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ACETYL-L-CARNITINE AFFECTS THE ELECTRICAL ACTIVITY OF MECHANOSENSORY NEURONS IN *HIRUDO MEDICINALIS* GANGLIA

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ABSTRACT

Was previously discovered that in the leech *Hirudo medicinalis*, acetyl-l-carnitine (ALC) affects forms of non-associative learning, such as sensitization and dishabituation, due to nociceptive stimulation of the dorsal skin in the swim induction behavioural paradigm, likely through modulating the activity of the mechanosensory tactile (T) neurons, which initiate swimming. Since was found that ALC impaired sensitization and dishabituation, both of which are mediated by the neurotransmitter serotonin, the present study analyzed how ALC may interfere with the sensitizing response.
Was already found that ALC reduced the activity of nociceptive (N) neurons, which modulate T cell activity through serotonergic mediation.

1. Introduction

Acetyl-l-carnitine (ALC), the short-chain ester of carnitine, is endogenously produced within mitochondria and peroxisomes, and involved in the transport of acetyl-moieties across the membranes of these organelles. Several studies have been carried out on the effects of ALC on the nervous system [1-11]. It is known that ALC has antioxidant, cytoprotective, neuromodulatory, anti-apoptotic effects and that it improves the cognitive capability in aged animals [12,13]. Previously, we observed that ALC affects simple forms of non-associative short-term learning, such as sensitization and dishabituation in the swim induction (SI) of the leech *Hirudo medicinalis*. SI is a behavioural paradigm used to investigate the mechanisms underlying learning and memory processes in invertebrate models [10,11,14]. Studies on elementary animal models offer various experimental advantages for explaining mechanisms underlying short-term changes and investigating the cellular and molecular activity of drugs. In the leech, swimming is an episodic behaviour initiated by sensory stimulation. Weak electrical shocks delivered into the caudal portion of the body wall trigger swimming activity by recruiting tactile (T) neurons [15]. These cells drive the sensory information to the swimming muscles through a complex neuronal network [16]. In the leech, it is possible to induce habituation in SI by delivering repetitive weak electrical stimulation onto the skin, whereas sensitization and dishabituation can be produced through nociceptive stimulation, such as brushing the body wall [17]. In particular, brush strokes induce both sensitization and dishabituation [14,17-18] by recruiting nociceptive (N) neurons. The neurotransmitter serotonin (5-HT) is involved in these forms of non-associative learning [17,19]. Moreover, pre-treatment with methysergide, a 5-HT receptor antagonist, prevents both sensitization and dishabituation, and sensitizing effect of 5-HT is mediated by cAMP [19]. The data obtained from previous studies showed that a single ALC treatment inhibited sensitization in a dose- and time-dependent manner, while not totally preventing dishabituation [14]. Moreover, a single ALC treatment affects T cell activity, increasing the amplitude of the afterhyperpolarization (AHP) that accompanies the firing of these neurons [20]. In T cells, changes in synaptic output occurred as a consequence of the AHP amplitude modulation [15,21], thus modulating the cascade of interactions that converts skin stimulation into the rhythmic swimming activity. These observations suggest that ALC may interfere with nociceptive perception, making the animals less susceptible to the stressful action of sensitizing stimuli. Therefore, in the present study we

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focused on N cells to gain additional insight into the effects of ALC in reducing sensitization.

2. Material and methods

Animals

Adult medicinal leeches (*Hirudo medicinalis*) (8–10 months old) weighing about 1.5 g were purchased from Ricarimpex® (Eysines, France) and maintained in commercially available bottled mineral water (Acqua Panna®, Firenze, Italy), with a natural daylight rhythm at 15-16°C.

Pharmacological treatment

ALC was freshly prepared, dissolved in saline solution and, if needed, buffered to pH 7.4 with NaOH before use. Saline solution contained 115 mM NaCl, 4 mM KCl, 1.8 mM CaCl2, and 10 mM glucose, and was 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.

Electrophysiological recordings

Single ganglia were dissected in leech Ringer's solution (saline solution, see above), and placed in a recording chamber that was constantly perfused with saline solution (1.5 mL/min). For some experiments, methysergide (100 µM) was added to block serotonergic transmission.

