**Title**

Expression of ARPP21 Protein in Zebra Finch Striatal Song Control Nucleus Area X



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# Expression of ARPP21 Protein in Zebra Finch Striatal Song Control Nucleus Area X

A thesis submitted in partial
satisfaction of the requirements
for the degree Master of Science
in Physiological Science

by

Yaochi Li

2024

ABSTRACT OF THE THESIS

Expression of ARPP21 Protein in

Zebra Finch Striatal Song Control Nucleus Area X

by

Yaochi Li

Master of Science in Physiological Science

University of California, Los Angeles, 2024

Professor Stephanie Ann White, Chair

Autism spectrum disorder (ASD) is characterized by impairment in social communication along with additional endophenotypes. Songbirds offer a relevant animal model for investigating communication deficits because like humans, but unlike most mammalian research models, a substantial portion of their communication signals are learned using similar cortical-striatal circuitry. Cyclic-AMP-Regulated Phosphoprotein 21kDa (ARPP21) is the host gene of miRNA-128 that has been associated with ASD. Computational models suggest that the collaboration between ARPP21 and Protein Phosphatase 1 Regulatory Inhibitor Subunit 1B (PPP1R1B, or DARPP32) can enhance cortical-striatal synaptic strength through the activation of CaMKII. Previous work has demonstrated enhanced song learning through inhibiting miRNA-128 within Area X, the song-dedicated region of the songbird striatum. Follow-up work discovered a downregulation of a striatal gene co-expression module following two hours of morning singing in a cluster of ARPP21-expressing medium spiny neurons. Together, these findings suggest a role for the miR-128 host gene, ARPP21, in learned vocalizations. Yet, the potential link

between ARPP21 expression levels and vocal learning in songbirds and humans remains unexplored. The present investigation aimed to determine whether ARPP21 and DARPP32 proteins are co-expressed in a subset of medium spiny neurons in the striatal Area X of adult male zebra finches. The results provide biological support for the computational model mentioned above. Additionally, they may reveal whether the co-expression of ARPP21 and DARPP32 is associated with vocal learning in both songbirds and humans.

The thesis of Yaochi Li is approved.

Patricia E. Phelps

Barnett Schlinger

Stephanie Ann White, Committee Chair

University of California, Los Angeles

2024

DEDICATION

I dedicate this thesis to my family for their constant support and encouragement.

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## BACKGROUND

Autism spectrum disorder (ASD) is a term used to characterize individuals who exhibit a unique combination of challenges in social communication, along with repetitive behaviors, and intensely focused interests from an early age [6]. A recent report indicates the disease affects approximately 2% of children in the United States, representing a 200% increase since 2000 [7]. This surge highlights ASD as one of the most prevalent developmental disorders among children [7]. Understanding the mechanisms underlying these behaviors and the factors contributing to the rise in prevalence is crucial for developing effective interventions and support strategies.

The pathology of ASD involves an integration of genetic, environmental, and neurological factors. Recent studies have identified numerous genetic variants associated with ASD, suggesting a vital hereditary component. In the previous research, genetic factors that significantly contribute to the development of autistic disorder include mutations in synaptic proteins such as Shanks/ProSAPs and neuroligins [8]. However, the mechanisms by which these mutations lead to ASD phenotypes remain largely unclear. Beyond that, genes linked to ASD are also associated with other neurological and psychiatric disorders. For instance, RELN and GluR6 have been correlated with schizophrenia [8]. Understanding how a single gene or protein leads to a specific neuropathology remains a significant challenge in the field.

Interestingly, ARPP21 appears strongly associated with intellectual disability. A growing body of research suggests that the ARPP21 protein is related to human intelligence and cognitive function. For example, in a prospective clinical report, four protein-coding genes including ARPP21, a modulator of calmodulin signaling, are depleted in the genome database of a family with intellectual disability [3]. Furthermore, within ARPP21, an intron-containing miR-128, a microRNA highly concentrated in the brain, has been linked to procedural learning, memory, and

neurodevelopment. In C57/Bl6J mice, fear-extinction learning resulted in a rise in the expression of the brain-specific microRNA miR-128b [4]. This increase destabilized several plasticity-related target genes and played a vital role in developing fear-extinction memory [4].

Additionally, miR-128 is one of 28 miRNAs found to be expressed at significantly different levels in postmortem cerebellar tissues related to autism [5]. A previous experiment demonstrated that while the majority of miRNA levels showed minimal variation across all samples—indicating that autism does not cause a widespread disruption of miRNA expression—certain miRNAs in the autistic samples were expressed at levels significantly different from the mean control values. Specifically, twenty-eight miRNAs, including miR-128, exhibited significantly different expression levels compared to the non-autism control group in at least one of the autism samples [5]. Consequently, multiple studies suggest that ARPP21 and miR-128 play critical roles in human cognitive development, with their dysregulation potentially leading to neurological disorders.

