RESEARCH ARTICLE

Functional Role of \(\gamma\)-Crystallin N in the Auditory Hindbrain

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Abstract

\(\gamma\)-crystallins are major components of the vertebrate lens but show expression in other tissues as well. Their extralenticular functions remain so far unclear. Here, we explored such roles in the rodent superior olivary complex in which previous analysis demonstrated developmentally regulated expression of \(\text{Crygd, Cryge}\) and \(\text{Crygn}\). Immunohistochemistry with novel antibodies against \(\text{Crygd/e and Crygn}\) indicate that expression of \(\text{Crygd/e}\) was moderate and varied between the perinatal superior olivary complex of mice, rats, and gerbils. Crygn-immunoreactivity was more robust and consistently highest in the medial nucleus of the trapezoid body, but also present in other nuclei of the superior olivary complex. To analyze the function of Crygn in the auditory hindbrain, we used a \(\text{Crygn}\) allele with a floxed exon 2. Upon pairing with \(\text{Egr2::Cre}\) mice, exon 2, encoding the first two greek key motifs of Crygn, was deleted in the developing auditory hindbrain. Anatomical analysis of these mice revealed a 20\% volume reduction in the medial nucleus of the trapezoid body and a 7\% reduction in the lateral superior olive at postnatal day 25. This was due to cell loss between postnatal days 4 and 25, whereas cell size was unaffected. Auditory brainstem responses showed normal threshold but a significant increase in the amplitude of wave IV. Crygn is hence required for postmigratory survival and proper function of auditory hindbrain neurons. These results ascertain for the first time an essential extralenticular role for \(\gamma\)-crystallins in vivo.

Introduction

\(\gamma\)-crystallins are small intracellular proteins of 174 to 182 amino acids with a molecular mass of ~21 kDa [1,2]. In vertebrates, four different classes of \(\gamma\)-crystallins exist: \(\text{Cryga-f, Crygm, Crygn, and Crygs}\) [1,2]. All family members share a highly symmetrical structure built from four characteristic Greek key motifs arranged into two similar domains [3]. Each Greek key motif consists of around 40 amino acids that fold into four anti-parallel \(\beta\)-strands [3]. Together with \(\beta\)-crystallins, \(\gamma\)-crystallins form the ancient \(\beta\gamma\) superfamily of crystallins, which is related to
microbial spore coat proteins. In vertebrates, they account for the majority of the soluble proteins in lens [4,5]. Accordingly, γ-cry stallins play a key role in determining the optical properties of this structure and mutations in several family members are associated with cataracts (Crygb-e, Crygs) or opacity (Crygf) in both humans and mice [2,6].

Outside the lens, γ-cry stallins are expressed in the retina and in the nervous system such as the hippocampus [7], yet little is known concerning their function in these tissues. Large variations in expression levels between mouse strains and between eyes in the same animal as well as poor correlation of RNA and protein levels [7,8] even put in question the functional importance of non-lens expression [9]. We recently observed high perinatal expression of Crygd, Cryge, and Crygn in the rat superior olivary complex (SOC) [10]. The SOC is an important second-order auditory center in the hindbrain, involved in sound localization and processing of temporal information [11–14]. Of note, the three γ-cry stallin genes showed down-regulation during postnatal development and had higher expression in the perinatal SOC compared to the age-matched total brain [10], pointing to an important role during development. This makes the SOC a promising system to study the role of γ-cry stallins outside vision. We therefore set out to characterize in detail the expression pattern of γ-cry stallins in the SOC on the protein level in three different rodents, using newly generated antibodies. To gain insight into the function of γ-cry stallins, we also generated and analyzed a region-specific Crygn knockout mouse. Together, the data revealed that Crygn is required for integrity and function of the auditory brainstem. This describes for the first time an essential extralenticular role for γ-cry stallins.

**Results**

**Generation and validation of γ-cry stallin antibodies**

To gain insight into the role of γ-cry stallins in the developing SOC, we wished to perform immunohistochemistry. This approach is hampered by the lack of isoform-specific antibodies. We therefore generated two different antibodies: one antibody against Crygd and Cryge (anti-Crygd/e, no immunogenic peptide is specific to Cryge) and one specifically recognizing Crygn (anti-Crygn). To validate the antibodies, the open reading frames of Crygd or Crygn were cloned into expression vectors, which were transiently transfected into HEK293 cells. Subsequent immunocytochemistry revealed that anti-Cryg, previously been used in our analysis of the SOC [10] strongly recognized Crygd (Fig 1A). In contrast, the immunoreactivity against Crygn was close to background (Fig 1A). In the immunoblot analysis, the antibody only bound to Crygd, detecting both a 17 kDa and a 55 kDa band (Fig 1C). As none of the two signals was present in Crygn transfected cells, they represent monomeric and likely complexed Crygd. Anti-Crygd/e recognized Crygd and not Crygn (Fig 1A and 1B), whereas anti-Crygn recognized Crygn and not Crygd (Fig 1A and 1B). These specificities were also observed in immunoblot analyses. Anti-Crygd/e detected a band of approximately 16 kDa, corresponding to the monomer, and an additional band around 55 kDa only in Crygd transfected cells (Fig 1D), similar to the antibody anti-Cryg. Anti-Crygd detected a 16 kDa band, corresponding to the monomer, only after transfection of HEK293 cells with a Crygn expression clone (Fig 1E). These data reveal that the two novel antibodies allow distinction between Crygd/e and Crygn.

