ORIGINAL ARTICLE

Liver
INTERNATIONAL

WILEY

Golexanolone improves fatigue, motor incoordination and gait and memory in rats with bile duct ligation

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Abstract

Background and Aims: Many patients with the chronic cholestatic liver disease primary biliary cholangitis (PBC) show fatigue and cognitive impairment that reduces their quality of life. Likewise, rats with bile duct ligation (BDL) are a model of cholestatic liver disease. Current PBC treatments do not improve symptomatic alterations such as fatigue or cognitive impairment and new, more effective treatments are therefore required. Golexanolone reduces the potentiation of $GABA_A$ receptors activation by neurosteroids. Golexanolone reduces peripheral inflammation and neuroinflammation and improves cognitive and motor function in rats with chronic hyperammonemia. The aims of the present study were to assess if golexanolone treatment improves fatigue and cognitive and motor function in cholestatic BDL rats and if this is associated with improvement of peripheral inflammation, neuroinflammation, and GABAergic neurotransmission in the cerebellum.

Methods: Rats were subjected to bile duct ligation. One week after surgery, oral golexanolone was administered daily to BDL and sham-operated controls. Fatigue was analysed in the treadmill, motor coordination in the motorater, locomotor gait in the Catwalk, and short-term memory in the Y-maze. We also analysed peripheral inflammation, neuroinflammation, and GABAergic neurotransmission markers by immunohistochemistry and Western blot.

Results: BDL induces fatigue, impairs memory and motor coordination, and alters locomotor gait in cholestatic rats. Golexanolone improves these alterations, and this was associated with improvement of peripheral inflammation, neuroinflammation, and GABAergic neurotransmission in the cerebellum.

Conclusion: Golexanolone may have beneficial effects to treat fatigue, and motor and cognitive impairment in patients with the chronic cholestatic liver disease PBC.

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1 | INTRODUCTION

Primary biliary cholangitis (PBC) is a cholestatic liver disease associated with behavioural symptoms such as fatigue and reduced memory and cognitive function. Fatigue is frequent in PBC patients and impairs their quality of life.[1] Long-term fatigue affects 68% of PBC patients but it is not related to the severity of liver disease.[2] Fatigue in patients with PBC remains stable over years and is independently associated with increased risk of death in general, and of cardiac death in particular.[3] High fatigue levels would be a predictor of risk of liver-related mortality.[4]

Newton et al[5] showed that 53% of PBC patients show cognitive symptoms with moderate or severe problems of concentration and/or memory, unrelated to histological markers of liver disease severity.

The only widely approved therapy for PBC is ursodeoxycholic acid (UDCA). UDCA may delay disease progression but has little impact on behavioural symptoms.[6] New treatments are therefore necessary to improve symptoms such as fatigue or cognitive impairment, and the approach to fatigue and its management, therefore, needs to run in parallel with the management of the underlying disease process.[7]

Fatigue in PBC has both cerebral and peripheral components. An association between increased peripheral inflammation and fatigue has been reported in various pathologies, including patients with rheumatoid arthritis, Parkinson's disease, multiple sclerosis or cancer.[8,9] Induction of fatigue by peripheral inflammation seems to be mediated by induction of neuroinflammation.[10,11] Morris et al[12] reviewed the contribution of immune dysregulations, associated cytokine abnormalities, and neuroinflammation to fatigue in diseases, such as Parkinson's disease, multiple sclerosis, cancer, or autoimmune diseases. They propose that enhanced pro-inflammatory factors alter neurotransmission leading to the induction of fatigue, which is associated with brain function alterations that can be observed by magnetic resonance imaging or PET scan studies.

PBC is characterized by brain abnormalities on cerebral magnetic resonance imaging.[13] These cerebral alterations may contribute to fatigue.

Alterations in GABAergic neurotransmission have been proposed to contribute to fatigue induction. Plasma levels of progesterone metabolites such as allopregnanolone and isopregnanolone (two inhibitory neuroactive steroids, neurosteroids) are increased in patients with chronic fatigue syndrome[14] and are higher than that in control subjects in PBC patients with fatigue, but not in those without fatigue.[15] Allopregnanolone and isopregnanolone are positive allosteric modulators of $GABA_A$ receptors and readily cross the blood–brain barrier. Enhanced activation of $GABA_A$ receptors by neuroinhibitory steroids may represent an important pathophysiological mechanism of fatigue in PBC.[15] Elevated serum allopregnanolone is associated with cognitive and emotional symptoms in PBC patients.[16] These authors proposed

Key points

Patients with the chronic cholestatic liver disease primary biliary cholangitis (PBC) show fatigue and cognitive impairment which strongly reduce their quality of life. There are no effective treatments for these alterations. We show that golexanolone, which reduces the potentiation of $GABA_A$ receptors activation by neurosteroids, improves fatigue and cognitive and motor impairment in a rat model of cholestasis. Golexanolone may be shown to be used in patients with cholestatic liver disease to improve these alterations which would greatly improve their quality of life.

acting on neurosteroids activation of $GABA_A$ receptors as a target to improve emotional and cognitive symptoms in PBC.[16]

The above reports support that peripheral inflammation, neuroinflammation, and altered GABAergic neurotransmission would contribute to fatigue in different pathologies, including PBC.

Rodents with bile duct ligation (BDL) are the most extensively used model of cholestasis.[17] Rats with bile duct ligation show cholestasis and fatigue.[18] Rats and mice with BDL also show cognitive and motor impairment.[19,20]

BDL rats also show neuroinflammation, with microglia activation and increased content of pro-inflammatory markers such as IL-1β, which are associated with impaired cognitive and motor function.[20] Neuroinflammation in the hippocampus and cerebellum, which alters neurotransmission, leading to neurological alterations, has previously been shown to contribute to cognitive and motor impairment in rats with hyperammonemia and hepatic encephalopathy.[21–24]

It has been shown in different animal models of hyperammonemia and hepatic encephalopathy that changes in peripheral inflammation and neuroinflammation are responsible for the cognitive and motor impairment. Preventing or reversing peripheral inflammation or neuroinflammation prevents or reverses cognitive and motor impairment.[20–27]

The aim of the present work was to assess in an animal model of cholestasis if reducing activation of $GABA_A$ receptors induced by allopregnanolone, improves BDL-associated symptoms, such as fatigue and cognitive and motor impairment and if this is associated with reduced peripheral inflammation and neuroinflammation.

To reduce the activation of $GABA_A$ receptors we have used golexanolone (GR3027), a novel drug in clinical development. Golexanolone is a $GABA_A$ receptor-modulating steroid antagonist which reduces the potentiation of $GABA_A$ receptors by allopregnanolone in animal models and in humans. Golexanolone is a promising therapeutic tool to improve cognitive and motor function in patients with hepatic encephalopathy.[28–30] The U.S. Food and Drug Administration (FDA)

recently granted golexanolone, a novel neurosteroid-based drug candidate for the treatment of PBC, with an Orphan Drug Designation (ODD). The present study is a preclinical modelling study to inform potential intervention studies in PBC patients.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Vehicle of the golexanolone treatment was CAPMUL MCM EP/NF (Glycerol monocaprylocaprate (Type I)), Medium Chain Mono- and Diglycerides, Ref.: 26402-22-2, 26402-26-6 from ABITEC Corporation. WI, USA. Golexanolone was obtained from Umecrine Cognition AB. Tris Base (BP152-1) from Fisher Scientific (ThermoFisher), Alcobendas, Madrid, Spain. NaCl (PHR1321), Supelco, Merck. EGTA (E4378) and EDTA (E4884) from Sigma-Aldrich. Protease inhibitor cocktail Set III, ref.: 539134, from Calbiochem, MerckMillipore. Sodium orthovanadate, S6508 and Sodium fluoride, 201154 from Sigma-Aldrich. Mayer's haematoxylin solution (MH532), from Sigma-Aldrich. EnVision™ Flex+ High pH kit (K8010) from DAKO. TNF alpha Rat Uncoated ELISA Kit (88-7340-22) from Invitrogen, ThermoFisher Sci., Alcobendas, Madrid, Spain. Sodium pentobarbital, dolethal, vetoquinol especialidades veterinarias, S.A. Madrid – España. The data on the antibodies used are cited in the corresponding sections.

