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L-Acetylcarnitine Induces Analgesia by Selectively Up-Regulating mGlu2 Metabotropic Glutamate Receptors

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ABSTRACT

L-Acetylcarnitine (LAC, 100 mg/kg, s.c.), a drug commonly used for the treatment of painful neuropathies, substantially reduced mechanical allodynia in rats subjected to monolateral chronic constriction injury (CCI) of the sciatic nerve and also attenuated acute thermal pain in intact rats. In both cases, induction of analgesia required repeated injections of LAC, suggesting that the drug induces plastic changes within the nociceptive pathway. In both CCI- and sham-operated rats, a 24-day treatment with LAC increased the expression of metabotropic glutamate (mGlu) receptors 2 and 3 in the lumbar segment of the spinal cord, without changing the expression of mGlu1a or -5 receptors. A similar up-regulation of mGlu2/3 receptors was detected in the dorsal horns and dorsal root ganglia of intact rats treated with LAC for 5–7 days, a time sufficient for the induction of thermal analgesia. Immunohistochemical analysis showed that LAC treatment enhanced mGlu2/3 immunoreactivity in the inner part of lamina II and in laminae III and IV of the spinal cord. An increased mGlu2/3 receptor expression was also observed in the cerebral cortex but not in the hippocampus or cerebellum of LAC-treated animals. Reverse transcription-polymerase chain reaction combined with Northern blot analysis showed that repeated LAC injections selectively induced mGlu2 mRNA in the dorsal horns and cerebral cortex (but not in the hippocampus). mGlu3 mRNA levels did not change in any brain region of LAC-treated animals. To examine whether the selective up-regulation of mGlu2 receptors had any role in LAC-induced analgesia, we have used the novel compound LY 341495, which is a potent and systemically active mGlu2/3 receptor antagonist. LAC-induced analgesia was largely reduced 45 to 75 min after a single injection of LY 341495 (1 mg/kg, i.p.) in both CCI rats tested for mechanical allodynia and intact rats tested for thermal pain. We conclude that LAC produces analgesia against chronic pain produced not only by peripheral nerve injury but also by acute pain in intact animals and that LAC-induced analgesia is associated with and causally related to a selective up-regulation of mGlu2 receptors. This offers the first example of a selective induction of mGlu2 receptors and discloses a novel mechanism for drug-induced analgesia.

Neuropathic pain is characterized by spontaneous pain, allodynia, hyperalgesia, and pain summation and is a hallmark of neuropathies caused by traumatic injury, diabetes, and viral infections. An enhanced sensitivity of dorsal horn neurons to sensory stimulation (a phenomenon referred as "central sensitization") is commonly recognized as one of the mechanisms underlying chronic pain. In animal models of neuropathic pain, a sustained release of glutamate and peptides induces long-term potentiation at the synapses between primary afferent fibers and second-order neurons in the dorsal horns (reviewed by Sandkuhler, 2000). Induction of long-term potentiation requires the simultaneous activation of neurokinin receptors, NMDA receptors, and metabotropic glutamate (mGlu) receptors (Randic et al., 1993; reviewed by Sandkuhler, 2000). Particular attention is currently focused on mGlu receptors because these receptors modulate rather than mediate excitatory synaptic transmission and are therefore ideal targets for "safe" drugs of potential use in the treatment of chronic pain. mGlu receptors form a family of eight subtypes (named mGlu1 to mGlu8) subdivided into three groups on the basis of sequence homology, pharmacological profile and transduction pathways. Group I mGlu receptors (mGlu1 and -5) are coupled to polyphosphoinositide hydrolysis, whereas members of group II (mGlu2 and -3) or group III (mGlu4, -6, -7, and -8) receptors are coupled to Gi proteins in heterologous expression systems (reviewed by De Blasi et al., 2001). In the dorsal horns, mGlu1 and mGlu5 receptors are localized in laminae I and II, whereas mGlu2/3 receptors are mainly found in the inner part of lamina II (Valerio et al., 1997; Berthele et al., 1999; Jia et al., 1999; Azkue et al., 2000; Tao et al., 2000). mGlu4 and -7 receptors are also present in the superficial laminae of dorsal horns (Li et al., 1997; Azkue et al., 2001). The use of neutralizing antibodies, antisense oligonucleotides, and novel subtype-

ABBREVIATIONS: mGlu, metabotropic glutamate; LAC, L-acetylcarnitine; LY 341495, (αS)-α-Amino-α-[(1S,2S)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid; CCI, chronic constriction injury; TBS, Tris-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction.

selective antagonists has shown that endogenous activation of mGlu1 or -5 receptors reduces pain threshold and is required for the development of chronic pain (Moroni et al., 1997; Young et al., 1998; Fundytus et al., 1998; 2001; Neugebauer et al., 1999; Bhave et al., 2001; Karim et al., 2001; Walker et al., 2001a,b). Pharmacological activation of group II or III mGlu receptors depresses excitatory synaptic responses in dorsal horn neurons (Gerber et al., 2000). However, how endogenous activation of these receptors regulates nociceptive transmission and contributes to responses to analgesic drugs is unknown. We now report the serendipitous finding that L-acetylcarnitine (LAC), a drug commonly used for the treatment of painful neuropathies, induces analgesia by selectively up-regulating the expression of the mGlu2 receptor subtype. This offers an unusual example of selectivity in the induction of a particular mGlu receptor subtype and provides an entirely novel mechanism for the induction of analgesia.

