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Research report

Early postnatal tobacco smoke exposure triggers anxiety-like behavior and decreases synaptic proteins even after a long exposure-free period in mice

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HIGHLIGHTS

- ETS exposure during a critical development triggers anxiety-like behavior.
- Exposure to ETS during the early postnatal period induces a decrease in the locomotor activity in mice.
- Postnatal ETS exposure decreases presynaptic proteins even after a long exposure-free period.
- Postnatal tobacco smoke exposure leads to a decrease in BDNF levels in different regions of the brain.

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ABSTRACT

Although environmental tobacco smoke (ETS) is mainly associated to cardiorespiratory disease, clinical and preclinical studies have showed that ETS induces behavioral disorders and deleterious effects in the brain. Our aim was to investigate the effects of ETS during the early postnatal period on locomotor activity and anxiety and in the presynaptic proteins and brain-derived neurotrophic factor (BDNF) in distinct brain regions. BALB/c mice were exposed to ETS generated from 3R4F reference research cigarettes from the third to the fourteenth days of life. Behavioral and biochemical analyzes were performed during infancy, adolescence, and adulthood. ETS exposure induced a decrease in the locomotor activity in both female and male mice during infancy and in male mice during adolescence. Mice exposed to ETS showed lower distance traveled in the open arms of the elevated plus maze than control group. We also observed a decrease in synapsin levels in the cerebellum and striatum during infancy and adolescence, which persisted during the adulthood only in the cerebellum. Synaptophysin levels were low in all brain regions studied during the infancy, which remained reduced in the cerebellum and prefrontal cortex during adolescence and in the prefrontal cortex during adulthood. BDNF levels were reduced in the striatum and prefrontal cortex during infancy. These behavioral and biochemical data indicate that exposure to ETS during a critical development period leads to anxiety-like behavior and blunted synaptic proteins levels in different regions of the brain. More important, several of these effects were not reversed even after a long exposure-free period.

1. Introduction

The environmental tobacco smoke (ETS), containing about 7000 substances, is composed of both mainstream (10–20%) and sidestream smoke (80–90%), the last one being more toxic and having higher levels of nicotine, carbon monoxide, ammonia, aldehydes and carcinogenic

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components (Rodgman and Perfetti, 2013). Despite the decline of global smoking prevalence, effects related to tobacco smoke are still a public health problem. The global tobacco smoking prevalence declined from 23.5% in 2007 to 20.7% in 2015. Regarding the low-income countries, tobacco consumption prevalence decreased from 15.0% to 13.2% in the same period. The most recent global report indicates that, by 2030, the rate of tobacco use will be around 17.0% for high-income countries and 11.5% for low-income countries (WHO, 2017).

Children are significantly affected by ETS exposure. They are exposed to ETS mainly inside their homes, where they often stay around their relatives or caregivers, and they are more sensitive than adults to environmental toxic substances. This can be explained by their immature physiological status, with important processes still under development, such as enzymes involved in clearance mechanisms, what makes children's detoxifying capability less effective than adults (Ginsberg et al., 2002). ETS exposure during perinatal period is related to attention deficits, hyperactive behavior, sleep and wake disruption, decreased academic performance and brain tumors (Banderali et al., 2015; Hwang et al., 2012; Pagani, 2014; Plichart et al., 2008). Exposure to passive smoke from infancy to adulthood was associated to higher risk for depression and anxiety disorders, such as panic attack (Taha and Goodwin, 2014). The age is a significant factor for the development of addiction. It is important to highlight that 17.3% of young people between 12 and 17 years have used tobacco products throughout their lives (Ahrnsbrak et al., 2017).

Rodents show marked brain growth spurt during the early postnatal development, while in humans this process occurs mainly during the prenatal period. The third trimester of gestation in humans is characterized by intense neurogenesis, cell migration, gliogenesis, synaptogenesis and myelination, processes that occur during the first 2–3 postnatal weeks in rodents (Monk et al., 2012; Rice and Barone, 2000). Preclinical studies showed that long-term exposure to ETS impairs the hippocampal neurogenesis in adult mice, while the exposure to ETS during lactation induces fat food preference in adulthood (Csabai et al., 2016; Pinheiro et al., 2015). Exposure to ETS during the early postnatal period of mice disturbs myelination, learning and memory, and decrease synapsin, synaptophysin and PSD95 in hippocampus of mice (Torres et al., 2015a,b). Evidence of the relationship between synaptic proteins and locomotor activity have been described in knockout mice, in which synapsin-I- and synapsin-II-null mice exhibited higher locomotor activity (Corradi et al., 2008). Synaptic proteins seems also to have a role in anxiety behaviors. Rats submitted to maternal separation display a downregulation of synapsin 1 (Syn1) gene in the amydgala, which was associated with emotional experience, including fear and anxiety (Park et al., 2014). BDNF Val66Met mice, a model of altered anxiety-related behavior, display poor maturation of the serotonergic transmission (Dincheva et al., 2017).

The aim of the present study was to evaluate the effects of exposure to ETS on locomotor activity and anxiety during infancy, adolescence, and adulthood. We also evaluated brain-derived neurotrophic factor (BDNF) and presynaptic proteins levels in the cerebellum, striatum and prefrontal cortex, which are regions related to motor and emotional functions and to addiction.

2. Results

BALB/c pups were exposed to ETS from the 3rd (P3) to the 14th (P14) days of life. For immunoblotting analysis, animals were euthanized in P15 (infancy), P35 (adolescence) and P65 (adulthood). The locomotor activity was assessed on P18, P35 and P65, and elevated plus maze was performed 24 h after the open field, i.e., on P19, P36 and P66. The experimental design is shown in Fig. 1.

Mice that were exposed to ETS during the early postnatal period gained less weight compared with controls during infancy ($p < 0.001$; Fig. 2A) and adolescence ($p < 0.01$; Fig. 2B). This effect was reversed in adulthood (Fig. 2C).

Fig. 3 shows the performance of ETS and control groups in the open field. For total locomotor activity and distance traveled in the central zone, the ANOVA revealed significant treatment ($F_{1,44} = 87.34$; $F_{1,44} = 23.08$, respectively; $p < 0.0001$ for both), sex ($F_{1,44} = 5.45$; $F_{1,44} = 4.71$, respectively; $p < 0.05$ for both) and age ($F_{2,88} = 20.8$; $F_{2,88} = 205.3$, respectively; $p < 0.0001$ for both) effects. In addition, there were significant age × treatment interaction for both total locomotor activity ($F_{2,88} = 22.4$; $p < 0.0001$) and distance traveled in the central zone ($F_{2,88} = 4.25$; $p < 0.05$). The exposure to ETS during the early postnatal period decreased the total locomotor activity (Fig. 3A) in both female ($p < 0.0001$) and male ($p < 0.0001$) mice during infancy when compared with their respective controls. The same effect was also observed in male mice during adolescence ($p < 0.01$) when compared with the control group. Also, ETS exposure reduced the distance traveled in the central zone (Fig. 3B) in male mice during infancy ($p < 0.05$) and in female mice during adolescence ($p < 0.05$) when compared with their respective controls.

