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RESEARCH ARTICLE

Molecular Characterization and Gene Expression of Midkine (Mdk) In Red-Bellied Pacu (*Piaractus orinoquensis*); Escobar, 2019

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A B S T R A C T

Midkine (Mdk) is a heparin-binding growth factor in development, reproduction, and repair. It plays a pivotal role in the etiology of inflammatory and malignant diseases. Thus, Mdk secretion and underlying gene transcription may serve as a biomarker for disease detection and monitoring. In the present work, the midkine gene in *Piaractus orinoquensis* (red-bellied pacu) was characterized using different sequencing techniques and bioinformatic analysis. Gene expression was also evaluated using RT-PCR and qPCR under a brain injury model. As a result, the complete open reading frame of midkine for red-bellied pacu was obtained with the presence of two orthologs (*Mdka* and *Mdkb*). Phylogenetic analysis grouped midkine with its other orthologs in teleost fish. In the sequence analysis, both *P. orinoquensis* midkines share highly conserved regions with teleosts and tetrapods. Both *Mdka* and *Mdkb* were expressed across all brain segments. For the expression analysis after brain injury, differential expression was obtained in brain segments with acute and chronic responses, suggesting its significant role in inflammatory and regenerative processes in the central nervous system. Together, these results support the role of midkine as a potential biomarker and highlight the possibility of the use of *P. orinoquensis* for the study of a brain injury model.

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INTRODUCTION

Growth factors and cytokines play a key role in the regulation of cellular activities and several pathological processes. Midkine (Mdk) is a heparin-binding growth factor (HBGF) /cytokine involved in cell development, reproduction, and repair, with an important role in the etiology of inflammatory and malignant diseases (Muramatsu, 2014). The *Mdk* gene promotes inflammatory cell recruitment, chemokine expression, endothelial cell proliferation, smooth muscle cell migration and proliferation, and anti-apoptotic effects (Horiba *et al.,* 2006; Weckbach *et al.,* 2011). Circulating Mdk is elevated in inflammatory diseases, being a useful biomarker of chronic autoimmune inflammatory diseases (Neumaier *et al.,* 2023). Mdk and its inhibitors show promise for treating renal and heart failure (Kosugi and Sato, 2012). Mdk could also be a biomarker and therapeutic target in sepsis, cardiac diseases, and inflammation (Yazihan, 2013).

Animal models are used to discover gene/protein biomarkers, and piscine models have become a suitable vertebrate model for biomedical research and drug discovery (Winkler and Yao, 2014). In zebrafish, Mdk has been reported with a role in neural development at embryonic stages (Le *et al.,* 2025) as well as in the retina photoreceptor regeneration, being a model to study human photoreceptor dystrophies (Gramage *et al.,* 2015). Thus, the red-bellied pacu (*Piaractus orinoquensis*), an endemic freshwater fish species and the second most produced in Colombia (Roca-Lanao *et al.,* 2021), has served as a biological model candidate in various pharmacological, neurological, and immunotoxicological studies (Zapata-Guerra *et al.,* 2020; Petano-Duque *et al.,* 2022; Carrillo-Godoy and Rondón-Barragán, 2023; Rueda-García and Rondón-Barragán, 2023). To be utilized as a model for neuropathophysiological studies, it is necessary to understand the response to brain injury or neural tissue damage. This can be achieved by characterizing biomarkers of neurorepair or neurodegeneration, such as glial acidic fibrillar protein, OX-42, and midkine. Accordingly, the purpose of this study was to characterize the *Mdk* gene in the *P. orinoquensis* and to evaluate its

expression as a possible biomarker under a brain injury model, as a bio-model to study brain pathophysiology.

MATERIALS AND METHODS

Experimental animals: Red-bellied pacu (*Piaractus orinoquensis*) fingerlings from the same spawning were housed in glass aquaria with thermostats and constant aeration at <1g/L density and 25±1°C temperature. They were fed twice daily with a diet equal to 2% of their BW, acclimatized for 3 weeks, and treated with NaCl (1%) for parasite removal. Fish were used for basal tissue expression with clinically healthy individuals (n=5) and for a brain injury experiment up to 14 days after a stab wound. Fish were anesthetized by immersion in a glass tank with eugenol (50mg/L), then sacrificed by cervical dislocation. The tissue samples were rapidly snap frozen in liquid nitrogen for subsequent use.

Experimental procedures adhered to the University of Tolima's Local Bioethics Committee Guidelines, based on Law 84/1989, Resolution 8430/1993, and Law 576/2000. They complied with the Colombian Network of CICUALES regulations for research animals and followed international guidelines (Jenkins *et al.,* 2014).

Open reading frame (ORF) sequencing of the *Mdk*: *Mdk* ORF was obtained by nano sequencing of cDNA obtained from red-bellied pacu brain samples using a Direct cDNA sequencing kit and a flow cell (R9.4.1) in a MinION sequencing device (Oxford Nanopore Technologies, UK). Raw data was analyzed and basecalling was performed with Guppy software (Oxford Nanopore Technologies, UK). The *Mdka* gene was mapped to the *Mdka* reference sequences reported for *Pygocentrus nattereri* (XP_017565070.1), and the *Mdkb* gene was mapped to the *Mdkb* sequence reported for *Colossoma macropomum* (XP_036432346.1). Primers were designed (Table 1) using Geneious Prime v2024.0.2 (Biomatters Ltd, USA) for subsequent confirmation by Sanger sequencing.

Table 1: Sequences of primers for RT-PCR and qPCR of the *Mdk* gene in *Piaractus orinoquensis*.

