Early Signs of Neuroinflammation in the Postnatal Wobbler Mice Model of Amyotrophic Lateral Sclerosis

Maria Meyer
Instituto de Biología y Medicina Experimental-CONICET
Analia Lima
Instituto de Biología y Medicina Experimental-CONICET
Maria Claudia Gonzalez Deniselle
Instituto de Biología y Medicina Experimental-CONICET
Alejandro F. De Nicola (alejandrodenicola@gmail.com)
Instituto de Biología y Medicina Experimental-CONICET

Research Article

Keywords: amyotrophic lateral sclerosis, Wobbler pups, astrogliosis, microgliosis, neuroinflammation, non-cell autonomous disease

Posted Date: June 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1746673/v1

Abstract

The Wobbler mouse is an accepted model of sporadic amyotrophic lateral sclerosis. The spinal cord of clinically symptomatic animals (3-5 months old) show vacuolar motoneuron degeneration, inflammation and gliosis accompanied by motor impairment. However, data are not conclusive concerning pathological changes appearing early after birth. To answer this question, we used postnatal day (PND) 6 genotyped Wobbler pups to determine abnormalities of glia and neurons at this early age period. We found astrogliosis, microgliosis with morphophenotypic changes pointing to active ameboid microglia, enhanced expression of the proinflammatory markers TLR4, NFkB, TNFα and inducible nitric oxide synthase. The astrocytic enzyme glutamine synthase and the glutamate-aspartate transporter GLAST were also reduced in PND 6 Wobbler pups, suggesting excitotoxicity due to impaired glutamate homeostasis. At the neuronal level, PND 6 Wobblers showed swollen soma, increased choline acetyltransferase immunofluorescence staining and low expression of the neuronal nuclear antigen NeuN. However, vacuolated motoneurons, a typical signature of older clinically symptomatic Wobbler mice were absent in the spinal cord of PND 6 Wobblers. The results suggest predominance of neuroinflammation and abnormalities of microglia and astrocytes at this early period of Wobbler life, accompanied by some neuronal changes. Data support the non-cell autonomous hypothesis of the Wobbler disorder, and bring useful information with regards to intervening molecular inflammatory mechanisms at the beginning stage of human motoneuron degenerative diseases.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal disorder characterized by motoneuron degeneration in motor cortex, brainstem and cervical spinal cord accompanied by astrogliosis and microgliosis (Kiernan et al. 2011). These abnormalities coincide with neuronal loss, neuroinflammation, increased oxidative stress, mitochondrial dysfunction, slow axonal transport and glutamate dyshomeostasis. Clinically, ALS patients show muscle wasting, motor impairment, respiratory failure and death within 5 years of diagnosis. The majority of ALS cases (80–90%) are sporadic (sALS), while the remaining 5–10% has a family history of the disease implicating several genes (familial ALS, fALS). (Gibson et al. 2014). In both cases, treatment with antioxidant or antiglutamatergic drugs only delays for a few months disease progression (Bensimon et al. 2009; Mora 2017). Because a typical histopathological feature is the pronounced gliosis, ALS is considered a non-cell autonomous disease (Filipi et al. 2020; Van Harten et al. 2021). Originally, gliosis has been considered a reactive response to neurodegeneration, although other studies postulate that pathological astrocytes and microglia play a pivotal role at the early beginning and progression stages of the disease. As such, astrocytes from both sALS and fALS secrete toxic factors for neurons, whereas microglia imposes an inflammatory environment (Brettschneider et al. 2012; Pirooznia et al. 2014; Rizzo et al. 2014).

The Wobbler mouse model of sALS provides important information regarding the role of different cells in this disease. Wobblers suffer a mutation of the vacuolar/vesicular protein sorting 54 gene (Vps 54) affecting the Golgi-associated retrograde protein (GARP) complex (Moser et al. 2013). Fully symptomatic

(> 2 month old) Wobblers of the NFR/NFR strain present a typical motoneuron vacuolation, impaired retrograde transport of vesicles, neuroinflammation, decreased glutamate transporters linked to excitotoxicity, changes of mitochondrial electron transport chain, decreased growth factor production, forelimb muscle atrophy, weak performance in tests of motor function, a wobbly gait and head tremor (Cihankaya et al. 2021; De Nicola et al. 2013; Moser et al. 2013). A time-dependent dysfunction of different cell types has been reported for Wobbler mice, but conclusions are unclear because reports covered different postnatal day (PND) periods. However, most authors support the non-cell autonomous hypothesis (Van Harten et al. 2021). Early studies by Hantaz-Ambroise et al (2001) and Laage et al (1988) have shown that astrogliosis is already present in 1 month-old Wobbler mice (Hantaz-Ambroise et al. 2001; Laage et al. 1988). Furthermore, Rathke-Hartlieb et al (1999) have found astrocyte reactivity starting on PND 17 and microgliosis from day 23 onwards (Rathke-Hartlieb et al. 1999). In Wobbler mice killed at PND 30, we have shown abundant vacuolated motoneurons in synchrony with astrogliosis and decreased expression of glutamine synthase (GS, EC 6.3.1.2), the enzyme preventing glutamate excitotoxicity (Meyer et al. 2010). Additional reports demonstrate that astrocytes from different ages show pathological features such as increased release of IL1β and tumor necrosis factor alpha (TNFα) in culture (6–7 week-old mice), glutamate dyshomeostasis (PND 20), increased nitric oxide synthase (NOS, EC 1.14.13.39) activity (PND 60) and lack of GS expression (PND 20) (Ait-Ikhlef et al. 1999; Boillee et al. 2001; Gonzalez Deniselle et al. 2004; Hantaz-Ambroise et al. 2001). Regarding microglia, Boillee et al (2001) have shown on PND 21 Wobblers microglial processes ensheathing vacuolated motoneurons followed by increased microglia density at later stages, whereas Diana et al (2010) have shown increased number of the ionized calcium-binding adaptor molecule 1 (Iba1) + microglia in the motor cortex of Wobblers at PND 40 (Diana et al. 2010). Recently Cihankaya et al. (2021) employing both symptomatic and non-symptomatic mice models of both sALS and fALS describe activated amoeboid microglia in the spinal cord and motor cortex (Cihankaya et al. 2021). Neuroinflammation is also a constant feature of 3–5 month-old Wobblers. These mice present increased levels of Iba1 and cluster of differentiation molecule 11B (CD11b) positive microgliosis and increased expression of the proinflammatory molecules tumor necrosis alpha /TNFα), toll-like receptor 4 (TLR4), inducible NOS (iNOS), the high mobility group 1 protein (HMGB1) and nuclear factor kappa B (NFkB) (De Nicola et al. 2020; Meyer et al. 2015; Meyer et al. 2018).

