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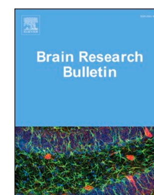

Research report

Lower ΔFosB expression in the dopaminergic system after stevia consumption in rats housed under environmental enrichment conditions

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ABSTRACT

Environmental enrichment (EE) has been proven to reduce drug seeking and the development of addiction-related behaviors in rodent models, but the effects of EE on natural reward acquisition in the form of sweet beverages are poorly understood. Accumulating evidence shows that the intake of sugar, the main ingredient of sweet beverages, alters the dopaminergic system, leading to addiction-related physiological and molecular changes. Sugar in sweet beverages has been replaced with natural sweeteners, such as stevia extract, which has greater sweetener potential but no energy content. Our research group found that sucralose consumption increased the expression of ΔFosB in reward-related nuclei, suggesting activation of the dopaminergic system. The present study assessed the effects of EE on stevia consumption and the expression of ΔFosB in the nucleus accumbens, caudate putamen, and prefrontal cortex. Sixteen male Wistar rats, 21 days old, were randomly assigned to an EE group ($n = 8$) or standard environment (SE) group ($n = 8$) and reared for 30 days. On postnatal day 52 (PND52), the brains of four animals in each housing condition were extracted to determine basal ΔFosB levels. Stevia consumption with intermittent access and ΔFosB immunoreactivity were measured for 21 days in the remainder of the rats. Compared with SE animals, EE animals exhibited a reduction of stevia consumption and alterations of ΔFosB immunoreactivity in the reward system. These results indicate that EE reduces stevia consumption and the stevia-induced ΔFosB expression, suggesting addiction-related changes in dopaminergic nuclei, which may be interpreted as a neuroprotective effect.

1. Introduction

Environmental enrichment (EE) consists of a combination of sensorial, motor, social, and cognitive stimuli that enhance animal wellbeing and induce a wide range of morphological, physiological, and behavioral changes (Nithianantharajah and Hannan, 2006). In rodents, EE consists of larger cages, running wheels, toys, tunnels, mazes, ladders, and social interactions, all of which elicit brain plasticity and recovery processes at multiple levels of neural organization in the brain compared with a standard environment (SE; Sampedro-Piquero and Begega, 2017). Moreover, EE has been proposed to be a potential therapy because of evidence that it elicits improvements in molecular, cellular, and behavioral deficits in rodent models of neurodegenerative diseases (Lazarov et al., 2005; Silva and Ferrari, 2020), brain injury (Bengoetxea et al., 2012; Livingston-Thomas et al., 2016), psychiatric disorders (Scarola et al., 2019; Rule et al., 2020), and drug addiction (Solinas et al., 2010; Sikora et al., 2018). Several studies show that EE improves rat performance in cognitive and emotional-reactivity tasks (Wood et al., 2006; Bhagya et al., 2017). These behavioral changes are associated with the cerebral rearrangement of neural circuits, such as

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monoaminergic systems (Novak et al., 2012), the hippocampal system (Mahati et al., 2016; Seong et al., 2017), and regulation of the hypothalamic-pituitary-adrenal axis (Smith et al., 2017). Furthermore, housing rats under EE conditions from weaning to adulthood reduced the rewarding effects of many drugs of abuse, such as nicotine (Sikora et al., 2018) and cocaine (Gipson et al., 2011). These effects include reductions of drug seeking (Solinas et al., 2009), locomotor activity (Bezard et al., 2003), and relapse (Grimm et al., 2008) and prevention of the accumulation of ΔFosB in reward-related nuclei after drug exposure (Solinas et al., 2009; Lafragette et al., 2017), which are related to the establishment of addictive behaviors (Nestler et al., 2001).

Recent studies focused on the modulation of natural rewards by EE (Moody et al., 2015; Gergerlioglu et al., 2016). Natural rewards are essential to maintain homeostatic processes and evolutionarily important for survival, reproduction, and fitness (Kelley and Berridge, 2002). Glucose is a natural reward and common component of many foods. It is essential for the brain to function properly and maintain elementary processes (Koekkoek et al., 2017). Nevertheless, sugar abuse through sweet beverages may increase the vulnerability to pathological conditions, such as metabolic diseases (Tryon et al., 2015). In rodents, sugar intake is associated with ΔFosB overexpression in reward-related nuclei, such as the nucleus accumbens (NAc; Wallace et al., 2008), and the establishment of addictive behaviors (Avena et al., 2005), including drugs of abuse. Environmental enrichment has been shown to decrease sucrose consumption by reducing activity in the NAc (Brenes and Fornaguera, 2008), a key reward-related nucleus that is involved in hedonic properties of rewards (Arias-Carrión et al., 2010). Sugar in sweet beverages has been replaced by natural and artificial sweeteners, which have higher sweetener potential than sugar but no energy content (Yang, 2010). Stevia extract is a natural sweetener that is used in various "dietary" products. Stevia does not trigger postprandial processes or higher blood glucose and plasma insulin levels that are usually caused by sugar consumption (Nettleton et al., 2019). Unfortunately, the effects of stevia consumption on the dopaminergic system have been scarcely studied, in contrast to other sweeteners. The intense sweetener saccharin generates a reward signal in the brain with the potential to promote addiction-related behaviors (Lenoir et al., 2007). Manganese-enhanced magnetic resonance imaging studies reported that voluntary saccharin consumption activated striatal and cortical areas, such as the NAc, caudate putamen (CPu), and prelimbic cortex (PrL; Dudek et al., 2015). The consumption of sucralose, another artificial sweetener, activates the dopaminergic system, reflected by the expression of ΔFosB in the NAc (Salaya-Velazquez et al., 2020), suggesting that orosensory properties of these sweeteners are sufficient to activate the dopaminergic system.

