Differential activity pattern of c-Fos in the nucleus accumbens between adult and aged rats during flavor recognition memory.

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1. Abstract

Previous studies have addressed the role of the nucleus accumbens core (NAcbC) and shell (NAcb-Shell) in taste aversion learning and in the processing of taste palatability which is affected by aging. However, little is known about its implication in safe taste memory and the aging impact. To explore the role of the NAcb in flavor neophobia and its attenuation during aging, we applied c-Fos immunohistochemistry as an index of neural activity of the NAcbC and NAcb-Shell. Twenty one adult (5-month-old) and 24 aged (24-month-old) male Wistar rats were exposed to a 3% cider vinegar solution for 1, 2 or 6 consecutive days (*n*=7 adult and *n*=8 aged rats per group). Aged rats exhibited slower attenuation of flavor neophobia than adult rats. Adult rats showed increased NAcb-Shell c-Fos activity on day 2 compared to days 1 and 6, while this increase was delayed to day 6 in aged rats. There were no differences in the number of NAcbC c-Fos positive cells. This suggests that changes in the activity of neural circuits of palatability processing during normal aging could contribute to the slower attenuation of flavor neophobia in aged rats.

Key Words: Accumbens; Aging; Attenuation of Neophobia; Flavor; c-Fos; Recognition Memory; Taste.

2. Introduction

The ingestion of novel flavors is a model of recognition memory useful to study the neural and molecular mechanisms of memory (Federico Bermúdez-Rattoni, 2004). After having ingested a novel substance, a taste memory of its flavor is generated and stored among several brain regions in parallel (Bermudez-Rattoni, 2014; De la Cruz et al., 2008). If the flavor is not followed by negative consequences, a safe taste memory is generated and it allows the recognition of the flavor as safe. Therefore the animal increases consumption of the flavor over repeated exposures, showing an attenuation of neophobia (Federico Bermúdez-Rattoni, 2004).

Different brain areas have been related with the formation of the safe taste memory, such as the hippocampus (Balderas, Morin, Rodriguez-Ortiz, & Bermudez-Rattoni, 2012; Grau-Perales, Levy, Fenton & Gallo, 2019b), the insular cortex (Bahar, Dudai, & Ahissar, 2004; Bermudez-Rattoni, 2014; Lin, Roman, Arthurs, & Reilly, 2012; Miranda, Ferreira, Ramírez-Lugo, & Bermúdez-Rattoni, 2003; Moraga-Amaro, Cortés-Rojas, Simon, & Stehberg, 2014), the basolateral amygdala (Gómez-Chacón, Gámiz, & Gallo, 2012), the piriform cortex(Grau-Perales et al., 2019a), the perirhinal cortex (Gómez-Chacón, Morillas, & Gallo, 2015; Gutiérrez, De la Cruz, Rodriguez-Ortiz, & Bermudez-Rattoni, 2004; Morillas, Gómez-Chacón, & Gallo, 2017) and also the nucleus accumbens (Pedroza-Llinás et al., 2009; Ramírez-Lugo et al., 2007).

The nucleus accumbens (NAcb), a well known component of the reward system, has been involved in different processes related to addiction and feeding (Bassareo & Di Chiara, 1999; Bassareo, De Luca, & Di Chiara, 2002; Yamamoto, 2006) as well as in the hedonic shifting of flavors (Shimura, Kamada, and Yamamoto, 2002; Yamamoto, 2006). Regarding the implication of the NAcb in the formation of taste memories, pharmacological studies have shown that both the nucleus accumbens

core (NAcbC) and shell (NAcb-Shell) are involved in aversive taste responses(S. Fenu et al., 2001), but only NAcb-Shell seems to be involved in the case of safe taste memory formation(Pedroza-Llinás et al., 2009; Ramírez-Lugo et al., 2007).

