RARβ Regulates Neuronal Cell Death and Differentiation in the Avian Ciliary Ganglion

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ABSTRACT: Programmed cell death during chicken ciliary ganglion (CG) development is mostly discussed as an extrinsically regulated process, guided either by the establishment of a functional balance between preganglionic and postganglionic activity or the availability of target-derived neurotrophic factors. We found that the expression of the gene coding for the nuclear retinoic acid receptor β (RARB) is transiently upregulated prior to and during the execution phase of cell death in the CG. Using retroviral vectors, the expression of RARB was knocked down during embryonic development in ovo. The knockdown led to a significant increase in CG neuron number after the cell death phase. BrdU injections and active caspase-3 staining revealed that this increase in neuron number was due to an inhibition of apoptosis during the normal cell death phase. Furthermore, apoptotic neuron numbers were significantly increased at a stage when cell death is normally completed. While the cholinergic phenotype of the neurons remained unchanged after RARB knockdown, the expression of the proneural gene Cash1 was increased, but somatostatin-like immunoreactivity, a hallmark of the mature choroid neuron population, was decreased. Taken together, these results point toward a delay in neuronal differentiation as well as cell death. The availability of nuclear retinoic acid receptor β (RARβ) and RARβ-induced transcription of genes could therefore be a new intrinsic cue for the maturation of CG neurons and their predisposition to undergo cell death.

Keywords: programmed cell death; ciliary ganglion; retinoic acid; RARβ; neuronal development

INTRODUCTION

In the developing nervous system, programmed cell death (PCD) plays a crucial role in the optimization of neuronal connectivity and error correction via the regulation of neuronal numbers; the elimination of
PCD leads to gross anatomical malformations (Buss et al., 2006). The chicken ciliary ganglion (CG) is a classical model to study PCD and neuronal differentiation during development, with a well-characterized 50% decline in neuron number between embryonic day 8 and 14 (E8–E14) (Landmesser and Pilar, 1974; Hamburger, 1975). There is a vast body of literature concerning PCD in the CG, discussing it as an extrinsically mediated process, guided either by the availability of target-derived neurotrophic factors, such as CNTF, BDNF, and GDNF (Barbin et al., 1987; Meriney et al., 1987; Collins et al., 1991) or the intrinsic factors, like Hox genes or bHLH and zinc finger transcription factors, are required for the proper specification of programmed cell deaths (Thellmann et al., 2003; Hoeppner et al., 2004; Simpson et al., 2013). Moreover, TGFβs were shown to be modulators of cell death in the CG (Kriegstein et al., 2000). Nevertheless, while PCD in vertebrates is commonly discussed as an extrinsically mediated mechanism, the opposite is seen in research about invertebrate development. In C. elegans, the specification of cell death has been studied for several cell types and the findings from these studies suggest that a diverse set of cell intrinsic factors, like Hox genes or bHLH and zinc finger transcription factors, are required for the proper specification of programmed cell deaths (Thellmann et al., 2003; Hoeppner et al., 2004; Liu et al., 2006).

The vitamin-A metabolite retinoic acid (RA) regulates the transcription of multiple target genes via binding to the nuclear retinoid receptors. These receptors can belong to two major families, the retinoid X receptors or the RA receptors (RARα, β, and γ), and the genes encoding these receptors are developmentally regulated and differentially expressed in tissues (Smith and Eichele, 1991). Several RAR-controlled genes are known to mediate proapoptotic activities, including caspases, Bcl-2 proteins, transcription factors that regulate apoptosis, and genes involved in DNA fragmentation (Noy, 2010). Moreover, in vitro and in vivo studies have shown that RA is involved in neuronal differentiation, cell type specification and survival (Wyatt et al., 1999; Maden, 2001; Ji et al., 2006). In the CG, RA has been shown to upregulate the expression of CNTF receptors in vitro (Wang and Halvorsen, 1998), raising the question, whether the level of RA receptors in neurons could influence survival via the regulation of CNTF responsiveness.

Concerning the plethora of signals, neurons need to integrate during the phase of PCD, the question has been raised, whether neuronal PCD is a mere stochastic process (Pettmann and Henderson, 1998). A cellular predisposition concerning its fate would help to understand the reproducibility of cell death events. Thus, an intrinsic, potentially transcription factor-driven program for PCD could also exist in vertebrates. Recently, this hypothesis gained support by a study in rodents, demonstrating that the death of interneurons during development is specified intrinsically, independently of trophic signaling (Southwell et al., 2012).

Screening the transcriptome of the developing CG, RARB expression was found to be transiently upregulated prior to and during the execution phase of PCD. Therefore, its function during CG development and neuronal cell death was investigated and challenged via RNAi-mediated knockdown experiments.

