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Investigating the expression profiles of cysteine string proteins (CSPs) in cochlear tissue

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ABSTRACT

Objective: This study aims to explore the expression patterns of cysteine string protein alpha (CSPα) and cysteine string protein beta (CSPβ) in the mammalian inner ear, with an emphasis on their temporal dynamics during the developmental stages of C57BL/6 mice.

Methods: We utilized immunofluorescence staining to assess the localization and distribution of CSPα and CSPβ within the inner ears of C57BL/6 mice and miniature pigs. Additionally, this method facilitated the investigation of their temporal expression profiles.

Results: In adult C57BL/6 mice and miniature pigs, CSPα and CSPβ were identified in the cytoplasm of inner hair cells and spiral ganglion cells, yet were absent in outer hair cells. Both proteins were found to colocalize with Ctbp2 on the basal side of the cytoplasm in inner hair cells' basilar membrane. Expression of CSPα was observed at the nerve fiber termini at the basilar membrane's base of inner and outer hair cells 10 days postnatally in C57BL/6 mice. Notably, expression of both CSPα and CSPβ in the cytoplasm of inner hair cells emerged on the 12th day post-birth, aligning with the timeline for registering cochlear potentials. The expression levels of both proteins increased with age, but were consistently absent in outer hair cells. Contrastingly, expression of CSPα and CSPβ was present in the cytoplasm of inner hair cells in miniature pigs as early as one day post-birth, yet remained absent in the three rows of outer hair cells.

Conclusion: CSPα and CSPβ exhibit predominant and specific expression in inner hair cells and spiral ganglion cells. A unique expression pattern was observed for CSPα, which was also present at the nerve fiber endings of both inner and outer hair cells. The developmental expression trajectory of CSPα and CSPβ in mouse inner hair cells is characterized by an initial absence, followed by a gradual increase. Moreover, the timing of expression onset between mice and miniature pigs indicates distinct temporal dynamics, suggesting a potential role in auditory development.

1. Introduction

Cysteine string proteins (CSPs) are part of the conserved DnaJ/Hsp40 family of cochaperones, primarily identified in neuronal synaptic vesicles (Mastrogiacomo et al., 1994; Burgoyne and Morgan, 2015). These proteins are ubiquitous across both invertebrates and mammals. Invertebrates harbor a single gene encoding for CSP, whereas mammals possess three distinct genes—DNAJC5a, DNAJC5b, and DNAJC5g—responsible for the synthesis of CSPα, CSPβ, and CSPγ, respectively. CSPα is the predominant isoform, extensively distributed and highly abundant in neuronal cells. In contrast, CSPβ and CSPγ are primarily localized in the testis. However, CSPβ has also been uniquely identified in auditory hair cells, highlighting its distinct tissue-specific presence (Schmitz et al., 2006; Liu et al., 2014). Sequence analysis has

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revealed a high homology between CSPβ and CSPα, suggesting similar biological functions, though the precise roles of CSPβ and CSPγ remain to be fully elucidated (Evans et al., 2003; Gundersen et al., 2010).

CSPs play critical roles in neuroprotection and are associated with several neurodegenerative conditions. Research involving CSPα knockout mice has demonstrated a significant neuroprotective effect of this protein. Initially, these knockout mice display normal behavior and do not exhibit any neurotransmitter loss within the first two weeks post-birth. However, they subsequently develop severe, progressive sensorimotor impairments and succumb approximately eight weeks after birth (Fernández-Chacón et al., 2004). Further investigations have shown exclusive expression of CSPα in retinal photoreceptor cells, with CSPα knockout leading to rapid, progressive blindness, although auditory functions remain unaffected. This indicates a particular vulnerability of tonically active ribbon synapses in the retina to the absence of CSPα, underscoring the necessity for at least one CSP isoform to prevent synaptic degeneration (Schmitz et al., 2006). Detailed analyses have also revealed disruptions in synaptic vesicle trafficking at neuromuscular junctions in these mice (Rozas et al., 2012). Recent findings have linked CSPα to adult-onset neuronal ceroid lipofuscinosis (ANCL) through mutations in the DNAJC5a gene and highlighted its protective role in Alzheimer's disease (Noskova et al., 2011; Tiwari et al., 2015).

