

Human cathepsin L rescues the neurodegeneration and lethality in cathepsin B / L double deficient mice

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Summary

Cathepsin B (CTSB) and cathepsin L (CTSL) are two widely expressed cysteine proteases thought to predominantly reside within lysosomes. Functional analysis of CTSL in humans is complicated by the existence of two CTSL-like homologues (CTSL and CTSL2), in contrast to mice which contain only one CTSL enzyme. Thus transgenic expression of human CTSL in CTSL deficient mice provides an opportunity to study the *in vivo* functions of this human protease without interference by its highly related homologue. While mice with single gene deficiencies for murine CTSB or CTSL survive without apparent neuromuscular impairment, murine CTSB/CTSL double deficient mice display degeneration of cerebellar Purkinje cells and neurons of the cerebral cortex, resulting in severe hypotrophy, motility defects, and lethality during their third to fourth week of life. Here we show that expression of human CTSL through a genomic transgene results in widespread expression of human CTSL in the mouse which is capable of rescuing the lethality found in CTSB/CTSL double-deficient animals. Human CTSL is expressed in the brain of these compound mutants predominantly in neurons of the cerebral cortex and in Purkinje cells of the cerebellum, where it appears to prevent neuronal cell death.

Key words:

Cathepsin, Neurodegeneration, Purkinje cell

Introduction

Cathepsins are proteolytic enzymes with a principal subcellular localization in the endosomal/lysosomal compartment. The largest family of cathepsins comprises the papain-like cysteine proteases with 11 human enzymes (Turk et al., 2001). As a result of a gene duplication, the human genome encodes for two cathepsin L-like proteases, namely the “classic” human cathepsin L (CTSL), which is produced by a wide range of tissue and cell types, as well as cathepsin L2 (CTSL2; alternatively termed cathepsin V) with a more restricted expression pattern (Brömme and Kaleta, 2002). In contrast, the mouse genome contains only one CTSL gene, which is ubiquitously expressed (Brömme and Kaleta, 2002). All three enzymes, murine CTSL, human CTSL and human CTSL2, are highly homologous with about 75% amino acid identity, which complicates the assignment of biological functions to the human enzymes (Brömme and Kaleta, 2002; Hagemann et al., 2004). Thus we sought to study the expression of human CTSL in CTSL-deficient mice to assess *in vivo* functions of the human protease without interference of its highly related homologue.

CTSL-deficient mice develop dilated cardiomyopathy (Stypmann et al., 2002) as well as epidermal hyperproliferation, retardation of hair follicle morphogenesis and alterations of the hair cycle (Reinheckel et al., 2005; Roth et al., 2000; Tobin et al., 2002). Murine CTSL is critical for MHC class II mediated antigen presentation in cortical epithelial cells of the thymus (Honey et al., 2002; Nakagawa et al., 1998). Furthermore, CTSL is involved in generating hypophyseal neuropeptides (i.e. enkephalin) and, in combination with cathepsin K and B, is responsible for solubilization of thyroglobulin and the release of thyroid hormones (Friedrichs et al., 2003; Yasothornsrikul et al., 2003). Recently, the critical role of CTSL for endothelial

progenitor cell–induced neovascularization in a hind limb ischemia model was demonstrated (Urbich et al., 2005). However, CTSL knockout mice are fertile, reproduce normally and do not develop a pathological neuromuscular phenotype. In contrast to CTSL deficient animals, CTSB knockout mice do not show any obvious spontaneous phenotype (Reinheckel et al., 2001). Nevertheless, under environment stress experiments CTSB proved to have a major role in pathological trypsinogen activation in the early course of experimental pancreatitis (Halangk et al., 2000), as well as to contribute significantly to TNF- α induced hepatocyte apoptosis (Guicciardi et al., 2000). In contrast to the single gene mutations, CTSB/CTSL double mutant mice exhibit a severely impaired life expectancy. While mice with CTSB/CTSL double deficiency are born at the expected Mendelian ratios, they die during their third to fourth week of life (Felbor et al., 2002). Felbor et al proposed that this lethality is most likely due to degeneration of cerebellar Purkinje cells and neurons of the cerebral cortex, which results in severe hypotrophy, motility defects, and ultimately death (Felbor et al., 2002).

