

## Article

# A Flupirtine Benzyl Carbamate Improves Neurocognitive Deficits and Molecular Pathology in the *Cln6<sup>nclf</sup>* Mouse

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## Abstract

Neuronal ceroid lipofuscinosis type 6 (CLN6) is a fatal, autosomal recessive neurodegenerative disorder characterized by cognitive/motor impairment, vision loss, as well as neuronal loss and gliosis in the brain, and premature death. Onset typically occurs in childhood. No approved pharmacological treatments exist that halt or reverse disease progression. A novel flupirtine benzyl carbamate was orally administered to male and female *Cln6<sup>nclf</sup>* mice from 4 to 28 weeks of age to evaluate its neuroprotective and antispastic effects. Drug treatment produced significant, sex-dependent phenotypic improvements. Treated mice of both sexes exhibited reduced hindlimb spasticity, but only treated males demonstrated diminution in locomotor hyperactivity and recovery of visuospatial performance. In the brains of male and female *Cln6<sup>nclf</sup>* mice, flupirtine benzyl carbamate significantly decreased astrogliosis, microgliosis and mitochondrial ATP synthase subunit C (SCMAS) accumulation, increased neuronal marker expression and reduced the number of TUNEL-positive cells. The treatment failed to rescue photoreceptor loss or clear retinal SCMAS storage. These outcomes result in distinct sex-specific differences in neuronal vulnerability and drug responsiveness. Overall, these findings demonstrate that flupirtine benzyl carbamate diminishes key motor, visual and pathological deficits in CLN6 disease, highlighting its promise as a potential disease-modifying therapy for CLN6 in humans despite sex-specific differences.

**Keywords:** neuronal ceroid lipofuscinosis type 6 (CLN6 disease); *Cln6<sup>nclf</sup>* mouse model; flupirtine benzyl carbamate; behavioral phenotype; apoptosis; gliosis

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## 1. Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a group of fatal pediatric neurodegenerative storage disorders, manifesting the accumulation of mitochondrial ATP synthase subunit C (SCMAS) in the lysosomes of neurons and other cells [1–3]. NCLs are characterized by accelerated cell death rates in the brain and retina [4–8]. Clinically, they present in childhood with progressive vision loss, seizures, and cognitive and motor decline [9]. Fourteen NCL subtypes have been delineated based on distinct genetic/clinical phenotypes and specific cellular ultrastructural inclusions [10].

Autosomal recessive mutations in the *CLN6* gene underlie two genetically and clinically distinct phenotypes: a pediatric form referred to as variant late-infantile type (vLINCL) and a rare adult-onset form called Kufs disease, type A [11,12]. Children with *CLN6* disease typically develop symptoms between 2 and 4 years of age, including speech and developmental delay, seizures, vision loss, progressive cognitive decline, spasticity and loss of motor function [13]. Brain imaging shows cortical and cerebellar atrophy [14]. At the cellular level, *CLN6* mutations manifest the accumulation of storage material in neurons [15]. In particular, SCMAS accumulates in the lysosomes of *CLN6*-deficient cells [16]. Early-onset apoptosis and glial activation in the brain and retina are pathological hallmarks in humans and animal models of *CLN6* disease [5,17,18].

The human *CLN6* gene maps to chromosome 15q23 and encodes a highly conserved endoplasmic reticulum (ER)-resident membrane protein that consists of 311 amino acids with seven transmembrane domains [19–21]. Although its function is not fully defined, the *CLN6* protein is implicated in ER-to-Golgi trafficking of proteins and in maintaining cellular homeostasis [22]. Indeed, *CLN6* forms a complex with *CLN8* in the ER to facilitate anterograde transport of lysosomal proteins and also binds to collapsin response mediator protein 2 (CRMP2), implicating a role in cytoskeletal organization [22–24]. Most disease-causing *CLN6* mutations lead to a severely reduced or truncated protein that is unstable and rapidly degraded, resulting in a severe reduction in functional *CLN6* protein [15,25,26]. A naturally occurring *CLN6* animal model is the *Cln6<sup>nclf</sup>* mouse. It harbors a single-base insertion (c.307insC in exon 4) [21,27]. *Cln6<sup>nclf</sup>* mice recapitulate key features of human disease, including intracellular inclusions, retinal degeneration, limb paralysis, visual loss and premature death [5,27].

There is currently no approved therapy for *CLN6* disease. Treatment is supportive and aims to manage symptoms. While a clinical trial (ClinicalTrials.gov Identifier NCT02725580, first posted on March 1st, 2016) for *CLN6* disease evaluating the ICV delivery of *CLN6* by self-complementary AAV9 has been completed, no disease-modifying treatments are approved [28]. Given the scarcity of options, repurposing and optimizing small molecules that promote neuronal survival in the NCLs provides a promising pharmacological approach.

Flupirtine is a non-opioid analgesic with neuroprotective properties. It functions as a selective neuronal potassium channel activator that suppresses neuronal hyperexcitability with anti-apoptotic and neuroprotective effects [29–33]. Flupirtine has been investigated in various neurodegenerative contexts, including Creutzfeldt–Jakob, Alzheimer’s disease, and Multiple Sclerosis [34–37], and proposed for use in other NCLs [30,38,39]. Its pharmacologic properties sparked interest in testing flupirtine derivatives as a potential therapy in NCL, which has previously been limited by potential hepatotoxicity [40–43]. Safer analogues of flupirtine have been synthesized and tested [44,45]. Aromatic carbamate derivatives of flupirtine reduced pathogenic ceramide accumulation and rescued neuronal precursor cells from accelerated apoptosis in *CLN1*-, *CLN2*-, *CLN3*-, *CLN6*- and *CLN8*-derived lymphoblasts. Interestingly, *CLN6*-derived lymphoblasts treated with flupirtine benzyl carbamate demonstrated the highest viability rates [39].

Here, flupirtine benzyl carbamate (FBC) is explored as a therapeutic candidate for CLN6 disease *in vivo* using the naturally occurring *Cln6<sup>nclf</sup>* mouse model. No signs of hepatotoxicity were detected after oral drug treatment of male and female wild-type mice (WT) at a dose of 7 mg/kg for 13 weeks. Functionally, 28-week-old treated male and female mice exhibited significantly diminished spasticity. Yet, reduced hyperactivity and preserved behavioral aspects of vision were documented only in *Cln6<sup>nclf</sup>*-treated male but not in female mice. Evidence shows that flupirtine benzyl carbamate markedly reduced apoptosis in the brain of affected mice but not in the retinas. Reactive gliosis was substantially decreased in multiple brain regions in both sexes, whereas astrogliosis was not reduced in the retinas of male and female mice. Flupirtine benzyl carbamate diminished levels of accumulated SCMAS in the brains of males and females, but it had no effect on SCMAS accumulation in the retinas in both sexes. This work represents the first *in vivo* examination of the therapeutic effect of flupirtine benzyl carbamate in CLN6 disease and provides a foundation for an urgently needed pharmacological therapy for this fatal neurodegenerative disorder in humans.

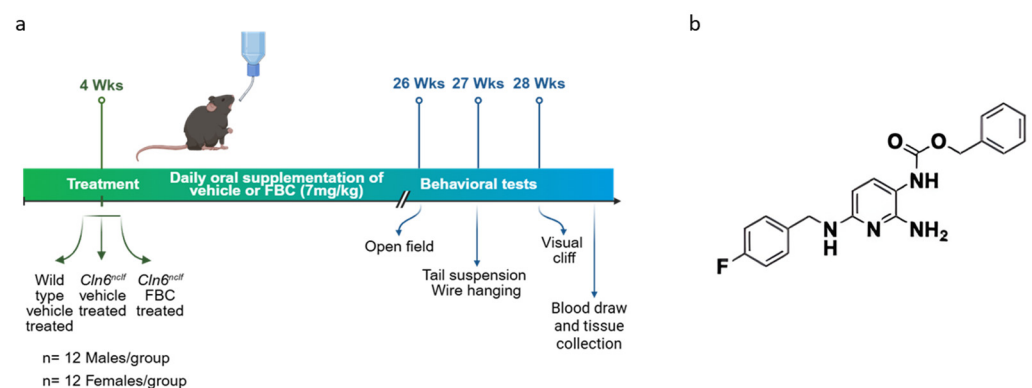
## 2. Materials and Methods

### 2.1. Animal Husbandry

All experimental procedures were performed in accordance with the American University of Beirut Institutional Animal Care and Use Committee (IACUC) guidelines (IACUC approval number: 24-10-RN497/642). *Cln6<sup>nclf</sup>* homozygous mice (stock number 002648) and WT C57BL/6J mice (stock number 000664) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Male and female mice were housed in a 12-hour light/12-hour dark cycle with *ad libitum* access to food and water. Ambient temperature was maintained daily between 18 and 26 °C and relative humidity between 30% and 70%. Animals were monitored weekly for general health and behavior, with body weights recorded. All adverse events were handled according to veterinary guidelines.

### 2.2. Flupirtine Benzyl Carbamate Treatment

At 4 weeks of age, mice were randomly assigned to three groups: WT control mice and *Cln6<sup>nclf</sup>* mice receiving a vehicle solution of 0.5% dimethyl sulfoxide (DMSO; AMRESCO, Solon, OH, USA), and *Cln6<sup>nclf</sup>* mice treated orally with 7 mg/kg of flupirtine benzyl carbamate (synthesized in the laboratory of Dr. Paul C. Trippier, Figure 1b) dissolved in 0.5% DMSO. Each group comprised 12 male and 12 female mice, totaling 72 mice. Mice were dosed orally for 24 weeks by supplying the compound in the drinking water *ad libitum*, with an average daily consumption of  $\approx 8$  mL/mouse. Drug exposure was supported by a single-dose pharmacokinetic characterization of 7 mg/kg in WT mice, which indicated rapid absorption and measurable systemic exposure ( $T_{max}$  30 min;  $C_{max}$  1154.8 ng/mL; absolute oral bioavailability 64.4%).



**Figure 1.** Timeline for experimental design and FBC chemical structure. (a) *Cln6<sup>nclf</sup>* mutant mice and WT C57BL/6J mice of both sexes were supplemented daily with vehicle or FBC (flupirtine benzyl carbamate) at 7 mg/kg, beginning at 4 weeks of age. Behavioral assessments were conducted at 26 weeks (open-field test), 27 weeks (wire hanging and tail suspension tests) and 28 weeks (visual cliff test). At 28 weeks of age, the study endpoint, mice were euthanized and tissues collected for molecular and histochemical analyses. This treatment and testing schedule were designed for evaluation of drug effect on disease progression in the *Cln6<sup>nclf</sup>* model, with WT mice included as controls. (b) Chemical structure of benzyl (2-amino-6-[(4-fluorobenzyl)amino]pyridin-3-yl)carbamate, the flupirtine-based small-molecule analogue used in this study.

### 2.3. Toxicity Assessment

A subacute 17-week toxicity study was conducted in WT mice to evaluate the safety profile of flupirtine benzyl carbamate. The animals were equally divided into vehicle-treated controls ( $n = 8$ ; 4 males, 4 females) and treatment groups ( $n = 8$ ; 4 males, 4 females), with the test drug administered via drinking water, while control animals received vehicle only. Throughout the dosing period, mice were observed daily for clinical signs of toxicity and were weighed three times a week to monitor drug-related effects on body weight. At study termination, blood samples were collected for serum chemistry analyses of standard hepatic (Aspartate Aminotransferase; AST, Alanine Aminotransferase; ALT) and renal (Blood Urea Nitrogen; BUN, creatinine) biomarkers. Livers and kidneys were harvested, fixed and processed for histopathological examination. Tissue sections were stained with hematoxylin and eosin (H&E).

### 2.4. Behavioral Tests

WT vehicle-treated, *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated male and female mice were subjected to behavioral assays to assess locomotion, exploratory and anxiety behavior, motor strength and visual perception. All tests were conducted using established protocols. Mice were acclimated to the testing room for 60 min before tests, and experiments were performed between 9:00 am and 12:00 pm under bright, uniform lighting. Male and female groups were tested in separate cohorts.

The open-field test assesses mouse locomotor activity, anxiety and exploratory ability. As previously described [46], 26-week-old mice were placed in the periphery of an open-field chamber (50 cm × 50 cm × 30 cm high), isolated from the researchers. During 10 min of exploration, the number of walling and rearing events was manually counted, and parameters, including total distance traveled (cm) and velocity (cm/s), were recorded by an overlying camera and displayed on the EthoVisionXT software v.17.5 (Noldus Information Technology, Wageningen, The Netherlands).