Proteins of the Fos family, including the transcription factors c-Fos, FosB, Fra1, Fra2, and ΔFosB, are characterized by rapid transient expression in specific brain regions after the administration of many drugs of abuse. These transcription factors are considered markers of neuronal activity (Nestler et al., 2001). The expression of ΔFosB increases in dynorphin/substance P-containing cells, a subset of medium spiny neurons that are located in the dorsal and ventral striatum after the chronic administration of numerous addictive drugs and substances (Nestler et al., 2001), including sucrose (Wallace et al., 2008), thereby promoting the development of addictive behaviors. If stevia consumption activates dopaminergic regions through the expression of ΔFosB similarly to sugar and drugs of abuse, then housing animals in enriched environments may be hypothesized to decrease stevia consumption and ΔFosB immunoreactivity in the reward system after stevia consumption. To test this hypothesis, rats were housed under EE conditions and then given intermittent access to stevia consumption. We assessed addiction-like effects and changes in ΔFosB in three brain structures that are related to the reward system in male Wistar rats.

2. Materials and methods

2.1. Animals

Male Wistar rats were obtained by controlled crossbreeding and weaned on postnatal day 21 (PND21). They were given *ad libitum* access to standard laboratory food and water except during stevia consumption. They were housed under controlled temperature and humidity and a 12 h/12 h reverse dark/light cycle (lights off at 9:00 AM). All the experimental procedures were strictly conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and the official Mexican guidelines (NOM-062-ZOO-1999, 1999). Every effort was made to minimize animal discomfort and sample sizes according to the Reduce, Replace, and Refine (3 R) principles of preclinical research.

2.2. Experimental design

On PND21, 28 rats were randomly assigned to either EE ($n = 16$) or a SE ($n = 12$) for 30 days (PND21–51) with *ad libitum* access to water and food (Nutri-cubos, Purina, Agribrands Purina Mexico, Mexico City, Mexico). On experimental day 31 (PND52), four animals from each housing group were anesthetized and perfused to obtain brains and determine basal ΔFosB immunoreactivity by immunohistochemistry. The remaining animals ($n = 8$ EE, $n = 8$ SE) were used to measure stevia consumption *via* the two-bottle intermittent access paradigm for 21 days (*i.e.*, on PND52, PND54, PND56, PND59, PND61, PND63, PND66, PND68, and PND70). The remaining four rats in the EE group were used for other experiments. On PND73, 48 h after the last stevia consumption session, brains of four rats from both the EE and SE groups were obtained to measure ΔFosB-immunoreactive cells in reward-related brain areas, including the NAc (core and shell subregions), CPu (medial and lateral subregions), and prefrontal cortex (PFC; PrL and infralimbic cortex [IL] subregions). The number of ΔFosB-positive cells was compared between housing conditions (basal EE *vs*. basal SE) and between treatments (basal *vs*. stevia consumption), with analyses of interactions between these factors (Fig. 1).

2.3. Housing conditions

Animals were exposed to SE or EE housing conditions from PND21 to PND51. Animals in the SE group were housed in groups of four animals per cage in transparent acrylic boxes (45 cm length × 30 cm width × 20 cm height) under standard housing conditions. Animals in the EE group were housed in groups of eight animals per cage (145 cm length × 60 cm width × 55 cm height). The EE cages were larger than in previous studies (Veena et al., 2009; Venebra-Muñoz et al., 2014; Scarola et al., 2019) to provide extra space for the animals. The EE animals had access to various non-toxic plastic objects, such as colored balls, toys, ladders, polyvinylchloride tunnels, two running wheels, climbing ropes, and nesting material that stimulated exploratory behavior. The objects were changed twice weekly to maintain novelty. Rats in the SE group were reared in social groups of four per cage as commonly used for standard conditions. Rats in the EE group had greater social interaction because they were housed in groups of eight animals per cage, similar to previous reports (Stairs et al., 2006; Venebra-Muñoz et al., 2014), from PND21 to PND51. Then these rats in the EE group were then housed in groups of four animals per cage from PND52 to PND73 in the same enrichment cage.

2.4. Stevia consumption

Based on our previous experiments (unpublished data), stevia extract (Gapélli Ingredients, Mexico City, Mexico) was dissolved in fresh water to produce a 0.2% solution, which was prepared daily to preserve its taste. Stevia consumption was measured using an intermittent access

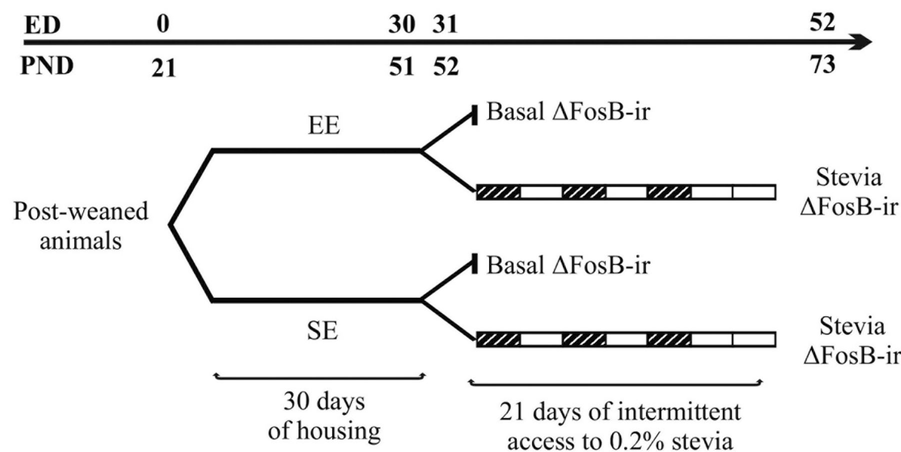

Fig. 1. Schematic representation of the experimental design. EE, enriched environment; SE, standard environment; ED, experimental day; PND, postnatal day; ir, immunoreactivity. Striped rectangles during intermittent access represent 24-h stevia consumption for 3 weeks.

paradigm that was adapted from Simms et al. (2008). This paradigm induces higher voluntary consumption compared with daily consumption paradigms (Simms et al., 2008). On PND52, the animals were given access to two bottles of stevia in three 24-h sessions per week for 3 weeks, starting on Monday. Rats consumed stevia and food *ad libitum*. Rats in the SE group were individually placed in contiguous cages of the same dimensions as their group assignment. Rats in the EE group were separated inside the same EE cage using perforated acrylic subdivisions to delimit similar areas as the cages of SE animals, allowing sensorial contact through smelling, hearing, and seeing other rats. After 24 h of stevia intake, the animals were returned to their respective housing conditions and given *ad libitum* access to food and water for 24 h before the next session of stevia consumption. This pattern was repeated on Wednesdays and Fridays for 21 days for a total of nine stevia consumption sessions (PND52, PND54, PND56, PND59, PND61, PND63, PND66, PND68, and PND70). Average consumption per day and total average consumption were compared between housing conditions.