Experimental Procedures

Materials

LAC was synthesized and provided by Sigma Tau Laboratories (Pomezia, Italy). (*αS*)-*α*-Amino-*α*-[(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xantine-9-propanoic acid (LY 341495) was purchased from Tocris Cookson (Avonmouth, Bristol, UK). All drugs were dissolved in saline and administered in a volume of 1 ml/kg.

Induction of Chronic Constriction Injury of the Sciatic Nerve

Sciatic constriction injury was induced in male Sprague-Dawley rats (300–350 g) under pentobarbital anesthesia (50 mg/kg, i.p.), as described by Bennett and Xie (1988). The left common sciatic nerve was isolated from surrounding tissues and four loose ligatures (4.0 chromie gut) were placed around the nerve with about 1-mm spacing, proximally to the nerve's trifurcation. In sham-operated rats, the sciatic nerve was only exposed and freed of adhering tissues, but no ligatures were placed on it.

Behavioral Studies

Mechanical Allodynia in Rats Undergoing CCI of the Sciatic Nerve. LAC (100 mg/kg, s.c.) or saline were injected twice a day in CCI- and sham-operated animals starting from the same day of surgery. The treatment was continued for 24 days. For the assessment of mechanical sensitivity, rats were placed in individual cages with elevated mesh floor 1 h before all behavioral testing. Mechanical allodynia was determined with a set of calibrated von Frey filaments used to stimulate the dorsal side of both right and left paws. Animals were tested for hind paw withdrawal both before and every week after CCI. The minimum value for the initial response of the right (nonligated) paw was subtracted from the score of the left (ligated) paw. Five applications of the von Frey filaments were repeated at 3-min intervals on each hind paw.

Thermosensitivity in Intact Animals. LAC (100 mg/kg, s.c.) or saline were also injected twice a day for 7 days in intact rats. Changes in thermal sensitivity were evaluated using a plantar test apparatus (Ugo Basile, Comerio, Italy). The latency of nociceptive response was measured every day starting from the first day of treatment. No tolerance to the test developed in rats treated with saline. Two independent groups of animals treated with saline or LAC received a single i.p. injection with the mGlu2/3 receptor antagonist LY 341495 (1 mg/kg). The two remaining groups received a single injection of saline. The latency of nociceptive response was measured before injection and at 5, 15, 30, 45, 60, and 75 min after the single injection of LY 341495 or saline. Plantar test results are expressed as percentage of maximum possible effect according to the following formula: $(PT_{post\ treatment} - PT_{basal}) / (\text{cut-off time} - PT_{basal}) \times 100$. A cut-off time of 20 s was set toa void tissue damage.

Western Blot Analysis

Tissues (lumbar segments of the spinal cord, dorsal horns, dorsal root ganglia, cerebral cortices, hippocampi, and cerebella) were homogenized on ice in 20 mM Tris-HCl, pH 7.4, containing 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 1% aprotinin. Homogenates were centrifuged at 3,000*g* for 20 min, and the supernatant was centrifuged at 10,000*g* to obtained the P2 fraction. After washing by centrifugation, the resulting pellets were resuspended in SDS-bromphenol blue buffer containing 40 mM dithiothreitol to limit the formation of receptor aggregates. Proteins (30 μg) were electrophoresed on 8% SDS-polyacrylamide gel electrophoresis and electroblotted on nitrocellulose paper (Bio-Rad, Hercules, CA). Filters were blocked overnight at 4°C with Tris-buffered saline/Tween 20 (100 mM Tris-HCl, pH 7.4, 0.9% NaCl, and 1% Tween 20) containing 3% nonfat dry milk, and then incubated for 1 h at room temperature with 1 μg/ml of anti-mGlu1a, -2/3, or -5 receptor antibodies (Upstate Biotechnology, Lake Placid, NY). Filters were then incubated for 1 h at room temperature with an anti-rabbit peroxidase-coupled secondary antibody diluted 1:10,000 in Tris-buffered saline/Tween 20. Immunostaining was revealed by enhanced chemiluminescence (Amersham Biosciences, Milan, Italy). Changes in mGlu receptor expression were expressed as ratio of densitometric measurements of the receptor bands and the relative β-actin bands.

Immunohistochemistry

Intact rats treated for 7 days with saline or LAC were used for immunohistochemistry. Animals were perfused intracardially with 400 ml of 4% paraformaldehyde in PBS, pH 7.4; the lumbar segment of the spinal cord was removed and cut into 50-μm transverse sections on a vibrotome. Sections were washed in Tris-buffered saline (TBS; 100 mM Tris, 0.9% NaCl), and then incubated in TBS containing 2% of normal goat serum for 30 min. Sections were then incubated overnight at 4°C with mGlu2/3 antibodies (1:100) and, after three washes in TBS, were incubated for 1.5 h with biotin-conjugated goat anti-rabbit secondary antibodies (1:200; Vector Laboratories, Burlingame, CA). Immunostaining was revealed by the avidin-biotin-peroxidase method.

Sections from control and LAC-treated animals were processed in parallel and the time of 3,3′-diaminobenzidine/H_2O_2 development was identical for each section. mGlu2/3 expression in dorsal horn was quantified by computer-assisted densitometry, using the MCID system (Imaging Research, St Catharine's, Ontario, Canada). Images were visualized under the same light conditions on a video monitor connected to the microscope through a video camera. The integrated optical density was obtained by software conversion of absolute gray values in arbitrary optical density units. This computation was done after obtaining a linear calibration curve generated by the system and attributing the arbitrary value of 0 to the lightest gray value and 3 to the highest value. These values were averaged from several readings in

different sections. Values obtained in the posterior white matter did not vary in different sections. Background values (ranging from 0.5 to 0.8 arbitrary units) were considered those obtained in the posterior white matter and were always subtracted.

