Morphologic and Functional Correlates of Synaptic Pathology in the Cathepsin D Knockout Mouse Model of Congenital Neuronal Ceroid Lipofuscinosis

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Abstract

Mutations in the cathepsin D (CTSD) gene cause an aggressive neurodegenerative disease (congenital neuronal ceroid lipofuscinosis) that leads to early death. Recent evidence suggests that presynaptic abnormalities play a major role in the pathogenesis of CTSD deficiencies. To identify the early events that lead to synaptic alterations, we investigated synaptic ultrastructure and function in presymptomatic CTSD knockout (Ctsd<sup>−/−</sup>) mice. Electron microscopy revealed that there were significantly greater numbers of readily releasable synaptic vesicles present in Ctsd<sup>−/−</sup> mice than in wild-type control mice as early as postnatal day 16. The size of this synaptic vesicle pool continued to increase with disease progression in the hippocampus and thalamus of the Ctsd<sup>−/−</sup> mice. Electrophysiology revealed a markedly decreased frequency of miniature excitatory postsynaptic currents (mEPSCs) with no effect on paired-pulse modulation of the evoked excitatory post synaptic potentials in the hippocampus of Ctsd<sup>−/−</sup> mice. The reduced mEPSCs frequency was observed before the appearance of epilepsy or any morphologic sign of synaptic degeneration. Taken together, these data indicate that CTSD is required for normal synaptic function and that a failure in synaptic trafficking or recycling may be an early and important pathologic mechanism in Ctsd<sup>−/−</sup> mice; these presynaptic abnormalities may initiate synaptic degeneration in advance of subsequent neuronal loss.

Key Words: Cathepsin D, Electron microscopy, Electrophysiology, Hippocampus, Synapse, Synaptic vesicle.

INTRODUCTION

Cathepsin D (CTSD) deficiency causes a devastating inherited neurologic disease in humans, known as congenital neuronal ceroid lipofuscinosis (cNCL, also called CLN10; Mendelian Inheritance in Man [MIM] no. 610127) (1). Affected babies have epilepsy, extreme brain atrophy, and die of cardiac failure within the first few days of life (1–3). The disease is characterized neuropathologically by profound loss of neurons and myelin, together with pronounced glial activation throughout the neocortex (1–3). Cathepsin D deficiency also occurs in sheep, in which it causes a congenital disease with neonatal death, and in American bulldogs, in which the disease has a more prolonged course (4, 5). In addition, models of CTSD deficiency have been created in Drosophila and mice by genetic modification (6, 7).

Ctsd knockout (Ctsd<sup>−/−</sup>) mice, generated by gene targeting, provide an excellent tool for studying the basic pathophysiology of cNCL (7). Cathepsin D–deficient mice appear phenotypically normal at birth but develop a rapidly progressive neurodegenerative disease with epileptic seizures and death at postnatal day (P) 26 ± 2 (7–9). Many of the neuropathologic features typical of human cNCL are recapitulated in Ctsd<sup>−/−</sup> mice, including accumulation of electron-dense storage material in neurons, extensive glial activation, dysmyelination, and loss of neurons (8, 10, 11).

Recent data from Ctsd<sup>−/−</sup> mice, and from mice that model other forms of NCL (12–15), suggest that early synaptic alterations are characteristic of these disorders. In Ctsd<sup>−/−</sup> mice, synaptic pathology was particularly prevalent in the somatosensory cortex and the related thalamic nuclei (ventral posteromedial and ventral posterolateral nuclei, VPM/VPL); the synaptic density in these areas is also reduced at late-symptomatic stages of disease (10). Loss of synapses was accompanied by aggregation of presynaptic proteins, including α-synuclein and SNARE proteins (10, 16). Similar pathologic alterations at the synapse have been noted in autopsy samples from CTSD-deficient patients (unpublished observation), indicating that presynaptic mechanisms play an important role in the pathogenesis of CTSD deficiencies.

In this study, we have undertaken a correlated ultrastructural and functional analysis of hippocampal synapses from Ctsd<sup>−/−</sup> mice to investigate the early events that may underlie synaptic pathology. We identified morphologic and functional changes that occur in synapses of Ctsd<sup>−/−</sup> mice at presymptomatic time points. The most notable changes...
included an increase in the number of docked synaptic vesicles and a decreased frequency of miniature excitatory postsynaptic currents (mEPSCs).

