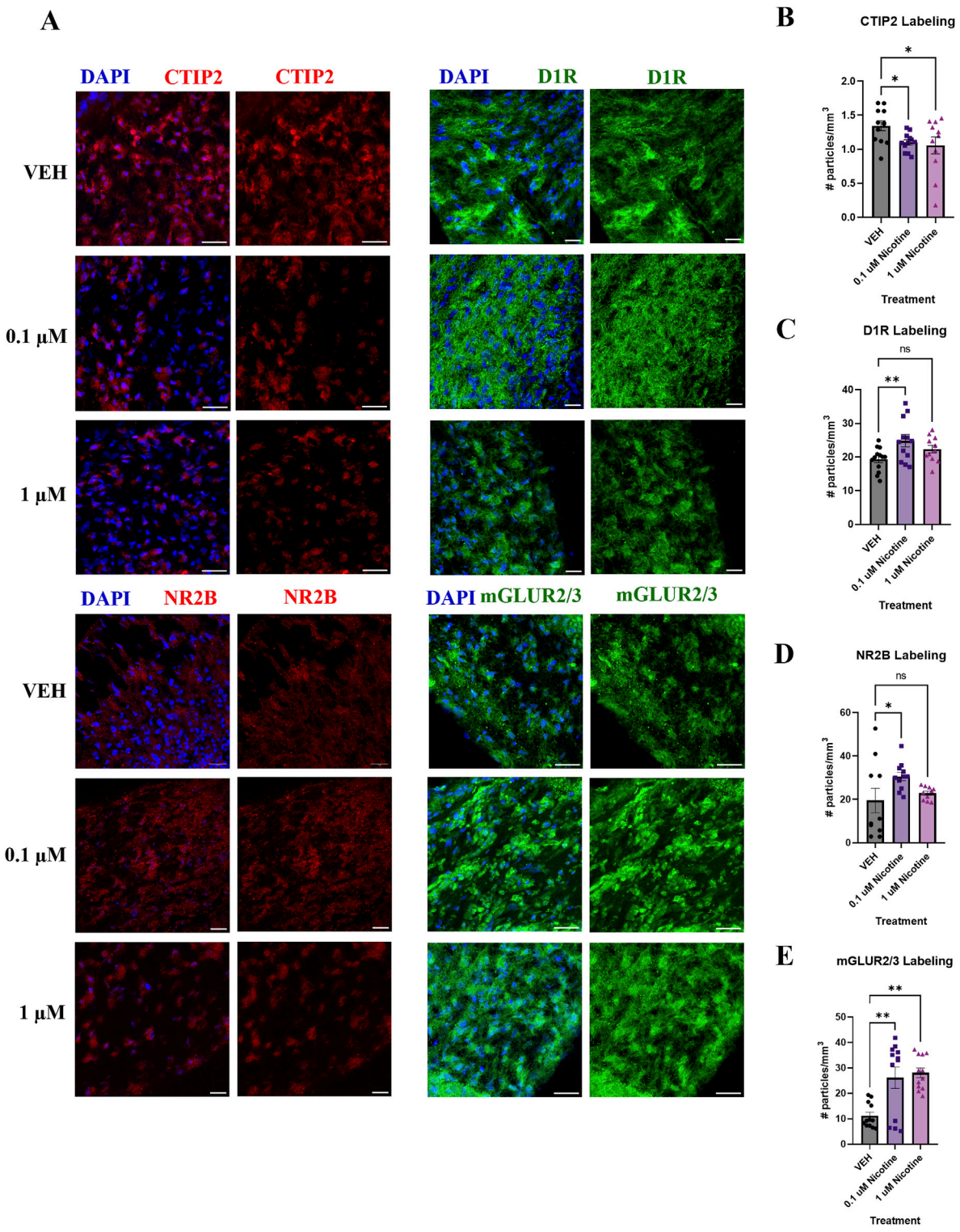

FIGURE 11
Nicotine induces long-term alterations in neuronal differentiation, dopaminergic and glutamatergic markers at D180. **(A)** Immunofluorescent images captured using confocal microscopy of CTIP2 (red), D1R (green), NR2B (red) and mGLUR2/3 (green) in brain organoids treated with (0.1 or 1 µM) nicotine or without (VEH) for 14 days. Scale bar = 50 µm. **(B–E)** Quantification of immunofluorescent images by the number of particles per area (mm^3). Compared to VEH, organoids treated with nicotine had significantly decreased cortical layer marker CTIP2 levels at 0.1 and 1.0 µM **(B)**. Nicotine also significantly increased D1R **(C)** and glutamatergic markers NR2B **(D)** and mGLUR2/3 **(E)**. Comparisons were made with one-way ANOVA or Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 3 organoids per group; 31–36 total ROIs per marker, $^{**}p < 0.01$, $^{*}p < 0.05$, trending = $p < 0.1$, ns = not significant, $p > 0.05$. Each data point represents one ROI.

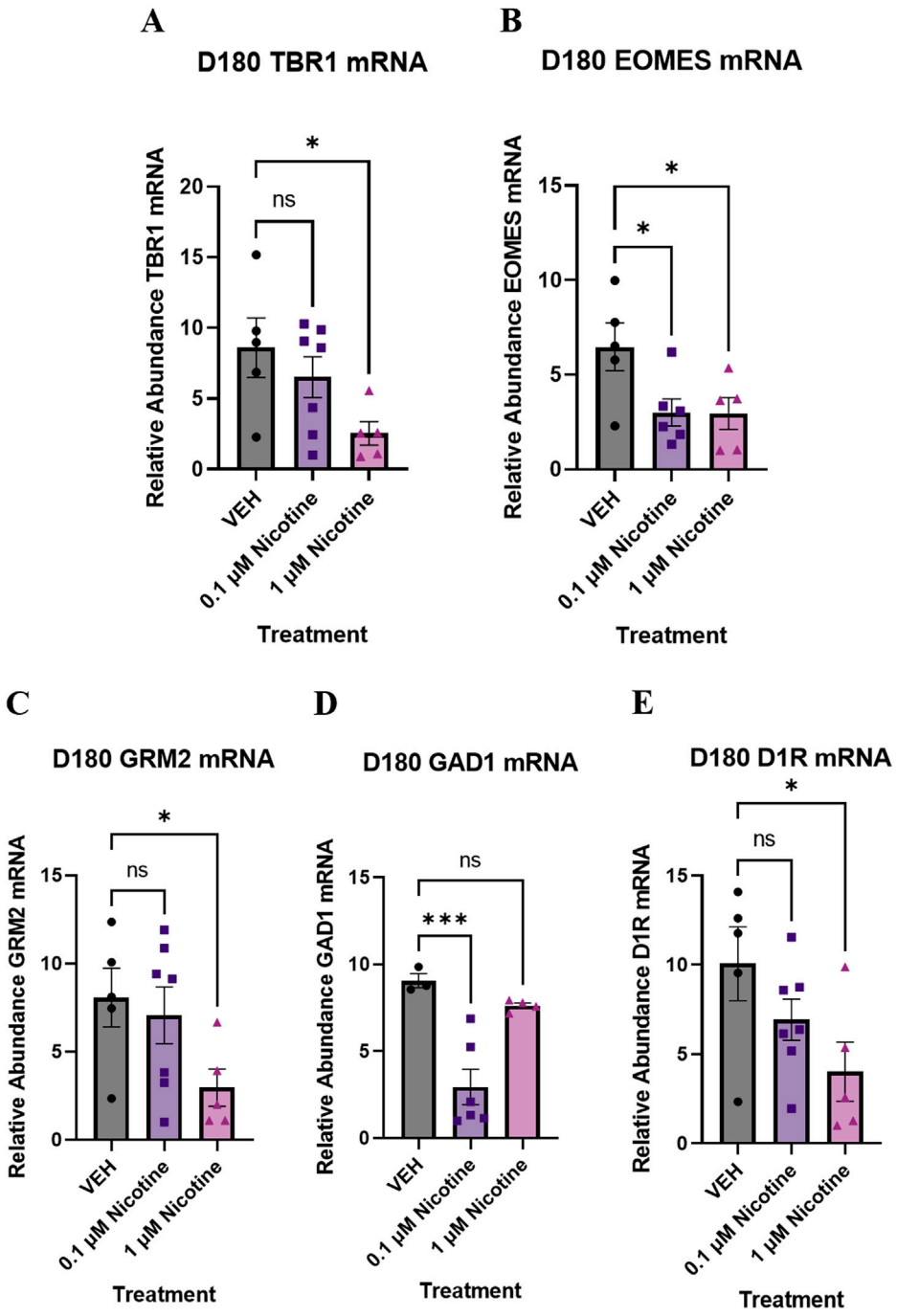

FIGURE 12
Nicotine elicits persistent, long-term changes in neural identity markers, glutamatergic, GABAergic and dopaminergic markers implicated in mood and anxiety disorders at D180. **(A–E)** Expression of relative abundance of mRNA by qPCR in mature D180 brain organoids exposed to nicotine (0.1, or 1 μM) or without (VEH) for 14 days. Relative mRNA abundance was calculated by normalizing the marker of interest to the geometric means of two housekeeping genes, *GAPDH* and *ACTB*. Nicotine was associated with a trending decrease in pre-plate marker *TBR1* **(A)** and a significant decrease in progenitor marker *EOMES* **(B)**. **(C)** Nicotine induced a trending, dose-dependent decrease in glutamatergic receptor *GRM2*. **(D)** Nicotine significantly downregulated the expression of GABA synthesis marker *GAD1*. **(E)** There was a significant decrease in *D1R* expression following nicotine exposure. Comparisons were made with one-way ANOVA or Kruskal Wallis followed by Fisher's LSD *post hoc* test. Data are mean ± SEM, n = 3–7 organoids per group, ***$p < 0.001$, *$p < 0.05$, trending = $p < 0.1$, ns = not significant, $p > 0.05$.

at D42 and D180. In terms of nicotine's effect on its target receptors, there was short-term upregulation of α_4 and β_2 nAchR subunits. However, nicotine had no significant effect on α_7 expression. Nicotine also dysregulated the expression of dopaminergic receptors, with long-lasting alterations in D1R expression persisting until D180. With regards to other neurotransmitter systems, nicotine affected glutamatergic and GABAergic markers of interest, which implies shifted E/I balance in the cortex and remained until the later stages of organoid maturation. Finally, RNA-Seq revealed substantial transcriptomic changes in organoids treated with 0.1 μM nicotine. Numerous BPs and MFs were differentially expressed, specifically in targets involving nervous system development, neurogenesis, and transcription activity.