The time of appearance of astrocyte and microglia pathology in relation to neuronal degeneration has been also analyzed in the superoxide dismutase type I (SOD1, EC 1.15.1.1) transgenic mice model of fALS. In early symptomatic SOD1 mice, astrogliosis show a temporal relationship with neuronal degeneration according to Levine et al (1999) (Levine et al. 1999). Astrocytes expressing mutant SOD1 up-regulate iNOS expression and exacerbate motoneuron death (Julien and Kriz 2006). However, Hall et al (1998) report late development of astrogliosis in 100-day old SOD1G93 mice, although activated microglia and astrocytes show first an anti-inflammatory phenotype and later on a proinflammatory phenotype (Beers and Appel 2019; Hall et al. 1998). A time-related dissociation between appearance of astrogliosis (60 days) and microgliosis (late stage) has been published by Yang et al (2011) (Yang et al. 2011). Gliosis and neurodegeneration may also depend on the strain of SOD1 mice employed (Vargas

and Johnson 2010). Interestingly, Trias et al (2017) have reported an aberrant glial phenotype called the AbA cell showing proinflammatory activity and expression of both astrocyte and microglia markers in SOD1 transgenic mice (Trias et al. 2017). In a recent report, Cihankaya et al (2021) describe in both symptomatic and non-symptomatic transgenic mice, microglia adopt an activated amoeboid morphology in the spinal cord and other brain regions

In the present work, we aimed to compare in early PND control and Wobbler mice the number and activation of glial cells, expression of inflammatory factors and some neuronal parameters. The results demonstrated strong inflammation and gliosis at this early period of the Wobbler disease, accompanied by neuronal changes but without evidence of vacuolar degeneration. These data may bring useful information for pathogenic mechanisms developing in human neurodegenerative disorders.

Materials And Methods

Experimental animals

Newborn mice from the parental NFR/wr strain were genotyped from a tail biopsy, using published procedures (Meyer et al. 2015; Schmitt-John 2015). On PND 6 genetically identified Wobbler mice (wr/wr) and wild type control mice (NFR/NFR) were used for the different experiments. Because neither disease onset nor the disease progression differ between male and female Wobbler mice (Meyer et al. 2015) mice of both sexes were used in similar numbers in all experiments. Wobblers comprised ~20% of each litter, which made necessary to mate large number of breeding pairs. For immunocytochemistry pups were anesthetized with a mixture of ketamine (75 mg/kg, i.p.) and xylazine (6 mg/kg, i.p) and perfused intracardially with 2.5 ml of 0.9% NaCl, followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.2. Afterwards spinal cords were dissected, postfixed for 2.5 h at 4^{o}C in the same fixative and finally embedded in paraffin. Five µm sections of cervical spinal cords were cut in a microtome and placed on microscope slides. For immunofluorescence, cervical spinal cords from heart-perfused mice were embedded in Tissue-Tek (OCT compound, Miles Inc., USA), and 16-30 µm sections cut in the horizontal plane in a cryostat maintained at -20 ^{o}C. For mRNA analysis by qPCR, mice were killed by decapitation and spinal cords stored at -80 ^{o} C until used for RNA extraction. About 5-6 samples were studied per mice in all measurements.At this early age period, anatomical or behavioral differences were not apparent between control and Wobbler mice. To prevent bias in the results of immunocytochemistry, immunofluorescence and PCR analysis, researchers were blinded to experimental protocols.

Immunocytochemistry for labeling astrocytes and microglia

For astrocyte staining in spinal cord sections, we used a rabbit polyclonal glial fibrillary acidic protein (GFAP) antibody (1:250 dilution, # G-9269 from SIGMA-Aldrich, MO, USA), followed by a goat anti-rabbit IgG conjugated to Alexa Red 555 (1:1000 dilution) (Invitrogen, Molecular Probes, Eugene, OR, USA). The final step included drying of sections at room temperature and cover slipped with Fluoromount-G

(Southern Biotech, Birmingham, AL, USA). GFAP + cells were counted in the ventral horn gray matter. Images produced by confocal microscopy were analyzed using Image J (Image Processing and Analysis in Java, NIH, MD, USA) at 200X from at least 6 sections per mice . The number of GFAP+ astrocytes was quantified by this program and results were averaged per mice. Data was expressed as the mean number of GFAP+ cells ± SEM per unit area (mm^2). The quantity of Iba1+ microglia was determined by immunofluorescence staining (Meyer et al. 2015). Spinal cord sections from PND 6 Wobbler and control pups embedded in Tissue-Tek were stained for resting and reactive microglia using a rabbit anti-Iba1 antibody (1:1000, cat. #019-19741, Wako, Japan). The secondary antibody was a goat anti-rabbit IgG conjugated to Alexa Red 555. Sections were cover-slipped with Fluoromont-G. Iba1+ immunofluorescent microglial cells were counted in the gray matter of the ventral horn of the spinal cord (Meyer et al. 2015) using methods described above for GFAP+ astrocytes. The number of Iba1+ microglia was expressed per unit area (mm^2). Cells were counted in 5-6 sections from each animal. In addition to cell density, we analyzed the morphological immunophenotype of Iba1+ cells. For this purpose, cells were classified as ramified, showing small body with long processes, or ameboid with round body and scarce short processes.

Immunocytochemistry for labeling Glutamine synthase (GS) and GLAST

For GS, 5-µm-thick spinal cord sections were treated with mouse IgG blocking reagent (Vector M.O.M. Immunodetection Kit, Vector Labs, Burlingame, CA, USA), washed and incubated overnight with a 1/200 dilution of a purified monoclonal mouse anti-GS (#610517, BD Biosciences). As second antibody we used a 1/1000 dilution of a goat antimouse IgG conjugated to Alexa Green 488 (Invitrogen). Sections were cover-slipped with Fluoromount-G and the number of GS+ cells counted using Image J program. For GLAST staining sections were first blocked with 1% H_2O_2, then with 10% goat serum in PBS and exposed to a primary goat EAAT1 polyclonal antibody (sc-7757, Santa Cruz Biotech, TX, USA). The second antibody was a rabbit anti-goat from Sigma (cat.7014). Sections were then processed according to the ABC kit instructions (Vector). Determination of GLAST immunoreactive was carried out by computerized image analysis, as detailed in Meyer et al (2018).

