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Acetyl-L-carnitine induces muscarinic antinociception in mice and rats

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Abstract

The analgesic activity of acetyl-L-carnitine (ALCAR) in neuropathic pain is well established. By contrast, its potential efficacy in the relief of acute pain has not been reported. The antinociceptive effect of ALCAR was, therefore, examined in the mouse hot-plate and abdominal constriction tests, and in the rat paw-pressure test. ALCAR (100 mg kg⁻¹ s.c. twice daily for seven days) produced an increase of the pain threshold in both mice and rats. ALCAR was also able to reverse hyperalgesia induced by kainic acid and NMDA administration in the mouse hot-plate test. The antinociception produced by ALCAR was prevented by the unselective muscarinic antagonist atropine, the M₁ selective antagonists pirenzepine and S(-)-ET126, and by the choline uptake inhibitor hemicholinium-3 (HC-3). By contrast the analgesic effect of ALCAR was not prevented by the opioid antagonist naloxone, the GABA_B antagonist CGP 35348, the monoamine synthesis inhibitor (α)-methyl-*p*-tyrosine, and the Gi-protein inactivator pertussis toxin. Moreover, ALCAR antinociception was abolished by pretreatment with an antisense oligonucleotide (aODN) against the M₁ receptor subtype, administered at the dose of 2 nmol per single i.c.v injection. On the basis of the above data, it can be postulated that ALCAR exerted an antinociceptive effect mediated by a central indirect cholinergic mechanism. In the antinociceptive dose-range, ALCAR did not impair mouse performance evaluated by the rota-rod and hole-board tests.

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Keywords: Acetyl-L-carnitine; Acetylcholine; M₁ muscarinic receptor subtype; Analgesia; Pain; Hyperalgesia

1. Introduction

Acetyl-L-carnitine (γ -trimethyl- β -acetylbutyrobetaine; ALCAR), the acetyl ester of carnitine, is a small water-soluble molecule naturally present in the central nervous system. ALCAR exerts a neuroprotective effect by increasing glutathione and by decreasing the concentration of malondialdehyde (Fariello et al., 1987), promoting nerve growth factor receptor synthesis and increasing its levels in the hippocampus (Tagliatela et al., 1991, 1992).

Several studies have indicated that ALCAR is involved in different aspects of neuronal activity. It has

been demonstrated to improve cognitive deficits in some experimental paradigms (Pettegrew et al., 1995; Dell'Anna et al., 1997), to modify the cerebral electrical activity (Mancia et al., 1990); and to induce an antidepressant-like effect (Pulvirenti et al., 1990). Furthermore, ALCAR modulates the release of various neurotransmitters such as dopamine (Harsing et al., 1992), excitatory amino acids, taurine (Toth et al., 1993), acetylcholine (Imperato et al., 1989), and GABA (Fariello et al., 1988).

ALCAR has also been demonstrated to be significantly effective in reducing neuropathic pain, a condition characterised by spontaneous pain, allodynia and hyperalgesia caused by traumatic injury, diabetes, and viral infections. Intramuscular chronic treatment with ALCAR significantly improves the outcome of painful neuropathies or radiculopathies (Onofrij et al., 1995). A beneficial effect of ALCAR has been reported in the treatment of symptomatic diabetic neuropathy (Quatraro

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et al., 1995) and in the treatment of pain in distal symmetrical polyneuropathy related to HIV infection (Scarpini et al., 1997). However, the mechanism underlying the analgesic effect of ALCAR is still obscure.

By contrast, its potential efficacy in the relief of acute pain has not been reported. On these bases, the aim of the present study was to first investigate the analgesic properties of ALCAR in animal models of acute pain by using different nociceptive stimuli, such as thermal, chemical and mechanical. We also investigated the receptor subtypes involved in the increase of pain threshold induced by ALCAR to elucidate its mechanism of action.

In order to exclude that the effects produced by ALCAR treatment were due to the induction of side effects, some additional behavioural tests (rota rod, hole board) were performed.

2. Methods

2.1. Animals

Maple Swiss albino mice (23–25 g) and Wistar rats (150–200 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Fifteen mice or five rats were housed per cage (26 × 41 cm). The cages were placed in the experimental room 24 h before the test for acclimatisation. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at $23 \pm 1^\circ\text{C}$ with a 12 h light/dark cycle, light on at 7 a.m. Each animal was used only once and was killed immediately after the end of the experiment by exposure to diethyl ether. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care. All efforts were made to minimise animal suffering, and to reduce the number of animals used.