RNA Extraction and Northern Blotting

Total RNA was prepared from freshly isolated tissue according to Auffray and Rougeon (1980), with modifications. Briefly, tissue was homogenized in a solution of 3 M LiCl, 6 M urea (5-ml volume per gram of tissue) for 2 min on ice, using a Polytron homogenizer. After an overnight incubation at 4°C, the homogenate was centrifuged at 14,000*g* for 15 min at 4°C. The pellet was then resuspended in half of the original volume of 3 M LiCl, 6 M urea, and centrifuged as before. The pellet was then resuspended in 5 ml of 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 5% SDS per gram of original tissue and immediately extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was then extracted with an equal volume of chloroform, and subjected to ethanol precipitation to recover the RNA. The pellet was air-dried, and resuspended in sterile, distilled water containing 20 units of RNasin (Promega, Madison, WI) and 5 units of RNase-free DNaseI (Roche Applied Science, Mannheim, Germany). After a 15-min incubation at 37°C, the sample was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform, and finally precipitated in ethanol. The pellet was then resuspended in sterile, distilled water containing 20 units of RNasin (Promega), quantified by spectrophotometry and analyzed for integrity by agarose gel electrophoresis. Northern blotting was carried out as described previously (Caricasole and Ward, 1993), using 20 μg RNA/sample. A specific full-length cDNA probe (Tanabe et al., 1992; kindly provided by Prof. S. Nakanishi, Kyoto, Japan) was used for the detection of mGlu mRNA. To detect mGlu3 mRNA, we used the following antisense oligoprobe: CAGGATGACTGTTTCCCGCTTCTCTGGAAGGGTGTATCTTCTAGT. Hybridization signals were analyzed and quantified using a filmless autoradiographic imaging (Packard Instruments, Meriden, CT).

PCR Analysis

cDNA synthesis and RT-PCR was carried out as described in Caricasole et al. (2000). PCR analysis was performed in a final volume of 30 μl, using an estimated 0.15 ng of cDNA and primer oligonucleotides specific for rat mGlu2 receptors (forward, 5′-CTACAGTGATGTCTCCATCC-3′; reverse, AAAGCCTCAATGCCTGTCTC), mGlu3 receptors (forward, 5′-CAAGTGACTACAGAGTGCAG-3′; reverse, 5′-CTGTCACCAATGCTCAGCTC-3′) or β-actin (Roelen et al., 1994). Reaction conditions included an initial denaturation step (94°C/3 min) followed by 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension step (72°C/10 min) concluded the reaction. PCR products (one third of the reaction) were analyzed electrophoretically on 2% agarose gels.

Results

Analgesic Activity of LAC

Thermosensitivity in Intact Rats. Repeated injections of LAC (100 mg/kg, s.c., twice daily for 7 days) induced thermal analgesia in intact rats, as reflected by an increased latency in the plantar test. Analgesia was substantial after 5 days of treatment with LAC, whereas shorter treatments were ineffective (Fig. 1A). Analgesia induced by a 7-day treatment with LAC was substantially reduced 45 to 75 min after a single injection with the mGlu2/3 receptor antagonist LY 341495 (1 mg/kg, i.p.). LY 341495 did not affect the pain threshold in rats that underwent long-term treatment with saline (Fig. 1B).

Mechanical Allodynia in Rats Subjected to Chronic Constriction Injury (CCI) of the Sciatic Nerve. In rats subjected to unilateral CCI of the sciatic nerve, mechanical allodynia developed between 3 and 10 days after surgery and