Double immunofluorescence staining for GFAP and GS

To study if GS colocalized with GFAP in control and Wobbler astrocytes, 5 µm thick spinal cord sections were treated with mouse IgG blocking reagent (Vector M.O.M. Immunodetection Kit), washed and incubated overnight with a 1/200 dilution of a purified monoclonal mouse anti-GS (#610517, BD Biosciences, Franklin Lakes, NJ , USA.). As second antibody, we used a 1/1000 dilution of a goat antimouse IgG conjugated to Alexa Green 488 (Invitrogen). Afterwards, staining of astrocytes was performed with a rabbit GFAP antibody (1/250 dilution; same antibody shown above). Sections were then incubated with a goat anti-rabbit IgG conjugated to Alexa Red 555 (Invitrogen). The final step included drying of sections at room temperature and cover slipped with Fluoromount-G. This antibody combination produced green and red fluorescent labeling of each antigen, respectively. Dual-labeled immunofluorescence microscopy was analyzed with an Olympus IX83 inverted microscope equipped

with a disk-spinning unit (Olympus Corporation, Japan). Digital images were acquired using Cell Sens Dimensions software from Olympus and photographed with a Hamamatsu Orca Flash 4.0 monochromatic camera (Hamamatsu Photonics K.K, Japan*).*

Gene expression in PND 6 control and Wobbler mice

Analysis of mRNA expression by qPCR was performed following published procedures (Garay et al. 2012; Meyer et al. 2018). Total RNA from the spinal cord of control and Wobbler pups was extracted with Trizol (Life Technologies-Invitrogen, CA, USA), and residual DNA degraded by deoxyribonuclease 1 (DNase1, EC 3.1.21.1) (Promega, Madison, WI, USA) treatment. A M1705 MMLV reverse transcriptase (EC 2.2.2.49; Promega) was used for PCR amplification of DNA templates in the presence of random hexamer primers. Sequences of forward and reverse primers for the microglial marker CD11b and proinflammatory factors TNFα, iNOS, TLR4 and p65NFkB were those previously published (Garay et al. 2012; Meyer et al. 2018). Cyclophilin was used as the house keeping gene. Gene expression profiles were assessed by a real Time Step-one Plus sequence Detection System (Applied Biosystems, Foster City, CA, USA) and analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Results were expressed as fold induction over control.

Immunocytochemistry for choline acetyltransferase (ChAT).

For ChAT immunostaining (Meyer et al. 2010), 16 µm cryostat sections of the spinal cord were postfixed in 4% paraformaldehyde and kept at −80°C until used. After defrosting, sections received 1% H_2O_2 in methanol to block endogenous peroxidases and incubated at 4°C overnight with a 1/500 dilution of a ChAT goat polyclonal antibody (AB 144P, Chemicon, CA, USA). Sections were then incubated for 60 min with a biotinylated rabbit anti-goat secondary antibody (1/200 dilution) and then with a 1/100 dilution of the ABC complex (ABC kit, Vector Labs, CA, USA). The peroxidase activity was revealed using diaminobenzidine (DAB, 0.25 mg/ml) in the presence of 0.01% H_2O_2 in the dark. The number of ChAT+ cells was quantified in 10 sections per animal in the ventral horn using a computerized image analysis system (Bioscan Optimas 6.02). Digital images (digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope of tissue sections were displayed on the video screen under identical lighting conditions and grey-scale threshold.

Cresyl violet staining for light microscopy

Following laminectomy, cervical spinal cords were removed and small blocks of tissue were obtained by cutting transverse sections of 2–3 mm maximum length. Blocks were immersed for 2.5 h in 4% PFA, postfixed in graded series of ethanols and embedded in paraffin. Paraffin sections (5 µm) were hydrated and stained with cresyl violet (0.5% aqueous solution) and then dehydrated in graded ethanols and xylene, and mounted with Permount.

Immunofluorescence staining for NeuN

The phenotype of neurons of the ventral spinal cord was investigated using immunostaining for NeuN. 30-µm cryostat sections were blocked with 3% donkey serum in PBS 0.5% Triton X-100 for 10 min at 37 °C, then incubated overnight with the monoclonal NeuN antibody (anti-Neuronal Nuclei MAB 2377, Chemicon-Millipore, Billerica, CA, USA) at a 1/200 dilution. Slices were rinsed three times in PBS 0.1% Triton X-100 for 5 min before application of the second antibody donkey anti-mouse IgG conjugated to Alexa Fluor 488 (dilution 1/500). Incubation with the second antibody was followed by three rinses in PBS. Sections were mounted with Fluoromount G and kept in the dark at 4 °C until analysis by confocal microscopy. Cells were examined under an Olympus IX83 inverted microscope equipped with a disk-spinning unit (Olympus Corporation, Japan). Digital images were acquired using Cell Sens Dimensions software from Olympus and photographed with a Hamamatsu Orca Flash 4.0 monochromatic camera (Hamamatsu Photonics K.K, Japan*).* Quantification was performed using the Image J system, as explained above.

Double immunofluorescence analysis of ChAT/NeuN+ cells by confocal microscopy

To localize ChAT with NeuN immunoreactive motoneurons, 30 µm cryostat sections were heated for 10 min at 120 °C to retrieve antigens in 10 mM sodium citrate buffer pH 6.0. Afterwards, sections were blocked with 3% donkey serum in PBS for 10 min at 37 °C and incubated overnight at 4 °C with 1/100 dilution of the ChAT goat polyclonal antibody (AB 144P) and NeuN antibody (anti-Neuronal Nuclei MAB 2377, Chemicon-Millipore, Billerica, CA, USA) at a 1/200 dilution in 2% donkey serum, and 0.5% Triton-X100 in PBS. Slices were rinsed three times in PBS 0.1% Triton X-100 for 5 min before application of the second antibody donkey anti-goat IgG conjugated to Alexa Fluor 555 (dilution1/500) and donkey anti-mouse IgG conjugated to Alexa Fluor 488 (dilution 1/500). After several washes in PBS, sections were mounted with Fluoromount-G (0100-01), and examined under an Olympus IX83 inverted microscope equipped with a disk-spinning unit (Olympus Corporation, Japan). Confocal images were acquired, photograph and analyzed as described above for NeuN cells. We determined double-labeled ChAT+/NeuN+ neurons in the ventral horn and results expressed as the percentage of ChAT+/NeuN+ neurons per unit area (mm^2).

Statistical analysis

Results were expressed as mean ± S.E.M. Data were analyzed by two-tailed Student`s “t” test since only two groups (control and Wobbler) were compared. Statistical analyses were performed with Prism 6 GraphPad software (San Diego, CA, USA) and significance was shown by the following asterisks: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

Changes of GFAP+ astrocytes, GS+ cells and GLAST in the spinal cord of PND 6 control and Wobbler mice.

Astrogliosis is a recognized feature of Wobbler mice spinal cord, with reported variability regarding its time of appearance (Blondet et al. 1995; Duchen and Strich 1968; Laage et al. 1988). In our experiments, quantitative determination of GFAP+ astrocytes in the ventral horn of the spinal cord of PND 6 mice, revealed a 4-fold increase in astrocyte number in Wobblers compared to controls ($p<0.01$, Fig.1A). The immunofluorescence images of Fig.1B illustrate the mentioned differences in GFAP+ cell density between the two groups of mice. Thus, astrogliosis was already prominent at this early age period.

