L-Acetylcarnitine causes analgesia in mice modeling Fabry disease by up-regulating type-2 metabotropic glutamate receptors

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Abstract
Fabry disease (FD) is a X-linked lysosomal storage disorder caused by deficient function of the alpha-galactosidase A (α-GalA) enzyme. α-GalA deficiency leads to multisystemic clinical manifestations caused by the preferential accumulation of globo-triacylglycerol (Gb3). A hallmark symptom of FD patients is neuropathic pain that appears in the early stage of the disease as a result of peripheral small fiber damage. Previous studies have shown that Acetyl-L-carnitine (ALC) has neuroprotective, neurotrophic, and analgesic activity in animal models of neuropathic pain. To study the action of ALC on neuropathic pain associated with FD, we treated α-GalA gene null mice (α-GalA(-/0)) with ALC for 30 days. In α-Gal KO mice, ALC treatment induced acute and long-lasting analgesia, which persisted 1 month after drug withdrawal. This effect was antagonized by single administration of LY341495, an orthosteric antagonist of mGlu2/3 metabotropic glutamate receptors. We also found an up-regulation of mGlu2 receptors in cultured DRG neurons isolated from 30-day ALC-treated α-GalA KO mice. However, the up-regulation of mGlu2 receptors was no longer present in DRG neurons isolated 30 days after the end of treatment. Taken together, these findings suggest that ALC induces analgesia in an animal model of FD by up-regulating mGlu2 receptors, and that analgesia is maintained by additional mechanisms after ALC withdrawal. ALC might represent a valuable pharmacological strategy to reduce pain in FD patients.

Keywords
Fabry disease, α-GalA null mice, pain, nociception, mGluR

Introduction
Neuropathic pain is induced by morphological and/or functional abnormalities of the somatosensory system, and develops as a result of a cascade of biochemical events and adaptive mechanisms, which cause dysregulation of sensory neurons and maladaptive plasticity within the nociceptive system. Chronic pain is sustained by a malfunction of the pain neuraxis, which alters the processing of nociceptive signaling in such a way that pain is felt even in the absence of detectable noxious or inflammatory inputs, and responses to innocuous and noxious stimuli are enhanced.1 These alterations are known as peripheral and central sensitization, which reflect an amplified response of peripheral nociceptors and an increased synaptic transmission in the pain pathways, respectively, resulting in a reduction in pain thresholds and an
amplification of pain responses. Neuropathic pain is a hallmark of a wide group of peripheral neuropathies, including Anderson-Fabry disease (FD). FD is a multi-systemic X-linked lysosomal storage disorder (LSD), caused by a reduced or absent function of the enzyme α-galactosidase A (α-GalA), a lysosomal hydrolase responsible for glycosphingolipid metabolism. To date, over 1000 mutations of GLA gene with a possible linkage to FD have been identified.4

Neuropathic pain is directly related to the loss of enzyme activity and it is considered the earliest symptom of FD. Furthermore, it could be acute/episodic or chronic.5–7 Episodic pain includes allodynia (i.e., pain caused by a normally non-painful stimulus) and hyperalgesia (i.e., an exaggerated painful sensation in response to a noxious stimulus).8,9 Chronic pain associated with FD form appears as acroparasthesia (i.e., a burning or tingling sensation in both hands and feet).10

Glutamate, the major excitatory neurotransmitter in the mammalian CNS, plays a key role in pain transmission and in nociceptive sensitization. Glutamate activates both ionotropic and metabotropic receptors (iGlu and mGlu receptors, respectively). Nowadays, eight mGlu receptor subtypes have been cloned and subdivided into three groups on the basis of their sequence similarity, pharmacological profile, and transduction mechanisms.11,12 Group I mGlu receptors (mGlu1 and-5 receptors) are coupled to Gq/11 proteins, and their activation likely contributes to the induction and expression of nociceptive sensitization as a result of intracellular Ca2+ mobilization and protein kinase C activation. In contrast, mGlu2 and mGlu4 receptors negatively modulate pain transmission by restraining glutamate release from presynaptic terminals in the dorsal horns of the spinal cord.14 As a result of an epigenetic mechanism, ALC-induced analgesia is long-lasting and persists weeks after drug withdrawal in models of chronic inflammatory or neuropathic pain.31

Based on these evidences, the first aim of our study was to ascertain whether ALC could induce a long-lasting analgesia in a mouse model of FD. The second goal of this study was to establish whether this long-lasting analgesia is correlated to an increased expression of mGlu2 receptors in the spinal cord.