The wire hanging test assesses neuromuscular strength and motor coordination [47]. Twenty-seven-week-old mice were placed in the center of a hanging wire, where movement was monitored for 90 s. The latency for the mouse to fall off the wire was recorded. Each mouse was tested in five trials, with 15 min rest between trials.

The tail suspension test measures hindlimb claspings as a readout of spasticity [48,49]. Twenty-seven-week-old mice were held by their tails for a period of 30 s and observed for any splaying (less spasticity) or flexing (more spasticity) before being placed back in their cages. Hindlimb claspings was evaluated using a 0–3 scoring system: 3, both hindlimbs fully clasped; 2, hindlimbs completely retracted and contacting the abdomen for >50% of the observation period; 1, both hindlimbs partially retracted toward the abdomen for > 50% of the observation period; and 0, hindlimbs consistently splayed outward away from the abdomen, with toes splayed.

The visual cliff test evaluates mouse vision and depth perception [50,51]. Twenty-eight-week-old mice were placed in an open-field chamber divided into a safe

(checkerboard pattern) zone and an unsafe (clear plexiglass overlying a “cliff”) zone. Mice were placed in the corner of the safe zone and allowed to roam the chamber freely for 5 min. An overlying camera recorded movement and displayed data on the EthoVisionXT software for further analysis. The time spent in both zones was recorded.

## 2.5. Tissue Collection and Histological Processing

Twenty-eight-week-old adult male and female mice were anesthetized by intraperitoneal injection of a 2:1 mixture of ketamine to xylazine (100 mg/kg and 10 mg/kg, respectively). Following blood collection (1 mL) from the inferior vena cava, mice were immediately prepared for cardiac perfusion. An incision was made in the right atrium, and phosphate-buffer solution (PBS; Corning®, Mediatech Inc., Manassas, VA, USA) was injected into the left ventricle, followed by fixation with 4% paraformaldehyde (PFA; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) solution. Mouse brains and eyes were post-fixed for 2 h in 4% PFA at 4°C. After fixation, tissues were cryoprotected by immersion in 20% sucrose to prevent freezing artifacts. Organs were then embedded in optimal cutting temperature (OCT; Kaltek S.r.l., Saonara, Italy) at -40°C. Frozen brains and eyes were sectioned in the coronal plane into 15 µm-thick sections via a cryostat at -20°C, mounted onto Superfrost Plus microscope slides, air-dried and stored at -20°C until further processing.

## 2.6. Corticosterone Assay

Total serum corticosterone was quantified by ELISA using the DetectX® Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI, USA) per manufacturer instructions. A 5 µL aliquot of serum was treated with an equal volume of kit dissociation reagent, incubated at room temperature (RT) for 5 min, then diluted with 490 µL of 1X assay buffer. Standards, controls, and samples were assayed in duplicates in 96-well microplates. Absorbance was measured at 450 nm. Concentrations were calculated using a four-parameter logistic (4PLC) standard curve, subtracting the mean non-specific binding (NSB) signal.

## 2.7. Hematoxylin and Eosin (H&E) Staining

Hematoxylin and Eosin (H&E) staining was performed on 5 µm-thick paraffin-embedded mouse kidney and liver sections to assess flupirtine benzyl carbamate toxicity. Sections were deparaffinized in xylene and rehydrated through a graded ethanol series of 100% to 70%. After rehydration, sections were stained with hematoxylin to visualize nuclei and rinsed in running water to remove excess stain. Sections were then counterstained with eosin to highlight cytoplasmic elements, dehydrated in ascending ethanol concentrations, cleared in xylene and mounted on coverslips with DPX mounting medium (Sigma-Aldrich, Saint Louis, MO, USA). Bright-field images were acquired with a Leica light microscope (Germany).

## 2.8. Immunohistochemistry

Brain and retinal cryosections were air-dried at RT for 15 min, washed in 1X PBS (2 × 5 min) and permeabilized in 1X PBS containing 0.1% Triton X-100 (PBST, AMRESCO, Solon, OH, USA) twice for 10 min. Sections were blocked for 1 hour in 10% fetal bovine serum diluted in PBST and incubated with the primary antibody overnight at 4 °C. After three washes in PBS (3 × 5 min), sections were treated with 0.01% (w/v) Sudan Black (MICROME/Edward Gurr Ltd., London, UK) in 70% ethanol for 40 min to reduce autofluorescence. The tissues were washed in PBST (3 × 10 min) and incubated with the appropriate secondary antibody for an hour at RT. After two final PBS washes (2 × 5 min), nuclei were counterstained with Hoechst (1 µg/ml in 1X PBS), and sections were mounted using Fluoromount (Sigma-Aldrich, Saint Louis, MO, USA). The following primary antibodies

were used: rabbit anti-NeuN (Abcam, ab104225; 1:500), rabbit anti-GFAP (Abcam, ab7260; 1:1000), rabbit anti-SCMAS (Abcam, ab181243; 1:200), rabbit anti-IBA1 (Abcam, ab178846; 1:200), rabbit anti-rhodopsin (Abcam, ab221664; 1:500) (Abcam, UK) and rabbit anti-cone-arrestin (Sigma-Aldrich, AB15282; 1:500) (Chemicon, Germany). Images were acquired via a Leica fluorescence microscope and processed with ImageJ software v1.54r. Fluorescence intensity was quantified as the ratio of integrated density to total image area. NeuN-positive cells were manually counted.

### 2.9. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The TUNEL assay labels 3'-hydroxyl termini of DNA strand breaks generated during apoptosis. Brain and retinal cryosections were fixed in 4% PFA for 15 min at RT and permeabilized with proteinase K (20 µg/mL) for 5 min at RT. Sections were treated with 50 µl TUNEL reaction mixture from the In Situ BrdU-Red DNA Fragmentation Kit (Abcam, ab66110, UK), freshly prepared by combining the enzyme and label solutions at a 1:10 ratio. Sections were incubated with TdT enzymes for one hour at 37 °C in a humidified dark chamber, followed by PBS washes to remove residual TdT enzyme. To visualize apoptotic nuclei, sections were incubated with Anti-BrdU-Red antibody for 30 min at RT in a dark chamber and imaged by fluorescence microscopy. TUNEL-positive cells were counted (ImageJ software).

### 2.10. Western Blot

Frozen mouse brain tissue (30 mg) was crushed and homogenized in RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with a proteinase inhibitor cocktail. Lysates were incubated on ice for 30 min and centrifuged at 15,000 × g for 10 min at 4 °C. Supernatants were collected, and protein concentration was determined via a BCA assay. Equal amounts of protein were resolved on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk prepared in Tris-buffered saline containing 0.1% Tween (TBST-0.1%) for 30 min at RT followed by overnight incubation at 4 °C with the following primary antibodies: rabbit anti-LC3 (Abcam, ab192890; 1:2000), rabbit anti-p62 (Abcam, ab91526; 1:1000) (Abcam, UK), rabbit anti-LAMP1 (Cell Signaling Technology, 3243S; 1:1000) and rabbit anti-GAPDH (Cell Signaling Technology, 2118S; 1:1000) (Danvers, MA, USA). Membranes were washed with TBST (3 × 5 min) and incubated with a secondary HRP-coupled anti-rabbit antibody (Cell signaling technology, 7074P2; 1:1000) for 1 hour at RT and washed again in TBST (5 × 5 min). Bound proteins were visualized via enhanced chemiluminescence and imaged on a ChemiDoc system (BioRad, Hercules, CA, USA). Band intensities were quantified by densitometric analysis in ImageJ and normalized to GAPDH levels.

### 2.11. Proteome Profiler Mouse Apoptosis Array

Expression of apoptosis-related proteins was examined with the Proteome Profiler Mouse Apoptosis Array Kit (R&D Systems, catalog ARY031) (Minneapolis, MN, USA), a membrane-based sandwich immunoassay validated for mouse tissue lysates. Tissue lysates (200–400 µg total protein) were prepared in Lysis Buffer 17 and applied to nitrocellulose membranes pre-spotted in duplicate with capture antibodies against 21 different apoptotic markers. After a one-hour block in Array Buffer 1 at RT, lysates were incubated overnight at 4 °C with gentle rocking. The membranes were washed, incubated sequentially with a biotinylated detection antibody cocktail and streptavidin-horseradish peroxidase, and then developed using the supplied chemiluminescent reagents. Spot intensities were captured using a ChemiDoc system (BioRad, Hercules, CA, USA) and quantified using Image Lab software (version 6.1.0 build 7). Duplicate signal intensities were averaged for each analyte and background was subtracted.

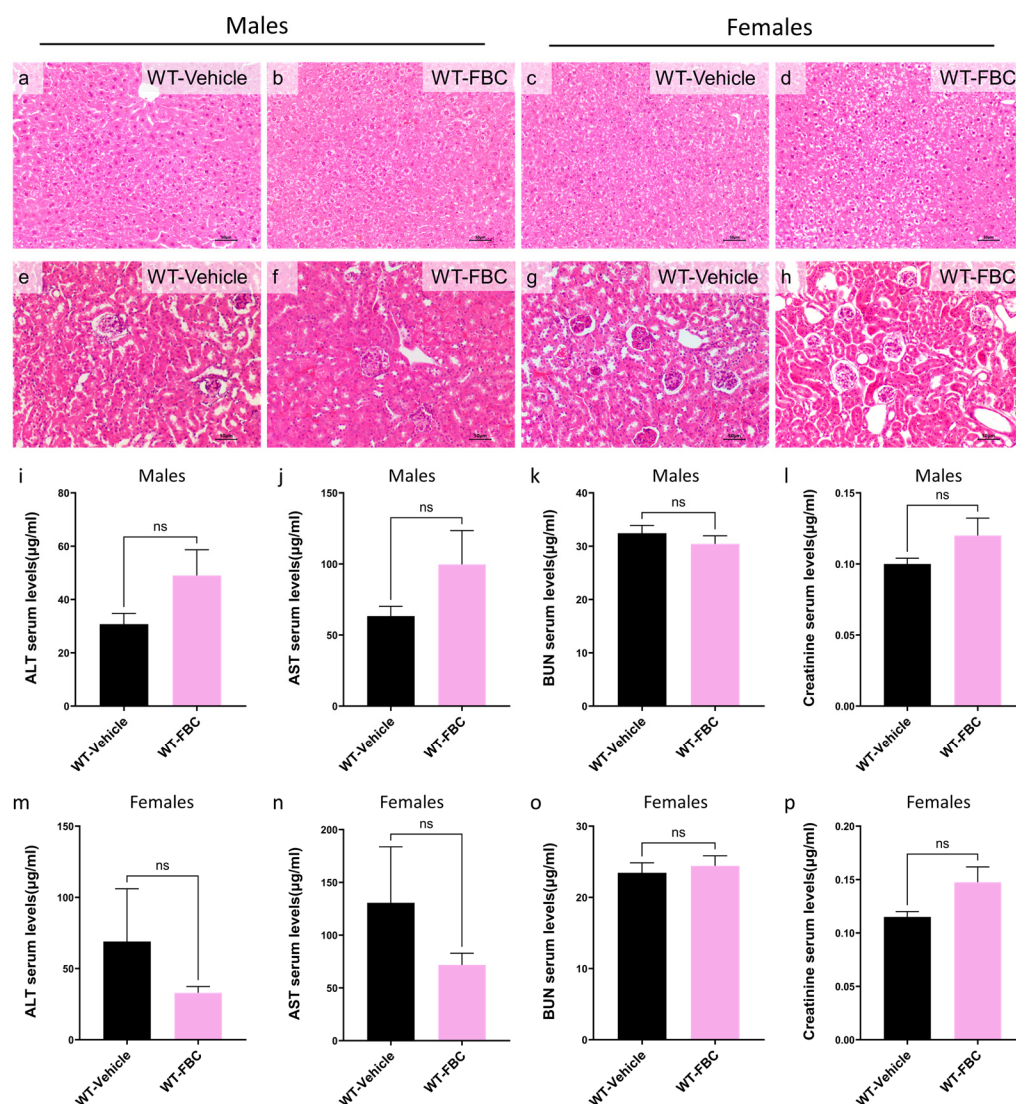
### 2.12. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 10.5.0 (GraphPad, Boston, MA, USA). For comparison between two groups, a two-tailed Mann–Whitney U test was used. For comparisons involving more than two groups, a Kruskal–Wallis test was performed, followed by Dunn’s post hoc multiple-comparisons test. To test for group  $\times$  region interactions, a two-way analysis of variance (ANOVA) was conducted to evaluate interactions between factors, followed by Fisher’s least significant difference (LSD) test for post-hoc comparisons. Data are presented as mean  $\pm$  standard error of the mean (SEM). A threshold of  $p < 0.05$  was considered statistically significant (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ).

