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Differential requirement of de novo Arc protein synthesis in the insular cortex and the amygdala for safe and aversive taste long-term memory formation

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Abstract

Several immediate early genes products are known to be involved in the facilitation of structural and functional modifications at distinct synapses activated through experience. The IEG-encoded protein Arc (activity regulated cytoskeletal-associated protein) has been widely implicated in long-term memory formation and stabilization. In this study, we sought to evaluate a possible role for de novo Arc protein synthesis in the insular cortex (IC) and in the amygdala (AMY) during long-term taste memory formation. We found that acute inhibition of Arc protein synthesis through the infusion of antisense oligonucleotides administered in the IC before a novel taste presentation, affected consolidation of a safe taste memory trace (ST) but spared consolidation of conditioned taste aversion (CTA). Conversely, blocking Arc synthesis within the AMY impaired CTA consolidation but had no effect on ST long-term memory formation. Our results suggest that Arc-dependent plasticity during taste learning is required within distinct structures of the medial temporal lobe, depending on the emotional valence of the memory trace.

Introduction

The nature of the information to be incorporated seems crucial in determining the structural and molecular events that are triggered to consolidate a memory trace. A very useful memory paradigm to study such plastic changes is taste memory, in which either "safe" or "aversive" memory traces will be formed based on the presence or absence of gastric malaise [1]. The conditioned taste aversion (CTA) is expressed as a diminished consumption of the food associated with gastric malaise, whereas if there are no negative outcomes from the food consumption, the animal will keep consuming it, and will be stored as a safe taste memory trace (ST) [1]. Acquisition and consolidation of taste memories are primarily dependent on the activity of the insular cortex (IC) [1] and the establishment of a taste memory trace on the long-term involves de novo translation of plasticity-related proteins in this structure such as expression of the transcription factor c-fos [2], the synthesis of the scaffolding protein PSD-95 [3] and the neurotrophic factor BDNF [4].

Another important structure for taste memory formation is the amygdala (AMY) both central and basolateral nuclei. For instance, it has been shown that the exposure to a novel saccharin solution induces c-fos synthesis in the central amygdala (CeA) [5,6] and gastric malaise induction is related to both the CeA and the basolateral amygdala (BLA) activity [6]. Interestingly, the pairing of a novel taste with gastric malaise induced a strong increase in c-fos-immunoreactive neurons in both CeA and BLA compared to gastric malaise induction after drinking a familiar taste [7].

Previously, we have shown that taste-induced synthesis of activity regulated cytoskeletal associated protein (Arc), an immediate early gene with a pivotal role in synaptic plasticity [9,10], is increased in dendritic regions of the IC after familiarization with the taste during ST [11], suggesting a role in this type of learning. In the case of AMY, gustatory and visceral stimuli association induce Arc mRNA expression that converge on the same amygdalar neurons during the establishment of taste aversion [8]; however, no study has addressed directly the requirement for Arc translation in the consolidation of both "safe" and "aversive" taste memory traces within the IC and the AMY. We propose that Arc protein in these structures is required differentially in the long-term taste memory formation depending on the kind of learning. To evaluate this, we conducted Arc protein knockdown experiments on the afore mentioned brain

structures with antisense oligonucleotides (ODN) during the acquisition of ST or CTA. Our results show that Arc protein synthesis in the IC is involved in the safe taste long-term memory impeding the familiarization process; this may suggest that Arc participates in safe taste storage. Conversely, Arc synthesis within the AMY appears to be involved only in the CTA stabilization, which shows a versatile role of Arc depending on the outcome of the food ingestion to form either safe or aversive memory traces.

Ninety days-old male Wistar rats, weighing 260–280 g, were housed individually in a temperature-controlled environment and maintained on a 12-h light/dark cycle. They had ad libitum access to food and water, except when noted otherwise in the experimental procedures. Studies were performed in accordance with the standards of the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Instituto de Fisiología Celular and the Institutional Animal Care and Use Committee SAAC from Texas A& M University under AUP 201141.