METHODS

Embryos, Fixation, and Histology

Fertilized white leghorn chicken eggs (Gallus gallus domesticus) were obtained from a local farm and incubated at 38.5°C and 70% humidity. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). At the desired stage, embryos were killed by decapitation and the heads were fixed in 4% paraformaldehyde over night, dehydrated in ascending ethanol concentrations and embedded in paraffin. Microtome sections of 10 μm were collected for hematoxylin eosin (HE) staining, immunohistochemistry and in situ hybridization. Total neuron numbers of wild type and RCASBP(B)-infected CGs were counted on HE stainings as described before (Oppenheim et al., 1989).

Immunohistochemistry

Sections were deparaffinized and heated in citrate buffer for improved antigen retrieval and further incubated with anti-islet-1/2 (40.2D6) 1:50; anti-gag (AMV3C2, 1:200; both antibodies from Developmental Studies Hybridoma Bank, University of Iowa), anti-somatostatin (Millipore; 1:100); anti-BrdU (Sigma; 1:1000); anti-active caspase 3 (R&D; 1:500) and visualized using biotinylated secondary antibodies (donkey-anti-mouse; –anti-rabbit; -anti-rat; 1:100; Dianova, Hamburg, Germany) and diaminobenzidine (Vectastain Peroxidase ABC-kit 6100, Vector Laboratories, CA, USA).

In Situ Hybridization

Sections were deparaffinized and in situ hybridization and preparation of digoxigenin-labeled probes for Cash1, chicken Islet-1, chicken ChAT, and chicken RARB were performed as described previously (Ernsberger et al., 1997). Cash1, ChAT, and Islet-1 probes were generously provided by K. Huber (Institute of Anatomy, Albert Ludwigs University Freiburg). Chicken RARB was cloned by RT-PCR using a pGEM-T vector system (Promega, Mannheim, Germany) following the manufacturer’s instruction.

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The sequence of the forward primer was CTCCAGAGTCACCCACCAAC, and the reverse primer sequence was TCCTGCGGAAAAAGCCCTTA.

**Microarray**

Ciliary ganglia were dissected from E6-E10 and E14 chicken embryos. For each analyzed time point, ganglia from at least 40 embryos were pooled for RNA extraction using the Qiagen RNeasy micro kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Four replicate sample of each time point were obtained. For transcriptome analysis, 4×44K Agilent (Santa Clara, CA, USA) whole transcriptome arrays were used. The raw intensity reads for each array were background corrected by spatial detrending and subsequently quantile normalized for comparison across arrays using the limma package from Bioconductor (http://www.bioconductor.org). Probes with low interquartile range across arrays and probes with low intensities were filtered out. Subsequent analysis considered only genes having a corresponding EntrezID annotation. If multiple probes mapped to the same EntrezID, the probe having the largest IQR was chosen and all others discarded, leaving 13533 transcripts for analysis. Gene expression analysis was carried out using a custom-made script in collaboration with H. Busch and M. Boerries. Differentially expressed genes were further analyzed using the Ingenuity software package (Qiagen, CA, USA).

**Quantitative Real-Time PCR**

RNA was isolated from ciliary ganglia using the RNeasy micro kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Schwerte, Germany). Quantitative RT-PCR analysis was performed with the MyiQ™ (BIO-RAD, München, Germany) and the GoTaq® qPCR Master Mix (Promega, Mannheim, Germany) with 5 ng of cDNA template in a 12.5 μL reaction mixture. RARB expression level was assessed at three different time points during development (E7, E9, and E14), with three independent biological replicates for every time point and three technical replicates. The expression levels were normalized to chicken GAPDH and results were analyzed with the comparative CT method. Data are expressed as 2^(-ΔΔCT) normalized to GAPDH and presented as fold change of expression values at E7 and E9 relative to the expression value at E14.

**Cell Culture and siRNA Transfection**

siRNAs were designed as 19mers with the help of eurofins mwg operon siRNA-design tools (eurofins, Ebersberg, Germany). Three different siRNAs were chosen to cover different regions of the RARB mRNA, according to the pattern AA(N19) and with a GC-content between 36 and 52%. Furthermore, one control siRNA with a Cy3-tag was ordered, that does not bind to any mRNA within the cell. Sequences were siRARB1: UCAUCUGGAUACACUAUG, siRARB2: UUACCCUUCUACACUAACA, siRARB3: CCACUGACACCAACCUCAA, siControlCy3: AAGUAGUGUAAUGCCCUUGUU. DF-1 cells (Himly et al., 1998) were cultured in DMEM medium containing 10% fetal calf serum and 1% penicillin, streptomycin and neomycin and maintained in a humidified 5% CO₂/95% humidified air atmosphere at 37°C. The cells were transfected with the RARB siRNAs using either Dharmafect reagent (Thermo Fisher Scientific, Schwerte, Germany) or Nanofectin (PAA Laboratories, GE Healthcare, Freiburg, Germany) according to manufacturer’s instructions.

