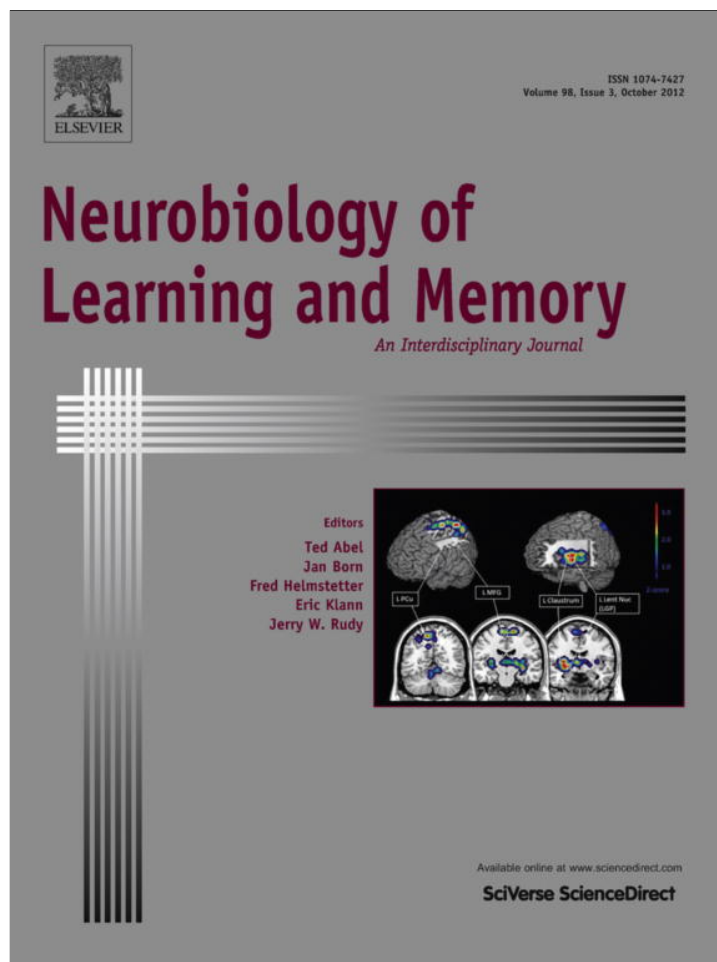


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

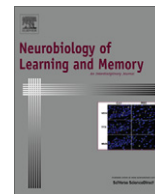
In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at SciVerse ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Maintenance of long-term memory storage is dependent on late posttraining Egr-1 expression

Cynthia Katche^{a,1}, Andrea Goldin^{a,1}, Carolina Gonzalez^a, Pedro Bekinschtein^{a,2}, Jorge H. Medina^{a,b,*}

^aInstituto de Biología Celular y Neurociencias "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 3rd Floor, C1121ABG Buenos Aires, Argentina
^bDepartamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 3rd Floor, C1121ABG Buenos Aires, Argentina

ARTICLE INFO

Article history:

Received 29 May 2012

Revised 31 July 2012

Accepted 2 August 2012

Available online 10 August 2012

Keywords:

Persistence of LTM storage

Egr-1

Immediate-early gene

Hippocampus

Inhibitory avoidance

ABSTRACT

Expression of immediate-early genes, like Egr-1, has been shown to be induced by activity-dependent synaptic plasticity or behavioral training and is widely thought to play an important role in long-term memory (LTM) formation. However, little is known about the role of Egr-1 in the maintenance of memory storage. Here we show that dorsal hippocampal Egr-1 protein expression is upregulated between 12 and 24 h after strong inhibitory avoidance (IA) training in rats. Local infusion of antisense oligodeoxynucleotide (ASO) to specifically knockdown Egr-1 in the dorsal hippocampus 8 h posttraining impairs LTM tested 7 days, but not 1 day after training, indicating that a delayed learning-associated expression of Egr-1 is necessary for the persistence of LTM storage. In addition, we show that consolidation of the IA memory is accompanied by an increase in Egr-1 protein levels 3 h, but not immediately or 1 h after training. Local infusion of *egr-1* ASO 30 min before training in the dorsal hippocampus persistently hinders memory formation measured 1 and 7 days after IA training, indicating the crucial role of Egr-1 in memory formation. Our findings demonstrate that there are at least two waves of Egr-1 expression in the dorsal hippocampus after IA training, an early wave which is involved in IA LTM formation, and a lasting late wave that peaks around 12–24 h after a strong training protocol which is specifically involved in the maintenance of LTM storage.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Early Growth Response-1 (Egr-1), also known as Zif-268 or Zenk, is a member of the zinc finger family of transcriptional factors induced by stress or injury, differentiation factors, and a variety of extracellular signals including neurotransmitters, peptides and growth factors (reviewed in Davis, Bozon, and Laroche (2003), Herdegen and Leah (1998) and O'Donovan et al. (1999)). Egr-1 regulates the expression of a number of late-response genes involved in growth control, survival and in plasticity-related processes in the brain (Bozon et al., 2003; Maddox, Monsey, & Schafe, 2011; Sukhatme et al., 1988; Williams et al., 2000). Egr-1 is rapidly induced by behavioral training in the amygdala (Rosen, Fanselow, Young, Sircoske, & Maren, 1998) and hippocampus (Guzowski, Setlow, Wagner, & McGaugh, 2001; Miyashita, Kameyama, Hasegawa, &

Abbreviations: LTM, long-term memory; IA, inhibitory avoidance; ASO, antisense oligodeoxynucleotide; MSO, missense oligodeoxynucleotide.

* Corresponding author at: Instituto de Biología Celular y Neurociencias, Facultad de Medicina, Paraguay 2155, 3rd Floor, C1121ABG Buenos Aires, Argentina. Fax: +54 11 59509626.

E-mail address: jmedina@fmed.uba.ar (J.H. Medina).

¹ These authors contributed equally to this work.

² Present address: Department of Experimental Psychology, University of Cambridge, Cambridge, United Kingdom.

1074-7427/\$ - see front matter © 2012 Elsevier Inc. All rights reserved.

<http://dx.doi.org/10.1016/j.nlm.2012.08.001>

Fukushima, 1998; Nikolaev, Kaminska, Tischmeyer, Matthies, & Kaczmarek, 1992).

