



A Bird's Eye View of Vertebrate Forebrain Development

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Abstract | Development of the vertebrate forebrain is a complex process involving the interplay of many different signaling pathways and their downstream effectors. Despite morphological differences among the forebrains of different vertebrate classes there is a remarkable degree of conservation in the basic molecular players and the mechanisms involved in its development. A large body of knowledge about this process has been built up over the years through studies carried out in diverse model systems ranging from fishes to mammals. However, several important aspects of forebrain development remain unexplored till today. In this review we have summarized the information currently available on the molecular mechanisms involved in vertebrate forebrain development with particular emphasis on early events that pattern the forebrain along the anterior-posterior as well as the dorsal-ventral axis. We have highlighted the contribution of the developing chick embryo towards the understanding of mechanisms regulating forebrain development. We have also described the recent advances made in molecular techniques that have made the avian model system an attractive option for studying vertebrate forebrain development.

1 Introduction

The forebrain, which is the anterior-most region of the Central Nervous System (CNS) in vertebrates, orchestrates many higher order neuro-cognitive functions resulting in generation of thoughts, emotions and memory. Leaving aside a few human-specific attributes of the forebrain, the basic organization of the forebrain is remarkably conserved in evolution. This is manifested in the form of evolving forebrain structure and function, represented not only by an increase in the number of anatomical regions, but also by an increase in complexity of each of these regions across vertebrate classes. One of the major research goals of developmental neurobiologists is to understand the genetic basis of development of the architecture of the vertebrate forebrain and to identify the factors responsible for this increase in complexity. Taking advantage of the fact that early forebrain development is largely conserved in vertebrates; experiments have been carried out on various

model organisms including fish, frog, chick and mouse to elucidate the molecular mechanisms regulating forebrain development. The wealth of information that has become available due to extensive research carried out to date in this field together with future research in this area will provide key insight into the development of the forebrain, and may also shed light on the etiology of developmental disorders of the forebrain such as **holoprosencephaly**, **atelencephaly**, **arhinencephaly** etc.

The forebrain can be divided into two primary subdivisions: 1) the telencephalon in the anterior which gives rise to the cerebral cortex, hippocampal formation, amygdala, basal ganglia and olfactory bulb and 2) the diencephalon in the posterior which gives rise to the neural retina, thalamus, hypothalamus and epithalamus. Development of the forebrain begins with a sheet of **neuroepithelium** which becomes specified as anterior neuroectoderm. Acquisition of anterior neural

Holoprosencephaly: A developmental disorder where the prosencephalon fails to divide into two hemispheres.

Atelencephaly: A developmental disorder leading to the congenital absence of the telencephalon.

Arhinencephaly: A developmental disorder leading to the congenital absence of olfactory bulb, olfactory tract and/or nerves.

Neuroepithelium: This is the layer of epithelial cells in the embryo which are composed of the progenitor cells that will give rise to neurons during the course of development.

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Patterning: In the developing embryo this refers to the complex process resulting in the organization of tissues and organs around the anterior-posterior axis, dorsal-ventral axis and medial-lateral axis of the organism.

Gastrulation: This is the process taking place at the single layered blastula stage embryo early in development whereby embryonic cells move and reorganize themselves to give rise to the three germ layers of the embryo.

Somitogenesis: The process of generation of somites, which are balls of mesoderm that will later give rise to skin, muscles and vertebrae.

Mesoendoderm: An embryonic tissue that is destined to give rise to both the mesoderm and endoderm.

characteristics is a result of timed and combinatorial activity of many signaling molecules such as BMPs, Wnts and Fgf8. Although these molecules have been implicated as factors determining neural fate, the individual roles played by these molecules are still under extensive investigation. Another important event taking place simultaneously with the rapid growth of the forebrain, is the **pattern-ing** of the forebrain along the dorso-ventral (D-V) axis which utilizes similar sets of signaling molecules as anterior-posterior (A-P) patterning but in different combinations. After initial specification, the dorsal and ventral compartments of the forebrain become specialized to give rise to morphologically distinct but functionally similar regions in different vertebrate classes.

