Retinoic acid signaling regulates development of the dorsal forebrain midline and the choroid plexus in the chick

Sandeep Gupta and Jonaki Sen*

ABSTRACT
The developing forebrain roof plate (RP) contains a transient signaling center, perturbations in which have been linked to holoprosencephaly (HPE). Here, we describe a novel domain of retinoic acid (RA) signaling that is specific to the chick RP and demonstrate that RA signaling is sufficient for inducing characteristics of the RP in ectopic locations. We further demonstrate that, unlike what has been observed in the mouse, RA signaling is essential for invagination of the RP in chick, failure of which leads to an HPE-like phenotype. In addition, we found that RA exerts a negative influence on choroid plexus differentiation. Thus, our findings identify RA as a novel regulator of chick forebrain RP development.

KEY WORDS: Retinoic acid, Forebrain roof plate, Invagination, Choroid plexus, Chick

INTRODUCTION
The formation of two cerebral hemispheres from a single telencephalic vesicle in vertebrates begins with the process of invagination in the forebrain roof plate (RP). This process is tightly regulated both spatially and temporally, and any perturbation results in severe congenital anomalies, including holoprosencephaly (HPE) (Klingermsmith et al., 2010; Roessler and Muenke, 2010). A signaling center located in the RP of the dorsal telencephalon patterns the dorsal forebrain along the medial-lateral (M-L) axis resulting in the development of the choroid plexus (ChP) medially and the hippocampus laterally (Currel et al., 2005; Cheng et al., 2006). This is achieved through the expression of specific signaling molecules in the dorsal forebrain, such as FGFs, BMPs and Wnts (Furuta et al., 1997; Grove et al., 1998; Crossley et al., 2001). In addition, transcription factors such as Six3, Tgif, Gli3 and Zic2, which are expressed by the RP, have also been implicated as regulators of dorsal forebrain development (Theil et al., 1999; Wallis et al., 1999; Warr et al., 2008; Taniguchi et al., 2012). Perturbation in the activity of these signaling molecules and transcription factors adversely affects the development of the dorso-medial forebrain-derived structures and may also lead to HPE.

Although several molecules have been implicated as regulators of dorsal forebrain development, there are likely to be other molecules that regulate this process. One such candidate is retinoic acid (RA), a known developmental regulator of many tissues and organs, including the forebrain (Hallilagic et al., 2003, 2007). Initial studies, where pan-RA receptor antagonist-soaked beads were implanted in the forebrain of early chick embryos, resulted in dramatic abnormalities due to disturbed anterior-posterior (A-P) patterning and reduced cell survival (Schneider et al., 2001). Similar patterning defects were found in VAD quail embryos (Hallilagic et al., 2003, 2007). However, mice with genetic deletions of RA-synthesizing enzymes did not exhibit defects in forebrain A-P patterning (Molotkova et al., 2007; Chatzi et al., 2013) but instead displayed abnormalities in optic vesicle and craniofacial development (Mic et al., 2004; Hallilagic et al., 2007), as well as defects in development of the LGE-derived dopaminergic neurons (Molotkova et al., 2007).

In previous studies, RA signaling was manipulated from the earliest stage, precluding the discovery of later roles of RA in forebrain development. Furthermore, there was a lack of understanding about the specific developmental processes regulated by RA in this context. Thus, our discovery of a specific domain of RA signaling in the center of the forebrain RP motivated us to investigate additional role(s) of RA signaling during forebrain development. Here, we demonstrate that RA signaling is necessary for invagination of the forebrain RP in chick and there is a negative influence of RA signaling on ChP development. Thus, through this study we have uncovered temporally distinct roles of RA signaling in the developing chick forebrain.