**MATERIALS AND METHODS**

**Animals**

*Ctsd*<sup>−/−</sup> mice (7) were maintained on a mixed C57BL/6J and wt littermate mice (P0 = day of birth) were rapidly killed by decapitation in accordance with the University of Helsinki animal welfare guidelines. Hippocampal slices (400 μm thick) were cut with a vibratome using standard methods (18). The slices were used 1 to 4 hours after cutting.

**Electrophysiology**

For electrophysiological recordings, the slices were placed in a submerged chamber and superfused with artificial cerebrospinal fluid containing 124 mmol/L NaCl, 3 mmol/L KCl, 1.25 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 1 mmol/L MgSO<sub>4</sub>, 26 mmol/L NaHCO<sub>3</sub>, 15 mmol/L d-glucose, and 2 mmol/L CaCl<sub>2</sub>, with 5% CO<sub>2</sub> and 95% O<sub>2</sub>, at a rate of 2 to 3 mL/min (32°C). Whole-cell recordings were obtained from CA1 pyramidal neurons using the Axopatch 200 B amplifier (Axon Instruments, Union City, CA). Cells were voltage-clamped at −70 mV with 3- to 5-MΩ pipettes filled with a solution containing 130 mmol/L CsMeSO<sub>4</sub>, 10 mmol/L HEPES, 0.5 mmol/L EGTA, 4 mmol/L Mg-ATP, 0.3 mmol/L Na-GTP, 5 mmol/L N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride, and 8 mmol/L NaCl (285 mosm), pH 7.2. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated mEPSCs were recorded from CA1 pyramidal neurons in the presence of antagonists of GABAA and voltage-dependent sodium channels (picrotoxin [100 μmol/L] and TTX [1 μmol/L], respectively). To measure the paired-pulse facilitation (PPF), the pipette filling solution was changed: 115 mmol/L CsMeSO<sub>4</sub>, 10 mmol/L HEPES, 10 mmol/L EGTA, 4 mmol/L Mg-ATP, 0.3 mmol/L Na-GTP, 5 mmol/L N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride, and 8 mmol/L NaCl (285 mosm), pH 7.2. A bipolar electrode was used for afferent stimulation. Excitatory postsynaptic currents were evoked by stimulation of Schaffer collateral-commissural fibers and recorded from CA1 pyramidal neurons in the presence of GABAA and N-Methyl-d-aspartate receptor (NMDAR) antagonists (100 μmol/L picrotoxin and 50 μmol/L AP5, respectively). All compounds were from Tocris (Bristol, UK).

WinLTP (0.95 b or 0.96, www.winltp.com [19]) or Axoscope 9.2 (Axon Instruments) was used for data acquisition. Offline analysis was performed using WinLTP or MiniAnalysis 6.0.3 program (Synaptosoft, Inc, Decatur, GA). Spontaneous events were detected using peak detector algorithm, and all events were confirmed visually. The cumulative distributions of mEPSCs were constructed from at least 10 minutes of recording (at least 50 events) from each cell, using a bin width of 100 milliseconds for interevent interval and 1 pA for amplitude. Pooled data are given as mean ± SEM for the number of cells indicated. Student 2-tailed *t*-test was used for statistical analysis. The level of significance was set as *p* < 0.05.

**RESULTS**

**Synaptic Density Is Markedly Decreased in the Hippocampus and Thalamus of Late-Symptomatic *Ctsd*<sup>−/−</sup> Mice**

Our previous semi-quantitative analyses revealed reduced numbers of synapses in the VPM/VPL thalamic nuclei of *Ctsd*<sup>−/−</sup> mice at P24 (10). We validated these findings using a more robust quantitative method, confirming a significant loss of synapses at P24 (***, *p* < 0.001; ANOVA with Tukey post hoc test; Fig. 1A). A similar analysis at presymptomatic age (P16) revealed a clear, but not statistically significant, reduction in synaptic density in VPM/VPL (*p > 0.05; Fig. 1A), indicating that synaptic pathology was only in its early stages at P16.