In the history of elucidation of the mechanism of forebrain development, the avian model system has played a significant role. It has been extensively used by developmental biologists due to several unique advantages afforded by the system such as accessibility for genetic and surgical manipulation from the earliest stages. Recent advances in genetic manipulation techniques in combination with the classical techniques such as generation of quail-chick chimera have now made the avian model system even more powerful and it is thus suitable for investigating the mechanisms of forebrain development. In this review we discuss the current advances made towards gaining insight into the molecular mechanisms of vertebrate forebrain development highlighting the contributions of studies carried out in the avian model system. In addition, we discuss the recently developed molecular manipulation techniques in the chicken embryo which will add considerable ammunition to the arsenal of developmental biologists investigating vertebrate forebrain development.

2 Anterior-Posterior Patterning of the Developing Forebrain

Anterior-posterior patterning of vertebrate embryos begins very early at the neuroectodermal stage. For specification of anterior neural character, neuroectodermal cells must be protected from the actions of caudalizing (posteriorizing) factors. This process can be achieved in three ways, all of which are employed in vertebrate embryos for proper patterning namely: 1) restricted expression of caudalizing factors, 2) restricted expression of antagonists of caudalizing factors and 3) early morphogenetic movements keeping anterior tissue at a distance from caudalizing factors. Many signaling molecules had been identified to play a role in this process, the chief among them being Wnts, Fgfs and Retinoic Acid.

Prior to formation of the neural plate, the ectoderm must be first directed towards a neural fate. BMP and FGF signaling pathways have been demonstrated to play a crucial role in specifying the neural fate of the early ectoderm. It has been proposed that in neural tissue, antagonists of BMP signaling maintain low levels of BMP signaling allowing conversion of ectodermal cells into neural tissue.¹ However, studies carried out in the chick indicated the presence of another pre-specifying signal, as BMP alone was not sufficient to induce neural characteristics.²⁻⁵ This pre-specifying signal turned out to be Fgf, which is secreted from the epiblast and node cells in the chicken^{3,6} where it suppresses BMP signaling, allowing neural tissue to develop.³

Studies conducted primarily in chick and zebrafish embryos have established Wnt as a potent caudalizing factor.^{7,8} Wnts and their antagonists display restricted expression patterns with Wnts expressed at the posterior side and Wnt-antagonists such as sFRPs at the anterior side of neural plate in a group of cells called the anterior neural border (ANB).⁹ There are many lines of evidence suggesting the requirement of Wnt-antagonism for specifying telencephalic fate. Zebrafish mutants for Axin, a Wnt pathway scaffolding protein and suppressor of Wnt activity, as well as mutants for TCF3, a transcriptional repressor of Wnt signaling, do not develop a telencephalon.^{10,11} During **gastrulation** in chick, Wnt8C and Wnt11 are expressed in the posterior pre-somitic mesoderm, Wnt8C is also expressed in neural cells generating a posterior gradient of Wnt activity which specifies this region of the neural plate to a caudal fate. On the other hand, implantation of a Wnt3a bead in the anterior plate causes lack of rostral (anterior)-most forebrain character and a rostral shift in the caudal midbrain and hindbrain gene signature.⁸ One of the downstream effects of Wnt-antagonism in the anterior neural boundary (ANB) is the expression of Fgf8 and Fgf3. However, Fgfs at this point do not play a major role in patterning of telencephalon. Rather, they are involved in regulating cell proliferation or cell survival in a dose dependent manner.^{12,13}

3 Dorsal-Ventral Patterning of the Vertebrate Forebrain

By the end of gastrulation and beginning of **somitogenesis**, the early forebrain begins to get patterned along the dorsal-ventral axis. Signals released from the **mesoendoderm**, underlying early forebrain, pattern the neural tube ventrally and specify the hypothalamus and the ventral retina.^{14,15} On the other hand, signals from surface

ectoderm and roof plate specify dorsal identity of forebrain which later give rise to future telencephalon and dorsal retina. A major signaling pathway implicated in the ventral patterning of the forebrain is the Hedgehog pathway. The expression of Sonic hedgehog (Shh) in the **prechordal plate** induces the expression of Shh in the floor plate (Fig. 1). Mutations in the sonic hedgehog signaling pathway as well the cholesterol pathway result in lack of all ventral structures^{16,17} leading to severe developmental disorders such as cyclopia and holoprocencephaly.