The zebra finch songbird (Taeniopygia guttata) serves as an exemplary model for investigating the mechanisms underlying human speech acquisition. Birdsong, like human speech, involves the rapid production of syllables with varying acoustic properties [9]. These vocalizations are not innate but are learned through imitation of a tutor, a process that substantially mirrors human language acquisition. Tutors modify their song patterns when singing in the presence of young learners, thereby affecting the song-learning process in young zebra finches. This behavior is similar to the way humans adjust their speech when communicating with infants [10]. Hence, understanding the neural and molecular mechanisms of birdsong learning in zebra finches may provide valuable insights into the biological foundations of speech and language in humans.

Moreover, studying ARPP21 holds significant potential for advancing our understanding of human speech development. Previous research implicates ARPP21 in the vocal learning process within zebra finches [11]. In computational modeling, the timing of calcium and dopamine signal integration in basal ganglia was synchronized through cooperation of ARPP21 and DARPP32, downstream effectors of cyclic AMP [1]. On top of that, in a study that used iCLIP to downregulate ARPP21, dendritic complexity was reduced, whereas the overexpression of the protein led to elevation in the complexity of dendritic arbors [2].

Research conducted by White and colleagues demonstrated that overexpression of the FOXP2 gene leads to inaccuracies in song learning in zebra finches [12]. Another study from the same lab revealed that miR-128 is associated with songbird vocalization, suggesting a potential role of miR-128 in human speech [11]. Building on these projects, the present study investigates the connection between ARPP21 expression and vocal-dedicated medium spiny neurons. By examining the expression of ARPP21 protein in these neurons, this study seeks to provide additional evidence supporting the computational model for dopaminergic modulation of cortical inputs to the striatum that guides procedural learning. Furthermore, the findings will enhance our understanding of the molecular similarities between human language and songbird vocal learning, offering valuable insights into the developmental processes of vocal learning and the underlying pathology of speech disorders.

# CHAPTER 1: DETECTION OF ARPP21 IN ZEBRA FINCH BRAIN

## INTRODUCTION

The primary objective of this study was to validate the primary antibody for accurately detecting zebra finch ARPP21 protein. To address this, we utilized two primary antibodies and employed two animal models to troubleshoot the experiment. To ensure the specificity of the antibody for the target protein, a constraint-based alignment tool (COBALT) was used, allowing for the simultaneous alignment of multiple protein sequences and ensuring optimal antibody selection [14]. Additionally, the study aimed to elucidate the distribution of ARPP21 in the brains of adult male zebra finches, with a particular focus on Area X in the basal ganglia, a region involved in song learning and production [15]. The same methodology was applied in the primary antibody validation process to confirm the presence and quantify the expression of ARPP21 within specific brain regions.

## MATERIALS AND METHODS

### *Constraint-based multiple alignment tool (COBALT)*

The alignment of the protein sequences between the antibodies' immunogen and each isoform of ARPP21 in zebra finches was verified using the constraint-based multiple alignment tool (COBALT). This verification ensured the antibodies' specificity and compatibility for detecting ARPP21 isoforms in zebra finch brain tissues. COBALT, a tool that facilitates accurate alignment of given protein sequences, thereby supports precise immunohistochemical analysis [14].

All animal use was in accordance with National Institutes of Health and American Veterinary Medical Association guidelines for experiments involving vertebrate animals and approved by the University of California, Los Angeles Chancellor’s Institutional Animal Care

and Use Committee (ARC-2001-054). A total of 12 adult male zebra finches were used for this work. Individual birds were perfused to obtain fixed brain tissue for the experiment. The birds were overdosed using isoflurane inhalation. Once dead, the body was rapidly secured supine in a dissecting tray, and a midline incision was made to expose the heart. A perfusion needle was carefully inserted into the left ventricle carrying perfusion fluids, and an incision was made in the right atrium to allow blood outflow. The vascular system was flushed with phosphate-buffered saline (PBS) to clear the blood, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer to fix the brain tissue and preserve the cellular architecture through cross-linking [17,18]. Subsequently, a 20% sucrose solution was applied to cryoprotect the brain tissues overnight [16]. The frozen tissues were sectioned sagittally into 30-40 μm thick slices using a cryostat. The sections were mounted onto charged slides (Superfrost Plus, Fisher) and stored at -80°C until use [19].

The vast majority of commercial antibodies are prepared against mammalian protein sequences. Because zebra finches served as the primary animal models for our experiments, coronal sections of anterior brain regions containing the striatum from adult mice, generously provided by Dr. Portera Cailliau's lab, served as a positive control. These 50 μm thick sections were maintained as free-floating specimens. The primary purpose of these mouse sections was to test our primary antibody obtained from Proteintech. The immunohistochemistry protocol for floating tissues is similar to that for slide-mounted tissues, with the additional step of mounting them onto charged slides before beginning the experiment.

