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Research report

Flavor recognition memory related activity of the posterior piriform cortex in adult and aged rats

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ABSTRACT

The relationship between the piriform cortex and flavor recognition memory was investigated in adult and aged rats. By using c-Fos immunohistochemistry, we assessed the piriform cortex activity induced by flavor familiarity. The results indicated increased activity in the rostral region of the posterior piriform cortex elicited by the most familiar cider vinegar solution after six exposures. Aged rats exhibited overall increased activity in the posterior, but not the anterior piriform cortex, which was not related to flavor familiarity. This suggests that the posterior piriform cortex is related to flavor recognition memory and that aging modifies its activity pattern which might underlie their slower attenuation of flavor neophobia.

1. Introduction

The general tendency to avoid novel tastes called taste neophobia plays a very important role for survival. Safe taste recognition memory refers to the ability of assessing the familiarity of a previously ingested food or drink not followed by negative consequences [1]. As the novel taste becomes familiar, it is classified as safe and its consumption increases, thus evidencing attenuation of the neophobic response. Taste is the critical cue for this type of memory but, due to the multimodal integration involved in ingestion, other sensory cues might become relevant. This is what happens with the olfactory cues in case of flavors since they combine taste and smell.

In spite of their independent sensory pathways, taste-odor interaction is evident in behavioral effects such as odor taste potentiated aversion [2–4] which depends on the amygdala [5].

Regarding taste recognition memory, the amygdala has been related to the neophobic response to both taste and flavored solutions [6,7] and the perirhinal cortex with the attenuation of flavor neophobia [8,9]. However, the specific role of the olfactory component of flavor has not been studied yet. Other cortical areas that might be relevant for recognition memory if an odorant component is added to taste are those receiving projections from both gustatory and olfactory pathways such as some prefrontal cortex regions [10,11]. In fact, the orbitofrontal cortex has received special attention in animals and humans [12].

Although not previously related to flavor recognition memory, the piriform cortex (PirCx) might be proposed as relevant for taste-odor integration [12] since it sends projections to both amygdala and orbitofrontal cortex among other brain areas [13]. PirCx is an anatomically and functionally complex region [14,15] consisting of two main divisions with different functions: the anterior piriform cortex (aPirCx) and the posterior piriform cortex (pPirCx) being the boundary placed at the level of the anterior commissure [16].

On the one hand, the aPirCx is mainly related to the detection and discrimination of odors and purely the smell sensation. Accordingly, it receives inputs from the primary olfactory neurons and is also well connected with the orbitofrontal cortex [17–19]. On the other hand, many studies suggest that the pPirCx is an area not only involved in odor information processing but it is also important for multisensory integration. In addition to neurons that respond selectively to odors, neurons that respond selectively to gustatory information and neurons that respond to both senses with different firing patterns have been described [20]. The pPirCx is connected not only with the aPirCx but also with areas involved in taste and flavor recognition memory such as the amygdala, the perirhinal cortex and the orbitofrontal cortex. Electrophysiological studies have shown that the activity of pPirCx neurons is highly plastic during a reversal learning task. They exhibit both earlier and more pronounced phasic electrical activity to positive than to negative odor cues [21]. Also, Dardou et al. [16] found a different pattern of expression of Egr1 protein, used as a marker of neural activity, between the aPirCx and the pPirCx in a typical task of odor taste potentiated aversion.

However, to our knowledge, there are no data on the potential relationship between the PirCx and safe flavor recognition memory. If the pPirCx processes flavor information and has higher order sensory

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plastic functions, then its activity might be sensitive to flavor familiarity. A feasible hypothesis is that increases in flavor familiarity during the attenuation of neophobia would induce changes in the activity of the pPirCx. This would support an involvement of the area in flavor recognition memory. Moreover, if this were so, the activity of pPirCx related to flavor familiarity could be modified by aging since it has been reported slower attenuation of neophobia in aged than in adult rats together with a different pattern of perirhinal cortex activity [8]. Hence, adult rats exhibited increased c-Fos activity in the perirhinal cortex after the exposure to a novel flavor meanwhile aged rats showed increased c-Fos activity after the exposure to the most familiar flavor, suggesting functional brain reorganization by aging leading to enhancement of the positive consequences of safe flavors. Accordingly, Dardou and Catarelli [22] found an increased expression of Egr1in the pPirCx of senescent compared to adult rats after odor taste potentiated aversion, suggesting a potentiation of the negative consequences of odor stimuli in aged rats.