2.2. Intracerebroventricular injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anaesthesia, according to the method described by Haley and McCormick (1957). Briefly, during anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl were then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5 μl of diluted 1:10 India ink and their brains examined macro-

scopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was 95.

2.3. Intrathecal injection technique

Intrathecal injections were performed under ether anaesthesia as described by Hylden and Wilcox (1980) in which the mouse was gently restrained and a 30-gauge, 1/2-inch needle mated to a 50 μl Hamilton syringe was inserted between L5 and L6 of the mouse spinal column. A volume of 5 μl was used for i.t. injection.

2.4. Hot-plate test

The method adopted was described by O'Callaghan and Holzman (1975). Mice were placed inside a stainless steel container, thermostatically set at $52.5 \pm 0.1^\circ\text{C}$ in a precision water-bath from KW Mechanical Workshop, Sienna, Italy. Reaction times (s), were measured with a stop-watch before and at regular intervals up to a maximum of 120 min after treatment. The endpoint used was the licking of the fore or hind paws. Before pretreating animals with ALCAR, a pretest was performed: those mice scoring below 12 and over 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

2.5. Abdominal constriction test

Mice were injected i.p. with a 0.6% solution of acetic acid (10 ml kg^{-1}), according to Koster et al. (1959). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

2.6. Paw pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. (1988). Threshold pressure was measured before and 15, 30, 45 and 60 min after treatment. Before pretreating animals with ALCAR a pretest was performed: rats scoring below 30 g or over 85 g were rejected (25%). An arbitrary cut-off value of 250 g was adopted.

2.7. Hole-board test

The hole board test consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4×4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to mid-point of opposite sides, thus dividing the plane into four equal quadrants, automatically signalled

the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

2.8. Rota-rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. (1985). Before pretreating animals with ALCAR a pretest was performed: mice scoring less than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30 and 45 min after s.c. treatment.

2.9. Antisense oligonucleotides

Low cell permeability and the high degradation of natural phosphodiester oligomers are considerable drawbacks in the application of aODNs both in vitro and in vivo. To overcome these drawbacks, phosphorothioate-capped phosphodiester oligonucleotides were used. The above-mentioned compounds are a class of ODN derivatives shown to maintain more stable and effective concentrations in the brain when compared with their unmodified counterpart (Whitesell et al., 1993). Phosphodiester oligonucleotides (ODNs) protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Genosys (Cambridge, England) and purified by high-performance liquid chromatography (HPLC). The 18-mer antisense ODN (aODN) 5'-CAC TGA GGT GTT CAT TGC-3' (phosphorothioate residues are underlined) complementary to the residues 112–129 of the published mouse M_1 cDNA sequence (Shapiro et al., 1988) and the 18-mer fully degenerated ODN (dODN) 5'-NNN NNN NNN NNN NNN NNN-3' (where N is G, or C, or A, or T and phosphorothioate residues are underlined) were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Boehringer-Mannheim, Germany) to enhance both uptake and stability. aODN or dODN (100–400 μ M) were preincubated at 37°C for 30 min with 13 μ M DOTAP, sterilised through a 0.2 μ m filter and supplied to mice by i.c.v. injection of a 5 μ l solution as described in the next section.

The accession number of the cDNA sequence for the

mouse muscarinic receptor subtype reported in this paper (M_1) is J04192.

2.10. Drugs

The following drugs were used: Acetyl-L-carnitine, Acetyl-D-carnitine, L-carnitine, D-carnitine (Sigma-Tau, Italy); hemicholinium-3 hydrobromide (HC-3), pirenzepine dihydrochloride, naloxone hydrochloride, pertussis toxin (RBI); atropine sulfate, α -methyl p-tyrosine methyl ester hydrochloride, kainic acid, N-methyl-D-aspartic acid (NMDA), DOTAP (Sigma); CGP 35348 (Ciba Geigy); S-(-)-ET 126 (S-(-)- α -(hydroxymethyl)benzene-acetic acid 1-methyl-4-piperidinyl ester) prepared in the Department of Pharmaceutical Sciences of Florence; D-amphetamine hydrochloride (De Angeli). All drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use, except for pertussis toxin which was dissolved in a water solution containing 0.01 M sodium phosphate buffer, pH = 7.0, with 0.05 M sodium chloride. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by s.c. or i.p. injection or 5 μ l per mouse by i.c.v. or i.t. injection.