**Different immunoreactivity patterns of γ-cry stallins in the perinatal rat, mouse, and gerbil SOC**

We next employed the three antibodies to study the expression of γ-cry stallins in the rat and mouse perinatal SOC at P4. To clearly detect SOC structures, we co-labeled with an antibody

superior olive; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; P, postnatal; SOC, superior olivary complex; wt, wild-type.
against the vesicular glutamate transporter VGluT1, a presynaptic marker for SOC nuclei [15–17]. We focused our analysis on the lateral and medial superior olive (LSO and MSO), respectively, and the medial nucleus of the trapezoid body (MNTB), as they are major nuclei of sound localization pathways. In the rat, anti-Cryg labeled prominently the MNTB, the fibers of the ventral acoustic stria, the MSO, and a large population of neurons in the LSO (Fig 2A and 2B). In the mouse, immunoreactivity was clearly seen in the MNTB and weak in the LSO, MSO, and the acoustic stria (Fig 2C and 2D). For the newly generated anti-Crygd/e antibody, labeling was observed throughout the SOC in the rat and mouse. Similar to anti-Cryg, fibers of the ventral acoustic stria were labeled in the rat but not in the mouse (Fig 3A–3D). Finally, anti-Crygn clearly labeled the rat MNTB, MSO, and neurons of the LSO (Fig 4A and 4B). In the mouse, MNTB showed a moderate immunoreactivity and the LSO and MSO were weakly labeled (Fig 4C and 4D).

In general, immunoreactivity for all three antibodies was weaker in the mouse compared to the rat. We therefore included the gerbil as a third rodent to obtain a broader view on γ-crystallin expression in rodents. For both anti-Cryg and anti Crygd/e, immunoreactivity was even weaker than in the mouse (Figs 2E, 2F, 3E and 3F). Anti-Crygn immunoreactivity was strongest in the gerbil MNTB (Fig 4E), but also present in the MSO and LSO (Fig 4F). One explanation for the weaker expression of γ-crystallins in mice compared to rats might be differently timed developmental processes [18]. To explore this possibility, we also immunolabeled prenatal mice between embryonic days 15 to 18. This analysis yielded only immunoreactivity close to background (data not shown). Finally, we investigated the expression of the different γ-crystallins in the adult SOC by applying the three antibodies to sections of P25 old mice. No significant labeling was obtained (Fig 5), confirming postnatal down-regulation of γ-crystallins in the SOC [19].
Our analyses so far revealed, that anti-Crygn clearly labeled the SOC in all three rodents at P4, whereas the other two antibodies mainly directed against Crygd/e gave only modest immunoreactivity in mouse and gerbil. Furthermore, Crygd and Cryge are pseudogenes in humans [6], which renders the extrapolation of functional data obtained in mice to other mammals difficult. We therefore focused our functional analysis on Crygn using a transgenic mouse line with a floxed Crygn allele generated by the International Knockout Mouse Consortium [19] (Fig 6A). The modified Crygn locus harbors a lacZ gene and a Neo cassette after exon 1 and loxP sites flanking exon 2 (Fig 6A). This allele design represents a so-called "knockout-first" allele due to the insertion of LacZ and the Neo cassette, which interrupts transcription of Crygn [19].
This cassette design should result in expression of lacZ, encoding β-galactosidase under the Crygn promoter. To corroborate our immunohistochemical data, we wished to exploit this reporter activity. Yet, X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) staining failed to detect β-galactosidase activity throughout the entire brain (data not shown). Preliminary PCR analyses indicate that part of the 5’ end of the LacZ gene has been lost (data not shown). As also Crygn mRNA could be detected in the brain of homozygous Crygnfl/fl mice (data not shown and Fig 6C), indicating a corrupted 5’ end of the inserted construct, we decided to employ a conditional knockout strategy by crossing the floxed Crygnfl allele with the Egr2::Cre driver line, which recombines in rhombomeres 3 and 5 derived neurons [20] (Fig 6A). In this cross, named CrygnEgr2, exon 2, encoding the first two greek key motifs, should be absent in most parts of the SOC, the anterior ventral cochlear nucleus, harboring the major input neurons into the SOC, and the dorsal cochlear nucleus, projecting to the inferior colliculus [17,21–25].

To confirm recombination, a PCR strategy was designed which amplifies a 604 bp long product upon recombination, whereas the non-recombined locus with 3,411 bp in length will not be amplified under the PCR conditions used. We used this strategy to probe genomic DNA

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**Fig 3. Crygd/e-ir in the rat, mouse, and gerbil SOC at P4.** (A) Crygd/e labeling is observed in the rat MNTB and fibers of the acoustic stria. (B) LSO and MSO display moderate Cryge-ir. In the mouse, MNTB (C), LSO and MSO (D) are also labeled. (E-F) The gerbil MNTB, LSO and MSO show Crygd/e-ir similar to background. Dorsal is up and medial to the left. n = 3, scale bar is 100 μm.

doi:10.1371/journal.pone.0161140.g003
isolated from the SOC of control or CrygnEgr2 mice. A 604 bp PCR product was clearly present when using genomic DNA from the SOC of CrygnEgr2 mice, whereas no product was obtained for SOC DNA from control mice (Fig 6B). The absence of exon 2 was confirmed by RNA in situ hybridization. A probe complementary only to the sequence of exon 2 hybridized to sections derived from control mice but yielded no signals from corresponding sections of CrygnEgr2 mice (Fig 6C). In contrast, a probe complementary to exons 2–4 hybridized to the SOC from CrygnEgr2 mice, albeit yielding weaker signals than in heterozygous Crygnwt/fl control mice (Fig 6C). These results reveal that exons 1, 3, and 4 are still transcribed from the modified locus and that the original allele in Crygnfl mice represents thus not a knockout-first allele. This is in agreement with the observation of Crygn mRNA in homozygous Crygnfl/fl mice and the lack of functional β-galactosidase.

