Early Microglial Activation Precedes Neuronal Loss in the Brain of the Cstb\(^{-/-}\) Mouse Model of Progressive Myoclonus Epilepsy, EPM1

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**Abstract**

Progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1) is a hereditary neurodegenerative disorder caused by mutations in the cystatin B (CSTB) gene encoding an inhibitor of cysteine proteases. Here, we provide the first detailed description of the onset and progression of pathologic changes in the CNS of Cstb-deficient (Cstb\(^{-/-}\)) mice. Our data reveal early and localized glial activation in brain regions where neuron loss subsequently occurs. These changes are most pronounced in the thalamocortical system, with neuron loss occurring first within the cortex and only subsequently in the corresponding thalamic relay nucleus. Microglial activation precedes the emergence of myoclonia and is followed by successive astrocytosis and selective neuron loss. Neuron loss was not detected in thalamic relay nuclei that displayed no glial activation. Microglia showed morphologic changes during disease progression from that of phagocytic brain macrophages in young animals to having thickened branched processes in older animals. These novel data on the timing of pathologic events in the CSTB-deficient brain highlight the potential role of glial activation at the initial stages of the disease. Determining the precise sequence of the neurodegenerative events in Cstb\(^{-/-}\) mouse brains will lay the basis for understanding the pathophysiology of EPM1.

**Key Words:** Astrocyte activation, Cerebellum, Cerebral cortex, Cerebrum, Microglia, Myoclonus epilepsy, Neurodegeneration, Posterior thalamic nuclei, Somatosensory cortex.

**INTRODUCTION**

The progressive myoclonus epilepsies (PMEs) are a heterogeneous group of inherited disorders characterized by myoclonus, epilepsy, and progressive neuronal degeneration (1, 2). The pathogenesis of the PMEs is poorly understood, and these disorders are generally refractory to treatment. The most common single cause of PME is Unverricht-Lundborg disease (EPM1, OMIM no. 254800), an autosomal recessively inherited disorder with an onset at 6 to 16 years, usually with stimulus-sensitive myoclonus or tonic-clonic seizures. Other neurologic symptoms, including ataxia and dysarthria, appear as the disease progresses but cognition is essentially preserved (3–5). Symptomatic pharmacologic and rehabilitative management including psychosocial support are the mainstay of EPM1 patient care (5).

EPM1 is caused by loss-of-function mutations in the cystatin B gene (CSTB; OMIM no. 601145) encoding an inhibitor of cysteine proteases (6–8). These proteases include lysosomal cysteine cathepsins, and based on studies on EPM1 patient lymphoblastoid cells, it has been suggested that their increased activity is related to EPM1 pathogenesis (9). However, the precise role of CSTB and the mechanisms by which its loss leads to EPM1 remain poorly understood.

A mouse model for EPM1 has been generated by targeted disruption of the mouse Cstb gene (Cstb\(^{-/-}\) mice) (10). These mice recapitulate the key clinical features of EPM1, including appearance of myoclonic seizures at 1 month of age and progressive ataxia (10). Their major neuropathologic phenotype is a severe loss of cerebellar granule neurons due to apoptotic death. Widespread gliosis accompanied by increased expression of apoptosis- and glial activation-related genes have been also reported in aged Cstb\(^{-/-}\) mice (11–13). Deficiency of Cstb on either seizure-prone or seizure-resistant backgrounds displays similar neuropathologic changes, indicating that these events are independent of seizures (10, 12). Our recent findings from cultured cerebellar granule neurons from Cstb\(^{-/-}\) mice point to an elevated susceptibility to oxidative stress that is at least partially mediated through elevated cathepsin B activity (14). Furthermore, mice deficient for both CSTB and cathepsin B show significantly less cerebellar apoptosis but no changes in their seizure or ataxia phenotypes (15). These results suggest that, although cathepsin B seems to contribute to the pathogenesis of EPM1, cathepsin inhibition is not the sole...
function of CSTB and that CSTB has a wider role in maintaining normal neuronal function and architecture.

In this study, we have systematically investigated the onset and progression of neuron loss and glial activation in the Cstb\(^{-/-}\) mouse brain at different stages of the disease. Our results show early microglial activation followed by astrogliosis and neuron loss, suggesting a central role for microglia in the pathogenesis of EPM1.

**MATERIALS AND METHODS**

**Mice**

The cystatin B–deficient (Cstb\(^{-/-}\)) mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME; 129-Cstb\(^{tm1Rm}\)/J; stock no. 003486) (10). Wild-type offspring from heterozygous matings were used as controls. Animal research protocols were approved by the Animal Ethics Committee of the State Provincial Office of Southern Finland (decision no. STU376A, no. STH660A and no. STH524A). All animals used were males.

