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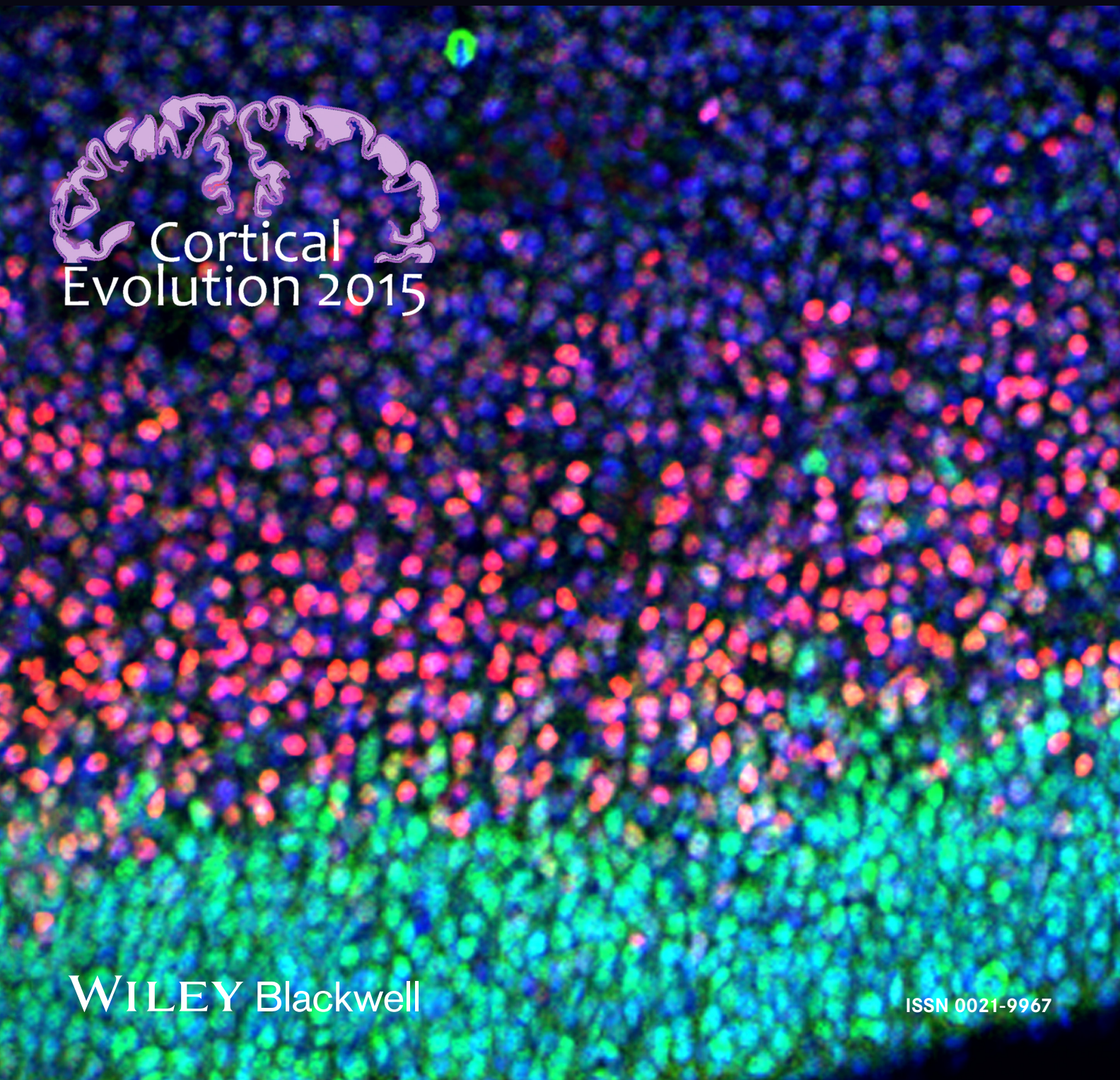

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Evolutionary Origin of Tbr2-Expressing Precursor Cells and the Subventricular Zone in the Developing Cortex

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ABSTRACT

The subventricular zone (SVZ) is greatly expanded in primates with gyrencephalic cortices and is thought to be absent from vertebrates with three-layered, lissencephalic cortices, such as the turtle. Recent work in rodents has shown that Tbr2-expressing neural precursor cells in the SVZ produce excitatory neurons for each cortical layer in the neocortex. Many excitatory neurons are generated through a two-step process in which Pax6-expressing radial glial cells divide in the VZ to produce Tbr2-expressing intermediate progenitor cells, which divide in the SVZ to produce cortical neurons. We investigated the evolutionary origin of SVZ neural precursor cells in the prenatal cerebral cortex by testing for the presence and distribution of Tbr2-expressing cells in the prenatal cortex of reptilian and avian species. We found that mitotic $Tbr2^+$ cells are present in the prenatal cortex of lizard, turtle, chicken, and dove. Furthermore, $Tbr2^+$ cells are organized into a distinct SVZ in the dorsal ventricular ridge (DVR) of turtle forebrain and in the cortices of chicken and dove. Our results are consistent with the concept that $Tbr2^+$ neural precursor cells were present in the common ancestor of mammals and reptiles. Our data also suggest that the organizing principle guiding the assembly of $Tbr2^+$ cells into an anatomically distinct SVZ, both developmentally and evolutionarily, may be shared across vertebrates. Finally, our results indicate that Tbr2 expression can be used to test for the presence of a distinct SVZ and to define the boundaries of the SVZ in developing cortices. J. Comp. Neurol. 524:433–447, 2016.

INDEXING TERMS: Tbr2; subventricular zone; Pax6; cortical neurons; neural precursor cell types

Work over the past 2 decades has identified and characterized neural precursor cell (NPC) classes that produce cortical neurons in the developing rodent forebrain. Radial glial (RG) cells are the primary NPCs that reside in the proliferative ventricular zone (VZ) surrounding the lumen of the forebrain (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001, 2002; Tamamaki et al., 2001). RG cells can be identified by their characteristic bipolar morphology. These cells possess a cell body in the VZ, a single process that contacts the lumen of the ventricle, and a long, thin pial process that extends to the surface of the growing brain (Rakic, 1972). RG cells are also identified by expression of the Pax6 transcription factor (Gotz et al., 1998; Englund et al., 2005). RG cells undergo divisions that produce additional RG cells, cortical neurons, intermediate progenitor (IP) stem cells, and astrocytes (Noctor et al., 2001, 2004, 2008; Haubensak et al., 2004; Miyata et al., 2004; Martinez-Cerdeno et al., 2012).

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During the neurogenic stages of cortical development, RG stem cells undergo divisions that produce IP cells, the secondary NPCs. IP cells migrate to a position just superficial to the VZ, establish the subventricular zone (SVZ), and can be distinguished from RG cells by their location, multipolar morphology, lack of pial attachments, and expression of the transcription factor Tbr2 (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004, 208; Englund et al., 2005). IP cells undergo symmetric divisions in the SVZ that generate pairs of cortical neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004, 2008). Thus, the output of each RG cell division is amplified from just one neuron per RG division to at least two cortical neurons via the IP cell divisions. This two-step neurogenic process could allow for more efficient control of cell genesis by reducing the number of primary NPCs required for brain growth (Martínez-Cerdeño et al., 2006). This process could also promote a rapid increase in cell production during cortical development via IP cell amplification. Evidence now suggests that $Tbr2^+$ IP cells produce excitatory cortical neurons destined for each of the cortical layers (Sessa et al., 2008; Kowalczyk et al., 2009), stressing the importance of fully understanding this cell production pathway during brain development.

Recent work has extended the initial findings on NPCs in lissencephalic rodent cortex into species with gyrencephalic cortices, including human (Fietz et al., 2010; Hansen et al., 2010), non-human primate (Martinez-Cerdeno et al., 2012), and ferret (Martinez-Cerdeno et al., 2012; Reillo and Borrell, 2012; Poluch and Juliano, 2015). These studies have shown that neurogenesis follows the same basic sequence in species with gyrencephalic cortices: $Pax6^+$ RG cells in the VZ produce $Tbr2^+$ IP cells, which then produce $NeuN^+$ excitatory cortical neurons in the SVZ. However, important differences were discovered concerning the translocation of RG cells. Earlier work had shown that RG cells detach from the ventricle, translocate out of the VZ, and express GFAP in fetal monkey (Schmechel and Rakic, 1979), developing ferret (Voigt, 1989), fetal human (deAzevedo et al., 2003), and embryonic rat (Noctor et al., 2004, 2008). Live time-lapse imaging in fetal rodent neocortex showed that translocating RG cells remained mitotic and produced daughter glial cells (Noctor et al., 2004, 2008). Exciting new work has shown that the translocating RG cells (called *tRGs*; *oRGs* [outer RGCs], *bRGs* [basal RGCs], and *IRGCs* [intermediate RGCs]) maintain Pax6 expression, as do RG cells in the VZ. The tRGs in the primate brain appear to be more numerous than in other mammals, exhibit unique migratory movements related to cell division, play an important role in cell genesis, and have been proposed as a cellular mechanism that contributes to cortical expansion in the gyrencephalic brain (Fietz et al., 2010; Hansen et al., 2010; Reillo and Borrell, 2012; Gertz et al., 2014). However, the exact role that tRGs play in cortical development remains to be determined (Hevner and Haydar, 2012).