Together, these data demonstrate that CrygnEgr2 mice lack exon 2 in the auditory hindbrain, that encodes the first two greek motifs in domain 1 except for the first two amino acids [1]. However, we note that lack of exon 2 does not disrupt the open reading frame, as it represents a phase 0–0 symmetric exon [26]. Due to transcription of the truncated Crygn mRNA, there is

Fig 4. Crygn-ir in the rat, mouse, and gerbil SOC at P4. Crygn clearly labels the MNTB (A), the LSO and the MSO (B) of rat. (C) The mouse MNTB shows a moderate labeling and the LSO and MSO a weak immunoreactivity (D). (E) Gerbils show a similar pattern of Crygn-ir as the mouse with the MNTB being strongest labeled. Dorsal is up and medial to the left. n = 3, scale bar is 100 μm.

doi:10.1371/journal.pone.0161140.g004
some residual truncated Crygn protein present within the auditory hindbrain. This is supported by the fact that our Crygn antibody labeled the SOC in CrygnEgr2 mice (data not shown). This prevented testing the specificity of the Crygn antibody in this mouse model.

Structural abnormalities in the adult SOC of CrygnEgr2 mice

To probe the functional consequences resulting from the loss of Crygn exon 2, we first investigated the integrity of the SOC in young-adult P25 mice by fluorescent immunohistochemistry for VGluT1 and the glycine transporter GlyT2, a presynaptic marker for inhibitory synapses in the auditory system [27–30]. Immunohistochemical analysis of the SOC using these two antibodies revealed no difference between CrygnEgr2 and control littermates containing one wt-allele and the Egr2::Cre allele (Fig 7A–7D). All major nuclei of the SOC were clearly labeled and no gross abnormalities in size or shape were observed. However, quantitative analysis of Nissl-stained sections demonstrated a significant volume reduction of the MNTB by 19.6% (control: 0.0392 ± 0.0013 mm³; CrygnEgr2; 0.0315 ± 0.0019 mm³, p = 0.0000094), whereas the LSO showed a small but significant reduction of 7% (control: 0.0444 ± 0.0025 mm³; CrygnEgr2; 0.0413 ± 0.0016 mm³, p = 0.0285) (Fig 7E).
Reduced volume could be the consequence of decreased cell number, reduced cell size, or changes in cell density. To distinguish between these possibilities we focused on the MNTB, showing the strongest phenotype. Cell counts revealed that the decreased volume in the MNTB was due to a 19.6% reduction in the number of neurons (control: 3,085.8 ± 108.3; CrygnEgr2: 2,481.8 ± 182.8, \( p = 0.00004 \)) (Fig 7E). In contrast, analysis of cross sectional area of MNTB soma revealed no reduction (control: 153.31 ± 31.71 \( \mu \text{m}^2 \); CrygnEgr2: 151.73 ± 44.17 \( \mu \text{m}^2 \), \( p = 0.7136 \)) (Fig 7E).

The observed reduction in cell number in the MNTB can be due to defects in cell birth, migration, or postmigratory survival of neurons. To assess which of these steps requires Crygn, we analyzed the MNTB at P4, five to six days after completion of migration [31]. This nucleus showed the strongest volume reduction and can easily be delineated at perinatal stages in the mouse [31]. Quantitative analysis of Nissl-stained sections revealed normal volume in
CrygnEgr2 mice (control: 0.0216 ± 0.0003 mm³; CrygnEgr2: 0.0209 ± 0.0005 mm³, p = 0.0615) (Fig 7E). Together, these data demonstrate that Crygn is required for postnatal integrity of second-order auditory hindbrain neurons. Furthermore, the postmigratory effect observed in CrygnEgr2 mice argue against a contribution of Egr2 haploinsufficiency in the Egr2::Cre driver line to the observed phenotype. This is in agreement with the lack of anatomical abnormalities in heterozygous Egr2::Cre;Cacna1d™/+ mice [22].

Altered auditory brainstem responses in CrygnEgr2 animals

To assess the functional consequences of the loss of Crygn exon 2 and the reduction in cell numbers of distinct SOC nuclei for auditory information processing, we audiometrically
analyzed the hearing function of CrygnFgr2 and control mice from their auditory brainstem responses (ABR), otoacoustic emissions (DPOAE), and auditory steady state responses (ASSR). From ABR to click, noise burst, and pure tone stimuli, hearing thresholds (Fig 8A) and amplitude and latency growth functions for individual waves (Fig 8D) were determined. Thresholds for click and noise burst stimuli were similar in both genotypes and did not significantly differ \( (p = 0.16 \text{ and } p = 0.09 \text{ for click and noise stimuli, respectively, 2-sided t-test}). \]

There was a tendency for even slightly better thresholds upon pure tone stimuli in the CrygnFgr2 mice \( (p = 0.0279 \text{ for factor genotype, F(1, 7) = 7.64, 2-way ANOVA}). \] However, differences were small (ca. 8 dB on average, refers to an effect size of \( D = 1.5 \)) and post-tests did not disclose a particular stimulus frequency at which thresholds were different \( (p > 0.05, \text{Holm-Sidak's multiple comparisons test}). \]

