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RAPID COMMUNICATION

Consolidation of Object Recognition Memory Requires HRI Kinase-Dependent Phosphorylation of eIF2α in the Hippocampus

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ABSTRACT: Local control of protein synthesis at synapses is crucial for synaptic plasticity and memory formation. However, little is known about the signals coupling neurotransmitter release with the translational machinery during these processes. Here, we studied the involvement of heme-regulated inhibitor (HRI) kinase, a kinase activated by nitric oxide that phosphorylates eukaryotic initiation factor 2α (eIF2α), in object recognition (OR) memory consolidation. Phosphorylated eIF2α mediates two opposing effects upon translation: translational arrest of most mRNAs and translational activation of selected mRNAs bearing specific features in their 5′untranslated regions (5′UTRs). We found that HRI kinase activation in the CA1 region of the dorsal hippocampus is necessary for retention of OR memory in rats. Accordingly, learning induced a transient increase in the phosphorylation state of eIF2α in dorsal CA1 which was abolished by the HRI kinase inhibitor *N*-(2,6-dimethylbenzyl)-6,7-dimethoxy-2H-[1]benzofuro[3,2-c]pyrazol-3-amine hydrochloride (AMI). The increase in p-eIF2α was associated with increased expression of BACE1 and activating transcription factor 4, two proteins containing eIF2α-responsive 5′UTRs in their mRNAs that play a key role in synaptic plasticity. Our data suggests that learning promotes the transient phosphorylation of eIF2α to allow for translation of specific 5′UTR-mRNAs through a process requiring HRI kinase activation. © **2013 Wiley Periodicals, Inc.**

KEY WORDS: eIF2α; memory; hippocampus; NO; HRI kinase; BACE1; ATF4; Sal003; *N*-(2,6-dimethylbenzyl)-6,7-dimethoxy-2*H*-[1]benzofuro [3,2-*c*]pyrazol-3-amine hydrochloride

Post-training intracerebral administration of general protein synthesis inhibitors impairs long-term memory (LTM) consolidation (Sutton and Schuman, (2005) J Neurobiol 64:116–131). In spite of this, it has been suggested that translation of particular mRNAs in neuronal dendrites, rather than global mRNA translation, is key for LTM storage (Jiang et al., (2010) J Neurosci 30:2582–2594). However, it is unknown how synaptic signaling couples to the dendritic translational machinery for controlling local synthesis of specific proteins.

Nitric oxide (NO) plays an important role in memory consolidation (Susswein et al., 2004), acting mostly through the sGC/cGMP pathway to modulate neurotransmitter release (Ignarro, 1991; Park et al., 1998). However, evidence suggests that there are sGC/cGMP-independent mechanisms through which NO may affect memory (Edwards and Rickard, 2007). In this respect, the heme-regulated inhibitor (HRI) kinase is a plausible link between NO signaling and translational control of gene expression. HRI kinase was first identified in rabbit reticulocytes (Mellor et al., 1994), where it regulates hemoglobin synthesis (Chen and London, 1995). Recently, its presence has been also demonstrated in dendritic spines of hippocampal neurons (ILL-Raga et al, submitted). HRI kinase is activated by NO binding to the heme motifs acting as prosthetic groups in its N-terminus (Ishikawa et al., 2002). Upon activation, HRI kinase phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) at Ser-51 (de Haro et al., 1996). Together with GTP and the initiator tRNA, eIF2α is part of the ternary complex that loads the ribosome with methionine to initiate mRNA translation. When eIF2α is phosphorylated GTP/GDP cannot be recycled resulting in a drop of active ternary complexes. As a consequence translation initiation is stopped leading to a global arrest in protein synthesis. Paradoxically this decrease in ternary complexes stimulates translation of a specific subset of mRNAs containing upstream open reading frames (uORFs) in their characteristically long and highly structured 5′untranslated regions (5′UTRs). Under normal conditions such 5′UTRs repress mRNA translation, a condition that is reversed when eIF2α is phosphorylated. Therefore, eIF2α phosphorylation is also a mechanism to stimulate the synthesis of specific proteins that would normally remain translationally silenced through the 5′UTRs in their RNA messengers. Such proteins include activating transcription factor 4 (ATF4) (Harding et al., 2000) and β-site amyloid precursor pro-