RESULTS AND DISCUSSION
A restricted domain of RA signaling in the center of the chick forebrain roof plate
In many developmental contexts, a source of RA (RA-synthesizing enzyme) and a sink of RA (RA-degrading enzyme) are in close proximity (Swindell et al., 1999). To investigate comprehensively the function(s) of RA during forebrain development, we first sought to identify the source(s) and sink(s) of RA in the developing chick forebrain between HH18 and HH22. Among the three RA-synthesizing enzymes (Raldh1, Raldh2 and Raldh3), we found that only Raldh2 was expressed in the mesenchyme overlying the dorsal forebrain at HH20 and HH22 (Fig. 1A-C). Furthermore, we observed that Raldh2 was expressed in an anterior-low to posterior-high gradient in the forebrain at HH22 (Fig. 1E-G). Among the three RA-degrading enzymes (Cyp26a1, Cyp26b1, Cyp26c1), two, namely Cyp26a1 and Cyp26c1, were expressed in bilateral domains flanking the center of the invaginating RP (Fig. 1A-C, supplementary material Fig. S1A,B). The temporal and spatial expression pattern of Cyp26a1 (Fig. 1A-C, I-K, red arrowheads) was very similar to that of Raldh2 (Fig. 1A-C, E-G, red arrowheads). Based on the juxtaposed expression domains of Raldh2 and Cyp26a1, we hypothesized that a narrow domain of RA signaling is likely to exist at the center of the invaginating RP. Indeed, when we electroporated the RA reporter construct pRARE-AP (see methods in the supplementary material) in the dorsal chick forebrain we observed alkaline phosphatase (AP) activity specifically in the center of the RP at HH22 (Fig. 1O, yellow arrowheads), whereas there was none within the region...
expressing Cyp26a1 (Fig. 1N, yellow arrowheads; Fig. 1R, black arrowheads). At later stages, such as HH24, AP activity was also found to be restricted to the center of the invaginating RP (supplementary material Fig. S1C-E). We compared the expression domains of previously reported RP markers such as Bmp7, Zic2, Otx2 and Wnt7b with the domain of RA activity. Although the expression domain of Bmp7 coincided perfectly with that of RA activity (Fig. 1T,U), the expression of Zic2 extended beyond it on either side (Fig. 1T,V). In fact, Bmp7 was expressed in an anterior-posterior gradient similar to that of Raldh2 and Cyp26a1 (supplementary material Fig. S2A-I). However, the expression of Otx2 and Wnt7b coincided more with that of Cyp26a1 (Fig. 1R,W,X).

The central region of the mouse forebrain RP reportedly exhibits low proliferation and high apoptosis (Furuta et al., 1997). Indeed, we found that in the chick forebrain RP, the domain of RA activity is largely devoid of proliferating cells, as assessed by phospho-histone 3 (PH3) immunohistochemistry (Fig. 1T,S), whereas the neuroepithelium on either side has a much higher number of proliferating cells. However, the number of apoptotic cells in the forebrain RP of the chick between HH18 and HH22 was insignificant (data not shown). Thus, the domain of active RA signaling in the forebrain RP is characterized by the expression of Bmp7 and Zic2, and by a low proliferation profile. We will henceforth refer to this domain of active RA signaling as the dorsal forebrain midline (DFM).

Activation of RA signaling in ectopic locations in the chick forebrain is sufficient for inducing some characteristics of the DFM

To shed light on the possible function(s) of RA signaling in the DFM, we electroporated the pCIG-VP16-RARα-IRES-nGFP construct in the chick dorsal forebrain, which would lead to expression of VP16-RARα, a constitutively active RA receptor (Novitch et al., 2003). The ability of VP16-RARα to activate RA signaling independent of RA was demonstrated by co-electroporating the RA reporter pRARE-AP with pCIG-VP16-RARα-IRES-nGFP (supplementary material Fig. S3E,F). Electroporation of pCIG-VP16-RARα-IRES-nGFP at HH10 in the dorsal forebrain resulted in perturbed dorsal-ventral (D-V) patterning and loss of expression of RP markers at HH22 (supplementary material Fig. S4A-H). This was similar to a previous study where implantation of a RA-soaked bead in HH10 chick forebrain led to defects in D-V patterning manifested as adoption of intermediate identity by the dorsal forebrain (Marklund et al., 2004). Thus, we propose that correct establishment of the D-V identity dictated by appropriate RA signaling in the forebrain is a prerequisite for the establishment of the RP and DFM in the chick forebrain.

To circumvent the forebrain defects caused by early activation of RA signaling, we electroporated pCAG-VP16RARα in the lateral forebrain at HH22, when D-V identity of the forebrain has been already established. At HH24, we examined the expression of the
RP and DFM markers, namely Bmp7, Zic2, Wnt7b and Otx2 in the pCAG-VP16RARα and control electroporated embryos. We observed ectopic expression of Bmp7 and Zic2 but not Otx2 and Wnt7b in the lateral regions of the pCAG-VP16RARα electroporated forebrains (Fig. 2B-D″). This is expected because only the expression of Bmp7 and Zic2 overlap with the domain of active RA signaling, whereas the expression of Wnt7b and Otx2 overlap with that of Cyp26a1. Another feature of the DFM region is the low number of proliferating cells (Fig. 1S). In keeping with this, we observed an 80% reduction in the number of PH3-positive cells in the lateral regions of the forebrains electroporated with the pCAG-VP16RARα when compared with the control (Fig. 2G-I). However, we did not observe any ectopic invagination in the lateral forebrain, suggesting that constitutive activation of RA signaling is sufficient to induce some but not all features of the DFM in ectopic locations.