Intracellular recordings of individual T and N neurons were made by impaling the cell with a sharp microelectrode made of borosilicate glass (1.5 mm outer diameter, 1 mm inner diameter, Hilgerberg GmbH, Germany) and pulled to tip resistance of 80-120 MΩ using a Sutter P-97 puller (Sutter Instruments). The microelectrodes contained 4M K-Acetate solution. Recordings were made with an Axoclamp 2B amplifier (Axon Instruments Inc., Union City, CA) in bridge mode. Signals were filtered at 30kHz, digitized and stored on a computer and sampled at 100 kHz using a BNC-2090 National Instruments interface. Data were subsequently analyzed using the LabView software (National instruments S.R.L., Milan, Italy). The experiments assessed the modulation of the AHP amplitude in T neurons during N cell activity, and were carried out by simultaneously impaling a T neuron and an ipsilateral N cell. The intracellular stimulation of the N cell consisted of an 18-s train of intracellular depolarizing pulses (300 ms, 2.5 Hz). In the T cell, the AHP was elicited by a 3-s train of intracellular depolarizing pulses (200 ms, 2.5 Hz) delivered before N stimulation (Control), during the last 3 seconds of N stimulation, and 10 and 20 min after N stimulation. A single 200-ms pulse induced T neurons to fire a burst of 7-8 spikes, which reliably reproduced the pattern of discharge evoked by the stimulation of their receptive field. The discharge frequency of the T neurons was kept constant during each series of trials by adjusting the amount of injected current, ranging from 0.6 to 0.8 nA.

Data analysis

Descriptive statistics are reported as mean ± SEM. The AHP amplitude was measured from the baseline of Vm to the peak of the maximal hyperpolarization. The changes in AHP amplitude were analyzed using a repeated measures ANOVA test, with $P < 0.05$ considered as statistically significant. Tukey's multiple comparison test was used to detect significant differences between the various experimental conditions and the control (before N stimulation) where the ANOVA analysis indicated a significant difference between groups (denoted on the histograms with an asterisk).

Vm, Rm and the discharge threshold (i.e. the minimal intensity of the current pulses leading to the neuron firing a spike) recorded in N neurons of animals treated with ALC or saline were compared using the Mann–Whitney U test (P level < 0.05).

All the statistical analyses were carried out with the Statistica software package (StatSoft, Tulsa, OK, USA).

3. Results

ALC inhibits N neurons activity

In a previous study, we demonstrated that a single ALC treatment blocked the behavioural sensitization and impaired the dishabituation induced by nociceptive stimuli, starting as early as two hours after treatment [14]. For the present study, we carried out intracellular recordings in medial and lateral N cells from single ganglia dissected from leeches that have been treated either 2 h or 6 days earlier with 2 mM ALC (n = 9 cells and n = 13 cells, respectively) or saline (Control, n=9 cells and n=6 cells, respectively). N neurons of ganglia from ALC-treated leeches exhibited a significant hyperpolarization of the resting potential when compared to the control, at both 2 h and 6 days (Mann Whitney U test $P < 0.01$ in both cases) (Figure 1A).

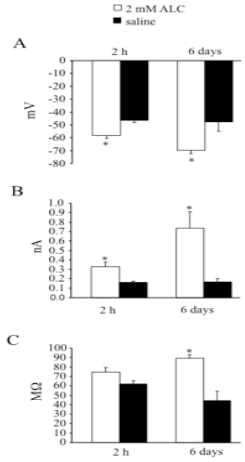

Figure 1 - Effects of 2 mM ALC on the electrophysiological properties of N cells. At both 2 h and 6 days, the resting potential (Vm) was significantly hyperpolarized in N cells of ALC-treated leeches (white column), in comparison with Vm in N cells of saline-treated leeches (black column). (B) At both 2 h and 6 days, the intensity of the current pulse capable of inducing firing in N cells of ALC-treated leeches (white column) was significantly higher than in N cells of saline-treated leeches (black column). (C) At 6 days, the input resistance was significantly higher in N cells of ALC-treated leeches (white column) than in N cells of saline-treated leeches (black column).*, p < 0.05.