2.2 | Bile duct ligation in rats and treatment with golexanolone

Male Wistar rats (175–200 g) from Charles River were used. Bile Duct Ligation was performed under isoflurane anaesthesia. A midline incision was made, and the common bile duct was localized, doubly ligated, and cut between these two ligatures. In sham animals, a midline incision was performed, but without BDL. The model is described in the International Society for Hepatic Encephalopathy (ISHEN) guidelines on animal models of hepatic encephalopathy.[31]

Rats were divided into four groups of six rats: sham-operated controls treated with vehicle (Sham VH), controls treated with golexanolone (Sham-GR); BDL rats treated with vehicle (BDL-VH), and BDL rats treated with golexanolone (BDL-GR). Golexanolone (40 mg/mL in CAPMUL) was administered daily using intra-gastric probes at 50 mg/kg (1.25 mL/kg) as in Mincheva et al[30] during 5 weeks. The experiment was repeated twice. A total of 12 rats per group were analysed. The experiments were approved by Comité Ético de Experimentación Animal (CEEA) of our centre and by Conselleria de Agricultura, Generalitat Valenciana, and performed according to the Directive of the European Commission (2010/63/EU) for the care and management of experimental animals and complied with the ARRIVE guidelines for animal research. The experimental design is shown in Figure 1.

2.3 | Behavioural tests

2.3.1 | Fatigue

Fatigue was measured in a treadmill. The procedure was a modification of Butterworth et al.[18] Rats were pre-trained on a single day and tested on the following day. Pre-training consisted of a first exploration for 3 min with the stationary belt inclined at 5 degrees and stopped. Then treadmill was started at 10 cm/s for 5 min and then at 20 cm/s for 5 min. For the test, rats were placed on the stationary belt inclined at 5 degrees with a gradually increasing speed of up to 30 cm/s during a 5-min period and continued at that speed for a further 15 min. The Anymaze video tracking software
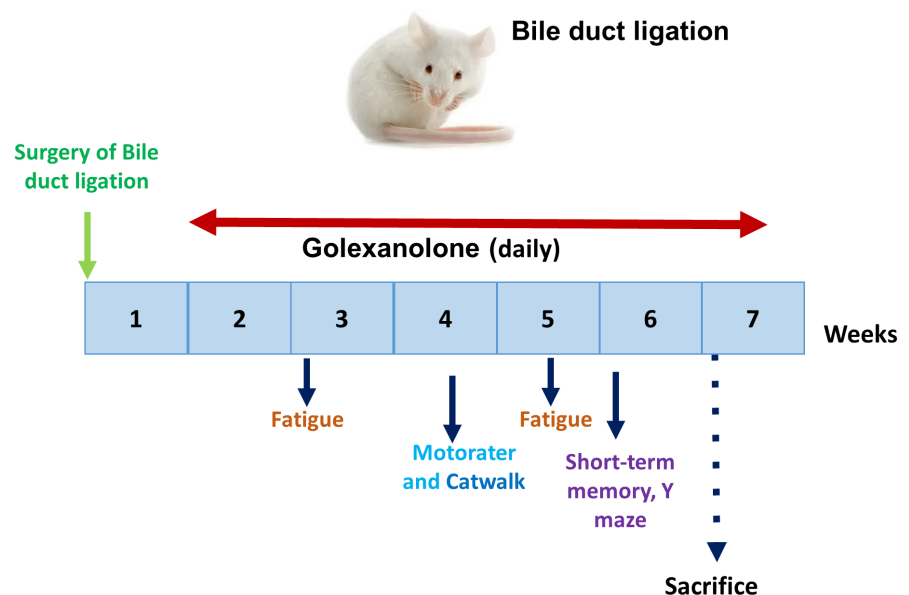

FIGURE 1 Scheme showing the experimental design.

was used to record and quantify the time spent on the grid (shock zone) and the number of times that the rats fall. Values recorded during the final 15 min of each trial were noted and referred to as fatigue scores.

2.3.2 | Short-term spatial memory

Short-term spatial recognition memory was analysed using a Y-maze. The rat was placed into one arm (start arm) and allowed to explore the maze with one arm closed, for 2 min (training trial) two times, with 1 min of inter-trial interval. In the test trial, performed after 1 min, the rat was allowed to explore both arms for 2 min. The number of entries into and the time spent in each arm were registered and a discrimination ratio [(time spent in the novel arm–time spent in the familiar arm)/total time passed in the two arms] was calculated.

2.4 | Motor function

Motor coordination and locomotor gait parameters were assessed after 2 weeks of golexanolone administration.

2.4.1 | Footprint analysis of locomotor gait in the CatWalk™

This is a video-based automated gait analysis system (Noldus, Wageningen, The Netherlands). Three trials were recorded each day during 2 days as in Ref. [30]. Data were analysed using the CatWalk analysis software (v 7.1) and are the mean of six runs.

2.4.2 | Motor coordination in the MotoRater

A kinematic analysis of motor coordination was conducted using MotoRater (TSE Systems, Germany) as in Ref. [32]. Each day for 3 days, three uninterrupted runs were recorded for each rat. The runs were analysed by counting and classifying the steps as correct or wrong paw placements. The results are expressed as a percentage of total steps and are the mean of nine runs.

2.5 | Liver function assessment

(a) Histological analysis of liver damage. Liver damage was analysed by histology using haematoxylin and eosin and Masson's trichrome staining of paraformaldehyde-fixed paraffin-embedded livers. The grade of steatosis (0–3), lobular inflammation (0–3), and fibrosis (0–4) were scored as in.[33] Grade of cholangitis (0–4) was scored as in.[34]

(b) Serum markers of liver damage. Serum markers were analysed in blood from the saphenous vein 5 weeks after BDL surgery. Activities of liver transaminases: GOT and GPT, and alkaline phosphatase, and the content of bilirubin and bile acids in these serum samples were analysed at Echevarne Laboratories.

(c) Ammonia in blood. Blood ammonia was measured with a commercial Kit in 20 μL of blood taken from the saphenous vein.

2.6 | Peripheral inflammation

Peripheral inflammation was analysed in plasma from the saphenous vein. TNFα was measured using an ELISA kit from Invitrogen. All other cytokines in plasma were analysed by Western blot (see below).

