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

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A B S T R A C T

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.

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.

3. Results

3.1. Actinomycin D, an inhibitor of transcription, antagonizes ALC effects on the sensitization process

Due to the long-term effects of ALC on both sensitization and dishabituation, as previously reported by Ristori et al. (2006), we analyzed whether ALC effects on these behavioral processes were due to transcription and protein synthesis. First, we tested the effects of an inhibitor of the transcription, actinomycin D. Therefore, 24 leeches were treated with 2 mM ALC and 30 min later, 14 leeches were injected with 2 mM actinomycin D, whereas 10 leeches received drug vehicle. Two other groups of animals were considered: saline plus 2 mM actinomycin D in DMSO (n = 11) and saline plus DMSO-treated leeches (n = 10). Two hours after ALC treatment, all the animals were trained by a typical brush-induced sensitization session (Ristori et al., 2006) (Fig. 3A). In ALC plus actinomycin D-treated leeches the sensitization was blocked (L_1: 1.67 ± 0.07 s; L_8: 1.35 ± 0.04 s, Wilcoxon test, $p < 0.001$) as well as in ALC-treated leeches (L_1: 1.76 ± 0.11 s, L_8: 1.32 ± 0.05 s, Wilcoxon test, $p = 0.005$), while saline plus DMSO-treated leeches and saline plus DMSO and 1 mM actinomycin D-treated leeches exhibited sensitization because L_1 (0.96 ± 0.06 s and 0.99 ± 0.07 s, respectively) was significantly lower than L_8 (1.30 ± 0.04 and 1.34 ± 0.03 s, Wilcoxon test, $p = 0.0033$ and $p = 0.005$, respectively). As Fig. 1 shows, the habituation curve obtained after the treatment with ALC alone is faster than that with ALC plus actinomycin D, meaning that the transcription inhibitor began to influence the ALC effects on learning processes.

Afterwards, six days after treatments, we again tested all the groups of animals (Fig. 1B) and we observed that ALC plus actinomycin D-treated leeches exhibited sensitization whereas ALC-treated leeches did not (Fig. 1B). At this time, in ALC-treated leeches L_1 (1.88 ± 0.08 s) was significantly higher (Wilcoxon test $p = 0.0076$) than L_8 (1.28 ± 0.04 s) whereas in ALC plus actinomycin D-treated leeches L_1 (1.22 ± 0.04 s) was significantly lower (Wilcoxon test $p = 0.0037$) than L_8 (1.42 ± 0.03 s) indicating that sensitization occurred as well as in saline plus DMSO, and saline plus 2 mM actinomycin D-treated leeches which exhibited L_1 (1.02 ± 0.04 s and 1.02 ± 0.03 s, respectively) significantly lower than L_8 (1.30 ± 0.06 s and 1.39 ± 0.02 s, Wilcoxon test, $p = 0.0117$ and $p = 0.005$, respectively) (Fig. 1B).

As at 6 days the ALC effects were totally counteracted by the inhibitor, we chose not to test the animals further in the following days.

3.2. Cycloheximide, a protein synthesis inhibitor, antagonizes ALC effects on the sensitization process

We also tested the effects of cycloheximide, an inhibitor of protein synthesis, on the sensitization process in leeches previously treated with 2 mM ALC. Therefore, 28 leeches were treated with 2 mM ALC and 30 min later, 13 leeches were injected with 1 mM cycloheximide; the remaining 15 leeches received the drug vehicle. All the animals were trained by a typical brush-induced sensitization session 2 h after ALC treatment (Fig. 2A). Two other groups of animals were considered: saline plus 1 mM cycloheximide (n = 15), and saline-treated leeches (Control, n = 10), which received saline instead of 2 mM ALC. Fig. 2A shows the sensitization process measured in the different groups. In ALC-treated leeches (L_1: 1.63 ± 0.11 s, L_8: 1.16 ± 0.05 s, Wilcoxon test, $p = 0.0033$) and in ALC plus cycloheximide-treated leeches the sensitization was blocked (L_1: 1.81 ± 0.06 s; L_8: 1.40 ± 0.03 s, Wilcoxon test, $p = 0.0015$), while saline-treated leeches and saline plus 1 mM cycloheximide-treated leeches exhibited sensitization because L_1 (0.97 ± 0.06 s and 1.11 ± 0.06 s, respectively) was significantly lower than L_8 (1.33 ± 0.05 and 1.36 ± 0.04 s, respectively, Wilcoxon test, $p < 0.001$ in both cases). As Fig. 2 shows, also in the case of the treatment with cycloheximide, the protein synthesis inhibitor seems to begin to affect the ALC effects on the learning process at two hours, because the habituation curve obtained after the treatment with ALC alone is faster than that obtained after ALC plus cycloheximide.