2.5. *ΔFosB immunoreactivity*

To obtain the detection of only ΔFosB immunoreactivity and avoid full-length FosB detection, the immunohistochemistry procedure was performed 48 h after the last stevia session. On PND73, after the paradigm of intermittent access to stevia consumption, four rats from SE and EE were deeply anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg) and transcardially perfused with a 0.9% saline solution, followed by 4% paraformaldehyde. Their brains were removed and postfixed for 24 h in the same fixative and then equilibrated to sucrose solutions of various gradients (10%, 20%, and 30%, 5 days each). Four coronal sections (40 μm) per rat from the NAc (bregma: +1.8–1.6 mm), CPu (bregma: +1.8–1.6 mm), and PFC (bregma: +3.2–3 mm) were obtained (Paxinos, 2004) in two pairs of two-step separated sections. The tissue sections were extensively washed in phosphate-buffered saline (PBS) solution (5 × 5 min) and incubated in 0.5% hydrogen peroxidase for 10 min. The sections were then incubated in blocking solution that contained PBS, 0.3% Triton X-100%, and 3% normal goat serum for 1 h. The tissue sections were incubated in the same solution with the addition of ΔFosB antibody (1:500; catalog no. sc-48; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 48 h at 4 °C. The sections were washed in PBS and incubated with biotinylated goat anti-rabbit antibody (1:250; catalog no. sc-240, Santa Cruz Biotechnology; Venebra-Muñoz et al., 2014) diluted in blocking solution for 2 h at room temperature. The reaction was visualized with a solution of PBS with 0.06% diaminobenzidine, 1% nickel sulfate, and 1% cobalt chloride. Finally, the sections were mounted on gelatin-coated slides, dried, and dehydrated before being covered with a microscope slide.

2.6. *Cell counting*

ΔFosB-positive cells were identified as black-purple precipitate in the cell nucleus. Photomicrographs were acquired with a digital compound microscope (DMB3–223) with a 40 × objective (14356 μm^2). The number of ΔFosB-immunoreactive cells was counted in both hemispheres by two observers who were blind to the experimental conditions in the following subregions: NAc core and shell, medial and lateral CPu, and PrL and IL of the PFC. Importantly, the striatum is a heterogeneous brain region. Therefore, the CPu was regionalized as medial and lateral according to its associative and sensorimotor functions, respectively (Lafragette et al., 2017). Several sections per structure were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. *Statistical analysis*

The stevia consumption data were analyzed using a two-factor linear model, with time (nine sessions of stevia consumption) as the within-subjects factor and housing condition (SE and EE) as between-subjects factor. The ΔFosB immunoreactivity data were analyzed using two-way ANOVA, with housing condition (EE and SE) and treatment (basal and stevia consumption) as between-subjects factors. Significant effects in the ANOVA were followed by the Holm-Sidak *post hoc* test. Values of $p \leq 0.05$ were considered statistically significant. All analyses used the R open source software environment (R Core Team, 2018). The results are expressed as mean ± SEM.

3. Results

3.1. *Stevia consumption*

The statistical analyses revealed significant effects of time ($F_{8,112} = 7.403$, $p < 0.001$) and housing ($F_{1,112} = 18.758$, $p < 0.001$) and a significant time × housing interaction ($F_{8,112} = 2.850$, $p = 0.006$). The *post hoc* analysis of the time factor revealed that the lowest stevia consumption occurred on the first experimental day (36.3 ± 3.74 ml on experimental day 31) compared with the subsequent days (50.1 ± 4.19 ml on day 33, 46.8 ± 4.81 ml on day 35, 50.9 ± 3.27 ml on day 38, 50.4 ± 2.79 ml on day 40, 52.2 ± 5.64 ml on day 42, 56.4 ± 1.56 ml on day 45, 59.1 ± 3.08 ml on day 47, 54.7 ± 2.84 ml on day 49). The minimum consumption of stevia on day 31 significantly differed from days 33, 38, 40, 42, 45, 47, and 49.

The average total stevia intake in EE rats was 28% lower than in SE rats (Fig. 2B). The analysis of interactions between factors showed that the SE group consumed higher volumes of stevia on all experimental