The aim of the present study was to express human CTSL from a genomic transgene and to compare postnatal development, brain histology and cathepsin L expression of the triple mutant Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} with *Ctsb*^{-/-}*Ctsl*^{-/-} double knockouts and wild-type controls.

Results

Expression of transgenic human CTSL.

We previously generated a human CTSL transgenic through the use of a publicly available Bacterial Artificial Chromosome clone (CTD-2062O4) (Houseweart et al., 2003). In order to assess the expression of the transgene, we examined human CTSL mRNA and protein levels as well as enzymatic activity in a panel of mouse tissues (Figure 1). Amplification of a specific 152 bp fragment of human CTSL cDNA was observed after RT-PCR of RNAs isolated from the human cell line HEK293 or from liver of transgenic Tg(hCTSL) mice, while material from nontransgenic mice did not show a specific RT-PCR product (Figure 1A). By using these primers in quantitative “real time” RT-PCR, we determined the relative level of the hCTSL transcript in several tissues from these transgenic mice (Figure 1B). Liver, followed by kidney, showed the highest level of human CTSL mRNA expression, while heart, skeletal muscle and brain displayed lower expression. This expression pattern was largely confirmed by Western blotting, although kidney revealed somewhat higher abundance of human CTSL than liver, suggesting a difference in their post-transcriptional processing (Figure 1C). Interestingly, pro-CTSL was almost undetectable in brain samples indicating that the protease is predominantly activated in the central nervous system, in contrast to other tissues???. Furthermore, we compared the degradation of the common CTSL substrate z-Phe-Arg-AMC (in presence of the specific CTSB inhibitor CA074) in samples of wild-type and triple mutant Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} mice, which are deficient for the murine proteases CTSB and CTSL (Figure 1D). Comparison of wild-type and transgenic mice revealed that the degradation of the fluorogenic peptide was widely different with livers and kidneys of transgenic mice showing 4-fold and 10-fold increases of z-Phe-Arg-AMC cleavage as compared to wild-type mice. In contrast, CTSL activities in cerebral

cortex and cerebellum were similar to control animals (Figure 1D). In summary these data indicate that the genomic transgene exerts control on tissue specific expression quantities of the human CTSL in the mouse, and a proteolytic active human CTSL is produced. However, these transgenic mice appear outwardly normal with no apparent effects on fertility, reproduction or viability. In order to determine if the human transgene could complement the mouse CTSB/CTSL deficient phenotypes, we crossed them consecutively with CTSB and CTSL knockout mice in order to obtain the triple mutant Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-}.

The lethal phenotype of CTSB/CTSL double deficient mice is rescued by the expression of human CTSL.

In contrast to the study of Felbor et al, which used CTSB/CTSL double deficient mice of a mixed C57BL/6 x 129/Sv genetic background (Felbor et al., 2002), the CTSB/CTSL double deficient mice used in the present study were backcrossed for at least 6 generations to the FVB/n background resulting in a more uniform genetic background since their original generation (Halangk et al., 2000, Roth et al., 2000). At the age of 16 days *Ctsb*^{-/-}*Ctsl*^{-/-} as well as Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} exhibited retarded fur growth (Figure 2A), while body weight was similar to the *Ctsb*^{-/-}*Ctsl*^{+/-} control mice that showed a weight gain identical to FVB/n wild-type mice (Figure 2B). However, CTSB/CTSL double deficient mice developed severe hypotrophy and neuromuscular dysfunction, characterized by ataxia, mild rear limb spasticity, and physical inactivity, during the next 7-10 days of life. This phenotype was completely rescued by expression of human CTSL in Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} mice (Figure 2A, B). Body weight of *Ctsb*^{-/-}*Ctsl*^{-/-} males and females ranged between 3-5 g in the fourth week of life, and animals were sacrificed at this stage. In contrast, the males and females of the triple mutant Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} showed a slightly

retarded weight gain during the third to fifth week of life, however, at 35 days body weights reached the control group and mice were viable for observation periods of up to one year (Figure 2B). Interestingly, Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} showed the periodic hair loss that is characteristic for mice with single deficiency for the murine CTSL (Figure 2A).

