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Forebrain roof plate morphogenesis and hippocampus development in the chick embryo

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ABSTRACT The forebrain roof plate undergoes dramatic morphogenetic changes to invaginate, and this leads to formation of the two cerebral hemispheres. While many genetic factors are known to regulate this process, the mechanism of forebrain roof plate invagination remains unknown. In a recent study we have identified retinoic acid as a signal from the dorsal mesenchyme that regulates the invagination of the roof plate. This has brought into focus the importance of the interaction between the dorsal mesenchyme and the underlying roof plate. One of the structures derived from the dorso-medial forebrain after roof plate invagination is the hippocampus. While the functions of the hippocampus are conserved between birds and mammals, there are distinct structural differences. We have studied hippocampus development in the chick embryo and uncovered several similarities and differences between the process in mammals and birds. This study has also lent support to one of the prevalent models of structural homology between the avian and mammalian hippocampus. In this review, we have underscored the importance of the chick embryo as a model for studying forebrain roof plate morphogenesis and hippocampus development.

KEY WORDS: *forebrain, roof plate, invagination, hippocampus, chick*

Introduction

The adult human nervous system, which comprises of thousands of neurons and supporting cells, connected to each other in an ordered fashion, is the most complex of all organ systems. The neuronal cells are organized in a vast network that not only coordinates all the functions within the organism but also determines its interaction with the environment. Needless to say, in order to establish this elaborate network, the development of the nervous system must occur through a sequence of highly complex phenomena. The process begins with a portion of the ectoderm (one of the three germ layers) being specified to adopt a neural fate. Subsequently, the neural ectoderm undergoes neurulation giving rise to the cylindrical neural tube. Patterning of the neural tube, as well as morphological changes, lead to distinct fates and shapes being imparted to different portions of the neural tube. Initially, the entire neural tube is comprised of highly proliferative progenitor cells arranged to form a pseudostratified neuroepithelium (Miyata, 2008, Sauer, 1935). These neural progenitor cells then undergo the process of differentiation to give rise to specific types of neuronal and glial cells, which populate the entire nervous system. Studies carried out in various vertebrate models ranging from zebrafish to mouse have revealed that the basic molecular mechanisms of neural development are largely conserved. These studies have been instrumental in shedding light on the normal development of the human nervous system as well as on the etiology of various neurodevelopmental disorders.

Patterning of the neural tube imparts distinct identities along its anterior-posterior and dorsal-ventral extent (Dessaud *et al.*, 2008). For example, the posterior region of the neural tube forms the spinal

Abbreviations used in this paper: APH, parahippocampal; Bmp7, bone morphogenetic protein 7; CA, cornu ammonis; DG, dentate gyrus; DL, dorsolateral; DM, dorsomedial; Eph4a, ephrin receptor 4a; Etv1, ETS variant 1; Fgf, fibroblast growth factor; Hes, hairy and enhancer of split; HNZ, hippocampal neurogenic zone; HPE, holoprosencephaly; Lef1, lymphoid enhancer binding factor 1; LHP, lateral hinge point; Lhx, LIM homeobox; LNZ, lateral neurogenic zone; LTP, long term potentiation; MHP, medial hinge point; MIH, midline interhemispheric, holoprosencephaly; MMTV, murine mammary tumour virus ; NeuroD, neurogenic differentiation 1; Ngn, neurogenin; Prox1, prospero homeobox 1; RA, retinoic acid; Raldh, retinaldehyde dehydrogenase; Shh, sonic hedgehog; Six3, SIX homeobox 3; SMAD, small and mothers against decapentaplegic; TGF, transforming growth factor; TGIF, TGFB induced factor homeobox 1; VL, ventrolateral; VM, ventromedial; Wnts, wingless and MMTV integration factors; Zic2, ZIC family member 2.

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cord while the anterior region gives rise to different subdivisions of the brain such as the forebrain, midbrain, and the hindbrain. Further, the forebrain is divided into the anterior telencephalon and posterior diencephalon. The cerebral hemispheres in the adult human, which are the seat of various higher cognitive functions such as thinking, reasoning and decision making, emerge from the telencephalic vesicles in the embryo. These higher cognitive functions of the cerebrum are made possible by the large diversity of resident neurons that are interconnected through complex networks. It is thus expected that the formation of an elaborate structure such as the cerebrum from the simple telencephalon, would be orchestrated through several sequential developmental events which are described in the following sections.

Establishment of the forebrain fate in the neural ectoderm

The initiation of forebrain development takes place when the naïve neuroectoderm is patterned along the anterior-posterior (A-P) axis and the rostrally positioned cells adopt anterior characteristics. The prospective forebrain region within the neural ectoderm acquires anterior characteristics by protecting itself from caudalizing factors such as Wingless and MMTV integration factors (Wnts), bone morphogenetic proteins (Bmps) and retinoic acid (RA) (Munoz-Sanjuan and A, 2001, Shimogori *et al.*, 2004). This is achieved by restricting the expression of caudalizing signals such as Wnts to the posterior as well as expression of Wnt antagonists in the anterior neural border cells (Erter *et al.*, 2001, Houart *et al.*, 2002, Nordstrom *et al.*, 2002). The fact that there is a requirement of a region with low Wnt signaling for telencephalic development is supported by several lines of evidence obtained from various model organisms including zebrafish, frog, chicken and mouse (Heisenberg *et al.*, 2001, Houart *et al.*, 2002, Kiecker and Niehrs, 2001, Lagutin *et al.*, 2003, Nordstrom *et al.*, 2002). Another strategy adopted to restrict the caudalizing influence to the posterior of the embryo is through early morphogenetic movements which result in separation of the prospective forebrain region from the posterior (Chuang and Raymond, 2002, Foley and Stern, 2001, Kenyon *et al.*, 2001). Subsequent to the prospective forebrain region acquiring its anterior identity, the process of formation of the neural tube known as neurulation occurs.