Even though changes in performance of learning and memory tasks related to aging have been described in terms of functionality decay, this is not applicable to taste memory, which in fact, seems to be spared -or even enhanced- in the case of aged rats (Gámiz & Gallo, 2011; Gallo, 2018). In fact, the progressive memory decay traditionally related to aging has been selectively associated to spatial memory (Dardou, Dariche & Catarelli, 2008) and hippocampal-dependent tasks. (Manrique, Morón, Ballesteros, Guerrero, & Gallo, 2007; Manrique, Morón, Ballesteros, Guerrero, Fenton & Gallo, 2009).However, aged rats continue to show neophobia when a novel flavor is encountered(Morón & Gallo, 2007a), although the exhibit a slower attenuation of neophobia than younger rats (Gómez-Chacón et al., 2015). This evidences that taste learning proceeds differently throughout life. However, little is known about the neural mechanisms underlying taste recognition memory in aged rats. It has been described a different pattern of c-Fos expression in some brain areas that belong to the taste recognition memory circuit in the case of aged rats, such as the perirhinal cortex (Gómez-Chacón et al., 2015) as well as the posterior piriform cortex (Grau-Perales, et al., 2019a). The aged brains exhibited a higher number of c-Fos positive cells in these areas than the adult brains after the exposure to a highly familiar rather than a novel flavor.

In order to explore the impact of normal aging in the function of nucleus accumbens related to flavor neophobia and its attenuation, we applied c-Fos immunohistochemistry as an index of neural activity of the nucleus accumbens core and shell of adult and aged male Wistar rats which were exposed to a novel flavor solution

for one, two or six sessions. Therefore, a 2 x 3 (age x familiarity) design was applied and the number of c-Fos positive cells was measured in NAcbC and NAcb-Shell separately.

Given the proposed role of NAcb-Shell but not NAcbC in the formation of safe taste memories, an increased number of c-Fos positive cells in NAcb-Shell during the second exposure session rather than the first and the sixth exposure might be expected in adult rats. In addition, as slower attenuation of flavor neophobia revealing slower taste memory formation has been found in aged rats, it can be hypothesized a delay of such increase in the aged NAcb-Shell that would be evident later, during the sixth but not the second exposure to the flavor.

3. Materials and methods

2.1. Subjects

Twenty-one adult (5-month-old) and twenty-four aged (24-month-old) male Wistar rats were used in this experiment. Housing, grouping and general management conditions have been described elsewhere since these are the same animals' brains previously used in Gómez-Chacón et al. (2015). This is in accordance with the reuse rule for reducing the number of animals in research.

All the experimental procedures were performed during the light cycle at the same time each morning in the home cage. Rats were given *ad libitum* food and water until the experiment started, when access to water was restricted. All procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (17-02-15-195), and were in accordance with the European Communities Council Directive 86/609/EEC.

3.2. Behavioral Procedure

The procedure of attenuation of neophobia has been described elsewhere (Gómez-Chacón et al., 2015). Briefly, the behavioral procedure consisted on 15-minute daily drinking sessions. Water intake during the morning drinking period was recorded for five days during the acclimation period in order to get the animals used to the deprivation schedule. Once the water intake was stabilized as baseline consumption (BL) all rats had access to a novel 3% cider vinegar solution during the morning 15-minute drinking session. The amount ingested was recorded by weighing the tubes containing the solution before and after each drinking session.

All the adult and aged rats were randomly assigned to the following familiarity groups: 15 rats were euthanized after drinking the vinegar solution on day 1 (*Novel: n*=7 *Adult; n=8 Aged*); 15 rats were euthanized after drinking the vinegar solution on day 2 (*Familiar-1: n*=7 *Adult*; *n=8 Aged*); and the remaining 15 rats were euthanized after drinking the vinegar solution on day 6 (*Familiar-2: n*=7 *Adult*; *n=8 Aged*).

3.3. Immunohistochemistry.

All the animals were euthanized 90 minutes after the corresponding drinking session depending on the c-Fos immunohistochemical group they were assigned. They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde 90 minutes after drinking the vinegar solution the first (Novel group), second (Familiar-1) and sixth day (Familiar-2). The brains were removed and placed in a 4% paraformaldehyde solution for 4 h at 4ºC before being transferred to a 30% sucrose solution until they sank for cryoprotection. Coronal sections were cut at 20µm in a cryostat (Leica CM1900). Some of the brains from each groups of age were randomly selected so that some sections

were stained with cresyl violet for general morphological study while immunohistochemistry for c-Fos was applied to the rest of sections.