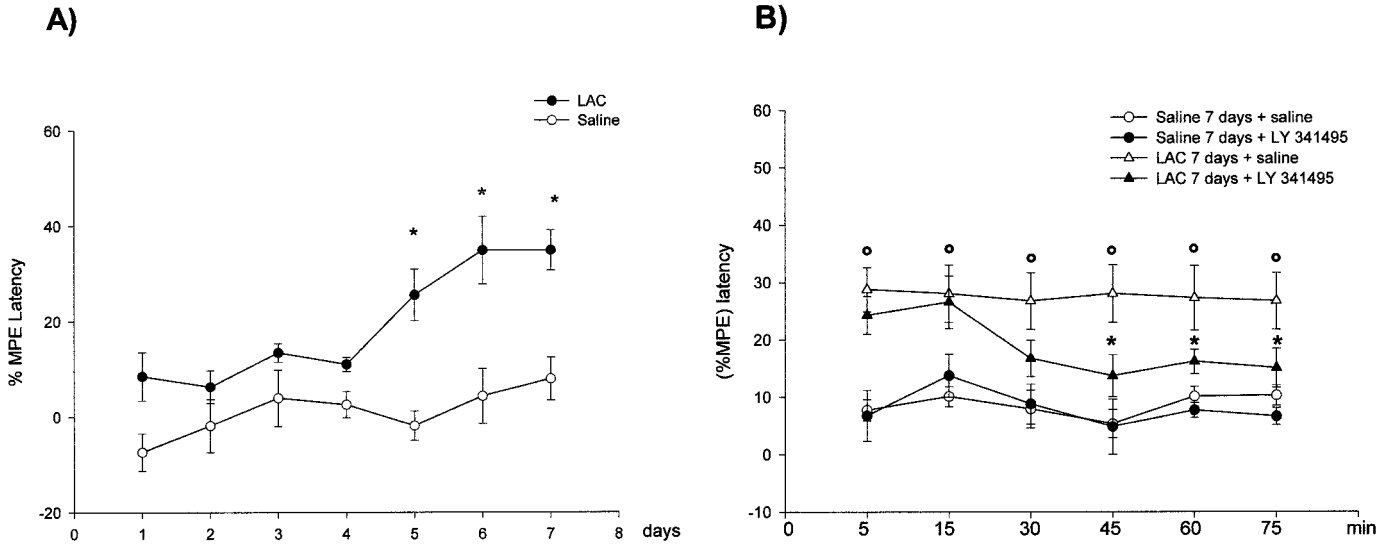

Fig. 1. A, repeated injections of LAC (100 mg/kg, s.c., twice daily) induces analgesia in intact rats subjected to an acute thermal stimulation in the plantar test. Note that a treatment with LAC of at least 5 days is required for the induction of analgesia. Data are means ± S.E.M. from six animals and are expressed as percentage of maximum possible effect (%MPE) (see *Experimental Procedures*). *, $p < 0.05$ (Student's t test) versus the corresponding value obtained in rats treated with saline. B, a single injection of LY 341495 (1 mg/kg, i.p.) reduces LAC-induced analgesia in a time-dependent manner. °, $p < 0.05$ versus the corresponding values of animals treated with saline for 7 days. *, $p < 0.05$ versus the corresponding values of animals treated with LAC for 7 days + saline (one-way analysis of variance + Fisher's protected least significant difference).

remained unchanged for the subsequent 2 weeks. Two groups of rats were injected once daily with saline or LAC (100 mg/kg, s.c.) starting 24 h after surgery and tested for mechanical allodynia 23 days later. LAC treatment completely abolished allodynia in CCI rats. LAC was ineffective when injected only once 30 to 60 min before the behavioral test. The analgesic effect of a 23-day treatment with LAC was prevented by a single injection of LY 341495 (1 mg/kg, i.p.). LY 341495 did not affect allodynia in CCI rats that had not been treated with LAC (Fig. 2).

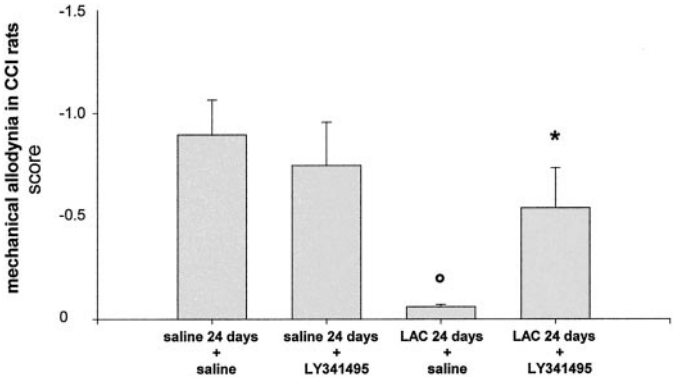

Fig. 2. The analgesic activity of a 24-day treatment with LAC (100 mg/kg, s.c., once daily) against mechanical allodynia in CCI rats is prevented by a single injection of LY 341495 (1 mg/kg, i.p.). Animals were tested for mechanical allodynia 45 min after the acute injection of LY 341495 or saline. The minimum value of the initial response for the right (nonligated) paw was subtracted from the score of the left (ligated) paw. Values are means ± S.E.M. of 10 to 14 determinations. °, $p < 0.05$ versus all other values. ∗, $p < 0.05$ versus LAC, 24 days + acute saline (one-way analysis of variance + Fisher's protected least significant difference).

Expression of mGlu Receptor Subtypes in Rats Treated with LAC

Initially, we have examined the expression of mGlu1, -2/3, and -5 receptor proteins in the lumbar segment of the spinal cord of sham-operated or CCI rats treated with LAC or saline for 23 days. Western blot analysis of mGlu1 and -5 receptors showed a major band at about 140 kDa, corresponding to receptor monomers. Immunoblots of mGlu2/3 receptors revealed a band at 100 kDa, corresponding to receptor monomers, and an additional high-molecular-mass band, corresponding to receptor dimers (Fig. 3A). CCI of the sciatic nerve induced a trend to an increase in the expression of mGlu2/3 receptors in the spinal cord without changing the expression of mGlu1 or -5 receptors. Expression of spinal mGlu2/3 receptors was markedly up-regulated by LAC treatment in both sham-operated and CCI rats. LAC had no detectable effect on mGlu1 or -5 receptor expression (Fig. 3, A and B).