Nicotine exposure has been shown to disrupt many facets of fetal neurogenesis, including changes in neuronal differentiation and apoptosis (Aoyama et al., 2016; Dwyer et al., 2009; Smith et al., 2010; Wang et al., 2018). For instance, chronic PNE has been shown to disrupt the cell cycle of neural progenitors and accelerate neuronal differentiation (Aoyama et al., 2016; Takarada et al., 2012). To further support this claim, compared to controls, 1 μM and 10 μM nicotine-treated organoids on a chip demonstrated increased short-term expression of CTIP2, an early-born neuronal marker, and CCasp3, a member of the caspase family implicated in neuronal death in neural syndromes (Wang et al., 2018). These alterations were accompanied by no change in the number of neural progenitor cells, which suggests nicotine disrupted cortical neuronal layering by the induction of premature differentiation. Wang's dose-dependent descriptions of increased differentiation and apoptosis at early developmental stages align with our D42 IF findings. Similarly, at D42, we report increased CCasp3 and CTIP2 at 10 μM nicotine, which also indicates short-term, dose-dependent increases in apoptosis and neuronal differentiation at higher nicotine concentrations. These findings suggest nicotine exposure has a dual effect on the developing brain, simultaneous apoptosis, and compensatory neuronal differentiation. Unlike Wang's study, which focused on early developmental stages of nicotine exposure, we also report long-term neurodevelopmental changes at D180, showing a significant reduction of CTIP2 at 1 μM, indicating a different dose response relationship over time. Although there is an initial compensatory response at D42, these findings suggest this mechanism may not be sufficient to sustain long-term neuronal health, leading to different outcomes over prolonged exposure. The differential response may indicate a biphasic effect, where high doses initially stimulate differentiation, but long-term exposure to lower doses disrupts it. Our findings provide insight to both dose-dependent and time-dependent effects of nicotine on neurodevelopment, emphasizing the need to carefully consider exposure levels during gestation. Understanding the impact of nicotine exposure at various stages of development can provide further insight to its lasting effects, potentially influencing mood and anxiety disorders. Apart from alterations in neurogenesis, PNE has also been found to impact the expression of various categories of neural identity markers. For example, the same study by Wang and colleagues (2018) reported differential expression of preplate marker *TBR1*, forebrain marker *FOXG1* and increased expression of hindbrain marker *ISL1* in their brain organoids (Wang et al., 2018). Indeed, mice with deficiencies in certain neural identity markers, like cortical marker *EMX1*, demonstrate lower levels of depressive behaviors, denoted by reduced immobility time in the forced swim test and reduced anxiety in the light/dark box and elevated plus maze (Cao and Li, 2002). Additionally, mice exposed to PNE demonstrate decreased PFC expression of *EOMES*, or *TBR2*, as well as cognitive and emotional deficits in adulthood (Aoyama et al., 2016). Our qPCR data confirms a significant increase in *EMX1* and *FOXG1* alongside a significant decrease in *ISL1* at D42. This suggests dysregulated development of neuronal populations comprising the forebrain and hindbrain. Additionally, we report significant reductions in *TBR1* and *EOMES* at D180 signifying long-term changes in cortical development. Overall, our qPCR results suggest nicotine-induced dysregulation of neurogenesis and numerous cortical markers that persist until D180. Thus, further studies examining these alterations underlying cortical development may help to understand the impact of PNE on the behavioral dysfunctions of the offspring when these developmental pathways mature.

$\alpha_4\beta_2$ and α_7 are the most abundant nAchRs in the cortex and are implicated in various cognitive and attentional functions (Alkam and Nabeshima, 2019; Livingstone et al., 2010). Results from preclinical studies have demonstrated that chronic PNE from gestational day 7–21 elevates α_4, α_7 and β_2 mRNA expression in the rat cortex and hippocampus (Shacka and Robinson, 1998). This has also been seen in α_4 and α_7 mRNA of human fetuses exposed to nicotine during pregnancy (Falk et al., 2005). Our IF results revealed significantly increased α_4 and β_2 nAchR protein expression following nicotine exposure at D42, which can have functional implications in the development of mood and anxiety disorders (Saricicek et al., 2012). For instance, abnormalities in $\alpha_4\beta_2$ nAchR expression and function, specifically in the PFC and hippocampus, may contribute to these disorders due to their ability to modulate GABA release (Fogaça and Duman, 2019; Freund et al., 1988; Kutlu and Gould, 2015; Lu et al., 1998). Therefore, altered nAchR levels could lead to an imbalance in GABAergic neurotransmission, alter mood and anxiety-related brain circuitry and lay the foundation for altered E/I levels previously reported in the literature (Fogaça and Duman, 2019; Sequeira et al., 2009). Interestingly, there was no change in α_7 expression which was unexpected due to its role in regulating cortical glutamate release (Livingstone et al., 2010). This insignificant effect in α_7 was especially surprising given our reports of immediate and long-term perturbations in GABA receptor expression in our IF and qPCR analyses. However, α_7 nAchR subunits desensitize more rapidly than α_4 and β_2 and have a lower affinity for nicotine (Dwyer et al., 2019; Fenster et al., 1999). Additionally, to our knowledge, the specific timing that these subtypes of nAchRs appear in development has yet to be identified in cerebral organoids, which further complicates temporal analyses in this PNE model. Nevertheless, the present findings may help elucidate the correlation between the temporal specificity of nicotine exposure on the circuitry of the developing fetal brain and the future emergence of mood and anxiety behaviors.

In comparison to other neurotransmitter systems, the effect of PNE on dopaminergic receptors in the fetal brain is unclear. However, as reported in a preclinical rodent model of adolescent nicotine exposure, another critical period of neurodevelopment, rodents chronically exposed to nicotine demonstrated significantly less D1R expression levels in the PFC compared to VEH (Jobson et al., 2019; Laviolette, 2021). This was concurrent

with no significant changes in D2R expression. Likewise, other animal studies have demonstrated dopaminergic hypofunction in the neocortex resulting from PNE as well as decreased dopaminergic metabolites (Ernst et al., 2001; Muneoka et al., 1997; Muneoka et al., 1999). Dopamine is suggested to be involved in anxiety-like behaviors and is involved in the regulation of emotion, therefore, this hypofrontality is also linked to the pathology of anxiety and depression (Muneoka et al., 1999; Zarrindast and Khakpai, 2015). Our D42 IF findings are consistent with previous reports of decreased D1R expression following nicotine exposure. Unlike previous preclinical studies that have failed to detect differences in D2R following nicotine exposure, we also report significant decreases in D2R at D42. Our qPCR data also revealed that reductions in *D1R* persist until D180, while IF demonstrated an increase in D1R protein expression. This may suggest that D1R is more vulnerable long-term to the effects of nicotine in comparison to D2R and altered dopaminergic signaling persists past the point of initial exposure. Given that various neurotransmitter systems, including acetylcholine and dopamine, occupy trophic roles in the development of the central nervous system, it is important to characterize when these receptors are susceptible to manipulation in the organoid model and in turn, how they could evoke long-term neurodevelopmental consequences for the offspring (Wickström, 2007).