**Production of shRNA Virus**

RCAS(BP)B virus carrying an shRNA construct against RARB was produced according to (Das et al., 2006). The primers used for the RARB hairpin in the cloning oligos were shRARB forward: gagaggtgctgctgacggccctgacacccactaggcaaccactgacaccaacctcaattagtgaagccacagatgta and shRARB reverse: attcaccacactggcaacctgaacctcaacctaatctgatggtgacatt. DF-1 cells were transfected with the RCAS(BP)B shRARB plasmids and RCAS(BP)B plasmids without an shRNA insert using Lipofectamine 2000 (Invitrogen, Life Technologies, Darmstadt, Germany). Virus was concentrated from the culture medium using Amicon ultra-4 centrifugal filter units (Merck Millipore, Darmstadt, Germany) with a 100 kDa cutoff. Virus stock aliquots were stored at −20°C until use.

**Injection of Virus into Chick Embryos**

Eggs were incubated until the desired stage (HH9). Virus stock was mixed with a vital dye (Fast Green, Sigma) and backfilled into a glass capillary, prepared in a Flaming/Brown micropipette puller (Shutter Instruments Co, USA). The virus was injected into the neural tube of the embryo at the level of the mesencephalon, where the progenitors of the CG neurons will delaminate (Narayanan and Narayanan, 1981). The eggs were further incubated and the embryos were sacrificed by decapitation at day 14. BrdU injections were performed according to (Striedter and Keeser, 2000), shortly, BrdU (Sigma) was injected at a concentration of 20 μg/μL into one of the embryo’s vitelline veins. The embryos were further incubated for 3 h before fixation at E7 and E9 or until E14 to visualize all cells proliferating between E7 and E14.

**Statistics**

Data are expressed as means ± SEM. Two-group analysis was performed using Student’s t-test. Values of p < 0.05 were considered as statistically significant. All statistical analyses were performed using the GraphPad Prism6 software (Graphpad Software Inc.).
RESULTS

RARβ Expression Is Regulated during PCD in the CG

Cell counts of CG neurons between E7 and E14 revealed a 56% reduction in neuron number during the time of PCD from approximately 6700 (6745 ± 308) neurons at E7 to a final number of approximately 3000 neurons (2972 ± 204) at E14 [Fig. 1(A)], a finding that is in line with the classical literature (Landmesser and Pilar, 1974). Phenotypically, the ganglion itself as well as the neurons notably increases in size during differentiation [Fig 1(B)]. The largest number of active caspase-3 positive apoptotic neurons are seen at E9 and E10 as seen in a staining against active caspase 3. (D) Time course of the expression of retinoic acid signaling components. Heatmap of z-scores of gene expression during development. Multiple components are upregulated during the induction and specification phase of ontogenetic PCD. The expression of the gene coding for RARβ is found to be significantly upregulated at E8 and E9, while expression levels are low at early (E6, E7) and late (E10, E14) stages of development. This regulatory pattern could be corroborated by qPCR (E) and (F). In situ hybridization (scale bar = 50 μm). (G) Higher magnification of the RARB in situ signal at E9. RARB appears to be located in all CG neurons (scale bar = 20 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Therefore, the six developmental time points chosen for transcriptomic analysis cover the specification and induction phase of PCD (E6–E8) as well as the early executive phase (E9/10). Gene expression levels at E14 were chosen to serve as a steady-state reference point in the analysis. The initial screen of the data yielded a wide array of differentially regulated genes, that is reviewed elsewhere (Koszinowski et al., in preparation). Focusing on genes that show a differential expression during specification and induction of cell death, components of RA signaling were found to be upregulated at the corresponding developmental stages, according to a z-score analysis [Fig. 1(D)]. The expression level of the gene coding for the nuclear retinoic acid receptor beta (RARB) was found to peak at E8 and E9 [Fig. 1(D)] with a 1.9-fold increase \( p = 0.0018 \) as compared to E14. This regulatory pattern could further be corroborated by qPCR analysis on independently collected cDNA samples [Fig. 1(E)]. To confirm the neuronal origin of this increase in RARB expression, an in situ hybridization against RARB mRNA was performed [Fig. 1(F)]. At all stages of development studied, the RARB in situ signal was located in the ganglion neurons. Moreover, the peak of RARB expression at early stages of PCD, namely at E9, could also be observed in the in situ hybridization. RARB appears to be expressed in all CG neurons [Fig. 1(G)]. Individual neurons on the sections although seem to exhibit higher levels of RARB expression than neighboring cells. But this observation could also be assigned to differences in the cutting levels of neurons and thickness of the material in general and does not provide conclusive evidence to identify a subpopulation of neurons expressing higher levels of RARB.

Nevertheless, the expression pattern of RARB during development detected in the microarray, qPCR and in situ hybridization argued for a role of RA signaling in the regulation of PCD in the CG and pointed toward the nuclear RA receptor beta as a central mediator of the pathway.

