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# Inositol 1,4,5-triphosphate receptor is selectively expressed in cerebellum but not cerebellum-like structures of the elasmobranch fish, *Leucoraja erinacea*

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## Abstract

The Inositol 1,4,5-trisphosphate receptor type 1 protein (Ip3r1) performs an essential role for the induction of cerebellar long-term depression. Here, I describe the use of RT-PCR, qPCR, western blotting and immunohistochemistry to assay *Ip3r1* gene expression and localize Ip3r1 protein in the hindbrain of the elasmobranch fish, *Leucoraja erinacea*. Elasmobranchs are representatives of the most basal, yet extant lineage of gnathostomes, or jawed vertebrates. The cerebellum is a synapomorphy for gnathostomes and thus elasmobranch cerebellar physiology may serve as a proxy for the ancestral state of other jawed vertebrates. *Lelp3r1* is selectively expressed in the cerebellum of the little skate and the resultant protein is localized to Purkinje cells. If Ip3r1 performs the same functions in the skate cerebellum as in the mammalian cerebellum, then parallel fiber-Purkinje cell long-term depression through Ip3r1 mediated intracellular calcium regulation may be a conserved feature of cerebellar physiology. Cerebellum and surrounding hindbrain regions termed cerebellum-like structures

share a common developmental genetic toolkit. *LeIp3r1* expression was lowly detected in cerebellum-like structures indicating that although generatively homologous, the cerebellum and cerebellum-like structures do not share a complete overlap of common expression. Because of the little skate's important phylogenetic placement, performing molecular methodologies to assay targeted gene expression and determine protein localization in the hindbrain can be valuable for our understanding of cerebellar evolution and comparative neural development.

Keywords: Neuroscience, Cell biology, Anatomy, Evolution

## 1. Introduction

The cerebellar cortex is a well conserved trilaminar structure in the gnathostome hindbrain containing a molecular layer, Purkinje cell layer and granule cell layer. Although differences exist in cerebellar organization across gnathostomes, the circuitry that comprises the molecular layer remains largely similar. In the molecular layer, the parallel fiber-Purkinje cell synapse undergoes long-term depression resulting in a change of cerebellar cortex output to the deep cerebellar nuclei. The cerebellum's most defined role is in motor functioning, although it has also been implicated in sensory processing and cognition [1, 2]. The elasmobranch fish, *Leucoraja erinacea*, is a representative of the most basal extant lineage of vertebrates that possess a cerebellum. Thus, cerebellar physiology in the little skate may serve as a proxy for the ancestral state of other jawed vertebrates.

The little skate possesses two hindbrain nuclei that are similar to the cerebellum in terms of shared gene expression, anatomy and physiology, termed cerebellum-like structures. These cerebellum-like structures are first order sensory processing centers in the dorsal hindbrain, termed the dorsal (DON) and medial (MON) octavolateralis nuclei [3]. Due to a shared developmental genetic toolkit, the cerebellum and cerebellum-like structures are considered generatively homologous [4, 5, 6]. Based on this data and the phylogenetic presence of these structures, it has been hypothesized that cerebellum-like structures are the evolutionary antecedent and the cerebellum evolved from duplication of the cerebellar-like developmental genetic toolkit [3, 5, 6, 7]. Thus, it is expected that proteins necessary for cerebellar physiology would also be present in the cerebellum-like structures. Of particular importance for cerebellar functioning are proteins that are necessary for parallel fiber-Purkinje cell long-term depression.

*Ip3r1* codes for the Inositol 1,4,5-trisphosphate receptor type 1 (Ip3r1) protein and is expressed by Purkinje cells in the mammalian, frog, zebrafish and skate cerebellum [8, 9]. In mammals, activation of Purkinje cell metabotropic glutamate receptors

results in the production of inositol triphosphate (Ip3), which binds to Ip3r1, releasing calcium from the endoplasmic reticulum to the cytosol. This results in downstream signaling cascades and the induction of long-term depression at the parallel fiber-Purkinje cell synapse leading to a change in cerebellar cortex output to the deep cerebellar nuclei [10]. The results presented here confirms a previous report of Ip3r1 localization in the skate cerebellum [9] and further examines the shared genetic toolkit of cerebellum and cerebellum-like structures by testing for *L. erinacea* Ip3r1 (*Lelp3r1*) expression in cerebellum-like structures. Interestingly, very little *Lelp3r1* mRNA and protein was detected in cerebellum-like structures. The evolutionary implications of cerebellar-specific *Lelp3r1* expression and localization in the skate is discussed.