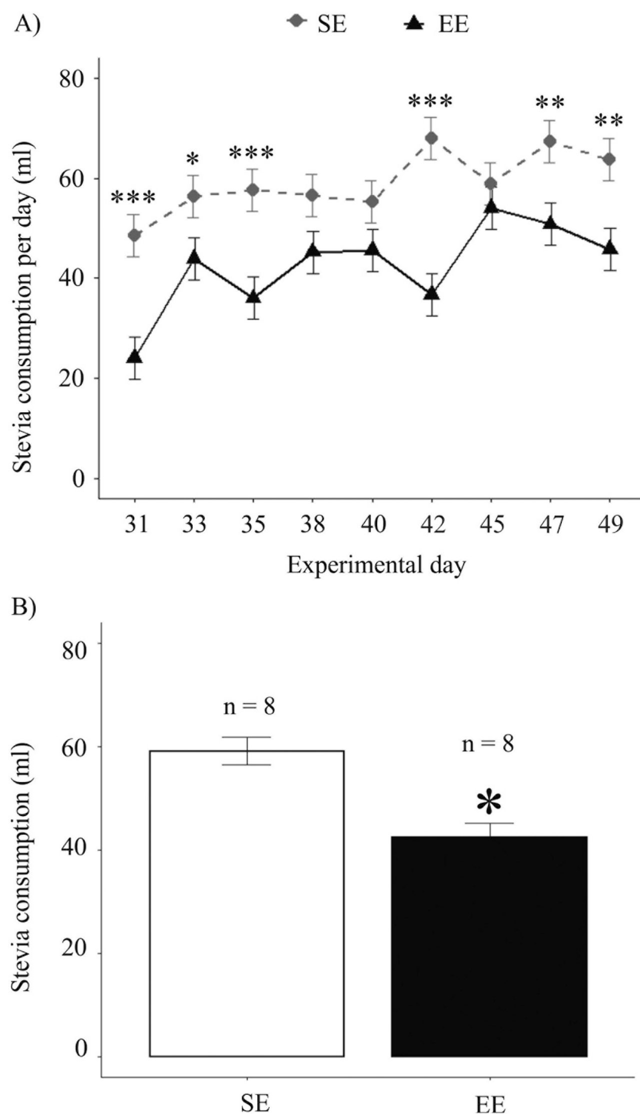

Fig. 2. Differences in stevia consumption between rats in the standard environment (SE) and enriched environment (EE) groups. (A) Rats in the EE group consumed less Stevia solution than rats in the SE group over the nine days of intermittent access. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *vs*. EE group on same day. (B) The overall mean of stevia consumption was lower in the EE group than in the SE group. * $p < 0.05$, *vs*. SE group (Holm-Sidak test). $n = 8$ rats/group.

days (48–68 ml) compared with the EE group (24–54 ml). Rats in the EE group consumed 12–31 ml less stevia than SE rats. Intake was statistically different between groups on experimental days 31, 33, 35, 42, 45, 47, and 49 (Fig. 2A).

3.2. ΔFosB-positive cells

ΔFosB expression in the NAc core and shell, medial and lateral CPu, and PrL and IL of the PFC was compared between the SE and EE groups before (basal) and after stevia consumption.

3.2.1. Number of ΔFosB-positive cells in the NAc

ΔFosB immunoreactivity in the NAc core significantly differed between treatments, in which stevia produced a higher number of positive cells compared with the basal condition ($F_{1,60} = 52.795$, $p < 0.001$), with a significant housing × treatment interaction ($F_{1,60} = 75.525$, $p < 0.001$) but no main effect of housing ($F_{1,60} = 0.469$, $p = 0.49$). Similar effects were observed for the NAc shell, with a significant effect of treatment ($F_{1,60} = 11.873$, $p = 0.001$) and a significant housing × treatment interaction ($F_{1,60} = 89.123$, $p < 0.001$) but no main effect of housing ($F_{1,60} = 2.996$, $p = 0.08$). The *post hoc* tests revealed that the number of ΔFosB-positive cells in the NAc was 80–86% higher in the EE group than in the SE group under basal conditions in the NAc core and shell. After consuming stevia, the EE group exhibited 36% and 30% lower ΔFosB-immunoreactive cells in the NAc core and shell, respectively, compared with the SE group. ΔFosB-immunoreactive cells in the EE group did not change in the NAc core with stevia consumption compared with the basal condition but were 25% lower in the NAc shell after stevia consumption. Increases of 160% in the NAc core and 100% in the NAc shell were observed in SE animals (Fig. 3A, B, 4).

3.2.2. Number of ΔFosB-positive cells in the CPu

ΔFosB immunoreactivity in the medial CPu significantly differed between housing conditions (SE group: 71.6 ± 5.41, EE: 59.5 ± 3.02; $F_{1,60} = 8.144$, $p = 0.006$) and treatments (basal: 55.2 ± 2.95, stevia: 75.9 ± 5.02; $F_{1,60} = 23.565$, $p < 0.001$), with a significant housing × treatment interaction ($F_{1,60} = 48.413$, $p < 0.001$; Fig. 3C). ΔFosB immunoreactivity in the lateral CPu also significantly differed between housing conditions (SE: 38.3 ± 3.74, EE: 20.7 ± 1.39; $F_{1,60} = 32.099$, $p < 0.001$) and treatments (basal: 21.1 ± 1.50, stevia: 37.8 ± 3.76; $F_{1,60} = 28.986$, $p < 0.001$), with a significant housing × treatment interaction ($F_{1,60} = 13.492$, $p < 0.001$; Fig. 3D). The number of ΔFosB-positive cells in the medial CPu was 39% higher in the EE group than in the SE group under basal conditions, with no such effect in the lateral CPu. After stevia consumption, ΔFosB immunoreactivity was 43% lower in the medial CPu in the EE group compared with the SE group and 55% lower in the lateral CPu. ΔFosB-immunoreactive cells in the EE group did not change in the medial or lateral CPu after stevia consumption, whereas ΔFosB expression increased 108% in the medial CPu and 116% in the lateral CPu in the SE group (Fig. 3C, D, 4).

3.2.3. Number of ΔFosB-positive cells in the PFC

ΔFosB immunoreactivity in the PrL significantly differed between housing conditions ($F_{1,60} = 10.742$, $p = 0.002$) and treatments ($F_{1,60} = 4.497$, $p = 0.03$), with no housing × treatment interaction ($F_{1,60} = 2.897$, $p = 0.09$). ΔFosB immunoreactivity in the IL significantly differed between housing conditions ($F_{1,60} = 4.247$, $p = 0.04$) and treatments ($F_{1,60} = 4.061$, $p = 0.04$), with no housing × treatment interaction ($F_{1,60} = 3.402$, $p = 0.07$). The *post hoc* tests showed that both PFC subregions had a higher number of ΔFosB-immunoreactive cells ($p < 0.05$) in the EE group compared with the SE group (EE IL: 60.4 ± 4.79, SE IL: 49.1 ± 3.12, EE PrL: 64.8 ± 5.34, SE PrL: 46.2 ± 2.52). The number of ΔFosB-immunoreactive cells significantly decreased ($p < 0.5$) after stevia intake compared with the basal condition (stevia IL: 49.2 ± 2.64, basal IL: 60.3 ± 5.08, stevia PrL: 49.5 ± 2.26, basal PrL: 61.5 ± 5.62). The trend of differences was attributable to the higher number of ΔFosB-immunoreactive cells in the EE basal condition, which significantly differed ($p < 0.05$) from the SE basal and EE stevia conditions in both PFC subregions (Fig. 3E, F, 4).