Wobbler astrocytes also show pathological features such as low expression of GS, the enzyme that prevents glutamatergic excitotoxicity by conversion of excess glutamate into inert glutamine (Blondet et al. 1995; Meyer et al. 2018). In consonance with findings in older symptomatic Wobbler mice (Blondet et al. 1995; Meyer et al. 2010), PND 6 Wobblers showed lower number of GS+ cells compared to control mice ($p<0.001$) (Fig. 1C). Representative images of GS labeling of both groups are shown in Fig. 1D. To identify the cell type expressing GFAP and GS, we performed double labeling immunofluorescence localization, and found that control mice showed higher number of double labeled GS+/GFAP+ cells compared to Wobbler pups ($p<0.01$). This abnormality of Wobbler astrocytes was determined by quantitative analysis (Fig. 1E) and illustrated in fluorescence microscope images (Fig. 1F). These results indicated that PND 6 Wobblers already expressed a population of GFAP+ GS - astrocytes, in agreement with results produced in mature Wobbler mice (Blondet et al. 1995; Diana et al. 2010; Meyer et al. 2010).

Furthermore, we also determined immunoreactivity for GLAST, an astrocyte transporter that takes up synaptic glutamate into the axonal terminal. GLAST immunolabeling was spread over the ventral horn of the spinal cord in both control and Wobbler pups. Quantitative analysis showed significantly reduced % immunoreactive area in PND 6 Wobblers vs control pups ($p<0.01$). Fig.1H points out the reduced GLAST immunostaining in the Wobbler pups.

Changes of microglia in PND 6 Wobbler and control pups

Microgliosis is a key finding of mature symptomatic Wobbler mice spinal cord (Blondet et al. 1995; Cihankaya et al. 2021; Meyer et al. 2018). According to our results, microgliosis was an early event of the Wobbler disorder. Thus, the number of Iba1+ cells was higher in the ventral horn of 6-day old Wobblers ($174\pm13/mm^2$) compared to control pups ($132\pm1/mm^2$) ($p<0.001$) according to quantitative analysis (Fig. 2A). Low power images of Iba1+ cells are shown in Fig.2B. In addition to Iba1 quantitative analysis, microgliosis was verified by measuring the microglial marker CD11b. Hence, mRNA levels of this marker in PND 6 Wobblers were significantly higher than in NFR/NFR control pups (fold increase 1.403 ± 0.14 in Wobblers vs 0.963 ± 0.10 in controls, $p<0.01$) (Fig. 2C). Furthermore, high power microscopy for Iba1+ cells demonstrated morphological differences between the two groups. Whereas control pups showed predominance of a ramified M1 phenotype, microglia in the Wobbler pups showed a more rounded body with short thick processes suggesting a M2 phenotype (Fig. 2D). Similar morphological variations were previously described for mature Wobbler mice, suggesting a higher degree of microglia activation (Meyer et al. 2018).

Proinflammatory mediators in PND 6 control and Wobbler mice.

Gliosis of symptomatic Wobbler mice is accompanied by neuroinflammation, with enhanced expression of proinflammatory mediators (Gargiulo-Monachelli et al. 2019; Meyer et al. 2018). To elucidate if inflammation was already present in PND 6 Wobbler mice we determined the mRNA expression of three components of the TLR-NFkB-iNOS pathway plus TNFα. As shown in Fig. 3, expression of the mRNA for NFkbp65, iNOS, TNFα and TLR4 was almost double in Wobblers compared to control pups. Statistical analysis using the Student`s “t” test showed significant increases for NFkBp65 ($p<0.01$), iNOS ($p<0.01$),), TNFα ($p<0.05$) and TLR4 ($p<0.05$). Thus, inflammatory markers were up regulated at this early age period of Wobbler mice, in consonance with data described in 5 month old Wobbler mice (Meyer et al. 2018).

Spinal motoneurons in PND 6 control and Wobbler mice

So far, changes of glial cells in PND 6 Wobblers resembled those at the early progressive (1-2 month old) and older stages of the disease, but in addition, the last two age periods show vacuolated (degenerating) motoneurons (Meyer et al. 2010). In contrast, cresyl violet staining of paraffin sections of PND 6 Wobbler mice did not detect vacuolated motoneurons (Fig.4B, left hand and middle images), although this histochemical procedure easily detects this abnormality in older mice (Fig. 4B, right hand images arrow). In PND 6 Wobblers, we observed swollen neurons in Lamina IX. Using Optimas software the area of control motoneurons measured 256 ± 13 μm^2 (n=8), whereas those from Wobbler pups were significantly larger (369±26 μm^2, n= 9; $p<0.01$) (Fig. 4 A).

Next, we performed immunocytochemistry for ChaT, the cholinergic marker enzyme. Quantitative analysis showed higher number of ChAT+ motoneurons in the Wobbler vs control pups (Fig.4C; $p<0.01$). Besides, ChAT immunofluorescent motoneurons of larger size appeared in Wobblers than in PND 6 controls, in similarity with results of cresyl violet staining (Fig 4D). Another finding in PND 6 Wobblers was a reduced number of NeuN+ neurons in ventral horn (Fig. 4E; $p<0.01$ vs control), suggesting abnormal protein expression in α-motoneurons that normally express ChAT and NeuN (Friese et al. 2009). Double-labeling immunofluorescence staining using different dyes demonstrated that % colocalization of ChAT+ neurons / NeuN + neurons was higher in PND 6 Wobblers vs control mice (Fig. 4F; $p<0.01$) and Fig. 4G (arrows). In association with the lower number of NeuN+ cells in the Wobbler spinal cord, these results supported that some ChAT + neurons in the Wobbler group did not express NeuN (Fig 4G, lower middle image, arrowhead). Fig.4G further showed in Wobbler pups decreased total number of NeuN cells (green), increased ChAT+ neurons (red), and colocalization of ChaT with remaining NeuN+ cells (arrows in merge image).

Discussion

The present report demonstrated that soon after birth, Wobbler mice showed stronger glial activation, neuroinflammation and incipient changes of ventral horn neurons compared to age-control mice. These statements were based on quantitative analysis of glial cell number and mRNA of the microglial marker

CD11b, morphophenotypic changes of microglia, and determination of proinflammatory markers. On the neuronal side, PND 6 Wobbler pups showed opposite changes of ChAT and NeuN labeling, swollen neuronal soma without evidence of vacuolar degeneration (paraptosis). This form of cell death is considered the typical signature of symptomatic Wobbler mice. Since neurodegeneration results from the combined effects of oxidative stress, mitochondrial respiratory chain dysfunction, blockage of axonal transport and high nitrergic activity (Dave et al. 2003; Fricker et al. 2018; Gonzalez Deniselle et al. 2002; Moser et al. 2013; Santoro et al. 2004) vacuolation probably emerges after longer exposure to pathological mediators.