## 3. Results

### 3.1. Flupirtine Benzyl Carbamate Hepatotoxicity/Nephrotoxicity in WT Mice

Tolerability of prolonged flupirtine benzyl carbamate (7 mg/kg) treatment following 90-day oral exposure was evaluated in WT C57BL/6L mice. Histopathological examination of H&E-stained liver and kidney sections at 17 weeks of age revealed intact tissue architecture in FBC-treated WT mice, with no lesions or abnormalities relative to age/sex-matched vehicle-treated controls. Representative liver sections from male and female mice showed normal histological integrity, lacking signs of hepatocellular necrosis, inflammatory infiltrates or other pathology in the drug-treated cohort (Figure 2a–d). Kidney sections from male and female FBC-treated WT mice displayed no overt histopathological changes or structural damage indicative of nephrotoxicity compared to controls (Figure 2e–h). Consistent with these findings, serum biochemistry analyses demonstrated that sub-chronic FBC exposure did not adversely affect liver or kidney function. Circulating levels of ALT and AST in male and female FBC-treated mice remained comparable to vehicle-treated groups, as did concentrations of BUN and creatinine (Figure 2i–p). Together, these data indicate that flupirtine benzyl carbamate is well-tolerated in mice without detectable hepatotoxicity and nephrotoxicity in either sex.

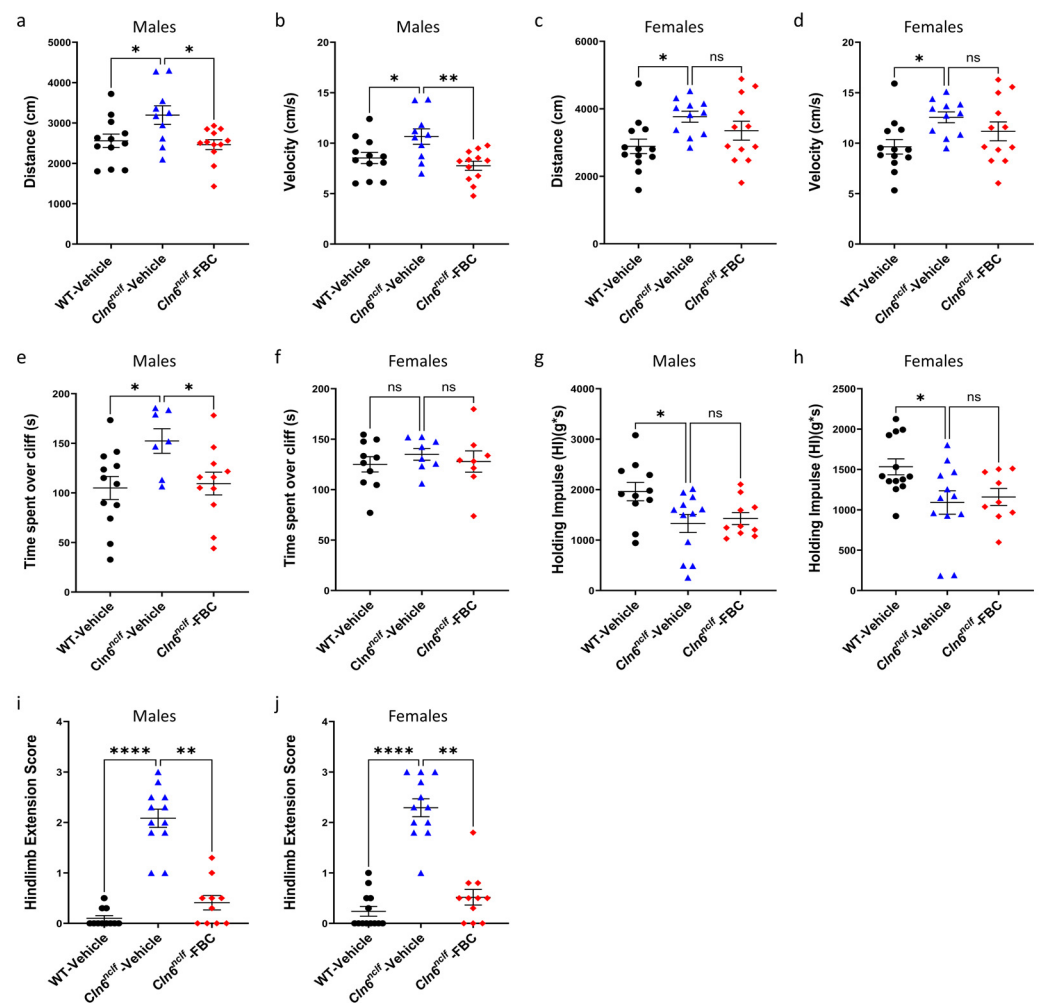


**Figure 2.** Evaluation of FBC toxicity in livers and kidneys of WT mice. Hematoxylin and Eosin staining of liver (a–d) and kidney (e–h) sections from 17-week-old WT-Vehicle (a,e) vs. WT-FBC (b,f) male mice, and WT-Vehicle (c,g) vs. WT-FBC (d,h) female mice. Serum biochemical parameters measured in WT-vehicle and WT-FBC-treated male (i–l) and female (m–p) mice. Panels show levels of ALT (i,m), AST (j,n), BUN (k,o), and creatinine (l,p) (n = 4/group/sex). Data are expressed as mean ± SEM. Statistical significance was determined using a two-tailed Mann–Whitney U test. ns: not significant. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen.

### 3.2. Impact of Flupirtine Benzyl Carbamate on Spasticity, Hyperactivity and Vision in Male and Female *Cln6<sup>nclf</sup>* Mice

Disease progression in *Cln6<sup>nclf</sup>* mice includes early onset of motor dysfunction characterized by impaired coordination, hyperactivity and spasticity versus WT controls [5]. Therapeutic efficacy of flupirtine benzyl carbamate on behavioral impairments in *Cln6<sup>nclf</sup>* mice was evaluated by various behavioral tests. In the open-field assay, 26-week-old vehicle-treated *Cln6<sup>nclf</sup>* male and female mice exhibited pronounced locomotor hyperactivity, with significantly greater distance traveled and higher velocity compared to WT controls. FBC-treated mutants attenuated this hyperactivity in male *Cln6<sup>nclf</sup>* mice, significantly reducing distance moved and average velocity to near WT levels (Figure 3a,b). Female *Cln6<sup>nclf</sup>* mice did not demonstrate a reduction in locomotor hyperactivity following the drug (Figure 3c,d), indicating a sex-specific response. FBC improved visual performance

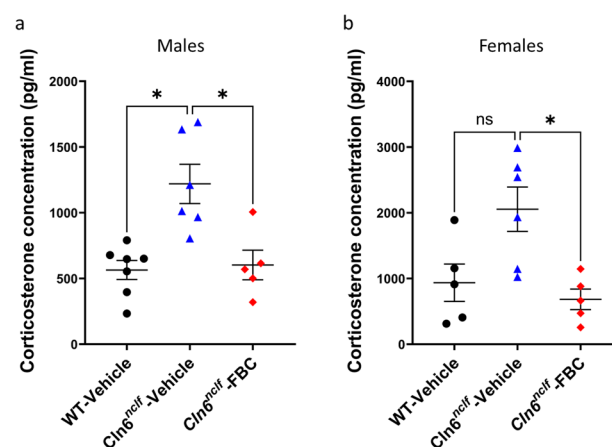
in the visual cliff assay in 28-week-old males with a significant decrease in time spent over the cliff, whereas females did not, underscoring sex-specific differences in therapeutic efficacy (Figure 3e,f, respectively). Motor coordination and endurance, assessed by the wire hanging test, were not affected by FBC in either sex: *Cln6<sup>ncl</sup>* mice showed no improvement in holding impulse with treatment (Figure 3g,h). Spasticity was evident in *Cln6<sup>ncl</sup>* mice in the tail suspension test, with 27-week-old vehicle-treated male and female mutants displaying significantly elevated hindlimb extension scores (spasticity index) relative to age-matched WT male and female mice. Notably, drug treatment significantly lowered hindlimb extension scores in male and female *Cln6<sup>ncl</sup>* mice (Figure 3i,j), indicating amelioration of hindlimb spasticity in both sexes.



**Figure 3.** Representative dot plots of behavioral assessment tests for anxiety/motor activity/spasticity/visual acuity. Distances moved (cm) and mean velocities (cm/s) for male (a,b) and female (c,d) WT vehicle- (black circles) vs. *Cln6<sup>ncl</sup>* vehicle- (blue triangles) vs. *Cln6<sup>ncl</sup>* FBC-treated (red diamonds) mice are displayed. Visual cliff performance is shown as time spent over the cliff for male (e) and female (f) WT vehicle- vs. *Cln6<sup>ncl</sup>* vehicle- vs. *Cln6<sup>ncl</sup>* FBC-treated mice. Motor function is evaluated in the wire hanging test by measuring holding impulses (g\*s) of male (g) and female (h) WT vehicle- vs. *Cln6<sup>ncl</sup>* vehicle- vs. *Cln6<sup>ncl</sup>* FBC-treated mice. Spasticity in the tail suspension test is documented as hindlimb extension scores of male (i) and female (j) WT vehicle- vs. *Cln6<sup>ncl</sup>* vehicle- vs. *Cln6<sup>ncl</sup>* FBC-treated mice. Each symbol represents an individual animal (n = 10–12/group/sex). Data are shown as mean  $\pm$  SEM. Statistical significance was determined via a Kruskal–Wallis test followed by Dunn’s post hoc multiple-comparisons test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ . ns: not significant.

### 3.3. Effect of Flupirtine Benzyl Carbamate on Serum Corticosterone Levels in Male and Female *Cln6<sup>nclf</sup>* Mice

Serum corticosterone was quantified by ELISA in 28-week-old WT vehicle-treated, *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated mice ( $n = 5\text{--}7$  per group/per sex) after 24 weeks. Only vehicle-treated *Cln6<sup>nclf</sup>* male mice exhibited markedly elevated corticosterone levels ( $1123.9 \pm 158.3$  pg/ml) relative to age- and sex-matched WT controls, while vehicle-treated *Cln6<sup>nclf</sup>* female mice trended towards an elevation in corticosterone levels but were not significant. Administration of flupirtine benzyl carbamate significantly reduced corticosterone levels in *Cln6<sup>nclf</sup>* mice: Serum corticosterone in treated males and females was lowered to values approaching those of WT animals ( $977.1 \pm 208.1$  pg/ml in FBC-treated males and  $683.3 \pm 122.5$  pg/ml in FBC-treated females) (Figure 4). These results demonstrate that drug treatment normalizes the hypercorticotestonemia observed in the *Cln6<sup>nclf</sup>* mouse.