In tandem with dopaminergic alterations, other cortical biomarkers of MDD resulting from excess cholinergic signaling are hyperglutamatergia and decreased GABAergic signaling, which subsequently disrupts E/I balance (Dwyer et al., 2009; Fogaça and Duman, 2019; Hashimoto, 2009; Livingstone et al., 2010; Martin et al., 2020; Nobis et al., 2020). Increased glutamate levels have been reported in *postmortem* cortical tissue of individuals with MDD, suggesting aberrant glutamatergic transmission underlying features of MDD (Hashimoto, 2009). In recent years, animal studies have reported antidepressant effects resulting from ketamine, an N-methyl-D-aspartate antagonist, in reducing immobility time in the forced swim test and shock-induced behavioral changes (Chaturvedi et al., 1999; Hashimoto, 2009; Yilmaz et al., 2002). Notably, clinical studies have demonstrated the antidepressant effect of ketamine, in treatment resistant MDD, and are investigating other glutamatergic receptors (e.g., α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, mGLURs) as additional therapeutic targets (Hashimoto, 2009; Zarate et al., 2006). In terms of reduced inhibitory synaptic transmission, marked reductions in GABA synthesis enzymes and decreased size and density of GABAergic interneurons have been found in the dorsolateral PFC of depressed individuals (Fogaça and Duman, 2019; Karolewicz et al., 2010). Likewise, a mouse model of PNE confirmed a shift towards excitation in the E/I balance, denoted by a dose-dependent decrease in cortical GABAergic neurons (Martin et al., 2020). Similar findings of reduced GABA and receptor functioning have been described in preclinical stress models (Sanacora et al., 1999). Our results are consistent with previously reported GABA and glutamate dysfunction in MDD. Our IF analysis exhibited GABAergic deficits, specifically decreased GAT-1, PV and GAD67 at D42 and increased expression of glutamatergic markers NR2B and mGLUR2/3 at D180. Our D180 qPCR results also revealed alterations in *GRM2* and *GAD1*, indicating that long-lasting changes are occurring at the level of gene transcription. Nonetheless, to strengthen the causal link between nicotine-related errors in E/I neurotransmission and phenotypes of mood and anxiety disorders, future studies are required to explore the electrophysiological impacts on neuronal activity states within nicotine-exposed cerebral organoids, to fully understand the impacts of these molecular alterations on neuronal activity states.

Finally, research has shown that PNE significantly impacts aspects of nervous system development such as the generation, proliferation, differentiation, and migration of neurons (Mizrak, 2019; Wang et al., 2018). This has been documented in RNA-Seq analysis of *postmortem* PFC tissue from fetuses of smoking mothers which revealed increased expression of genes involved in neurodevelopment (Semick et al., 2020; Sherafat et al., 2021). This exposure to nicotine underlies changes in various neurotrophic factors, such as brain-derived neurotrophic factor and NGF that are essential for the growth and survival of neurons (Lauterstein et al., 2016). In turn, these modifications have the capacity to influence the human genome and epigenome, which may increase the occurrence of MDD, and suggests a genetic overlap between nicotine exposure and mood disorders (Dome et al., 2010; Lauterstein et al., 2016). For example, there is evolving evidence that suggests abnormal transcriptional regulation is a crucial component of mood disorders (Hobara et al., 2010). Mainly, a theory surrounding the evolution of MDD is that chronic stress induces alterations in the transcriptional regulation of growth factors, which leads to impaired neurogenesis (Malki et al., 2015). Similar findings were reported in human *postmortem* brain tissue where significant DEGs were enriched in pathways relating to neurodevelopment such as NGF, neurotrophin, and integrin signaling (Yoshino et al., 2021). There were also significant genes in specific function and disease pathways such as psychological disorders and nervous system development (Yoshino et al., 2021). Furthermore, repeated nicotine exposure can exert various epigenetic modifications such as the activity of nicotine-responsive transcription factors and inhibition of histone deacetylases (HDACs), which greatly modify gene expression (Volkow, 2011). Changes in HDACs are also seen in MDD. For instance, compared to nonpsychiatric controls, Hobara et al., 2010 reported decreased expression of HDAC9 mRNA in patients with mood disorders. This further associates transcriptional alterations as a focal point within mood disorders. Our RNA-seq results are consistent with previous findings in transcriptional studies of PNE and MDD, with many of our GO BP terms representing nervous system development, neurogenesis, and regulation of developmental/transcriptional processes. As for GO MF, all the terms were related to transcription factor activity, DNA, integrin or signaling receptor binding. This provides a better understanding of how nicotine influences MFs that are also altered in mood and anxiety disorders. We also reported five overlapping DEGs shared between nicotine, anxiety and MDD phenotypes: *SLC6A3, SPP1, NGFR, HDAC9* and *IGF2*. These genes were altered following nicotine exposure but also have a role in neurodevelopment, mood and anxiety disorders or closely interact with genes related to these phenotypes (Fan et al., 2020; Hobara et al., 2010; Lauterstein et al., 2016; Luo et al., 2015; Rafikova et al., 2021). Comprehensively, our results strengthen the genetic association between neurodevelopmental and transcriptional abnormalities resulting from PNE and the basis of mood and anxiety molecular

endophenotypes. Future efforts are required to fully validate and characterize novel DE transcripts, their underlying function in the cortical transcriptome and their role in neuropsychiatric disorders.

Conclusion

Using cerebral organoids, the present study aimed to validate a novel application of a human-derived *in vitro* model, to better comprehend the emergence of neurodevelopmental abnormalities and the manifestation of neuropsychiatric molecular endophenotypes resulting from chronic PNE. The advent of iPSC technology coupled with molecular analyses provided a framework to examine long-lasting alterations in fetal neurodevelopment, modifications in receptors vital to mood and anxiety pathophysiology and changes to the cortical transcriptome. Understanding how environmental drug exposure during pregnancy alters early cortical development and the resulting changes in biomarkers may raise awareness to the dangers of electronic nicotine delivery systems and provide a basis for the etiology of mood and anxiety disorders in human-based models. In the future, this will provide a platform for patient-specific treatments and finding appropriate and efficacious interventions to improve the outcomes of the offspring, who without choice, struggle with these neuropsychiatric disorders long-term.

Data availability statement

The datasets generated for this study can be found in the National Library of Medicine, Accession: PRJNA1137246; ID: 1137246: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1137246/.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

EP: Data curation, Formal Analysis, Investigation, Writing–original draft, Writing–review and editing. MR-R: Conceptualization, Investigation, Methodology, Supervision, Writing–review and editing. DG: Data curation, Formal Analysis, Investigation, Writing–review and editing. SV: Data curation, Formal Analysis, Writing–review and editing. DH: Conceptualization, Methodology, Supervision, Writing–review and editing. WR: Conceptualization, Data curation, Formal Analysis, Investigation, Resources, Writing–review and editing. SL: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Glossary

ANOVA	Analysis of variance
BP	Biological process
CCasp3	Cleaved caspase 3
CDH13	Cadherin-13
cDNA	Complementary DNA
CTIP-2	COUP-TF-interacting protein 2
Cq	Cycle quantification
DEGs	Differentially expressed genes
DEPC	Diethyl pyrocarbonate
D1R	Dopamine 1 receptor
D2R	Dopamine 2 receptor
D42	Day 42
D180	Day 180
EB	Embryoid body
E/I	Excitatory/inhibitory
EOMES	Eomesodermin
FDR	False discovery rate
FGFR1	Fibroblast growth factor receptor 1
FOXG1	Forkhead-box G1
FZD9	Frizzled-9
GABA	γ-aminobutyric acid
GAD1	Glutamate decarboxylase 1
GAD67	Glutamic Acid Decarboxylase 67
GAT-1	GABA transporter type 1
GO	Gene ontology
GRM2	Metabotropic glutamate receptor 2
HDAC	Histone deacetylase
IF	Immunofluorescence
iPSCs	Induced pluripotent stem cells
ISL1	ISL LIM homeobox 1
Ki67	Antigen Kiel 67
LSD	Least squared difference
MAP2	Microtubule-associated protein 2
MDD	Major depressive disorder
MF	Molecular function
mGLUR2/3	Metabotropic glutamate receptor 2/3
nAchRs	Nicotinic acetylcholine receptors
NGF	Nerve growth factor
NR2B	NMDA receptor subunit 2B
PBS-T	Phosphate buffered saline with Tween 20
PFC	Prefrontal cortex
PNE	Prenatal nicotine exposure
PROX1	Prospero homeobox 1 protein
PV	Parvalbumin
qPCR	Real-time quantitative polymerase chain reaction
RNA-Seq	Ribonucleic acid sequencing/RNA sequencing
ROI	Region of interest
VEH	Vehicle

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EDITED BY
Nicholas M. Barnes,
University of Birmingham, United Kingdom

REVIEWED BY
Qihai Gu,
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Kabirullah Lutfy,
Western University of Health Sciences, United States
Intan Suhana Munira Mat Azmi,
Sultan Zainal Abidin University, Malaysia

*CORRESPONDENCE
Jacob Raber,
raberj@ohsu.edu
Glen Kisby,
gkisby@westernu.edu

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World no-tobacco: effects of second-hand smoke (SHS) and vapors on the developing and adult brain

Glen Kisby[1]* and Jacob Raber[1,2,3]*

[1]Department of Biomedical Sciences, College of Osteopathic Medicine of the Pacific Northwest, Western University of Health Sciences, Lebanon, OR, United States, [2]Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR, United States, [3]Departments of Behavioral Neuroscience, Neurology, Psychiatry, and Radiation Medicine, Division of Neuroscience ONPRC, Oregon Health and Science University, Portland, OR, United States

The goal of this review is to highlight the role of second-hand smoke (SHS) or environmental tobacco smoke (ETS) and e-cigarette (EC) vapors on brain integrity and function during development and adulthood, including how it relates to increasing the risk for age-related neurodegenerative disorders. A systematic review of the literature of the effect of SHS or ETS and e-cigarette vapors on the brain revealed a total of 284 or 372 publications and 312 publications, respectively. After taking into account duplicate publications or publications focused on policy, surveys or other organs than brain, there are limited studies on the effects of SHS, ETS or EC vapors on brain structure and function. In this review, we examine the major constituents in SHS or EC vapors and their effects on brain health, mechanisms by which SHS or vapors alters brain integrity and function, including behavioral and cognitive performance. We hope that this review will encourage investigators to explore further the short-as well long-term effects of SHS or vapor exposure on the developing and adult brain to better understand its role in neurodevelopmental disorders and neurodegenerative diseases and ultimately to develop therapeutic modalities to reduce or even prevent the short- and long-term detrimental effects on brain health.