4. Discussion

The present study assessed the effects of EE on the consumption of a natural sweetener, stevia, and expression of the transcription factor ΔFosB in brain structures that are related to the reward system in male Wistar rats. Housing under EE conditions from weaning through adolescence decreased stevia consumption in adulthood and reduced the number of ΔFosB-immunoreactive cells that were induced by stevia consumption in the NAc and CPu but not in the PFC. Fig. 4.

The intermittent access paradigm is useful for inducing high voluntary consumption compared with daily consumption paradigms (Simms et al., 2008). The intake of higher volumes of test substances under intermittent access conditions is interpreted as an addiction-related

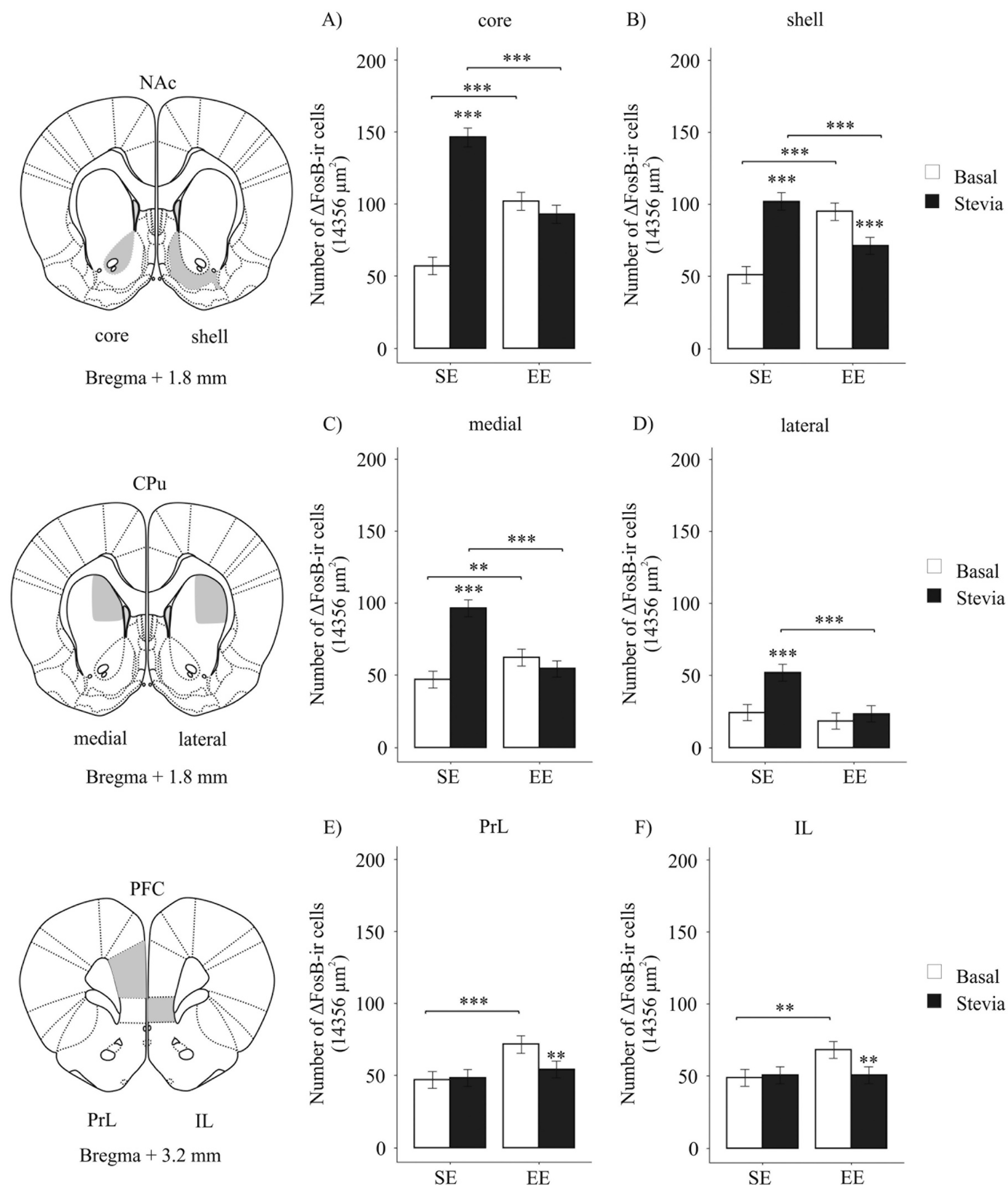

Fig. 3. Number of ΔFosB-positive cells in the enriched environment (EE) and standard environment (SE) groups. The gray areas in the plates show where ΔFosB-immunoreactive cells were counted in the nucleus accumbens (NAc), caudate putamen (CPu), and prefrontal cortex (PFC). The histograms show the results of basal (white bars) and stevia (black bars) consumption. $n = 16$ sections/group. Asterisks directly over the black bars show significant differences between treatments within each environment. Asterisks over the horizontal lines show significant differences between environments. $^{**}p < 0.01$, $^{***}p < 0.001$ (Holm-Sidak test).

behavior (Rada et al., 2005; Simms et al., 2008). We hypothesize that the orosensory properties of stevia, a natural non-caloric sweetener, promote consumption through the activation of dopaminergic systems, similar to glucose and other sweetened substances (Avena et al., 2006; Salaya-Velazquez et al., 2020).