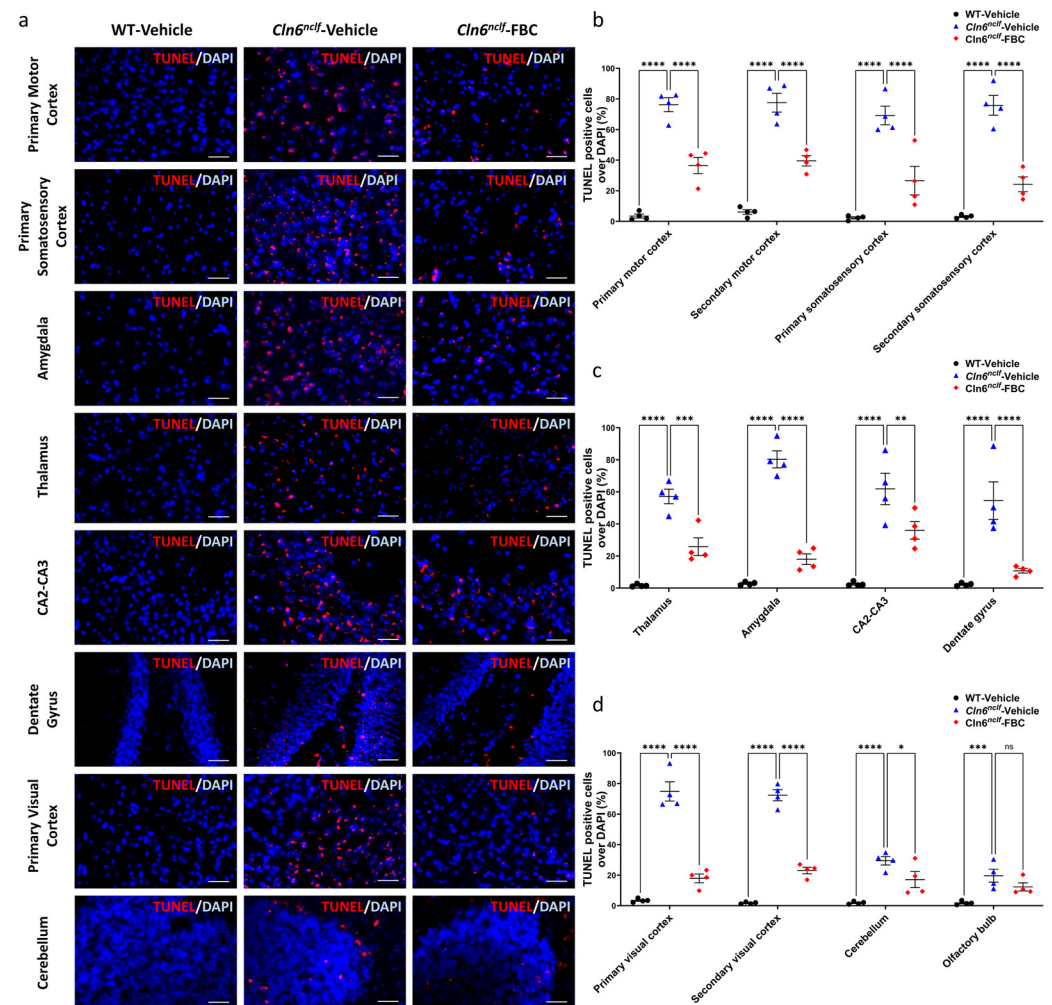


**Figure 4.** Effect of flupirtine benzyl carbamate on serum corticosterone concentrations in male and female *Cln6<sup>nclf</sup>* mice. Representative dot plots of serum corticosterone levels in 28-week-old male (a) and female (b) WT vehicle- (black circles) vs. *Cln6<sup>nclf</sup>* vehicle- (blue triangles) vs. *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) mice, determined by ELISA. Each symbol represents an individual animal ( $n = 5\text{--}7$ /group/sex). Data are displayed as mean  $\pm$  SEM. Statistical significance was determined via Kruskal–Wallis test followed by Dunn’s post hoc multiple-comparisons test. \*  $p < 0.05$ . ns: not significant.

### 3.4. Impact of Flupirtine Benzyl Carbamate on Neuronal Cell Death in the Brains of Male and Female *Cln6<sup>nclf</sup>* Mice

Apoptosis was quantified in frozen brain sections from 28-week-old male mice using TUNEL assay (detected via red fluorescence) with DAPI counterstaining of all nuclei (blue) (representative images, Figure 5a). Apoptotic index for each region was calculated as the fraction of TUNEL-positive nuclei relative to total DAPI-positive nuclei. Compared to sex- and age-matched WT controls, vehicle-treated *Cln6<sup>nclf</sup>* male mice exhibited a significant increase in apoptosis across all regions examined. Statistical analysis confirmed that the percentage of TUNEL-positive nuclei was markedly higher in *Cln6<sup>nclf</sup>* vehicle-treated mice, reaching a peak of  $57.1 \pm 4.6\%$  in the thalamus and  $61.8 \pm 9.8\%$  in the CA2–CA3 hippocampal subregion, relative to baseline WT levels (Figure 5b–d). Treatment with FBC significantly attenuated this pathological increase in apoptotic nuclei across all major affected regions. Percentage of TUNEL-positive cells in FBC-treated *Cln6<sup>nclf</sup>* mice was reduced to  $25.8 \pm 5.5\%$  in the thalamus and  $36 \pm 5.5\%$  in the CA2–CA3 hippocampal subregions (Figure 5b–d). To complement these histological findings, the mouse apoptosis proteome profiler array was performed on brain lysates from WT vehicle-, *Cln6<sup>nclf</sup>* vehicle-

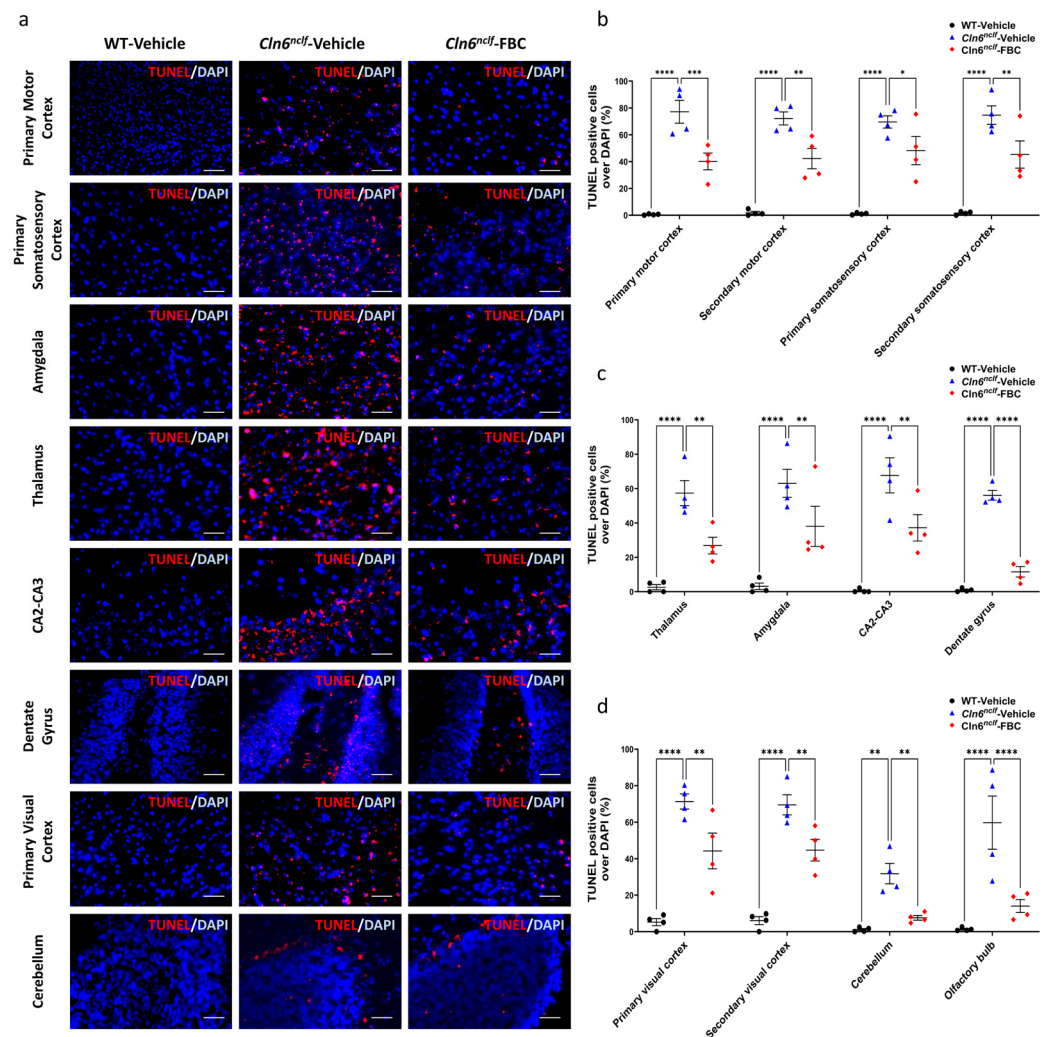
and *Cln6<sup>nclf</sup>* FBC-treated male mice. Levels of apoptosis-related proteins were measured, but no changes were noted (data shown in Supplementary Figure S1).



**Figure 5.** Apoptosis in 28-week-old WT/*Cln6<sup>nclf</sup>* male mice in vehicle- or flupirtine benzyl carbamate-treated mice groups. (a) Representative immunofluorescence images of coronal brain sections from 28-week-old WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated male mice (red diamonds). Cell death was determined using the TUNEL assay (red). All nuclei were counterstained with DAPI (blue). Images from the primary motor cortex, primary somatosensory cortex, amygdala, thalamus, hippocampal CA2–CA3 regions, dentate gyrus, primary visual cortex and cerebellum are shown. Scale bars: 50  $\mu$ m. (b–d) Dot plots representing percentages of TUNEL-positive cells across brain regions in WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) male mice. Each symbol represents an individual animal ( $n = 4$  per group). Data are displayed as mean  $\pm$  SEM. Statistical comparisons were performed by two-way ANOVA followed by Fisher's LSD post hoc test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . ns: not significant.

Cell death was quantified in frozen brain sections from 28-week-old female mice using TUNEL assay (red fluorescence) and DAPI counterstaining (blue fluorescence) (see Figure 6a). Vehicle-treated *Cln6<sup>nclf</sup>* female mice exhibited abundant TUNEL-positive nuclei in the motor cortex, thalamus, amygdala, CA2–CA3 subregion of the hippocampus and dentate gyrus, versus sparse staining found in age- and sex-matched WT controls. Quantitation of percentage of TUNEL-positive cells over total cells confirmed significant apoptosis in all regions examined in *Cln6<sup>nclf</sup>* vehicle-treated groups (Figure 6b–d). *Cln6<sup>nclf</sup>* vehicle-treated females displayed substantial apoptosis in the thalamus ( $57.4 \pm 7.3\%$ ) and

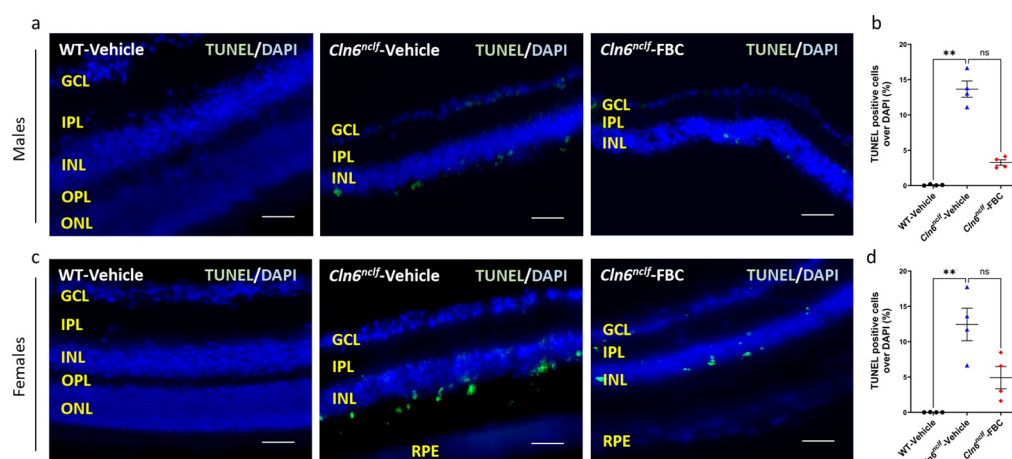
hippocampal CA2–CA3 regions ( $67.7 \pm 10.2\%$ ) versus age/sex-matched WT controls (Figure 6b–d). FBC therapeutic efficacy was confirmed by attenuation of TUNEL-positive nuclei in brain regions, including the thalamus ( $26.8 \pm 4.9\%$ ) and hippocampal CA2–CA3 regions ( $37.2 \pm 7.7\%$ ) (Figure 6b–d). To complement these histological findings, apoptosis-related proteins were profiled in female brain lysates. No noted differences were demonstrated in females after FBC treatment (see Supplementary Figure S2).



**Figure 6.** Apoptosis in 28-week-old WT/*Cln6<sup>nclf</sup>* female mice after flupirtine benzyl carbamate treatment. (a) Representative immunofluorescence images of coronal brain sections from 28-week-old WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated male mice (red diamonds). Cell death was detected with the TUNEL assay (red), and nuclei were counterstained with DAPI (blue). Images represent the primary motor cortex, primary somatosensory cortex, amygdala, thalamus, hippocampal CA2–CA3 regions, dentate gyrus, primary visual cortex and cerebellum. Scale bars: 50 µm. (b–d) Dot plots representing percentages of TUNEL-positive cells in brain regions in WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) female mice. Each symbol represents an individual animal (n = 4 per group). Data are displayed as mean ± SEM. Statistical comparisons were performed by two-way ANOVA followed by Fisher's LSD post hoc test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . ns: not significant.