Egr-1 has an important role in learning and memory. Deletion of Egr-1 leads to a reduction of hippocampal late-long-term potentiation (L-LTP) as well as a LTM impairing for several tasks including spatial navigation in the Morris water maze, conditioned taste aversion, social transmission of food preference and object recognition (Jones et al., 2001). In addition, Egr-1 mutant mice are unable to consolidate information about the spatial location or the features of objects (Bozon et al., 2003). By using *egr-1* antisense oligonucleotides (ASO) infused into the amygdala, Malkani, Wallace, Donley, and Rosen (2004) found impaired expression of fear conditioning. Furthermore, Yang et al. (2012) infused *egr-1* ASO in the hippocampus and found an impairment in spatial memory consolidation and also the infusion before retrieval impaired reconsolidation of contextual fear conditioning memory (Lee, Everitt, & Thomas, 2004).

Despite the general consensus that Egr-1 participates in the mechanisms involved in memory consolidation and reconsolidation, there is no information concerning the role of Egr-1 in the mechanisms involved in the persistence of LTM storage. In a series of previous experiments carried out by our group we demonstrated a novel BDNF- and protein synthesis-dependent late consolidation phase in the dorsal hippocampus important for the persistence of

memory storage (Bekinschtein et al., 2007). We also found that this phase is accompanied by an increase in Egr-1 protein expression 24 h after training. Therefore, the present study was designed to determine whether or not this late wave of Egr-1 expression in the dorsal hippocampus is required for maintaining the memory trace of a one-trial IA LTM in rats.

2. Material and methods

2.1. Subjects

Male Wistar rats (2.5 months/220–250 g) from our own breeding colony were used. Animals were housed five to a cage at 23 °C, with water and food *ad libitum*, under a 12 h light/dark cycle (lights on at 7:00 a.m.). The procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory

Animals and were approved by the Animal Care and Use Committees of the University of Buenos Aires.

2.2. Inhibitory avoidance training and testing

Animals were trained in a one-trial step-down inhibitory avoidance task (IA) as previously described (Bekinschtein et al., 2007). Briefly, the apparatus was a 50 × 25 × 25 cm acrylic box with a 5 cm high, 7 cm wide, and 25 cm long platform on the left end of a series of stainless steel bars that made up the floor of the box. For training, animals were gently placed on the platform; as they stepped down to the grid they received either a 3 s, 0.7 mA scrambled foot-shock (strong training) or a 3 s, 0.3 mA scrambled foot-shock (weak training). Rats were tested for retention 1 or 7 days after training. All animals were tested only once. In the test sessions the foot-shock was omitted. Significant differences on latency to step down between training and test sessions were