In the present study, lower stevia consumption in EE rats suggests that housing conditions with multisensory stimuli can attenuate the higher consumption of solutions that are sweetened with a non-caloric sweetener. These results are consistent with previous studies in which exposure to physical activity reduced the consumption of high-fat and high-sucrose foods (Moody et al., 2015). In contrast, rats that are housed under SE conditions exhibit an increase in sucrose consumption compared with rats that are housed under EE conditions (Grimm et al., 2016). The novel context of EE and social and cognitive interactions reduce the hedonic drive to consume sucrose or drugs of abuse (Glueck et al., 2018; Sikora et al., 2018). Rats that are housed under EE conditions exhibit a lower preference for these substances. Furthermore, several studies found that EE reduced the levels of corticosterone and adrenocorticotropic hormone, stress-related hormones that at high concentrations are correlated with the high consumption of natural and artificial rewards, such as sugars and drugs of abuse (Grimm et al., 2016; Mahati et al., 2016; Lopes et al., 2018). The decrease in stevia

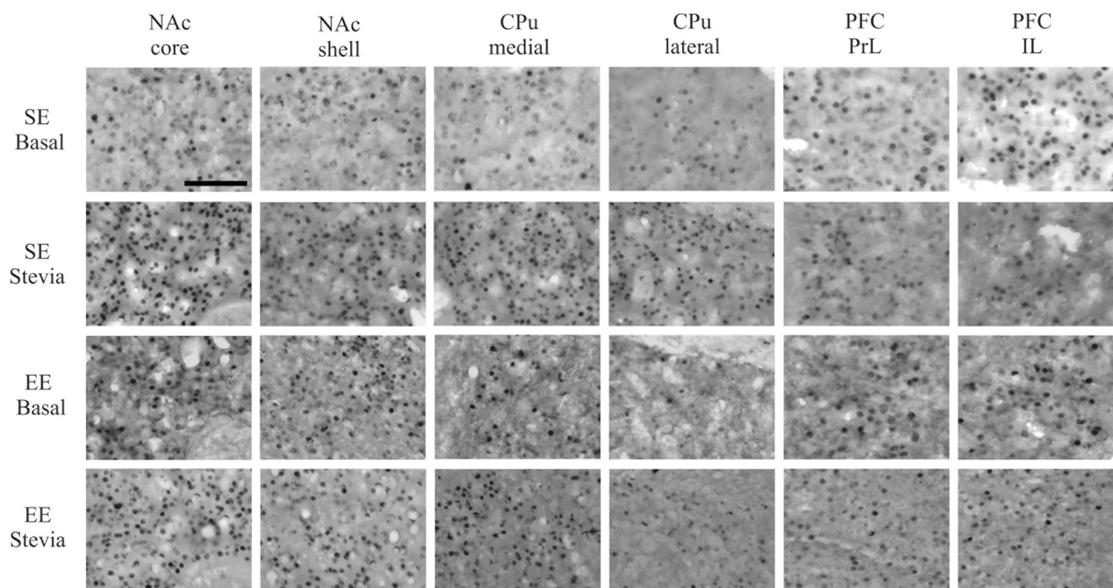

Fig. 4. Photomicrographs of ΔFosB immunoreactivity in various brain regions in the enriched environment (EE) and standard environment (SE) groups. Columns represent each subregion where ΔFosB-positive cells were quantified (40 × magnification). ΔFosB-positive cells were identified as black-purple precipitate in the cell nucleus. NAc, nucleus accumbens; CPu, caudate putamen; PFC, prefrontal cortex; PrL, prelimbic cortex; IL, infralimbic cortex. Scale bar = 50 μm.

consumption in EE animals may have occurred because of decreases in stress-related hormones that were induced by this environment. This possibility requires further investigation.

We observed no escalation of stevia consumption in SE animals. Therefore, we cannot make any definitive conclusions about the addictive potential of this sweetener. An addiction-related behavior is associated with higher rates of drug self-administration. Similar to drugs of abuse, sugar releases opioids and dopamine, and rats prefer sweeteners like saccharin when they are allowed a free choice between sweetened water *vs*. intravenous cocaine (Lenoir et al., 2007). Thus, stevia may indeed have a similar addictive potential as sugar (Avena et al., 2008). Although there are similarities between sweeteners and drugs of abuse, the addictive potential of stevia is currently unknown. The addictive potential of stevia requires further assessment in operant self-administration paradigms in animals that are exposed to food deprivation, abstinence, and other experimental conditions. The intermittent access paradigm is an unconditioned procedure that has been used to observe an escalated intake of sucrose in rats under a 12 h-daily privation of food for 21 days leading to constant dopamine release *versus* daily *ad libitum* sucrose intake (Rada et al., 2005). We used a modified access procedure which consisted in 24/24 h intermittent access in non-private rats, and we observed no escalation of stevia consumption. These findings do not allow us to conclude that there was addiction-related behavior. The relative salience of drugs is further augmented by negative affective states (Hogarth and Field, 2020). We speculate that our rats that were housed under SE conditions were not in a negative affective state (*e.g.*, non-food-deprived and not switched to unfamiliar cages) and thus did not escalate their stevia intake. The acute effect in the stevia intake on experimental day 31 revealed that the taste of stevia at the first exposure was not as appetitive for EE rats as for SE rats. Intermittent exposure to stevia during the subsequent intake sessions revealed a sustained higher preference for the sweetener in SE rats compared with EE that was significant on most experimental days, suggesting a protective effect of EE against the increase in stevia consumption. Three weeks could be considered a relatively short interval of testing, but we believe it was sufficient to differentiate effects of SE *vs.* EE housing. The present results suggest that EE decreases natural reward intake, likely by decreasing stevia's hedonic threshold, which leads to greater sensitivity to the sweetener, similar to sugar and drugs of abuse.