### 3.5. Impact of Flupirtine Benzyl Carbamate on Apoptosis in the Retina of Male and Female *Cln6<sup>nclf</sup>* Mice

Retinal pathology begins early in the naturally occurring *Cln6<sup>nclf</sup>* mouse, with marked thinning of the outer nuclear layer (ONL) and loss of photoreceptors by post-natal day 14 (P14), accompanied by apoptotic photoreceptor nuclei confined to the ONL by P12 [5]. To determine whether pharmacological intervention can modify this established degenerative process at a later stage, TUNEL-positive cells were quantified in frozen retinal sections of 28-week-old WT vehicle-treated, *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated male and female mice. Retinas of vehicle-treated *Cln6<sup>nclf</sup>* male and female mice exhibited a significantly higher density of TUNEL-positive nuclei in the ONL compared to age- and sex-matched WT controls. Retinas of FBC-treated *Cln6<sup>nclf</sup>* mice displayed a slight reduction in apoptotic cells (Figure 7a,c). Consistent with the micrographs, quantification of TUNEL-positive cells displayed a significant abundance of apoptotic cells in the retina of *Cln6<sup>nclf</sup>* vehicle-treated male and female mice. The reduction observed after drug treatment was not significant (Figure 7b,d). These findings indicate that flupirtine benzyl carbamate did not preserve retinal cell survival in this CLN6 disease model.

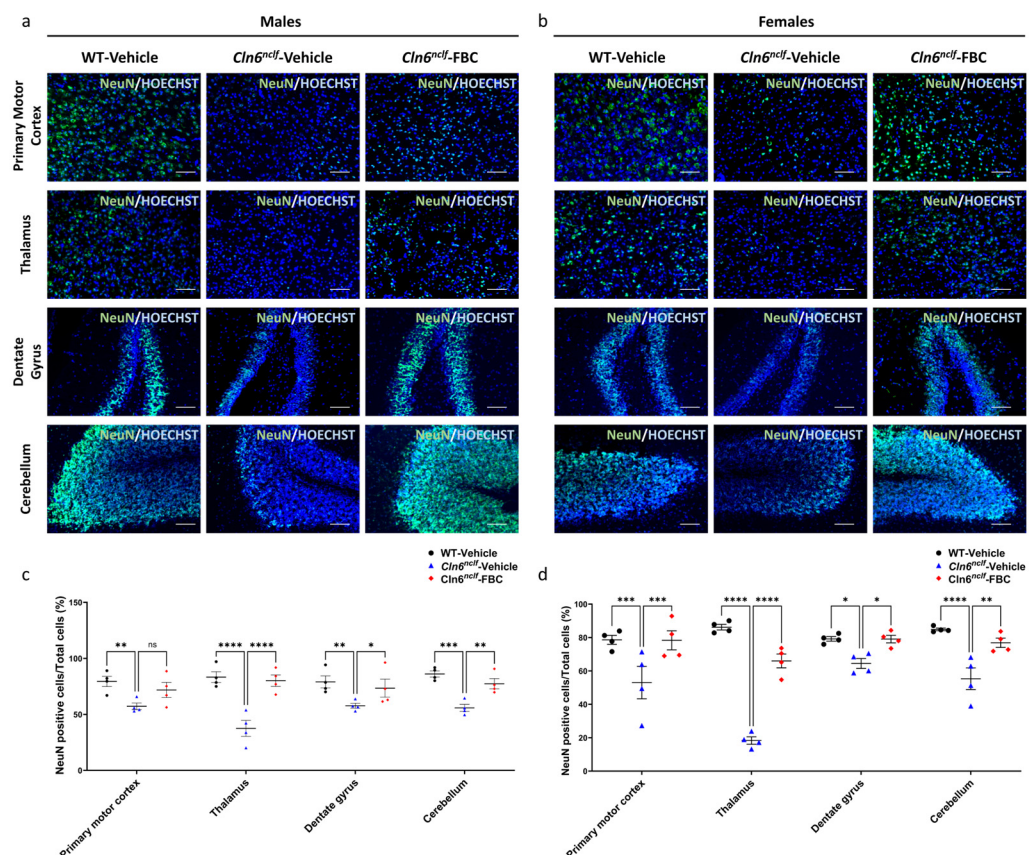


**Figure 7.** Retinal cell death in male and female *Cln6<sup>nclf</sup>* mice. Representative fluorescence micrographs of retinal cross-sections from male (a) and female (c) WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated male mice (red diamonds) groups. Apoptotic cells appear as TUNEL-positive nuclei (green) and cell nuclei are counterstained with DAPI (blue). Retinal layers indicated: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and retinal pigment epithelium (RPE). Scale bars: 50  $\mu$ m. Quantification of apoptotic retinal cells in male (b) and female (d) mice, expressed as a percentage of TUNEL-positive nuclei versus total DAPI-stained nuclei across the whole retina. Each symbol represents an individual animal (n = 4 per group). Data are displayed as mean  $\pm$  SEM. Statistical significance was determined via Kruskal–Wallis test followed by Dunn’s post hoc multiple-comparisons test. \*\*  $p < 0.01$ . ns: not significant.

### 3.6. Effect of Flupirtine Benzyl Carbamate on Mature Neuronal Counts in *Cln6<sup>nclf</sup>* Brains

Mature neuronal populations were assessed by immunolabelling for neuron-specific nuclear protein neuronal nuclei (NeuN) in the brains of 28-week-old male and female WT vehicle-treated, *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated mice. Representative NeuN micrographs (green) revealed marked loss of NeuN-positive cells in *Cln6<sup>nclf</sup>* vehicle-treated brains in the primary motor cortex, thalamus, dentate gyrus and cerebellum relative to age- and sex-matched WT controls (Figure 8a,b). Quantitative analysis of NeuN-positive cells over total number of cells in these regions (Figure 8c,d) confirmed significant reductions in NeuN-positive cell number in *Cln6<sup>nclf</sup>* vehicle-treated males and females

compared to age- and sex-matched WT vehicle-treated mice. In both sexes, treatment with FBC substantially increased NeuN immunoreactivity in most regions, restoring NeuN levels to those in WT animals (data shown in Supplementary Figure S5). The data indicate that flupirtine benzyl carbamate leads to an increase in neuronal populations in male and female *Cln6<sup>ncl</sup>* brains.

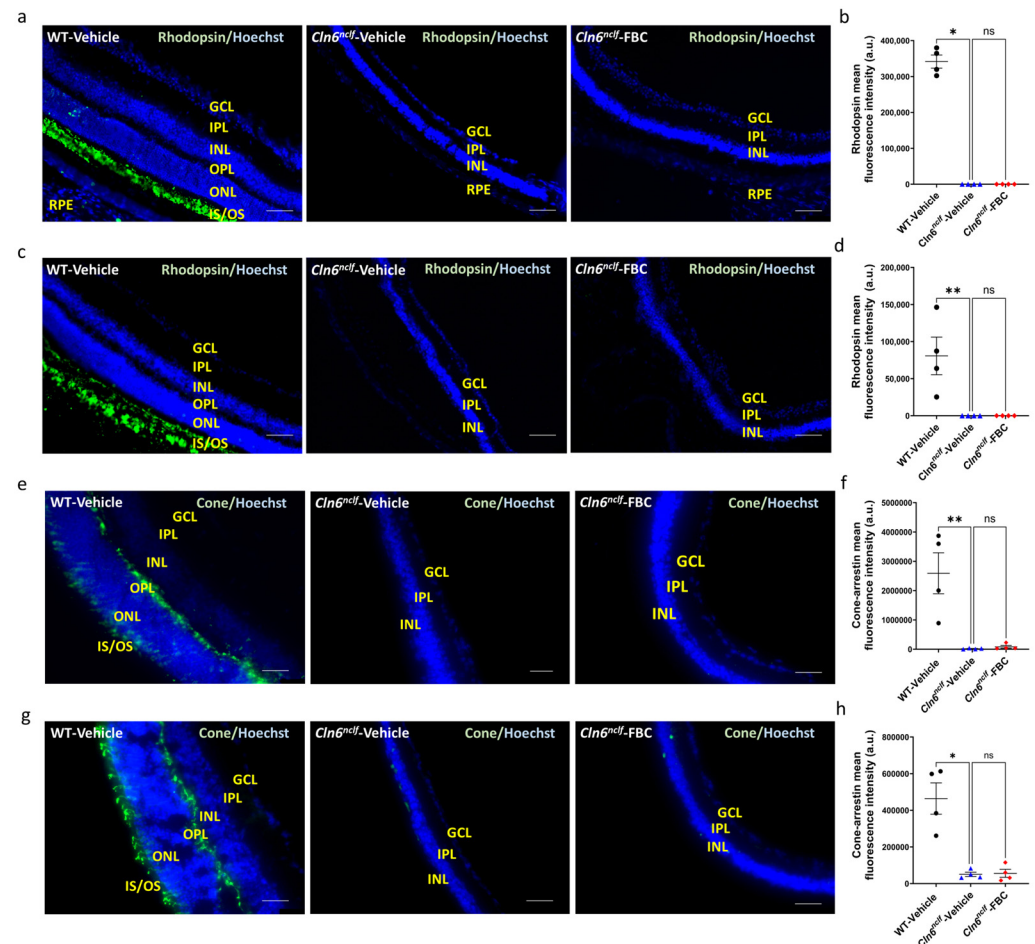


**Figure 8.** Neuronal nuclei immunoreactivity in the brains of male and female *Cln6<sup>ncl</sup>* mice treated with FBC. Representative images of NeuN (green) immunofluorescence with Hoechst nuclear counterstain (blue) in coronal brain sections from 28-week-old male (a) and female (b) WT vehicle- (black circles), *Cln6<sup>ncl</sup>* vehicle- (blue triangles) and *Cln6<sup>ncl</sup>* FBC-treated mice (red diamonds). Images represent the primary motor cortex, thalamus, dentate gyrus and cerebellum. Scale bars: 100  $\mu$ m. Quantification of NeuN-positive cells divided by the total number of cells in indicated regions in male (c) and female (d) mice. Each symbol represents an individual animal (n = 4 per group). Data are displayed as mean  $\pm$  SEM. Statistical comparisons were performed using two-way ANOVA followed by Fisher's LSD post hoc test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . ns: not significant.

### 3.7. Effect of Flupirtine Benzyl Carbamate on Retinal Cones and Rods of Male and Female *Cln6<sup>ncl</sup>* Mice

Degeneration of photoreceptors was assessed by immunolabelling for cone-specific arrestin and rod-specific rhodopsin in retinal sections from 28-week-old WT vehicle-treated, *Cln6<sup>ncl</sup>* vehicle-treated and *Cln6<sup>ncl</sup>* FBC-treated male and female mice. In WT vehicle-treated retinas, robust rhodopsin and cone-arrestin immunofluorescence was confined to the rod inner/outer segment layer overlying the ONL, delineating a well-organized photoreceptor band in male and female mice. In contrast, male and female *Cln6<sup>ncl</sup>* vehicle-treated mice exhibited markedly attenuated rhodopsin and cone-arrestin staining consistent with substantial rod photoreceptor loss (Figure 9a,c and Figure 9e,g, respectively). Quantification of rhodopsin and cone-arrestin mean fluorescence intensity confirmed a significant reduction in male and female *Cln6<sup>ncl</sup>* vehicle-treated mice relative to

age- and sex-matched WT vehicle-treated controls. Treatment with FBC did not increase rhodopsin and cone-arrestin immunoreactivity in male and female *Cln6<sup>nclf</sup>* mice (Figure 9b,d for rhodopsin and Figure 9f,h for cone-arrestin). Flupirtine benzyl carbamate did not reverse rod and cone photoreceptor degeneration in *Cln6<sup>nclf</sup>* retina.