***Immunohistochemistry***

Frozen tissues were equilibrated to room temperature for approximately 10 minutes, then rehydrated in TBS containing 0.3% Triton X-100 (TBSTx) for 15 minutes [22]. Based on prior

research conducted in our laboratory, ammonium chloride ($NH_4Cl$) was utilized to reduce autofluorescence, thereby optimizing experimental outcomes [16]. Following a 10-minute incubation with $NH_4Cl$, the sections were thoroughly rinsed with TBS and subsequently processed for the blocking step, during which 10% goat serum in TBSTx solution was applied to the brain sections. The ARPP21 polyclonal antibody (Thermo Fisher Scientific) was then used at a 1:100 dilution following the blocking step, and slides were incubated in the primary antibody overnight at 4°C [16]. The control group was incubated in a regular TBSTx solution to rule out any effects such as tissue drying. The following day, the slides were removed from refrigeration and washed five times for 5 minutes each with TBSTx solution [16]. Subsequently, the slides were incubated for 3 hours with goat anti-rabbit secondary antibody (Lot# 2539808) at a 1:1000 dilution. The sections were washed thoroughly five times for 5 minutes each in TBS saline solution and subsequently mounted with a coverslip using ProLong Gold Antifade Reagent (Molecular Probes). Completed slides were imaged immediately using a fluorescence microscope or stored in a slide box at 4°C until imaging.

In this study, the utilization of antibodies from Proteintech was characterized by the implementation of antigen retrieval, a process that enhances epitope exposure. Specifically, the procedure involved heating 0.1 M Tris-EDTA Buffer (pH 9.0) to its boiling point [33]. The slides were subsequently immersed in the heated buffer for 5 minutes, followed by a 5-minute cooling period. Afterwards, we adhered to a nearly identical protocol with two exceptions: we omitted ammonium chloride and utilized a primary antibody from Proteintech at a 1:1000 dilution to achieve optimal results.

***Imaging***

Images were captured using a Zeiss-Imager.A2 microscope (Carl Zeiss MicroImaging, Thornwood, NY) equipped with fluorescence capabilities and the Basler pylon Viewer software. A 40x objective was employed, with exposure times ranging from 6 ms to 600 ms, depending on the fluorescence color and signal intensity. The quantification of ARPP21 staining was performed using ImageJ2 software, employing the measurement feature after optimizing the threshold for each image. To ensure consistency, all images were processed under identical conditions. The fluorescence intensity of ARPP21 was measured and normalized against background signals.

## RESULTS

COBALT analysis of mouse and zebra finch ARPP21 isoforms compared with the antigenic peptide sequence used for the Rabbit Polyclonal antibody from Proteintech was a good match for mouse ARPP21 but not for zebra finch ARPP21 (Table 1). Accordingly, only faint ARPP21 staining was visible in the anterior regions of the zebra finch brain whereas there was strong signal in the mouse tissues (data not shown). This latter result serves as a positive control for the staining procedure. However, upon realizing the lack of similarity between this antibody's immunizing sequence and the sequences for zebra finch ARPP21, further experimentation was not pursued. In contrast, COBALT analysis revealed a good match with the immunizing sequence for the Thermo Fisher antibody (Table 1). Consequently, the following results pertain to the antibody from Thermo Fisher.

Immunohistochemical analysis revealed significant expression of ARPP21 in the cerebellum of an adult male zebra finches relative to the blank controls. Quantitative measurements demonstrated a marked increase in ARPP21 levels compared to the blank control group (Fig 2A). Notably, exposure time emerged as a critical parameter in this analysis. Short

exposure times were employed to avoid potential photobleaching and suggested robust cellular expression [13]. Specifically, the exposure time for the experimental group was set at 60 ms (Fig 2A), during which a substantial number of signals were detected, with a mean value of 121.46. In contrast, the DAPI-stained cell nuclei in the experimental group exhibited a mean value of 49.38 (fig 2C). The control group, with an exposure time of 600 ms, showed a mean value of 58.32 (fig 2B), underscoring the importance of exposure time in accurately measuring expression levels.

## DISCUSSION

I performed protein sequence alignment for the primary antibody acquired from Proteintech, which showed only partial alignment (37% to 64%; Table 1) between ARPP21 protein sequences of human and zebra finches. Despite recognizing this partial alignment, we proceeded with the experiment, incorporating an antigen retrieval step to enhance epitope unmasking. During the early stages, this additional step allowed us to detect signals, albeit with some limitations. Conversely, the pronounced signals observed in the cerebellum regions of adult zebra finches indicated the presence of ARPP21 within the brain, thereby validating the efficacy of the primary antibody from Thermo Fisher Scientific in targeting ARPP21 in avian species. Although the presence of ARPP21 protein within Area X of the basal ganglia—critical for song production—was not initially confirmed (see Chapter 3), the successful detection of ARPP21 in the cerebellum demonstrates the reliability of our immunohistochemical protocol.

The significant expression of ARPP21 in the cerebellum, especially granular regions, as revealed by immunohistochemical analysis, underscores its potential role in cerebellar function. The marked increase in ARPP21 signals compared to the blank control group suggests that ARPP21 may be involved in specific regulatory mechanisms within the cerebellum. Future research should investigate the specific functional roles of ARPP21 in striatal Area X,

particularly in relation to motor coordination and learning processes. Additionally, exploring the interaction of ARPP21 with other signaling molecules and pathways could provide further insights into its mechanistic roles. Longitudinal studies examining ARPP21 expression across different developmental stages, sexes and behavioral states could also elucidate its dynamic regulatory functions.