**Materials and methods**

**Animal model**

Heterozygous female α-Gal A (+/−) and wild type α-Gal A (+/0) male mice (same JAX strain B6; 129Gla.im1Kul/J) purchased from Charles River Laboratories Italia s.r.l. (Jackson Laboratory; Bar Harbor, ME, USA) were crossed to give the F1 generation.31,32 These mice were used from the F1 to the F4 generation as heterozygous females (+/−, B6; 129-Glatm1Kul/J) crossed with wild type males (+/0, B6; 129-Glatm1Kul/J) of the same genetic background (B6; 129-Glatm1Kul/J). From the F4 generation, we obtained homozygous females α-Gal A (−/−) and hemizygous males α-Gal A(+/−) mice that were compared to α-Gal (+/+) and +/0 as controls. The two homozygous/hemizygous knock-out and wild type groups were separated after at least 4 generations that is known to be enough to stabilize the background and all the experiments were performed after more than 10 generations.33 Therefore, well-established homozygous α-Gal A (−/−) and hemizygous α-Gal A(+/−) mice compared to α-gal (+/+) and +/0 controls were used. Because the X inactivation process might have impaired the reproducibility of our model, and because FD is more severe in male patients,34,35 we decided to use exclusively male α-Gal A (−/0) mice in our study.

Mice were housed in groups of six in individually ventilated cages (Tecniplast, Italia) with water and food ad libitum in controlled environmental conditions: lights on from 7.00 a.m. to 7.00 p.m., 22 ± 2°C temperature and 65% humidity. Only α-GalA (+/0) (WT) and α-GalA (−/0) (KO) male mice were used. Behavioral experiments were carried out at the Department of Medical and Clinical Sciences (DIMEC), University of Bologna, with the approval of the local ethical committee (Veterinary Service of the University of Bologna) and in agreement with the National Animal Welfare Act. All efforts were made to minimize animal
suffering and the number of animals used was kept to a minimum by the experimental design. All the procedures followed in this work complied with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethical committee of the University of Bologna (prot. N. 141/2019PR). The age of animals used for behavioral and immunohistochemistry experiments was in the range of two and three months of age.

**Treatments**

Starting from 1-month of age α-GalA (+/0) (WT) and α-GalA (-/0) (KO) male mice were treated with vehicle (saline 0.9%) or L-acetyl-carnitine (ALC) (100 mg/kg body weight; Alpha-Sigma, Bologna, Italy) administered via intraperitoneal injection (ip) every day for 30 days (Table 1). The dose of 100 mg/kg has been selected because it was proven to cause analgesia in mouse models of chronic inflammatory and neuropathic pain. During the 30-day period, animals were injected in the morning between 9.00 am and 11.00. LY341495 (1 mg/kg body weight, Tocris Cookson, UK) was dissolved in DMSO (its final concentration of 3%) and then diluted in saline, and administered i.p. 45 min prior to the onset of behavioral experiments. Fifty-six GalA (+/0) (WT) and 77 α-GalA (-/0) (KO) male mice were randomly assigned to the different treatments. All compounds were administered in a volume of 10 mL/kg body weight. Behavioral and in vitro experiments were performed in the following order and in the same group of animals: von Frey test, hot plate test and in vitro tests. The number of mice included in individual analyses is reported in figure legends and table.

**Behavioral procedures**

Behavioral tests were conducted between 09:00 am and 17:00 PM. Mice were habituated to the experimental room 1 h before the tests. Behavioral analysis was performed with a blind procedure.