Expanding into otology, investigations have confirmed the expression of CSPα and CSPβ in the mouse cochlea, although detailed expression patterns within the cochlea remain uncharacterized (Schmitz et al., 2006). The C57BL/6 mouse, a commonly used experimental model, has been extensively employed in inner ear research. Unlike mice, which are late-maturing and lack auditory function at birth, pigs display nearly fully developed inner ear structures and mature auditory functions immediately post-birth, making them an ideal model for such studies. In this research, we utilized both wild-type C57BL/6 mice and newborn miniature pigs to explore and compare the expression patterns of CSPα and CSPβ in the cochlea. Our findings indicate that both proteins are expressed in the inner hair cells but not in the outer hair cells. Notably, CSPα is also expressed at the nerve fiber endings at the base of both inner and outer hair cells. The differential temporal expression of CSPα and CSPβ in the cochlear inner hair cells of mice and miniature pigs suggests their potential involvement in the development of auditory functions.

2. Materials and methods

2.1. Animals

C57BL/6 mice were acquired at various postnatal stages (P1, P10, P12, P14, P21, P28, P60) from the Academy of Military Medical Sciences (Beijing, China), with ten mice at each time point. Additionally, three newborn miniature pigs were sourced from the Beijing Farm Animal Research Center (BFARC, Beijing, China). All animal-related procedures complied with the ethical guidelines approved by the Animal Research Committee of the Institute of Audiology and Speech Science under the oversight of the Xuzhou Medical University's Committee. The National Institutes of Health guidelines on the care and use of laboratory animals were also adhered to in conducting these experiments.

2.2. Preparation of cochlear sections

On the 60th postnatal day (P60), the cochleae of C57BL/6 mice were dissected and placed in a Petri dish containing 4% paraformaldehyde precooled to 4 °C. The apical cap, as well as the round and oval windows of the cochleae, were then unsealed. The cochleae were perfused 3–4 times with 4% paraformaldehyde through the apical cap until a transparent fluid was released from both windows, followed by overnight immersion in the same fixative at 4 °C. Post-fixation, the cochleae were washed with 0.1M PBS and decalcified in 10% EDTA for 6–12 h at room temperature. Subsequently, the decalcified cochleae were dehydrated in 15% and 30% sucrose solutions, embedded in OCT compound (Tissue-Tek, Japan), frozen, and sectioned into 10 μm slices. The sections were stored at −20 °C until further use.

2.3. Preparation of cochlear basilar membrane

The procedure for cochlear fixation and decalcification was identical to that described for cochlear section preparation. The cochlea was positioned in a Petri dish filled with 0.01 mmol/L PBS, and the decalcified osseous labyrinth was carefully separated from the cochlear apex using fine forceps under a stereoscopic microscope. The basilar membrane was then delicately separated from the modiolus and tectorial membranes, and Reissner's membrane was carefully removed. The isolated basilar membrane was subsequently placed in an EP tube containing PBS for further processing.

2.4. Immunofluorescence

Dissected cochlear basilar membranes or cochlear sections were incubated for 30 min in 0.25% Triton X-100 mixed with 0.01M PBS. After three PBS washes, the samples were blocked in 10% goat serum in 0.01M PBS at room temperature. They were then incubated overnight at 4 °C with primary antibodies diluted between 1:100 and 1:200. The primary antibodies included rabbit anti-Dnajc5a (ADI-VAP-SV003-E, Enzo Life Science), rabbit anti-Dnajc5b (17364-1-AP, Proteintech), mouse anti-parvalbumin (P3088, Sigma), mouse anti-synaptophysin (ab-8049, Abcam), mouse anti-CtBP2 (612044, BD Biosciences), and mouse anti-NF200 (1:100, Santa Cruz). Following primary antibody incubation, the samples were rinsed four times with 0.01M PBS for 15 min each. Subsequently, the sections were incubated with secondary antibodies at 37 °C for 40 min, including Alexa Fluor 647 goat anti-rabbit IgG (1:400; A21244, Invitrogen), Alexa Fluor 488 goat anti-mouse IgG (1:400; A11001, Invitrogen), and phalloidin (1:400, Sigma, P-1951). After four thorough washes with 0.01M PBS, samples were stained with DAPI (40,6-diamidino-2-phenylindole; Santa Cruz) at room temperature to highlight nuclei alongside immunofluorescence. Specimens were then examined under a confocal microscope.