# CHAPTER 2: ARPP21 IS CO-LOCALIZED WITH THE INTRACELLULAR SIGNALING PROTEIN DARPP32

## INTRODUCTION

DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa) is expressed within medium spiny neurons of the striatum of humans, rats, and birds. Activation of dopamine D1 receptors leads to its phosphorylation by protein kinase A (PKA), significantly influencing the electrophysiological, transcriptional, and behavioral responses of medium spiny neurons (MSNs) to various stimuli, including antidepressants [26]. Prior evidence has demonstrated that using DARPP-32 and EGR-1 (early growth response protein 1) as markers for neural maturation and activation, approximately 80% of newly labeled neurons at 42 days post-labeling had matured into medium spiny neurons (MSNs) and could be activated by singing behavior [27]. Additionally, DARPP-32 expression is strongly correlated with the singing behavior of zebra finches [28].

In recent years, the interaction between DARPP-32 and other signaling proteins has garnered significant attention. One such protein is ARPP21, which is involved in the cAMP signaling pathway and plays a role in modulating neuronal responses to neurotransmitters. Studies suggest that ARPP21 and DARPP-32 may co-localize within specific cellular compartments, potentially indicating a synergistic relationship in regulating neuronal function [25]. Understanding the spatial and functional interplay between these proteins could provide deeper insights into the molecular mechanisms underlying synaptic plasticity and neuronal signaling. This study aims to explore the co-localization of ARPP21 with DARPP-32 in neurons, utilizing imaging techniques and immunostaining. By elucidating the potential for their

interaction, we hope to shed light on their combined role in mediating intracellular signaling processes important for vocal learning.

For the purpose of this study, I aim to co-stain ARPP21 and DARPP-32 to further characterize and understand the mechanisms underlying singing behavior in area X of the basal ganglia in zebra finches. Studies from showed that the majority of medium spiny neurons (MSNs) in rats co-express DARPP-32 and ARPP-21, with DARPP-32 being the most commonly used marker for MSNs in rats [25].

## MATERIALS AND METHODS

### *Nissl staining*

The Nissl staining technique, commonly employed for examining brain morphology, entails immersing slides in 95% and 75% ethanol (EtOH) for 2 minutes each, followed by a brief 1-minute rinse in diluted water. The slides were then stained with Cresyl Violet for 5 minutes and subsequently rinsed in distilled water six times [29, 30]. After this, the slides were immersed in 70% EtOH with acetic acid three times. The procedure continues with the dehydration of the slides through a graded series of ethanol concentrations: 95% EtOH for 2 minutes, 100% EtOH for 2 minutes, and finally, the slides are cleared in Citrasol for 3, 4, and 5 minutes. The slides are then coverslipped with a mounting medium and allowed to dry overnight before examination under a microscope.

### *Immunohistochemistry staining (Dual)*

The overall procedure for dual immunohistochemistry adhered closely to the established protocol for ARPP21 staining. The primary deviation involved the addition of mouse anti-DARPP 32 antibody (a gift from Charles Hemmings) at a 1:2500 dilution alongside the ARPP21 polyclonal antibody (Thermo Fisher Scientific) at a 1:100 dilution during the initial day of the

IHC experiment. Subsequently, the slides were incubated for 3 hours at room temperature with both goat anti-rabbit secondary antibody and goat anti-mouse secondary antibody, each at a 1:1000 dilution.

## RESULTS

Using Nissl staining, Area X was visually identified in cryosections from the adult zebra finch brain. All images were captured within Area X. Five sets of slides containing adjacent sections were generated. One set of replicates was used for Nissl staining to identify anatomical landmarks. Another served as the experimental set for exposure to primary antibodies. A third was used as the control set, or 'blank', that was not exposed to primary antibodies. The remaining sets provided the opportunity for additional immunohistochemical experiments. Figure 3D, 3E and 3F illustrates the coronal sections of the brains analyzed using a 20X objective. Figure 3D comprises the control group, which served to reveal background staining and thereby verify the specificity of the immunohistochemical methods used. As anticipated, there were no detectable signals for either DARPP32 or ARPP21, confirming the absence of detection of these markers in the control specimens and validating the reliability of the experimental setup. In contrast, figure 3E and F depict striatal neurons obtained from an adult male zebra finch, where a pronounced expression of both DARPP32 and ARPP21 was observed. This robust expression indicates the presence of these proteins in the striatal neurons, as expected based on prior research. Notably, figure 3H through 3K further illustrates a strong co-localization of DARPP32 and ARPP21 signals within the same neurons with 40X objectives. This finding is consistent with the hypothesis that these two proteins interact or are co-expressed in specific neuronal populations, thereby reinforcing the experimental outcomes and supporting the broader objectives of the study.

## DISCUSSION

Previous studies have demonstrated extensive expression of DARPP32 protein within the medial and lateral striatum [31]. This widespread distribution highlights DARPP32's critical role in modulating dopaminergic signaling, essential for various neuronal processes [32]. Therefore, understanding DARPP32 through immunohistochemistry or other cell assays is crucial, as it aids in elucidating its roles in neural processes and how deficiencies in this protein contribute to pathologies such as autism and schizophrenia.

