Neuropathologic and Biochemical Changes During Disease Progression in Liver X Receptor β<sup>−/−</sup> Mice, A Model of Adult Neuron Disease

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a disorder of the central nervous system (CNS) that affects motor neurons and leads to muscular atrophy and paralysis (1). The onset of ALS is highly variable, and the worst prognosis is associated with a younger age at onset of symptoms (2, 3). The lack of early pathological markers in patients requires alternative strategies of investigation, including the use of animal and cellular models. Transgenic mice that overexpress the mutated form(s) of the human Cu/Zn superoxide dismutase 1 (SOD-1) (which is related to the familial form, fALS1) and wobbler mice (which carry a spontaneous mutation in the Vps54 gene) are the 2 most studied models of motor neuron death (4–7). Although these mice share many clinical and neuropathologic features with ALS patients, the development and characterization of new ALS animal models would enhance understanding of possible risk factors that lead to the sporadic forms of ALS.

We recently characterized a murine model of adult-onset motor neuron disorder related to the deletion of the oxysterol receptor, liver X receptor β (LXRβ) (8). The LR<sub>α</sub> and LXRβ are oxysterol-activated nuclear receptors with important functions in regulating cholesterol homeostasis; they participate in lipid metabolism and attenuate inflammatory responses in peripheral organs and in the CNS. The activation of LXR decreases the severity of the animal model experimental autoimmune encephalomyelitis (9), attenuates neuroinflammation and amyloid plaque deposition in amyloid precursor protein/presenilin 1 transgenic mice (10), and inhibits inflammatory responses of microglia and astrocytes (11). The LR<sub>α</sub> is almost exclusively expressed in metabolically active tissues, whereas LXRβ is ubiquitously expressed, with high levels in the CNS (10). We previously described impaired learning and coordination ability, loss of motor neurons, reactive gliosis, and lipid accumulation in spinal motor neurons in 8-month-old LXRβ<sup>−/−</sup> male mice (8), but further characterization of the neurological phenotype of these mice is needed. Here, we evaluated the long-term progression of neuromuscular decay and investigated molecular mechanisms occurring in the spinal cord of LXRβ<sup>−/−</sup> mice from the presymptomatic (aged 3 months) to the latest stage of the disease (aged 24 months). We found that presymptomatic LXRβ<sup>−/−</sup> mice selectively accumulate cholesterol in...
spinal cord but not in peripheral tissues and develop marked spinal cord inflammation. We speculate that the loss of spinal motor neurons and decline in motor function are related to cholesterol metabolism, neuroinflammation, and increased susceptibility to motor neuron damage during aging of these mice.

**MATERIALS AND METHODS**

**Animals**

The LXRβ⁻/⁻ mice were generated as previously described and backcrossed for 10 generations to a C57BL/6J background (12). The mice were housed with a regular 12-hour light/12-hour dark cycle and given free access to water and standard rodent chow. Procedures involving animals and their welfare were conducted according to institutional guidelines (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council 1996). All experiments were approved by the local ethical committee. Based on our previous characterization (8), this study was carried out exclusively in male mice.

**Motor Performance Tests**

A single individual who was blinded to the mouse genotype during the test scored neuromuscular performances on Rotarod, grip strength, and running time tests (13). To measure the foreleg muscle strength (grip strength), mice were lifted by the tail and positioned slightly above a horizontal bar that was connected to a mechanoelectric transducer (Ugo Basile Instruments, Comerio, Italy). They

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**FIGURE 1.** Abnormalities in liver X receptor β⁻/⁻ (LXRβ⁻/⁻) mice include impaired motor performance. Body weight (A), Rotarod (B), running time (C), and grip strength (D) were analyzed once a week in LXRβ⁻/⁻ (broken lines) and wild-type (WT) (black lines) mice from ages 27 to 100 weeks. Each point represents the mean ± SD obtained for each experimental group (n = 15). p < 0.001 between the WT and LXRβ⁻/⁻ groups for all performance tests. (E) Representative figure showing the correct procedure of mouse positioning and traction to minimize the effect of the body weight in the grip strength evaluation.
were allowed to grasp a small triangle tied to the bar with both forelegs. The correct positioning of mice minimized the influence of body weight (Fig. 1E). The grip strength value was recorded at the moment when the mouse released the horizontal bar, as a result of a gentle and continuous traction applied by the operator. Because reduced lipogenesis and lesser fat accumulation led to lower gains of body weight of LXRβ−/− mice compared with age-matched wild-type (WT) mice, normalization of grip strength values by body weight was not done in this study. This strategy avoided any possible overestimation of muscle strength of the LXRβ−/− mice. Indeed, we observed that there was a significant increase of normalized grip strength in LXRβ−/− compared with WT mice from age 3 to 7 months; this difference was exclusively related to the lower increase of body weight and was unrelated to the grip strength scores. To minimize anxiety or stress, the mice were transferred to a room dedicated to behavioral performances 15 minutes before the test. Before recording of scores, each mouse was trained to the operator’s handling (ages 4-6 months). Behavioral trials were performed once a week and lasted until the end of the 24th month of life.