2.7 | Analysis of protein content

Protein content was analysed by Western blot in plasma and homogenates of the cerebellum. After 5 weeks of golexanolone treatment, cerebellum was dissected from 6 rats per group and homogenized in 50 mM TRIS–HCl pH 7.5, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA and protease and phosphatase inhibitors. Protein content was analysed by Western blot as in.[35] Primary antibodies used were anti-TNFα (AF-510-NA), anti-IL1β (AF-501-NA) 1:250 and anti-IFN-γ (MAB5851) 1:1000 from R&D Systems; anti-IL-6 (ARC0062) 1:500 from BIOSOURCE; anti-IL-17 (ab79056), anti-IL-18 (ab19160), anti-HMGB1 (ab18256) 1:1000 and anti- β-actin (ab6276-100) 1:5000 from ABCAM; anti-IL-15 (1829R) 1:2000 from BIOSS; anti-TGFβ (PA5-99186) 1:1000, anti-IL-10 (ab9969) and anti-CCL20 (AB9829) 1:1000 from ABCAM; anti-CCL5 (710001) 1:500 from Invitrogen and anti-CCL2 (66272-1-Ig) 1:2000 from Proteintech. Secondary antibodies (1:4000) against rabbit, mouse, or goat were IgGs conjugated with alkaline phosphatase (Sigma). The images were captured using a Hewlett Packard ScanJet 5300C and band intensities were quantified using AlphaImager 2200 software.

2.8 | Immunohistochemistry in brain sections

Rats were anaesthetized with sodium pentobarbital and subjected to transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and post-fixed in the same fixative for 24 h at 4°C. Paraffin-embedded sections (5-μm thick) were cut and mounted on coated glass slides, processed with the Envision Flex+ Kit (Dako) to block endogenous peroxidase activity for 5 min, and incubated with an anti-Iba-1 (1:300 Wako) or anti-GFAP (1:400 Sigma) antibody. The reaction was visualized by incubation with Envision Flex+ horseradish peroxidase for 20 min and then with diaminobenzidine for 10 min. The sections were counterstained with Mayer's haematoxylin for 3 min.

2.9 | Analysis of microglia and astrocytes activation

Analysis of Iba1 and GFAP staining was performed in the white matter and molecular layer of cerebellum from four rats per group using the Image J software. Microglial activation was analysed by measuring the area of Iba1-stained cells in 10 randomly selected 40× fields per section. The results are expressed in square micrometres. For GFAP quantification, the area of interest was selected. Using Auto Local Threshold and 'analyse particles' functions, the intensity thresholds and size filter were applied. To measure the total amount of GFAP, no size filter was applied. For each rat, at least 10 40× fields were quantified. The result was expressed as the percentage of area stained by GFAP.

2.10 | Statistical analysis

Results are expressed as mean±standard error. Statistical analyses were performed using the software GraphPad Prism 8.0. Normality was assessed using the D'Agostino and Pearson and the Shapiro–Wilk normality tests. Differences in variances of normally distributed data were assessed using Bartlett's test. Data with the same variance across groups were analysed by a parametric one-way analysis of variance (ANOVA) followed by Turkey post hoc test. Data with different variances across groups were analysed using Brown-Forsythe and Welch ANOVA tests followed by Dunnett's T3 multiple comparisons test. Data that did not pass normality tests were analysed with the non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. A confidence level of 95% was accepted as significant.

3 | RESULTS

3.1 | Golexanolone reduces fatigue in BDL rats at 2 and 5 weeks after surgery

Two weeks after surgery, BDL rats show more fatigue than controls, with increased number of shocks received (Figure 2A) and in the time remaining in the shock zone (Figure 2B). Golexanolone treatment for 1 week reduces fatigue in BDL rats, normalizing both the number of shocks (Figure 2A) and the time in the shock zone (Figure 2B). BDL rats also show increased fatigue at 4–5 weeks of surgery, with increased number of shocks (Figure 2C) and time in the shock zone (Figure 2D). Golexanolone treatment improved fatigue also in BDL rats at 4–5 weeks of surgery (Figure 2C,D), indicating sustained protection against fatigue by golexanolone.

3.2 | Golexanolone improves short-term memory in a Y-maze in BDL rats

BDL rats explore less time in the new arm, showing a lower discrimination index (Figure 2E), and therefore impaired short-term memory.
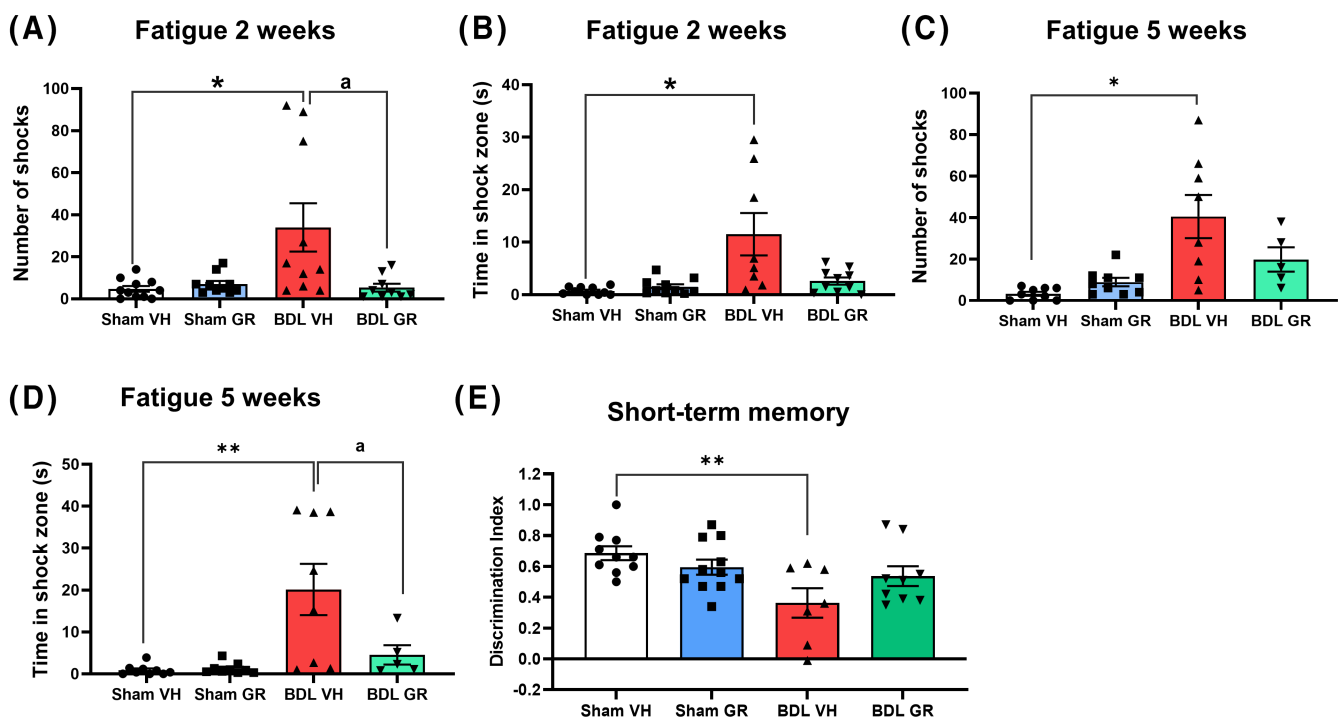

FIGURE 2 Golexanolone eliminates fatigue and improves short-term memory in BDL rats. Fatigue was analysed at 2 and 5 weeks after surgery. The number of shocks (A, C) and the time in the shock zone (B, D) were quantified. Discrimination ratio was calculated as indicated in Methods for short-term memory in the Y-Maze. (E) Values are the mean±SEM of 5–6 rats per group. Values significantly different from control rats are indicated by asterisk and from BDL-VH rats by 'a'. *$p<.05$; **$p<.01$; $^a p<.05$. GR, treated with golexanolone; VH, vehicle.

Golexanolone treatment induces a relevant improvement in the time exploring the new arm. Their discrimination index is not different from control rats (Figure 2E).