The ODN for Arc/Arg 3.1 mRNA and a scrambled oligonucleotides sequence (SCR) (Midland Certified Reagent Company, TX, USA) were custom made as following the nucleotides sequence described by Guzowski et al. [12]. Guide cannulae were implanted bilaterally into the IC and the AMY in independent groups using standard stereotaxic procedures. The coordinates were determined from bregma (in mm): AP +1.2, L ± 5.5 and DV –4.5 for IC, and AP −2.8 mm; L ± 4.8 mm; DV –6.5 mm to aim a point where the infusion would include CeA and BLA according to Paxinos and Watson [13].

One week after surgery, behavioral experiments were performed to assess the functional role of Arc synthesis within the IC and the AMY in taste memory. Rats were water-restricted for 24 h and a baseline water consumption was determined by allowing access to tap water for 15 min in the morning (around 10:00 a.m.) and for 15 min in the afternoon (around 6:00 p.m.). After five days of baseline consumption recording, these animals were then divided into two groups with similar baseline consumption of water: aversive (SAC+ LiCl) and safe (SAC + NaCl). Half of the aversive group received injections of ODN and half received SCR (1 µl (2 nmol)). ODN or SCR were injected one hour prior to the taste exposure using 30-gauge infusion needles that protruded 1.5 mm from the cannula tip; the injection needles remained in the guide cannulae for an additional minute to allow diffusion of fluid into the tissue. Rats in the aversive group were provided with 0.1% (w/v) saccharin solution for 15 min. After 30 min, 7.5 ml/kg of LiCl 0.15M was injected intraperitoneally. This procedure results in acquisition of CTA. For acquisition of safe taste memory, the outlined behavioral protocol was followed, except that 0.15M NaCl injections
replaced LiCl. Twenty-four hours after the acquisition of either CTA or ST, a 0.1% saccharin solution was provided during 15 min to assess long-term memory.

To evaluate the extent of Arc protein knockdown after ODN treatment, three rats were administered with ODN in one hemisphere and SCR on the other one hour before the consumption of a saccharin solution during either ST protocol for the IC analysis or CTA for the AMY. One hour after the corresponding protocol, animals were decapitated and their brains flash-frozen and stored at −80 °C until use. Coronal sections of 20 µm were incubated with Arc primary antibody (1:500 in TBS Affinity purified rabbit anti-activity regulated cytoskeletal-associated protein, Synaptic Systems, Gottingen, Germany) overnight at room temperature. On the following day, slices were incubated with biotinylated goat-antirabbit (1:2000, Vector Laboratories, CA, USA) for 2 h. Finally, sections were incubated in ABC amplification kit (Vector laboratories, CA, USA), washed thoroughly and the diaminobenzidine reaction was performed.

To perform the Arc synthesis analysis, between 3 and 5 images per hemisphere were obtained with a Nikon Diaphot 300 microscope and photomicrographs were digitalized with the help of Nikon's NEF coder software. For the study of the IC and AMY, images were obtained with a 4X or 10X objective and included whole coronal hemisections. All image processing and analyses were performed using NIH ImageJ software. Masks generated by the threshold were examined in search for putative staining artifacts that may have been included in the calculation and on these rare instances, these objects were removed from the analyses.

A Mann-Whitney test indicated that the count of Arc positive cells was higher for SCR-infused tissue than for ODN-infused tissue in both IC and AMY ($p = 0.0495$ and $p = 0.0228$, respectively). We observed that administration of ODN significantly decreases Arc levels within the IC and the AMY compared to the contralateral SCR infusion (Fig. 1A–E). To confirm that the extension of the Arc synthesis inhibition did not affect beyond the intended brain structures, we analyzed adjacent areas as outlined in Fig. 1A marked as 1 and 2 for the IC and in Fig. 1D marked as 1–4 for the AMY area comprising basolateral and central amygdala. There were no significant differences on the number of Arc + cells on the marked areas between the SCR and the ODN groups (Fig. 1C, area 1 $p = 0.8273$ and

area 2 p = 0.8273; Fig. 1F, area 1 p = 0.8273, area 2 p = 0.1904, area 3 p = 0.6625, area 4 p = 0.5127) indicating that the behavioral effects described after the Arc synthesis blockage are due to the decrease of Arc protein necessary for the memory trace stabilization within the AMY and the IC.

