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# 5-LIPOXYGENASE IN MOUSE CEREBELLAR Purkinje CELLS

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**Abstract—It has been suggested that the enzymatic pathway of 5-lipoxygenase (5-LOX) influences brain functioning and pathobiology. The mRNAs for both the enzyme 5-LOX and its activating protein FLAP have been found in the cerebellum. In this work, we investigated the cellular expression of 5-LOX in the adult mouse cerebellar cortex. We used the *in situ* mRNA hybridization assay, immunocytochemistry, laser capture microdissection, and our previously developed method for assaying the DNA methylation status of a putative mouse 5-LOX promoter. Since both 5-LOX mRNA *in situ* hybridization signal and FLAP immunoreactivity co-localize with calbindin 28 kD immunoreactivity (a Purkinje cell marker) but not with S-100β immunoreactivity (a Bergmann glia marker), the suggestion is that the 5-LOX pathway is expressed in cerebellar Purkinje cells. We found that methylation in the sites targeted by methylation-sensitive restriction endonucleases AciI and HinP1I but not BstUI and HpaII was greater in DNA samples obtained from a high-5-LOX-expressing cerebellar region (Purkinje cells) versus a low-5-LOX-expressing region (the molecular cell layer), suggesting a possible epigenetic contribution to the cell-specific 5-LOX expression in the cerebellum. We propose that Purkinje cell-localized 5-LOX and FLAP expression may be involved in the cerebellar synthesis of leukotrienes and/or could influence the Dicer-mediated microRNA formation and processes of neuroplasticity.** 

**Key words: 5-Lipoxygenase, cerebellum, Purkinje cell, Dicer, DNA methylation, FLAP.**

Several lines of research have established the presence of the 5-lipoxygenase (5-LOX; EC 1.13.11.34) pathway in the CNS (Chinnici et al., 2007; Chu and Praticô, 2009; Lindgren et al., 1984; Miyamoto et al., 1987; Ohtsuki et al., 1995). Information about the site and the cell types responsible for the synthesis of 5-LOX products, the inflammatory leukotrienes and the anti-inflammatory lipoxins (Rådmark et al., 2007; Serhan et al., 2008) in the CNS points to brain region differences in leukotriene formation (Chinnici et al., 2007; Miyamoto et al., 1987) and indicates the possibility of transcellular biosynthesis of cysteinyl leukotrienes in neuronal and glial cells (Farias et al., 2007).

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*Abbreviations:* CaBP-28, calbindin 28-kDa protein; DEPC, diethylpyrocarbonate; DIG, digoxigenin; FLAP, 5-lipoxygenase activating protein; LCM, laser capture microdissection; LTB4, leukotriene B4; M-MLV, Moloney murine leukemia virus; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SEM, standard error mean; siRNA, small interfering RNA; SSC, saline sodium citrate; TRPV1, transient receptor potential vanilloid type 1; 5-HPETEs, 5-(S)-hydroxy-eicosatetraenoic acids; 5-LOX, 5-lipoxygenase.

5-LOX expression is relatively high in neuronal precursor cells (Wada et al., 2006), including immature cerebellar granule neurons grown *in vitro* (Uz et al., 2001). In these *in vitro* models, 5-LOX appears to be involved in mechanisms of cell proliferation and differentiation and in neuroplasticity, such as hedgehog-dependent neurite projection (Bijlsma et al., 2008). In the adult brain, in addition to their strong hippocampal expression, the mRNAs for both 5-LOX and its activating protein FLAP (5-lipoxygenase activating protein) were found in the cerebellum (Lammers et al., 1996) but their cellular distribution has not been studied. Recently, the expression of cerebellar 5-LOX was confirmed in samples of mouse cerebellum (Chinnici et al., 2007) and in post-mortem human cerebellum (Zhang et al., 2006). In a human cerebellar cortex, 5-LOX immunoreactivity was predominantly found in the cytosol of the Purkinje cells (Zhang et al., 2006).

