Function of Cathepsin K in the Central Nervous System of Male Mice is Independent of its Role in the Thyroid Gland

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Abstract

Background: Cathepsin K deficiency in male mice (*Ctsk*^{-/-}) results in decreased numbers of hippocampal astrocytes and altered neuronal patterning as well as learning and memory deficits. Additionally, cathepsin K carries essential roles in the thyroid gland where it contributes to the liberation of thyroid hormones (TH). Because TH are essential for brain development, in particular for the cerebellum, we investigated whether cathepsin K's function in the thyroid is directly linked to the brain phenotype of *Ctsk*^{-/-} mice.

Results: Serum levels of thyroid stimulating hormone, brain concentrations of free TH, and deiodinase 2 (Dio2) activity in brain parenchyma as well as cerebellar development were comparable in *Ctsk*^{-/-} and WT animals, suggesting regular thyroid states and TH metabolism. Despite unaltered transcript levels, protein expression of two TH transporters was enhanced in specific brain regions in *Ctsk*^{-/-} mice, suggesting altered TH supply to these regions. Thyrotropin releasing hormone (Trh) mRNA levels were enhanced 3-fold in the hippocampus of *Ctsk*^{-/-} mice. In the striatum of *Ctsk*^{-/-} mice the mRNA for *Dio2* and *hairless* were approximately 1.3-fold enhanced, while mRNA levels for monocarboxylate transporter 8 and Trh were reduced to 60% and 40%, respectively, pointing to altered striatal physiology.

Conclusion: We conclude that the role of cathepsin K in the thyroid gland is not directly associated with its function in the central nervous system (CNS) of mice. Future studies will show whether the brain region-specific alterations in Trh mRNA may eventually result in altered neuroprotection that could explain the neurobehavioral defects of *Ctsk*^{-/-} mice.

Key-words

cathepsin K; thyroid gland function; thyroid hormone transporters; thyroid hormone target genes; brain function

Introduction

Cathepsin K is an endo-lysosomal cysteine peptidase of epithelial cells, macrophages, and osteoclasts among other cell types. In the past, the enzyme was mainly considered for its importance in bone remodelling enabled by high expression levels of cathepsin K in osteoclasts (Brömme and Okamoto 1995). Indeed, a lack of cathepsin K has been linked to bone disorders such as pycnodysostosis in humans and osteopetrosis in mice, both featuring abnormally dense bones (Gelb et al. 1996; Saftig et al. 1998). Excessive proteolytic activity of cathepsin K on collagen I may account for osteoporosis, rendering the protease a prominent target for rational drug design (Desmarais et al. 2009; Jordans et al. 2009). However, the development of the cathepsin K inhibitor Odanacatib for osteoporosis was discontinued recently after 12 years in clinical exploitation and after its pivotal trial enrolling more than 16,000 patients, because Odanacatib increased the risk of stroke and other cerebrovascular pathologies (Drake et al. 2017; Mullard 2016). Beside bone osteoclasts and macrophages associated with blood vessel lesions (Weiss-Sadan et al. 2017), cathepsin K has also been shown to be present in alveolar and bronchial epithelia (Buhling et al. 1999), urothelia, gastrointestinal mucosa (Haeckel et al. 1999), and thyroid epithelial cells (Tepel et al. 2000). Since the distribution of cathepsin K is by far not as clearly delineated as originally thought (Brix et al. 2014; Dauth et al. 2011a), understanding cathepsin K's functions in different body tissues are of substantial interest in order to better understand its physiological tasks.

Importantly, cathepsin K contributes to the intra-thyroidal processing of thyroglobulin (Tg), the protein precursor of TH (Brix et al. 2001; Tepel et al. 2000). Moreover, we have shown previously that cathepsin K and L double-deficient mice (*Ctsk*^{-/-}/*Ctsl*^{-/-}) are characterized by a goiter-like enlargement of the thyroid gland accompanied by significantly decreased levels of serum free T₄ (fT₄) (Friedrichs et al. 2003). Thus, *Ctsk*^{-/-}/*Ctsl*^{-/-} mice are considered hypothyroid. While a pheno-copy of the tissue alterations within the thyroid was also observed in mice lacking only cathepsin K, Tg processing and serum fT₄ levels remained similar in *Ctsk*^{-/-} animals compared to WT controls as observed in our previous study (Friedrichs et al. 2003). This is due to the fact that lacking cathepsin K-mediated Tg processing is compensated by upregulated cathepsin L activity in the thyroid gland of *Ctsk*^{-/-} mice (Friedrichs et al. 2003). Moreover, enhanced presence of the TH transporter monocarboxylate transporter 8 (Mct8) at the basolateral plasma membrane domain of thyrocytes further compensates, such that normal serum TH concentrations can be maintained by enhanced T₄ release from thyroid follicles upon cathepsin K deficiency (Wirth et al. 2009). However, we observed a possible loss of TH-sensing capability in the thyroid gland of *Ctsk*^{-/-} animals (Weber et al. 2017).

It remains unclear to date whether the impaired TH sensing of *Ctsk*^{-/-} mice is restricted to the TH-generating thyroid gland itself, or whether similar defects are manifest at the level of TH target organs such as the brain. Interestingly, our previous work on *Ctsk*^{-/-} mice suggests that deficiency in cathepsin K impairs learning and memory and cell differentiation in the brain (Dauth et al. 2012; Dauth et al. 2011b). Because of the known involvement of cathepsin K in the liberation of TH in the thyroid gland (Friedrichs et al. 2003) and in TH sensing (Weber et al. 2017), we tested the possibility that the *Ctsk*^{-/-} brain is functionally hypothyroid. In particular, we were interested to determine whether signs of systemic or local hypothyroidism became detectable in the brain of *Ctsk*^{-/-} mice. Therefore, thyroid states, TH transporter expression and deiodinase activity were tested as indicators of adequate TH supply and TH metabolism in the brain of adult male mice. Since some alterations in TH transporters were observed, we tested for cerebellar development, a TH-dependent process, and found this unaffected. While TH target gene expression remained largely unaffected in *Ctsk*^{-/-} mice, gene transcription of the thyroid stimulating hormone

(TSH) releasing hormone (*Trh*) was deregulated in striatum and hippocampus, indicating its brain region-specific role as a neuromodulator which is independent from its function in regulating the HPT axis of *Ctsk*^{-/-} mice.

Materials and Methods

Animals

All studies were performed using male *Ctsk*^{-/-} (RRID:MGI_MGI:3695740) or WT C57Bl/6J mice (RRID:IMSR_JAX:000664). For most experiments adult mice (4-6 months old) were used, except for investigating cerebellar development for which 12-day, 21-day and 120-day old mice were used. The mice were back-crossed to a congenic C57Bl/6J background in the animal facility of Jacobs University Bremen, Germany. Back-crossing was performed for at least 6 generations. Generation of the founder *Ctsk*^{-/-} mice at the University of Göttingen, Germany, and the genotyping methods are described elsewhere (Saftig et al. 1998). For the experiments, mice were housed under standard conditions, with a 12h/12h light/dark cycle with lights out at 07:00 p.m. and *ad libitum* water and food. Body weight gain was assessed for WT (n=7) and *Ctsk*^{-/-} (n=10) animals, respectively, from 2.3 to 7.3 months of age (Figure 2 f). All procedures performed in studies involving animals were conducted in accordance with institutional guidelines in S1-laboratories of Jacobs University Bremen (Senator für Arbeit, Frauen, Gesundheit, Jugend und Soziales der Hansestadt Bremen Az. 513-30-00/2-15-32 including KB as project leader, and Az. 0515_2040_15 to KB allowing breeding, housing and sacrifice of genetically engineered mice).