**RNAi Mediated Knockdown of RARB In Vitro**

To address the role of RA signaling in CG development, we opted for an RNAi-based knockdown approach, targeting the RARβ. Before starting the in vivo RNAi experiments, however, a pilot in vitro study was performed, testing multiple siRNAs designed against RARB in DF-1 cell culture. RARB is expressed in DF-1 cells [Fig. 2(A)], showing that cell viability is not affected by siRNA transfection and the DF-1 cells are readily transfected with the siRNA construct. DF-1 transfection with the RARB-specific siRNAs was conducted according to the control treatments. After 24 h of exposure to the respective siRNA, RNA was extracted from the DF-1 cells and knockdown success was validated using qPCR [Fig. 2(C)]. At the recommended concentration of 25 nm, all RARB siRNA constructs yielded a significant knockdown of gene expression with only minor differences in effectiveness when compared to cells sham-transfected with the unspecific control siRNA or cells treated with the respective transfection reagent alone. Out of the three siRNA constructs designed for each gene, the most effective siRNA was tested at a lower concentration (5 nm) and was also validated using a different transfection reagent (Nanofect). In all of these experiments, the respective siRNAs proved to significantly downregulate RARB expression [Fig. 2(C)]. Therefore, siRNA-design parameters were correctly chosen and these parameters were further used in the design of the cloning oligos that, together with generic flanking sequences, would generate the endogenously expressed shRNA-hairpins in the in vivo RNAi experiments.

**In Vivo Knockdown of RARB Leads to an Increase in CG Neuron Number**

The RCAS(BP)B-plasmid carrying the micro-RNA operon expression cassettes with the shRNA-hairpin insert against RARB (RCASBshRARB) was constructed according to (Das et al., 2006) and transfected into DF-1 cells. After one week of incubation, the virus-containing culture medium was harvested and concentrated. The concentrated virus-solution was injected into the neural tube of HH stage 9 chicken embryos at the level of the mesencephalon [Fig. 3(B)]. Progenitors of CG neurons delaminate from the neural crest at this time point and were, therefore, targeted with the injection (Narayan and Narayan, 1981), an approach that has been proven successful (Hruska and Nishi, 2007). The overall survival rate of injected embryos was relatively poor;
generally only about 20% of the injected embryos survived until E14. Nevertheless, embryos that survived until E14 did not show physical malformations or other obvious changes in morphology. The viral infection of the CG was always bilateral, due to the distribution of viral particles throughout the neural tube upon injection. Only ganglia showing a complete infection of all neurons, as demonstrated by similar

Figure 2  RNAi mediated knockdown of RARB in vitro. (A) DF-1 exposure to the transfection agent as well as scramble Cy3-tagged control siRNA exposure did not affect cell viability. Twenty-four hours after transfection, all cells are positive for Cy3 fluorescence. (Scale bar = 20 μm). (B) Agarose gel electrophoresis of RARB PCR product as detected in E9 CG and DF-1 cell lysate-derived cDNA. Marker is a 100-bp ladder of molecular weight markers. (C) RARB siRNA-mediated knockdown. All tested siRNA concentrations yield a significant knockdown of RARB expression and levels of knockdown are comparable across all samples. (light gray bars: Transfection with Dharmafect; dark gray bar: Transfection with Nanofectin) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
numbers of islet-1/2 and gag-positive neurons [Fig. 3(C)], were subjected to further analysis. Approximately 50% of the surviving embryos showed such a successful infection of the ganglion that lasted and could be detected until E14 [Fig. 3(A)]. The RARB mRNA level was clearly decreased in ganglia after shRARB infection [Fig. 3(E)]. CG neurons were counted on HE-stained sections of E14 virus-infected CGs and the total cell number was calculated, as previously described (Krieglstein et al. 2000). The numbers were compared to uninjected wild type embryos of the same stage as well as to embryos, which were injected with RCASB virus without any shRNA insert (RCASB CTL). The injection of RCASB CTL did not alter the number of CG neurons at E14. Therefore, the injection with the virus as such, and the subsequent infection and spread of the viral particles does not influence PCD during embryonic development. The overall size of the ganglion was not increased after RARB knockdown [Fig. 1(A)], but infected CGs showed a striking increase in neuron number at E14 as compared to control embryos [Fig. 3(D)]. Precisely, the neuron number at E14 increased by 66% after RCASBshRARB injection (4949 ± 312, in the knockdown vs. 2973 ± 204.8 in the wild type control), suggesting a role for RARB in the regulation of neuronal PCD in the CG.

**The Increased Neuron Number after RARB Knockdown Is Due to a Delay in Neuronal Programmed Cell Death**

Neurons undergoing apoptosis were visualized with a staining against active caspase 3 on E7, E9, and E14 wild type and RCASBshRARB infected ganglia.