We again tested all the groups of animals at six days from ALC administration (Fig. 2B), and we observed that in ALC-treated leeches L_1 (1.62 ± 0.02 s) was significantly higher (Wilcoxon test $p = 0.017$) than L_8 (1.25 ± 0.05 s), while in ALC plus cycloheximide-treated leeches L_1 (1.39 ± 0.02 s) did not significantly differ (Wilcoxon test $p = 0.530$) from

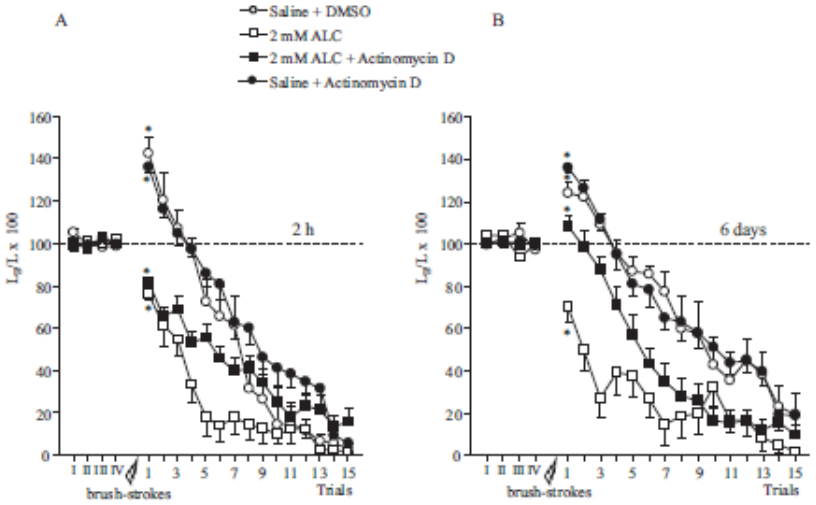

Fig. 1. Actinomycin D antagonizes the ALC effects on the sensitization process. In this and the following figure, the inverse latencies of each response normalized to the inverse of the baseline latency (L_8) have been plotted. (A) Two hours after the ALC or saline treatment, only the leeches treated with saline plus DMSO or saline plus actinomycin D showed sensitization whereas the leeches treated with 2 mM ALC or 2 mM ALC plus actinomycin D did not. (B) At 6 days only the 2 mM ALC-treated leeches (white square) exhibited a strong impairment of the brush strokes-induced sensitization. Data are presented as mean ± SEM. *Indicates significant differences within each group between L_8 and L_1, $p < 0.05$.