Cell type specific expression of human CTSL in the central nervous system is associated with prevention of Purkinje cell degeneration.

Purkinje cells possess the only efferent neurites of the cerebellar cortex and control muscle movements by their inhibitory action on cerebellar nuclei. Strikingly, CTSB/CTSL double deficient mice revealed a specific loss of Purkinje cells coinciding with neuromuscular impairment, while cerebella of mice with single deficiencies for CTSB or CTSL showed a normal Purkinje cell layer (Figure 3 A-E). The other layers of the cerebellar cortex, namely the molecular and granular cell layers, did not exhibit histomorphological alterations. Strikingly, the triple mutant Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} showed a complete rescue of Purkinje cells supporting a highly conserved function of CTSL activity for maintenance of this cell layer (Figure 3E).

In order to localize CTSL expression in mouse cerebral and cerebellar cortex, immunohistochemistry for murine CTSL and for transgenic human CTSL was performed (Figures 4 and 5). In the cerebral cortex murine CTSL is highly expressed in the pyramidal cell neurons (Figure 4A, B). This cerebral expression pattern is clearly recapitulated by the expression of human CTSL in Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} mice (Figure 5 A,B). However, histopathological lesions have not been detected in the cerebral cortex of CTSB/CTSL double deficient FVB/n congenic mice (Figure 5 C, D). Murine CTSL is highly abundant in cerebellar Purkinje cells with a staining intensity clearly exceeding the decoration of the other cell layers (Figure 4 D). Strikingly, human CTSL is strongly expressed by the cerebellar Purkinje cell layer of

Tg(hCTSL);*Ctsb*^{-/-}*Ctsr*^{-/-} mice (Figure 5E, F). Thus, both the mouse CTSL and human transgenic human CTSL express in similar patterns in the brain. Furthermore, transgene expression in Purkinje cells is associated with the prevention of Purkinje cell death supporting the intrinsic need for CTSL in these specific cells of the cerebellum.

Discussion

Because of their principal endosomal/lysosomal localizations and redundant substrate specificities, cysteine cathepsins are thought to cooperate in terminal degradation of proteins upon delivery to lysosomes by endocytosis or autophagy (Ciechanover, 2005). However, individual cell types show a differential requirement for the presence of various cysteine cathepsins. Cerebellar Purkinje cells are highly susceptible to the loss of cathepsins B and L. Although present after birth (Felbor et al., 2002), these cells degenerate during the first weeks of life. In contrast to Felbor et al. cerebral atrophy and astrogliosis were not observed in our analysis of *Ctsb*^{-/-}*Ctsl*^{-/-} mice (Felbor et al., 2002). This might be explained by differences in the mouse strain, congenic FVB (this study) versus mixed C57BL/6 x 129/Sv, or since Felbor et al. nursed some *Ctsb*^{-/-}*Ctsl*^{-/-} mice up to an age of 50 days at which marked cerebral atrophy was found (Felbor et al., 2002). Thus it appears that degeneration of Purkinje cells represents an early pathogenic event in the brain of CTSB/CTSL double deficient mice while cerebral atrophy occurs later in pathogenesis. Since Purkinje cells are critical for the refinement of movement, the loss of these cells is likely to cause the neuromuscular phenotype of CTSB/CTSL double knockout mice. In extension of the study of Felbor et al. (Felbor et al., 2002), we report an expression pattern of murine CTSL in wild-type brain that corresponds to the cell types that are highly susceptible to the loss of cathepsins, namely the cerebellar Purkinje cells and the neurons of the cerebral pyramidal cell layer. The brain phenotype of CTSB/CTSL double deficient mice provides a clear example for the functional redundancy among lysosomal cathepsins, since mice with single deficiencies of CTSB or CTSL do not show neuronal loss or neuromuscular symptoms. This is further supported by the finding of a rescue of Purkinje cell loss by transgenic expression of human CTSL in CTSB/CTSL double deficient mice. Consistent with this observation, in the brain the