RA signaling regulates the invagination of the chick RP
To ascertain the necessity of RA signaling for the establishment of the DFM, we electroporated the pCAGEN-RAR403 construct encoding the dominant-negative RAR receptor (RAR403) (Novitch et al., 2003; Sen et al., 2005) in the chick dorsal forebrain. The ability of this construct to block RA signaling was confirmed by co-electroporating pCAGEN-RAR403 with the RA reporter pRARE-AP in the dorsal chick forebrain (supplementary material Fig. S3A-D). Electroporation of pCAGEN-RAR403 in the dorsal forebrain at HH10 resulted in a flat forebrain RP with no invagination at HH22 (n=5/8). Subsequently, the analysis of RP and DFM marker expression revealed a loss of Bmp7 expression (n=3/5), while the expression domains of Zic2, Wnt7b and Otx2 were found to be greatly reduced in size when compared with that in controls (n=4/5) (Fig. 3A-E′). Moreover, we found a sixfold increase in the percentage of PH3-positive cells in the pCAGEN-RAR403-electroporated region when compared with controls (Fig. 3F-H). RAR403 may have indirect effects due to sequestering of RXR, a common co-receptor for other nuclear hormone signaling pathways such as thyroid hormone (TR). Thus, as a control, a dominant-negative thyroid hormone receptor (dnTR) (Sen et al., 2005) was expressed in the RP at HH10. This did not lead to perturbations in the invagination or development of the RP (supplementary material Fig. S5). Furthermore, to rule out the possibility that the observed phenotype is due to off-target effects of RAR403, we mis-expressed Cyp26a1 in the developing chick RP at HH10. This resulted in a completely flat forebrain roof plate at HH22 (Fig. 3I-S), similar to that observed with electroporation of pCAGEN-RAR403. Thus, from the phenotypes obtained with two independent methods of reducing RA signaling, we concluded that RA plays a specific role in regulating the invagination of the chick forebrain RP.

RA has been implicated in various aspects of early forebrain development through studies primarily conducted in chick. VAD quail embryos, deficient in RA from the beginning of development, exhibit a lack of RP invagination (Halilagic et al., 2003) with features similar to a specific subtype of holoprocencephaly (Simon et al., 2002; Fernandes et al., 2007). As RA is a caudalizing factor, this may be attributed to abnormal A-P patterning during gastrulation (Chen et al., 1994; Halilagic et al., 2003). Although these studies implicated RA as a regulator of the separation of telencephalic vesicles, the specific role of RA signaling in this context was not known. Through this study, we have identified the specific event regulated by RA, which is the invagination of the chick forebrain RP.

Contrary to the defects observed in the present study and in the VAD quail embryos, RP invagination defects were never observed in mouse mutants lacking either Rdh10 (Sandell et al., 2007) or both Raldh2 and Raldh3, the major sources of RA in the forebrain

Fig. 2. Effects of ectopic activation of RA signaling in the chick lateral forebrain. (A) Schematic of coronal section of HH21 electroporated forebrain (electroporated area in green). Expression of RP/DFM markers (C-F′; C″,D″ are higher magnification images of boxed areas in C″,D″) and PH3 immunohistochemistry (G-H′) and quantification (I) of forebrains electroporated with pCAG-GFP or pCAG-VP16RARα. Data are means±s.d. Scale bars: 200 µm in B-F′; 100 µm in C″,D″,G-H′.
(Molotkova et al., 2007). This may reflect the differences between mammals and birds with respect to developmental events regulated by RA. In keeping with this, a specific domain of RA signaling has never been observed in the center of the mouse RP (Rajaii et al., 2008). In addition, we did not detect any source (Raldh) or sink (Cyp26) of RA in the RP of the early mouse forebrain (data not shown). This explains the absence of forebrain RP invagination defects in Raldh1 knockout mice, which lack all RA signaling in the forebrain (Sandell et al., 2007). Thus, RA appears to be a chick-specific regulator of forebrain RP invagination. Nonetheless, this study highlights the importance of the dorsal mesenchyme in regulating the morphogenesis of forebrain RP in both species. In agreement with this, a recent report described the failure of RP invagination upon reduced dorsal mesenchymal expansion (Choe et al., 2014). This may be attributed either to a decreased number of mesenchymal cells applying a lesser downward force on the underlying RP or to the decrease in secreted signal(s) emanating from the mesenchyme that direct RP invagination. We propose that such a dorsal mesenchyme-derived signal is likely to be RA in the chick, whereas the identity of such a signal, if any, in the mouse is yet to be discovered. Thus, through this study we have uncovered a
novel mechanism by which forebrain RP morphogenesis can be regulated.