Gene	Sequence (5´-3´)	Tm (°C)	Amplicon size (pb)
EF1a	F- ACTGAGGTCAAGTCTGTGGA	57.91	110
	R – CCACGACGGATGTCTTTAA	54.96	
Mdka	F - GAGATGCGTGGCTTGTTTTCCA	58.3	435
(ORF)	R - CTCCTCATGCCTTTGTTGGGTTTC	58.5	
Mdka	F- GCTAGTGGCCTTAATGATCGTC	55.4	150
(qPCR)	R- CCTTCCCTCACACCTGTTCC	57.7	
Mdkb	F- AGGAGTGTATTCTCAACTACGATTG	54.2	456
(ORF)	R-GCCAGCAACACTTAGTTTTCTTTCCCTTTC	60	
Mdkb	F- TACGATTGTGCTGCTGGTGGCTC	61.7	126
(qPCR)	R- CTGTTCGGCACACATTTCCCAAACTT	60.1	

Basal tissue expression of *Mdk*: The complete ORF of the *Mdka* and *Mdkb* genes was amplified from cDNA samples of red-bellied pacu fingerlings. RNA was extracted from homogenized tissue using by using F6/10 handheld homogenizer (Jingxin, China), followed by the addition of RNA-Solv® Reagent (Omega Bio-Tek, USA) and vortexing. Briefly, 200µL of chloroform (J.T.Baker®, USA) was added, followed by centrifugation at 12,000 rpm for 15 minutes at 4°C, and the aqueous phase was transferred to a clean tube. For the precipitation stage, two volumes of isopropanol were added to the recovered aqueous phase, centrifuged at 12,000 rpm for 10 minutes at 4°C to obtain a pellet, which was washed twice in ethanol (75%, Merck, Germany). After centrifuging at 12,000 rpm for 10 minutes at 4°C and discarding the supernatant, the pellet was dried at room temperature for 5 minutes and dissolved in 21µL of DEPC water. RNA quality was measured by spectrophotometry using the Nano500 (Allsheng, China). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, USA) following the manufacturer´s instructions.

Endpoint PCR was carried out in a 25µL volume of reaction with 1µL of cDNA as template. PCR conditions included 1 step of pre-denaturation (95°C) for 3 min, 1 step of 35 cycles of denaturation (95°C) for 30s, annealing (55°C) for 30s, and extension (72°C) for 1 min, and 1 step for final extension (72°C) for 7min. PCR amplification products were separated by agarose gel electrophoresis. Hydragreen™ (ACTGene, Piscataway, NJ) was used as a DNA dye and visualized with an ultraviolet transilluminator (Enduro™ GDS, Labnet International, USA). Amplicons were sequenced by Sanger sequencing (Macrogen Inc, South Korea).

Sequence analysis and construction of model structures: Bioinformatic analyses were done using Geneious Prime v2024.0.2 (Biomatters Ltd, USA), and sequences were submitted to GenBank (NCBI, USA). Multiple sequence alignments of the peptides were generated using Geneious Prime v2024.0.2 software (Biomatters Ltd, USA). Signal IP 6.0 (http://www.cbs.dtu.dk/services/SignalP/) was used for signal peptide detection, InterproScan, and the conserved domain tool of NCBI for domain prediction (Blum *et al.,* 2021). N- and O-glycosylation sites were detected using bioinformatic services of DTU Tech (https://services.healthtech.dtu.dk). The structural model of the red-bellied pacu Mdk was built in SWISS-MODEL (Waterhouse *et al.,* 2018) with Mdk sequence from *Hucho hucho* as a template; the model was modified in PyMOL 2.1 (DeLano Scientific, San Carlos, CA, USA), to improve its interpretation.

Sequence alignment and phylogenetic analysis: Based on confirmed sequences, multiple alignment was performed (MUSCLE algorithm) and the phylogenetic tree was built by the Neighbor-Joining method (1000 iterations bootstrap) in Geneious Prime v2024.0.2 software (Biomatters Ltd, USA) using the Mdk ortholog for *Drosophila melanogaster* Miple 1 as an outgroup and the amino acid (aa) sequences (Mdka, Mdkb) reported by GenBank as follows red-bellied pacu - *Piaractus orinoquensis* (QZS37423.1, WNH42905.1), tambaqui - *Colossoma macropomum* (XP_036439865.1, XP_036432346.1), Mexican tetra - *Astyanax mexicanus* (XP_007244983.2, XP_007235224.1), piranha - *Pygocentrus nattereri* (XP_017565070.1, XP_037398523.1), iridescent shark catfish - *Pangasionodon hypophthalmus* (XP_026802572.1, XP_026774923.1), channel catfish - *Ictalurus punctatus* (XP_017313961.1, XP_017340922.1), blue catfish - *Ictalurus furcatus* (XP_053473164.1, XP_053497098.1),

common carp - *Cyprinus carpio* (XP_018939704.1, XP_018962613.1), zebrafish - *Danio rerio* (NP_571145.1, NP_571791.1), stone loach - *Triplophysa dalaica* (XP_056605632.1, XP_056596654.1), Prussian carp - *Carassius gibelio* (XP_052417647.1, ABC67288.1), Rohu - *Labeo rohita* (XP_050970836.1, XP_050955207.1), tiger barb - *Puntigrus tetrazona* (XP_043101050.1, XP_043084604.1), Yangtze catfish - *Silurus meridionalis* (XP_046696606.1, XP_046714517.1), African catfish - *Clarias gariepinus* (XP_053348418.1, XP_053368757.1), western clawed frog - *Xenopus tropicalis* (NP_989074.1), Gaboon caecilian - *Geotrypetes seraphini* (XP_033783762.1), Schlegel's Japanese gecko - *Gekko japonicus* (XP_015263459.1*),* corn snake - *Pantherophis guttatus* (XP_034273350.1), chestnut-collared longspur - *Calcarius ornatus* (NXE62500.1), rooster - *Gallus gallus* (NP_001385102.1), mouse - *Mus musculus* (NP_001012335.1), human - *Homo sapiens* (NP_001012333.1) and common fruit fly -*Drosophila melanogaster* Miple 1 (NP_612022.1).

Expression of *Mdk* transcript in a model of brain injury: Fingerlings of *P. orinoquensis* were used in a brain injury model (Kishimoto *et al.,* 2012; Schmidt *et al.,* 2014). Fish in the control group (0 h, n=3) and brain injury group (n=3 per sampling time) were housed in glass tanks under identical conditions as previously described. Prior to the puncture procedure, all fish were anesthetized using eugenol. Brain injury was induced in the frontal region using a sterile 000-gauge entomological needle to a depth of 5 mm on the left lateral side of the skull, targeting the telencephalon (TE) and optic lobe. The first group of brain-injured fish was sacrificed 24 h post-injury, the second group after 7 days, and the third group after 14 days. Control group (0 h) brain samples were taken from fish without puncture. Brain samples from TE, optic chiasm (OC), optic bulb (OP), olfactory bulb (OB), cerebellum (CB), hypothalamus (HP), and medulla oblongata (MO) were taken from the left hemisphere. Extraction of RNA and synthesis of cDNA were performed as previously described.