The main aim of this study was to investigate if the piriform cortex of rats exhibits activity changes over repeated exposures to a novel vinegar solution. In order to establish comparisons with previous data on other brain areas [6–8] we measured the number of c-Fos positive cells during the first, second and sixth flavor exposure. We also assessed the piriform cortex activity in aged rats during the attenuation of flavor neophobia.

2. Materials and methods

2.1. Subjects

The brains of 21 adult (5-month-old) and 24 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-Chacon et al. [8]. This is in accordance with the reuse rule for reducing the number of animals in research.

In brief, depending on the day in which the animals were euthanized to remove their brains, each rat was assigned to one of the following groups: Novel (with one exposure to vinegar; adult: n = 7; aged: n = 8), F–I ("Familiar-I" groups with two exposures to vinegar: adult; n = 7; aged: n = 8) and F–II ("Familiar-II" groups with six exposures to vinegar; adult: n = 7; aged: n = 8). Four brains (Novel = 1, F–I = 2 and F–II = 1) were discarded for the data analyses due to tissue damage during the immunohistochemical procedure. All procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (17-02-15-195).

2.2. Behavioral procedure

All the subjects were subjected to the same behavioral procedure, which consisted of daily 15-minute drinking sessions in which consumption was recorded. Water intake during the morning drinking period was recorded for five days during the acclimation period to the deprivation schedule. Once the water intake baseline (BL) was stabilized all rats had access to a 3% (vol/vol) cider vinegar solution instead of water during the morning daily drinking session. Consumption (ml) was recorded after each session (see Gómez-Chacón et al., 2015 [8]).

2.3. Immunohistochemical procedure

All the animals were euthanized 90 min after the drinking the novel cider vinegar solution on Day 1 for the first time (Novel), the already familiar solution on Day 2 (F–I) or the most familiar solution on Day 6 (F–II). The immunohistochemical procedure has been described elsewhere [8]. In brief, deeply anesthetized rats were transcardially perfused. The brains were removed and coronal sections were cut at 20 μm in a cryostat (Leica CM1900). Tissue sections were then rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4), incubated for 15 min 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 min. Slices were transferred to a c-Fos primary antibody (1:10,000; Cal-biochem) for 48 h at 4 °C. After being rinsed with PBS, they were incubated in a secondary antibody (Biotinylated goat anti-rabbit IgG, 1:500; Calbiochem) for 120 min 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.

2.4. Data acquisition

In order to quantify the number of c-Fos positive cells, coronal sections of the brain containing the anterior and posterior PirCx were identified using *Neurolucida Software.* Two images of both hemispheres were captured using a light microscope (Olympus BX41) at 20X magnification. The coronal sections were separated into three regions following a rostro-caudal division according to the different areas that were adjacent to the PirCx. Images of the rostral, central and caudal regions of aPirCx were captured approximately at + 3 mm, + 2 mm and + 1 mm, respectively, relative to Bregma according to Paxinos and Watson (2009). Images of the rostral, central and caudal regions of the pPirCx were captured at -2.5 mm, -3.00 mm and -3.5 mm relative to bregma according to Paxinos and Watson (2009). See Fig. 1.

The number of c-Fos positive cells was counted using the Image *J* Software (National Institute of Mental Health) automatically. For each image threshold objects having specific area (20–150) and circularity (0.25–1.00) values matching those c-Fos positive nuclei were automatically counted by the software. In order to equalize all images and cancel out background noise, they were previously converted into 8-bit type image and the background was lightened (50.0 pixels). Mean values were calculated for both hemispheres.