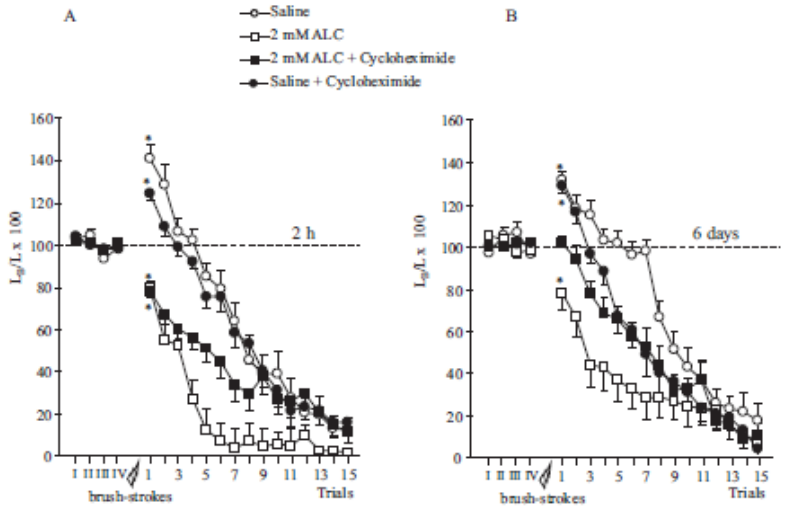

Fig. 2. Cycloheximide antagonizes the ALC effects on the sensitization process. (A) Two hours after 2 mM ALC or saline treatment the sensitization process did not occur in leeches treated with 2 mM ALC, as well as in leeches treated with 2 mM ALC and 30 min later with 1 mM cycloheximide, whereas in saline, or saline plus 1 mM cycloheximide-treated leeches sensitization occurred. After brushing, the leeches treated with ALC exhibited reduced behavioral responses compared to the baseline response. (B) Also 6 days after the ALC or saline treatment, the animals treated with 2 mM ALC and the leeches treated with 2 mM ALC and 30 min later with cycloheximide did not exhibit sensitization, but the leeches which received the inhibitor exhibited behavioral responses after brushing close to the baseline response: although not totally, cycloheximide seems to counteract the ALC effect. Data are presented as mean ± SEM. *Indicates significant differences within each group between L_8 and L_1, $p < 0.05$.

L_8 (1.39 ± 0.05 s). These animals still showed no sensitization. However, their responses to the test stimulus approached the baseline response and they were more rapid than those shown at 2 h, meaning that cycloheximide counteracted the effect of ALC on the sensitization process over time. In saline-treated leeches ($L_8 = 1.31 \pm 0.07$; $L_1 = 1 \pm 0.05$, Wilcoxon test, $p = 0.005$) and in saline plus 1 mM cycloheximide-treated leeches ($L_8 = 1.47 \pm 0.03$; $L_1 = 1.15 \pm 0.03$, Wilcoxon test, $p < 0.001$) sensitization occurred, as expected.

Fig. 3 matches the sensitization process measured in the animals treated with 2 mM ALC *vs* both ALC plus cycloheximide- and ALC plus actinomycin D-treated leeches. The histograms plot the entity of sensitization as the difference between the percentage of the inverse of the latency to the 1st trial following brush strokes and the inverse of baseline latency taken as 100% at 2 h and 6 days. At 2 h, in both ALC plus cycloheximide- and ALC plus actinomycin D-treated leeches the sensitization was blocked as in ALC-treated leeches (Kruskal–Wallis test: $p = 0.3285$), while at 6 days both ALC plus cycloheximide- and ALC plus actinomycin D-treated leeches exhibited a response to nociceptive stimulation significantly different from the ones exhibited by ALC-treated leeches (Kruskal–Wallis test: $p < 0.001$; post-hoc Dunn's Multiple Comparison, $p < 0.05$ in both cases): only the animals treated with ALC alone did not exhibit sensitization.

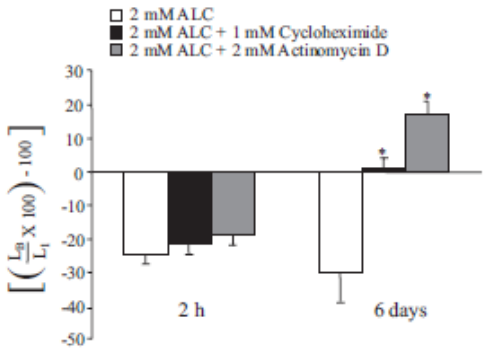

Fig. 3. Effects of ALC after cycloheximide or actinomycin D administration on the sensitization process. Each column represents the percentage difference between the response to the 1st test stimulus applied after brush strokes and the baseline response taken as 100% at 2 h and 6 days after ALC treatment. The lower zero bars indicate a reduced response after brushing with respect to the baseline response. The upper zero bars indicate a sensitized response.*Indicates significant difference among groups, $p < 0.05$.

3.3. *Both Actinomycin D and cycloheximide antagonize ALC effects on the dishabituation process*

As previously shown, the 2 mM ALC treatment did not completely block but did provoke a reduction of the potentiation of the response to the test stimulus delivered soon after brush strokes in the dishabituation process (Ristori et al., 2006). Herein, we tested the effects of the inhibition of protein synthesis in leeches previously treated with ALC and subjected to the dishabituation process of SI. Twenty-one leeches were treated with 2 mM ALC, and 30 min later, 12 leeches were injected with 2 mM actinomycin D and 9 leeches received drug vehicle. We also considered two other groups of animals: saline plus 2 mM actinomycin D (n = 11) and saline plus DMSO (Control, n = 8) -treated leeches which received saline plus DMSO instead of 2 mM ALC. Two hours and 6 days after either ALC or saline plus DMSO administration all leeches exhibited dishabituation because L_{16} resulted significantly shorter than L_{15} at both 2 h (Wilcoxon test $p < 0.01$ in all cases) and 6 days (Wilcoxon test $p < 0.01$ in all cases) as shown in Fig. 4.

