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Rapamycin inhibits mTOR/p70S6K activation in CA3 region of the hippocampus of the rat and impairs long term memory

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ABSTRACT

The present study was aimed at establishing whether the mTOR pathway and its downstream effector p70S6K in CA3 pyramidal neurons are under the modulation of the cholinergic input to trigger the formation of long term memories, similar to what we demonstrated in CA1 hippocampus. We performed *in vivo* behavioral experiments using the step down inhibitory avoidance test in adult Wistar rats to evaluate memory formation under different conditions. We examined the effects of rapamycin, an inhibitor of mTORC1 formation, scopolamine, a muscarinic receptor antagonist or mecamylamine, a nicotinic receptor antagonist, on short and long term memory formation and on the functionality of the mTOR pathway. Acquisition was conducted 30 min after i.c.v. injection of rapamycin. Recall testing was performed 1 h, 4 h or 24 h after acquisition. We found that (1) mTOR and p70S6K activation in CA3 pyramidal neurons were involved in long term memory formation; (2) rapamycin significantly inhibited mTOR and of p70S6K activation at 4 h, and long term memory impairment 24 h after acquisition; (3) scopolamine impaired short but not long term memory, with an early increase of mTOR/p70S6K activation at 1 h followed by stabilization at longer times; (4) mecamylamine and scopolamine co-administration impaired short term memory at 1 h and 4 h and reduced the scopolamine-induced increase of mTOR/p70S6K activation at 1 h and 4 h; (5) mecamylamine and scopolamine treatment did not impair long term memory formation; (6) unexpectedly, rapamycin increased mTORC2 activation in microglial cells. Our results demonstrate that in CA3 pyramidal neurons the mTOR/p70S6K pathway is under the modulation of the cholinergic system and is involved in long-term memory encoding, and are consistent with the hypothesis that the CA3 region of the hippocampus is involved in memory mechanisms based on rapid, one-trial object–place learning and recall. Furthermore, our results are in accordance with previous reports that selective molecular mechanisms underlie either short term memory, long term memory, or both. Furthermore, our discovery that administration of rapamycin increased the activation of mTORC2 in microglial cells supports a reappraisal of the beneficial/adverse effects of rapamycin administration.

1. Introduction

The hippocampus is critical for learning and memory. Age related decline in hippocampal function may underlie impaired memory abilities in about half of the population over 60 years of age (Hedden & Gabrieli, 2004; Small, Tsai, DeLaPaz, Mayeux, & Stern 2002). The hippocampal regions CA1, CA3 and dentate gyrus, although interconnected *via* the trisynaptic pathway, display striking anatomical differences (Amaral & Witter, 1989) and show distinct functions, contributing to specific types of information processing such as novelty detection, encoding, short-term

Abbreviations: ACQ, acquisition; ACh, acetylcholine; DMSO, dimethyl sulfoxide; IA, inhibitory avoidance; i.c.v., intracerebroventricular; i.p., intraperitoneal; mTOR, mammalian Target of Rapamycin; mAChR, muscarinic acetylcholine receptors; mTORC1,2, mammalian Target of Rapamycin Complex 1,2; MEC, mecamylamine; nAChR, nicotinic acetylcholine receptors; p70S6K, p70S6Kinase; P-mTOR, phospho-mTOR; P-p70S6K, phospho-p70S6K; RAPA, rapamycin; SCOP, scopolamine; SEM, standard error of the mean.

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memory, intermediate-term memory and retrieval. In particular, CA3 and CA1 pyramidal neurons perform distinct, yet complementary, functions in the processing of spatial and contextual information (Vazdarjanova & Guzowski, 2004). The CA3 hippocampus supports processes associated with the rapid formation of spatial or contextual memory (Kesner, Lee, & Gilbert, 2004; Lee & Kesner, 2002, 2003; Nakazawa et al., 2003). However, CA3 lesions, or experimentally-induced dysfunctions of CA3, also impair spatial memory (Lee & Kesner, 2004; Nakazawa et al., 2003) and object-place associations (Hunsaker & Kesner, 2008; Langston, Stevenson, Wilson, Saunders, & Wood, 2010). Intrahippocampal CA3 information processing is also important for memory-based behavior and can modulate activity in the CA1 (O'Reilly, Alarcon, & Ferbinteanu, 2014). The relative contribution of CA3 and CA1 regions to memory is not completely understood. A recent review of the learning and memory literature suggested (Stokes, Kyle, & Ekstrom, 2015) that CA3/DG and CA1 have distinct and separate roles in the representation of a spatial context during the formation of memories.

Long term memory requires protein synthesis; mTOR signaling is of crucial importance in this process, especially at the level of neuronal synaptodendritic compartment (Giovannini & Lana, 2016). Activation of mTOR/p70S6K pathway in CA1 hippocampal pyramidal neurons is instrumental to the process of formation of a long term inhibitory avoidance (IA) memory, and the cholinergic input through muscarinic and nicotinic receptor blockade impairs short term, but not long term IA memory (Lana et al., 2013).