**Histologic Processing**

For histologic analysis, Cstb\(^{-/-}\) and control mice (n = 6 per genotype and age, except n = 4 for P14) were killed at 2 weeks (P14) and at 1, 2, 4, and 6 months of age. Brains were removed, bisected along the midline, and immersion fixed in 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer pH 7.4 for at least 1 week before cryoprotection in 30% sucrose in 50 mmol/L Tris-buffered saline (TBS) containing 0.05% sodium azide. Forty-micrometer frozen coronal sections were cut through the cerebral with Microm HM430 Sliding Microtome (Microm International, Walldorf, Denmark), and cerebella were cut sagittally. Sections were collected, 1 per well, into 96-well plates containing cryoprotectant solution (TBS/30% ethylene glycol/15% sucrose/0.05% sodium azide), as described (16, 17), and stored at \(-80^\circ\text{C}\) before histologic processing. All histologic processing and subsequent analyses were done using all these samples unless otherwise stated and were performed with no prior knowledge of genotype.
Nissl Staining

To provide direct visualization of neuronal morphology, every sixth section was Nissl stained as previously described (16, 17). Sections were mounted onto gelatin-chrome alun–coated slides, air-dried overnight, and incubated for 45 minutes at 60°C in a solution of 0.05% Cresyl fast violet (VWR, Dorset, UK)/0.05% acetic acid, rinsed in distilled water, and differentiated through a graded series of alcohols before clearing in xylene (VWR) and coverslipped with DPX (VWR).

Regional Volume Measurements

Unbiased Cavalieri estimates of the volume of cerebral cortex, cerebellum, and cerebellar layers (molecular and granular cell layer, white matter) were made in Nissl-stained sections using StereoInvestigator software (Microbrightfield, Inc, Williston, VT), as previously described (16, 17). A sampling grid with appropriate spacing (200 μm for cerebral cortex and 150 μm for cerebellar layers) was superimposed over Nissl-stained sections, and the number of points covering the relevant areas was counted using a ×1.25 objective. Regional volumes were expressed in cubic micrometers, and mean volume was calculated for control and Cstb−/− mice at each age.

Cerebral Cortex Thickness Measurements

To explore the extent of cortical atrophy, cortical thickness measurements were made in Nissl-stained sections through cortical regions that serve different functional roles, including the primary somatosensory (S1), primary somatosensory barrel field (S1BF), primary visual (V1), primary motor (M1), and lateral entorhinal (LEnt) cortices defined according to the landmarks described by Paxinos and Franklin (18) (Fig. 1). Ten perpendicular lines extending from white matter to pial surface were placed on 4 consecutive sections using a ×5 objective. Lines were measured using either StereoInvestigator or Fiji (ImageJ 1.44a) software. Results were expressed as the mean cortical thickness in micrometer per region for Cstb−/− and control mice.

Counts of Neuronal Number and Volume

To examine neuronal survival within individual thalamic nuclei and their target cortical regions, we used StereoInvestigator software to obtain unbiased optical fractionator estimates of neuronal numbers and nucleator estimates of neuronal volumes from Nissl-stained sections. Estimates of neuronal number were obtained in posterior thalamic nucleus (Po) and dorsal lateral geniculate nucleus (LGNd), and their target areas S1 and V1 (laminae IV, V, and VI), respectively. Estimates for neuronal volume were obtained only for neurons in Po and lamina V of S1 of P14 and 6-month-old animals. Measurements were performed as previously described (19), with random starting section followed by every 6th (Po, LGNd, and V1) or every 12th (S1) section thereafter. The boundaries of selected areas were defined by reference to landmarks in Paxinos and Franklin (18). Only neurons with clearly identifiable nucleolus were sampled, and all counts were carried out using a ×100 oil objective (NA 1.4). The following sampling scheme was applied to the regions of interest: grid area for Po 30,625 μm²; frame area 2400 μm²; grid area for LGNd 15,625 μm²; frame area 2400 μm²; grid area for S1 and laminae IV and V 50,625 μm²; frame area 2400 μm²; grid area for S1 and lamina VI 50,625 μm²; frame area 1000 μm²; grid area for V1 and laminae IV, V, and VI 30,625 μm², frame area 1000 μm².