The expression of the *ALOX5* gene, which encodes 5-LOX is influenced by epigenetic mechanisms such as DNA methylation (Katryniok et al., 2010; Uhl et al., 2002, 2003; Vikman et al., 2009; Zhang et al., 2004). Most current knowledge about the role of DNA methylation in regulating 5-LOX expression has been obtained from studies of the human 5-LOX gene (on chromosome 10) and its promoter which possesses a unique GC (guanine-cytosine) -rich region and is a target of methylation modifications (Katryniok et al., 2010). In contrast to the human gene, the mouse 5-LOX gene (on chromosome 6) lacks the typical CpG islands found in the human sequence. Nevertheless, the methylation status of the non-island CpG loci appears to be equally relevant for gene regulation (Oakes et al., 2007). With respect to the mouse 5-LOX gene, this type of DNA methylation in cerebellar cells *in vitro* (Imbesi et al., 2009) and *in vivo* (Dzitoyeva et al., 2009) is influenced by developmental and aging-related mechanisms. The available data on cerebellar 5-LOX DNA methylation only report results from studies in brain homogenates and lack information regarding region or cell specificity.

In this work, we investigated the cellular expression of 5-LOX in mouse cerebellar cortex. Furthermore, using a method of laser capture microdissection and our previously developed method for assaying the DNA methylation status of mouse 5-LOX (Dzitoyeva et al., 2009), we characterized DNA methylation of 5-LOX-high- vs. 5-LOX-low-expressing cerebellar samples.

## EXPERIMENTAL PROCEDURES

### Animals

Male C57BL/6J mice (2 months old, weighing 25–30 g) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and

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were housed in a temperature controlled room on a 12 h light/dark cycle. They had free access to laboratory chow and water. The experimental protocol was approved by the Institutional Animal Care Committee.

### *In situ* hybridization and immunofluorescence

Mice were killed by a lethal anesthesia followed by transcardial perfusion with phosphate buffered saline (PBS) and fixation (4% formaldehyde). Cerebella were removed and postfixed overnight at 4 °C. After cryoprotection with 30% sucrose in PBS at 4 °C, the cerebella were cut into 30 $\mu$m coronal sections for subsequent *in situ* hybridization and immunofluorescence analyses. Briefly, free-floating sections were incubated for 30 min in 0.25% Triton X-100, digested with 5 $\mu$g/mL proteinase K (15 min at 37 °C) and the enzymatic reaction was terminated by addition of 0.75% glycine. After a brief fixation with 4% formaldehyde, sections were acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine for 15 min, pre-hybridized for 1 h at 55 °C in hybridization buffer [4× saline sodium citrate (SSC), 5 mM EDTA pH 8, 250 $\mu$g/mL yeast tRNA, 250 $\mu$g/mL salmon sperm DNA, 1× Denhardt's solution, 10% dextran sulfate, and 50% formamide], then incubated overnight at 55 °C with 1 $\mu$g/mL sense or antisense digoxigenin (DIG)-labeled RNA probes in hybridization buffer. After two washes at 55 °C in 4× SSC, 2× SSC, 0.2× SSC for 30 min, hybridized sections were blocked in blocking reagent (5% donkey serum with 0.25% Triton X-100) for 1 h. Subsequently the hybridized sections were incubated with sheep anti-DIG antibody (1:1000, Roche Applied Science, Indianapolis, IN, USA) overnight at 4 °C, then with fluorescein-conjugated donkey anti-sheep secondary antibody (1:100, Jackson Immunoresearch, West Grove, PA, USA) for 1 h. Sections were re-blocked again, incubated with mouse anti-calbindin 28-kDa protein (CaBP-28; 1:2000, Sigma), or mouse anti-S-100$\beta$ (1:100, Abcam, Cambridge, MA, USA) antibodies for 1 h at room temperature. Samples were probed with the appropriate rhodamine-conjugated secondary antibody for another 1 h. For FLAP immunostaining, we used rabbit primary antibody (1:100, Santa Cruz, Santa Cruz, CA, USA) to allow for double-labeling with CaBP-28 or S-100$\beta$ antibodies. Images of labeled sections were obtained with a computer-linked fluorescence microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a ×20 objective lens (Plan-Neofluar, NA=0.5, Carl Zeiss).

### Laser capture microdissection

Coronal sections (30 $\mu$m thick) were cut at −20 °C and mounted on PET-Membrane Slides (Leica, Bannockburn, IL, USA). Sections were thawed at room temperature for 30 s, washed briefly in diethylpyrocarbonate (DEPC)-treated water and stained with Hematoxylin for 1 min, 0.1% $NH_4OH$ Eosin solution for 30 s, and dehydrated in a series of ethanol baths (30 s in 75%, 95% and 100% ethanol). Immediately after dehydration, laser capture microdissection (LCM) was performed using a laser capture microscope (Leica). The Purkinje cell layers and molecular layers (approximately 2 $mm^2$ tissue per animal; around 2500 Purkinje cells total) were selectively captured into caps of 0.5 ml polymerase chain reaction (PCR) tubes containing 30 $\mu$l RNA extraction buffer (for RNA, 1% triton-X100, 0.8% 2-mercaptoethanol in guanidine isothiocyanate solution) or 30 $\mu$l sample loading buffer (for protein Western blot assay, 50 mM tris pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.2‰ Bromophenol Blue). Genomic DNA for the methylation assay was extracted using Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA).