RT-PCR and qPCR: RT-PCR was performed using qPCR primers designed based on the nucleotide sequence to amplify a fragment of the *Mdka* and *Mdkb* genes from cDNA from different tissues, and ipsilateral brain samples in the brain injury model. RT-PCR was performed as previously described.

For qPCR, melting curve analysis and gel electrophoresis were carried out to assess the presence of gDNA and primer specificity. QuantStudio™ 3 real-time thermal cycler (ThermoFisher Scientific, USA) was used for qPCR assays, using Luna Universal qPCR master mix (New England Biolabs, USA) and running in duplicate. Relative gene expression (fold change) was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and elongation factor 1 alpha (*EF1a*) was set as a normalization gene (Table 1).

Statistical analysis: Descriptive statistics were applied to the data, and statistical assumptions of multiple comparison tests were validated, including normality through the Shapiro-Wilk test. Differences in gene expression were assessed by Kruskal-Wallis's test, followed by Dunn's test as post hoc. Statistical analyses were performed with GraphPad Prism v 10.4.1 (532) for MacOS (La Jolla, CA, USA). In all cases, $P<0.05$ was considered statistically significant.

RESULTS

Tissue expression of *Mdk* gene in *Piaractus orinoquensis*: Full ORF of *Mdka* and *Mdkb* genes was obtained from *Piaractus orinoquensis* tissues. The expected size bands for *Mdka* were detected in the brain, liver, and gills. Regarding *Mdkb*, a band was observed in the brain sample (Fig. 1).

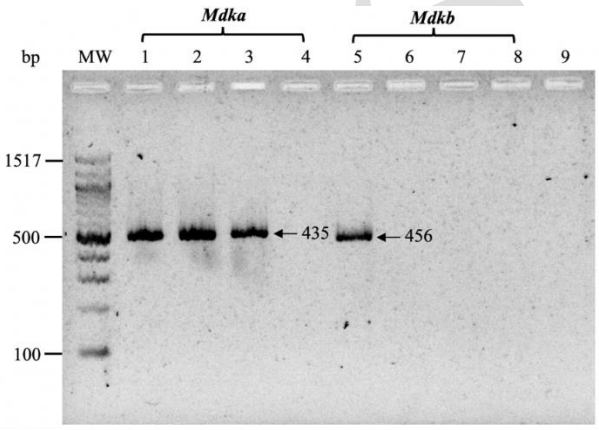

Fig. 1: Detection of the *Mdka* (435 bp) and *Mdkb* (456 bp) genes in red-bellied pacu tissues with RT-PCR. A representative sample (1 out of 5). bp: Base pairs; MW: Molecular weight. 1 and 5: brain, 2 and 6: gills, 3 and 7: liver, 4 and 8: blood, and 9: negative control. MW: 100 bp molecular weight marker. 2% agarose electrophoresis gel.

Mdk bioinformatic analysis: The ORF of *Mdka* from *P. orinoquensis* contains 435 nucleotides encoding 144 aa, with a molecular weight (MW) of 15.57023kDa, a theoretical isoelectric point of 7.65, a total number of negatively charged residues (Asp + Glu) of 11, and positive residues (Arg + Lys) of 29. The instability index was 6.78, and the GRAVY index was –0.537. Five O-glycosylation sites were predicted: Thr_{19}, Ser_{37}, Thr_{50}, Ser_{125}, and Ser_{132}. In the case of *Mdkb*, the full ORF contains 456 nucleotides encoding 146 aa with a MW of 15.99865 kDa, a theoretical isoelectric point of 9.64, a total number of negatively charged residues of 16, and positive residues of 34. The instability index was 26.40, and the GRAVY index was –0.823. Five O-glycosylation sites were predicted: Ile_{18}, Lys_{100}, Thr_{102}, Ser_{104} and Gln_{119}, Lys_{123}, Pro_{127}, Ile_{130}.

A consensus model of the protein was predicted using the *Hucho hucho* Mdk structure as a template, with an identity of 74.31% and an overall model quality estimate of 0.73. Mdk from *P. orinoquensis* (Mdka and Mdkb) presents two domains with three antiparallel β-sheets (Fig. 2a). Ten cysteines were found involved in disulfide bonds. In this way, six cysteines involved in the formation of three pairs of disulfide bonds in the N-terminal domain and four cysteines involved in two disulfide bonds are presented (Fig. 2c), and two heparin binding groups in the C-terminal domain (Fig. 2b).