A further 20 leeches were treated with 2 mM ALC and 30 min later, 10 leeches were injected with 1 mM cycloheximide, and 10 leeches received drug vehicle (Fig. 5). Also in this case, two other groups of animals were considered: saline plus 1 mM cycloheximide (n = 9), and saline (Control, n = 8) -treated leeches which

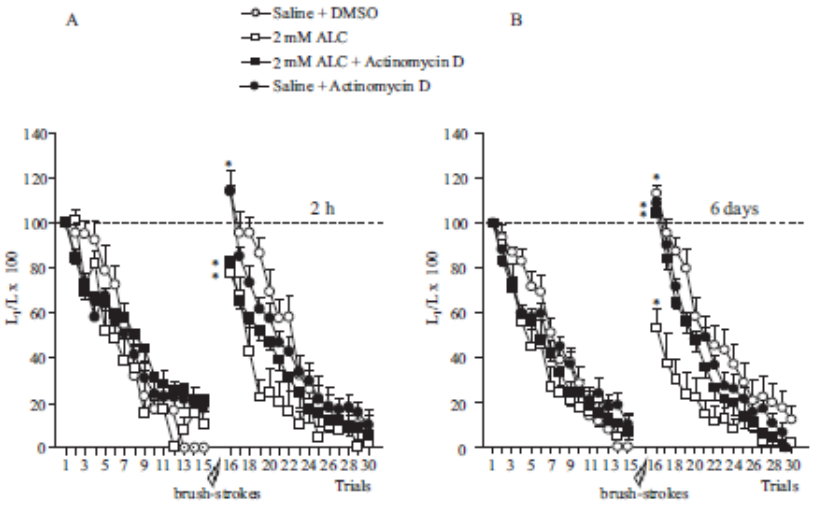

Fig. 4. Actinomycin D antagonizes the ALC effects on the dishabituation process. In this and the following figure, the inverse latencies of each response normalized to the inverse of the latency (L_1) recorded after the 1st trial in the 1st habituation session have been plotted. (A) 2 h after ALC or saline administration all leeches exhibited dishabituation because L_{16} resulted significantly shorter than L_{15} (Wilcoxon test $p < 0.01$ in all cases). (B) Also at 6 days dishabituation occurred (Wilcoxon test $p < 0.01$ in all cases); nevertheless, ALC-treated leeches exhibited a strong impairment of dishabituation, whereas 2 mM ALC plus actinomycin D group showed a dishabituation comparable to that exhibited by the other control groups. *Indicates significant differences within each group between L_{15} and L_{16}, $p < 0.05$.

received saline instead of 2 mM ALC. All the animals were trained by a typical dishabituation session 2 h and 6 days after either ALC or saline administration. At the two time points considered all leeches exhibited dishabituation because L_{16} resulted significantly shorter than L_{15} at both 2 h (Wilcoxon test $p < 0.01$ in all cases) and 6 days (Wilcoxon test $p < 0.01$ in all cases) as shown in Fig. 5.

Nevertheless, by evaluating the entity of the dishabituation process measured as the ratio L_{16}/L_1 (Fig. 6), it is possible to note that this ratio was about 1 at 2 h for all considered groups of animals, indicating that the dishabituated responses were similar to the initial responses, whereas at 6 days ALC-treated leeches exhibited a ratio significantly higher (Kruskal–Wallis test: $p < 0.001$) than the one exhibited by both 1 mM cycloheximide plus 2 mM ALC-treated leeches (post-hoc Dunn's Multiple Comparison test, $p < 0.01$) and 2 mM actinomycin D plus 2 mM ALC-treated leeches (post-hoc Dunn's Multiple Comparison test, $p < 0.001$), indicating that in the leeches treated with ALC alone dishabituation was more impaired than in the other groups of animals considered.