Tissue sampling

Adult male mice were deeply anesthetized using the inhalation anesthetic IsoFlo® (Abbott GmbH & Co. KG, Wiesbaden-Delkenheim, Germany) and subsequently 100 µL of 10% ketamine (CEVA SanteAnimale, Düsseldorf, Germany) was injected intra-peritoneally. The abdominal and thoracic cavities were opened and the abdominal aorta was cut, allowing the collection of a sufficient blood sample for serum extraction. Perfusion was carried out through the heart with 0.9% NaCl including 200 IU heparin until the liver became light-colored. The head and neck regions were immediately separated and placed on ice for the entire dissection procedure.

Brain samples. The brain was removed and the two hemispheres were separated. One hemisphere was placed in 4% PFA in 200 mM HEPES, pH 7.4 overnight at 4°C, then cryo-preservation was carried out by incubating in 0.5 M and afterwards in 1 M sucrose solution in phosphate-buffered saline (PBS), pH 7.4, each for another 24h at 4°C. Tissue samples were embedded in Tissue Freezing Medium (Jung, through Leica Microsystems) and stored at -20°C. Alternatively, tissue was frozen using the liquid nitrogen-isopentane method and stored at -80°C until sectioning. The other hemisphere was immediately dissected on ice and the cortex, hippocampus, cerebellum, and striatum together with the mesencephalon and diencephalon (referred to as striatum/mesencephalon fraction) were separated and snap-frozen in liquid nitrogen.

Measurement of fT₃, fT₄ and TSH in serum

Blood samples of 5-5.5 months old male mice were allowed to clot. Clotted material was removed and sera were cleared by centrifugation at 10,000 × g at 4°C for 10 min, and stored at -80°C until further analysis.

For determining levels of fT₃, fT₄ and TSH commercially available ELISA kits were used. Serum samples were used for determination of fT₄ (fT₄ ELISA, DRG EIA-2386, DRG Instruments GmbH, Marburg/Lahn, Germany), fT₃ (fT₃ ELISA, DRG EIA-2385, DRG Instruments GmbH, Marburg/Lahn, Germany) and TSH (TSH ELISA,

AKRTS-010R, Shibayagi, Japan) according to the manufacturer's protocols. The assay range of the TSH kit is 0.288-36 ng/mL (for 5x sample dilution 1.44 – 180 ng/mL). The mean coefficient of variation (CV) for the assays' precision is less than 5%. Assay reproducible has a CV less than 5% as well.

Measurement of fT_4 and tT_3 in brain lysates

For determining levels of fT_4 and tT_3 , commercially available ELISA kits were used. Tissue extracts from brain (method for retrieving tissue see above) fractions including the dopaminergic system were used for determinations of free T_4 (see above) and total T_3 (total T_3 ELISA, EIA-1780, DRG Instruments), according to the manufacturer's protocols, with the only exception of extended washing steps in order to avoid any residual extraction buffer (enzyme conjugate reagents). The assay range of the tT_3 ELISA is 0.14 ng/mL to 6.20 ng/mL. The accuracy of the test was reported with a correlation coefficient of 0.906 (comparison with the Abbott AxSym Total T_3 kit) and 0.993 (compared with the Monobind Total T_3 assay). This assay has been reported to detect T_3 concentrations reliably as low as 0.2 ng/mL. Running 3 replicates intra-assay precision was reported with a CV of 3.2% and inter-assay precision with a CV of 1.4%. fT_4 and tT_3 ELISA values were normalized to the protein content of each sample (protein content determined by Neuhoff).

Immunohistochemistry of brain sections

Brain hemispheres were cut in the sagittal plane on a cryostat (Leica CM1900, Leica Microsystems) into 25 μ m-thick sections and thaw-mounted serially on microscope slides. Residual embedding material was washed out prior to the staining procedure by incubating slides overnight in PBS at 4°C. Blocking of non-specific binding sites on sections was performed at room temperature through a 4h-incubation with 3% bovine serum albumin (Albumin Fraction V, Roth, Karlsruhe, Germany) including 0.3% Triton X-100 in CMF-PBS, respectively. Primary antibodies, mouse anti-GFAP (Sigma-Aldrich Cat# G3893 RRID:AB_477010) were applied overnight at 4°C. The appropriate secondary antibodies, goat anti-mouse IgG conjugated with Alexa 546 (Life Technologies Cat# A11018 RRID:AB_10393166; through Molecular Probes), or Alexa 488 (Life Technologies Cat# A11017 RRID:AB_10562367) were applied for 4h at room temperature in a moisturized chamber. As a counter-stain of nuclear DNA, Draq5™ (Biostatus Ltd., Shepshed Leicestershire, UK) was applied together with the secondary antibody. For the structural analysis of the cerebellum sections, only Draq5™ staining was used.

Parvalbumin staining. The sections were washed with PBS and blocked with M.O.M. (Mouse on mouse, Vector Laboratories, Burlingame, CA) blocking reagent for 60 min at RT. Primary antibody mouse anti-parvalbumin (Swant Cat# 235 RRID:AB_10000343) was applied overnight at 4°C. Sections were then washed and blocked again before the secondary antibody Cy3-coupled goat anti-mouse IgG (Jackson ImmunoResearch Cat# 115-166-003 RRID:AB_2338699; 115-166-003) was applied in 0.01 M PBS including 0.25 M NaCl for 45 min at RT. Draq5™ was used as a counterstain. Sections were mounted with Immumount (Thermo Scientific, Pittsburgh, PA, USA).

Thyroid morphology

Thyroid gland tissue samples from 8 WT and 4 *Ctsk*^{-/-} mice were fixed in 4% paraformaldehyde (PFA) in 200 mM HEPES, pH 7.4, and left overnight at 4°C. Cryo-preservation was carried out by incubation in tissue freezing medium (Jung, through Leica Microsystems, Nussloch, Germany) overnight at 4°C, then frozen and stored at -20°C until sectioning on a cryostat (Leica CM1900, Leica Microsystems, Wetzlar, Germany). Transverse sections

of 5 μm were prepared from the middle part of the thyroid gland and thaw-mounted on microscope slides before analyses as previously described (Weber et al. 2015; Qatato/Szumska et al. 2018). In brief, the sections were stained with rabbit anti-mouse collagen type IV (Novotech, through BioLogo, Kronshagen, Germany; CO20451), which was visualized by incubation with Alexa 488TM-coupled goat anti-rabbit antibodies (Molecular Probes, through Invitrogen, Darmstadt, Germany; A-11070), and counter-stained for cytoplasm and nuclei, respectively, with HCS Cell Mask OrangeTM (CMO) (Molecular ProbesTM; H32713) and Draq5TM (BioStatus, Leicestershire, UK; DR05500). The epithelial extensions, follicle areas, follicle counts, and follicle luminal areas were analyzed with the aid of the open source software Cell Profiler (version 2.1.1.; available from the Broad Institute at www.cellprofiler.org, Lamprecht et al. 2007) following our established pipelines described elsewhere (Weber et al., 2015). 1111 (WT) and 1526 (*Ctsk*^{-/-}) follicles were analysed.