The telencephalon is divided into two hemispheres which pinch off from the developing neural tube after the dorsal fate of the neural tube is established. The telencephalon is also patterned along the medio-lateral axis giving rise to different regions consisting of a midline. This midline acts as the signaling center to pattern the telencephalon along the medial-lateral axis. In addition to this, signals emanating from the midline also

contribute considerably towards the establishment of the dorsal-ventral patterning of the early forebrain. Separation of the two hemispheres is a complex process and involves the action of many signaling molecules such as Wnts,^{18,19} Fgfs,¹⁸ BMPs²⁰ and Shh,^{21,22} transcription factors such as Zic2 and receptors for lipid molecules such as LRP2.²³ Perturbations in the process of hemisphere separation lead to the congenital developmental disorder known as holoprocencephaly which is very common in humans.²⁴ The roles of many signaling molecules such as Wnts, FGFs, BMPs and Shh in the etiology of holoprocencephaly (HPE) have been investigated in great detail. It is now an established fact that HPE is a multifactorial disorder, resulting from mutations in one or more signaling pathways as well as exposure to certain environmental factors. However, the complete mechanism is still not clear. Understanding the etiology of holoprocencephaly has been greatly enhanced within the last decade by studying animal models

Prechordal plate: This is an embryonic tissue composed of a thickening of the endoderm that is present anterior to the notochord.

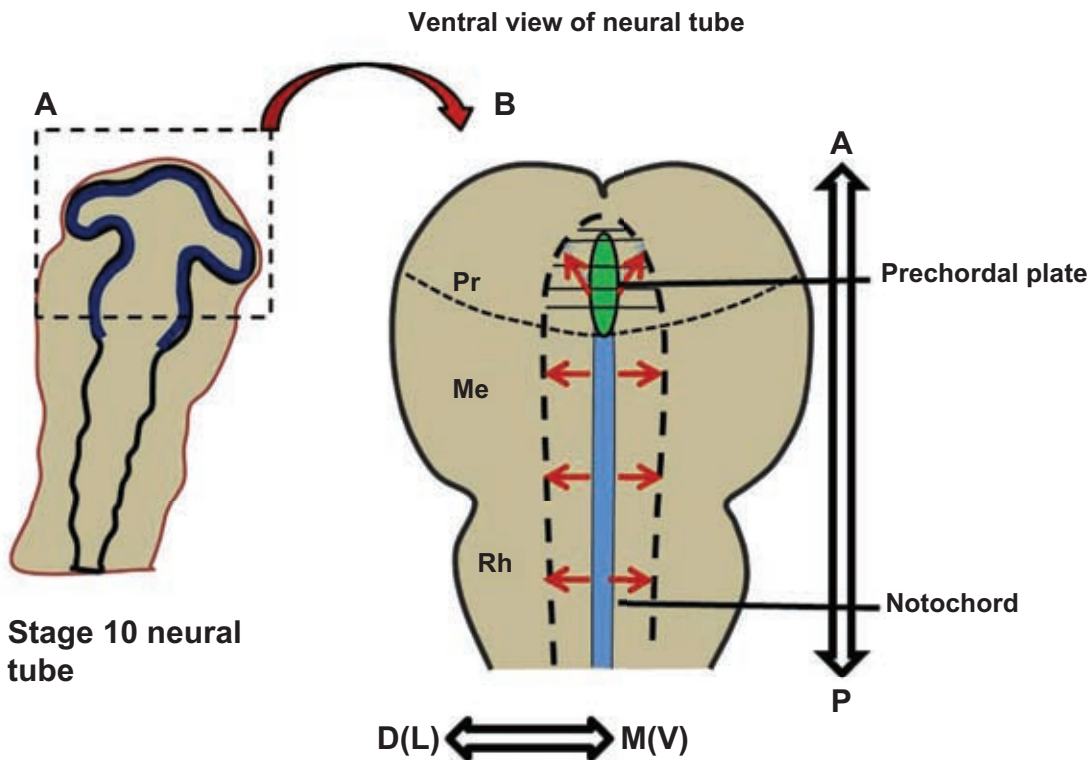


Figure 1: Schematic showing anterior region of Hamburger and Hamilton stage 10 (HH stage 10) chicken embryo. (A) low magnification view of HH stage 10 chicken embryo. Procencephalic region is within the boxed area and the ventral view of boxed area is shown in B. (B) Ventral region of procencephalon showing the major signaling centers which pattern the neural tube along the dorsal-ventral axes. Prechordal plate and notochord are the two major regions releasing secreted signaling molecules (red arrows) such as Shh which establishes ventral fate of the neural tube. (Pr-procencephalon, Me-Mesencephalon, Rh-rhombencephalon, D(L)-Dorso/lateral, M(V)-medial/ventral).