Because assessing synaptic function using electrophysiologic methods in VPM/VPL is difficult, we extended...
Synaptic Function and Vesicle Pool in Ctsd<sup>−/−</sup> Mice

FIGURE 1. Synaptic density in the ventral posteromedial and ventral posterolateral nuclei (VPM/VPL) nuclei of the thalamus and the CA1 region of hippocampus in Ctsd<sup>−/−</sup> and control mice. (A) Bar chart (mean ± SEM) showing synaptic density in the VPM/VPL nuclei quantified from electron micrographs from Ctsd<sup>−/−</sup> mice (−/−; black bars) and control mice (+/+; white bars). There is a subtle reduction in the number of synapses at postnatal day (P) 16 (presymptomatic; not significant, p > 0.05) and a significant loss of synapses by P24 (late-symptomatic; **, p < 0.001). (B) Bar chart showing synaptic density in the CA1 region of hippocampus quantified from electron micrographs. There was no reduction in the number of synapses at P16 (presymptomatic; not significant, p > 0.05), but there was a significant loss of synapses by P24 (late-symptomatic; **, p < 0.01).

n = number of mice per genotype/age.

Synaptic Vesicle Density Is Increased in Ctsd<sup>−/−</sup> Mice

Qualitative observations of synapses in the hippocampal CA1 region from Ctsd<sup>−/−</sup> mice suggested that there was no obvious change in the size of synaptic boutons at either presymptomatic or late-symptomatic time points (Figs. 2A, B). Quantitative analyses confirmed these observations (Fig. 2C). However, synaptic vesicle pools seemed to be changed qualitatively in CA1 synapses at both presymptomatic and late-symptomatic time points, that is, denser clustering of vesicles around presynaptic membranes was routinely observed at P24 in Ctsd<sup>−/−</sup> mice (Fig. 2B). Quantitative analysis of total synaptic vesicle densities in CA1 showed no difference between genotypes at P16, but at P24, there was a significant increase in Ctsd<sup>−/−</sup> mice (*, p < 0.05; ANOVA with Tukey post hoc test; Fig. 2D). Most strikingly, however, the number of docked vesicles was significantly increased in CA1 synapses from Ctsd<sup>−/−</sup> mice, with significant changes already present at P16 (p < 0.05; Fig. 2E). The number of docked vesicles was almost double that in control mice by P24 (p < 0.05; Fig. 2E). Although not statistically significant, a similar trend was observed for the numbers of undocked vesicles in these mutant mice (Fig. 2F). These data indicate that disruption of hippocampal synaptic vesicle localization and/or recycling is an early pathologic event in Ctsd<sup>−/−</sup> mice.

To determine whether the changes observed in synaptic vesicle densities in CA1 were a common feature of synaptic pathology in Ctsd<sup>−/−</sup> mice, we quantified synaptic vesicle densities in VPM/VPL at P16 and P24 (Figs. 3A, B). The number of docked synaptic vesicles and the total number of vesicles in VPM/VPL were significantly increased (**, p < 0.01; ***p < 0.001; Figs. 3C, D) at P24, but not at P16.