**Mechanical hyperalgesia**

Mice were placed in test cages with a metal grid bottom at least 2 h prior to testing to allow accommodation in the novel environment. Paw withdrawal latency to mechanical stimulation was assessed with an automated testing device consisting of a steel rod (2 mm) that was pushed with electronic ascending force against the plantar surface of the hind paw with increasing force until the paw was withdrawn (Dynamic Plantar Aesthesiometer, Ugo Basile, Varese, Italy). A linear increase in force to 5 g was applied over 10 s after which the force remained constant. An interval of 120 sec was used during testing. When a mouse withdrew its hind paw, the mechanical stimulus was automatically withdrawn and the force recorded to the nearest 0.1 g. The paw withdrawal latency and actual force at the time of paw withdrawal reflex were calculated as the mean of 5 consecutive trials.

**Hot plate test**

Mice were positioned in the experimental room 1 h before the test. Each mouse was placed into a transparent Plexiglas beaker of 60 × 18 cm to avoid animals escaped from the plate, the temperature of which was set at 52 ± 0.1°C by using a thermo-regulated heated plate (Ugo Basile, Varese, Italy). The time (in seconds) between the placement of the animal and the first response: paw licking/fanning or jumping was measured as latency. A 30-s cut-off was used to prevent tissue damage. Measurements started 45 min after administration of LY 341495. Mice were investigated by observers who were blinded to animal treatment.

**Preparation of rat dorsal root ganglion neuron**

Primary cultures of DRG neurons were prepared from adult 8–12 weeks old males, according to previously described protocols with some modifications. Mice were anesthetized by halothane prior to decapitation. All ganglia were removed from each mouse and transferred in ice cold DPBS 1x (Gibco) and the roots were cut using microdissecting scissors. After
rinsing in DMEM (Gibco), the ganglia were placed in DMEM containing 5000 U/mL type IV collagenase (Worthington) for 45–75 min at 37°C, 5% CO2, washed twice with FBS-containing medium, and then gently mechanically dissociated with passages through 0.5 mm and 0.6 mm sterile needles. Cells were centrifuged for 10 min at low speed and then appropriately diluted in 1 mL of DMEM containing 10% FBS (Gibco), 50 ng/mL NGF (Gibco), and 1.5 μg/mL cytosine β-D-arabinofuranoside, (AraC, Sigma). For protein extraction, 60,000 cells/5 mL were plated onto Poly-Lysine pre-coated culture dishes. For immunocytochemistry, 15,000 cells/ml were plated onto 18 mm round glass cover slips, pre-coated with poly-L-lysine and Laminin. Cell cultures were maintained in an incubator at 37°C, with 5% CO2 for different periods of time (for electrophysiological experiments, cells were used within the first 4 days after plating (DIV); for WB analysis and immunocytochemistry cells at 4 or 6 DIV were used. Cells were maintained in DMEM, supplemented with 10% FBS in the presence of 50 ng/mL NGF, and 1.5 μg/mL cytosine β-D-arabinofuranoside, (AraC, Sigma) to reduce glial cell expression. Half volume of medium was changed every second day.

**Immunofluorescence analysis**

DRG neurons were isolated from WT and KO mice treated with ALC or saline. Cells were seeded on coverslips coated with poly-d-lysine; after 48 h, cells were fixed for 10 min with 4% formaldehyde at room temperature. After washing (2 times for 10 min each) in PBS, they were blocked with 5% BSA, 0.05% Triton X-100, PBS 1X for 1 hr at RT, and then incubated overnight at 4°C with rabbit anti-mGlu2/3 (1:50, Millipore) in 1% BSA in 0.3% H2O2 in methanol. After 2 washes in 10 mM PBS, the slides were blocked with 5% BSA, 0.1% Triton X-100, PBS 1X for 1 hr at RT, and then incubated overnight at 4°C with rabbit anti-mGlu2/3 (1:50, Millipore) in 1% BSA in 1X PBS with 0.1% Triton X-100. On the following day, preparations were washed 3x10 min with PBS 1X and the following secondary antibody was applied for 1.5 h at RT in the dark: goat anti rabbit-HRP (Santa Crutz) diluted in 1% BSA in 1X PBS with 0.1% Triton X-100. After three washings of 10 min with PBS 1X, the signal was revealed through the DAB Substrate Kit (Vector Laboratories) for 5 minutes and washed in H2O. Afterward, slides were mounted with glycerol and images were taken by microscope (AxioImager M1, (Carl Zeiss, Oberkochen, Germany) and analyzed (AxioCam MRc5, Carl Zeiss) software (AxioVision Rel 4.8, Carl Zeiss).

