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

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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.

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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, Sitcoske, & Maren, 1998) and hippocampus (Guzowski, Setlow, Wagner, & McGaugh, 2001; Miyashita, Kameyama, Hasegawa, & Fukushima, 1998; Nikolaev, Kaminska, Tischmeyer, Matthias, & 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
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 μM/ml aprotilin, 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 (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The reaction product was then visualized using the nickel-DAB technique.

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’

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>100 ± 5.6</td>
</tr>
<tr>
<td>Shock</td>
<td>102.3 ± 23.8</td>
</tr>
<tr>
<td>Context no foot-shock</td>
<td>97.7 ± 2.6</td>
</tr>
<tr>
<td>Delayed foot-shock</td>
<td>102.9 ± 4.7</td>
</tr>
<tr>
<td>Strong IA training</td>
<td>186.9 ± 12**</td>
</tr>
</tbody>
</table>

Newman–Keuls test after ANOVA; *n = 5 per group. Data are expressed as mean ± SEM. **p < 0.0001 vs. TR; Student’s t test, *p = 0.001 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. 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.
ends. This modification results in increased stability and less toxicity of the ODN. egr-1 ASO 5'-GGT AGT TGT CCA TGG TGG-3' and 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 ± 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 hippocampus 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.
(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, Mileikic, 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 ± 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 posttraining. 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. 4. Infusion of egr-1 ASO 8 h after IA training does not affect locomotor activity, anxiety state, or exploratory behavior.](image-url)
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 Egr-1 protein expression 3 h after training (Fig. 5B). No changes in Egr-1 expression were observed immediately or 1 h after training.

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 ± 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 naïve 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 ± SEM of Egr-1/Actin ratio. *p < 0.05, Newman–Keuls test after ANOVA, n = 5–6 per group.

What are the downstream "effector" genes regulated by the increased expression of Egr-1 late after training? It has been shown that Egr-1 overexpression 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, 2008; Eckel-Mahan et al., 2008); 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., 2008; 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, Chagnon, & Larroche, 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, 2005) who demonstrated that glucocorticoids-induced overexpression of Arc is necessary for the expression of fear memories. Thus, there are conflicting findings regarding the role of Egr-1 in fear-motivated learning tasks that depend on the hippocampus. While Egr-1 is 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, 2005) who demonstrated that glucocorticoids-induced overexpression of Arc is necessary for the expression of fear memories. Thus, there are conflicting findings regarding the role of Egr-1 in fear-motivated learning tasks that depend on the hippocampus. In addition, 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).

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.

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