Chondrichthyes, or cartilaginous fish, are comprised of both elasmobranchs and holocephalans and are the most basal lineage of vertebrates that possess a cerebellum. Although the little skate occupies an important phylogenetic position for the study of vertebrate brain evolution, it is not a well characterized model organism. Thus, I developed custom primers for RT-PCR and qPCR and validated that a commercially available antibody was specifically binding to Ip3r1 in the skate. This was made possible through a publicly accessible online resource called SkateBase [11, 12, 13]. Here, I detail my use of this resource and describe methods for performing sequence analysis, RT-PCR, qPCR, western blotting and immunohistochemistry in the little skate to localize *Lelp3r1* expression throughout the cerebellum and cerebellum-like structures.

Following the custom, when referring to the gene *Ip3r1*, the name will be italicized and when referring to the protein Ip3r1, the name will not be italicized.

## 2. Materials and methods

### 2.1. Animals and dissection

Wild caught little skates (*Leucoraja erinacea*) were obtained from the Marine Biological Laboratory in Woods Hole, MA. This research was performed under guidelines established by NIH and approved by Wesleyan University IACUC. Animals were anesthetized in benzocaine, and a craniotomy was performed to visualize the desired brain regions. For RNA and protein analysis, desired brain regions (cerebellum and cerebellum-like structures) were excised from three animals using dedicated surgical tools to avoid contamination and dissections were performed in under 10 minutes to avoid degradation. RNA and western blot samples were flash-frozen after excision without fixative. For immunohistochemistry, animals (skate and mouse) were perfused and brains were post-fixed in 4% PFA overnight, cryoprotected in 20% sucrose, embedded in tissue freezing medium and sectioned by cryostat.

## 2.2. Gene expression assay

### 2.2.1. Finding the *Ip3r1* homolog for sequence analysis

Sequence analysis was done to determine if genes homologous to *Ip3r1* are present in the skate, determine immunogen sequence similarity for suitable antibodies and design custom primers. First, the *Ip3r1* amino acid sequence was obtained from a well characterized species (*Homo sapiens* IP3R1) [14]. The whole amino acid sequence was then entered into SkateBLAST [11, 12, 13, 15]. Translated Nucleotide Database and Little Skate Genomic Build2 or Transcriptomic Contigs Build2 were selected and results were viewed under “Raw BLAST output report”. Sequences similar to *H. sapiens* IP3R1 were identified by contig number (transcriptomic contig 16904) and retrieved through the Contig Lookup Tool. The nucleotide sequence from transcriptomic contig 16904 was translated through EMBOSS Transeq [16] and together with *H. sapiens* IP3R1, entered into Clustal Omega multiple sequence alignment [17, 18, 19, 20] to determine which domains were contained within the contig. The alignment was exported to Jalview for editing and adjustment [21, 22], then exported to Adobe Photoshop.

### 2.2.2. Primer design

First, I determined the desired region of *LeIp3r1* for primer production. I aimed to have the primer derived from the same region of the gene that codes for the *Ip3r1* antibody immunogen used in this study to ensure that I likely measured the same target in both mRNA and protein level analyses. The specific region of transcriptomic contig 16904 that translates to the antibody immunogen sequence was entered into the PrimerQuest tool at IDT and several primers were tested for specificity [23]. *LeIp3r1* primer sequences can be seen in Table 1.

### 2.2.3. RNA extraction

RNA was extracted from desired brain regions using the Promega SV RNA isolation kit (*Promega Catalog # Z3100*). Brain tissue was homogenized in 175µl SV RNA lysis buffer for 5 minutes on dry ice. 350µl SV RNA dilution buffer was added and vortexed for 1 minute. Samples were centrifuged for 10 minutes at 15,000rpm, supernatant was transferred to a tube containing 375µl 95% EtOH, inverted 10 times, then transferred to new spin column assembly. After standing for 1 minute, samples

