Mol Neurobiol
DOI 10.1007/s12035-017-0713-1

DREAM-Dependent Activation of Astrocytes in Amyotrophic Lateral Sclerosis

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Abstract Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of unknown origin and characterized by a relentless loss of motor neurons that causes a progressive muscle weakness until death. Among the several pathogenic mechanisms that have been related to ALS, a dysregulation of calcium-buffering proteins in motor neurons of the brain and spinal cord can make these neurons more vulnerable to disease progression. Downstream regulatory element antagonist modulator (DREAM) is a neuronal calcium-binding protein that plays multiple roles in the nucleus and cytosol. The main aim of this study was focused on the characterization of DREAM and glial fibrillary acid protein (GFAP) in the brain and spinal cord tissues from transgenic SOD1^{G93A} mice and ALS patients to unravel its potential role under neurodegenerative conditions. The DREAM and GFAP levels in the spinal cord and different brain areas from transgenic SOD1^{G93A} mice and ALS patients were analyzed by Western blot and immunohistochemistry. Our findings suggest that the calcium-dependent excitotoxicity progressively enhanced in the CNS in ALS could modulate the multifunctional nature of DREAM, strengthening its apoptotic way of action in both motor neurons and astrocytes, which could act as an additional factor to increase neuronal damage. The direct crosstalk between astrocytes and motor neurons can become vulnerable under neurodegenerative conditions, and DREAM could act as an additional switch to enhance motor neuron loss. Together, these findings could pave the way to further study the molecular targets of DREAM to find novel therapeutic strategies to fight ALS.

Keywords ALS · Astrocyte · Calcium · DREAM · Motor neuron disease · Neurodegeneration

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease in which a relentless loss of motor neurons follows a fatal evolution in few years. From the clinical point of view, ALS is characterized by a muscle weakness that reflects the great motor system degeneration at both superior motor neuron level in motor cortex and inferior motor neuron level in brainstem and spinal cord. The most common onset of the disease is called spinal ALS that follows with muscle weakness which begins focally in one limb. Albeit the bulbar ALS is less frequent, it also begins with focal weakness of oropharyngeal muscles showing dysarthria and dysphagia. In both ALS cases, the main characteristic of the disease is the nonstop and progressive spread to the whole bulbar and spinal motor system [1, 2]. Therefore, the diagnosis of ALS is based on superior and inferior motor neuron tests as well as on the characteristic progression of symptoms to skeletal muscles, according to "El Escorial Criteria" and defined by the Mundial Federation of Neurology [3].

The origin of ALS remains unknown albeit the majority of ALS cases are sporadic (SALS) and 10% of the cases have family history (FALS). Regarding FALS, different mutations have been found in the SOD1 enzyme in 20% of the cases.

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Other genetic loci and genes have been also identified, although the pathophysiology of *SOD1* mutations is the most common cause of classical autosomal dominant ALS [4, 5], together with Tar DNA-binding protein gene (*TDP-43*) and the most recent discovered DNA/RNA-binding protein called *FUS* (fused in sarcoma) or *TLS* (translocation in liposarcoma), suggesting that alterations in RNA processing could play a central role in ALS pathogenesis [6].

Several important mechanisms and not mutually exclusive can be involved in the neurodegeneration process, including a complex interaction of genetic factors, mitochondrial dysfunction, a break in the assembly of neurofilament network, aggregation of aberrant proteins, oxidative stress, excitotoxicity mediated by glutamate, and the action of neighbor nonneuronal cells (glial cells) to motor neurons. The oxidative stress is considered a main effector and the common mechanism, by which motor neuron death takes place, and therefore, these neurons become more selectively vulnerable as they are exceptionally large, post-mitotic, with a higher energetic demand, and they received a high activated level of glutamate. The interaction between motor neurons and glial cells is essential in the clinical progression of both SALS and FALS diseases and the release of reactive oxygen species or cytokines by glial cells could contribute to motor neuron death [7–12].

One of the main hypotheses related to ALS is the glutamatergic toxicity due to the high expression of glutamate transporters in motor neurons, and as a consequence, these cells are more vulnerable to calcium homeostasis dysfunction [13]. An increase of the cytoplasmic calcium concentration could prompt the majority of the pathogenic pathways above mentioned, even the ones that are no dependent on glutamate excitotoxicity [14]. Moreover, human spinal motor neurons, especially when they carry *SOD1* gene mutation that prompts ALS, are selectively vulnerable to glial toxic effect, in particular astrocytes [15]. In absence of SOD1 gene mutation, astrocytes can even increase motor neuron vulnerability to other neurotoxic mechanisms involved in the ALS pathogenesis. As previously described in postmortem spinal cord samples from ALS patients [16], excessive calcium-dependent proliferation of astrocytes could be an acceptable neurodegenerative mechanism. In fact, the multifactorial component of this disease makes it difficult to establish the first link of the neurodegenerative chain in ALS.

Notwithstanding, reactive astrogliosis has been widely described in ALS disease [17, 18]. Consequently, the main aim of our study was based on the analysis of the potential role of downstream regulatory element antagonist modulator (DREAM) in the neurodegenerative progression of the disease due to the fact that DREAM can stimulate the expression of glial fibrillary acid protein (GFAP) in astrocytes [15]. To achieve this aim, we performed DREAM and GFAP immunodetection in spinal cord and brain from transgenic $SOD1^{G93A}$ mice, one of the best characterized murine models of ALS, and in the frontal cortex and bulbar regions of the brain, as well as the lumbar region of the spinal cord from ALS patients. The findings could shed light to identify new potential targets of the disease and to understand its role under neurodegenerative conditions.

Methods

Animals

Wild-type (WT) mice on a B6SJL genetic background and $SOD1^{G93A}$ mutant mice on a mixed B6SJL background used for the experimental procedures were provided by The Jackson Laboratory (Bar Harbor, ME; Sacramento, CA). All the experimental procedures were approved by the Ethic Committee for Animal Experiments of the University of Zaragoza. Animal care and experimentation were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE on the protection of animals used for experimental and other scientific purposes. Food and water was administered ad libitum.

A total of 32 animals were included in this study: the group of transgenic $SOD1^{G93A}$ mice (n = 16 animals, sex balanced) and their littermate WT mice (n = 16, sex balanced).