**Lipopolysaccharide Treatment and Enzyme-Linked Immunosorbent Assay**

Plasma levels of tumor necrosis factor (TNF) and interleukin 6 (IL-6) were measured at ages 3, 10, and 24 months. Both cytokines were measured 90 minutes after lipopolysaccharide administration (35 µg/kg), diluted in saline solution (10 µL/g body weight, intraperitoneally), or after administration of the same volume of vehicle. Animals were killed by decapitation. Blood was collected in heparinized vials and centrifuged at 2,000 × g for 10 minutes at 4°C. Tumor necrosis factor and IL-6 were measured in the supernatants by enzyme-linked immunosorbent assay (ELISA) using commercial kits according to the suppliers’ protocols (TNF, DuoSet ELISA, R&D Systems, Minneapolis, MN; IL-6, mouse IL-6 ELISA Ready-Set-Go, e-Bioscience, Rome, Italy).

**Cholesterol Measurement**

Total plasma cholesterol levels were measured by colorimetric analysis (500 nm) using a commercial kit (Cholesterol 20; Sigma Diagnostic, St Louis, MO). Liver and spinal cord were homogenized and lipids were extracted overnight with chloroform/methanol mixture. Spinal and liver cholesterol values were normalized to the relative protein amount (14). All experiments were performed using tissues from 3-month-old LXRβ−/− and age-matched WT mice.

**Quantitative Polymerase Chain Reaction**

Cervical spinal cords from 3-month-old LXRβ−/− and WT mice were collected. Total RNA was isolated using the E.Z.N.A. Total RNA kit I (Omega Bio-Tek, Inc, Norcross, GA). The RNA was reverse-transcribed using the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction was performed using the SYBR Green technology with the Power SYBR Fast Master Mix (Applied Biosystems, Foster City, CA) using the 7500 Fast Real-Time PCR System (Applied Biosystems). Primers were designed using Primer Express software. The relative changes in gene expression were calculated by the comparative Ct method using 18S (20 × ribosomal 18S, ABI; Part-4310875, Applied Biosystems) as the internal reference gene. All primer sequences are available upon request.

**Immunohistochemistry**

At the end of behavioral trials, LXRβ−/− and WT mice that had been randomly selected before starting behavioral observations were transcardially perfused, and the spinal cord was collected as previously described (15). The same procedure was used for the spinal cords of 3- and 10-month-old mice not included in the behavioral observation. Thirty-micrometer-thick sections were cut and immunostained for glial fibrillary acidic protein ([GFAP] dilution 1:1000, mouse monoclonal antibody; Immunological Sciences, Rome, Italy), CD11b (dilution 1:50, mouse monoclonal antibody OX 42; Serotec, Oxford, UK), TNF (dilution 1:200, mouse monoclonal antibody; HyCult Biotechnology, Udend, the Netherlands), and IL-1β (1:200, goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (16). For colocalization experiments, the following markers were analyzed: GFAP, CD11b, TNF (dilution 1:200, mouse monoclonal antibody; HyCult Biotechnology), phosphorylated JNK (dilution 1:200, mouse monoclonal antibody; Santa Cruz Biotechnology), phosphorylated p38MAPK ([Pp38MAPK] dilution 1:200, goat polyclonal antibody; Cell Signaling Technology, Beverly, MA). To stain neurons, sections were incubated for 30 minutes at room temperature with 530/615 NeuroTrace fluorescent Nissl reagent (dilution 1:1000; Molecular Probes, Eugene, OR). Secondary antibodies (1:1000, Alexa-488, Alexa-546, Alexa-Cy5; Molecular Probes) were incubated for 1 hour at room temperature. Sections were analyzed with an Olympus Fluoview microscope BX61 with confocal system FV500. Images were pseudocolored, and the signals from the 3 different channels were automatically merged by Olympus Fluoview software.