that mimic some of the features of human forms of HPE. It has been demonstrated by genetic linkage analysis of HPE patients that multiple mutations in the Shh pathway are linked to human HPE.²⁵ In mouse, mutations in sonic hedgehog leads to lack of all the ventral structures and indeed exhibits **cyctopia** and holoprocencephaly indicating its importance for maintenance and survival of ventral structures.¹⁶

BMP has also been implicated in the development of the midline structures and is one of the signaling molecules linked to holoprocencephaly. Experiments conducted on chick embryos involving application of BMP beads in the early forebrain resulted in severe defects in dorsal-ventral patterning of the forebrain and holoprocencephaly.²⁶ In the mouse, removal of BMPR1a from the dorsal midline resulted in loss of most medial structure such as the **choroid plexus**,²⁰ however, the patterning centre for the hippocampus, i.e. the **cortical hem**, seems to have been specified normally in these mutants. On the other hand, overexpression of the constitutively active BMPR1a resulted in conversion of the lateral ventricle cells to a more medial fate, e.g. the choroid plexus.²⁷ This was very similar to the thinning of neuroepithelium observed in the chick embryo treated with ectopic BMP4 and BMP5.²⁶ However, the patterning of the dorsal neuroepithelium in this case was not investigated using molecular markers. Moreover, removal of both BMPR1a and BMPR1b resulted in classical HPE without perturbing ventral patterning.

Although the involvement of secreted signaling molecules in specification of dorsal-ventral identity in the forebrain is well recognized in literature, recently, some transcription factors have been identified primarily to be playing a role in the process of dorsal-ventral patterning, and mutations of these transcription factors result in HPE. One such transcription factor is Zic2 which has been genetically linked to human form of HPE.²⁸ In the mouse, **hypomorphic** mutations in Zic2 result in a somewhat milder form of HPE, but the complete loss of Zic2 exhibits the characteristics of classical HPE as observed in Shh mutants. However, it has been demonstrated that Zic2 does not interact with Shh genetically and functions independently of Shh activity. Recently Arkel et, al.²⁹ established the role of Zic2 in prechordal plate development, which later becomes the major source of Shh required to pattern the floor plate. In the zebrafish, loss of function of Zic1, a close homolog of the mammalian Zic2, causes HPE by interfering with the Shh and Nodal pathway ventrally and increasing

retinoic acid signaling dorsally.³⁰ However this mechanism may be limited to zebrafish as it is yet to be demonstrated in other vertebrates. The growing body of literature providing insight into the mechanism of the dorsal-ventral patterning and associated HPE indicates two principal mechanisms:³¹ 1) involving mutations in genes specifying ventral fate such as Shh, PTCH1 etc. which results in classic HPE and (2) involving mutations in genes specifying the dorsal fate such as BMPs.

4 The Chick Embryo as an Attractive Model System for Studying Forebrain Development

The chick embryo has been a favorite model system for developmental biologists for a very long time, starting from Aristotle's observation of the live chick embryo in the egg. Since then studies in the chick embryo have contributed towards many remarkable discoveries in the field of developmental biology such as the discovery of patterning centers like the Zone of Polarizing Activity (ZPA) and the Apical Ectodermal Ridge (AER) in the limb, migration of neural crest cells and the role of the notochord in establishment of dorsal-ventral polarity and fate-specification of neuronal subpopulation in the neural tube. The chick embryo has certain obvious advantages compared to the mouse such as accessibility of the embryo from the earliest stages of development and the fact that genetic manipulations can be carried out in a more cost-effective and time-efficient manner. Recent advances in gene transfer technology in the early chick embryo have made it an extremely powerful and versatile system for developmental biological studies. In order to understand the early patterning events of forebrain development, manipulation of the early forebrain **primordia** is necessary. This can be easily carried out in the chick embryo either by surgical ablations or grafting techniques such as generation of quail-chick **chimera**. Besides that recent gene transfer techniques such as electroporation and virus-mediated gene transfer, may also be used for carrying out gain and loss of function studies.