Because the effect of LAC on mGlu2/3 receptor expression

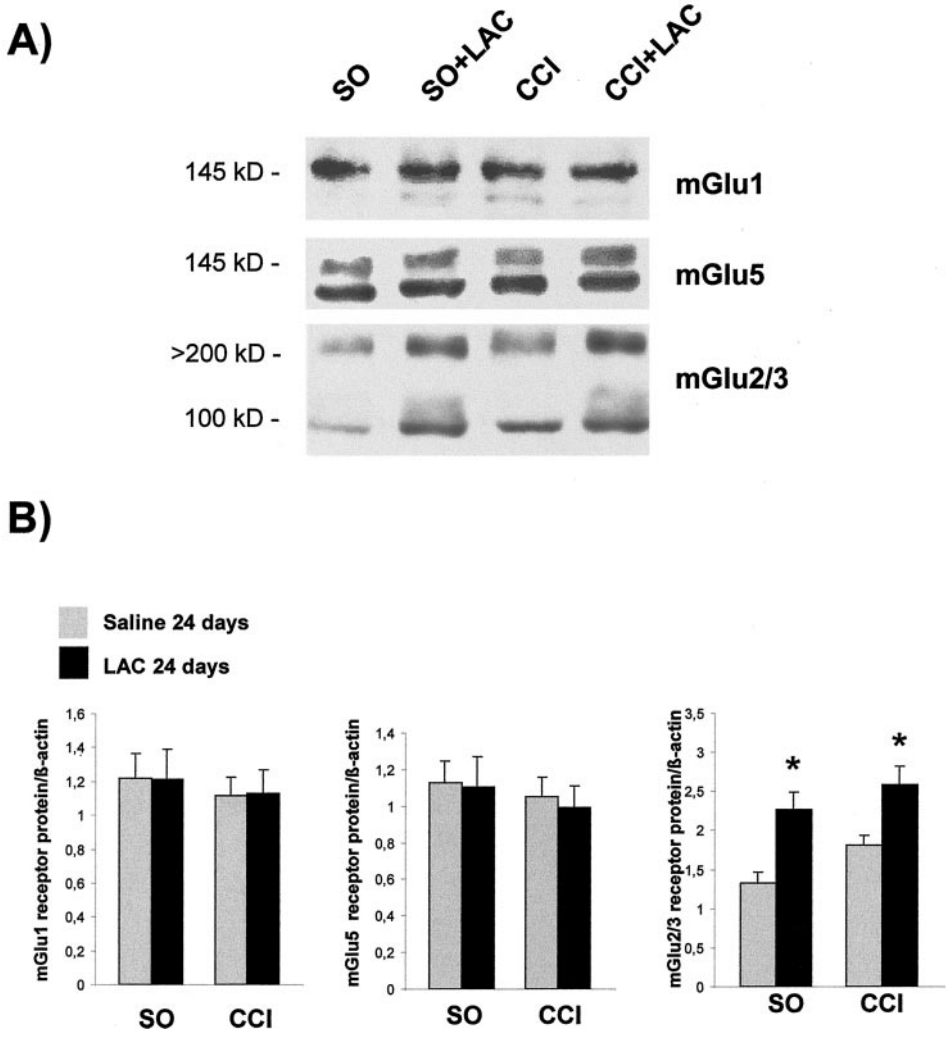

Fig. 3. A, Representative immunoblots of mGlu1, -5, and -2/3 receptors in the lumbar spinal cord of sham operated animals (SO), SO animals treated with LAC (100 mg/kg, s.c., once daily for 24 days), CCI animals, and CCI animals treated with LAC. Densitometric analysis of immunoblots is shown in B, where values ($n = 6–8$) were normalized by the expression of β-actin. Note that LAC treatment selectively up-regulates the expression of mGlu2/3 receptors in both SO and CCI animals. ∗, $p < 0.05$ (Student's t test) versus values obtained in animals treated with saline for 24 days.

was independent of the induction of neuropathic pain, we decided to use intact rats repeatedly injected with LAC for further characterization. Western blot analysis carried out in extracts prepared from the lumbar spinal cord showed a substantial up-regulation of mGlu2/3 receptor protein after a 5- or 7-day treatment with LAC. A slight up-regulation of mGlu2/3 receptors was seen in only 2 of 5 rats treated with LAC for 3 days, but the overall difference from controls was not statistically significant (Fig. 4A). Thus, the increased expression of mGlu2/3 receptors in the spinal cords showed a good relation with the analgesic activity of LAC in the plantar test (which was observed after 5 or 7 days of treatment but not after 3 days). An increased mGlu2/3 receptor expression was also observed in isolated dorsal horns and in the cerebral cortex, but not in the cerebellum or hippocampus, of animals treated with LAC for 7 days (Fig. 4B). Immunohistochemical analysis of mGlu2/3 receptors in control rats showed a predominant labeling in the inner portion of lamina II of the dorsal horns, as expected. In rats treated with LAC for 7 days, mGlu2/3 immunolabeling was more intense in the inner portion of lamina II and in laminae III and IV (Fig. 5, A-C). At higher magnification, mGlu2/3 immunostaining was mostly punctate in the dorsal horns of LAC-treated animals (not shown), suggesting that up-regulated receptors were preferentially localized on nerve terminals. To examine the contribution of primary afferent fibers to the up-regulation of mGlu2/3 receptors, we performed Western blot analysis in dorsal root ganglia isolated from the lumbar tract of the spinal cord. Immunoblots showed an up-regulation of mGlu2/3 receptors in the dorsal root ganglia of animals treated with LAC for 5 or 7 days (Fig. 6).

We also examined the expression of mGlu2 and -3 receptor mRNA by combining RT-PCR and Northern blot analysis. mGlu2 mRNA in the dorsal horns was below the detection levels by RT-PCR in control rats but was clearly induced in rats injected repeatedly with LAC. RT-PCR analysis suggested that