3. Results

3.1. Expression of CSPα and CSPβ in the cochlea of C57BL/6 mice

Immunofluorescent staining was utilized to examine the localization of CSPα and CSPβ within the cochlear basilar membrane of adult (P60) C57BL/6 mice (Fig. 1). Parvalbumin was employed to specifically label hair cells, providing a clear delineation (Pangršič et al., 2015), while synaptophysin was used to predominantly mark efferent auditory fibers (Akil et al., 2008). CSPα was found predominantly in the cytoplasm of inner hair cells (IHCs) and not in outer hair cells (OHCs)(Fig. 1A), and it co-localized with synaptophysin at the synaptic bases of both IHCs and OHCs (Fig. 1B). In contrast, CSPβ was localized exclusively within the cytoplasm of IHCs(Fig. 1C), with no presence in OHCs or at the synaptic bases(Fig. 1D). Additional immunofluorescence staining on frozen cochlear sections revealed that CSPα and CSPβ are also present in the cytoplasm of spiral ganglion cells (Fig. 2C). CSPβ was observed on the basement membrane, while neither CSP subtype was detected on the spiral ligaments, stria vascularis, or other cochlear structures (Fig. 2).

3.2. Co-localization of CSPα and CSPβ with Ctbp2 in IHCs

Double immunofluorescence staining of the adult C57BL/6 mouse cochlear basilar membrane revealed that Ctbp2, a protein marker for ribbon synapses, was predominantly expressed in the basolateral membrane of IHCs, occasionally below the nuclei of these cells. The green fluorescent signal of Ctbp2 overlapped with the red fluorescent signals of CSPα and CSPβ, primarily expressed in the cytoplasm of IHCs,