As described before, the number of CG neurons undergoing PCD during development peaks at E9 while at E7 and E14 only very few apoptotic neurons can be found in wild type CGs [Fig. 1(C), Fig. 4(A, C, E)]. After RARB knockdown, similar numbers of apoptotic neurons were found in E7 CGs, but at E9 and E14, the numbers significantly differed from the control [Fig. 4(G)]. At E9, there were hardly any neurons found undergoing PCD in the RCASBshRARB infected ganglia [Fig. 4(D)], while at E14, significantly more apoptotic neurons were found than in the control CGs [Fig. 4(F)]. In fact, the number of neurons undergoing apoptosis in RCASBshRARB infected ganglia at E14 was similar to the number counted in the E9 control animals [Fig. 4(G)]. This finding suggests that PCD in the developing CG could be delayed after knockdown of RARB.

CG neurons become postmitotic shortly after their migration from the neural crest (Landmesser and...
Figure 4.

**RARβ Regulates Neuronal Cell Death**
Moreover, at E7–E9, the ganglion also contains non-neuronal neural crest derived cells, being yet undifferentiated or expressing glial properties (Lee et al., 2001). Nevertheless, a small pool of neuronal precursors has been shown to retain the ability to divide and generate neurons until E7–E8 at least in vitro (Gilardino et al., 2000). To exclude the possibility, that the increase in CG neuron number after RARB knockdown is due to the proliferation of such ganglion neuron progenitors, an anti-BrdU staining on RCASBshRARB and control ganglia was performed. Chicken embryos were injected with BrdU at E7 and E9 into one of the vitelline veins and fixed and processed for paraffin embedding 3 h after the injection. Wild type ganglia and RCASBshRARB-infected ganglia were stained for BrdU and cells within the ganglion, having incorporated BrdU, were counted on every tenth section. The mean number of proliferating cells per section and ganglion was calculated. There was no difference in the number of BrdU positive cells counted in the control and the RCASBshRARB infected ganglia [Fig. 4(H)]. Roughly the same number of cells had incorporated BrdU at E7, as well as at E9. The cells that were found to have incorporated BrdU were satellite cells; no trace of BrdU staining could be detected in the nucleus of a CG neuron at either time point. When injecting embryos with BrdU at E7 and incubating them until E14, numerous BrdU-positive nuclei can be detected that are evenly distributed in the ganglion [Fig. 4(I,J)] and are surrounding the neurons [Fig. 4(I′,J′)]. It can therefore be concluded that only glia and other non-neuronal cell types divide in significant numbers after E7 and RARB-deficiency does not affect the proliferation within the CG or lead to aberrant neuronal proliferation. Thus, the increase in neuron number after RARB knockdown is a result of a delay in naturally occurring PCD, as observed in the caspase-3 staining.

**Figure 4** The increase in neuron number after RARB knockdown is due to a delay in neuronal programmed cell death. (A–F) Wild type control and RCASBshRARB infected CGs were stained for active caspase 3 at E7, E9, and E14 and apoptotic neurons (arrows) were counted. (G) After knockdown of RARB, neuronal apoptosis did not take place at E9 (D), as is seen in the wild type ganglia (C) (n(WT E9) = 3; n(shRARB E9) = 3; *p = 0.031). Apoptotic cell death in shRARB infected ganglia was seen to occur at E14 (F) (n(WT E14) = 3; n(shRARB E14) = 3; *p = 0.023), when similar numbers of apoptotic cells were counted as in E9 wild type ganglia. (H) RARB knockdown has no effect on the number of BrdU-positive proliferating cells within the CG at E7 and E9. (I,J) There is no abnormal neuronal proliferation during the time of PCD in the CG. shRARB infected embryos, injected with BrdU at E7 and fixed at E14(J) show a similar pattern of BrdU-positive satellite cells as the wild type control (I). Scale bars correspond to 50 μm (A–F) and 20μm (I′, J′), respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
the expression of somatostatin in the CG became questionable, when PSS1 mRNA, encoding somatostatin, could not be detected in the CG, but PSS2, coding for cortistatin, was found to be expressed (Nishi et al., 2010). Somatostatin and cortistatin are structurally closely related and share many pharmacological and functional properties (Spier and de Lecea, 2000).

To investigate a possible association between Cash1-positivity and choroid neuronal subtype, sections were therefore stained with an antibody detecting both somatostatin and cortistatin, hence the signal