2.11. Statistical analysis

All experimental results are given as the mean $\pm$ S.E.M. Analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) procedure for post-hoc comparison, was used to verify significance between two means. Data were analysed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

3. Results

3.1. Antinociceptive activity of ALCAR

ALCAR, as shown in Fig. 1, increased the pain threshold in the mouse hot-plate test after s.c. administration (100–200 mg kg^{-1}) twice daily for seven days (panel b). After 14 day-treatment, the licking latency values recorded in the ALCAR-treated group were similar to those measured after 7-day treatment (data not shown). The analgesic effect of ALCAR persisted up to seven days after the end of the 7-day treatment. The increase of the pain threshold induced by ALCAR disappeared 21 days after the end of the treatment (data not shown). Acute administration of ALCAR (100–200 mg kg^{-1} s.c.) was not able to produce any statistically significant increase of the pain threshold (Fig. 1, panel a). Similarly, the dose of 30 mg kg^{-1} s.c. was devoid of any effect after acute (Fig. 1, panel a) or subchronic (Fig. 1,

Fig. 1. Dose-response curves of ALCAR after acute (panel a) and subchronic treatment (twice daily for 7 days: panel b) in the hot-plate test. The doses are expressed as mg kg^{-1} s.c.. The licking latency values were recorded every 15 min up to 120 min from the beginning of the test. In panel a) ALCAR was injected at time 0; in panel b) the last injection of ALCAR was performed 24 h after the last injection. Vertical lines give s.e. mean. Each point is the mean of at least 12 mice. * $P < 0.01$ in comparison with the respective saline controls.

panel b) treatment. The administration of acetyl-D-carnitine, L-carnitine and D-carnitine, twice daily for seven days at the dose of 100 mg kg^{-1} s.c., did not produce any analgesic effect in the hot-plate (data not shown).

ALCAR was able to induce antinociception also in the mouse abdominal constriction test. After 14-day treat-

ment at the dose of 100 mg kg^{-1} s.c., ALCAR induced antinociception (Fig. 2), whereas, after 7-day treatment, the investigated compound produced a reduction of the number of abdominal constrictions that did not reach statistical significance (Fig. 2). A lower dose of ALCAR (30 mg kg^{-1} s.c.) was devoid of any effect regardless of the administration procedure employed (Fig. 2). Similarly to the results obtained in the mouse hot-plate test, ALCAR was ineffective after acute administration in the abdominal constriction test (Fig. 2). Pretreatment with acetyl-D-carnitine, L-carnitine and D-carnitine, twice daily for 14 days at the dose of 100 mg kg^{-1} s.c., did not produce any analgesic effect in comparison with ALCAR (Fig. 2).

In the paw pressure test ALCAR, administered i.p. at the dose of 100 mg kg^{-1} s.c. in the rat, induced antinociception after subchronic treatment (twice daily for seven days) (data not shown).

ALCAR also had antihyperalgesic activity. At 100 mg kg^{-1} s.c. it reversed hyperalgesia induced by administration of kainic acid (20 mg kg^{-1} i.p.) or NMDA ($1.64 \mu\text{g}$ per mouse i.t.) in the mouse hot-plate test after subchronic treatment (twice daily for seven days). After acute treatment ALCAR was devoid of any effect (Fig. 3). No significant antihyperalgesic effect was observed at 30 mg kg^{-1} s.c. (Fig. 3).

3.2. Antagonism of ALCAR antinociception

In the mouse hot-plate test, the antinociceptive effect of ALCAR (100 mg kg^{-1} s.c., twice daily for seven days) was antagonised by atropine (5 mg kg^{-1} i.p.), pirenzepine ($0.1 \mu\text{g}$ per mouse i.c.v.), S-(-)-ET126 ($0.1 \mu\text{g}$ per mouse i.c.v.), and hemicholinium-3 ($1 \mu\text{g}$ per mouse i.c.v.) (Fig. 4). By contrast, naloxone (1 mg kg^{-1} i.p.), CGP-35348 (100 mg kg^{-1} i.p.), α -methyl-*p*-tyrosine (200 mg kg^{-1} i.p.), and pertussis toxin ($0.25 \mu\text{g}$ per mouse i.c.v.) did not prevent ALCAR antinociception (Fig. 4).

