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Transcription and protein synthesis inhibitors influence long-term effects of acetyl-L-carnitine on non-associative learning in the leech

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ABSTRACT

Acetyl-L-carnitine (ALC) is the principal acetyl ester of L-carnitine and it plays an essential role in intermediary metabolism. ALC affects several targets in the nervous system. Along this line of investigation, we analyzed the long-term effects of ALC on elementary nonassociative learning in the swimming induction model of the leech *Hirudo medicinalis*, in which nociceptive stimulation of the dorsal skin produces a more rapid swim response to a test stimulus (sensitization). In this simplified model a single ALC administration blocked the sensitizing effects of nociceptive stimulation in swim induction showing increasingly long lasting effects. Herein, we have analyzed the long-term effects of ALC on sensitization and dishabituation. Leeches were treated with inhibitors of either transcription or protein synthesis 30 min after the administration of ALC and, subsequently, subjected to noxious stimuli: the animals exhibited a sensitized swimming response 6 days after ALC treatment but not after 2 hours indicating that the long-term suppressive effects of ALC on sensitization/dishabituation needed mRNA and protein synthesis.

1. Introduction

Acetyl-L-carnitine (ALC) is an acetyl derivative of carnitine synthesized in the nervous system. It is a cofactor facilitating the utilization of fats as an energy source. When exogenously administered, ALC induces a cascade of actions influencing different aspects of the neuronal activity from metabolism to behavior (for a review, see Traina, 2011). ALC is known to affect the activity of enzymes involved in the energy turnover, thus modulating either different neurotransmitter systems (Picconi et al., 2006; Tolu et al., 2002) or intracellular pathways (Galeotti et al., 2004; Pérez-De La Cruz et al., 2008). A relevant action ascribed to ALC is its anti-nociceptive effect observed in the treatment of painful neuropathies of various origins (Chiechio et al., 2010; Di Cesare Mannelli et al., 2009; Janiri et al., 2009; Memeo and Loiero, 2008; Sima, 2007; Sima et al., 2005). In addition, there is evidence that ALC improves cognitive capabilities (Adriani et al., 2004; Ando et al., 2001; Marini et al., 2006; Shea, 2007).

Starting from 2004, we have exploited the experimental advantages of the leech *Hirudo medicinalis* in order to investigate some mechanisms through which ALC exerts its actions on the nervous system (Lombardo et al., 2004; Ristori et al., 2006; Traina et al., 2013). In this Invertebrate, the nociceptive stimulation of the dorsal skin induces changes in the swim initiation (SI) by producing a more rapid response to a test stimulus (sensitization) (Zaccardi et al., 2001). Leech swimming is an episodic behavior triggered by sensory stimulations. Previously, we have demonstrated that a single treatment with ALC blocks non-associative learning processes such as sensitization and that it partially prevents dishabituation triggered by brush strokes in a dose and time-dependent manner (Ristori et al., 2006); moreover, ALC is capable of modulating the electrical activity of the sensory T neurons (Lombardo et al., 2004) which in SI drive the sensory information to the swim-related muscles through a complex neuronal network initiating swimming activity. The most effective dose of ALC was 2 mM and the effects of the drug at this concentration on the non-associative learning processes were long-lasting suggesting the hypothesis that the ALC effects on sensitization and dishabituation processes might involve mechanisms related to qualitative changes in the gene expression or the modulation of protein synthesis. Recently, we have reported that a single administration of ALC is able to modulate positively the expression of genes coding for functions that reveal a lasting effect of ALC on the leech, and to confirm the neuroprotective and neuromodulatory role of the substance (Federighi et al., 2013). In particular, in that study, after ALC treatment, we identified, as differentially expressed, the genes coding for actinin, Hsp90, and the biosynthetic enzyme for thiazole.

Abbreviations: ALC, acetyl-L-carnitine; SI, swim initiation; ITI, interstimulus interval; L, latency; L_B, baseline latency.

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In the present paper we investigated the effects of the inhibition of both transcription and protein synthesis in the learning behavior of leeches subjected to ALC treatment.

2. Materials and methods

2.1. Animals

Adult medicinal leeches (*H. medicinalis*) 8–10 months old and weighing about 1.5 g were purchased from Ricarimpex (Eysines, France). The animals were maintained in commercially available mineral water (Acqua Panna, Firenze, Italy) under natural daylight conditions at 15–16 °C.