3.3 | Golexanolone improves motor incoordination in BDL rats

In the Motorater, BDL rats show a higher percentage of wrong foot placements than controls (Figure 3A) and a higher number of errors per run (Figure 3B), indicating motor incoordination. Treatment with golexanolone completely reversed motor incoordination (Figure 3A,B), restoring normal motor coordination.

3.4 | Golexanolone improves locomotor gait in BDL rats

The Catwalk provides many parameters that very finely characterize the gait, speed, position, etc. of the walking of rats. Some of these parameters are altered in BDL rats, indicating impaired locomotor gait (Figure S1). Golexanolone treatment improves many of these alterations (Figure S1).

The above data show that golexanolone improves fatigue, memory and motor coordination and gait in BDL rats. We then analysed if this is associated with improvement of liver damage, peripheral inflammation, and/or neuroinflammation.

3.5 | Golexanolone does not improve histological parameters of liver damage

The analysis shows the presence of several histopathological changes characteristic of a cirrhotic/cholestatic pathology in BDL

rats (Figure 4), that presented massive lobular inflammation, mainly in the vicinity of the portal area and large deposits of collagen (yellow arrows), leading to cirrhosis (asterisk). A massive ductal proliferation (circle) and several bile plugs (black arrowheads) were also observed in BDL rats (Figure 4A). Fibrosis, lobular inflammation, and cholestasis scores were significantly ($p<.0001$) increased in BDL rats treated or not with golexanolone. Golexanolone treatment did not have any effect on histopathological characteristics of the livers of BDL rats (Figure 4B). Golexanolone did not improve serum markers of liver damage (Figure S2).

BDL increases ammonia levels in the blood at 6 weeks after surgery to $119\pm20\,\mu M$, compared with $40\pm13\,\mu M$ in controls. Treatment with golexanolone does not affect ammonia levels, which remained at $101\pm28\,\mu M$.

3.6 | BDL induces peripheral inflammation, with increased levels of pro-inflammatory cytokines. Golexanolone treatment reduces the increase in some pro-inflammatory cytokines

As mentioned in the Introduction, BDL-induced fatigue and behavioural alterations may be mediated by peripheral inflammation. We therefore analysed if golexanolone reduces peripheral inflammation in BDL rats. BDL increases the plasma levels of pro-inflammatory cytokines TNF-α, IL-6, IL-17, IL-18, IFN-γ, HMGB1, and CCl2 (Table 1); reduces the levels of the anti-inflammatory IL-10 and does not affect IL-15, TGFβ, CCL20 or CCL5 (Table 1).

Golexanolone has beneficial effects on some aspects of peripheral inflammation. Golexanolone reduces plasma levels of TNF-α, IL-6, IL-17, and IL-18 and tends to reduce HMGB1 (Table 1) in BDL rats. Golexanolone does not affect IFN-γ, CCL2, or IL-10 (Table 1).

BDL-induced fatigue and behavioural alterations may be mediated also by induction of neuroinflammation. We therefore analysed
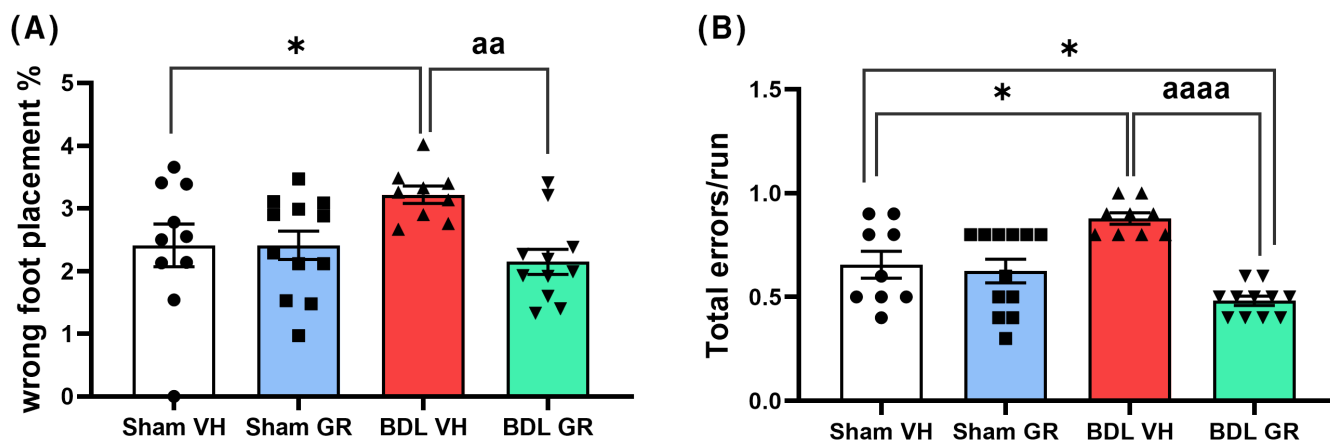

FIGURE 3 Golexanolone treatment improves the impairment of motor coordination in the Motorater. Motor coordination was assessed in the Motorater by analysing wrong foot placements (slips) when the rat crossed a ladder (A) and the total errors per run (B). Values are the mean±SEM of 6 rats per group. Values significantly different from control rats are indicated by asterisk and from BDL-VH rats by 'a'. *$p<.05$; $^a p<.05$; $^{aaaa}p<.0001$. GR, treated with golexanolone; VH, vehicle.
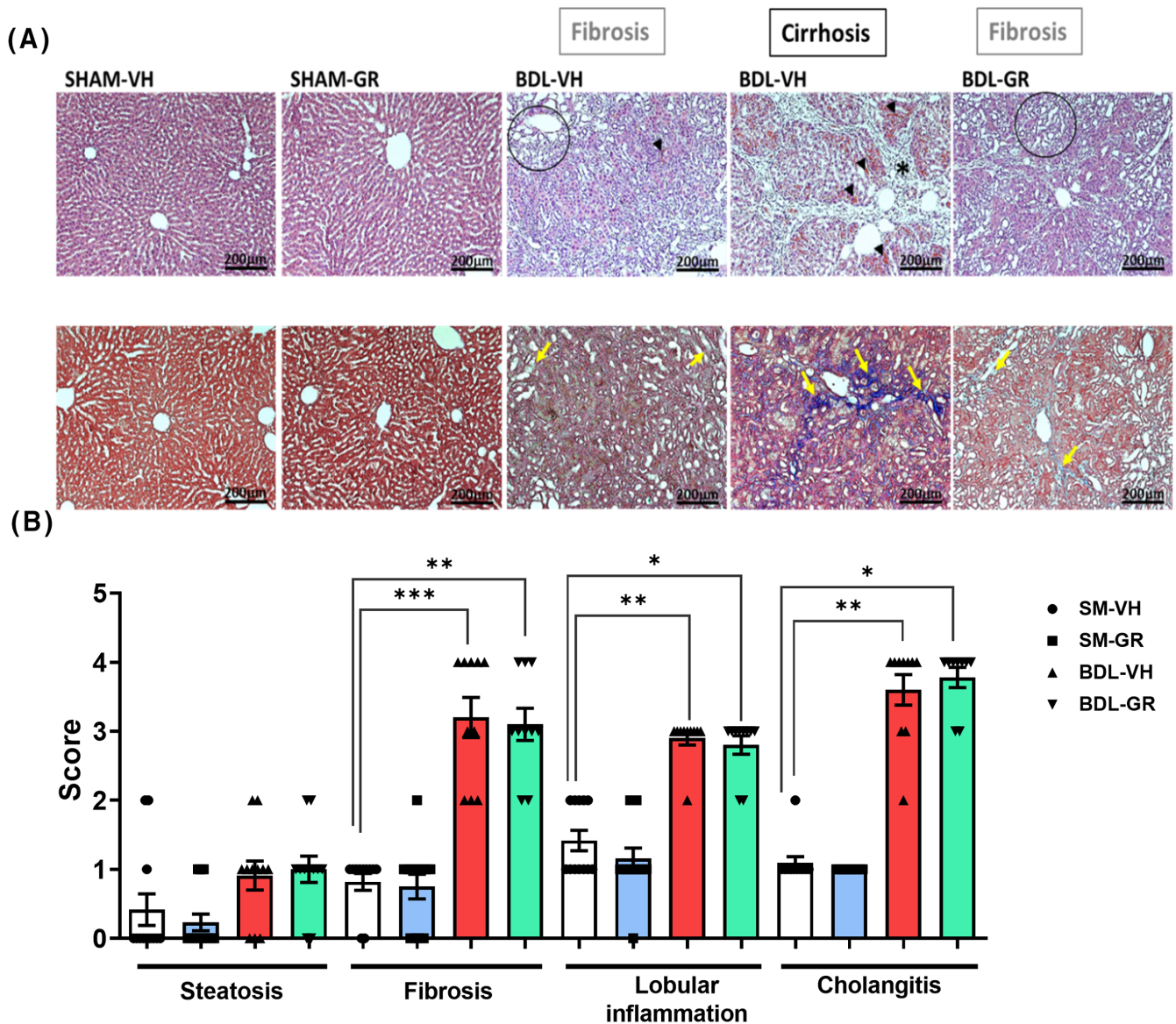
(A)