Less is known about neurogenesis, and production of cortical cells in general, in other vertebrates such as reptiles. The mature turtle forebrain has a three-layered organization that is simple in comparison with the six-layered cortex present in mammals. Turtles are thought to be the most closely related living animal to the stem vertebrate that was the common ancestor of mammals and reptiles (Nieuwenhuys, 1994). As such, the organization of turtle cortex may represent that of the earliest mammals, and understanding features of turtle cortical development could lay the foundation for shedding light on mechanisms that guided evolutionary expansion of the cerebral cortex.

The three layers of turtle cortex include an outer molecular layer that contains some inhibitory interneurons, a pyramidal cell layer that consists primarily of excitatory pyramidal neurons, and a subcellular layer that is adjacent to the lateral ventricle and contains interneurons, some pyramidal cells, and $GFAP^+$ astroglial cells (Connors and Kriegstein, 1986; Blanton et al., 1987). The discrete cortical regions in turtle forebrain are readily distinguished in Nissl-stained tissue and include the medial cortex, dorsomedial cortex, dorsal cortex, lateral cortex, and dorsal ventricular ridge (DVR; Nieuwenhuys et al., 1998). In coronal sections of the brain, the DVR resembles the ganglionic eminence (GE) of the developing rodent brain or the caudoputamen of the mature rodent brain because it has the same relative position and shape. However, the DVR is not a proliferative region for GABAergic interneurons or a component of the basal ganglia, but rather a cortical structure. Although the DVR lacks laminar organization and is not located on the surface of the turtle forebrain, it is widely recognized as a cortical structure. The turtle DVR has been proposed as an evolutionary forerunner of mammalian cortex or, alternatively, as a derivative of the structure that gave rise to the mammalian neocortex (Reiner, 1993). The DVR is present in turtles, other reptiles, and birds.

Our previous work showed that mitotic RG cells are present in the VZ of the developing turtle forebrain (Weissman et al., 2003). We have also shown that the turtle cortex shares additional features with that of mammals, including mitotic RG cells at the ventricle and the presence of mitotic cells away from the ventricular lumen during neurogenesis (Martínez-Cerdeño

et al., 2006; Clinton et al., 2014). We noted that abventricular mitoses were most numerous in lateral regions in the dorsal cortex and, based on cytological organization, proposed that the lateral dorsal cortex of the turtle possessed what we termed a *rudimentary SVZ* (Martínez-Cerdeño et al., 2006). However, previous studies have also indicated that the turtle dorsal cortex does not possess an SVZ (Cheung et al., 2007). To integrate these findings, we further explored the characteristics of precursor cells in the developing turtle cortex, focusing primarily on abventricular precursor cells. We examined four stages of the developing forebrain of turtle (*Trachemys scripta elegans*). To determine whether the proliferative characteristics of the developing turtle forebrain are common to other reptiles, we compared the distribution and transcription factor expression of precursor cells in the lizard *Anolis carolinensis*.

Previous analyses had placed turtles outside the clade Archosauria, which includes birds, crocodiles, and dinosaurs. However, recent genetic analysis indicates that turtles are more closely related to birds than to other reptiles, such as lizards or snakes (Crawford et al., 2014; see Fig. 1). Crawford et al proposed a new clade, Archelosauria, which includes turtles and Archosaurs, but does not include Lepidosaurs, such as lizards (Crawford et al., 2014). We therefore examined the distribution and expression characteristics of precursor cells in the developing forebrains of two avian species, chicken (*Gallus gallus domesticus*) and dove (*Zenaida macroura*). These comparisons allowed us to place turtle precursor cells in evolutionary context with other relevant vertebrates to provide new insight on the evolutionary origin of SVZ precursor cells in the mammalian brain.

We show here that the developing turtle forebrain shares several key features with the developing mammalian brain. We find that Tbr2-expressing IP cells are present in the proliferative zones of turtle dorsal cortex. Furthermore, we show that Tbr2 cells are organized into a tight SVZ band in the turtle DVR, which is superficial to the VZ as in developing rodent forebrain. We show that small numbers of Tbr2-expressing cells are scattered throughout the VZ in the developing lizard forebrain but that a distinct SVZ is not present. We also examined the developing forebrain of chick and dove and show that both possess numerous Tbr2-expressing precursor cells that are organized into a distinct SVZ in a manner that is indistinguishable from that of developing rodent forebrain. These data support the concept that the DVR in the developing turtle cortex and the forebrain of chick and dove possess a distinct SVZ. These data also corroborate the cortical nature of the DVR. Finally, our data demonstrating that Tbr2 cells and a distinct SVZ are present in mammals, birds, and some regions of the developing turtle brain suggest that the principal cellular elements of the mammalian SVZ evolved prior to the appearance of modern-day mammals and were likely present in the ancestor to both mammals and reptiles.

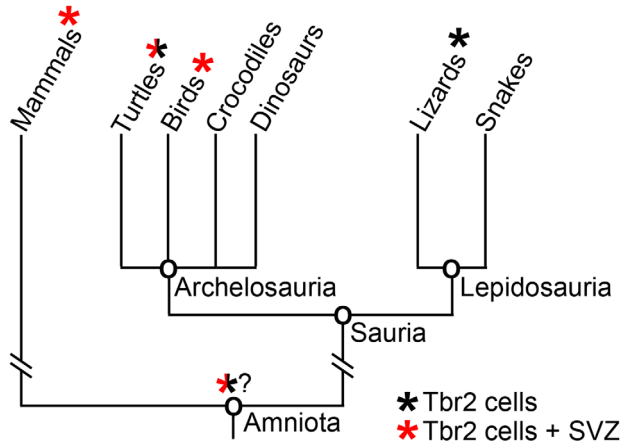

Figure 1. Tbr2-expressing cells may have been present in the developing forebrain of the common ancestor for mammals and reptiles. The cladogram shows the relationship among mammals, turtles, birds, and lizards based on recent genetic analysis (Crawford et al., 2014). Turtles and birds have been placed in the recently proposed clade Archelosauria, whereas lizards and other reptiles, such as snakes, are in the superorder Lepidosauria. Tbr2-expressing cells are found in the developing forebrain of each vertebrate that we examined: mammals, turtles, birds, and lizards. Mammals and birds possess a distinct SVZ based on the distribution of $Tbr2^+$ cells (red asterisks). $Tbr2^+$ cells are present in both the dorsal cortex and the dorsal ventricular ridge (DVR) of developing turtles, but only the DVR shows evidence of a distinct SVZ-like structure (red/black asterisk). $Tbr2^+$ cells are also present in the developing lizard forebrain, but there is no evidence of abventricular divisions or an anatomically defined SVZ (black asterisk). The presence of $Tbr2^+$ cells in mammals and both reptilian clades, Archelosauria and Lepidosauria, supports the concept that the common ancestor for mammals and reptiles possessed $Tbr2^+$ cells and possibly a distinct SVZ.