Immunohistochemistry

To assess the extent of glial activation, adjacent 1-in-6 series of free-floating sections were immunostained, as described previously (17) for detection of astrocytic (rabbit anti–glial fibrillary acidic protein [GFAP], 1:5000; DAKO, Cambridge, UK) and microglial (rat anti-F4/80, 1:100; Serotec, Oxford, UK) markers. Briefly, sections were incubated for 15 minutes in 1% H2O2 in TBS to quench endogenous peroxidase activity, rinsed in TBS, and incubated for 40 minutes in TBS/0.3% Triton X-100 (TBS-T)/15% normal serum. Sections were incubated overnight at 4°C in primary antibody diluted with 10% normal serum in TBS-T and subsequently rinsed in TBS and incubated for 2 hours in biotinylated secondary antiserum (Vector Laboratories, Peterborough, UK) diluted with 10% normal serum in TBS-T. After rinsing with TBS, sections were incubated for 2 hours in Vectastain avidin-biotin-peroxidase complex (Vectastain Elite APCKit; Vector Laboratories), diluted with 10% normal serum in TBS-T, and rinsed again in TBS. Immunoreactivity was visualized by a standard diaminobenzidine–hydrogen peroxide reaction (Sigma, Dorset, UK), after which the sections were transferred to excess ice-cold TBS, mounted onto gelatin-chrome alun–coated SuperFrost microscope slides (VWR), air-dried overnight, and passed through a graded series of alcohols before clearing in xylene and coverslipping with DPX mounting medium (VWR).

Statistical Analysis

The statistical significance of differences between genotypes of all quantitative data was assessed using 1-way analysis of variance with Bonferroni correction (GraphPad Prism 5.0c; GraphPad Software, La Jolla, CA) with p ≤ 0.05 considered as statistically significant. The mean coefficient of error for all individual optical fractionator and Cavalieri estimates was calculated according to the method of Gundersen and Jensen (20) and was less than 0.08 in all of the analyses.

FIGURE 3. Progressive atrophy of cerebral cortex in Cstb−/− mice. (A) Cavalieri estimates of the volume of cerebral cortex in Cstb−/− mice compared with control (ctrl) mice. Progressive cortical atrophy was seen in Cstb−/− mice from 2 months of age onward. (B) Primary somatosensory cortex (S1) in Nissl-stained sections from Cstb−/− and ctrl mice showed progressive atrophy in Cstb−/− mice, which occurred to a similar extent across all cortical laminae. Scale bars = 100 μm. (C–G) Cortical thickness measurements reveal progressive thinning of the cortex, at 2 months in S1, S1BF, and V1; at 4 months in M1; and at 6 months in LEnt. Data are expressed as mean regional volume in cubic micrometers ± SEM (A) or mean thickness in micrometers ± SEM (C–G). One-way analysis of variance: *, p ≤ 0.05; **, p ≤ 0.01, ***, p ≤ 0.001.
Image Analysis

All microphotographs were obtained as color images with AxioCam HRc (Zeiss, Oberkochen, Germany) and converted to black-and-white images using Adobe Photoshop CS4 software (Adobe Systems, Inc, San Jose, CA). Images of F4/80 staining were converted using blue filter to enhance the visualization of the staining.

RESULTS

Progressive Atrophy and Cortical Thinning in Cstb⁻/⁻ Mouse Brain

Previous studies have reported a progressive loss of cerebellar granule cells in Cstb⁻/⁻ mice (10). Our analyses also show marked progressive loss of cerebellar volume in Cstb⁻/⁻ mice compared to control mice (Fig. 2A). Cavalieri estimates of regional volumes revealed significant and progressive atrophy of the cerebellum of Cstb⁻/⁻ mice from 2 months of age onward, reaching almost 50% reduction in cerebellar volume by the age of 6 months (Fig. 2B). No significant differences in the rate of volume loss between molecular layer, cerebellar granule cell layer, and white matter were observed (data not shown).

The occurrence of pathologic changes in the cerebrum of Cstb⁻/⁻ mice, particularly in young animals, has not yet been systematically analyzed. Our data reveal progressive atrophy of the cerebral cortex of Cstb⁻/⁻ mice from 2 months of age onward (Figs. 3A, B). To assess whether any sub-regions of the cortex may be preferentially affected, we carried out thickness measurements of different cortical regions as representative areas that receive information from different sensory modalities (Figs. 3C–G). Significant and progressive thinning was observed in S1, S1BF, and V1 from 2 months of age onward in Cstb⁻/⁻ compared to control mice. In M1, the difference was significant at 4 months, and in LEnt, the difference was significant only at 6 months of age. Cortical thinning at 2 months of age coincided with the onset of atrophy in the Cstb⁻/⁻ brains, but no differences were seen in rate of thinning between individual cortical laminae (Fig. 3B, numerical data not shown).