**Statistical analysis**

Data from single animals represented the unity of analysis. Results are presented as means ± S.E.M. Statistical analysis was performed with SPSS (version 25) or GraphPad Prism (version 7). All datasets were analyzed using the ROUT method (Q = 1%) to identify significant outliers and the Kolmogorov–Smirnov test for normality. Datasets with normal distribution were analyzed for significance using mixed model analysis of variance (ANOVA) with genotype (WT, KO) and treatment (saline, ALC, LY341495) as independent factors, and experimental session (1 and 30 after the end of treatments) as within subjects. Post hoc multiple comparisons were carried out using LSD Fisher’s post hoc test. A probability level of p < 0.05 was considered to be statistically significant. All behavioral data were normally distributed and no significant outliers were found in the dataset (see Table 2). Western blots data obtained from densitometric analysis were evaluated by performing analysis of variance followed by LSD Fisher’s post hoc test.

**Results**

Effect of 30-day treatment with ALC on mechanical pain threshold in α-GalA (+/0) (WT) and α-GalA (−/0) (KO) mice

We assessed mechanical pain thresholds in α-GalA (+/0) (WT) and α-GalA (−/0) (KO) mice treated daily for 30 days with LAC (100 mg/kg) or saline, i.p. (Figure 1 A and B). Measurements were carried out 24 hours and 30 days following ALC withdrawal. In some experiments, the mGlu2/3 receptor antagonist, LY341495 (1 mg/kg) was acutely injected i.p. 30 min before pain measurements.
Table 2. Statistical analysis.

| Data                        | Figure  | Normal distribution (Parametricity)                      | Statistical analysis (Overall effects)                                                                 | Post hoc analysis                |
|-----------------------------|---------|----------------------------------------------------------|-------------------------------------------------------------------------------------------------------|----------------------------------|
| VonFrey PWL 1-day after end of treatment | Figure 1A | Yes, Kolmogorov–Smirnov test for normality                | Mixed ANOVA: Genotype F (1, 129) = 86.736, p < 0.000001, Treatment F (3, 129) = 12.164, p < 0.000001 | LSD Fisher’s post hoc test: |
|                             |         | WTsal/sal KS = 0.170, p > 0.1, n = 16                   | Genotype*Treatment F (3, 129) = 14.12, p < 0.000001                                                   | Saline l-day: WTsal/sal vs KOsal/sal p = 0.000003 |
|                             |         | WTsal/LY KS = 0.158, p > 0.1, n = 13                    | Within-subjects (1-day and 30-day): Session F (1, 129) = 8.408, p < 0.0043, Session*Genotype F (1, 129) = 0.536, p = 0.46 | 30-day: WTsal/sal vs KOsal/sal p = 0.000004 |
|                             |         | WTsal/ALC KS = 0.223, p = 0.19, n = 18                   | Session*Treatment F (3, 129) = 1.132, p = 0.33, Session*Genotype*Treatment F (3, 129) = 0.88, p = 0.45 | Within-subjects l-day vs 30-day: WTsal/sal 1-day vs WTsal/sal 30-day p = 0.72 |
|                             |         | WTLY/ALC KS = 0.259, p = 0.06, n = 10                    |                                                                                                       | KOsal/sal 1-day vs KOsal/sal 30-day p = 0.64 |
|                             |         | KOsal/sal KS = 0.148, p > 0.1, n = 21                    |                                                                                                       | ALC l-day: WTsal/sal vs KOsal/ALC p = 0.51 |
|                             |         | KOsal/LY KS = 0.136, p > 0.1, n = 19                     |                                                                                                       | KOsal/sal vs KOsal/ALC p = 0.73 |
|                             |         | KOsal/ALC KS = 0.181, p > 0.1, n = 19                   |                                                                                                       | 30-day: WTsal/sal vs KOsal/ALC p = 0.73 |
|                             |         | KOLY/ALC KS = 0.121, p > 0.1, n = 21                    |                                                                                                       | LY341495 l-day: WTsal/sal vs WTsal/LY341495 p = 0.999 |
|                             |         |                                                                 |                                                                                                       | KOsal/sal vs KOsal/LY p = 0.4 |
|                             |         |                                                                 |                                                                                                       | 30-day: WTsal/sal vs WTsal/LY341495 p = 0.38 |
|                             |         |                                                                 |                                                                                                       | KOsal/sal vs KOsal/LY p = 0.11 |
|                             |         |                                                                 |                                                                                                       | ALC l-day: KOsal/sal vs KOsal/LY p = 0.011 |
| VonFrey PWL 30-day after end of treatment | Figure 1A | Yes, Kolmogorov–Smirnov test for normality                |                                                                                                       |                                                                 |
|                             |         | WTsal/sal KS = 0.201, p > 0.1, n = 15                   |                                                                                                       |                                                                 |
|                             |         | WTsal/LY KS = 0.201, p > 0.1, n = 12                    |                                                                                                       |                                                                 |
|                             |         | WTsal/ALC KS = 0.253, p > 0.1, n = 11                   |                                                                                                       |                                                                 |
|                             |         | WTLY/ALC KS = 0.140, p > 0.1, n = 10                    |                                                                                                       |                                                                 |
|                             |         | KOsal/sal KS = 0.164, p > 0.1, n = 12                    |                                                                                                       |                                                                 |
|                             |         | KOsal/LY KS = 0.169, p > 0.1, n = 12                     |                                                                                                       |                                                                 |
|                             |         | KOsal/ALC KS = 0.230, p > 0.1, n = 12                   |                                                                                                       |                                                                 |
|                             |         | KOLY/ALC KS = 0.122, p > 0.1, n = 21                    |                                                                                                       |                                                                 |