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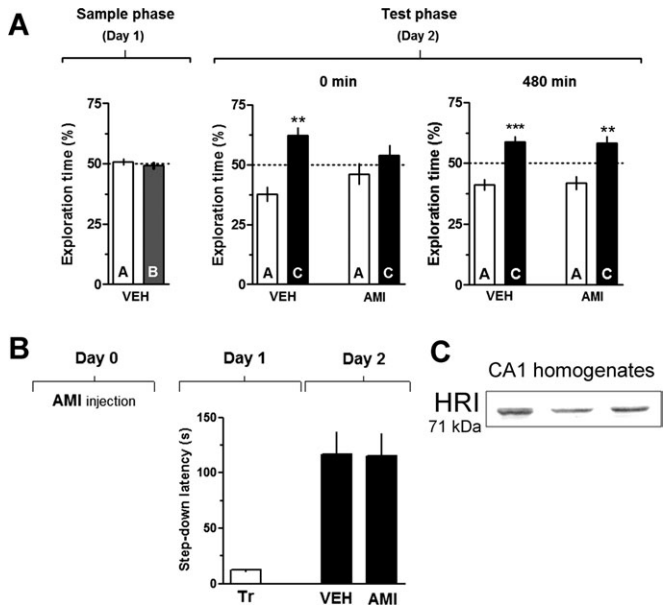

FIGURE 1. OR memory consolidation requires HRI kinase activation in the CA1 region of the dorsal hippocampus. A. On Day 1 (Sample Phase) rats ($n = 53$) were exposed to two different objects (A and B) for 5 min and at different times after that (0 or 480 min) received bilateral infusions of vehicle (VEH; saline) or N-(2,6-Dimethylbenzyl)-6,7-dimethoxy-2H-[1]benzofuro[3,2-c] pyrazol-3-amine hydrochloride (AMI; 0.01 nmol/side) in the CA1 region of the dorsal hippocampus. On Day 2 (Test phase) animals were exposed to a familiar (A) and a novel object (C) for five additional minutes. Data are presented as mean (±SEM) of the percentage of time exploring a particular object over the total time of object exploration. $t_{(9)} = 4.081$ for 0 min-VEH, $t_{(6)} = 0.928$ for 0 min-AMI, $t_{(19)} = 4.171$ for 480 min-VEH; $t_{(12)} = 3.084$ for 480 min-AMI; $^{**}P < 0.01$, $^{***}P < 0.001$ in one-sample Student's t-test (reference value = 50%); $n = 7$–20 per group. Note that animals that received AMI immediately after training spent the same amount of time exploring objects A and C during the test phase (Day 2; 0 min-AMI) indicating that recognition memory was impaired. B. Rats received bilateral intra-CA1 microinfusions of AMI (0.01 nmol/side) and 24 h later were trained in a step-down inhibitory avoidance learning task using a 0.4 mA/2 s footshock as unconditioned stimulus. Bars represent mean ± SEM of step-down latencies during a retention test session carried out 24 h after training. $t_{(20)} = 0.048$, $p = 0.96$ in unpaired Student's t-test; $n = 10$–12 per group. C. Representative immunoblot showing the presence of HRI kinase (Abcam ab77775) in total homogenates obtained from the dorsal CA1 region of three different animals.

