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# Carbonic anhydrase activation enhances object recognition memory in mice through phosphorylation of the extracellular signal-regulated kinase in the cortex and the hippocampus

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ABSTRACT

Rats injected with by D-phenylalanine, a carbonic anhydrase (CA) activator, enhanced spatial learning, whereas rats given acetazolamide, a CA inhibitor, exhibited impairments of fear memory consolidation. However, the related mechanisms are unclear. We investigated if CAs are involved in a non-spatial recognition memory task assessed using the object recognition test (ORT). Systemic administration of acetazolamide to male CD1 mice caused amnesia in the ORT and reduced CA activity in brain homogenates, while treatment with D-phenylalanine enhanced memory and increased CA activity. We provided also the first evidence that D-phenylalanine administration rapidly activated extracellular signal-regulated kinase (ERK) pathways, a critical step for memory formation, in the cortex and the hippocampus, two brain areas involved in memory processing. Effects elicited by D-phenylalanine were completely blunted by co-administration of acetazolamide, but not of 1-*N*-(4-sulfamoylphenyl-ethyl)-2,4,6-trimethylpyridinium perchlorate (C18), a CA inhibitor that, differently from acetazolamide, does not cross the blood brain barrier. Our results strongly suggest that brain but not peripheral CAs activation potentiates memory as a result of ERK pathway enhanced activation.



## 1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are a family of metalloenzymes of ancient origin, that catalyze the reversible conversion of $CO_2$ to bicarbonate and protons (Supuran, 2008). Sixteen isomers of CAs have been discovered in mammals, and they differ for catalytic activity, cellular/subcellular localization and tissue distribution (Supuran and Scozzafava, 2007). CAs exert a role in a number of physiological processes such as pH regulation, $CO_2$ and $HCO_3^-$ transport, bone resorption, gluconeogenesis, ureagenesis and lipogenesis (Supuran, 2008). Inhibitors of CAs have been proposed as effective compounds for the treatment of glaucoma, epilepsy, cancer and infections (Supuran, 2016). Few studies have investigated the role of CAs in cognition, although a significant decline of CAs in the brain occurs during aging (Meier-Ruge et al., 1980), and this decline is even more dramatic in brains of Alzheimer's disease patients (Meier-Ruge et al., 1984). Sparse evidence suggests that CAs influence the formation of some types of memory, specifically spatial and fear memory (Sun and Alkon, 2001; Yang et al., 2013). Indeed, rats given i.c.v. phenylalanine, a CA activator (Temperini et al., 2006), ameliorated the performance to find the hidden platform in the Morris water maze. This effect is abolished by

*Abbreviations:* CA, Carbonic anhydrases; DI, discrimination index; tF, exploration period of the familiar object; tN, exploration period of new object; ERK, extracellular signal-regulated kinase; i.c.v., intracerebroventricular; i.p., intraperitoneal; LTM, long-term memory; MCT, multiple comparison test; C18, 1-*N*-(4-sulfamoylphenyl-ethyl)-2,4,6-trimethylpyridinium perchlorate; ORT, object recognition paradigm; pERK, phosphorylated ERK; STM, short-term memory; T2, testing phase; T1, training phase.

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treatment with acetazolamide, a CA inhibitor (Sun and Alkon, 2001). In keeping with these findings, mice genetically deficient of the CA IX isoform, when exposed to the Morris water maze test, have difficulties in learning to find the hidden platform, as well as in remembering the site of the underwater platform compared to wild type littermates (Pan et al., 2012). More recently, it was reported that rats receiving acetazolamide displayed impairment of fear memory consolidation, as demonstrated by diminished avoidance rates in the shuttle avoidance test, and step-through latencies in the passive avoidance test compared to controls (Yang et al., 2013). The mechanisms underlying CAs effects on memory remain mostly unknown. As CAs activation raises $HCO_3^-$ concentrations in the hippocampus (Staley et al., 1995; Sun and Alkon, 2001), thus triggering a switch of GABAergic synaptic outputs from inhibitory to excitatory (Kaila et al., 1997), it has been suggested that under these conditions GABA may activate a subset of pyramidal neurons, thus enhancing associative memories (Sun and Alkon, 2002). More recently it has been reported that acetazolamide inhibited fear conditioning-induced extracellular signal-regulated kinase (ERK) phosphorylation in the amygdala (Yang et al., 2013), that is consistent with acetazolamide-induced impairments in the consolidation of fear memory (Yang et al., 2013). Indeed, several studies have identified ERK's pivotal role in the consolidation of fear memory (Atkins et al., 1998; Giovannini et al., 2003). Nevertheless, the effects of CAs on other types of memory and the underlying molecular mechanisms have not been investigated. Reports that similar treatments may result in discrepant responses in different learning paradigms (Ambrogini et al., 2011; Cho et al., 1999), prompted us to learn the role of CAs in the object recognition paradigm (ORT). This test is a validated task to study a non-spatial working memory type based on the natural tendency of animals to explore novel objects, and not involving primary reinforcement such as food or electric shocks. We tested in the present study the effects of systemic administration of two CA inhibitors, acetazolamide, that crosses the blood-brain barrier, and 1-*N*-(4-sulfamoylphenyl-ethyl)-2,4,6-trimethylpyridinium perchlorate (C18), that does not (Menchise et al., 2005), on novel object recognition, and completed our observations evaluating the effects produced by the acute administration of phenylalanine, that activates CAs. We correlated the effects of these compounds on novel object recognition with those on CA activity measured in brain homogenates. ERK pathway is a central cellular signaling pathway that connects the numerous extracellular signals to the membrane receptors, cascading down to transcription factors and eventually controlling gene regulation, and has a critical role in synaptic plasticity, learning and memory (Sweatt, 2001; Thomas and Huganir, 2004). As ERK phosphorylation increased in the cortex immediately after exposure to novel objects in the training session of the ORT (Nagai et al., 2007; Orr et al., 2012), and inhibition of ERK pathway in the hippocampus (Wang et al., 2016) or the prefrontal cortex (Nagai et al., 2007) impaired the expression of long-term object recognition, we examined the effects of phenylalanine in the presence and in the absence of CA inhibitors on the phosphorylation of ERK.