(continued)
| Data | Figure | Normal distribution (Parametricity) | Statistical analysis (Overall effects) | Post hoc analysis |
|------|--------|------------------------------------|---------------------------------------|------------------|
| VonFrey force 1-day after end of treatment | Figure 1B Yes Kolmogorov–Smirnov test for normality | Genotype: F (1, 129) = 131.45, p < 0.00001; Treatments F (3, 129) = 13.14, p < 0.00001; Genotype*Treatment: F (3, 129) = 16.85, p < 0.00001. | LSD Fisher’s post hoc test: Saline 1-day WTsal/sal vs KOsal/sal p = 0.00025 30-day WTsal/sal vs KOsal/sal p < 0.00001 1-day vs 30-day WTsal/sal 1-day vs WTsal/sal 30-day p = 0.06 KOsal/sal 1-day vs KOsal/sal 30-day p = 0.64 ALC 1-day WTsal/sal vs KOsal/ALC p = 0.7 KOsal/sal vs KOsal/ALC p = 0.000041 30-day WTsal/sal vs KOsal/ALC p = 0.6 KOsal/sal vs KOsal/ALC p < 0.000001 LY341495 1-day KOsal/sal vs KOsal/LY p = 0.07 WTsal/sal vs WTsal/LY p = 0.44 30-day KOsal/sal vs KOsal/LY p = 0.811 WTsal/sal vs WTsal/LY p = 0.48 p = 0.04; 30-day PWL p = 0.076, force p = 0.04 WTsal/sal vs WTsal/ALC p = 0.9999 LY341495/ALC 1-day WTsal/sal vs WTsal/ALC p = 0.000004 KOsal/sal vs KOsal/LY/ALC p = 0.89 30-day WTsal/sal vs WTsal/ALC p = 0.002 | |
| VonFrey force 30-day after end of treatment | Figure 1B Kolmogorov–Smirnov test for normality | | |