2.2. Behavioral procedure

Behavioral procedure has been previously described by Zaccardi et al. (2001, 2004) and Ristori et al. (2006). Briefly, before testing the animals, the connection between the cephalic and the first segmental ganglion was cut to remove the tonic inhibition exerted by the head ganglion on swimming activity making a behaving animal able to respond consistently to the training protocol (Brodfuehrer and Burns, 1995; Ristori et al., 2006; Zaccardi et al., 2001). Each experimental session started two days after surgery. To induce swimming, a weak electrical stimulus (1.6 s – duration train of 5 ms current pulses at a frequency of 8.3 Hz) was applied onto the caudal portion of the body set on a bipolar Ag–AgCl electrode connected to a stimulus isolation unit. The leeches were restrained to swimming in a plexiglas chamber containing mineral water in response to the electrical stimulus (test stimulus) whose intensity was set at the lowest voltage capable of producing a steady swimming cycle. The voltage chosen for each animal (0.8 V–1.4 V) was kept constant during the whole experiment. Following published protocols (Ristori et al., 2006; Zaccardi et al., 2004), as a response index we chose the interval between the start of the electrical shocks and the onset of the swimming cycle (i.e., latency, L). We considered the onset of the swimming cycle when the animal started to undulate its body in the dorsoventral plane forming a wave that travels from the head to the tail. The operator delivered the test stimulus and signaled the onset of the swimming cycle through an on/off button connected to a computerized system that simultaneously measured the latencies by means of a customized software.

As reported in Zaccardi et al. (2001, 2004), the training protocol for sensitization was the following: each animal was first subjected to four test stimuli applied at variable inter-stimulus intervals (ITIs), ranging from 2 to 10 min, to avoid inducing habituation; then, 15 brush strokes (1/s) were administered on the back of the animal and, immediately afterwards, the animal was again placed in the recording chamber and trained with a typical habituation session consisted of 15 test stimuli delivered at 1 min ITI. For each animal the average of the latencies measured in response to the first four test stimuli was considered as the baseline response (baseline latency, L_B).

A dishabituation session consisted of a first habituation training, followed by brush strokes administration and a second habituation training (Zaccardi et al., 2004).

2.3. Pharmacological treatment

ALC, cycloheximide, actinomycin D were freshly prepared and before use diluted to their final concentrations in saline. Actinomycin D had been previously dissolved in DMSO 1: 1000. ALC was freshly prepared, dissolved in saline solution and, if needed, buffered to 7.4 pH with NaOH before use. The saline solution contained: 115 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 10 mM glucose, buffered to 7.4 pH by 10 mM Tris-maleate. ALC was supplied by Sigma-Tau (Pomezia, Italy). All other chemicals were purchased from commercial sources.

In order to assure their distribution in the nervous system (Zaccardi et al., 2004), ALC, saline, cycloheximide, actinomycin D were supplied dorsally by two injections (one in the rostral and the other one in the caudal part of the body of the leech), each one of 100 μl/g animal. ALC or saline were injected, and 2 hours (h) and 6 days later the animals were subjected to the training sessions. Cycloheximide or actinomycin D or drug vehicle were injected 30 min after ALC administration. All animals were handled and processed in the same manner, so that the only difference was the pharmacological treatment.

All experiments were performed in blind conditions: the researcher doing the behavioral testing was not aware of the treatment that each animal had received.

2.4. Data analysis

Descriptive statistics are given as mean ± SE. As previously described (Ristori et al., 2006; Zaccardi et al., 2001, 2004) in order to represent sensitization as an increase in the amplitude of the response to the test stimulus delivered immediately after the noxious stimulus in comparison with the baseline response (see above), we plotted the inverse latency of each response recorded after brush strokes normalized to the inverse of the baseline latency, using the formula: $[(1/\text{latency})/(1/\text{baseline latency})] \times 100$. To compare the effects of ALC after actinomycin D or cycloheximide treatments on sensitization induced by noxious stimuli, the percentage differences between the normalized inverse latency to the 1st test stimulus after brush strokes and the value 100 (which represented the inverse of baseline latency) were plotted in the different conditions (see Fig. 3).

In the experiments of dishabituation the inverse latency of each response was considered (Zaccardi et al., 2001). For each animal, all inverse latencies were normalized by expressing them as a percentage of the inverse of the response to the 1st trial in the 1st habituation session (latency1), taken as 100% by using the formula: $[(1/\text{latency})/(1/\text{latency1})] \times 100$, so that habituation has been described as a progressive reduction of the response to the test stimulus repetitively delivered at constant ITI. Dishabituation has been evaluated as an increase of the response to the 1st test stimulus after brush strokes (latency16), in comparison with the habituated response represented by the response to the 15th trial in the 1st habituation training (latency15). The effects of ALC after actinomycin D or cycloheximide treatments on dishabituation induced by brush stroke are described as ratio latency16/latency1 (L_{16}/L_1), with a ratio of about 1, if the dishabituated response was similar to the initial response, >1 if the dishabituated response exhibited a latency longer than the initial response. In this latter case, dishabituation was impaired (see Fig. 6).

Due to the non-normality of our data (latencies measured in seconds) (Kolmogorov–Smirnov test), statistical analysis was performed by means of non-parametric tests. The Wilcoxon test was used within each group of animals to compare the baseline response with the 1st response after brush strokes in sensitization experiments and the response to the 15th trial in the 1st habituation training with the response to the 1st test stimulus after brush strokes (16th trial) in dishabituation experiments. To compare the amount of sensitization or dishabituation in the different experimental conditions (Figs. 3, 6) the Kruskal–Wallis test and the post hoc Dunn's multiple comparison test were done.

The software package GraphPad Prism (version 4.0, GraphPad Software, San Diego, CA, USA) was used. Differences with $p < 0.05$ were considered statistically significant.