As expected from the ABR thresholds, outer hair cells function as determined by DPOAE growth functions, amplitudes, and thresholds (Fig 8B) also were slightly though non-significantly better in CrygnFgr2 mice than in controls \( (growth \text{ function at } f_2 = 11.3 \text{ kHz}: p = 0.1048 \text{ for factor genotype, F(1, 15) = 2.98, 2-way ANOVA}). \]

Pairwise comparison revealed larger responses in CrygnFgr2 mice than in controls only at 45 dB SPL \( (p < 0.05, \text{Holm-Sidak's multiple comparisons test}). \] The maximal emission strength was not significantly different \( (50 \text{ dB SPL L2: } p = 0.4, \text{ 2-sided t-test}). \] Corresponding to the slightly improved ABR thresholds in CrygnFgr2 mice, DPOAE thresholds were slightly but non-significantly improved in CrygnFgr2 mice \( (p = 0.1013 \text{ for factor genotype, F(1, 14) = 3.08, 2-way ANOVA}), \]

proposing an increased outer hair cell response as the origin of the slightly better ABR (hearing) thresholds in CrygnFgr2 mice. This slightly improved responsiveness of the sensory structures in the cochlea may be due to a reduced activity within the inhibitory olivo-cochlear efferent feedback upon outer hair cells and inner hair cell afferents, resulting in marginally better thresholds, and stronger outer hair cell responses at close threshold and moderate stimulation levels \( (45 \text{ dB SPL}). \]

Since temporal processing depends on the fast and synchronous responses of neurons in hindbrain and midbrain auditory structures, the observed anatomical alterations in CrygnFgr2 mice might impair temporal auditory processing in these mice. Therefore the temporal responses were recorded in CrygnFgr2 and control mice by auditory steady state responses (ASSR) to amplitude modulated stimuli (AM) and by calculating the amplitudes and latencies of subsequent waves in the auditory brainstem response (waves I, II, III, and IV) that are generated by synchronous neuronal activation within the hierarchically organized succeeding auditory brainstem nuclei (wave I: auditory nerve, wave II: cochlear nucleus complex, wave III: SOC, wave IV: lateral lemniscus and inferior colliculus [32,33]). ASSR for amplitude modulated stimuli of increasing modulation frequency \( (64–2,028 \text{ Hz}), \]

increasing modulation depth (index 1%-100%), and increasing carrier stimulus level did not reveal any deficits in the capacity of CrygnFgr2 mice to process temporally modulated auditory stimuli (Fig 8C). Best responses in the temporal modulation transfer function occurred within the same modulation frequencies as for control mice \( (ca. 128 \text{ Hz}) \) and responses brake off at similar high modulation frequencies \( (> 1,024 \text{ Hz}). \]

Modulation thresholds were not different for both groups \( (ca. 3–4\% \text{ modulation index} \) and modulation responses extended to the same range in both genotypes \( (carrier \text{ levels up to } 40 \text{ dB hearing level}). \] There was a tendency for a signal compression in CrygnFgr2 mice at higher carrier levels while the response in control mice still grew \( (50–60 \text{ dB hearing level, Fig 7C, right panel}). \]

Corroborating the normal or slightly improved cochlear and cochlear nucleus function, waves I and II amplitudes of ABRs were normal or even slightly increased for CrygnFgr2 mice \( (Fig 8D), \]

wave I: \( p = 0.3287 \text{ for factor genotype, F(1, 15) = 1.02, wave II: } p = 0.2476 \text{ for factor genotype, F(1, 15) = 1.45, 2-way ANOVA}, \]

no significance for pair-wise comparison in post-test). Wave III amplitudes were not different for CrygnFgr2 and control mice \( (p = 0.8461 \text{ for factor genotype, F(1, 13) = 0.0392}). \] In contrast, the amplitudes of
Taken together these physiological data reveal that later postnatal stages. This is in agreement with the observation in mice lacking the neural cell

therefore extend the vulnerable period of second-order neurons of the auditory hindbrain to

p

Crygn

sensitivity of the inner ear (as demonstrated by ABR thresholds and DPOAE functions), but that was due to cell death (Fig 7). This cell death occurs during terminal postnatal differentiation, specific knockout strategy, our data demonstrate that lack of exon 2 of

CrygnEgr2

Our anatomical analysis revealed volume reduction in both the MNTB (~20%) and LSO (7%) of CrygnEgr2 mice. A detailed analysis demonstrated that the volume reduction in the MNTB was due to cell death (Fig 7). This cell death occurs during terminal postnatal differentiation, after completion of migration [12]. Interestingly, Crygn is thus required for survival after the previously established period between P0 and P4, in which a significant number of SOC neurons die in the absence of innervation [23] or postsynaptic Ca^{2+} signaling [21,22]. Our data therefore extend the vulnerable period of second-order neurons of the auditory hindbrain to later postnatal stages. This is in agreement with the observation in mice lacking the neural cell
adhesion molecule contactin 5 [34]. These mice showed cell death of MNTB neurons between P6 and 1 month of age [34]. This was likely due to a reduced number of calyces of Held, which represent the large presynaptic terminals of MNTB neurons [35,36]. A prolonged period of vulnerability beyond P4 was also shown for the anterior ventral cochlear nucleus, a first-order auditory nucleus in the hindbrain. Timed cochlear ablation experiments demonstrated an abrupt age-dependent change in sensitivity of these neurons to disruption of afferent input. Deafferentation before P11 resulted in significant cell loss, whereas the same procedure done by age P14 had no effect on survival [37]. Thus, both first-order cochlear nucleus neurons and second-order SOC neurons display a period of vulnerability beyond the first days post-migration.