Protein Extraction and Western Blot Analysis in Mice Samples

Control mice (wild type) and transgenic $SOD1^{G93A}$ mice at 8, 12, and 16 weeks of age (n = 12 mice per group, n = 4 mice per age and group) were euthanized by CO_2 inhalation, and the spinal cord and brain tissues were rapidly removed and stored at 80 °C until the experiment. In a group of eight animals of 16 weeks (n = 4 wild type and n = 4 $SOD1^{G93A}$ mice, sex balanced), the whole brain was dissected and the temporal lobe, motor cortex, and brain stem areas were extracted to study DREAM and GFAP protein levels. Powdered spinal cord and brain tissues for protein extraction were resuspended in RIPA lysis buffer together with protease inhibitors (SC-24948, Santa Cruz Biotechnology, Inc., CA, USA) according to manufacturer's protocol. After centrifugation, supernatants were collected and total protein was quantified using the BCA method (Sigma-Aldrich). Next, 25 μg of protein were loaded to each lane of a 10% SDS-PAGE gel for DREAM and GFAP analysis. After proper protein resolving, proteins were transferred to a PVDF membrane (Amersham™, GE Healthcare Life Sciences) and subsequently blocked with a Tris-buffered saline solution containing 5% skimmed milk and 0.1% Tween as supplement for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with the selected primary antibodies: 1:1000 DREAM (sc-9142, Santa Cruz Biotechnology,

Inc., CA, USA) and 1:1000 GFAP (sc-51601, Santa Cruz Biotechnology, Santa Cruz, CA, USA). GAPDH (sc-25778, Santa Cruz Biotechnology, Inc., CA, USA) was selected as normalization protein in accordance with previous studies [19]. The secondary antibody was diluted 1:5000 in blocking buffer (goat anti-rabbit IgG-HRP (sc-2004) or goat anti-mouse IgG-HRP (sc-2005), Santa Cruz Biotechnology, Inc.). The Western blots were developed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc., CA 95060) and exposed to Agfa X-ray films (Agfa, 2640 Mortsel, Belgium). The computer-assisted analysis of the bands was performed with AlphaEase FC software (Bonsai Technologies Group, S.A., Madrid, Spain).

Selection of ALS Patients

We studied the clinical and pathological manifestation in deceased patients which were diagnosed as ALS and were randomly selected, as well as in control patients deceased by another cause without evidence of neither neurological disease nor cognitive dysfunction. In all the cases, the corresponding informed consent was obtained from their legal representatives in order to carry out the postmortem analysis, following the ethical rule of the Hospital Clínico Universitario from Zaragoza (Spain).

In this study, four male deceased ALS patients were evaluated (Table 1). These patients were diagnosed as ALS patients under El Escorial Criteria, and their age at death time ranged between 56 and 73 years (mean value 65.25 ± 7.18 years old). The progression of the disease varied between 18 and 48 months (mean value 30 ± 14.77 months). One of the ALS patients suffered from a bulbar onset while in three of them the onset came from a spinal origin (cervical onset in two of them and lumbar onset in one patient). The ALS Functional Rating Scale-revised (ALSFRS-r) ranged between 3 and 16 (mean value 8.75 ± 5.62).

Two male control patients of 65 and 67 years old at death (mean value 66 ± 1.41 years old) were also enrolled in this study (Table 1). No significant differences were found respect to the age at death of ALS patients, which died under hemorrhagic and septic shock due to different causes. The disease lasted 1 month, and there was no evidence of either neurological disease or cognitive dysfunction.

Histology and Immunohistochemistry Analysis

Spinal cord samples from control mice (wild type) and transgenic SOD1^{G93A} mice at late symptomatic stage were removed and fixed in buffered p-formaldehyde to be finally embedded in paraffin. Spinal cord sections were blocked with peroxidase-blocking solution buffer and incubated overnight at 4 °C with primary antibody anti-DREAM (1:100, sc-9142, Santa Cruz Biotechnology, Inc., CA, USA). Antigen detection was carried out using Dako REAL™ EnVision™ Detection System (Denmark A/S). Microphotographs of the gray matter of the ventral horn were taken at × 60 to determine the immunolabeling of DREAM.

Fresh tissue samples from brain (motor, frontal and temporal cortex, and brainstem) and spinal cord (cervical, thoracic, and lumbar) were obtained postmortem from both ALS patients and deceased control individuals without evidence of neurodegenerative disease, sharing a similar age and postmortem interval. The samples were fixed in neutral buffer with formol at 10% (fixed in buffered p-formaldehyde, no buffered saline formol or Bouin fixing) and embedded in paraffin. Cuts were displayed in serial sections of 3 μm, and they were mounted in glass slides (FLEX IHC Microscope Slides, Code K8020, Dako, Denmark A/S).

Table 1 Clinical data of the participants enrolled in this study

Cases	ALS-1	ALS-2	ALS-3	ALS-4	C-1	C-2
Sex	M	M	M	M	M	M
Family history neurodegenerative disorder	No	ALS in mother	No	Dementia in mother	No	No
Age onset (years)	67	52	63	70		
Age death (years)	68	56	64	73	65	67
Duration of disease (months)	18	48	18	36	–	–
Initial signs	Spinal	Spinal	Bulbar	Spinal	–	–
LMN/UMN signs	UMN = LMN	LMN > UMN	UMN = LMN	LMN > UMN	–	–
ALSFRS-r	3	10	16	6	–	–
Clinical diagnosis	ALS	ALS	ALS	ALS + FTD	Kidney cancer	Lung cancer
Cause of death	Respiratory failure	Respiratory failure	Traumatic cerebral hemorragy	Respiratory failure	Septic shock	Hemo shock

The four of the ALS patients are identified as ALS-1, ALS-2, ALS-3, and ALS-4, and the control individuals are identified as C-1 and C-2. Patients ALS-2 and ALS-4 showed LMN more affected than UMN

LMN lower motor neuron, *UMN* upper motor neuron, *FTD* fronto-temporal dementia, *ALSFRS-r* ALS Functional Rating Scale-revised

The antigens were exposed by epitope recovering induced by heat. Antigen detection was carried out using Dako REAL™ EnVision™ Detection System (Denmark A/S), following the techniques and incubation times for each of the primary antibodies used. The slides were analyzed by a light microscope. Some slides were processed without primary antibody or with an isotype control anti-serum to confirm the specificity of the staining. Approximately ten samples from serial sections were randomly selected. Primary antibodies to detect molecular markers of apoptosis (Bax, sc-526, Santa Cruz Biotechnology, Inc., CA, USA, and caspase-3, AM65, Calbiochem, San Diego, CA, USA), gliosis (GFAP, FLEX polyclonal rabbit anti-glial fibrillary acidic protein, code IR524, Dako, Denmark A/S) and DREAM protein (sc-9142, Santa Cruz Biotechnology, Inc., CA, USA) were used.