**Morphometric and Morphological Analyses**

Cholinergic spinal neurons were counted in 24-month-old LXRβ−/− and WT mice either in the lumbar (L1–L3) or the cervical (C2–C6) region. In 3- and 10-month-old mice, counting was limited to the cervical cord level (C2–C6). In all experiments, a specific antibody against choline acetyl transferase ([ChAT] dilution 1:1000, rabbit polyclonal antibody; Immunological Sciences) (17) was used. In addition, the total number of Nissl-stained neurons in cervical cord (C2–C5) of 3- and 24-month-old LXRβ−/− and WT mice was determined using TissueQuest analysis software (TissueGnostics, Vienna, Austria) (18). The analysis included processing of at least 30 sections per mouse. Bright-field colored images were converted into 8-bit pictures (gray scale) and transformed into negative images (Figure, Supplemental Digital Content 1, parts A and B, http://links.lww.com/NEN/A114). Before starting the automatic counting, the main parameters (nuclear size, discrimination area, discrimination gray) were manually set (Figure, Supplemental Digital Content 1, parts C and D,
http://links.lww.com/NEN/A114). For each section, all values representing each single cell were automatically plotted in a scattergram based on the values of the cell size (y value) and the intensity of staining (x value). A threshold of 200 μm$^2$ was automatically introduced, and the total number of events (>200 μm$^2$ and <200 μm$^2$) was visualized (Figure, Supplemental Digital Content 1, part E, http://links.lww.com/NEN/A114).

Finally, evaluation of Nissl-stained large-sized neurons was done in the ventral horn (Lamina IX) to quantify the number of healthy cells. Neurons altered in shape, size, and with weaker cytoplasmic staining (chromatolysis) were excluded from the cell counting. The same operator performed each counting in a blinded way. The number of neurons measured in LXR$^{--/--}$ mice was expressed as percentage of the number of neurons in the WT group (normalized to 100).

To identify active neuromuscular junctions, biceps muscles were removed from both forepaws by cutting their tendons. This tissue was immediately postfixed in 4% paraformaldehyde at 4°C for 90 minutes, washed in 3 sucrose-graded solutions in a 0.1 mol/L phosphate buffer at 4°C, and frozen by submersion into 2-methylbutane cooled in liquid nitrogen (~160°C). Serial longitudinal sections 20-μm thick (n = 20) were cut and stained with bromoindoxyl acetate (Sigma). Biceps muscles from WT and LXR$^{--/--}$ mice were analyzed at ages 3, 10, and 24 months (6 per group). The counting was performed with the Tissue Quest system as previously described.

Free cholesterol was stained by Filipin III (5 μg/mL; Sigma), as previously reported (19). To investigate the presence of cholesterol in neurons, spinal cord sections 25-μm thick were processed with 530/615 NeuroTrace fluorescent Nissl reagent (1:1000; Molecular Probes) and Filipin III in 10 spinal cord sections/mouse; 4 animals for each group at the 3 ages (3, 10, and 24 months) were used. For each section, 2 fields corresponding to the Lamina IX of right and left ventral horns were sampled. Each field sampled was a rectangle measuring 512 μm x 376 μm. Olympus-DB software, coupled to the camera of microscope, was used to perform the counting of neurons stained with Filipin III.

Statistical Analysis

All data were expressed as mean ± SD. Two-way analysis of variance (ANOVA), repeated measures, with Bonferroni post-test analysis, was used for studying the interaction between the 2 groups during clinical observation and to compare the trend of behavioral trials at any single point. To evaluate the influence of time for each group, 1-way ANOVA test was used. Linear regression analysis was used to determine if the slope of body weight differed between the 2 experimental groups. Numbers of motor neurons and levels of proinflammatory cytokines were analyzed by the unpaired Student t-test. Values of p ≤ 0.05 were considered significant. All statistical analyses were done using the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

LXR$^{--/--}$ Mice Have Progressively Impaired Neuromuscular Function

No differences in body weight were found between LXR$^{--}$ mice and WT mice at 3 months of age (26.3 ± 1.3 g vs 24.9 ± 0.9 g, respectively). At the beginning of the behavioral observations (27th week), the weights of LXR$^{--/--}$ mice were significantly lower than those of WT mice (32.6 ± 1.2 g vs 35.4 ± 0.8 g, ***p < 0.0001) (Fig. 1A). The LXR$^{--/--}$ mice did not gain weight during the study, whereas the weight gain over time was significant in WT mice (1-way ANOVA, p < 0.0001). The body weight difference between LXR$^{--/--}$ and WT mice was highly significant (2-way ANOVA, p < 0.0001).