MATERIALS AND METHODS

Animals

Avian eggs were obtained from the UC Davis Animal Science Department Avian Facilities. Lizards (*Anolis carolinensis*) were purchased from Charles Sullivan Co. (Nashville, TN) by the Lovern laboratory. They were bred and embryos collected as previously described (Lovern and Wade, 2003). Turtle eggs (*Trachemys scripta elegans*) were purchased from Harvey Kliebert's Reptile Farm (Hammond, LA). All experimental protocols were approved and in accordance with IACUC regulations and guidelines. Eggs were incubated in a Hova-Bator thermal incubator. Embryos were removed from

TABLE 1.
Primary Antibodies Used

Antigen	Description of immunogen	Source, host species, catalog No., clone or lot No., RRID	Concentration used
Pax6	Recombinant full-length human Pax6	Abcam, mouse monoclonal, ab78545, AD2.38, RRID:AB_1566562	1:50
Pax6	Antibody generated against the peptide (QVPGSEPDMSQYWPRLQ) derived from the C-terminus of the mouse Pax-6 protein	Covance, rabbit polyclonal, PRB-278P-100, RRID:AB_291612	1:100
Phosphorylated vimentin (Ser55)	Synthetic MPV55 phophopeptide corresponding to mouse phophorylated vimentin Ser55 (SLYSS-phosphoS55-PGGAYC-KLH)	MBL, mouse monoclonal, DO76-3S, clone 4A4, AB_592962	1:500
Tbr2	Synthetic peptide conjugated to KLH derived from within residues 650 to the C-terminus of mouse TBR2/Eomes	Abcam, rabbit polyclonal, ab23345, RRID:AB_778267	1:500
Tbr2	KLH-conjugated linear peptide corresponding to mouse Tbr2	Millipore, chicken polyclonal, 15894, RRID:AB_10615604	1:100
PCNA	Rat PCNA made in the protein A vector pR1T2T	Millipore, mouse monoclonal, MAB424, clone PC-10, RRID:AB_95106	1:50

eggs, and brains were removed and immersion fixed in paraformaldehyde (PFA) overnight, then stored in PBS until histologic processing. Dove (*Zenaida macroura*) embryos were staged according to Muller et al. (1984). Chicken (*Gallus gallus domesticus*) embryos were staged according Hamburger and Hamilton (1992). Turtle embryos were staged according to Yntema (1968).

Nissl staining

Cryostat-sectioned coronal sections (20 μm) from embryonic turtle and lizard were mounted on Superfrost Plus slides. Slides were hydrated in a series of 2-minute incubations as follows: 100% ethyl alcohol (EtOH), 96% EtOH, 70% EtOH, 50% EtOH, and two incubations in Milli-Q H_2O. Slides were then incubated for 2 minutes in a 0.1% cresyl violet solution. Slides were dehydrated in a series of 2-minute incubations as follows: Milli-Q H_2O, Milli-Q H_2O, 50% EtOH, 70% EtOH, and 96% EtOH. Slides were then incubated in 100% chloroform for 2 minutes on a shaker. Cresyl violet stain was differentiated in 95% EtOH + glacial acetic acid until nucleoli were clearly visible (2–10 minutes). Slides were placed in 100% EtOH for 2 minutes, incubated in Safeclear (Fisher, Fair Lawn, NJ) for 5 minutes, and coverslipped in DPX mounting medium (EMS, Hatfield, PA).

Immunohistochemistry

See Table 1 for a list of all antibodies used. Immunostaining was performed as previously described (Martinez-Cerdeno et al., 2012). Briefly, antigen retrieval was performed on slide-mounted tissue by boiling sections in 10 mM citrate buffer (pH 6.0) containing 10 mM citric acid (Fisher) and (v/v) 0.5% Tween-20 (Acros) for 15 minutes. Sections were blocked in blocking buffer containing (v/v) 10% fetal donkey serum, 0.1% Triton X-100 (Acros), and (w/v) 0.2% gelatin (Acros) for a minimum of 1 hour at room temperature (RT). Sections were incubated in primary antibody buffer containing primary antibodies (see Table 1), (v/v) 2% fetal donkey serum, 0.02% Triton X-100, and (w/v) 0.04% gelatin overnight at RT. Sections were rinsed in 0.1 M PBS, then incubated for 1 hour at RT in secondary antibody buffer, which contained secondary antibodies (see below), (v/v) 2% fetal donkey serum, 0.02% Triton X-100, (w/v) 0.04% gelatin, and in some cases DAPI 1:1,000 (Roche, Indianapois, IN). Primary antibodies: mouse anti-Pax6 (1:50; Abcam, Cambridge, MA), antiphosphorylated vimentin (4A4; 1:500; MBL Intl., Woburn, MA), and PCNA (1:50; Millipore, Bilerica, MA); rabbit anti-Pax6 (1:100; Covance, Berkeley, CA) and Tbr2 (1:500; Abcam); chicken anti-Tbr2 (1:100; Millipore); and DAPI (1:1,000; Roche). Secondary antibodies: (1:200; Jackson Immunoresearch, West Grove, PA). Imaging was performed on an Olympus FV1000 confocal microscope, with analysis of Z-stack images through the entire extent of immunopositive cells to test for coexpression of markers. Figures show projections from sequential confocal planes.

Antibody characterization

See Table 1 for a list of all antibodies used. The mouse monoclonal to the recombinant full-length protein human PAX6 (paired box 6), clone number AD2.38, recognizes both products of the two major alternatively spliced forms, reacts with mouse and human, and is tissue specific for fetal eye, brain, spinal cord, and olfactory epithelium. Western blot analysis showed a band of 47–50 kDa, which was characterized by using immunohistochemistry in mouse (Stevens et al., 2010). Our

staining with this antibody matched that from original descriptions of Pax6 immunostaining in the developing cortex (Gotz et al., 1998), our previous publications (Martinez-Cerdeno et al., 2012; Cunningham et al., 2013a), and more recent work from other laboratories (Fukushima et al., 2014; Maury et al., 2015).

The rabbit anti-Pax6 polyclonal antibody was generated against the peptide (QVPGSEPDMSQYWPRLQ) derived from the C-terminus of the mouse Pax-6 protein. The antibody was subsequently purified on a protein A column. Pax-6 antibody detected two closely migrating bands of 50 kDa on SDS-polyacrylamide gels in tissue extracts from adult brain, olfactory bulb, eye, and olfactory turbinates but not in liver, and expression was initially characterized by Davis and Reed (1996) from the adult brain. Our staining matches that obtained in previous studies (Yamamoto and Jeffery, 2000; Marquardt et al., 2001). The mouse monoclonal and rabbit polyclonal anti-Pax6 antibodies produced the same pattern of immunostaining.

The monoclonal antiphosphorylated vimentin (Ser55) IgG2b antibody, also known as *4A4*, reacts specifically with the phosphorylated MPV55 peptide but not the nonphosphorylated peptide. This antibody detects vimentin phosphorylated by cdc2 kinase and does not detect nonphosphorylated vimentin or phosphorylated vimentin by cAMP-dependent kinase, protein kinase C, or Ca^{2+}-calmodulin-dependent protein kinase II on Western blotting. This antibody was purified from mouse ascites fluid using protein A agarose. This hybridoma was established by fusion of mouse myeloma cell SP2/0-Ag14 with Balb/c mouse splenocyte immunized with synthetic phosphopeptide MPV55 corresponding to mouse phosphorylated PGGAYC-KLH. Our results match those from previously published independent studies performed by Malik and colleagues (2013) in human and rabbit telencephalon, Chang and colleagues (2012) in the peripheral nervous system, and Shitamukai and colleagues (2011) and Hevner and colleagues (Englund et al., 2005) in the developing mouse cortex. Our present results also match our previous descriptions obtained with this antibody in telencephalon of rat, ferret, and macaque (Martinez-Cerdeno et al., 2012).

Rabbit polyclonal antibody to Tbr2 was produced against the synthetic peptide conjugated to KLH derived from within residues 650 to the C-terminus of mouse TBR2/Eomes. As a positive control, human mesendoderm (day 2) whole-cell lysate and mouse embryonic brain (E14) tissue lysate were used. Western blotting of mouse brain homogenates shows a 73-kDa band, matching the predicted molecular weight of Tbr2 (Quinn et al., 2007). Our immunostaining matches that of previous publications showing results in ferret and human cerebral cortex (Gertz et al., 2014) as well as our previous work (Martinez-Cerdeno et al., 2012).

The chicken polyclonal anti-Tbr2 antibody was produced against KLH-conjugated linear peptide corresponding to mouse Tbr2 and reacts with mouse, rat, and human. It was tested for specificity through Western blotting in E13–14 mouse brain lysate. The pattern of immunostaining that we obtained matched that from previous publications describing immunostaining in mouse telencephalon (Avila et al., 2014; Toyo-oka et al., 2014), the immunostaining we obtained with the rabbit polyclonal in this study, and the immunostaining we had obtained from other Tbr2 antibodies in previous studies (Noctor et al., 2008; Cunningham et al., 2013b).

The monoclonal anti-PCNA IgG2a antibody, clone PC10, against human PCNA was validated by ELISA, Western blot, and immunohistochemistry. This antibody reacted strongly with the predicted single 36 kDa band on Western blots of protein purified from HeLa cells (Waseem and Lane, 1990). This antibody has been used in previous investigations of mouse embryonic cerebral cortex (Zimmer et al., 2004; Ohtaka-Maruyama et al., 2007). Our staining matched these previous reports.