(continued)
| Data | Figure | Normal distribution (Parametricity) | Statistical analysis (Overall effects) | Post hoc analysis |
|------|--------|------------------------------------|----------------------------------------|-------------------|
| Hot plate latency time 1-day after end of treatment | Figure 2 | Yes | Kolmogorov–Smirnov test for normality | Mixed ANOVA:  
Genotype F (1, 129) = 22.453, p = 0.000006  
Treatments F (3, 129) = 3.368, p = 0.014  
Genotype*Treatment F (3, 129) = 7.429, p = 0.00012  
Within-subjects (1-day and 30-day)  
Session F (1, 129) = 16.676, p = 0.000077  
Session*Genotype F (1, 129) = 0.000, p = 0.98  
Session*Treatment F (3, 129) = 0.536, p = 0.658  
Session*Genotype*Treatment F (3, 129) = 1.945, p = 0.125 | LSD Fisher’s post hoc test:  
Saline  
WTsal/sal vs KOsal/sal  
P = 0.0011  
30-day  
WTsal/sal vs KOsal/sal  
P = 0.0012  
ALC  
WTsal/ALC vs KOsal/ALC  
P = 0.58  
KOsal/sal versus KOsal/ALC  
P = 0.00003  
30-day  
WTsal/ALC vs KOsal/ALC  
P = 0.49  
KOsal/sal vs KOsal/ALC  
P = 0.000027  
LY341495  
WTsal/LY vs KOsal/LY  
P = 0.0001  
KOsal/sal vs KOsal/LY  
P = 0.75  
30-day  
WTsal/LY vs KOsal/LY  
P = 0.05  
KOsal/sal vs KOsal/LY  
P = 0.5  
LY341495/ALC  
1-day  
WTsal/sal vs KOsal/LY  
P = 0.04  
KOsal/sal vs KOsal/LY  
P = 0.46  
30-day  
WTsal/sal vs KOsal/LY  
P = 0.002  
KOsal/sal vs KOsal/LY  
P = 0.97 |
| Hot plate latency time 30-day after end of treatment | Figure 2 | Yes | Kolmogorov–Smirnov test for normality |  
WTsal/sal KS = 0.202, p = 0.06, n = 21  
WTsal/LY KS = 0.151, p > 0.1, n = 13  
WTsal/ALC KS = 0.151, p > 0.1, n = 18  
WTLY/ALC KS = 0.143, p > 0.1, n = 10  
Kosal/sal KS = 0.110, p > 0.1, n = 21  
Kosal/LY KS = 0.161, p > 0.1, n = 19  
Kosal/ALC KS = 0.082, p > 0.1, n = 19  
KOLY/ALC KS = 0.159, p > 0.1, n = 21  
KOsal/sal KS = 0.125, p > 0.1, n = 12  
KOsal/LY KS = 0.190, p > 0.1, n = 12  
KOsal/ALC KS = 0.129, p > 0.1, n = 12  
KOLY/ALC KS = 0.125, p > 0.1, n = 12 |
A mixed ANOVA on latency time of paw withdrawal latency (PWL), with genotype and treatment as grouping factors and sessions (1 and 30 days after the end of treatment, Figure 1(A), left and right graphs, respectively) as dependent variables, showed significant main effect of genotype [F (1, 129) = 86.736, \(p < .000,001\)], treatments [F(3, 129) = 12.164, \(p < .00,001\)] and interaction genotype*treatment [F(3, 129) = 14.12, \(p < .000,001\)]. When we analyzed the within effects (1-day and 30-day after end of treatment), statistical analysis revealed a significant main of session [F (1, 129) = 8.408, \(p = .00,043\)]. No significance was observed in the interaction session*genotype [F (1, 129) = 0.536, \(p = .46\)], session*treatment [F (3, 129) = 1.132, \(p = .33\)] and session*genotype*treatment [F (3, 129) = 0.88, \(p = .45\)].

Similar results were observed with regard to the applied force, as shown in figure 1B (main effects: genotype: F(1, 129) = 131.45, \(p < 0.000,001\); treatments F(3, 129) = 16.85, \(p < 0.000,001\); session [F(1, 129) = 35.53, \(p < 0.0001\]); session*genotype [F(1, 129) = 0.56, \(p = 0.456\)], session*treatment [F (3, 129) = 1.16, \(p = 0.327\)] and session*genotype*treatment [F (3, 129) = 1.52, \(p = 0.213\)].