What might be the underlying mechanism of cell death in mice lacking Crygn? Recent analysis in mice lacking L-type Ca2+ channels revealed that MNTB neurons crucially depend on Ca2+ signaling. This was shown both in vivo by the use of mice lacking L-type Ca2+ channels [21,22,38] or in vitro by pharmacological approaches [39]. Due to the importance of Ca2+ signaling and homeostasis, developing auditory neurons express various Ca2+-binding proteins such as parvalbumin, calbindin, and calretinin [40,41]. Of note, in diverse microorganisms, members of the crystallin βγ superfamily bind Ca2+ [42,43]. Yet, the Ca2+ binding site is degenerated in vertebrate family members, resulting in a low affinity Ca2+ binding site [44]. Consequently, no bound Ca2+ has ever been observed in crystal structures of lens βγ-crystallins [45]. Vertebrate βγ-crystallins likely traded the Ca2+ binding site for improved stability [44,45]. It is therefore unlikely that Crygn is critical for Ca2+ homeostasis in SOC neurons. This is in agreement with the different time periods of cell death in mice lacking Crygn (after P4) or L-type Ca2+ channels (P0—P4).

Alternatively, Crygn might have a presynaptic role. B-crystallin family members were previously shown to be present in filopodial protrusions and axons of retinal ganglion cells and to promote axon regeneration [46–48]. Furthermore, β-crystallins were observed in neurites and growth cones of hippocampal neurons [46]. We did not observe Crygn in growth cones during prenatal development (data not shown) and only in the acoustic stria of rats, but not in other species. This renders such a presynaptic role questionable. The identification of the precise function of Crygn therefore requires further studies.

**Altered ABR in CrygnEgr2 mice**

The analysis of auditory brainstem responses revealed a significant increase of the amplitude of wave IV, without detectable differences in the ABR thresholds or peak latencies (Fig 8). Wave IV likely reflects activity of the nuclei of the lateral lemniscus and the inferior colliculus [32,33]. Both nuclei are not targeted by the Egr2::Cre driver line [17], as their neurons are born outside r3 and r5 [49]. This suggests that the observed increase in amplitude reflects altered input from lower auditory centers. This assumption is compatible with the reduced number of MNTB neurons in CrygnEgr2 animals. MNTB neurons make inhibitory projections to various nuclei, including the LSO, the MSO, and the superior paroalivary nucleus within the SOC, and to the nuclei of the lateral lemniscus, which all project to the inferior colliculus [50–53]. Lack of inhibitory MNTB neurons therefore likely causes increased activity in the lateral lemniscus and inferior colliculus. However, this explanation cannot fully account for the phenotype, as indicated by anatomical and functional analyses in contactin 5 null mice and En1Egr2 conditional knockout animals. Contactin 5 null mice, which lack 10% of MNTB neurons, show mainly an increased interpeak latency between waves III and IV, whereas the amplitude of wave IV was unchanged [34]. In En1Egr2 mice, which lack the transcription factor En1 in r3 and r5 derived cells, the MNTB is entirely absent [54]. These animals exhibit a decreased wave
III amplitude, whereas waves I, II, and IV were unaffected [54]. Thus cell loss in the MNTB alone cannot account for the altered amplitude of wave IV in our mouse model. It is thus likely, that molecular alterations contribute to the observed phenotype in CrygnErg2 mice. This is in agreement with a comparative analysis of Canca1cErg2 and Cacna1dEgr2 mice, which lack the L-type Ca2+ channels Ca1.2 or Ca1.3, respectively, in the auditory hindbrain. Despite a similar cell loss in major nuclei of the SOC such as 46% (Canca1cErg) and 53% (Cacna1dEgr2) in the LSO, alterations in ABRs markedly differed between these two mouse lines. Loss of Ca1.2 increased interindividual variation in latency of the negative peaks III and IV [21], whereas lack of Ca1.3 resulted in increased amplitudes of waves II and III [22]. Altogether, these data support the notion that altered ABRs do often only poorly correlate with structural abnormalities in the auditory hindbrain.

Species-specific expression pattern of γ-crystallins

Our immunohistochemical analyses revealed considerable differences in the immunoreactivity pattern and signal strength between rats, mice, and gerbils. This could reflect species-specific differences in the affinity of the antibodies used. However, crystallins are highly conserved and species-specific immunolabeling patterns were observed for all three antibodies (Figs 2–4). It is therefore more likely that our species-specific differences add to the reported highly variable expression of γ-crystallins between species or even strains. Considerable variability is observed between vertebrates, with fish and rodents showing high levels, whereas other terrestrial species show much lower levels [1]. Some γ-crystallins have even been converted to pseudogenes during evolution such as Cryge and Crygf in humans [1]. Finally, even mouse strain-specific differences exist with high levels of γ-crystallins in the retina of C57BL/6 mice and relatively low levels in DBA/2J animals [8]. It will therefore be interesting to study whether Crygn has a similar function in the auditory brainstem of other mammals such as the gerbil or the rat.

γ-crystallins expression in the retina was suggested to be regulated by Maf transcription factors, based on a bioinformatic analysis [7]. Subsequent studies in various Maf transcription factor knockout mice such as a MafA/MafB double knockout mouse line observed no alteration in the expression of γ-crystallins [55]. This points to a Maf-independent expression of γ-crystallins in the retina. Our data in the brainstem conform to this assumption. Expression of γ-crystallins is generally higher in the MNTB compared to the LSO and MSO, whereas expression of MafB is high in the latter two nuclei, but absent in the MNTB [56]. Thus, other transcription factors than MafB have to account for the different expression of γ-crystallins within the SOC and between rodents.