## 2. Experimental procedure

### 2.1. Animals

Male CD1 mice (Harlan, Italy) were 25–35 g (b.w.) at the time of the experiment and had free access to food (4RF21; Mucedola s.r.l., Italy) and water in their home cages. They were housed in a humidity- and temperature-controlled room (22–24 °C) in the animal facility of Dipartimento di Neuroscienze, Psicologia, Area del Farmaco e Salute del Bambino – Universitá degli Studi di Firenze (I). Lights were maintained on a 12-h light/12-h dark cycle (lights start at 7:00 a.m.), with all behavioral testing carried out during the light phase of the cycle. Housing, animal maintenance and all experiments were conducted in accordance with the Council Directive of the European Community (2010/63/EU) and the Italian Decreto Legislativo 26 (13/03/2014), NIH guidelines on animal care and approved and supervised by a veterinarian.

### 2.2. Object recognition test

Object recognition paradigm measures a form of memory based on short and unrepeated experiments without any reinforcement, such as food or electric shocks (Ennaceur and Delacour, 1988). Object recognition is a one-trial task, and does not involve the learning of any rule, being entirely based on the spontaneous exploratory behavior of rodents toward objects. The detailed procedure has been published previously (Provensi et al., 2016). In brief, mice were placed in a white polyvinylchloride box (70 × 60 cm and 30 cm high) with a grid floor that is easily cleaned, and illuminated by a 75-W lamp suspended 50 cm above the box. The objects to be discriminated were gray polyvinyl chloride shapes: cubes of 8 cm side, pyramids and cylinders of 8 cm height. The object recognition task consisted of a training phase (T1) and a testing phase (T2). Before T1, all mice were handled 1–2 min daily for 5 days. Twenty-four h prior to T1, they were habituated for 10 min to the experimental apparatus in the absence of any object. Each mouse was subjected to the procedure separately and care was taken to remove any olfactory/taste cues by cleaning carefully the arena and test objects between trials. On the day of the experiment, the mouse was placed for 5 min into the test arena facing the same direction and in the same position in the presence of two identical plastic objects such as cubes, cylinders or pyramids (T1). The behavior of mice was videotaped, and the time spent actively exploring the objects was measured by two experienced observers unaware of the pharmacological treatment. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. T2 was performed 2 or 24 h after T1, during which, each mouse was again placed in the test arena for 5 min in the presence of one of the familiar objects and a novel object. The position of the objects (left/right) was randomized to prevent bias from order or place preference. Mice were placed in their home cage between trials. The behavior of mice during T2 was videotaped, and the exploration periods of the familiar (tF) and the new object (tN) were measured by an experienced observer unaware of the pharmacological treatment. A discrimination index (DI) was calculated according to the formula (tN - tF)/(tN + tF). Care was taken to avoid place preference and olfactory stimuli by randomly changing the role (familiar or new object), and the position of the two objects during T2, and cleaning them carefully. Object recognition was carried out in an insulated room to avoid any noise that could impair the performance of the mouse. To examine the effects of CA inhibitors or activators on the object recognition memory, two separate tests examining short-term (2-h; STM) and long-term (24-h; LTM) memory for objects were given in the present study. In STM experiments T2 was performed 2 h after T1, whereas in LTM experiments the inter-trial interval was 24 h. Animals were arbitrarily allocated to different experimental groups and behavioral tests with controls and mice treated with the different compounds were run in the same session.

### 2.3. General motor activity

The general motor activity was determined by measuring the time in seconds in which mice were moving in the arena during T2,