KEYWORDS

environmental tobacco smoke (ETS), nicotine, oxidative stress, e-cigarettes, DNA damage/repair

1 Introduction

Please see Figure 1 for the Prisma statement.

Tobacco use is one of the leading risk factors for disease burden and mortality worldwide, contributing to 229.8 million (95% uncertainty interval: 213.1–246.4 million) disability-adjusted life years and 8.7 million (8.1–9.3 million) deaths in 2019 (Flor et al., 2024). Second-hand smoke (SHS) exposure, also referred to as passive or environmental tobacco smoke (ETS), is a major tobacco-related public health concern for nonsmokers. SHS increases the risk of nine health outcomes, including ischemic heart disease stroke, diabetes and lung cancer, but the effects on the nervous system have not been extensively examined (Flor et al., 2024). Although smoking rates have gradually declined over the past 50 years, ~37% of the global population is still being exposed to the smoke emitted from the burning end of tobacco products (sidestream smoke, SS)

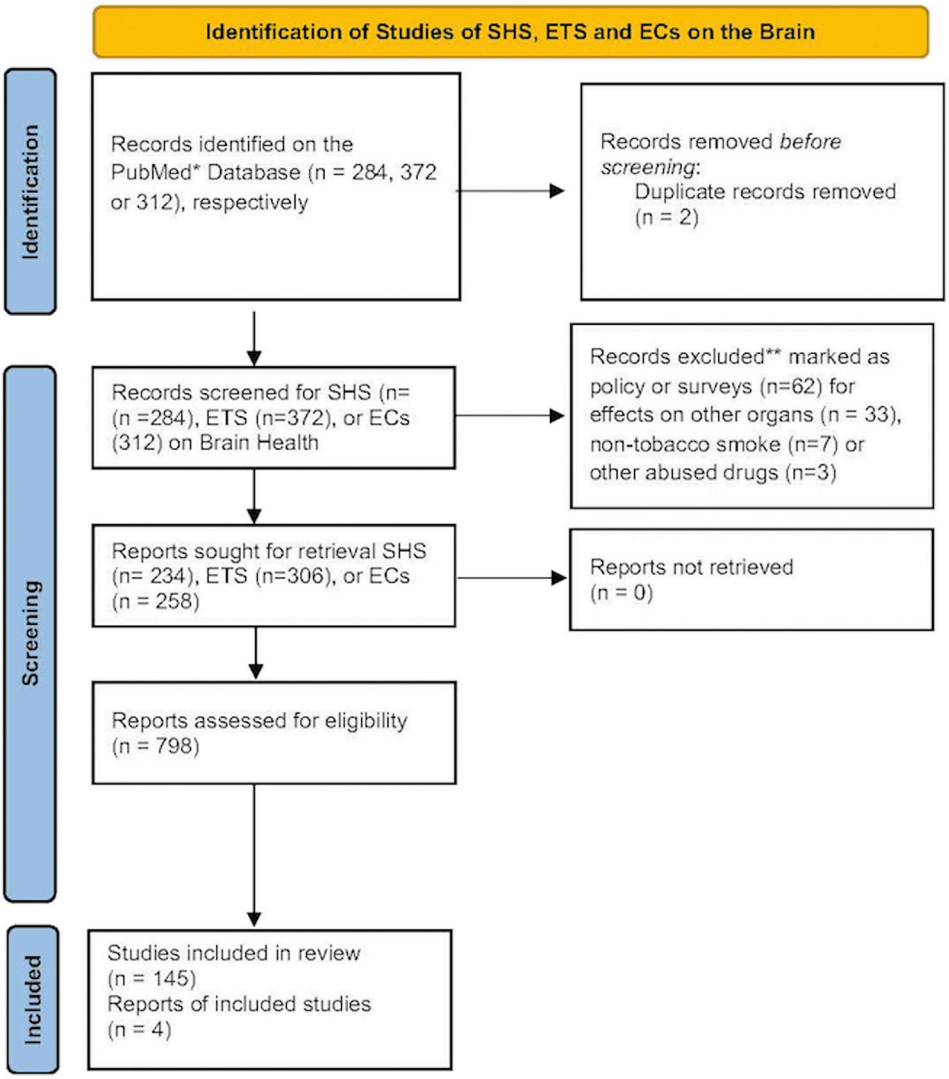

FIGURE 1
The number of records identified from the PubMed database was searched using the terms SHS, ETS or EC and brain. Records that were excluded from this review were those focusing primarily on policy or surveys, effects on non-nervous tissues, non-tobacco smoke or from other abused drugs. These reduced the number of records for each term that focused primarily on brain health. A document from the CDC on a visual dictionary of electronic cigarettes or vaping was also included in this review (Centers for Disease Control and Prevention, 2024).

or exhaled from smokers (mainstream smoke, MS), with higher rates of exposure reported among women and children compared to men (Flor et al., 2024). This is concerning, since tobacco smoke is composed of thousands of toxic chemicals and compounds, including many carcinogens, which when inhaled can lead to disease and death, especially among vulnerable populations.

SHS or ETS consists of sidestream (~85%) and mainstream smoke (~15%) (Soleimani et al., 2022). Mainstream smoke is exhaled from a smoker, while sidestream smoke is smoke emitted from a burning cigarette. Indoors, SHS can persist for hours to become more toxic with time and duration, a process known as aging (Schick and Glantz, 2005; Schick and Glantz, 2007). When second-hand smoke is released into the open air, it changes both chemically and physically. Human exposure to SHS depends on airflow patterns, dilution volume, the distance between smokers and non-smokers, and smoking prevalence (Public Health Service, 2006). The toxic chemicals in SHS can also react with atmospheric air to generate other toxins that can be inhaled (Centers for Disease Control and Prevention, 2010; Fu et al., 2012). SHS contains >7,000 chemicals with at least 70 of them being carcinogenic (Li and Hecht, 2022). Nicotine, polycyclic aromatic hydrocarbons (PAHs) and aldehydes (formaldehyde, acetaldehyde and propionaldehyde) are the most abundant chemical constituents commonly found in SHS. SHS also contains smaller amounts of metals and nitrosamines (Soleimani et al., 2022). However, aldehyde concentrations in SHS can exceed those of nicotine, depending upon exposure conditions (*e.g.*, indoor vs outdoor places). PAHs, formaldehyde, and acetaldehyde all have the potential to damage DNA (genotoxins) associated with neurodevelopmental and neurological disorders (Perera et al., 2014; Joshi et al., 2019; Rana et al., 2021; Kou et al., 2022; Yuan et al., 2023). These genotoxic effects might also vary among different

ethnic populations. For example, recent studies indicate that a mutation of the enzyme that metabolizes formaldehyde and acetaldehyde (aldehyde dehydrogenase 2, ALDH2) is highly prevalent (30%–50%) among the East Asian population (Chen et al., 2015; Wang X. et al., 2024), which can increase the susceptibility to second-hand exposure to toxins (Ma et al., 2024).

Electronic cigarettes (e.g., e-cigarettes, ECs, vape pens, etc.) or electronic nicotine delivery systems (ENDS) contain a liquid solution ("pods") composed of various amounts of nicotine, flavoring substances, and other chemicals that is vaporized upon activation of an electronic heating element that is triggered by inhalation (Grana et al., 2014; Lopez-Ojeda and Hurley, 2024). Using ECs, is commonly known as 'vaping. Details about the different types of ECs, their components and aerosols can be found in recent reviews by the Center for Disease Control (Centers for Disease Control and Prevention, 2024) and other investigators (Lopez-Ojeda and Hurley, 2024; Omaiye et al., 2022; Heywood et al., 2024). ECs have been promoted as containing fewer toxic chemicals than conventional cigarettes suggesting that these type of cigarettes are less harmful (Heywood et al., 2024; Hamann et al., 2023; Izquierdo-Condoy et al., 2024). However, the exhaled vapors from e-cigarettes also contains toxic chemicals and carcinogens (*e.g.*, acrolein, benzene, diacetyl, formaldehyde) following second-hand exposure of individuals (Lopez-Ojeda and Hurley, 2024; Armendariz-Castillo et al., 2019; Yan et al., 2021). These findings suggest that e-cigarettes may be less harmful for smokers, but they are not safe for non-smokers (Izquierdo-Condoy et al., 2024). While there are many studies examining the neurological effects of e-cig vapors in humans (Hamann et al., 2023), there have been limited studies to investigate the direct effects of e-cigarette vapors on brain function and structure using animal models (Siegel et al., 2022; Ruszkiewicz et al., 2020). The vapors from e-cigarettes also contain significant amounts of nicotine, as well as other constituents found in SHS, but at lower concentrations (Ebersole et al., 2020) (Table 1). Formaldehyde can also be formed during the partial combustion of propylene glycol and glycerol liquids in e-cigarettes (Strongin et al., 2024), which reaches more deeply into the lungs than gaseous formaldehyde (Pankow, 2017).