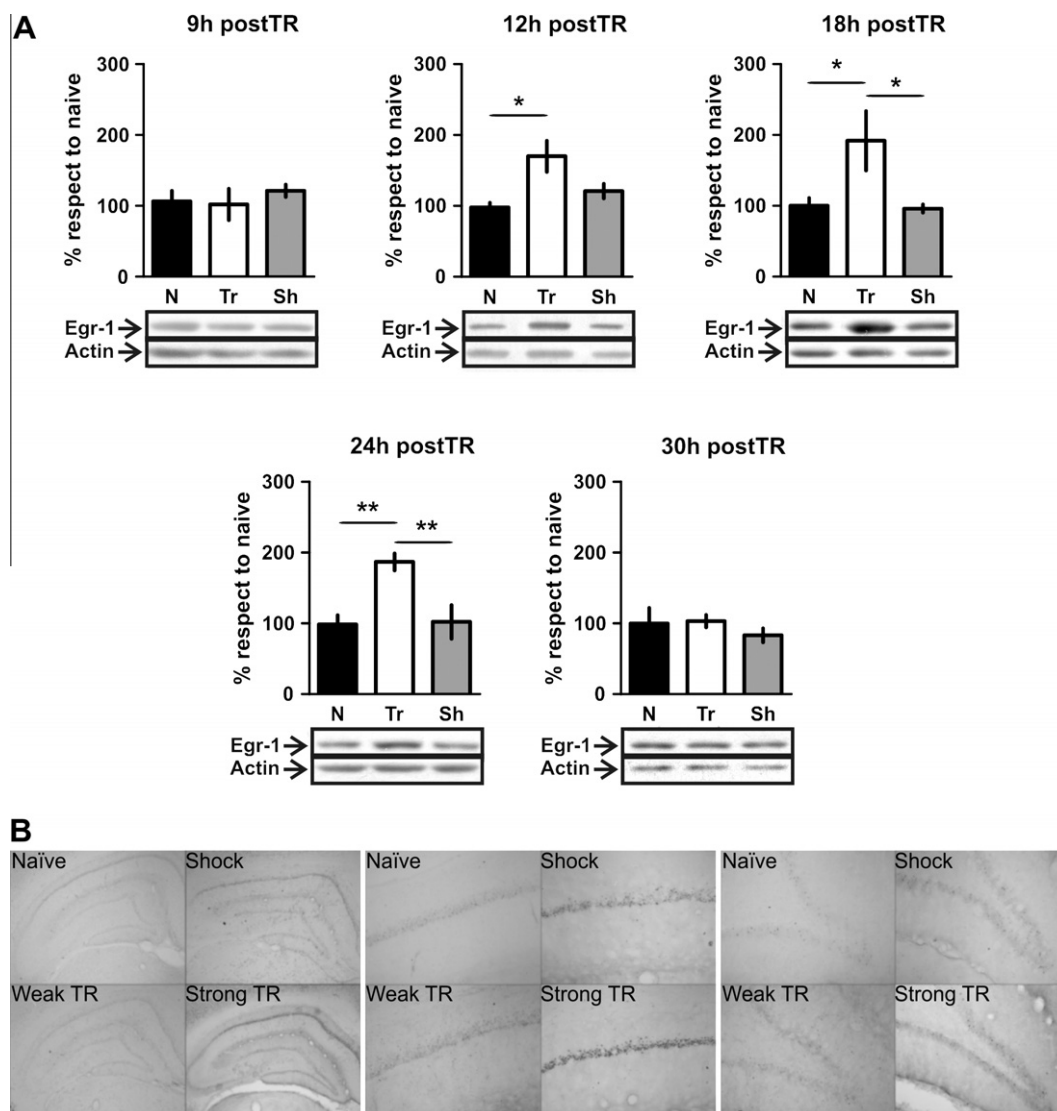


Fig. 1. IA training induces a delayed wave of Egr-1 in the CA1 of dorsal hippocampus. (A) Time course of hippocampal Egr-1 levels late after strong IA training. Bars indicate the percentage of change respect to the naive (N) and shocked (Sh) groups for rats trained (Tr) and sacrificed 9, 12, 18, 24 or 30 h after the behavioral procedure. Data are expressed as mean ± SEM of Egr-1/Actin ratio. **p* < 0.05; ***p* < 0.01; Newman–Keuls test after ANOVA, *n* = 5–6 per group. (B) Egr-1 immunoreactivity is increased in CA1 region of dorsal hippocampus after strong IA training. Rats were sacrificed 12 h after IA training, coronal sections of the brain were subjected to immunohistochemical analysis using antibodies against Egr-1. Representative photomicrographs show Egr-1 immunoreactivity in the dorsal hippocampus (Hp, left panel). Inset at a high magnification showing the CA1 region of the dorsal hippocampus (middle panel) and dentate gyrus (DG, right panel).

taken as a measure of memory retention. In all experiments, the animals were trained between 7:00 a.m. and 9:00 a.m.

2.3. Open field and elevated plus maze tests

The open field was a 50 × 50 × 39 cm arena with black plywood walls and a brown floor divided into nine squares by black lines. The number of line crossings and rearings were measured during 5-min long test session. To evaluate their anxiety state, animals were exposed to an elevated plus maze. The total number of entries into the four arms, the number of entries, and time spent in the open arms were recorded over a 5 min session.

2.4. Immunoblot assays

The animals utilized in the biochemical experiments were divided in three experimental groups: (1) animals trained in the inhibitory avoidance task and killed at different times after training (trained group, TR); (2) animals received a foot-shock identical to that given to the trained ones but were not submitted to the IA training procedure (the platform was not inside the box, and the animals were put directly over the grid) and killed at the same time points than the trained group (shocked group, S); and (3) animals withdrawn from their home cages at the same time points than the other two groups and killed immediately thereafter (naïve group, N); Two additional control groups were done at 24 h: (1) context-no footshock group, animals were placed on the platform but did not receive a foot shock when they stepped down to the grid and, (2) delayed foot-shock group, animals were placed on the platform, no foot shock was given, the subjects were returned to their home cage, and then, 1 h later, they received the foot shock. The dorsal hippocampus was dissected out and rapidly homogenized in ice-chilled buffer (20 mM Tris-HCl (pH 7.4), 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin, 15 µg/ml leupeptin, 50 mM NaF and 1 mM sodium) as described previously (Bekinschtein et al., 2007). Tissue was homogenized and samples of homogenates were subjected to SDS-PAGE as described before. PVDF membranes were incubated first with anti-Egr1 antibody (1:2000; Santa Cruz Biotechnology Inc, Santa Cruz, CA), then stripped and incubated with anti-Actin antibody (1:5000, Santa Cruz Biotechnology Inc, Santa Cruz, CA). Film densitometry analysis was performed by using Gel-Pro Analyzer (version 4.0, Media Cybernetics, Inc., MD).

Table 1

Strong IA training induces a late increase in Egr-1 expression.

Group	% Change
Naïve	100 ± 5.6
Shock	102.3 ± 23.8
Context no foot-shock	97.7 ± 2.6
Delayed foot-shock	102.9 ± 4.7
Strong IA training	186.9 ± 12***

Newman-Keuls test after ANOVA; n = 5 per group. Data are expressed as mean ± SEM.

*** p < 0.001.

2.5. Immunohistochemistry

Rats were anesthetized 12 h after IA training and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde. Brains were isolated and sliced. The brain sections were subjected to an immunohistochemical assay with an anti-Egr-1 antibody (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The reaction product was then visualized using the nickel-DAB technique.

2.6. Surgery and infusion procedures

Rats were implanted under deep ketamine/xilacine anesthesia with 22-g guide cannulae in the dorsal CA1 region of the hippocampus at coordinates A -4.3, L ±3.0, V 1.4 of the atlas of Paxinos and Watson (1997). The cannulae were fixed to the skull with dental acrylic. After recovery from surgery, the animals were handled once a day for 2 days and then trained in IA. In all cases, infusions were bilateral and had a volume of 1 µl. The entire infusion procedure took ~2 min, including 45 s for the infusions themselves, first on one side and then on the other, and the handling. Histological examination of cannula placements was performed and only the behavioral data from animals with the cannula located in the intended site were included in the final analysis.

2.7. Drugs

Oligonucleotides (ODN) (Genbiotech, S.R.L.) were HPLC-purified phosphorothioated end-capped 18-mer sequences, resuspended in sterile saline to a concentration of 2 nmol/µl. Both ODNs were phosphorothioated on the three terminal bases of both 5' and 3'

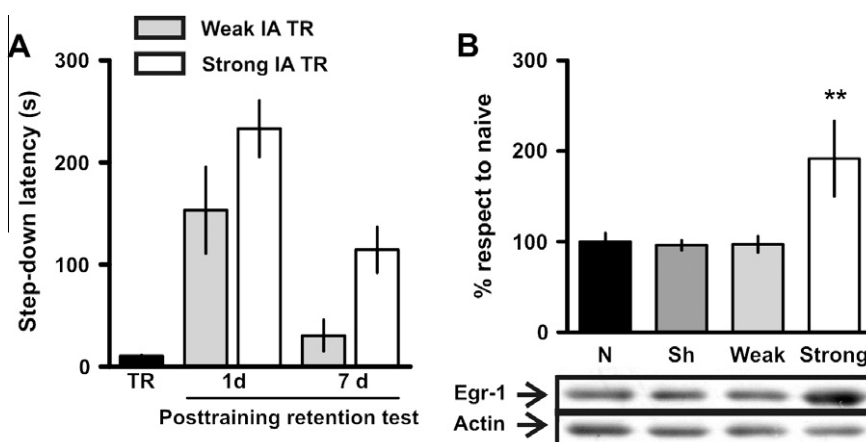


Fig. 2. Egr-1 increase is associated with a strong, but not weak, IA training. (A) Strong (0.7 mA), but not weak (0.3 mA), IA training generates a persistent LTM. Data are expressed as mean ± SEM of TR (black bars) or test-session step-down latency at 1 or 7 days after weak (gray bars) or strong (white bars) IA training. ***p < 0.0001 vs. TR; Student's *t* test, n = 10 per group. (B) Strong, but not weak, training is associated with an increase in Egr-1 in the dorsal hippocampus 18 h after IA training. Bars show normalized mean percentage level of Egr-1 respect to the naïve group. Data are expressed as mean ± SEM. **p < 0.01 vs. naïve in Newman-Keuls test after ANOVA, n = 6 per group.