tein cleaving enzyme 1 (BACE1) (O'Connor et al., 2008). Interestingly, it has been recently proposed that eIF2α activity-dependent phosphorylation constitutes a molecular switch essential for fear memory formation (Costa-Mattioli et al., 2005, 2007; Jiang et al., 2010). We have previously shown that protein synthesis and NO signaling are necessary in the dorsal hippocampus for consolidation of object recognition (OR) memory (Furini et al., 2010; Rossato et al., 2007), a type of declarative memory conferring the ability to discriminate between novel and familiar entities. Therefore, we wondered whether HRI kinase activation is also involved in this process. To address this question, male Wistar rats (3-month-old) implanted with 22-gauge guides aimed to the CA1 region of the dorsal hippocampus [stereotaxic coordinates AP −4.2/ LL ±3.0/ DV −3.0 in accordance with (Paxinos and Watson, 1986)] were trained in an OR learning task involving a 5-min exposure to two novel stimuli objects in an open field arena. Before that, animals were habituated to the training arena by allowing them to freely explore it during 20 min per day for 4 days in the absence of any other behaviorally relevant stimulus. The stimuli objects were made of metal, glass or glazed ceramic, and their role (familiar or novel) and relative position were counterbalanced and randomly permuted for each experimental animal. Exploration was defined as sniffing or touching the stimuli objects with the muzzle and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. Immediately after training, animals received bilateral intra-CA1 microinfusions (1 μl/side) of vehicle (VEH; saline) or of the specific HRI kinase inhibitor (AMI; 0.01 nmol/side; Rosen et al., 2009). Long-term memory (LTM) was assessed 24 h later. In the LTM test session, animals were re-exposed for 5 min to one of the objects presented during the training session alongside a novel one. Rats that received VEH explored the novel object significantly longer than the familiar one. Conversely, animals that received AMI immediately after training spent the same amount of time exploring the novel and the familiar objects. AMI had no effect on memory when given in dorsal CA1 8 h posttraining (Fig. 1A). Moreover, when injected in dorsal CA1 24 h before training, AMI did not affect acquisition or retention of inhibitory avoidance memory (Fig. 1B), a learning task that requires the functional integrity of the hippocampus. These results indicate that HRI kinase is necessary early after training for retention of OR LTM, and demonstrate that the amnesia caused by AMI is in fact due to impairment of the consolidation process and not to a protracted effect of this inhibitor on hippocampal function able to hinder retrieval of recognition memory. AMI specificity has been previously tested in a commercially available selectivity panel (EMD Millipore) of over 100 kinase, enzyme, GPCR, and ion channel targets, and showed >100-fold selectivity for HRI kinase versus these pharmacological loci (Rosen et al., 2009). As shown in Figure 1C, HRI kinase is present in the rat hippocampus.

To investigate whether OR training modifies the phosphorylation state of eIF2α at Ser-51 (p-eIF2α), rats were euthanized at different times after OR training and the dorsal CA1 region was dissected out and processed for immunoblot. p-eIF2α levels were low in naïve control animals, but increased rapidly after training. This increase was already detectable 30 min after training, peaked 2 h thereafter, and lost intensity within 8 h. Intra-CA1 infusion of AMI immediately after OR training fully inhibited the learning-induced increase in p-eIF2α. We could not detect any training-induced modification in the total levels of eIF2α (Fig. 2).

Two of the best known mRNAs activated by p-eIF2α are those coding for BACE1 and ATF4 (De Pietri et al., 2004; Vattem and Wek, 2004). BACE1 is an aspartic-acid protease whose mRNA is present in dendritic spines of hippocampal pyramidal neurons (Smalheiser et al., 2008). Although it is essential for