Exposure to second-hand smoke (SHS) at different times during brain development (fetal, infant, and adolescence) can produce short- or long-term effects on brain structure and function leading to neurodevelopmental disorders (NDDs) (Slotkin et al., 2015; Lin et al., 2021; Ou et al., 2024). Children exposed from pregnancy to childhood have a higher risk of developing Attention Deficit Hyperactivity Disorder (ADHD) during school-aged years and this risk is somewhat stronger for SHS exposure during the prenatal and postnatal periods (Lin et al., 2021). A more recent study of SHS exposure and neurodevelopmental disorders also revealed that exposure is associated with higher risk of ADHD and other learning disabilities (Ou et al., 2024). Collectively, these studies demonstrate that SHS can induce short- and long-term structural effects on the developing brain to cause permanent functional changes. The contribution of individual toxins in cigarette on both brain development and function appears to be greater when they are combined (Slotkin et al., 2019). Combined exposure of pregnant rats to both a PAH (*i.e.*, benzo [a]pyrene) and nicotine impairs acetylcholine presynaptic activity and upregulates acetylcholine and serotonin receptors in adolescent rats when compared with exposure to either agent alone. These studies demonstrate that exposure to combinations of SHS chemicals is more detrimental to the developing brain than single exposure to SHS chemicals. Since SHS contains more than 4,000 chemicals (Arfaeinia et al., 2023), two or more of them may be more detrimental to the developing brain, but studies assessing combinations of SHS chemicals have yet to been conducted.

2 Pharmacology and pathology of nicotine in the brain

Nicotine is one of the most abundant toxins in SHS (mg to μg quantities), next to aldehydes and polycyclic aromatic hydrocarbons (Arfaeinia et al., 2023), and is also produced after the heating of tobacco products (*e.g.*, e-cigarettes) (Upadhyay et al., 2023). EC cigarette pods contain approximately 59.2–66.7 mg/mL of nicotine, which is comparable to one pack of 20 conventional cigarettes (Lopez-Ojeda and Hurley, 2024). Nicotine levels in e-cigarette aerosols can range anywhere from 0–50 mg/mL of liquid (Yan et al., 2021; Goniewicz et al., 2018; Prochaska et al., 2022) and are reportedly lower following exposure of individuals to vapors vs SHS (Tattan-Birch et al., 2024). This difference may be explained by the 99% retention of nicotine by vapors following inhalation (Czogala et al., 2014; St Helen et al., 2016). SHS inhaled from conventional cigarettes or e-cigarette vapors is absorbed into the pulmonary circulation where it binds to neuronal nicotinic acetylcholine receptors (nAChRs) that mediate fast neurotransmission in both the central and peripheral nervous system (Wells and Lotfipour, 2023). The inhaled nicotine causes the release of multiple neurotransmitters (*e.g.*, dopamine, norepinepinephrine, acetylcholine, GABA and glutamate) in the reward/addiction pathways and involved in cognition, as well as activation of nicotinic receptors at the neuromuscular junction (Figure 2). Cholinergic receptors are located in several brain regions notably the midbrain tegmentum, the striatum, nucleus accumbens and the ventral tegmentum (VTA). The addictive properties of nicotine are reportedly due to activation of nACHRs in the brain to cause the release acetylcholine and dopamine in the nucleus acccumbens (Tiwari et al., 2020). GABAergic, serotonergic, noradrenergic, and brain stem cholinergic may also mediate the actions of nicotine on the brain. The addictive properties of nicotine may also be related to its activation of both dopaminergic neurons of VTA as well the GABA-ergic neurons (Varani et al., 2018). The activation of nicotinic receptors at the neuromuscular junction by SHS can also cause degeneration, consistent with a key role for smoke exposure causing denervation in patients with chronic pulmonary disease (Kapchinsky et al., 2018). In the CNS, nicotine modulates the reward/addiction pathways and cognition through activation of nAChRs in the mesocortical and mesolimbic dopaminergic (DA) pathways (Ikemoto and Bonci, 2014). The rewarding and cognitive effects of nicotine are mediated through the activation of mesocortical DA receptors in the prefrontal cortex and anterior cingulate cortex, while the activation of DA receptors by nicotine in the nucleus accumbens and amygdala modulate synaptic plasticity and long-term potentiation that are more important in addiction.

Inhaled nicotine can also have long-term effects on brain development, because nicotinic acetylcholine receptors (nAChRs)

TABLE 1 Chemical components found in SHS from Conventional and Electronic cigarettes.

Chemical	[a]Conventional	[b]Electronic (E-cigs)
Nicotine[c]	0.85–100 μg/m^3 (6.80 μg/m^3)	0–50 mg/mL
Formaldehyde	49 μg/m^3	0–11 μg/10 puffs
Acetaldehyde	1,390 μg/m^3	0–4.5 μg/10 puffs (341 μg/m^3)
Propionaldehyde	120 μg/m^3	ND (87 μg/m^3)
Acrolein	2.3–275 μg/m^3	0–1.0 μg/10 puffs
AA	43.43–155.11 ng/m^3	147 ng/m^3
Metals (Cd, Cr, Ni)	0.03–0.01 μg/m^3 0.0012–0.009 μg/m^3 0.0025–0.007 μg/m^3	ND 0.0846 mg/m^3 0.04 mg/m^3
Nitrosoamines (NNN, NNK, NDMA, NPYR)	ND – 0.006 μg/m^3 ND – 0.0135 μg/m^3 0.008–0.045 μg/m^3 0.0025–0.007 μg/m^3	ND – 0.06 ng/g ND – 0.06 ng/g Not determined Not determined

[a]Adapted from Arfaeinia et al. (2023).
[b]Nicotine varied depending on the source of exposure (i.e., indoor air, restaurants, bars or discotheques). Mean concentrations in parentheses were from Soleimani et al. (2022).
[c]Adapted from multiple sources (Goniewicz et al., 2018; Vivarelli et al., 2024; Farsalinos and Gillman, 2017; Schober et al., 2014; Olmedo et al., 2018; Quintana et al., 2021). ND, below detection limits.

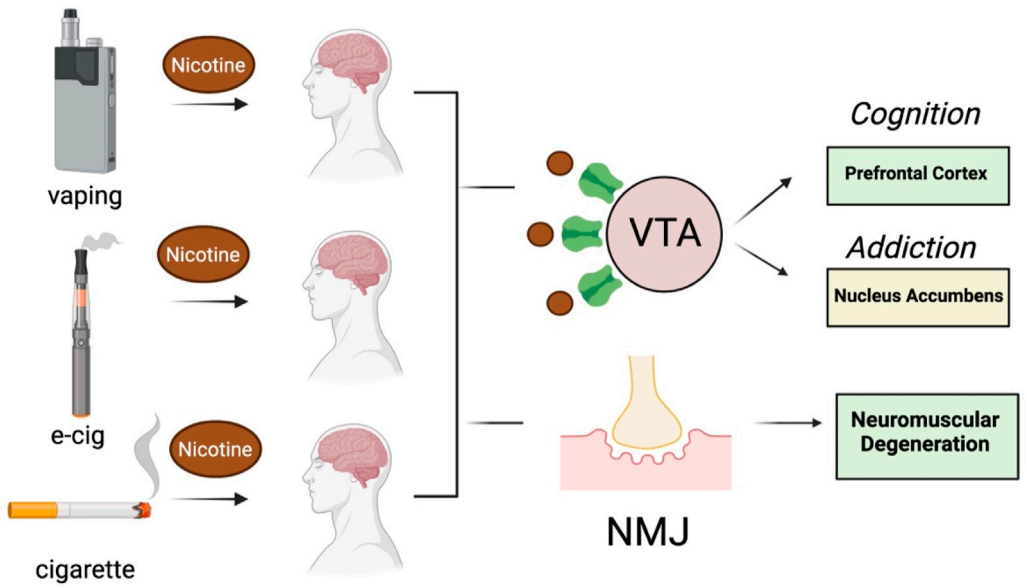

FIGURE 2
Effect of nicotine on brain and neuromuscular function following exposure to vapors or SHS generated by e-cigarettes and conventional cigarettes (respectively). VTA, ventral tegmentum area, NMJ, neuromuscular junction. Images were created with BioRender.

play a very important role in modulating the release of neurotransmitters during key stages of neurodevelopment (England et al., 2017). Nicotinic receptors regulate critical aspects of brain maturation during the prenatal, early postnatal, and adolescent periods (Dwyer et al., 2009). Nicotine interferes with catecholamine and brainstem autonomic nuclei development during the rodent prenatal period (first and second trimester in humans), alters the neocortex, hippocampus, and cerebellum during the early rodent postnatal period (third trimester in humans) and influences the limbic system and later monoamine-containing neuron maturation during adolescence (Dwyer et al., 2009). SHS exposure during fetal (prenatal) or neonatal (postnatal) brain development can also produce long-term effects on the developing brain to disrupt brain plasticity and overall brain structure (*e.g.*, volume, thinning) to lead to neurodevelopmental disorders (*e.g.*, ADHD, schizophrenia, ASD, and anxiety) (Ou et al., 2024; Herrmann et al., 2008; Colyer-Patel et al., 2023; Greenwood et al., 2024).