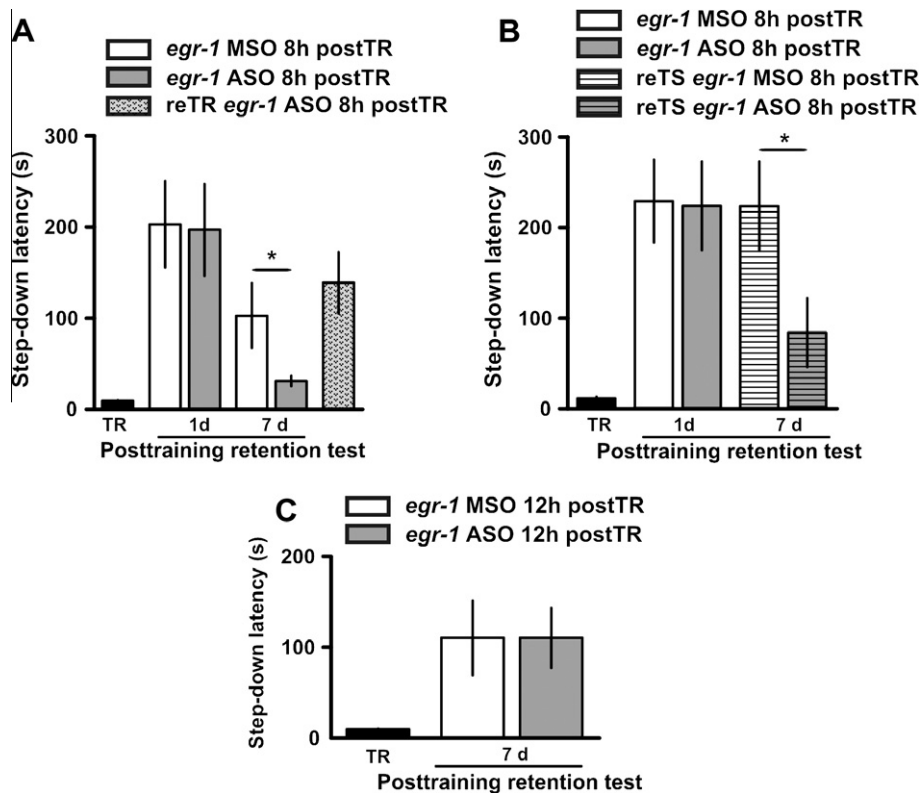


Fig. 3. Delayed Egr-1 expression is required for the persistence of LTM storage. (A) Intrahippocampal infusion of *egr-1* antisense oligonucleotide (*egr-1* ASO), but not *egr-1* missense oligonucleotide (*egr-1* MSO), 8 h after IA training hinders the persistence of LTM storage at 7 days. Animals were infused into the dorsal hippocampus with *egr-1* MSO (2 nmol per side) (white bars) or *egr-1* ASO (2 nmol per side) (gray bars) 8 h after training. Data are expressed as mean \pm SEM of training (TR, black bars) or test session step-down latency 1 or 7 days after IA training or 1 day after IA retraining of the *egr-1* ASO group (reTR) (dotted gray bar). * $p < 0.05$; ASO vs. MSO at 7 days; Student's *t* test, $n = 10$ –12 per group. (B) Infusion of *egr-1* ASO 8 h after training leaves memory intact at 1 day, but impairs it when retested at 7 days. Animals were infused into the dorsal hippocampus with *egr-1* MSO (2 nmol per side) (white bars) or *egr-1* ASO (2 nmol per side) (gray bars) 8 h after training, tested at 1 day and retested at 7 days. Data are expressed as mean \pm SEM of training (TR, black bars) or test session step-down latency 1 or 7 days after IA training. $p > 0.5$; ASO vs. MSO at 1 day and, * $p < 0.05$; ASO vs. MSO at 7 days; Student's *t* test, $n = 8$ per group. (C) Infusion of *egr-1* ASO 12 h after IA training does not affect the persistence of LTM storage. Animals were infused into the dorsal hippocampus with *egr-1* MSO (2 nmol per side) (white bars) or *egr-1* ASO (2 nmol per side) (gray bars) 12 h after training. Data are expressed as mean \pm SEM of training (TR, black bars) or test session step-down latency 7 days after IA training. $p > 0.5$; ASO vs. MSO at 7 days; Student's *t* test, $n = 8$ per group.

ends. This modification results in increased stability and less toxicity of the ODN. *egr-1* ASO 5'-GGT AGT TGT CCA TGG TGG-3', *egr-1* MSO 5'-GTG TTC GGT AGG GTG TCA-3'. Both ODN sequences were subjected to a BLAST search on the National Center for Biotechnology Information BLAST server using the Genbank database. ASO is specific for rat *egr-1* mRNA. Control missense (MSO) sequence, which included the same 18 nucleotides as the ASO but in a scrambled order, did not generate any full matches to identified gene sequences in the database. To determine the degree of inhibition of Egr-1 protein expression, *egr-1* ASO and MSO was infused 4 h before sacrifice trained animals in IA task and immunoblots assays were carried out as described above.

2.8. Data analysis

In all behavioral experiments statistical analysis was performed by unpaired Student's *t* test or one-way ANOVA comparing mean step-down latencies of drug-treated groups and vehicle at each time point studied. Immunoblot data were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. All data are presented as mean \pm SEM.

3. Results

To determine whether IA training results in a learning-specific alteration in Egr-1 protein expression in the dorsal hippocampus, we measured Egr-1 levels by immunoblotting in the dorsal hip-

campus at 9, 12, 18, 24 and 30 h after training. Using a strong training protocol that yielded persistent IA memories (Bekinschtein et al., 2007, 2008 and Fig. 2A) we found a late wave of Egr-1 increased protein expression between 12 h and 24 h following training (Fig. 1). This wave was transient because no changes were found at the 30th hour time point. Immunohistochemical assays revealed that this increase was mainly due to changes in Egr-1 immunoreactivity in the CA1 region of the dorsal hippocampus (Fig. 1B).