IA is an emotionally-arousing paradigm (Giovannini et al., 2005, 2008; Izquierdo et al., 1997a; Lana et al., 2013; Maren, 2001) that involves a spatial memory component as the animal remembers the location where the noxious stimulus was given during acquisition (Cammarota, Bevilaqua, Medina, & Izquierdo, 2007), an explicit, associative component to the context, and an operant-like conditioning component to the shock as the animal may avoid the aversive stimulus (Wilensky, Schafe, & LeDoux, 2000). The IA response is a learning task that depends upon the activation of the hippocampal cholinergic system (Giovannini et al., 2005), as shown by the impairment by pre-training (Giovannini, Bartolini, Bacciottini, Greco, & Blandina, 1999; Izquierdo et al., 1998a) or post-training administration of muscarinic receptor antagonists (Giovannini et al., 1999; Izquierdo et al., 1998b; McGaugh & Izquierdo, 2000). The recall test, performed at different times after acquisition, offers insight into the mechanisms involved in short term (Izquierdo et al., 1998a,b) and long term memory (Izquierdo et al., 2002). The step-down IA is acquired in one trial by activation of different brain structures by sensorial stimuli, including spatial and visual perceptions, pain, and fear (Izquierdo, 1989; Izquierdo & Medina, 1997b).

In this paper we will extend our analysis on mTOR pathway dynamics in the CA3 region of the hippocampus during the formation of an IA memory. By comparing the similarities and differences between CA1 and CA3 we will be better able to define the relative contribution of these two hippocampal regions in the encoding of an IA memory. Furthermore, we will define whether in CA3 pyramidal neurons the mTOR pathway is modulated by the cholinergic input and whether activation of this pathway triggers the encoding of long term memories in a similar manner to what we had demonstrated in CA1 hippocampus (Lana et al., 2013).

2. Methods

2.1. Animals

Male adult (3 months old) Wistar rats, weighing 200–225 g, were (Harlan Nossan, Milano, Italy) housed in macrolon cages until experiment with ad libitum food and water and maintained on a 16 h light – 8 h dark cycle with light at 7:00 am. The room temperature was 23 ± 1 °C. All rats were kept for at least 1 week in the animal house facility of the University of Florence before initiating the experiment and were frequently handled. All animal manipulations were carried out according to the European Community guidelines for animal care. All efforts were made to minimize animal sufferings and to use only the number of animals necessary to produce reliable scientific data. No alternatives to *in vivo* techniques are available for this type of experiments.

2.2. Surgery

For the intracerebroventricular (i.c.v.) injection of rapamycin or mecamylamine, rats were deeply anaesthethized with Zoletyl 100, i.p. and placed in a stereotaxic frame (Stellar, Stoelting Co., Wood Dale, IL, USA) for surgery. A stainless steel cannula was implanted in the right lateral ventricle (coordinates from bregma: AP: −1.5; L: −1.5; H: 4.0 mm). Coordinates were taken from (Paxinos & Watson, 1982) and are relative to bregma and dural surface. The cannula was secured to the parietal bone with acrylic dental cement and the skin sutured closed. After surgery the rats were treated with Amplital 5 mg/rat s.c. Injection of rapamycin or mecamylamine was performed i.c.v. 7 days after surgery.

2.3. Drug treatments

Rapamycin (Calbiochem, EMD Biosciences, La Jolla, CA, USA), an inhibitor of mTORC1, was dissolved in a H_2O/DMSO solution (50% H_2O, 50% DMSO) and administered i.c.v. (1.5 nmol/5 μl of vehicle, 5.49 μg/kg) to rats 30 min before acquisition of the step down IA test. Control animals received injection of vehicle alone (H_2O/DMSO solution, 5 μl). The amount of rapamycin was chosen in order to obtain a tissue concentration ranging between 2 and 3 μM assuming the drug distributes evenly throughout the brain (1–1.5 g total). Scopolamine hydrochloride (SIGMA, St. Louis, MO, USA), a non-selective antagonist of cholinergic muscarinic receptors, was dissolved in saline and administered i.p. (1.5 mg/kg) 30 min before the acquisition trial of the step down IA test. Mecamylamine (SIGMA, St. Louis, MO, USA), a non-selective antagonist of cholinergic nicotinic receptors, was dissolved in saline and administered i.c.v (15 nmol/5 μl of saline, 10.04 μg/kg) 40 min before acquisition of the step down IA test.

For i.c.v. injection a micro syringe was connected to the cannula and rapamycin or mecamylamine was injected over a 2 min period. The syringe was then left in place for one additional min to avoid back diffusion of the solution.

2.4. Step down inhibitory avoidance test

In the step down IA test the rats, put on an elevated platform placed by one wall of an arena, learn to associate exploration of the adjacent compartment with a foot shock delivered through the floor grid. On a subsequent exposure to the same environment, the animal will avoid to step down onto the floor grid or will increase the latency before stepping down. We used a standard step down apparatus placed in a soundproof room. Rats were handled and habituated to the experimenter and to the handling procedure the day before the acquisition trial. Rats were positioned on an elevated platform placed in a dark compartment facing an open arena equipped with an electrified floor grid. The time spent to step down onto the grid where the aversive stimulus (10 electric shocks, 20 ms/0.5 mA/5 Hz) was delivered was recorded (Acquisition Latency). After the aversive stimulus, rats were immediately removed from the arena and placed in their home cage for consolidation (encoding). Recall trial, performed 1 h, 4 h or 24 h after the