The elevated plus maze test showed that exposure to ETS during the early postnatal period leads to anxiogenic effects in the animals (Fig. 4). The ANOVA revealed significant treatment ($F_{1,44} = 25.01$, $p < 0.0001$) and age ($F_{2,88} = 18.24$, $p < 0.0001$) effects for the percentage of distance traveled in the open arms (Fig. 4A) and a significant treatment effect for percentage of entries into the open arms ($F_{1,44} = 28.53$; $p < 0.0001$; Fig. 4B). There was no statistical effect for sex regarding the percentage of distance traveled in the open arms ($F_{1,44} = 4.00$; $p = 0.052$) and for the percentage of entries into the open arms ($F_{1,44} = 0.01$; $p = 0.93$).

The Western blot analyses in infancy showed that the exposure to ETS during the early postnatal period decreased synapsin I levels in cerebellum ($p < 0.05$; Fig. 5A) and striatum ($p < 0.05$; Fig. 5G) and synaptophysin levels in all the brain regions evaluated ($p < 0.05$; Fig. 5D, 5J, 5P). In adolescence, ETS decreased synapsin I levels in cerebellum ($p < 0.05$; Fig. 5B) and striatum ($p < 0.05$; Fig. 5H). Synaptophysin levels were reduced in cerebellum ($p < 0.05$; Fig. 5E) and prefrontal cortex ($p < 0.001$; Fig. 5Q) during adolescence. In adulthood, ETS induced a decrease in synapsin I levels in the cerebellum ($p < 0.05$; Fig. 5C) and in synaptophysin in the prefrontal cortex ($p < 0.01$; Fig. 5R) compared with the control group. Also, mice exposed to ETS showed lower levels of BDNF in the striatum ($p < 0.01$; Fig. 6D) and prefrontal cortex ($p < 0.001$; Fig. 6G) during infancy when compared with control animals. No changes in BDNF levels were detected in adolescence and adulthood in all brain regions studied (Fig. 6).

3. Discussion

Several studies describe the adverse effects of nicotine during a critical period of brain development (Dwyer et al., 2009; Valentine and Sofuoglu, 2018; Yuan et al., 2015). However, the toxic effects of exposure to tobacco smoke are not only due to nicotine. The ETS is a mixture containing more than 7000 substances, including nicotine. In fact, clinical and preclinical studies have showed that ETS triggers behavioral disorders and deleterious effects in the brain (Pagani, 2014; Slotkin et al., 2015). Previous studies of our group showed that ETS leads to oxidative stress in the brain of infant mice (Lobo-Torres et al., 2012), impairs spatial learning and memory (Torres et al., 2015b) and also brain myelination (Torres et al., 2015a). The current study corroborates these findings since the animals exposed to ETS during the first two postnatal weeks showed lower locomotor activity, anxiety-like behavior and diminished levels of synaptic proteins and BDNF in the cerebellum, striatum and prefrontal cortex. Our results also showed that mice exposed to ETS gained less weight during infancy and adolescence. These results are consistent with several studies that showed an inverse relationship between the use of tobacco products and body weight in humans and rodents (Abreu-Villaça et al., 2010; Banderali et al., 2015; Chen et al., 2005; Obot et al., 2004).

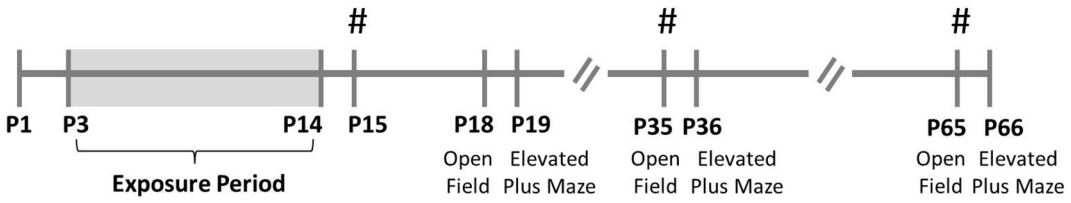

Fig. 1. Summary of the experimental design. Animals were exposed to a mixture of mainstream and sidestream tobacco smoke of reference cigarettes 3R4F from the P3 to the P14. For immunoblotting analysis, animals were euthanized (#) in P15 (infancy), P35 (adolescence) and P65 (adulthood). The locomotor activity was assessed on P18, P35 and P65, and elevated plus maze was performed 24 h after the open field, i.e., on P19, P36 and P66.

The exposure biomarkers we obtained were similar to previous preclinical studies (Amos-Kroohs et al., 2013; Obot et al., 2004; Torres et al., 2015a,b). In humans, children and adolescent (under 17 years old) have been classified as passive or active smoking according to serum cotinine levels. Serum cotinine levels between 0.05 and 10 ng/mL were considered as passive smoking and greater than 10 ng/mL as active smoking (Nwosu, 2018). It is important to note that the half-life for cotinine in human plasma (12–15 h) is longer than in rodents (5.5 h) (Li et al., 2015). In the present study, the animals were euthanized immediately after the last exposure to ETS and blood was collected to quantify the biological exposure markers. Thus, based on a half-life of 0.9 to 1.1 h for plasma nicotine in rodents, the results for cotinine levels found in our study reflect the peak of cotinine formed after 1 h of the exposure to tobacco smoke.

The cerebellum is strongly associated with motor coordination. Nevertheless, several studies have shown that cerebellum is also involved in cognitive and emotional functions, such as anxiety disorders, unipolar depression, schizophrenia and neurodegenerative diseases (Baldaçara et al., 2008; Phillips et al., 2015). The striatum, constituted by the caudate nucleus and putamen, plays an important role in motor, cognitive and limbic functions (Burton et al., 2015), while prefrontal cortex acts in cognitive control and abstract thinking (Dumontheil, 2014). Tobacco smoke and nicotine can modulate synaptic transmitter elements in different brain regions. Environmental tobacco smoke leads to higher serotonin synaptic proteins in the midbrain and frontal, temporal and occipital cortex of Rhesus monkeys (Slotkin et al., 2006); cholinergic synaptic proteins alterations in the cerebral cortex and midbrain of mice (Abreu-Villaça et al., 2016); and to lower synapsin and synaptophysin levels in the hippocampus of mice (Torres et al., 2015b). Parameshwaran et al. (2012) revealed that 3-week-old rats exposed prenatally to nicotine showed a decrease in vesicular glutamate transporter 1, synaptophysin, AMPA receptor subunit GluR1 and PSD-95 in the hippocampus (Parameshwaran et al., 2012). Nicotine infusion during 14 days increased synapsin levels in the nucleus accumbens, while spontaneous nicotine withdrawal induced a decrease in synapsin levels 24 h after cessation (Jackson and Imad Damaj, 2013). In the present study, presynaptic proteins levels in the cerebellum, striatum and prefrontal cortex were reduced in different period of development after exposure to ETS during the early postnatal period.