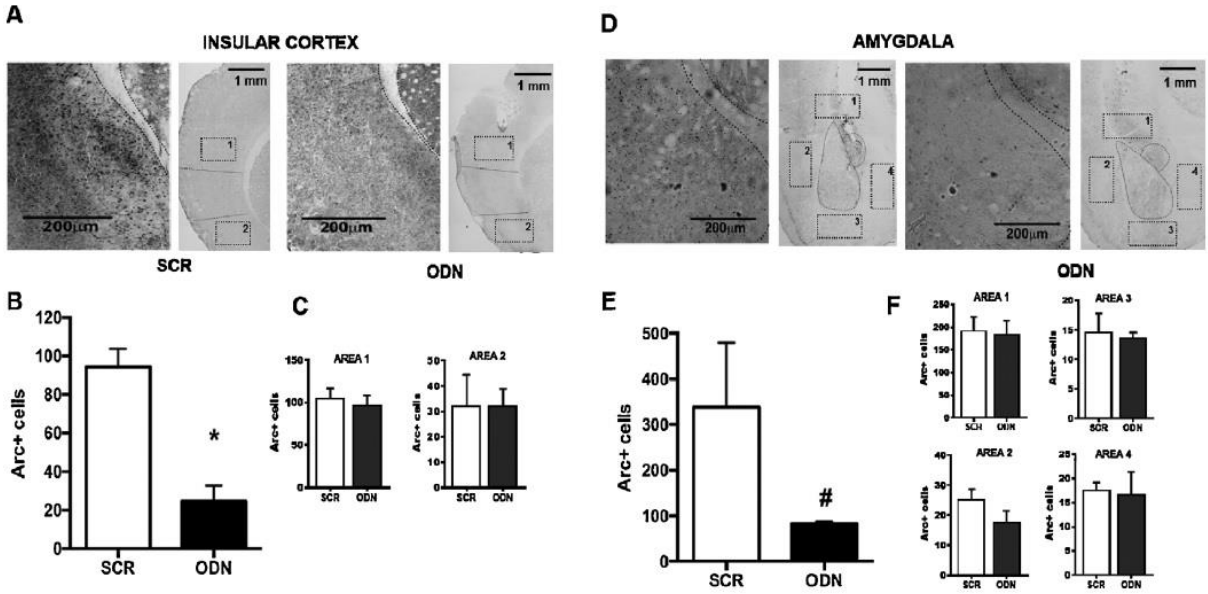

Fig. 1. (A) Representative images from IC sections 10X and 4X reconstruction immunostained for Arc protein showing the effect of scrambled oligodeoxynucleotide (SCR) injection and contralateral hemisphere administration of Arc antisense oligodeoxynucleotide (ODN). (B) Quantification of Arc + cells ± S.E.M in the IC 1 h after the safe taste memory protocol is substantially reduced in the ODN group as compared with the SCR group (*p=0.0495). (C) Quantification of Arc + cells ± S.E.M on the outlined areas adjacent to the IC: area 1 somatosensory cortex and area 2 piriform cortex showing no significant difference. (D) Representative images from AMY sections 10X and 4X reconstruction immunostained for Arc protein on SCR and ODN groups. (E) Arc + cells ± S.E.M. quantification on the region of the amygdala where the injection was performed (SCR vs. ODN group #p=0.0228). (F) Quantification of Arc+cells ± S.E.M on the outlined areas adjacent to the basolateral and central amygdala: area 1 amygdalostriatal transition area and ventral caudate, area 2 dorsal endopiriform nucleus, area 3 basomedial amygdaloid nucleus, area 4 bed nucleus of the stria terminalis; showing no significant decrease of Arc+cells on the ODN group.

To verify the site of injection, histological analysis of the brains was performed at the end of the experimental period using cresyl violet staining of 40-µm thick coronal brain slices for microscopic observation. Rats with an incorrect cannulae placement were removed from the experiment. Final number of animals per groups is specified in Fig. 2 legend; cannula placement is shown in Fig. 3.