RESULTS

We first plotted the position of mitotic cells within the proliferative zone of turtle and lizard at multiple developmental stages in Nissl-stained tissue, which provides a rapid and accurate assessment of the location of actively dividing cells. Prophase, metaphase, anaphase, and telophase cells are easily identified in Nissl-stained tissue (Martínez-Cerdeño et al., 2006; Noctor et al., 2008). At early stages of development the turtle (*Trachemys scripta elegans*) possesses a relatively thick proliferative VZ, but the VZ becomes appreciably thinner by E25 as cell genesis slows (Fig. 2). In the developing turtle cortex most mitotic cells were located at the surface of the ventricle, but we also observed mitotic cells undergoing division away from the ventricle (Fig. 3). We have previously shown that mitotic cells at the surface of the ventricle in the developing turtle cortex share features with mammalian RG cells, including morphological features such as an ascending pial fiber, and expression of phosphorylated vimentin during mitosis (Martínez-Cerdeño et al., 2006; Clinton et al., 2014). Our previous work also showed that dividing cells away from the ventricle express phosphorylated vimentin in the turtle cortex (Martínez-Cerdeño et al., 2006). In addition, we showed that the proportion of

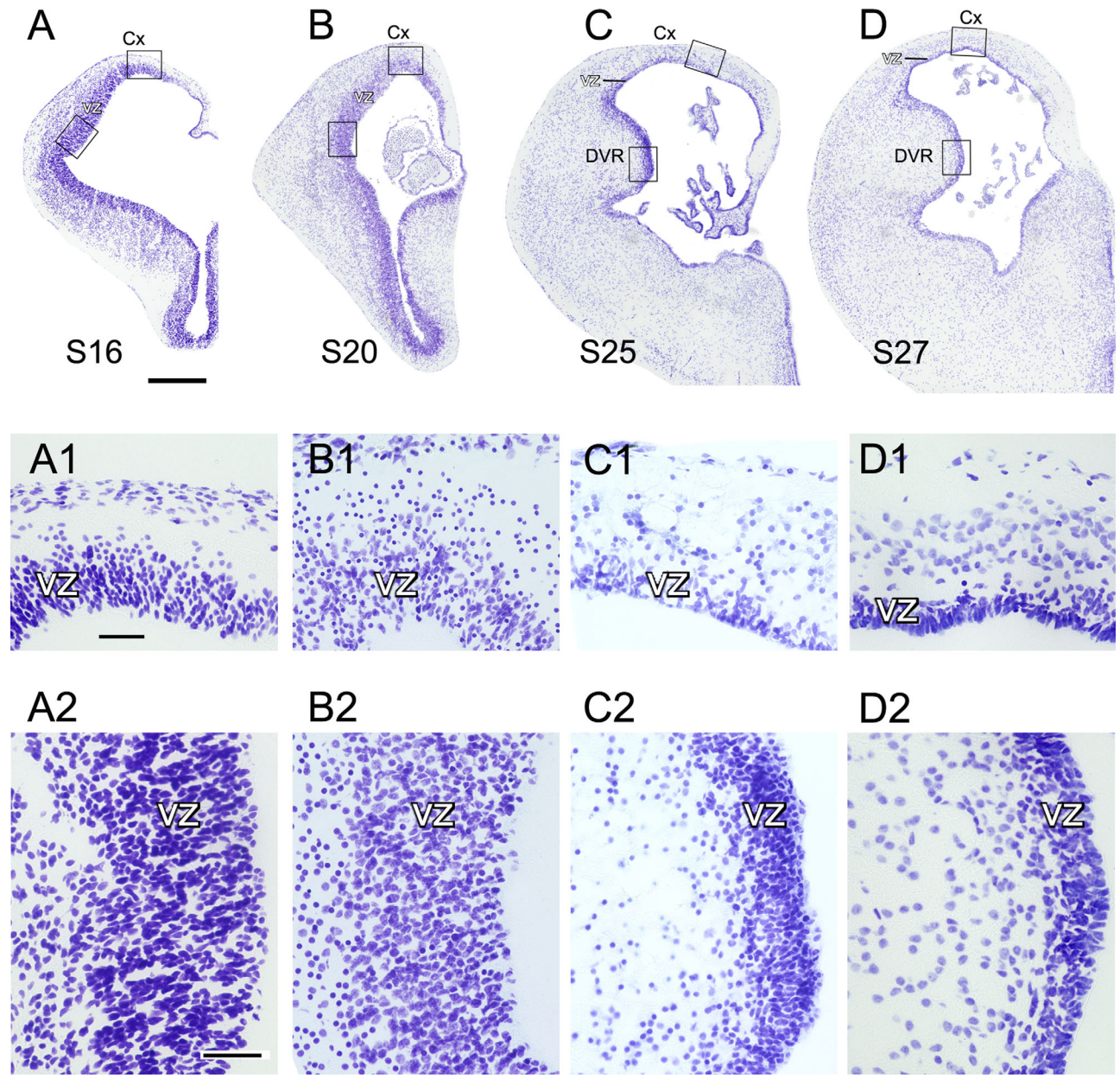

Figure 2. **A–D:** Nissl-stained sections of the embryonic turtle forebrain from stage 16 through stage 27 (Yntema, 1968), showing development of the dorsal cortex and dorsal ventricular ridge (DVR). The DVR becomes prominent after S19. Each stage is shown at higher magnification for the dorsal cortex (**A1–D1**) and for the DVR (**A2–D2**). The proliferative ventricular zone (VZ) is thicker in the DVR than in the dorsal cortex. The VZ becomes thinner in the dorsal cortex after S20 but remains relatively thicker in the DVR for a longer span of development. Scale bars in A = 500 μm (applies to A–D); 50 μm in A1 (applies to A1–D1, A2–D2).

abventricular mitoses does not change during cortical development in the turtle, although the proportion of SVZ mitoses steadily increases during cortical development in mammals (Martínez-Cerdeño et al., 2006), as originally demonstrated by Hamilton (1901). Our current data are consistent with those findings. We find many surface divisions with a few scattered abventricular divisions, particularly in the lateral cortex of turtle.

We next examined Nissl-stained tissue prepared from the developing forebrain of the lizard *Anolis carolinensis* at four embryonic stages. There were very few, if any, abventricular mitoses across the four stages of lizard development that we examined. The proliferative zone in the lizard forebrain is thinner than that of the turtle, approximately 25 μm thick in most regions of the forebrain (Fig. 4). Mitotic cells at the surface of the ventricle