4.1 Generation of quail-chick chimera

Development of the vertebrate forebrain is a complex phenomenon which begins from a simple neuroepithelium. During this process, cells originating from different anterior-posterior and dorsal-ventral locations, interweave together to form compartments of the mature forebrain. Thus, by studying the mature forebrain structures one cannot predict the exact origin of these

Choroid plexus: It is the structure composed of modified ependymal cells located within the brain ventricles that is responsible for the production of the cerebrospinal fluid.

Cortical hem: A small region of neuroepithelium located in the caudo-medial region of the developing telencephalon which acts as an important signaling center for forebrain development.

Primordia: In embryology this means the earliest stage of a particular tissue or organ before it has undergone differentiation.

Chimera: An organism composed of at least two different populations of cells that are genetically distinct.

Hypomorphic: This is the phenotype cause by a partial loss of function of a particular gene.

cells and the migratory path followed by them to reach their final destination during development. Many techniques have been developed for labeling the cells at the neuroepithelial stage followed by tracking these labeled cells at different time points of forebrain morphogenesis. Some of these techniques involve the application of dyes to cells and subsequently following the dye-labeled cells during development. These techniques, although useful, are limited by the fact that the dye gets diluted over time and the labeling is neither very precise nor reproducible. These issues were tackled by the generation of quail-chick chimera embryos, a technique pioneered by Nicole Marthe Le Douarin.³² In this approach, a small piece of the neuroepithelium is transplanted from a quail embryo to the same location (homotopic graft) or a different location (heterotopic grafts) of a stage-matched chicken embryo (Fig. 2). These embryos are then analyzed at later time points by identifying the quail cells by immunostaining with a quail-specific antibody, allowing visualizing of the region which is derived from transplanted piece of the quail neuroepithelial graft. The Origin of multiple regions of the mature brain have been identified through quail-chick chimera analysis, which resulted in generation of a comprehensive

fate map of the mature forebrain at the neural fold stage³³ and the neural tube stages of the chick embryo.³⁴

4.2 Electroporation mediated gene transfer

This technique was first established in the lab of Dr. Nakamura in Japan³⁵ whereby a low voltage electric field is applied across a particular tissue after its lumen is filled with the DNA solution corresponding to the gene to be electroporated (Fig. 3). By virtue of the negative charge present on DNA it migrates towards the positive electrode, and by placing the electrodes in the desired direction the DNA can be directed to enter the cells of one's choice. This method is rapid and with this one can introduce any gene under the control of a strong promoter to drive its expression in the tissue of one's choice. An extension of this method can be utilized to screen for the presence of regulatory elements for the desired gene. This may be achieved by placing the fragment of DNA to be screened for putative regulatory elements upstream of a minimal promoter driving the expression of a reporter gene such as green fluorescent protein (GFP), alkaline phosphatase (AP) or β -galactosidase (β -gal). On electroporation of

Fate map: A map of an organism or a tissue indicating the embryological origin of the different sub-regions. This is generated by labeling a cell or a subpopulation of cells at embryonic stages and determining the position of these cells and their progeny at later developmental stages.