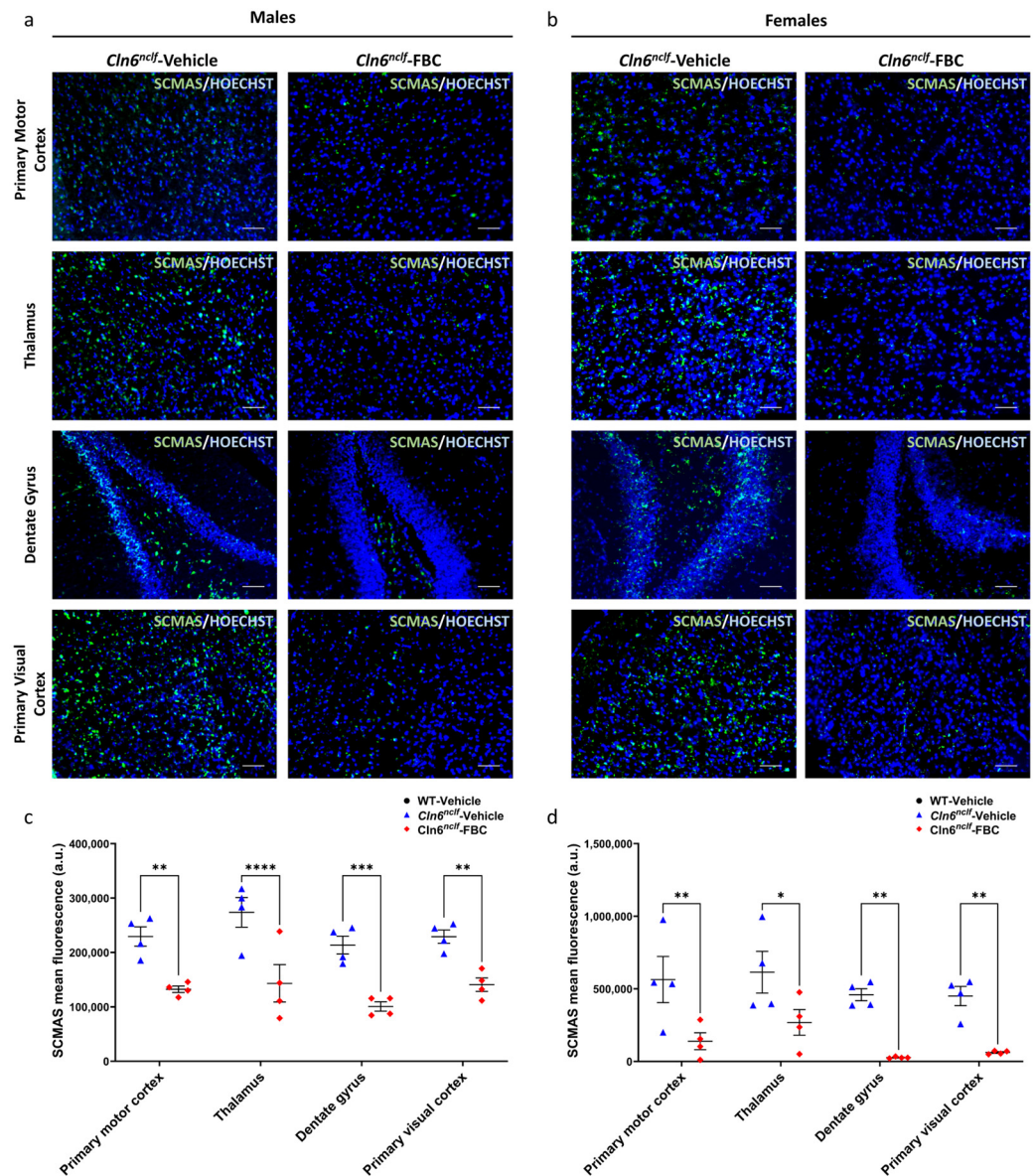


**Figure 9.** Rhodopsin and cone-arrestin assessment in the retinas of *Cln6<sup>nclf</sup>* mice after flupirtine benzyl carbamate treatment. Representative images of rhodopsin (a,c) and cone-arrestin (e,g) (green) immunolabelling with Hoechst nuclear counterstain (blue) in retinal sections from 28-week-old male WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) male (a,e) and female (c,g) mice. Retinal layers include the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner/outer segments (IS/OS) and retinal pigment epithelium (RPE). Scale bars: 50 μm. Quantification of mean rhodopsin fluorescence intensity in male (b) and female (d) retinas and cone-arrestin mean fluorescence intensity in male (f) and female (h) retinas. Each symbol represents an individual animal (n = 4/group/sex). Data are displayed as mean ± SEM. Statistical significance was determined via Kruskal–Wallis test followed by Dunn’s post hoc multiple-comparisons test. \* p < 0.05, \*\* p < 0.01. ns: not significant.

### 3.8. Flupirtine Benzyl Carbamate and SCMAS Accumulation in the Brains of Male and Female *Cln6<sup>nclf</sup>* Mice

Accumulation of SCMAS was determined in the brains of 28-week-old *Cln6<sup>nclf</sup>* vehicle- and *Cln6<sup>nclf</sup>* FBC-treated male and female mice. In the brains of male and female vehicle-treated *Cln6<sup>nclf</sup>* mice, SCMAS immunofluorescence (green) was intense in the primary motor and visual cortices, thalamus and dentate gyrus (Figure 10a,b, respectively). Brains of male and female *Cln6<sup>nclf</sup>* mice treated with FBC exhibited a significant reduction in

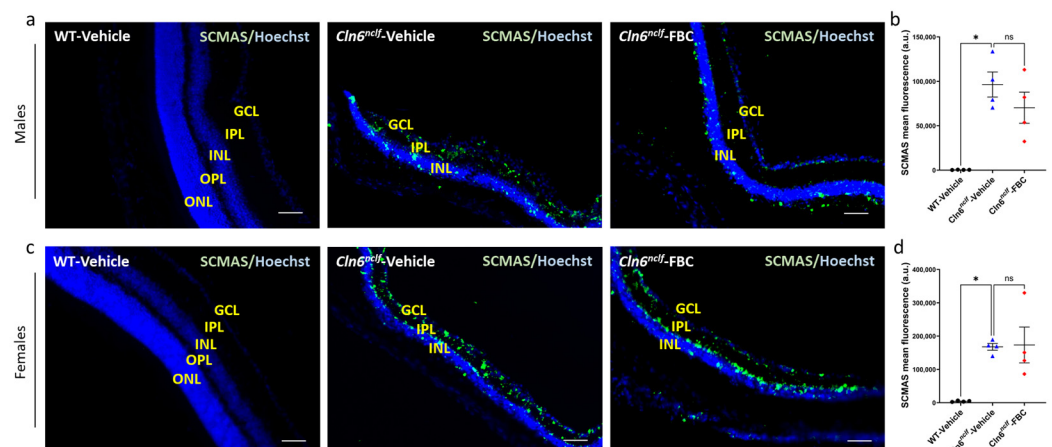
SCMAS immunoreactivity in the same regions. Quantitative analysis in brain regions confirmed a significant decrease in SCMAS mean fluorescence in the brains of male and female *Cln6<sup>ncl</sup>* FBC-treated mice compared to the age- and sex-matched *Cln6<sup>ncl</sup>* vehicle-treated group (Figure 10c,d, respectively). SCMAS mean fluorescence was also measured in additional brain regions, and these data are provided in the Supplementary Figure S6. The data confirm that flupirtine benzyl carbamate diminishes SCMAS storage in male and female *Cln6<sup>ncl</sup>* brains.



**Figure 10.** Accumulation of SCMAS in the brains of male and female *Cln6<sup>ncl</sup>* mice. Immunofluorescence images show SCMAS accumulation (green) in cells counterstained with Hoechst (blue) in coronal brain sections of 28-week-old male (a) and female (b) *Cln6<sup>ncl</sup>* vehicle- (blue triangles) and *Cln6<sup>ncl</sup>* FBC-treated (red diamonds) mice. Regions examined include the primary motor and visual cortices, thalamus and dentate gyrus. Scale bars: 100  $\mu$ m. Dot plots summarizing SCMAS mean fluorescence intensity in brain regions of 28-week-old *Cln6<sup>ncl</sup>* vehicle- and *Cln6<sup>ncl</sup>* FBC-treated male (c) and female (d) mice. Each symbol represents an individual animal (n = 4 per group). Data are displayed as mean  $\pm$  SEM. Statistical comparisons were performed using two-way ANOVA followed by Fisher's post hoc test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

### 3.9. Flupirtine Benzyl Carbamate and SCMAS Accumulation in the Retina of Male and Female *Cln6<sup>nclf</sup>* Mice

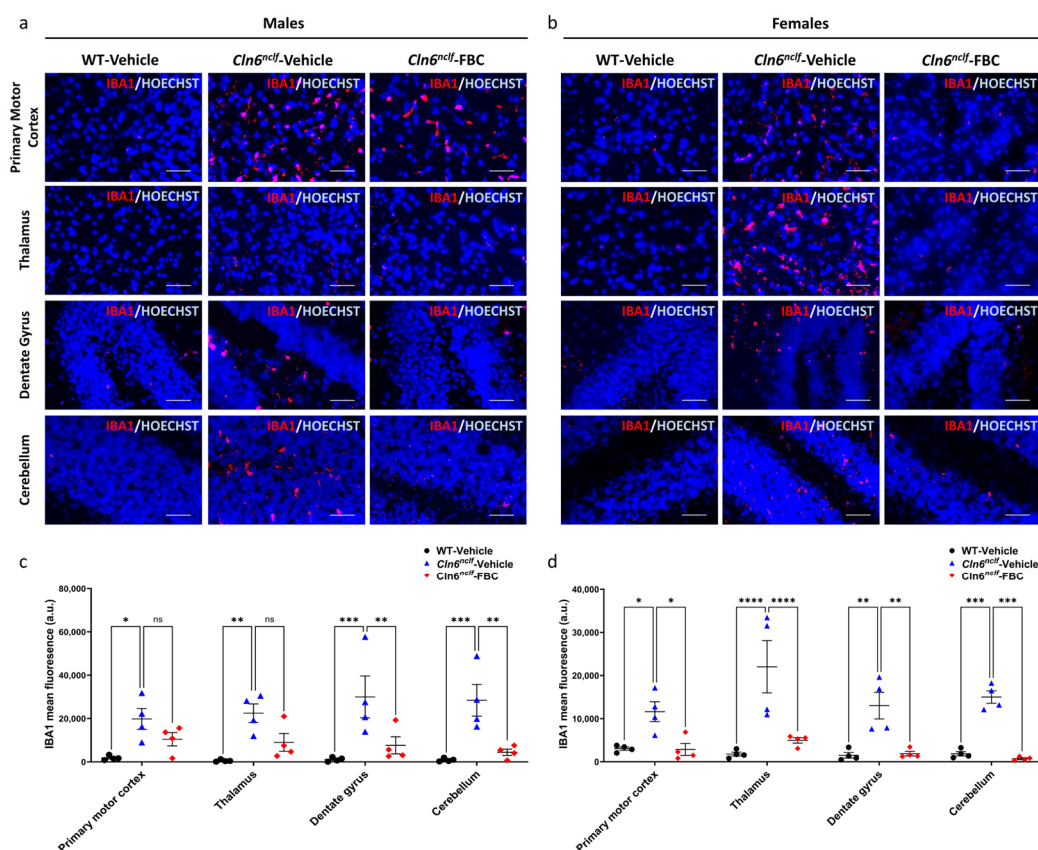
Retinal sections of WT vehicle-treated mice were devoid of SCMAS storage. *Cln6<sup>nclf</sup>* vehicle-treated mice retinas exhibited pronounced SCMAS (green) accumulation in male and female mice in all retinal layers. *Cln6<sup>nclf</sup>* FBC-treated mice displayed a similar pattern and intensity of SCMAS staining with no qualitative reduction relative to vehicle-treated mutants (Figure 11a,c). Quantification of SCMAS mean fluorescence confirmed the absence of treatment effect in both sexes with no significant difference detected between *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated mice (Figure 11b,d). These data indicate that flupirtine benzyl carbamate fails to reduce retinal SCMAS storage in the retinas of male and female *Cln6<sup>nclf</sup>* mice.



**Figure 11.** Accumulation of SCMAS in the retinas of male and female *Cln6<sup>nclf</sup>* mice. Representative images of SCMAS (green) accumulation counterstained with Hoechst (blue) in retinas of WT vehicle- vs. *Cln6<sup>nclf</sup>* vehicle- vs. *Cln6<sup>nclf</sup>* FBC-treated male (a) and female (c) mice. Retinal layers include the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL). Scale bars: 100 μm. Quantification of SCMAS mean fluorescence in male (b) and female (d) retinas of WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) mice. Each symbol represents an individual animal (n = 4/group/sex). Data are displayed as mean ± SEM. Statistical significance was determined via Kruskal–Wallis test followed by Dunn’s post hoc multiple-comparisons test. \* p < 0.05. ns: not significant.