The role of glial cells in neurodegeneration has been studied in both human patients and animal models of the disease (Lasiene and Yamanaka 2011). There is general consensus that in presymptomatic and symptomatic periods of the Wobbler disease as well as in SOD1 transgenic mice, there is a close association between gliosis and neuronal degeneration (Gonzalez Deniselle et al. 2004; Hall et al. 1998; Levine et al. 1999; MacLean et al. 2021). Wobbler spinal cord astrocytes show negative staining for GS and glutamatergic dyshomeostasis at PND 20, whereas those showing increased NADPH-diaphorase activity (nitric oxide synthase) appear at PND 60 (Blondet et al. 1995; Diana et al. 2010; Gonzalez Deniselle et al. 2004). The present study demonstrated that glial activation and high expression of proinflammatory factors occurred at earlier time points. The PND 6 period was selected because methodology used for mRNA extraction, retrotranscription and final identification of the Wobbler mutation takes about 3–5 working days. Genotyping is the accurate procedure to differentiate mutant mice from heterozygotes and control mice, since reliable methods to test motor impairment and paw deformity require 1 month or longer time. Our data agreed with pioneer studies by Laage et al (1998) and Hantaz-Ambroise et al (1994) that studied astrocyte number and concluded they may be the primary cell affected by the Wobbler mutation. Additionally to astrocyte changes, we and others have shown (1) microgliosis with high labeling for Iba1 and high expression of the CD11b marker, and (2) activated microglia and astrocytes associated to increased gene transcription of iNOS, TLR4, TNFα and NFkB (Meyer et al. 2015; Meyer et al. 2018). Published work have shown increased mRNA levels of TLR4 in young and mature Wobbler mice (De Paola et al. 2012; Meyer et al. 2018). TLR4 mediates neurotoxicity of Wobbler spinal cord (De Paola et al. 2012) and enhances NFkB-dependent transcription of key inflammatory molecules including iNOS. The enhanced NO-producing enzyme results in neurotoxic levels of NO, which damages membranes and inhibits mitochondrial respiratory complexes (Gonzalez Deniselle et al. 2012). Activated microglial cells also produce neurotoxic levels of TNFα, which in turn activates caspase 3 and exacerbates neurodegeneration (Dahlke et al. 2015). Although TNFα, iNOS, NFkB and TLR4 were already upregulated in PND 6 Wobblers, vacuolar degeneration of neurons was missing. In contrast, vacuolated neurons can already be detected in 1 month old Wobbler mice by common histochemical procedures (Gonzalez Deniselle et al. 2007).

Therefore, precocious glial activation with synthesis and release of proinflammatory molecules are found in the Wobbler mutation, supporting the non-cell autonomous hypothesis of neurodegenerative diseases (Cihankaya et al. 2021; Van Harten et al. 2021). This hypothesis is sustained by the increased number of GFAP + astrocytes and increased staining for Iba1 and enhanced CD11b mRNA levels in microglia of PND

6 Wobbler mice. During inflammation, these glial cell types change their functional and morphological phenotype from neuroprotective to neurotoxic, producing proinflammatory mediators leading to motoneuron degeneration (Frank-Cannon et al. 2009). At the morphological level, PND 6 Wobbler astrocytes became reactive with expression of GFAP in their processes, whereas microglia changed their quiescent phenotype with small soma and fine ramifications into activated ameboid forms bearing thick short processes. Thus, the local immune system of the spinal cord could play a pivotal role in progression of the disease. Interestingly, these changes in the mouse simulates the dysregulated immune system of ALS patients (Beers and Appel 2019).

Thus, changes of glial cells without vacuolar degeneration were readily detected in PND 6 Wobblers, indicating differences with late stage Wobblers. It is known that 1 month old and mature Wobbler mice show motoneuron vacuolation, and a general decrease of ChAT immunoreactive protein and enzyme activity in whole ventral horn (Gonzalez Deniselle et al. 2007). This would decreased acetylcholine synthesis in soma and its release at the neuromuscular plate, resulting in muscle weakness, paw atrophy, the “wobbling” gait and motor impairment shown by symptomatic Wobblers (Gonzalez Deniselle et al. 2007). In contrast, the PND 6 Wobblers of the present study showed increased ChAT staining and larger size motoneurons, findings opposite to those in older animals. However, cultures from 7 day old (Boillee et al. 2003), 16 day old (Blondet et al. 2002) and 18–22 day old Wobblers (Dockery et al. 1997) showed large swollen motoneurons. Therefore, increased ChAT labeling as shown here, could be due to an expanding soma (Boillee et al. 2003). In the SOD1-G93 mouse model of fALS, Dukkipati et al (2018) postulate that vulnerable motoneurons of PND 10 and 30 mice show increased soma size (Dukkipati et al. 2018). Another interpretation for this phenomenon could be that larger neurons are those that matured earlier under the influence of neurotrophic factors. In this regard, BDNF could play a compensatory role for an incoming degeneration. On PND 6 Wobblers show normal levels of BDNF measured by immunocytochemistry and in situ hybridization (results not shown) in contrast to the pronounced depletion of this factor in mature animals (Gonzalez Deniselle et al. 2007). One study demonstrate upregulation of BDNF (mRNA and protein) and its receptor TrKB in motoneurons from PND 28 Wobblers (Tsuzaka et al. 2001), although in our experience this neurotrophic factor was depleted in 1–3 month old Wobbler mice (Meyer et al. 2012). BDNF exerts positive effects on neurons, including survival, soma size and ChAT expression (Kishino et al. 1997), and may be an important factor that opposes vulnerability of young neurons. In the course of time, however, BDNF appears to be inadequate to stop motoneuron degeneration and neuroinflammation.

In addition, other neuronal differences were noted between control and Wobbler pups. For example, PND 6 Wobblers showed lower labeling for NeuN, a marker for α-motoneurons but not for γ-motoneurons, which normally stain negative. Under normal conditions, α-motoneurons are involved in muscle contraction and voluntary movement whereas γ-motoneurons are involved in the stretch reflex. Therefore, the misbalance between ChAT+, NeuN + α-motoneurons in controls and ChAT+, NeuN- γ motoneurons in PND 6 Wobbler mice could in the long run impair neuromuscular function. Finally, Wobbler pups showed abnormalities of GS in cells that did not bear GFAP staining. This abnormality of Wobbler astrocytes has been reported for mature animals (Blondet et al. 1995; Diana et al. 2010; Meyer et al. 2010) and replicated

here for early postnatal mice. Slow clearance of glutamate at the synapsis due to reduced expression of GS and GLAST likely produces excitotoxicity, that adversely affects neurons expressing ionotropic receptors (Diana et al. 2010). Thus, reduction of glutamate uptake by astrocytes and by the neuronal termini constitutes further evidence of early glial dysfunction in early postnatal Wobbler mice.

Concluding remarks

Data produced in this work demonstrated that during early days of life, Wobbler mice already showed inflammation of the spinal cord. Abnormalities in the function of glial cell types including astrocytes and microglia may bias neurons towards degeneration as the disease goes on unchecked. Neurons of early Wobblers showed abnormalities in markers of neuronal function and type, associated to early glial dysfunction. The profound changes of glial cells suggest that identification of neuroinflammatory markers in blood or peripheral tissues may help to design new therapeutic formulations. Altogether, our experiments support the non-cell autonomous hypothesis for ALS and other neurodegenerative diseases.