At 3 weeks after initiation of the training period, both LXR$^{--/--}$ and WT mice were able to complete the performance test. From the 27th to the 45th week, there were no significant differences between LXR$^{--/--}$ and WT mice (Fig. 1B). Thereafter, LXR$^{--/--}$ mice showed markedly reduced performance, as determined by the time spent on the rotating bar from 179 seconds recorded at the 45th week to 50 seconds at the 100th week. The impaired motor function affecting LXR$^{--/--}$ mice was further confirmed by running time tests: LXR$^{--/--}$ mice worsened their performances, whereas WT mice showed a mild trend toward improvement in the first phase of observation (Fig. 1C). At the end point, performance was significantly worse in LXR$^{--/--}$ than in WT mice (WT, 2.96 ± 0.16 seconds; LXR$^{--/--}$, 5.83 ± 0.20 seconds; ***p < 0.0001).

Grip strength performances in WT mice progressively increased during the first phase of behavioral observations.
LXRβ−/− mice were markedly reduced, that is, from 115 to 79 g during the same time range (Fig. 1D). In the second half of the behavioral tests, both LXRβ−/− and WT mice reduced their performances, but a significant difference persisted between the groups until the end point of the study (WT, 101 ± 3.0 g; LXRβ−/−, 48 ± 2.2 g; ***p < 0.0001). In contrast to other models of neurodegenerative diseases, LXRβ−/− mice did not show alteration of the paw position throughout the whole duration of the study.

**LXRβ−/− Mice Show Progressive Loss of Motor Neurons and Neuromuscular Innervation**

The morphological changes affecting spinal cord neurons and neuromuscular districts of LXRβ−/− mice were evaluated in preclinical (3 months), early clinical (10 months), and late clinical (24 months) phases. The Chat immunohistochemistry and active neuromuscular junctions in 10-month-old LXRβ−/− mice are illustrated in Figures 2A and C, respectively.

At 3 months, the mean numbers of Nissl-positive large neurons (WT, 100.0 ± 11.9; LXRβ−/−, 101.3 ± 9.7; not significant [NS]) (Fig. 2H) and small neurons (WT, 100.0 ± 3.7; LXRβ−/−, 100.9 ± 5.2; NS) and numbers of active neuromuscular junctions (WT, 100.0 ± 8.4; LXRβ−/−, 102.1 ± 7.9; NS) (Fig. 2D, left columns) were not different between the 2 groups. Counts of cholinergic neurons confirmed these results. No difference in the total number of Chat-positive neurons was observed in 3-month-old LXRβ−/− mice versus WT mice (WT, 100.0 ± 6.9; LXRβ−/−, 102.3 ± 6.1; NS) (Fig. 2B, left columns). The mean number of neuromuscular junctions in biceps muscles of 3-month-old LXRβ−/− mice also did not differ with the number in WT mice (Fig. 2D, left columns).

At 10 months, LXRβ−/− mice had a mild but significant loss of cholinergic neurons compared with WT mice (WT, 100.0 ± 7.4; LXRβ−/−, 86.6 ± 8.3; *p < 0.05) (Fig. 2B, middle columns), but there was no significant difference in mean numbers of active neuromuscular junctions in biceps at this age (WT, 100.0 ± 6.9; LXRβ−/−, 91.1 ± 7.9; NS) (Fig. 2D, middle columns).

At 24 months, there was an overall loss of spinal neurons in the LXRβ−/− mice compared with WT mice (WT, 100.0 ± 1.8; LXRβ−/−, 88.0 ± 3.2; ***p < 0.01). There were differences in both large neurons (WT, 100.0 ± 3.0; LXRβ−/−, 78.3 ± 3.1; ***p < 0.01) (Fig. 2F, right columns) and smaller neurons (WT, 100.0 ± 1.8; LXRβ−/−, 88.3 ± 3.9; ***p < 0.01) (Fig. 2E, left columns). In 24-month-old LXRβ−/− mice, the rate of loss of cholinergic neurons at the cervical spinal cord level was similar to that measured by counting large (>200 μm²) Nissl-positive neurons (WT, 100.0 ± 12.5; LXRβ−/−, 79.8 ± 2.6; **p < 0.01) (Fig. 2B, right columns). A similar result was observed by comparing the number of Chat-positive cells at the lumbar level (WT, 100.0 ± 7.3; LXRβ−/−, 74.3 ± 7.9; ***p < 0.001), indicating widespread motor neuron loss along the whole spinal axis in LXRβ−/− mice at 24 months. There was also a dramatic loss of active neuromuscular junctions (WT, 100.0 ± 9.8; LXRβ−/−, 68.9 ± 10.3; ***p < 0.001).