(B)

FIGURE 4 Golexanolone does not improve liver damage. Steatosis, fibrosis, lobular inflammation, and cholangitis were analysed in liver by immunohistochemistry (A, B). Representative images are shown in (A). Values are the mean $\pm$ SEM of 6 rats per group. Values significantly different from control rats are indicated by asterisk. **** $p < .0001$; GR, treated with golexanolone; VH, vehicle.

the effects of golexanolone on the activation of astrocytes and microglia and some pro-inflammatory markers in cerebellum.

3.7 | Golexanolone prevents the loss of GFAP in cerebellum of BDL rats

BDL rats sacrificed 6 weeks after surgery, a very advanced stage, showed a loss of GFAP staining in the cerebellum indicating late stage astrocyte damage. The area covered by GFAP staining was reduced to $81 \pm 6\%$ ($p < .05$) of controls. This loss of GFAP was reversed ($p < .05$) by treatment with golexanolone. In BDL rats treated with golexanolone, the area covered by GFAP returned to $99 \pm 2\%$ of controls (Figure 5A,D).

3.8 | Golexanolone reduces microglia activation in the molecular layer but not in the white matter of BDL rats

BDL rats show activation of microglia in the molecular layer. The area of microglial cells was reduced to $72 \pm 2\%$ of the area in controls ($p < .0001$). Treatment with golexanolone reduced microglia activation in the molecular layer ($p < .05$, compared with BDL without treatment) increasing the area of microglia to $87 \pm 1\%$ of controls ($p < .05$, compared with controls) (Figure 5B,E). Microglia was also activated in the white matter of BDL rats. The area of microglial cells was reduced to $85 \pm 2\%$ of the area in controls ($p < .01$). Treatment with golexanolone did not reverse microglia activation in white matter. The area was reduced to $77 \pm 5\%$ of controls ($p < .05$) (Figure 5C,F).

TABLE 1 Golexanolone treatment reduces the increase in some pro-inflammatory cytokines in plasma of BDL rats.

	Content in plasma		
Inflammatory marker	Sham-GR	BDL	BDL-GR
TNF-α (pg/mL)	2.4 ± 0.3 n = 12	7.5 ± 1.4** n = 8	3.9 ± 0.6*,a n = 6
IL-6	109 ± 5 n = 8	225 ± 16**** n = 8	182 ± 7****,a n = 10
IL-17	108 ± 7 n = 12	192 ± 13**** n = 7	144 ± 11*,a n = 6
IL-18	104 ± 8 n = 11	431 ± 64*** n = 10	227 ± 32*,a n = 6
IFN-γ	108 ± 6 n = 8	192 ± 17**** n = 6	175 ± 12*** n = 6
HMGB1	97 ± 9 n = 12	248 ± 30** n = 9	175 ± 20* n = 11
CCL2	98 ± 9 n = 11	166 ± 13** n = 6	138 ± 15* n = 6
IL-10	101 ± 10 n = 11	52 ± 10** n = 10	49 ± 6** n = 9
IL-15	111 ± 5 n = 12	94 ± 9 n = 10	103 ± 9 n = 11
TGF-β	120 ± 15 n = 12	108 ± 12 n = 11	99 ± 11 n = 11
CCL20	113 ± 10 n = 12	107 ± 9 n = 12	94 ± 12 n = 12
CCL5	110 ± 13 n = 10	130 ± 15 n = 12	117 ± 21 n = 10

Note: TNF-α was analysed in plasma using an ELISA kit. The remaining cytokines were analysed by Western blot and the data are expressed as percentage of control rats. Golexanolone reduces plasma levels of TNF-α, IL-6, IL-17 and IL-18. Values are the mean ± SEM of the indicated number of rats. Values significantly different from control rats are indicated by asterisk and from BDL-VH rats by 'a'.

Abbreviations: GR, treated with golexanolone; VH, vehicle.

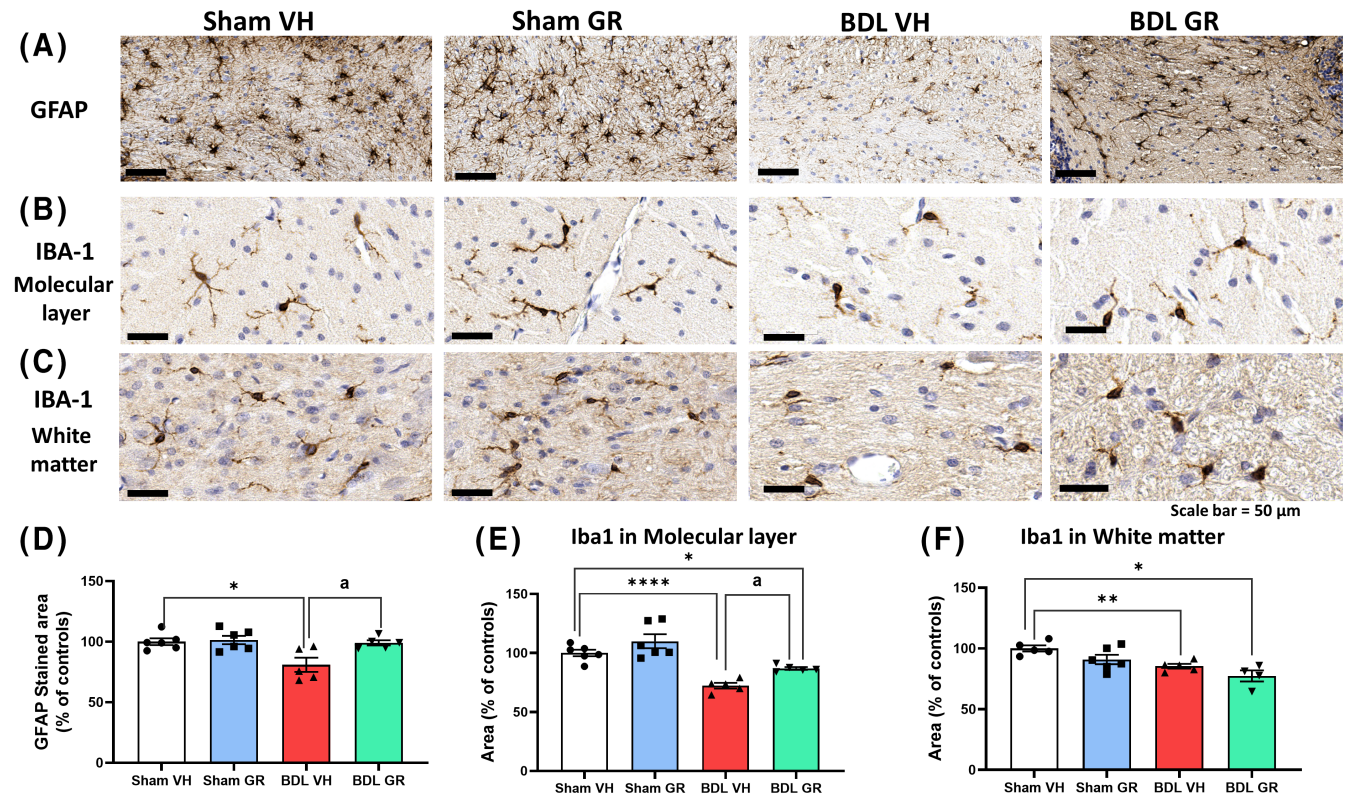*$p < .05$. **$p < .01$. ***$p < .001$. ****$p < .0001$.