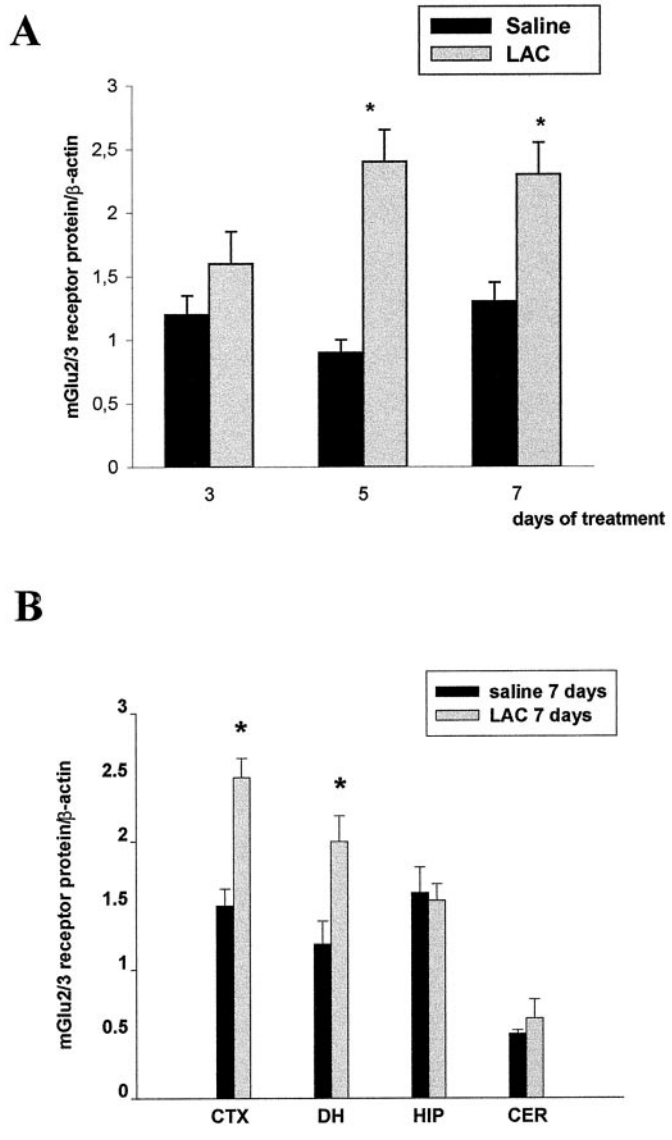

Fig. 4. Densitometric analysis of mGlu2/3 receptor proteins in immunoblots obtained: from the lumbar spinal cord of intact rats treated with saline or LAC (100 mg/kg, i.p., twice daily) for 3, 5, or 7 days (A) or from the cerebral cortex (CTX), dorsal horns (DH), hippocampus (HIP), and cerebellum (CER) of intact rats treated for with saline or LAC for 7 days (B). Values are means + S.E.M. of five (A) or six (B) determinations. *, $p < 0.05$ (Student's t test) versus the corresponding values from rats treated with saline. Densitometric analysis of samples in A was performed after two different exposure times, as well as after loading 30 instead of 60 μg of protein per lane, obtaining similar results.

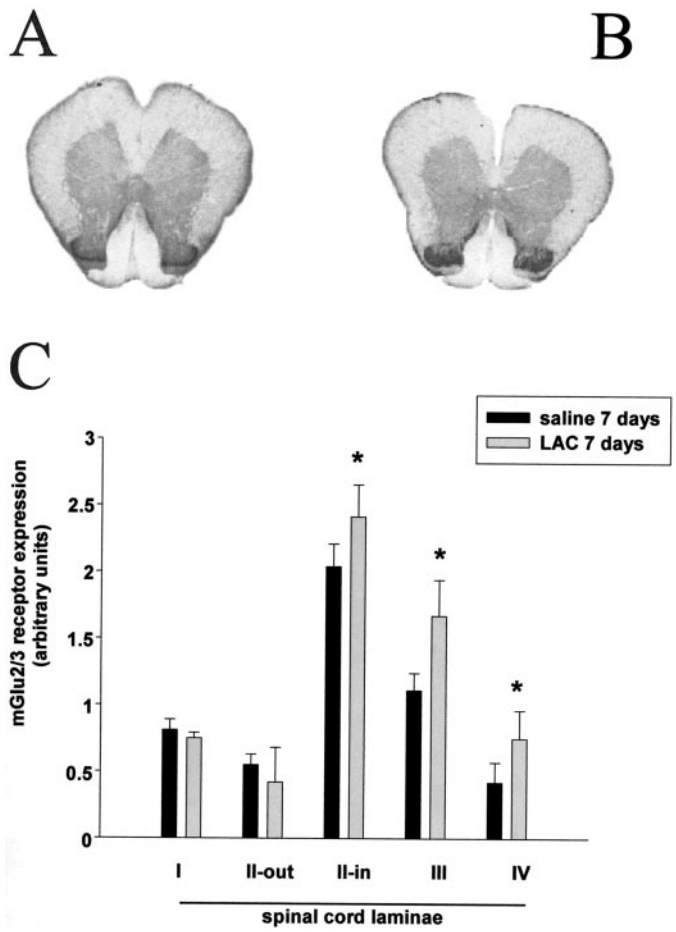

Fig. 5. Immunohistochemical analysis of mGlu2/3 receptors in a representative section of the lumbar spinal cord from intact rats treated with saline (A) or LAC (B) for 7 days. Image analysis of mGlu2/3 receptor expression in laminae I-IV is shown in C. Values (means ± S.E.M.) were calculated from 12 sections from four animals per group. Immunostaining in the posterior white matter was considered as a blank and subtracted from all values. *, $p < 0.05$ (Student's t test) versus the corresponding value from rats treated with saline for 7 days.

a similar induction was present in the cerebral cortex but not in the cerebellum or hippocampus (Fig. 7A). These data were confirmed by Northern blot analysis, which showed an increased expression of mGlu2 mRNA levels in the cerebral cortex but not in the hippocampus of rats treated with LAC. mGlu2 mRNA levels in the dorsal horns could not be detected by Northern blot analysis (Fig. 7B). The effect of LAC treatment on mGlu2 receptors was specific because no changes in mGlu3 mRNA levels were observed in any brain region (Fig. 7C).