**FIGURE 3.** Proinflammatory factor expression is increased in liver X receptor β−/− (LXRβ−/−) mice. The RNA from 3-month-old wild-type (WT) and LXRβ−/− mice spinal cord and mRNA expression of tumor necrosis factor (TNF) (A), interleukin 6 (IL-6) (B), interleukin 1β (IL-1β) (C), and monocyte chemotactic protein 1 (MCP-1) (D). Levels of TNF (E) and IL-6 (F) were determined in plasma from 3-, 10-, and 24-month-old WT and LXRβ−/− mice 90 minutes after lipopolysaccharide (75 μg/kg) stimulation. The relative expression level in WT mice was set to 1.0 for all genes. Data are the mean ± SD (n = 6 per group). *p < 0.05; ***p < 0.0001.
In 24-month-old mice, large Nissl-positive α-motor neurons were identified as central-column motor neurons and lateral-column motor neurons (Fig. 2G). Large neurons in WT mice did not show any apparent morphological abnormalities (Fig. 2H), whereas in LXRβ−/− mice, there were many large neurons in the ventral horns with altered shapes and sizes and weaker nonhomogeneous staining (chromatolysis) (Fig. 2I); this feature resembles postmortem motor neurons in ALS patients (20).

Quantification of morphologically healthy neurons further confirmed the difference between the 2 groups (WT, 100.0 ± 16.6; LXRβ−/−, 48.4 ± 21.7; ***p < 0.001) (Fig. 2J). Interestingly, in 10-month-old LXRβ−/− mice, there was a high percentage of healthy motor neurons (WT, 100.0 ± 3.4; LXRβ−/−, 79.9 ± 11.3; **p < 0.01), indicating that there is progressive neurodegeneration between the 10th and the 24th month in cervical motor neurons of LXRβ−/− mice. Furthermore, there was a continuous impairment of neuromuscular units during the second year of life.

**LXRβ−/− Mice Have Progressive Inflammation in the Spinal Cord**

There was significantly greater mRNA expression of IL-6, TNF, IL-1β, and monocyte chemotactic protein 1 (MCP-1) in the spinal cords of 3-month-old LXRβ−/− versus WT mice, indicating the presence of an inflammatory response (Figs. 3A–D). Plasma TNF and IL-6 levels in LXRβ−/− mice were below the threshold of ELISA sensitivity for both LXRβ−/− and WT mice at any stage of life (results not shown), but when the mice were stimulated with lipopolysaccharide, LXRβ−/− mice showed a stronger activation of both plasma cytokines compared with WT mice (Figs. 3E, F).

Immunohistochemical analyses of glial markers in the cervical spinal cords of 3-, 10-, and 24-month-old WT mice showed very low expression of GFAP (Figs. 4A, E) and CD11b (Figs. 4I, M) throughout the ventral horn gray matter. In contrast, there was more immunoreactivity of both glial markers in LXRβ−/− mice (Fig. 4) even at age 3 months, before clinical disease onset. At 10 months, there

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**FIGURE 4.** Liver X receptor β−/− (LXRβ−/−) mice show progressive astrogliosis in the CNS. Glial fibrillary acidic protein (GFAP) staining of cervical spinal cord of 24-month-old wild-type (WT) mice (A, E) and LXRβ−/− mice at 3 months (B, F), 10 months (C, G), and 24 months (D, H) of age. CD11b staining of activated microglia in the cervical spinal cord sections of 24-month-old WT mice (I, M) and LXRβ−/− mice at 3 months (J, N), 10 months (K, O), and 24 months (L, P) of age. Scale bars = (A–D, I–L) 100 μm; (E–H, M–P) 20 μm.
were morphological changes in astrocytes (Figs. 4C, G) and microglia (Figs. 4K, O) in the spinal cord, and further changes were found at 24 months. Astrocytes in the anterior horns were shorter and thicker with high expressions of GFAP (Figs. 4D, H), and marked alterations in microglial morphology were observed at this later phase of the disease. They showed thickening of proximal processes and transformation from fibrillar to phagocyte-like cells mainly in cells close to the Lamina IX (Figs. 4L, P). Although LXRβ−/− mice had slower and milder courses of motor neuron degeneration, these morphological changes are similar to those found in astrocytes and microglia in wobbler mice (21) and in SOD1 mice (22, 23).