The possible effects of interrupting EE should be considered. Nader et al. (2012) switched 2-month EE mice to 1 week SE housing (*i.e.*, change from 60 cm × 38 cm × 20 cm cage to 25 cm × 20 cm × 15 cm cage). These mice exhibited emotional distress in the open field, forced swim, and splash tests (Nader et al., 2012). However, the possibility that an interruption of EE occurred in the present study when we tested the animals in the intermittent access paradigm can be discarded because EE was not interrupted. Our EE rats were never switched to a standard cage. They continued to be allowed individual access to stevia bottles within the same EE cage in delimited areas to record individual intake. These rats were only confined to individual areas that were delimited by perforated acrylic subdivisions within the EE cage. These smaller areas were the same size as the standard cages that were used for SE rats during the intermittent access protocol. The EE rats were not deprived of other types of sensory interaction, such as contact through nostrils, communication by vocalization, and visibility, which could maintain the protective effect of EE. Moreover, the observed behaviors after returning EE rats to EE housing conditions (*i.e.*, removal of the acrylic subdivisions in the EE cage) did not suggest signs of distress (data not available). Unfortunately, we did not measure distress-related behaviors or stress-related hormones because we assumed the low probability of stress-induced responses under our experimental conditions. We also did not subject the EE rats to social isolation. Social isolation is known to enhance the rewarding effects of drugs (Solinas et al., 2009) and sugar (Brenes and Fornaguera, 2008), leading to an increase in ΔFosB immunoreactivity. Our results were the opposite (*i.e.*, decrease in ΔFosB immunoreactivity) and agree with the protective effect of EE. Thus, the ΔFosB results are consistent with the absence of stress in EE rats.

The expression of ΔFosB in reward-related nuclei is one of several mechanisms whereby drugs of abuse produce stable changes in the brain and contribute to the establishment of addictive-related behaviors (Nestler et al., 2001). Although little is known about the effects of natural reward consumption *via* sweet beverages on the expression of ΔFosB and its modulation by EE, EE is well known to regulate the expression of ΔFosB, which prevents these behaviors (Stairs and Bardo, 2009). In the present study, EE rats under basal conditions exhibited an increase in the number of ΔFosB-positive cells in the NAc core and shell, medial CPu, and PrL and IL of the PFC compared with SE rats under basal conditions. These results are consistent with previous studies that reported that animals that were housed under EE conditions exhibited higher basal ΔFosB immunoreactivity in the ventral and dorsal striatum

(Solinas et al., 2009; Zhang et al., 2014; Lafragette et al., 2017), PFC (Lafragette et al., 2017; Watanasriyakul et al., 2019), and other brain regions that participate in the reward system (Arias-Carrión et al., 2010). This system comprises dopaminergic neurons in the ventral tegmental area and its projections to limbic regions (NAc, CPu, hippocampus, and amygdaloid nuclei) and cortical regions (prefrontal, cingulate, and perirhinal cortex). The dopaminergic system is involved in motor control, learning, motivation, and reward (Arias-Carrión and Pöppel, 2007; Ikemoto et al., 2015). Our results suggest that exposure to motor, sensory, social, and cognitive stimuli under EE conditions increases extracellular dopamine currents from the ventral tegmental area to forebrain and possibly cortical regions, which in turn triggers such molecular mechanisms as the expression of ΔFosB, a transcription factor that is used as an index of long-term neuronal activity that is responsible for the establishment of compulsive behaviors (Nestler et al., 2001). The results from rats that were exposed to EE housing conditions may support activation of the dopaminergic system, reflected by an increase in ΔFosB immunoreactivity in the striatum and PFC.

After chronic stevia consumption, we found that ΔFosB immunoreactivity was higher in subregions of the NAc and CPu in SE animals compared with EE animals. These results are similar to other studies of drugs and natural rewards, in which ΔFosB immunoreactivity increased in the ventral and dorsal striatum in SE animals after reward exposure, coupled with an increase in cocaine (Solinas et al., 2008, 2009; Zhang et al., 2014; Lafragette et al., 2017), and motivated-hunger rats (Zhang et al., 2014), nicotine (Venebra-Muñoz et al., 2014), and sugar intake and sexual behavior (Wallace et al., 2008) compared with EE animals. Our data suggest that stevia consumption increases activity of the dopaminergic system in SE rats, reflected by an increase in ΔFosB-positive cells in reward-related nuclei, similar to drugs of abuse. However, the mechanisms that underlie activation of the dopaminergic system by stevia consumption are not well understood. Several studies have reported that the sweet taste of sugar increases extracellular surges of dopamine from the ventral tegmental area to NAc (Rada et al., 2005), and sugar intake increases ΔFosB expression in the NAc (Wallace et al., 2008; Christiansen et al., 2011), suggesting activation of the dopaminergic system by means of gustatory signals (Avena et al., 2006). Sugar and artificial sweeteners, such as sucrose and saccharin molecules (Chen et al., 2011), interact with sweet-taste receptors, specifically T1R2 and T1R3 G protein-coupled receptor subunits (Margolskee, 2002; Zhang et al., 2003). These signals travel throughout facial and glossopharyngeal afferent nerves to the geniculate and petrosal ganglia. Taste signals from the ganglia travel to the medulla oblongata, the pons, limbic nuclei, the thalamus, and the primary and secondary gustative cortex (Yarmolinsky et al., 2009). Additionally, some studies have shown that activation of the dopaminergic system that is induced by sweet taste can occur directly through catecholaminergic neurons from the nucleus of the solitary tract in the medulla oblongata and directly from the lateral hypothalamic area to the ventral tegmental area and NAc (Roberts et al., 2017; Leigh and Morris, 2018). Striatal neurons express two types of dopaminergic receptor subunits: D_1 receptors (which are positively coupled to adenylate cyclase) and D_2 receptors (which leads to the inhibition of adenylate cyclase; Jones et al., 1992; Hoebel et al., 2009). Moreover, several studies have found that sugar consumption increases dopamine currents and dopamine binding to D_1 receptors in the NAc, coupled with a decrease in D_2 receptor binding (de Araujo, 2016; Leigh and Morris, 2018). Interestingly, this pattern of antagonism between D_1 and D_2 receptors has also been reported in studies of drugs of abuse (*e.g.*, cocaine), in which the accumulation of ΔFosB in striatal D_1 receptors by chronic cocaine administration is higher than the accumulation of ΔFosB in D_2 receptors. These findings suggest that the accumulation of ΔFosB in D_1 receptor-expressing neurons is related to the rewarding properties of both natural and artificial rewards (Lafragette et al., 2017). Studies have shown that voluntary gustative stimulation by saccharin increases c-Fos-like immunoreactivity (an immediately-early gene response to multiple extracellular stimuli that presents specific, rapid, and transient expression) in the nucleus of the solitary tract and parabrachial nucleus (Chen et al., 2011) and in the NAc and insular cortex after intraperitoneal administration of this sweetener (Soto et al., 2017). A computational approach showed that steviol glycosides interact with sweet taste receptors in the tongue (Mayank and Jaitak, 2015). Thus, the orosensory properties of stevia may trigger gustatory signals to the primary gustative cortex and dopaminergic nuclei, similar to the actions of sugar. A previous study showed that sucralose consumption increased ΔFosB levels in the NAc, PFC, and amygdala in adult male Wistar rats, suggesting activation of the dopaminergic system (Salaya-Velazquez et al., 2020). Therefore, the increase in ΔFosB immunoreactivity that is caused by intermittent stevia consumption in SE animals in the present study may be related to the activation of dopamine D_1 receptors and concomitant ΔFosB expression through the relaying of gustative signals, similar to the actions of sugar and drugs of abuse despite differences in their chemical structures.