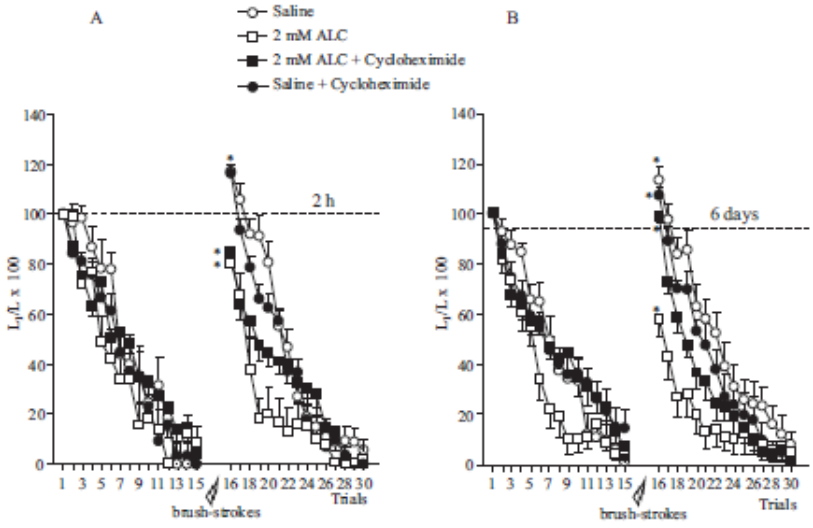

Fig. 5. Cycloheximide antagonizes the ALC effects on the dishabituation process. (A) 2 h after ALC or saline treatment all leeches exhibited dishabituation because L_{16} was significantly shorter than L_{15} (Wilcoxon test $p < 0.01$ in all cases). (B) At 6 days ALC-treated leeches exhibited a strong impairment of dishabituation, whereas the 2 mM ALC plus cycloheximide group showed a dishabituation comparable to the one exhibited by the other control groups. *Indicates significant differences within each group between L_{15} and L_{16}, $p < 0.001$.

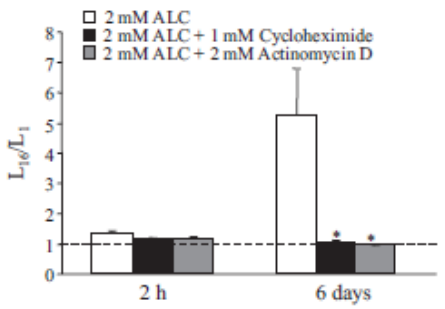

Fig. 6. Effects of ALC after cycloheximide or actinomycin D administration on the dishabituation at different time points. The ratio between the latencies exhibited by the animals with different treatments to the 16th and 1st test stimuli is plotted. Two hours after the ALC treatment, no significant differences between treatments were found. After 6 days, 2 mM ALC-treated animals showed a significant difference with respect to ALC plus cycloheximide or ALC plus actinomycin D-treated ones. *Indicates Dunn's post hoc test: $p < 0.001$, after Kruskal–Wallis test.

All together, the data collected indicates that when protein and mRNA synthesis were inhibited ALC was less able to affect the dishabituation process.

4. Discussion

Several studies carried out in vertebrate models and humans illustrate the effects of ALC in the nervous system including its effect in improving the cognitive capability likely due to its protective actions against several physiological and pathological brain modifications induced by aging (Calabrese et al., 2012; Tolu et al., 2002), diseases (Shea, 2007; Torrioli et al., 2008; Traina et al., 2006, 2008a, 2008b, 2009, 2011) and drug abuse (Coccurello et al., 2007; Scheggi et al., 2004) rather than to its actual action on the mechanisms underlying learning processes. Recently, a role of ALC in the modulation of pain perception has been demonstrated mostly in the treatment of neuropathies of various origins (Chiechio et al., 2010; Sima, 2007).

In the present paper, we use a simple animal model, the leech *H. medicinalis* to investigate the ALC-induced modulation of elementary forms of learning and, we provide evidence for interesting long-term effects of the drug on non-associative learning processes triggered by brush strokes. In particular, this study has been performed with the aim of unveiling some mechanisms underlying the long-lasting effects of ALC on sensitization and dishabituation by exploiting the experimental advantages of the leech. There are very few studies on the effects of ALC in invertebrate models, but we chose to use the leech because this invertebrate exhibits simple behaviors that are sustained by neural circuits easily accessible to experimentation and already complex enough to provide information on the mechanisms that govern the function of the most evolved nervous system in the vertebrates.