In summary, our data add Crygn to genes such as Cacna1c [21], Cacna1d [22], contactin-5 [34] or En1 [57,58], that are important for maintenance of auditory hindbrain structures after completion of migration. Our results thus demonstrate that γ-crystallins can serve important extralenticular function as well. The observed cell death after P4 extends the previously established vulnerable period between P0 and P4 in second-order auditory neurons to later stages of terminal differentiation.

Materials and Methods

Animals

The Crygn transgenic mouse (Crygn<sup>β</sup>, GenBank accession number JN950846.1) was provided by the International Knockout Mouse Consortium (IKMC project 41021) and carries a floxed exon II Crygn allele (Fig 6A). The locus was genotyped with the primers 5’-TTAGGACCTTCTCCTGAACACC-3’, 5’-CCTCGTACAGAGTGATCTGG-3’ and 5’-CTACATAGTTGGCAGTGTGTTGG-3’, which amplify in the wild-type (wt) a 524 bp band and in the mutant locus a
207 bp band. Homozygous Crygn<sup>fl/fl</sup> mice were crossed with mice containing the locus Egr2::Cre [20] in the heterozygous state. Mice carrying two floxed Crygn alleles and one Egr2::Cre allele were designated Crygn<sup>Egr2</sup>. Mice of both sexes were used and littermates served as controls. Control littermates were either Crygn<sup>wt/fl</sup> or Egr2::Cre;Crygn<sup>wt/fl</sup>, as no differences between these genotypes were observed throughout the study. The day of birth was taken as P0. All protocols were approved by the local animal care and use committee (LAVES, Oldenburg or the Animal Care and Ethics Committee of the regional board of the Federal State Government of Baden-Württemberg, Germany). All experiments were in accordance with the regulations of the German federal law on the care and use of laboratory animals, and followed the guidelines of the EU Directive 2010/63/EU for animal experiments.

To confirm recombination in the auditory hindbrain, adult Crygn<sup>fl/fl</sup> transgenic mice with and without Egr2::Cre were killed by CO2 gassing. Brains were removed from the skull and the SOC region was cut out from 100-μm-thick coronal cryosections. Isolated genomic DNA from this tissue was subjected to PCR using the primers 5’-CCATCGCCATCTGCTGCACG-3’ binding at the very 3’ end of the lacZ gene and 5’-AGGCATTTACAAACATCTCCCCGGG-3’ binding downstream to the floxed exon 2 (Fig 6A and 6B). The expected product size after recombination is 604 bp, whereas the unrecombined sequence is 3,411 bp in length and not amplified under the PCR conditions used (30 s extension).

Molecular cloning of Cryg flag fusion constructs

PCR products including flag coding sequences at the 5’-end were generated with the primers 5’-ATTCTAGATGGACCTACAAAGGCGAGGAGGGAAGATCACCTTCTATGAG-3’ and 5’-ATAGAATTCTCATAGAAATCCATGACG-3’ for Crygd and 5’-ATTCTAGAATTCTCACAAAGGCGAGGAGGGAAGATCACCTTCTATGAG-3’ and 5’-ATAGAATTCTCATAGAAATCCATGACG-3’ for Crygn using Phusion polymerase (Thermo Fisher), phosphorylated with polynucleotide kinase (Thermo Fisher), and ligated into the EcoRV site of pcDNA3.1/Zeo- (Thermo Fisher, Darmstadt, Germany).

Primary Antibodies

The mouse Crygn peptide CDNFQDQGFMNRVN that was predicted to be antigenic and to discriminate among related proteins was synthesized at Pineda (Berlin, Germany), conjugated to carrier, and used to immunize rabbits. The rabbit anti-Crygd/e was produced by Genescript (Piscataway Township, NJ, USA) by immunization with the peptide RGFQGRHYECSTDHC that is present in both Crygd and Cryg/e. Antibodies were cleaned up by affinity purification. The primary antibody rabbit anti-Cryg was kindly provided by Dr. Samuel Zigler. All three primary antibodies were used in the following dilutions: immunohistochemistry: 1:50 for anti-Crygd/e and Crygn; 1:200 for anti-Cryg, immunocytochemistry: 1:100, and immunoblotting 1:250 for all three antibodies. The primary antibodies guinea pig anti-GlyT2 (Millipore; Darmstadt, Germany) (1:1,000), guinea pig anti-VGluT1 (Millipore) (1:5,000) and the rabbit anti-GlyT2 (1:1,000) have been characterized previously [59,60]. Mouse anti-flag was obtained from Genescript (1:500).

Immunolabeling

Immunohistochemistry was performed in wt mice, Crygn transgenic mice, gerbils and rats of both genders. Three animals were used for each species and age. Animals were deeply anesthetized with chloral hydrate (700 mg per kg body weight i.p.) and perfused transcardially with 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% PFA (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed and stored in fixative overnight at 7°C.
incubation in 30% sucrose/PBS for cryoprotection, 40-μm-thick (P4) or 30-μm-thick (>P4) coronal sections were cut through the hindbrain, collected in 15% sucrose/PBS, thoroughly rinsed in PBS, and blocked for 1 h in blocking solution (2% bovine serum albumine (BSA), 10% goat serum, and 0.3% Triton in TRIS-buffered saline (pH 7.4)). Primary antibodies were added to the blocking solution. Incubation with agitation was carried out at 4°C overnight. After three rinses in PBS, sections were transferred to carrier solution (0.3% Triton, 1% BSA, 1% goat serum) and treated with the secondary antibodies, goat anti-rabbit conjugated to Alexa Fluor 488, goat anti-rabbit conjugated to Alexa Fluor 568, goat anti-guinea pig conjugated to Alexa Fluor 568, or goat anti-guinea pig conjugated to Alexa Fluor 488 (all diluted 1:500, Thermo Fisher). Sections were incubated 1.5 hrs at RT, rinsed again with PBS, mounted on slides, air dried, and coverslips were mounted with Mowiol. Control experiments were performed by omission of secondary antibodies and resulted in the absence of immunosignals. Images were taken with a BZ 8100 E fluorescence microscope (Keyence, Neu-Isenburg, Germany).