The inhibition of RA signaling in the bilateral domains flanking the chick DFM is essential for choroid plexus development

The bilateral domains of Cyp26a1 expression flanking the chick DFM at HH22 later form the choroid plexus (ChP). We observed that the expression of Cyp26A1 persists until the advent of choroid plexus differentiation at HH12, and its expression colocalizes with a marker of ChP differentiation, transthyretin (Ttr) (Fig. 4B-D). Although Cyp26a1 and Ttr are expressed in overlapping domains at HH29, the onset of Cyp26a1 expression precedes Ttr expression by 3 days. This indicates that inhibition of RA signaling is probably required from the earliest stage of ChP development. Indeed, when we forcibly activated RA signaling in the domain of Cyp26A1 expression at HH22 by electroporating pCAG-VP16RARα, we observed absence of Ttr expression in the electroporated region at HH29 (Fig. 4F-G'). Thus, our data suggest that RA signaling must be inhibited in the bilateral regions flanking the DFM to ensure proper ChP development. There are several possible mechanisms by which RA may negatively influence ChP development. At the progenitor stage, RA may influence the fate determination of the ChP cells by altering the Hes versus neurogenin 2 balance, which has been reported to influence the neural versus non-neural fate choice in the mammalian forebrain (Imayoshi et al., 2008). However, it is not known whether a similar Hes-neurogenin 2 system functions in the chick forebrain. Alternatively, RA may affect cell survival by modulating Otx2, a major determinant of differentiation and survival of mammalian ChP cells (Johansson et al., 2013). However, this appears to be unlikely as Otx2 expression was not altered when RA signaling was activated in the bilateral domains flanking the DFM in this study (data not shown). RA may also influence ChP development through affecting BMP signaling, as the absence of BMP signaling leads to failure of ChP development in mice (Hébert et al., 2002; Fernandez et al., 2007). In keeping with this, we observed altered levels of Bmp7 expression in the DFM upon modulation of the RA signaling, indicating the possible interaction between RA and BMP signaling pathways.

In this study, we have discovered chick-specific novel functions of RA during forebrain RP morphogenesis and ChP development. Our data, together with a previous study in the mouse, indicate that there exists a conserved theme in the invagination of the forebrain RP between different species. This involves crosstalk between the dorsal mesenchyme and the underlying forebrain RP. We have identified RA as one of the mediators of this crosstalk in the chick, whereas the identity of such a mediator in the mouse remains unknown. This study will provide the impetus for the identification of this mediator in the mouse. Furthermore, it would be informative to study the similarities and differences in the cellular and molecular bases of this invagination process between the chick and the mouse. Such comparative studies are likely to reveal how regulatory mechanisms evolved to bring about the same morphogenetic changes in different species.

MATERIALS AND METHODS

Fertilized chicken eggs and embryos
Fertilized chicken eggs were obtained from Santosh’s Poultry Farm (Kanpur, India) and CARI (Bareilly, India). Eggs were incubated at 38°C in a humidified incubator until the desired HH stages (Hamburger and Hamilton, 1951).

Plasmids
Constructs used were: (1) pCIG-VP16RARα-IRES-nGFP (Addgene; Novitch et al., 2003); (2) pCAG-VP16RARα; (3) pCAGEN-RAR403, (4) pCAG-GFP, (5) RCAS-dnTR and (6) RCAS-Cyp26A1 (gifts from Prof. Constance Cepko, Harvard Medical School, Boston, MA, USA); (7) pCIG vector (a gift from Dr Axia Morales, Instituto Cajal, Madrid, Spain); and (8) pRARE-AP. See methods in the supplementary material for constructs (2) and (8).

In ovo electroporation and alkaline phosphatase staining
In ovo electroporation of HH10 and HH22 chick forebrains as well as AP staining on frozen sections of pRARE-AP electroporated forebrains...
were carried out according to protocols described in the methods in the supplementary material. Imaging was carried out using a Leica stereomicroscope (DM5000B) equipped with a DFC500 camera.