Free floating tissue sections were rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4), incubated for 15 minutes with 3% hydrogen peroxide, rinsed again and incubated in a solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 30 minutes. Slices were transferred to a c-Fos primary antibody (1:10,000; Calbiochem) for 48 h at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Biotinylated goat anti-rabbit IgG, 1:500; Calbiochem) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, 0.4% Triton X-100 and PBS. The sections were rinsed, then processed using the ABC-kit (Vector Laboratories, Burlingame, CA), and the reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA). Finally they were rinsed, mounted on gelatine-bubbed slides, rehydrated with ethanol and xylenes and finally they were cover-slipped.

3.4. Data Acquisition

Images of both hemispheres from brain slices containing the nucleus accumbens core and shell (NAcbC and NAcb-Shell) were captured using a light microscope (Olympus BX41). Slices containing the NAcbC and NAcb-Shell were identified using the *Stereo Investigator* Software (mbf Bioscience) from two coronal sections of the NAcb located at the level of the apparition of the lateral ventricles, approximately at +2,52 and +2,28mm relative to Bregma (Section 1 and Section 2 respectively) according to Paxinos and Watson (2009). Within each section 5 microphotographs at 40X magnification were captured for the NAcbC and the NAcb-Shell according to a dorso-ventral-medio-lateral axis in order to cover the entire extension of both nuclei.

The microphotographs were labeled with the number of its position within each nucleus (ranging from 1 to 5) (See **Figure 1A**).

The number of c-Fos positive cells was obtained using the *Image J* Software (National Institute of Mental Health). For each microphotograph threshold objects (black circular dots over the white background) having specific size (35-150 μm^2) and circularity (0.35-1.00) values matching those c-Fos positive nuclei were automatically identified by the software as c-Fos positive cells. In order to equalize all the microphotographs and cancel out possible background noise, they were previously converted into 8-bit type image and the background was lightened (150.0 pixels). Representative microphotographs of the different experimental groups are shown in **Figure 1B**.

Due to the immunohistochemical procedure used when the brain slices were mounted in the slides, it was impossible to determine to which hemisphere belonged. For this reason, in order to randomize a possible confounding effect, for each section the mean number of c-Fos positive cells was calculated in every microphotograph for both hemispheres.

4. Results.

To assess the correct location of the microphotographs, the perimeter (µm) and area (μm^2) of every region selected as NAcbC and NAcb-Shell was measured. A global mixed ANOVA that included all the groups of animals and the two nuclei only revealed an effect of size [$F(1, 33)=2219.4$; $p<.0001$] and nucleus [$F(1, 33)=9,32$; $p<.01$] that corresponded to a bigger size of the NAcbC relative to the NAcb-Shell. No other effect or interaction was found. This allowed us to ensure that all the microphotographs were captured in the correct location as all the regions selected for every group had the same

perimeter (NAcbC perimeter = 4439,64 ± 20,22 µm; NAcb-Shell perimeter = 5251,78 ± 21,35 µm) and area (NAcbC area = 1091601,13 ± 417 μm^2; NAcb-Shell area = 1023460,59 ± 368 μm^2).

4.1. Behavioral results

The behavioral results are available elsewhere (Gómez-Chacón et al., 2015; Grau-Perales et al., 2019a). In brief, both adult and aged groups exhibited a similar neophobic response to vinegar. However, the attenuation of neophobia was delayed in the aged group in comparison with the adult group. While adult rats required a single exposure to reach the asymptote by the second exposure to vinegar on Day 2, aged rats required two more exposures as there were no differences between the amounts of vinegar drank on Day 4 and the subsequent exposures on Days 5 and 6.