mEPSC Frequency Is Decreased in Ctsd<sup>−/−</sup> Mice

To determine the functional effects of the increased numbers of docked synaptic vesicles in the hippocampal CA1 region of Ctsd<sup>−/−</sup> mice, pharmacologically isolated AMPA mEPSCs were recorded from hippocampal pyramidal cells in the CA1 subfield (Figs. 4A–D). At P16 to P18, there was a lower mEPSC frequency in Ctsd<sup>−/−</sup> mice versus controls. The mEPSC interevent interval in the mutants was about twice as long as in wild-type mice (wt: 2.74 ± 0.31 seconds; Ctsd<sup>−/−</sup>: 5.89 ± 1.17 seconds; p < 0.05; Fig. 4E). There was, however, no difference between genotypes in the AMPA
FIGURE 2. Synaptic vesicle pools in the CA1 region of hippocampus in Ctsd−/− and control mice. (A, B) Representative electron micrographs showing synapses in the CA1 of control (A) and Ctsd−/− (B) mice at postnatal day (P) 24. Note the clustering of synaptic vesicles toward the presynaptic membrane in the Ctsd−/− synapses (B). Scale bars: 250 nm (A) and 700 nm (B). (C) Bar chart (mean ± SEM) showing synaptic bouton areas in CA1 quantified from electron micrographs of Ctsd−/− (−/−; black bars) and wild-type control mice (+/+; white bars). (D) Bar chart showing synaptic vesicle densities in CA1, revealing a significant increase in Ctsd−/− mice at P24 (*, p < 0.05). (E) Bar chart showing average numbers of docked synaptic vesicles per synapse in CA1, revealing a significant increase in Ctsd−/− mice at presymptomatic (P16) and late-symptomatic (P24) time points (*, p < 0.05). (F) Bar chart showing average numbers of undocked synaptic vesicles per synapse in CA1. See Figure 1 for the number of mice in each group.
mEPSC amplitude (wt: 14.04 ± 0.80 pA; Ctsd−/−: 14.48 ± 0.99 pA; p > 0.05; Fig. 4F).

Release Probability Is Unaltered in Ctsd−/− Mice

Because more docked synaptic vesicles may be associated with increased neurotransmitter release (20), we next measured PPF in the CA3-CA1 synapse because it is inversely correlated with a change in the initial release probability (Pr) (20–22). To assess PPF, we used interpulse intervals of 20 milliseconds (50 Hz), 50 milliseconds (20 Hz), and 150 milliseconds (6, 7 Hz). We found no difference in PPF between the genotypes at any of the interpulse intervals used (paired-pulse ratio, 20-millisecond interval: wt 2.54 ± 0.08, Ctsd−/− 2.61 ± 0.19; 50-millisecond interval: wt 2.13 ± 0.14, Ctsd−/− 2.17 ± 0.22; 150-millisecond interval: wt 1.50 ± 0.05, Ctsd−/− 1.27 ± 0.19; p > 0.05; Fig. 5), indicating that the release probability is unaltered in Ctsd−/− mice.

DISCUSSION

Readily Releasable Synaptic Vesicle Pool Size Increases With Disease Progression in Ctsd−/− Mice

Our most important ultrastructural finding in the synapses of Ctsd−/− mice was a marked increase in vesicle pool size compared with controls. Indeed, the readily releasable vesicle pool size was already significantly increased (~1.2-fold) in hippocampal neurons of Ctsd−/− mice at P16, which represents a presymptomatic stage of the disease. If there were a significant loss of synapses at
P16, more synaptic vesicles could reflect a compensatory mechanism as a response to synaptic loss. However, at this stage, synaptic loss was not yet evident in CA1 of these affected mice. By P24, the size of the readily releasable vesicle pool had increased markedly not only in the CA1 (~1.7-fold) but also in the thalamus (~1.5-fold). In addition, the total number of synaptic vesicles per bouton was significantly increased in both of these brain regions, suggesting that the synaptic vesicle pool undergoes abnormal changes throughout the brain of Ctsd<sup>−/−</sup> mice. Inasmuch as the changes in the vesicle pool size occurred in advance of synaptic degeneration in the hippocampus of Ctsd<sup>−/−</sup> mice, these data raise the possibility that alterations in synaptic vesicle recycling may contribute to the initiation and progression of synaptic degeneration in these mice. These findings are in line with our previously reported alterations in the distribution of presynaptic proteins in Ctsd<sup>−/−</sup> mice (10).

**Electrophysiologic Data Indicate Early Dysfunction of Synapses in Ctsd<sup>−/−</sup> Mice**

The synaptic vesicle pool size and, particularly, the number of readily releasable synaptic vesicles (so-called docked vesicles) affect the efficacy of synaptic transmission (20). The increased number of docked vesicles may lead to an increased neurotransmitter release and to a consequent increase in postsynaptic currents (20–22). Somewhat surprisingly, our observations contradict this principle, that is, despite the increased number of docked vesicles in Ctsd<sup>−/−</sup> mice, the paired-pulse data suggested no change in the P<sub>r</sub>. Rather, the increased number of readily releasable vesicles