All antagonists were injected 15 min before the test, with the exception of CGP 35348, α -methyl-*p*-tyrosine, hemicholinium-3 and pertussis toxin injected respectively 5 min, 1 h, 5 h and 11 days before the test.

The doses and administration schedules of the above-mentioned antagonists were selected for preventing antinociception induced by the corresponding agonists without modifying the increase of pain threshold induced by activation of other neurotransmission systems (Bartolini et al., 1987; Malcangio et al., 1991; Ghelardini et al., 1992; Ghelardini et al., 1996). Furthermore, previous results indicated that an 11-day latency was required to abolish analgesia mediated through activation of the Gi protein-mediated system.

Fig. 2. Effect of ALCAR, L-carnitine, D-carnitine and acetyl-D-carnitine in mouse abdominal constriction test. Each column represents the mean of at least 12 mice. Nociceptive response was recorded 30 min (acute treatment) and 24 h (subchronic treatment) after the last s.c. injection of ALCAR and structurally related compounds. * $P < 0.01$ in comparison with saline-treated mice.

Fig. 3. Effect of ALCAR on hyperalgesia induced by kainic acid (20 mg kg⁻¹ i.p.) and NMDA (1.64 µg per mouse i.t.) in mouse hot-plate test. Nociceptive responses were recorded 30 min (acute treatment) and 24 h (subchronic treatment) after the last ALCAR injection. Kainic acid and NMDA were administered respectively 48 h and 15 min before ALCAR administration. * $P < 0.01$ in comparison with kainic acid/NMDA-treated mice. Each column represents the mean of at least 8 mice.

3.3. Effect of anti- M_1 treatment on ALCAR antinociception

Mice were pretreated with a single i.c.v. injection of antisense ODN (aODN) to the M_1 gene, degenerate ODN (dODN), or vehicle, on days 1, 4 and 7. The effect of aODN pretreatment on ALCAR (100 mg kg⁻¹ s.c., twice daily for seven days) induced antinociception was then evaluated in the mouse hot-plate test. aODN, at the dose of 2 nmol per i.c.v. injection, prevented the ALCAR-induced increase of pain threshold (Fig. 4). This antagonistic effect was detected 24 h after the last i.c.v. injection.

Seven days after the end of the aODN treatment, ALCAR induced an antinociceptive effect of the same intensity in aODN-, dODN- and vehicle-treated mice,

Fig. 4. Effect of atropine, hemicholinium-3 (HC-3), pirenzepine, S-(-)-ET-126, aODN to M_1 gene, naloxone, CGP-35348, α -methyl-*p*-tyrosine (α -M-*p*-T) and pertussis toxin (PTX) on antinociception induced by ALCAR (100 mg kg⁻¹ s.c. twice daily $\times$ 7 days) in mouse hot-plate test. All antagonists were injected 15 min before the test, with the exception of CGP-35348, α -methyl-*p*-tyrosine, hemicholinium-3 and pertussis toxin injected respectively 5 min, 1 h, 5 h and 11 days before test. Each value represents the mean of at least 10 mice. Nociceptive responses were recorded 24 h after the last s.c. injection of ALCAR. Mice were i.c.v. injected with aODN at the dose of 2.0 nmol per single i.c.v. injection on days 1, 4 and 7 and the hot plate test was performed 24 h after the last i.c.v. injection of aODN. Vertical lines give S.E.M. * $P < 0.01$ in comparison with ALCAR treated-mice.

indicating the loss of antagonistic activity by the anti- M_1 aODN (data not shown). The aODN, when injected alone, did not alter the pain threshold in mice, showing a lack of any hyperalgesic effect. Pretreatment with dODN did not modify ALCAR-induced antinociception

in comparison with mice injected with vehicle (data not shown).

The dose and administration schedule for aODN was ideal for selectively preventing analgesia induced by direct and indirect muscarinic cholinomimetics (Ghelardini et al., 2000).

3.4. Effect of ALCAR on mouse behaviour

The spontaneous motility and exploratory behaviour of mice were not modified by treatment with ALCAR (100 mg kg⁻¹ s.c. twice daily for 14 days) as revealed by the hole-board test (Table 1). In the same experimental conditions, D-amphetamine (2 mg kg⁻¹ s.c.), used as the reference drug, increased both parameters evaluated.