3. Results

3.1. Behavioral results

A two-way ANOVA analysis (*Familiarity* X *Age*) of the water amount drank during the last baseline day yielded a significant effect of *Age* [$F(1, 38) = 5.22$; $p = .027$] but not *Familiarity* [$F(2, 38) = 0.06$; $p = .941$] or the interaction *Age* X *Familiarity* [$F(2, 38) = 0.6$; $p = .55$]. Aged rats drank more water (Mean: 11.02 ± 0.71 ml) than adult rats (Mean: 13.29 ± 0.68 ml). This can be attributed to the higher weight of the aged rats. However, no significant differences between the different age groups in the amount of vinegar solution drank were found during the novel flavor exposure. A two-way ANOVA analysis (*Familiarity* X *Age*) of the vinegar solution drank on Vin1did not yield significant effects of *Age* [$F(1, 38) = 0.002$; $p = 0.96$], *Familiarity* [$F(2, 38) = 0.093$; $p = .91$] or the interaction *Age* X *Familiarity* [$F(2, 38) = 0.36$; $p = 0.76$]. Moreover, during the second exposure to the vinegar solution the effect of Age was opposite to that found in the baseline since aged rats (Mean: 7.40 ± 0.64 ml) drank lower amounts than adult rats (Mean: 9.42 ± 0.68 ml). A two-way ANOVA analysis (*Familiarity* X *Age*) of the vinegar solution drank in Vin 2 yielded a significant effects of *Age* [$F(1, 26) = 4.68$; $p = .04$] but not *Familiarity* [$F(1, 26) = 0.003$; $p = .96$] or the interaction *Age* X *Familiarity* [$F(1, 26) = 0.275$; $p = .61$]. Therefore, adult rats but not aged rats showed attenuation of neophobia after the first exposure to vinegar as intake of vinegar on day 2 increased in comparison with day 1 in adult but not in aged rats.

The behavioral results of Familiar-II groups have been previously described [8]. In brief, both adult and aged groups exhibited the

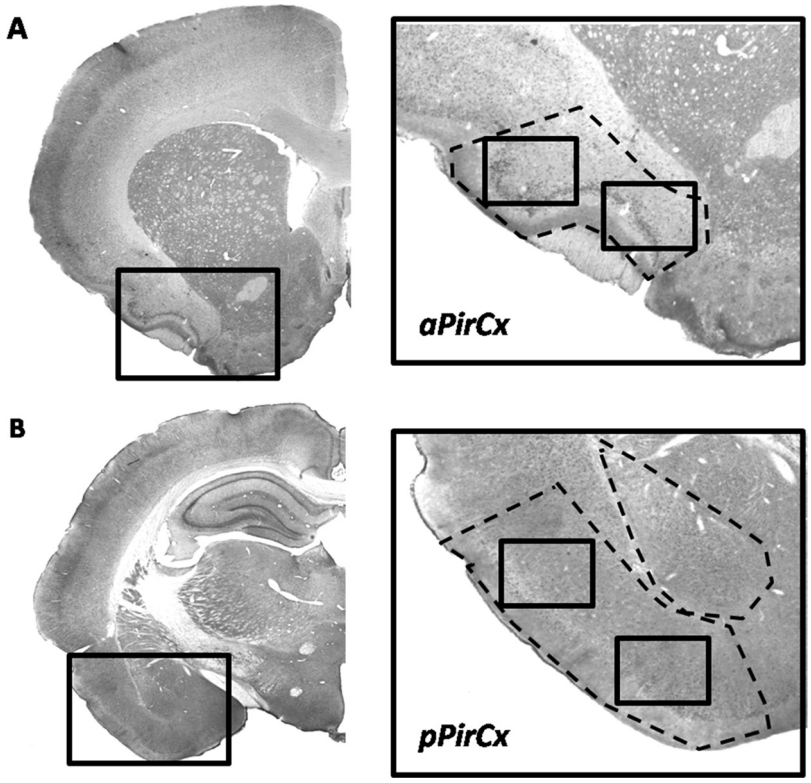

Fig. 1. Representation of the location of the images containing the aPirCx (**A**) and pPirCx (**B**) captured with the light microscope at 20X magnification.

Table 1
Mean (± SEM) consumption (ml.).