**Figure 5** Knockdown of RARB affects the differentiation of CG neurons. During normal development, the expression of Cash1 in CG neurons decreases continuously between E7 (A), E9 (C), and E14 (B), when the expression of Cash1 is restricted to very few neurons. In RCASBshRARB infected ganglia, more neurons show Cash1 expression (D). Across all samples, significantly more neurons express Cash1 at E14 after RARB knockdown as compared to control (I). (n(CTL) = 3; n(RCASBshRARB) = 5; **p = 0.0088.) At E14, ciliary ganglia show a prominent SOM-LIR in the choroid neuron population (E). In RCASBshRARB infected ganglia, less neurons show SOM-LIR (F) and the signal is generally fainter. (I) Quantification of the decrease in the number of SOM-LIR positive neurons after RARB-knockdown (n(CTL) = 3; n(RCASBshRARB) = 5; #p = 0.057). ChAT expression remains unchanged by RARB knockdown (G,H). Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
detected will further be called somatostatin-like immunoreactivity (SOM-LIR), according to literature. During development, the number of neurons showing SOM-LIR increases, corresponding to the maturation of the choroid neuron population (Darland and Nishi, 1998). At E14 [Fig. 5(E)], the choroid neuron population shows a mature phenotype with a clear SOM-LIR. The smaller size and peripheral location of the choroid subpopulation could also be confirmed [Fig. 5(E)]. Nevertheless, when comparing the Cash1-signal [Fig. 5(B)] to the somatostatin-like expression, too few neurons appear to show positivity for Cash1 to represent the entire choroid population.

To better compare the two signals, SOM-LIR and Cash1 positive neurons were again counted on directly adjacent sections [Fig. 5(J), white bars], showing that there are indeed far more SOM-like positive choroid neurons detectable, even on adjacent CG sections, than neurons expressing Cash1. Thus, no direct relationship between choroid phenotype and Cash1-positivity at E14 can be concluded, but based on the overlapping locations of the signals; a partial concurrence of these features remains possible. The final differentiation and maturation of the choroid neuron population could be completed later than expected, and potentially a small subset of neurons might even remain undifferentiated altogether.

**Knockdown of RARB Affects Neuronal Differentiation**

As mentioned before, the overall size of the CG was unchanged by RARB knockdown, although neuron numbers were substantially increased. Studying the morphology of the ganglion after knockdown, it appeared that a large number of neurons rather seemed to be smaller than in the wild type and therefore potentially immature. To investigate whether RARB knockdown affects the differentiation state of CG neurons, in situ hybridization against Cash1 was conducted.

More neurons than in the control situation were found to show a Cash1-signal after the knockdown [Fig. 5(D)], and these cells were located lining the edges of the ganglion, like observed in the E14 wild type before. A cell count comparing Cash1-positive neurons on control sections and RCASBshRARB infected ganglia showed a significant increase in the number of neurons still expressing Cash1 at E14 in the knockdown [Fig. 5(I)]. This finding clearly argues for a delay in the differentiation of these neurons after RARB knockdown.

Coming back to the previous assumption, that there could be a connection between choroid neuron phenotype and Cash1-expression, the SOM-like signal was investigated on E14 ganglia from RCASBshRARB-injected embryos. Fewer neurons appear SOM-LIR positive [Fig. 5(F)], a tendency that could also be corroborated by SOM-LIR \(^+\) neuron counts [Fig. 5(I)]. This decrease in SOM-LIR \(^+\) neuron number is surprising as the RCASBshRARB infected ganglia generally contain more neurons than the control and could, therefore, be expected to contain increased numbers of choroid neurons. Moreover, the SOM-like signal appeared to be much fainter in RCASBshRARB-injected ganglia in comparison to all control sections stained.

SOM-LIR and Cash1 positive neurons were again counted on directly adjacent sections [Fig. 5(J)]. As already mentioned, very few Cash1 positive neurons are still detected on E14 control sections and there are indeed far more choroid neurons detectable on adjacent CG sections. After knockdown of RARB, these numbers are reversed. On neighboring sections, far more Cash1-positive neurons can be detected than mature choroid neurons [Fig. 5(J), filled bars]. Consequently, with Cash1 being a marker for immature neurons, the additional Cash1-positive neurons seen after RARB knockdown could in fact be immature choroid neurons and thus account for the loss of SOM-LIR-positive neurons shown in Figure 5(I).

Albeit these changes, the cholinergic phenotype of all CG neurons appeared to be unaffected by RARB knockdown as RCASBshRARB-infected ganglia showed levels of choline acetyltransferase (ChAT) in situ-signal that were comparable to the control and located in all of the neurons of the respective ganglia [Fig. 5(G,H)].

Taken together, all these findings point toward a delay in neuronal differentiation after RARB knockdown. While the general cholinergic phenotype of the CG neurons remained unchanged, the decrease in RARB expression impaired the maturation of the choroid neuron population, based on a decreased SOM-like immunoreactivity, and led to a prolonged expression of the proneural gene Cash1. The delayed PCD of these neurons could, therefore, be a result of their immature state. When the expression of RAR\(\beta\) does not get upregulated at E8 and E9, the maturation state of the CG neurons is affected and neurons fail to undergo PCD at the appropriate time point.