SDS-PAGE, immunoblotting, immunodetection and densitometry

Protein extraction for analysis of TH transporters. Tissue samples were grounded to powder in liquid nitrogen. The tissue powder was resuspended in homogenization buffer consisting of 20 mM HEPES, 250 mM sucrose, and 1 mM EDTA, pH 7.4, and protease inhibitor cocktail (539134, Calbiochem/Merck, Darmstadt, Germany) before sonication (Sonoplus GM70, type UW70, Bandelin, Berlin, Germany) with an amplitude of 70 and cycle 0.6, thrice for 15 sec each with 30 sec cooling cycles in between. Homogenates were cleared by centrifugation for 15 min at $13,000 \times g$ and 4°C. Pellets were resuspended in fresh homogenization buffer and again sonicated on ice for 15 sec. Homogenates were stored at -20°C.

Gel electrophoresis. The protein content of all samples was determined using the Neuhoff assay (Neuhoff et al. 1979). Homogenates were normalized to equal amounts of protein, boiled in sample buffer and separated on 12.5% acrylamide gels.

Immunoblotting and immunodetection. Separated proteins were semi-dry blotted onto nitrocellulose membranes. Blocking was done overnight at 4°C using 5% milk powder in PBS containing 0.3% Tween-20 (PBST). Primary antibodies, rabbit anti-mouse Mct8 (Sigma-Aldrich Cat# HPA003353 RRID:AB_1079343; Atlas Antibodies AB, Stockholm, Sweden) and rabbit anti-mouse Lat2 (#0421-10, Immunoglobulin, Himmelstadt, Germany) were applied for 90 min at room temperature. The appropriate secondary antibody, HRP-conjugated goat anti-rabbit IgG (4050-05, Southern Biotech, Birmingham, Alabama, USA) was applied for 60 min at room temperature. As a loading control, membranes were stripped and re-probed for β -tubulin, using a rabbit anti- β -tubulin primary antibody (Sigma-Aldrich Cat# T3526 RRID:AB_261659), and an HRP-conjugated goat anti-rabbit secondary antibody, as described above. Immunoreactions were visualized by enhanced chemiluminescence on CL-XPosure film (Pierce through Perbio Science Europe, Bonn, Germany) or on Hyperfilm MP (GE Healthcare Europe GmbH, Munich, Germany).

Densitometry. Films from two to four different blots for each immunodetection and each animal were scanned with a transmitted-light scanner (Desk Scan II version 2.9; Hewlett-Packard Co., Palo Alto, California, USA) and evaluated densitometrically using TINA version 2.09d (Raytest Isotopen-Messgeräte GmbH, Straubenhardt, Germany). The measured optical density/mm² values, after background subtraction, were normalized within each blot by dividing individual values to the average of the WT mice values in the respective blot. For normalization β -tubulin was used. For averaging over different immunoblot intensities varying from membrane to membrane values are given as percent of control, i.e. the average of the WT values was set to 100% and each single value (WT and *Ctsk*^{-/-}) was then compared to this average value.

Deiodinase 2 activity

Frozen brain tissue was ground in liquid nitrogen, and the tissue powder was sonicated (Labsonic M, Braun Biotech, Melsungen, Germany) in homogenization buffer containing 20 mM HEPES, 250 mM sucrose, 1 mM EDTA and 1 mM DTT, at pH 7.4. After centrifugation for 15 min at $13,000 \times g$ at 4°C, the supernatants were collected and the pellets were resuspended in the same buffer for an additional round of sonication. Protein contents of the tissue lysates were determined by the Bradford method (Bradford 1976). The specific activities of type II deiodinase (Dio2) were analyzed as described previously (Schmutzler et al. 2004; Streckfuss et al. 2005). Briefly, the activities were determined measuring the release of ^{125}I from radiolabeled reverse T_3 (rT_3) used as a tracer in the presence of 1 nM rT_3 . In addition, samples pre-treated with 6-n-propylthiouracil (PTU; 102748, MP Biomedicals, Eschwege, Germany), a specific inhibitor of Dio1, were run in parallel allowing to determine activity levels as calculated by the difference between samples treated with and without PTU. Samples were measured in triplicates, and 2 (cerebellum and hippocampus) or 3 independent experiments (cortex and striatum) were performed.

Quantitative real-time PCR

Total RNA from cortex, cerebellum, hippocampus and striatum was isolated using RNeasy Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany) and stored at -80°C as previously described (Engels et al. 2015). RNA was reverse transcribed into cDNA with SuperScriptIII (Life Technologies, Darmstadt, Germany) and hexamer primers. Quantitative real-time PCR (qRT-PCR) was performed using Roche SYBR Green I master mix (Roche, Mannheim, Germany). The primer sequences are provided in Supplementary Table 1. In compliance with the MIQE guidelines for RT-PCR (Bustin et al. 2009), we used a set of 3 reference genes per tissue to assure accurate normalization and calculation (18S (18S ribosomal RNA), *Ppia* (peptidylprolyl isomerase A, cyclophilin A) and *Hprt* (hypoxanthine guanine phosphoribosyl transferase)). Analysis and calculation of the fold-change in gene expression were done on Ct-values ≤ 35 using the efficiency-corrected $\Delta\Delta\text{Ct}$ method (Pfaffl 2001). For statistical analysis of real-time PCR results (in figures presented as fold changes) antilogarithmic data of *Ctsk*^{-/-} mice and WT controls were used.

Microscopy and analysis

Confocal images were taken using a Zeiss LSM 510 META laser-scanning microscope equipped with Argon and Helium-Neon lasers (Carl Zeiss GmbH, Oberkochen, Germany). Optical sections were taken with a pinhole opening of 1 Airy unit and at a resolution of $1,024 \times 1,024$ pixels, using LSM 5 software (version 3.2; Carl Zeiss).

Statistical analysis

All data were assessed for normality using the Shapiro-Wilk or D'Agostino-Pearson test, and passed. Normalized data from densitometry analysis of immunoblots were analyzed using a linear mixed effects model with one between-subjects factor (genotype), two within-subjects factors (marker and brain region), and one random factor (individual). The model included all main effects and all two-way and three-way interactions between fixed factors. Post hoc tests were adjusted for multiple comparisons using the Bonferroni method. Residuals generated by the model were verified to be normally distributed. Serum measurements of TSH, fT_4 , and T_3 as well as deiodinase 2 activity data across brain regions were analyzed using a mixed-effects model (residual maximum likelihood,

REML), followed by Sidak's multiple comparisons test to assess whether significant differences may be present in specified brain regions between WT and *Ctsk*^{-/-} mice. Quantitative real-time PCR data were assessed using either unpaired Student's t-test (genotype comparison) one-way ANOVA (brain regions comparison) or two-way ANOVA (genotype and brain regions comparison) followed by Tukey's post-hoc test for multiple comparisons. Levels of significance of non-paired observations for thyroid morphology data were calculated by two-tailed t-test or 1-way ANOVA for multiple columns. Statistical analyses were conducted using GraphPad Prism 8.1.2 (GraphPad Software Inc.) and SPSS Statistics version 23 (IBM Inc.).