**Table 1.** Primers used in *LeIp3r1* analysis.

| Primer         | Forward/Sense           | Reverse/Anti-sense    |
|----------------|-------------------------|-----------------------|
| <i>LeTuba</i>  | CATGGCTTGTGTCTGCTTTAC   | CTGGATGGTACGTTTGGTCTT |
| <i>LeIp3r1</i> | GAGTCTACGATGAAGCTGGTTAC | GAGGAGACCAATACGTTGCTT |

were centrifuged for 1.5 minutes. 600µl RNA wash buffer was added and centrifuged for 1 minute. Flow through was discarded. 5µl DNase was added to 40µl yellow core buffer and 5µl MnCl<sub>2</sub> and then applied to the membrane in the spin column for 15 minutes. 200µl SV DNase stop solution was added for 1 minute and spun for 1 minute. 600µl SV RNA wash solution was added and spun for 1 minute. 250µl SV RNA wash solution was added and spun for 2 minutes. The column was transferred to the elution tube and RNA was eluted with 100µl nuclease free water by standing for 5 minutes and then spun for 1.5 minutes. Aliquots were made and immediately frozen at -80 °C.

#### 2.2.4. cDNA synthesis

8µl sample RNA, 8.4µl 5x first-strand buffer (Fisher Scientific Catalog # 18064022) and 2µl DNase was added and incubated for 1 hour at 37 °C. The samples were then heated to 65 °C for 15 minutes. 5.4µl Oligo dT<sub>(16)</sub> primer was added and cooled to 37 °C over 20 minutes. 4.2µl dNTP mix (10mM), 1µl RNAsin (Fisher Scientific Catalog # PRN2511) and 1µl SuperScriptII-RT (Fisher Scientific Catalog # 18064022) was added and incubated at 37 °C for 1 hour. cDNA was stored at 4°. A control RT-PCR was run on a 1% agarose gel using reference gene primers to confirm that cDNA synthesis was successful.

#### 2.2.5. RT-PCR

RT-PCR was done to assay *Ip3r1* gene expression. A Master Mix of 2µl taq buffer, 0.1µl taq polymerase (Fisher Scientific Catalog # PRM3001), and 3.9µl UltraPure water (Thermo-Fisher Catalog # 10977-015) was made for each tube. 1µl cDNA or 1µl water was added to respective experimental or blank tubes and run for 35 cycles.

#### 2.2.6. qPCR

qPCR was used to quantify relative *LeIp3r1* expression levels between the cerebellum and cerebellum-like structures. A Master Mix of 5µl SYBR Green Master Mix (Life Technologies Catalog # A25742), 0.1µl taq polymerase and 1.9µl Ultra-Pure water was made for each sample. 1µl primer mix and 2µl cDNA was added to each well and run for 50 cycles in an Applied Biosystems QuantStudio3 system. Melt curves were done to establish that quality of the amplicons and data was exported to Excel for analysis.  $\Delta C_T$  values were created by subtracting the reference gene's  $C_T$  value (*LeTuba*) from *LeIp3r1*'s  $C_T$  value to control for differences in sample size.  $\Delta C_T$  values were then taken out of log by transforming by  $2^{(-\Delta C_T)}$ . A Shapiro-Wilk test for normality and Grubb's outlier test were performed. A one-tailed t-test was done to determine if the difference between *LeIp3r1* expression in the cerebellum and cerebellum-like structures was significantly different ( $p < 0.05$ ).

## 2.3. Protein analysis

### 2.3.1. Western blotting

Western blotting was done to determine if the antibody selectively binds to the target protein, and to determine if gene expression shown through RT-PCR is translated to protein. The SkateBLAST protocol mentioned above was used to select an antibody that has a similar immunogen sequence between the host species and the skate. Samples containing the cerebellum and cerebellum-like structures were homogenized, and protein concentration was determined by BCA analysis (20µg per lane). Samples were diluted in Sample Buffer and boiled for 5 minutes. SDS-Page electrophoresis was run at 100V for 1 hour. Proteins were transferred from the gel to the PVDF membrane at 350mA for 1 hour. All proteins were visualized by Ponceau Stain to determine transfer success. Vertical strips were cut containing all kDa for each lane and blocked in 5% BSA in TBST for 1 hour at room temperature. Primary antibody (*Novus Biologicals catalog # NBP1-21398*) was applied in 5% BSA blocking buffer for 18–24 hours at 4°. A no-primary negative control was done to determine if the band was a product of abnormal secondary antibody binding. Membranes were washed 3 times for 5 minutes in TBST and secondary antibody was applied in 5% BSA in TBST for 1.5 hours at room temperature. Membranes were washed 5 times for 5 minutes, then immersed in a Femto substrate for 15–30 seconds (*Thermo Scientific Catalog # 34096*). Images were taken in a Syngene Gel Box using Geneseq software.