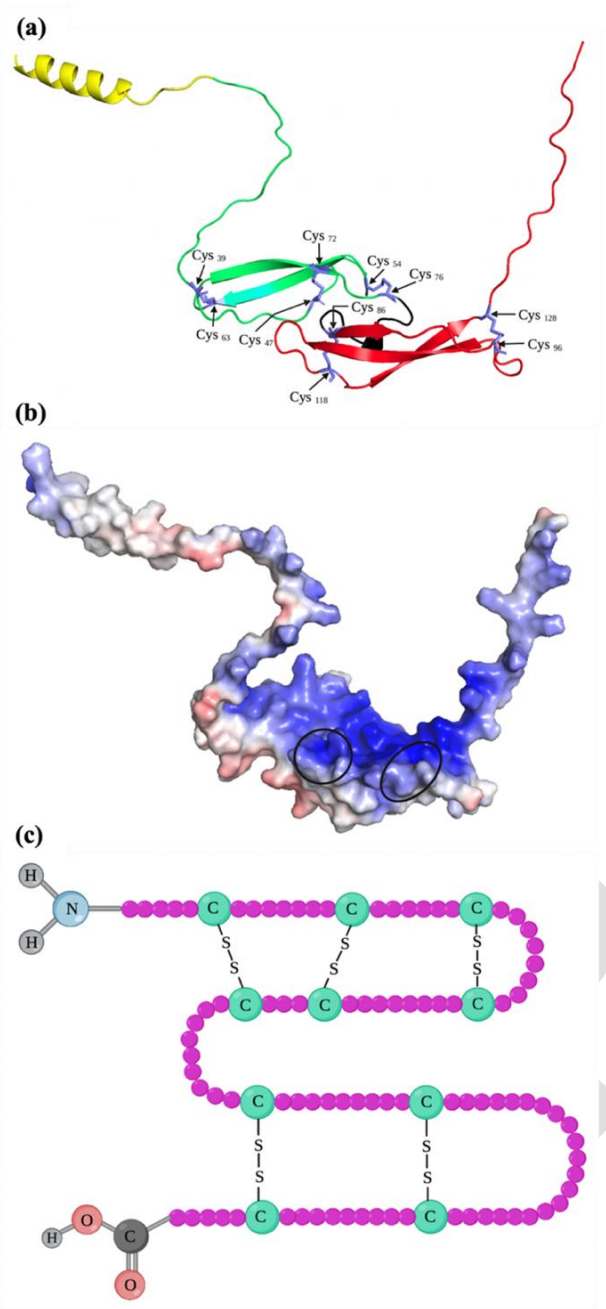

Fig. 2: Predicted model for *Piaractus orinoquensis* Mdk. (a) Ribbon diagram of Mdk from *P. orinoquensis*: signal peptide (yellow), N-terminal domain (Green), C-terminal domain (Red) with presence of b-helices; the hinge region (black) and cysteines (Cys) (purple). (b) Surface model of Mdk showing the distribution of positively charged (blue) and negatively charged (red) residues. Heparin binding sites are indicated in the C-terminal domain (circles). (c) Schematic model of Mdk showing the distribution of cysteines (green) in both domains and disulfide bond formation.

Sequence alignment and phylogenetic analysis: Multiple alignment homology analyses indicated that the Mdka and Mdkb proteins of *P. orinoquensis* are 59.46% identical; additionally, the aa sequence identity with human Mdk was 53.42% and 51.35%, respectively. Likewise, both Mdka and Mdkb from *P. orinoquensis* presented the highest percentage of identity with Mdka and Mdkb from *Colossoma macropomum* (93.05% and 97.95%, respectively). This secreted protein is rich in cysteine and basic aa and has the typical structure of the pleitrophin (PTN)/Mdk family including a highly conserved hinge region, conserved cysteine residues (n=10), two putative heparin-binding clusters: cluster I (K_{103} and R_{105}) and cluster II (K_{110}, K_{111} and L_{113}) and a highly conserved arginine residue (R_{105}) required for the protein tyrosine phosphatase ζ (PTPζ) receptor (Fig. 3). There are two glutamine residues (Q_{66} and Q_{119}) highly conserved among the different fish species.

As for the phylogenetic analysis of the Mdka and Mdkb sequences of *Piaractus orinoquensis*, two major clades are found: teleost fishes and tetrapods. The teleost fishes are divided into 3 orders: Characiformes, Siluriformes, and Cypriniformes, where *P. orinoquensis* is grouped with *Pygocentrus nattereri*, *Colossoma macropomum,* and *Astyanax mexicanus* in the Characiformes order and are in turn separated between Mdka and Mdkb. The other clade groups, the different classes of tetrapods, where species of amphibians, reptiles, birds, and mammals are found, with only one Mdk (Fig. 4).

Basal expression of *Mdka* and *Mdkb* mRNA from *P. orinoquensis*: Basal expression of *Mdk* genes was measured in brain regions of *P. orinoquensis* (Fig. 5c). Higher expressions were evident in the OP, CB, and MO for *Mdka* (Fig. 5a). Expression in the OP and MO was higher compared to TE and OC, and in CB it was higher compared to HP and TE. In the case of *Mdkb* (Fig. 5b), higher expressions were observed in the OB, TE, and CB. Expression in the OB and CB was higher compared to HP and OC, and in the TE it was higher compared to OC.

Relative mRNA expression of *Mdka* and *Mdkb* from *P. orinoquensis* under a brain injury model: In the brain injury assay, differential *Mdka* expression was detected in all brain segments. In OB, there was an *Mdka* upregulation at 14 days compared to 7 days ($P<0.001$) (Fig. 6a). In the TE, upregulation was evident at 24 h ($P<0.05$) vs. 0 h. In the CB, downregulation was detected at 7 and 14 days ($P<0.05$) compared to 0 h. Finally, in the OC, upregulation occurred at 24 h ($P<0.01$) and downregulation at 7 days ($P<0.01$) compared to 24 h (Fig. 6a).

Mdkb expression showed significant differences in OB and TE. In OB, *Mdkb* mRNA transcript levels were higher at 7 ($P<0.05$) and 14 days ($P<0.001$) compared to 0h (Fig. 6b). In the TE, a downregulation of *Mdkb* was evident at 7 ($P<0.05$) and 14 days ($P<0.001$) post injury; in addition, *Mdkb* showed a significant difference between 24 h and 14 days ($P<0.05$) (Fig. 6b).

DISCUSSION

In mammals, the single *Mdk* gene encodes a secreted heparin-binding growth factor with neurotrophic activity (Winkler *et al.,* 2003), with high expression in neural precursor cells, promoting neurite outgrowth, cell survival, and differentiation (Togo *et al.,* 2014). In addition, this cytokine is involved in the regulation of tissue repair, growth promotion, cell migration, and embryogenesis (Muramatsu, 2014). Midkine reduces fibril assembly and plaque formation in Alzheimer's disease (Peng *et al.,* 2024) and enhances neurorepair after traumatic brain injury (Takada *et al.,* 2020), indicating its potential for neurological disease therapy. In birds, Mdk

mediates glial activity, survival, and neuronal reprogramming of Müller glia (Campbell *et al.,* 2021). Moreover, in reptiles is considered a healing marker gene involved in blastema formation and chondrogenesis in lizards after tail amputation (Vonk *et al.,* 2023). In amphibians, Mdk participates in limb regeneration in salamanders (Tsai *et al.,* 2020) and in anurans has been related to skin physiology, including immunity and respiration (Huang *et al.,* 2016).