The motor coordination of mice treated with ALCAR was evaluated by using the rota-rod test (Table 1). The rota-rod performance of mice treated with ALCAR at the dose of 100 mg kg⁻¹ s.c. twice daily for seven (data not shown) or 14 days was not impaired in comparison with controls (Table 1). The number of falls by control animals progressively decreased at every measurement since the mice learnt how to balance on the rotating rod.

4. Discussion

ALCAR was able to induce antinociception in mice and rats. The increase of the pain threshold was elicited regardless of which noxious stimulus was used: thermal (hot-plate test), chemical (abdominal constriction test) and mechanical (paw pressure test). ALCAR was also able to reverse hyperalgesia induced by kainic acid and NMDA administration, in the mouse hot-plate test. ALCAR antinociception was obtained without producing

any visible modification of animal gross behaviour. Moreover, ALCAR treated mice showed a complete integrity of motor coordination on the rota-rod test, normal spontaneous motility, as well as exploratory behaviour, as revealed by the hole-board test.

The antinociceptive effect of ALCAR did not reach statistical significance after acute administration. After 7-day treatment (twice daily) there was an increase of the pain threshold. The only exception was the abdominal constriction test, in which the ALCAR analgesia was significant only after 14-day treatment. The hot-plate, paw pressure and abdominal constriction tests involve three different nociceptive stimuli. The detection of the analgesic effect of ALCAR after long term treatment is in agreement with results obtained in clinical studies in which ALCAR was used to relieve neuropathic pain (Onofri et al., 1995; Quatraro et al., 1995; Scarpini et al., 1997). The enhancement of the pain threshold induced by ALCAR was still present seven days after the end of the subchronic treatment. This long-term analgesic effect, together with the complete absence of induction of any side effect after 14-day treatment, confers to ALCAR an advantageous pharmacological profile.

The analgesia induced by ALCAR is stereospecific since acetyl-D-carnitine is devoid of any antinociceptive activity. Moreover, related compounds such L-carnitine and D-carnitine did not induce any increase of response threshold.

ALCAR antinociception was found to be dependent on central cholinergic activation since it was prevented by the non-selective muscarinic antagonist atropine, the selective M₁-antagonists pirenzepine and S-(-)-ET-126, the ACh depletor hemicholinium-3 and an aODN to the M₁ receptor subtype. The aODN treatment induces a

Table 1

Lack of effect of ALCAR (100 mg kg⁻¹ s.c. twice daily for 14 days) in mouse hole-board (panel A) in comparison with D-amphetamine (2 mg kg⁻¹ s.c.), and in Rota-rod test (panel B)^a

Panel A		Locomotory activity		Explorative activity	
Treatment	Number of mice	Number of movements		Number of explorations	
Saline	7	59.4±7.7		21.3±5.6	
ALCAR	10	52.3±6.5		24.0±5.1	
D-Amphetamine	10	82.3±4.8*		34.1±3.9*	
Panel B		Rota-rod—Falls in 30 s			
Treatment	Number of mice	Before treament		After treatment	
			15 min	30 min	45 min
Saline	5	4.2±0.4	3.1±0.4	2.2±0.2	1.6±0.3
ALCAR	5	3.9±0.5	2.9±0.3	1.7±0.4	1.0±0.3

^a Rota rod and hole-board test was performed 30 min after the last ALCAR administration. Amphetamine was injected 15 min before test.

**P*<0.01 in comparison with saline controls.

transient prevention of muscarinic antinociception since the inhibition of the ALCAR effect disappeared seven days after the last i.c.v. injection of the aODN. This return to normal sensitivity to analgesic treatments implies both the total reversal of aODN-induced specific inhibition of M_1 gene expression and a lack of damage or toxicity associated with aODN treatment.

Cholinergic antinociception in mice and rats is mediated by M_1 receptor stimulation (Bartolini et al., 1992; Iwamoto and Marion, 1993). Furthermore, cholinergic antinociception induced both directly, through muscarinic agonists, and indirectly, by enhancing ACh extracellular levels through cholinesterase inhibitors, is prevented by i.c.v. administration of an antisense to the M_1 gene coding for the mouse M_1 receptor. The efficacy and selectivity of the aODN employed towards M_1 receptor was previously demonstrated by us (Ghelardini et al., 2000).