	Group	Water	Vin 1 (Novel)	Vin 2 (F-I)	Vin 3	Vin 4	Vin 5	Vin 6 (F-II)
	Novel	10,42 (± 1,46)	6,32 (± 0,53)					
Adult	F-I	10,78 (± 1,43)	5,59 (± 0,95)#	9,64 (± 0,87)*				
	F-II	11,85 (± 0,94)	5,72 (± 0,77)# +	9,20 (± 0,80)*	10,89 (± 1,43)*	12,16 (± 1,22)*	10,99 (± 1,46)*	11,73 (± 0,78)*
	Novel	13,42 (± 0,95)	5,65 (± 0,8)#					
Aged	F-I	14,75 (± 0,67)	6,18 (± 1,79)#	7,01 (± 0,8)#				
	F-II	13,00 (± 1,21)	5,44 (± 0,96)# +	7,43 (± 1,21)# +	8,65 (± 0,64)#*	9,26 (± 0,53)#*	10,57 (± 0,8)*	11,51 (± 0,79)*

Water (last day of Baseline); Vin = 3% cider vinegar solution; # = $p < .05$ compared to Water of the same age group; + = $p < .05$ compared to Vin6 of the same age group; * = $p < .05$ compared to Vin1 of the same group; # = p $< .05$ compared to Water of the same group.

neophobic response to vinegar. Attenuation of neophobia was evident in both age groups as vinegar intake increased over repeated exposures until there were no differences along the last days. However, it was slower in the aged group than in the adult group. While adult rats did not exhibit increased vinegar consumption from Day 2 after two vinegar exposures, aged rats required one more vinegar exposures since there were no differences with Vin6 from Day 3and no differences were found between Days 1 and 2 (Table 1). The slower attenuation of neophobia in the aged group is evident also including the baseline water in the analysis. One-way ANOVA analysis of the F–II adult group consumption along the drinking sessions indicated lower vinegar than baseline water intake only in Vin1 ($p = .022$). A similar analysis of the FII aged group showed lower vinegar intake in comparison with water consumption during the last baseline day in Vin1 ($p = .011$), Vin2 ($p = .02$), Vin3 ($p = .011$) and Vin4 ($p = .011$).

3.2. Immunohistochemical results

3.2.1. Anterior piriform cortex

A mixed repeated measures ANOVA (*Region* X *Familiarity* X *Age*) was used to compare differences in c-Fos positive cells in the aPirCx between adult and aged rats. This ANOVA revealed a significant effect of the main factor *Region* [$F(2, 43) = 4.19$; $p = .021$] and no other effect or interaction was significant (all $p > .1$). Further post-hoc analyses of the main effect using Bonferroni tests revealed a higher number of c-Fos positive cells in the most rostral region of the aPirCx compared to the other two regions (Fig. 2B).

3.2.2. Posterior piriform cortex

A repeated-measures ANOVA (*Region* X *Familiarity* X *Age*) was applied to compare differences in c-Fos positive cells in the pPirCx between adult and aged rats. This ANOVA revealed significant effects of the main factor *Age* [$F(1, 34) = 35.56$; $p < .001$] and the interaction *Region* X *Familiarity* [$F(4, 34) = 5.16$; $p < .003$]. Further post-hoc analyses of the main effect *Age* using Bonferroni tests revealed an overall increased number of c-Fos positive cells in the aged rats compared to adult rats regardless the familiarity or the region of the posterior piriform cortex studied (Fig. 2A).

In order to understand the second order interaction *Region* X *Familiarity,* a one-way ANOVA was performed for each of the three Regions. The analysis performed for the rostral Region revealed a significant effect of *Familiarity* [$F(2, 34) = 7.79$; $p < .002$]. Further post-hoc analyses using Bonferroni tests revealed a higher number of c-Fos positive cells in the F–II group compared to the Novel Group ($p = .016$)