In teleost fish, Mdk from *Danio rerio*, *Carassius auratus gibelio*, *Oncorhynchus mykiss* (Yin *et al.,* 2007), *Cynoglossus semilaevis* (Zhang *et al.,* 2018), and *Megalobrama amblycephala* (Guo *et al.,* 2018) have been reported. *Mdka* and *Mdkb*, have been characterized in zebrafish. *Mdka* helps form the medial floor plate (Schäfer *et al.,* 2005), and *Mdkb* is involved in posterior neural development (Winkler *et al.,* 2003).

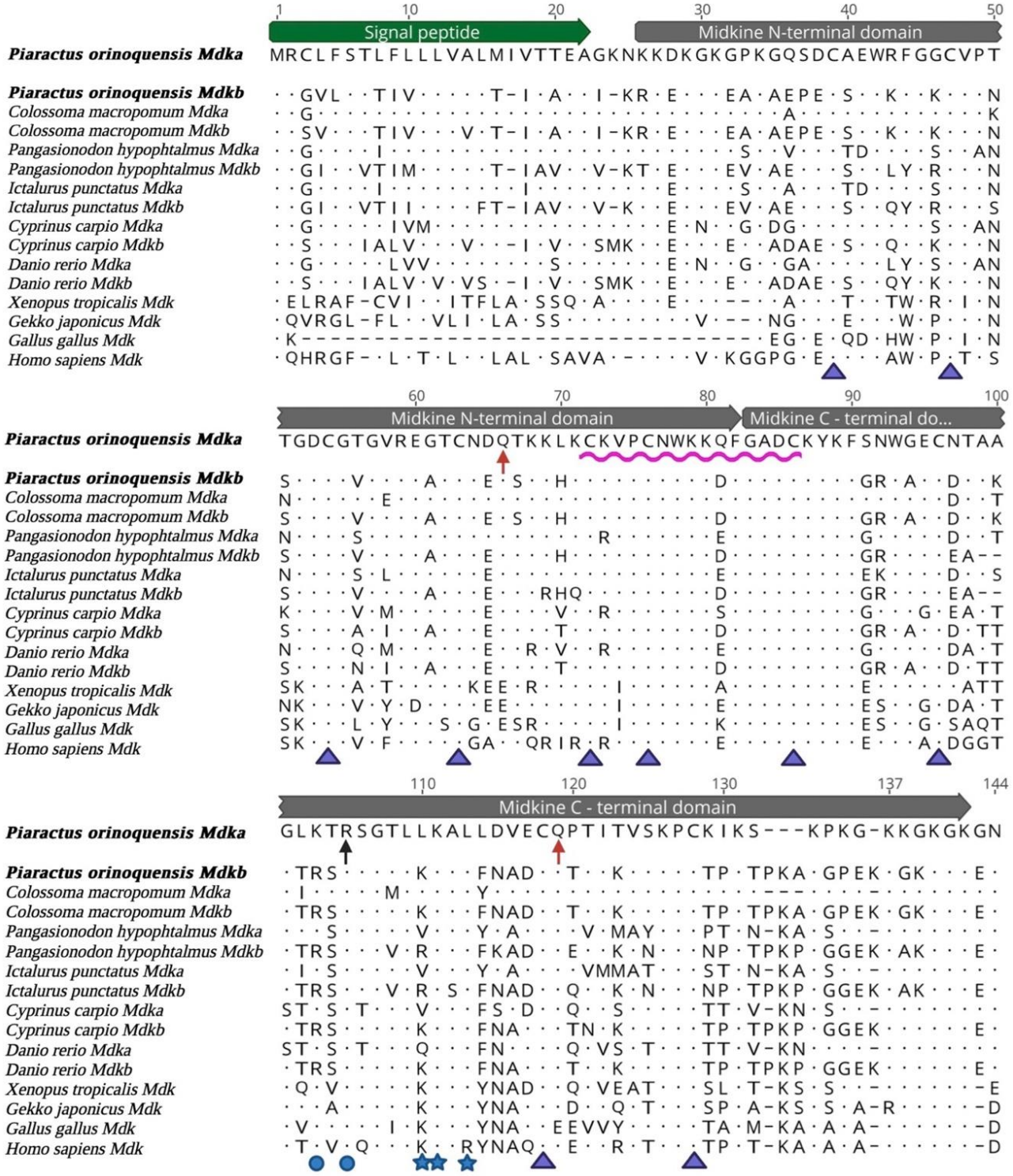

Fig. 3: Multiple amino acid alignment of Mdka and Mdkb from *P. orinoquensis* with teleost and tetrapods. Mdka from *P. orinoquensis* was used as the reference sequence. Amino acids are designated by single letter codes. The hinge region is indicated by a pink coil. Conserved cysteines are indicated by purple triangles. Heparin-binding clusters are indicated by blue circles (cluster I) and blue stars (cluster II). Arginine residue (black arrow) and glutamine residues (red arrows).