The behavioral effect of ODN and SCR administrations for each behavioral paradigm was analyzed using one-way ANOVA assessing the effect of the ODN infusion on either IC or AMY on the long-term memory formation. Bonferroni post hoc analyses were conducted, and statistical significance was determined if p-value < 0.05 in all cases. Values are presented as mean ± S.E.M. All statistical analyses were performed with StatView (Abacus Concepts Inc.).

Milliliters consumed during the first saccharin presentation were analyzed to rule out a behavioral effect of the ODN or SCR injections on the day of the task acquisition (Fig. 2A and C). One-way ANOVA showed that there were no significant statistical differences in the mean consumption of the novel saccharin during the ST task ($F_{3,23} = 2.423$, $p = 0.0917$), nor the CTA task ($F_{3,22} = 1.081$, $p = 0.3776$). The effect of the ODN or SCR injection on the second presentation of saccharin was expressed as

the percentage of the first saccharin consumption; i.e. SAC DAY2 = [(saccharin mL day 2 × 100)/saccharin mL day 1]. As shown in the Fig. 2B regarding the ST, there was a significant effect of ODN infusion before a novel taste presentation that was not paired with malaise ($F_{3,23} = 7.035$, $p = 0.0016$), Bonferroni/Dunn post hoc revealed that saccharin consumption on day 2 was significantly different in the ODN IC group than in the SCR IC group ($p = 0.0022$); no statistical differences were observed on the AMY groups, suggesting a specific role of Arc translation in the IC, but not in the AMY for safe

taste long-term memory formation.

For CTA, one-way ANOVA analysis shows a significant difference on the second day saccharin consumption between groups ($F_{3,22} = 4.289$, $p = 0.0158$), and post-hoc analysis revealed that only the AMY ODN group showed hindered

CTA (p=0.0028); indicating that blockade of Arc synthesis affects only the stabilization of the aversive memory trace within the AMY, but not within the IC (Fig. 2D).

In the last few years, the role of Arc in memory consolidation has been established for distinct types of memory [12,20]. Here we provide the first evidence for a requirement of cortical and amygdalar de novo Arc protein synthesis as part of the molecular mechanisms involved in taste long-term memory formation. We observed that infusion of Arcantisense ODN in the IC 1 h before the intake of a novel taste affected long-term safe taste memory formation but spared CTA consolidation. On the other hand, the same treatment performed in the AMY, alters CTA memory formation, but leaves safe memory intact.

The differential involvement of Arc in the AMY and the IC goes in accordance with an earlier study that used the catFISH technique which showed that neurons of the AMY, but not the IC integrated the information related to the taste stimulus and the gastric malaise [8]. Furthermore, another group found that conditioned odor preference produced convergence of odor and taste information onto BLA but not IC neurons [14]. This absence of evidence for stimuli integration in the IC is in line with the present study that observed no effect of Arc protein knockdown on CTA consolidation. It is important to mention however that a massive amount of evidence supports a role of the IC in aversive

taste memory such as the one that occurs after CTA. For instance, lesions of specific regions within the IC are known to impair CTA learning [15]. A remapping of neural activity has also been shown to occur during a taste's shifts in hedonic value, such as those that occur during CTA and CTA extinction [16]. Also, protein synthesis inhibition in the IC has long been known to impair long-term memory of CTA [17].

Furthermore, Erk and mTOR-dependent de novo translation of PSD-95 in the gustatory part of the IC has been shown to be essential for aversive taste memory consolidation [3,18]. All in all, these data suggest that aversive associative memory processes do take place in the IC that allow CTA formation, although the present results suggest that these operate through Arc-independent mechanism. However, participation of Arc in the IC for CTA consolidation during time points beyond the one analyzed in the current study cannot be ruled out.

Our observation of a requirement for de novo Arc protein synthesis in the AMY for CTA long-term memory formation agrees with previous reports showing that Arc translation in this region is essential for fear conditioning. For instance, knockdown of Arc within the BLA affected long-term memory of an auditory fear conditioning, leaving short-term memory intact [19] and synthesis of Arc in the BLA is involved in persistence of a contextual fear conditioning [20]. Furthermore, genetically blocking the function of a transcription factor involved in Arc expression such as CREB in the BLA has been shown to impair CTA memory [21]. Also, metabotropic glutamate receptors, whose activation is involved in Arc translation [22], are also known to be involved in the AMY for CTA memory formation [23]. Together, these findings suggest that Arc-dependent synaptic plasticity mechanisms in the AMY are essential for long-term memory of aversive associative memory traces.