BDNF plays an important role in the anxiety behaviors, mainly during development (Duman, 2017). Nicotine and tobacco smoke exposure can interfere with BDNF expression in humans and rodents (for review: Machaalani and Chen, 2018). As examples, Bhang et al., (2010) showed that peripheral BDNF levels are lower in active smokers than in nonsmokers, however there is an increase from baseline in BDNF levels after smoking cessation, although other studies are not in agreement with those findings (Jamal et al., 2015; Neves et al., 2017). Studies with rodents showed that changes in BDNF expression induced by nicotine or tobacco smoke depend on age, brain regions and the period after exposure (Machaalani and Chen, 2018). Here, we found that, in infancy, tobacco smoke exposure during the early postnatal period decreases BDNF levels in the striatum and prefrontal cortex. Exposure to tobacco smoke during prenatal and the early postnatal period is related to aggression and depression-like behaviors, decrease in striatal catecholamines and reduction in BDNF levels and tyrosine kinase receptor B expression in the brain of infant, adolescent and adult mice (Torres et al., 2015b; Xiao et al., 2016; Yochum et al., 2014). Furthermore, BDNF *knock-in* mouse containing the BDNF Val66Met polymorphism exhibited a reduced anxiety-like behavior during nicotine withdrawal (Lee et al., 2015).

Perinatal exposure to nicotine increased oxidative stress in the cerebellum and medulla oblongata, impaired locomotor activity and induced anxiety in infant mice (Ajarem et al., 2017). Regarding prenatal exposure, nicotine induced depression, anxiety-like behavior and impairment in the spatial memory in infant rats (Parameshwaran et al., 2012). In the present study, we showed that ETS exposure during the postnatal period reduces locomotor activity. Wiley et al. (2015) observed that nicotine induced a biphasic effect in adult male rats, with a hypoactivity in the first 10 min followed by a hyperactivity at 40–50 min, while cotinine, a nicotine metabolite, produced hypoactivity (Wiley et al., 2015). Alternatively, locomotor activity was increased in adult rats exposed to tobacco smoke during the prenatal period (Zugno et al., 2013) and in adolescent rats during the withdrawal period (de la Peña et al., 2016).

Regarding anxiety-like behavior, our data are in accordance with Abreu-Villaça et al. (2015) which showed that tobacco smoke produces anxiogenic effect in adolescent mice (Abreu-Villaça et al., 2015). The authors used 2R1F (1.74 mg of nicotine) and 4A1 (0.14 mg nicotine) cigarettes and demonstrated that anxiety-like behavior pattern induced by tobacco smoke depends on the amount of nicotine in the cigarette.

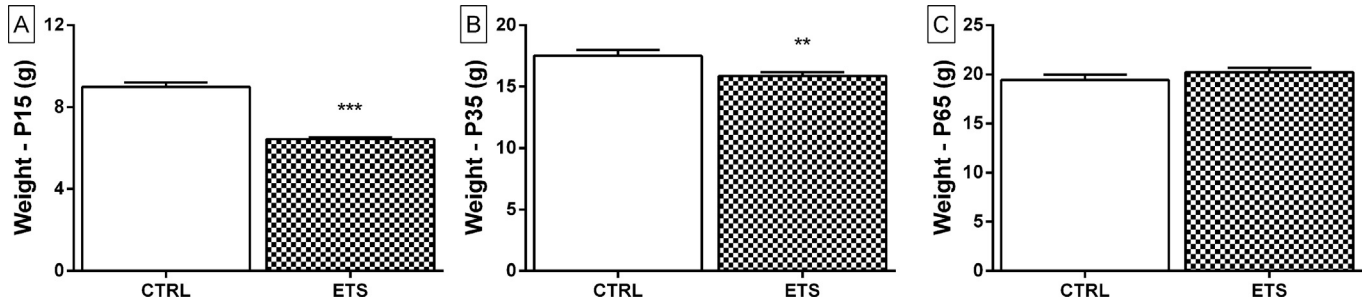

Fig. 2. Weight of mice exposed to ETS during the early postnatal period compared with those of control animals (n = 24). Data expressed in g (means ± SEM). $^{**}p < 0.01$; $^{***}p < 0.001$. A – Infancy, B – adolescence and C – adulthood.

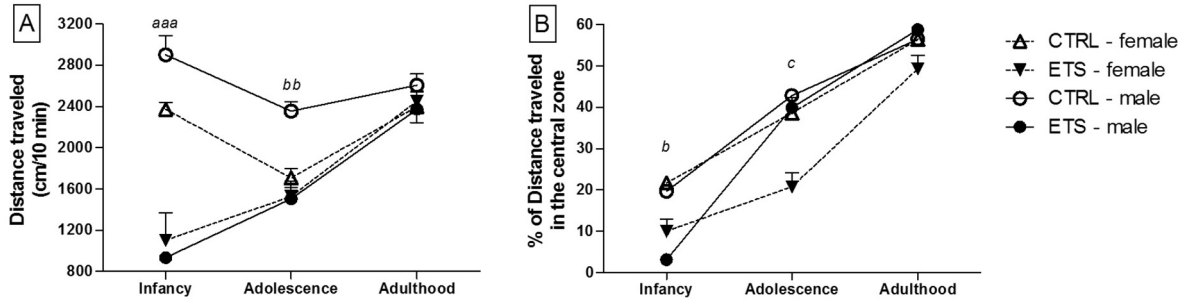

Fig. 3. Total locomotor activity (A) and distance traveled in the central zone (B) during open field test (n = 12). Data expressed in cm (means ± SEM) and in percentage (means ± SEM), respectively. $^{aaa}p < 0.001$ – Both male and female ETS-exposed mice compared with the control animals during infancy; $^{bb}p < 0.01$ Male ETS-exposed mice compared with the control animals in adolescence; $^{b}p < 0.05$ Male ETS-exposed mice compared with the control animals in infancy; $^{c}p < 0.05$ – Female ETS-exposed mice compared with the control animals in adolescence.