Prenatal exposure to nicotine from tobacco products might also produce neurodevelopmental delay through epigenetic changes (Buck et al., 2020; Gould, 2023; Hoang et al., 2024). DNA methylation changes are observed in mothers who are exposed to

cigarette smoke during pregnancy (Markunas et al., 2014). Markunas and colleagues (Markunas et al., 2014) showed that DNA methylation is changed in 110 gene regions and notably *FRMD4A*, a gene associated with Alzheimer disease (AD) and nicotine dependence and *CNTNAP2*, a gene associates with neural development, autism spectrum disorder, schizophrenia, and language impairment. Another study (Rauschert et al., 2019) also revealed that tobacco use is associated with differential DNA methylation of both *FRMD4A* and *CNTNAP2*. More recently, Hoang and colleagues (Hoang et al., 2024) reported that *in utero* exposure to environmental tobacco smoke (ETS) alters DNA methylation of both *CNTNAP2* and *FRMD4a* that might persist into adulthood. Collectively, these studies provide evidence that prenatal exposure to nicotine in tobacco smoke can alter brain processes involved in neural development, age-related neurodegenerative conditions like Alzheimer's disease, and addiction.

Neurotrophins, like nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), play important roles in neuronal development, function, and survival during early stages of both the central and peripheral nervous system (Ferraguti et al., 2023). In general, the brain peptide system plays an important role in nicotine addiction and drugs that target this system prevent the activation of reward systems (Bruijnzeel, 2017; Boiangiu et al., 2023). Exposure of the fetus to SHS during pregnancy appears to alter the expression of neuropeptides (BDNF, PCAP) by the region-specific activation of nicotinic receptors (Machaalani et al., 2019). Nicotine also increases brain-derived neurotrophic factor (BDNF) levels in the hippocampus and neocortex. Mice exposed to environmental tobacco smoke (ETS) during the first two postnatal weeks show lower locomotor activity, anxiety-like behavior and corresponding reduced levels of synaptic proteins and BDNF in the cerebellum, striatum and prefrontal cortex (Torres et al., 2018). Exposure to tobacco smoke during various periods of brain development also alters synaptic and neurotrophin levels in different brain regions that appear to last long after early life exposure to SHS.

3 Pharmacology and pathology of other SHS chemicals on the brain

As discussed above, smoke from cigarettes or e-cigarettes contains many chemicals at levels comparable to nicotine (*see* Table 1). SHS also contains aldehydes and polycyclic aromatic hydrocarbons (PAHs), as well as lower concentrations of other constituents (Arfaeinia et al., 2023), which have also been detected in e-cigarette vapors or even after the heating (*versus* combustion) of tobacco products (HTP) (Upadhyay et al., 2023; Vivarelli et al., 2022). HTPs are a newer category of tobacco products that generate nicotine and other chemicals, but at lower amounts than conventional cigarettes. Sufficient evidence supports that prenatal PAH exposure negatively impacts cognitive development with specific regard to child intelligence (Humphreys and Valdes Hernandez, 2022). PAH during childhood and as an adult also was associated with an increase in biomarkers of neuroinflammation found in neurodegenerative diseases like AD and PD (Humphreys and Valdes Hernandez, 2022). There are ethnicity differences in SHS exposure that need to be considered as well. Smoking and SHS exposure accounts for the largest exposure to PAHs of non-Hispanic Whites vs other age-matched ethnic groups as well as older age groups (Gearhart-Serna et al., 2021). This large study indicates that there are vulnerable subpopulations with high PAH intake as a result of different smoking behaviors and potentially other exposures. Prenatal exposure to PAHs might alter mitochondrial copy number as a mechanism to explain its ability to impair neurodevelopment in children (Cao et al., 2020). Cord blood levels of benzo [a]pyrene DNA adducts (marker of PAH exposure) are inversely associated with the development of infants born to pregnant mothers who are exposed to PAHs from a Chinese coal burning power plant (Kalia et al., 2017). Benzo [a]pyrene DNA adducts levels are also negatively associated with BDNF levels in cord blood, suggesting that PAHs might cause neurodevelopmental delay through a DNA damage-mediated mechanism. The PAH concentration in cord blood after exposure to SHS would be expected to be much lower than after exposure of mothers to PAHs from a coal-burning plant.

Reactive aldehydes like acetaldehyde, acrolein and formaldehyde are formed during the combustion of tobacco products (Tulen et al., 2022). Acetaldehyde induces cytotoxicity by disrupting mitochondrial function to cause oxidative stress in neural cells (Yan et al., 2022), while acrolein causes DNA damage and oxidative stress in non-neural cells (Bellamri et al., 2022; Hikisz and Jacenik, 2023) and is elevated in the brain of patients with neurodegenerative diseases (Chang et al., 2022). Acrolein and formaldehyde induce Alzheimer-like disease pathology when administered to rodents (Liu et al., 2018; Chen et al., 2022) or primates (Zhai et al., 2018). Adult male rats treated for 3 months with acrolein show neurobehavioral alterations and cognitive impairments that are associated with electrophysiological disturbances (Khoramjouy et al., 2021). Endogenous acrolein plays a significant role in the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease (Chang et al., 2022), possibly through inducing oxidative stress reducing brain antioxidant levels and activating the MAPK pathway resulting in the hyperphosphorylation of tau and increasing amyloid-β levels, both biomarkers of neuropathology (Dhapola et al., 2023; Jallow et al., 2024). Thus, environmental exposure of humans to tobacco smoke and endogenous antioxidant levels could be important risk factors for the developing as well as adult brain (Chang et al., 2022) (Figure 3).

Formaldehyde (FA) is as environmental contaminant with toxic potential that also serves as an indispensable and thus normal physiological metabolite in the healthy brain, where it is hypothesized to regulate learning and memory via the *N*-methyl-D-aspartate receptor (Ai et al., 2019). FA is also a product of various metabolic pathways that participate in the one-carbon cycle, which provides carbon for the synthesis and modification of bio-compounds, such as DNA, RNA, and amino acids (Li et al., 2021). Endogenous FA plays a role in epigenetic regulation by regulating the methylation and demethylation of DNA, histones, and RNA (Li et al., 2021). At high levels, FA can pose a significant threat to genomic stability (Reingruber and Pontel, 2018), DNA repair (Nadalutti et al., 2021; Weng et al., 2018; Tang et al., 2022) and impede transcription, with negative physiological consequences (Mulderrig et al., 2021), through epigenetic alteration (Li et al., 2021), including neuronal and endothelial damage, (Chen et al.,

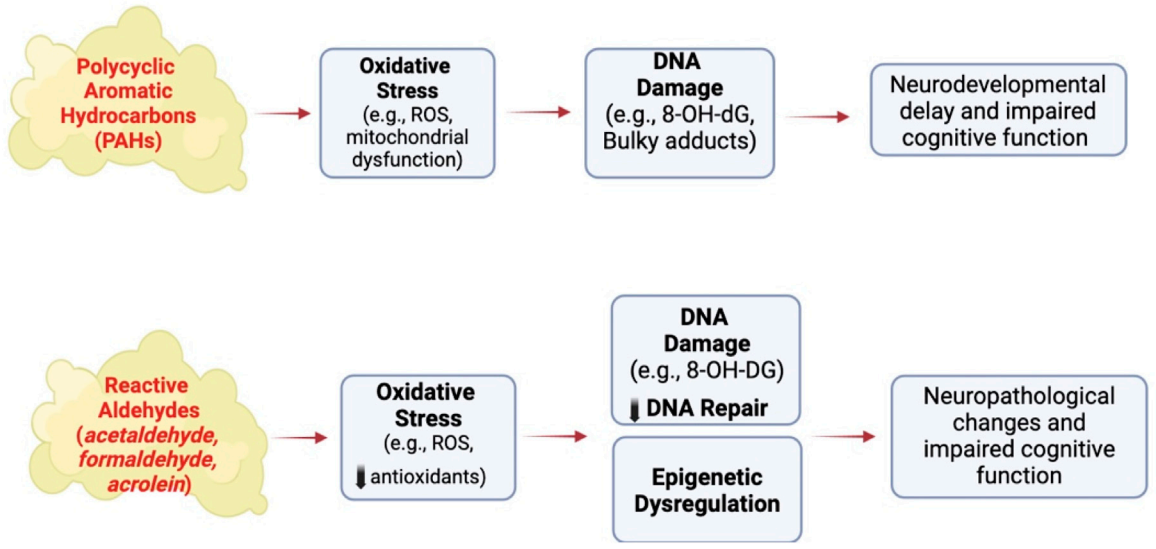

FIGURE 3
Effect of other SHS chemicals on brain structure and function following exposure to conventional cigarettes. ROS, reactive oxygen species, 8-OH-dG, 8-hydroxy-2′-deoxyguaonsine,. Images were created using BioRender software.

2024). Notably, impaired memory is observed in mice with elevated endogenous FA, induced by knock-out of the gene coding for aldehyde dehydrogenase-2, a key mitochondrial enzyme for the effective metabolism of alcohol and acetaldehyde (Ai et al., 2019). Exposure to high levels of FA by inhalation (3.0 mg/m^3) impairs cognitive function, including memory, in humans, causing neuronal damage and oxidative stress in the cerebellum of experimental animals, and inducing the misfolding of neuronal tau and related proteins *in vitro* (Rana et al., 2021). The balance between genotoxins and benign metabolites is presumed to depend on concentration, localization, pH and redox state, features that are or potentially altered during disease progression (Hopkinson and Schofield, 2018).