In this study, we aimed to demonstrate that ARPP21 and DARPP32 are co-expressed within Area X of the basal ganglia, as co-staining could further elucidate the function of ARPP21 in songbird singing. The results obtained allow us to conclude definitively that both ARPP21 and DARPP32 coexist within the Area X medium spiny neurons. It is evident that both proteins are expressed in the striatum, a region where they are hypothesized to play complementary roles in neuronal signaling and plasticity. Further investigations, including more refined co-localization studies and functional assays, will be necessary to elucidate the precise relationship and potential interaction between ARPP21 and DARPP32 in this context. Future work will aim to determine whether these proteins can indeed co-exist within the same cellular compartments and contribute collectively to the modulation of neural circuits involved in song production.

# CHAPTER 3: CONCLUSION

This project sought to identify the role of ARPP21 in the singing of songbirds via detection of the protein within the basal ganglia of our bird model. The primary antibody from Thermo Fisher was confirmed to target the protein of interest, as suggested by both COBALT protein alignment and immunohistochemistry (IHC) results. Additionally, ongoing co-staining experiments of ARPP21 and DARPP32 have revealed the presence of both proteins in Area X, a region critical for song learning and production. Future research will focus on the upregulation or knockout of ARPP21 to investigate its direct correlation with vocalization. Furthermore, it is essential to examine ARPP21 expression levels across different age groups of zebra finches to understand its role throughout development.

Despite these promising findings, the study faced several limitations. A major limitation was the lack of commercially available antibodies specifically targeting zebra finch proteins, given the less common use of this animal model. Moreover, while co-staining results suggest the presence of both proteins in Area X medium spiny neurons, they do not confirm functional interaction or subcellular co-localization. Further studies are needed to address these limitations and provide a more comprehensive understanding of ARPP21's role in neural processes and vocal learning.

# TABLE AND FIGURES

## Table 1

***COBALT Alignment Validates Antibody Specificity for ARPP21 Isoforms in Zebra Finch Brain***

***Tissue preparation for protein study***

| **Invitrogen ARPP21 Polyclonal Antibody** | | | **ARPP21 Fusion Protein** | | |
|---|---|---|---|---|---|
| Human Immunogen (Isoform#) | Zebra Finch Sequence (Isoform#) | Alignment Percentage (%) | Human Immunogen (Isoform#) | Zebra Finch Sequence (Isoform#) | Alignment Percentage (%) |
| Q9UBL0.2 | XP_041570154.1 | 23.00 | AAH17805 | XP_041570154.1 | 64.04 |
| Q9UBL0.2 | XP_041570153.1 | 96.67 | AAH17805 | XP_041570153.1 | 64.04 |
| Q9UBL0.2 | XP_041570151.1 | 96.67 | AAH17805 | XP_041570151.1 | 64.04 |
| Q9UBL0.2 | XP_041570150.1 | 96.67 | AAH17805 | XP_041570150.1 | 62.92 |
| Q9UBL0.2 | XP_030122885.1 | 96.67 | AAH17805 | XP_030122885.1 | 64.04 |
| Q9UBL0.2 | XP_012426959 | 23.00 | AAH17805 | XP_012426959 | 62.92 |
| Q9UBL0.2 | XP_030122880 | 96.67 | AAH17805 | XP_030122880 | 64.04 |
| Q9UBL0.2 | XP_041570149 | 96.67 | AAH17805 | XP_041570149 | 62.92 |
| Q9UBL0.2 | XP_041570148 | 16.67 | AAH17805 | XP_041570148 | 64.04 |
| Q9UBL0.2 | XP_030122877 | 23.33 | AAH17805 | XP_030122877 | 62.92 |
| Q9UBL0.2 | XP_030122876 | 16.67 | AAH17805 | XP_030122876 | 62.92 |
| Q9UBL0.2 | XP_041570147 | 96.67 | AAH17805 | XP_041570147 | 51.685 |
| Q9UBL0.2 | XP_041570146 | 96.67 | AAH17805 | XP_041570146 | 51.685 |