The activation of the muscarinic system by ALCAR in the induction of its pharmacological effects has been reported. Cortical application of ALCAR exerted an excitatory action on cells excited by acetylcholine and changed the components of the visual evoked potentials in rats by a mechanism that was prevented by atropine (Onofri et al., 1983). The microiontophoretic application of ALCAR increased the firing rate of single cholinceptive neurons of the somatosensory cortex in rats in an atropine-sensitive manner (Janiri et al., 1991). ALCAR has also been reported to produce atropine-sensitive muscarinic effects induced by application of ACh such as increase of salivation, reduction of pupil diameter and blood pressure (Blum et al., 1971).

Taking into account that HC-3, pirenzepine and S(-)-ET126 were able to antagonise ALCAR antinociception after i.c.v. injection, this indicates that the analgesic site of action of ALCAR is localised in the CNS.

A presynaptic mechanism facilitating cholinergic transmission is involved in ALCAR antinociception as revealed by the antagonism by HC-3. A postsynaptic mechanism of action can be ruled out since, as reported by Bartolini et al. (1987, 1992), HC-3 was not able to antagonise antinociception induced by agonists of postsynaptic muscarinic receptors such as oxotremorine, McN-A-343 and AF-102B.

The hypothesis of a presynaptic cholinergic mechanism for ALCAR is in agreement with previous results demonstrating, by microdialysis studies, an increase in ACh release from rat striatum and hippocampus induced by ALCAR administration (Imperato et al., 1989). This effect occurred in the same range of doses (50–200 mg kg⁻¹ s.c.) in which the above-mentioned compound exerted its antinociceptive activity. The increase of extracellular ACh levels by ALCAR can arise from the capability of this compound to be a precursor of ACh. The synthesis of [¹⁴C]ACh from [¹⁴C]ALCAR was observed in rat brain caudate nuclei slices (Dolezal and

Tucek, 1981) and in synaptosomal membrane preparation from rat brain (White and Scates, 1990).

Other neurotransmission systems able to modulate pain threshold are not involved in ALCAR antinociception since the opioid antagonist naloxone, the GABA_B antagonist CGP-35348 and the monoamine synthesis inhibitor (α)-methyl-*p*-tyrosine, were all unable to prevent the effect of ALCAR. The doses and administration schedules of the above-mentioned drugs were appropriate for preventing antinociception induced respectively by morphine (Ghelardini et al., 1992), the GABA_B agonist baclofen (Malcangio et al., 1991) and amphetamine (Bartolini et al., 1987). Furthermore, pretreatment with PTX did not prevent the analgesia induced by ALCAR. It has been reported that PTX is able to antagonise the analgesia induced by the most common analgesic drugs such as morphine (Parenti et al., 1986), α_2 -adrenoceptor agonists (Sanchez-Blazquez and Garzon, 1991), GABA_B agonists (Hoehn et al., 1988), tricyclic antidepressants (Galeotti et al., 1996), with the exception of that induced by cholinomimetic drugs (Galeotti et al., 1996). These data further support the hypothesis not only of the lack of involvement of non-cholinergic neurotransmitter systems, but also of the involvement of M_1 receptors, which are not coupled to Gi proteins, in the mechanism of analgesic action of ALCAR.

Finally, we can exclude that the antinociceptive effect of ALCAR is a consequence of an irreversible change in afferent function since the increase of the pain threshold induced by the investigated compound disappeared three weeks after the end of the treatment. Moreover, the observed reversion of the analgesia after treatment with antimuscarinic compounds further indicates the lack of induction of irreversible neuronal changes.

In summary, our results have shown that ALCAR is able to produce antinociception in rodents after repeated administration, without impairing motor coordination, by potentiating endogenous cholinergic activity.

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References

- Bartolini, A., Galli, A., Ghelardini, C., Giotti, A., Malcangio, M., Malmberg-Aiello, P., Zucchi, P.L., 1987. Antinociception induced by systemic administration of local anaesthetics depends on a cholinergic mechanism. *British Journal of Pharmacology* 105 (92), 711–721.
- Bartolini, A., Ghelardini, C., Fantetti, L., Malcangio, M., Malmberg-Aiello, P., Giotti, A., 1992. Role of muscarinic receptor subtypes in central antinociception. *British Journal of Pharmacology* 105, 77–82.