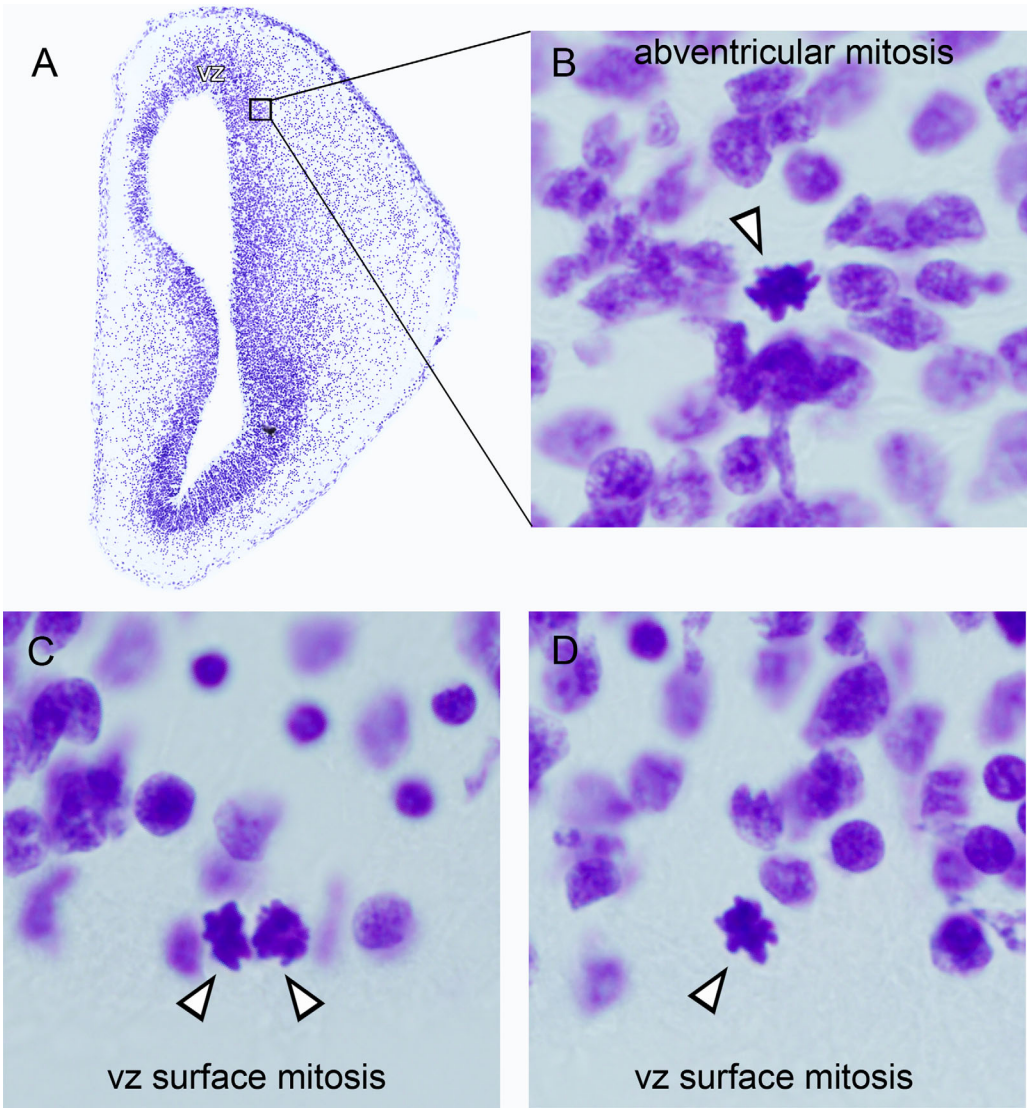

Figure 3. Nissl-stained sections of S20 turtle forebrain (**A**) showing that mitotic cells undergo division at the surface of the ventricle (arrowheads, **C,D**) and away from the ventricle (arrowhead, **B**), as in mammals.

in the lizard forebrain labeled very strongly with the M-phase RG cell marker 4A4. The $4A4^+$ cells in the lizard forebrain closely resembled RG cells in the developing turtle forebrain: mitotic cell bodies located at the surface of the ventricle, with a pial fiber coursing toward the pial surface (Fig. 5), as we previously described for rat (Noctor et al., 2002) and other mammals (Weissman et al., 2003; Martinez-Cerdeno et al., 2012). These data demonstrate that lizard RG cells share basic features with RG cells in turtle and mammals. We did not observe $4A4^+$ abventricular mitoses in the lizard.

We next tested the molecular identity of the mitotic cells in the proliferative zone of developing reptile cortex. We tested whether the mitotic figures located away from the ventricle in the turtle cortex were Pax6-expressing RG cells, Tbr2-expressing IP cells, or neither. We also tested whether Tbr2-expressing cells were present in the lizard cortex despite the absence of abventricular divisions. We prepared coronal sections of turtle and lizard forebrain for immunostaining with antibodies directed against RG cells (Pax6), IP cells (Tbr2), and mitotic cells (PCNA or phosphorylated vimentin). In both reptiles, $Pax6^+$ cells were located in the VZ (Fig. 6), as in many mammals (Gotz et al., 1998; Englund et al., 2005; Fietz et al., 2010; Hansen et al., 2010; Martinez-Cerdeno et al., 2012; Betizeau et al., 2013; Poluch and Juliano, 2015). We used anti-Tbr2 antibodies that label IP cells in mammals to examine the dorsal cortex and the DVR of turtles. We found that in turtle numerous $Tbr2^+$ cells were distributed throughout the

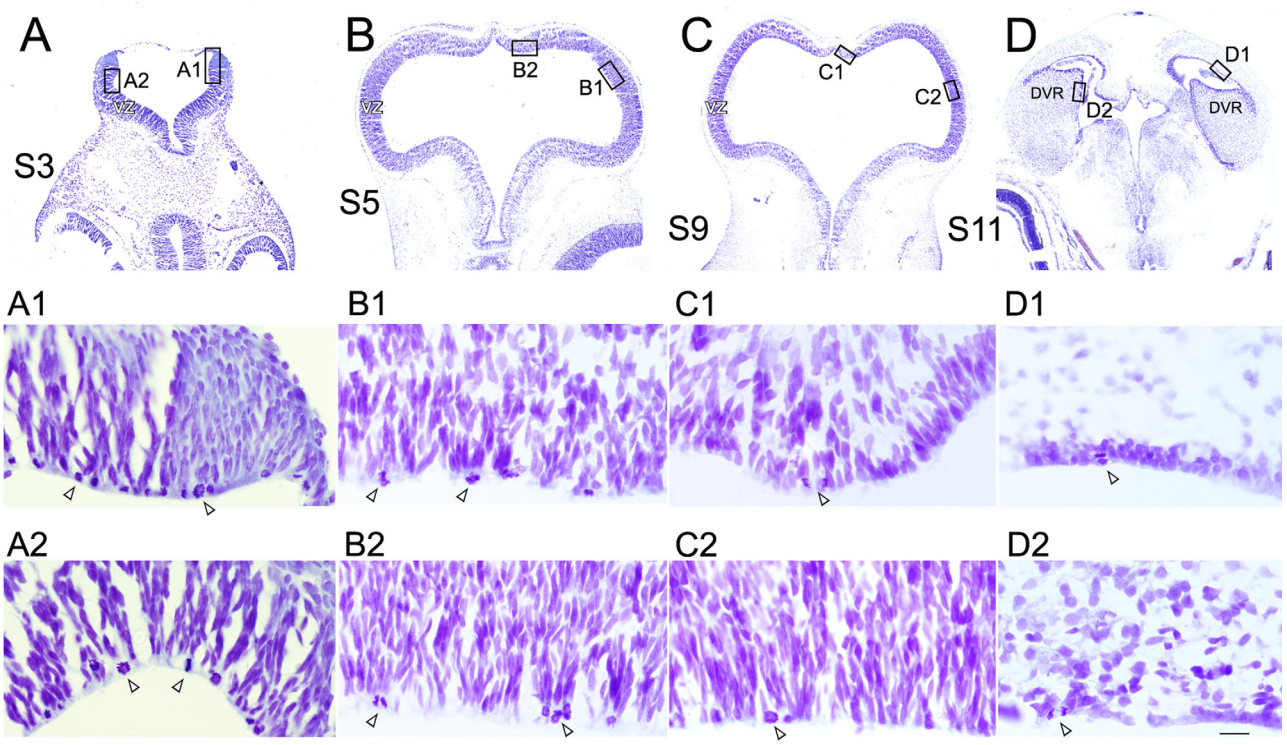

Figure 4. **A–D:**Nissl-stained sections from embryonic lizard forebrain showing development of the dorsal cortex and dorsal ventricular ridge (DVR) over Carnegie stages CS3, CS5, CS9, and CS11. Through CS9, the proliferative VZ remains at a relative constant thickness but becomes noticeably thinner by CS11. **A1–D1, A2–D2:** Higher magnification images of lizard VZ cells. Many mitotic cells were observed undergoing division at the surface of the ventricle (arrowheads), but we did not observe any cells undergoing division away from the ventricle at any stage of development in the dorsal cortex or DVR of the lizard forebrain.

VZ. The Tbr2-expressing cells were located at the surface of the ventricle and throughout the VZ (Fig. 7A). We noted fewer $Tbr2^+$ cells in medial cortex and more $Tbr2^+$ cells in lateral regions of the dorsal cortex. This was not surprising because the proliferative zone in lateral portions of the cortex was nearly twice as thick in the radial dimension (Fig. 7B). The same pattern of $Tbr2^+$ cell distribution was present at each stage of turtle development that we examined. The distribution of $Tbr2^+$ cells in the turtle dorsal cortex resembled the distribution of $Tbr2^+$ cells in rat neocortex before formation of the SVZ, when $Tbr2^+$ cells are distributed throughout the VZ (Noctor et al., 2008). In the dorsal cortex of the developing turtle brain, the $Tbr2^+$ cells did not collect into a tight band at any stage that we examined. However, we found that in the turtle DVR $Tbr2^+$ cells were organized into a distinct SVZ-like structure (Fig. 8). Despite the different structural organization of the DVR in comparison with mammalian neocortex, the DVR shares key features with mammalian cortex and has been proposed as a forerunner of mammalian cortex (Reiner, 1993). Tbr2 expression in the DVR supports this concept. We tested whether the $Tbr2^+$ cells were mitotic by costaining tissue with anti-PCNA antibodies, a marker of cells in all phases of the cell cycle. We found $Tbr2^+$ cells that were $PCNA^+$ in both dorsal cortex and in the DVR (see Figs. 7C, 8C). Not all $Tbr2^+$ cells in the turtle VZ obviously coexpressed PCNA. This might reflect slower cell cycle dynamics in the turtle, with a prolonged G0 stage when PCNA may not be expressed, or perhaps downregulation of Tbr2 expression in cells differentiating into neurons as originally demonstrated by Hevner and colleagues (Englund et al., 2005). $PCNA^+$ cells were located both at the ventricle and away from the ventricle. However, because PCNA labels cells in all phases of the cell cycle, we were not able to confirm in this data set whether the $Tbr2^+$ cells in the turtle dorsal cortex represented only the abventricular dividing cells that we observed in Nissl-stained tissue or both ventricular and abventricular dividing cell populations. Our finding that $Tbr2^+$ cells in the turtle DVR are organized into a well-developed SVZ suggests another similarity with mammalian neocortex.