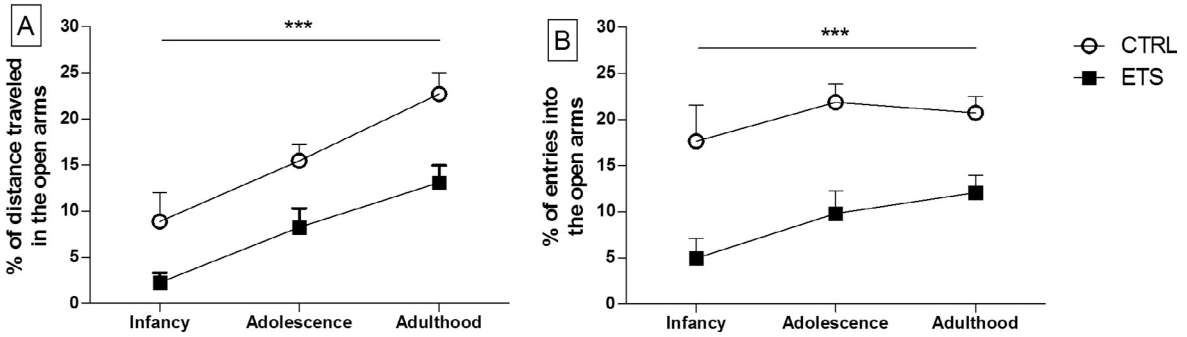

Fig. 4. Percentage of distance traveled in the open arms (A) and percentage of entries into the open arms (B) during elevated plus maze test (n = 24). Data expressed in percentage (means ± SEM). $^{***}p < 0.001$ – ETS-exposed mice compared with the control animals.

de la Peña et al. (2016) also showed that tobacco smoke increased anxiety in adolescent rats during the withdrawal period (de la Peña et al., 2016). It is important to point out that in our study, anxiety-like behavior does not reflect changes in locomotion, since we evaluated the percentage of distance traveled in the open arms relative to the total distance traveled in the elevated plus maze. Moreover, adult mice did not show any change in locomotor activity while they also showed lower percentage of distance travelled and entries as observed in infants and adolescents.

In summary, we showed that exposure to ETS during a critical development period interferes with behavioral responses in mice and changes synaptic protein levels in different brain regions. Importantly, several of these effects were not reversed even after a long exposure-free period.

4. Experimental procedure

4.1. Animals

BALB/c mice were obtained from the animal facility of the School of Medicine of University of São Paulo. This study was approved by the Ethics Committee of the School of Medicine (no.1038/09) and the School of Pharmaceutical Sciences (no. 260/10), University of São Paulo. Animals were housed at 22–26 °C with a 12 h/12 h light/dark cycle (light starting at 7 a.m.) and received water and commercial pellet food for small rodents from Nuvital (Nuvilab CR-1; Colombo, Brazil) *ad libitum*. All procedures were in compliance with the "Principles of Laboratory Animal Care" published by the National Institutes of Health.

4.2. Procedures

A summary of the experimental schedule is shown in Fig. 1. The size of each litter was randomly adjusted to 6–7 pups within the first day after delivery, as previously described by Torres et al. (2015b). To avoid litter effect, 1 to 2 mice from different litters were used to form a group. The BALB/c pups were exposed, together with their mothers, to a mixture of mainstream and sidestream tobacco smoke of reference cigarettes 3R4F from the 3rd (P3) to the 14th (P14) day of life during 2 h/day (1 h at 8 a.m. and 1 h at 5p.m.). The control group inhaled compressed air only. Immediately after the last exposure, the animals (n = 5) were anesthetized and the blood was collected by transcardiac puncture to quantify the biological exposure markers. The exposure parameters were similar to our previous study (Torres et al., 2015b): CO levels in the chamber were 361.8 ± 77.3 ppm; COHb were 12.5 ± 1.9%; plasma nicotine and cotinine levels were 126.1 ± 17.2 and 100.8 ± 14.5 ng/ml, respectively. Forty-eight animals (24 females and 24 males) were used from P18 to P66 for the behavioral tests. Locomotor activity (n = 12 females and n = 12 males) was assessed on P18, P35 and P65 and elevated plus maze (n = 12 females and n = 12 males) was assessed 24 h after the open field (P19, P36 and P66). Different sets of pups (36 animals) were euthanized by cervical dislocation for Western blot analysis; both male and female pups were randomly assigned to three groups (n = 6): P15 (infancy), P35 (adolescence) and P65 (adulthood).

4.3. Behavioral evaluation

All behavioral procedures were performed during the light phase of the light/dark cycle. The apparatus used for open-field test consisted of a wooden round, 40 cm diameter surrounded by a 50 cm wall. Total locomotor activity and distance traveled in the central zone of the arena was evaluated for 10 min in the open-field test. Anxiety was measured in an elevated plus maze that consists in two open and two closed arms (30 × 5 × 0.25) positioned 50 cm above the floor. Animals were placed on the central platform of the apparatus facing a closed arm. Distance traveled on open and closed arms and number of entries into the open arms were recorded. All the behavioral trials were recorded with a digital camera and the analysis was performed with EthoVision 3.1