Microglial Activation

To examine glial activation in Cstb⁻/⁻ mice, we surveyed the distribution of cells immunoreactive for the microglial marker F4/80 in the brains of Cstb⁻/⁻ and control mice at different ages (Fig. 4). Intensely stained F4/80 immunoreactive microglia were already apparent in restricted regions of the Cstb⁻/⁻ CNS at P14 and became more widespread and intense with disease progression (Table). The most pronounced microglial activation in Cstb⁻/⁻ brains was seen in midline and posterior thalamic nuclei, substantia nigra, claustrum, amygdaloid nuclei, and cingulate cortex (Figs. 4A–E). The hippocampal formation of Cstb⁻/⁻ mice contained considerable amounts of activated microglia at P14, but at later stages (with the exception of the hippocampal dentate gyrus, which displayed activated microglia throughout disease progression), it was almost completely devoid of activated microglia (Fig. 4F). Throughout the cerebral cortex, many more intensely stained microglia were present in the most dorsal (I, II) and ventral laminae (VI) than in intervening laminae III-V (Fig. 4G). In the cerebellum of Cstb⁻/⁻ mice, activated microglia were evenly distributed with no consistent differences between individual layers (white matter, granule cell layer, or molecular layer) or individual lobules (data not shown). In contrast to gray matter, white matter of Cstb⁻/⁻ mice only displayed microglial activation at 6 months of age.

A striking feature of Cstb⁻/⁻ mice was the change in morphology of microglia during disease progression (Fig. 4H). At P14, microglial cells in Cstb⁻/⁻ brain show the classic, activated phenotype with thickened processes and round somata, whereas in control brains, microglia were much less darkly stained with many thin ramified processes. In 1-month-old Cstb⁻/⁻ mice, most microglia displayed intense F4/80 immunoreactivity with a dramatically enlarged cell soma and numerous short tufted processes, with a subset exhibiting an amoeboid brain macrophage-like morphology. The occurrence of darkly stained F4/80–immunoreactive microglia in Cstb⁻/⁻ mice declined from 2 to 4 months of age, with many cells displaying more branched processes. At 6 months, microglia in Cstb⁻/⁻ mice displayed much less intense F4/80 staining but frequently displayed a characteristic morphology with a large cell soma and thick processes that branched several times. In contrast to the Cstb⁻/⁻ mice, in the control brains, F4/80 staining was barely detectable above background, with a network of faintly stained ramified processes evident in the neuropil. This was most evident at P14, in the same regions as the most intensive F4/80 immunoreactivity in P14 Cstb⁻/⁻ brains. Control brains displayed only sporadic activated microglia from 1 month onward (Fig. 4G).

Astrocytosis

In contrast to microglial staining, GFAP immunoreactivity revealed no clear differences between Cstb⁻/⁻ and control mice at P14 (Fig. 5A). However, at 1 month of age, there was a

FIGURE 4. Progressive microglial activation in Cstb⁻/⁻ brain. (A–H) Patchy immunohistochemical staining for the microglial marker F4/80 revealed the progressive activation of microglia in selected brain regions of Cstb⁻/⁻ mice but not in age-matched controls (also see the Table). (A) Midline and posterior thalamic nuclei, P14. (B) Substantia nigra, P14. (C) Claustrum, 1 month. (D) Nuclei of the amygdala, 4 months. (E) Cingulate cortex, 6 months. (F) The hippocampal formation displayed no microgliosis even at 6 months of age, except for the dentate gyrus (arrowhead). (G) Laminar-specific microglial activation in primary somatosensory cortex (S1) of Cstb⁻/⁻, but not control (ctrl) mice. (H) Progressive changes in microglial morphology in Cstb⁻/⁻ during disease progression. Darkly stained activated microglia with a brain macrophage-like morphology could be seen in Cstb⁻/⁻ mice at P14 and were more pronounced at 1 month of age, whereas age-matched controls show much paler-stained microglia with many thin ramified processes. By 6 months of age, microglia in Cstb⁻/⁻ mice lose their activated phenotype with a pronounced decline in the intensity of F4/80 staining, but displaying distinctive thickened and branched processes. Cells are from the midline thalamus. Arrowheads indicate the regions of interest. Scale bars = (A–F) 200 μm; (G, H) 100 μm.
### TABLE. Distribution of F4/80-Immunoreactive Microglia