Based on our previous results, we hypothesized that this late IA training-associated upregulation of Egr-1 was related to the persistence of LTM storage. We reasoned that if the late wave of Egr-1 expression is specifically required for maintenance of a persistent LTM, it should not occur after a training session unable to induce a long-lasting LTM trace. IA training using a strong foot-shock (0.7 mA, 3 s; strong training, Fig. 2A), which generates a persistent LTM as tested 7 days after training, increased Egr-1 expression in the dorsal hippocampus 18 h posttraining ($p < 0.01$ compared to naïve, Fig. 2B). On the other hand, training with a mild foot-shock (0.3 mA, 3 s; weak training), which yields a rapidly decaying LTM (Fig. 2A), did not change Egr-1 levels at 18 h posttraining (Fig. 2B). Additional control experiments included a context-no footshock group, in which the animals were placed on the platform, but did not receive a footshock when stepped down to the grid and also a delayed foot-shock group (see Section 2). Immunoblot analysis revealed significant differences in Egr-1 levels 24 h after strong IA training with respect to all control groups

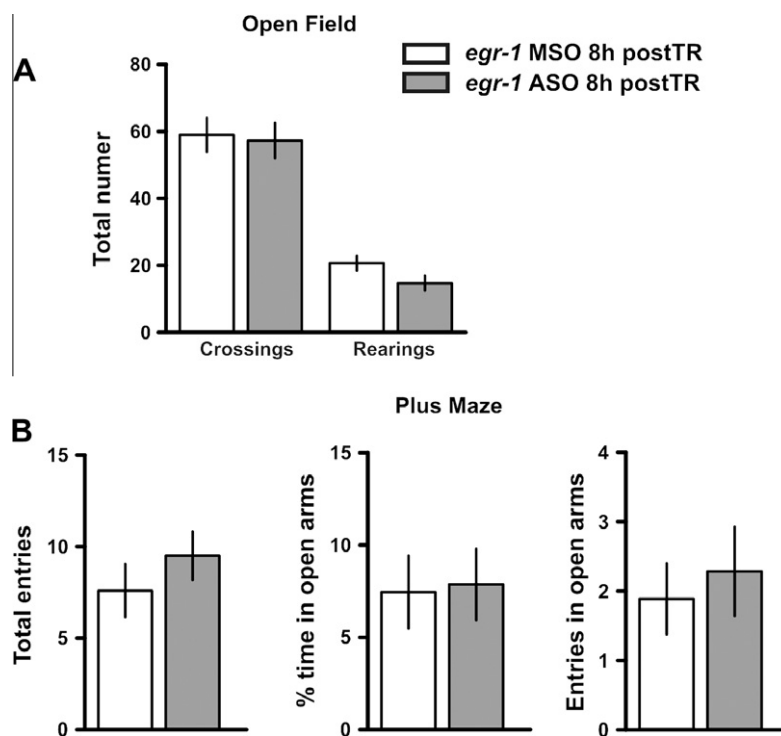


Fig. 4. Infusion of *egr-1* ASO 8 h after IA training does not affect locomotor activity, anxiety state, or exploratory behavior. (A) Total number of rearings and crossings during a 5 min open-field (OF) session for animals that had received bilateral infusion of *egr-1* MSO (2 nmol per side) (white bars) or *egr-1* ASO (2 nmol per side) in dorsal CA1 8 h posttraining 7 days before. Data are expressed as mean \pm SEM number of crossings or rearings $p > 0.1$; Student's *t* test, $n = 8$ per group. (B) Total number of entries (left), time spent in open arms (center), and number of entries into the open arms (right) during a 5 min plus maze session for rats that had received bilateral intra-CA1 infusion of *egr-1* MSO (2 nmol per side) (white bars) or *egr-1* ASO (2 nmol per side) (gray bars) 7 days before $p > 0.1$; Student's *t* test, $n = 8$ per group.

($p < 0.001$, Table 1), confirming that late Egr-1 expression is associated with a training experience generating a persistent memory.

The experiments presented above indicate that hippocampal Egr-1 protein levels were increased 12–24 h after IA training that generates a long-lasting LTM, but they do not address the question of whether Egr-1 expression is in fact required for the persistence of LTM storage. We used an antisense oligonucleotide (ASO) (Lee et al., 2004) to specifically knockdown *de novo* Egr-1 expression in the hippocampus. After assessing that the distribution and stability of the ASO infused into the dorsal hippocampus are similar to our own previous studies and other reports describing the use of intra-hippocampal oligonucleotide injections (Guzowski et al., 2000; Lee et al., 2004; Taubenfeld, Milekic, Monti, & Alberini, 2001; Bekinschtein et al., 2007, 2008; Katche et al., 2010), we found that local infusion of *egr-1* ASO abolished IA training-induced increase in Egr-1 protein expression (MSO infused rats: $191 \pm 11\%$, $n = 5$, $**p < 0.01$ respect to naïve rats, and ASO infused rats: 135 ± 15 , $n = 5$, $p > 0.05$ respect to naïve rats; $*p < 0.05$ MSO vs. ASO; in Newman–Keuls test after ANOVA, $n = 6$ per group).

The infusion of *egr-1* ASO, but not *egr-1* MSO 8 h after IA training caused a severe memory impairment 7 days posttraining but left memory intact at 1 day after acquisition (Fig. 3A, $t = 2.96$, $p = 0.008$, $n = 10$, ASO vs. MSO at 7 days, Student's *t* test). Two days after the last test session (9th day), animals previously infused with *egr-1* ASO were submitted again to a strong training protocol and tested 24 h thereafter. As shown in Fig. 3A, rats exhibited similar retention latencies to those observed 1 day after original training, suggesting that ASO infusions did not affect the functionality of hippocampus.

Since the experiments shown in Fig. 3A involved testing animals 1 or 7 days after training, i.e., different groups of animals tested at the two time points, we asked whether the selective deficit in long-lasting memory storage seen after bilateral infusion of

egr-1 ASO is also observed when the same group of rats was tested at both 1 and 7 days following training. As shown in Fig. 3B, rats microinfused with *egr-1* ASO at 8 h posttraining showed no alterations in memory retention when tested 1 day after training, but exhibited a marked memory deficit when tested again 7 days post-training. No changes in the persistence of LTM storage were found when *egr-1* ASO was infused 12 h after training (Fig. 3C).