Morphometric Study

Stained sections in hematoxilin and eosin of four neuroaxis levels were studied in light microscope: brainstem at the hypoglossal nucleus level, medium enlargement of cervical cord, medium region of dorsal spinal, and medium enlargement of lumbar cord. Due to the fact that the number of motor neurons can vary from one histological preparation to another one, two sections of each level of the neuroaxis were evaluated. Both hypoglossal nucleus and anterior horns of the spinal levels above mentioned were photographed separately by a microscope Leica-DM2500 (× 50 and × 100 magnifications), and each image was stored and labeled in image format extension.

The motor neuron "particles" counting in each image archive followed the criteria used by other authors [20, 21]: localization in the anterior part of anterior horn or inside the hypoglossal nucleus, Nissl intense staining, multiconcave morphology and enough size (> 25 μm in the cervical and lumbar region and > 15–20 μm in thoracic and hypoglossal regions). The neuronal particles were counted in both sides of two sections at each level (bulbar, cervical, dorsal, and lumbar) in each of the four ALS patients and in two control individuals. The medium value of the different levels from the two controls was calculated to establish only one value of reference to each level. As other authors have described [20, 21], the estimation of the neuronal loss in each level was carried out comparing the medium counts of neurons between ALS cases and controls, in such a way that the percentage of neuronal loss was referred in ALS cases to control value. In this way, the loss of neurons in each level in ALS cases was expressed as a percentage of neuron counting at the same level as in control cases.

Statistical Analysis

All values are expressed as the mean ± SEM. The associations between quantitative variables were examined using Student's t test (test of Levene was used to calculate the variance analysis). Previously, the normal distribution was determined using Kolmogorov-Smirnov test (SPSS 16.0 software). Comparisons regarding target protein levels were made using ANOVA followed by a Tukey post hoc test. Statistical differences were considered significant at $p < 0.05$ level.

Results

DREAM-Enhanced GFAP Protein Expression at the Late Symptomatic Stage in the Spinal Cord from Transgenic SOD1^{G93A} Mice

Spinal cord samples from transgenic SOD1^{G93A} mice were analyzed by Western blot to study DREAM and GFAP protein levels at the main stages of the disease: early symptomatic (8 weeks), late symptomatic (12 weeks), and terminal (16 weeks) stages. DREAM protein levels were found significantly upregulated in the spinal cord from transgenic SOD1^{G93A} mice at the late symptomatic and terminal stages of the disease respect to their littermate control mice. Interestingly, a similar profile pattern was observed in the GFAP levels, which were only significantly upregulated at the late symptomatic stage in transgenic SOD1^{G93A} mice (Fig. 1). Considering that DREAM is an activator of GFAP

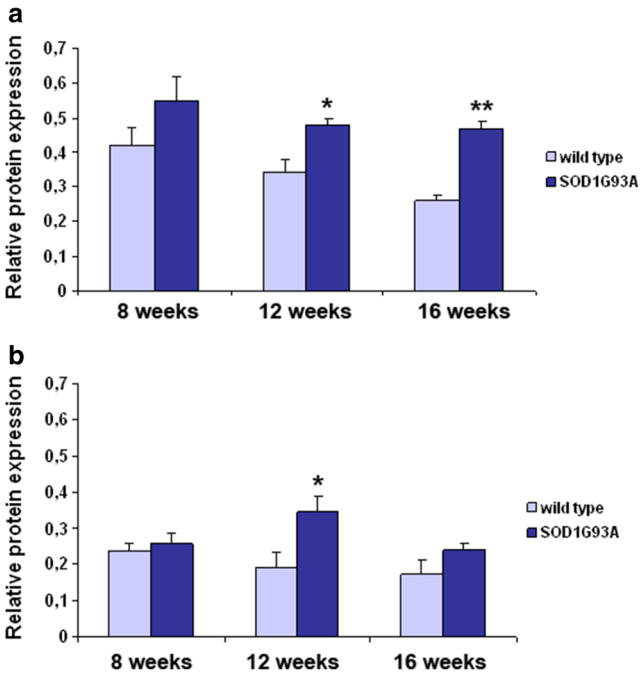

Fig. 1 DREAM and GFAP protein expression in the spinal cord from transgenic SOD1^{G93A} mice. Western blot analysis showed protein expression profiles of DREAM (**a**) and GFAP (**b**) in 8-, 12-, and 16-week-old transgenic SOD1^{G93A} mice. Wild-type littermate mice were used as control mice. ANOVA test showed significant differences in DREAM and GFAP levels from the late symptomatic stage (12 weeks), $n = 8$ wild-type and transgenic mice per age, sex balanced, $^{*}p < 0.05$, $^{**}p < 0.001$

gene expression, these findings could suggest that reactive astrocytes were actively enhancing the motor neuron degeneration, especially in the symptomatic stage when denervation and motor neuron loss had started in this animal model, as previously reported [22, 23]. In line with these findings, immunohistochemistry analysis revealed intense immunostaining in the cytoplasm and nucleus of motor neurons and astrocytes in the spinal cord tissue from transgenic SOD1^{G93A} mice at late symptomatic stage (Fig. 2). Interestingly, DREAM immunostaining was more intense in the cytoplasm and near the cytoplasmic membrane of still alive motor neurons in transgenic mice (Fig. 2a), while in damaged motor neurons, DREAM immunostaining was predominantly detected in their nucleus (Fig. 2b). Surrounding reactive astrocytes also showed positive immunostaining that were even observed in WT mice around dead motor neurons (Fig. 2d), suggesting that the localization of DREAM inside motor neurons could be indicative of their state of degeneration. In addition, previous studies in our group demonstrated the presence of astrocyte reactivity in spinal cords' ventral horns from transgenic SOD1^{G93A} mice in relation to WT mice at terminal stage [23], suggesting that DREAM response in astrocytes could finally enhance motor neuron death.