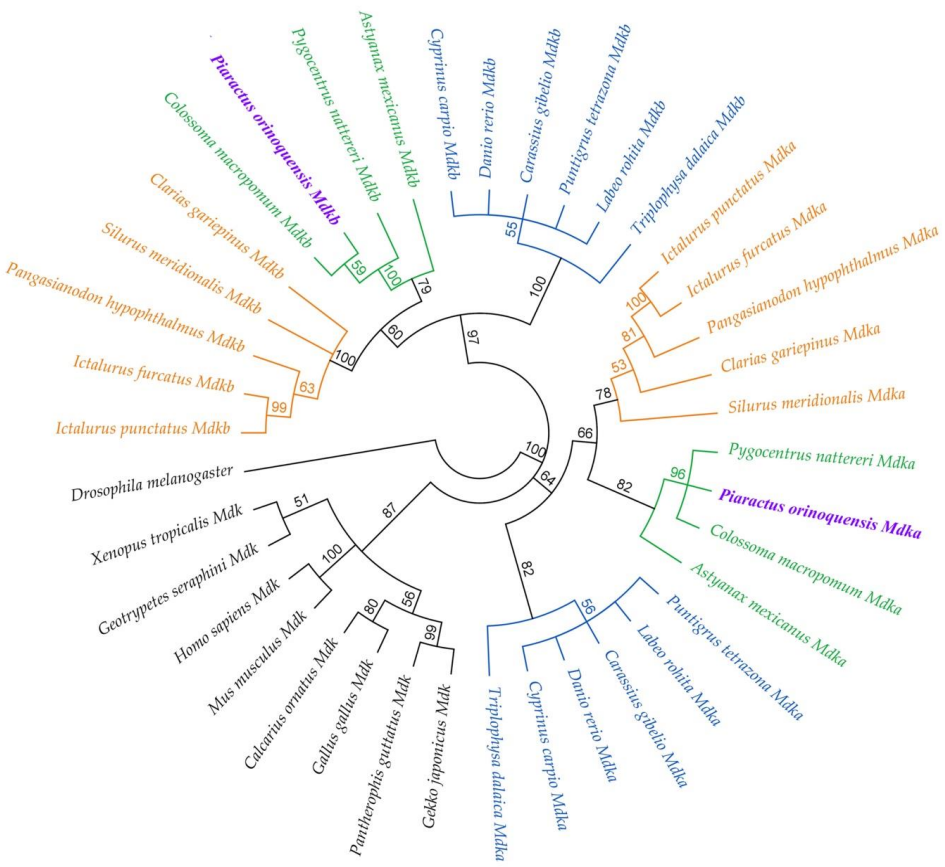

Fig. 4: Phylogenetic analysis of the Mdka and Mdkb of *P. orinoquensis*. Phylogenetic tree shows two major clades, one for teleost fishes and one for tetrapods (black). The fish group is separated into three orders: Characiformes (green), Siluriformes (orange), and Cypriniformes (blue), as well as Mdka and Mdkb. *P. orinoquensis* Mdk OTU is shown in purple. For the tetrapod clade, only one Mdk is presented. The phylogenetic tree was built using the Neighbor-Joining method with bootstrap values from 1000 iterations.

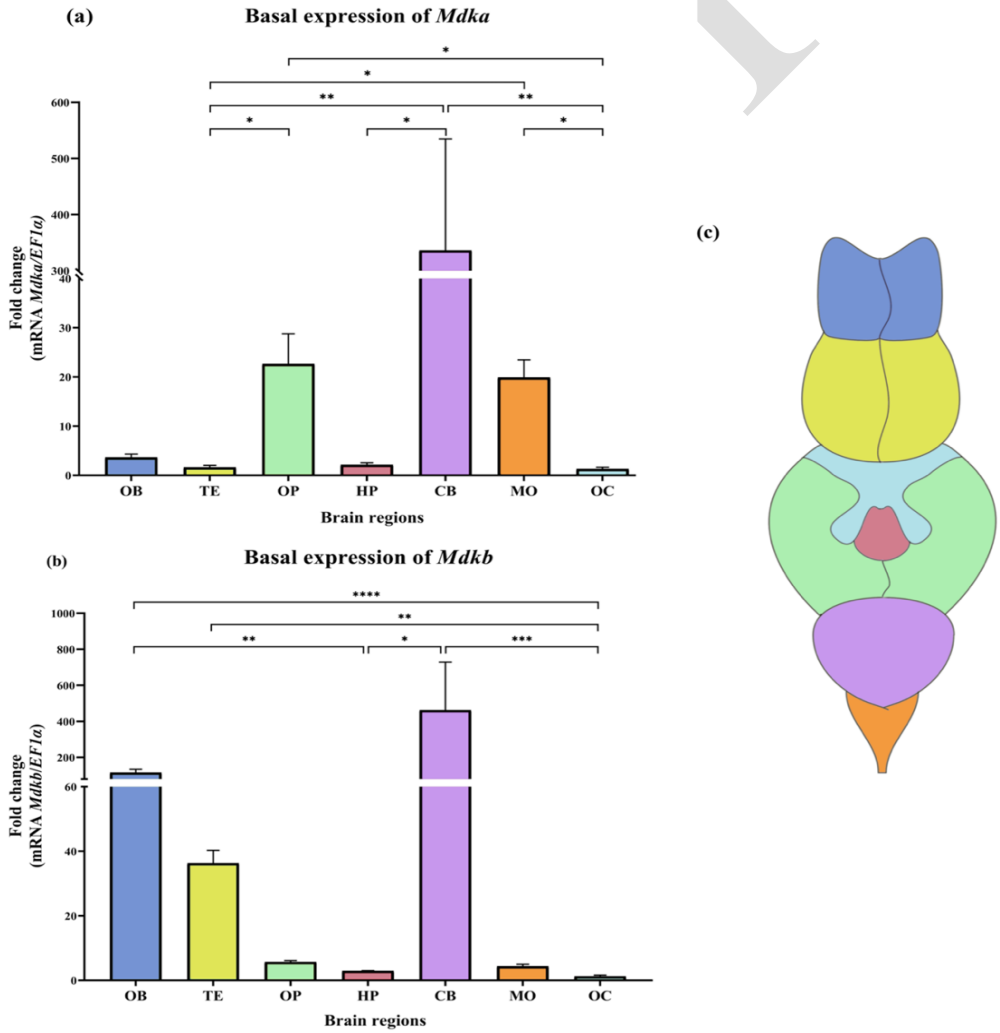

Fig. 5: Relative basal expression of *Mdka* and *Mdkb* in different brain segments. (a-b) OB: olfactory bulb; OC: optic chiasm; HP: hypothalamus, TE: telencephalon; OP: optic bulb; CB: cerebellum; MO: medulla oblongata. *EF1α* was used as a reference gene. Data (n=5) are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (c) Schematic of the anatomical regions of the brain (ventrodorsal view) of *P. orinoquensis* where *Mdka* and *Mdkb* are expressed.