4.2. Attenuation of neophobia: c-Fos analyses.

The mean number of c-Fos positive cells (±SEM) for the three familiarity groups of both ages during the vinegar drinking sessions is shown in the **Figure 2**for both NAcbC and NAcb-Shell. A 2 x 3 bifactorial ANOVA (*Age* X *Familiarity*) was used to compare differences in c-Fos positive cells in the NAcbC and NAcb-Shell.

A 2 x 3 (*Age* X *Familiarity*) two-way ANOVA of the c-Fos positive cells number in the NAcbC did not reveal any significant effect (all *p´*s>.05) (See **Figure 2A**). The same two-way ANOVA 2 x 3 (*Age* X *Familiarity*) of the c-Fos positive cells number in the NAcb-Shell revealed significant effects of *Familiarity* [F(2, 35)=4,021; $p<.05$] and the interaction *Age* X *Familiarity* [F(2,35)=4, 877; $p<.05$]. In order to understand this interaction, further one way ANOVAs were used for each group of age separately. The analysis performed for the adult rats revealed a significant effect of

Familiarity [F(2,16)=5,59; $p<.05$] and further comparisons using Bonferroni-corrected tests revealed a statistically significant higher number of c-Fos positive cells in the Familiar-1 group compared to Novel ($p=.027$) and Familiar-2 ($p=.034$), but no differences between Novel and Familiar-2 ($p=1$). The analysis performed for the aged rats revealed a significant effect of *Familiarity* [F(2,18)=3,72; $p<.05$] and further comparisons using Bonferroni-corrected tests revealed a statistically significant higher number of c-Fos positive cells in the Familiar-2 group compared to Novel ($p=.04$) but not with Familiar-1 ($p=.2$). In brief, adult rats showed higher number of c-Fos positive cells in the NAcb-Shell after drinking the vinegar solution on day 2 compared to days 1 and 6, while aged rats showed higher c-Fos activity on day 6 compared to day 1. Consistently, there were significant differences between adult and aged groups in the number of c-Fos positive cells found in Familiar-1 (p=.012) and Familiar-2 (p=.03) groups in the NAcb-Shell, but not in the NAcbC (all p´s > .3). However, no differences related with taste familiarity were found in the NAcbC of adult and aged rats (See **Figure 2B**).