In agreement with our previous data,\(^3\)\(^2\) Fisher’s LSD post hoc test showed that KO mice had a reduction in PWL and applied force compared to WT mice (PWL: 1-day \(p = 0.000,003\); 30-day \(p < 0.000,001\)). Furthermore, no differences between sessions were observed (WTsal/sal 1-day vs WTsal/sal 30-day \(p = 0.72\); KOsal/sal 1-day vs KOsal/ALC 30-day \(p = 0.64\)).

Thirty-day treatment with ALC induced a significant decrease in plantar sensitivity in KO mice to levels similar to those found in WT mice (WTsal/sal vs KOsal/ALC PWL p =
Figure 2. Efficacy of long-term Acetyl-L-carnitine treatment on thermal hyperalgesia in α-GalA (+/0) (WT) and α-GalA (-/0) (KO) mice. KO mice show an increase in thermal hyperalgesia compared to WT mice. Acetyl-L-carnitine (Acetyl-L-carnitine, 100 mg/kg, ip, 30 days) treatment produced significant increases in latency to response to a thermal stimulus in treated KO mice compared to the saline group (Sal). LY341495 (LY, 1 mg/kg, ip, 30 min prior onset experiment) counteracted this effect. Pharmacological treatments did not induce any effects on WT mice. Data are representative of at least three independent experiments. Values are means ± SEM. Mixed-Way ANOVA followed by LSD Fisher’s post hoc test. ***p < .001 vs. WT### p < .0001 vs. saline KO.

Expression of mGlu2 receptors in cultured DRG neurons

To study whether ALC was able to enhance mGlu2 receptor expression in FD mice, we first assessed mGlu2 receptor protein levels in cultured DRG neurons isolated from α-GalA (+/0) (WT) and α-GalA (-/0) (KO) mice. Using cultured DRG neurons we were able to demonstrate an overexpression of ion channels in FD mice.38 Hence, this experimental paradigm was considered appropriate for the study of mGlu2 receptors. Mice were sacrificed one day after a 30-day ALC or Saline treatment and DRG neurons were isolated and plated in culture. Total proteins extracts were prepared 48 h after plating, and mGlu2 receptor protein levels were assessed by Western blot using a specific anti-mGlu2 receptor antibody. Immuno blot showed a clear band at approximately 95 kDa, corresponding to the deduced molecular size of receptor monomers (Figure 3(A), left panel). A significant
increase in mGlu2 receptor protein levels was detectable in DRG neurons prepared from ALC-treated KO mice compared to KO mice treated with saline (Figure 3(A), right panel, *p* = .0021). A trend to a non-significant increase in mGlu2 receptor expression was also observed in DRG neurons isolated from WT mice treated with ALC as compared to the corresponding group of mice treated with saline ((a), *p* = .0549). Interestingly, the KO mice treated with saline solution display a decrease in the total expression of the protein, suggesting a possible involvement of the protein in the pathophysiology of the disease. In line with these evidences, we revealed an increase in mGlu2 receptors immunoreactivity in cultured DRG neurons. As shown in Figure 4 panel A, ALC treatment qualitatively increases the fluorescence of mGlu2 receptor immunoreactivity, both in WT and KO DRG and positive for the neuronal marker PGP 9.5. These up-regulation was not long lasting since no increase in protein level nor increase in mGlu2 receptor immunoreactivity was observed in DRG neurons isolated from both genotypes treated with ALC or saline, sacrificed 30 days after the end of the treatment (Figure 3(B), KO SAL vs KO ALC, *p* = .7534). On the contrary, 2 receptor protein levels and immunofluorescence were significantly lower in DRG isolated from KO mice with respect to WT (Figure 3(B), *p* = .0069).

Expression of mGlu2 receptors in the lumbar spinal cord

We performed a qualitatively immunohistochemical analysis of mGlu2 receptors in lumbar (L4-L5) spinal cord sections from
WT and KO mice 1 day after treatment with ALC or saline. mGlu2 receptor immunoreactivity was nearly undetectable in laminae I-IV of the dorsal horns of WT and KO mice treated with saline (Figure 5A, B, C), and increased substantially after ALC treatment in both genotypes (Figures 5(D) and (E)).