### 3.10. Impact of Flupirtine Benzyl Carbamate on Microgliosis in Male and Female *Cln6<sup>nclf</sup>* Mouse Brains

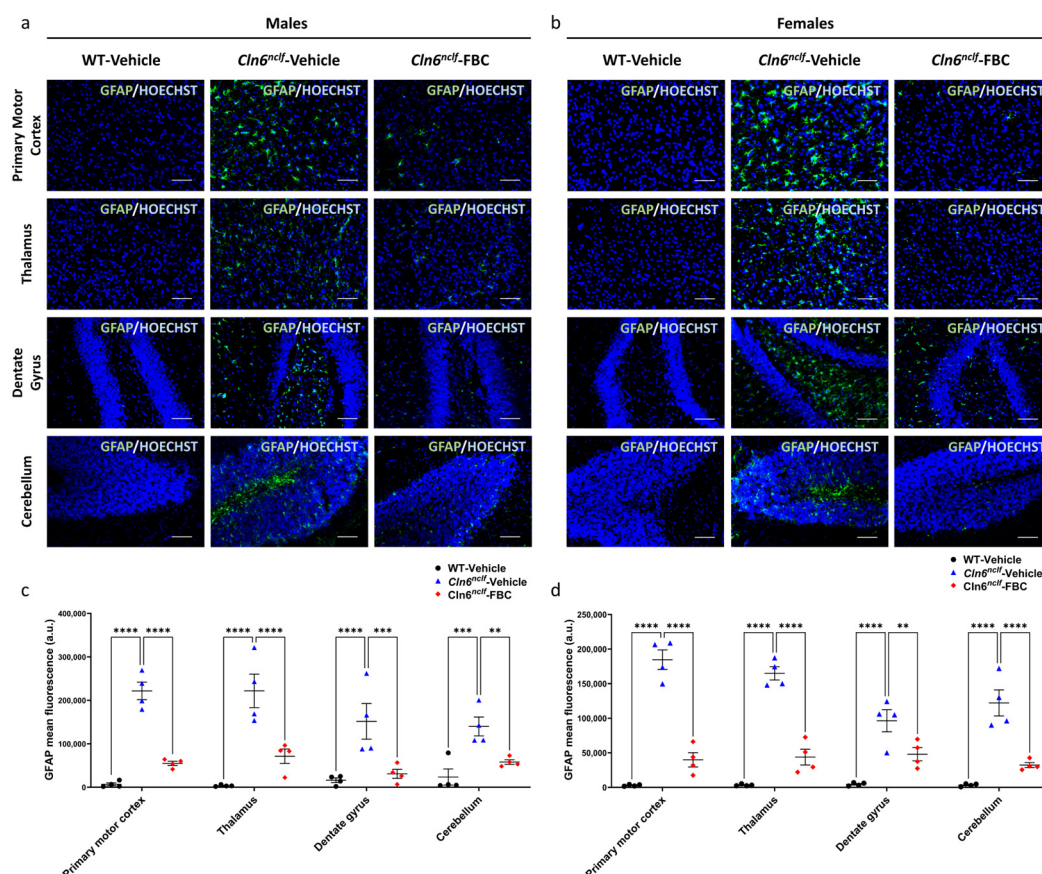
To evaluate the extent of microglia-mediated neuroinflammation, ionized calcium-binding adapter molecule 1 (IBA1) levels were determined in brain sections of 28-week-old WT vehicle-treated, *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated male and female mice. Immunofluorescence micrographs for vehicle-treated male and female *Cln6<sup>nclf</sup>* mice exhibited dense clusters of IBA1-positive microglia in various brain regions, including the primary motor cortex, thalamus, dentate gyrus and cerebellum (Figure 12a,b, respectively). *Cln6<sup>nclf</sup>* male mice treated with FBC showed a significant reduction in IBA1 staining in the dentate gyrus and the cerebellum (Figure 12c). On the other hand, *Cln6<sup>nclf</sup>* female mice treated with FBC showed a significant reduction in IBA1 signal in the primary motor cortex, thalamus, dentate gyrus and cerebellum (Figure 12d). Flupirtine benzyl carbamate effectively diminishes microglial activation in many brain areas that had exhibited increased inflammation in the *Cln6<sup>nclf</sup>* mouse brain (other regions shown in Supplementary Figure S7).



**Figure 12.** Microgliosis in *Cln6<sup>nclf</sup>* male and female mouse brains treated with flupirtine benzyl carbamate. Immunofluorescence images of 28-week-old WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) male (a) and female (b) mouse brain sections stained with IBA1 (red) and nuclear counterstain (Hoechst, blue) in the primary motor cortex, thalamus, dentate gyrus and cerebellum. Scale bars: 50 μm. Dot plots depicting IBA1 mean fluorescence intensity for each brain area for (c) males and (d) females, with each symbol representing an individual animal (n = 4 per group). Data are displayed as mean ± SEM. Statistical comparisons were performed using two-way ANOVA followed by Fisher's LSD post hoc test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . ns: not significant.

### 3.11. Brain Astrogliosis After Flupirtine Benzyl Carbamate Treatment in Male and Female *Cln6<sup>nclf</sup>* Mice

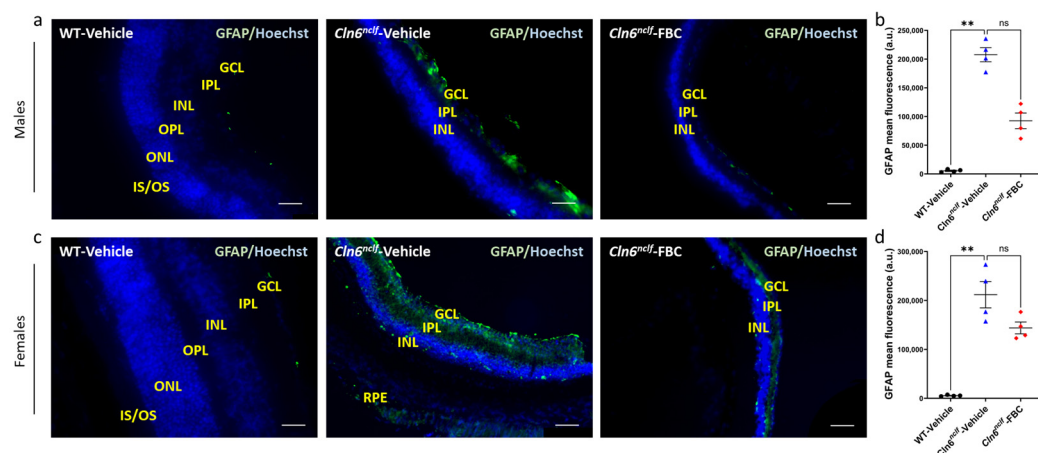
To examine the impact of flupirtine benzyl carbamate on reactive astrogliosis in *Cln6<sup>nclf</sup>* male mice, glial fibrillary acidic protein (GFAP) immunoreactivity was determined in multiple brain regions of 28-week-old WT vehicle-treated, *Cln6<sup>nclf</sup>* vehicle-treated and *Cln6<sup>nclf</sup>* FBC-treated male and female mice. In vehicle-treated mutants, GFAP staining (green fluorescence) was markedly elevated in the primary motor cortex, thalamus, dentate gyrus and cerebellum. Brains of FBC-treated *Cln6<sup>nclf</sup>* male and female mice exhibited reduced GFAP signal in each of these regions (Figure 13a,b). Quantification of GFAP mean fluorescence confirmed a robust treatment effect of FBC, with significant reductions in astrocyte activation in all brain regions (further brain regions added in Supplementary Figure S8) compared to vehicle-treated *Cln6<sup>nclf</sup>* male and female mice (Figure 13c,d). This indicates that flupirtine benzyl carbamate elicits a broad, brain-wide reduction in astrogliosis in the brains of *Cln6<sup>nclf</sup>* male and female mice.



**Figure 13.** Astroglial activation in *Cln6<sup>nclf</sup>* male and female mouse brains after treatment with FBC. Immunofluorescence images of 28-week-old (a) male and (b) female WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) mouse brains confirm GFAP (green) with nuclear counterstaining (Hoechst, blue) in the primary motor cortex, thalamus, dentate gyrus and cerebellum. Scale bars: 100 μm. Dot plots depict GFAP mean fluorescence intensity for brain areas in (c) males and (d) females. Each symbol represents an individual animal (n = 4 per group). Data are displayed as mean ± SEM. Statistical comparisons were performed using two-way ANOVA followed by Fisher's LSD post hoc test. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

### 3.12. Effect of Flupirtine Benzyl Carbamate on Retinal Astroglial Activation in Male and Female *Cln6<sup>nclf</sup>* Mice

GFAP immunoreactivity in the mouse retinas was determined to illustrate flupirtine benzyl carbamate modulation of Müller glial/astrocytic activation of *Cln6<sup>nclf</sup>* mice. *Cln6<sup>nclf</sup>* vehicle-treated male and female retinas exhibited a pronounced increase in GFAP signal extending from the ganglion cell layer into the inner plexiform and inner nuclear layers relative to WT vehicle-treated mice. FBC-treated *Cln6<sup>nclf</sup>* males and females exhibited a non-significant reduction in GFAP staining across these retinal layers (Figure 14a). This indicates that flupirtine benzyl carbamate did not suppress retinal astroglial activation in male and female *Cln6<sup>nclf</sup>* mice.



**Figure 14.** Retinal GFAP immunoreactivity in male and female *Cln6<sup>nclf</sup>* mice following flupirtine benzyl carbamate treatment. Representative GFAP (green) immunofluorescence with Hoechst nuclear counterstain (blue) of retinal sections from 28-week-old male (a) and female (c) WT vehicle- (black circles), *Cln6<sup>nclf</sup>* vehicle- (blue triangles) and *Cln6<sup>nclf</sup>* FBC-treated (red diamonds) mice. Retinal layers: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner/outer segments (IS/OS), and retinal pigment epithelium (RPE). Scale bars: 100 μm. Quantification of GFAP mean fluorescence intensity in male (b) and female (d) retinas. Each symbol represents an individual animal (n = 4 per group). Data are displayed as mean ± SEM. Statistical significance was determined via Kruskal–Wallis test followed by Dunn’s post hoc multiple-comparisons test. \*\*  $p < 0.01$ . ns: not significant.

#### 4. Discussion

This study investigates the long-term therapeutic efficacy of a novel flupirtine benzyl carbamate administered orally to the naturally occurring *Cln6<sup>nclf</sup>* mouse model that closely recapitulates human CLN6 disease [5,17]. Findings prove that this drug achieves widespread molecular and cellular rescue across the central nervous system (CNS) of both sexes, while it could not reverse or halt retinal gliosis and photoreceptor loss. Functional motor recovery was sex- and task-specific, highlighting a crucial sex-dependent vulnerability to therapeutic intervention in CLN6 disease progression in these mice.

A core requirement for any chronic therapeutic targeting pediatric neurodegeneration is a favorable safety and tolerability profile. The oral administration of flupirtine benzyl carbamate over 17 weeks yielded no signs of systemic toxicity. Histopathological H&E staining of the kidneys and liver confirms normal morphology without pathological changes. Measurements of serum biochemical parameters remained within normal ranges, demonstrating the drug’s systemic safety and the absence of hepatotoxicity or nephrotoxicity following prolonged dosing, addressing a critical point, i.e., hepatotoxicity [36,52], despite a favorable neuroprotective and analgesic profile in other similar compounds.

Flupirtine benzyl carbamate normalized the abnormally elevated corticosterone levels in male and female *Cln6<sup>nclf</sup>* mice. This endocrine correction is significant because chronic glucocorticoid elevation is tightly linked to sustained stress, heightened neuroinflammation and accelerated neurodegeneration [53,54]. Lowering corticosterone in treated *Cln6<sup>nclf</sup>* mice suggests relief of systemic stress and potentially neuroprotective effects on disease progression. Consistent with this broad action, male and female *Cln6<sup>nclf</sup>* mice showed reduced hindlimb spasticity in the tail suspension test. The mechanism is likely attributable to flupirtine’s muscle relaxant effect in spastic conditions [29,55]. KCNQ2/3-encoded voltage-gated K<sup>+</sup> channels (KV7) are key regulators of neuronal excitability and network stability, and pharmacological openers reduce hyperexcitability

across multiple contexts [56,57]. Higher-order behavioral improvements under flupirtine benzyl carbamate were sex-dependent. Open-field hyperactivity in *Cln6<sup>nclf</sup>* mice is consistent with prior NCL models, particularly the *Cln3<sup>Δex7/8</sup>* mouse model [5,38,58]. FBC's ability to normalize locomotor activity selectively in males, together with improved behavioral performance in the visual cliff test, suggests effective stabilization of limbic and visuospatial circuits in male *Cln6<sup>nclf</sup>* mice. In contrast, female littermates failed to demonstrate substantial recovery in open-field activity and vision, despite evidence of improved retinal pathology, normalized corticosterone levels and improved hindlimb tone. The lack of benefit in the wire-hanging test supports a distinction between modulation of reflex/spasticity phenotypes and restoration of complex motor coordination and endurance, which likely require more extensive structural rescue in cerebellar, corticospinal and neuromuscular systems.