Our data seem to agree with a model of gustatory memory consolidation in which Arc-dependent plasticity in the IC, is essential for familiarity processing in the long-term. In a previous work [11], we showed that Arc protein accumulation in the IC positively correlated with taste familiarity. Similarly, a more recent study found that both water and familiar (i.e. non neophobic) saccharin produced higher Arc transcript levels in the gustatory cortex than when rats were exposed to novel saccharin, showing that taste novelty actually down-regulates Arc mRNA expression produced by fluid consumption [24]. Along with our current results describing a role for Arc in the IC for safe, but not aversive, taste memory consolidation, these studies bring up the intriguing possibility that Arc protein synthesis and requirement in the IC may correlate with the appetitive character of the presented taste stimulus rather than familiarity per se, with neophobic taste inducing only weak Arc synthesis while a familiar safe taste induces peak levels. Notably, some studies have shown that IC activity is involved in reward signaling that is not necessarily linked to the gustatory modality. Indeed electrical stimulation of the IC has rewarding effects under certain conditions since it has been shown to promote conditioned place preference [25]. Studies evaluating Arc protein synthesis pattern and requirement using taste stimuli of varying hedonic properties could help clarify this issue.

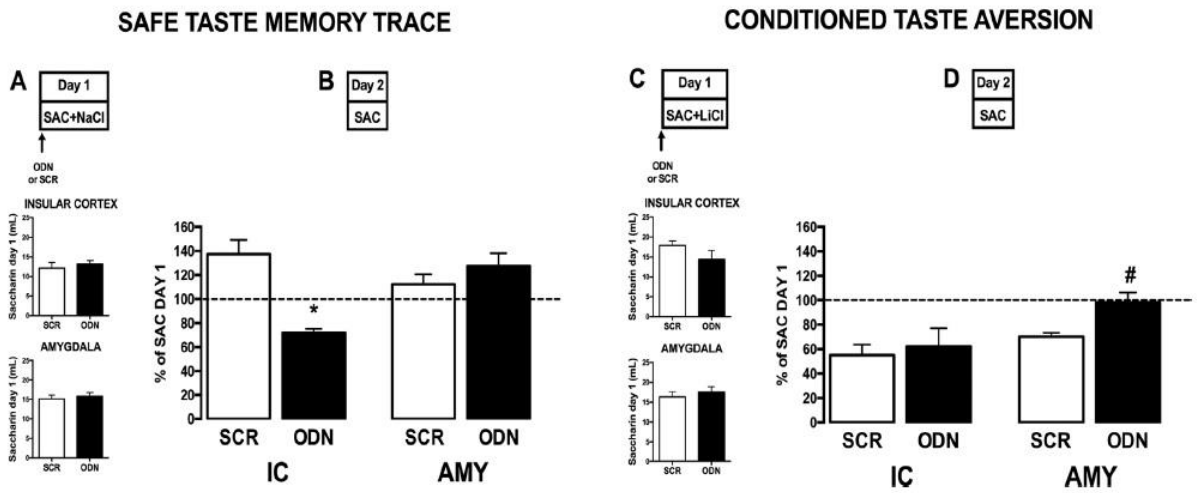

Fig. 2. (A) Saccharin consumption (mL) on Day 1 during ST acquisition phase. (B) Effect of Arc ODN infusions on the formation of long-term safe taste memory in the IC (SCR n=7, ODN n=6) and in the AMY (SCR n=6, ODN n=8). Saccharin consumption of day 2 is represented as % of consumption on the previous day. (C) Saccharin consumption (mL) on Day 1 during CTA acquisition phase. (D) Effect of Arc ODN infusions on the formation long-term aversive memory in the IC (SCR n=7, ODN n=5) and the AMY (SCR n=6, ODN n=7). *p=0.0022, #p=0.0028.