$Tbr2^+$ cells were also present in the lizard proliferative zone. However, the small number of $Tbr2^+$ cells in

lizard forebrain were randomly scattered throughout the VZ, with some Tbr2$^+$ cells located at the surface of the ventricle, a few within the VZ, and a few at the superficial aspect of the VZ. The same pattern was present in the lizard DVR (Fig. 9). We did not observe any features resembling the SVZ at any stage of development or in any regions of the developing lizard forebrain.

Recent studies have shown that the turtle is more closely related to birds, dinosaurs, and crocodiles than to other reptiles such as lizards (Crawford et al., 2014). To determine whether turtle cortex shares more features with avian cortex than with that of other reptiles, we examined the distribution of Tbr2$^+$ cells in the developing avian forebrain. We obtained chicken and dove embryos at several stages of development and prepared coronal sections of the forebrain, including the hyperpallium and the mesopallium, for double immunostaining with Pax6 and Tbr2 antibodies. As in all other vertebrates, Pax6$^+$ cells in chicken forebrain were located in the VZ (Fig. 10A,B). Double labeling showed that Tbr2$^+$ cells were also present in the E8 chicken forebrain (Fig. 10A,B) and also the E5 dove forebrain (Fig. 10C). In both species, Tbr2$^+$ cells were concentrated in a tight SVZ-like band superficial to the VZ, as in rodents. Triple labeling of the chick forebrain with Pax6, Tbr2, and DAPI showed that many of the Tbr2$^+$ cells in the chick SVZ also expressed Pax6, as in the mammalian cortex (Fietz et al., 2010; Hansen et al., 2010; Martinez-Cerdeno et al., 2012). Visible condensed chromatin indicated that some of the Tbr2$^+$ cells were actively dividing in the chick, as in mammals. This is consistent with the findings of Molnar and colleagues, who reported abventricular mitoses in the chick forebrain (Cheung et al., 2007). Furthermore, we also observed individual Pax6$^+$ cells that were superficial to the VZ and SVZ at a substantial distance from the ventricle. These Pax6$^+$ cells were located superficial to the Tbr2$^+$ band, did not express Tbr2, and were actively dividing (Fig. 10B, arrow). This evidence is consistent with the possibility that translocating RG cells are present in the developing chick brain, suggesting that fundamental characteristics of precursor cells in the developing forebrain are shared across a broad spectrum of vertebrates. Taken together, these data

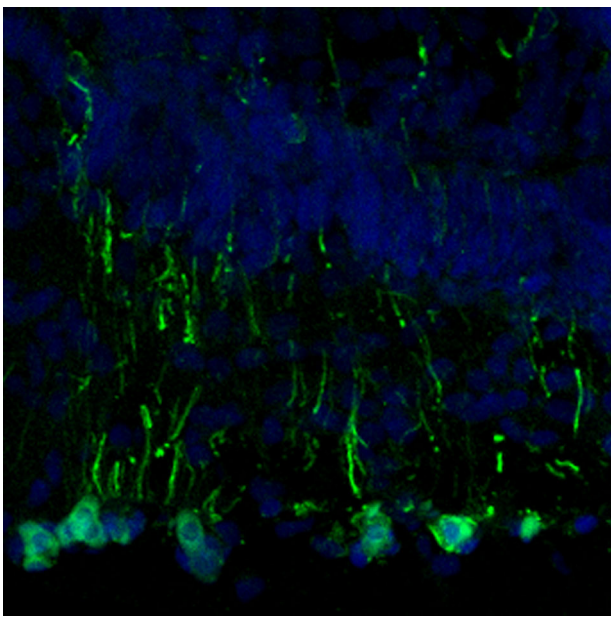

Figure 5. Mitotic cells undergoing division at the surface of the ventricle in the lizard forebrain express the mitotic cell marker phosphorylated vimentin (4A4). The 4A4$^+$ mitotic cells in lizard possess a pial process that courses toward the pial surface of the developing brain, as in mammals.

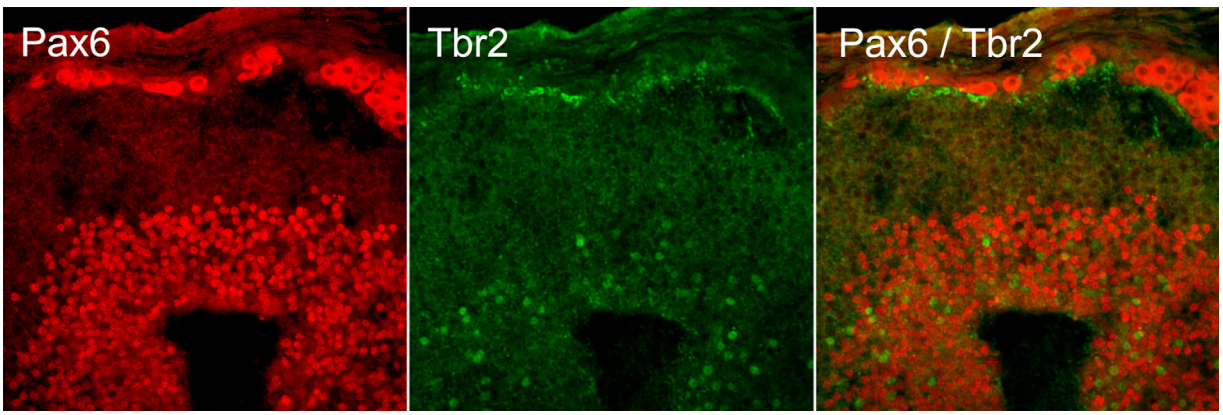

Figure 6. VZ cells in the dorsal cortex of the turtle forebrain express the transcription factor Pax6 (red), as in mammals. Proliferative cells that express the intermediate progenitor cell marker Tbr2 (green) are also present in the VZ. A magenta-green version of this figure can be viewed online as Supporting Figure 1.

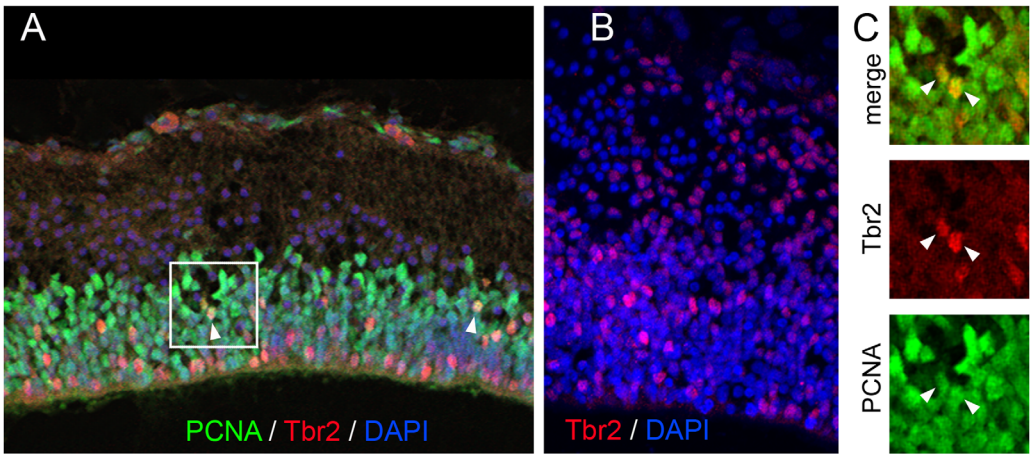

Figure 7. **A:** Tbr2-expressing cells (red) in the VZ of the turtle dorsal cortex also express the mitotic cell marker PCNA (green), indicating that they are in the mitotic cell cycle (arrowheads). **B:** The Tbr2$^+$ cells are scattered throughout the VZ in lateral portions of the turtle dorsal cortex. **C:** Higher magnification images from A showing that some Tbr2$^+$ cells (arrowheads) in the dorsal cortex also express the mitotic cell marker PCNA. A magenta-green version of this figure can be viewed online as Supporting Figure 2.