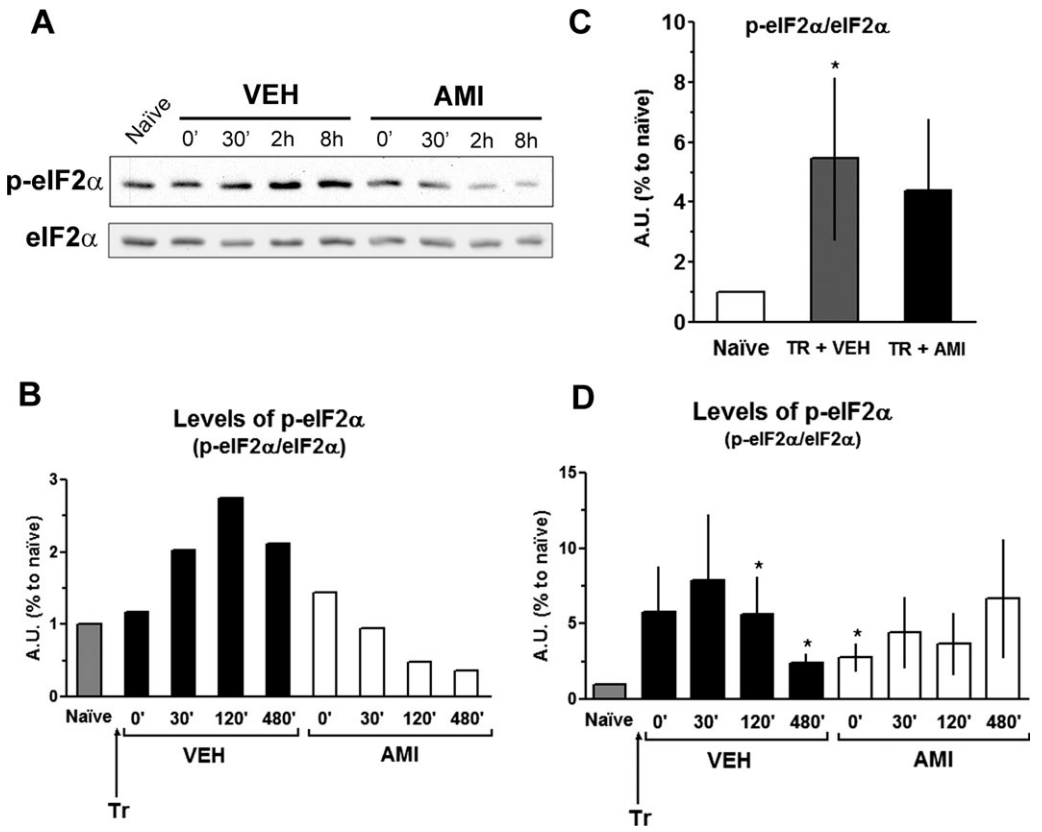

FIGURE 2. OR training increases p-eIF2α levels in dorsal CA1 through a mechanism involving HRI kinase. A. Representative blots showing a progressive increase in the levels of dorsal CA1 p-eIF2α induced by OR training. AMI injection (0.01 nmol/side) immediately after training blocks the increase in p-eIF2α levels induced by OR training, without affecting total eIF2α levels. Blots were first processed for p-eIF2α immunodetection (Abcam ab32157), stripped, and reprobed with anti-elF2 antibody (Cell Signaling L57A5). B. Quantification of p-eIF2α/eIF2α ratio for the blots shown in A. C. p-eIF2α levels are significantly higher in OR-trained than in nontrained animals. Bars represent the mean ± SEM of 20 independent values obtained from four time points (0', 30', 120', 480') in five independent experiments. *$P = 0.05$ versus naïve in unpaired Student's *t*-test. D. Quantification of p-eIF2α/eIF2α ratio of five independent experiments summarized in C. Note the transient increase in p-eIF2α induced by OR training, which is prevented by AMI. *$P < 0.05$ versus naïve in unpaired Student's *t*-test.

normal memory processing (Laird et al., 2005; Ma et al., 2007), its levels are aberrantly upregulated in sporadic AD (Holsinger et al., 2002). ATF4 is a repressor of CREB-mediated transcription which is involved in memory formation (Bartsch et al., 1995) and is locally translated at dendrites from where it can be transported back to the nucleus (Lai et al., 2008; Wagatsuma et al., 2006). We found that BACE1 and ATF4 protein levels were low in the CA1 region of naïve control rats. However, OR training increased BACE1 and ATF4 expression, an effect blocked by intra-CA1 administration of AMI immediately posttraining (Fig. 3).

Our results indicate that expression of p-eIF2α and its downstream effectors BACE1 and ATF4 is low in nontrained animals, and that OR training triggers eIF2α phosphorylation (Fig. 2) and synthesis of BACE1 and ATF4 (Fig. 3) through a mechanism involving HRI kinase. To be functional, such switch-like response would require a low basal state of eIF2α phosphorylation. In other words: to switch on gene expression through eIF2α phosphorylation, eIF2α must be first in its nonphosphorylated "off state." To examine the hypothesis that disruption of eIF2α molecular switch would impair memory consolidation, we used Sal003, a potent and cell-permeable analog of the eIF2α phosphatase inhibitor salubrinal (Boyce et al., 2005) that rapidly increases eIF2α phosphorylation in vivo (Fig 4A). Intra-CA1 infusion of Sal003 (45 nmol/side) immediately after training hindered OR memory retention when tested 24 h later (Fig 4B). Sal003 did not affect inhibitory avoidance memory when injected in dorsal CA1 24 h before training (Fig 4C).