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<tr>
<th>Brain Region</th>
<th>ctrl</th>
<th>Csb &quot;−/−&quot;</th>
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<tbody>
<tr>
<td></td>
<td>P14</td>
<td>P14 1 mo</td>
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<tr>
<td><strong>Cortex</strong></td>
<td></td>
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</tr>
<tr>
<td>Prelimbic</td>
<td>PrL</td>
<td>+</td>
</tr>
<tr>
<td>Primary motor</td>
<td>M1</td>
<td>+</td>
</tr>
<tr>
<td>Secondary motor</td>
<td>M2</td>
<td>++</td>
</tr>
<tr>
<td>Infalimbic</td>
<td>IL</td>
<td>+</td>
</tr>
<tr>
<td>Dorsal peduncular</td>
<td>DP</td>
<td>+</td>
</tr>
<tr>
<td>Lateral orbital</td>
<td>LO</td>
<td>+</td>
</tr>
<tr>
<td>Primary sensory</td>
<td>S1</td>
<td>–</td>
</tr>
<tr>
<td>Secondary sensory</td>
<td>S2</td>
<td>–</td>
</tr>
<tr>
<td>Somatosensory barrel field</td>
<td>S1BF</td>
<td>–</td>
</tr>
<tr>
<td>Agranular insular</td>
<td>AI</td>
<td>–</td>
</tr>
<tr>
<td>Granular insular</td>
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<td>–</td>
</tr>
<tr>
<td>Piriform</td>
<td>Pir</td>
<td>+</td>
</tr>
<tr>
<td>Cingulate</td>
<td>Cg</td>
<td>+</td>
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<tr>
<td>Auditory</td>
<td>Au</td>
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</tr>
<tr>
<td>Primary visual</td>
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<tr>
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<td>+</td>
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<tr>
<td>Ectorhinal</td>
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<tr>
<td>Perirhinal</td>
<td>PRh</td>
<td>+</td>
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<tr>
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<tr>
<td>Diagonal band vertical</td>
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<td><strong>Basal ganglia</strong></td>
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<tr>
<td>Caudate putamen</td>
<td>CPu</td>
<td>+</td>
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<tr>
<td>Substantia innominata</td>
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<tr>
<td>Interstitial nucleus of posterior limb of anterior commissure</td>
<td>IPAC</td>
<td>++</td>
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<tr>
<td>Bed nucleus—stria terminalis</td>
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<tr>
<td>Nucleus accumbens</td>
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<tr>
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<td>Dorsal endopiriform claustrum</td>
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<td>Lateral accumbens shell</td>
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<td>++</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td><strong>Amygdala</strong></td>
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<tr>
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<tr>
<td>CA1</td>
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<td>CA3</td>
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<tr>
<td>Dentate gyrus</td>
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<tr>
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<tr>
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<td>Lateral nucleus</td>
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<tr>
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TABLE. Continued

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<td>Thalamic nuclei</td>
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<tr>
<td>Reunions</td>
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<tr>
<td>Subparafascicular thalamic</td>
<td>SPF</td>
<td>+</td>
</tr>
<tr>
<td>Lateral geniculate (ventral part)</td>
<td>VLG</td>
<td>−</td>
</tr>
<tr>
<td>Medial geniculate (dorsal)</td>
<td>MGD</td>
<td>+</td>
</tr>
<tr>
<td>Medial geniculate (ventral)</td>
<td>MGV</td>
<td>−</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precommissural nucleus</td>
<td>PrC</td>
<td>−</td>
</tr>
<tr>
<td>Prenasal field</td>
<td>PR</td>
<td>−</td>
</tr>
<tr>
<td>Deep mesencephalic nuclei</td>
<td>DpMe</td>
<td>−</td>
</tr>
<tr>
<td>Substantia nigra reticular</td>
<td>SNR</td>
<td>++</td>
</tr>
<tr>
<td>Substantia nigra compacta</td>
<td>SNC</td>
<td>++</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>VTA</td>
<td>−</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>IP</td>
<td>−</td>
</tr>
<tr>
<td>Deep gray layer of SC</td>
<td>DpG</td>
<td>−</td>
</tr>
<tr>
<td>Deep white layer of SC</td>
<td>DpWh</td>
<td>−</td>
</tr>
<tr>
<td>Optic nerve layer of SC</td>
<td>Op</td>
<td>−</td>
</tr>
<tr>
<td>Ventral periaqueductal gray</td>
<td>PAG</td>
<td>−</td>
</tr>
<tr>
<td>Cerebellum</td>
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<td></td>
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<tr>
<td>Granule cell layer</td>
<td>GCL</td>
<td>−</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>ML</td>
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</tr>
<tr>
<td>Deep cerebellar nuclei</td>
<td>DCN</td>
<td>−</td>
</tr>
<tr>
<td>White matter</td>
<td>WM</td>
<td>−</td>
</tr>
<tr>
<td>White matter tracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fimbria</td>
<td>fi</td>
<td>++</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>cc</td>
<td>++</td>
</tr>
<tr>
<td>Internal capsule</td>
<td>ic</td>
<td>+</td>
</tr>
<tr>
<td>Cerebral peduncle</td>
<td>cp</td>
<td>+</td>
</tr>
<tr>
<td>Anterior commissure (anterior part)</td>
<td>aca</td>
<td>++</td>
</tr>
<tr>
<td>Periventricular fiber system</td>
<td>pv</td>
<td>+++</td>
</tr>
<tr>
<td>Rostral interstitial nucleus of medial longitudinal fasciculus</td>
<td>RI</td>
<td>+</td>
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</table>

The distribution of F4/80-immunoreactive microglia in different brain regions of Cstb−/− mice from P14 to 6 months of age and control (ctrl) mice at P14.