Excitotoxicity Modulated DREAM Protein Levels in Specific Brain Regions from Transgenic SOD1^{G93A} Mice

Brain samples from transgenic SOD1^{G93A} mice were analyzed by Western blot to study DREAM and GFAP protein levels at the main stages of the disease in transgenic SOD1^{G93A} mice. No significant changes were observed in DREAM protein levels in brain samples from transgenic SOD1^{G93A} mice along disease progression. However, upregulated GFAP levels were found at the terminal stage of transgenic SOD1^{G93A} mice, suggesting that astrogliosis could be enhanced later than in the spinal cord (Fig. 3). Additionally, this astrogliosis was not coincident with an upregulation of DREAM levels at this stage, but DREAM levels showed an opposed tendency to GFAP levels along disease progression. In particular, at the terminal stage, both DREAM and GFAP protein levels showed different profile patterns. Consequently, we tested specifically DREAM and GFAP protein levels in the temporal lobe, motor cortex, and brainstem areas from 16-week-old transgenic SOD1^{G93A} mice to study more accurately the brain regions mostly affected by the motor neuron loss due to the disease progression (Fig. 4).

In the temporal lobe and motor cortex areas, DREAM protein levels were significantly downregulated in 16-week-old transgenic SOD1^{G93A} mice, while in the brain stem, no significant changes were found. This different response observed in the brain with respect to the spinal cord tissue from transgenic SOD1^{G93A} mice could probably indicate, on the one hand, an anti-apoptotic role of DREAM conferred by its transcriptional repressor activity on the apoptotic protein Hrk, or on the other hand, it could suggest tissue damage, in accordance with previous studies on a different murine model of neurodegeneration [24]. Considering this dual role of DREAM, we extended the study to human postmortem samples to better define the role of DREAM in ALS at the very end of the progression of

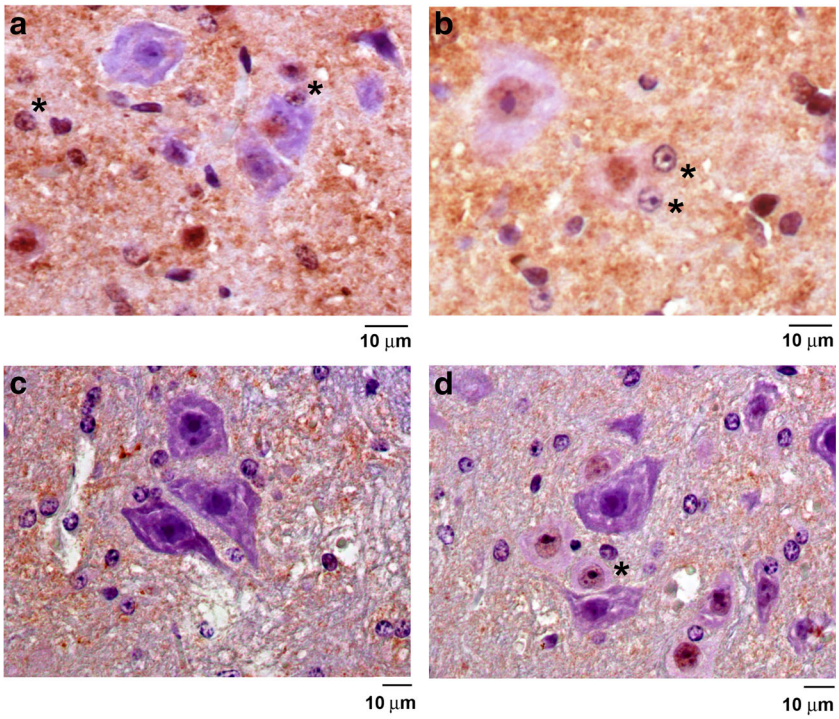

Fig. 2 DREAM immunostaining in the spinal cord from transgenic SOD1^{G93A} mice. Serial cross sections of spinal cord tissue from 12-week-old transgenic SOD1^{G93A} and wild type were analyzed. DREAM-positive immunostaining was observed in the cytoplasm and nucleus of motor neurons from 12-week-old transgenic SOD1^{G93A} mice and in reactive astrocytes surrounding motor neurons (asterisks) (**a**, **b**). Wild-type littermate mice were used as control mice (**c**, **d**). DREAM reactivity was also found in the nucleus of dead motor neurons from wild-type mice (asterisk)

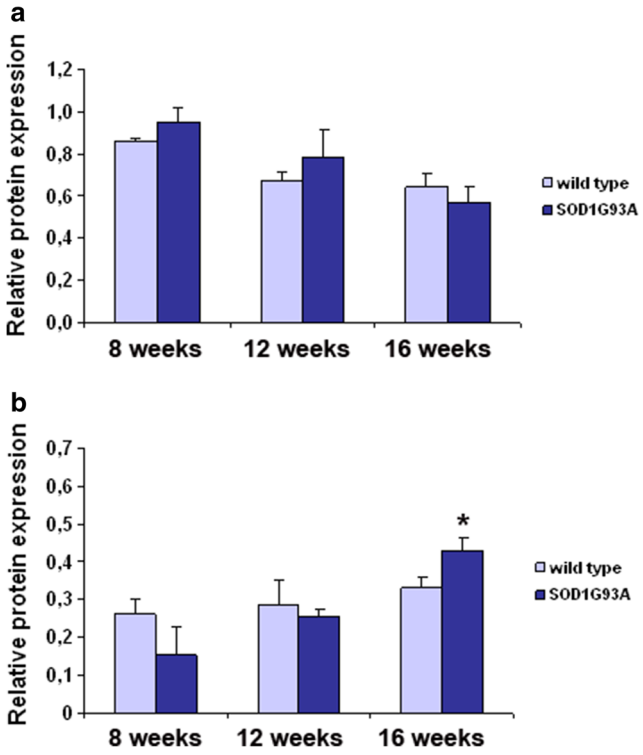

Fig. 3 DREAM and GFAP protein expression in the brain from transgenic SOD1^{G93A} mice. Western blot analysis showed protein expression profiles of DREAM (**a**) and GFAP (**b**) in 8-, 12-, and 16-week-old transgenic SOD1^{G93A} mice. Wild-type littermate mice were used as control mice. ANOVA test showed significant differences in GFAP levels at the late terminal stage (16 weeks), $n = 8$ wild-type and transgenic mice per age, sex balanced, $^{*}p < 0.05$

the disease, since the transgenic SOD1^{G93A} mice were not kept alive until their real survival time.