Notwithstanding the obvious differences between the learning tasks employed, our results are apparently at odds with previous reports suggesting that the basal level of p-eIF2α is high, and that this protein is dephosphorylated during memory consolidation (Costa-Mattioli et al., 2005, 2007) to inhibit expression of the CREB repressor ATF4 and allow for general protein synthesis. This assertion is based mainly on results showing the amnesic effect of Sal0003, and more recently, on experiments with transgenic mice overexpressing a constitutively active form of PKR (Jiang et al., 2010). However, PKR, an interferon-inducible eIF2α kinase that is not expressed in the healthy brain, is involved in neurodegeneration, including AD, (Chang et al., 2002) and its sustained activation leads to apoptosis (Peel and Bredesen, 2003). More recently, activation of PKR in AD brains

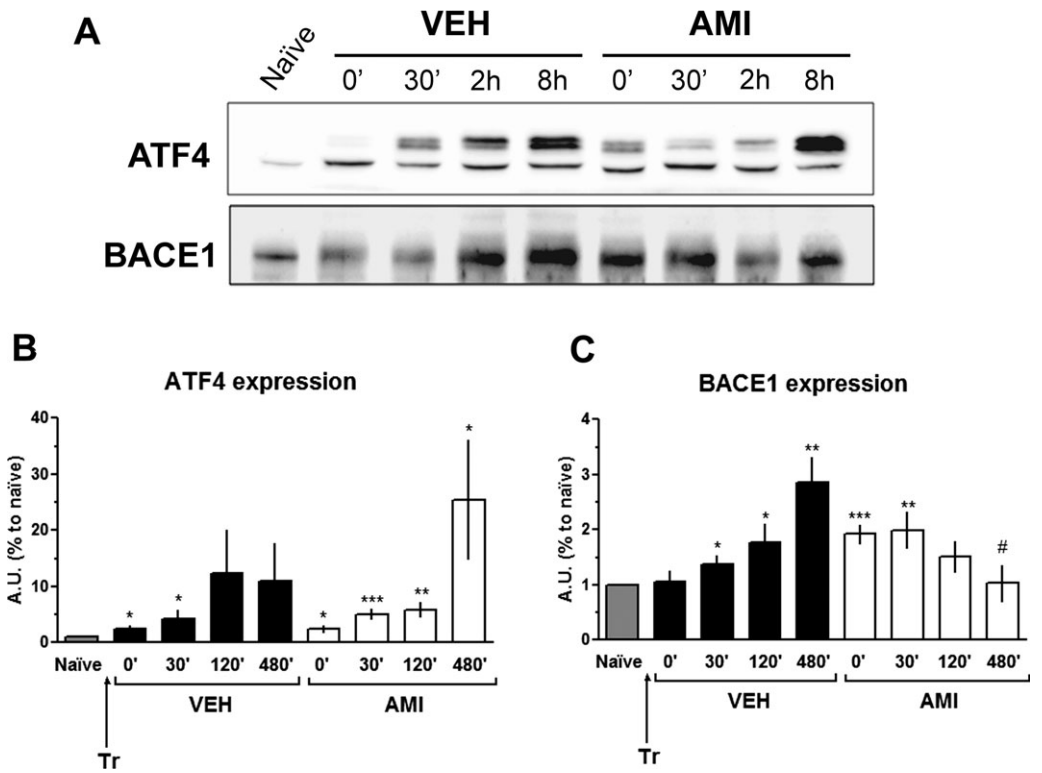

FIGURE 3. OR training induces the HRI kinase-dependent expression of ATF4 and BACE1 in dorsal CA1. A and B. Rats were trained in the OR paradigm and, immediately after that, received bilateral infusions of vehicle (VEH, saline) or of the HRI kinase inhibitor (AMI, 10 μM) in dorsal CA1. At different posttraining times (0', 30', 120', 480') the dorsal CA1 region was dissected out and processed for SDS-PAGE. A. Representative blot of ATF4 (Abcam ab23760) and quantification of seven independent experiments. Data are presented as mean ± SEM; *$P < 0.05$; **$P < 0.01$ versus naïve; # $P < 0.05$ versus 120' VEH in unpaired Student's *t*-test. B. Representative blot of BACE1 (Millipore 485-501) and quantification of five independent experiments. Data are presented as mean ± SEM; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ versus naïve; # $P < 0.01$ versus 480' VEH in unpaired Student's *t*-test.