The sex-specific pattern of behavioral rescue aligns with the established sexual dimorphism in human CLN6 disease. Female *Cln6<sup>nclf</sup>* mice show earlier onset and greater severity of motor and visual deficits, higher early SCMAS load in thalamus and cortex and accelerated neurodegeneration compared with males [59]. Within this framework, the male-restricted normalization of hyperactivity and visuomotor behavior likely reflects treatment of circuits still within a reversible window, whereas female circuits at the same age may already be functionally compromised. In addition, sex-dependent pharmacodynamics cannot be excluded: flupirtine maleate induces sex-specific gene-expression changes in *Cln3<sup>Δex7/8</sup>* mouse brains [47], suggesting that downstream signaling and circuit-level responses to Kv7 activation differ between males and females. Sex hormones regulate multiple potassium channel systems and can alter channel expression, subunit coupling and drug sensitivity [60,61]. Thus, KV7 opener efficacy could plausibly differ between sexes at the level of intrinsic excitability. These data argue that future preclinical and clinical development should include sex-stratified analyses and evaluation of earlier initiation or adjusted dosing paradigms in females.

The most striking histological aspect of the impact of flupirtine benzyl carbamate is a reduction in apoptotic TUNEL-positive cells throughout the brain of both male and female *Cln6<sup>nclf</sup>* mice, also confirmed by increased NeuN immunoreactivity in viable neurons. These findings fit well with known anti-apoptotic actions of flupirtine maleate and derivatives. Flupirtine maleate increases expression of the anti-apoptotic protein Bcl-2, reduces pro-apoptotic Bax, preserves mitochondrial function and elevates glutathione levels in neuronal and retinal cells exposed to glutamatergic or oxidative insults [33,62–64]. Importantly, flupirtine maleate blocks apoptosis in lymphoblasts from patients with several NCL genotypes (CLN1, CLN2, CLN3, CLN6) and protects CLN2- and CLN3-deficient post-mitotic neurons in vitro [30,39]. In this study, *in vivo* data extends these mechanistic observations to the naturally occurring *Cln6<sup>nclf</sup>* mouse model. An increase in NeuN-positive neurons in multiple brain regions suggests that flupirtine benzyl carbamate prevents loss of vulnerable neuronal populations, promoting survival of neurons that would otherwise be destined to die.

Accumulation of SCMAS within cells is a biochemical hallmark of many NCL forms, including CLN6 disease in humans [65]. In *Cln6* mouse and ovine models, widespread neuronal degeneration and SCMAS deposition correlate with progressive motor, cognitive and visual decline [17,66,67]. Here, reduction of SCMAS immunoreactivity across brain regions of male and female *Cln6<sup>nclf</sup>* FBC-treated mice provides additional support that the drug acts as a disease-modifying agent.

In the *Cln6<sup>nclf</sup>* mouse, neuropathology has been linked to autophagy-lysosome pathway alterations, including LC3/p62 changes and defective clearance consistent with disrupted flux [15]. Western blot analysis of autophagy markers in brain lysates (see Supplementary Figure S3a–d and Figure S4a–d) in both sexes of *Cln6<sup>nclf</sup>* vehicle-treated mice

confirms non-significant trends for reduced expression of LC3-I and LC3-II and for increased p62/sequestosome1 levels when compared to age- and sex-matched WT mice. Because changes in LC3 and p62 are non-significant, these preliminary results do not permit firm conclusions regarding autophagic flux in *Cln6<sup>nclf</sup>* mice, unlike previously published results [15].

Consistent with prior work in CLN6 mouse models, untreated *Cln6<sup>nclf</sup>* mice display enhanced astrocytosis (marked by GFAP) and microgliosis (marked by IBA1) across brain regions [67,68]. Flupirtine benzyl carbamate treatment significantly reduces GFAP and IBA1 immunoreactivity in the brains of both sexes. These findings are highly relevant in light of accumulating evidence that glial dysfunction is not merely reactive but contributes causally to NCL pathogenesis [18,69]. The generalized reduction in astro- and microgliosis observed with flupirtine benzyl carbamate treatment aligns with this concept and suggests that flupirtine analogues may exert part of their benefit by modulating glial-mediated neuroinflammation.

In the retina, flupirtine benzyl carbamate did not reduce TUNEL-positive cells nor alter the ongoing loss of rods and cones marked by reduced rhodopsin and cone-arrestin immunoreactivity. This pattern suggests that the drug does not reverse established photoreceptor loss or halt outer-segment degeneration. It is noteworthy that retinal GFAP trended lower with FBC treatment in *Cln6<sup>nclf</sup>* mice in both males and females, but this did not reach significance. A similar partial dissociation between glial modulation and photoreceptor preservation has been observed in *Cln6<sup>nclf</sup>* mice treated with DHA or curcumin, where microgliosis and Müller cell activation were reduced, and photoreceptor structure and function were only partially maintained [67]. Despite robust SCMAS reduction across brain regions after treatment, these levels were not diminished in the retina of male and female *Cln6<sup>nclf</sup>* mice. Given that CLN6 disease is characterized by early-onset visual failure driven by photoreceptor degeneration and cortical involvement [5,14,67], initiating flupirtine benzyl carbamate treatment at or before the onset of retinal cell death may be required to translate anti-apoptotic and anti-inflammatory effects into long-term visual preservation. Alternatively, direct delivery of the drug into the eye may be necessary to overcome this, as has been observed in treatment of retinal degeneration in CLN2 disease [70].

Within the current NCL therapeutic landscape, an orally deliverable small molecule with sustained CNS benefit and long-term tolerability would address a central translational challenge in pediatric neurodegeneration. In this context, oral small-molecule strategies already being evaluated clinically include miglustat in CLN3 [71], oral trehalose across NCLs [72] and mycophenolate mofetil in CLN3 [73]. FBC's putative advantages, in addition to oral administration, are chronic tolerability *in vivo*, spasticity-relevant KV7/KCNQ2/3 excitability-stabilizing end-target and CNS-relevant anti-apoptotic and anti-inflammatory effects.

The presented findings build directly on an emerging line of work proposing flupirtine benzyl carbamate as a candidate therapy for human CLN6 disease. This study is the first to show that flupirtine benzyl carbamate can be safely administered orally from early postnatal life to adulthood in an *in vivo* *Cln6<sup>nclf</sup>* model, impacting both behavioral and corticosterone endpoints, diminishing CNS apoptosis, reducing SCMAS accumulation in the brain and modulating glial activation.

## 5. Conclusions

In summary, chronic oral treatment with flupirtine benzyl carbamate in *Cln6<sup>nclf</sup>* mice is well tolerated and produces broad CNS neuroprotection. It improves behavior in a sex- and task-dependent manner, normalizes corticosterone levels, reduces apoptosis and SCMAS storage, preserves neurons, and attenuates astrocytosis and microgliosis in the

brains of male and female *Cln6<sup>nclf</sup>* mice. However, the failure to restore complex motor function in females, despite correction of cellular and molecular pathology, highlights a narrow therapeutic window, likely due to faster and more severe disease progression. Notably, flupirtine benzyl carbamate had no detectable effect in the retina, failing to reduce apoptosis or prevent photoreceptor loss in either sex. Overall, this compound emerges as a promising disease-modifying candidate for CLN6-Batten disease and supports further development of optimized flupirtine derivatives for NCL.

## 6. Patents

- Application for Method of Treating Batten Disease. Inventor: Rose-Mary Boustany. Provisional filing date: 12 January 1999, Duke Ref. No. 1684. Myers, Bigel Sibley and Sajovek, P.A. (File No. 5405-240 PR). Met filing requirements of US Patent and Trademark Office on 10 July 2002. Assigned Serial No.10/148,859 (U.S. National Phase); US Patent issued 23 November 2004, US Pat # 6 821 995, expired 23 November 2014.
- Functionalized Pyridine Carbamates with Enhanced Neuroprotective Activity. Inventors: P. Trippier, N. Kinarivala, R.-M. Boustany (Texas Tech University and AUB). Application serial number 16/630522. Filing date: 14 July 2017. Receipt date: 13 January 2020, US National Stage under 35 USC 371. International Filing date: 13 July 2018. D-1365 NATL US Official Filing Receipt Ref TTU-1365; File No. TECH: 1166 US. Application Publication 28 January 2021; Publication No. US. -2021-0023064-A1. US Patent issued US 11,369,593 B2.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15050442/s1>, Supplementary Figure S1: Profiling of apoptosis-related protein markers in the brains of male mice; Supplementary Figure S2: Profiling of apoptosis-related protein markers in the brains of female mice; Supplementary Figure S3: Autophagy marker protein expression levels in the brains of male mice; Supplementary Figure S4: Autophagy marker protein expression levels in the brains of female mice; Supplementary Figure S5: Neuronal nuclei immunoreactivity in the brains of male and female *Cln6<sup>nclf</sup>* mice treated with FBC; Supplementary Figure S6: Accumulation of SCMAS in the brains of male and female *Cln6<sup>nclf</sup>* mice; Supplementary Figure S7: Astrogliosis in *Cln6<sup>nclf</sup>* male and female mouse brains after treatment with FBC; Supplementary Figure S8: Microgliosis in *Cln6<sup>nclf</sup>* male and female mouse brains treated with flupirtine benzyl carbamate.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The original data presented in this study are included in the article or uploaded as online supplemental material. Further inquiries can be directed to the corresponding author.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|        |                                                              |
|--------|--------------------------------------------------------------|
| ALT    | Alanine aminotransferase                                     |
| AST    | Aspartate aminotransferase                                   |
| ANOVA  | Two-way analysis of variance                                 |
| Bad    | BCL2 associated agonist of cell death                        |
| BUN    | Blood urea nitrogen                                          |
| CLN6   | Neuronal ceroid lipofuscinosis type 6                        |
| CNS    | Central nervous system                                       |
| CRMP2  | Collapsin response mediator protein 2                        |
| Cyt C  | Cytochrome C                                                 |
| DAPI   | 4',6-diamidino-2-phenylindole                                |
| DMSO   | Dimethyl sulfoxide                                           |
| ER     | Endoplasmic reticulum                                        |
| GAPDH  | Glyceraldehyde-3-phosphate dehydrogenase                     |
| GCL    | Ganglion cell layer                                          |
| GFAP   | Glial Fibrillary Acidic Protein                              |
| H&E    | Hematoxylin and eosin                                        |
| HO-2   | Heme oxygenase-2                                             |
| HSP70  | Heat shock protein 70                                        |
| IBA1   | Ionized calcium-binding adaptor molecule 1                   |
| INL    | Inner nuclear layer                                          |
| IPL    | Inner plexiform layer                                        |
| LAMP1  | Lysosome-Associated Membrane Protein 1                       |
| LC3    | Microtubule-associated protein 1 light chain 3               |
| NCL    | Neuronal ceroid lipofuscinoses                               |
| NeuN   | Neuron-specific nuclear protein                              |
| OCT    | Optimal cutting temperature                                  |
| ONL    | Outer nuclear layer                                          |
| OPL    | Outer plexiform layer                                        |
| PBS    | Phosphate-buffer solution                                    |
| PBST   | Phosphate-buffer solution containing 0.1% Triton X-100       |
| PFA    | Paraformaldehyde                                             |
| RPE    | Retinal pigment epithelium                                   |
| RT     | Room temperature                                             |
| SEM    | Mean $\pm$ standard error of mean                            |
| SMAC   | Second Mitochondria-derived Activator of Caspases            |
| SCMAS  | Mitochondrial ATP synthase subunit C                         |
| TBST   | Tris-buffered saline containing 0.1% Tween                   |
| TUNEL  | Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling |
| vLINCL | Variant late-infantile                                       |
| WT     | Wild-type                                                    |

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