4 Pathway changes associated with cellular damage and inflammation in brain

A systematic review of the effects of active and passive smoking of conventional cigarettes, electronic cigarettes and tobacco heating products indicated that active and passive smoking induce oxidative stress and inflammatory responses in peripheral tissues (Kopa-Stojak and Pawliczak, 2024; Zieba et al., 2024), but the nervous system was not examined (Kopa-Stojak and Pawliczak, 2024). Oxidative stress-induced DNA damage and inflammation are also emerging as key triggers of dementia and related neurological disorders (Houldsworth, 2024; Giri et al., 2024; Neven et al., 2024; Firdous et al., 2024), as well as neurodevelopmental disorders (Qing et al., 2023; Lubrano et al., 2024; Xu et al., 2024). Since 90% of neurodegenerative diseases (*e.g.*, MCI, dementia) are sporadic, this suggests that environmental factors like tobacco smoke might play an important, but undefined, role in their etiology (Ourry et al., 2024; Pandics et al., 2023). Early life exposure to SHS may also be an important risk factor for dementia (Chen, 2012; Zhou and Wang, 2021; Wan et al., 2024), as well as neurodevelopmental disorders (Hall et al., 2016; Julvez et al., 2021; Mukhopadhyay et al., 2010; Wade et al., 2023). Studies with animal models suggest that the effect of SHS on the brain may be due to increased oxidative stress and inflammation during brain development, leading to increased brain cell apoptosis in adulthood (Vivarelli et al., 2024; Raber et al., 2021; Raber et al., 2023; Lopes et al., 2023). Short-term exposure of 2-month-old mice (6h/day x 5 days/wk x four or 8 weeks) to a mixture of sidestream/mainstream cigarette smoke impairs brain insulin signaling and induces the accumulation of neuropathological proteins (Deochand et al., 2015; Deochand et al., 2016). Shorter durations of mainstream/sidestream smoke (1h/day x 1 month) induces lipid peroxides, DNA damage, and tau dysregulation (tau isomers, phosphotau) in the brain of neonatal mice (La Maestra et al., 2011), markers of neuropathology frequently observed in MCI and patients with dementia (Lovell and Markesbery, 2007; Simpson et al., 2016; Wirz et al., 2014). Longer exposures of 2-month-old rats or 3-month-old APP/PS1 transgenic mice to sidestream cigarette smoke (1h/day x 5 days/wk x two or 4 months) induces tau and amyloid pathology like that reported in MCI and patients with dementia (Ho et al., 2012; Moreno-Gonzalez et al., 2013). These studies strongly suggest that SHS increases the risk of developing MCI and dementia by perturbing brain metabolism (*i.e.*, insulin signaling, oxidative stress) and the accumulation of neuropathological proteins (*i.e.*, tau, amyloid). SHS induces a distinct brain metabolic profile characterized by oxidative stress (Raber et al., 2021; Raber et al., 2023; Neal et al., 2016) and inflammation (Lopes et al., 2023; D et al., 2019; Chan et al., 2020). The cortex and hippocampus in the brains from the offspring of female C57BL/6 mice exposed to air or SHS (50 μg/m^3; 5h/day, 5 days/week for 5 weeks and 2 days) were examined by untargeted metabolomics. Insulin signaling, which regulates an abundance of metabolic proteins, is altered in the hippocampus of the offspring exposed throughout development to SHS. An

increase in glutathione-S-transferase is also detected, and a trend towards increased glutathione reductase activity, increases GSSG, and a decreased GSH/GSSG ratio is observed. In a systematic review of the literature, it was also reported that cigarette smoking (active and passive) induces oxidative stress and an inflammatory response in peripheral (*i.e.*, non-neurological) tissues (Kopa-Stojak and Pawliczak, 2024; Kanithi et al., 2022; Prasad and Bondy, 2022; Mukharjee et al., 2020). Thus, exposure to SHS (passive smoking) on the brain and non-neurological tissues induces both oxidative stress and inflammation.

A multi-center study of six European countries that examined the relationship between early life environmental exposures and child cognitive function found that ETS exposures adversely and cross sectionally associate with cognitive function (Julvez et al., 2021). In human studies, maternal exposure to SHS is also closely linked to small brain size and changes in brain structure that associated with a higher risk of cognitive impairments (Colyer-Patel et al., 2023; Greenwood et al., 2024; Chan et al., 2020) and psychotic experiences (Wang D. et al., 2024). SHS also induces oxidative stress and inflammation in the developing brain (D et al., 2019; Chan et al., 2020; Lobo Torres et al., 2012; Mohamed et al., 2022; Church et al., 2020). Acute exposure to SHS on postnatal day 18 increased GST activity and malondialdehyde (MDA levels in the hippocampus, GPx and SOD activity in the prefrontal cortex and GST activity and MDA levels in the striatum and cerebellum of postnatal mice (Lobo Torres et al., 2012). Three hours later, SOD activity and MDA levels increased in the hippocampus and the activity of all enzymes decreased in the prefrontal cortex. This study shows that SHS induces oxidative stress by perturbing antioxidant enzymes in distinct brain regions during early brain development like that reported in older animals. Thus, oxidative stress appears to be an early event following exposure to ETS or SHS from tobacco products. Pregnant mice were exposed to e-cig vapor (2.4% nicotine) from GD five until postnatal day 7 (PD7) and the brain of mice at PD7 and PD 90 examined for reactive oxygen species (ROS) and pro-inflammatory cytokines (Archie et al., 2023). E-cig vapor reduced antioxidant marker expression and increased the expression of pro-inflammatory and cytokine markers in the PD7 brain, but not the PD 90 brain. Pregnant mice were also exposed daily to e-cigarette chemicals (propylene glycol, vegetable glycol) and 16 mg/mL of nicotine for 3 h/d, 7 days a week from gestational day (GD) 0.5 until GD 17.5 (Church et al., 2020). Male and female offspring of e-cigarette exposed mice had lower scores on the novel object recognition task and reduced inflammatory markers in the diencephalon (IL-4, IFNγ) and hippocampus (IFNγ; females only). This experimental study demonstrates that e-cigarette vapors can also persistently alter the neuroimmunology and behavior following maternal exposure. Thus, oxidative stress and inflammatory markers are also increased in the brain of mice after *in utero* exposure to SHS from both conventional cigarettes and e-cigarettes (Figure 4).

5 MCI, neurodegeneration, and other neurological disorders

As described earlier, there is growing evidence that non-smokers exposed to SHS are at an increased risk of developing MCI (Chen, 2012; Llewellyn et al., 2009; Yolton et al., 2005; Murphy et al., 2020; Akhtar et al., 2013; Cataldo et al., 2010; Chang et al., 2012) and dementia (Chen, 2012; Cataldo et al., 2010; Chang et al., 2012), as well as neurodevelopmental disorders (Ou et al., 2024; Chan et al., 2020; Pagani, 2014). Exposure to SHS increases the risk for dementia among individuals who never smoked (Zhou and Wang, 2021; Wan et al., 2024; He et al., 2020) and it is 2–6 times more toxic and tumorigenic to humans than mainstream smoke (Schick and Glantz, 2005; Akhtar et al., 2013). Cholinergic dysfunction of the nucleus basalis of Meynert (NBM) is hypothesized to be an important factor for the increased risk of AD (Slotkin et al., 2015; Slotkin et al., 2019; Toro et al., 2008). Chronic exposure to nicotine through smoking may lead to atrophy of cholinergic input areas of the basal forebrain. Chronic exposure to nicotine through smoking disrupts the functional connectivity between the NBM and precuneus in MCI patients (Qiu et al., 2022). The ability of cigarette smoke to disrupt the connectivity in both non-smokers and those with MCI suggests that exposure to cigarette smoke disrupts cognition. Yet, 6 months of transdermal nicotine administration (16 mg/day) to MCI patients improves primary and secondary cognitive measures of attention, memory, and mental processing (Heffernan and O'Neill, 2013). These studies demonstrate that exposure to nicotine through cigarette smoke can either disrupt cholinergic function or be protective. Early life exposure to nicotine and other SHS constituents might be the key to understanding how the toxins in cigarette smoke induce their long-term effects on learning and memory, executive function, and the reward circuitry (Hall et al., 2016; Cauley et al., 2018; Ponzoni et al., 2020).