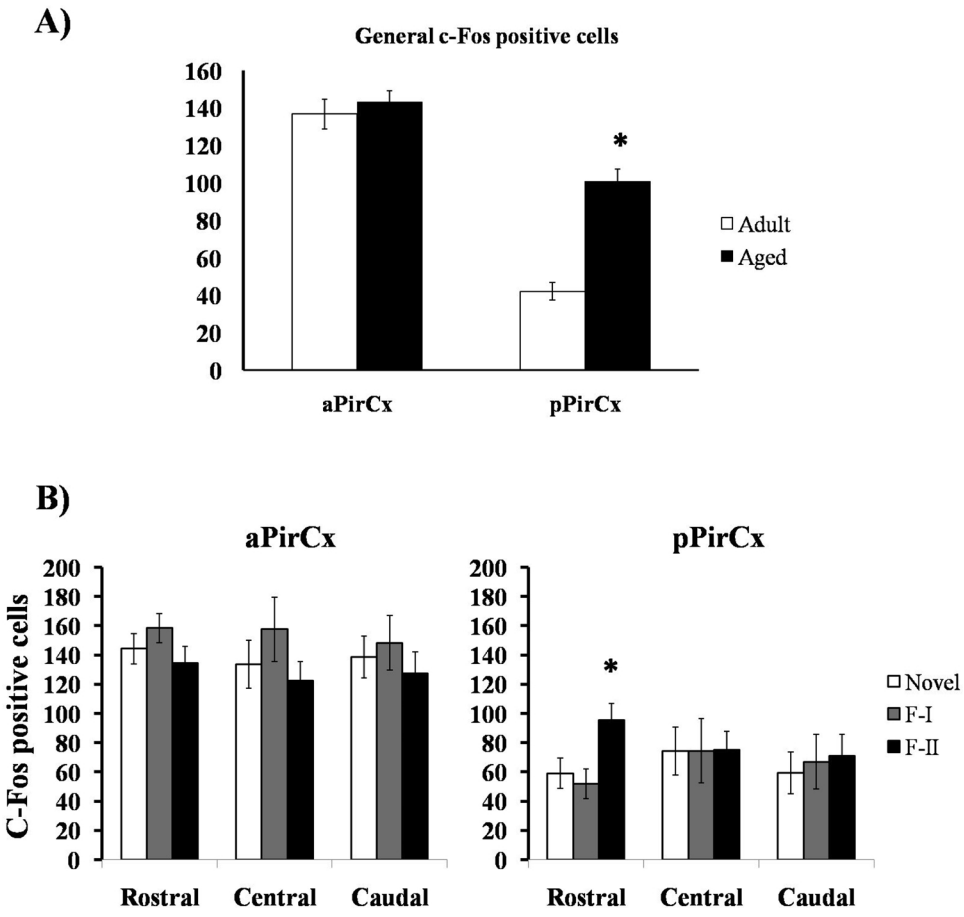

Fig. 2. Panel **A** represents number of c-Fos positive cells ± SEM in both regions of the Piriform Cortex. The symbol * represents statistically significant difference ($p < .05$) compared to the adult group. Panel **B** represents the number of c-Fos positive cells in the three sub-regions of the aPirCx and the pPirCx of both adult and aged rats. The symbol * represents statistically significant differences ($p < .05$) compared to the Novel and F–I groups.

and the F–I Group ($p = .003$) and no differences in c-Fos positive cells were found between Novel and F–I Groups ($p = 1$). Analyses performed for the central and caudal Regions of the pPirCx did not reveal any significant effect of Familiarity (all $p > .9$ and $p > .8$ respectively). Fig. 3 represents the results of the adult and aged groups.

Additionally, another one-way ANOVA *Region* X *Familiarity* was performed for each familiarity group separately. There were no significant effects in the Novel and F–I groups. There was a significant effect of *Region* in the F–II group [$F(2, 25) = 14.4$; $p < .001$]. Post-hoc analyses by Bonferroni tests revealed an increased number of c-Fos positive cells in the rostral region compared to the central ($p = .002$) and caudal ($p = .001$) regions.

Thus, the relevant significant differences can be attributed to a higher number of c-Fos positive cells in the pPirCx after drinking the familiar vinegar solution during the sixth exposure in the F–II group. This increase was found in the most rostral region of the pPirCx. We also found an overall increased number of c-Fos positive cells in the pPirCx of aged rats compared to adult rats, regardless the number of exposures to the flavor.

4. Discussion

The main finding reported in the present study is that the activity of the pPirCx is selectively modified during repeated flavor exposures. This is to our knowledge the first evidence reporting a potential role of the PirCx in flavor memory. Immunohistochemistry data revealed selective activity changes in the rostral pPirCx related to the familiarity of the flavor while no changes were found within the aPirCx. The number of c-Fos positive cells in this portion of the pPirCx was higher in the F–II group of adult rats than in Novel and F–I groups. Thus, the activity of this region increased significantly when the rats drank the most familiar vinegar solution after six exposures. In addition, there were no differences between Novel and F–I groups in the number of c-Fos positive cells.