Consequently, the firing threshold resulted significantly higher in the N cells of ALC-treated leeches than in the N cells of control leeches at both 2 h and 6 days (Mann Whitney U test $P < 0.01$ in both cases). As shown in Figure 1B, the intensity of the current pulse capable of inducing a spike in N cells of ALC-treated leeches was 0.33 ± 0.05 nA at 2 h and 0.73 ± 0.18 nA at 6 days, whereas in N cells of control leeches, the intensity was practically constant, resulting 0.16 ± 0.01 nA at 2 h and 0.17 ± 0.04 at 6 days.

The firing threshold did not change by maintaining the N membrane potential at about -50 mV in both ALC and saline-treated leeches (data not shown). The input resistance was also evaluated and found to be significantly higher in N cells of ALC-treated leeches than in those of control leeches at 6 days only (Mann Whitney U test $p = 0.002$) (Figure 1C).

The modulation of AHP in T neuron by N cell represents a basic mechanism for sensitization

The data reported above suggest that after ALC treatment, N cells might be less responsive to noxious stimuli, thus impairing sensitization and dishabituation processes induced by brush strokes. Moreover, the reduced sensitized response observed after ALC administration might also depend on the fact that N cell activity affected T cell activity through the modulation of the T cell AHP amplitude that regulates the synaptic efficacy of T neuron on follower neurons in the swimming circuit, as previously demonstrated [15,21]. To test the possibility that the nociceptive stimulation may affect T cell activity, we carried out electrophysiological recordings by simultaneously impaling a T neuron and an ipsilateral nociceptive N cell (n=7 pair of neurons). The N neuron was intracellularly stimulated for 18-s (see Materials and Methods), while intracellular recordings were performed in T neurons to measure the AHP amplitude elicited by a 3-s train of depolarizing pulses before (Control), during the last 3-s and every 10 min after N cell stimulation. As shown in Fig. 2A, the AHP amplitude significantly changed (ANOVA for repeated measures, $F_{2,19} = 16.56$, $p < 0.001$); Tukey's multiple comparison test showed that the AHP amplitude during N cell stimulation was significantly reduced (5.48 ± 0.99 mV) in comparison with the control value (7.68 ± 0.97 mV), and that it recovered to its initial value 20 min later (8.3 ± 1.42 mV, $p > 0.05$). In all phases of the experiments, we also monitored the membrane voltage (Vm) and the input resistance (Rm) of both T and N neurons, which did not show any change (data not shown).

When the stimulation of N neurons was preceded by incubation for 15 min with 100 µM of methysergide, a 5-HT receptors antagonist, no changes of AHP amplitude occurred (n=7 pairs of neurons, ANOVA for repeated measures, $F_{2,19} = 1.213$, $p = 0.331$) (Fig. 2B) both during N stimulation (8.7 ± 0.76 mV) and 20 min after (7.87 ± 0.82 mV), when compared to the control value (8.5 ± 0.75 mV), indicating that nociceptive stimulation induced changes in the AHP amplitude in T neurons and that the 5-HT mediated such effect.

We also tried to simultaneously record the activity of the N and T cells in leeches previously treated with ALC, but the effects of the drug on the N cells were so strong that the duration and intensity of N cell stimulation prevented a reliable recording in T cells.