Since performance in the IA task could be modified by factors such as basal locomotor activity and anxiety, which can be potentially affected by *egr-1* ASO we analyzed the behavior of animals that had been injected with *egr-1* ASO 8 h after IA training in the open field and elevated plus maze tests. Intra-CA1 infusion of ASO after IA training did not affect anxiety state or exploratory behavior in a novel environment and did not modify basal locomotor activity as evaluated 7 days after *egr-1* ASO administration (Fig. 4), strongly suggesting that the observed memory deficit is directly caused by blocking Egr-1 expression required for persistence of the memory trace. Moreover, it is not likely that the lower retention score at 7 days was due to modifications in performance, since ASO infusion at posttraining time later than 8 h did not cause any deficit in memory retention at 7 days (see Fig. 3A).

Given that some controversy emerged concerning the role of hippocampal Egr-1 in memory consolidation of some hippocampus-dependent learning tasks (Hall, Thomas, & Everitt, 2000; Jones et al., 2001; Lee et al., 2004), we next determined whether or not Egr-1 in the dorsal hippocampus is required for IA memory formation. Therefore, we infused *egr-1* ASO or MSO 30 min before a strong IA training and tested rats at 1 or 7 days following training. As shown in Fig. 5A, *egr-1* ASO provoked a marked and persistent deficit in retention scores. Two days after rats were tested at 7 days time point they were again subjected to IA training and tested 24 h thereafter. Rats showed similar retention scores to those obtained 24 h following the original training, indicating that ASO infusion

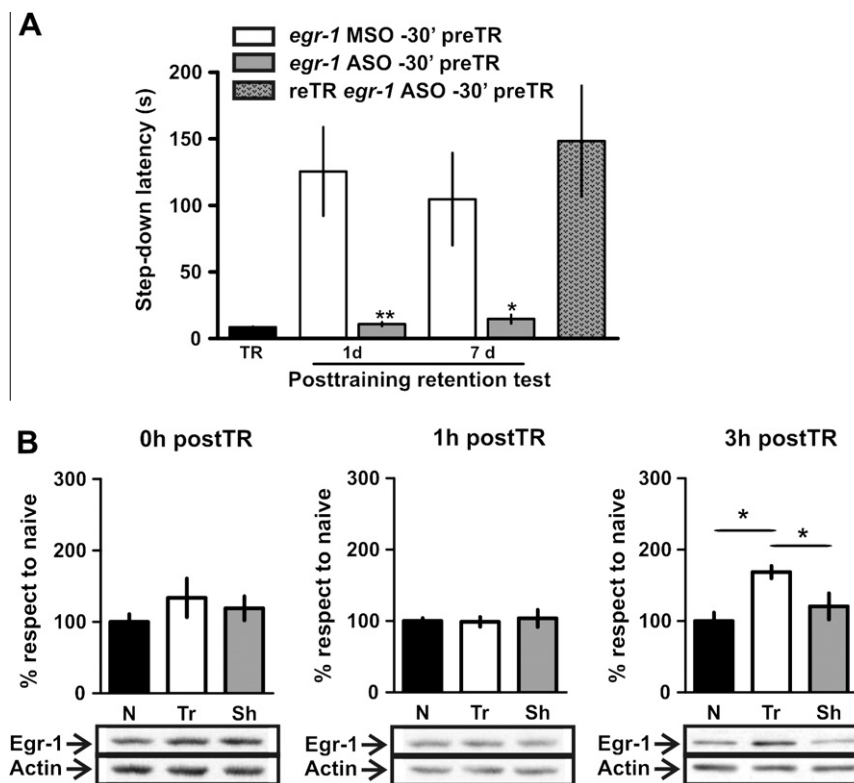


Fig. 5. Early Egr-1 expression is required for memory formation. (A) Infusion of *egr-1* ASO 30' pre-training prevent IA memory formation. Animals were infused into the dorsal hippocampus with *egr-1* MSO (2 nmol per side) (white bars) or *egr-1* ASO (2 nmol per side) (gray bars) 30 min before IA training. Data are expressed as mean \pm SEM of TR (black bars) or test-session step-down latency at 1 or 7 days after IA training or 1 day after IA retraining of the *egr-1* ASO group (reTR) (dotted gray bar). ** $p < 0.01$; ASO vs. MSO at 1 day and, * $p < 0.05$; ASO vs. MSO at 7 days. (B) Time course of hippocampal Egr-1 levels early after strong IA training. Bars indicate the percentage of change respect to the naive group (N) for rats trained (TR), or shocked (Sh) and sacrificed 0, 1 or 3 h after the behavioral procedure. Data are expressed as mean \pm SEM of Egr-1/Actin ratio. * $p < 0.05$, Newman-Keuls test after ANOVA, $n = 5-6$ per group.

did not affect the functionality of the hippocampus. Interestingly, IA training resulted in an increased Egr-1 protein expression 3 h after training (Fig. 5B). No changes in Egr-1 expression were observed immediately or 1 h after training.

4. Discussion

The main finding of the present study is that Egr-1 protein expression during a late and restricted posttraining time window is required for the maintenance of IA LTM storage, but not IA memory formation. These results demonstrate that the hippocampus is still engaged in memory processing after LTM is already formed and that a previously unknown phase of Egr-1 expression plays an important role in maintenance of a memory trace over time. Taking together with a recent study (Katche et al., 2010), the present findings strongly suggest that a delayed wave of IEGs in the hippocampus is part of the molecular mechanisms responsible for the establishment of persistent memories. In addition our experiments showed that an IA training protocol that induced persistent IA LTM resulted in an increased expression of Egr-1 that begins around 12 h after training and ends about 24 h posttraining. In contrast, an IA training protocol that induced transient IA LTM was not associated with an upregulation of Egr-1 protein expression. Although no alterations in Egr-1 protein levels were found at 30 h after training (Fig. 1), we cannot rule out the possibility that after that time point other waves of Egr-1 might take place. In this context, Baumgartel et al. (2008) observed an increase in Egr-1 mRNA expression in the amygdala of rats trained in a conditioning taste aversion task 72 h posttraining. Interestingly, Egr-1 overex-

pression in the mouse forebrain is accompanied by a more persistent CTA memory during extinction training (Baumgartel et al., 2008).

