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Research report

Phosphorylation of extra-nuclear ERK/MAPK is required for long-term memory consolidation in the crab *Chasmagnathus*

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Abstract

It was previously demonstrated that mitogen-activated protein kinase (MAPK) signaling plays a pivotal role in neural plasticity and memory processes both in rodents and mollusks. Although the MAPK pathways are highly conserved, no evidence was found for its participation in memory models in other animal groups. Here we found ERK-like and JNK-like cross-immunoreactivity in the crab brain with phospho-specific antibodies and we estimated ERK and JNK activity during long-term memory consolidation in the context–signal learning paradigm of the crab *Chasmagnathus*. At 0, 1, 3 and 6 h after training ERK and JNK activity was measured. ERK-like activation was found 1 h after spaced training in cytosolic but not in nuclear fractions of brain homogenates, while JNK activity remained unchanged in both fractions. Passive (context exposure) and active (continuous stimulation) controls showed cytosolic ERK and JNK activation immediately after training, which decayed 1 h later. In coincidence with this time course of activity, an ERK1/2 pathway inhibitor, PD098059, induced amnesia only when administered 45 min after training but not when administered immediately pre- or post-training. These data support that: (1) cytoplasmic but not nuclear ERK substrates must be differentially phosphorylated during memory consolidation, and (2) ERK phosphorylation and consequent activation 1 h after training is necessary for long-term memory consolidation in this arthropod model.

Keywords: Long-term memory; MAPK; ERK; JNK; PD098059; Crab

1. Introduction

Changes in the balance between the activity of protein kinases and protein phosphatases are part of the molecular mechanisms that subserve memory storage [48,49]. A substantial body of evidence supports the important role of some protein kinases in memory consolidation, acting in neurons both locally in synapses and translocating to the nucleus to regulate gene expression.

Mitogen-activated protein kinases (MAPKs) are a family of Ser/Thr protein kinases present in a variety of tissues, regulating development, mitogenesis and the stress response. MAPKs are activated when phosphorylated (at threonine and tyrosine residues) by MAPK kinases (MAPKK) such as MAPK/ERK kinases (MEKs). Mainly, three subgroups of MAPKs have been identified: extracellular-signal regulated kinase 1/2 (ERK1/2, also known as p42/p44^{MAPK}), involved in proliferation, differentiation and development [25] and the stress-activated protein kinases (SAPKs), c-Jun N-terminal kinase (JNK) and p38 MAPKs, both involved in development, stress and inflammatory response [11,47,36]. The ERK1/2 pathway is strongly activated by growth factors acting through tyrosine kinase receptors, while SAPKs are typically activated by cytokines. In response to such extracellular signals, these MAPKs normally translocate from the cytoplasm to the nucleus regulating the activity of several transcription factors, including Elk-1, AP-1, ATF-2 and

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p53 [10]. However, apart from nuclear targets, ERK1/2 can phosphorylate membrane, cytoskeletal and cytoplasmic proteins and thus might be involved in other functions such as regulation of A-type K^+ channels [2] and internalization of cell-adhesion molecules [5].

MAPKs are a highly conserved family of kinases, present in species as different as yeast, worms, mollusks, insects and mammals [30,52]. In Drosophila, only one ERK isoform has been described with a molecular weight similar to that found in mammals, 44 kDa [8], one JNK isoform (DJNK, basket) [30] and two p38 isoforms (Drosophila-p38 MAPK, D-p38a and b [18]). In *Aplysia*, a homologue of mammalian ERK2 with a molecular weight of 43 kDa was described [28] and *Aplysia* p38 (Ap-p38) was recently identified [17]. Both kinases showed 85 and 80% homology with mammalian ERK2 and p38, respectively. In *Hermissenda*, two ERK isoforms with the same molecular weight than mammalian ERK1 and ERK2 have been detected [12].