Results

Thyroid gland morphology

We investigated the effect of cathepsin K deficiency on thyroid morphology and compared the results to previous analyses on thyroid gland size and epithelial extensions (Friedrichs et al. 2003). The average lumen sizes (Figure 1a-d) were comparable between *Ctsk*^{-/-} mice ($2849 \mu\text{m}^2 \pm 69.91$; mean $\pm$ SD) and WT animals ($3511 \mu\text{m}^2 \pm 144.0$; mean $\pm$ SD), which does not reproduce the previous results. However, in our previous study (Friedrichs et al. 2003), animals of both sex, different ages and on a mixed genetic background, i.e. 129Ola and C57BL/6J (Friedrichs et al. 2003) were analyzed. In the present study, we only used male mice on a C57BL/6J background back-crossed for at least 6 generations. Interestingly, thyroid glands of cathepsin K-deficient animals displayed a decrease in thyroid follicle size heterogeneity when compared to WT controls (Figure 1b and c) and a marginal, non-significant decrease (0.81- fold $\pm$ 0.14) in whole lumen area (Figure 1d). The count of follicles per thyroid middle section (Figure 1e) was not significantly increased in *Ctsk*^{-/-} mice and the epithelial extension was comparable to the WT (Figure 1f). These data suggest largely unaltered thyroid morphology upon cathepsin K deficiency in C57BL/6J mice.

TH supply and TH metabolism in the brain of *Ctsk*^{-/-} mice

We found that TSH serum concentrations as well as serum fT₃ and fT₄ concentrations are comparable between *Ctsk*^{-/-} mice and WT animals (Fig. 2a-c). Moreover, the brain as one of the main TH target tissues, exhibited no signs of altered fT₄ or tT₃ concentrations in *Ctsk*^{-/-} mice (Fig. 2d and 2e). These data indicate comparable thyroid states in *Ctsk*^{-/-} mice and WT animals, correlating well with the unchanged body weight gain (Fig. 2f) and unaltered brain and thyroid weights of *Ctsk*^{-/-} vs. WT mice (Table 1), respectively.

To determine whether local changes in TH metabolism may occur in the brain of *Ctsk*^{-/-} mice, we measured the availability and activity of Dio2, the main T₄ to T₃-converting enzyme, in different brain regions and compared *Ctsk*^{-/-} mice with WT controls (Fig. 3). Expression of *Dio2* showed no significant effect of genotype ($F_{(1,32)}=2.534$, $p=0.1213$), but a significant effect of brain regions ($F_{(3,32)}=80.13$, $p<0.0001$, striatum < hippocampus < cortex < cerebellum). However, interaction of genotype x brain regions was unaltered ($F_{(1,32)}=0.6970$, $p=0.5607$) and concludes no changes of *Dio2* transcripts in the cortex, striatum, cerebellum and hippocampus of *Ctsk*^{-/-} (Fig. 3a), comparable to Dio2 enzymatic activity (Fig. 3b). These data indicate that TH metabolism is not affected in the CNS of adult male *Ctsk*^{-/-} mice.

TH transporter mRNA and protein amounts suggest subtle changes in TH supply to specific brain regions

The TH transporter families that are important in the brain belong to the organic anion transporting polypeptides (Oatp), the monocarboxylate transporters (Mct) and the L-type amino-acid transporters (Lat) (Chiamolera and Wondisford 2009; Wirth et al. 2009). *Lat1*, *Lat2* and *Oatp1c1* expression was unaltered in all brain regions analysed in *Ctsk*^{-/-} mice in comparison to WT (Fig. 4a-d). *Mct8* mRNA levels were decreased by approx. 40% in the striatum, and not altered in the cortex, cerebellum and hippocampus of *Ctsk*^{-/-} mice in comparison with WT (Fig. 4a-d). In addition, *Mct10* expression was decreased in the cerebellum (Fig. 4c) but it was not changed in any of the other brain regions of *Ctsk*^{-/-} mice when compared to WT controls (Fig. 4a, b, d). *Mct8* expression is highest in the hippocampus and lowest in the striatum compared to the other three brain regions. This expression pattern was seen in both *Ctsk*^{-/-} mice and WT animals (Sup. Fig. 1). Of all four brain regions analysed, *Mct10* expression

was lowest in striatum and cortex in both *Ctsk*^{-/-} mice and WT controls (Sup. Fig. 1). It is of note that no specific Mct10 antibodies were available so that we could not test for the protein expression levels in the CNS of mice.

Analysis of the protein levels of Mct8 and Lat2 showed an overall significant effect of genotype ($F_{(1,78)} = 25.4$, $p < 0.001$; *Ctsk*^{-/-} > WT) and brain region ($F_{(3,78)} = 3.4$, $p < 0.05$; cortex > hippocampus), as well as a marginal effect of marker ($F_{(1,78)} = 2.9$, $p = 0.096$; Mct8 > Lat2). There was a significant genotype $\times$ brain region interaction ($F_{(3,78)} = 3.4$, $p < 0.05$), indicating that both proteins were more highly expressed in *Ctsk*^{-/-} animals in the cortex and cerebellum, and marginally in striatum, but not in hippocampus. Moreover, in *Ctsk*^{-/-} animals expression of both Mct8 and Lat2 was higher in the cortex fraction than in all other brain regions. While the three-way interaction was not significant ($p = 0.18$), pairwise comparisons confirmed that in *Ctsk*^{-/-} animals both markers were significantly increased in the cortex ($p < 0.01$), and Mct8 was also elevated in cerebellum and striatum ($p < 0.05$). Collectively, these data indicated subtle alterations in TH supply to and TH distribution in specific brain regions upon cathepsin K deficiency, which would possibly be more obvious when inspecting early brain development that is known to be highly sensitive to challenges in thyroid states.

Development of the cerebellum and differentiation of Purkinje neurons and Bergmann glia are not affected by cathepsin K deficiency

Cerebellar development is highly sensitive to TH dysregulation and insufficiencies, thus presenting with well distinguishable structural alterations (Horn and Heuer 2010; Koibuchi and Chin 2000). To assess if cathepsin K deficiency affects cerebellar development in a similar way as hypothyroidism, cryo-sections were investigated by Draq5TM staining of nuclear DNA and compared between WT and *Ctsk*^{-/-} animals at the ages of postnatal days (P) 12, 21, and 120. At P12, the mouse cerebellum still contains an external granular cell layer (EGL) which is visible as a separate layer of cells that will migrate to form the internal granular cell layer (IGL). The EGL was present to a similar extent in WT and *Ctsk*^{-/-} cerebellum at P12 (Figure 5a – c', arrows). Cells in the EGL region were no longer detectable at P21 in both WT controls and *Ctsk*^{-/-} mice (Figure 5d - f'), indicating exhaustive migration of most cells to the IGL comparable in both, *Ctsk*^{-/-} mice and WT controls. Cerebellar foliation was indistinguishable between adult *Ctsk*^{-/-} and WT mice inspected at P120 (Figure 5g - j'). These results indicated that development of the cerebellum was generally not affected by cathepsin K deficiency.

The maturation state of Purkinje cells serves as another indication for subtle delays in cerebellar development (Horn and Heuer 2010). Sagittal sections prepared from cerebellum of WT and *Ctsk*^{-/-} animals were immunolabeled with antibodies specific for parvalbumin, a marker protein for differentiated Purkinje cells (Bastianelli 2003). The expression of parvalbumin and the dendrite branching of Purkinje cells were comparable in WT and *Ctsk*^{-/-} mice at P12 and P21 (Figure 6a, a', and b, b', respectively).

Bergmann glial cell processes provide the structural scaffold for TH-dependent migration of neurons in the developing cerebellum (Barres 2008; He and Sun 2007). Their long fiber-like projections span the molecular layer (ML) between the EGL and the IGL (Ango et al. 2008). Bergmann glial cells are characterized by the expression of glial fibrillary acidic protein (GFAP) (Ango et al. 2008; Galou et al. 1996). The extension of Bergmann glia across the ML of the cerebellum was determined by morphometry at different regions in the cerebellum of WT and *Ctsk*^{-/-} mice at P12, P21 and P120. No significant differences were detectable between the groups (Figure 7a and f), and the differentiation state of the Bergmann glia at P21 as assessed by GFAP immunolabeling was comparable between the genotypes (Figure 7b – e').