Intense DREAM Staining Was Observed in the Cytoplasm and Nucleus of Motor Neurons and in Astrocytes in the Spinal Cord and Frontal Cortex from ALS Patients

DREAM and GFAP staining was performed in the frontal cortex and bulbar regions of the brain. In addition, lumbar region of the spinal cord from the ALS patients was also included in this study. Remarkably, DREAM staining showed astrocyte activation surrounding motor neurons in the spinal anterior horn and frontal cortex and bulbar brain regions (Fig. 5). DREAM localization was mainly found in the cytoplasm of motor neurons, very close to the nucleus of these cells, and even in the nucleus of some motor neurons. Additionally, it was also detected in the surrounding astrocytes, suggesting that the higher excitotoxicity generated at this terminal stage enhanced DREAM calcium-binding activity in different cells of the CNS and in different compartments inside these cells, especially in motor neurons. Therefore, DREAM could be acting as a calcium-binding protein in the cytoplasm, as a modulator of the surface expression and gating kinetics of Kv4 channels or as a transcriptional activator in the nucleus [25, 26].

Additionally, GFAP staining showed an intense astrocyte gliosis in the medullar anterior horn around motor neurons and in the frontal cortex (Fig. 6), which is in accordance with previous studies [27]. This increased immunoreactivity for GFAP could be enhanced by DREAM, reaching its highest levels at the end of the disease. These findings showed for the first time that in both spinal cord and brain from ALS patients, DREAM was not only linked to motor neurons but also to astrocytes, which contributed to reactive astrogliosis due to the neurodegenerative progression of the disease.

In connection with these results, the localization of DREAM out of the nucleus in the motor neurons suggested that excitotoxicity inherent to disease progression could prevent DREAM to act as a transcriptional repressor in the nucleus, thus favoring the activation of apoptotic genes [25, 28]. To further test this hypothesis, the number of surviving motor neurons was counted in the bulbar region of the brain and in cervical, thoracic, and lumbar anterior horn of spinal cord in ALS patients (Table 2). The percentage of motor neuron loss in the bulbar region of the brain was more evident in the patient that developed a bulbar form of the disease, while in the rest of the patients that presented a spinal form of the disease, both upper and lower motor neurons affected showed the highest motor neuron loss in the spinal cord regions. Moreover, caspase-3 and Bax staining was performed in the brain and spinal cord sections (Fig. 7). Intense staining of these apoptotic markers in the cytoplasm of the motor neurons supported the inhibition of anti-apoptotic activity of DREAM, prompting motor neuron loss.

Discussion

The fine-tuned among astrocytes and motor neurons is essential to maintain normally functioning synapses. Focusing on ALS, compelling evidence shows that mutant SOD1 astrocytes from mouse, rat, and humans effectively and selectively induce motor neuron death [29–31]. In addition, astrocytes from postmortem spinal cord tissue or from skin biopsies from FALS and SALS patients also induce motor neuron death by using a non-cell-autonomous toxicity [18, 29]. One of the altered molecular mechanisms that has been described in ALS is glutamate toxicity, which triggers an overstimulation of neuronal excitability, leading to an increase of intracellular Ca^{2+} concentration and consequently, to an amplification of excitotoxic damage. In this sense, one of the effects of riluzole, the current pharmacologic treatment used to enhance neuronal survival in ALS patients, has proven effect on the reduction of glutamate-induced excitotoxicity [32]. In particular, this glutamate excitotoxicity can be prompted in motor neurons by an excessive calcium-dependent release of glutamate from astrocytes [33].