### 2.3.2. Immunohistochemistry

Immunohistochemistry was performed to localize protein expression shown through western blots to specific cell types in the brain. Sections were blocked for 1 hour in 5% NRS blocking buffer or 5% NGS blocking buffer (*Vector Laboratories Vectastain Elite ABC Kit Catalog # PK-6101*) in PBST at room temperature. Sections were incubated in primary antibody for 12–15 hours at room temperature followed by biotinylated secondary antibody (*Vector Laboratories goatarrabbit Catalog # PK-6101*) in blocking buffer for 30 minutes at room temperature. Slides were incubated in ABC reagent for 30 minutes, then in a DAB peroxidase substrate (*Vector Laboratories ImmPACT DAB peroxidase (HRP) substrate Catalog # SK-4105*) for two minutes and dehydrated in an ethyl alcohol series and cleared in xylene. A no-primary negative control was done for each protein. A positive control was done on mouse cerebellar sections using the same protocols detailed above with a fluorescent secondary antibody (*Life Technologies Corporation Catalog # A10042*). All photos underwent the same modifications. Ip3r1 antibody information can be seen in [Table 2](#).

**Table 2.** Antibodies used for Ip3r1 analysis.

| Antibody                       | Immunogen             | Supplier                                                       |
|--------------------------------|-----------------------|----------------------------------------------------------------|
| Rabbitzhuman IP3R1             | Amino Acids 2708-2758 | Novus Biologicals catalog # NBP1-21398                         |
| Donkeyzrabbit 568              | Gamma immunoglobulin  | Life Technologies Corporation Catalog # A10042                 |
| Biotinylated goatzrabbit IgG   | Rabbit IgG            | Vector Laboratories Vectastain Elite ABC Kit Catalog # PK-6101 |
| HRP conjugated goatzrabbit IgG | Heavy and light chain | Cell Signaling Technology Catalog # 7074                       |

### 3. Results

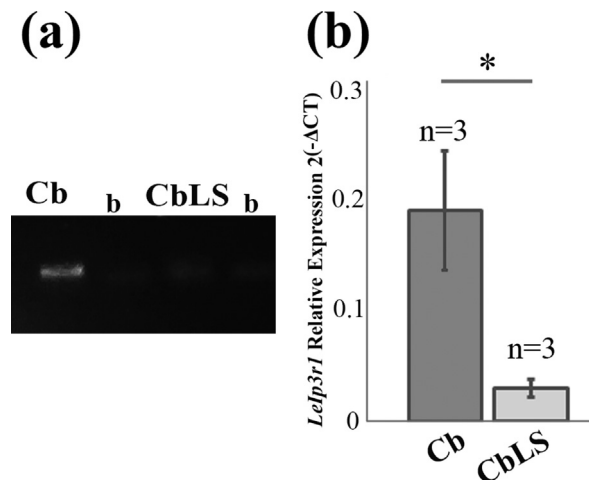
#### 3.1. Detecting and quantifying *LeIp3r1* mRNA in the cerebellum and cerebellum-like structures

I first determined if the little skate possesses an *IP3R1* homolog. This was done by searching the little skate transcriptome for sequences similar to *H. sapiens* IP3R1 using SkateBLAST via the protocol detailed above. Transcriptomic contig 16904 contained a region that is exclusively similar to human IP3R1 with a percent identity matrix score of 96. This sequence was not similar to IP3R2 or IP3R3, with percent identity matrix scores of 62.75 and 60, respectively.

The region of *LeIp3r1* that was used for primer design was taken from the same region of the gene that codes for the anti-IP3R1 antibody immunogen used in Figs. 2b and 3a–b. This sequence is GAGAAGCTGGAGTCTACGATGAAGCTGGTTAC-TAATTATCTGGCCAACTGTCAGAGCTCAAGGAGCAGATGACTGAGCAAAG-GAAACAAAAGCAACGTATTGGTCTCCTCGGGCACCCTCCGCACATGAACAT-TAACCCACAGCAGCCGGCC. The use of this partial sequence was done to ensure that I was measuring the same target in both RNA and protein levels of analysis. *Le-Tuba* primers were taken from Suriano and Bodznick 2018. The resulting primers can be seen in Table 1.