### Quantitative 5-LOX mRNA assay

Total RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To eliminate possible DNA contamination, RNA samples were treated with a DNase reagent, DNA-free™ (Ambion, Inc., Austin, TX, USA). Total RNA was reverse-transcribed with 200 U of cloned Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco BRL, Carlsbad, CA, USA). The quantitative PCR was performed in a Stratagene Mx3005P QPCR System (Stratagene, La Jolla, CA, USA) using Maxima™ SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD, USA) in a three-step cycling protocol as described by the manufacturer. The PCR results were normalized against the corresponding cyclophilin contents. Data are presented in units calculated as a coefficient of variation $2^{-[\Delta Ct(target)-\Delta Ct(input)]}$ (Imbesi et al., 2009). Primers employed were 5-LOX: forward 5′-ATT GCC ATC CAG CTC AAC CAA ACC-3′, reverse 3′-TGG CGA TAC CAA ACA CCT CAG ACA-3′; cyclophilin: forward 5′-AGC ATA CAG GTC CTG GCA TCT TGT-3′, reverse 5′-AAA CGC TCC ATG GCT TCC ACA ATG-3′.

### 5-LOX DNA methylation assay

This assay was performed as previously described (Dzitoyeva et al., 2009). Briefly, multiple methylation-sensitive endonuclease recognition sites are located in the 300 nt region upstream of the ATG translation start codon of the mouse ALOX5 gene (a putative promoter region). Restriction digest-quantitative PCR was utilized to characterize the CpG methylation rate of that region with the aid of four methylation-sensitive endonucleases: AciI (C ↓ CGC), BstUI (CG ↓ CG), HinP1I (G ↓ CGC), and HpaII (C ↓ CGG). In this assay the methylation-sensitive endonucleases digest only unmethylated recognition sites and are inactive on sites with methylated cytosines. Genomic DNA was extracted from LCM samples by the Genomoic DNA Purification Kit (Gentra) according to manufacturer's instructions. The concentration of DNA was measured with a NanoDrop Spectrophotometer (Fishersci). The extracted genomic DNA was subjected to a restriction digest with the above-noted methylation-sensitive endonucleases in a separate reaction for each endonuclease. To measure methylation levels, the following primers were used for PCR: forward 5′-AGA GAA GGA TGC GTT GGA AGG T-3′, reverse 5′-GAC TCC GGG CAA GTG AGT GCT-3′. These primers amplify a 238 nt region upstream from the first ATG translation start codon. This region contains two recognition sites for each endonuclease used in the assay. For the input control, a 394 nt region in the first intron was chosen and amplified with the following primers: forward 5′-TGA TGT GGC TGG CCT CTT ATG TGA-3′, reverse 5′-ACT GGG ACT GAG TGC AGG AAA TGT-3′. This region does not contain recognition sites for the selected methylation-sensitive endonucleases. PCR reactions with two different primer sets (target and input) were run in separate tubes and the coefficient of variation (CV) for the relative amount of target sequence was calculated and is reported in units (Dzitoyeva et al., 2009).

### Quantitative western blotting

Before boiling for 10 min, the LCM samples were quickly frozen/thawed five times. The proteins were loaded on 7.5% Tris–HCl gels, transferred to nitrocellulose membranes (Amersham Piscataway, NJ, USA), and blocked by 5% non-fat milk in TBST buffer (Tris buffer saline Tween 20) for 1 h, then incubated overnight at 4 °C with mouse anti 5-LOX antibody (1:1000, BD Bioscience, San Jose, CA, USA). Thereafter, the membranes were incubated with horseradish-peroxidase-linked secondary antibody (1:1000 Amersham). To normalize the signals for 5-LOX proteins, the corresponding signals obtained with an anti-$\beta$-actin antibody (1:2000, Sigma) were measured on the same membranes. An ECL plus Kit (Amersham) was used for band visualization. The intensity of the bands was quantified using the NIH ImageJ system.