has been found to be mechanistically linked to Aβ peptide production (ILL-Raga et al., 2011; Mouton-Liger et al., 2012). Therefore, it is not surprising that overexpression of PKR in hippocampal pyramidal neurons results in memory impairment through an aberrant increase in p-eIF2α (Jiang et al., 2010). In fact, phosphorylation of eIF2α occurs mainly as a consequence of cellular stress compromising cell viability (Wek et al., 2006). Stress in dendrites induces eIF2α phosphorylation through the eIF2α kinase PERK (Murakami et al., 2007). PERK also mediates eIF2α phosphorylation in response to the cellular stress induced by glucose deprivation in neurons (O'Connor et al., 2008) or by transient global brain ischemia (Owen et al., 2005) leading to severe inhibition of protein synthesis. Finally, the unfolded protein response, a cell adaptive response to endoplasmic reticulum stress that includes PERK activation and eIF2α phosphorylation (Kaufman, 2002) is activated in AD (Hoozemans et al., 2005) and is a hallmark of neurodegenerative diseases (Lindholm et al., 2006). Taking these evidences into account, we believe it is unlikely that p-eIF2α basal levels are constitutively high in the hippocampus of healthy, nontrained animals. Besides, persistent phosphorylation of eIF2α would imply a permanent arrest in protein synthesis incompatible with metabolism, and would indirectly lead to aberrant overexpression of BACE1 and amyloidogenesis (O'Connor et al., 2008; ILL-Raga et al., 2011; Mouton-Liger et al., 2012), a neurochemical scenario typical of AD pathogenesis and certainly not suitable for normal memory consolidation.

As reported earlier, we also found that intrahippocampal administration of Sal003 is amnesic, as should be expected from a pharmacological treatment inducing a massive and ill-timed increase in the phosphorylation state of a protein controlling a strictly regulated mechanism. However, our experiments show that hippocampal p-eIF2α is expressed at trace levels in naïve animals, increases rapidly after training and then slowly returns to control values to promote the timely expression of ATF4 and BACE1. Based on these results we proposed that learning induces the HRI kinase-mediated phosphorylation of eIF2α to activate the translation of specific latent mRNAs bearing 5'UTRs with uORFs homologous to ATF4 and BACE1.

The question remains unanswered, however, as to why a CREB repressor and a protease involved in AD pathogenesis would ever be needed for memory consolidation. The dominant hypothesis about the role of ATF4 in memory processing states that this protein inhibits memory consolidation by blocking CREB-mediated transcription (Gkogkas et al., 2010). Nonetheless, it has been recently reported that diminished ATF4 expression is associated with decreased prepulse inhibition and reduced fear extinction (Trinh et al., 2012), indicating