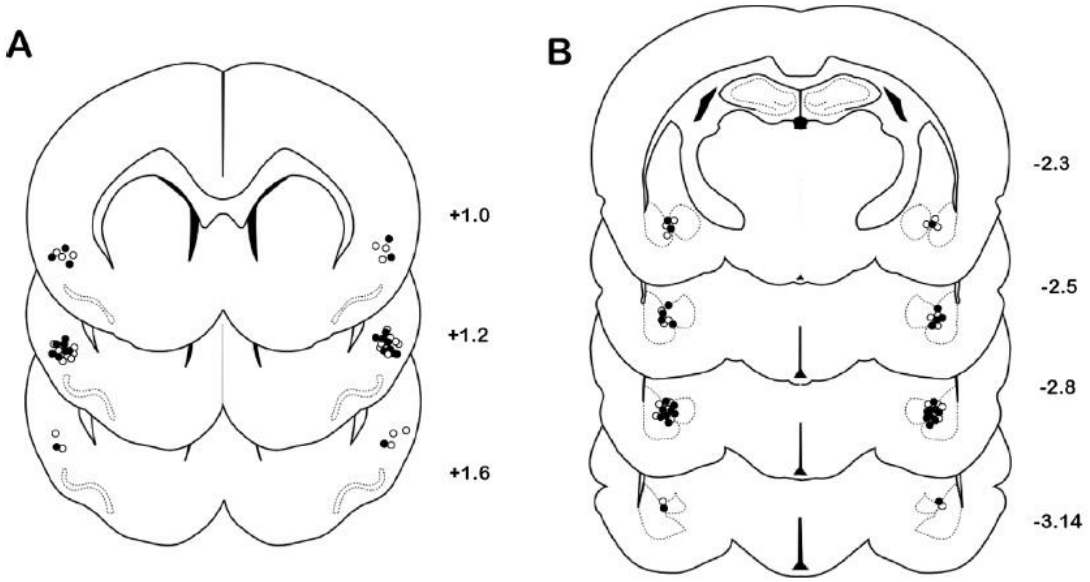

Fig. 3. Infusion cannula placement as verified on Nissl stained sections of the IC (A) and AMY (B). SCR (○) and ODN (●) injector tips. Sections based on Paxinos and Watson [13].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

[1] F. Bermudez-Rattoni, Molecular mechanisms of taste-recognition memory, Nat. Rev. Neurosci. 5 (3) (2004) 209–217.