The results are in accordance with previous reports relating pPirCx but not aPirCx with learning and memory processes. The fact that the increased number of c-Fos positive cells reaches significance after sixth exposures when the flavor familiarity is consolidated suggests a potential role of pPirCx in long-term flavor recognition memory. Alternative explanations in terms of increased activity induced by drinking-related sensory, motor and motivational processes cannot be ruled out but they do not seem feasible. First, the increase in the number of c-Fos positive cells is not parallel to the consumption pattern of the flavored solution that shows a significant increase at the second exposure (F–I group). Second, the animals could smell the odor during the entire 15 min drinking sessions including the periods in which they did not drink so that the odor exposure did not differ among the groups. Third, if higher intake or longer odor exposure were the reasons for the increased PirCx activity it would be evident in all the regions and the differences appeared only in rostral pPirCx. A similar argument applies for changes in the motor and motivational state. There are no data to support a selective role of pPirCx in these processes.

An overall increased expression of c-Fos was found in the pPirCx of aged rats indicating a hyper-activation regardless the number of vinegar exposures. Such over-expression of c-Fos was not found in aPirCx, which is the most pure sensory area of the secondary olfactory cortex. In fact, it has been reported that the aPirCx receiving olfactory information from the tufted and mitral cells of the Olfactory Bulb, does not exhibit learning-related activity changes. Roesch et al. [19] found no changes in the firing patterns of the aPirCx neurons after a reversal learning task. In contrast, Calu et al. [21] reported that the electrical activity of pPirCx neurons was highly plastic during a reversal learning