Once the IP3R1 skate homolog was found, I determined the spatial restriction of *LeIp3r1* expression by testing regions from the hindbrain (cerebellum and cerebellum-like structures) using RT-PCR. The cerebellum and cerebellum-like structures are considered generatively homologous, so they should share common gene expression [5]. Surprisingly, *LeIp3r1* is expressed in the cerebellum at much higher levels than in the cerebellum-like structures of the skate (Fig. 1a, Supplemental Fig. 1). Blank lanes, containing all reagents except cDNA from the desired region, show that the band is not the result of primer self-dimer or hetero-dimer formation.

*LeIp3r1* expression was then quantified using qPCR. Cerebellar *LeIp3r1* expression was significantly higher than cerebellar-like expression indicating that Ip3r1 may be



**Fig. 1.** *Lelp3r1* expression in the cerebellum. (a) RT-PCR showing presence of *Lelp3r1* mRNA in the cerebellum (Cb) but not cerebellum-like structures (CbLS). Blank lanes contained all reagents except cDNA. (b) qPCR showing *Lelp3r1* expression at higher levels in the Cb than in the CbLS. Data is transformed by  $2^{(-\Delta CT)}$  to show relative expression. Bars represent the mean with S.E.M. \* $p < 0.05$ .

selectively utilized by the cerebellum in the skate (Fig. 1b). *Lelp3r1*  $C_T$  values were rooted in *LeTuba*  $C_T$  values to control for sample size, then transformed by  $2^{(-\Delta CT)}$ . Once expression for *Lelp3r1* in the cerebellum was detected and quantified, it was necessary to verify that this mRNA is translated into protein.

### 3.2. Detecting Ip3r1 protein in the skate cerebellum and validating antibody usage

Brain tissue containing the cerebellum was tested for the presence of Ip3r1 protein by western blot. Blasting the antibody immunogen against the skate transcriptomic-build2.0 revealed that the immunogen was well conserved between skate and human. Fig. 2a shows an alignment of the antibody immunogen with skate Ip3r1 (top) and human IP3R1 (bottom).

The antibody used for western blotting and immunohistochemistry was rabbitαhuman IP3R1 using a concentration of 1:300. The western blot showed a single immunoreactive band of approximately the appropriate mass, 300kDa (Fig. 2b, Supplementary Fig. 2). A no-primary negative control shows the immunoreactive band was not a product of abnormal secondary antibody binding.

### 3.3. Localizing Ip3r1 protein to specific cell types within the skate cerebellum

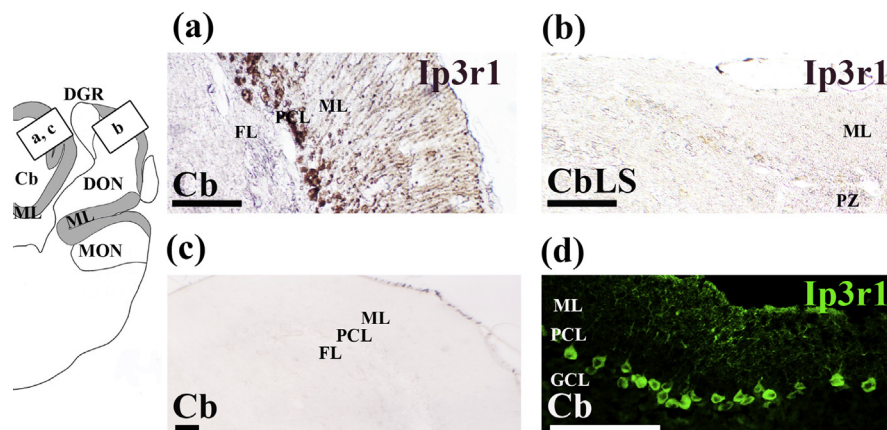
Once the presence of Ip3r1 protein was detected, I localized this protein to specific cell types within the cerebellum and tested for cerebellar-like Ip3r1 localization using immunohistochemistry. An ABC-DAB reaction showed Ip3r1 was localized to



**Fig. 2.** Ip3r1 protein is present in the cerebellum. (a) Conservation of the Ip3r1 immunogen. Partial sequence alignment of skate Ip3r1 (top) and human IP3R1 (bottom) showing high sequence similarity of the antibody immunogen. (b) Western blot shows a single Ip3r1 immunoreactive band of approximately 300kDa in the cerebellum with no-primary control.