The morphology of the cerebellum, Purkinje cells and Bergmann glia appear to be comparable between *Ctsk*^{-/-} mice and WT controls; no obvious alterations were observed. Thus, we conclude that development of the cerebellum is not affected by cathepsin K deficiency.

Impact of cathepsin K deficiency on TH associated genes in the brain

TH regulate the expression of many target genes, depending on the presence of tissue-specific availability of nuclear TH receptors (TR). The TRs are expressed in different isoforms depending on the tissue or cell type (Anyetee-Anum et al. 2018). Here, we analysed the expression of TR α and TR β isoforms and found these to be unaffected by cathepsin K deficiency ($F_{(1,32)}=1.622$, $p=0.2119$ for TR α , $F_{(1,32)}=1.493$, $p=0.2307$ for TR β), in contrast to brain regions ($F_{(3,32)}=128.9$, $p<0.0001$ for TR α , striatum < cortex < hippocampus < cerebellum, $F_{(3,32)}=35.15$, $p<0.0001$ for TR β striatum < hippocampus < cortex < cerebellum) (Fig. 8a, b). Interaction of both factors was not significant ($F_{(3,32)}=0.3406$, $p=0.7961$ for TR α , $F_{(3,32)}=1.525$, $p=0.2269$ for TR β).

Expression of the hairless gene (*Hr*) is considered an internal sensor for proper T₃ signalling in the brain (Engelhard and Christiano 2004). The transcription of *Hr* was not changed in any of the brain regions analyzed in *Ctsk*^{-/-} mice ($F_{(1,32)}=4.12$, $p=0.0508$, Fig. 8c). *Hr* mRNA levels were lowest in hippocampus and highest in cerebellum in both *Ctsk*^{-/-} mice and WT animals ($F_{(3,32)}=1249$, $p<0.0001$, Sup. Fig. 1), but the interaction of genotype x brain region was not significant ($F_{(3,32)}=1.622$, $p=0.2037$).

Since the T₃ signalling sensor *Hr* was not altered at all in hippocampus, we considered alternative mechanisms responsible for the changes in hippocampal architecture and function upon cathepsin K deficiency. Such an alternative explanation might be provided by the presence of neuroprotective factors which could be deregulated in *Ctsk*^{-/-} mice. Interestingly, gene expression levels of *Trh* were observed to be decreased in the striatum and increased in hippocampus of *Ctsk*^{-/-} mice ($F_{(1,32)}=0.05557$, $p=0.8151$ for genotype, $F_{(3,32)}=1679$, $p<0.0001$ for brain regions and $F_{(3,32)}=3.754$, $p=0.0204$ for interaction Fig. 8c). Taking into consideration that *Trh* bears neuromodulatory and neuroprotective functions, the hippocampal enhancement and the striatal decrease in *Trh* message levels in *Ctsk*^{-/-} animals may indicate a brain-wide deregulation of *Trh* amounts upon cathepsin K deficiency. *Trh* mRNA levels were lowest in cerebellum and highest in striatum in both *Ctsk*^{-/-} mice and WT animals (Sup. Fig. 1).

Discussion

Cathepsin K is present and active in neurons and glial cells of the CNS (Bernstein et al. 2007; Feher et al. 2005; Ko et al. 2006). Considering the effects of cathepsin K deficiency in mice on early differentiation of glial cells (Dauth et al. 2012) as well as the altered numbers of hippocampal astrocytes, neuronal rearrangements, and the severe learning and memory deficits in adult mice (Dauth et al. 2011b), we were interested to find out whether the function of cathepsin K in the CNS of male mice is connected to its role in the thyroid gland. While no obvious signs for a hypothyroid state were observed, TH transporter mRNA and protein amounts were altered in the CNS of *Ctsk*^{-/-} mice vs. WT controls in a subtle and brain region-specific manner. Thus, we were further interested to investigate whether cathepsin K deficiency affects TH-dependent CNS development (Richard and Flamant 2018) early after birth, such that possible developmental defects would manifest in adult mice thereby explaining their learning and memory impairments. In the cerebellum, a primary TH target tissue, overall structural development and its specific cell types were unaffected by cathepsin K deficiency, consistent with unaltered expression of *Hr*, a sensor of T₃ signalling, in all brain regions tested including cerebellum and hippocampus. We conclude that cathepsin K does not disrupt cerebellar development in a manner pheno-copying a lack of TH supply. Since the expression levels of *Hr* were also unchanged in all tested brain regions of adult mice, the same conclusion might hold true for the development of the brain at large, but this proposal would need to be tested further. Taking further into account that the serum fT₃, fT₄ and TSH levels of *Ctsk*^{-/-} mice are not different from WT controls (Friedrichs et al. 2003), we conclude that the role of cathepsin K in the thyroid gland is independent of its function in the CNS of mice.

Thus, neither altered thyroid states or brain TH levels nor cerebellar developmental defects suggestive of hypothyroidism were observed in *Ctsk*^{-/-} mice. However, transcription of the *Trh* gene was deregulated in a brain region-specific manner in *Ctsk*^{-/-} mice. TRH is a well-known regulator of the HPT (hypothalamus-pituitary-thyroid) axis (Muller-Fielitz et al. 2017; Ortiga-Carvalho et al. 2016). In the presence of its ligand, T₃, the nuclear TR mediates ligand-dependent repression of the transcription of the *Trh* gene, and in the absence of T₃, the transcription rate is not simply returned to baseline, but ligand-independent activation is observed (Chiamolera and Wondisford 2009). In particular, at high serum T₃ concentrations TRH is down-regulated, constituting a negative feedback loop that depends on Dio2-mediated T₄-to-T₃ conversion in tanycytes (Fonseca et al. 2013). Specifically, β 2-tanycytes of the median eminence are critical for HPT axis regulation through modulation of the TRH flux into portal vessels (Rodriguez-Rodriguez et al. 2019). It is interesting to note, however, that multiple other types of tanycytes exist in the brain of mammals, and specifically the tanycytes and ependymal cells of the blood-cerebrospinal fluid barrier (BCSFB) and the choroid plexus belong to the cathepsin K-expressing cells (Haeckel et al. 1999). Tanycytes and ependymal cells might therefore be involved in the complex sequence of proteolytic processing of the TRH precursor proTRH, which finally requires glutaminyl cyclase (QC)-mediated conversion resulting in N-terminal pyroglutaminylation for acquisition of its bioactivity (Monga et al. 2008; Nillni 1999; Schilling et al. 2011). Moreover, TRH is found in several regions of the CNS including the hippocampus (Hokfelt et al. 1989; Low et al. 1989; Merchenthaler et al. 1988; Morley 1979). TRH thus carries a number of highly important roles in the CNS, which are independent of its HPT axis regulatory tasks (Schilling et al. 2011). In particular, TRH acts as a neuromodulator and was shown to have neuroprotective functions (Waniewski et al. 2015). Hence, the observed deregulated *Trh* transcription may contribute to the CNS alterations observed in *Ctsk*^{-/-} mice.