a$p < .05$.

FIGURE 5 Golexanolone treatment prevents the loss astrocytes GFAP and reduces activation microglia in white matter of cerebellum of BDL rats. Representative immunohistochemistry images of GFAP staining are shown in (A) and of Iba1 in (B) and (C). The area stained by anti-GFAP was quantified (D) as well as the area of Iba1+ cells in white matter (E) and molecular layer (F). Values are the mean ± SEM of 4 rats per group. Values significantly different from control rats are indicated by asterisk and from BDL-VH rats by 'a'. *$p < .05$; **$p < .01$; ****$p < .0001$; a$p < .05$. GR, treated with golexanolone; VH, vehicle.

3.9 | Golexanolone reduces the content of pro-inflammatory factors in the cerebellum of BDL rats

BDL increases the content of pro-inflammatory cytokines IL-1β (Figure 6A); TNFα (Figure 6B) and IL-6 (Figure 6C) and glutaminase (Figure 6D) in the cerebellum. Treatment with golexanolone reversed the increase of these proteins (Figure 6A–D) in BDL rats. Golexanolone also increased the content of anti-inflammatory IL-10 in the cerebellum (Figure 6E).

3.10 | Golexanolone improves some alterations in GABAergic neurotransmission in the cerebellum of BDL rats

We also analysed the effects of BDL and golexanolone on key factors modulating GABAergic neurotransmission. GABA is synthesized by the glutamate decarboxylases GAD65 and GAD67. BDL or treatment with golexanolone did not affect the amount of GAD65 (Figure 7A). However, BDL increased GAD67 content and this increase was reversed by golexanolone (Figure 7B).

Extracellular GABA concentration is mainly modulated by GABA transporters GAT1 and GAT3. BDL reduced GAT1 content, and this reduction was partially but significantly reversed by golexanolone (Figure 7C). The content of GAT3 was not affected in BDL rats treated or not with golexanolone (Figure 7D).

The intensity of GABA$_A$ receptors activation is also modulated by the amount of GABA$_A$ receptors. BDL increased the content of the α2 (Figure 7E); β3 (Figure 7F) and γ2 (Figure 7G) subunits of GABA$_A$ receptors. Treatment with golexanolone reversed the increase in the β3 subunit (Figure 7F), but not in the α2 (Figure 7E) or γ2 (Figure 7G) subunits.

4 | DISCUSSION

The results reported here are summarized in Figure 8 and show that treatment with golexanolone eliminates fatigue and improves memory impairment and motor incoordination and improves locomotor gait in rats with BDL.

These preclinical data support clinical evaluation of the beneficial effects of golexanolone in PBC patients. Improvement of fatigue and of cognitive and motor function would greatly improve the quality of life of these patients.

Hepatic encephalopathy is defined as an alteration in cerebral function which is a consequence of previous liver failure.[36] The alterations reported here are consequence of liver failure and therefore are also considered as hepatic encephalopathy.

As commented in the Introduction, three main factors that may contribute to the induction of fatigue and cognitive and motor impairment in PBC patients and in BDL rats are peripheral inflammation, neuroinflammation, and altered GABAergic
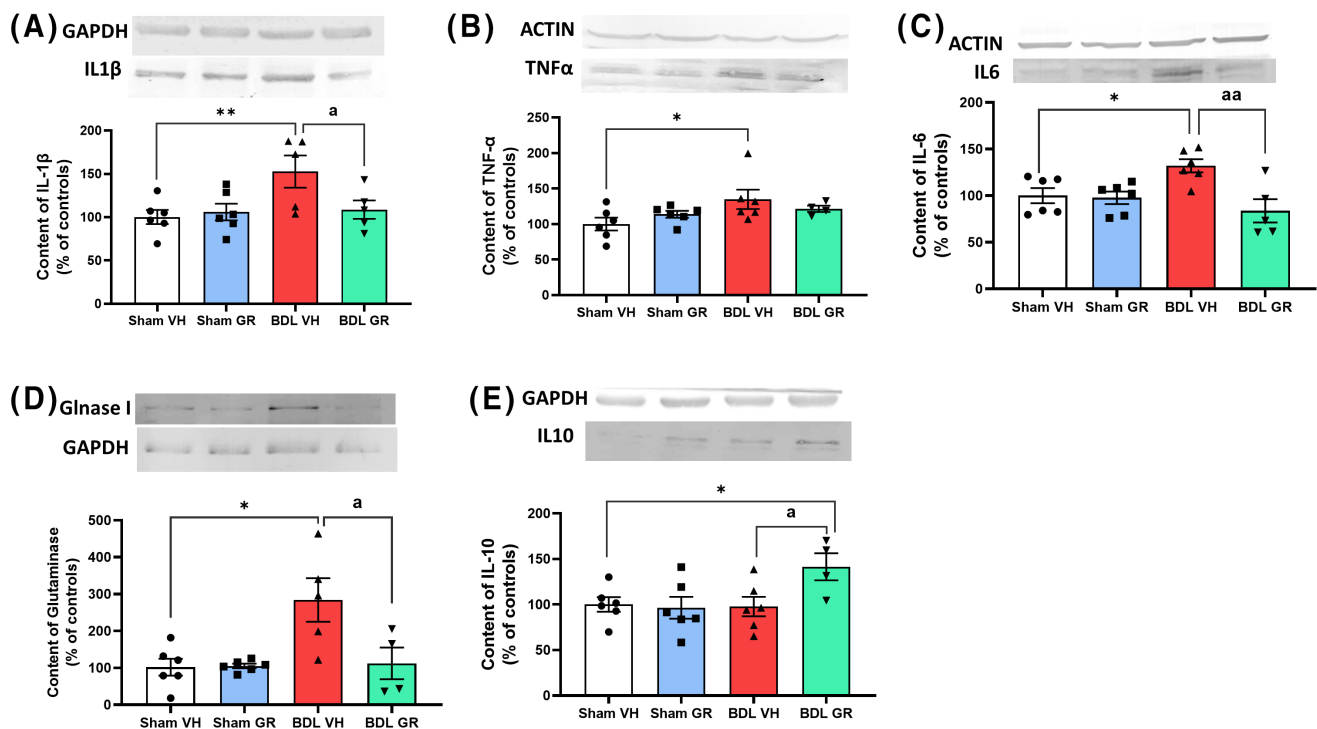