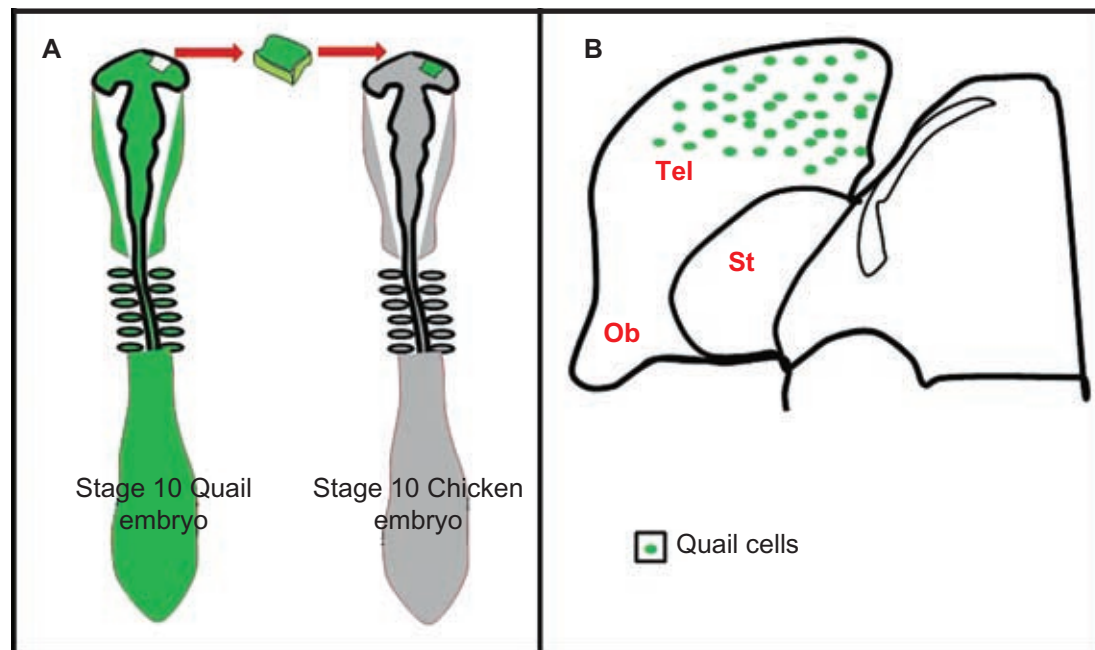


Figure 2: Schematic of quail-chick graft. (A) Schematic showing low magnification view of HH stage 10 quail (green) and chick (grey) embryos. A small piece of the quail procencephalic neural tube is excised and transplanted at the same location (homotopic) in the stage-matched chick procencephalon (green segment). (B) Sagittal section of mature forebrain of the chimeric chick brain where the quail cells are depicted in green and can be identified by a quail specific antibody (QCPN). (Tel-Telencephalon, St-Striatum, Ob-Olfactory bulb).

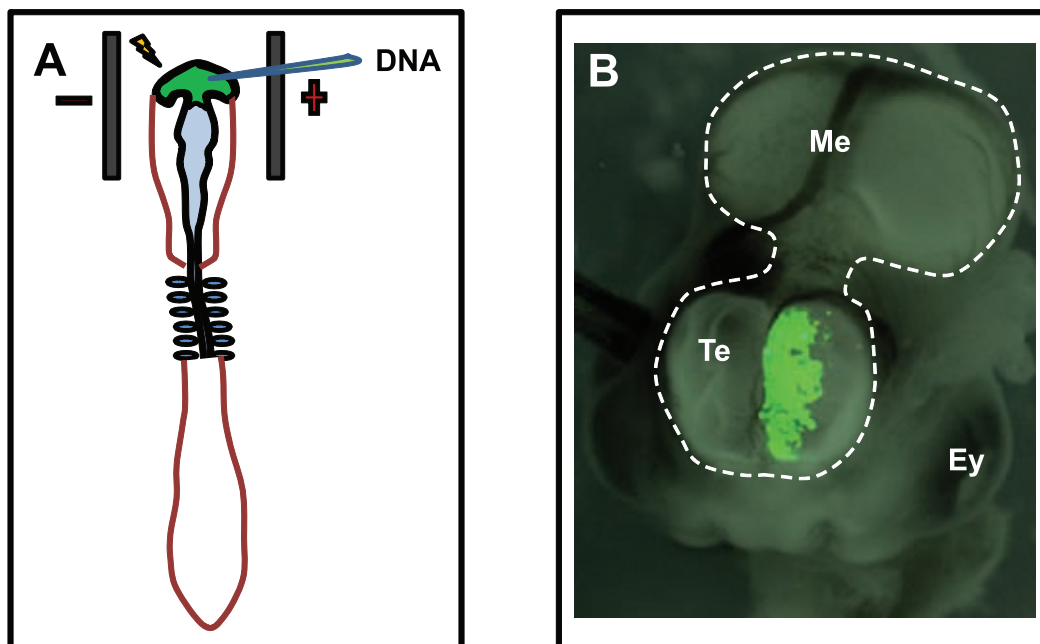


Figure 3: Schematic of electroporation of the procencephalon of a HH stage 10 chicken embryo. (A) Schematic illustrating the procedure of electroporation in the forebrain primordium. DNA solution is injected in anterior region of neural tube and a low voltage electric field is applied across the anterior neural tube which results in migration of DNA into the cells positioned near the positive electrode. (B) The image of a brain of an electroporated embryo at embryonic day 6 where green cells express the GFP transgene that was electroporated. (Te-Telencephalon, Me-Mesencephalon, Ey-Eye).