Declarations

AUTHOR CONTRIBUTION

.Maria Meyer and Maria Claudia Gonzalez Deniselle performed the experiments, carried out statistical analysis and made substantial contributions to interpretation of the data. Analia Lima provided technical support. Maria Claudia Gonzalez Deniselle and Alejandro F. De Nicola designed the experiments and performed writing and editing of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest The authors declare no conflict of interest

CONSENT TO PARTICIPATE All authors have given their consent to participate

Ethical Approval Animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) following the Guide for the Care and Use of Laboratory Animals (Animal Welfare Assurance, NIH certificate # F16-00065 A5072-01). Every effort was made to avoid animal suffering and treatments were supervised by IACUC personnel. .

References

1. Ait-Ikhlef A, Hantaz-Ambroise D, Jacque C, Belkadi L, Rieger F (1999) Astrocyte proliferation induced by wobbler astrocyte conditioned medium is blocked by tumor necrosis factor-alpha (TNF-alpha) and interleukin-1beta (IL-1beta) neutralizing antibodies in vitro. Cellular and molecular biology 45 (4):393–400
2. Beers DR, Appel SH (2019) Immune dysregulation in amyotrophic lateral sclerosis: mechanisms and emerging therapies. The Lancet Neurology 18 (2):211–220. doi:10.1016/S1474-4422(18)30394-6

3. Bensimon G, Ludolph A, Agid Y, Vidailhet M, Payan C, Leigh PN, Group NS (2009) Riluzole treatment, survival and diagnostic criteria in Parkinson plus disorders: the NNIPPS study. Brain: a journal of neurology 132 (Pt 1):156–171. doi:10.1093/brain/awn291
4. Blondet B, Carpentier G, Ait-Ikhlef A, Murawsky M, Rieger F (2002) Motoneuron morphological alterations before and after the onset of the disease in the wobbler mouse. Brain research 930 (1–2):53–57. doi:10.1016/s0006-8993(01)03405-9
5. Blondet B, Hantaz-Ambroise D, Ait-Ikhlef A, Cambier D, Murawsky M, Rieger F (1995) Astrocytosis in wobbler mouse spinal cord involves a population of astrocytes which is glutamine synthetase-negative. Neuroscience letters 183 (3):179–182
6. Boillee S, Peschanski M, Junier MP (2003) The wobbler mouse: a neurodegeneration jigsaw puzzle. Molecular neurobiology 28 (1):65–106. doi:10.1385/MN:28:1:65
7. Boillee S, Viala L, Peschanski M, Dreyfus PA (2001) Differential microglial response to progressive neurodegeneration in the murine mutant Wobbler. Glia 33 (4):277–287
8. Brettschneider J, Toledo JB, Van Deerlin VM, Elman L, McCluskey L, Lee VM, Trojanowski JQ (2012) Microglial activation correlates with disease progression and upper motor neuron clinical symptoms in amyotrophic lateral sclerosis. PloS one 7 (6):e39216. doi:10.1371/journal.pone.0039216
9. Cihankaya H, Theiss C, Matschke V (2021) Little Helpers or Mean Rogue-Role of Microglia in Animal Models of Amyotrophic Lateral Sclerosis. International journal of molecular sciences 22 (3). doi:10.3390/ijms22030993
10. Dahlke C, Saberi D, Ott B, Brand-Saberi B, Schmitt-John T, Theiss C (2015) Inflammation and neuronal death in the motor cortex of the wobbler mouse, an ALS animal model. Journal of neuroinflammation 12:215. doi:10.1186/s12974-015-0435-0
11. Dave KR, Bradley WG, Perez-Pinzon MA (2003) Early mitochondrial dysfunction occurs in motor cortex and spinal cord at the onset of disease in the Wobbler mouse. Experimental neurology 182 (2):412–420. doi:10.1016/s0014-4886(03)00091-8
12. De Nicola AF, Coronel F, Garay LI, Gargiulo-Monachelli G, Gonzalez Deniselle MC, Gonzalez SL, Labombarda F, Meyer M, Guennoun R, Schumacher M (2013) Therapeutic effects of progesterone in animal models of neurological disorders. CNS & neurological disorders drug targets 12 (8):1205–1218
13. De Nicola AF, Meyer M, Guennoun R, Schumacher M, Hunt H, Belanoff J, de Kloet ER, Gonzalez Deniselle MC (2020) Insights into the Therapeutic Potential of Glucocorticoid Receptor Modulators for Neurodegenerative Diseases. International journal of molecular sciences 21 (6). doi:10.3390/ijms21062137
14. De Paola M, Mariani A, Bigini P, Peviani M, Ferrara G, Molteni M, Gemma S, Veglianese P, Castellaneta V, Boldrin V, Rossetti C, Chiabrando C, Forloni G, Mennini T, Fanelli R (2012) Neuroprotective effects of toll-like receptor 4 antagonism in spinal cord cultures and in a mouse model of motor neuron degeneration. Molecular medicine 18:971–981. doi:10.2119/molmed.2012.00020