Substantial evidence has implicated ERK in synaptic plasticity since the initial works of English and Sweatt [13] in long-term potentiation (LTP) and Martin et al. [26] in long-term facilitation in *Aplysia* neurons. Subsequent studies showed the involvement of this kinase in memory processes in rodents (e.g., [3,4,9,45,54]), *Hermissenda* [12] and more recently in *Aplysia* [35,46]. ERK translocation to the nucleus and transcription factor CREB indirect activation was initially proposed as the mechanism by which this MAPK acts in memory consolidation. However, in a recent report this kinase has been implicated in the regulation of membrane permeability in dendrites in spatial memory [29].

There are only two reports linking JNK with memory processes, both in rat models. In the taste aversion paradigm, JNK is activated in the insular cortex after conditioned stimulus presentation [6]. In the passive avoidance paradigm, intrahippocampal injections of a JNK inhibitor blocked LTM [7]. In the rabbit classical conditioning of the eye-blink response, activation of p38 and ERK, but not of JNK, was detected in cerebellum. p38 MAPK inhibition, but not ERK inhibition, delayed the acquisition of the task [55], suggesting that only p38 activation is required in that model.

In the context–signal learning paradigm of the crab *Chasmagnathus*, the repeated presentation of a danger stimulus (an opaque figure passing overhead) provokes the fading of the escape response that was initially elicited [24]. Spaced training (e.g., 15 trials, 171 s inter-trial interval, ITI) induces a LTM (context–signal memory, CSM) that lasts for at least a week, and entails an association between the iterated stimulus and contextual features [50]. CSM consolidation is dependent on protein and mRNA synthesis [31,32]. Protein synthesis is also required for CSM reconsolidation induced by re-exposition to the training context [34]. On the other hand, massed training (e.g., 300 trials of stimulus presentations, with 4 s ITI) induces non-associative habituation learning. This training protocol yields an intermediate-term memory, so called signal memory (SM) that lasts no more than 3 days and is not dependent on protein synthesis [19,33]. An extreme case of massed training, the continuous stimulation training protocol (ITI less than 1 s), does not entail any kind of retention at 24 h in spite of the enormous amount of trials presented (more than 1000) [14]. This protocol is used in active control groups, proving to be useful for controlling the effects of visual stimulation, motor activity, stress, novelty, etc., that could enhance molecular changes in a way not directly associated with memory formation.

In the present report we studied the temporal course of ERK and JNK phosphorylation in *Chasmagnathus* brain, in order to estimate their activity during CSM consolidation induced by spaced training in comparison with the activity induced by continuous stimulation and context exposition. We also evaluated the effect on memory retention of the ERK kinase (MEK) inhibitor PD098059.

2. Matherials and methods

2.1. Animals

Adult male *Chasmagnathus granulatus* intertidal crabs, 2.6–2.9 cm across the carapace, weighting 17 ± 0.2 g ($n = 60$), were collected from water less than 1 m deep in the estuarine coasts of San Clemente del Tuyú, Argentina, and transported to the laboratory, where they were lodged in plastic tanks ($30 \times 45 \times 20$ cm) filled to 0.5 cm depth with diluted (12‰, pH 7.4–7.6) marine water (prepared from Cristalsea Marinemix salts, USA), to a density of 20 crabs per tank. The holding room was maintained on a 12-h light:12-h dark cycle (light on 07:00–19:00 h). Animals were fed rabbit pellets (Nutrientes S.A., Argentina) every 3 days and water was changed after feeding. Temperature of both holding and experimental rooms was maintained within a range of 22–24 °C. Experiments were carried out within the first week after the animals arrival. Each crab was used in only one experiment.

2.2. Training-testing apparatus

The experimental unit, the *actometer*, was described in detail elsewhere [39]. Briefly, it consists of a bowl-shaped plastic container where the crab is lodged and an opaque rectangular screen, which moves horizontally over the animal. Screen displacements provoke a crab's running response and, as a consequence, container oscillations which induce electrical signals through a piezoelectric transducer. Signals recorded during a trial are translated into numerical units ranging from 0 to 5000. A computer was employed to program trial sequences, trial duration and inter-trial intervals, as well as to monitor experimental events.

2.3. Drugs and injection procedure

Five milligrams of 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD098059) (Sigma, USA) were freshly dissolved in 250 μl of dimethyl sulfoxide (DMSO) (Sigma,