Purkinje cell somas and dendrites as well as molecular layer interneurons of the cerebellum (Fig. 3a). This interneuron staining is different than results reported in Koulen et al. 2000, which did not observe Ip3r1 molecular layer interneuron immunoreactivity.

Little to no Ip3r1 immunoreactivity was found in the dorsal nucleus, a cerebellum-like structure (Fig. 3b), which corresponds with previously stated mRNA results (Fig. 1a–b). A no-primary negative control in the skate cerebellum shows that staining was not a result of improper secondary antibody binding (Fig. 3c). A positive



**Fig. 3.** Ip3r1 immunoreactivity in the cerebellum. Schematic (left) shows the location of images in a, b and c. (a) ABC-DAB reaction using Ip3r1 antibody from Fig. 2 shows cellular localization in Purkinje cell somas and dendrites as well as some molecular layer interneurons of the skate cerebellum (Cb). (b) ABC-DAB reaction showing little to no Ip3r1 immunoreactivity in the dorsal nucleus, a cerebellum-like structure (CbLS). (c) No-primary negative control in skate cerebellar section. (d) Positive control in mouse cerebellar section shows immunoreactive Purkinje cell somas and dendrites. Scale bars equal 100µm. Abbreviations: FL, fibrous layer; GCL, granule cell layer; PCL, Purkinje cell layer; PZ, Principal zone; ML, molecular layer.

control using fluorescent immunohistochemistry on mouse cerebellar sections shows selective Ip3r1 localization to Purkinje cell somas and dendrites (Fig. 3d).

## 4. Discussion

### 4.1. Ip3r1 antibody specificity in the little skate

*LeIp3r1* expression in the cerebellum is shown through both mRNA and protein levels of analysis using custom primers and an antibody that was specific to Ip3r1. A western blot with a single immunoreactive band of the appropriate mass, a well conserved antibody immunogen, immunostaining that is consistent with the existing literature and the presence of *LeIp3r1* mRNA in the cerebellum show that this antibody likely binds selectively to the target protein in the little skate. Custom primer design and determining antibody immunogen conservation was made possible through transcriptomic data that can be accessed through SkateBase [11, 12, 13].

### 4.2. *LeIp3r1* expression in the cerebellum and its evolutionary implications

The little skate *IP3R1* homolog, *LeIp3r1*, is expressed by Purkinje cells of the cerebellum, confirming a previous report by Koulen et al. 2000 [9]. If Ip3r1 performs the same functions in the skate cerebellum as in the mammalian cerebellum, then Ip3 mediated intracellular calcium signaling may be a conserved feature of Purkinje cell physiology, from its evolutionary inception throughout gnathostomes.

### 4.3. Generative homology of cerebellum and cerebellum-like structures with regards to *LeIp3r1* expression

Due to their complex evolutionary relationship, it was expected that proteins necessary for cerebellar physiology would also be present in the cerebellum-like structures. Although *LeIp3r1* mRNA and protein was not detected in the cerebellum-like structures of the skate at levels approximating cerebellar expression, Ip3 receptors are present in several cerebellum-like structures across vertebrate phylogeny. These include the electrosensory lateral line lobe and the optic tectum of the weakly electric fish, *Apteronotus leptorhynchus* [24], and the dorsal cochlear nucleus of several mammals [25]. Even though the cerebellum and cerebellum-like structures in the skate do not share a complete overlap of common expression, they should still be considered generatively homologous as their developmental toolkits, anatomy and physiology are more alike to each other than other nuclei.

It is possible that Ip3r1 functionality was not utilized by the ancestral cerebellum-like structures and cerebellar/cerebellar-like Ip3r1 functionality was introduced to

the shared genetic toolkit after the evolutionary genesis of the cerebellum. This newly expressed cerebellar protein may then be subsequently introduced to cerebellar-like structures as they independently arose throughout vertebrate phylogeny. It also remains possible that *LeIp3r1* is expressed in the cerebellum-like structures, but tightly developmentally regulated or expression was missed due to small sample size and heterogeneity of expression.

Elasmobranchs are representatives of the most basal, yet extant, lineage of gnathostomes, or jawed vertebrates. They are also representatives of the most basal lineage of vertebrates that possess a cerebellum. Due to their phylogenetic placement, performing molecular methodologies to assay targeted gene expression and localize proteins in the cerebellum and cerebellum-like structures can be valuable for our understanding of comparative cerebellar physiology and neural development.

## Declarations

### Author contribution statement

Christos Michael Suriano: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

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