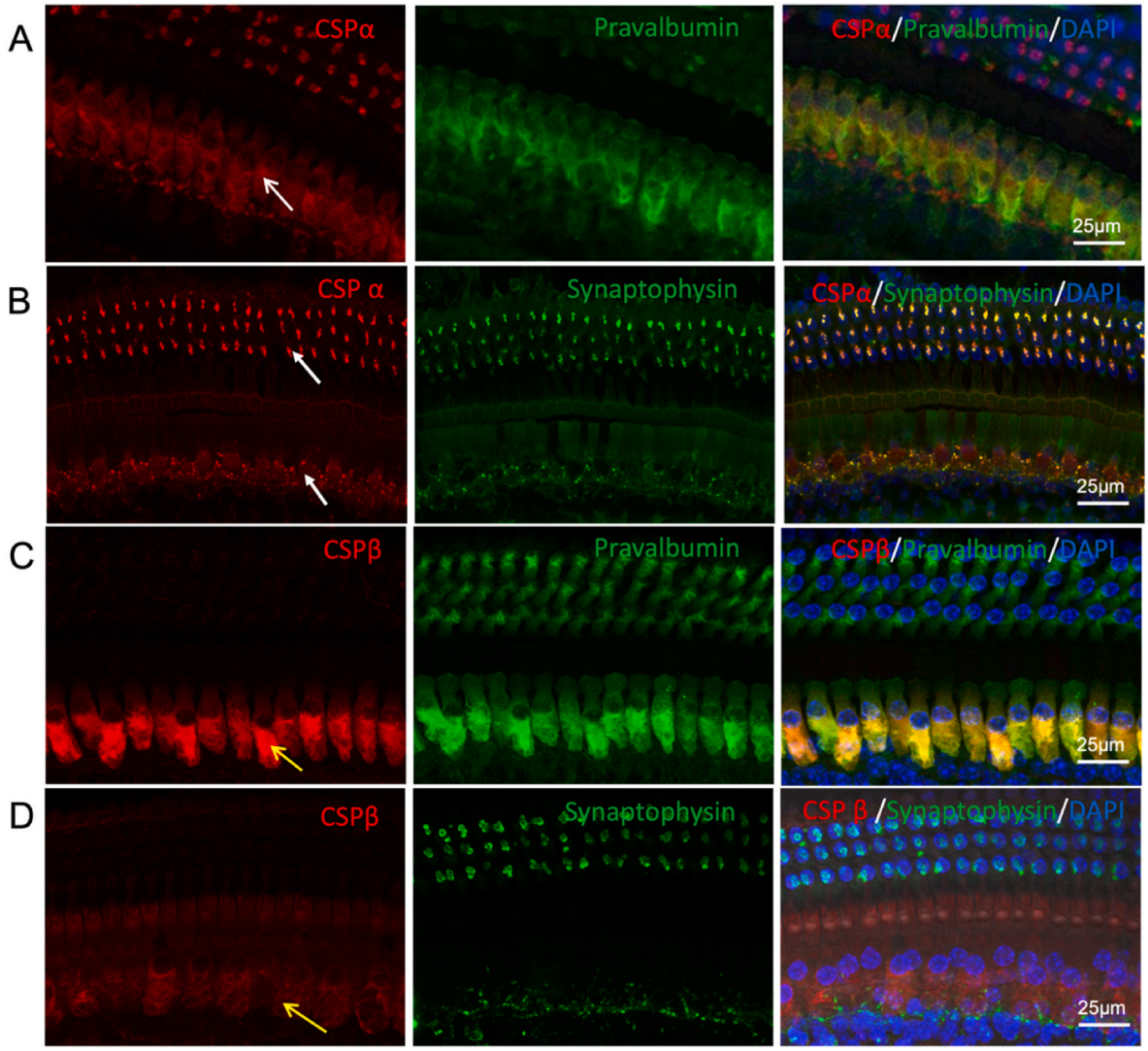

Fig. 1. Localization of CSPα and CSPβ in the Cochlea of Adult C57BL/6 Mice at P60. CSPα is distinctly expressed in the inner hair cells (IHCs), but it is not present in the OHCs themselves, as indicated by the white arrow (A). CSPα also expressed at the presynaptic nerve fiber endings of both IHCs and outer hair cells (OHCs), as indicated by the white arrow (B). In contrast, CSPβ is exclusively found within the IHCs, absent from both the OHCs and the nerve fiber endings, as indicated by the yellow arrow (C). CSPβ is not expressed in the presynaptic nerve terminals (D) . The hair cells are labeled with parvalbumin, emitting green fluorescence, which identifies their structural outline. Synaptophysin, marking the presynaptic nerve fiber endings at the bases of both IHCs and OHCs, also exhibits green fluorescence. Nuclei are stained with DAPI, displaying blue fluorescence, while CSPα and CSPβ are visualized in red fluorescence, highlighting their specific cellular locations.

resulting in orange fluorescent signals (Fig. 3)

3.3. Developmental expression patterns of CSPα and CSPβ in the cochlear basilar membrane of mice

Investigations into the temporal expression of CSPα and CSPβ during mouse development revealed no detectable immunolabeling in IHCs or other regions at P1. CSPα labeling was first observed weakly at the efferent nerve fiber terminals below the IHCs and OHCs by P10, with clear signals appearing within the IHCs by P12. CSPβ expression was first noted at P12, initially within the IHCs. Subsequent observations indicated that the expression levels of both proteins increased progressively with the development of the mice, although no signals were noted in the OHCs at any developmental stage (Fig. 4) .

3.4. Early Expression of CSPα and CSPβ in the cochlear basilar membrane of miniature pigs

In the cochlear basilar membrane of miniature pigs, CSPα and CSPβ were found expressed at P1, significantly earlier than in mice. The expression patterns in miniature pigs mirrored those observed in adult C57BL/6 mice; CSPα was primarily localized in the cytoplasm of IHCs and the synaptic base of both IHCs and OHCs, while CSPβ was restricted to the cytoplasm of IHCs, with no presence at the synaptic bases or in OHCs (Fig. 5). Furthermore, Ctbp2 colocalization immunofluorescence staining demonstrated that Ctbp2 was also expressed in the cochlear basilar membrane of miniature pigs at P1, predominantly concentrated at the base of IHCs (Fig. 5).

4. Discussion

Previous studies have established that cysteine string proteins (CSPs), known as synaptic vesicle proteins, are present in both neuronal and non-neuronal cells. There is compelling evidence to suggest that CSPs play a crucial role in preventing neurodegeneration, and recent findings have linked them to neurodegenerative diseases such as adult-onset neuronal ceroid lipofuscinosis (ANCL) and Parkinson's disease (Burgoyne and Morgan, 2015). The identification of CSPs in the auditory