Neuroprotection – another task of the HPT axis regulator TRH

Interestingly, our previous studies (Dauth et al. 2011b) have pointed out that *Ctsk*^{-/-} mice exhibit an overall induction of the dopaminergic system, which is a function of the striatum/mesencephalon. In this study, the most obvious changes in gene expression seen in the striatum of *Ctsk*^{-/-} mice was a decreased *Trh* expression. In addition, striatal *Trh* expression is highest among the analyzed brain regions in WT mice as expected, and this does not change in *Ctsk*^{-/-} mice. However, we propose that the change in gene transcription upon cathepsin K deficiency affects the amount of neuroprotective Trh among others. In the hippocampus, *Trh* expression is higher in *Ctsk*^{-/-} mice compared to WT controls (this study), and could possibly protect hippocampal neurons in *Ctsk*^{-/-} mice that are largely deprived of the supportive input from astroglial cells, because astroglial cells are fewer in the hippocampus of *Ctsk*^{-/-} mice (Dauth et al. 2011b). Hence, deregulation of Trh and possibly other neuromodulators may result in alterations that become manifest as altered neuronal patterning in the hippocampus, and may eventually also result in disturbed hippocampal (learning and memory, novelty-seeking) and striatal activities (reward) such as observed in cathepsin K deficiency (Dauth et al. 2011b). In conclusion, since the HPT axis does not seem to be affected in *Ctsk*^{-/-} mice, the observed alterations in TRH availability could possibly cause changes in neuroprotection, thereby affecting e.g. the survival of neurons in the hippocampus.

In addition, cathepsin K deficiency might result in alterations of the BCSFB structurally and functionally, a proposal which has to be addressed in future by a detailed analysis of the cerebrovascular lining, including a thorough study of the choroid plexus. More precisely, defects in tanycytes and ependymal cells may contribute to the neurobehavioral phenotypes observed in *Ctsk*^{-/-} mice, because an important task of these cells at the BCSFB and in the choroid plexus is to establish and maintain the composition of intrathecal fluid and to allow for waste disposal from the brain to the blood.

A proposed function for cathepsin K in the CNS

It is now well established that through proteolytic processing the endolysosomal system is involved in numerous cellular processes responsible for homeostasis and cell differentiation in various organ systems (Turk and Turk 2009). Any dysregulation in protease expression and/or their proteolytic activities disrupts cellular and tissue homeostasis (Brix et al. 2008; Brix et al. 2014). In the case of cathepsin K deficiency, either an imbalance of proteolytic activities as a response to the lack of cathepsin K, or the missing proteolytic activity of cathepsin K specifically for cleavage of a possible substrate could lead to pathological alterations in the brain. Interestingly, cathepsin K has been associated with schizophrenia in human patients (Bernstein et al. 2007) and it has been suggested to contribute to opioid metabolism by releasing met-enkephalin through the cleavage of its precursor, β -endorphin (Lendeckel et al. 2009). Hence, we propose that the main role of cathepsin K in the CNS of mice may be through a direct involvement in pro-protein processing, both within endo-lysosomes and in the peri-cellular environment of glial cells and neurons of the brain (Brix et al. 2014; Brix et al. 2013; Dauth et al. 2011a).

In particular, proTRH is a good candidate of cathepsin-mediated processing because it requires several endo- and exopeptidase cleavages to generate the tripeptide TRH, which is then further subjected to glutaminy cyclase modification at the N-terminus to yield bioactive TRH (Monga et al. 2008; Nillni 1999; Schilling et al. 2011). The precursor of bioactive TRH is proTRH which contains several repetitive sequences of QHPG that must be cleaved out of the polypeptide backbone by proteolysis after Lys-Arg and Arg-Arg flanking motifs. Such cleavages are preferentially mediated by cysteine cathepsin B acting as an endo- and exopeptidase and by cathepsin L serving as endopeptidase. Both enzymes are less active in the striatum/mesencephalon of *Ctsk*^{-/-} mice (Dauth et al. 2011b)

in which the endogenous cysteine peptidase inhibitor cystatin C, a secretory protein, is present in enhanced amounts. However, in other regions of the *Ctsk*^{-/-} brain, cathepsin B and L activities were unaltered, whereas hippocampal cystatin C was enhanced (Dauth et al. 2011b). Thus, one might argue that initial proTRH processing might be altered in *Ctsk*^{-/-} mice. Eventually, the C-terminal glycine of the QHPG peptides cut out of proTRH is subjected to the action of PAM monooxygenase, while the N-terminal glutamine is converted to cyclic pyroglutamate by QC, thus, yielding bioactive TRH (Schilling et al. 2011). In an AD mouse model, the neuroprotective role of such pGlu-modified TRH has been shown, and importantly, evidence was provided that especially hippocampal neurons and Aβ plaque-activated astrocytes use this pathway of QC-mediated TRH modification for cytoprotection and survival (Wanek et al. 2015).

Similarly, in *Ctsk*^{-/-} hippocampal neurons the activities of cathepsins B and L are mildly decreased, while the endogenous cysteine peptidase inhibitor cystatin C is significantly enhanced, and astrocyte numbers are significantly reduced in this region (Dauth et al. 2011b). Such alterations could, in principle, result in less extensive proTRH processing and may therefore explain why neuroprotective functions of TH are different in *Ctsk*^{-/-} mice. Indeed, neuronal patterning differs significantly in *Ctsk*^{-/-} mice, with overall fewer and sparser neurons being found in the ventral hippocampus in particular (Dauth et al. 2011b). Interestingly, the *Trh* transcript levels are enhanced in the hippocampus of *Ctsk*^{-/-} mice, which might be the regulatory response to a lack of bioactive Trh. In striatum/mesencephalon, however, *Trh* mRNA amounts were decreased to approx. 40% of WT control levels indicating that less proTRH would be available, resulting in altered neuro-modulatory and neuroprotective abilities in the *Ctsk*^{-/-} brain. This hypothesis will be evaluated in future studies. It will be important to investigate whether and where precisely TRH bioactivity might be altered in *Ctsk*^{-/-} mice, and to identify specific substrates of cathepsin K in the CNS in order to clarify the precise physiological role of this protease in the brain.

Conclusions

Overall, the results of this study suggest that the intrinsic function of cathepsin K in the brain is independent from its function in the thyroid gland. Whether the lack of cathepsin K affects the CNS indirectly by an altered proteolytic network or directly by non-cleavage of its yet unknown substrate(s) will require further investigation. Delineating the functions of cathepsin K in the brain is important, however, because inhibitors targeting this protease are of interest clinically for prevention and alleviation of disease symptoms caused by deregulated or excessive proteolytic degradation of ECM constituents as in atherosclerosis, osteoarthritis, bone metastasis, and osteoporosis (Blum 2008; Le Gall et al. 2008; Podgorski 2009). Moreover, the functions that cathepsin K might play in the cerebrovascular lining cells (ependymal cells and tanycytes) is not even approaching the spotlight of recent research. We suggest that it is worthwhile to study in more detail how the cathepsin K-tanycyte/ependymal cell-TRH interrelationship may affect brain homeostasis and metabolism besides the prominent role that TRH plays in HPT axis regulation.

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Conflict of Interest

The authors declare that they have no competing interests.

Ethical approval

All procedures performed in studies involving animals were conducted in accordance with institutional guidelines in S1-laboratories of Jacobs University Bremen (Senator für Arbeit, Frauen, Gesundheit, Jugend und Soziales der Hansestadt Bremen Az. 513-30-00/2-15-32 including KB as project leader, and Az. 0515_2040_15 to KB allowing breeding, housing and sacrifice of genetically engineered mice).