Here we show that the infusion of *egr-1* ASO 8 h after training impairs long-lasting memory storage at 7 days, but not memory formation at 1 day. This indicates that the late wave of Egr-1 expression is not involved in the mechanism of memory formation and it is critical for long-lasting storage of LTM in the hippocampus. This statement is also supported by the fact that in contrast to what occurs when animals generate a persistent LTM, a weak IA training which produces a visible LTM at 1 day, but not at 7 days, does not induce the late increase in Egr-1 levels. On the other hand, the infusion of *egr-1* ASO 12 h after training, has no effect on long-lasting LTM storage despite the of increased Egr-1 expression is between 12 and 24 h. This is an expected finding, because ASO infusions need at least a couple of hours to affect target protein levels (Bekinschtein et al., 2007; Katche et al., 2010); therefore, our findings indicate that the induction of a late wave of Egr-1 about 12 h after training is crucial for persistence of LTM storage.

Given that ERK 1/2 activation is part of the upstream cascade involved in Egr-1 regulated transcription (see Davis et al., 2003; Revest et al., 2005), our present results are consistent with the demonstration that a late BDNF-induced activation of ERK1/2 in the hippocampus is also crucial for the persistence of the memory trace (Bekinschtein et al., 2007, 2008; Eckel-Mahan et al., 2008).

What are the downstream "effector" genes regulated by the increased expression of Egr-1 late after training? It has been shown that Egr-1 regulates a diversity of substrates with different biological roles including protein degradation (Baumgartel et al., 2009; James, Conway, & Morris, 2006), cell division, metabolism, sensory

perception (Baumgartel et al., 2009). In addition, Egr-1 controls the expression of Arc, another IEG that behaves as a late-response gene (Penke, Chagneau, & Laroche, 2011). Recent studies provide evidence that memory storage is associated with synapse and dendritic spine remodeling and also growth of new synaptic connections (Lai, Franke, & Gan, 2012; Lamprecht & LeDoux, 2004; Miniaci et al., 2008). Thus, late Egr-1-dependent transcription could be necessary for the expression of effector genes involved in the synaptic remodeling related to the persistence of LTM storage. In line with this suggestion, there are reports showing that Egr-1 controls the neuronal machinery of protein degradation (James et al., 2006) and that 48 h after a fear-motivated training there is a significant increase in the number of CA1 dendritic spines (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009).

While Egr-1 is widely assumed to play a selective role in LTM formation, there are conflicting findings regarding the role of Egr-1 in fear-motivated learning tasks that depend on the hippocampus, like the contextual fear conditioning (Lee et al., 2004, but see Revest et al., 2005). Although, Egr-1 regulated transcription in the hippocampus is associated with the late phase of LTP and correlated well with the maintenance of LTP (Abraham, Dragunow, & Tate, 1991; Jones et al., 2001), behavioral studies that investigated whether contextual fear conditioning or step-through IA training, are associated with a learning-specific increase in *egr-1* mRNA in the CA1, gave negative results (Cheval et al., 2012; Hall et al., 2000; Malkani & Rosen, 2000). Our results showing no increase in hippocampal Egr-1 protein levels immediately after or 1 h posttraining are in line with those studies. In contrast, Cheval et al. (2012) found that Egr-1 DNA binding activity to the Egr response element is indeed increased in the CA1 in a learning-specific manner 2 h after training. In the present study a learning-specific increase in dorsal hippocampal Egr-1 levels was observed 3 h posttraining. This change occurred mainly in the CA1 region, but not in the dentate gyrus. Consistent with this finding, we observed that CA1 infusion of Egr-1 ASO around training blocked IA memory consolidation as tested 1 or 7 days after training (Fig. 5). Our results are in well agreement with Piazza and colleagues (Revest et al., 2005) who demonstrated that glucocorticoids-induced enhancement of contextual fear memory depends on Egr-1 under the control of ERK1/2.

In conclusion, our findings reveal that Egr-1 plays a pivotal role in different stages of memory processing, not only in memory consolidation and reconsolidation but also in the maintenance of memory storage. We suggest that Egr-1-dependent transcriptional processes occurring late after training in the hippocampus are at the interface between cellular- and systems-level memory consolidation.

Acknowledgments

We thank Leandro Slipczuk and Guido Dorman for help with some experiments. This work was supported by research grants from the National Agency of Scientific and Technological Promotion of Argentina (ANPCyT) and University of Buenos Aires (UBACyT).