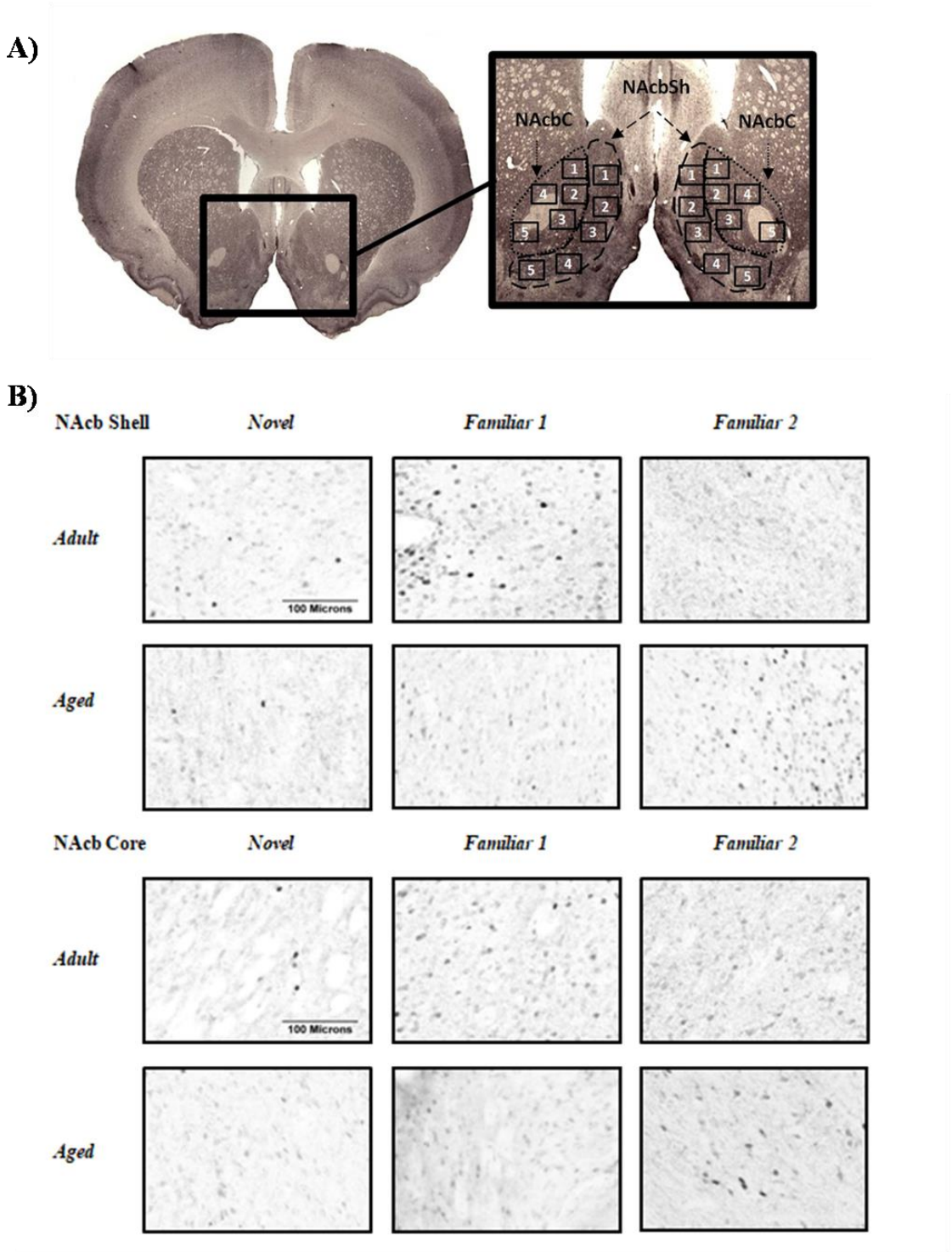

Figure 1:A) Schematics of the microphotographs (40X) of the nucleus accumbens core and Shell. All the microphotographs were captured following a dorso-ventral and medio-lateral axis and were labeled with numbers from 1 to 5 in order to cover the entire extension of both nuclei. **B)**Representative microphotographs obtained at 40X magnification of the medial part of the nucleus accumbens shell (top panel) and the medial part of the nucleus accumbens core (bottom panel).

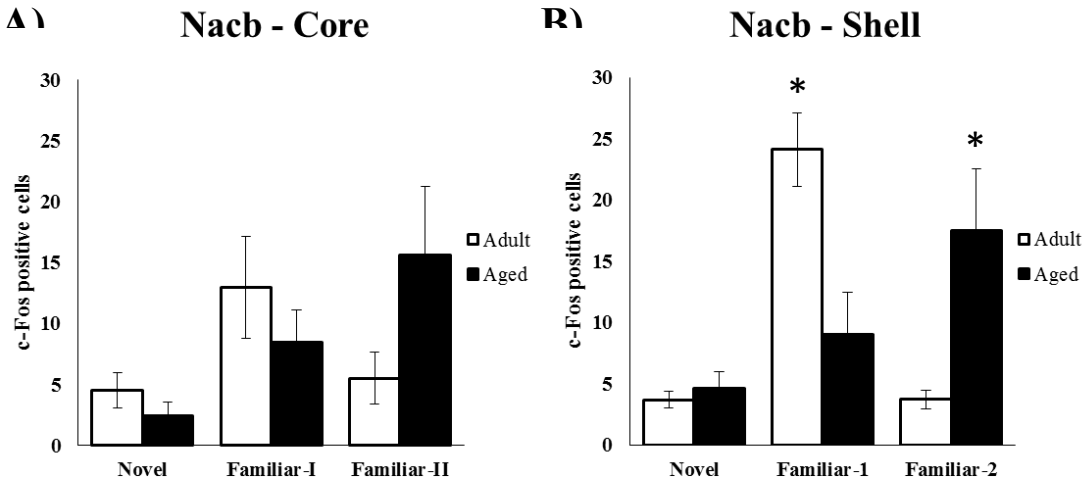

Figure 2: Mean (±SEM) of c-Fos positive cells in the five subregions of the nucleus accumbens core (A) and shell (B) for both groups of age. * symbol represents statistically significant differences (p<.05) compared to Novel group
of the same group of age