Furthermore, EE blunted ΔFosB immunoreactivity in the NAc and CPu, coupled with a decrease in stevia consumption, compared with SE animals. These results are similar to studies that reported that EE decreased sugar (Brenes and Fornaguera, 2008), cocaine (Lafragette et al., 2017), heroin, and nicotine (Sikora et al., 2018) administration and decreased ΔFos immunoreactivity in reward-related brain regions (Venebra-Muñoz et al., 2014; Grimm et al., 2016; Lafragette et al., 2017). Therefore, EE appears to confer a neuroprotective effect against the development of addiction-related behaviors, but the molecular mechanisms that underlie this protective effect require further study. Solinas et al. proposed that chronic cocaine administration in EE animals alters kinase and phosphatase activity, which dephosphorylates ΔFosB, leading to a decrease in ΔFosB levels (Solinas et al., 2009). The stimulation of D_2 receptors in the striatum in EE animals was reported to produce an aversive response to cocaine (Lafragette et al., 2017). It has been suggested that the accumulation of ΔFosB might repress further induction of ΔFosB after cocaine use, perhaps indicating a negative feedback loop (Zhang et al., 2014). These findings could explain the neuroprotective effect of EE against stevia consumption that was observed in the present study.

With regard to the effect of stevia consumption on ΔFosB immunoreactivity in the PrL and IL subregions of the PFC, the results showed that EE produced higher basal levels of ΔFosB in both subregions compared with SE animals, though these levels decreased in EE animals after stevia consumption. The PFC integrates information from several brain structures, including the mesolimbic pathway, which plays an important role in addiction (Solinas et al., 2010). The PFC also regulates activity of the hypothalamic-pituitary-adrenal axis in response to stressors (Ronzoni et al., 2016; Watanasriyakul et al., 2019) and is related to decision making (Kirkpatrick et al., 2013). The higher basal ΔFosB immunoreactivity in the PrL and IL in EE animals in the present study may be related to higher information integration and plasticity processes that derived from multisensory stimuli to which EE animals were exposed. Neural activity of the mPFC, including its PrL and IL subregions, increases during the ingestion of a sucrose solution, suggesting an increase in extracellular dopamine (Petykó et al., 2009). Moreover, sucralose intake increases ΔFosB immunoreactivity in the IL (Salaya-Velazquez et al., 2020). Our results contrast with these observations, in which ΔFosB immunoreactivity did not increase in the PFC in SE and apparently decreased in EE animals after stevia consumption. Nevertheless, this observation is consistent with Venebra-Muñoz et al. (2014), who found that ΔFosB immunoreactivity in the PFC decreased in EE animals after nicotine intake (Venebra-Muñoz et al., 2014). The increase in PFC activity that was induced by sucrose consumption may be related to caloric and orosensory properties of sugar relative to only orosensory properties of stevia. Increases in ΔFosB-positive cells in such studies may be related to daily access (rather than more intermittent access) to sucralose solution. Further studies are needed to elucidate the role of the PFC in processing sweet taste signals.

The present study has limitations. The present study evaluated

changes before and after stevia consumption (PND52 and PND74, respectively), which implies a 3-week interval in a within-subjects design. Although we cannot discard the possible influence of age on behavior and immunoreactive cells, all of the rats were in young adulthood and underwent the same manipulations. The assessment of water *vs*. stevia consumption was not considered. Therefore, future studies are needed to understand the mechanisms that underlie the possible hedonic actions of stevia and its activation of the reward system. Although the experimental design was presumed to not subject the rats to stress, the measurement of stress-related hormones would confirm this assumption. Finally, the present study had a relatively low number of rats per group ($n = 4$) for the assessment of ΔFosB immunoreactivity. Nonetheless, we obtained four brain sections per rat and thus 16 sections per group, which was sufficient to identify significant changes in immunoreactivity and is consistent with the 3 R principles (Russell and Burch, 2005).

Our findings may hold clinical relevance for the development of addiction therapies in animal models and humans because stevia extract could be used instead of sugar after periods of drug withdrawal, based on its rewarding properties that were observed in the present study. The use of stevia may avoid concomitant metabolic issues that are associated with chronic sugar consumption.

5. Conclusions

The environment is a critical factor in reward-seeking behavior, particularly natural rewards. Animals that were housed under EE conditions consumed less stevia compared with animals that were housed under SE conditions, demonstrating the rewarding properties of EE. The expression of ΔFosB was lower after stevia consumption in dopaminergic regions in EE animals compared with SE animals, similar to drugs of abuse despite differences in their chemical structures. The present findings suggest that the orosensory properties of stevia concomitantly activate the dopaminergic system and induce ΔFosB, and EE confers neuroprotection against chronic exposure to natural rewards.

CRediT authorship contribution statement

I.D.S.V: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Verification. **P.S.A:** Resources, Writing – review & editing. **L.I.P.M:** Resources, Writing – review & editing. **P.P.C:** Data curation, Writing – review & editing, Supervision. **B.B.M:** Methodology, Formal analysis, Data curation, Writing – review & editing, Supervision, Verification. **A.V.M:** Conceptualization, Methodology, Writing – review & editing, Supervision. All authors reviewed, discussed, and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare there are no conflicts of interest.

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