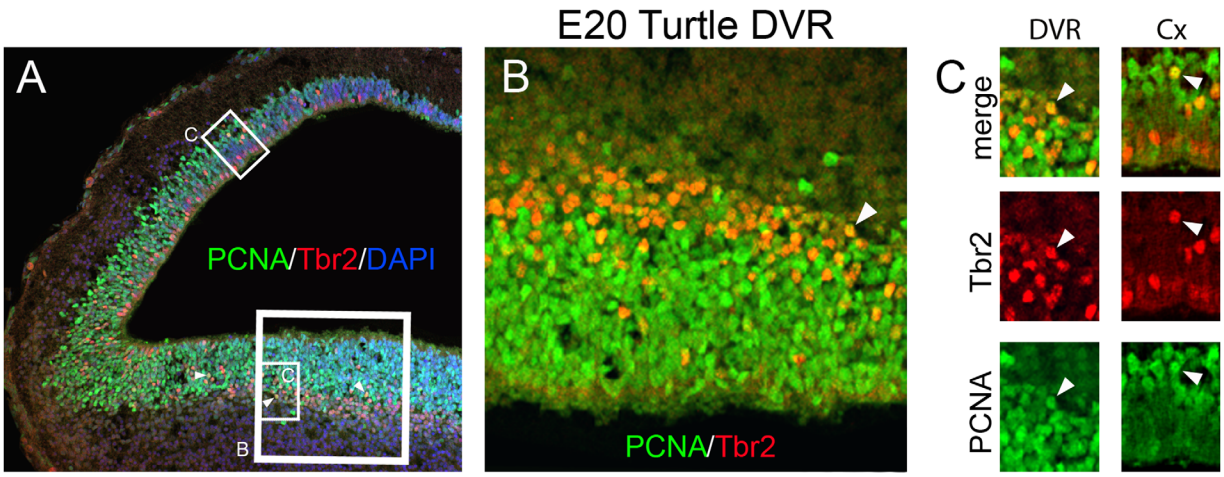

Figure 8. **A:** Tbr2-expressing cells (red) in the DVR of the developing turtle forebrain form an SVZ and express the mitotic cell marker PCNA (green). **B:** Boxed area from A showing a higher magnification image of Tbr2$^+$ cells assembling into a distinct SVZ-like structure in the DVR. A PCNA$^+$/Tbr2$^+$ cell is indicated with the arrowhead and shown at higher magnification in C. **C:** Some Tbr2$^+$ cells in the DVR and the dorsal cortex (Cx) also express PCNA, indicating that they are mitotic. A magenta-green version of this figure can be viewed online as Supporting Figure 3.

indicate that the developing forebrain of some reptilian and avian species possesses key elements of the mammalian SVZ.

DISCUSSION

The transition from three-layered lissencephalic cortex to six-layered gyrencephalic cortex, from both an evolutionary and developmental perspective, remains a central question in studies of cortical formation. Recent advances have produced great strides in our understanding of factors that regulate cortical growth and expansion. The demonstration that excitatory cortical neurons are generated by precursor cells residing in the prenatal SVZ (Tarabykin, 2001; Smart et al., 2002; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004) ushered in a new era of research on factors that control growth of the cerebral cortex. The SVZ exhibits different characteristic features across mammalian species, perhaps most notably a substantial increase in thickness in primates (Smart et al., 2002; Fietz et al., 2010; Hansen et al., 2010; Martinez-

Cerdeno et al., 2012; Betizeau et al., 2013). This notable difference supports the hypotheses that the SVZ is required for expansion of cortical surface area and that local amplification of IP cell number in the SVZ could underlie expansion of the cortical surface area in forming cortical gyri (Kriegstein et al., 2006). Furthermore, it has been proposed that regulation of IP cell number may determine cortical sheet size across species (Martínez-Cerdeño et al., 2006; Cheung et al., 2010). It has also been proposed that the evolutionary advent of IP cells was crucial for the radial expansion of the forebrain from a three-layered cortex characteristic of the reptilian forebrain to the six-layered mammalian neocortex (Martínez-Cerdeño et al., 2006). This idea is supported by the finding that reptiles, such as the turtle, possess a simple three-layered cortex but lack an anatomically defined SVZ in the dorsal cortex.

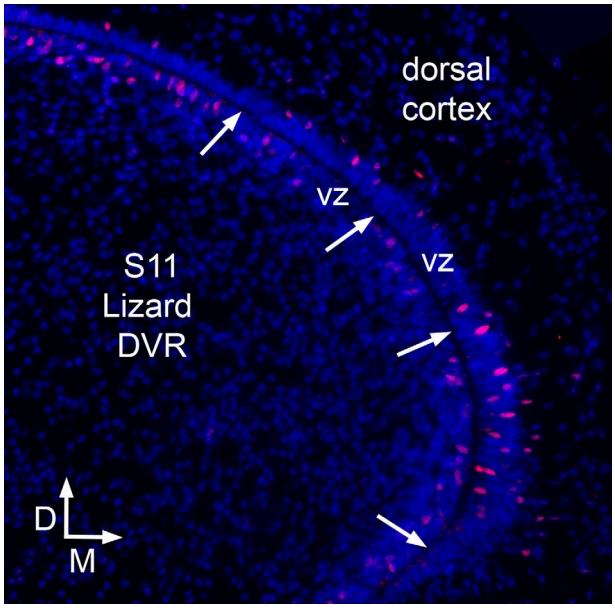

Figure 9. Tbr2-expressing cells are present in the developing forebrain of the lizard *Anolis carolinensis*. The Tbr2$^+$ cells (red/magenta) are located in the thin VZ of both the dorsal cortex and the DVR but are not organized into a subventricular band superficial to the VZ. Arrows indicate the lateral ventricle. Dorsal (D) and medial (M) orientations are indicated. Blue, DAPI.

Defining the SVZ

Determining whether a given species possesses an SVZ depends ultimately on defining the SVZ. Research for over 100 years has examined embryonic proliferative zones, including what today we call the SVZ. For example, in 1901, Alice Hamilton compared the proportion of divisions in the VZ and SVZ (which she termed *extraventricular*) and showed that the proportion of SVZ divisions in the rat increases steadily and becomes the primary proliferative zone by birth. Starting in the 1960s Smart began a series of detailed studies on the SVZ, which he termed the *subependymal zone*. In 1961, he defined the "SVZ" as superficial to the VZ, and defined SVZ cells as "a collection of undifferentiated, mitotically active cells which appear during embryonic development" and which play "an important part in the production of cells for the cerebral cortex and persists into adult life retaining, at least in rats and mice, its ability to form new cells" (Smart, 1961). In 1970, The Boulder Committee published a special communication

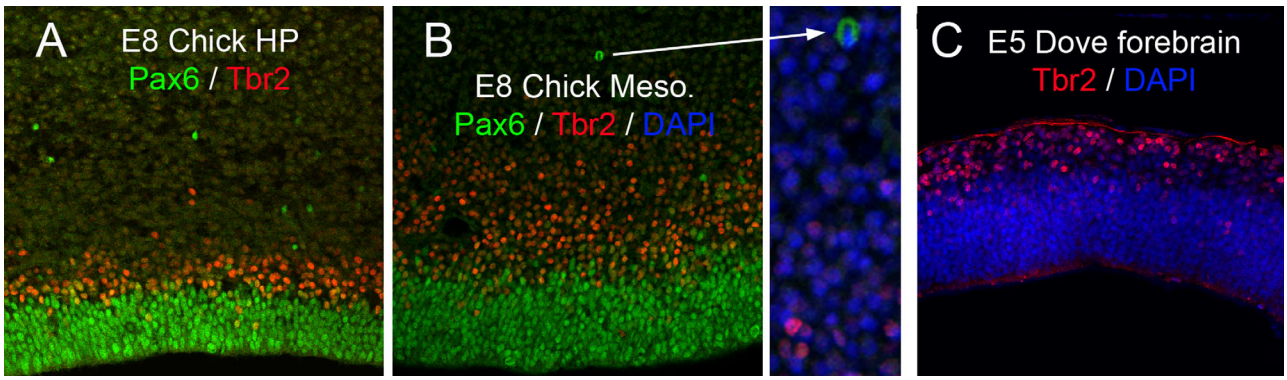

Figure 10. Tbr2-expressing cells in two avian species, chick and dove, are organized into an SVZ structure. **A,B:** Tbr2$^+$ cells (red) are located superficial to Pax6-expressing cells (green) in the VZ of the chick hyperpallium (HP) and mesopallium (Meso). Mitotic Pax6-expressing cells were also observed superficial to the SVZ (arrow, inset in B), as described for translocating cells in the mammalian neocortex. **C:** Tbr2$^+$ cells (red) are concentrated in a tight band located superficial to the VZ in the developing forebrain of the dove *Zenaida macroura*. The distribution of Tbr2-expressing cells in the developing avian forebrain supports the concept that these species possess a distinct SVZ. A magenta-green version of this figure can be viewed online as Supporting Figure 4.

to recommend revised terminology for the embryonic vertebrate brain (The Boulder Committee: Angevine et al., 1970). The Boulder Committee recognized first and foremost that terminological problems are inevitable and even a desirable consequence of scientific advance (The Boulder Committee: Angevine et al., 1970). The committee listed four fundamental zones, ventricular, subventricular, intermediate, and marginal. The SVZ was defined as being located between the junction of the ventricular and intermediate zones. SVZ cells were described as small and round or oval; distinguished from neurons by their proliferative activity, and distinguished from VZ cells by their stable position and lack of an interkinetic nuclear movement during the cell cycle. The Boulder Committee suggested that the SVZ might be present in most regions of the developing CNS but was especially prominent in the developing cerebral cortex (The Boulder Committee: Angevine et al., 1970). Further defining the characteristics of SVZ cells, such as estimating the length of the cell cycle (Takahashi et al., 1995), has been instrumental for further advances in the field.