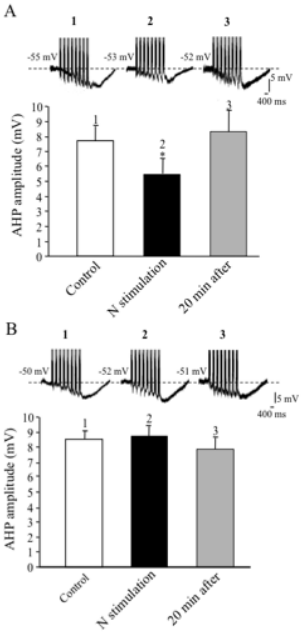

Figure 2 - The mean ± SEM of the AHP amplitude recorded during N cell stimulation was significantly reduced in comparison with the initial value (Control). In the 20 min after N cell stimulation time point, AHP had recovered to the control value. Upper traces are the AHP recorded in a single T cell. The AHP amplitude measured from the baseline (-53 mV) to the peak of the hyperpolarization during the stimulation of an ipsilateral N cell was shorter (2) than in both Control (1) and 20 min after N stimulation (3) groups. (B) When ganglia were incubated with 100 µM methysergide for 15 min, the N cell stimulation did not induce changes in the AHP amplitude in T cells. Upper traces are AHP recorded from a single T cell in Control (1), during the stimulation of an ipsilateral N cell (2) and 20 min after (3). As in A, for graphical convenience, the action potentials generating AHP have been clipped and they are indistinguishable from each other because of the time scale used. *, $p < 0.05$.

4. Discussion

Previously, we studied sensitization in the leech *H. medicinalis*. Application of nociceptive stimuli, such as brushing, induced potentiation of swim induction [17] and such effect was mimicked by 5-HT application [19]. In a restrained animal, weak tactile (or electrical) stimuli onto the back induced swim activity initiated through the activation of T neurons [15] that interact with swim motor neurons. The stimulation induced a reduction in the action potential of T cells, followed by AHP, generated by two factors: a) activation of the Na/K-ATPase, and b) $I_{K/Ca}$ current [22]. The AHP amplitude in T neurons plays a crucial role in controlling the traffic of action potentials (AP).

When AHP amplitude is reduced, AP traffic along T cell neurites is facilitated, facilitating access to the synaptic terminals, and an enhancement of synaptic potential can be observed [21]. When AHP amplitude increases, a reduction in APs traveling along T cell neurites occurs, and a decrease of EPSP at the synaptic level of follower neurons is observed [21].

In the present study, we show that the N cell activation brings about a reduction of the AHP amplitude in T neurons. It has been observed that T and N neurons converge on a common target, the motor neuron that innervates the longitudinal muscles [23]. These data suggest that during sensitization, N cells are activated, and might modulate the motor response by affecting the T cells' electrical activity. Moreover, previous studies have demonstrated that 5-HT produces a reduction of AHP amplitude in T cells via the cAMP pathway, and an increase in sensitization [24]. Therefore, the sensitizing effect produced by 5-HT could be due to a direct action of 5-HT on T neurons, leading to a reduction of AHP and an enhancement of T cell activity. We postulate that in our sensitization or dishabituation paradigm, brush strokes activate the N cell that in turn impinges on T cell action through 5-HT-induced potentiation of the capability of T cells to stimulate the follower neurons, thus producing the sensitizing effect. Indeed, N neurons act through a serotonergic pathway. Administration of methysergide, a 5-HT blocking agent, inhibited the AHP decrease into T cells after N stimulation.

In the present study, we analyzed the effects of ALC in N cells. Our observations show that in the N cells of ganglia dissected from leeches previously treated with 2 mM ALC:

i) the membrane potential shifted closer to that of potassium, suggesting a decrease of sodium conductance at rest; *ii*) the input resistance was higher, implying that leak channels were closed or removed; *iii*) more current was necessary to activate N cell spikes despite having the same firing threshold. These effects produced a significant hyperpolarization of the resting potential and, consequently, an enhancement of the firing threshold, and they increased over time, as confirmed by our experiments with N cells of leeches treated with ALC at 6 days before the observations.

In the leech, we have previously observed that ALC was capable of blocking sensitization and impairing dishabituation induced by the administration of 5-HT and that these effects were long-lasting, being observable to up to 11 days after the treatment [10]. Therefore, our data suggest that the reduced sensitizing effect produced by brush strokes in our sensitization (or dishabituation) paradigm was brought on by the ALC-induced decrease in 5-HT levels in the leech ganglia due to the reduction of N cell activity stimulated by brush strokes, that lead to the impairment of the sensitization and dishabituation processes. Our hypothesis is supported by the circuit model proposed by Velázquez-Ulloa and colleagues [25], characterized by a convergence of mechanosensory inputs onto serotonergic neurons. In view of these considerations, N cell stimulation modulates serotonergic activity. Therefore, we can assume that ALC affects N cell activity, reducing the capacity of the animals to respond to the test stimulus and inducing an impairment of the sensitization and dishabituation processes [14].

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