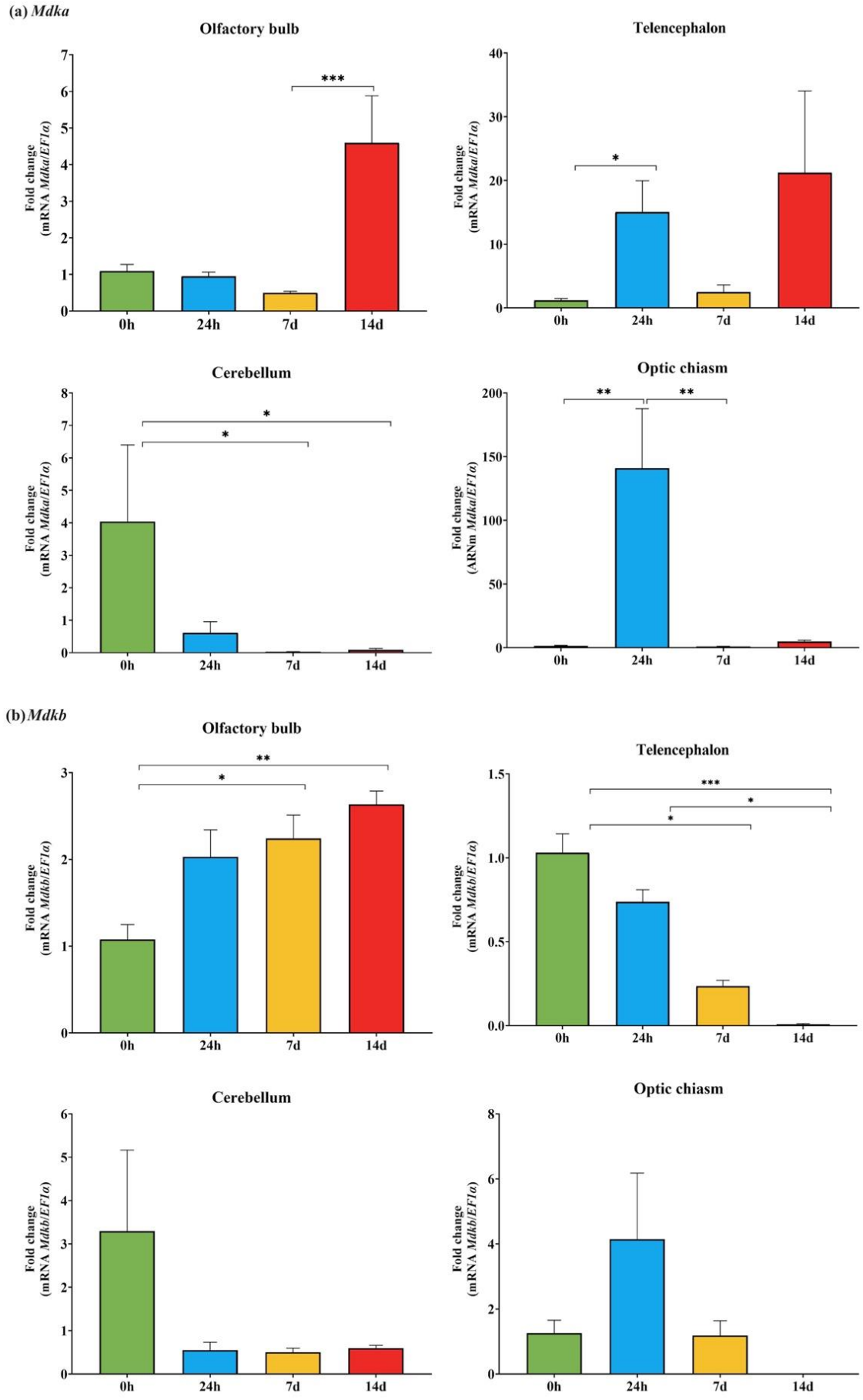

Fig. 6: Relative mRNA expression of *Mdka* and *Mdkb* genes in different fish brain segments under a brain injury model relative to time. (a) *Mdka*, (b) *Mdkb*. *EF1α* was used as a reference gene. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Like other vertebrates, in *P. orinoquensis* the mature Mdk protein consists of two domains with three antiparallel β-sheets (Fig. 2a), which are more stable compared to parallel β-sheets (Balamurugan and Subramanian, 2022). The C-domain contains two disulfide bridges, and the N-domain contains three disulfide bridges, which are important post-translational modifications essential for proper folding and full activity of the protein (Manta *et al.,* 2022), participating in enzymatic catalysis and facilitating their translocation across membranes (Weiss *et al.,* 2022).

The C-terminal domain is involved in synaptic development, contains the heparin-binding site necessary for plasminogen activity, and shows high antibacterial activity (Svensson *et al.,* 2010; Weckbach *et al.,* 2011). The two glutamine residues are important for dimerization (Guo *et al.,* 2018), which occurs by spontaneous association and by crosslinking with transglutaminase, being promoted by heparin. Likewise, the N-terminal half is also necessary for dimer formation (Muramatsu, 2011). Similarly, two basic residue clusters are found in the C-terminal domain: heparin-binding regions (cluster I and II), which were evidenced in the predicted model and in the multiple alignment of the predicted Mdka and Mdkb of *P. orinoquensis* with other fish and higher vertebrates, showing that Mdk is highly conserved and functional in vertebrates.

As previously described (Winkler *et al.,* 2003), there are ten highly conserved cysteine residues and a hinge region separating the amino- and carboxy-terminal domains. At the C-terminal domain, a highly conserved arginine residue was found, which is required for the PTPζ receptor; involved in neuronal migration (Maeda *et al.,* 1999). In addition, five O-glycosylation sites were found for Mdka and Mdkb, which play a role in cell trafficking, cell differentiation and cell-to-cell interactions (Apweiler *et al.,* 1999). O-GlcNAc-type glycosylations (O-GlucosylNAcetylation) are the most common in eukaryotes as the O-glycosylations found in the sequence of both midkines in *P. orinoquensis*.