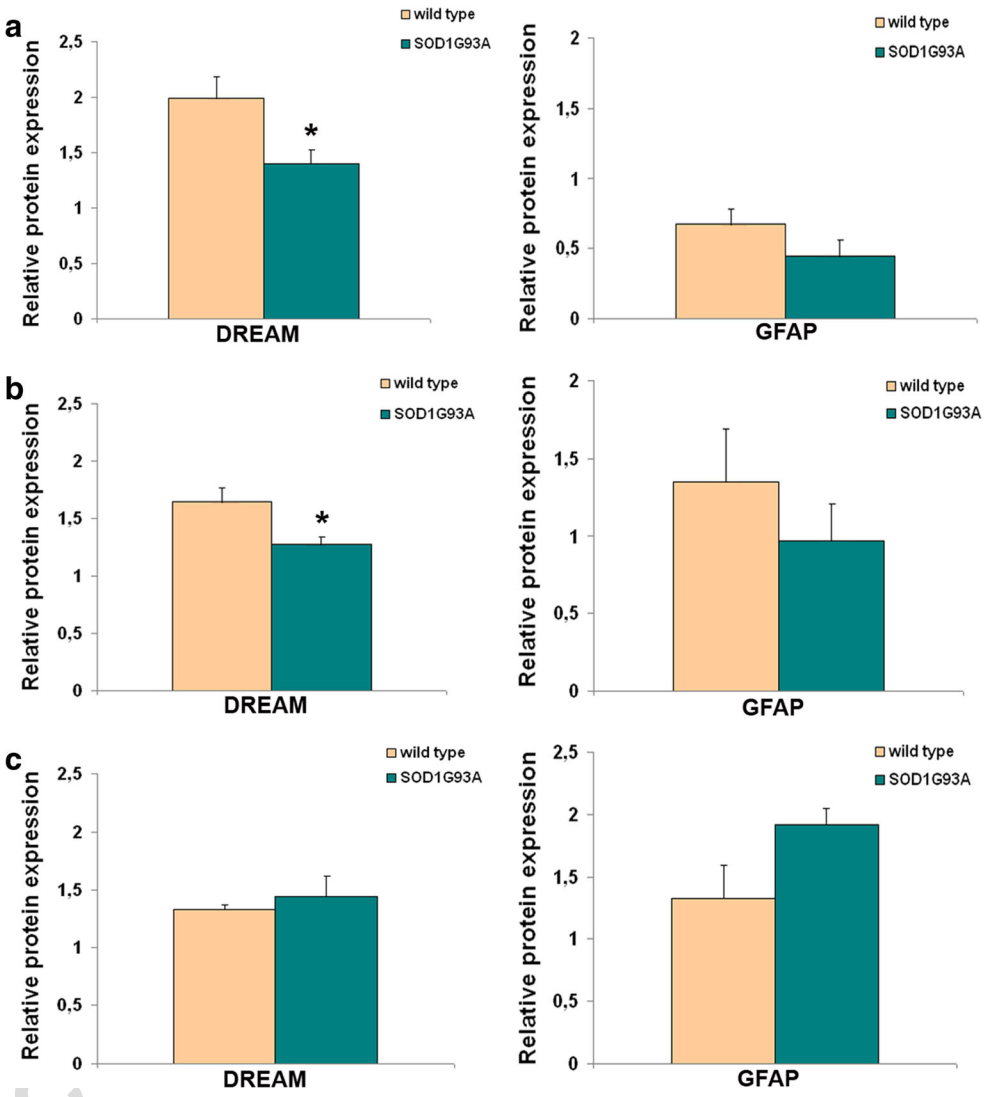

Fig. 4 DREAM and GFAP protein expression in the temporal lobe, motor cortex and brainstem areas from 16-week-old transgenic SOD1^{G93A} mice. Protein expression profiles of DREAM and GFAP in the temporal lobe (**a**), motor cortex (**b**), and brainstem (**c**) areas from 16-week-old transgenic SOD1^{G93A} mice. Wild-type littermate mice were used as control mice. ANOVA test showed significant differences in DREAM levels in the temporal lobe and motor cortex, $n = 4$ wild-type and $n = 4$ transgenic mice,*$p < 0.05$

Impairment in the synaptic glutamate concentration could enhance the presence of reactive astrocytes, which would participate actively in the neuronal degeneration and loss [27, 34]. One of the features that characterize this reactive phenotype in astrocytes is the increased content of GFAP [27, 34]. From the molecular point of view, GFAP gene expression can be activated by the transcriptional repressor DREAM, also known as calsenilin and KChIP3, during astrocyte differentiation [15]. DREAM is a calcium-binding protein that binds to a regulatory element called DRE and localized downstream from the transcription initiation site. This binding represses the transcription in the nucleus of target genes, such as prodynorphin, Na^+/Ca^+ exchanger *NCX3* gene, *c-fos*, and Fos-related antigen-2 (*fra-2*) [35, 36]. Outside the nucleus, DREAM can interact directly with calcium-dependent proteins and even inhibit *N*-methyl-D-aspartate receptor (NMDAR) function and its surface expression [36]. In connection with this point, DREAM could represent an alternative to significantly ameliorate NMDAR-mediated excitotoxicity, which has been described in ALS. In particular, reactive astrocytes by IL-1β can promote NMDA-mediated neurotoxicity in cortical cultures [34]. On the other hand, due to the multifunctional nature of DREAM, this protein can also play a proapoptotic role, enhancing the cleavage of Notch and contributing to neuronal death under ischemia-like conditions [37]. Considering that activated astrocytes may contribute to motor neuron death, we aimed to study the potential role of DREAM in the spinal cord and brain from both transgenic SOD1^{G93A} mice and post-mortem ALS patient samples.

In the spinal cord tissue from transgenic SOD1^{G93A} mice, DREAM was found significantly upregulated in the late symptomatic and terminal stages. This upregulation was coincident with an active activation of GFAP levels at the late symptomatic stage, suggesting that in this tissue, the high reactive response of astrocytes could promote an upregulation of DREAM till the terminal stage along disease progression. Since the denervation and neuronal loss has been described in earlier stages of the disease in this

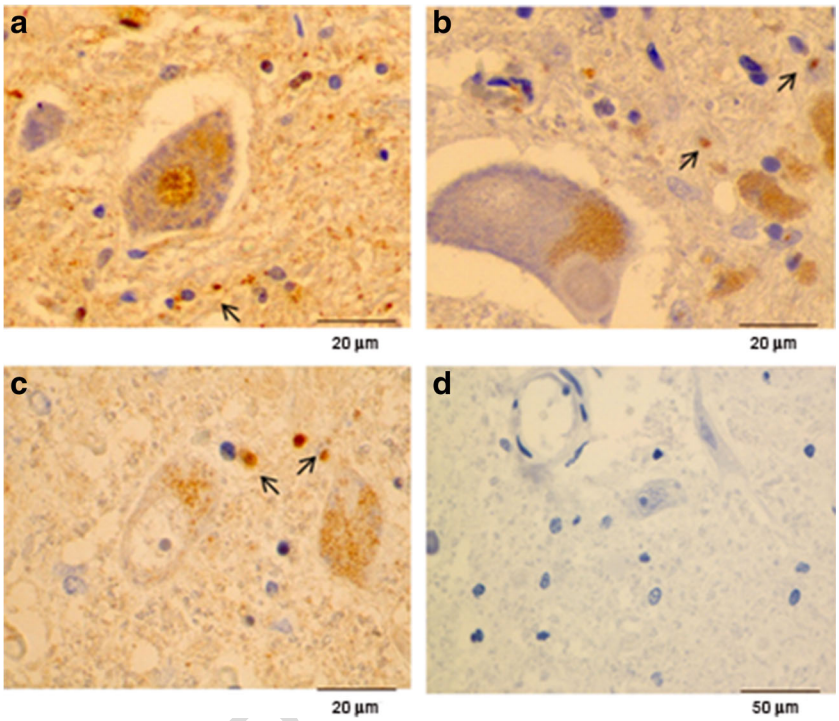

Fig. 5 Immunohistochemical staining of DREAM in brain and spinal cord samples from ALS patients. Strong positive expression of DREAM is localized inside and around the nucleus of the motor neurons, and in astrocytes (arrows). DREAM immunostaining in **a** frontal cortex, **b** dorsal region in brain, **c** anterior horn of spinal cord, and **d** control, frontal cortex

animal model [22, 23], the upregulated levels of DREAM run in parallel to the neuronal loss, especially during the later stages of the disease. At this step, two ways of action of DREAM could be possible: on the one hand, DREAM could promote neuronal death by modulating Ca^{2+} signaling, which is largely altered in ALS; and on the other hand, DREAM could repress apoptotic genes in the nucleus and it could also inactivate NMDAR, which are actively expressed in motor neurons. Considering that astrocytes participate actively in the neuronal degeneration process [27], the first hypothesis seemed more plausible in this animal model. To confirm this hypothesis, DREAM