For immunocytochemistry, Crygd or Crygn flag fusion constructs were transiently transfected into HEK293 cells grown on cover slips. After two days of incubation at 37°C and 5% CO₂ cells were rinsed with PBS, incubated for 10 min with 4% PFA, rinsed three times in PBS, and blocked for 30 min in blocking solution. Primary antibodies were applied in carrier solution and incubated for 1 h at RT. After three rinses in PBS, the secondary antibodies anti-rabbit conjugated to Alexa Fluor 488 and anti-mouse conjugated to Alexa Fluor 568 were added (both diluted 1:500, Molecular Probes, The Netherlands). Cover slips were incubated 1.5 hrs at RT, rinsed again with PBS, mounted on slides with Mowiol and air dried. Images were taken with a BZ 8100 E fluorescence microscope (Keyence).

Protein isolation and immunoblot

Brains were lysed in a buffer containing 150 mM NaCl, 15 mM Tris, 1% dodecyl-β-D-maltoside, and 0.4% iodoacetamide. After incubation for 5 min at 25°C, samples were centrifuged for 5 min at 125,000 g. Protein amount of the supernatant was determined using the Bradford assay. 10 μg of each sample were loaded onto a 10% SDS-polyacrylamide gel system. After separation and electrotransfer onto PVDF membranes, membranes were incubated with primary antibodies (dilution 1:250). After incubation for 2 hrs at room temperature, membranes were washed four times with TBS-T (20 mM Tris, 150 mM NaCl, 1% Tween, pH 7.5) and the secondary antibody donkey anti-rabbit IgG-HP (Santa Cruz Biotechnology, Heidelberg, Germany) was applied for 1 h. After washing, bound antibodies were detected using an enhanced chemiluminescence assay (GE Healthcare, Hamburg, Germany) and a LAS-3000 documentation system (Fujifilm, Düsseldorf, Germany).

RNA in situ hybridization

Two different Crygn-derived PCR products were generated. One PCR product (primers 5'-GGAGCATGTTGACTACCTG-3' and 5'-CTGGATCTTTATTGCCTCGT-3') amplified sequences from exon 2 to exon 4, the other product (primers 5'-ATCACTCTGTACAGAGGCAAG-3' and 5'-CATGCCACAGCCGACAG-3') amplified only exon 2. Both products were cloned into pGEMTeasy (Promega). Sequence verified clones were used as template for transcription of RNA in situ hybridization probes in the presence of digoxigenin-11-UTP (Roche Applied Science, Mannheim, Germany). 30-μm-thick coronal cryosections were cut in a cryostate (Leica, Wetzlar, Germany) using 4% PFA fixed brains. After proteinase K treatment (10 μg/ml) and deacetylation (12.5 μl acetate anhydride in 5 ml 0.1 M Triethanolamin + 0.9% NaCl), slices were incubated in hybridization buffer (50% v/v formamide, 5x SSC, 20% v/v
blocking solution, 0.2% SDS, 1% n-lauroyl sarcosinate) for 1 h, followed by an incubation overnight with the probes (1 μg/ml in hybridization buffer). Both steps were performed at 55°C. After washing for 30 min each at 45°C with 2xSSC, 0.5xSSC and PBS-0.1%-Tween, slices were incubated for 1 h with blocking solution (1% blocking reagent (Roche Applied Science) in maleic acid buffer (pH 7.5) at RT, followed by incubation with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Applied Science) (1:1,000 in blocking solution) for 1.5 hrs at RT. Detection occurred with the substrate NBT/BCIP Stock Solution (Roche Applied Science) diluted 1:50 in AP-Buffer (100 mM Tris-HCl, pH 9.5, 150 mM NaCl, 5 mM MgCl₂) at RT. Sense probes served as negative controls and yielded no signals.

Anatomical analyses

Nissl staining was performed on consecutive 30-μm-thick coronal cryosections. The volume of auditory nuclei was calculated by multiplying the outlined area with the thickness of each section [38, 61]. Three animals aged P4 or P25 were used from each genotype and the SOC of both sites were analyzed. Analysis was carried out blind to the respective genotype. Sections were then analyzed using ImageJ, and statistical analysis was performed using two-tailed Student’s t test after testing for Gaussian distribution of the datasets.

Auditory Evoked Brainstem Responses

Five Crygn<sup>Er2</sup> mice and four littermate controls (aged 8–10 weeks) were used to test cochlear and brainstem auditory responses. Experiments were performed in a soundproof chamber (IAC) as described [62]. In short, for stimulus generation and recording a multi-function IO-Card (PCI-6052E, National Instruments, USA) was used, housed in an IBM compatible computer. Sound pressure level was controlled with attenuators and amplifiers (Wulf Elektronik, Frankfurt, Germany). Stimuli were delivered open field to the ear by loudspeakers either placed 3 cm lateral to the animal’s pinna or placed as closed field probe for otoacoustic measurements. Sound pressures were calibrated online prior to each measurement.