human CTSL genomic transgene shows a similar expression pattern and CTSL activity as endogenous mouse CTSL. In contrast, in other tissues (i.e. kidney and liver) the transgene construct results in a 4-10 fold excess of CTSL activity as compared to wild-type mice. However, this over-expression did not result in a gross phenotype. Interestingly, not all phenotypes of *Ctsb*^{-/-}*Ctsl*^{-/-} mice are rescued by the human CTSL transgene. The early postnatal phenotype of mice with CTSB/CTSL double deficiency is characterized by a delay of hair development, which is also a hallmark of mice with single deficiency of CTSL (Roth et al., 2000; Tobin et al., 2002). As shown in figure 2, Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} mice still show this characteristic pattern of hair loss. It has been shown that this skin phenotype of CTSL deficient mice depends on CTSL activity in keratinocytes of hair follicles and of the basal epidermal layer (Hagemann et al., 2004; Reinheckel et al., 2005). The question of whether active human CTSL is expressed in keratinocytes of Tg(hCTSL) mice or if human CTSL is not capable of executing the murine CTSL functions in these cell types is currently under investigation.

Over the past years evidence of cell type specific functions of lysosomal cysteine cathepsins in the brain has been accumulating. As discussed above, cerebellar Purkinje cells are affected by the combined loss of cathepsins B and L. Interestingly, mutations of the cysteine protease inhibitor cystatin B (stefin B) result in apoptosis of cerebellar granule cells and cause progressive ataxia and a progressive myoclonus epilepsy (EPM1; Unverricht-Lundborg disease) in humans and in a mouse model (Lieuallen et al., 2001; Pennacchio et al., 1996; Pennacchio et al., 1998). Breeding of cathepsin B deficient mice to cystatin B knockout mice could ameliorate the neurological phenotype (Houseweart et al., 2003). Thus, an excess of cysteine cathepsin activity, i.e. CTSB activity, in the cerebellum contributes to the neurodegeneration of granule cells, while the loss of cysteine cathepsins results in

degeneration of the neighbouring Purkinje cells. Deletion of another cysteine cathepsin inhibitor, cystatin C, promotes neuronal injury after focal brain ischemia but is protective after global ischemia, indicating a fine balance of cysteine cathepsins and their inhibitors in pathological processes of the brain (Olsson et al., 2004). Furthermore, CTSB has been implicated in the generation of β -amyloid in the regulated secretory pathway of neuronal chromaffin cells and, therefore, in Alzheimers neurodegenerative disease (Hook et al., 2005). CTSL has been shown to be involved in the processing of hypophyseal neuropeptides, i.e. enkephalin, from their precursor molecule (Hook et al., 2004; Yasothornsrikul et al., 2003).

In summary, present evidence indicates cell type specific functions of cysteine cathepsins in various physiological and pathological processes of the central nervous system. The study of transgene expressed human cathepsins in absence of their murine orthologues provides a valuable tool for investigation of the *in vivo* functions of the human enzymes in the brain. Our present results correlate the expression of human CTSL to the prevention of Purkinje cell degeneration and to the rescue of the severe neuromuscular phenotype of mice with double deficiency for CTSB and CTSL.

Materials and Methods

Animals

Generation of Tg(hCTSL) mice has been described by Houseweart et al (Houseweart et al., 2003). Briefly, a human CTSL BAC clone (CTD-2062O4) was identified by sequence homology search, and BAC DNA was micro-injected into fertilized FVB blastocysts using standard procedures. Cathepsin B and cathepsin L deficient mice (Halangk et al., 2000, Roth et al., 2000) were backcrossed for at least 6 generations on FVB/n background. Transgenic Tg(hCTSL) mice were crossed with the congenic cathepsin L deficient mouse line to generate Tg(hCTSL);*Ctsl*^{-/-} mice. *Ctsb*^{-/-}*Ctsl*^{+/-} were crossed with Tg(hCTSL);*Ctsl*^{-/-} to obtain mice with the genotype Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-}.