Two Mdk paralogs of *P. orinoquensis* were obtained: Mdka and Mdkb. Multiple alignment revealed high identity between Mdk sequences from teleost fish. The phylogenetic tree shows the divergence of Mdk between teleost fishes and tetrapods, where the former is separated by orders, where Mdk from *P. orinoquensis* shows a close relationship with Mdk from *Colossoma macropomum* and *Pygocentrus nattereri*. Likewise, Mdka and Mdkb are grouped in different clades. The presence of two midkines in teleost fish may be due to a large duplication of fish-specific chromosomal blocks, due to a whole genome duplication (Chiang *et al.,* 2001), and both genes encode proteins that are related to *Mdk* orthologs reported in tetrapods (Winkler *et al.,* 2003).

After teleosts diverged from tetrapods around 300 million years ago, a fish-specific genome duplication occurred (Winkler *et al.,* 2003). This event is believed to drive morphological variation and functional innovation in fish, contributing to their diversity. Typically, one duplicate gene retains its original function while the other accumulates mutations and degenerates over time (Guo *et al.,* 2018).

Mdk is highly expressed in embryonic tissues, particularly within the nervous system. In adult tissues of both mice and humans, however, *Mdk* expression diminishes to very low levels, except for the kidney, where high expression continues into adulthood (Kadomatsu *et al.,* 1990). Additionally, *Mdk* can serve as a biomarker in certain tumors (Yildirim *et al.,* 2024).

In red-bellied pacu, *Mdka* and *Mdkb* were detected in the brain of fingerlings, and only *Mdka* was detected in gills and liver, similar to the report in *Megalobrama amblycephala*, nevertheless, contrary to our results, *Mdkb* was also expressed in these tissues (Guo *et al.,* 2018). In blood tissue, none of the midkines were detected. In case of the brain, our results agree with those reported for zebrafish where both *Mdka* and *Mdkb* were expressed in the adult brain, in a highly restricted pattern and without overlap (Winkler *et al.,* 2003), however, in red-bellied pacu an overlap in the expression of both midkines was observed showing the highest expression pattern in CB.

Furthermore, *Mdka* is expressed mostly in OP and MO in addition to CB while *Mdkb* in addition to CB, is highly expressed in OB and TE, suggesting that the expression of *Mdk* genes in *P. orinoquensis* is not restricted to embryogenesis and that they might be involved in processes that are different in teleost brains with respect to higher vertebrates. Furthermore, based on differences in expression patterns, both genes could play independent or complementary roles.

The brain injury model used in our study corresponds to a telencephalic lesion, at the telencephalic ventricular zone, which showed a physiological response similar to the subventricular zone in mammals, mainly in humans, where the neurogenesis can occur and contribute to brain remodeling following these injuries (Brockman *et al.,* 2021; Magrinelli *et al.*, 2022; Li *et al.*, 2023). In this model, *Mdk* expression showed an upregulation of *Mdka* at 24h post-injury in the TE and OC and a time-dependent downregulation of Mdkb, specifically in the TE. This may occur due to the role of Mdk as a proinflammatory cytokine, promoting immune cell migration, mainly in the early stages of tissue injury (Takada *et al.,* 2020). In addition, *Mdka* showed upregulation 14 days after injury in OB, and a trend to higher values of transcription in TE, which may correspond to a remote response related with chronic inflammatory response or repairing process, since Mdk have been related with polarization of M1 cells to M2 phenotype, promoting repair and regeneration processes (Takada *et al.,* 2020).

Likewise, in the OB, *Mdkb* showed an upregulation at 7 and 14 days post injury, which may be due to the fact that sensory neurons present in this segment are continuously renewed throughout of fish life (Calvo-Ochoa and Byrd-Jacobs, 2019; Var and Byrd-Jacobs, 2019) and are considered the stem cells of the TE in fish (Calvo-Ochoa and Byrd-Jacobs, 2019; Rheinsmith *et al.,* 2023), which can be an adaptive response to cell repopulation of TE.

Teleosts, like *P. orinoquensis*, maintain neurogenesis in adult stages because they grow continuously and required an abundant neurogenesis (Lübke *et al.,* 2022), it could be presumed that *Mdka* and *Mdkb* play complementary roles depending on the brain segment that was stimulated by the lesion; in the particular case of the OB that has direct interaction with the environment and is

related to obtaining food, defense from predators and reproduction (Hamdani and Døving, 2007) it requires constant regeneration as does the TE and its importance with radial glia cells.

This would indicate that the *Mdka* response to injury is not instantaneous in the TE and OB. Therefore, it is likely that this gene is not involved in responses that occur immediately after injury, such as the inflammatory response in these two segments (Kyritsis *et al.,* 2012). Rather, Mdka appears to be involved in mechanisms activated with a delay. This is an important mechanism to prevent the depletion of the brain stem cell pool in the TE (Lübke *et al.,* 2022). Considering the results, both *Mdka* and *Mdkb* show differential expression in the brain regions, further studies are required to describe the role of midkines in fish brain pathophysiology.

Previous studies in our lab with *P. orinoquensis* have shown its suitability to study gene expression in pharmacological and toxicological experiments (Zapata-Guerra *et al.,* 2020), as well as expression of transcript of immune-related genes such as hepcidin-1 (Petano-Duque *et al.,* 2022), high-mobility group box-1 (Carrillo-Godoy and Rondón-Barragán, 2023), neurogranin (Rueda-García and Rondón-Barragán, 2023), glial acidic fibrillar protein (Holguín-Cespedes *et al.,* 2022), myelin basic protein (Cruz-Mendez *et al.,* 2022a), as well antioxidants (Cruz-Mendez *et al.,* 2022b; Ortiz-Muñoz *et al.,* 2023) in models of neurological damage. Since *P. orinoquensis* is a Colombian native freshwater fish, it is easy to find in natural sources, and its biology is well known for reproduction and handling. Moreover, we proposed its use as a bioindicator of environmental contamination, mainly for organophosphates and carbamates (Holguín-Cespedes *et al.,* 2022).

Conclusions: The ORF of the *Mdk* gene was characterized, finding two paralogs in *P. orinoquensis*: *Mdka* and *Mdkb*, which share highly conserved regions with the other orthologs of teleost fishes and higher vertebrates. *Mdka* and *Mdkb* transcripts showed differential expression in brain regions and under a brain injury in a time-dependent manner, which may be considered for their use as a possible biomarker/therapeutic target for neuroinflammatory response in this biomodel.

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