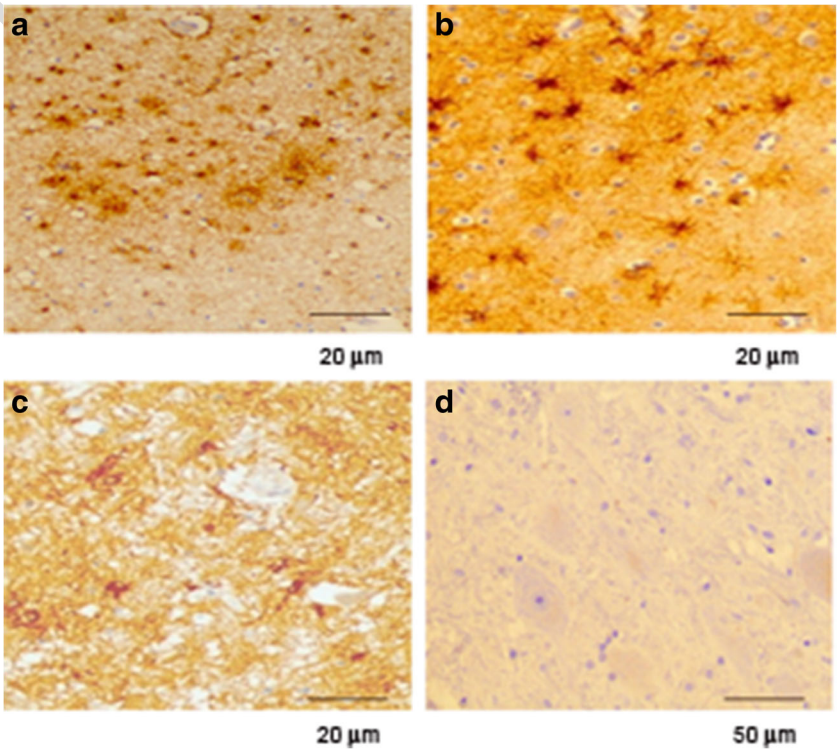

Fig. 6 Immunohistochemical staining of GFAP in brain and spinal cord samples from ALS patients. Strong positive expression of GFAP is localized in astrocytes. GFAP immunostaining in **a**, **b** frontal cortex, **c** anterior horn of spinal cord, and **d** control, frontal cortex

t2.1 **Table 2** Percentage of motor neuron loss in each region (bulbar, cervical anterior, thoracic anterior, and lumbar anterior) from ALS patients

t2.2 Cases	ALS-1	ALS-2	ALS-3	ALS-4
t2.3 Bulbar (hypoglosal nucleus)	70%	65%	83%	50%
t2.4 Cervical (anterior horn)	84%	86%	84%	34%
t2.5 Thoracic (anterior horn)	84%	81%	78%	56%
t2.6 Lumbar (anterior horn)	76%	70%	67%	87%

The four of the ALS patients are identified as ALS-1, ALS-2, ALS-3, and ALS-4

immunostaining was tested in spinal cord tissues from transgenic SOD1^{G93A} mice at the late symptomatic stage, when the motor neuron loss and denervation had started and the crosstalk between motor neurons and reactive astrocytes could be analyzed under the neurodegenerative conditions of the disease just before reaching its terminal stage. Intense and positive DREAM immunostaining in motor neurons and astrocytes supported the dual role of DREAM in this tissue, finally enhancing motor neuron death. In particular, as shown in Fig. 2, the presence of DREAM in the cytoplasm of motor neurons could be the first signs of motor neuron degeneration, acting as a compensatory response to Ca^{2+}-mediated cytotoxicity, to finally prompt neuronal death when DREAM was mainly localized in the nucleus of the cells. This finding is in clear connection with previous studies that showed a nuclear localization of DREAM in ipsilateral spinal dorsal horn neurons from a murine model of inflammatory pain [38, 39]. During the neurodegenerative progression of the disease, overactivation of NMDAR could trigger an increase in cytosolic free calcium concentration of motor neurons, prompting a translocation of DREAM protein to the nucleus and leading to the motor neuron death. However, the localization of DREAM in the cytoplasm near the cytoplasmic membrane could favor its potential role as a channel modulator, influencing on the biochemical and electrophysiological properties of membrane channels. Therefore, DREAM localization outside the nucleus could finally counteract the neurodegenerative and inflammatory response due to the progression of the disease.

The scenario in brain samples from transgenic SOD1^{G93A} mice was quite different. No alteration in DREAM levels was found, and a late significant upregulation in GFAP levels was observed at the terminal stage. This finding could indicate the presence of astrogliosis, especially at the end of the neurodegenerative progression of the disease, which has been described in this animal model in the ongoing progression of the disease [27]. For this reason, we analyzed DREAM and GFAP expression in three different regions of the brain from 16-week-old transgenic SOD1^{G93A} mice, the temporal lobe, motor cortex, and brainstem regions, which can be mainly affected by disease progression since these regions participate in the motor area of the brain, severely affected by the disease. Interestingly, GFAP levels did not vary at this stage in these brain regions, although DREAM levels were significantly downregulated, especially in the temporal lobe and motor cortex. Taking into consideration