RT-PCR and quantitative real time PCR

Total RNA from the human cell line HEK293 and murine tissues was prepared using the "RNeasy Mini kit" (Qiagen, Hilden, Germany). Reverse transcription for the generation of cDNA from total RNA was performed by using a first strand cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). For expression analysis of human cathepsin L and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PCR amplification was performed using equivalent amounts of the intercalating SYBR-green dye, cDNA/RNA, Taq-polymerase, and specific primers (hCTSL: 5'-gaatcctacactcatccttgctgcc-3' and 5'-acactgctctcctccactcttcttc-3'; GAPDH: 5'-tgcaccaccaactgctta-3' and 5'-gatgcagggatgatgttc-3') under the following conditions: 1 cycle for 1 minute at 72°C, 50 cycles (94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds) and 1 cycle at 72°C for 7 minutes in the MyiQ™ single-color real-time PCR detection system (BioRad, München, Germany). The resulting PCR products were visualized by ethidium bromide staining after separation on 1.5% (w/v) agarose gels.

Detection of human cathepsin L by Western blotting

Murine tissues (100 mg) were homogenized in 500 µl homogenization buffer (10 mM Tris, 128 mM NaCl, 1 mM EDTA, 1 mM Pefablock, 100 µM Pepsatin A, 10 µM Leupeptin, pH 7.5) using an Ultrathurrax followed by Dounce homogenization and centrifuged at 1000 g for 15 min. Postnuclear supernatants (75 µg protein) were applied to 15% SDS-PAGE under reducing conditions and subsequently blotted to a PVDF membrane. Goat anti-human CTSL antibody (R&D Systems, Wiesbaden, Germany) at a 1:500 dilution and mouse anti-mouse actin antibody (ICN, Biochemicals, Aurora, OH) at 1:5000 dilution were used. The binding of secondary antibody anti-goat IgG-POD (Sigma, Saint Louis, USA) and anti-mouse IgG-POD (Calbiochem) at 1:5000 dilution was detected by the SuperSignal™ Chemiluminescent Substrate (Pierce, Rockford, IL).

Detection of CTSL enzyme activity

Fresh tissues (100 mg) were homogenized in 500 µl NaAc-Buffer (200 mM Sodiumacetate, 1 mM EDTA, 0,05% Brij) using an Ultrathurrax followed by Dounce homogenization and centrifuged at 1000 g for 15 min. CTSL proteolytic activity was determined by degradation of the fluoropeptide z-Phe-Arg-4-methyl-coumarin-7-amide (20 µM; Bachem) in the presence of the CTSB-specific inhibitor CA074 (1.5 µM; Bachem) at pH 5.5. The release of 7-amino-4-methyl-coumarin was continuously monitored for 45 minutes by spectrofluorometry at excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Histology and Immunohistochemistry

For histological assessment sagittal brain sections of 5 µm thickness were deparaffinized in xylene, hydrated in graded ethanol solutions and stained with hematoxylin/eosin. Goat anti-mouse CTSL antibody (R&D Systems, Wiesbaden, Germany) at 1:500 dilution and goat anti-human CTSL antibody (R&D Systems,

Wiesbaden, Germany) at 1:50 dilution were used for the detection of murine and human CTSL, respectively. Peroxidase-based detection of the primary antibodies was performed according to the instructions of the "Vectastain Elite ABC Kit" (Vector Laboratories, Burlingame, CA). Microscopy was performed with an Axioplan microscope (Zeiss, Stuttgart, Germany) and digital images were obtained with an Axiocam camera (Zeiss, Stuttgart, Germany).