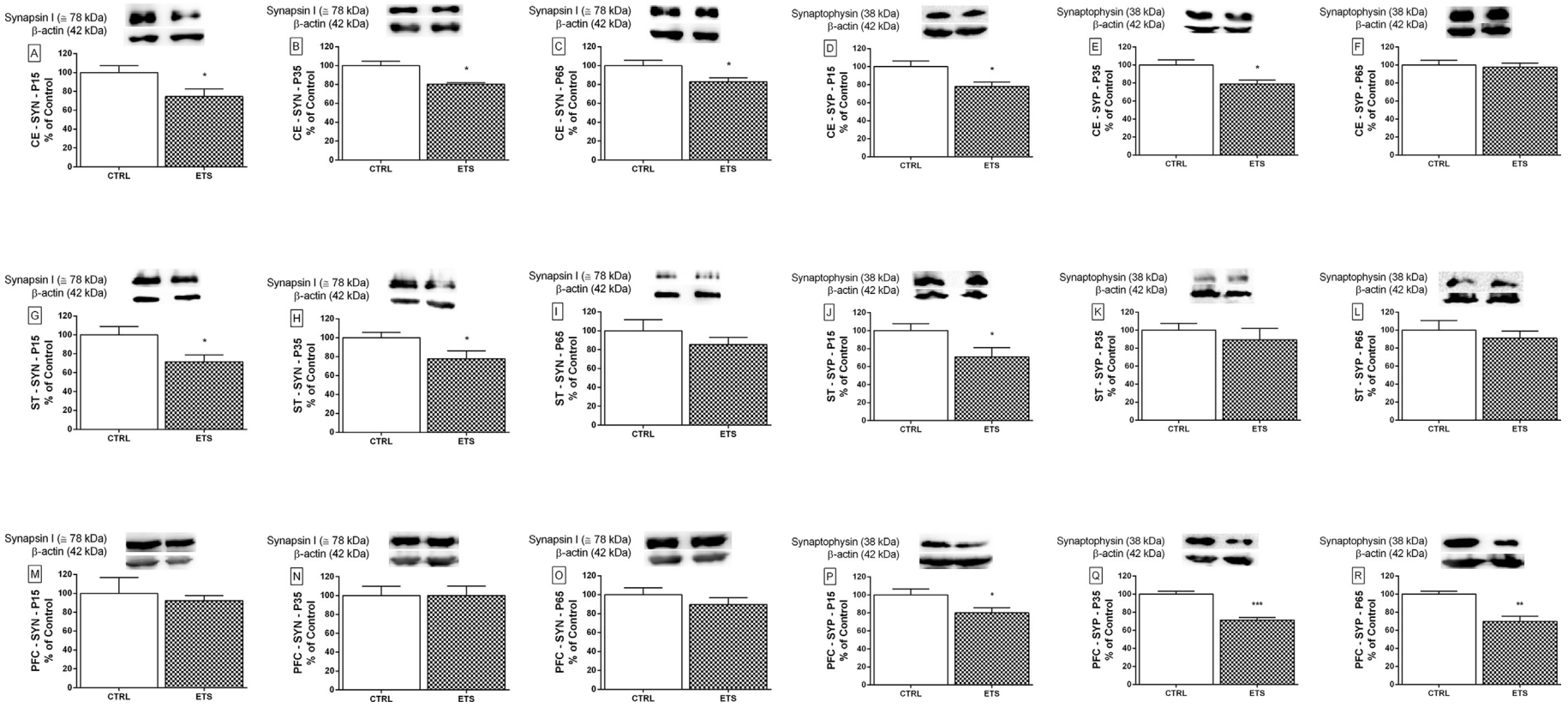

Fig. 5. Quantification of Synapsin I (SYN) and synaptophysin (SYP) in cerebellum (CE), striatum (ST) and prefrontal cortex (PFC) during in infancy (P15), adolescence (P35) and adulthood (P65) by Western blot (n = 6). Data are expressed as the percent of control (mean ± SEM). $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$ – ETS-exposed mice compared with the control animals. Synapsin I (≅78 kDa), Synaptophysin (38 kDa), β-actin (42 kDa). A: CE-SYN-P15; B: CE-SYN-P35; C: CE-SYN-P65; D: CE-SYP-P15; E: CE-SYP-P35; F: CE-SYP-P65; G: ST-SYN-P15; H: ST-SYN-P35; I: ST-SYN-P65; J: ST-SYP-P15; K: ST-SYP-P35; L: ST-SYP-P65; M: PFC-SYN-P15; N: PFC-SYN-P35; O: PFC-SYN-P65; P: PFC-SYP-P15; Q: PFC-SYP-P35; R: PFC-SYP-P65.

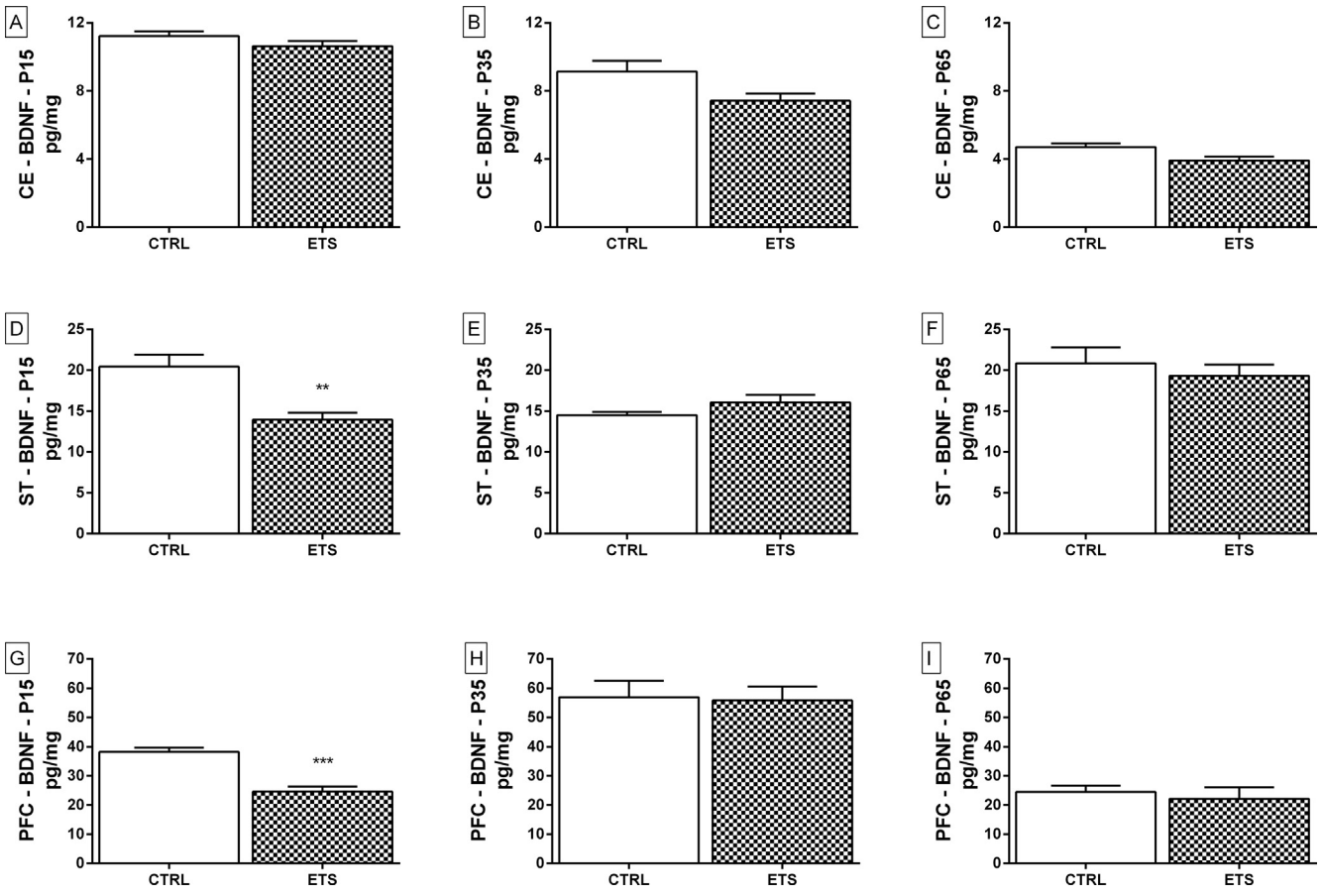

Fig. 6. Quantification of BDNF in cerebellum (CE), striatum (ST) and prefrontal cortex (PFC) during infancy (A, D, G), adolescence (B, E, H) and adulthood (C, F, I) by BDNF Emax ImmunoAssay System kit (n = 6). Data are expressed in pg/mL (mean ± SEM). $^{**}p < 0.01$ $^{***}p < 0.001$ – ETS-exposed mice compared with the control animals.

software (Noldus, Wageningen, the Netherlands).