Neurulation

The process of folding of the neural plate to give rise to the neural tube requires complex morphogenetic movements which lead to elongation, bending and merging of cells (Keller *et al.*, 1992). The cells from the head mesoderm and pharyngeal endoderm which lie below the neural plate signal the overlying ectodermal cells to adopt a columnar shape (Smith and Schoenwolf, 1989). Following this, the neural plate elongates through the convergent extension along the anterior-posterior axis (Schoenwolf and Alvarez, 1989). The cells along the midline of the neural plate then thicken and this acts a pivot for bending of the neural plate in a V-shape at the medial hinge point (MHP) (Catala *et al.*, 1996). At the same time, the lateral edges of the neural plate are elevated to form the lateral hinge points (LHPs), on either side at the junction between the neural ectoderm and the prospective surface ectoderm. Forces that push the lateral ectoderm inwards cause further elevation of the LHPs, resulting in their meeting at the dorsal midline to give rise to a closed cylindrical neural tube. Finally, the neural tube is separated from the overlying surface ectoderm through differential expression of cell adhesion molecules such as N-cadherin in the neural tube and E-cadherin in the surface ectoderm (Detrick *et al.*, 1990, Fujimori *et al.*, 1990). The closure of the neural tube occurs at multiple points along its length (Van Allen *et al.*, 1993), perturbations in which can cause a variety of congenital defects such as exencephaly, anencephaly and spina bifida.

After the formation of the neural tube, the cells of the medial hinge point give rise to a distinct structure known as the floor plate, located at the ventral-most region of the neural tube. Similarly, the cells of the dorsal-most region of the neural tube after its separation from the surface ectoderm give rise to a structure known as the roof plate. Both the roof plate and the floor plate act as signaling centers to pattern the neural tube along the dorsal-ventral (D-V) axis, which has been extensively studied in the developing spinal cord. In this context it is known that Sonic hedgehog (Shh) secreted by the floor plate (Briscoe and Ericson, 1999) and Bmp ligands secreted by the roof plate (Liem *et al.*, 1997, Liem *et al.*, 1995) confer distinct identities to groups of neuroepithelial progenitor cells along the D-V axis of the spinal cord, which ultimately give rise to distinct neuronal populations. A similar paradigm of signaling appears to be operational in the forebrain anlagen for patterning along the D-V axis wherein BMPs and Wnts are known to be expressed in the dorsally located roof plate whereas Shh is expressed at first in the anterior mesendoderm and later in the anterior hypothalamus ventrally (Fig. 1). Since this review is focused on the role of the roof plate, we will restrict the discussion to the roof plate in the subsequent sections.

Morphogenesis and patterning of the forebrain directed by the roof plate

After the closure of the neural tube, the differentiation of a specialized group of cells known as the roof plate occurs along its length on the dorsal side. This event is observed in both, chick (Liu *et al.*, 2004, Chizhikov and Millen, 2004a, Chizhikov and Millen, 2004b) and mouse embryos (Millonig *et al.*, 2000, Millen *et al.*, 2004). These cells are first to exit the cell cycle and form a single layer (Kahane and Kalcheim, 1998). The process of roof plate formation is complex and requires the prior establishment of dorsal identity in the forebrain through Wnt signaling and Fgf8 activity (Gunhaga *et al.*, 2003). Subsequently, RA signaling from the mesenchyme adjacent to the lateral forebrain on either side suppresses dorsal identity and induces intermediate fate in the lateral region, as shown by studies in the chick embryo from our group and others (Gupta and Sen, 2015, Marklund *et al.*, 2004). The roof plate acts as a signaling center to pattern the neural tube along its entire length, however, in the forebrain region, in addition to patterning, the roof plate undergoes morphogenesis. Signals emanating from the forebrain roof plate direct the process of invagination in the dorsal midline which leads to eventual separation of the two cerebral hemispheres from the single forebrain vesicle. Studies carried out in the mouse model have revealed that either lack of proper roof plate formation or the lack of roof plate mediated patterning often leads to neurodevelopmental disorders (Cheng *et al.*, 2006, Hebert *et al.*, 2002, Panchision *et al.*, 2001). One example of such a disorder is a variant of holoprosencephaly (HPE) known as the midline interhemispheric holoprosencephaly

(MIH-HPE) (Fernandes *et al.*, 2007).

The roof plate acts as a secondary signaling center producing Wnt and Bmp ligands, that is responsible for patterning the forebrain along the medial-lateral (M-L) axis. This has been demonstrated both in chick (Quinlan *et al.*, 2009) and mouse model systems. (Furuta *et al.*, 1997, Quinlan *et al.*, 2009, Shimogori *et al.*, 2004).This results in the positioning of the choroid plexus, the hippocampus and the cerebral cortex from the medial to the lateral end of the dorsal forebrain. In mammals this process begins with the invagination of the roof plate, within which the choroid plexus epithelium is specified medially, while lateral to it the cortical hem is specified. The cortical hem then acts as a source of inductive signals which impart the hippocampus fate to the adjacent lateral neuroepithelium (Lee *et al.*, 2000, Mangale *et al.*, 2008). Studies involving mouse mutants have revealed that in the MIH variant of HPE, there is absence of the dorsal midline derived structures such as the choroid plexus, the cortical hem, and hippocampus, while the ventral forebrain is not affected (Fernandes *et al.*, 2007, Klingensmith *et al.*, 2010, Sato *et al.*, 2001). Through these studies in the mouse, several genetic factors have been identified which include members of several signaling pathways as well as transcription factors, that influence patterning and morphogenesis in the dorsal forebrain. Although the above-mentioned studies have linked proper functioning of the roof plate to the MIH variant of HPE, they have not provided any information on the mechanism(s) through which the roof plate functions to regulate the invagination of the dorsal forebrain midline and the subsequent separation of the cerebral hemispheres.

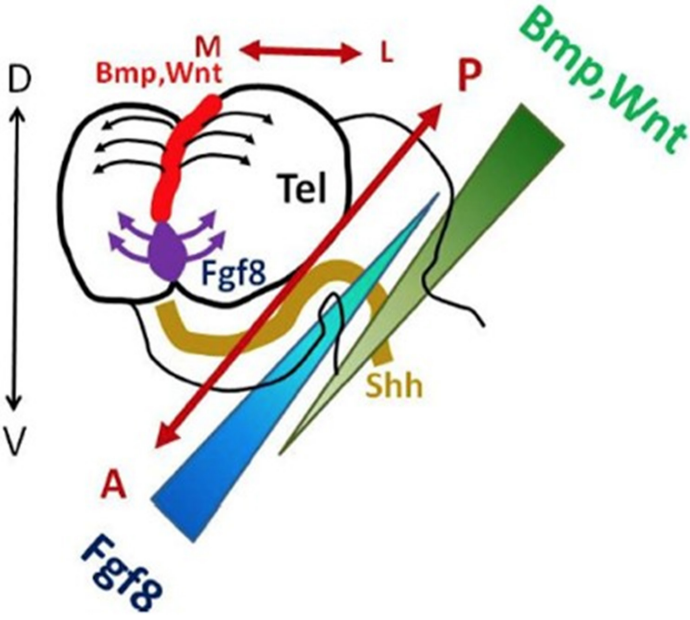

Fig 1. A three-dimensional representation of regional signaling pathways in the forebrain. *Fgf8 is expressed in the anterior neural ridge towards the rostral end of the roof plate (purple), Bmps and Wnts are expressed towards the caudal end of the roof plate (red) and Shh is expressed in the ventral compartment of the forebrain (brown). Abbreviations: M: medial, L: lateral, D: dorsal, V: ventral, A: anterior, P: posterior, Tel: telencephalon. Figure adapted from Gupta and Sen, 2016.*