**Table 1 (Continued)**

| | | | | | |
|---|---|---|---|---|---|
| Q9UBL0.2 | XP_030122868 | 96.67 | AAH17805 | XP_030122868 | 51.685 |
| Q9UBL0.2 | XP_012426958 | 96.67 | AAH17805 | XP_012426958 | 62.92 |
| Q9UBL0.2 | XP_030122866 | 96.67 | AAH17805 | XP_030122866 | 37.10 |
| Q9UBL0.2 | XP_030122865 | 96.67 | AAH17805 | XP_030122865 | 37.10 |
| Q9UBL0.2 | XP_030122864 | 96.67 | AAH17805 | XP_030122864 | 64.04 |
| Q9UBL0.2 | XP_030122863 | 96.67 | AAH17805 | XP_030122863 | 62.92 |
| Q9UBL0.2 | XP_041570145 | 96.67 | AAH17805 | XP_041570145 | 64.04 |
| Q9UBL0.2 | XP_041570144.1 | 96.67 | AAH17805 | XP_041570144.1 | 64.04 |
| Q9UBL0.2 | XP_030122862 | 96.67 | AAH17805 | XP_030122862 | 64.04 |
| Q9UBL0.2 | XP_041570143 | 96.67 | AAH17805 | XP_041570143 | 64.04 |
| Q9UBL0.2 | XP_030122860 | 96.67 | AAH17805 | XP_030122860 | 53.93 |
| Q9UBL0.2 | XP_030122859 | 96.67 | AAH17805 | XP_030122859 | 62.92 |
| Q9UBL0.2 | XP_030122858 | 96.67 | AAH17805 | XP_030122858 | 62.92 |
| Q9UBL0.2 | XP_012426957 | 96.67 | AAH17805 | XP_012426957 | 62.92 |
| Q9UBL0.2 | XP_030122857 | 96.67 | AAH17805 | XP_030122857 | 62.92 |
| Q9UBL0.2 | XP_030122856 | 96.67 | AAH17805 | XP_030122856 | 64.04 |
| Q9UBL0.2 | XP_030122851 | 96.67 | AAH17805 | XP_030122851 | 62.92 |
| Q9UBL0.2 | XP_030122850 | 96.67 | AAH17805 | XP_030122850 | 62.92 |
| Q9UBL0.2 | XP_030122849 | 96.67 | AAH17805 | XP_030122849 | 62.92 |

| Alignment General Statistic | **Invitrogen ARPP21 Polyclonal Antibody (Fisher)** | **ARPP21 Fusion Protein (Proteintech)** |
|---|---|---|
| Average | 84.77 | 60.39 |

**Table 1 (Continued)**

| | | |
|---|---|---|
| Max | 96.67 | 64.04 |
| Min | 16.67 | 37.10 |
| SD | 28.11 | 7.19 |

* Note: The protein sequence provided by Thermo Fisher is a synthesized peptide derived from the human ARPP21 protein (Accession Q9UBL0), corresponding to amino acid residues S218 to R268. In contrast, the peptide offered by Proteintech is derived from the human ARPP21 protein (Accession BC017805) and corresponds to amino acid residues 1-89.