Despite a large increase of IL-1β mRNA levels in spinal cords of 3-month-old LXRβ−/− mice, low expressions of IL-1β were observed in both experimental groups. The expressions of IL-1β also did not increase in either 10- or 24-month-old LXRβ−/− mice (not shown). The expression of TNF was nearly absent in WT mice at all ages (Fig. 5A), whereas there was selective expression in the gray matter of LXRβ−/− mice (Fig. 5B). High-magnification pictures confirmed the expression of TNF in both large and smaller neurons (Fig. 5C).

Colocalization experiments were carried out to determine whether the increased expression of proinflammatory markers was exclusive to motor neurons or was also in glial cells. In 3-month-old LXRβ−/− mice, TNF expression was mainly observed in the ventral horns (Figs. 5D, G) and colocalized with the neuronal marker NeuroTrace in large neurons (Figs. 5E, F). Neither GFAP-positive cells (Fig. 5H) nor CD11b+ cells (not shown) showed significant colocalization with TNF (Fig. 5I). Strong TNF expression was also found in 10- and 24-month-old LXRβ−/− mice as well as in other neuronal subtypes (not shown). The TNF, GFAP, or CD11b immunoactivities in WT mice were very faint or were almost undetectable (not shown).

The Pp38MAPK expression was observed in large neurons in 10-month-old LXRβ−/− mice that colocalized with NeuroTrace in a small number of neurons (Figs. 5J–L). The expression of Pp38MAPK (Fig. 5N) was very low in astrocytes (Figs. 5M, O) and almost absent in microglia (not shown). The phosphorylated INK was almost undetectable in both experimental groups at any age (results not shown).

**LXRβ−/− Mice Accumulate Cholesterol in Spinal Cord Neurons**

The relative expression of multiple genes related to biosynthesis, esterification, and transport of cholesterol was measured in the spinal cords of 3-month-old WT and LXRβ−/− mice. No differences in the mRNA levels of genes involved in either biosynthesis or esterification were found (Figs. 6A, B). By contrast, expression of apolipoprotein E (ApoE) that is involved in cholesterol transport was markedly increased in LXRβ−/− mice. Other cholesterol transport mRNA levels did not differ from those of WT mice (Fig. 6C). Consistent with this observation, LXRβ−/− mice selectively accumulated cholesterol in the spinal cord (even in the preclinical phase; WT, 0.06 ± 0.01 mg/μg protein; LXRβ−/−, 0.10 ± 0.01 mg/μg protein; ***p < 0.0001) (Fig. 6F), whereas systemic cholesterol levels did not differ between WT and LXRβ−/− mice (plasma: WT, 31.1 ± 1.8 μg/dL; LXRβ−/−, 31.0 ± 1.4 μg/dL; liver: WT, 0.05 ± 0.08 mg/μg protein; LXRβ−/−, 0.07 ± 0.02 mg/μg protein) (Figs. 6D, E). The ApoE is an important apolipoprotein necessary for cholesterol transport from glial cells into neurons. The selective increase in spinal cholesterol levels and overexpression of ApoE are consistent with accumulation of cholesterol in neurons of LXRβ−/− mice, which was demonstrated by double staining with Filipin III and the neuronal marker NeuroTrace in 3-month-old LXRβ−/− mice (Fig. 5P), but not in age-matched WT mice (not shown). The mean number of Filipin III–positive large neurons was increased in the ventral spinal cord of LXRβ−/− mice compared with WT mice at all ages (Fig. 6H). Although the mean number of neurons with a high cytoplasmic content of free cholesterol did not increase until the late phase, it should be noted that a large number of neurons in the spinal cord of 24-month-old LXRβ−/− mice were lost or showed marked alterations, including loss of cytoplasmic content. The present characterization cannot exclude additional mechanisms, but marked accumulation of free cholesterol in TNF-immunoreactive ventral horn neurons (Figs. 5Q–R) suggests a possible link between cholesterol influx, neuroinflammation, and neurodegeneration.