this DNA construct, the expression pattern of the electroporated reporter gene would be compared to the endogenous expression pattern of the gene being investigated³⁶ in order to determine the presence of putative regulatory elements. Analysis of this kind would enable the rapid screening of many putative enhancer elements or combinations of them in the chick embryo. This is considerably economical and time-efficient compared to carrying out a similar screen in the mouse, since in this case it would require the generation of transgenic mice corresponding to each putative enhancer. Recently a *Tol2* transposon based strategy has been developed for electroporation-mediated gene delivery in the chick embryo. When two constructs, one with the gene of choice flanked by *Tol2* transposon sites and another expressing transposase are co-electroporated into the desired tissue in the chick embryo, it results in the gene of choice being integrated into the genome of the electroporated cells. Using this system the expression of the gene of choice can be spatially regulated by placing a tissue-specific enhancer upstream of the gene. Temporal control may also be introduced in the *Tol2* transposon based gene transduction system by placing the gene of choice under the control of the Tetracyclin Responsive Element (TRE) such that

it is expressed only when the embryo is injected with doxycyclin (a tetracyclin analogue)³⁷

4.3 Virus-mediated gene delivery

Another efficient method for transfer of exogenous genes in the avian system is virus-mediated gene delivery. Retroviruses have the ability to infect dividing as well as non-dividing cells and integrate their genome into host cell genome by a two step process of reverse transcription and integration. This property of retroviruses makes them excellent tools for introducing a gene permanently in the chicken genome. In developing embryos most of the cells are undergoing proliferation and thus an avian specific retrovirus which is able to produce new viral particles upon infection known as the Replication Competent Avian Splice (RCAS) virus, can be efficiently used for gene delivery in the chick embryo. The replication competent nature of the RCAS virus facilitates the horizontal transfer of the viral genome where new virus particles released from the initially infected cells, infect the dividing cells in the neighborhood. Horizontal transfer of the viral genome ensures widespread expression of the gene delivered by this viral vector. (For detailed information about the RCAS system, please refer to the excellent

Replication competent virus:

Those retroviruses which are capable of producing and releasing fully infective virus particles from the cell that was initially infected, resulting in infection of neighboring cells.

review by S. Hughes).³⁸ Using the RCAS system one can misexpress any gene in the desired location in the chick embryo,^{39,40} or misexpress a gene in ectopic locations.⁴¹ In addition to this, loss of function studies can be carried out using RCAS viral vectors to transduce either the dominant negative form of a gene^{42–44} or **RNAi constructs** targeting a particular gene.^{45,46}

Although RCAS is a powerful tool for introducing genes into the avian genome, due to its replication competence nature it cannot be used for determining the lineage of a particular cell or group of cells. Lineage analysis at the cellular level can be achieved by using replication defective viruses which upon infection, integrate into cell's genome but are unable to infect neighboring cells. Thus, only daughter cells derived from the initially infected cell carry the genetic signature of the virus. **Lineage tracing** by replication defective viruses has been successfully applied to determine clonal relationships in the chick diencephalon⁴⁷ and for unraveling tangential migration pathway of cells in the chick telencephalon⁴⁸ and diencephalon.⁴⁹

5 Conclusions and Future Directions

Development of the vertebrate forebrain is a complex and dynamic process where multiple signaling cascades shape the initial neuroepithelium to give rise to a functional forebrain. Our current knowledge about forebrain development is a result of decades of scientific research resulting in the unraveling of complex interactions occurring during forebrain morphogenesis. In summary, considerable progress has been made in understanding how initial patterning events operate in order to make a functional forebrain. However, there are several unanswered questions that remain, such as how individual signaling molecules interact with each other and how independent these patterning events are, in space and time. We still lack all the mechanistic details about how morphogenesis of this complex structure occurs as well as information about the factors determining the observed morphological differences in the forebrain among different vertebrate species. In the context of forebrain development, the avian model system embodied by the chick embryo has played a significant role. With all the recent technical advances in gene transduction systems developed for the chick embryo it is now poised to help provide answers to some of the critical questions with regard to the mechanisms regulating vertebrate forebrain development.

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RNAi constructs: Plasmid expression vectors encoding short RNA hairpins that are complementary to a certain region of the target gene. These RNA hairpins target the mRNA of the gene for degradation leading to decrease in its expression. This phenomenon is known as RNA interference (RNAi).

Lineage tracing: The process of fate mapping carried out at the level of a single cell. A single cell is labeled genetically at an early stage in development and the inheritance of this label by the progeny of this cell can be used to trace the kind of cells produced from it at later stages.

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