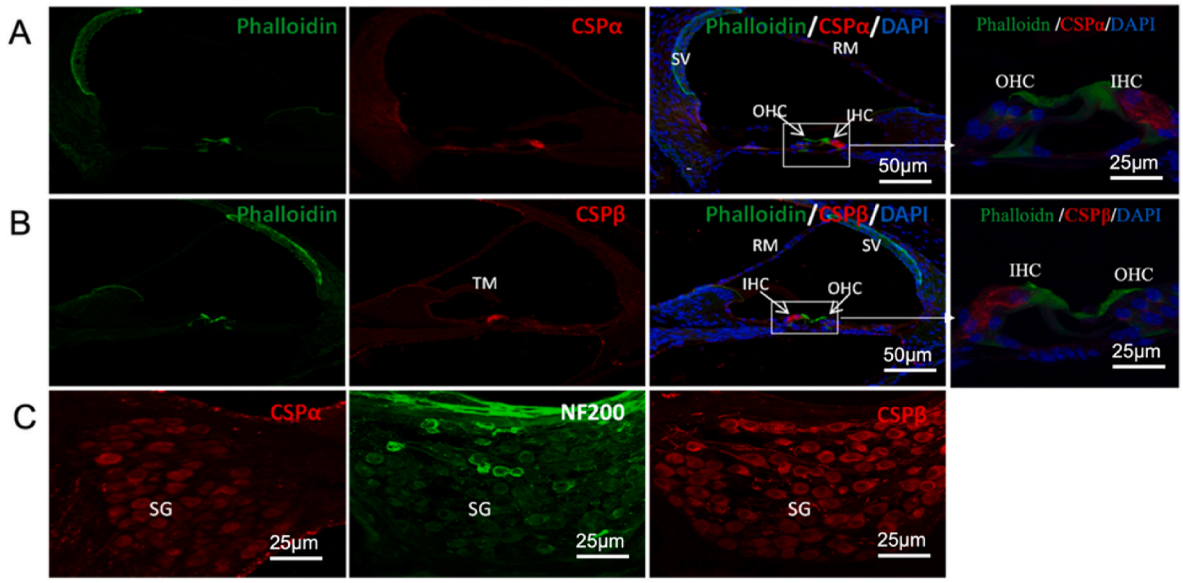

Fig. 2. Distribution of CSPα and CSPβ Proteins in the Cochlea of Adult C57BL/6 Mice at P60. CSPα is extensively expressed in the inner hair cells (IHCs) and at the presynaptic nerve fiber endings at the bases of both inner and outer hair cells (OHCs); notably, it is absent from the OHCs (A). In contrast, CSPβ is exclusively localized within the IHCs, without any detectable expression in the OHCs or at the presynaptic nerve fiber endings (B). CSPα and CSPβ is also expressed in spiral ganglion cells (C). Phalloidin labels the hair cell cilia, shown in green (A, B); NF200 labels spiral ganglion cells, also shown in green(C). Cell nuclei are stained with DAPI, resulting in blue fluorescence. Both CSPα and CSPβ proteins are visualized in red fluorescence, clearly differentiating their specific locations within the cochlear structure. SV: vascular stripe, TM: tectorial membrane, RM: Reissner's membrane, SG: spiral ganglion.