4.3. Cell quantification: Cresyl Violet staining.

Some of the brains were chosen for cell quantification semi-randomly, so 2 Cresyl Violet stained brains of each familiarity group (6 adult and 6 aged) were selected for each group of age. A two-way ANOVA 2 x 2 (*Age* X *Region*)was used to compare the number of Cresyl Violet stained cells between adult and aged rats in both NAcbC and NAcb-Shell. The analysis revealedno significant effect of any of the factors nor its interaction (all p´s > .3). The fact that no differences in the total number of stained cells were found in the NAcb-Shell allows us to exclude that the differences found in the number of c-Fos positive cells between adult and aged groups could be due to the effect of age on neuron survival (**Figure 3**).

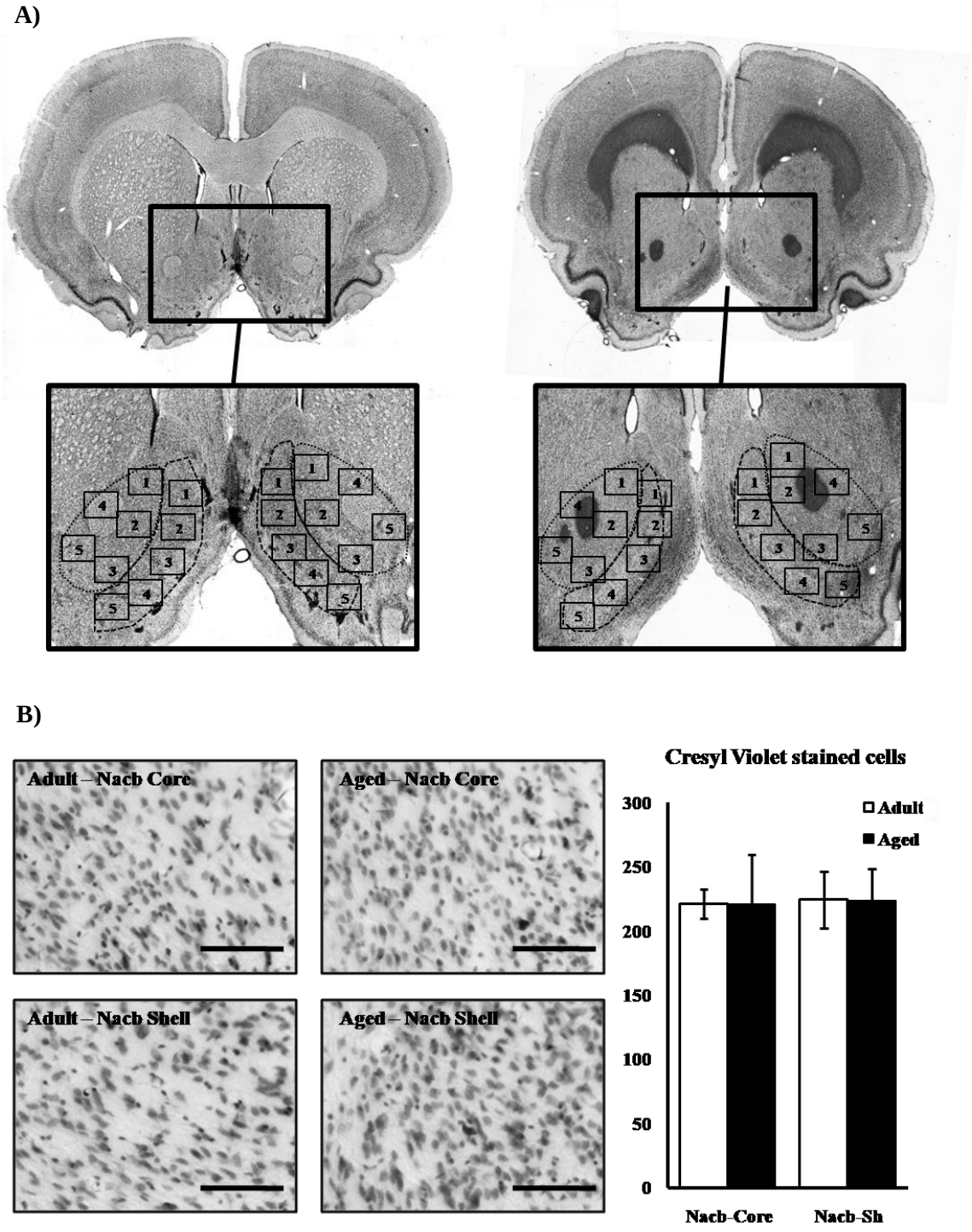

Figure 3: (A) Schematics of the microphotographs (40X) of the nucleus accumbens core and Shell that were stained with Cresyl Violet. All the microphotographs were captured following a dorso-ventral and medio-lateral axis and were labeled with numbers from 1 to 5 in order to cover the entire extension of both nuclei for the adult (left panel) and aged (right panel) groups of age. **B)**Representative microphotographs obtained at 40X magnification of the nucleus accumbens core for adult and aged rats (top panel) and the nucleus accumbens shell for adult and aged rats (bottom panel). On the right, no differences in the counted cells stained with Cresyl Violet. Scale bars = 100 μm.

4. Discussion.

In this experiment we compared the number of c-Fos positive cells in the nucleus accumbens core and shell of adult and aged rats that were exposed to a novel flavor solution for one, two or six consecutive days. Consistent with the fact that the nucleus accumbens has been proposed to participate in flavor memory and the processing of flavor palatability, in adult rats we found an increased number of c-Fos positive cells in the NAcb-Shell after drinking the novel solution twice compared to the first and the sixth time. This increase was not evident in NAcbC, thus confirming our first hypothesis in adult rats. This is consistent with previous findings showing that reversible inactivation by anisomycin(Pedroza-Llinás et al., 2009) and neurotransmitter antagonists(Ramírez-Lugo et al., 2007) of NAcb-Shell prevented the attenuation of taste neophobia.