**DISCUSSION**

During embryonic development, PCD is a crucial mechanism for error correction and the optimization of neuronal connectivity, which, if inhibited, leads to
In wild type CGs, the upregulation of RARB expression shortly precedes the onset of PCD. Does RA signaling via RARβ, therefore, prime the neurons to undergo cell death? RA is known to have diverse and sometimes conflicting functions in different cell types. In oncology, RA is widely used as an antiproliferative agent and this effect is believed to be mediated by RARs. Several mechanisms of RA-induced growth inhibition have been suggested, such as the upregulation of cell-cycle-regulating and proapoptotic genes (Donato et al., 2007), including caspases, Bcl-2 proteins, and transcription factors regulating apoptosis (Noy, 2010). However, neurons from avian sympathetic ganglia respond to RA treatment with an induction of NGF-receptors, thus increasing neuronal survival (Rodriguez-Tébar and Rohrer, 1991). Similarly, when CG neurons are treated with RA in vitro, the expression of CNTF receptor mRNA increases (Wang and Halvorsen, 1998). Moreover, this treatment also resulted in a CNTF-induced phosphorylation of STAT3, suggesting that in CG development, RA regulates the sensitivity of the neurons toward cytokines (Wang and Halvorsen, 1998). However, this study partly contradicts this model. Significantly fewer cells were seen to undergo PCD between E9 and E14 after RARB knockdown, arguing against a sensitizing effect of RA signaling in the CG, at least via RARβ. However, another recent study revealed that in retinal neuron cultures, RA selectively increases apoptosis in photoreceptors, when applied while cells still proliferated; regardless of the developmental stage although, RA promoted photoreceptor differentiation, shown by the expression of opsins and neurite outgrowth (De Genaro et al., 2013).

Knockdown of RARB Expression during Development Leads to a Delay in Cell Death

A genome-wide transcriptomic screen during CG development was performed, covering the induction, execution and termination phase of cell death in the CG. To identify potential cell-intrinsic determinants of PCD, there was a special focus on genes showing a differential regulation pattern during the induction and early executional phase of PCD. The expression of RARB, the gene coding for the RARβ, was found to be differentially regulated during these time points in CG development, being most significantly upregulated at E9, with a general transient peak in RARB expression seen between E8 and E10. To elucidate whether this expression pattern plays a role in the specification of cell fate during PCD in the CG, RARB was knocked down in vivo, resulting in a significant delay in cell death execution.

The Role of RA Signaling in Neuronal Differentiation and Cell Death

A similar effect of RA signaling was found in this study. After the disruption of RA signaling via RARβ, CG neurons showed an increased expression of the proneural gene Cash1, but a decrease in somatostatin-like immunoreactivity (SOM-LIR).

The expression of Cash1 is known to precede cholinergic differentiation (Müller and Rohrer, 2002). In wild type CGs, Cash1 expression is continuously decreased during development to become nearly undetectable at E14 (Müller and Rohrer, 2002). The striking increase in CG neurons still expressing Cash1 at E14 after RARB knockdown, therefore, adds further evidence to a potential role of RARβ-mediated signaling in neuronal differentiation.

This increase in Cash1 expression could argue for a delay in differentiation that equally affects both morphological malformations and severe malfunctions of the nervous system (Buss et al., 2006). In this study, the chicken CG was used to study developmental PCD. In the majority of publications, PCD in the CG has been discussed as an extrinsically regulated process, dependent on the establishment of functional synapses (Meriney et al., 1987; Collins et al., 1991) or the availability of target-derived neurotrophic factors (Barbin et al., 1984; Dreyer et al., 1989; Simpson et al., 2013). The complexity of signals, that need to integrated by developing neurons during the time of PCD has moreover led to the hypothesis of an intrinsic predisposition of neurons toward a certain cell fate (Pettmann and Henderson, 1998). Recently, evidence of a potential intrinsic determination of cell death in vertebrates is growing (Dekkers and Barde, 2013), after a study showed that murine cortical interneurons undergo cell death by a cell-autonomous mechanism, independent from the presence of trophic factors (Southwell et al., 2012).

This study is contributing to the investigation of novel determinants of neuronal PCD, aiming to discover and investigate processes that are able to specify cell fate during the development of the chicken CG. In a gene expression screen covering the entire time span of PCD, the expression of the nuclear retinoic acid receptor RARβ was found to be tightly regulated. Challenging the role of RARB expression in the regulation of PCD via an in vivo viral knockdown approach, RARB expression was found to be vital for the timely execution of PCD and was furthermore seen to have an influence on the differentiation state of the CG neurons.
ciliary and choroid neurons. The expression of the neuropeptide somatostatin/cortistatin is a hallmark of mature choroid neurons, which are known to represent half of the CGs neuronal population and undergo cell death in similar numbers as the ciliary neuron subpopulation (Landmesser and Pilar, 1974; Nishi et al., 2010). During normal embryonic development, somatostatin-like signal was seen to increase in the choroid neuron population, until at E14, choroid neurons showed a mature phenotype with a clear SOM-LIR. The striking decrease in somatostatin-like signal after RARB knockdown was, therefore, rather surprising. With an overall increase in cell number and a larger number of Cash1-positive neurons, an increase in the number of choroid neurons would have been expected. The increased Cash1 expression and concomitant decrease in the number of mature choroid neurons after RARB knockdown thus suggests that at least a part of the additional neurons could in fact be immature choroid neurons. Another possibility to explain the phenotype would be a switch in cell fate from choroid to ciliary neuron after RARB knockdown. Such a fate-change could explain the rise in neuron numbers and the loss of somatostatin-like signal. Nevertheless, such a potential change of phenotype cannot account for the delay in cell death observed after RARB knockdown. It appears to be more likely, that the knockdown predominantly delays the differentiation of the choroid neuron population and thus indirectly leads to a delay in programmed cell death.