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GFAP

A

B

1 mo
ctrl

1 mo
Cstb-/-

C: 1 mo
Cstb-/-

D: 1 mo
Cstb-/-

E: 1 mo
Cstb-/-

F: 4 mo
Cstb-/-

G: 6 mo
Cstb-/-

H: 6 mo
ctrl

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Glial Activation and Neuron Loss in Mouse EPM1

marked increase in intensely GFAP-immunopositive cells in Cstb−/− brains. These activated astrocytes displayed hypertrophy with enlarged soma and thickened, branched processes compared to the sporadic GFAP-positive astrocytes in controls (Fig. 5B).

Glia fibrillary acidic protein immunoreactivity in Cstb−/− brains became more pronounced and widespread with increased age. The distribution of the astrocytes overlapped with that of microglia, that is, staining for GFAP again was more intense in the most dorsal and ventral cortical laminae but was less evident in the intervening laminae (Fig. 5A). Prominent GFAP staining was present within midline and posterior thalamic nuclei, the substantia nigra, claustrum, and amygdaloid nuclei (Figs. 5C–F) in the Cstb−/− but not the control brains. However, the extent of GFAP immunoreactivity in hippocampal formation was similar in both Cstb−/− and control mice (Figs. 5G, H).

Neuronal Loss

To determine whether there was any spatiotemporal correlation between gliosis and neuron survival, we used unbiased stereology to document the onset and progression of neuronal loss in Nissl-stained sections through the thalamocortical system. On the basis of the pattern of glial activation, we focused this analysis on the Po and the LGNd, together with their corresponding cortical projection areas, S1 and V1, respectively. Po was one of the first areas to display microglial activation at P14 (Fig. 4A) and also displayed pronounced astrocytosis from 1 month onward (Fig. 6A). In contrast, LGNd remained devoid of activated microglia and astrocytes even in 6-month-old Cstb−/− mice (Fig. 6B).

Significant neuron loss was first evident within lamina V of S1 in 1-month-old Cstb−/− mice (Figs. 7A–C) and progressed to all laminae analyzed (Lam IV, V, and VI) by 2 months. In contrast, neuron loss within Po (the corresponding thalamic relay nucleus) did not become significant in Cstb−/− mice until 2 months of age (Fig. 7D), occurring after the onset of neuron loss within S1. Neuron loss within these somatosensory nuclei of Cstb−/− mice occurred after the onset of microglial activation and coincided with the beginning of astrocyte activation within these pathways. In contrast, neuron loss in the primary visual cortex (V1) of Cstb−/− mice only became apparent at 6 months of age (Figs. 7E–G), with no differences in the degree of neuronal loss between individual laminae in V1. The visual relay nucleus LGNd did not show significant neuron loss, even in 6-month-old Cstb−/− mice (Fig. 7H). No differences were detected in neuronal volumes between Cstb−/− and control mice at P14 or 6-month-old animals at Po and lamina V of S1 (data not shown).

Taken together, these data reveal that cortical neurons are consistently lost in Cstb−/− mice before any loss of thalamic

FIGURE 5. Extensive and progressive astrocytosis in Cstb−/− mice. Immunostaining for the astrocyte marker glial fibrillary acidic protein (GFAP) reveals progressive astrocytosis in selected brain regions of Cstb−/− mice but not in age-matched control (ctrl) mice. (A) Laminar-specific astrocyte activation in primary somatosensory cortex (S1) of Cstb−/− but not ctrl mice. (B) Astrocytes show an activated phenotype in 1-month-old Cstb−/− mouse midline thalamus but not in the ctrl. (C) Midline and posterior thalamic nuclei, 1 month. (D) Substantia nigra, 1 month. (E) Claustrum, 1 month. (F) Nuclei of amygdala, 4 months. (G, H) The hippocampal formation displayed similar level of GFAP immunoreactivity in both Cstb−/− (G) and control mouse (H) at 6 months of age. Arrowheads indicate the regions of interest. Scale bars = (A, B) 100 μm; (C–H) 200 μm.