This article does not contain any studies with human participants performed by any of the authors.

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Table 1: Brain and thyroid weights of male WT and *Ctsk*^{-/-} mice (4-6 months)

	Brain weight [g]	Thyroid weight [mg]
WT (average $\pm$ SEM)	0.47 $\pm$ 0.01	3.2 $\pm$ 0.2
<i>Ctsk</i>^{-/-} (average $\pm$ SEM)	0.43 $\pm$ 0.009	4.0 $\pm$ 0,4

Brain weights: WT n=15, *Ctsk*^{-/-} n=10; thyroid weights: WT n=10, *Ctsk*^{-/-} n=12

Fig. 1: Morphological assessment of the thyroid gland in WT and *Ctsk*^{-/-} animals.

The analysis was performed using middle sections through an entire thyroid lobe (**a, top panel**) upon staining of the cytoplasm with Cell Mask OrangeTM (red), the thyroid follicle-surrounding extracellular matrix with collagen type IV-specific antibodies (green), and the nuclei of follicle cells with Draq5TM (blue); scale bars represent 500 μm . Thyroids from 3-5 mice per genotype were analyzed and the follicle lumina are displayed in a color code from blue to red according to their sizes as indicated (**a, left and bottom panel**). In comparison to WT, the follicle population does not follow a normal distribution but rather a left skewed distribution when frequencies are plotted against deviation of the means (**b**). Although the mean lumen size does not differ (**c, red lines indicate median**), the heterogeneity of individual follicles is reduced in *Ctsk*^{-/-} mice (**b and c, dots denote individual follicle lumina**). 1111 (WT) and 1526 (*Ctsk*^{-/-}) follicles from 8 (WT) and 4 (*Ctsk*^{-/-}) animals were analysed. The overall lumen size of *Ctsk*^{-/-} thyroid lobes (**d**) was comparable to the WT, but slightly decreased, and the follicle count per middle section shows a corresponding, but also not significant increase (**e**). The epithelial extensions of *Ctsk*^{-/-} thyroid follicles (**f**) are not altered when compared to the WT. Bar charts denote mean values $\pm$ standard deviation.

Fig. 2 Serum levels of TSH, fT₄ and fT₃, brain levels of fT₄ and tT₃, and body weight gain over time in WT and *Ctsk*^{-/-} mice

Serum TSH, fT₄ and fT₃ levels of adult mice (5 – 5.5 months old) were measured using ELISA tests. Serum levels of TSH, fT₄ and fT₃ of *Ctsk*^{-/-} (n=3) mice were comparable to those of WT (n=14, 16, 16, respectively) mice (**a-c**). The levels of fT₄ (**d**) and tT₃ (**e**) in brain lysates as determined by ELISA and normalized to tissue protein were also similar between *Ctsk*^{-/-} (n=10) and WT (n=13) animals. Body weight gain was also comparable between *Ctsk*^{-/-} (n=9) and WT (n=7) animals. Bar charts denote mean values ± SEM.

Fig. 3 Deiodinase 2 mRNA and activity levels in *Ctsk*^{-/-} and wild type animals

Quantitative real-time PCR was used to assess gene expression levels of Dio2 in tissue lysates of cortex, striatum/mesencephalon, cerebellum and hippocampus from adult mice (4-5 months) (**a**). mRNA levels of Dio2 were comparable between *Ctsk*^{-/-} mice and WT controls. Bar charts denote fold-change in WT/WT and *Ctsk*^{-/-}/WT $\pm$ SEM; antilogarithmic values of fold-changes were used for statistical analysis by two-way ANOVA with Tukey's post-hoc test for multiple correction, n=5. Dio2 activity (**b**) was determined in tissue lysates of cortex, striatum/mesencephalon, cerebellum and hippocampus from adult mice (4-5 months). No significant differences were detectable between *Ctsk*^{-/-} mice (n = 3-5) and WT controls (n = 3-4). Bar charts denote mean values expressed as percent of control $\pm$ SEM.

Alternative for n/o:

Fig. 4 Gene expression and protein amount of TH transporters in *Ctsk*^{-/-} mice and wild type controls

Quantitative real-time PCR was used to assess gene expression levels of TH transporters in tissue lysates of cortex, striatum/mesencephalon, cerebellum and hippocampus from adult mice (4-5 months) (**a-d**). In striatum, *Mct8* levels are decreased, while *Mct10* levels are decreased in the cerebellum of *Ctsk*^{-/-} mice compared to WT. Bar charts denote fold-change in WT/WT and *Ctsk*^{-/-}/WT $\pm$ SEM; antilogarithmic values of fold-changes were used for statistical analysis and unpaired Student's t-test applied to each brain region and transcript of interest. Levels of significance are denoted as * for $p < 0.05$, $n = 5$. Representative immunoblots prepared from homogenates of cerebral cortex, striatum/mesencephalon, cerebellum and hippocampus of WT (white bars) and *Ctsk*^{-/-} mice (grey bars) as indicated were probed for Lat 2 (**e-h**) and Mct8 (**i-m**); lanes represent separate individuals. Densitometry analysis revealed significantly increased Lat2 (**n**) and Mct8 (**o**) amounts in the cortex and cerebellum of *Ctsk*^{-/-} mice ($n = 5$) compared to WT controls ($n = 6$). Protein levels were determined by densitometry and grey values per area were normalized to the loading control β -tubulin. Box plots charts show the range from the 25th to 75th

percentiles, with the middle line indicating the median and the whiskers min to max spread of data. Values of individual animals are plotted as circles for WT and squares for *Ctsk*^{-/-} values (n and o). Levels of significance are denoted as * for $p < 0.05$ and ** for $p < 0.01$.

Fig. 5 Structure of the cerebellum at different post-natal stages

Sagittal cryo-sections through brain hemispheres of *Ctsk*^{-/-} mice and WT mice sacrificed at postnatal day (P) 12, 21, and 120 were stained with Draq5TM and analyzed through confocal laser scanning microscopy. Three separate regions of the cerebellum were compared for each of the two genotypes. Depicted are three separate cerebellum regions at P12 in WT mice (**a, b, and c**) and the corresponding regions at P12 for *Ctsk*^{-/-} (**a', b', and c'**). The same regions are shown for WT animals at P21 (**d, e, and f**) and *Ctsk*^{-/-} mice (**d', e', and f'**). For P120 the same regions are shown for WT mice (**g, h, and j**) and *Ctsk*^{-/-} mice (**g', h', and j'**). The overall structure of the cerebellum was not altered in *Ctsk*^{-/-} mice compared to WT animals, independent of the age of the animal and the region investigated. EGL = external granule cell layer, IGL = internal granule cell layer, ML = molecular layer. Bar – 400 μm.

Fig. 6 Differentiation states of Purkinje cells

Sagittal cryo-sections through brain hemispheres of *Ctsk*^{-/-} mice and WT mice sacrificed at postnatal day 12 and 21 were stained with antibodies specific for parvalbumin (green) and nuclear DNA was counter-stained by Draq5TM (red). Purkinje cells are depicted at P12 in WT animals (**a**) and *Ctsk*^{-/-} mice (**a'**), and at P21 (**b** and **b'**, **respectively**). Purkinje cells and their dendritic branching patterns were not altered in *Ctsk*^{-/-} mice. EGL = external granule cell layer, IGL = internal granule cell layer, ML = molecular layer. Bar – 80 μ m.