**DISCUSSION**

We provide a detailed longitudinal investigation of clinical and neuropathologic features of LXRβ-deficient mice until the end of the second year of life. The results suggest that lack of the LXRβ gene leads to impairment of cholesterol homeostasis, accumulation of cholesterol in neurons, neuroinflammation, susceptibility to motor neuron degeneration, loss of neuromuscular junctions, muscular weakness, and motor function deficits. These observations make LXRβ−/− mice a potentially useful model for further investigations into motor neurodegenerative pathogenesis that likely are

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**FIGURE 5.** Tumor necrosis factor (TNF) and phosphorylated p38MAPK (Pp38MAPK) are selectively overexpressed in neurons of liver X receptor β−/− (LXRβ−/−) mice. The TNF expression in the cervical spinal cord of 24-month-old wild-type (WT) (A) and LXRβ−/− (B, C) mice. The TNF (D), Nissl NeuroTrace (E), and colocalization (F) in the spinal cord of a 3-month-old LXRβ−/− mouse. Spinal cord of a 3-month-old LXRβ−/− mouse showing TNF (G), glial fibrillary acidic protein (GFAP) (H), and colocalization (I). Nissl NeuroTrace (J), activated Pp38MAPK (K), and colocalization (L) in the spinal cord of a 10-month-old LXRβ−/− mouse. The GFAP (M), activated Pp38MAPK (N), and colocalization (O) in the spinal cord of a 10-month-old LXRβ−/− mouse. (P) Cervical spinal cord of a 3-month-old LXRβ−/− mouse shows marked accumulation of free cholesterol (yellow) in neurons (red, arrows). (Q) Staining for free cholesterol (yellow, Filipin III) in TNF-positive neurons (red). Scale bars = (A, B) 100 μm; (C, J–L) 25 μm; (D–I) 40 μm; (M–O, Q) 50 μm; (P) 30 μm; (R) 15 μm.
unrelated to a specific gene mutation. In ALS patients and in wobbler and SOD1 mice, loss of motor neurons precedes the onset of disease and progresses during clinical phases, leading to an almost complete muscle paralysis of affected regions (5, 6). However, unlike sporadic ALS patients, these mouse models show early onset of signs and progression in juvenile mice. A late adult-onset motor neuron disorder has been identified in male mice lacking the LXRβ gene (8, 24), characterized by a progressive decay in neuromuscular function that does not lead to muscle paralysis or premature death. Our analyses of spinal motor neurons in 24-month-old LXRβ−/− mice show less motor neuron loss than is generally seen in human postmortem samples (25) and in both mouse models (6, 26, 27). The moderate decrease of active neuromuscular junctions, occurring from the early to the late symptomatic stage of the disease and likely after motor neuron loss, fits well with neuropathologic and behavioral observations of a late, slight, and progressive motor neuron degenerative process associated with the lack of the LXRβ gene. This “mild” clinical worsening can be dramatically modified in the presence of exogenous stimuli, that is, β-sitosterol administration (24). The hypersusceptibility of LXRβ−/− mice to disease worsening might allow investigations of possible risk factors that have been suggested to be responsible for sporadic ALS cases. Another important point of dissimilarity between LXRβ−/− mice and ALS patients is the degeneration of smaller spinal neurons observed in the spinal cord of 24-month-old LXRβ−/− mice. However, lack of a selective motor neuron vulnerability, already reported even in younger LXRβ−/− mice (24), has also been also been observed in wobbler and SOD1 mice (28, 29).

In LXRβ−/− mice, glial activation and increased expression of proinflammatory cytokines in the spinal cord occur before neuronal loss and clinical disease onset, as in SOD1 mice (20, 30). The selective activation of TNF could lead to the phosphorylation of 2 important stress kinases (p38MAPK and JNK), thereby activating cell death pathway (23). The colocalization and increased expression of both Pp38MAPK and TNF observed in spinal neurons of LXRβ−/− mice suggest that a similar mechanism may be involved.

The lack of normal LXR function increases in the expression of several cytokines and activates monocytes or microglia recruitment, worsening the inflammatory state in CNS and in peripheral organs (31). Furthermore, treatment with LXR agonists reduces the in vivo and in vitro response to inflammatory stimuli (32). We found increases in expression of proinflammatory cytokines and monocyte chemotactic protein 1 in the deficient mice, suggesting that LXRβ plays a fundamental role in the CNS by reducing levels of cytokines and therefore reducing the development of neuroinflammation. In contrast to other mutant mice models in which adaptive processes somehow mask gene deletion(s), our results suggest that a compensatory effect mediated by increased expression of the other LXR isoform, LXRα, does not occur in the CNS of LXRβ−/− mice.