The genetic factors that mediate the functions of the roof plate

The major signaling molecules that are known to regulate patterning and morphogenesis in the forebrain are Fgfs, Wnts, Bmps, and Shh (Fig. 1). Among these, the Fgf ligands are known to be produced from the signaling center located in the anterior forebrain midline, known as the anterior neural ridge (ANR) (Crossley *et al.*, 2001). Fgf signaling in the midline has been shown to be important for cell survival (Storm *et al.*, 2003), for proper development of the septum and also for the forebrain commissures to cross the midline (Tole *et al.*, 2006). Moreover, Fgf8 regulates the expression of some transcription factors expressed by the roof plate such as Zic2 and Lhx5 and thereby influence the development of the forebrain midline (Okada *et al.*, 2008). Although many Wnt ligands and other members of the Wnt signaling pathway are expressed in the dorsal forebrain from an early stage (Quinlan *et al.*, 2009), the role of Wnt signaling in this context becomes apparent at a much later stage in development. Wnt ligands emanating from the cortical hem have been implicated in regulating the growth and patterning of the hippocampus (Lee *et al.*, 2000) mostly through the regulation of proliferation of hippocampal progenitors (Galceran *et al.*, 2000, Zhou *et al.*, 2004).

Bmp ligands are produced from the developing forebrain roof plate and Bmp signaling in this context been associated with low cell proliferation and a relatively higher rate of cell death (Doan *et al.*, 2012, Furuta *et al.*, 1997, Hebert *et al.*, 2002). Bmps have also been implicated in the regulation of choroid plexus development (Cheng *et al.*, 2006). Moreover, Bmps seem to play an important role in roof plate morphogenesis. This is evident from mice where both Bmp receptor 1a and 1b have been knocked out. These mice exhibit a phenotype similar to the MIH variant of HPE (Fernandes *et al.*, 2007). Shh is expressed in the floor plate along the entire length of the neural tube and is required for the development of ventral neural tube-derived structures (Chiang *et al.*, 1996). The classic form of HPE has also been linked to the loss of Shh signaling in humans (Roessler *et al.*, 1996). Comparison between the phenotype observed in the *Shh* mutant mice with the *Shh*/*Gli3* double mutant mice indicates that Shh regulates the positioning and establishment of the roof plate signaling center through its interaction with Gli3 (Franz, 1994, Grove *et al.*, 1998, Tole *et al.*, 2000)

Individual roles played by each of the above-mentioned signaling pathways have been identified in roof plate morphogenesis and patterning of the forebrain. In addition, these signaling pathways also interact with each other in order to regulate these processes. For example, Shh regulates the expression of Fgf8, in the anterior forebrain (Rash and Grove, 2007) and Wnt1/Wnt3a, in the ventral neural tube of the chick through its interaction with Gli3 (Alvarez-Medina *et al.*, 2008). Also, Fgf8 emanating from the anterior forebrain has an antagonistic relationship with Bmps and Wnts secreted from the roof plate in the posterior dorsal forebrain and they limit each other's expression to their respective domains (Crossley *et al.*, 2001, Ohkubo *et al.*, 2002, Shimogori *et al.*, 2004).

Several transcription factors namely members of the Hairy and enhancer of split (Hes) family, Zic2, Six3, and TGIF1, are also known to influence the functions of the roof plate. In the mouse embryo, antagonistic interactions between Hes family members and Neurogenin 2 (Ngn2) determine whether neural (Cajal-retzius

cells) or non-neural (choroid plexus) fate will be adopted by the cells in the dorsal midline region of the forebrain (Imayoshi *et al.*, 2008). Mutations in the DNA-binding domain, as well as the transactivation domains of Zic2, have been linked with HPE (Warr *et al.*, 2008). However, this is due to an early defect in prechordal plate formation. Zic2 continues to be expressed in the dorsal forebrain midline in the chick embryo at later stages and its expression appears to be dependent on active RA signaling (Cheng *et al.*, 2006, Gupta and Sen, 2015). The possible later role of Zic2 in this context remains to be explored. In humans, mutations in the *Six3* gene have been linked to HPE (Domene *et al.*, 2008) while in zebrafish, Six3 has been shown to be a regulator of Shh expression (Carlin *et al.*, 2012, Geng *et al.*, 2008). Based on this and several other lines of evidence it is hypothesized that mutations in *Six3* are associated with HPE because it perturbs Shh expression, which in turn causes HPE (Geng *et al.*, 2008). TGIF transcription factors are known to interact with SMAD proteins activated by TGF-β signaling (Bertolino *et al.*, 1995, Wotton *et al.*, 1999). Indeed, deletion of TGIF1 and TGIF2 in mice causes a severe HPE like phenotype possibly due to alterations in Shh signaling (Taniguchi *et al.*, 2012).

Interaction of the dorsal mesenchyme with the roof plate influences morphogenesis

There is a large body of literature that implicates various signaling molecules and transcription factors as regulators of forebrain roof plate morphogenesis. Despite this, till today, there is very little mechanistic insight on how these various factors bring about the process of dorsal midline invagination which leads to separation of the two cerebral hemispheres. In the search for molecular players regulating this process, the possible influence of tissue-tissue interaction on midline invagination in the dorsal forebrain has been mostly overlooked. It is well known that the mesenchyme in the forebrain interacts closely with the underlying neuroepithelium as well as the overlying skull bones, such that the growth of these tissues can occur in a coordinated manner (Richtsmeier and Flaherty, 2013). Although the interaction between the mesenchyme and the forebrain neuroepithelium is well known, most of the previous studies have focused only on understanding how the signals from the neuroepithelium influence the development of the mesenchyme (Choe *et al.*, 2014, Nielsen and Dymecki, 2010).