As it has been previously reported (Gómez-Chacón et al., 2015; Grau-Perales et al., 2019a), the behavioral results indicated that there was attenuation of the neophobic response to the novel flavor in both groups of age. It can be conceived that since the novel flavor was not followed by negative consequences after the first exposure it was classified by the animals as safe so that the neophobic response was attenuated, increasing the biological significance of the flavor (Federico Bermúdez-Rattoni, 2004). The increased activity found in the NAcb-Shell during the second exposure supports its involvement in the formation of the safe flavor memory. Nevertheless, the attenuation of flavor neophobia was delayed in the case of aged rats. Aged rats did not increase consumption in the second exposure to the flavor, suggesting impairment of memory processes involved in familiarization. In fact, the attenuation of neophobia was evident

at the third exposure and the neophobic response attenuated completely on day four while adult rats did it on day two.

The nucleus accumbens shell, but not the nucleus accumbens core, showed increased activity of the protein c-Fos when adult animals drank the novel flavored solution twice. This is relevant as in that specific day the animals showed an increase in consumption, indicating the existence of attenuation of the neophobic response. Moreover, once the neophobic response was completely attenuated on day six, the c-Fos activity was similar to that of the Novel group. This indicates that the activity found on the nucleus accumbens would not be related with the processing of flavor features such as taste, and also that the activity is not related to the detection of novelty or familiarity. Indeed, this activity seems to be related to the formation of flavor memory and the consolidation of the safe taste memory. Similar patterns of dopamine activity have been described in the nucleus accumbens, as Bassareo and Di Chiara (1997) using microdialisys found a release of dopamine in the NAcb after the exposure to palatable and high-preferred food, but this response attenuated after two consecutive exposures to the same food. An alternative interpretation in terms of increased c-Fos expression induced by the motor activity during drinking is not feasible. As a matter of fact, the increase in the number of c-Fos positive cells does not parallel the consumption pattern of the flavored solution. The flavor solution intake increases throughout successive drinking sessions being significant such increase at the second exposure. However, the increase in c-Fos positive cells was only found at the second exposure (Familiar 1 group) while no differences were observed between Novel and Familiar 2 groups.