Moreover, studies in pancreatic cell lines hint toward a potential connection between RA and somatostatin expression as RA treatment was shown to induce markers of endocrine differentiation, among them somatostatin (El-Metwally et al., 2006). Moreover, in the absence of RARβ, the expression of important islet cell endocrine markers was greatly reduced (Pérez et al., 2013). But on the other hand, somatostatin expression was unchanged in the RARβ/KO cells.

The role of RA in neuronal differentiation has mostly been studied in in vitro models, where it has been shown to induce differentiation toward neurons or glia, as well as photoreceptor subtypes (Jones-Villemune et al., 1982; Thompson et al., 1984; De Genaro et al., 2013). In vivo, RA contribution to neuronal differentiation has been thoroughly investigated in the motor neurons of the chicken. Without RA, motor neuron number in the spinal cord is reduced and dorsoventral patterning is disrupted (Wilson et al., 2004). The amount of RA present in the respective level of the spinal cord is important for the specification of motor neuron subtype. Moreover, neuronally synthesized RA is also involved in motor neuron maintenance and survival (Ensini et al., 1998; Ji et al., 2006). In the adult CNS, RA has been shown to mediate neurogenesis in the hippocampus, where the rate of cell proliferation again depends on the relative RA concentration (Goodman et al., 2012). Conversely, in the hypothalamus, RA was found to inhibit proliferation of progenitor cells lining the third ventricle (Shearer et al., 2012).

CG neurons in vitro respond to RA treatment with an enhanced expression of CNTF receptor mRNA (Wang and Halvorsen, 1998). But a potential loss in CNTF receptor density cannot explain the delay in differentiation observed in this study as CNTF does not influence the CG neuron differentiation (Smet and Rush, 1993). Nevertheless, RA has been shown to have differential effects, dependent on the state of cellular maturation. RA treatment activated the apoptotic program in embryoid bodies, but when applied to ES cells, it was found to enhance neuronal differentiation (Castro-Obregón and Covarrubias, 1996). A similar regulatory function of RA in the chicken CG could be possible. Upregulated shortly before the peak of cell death in the CG, RARβ-induced genes could enhance the maturation of CG choroid neurons. These neurons, when reaching a certain maturation state, could in turn be more susceptible to death-inducing signals. The notion that the predisposition of a neuron to undergo cell death can be age and, therefore, differentiation-related comes from a recent publication, showing that cortical interneurons undergo cell death by a cell-autonomous mechanism, independent from the presence of trophic factors at a certain cellular age (Southwell et al., 2012). In this study, the availability of trophic factors was unchanged; nevertheless, significantly more neurons survived the natural period of cell death. Surprisingly although, these supernumerary CG neurons were then found to undergo cell death with a significant delay at E14. As RA induces the expression of pro-apoptotic genes (Noy, 2010), the knockdown of RARβ might reduce the presence of their gene products and thus impair cell death execution until other RAR subtypes compensate for the loss of RARβ. The delay in cell death could also be explained by a potentially reduced sensitivity for CNTF-withdrawal after RARB knockdown, based on a decrease in CNTF-receptor expression in the neurons lacking RARB. Nevertheless, the efficiency of the viral knockdown could also decrease with time, so that the delayed induction of cell death in the CG might also reflect a slow regeneration of native RA signaling.

Most current studies focus on the general action of RA, but do not specify the respective receptor
mediating the observed effects. Nevertheless, the answer to this question might be crucial as hybridization studies have shown that all RAR subtypes show very distinct spatially and temporally restricted expression patterns during development (Zelent et al., 1991). This finding argues for specific functions of every RAR subtype in the respective tissue and these functions still remain to be elucidated. This study shows for the first time that RARB expression is tightly regulated during CG neuronal development and signaling via RARB has a significant impact on, most probably, cholinergic neuron differentiation and the timing of PCD execution. By reducing the levels of RARB in CG neurons prior to and during the time of PCD, neuronal differentiation was significantly delayed, and so was cell death. The level of RARB-induced transcription of genes could, therefore, be a decisive intrinsic cue for the maturation of CG neurons and also their predisposition to undergo cell death.

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