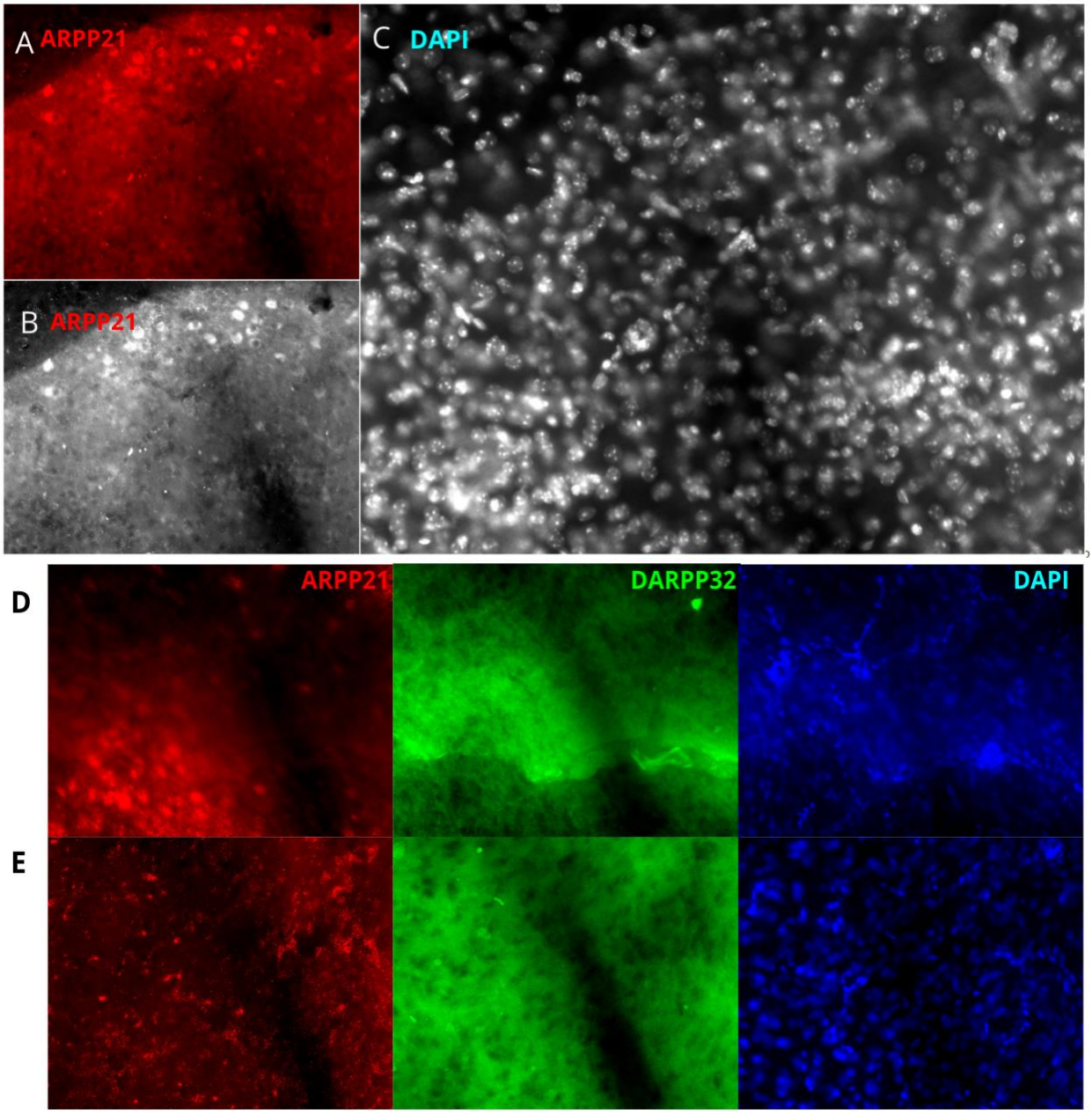


**Figure 1:**
Figures A-C represent ARPP21 expression in the cerebellum of mice brain sections with labels in cytosol. (A) ARPP21 expression in the cerebellum is shown with red fluorescence following look up table (LUT) adjustment. (B) ARPP21 expression displayed with a black and white background for enhanced contrast. (C) DAPI staining indicates the cell nuclei within the cerebellum. Figures D-E represent the result of ARPP21 protein expression in zebra finches with antigen retrieval.

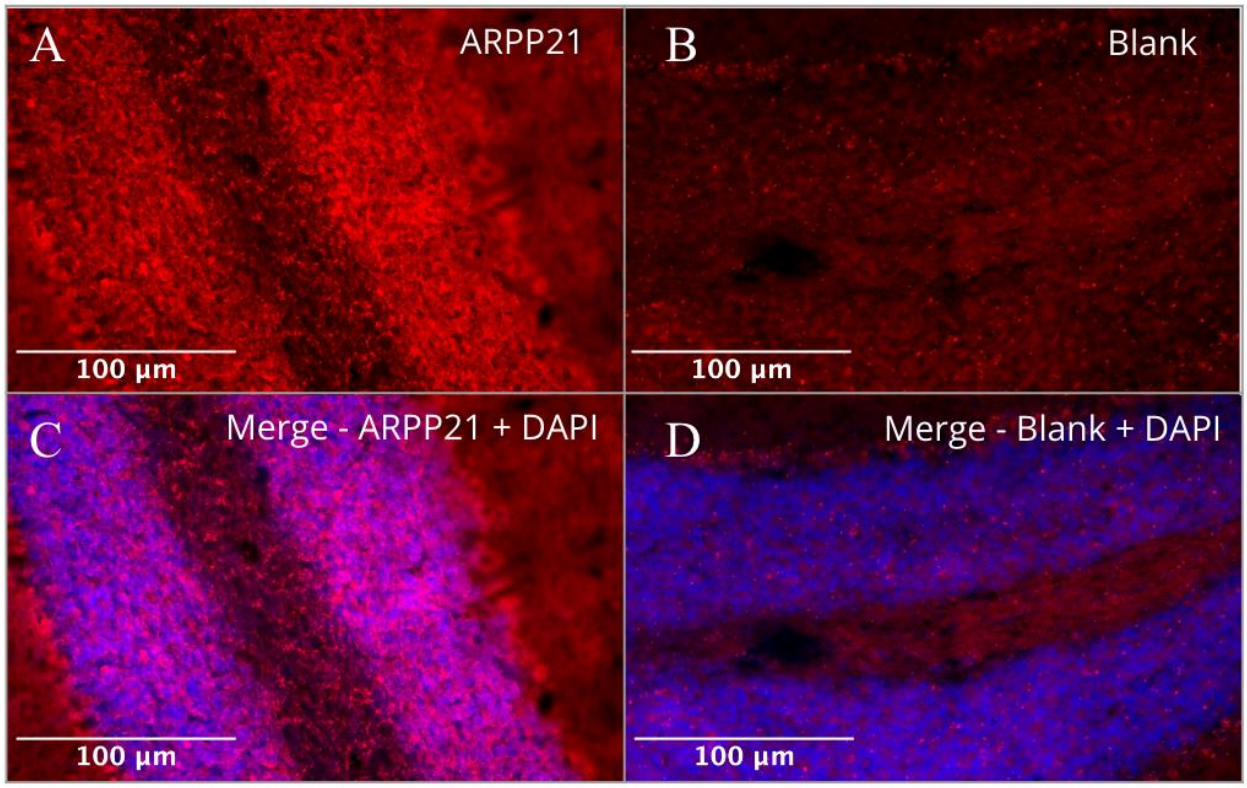


**Figure 2:** Photomicrographs show sagittal cerebellar sections from an adult male zebra finch (A) The anti-ARPP21 antibody reveals robust expression (red) using a 60 ms exposure time. (B) Control section that was not exposed to the anti-ARPP21 antibody imaged with a 600 ms exposure time exhibited no target expression. (C) ARPP21 signals are shown together with those from DAPI staining (blue), with the latter conducted under identical conditions as described for A, except with an exposure time of 6 ms for DAPI. (D) Control setup (without primary antibody dilution), shows no target signal in the DAPI-stained tissue.