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**FIGURE 4.** (A–D) Characteristics of miniature excitatory postsynaptic currents (EPSCs) recorded from hippocampal CA1 pyramidal neurons of wild-type (A, B) and Ctsd<sup>−/−</sup> mice (C, D) at postnatal days (P) 16 and 17. Asterisks in example recordings (A, C) indicate regions presented with the expanded time scale (B, D). (E) Cumulative distribution and averaged values of mEPSC interevent interval; *, p < 0.05. (F) Cumulative distribution and averaged values of mEPSC amplitude.
Comparison With Other Forms of NCL

The NCLs are a genetically heterogeneous group of fatal neurodegenerative diseases, sharing common clinical symptoms and pathologic events (24, 25). The existence of several diverse mouse models of NCL allows comparison of the basic mechanisms underlying the diseases (24, 25). In contrast to our findings in Ctsd<sup>−/−</sup> mice, the size of the readily releasable synaptic vesicle pool has been shown to progressively decline in cultured neurons of palmitoyl-protein thioesterase 1 (Ppt1) knockout mice, a model of infantile NCL disease (12). This reduction was associated with decreased frequency of spontaneous mEPSCs (12). Whether the differences observed between this study and ours reflect different roles of PPT1 and CTSD in regulating synaptic vesicle dynamics or whether other factors affected the results (e.g., comparing in vitro changes with in vivo pathology) remains unclear. The major neuropathologic changes in Ppt1 knockout mice closely resemble those in Ctsd<sup>−/−</sup> mice, with synaptic pathology occurring in a regionally selective manner, preceding the onset of neuronal loss in the thalamus and cortex (10, 15). Similar pathologic changes are evident in autopsy material of infantile NCL patients with PPT1 deficiency and it has been suggested that the lack of PPT1 activity leads to altered synaptic vesicle trafficking at nerve terminals (14). Thus, whatever the basis for these events, it is becoming apparent that the presynaptic compartment is targeted early in the pathogenesis of the NCLs. Although the precise nature of the early events underlying synaptic pathology may differ between the forms of NCL, invariably these changes lead to synaptic degeneration and subsequent neuronal loss.

Decreased frequency of spontaneous mEPSCs at an early age has only been reported in a few mouse models. These models have genetic defects affecting elementary functions of the brain and/or causing severe neurologic diseases, such as neocortical maturation and autism (26), frontotemporal dementia (27), and Huntington disease (28). The early appearance of functional alterations in the synapses of Ctsd<sup>−/−</sup> mice suggests that CTSD has a fundamental role in maintenance and perhaps even development of synapses and neuronal networks. This possibility is supported by our previous findings showing that the somatosensory thalamocortical system is a focus of major pathologic alterations in these mice (10). The abnormal accumulation of synaptic vesicles in Ctsd<sup>−/−</sup> brains indicates that the function of CTSD may be related to recycling or trafficking of presynaptic vesicles.

Relationship to Other “Synaptopathies”

In the most common forms of age-related dementia, such as Alzheimer disease and Parkinson disease, the role of synaptic loss in the neurodegenerative process has been recognized since the early 1990s (29, 30). Subsequently, there has been growing evidence of early synaptic vulnerability in a number of different neurodegenerative conditions affecting both central and peripheral nervous systems, including Alzheimer and Huntington disease, prion diseases, and spinal muscular atrophy (31–36). In particular, disruption of synaptic functions may precede synaptic degeneration and loss in...
many of these diseases (31–34), whereas the exact functional mechanisms may vary from one disease to another.

SUMMARY

We have quantitatively assessed synaptic pathology in the thalamus and hippocampus of \( Ctd \) mice at an ultrastructural and functional level. We observed a significant increase in the number of docked vesicles at individual synapses occurring in advance of synaptic degeneration and neuron loss. These structural changes were accompanied by a markedly decreased frequency of mEPSCs but no effects on evoked excitatory postsynaptic potentials. Taken together, the present data provide the first direct evidence for functional and structural changes at the synapse in presymptomatic \( Ctd \) mice and strongly suggest that a failure in the presynaptic function initiates synaptic degeneration, which leads to neuronal degeneration in these mice. These findings further emphasize the role of \( CTD \) in maintaining synaptic integrity in vivo.

REFERENCES

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