Impaired cholesterol homeostasis in the CNS is common to several neurodegenerative diseases, but the possible contribution of cholesterol in ALS is controversial. Despite a recent study hypothesizing a protective role of systemic dyslipidemia (33), previous data suggest that accumulation of ceramides and cholesterol esters mediates oxidative stress–induced death of motor neurons in ALS (34, 35). In mammals, CNS is the most cholesterol-rich region of the body. In adults, synthesis of cholesterol in the spinal cord is 5-fold higher than in the brain, but its concentration is only 2-fold higher (36), indicating that the spinal cord needs a higher efficiency of cholesterol excretion than other regions in the brain. Although cholesterol cannot cross through the blood-brain barrier, its metabolites, 24(S)-hydroxycholesterol (24S-OHC) and 27-OHC, have this ability (37). The 27-OHC was identified as a potent LXR agonist (38), and activation of LXR in the brain induces expression of several target genes including the cholesterol transporters, ABCA1 and ABCG1, in neurons (39) and ApoE in astrocytes (40). Neurons are believed to meet their demand for cholesterol by uptake from ApoE-containing lipoproteins secreted from astrocytes (41) and to remove excess cholesterol by converting it to 24S-OHC (via Cyp4p6), which is released into the circulation by the ATP-binding cassette transporter–cholesterol transporters. Disruption of this pathway would lead to altered cholesterol levels in neurons. In the spinal cord of LXRβ−/− mice, we found a significant and specific induced expression of ApoE, but not of other genes involved in biosynthesis, esterification, or transport of cholesterol. Our results suggest that cholesterol is correctly transported into the neurons but not removed from the cerebrospinal fluid. This is in agreement with our previous observation of selective lipid accumulation in spinal motor neurons of LXRβ−/− mice (8) and to the selective increase of cholesterol levels in spinal cord neurons.

Increased cholesterol levels are also associated with a proinflammatory response, as seen in atherosclerosis, via cytokine production from macrophages. Microglia are

FIGURE 6. Selective increase of cholesterol levels in the spinal cord of liver X receptor β−/− (LXRβ−/−) mice. (A–C) The mRNA in spinal cord from 3-month-old wild-type (WT) and LXRβ−/− mice measured. (A) Cholesterol biosynthesis; sterol regulatory element-binding protein (SREBP) 1a and 2, 3-hydroxy-3-methyl-glutaryl-CoA synthase (HMG-CoS), squalene synthase (SqS). (B) Cholesterol esterification; sterol O-acetyltransferase acyl-Coenzyme A: cholesterol acyltransferase (SOAT) 1 and 2. (C) Cholesterol transport: apolipoprotein E (ApoE), ATP-binding cassette transporter (ABC) A1/G1/G4/G5/G8, low-density lipoprotein receptor (LDL-R), low-density lipoprotein receptor–related protein 1 (LRP1), Proprotein convertase subtilisin/kexin type 9 (PCSK9), very-low-density lipoprotein receptor (VLDL-R). Data are expressed as the ratio of levels in LXRβ−/− compared with WT mice. Cholesterol levels in plasma (D), liver (E), and spinal cord (F) of 3-month-old WT and LXRβ−/− mice (G). Representative fields in ventral horns (white boxes) used for counting neurons that showed a high expression of Filipin III. (H) Numbers of neurons (mean ± SD) showing high Filipin III expression in WT and LXRβ−/− mice (n = 6 per group). *p < 0.05; **p < 0.005.

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capable of producing an array of cytokines (42), and it is therefore possible that increased levels of cholesterol in the spinal cord of LXRβ−/− mice may make them prone to an early inflammatory response (as demonstrated by glial activation in presymptomatic mice), and that the lack of anti-inflammatory activity further exacerbates this process.

In summary, we report here that LXRβ plays an important role not only in the maintenance of CNS cholesterol homeostasis, but also to the inhibition of the glia-associated inflammatory response. Many features in the CNS of LXRβ−/− mice resemble those in ALS patients and, despite the lack of selective motor neuron vulnerability, these mice may represent a novel and interesting model to test the susceptibility of motor neurons to detrimental stimuli that may be hypothesized to be involved in ALS pathogenesis.

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604 © 2010 American Association of Neuropathologists, Inc.
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