Previously, in nearly-intact leeches we tested the effects of a single treatment with ALC on the sensitization and dishabituation processes induced by brush strokes delivered onto the dorsal skin of the animal, and we observed that ALC strongly impaired sensitization in a dose and time-dependent manner (Ristori et al., 2006). As regards the dishabituation process, ALC treatment did not block this form of learning but it only provoked a reduction of the potentiation of the response to the test stimulus delivered immediately after the nociceptive stimulation (Ristori et al., 2006).

Different effects of ALC on sensitization and dishabituation were not unexpected. In fact, these two forms of non-associative learning share some molecular and cellular mechanisms (Zaccardi et al., 2004), but differ for a different integration and convergence of multiple physiological mechanisms which act at different sites of the neural circuit underlying the swim activity with different time courses (Zaccardi et al., 2001).

The ALC effects on both sensitization and dishabituation show a common feature: they arose 2 h after ALC treatment, and increased over time, showing a long-lasting effect of the drug (Ristori et al., 2006).

In the present study, we demonstrated that these long-term ALC effects require transcription and protein synthesis. In fact, the injection of inhibitors of either transcription or protein synthesis after the administration of 2 mM ALC prevented the block of sensitization and the impairment of dishabituation induced by ALC 6 days after the treatment, while it did not after 2 hours suggesting distinct mechanisms through which ALC exerted its action in the short- and long-term. It is not surprising that similar effects are due to different phenomena: in learning processes, the short-term processes are mainly supported by covalent modifications and long-term processes are due to transcription and translation phenomena. In fact, long-term effects often require qualitative and quantitative changes of gene expression and proteins synthesis. Previous reports on rats have shown that ALC administration mediated neuroprotection during hypoxia through peroxisome proliferator-activated receptor γ coactivator-1α and nuclear respiratory factor-1-induced mitochondrial biogenesis. The expression of these genes was regulated by a related kinase-nuclear factor erythroid 2-related factor 2-mediated mechanism (Barhwal et al., 2009; Hota et al., 2012). Behavioral studies on hypoxic rats report that ALC supplement improves memory impairment (Hota et al., 2012). Such evidence suggests a relevant therapeutic potential of ALC for the treatment of ischemia, stroke, and other neurodegenerative disorders associated with hypoxic stress and excitotoxicity. Despite the increasing body of evidence that in the vertebrate nervous system ALC modulates gene expression (Abdul et al., 2006; Traina et al., 2004, 2006, 2008a, 2008b, 2009) so far little is known, to our knowledge, in the leech nervous system except what has been reported in our previous paper. In particular, we analyzed the modulation of gene expression induced by ALC treatment in the leech, utilizing the suppression subtractive hybridization procedure, one of the most powerful methods for generating subtractive differential cDNA libraries (Federighi et al., 2013). In that study, we showed that a single administration of ALC was able to modulate positively the expression of some genes coding for proteins whose functions revealed and confirmed the neuroprotective and neuromodulatory role of the molecule. In particular, we evidenced an up-regulation of the expression of the gene coding for thiazole, an enzyme involved in the biosynthesis of thiamine (vitamin B1) that, like other B group vitamins, seems to have antinociceptive effects. In this context, ALC is a drug marketed for the treatment of neuropathic pain (Youle, 2007) and causes analgesia by increasing type-2 metabotropic glutamate receptor (mGlu2) expression (Chiechio et al., 2010). We cannot exclude that the leeches present a reduction in the responsiveness to stroke stimuli, according to an analgesic role of ALC. The reduction of the responsiveness to brush strokes exhibited by the ALC-treated leeches might be due to changes in the integration of nociceptive stimuli, and mediated by transcription and translation processes. Finally, the rapid and long-lasting action of ALC strongly suggests the appropriateness of examining the epigenetic hypothesis. Further experiments will aim at clarifying which proteins mediate the effects of ALC on the mechanisms underlying sensitization/dishabituation processes.

Conflict of interest

The authors declare no conflict of interest.

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