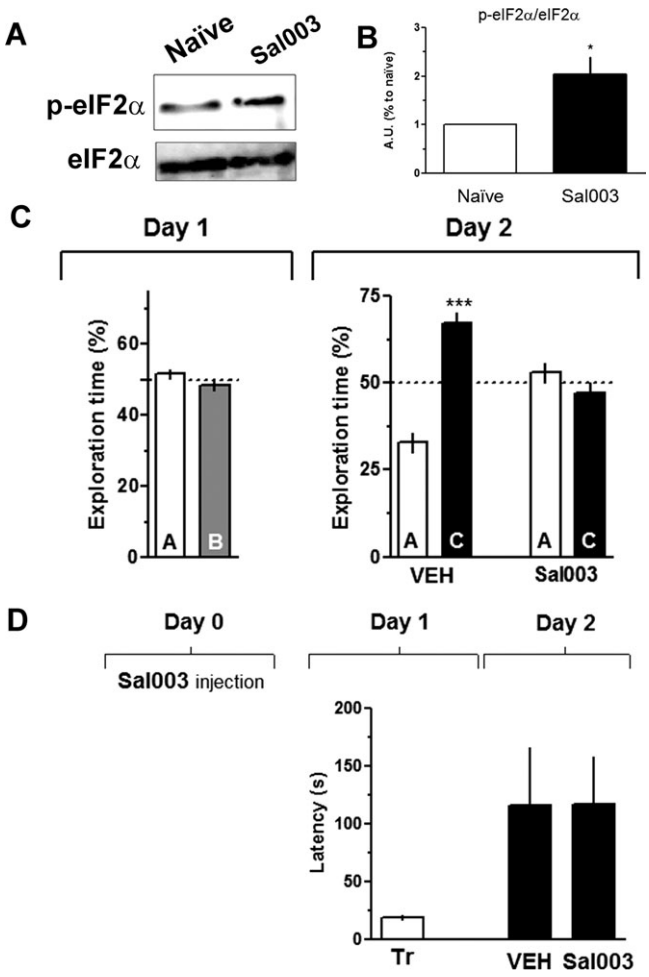

FIGURE 4. Sal003-induced eIF2α phosphorylation impairs OR memory formation. A. Injection of Sal003 rapidly increases p-eIF2α in the CA1 region. Sal003 (45 nmol/side) was injected in the dorsal CA1 region of naive animals. Forty minutes later, dorsal CA1 was dissected out and the levels of p-eIF2α were analyzed by western blot. B. Quantification of p-eIF2α/eIF2α ratio depicted in A. Data are presented as mean ± SEM of five independent experiments; *$P < 0.05$ versus naïve in unpaired Student's *t*-test. C. Alteration of p-eIF2α basal state by Sal003 impairs OR memory consolidation. On Day 1 rats ($n = 16$) were exposed to two different objects (A and B) for 5 min and immediately after that received bilateral intra-CA1 infusions of vehicle (VEH; saline) or Sal003 (45 nmol/side). On Day 2 animals were exposed to a familiar (A) and a novel object (C) for five additional minutes. Data are presented as mean ± SEM of the percentage of time exploring a particular object over the total time of object exploration. $t_{(9)} = 5.963$ for VEH, $t_{(5)} = 1.009$ for Sal003; ***$P < 0.001$ in one-sample Student's *t*-test (reference value = 50%); $n = 6$–10 per group. Note that animals that received Sal003 immediately after training spent the same amount of time exploring objects A and C in the test day (Day 2, Sal003) indicating that recognition memory was impaired. D. Sal003 microinjection does not impair hippocampus function. Rats received bilateral intra-CA1 microinfusions of Sal003 (45 nmol/side) or VEH and 24 h later were submitted to inhibitory avoidance training. Memory retention was evaluated 24 h after training. Bars represent mean ± SEM of step-down latencies. Note that both VEH and Sal003 animals normally acquired the aversive memory associated with inhibitory avoidance training as reflected by the increase in step-down latencies during the test session. $t_{(11)} = 0.015$, $P = 0.99$ in unpaired Student's *t*-test; $n = 6$–7 per group.

that the participation of this transcription factor in cognition is not as simple as earlier thought, but must be tightly controlled for the normal formation of LTM (Klann and Sweatt, 2008; Viosca et al., 2009). Although BACE1 is involved in Aβ generation and in the cognitive deficit observed in animal models of AD (Ohno et al., 2004), evidence suggests that this protein is also essential for normal memory processing. Thus, BACE1 null mice show impaired hippocampal synaptic plasticity and memory acquisition (Laird et al., 2005), and it has been shown that BACE1-mediated cleavage of amyloid precursor protein facilitates in vivo long-term potentiation in the hippocampus as well as spatial learning (Ma et al., 2007), maybe controlling voltage-dependent sodium channels and neuronal activity (Kim et al., 2007; Huth et al., 2009) and/or spine density in hippocampal pyramidal neurons (Savonenko et al., 2008).

In conclusion, our results indicate that OR memory consolidation requires HRI kinase-dependent phosphorylation of eIF2α and induces the expression of BACE-1 and ATF-4 in the CA1 region of the dorsal hippocampus. Importantly, they also suggest that eIF2α phosphorylation is a double-edged plasticity sword, essential for memory consolidation but easily shifted to promote neurodegeneration by external neuronal stressors.

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