**RNA in situ hybridization and immunohistochemistry**

*In situ* hybridization was carried out according to established protocols (Gupta et al., 2012). RNA probes were prepared from the following cDNA clones: (1) Bmp7, Meis1 and Wnt7b (gifts from Prof. Cliford Tabin, Harvard Medical School, Boston, MA, USA); (2) Raldh2 and Cyp26a1 (gifts from Prof. Constance Cepko); (3) Zic2, Cyp26c1 and Otx2 (RT-PCR amplified); and (4) transfhretin (Ttr) (ChEST 735F8, Source Bioscience). For PH3 analysis. The manuscript was prepared and edited by S.G. and J.S.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

S.G. and J.S. designed the experiments. S.G. performed experiments and data analysis. The manuscript was prepared and edited by S.G. and J.S.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.orglookup/suppl doi:10.1242/dev.122390/DC1

**References**


Supplementary materials and methods

Construction of plasmids: (2) pCAG-VP16RARα: was generated by sub-cloning the VP16RARα from the pCIG-VP16RARα-IRES-nGFP into the pCAG vector. For cloning of VP16RARα into the pCAG vector backbone, VP16RARα was excised from pCIG-VP16RARα-IRES-nGFP by digestion with the XhoI and EcoRV restriction enzymes. pCAG was modified previously by introducing XhoI and EcoRV restriction sites to accommodate the insert. (8) RARE-AP: For generation of the RA reporter construct, pRARE-AP, oligonucleotides encoding the RA response element (RARE) present upstream of the RARβ gene (Wagner et al., 1992) were synthesized, annealed and cloned upstream of the SV40 minimal promoter of pGL3-AP between Sall and XhoI restriction sites. pGL3-AP was previously generated by replacing the GFP in pGL3-GFP (gift from Prof. Constance Cepko, Harvard Medical School, USA) with alkaline phosphatase reporter gene in between NotI and KpnI restriction enzymes sites. RARE sequence used in the construct:

5'-AGTTGGGCATTTGAAGTTAGCAGCCCGGTAGGTTACCGAAAGTTCACTCGC-3'

In-ovo electroporation: For early electroporations, DNA mixed with 0.01% Fast Green was injected in the forebrain anlage of the Hamilton Hamburger stage 10 (HH10) chick embryo. Platinum joystick electrodes (Nepagene, Japan) were used for electroporations. The negative electrode was placed underneath the yolk while positive electrode was placed over the forebrain anlagen. 5 pulses of 11V each were delivered for 50 ms at 950 ms interval. The following constructs in the concentrations indicated were used for early electroporation: (1) pCIG-VP16RARα (2 µg/µl), (2) pCIG (2 µg/µl), (3) pRARE-AP: pCAG-GFP (1.5 µg/µl: 0.7 µg/µl) (4) pCAGEN-RAR403: pCAG-GFP (2 µg/µl: 0.8 µg/µl). For late stage electroporations, DNA corresponding to the desired construct along with fast green was
injected in one of the forebrain vesicles of HH22 chick embryos. Sterile HBSS (Gibco) with antibiotic and antimycotic (1X) was layered over the embryos and the head was positioned between platinum joystick electrodes. 5 cycles of electric pulse of 14 volts each were delivered with each cycle consisting of 50 msec pulses at 950 ms interval. For pCIG-VP16RARα electroporations, pCIG was used as a control and for pCAG-VP16RARα and pCAGEN-RAR403 electroporations, pCAG-GFP was used as a control.

**Alkaline phosphatase staining:** Frozen sections were postfixed in 4% paraformaldehyde for 10 minutes. Endogenous alkaline phosphatase was inactivated by incubating the sections in 1X PBS at 65°C for 30 minutes followed by incubation in NTM buffer (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂) pH 9.5 for 10 minutes. Sections were then incubated with alkaline phosphatase staining solution (NBT+BCIP in NTM, pH 9.5) until the appearance of signal. Sections were counterstained with DAPI and mounted with Vectashield mounting medium (Vector Laboratory: H-1000) to visualize GFP.