4.4. Western blot analysis

The cerebellum, striatum and prefrontal cortex were dissected and stored at -80 °C until preparation of the homogenate in ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 1.0 mM PMFS, 10 µg/ml leupeptin, and 1.0 mM L-cit. The extracts were then centrifuged at 1,000 g at 4 °C for 5 min. Protein determination in the supernatants was performed with the Bradford dye method using the Bio-Rad reagent. The whole extracts were treated with Laemmli sample buffer containing dithiothreitol and boiled for 5 min. Equal quantities of protein from each sample were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) and transferred onto a nitrocellulose membrane. After blocking the nonspecific sites with 0.2% (w/v) casein, membranes were incubated overnight at 18 °C with primary antibodies, rabbit polyclonal anti-synapsin, 1:1000 (Millipore, Temecula, United States) and mouse monoclonal anti-synaptophysin, 1:1000 (DakoCytomation, Glostrup, Denmark). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and subsequently incubated with a peroxidase-conjugated secondary antibody for 1 h. After 3 washes, the immunoreactive bands were visualized using the ECL detection system (Thermo Scientific, Rockford, USA), and the images were captured with ImageQuantTM 400® v.1.0.0 (Amersham Biosciences, Pittsburg, USA). The band intensities were quantified with ImageJ 1.43u (National Institutes of Health, USA), and the results were normalized to the intensity of β-actin (Sigma-Aldrich, St. Louis, USA)

4.5. BDNF

BDNF levels were quantified using the BDNF Emax ImmunoAssay System (Promega, Madison, WI, USA). Briefly, each well of a 96-well microplate was covered with 100 µL anti-BDNF monoclonal antibody diluted 1:1000 in carbonate buffer (25 mM $NaHCO_3$, 25 mM Na_2CO_3, pH 9.7). The plate was sealed, incubated overnight at 4 °C and washed with Tris-buffered saline containing 0.05% Tween 20. Then 200 µL/well of 1x Block & Sample buffer was added and the plate was incubated at room temperature for 1 h. Following additional washes, 100 µL of the sample (1:4 dilution) or BDNF standard (serial 1:2 dilutions ranging from 0 to 500 pg BDNF/mL) were added and the plate was incubated for 2 h at room temperature with shaking. After additional washes, 100 µL of anti-BDNF polyclonal antibody diluted 1:500 in 1x Block &Sample buffer was added and the plate incubated for 2 h at room temperature with shaking. The plate was washed and 100 µL of anti-IgY horseradish peroxidase conjugate (diluted 1:200 in 1x Block & Sample buffer) was added. The plate was incubated for 1 h at room temperature with shaking and protected from light. Following additional washes, the plate was then incubated for 10 min with shaking at room temperature with 100 µL/well of TMB One solution. The reaction was stopped by adding 100 µL of HCl 1 N, and absorbance was measured at 450 nm.

4.6. Statistical analysis

The open field and elevated plus maze data were analyzed using repeated-measures analysis of variance (ANOVA) with the treatments and sex as the between-subject factors and age as the within-subject

factor. *Post-hoc* analysis was performed with the Bonferroni test. The weight, Western blot and BDNF data were analyzed using Student's *t*-test for independent samples. The data were presented as the means ± SEM. Differences with a probability of 95% ($p < 0.05$) were considered significant.

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References

Abreu-Villaça, Y., Correa-Santos, M., Dutra-Tavares, A.C., Paes-Branco, D., Nunes-Freitas, A., Manhães, A.C., Filgueiras, C.C., Ribeiro-Carvalho, A., 2016. A ten fold reduction of nicotine yield in tobacco smoke does not spare the central cholinergic system in adolescent mice. Int. J. Dev. Neurosci. 52, 93–103. https://doi.org/10.1016/j.ijdevneu.2016.06.002.

Abreu-Villaça, Y., Filgueiras, C.C., Correa-Santos, M., Cavina, C.C., Naiff, V.F., Krahe, T.E., Manhães, A.C., Ribeiro-Carvalho, A., 2015. Tobacco smoke containing high or low levels of nicotine during adolescence: effects on novelty-seeking and anxiety-like behaviors in mice. Psychopharmacology (Berl). 232, 1693–1703. https://doi.org/10.1007/s00213-014-3801-1.

Abreu-Villaça, Y., Filgueiras, C.C., Guthierrez, M., Medeiros, A.H. De, Mattos, M.A., Pereira, M.D.S., Manhães, A.C., Kubrusly, R.C.C., 2010. Exposure to tobacco smoke containing either high or low levels of nicotine during adolescence: differential effects on choline uptake in the cerebral cortex and hippocampus. Nicotine Tob. Res. 12, 776–780. https://doi.org/10.1093/ntr/ntq075.

Ahrnsbrak, R., Bose, J., Hedden, S., Lipari, R., Park-Lee, E., 2017. Key Substance Use and Mental Health Indicators in the United States: Results from the 2016 National Survey on Drug Use and Health. Substance Abuse Mental Health Serv. Administration. https://doi.org/10.1016/j.drugalcdep.2016.10.042.

Ajarem, J.S., Al-Basher, G., Allam, A.A., Mahmoud, A.M., 2017. Camellia sinensis Prevents Perinatal Nicotine-Induced Neurobehavioral Alterations, Tissue Injury, and Oxidative Stress in Male and Female Mice Newborns. Oxid. Med. Cell. Longev. 5985219, 1–16. https://doi.org/10.1155/2017/5985219.

Amos-Kroohs, R.M., Williams, M.T., Braun, A.A., Graham, D.L., Webb, C.L., Birtles, T.S., Greene, R.M., Vorhees, C.V., Pisano, M.M., 2013. Neurobehavioral phenotype of C57BL/6J mice prenatally and neonatally exposed to cigarette smoke. Neurotoxicol. Teratol. 35, 34–45. https://doi.org/10.1016/j.ntt.2013.01.001.

Baldaçara, L., Guilherme, J., Borgio, F., Luiz, A., Lacerda, T. De, Jackowski, A.P., 2008. Cerebellum and psychiatric disorders O cerebelo e os transtornos psiquiátricos. Rev. Bras. Psiquiatr. 30, 281–289. https://doi.org/10.1590/S1516-44462008000300016.

Banderali, G., Martelli, A., Landi, M., Moretti, F., Betti, F., Radaelli, G., Lassandro, C., Verduci, E., 2015. Short and long term health effects of parental tobacco smoking during pregnancy and lactation: a descriptive review. J. Transl. Med. 13, 1–7. https://doi.org/10.1186/s12967-015-0690-y.