One notable advance in the past decade was the demonstration that neurogenic SVZ cells can be identified by expression of the Tbr2 transcription factor (Englund et al., 2005). Analysis of the distribution of Tbr2-expressing cells in rodent supports the concept that the location of $Tbr2^+$ cells matches the location of SVZ cells (Noctor et al., 2008), as previously defined by Smart (1961), (The Boulder Committee :Angevine et al., 1970), Bayer and Altman (1991), and other researchers (see, e.g., Takahashi et al., 1995). An important point to keep in mind is that the SVZ does not arise as a fully formed structure during cortical histogenesis but rather forms over a period of several days from a group of cells initially distributed throughout the VZ. We previously analyzed the temporal gradient of $Tbr2^+$ cell position in the developing rat neocortex and showed that $Tbr2^+$ cells appear early, by E12, but until E15 remain scattered throughout the VZ. By E17, $Tbr2^+$ cells have coalesced into a tight band located between the VZ and the IZ (Noctor et al., 2008). The development and distribution of $Tbr2^+$ cells matches the appearance of the SVZ as described in previous work (see, e.g., Bayer and Altman, 1991). We therefore propose that Tbr2 immunoreactivity can be used to identify SVZ cells and define the boundaries of the SVZ itself. We recognize that additional precursor cell types are located within the SVZ during these developmental stages. Nonetheless, evidence indicates that Tbr2-expressing cells provide a viable means of identifying the boundaries of the SVZ.

Developing turtle and avian forebrain exhibits features of the SVZ

Living turtles may share key features with the stem vertebrate that was the common ancestor for both mammals and reptiles (Goffinet, 1983; Nieuwenhuys, 1994). It is difficult to estimate how the brain of living turtles may have changed from that ancestor. Nevertheless, we show here that the developing turtle brain shares fundamental features with the mammalian brain. For example, the VZ contains $Pax6^+$/$vimentin^+$ mitotic RG precursor cells. Furthermore, we show via Tbr2 expression that SVZ cells are present in the developing turtle brain. In previous work we showed the presence of mitotic cells away from the surface of the ventricle in the dorsal forebrain of the turtle cortex and that abventricular mitoses were more frequent in lateral portions of the dorsal cortex in an anatomical structure we termed the *rudimentary SVZ* (Martínez-Cerdeño et al., 2006). Here we further those findings by showing that $Tbr2^+$ cells are present in the turtle dorsal cortex but are scattered throughout the VZ rather than collected into a distinct SVZ. We show that $Tbr2^+$ cells are mitotic via PCNA coexpression (Figs. 7 and 8). Furthermore, we show that in the turtle DVR $Tbr2^+$ cells are organized into a tight band superficial to the VZ that resembles rodent SVZ (Fig. 8). We examined developing lizard forebrain and found that $Tbr2^+$ cells are present in the lizard cortex but exhibit a scattered distribution throughout the VZ, as in the turtle dorsal cortex (see Fig. 9). Therefore, we conclude that the SVZ is not present in lizards.

We also examined $Tbr2^+$ cell expression in the developing chick and dove because recent data indicate that turtles are more closely related to birds than to other reptiles such as lizards (Crawford et al., 2014). We found that $Tbr2^+$ cells are organized in a thick, tight band superficial to the VZ in these birds, exactly as in mammals (see Fig. 10). These data are consistent with a previous report that abventricular mitoses and an SVZ are present in some regions of the chick forebrain (Cheung et al., 2007). We further that finding by showing that the chick SVZ is formed by Tbr2-expressing precursor cells, as in mammals. These data indicate that the avian forebrain possesses a distinct SVZ, and that some regions of the developing turtle brain share this developmental feature. Together these data are consistent with the concept that the evolutionary appearance of $Tbr2^+$ cells occurred prior to the split of reptiles and mammals. Based on Tbr2 expression, the dorsal cortex of lizard and turtle resembles the embryonic rat brain at early stages of development, whereas the chick brain shares a more robust SVZ similar to

that in the rat brain at later stages of cortical development.

One concept that was proposed soon after the demonstration that the SVZ is a neurogenic structure, is that RG cells directly produce projection neurons destined for the lower cortical layers, whereas IP cells in the SVZ produce neurons that populate the upper cortical layers. This attractive hypothesis was supported by data from multiple species. For example, because the single pyramidal cell layer in turtle cortex shares morphological and functional characteristics with lower cortical layers of mammalian cortex, and because the turtle dorsal cortex does not possess an observable SVZ, as anatomically defined, it has been proposed that RG cells in the VZ produce lower layer neurons whereas precursor cells in SVZ produces the more extensive upper layer neurons. However, more recent data has shown that $Tbr2^+$ cells in the mouse produce cortical neurons destined for each cortical layer (Sessa et al., 2008; Kowalczyk et al., 2009). The data that we present here show that $Tbr2^+$ cells are present in the lizard and turtle dorsal cortex, some evidently mitotic, during neurogenic stages of cortical development. Our data support the concept that many, if not all, excitatory cortical neurons are produced by Tbr2-expressing IP cells and that these cells need not be organized into a distinct, recognizable SVZ to produce cortical neurons. This raises the question of what function, if any, is served by SVZ cells coalescing into a discrete band, both evolutionarily and developmentally. Undoubtedly, the concentration of IP cells in a distinct anatomical compartment could facilitate a more effective regulation of the IP cells' function during neurogenesis.

Evolutionary emergence of the SVZ

The data that we present here, taken together with previously published evidence, is consistent with the idea that the appearance of SVZ cells in the developing brain presaged the emergence of the SVZ as a distinct proliferative zone. $Tbr2^+$ cells are present in every mammal that has been examined to date. Here we show that $Tbr2^+$ cells are present in two species of reptile and in the developing forebrain of two avian species. This suggests that $Tbr2^+$ cells existed in the common ancestor of both reptiles and mammals. $Tbr2^+$ cells in mammals emerge in the VZ and later collect into a tight band that is superficial to the VZ (Noctor et al., 2008). However, in the dorsal cortex of turtle and lizard, $Tbr2^+$ cells are scattered throughout the VZ. This raises the possibility that $Tbr2^+$ cells in some regions of the reptilian brain have not acquired the capacity to delaminate and therefore remain attached to the ventricle and incorporated within the VZ. Smart (1965) described a limit whereby an increase in precursor cell number would be restricted if there was not a corresponding increase in ventricular surface area to accommodate cytokinesis for the precursor cells at the ventricular lumen (Smart, 1965). He proposed that this phenomenon, which he termed *ventricular choke*, could be avoided by the delamination of precursor cells from the ventricle, allowing cell division to occur away from the ventricle, with the end result that each unit of ventricular surface area could produce more cells (Smart, 1965), leading to greater expansion of the cortex in both radial and tangential dimensions (Smart, 1965). Future studies could test this idea by comparing the capacity of $Tbr2^+$ cells to delaminate from the ventricle across species and brain regions.

SUMMARY

In conclusion, we present data here that are consistent with the concept that the production of excitatory cortical neurons through a two-step process, RG $>$ IP $>$ neurons (Martínez-Cerdeño et al., 2006), evolved very early in the vertebrate brain, perhaps before mammals and reptiles diverged. These data indicate that a broad variety of species may serve as useful models for understanding how $Tbr2^+$ cell number and cortical neuron production are regulated in the developing forebrain. A central question that remains to be answered is whether the common ancestor to mammals and reptiles possessed $Tbr2^+$ cells and a distinct SVZ. Future studies can begin to address this question by simply testing for the presence of Tbr2-expressing cells in the developing forebrain of extant reptiles.

CONFLICT OF INTEREST STATEMENT

All authors declare that there were no conflicts of interest involved in the production of this work.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: VM-C, SCN. Acquisition of data: CLC, JC, JAK, JA. Analysis and interpretation of data: VM-C, CLC, SCN. Drafting of the manuscript: VM-C, SCN. Critical revision of the manuscript for important intellectual content: CLC and Anna Kreutz. Obtained funding: VM-C, ML, SCN. Study supervision: VM-C, SCN.

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