- Blum, K., Seifter, E., Seifter, J., 1971. The pharmacology of *D*- and *L*-carnitine and *D*- and *L*-acetylcarnitine: comparison with choline and acetylcholine. *Journal of Pharmacology and Experimental Therapeutics* 178, 331–338.
- Dell'Anna, E., Iuvone, L., Calzolari, S., Geloso, M.C., 1997. Effect of acetyl-L-carnitine on hyperactivity and spatial memory deficits of rats exposed to neonatal anoxia. *Neuroscience Letters* 223, 201–205.
- Dolezal, V., Tucek, S., 1981. Utilization of citrate acetylcarnitine, acetate, piruvate and glucose for the synthesis of acetylcholine in rat brain slices. *Journal of Neurochemistry* 36, 1323–1330.
- Fariello, R.G., Ferraro, T.N., Golden, G.T., DeMattei, M., 1988. Systemic acetyl-L-carnitine elevates nigral levels of glutathione and GABA. *Life Sciences* 43, 289–292.
- Fariello, R.G., Ghirardi, O., Paschera, A., Ramacci, M.T., Angelucci, L., 1987. Transient nigral ubiquinone depletion after single MPTP administration in mice. *Neuropharmacology* 26, 1799–1802.
- Galeotti, N., Ghelardini, C., Bartolini, A., 1996. Effect of pertussis toxin on morphine, diphenhydramine, baclofen, clomipramine and physostigmine antinociception. *European Journal of Pharmacology* 308, 125–133.
- Ghelardini, C., Bartolini, A., Galeotti, N., Teodori, E., Gualtieri, F., 1996. S-(+)-ET126: a potent and selective M₁ antagonist in vitro and in vivo. *Life Sciences* 58, 991–1000.
- Ghelardini, C., Galeotti, N., Bartolini, A., 2000. Loss of muscarinic antinociception by antisense inhibition of M₁ receptors. *British Journal of Pharmacology* 129, 1633–1640.
- Ghelardini, C., Giotti, A., Gualtieri, F., Matucci, R., Romanelli, M.N., Scapechi, S., Teodori, E., Bartolini, A., 1992. Presynaptic auto- and hetero-receptors in the cholinergic regulation of pain. in: Angeli, P., Gulini, U., Quaglia, W. (Eds.), *Trends in Receptor Research*. Elsevier Science Publisher B.V., Amsterdam, NL, pp. 95–114.
- Haley, T.J., McCormick, W.G., 1957. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *British Journal of Pharmacology and Chemotherapy* 12, 12–15.
- Harsing Jr, L.G., Sershen, H., Toth, E., Hashim, A., Ramacci, M.T., Lajtha, A., 1992. Acetyl-L-carnitine releases dopamine in rat corpus striatum: an in vivo microdialysis study. *European Journal of Pharmacology* 218, 117–121.
- Hoehn, K., Reid, A., Sawynok, J., 1988. Pertussis toxin inhibits antinociception produced by intrathecal injection of morphine, noradrenaline and baclofen. *European Journal of Pharmacology* 146, 65–72.
- Hylden, J.L.K., Wilcox, G., 1980. Intrathecal morphine in mice: a new technique. *European Journal of Pharmacology* 67, 313–316.
- Imperato, A., Ramacci, M.T., Angelucci, L., 1989. Acetyl-L-carnitine enhances acetylcholine release in the striatum and hippocampus of awake freely moving rats. *Neuroscience Letters* 107, 251–255.
- Iwamoto, E.T., Marion, L., 1993. Characterization of the antinociception produced by intrathecally administered muscarinic agonists in rats. *Journal of Pharmacology and Experimental Therapeutics* 266, 329–338.
- Janiri, J., Falcone, M., Persico, A., Tempesta, E., 1991. Activity of L-carnitine and L-acetylcarnitine on cholinceptive neocortical neurons of the rat in vivo. *Journal of Neural Transmission. General Section* 86, 135–146.
- Koster, R., Anderson, M., De Beer, E.J., 1959. Acetic acid for analgesic screening. *Federation Proceedings* 18, 412.
- Leighton, G.E., Rodriguez, R.E., Hill, R.G., Hughes, J., 1988. κ -opioid agonists produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. *British Journal of Pharmacology* 93, 553–560.
- Malcangio, M., Ghelardini, C., Giotti, A., Malmberg-Aiello, P., Bartolini, A., 1991. CGP 35348, a new GABA_B antagonist, prevents antinociception and muscle-relaxant effect induced by baclofen. *British Journal of Pharmacology* 103, 1303–1308.