FIGURE 6. Activation of microglia and astrocytes in the posterior thalamic nuclei (Po) and the dorsal lateral geniculate nucleus (LGNd). (A) Po of a Cstb−/−, but not a control (ctrl) mouse, shows microglial and astrocyte activation at 1 month of age. (B) LGNd remained devoid of activated microglia and astrocytes even in a 6-month-old Cstb−/− mouse, as well as in control mouse. F4/80 was used as a marker for microglia, and glial fibrillary acidic protein (GFAP) was used as a marker for astrocytes. Scale bars = 200 μm.

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FIGURE 7. Progressive region-specific neuron loss in Cstb−/− mice. (A–H) Histograms show unbiased optical fractionator estimates of numbers of Nissl stained neurons in Cstb−/− and control (ctrl) mice during disease progression. Loss of neurons is first seen in the lamina V of the primary somatosensory cortex (S1) (B), followed by the laminae IV and VI (A, C) and the corresponding thalamic relay nuclei, the posterior thalamic nuclear group (Po) (D). In contrast, the primary visual cortex (E–G) showed later neuron loss at 6 months of age and the corresponding thalamic relay nuclei, the dorsal lateral geniculate nucleus (LGNd) (H) retained neurons even at this time. Data are expressed as mean neuron number ± SEM. One-way analysis of variance: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
relay neurons occurs and suggest a correlation between the extent of glial activation and neuron loss within the thalamo-cortical system.

DISCUSSION

We here provide the first detailed description of the extent and sequence of pathologic changes in the CNS of Cstb<sup>−/−</sup> mice, from young presymptomatic mice, to the stage when ataxia starts to occur at 6 months of age. Previous studies of these mice have largely focused on cerebellum (10) or have studied older mice with more advanced disease (12, 13).

Our results agree with the suggestion that EPM1 is a neurodegenerative disorder (10). However, we show that the disease pathogenesis is associated with a remarkable and early glial involvement. Indeed, our data reveal localized microglial activation as early as P14 in Cstb<sup>−/−</sup> mice, when these mice are still clinically normal. This microglial activation is followed successively by intense astrogliosis, but the first signs of neuron loss in the cerebral cortex only become evident at 1 month, coinciding with the appearance of myoclonic seizures (10) (Fig. 8).

Our data also reveal that atrophy of the cerebral cortex parallels that previously described in the cerebellum (10). This atrophy and glial activation within the cerebral cortex is widespread, although not uniform, but neuron counts reveal substantial region-specific effects, suggesting no direct relationship between glial activation and neuron survival within the cerebral cortex.

The region-specific neuron loss in Cstb<sup>−/−</sup> mice also extends to subcortical structures, with loss of afferent relay neurons in the corresponding thalamic relay nucleus, which was only observed after the onset of cortical neuron loss. In contrast to the cortex, the distribution of thalamic neuron loss corresponded closely with the degree of glial activation within these nuclei. For example, neuron loss was pronounced within the somatosensory relay nucleus Po, which displayed prominent early microglial activation, whereas in those without evident glial activation (eg, visual relay system), neurons were not significantly lost.

Our data for selective effects on the somatosensory thalamocortical system are consistent with magnetoencephalography studies on EPM1 patients, which suggest that EPM1 is characterized by hyperreactivity of the sensorimotor thalamocortical loop but not of the cortex in general (21). Neuropathologic studies on EPM1 patients are sparse, with neuron loss reported in medial thalamic nucleus and the striatum, but not in the hippocampal formation, in 3 patients with EPM1 (22). The interpretation of such pathologic findings in EPM1 patients have been complicated by the effects of antiepileptic drug treatment, especially the association of phenytoin treatment with loss of Purkinje cells (23). However, our histopathologic findings in Cstb<sup>−/−</sup> mice are remarkably similar to

FIGURE 8. Schematic representation of the pathologic changes in the Cstb<sup>−/−</sup> mouse brain during disease progression. Extensive microglial activation is evident at P14 in the Cstb<sup>−/−</sup> brain. This is followed by astrogliosis at 1 month of age, when the first signs of myoclonus appear. Region-specific neuron loss in the primary somatosensory cortex (S1) also begins at this time and is followed by neuron loss in the corresponding thalamic relay nucleus, the posterior thalamic nuclear group (Po). Evidence of regional atrophy and cortical thinning can be seen from 2 months of age onward. Ataxia appears by 6 months of age, at which time the Cstb<sup>−/−</sup> brain is severely shrunken and gliotic.
the pathology reported in human patients, suggesting that this neuron loss may be the direct result of deficient CSTB function.