**Discussion**

ALC, an endogenous molecule derived from acetylation of carnitine in the mitochondria, has been extensively studied as a valuable therapeutic option to improve the symptoms and slow the progression of painful neuropathies. The antinociceptive and neuroprotective action of ALC has been demonstrated in a variety of experimental models of neuropathic pain, such as streptozotocin- and chemotherapy-induced neuropathy, chronic constriction injury of the sciatic nerve, and diabetic or HIV neuropathies. The therapeutic efficacy of ALC has been consistently demonstrated in patients affected by diabetic or HIV painful neuropathy. ALC causes analgesia by enhancing acetylation of p65/RelA, a member of the Nuclear factor-κB (NF-κB) family of transcription factors. This amplifies Grm2 gene transcription, with a resulting expression of mGlu2 receptors in DRGs and dorsal horns of the spinal cord. The mGlu2 receptor, which is coupled to Gi/o
Figure 5. Acetyl-L-carnitine treatment up-regulates mGlu2 receptors in the lumbar spinal cord. A schematic representation of the structures analyzed is shown in (A). Immunohistochemical analysis of mGlu2 receptors in a representative section of the lumbar spinal cord from mice treated with saline or Acetyl-L-carnitine, 1 day post-treatment, is shown in (B,D), and (C,E), respectively. Images show a more intense immunoreactive signal (black arrows) in the dorsal horn, especially in layers 1,2, and 3 (indicated by black hatching in the right horn, panels C,E) of the sections prepared from ALC-treated animals both in WT (C) and KO (E) genotypes compared to saline-injected controls (B and D, WT, and KO, respectively).
proteins, is strategically positioned to restrain glutamate release from sensitized primary afferent sensory fibers. Remarkably, ALC-induced analgesia and up-regulation of mGlu2 receptors persist for a long time after drug withdrawal, perhaps as a result of H3 histone acetylation at Grm2 gene promoter. Thus, ALC can be considered as first-in-class of epigenetic drugs used in the treatment of chronic pain.

Pain is one of the earliest clinical symptoms reported by children and young adults affected by FD, and although some improvement may be obtained by enzyme replacement therapy, pain may still be present, and requires the use of analgesics. Similarly to patients affected by FD, α-Gal KO mice show mechanical and heat hypersensitivity associated with cold hyposensitivity, although heat hypersensitivity turns into hyposensitivity with age. We wish to point out that pain-related studies performed on FD mouse models are not homogeneous. Accordingly, two studies found that FD mice show increased sensitivity to radiant heat, while other studies found decreased sensitivity to heat in these mice. Contrasting data may arise from the mixed genetic background of the offspring of FD mice, making difficult to obtain strain-matched healthy controls. In our model, we separated homozygous α-Gal KO and WT mice after at least 4 generations and performed all experiments after more than 10 generations, thus eliminating any strain variability in the two genotypes. Our FD mice therefore provide a reliable model for the study of novel therapeutic strategies to relieve pain associated with FD.

The following aspects should be highlighted: (1) mGlu2 receptors have been detected in human DRGs, where its activation prevents nociceptive sensitization of sensory neurons; (2) No tolerance develops to the action of ALC; (3) ALC is an endogenous compound and has an excellent profile of safety and tolerability (see all references of clinical studies with ALC); (4) ALC has no interaction with cytochrome-P450 or other drug metabolizing enzymes, as opposed to other analgesics used in the treatment of neuropathic pain, such as amtryptiline, duloxetine, or venlafaxine; (5) ALC has been shown to display fast antidepressant effect in experimental animals and humans and, therefore, ALC may have the added value to improve depressive symptoms in patients affected by FD; and, (6) the long-lasting effect of ALC might prevent fluctuations in the control of pain. It will be important to examine whether, and to what extent, ALC treatment affects the primary pathological mechanism associated with FD, which is the accumulation of glycosphingolipids as a result of α-Gal deficiency.

Author contributions

F.F and C.D and R.R managed the maintaining of the mice colony, designed and performed all the molecular, behavioral and immunoassaying experiments and their analyses, revised critically the manuscript; R.R., R.L revised critically the manuscript and contributed reagents; M.C. and F.N conceived, designed and supervised the project, wrote the paper. All authors read and approved the final manuscript.
Declaration of conflicting interests
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