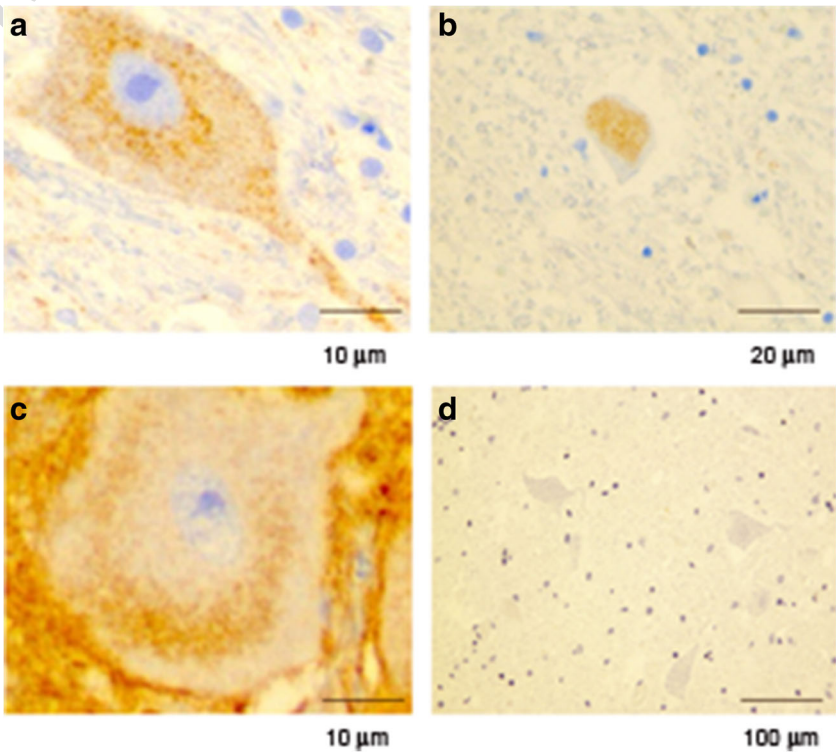

Fig. 7 Immunohistochemical staining of caspase-3 and Bax in brain and spinal cord samples from ALS patients. Strong positive expression of caspase-3 and Bax is localized in the cytoplasm and axons of motor neurons. GFAP caspase-3 immunostaining in **a**, **b** frontal cortex, Bax immunostaining, **c** anterior horn of spinal cord, and **d** control, frontal cortex

that these two brain regions are enriched in NMDAR [25], which can promote neuronal damage in ALS, excitotoxicity could influence NMDAR expression and activation, and therefore, DREAM could not exert its inhibitory effect on these receptors. These findings could indicate that at this terminal stage, the excitotoxicity, prompted by the disease progression, overactivated NMDA receptors (NMDAR), which are predominantly expressed in these brain regions, resulting in a downregulation of DREAM levels, in accordance with previous studies [25]. This downregulation of DREAM levels was coincident with a tendency to decreasing GFAP levels in these brain areas. In this sense, the NMDAR-mediated excitotoxicity in these specific areas could be exacerbated, prompting excitotoxic injury but not enhancing a higher astrogliosis response. Furthermore, dysfunction of astrocytic glutamate transporters, previously reported in SALS patients could favor a rise in the intracellular Ca^{2+} concentration, promoting an upregulation in GFAP protein in the whole tissue [40]. In particular, astrocytes produce an excess of transforming growth factor-β1 (TGF-β1) in spinal cord astrocytes from ALS patients and symptomatic mSOD1 mice, which deactivates microglia and accelerates disease progression [41].

These findings suggested that in the spinal cord from transgenic $SOD1^{G93A}$ mice, DREAM protein could exhibit its multifunctional properties depending on its localization in motor neurons, finally enhancing neuronal damage in coordination with astrocytes. In brain tissue from transgenic $SOD1^{G93A}$ mice, reactive astrogliosis was detected at the terminal stage of the disease in the whole tissue, probably favored by calcium-dependent cytotoxicity exacerbated at this last stage. Due to the fact that the animals were not kept alive till their real survival, we wanted to explore more in depth the implication of DREAM in ALS in human samples as well as to define the localization of this molecular marker inside the neurons and astrocytes in the very end of the progression of the disease. For this purpose, DREAM and GFAP staining was performed in the frontal cortex and bulbar regions of the brain, and in the lumbar region of the spinal cord from ALS patients. DREAM staining was detected in the cytoplasm of motor neurons, very closed to the nucleus of these cells, and even in the nucleus of some motor neurons. In addition, it was also detected in the nucleus of surrounding astrocytes, which was in accordance with the intense astrocyte gliosis observed in the samples. These findings suggested that astrocytes could express DREAM, which enhanced GFAP expression and finally astrogliosis. In addition, the specific localization of DREAM inside the nucleus and in the cytoplasm, near the cytoplasmic membrane in motor neurons, probably indicated that its calcium-binding activity in this cellular compartment was exacerbated and could be the consequence of the calcium-mediated excitotoxicity that reached higher levels in this terminal stage. In this sense, DREAM could be acting as a transcriptional repressor in the nucleus, thus favoring the activation of apoptotic genes, as well as a membrane channel modulator, as previously observed in the spinal cord from transgenic $SOD1^{G93A}$ mice [25, 28]. The percentage of motor neuron loss in the bulbar region of the brain and in cervical, thoracic, and lumbar anterior horn from spinal cord in ALS patients, together with the intense caspase-3 and Bax staining in motor neurons, support this hypothesis. These findings were in line with the ones obtained in the transgenic $SOD1^{G93A}$ mice, suggesting that DREAM could play a relevant role in the crosstalk between astrocyte and motor neuron. This crosstalk could be modulated under excitotoxicity along disease progression, favoring the anti-apoptotic nature of DREAM, resembling astrocyte TGF-β1 [41].

In summary, the direct interaction of astrocytes with motor neurons can become particularly vulnerable under neurodegenerative conditions in ALS. This altered interaction can involve a complex network of different cells, promoting the propagation of motor neuron loss. The findings obtained identified DREAM as a novel marker in motor neurons and astrocytes from transgenic $SOD1^{G93A}$ mice and postmortem ALS patient's samples. The calcium-dependent excitotoxicity progressively enhanced in the CNS in ALS could modulate the multifunctional nature of DREAM and its localization inside motor neurons, strengthening its apoptotic way of action in both motor neurons and astrocytes and finally acting as an additional factor to increase neuronal damage. The identification of this novel marker opens the door to future studies to characterize the specific upstream and downstream targets of DREAM to find new therapeutic strategies based on neuroprotection.

Acknowledgements This work was supported by the Instituto de Salud Carlos III y Fondos Feder: Grant PI14/00947, and Consolidated Groups from Gobierno de Aragón.

Compliance with Ethical Standards

Statement on the Welfare of Animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Competing Interests The authors declare that they have no competing interests.

Statement on Sample Extraction from ALS Patients Postmortem samples from ALS patients were obtained with written informed consent prior to inclusion in the study, which has been conducted according to Declaration of Helsinki principles, following the ethical rule of the Hospital Clínico Universitario from Zaragoza (Spain) and according to the Directive 2004/23/EC of the European Parliament and of the Council.

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