15. Diana V, Ottolina A, Botti F, Fumagalli E, Calcagno E, De Paola M, Cagnotto A, Invernici G, Parati E, Curti D, Mennini T (2010) Neural precursor-derived astrocytes of wobbler mice induce apoptotic death of motor neurons through reduced glutamate uptake. Experimental neurology 225 (1):163–172. doi:10.1016/j.expneurol.2010.06.008
16. Dockery P, Tang Y, Morais M, Vacca-Galloway LL (1997) Neuron volume in the ventral horn in Wobbler mouse motoneuron disease: a light microscope stereological study. Journal of anatomy 191 (Pt 1):89–98. doi:10.1046/j.1469-7580.1997.19110089.x
17. Duchen LW, Strich SJ (1968) An hereditary motor neurone disease with progressive denervation of muscle in the mouse: the mutant 'wobbler'. Journal of neurology, neurosurgery, and psychiatry 31 (6):535–542. doi:10.1136/jnnp.31.6.535
18. Dukkipati SS, Garrett TL, Elbasiouny SM (2018) The vulnerability of spinal motoneurons and soma size plasticity in a mouse model of amyotrophic lateral sclerosis. The Journal of physiology 596 (9):1723–1745. doi:10.1113/JP275498
19. Filipi T, Hermanova Z, Tureckova J, Vanatko O, Anderova AM (2020) Glial Cells-The Strategic Targets in Amyotrophic Lateral Sclerosis Treatment. Journal of clinical medicine 9 (1). doi:10.3390/jcm9010261
20. Frank-Cannon TC, Alto LT, McAlpine FE, Tansey MG (2009) Does neuroinflammation fan the flame in neurodegenerative diseases? Molecular neurodegeneration 4:47. doi:10.1186/1750-1326-4-47
21. Fricker M, Tolkovsky AM, Borutaite V, Coleman M, Brown GC (2018) Neuronal Cell Death. Physiological reviews 98 (2):813–880. doi:10.1152/physrev.00011.2017
22. Friese A, Kaltschmidt JA, Ladle DR, Sigrist M, Jessell TM, Arber S (2009) Gamma and alpha motor neurons distinguished by expression of transcription factor Err3. Proceedings of the National Academy of Sciences of the United States of America 106 (32):13588–13593. doi:10.1073/pnas.0906809106
23. Garay LI, Gonzalez Deniselle MC, Brocca ME, Lima A, Roig P, De Nicola AF (2012) Progesterone down-regulates spinal cord inflammatory mediators and increases myelination in experimental autoimmune encephalomyelitis. Neuroscience 226:40–50. doi:10.1016/j.neuroscience.2012.09.032
24. Gargiulo-Monachelli G, Meyer M, Lara A, Garay L, Lima A, Roig P, De Nicola AF, Gonzalez Deniselle MC (2019) Comparative effects of progesterone and the synthetic progestin norethindrone on neuroprotection in a model of spontaneous motoneuron degeneration. The Journal of steroid biochemistry and molecular biology 192:105385. doi:10.1016/j.jsbmb.2019.105385
25. Gibson SB, Figueroa KP, Bromberg MB, Pulst SM, Cannon-Albright L (2014) Familial clustering of ALS in a population-based resource. Neurology 82 (1):17–22. doi:10.1212/01.wnl.0000438219.39061.da
26. Gonzalez Deniselle MC, Carreras MC, Garay L, Gargiulo-Monachelli G, Meyer M, Poderoso JJ, De Nicola AF (2012) Progesterone prevents mitochondrial dysfunction in the spinal cord of wobbler mice. Journal of neurochemistry 122 (1):185–195. doi:10.1111/j.1471-4159.2012.07753.x
27. Gonzalez Deniselle MC, Garay L, Gonzalez S, Saravia F, Labombarda F, Guennoun R, Schumacher M, De Nicola AF (2007) Progesterone modulates brain-derived neurotrophic factor and choline

acetyltransferase in degenerating Wobbler motoneurons. Experimental neurology 203 (2):406–414. doi:10.1016/j.expneurol.2006.08.019

28. Gonzalez Deniselle MC, Garay L, Lopez-Costa JJ, Gonzalez S, Mougel A, Guennoun R, Schumacher M, De Nicola AF (2004) Progesterone treatment reduces NADPH-diaphorase/nitric oxide synthase in Wobbler mouse motoneuron disease. Brain research 1014 (1–2):71–79. doi:10.1016/j.brainres.2004.04.004
29. Gonzalez Deniselle MC, Lopez-Costa JJ, Saavedra JP, Pietranera L, Gonzalez SL, Garay L, Guennoun R, Schumacher M, De Nicola AF (2002) Progesterone neuroprotection in the Wobbler mouse, a genetic model of spinal cord motor neuron disease. Neurobiology of disease 11 (3):457–468
30. Hall ED, Andrus PK, Oostveen JA, Fleck TJ, Gurney ME (1998) Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS. Journal of neuroscience research 53 (1):66–77. doi:10.1002/(SICI)1097-4547(19980701)53:1<66::AID-JNR7>3.0.CO;2-H
31. Hantaz-Ambroise D, Jacque C, Ait Ikhlef A, Parmentier C, Leclerc P, Cambier D, Zadigue G, Rieger F (2001) Specific features of chronic astrocyte gliosis after experimental central nervous system (CNS) xenografting and in Wobbler neurological mutant CNS. Differentiation; research in biological diversity 69 (2–3):100–107
32. Julien JP, Kriz J (2006) Transgenic mouse models of amyotrophic lateral sclerosis. Biochimica et biophysica acta 1762 (11–12):1013–1024. doi:10.1016/j.bbadis.2006.03.006
33. Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burrell JR, Zoing MC (2011) Amyotrophic lateral sclerosis. Lancet 377 (9769):942–955. doi:10.1016/S0140-6736(10)61156-7
34. Kishino A, Ishige Y, Tatsuno T, Nakayama C, Noguchi H (1997) BDNF prevents and reverses adult rat motor neuron degeneration and induces axonal outgrowth. Experimental neurology 144 (2):273–286. doi:10.1006/exnr.1996.6367
35. Laage S, Zobel G, Jockusch H (1988) Astrocyte overgrowth in the brain stem and spinal cord of mice affected by spinal atrophy, wobbler. Developmental neuroscience 10 (3):190–198
36. Lasiene J, Yamanaka K (2011) Glial cells in amyotrophic lateral sclerosis. Neurology research international 2011:718987. doi:10.1155/2011/718987
37. Levine JB, Kong J, Nadler M, Xu Z (1999) Astrocytes interact intimately with degenerating motor neurons in mouse amyotrophic lateral sclerosis (ALS). Glia 28 (3):215–224
38. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25 (4):402–408. doi:10.1006/meth.2001.1262
39. MacLean M, Juranek J, Cuddapah S, Lopez-Diez R, Ruiz HH, Hu J, Frye L, Li H, Gugger PF, Schmidt AM (2021) Microglia RAGE exacerbates the progression of neurodegeneration within the SOD1(G93A) murine model of amyotrophic lateral sclerosis in a sex-dependent manner. Journal of neuroinflammation 18 (1):139. doi:10.1186/s12974-021-02191-2