Retinoic acid from the mesenchyme regulates the invagination of the dorsal forebrain midline in chick embryo

A recent study undertaken by our group revealed that there is a restricted expression of a retinoic acid (RA) synthesizing enzyme, Raldh2 (source of RA) in the dorsal mesenchyme, overlying the invaginating roof plate in the chick embryo (Gupta and Sen, 2015). In addition, we identified two domains of expression of an RA degrading enzyme Cyp26A1 (sink of RA) flanking the center of the invagination. Thus, the closely placed source and sink of RA resulted in a narrow domain of active RA signaling in the center of the invaginating roof plate (Fig. 2). We were intrigued by this elaborate mechanism of juxtaposing a source and sinks of RA in order to restrict RA signaling to such a narrow domain and wondered about the role of RA signaling in this context. To investigate this, we blocked RA signaling in this domain by delivering a construct expressing the dominant negative version of the retinoic acid receptor (RAR403) through *in ovo* electroporation in the forebrain of the chick embryo. We observed that this led to a complete failure in the process of invagination of the roof plate (Fig. 2), which was very similar to the phenotype observed in humans with HPE. Not only this, with the blocking of RA signaling in the dorsal midline, the following two characteristic features of this region were lost: i) a domain of very low cell proliferation and ii) expression of some genes such as Bmp7 and Zic2 which are expressed in the midline (Fig. 2).

These data indicate that there is a signal from the dorsal mesenchyme which instructs the underlying neuroepithelium to undergo invagination in the chick embryo. Since the process of roof plate invagination is conserved between birds and mammals, we investigated whether there is a similar expression of a source of RA in the roof plate of the mouse embryo. Surprisingly, we were unable to detect the expression of any Raldh in the dorsal mesenchyme nor any RA degrading enzyme in the bilateral domain flanking the invaginating midline in the mouse embryo. Nonetheless, it seems extremely likely that even in mammals the dorsal mesenchyme is the source of a signal that instructs the underlying neuroepithelium to invaginate. Future studies should be directed towards identifying such a molecule in the mouse embryo. Moreover, the mechanism through which the various genetic factors in the roof plate initiate and regulate the process of dorsal forebrain midline invagination needs to be thoroughly investigated. The chick embryo has been established as a good model system for such investigations, as it provides access for manipulation from the earliest stages of development.

The hippocampus: a dorsal forebrain midline derived structure

The anterior-most region of the vertebrate brain, known as the telencephalon, comprises of a dorsal pallium (the center of higher cognitive functions) and a ventral subpallium. In mammals, the caudomedial region within the dorsal pallium, known as the hippocampus, is the seat of memory, learning, and spatial navigation (Bingman and Yates, 1992, Scoville and Milner, 2000). The mammalian hippocampus is sub-divided anatomically into cornu amonis (CA) regions comprising of the CA fields (CA1-3), the subiculum and the dentate gyrus (DG) (Lorente De Nó, 1934). The dentate gyrus of the hippocampus is also one of two major centers of neurogenesis in the adult brain. The hippocampus is thus an important model for understanding how the stem cell niche is established and maintained in the adult. Moreover, since perturbations in adult neurogenesis can lead to epilepsy, Fragile-X syndrome and other neurological disorders (Drew *et al.*, 2013), knowledge of how the hippocampus forms and functions are essential for shedding light on the etiology of such disorders.

Development of the hippocampus

The development of hippocampus has been most extensively studied in mammals, where a transient structure with organizing activity known as the cortical hem located in the medial forebrain is responsible for the induction of the hippocampus. The cortical hem is a derivative of the invaginating roof plate and is sandwiched between the region specified to form the choroid plexus and the cortical neuroepithelium (Grove and Tole, 1999). Signals emanating

from the hem such as Wnt ligands are essential for proliferation and imparting hippocampal fate to the adjacent forebrain regions. Genetic ablation of either the hem (Caronia-Brown *et al.*, 2014) or Wnt3a (Lee *et al.*, 2000) that is specifically expressed in the hem leads to the decrease in the hippocampal field. Further evidence in support of the sufficiency of the hem for induction of hippocampal fate came from Lhx2 mouse chimeras, wherein islands of Lhx2 null cells were interspersed with Lhx2 positive cells in the forebrain. The Lhx2 null cells functioned as ectopic hem tissue capable of inducing hippocampal fate in its surrounding region thus, demonstrating the sufficiency of the hem to induce hippocampal fate (Mangale *et al.*, 2008).

A fully developed hippocampus is formed as a result of three sequential events; specification, migration, and differentiation. The adult mammalian hippocampus consists of the dentate gyrus (DG) and three CA (*cornu ammonis*) fields; CA1, CA2, and CA3. Each of these sub-fields contains characteristic neuronal populations and express unique sets of molecular markers (Lein *et al.*, 2004). Evidence from some studies suggest that these sub-fields are specified autonomously without input from the surrounding regions, however, the molecular details about how they are specified are not known (Lee *et al.*, 2000, Tole and Grove, 2001). Once the specification of the hippocampus takes place, the process of neuronal migration commences. Appropriate migration of the neuronal precursors to their destinations leads to the formation of the different subfields of the hippocampus. Tracking the migrating cells by labeling them with green fluorescent protein (GFP) has revealed that the pyramidal neurons that will populate the CA fields are born at 14.5 days post coitus (dpc) and these neuronal precursors undergo radial migration. Meanwhile, the granule neurons that populate the DG are born later at 16.5 dpc and these neuronal precursors undergo tangential migration to reach the dentate matrix (Nakahira and Yuasa, 2005). The presence of these two modes of migration during mammalian hippocampus development is thought to contribute to the morphology of the adult hippocampus.