Discussion

LAC, the acetyl ester of carnitine, is a compound of considerable interest for its wide clinical application in a number of neurological disorders, including diabetic and herpes- or HIV-associated neuropathies (Schoemaker, 1994; Quatraro et al., 1995; Scarpini et al., 1996; 1997). LAC is a donor of acetyl groups and increases the intracellular levels of carnitine, which serves as a major transporter of fatty acids across the mitochondrial membranes (Dolezal and Tucek, 1981; Farrell et al., 1986). Hence, the beneficial effect of LAC on neuropathic pain is generally considered secondary to an improved energy metabolism in the injured peripheral nerves. Present results show that LAC acts as a potent analgesic agent and is active not only in a model of neuropathic pain (i.e., in rats subjected to CCI of the sciatic nerve) but also in intact animals subjected to acute pain. This suggests that LAC produces analgesia independently of its neurotrophic activity and provides the basis for the use of LAC in the treatment of non-neuropathic pain. Induction of analgesia required multiple injections of LAC, as opposed to what we observed with classical analgesic agents (such as opiates), which are active after the first administration but then progressively loose efficacy, owing to the development of toler-

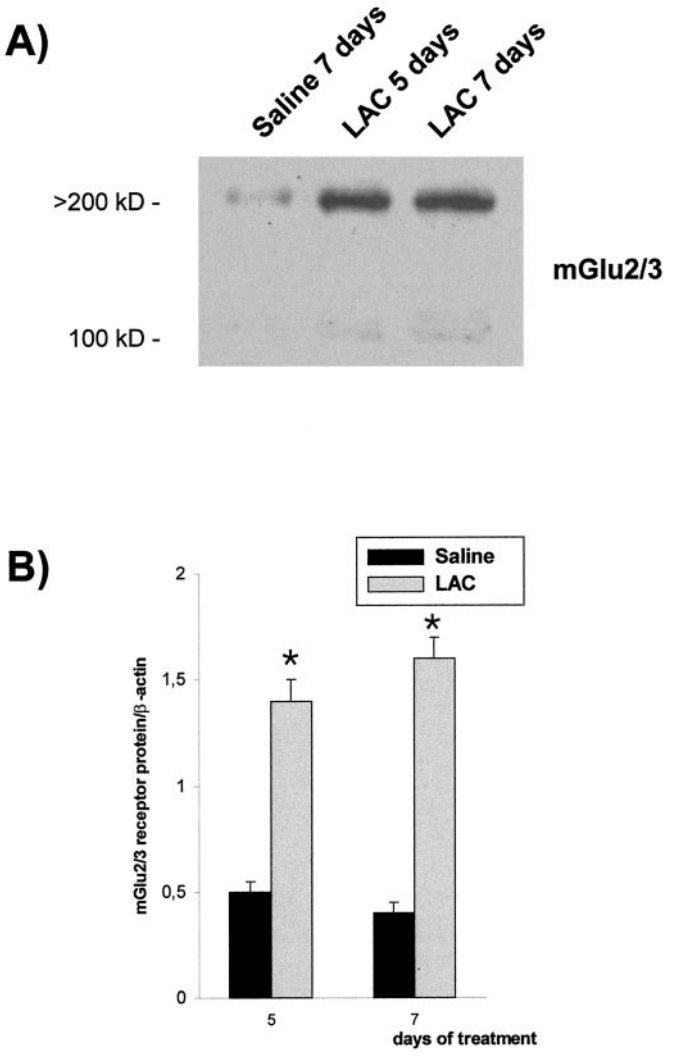

Fig. 6. A, representative immunoblot of mGlu2/3 receptors in the dorsal root ganglia of intact rats treated with saline for 7 days or with LAC (100 mg/kg, i.p., twice daily) for 5 or 7 days. Densitometric analysis of immunoblots is shown in B, where values ($n = 4$) were normalized by the expression of β-actin. *, $p < 0.05$ (Student's t test) versus the corresponding values obtained from animals treated with saline.