FIGURE 6 Golexanolone reduces the content of pro-inflammatory factors in cerebellum of BDL rats. The content in cerebellum of pro-inflammatory cytokines IL-1β (A); TNFα (B) and IL-6 (C), glutaminase (D) and IL-10 (E) were analysed by Western blot as described in Methods. Values are given as percentage of controls and are the mean±SEM of 6 rats per group. Values significantly different from control rats are indicated by asterisk and from BDL-VH rats by 'a'. *$p < .05$; **$p < .01$; $^a p < .05$; $^{aa} p < .01$. GR, treated with golexanolone; VH, vehicle.
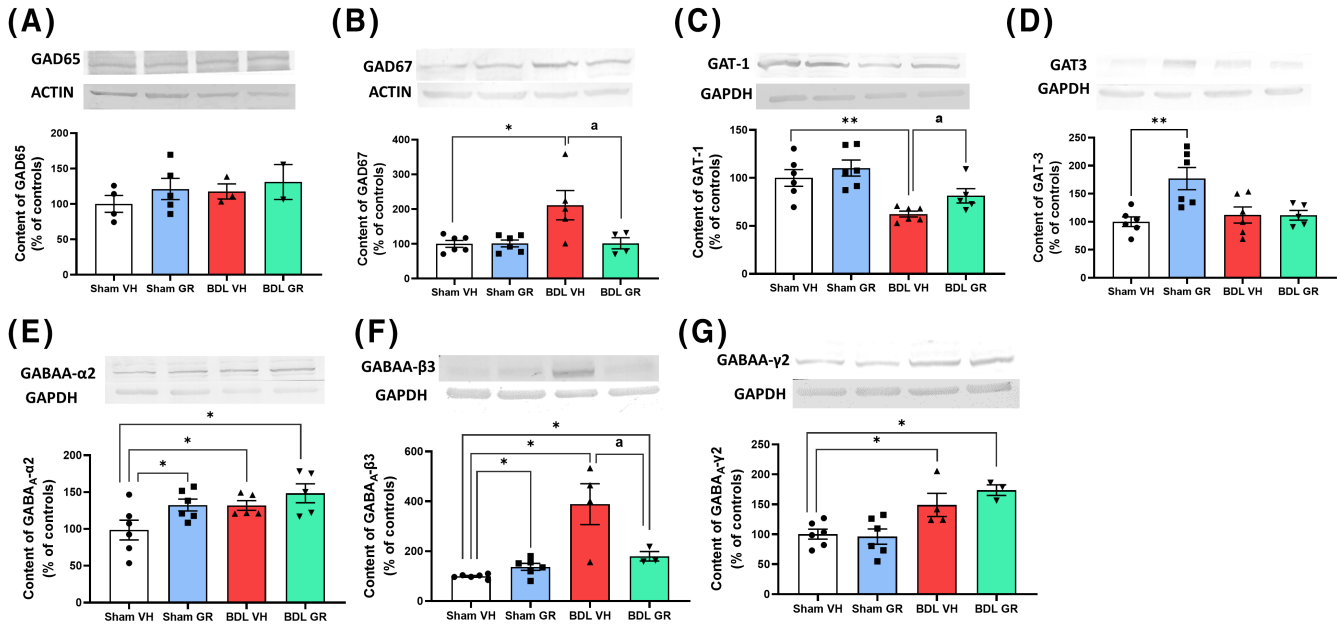
FIGURE 7 Golexanolone improves some alterations in GABAergic neurotransmission in cerebellum of BDL rats. We also analysed by Western blot the content in cerebellum of some key factors modulating GABAergic neurotransmission: GAD65 (A); GAD67 (B); GABA transporters GAT1 (C) and GAT3 (D) and the GABA$_A$ receptor subunits α2 (E); β3 (F) and γ2 (G). Values are given as percentage of controls and are the mean ± SEM of 6 rats per group. Values significantly different from control rats are indicated by asterisk and from BDL-VH rats by 'a'. $*p < .05$; $**p < .01$; $***p < .001$; $^a p < .05$. GR, treated with golexanolone; VH, vehicle.
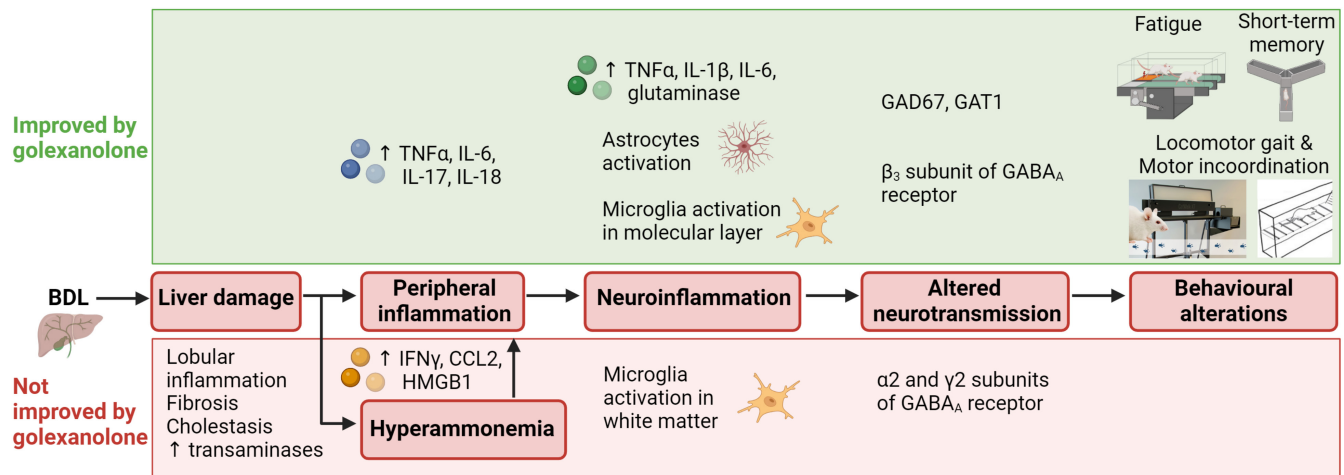

FIGURE 8 Scheme summarizing the effects of golexanolone on the different steps of the process leading to fatigue and cognitive and motor impairment.

neurotransmission. We show here that these three factors are all improved by golexanolone in BDL rats. Improvement of fatigue and of cognitive and motor function is associated with reduction of neuroinflammation, with the improvement of microglia activation and of astrocytes damage and reduction of pro-inflammatory IL-1β, TNFα, IL-6, and glutaminase and increase of anti-inflammatory IL-10 in the cerebellum. Behavioural and neuroinflammation improvements are also associated with the reduction of plasma levels of pro-inflammatory TNFα, IL-6, IL-17, and IL-18. Moreover, golexanolone treatment also improves some key changes which enhance GABAergic neurotransmission in the cerebellum of BDL rats: the increase in the GABA-synthesizing enzyme GAD67,

which would increase GABA levels; the reduction in the GABA transporter GAT1, which would increase extracellular GABA and the increase in the GABA β3 subunit of GABA$_A$ receptors, which would enhance activation of these receptors.