Functional conservation of the hippocampus across vertebrate classes

The vertebrate forebrain exhibits an impressive range of variations and adaptive specializations in terms of morphology and function (Northcutt and Kaas, 1995). Despite the variations, the fundamental functioning of the forebrain has been found to be more conserved across vertebrate species than previously thought. This holds true for the hippocampus as well. Evidence from developmental and neuroanatomical studies indicate that a forebrain region that is functionally homologous to the mammalian hippocampus exists in all vertebrate classes from agnathans to aves (Butler and Hodos, 2005, Striedter, 2005). Although some limited studies have been carried out in cartilaginous fishes and amphibians to show that they have significant spatial navigation abilities (Papastamatiou *et al.*, 2011, Pasukonis *et al.*, 2014,

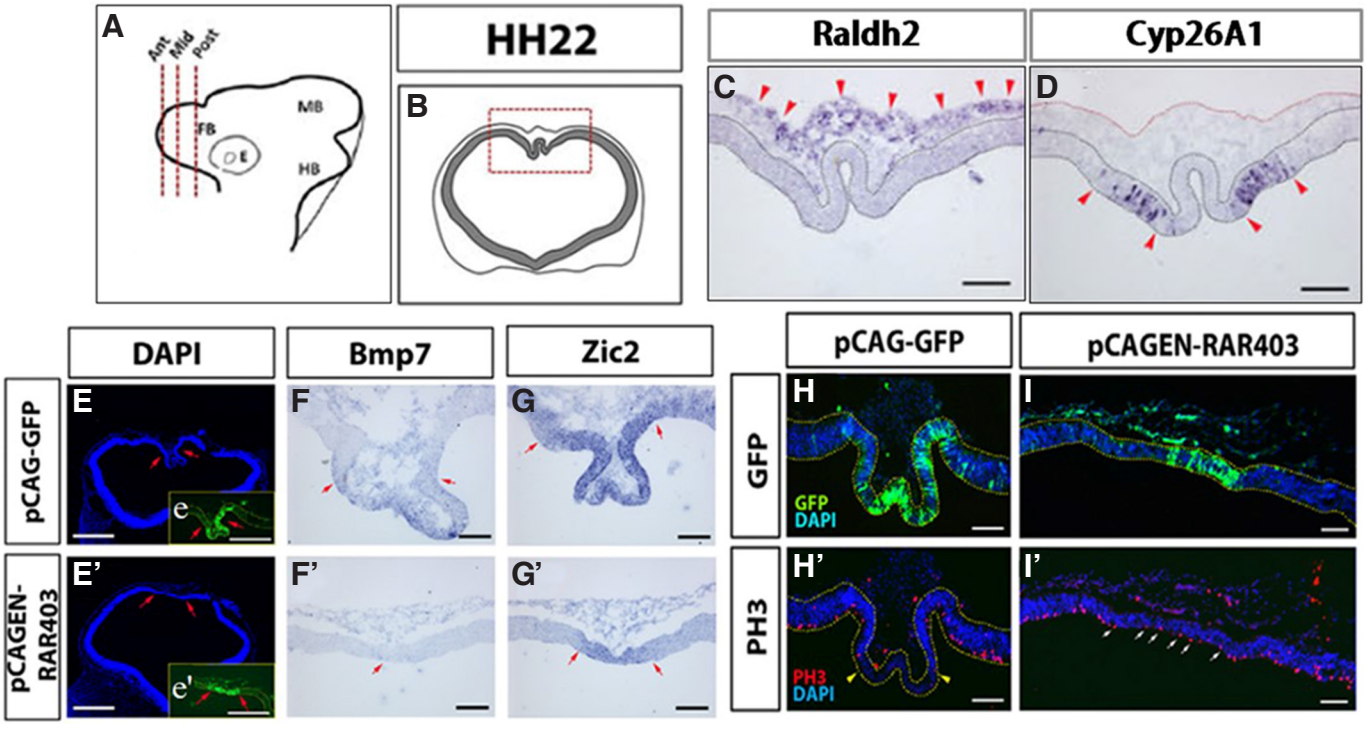

Fig. 2. Source, sink and the effects of loss of function of RA signaling in the chick RP. **(A)** *Schematic of plane of section of the chick forebrain.* **(B)** *Boxed region indicates the region of dorsal forebrain midline invagination at HH22.* **(C)** *Expression (red arrows) of Raldh2 and* **(D)** *Cyp26A1 at HH22.* **(E)** *DAPI stained images of control, pCAG GFP electroporated forebrain.* **(E')** *DAPI stained images of test, pCAGEN-dnRAR403 electroporated forebrain.* **(e)** *High magnification images of region of electroporation in control, pCAG-GFP.* **(e')** *High magnification images of region of electroporation in test, pCAGEN-dnRAR403 in HH22 chick forebrains.* **(F')** *Loss of RA signaling via electroporation of dnRAR403 leads to loss of invagination and roof plate markers, Bmp7 and* **(G')** *Zic 2 as compared to control* **(F, G)**. *Increase in cell proliferation in pCAGEN-dnRAR403 electroporated regions* **(green, H)** *as indicated by pH3 positive cells (***red***,* **H')** *as compared to pCAG-GFP electroporated regions* **(green, I)** *as indicated by pH3 positive cells* **(red, I')**. *Scale bar, 100μm in C-I', 500μm in e and e'. Figure adapted with permission from Gupta and Sen,2015.*

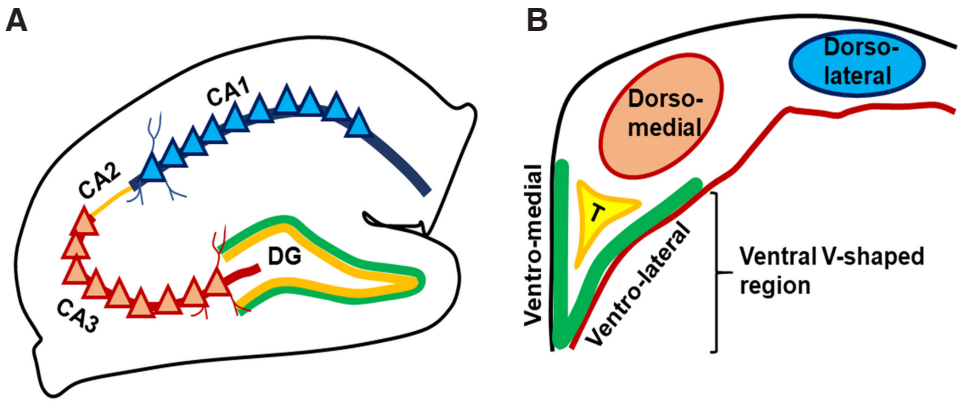

Fig. 3. Comparison between the subdivisions of mammalian and avian hippocampus. (A) *The schematic of the hippocampus in adult mice.* **(B)** *The adult avian hippocampus. Blue and red represents cornu amonis regions (CA1 and CA3) and its homolog the dorso-lateral region of the avian hippocampus. Green represents the dentate gyrus region in the mammalian and avian hippocampus respectively. Figure adapted with permission from Gupta and Sen, 2012.*

Schluessel and Bleckmann, 2005, Schluessel and Bleckmann, 2012, Sotelo *et al.*, 2015), none link these abilities to the medial pallial region proposed to be homologous to the hippocampus. On the other hand several studies linking lesions in the medial pallium to spatial learning and navigation have been carried out in non-amniotes such as teleost fishes as well as in amniotes including reptiles (Day *et al.*, 2001, Lopez *et al.*, 2003), birds (Coppola *et al.*, 2014, Fremouw *et al.*, 1997, Good and Macphail, 1994) and mammals (Hampton *et al.*, 2004).