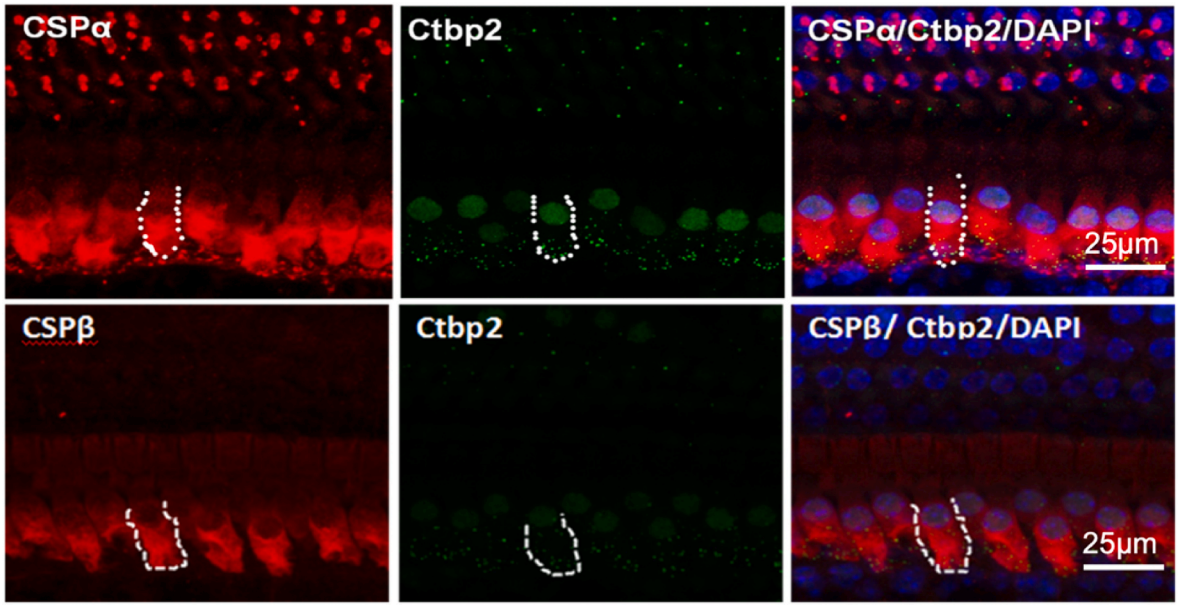

Fig. 3. Co-localization of CSPα and CSPβ with Ctbp2 in Inner Hair Cells of Adult C57BL/6 Mice at P60. CSPα and CSPβ are indicated by red fluorescence, whereas Ctbp2, marking synaptic ribbons, is denoted by green fluorescence. The outlines of the IHCs are clearly delineated with white dotted lines, emphasizing the specific cellular architecture and the spatial distribution of these proteins.

neuron system raises intriguing questions about their potential involvement in neurodegenerative processes within the inner ear.

Our research identified CSPα and CSPβ within the mammalian cochlea through immunofluorescent staining, revealing their predominant presence in the inner hair cells (IHCs) and spiral ganglion cells, with no expression noted in outer hair cells (OHCs). This pattern suggests a primary expression of CSPs in sensory nerve cells. IHCs are primarily innervated by type I spiral ganglion cells that convert acoustic signals into nerve impulses relayed to the brain, whereas OHCs, predominantly associated with type II spiral ganglion cells, function to amplify the vibration of the basilar membrane and enhance the sensitivity of auditory nerves (Robertson, 1984). The differential expression of proteins in IHCs and OHCs likely underpins their distinct functional roles.

In the IHCs, unique ribbon synapses form with the dendrites of afferent neurons, surrounded by dense neurotransmitter vesicles—a distinctive feature related to neurotransmitter release (Chamberlain and Burgoyne, 1997; Zhang et al., 1999). In our study, these ribbon synapses, marked by the scaffold protein RIBEYE/CtBP2, were co-stained with CSPα and CSPβ on the basolateral membrane of IHCs by double immunofluorescence staining. This co-localization suggests a role for CSPs in