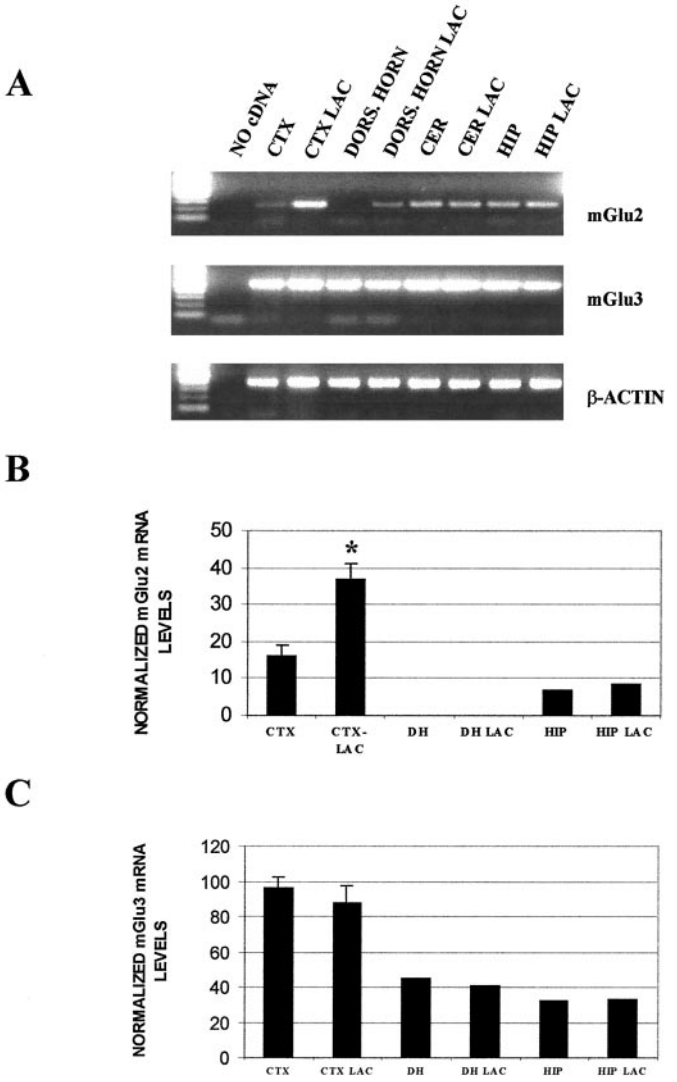

Fig. 7. A, RT-PCR analysis of mGlu2, mGlu3, and β-actin mRNA in the cerebral cortex (CTX), dorsal horns, cerebellum (CER), and hippocampus (HIP) of rats treated with saline (none) or LAC for 7 days. The size of the β-actin amplimer excludes any contamination by genomic DNA. Northern blot analysis of mGlu2 and mGlu3 mRNA is shown in B and C, respectively. Values are expressed as percentage of the corresponding β-actin mRNA and represent the means ± S.E.M. of four to five determinations for the cerebral cortex. For hippocampi and dorsal horns, samples were pooled and the bars are representative of two similar determinations. *, $p < 0.05$ (Student's t test), versus the corresponding value in the absence of LAC.

ance. Hence, we have speculated that LAC modulates nociceptive transmission by inducing plastic changes within the dorsal horns of the spinal cord. Searching for a mechanism underlying LAC-induced analgesia, we focused on mGlu receptors, which are largely implicated in the regulation of pain transmission (Baranauskas and Nistri, 1998; Budai and Larson, 1998; Maione et al., 1998; Bordi and Ugolini, 1999). Recent studies have disclosed a role for mGlu1 and -5 receptors in pain transmission (Fundytus et al., 1998, 2001; Bordi and Ugolini, 2000; Chen et al., 2000; Dogrul et al., 2000; Bhave et al., 2001; Karim et al., 2001; Walker et al., 2001). However, LAC induced a selective increase in mGlu2/3 receptors that was temporally related to the analgesic effect of the drug. In the dorsal horns, mGlu2/3 receptors are known to be localized in postsynaptic densities, vesicle-containing profiles, and glial cells of the inner part of lamina II (Jia et al., 1999). In vesicle-containing profiles, mGlu2/3 immunoreactivity is located to membrane compartments distinct from active release sites (Azkue et al., 2000). Studies of mRNA levels in the dorsal horns indicate that mGlu3 receptors are much more expressed than mGlu2 receptors in resident neurons or glial cells (Boxall et al., 1998; Berthele et al., 1999). An increased expression of mGlu3 mRNA has been reported in the spinal cord after peripheral inflammation (Boxall et al., 1998). In our study, LAC treatment did not affect mGlu3 mRNA levels, whereas it clearly induced the expression of mGlu2 mRNA in the dorsal horns and cerebral cortex. This suggests that enhanced expression of mGlu2 receptors accounts for the increased mGlu2/3 immunoreactivity in LAC-treated animals. Because the induction of mGlu2 mRNA by LAC in the dorsal horns could be detected only after amplification by RT-PCR, it is likely that most of the receptor protein is synthesized outside the spinal cord and conveyed inside the dorsal horns through the afferent fibers, as suggested also by the punctate profile of mGlu2/3 immunoreactivity. It is consistent with this hypothesis that LAC treatment also induced an up-regulation of mGlu2/3 receptors in the lumbar dorsal root ganglia. To examine whether the induction of mGlu2 receptors had any role in LAC-induced analgesia, we have treated animals with the compound LY 341495, which behaves as a potent and systemically active mGlu2/3 receptor antagonist (reviewed by Schoepp et al., 1999). A single injection of LY 341495 did not affect acute pain or mechanical hyperalgesia in the absence of LAC, indicating that endogenously activated mGlu2/3 receptors do not contribute to pain transmission. Interestingly, however, LY 341495 largely reduced the effect of LAC in both intact and CCI animals, suggesting that it is the up-regulation of mGlu2 receptors that mediates LAC-induced analgesia. We speculate that endogenous activation of mGlu2 receptors acquires the ability to control the release of glutamate (and/or peptides) from primary afferent fibers only in animals treated with LAC in which there is an increased probability that the endogenously released glutamate recruits presynaptic mGlu2 autoreceptors. However, we cannot exclude that the up-regulation of mGlu2 receptors in upper brain regions (such as the cerebral cortex) participates in the analgesic effect of LAC. Studies with local injections of mGlu2/3 receptor antagonists are necessary to specifically address this point. The mechanism by which LAC induces the expression of mGlu2 receptors is still obscure. The regional selectivity of the effect of LAC suggests that the mGlu2 receptor gene is not a sensor of the metabolic changes induced by LAC treatment in nerve cells. We rather believe that LAC specifically affects the expression of the mGlu2 receptor gene in brain regions that are directly involved in pain sensation, such as the dorsal horns of the spinal cord and the cerebral cortex.

In conclusion, three novel findings emerge from the present data: 1) LAC treatment induces analgesia not only in models of neuropathic pain but also in models of acute pain in intact animals; 2) LAC is the only known drug that selectively induces mGlu2 receptors. This is relevant from a pharmacological standpoint, because it is hard to differentiate between mGlu2 and -3 receptors; and 3) an enhanced expression of mGlu2 receptors in particular brain regions (such as the spinal cord) can be instrumental for the induction of analgesia. LAC can be therefore considered as the prototype of a novel class of drugs that induce analgesia by up-regulating a specific mGlu receptor subtype.

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