Bhang, S.-Y., Choi, S.-W., Ahn, J.-H., 2010. Changes in plasma brain-derived neurotrophic factor levels in smokers after smoking cessation. Neurosci. Lett. 468, 7–11. https://doi.org/10.1016/J.NEULET.2009.10.046.

Burton, A., Nakamura, K., Roesch, M., 2015. From ventral-medial to dorsal-lateral striatum: Neural correlates of reward-guided decision-making. Neurobiol. Learn. Mem. 6, 51–59. https://doi.org/10.1111/j.1743-6109.2008.01122.x.Endothelial.

Chen, H., Vlahos, R., Bozinovski, S., Jones, J., Anderson, G.P., Morris, M.J., 2005. Effect of short-term cigarette smoke exposure on body weight, appetite and brain neuropeptide Y in mice. Neuropsychopharmacology 30, 713–719. https://doi.org/10.1038/sj.npp.1300597.

Corradi, A., Zanardi, A., Giacomini, C., Onofri, F., Valtorta, F., Zoli, M., Benfenati, F., 2008. Synapsin-I- and synapsin-II-null mice display an increased age-dependent cognitive impairment. J. Cell Sci. https://doi.org/10.1242/jcs.035063.

Csabai, D., Csekő, K., Szaiff, L., Varga, Z., Miseta, A., Helyes, Z., Czéh, B., 2016. Low intensity, long term exposure to tobacco smoke inhibits hippocampal neurogenesis in adult mice. Behav. Brain Res. 302, 44–52. https://doi.org/10.1016/j.bbr.2016.01.022.

de la Peña, J.B., Ahsan, H.M., Botanas, C.J., dela Peña, I.J., Woo, T., Kim, H.J., Cheong, J.H., 2016. Cigarette smoke exposure during adolescence but not adulthood induces anxiety-like behavior and locomotor stimulation in rats during withdrawal. Int. J. Dev. Neurosci. 55, 49–55. https://doi.org/10.1016/j.ijdevneu.2016.09.007.

Dincheva, I., Yang, J., Li, A., Marinic, T., Freilingsdorf, H., Huang, C., Casey, B.J., Hempstead, B., Glatt, C.E., Lee, F.S., Bath, K.G., Jing, D., 2017. Effect of early-life fluoxetine on anxiety-like behaviors in BDNF Val66Met mice. Am. J. Psychiatry. https://doi.org/10.1176/appi.ajp.2017.15121592.

Duman, R.S., 2017. BDNF, 5-HT, and Anxiety: Identification of a Critical Periadolescent Developmental Period. Am. J. Psychiatry 174, 1137–1139.

Dumontheil, I., 2014. Development of abstract thinking during childhood and adolescence: the role of rostrolateral prefrontal cortex. Dev. Cogn. Neurosci. 10, 57–76. https://doi.org/10.1016/j.dcn.2014.07.009.

Dwyer, J.B., Mcquown, S.C., Leslie, F.M., 2009. The dynamic effects of nicotine on the developing brain. Pharmacol. Ther. 122, 125–139. https://doi.org/10.1016/j.pharmthera.2009.02.003.

Ginsberg, G., Hattis, D., Sonawane, B., Russ, A., Banati, P., Kozlak, M., Smolenski, S., Goble, R., 2002. Evaluation of child/adult pharmacokinetic differences from a database derived from the therapeutic drug literature. Toxicol. Sci. 66, 185–200. https://doi.org/10.1093/toxsci/66.2.185.

Hwang, S.-H., Hwang, J.H., Moon, J.S., Lee, D.-H., 2012. Environmental tobacco smoke and children's health. Korean J. Pediatr. 55, 35. https://doi.org/10.3345/kjp.2012.55.2.35.

Jackson, K.J., Imad Damaj, M., 2013. Beta2-containing nicotinic acetylcholine receptors mediate calcium/calmodulin-dependent protein kinase-II and synapsin I protein levels in the nucleus accumbens after nicotine withdrawal in mice. Eur. J. Pharmacol. 701, 1–6. https://doi.org/10.1016/J.EJPHAR.2012.12.005.

Jamal, M., Van der Does, W., Elzinga, B.M., Molendijk, M.L., Penninx, B.W.J.H., 2015. Association Between Smoking, Nicotine Dependence, and BDNF Val66Met Polymorphism with BDNF Concentrations in Serum. Nicotine Tob. Res. 17, 323–329. https://doi.org/10.1093/ntr/ntu151.

Lee, B.G., Anastasia, A., Hempstead, B.L., Lee, F.S., Blendy, J.A., 2015. Effects of the BDNF Val66Met Polymorphism on Anxiety-Like Behavior Following Nicotine Withdrawal in Mice. Nicotine Tob. Res. 17, 1428–1435. https://doi.org/10.1093/ntr/ntv047.

Li, P., Beck, W.D., Callahan, P.M., Terry, A.V., Bartlett, M.G., 2015. Pharmacokinetics of cotinine in rats: a potential therapeutic agent for disorders of cognitive function. Pharmacol. Reports 67, 494–500. https://doi.org/10.1016/j.pharep.2014.12.004.

Lobo-Torres, L.H., Moreira, W.L., Garcia, R.C.T., Annoni, R., Nicoletti Carvalho, A.L., Teixeira, S.A., Pacheco-Neto, M., Muscará, M.N., Camarini, R., de Melo Loureiro, A.P., Yonamine, M., Mauad, T., Marcourakis, T., 2012. Environmental tobacco smoke induces oxidative stress in distinct brain regions of infant mice. J. Toxicol. Environ. Health. A 75, 971–980. https://doi.org/10.1080/15287394.2012.695985.

Machaalani, R., Chen, H., 2018. Brain derived neurotrophic factor (BDNF), its tyrosine kinase receptor B (TrkB) and nicotine. Neurotoxicology 65, 186–195. https://doi.org/10.1016/j.neuro.2018.02.014.

Monk, B.R., Leslie, F.M., Thomas, J.D., 2012. The effects of perinatal choline supplementation on hippocampal cholinergic development in rats exposed to alcohol during the brain growth spurt. Hippocampus 22, 1750–1757. https://doi.org/10.1002/hipo.22009.

Neves, C.D.C., Lacerda, A.C.R., Lima, L.P., Lage, V.K.S., Balthazar, C.H., Leite, H.R., Mendonça, V.A., 2017. Different levels of brain-derived neurotrophic factor and cortisol in healthy heavy smokers. Brazilian J. Med. Biol. Res. 50, 1–5. https://doi.org/10.1590/1414-431X20176424.

Nwosu, B.U., 2018. Tobacco smoke exposure is an independent predictor of vitamin D deciency in US children. PLoS One 13, 1–8. https://doi.org/10.1371/journal.pone.0205342.