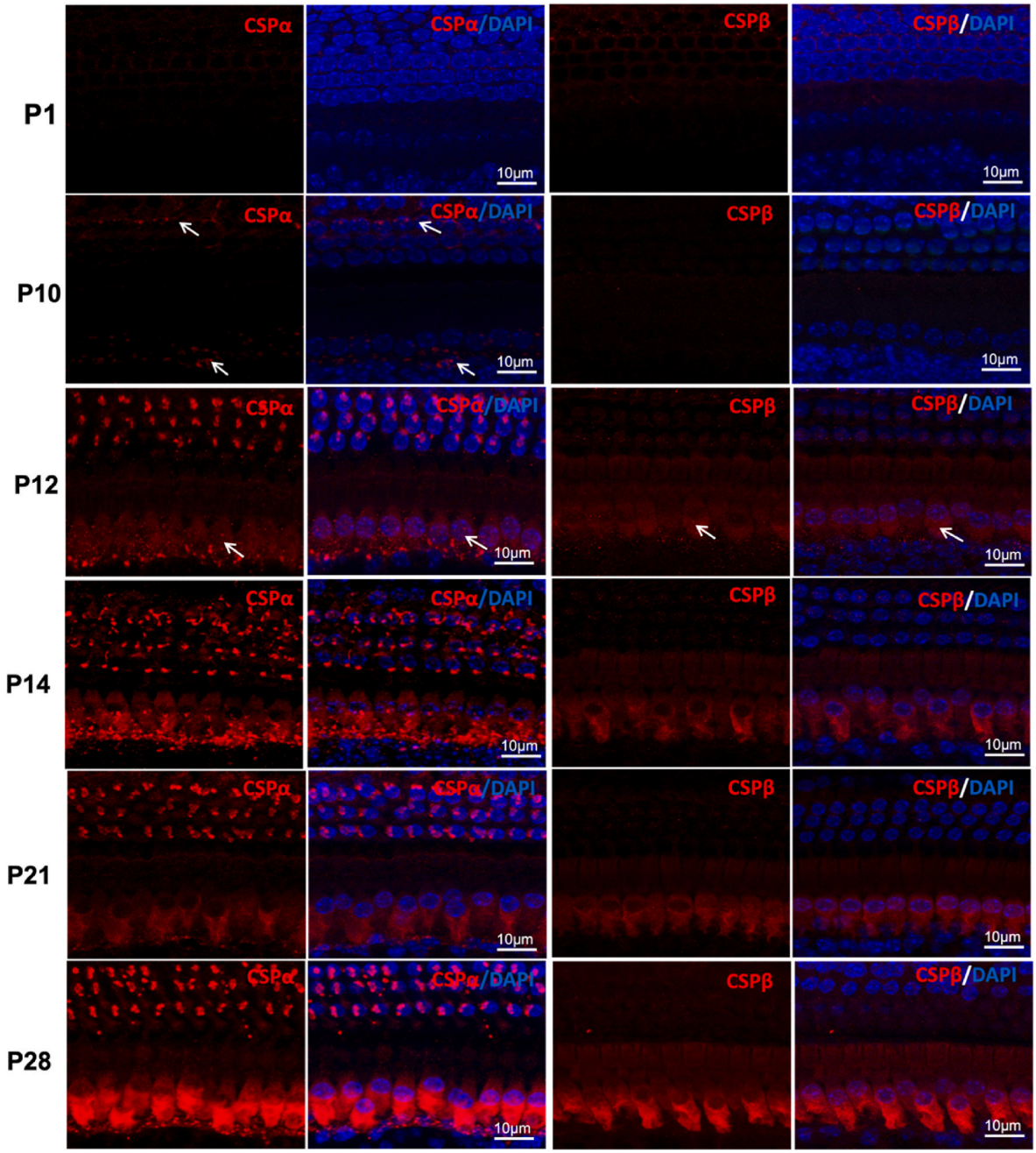

Fig. 4. Developmental Expression Dynamics of CSPα and CSPβ in the Cochlear Basilar Membrane of C57BL/6 Mice. At postnatal day 1 (P1), there is no detectable expression of CSPα or CSPβ in hair cells. By P10, faint signals of CSPα (indicated by white arrows) are observed primarily in the region below the inner hair cells (IHCs) and outer hair cells (OHCs), while CSPβ remains undetected. Initial detection of both CSPα and CSPβ in IHCs occurs around P12. The expression of these proteins peaks at P28, showing a significant increase between days 12 and 28 post-birth. Throughout this developmental timeline, the expression levels of CSPα and CSPβ gradually rise, but remain absent in all OHCs, highlighting a specific pattern of maturation and localization within the cochlea. CSPα and CSPβ are labeled in red, DAPI labels the nuclei, displayed as blue fluorescence.

neurotransmitter release and synaptic transmission in IHCs. Prior studies have shown that CSPα knockout mice experience rapid deterioration of photoreceptor synapses, leading to blindness, highlighting the importance of CSP in synaptic maintenance (Schmitz et al., 2006).

During developmental studies, we observed that CSPα and CSPβ were not expressed in IHCs and OHCs of C57BL/6 mice until 12 days post-birth. Despite the presence of labeled ribbon synapses and GluR2/3 AMPA receptors at birth (Huang et al., 2012), these mice initially lacked hearing, indicating that while synaptic structures are formed early, they are functionally immature. As the mice developed, CSPα and CSPβ expressions began in the IHCs by P12, coinciding with the first recordings of cochlear action potentials (Puel and Uziel, 1987). This timing aligns with the maturation of the cochlea and stria vascularis by P14 and the average onset of auditory functions around 13.2 days post-birth in C57BL/6 mice (Yang et al., 2017).