A different pattern of c-Fos activity was found in aged rats. While there were no differences between Novel and Familiar 1, c-Fos activity in NAcb-Shell but not NAcbC increased in the Familiar 2 group. An interpretation in terms of a delayed formation of

the safe taste memory is consistent with the behavioral pattern as, they did not show a significant increase in vinegar consumption between days one and two. It can be conceived that, as vinegar is a non palatable flavor, the shifting in the hedonic value of the flavor would take more time to occur within the aged rat brain. This would directly affect the attenuation of flavor neophobia, which would not happen at the second exposure to the flavor. Thus, recognition of the flavor as safe might require additional exposures to the flavor, resulting in an increased palatability of the flavor and thus, increasing the activity of the nucleus accumbens. However, it is important to note that aged rats as well as adult rats completely attenuated the neophobic response on day 6, but only aged rats exhibited that day increased number of c-Fos positive cells compared to the first day of exposure. It seems as if the nucleus accumbens of aged rats would not be processing only changes in flavor palatability but would be responding to the detection of flavor familiarity. In that case, it might be plausible that the nucleus accumbens would be participating in compensatory mechanisms and its activity would be necessary in order to achieve a behavioral response similar to that observed in adult rats.

The differential pattern of NAcb-Shell c-Fos activity in adult and aged rats allows us to exclude non-specific decline of brain activity induced by aging. In addition, cell quantification of Nissl stained cells using Cresyl Violet indicated no cell loss associated to aging. Therefore, the change of the brain activity patterns associated to aging could be interpreted as a reorganization of the brain circuit involved in processing flavor familiarity at advanced age. Such a reorganization could be expected taking into account the effect of the experience on a plastic brain along the life which is evident also in taste related behaviors(Gámiz & Gallo, 2011; Morón & Gallo, 2007b). We have previously reported a similar change of the c-Fos activity pattern induced by flavor

familiarity in the perirhinal cortex of aged rats (Gómez-Chacón et al., 2015). However, not all the areas involved in the attenuation of flavor neophobia are affected by aging in the same way since the pattern of changes is not uniform. In fact, we have also reported an overall increase of c-Fos activity of the posterior but not the anterior piriform cortex of aged rats exposed to a vinegar solution (Grau-Perales et al., 2019a).

In summary, it is conceivable that NAcb-Shell might be included together with amygdala (Gómez-Chacón, Gámiz, Foster, & Gallo, 2016; Gómez-Chacón et al., 2012), insular cortex (Bahar, Dudai, & Ahissar, 2004; Bermudez-Rattoni, 2014; Lin, Roman, Arthurs, & Reilly, 2012; Miranda, Ferreira, Ramírez-Lugo, & Bermúdez-Rattoni, 2003; Moraga-Amaro, Cortés-Rojas, Simon, & Stehberg, 2014), perirhinal cortex (Balderas et al., 2012; Gómez-Chacón et al., 2015; Gómez-Chacón et al., 2016; Morillas et al., 2017), piriform cortex (Grau-Perales et al., 2019a) and hippocampus (Grau-Perales et al., 2019b) as part of a flavor recognition memory brain circuit which shows complex reorganization throughout the lifespan. Further research on the specific role of the NAcb in recognition memory could help to understand dysfunctional restriction of dietary intake described in eating disorders often affecting to the older population (Gallo, 2018) and it will contribute to advancements in diagnosis, assessment, behavioral intervention and nutritional management.

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