**3C2 immunostaining:** Forebrain sections were postfixed in 4% paraformaldehyde for 10 minutes followed by three 1X PBS washes for 5 minutes each. Sections were then incubated in MST (DMEM+10% FBS+ 0.1% Triton) for 1 hour. Sections were further incubated in 3C2 monoclonal antibody (1:5 dilution in MST) overnight at 4°C. After several washes with 1X PBS, sections were incubated with biotinylated anti-mouse secondary antibody (1:250, Vector Labs) for 1 hour. Signal was detected with Vectastain ABC kit (Vector Labs) using DAB as a chromogenic substrate according to manufacturer's protocol.
Supplementary figure S1: (A-B) Expression analysis of Cyp26A1 (A, red arrowheads) and Cyp26C1 (B, red arrowheads) on serial transverse sections of the HH22 forebrain. (C-E) Detection of the region of active RA signaling in the dorsal forebrain at HH24 after co-electroporation of the forebrain with pCAG-GFP and pRARE-AP. GFP indicating the extent of electroporation (C, yellow arrowheads) and pseudo-colored red signal indicating the cells with AP activity (D, yellow arrowheads) in the invaginating roof plate. Merged image of GFP expression and AP staining on same section (C). Scale bar = 100 µm.
Supplementary figure S2: Expression analysis of Raldh2, Cyp26A1 and Bmp7 in the anterior (A, D and G), middle (B, E and H, red arrowheads) and posterior (C, F and I, red arrowheads) transverse sections of the HH22 forebrain respectively. An asterisk marks the expression of Bmp7 in the mesenchyme (H-I). In each image dorsal is towards the top and ventral is towards the bottom. Anterior (Ant), middle (Mid), posterior (Post). Scale bar = 100 µm.
Supplementary figure S3: Testing the efficacy of the dominant negative (pCAGEN-RAR403) and constitutive active (pCIG-VP16RARα) RAR constructs used in the study. Images of transverse sections of the forebrain electroporated with pCAG-GFP and pRARE-AP with AP activity detected (blue colored signal, A). Merged image of GFP, indicating the electroporated region and AP, pseudo-colored red fluorescent signal using Photoshop (B). Images of the transverse sections of the forebrain electroporated with pCAGEN-RAR403, pCAG-GFP and pRARE-AP with AP activity detected (faint purple signal, C). Merged image of GFP and AP, pseudo-colored red fluorescent signal using Photoshop (D). Images of the transverse sections of the forebrain electroporated with pCIG-VP16RARα and pRARE-AP with AP activity detected at ectopic locations (purple signal, E, red arrowheads). GFP expression indicates the region of electroporation as well as region expressing VP16RARα (F, yellow arrowheads). In each image dorsal is towards the top and ventral is towards the bottom. Scale bar = 100 µm.
**Supplementary figure S4:** Early ectopic activation of RA signaling in the dorsal forebrain. (A) Schematic depicting the site of electroporation performed at HH10 (area marked in red). Embryos were harvested at HH22 post electroporation. (B-C) Images of whole mount chick
embryos electroporated with the pCIG (B) and pCIG-VP16RARα constructs (C) at HH10 and imaged at HH22. (D-F, D'-F' and F'') Expression analysis of roof plate/DFM markers- Bmp7, Wnt7b and Otx2 on transverse sections of HH22 forebrains electroporated with either pCIG construct (D, E and F) or the pCIG-VP16RARα construct (D', E' and F') respectively. GFP indicates the cells electroporated with pCIG-VP16RARα (F'', yellow arrowheads). (G-I, G'-H', G'' and H'') Expression analysis of dorsal forebrain marker-Emx1 (G and G') and intermediate forebrain marker-Meis1 (H,H',I) on transverse sections of HH22 forebrains electroporated with either pCIG construct (G and H) or the pCIG-VP16RARα construct (G’ and H’). Magnified image of boxed area in H' with ectopic expression of Meis1 (I, black arrowheads). GFP indicates the cells electroporated with the pCIG-VP16RARα construct (H’’ and G’’). In each image dorsal is towards the top and ventral is towards the bottom. Forebrain (FB), midbrain (MB) and eye (E). Scale bar = 100 µm.
Supplementary figure S5: Overexpression of dominant negative thyroid hormone receptor (dnTR) in the RP. (A-C) Images of transverse sections of the RCAS-dnTR electroporated forebrain at HH22. Extent of electroporation is detected by GFP expression (A), expression of dnTR is detected by immunohistochemistry with the 3c2 antibody for the viral Gag protein (B and B’) and the merged image of GFP and 3c2 immunostaining (C). Red fluorescent signal in B is a pseudocoloured version of the original brown signal of 3c2 immunostaining in B’ using the Photoshop software. Scale bar = 100 µm.