Comparing the mammalian and avian hippocampus

The mammalian hippocampus has morphologically distinct subdivisions namely the dentate gyrus and the Ammon's horn region but in contrast, the avian hippocampus is composed of a homogeneous arrangement of densely packed neurons which merge into the parahippocampal (APH) region with no precise demarcations. Despite these structural dissimilarities, the avian hippocampus is functionally similar to the mammalian hippocampus (Sandi *et al.*, 1992). Experiments carried out in hippocampal slices demonstrated the presence of long-term potentiation (LTP) in chicken (Margrie *et al.*, 1998). Moreover, damage in the hippocampal area led to spatial learning defects (Bingman and Yates, 1992), which is one of the basic functions of the hippocampus. The avian hippocampal formation is the center for learning and memory which the birds utilize to navigate long distances, retrieve stored food, and homing (Bingman *et al.*, 2005, Budzynski *et al.*, 2002, Sherry *et al.*, 1989). Various criteria have been used to divide the region of the hippocampal formation in different bird species such as the zebra finch and pigeon (Craigie, 1935, Karten and Hodos, 1967, Szekely and Krebs, 1996). The earliest studies, based on histological analysis divided the pigeon hippocampal formation into two sub-regions namely the hippocampus and the APH (Karten and Hodos, 1967). More recently, experiments involving tract tracing and histological analysis (Atoji and Wild, 2004, Atoji and Wild, 2006) aided in dividing the pigeon hippocampus into the following major subdivisions namely the dorsomedial region (DM), the dorsolateral region (DL), the triangular region (T) and the ventromedial (VM) and ventrolateral (VL) arms of the ventral v-shaped region. Various attempts and models have been proposed to determine the structural homology between the mammalian and avian hippocampus (Erichsen *et al.*, 1991, Kuhlenbeck, 1938, Margrie *et al.*, 1998, Montagnese *et al.*, 1996, Redies *et al.*, 2001, Siegel *et al.*, 2002), however, these models were unable to precisely determine the regional homology. In a study based on tract tracing experiments, the DM region of the avian hippocampus was proposed to be homologous to the mammalian dentate gyrus and the ventral v-shaped region, homologous to Ammon's horn(Kahn *et al.*, 2003). Later through another study, it was demonstrated that the DM subdivision exhibits properties of both the mammalian Ammon's horn and the mammalian subiculum (Atoji and Wild, 2006). Further, the connections in the ventral v-shaped region project to the DM area of the avian hippocampus. The neuronal connections of the V-shaped region resemble the mossy fiber pathway of the mammalian dentate gyrus and thus this region is proposed to be similar to the mammalian dentate gyrus (Atoji and Wild, 2004).

Although the hippocampus is evolutionarily conserved between aves and mammals, the avian hippocampus differs from its mammalian counterpart in terms of morphology and cell architecture (Fig. 3). The avian hippocampus also consists of homogenously packed cells with no demarcations of subregions (Szekely and Krebs, 1996). Despite several attempts made to identify the structural homology between the avian and the mammalian hippocampus, this issue remained controversial and inconclusive. Moreover, many aspects pertaining to the development of the mammalian hippocampus such as the regulation of the mode of neuronal migration, the establishment of differential gene expression patterns in the subregions of the hippocampus as well as a comprehensive understanding of molecular mechanisms involved in hippocampus development still remain unknown.

Establishing the chick embryo as a model to study hippocampus development and structural homology between the avian and mammalian hippocampus

In order to understand the process of hippocampus development in greater molecular detail, we initiated a comprehensive study in the chick embryo (Gupta *et al.*, 2012). Since this was one of the first investigations of avian hippocampus development at the molecular level, it was expected to shed light on the molecular mechanisms

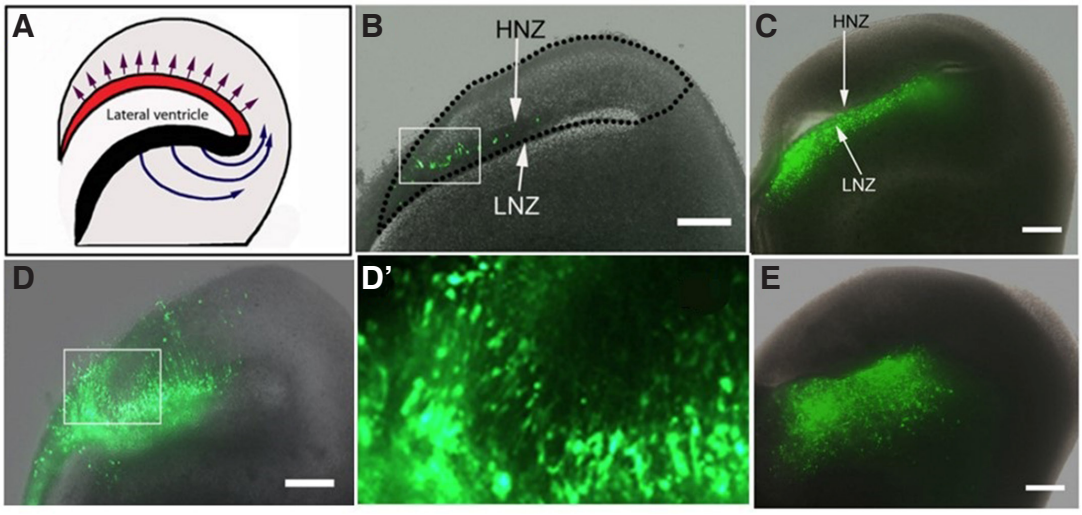

Fig. 4. Modes of migration adopted by the hippocampal neurons. **(A)** *Schematic showing the possible routes of migration of neuroepithelial cells to form the avian hippocampus.* **(B)** *Image of a slice of the chick embryo forebrain after electroporation of pCAG-GFP in the HNZ followed by 4h in culture.* **(C)** *Image of a slice of the chick embryo forebrain after Electroporation of pCAG-GFP in the LNZ region, followed by 24h in culture.* **(D)** *Image of the slice of chick forebrain showing migration of the electroporated neurons in HNZ after 68h in culture.* **(D')** *Higher magnification image of boxed region in D.* **(E)** *Image of a slice of the forebrain showing migration of the electroporated neurons from LNZ after 168h in culture. Figure adapted from Gupta and Sen, 2012. Scale bar, 200μm.*

and the players involved in hippocampus development. In addition, this study was likely to provide insight into the structural homology between the avian and mammalian hippocampus.