- Mancia, M., Imeri, L., Giglio, R., 1990. Modifications of the cerebral electric activity induced by the intracerebroventricular injection of acetyl-L-carnitine in cats. *International Journal of Clinical Pharmacology Research* 10, 115–121.
- O'Callaghan, J.P., Holtzman, S.G., 1975. Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. *Journal of Pharmacology and Experimental Therapeutics* 192, 497–505.
- Onofri, M., Bodis-Wollner, I., Pola, P., Calvani, M., 1983. Central cholinergic effects of levo-acetylcarnitine. *Drugs Experimental Clinical Research* 9, 161–169.
- Onofri, M., Fulgente, T., Melchionda, D., Marchionni, A., Tomasello, F., Salpietro, F.M., Alafaci, C., De Sanctis, E., Pennisi, G., Bella, R., Ventura, F., Morganti, A., 1995. L-acetyl-carnitine as a new therapeutic approach for peripheral neuropathies with pain. *International Journal of Clinical Pharmacology Research* 15, 9–15.
- Parenti, M., Tirone, F., Giagnoni, G., Pecora, N., Parolaro, D., 1986. Pertussis toxin inhibits the antinociceptive action of morphine in the rat. *European Journal of Pharmacology* 124, 357–359.
- Pettegrew, J.W., Klunk, W.E., Panchalingam, K., Kanfer, J.N., McClure, R.J., 1995. Clinical and neurochemical effects of acetyl-L-carnitine in Alzheimer's disease. *Neurobiology of Aging* 16, 1–4.
- Pulvirenti, G., Valerio, C., Spadaro, F., D'Agata, V., Freni, V., Nardo, L., Drago, F., 1990. Acetylcarnitine reduces the immobility of rats in a despair test (constrained swim). *Behavioral and Neural Biology* 54, 110–114.
- Quatraro, A., Roca, P., Donzella, C., Acampora, R., Marfella, R., Giugliano, D., 1995. Acetyl-L-carnitine for symptomatic neuropathy. *Diabetologia* 38 (123).
- Sanchez-Blazquez, P., Garzon, J., 1991. Cholera toxin and pertussis toxin on opioid- and α_2 -mediated supraspinal analgesia in mice. *Life Sciences* 48, 1721–1727.
- Scarpini, E., Sacilotto, G., Baron, P., Cusini, M., Scarlato, G., 1997. Effect of acetyl-L-carnitine in the treatment of painful peripheral neuropathies in HIV+patients. *Journal of the Peripheral Nervous System* 2, 250–252.
- Shapiro, R.A., Scherer, N.M., Habecker, B.A., Subers, E.M., Nathanson, N.M., 1988. Isolation, sequence, and functional expression of the mouse M₁ muscarinic acetylcholine receptor gene. *The Journal of Biological Chemistry* 263, 18397–18403.
- Tagliatela, G., Angelucci, L., Ramacci, M.T., Werrbach-Perez, K., Jackson, G.R., Perez-Polo, J.R., 1991. Acetyl-L-carnitine enhances the response of PC12 cells to nerve growth factor. *Brain Research, Developmental Brain Research* 59, 221–230.
- Tagliatela, G., Navarra, D., Cruciana, R., Ramacci, M.T., Angelucci, L., 1992. Treatment with acetyl-L-carnitine (ALCAR) elevates NGF levels in the CNS of aged rats. *Society for Neurosciences* 18, 776.
- Toth, E., Harsing Jr, L.G., Sershen, H., Ramacci, M.T., Lajtha, A., 1993. Effect of acetyl-L-carnitine on extracellular amino acid levels in vivo in rat brain regions. *Neurochemical Research* 18, 573–578.
- Vaught, J., Pelley, K., Costa, L.G., Sether, P., Enna, S.J., 1985. A comparison of the antinociceptive responses to GABA-receptor agonists THIP and baclofen. *Neuropharmacology* 24, 211–216.
- White, H.L., Scates, P.W., 1990. Acetyl-L-carnitine as precursor of acetylcholine. *Neurochemical Research* 15, 597–601.
- Whitesell, L., Geselowitz, D., Chavany, C., Fahmy, B., Walbridge, S., Alger, J., Neckers, L.M., 1993. Stability, clearance, and disposition of intraventricularly administered oligodeoxynucleotides: Implications for therapeutic application within the central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* 90, 4665–4669.