[2] Y. Yasoshima, N. Sako, E. Senba, T. Yamamoto, Acute suppression, but not chronic genetic deficiency, of c-fos gene expression impairs long-term memory in aversive
taste learning, Proc. National Acad. Sci. U. S. A. 103 (18) (2006) 7106–7111.
[3] A. Elkobi, I. Ehrlich, K. Belelovsky, L. Barki-Harrington, K. Rosenblum, ERK-dependent PSD-95 induction in the gustatory cortex is necessary for taste learning, but not retrieval, Nat. Neurosci. 11 (10) (2008) 1149–1151.
[4] D.V. Castillo, Y. Figueroa-Guzman, M.L. Escobar, Brain-derived neurotrophic factor enhances conditioned taste aversion retention, Brain Res. 1067 (1) (2006) 250–255.
[5] M.T. Koh, E.E. Wilkins, I.L. Bernstein, Novel tastes elevate c-fos expression in the central amygdala and insular cortex: implication for taste aversion learning, Behav.
Neurosci. 117 (6) (2003) 1416–1422.
[6] T. Yamamoto, N. Sako, N. Sakai, A. Iwafune, Gustatory and visceral inputs to the amygdala of the rat: conditioned taste aversion and induction of c-fos-like immunoreactivity, Neurosci. Lett. 226 (2) (1997) 127–130.
[7] E.E. Wilkins, I.L. Bernstein, Conditioning method determines patterns of c-fos expression following novel taste-illness pairing, Behav. Brain Res. 169 (1) (2006) 93–97.
[8] S.K. Barot, Y. Kyono, E.W. Clark, I.L. Bernstein, Visualizing stimulus convergence in
amygdala neurons during associative learning, Proc. Natl. Acad. Sci. U. S. A. 105 (52) (2008) 20959–20963.
[9] H. Okuno, Regulation and function of immediate-early genes in the brain: beyond neuronal activity markers, Neurosci. Res. 69 (3) (2011) 175–186.
[10] J.D. Shepherd, M.F. Bear, New views of Arc, a master regulator of synaptic plasticity,
Nat. Neurosci. 14 (3) (2011) 279–284.
[11] J.P. Morin, C. Quiroz, L. Mendoza-Viveros, V. Ramirez-Amaya, F. Bermudez- Rattoni, Familiar taste induces higher dendritic levels of activity-regulated cytoskeleton- associated protein in the insular cortex than a novel one, Learn. Mem. 18 (10) (2011) 610–616.
[12] J.F. Guzowski, G.L. Lyford, G.D. Stevenson, F.P. Houston, J.L. McGaugh, P.F. Worley, C.A. Barnes, Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory, J. Neurosci. 20 (11) (2000) 3993–4001.
[13] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press,
2007.
[14] B. Desgranges, V. Ramirez-Amaya, I. Ricano-Cornejo, F. Levy, G. Ferreira, Flavor preference learning increases olfactory and gustatory convergence onto single neurons in the basolateral amygdala but not in the insular cortex in rats, PloS One 5 (4) (2010) e10097.
[15] T. Yamamoto, T. Shimura, N. Sako, Y. Yasoshima, N. Sakai, Neural substrates for conditioned taste aversion in the rat, Behav. Brain Res. 65 (2) (1994) 123–137.
[16] R. Accolla, A. Carleton, Internal body state influences topographical plasticity of sensory representations in the rat gustatory cortex, Proc. Natl. Acad. Sci. U. S. A. 105 (10) (2008) 4010–4015.
[17] K. Rosenblum, N. Meiri, Y. Dudai, Taste memory: the role of protein synthesis in gustatory cortex, Behav. Neural Biol. 59 (1) (1993) 49–56.
[18] K. Belelovsky, H. Kaphzan, A. Elkobi, K. Rosenblum, Biphasic activation of the mTOR pathway in the gustatory cortex is correlated with and necessary for taste learning, J. Neurosci. 29 (23) (2009) 7424–7431.
[19] J.E. Ploski, V.J. Pierre, J. Smucny, K. Park, M.S. Monsey, K.A. Overeem, G.E. Schafe, The activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) is required for
memory consolidation of pavlovian fear conditioning in the lateral amygdala, J. Neurosci. 28 (47) (2008) 12383–12395.
[20] D. Nakayama, Y. Hashikawa-Yamasaki, Y. Ikegaya, N. Matsuki, H. Nomura, Late Arc/Arg3.1 expression in the basolateral amygdala is essential for persistence of newly-acquired and reactivated contextual fear memories, Sci. Rep. 6 (2016) 21007.
[21] S.A. Josselyn, S. Kida, A.J. Silva, Inducible repression of CREB function disrupts amygdala-dependent memory, Neurobiol. Learn. Mem. 82 (2) (2004) 159–163.
[22] S. Park, J.M. Park, S. Kim, J.A. Kim, J.D. Shepherd, C.L. Smith-Hicks, S. Chowdhury, W. Kaufmann, D. Kuhl, A.G. Ryazanov, R.L. Huganir, D.J. Linden, P.F. Worley, Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD, Neuron 59 (1) (2008) 70–83.
[23] Y. Yasoshima, T. Yamamoto, K. Kobayashi, Amygdala-dependent mechanisms underlying memory retrieval of conditioned taste aversion, Chem. Senses 30 (Suppl. 1)
(2005) i158–i159.
[24] S. Inberg, E. Jacob, A. Elkobi, E. Edry, A. Rappaport, T.I. Simpson, J.D. Armstrong,
N. Shomron, M. Pasmanik-Chor, K. Rosenblum, Fluid consumption and taste novelty determines transcription temporal dynamics in the gustatory cortex, Mol. Brain 9 (2016) 13.
[25] R. Garcia, M.J. Simon, A. Puerto, Conditioned place preference induced by electrical
stimulation of the insular cortex: effects of naloxone, Exp. Brain Res. 226 (2) (2013) 165–174.