Obot, C.J., Lee, K.M., Fuciarelli, A.F., Renne, R.A., Mckinney, W.J., 2004. Characterization of Mainstream Cigarette Smoke-Induced Biomarker Responses in ICR and C57Bl / 6 Mice 701–719. https://doi.org/10.1080/08958370490476604.

Pagani, L.S., 2014. Environmental tobacco smoke exposure and brain development: the case of attention deficit/hyperactivity disorder. Neurosci. Biobehav. Rev. 44, 195–205. https://doi.org/https://doi.org/10.1016/j.neubiorev.2013.03.008.

Parameshwaran, K., Buabeid, M.a., Karuppagounder, S.S., Uthayathas, S., Thiruchelvam, K., Shonesy, B., Dityatev, A., Escobar, M.C., Dhanasekaran, M., Suppiramaniam, V., 2012. Developmental nicotine exposure induced alterations in behavior and glutamate receptor function in hippocampus. Cell. Mol. Life Sci. 69, 829–841. https://doi.org/10.1007/s00018-011-0805-4.

Park, H.J., Kim, S.K., Kang, W.S., Chung, J.H., Kim, J.W., 2014. Increased activation of synapsin 1 and mitogen-activated protein kinases/extracellular signal-regulated kinase in the amygdala of maternal separation rats. CNS Neurosci. Ther. https://doi.org/10.1111/cns.12202.

Phillips, J.R., Hewedi, D.H., Eissa, A.M., Moustafa, A.A., 2015. The Cerebellum and Psychiatric Disorders. Front. Public Heal. 3, 1–8. https://doi.org/10.3389/fpubh.2015.00066.

Pinheiro, C.R., Moura, E.G., Manha, A.C., Fraga, M.C., Oliveira, E., Lisboa, P.C., 2015. Concurrent Maternal And Pup Postnatal Tobacco Smoke Exposure In Wistar Rats Changes Food Preference And Dopaminergic Reward System Parameters In The Adult. Neuroscience 301, 178–192. https://doi.org/10.1016/j.neuroscience.2015.06.001.

Plichart, M., Menegaux, F., Lacour, B., Hartmann, O., Frappaz, D., Doz, F., Bertozzi, A.I., Defaschelles, A.S., Pierre-Kahn, A., Icher, C., Chastagner, P., Plantaz, D., Rialland, X., Hémon, D., Clavel, J., 2008. Parental smoking, maternal alcohol, coffee and tea consumption during pregnancy and childhood malignant central nervous system tumours: The ESCALE Study (SFCE). Eur. J. Cancer Prev. 17, 376–383. https://doi.org/10.1097/CEJ.0b013e3282f75e6f.

Rice, D., Barone, S., 2000. Critical Periods of Vulnerability for the Developing Nervous System : Evidence from Humans and Animal. Models Birth 108.

Rodgman, A., Perfetti, T.A., 2013. The Chemical Components of Tobacco and Tobacco Smoke, Second. ed. Taylor & Francis Group, LLC, Boca Raton.

Slotkin, T.A., Pinkerton, K.E., Tate, C.A., Seidler, F.J., 2006. Alterations of serotonin synaptic proteins in brain regions of neonatal Rhesus monkeys exposed to perinatal environmental tobacco smoke. Brain Res. 1111, 30–35. https://doi.org/10.1016/J.BRAINRES.2006.06.094.

Slotkin, T.A., Skavicus, S., Card, J., Stadler, A., Levin, E.D., Seidler, F.J., 2015. Developmental neurotoxicity of tobacco smoke directed toward cholinergic and

serotonergic systems: more than just nicotine. Sci. Toxicol. https://doi.org/10.1093/toxsci/kfv123.

Taha, F., Goodwin, R.D., 2014. Secondhand smoke exposure across the life course and the risk of adult-onset depression and anxiety disorder. J. Affect. Disord. 168, 367–372 10.1016/j.jad.2014.07.014.

Torres, L.H., Annoni, R., Balestrin, N.T., Coleto, P.L., Duro, S.O., Garcia, R.C.T., Pacheco-Neto, M., Mauad, T., Camarini, R., Britto, L.R.G., Marcourakis, T., 2015a. Environmental tobacco smoke in the early postnatal period induces impairment in brain myelination. Arch. Toxicol. 89, 2051–2058. https://doi.org/10.1007/s00204-014-1343-2.

Torres, L.H., Garcia, R.C.T., Blois, A.M.M., Dati, L.M.M., Durão, A.C., Alves, A.S., Pacheco-Neto, M., Mauad, T., Britto, L.R.G., Xavier, G.F., Camarini, R., Marcourakis, T., 2015b. Exposure of neonatal mice to tobacco smoke disturbs synaptic proteins and spatial learning and memory from late infancy to early adulthood. PLoS One 10, e0136399. https://doi.org/10.1371/journal.pone.0136399.

Valentine, G., Sofuoglu, M., 2018. Cognitive Effects of Nicotine: Recent Progress. Curr. Neuropharmacol. 16, 403–414.

WHO, 2017. WHO Report on the Global Tobacco Epidemic, 2017, World Health Organization. https:// 2017 doi.org/Licence: CC BY-NC-SA 3.0 IGO.

Wiley, J.L., Marusich, J.A., Thomas, B.F., Jackson, K.J., 2015. Determination of behaviorally effective tobacco constituent doses in rats. Nicotine Tob. Res. 17, 368–371. https://doi.org/10.1093/ntr/ntu194.

Xiao, L., Kish, V.L., Benders, K.M., Wu, Z.X., 2016. Prenatal and early postnatal exposure to cigarette smoke decreases BDNF/TrkB signaling and increases abnormal behaviors later in life. Int. J. Neuropsychopharmacol. 19, 1–11. https://doi.org/10.1093/ijnp/pyv117.

Yochum, C., Doherty-Lyon, S., Hoffman, C., Hossain, M.M., Zelikoff, J.T., Richardson, J.R., 2014. Prenatal cigarette smoke exposure causes hyperactivity and aggressive behavior: role of altered catecholamines and BDNF. Exp. Neurol. 254, 145–152. https://doi.org/10.1016/j.expneurol.2014.01.016.

Yuan, M., Cross, S.J., Loughlin, S.E., Leslie, F.M., 2015. Nicotine and the adolescent brain. J. Physiol. https://doi.org/10.1113/JP270492.

Zugno, A.I., Fraga, D.B., De Luca, R.D., Ghedim, F.V., Deroza, P.F., Cipriano, A.L., Oliveira, M.B., Heylmann, A.S.A., Budni, J., Souza, R.P., Quevedo, J., 2013. Chronic exposure to cigarette smoke during gestation results in altered cholinesterase enzyme activity and behavioral deficits in adult rat offspring: Potential relevance to schizophrenia. J. Psychiatr. Res. 47, 740–746. https://doi.org/10.1016/j.jpsychires.2013.02.001.