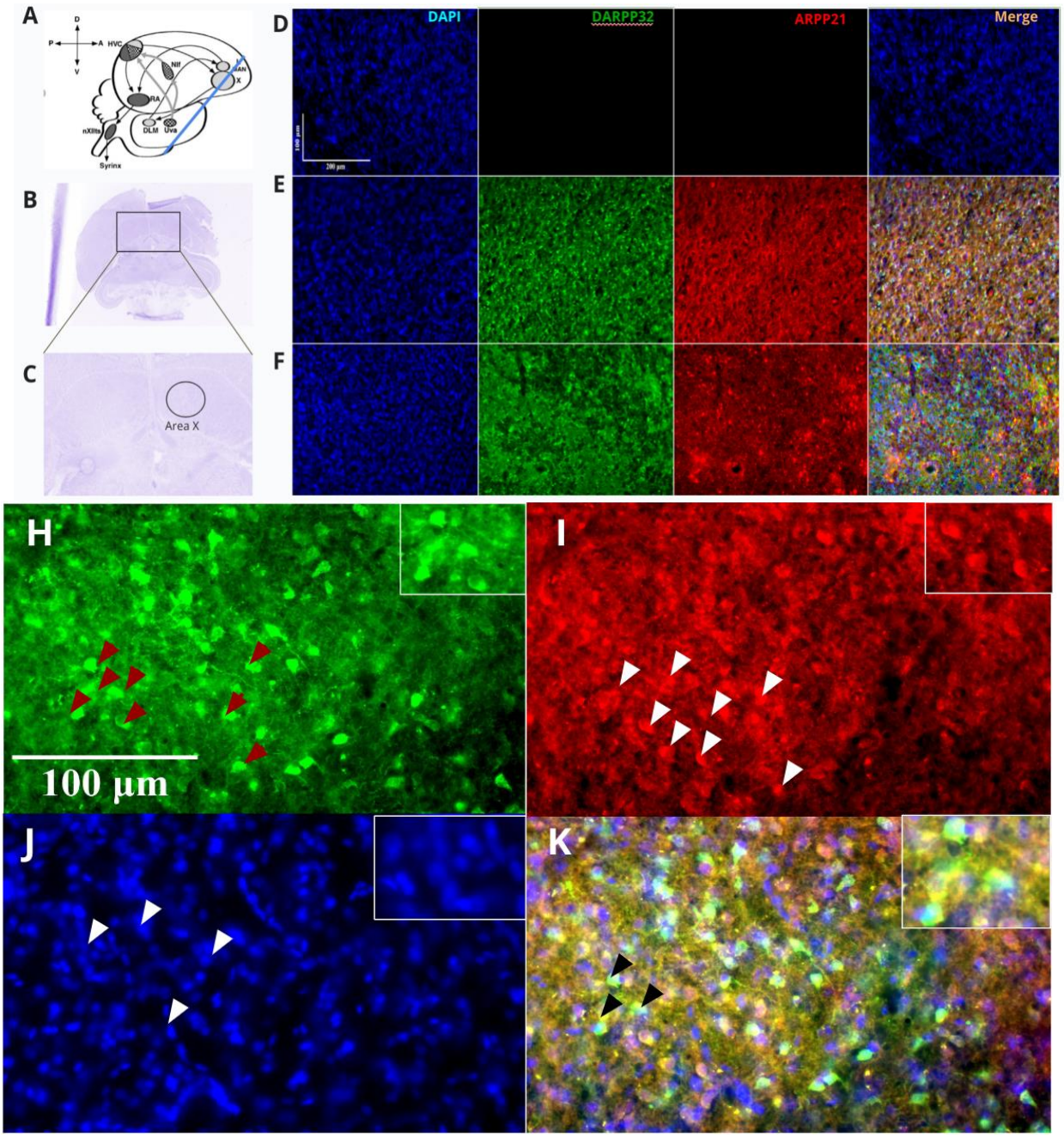


**Figure 3: Analysis of ARPP21 and DARPP32 Expression in Area X**

All images in this figure were captured from the Area X region of zebra finch brains. (A) a schematic representation of zebra finch brain in sagittal view. (B)-(C) Nissel staining of the brain of zebra finches where it shows the location of area X within the striatum. (D) the control groups, where, as expected, no DARPP32 or ARPP21 signals were detected. In contrast, sets (B) and (C) display striatal neurons from an adult male zebra finch, showing robust expression of both DARPP32 and ARPP21. Additionally, set (C) demonstrates strong co-localization of DARPP32 and ARPP21 signals, consistent with the expected results. Figures (H)-(K) provide enlarged views of Figure E, highlighting the specific expression patterns of DARPP32 and ARPP21, as well as their co-expression in Figure K. The top right corner of each figure displays the categorization of labeled cells, while the micron-scale arrows indicate the expression localization of each protein.

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