References

- Abraham, W. C., Dragunow, M., & Tate, W. P. (1991). The role of immediate early genes in the stabilization of long-term potentiation. *Molecular Neurobiology*, 5(2–4), 297–314.
- Baumgartel, K., Genoux, D., Welzl, H., Tweedie-Cullen, R. Y., Koshibu, K., Livingstone-Zatchej, M., et al. (2008). Control of the establishment of aversive memory by calcineurin and Zif268. *Nature Neuroscience*, 11(5), 572–578.
- Baumgartel, K., Tweedie-Cullen, R. Y., Grossmann, J., Gehrig, P., Livingstone-Zatchej, M., & Mansuy, I. M. (2009). Changes in the proteome after neuronal zif268 overexpression. *Journal of Proteome Research*, 8(7), 3298–3316.
- Bekinschtein, P., Cammarota, M., Izag, L. M., Bevilaqua, L. R., Izquierdo, I., & Medina, J. H. (2007). Persistence of long-term memory storage requires a late protein synthesis- and BDNF-dependent phase in the hippocampus. *Neuron*, 53(2), 261–277.
- Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J. I., Goldin, A., et al. (2008). BDNF is essential to promote persistence of long-term memory storage. *Proceedings of the National Academy of Sciences of the United States of America*, 105(7), 2711–2716.
- Bozon, B., Kelly, A., Josselyn, S. A., Silva, A. J., Davis, S., & Laroche, S. (2003). MAPK, CREB and zif268 are all required for the consolidation of recognition memory. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, 358(1432), 805–814.
- Cheval, H., Chagneau, C., Levasseur, G., Veyrac, A., Faucon-Biguier, N., Laroche, S., et al. (2012). Distinctive features of Egr transcription factor regulation and DNA binding activity in CA1 of the hippocampus in synaptic plasticity and consolidation and reconsolidation of fear memory. *Hippocampus*, 22(3), 631–642.
- Davis, S., Bozon, B., & Laroche, S. (2003). How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? *Behavioural Brain Research*, 142(1–2), 17–30.
- Eckel-Mahan, K. L., Phan, T., Han, S., Wang, H., Chan, G. C., Scheiner, Z. S., et al. (2008). Circadian oscillation of hippocampal MAPK activity and cAmp: Implications for memory persistence. *Nature Neuroscience*, 11(9), 1074–1082.
- Guzowski, J. F., Lyford, G. L., Stevenson, G. D., Houston, F. P., McGaugh, J. L., Worley, P. F., et al. (2000). 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. *Journal of Neuroscience*, 20(11), 3993–4001.
- Guzowski, J. F., Setlow, B., Wagner, E. K., & McGaugh, J. L. (2001). Experience-dependent gene expression in the rat hippocampus after spatial learning: A comparison of the immediate-early genes Arc, c-fos, and zif268. *Journal of Neuroscience*, 21(14), 5089–5098.
- Hall, J., Thomas, K. L., & Everitt, B. J. (2000). Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nature Neuroscience*, 3(6), 533–535.
- Herdegen, T., & Leah, J. D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: Control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Research. Brain Research Reviews*, 28(3), 370–490.
- James, A. B., Conway, A.-marie, & Morris, B. J. (2006). Regulation of the neuronal proteasome by Zif268 (Egr1). *Journal of Neuroscience*, 26(5), 1624–1634.
- Jones, M. W., Errington, M. L., French, P. J., Fine, A., Bliss, T. V., Garel, S., et al. (2001). A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nature Neuroscience*, 4(3), 289–296.
- Katche, C., Bekinschtein, P., Slipczuk, L., Goldin, A., Izquierdo, I. A., Cammarota, M., et al. (2010). Delayed wave of c-Fos expression in the dorsal hippocampus involved specifically in persistence of long-term memory storage. *Proceedings of the National Academy of Sciences of the United States of America*, 107(1), 349–354.
- Lai, C. S., Franke, T. F., & Gan, W. B. (2012). Opposite effects of fear conditioning and extinction on dendritic spine remodeling. *Nature*, 483(7387), 87–91.
- Lamprecht, R., & LeDoux, J. (2004). Structural plasticity and memory. *Nature Reviews Neuroscience*, 5(1), 45–54.
- Lee, J. L. C., Everitt, B. J., & Thomas, K. L. (2004). Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science (New York, NY)*, 304(5672), 839–843.
- Maddox, S. A., Monsey, M. S., & Schafe, G. E. (2011). Early growth response gene 1 (Egr-1) is required for new and reactivated fear memories in the lateral amygdala. *Learning & Memory*, 18(1), 24–38.
- Malkani, S., & Rosen, J. B. (2000). Specific induction of early growth response gene 1 in the lateral nucleus of the amygdala following contextual fear conditioning in rats. *Neuroscience*, 97(4), 693–702.
- Malkani, S., Wallace, K. J., Donley, M. P., & Rosen, J. B. (2004). An egr-1 (zif268) antisense oligodeoxynucleotide infused into the amygdala disrupts fear conditioning. *Learning & Memory*, 11(5), 617–624.
- Miniaci, M. C., Kim, J.-H., Puthanveetil, S. V., Si, K., Zhu, H., Kandel, E. R., et al. (2008). Sustained CPEB-dependent local protein synthesis is required to stabilize synaptic growth for persistence of long-term facilitation in Aplysia. *Neuron*, 59(6), 1024–1036.
- Miyashita, Y., Kameyama, M., Hasegawa, I., & Fukushima, T. (1998). Consolidation of visual associative long-term memory in the temporal cortex of primates. *Neurobiology of Learning and Memory*, 70(1–2), 197–211.
- Nikolaev, E., Kaminska, B., Tischmeyer, W., Matthies, H., & Kaczmarek, L. (1992). Induction of expression of genes encoding transcription factors in the rat brain elicited by behavioral training. *Brain Research Bulletin*, 28(3), 479–484.
- O'Donovan, K. J., Tourtellotte, W. G., Millbrandt, J., & Baraban, J. M. (1999). The EGR family of transcription-regulatory factors: Progress at the interface of molecular and systems neuroscience. *Trends in Neurosciences*, 22(4), 167–173.
- Paxinos, G., & Watson, C. (1997). *The rat brain in stereotaxic coordinates* (compact 3rd ed., Vol. 1, pp. 30–37). San Diego: Academic Press.
- Penke, Z., Chagneau, C., & Laroche, S. (2011). Contribution of Egr1/zif268 to activity-dependent Arc/Arg3.1 transcription in the dentate gyrus and area CA1 of the hippocampus. *Frontiers in Behavioral Neuroscience*, 5, 48.
- Restivo, L., Vetere, G., Bontempi, B., & Ammassari-Teule, M. (2009). The formation of recent and remote memory is associated with time-dependent formation of dendritic spines in the hippocampus and anterior cingulate cortex. *Journal of Neuroscience*, 29(25), 8206–8214.

- Revest, J. M., Di Blasi, F., Kitchener, P., Rouge-Pont, F., Desmedt, A., Turiault, M., et al. (2005). The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. *Nature Neuroscience*, 8(5), 664–672.
- Rosen, J. B., Fanselow, M. S., Young, S. L., Sitcoske, M., & Maren, S. (1998). Immediate-early gene expression in the amygdala following footshock stress and contextual fear conditioning. *Brain Research*, 796(1–2), 132–142.
- Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C., et al. (1988). A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell*, 53(1), 37–43.
- Taubenfeld, S. M., Milekic, M. H., Monti, B., & Alberini, C. M. (2001). The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta. *Nature Neuroscience*, 4(8), 813–818.
- Williams, J. M., Beckmann, A. M., Mason-Parker, S. E., Abraham, W. C., Wilce, P. A., & Tate, W. P. (2000). Sequential increase in Egr-1 and AP-1 DNA binding activity in the dentate gyrus following the induction of long-term potentiation. *Brain Research. Molecular Brain Research*, 77(2), 258–266.
- Yang, Y., Shu, X., Liu, D., Shang, Y., Wu, Y., Pei, L., et al. (2012). EPAC null mutation impairs learning and social interactions via aberrant regulation of miR-124 and Zif268 translation. *Neuron*, 73(4), 774–788.