Identifying the modes of neuronal migration in the chick hippocampus

It is essential to identify the origin and migratory route followed by the hippocampal neurons in birds in order to obtain a comprehensive picture of avian hippocampus development. In mammals, the migratory route of the hippocampal neurons has been determined by tracing GFP labeled neurons. These studies have established that in mammals, there exist two routes of hippocampal neuronal migration, which contribute to the formation of different subdivisions of the hippocampus. For instance, the CA regions of the hippocampus are formed by neurons that migrate radially, while the dentate gyrus is formed by the hippocampal neurons that migrate tangentially. In fact, the morphology of the adult mammalian hippocampus may be attributed to the two migratory routes adopted by the hippocampal neurons (Nakahira and Yuasa, 2005). On the other hand, no investigations have been carried out to determine whether these two modes of neuronal migration are present in the avian hippocampus as well.

By utilizing the technique of *in ovo* electroporation to label cells, followed by slice culture of the chick forebrain, we attempted to identify the place of origin and the routes of migration of the hippocampal neurons. For the sake of simplicity, we divided the forebrain neuroepithelium into two zones based on their location; the hippocampal neurogenic zone (HNZ) and the lateral neurogenic zone (LNZ), which lie adjacent and opposite to the hippocampal primordium, respectively (Fig 4A). We injected the DNA encoding an expression construct for GFP (pCAG-GFP) into the ventricular space of the developing forebrain in the chick embryo. By placing the electrodes in appropriate positions during *in ovo* electroporation of pCAG-GFP, we labeled either the neurons of the HNZ (Fig 4B) or the LNZ (Fig 4C) separately. Following electroporation, slices of the forebrain were made and imaged at regular intervals to assess the modes of migration of the GFP labeled hippocampal neurons. Our experiments revealed that the neurons both in the HNZ and LNZ migrate radially and the progenitor cells from the HNZ give rise to the majority of the neurons of the hippocampus. Thus, radial migration seems to be the dominant mode of migration in the chick hippocampus (Fig .4 D,E) (Gupta *et al*., 2012). This is in contrast to the mammalian hippocampus where both the tangential and radial modes of migration are used by the hippocampal neurons.

Identification of spatiotemporal gene expression patterns in the chick embryonic hippocampus

There have been several attempts to morphologically and functionally characterize the adult avian hippocampus (Molla *et al*., 1986, Sandi *et al*., 1992), but no studies have been performed to molecularly characterize the developing hippocampus in birds. We used a candidate gene approach, to identify genes that could be involved in regulating neuronal migration, differentiation, and development in the chick hippocampus, assuming that such regulatory genes would exhibit conserved expression between birds and mammals. A list of 37 such genes that were known to be expressed in the developing and adult mouse hippocampus was generated, of which, chick orthologs were available for ten. Spatial and temporal expression profiling by RNA *in situ* hybridization was performed across several developmental stages in the embryonic chick hippocampal primordium.

Based on the expression patterns obtained, the genes could be categorized into the following four groups: (1) Genes specifically expressed across the entire chick hippocampus such as Neuropilin2 and Eph4a, which are also expressed across the entire hippocampus in mouse (Chen *et al*., 1997, Liebl *et al*., 2003); (2) Genes expressed in the prospective ventral V-region, such as Prox1 and NeuroD (Fig 5 B,C), which are expressed in the dentate gyrus region of the developing and adult mouse hippocampus (Galeeva *et al*., 2007, Lee *et al*., 1995); (3) Genes such as Etv1 and Lef1 expressed in the prospective D-M region of the chick hippocampus (Fig 5 D,E). Etv1 is expressed in the CA1 and subiculum while Lef1 is expressed in the ventricular zone of the dentate gyrus and embedded blood vessels of the mouse hippocampus (Galceran