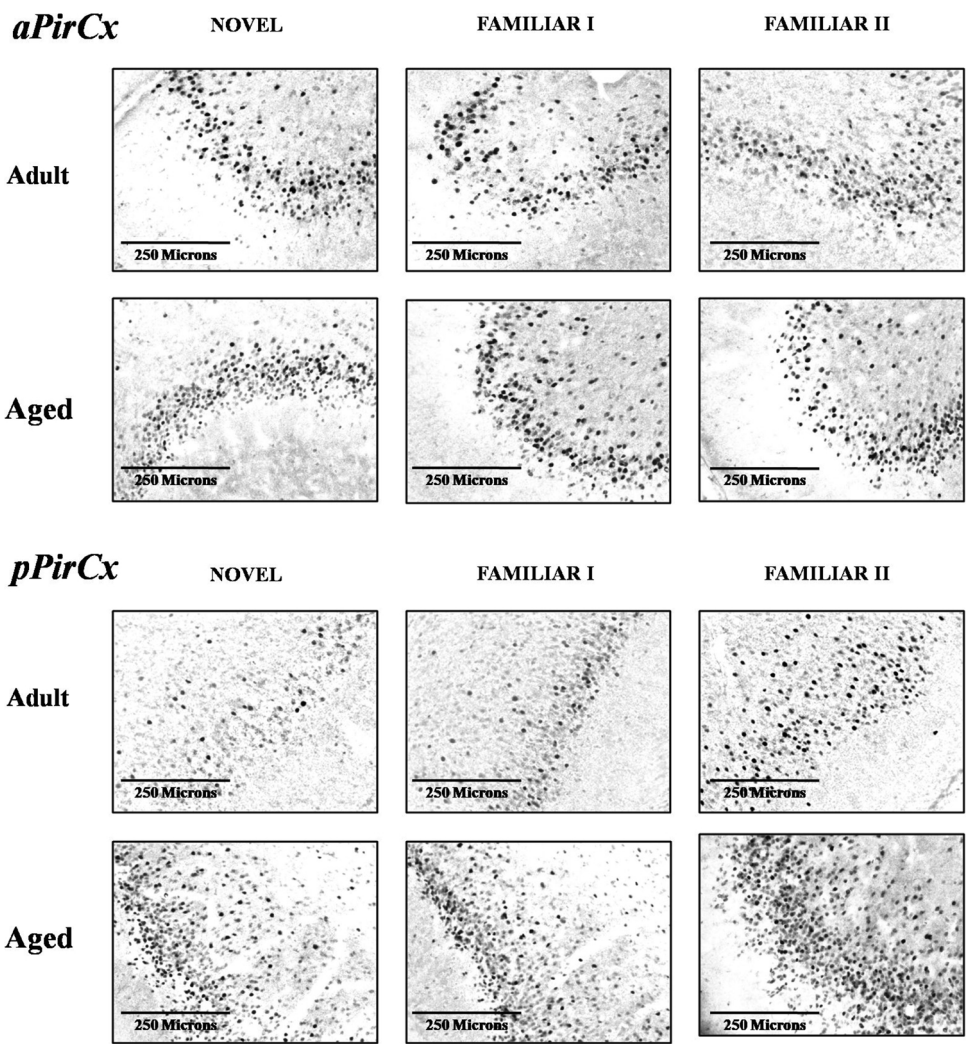

Fig. 3. Panel **A** shows representative microphotographs at 20X magnification of the Rostral aPirCx for the three familiarity groups (Novel, Familiar I and Familiar II) of adult and aged rats. Panel **B** shows representative microphotographs at 20X magnification of the Rostral pPirCx for the three familiarity groups (Novel, Familiar I and Familiar II) of adult and aged rats.

task. This evidence together with the anatomical connections of the pPirCx with areas involved in taste learning, such as basolateral amygdala and perirhinal cortex, as well as the present data suggest that the aPirCx might be involved in smell sensation while the pPirCx would be more likely involved in flavor learning being more sensitive to the effect of aging on memory.

Our results are in accordance with previous reports of increased protein expression in the pPirCx of aged rats [16]. This over-expression could be reflecting compensatory mechanisms to deal with aging-related memory difficulties. In this line, Foster et al. [23] found excitatory changes in glutamatergic neurons of the cortex proposed as early markers of cognitive decline. This could be consistent with the delayed attenuation of flavor neophobia exhibited by aged animals. The evidence indicating that aged and adult rats did not differ in the neophobic response in spite of exhibiting pPirCx over-activation further supports a selective involvement of this region in the attenuation of neophobia, but not in neophobia itself. The fact that flavor neophobia was present in both age groups is in accordance with previous findings on the effect of aging in taste memory. In contrast to the deleterious effect of aging in other learning and memory tasks, taste learning is largely preserved at advanced ages (for reviews see [24,27]. Taste neophobia might exhibit age-related changes [25], but they seem to depend on previous life experiences more than on aging itself [26].

Also, as it has been previously reported [8], aged rats exhibited slower attenuation of neophobia than adult rats. It cannot be ruled out that during the familiarization process different learning effects such as habituation, appetitive learning, habit learning or aversion counter-conditioning had taken place. In any case, the increase in vinegar consumption with repeated exposures indicates attenuation of neophobia.

The finding relating the pPirCx with the attenuation of flavor neophobia is particularly relevant because it has been demonstrated to be a multisensory integration area that could be receiving afferents related to taste memory. In fact, the rostral portion of pPirCx borders with the insular cortex. Areas involved in other types of memory, such as perirhinal cortex, entorhinal cortex, and amygdala are also bordering with other regions of the pPirCx. Thus, we consider that the selective increased activity of the rostral pPirCx could be related with a role of this region in taste memory. Consistently, electrophysiological findings have demonstrated responsiveness of pPirCx neurons to taste stimulation [20]. Interestingly, the recording electrodes were located at -1,4 mm posterior to bregma, i.e., a region of the PirCx bordering with the insular cortex. Also, reciprocal connectivity between insular cortex and PirCx could be responsible for taste-odor interactions relevant for memory [12,20].

In summary, it is conceivable that pPirCx might be included together with amygdala, insular cortex, perirhinal cortex and orbitofrontal cortex as part of a flavor memory brain circuit. Further research

on the specific role of the piriform cortex in recognition memory could help to understand dysfunctional restriction of dietary intake described in eating disorders often affecting to the older population [27] and it will contribute to advancements in diagnosis assessment, behavioral intervention and nutritional management.

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