In contrast, immunofluorescence staining of the cochlear basilar membrane in miniature pigs at P0 revealed that unlike in mice, CSPα and CSPβ are expressed in IHCs from birth. This supports findings that pigs, similar to humans, exhibit precocial development with a mature auditory system at birth, in contrast to the postnatal maturation seen in rodents (Guo et al., 2015). This difference in the timing of CSP expression between species underscores the variability in cochlear

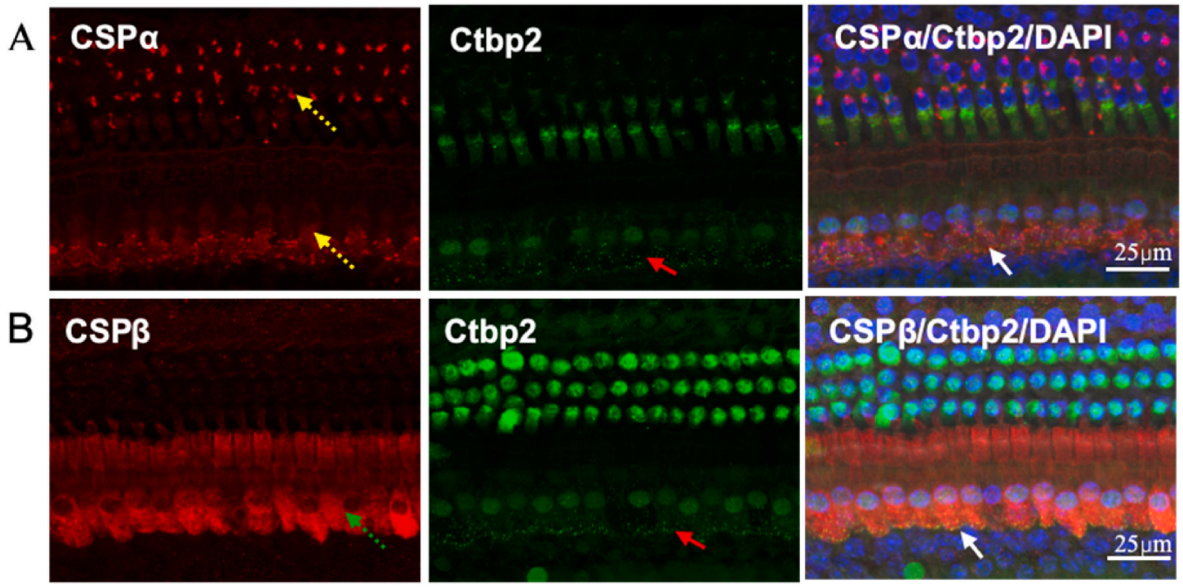

Fig. 5. Early Expression of CSPα, CSPβ, and Ctbp2 in the Cochlear Basilar Membrane of Miniature Pigs at P1. CSPα is observed in the cytoplasm of inner hair cells (IHCs) and at the synaptic bases of both IHCs and outer hair cells (OHCs), indicated by yellow arrows (A). In contrast, CSPβ expression is restricted to the cytoplasm of IHCs alone, as indicated by green arrows (B). Additionally, Ctbp2 is prominently expressed at this early stage, localized primarily at the basal part of IHCs, marked by red arrows. This demonstrates the early and distinct localization of these proteins within the cochlear structures of neonatal miniature pigs. CSPα and CSPβ are indicated by red fluorescence, whereas Ctbp2 is labled by green fluorescence.

development and suggests a correlation between CSP expression and early auditory function.

Our findings also show distinct expression patterns for CSPα and CSPβ. While CSPα was found in efferent presynaptic terminals marked by synaptophysin as early as P10, CSPβ did not show similar expression, which may indicate functional differences between the isoforms (Schmitz et al., 2006). Despite their nearly 70% amino acid identity, certain domain variations could account for these differences (Boal et al., 2007). Although CSPβ has demonstrated the ability to interact with CSPα chaperone associates like Hsc70 and SGT, suggesting similar biological functions, the exclusive expression of CSPβ in IHCs indicates it may not fully compensate for the loss of CSPα in other cochlear regions.

In summary, our study elucidates the expression and potential roles of CSPα and CSPβ in the cochlea of mice and miniature pigs. While both proteins are closely related to neurotransmitter release and auditory maturation, they also exhibit notable differences that could influence their function within synaptic vesicles of IHCs and possibly other cochlear regions. These findings lay a morphological foundation for future research into the mechanisms underlying CSP function in the cochlea.

Acknowledgments

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