Base excision repair (BER) is the primary cellular pathway for repairing oxidative DNA damage *(e.g.*, 8-oxo-deoxyguanosine, 8-oxodG) (Oka et al., 2021) that is reportedly impaired in both MCI individuals and those with AD (Chang et al., 2022; Cherbuin et al., 2024). Furthermore, the brain of dementia subjects also exhibits activation of the DNA damage response (DDR) pathway, inflammatory changes and cellular senescence (Schwab et al., 2021). Exposing mice for 4 months to active (La Maestra et al., 2011) or passive (Moreno-Gonzalez et al., 2013) cigarette smoke induces oxidative stress, DNA damage and neuropathology in mice. Chronic exposure of mice to SHS (90% side stream, 10% mainstream smoke x 2.8 h/day x 7 days/wk x 10.4 mos) induces dark, shrunken cells, hippocampal thinning, and the presence of activated astrocytes and prominent 8-oxoG staining in the prefrontal cortex (PFC) and hippocampus (HIPP) (Raber et al., 2021; Lopes et al., 2023). 8-oxoguanine DNA glycosylase (Ogg1) staining is also reduced in the PFC and CA3 hippocampal neurons of SHS chronically exposed mice. Apurinic/apyrimdinic endonuclease (Ape1) staining is more prominent in the PFC and the HIPP in SHS chronically exposed mice. These studies demonstrate that oxidative DNA damage (8-oxoG) is elevated and oxidative DNA repair (Ogg1 and Ape1) is altered in the brain of SHS exposed mice, as well as activation of reactive astrocytes. The percentage of 8-OHdG-labeled cells in the CA1 region of the hippocampus is associated with performance in the novel object recognition test, consistent with urine and serum levels of 8-OHdG serving as a biomarker of cognitive performance in humans. Therefore, SHS induces both oxidative DNA damage and repair, as well as inflammation as possible underlying mechanism(s) of the behavioral and cognitive function and metabolic changes that were observed in chronically exposed mice (Raber et al., 2021). These findings suggest that human exposure to cigarette smoke induces oxidative stress, genomic

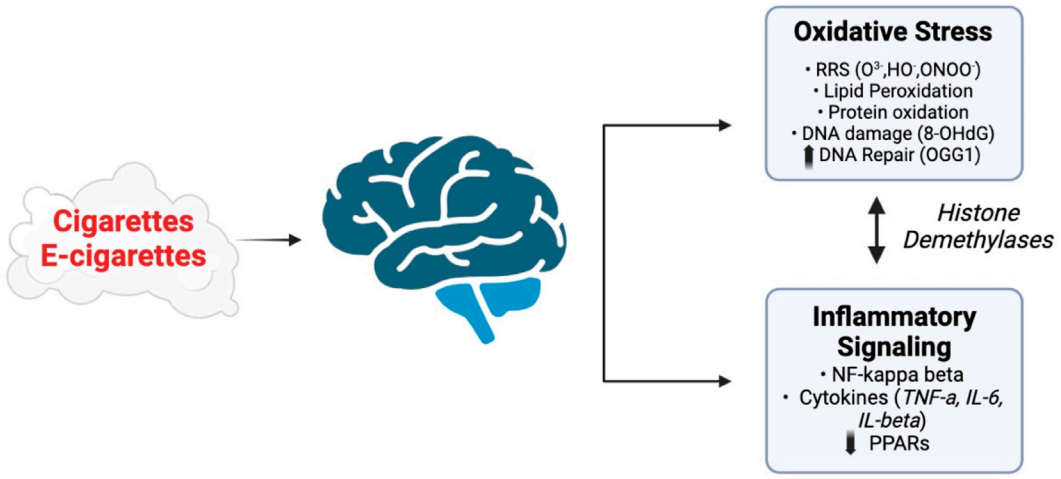

FIGURE 4
Potential pathways affected by exposure of the CNS to vapors or SHS generated by e-cigarettes and conventional cigarettes. Adapted from Vivarelli et al. (2024), Arfaeinia et al. (2023), Addissouky et al. (2024), and Auschwitz et al. (2023). Images were created using BioRender software.

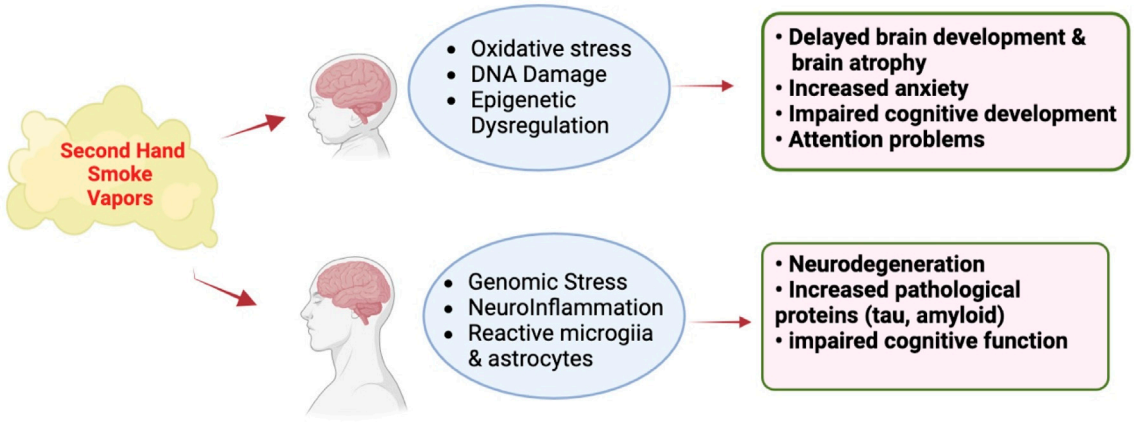

FIGURE 5
Effect of vapors or SHS generated by e-cigarettes and conventional cigarettes (respectively) on the developing and mature CNS. Adapted from Merecz-Sadowska et al. (2020), Prasedya et al. (2020), Chan et al. (2020), and Garza et al. (2023). Images were generated using BioRender software.

stress (i.e., DNA damage and repair) and enhances neuroinflammation like that in age-related neurodegenerative diseases (Simpson et al., 2016; Shadfar et al., 2023) (Figure 5).

With regard to neurodevelopmental disorders, a recent META analysis involving 54 studies revealed associations between SHS exposure and the risks of developing attention deficit hyperactivity disorder (ADHD) and learning disabilities (LD) (Ou et al., 2024). There is also an association between cotinine exposure and ADHD. Consistent with the increased ADHD risk, prenatal SHS and postnatal maternal distress alter the efficiency of the cingulo-opercular (CO) network, which is involved in task control and executive function (Freedman et al., 2020). In addition to SHS, third hand smoke, consisting of residual tobacco smoke pollutants that remain on surfaces and in dust after tobacco has been smoked, which are re-emitted into the gas phase or react with oxidants and other compounds in the environment to yield secondary pollutants and include nicotine, three-ethenylpyridine (3-EP), phenol, cresols, naphthalene, formaldehyde, and tobacco-specific nitrosamines that have detrimental effects on the developing brain (Matt et al., 2011). Third hand smoke can be inhaled through inhalation, ingestion, or dermal uptake from the air, dust, and from surfaces. Consistent with the human data, third hand smoke for 4 weeks in mice increased inflammatory cytokines in plasma and increased epinephrine and aspartate aminotransferase, a biomarker of liver damage (Adhami et al., 2017). These detrimental effects are more pronounced when the mice were chronically exposed to third hand smoke for 8, 16, and 24 weeks. With longer third hand smoke exposure, mice become hyperglycemic and hyperinsulinimic, indicating an important role for impaired insulin sensitivity after third hand

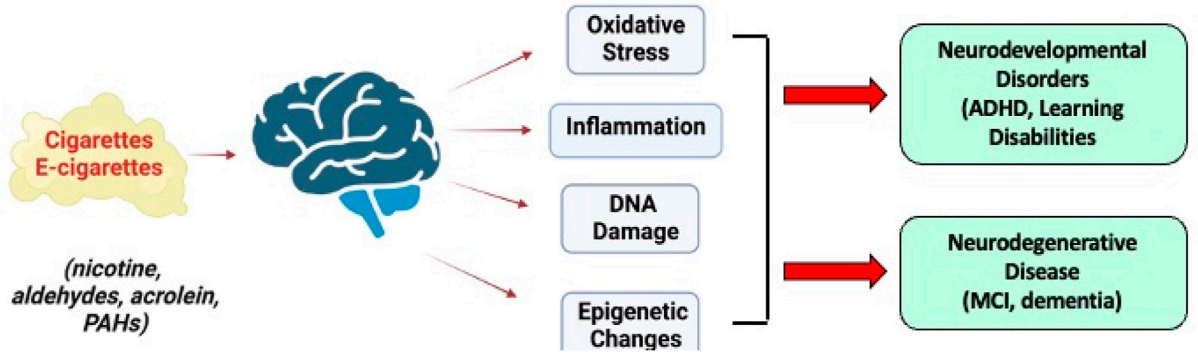

FIGURE 6
Long-term effects of SHS constituents from conventional and e-cigarettes on the brain and their role in neurodevelopmental disorders and neurodegenerative disease. Images were created with BioRender.

smoke exposure. Pre- and post-natally, there can also be a combination of SHS and third hand smoke.

6 Conclusion

The above studies of SHS exposure in animals and humans demonstrate that the brain is a key target of nicotine and other constituents following exposure to tobacco products. Early life exposure to SHS disrupts brain development to increase the risk for neurodevelopmental disorders (ADHD, learning disabilities). On the other hand, exposure of the mature brain to SHS is considered a risk factor mild cognitive impairment and dementia. A common target for SHS in the developing and adult brain is oxidative stress, inflammation responses and genomic stress that might be responsible for triggering both neurodevelopmental disorders as well as dementia (Figure 6). Given the world-wide exposure of pregnant women, children and adults to SHS, additional research will be required to pinpoint the mechanism by which SHS is a risk factor for both neurodevelopmental and neurodegenerative disorders with the goal of protecting the most vulnerable to these environmental exposures.

Author contributions

JR: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing. GK: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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