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Figure legends

Fig.1. Expression of human CTSL in the transgenic Tg(hCTSL) mouse line. (A) RT-PCR for detection of hCTSL and GAPDH mRNA in the human cell line HEK293 (lane 1-2) and liver of wildtype (lane 3-4) and Tg(hCTSL);*Ctsl*^{-/-} mice (lane 5-6)+RT: cDNA samples with reverse transcription; -RT: total RNA without reverse transcription; M: 100 bp ladder. (B) Quantitative real time PCR for quantification of transgenic human CTSL and GAPDH mRNA in liver, kidney, heart, brain and skeletal muscle of Tg(hCTSL);*Ctsl*^{-/-} mice (n=3). (C) Western blot for detection of transgenic human CTSL in liver of wildtype (*Ctsl*^{+/+}), cathepsin L deficient mice (*Ctsl*^{-/-}) and in liver, kidney, heart, brain and skeletal muscle of Tg(hCTSL);*Ctsl*^{-/-} mice. (D) Proteolytic activity of murine CTSL and transgenic human CTSL in liver, kidney, cerebral cortex and cerebellum of wildtype (*Ctsb*^{+/+}*Ctsl*^{+/+}) and Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} mice (n=3). Data in graphs are expressed as means ± s.e.m.

Fig.2. Postnatal development of CTSB/CTSL double deficient mice. (A) Littermates were photographed 16 days, 26 days and 43 days after birth. Control mice (*Ctsb*^{-/-}*Ctsl*^{+/+}; triangle), CTSB/CTSL double deficient mice (*Ctsb*^{-/-}*Ctsl*^{-/-}; star), and Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} (arrow). (B) Weight gain of Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} and *Ctsb*^{-/-}*Ctsl*^{-/-} in comparison to *Ctsb*^{-/-}*Ctsl*^{+/+} control mice. Body weight was measured from day 15 to day 48 after birth. *Ctsb*^{-/-}*Ctsl*^{-/-} were sacrificed on day 26 after birth at the latest. Data in graphs are expressed as means ± s.e.m.

Fig.3. Loss of Purkinje cells in CTSB/CTSL double deficient mice and complete rescue of the Purkinje cells in Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-}. H&E stained sagittal sections of the cerebellar cortex of wildtype (*Ctsb*^{+/+}*Ctsl*^{+/+}; A), CTSB single deficient mice (*Ctsb*^{-/-}*Ctsl*^{+/+}; B), CTSL single deficient mice (*Ctsb*^{+/+}*Ctsl*^{-/-}; C), CTSB/CTSL double

deficient mice (*Ctsb*^{-/-}*Ctsl*^{-/-}; D) and Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} mice (E). MC: molecular cell layer; PC: Purkinje cell layer; GC: granular cell layer.

Fig.4. Expression of CTSL in the mouse brain. Immunohistochemical detection of murine CTSL in the cerebral (A-C) and cerebellar cortex (D-E) of wildtype (*Ctsl*^{+/+}) (A-B; D) and CTSL single deficient mice (*Ctsl*^{-/-}) (C; E). CTSL is highly abundant in the pyramidal cell neurons of the inner and outer pyramidal cell layer of the cerebral cortex (A-B) and in the cerebellar Purkinje cell layer (D) OPC: outer pyramidal cell layer; IPC: inner pyramidal cell layer; MC: molecular cell layer; PC: Purkinje cell layer; GC: granular cell layer.

Fig.5. Recapitulation of the CTSL expression pattern by the transgene within the mouse brain. Immunohistochemical detection of human CTSL in the cerebral (A-D) and cerebellar cortex (E-H) of Tg(hCTSL);*Ctsb*^{-/-}*Ctsl*^{-/-} (Tg(hCTSL);dKO, A-B; E-F) and *Ctsb*^{-/-}*Ctsl*^{-/-} (C-D; G-H). The transgenic human CTSL is highly abundant in the the pyramidal cell neurons of the inner and outer pyramidal cell layer of the cerebral cortex (A-B) and in the cerebellar Purkinje cells (E-F). OPC: outer pyramidal cell layer; IPC: inner pyramidal cell layer; MC: molecular cell layer; PC: Purkinje cell layer; GC: granular cell layer.