Animals were anaesthetized by intraperitoneal injection with a mixture of 0.05 mg/kg b.w. fentanyl dihydrogen citrate (Fentanyl Ratiopharm, Ulm, Germany), 5 mg/kg b.w. midazolam (Dormicum Roche Pharma AG, Grenzach-Wyhlen, Germany Germany), 0.5 mg/kg b.w. medetomidin hydrochloride (Sedator Eurovet Animal Health B.V., Aulendorf Germany). To support the heartbeat and to prevent circulation depression, a single dose of 0.2 mg/kg b.w. atropine sulfate (Atropin B.Braun, Melsungen, Germany) was applied within the first 30 min of anaesthesia. Additional doses of anaesthetics were applied in amounts of 1/5 to 1/3 of the initial dose when needed, usually every hour.

Auditory brainstem responses (ABRs) were measured by averaging the evoked electrical response recorded via subcutaneous silver wire electrodes at the ear (active), vertex (reference), and the back (ground) of the animal. Briefly, ABRs were evoked by click (100 μs), noise burst (1 ms static random phase noise) or pure tone stimuli (3 ms duration, 1 ms rise/fall times, frequencies: 2–45.3 kHz, two septs per octave) of gradually increasing sound pressure in 5 dB steps of intensity from 0 to 100 dB SPL at a repetition rate of 60/sec.

Stimuli were presented in open field by a loudspeaker (DT-911, Beyerdynamic, Heilbronn, Germany) and online calibrated with a microphone (B&K 4191, Bruel & Kjaer, Naerum, Denmark) placed near the animals’ ear. The recorded signal was amplified (80 dB), filtered (0.2–5 kHz) and added for alternating phase or polarity to omit the stimulus artefact and cochlear microphonics. Hearing thresholds were determined for click, noise, and each stimulus frequency as the minimal sound pressure evoking a noticeable potential peak in the expected time window of the recorded signal (for details see [61]). From the ABR recordings to increasing
stimulus levels, thresholds were defined as the sound pressure level where a stimulus-correlated response was clearly identified by visual inspection of the averaged signal. ABR waveforms were analysed for consecutive amplitude deflections (waves), with each wave consisting of a starting negative (n) peak and the following positive (p) peak. Peak amplitudes of ABR waves I, II, III, and IV were extracted with a customized program based on peak time and amplitude. ABR wave amplitude (μV) growth functions were constructed for individual ears based on the extracted peaks and plotted as I/O function with increasing stimulus level (from −20 to a maximum of 75 dB above threshold).

Cochlear outer hair cell (OHC) function was assessed by the response strength and response threshold from the growth function and the distortion product audiogram of the cubic distortion product otoacoustic emission (DPOAE). The cubic 2×f1−f2 distortion product of the otoacoustic emission (DPOAE) for f2 = 1.24×f1 and L2 = L1−10 dB were recorded in a soundproof chamber (IAC, Niederkrüchten, Germany) as previously described [63]. In short, frequency pairs of tones were between f2 = 4 kHz and f2 = 32 kHz were presented directly into the ear canal by means of a metal coupler connected to two loudspeakers (DT-911, Beyerdynamic). The emission signals were recorded by a microphone (MK 231, Microtech, Gefell, Germany; Preamplifier Brüel & Kjaer 2670, Naerum, Denmark) connected to the coupler. Emission signals were recorded during sound presentation of 260 ms and averaged four times for each sound pressure and frequency presented. For the DP audiogram, the 2f1−f2 distortion product amplitude was measured at constant L2 of 50 dB SPL with f2 varying from 4 to 32 kHz in four steps per octave. The growth function of the 2f1−f2 distortion product amplitude was measured for L1 ranging from 0 to 65 dB SPL with frequencies f2 ranged between 4 and 32 kHz in half octave steps. Threshold was determined as the L1 sound pressure that could generate a 2f1−f2 signal reliably exceeding about 5–10 dB above noise level with noise level typically at -20 dB SPL.

Auditory steady state responses (ASSRs) were used to investigate the temporal capacity of auditory neurons in the auditory pathways. ASSRs are generated by synchronous neuron discharges phase-locked to the modulation frequency of an amplitude or frequency modulated pure tone [64]. ASSRs were measured with amplitude modulated sinusoidal stimuli (carrier frequency 11.3 kHz). The stimuli were presented between -20 and +40 dB around threshold (dB hearing level) in steps of 20 dB. Stimuli were amplitude modulated between 0.8% and 100% and presented for modulation frequencies between 64 Hz and 2,048 Hz in half-octave steps. Unmodulated stimuli (0%) were presented for control. Responses to 1,114 ms long presentations were averaged for 32 repetitions and transformed into frequency energy spectra by fast fourier transformation. From the fast fourier transformation, signal strength at a given modulation frequency was calculated as signal above noise (in nV) within a 16 Hz frequency window. For statistical analysis, data were compared for statistical difference by means of 2-way repeated measures ANOVA (2-way ANOVA) for the factors genotype and stimulus frequency (Hz) or the factors genotype and stimulation level (dB SPL) (Graphpad Prism 6.0, San Diego, USA) and pair-wise Holm-Sidak’s multiple comparisons test as posthoc-test (alpha level corrected for repeated measurements). Mean values are quoted ± standard deviation (SD) or standard error of the mean (s.e.m.) unless otherwise stated. P < 0.05 was interpreted as statistical significance.

Acknowledgments
We would like to thank Martina Reents for excellent technical assistance and S. Zigler for providing the Cryg antibody.

Author Contributions

Conceptualization: HH LR HGN.
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Software: LR.
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