40. Meyer M, Gonzalez Deniselle MC, Garay L, Sitruk-Ware R, Guennoun R, Schumacher M, De Nicola AF (2015) The progesterone receptor agonist Nestorone holds back proinflammatory mediators and neuropathology in the wobbler mouse model of motoneuron degeneration. Neuroscience 308:51–63. doi:10.1016/j.neuroscience.2015.09.007
41. Meyer M, Gonzalez Deniselle MC, Garay LI, Monachelli GG, Lima A, Roig P, Guennoun R, Schumacher M, De Nicola AF (2010) Stage dependent effects of progesterone on motoneurons and glial cells of wobbler mouse spinal cord degeneration. Cellular and molecular neurobiology 30 (1):123–135. doi:10.1007/s10571-009-9437-8
42. Meyer M, Gonzalez Deniselle MC, Gargiulo-Monachelli G, Garay LI, Schumacher M, Guennoun R, De Nicola AF (2012) Progesterone effects on neuronal brain-derived neurotrophic factor and glial cells during progression of Wobbler mouse neurodegeneration. Neuroscience 201:267–279. doi:10.1016/j.neuroscience.2011.11.034
43. Meyer M, Lara A, Hunt H, Belanoff J, de Kloet ER, Gonzalez Deniselle MC, De Nicola AF (2018) The Selective Glucocorticoid Receptor Modulator Cort 113176 Reduces Neurodegeneration and Neuroinflammation in Wobbler Mice Spinal Cord. Neuroscience 384:384–396. doi:10.1016/j.neuroscience.2018.05.042
44. Mora JS (2017) Edaravone for treatment of early-stage ALS. The Lancet Neurology 16 (10):772. doi:10.1016/S1474-4422(17)30289-2
45. Moser JM, Bigini P, Schmitt-John T (2013) The wobbler mouse, an ALS animal model. Molecular genetics and genomics: MGG 288 (5–6):207–229. doi:10.1007/s00438-013-0741-0
46. Pirooznia SK, Dawson VL, Dawson TM (2014) Motor neuron death in ALS: programmed by astrocytes? Neuron 81 (5):961–963. doi:10.1016/j.neuron.2014.02.024
47. Rathke-Hartlieb S, Schmidt VC, Jockusch H, Schmitt-John T, Bartsch JW (1999) Spatiotemporal progression of neurodegeneration and glia activation in the wobbler neuropathy of the mouse. Neuroreport 10 (16):3411–3416
48. Rizzo F, Riboldi G, Salani S, Nizzardo M, Simone C, Corti S, Hedlund E (2014) Cellular therapy to target neuroinflammation in amyotrophic lateral sclerosis. Cellular and molecular life sciences: CMLS 71 (6):999–1015. doi:10.1007/s00018-013-1480-4
49. Santoro B, Bigini P, Levandis G, Nobile V, Biggiogera M, Botti F, Mennini T, Curti D (2004) Evidence for chronic mitochondrial impairment in the cervical spinal cord of a murine model of motor neuron disease. Neurobiology of disease 17 (2):349–357. doi:10.1016/j.nbd.2004.07.003
50. Schmitt-John T (2015) VPS54 and the wobbler mouse. Frontiers in neuroscience 9:381. doi:10.3389/fnins.2015.00381
51. Trias E, Ibarburu S, Barreto-Nunez R, Barbeito L (2017) Significance of aberrant glial cell phenotypes in pathophysiology of amyotrophic lateral sclerosis. Neuroscience letters 636:27–31. doi:10.1016/j.neulet.2016.07.052
52. Tsuzaka K, Ishiyama T, Pioro EP, Mitsumoto H (2001) Role of brain-derived neurotrophic factor in wobbler mouse motor neuron disease. Muscle & nerve 24 (4):474–480. doi:10.1002/mus.1029

53. Van Harten ACM, Phatnani H, Przedborski S (2021) Non-cell-autonomous pathogenic mechanisms in amyotrophic lateral sclerosis. Trends in neurosciences 44 (8):658–668. doi:10.1016/j.tins.2021.04.008
54. Vargas MR, Johnson JA (2010) Astrogliosis in amyotrophic lateral sclerosis: role and therapeutic potential of astrocytes. Neurotherapeutics: the journal of the American Society for Experimental NeuroTherapeutics 7 (4):471–481. doi:10.1016/j.nurt.2010.05.012
55. Yang EJ, Kim SH, Yang SC, Lee SM, Choi SM (2011) Melittin restores proteasome function in an animal model of ALS. Journal of neuroinflammation 8:69. doi:10.1186/1742-2094-8-69

Figures

Figure 1

Effects of the Wobbler mutation on GFAP, glutamine synthase (GS) and GLAST labeling in PND 6 control and Wobbler mice. Wobblers (Wr) showed increased number of GFAP+ astrocytes (A, ** p<0.01), decreased labeling of GS (C, *** p<0.001) and reduced GLAST immunoreactive area (**p<0.01) vs. control (Ctl) mice. B and D represent immunofluorescence microscope images of the mentioned changes in GFAP+ and GS+ cells. E: Double-labeled staining for GS/GFAP showed decreased % colocalization in Wr vs. Ctl mice (** p<0.01). The arrows in F point to cells bearing both markers. Arrowheads point to GS - / GFAP+ cells in Wobbler´s spinal cord. Scale bar: 100 µm. H: GLAST immunolabeling of the ventral horn showed reduced GLAST protein expression in Wr. Scale bar:20 µm. (For GFAP n= 5 mice per group; for GS n=4 Ctl and 6 Wr mice; for GS/GFAP colocalizacion n= 5 mice per group; for % GLAST immunoreactive area n=5 mice per group).

Figure 2

Immunostaining for microglia and expression of the microglial marker CD11b in PND 6 control and Wobbler mice. Wobbler (Wr) mice showed Iba1+ microgliosis (A, ***p<0.001) and higher CD11b mRNA (C, **p<0.01) vs control (Ctl) mice. B: Low power immunofluorescence images of Iba1+ microglia in Ctl and Wr mice spinal cord. Scale bar: 100µm. Higher power microscopy (D) distinguished resting ramified microglia in Ctl mice vs active microglia in Wr mice. Scale bar: 50µm. Results represent the mean ± S.E.M. (For Iba1 n=6 Ctl and 5 Wr mice; for CD11b mRNA n=7 Ctl and 6 Wr mice, respectively).

Figure 3

Expression of proinflammatory markers in PND 6 control and Wobbler mice. Wobblers (Wr) showed significantly higher mRNA levels than Ctl for NFkBp65 (** p<0.01), iNOS (**<0.01), TNFα (* P<0.05) and TLR4 (*p<0.01). (Student “t” test”). Results represent the mean ± S.E.M. (For NFkB n= 7 Ctl and 6 Wr mice; for iNOS and TNFα n=5 mice per group; for TLR4 n=7 Ctl and 6 Wr mice, respectively).

Figure 4

Neuronal markers in PND 6 control and Wobbler mice. A: Cresyl violet staining showed increased area of motoneurons (MTN) in Wobbler (Wr) pups vs. control (A, **p<0.01). Furthermore, vacuolated neurons were absent in Wr (B, middle image) and Ctl mice (B, left-hand image). For comparison, the arrow on the right-hand image (B) point to a vacuolated neuron in a 5 month-old Wobbler mouse. Scale bar: 50μm. C: ChAT immunostaining showed higher labeling in Wr vs. Ctl (***p<.001); this change is reproduced in image form in D. Scale bar: 50μm. E: NeuN+ cells were decreased in Wr (**p<0.01), whereas % colocalization of ChAT+ / NeuN+ cells are higher in Wr (F, **p<0.01). G: Double-labeled colocalization of ChAT (red) and NeuN (green) demonstrated that a higher % of ChAT+ neurons colocalize with NeuN in Wr mice (lower right image, arrows) in comparison to Ctl mice (upper right image, arrows). Arrowheads depict ChAT+/NeuN neurons. The three upper images correspond to PND 6 control mice. Scale bar: 50μm. (For cresyl violet staining n=8 Ctl and 9 Wr mice; for ChAT n=5 mice per group; for double labeled colocalization n= 4 Ctl and 5 Wr mice, respectively).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Onlinefloatimage1.png