Fig. 7 Differentiation states of Bergmann glia and thickness of the molecular layer

An overview sketch of the structure of the cerebellum is depicted in **a**. Areas which are shown in higher magnification in **b – e'** and which were analyzed by morphometry (**f**) are indicated by the respective boxes. Sagittal cryo-sections through brain hemispheres of *Ctsk*^{-/-} mice and WT animals sacrificed at postnatal day (P) 12, 21, and 120 were stained with antibodies specific for GFAP (green) and nuclear DNA was counter-stained by Draq5TM (red). Representative examples of immunolabeled sections are given for P21, only. Fibers of Bergmann glial cells were not changed in length or number in *Ctsk*^{-/-} mice compared to WT animals. (**f**) The thickness of the ML was measured in three to four different individuals per genotype and 7-13 pictures were investigated per age and genotype. Bar charts denote mean values expressed as $\mu\text{m} \pm \text{SEM}$; no alterations were noted in the thickness of the ML in the cerebellum of WT or *Ctsk*^{-/-} mice. EGL = external granule cell layer, IGL = internal granule cell layer, ML = molecular layer. Bar – 80 μm .

Fig. 8 Gene expression of *Tra*, *Trβ*, *hairless* and *Trh* in different brain regions of *Ctsk*^{-/-} mice compared to WT animals

Quantitative real-time PCR was used to assess gene expression levels of TH receptor α (*Trα*) (a), TH receptor β (*Trβ*) (b), *hairless* (*Hr*) (c) and TSH releasing hormone (*Trh*) (d) in tissue lysates of the cortex, striatum, cerebellum and hippocampus from adult mice (4-5 months). Note the increase in *Trh* mRNA upon cathepsin K deficiency (grey bar) compared to WT (white bar) specifically in the hippocampus (d) Bars represent the fold-change of gene expression in WT/WT and *Ctsk*^{-/-}/WT mice $\pm$ SEM. Antilogarithmic values of fold changes were used for statistical analysis by two-way ANOVA with Tukey's post-hoc test for multiple correction, n=5.

Supplementary Fig. 1 Gene expression in the cortex, striatum, and hippocampus compared to the cerebellum in *Ctsk*^{-/-} mice and WT animals

Quantitative real-time PCR was used to assess gene expression levels of hairless (*Hr*), TSH releasing hormone (*Trh*), deiodinase 2 (*Dio2*), TH receptor α (*Tr\alpha*), TH receptor β (*Tr\beta*), monocarboxylate transporter 8 (*Mct8*), *Mct10*, L-type amino acid transporter 1 and 2 (*Lat1* and *Lat2*), and organic anion transporter protein 1c1 (*Oatp1c1*) in tissue lysate of the cortex, striatum, cerebellum and hippocampus from adult WT mice (**a**) and *Ctsk*^{-/-} mice (**b**). Gene expression levels were normalized to the cerebellum in order to compare mRNA levels between different brain regions. Bars represent the fold-change of gene expression in different brain regions compared to the cerebellum in *Ctsk*^{-/-} mice and WT controls $\pm$ SEM (n=5). Anti-logarithmic values of fold changes were used for statistical analysis by one-way ANOVA with Dunnet's post-hoc test for multiple correction. Significance levels are depicted by * = p-value $\leq$ 0.05, ** = p-value $\leq$ 0.01, *** = p-value $\leq$ 0.001, and # = p-value $\leq$ 0.0001, while difference to cerebellum are denoted by symbols over the bars, and differences between brain areas are denoted by symbols on half-tick down lines. ANOVA results wt: $F_{(3,16)}=72.69$, $p<0.0001$ for *Hr*, $F_{(3,16)}=45.89$, $p<0.0001$ for *Trh*, $F_{(3,16)}=62.24$, $p<0.0001$ for *Dio2*, $F_{(3,16)}=27.82$, $p<0.0001$ for *Tr\alpha*, $F_{(3,16)}=20.19$, $p<0.0001$ for *Tr\beta*, $F_{(3,16)}=11.88$, $p=0.0002$ for *Mct8*, $F_{(3,16)}=65.08$, $p<0.0001$ for *Mct10*, $F_{(3,16)}=36.73$, $p<0.0001$ for *Lat1*, $F_{(3,16)}=11.24$, $p=0.0003$ for *Lat2*, $F_{(3,16)}=8.546$, $p=0.0013$ for *Oatp1c1*; ko: $F_{(3,16)}=51.77$, $p<0.0001$ for *Hr*, $F_{(3,16)}=24.26$, $p<0.0001$ for *Trh*, $F_{(3,16)}=19.31$, $p<0.0001$ for *Dio2*, $F_{(3,16)}=39.96$, $p<0.0001$ for *Tr\alpha*, $F_{(3,16)}=64.42$, $p<0.0001$ for *Tr\beta*, $F_{(3,16)}=47.67$, $p<0.0001$ for *Mct8*, $F_{(3,16)}=110.5$, $p<0.0001$ for *Mct10*, $F_{(3,16)}=21.71$, $p<0.0001$ for *Lat1*, $F_{(3,16)}=59.12$, $p<0.0001$ for *Lat2*, $F_{(3,16)}=11.6$, $p=0.0003$ for *Oatp1c1*.

Supplementary Table 1

Oligonucleotides for quantitative RT-PCR. Oligonucleotides were designed using PrimerBlast (NCBI) and synthesized by Eurofins (Eurofins MWG Synthesis, Ebersberg, Germany).

Gene name	Forward primer	Reverse primer
<i>18S</i>	<i>CGGCTACCACATCCAAGGAA</i>	<i>GCTGGAATTACCGCGGCT</i>
<i>Ppia</i>	<i>CTTGGGCCGCGTCTCCTTCG</i>	<i>GCGTGTAAGTCACCACCCTGGC</i>
<i>Hprt</i>	<i>TGGGCTTACCTCACTGCTTT</i>	<i>TCATCGCTAATCACGACGCT</i>
<i>Hr</i>	<i>CCCTGACCACCGTATGCTTT</i>	<i>CCACACCGTCTAAGTTCCCC</i>
<i>Dio2</i>	<i>GTGACTGGGGAAGCAGAGTG</i>	<i>AGTTTAACCTGTTTGTAGGCATC</i>
<i>Trh</i>	<i>GCTTGGCCTCTGAGAACTAAAGA</i>	<i>TCCCAGTATGTAACTTGCCTGA</i>
<i>Tralpha</i>	<i>GAAAAGCAGCATGTCAGGGTA</i>	<i>GGATTGTGCGGCCAAAGAAG</i>
<i>Trbeta</i>	<i>GGGAAATGGCAGTGACACGA</i>	<i>CCTGGGCGATCTGAAGACAT</i>
<i>Mct8</i>	<i>CTCCTTCACCAGCTCCCTAAG</i>	<i>ACTTCCAGCAGATACCACACC</i>
<i>Mct10</i>	<i>CGTGAGTGTCTTCACGGACA</i>	<i>CGATGGAGCTTACAAAAGAACTGG</i>
<i>Lat1</i>	<i>AGCGTCCCATCAAGGTGAAT</i>	<i>GGGCTTGTTCTTCCACCAGA</i>
<i>Lat2</i>	<i>AACAACACCGCGAAGAACCA</i>	<i>GGAGCCAATGATGTTCCCTACAA</i>
<i>Oatp1c1</i>	<i>TTTGCTCTGGGTATCTACACCTT</i>	<i>ATATGTGTCTGAAAGCGTGGGA</i>