Localized glial activation and selective neuron loss has also been documented in mouse models of neuronal ceroid lipofuscinosis (NCLs), another group of disorders that display PME (19, 24–26). These disorders also consistently display early glial activation before neuron loss, but the precise relationship between astrocytosis, microglial activation, and whether neuron loss begins in the thalamus or cortex varies markedly among the different forms of NCL (27). However, the characteristic pattern of microglial activation, its early intense phase and later decline in Cstb<sup>−/−</sup> mice, has not been seen in any form of NCL. Although present in many widespread brain regions, glial activation in Cstb<sup>−/−</sup> mice is in several instances remarkably localized to individual brain structures. For example, in 1-month-old Cstb<sup>−/−</sup> mice, the claustrum shows extremely intense and sharply demarcated activation of both microglia and astrocytes. The claustrum is a telencephalic subcortical structure with largely unknown physiological function, but it has been suggested to be critical to coordinating information within and across sensory and motor modalities (28), as well as epileptiform activity and spreading and generalization of the seizures (29). Interestingly, the claustrum connects several brain areas that display pronounced gliosis and neuron loss in Cstb<sup>−/−</sup> mice, including somatosensory and motor cortex (30), anterior cingulate cortex (31) and substantia innominata (32), amygdala (33), striatum (34), substantia nigra (34), and thalamus, particularly the posterior thalamic nuclei and midline thalamus (35).

A characteristic feature of our data is the activation of both microglia and astrocytes before the onset of regional atrophy or significant neuron loss in Cstb<sup>−/−</sup> mice. Although gliosis has long been considered a hallmark of neurodegenerative changes, it is now apparent that glial responses often precede neuron loss and may indicate neuronal dysfunction (36–39). Reactive changes in microglia and astrocyte populations in older Cstb<sup>−/−</sup> mice have been reported previously (12, 13), as well as hyperexcitability possibly caused by loss of GABAergic inhibition (13). We did not detect changes in neuron volumes at P14 when there is intense microgliosis but no neuron loss or in severely affected 6-month-old Cstb<sup>−/−</sup> mice in the areas with the greatest neuron loss and most intense gliosis. In line with our results, Shannon et al (12) reported no significant difference in cross-sectional areas of superficial granular cell neurons of the parasubiculum and prosubiculum between 3- to 6-month-old control and Cstb<sup>−/−</sup> mice. Significant change in neuron size was seen only in substantially older animals of 16 to 20 months of age (12). They also reported region-specific neuron loss despite difficulties in detecting apoptosis. However, we cannot exclude the role of early neuronal changes, such as abnormal synaptic activity in inducing early glial activation in Cstb<sup>−/−</sup> mice. Notably, microglia and astrocytes are central regulators of both synaptogenesis and normal synaptic function (40). Therefore, further studies are needed to unravel the neurodegenerative mechanisms in Cstb<sup>−/−</sup> mice.

Our results from Cstb<sup>−/−</sup> mice suggest a key role for microglia in the pathogenesis of EPM1. Several lines of evidence show that cathepsins, secreted by activated microglia, directly induce neuron loss (41, 42). Because CSTB is an inhibitor of cysteine cathepsins (9), it is plausible that an imbalance in the cathepsin homeostasis in Cstb-deficient microglia may provoke neuronal death in Cstb<sup>−/−</sup> mice, although this remains to be proven. Although microglia are traditionally thought to be negative players in neurodegeneration by causing neuroinflammation, this view has been challenged recently by the suggestion that a loss of microglia-mediated neuroprotection may be an important contributor to neuropathology. Indeed, there is a wealth of new evidence for a compromised protective role of microglia in several neurodegenerative disorders, including Alzheimer disease (43) and amyotrophic lateral sclerosis (44). The progression of microglia from an activated brain macrophage-like morphology to a more ramified appearance with increased age in Cstb<sup>−/−</sup> mice may hint at microglial dysfunction. Alternatively, it may simply reflect that the molecular cues that caused their activation (presumably released from diseased neurons) are no longer present because these neurons have died. It will be important to distinguish between these possibilities, but both cell-autonomous changes and alterations in the extracellular milieu probably have a role in this distinct microglial phenotype.

The contribution of early microglia activation to subsequent pathologic changes in Cstb<sup>−/−</sup> mouse remains to be elucidated. Moreover, whether such microglial activation plays a role in the pathogenesis of human EPM1 remains unknown. Our study provides the first evidence for the early involvement of microglial activation in this disorder. Further studies should be directed at discovering disease-related changes in microglia to understand the mechanistic basis for the morphologic alterations. Indeed, a very topical question raised from this study is whether modulation of microglial function could be used as a therapeutic tool for preventing or combating neurodegeneration in EPM1.

ACKNOWLEDGMENT

The authors thank Dr Jaana Tyynelä for critical reading of the article.

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Cln3 deficient mice: Distinct effects in Finnish variant Cln5 Glial Activation and Neuron Loss in Mouse EPM1


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