Reduction of GABAergic neurotransmission would be a consequence of reduced neuroinflammation. In the cerebellum of hyperammonemic rats, increased TNFα levels and activation of its receptor TNFR1 increase the pro-inflammatory cytokine IL-1β, glutaminase, and HMGB1 and enhances GABAergic neurotransmission by increasing GAD67, altering the content of GABA transporters GAT1 and GAT3 and increasing the content of GABA$_A$ receptor subunits. This enhanced GABAergic neurotransmission is responsible for motor incoordination

and impairment of locomotor gait.[32,37–39] A similar process would occur in the cerebellum of BDL rats and reduction of TNFα in the cerebellum by golexanolone would contribute to the reduction of proinflammatory factors and of GABAergic neurotransmission.

In hyperammonemic or BDL rats, preventing the induction of peripheral inflammation by injecting anti-TNFα or ibuprofen prevents the appearance of neuroinflammation and cognitive impairment.[20,27] The reduction of plasma levels of TNFα, IL-6, IL-17, and IL-18 by golexanolone would contribute to the reduction of neuroinflammation in the cerebellum of BDL rats and to the subsequent improvement of GABAergic neurotransmission, fatigue, and cognitive and motor function.

A similar process has been reported in hyperammonemic rats treated with bicuculline, an antagonist of GABA$_A$ receptors.[39] Golexanolone also reduces peripheral inflammation (TNFα in plasma) and neuroinflammation (microglia and astrocytes activation and TNFα levels) and GABAergic neurotransmission (content of GAD67 and β3 subunit of GABA$_A$ receptors) in the cerebellum of hyperammonemic rats.[30]

How reducing GABAergic neurotransmission by treatment with golexanolone or bicuculline reduces peripheral inflammation remains unclear. Further studies to identify the mechanisms involved would provide useful information to improve the treatment of PBC and of other pathologies.

Reduced peripheral inflammation would contribute to the reduction of neuroinflammation in the cerebellum of BDL rats by golexanolone, as discussed above. However, a direct effect on glial activation may also contribute to this beneficial effect of golexanolone. GABA$_A$ receptors are expressed both by microglia[40] and astrocytes.[41] Activation of GABA$_A$ receptors seems to induce activation of astrocytes.[42,43] A direct effect of golexanolone on GABA$_A$ receptors in astrocytes may also contribute to the reduction of astrocyte damage in BDL rats. Astrocyte activation is reflected in an increase in the area stained by GFAP by immunohistochemistry. This occurs at the early stages of hyperammonemia or liver damage.[26,44] In this report, BDL rats were sacrificed at an advanced stage and show a reduction in GFAP staining, which would be a more advanced stage of astrocyte damage than early activation. A loss of GFAP has been reported at advanced stages of liver disease, both in patients and in animal models. A reduction of GFAP immunoreactivity in different brain areas of BDL rats has been reported.[45] A reduction of GFAP staining and content has been also reported in human patients with hepatic encephalopathy by immunohistochemistry, ELISA, and Western blot.[46] This reduction of GFAP would represent an advanced stage of astrocyte damage, which is completely prevented by treatment with golexanolone.

Hiba et al[45] propose that reduction of GFAP-expression in BDL rats seem to be related to elevated ammonia levels. The presence of hyperammonemia in BDL rats has been already repeatedly reported, for example in the ISHEN guidelines.[31,47,48] It has been shown that ammonia levels are also increased in brain of BDL rats.[20,48] Hyperammonemia in BDL rats seems to be associated to fibrosis. Heidari et al[48] showed that increased levels of ammonia in blood and brain in BDL rats are reduced by treatment with taurine. They attribute this ammonia-lowering effect to the anti-fibrotic properties of taurine which might prevent liver failure and its consequent deleterious effects such as increase in blood and brain ammonia. In the present work, BDL increases ammonia levels in the blood at 6 weeks after surgery to $119 \pm 20\mu M$, compared with $40 \pm 13\mu M$ in controls. Treatment with golexanolone does not affect ammonia levels, which remained at $101 \pm 28\mu M$. These values are similar to those reported by Bosoi et al[47] and by Gimenez-Garzo et al[49] at 6 weeks after surgery. The ammonia levels at the time of behavioural test was not measured in the current work, but we showed previously that ammonia levels at 4–5 weeks, when we performed most behavioural tests are similar to those at 6 weeks.[49] Therefore, it may be assumed that ammonia levels were increased around twice at the time of behavioural testing. However, it must be highlighted that golexanolone does not reduce ammonia levels. It improves peripheral inflammation, neuroinflammation, and GABAergic neurotransmission in the cerebellum and fatigue, memory and motor coordination, locomotor gait without reducing hyperammonemia. Similar improvement of neuroinflammation and cognitive and motor function without reducing hyperammonemia have been reported in other rat models of hyperammonemia and hepatic encephalopathy.[20,24,27,44] This is because the effects of hyperammonemia on cognitive and motor function are mediated by peripheral inflammation and neuroinflammation and may be reversed by acting on these subsequent steps of the process.[21,30]

A few studies suggest that activation of GABA$_A$ receptors may increase microglia activation.[41,50] A direct effect on GABA$_A$ receptors could also contribute to the reduction of microglia activation in the molecular layer of the cerebellum by golexanolone.

Reduction of microglia activation and of astrocyte damage would contribute to the normalization of the levels of pro-inflammatory IL-1β, TNFα, IL-6, and glutaminase in the cerebellum of BDL rats by golexanolone, which in turn, would be responsible for the improvement of fatigue and cognitive and motor function. Omdal[10] proposes that peripheral inflammation triggers the activation of brain microglia, which produces IL-1β, which activates its receptor in neurons, triggering the fatigue response. A key role for peripheral inflammation and subsequent neuroinflammation in the induction of fatigue in patients with liver diseases, including PBC, has also been proposed.[11] The reduction of glial activation and damage and of IL-1β induced by golexanolone in BDL rats would contribute to the improvement of fatigue.

Improvement of motor and cognitive function by golexanolone would be mediated by the reduction to normal levels of TNFα in the cerebellum, which would normalize GABAergic neurotransmission and restore motor coordination and gait, and cognitive function, as discussed above.

Chronic fatigue is common in PBC patients and in other neuroinflammatory and chronic inflammatory diseases and many patients consider it as their worst problem.[1,4,10] These patients may also suffer cognitive and motor impairment, which in the case of advanced liver disease is named hepatic encephalopathy. Fatigue and cognitive and motor impairment strongly reduce the quality of life and life expectancy in these patients. We show here that treatment with golexanolone improves fatigue and cognitive and motor

impairment in BDL rats, a model of cholestasis. This is associated with improvements of peripheral inflammation and of neuroinflammation and GABAergic neurotransmission in the cerebellum. Golexanolone has been already shown to be safe and may be used in patients.[29] The U.S. Food and Drug Administration (FDA) has recently grantedan Orphan Drug Designation (ODD) to golexanolone, a novel neurosteroid-based drug candidate for the treatment of PBC. This preclinical modelling study shows that golexanolone is a promising therapeutic tool to improve fatigue and cognitive and motor function in patients with PBC and supports intervention studies in these patients.

CONFLICT OF INTEREST STATEMENT
A conflict of interest declaration for all authors.

DATA AVAILABILITY STATEMENT
Data are available from the corresponding author upon reasonable request.

ETHICS APPROVAL STATEMENT
The experiments were approved by Comité Ético de Experimentación Animal (CEEA) of our centre and by Conselleria de Agricultura, Generalitat Valenciana and complied with the ARRIVE guidelines for animal research.

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