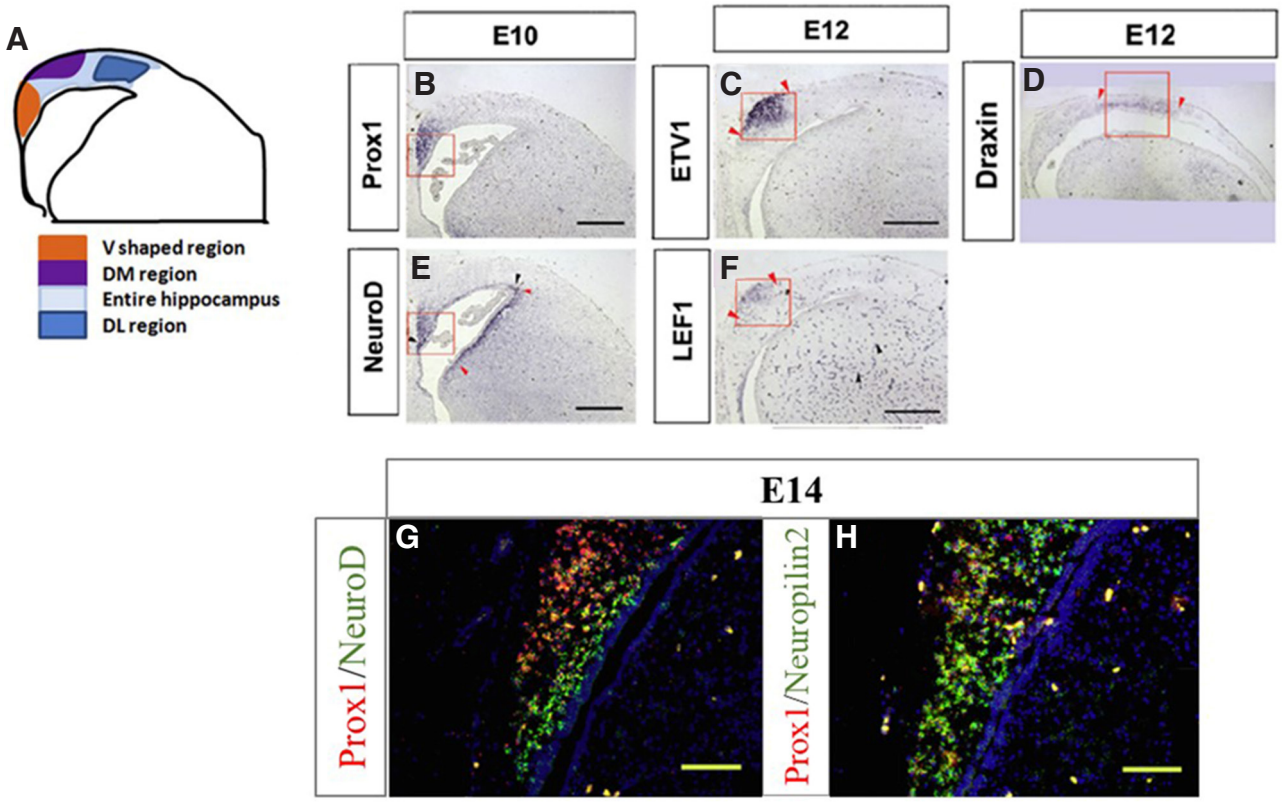

Fig. 5. Spatially and temporally restricted gene expression patterns in the developing hippocampus. (A) *Schematic showing the various gene expression domains in the avian hippocampus.* **(B)** *Expression patterns of Prox1 and* **(E)** *NeuroD in the ventral V-shaped region (boxed area) of E10 hippocampus.* **(C)** *Expression patterns of Etv1 and* **(D)** *Lef1 in the dorso-medial region (DM,boxed region) of the E12 hippocampus.***(F)** *Expression pattern of Draxin in the dorso-lateral (DL, boxed region) of the E12 hippocampus.***(G)** *Double fluorescent RNA* in situ *hybridization of Prox1(red) and NeuroD (green) indicating overlap of expression domains of the two genes.***(H)** *Double fluorescent RNA* in situ *hybridization of Prox1(red) and Neuropilin2 (green) indicating overlap of expression domains of the two genes. Figure adapted from Gupta and Sen, 2012.Scale bar, 500μm and 100μm in (G) and (H).*

et al., 2000, Lein *et al.*, 2004) and (4) Genes that were expressed in different regions at different time-points (transitory). Among these, Draxin (Fig 5F) was expressed in different regions of the hippocampus at different time-points. It is reported that in the mammalian hippocampus, Draxin is expressed in broad domains within the hippocampus (Lein *et al.*, 2004, Zhang *et al.*, 2010).

We also examined the overlaps in expression domains of the above-mentioned genes in the developing chick hippocampus. Double fluorescent RNA *in situ* hybridization analysis revealed that there is an overlap between the expression domains of Prox1 and NeuroD in the V-shaped region of the hippocampus which is homologous to the mammalian dentate gyrus (Fig 5G). Similarly, double fluorescent RNA *in situ* hybridizations carried out for detecting Prox1 and Neuropilin2 showed a broad overlap in their expression domains encompassing the v-region of the hippocampus (Fig 5H). On the other hand, there was no overlap in the expression domains between Prox1 with Etv1, Lef1, and Draxin (data not shown). Taken together these data indicate that there is significant conservation in the spatiotemporal gene expression patterns between the developing chick and mouse hippocampus (Gupta *et al.*, 2012). In fact, these genes expression patterns can be used to determine which sub-regions of the avian hippocampus are homologous to which sub-regions of the mammalian hippocampus. The fact that the ventral v-shaped region expresses Prox1 and NeuroD, both of which are markers of the mammalian dentate gyrus, lends support to the model proposing the ventral v-shaped region to be homologous to the DG of the mouse (Atoji and Wild, 2004).

Conclusions and future perspectives

Investigations carried out in several developmental models have indicated that the molecular mechanisms that direct roof plate morphogenesis is likely to be conserved between some of the vertebrate classes such as birds and mammals. Although a large body of such studies has implicated a host of molecular regulators of this process, yet, there is almost no insight gained into the mechanisms involved. In this review, we have described in a comprehensive manner all of the genetic factors and their known interactions that have been implicated in regulating the morphological processes in the roof plate. We have placed particular emphasis on the factors regulating the process of dorsal midline invagination in the forebrain that leads to separation of the two cerebral hemispheres, the failure of which leads to a phenotype resembling holoprosencephaly in humans. We have also highlighted the importance of the interaction between the dorsal mesenchyme and the underlying neuroepithelium in the orchestration of this critical process. Our recent study (Gupta and Sen, 2015) identifying the role of RA emanating from the dorsal mesenchyme in the chick,

regulating the process of dorsal midline invagination, has brought the mesenchyme-neuroepithelial interaction to the fore. The stage has been now set to address the following: 1) the identity of the signal from the dorsal mesenchyme in the mouse forebrain that performs a similar function. 2) The molecular mechanism of the morphological changes manifested as dorsal forebrain midline invagination and 3) the mechanism by which the known genetic factors function to regulate and/or initiate the morphological changes in the forebrain roof plate.

One of the brain structures that develops from the dorsomedial region of the forebrain is the hippocampus. While the mammalian hippocampus has been studied quite extensively, there are still several mechanistic details about its development that are missing. Moreover, the evolutionary conservation in the mechanisms of hippocampus development across vertebrate classes has not been thoroughly investigated. This is mostly due to the lack of information available about the development of the hippocampus of other amniotes such as birds and reptiles. Our study (Gupta *et al.*, 2012) investigating the development of the chick hippocampus at the molecular level was the first initiative taken to address this issue. Subsequently, another study has been carried out in the developing chick hippocampus to examine the expression of genes known to be important in the development of the mouse hippocampus (Abellan *et al.*, 2014). In addition, a review has recently compared the genoarchitechture between different classes of amniotes to provide insights into the evolution and development of the hippocampus (Medina *et al.*, 2017). All of these studies indicate the importance of further investigations at the molecular level in several vertebrate models, which is necessary in order to gain insight into the evolution and development of the hippocampus. Further, detailed studies need to be carried out in future to profile the chick hippocampal neuron subtypes. This will add to our overall understanding of hippocampus function. With all of the tools available for molecular manipulation, the chick embryo can thus serve as a powerful model for investigating the process of roof plate morphogenesis as well for studying avian hippocampus development.

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